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Degradability, Ecotoxicity and Bioaccumulation

**The determination of the possible effects
of chemicals and wastes
on the aquatic environment**

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Preface

On 15th February 1972 the 'Convention for the Prevention of Marine Pollution by Dumping from Ships and Aircraft' was signed in Oslo. In this convention, which relates to the north-eastern part of the Atlantic and the North Sea, a distinction was drawn between a 'black' list of substances that, in principle, must not be dumped into the sea and a 'grey' list of substances that can be dumped only when certain rules are observed. A very important group of substances on the black list comprises 'organohalogen compounds and compounds which may form such substances in the marine environment, excluding those which are non-toxic, or which are rapidly converted in the sea into substances which are biologically harmless'. No further explanation of the terms toxicity and degradability is given in the Oslo Convention. Moreover, at the first international meetings concerned with the execution of the Convention the term toxicity appeared to be interpreted in such a way that it must include not only the short-term but also the long-term effects. What this means is that in the execution of the regulations of the Convention attention must also be paid to the fact that some compounds can accumulate in living organisms (bio-accumulation), which in the long-term may lead to harmful consequences.

The three terms mentioned above – toxicity, degradability, and bio-accumulation – thus constitute important criteria for the assessment of whether given organohalogen compounds can be dumped into the sea. It is therefore necessary that methods (tests) are available by means of which these properties can be quantified. Work has already been carried out elsewhere (*inter alia*, in the United States and in international organizations such as the European Communities and the Organization for Economic Cooperation and Development) on the application of such test systems to a wide range of substances, but no detailed instructions have yet been produced.

In view of this situation the Netherlands Ministry of Transport and Public Works in 1973 started a research project directed at the extension, adaptation, and where necessary development of laboratory methods for the determination of the toxicity, degradability, and bio-accumulation of chlorine-containing waste materials in the marine environment.

The project was performed by the Netherlands Organization for Applied Scientific Research – TNO in its 'Central Laboratory' now known as 'Division of Technology for Society'. The research team was coordinated by Dr. H. J. Hueck and Dr. C. Pries. Main investigators were Mrs. D. M. M. Adema, Mr. H. Compaan, Drs. A. O. Hanstveit, Ir. J. F. de Kreuk and Ir. G. J. Vink, assisted by some 30 collaborators. This team was advised by a project-committee chaired by Ir. D. Tromp, Head of the Environmental Section of the North Sea Directorate, Ministry of Transport and Public Works. The investigations were carried out under the responsibility of a Steering Committee in which the following organizations were represented: the Government Institute for Sewage and Waste Water Treatment, the Netherlands Institute for Fishery Investigations, the National Public Health Institute, the North Sea Directorate of 'Rijkswaterstaat', and the TNO Organization. The composition of the Steering Committee is given at the end of this introduction.

The results of the project have been incorporated in the present report, which is divided into three parts.

- The first part (*General considerations*) goes into the background forming the foundation for the use of the test methods. It shows clearly that the tests depend upon one another and also that, in addition to the nature of the test substance, practical possibilities often determine the scope of the investigation and the test sequence.
- The second part (*Methods*) gives an exhaustive description of a number of test methods. In this part attention is also paid,

inter alia, to the cultivation of test organisms in the laboratory, the applicability of the methods, the analyses to be performed, and the treatment and presentation of the results. In practice the optimum methods must be selected as a function of the circumstances, but a certain amount of basic information must first be collected in each case.

- The third part (*A tabular review of the literature*) includes the already existing nationally or internationally standardized methods. These are directed mainly at the determination of the acute toxicity.

It must be emphatically, and perhaps superfluously, emphasized that the present handbook only specifies methods for the investigation of substances potentially harmful to the environment. It does not go into the standards to be established for these substances. Much more research experience is still needed to judge the permissibility of substances in the marine environment, while other criteria also play a part.

Since the investigation of methods as presented in this report does not claim to be complete, supplements are in preparation, especially in the field of the determination of degradability. Supplements will also be issued for methods that are still lacking, such as the detection of mutagenic properties as an indication of possible carcinogenicity.

In addition to this, the facts given are naturally limited to what is known in the present state of science. Finally, a loose-leaf form of presentation has been chosen so that the supplements can easily be inserted in their proper place.

The investigation for the development of the tests was performed with the aid of four model substances selected in such a way as to represent the group of organohalogen compounds as well as possible. The methods given are therefore applicable in broad outlines in studies directed at the permissibility of all organohalogenes and also of other substances with comparable properties in the marine environment.

The following remarks may be made about usefulness of the handbook in an international context:

- Within the framework of implementation of the Oslo Convention a preliminary consultation procedure has been set up: if a country proposes to dump black-listed substances, the other Parties to the Convention must be notified; they can then raise objections. The consultation procedure is associated with an exchange of the results of a number of tests that have been made obligatory. In the drafting of these tests, the Netherlands has already contributed the experience obtained in the investigation of methods, which was still not complete at the time. With the aid of the present handbook if international agreement concerning the necessity for it exists, more detailed and more standardized agreements can be made.

- The Oslo Convention recognizes a world variant of almost the same scope: the London Convention of 29th December 1972 on the prevention of marine pollution by dumping of wastes and other matter. It goes without saying that this handbook is also of importance for the implementation of the London Convention, which was also signed by the Netherlands.

- In addition to the Oslo and London Conventions there are various international regulations that control the dumping of wastes from land into fresh surface waters and into the sea. Three of them also recognize a 'black list' (the Rhine Chemical Agreement, the EEC guidelines concerning the aquatic environment, and the Paris Convention for the prevention of marine pollution from land-based sources). Substances already on the black list and ones possibly to be added to it must be identified mainly on the basis of the

The Steering Committee of the project was composed as follows:

Ir. C. v. d. Burgt (Chairman)	Chief Engineer Director of the North Sea Directorate, Ministry of Transport and Public Works.
Drs. E. H. Hueck-van der Plas (Secretary)	Study and Information Centre on Environmental Research – TNO
Dr. M. J. van Logten	Head of the Laboratory for General Toxicology, National Public Health Institute.
Dr. C. L. C. Meyer	Inspector of the Ministry of Health and Environment.
Dr. J. H. Parmentier	Director of the Division of Technology for Society – TNO.
Dr. A. Schors	Director in chief of the Organization of Industrial Research – TNO.
Ir. N. J. Spijker	Head of the Environmental Section of the North Sea Directorate, Ministry of Transport and Public Works.
Ir. K. C. Zijlstra	Chief Engineer Director of the Government Institute for Sewage and Waste Water Treatment.

Former members of the Steering Committee:

Drs. G. J. van Esch	Director of the Division for Toxicology and Chemical Analyses of Food, National Public Health Institute.
Prof. Dr. P. Korringa	Former Director of the Netherlands Institute for Fishery Investigations.
Ir. D. Tromp	Former Head of the Environmental Section of the North Sea Directorate, Ministry of Transport and Public Works.

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PART I

General considerations on a systematic evaluation of the ecological effects of chemicals and wastes in water

by H. J. Hueck

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by U. H. de Graaf

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PART I

General considerations on a systematic evaluation of the ecological effects of chemicals and wastes in water

by H. J. Hueck

INTRODUCTION

There is a growing conviction in many countries that the discharge of chemicals and wastes has reached an alarming level. In some cases the limits of disaster have been reached. Laws intended to keep this discharge within bounds have been issued or are being prepared in a number of countries, and to apply these laws it is desirable that the authorities should have at their disposal methods and standards to estimate the potential effect of a given discharge into the environment.

Zielhuis (1969, 1973) has pointed out for the area of industrial hygiene that three phases should be distinguished for the achievement of such an aim:

1. The laying down of **criteria** that establish the connection between the load and its effect. This usually amounts to a determination of dose-effect relationships in certain test systems.
2. The formulation of **guidelines** giving recommendations for the application of such criteria in practical situations.
3. The laying down of scientifically based **standards** in which policy considerations that may be based on social grounds other than the scientific grounds of phases 1 and 2 are discounted.

The aim of a test system can only be to provide information necessary for phase 1, while the instructions for the application of the results constitute part of phase 2. The present compilation of tests is intended to be a manual dealing with pollution of the environment by chemicals and wastes discharged into bodies of water deliberately or unintentionally.

The drawing up of standards in this field must be expressly reserved to government policy-makers, using the results obtained by the tests described in Part II of this book.

Similar test systems have been drawn up elsewhere. In the United States these are summarized in the report 'Principles of Evaluating Chemicals in the Environment' (National Academy of Sciences, Washington D.C., 1975). There is also a French law (Chemicals Control Act of 12th July 1977; cf. Notification to OECD, 35/NCP/ENV/77.1 of 20 September 1977), whereas already in 1973 a Japanese law (Anon., 1973) was published covering parts of the problem. Similar laws are in preparation in many countries. A review of the state of affairs within the European Communities is given by McLoughlin (1976). These acts themselves do not contain detached test specifications, but separate collections of test methods are being issued (e.g. Cabridenc and Chouroulinkov (1977)). A review of existing test specifications is given in Part III of the present study, as far as known to us up to 1976.

In the Netherlands the 'Oslo Convention' of 15 February, 1972 (Convention for the Prevention of Marine Pollution by Dumping from Ships and Aircraft) has stimulated efforts to develop such a test system (see Foreword). Although the Convention limits itself to rules for dumping wastes, it is also observed in a preliminary note that pollution has many sources, of which dumping is only one. It may be expected that the technical elaboration of this Convention, described in Annexes I to III, which include the 'black' and the 'grey' lists of substances, will act as a model for other international agreements in this field.

For the present compilation of tests the following parts of the above-mentioned Convention are relevant (Dutch Treaty Series No. 62, 1972).

In Article 5, it is stated that 'dumping of the substances listed in Annex I to this Convention is prohibited'. In Annex I (the so-called black list) under 1. are specified 'Organohalogen compounds and compounds which may form such substances in the marine environment, excluding those which are non-toxic or which are rapidly converted in the sea into substances which are biologically harmless'.

Characteristics of waste

In Annex III the characteristics of wastes are listed as follows:

- a. Amount and composition;
- b. amount of the substances and materials to be deposited per day (per week, per month);
- c. form in which it is presented for dumping, i.e. whether as a solid, sludge, or liquid;
- d. physical (especially solubility and specific gravity), chemical, biochemical (oxygen demand, nutrient production), and biological properties (presence of viruses, bacteria, yeasts, parasites, etc.);
- e. toxicity;
- f. persistence;
- g. accumulation in biological materials or sediments;
- h. chemical and physical changes of the waste after release, including possible formation of new compounds;
- i. probability of the production of taints reducing the marketability of resources (fish, shellfish etc.).

It is also stated in the preamble: 'mindful that the ecological equilibrium and the legitimate uses of the sea are increasingly threatened by pollution...' etc.

The legislators therefore intend that the evaluation of the consequences of dumping should also take into consideration the latter's ecological effects and not simply the infringement of 'legitimate use' (fisheries, shipping, recreation).

It is not easy to define damage to the environment on an ecological basis. An attempt at this was made by Hueck (1972) and it was worked out in greater detail by Hueck and Hueck-van der Plas (1976). The corresponding considerations formed the basis for the experimental work that has given rise to the present collection of tests, in which an attempt has been made to fill in the evaluation system with practical tests. The principles will be given briefly in the following section. For further details reference may be made to the above-mentioned articles.

In Sections 2-6 of Part I of the present study the use of the tests described in Part II will be related to a provisional evaluation system. Finally, it must also be pointed out that the tests included in Part II have usually been tried out thoroughly with four model substances, namely:

- 1,1,2-trichloroethane;
- Dieldrin;
- pentachlorophenol;
- 3,4-dichloroaniline;
- and in some cases also with a series of chlorophenols containing increasing amounts of chlorine.

The model substances have been chosen in such a way that a broad spectrum of chemical characteristics is covered and that they are of some environmental relevance.

1,1,2-Trichloroethane is a slightly polar, volatile, and neutral compound that is one of the main components of EDC-tar and can be used as an indicator component for this tar.

Dieldrin is a slightly polar, non-volatile, and neutral compound that is very toxic, has a high accumulation factor in organisms, and is widely used as a pesticide.

Pentachlorophenol is a polar, acidic, and non-volatile compound that is very toxic and is widely used as a fungicide.

3,4-Dichloroaniline is a polar, basic, and non-volatile compound considered to be very toxic in some respects. It is a waste substance of the chemical industry and the breakdown product of the widely used herbicide 'Diuron'. In the environment it is slowly transformed chemically.

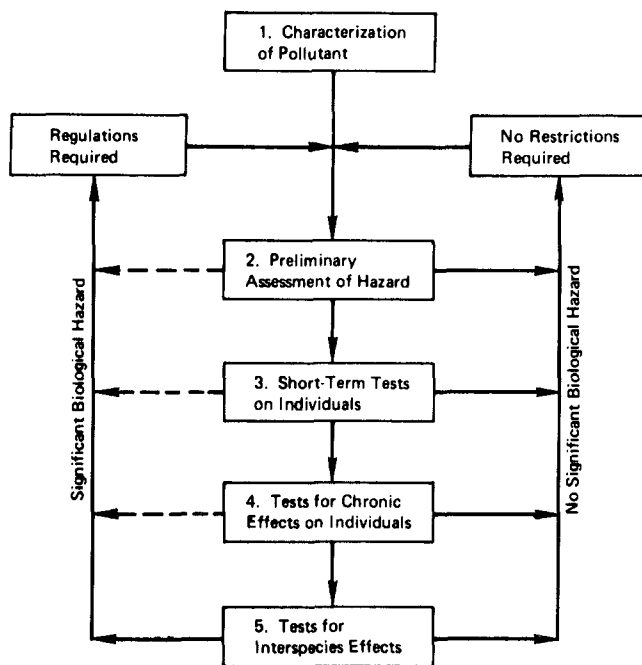


Fig. 1 Flow diagram for laboratory investigations of toxicity

safe handling of the substance. If the substance appears to be both toxic and difficultly degradable, bio-accumulation is included already in this phase of the investigation. On the basis of the over-all picture it can be decided whether further investigation is necessary, and if so, on what aspects. Substances that are produced in large amounts or form an important constituent of permanent large-scale discharges will in general be considered for further investigation even when the results of the preliminary toxicity and bio-degradation investigation are favourable.

b. Main Phase

In general, in this phase information will be sought on long-term effects. Subchronic and chronic toxicity tests in which attention must be directed at sublethal phenomena come into consideration. From the point of view of human toxicology, investigations for mutagenic and teratogenic effects will be desirable in some cases, depending on indications from the preliminary investigation.* The bio-degradation study will rather be directed at metabolites (bio-transformation). The investigation of bio-accumulation will in this phase extend to a greater number of organisms. The possibility of interaction with other environmental factors must be borne in mind, depending on the nature and use of the substance and/or the conditions of its discharge. In the case of the discharge of heavy metals the possibility of, for example, depot formation by adsorption on mud appears to be an interaction that cannot be neglected under practical conditions. Something similar cannot be excluded for other persistent compounds, but no rigid rules can be given. It appears desirable in this phase to develop physico-chemical methods making it possible to monitor discharges or the presence of the substance in the environment. This again depends on the amount of substance to which the investigation relates. After this phase generally, a decision should be possible concerning the permissibility of the use or discharge of the substance and the specific conditions to be imposed on it.

* Since rapid and simple tests for mutagenicity (e.g. the so-called Ames test) have become available, there is a tendency to include this type of investigation as early as the screening phase.

c. Confirmatory phase

In this phase an attempt is made to confirm (usually by field trials) the prognosis emerging from the preceding phases. Naturally, this can apply only to substances that are found in considerable amounts in the environment or which exhibit extreme properties of toxicity, etc., but that nevertheless are permitted for limited applications for all kinds of reasons. The principal measures here are chemical and biological monitoring of the threatened elements of the environment in cases of existing use or permitted discharge. Epidemiological investigations must be associated with such measures. Striking examples of the need for this have appeared in the recent past (vinyl chloride monomer). In some cases investigations in model systems (e.g. basin tests) are necessary to evaluate interactions between organisms and with abiotic factors of the environment. Such an investigation could be compared with the clinical investigation of drugs that precedes or accompanies the issue of agents with a high biological activity for general use. It goes without saying that only clearly indicated cases can be considered for such costly investigations. Generally speaking, the social need and the existence of alternatives must be weighed against the possible dangers of continued use or discharge. The development of such an outlook is primarily the province of policy-making authorities.

The course of events outlined above is fairly clear when the evaluation of pure substances (chemicals) is required. However, waste materials are mixtures of many substances. In such cases the possibility of testing is much more limited, since far-reaching studies (metabolites) and the investigation of accumulation depend on the availability of good and specific analytical methods. Here the preliminary (orienting) investigation will frequently give only general indications of a physico-chemical nature, general information on bio-degradation (e.g. BOD determinations), and simple facts relating to environmental toxicology. A decision must be made on the basis of these indications whether further investigation is required. If the answer is in the affirmative, separation of the mixture into the main components will sometimes be necessary. This does not give complete certainty for subsequent investigations, e.g. on bio-accumulation, but this applies to all prognostic investigations of the environment.

It would be wrong to believe that complete certainty can ever be achieved. However, the margin of risk can be reduced.

In the following section the tests described in Part II of this publication will be brought into relationship with the phasing of the investigation referred to above.

SCHEME FOR THE CLASSIFICATION AND PHASING OF THE INVESTIGATION

A large number of tests are described in Part II that can be used in investigations of the aquatic environment. As described in the preceding sections, these can be subdivided into four classes of information required for evaluation, namely:

- A. Substance-specific information
- B. Bio-degradation
- C. Bio-accumulation
- D. Environmental toxicity.

It is proposed to obtain this information in at least three phases. In the following table these phases are collected under the relevant headings. What is summarized in this table can be regarded as a guideline in which considerable variations are possible, and frequently even necessary, from case to case.

Evaluation of the results

The subjects given in the Table, as far as they are treated, can be found in the methodological part (Part II) as follows:

A. Substance-specific properties:

- Phase 1 Sections 1.1 to 1.4
- Phase 2 Sections 1.4 and 1.5; 2.1 to 2.5
- Phase 3 Section 1.4.

B. Bio-degradation:

- Phase 1 Sections 3.5.2 and 3.5.3
- Phase 2 Sections 3.5.4 and 3.5.5
- Phase 3 Chapter 7.

C. Bio-accumulation:

- Phase 1 Section 6.3.8
- Phase 2 Sections 6.3.8, 6.3.9.1, and 6.3.9.2
- Phase 3 Section 6.3.9.2 and Chapter 7.

D. Environmental toxicity:

- Phase 1 Section 5.9
- Phase 2 Sections 5.9 and 5.10
- Phase 3 Chapter 7.

It should be noted that the scheme is still incomplete. Hueck and Hueck-van der Plas (1976) have pointed out other aspects, such as the determination of adverse effects on odour (tainting) which is important for fish used for human consumption. Such specific methods are not (yet) included in the test compilation and are not treated in the table. Depending on demand and possibility, these aspects may be included in later supplementary publications. It should be mentioned that international efforts (e.g. by the OECD, Environment Committee, Chemicals Group) are under way to establish acceptable and well considered schemes of testing as indicated above.

EVALUATION OF THE RESULTS

1 Introduction

In the evaluation of the consequences of introducing into the environment a given substance that must be put through the system of tests as described in the preceding sections, we come up against two difficulties. In the first place, it is desired to assign to the result of each individual test a value that reflects the seriousness of the phenomenon being determined. The result of an acute toxicity test, for example expressed as $\mu\text{g.l}^{-1}$ for LC_{50} for a given test period in relation to a given test animal, must be interpreted as being high or low in the 'range' of usual results of this test. In addition, it must be possible to be able to evaluate the 'weight' of the experiment in the results as a whole. A toxicity test in a flow-through system during the whole life of a test organism carries objectively more weight and provides more information than a short-term (acute) toxicity test in a static system. While the relative

values of a result within a given test can still be quantified, since, for example, experience shows the upper and lower limits of such a test, this is practically impossible in a mutual comparison of tests. Yet it is necessary to review the results in their entirety. Two approaches can be used here.

1. The results of all the tests can be systematized by defining a limited number of result categories. This is done, for example, in the French law cited above (OECD, 1975 and 1977, loc. cit.). A tabular review can then be made from the various results, that gives the over-all picture in simplified form and visualizes the situation for an expert reviewer (cf. Hueck and Hueck-van der Plas, 1976, para. 4.5).
2. Use can be made of a comparison with a standard substance. Individual substances such as Dieldrin and DDT have already been subjected to far-reaching study. The results of the tests can be related to such 'standard substances'. Naturally this also has its limitations, but it can nevertheless serve as a guideline for the evaluation of the over-all picture. This in fact requires a comparison value in the specific test. For this purpose the values that we have found with the four model substances (see Section 1) with the tests described in Part II are being published *in extenso*.

Our knowledge has so far been insufficient to include all the desired parameters in a five-part scale. For some phenomena, especially those detected in chronic studies (occurrence of tumours, etc.), such arbitrary subdivision appears to be too simple. Here the subdivision of the ecological stress (relevance) should be left to the mature judgement of experts who must weigh the findings in the light of current knowledge. An arbitrary subdivision is then sometimes possible. In the French law (loc. cit.), for example, it is laid down that a finding of carcinogenicity must always lead to inclusion in the most severe category of environmental stress.

Individual indications will be given below for each parameter in relation to subdivision in the above sense. It must be expressly stated that here again much knowledge is lacking, and therefore the proposed arbitrary limitation must be regarded as a **very provisional** indication open to improvement. In agreement with the French law and as previously stated by Hueck and Hueck-van der Plas (1976), five categories are used as standard, A, B, C, D, and E, where A indicates the category least objectionable for the environment and E the most objectionable.

2 Substance-specific properties

The magnitudes that lend themselves to classification into categories are the amounts produced and the chemical stability. At the same time, the acute toxicity for warm-blooded animals can be included here as an indication of the problems of occupational hygiene. The whole field of human toxicity, which will certainly have to be taken into consideration by the policy-making authorities, falls outside the scope of the present work. Reference will be made to the previously cited American publication (NAS, 1975), where this field is treated in detail on pp. 93–216, the French law already mentioned several times, and Coulston's publication (1969).

Table 2 Categories of substance-specific properties

Parameter	Category A	B	C	D	E
Amount produced (tons/year)	<10 ²	10 ² –10 ³	10 ³ –10 ⁴	10 ⁴ –10 ⁵	>10 ⁵
Stability, chemical (half-life)	<1 week	1–4 weeks	1–3 months	3–12 months	>1 year
Acute toxicity (rat, oral, LD ₅₀ , mg/kg)	>5000	2000 to 5000	200 to 2000	100 to 200	<100

Remarks:

According to the French regulation the amount produced must be placed in a higher category if it relates to very toxic substances. If, however, both the toxicity and the amount are considered simultaneously and in combination with one another, which is urgently necessary for environmental questions, such arbitrary displacements can be avoided without difficulty.

The stability relates to the element of the environment in which the substance occurs. In our case this is water, and the values can be derived from the blank tests that accompany the bio-degradation tests (see the relevant section). In other cases the values can be approximated from specific laboratory data, taking into account the specific conditions (pH, temperature, etc.) of the receiving medium. For mixtures of substances such as occur in effluents an accurate determination is usually impossible.

In the French law the social need for the compound and the expansion expected for it are categorized. Although these attributes are certainly important, the classification appears to be still rather arbitrary. For further details, see the original publication (loc. cit.).

3 Bio-degradation

As set out in the introduction to the corresponding chapter in Part II, it is possible to regard as extremes substances that are fundamentally degradable and substances which are deemed to be recalcitrant (persistent) under the conditions of the receiving medium. These groups of compounds correspond to categories A and E of the proposed classification. Substances can be put in these categories by means of tests described in the appropriate chapter. With the test time given there one must still take the accuracy of the determination into consideration. Variations of 10–20 % degradation at a given test period are possible. Consequently, one can decide that a substance is degradable with certainty if 80–90 % or more of the substance is transformed in the test, while recalcitrance is assumed if less than 10–20 % (depending on the accuracy of the determination) undergoes transformation. It is less easy to categorize the result in the intermediate interval of 20–80 % conversion. When working with pure substances and with specific analyses, a classification into categories B, C, and D should still be possible. In the case of mixtures where global analyses (TOC, COD, biological titration) are used, this middle region can be characterized only as doubtful in relation to degradability. Category C could be used as an indication of this situation.

A gradation of the degradability must be sought in the type of test and in the greater range of conditions under which the degradation is caused to take place rather than in the test duration itself. **Thus, in any statement of degradability the test must be specified.** The usual time is given in Part II for each test.

Table 3 can therefore be treated only in connection with the corresponding specification.

In simple (ideal) cases a simple relationship exists between the percentage degradation in a given test (with a given test time) and the degradability.

The parameter used most often is probably the degradability expressed as the half-life of the substance under the conditions of the test. However, the present-day laboratory methods must be considered critically to see how far they are representative of the conditions prevailing in nature before this suggestion of degradation time (half-life) can be used for the actual breakdown in a natural environment.

The classification into categories therefore has to make do as best as possible with percentage degradations for each test specifically. The classification proposed in Table 2 must then also be regarded as very provisional. Not too much value should be attached to categories B and D. Summarizing, the classification of degradation can be systematized as follows. The percentage degradation here is the proportion of the substance converted in the test. A degradation of 20 % thus means that 80 % of the original amount was still found to be present at the end of the test.

Table 3 Classification of bio-degradation

Category	Pure substance, specific analysis	Mixtures and/or aspecific analysis
A (degradable)	>80 %	>80 %
B	80–60 %	–
C (doubtful)	60–40 %	<80 % >20 %
D	40–20 %	–
E (recalcitrant)	<20 %	<20 %

The limit of 20 % (or 80 %) mentioned can be read for experiments with a chemostat as 10 % (or 90 %). For this purpose the reader is referred to the introduction and description of the tests in the corresponding chapter of the manual.

4 Bio-accumulation and bio-elimination

The following factors are of practical importance for the evaluation of bio-accumulation, as explained in detail in the introduction to the corresponding chapter in Part II of this manual:

- rate of accumulation,
- level of accumulation reached,
- rate of elimination when the organism is introduced into water free from the agent under investigation.

The parameters mentioned under a) and b) depend on the concentration of the agent in the surrounding water. For the rate of accumulation a classification into a 5-point scale referred to above is renounced.

The much-used accumulation factor can be approximated by the quotient:

$$\frac{\text{concentration in the organism}}{\text{concentration in the surrounding water}}$$

after equilibrium with the surroundings has been reached.

The rate of elimination could be characterized by the half-life were it not for the fact that some substances after a rapid partial elimination show a much slower ensuing elimination behaviour, possibly owing to the presence of a multi-compartment system. It is therefore safer to use the total elimination as a measure. However, here one must take into account the inaccuracy of the chemical analyses upon which such a statement is based. Consequently, a practical measure is the T_{90} value, defined as the time after which 90 % of the amount present has been eliminated.

With the above reservation, the following categories can be distinguished (Table 4).

Table 4 Categories of bio-accumulation and bio-elimination

Category	Accumulation factor (for definition see text)	Elimination time, weeks, T_{90}
A	<10	<1
B	10–100	1–2
C	100–1000	2–4
D	1000–10,000	4–8
E	>10,000	>8

Remarks:

The division into categories depends, inter alia, on the accuracy of the determination of the respective parameters. Because of this, we renounce any excessively accurate indication of limits of the categories. It goes without saying that in any report the test animal and all other relevant circumstances must be specified. Relatively long elimination times (>1 month) cannot be determined accurately.

tely by simple experimental methods because of the complications of feeding and growth in the course of the trial.

In the example given, fish were used as the test animals. It may well be that for other test animals with shorter lifetimes the generation time could be selected as the unit, but more experience is desirable. In fact, only categories A and E give a clear indication in the case of elimination: category A indicates a rapid elimination and category E means that the substance is present practically permanently and cannot simply be eliminated with water. Categories B, C, and D express an increasing doubt about the possibility of elimination with water.

5 Ecotoxicity

The parameter that can reasonably be classified into five categories is the acute toxicity expressed as the LC_{50} . The correct description of the test concerned also requires a statement of the test duration and the test animal.

The $LC_{50,4d}$ value for a fish is one of the most-used parameters in this respect. The number of variations on this subject both in relation to the test animal and the test duration is very great. The species used e.g. in the United States are different from those used in Europe. The sensitivity of these species to different compounds may differ by an order of magnitude. Much greater differences can be observed when fish are compared, for example, with algae. It is therefore advisable to relate a still always arbitrary classification into toxicity categories to $LC_{50,4d}$ -values actually occurring in practice, and according to experience these lie mostly between 1 and 1000 $mg.l^{-1}$. The meaning of the categories is then that effects are to be expected in a range indicated by the scale. The test organism must be specified. As stated in the introduction (and in more detail by Hueck and Hueck-van der Plas, 1976), an ecologically justified evaluation of ecotoxicity cannot be based on tests with just one species. Individual types of organisms representative of different trophic levels should preferably be included in the investigation. The presentation of the results must then also be differentiated in relation to these organisms.

The following categories can then be maintained for the acute toxicity tests (Table 5).

Table 5 Classification of the acute toxicity

Category	C_{50} ($mg.l^{-1}$)
A	>1000
B	100–1000
C	10–100
D	1–10
E	<1

Remarks:

The above classification differs from the one proposed previously (Hueck and Hueck-van der Plas, loc. cit.); this is because of the objections described above. At the same time, reference may be made to the relevant introduction in Part II of this work. The customary unit of $mg.l^{-1} = ppm$ is retained because it is used most frequently in current investigations. In fact, it would be more correct if the concentrations were given systematically in $mole.l^{-1}$ (M). For the organic compounds that may be involved in this type of investigation the molecular weights may easily be between 100 and 500, so that comparative investigations are often rather confusing in this respect. However, the expression $mg.l^{-1}$ (ppm) has become customary for the concentrations actually occurring, to which the above table refers. The global indication has also been kept in view of the great variations from other sources.

PRACTICAL HANDLING OF THE TEST SCHEME

The classification given above for the evaluation of ecological effects of chemicals and wastes in the aquatic environment into

four main groups of effects, each again to be treated in three phases, is based on a theoretical consideration of the sequence of events expected on introduction of a substance: first physico-chemical reactivity, then bio-degradability followed by bio-accumulation, and finally the resulting toxic effect. Although this logical build-up is desirable for evaluating the over-all picture, the sequence of testing in the laboratory deviates from it. In general, tests cannot be carried out with organisms if it is not known in what concentrations of the substance under investigation they will survive. For this reason, the toxicity test is usually the first to be done after it has been established that the physico-chemical properties (stability, manageability) of the compounds do not present any special problems. Volatile compounds and readily soluble non-volatile compounds require different test arrangements, etc. As described above, the time-consuming investigation of bio-accumulation is frequently optimal.

The results of the bio-degradation test may be important for the interpretation of the other biological investigation, since both rapid degradation and the formation of metabolites have a strong influence on the determination of toxicity. This argues for a definitive or repeated toxicity study after the bio-degradation study which may take place in the toxicity investigation of phase 2.

A common test scheme then appears as follows:

1. Collection of substance-specific properties in relation to stability and manageability.
2. Preliminary toxicity study of phase 1, including toxicity with respect to bacteria ('range' determination), if reasonably stable compounds are involved.
3. Bio-degradation study.
4. Definitive toxicity study taking possible degradability into account. If long-term tests are undertaken in this connection (subchronic toxicity), this can sometimes be coupled with the following:
5. Bio-accumulation study.

Finally, the possibilities of evaluation according to the system discussed in the preceding pages will be described on the basis of an example of a fictitious substance. In the scheme below, as described above, A represents the ecologically least objectionable and E the ecologically most objectionable category.

Table 6 Example of an evaluation of impact made on the environment by a fictitious chemical compound

1 Parameter	Category				
	A	B	C	D	E
1. Substance-specific properties					
Amount produced					x
Stability			x		
Acute toxicity		x			
2. Bio-degradation					
Substrate-conversion test			x		
Activated sludge test	x				
Chemostat			x		
3. Bio-accumulation					
Concentration factor				x	
Elimination time (T_{90})		x			
4. Ecotoxicity					
Algae	x				
Crustaceans					x
Fish			x		
Bacteria	x				

On the basis of the above scheme it can be stated that the amount produced is such that it is impossible to be satisfied with only orienting determination according to phase 1.

The chemical stability and the acute toxicity to mammals indicate that no great problems need be expected in direct handling of the substance.

The bio-degradation in sewage purification systems (activated sludge test) is good, but the prolonged presence of the substance in surface water requires further attention (self-purification capacity, BOD loading). The discrepancy in the results of the bio-degradation tests indicates that a repeated and more intensive investigation is desirable, since complicating factors are apparently present. The bio-accumulation is considerable in its level, but the substance is also rapidly excreted. Therefore, problems are to be expected here particularly when this substance is permanently present in surface water. In the case of a single dumping the risk is slight because of the rapid elimination (and subsequent dilution).

The ecotoxicity shows that a purification plant (bacteria) will suffer no difficulties due to this substance. In the case of direct discharge into surface waters, problems are to be expected for the stock of fish and their food animals. Since the substance is toxic but not particularly persistent, permanent monitoring of its concentration with the aid of an automatic analyser at points of discharge is desirable. Random tests will be adequate for the remainder of the body of water. In view of the rapid accumulation of the substance, an investigation of its occurrence in fish for human consumption and crustaceans is desirable.

A disadvantage of putting forward a scheme for evaluation purposes is that excessive and sometimes absolute value is usually attached to such classifications. Reference has already been made to the relative value of the parameters given. Many others cannot be included in such a scheme, although they must also be taken into consideration.

It must therefore be emphasized that the described scheme is only an aid for part of the evaluation process. It should not encourage an automatic attitude in the acceptance or rejection of certain chemicals. The final evaluation must be made by experts with mature experience, on the basis of all the information obtained.

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PART II

Methods



CHAPTER 1

Properties of the substances to be investigated

by H. Compaan



1.1 INTRODUCTION

While the laboratory study directed at the formulation of test instructions was carried out mostly with pure model substances, wastes are generally made up of a large number of components with different toxic and other properties. This discrepancy, which particularly affects the present chapter, has led to a certain reserve in the formulation of detailed working procedures. On the one hand such procedures for pure compounds are already widely available in the analytical literature and, on the other hand, instructions of general validity can hardly be formulated for complicated mixtures or wastes. The limited experience with this last category,

and therefore also with practice, has even led to the situation that occasionally in Chapter 1 we have contented ourselves with suggestions for the design and performance of further studies (see, for example, Section 1.4).

The methods described in Section 1.3 and the proposal to **calculate** the partition coefficient between n-octanol and water by Rekker's method to determine lipophilicity (see Section 1.5.3.2) are fairly new, but we believe that within the framework of this handbook the information so obtained cannot be omitted.

1. Properties of the substances to be investigated

1.2 SAMPLING

Subject

The collection, handling, and storage of samples of waste materials to be investigated (see Note 1).

Field of application

Sampling of liquid wastes in which emulsions and/or solid material, suspended or otherwise, may be present. The waste mixtures are held in metal or plastic containers, large storage tanks, etc.

Principles

1. As much information as possible is obtained on the waste material prior to sampling.
2. The sample or samples must be representative.
3. After the sample has been collected, its composition must not change.
4. The sample must be large enough to permit the biological and chemical tests to be carried out at least twice.
5. At least two persons must be present at the sampling.

REQUIREMENTS

For sample collection

- protective clothing, especially good rubber gloves
- apparatus to lash and shake large vessels and carboys
- suitable first-aid equipment
- (foot-) pump with a suction nozzle and hose of suitable material
- glass or plastic funnels with a provision for venting
- glass bottles with well ground-in stoppers and stopper clips or, if need be, plastic bottles with loose attachable labels
- if necessary, a measuring instrument for the detection of phase discontinuities in storage tanks.

For sample transportation

- transport boxes in which the sample bottles can be carried without risk of breakage (shock-absorbent packing);
- a vehicle in which the samples can be carried in such a way that no risk to the driver can arise.

For sample storage

- A refrigerator or freezer, where necessary placed in a room specially reserved for the storage of dangerous chemicals.

SAMPLING INSTRUCTIONS

The sampling procedure must be governed by the prevailing conditions, and consequently no attempt can be made here to lay down a detailed procedure. Some general guidelines are given instead.

Collection of information before sampling

This is extremely important. The information is also necessary to determine the composition (see Section 1.4) and for the investigation described in Section 1.3. In most cases a certain amount of information is already available.

Before sampling, information must be obtained on the following points:

- a. Dangers that the substances present to those who collect, transport, and investigate the samples. If at all possible, the waste mixture should be put into one of the substance classes given in the Dangerous Substances Act (see reference list). Decisions concerning the protective clothing, first-aid equipment, method

of transportation, and the like must be decided on the basis of the information obtained (ISO 3165; 1976).

- b. Possible corrosive action on the materials used in sampling. The sample and the materials with which it comes into contact must not interact.

Representative nature of the sample

Problems can arise through:

- a. Inhomogeneity within a vessel or tank.
- b. Distribution of the waste over several vessels.
- c. Supply of the waste material in batches at different times

re a: Inhomogeneity within a vessel or tank

This will occur frequently. In many cases at the bottom of the tank there will be a layer of sediment, consisting of solid particles and/or tar-like material, or two layers of liquid may be present, frequently separated by an emulsion layer.

Since it will sometimes be impossible to homogenize the contents of a vessel or tank, it is recommended to collect the samples **both from the bottom and the top** of the vessel. It may also be useful to check with a measuring probe whether, and where, phase boundaries are present in the tank.

re b: The waste is distributed over several vessels

On statistical grounds, one will be inclined to sample the batch at random. However, it may also be useful to work selectively and, for example, to collect samples from the youngest and the oldest vessels and from those that are most difficult to reach.

re c: Batches supplied at different times

This too is a common situation. In some processes the waste has a reasonably constant composition, but this is by no means always the case.

It can be seen from what has been said above that sometimes it will be necessary to judge whether a number of samples have approximately the same composition. Since it is practically impossible to do this unless each sample is analysed completely, a number of rapid and informative identification techniques are mentioned in Section 1.4. See also Note 2.

Storage of the samples

It is wise, and has been found necessary in practice, to assume that the samples are chemically unstable. Storage at low temperatures can avoid this problem, or at least reduce it to a minimum. Care must of course be taken that the sample vessel is not attacked by the sample. After being thawed out to room temperature, the sample must usually be rehomogenized immediately before use.

The rapid identification techniques mentioned above (see Section 1.4) may be useful in evaluating the storage properties of the samples.

The size of the samples

Samples of a few litres are generally sufficient both to ensure that they are representative and to enable various tests to be performed.

Other precautions in sampling

The sampling should be carried out by at least two persons, mainly for the following three reasons:

- a. on purely technical and working grounds
- b. on the grounds of safety and health
- c. on legal grounds.

1.2 Sampling

The entire sampling procedure must be carefully recorded with statement of the:

- place
- date
- time
- names of persons present during the sampling
- numbers of the vessels and tanks sampled
- description of the samples (see below)
etc.

Description of the samples taken

At least the following information about a sample must be recorded immediately:

- its composition, to the extent that this is known
- its colour
- its odour and any evolution of irritant vapours on contact with air and water

- the extent to which the waste is liquid
- the presence of more than one liquid phase
- the presence of solid materials
- the nature of the process or processes from which the waste has come.

(For further details see 'Explanation', point 1).

Reporting of the sampling

The sampling procedure and the names of the sample collectors must also be recorded accurately in the final report on the tests and analyses performed on the samples.

NOTES

1. Sampling of water and test organisms is described in the various analytical procedures (see Chapters 5 and 6).
2. If it is necessary to reduce the number of samples to the statistically permissible minimum, advice must be obtained from specialists in the field of sampling statistics.

1.3 RAPID EVALUATION OF SOME IMPORTANT CHEMICAL AND PHYSICO-CHEMICAL PROPERTIES

Statement of the problem

It is often required to evaluate a waste of unknown composition. A thorough analysis of such a waste takes at least several weeks. Nevertheless, it will be desired to begin biological testing as soon as possible. It has been found that this can be done, and frequently it is even necessary, before the analytical composition has been completely established. In each investigation a number of chemical and physico-chemical properties of the mixed waste must be determined as rapidly as possible in the light of the method by which the biological tests are to be performed. It is obvious that, in this first biological evaluation of the waste, chemical analysis will be avoided as far as possible. This section will describe the procedure used at the TNO Division of Technology for Society in such situations. No detailed instructions can be given, and with each new type of waste the path must be explored step by step.

A distinction is made between **three** types of waste:

- mainly water (subsequently called 'effluent')
- concentrated chemical waste containing little water and usually of complex composition (subsequently called 'mixed waste')
- a single, reasonably pure substance whose identity is known (subsequently called 'waste substance').

Solubility

In the very first place, a rough idea of the solubility, especially in the test medium, is required. For an identified **waste substance**, it will mostly be possible to find its solubilities in water and in organic solvents from the literature, or these properties can be estimated with the aid of information on closely related compounds. During the tests concentrations higher than the solubility in the test medium are never used. Readily water-soluble compounds can be dissolved directly in water; sparingly soluble substances are dissolved in acetone and the solution is then poured into water. Complete dissolution sometimes requires a whole day. With very sparingly soluble substances it is not easy to decide whether a true solution or a very fine suspension has been obtained. It can be deduced from the molecular structure of the waste substance whether the pH of the test medium will affect the solubility. Moreover, attention must be paid to whether on dissolution precipitates are formed with the salts present in the test medium.

Effluents are investigated undiluted or, at the most, in tenfold dilution. In general, the presence and persistence of some suspended material cannot be avoided.

In the case of a **mixed waste** an attempt is first made to prepare a 0.1% solution in distilled water; higher concentrations are rarely used in the tests (sometimes 0.3%). If necessary, the dissolution is hastened by heating on a water bath, but then care must be taken that the whole remains in solution on cooling. Another possibility is ultrasonic vibration. If necessary, the concentration is lowered, but usually not below 10 mg.l⁻¹.

A solution in the test medium is made only after the determination of the pH variation of the solubility (see below). Again in the case of mixed wastes the presence of suspension usually cannot be avoided.

If it does not appear to be easy to dissolve the mixed waste directly in water, attempts are made to obtain the desired result via a solution, for example, in acetone, isopropanol, or tert.butanol; this last alcohol causes the least consumption of oxygen in the test medium. As a first attempt, a concentration of about 10 mg.l⁻¹ of the waste in water is prepared via one of these organic solvents. If a true solution is achieved at this concentration, an attempt is made to prepare solutions of higher concentrations. If not, concentrations lower than 10 mg.l⁻¹ are prepared, but the determination of true solubility can be difficult at these low concentrations.

pH, salt content and hardness

The pH of an **effluent** is determined in the usual way. In the case of a **waste substance** or a **mixed waste**, a saturated solution in water, but with a maximum concentration of 0.1%, is prepared and its pH is measured. Titration with acid or base to pH 7 (electrical measurement) gives an idea of the amount of base or acid in the waste. If the amounts are such that the pH of the test medium does not change by more than 0.3, no further problems arise in this connection. If too much acid or base is present in the waste, so that the pH changes by more than 0.3, neutralization is necessary (see Section 5.5). Care must then be taken that the salt content does not become too high for the test organism concerned, and it may sometimes be necessary to choose organisms that can tolerate a high salt content (see also Section 5.4.5). In practice, the pH must be checked repeatedly during the tests.

Influence on the amount of oxygen dissolved in the test medium

Even at low concentrations, many organic and inorganic compounds cause a marked fall in the concentration of oxygen dissolved in the test medium. Sometimes this is connected with direct chemical binding, e.g. by sulphite, sometimes with biological oxidation, e.g. that of ethanol but also – and much to our surprise – of pentachlorophenol. It cannot be emphasized strongly enough that the concentration of oxygen dissolved in the test media should be measured repeatedly (see Section 2.2.4).

This oxygen consumption must be investigated for all types of waste before the start of the biological tests. In an **effluent**, the concentration of dissolved oxygen is measured **directly** and, where appropriate, again after tenfold dilution within 5 min of making the dilution. An aqueous solution is made of a **waste substance** or a **mixed waste**, with a maximum concentration of 0.1%, and the amount of oxygen dissolved in this is likewise determined immediately. If a consumption of oxygen is found, the oxygen content is measured in a series of solutions of increasing dilution. If no consumption of oxygen is found by the method given above, the following procedure is used with all types of waste. A series of solutions of decreasing concentration is made up and stored in conical flasks covered with watchglasses at 25°C, and the oxygen concentrations are measured after 6, 24, and 48 h.

For an **effluent** concentrations of 100, 30, and 10% are selected, and for the other types of waste concentrations of 1000, 100, and 10 mg.l⁻¹. The amount of dissolved oxygen will often decrease. It is frequently found that at low concentrations of the waste a higher consumption is measured than at high concentrations, because the activity of oxygen-consuming microorganisms is inhibited by the higher waste concentrations. If no influence on the oxygen concentration in the test medium is found, no further problems are caused by this factor. If a distinctly measurable consumption of oxygen takes place, this has important consequences for the biological experiments, since it means that the aquaria must be aerated, which some test organisms may not tolerate (see Sections 5.4.5 and 5.9.7.1); furthermore, aeration leads to major losses of volatile substances from the waste material. Here again frequent oxygen measurements must be carried out in the course of the biological tests.

An alternative that is often relevant for practical conditions is that the microorganisms are first permitted to act on the oxidizable substances until no appreciable further oxygen consumption can be detected. The biological testing is then begun, and the procedure is specified in the report.

If rapid and intensive uptake of oxygen is taking place, this last alternative is the only method that has hitherto proved successful.

Volatility

During the biological testing, volatile substances may rapidly disappear from the test medium (see also Section 1.5.2). For **toxic**

1.3 Rapid evaluation of some important chemical and physico-chemical properties

volatile substances one can simply determine whether they occur in the waste to be evaluated. For this purpose, a simple toxicity test (6-, 12-, or 24-hour LC₅₀) can be carried out with *Daphnia* (juv.; Section 5.9.5.1), *Artemia* (Section 5.9.4.1), or *Gammarus* (Section 5.9.7.1), with **high** concentrations of the waste to be tested, in such a way that it is **certain** that volatile toxic substances are present in the medium (see Section 5.4.4).

These toxicity tests are now performed in two ways:

- **without previous** aeration of the test medium with the waste dissolved in it;
- **after** the test medium with the waste dissolved in it has been

aerated intensively for 3 h **beforehand**.

Clear differences in toxicity then indicate the presence of volatile toxic components.

In terms of effect, it is not always easy to distinguish between volatility and degradation of the substance; consequently, the content of oxygen dissolved in the test medium should be measured repeatedly.

Acute toxicity

After investigations carefully carried out according to the outline given above, reliable acute toxicity tests can be performed.

1. Properties of the substances to be investigated

1.4 DETERMINATION OF THE CHEMICAL COMPOSITION

Although the methods described in Section 1.3 already allow a biological investigation before the composition of the waste has been accurately established, a careful analysis must naturally be carried out as soon as possible. In general, for this purpose use must be made of a **combination** of modern analytical methods such as gas, liquid, thin-layer, and gel-filtration chromatography, together with mass, infrared, nuclear spin resonance, emission, and X-ray fluorescence spectrometry. Extensive literature is available on all these methods, and the latter's description would be outside the scope of this manual.

However, very little has been published on the application of the above methods to the analysis of industrial wastes. Up to the present only a limited number of waste mixtures have been investigated at the TNO Division of Technology for Society, and none of them completely. For this reason, it is not yet possible to give a general analytical procedure – if this should ever become possible – and only some important incidental observations can be made. The following aspects can be distinguished:

The analysis can be directed at:

- the most complete possible determination of the composition
- the fastest possible determination of changes in the composition of waste whose batches have previously been analysed exhaustively, or the determination of the homogeneity of a batch stored in a number of vessels, of which one sample has been analysed completely
- the detection of traces of highly toxic components in the waste.

The waste to be investigated may consist of:

- a fairly pure substance
- a complex mixture.

The most complete possible determination of the composition

The waste substance to be tested is occasionally so pure that the description 'chemically pure' is not inappropriate. Its identity will then usually be known and be easy to check by its melting or boiling point together with an infrared spectrum. The results of these measurements usually give a first idea of the purity of the substance.

However, the overwhelming bulk of the samples supplied for testing are complex mixed wastes which require a whole battery of analytical techniques for their composition to be determined.

It has been mentioned in Section 1.2 that before the analysis as much information as possible should be obtained concerning the waste to be investigated.

Mixed wastes have the following general properties:

- They are usually present as a single liquid phase, sometimes two phases (water and an organic liquid).
- In addition, a solid phase is almost invariably present, which may or may not be suspended. This may consist of inorganic and/or organic substances.
- The solid organic phase is often polymeric or tarry; sometimes it is difficult to distinguish it from the liquid phase.

In all operations on the samples one must start from the assumption that the sample may explode on heating; it is not exceptional to find that the sample polymerizes further on heating or undergoes other chemical reactions.

The dry residue (105°C) is determined by driving off carefully the volatile components **at the lowest possible pressure** (10⁻³ mm Hg).

The **distillate** is investigated separately. Often it will be suitable for direct gas-chromatographic analysis. There may be reasons for first carrying out a determination of the water content of the distillate. This relates not to traces but to appreciable concentrations.

The water content can be determined by one of the many standard methods based on azeotropic distillation with toluene. If the distillate of the waste sample contains a large amount of water, it can then be decided whether it should be dried over a large amount of sodium sulphate or be extracted with cyclohexane before the gas-chromatographic analysis.

The **residue** from the vacuum distillation is also investigated separately. On one portion the ash residue is determined, which gives an idea of the content of inorganic components. The ash residue can be analysed most rapidly qualitatively or semi-quantitatively by emission spectroscopy, and the important identified elements can then, if necessary, be determined more accurately by a method more suitable for the purpose.

(A number of elements can be determined quantitatively with the aid of X-ray fluorescence **directly** both in the original waste sample and in the fraction obtained).

The residue from the vacuum distillation is dissolved in acetone or in a mixture of acetone and methanol. The following then remain undissolved:

- inorganic constituents
- soot
- some organic polymers.

In most cases it will be possible to separate the solid and liquid phases by centrifuging, if necessary at a very high speed. The soluble constituents of the residue can be investigated by gas chromatography and thin-layer chromatography and, at a later stage, possibly by liquid chromatography (HPLC). Any organic polymers present will in general make dumping into the sea impermissible, and further analysis of this fraction is then rarely necessary, usually because some information on the nature of these polymers is already available. If it is nevertheless desired to analyse this fraction, gel-filtration chromatography (Hillman and Paul, 1975) and pyrolysis techniques, combined with infrared spectrometry, and the combination of gas chromatography and mass spectrometry, are very suitable.

Both the distillate and the part of the residue that is soluble in organic solvents must in general be considered to have such a complicated composition that it is better to carry out a preliminary functional-group separation before the application of chromatographic techniques.

The separation scheme proposed by Shriner, Fuson, and Curtin (1956) is probably the best; it makes use of various solubility classes. Some limitations of the method have been described by Davidson (1942). Ion-exchangers can give good service if it is desired to separate electrolytically dissociating compounds from neutral compounds (see, for example, Wilson *et al.*, 1967).

In practice it must be judged in each individual case which of the techniques mentioned above should be used.

Rapid evaluation of the composition

In the case of a 'pure' waste substance rapid identification will present no problems. The above-mentioned spectroscopic techniques, especially infrared spectrometry, will usually give adequate information. The method described by Corbett and Scullion (1976) may prove useful for determining some minor impurity. However, so far as complex waste mixtures are concerned the problem must be solved by the application of rapid instrumental methods that give as much information as possible on carefully selected fractions of the waste and, if possible, directly on the unfractionated waste. Gas chromatograms of the distillate on two different stationary phases (e.g. OV-101 and DEGS) and thin-layer chromatograms of the soluble part of the residue on two different adsorbents, together with an emission spectrum of the ash residue, are usually sufficient to determine whether significant changes have taken place in the composition. Frequently it may be possible to apply these techniques directly to the unfractionated waste.

Like the infrared spectra, the gas chromatograms can be investi-

1.4 Determination of the chemical composition

gated statistically with the aim of detecting small differences in identity between different samples (see, for example, Davies, 1975).

Determination of the presence of traces of highly toxic components

Both 'pure' waste substances and complex mixtures of waste may contain traces of compounds that are extremely toxic and therefore, in spite of their small amount, determine the toxicological properties of the waste with respect to the environment.

For the 'pure' substances the presence of chlorodioxins in chlorophenols is a notorious example, but it may be assumed that this situation frequently occurs for waste mixtures as well. This is confirmed, *inter alia*, by the fact that a waste made up synthetically

on the basis of the analytical results may have a much lower toxicity than the actual waste (see, for example, Boëtius, 1968).

The problem can perhaps be approached as follows. When 'pure' substances are involved, these can, for example, be recrystallized and after this the mother liquor and the purified substance can be compared toxicologically by the methods described in this report. If no difference is found, there is considerably less chance of the presence of traces of highly toxic impurities. In the case of a dissociating substance this can easily be separated from the neutral impurities and the same procedure be followed.

Mixed wastes can be treated similarly by testing the various fractions obtained by preliminary separation against one another.

A modest start in this direction has been made at the TNO Division of Technology for Society.

1.5 DETERMINATION OF SOME PHYSICO-CHEMICAL PROPERTIES

1.5.1 Solubility

No **accurate** solubility determinations were performed during the investigations forming the basis of this report.

Solubility in water (see also Sections 1.3 and 1.4)

For the **pure** (model) **substances** used it was possible to find solubility information in the literature (see, especially, Gunther *et al.*, 1968). It follows from the literature that it is very difficult to determine the solubility in water when this appears to be in the ppm region or lower (Gunther *et al.*, 1968; Biggar *et al.*, 1967; Bouwman *et al.*, 1960; and Schoor, 1975).

For **mixed wastes**, the situation is more complicated. Preferably, attempts should be made to test the total waste (see also Section 1.3). As a rule, a mixed waste contains insoluble inorganic constituents and soot, and often also dark-coloured high-molecular components. In practice, attempts will be made to test the waste at a 'no effect' concentration, when some of it will pass into solution and the remainder will be suspended or withdrawn from testing. The water solubility of many highly toxic substances is in the ppb range; whether such components are present in a waste mixture cannot be deduced from its solubility behaviour. However, an estimate of this can probably be made if the composition of the waste mixture is well known (see Section 1.4).

There is also another complication. In the environment itself the harmful substances with which this report is concerned are mostly adsorbed onto suspended solids and/or bound to humic acids and colloids in the water. No prediction about this can be made at present with the aid of solubility data. The magnitude of the influence exerted by this phenomenon on the ecotoxicological behaviour of organic compounds is unknown (see Matsuda *et al.*, 1971; Poirrier *et al.*, 1972; and Wershaw *et al.*, 1969).

Solubility in organic solvents

This information is important for two reasons.

In the first place, it is often necessary to introduce the substance into the test medium from a solution in an organic solvent. This applies particularly to substances that are sparingly soluble in water (see Section 1.3), when acetone, in which most organic substances are readily soluble, is usually selected. An accurate determination of the solubility is unnecessary.

In the second place, it is frequently very useful to know the solubility in certain **water-immiscible** solvents for the development of an analytical method. Here again, an accurate determination of the solubility is unnecessary. All that is checked is whether the substance dissolves reasonably well and rapidly in the following four solvents:

- cyclohexane
- toluene
- diisopropyl ether
- butyl acetate.

Extraction from water is usually possible with one of these four solvents. Diisopropyl ether and butyl acetate generally give a better extraction yield of polar compounds. Diethyl ether is too soluble in water. In general, organic halogen compounds should be avoided as solvents when a halogen-sensitive gas-chromatographic detector is used.

Benzene is still sometimes prescribed in publications, but must never be used for residue analysis since it is extremely toxic and causes leukaemia. It can be replaced by toluene.

1.5.2 Volatility

1.5.2.1 General introduction on the measurement and calculation of some volatility characteristics

The term 'volatility' may relate to various parameters.

Boiling point

Boiling point and boiling range are more significant for analytical purposes than in relation to environmental investigations.

The boiling points of many compounds are listed in countless standard tables. If a substance cannot be found in these, it is sometimes possible to estimate its boiling point from the boiling points of some related compounds or by another method (see, for example, Reid and Sherwood, 1958).

All handbooks of organic analysis describe a simple micromethod for the determination of boiling points. This is sufficiently accurate. However, in the case of a waste mixture, it is possible, at the most, to determine a boiling **range**. This must often be done at a low pressure in order to prevent decomposition (see Section 1.4).

Vapour pressure

This parameter has more relevance for environmental chemistry. For pure substances the vapour pressures at various temperatures can often be found in standard tables. If necessary, estimates can be made on the basis of information for related compounds, and it is sometimes possible to make use of Antoine's relationship:

$$\log_{10} P = A - \frac{B}{t + C}$$

where P is the vapour pressure in mm Hg at temperature t (in °C) and A, B, and C are constants that are also given in published tables; C is of the order of 230. (See Weissberger *et al.*, 1955 and 1959, and also Reid and Sherwood, 1958). The determination of a vapour pressure is work for specialists and is particularly difficult for compounds having a low volatility. Blaine's review (1976) should be consulted.

The great labour and the expensive apparatus that are necessary to obtain reliable vapour-pressure measurements appear to us to be justified only for those compounds, such as insecticides, that will persist for a long time in the environment and for which it is desired to draw up distribution models. For a waste **mixture** the vapour pressure is a very uninformative characteristic in the context of this report.

Evaporation losses from aqueous solutions

Within the framework of the present report this appears to be the most relevant factor both for estimating the losses to be expected in biological tests and analyses and for evaluating the residence time in surface waters.

If a substance obeys Raoult's first law, its concentration in the air above an aqueous solution can be calculated from the vapour pressure of the substance as such and its concentration in the water. In practice, however, this appears to apply only rarely. The loss of an organic substance from its solution in water is often much higher than would be expected. Thus, after a 5% evaporation loss, a stationary solution of DDT in water is found to contain only 20% of this compound (Mackay and Wolkoff, 1973). This problem has been discussed in detail by Haring (1974), Kuiper and Verdouw (1971), Mackay and Wolkoff (1973), and Mackay and Leinonen (1975).

Frequently for sparingly soluble compounds the partition coefficient in the water/air system can be approximated by the relationship:

$$K = 62.36 \cdot \frac{C_{w,m} \cdot T}{P}, \text{ where:}$$

K is the partition coefficient in the air/water system
or $\frac{\text{concentration in water}}{\text{concentration in air}}$

$C_{w,m}$ is the solubility of the substance in water (mole/l)

P is the vapour pressure of the substance in mm Hg, and

T is the temperature in °K.

This equation can be applied to volatile chlorinated hydrocarbons that are liquid at room temperature (see Section 1.5.2, Table 1).

1.5 Determination of physico-chemical properties

1.5.2 Table 1

Calculated and measured partition coefficients for certain chlorinated hydrocarbons in a water/air system according to McConnell *et al.* (1975); the figures in the last column were calculated from $K = 62.36 \frac{C_{w,m} \cdot T}{P}$ (see text).

Compound	Structure	Vapour pressure at 20° C, mm Hg	Solubility in water at 20° C, mole/l	Measured water/air partition coefficient	Calculated water/air partition coefficient
Chloroform	CHCl ₃	150.5	0.0686	8.6	8.4
Carbon tetrachloride	CCl ₄	90.0	0.0051	1.1	1.04
1,2-Dichloroethane	CH ₂ Cl-CH ₂ Cl	63.9	0.0888	26.4	25.5
Tetrachloroethylene	CCl ₂ =CCl ₂	14.0	0.00090	1.22	1.18

However, one can rarely obtain completely reliable information on the vapour pressure and the solubility in water, especially where substances of low volatility are concerned. For many substances very harmful to the environment verification of the relationship is extremely difficult – on the one hand because solubility and vapour pressure must be well known and, on the other hand, because the actual determination of the water/air partition coefficient is not yet possible for substances of low volatility.

Both for 'pure' waste substances of low volatility and for mixed wastes the direct determination of evaporation losses under practical conditions (Dilling *et al.*, 1975 and Section 1.3) is at present the only method that can be recommended.

In the laboratory, the loss of volatile substances from the aquaria is the greatest problem; particularly in the case of static tests in open aquaria, the losses of low-volatility substances can be very large (Acree Jr., *et al.*, 1963; Lichtenstein *et al.*, 1968; and Wheeler, 1969).

It has been possible to develop a simple method for the determination of the air/water partition coefficient for volatile compounds. The procedure is described in Section 1.5.2.2. A number of values have also been published by McConnell *et al.*, (1975).

1.5.2.2 Determination of the partition coefficient in the water/air system

Subject

Determination of the partition coefficients in water/air systems by gas chromatography.

Range of application

The method described here is suitable for determining the partition coefficients of neutral, fairly volatile, organic compounds in water/air systems.

Principle

A small amount of the compound whose partition coefficient is to be determined is placed in a special flask (see Section 1.5.2.2, Fig. 1) partly filled with water. When equilibrium has been reached in the closed flask, the compound is determined in both phases by gas chromatography.

EQUIPMENT

- Special conical flasks, 500 ml, with ground-in stoppers (see Section 1.5.2.2, Fig. 1)
- Kipp automatic dosing device, 100 ml
- plunger pipette, with disposable tips.

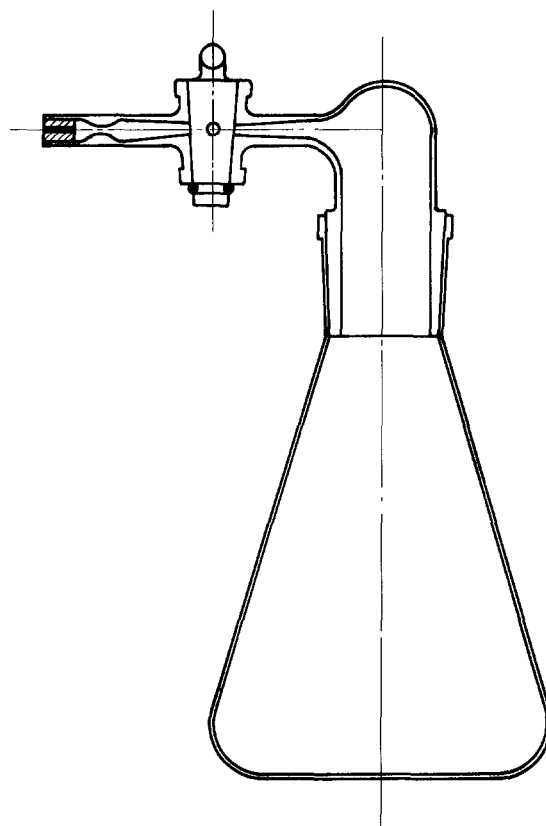
INTRODUCTION

The water/air partition coefficient is used mainly for estimating losses of test compounds from test aquaria and from aqueous solutions in the course of analysis. Further background information

concerning the method of determination has already been given in Section 1.5.2.1.

PROCEDURE

- Using a Kipp automatic dosing device, transfer 100 ml of distilled water into the conical flask (see Section 1.5.2.2, Fig. 1).
- After this, with the plunger pipette (with disposable tip) transfer 1 to 10 µl (or µg) of the compound under investigation to the conical flask (see Note 1).
- Close the conical flask with a moistened ground-in stopper with side-tube, using a pushing and turning movement.
- Shake the conical flask by hand for 1 min.
- Allow the flask to stand for 48 h (see Note 2).
- The gas-chromatographic analysis can then be performed.



1.5.2.2 Fig. 1. Conical flask (500 ml) for the determination of the water/air partition coefficient.

(The tap before the rubber injection tube, which is lubricated with water, is used to avoid losses of organic compounds by absorption in the silicone rubber)

1. Properties of the substances to be investigated

Set up the gas chromatograph as follows (see Note 3):

Column: 5% of OV-101 on Chromosorb G-AW-DMCS, 80–100 mesh. Stainless steel or glass, 4 m, external diameter 6.35 mm ($\frac{1}{4}$ "), internal about 4 mm.

Column temperature: this depends on the compound to be determined.

Detector: flame-ionization detector.

Detector temperature: 200°C.

With a gas-tight injection syringe inject, for example, 200 μ l of the gas phase into the chromatograph (see Note 4). Invert the flask and inject, for example, 5 μ l of the aqueous phase into the chromatograph (see Note 4).

The partition coefficient K in the water/air system can now be calculated directly from the volumes injected V and the peak areas S obtained (see Note 5).

$$K = \frac{S_w \cdot V_g}{S_g \cdot V_w}$$

Here the subscript 'w' refers to the aqueous phase and 'g' to the air phase. It is often possible to use the peak height instead of S : this saves time and incurs only slight loss of accuracy.

NOTES

1. The quantity of the substance to be investigated must be chosen in such a way that the concentration in water remains as low as possible but high enough for a quantitative determination; care must be taken that the concentration in the water remains considerably below the saturation level.
2. For volatile chloroalkanes it may first be necessary to adjust the water to pH 6 to prevent hydrolytic decomposition.
3. For details of gas-chromatographic analysis the reader is referred to Sections 2.1.1 and 2.4.2.1.
4. The amounts to be injected can of course be freely selected according to requirements.
5. Here it is assumed that the detector used is not disturbed by the injected water (see Section 2.4.2.1).
6. When the laboratory temperature varies by less than about 4°C it is not necessary to take the temperature into account.

1.5.3 Lipophilicity

1.5.3.1 General introduction on the determination and calculation of lipophilicity

From the very beginning of the investigations on which this book is based a search was made for an easily determined physico-chemical parameter for predicting the accumulation of test compounds in test organisms. Initially the fat/water partition coefficient was considered in this connection, and it has also been proposed for this purpose outside the Netherlands. However, there were two difficulties. In the first place, a 'standard fat' must be selected. This is not a new problem (see, for example, Koch, 1972), but one could perhaps with equal justification choose a **wax** (see Benson and Lee, 1975).

The second difficulty has also been encountered directly in practice, namely, that it is very difficult to measure partition coefficients greater than 10^4 (see Leo *et al.*, 1975). Since this often occurs in practice, the direct determination of the fat/water partition coefficient is no longer used.

The problem is a twofold one. In the first place, a suitable measure of lipophilicity has to be found that is reasonably easy to determine.

In the second place, we must investigate whether it is in fact possible to predict bio-accumulation factors with the parameters so determined. The first problem appears to have been solved by pharmaceutical chemists; with respect to the second problem, the first encouraging results have meanwhile been published by Brock Neeley *et al.* (1974).

A measure of lipophilicity

In pharmaceutical chemistry, use has already been made, on the basis of a proposal by C. Hansch, of the logarithm of the partition coefficient of the undissociated compound in the n-octanol/water system – $\log_{10} P$ – as a measure of lipophilicity (see Leo, Hansch, and Elkins, 1971). Hansch is developing a system that will make it possible to calculate $\log_{10} P$ on the basis of the structure.

Another system of calculation has been introduced by Rekker (Nijs and Rekker, 1973, 1974; Rekker, 1974), which in our opinion is more satisfactory and which has also been judged favourably by others (Tute, 1975). Instructions for Rekker's method of calculation are given in Section 1.5.3.2. The choice of n-octanol has proved a very fortunate one, even in the biological sense. Work on the partition coefficients between n-octanol and water has flourished, and a very large volume of information has been gathered on the subject. It therefore appears best to seek a connection with pharmaceutical chemistry in marine toxicological research, especially as Hansch and Rekker, among others, are studying the applicability of their systems to environmental-toxicological studies.

The determination of partition coefficients is a time-consuming task. Leo *et al.* (1971) have given instructions for its practical performance. However, for volatile materials the usual methods were unsatisfactory, since their reproducibility was inadequate. Since it is precisely for the small volatile molecules of chlorinated hydrocarbons that the method of calculation had not been satisfactorily worked out, we have developed a simple experimental method for these compounds; in general, this has an acceptable reproducibility (see Section 1.5.3.3). For 'pure' waste substances there is, therefore, a reasonable chance that the **determination** of the partition coefficient can be replaced by a **calculation**, which is in fact an extrapolation of known coefficients. Naturally, the same applies to any identified components of waste mixtures. This also solves the problem for compounds with a large value of $\log_{10} P$ (> 4), because, on the whole, experimental determination is in these cases unsuccessful with the techniques used so far.

The lipophilicity of waste mixtures

In order to evaluate waste mixtures it is relevant to know the partition coefficients of all harmful components. However, in most cases these are unknown. While the values of $\log_{10} P$ can be calculated separately for the known components, this is obviously impossible for the unknown ones. The dilemma can possibly be resolved in the following way.

The waste is added to a mixture of n-octanol and water and, when equilibrium has been achieved, the two liquids are analysed separately. The two fractions of the waste mixture are then compared toxicologically.

In addition, it is possible to compare the complete mixed waste toxicologically with the aqueous fraction after its partition in the n-octanol/water system.

Our first experiments in this direction have given encouraging results.

The relationship between $\log_{10} P$ $\frac{n\text{-octanol}}{\text{water}}$ and bio-accumulation

Brock Neely *et al.* (1974) showed for a number of **neutral** compounds a distinct connection between $\log_{10} P$ and the accumulation factor in fish. It is clear that this should be investigated further for other compounds and for other organisms.

1.5 Determination of physico-chemical properties

1.5.3.2 Estimation of the partition coefficient of organic compounds in the n-octanol/water system

Subject

An approximate empirical calculation of the partition coefficient in the n-octanol/water system for organic compounds from their structural formulae.

Field of application

The method makes it possible to calculate the partition coefficients mentioned for organic compounds of known chemical structure. Differences resulting from stereoisomerism and cis-trans isomerism are **not** expressed (see Note 1).

For dissociating compounds the partition coefficient is obtained at a pH at which the compound is **not** dissociated. In order to calculate the partition coefficient at the desired pH from this value, the pK_a value of the substance must be known (see Golumbic *et al.* 1949 and Tute, 1975).

For small aliphatic molecules with more than one functional group and for complex alicyclic compounds the method is still inadequately developed.

The value obtained gives an idea of lipophilicity that is sufficiently accurate for the aim pursued in this book (see Section 1.5.3.1), is useful in the preparation for a possible **measurement** of the partition coefficient, and can make the (difficult) measurement of very high partition coefficients unnecessary.

Principle

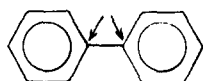
The structure of the compound is resolved into structural fragments whose additive contribution to the logarithm of the partition coefficient is known. In pharmaceutical chemistry it is customary to write this magnitude as $\log_{10} P$. The contribution from each fragment is independent of the compound in which this fragment occurs, apart from a few exceptions. Thus, there are differences for the same fragment in an aliphatic and in an aromatic molecule, and influences may be exerted by neighbouring functional groups. Correction factors have been introduced for this last effect and also for certain special configurations.

The method has been developed by Rakker and his associates (see Nijs and Rekker, 1973, 1974). The additive group contributions and the correction factors were found by multiple regression analysis of a large collection of measured partition coefficients (see Note 4). For details of the calculation procedure followed by Rekker and Nijs, we refer to the two above-mentioned publications.

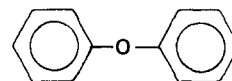
PROCEDURE

This has been taken from the publication of Nijs and Rekker (1974):

- Break down the structure of the compound into **the smallest possible number** of structural fragments chosen from Table 1.
- Use f_{Al} values for fragments directly bound to an aliphatic group and f_{Ar} values for fragments bound to an aromatic system.
- Use the f values with the numbers 27, 28, and 29* for mono-, di-, and trisubstituted benzene derivatives, respectively. For further-substituted derivatives, one $f(H)$ (No. 43)* is deducted for each substituent.
- A positive correction (No. 41)* must be applied for:
 - common C atoms in condensed ring systems;
 - C atoms that directly link two aromatic systems, as for example in biphenyl:



- C atoms that connect two aromatic systems via an S, O, or N atom as, for example, in diphenyl ether:



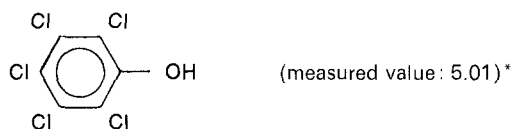
- For the case in which two electronegative groups separated from one another by only one C atom are present in one molecule, a positive correction of 0.80 is applied. When they are separated by two C atoms, this correction is +0.46.
- Calculate the **sum** of all the f values and corrections.

PRESENTATION OF THE RESULT

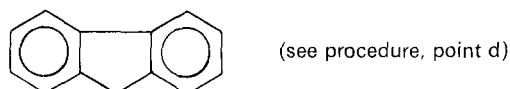
The number obtained is $\log_{10} P = \log_{10} \frac{\text{concn. in n-octanol}}{\text{concn. in water}}$ at a temperature of about 20° C (see Notes 3 and 5). In this connection it must be observed that, of course, the n-octanol is saturated with water and the water with n-octanol.

EXAMPLES OF CALCULATIONS

Pentachlorophenol $f(C_6H_3) + 5f(Cl_{Ar}) + f(OH_{Ar}) - 3f(H) = 5.17$



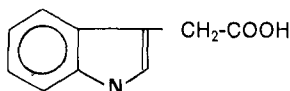
Fluorene $8f(CH_{Ar}) + 2f(C_{Ar}) + 2f(C_{Ar}) + f(CH_2) = 4.19$



or: $2f(C_6H_4) + f(CH_2) + 2f(C_{Ar}) - 2f(C_{Ar}) = 4.27$

(measured value: 4.18)*

3-Indolylacetic acid



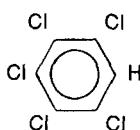
$5f(CH_{Ar}) + 2f(C_{Ar}) + f(C_{Ar}) + f(CH_2) + f(NH) \rightarrow f(COOH_{Al}) = 1.40$

(measured value: 1.41)*

1-Chlorobutane

$CH_3 - CH_2 - CH_2 - CH_2Cl$
 $f(CH_3) + 3f(CH_2) + f(Cl_{Al}) = 2.35$ (measured value: 2.39)*

Pentachlorobenzene $f(C_6H_3) + 5f(Cl_{Ar}) - 2f(H) = 5.74$

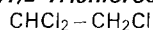


* See Table 1.

* The measured values have been taken from Nijs and Rekker (1974).

1. Properties of the substances to be investigated

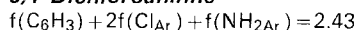
1,1,2-Trichloroethane



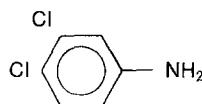
$$f(\text{CH}) + f(\text{CH}_2) + 3f(\text{Cl}_{\text{Al}}) + 0.80^* = 1.74$$

(value measured by us: 1.79).

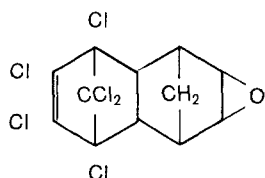
3,4-Dichloroaniline



(measured value: 2.69) (Leo *et al.*, 1971).



Dieldrin



$$f(\text{CH}_2 = \text{CH}) - 3f(\text{H}) + 3f(\text{C}) + 6f(\text{CH}) + f(\text{CH}_2) + f(\text{O}) + \rightarrow$$
$$6f(\text{Cl}_{\text{Al}}) + 0.80 = 2.34$$

The correction of 0.80 is introduced for the $\begin{array}{c} \text{Cl} \\ | \\ -\text{C}- \\ | \\ \text{Cl} \end{array}$ group.

This correction factor must presumably be modified somewhat in the future. A $\log_{10} P$ value of 2.34 for dieldrin seems much too low. The cause must be sought in the fact that this type of compound has not yet been studied by Rekker or others, so that the use of the available group contributions is presumably not permissible. No measured value is known.

NOTES

1. In many cases it is very laborious to determine the isomeric form of an unidentified substance analytically. However, this is usually not necessary for calculating the partition coefficient.
2. The influence of salts dissolved in the water (about 3.5% in sea water) on the partition coefficient can be neglected. However, the possibility of complex-formation with calcium, magnesium, and heavy metals must be taken into account.
3. The influence of the temperature is of the order of 0.01:

$$\frac{\Delta(\log_{10} P)}{\Delta T} = 0.01 \text{ per } ^\circ\text{C} \quad (\text{Leo, Hansch and Elkins, 1971}).$$

The influence of small changes in 'room' temperature can therefore be neglected.

4. Work is continuously in progress on further development and refinement of the method. In future, a number of minor modifications may therefore be made in the f values in Table 1, and the number of f values will be increased still further.

1.5.3.3 Determination of the partition coefficient in the n-octanol/water system

Subject

Determination of the partition coefficient in the n-octanol/water system by gas chromatography.

* See under dieldrin.

Field of application

The method described here is suitable for determining the partition coefficients of neutral and relatively volatile organic compounds in the n-octanol/water system.

Principle

A certain amount of water, in which the compound to be investigated is dissolved (Note 1), is shaken with n-octanol. The concentrations in both the octanol phase and the aqueous phase are then determined by gas chromatography.

EQUIPMENT AND MATERIALS

- Distilled water
- The compound(s) whose partition coefficient(s) is (are) to be measured
- n-Octanol, analytical grade
- Volumetric flask, 100 ml, with ground-in stopper
- Wash-bottle
- Plunger pipette with disposable tips
- Glass beads with a diameter of 3 to 4 mm.

INTRODUCTION

This partition coefficient is an important measure of lipophilicity. Further background information concerning the determination is given in Section 1.5.3.1.

PROCEDURE

- Place about 50 ml of distilled water (see Note 8) and a few glass beads into a 100-ml volumetric flask with stopper.
- Next, with a plunger pipette (with a disposable tip) add 5 to 10 μl (or μg) of the relevant compound to the volumetric flask (see Note 1).
- Make up to the mark.
- Shake the volumetric flask by hand (about 1 min).
- Allow the flask to stand for 24 h (see Notes 2 and 3).
- By means of a Kipp automatic dosing device introduce 2 ml of n-octanol into the volumetric flask (see Note 4).
- Shake the volumetric flask vigorously for 5 min six times and after each period of shaking wait until the phases have separated.
- Allow the well-stoppered volumetric flask to stand for at least an hour. The two phases can then be analysed by gas chromatography.

Set up the gas chromatograph as follows:

Column: 5% of OV-101 on Chromosorb G-AW-DMCS (see Note 5), 80–100 mesh.

Stainless steel or glass, 4 m, external diameter $\frac{1}{4}$ " , internal diameter about 4 mm.

Column temperature: depends on the compound to be determined.

Detector: flame-ionization detector.

Detector temperature: 200° C (see Note 6).

From the octanol phase inject the desired number of μl into the chromatograph.

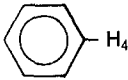
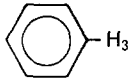
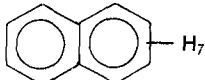
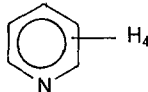
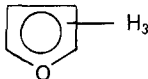
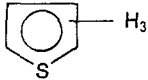
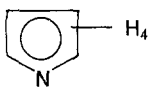
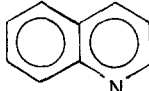
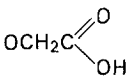
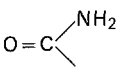
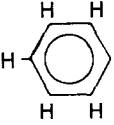
Do the same for the aqueous phase, in the **following way**.

Fill the injection syringe with distilled water and pass it through the octanol phase into the aqueous phase. Move the plunger up and down 10 times, with occasional careful stirring in the aqueous layer by means of the glass beads. Fill the injection syringe com-

1.5 Determination of physico-chemical properties

1.5.3.2 Table 1 $\log_{10} P$

Group contributions and increments of organic compounds in the n-octanol/water system, after Nijs and Rekker (1974).

No.	Fragment	aliph. ($f_{Al.}$)	f	arom. ($f_{Ar.}$)	No.	Fragment	aliph. ($f_{Al.}$)	f	arom. ($f_{Ar.}$)
1.	CH ₃		0.702		28.			1.732	
2.	CH ₂		0.527		29.			1.477	
3.	CH		0.236		30.			3.17	
4.	C		0.14		31.			0.543	
5.	CH ₂ =CH		0.93		32.			1.22	
6.	CH≡C		0.51		33.			1.62	
7.	F	-0.51		0.425	34.			0.59	
8.	Cl	0.06		0.930	35.			1.85	
9.	Br	0.24		1.169	36.	heterocycl. {NH}		-0.60	
10.	I	0.59		1.456	37.	heterocycl. {S}		0.44	
11.	OH	-1.440		-0.374	38.	heterocycl. {O}		0.10	
12.	O	-1.536		-0.458	39.	heterocycl. {N}		-0.98	
13.		-1.003		0.00	40.	CH _{Ar}		0.344	
14.		-1.281		-0.40	41.	C _{Ar}		0.297	
15.	NH ₂	-1.380		-0.911	42.	C _{Ar}		0.158	
16.	NH	-1.864		-0.93	43.	H		0.193	
17.	N	-2.133		-1.07	44.	Two electronegative groups separated by one C		0.80	
18.		-1.21		0.609	45.	Ditto, separated by two C		0.46	
19.	NO ₂	-1.06		-0.089					
20.		-1.99		-1.26					
21.		-1.69		-0.99					
22.	-CF ₃			1.28					
23.	CN	-1.13		-0.20					
24.	SH	0.00		0.62					
25.	S	-0.51		0.14					
26.	OCH ₃	-0.834		0.244					
27.			1.896						

1. Properties of the substances to be investigated

pletely and remove it from the flask. Clean the lower edge of the needle. Allow the injection syringe to empty until the desired volume for injection into the gas chromatograph remains and clean the needle again. Now inject the sample into the gas chromatograph.

- Calculate the partition coefficient as follows:

$$P = \frac{S_{\text{oct}}}{V_{\text{oct}}} \times \frac{V_w}{S_w}$$

Here S is the peak area and V is the injected volume; 'oct' denotes the octanol phase and 'w' the aqueous phase.

S can frequently be replaced by the peak height with less expenditure of labour and only slight loss of accuracy (see Note 7).

NOTES

1. In the selection of the amount of compound under investigation the sensitivity of the detector used and the solubilities of the compounds in both phases must be taken into account. In general the solubility in the water is the lower, so that if this is not exceeded no difficulties can arise. Take no more of the compound than can be determined satisfactorily.
2. After 24 h it may be assumed that the substance is completely dissolved
3. In order to ensure stability of the compounds to be investigated,

the pH of the water in which the compounds are dissolved must be adjusted to a value at which no hydrolysis takes place. For chlorinated hydrocarbons pH 6 is usually suitable.

4. These dissimilar volumes of water and n-octanol are so chosen that at a partition coefficient of about 100 the concentration in the aqueous phase after the addition of the octanol falls only slightly, so that it can still be determined satisfactorily. Different ratios can be chosen if other partition coefficients are expected. The aqueous layer must, however, remain easily reachable with a (long) injection needle.
5. This stationary phase is selected for the measurement of the partition coefficients of volatile chlorinated hydrocarbons. For other compounds a different stationary phase may be necessary. Care must be taken that the peak of the test compound does not coincide with that of n-octanol or of one of its impurities.
6. For details of gas-chromatographic analysis the reader is referred to Sections 2.1.1 and 2.4.2.1.
7. Here it is assumed that the water injected does not interfere with the analysis (see Section 2.4.2.1).
8. Shaking is carried out in a volumetric flask in order to limit the evaporation losses to a minimum. Moreover, the usually small amount of n-octanol in the neck of the volumetric flask is readily accessible for sampling.
9. For the influence of the temperature, the reader is referred to Section 1.5.3.1.

1.6 REFERENCES

1.2 Sampling

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1.4 Determination of the chemical composition

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1.5 Determination of physico-chemical properties

1.5.1 Solubility

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1.5.2 Volatility

1.5.2.1 General introduction on the measurement and calculation of some volatility characteristics

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1.5.3 Lipophilicity

1.5.3.1 General introduction on the determination and calculation of lipophilicity

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CHAPTER 2

**Analysis of the investigation of degradability, toxicity,
and accumulation**

by H. Compaan

with contributions by

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2.1 GENERAL PART

2.1.1 Some general instructions for the use of gas-chromatographic methods

2.1.1.1 Introduction

The gas-chromatographic methods described in this report largely belong to the domain of trace and residue analysis. It is one of the most difficult fields of application in gas chromatography – a field full of pitfalls. Here again we assume that those using the instructions given in this work will have a wide knowledge and experience in the field of gas chromatography.

The gas-chromatographic literature is very extensive but well organized and readily accessible. Many excellent handbooks and summarizing publications are available. At the end of this chapter a short list will be given of some books, journals, and articles that we have found indispensable in our practice.

We shall confine ourselves to giving general advice that does not appear in the literature, or perhaps not with sufficient emphasis.

2.1.1.2 Primary requirements for the gas chromatograph

The requirements that we believe a gas chromatograph must satisfy to be suitable for the work described in this report are strongly dependent on the way in which the instrument is used.

During the day the gas chromatograph is used for incidental analyses, for the development and evaluation of methods, for checking the purity of reagents, and the like. At night it is used to perform large series of automated routine analyses; 50 analyses per night is normal. This requires the setting up of an **automatic** sample injector that can take at least 50 sample bottles and, in addition, one or more bottles with rinsing liquid for the injection syringe (usually one or another 50). The injector must be designed in such a way that a rapid change-over from automatic to manual injection and vice versa is possible.

For many of the determinations it is desirable that the injection be carried out directly **onto the packing** in a **glass column**: the so-called 'on-column injection'. We prefer to place the column vertically in the column oven. The automatic injector must then be able to inject vertically downwards.

Almost invariably an **electron capture detector must be used**. This must be provided with a ^{63}Ni electron source and preferably have a **linear measuring range of at least 4 decades**.

It can be seen that the demands placed on the injector and the detector determine the choice of the instrument. The requirements printed in **heavy type** above are in our opinion indispensable, if a large number of samples is to be analysed, and considerably restrict the choice.

Some less strict demands will now be discussed and a number of practical hints will be given.

2.1.1.3 Injection

The injector has been described above, but we shall make three observations concerning its correct use:

- According to our experience, even for occasional analyses it is recommended to use the automatic injector as much as possible and not to inject manually, especially not in the case of quantitative analyses.
- A problem that can arise with 'on-column' injection is caused by an incorrect adjustment of the downward motion of the automatic injection syringe. If this causes the tip of the injection needle to descend too low and to come into contact with the column packing, a burr is formed at the tip. The septum is then torn and is no longer leakproof when the syringe is withdrawn.
- Although many different and sometimes very ingenious septa can be bought for the injector, the normal **blue** type has proved the best in our practice.

2.1.1.4 The carrier gas and the auxiliary gases

Very many of the interferences with an electron capture detector are caused by impurities in the gases used (see also Van de Wiel and Tommassen, 1972, and Karasek and Kane, 1973).

The necessary gases are marketed in various purity grades. According to our experience, they hardly need to be purified if the highest-purity grade is used. The necessary purification tube containing a molecular sieve is in many cases supplied as a standard accessory with the gas chromatograph.

It is perhaps appropriate to bear in mind that detailed measures for the purification of gases must be combined with careful monitoring of the effects of all the measures. In practice, this is often (wrongly) neglected.

In most cases it is sufficient to take care that the pure gases remain pure on their path to the column. For a stream of gas to the electron capture detector, use only reducing valves with metal membranes. Pass the gas through carefully cleaned tubes. Take measures such that when the gas cylinders are changed no air can penetrate into the system. Avoid plastics, rubber, and grease in the gas system as far as possible. Keep the tubes as short as possible. Carry out cleaning procedures at a point **immediately upstream** of the injector and/or the detector.

Traces of water in the gases do not always interfere, but oxygen does and, moreover, may adversely affect the stationary phase. When purchasing apparatus, an attempt must be made to ensure that the various gas **velocities** can be measured easily and without great effort. Never rely completely on a pressure measurement upstream of the column.

Carefully check the whole system for gas leaks.

Perhaps a good half of all disturbances in a gas chromatograph are due to such leaks.

2.1.1.5 Columns

Only three materials can be considered: glass, stainless steel, and nickel. For pesticide analysis we still prefer glass columns. Avoid dead spaces at the beginning and the end of the column. Always have packed and conditioned reserve columns ready.

In practice it has been found desirable to buy the column ready for use. At the present time any column can be supplied to order, mostly from stock.

2.1.1.6 Support materials

From our own experience, and according to various authors, acid-washed and silanized Chromosorb G is the most inert support. We have had insufficient experience with some newly-developed supports and with Chromosorb-750. The inertness of the support is necessary to prevent losses and asymmetric peaks of polar compounds.

Only exceptionally have we been forced to seek another support material (see Section 2.4.4.1).

2.1.1.7 The stationary phase

Whereas hundreds of different stationary phases are on the market, most gas-chromatographic separation problems can be solved if a considered choice is made of at the most 10 stationary phases.

As an apolar phase we prefer to use OV-101. This has the same selectivity as SE-30, OV-1, DC-200, and a number of other similar methylsilicones. However, we chose OV-101 because of its low 'bleeding', its wide temperature range, and the high plate numbers of the OV-101 columns.

For more polar columns and a different selectivity we use OV-210 (trifluoropropylsilicone), OV-225 (cyanoethylmethylphenylsilicone), stabilized diethylene glycolsuccinate, and Carbowax 20 M.

For a number of problems, especially the separation of volatile compounds, it is useful to have available column packings of the type of Porapak and the Chromosorb-100 series. In practice, there is hardly any need for other stationary phases.

It is sometimes recommended to add phosphoric acid to a stationary phase for chromatographing acidic compounds. However, the addition of phosphoric acid does not solve the problems if picograms of the substance to be separated are involved, and it also appears to us to be too risky for the electron capture detector. Consequently, we prefer to make from the substances to be separated derivatives (such as esters) that lend themselves better to gas-chromatographic analysis.

For basic compounds, we use the addition of, for example, KOH or K_2CO_3 to the stationary phase. This has given very good results. The stationary phases mentioned here can undoubtedly be replaced by similar ones without any objection.

Especially in the literature on pesticide analysis, mention is made of a great variety of **mixtures** of stationary phases. These may have great advantages when several substances have to be separated from one another in one run. Within the framework of this report, this problem, and therefore the need for such stationary phases, has not arisen so far.

2.1.1.8 The electron capture detector

In by far the most of the gas-chromatographic methods described in this report an electron capture detector can be used. Many compounds can then be detected in the subpicogram region. Its greatest advantages are that little effort has to be devoted to pre-concentration and that **small** samples are adequate. This last-mentioned fact has far-reaching and frequently even decisive consequences for the size of the biological test systems.

A very common misapprehension is that the electron capture detector is a chlorine-specific detector. This it definitely is NOT. The detector also has a very high sensitivity to substances with other highly electronegative groups in the molecule.

We should like to emphasize that much annoyance and time can be saved by using a detector with a ^{63}Ni source and a **wide** linear range of measurement (4 to 5 decades). A detector with a ^{63}Ni source can work at high temperatures, certainly up to $350^{\circ}C$, which considerably retards contamination. Moreover, no exchange with water can take place, as was frequently the case with the older tritium-containing sources. (We have had no experience with the modern scandium tritide sources; these should be able to cope with large amounts of water without interference).

For using such a detector with a radioactive source a special licence is required. In the Netherlands applications for this licence should be sent to the Ministerie van Volksgezondheid en Milieuhygiëne, Sector Straling, at Leidschendam, Dokter Reyerstraat 12. If it is desired to clean and dismantle the detector in-house, the laboratory must satisfy the demands that are placed on laboratories working with radioisotopes. In practice, it is best to have a reserve detector and have cleaning carried out by the supplier. As already mentioned, contamination of the detector can be slowed down by running it at the highest temperature possible, e.g. $300^{\circ}C$. This is not always the temperature at which the detector is most sensitive to the substances to be determined. Thus, it is sometimes necessary to weigh the advantages and disadvantages against one another.

Much loss of time due to breakdowns can be avoided if one routinely measures the base current of the detector every morning and records it in the apparatus logbook. In this way, one is warned **in good time** of an excessive contamination of the detector, and in most cases the latter can then be restored completely by an overnight conditioning. If the detector is allowed to become too contaminated it generally costs much trouble and time to make it operational again, and, moreover, a number of analyses have been spoiled.

All other facts must also be recorded in the logbook, such as the date on which a septum is renewed, date and temperature of the conditioning of the column and the detector, date of cleaning and changing of the detector, the measured gas velocities, replacement

of the first 10 cm of the column packing, trouble-shooting, maintenance, and so on. When the effort has been made to adjust the detector optimally for a substance to be determined, it is worth the trouble to record this information for possible future use.

Electron capture detectors from different manufacturers react very differently to shock-loading with a large amount of water, but the detectors of at least two makes are not affected by a deliberate introduction of about 20 ppm of moisture into the carrier gas.

Since the electron capture detector is not destructive, provision must be made to prevent the gases flowing **out** of the detector from finding their way into the laboratory.

2.1.1.9 The flame-ionization detector

This is used by us exclusively for determining **ppm** concentrations of test compounds in water. Both the low specificity and the fact that the sensitivity is a good three magnitudes smaller than that of the electron capture detector limits the applicability of flame-ionization detectors for trace and residue analysis.

Although the flame-ionization detector does not 'see' water, its sensitivity is substantially affected by moisture in the carrier gas.

2.1.1.10 Chlorine-specific detectors

So far as we are aware, all these have (for chlorine compounds!) a sensitivity that is comparable with that of the flame-ionization detector (Davis 1974; Sevcik, 1976). We have had no experience with the microcoulometric and the 'indium flame' detector.

We have carried out prolonged experiments with the Coulson detector in the form described by Hall (1974) and with the emission-spectrometric or helium-plasma detector (also called micro-wave powered plasma detector).

In addition to the still inadequate sensitivity of these four detectors for our purposes, the coupling of an automatic gas chromatograph working overnight is also insufficiently reliable in operation.

Furthermore, the specificity often leaves much to be desired, a very extensive pre-treatment of the samples thus being necessary, which is not acceptable within the framework of the investigations described in the present report.

2.1.1.11 Quantitative analysis

For an excellent review of the many pitfalls in quantitative gas-chromatographic analysis we refer to Kaiser's book (1965). When an automatic gas chromatograph is used, it has been found to be quite feasible in quantitative determinations to make use of the **peak height** rather than peak area. On the other hand, it saves much time if an automatic peak area integrator is available.

There are various integrators on the market, but it is absolutely necessary before buying to test such an instrument in the laboratory with the gas chromatograph to which it is to be connected and under conditions of the actual practical analyses concerned. Such a preliminary test can prevent much disappointment, and is even desirable for instruments of high reputation.

2.1.1.12 Derivatives of the compounds to be determined

Derivatives can be made of the compounds before they are chromatographed, for four reasons:

- in order to extract them better from water (1)
- in order to chromatograph them better (2)
- in order to detect them better (3)
- in order to distinguish them better from interfering substances (4)

Extraction (1)

For this purpose, the derivatives must be capable of being formed in an aqueous medium in a reproducible and preferably high yield. The determinations of pentachlorophenol in water by the 'acetyla-

tion method' and of dichloroaniline in water after bromination are good examples. In the first example the reaction takes place quantitatively, but in the second case it does not, which reduces the value of this treatment for analytical purposes.

The derivatives are less readily soluble in water than the initial substances and can therefore be better extracted.

Chromatography (2)

The first example mentioned under (1) simultaneously demonstrates the advantage to be obtained here. Free phenols are difficult to chromatograph, while their acetates chromatograph well.

Detection (3)

Likewise, the second case mentioned under (1) is an example of an improvement of the detection sensitivity. In fact, the electron capture detector is very much more sensitive to dibromodichloroaniline than to dichloroaniline itself.

This is probably an appropriate place to dispose of a frequently occurring misapprehension:

According to our experience, many workers expect that the introduction of a $-CF_3$ group will give a better detectability with an electron capture detector. However, this is by no means the case. It is true that the trifluoroacetates chromatograph very well, but for an appreciable gain in detectability at least the heptafluorobutyrate must be prepared.

Distinction from interfering substances (4)

A good example of this is the acetylation of phenols mentioned above; in this way these can be separated satisfactorily from carboxylic acids.

A good source of information concerning the preparation of derivatives suitable for gas-chromatographic analysis is formed by the various house magazines of the suppliers of reagents and other accessories for gas chromatography. In this connection, the following observation must be made:

We avoid the use of silicon-containing derivatives to the greatest possible extent in view of serious problems of contamination both in the flame-ionization detector and in the electron capture detector.

2.1.1.13 Contamination of reagents and glassware

'Everything is everywhere' is a well-known saying in biology. The chemist engaged in residue and trace analysis will have good reason to remember this when he is confronted in his daily practice with an almost inexhaustible variety of sources of contamination.

Detailed instructions for cleaning and the inclusion of blanks in **each** series of samples are the **minimum requirements** that must be fulfilled in this kind of work. Indeed, some easing of the task can be achieved by investigating for **each new analytical pro-**

cedure which precautions can be omitted and which are indispensable. Throughout the literature there are observations on all kinds of interference in residue analysis. A number of these publications are mentioned in the references.

2.1.1.14 Remarks

The project that has led to this review involved the analysis of large numbers of samples.

To keep the programme manageable, specific demands had to be made on the analytical equipment, leading to a high degree of automation of the analyses.

This automation also had certain side advantages, such as the possibility of analysing additional standards and blanks with relatively little extra effort.

As a result, the analytical techniques described in this chapter mostly relate to the automated methods used.

However, if similar work is carried out in a practical situation and a smaller number of samples has to be analysed, other apparatus and materials could be selected.

2.1.2 Some general instructions for the use of AutoAnalyzer methods

2.1.2.1 Introduction

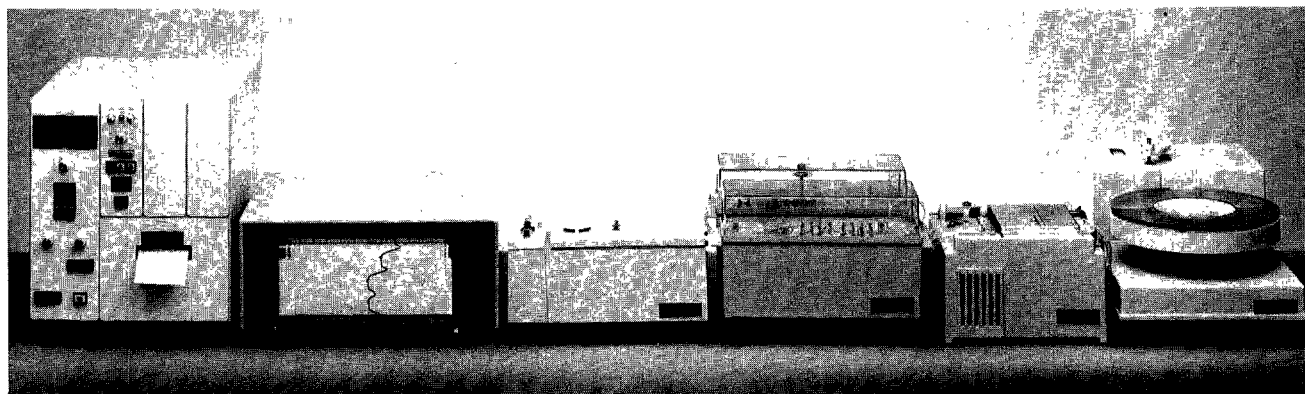
In order to analyse a large number of samples for several constituents in a limited time, it is sometimes possible to automate the analytical procedure. The possibility of this is provided, inter alia, by an automatic analyser. The AutoAnalyzer from Technicon Instruments Corporation, New York, has been selected for analyses that can be performed in this manner.

The AutoAnalyzer mechanizes an arbitrary manual method of analysis. This gives a high degree of reproducibility, because samples and standard solutions can both be analysed in an identical manner directly after one another.

In general, a large number of samples can be analysed in unit time (a minimum of 10 samples per hour). This is made possible, in part, by the fact that reactions taking place slowly are not completed. Since treatments are accurately repeated, reproducible results are nevertheless obtained and the relationship between the analytical result and the concentration in the sample can be determined by means of a calibration curve.

The analyser used in the investigations was of the AutoAnalyzer I type. A second-generation apparatus has meanwhile become available, the AutoAnalyzer II, and this is the type described here. An analyser machine of the type mentioned is built up from a number of units each of which has its own function and carries out operations that correspond to those of a manual method.

For the analyses that can be performed by an AutoAnalyzer the following units are required (see Section 2.1.2, Fig. 1):



2.1.2 Fig. 1 AutoAnalyzer

A: sample-feeding device

C: analytical cartridge

E: recorder

B: proportioning pump

D: detection unit

F: printer

The following units are shown in Fig. 1:

- Sample-feeding device (Fig. 1, A), which arranges that from a definite series of samples and standard solutions accurate 'weighed amounts' are introduced into the process and that the apparatus is rinsed between successive determinations.
- Proportioning pump (Fig. 1, B), mixes the sample solution and the reagents in the desired proportions and transports the solutions to the various units.
- Analytical cartridge (Fig. 1, C), contains a dialyser, a heating bath, and minor miscellaneous glassware. It is used for, among other processes, the addition, mixing, extraction, decantation, cooling, heating, or retardation of liquid streams. These treatments are also performed in a manual method, though in a different manner.
- Detection unit (Fig. 1, D). Among other instruments, a spectrophotometer, a flame photometer, or an atomic absorption spectrometer is used for this purpose. The most customary unit is a spectrophotometer. The difference of this unit from an instrument used in manual analysis is that in the latter case the measurements are carried out on separate solutions, while in the spectrophotometer of an AutoAnalyzer the flow of the coloured sample is continuous.
- Recorder (Fig. 1, E). This is an instrument by which the signal given by the detection unit is recorded as a peak.
- The printer (Fig. 1, F) presents the results in concentration units in digital form.

When a definite analysis has to be performed, it is not always necessary to use a combination of all the units.

Thus, if the analysis is to be carried out not on discrete samples but on a continuous stream, no sample-feeding device is used. When a clear solution is analysed which contains no components interfering with the determination to be performed, the dialyser can sometimes be omitted.

2.1.2.2 Procedure

Development

For the automation of an analytical method, and if an AutoAnalyzer is to be used, the analytical sequence used in the manual method must be subdivided into separate treatments.

In general, these are:

- supply of the sample
- addition of reagents
- mixing
- filtration
- heating
- detection
- indication of the concentration.

In the previous paragraphs we described the units that can perform one or more of these treatments. By a correct choice of the diameters of the pump tubes, the proportional pump can perform or govern a considerable part of these treatments.

Optimization

As soon as the system is working reasonably well, the method can be optimized by the introduction of stepwise changes. Thus, the length of the mixing spirals and of the spirals in the heating bath can be selected in such a way that, with the other conditions remaining constant, as large a signal as possible is obtained. After this, a sample size is sought that with the available colorimeter again gives a peak of the desired height on the recorder

chart. Here it must be borne in mind that it must be possible to analyse a concentration range covering one or two decades.

The sample-rate frequency is chosen in such a way that the peak height of one sample with a low concentration of the components to be determined is just not affected by a preceding sample with a high concentration.

The rinsing time between each successive sample is determined by the choice of the diameter of the pump tubes for aspiration of the samples and the setting of the sample-rate frequency.

Calibration

The calibration of the method is performed with standard solutions. These are solutions of the component to be determined, generally in a solvent that resembles the matrix of the sample as far as possible. These standard solutions, forming a series of increasing concentrations of the component to be determined, are analysed in the same way as the samples. This results in a series of peaks of increasing height on the recorder. The heights in millimetres plotted on graph paper against the corresponding component concentrations yield a calibration curve. The peak heights measured on real samples can then be 'fitted' to this calibration curve and the component concentrations read off.

In order to achieve a high degree of precision, it is necessary to analyse this series of standard solutions before and after the samples. With a large series of samples it has been found useful to record yet another series of standard solutions in the middle.

2.1.2.3 Remarks

Before proceeding to develop an analytical scheme for the AutoAnalyzer, it is advisable to ask Technicon if they already have such a scheme that could be used directly or after some adaptation.

The transformation of a manual method into an AutoAnalyzer method requires much experience, even if an existing scheme can be remodelled. In all cases the points given below must be borne in mind, since otherwise the chances of success become very small.

- When fitting AutoAnalyzer units together care must be taken that, *inter alia*, no dead spaces are formed at the joints.
- Take care that no air bubbles are drawn through the flow-through cell. These give 'false' peaks on the recorder chart.
- If highly aggressive reagents are used, care must be taken that the tubes cannot slip loose.

Push tubes about 1 cm onto the nipples and use hose clips where necessary.

Take care that in the chemical reactions that occur no precipitates are formed that can block up the system, especially in the connecting nipples. Precipitates in the flow-through cell of the colorimeter give rise to 'false' peaks.

- Use the correct tubing for each liquid. There are four types.
- polyvinyl chloride tubing for general use, 'solvaflex' and silicone tubing for organic liquids, and 'acidflex' tubing for concentrated acids.
- The dialyser membrane is easily damaged and should never be allowed to dry out.
- It is possible that the aspiration of aggressive samples may lead to corrosion of the stainless steel sampling needle and cause blockage. The use of a glass needle can be recommended in such cases.

2.1.2.4 Discussion

In our laboratory AutoAnalyzer methods have proved their worth in the determination of the degradability of pure organic compounds by various organisms. To follow the change of a concentration with time a large number of analyses are necessary. After the removal of organisms and other suspended particles, the clear

2 General part

sample solution is suitable for photometric analysis, which takes place in principle as follows:

To a known volume of a sample solution, in which the concentration of some dissolved substance S ($\equiv [S]$) is to be determined, is added an amount of reagent R that forms a coloured compound K with S:



After a preset time t , the colouration is determined photometrically in a standard cell. If the reaction has then gone to completion (R in excess), the following relationship holds:

$$\log \frac{I_0}{I} = C \cdot [S]$$

in which I_0 is the luminous intensity at $[S] = 0$ (pure water, but with the reagent added), and I is the luminous intensity of the samples to be investigated. The constant C can in principle be determined by means of one standard solution with known $[S]$. In practice, the curve of $\log I_0/I$ along the y-axis and $[S]$ along the x-axis is not always a simple straight line passing through the origin. Consequently, in most cases a number of standard solutions must be treated and a calibration curve be plotted from the results.

Frequently the incompleteness of the reaction after the preset reaction time causes breaks in the calibration curve.

This need not be harmful if the procedure is carried out with a high degree of reproducibility. Moreover, the large number of samples demands a short time of analysis.

The AutoAnalyzer, which carries out the photometric determination rapidly and highly reproducibly, satisfies both requirements. Slight breaks in the calibration graph (for example, due to incompleteness of the reaction) are not objectionable if a considerable number of standard solutions is included during the analysis.

If, however, we have to deal with a solution containing other substances in addition to S, that form coloured compounds with R, the method is **unsuitable**. This can be seen most clearly in the simple case of an excess of R and complete reaction with all the components. When such another substance forms a coloured product which, for example, has a ten-times higher extinction than K itself (cases that do occur in practice), this means that in the determination one molecule of this substance is counted as ten molecules of S. There is no possibility of any correction unless the light absorption is measured at several wavelengths.

The method is thus satisfactorily applicable in the determination of the degradability and toxicity of **pure** substances. In other cases the method cannot be used, unless we are dealing with a large series of closely related solutions, but then great caution is still required in the interpretation of the results.

A check on a limited number of samples, by chromatography for example, can allow a more realistic interpretation.

2.2 METHODS OF MONITORING THE TEST MEDIUM

2.2.1 Introduction

When the effects of chemicals on aquatic animals are studied in aquaria, the results are affected not only by the test compounds added but also by what could be called the 'quality' of the aquarium water. It may be fresh water, brackish water, or sea water; in this report for convenience we speak of the 'test medium'. The 'quality' of the test medium can be described by the numerical values of factors such as pH, temperature, dissolved oxygen, hardness, salt content, etc.

These factors affect the tests in three ways:

- by a direct influence on the test organisms;
- via the chemistry of the test compounds;
- by the occurrence of synergism with the test compounds.

Lee (1973) has gone into this in detail. The quality of the test medium is affected by:

- The test compound itself.

Here we refer to the side effects that it may have, in addition to its direct toxic action, on the quality of the test medium, such as causing a change in pH, a marked increase in the chemical oxygen demand, or a disturbance of an ion balance.

- The consumption of oxygen by the test organisms and also by their excretion products.

This effect will occur especially in static tests and usually becomes too large if the test organism is fed during the tests.

- The quality of the chemicals used for the preparation of the test media, in which mains water and distilled water may also be included.
- The vessels, pipes, pumps, filters, etc., used for the test medium.

It follows that in the performance of the biological tests it is impossible to make do with the determination of the test compound alone; the 'quality' of the test medium in the broader sense must also be monitored. Many of these quality checks must be performed frequently and in a number of cases it is preferable to carry them out *in situ*.

For many of the measurements of factors affecting quality there are more or less generally accepted instructions, which in many countries have already been standardized. The user of the present book is in principle referred to these instructions (see reference list), although we shall discuss certain determinations somewhat more deeply.

For a large number of water analyses the available methods vary from extremely rapid and simple field methods to highly refined and accurate laboratory techniques. In many cases the rapid field methods are sufficiently accurate even for aquarium tests. It should always be considered whether the methods used are sufficiently accurate, but not more accurate than is necessary. It is therefore necessary that one should have not only a qualitative knowledge of the factors to be controlled in relation to their various effects on the experiment but also at least a semi-quantitative knowledge insight.

In this introduction a review is given of the various water analyses and other measurements performed at TNO Division of Technology for Society to monitor the quality of the test media and of the methods used for this purpose, supplemented by a short discussion. Where a procedure to be followed has been investigated critically in our laboratory, the method most satisfactory in our opinion is described as accurately as possible in this report. When it was considered satisfactory to adopt published methods, these are referred to; they are supplemented by some practical hints where necessary.

It is assumed that the books by Strickland and Parsons (1968) and by Golterman and Clymo (1969) are available in every case, although, naturally, a choice of methods will also be found in the other books and publications mentioned. Nevertheless, the literature list also has the aim of providing analysts not well acquainted with the analysis of fresh water and sea water with an introduction into these two fields of science, which appear to be surprisingly sharply separated in the literature.

There is a number of manuals devoted to methods for water analysis that are known to be used successfully by a large number of laboratories. Where the title does not expressly refer to sea water or the sea, they relate exclusively to methods for the analysis of fresh water.

These methods generally cannot be applied directly to the analysis of brackish water and sea water. For the analysis of brackish water it is best to use methods that have been developed for sea water.

At the end of this chapter we mention three suppliers of a large variety of field methods that can be performed with simple and portable apparatus.

Review of the methods for monitoring the test medium:

• temperature	see Section 2.2.2
• pH	see Section 2.2.3
• dissolved oxygen	see Section 2.2.4
• chlorides, salt content, and electrical conductivity	see Section 2.2.5

Calcium and magnesium

We have so far determined these elements only in fresh water (permanent hardness). Many metals are less toxic in hard water than in soft. The bio-accumulation of toxic and/or radioactive metals is also affected by the water hardness.

The determination is usually carried out complexometrically, for example, according to the standard NEN 1056-III-8.

Sodium and potassium

These elements are determined in fresh water and sea water if there is reason to monitor the sodium-potassium ratio. An increased potassium content may be toxic, e.g. to shrimps. The accumulation of caesium, for example, can also be affected by it.

The determination is performed with a flame photometer in accordance with instructions of the instrument's manufacturers.

Sulphate

For a complete characterization of fresh water, in addition to the four cations just mentioned, sulphate and chloride must also be determined. The determination of chloride is described in Section 2.2.5. For the determination of sulphate we refer to the various handbooks. We have no clear preference for any particular method.

Copper

Copper frequently occurs as an impurity in mains water, but it is also found in distilled and demineralized water. The first few litres of water that come from a tap sometimes contain more than 1 ppm of copper. This can pass into the water by contact with pipes, taps, and pumps in which copper, brass, and bronze are used. Even dust from the laboratory atmosphere can measurably increase the copper content of water samples not kept in closed vessels.

Even in ppb concentrations copper can inhibit the reproduction of small crustaceans.

In our laboratory copper is determined with the aid of an AutoAnalyzer, with zinc dithiocarbamate as the reagent (Brouwer and Van Hooven, 1972).

Other toxic metals

In some cases it may be necessary to monitor the concentration of other metals as well. Where possible, we use atomic absorption spectrometry for this purpose.

'Active' chlorine

This is an impurity that occurs frequently in mains water in the form of free chlorine, hypochlorite, or hypochlorous acid, and also as chloramines, which presumably also occur as impurities in distilled water.

Hypobromite is formed with the bromides present in sea water. Manufacturers of salt mixtures for the preparation of artificial sea water often add sodium thiosulphate to remove any 'active' chlorine from the water used in the preparation.

Hypochlorite can be toxic to aquatic organisms in ppb concentrations.

It is determined by a simple colorimetric field method.

Remark:

There are two methods of determination; in the first o-toluidine is used as the reagent (→ yellow colour), and in the second diethyl-p-phenylenediamine (DPD) (→ pink colour); o-toluidine is **carcinogenic**, DPD is not.

Ammonia and ammonium salts

How much ammonia is present as NH_3 and how much as NH_4^+ is determined by the pH of the medium. With a change in circumstances the $\text{NH}_3 + \text{H}^+ \rightleftharpoons \text{NH}_4^+$ equilibrium is re-established instantaneously. In sea water, at the usual pH of 8.3, about one-tenth is present as NH_3 and the remainder as NH_4^+ (Trussell, 1972; Whitfield, 1974).

The toxicity is determined by the concentration of NH_3 . For some species of fish, 20 to 100 ppb are already distinctly toxic. Ammonium salts, i.e., NH_4^+ ions in solution, are not toxic up to high concentrations. Synergisms with inorganic and organic toxic compounds are known.

Test animals continuously excrete ammonia and ammonium salts even when they are not fed (although then to a smaller extent). Continuous monitoring of the ammonia content – partly with consideration of the toxic effects – is therefore of great importance.

In practice, it is found that in flow-through tests where no feeding is carried out the ammonia content remains negligibly low, well below toxic concentrations. On the other hand, in static tests where the animals are abundantly fed the occurrence of NH_3 toxicity must definitely be reckoned with.

In our laboratory the sum of the NH_3 and NH_4^+ concentrations is determined with the AutoAnalyzer (phenol-hypochlorite reagent) (Bradshaw *et al.*, 1970).

Nitrite

Nitrite arises through the biological oxidation of ammonia and occurs in chemical wastes. It is toxic, but less so than ammonia. Nitrite is also determined in our laboratory with the aid of an AutoAnalyzer (Armstrong *et al.*, 1967).

Note:

The α -naphthylamine frequently used as a reagent can cause **cancer** of the urinary tract. We have not yet had any experience with the substitute reagent 'Cleve's acid' (1-naphthylamine-7-sulphonic acid), which is said not to be carcinogenic (Crosby, 1967 and Bunton, 1969).

Apparatus for rapid water analysis

Three important manufacturers of apparatus for rapid and simple water analysis are:

- Hach. Chem. Comp., Ames, Iowa, USA.
- F. Hellige & Co., GmbH, Freiburg im Breisgau, GFR ('Neokomparator').

- Tintometer, GmbH, Dortmund, GFR ('Lovibond').

They make the greatest possible use of simple colorimetric **comparison** methods. These methods relate mostly to the analysis of **fresh** water. Perhaps superfluously, we again emphasize that analytical methods suitable for fresh water in many cases cannot be used directly for the analysis of brackish water or sea water.

2.2.2 Determination of the temperature

Subject

Determination of the temperature of the test medium.

Field of application

No closer indication is needed.

Principle

No closer indication is needed.

EQUIPMENT

Use can be made of:

- a. alcohol-filled thermometers
- b. electric thermometers
- c. mercury thermometers
- d. maximum-minimum thermometers.

INTRODUCTION

Temperature has a very strong influence on biological processes, on the positions of chemical equilibria, and on the effective toxicities of substances. Furthermore, a temperature measurement is necessary for a correct determination of many other parameters such as pH, conductivity, oxygen content, etc. A maximum-minimum thermometer can prove very useful in the monitoring of the temperature in systems for biological tests.

PROCEDURE

Check the thermometers to be used for correct reading:

- by comparison with a standard thermometer, or
- as follows:

Freeze distilled water in a refrigerator or a laboratory ice machine. Place this ice in a tall beaker and add distilled water. Stir the mixture of water and ice by passing air near the bottom so that a steady stream of air bubbles is produced.

Wait at least 5 min and measure the temperature of the water-ice mixture with the thermometer to be checked; the melt water has a temperature of 0.0°C (see also under 'Notes').

Thermometers that indicate a temperature of $0.0 \pm 0.25^\circ\text{C}$ can be used.

Thermometers with a greater deviation can be used after being marked with a correction factor. When using the thermometers mentioned under a, c, and d, the alcohol or mercury column must be completely under water during the reading.

Take the response time of the thermometer into account.

When using an electric thermometer, follow the manufacturer's instructions precisely.

REPORTING OF THE RESULTS OF THE MEASUREMENTS

Report the temperature rounded off to 0.5°C .

NOTES

NEVER use mercury thermometers for measuring the temperature in biological test systems.

Stirring with a stream of air bubbles during calibration is essential (Feiner, 1976).

With thermometers obtained commercially, deviations of up to 6°C may be found.

The handling process, or pushing back and forth in a drawer, can cause breaks in the mercury or alcohol column. This can be restored by cooling the thermometer to such an extent that all the alcohol or mercury draws back into the bulb. After this treatment the thermometer must be checked again for correct reading.

Electric thermometers must be checked frequently for the correctness of their reading.

2.2.3 Some remarks on the measurement of the pH of the test medium

2.2.3.1 Introduction

The pH has a very strong influence on the positions of many chemical equilibria and on the rates of many chemical reactions. The chemical stability, the solubility in water, the toxicity, and the accumulation of many substances are therefore highly pH-dependent. It is not easy to determine whether a pH change in the water has a direct influence on the organisms present; in any event, between pH 6.5 and 8.5 the effect will not be very pronounced. On the other hand, a pH change from 8.5 to 6.5 has an enormous influence on the chemical stability of, for example, pentachloroethane.

Any book on water analysis includes a chapter on pH measurement. We shall therefore satisfy ourselves with directing attention to a few practical aspects of pH measurement that appear to us to be important.

2.2.3.2 The various measurement techniques

To determine the pH, use can be made of:

- pH paper;
- colorimetric pH meter;
- simple portable electric pH meters;
- improved laboratory electric pH meters.

pH Paper

In general, the use of pH paper should be discouraged. While it is true that pH paper ensuring an accuracy of 0.2 pH units is available, this accuracy is achievable only under ideal conditions. Errors of measurement frequently exceeding one pH unit can be made, for example, owing to an incorrect method of storing the pH paper, when measurements are made in weakly buffered systems, when dissolved salts and organic compounds are present in the water, and when an incorrect measuring technique is used. However, such errors are not quickly detected unless control measurements are performed with an electric pH meter.

Colorimetric pH meter

The manufacturers mentioned in Section 2.2.1 supply simple portable instruments for a colorimetric pH measurement accurate to within 0.2 unit. Measurements with these instruments are reasonably reliable, and if necessary, compensation for the colour of the water itself is possible. If a set designed for use in fresh water is used for pH measurements in sea water, 'salt errors' amounting to a few tenths of a pH unit may occur. The presence of dissolved or colloidal organic substances can cause errors of more than one pH unit. Errors can also arise on measurements in weakly buffered systems.

Simple portable electric pH meters

The portable electric pH meters of compact design are the most practical in use. Moreover, they are not much more expensive than the colorimetric pH meters mentioned under b). With these instruments the pH can be measured to within 0.1 unit. The disturbances due to dissolved salts and organic compounds, to colloids, and to the intrinsic colour of the test medium mentioned under a) and b) do not occur.

Laboratory electric pH meters

With the larger laboratory pH meters, the pH can be measured with an error that is sometimes smaller than 0.01 pH unit. This is much more accurate than is necessary for the tests described in this book. A disadvantage is that samples must be removed from the test aquaria and transferred to the pH meter.

2.2.3.3 Important sources of error

In practice, a number of errors appear to be fairly frequent. These can be avoided by taking the following precautions:

- Store and handle the glass electrode strictly according to the manufacturer's instructions.
The electrode can actually be made completely unusable by incorrect storage.
- Calibrate the pH meter frequently.
This calibration should preferably take place with two different buffers, so that it is not only the correctness of one reading that is checked, but also the scale value.
For measuring the pH of sea water, it is possible, if necessary, to make do very well with a dilute aqueous solution of borax (sodium tetraborate; $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10 \text{H}_2\text{O}$) (see Section 2.2.1, Table 1).

2.2.1 Table 1 The pH of an aqueous 0.01 M solution of borax as a function of temperature (see: 'Standard methods for the examination of water and wastewater', 1971)

Temp. (°C)	pH
5	9.395
10	9.332
15	9.276
20	9.225
25	9.180
30	9.139
35	9.102

Report the temperature of the water in which the pH is measured. The temperature affects the position of the dissociation equilibrium in the water and also the glass electrode itself.

When the pH is measured with a glass electrode successively in different test media, care must be taken that the pH is measured first in the blank aquaria and subsequently in the aquaria with the lowest added concentrations of the test substance or substances, and so on. The aquaria with the highest concentration must be checked last!

2.2.3.4 Notes

- It must be borne in mind that calomel electrodes can give up mercury to the water.
- There is much misunderstanding concerning the concept of pH and its definition. On this, De Ligny's publication (1966) should be consulted.

2.2.4 Some remarks on measuring the concentration of dissolved oxygen in the test medium

INTRODUCTION

In the case of the biological experiments described in this report few parameters demand such attention as the dissolved oxygen content. The animals themselves naturally consume oxygen, but in addition ppm concentrations of a rapidly degradable test compound can drastically lower the oxygen content of the test medium, even in a flow-through test. Low oxygen contents influence strongly the course of toxicological experiments with aquatic organisms. It appeared to us, therefore, that it is absolutely necessary to devote close attention to monitoring the oxygen concentration of the test medium even in flow-through tests. The number of oxygen measurements may therefore easily amount to 100 per day (see also Section 2.7.1.4).

METHODS

In view of the large number of measurements per day, only one method is applicable in practice: the use of an 'oxygen electrode', two types of which are distinguished:

- 'electrodes' consisting of a polarographic measuring cell covered by a thin plastic membrane, the so-called 'Clarke electrode';
- 'electrodes' that consist of a galvanic cell covered by a thin plastic membrane.

We have had favourable experience with the first type; various makes are on the market (see Note).

The temperature has a strong influence on the oxygen concentration of the test medium and on the operation of the electrode (the oxygen permeability of the plastic membrane changes by about 5 % per °C).

NECESSARY PRECAUTIONS

- Whenever the amount of dissolved oxygen in the test aquaria is checked by means of an 'oxygen electrode', care must be taken to carry out the measurement first in blank aquaria, then in those with the lowest dosed concentration of the test substance or substances, and so on in succession, ending with the aquaria having the highest concentration.
- In any oxygen measurement the temperature of the test medium must be measured accurately and be reported.

NOTE

In the book by Golterman and Clymo (1969) it is reported that the galvanic cells covered by a membrane are more satisfactory than the Clarke cell. This is quite contrary to our experience, and is presumably illustrative of the developments of portable oxygen meters up to the present time.

2.2.5 Chlorides, salt content, and electrical conductivity

2.2.5.1 Introduction

The most important cations and anions in sea water, also known as the macro-components, occur in fixed mutual proportions (see Chapter 5). It is therefore possible to calculate the total salt concentration from the measured concentration of one ion or of a few ions together. The argentometric titration of **the sum of chlorides, bromides, and iodides** is the most practical method of those available.

The electrical conductivity of sea water can also be used as a measure of the total salt concentration. A good review has been given by Wallace (1974).

In view of the fact that the composition of the dissolved substances in sea water is fairly constant, the **specific gravity** of sea water can also be used as a measure of the salt content and thus of the concentrations of the separate ions. In fresh water there is no fixed ratio between the macro-components, so that both the measurement of the electrical conductivity and the measurement of density give only an over-all idea of the concentration of dissolved salts, while a measurement of the specific gravity is unsuitable for salt contents lower than about 2000 ppm. For brackish water a determination of the specific gravity can be useful to the extent that it gives a rough idea of the salt content.

The salt content, or better the 'salinity', denoted by *S*, is defined by international agreement in terms of the electrical conductivity:

$$S_{\text{‰}} = -0.08996 + 28.29720 R_{15} + 12.80832 R_{15}^2 - 10.67869 R_{15}^3 + 5.98624 R_{15}^4 - 1.32311 R_{15}^5$$

Here the dimensionless magnitude R_{15} is defined as the conductivity of the sample divided by the conductivity of water with a salinity of precisely 35‰. In the case of measuring instruments for oceanographic purposes, also called salinity meters, *S* can be read off directly. These are calibrated with so-called 'standard sea water' from the ICES in Copenhagen.

In this way the technical problems that arise in the measurement of the absolute value of conductivity are avoided.

On this, one should consult the publication by Johnston (1969) and with it the International Oceanographic Tables, and also the books by Strickland and Parsons (1960); Golterman and Clymo (1969); and Riley and Skirrow (1965, 1975).

The best approximation for the calculation of the chloride contents $[Cl^-]$ from the salinity *S* is given by the relationship

$$[Cl^-] = \frac{S}{1.80655}; ([Cl^-] \text{ and } S \text{ in } \text{‰}); \text{ see Cox } et al. (1967).$$

Toxicological experiments can be greatly affected by variations in the total salt content, and in fresh and brackish water also by variations in the ion ratios (see also Section 2.2.1).

A review of the various methods of determining the salt content of sea water was given by Johnston in 1964 and 1969.

2.2.5.2 Chloride content

A direct determination of the chloride ion is carried out in our laboratory only in the analysis of **fresh** water. Use is made of the Mohr titration. Naturally, the method can also be used for the investigation of sea water.

2.2.5.3 Salinity

In our laboratory the salinity of sea water is determined by means of an **electrical conductivity meter made especially for this purpose**. The salt content of sea water can also be checked with a hydrometer having a measuring range from 1.00 to 1.030. This method is particularly useful in the field and when the salt content has to be checked during the sampling of natural sea water. The correct use of hydrometers is not so simple as it may appear. Rands and Bigg (1961) have written a good monograph on this point. Conversion tables, including information on the temperature dependence, can be found in the books mentioned above.

2.2.5.4 Electrical conductivity

A conductivity meter suitable for the determination of the salinity of sea water in oceanographic investigations is a refined and expensive instrument. For measuring the conductivities of fresh water and sea water in the tests described in this book a cheap and simple type will prove adequate. The electrical conductivity of fresh water naturally depends on the salt content but, because

2. Analysis of the investigation of degradability, toxicity, and accumulation

of the variable ion ratios, it does not give such unambiguous information as in the case of sea water. For this reason we use this measurement exclusively to check the purity of demineralized and distilled water.

The official SI unit of conductivity is S.m^{-1} (Siemens per metre) (see Note 2).

2.2.5.5 Notes

1. In the measurement of the electrical conductivity and the specific gravity the temperature dependence of the results must naturally be taken into account.
2. Various units can be found in the literature for specifying the 'electrical conducting capacity' or 'electrical conductivity'. The origin of these various units can be summarized briefly as follows. The specific resistance of an electric conductor is the resistance of a specimen having a unit length and unit cross-section area. In older handbooks these are respectively 1 cm and 1 cm^2 , and the specific resistance or resistivity is expressed in ohm.cm ($\Omega\text{.cm}$). The conductivity is the reciprocal of the resistivity, and is then given in $\text{ohm}^{-1}\text{.cm}^{-1}$. Ohm^{-1} is also written as: mho, Siemens, and even Ω . These give rise to the units of mhos/cm, Siemens/cm, and Ω/cm . In the case of fresh water the conductivity is low and it is therefore expressed in $\mu\text{mhos/cm}$, $\mu\text{Siemens/cm}$, and $\mu\Omega/\text{cm}$.
3. When the electrical conductivities are measured with an immersion electrode successively in various test aquaria, care must be taken that the measurements are performed first in the blank aquaria and subsequently in the aquaria with the lowest dosed concentrations of the test substance or substances, and so on. The aquaria with the highest concentration must be checked last.

2.2.6 Some remarks on checking the water of the test medium for organic chlorine compounds

The water that is to be used as the test medium itself or for the preparation of test media must be checked for the possible presence of organic chlorine compounds. The **prepared** test medium must also be analysed for these substances. If there is a large amount of such compounds in the water, or if one or more is present in unacceptably high concentrations, the water must not be used as a test medium.

In this context 'unacceptably high' means of the same order (or greater) as that of the model substance.

1 litre of water is extracted with 5 ml of cyclohexane, if possible in duplicate, as laid down in Section 2.4.2.3.

If the model substance(s) for which the water will be used is (are) known, special attention must be paid to the possible previous presence in the water of this (these) substance(s) or of substances that have the same retention time in the gas chromatogram. This means that methylation, acetylation, or bromination may have to be used.

If it is necessary, for example, both to acetylate and to brominate, it is wise to acetylate one sample in duplicate and to brominate another in duplicate.

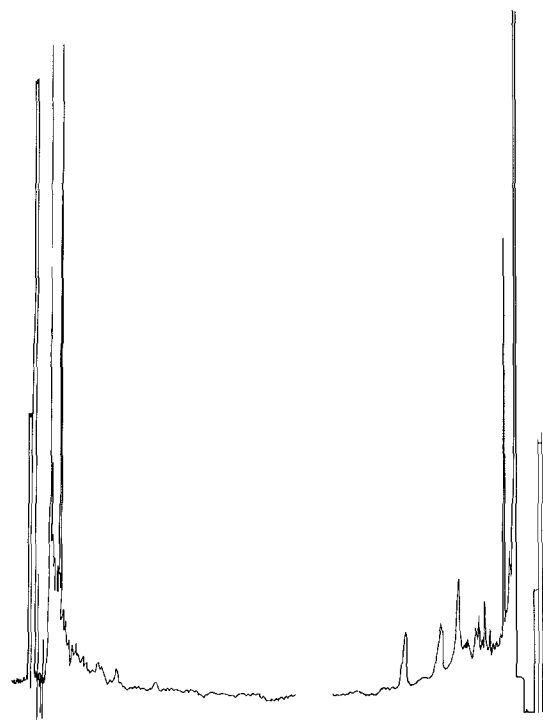
It goes without saying that, in addition to the samples, low dosed concentrations (i.e. $0.01\text{--}0.05 \mu\text{g.kg}^{-1}$) of the various compounds must be determined to establish their retention time and possible concentration in the water.

If there is a suspicion that a certain compound is present (for example because of a characteristic odour), particular attention can be paid to this compound by selecting gas-chromatographic conditions suitable for such a substance and also by determining standards.

The extracts from the water must be chromatographed on the same column as the samples from the biological tests. A blank of the hexane used for the extraction is also chromatographed. Furthermore, the column must be programmed from 50 to 200°C (or be run at constant temperatures of 50, 100, 150, and 200°C).

In addition, a toxicity test is carried out on the water with *Artemia* or *Daphnia* (see Sections 5.7.4).

It is thus possible to obtain an idea of the quality of the water so far as concerns the presence of a number of organic chlorine compounds.



2.2.6 Fig. 1 Example of a 'clean' test medium (left)

2.2.6 Fig. 2 Example of a 'dirty' test medium (right)

Limitation of the method

Attention is paid particularly to the presence of those compounds with which tests are to be performed.

Derivatives are made only of certain types of compounds

It is therefore possible that some compounds will escape attention. Above all, the electron capture detector is not sensitive to all compounds.

An example of a 'clean' test medium is given in 2.2.6, Fig. 1, and of a 'dirty' test medium in 2.2.6, Fig. 2.

2.3 METHODS FOR THE DETERMINATION OF GROUPS OF SUBSTANCES

2.3.1 Determination of the total organic carbon (TOC) in water

Subject

Determination of the amount of organic substances in water, expressed in mg of carbon per litre of water.

Field of application

The TOC determination can be performed in fresh water and in sea water in a range of concentrations from 1 to 1000 mg.l⁻¹ (see Note 1). In principle, the method has been designed for homogeneous solutions, but it can also be applied to fine homogeneously distributed suspensions and emulsions.

Principle

The organic constituents in a sample of water are oxidized catalytically at a high temperature. The carbon dioxide so formed is determined. The TOC is then calculated from a calibration curve determined with potassium biphthalate as the standard. It is corrected for the content of inorganic carbon (carbonate) by determining this separately or by blowing out the carbon dioxide formed after acidification.

EQUIPMENT AND MATERIALS

- TOC analyser (Note 2)
- potassium biphthalate (primary standard)
- sodium carbonate (analytical grade)
- sodium bicarbonate (analytical grade)

For the preparation of calibration solutions:

- analytical balance (accurate to 0.1 mg)
- carbon-dioxide-free water
- carbon-free water
- volumetric flasks and pipettes (depending on the desired concentration range).

For storing samples and calibration solutions:

deep-freeze cabinet (−20°C or −35°C).

INTRODUCTION

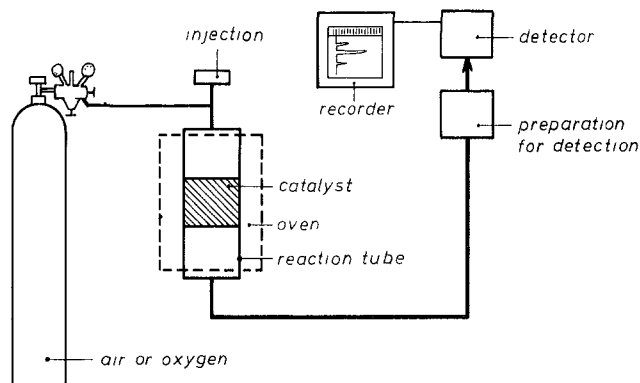
A TOC determination may consist of the following steps (Arin, 1974):

- injection of the sample;
- catalytic combustion of the organic material;
- pre-treatment of the combustion products for detection;
- detection and recording.

2.3.1 Fig. 1 shows the basic scheme of a TOC analyser.

So far as concerns the various types and brands of commercial apparatus, the following observations may be made:

- The introduction of the analytical sample into the apparatus can be carried out with an injection syringe or by means of a small-boat from which the sample is volatilized in a separate small oven and passes with the current of gas to the catalyst. A peak



2.3.1 Fig. 1 Basic scheme of a TOC analyser

is recorded whose height is proportional to the concentration of organic material in the sample (2.3.1, Fig. 2A) (peaks of different amounts and different substances have similar shapes). In addition, there are systems in which a constant stream of the sample is fed in for some time (Croll, 1972), so that a definite level (in the recording) is reached, which is proportional to the amount of organic substance in the sample (2.3.1, Fig. 2B).

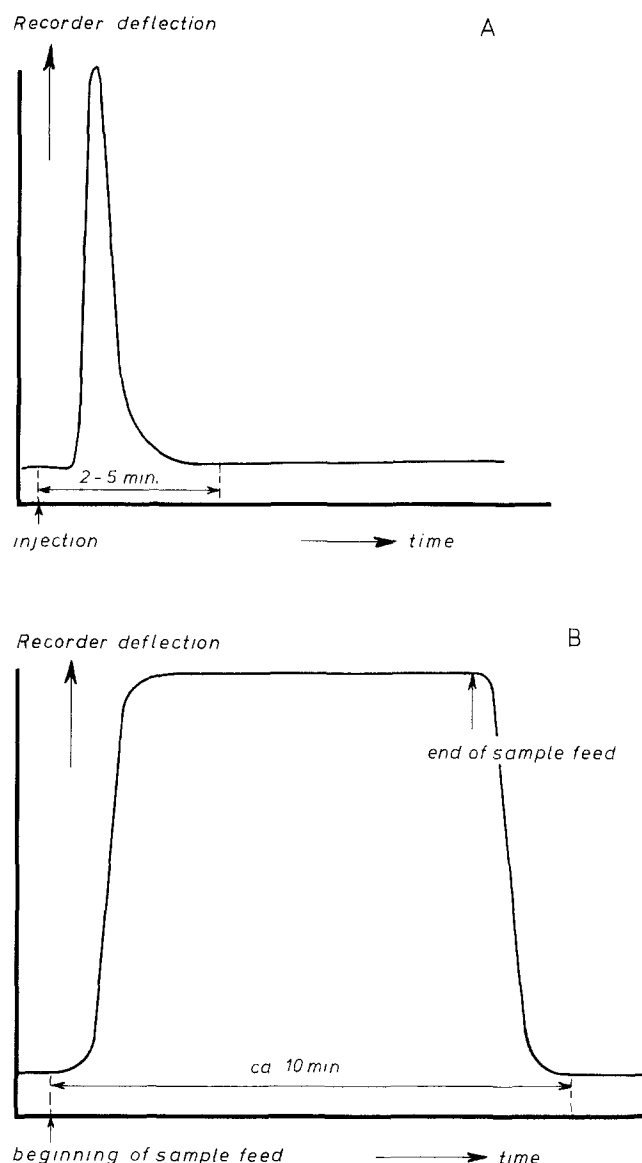
The advantages of the second method are the possibility of 'averaging out noise' and of automating the apparatus. However, account must be taken of the fact that it requires a larger amount of sample than the first method (10–15 ml instead of 1 ml) and that the determination takes longer.

- In all types of analysers the sample is burnt with the aid of an oxidation catalyst (cobalt oxide, palladium, copper oxide, etc.) at a temperature of 900–1000°C, so that all the carbon is oxidized to CO₂.
- The combustion products must be pre-treated in a manner that depends on the method of detection. The necessary pre-treatments are described below for the relevant detectors.
- In the detection of combustion products, in general, use is made of an infrared or a flame-ionization detector. In the former system it is sufficient to condense the water formed in combustion and to trap it. The carbon dioxide is then determined directly. For the flame-ionization detector the CO₂ formed must be converted into methane with the aid of a catalyst and hydrogen. The advantage of this method is that this detector has a low detection limit. On the other hand, the sensitivity of the hydrogenation catalyst to sulphur compounds, for example, is a drawback.

The TOC determination also includes the inorganic carbon (carbonate), since carbonates decompose at 900°C into oxide and CO₂. In order to correct for this, a sample is acidified and the resulting CO₂ is blown out (Van Hall *et al.*, 1965), or the carbonate carbon is determined separately. The determination is similar, but now the reaction tube is held at 150°C and is filled with an 'acid catalyst.' The determination of TOD (total oxygen demand) is closely related to the TOC determination. In the TOD determination the sample is placed in a reaction tube and is oxidized in a current of gas. Under these conditions carbon, hydrogen, nitrogen, sulphur, and sulphite are converted into carbon dioxide, water, nitrate, sulphate, and again sulphate, respectively. The oxygen content of the gas stream is measured before and after combustion. The TOD of the sample is calculated from the difference.

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Carbonates make no contribution to the TOD. Hydrogen, nitrogen, sulphur, etc. do not contribute to the TOC determination, since only CO_2 is detected.



2.3.1 Fig. 2

A. Recorder pattern with sample injection

B. Recorder pattern with constant sample feed

PROCEDURE

- Set up and calibrate the TOC analyser according to the instructions from the manufacturer.
- Prepare stock solutions for the calibration of the TOC analyser.
 - Prepare a stock solution for the calibration of the determination of the organic carbon content by weighing 2.125 g of anhydrous potassium biphthalate (primary standard grade) into a volumetric flask (1 litre) and dissolving it in CO_2 -free water (Note 3). After being made up to 1000 ml, this solution contains 1000 mg of organic carbon per litre.

- Prepare the stock solution for the calibration of the determination of the inorganic carbon content by weighing 4.404 g of anhydrous sodium carbonate (analytical grade) into a volumetric flask (1 litre) and dissolving it in about 0.5 litre of CO_2 -free water. Next, dissolve 3.497 g of anhydrous sodium bicarbonate (analytical grade) in the same solution and make up the volume to 1000 ml. The solution then contains 1000 mg of inorganic carbon per litre.

- Prepare the calibration solutions by diluting the stock solution (Note 4).

The calibration solutions for the determination of organic carbon must preferably be prepared with carbon-free water (Notes 5 and 6).

The calibration solutions for the determination of inorganic carbon must be prepared with CO_2 -free water (Note 3).
- Plot calibration curves for the determination of the organic and the inorganic carbon contents (Note 7).

The calibration curve must consist of a minimum of five points, which must be so chosen that the carbon contents of the sample are within the range of the calibration curve.

Determine the carbon content of the dilution water as well. Correct the points of the calibration curves by subtracting the peak heights for the dilution water from the peak heights of the measured points.

Construct the calibration curve by plotting the corrected peak heights (y-axis) against the concentrations of the calibration solutions (x-axis).

The calibration curve corrected in this way must pass through the origin (Note 4).
- Determine the inorganic carbon content of the sample or eliminate the carbonates (Note 7).
- Determine directly the total organic carbon content of the sample from which the carbonate has been eliminated.
- Determine the total carbon content of samples that contain carbonates and calculate the total organic carbon content by subtracting the inorganic carbon from the total carbon (Note 8).
- The use of calibration curves is described in Section 2.4.1.

NOTES

- The detection limits depend on the type of the apparatus. Usually the lower limit of detection is about 1 milligram per litre. However, there are TOC analysers with a lower detection limit. When TOC determinations are carried out in sea water, it may be necessary to prepare calibration solutions containing an amount of common salt (analytical grade) agreeing with that of sea water. This depends on the apparatus used.
- By the TOC analyser is meant the analyser and the apparatus necessary for introducing the sample into the oven. This may be an injection syringe, or a peristaltic pump with a sequential sampler, or the like.
- Carbon-dioxide-free water can be obtained by boiling distilled water in a conical flask for 20 min. Close the conical flask with a stopper provided with a 'calcium chloride tube' filled with soda-lime as the CO_2 absorber and let the boiling water cool. The water can be stored in glass bottles if these are well sealed.
- The response of the TOC analyser is frequently not quite proportional to the organic-material content of the sample. It is therefore recommended to choose the strength of the calibration solutions in such a way that these agree as far as possible with those of the samples to be determined.

Calibration solutions of potassium biphthalate are sensitive to biological degradation. After preparation, they should be stored

2.3 Methods for the determination of groups of substances

in portions of a few millilitres in a deep-freeze cabinet so that a 'new' solution can be used for each calibration.

- Water free from organic carbon can be prepared by redistilling distilled water after the addition of an alkaline permanganate solution and once more after the addition of dichromate and acid (Montgomery and Thom, 1962).

This water contains less than 0.1 mg of organic carbon per litre.

- The dilution water for the calibration solution must always be prepared in the manner described under 5 if the samples to be investigated have an organic carbon content lower than about 4 mg.l⁻¹. The correction of the calibration curve for the carbon content of the dilution water is then of the same order of magnitude as the carbon content of the samples. This can lead to relatively large errors.

- Various makes of TOC analysers are not suited for the determination of inorganic carbon. In these cases one can, of course, make do with a calibration curve for the determination of the organic carbon content.

The inorganic carbon (carbonate) can then be eliminated by Van Hall's method (1965) or by acidifying the sample to pH 1 and then blowing out the CO₂ with a stream of CO₂-free air for about 1 min.

However, it must be borne in mind that in the last-mentioned method **volatile organic compounds may also be lost**.

- When the inorganic carbon content is high in relation to the organic carbon, it is recommended to eliminate the carbonates before the determination (Note 7). In other words, the error in the total organic carbon may become relatively high because one large number has to be subtracted from another large number.

2.3.2 Dry weight/wet weight and ash weight/dry weight ratios

Subject

Determination of the dry weight/wet weight and ash weight/dry weight ratios in samples of biological tissue.

Field of application

The method is suitable for samples of biological tissue weighing about 1 gram.

Principle

The tissue is dried at 110°C to constant weight and is then ashed at 600°C to constant weight.

EQUIPMENT

- towel or filter paper
- scalpel
- tweezers
- spatula
- felt-tip pen
- porcelain dishes 4 cm in diameter and 1 cm high.
- desiccator (with silica gel as the desiccant)
- drying oven, 110°C
- oven, 600°C
- top-weighing balance, accurate to 0.01 g
- analytical balance, accurate to 0.1 mg, with a 50-ml beaker containing dry silica gel in the balance cabinet
- homogenizer (e.g. Ultra Turrax).

PROCEDURE

Preparation of the tissue sample

- Dry the animal with the towel or filter paper
- Dissect out the desired tissue; more than 1 gram is required.
- Homogenize the sample with the homogenizer.

Analysis of the tissue sample

Dry weight of the porcelain dish (= g₀).

- Dry the coded porcelain dish for 24 h at 110°C
- Allow the dried dish to cool in the desiccator for 1 h
- Weigh the dish to within 0.1 mg
- Repeat the drying (now for 1 h), cooling, and weighing, until the change in weight is less than 0.5 mg.

Dry weight of the tissue sample

- Weight about 1 gram of tissue with an accuracy of 0.01 g into a dish that has been dried to constant weight (g₁).
- Dry the tissue at 110°C for 24 h.
- Allow the sample to cool in a desiccator for 1 h.
- Weigh the dish with the sample to within 0.1 mg on the analytical balance.
- Repeat the drying, cooling, and weighing until constant weight (g₂) has been achieved (see Note 1).

Ash weight of the tissue sample

- Heat the dish containing the dry tissue (weight g₂) in an oven for 1 h at 600°C
- Allow the sample to cool in a desiccator for 1 h.
- Weigh the dish containing the ashed sample to within 0.1 mg.
- Repeat the ashing, cooling, and weighing until a constant weight (g₃) has been obtained (see Note 1).

TREATMENT OF THE MEASUREMENTS

Calculation of the ratio $\frac{\text{dry weight}}{\text{wet weight}} = \frac{g_2 - g_0}{g_1 - g_0}$

Calculation of the ratio $\frac{\text{ash weight}}{\text{dry weight}} = \frac{g_3 - g_0}{g_2 - g_0}$

NOTE

If the last and last but one weighings differ by not more than 0.5 mg, constancy of the weight is assumed. The last weighing is taken.

2.3.3 Lipid content (colorimetric method)

Subject

Vanillin determination of the total lipid content in samples of biological tissue I.

Field of application

The method is suitable for samples of biological tissue weighing about 100 mg (see Note 4) and for lipid contents of 1% and higher (see Note 1).

2. Analysis of the investigation of degradability, toxicity, and accumulation

Principle

On heating with concentrated sulphuric acid, phosphoric acid, and vanillin, lipids form pink-red compounds whose colour intensity is in principle proportional to the lipid concentration (see Barnes and Blackstock, 1973).

EQUIPMENT AND MATERIALS

Glassware

- round-bottomed flasks, 25 ml, with ground-glass stoppers (the ground joint must fit the freeze-drying apparatus)
- glass rod
- homogenizing tube and pestle
- funnels, dia. 3 cm
- test tubes
- Pasteur pipettes, with suitable teats
- glass bottles, 1 l, with ground-glass stoppers
- volumetric flasks, 10 ml
- measuring cylinders, 500 ml
- volumetric pipettes, 1 ml and 2 ml
- graduated pipettes, 10 ml and 5 ml

Various auxiliaries

- towel or filter paper
- Whatman No. 1 filter paper
- scalpel
- tweezers
- weighing paper
- spatula
- felt-tip pen
- closable tubes, ca. 5 ml
- pipetting bulb
- cotton wool
- thermometers, 0–110°C; –100–0°C
- water bath, 100°C
- Dewar vessel
- stirrer motor
- plunger pipettes, 100 µl and 1000 µl, with disposable tips
- homogenizer (e.g. Ultra Turrax)
- freeze-drying apparatus
- deep-freeze cabinet (–20°C)
- colorimeter suitable for measurements at 520 nm, with associated circular cells, 1 cm
- analytical balance, accurate to 0.1 mg
- fume cupboard.

Reagents

- solid CO₂
- acetone (analytical grade)
- chloroform (analytical grade)
- methanol (analytical grade)

- ethanol (analytical grade)
- H₂SO₄, concentrated
- E₁ (chloroform-methanol 1/1)
- Boehringer Biochemica test kit, Cat. No. 15991 (Total Lipids). This test kit consists of two solutions: BBT-1 contains 10 g of total lipids/l ethanol; BBT-2 contains 14 M phosphoric acid and 13 mM vanillin. The composition of the extraction liquid E₂ is chloroform-methanol 2/1 (v/v).

INTRODUCTION

The Boehringer Biochemica test kit has been developed for determinations of the total lipids in blood serum. In general, serum has a fairly constant composition so far as concerns the lipids. However, the various types of lipids have different colour yields. Some examples are given below:

standardized to cholesterol, the figures are:

cholesterol	1.00
triolein	1.27
stearic acid (18:0)	0.15
oleic acid (18:1)	1.48
linoleic acid (18:2)	1.23
linolenic acid (18:3)	0.93

Since the composition of the lipids in the sample is rarely known precisely, it is important to compare the results of the colorimetric methods with those of the gravimetric method (as described in Section 2.3.4) for a biological tissue. If necessary, a correction factor can be determined in this way, which is applicable to long series of closely related samples.

PROCEDURE

Although this procedure is described for one sample, in practice the analytical blank, the calibration points, and a large number of samples are treated together. Accurate coding of all flasks, test-tubes, etc. is then indispensable.

Preparation of the tissue sample (see Notes 2, 3, and 4)

- Dry the animal with a towel or filter paper
- Dissect out the desired tissue
- Homogenize the tissue with the homogenizer.

Freeze-drying of the tissue sample (see Note 7)

- Weigh about 100 mg of the tissue to within 0.1 mg in a previously weighed 25-ml round-bottomed flask (see Note 4).
- Cool this flask in CO₂/acetone to –60°C.
- Place the flask in the previously adjusted freeze-drying apparatus.
- Freeze-dry the sample over about 4 h.
- Weigh the flask and determine the freeze-dried weight.
- Crush the sample finely with a glass rod on a piece of weighing paper.
- Transfer the sample to a 5-ml closable tube.
- Keep this tube at –20°C until the analysis is to be performed.

Analysis of the sample (see Note 6)

- Weigh 10 mg of 'freeze-dried' tissue to within 0.1 mg into a homogenizing tube.

2.3 Methods for the determination of groups of substances

- Add 8 ml of E_1 with a graduated pipette.
- Homogenize this mixture (with a pestle and a stirrer motor).
- Filter the homogenate through Whatman No.1 filter paper previously moistened with E_1 into a 10-ml volumetric flask.
- Rinse the filter with E_1 .
- Make up the volume in the flask to 10 ml with E_1 .
- With a volumetric pipette, transfer 1 ml from the volumetric flask into a test-tube (see Note 1).
- Evaporate off the mixture of chloroform and methanol in a current of nitrogen.
- With a plunger pipette add 1 ml of concentrated sulphuric acid (see Note 5).
- Close the tube with a plug of cotton wool.
- Heat the test-tube in a water-bath for 10 min at 100°C.
- Cool the tube to room temperature.
- With a plunger pipette, transfer 0.1 ml from this test-tube into the 1-cm cell (see Note 5).
- Using a graduated pipette, add 2.5 ml of BBT-2 (see Note 5).
- After 30 min (at room temperature) measure the extinction $\left(= \log \frac{1}{I_0} \right)$ at 520 nm against the blank.
- Read the corresponding concentration off the calibration curve.

Blank determination (analytical blank)

- Using a graduated pipette, transfer 8 ml of E_1 into a homogenizing tube.
- Follow 3.3 from homogenization onwards.

Determination of the calibration curve

- Make a dilution series from BBT-1 in concentrations from 200 to 1000 μg of lipids/ml ethanol.
- Pipette 1 ml into the test-tubes (see Note 1).
- Evaporate off the ethanol in a current of nitrogen.
- Follow 3.3 from the addition of concentrated sulphuric acid onwards.
- Plot $\log \frac{1}{I_0}$ against the standard concentrations.

TREATMENT OF THE RESULTS

- Calculate the freeze-dried/wet weight ratio of the sample.
- With the aid of the calibration curve calculate the total lipid content of the samples.

Example

Freeze-dried weight/wet weight ratio = 0.30. The prepared tissue homogenate in chloroform/methanol was: 10 mg of tissue/10 ml of chloroform/methanol. 1 ml of extract was used. The extinction was 0.32.

Calculation

$0.32 = 300 \mu\text{g lipid/ml extract}$
(according to the calibration curve)
 $1 \text{ ml extract} = 1 \text{ mg freeze-dried weight (see above)}$

} $\rightarrow 300 \mu\text{g lipid/mg freeze-dried tissue}$

or: 0.09 g lipid/g wet tissue (= 9 %)

NOTES

1. It is possible to make the determination of total lipids suitable for contents of about 1 %. For this purpose procedure (3) must be somewhat modified. In place of 1 ml, in this case 2 ml must be pipetted into the test-tube. The consequence is that, after evaporation, the total lipid concentration in the sulphuric acid solution is twice that in the chloroform/ethanol solution. The content can be determined with the aid of a suitable calibration curve (25–200 μg of total lipids/ml).
2. If the sample to be analysed is highly perfused with blood, for example in the case of liver tissue, the tissue should previously be freed from blood. This considerably reduces the errors of measurement.
3. In addition to the determination of total lipids, other analyses are frequently carried out on the same organ. For a subsequent determination of enzyme activities it is important that the organ tissue should be kept cool (about +4°C). It is then impossible to homogenize the tissue previously with the Ultra Turrax homogenizer (development of too much heat). In this case a sample of the organ as representative as possible is taken with a scalpel, and this is subsequently freeze-dried and weighed out for the lipid determination. The remainder of the organ can be used for other determinations.
4. The amount of sample necessary for a determination of the total lipids depends on the freeze-dried weight. At least 30 mg of freeze-dried tissue is necessary for a determination in duplicate. The required amount of the sample can be calculated from the freeze-dried weight.
5. The phosphoric acid – vanillin reagent and the sulphuric acid are very viscous and must therefore be pipetted far down in the tube (close to the liquid surface).
6. Detergents interfere with the determination. Contamination of the apparatus, glassware, etc. with fats must be avoided.
7. The sample need not be freeze-dried if the extraction method described in 'Determination of the lipid content in tissue samples II' (Section 2.3.4) is used. The moisture is taken up by the methanol/chloroform mixture in a single-phase system. Since with small samples (about 100 mg) the weighing of the residue is too inaccurate, the total lipids are determined colorimetrically (with the aid of the phosphoric acid – vanillin reagent).

2.3.4 Lipid content (gravimetric method)

Subject

Determination of the lipid content in samples of biological tissue II.

Field of application

The method is suitable for both fresh and previously freeze-dried samples of biological tissue. The size of the sample required is about 1 gram.

Principle

The total lipid content is determined by weighing the lipids extracted from the tissue.

In principle, the method of Folch *et al.* (1957) and of Böttcher *et al.* (1959) is followed, with a number of improvements.

EQUIPMENT AND MATERIALS

Glassware

- centrifuge tubes, 15 ml, with ground-glass stoppers
- centrifuge tubes, 200 ml

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- conical flasks, 50 ml, with ground-glass stoppers
- beakers, 50 ml
- glass bottles, 1 l, with ground-glass stoppers
- sealable pots, 50 ml
- measuring cylinders, 500 ml
- graduated pipettes, 10 ml
- Kipp automatic metering devices, 20 ml, 10 ml, 5 ml with matching conical flasks, 250 ml.

Various auxiliaries

- towel or filter paper
- scalpel
- tweezers
- spatula
- felt-tip pen
- pipetting bulb
- top-weighing balance, accurate to 0.01 g
- analytical balance, accurate to 0.1 mg
- homogenizer (e.g. Ultra Turrax)
- vacuum desiccator
- vacuum pump (to 10 mm Hg)
- centrifuge
- fume cupboard.

Reagents

- ethanol
- chloroform (stabilized with 2 % of ethanol)
- methanol
- distilled water
- cylinder of nitrogen
- three extraction liquids with the following compositions in parts by volume:
 - E₁: chloroform-methanol 1/1
 - E₂: chloroform-ethanol 2/1
 - E₃: methanol-chloroform-water 48/5/47.

PROCEDURE

Preparation of the tissue sample (see Note 2)

- Dry the animal with a towel or filter paper.
- Dissect out the desired tissue; more than 1 gram is necessary.
- Homogenize the sample with the homogenizer.
- Store the homogenized tissue at -20°C until the analysis is performed.

Analysis of the tissue sample

- Weigh about 1 g of tissue with an accuracy of 0.01 g into a 15-ml centrifuge tube.

1st extraction

- With a Kipp automatic metering device, add 10 ml of E₁ and mix.
- Allow to stand at room temperature for 3 h.

- Centrifuge at about 1100 *g* for 15 min.
- Decant off the supernatant liquid into coded 50-ml collecting conical flasks with ground-glass stoppers.

2nd extraction

- With a Kipp automatic metering device, add 10 ml of E₂ to the sample in the centrifuge tube and mix.
- Allow the whole to stand at room temperature for 24 h.
- Centrifuge at about 1100 *g* for 15 min.
- Decant off the supernatant liquid into coded collecting conical flasks.

3rd extraction

- Repeat the 2nd extraction.
- Empty each coded conical flask into a correspondingly coded 200-ml centrifuge tube.
- Then rinse out each conical flask with 5 ml of chloroform.
- Add 8 ml of distilled water to each centrifuge tube and mix.
- Centrifuge at 1100 *g* for 15 min.
- Remove the uppermost water layer with a pipette.
- With a Kipp automatic metering device, add 20 ml of E₃ to the lowest layer and mix.
- Centrifuge at about 1100 *g* for 15 min.
- From each centrifuge tube transfer the lowest layer to a correspondingly coded 50-ml beaker (see Note 3).
- Evaporate off the chloroform under a current of nitrogen.
- Place the beakers with the residues in a vacuum desiccator (pressure 10 mm) for 20 min.
- Weigh the beakers with the residues.

TREATMENT OF THE MEASUREMENTS

The total lipid content as a percentage is $100 \times$ the weight of the residue divided by the initial weight of the tissue sample.

NOTES

1. The determination of the total lipids in tissue sample I can be used if the sample is smaller than 1 gram (see Section 2.3.3).
2. If the sample to be analysed is highly perfused with blood (for example liver tissue), the tissue must first be freed from blood to prevent errors in the weighing out of the sample.
3. The 50-ml beakers are placed in a vacuum desiccator (pressure = 10 mm) for 20 min and are then weighed.

2.3.5 Total protein content

Subject

Determination of the total protein content in biological tissue samples.

Field of application

The method is suitable for samples of biological tissue weighing about 100 mg.

Principle

In alkaline solution proteins form with copper ions a blue complex

2.3 Methods for the determination of groups of substances

whose colour intensity is proportional to the protein concentration (biuret method).

EQUIPMENT

Glassware

- Volumetric flasks, 1 l
- homogenizing tube and pestle
- measuring pipettes, 5 ml
- test tubes.

Various auxiliaries

- towel or filter paper
- scalpel
- tweezers
- spatula
- felt-tip pen
- plunger pipettes, 100 μ l and 5 ml, with disposable tips
- stirrer motor
- analytical balance, accurate to 0.1 mg
- (bench) centrifuge suitable for 1500 g
- colorimeter, suitable for measurements at 546 nm, with associated circular 1-cm cells
- pH meter.

Reagents

- 5×10^{-2} M Tris-HCl buffer, pH 7.4.
Weigh 24.2 g of aminotris (hydroxymethyl) methane into a 1-l volumetric flask and make up to 1 l with distilled water (solution A).
Introduce about 500 ml of distilled water into a 1-l volumetric flask, add 17 ml of concentrated hydrochloric acid (sp. gr. 1.19), and make up to 1 l with distilled water (solution B).
Transfer 250 ml of solution A and 207 ml of solution B into a 1-l measuring flask and make up to 1 l with distilled water.
Measure the pH of the last-mentioned solution.
If necessary, adjust the pH to 7.4 (with solution A or B).
- Homogenization liquid.
Weigh 85.5 g of sucrose and 372.2 mg of EDTA into a 1-l volumetric flask and make up to 1 l with 0.05 M Tris-HCl buffer.
- Boehringer Biochemica test kit.
Cat.No. 15899 (Total Protein) (see also the accompanying manual).

This test kit contains three solutions, coded in the following way:

BBT-1, BBT-2, and BBT-3.

BBT-1 contains 0.1 M NaOH, 16 mM Na-K tartrate, 15 mM KI, and 6 mM copper sulphate.

BBT-2 contains 0.1 M NaOH and 16 mM Na-K tartrate.

BBT-3 contains 6 g protein/100 ml (= standard solution).

PROCEDURE

Determination of the calibration curve for total protein (see Note 2)

- Make a dilution series of 2–6 g of protein/100 ml from BBT-3.

- With a plunger pipette, transfer 5 ml of BBT-1 into the 1-cm cells
- With a plunger pipette add 0.1 ml of one of the standard solutions (of the dilution series) to each cell.
- Allow this mixture to stand at room temperature for 30 min.
- Centrifuge the cells at 1500 g for 5 min.
- Measure the extinctions $\left(= \log \frac{I}{I_0} \right)$ at 546 nm against the blank (= BBT-1).
- Plot the measurements so obtained with the protein content along the x-axis and $\log (I/I_0)$ along the y-axis. Draw the calibration curve through the measurement points.

Preparation of the tissue sample (see Note 1)

- Dry the animal with a towel or filter paper.
- Dissect out the desired tissue.
- Weigh about 100 mg of tissue with an accuracy of 0.1 mg into a homogenizing tube.
- Add 3 ml of homogenization liquid per gram of tissue.
- Homogenize this mixture (with pestle and stirrer motor).

Analysis of the tissue sample

- With a plunger pipette, transfer 5 ml of BBT-1 into each of a number of cells.
- With a plunger pipette, transfer 0.1 ml of the homogenate prepared as in 2.2 into each 1-cm cell and mix.
- Allow this mixture to stand at room temperature for 30 min.
- Centrifuge the cells at 1500 g for 5 min.
- Measure the extinction $\left(= \log \frac{I}{I_0} \right)$ at 546 nm against BBT-1.
- Determine the protein concentration with the aid of the calibration curve.

TREATMENT OF THE MEASUREMENTS

With the aid of the calibration curve calculate the total protein content of each sample.

Example

The extinction was 0.20, starting with a homogenate containing 25% (w/w) of tissue.

Calculation:

$$0.20 = 3.4 \text{ g protein/100 ml homogenate (according to the calibration curve)} \left. \begin{array}{l} \\ \\ \end{array} \right\} \begin{array}{l} \text{protein} \\ \text{content} \\ \approx 13.6\% \end{array}$$

100 ml of homogenate contains 25 g of tissue

NOTES

1. If the sample to be analysed is highly perfused with blood (for example liver tissue), the tissue must previously be freed from blood to prevent errors in the weighing out.
2. If the sample has a protein content greater than 45% it must be diluted beforehand, since otherwise the amount of reagent specified may prove insufficient.

2.4 METHODS FOR THE DETERMINATION OF INDIVIDUAL TEST COMPOUNDS IN WATER

2.4.1 General

2.4.1.1 Introduction

This fourth part of Chapter 2 deals with the methods developed at TNO Division of Technology for Society for the determination of test compounds in fresh water, brackish water, and sea water. In this part the analytical procedure is discussed in detail; the separate sets of instructions given later therefore remain short and simple*.

Attention is devoted successively to:

- Grouping of the samples and administration.
- Treatments preceding the gas-chromatographic analysis.
- Analysis of the extracts obtained.
- Preparation of standard solutions and standards for gas chromatography.
- A general review of the requirements for glassware (Appendix 1), calibrated glassware (Appendix 2), auxiliary apparatus (Appendices 3 and 4), and reagents (Appendix 5) in tabular form with explanation.

The methods used for the determination of the test substances in water and organisms differ slightly from those found in the literature on residue analysis. This is due to the following facts.

In general, it is not necessary to determine more than one compound; the concentration of the compound to be determined is never lower than $0.1 \mu\text{g.l}^{-1}$ in water and rarely lower than 0.1 mg.kg^{-1} in animal tissue. The analysis is therefore less difficult than that of field samples, in which the concentrations are usually two or three orders of magnitude lower and the number and concentration of interfering substances are much higher. This means that the clean-up of the samples can be simplified.

The samples to be analysed usually must and can be of a limited size, e.g. not more than 1 l of water and a few grams of animal tissue.

The number of samples to be analysed amounts to a few hundred water samples and 40-50 tissue samples per week. For efficient planning of accumulation-elimination experiments, the analytical results must be available within 24 h.

These circumstances have made it necessary to carry out a far-reaching streamlining of the analytical procedures, which has fortunately been possible because of the fairly high concentrations and the fact that in every case only one substance is involved. This streamlining involves automation and mechanization where this is possible in a simple manner, the avoidance of complicated treatments and limitation of the number of treatments, the replacement of time-consuming treatments by rapid ones, limitation of contact with glassware as far as possible, etc. Various simple auxiliary devices not commercially available had to be constructed for this purpose.

One of the least reliable treatments in residue analysis is the evaporation of an extract down to a small volume. Major losses frequently occur in this process. However, because of the fairly high concentrations to be determined this treatment could almost invariably be omitted.

Extractions are carried out only once, with the smallest possible volume of the extraction solvent; this is contrary to the process frequently described in the literature, in which, for example, the same sample of water is extracted three times with fairly large amounts of the same extraction liquid. It has been found and can be calculated that there is frequently little point in carrying out

the last-mentioned procedure for readily extracted compounds: it causes more errors than it eliminates and is very time-consuming. It was not necessary to use clean-up on adsorption columns (alumina, silica gel, or Florisil). Such clean-up is very time-consuming and frequent standardization of the adsorbent is needed. It must be emphasized that, for the analysis of field samples, this method of cleaning up the samples generally cannot be omitted.

For quantitative work with a relative error of measurement of about 10% it is permissible to measure out with Kipp automatic metering devices, plunger pipettes with disposable tips, and measuring cylinders, while in the quantitative evaluation of gas chromatograms the **peak height** can be used.

A relative measurement error of about 10% is quite adequate for the aim pursued here. Furthermore, it is known from the theory of process control that the performance of a somewhat larger number of relatively more inaccurate but simple analyses is often more successful than the devotion of much labour to a smaller number of very accurate analyses, if the latter are possible at all. Each set of instructions states the range of concentrations and the size of the sample for which it is designed. It is possible to deviate from these within reasonable limits, provided that the lowest detection limit, which can be calculated from the figures given for the size of the sample and the concentration, is taken into account.

For the reasons given above, the sets of instructions given here are not directly applicable to the analysis of field samples. However, they constitute a good starting point for the development of methods for the determination of related materials.

The publications upon which the development of the procedures was based are mentioned with each procedure, and our own contributions to the final procedures are substantiated in other reports.

All the methods described, and especially those for the analysis of animal tissue, have been checked in complete model experiments with radioactively labelled compounds. They have not been published elsewhere. We therefore thought it useful to give the experimental details as completely as possible. Every experienced analytical chemist can judge for himself what deviations from our procedures will be acceptable.

The requirements for the analysis of water are summarized in Appendices 1 to 5 of Section 2.4.1. Appendices 1 to 5 of Section 2.5.1 apply to the procedures for tissue analysis. An explanation of the appendices is given **only** in Section 2.4.1. The requirement tables give only the requirements that relate to **more than one** procedure. The requirements for **only one** procedure are described in that procedure.

2.4.1.2 Grouping of the samples and their administration

Since the electron capture detectors in the gas chromatographs show slight changes in sensitivity from day to day, it is necessary to analyse a fairly large number of 'standard samples' at the same time.

The samples from biological experiments are divided into groups in a so-called 'series' in the following way:

- 8 samples at the most
- at least 5 'standard samples'
- at least 1 'analytical blank'.

- By a 'standard sample' we understand an amount of unused test medium of the same size as the samples to be analysed and with a known amount of the compound to be determined. This 'standard sample' is subjected to the same analytical procedure as the samples.

* Most of the material in Section 2.4.1 is also applicable to the analysis of organisms (see Section 2.5.1).

- By an 'analytical blank' we understand an amount of unused test medium of the same size as the samples to be analysed, to which the compound to be determined has **not** been added. This 'analytical blank' is subjected to the same analytical procedure as the samples.

A series therefore consists of 14 samples in the broadest sense of the word.

Among the 8 'true' samples, of course, some may occur that have been derived from 'biological blanks'.

In the 5 standard samples the concentrations of the compound to be determined are made up in such a way that a calibration curve can be obtained in a range of concentrations expected to include the samples to be analysed. The selected size of a series has practical reasons. The number of samples remains easy to handle and easy to survey, while it also follows in a logical manner from the design of the biological experiments. Three series of 14 samples fit into a sample plate of the automatic gas-chromatographic injector used. A few places are then still left for so-called 'gas-chromatographic standards'.

- By a 'gas-chromatographic standard' we understand a solution of the substance to be determined, or of a derivative of this substance to be chromatographed, in an organic solvent. The concentration is chosen in such a way that a peak with an easily measurable height is formed, the concentration to be expected

in the sample being taken into account.

At least four such 'standards' are chromatographed together with each three series in order to monitor the correct operation and sensitivity of the gas chromatograph.

The series forms a unit that is not broken up during the whole analytical procedure. This is achieved with the following aids:

- a rack holding of 2×14 separating funnels (2.4.1, Fig. 11)
- a shaking machine for 14 separating funnels (2.4.1, Fig. 13)
- wooden blocks holding 14 volumetric flasks (2.4.1, Fig. 14)
- an arrangement in which solvent can be blown off from 14 measuring flasks simultaneously with a current of nitrogen (2.4.1, Fig. 15)
- a wooden block in which 14 centrifuge tubes can be shaken simultaneously on a shaker (2.4.1, Fig. 16).

These aids will be described in detail below.

A 'serial record sheet' (2.4.1, Table 1) accompanies the series from the beginning to the end of the analysis. Just like the procedure just mentioned, these sheets are of an importance that should not be underestimated, particularly when many series of samples have to be analysed. The loss of information due to various mistakes is hereby reduced to a minimum (a few parts percent).

2. Analysis of the investigation of degradability, toxicity, and accumulation

2.4.1 Table 1 Example of a 'serial record sheet' used to record analytical results

Biology initials: W.R.			Serial No. G.Y.g		Analytical chemistry initials: v.V.		Remarks:			
Name: <i>Peter Brown</i>	Date of delivery: 23-4	Name of analyst for pretreatment: <i>John</i>	Date of pretreatment: 28-4	Name of GC analyst: <i>Susan</i>	Date: 6-5	GC code: <i>aut 3122</i>				
Order No. 12345	Test code: <i>Dd D28</i>	Type of water: <i>st ref.</i> pH on receipt: ± 8	Column: <i>5% OV-101</i> <i>G-AW-DMCS</i>	temp.: <i>200°C</i>	ATT: <i>X16</i> current: <i>8</i>	injected μ l: <i>0.5</i>				
Type of tissue:	Analysis required of: <i>Dieldrin</i>		Integrator:	Chart speed: <i>5 mm/min</i> flow rate of carrier gas: <i>50 ml/min</i>						
			Results calculated by: <i>Susan</i>		Date of calculations: 7-5-1976					
Sample code	Amount received g or l	Expected content μ g l ⁻¹	Substance:	extracted volume	volume extraction liquid	No. in table	peak height	Integrator counts:	μ g kg ⁻¹ acc. to calibration curve	μ g kg ⁻¹ corres. for weight
Date	Other marks									
23-4	0A	0.5 l	0	500 ml	10 ml	39	3		0.4	
	0B	"	0			41	4		0.5	
	56A	"	11			43	66		7.4	
	56B	"	11			45	84		9.4	
	100A	"	10			47	84		9.4	
	100B	"	10			49	62		7.0	
	180A	"	9			51	55		6.2	
	180B	"	9			53	50		5.6	
	anal.					27				
	st 1	20 μ l	25 ppm			29	11			
	st 2	50 μ l	25 "			31	20			
	st 3	100 μ l	25 "			33	45			
	st 4	100 μ l	25 "			35	46			
	st 5	200 μ l	25 "			37	88			
	st 6									
	st GC									
	st GC									
	st GC									

In each treatment that a series undergoes the corresponding information is reported on the serial record sheet with notes of any special features.

The system of administration is based on the following principles. Each series of 8 samples is given a serial number and a form. Whoever sends the samples for analysis records the following in a separate book (2.4.1, Table 2):

- the serial number
- the date of dispatch
- the name of the person dispatching the samples
- the number of samples
- the code of the test from which the samples originate
- the date of receipt of results.

2.4 Methods for the determination of individual test compounds in water

2.4.1 Table 2 Example of a page from the sample administration book in the biological part of the investigation

Date recd.	Date retained	No. of samples	Serial No.	Investigator	(Biol.) test code
29-10	3-11	8	HT 4	John	AB P3
1-11	8-11	6	HT 5	Peter	PQR G5
3-11	10-11	6	HT 6	Susan	CZ D5

After extraction, each series in its totality is placed in a wooden block provided with holes into which the volumetric flasks fit (2.4.1, Fig. 14). The serial number is also put on the flasks of the 'standard samples' and of the 'analytical blank'. The record sheet is folded in two and placed between the flasks.

If the gas chromatograph is an automatic one, it is convenient to use a separate 'GC-automat book' for recording the analysed samples (2.4.1, Table 3). Each page is numbered. In addition, the book may be numbered, e.g. aut. 236 (GC-automat book 2, p. 36). This is also recorded separately in the general section of the form (2.4.1, Table 1).

2.4.1 Table 3 Example of a page from the sample administration book in the analytical part of the investigation; one such book is attached to each automatic gas chromatograph ('GC-automat book'; *not to be confused with the logbook used with each automatic gas chromatograph to record the characteristics of the instrument*)

No. in the table	Samples	Peak height (mm)	$\mu\text{g.l}^{-1}$
1	GC standard 100 ppb Dieldrin	28	
3	Anal. blank DR5	0.5	
5	st 20 μl of 25 ppm	13	1 (measured out)
7	50 μl of 25 ppm	26	2.5 (measured out)
9	100 μl of 25 ppm	60	5 (measured out)
11	100 μl of 25 ppm	58	5 (measured out)
13	200 μl of 25 ppm	108	10 (measured out)
15	19-3 0	6	0.4 (calculated)
17	0 ^{ac})	1	0 (calculated)
19	10	56	5 (calculated)
21	18	40	3.5 (calculated)
23	32	68.5	6.1 (calculated)
25	56	73	6.5 (calculated)
27	100	70	6.3 (calculated)
29	GC standard 100 ppb Dieldrin	30	

*) ac=biological sample code

The date and the GC-automat book code is recorded on each page. The numbering on the sample holder of the automatic gas chromatograph is also written in. Behind each number is noted which sample has been put in that place and also the corresponding peak height and $\mu\text{g.kg}^{-1}$.

The serial record sheet is now also preserved in the GC-automat book. It is convenient to place the samples still to be analysed and those already analysed on separate shelves in a cupboard. After the results of the analysis of a series are known, these are entered on the sheet and the latter is copied.

The chromatograms of a series are kept together and folded and stored; the following data are noted on the chromatogram: serial number, GC-automat book code, amount injected, attenuation of the amplifier (2.4.1, Table 4).

2.4.1 Table 4 Information entered on each chromatogram (use preprinted labels or rubber stamps)

Date	: 9-11-'76
GC-automat book code	: aut. 23-12
Serial no.	: HS 8, HS 9
Amount injected	: 0.5 μl
Attenuation	: X16
Current	: 8
Column	: 5% OV-101, G-AW-DMCS
Column temperature	: 180°C

The calibration curve is stapled to the first fold of the chromatogram, and the copy of the sheet is fixed with a paper clip.

The original of the sheet returns to the person who sent in the samples.

The sheets of the series are stored in successive serial numbers in a hanging file; the file is coded either for the substance(s) to be determined or for the test code.

After a test has been rounded off, the analytical results are stored in an archive box.

Only after the whole series has been chromatographed, and when the results do not make it necessary to repeat the gas chromatographic analysis, can the flasks be emptied and cleaned.

It is sometimes practical, when a larger number of samples is supplied, to group them into series with the smallest possible ranges of concentrations. This can be done only for samples with the same test medium per series. Very close communication with the biologist who sent in the samples is then necessary to avoid administrative mistakes.

The system of administration for the samples that we have now used for more than 3 years has completely proved its value. Nevertheless, other systems are conceivable, which give at least equally good results. A marginal note may be made about these.

The described system of analytical administration relates to the system used by the biologists, as described in Sections 5.6.2 and 6.5.4. There are many points of agreement and overlap between the two administrations. At first sight this appears inefficient, but in practice this 'redundant' information makes the system as a whole less sensitive to disturbances such as mistakes, mislaying of sheets, accidental loss of coding, etc.

2.4.1.3 Treatments of the samples preceding gas-chromatographic analysis

The samples of water are supplied in glass volumetric flasks provided with the date, test code, and sample code. (The samples must come into contact only with glass). Each sample volume is therefore known with sufficient accuracy*. Fourteen 1 l conical separating funnels are suspended in a rack specially constructed for the purpose (see 2.4.1, Fig. 11). The separating funnels must have this conical shape (see 2.4.1, Fig. 8) so that a good mixing of water and extraction liquid is achieved in a shaking machine (see 2.4.1, Fig. 13). The stopper and the tap must be made of glass and ground so that they can be lubricated with water. **Greasing and the use of plastic taps and stoppers are out of the question;** they cause losses and contamination.

* These volumetric flasks are repeatedly heated in an oven to 300°C to eliminate traces of the substances to be determined. They are therefore no longer suitable for accurate volumetric work; for this purpose they should be recalibrated.

2. Analysis of the investigation of degradability, toxicity, and accumulation

8 samples are poured into 8 separating funnels without the use of a funnel. In each of the other 6 separating funnels an equal volume of test medium is placed. The desired amount of an acetone solution of the substance to be determined is introduced into 5 of these 6 separating funnels with the aid of a plunger pipette with a disposable tip. These amounts are chosen in such a way that a calibration curve is obtained in the range of concentrations to be expected. The same acetone as was used for the preparation of the standard solutions, and the maximum amount of it that was used for the five 'standard samples' is added to the sixth separating funnel.

The sample code number is recorded on the first eight separating funnels with a (blue) felt-tip pen whose ink cannot easily be removed by spilled water (but can be in the washing machine). The five 'standard samples' are coded in the following way:

(added:) 'x µl of y ppm' (in acetone).

The analytical blank is given the coding:

'anal. blank' + series number.

A plunger pipette with a disposable tip (see 2.4.1, Fig. 10) is used for metering out the standard solutions. The plunger pipettes are sufficiently accurate for this purpose (Van Ginkel and Van Ooijen, 1974).

When, for example, the aim is to make the following concentrations in separating funnels 9 to 14: 0.10–0.25–0.50–1.0–1.50 µg.l⁻¹ and the separating funnels contain 500 ml of the test medium (sample size 500 ml), the following amounts are metered out:

No. 9: 20 µl of 2.5 ppm in acetone → 0.10 ppb

No. 10: 50 µl of 2.5 ppm in acetone → 0.25 ppb

No. 11: 100 µl of 2.5 ppm in acetone → 0.50 ppb

No. 12: 200 µl of 2.5 ppm in acetone → 1.00 ppb

No. 13: 100 + 200 µl of 2.5 ppm in acetone → 1.50 ppb
(1 ppb = 1 µg per kg; 1 ppm = 1000 µg per kg).

A standard solution in acetone and, for example, two plunger pipettes with disposable tips are required. These tips are discarded immediately after use.

In some cases other reagents must then be added for the preparation of derivatives of the substances to be determined. In general, these reagents are liquid. They can be metered out with a Kipp automatic dosing device (2.4.1, Fig. 1) much faster than with a measuring cylinder or pipette.

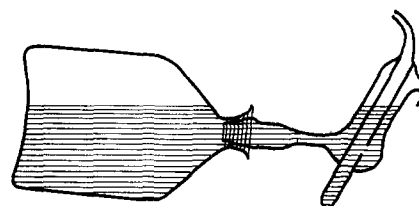
If the reaction requires more than a few minutes it is wise to make the reaction time long enough to be able to do something else during this time. The end of the waiting period is indicated by a kitchen timer. In this way a reproducible waiting time is obtained that can be utilized without trouble.

After the derivative-forming reaction, if any, extraction of the substance to be determined or of its derivative is carried out. For this purpose 5 ml of cyclohexane is transferred to each separating funnel by means of a Kipp automatic dosing device. The neck of the funnel is rinsed with a small amount of distilled water and then, with a powerful turning-thrusting motion, it is closed with the moistened glass stopper. After brief shaking by hand, the pressure is released through the tap, and the separating funnel is placed in the shaking machine; the latter is started up, the speed being adjusted in such a way that an intensive mixing is achieved. After an hour, for example, the machine is stopped by means of a time switch. Care must be taken that the separating funnels do not leak. This does happen occasionally, and the relevant sample must then be marked so that it remains recognizable; to avoid mistakes, however, it is advisable to continue with the whole analytical procedure in the normal way.

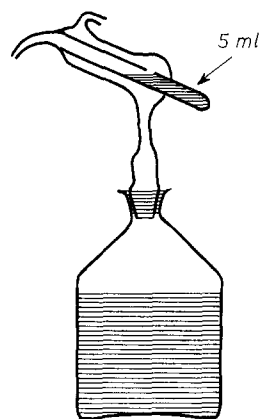
When 500 ml of water is brought into equilibrium with 5 ml of cyclohexane, about 50 % of a substance having a partition coefficient $K=100$ in this system is extracted. In most cases K is much higher and so is the extraction yield. The aim is to obtain a **reproducible yield**. Cyclohexane is chosen because n-hexane is somewhat too volatile and **benzene is much too toxic**.

Cyclohexane is somewhat more polar than hexane and can be bought in a sufficiently pure state. When a large amount of organic material is present in the sample – for example in algal cultures – or when organisms are analysed, n-hexane sometimes has the advantage that one is less troubled by the formation of emulsions. When an emulsion forms during extraction with cyclohexane, it is usually easy to break with ethanol or isopropanol **after** as much water as possible has been allowed to run from the separating funnel.

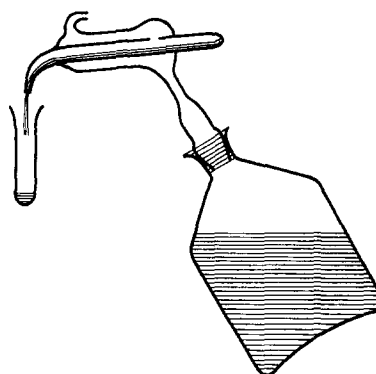
The stoppers and taps of the separating funnels and the centrifuge tubes may, if necessary, be loosened by knocking with a block of hard wood.



filling



measuring out
the volume to be metered



adding the volume
to be metered

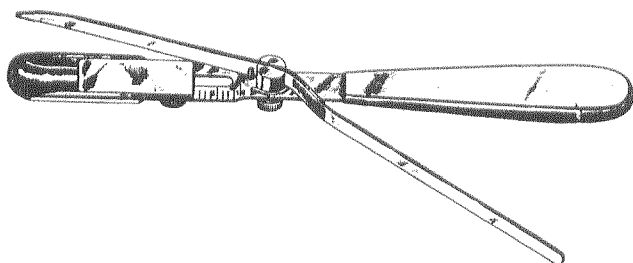
2.4.1 Fig. 1 Measuring out with a Kipp automatic metering device

The bulk of the extracted water is allowed to run into a plastic bucket. This is most conveniently placed under the separating-

funnel rack. After this, any emulsions are broken and the last traces of water are run into a small beaker. Both this beaker and the lower end of the separating funnel are held firmly with the left hand; the tap is operated with the right hand, in such a way that the interface of the two liquids comes **into** the bore of the tap.

The outlet tube of the separating funnel is next dried. This is done most easily with a pipe-cleaner. Now 10 ml volumetric flasks are placed under the separating funnels and into each of them is put a known amount of anhydrous sodium sulphate with the aid of a powder-dosing spoon (2.4.1, Fig. 2) through a small funnel. This must be done **beforehand**, when the flasks and the funnels are still dry. The sample code on the separating funnel is noted on the corresponding flask.

The cyclohexane is now run from the separating funnels and through the above-mentioned small funnels into the volumetric flasks. After this, the separating funnels are rinsed with about 4 ml of cyclohexane, along the wall, by means of a glass wash-bottle (see 2.4.1, Fig. 9). This cyclohexane is also run into the volumetric flask.



2.4.1 Fig. 2 Powder-dosing spoon

The flasks must be placed immediately in a wooden block, because they are easily knocked over (see 2.4.1, Fig. 14).

In a number of cases it is now possible to make the solutions up to the mark with cyclohexane and homogenize the contents of each flask, closed with a glass stopper, by inverting the flask **ten** times. After drying the extract for at least an hour, the solution is ready for gas-chromatographic analysis.

Sometimes it is first necessary to carry out methylation by means of a solution of diazomethane in diethyl ether (for preparation see Appendix 7 to this section). In this case the solution is not made up to the mark but the diazomethane solution is added in the fume cupboard with a suction pipette (2.4.1, Fig. 3).



2.4.1 Fig. 3 Suction pipette

Care must be taken that the liquid in the volumetric flask remains distinctly yellow. After half an hour the ether and the excess of diazomethane are blown off with a current of nitrogen in a simple arrangement made for the purpose (see 2.4.1, Fig. 15), until the liquid in the flask is colourless:

This cools down the liquid in the flask very considerably. The flasks are placed in their wooden block, and when they, with their contents, have returned to room temperature each is made up to the mark with cyclohexane. The contents are then homogenized by inverting this covered flask 10 times.

The folded serial record sheet is placed on the appropriate block between the flasks.

Tissue homogenates and very small samples of water are extracted in 10- or 25-ml centrifuge tubes which, closed with well-ground stoppers, fit into the centrifuge. The two liquid phases are brought into contact with one another by shaking the tubes for 2 min on a so-called 'Vortex' shaker (2.4.1, Fig. 4) and then for 30 min in a 'shaking block' (see 2.4.1, Fig. 16) on the shaker that is also

used for the separating funnels. Any emulsions are broken with ethanol or isopropanol.

The two liquid phases are separated by centrifugation. The organic upper layer can be transferred by means of a Pasteur pipette from the centrifuge tube into a volumetric flask or into a smaller centrifuge tube.

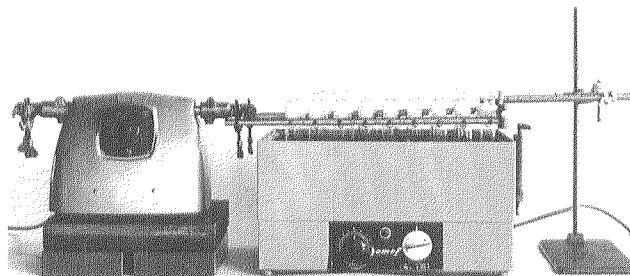
Alternatively, the aqueous layer can be frozen by placing the centrifuge tube in solid carbon dioxide. The aqueous layer then freezes rapidly and the hexane or cyclohexane layer can be decanted.

In some procedures saponification is carried out in the centrifuge tubes. These are shaken while they are suspended in a warm water bath (2.4.1, Fig. 5).

The coding on the centrifuge tubes must be applied with a (black) felt-tip pen containing **water-resistant** ink.



2.4.1 Fig. 4 'Vortex' test-tube shaker



2.4.1 Fig. 5 System for shaking centrifuge tubes in a water bath

2. Analysis of the investigation of degradability, toxicity, and accumulation

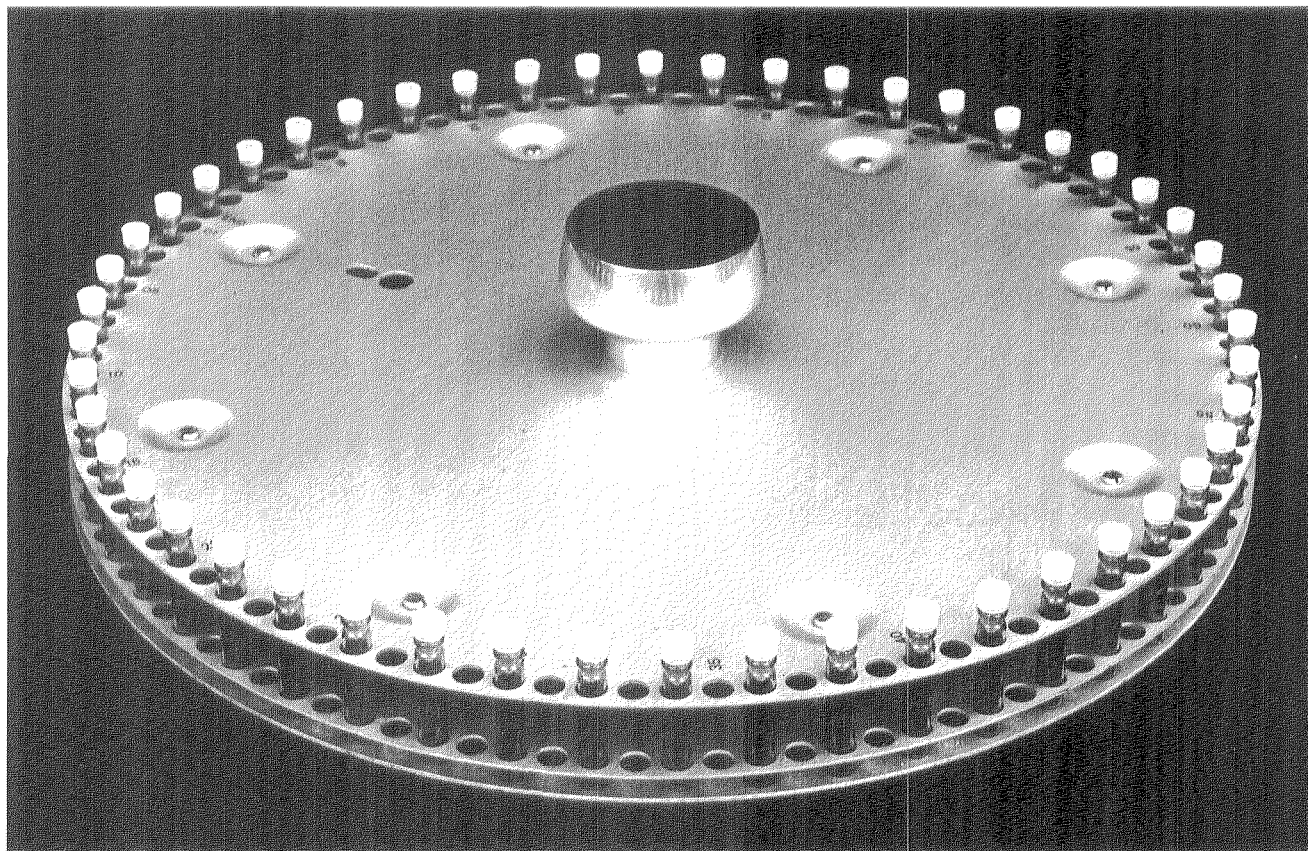
2.4.1.4 Gas-chromatographic analysis of the extracts obtained

The gas chromatograph to be used has been described in Section 2.4.1. It is assumed that the instrument has been fitted with an automatic sample injector. The injectors we use include an interchangeable circular sample plate (2.4.1, Fig. 6) taking 100 sample bottles of 1 or 0.1 ml.

Of these 100 places, 50 are occupied by sample bottles containing

For each series a separate calibration graph is plotted by means of the peak heights or areas for the five standard samples (2.4.1, Fig. 7).

In general, the calibration graph for one series cannot be used for another series. This can be done only if the values obtained for the gas-chromatographic standards differ very little, and if it is also established that there are no procedural differences between the series.



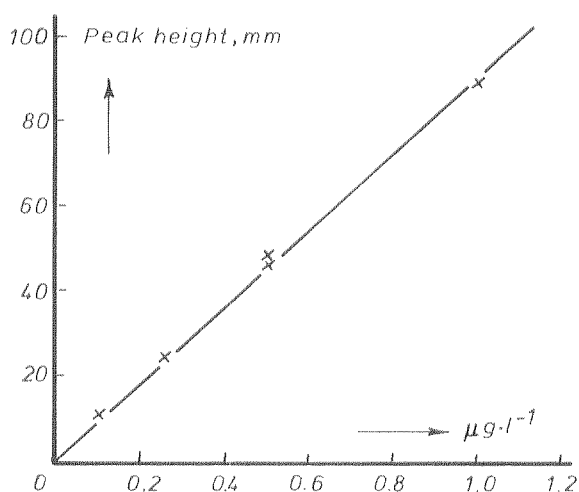
2.4.1 Fig. 6 Sample plate for an automatic gas chromatograph. Only bottles with rinsing acetone have been placed in the plate.

pure acetone, so that next to each acetone bottle a place remains open for a sample. The following are then placed successively in the plate:

- 1 'gas-chromatographic standard'
- 1 series of 8 (if necessary 9) samples, 5 standard samples, and 1 analytical blank
- 1 'gas-chromatographic standard'
- 1 series
- 1 'gas-chromatographic standard'
- 1 series
- 1 'gas-chromatographic standard'.

In this way a maximum of 49 gas chromatograms can be run off. If these each require about 20 min, the whole plate can be processed in one night.

By means of an automatic peak-area integrator the corresponding peak areas of the substance to be determined can be printed out on a strip of paper. When this is not possible with the apparatus used, one can make do with measuring the peak heights.



2.4.1 Fig. 7 Calibration graph obtained in the determination of, for example, dieldrin in water. The point for $0.5 \mu\text{g.l}^{-1}$ was recorded in duplicate

2.4.1.5 The preparation of 'standard solutions'

The 'standard samples' mentioned above consist of the test medium with various amounts of a known acetone solution (or a number of solutions) of the substance to be determined. Only exceptionally is a solvent other than acetone used. The concentration(s) of this solution or these solutions depend(s) on the concentration range in which the measurements are to be performed, on the sample volume, and on the available plunger pipettes. If at all possible, the number of the required concentrations should be limited to one: it is never necessary to have more than two. The concentration must also be calculated in such a way that a logical concentration series is obtained with a minimum of pipette volumes.

The solutions are made up in two steps. A 'standard stock solution' is first made up by dissolving 50 mg of the substance in acetone in a 100-ml volumetric flask. This solution therefore has a concentration of 500 mg.l^{-1} . The stock solution is coded and dated and is kept in the refrigerator.

Acetone dilutions of this stock solution are made up in 25-ml volumetric flasks with the concentrations necessary to prepare the 'standard samples' (e.g. 2.5 mg.l^{-1}). When making up these dilutions, a plunger pipette with disposable tips can be used (a model with a volume of, for example, 1 ml). The diluted solutions are also kept in the refrigerator. Small amounts, such as 5 ml, of the diluted solutions are kept in 25-ml conical flasks with well-ground-in stoppers in the dark at room temperature. These last-mentioned solutions at room temperature are used to prepare the 'standard samples'. If necessary, the standard substances must be purified by recrystallization.

2.4.1.6 Preparation of the 'gas-chromatographic standards'

When a litre of water with the substance to be determined at a concentration of, for example, $1 \mu\text{g.l}^{-1}$ is extracted with 10 ml of cyclohexane, the concentration in the latter is at the most $100 \mu\text{g.l}^{-1}$. Then, for the 'gas-chromatographic standard', a solution of the substance to be determined is made up in hexane at a concentration not exceeding $200 \mu\text{g.l}^{-1}$. This can also be done in several steps, while in many cases acetone must be used as the solvent for the first step(s) because of the relatively low solubility of the substance to be determined in cyclohexane.

2.4.1.7 Explanation to the tables of 'Glassware, general', 'Glassware, calibrated', and 'Auxiliary apparatus, calibrated' requirements

(Appendices 1, 2, and 4 of this section and Section 2.5.1).

A few points from these tables require some explanation. Whereas in general for the glassware that we mentioned the shape and dimensions can be chosen freely within certain limits, this is certainly not the case with the separating funnels. The only separating funnels with which we have been able to carry out a good extraction on shaking in an almost horizontal position in a shaker were those of the 'Squibb' type (2.4.1, Fig. 8).

The second important observation relates to the ground taps and stoppers in the separating funnels and other glassware. These must be glass and not plastic. They must be ground so well that they can be lubricated quite satisfactorily with distilled water. Glass can withstand the baking at 300°C which is absolutely necessary. Plastics, even polytetrafluoroethylene*, emit substances that interfere with the analysis and have a 'memory' for the (organic) substances with which they have been in contact.

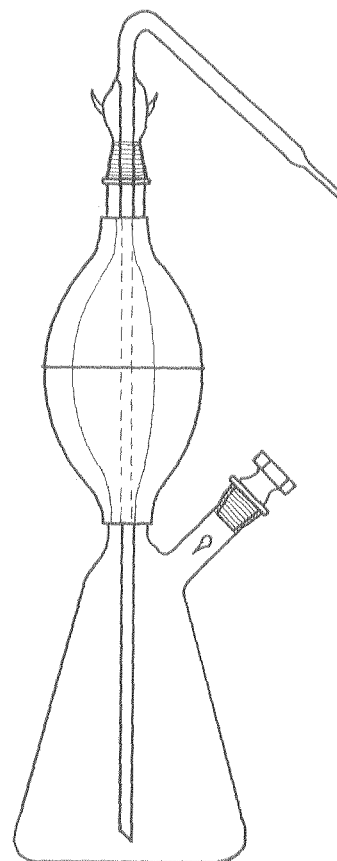
Commercial glass wash-bottles as such cannot be closed with a ground stopper. This must be ground in by a glass-blower (2.4.1, Fig. 9).

All the volumetric glassware is frequently heated overnight at 300°C ; it is therefore no longer suitable for high-precision volumetric analyses.

Where this was in any way possible, for measuring out the liquids to make up standard solutions, etc., we used so-called 'plunger pipettes' with disposable tips. These are sufficiently accurate for our purpose (2.4.1, Fig. 10) (Van Ginkel and Van Ooijen, 1974). There are two types: a cheap 'one-stroke' type and a more expensive type with a small 'stroke' for filling and a larger 'stroke' for emptying. We prefer the second type, since it is much more accurate (see also Siemer, 1976).

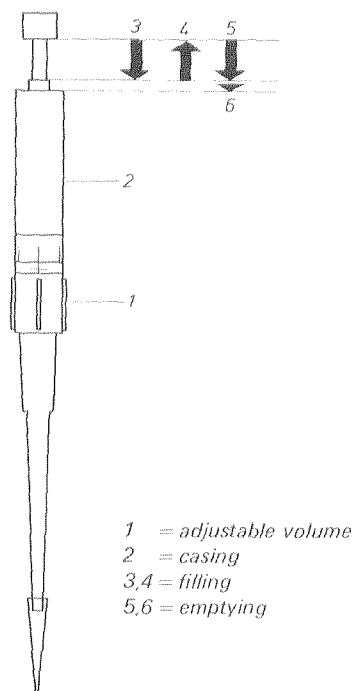


2.4.1 Fig. 8 Squibb conical separating funnel



2.4.1 Fig. 9 Glass wash-bottle in which the liquid comes into contact exclusively with glass

* Teflon, Hostafion, Fluon, Gafion.



2.4.1 Fig. 10 Plunger pipette with a disposable tip

A set of instructions for cleaning all glassware is included in Appendix 6 of this section.

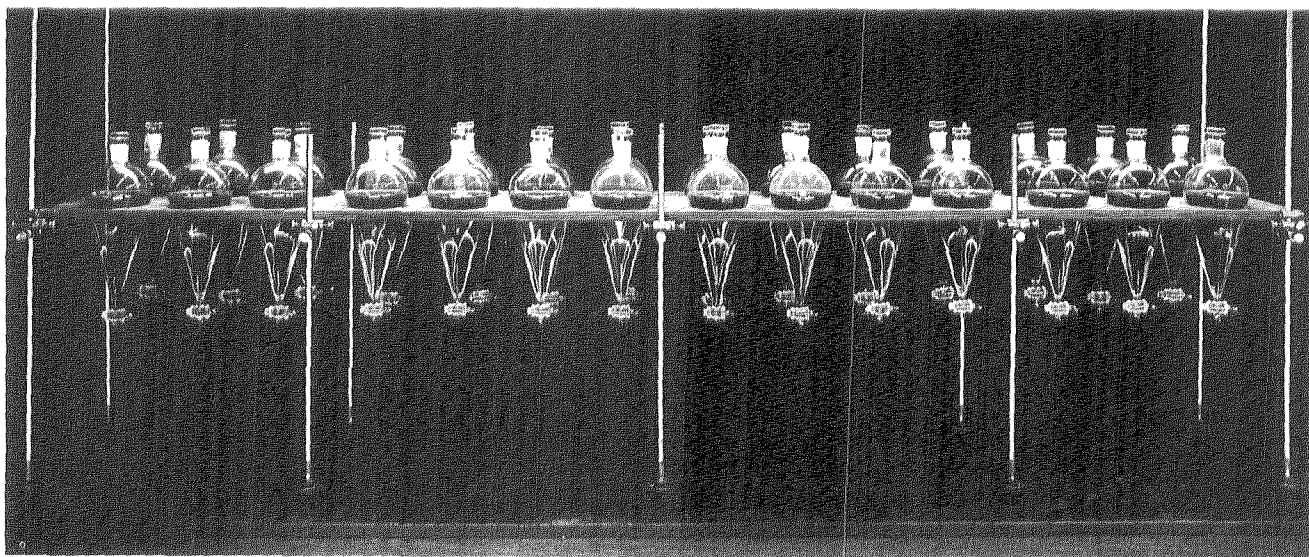
It is recommended to have some specimens of a number of types of glassware (such as separating funnels, Kipp automatic dosing devices) in reserve.

2.4.1.8 Explanation to the table of 'Auxiliary apparatus – general' requirements (Appendix 3 of this section and Section 2.5.1).

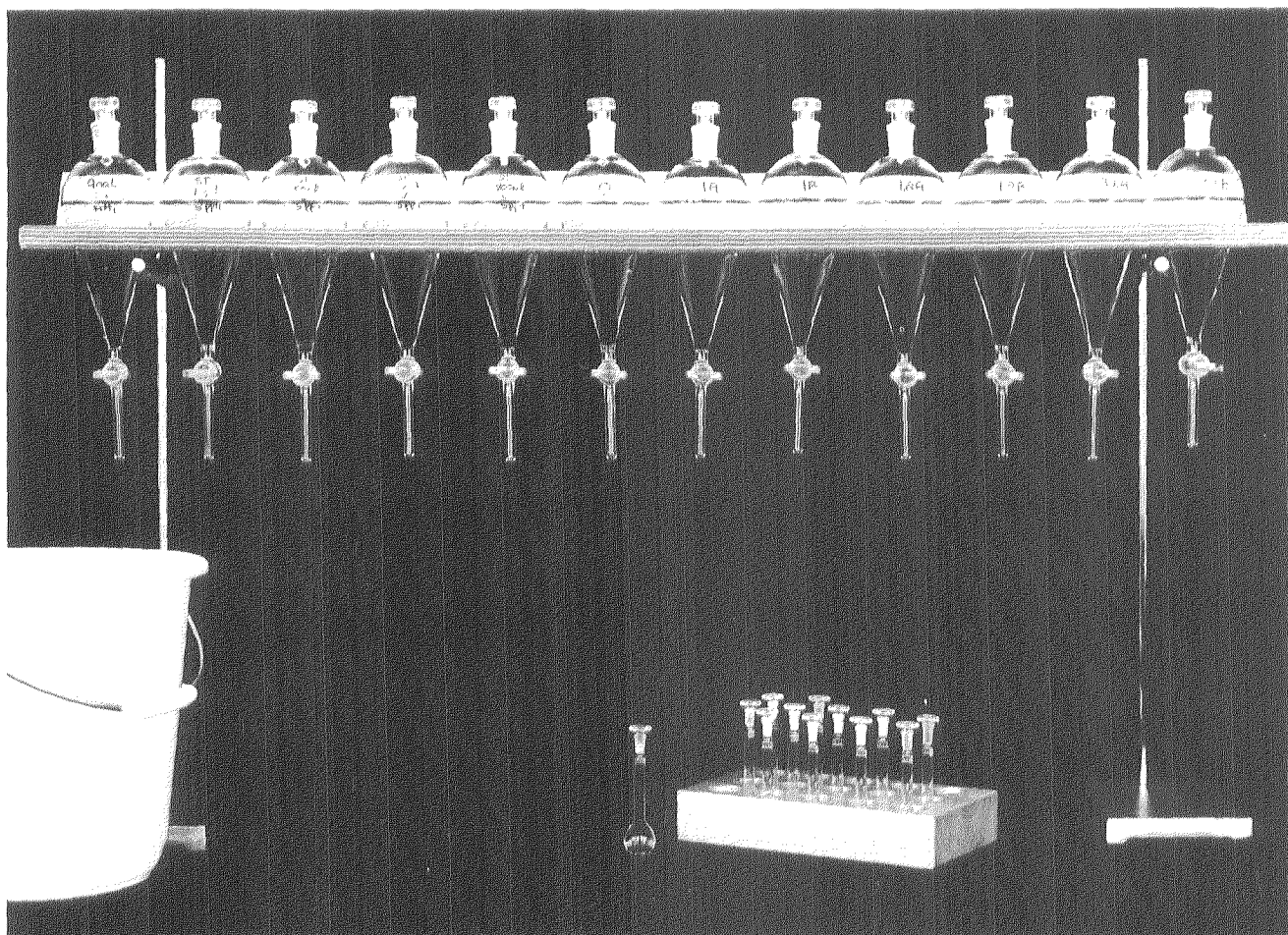
Rack for separating funnels

A rack such as we used for suspending the separating funnels is shown in 2.4.1, Figs. 11 and 12.

There are, for example, two rows of 14 holes with a diameter of 11.5 cm for 1-l separating funnels and of 7.5 cm for 0.25-l funnels. The funnels in use are located in the front row, and the back row may contain the funnels for the next series. The rack is made of metal scaffolding and a 2-cm-thick sheet of water-resistant plywood. It must be high enough for a 10-l plastic bucket to be placed under the separating funnels.

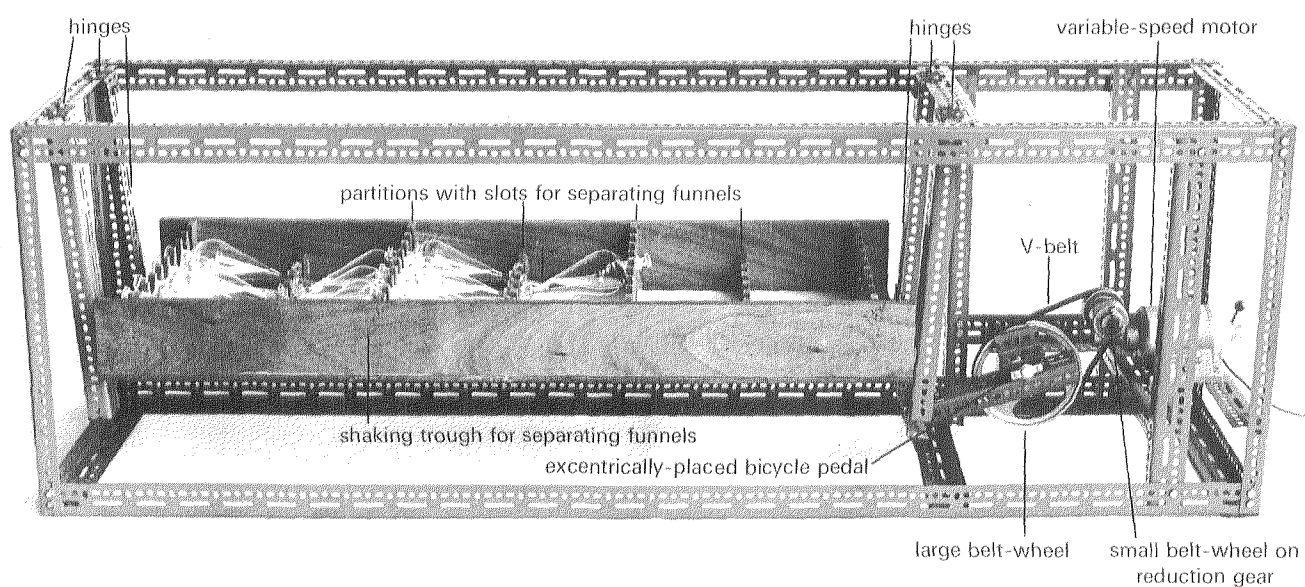


2.4.1 Fig. 11 Rack for two rows of fourteen 1-litre, 250-ml, or 100-ml funnels, to be used in the extraction of water samples



2.4.1 Fig. 12 Rack for 12 separating funnels that fits into the fume cupboard, to be used in the analysis of tissue samples

Shaker for separating funnels and centrifuge tubes This is shown in 2.4.1, Fig. 13.

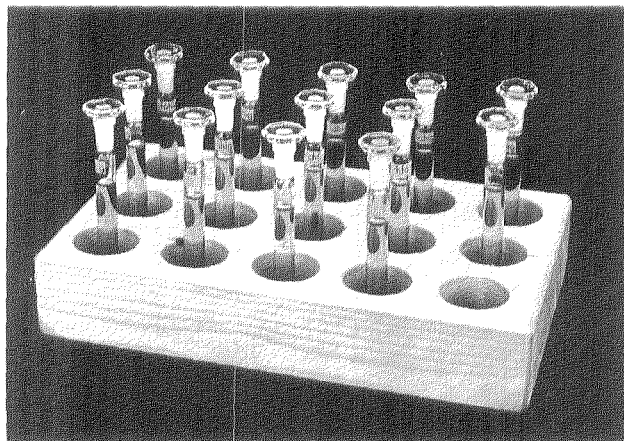


2.4.1 Fig. 13 Shaker for separating funnels

2. Analysis of the investigation of degradability, toxicity, and accumulation

The 'swinging trough', made from water-resistant plywood, is large enough to take sixteen 1 l separating funnels.

The speed of the motor must be adjustable. We have had satisfactory results with the 'Multifix' (Ahrin B.V.). The motor is provided with an interchangeable reduction gear with a small belt-wheel. The rotary motion of this belt-wheel is transmitted via a V-belt to a large metal belt-wheel fixed to a well-journaled shaft. This may be a bicycle axle. To the large belt-wheel, about 5 cm from the centre, a bicycle pedal is attached, to which is fixed the arm that moves the swinging trough back and forth. The speed of the large belt-wheel must be capable of adjustment between 0.5 and 2 revolutions per second. The hinges from which the swinging trough hangs and the various shafts must be oiled from time to time. Two such shakers, one for large and one for small separating funnels, have been in intensive use for more than 3 years and during that time only one breakdown has occurred, due to the seizing up of a bearing.



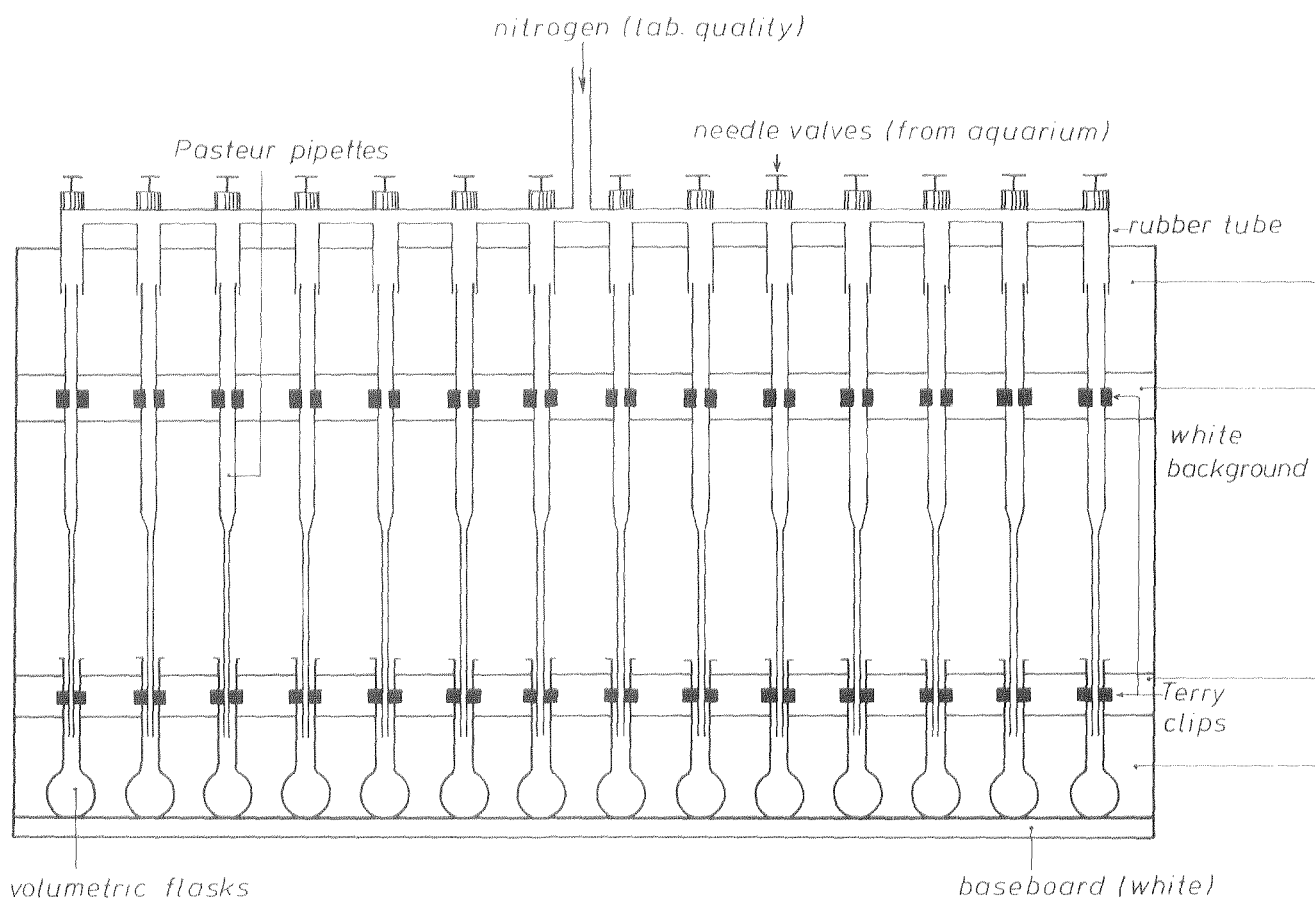
Wooden blocks for volumetric flasks

One of these is shown in 2.4.1, Fig. 14, which speaks for itself. A few dozen of these are required, so that it is possible to process several series simultaneously and still store the analysed extracts for a short time.

2.4.1 Fig. 14 Wooden block for fifteen 10-ml volumetric flasks

Arrangement for evaporating solvent from volumetric flasks

This is shown in 2.4.1, Fig. 15.

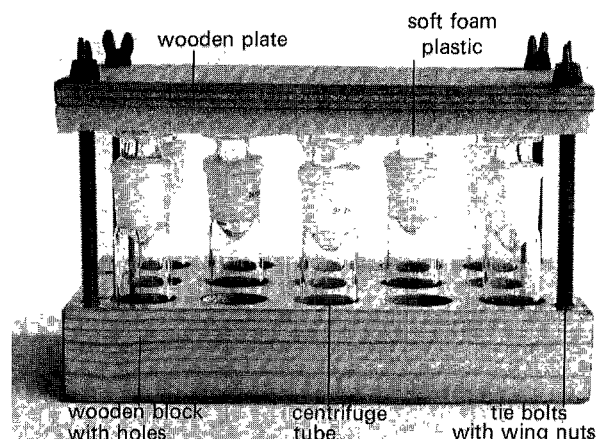


2.4.1 Fig. 15 Arrangement for evaporation with the aid of a current of nitrogen

2.4 Methods for the determination of individual test compounds in water

The Pasteur pipettes inserted into the volumetric flasks can be moved up and down in the Terry clips. This in principle makes it possible to keep the jet opening just above the surface of the liquid, which considerably accelerates evaporation. The current of nitrogen through the Pasteur pipettes must be capable of being adjusted with a simple needle valve. The needle valves can be bought in an aquarium shop.

Shaking blocks for centrifuge tubes



2.4.1 Fig. 16 Shaking block for centrifuge tubes

In these blocks 15 closed centrifuge tubes in the horizontal position can be shaken on the shaker (see 2.4.1, Fig. 13) which is also used for shaking the separating funnels.

2.4.1.9 Explanation to the 'Reagents' tables (Appendix 5 to this section and Section 2.5.1)

Pure and unused test medium

Each series should be accompanied by a quantity of pure test medium contained in a large conical flask and covered with a small beaker, sufficient for one 'analytical blank' and five 'standards'.

Distilled water

This water is demineralized and is then distilled twice in stainless steel apparatus. The water must not come into contact with plastics or rubber and must be stored in a glass bottle provided with a glass siphon that can be closed with a glass tap (not greased).

Organic solvents (analytical grade)

These may be:

acetone, cyclohexane, n-hexane, diethyl ether, diisopropyl ether, ethanol, isopropanol.

Each bottle, regardless of the batch number, is checked for purity before use by gas chromatography with the aid of an electron capture detector. In this process the gas-chromatographic conditions chosen are the same as those in the final analysis. If the analytical procedure includes an evaporation step, this must also be performed before the purity check.

If interfering peaks are found during the check, purification by distillation through a 10-plate column is generally sufficient.

Acetic anhydride (analytical grade)

The purity is checked with the aid of a complete analysis of a blank sample. If necessary, the compound can be purified by distillation through a 10-plate column.

Sodium tetraborate (analytical grade)

We used $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$ (borax) without further purification. When purification appears to be necessary, this can be done by heating overnight in a porcelain dish at 330°C. This yields anhydrous sodium tetraborate.

A solution is stored in a glass bottle with a plastic screw-cap. Contact between the liquid and the screw-cap must be minimized.

Calcium chloride

This is used only in drying tubes included in systems for the preparation of certain reagents (see Appendix 1 of Section 2.4.3.1 and Appendix 1 of Section 2.4.3.2).

Sodium sulphate, anhydrous (analytical grade)

This is heated overnight at 300°C before use. After this it may come into contact only with glass that has also been baked at 300°C.

Hydrochloric acid and sulphuric acid, concentrated (analytical grade)

A bottle of acid is selected that gives an acceptable blank on the complete analysis of a blank sample.

Diazomethane

A solution in diethyl ether prepared according to the instructions given in Appendix 7 of this section is used.

2. Analysis of the investigation of degradability, toxicity, and accumulation

2.4.1 Appendix 1

Glassware, general	TCE/DCE 2.4.2.1	TCE/DCE 2.4.2.2	Dieldrin 2.4.2.4	PCP (acetylation) 2.4.3.1	Synthesis of PCPA 2.4.3.1, Appendix 1	PCP (methylation) 2.4.3.2	Synthesis of PCA 2.4.3.2, Appendix 1	Chlorophenols 2.4.3.3	Chloroacetic acids 2.4.3.6	DCAN 2.4.4.1	Synthesis of 2,6 -dibromo- 3,4-DCAN 2.4.4.1, App. 1	Synthesis of DAM 2.4.1, Appendix 7
* at least 14		*	*	*		*			*			
Conical separating funnels, 1 litre, Squibb type, provided with glass taps and glass stoppers (see 2.4.1, Fig. 8)												
Beaker, 5 litre				2				1		3		
Beaker, 1 litre										1		1
Beaker 250 ml		5	5	6		6		4	6	8		
Beaker 100 ml					3					2		
Conical flask, 5 litre		3	3	3		3			3	2		
Conical flask, with ground-in stopper, 25 ml	4	4	4	4		4		4	4			
Buchner flask, 1 litre					1		1				1	
Glass wash-bottle (see 2.4.1, Fig. 9)												
Wide-mouth glass bottle, 1 litre, with ground-in stopper		1	1	1		1			1	1		1
Wide-mouth glass bottle, 50 ml, with ground-in stopper		2	2	2	2		2		2	2		2
Narrow-mouth glass bottle, 2.5 litre, with ground-in stopper									1	1		
Narrow-mouth glass bottle, 1 litre, with ground-in stopper						1				1		
Narrow-mouth glass bottle, 2.5 litre, with plastic screw-cap				2				3		3		
Glass funnel, 15 cm diameter				3		1		2	3	3		
Glass funnel, 6 cm diameter		3	3	3		3			3	3		
Glass funnel, 3 cm diameter		20	20	20		20		20	20	20		
Watchglass, 7 cm diameter					3		2					
Straight glass stirring rod, length 15 cm					2		1					
Weighing bottle for 1 g	2	2	2	3		3			2	2		
Pasteur pipettes, length 15 cm, in box				1	1							1
Porcelain dish, diameter 15cm		2	2	2		2			2			2
3-Necked round-bottomed flask, 500 ml (see 2.4.1, Fig. 18, and 2.4.3.2, Fig. 2)							1					1
250 ml double funnel with pressure compensation and NS 29 ground joint							1					1

2.4 Methods for the determination of individual test compounds in water

2.4.1 Appendix 2

Glassware, calibrated	TCE/DCE 2.4.2.1	TCE/DCE 2.4.2.2	Dieldrin 2.4.2.4	PCP (acetylation) 2.4.3.1	Synthesis of PCPA 2.4.3.1, Appendix 1	PCP (methylation) 2.4.3.2	Synthesis of PCA 2.4.3.2, Appendix 1	Chlorophenols 2.4.3.3	Chloroacetic acids 2.4.3.6	DCAN 2.4.4.1	Synthesis of 2,6 -dibromo- 3,4-DCAN 2.4.4.1, App. 1	Synthesis of DAM 2.4.1, Appendix 7
*multiples of 14												
Volumetric flasks with calibration mark and ground-in stoppers												
Volume: 100 ml	2	2	2	2		2		2	4	4		
50 ml	4	4	4	4		4		4	8	8		
25 ml	2	2	2	2		*		2	*	2		2
10 ml		*	*					*		*		
Measuring cylinder, 1000 ml				1		1			1	1		
Measuring cylinder, 500 ml			3	3		3			3			
Measuring cylinder, 100 ml								3	1	3	1	1
Measuring cylinder, 25 ml	2	2			2		2				1	2
Kipp automatic dosing device, 10 ml, with matching 500-ml conical flask (2.4.1, Fig. 1)						1			2	1		
Kipp automatic dosing device, 5 ml, with match- ing 250 ml conical flask		1	1	1		1		2		1		
Kipp automatic dosing device, 1 ml, with match- ing 250 ml conical flask				1				1				
Suction pipette, 5 ml (2.4.1, Fig. 3)						1			1			1

2. Analysis of the investigation of degradability, toxicity, and accumulation

2.4.1 Appendix 3

Auxiliary apparatus, general	TCE/DCE 2.4.2.1	TCE/DCE 2.4.2.2	Dieldrin 2.4.2.4	PCP (acetylation) 2.4.3.1	Synthesis of PCPA 2.4.3.1, Appendix 1	PCP (methylation) 2.4.3.2	Synthesis of PCA 2.4.3.2, Appendix 1	Chlorophenols 2.4.3.3	Chloroacetic acids 2.4.3.6	DCAN 2.4.4.1	Synthesis of 2,6 -dibromo- 3,4-DCAN 2.4.4.1, App. 1	Synthesis of DAM 2.4.1, Appendix 7
Laboratory glassware washing machine	x	x	x	x	x	x	x	x	x	x	x	x
Drying cabinet, adjustable to 300° C	x	x	x	x	x	x	x	x	x	x	x	x
Rinsing apparatus for bottles with a powerful jet of water (see 2.4.1, Fig. 17)		x	x	x		x		x	x	x		
Fume cupboard					x	x	x		x	x	x	x
Refrigerator	x	x	x	x	x	x	x	x	x	x	x	x
Closed cupboard for storing samples and stan- dards in the dark			x	x		x		x	x	x		
Cylinder of nitrogen with reducing valve					x	x	x		x			x
Evaporating device (see 2.4.1, Fig. 15)						x			x			x
Rack with 2 rows for at least 14 one-litre separat- ing funnels (see 2.4.1, Fig. 11)		x	x	x		x			x			
Shaker for at least 14 one-litre separating funnels (see 2.4.1, Fig. 13)		x	x	x		x			x			
Wooden block with holes for at least 14 25-ml volumetric flasks						x			x			
Wooden block with holes for at least 14 10-ml volumetric flasks (see 2.4.1, Fig. 14)		x	x	x				x		x		
Combination of magnetic stirrer and heating plate	x			x	x	x		x	x	x	x	
Heated vacuum desiccator					x		x				x	
'Stop-start' time switch plug		x	x	x		x		x	x	x		
Vacuum pump, water-driven					x		x				x	
Explosion screen							x					x
Centrifuge taking at least 12 centrifuge tubes, 25-ml or 10-ml					x		x					
Water bath					x	x						
Stainless-steel spoon-type spatula	2	3	2	3	2	2	2	2	2	4	2	2
10-l plastic bucket		2	2	2		2		2	2	2		
Thick blue felt-tip pen	x	x	x	x	x	x	x	x	x	x	x	x
Pipe cleaners, white, 4 mm × 15 cm in a pack of 25	x	x	x	x		x		x	x	x		
Disposable polythene gloves in a pack of 100	x	x	x	x	x	x	x	x	x	x	x	x

2.4 Methods for the determination of individual test compounds in water

2.4.1 Appendix 4

Auxiliary apparatus, calibrated	TCE/DCE 2.4.2.1	TCE/DCE 2.4.2.2	Dieldrin 2.4.2.4	PCP (acetylation) 2.4.3.1	Synthesis of PCPA 2.4.3.1, Appendix 1	PCP (methylation) 2.4.3.2	Synthesis of PCA 2.4.3.2, Appendix 1	Chlorophenols 2.4.3.3	Chloroacetic acids 2.4.3.6	DCAN 2.4.4.1	Synthesis of 2,6 -dibromo- 3,4-DCAN 2.4.4.1, App. 1	Synthesis of DAM 2.4.1, Appendix 7
Analytical balance, accurate to 0.1 mg	x	x	x	x		x		x	x	x		
Top-weighing balance, accurate to 0.01 g	x	x	x	x	x	x	x	x	x	x	x	x
2-4 Plunger pipettes with one or more fixed- adjustable metering volumes (e.g. 20, 50, 100, 200µl) (2.4.1, Fig. 10)	x	x	x	x		x		x	x	x		
500-1000 disposable tips for these pipettes	x	x	x	x		x		x	x	x		
Powder-dosing spoon (2.4.1, Fig. 2)		x	x	x		x			x	x		
Apparatus for melting-point determinations					x		x				x	

2.4.1 Appendix 5

Reagents	TCE/DCE 2.4.2.1	TCE/DCE 2.4.2.2	Dieldrin 2.4.2.4	PCP (acetylation) 2.4.3.1	Synthesis of PCPA 2.4.3.1, Appendix 1	PCP (methylation) 2.4.3.2	Synthesis of PCA 2.4.3.2, Appendix 1	Chlorophenols 2.4.3.3	Chloroacetic acids 2.4.3.6	DCAN 2.4.4.1	Synthesis of 2,6 -dibromo- 3,4-DCAN 2.4.4.1, App. 1	Synthesis of DAM 2.4.1, Appendix 7
Pure and unused test medium		x	x	x		x		x	x	x		
Distilled water	x	x	x	x	x	x	x	x	x	x	x	x
Acetone, analytical grade		x	x	x		x		x	x	x		
Cyclohexane, analytical grade		x	x	x		x		x	x	x		
Diethyl ether, analytical grade					x		x				x	x
Absolute ethanol, analytical grade		x	x	x	x	x	x	x	x	x		x
Acetic anhydride, analytical grade				x	x			x				
Borax, analytical grade				x	x			x				
Calcium chloride					x		x					
Sodium sulphate, anhydrous		x	x	x		x			x	x		x
Concentrated sulphuric acid						x				x		
Solution of diazomethane in ether (see Appendix 7 of 2.4.1)						x			x			
Ice					x		x					x
pH paper					x		x			x	x	



2.4.1 Appendix 6 Cleaning of the analytical glassware

Separating funnels

These are rinsed clean with a powerful jet of mains water with the aid of a rinsing apparatus (2.4.1, Fig. 17). The coding is removed with acetone.

After cleaning, they are suspended in the separating-funnel rack loosely closed with the stopper and filled with about 10 ml of mains water to above the closed tap.

Only exceptionally after rinsing with acetone are they cleaned in a laboratory glass washing machine and dried at 300° C.

Other glassware, spatulas, and porcelain vessels

These are cleaned in a laboratory glass washing machine with the following programme: (2.4.1, Table 5).

2.4.1 Table 5

Washing step	Amount of water, etc.	Time
1	10 litres of coldwater	2.5 min
2	10 litres of cold water	25 min
3	3 ml of defoamer NEO DISHER A 8 detergent (alkaline) thermostat→80° C	8 min
4	10 litres of warm water 10 ml of NEO DISHER N (acid)	3 min
5	10 litres of warm water	2 min
6	10 litres of warm water	2 min
7	10 litres of warm water	2 min
8	5 litres of distilled water 10 litres of distilled water thermostat→70° C	1 min 4 min

They are then dried for 6 h at 300° C in a large cabinet **set aside exclusively for this purpose**.

Only exceptionally is a preliminary cleaning with acetone used.

The cleaning procedures given are sufficient for the usual concentrations of substances to be determined in this investigation (not lower than 0.1 µg.l⁻¹). For lower concentrations – for example in the analysis of field samples – a more thorough cleaning procedure is often necessary.

2.4.1 Fig. 17 Rinsing apparatus for bottles and separating funnels with a powerful jet of water. This apparatus is of course used in a sink. The jet of water is started by pressure on the circular plate

2.4.1 Appendix 7 The synthesis of diazomethane

Subject:

Preparation of a solution of diazomethane in diethyl ether.

Field of application

A solution of diazomethane in ether is used for methylating phenols and carboxylic acids. The methyl ethers of the phenols and methyl esters of the carboxylic acids so formed are more suited to gas-chromatographic determination than the original phenols and carboxylic acids.

Principle

Diazomethane is formed by adding a solution of N-methyl-N-nitroso-p-toluenesulphonamide (see references) in ether to a warm solution of alcoholic alkali.

Diazomethane is extremely toxic and carcinogenic. It may explode spontaneously even in solution. Use clean and smooth glassware for the synthesis. Rough surfaces accelerate decomposition. Store the solution in a refrigerator. Always use disposable gloves.

Operations with diazomethane must be carried out in an efficient fume cupboard and behind a safety screen.

Wear a face mask.

EQUIPMENT AND MATERIALS

In addition to the equipment given in this section, the following reagents, glassware, and auxiliary apparatus are needed.

Reagents

- N-Methyl-N-nitroso-p-toluenesulphonamide (e.g. Diazald, made by Messrs. Aldrich)
- potassium hydroxide pellets, analytical grade
- benzoic acid, no special grade.

Glassware

See first Note 3.

- 1 round-bottomed flask, 500 ml, with NS 29 ground joint
- 1 round-bottomed flask, 100 ml, with NS 29 ground joint
- 1 coil condenser, length 30 cm, with NS 29 and NS 19 ground joints (see 2.4.1, Fig. 18).
- 1 vacuum adaptor with NS 19 and NS 29 ground joints
- 1 measuring cylinder, 250-ml
- 1 conical flask, 250 ml, with ground-in stopper.

Auxiliary apparatus

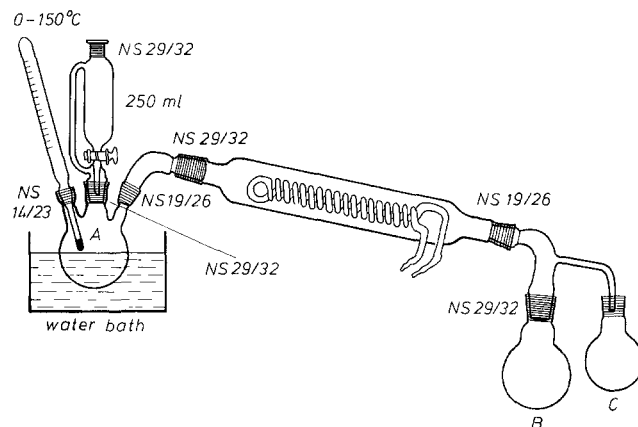
- 3 Suberit rings, dia. 13.5 cm
- 1 insertion thermometer, 0–150°C, with NS 14 ground joint.

PROCEDURE

The apparatus used is shown in 2.4.1, Fig. 18.

- Dissolve 5 g of potassium hydroxide in 8 ml of water in the three-necked round-bottomed flask A.
- Add to this 25 ml of 95 % ethanol.
- Introduce 20–30 ml of diethyl ether into flask C.
- Cool flasks B and C in ice water.
- By means of a water bath, warm the contents of flask A to 65°C.

- Dissolve 21.5 g of 'Diazald' in 200 ml of ether in a 250-ml conical flask.
- Transfer this solution into the dropping funnel.
- Add the Diazald solution to the warm alcoholic alkali dropwise over about 25 min.
Ether distils over with the yellow diazomethane into flask B.
- Take care that the rate of distillation is equal to the rate of addition of the Diazald solution.
- When the whole of the solution has been added, slowly run in another 40 ml of ether.
- Continue the distillation until the distillate dripping from the condenser is colourless.
The ethereal solution obtained contains about 3 g of diazomethane.
- Add about 10 g of anhydrous sodium sulphate to the solution.
- Store the solution in the **well-stoppered** flask B in the refrigerator.
- Destroy the contents of flask C by adding a small amount of benzoic acid until the solution is colourless.
- Check the purity of the diazomethane solution by adding, with a suction pipette, 5 ml of the diazomethane solution to 10 ml of cyclohexane in a 25-ml volumetric flask.
Allow it to stand for half an hour.
Evaporate off the excess of diazomethane under a current of nitrogen.
Make up to the mark and homogenize the contents of the volumetric flask.
Inject 1 µl of the solution into the gas chromatograph (for the conditions, see Section 2.4.3.2 or Section 2.4.3.6).
Check whether any unwanted peaks appear in the chromatogram.



2.4.1 Fig. 18 Apparatus for the synthesis of diazomethane

NOTES

1. Spontaneous detonation of the starting material, the Diazald, cannot be excluded. This must therefore be stored in a refrigerator, best in a chemicals vault.
For longer storage periods, it is wise first to recrystallize the Diazald from warm ether and then to store it in a cool place in the dark.
2. A solution of diazomethane can be safely stored in a refrigerator for 1 week.
3. As far as possible, use polished glass joints instead of ground joints (e.g. 'Klare Präzisions Verbindung', KPV, Duran 50, Schott).

2. Analysis of the investigation of degradability, toxicity, and accumulation

2.4.2 Neutral compounds

2.4.2.1 Direct gas-chromatographic determination of volatile neutral compounds in water

Subject

Direct gas-chromatographic determination of 1,2-dichloroethane and 1,1,1- and 1,1,2-trichloroethanes in water.

Field of application

The method is suitable for gas-chromatographic determination of 1,2-dichloroethane and 1,1,1- and 1,1,2-trichloroethanes in fresh water, brackish water, and sea water used in biological test systems. Range of concentrations: 0.2–100 mg.l⁻¹.

Sample size: at least ca. 0.1 ml

After slight adaptation, the instructions also apply to the determination of a number of other lower chloroalkanes (see Note 6).

Principle

The concentrations of 1,2-dichloroethane and of 1,1,1- and 1,1,2-trichloroethanes are determined by direct injection of the water to be analysed into the gas chromatograph.

A flame-ionization detector is used.

EQUIPMENT AND MATERIALS

In addition to the equipment stated in Section 2.4.1, the following are also needed:

- 1,2-dichloroethane, 99 % pure
- 1,1,1- and 1,1,2-trichloroethanes, 99 % pure.

PROCEDURE

- First read Section 2.4.1.
- In each series, make five calibration solutions in distilled water in the range of concentrations to be expected (see Note 7).
- Also make an analytical blank.
- From each calibration solution inject preferably not more than 5 µl into the gas chromatograph and subsequently the same volume from each sample (see Notes 3 and 4).

Set up the gas chromatograph as follows:

Column: 5 % of OV-101 on Chromosorb G-AW-DMCS, 80–100 mesh 4-metre stainless steel, external diameter $\frac{1}{8}$ ", internal ca. 4 mm.

Column temperature: for 1,1,1-trichloroethane and 1,2-dichloroethane 70°C;
for 1,1,2-trichloroethane 100°C.

Detector temperature: 200°C.

Inject directly above the column packing with a **gas-tight** injection syringe.

REPORTING OF THE RESULTS OF THE MEASUREMENTS

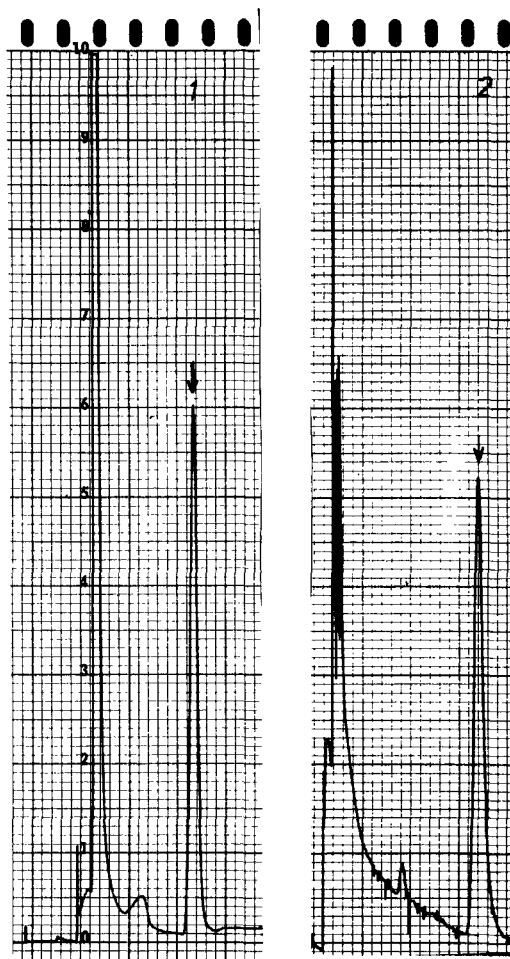
Give the concentrations in mg per litre of water.

Report any special features, such as the appearance of unexpected peaks in the chromatogram.

NOTES

1. 1,2-Dichloroethane, 1,1,1-trichloroethane, and 1,1,2-trichloroethane can, if necessary, be purified by distillation.
2. The samples must be kept in a refrigerator.

3. The maximum amount of water that can be injected depends on the type of the flame-ionization detector. Usually the injection of 25 µl of water is the maximum possible.
4. The same amount of water must always be injected both for the standards and for the samples, since the sensitivity of the flame-ionization detector can be affected by the presence of water. For example, if water and dichloroethane are present simultaneously the detector is less sensitive to the latter. Furthermore, the injection of water gives a disturbing signal that depends on the amount injected.
5. The first 10 cm of the column packing must be regularly renewed, since this part takes up the salt dissolved in the water and any other non-volatile constituents that may be present.
6. With a suitable stationary phase, a number of these compounds can frequently be determined simultaneously.
7. The standard solution in water must be made up at least 24 h before use.



2.4.2.1 Fig. 1 Example of a gas chromatogram obtained in the determination of 1,2-dichloroethane in water. The peak represents 440 ng of 1,2-dichloroethane derived from a sample of water with a concentration of 110 mg.l⁻¹.

2.4.2.1 Fig. 2 Example of a gas chromatogram obtained in the determination of 1,1,2-trichloroethane in water. The peak represents 352 ng of 1,1,2-trichloroethane derived from a sample of water with a concentration of 88 mg.l⁻¹.

2.4.2.2 Gas-chromatographic determination of volatile neutral compounds in water

Subject

Gas-chromatographic determination of 1,2-dichloroethanes and 1,1,1- and 1,1,2-trichloroethanes after their extraction from water.

Field of application

The method is suitable for the gas-chromatographic determination of 1,2-dichloroethane and 1,1,1- and 1,1,2-trichloroethanes in fresh water, brackish water, and sea water used in biological test systems.

Range of concentrations: 0.2–100 µg.l⁻¹.

Sample size: 100–1000 ml.

The instructions can be adapted to different sample sizes and/or concentrations (see Note 3).

After slight adaptation, the instructions are also suitable for the determination of chloro-, bromo-, and iodomethanes, -ethanes, and -ethenes.

Principle:

The 1,2-dichloroethane and/or 1,1,1- and/or 1,1,2-trichloroethane are extracted from the water with cyclohexane. After the extract has been dried, the concentrations of the above-mentioned compounds are determined by gas chromatography. Use is made of an electron capture detector.

EQUIPMENT AND MATERIALS

In addition to the equipment given in Section 2.4.1, the following are needed:

- 1,2-dichloroethane, 99% pure, and
- 1,1,1- and 1,1,2-trichloroethanes, 99% pure.

PROCEDURE

The procedure described here is suitable for 500-ml samples and a range of concentrations of 0.2–25 µg.l⁻¹ (see Note 3).

- First read Section 2.4.1.
- Place 14 one-litre separating funnels in the rack.
- Without using a funnel, transfer eight samples to eight separating funnels.
- Transfer the coding of the samples to the separating funnels.
- Using a measuring cylinder, transfer 500 ml of pure test medium into each of the six other separating funnels.
- Code these separating funnels as standards (× µl of γ ppm added) and as analytical blank + serial number.
- With a plunger pipette, add the desired amounts of standard solutions of the compound(s) to be determined in water to five of these six separating funnels. Choose five different concentrations for this purpose.
- Add no chlorohydrocarbons to the water in separating funnel 14. This is the analytical blank.
- Then, by means of a Kipp automatic dosing device, add 5 ml of cyclohexane to each of the separating funnels.
- Close each separating funnel firmly with the ground-in stopper, by placing it in the ground socket **wet** with a thrusting-turning motion.
- Shake briefly by hand and release the excess pressure through the tap.
- Place the separating funnels in the shaker.

- Shake for 60 min.
- Place fourteen 10-ml volumetric flasks under the separating-funnel rack.
- By means of a powder-dosing spoon and through a glass funnel (diameter 6 cm) add 1 g of anhydrous sodium sulphate to each volumetric flask.
- Then place on each volumetric flask a small glass funnel (diameter 3 cm).
- Apply to the volumetric flasks the same coding as on the separating funnels.
- After they have been shaken for an hour, replace the separating funnels in the rack.
- Allow the contents of each separating funnel to empty into a plastic bucket until there is about 1 cm of water above the tap.
- Break any emulsions present.
- Then carefully let the contents of each separating funnel run out into a 250-ml beaker until the interface of the two liquids is located in the bore of the tap.
- Dry the outflow tube of each separating funnel with a pipe cleaner.
- Allow the cyclohexane layers to run into the 10-ml volumetric flasks.
- Rinse each separating funnel with about 4 ml of cyclohexane from a glass wash-bottle.
- Run this cyclohexane also into the volumetric flasks.
- Place the volumetric flasks in a wooden block with suitable holes.
- Fill the volumetric flasks with cyclohexane up to the mark and close them.
- Homogenize the contents of each volumetric flask.
- Allow the extracts to stand over the sodium sulphate already present in the flask for **at least** an hour.
- The solutions are ready for gas-chromatographic analysis.

Set up the gas chromatograph as follows:

Column: 5% of OV-101 on Chromosorb G-AW-DMCS, 80–100 mesh
2-metre stainless steel column, external diameter $\frac{1}{8}$ ", internal diameter ca. 4 mm.

Column temperature: for 1,1,1-trichloroethane and 1,2-dichloroethane 70°C; for 1,1,2-trichloroethane 100°C.

Detector temperature: 200°C.

Carrier gas, auxiliary gas, and electric detector variables: optimum for dichloro- and/or trichloroethanes (see Section 2.1.1).

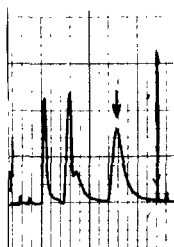
REPORTING OF THE RESULTS OF THE MEASUREMENTS

Give the concentrations in µg per litre of water.

Report any special features, such as the appearance of unexpected peaks in the chromatogram.

NOTES

1. 1,2-Dichloroethane and 1,1,1- and/or 1,1,2-trichloroethane can if necessary be purified by distillation.
2. The samples must be kept in the refrigerator.
3. When the volumes and/or concentrations differ, care must be taken that the amount of dichloro- and/or trichloroethane injected into the gas chromatograph is 25–4000 pg.



2.4.2.2 Fig. 1 Example of a gas chromatogram obtained in the determination of 1,1,2-trichloroethane in water. The peak corresponds to 1 ng of 1,1,2-trichloroethane derived from a sample of water with a concentration of $50 \mu\text{g.l}^{-1}$

2.4.2.3 Gas-chromatographic determination of non-volatile neutral organic compounds in water

Subject

Gas-chromatographic determination of dieldrin in water.

Field of application

The method is suitable for the determination of dieldrin in fresh water, brackish water, and sea water used in biological test systems. Range of concentrations: $0.05\text{--}50 \mu\text{g.l}^{-1}$.

Sample size: 100–1000 ml.

When the sample sizes and/or the concentrations differ, this procedure can be adapted (see Note 3).

After slight adaptation, the procedure is also suitable for the determination of other chlorine-containing pesticides (see Note 4).

Principle

Dieldrin is extracted from the water with cyclohexane. After drying of the extract, the concentration of dieldrin is determined gas-chromatographically with an electron capture detector.

EQUIPMENT AND MATERIALS

In addition to the requirements mentioned in Section 2.4.1, the following are needed:

- dieldrin, 99% pure
(1, 2, 3, 4, 10, 10-hexachloro-6, 7-epoxy-1, 4, 4a, 5, 6, 7, 8, 8a-octahydro-1,4-endo,exo-5,8-dimethanonaphthalene or HEOD).

PROCEDURE

The procedure described here is suitable for samples with a size of 500 ml in a range of concentrations of $0.1\text{--}10 \mu\text{g}$ per litre.

- First read Section 2.4.1.
- Place 14 one-litre separating funnels in the rack.
- Without using a funnel, transfer eight samples to eight separating funnels.
- Apply the coding of the samples to the separating funnels.
- Using a measuring cylinder, transfer 500 ml of pure test medium into each of the six remaining separating funnels.
- Code the separating funnels as standard ($x \mu\text{l}$ of $y \text{ ppm}$ added) and as analytical blank + serial number.
- Transfer the desired amounts of standard solutions of dieldrin in acetone into five of these six separating funnels.
- Choose five different concentrations for this purpose.

- Add no dieldrin to the water in separating funnel 14 but do add 200 μl of the acetone that is used for making the standard solution. This is the analytical blank.
- Subsequently, with a Kipp automatic dosing device, add 5 ml of cyclohexane to each of the separating funnels.
- Close each separating funnel firmly with the ground-in stopper by placing this **wet** in the ground socket with a thrusting-turning motion.
- Shake briefly by hand and relieve the excess pressure through the tap.
- Place the separating funnel in the shaker.
- Shake for 30 min.
- Place fourteen 10 ml volumetric flasks under the separating funnel rack.
- With a powder-dosing spoon, through a glass funnel (diameter 6 cm) add 1 g of anhydrous sodium sulphate to each volumetric flask.
- Place a smaller glass funnel (diameter 3 cm) on each volumetric flask.
- Transfer the coding from the separating funnels to the volumetric flask.
- After they have been shaken for half an hour, replace the separating funnels in the rack.
- Allow the contents of each separating funnel to run out into a plastic bucket until about 1 cm of water still remains above the tap.
- Break any emulsions that may be present.
- Then carefully allow the contents of each separating funnel to run into a 250-ml beaker until the interface of the two liquids is located in the bore of the tap.
- Dry the outflow tube of the separating funnel with a pipe cleaner.
- Allow the cyclohexane layer to run into the 10-ml volumetric flask.
- Rinse each separating funnel with about 4 ml of cyclohexane from a glass wash-bottle.
- Run this cyclohexane also into the volumetric flasks.
- Place the volumetric flasks in a wooden block with suitable holes.
- Fill the volumetric flasks with cyclohexane to the mark and close them.
- Homogenize the contents of each volumetric flask.
- Dry the extracts over sodium sulphate for **at least** 1 h.
- The extracts are now ready for gas-chromatographic analysis.

Set up the gas chromatograph as follows:

Column: 5% of OV-101 on Chromosorb G-AW-DMCS, 80–100 mesh

Glass; 220 cm $\times$ 3 mm internal diameter.

Column temperature: 200°C

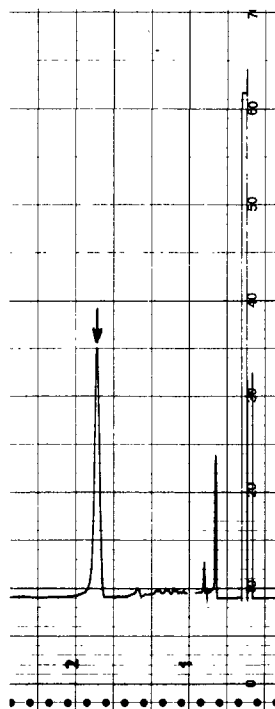
Detector temperature: 300°C

Carrier gas, auxiliary gas, and electric detector variables: optimum for dieldrin (see Section 2.1.1).

REPORTING OF THE RESULTS OF THE MEASUREMENTS

Give the concentrations in μg per litre of water.

Report any special features, such as the appearance of unexpected peaks in the chromatogram.



2.4.2.3 Fig. 1 Example of a gas chromatogram obtained in the determination of dieldrin in water. The peak represents 80 pg of dieldrin derived from a water sample of 500 ml with a concentration of $7.5 \mu\text{g.l}^{-1}$

NOTES

1. Dieldrin can be purified by recrystallization from ethanol.
2. According to our experience, samples can be stored safely for 2 weeks and their extracts safely for 4 weeks in the dark. They can best be processed within these periods.
3. When the volumes and/or concentrations differ, care must be taken that the amount of dieldrin injected into the gas chromatograph is between 2.5 and 250 pg.
4. After slight adaptation, the procedure is also suitable for the determination of other chlorinated pesticides (for example lindane, endrin, aldrin, Telodrin (isobenzan), heptachlor, heptachlor epoxide, endosulfan, pentachlorobenzene, hexachlorobenzene (HCB), pentachloronitrobenzene (PCNB), TDE, DDE, DDT, PCB's, octachlorostyrene, and hexachlorobutadiene. With a suitable stationary phase, it is often possible to determine a number of these compounds in the presence of one another.

2.4.3. Acidic compounds

2.4.3.1 Gas-chromatographic determination of highly electronegative phenols in water (acetylation method)

Subject

Gas-chromatographic determination of pentachlorophenol in water.

Field of application

The method is suitable for the determination of pentachlorophenol in fresh water, brackish water, or sea water used for biological tests.

Sample size: 100–1000 ml.

Range of concentrations: $0.05\text{--}50 \mu\text{g.l}^{-1}$.

When the sample sizes and/or concentrations differ, the procedure can be adapted (see Note 4). After slight adaptation, the procedure is also suitable for the determination of a number of other highly electronegative phenols (see Note 5).

Principle

The procedure is based on the work of Rudling (1970), Chau *et al.* (1974), and others.

The sample is buffered weakly alkaline by the addition of a borax solution. After the addition of acetic anhydride, pentachlorophenyl acetate is produced and this is extracted with cyclohexane. The pentachlorophenyl acetate is determined gas-chromatographically in the dried extract, using an electron capture detector.

EQUIPMENT AND MATERIALS

In addition to the equipment mentioned in Section 2.4.1, the following reagents and auxiliary glassware are needed:

Reagents

- Pentachlorophenol, 99 % pure.
- Pentachlorophenyl acetate, 99 % pure; the synthesis is described in Section 2.4.3.1, Appendix 1.
- Sodium tetraborate solution in water: ca. 40 g.l^{-1} .
With stirring and gentle heating, dissolve in 4 litres of distilled water in a large beaker:
163 g of anhydrous sodium tetraborate, $\text{Na}_2\text{B}_4\text{O}_7 \cdot \text{O H}_2\text{O}$ or
317 g of borax, $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10 \text{ H}_2\text{O}$.
Store the solution in 2.5-litre glass bottles with screw-caps. Avoid contact between the solution and the screw-cap as far as possible.

Glassware

- 1 Kipp automatic dosing device, 50-ml, with associated 1-l glass bottle.

PROCEDURE

The procedure described here is suitable for 500-ml samples and a range of concentrations of $0.1\text{--}10 \mu\text{g}$ per litre.

- First read Section 2.4.1.
- Place 14 one-litre separating funnels in the rack.
- Without using a funnel, transfer eight samples to eight separating funnels.
- Apply the coding of the samples to the separating funnels.
- Using a measuring cylinder, add 500 ml of pure test medium to each of the six other separating funnels.
- Code these separating funnels as standards ($x \mu\text{l}$ of $y \text{ ppm}$ added) and as analytical blank + serial number.
- By means of a plunger pipette, add to five of these six separating funnels the desired amounts of standard solutions of pentachlorophenol in acetone.
- Choose five different concentrations for this purpose.
- Add no pentachlorophenol to the water in separating funnel 14, but do add 200 μl of the acetone that has been used for preparing the standard solutions. This is the analytical blank.
- Using a Kipp automatic dosing device, then add successively to **each** separating funnel:

2. Analysis of the investigation of degradability, toxicity, and accumulation

- 50 ml of 4 % borax solution,
- 1 ml of acetic anhydride,
- 5 ml of cyclohexane.
- Rinse the neck of each separating funnel with a small amount of water from a glass wash-bottle.
- Firmly close each separating funnel with the ground-in stopper by placing this **wet** in the ground socket with a thrusting-turning movement.
- Shake briefly by hand and relieve the excess pressure through the tap.
- Place the separating funnels in the shaker.
- Shake for 30 min.
- Place fourteen 10-ml volumetric flasks under the separating funnel rack.
- Using a powder-dosing spoon, add through a glass funnel (diameter 6 cm) 1 g of anhydrous sodium sulphate to each volumetric flask.
- Place a small glass funnel (diameter 3 cm) on each volumetric flask.
- Transfer the coding from the separating funnels to the volumetric flasks.
- After they have been shaken for half an hour, replace the separating funnels in the rack.
- Allow the contents of each separating funnel to run into a plastic bucket until there is still about 1 cm of water above the tap.
- Break any emulsions that may be present.
- Then carefully let the contents of each separating funnel run into a 250-ml beaker until the interface of the two liquids is located in the bore of the tap.
- Dry the outflow tube of each separating funnel with a pipe cleaner.
- Allow the cyclohexane layers to run into the 10-ml volumetric flasks.
- Rinse each separating funnel with about 4 ml of cyclohexane from a glass wash-bottle.
- Run this cyclohexane also into the volumetric flasks.
- Place the volumetric flasks in a wooden block with suitable holes.
- Fill the volumetric flasks with cyclohexane up to the mark and close them.
- Homogenize the contents of each volumetric flask.
- Dry extracts over anhydrous sodium sulphate for **at least** 1 h.
- The extracts are now ready for gas-chromatographic analysis.

Set up the gas chromatograph as follows:

Column: 5 % OV-101 on Chromosorb G-AW-DMS, 80–100 mesh Glass, 220 mm × 3 mm internal diameter.

Column temperature: 180° C.

Detector temperature: 300° C.

Carrier gas, auxiliary gas, and electric detector variables: optimum for pentachlorophenyl acetate (see Section 2.1.1).

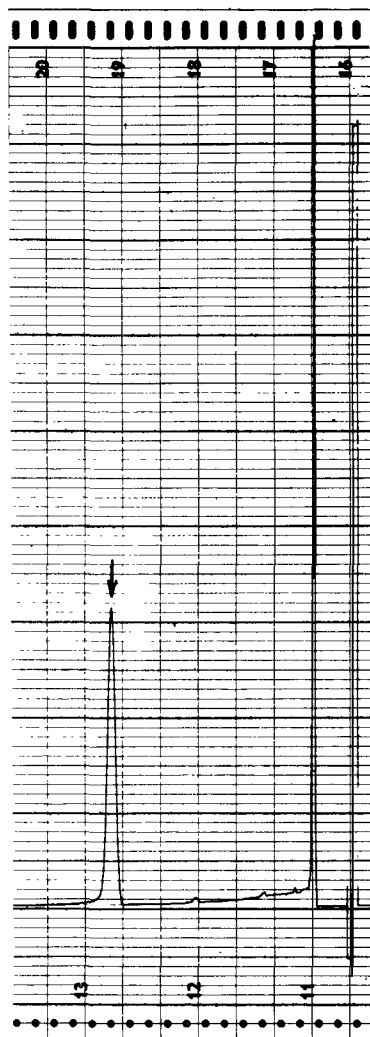
REPORTING OF THE RESULTS OF THE MEASUREMENTS

Give the concentrations in µg per litre of water.

Report any special features, such as the appearance of unexpected peaks in the chromatogram.

NOTES

1. The method is preferred to the methylation method (see Section 2.4.3.2) for reasons of occupational health, since the toxic and carcinogenic diazomethane must be used as a reagent in the latter.
2. If necessary, the pentachlorophenol can be purified by recrystallization from toluene.
3. According to our experience, samples can be stored safely for 2 weeks and extracts for 4 weeks in the dark. They can best be processed within these periods.
4. With different volumes and concentrations care must be taken that the amount of pentachlorophenyl acetate injected into the gas chromatograph is between 2.5 and 250 pg.
5. Only small adaptations of the procedure are necessary for the determination of tri- and tetrachlorophenols. With a suitable stationary phase a number of these compounds can frequently be determined in the presence of one another.



2.4.3.1 Fig. 1 Example of a gas chromatogram obtained in the determination of pentachlorophenol as pentachlorophenyl acetate in water. The peak represents 125 µg of pentachlorophenol, derived from a water sample with a concentration of 10 µg.l⁻¹

2.4.3.1 Appendix 1

Preparation of pentachlorophenyl acetate

Subject

Preparation of a small amount of pure pentachlorophenyl acetate.

Field of application

Pentachlorophenyl acetate is needed in the determination of pentachlorophenol by the acetylation method (Section 2.4.3.1). It is used as a standard in gas-chromatographic analyses, as a comparison material for identification, for the determination of extraction yields, etc.

Principle

Pentachlorophenol is dissolved in pyridine and is acetylated with acetic anhydride.

EQUIPMENT AND MATERIALS

In addition to the requirements mentioned in Section 2.4.1, the following reagents and auxiliary glassware are needed:

Reagents

- Pentachlorophenol, 99 % pure
- pyridine, analytical grade
- solution of sodium tetraborate in water, ca. 40 g.l⁻¹ (see Section 2.4.3.1)
- calcium chloride, anhydrous.

Glassware

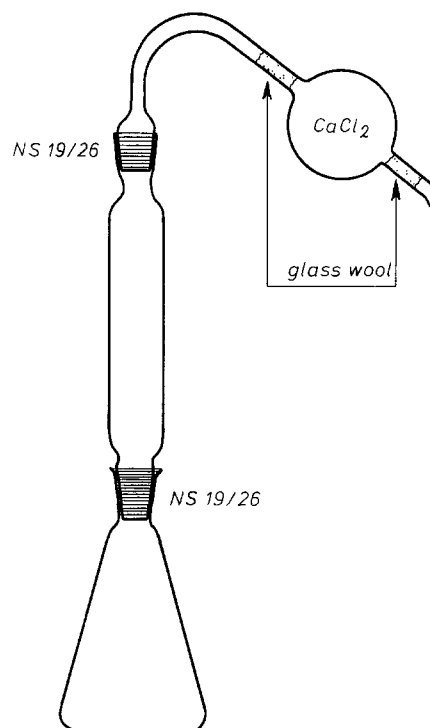
- 2 conical flasks, 100 ml, with ground-in stoppers
- air condenser, fitting on a 100-ml conical flask, length 30 cm, diameter 1.5–2 cm
- Squibb separating funnel, 100 ml, provided with a glass tap and glass stopper (see Section 2.4.1)
- calcium chloride tube.

PROCEDURE

The apparatus is shown in 2.4.3.1, Fig. 2.

- Dissolve 1 g of pentachlorophenol in 10 ml of pyridine in a 100-ml conical flask.
- With a measuring pipette, add 2 ml of acetic anhydride to this solution.
- Fit the air condenser, fitted with a calcium chloride tube, on the conical flask.
- Keep the mixture for 30 min in a water bath at 50°C with occasional shaking.
- Then cool in ice water to room temperature.
- Add 10 ml of distilled water.
- Extract the mixture with two 10-ml portions of diethyl ether in a 100-ml separating funnel.
- Wash the ether layer once with 15 ml of 4 % borax solution.
- Centrifuge the ether layer for 3 min at 2000 g.
- Transfer the ether layer into a 100-ml conical flask and evaporate the ether off at 60°C under a current of nitrogen.
- Take up the residue in a few ml of ethanol with warming on a water bath.
- If necessary, filter the solution in ethanol through a glass filter.
- Cool the solution in ice. White needle-like crystals appear.

- Filter off the crystals on a G3 sinter-glass filter.
- Dry the material in a vacuum desiccator at 80°C.
- Recrystallize from ethanol until the melting point of pentachlorophenyl acetate (149–150°C) has been reached.
- Store the substance in a refrigerator in a weighing bottle with ground-in stopper.
- Check the purity by gas chromatography and IR spectroscopy.



2.4.3.1 Fig. 2 Apparatus for the synthesis of pentachlorophenyl acetate

NOTE

The procedure is designed to yield a product of the highest possible purity. Consequently the yield is low: ca. 50%.

2.4.3.2 Gas-chromatographic determination of strongly electronegative phenols in water (methylation method)

Subject

Gas-chromatographic determination of pentachlorophenol in water.

Field of application

The method is suitable for the determination of pentachlorophenol in fresh water, brackish water, or sea water used for biological tests.

Sample size: 100–1000 ml.

Range of concentrations: 0.05–50 µg.l⁻¹.

When the sample sizes and/or concentrations are different, the procedure can be adapted (see Note 5). After slight adaptation, the procedure is also suitable for the determination of tri- and tetrachlorophenols (see Note 6).

The method can be used if for any reason the acetylation method (Section 2.4.3.1) is out of the question. This may be the case if, for example, in the latter method too many interfering peaks appear or when other compounds have to be determined at the same time.

2. Analysis of the investigation of degradability, toxicity, and accumulation

Principle

The sample is acidified to pH 3. Pentachlorophenol is extracted with cyclohexane. After the extract has been dried, the pentachlorophenol is methylated to pentachloroanisole with diazomethane. The pentachloroanisole is very suitable for gas-chromatographic analysis, provided that this is done with an electron capture detector.

Diazomethane is extremely toxic and carcinogenic. It may explode spontaneously, even in solution. Use clean and smooth glassware in the synthesis. Rough surfaces accelerate the decomposition. Store the solution in a refrigerator. Always use disposable gloves.

Operations with diazomethane must be performed in an efficient fume cupboard and behind a safety screen.

Wear a face mask.

MATERIALS

In addition to the materials mentioned in Section 2.4.1, the following are needed:

- pentachlorophenol, 99 % pure
- pentachloroanisole, 99 % pure
the synthesis is described in Section 2.4.3.2, Appendix 1
- 0.5 M sulphuric acid (see Section 2.5.4.1).

PROCEDURE

The procedure described here is suitable for 500-ml samples and a concentration range of 0.1–25 µg.l⁻¹ (see Note 5).

- First read Section 2.4.1.
- Place 14 one-litre separating funnels in the rack.
- Without using a funnel, transfer eight samples into eight separating funnels.
- Apply the coding of the samples to the separating funnels.
- Using a measuring cylinder, add 500 ml of pure test medium to each of the six remaining separating funnels.
- Code these separating funnels as standards (x µl of y ppm added) and as analytical blank + serial number.
- Using a plunger pipette, add the required amounts of standard solutions of pentachlorophenol in acetone to five of these six funnels.
- Choose five different concentrations for this purpose.
- Add no pentachlorophenol to the water in funnel 14 but do add 200 µl of the acetone that was used for preparing the standard solutions. This is the analytical blank.
- Using a Kipp automatic dosing device, add 5 ml of 0.5 M sulphuric acid to each of the separating funnels.
- Check the pH of the blank with a stirring rod and pH paper. The pH must not be higher than 3.
- Then, using a Kipp automatic dosing device, add 10 ml of cyclohexane to each of the separating funnels.
- Close each separating funnel tightly with the ground-in stopper by placing this **wet** in the ground socket with a thrusting-twisting movement.
- Shake briefly by hand and release the excess pressure through the tap.
- Place the separating funnels in the shaker.
- Shake for 30 min.
- Place fourteen 25-ml volumetric flasks below the separating funnel rack.

- With a powder-dosing spoon and through a glass funnel (diameter 6 cm) add 2 g of anhydrous sodium sulphate to each volumetric flask.
- Place a smaller glass funnel (diameter 3 cm) on each volumetric flask.
- Transfer the coding of the separating funnels to the volumetric flasks.
- After they have been shaken for half an hour, replace the separating funnels in the rack.
- Allow the contents of each separating funnel to run into a plastic bucket until there is still about 1 cm of water above the tap.
- Break any emulsions that may be present.
- Then carefully allow the contents of each separating funnel to run into a 250-ml beaker until the interface of the two liquids is located in the bore of the tap.
- Dry the outflow tube of each separating funnel with a pipe-cleaner.
- Allow the cyclohexane layers to run into the 25-ml volumetric flasks.
- Rinse each separating funnel with ca. 5 ml of cyclohexane from a glass wash-bottle.
- Run this cyclohexane also into the volumetric flasks.
- Place the volumetric flasks in a wooden block with suitable holes.
- Do NOT fill the volumetric flasks to the mark, but close them well.
- With a suction pipette (in the fume cupboard!) add 5 ml of a solution of diazomethane in ether to each volumetric flask.
- Allow the flasks to stand for 30 min.
- If the solution has become decolorized, add another 5 ml of diazomethane and continue in the same way until the yellow colour persists. Now pentachloroanisole has been formed.
- Blow off the excess of diazomethane with a current of nitrogen in a special apparatus in the fume cupboard until the contents of each flask become colourless. During this process the flasks cool down.
- Replace the flasks in the wooden block.
- Allow the contents of the flasks to warm up to room temperature.
- Fill each flask with cyclohexane to the mark.
- Close the flasks.
- Dry them over sodium sulphate for **at least** 1 h.
- Homogenize the contents of the volumetric flasks.

The extracts are now ready for gas-chromatographic analysis. Set up the gas chromatograph as follows:

Column: 5 % of OV-101 on Chromosorb G-AW-DMCS,
80–100 mesh
Glass; 220 cm × 3 mm internal diameter

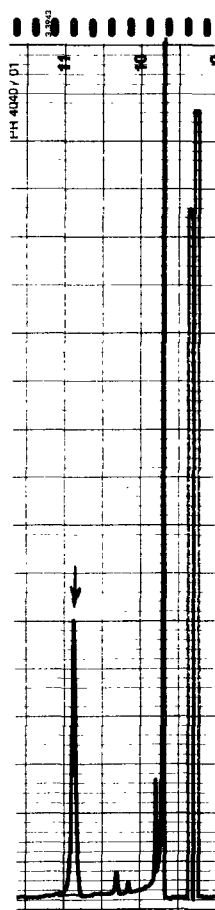
Column temperature: 180° C.

Detector temperature: 300° C.

Carrier gas, auxiliary gas, and electrical detector variables: optimum for pentachloroanisole (see Section 2.1.1).

REPORTING OF THE RESULTS OF THE MEASUREMENTS

Give the concentrations in µg per litre of water. Report any special features, such as the appearance of unexpected peaks in the chromatogram.



2.4.3.2 Fig. 1 Example of a gas chromatogram obtained in the determination of pentachlorophenol in the form of pentachloroanisole in water. The peak represents 120 pg of pentachlorophenol derived from a water sample having a concentration of $24 \mu\text{g.l}^{-1}$

NOTES

1. Only slight adaptations of the procedure are necessary for the determination of tri- and tetrachlorophenols (see Section 2.4.3.3).
2. Pure pentachloroanisole is necessary to determine the retention time of this substance and to optimize and check the sensitivity of the gas chromatograph. The substance is not available commercially. Its synthesis is described in Appendix 1 of this procedure.
3. If necessary, the pentachlorophenol can be purified by recrystallization from toluene.
4. When OV-101 is used as the stationary phase, hexachlorobenzene may interfere in the determination of pentachloroanisole, since these compounds have the same retention time on OV-101. The problem can be eliminated by using a different stationary phase.
5. When different volumes and/or concentrations are used, care must be taken that the amount of pentachloroanisole injected into the gas chromatograph is between 2.5 and 250 pg. In addition, the amounts of reagents must be adapted appropriately.
6. With a suitable stationary phase, a number of multiply-chlorinated phenols can frequently be determined simultaneously.

2.4.3.2 Appendix 1 Synthesis of pentachloroanisole

Subject:

Preparation of a small amount of pure pentachloroanisole.

Field of application:

Pentachloroanisole is an auxiliary in the determination of pentachlorophenol by the methylation method (Section 2.4.3.2). It is used as a standard in the gas-chromatographic analysis, as a comparison material for identification, as an aid for the determination of extraction yields, etc.

Principle

Pentachlorophenol is dissolved in dry ether and is methylated with a solution of diazomethane in ether.

Diazomethane is extremely toxic and carcinogenic. It may explode spontaneously, even in solution. Use clean and smooth glassware in the synthesis. Rough surfaces accelerate the decomposition. Store the solution in a refrigerator. Always use disposable gloves.

Operations with diazomethane must be performed in an efficient fume cupboard and behind a safety screen. Wear a face mask.

EQUIPMENT AND MATERIALS

In addition to the requirements mentioned in Section 2.4.1, the following reagents, glassware, and auxiliary apparatus are needed:

Reagents

- Pentachlorophenol, 99 % pure
- calcium chloride, anhydrous.

Glassware

- Coil condenser, length 30 cm, with NS 29 ground joints
- calcium chloride tube.

Auxiliary apparatus

- Insertion thermometer, -10 to 150°C , with NS 14 ground joint
- rotary evaporator, with vacuum attachment.

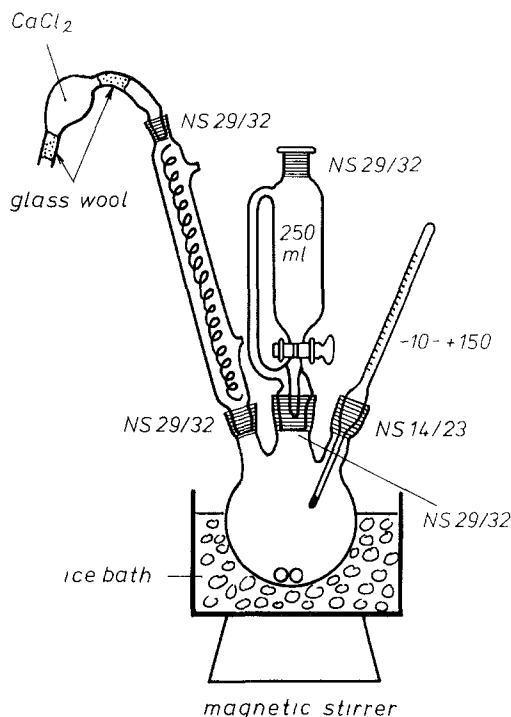
PROCEDURE

The apparatus used is shown in 2.4.3.2, Fig. 2.

- Place a tube containing calcium chloride on the coil condenser.
- Dissolve 2 g of pentachlorophenol in 50 ml of ether in a 500-ml three-necked flask.
- Cool this solution in an ice bath.
- With cooling and stirring on the magnetic stirrer, add through the dropping funnel a sufficient amount of a solution of diazomethane in ether (see Section 2.4.1) until the solution remains light yellow and no further evolution of gas takes place.
- Allow the mixture to stand for half an hour.
- Remove the dropping funnel.
- Place in the flask an inlet tube connected to a cylinder of nitrogen.
- Pass a slow current of nitrogen through the solution until this has become colourless again.
- Remove the condenser and the magnetic stirrer.
- Connect the flask to the rotary evaporator, applying a vacuum.
- Evaporate to dryness.

2. Analysis of the investigation of degradability, toxicity, and accumulation

- Dissolve the residue in the smallest possible volume of warm ethanol.
- Transfer the solution to a 100 ml beaker.
- Allow the pentachloroanisole to crystallize out.
- Recrystallize it from ethanol until the melting point of pentachloroanisole (108°C) has been reached.
- Filter off the residue under reduced pressure on a G3 sinter-glass filter.
- Dry it in a vacuum desiccator at 50°C.
- Check the purity by infrared spectrometry and gas chromatography.



2.4.3.2 Fig. 2 Apparatus for the synthesis of pentachloroanisole

2.4.3.3 Gas-chromatographic determination of weakly electronegative phenols in water

Subject:

Gas-chromatographic determination of (chlorinated) phenols in water.

Field of application:

The method is suitable for the determination of (chlorinated) phenols in fresh water, brackish water, or sea water used in biological test systems.

Sample size: minimum 50 ml.

Range of concentrations: 0.1–100 mg.l⁻¹

Principle:

After the addition of acetic anhydride to the sample, the phenols are converted into acetates which are then extracted with cyclohexane (see Notes 1 and 3).

These extracts are suitable for quantitative gas-chromatographic analysis with a flame-ionization detector.

EQUIPMENT AND MATERIALS

In addition to the requirements mentioned in Section 2.4.1, the following reagents, glassware, and auxiliary apparatus are also needed:

Reagents

- The (chloro)phenol to be determined, 99 % pure
- 4 % borax solution (see Section 2.4.3.1)
- 10 M caustic soda solution
 - with stirring, dissolve 420 g of sodium hydroxide (95 %) in water and make up to 1 litre
 - keep the solution in a glass bottle with a screw-cap. Avoid contact between the solution and the screw-cap as far as possible.

Glassware

- Volumetric flask, 1-litre, with ground-in stopper
- at least 14 Squibb conical separating funnels, 100 ml, with glass taps and glass stoppers
- plunger pipette, 0.5 ml, with matching disposable tips.

Auxiliary apparatus

- Separating funnel rack for at least fourteen 100-ml separating funnels
- shaker taking at least fourteen 100-ml separating funnels.

PROCEDURE

The procedure is based on the Schotten-Baumann reaction (see Higginbottom *et al.*, 1965, and Menalda, 1930).

The procedure described here is suitable for 50-ml samples.

- First read Section 2.4.1.
- To each 50-ml sample, immediately after the sample has been taken, add 0.5 ml of 10 M caustic soda with a plunger pipette (see Note 2).
- Place fourteen 100-ml conical separating funnels in a separating funnel rack.
- Without using a funnel, add eight samples to eight separating funnels.
- Transfer the coding from the samples to the separating funnels.
- Using a measuring cylinder, add 50 ml of pure test medium to each of the six other separating funnels.
- Code these separating funnels as well, as standards (x µl of y ppm added) and as analytical blank + serial number.

Using a pipette, add the desired amounts of standard solutions of the (chloro)phenol in acetone to five of the six funnels. Choose five different concentrations for this purpose.

- Add no (chloro)phenol to the water in funnel 14, but do add 200 µl of the acetone that was used for making up the standard solutions. This is the analytical blank.
- Add successively by means of a Kipp automatic dosing device to all the funnels:
 - 1 ml of acetic anhydride
 - 5 ml of 4 % borax solution, and
 - 5 ml of cyclohexane.
- Rinse the neck of each separating funnel with a small amount of water from a glass wash-bottle.
- Close each separating funnel firmly with the ground-in stopper by inserting this **wet** in the ground socket with a thrusting-turning movement.
- Shake briefly by hand and release the excess pressure through the tap.
- Place the separating funnels in the shaker.
- Shake for at least 3 h.
- Place fourteen 10-ml flasks below the separating funnel rack.

- Apply to these volumetric flasks the same coding as to the separating funnels.
- Place a glass funnel with a diameter of 3 cm on each volumetric flask.
- After shaking, replace the separating funnels in the rack.
- Allow the contents of each separating funnel to run into a plastic bucket until there is still about 1 cm of water above the tap.
- Break any emulsions present (see Section 2.4.1).
- Then carefully allow the contents of each separating funnel to run into a 250-ml beaker until the interface of the two liquids is located in the bore of the tap.
- Dry the outlet tube of each separating funnel with a pipe-cleaner.
- Allow the cyclohexane layers to run into the 10-ml volumetric flasks.
- Rinse each separating funnel with ca. 4 ml of cyclohexane from a glass wash-bottle.
- Run this cyclohexane also into the volumetric flasks.
- Fill each volumetric flask with cyclohexane to the mark and close it.
- Place the flasks in a wooden block with suitable holes.
- Homogenize the contents of each volumetric flask.
- The extracts are now ready for gas-chromatographic analysis.

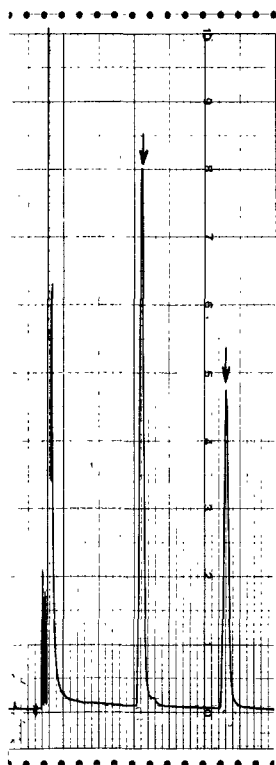
Set up the gas chromatograph as follows:

Column: 5% of OV-101 on Chromosorb G-AW-DMCS, 80–100 mesh.

Glass; 220 cm x 3 mm internal diameter.

Column temperature: 90°C.

Detector temperature: 250°C.



2.4.3.3 Fig. 1 Example of a gas chromatogram obtained in the determination of phenol and *p*-chlorophenol in the form of acetates in water. The peaks represent 1 µg each of phenol and *p*-chlorophenol derived from a water sample having a concentration of 50 mg.l⁻¹

REPORTING OF THE RESULTS OF THE MEASUREMENTS

Give the concentrations in mg per litre of water.

Report any special features such as the appearance of unexpected peaks in the chromatogram.

NOTES

1. The method described here is preferred to the methylation method, which requires the toxic and carcinogenic diazomethane.
2. The samples can be stored safely in the dark at room temperature for 2 weeks provided they are made alkaline.
3. Diisopropyl ether is also a very suitable solvent, particularly in the determination of polyhydric phenols.

2.4.3.4 Automated photometric determination of (chlorinated) phenols

Subject

Determination of (chlorinated) phenols in water.

Field of application

The method is directly applicable to the determination of (chlorinated) phenols in fresh water, brackish water, and sea water in the absence of interfering components. In the presence of interfering components the method may be usable after the application of extrapolation procedures.

Sample size: minimum 0.5 ml.

Range of concentrations: 200 µg.l⁻¹ and higher.

Principle

The (chlorinated) phenol present in the sample is nitrated with nitric acid. The subsequent treatment with alkali leads to the formation of picric acid or related coloured chloronitrophenols.

The optical density, which is a measure of the (chloro)phenol concentration of the sample, is compared with those of standard solutions treated under the same conditions.

EQUIPMENT AND MATERIALS

Glassware, general

- Weighing bottle
- Pasteur pipette.

Glassware, calibrated

- Volumetric flasks with calibration mark and ground-in stopper, 1000 ml
- volumetric flasks with calibration mark and ground-in stopper, 250 ml
- burette, 25 ml.

Various items of auxiliary apparatus, general

- Refrigerator
- closed cupboard for storing samples and solutions in the dark
- felt-tip pen for marking glassware; the ink must be capable of being removed in a washing machine
- spatula.

Various items of auxiliary apparatus, calibrated

- Analytical balance, accurate to 0.1 mg
- ruler
- millimetre paper.

2. Analysis of the investigation of degradability, toxicity, and accumulation

Analyser

AutoAnalyzer type, manufactured by Technicon, consisting of the following modules:

- a sample-feed device, 20 samples per hour; the sample is aspirated for 36 sec and rinsing is carried out for 144 sec
- sample vessels, 2 ml
- a proportional pump provided with 9 tubes of different diameters
- three glass mixing coils
- a dialyser, temperature 37°C
- a heating bath, temperature 90°C
- a cooler
- a spectrophotometer provided with an interference filter with maximum transmission at 400 nm and a flow-through cell with an optical path length of 50 mm
- a recorder.

The scheme of analysis is included in the 'Procedure'.

Reagents and auxiliary materials

- Water, distilled, de-ionized
- sea water, synthetic
- (chlorinated) phenols
- water with 0.5 ml of Aerosol-22* per litre
- sea water with 0.5 ml of Aerosol-22 per litre
- sodium hydroxide solution, 4 g per litre (water)
- sodium hydroxide solution, 4 g per litre (sea water)
- sodium hydroxide solution, 160 g per litre
- sodium hydroxide solution, 160 g per litre, with 0.5 ml of Aerosol-22 and 3.7 g of Na₂EDTA.2H₂O per litre

- dilute nitric acid (1:1) with 1 g of sodium nitrite per litre

- standard solutions of (chlorinated) phenols in water or sea water. Preparation of stock solutions: dissolve 50 mg of (chlorinated) phenol in sodium hydroxide solution (4 g per litre of water or sea water) in a 1000-ml volumetric flask. Make up with the same solution of alkali to 1000 ml.

Record on the flask:

- date of preparation of the solution
- solute
- solvent
- concentration.

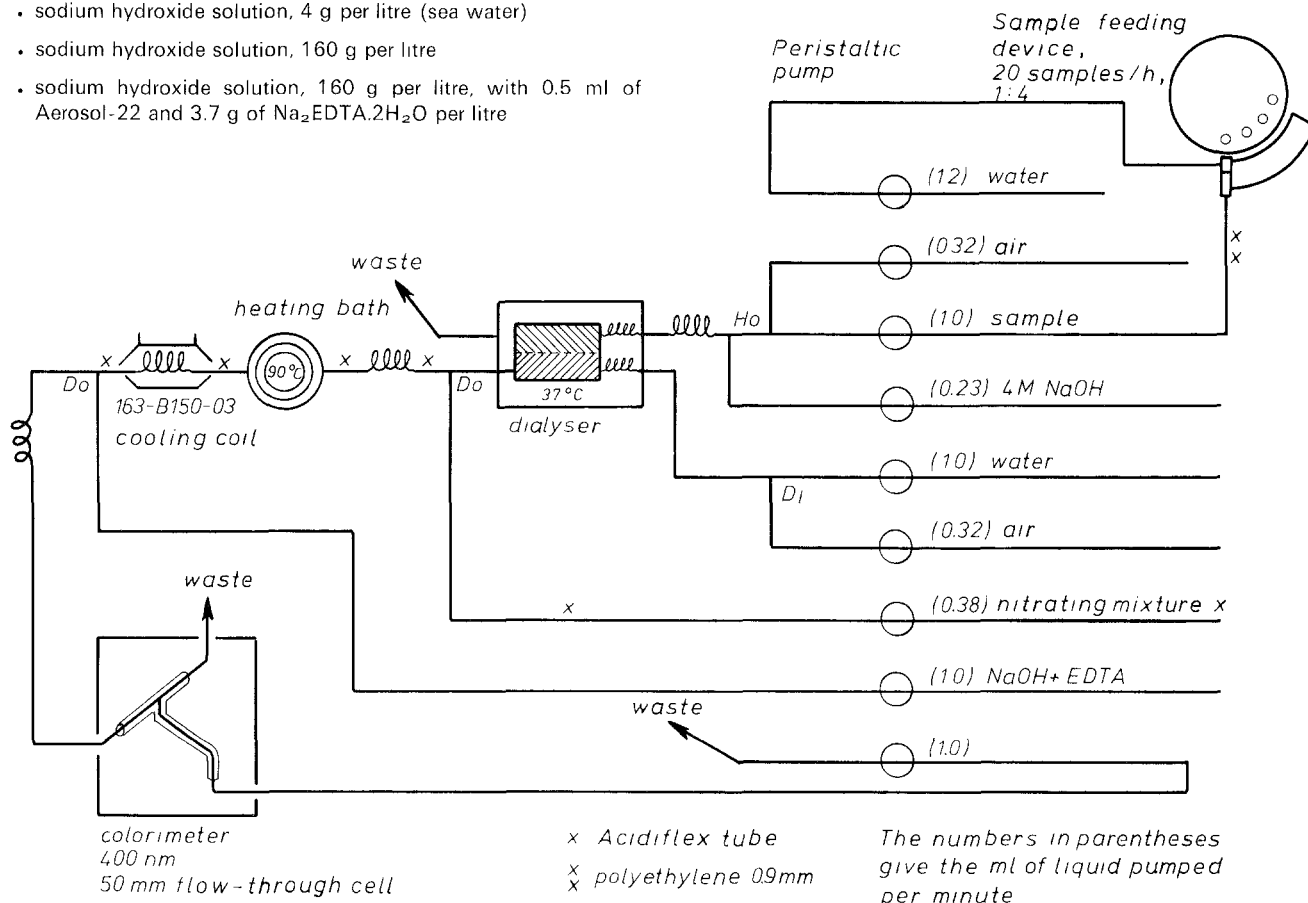
Store the stock solutions in a refrigerator. From these stock solutions, dilutions in sodium hydroxide solution (4 g per litre of water or sea water) are made up in 250-ml volumetric flasks to the concentrations necessary for the standard solutions to be used (see under 'Procedure').

These diluted solutions are also coded and stored in a refrigerator; they are renewed each month.

PROCEDURE

The procedure described here is suitable for samples of water of at least 0.5 ml with a concentration range of (chlorinated) phenols of 200 to 5000 µg.l⁻¹. At higher concentrations the samples must be diluted manually or, in the case of large numbers, by means of a special device provided in the AutoAnalyzer.

The scheme of analysis is shown in 2.4.3.4, Fig. 1.



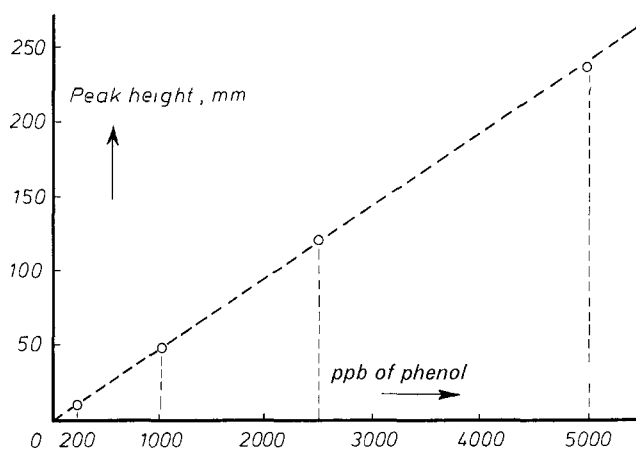
2.4.3.4 Fig. 1 Scheme of analysis for the AutoAnalyzer determination of (chloro)phenols in water

* Aerosol-22 is a wetting agent from Technicon.

2.4 Methods for the determination of individual test compounds in water

Preparation of standard solutions of (chlorinated) phenols

- Charge 250-ml volumetric flasks with 0, 1, 5, 12.5, and 25 ml of the stock solution of the (chlorinated) phenol and make up with sodium hydroxide solution (4 g per litre in water or sea water) to 250 ml. These standard solutions contain respectively 0, 200, 1000, 2500, and 5000 µg of (chlorinated) phenol per litre.
- If the samples to be analysed were kept in the refrigerator, allow them to warm up to room temperature on a laboratory bench.
- Then start the AutoAnalyzer except for the sample-feed device. The sample-aspiration needle must be located in the vessel with rinsing liquid.
- As soon as the baseline runs quite straight and parallel to the x-axis, insert a series of standard solutions of (chlorinated) phenols in increasing concentrations in coded 2-ml vessels into the sample-feeding device.
- After these, place the test-samples in the relevant coded vessels in the sample-feed device.
After each group of about 30 samples another series of standard solutions is inserted. After the last sample the group is completed with another series of standard solutions.
- Now start up the sample-feed device, whereupon all the standard solutions and samples are drawn in successively, which, after some time (the running time), results in an equal number of peaks on the recorder chart. Identify these peaks with the codes of the corresponding vessels.
- At the end of the analysis, measure the heights of the peaks relating to the standard solutions in each series and plot a calibration curve (see 2.4.3.4, Fig. 2).



2.4.3.4 Fig. 2 Calibration curve used in the AutoAnalyzer determination of (chloro)phenols in water

- Read off the sample concentrations from this curve.
(Some modern pocket calculators have the capability for linear regression analysis. With these it is possible to carry out linear interpolations without drawing the curve).

REPORTING OF THE RESULTS OF THE MEASUREMENTS

Give the concentrations in µg of (chlorinated) phenol per litre and report any special features observed during or before the analysis.

Check on errors of measurement due to interfering components

The most prominent sources of error are:

1. The colour of the sample.

2. The presence of other substances that, with the reagents, form compounds that also absorb at 400 nm.

For checking errors of measurement, see the discussion in Section 2.1.2.4.

Actually in a large series of closely related samples this check need not be repeated for each sample if errors of measurement have been found to be absent.

NOTES

1. On completion of the analyses, the AutoAnalyzer system must be rinsed with water. This is achieved by suspending all the feed tubes in water and continuing the pumping for a further 20 min.
2. After switching off of the AutoAnalyzer, the pump must be opened and the packet of tubes be relaxed.
3. The method is suitable for the determination of most (chlorinated) phenols; however, the following cannot be determined: 2,4,5-trichlorophenol, 2,4,6-trichlorophenol, 3,4,5-trichlorophenol, 2,3,4,5-tetrachlorophenol, 2,3,4,6-tetrachlorophenol, and pentachlorophenol.
4. If only one (chlorinated) phenol is present in a sample, it can be determined directly with the aid of a calibration curve based on standard solutions of the same compound. If samples containing mixtures of chlorophenols are involved, this is no longer quite the case. Each chlorophenol shows a molecular extinction and a maximum in the absorption spectrum the values of which differ for the different compounds.

2.4.3.5 Automated photometric determination of trichloroacetic acid

Subject

Determination of trichloroacetic acid in water.

Field of application

The method is directly applicable to the determination of trichloroacetic acid in fresh water, brackish water, and sea water in the absence of interfering components. In the presence of interfering components the method may be usable after the application of extrapolation procedures.

Sample size: minimum 1.5 ml.

Range of concentrations: 25 ppb = 25 µg.l⁻¹ and higher.

Principle

The trichloroacetic acid present in the sample is subjected to the Fujiwara reaction, in which it is coupled with pyridine under the influence of sodium hydroxide. The optical density of the water sample treated in this way is then measured and is compared with that of standard solutions treated under the same conditions.

EQUIPMENT

Glassware, general

- Weighing bottle.

Glassware, calibrated

- Volumetric flasks with calibration marks and ground-in stoppers, 1000 ml
- volumetric flasks with calibration marks and ground-in stoppers, 250 ml
- burette, 25 ml.

Various items of auxiliary apparatus, general

- Refrigerator
- closed cupboard for the storage of samples and solutions in the dark

2. Analysis of the investigation of degradability, toxicity, and accumulation

- felt-tip pen for marking glassware; the ink must be capable of being removed in a washing machine
- spatula.

Various items of auxiliary apparatus, calibrated

- Analytical balance, accurate to 0.1 mg
- ruler
- millimetre paper.

Analyser

AutoAnalyzer type, manufactured by Technicon, consisting of the following modules:

- a sample-feeding device, 20 samples per hour: the samples are aspirated for 108 sec and rinsing is carried out for 72 sec
- sample vessels, 2 ml
- a proportioning pump provided with eight tubes of different diameters
- two glass mixing coils (double length)
- a cooling spiral
- a heating bath, 95°C
- a spectrophotometer provided with an interference filter having a maximum transmission at 366 nm and a flow-through cell with an optical path length of 50 mm
- a recorder,

The scheme of analysis is included in the 'Procedure'.

Reagents and auxiliary material

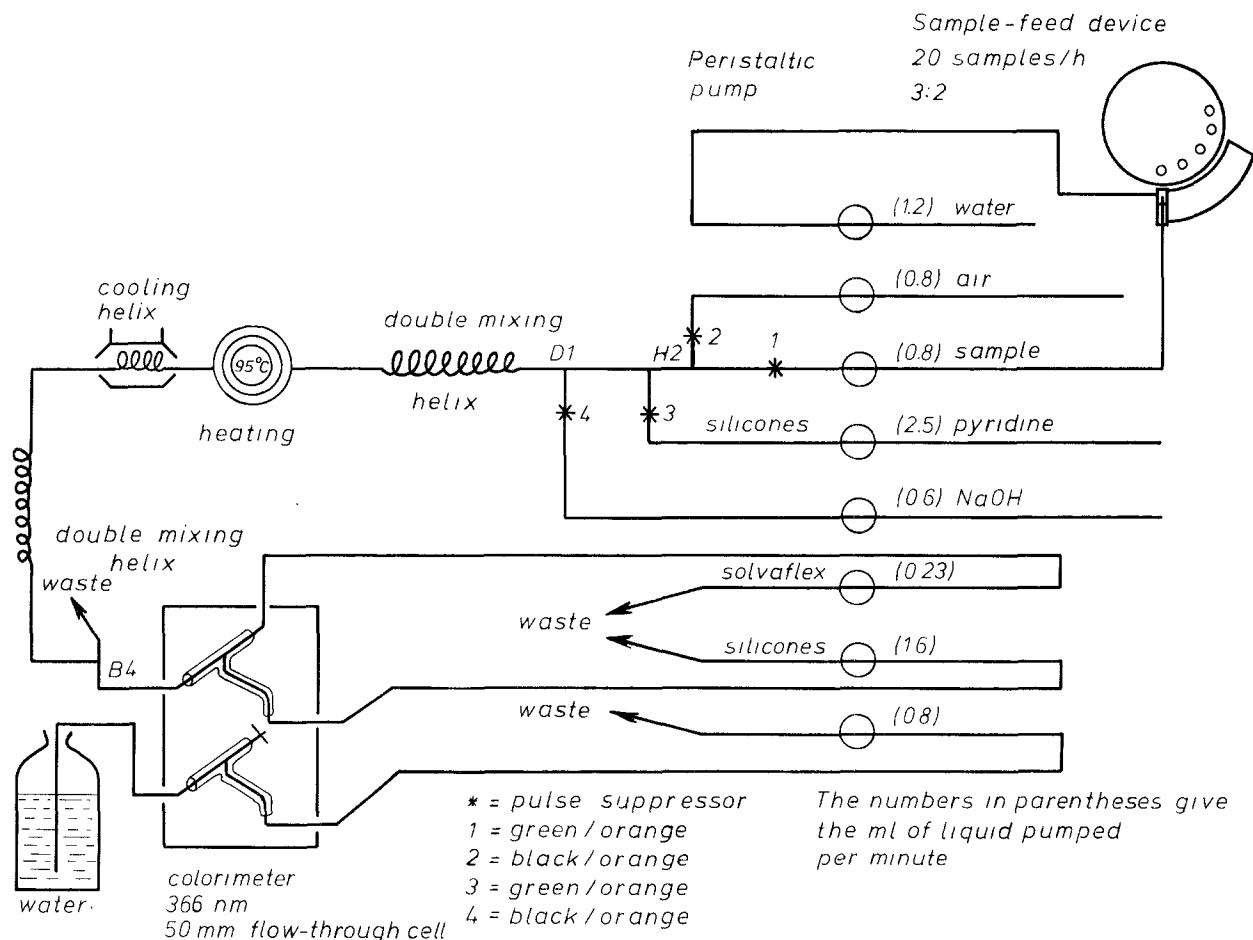
- Water, distilled or de-ionized
- trichloroacetic acid, analytical grade
- solution of sodium hydroxide in water, 3 g per litre
- standard solutions of trichloroacetic acid in water.
Make a stock solution of trichloroacetic acid. For this purpose dissolve 12.5 mg of the compound in water in a 1000-ml volumetric flask and make the volume up to precisely 1000 ml. Record on the measuring flask:
 - date of preparation of the solution
 - solute
 - solvent
 - concentration.

Store the stock solution in a refrigerator. From this stock solution, in 250-ml volumetric flasks, make up dilutions in water to the concentrations required for the standard solutions to be used (see under 'Procedure').

These diluted solutions are also coded and can be kept in a refrigerator for two weeks. After this, they must be discarded.

PROCEDURE

The procedure described here is suitable for water samples with a size of at least 1.5 ml and a range of concentrations of trichloroacetic acid of 25 to 500 $\mu\text{g.l}^{-1}$. At higher concentrations the samples must be diluted by hand or, in the case of large numbers, by means of a special device provided in the AutoAnalyzer.

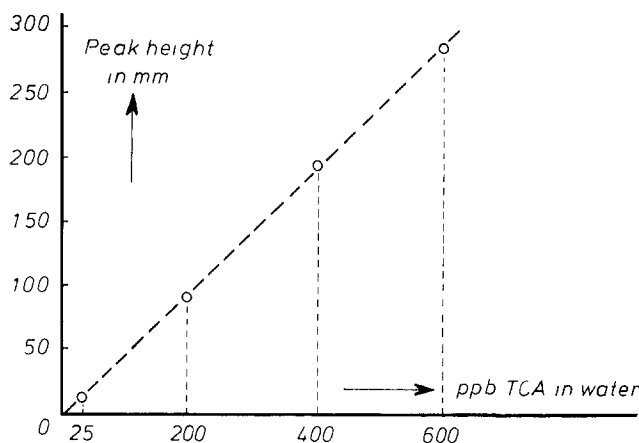


2.4.3.5 Fig. 1 Analytical scheme for the AutoAnalyzer determination of trichloroacetic acid in water

2.4 Methods for the determination of individual test compounds in water

Prepare standard solutions of trichloroacetic acid in water as follows:

- Charge 250-ml volumetric flasks with 0, 0.5, 4, 8, and 12 ml of the stock solution of trichloroacetic acid and make up with water to 250 ml. These standard solutions contain respectively 0, 25, 200, 400, and 600 $\mu\text{g.l}^{-1}$ of trichloroacetic acid.
- If the samples to be analysed have been stored in a refrigerator, allow them to warm up to room temperature on a laboratory bench.
- Then start the AutoAnalyzer except for the sample-feed device. The sample-aspiration needle must be present in the vessel with the rinsing liquid.
- As soon as the baseline is satisfactorily straight and parallel to the x-axis, transfer a series of standard solutions of trichloroacetic acid with increasing concentrations in coded 2-ml vessels into the sample-feed device.
- Then place the samples to be analysed in the coded vessels intended for them in the sample-feed device. After each group of approximately 30 samples another series of standard samples is included. After the last sample, the group is completed with another series of standard samples.
- Now start up the sample-feed device as well, so that all the standard solutions and samples are drawn in successively which, after some time (the running time) results in an equal number of peaks on the recorder chart. Identify these peaks with the codes of the corresponding vessels.
- On completion of the analyses, measure the heights of the peaks relating to each series of standard solutions and plot a calibration curve (see 2.4.3.5, Fig. 2).



2.4.3.5 Fig. 2 Calibration curve used in the AutoAnalyzer determination of trichloroacetic acid in water

- With the aid of this calibration curve read off the concentrations of trichloroacetic acid. (Some modern pocket calculators have the capability for linear regression analysis. It is then possible to carry out linear interpolations without drawing the curve).

REPORTING OF THE RESULTS OF THE MEASUREMENTS

Give the concentrations in μg of trichloroacetic acid per litre and report any special features observed during or before the analysis.

Check on errors of measurement due to interfering components

The most important sources of error are:

1. The colour of the sample;

2. Presence of other substances reacting with the reagents to form compounds that also absorb at 366 nm.

For checking the errors of measurement, see the discussion in Section 2.1.2.4.

Naturally, in a large series of closely related samples this check need not be repeated for each sample if errors of measurement appear to be absent.

NOTES

1. Take comprehensive measures against the toxic pyridine vapour. For this purpose place the AutoAnalyzer in a fume cupboard or draw off the vapour in some other way. Pass the tubes carrying concentrated pyridine through other tubes of larger diameter; this gives better protection.
2. The stock of pyridine must be stored in well-closed brown bottles.
3. At the end of the analyses the AutoAnalyzer system must be rinsed out with water. This is achieved by suspending all the feed tubes in water and continuing pumping for about 20 min.
4. After the switching off of the AutoAnalyzer, the pump must be opened and the bundle of tubes be relaxed.
5. If only trichloroacetic acid is present in a sample, it can be determined directly with the aid of a calibration graph constructed with standard solutions of the same compound. If the samples contain mixtures of chlorinated hydrocarbons, this is no longer quite the case. A number of chlorinated hydrocarbons show molecular extinction and a maximum in the absorption spectrum, the values of which differ for different compounds. (This relates particularly to chlorinated hydrocarbons in which 3 or 4 Cl atoms are bound to one C atom).

2.4.3.6 Gas-chromatographic determination of highly electronegative aliphatic carboxylic acids in water.

Subject

Gas-chromatographic determination of dichloro- and trichloroacetic acid in water.

Field of application

The method is suitable for the determination of dichloro- and trichloroacetic acid in fresh water, brackish water, or sea water used for biological tests.

Sample size: 100–1000 ml.

Range of concentration: dichloroacetic acid 0.5–50 $\mu\text{g.l}^{-1}$; trichloroacetic acid 0.1–30 $\mu\text{g.l}^{-1}$.

When the sizes and/or concentrations of the samples are different, the procedure can be adapted (see Note 4). After slight adaptation, this procedure is also suitable for the determination of a number of brominated, iodinated, and multiply-chlorinated acetic and propionic acids (see Note 5).

Principle

The sample is brought to a pH between 0 and 0.2. The dichloro- and/or trichloroacetic acids are then extracted with isobutyl acetate. The methyl esters of the above-mentioned compounds are formed with the aid of diazomethane. These can be determined by gas chromatography using an electron capture detector.

Diazomethane is extremely toxic and carcinogenic. It may explode spontaneously, even in solution. Use clean and smooth glassware in the synthesis. Rough surfaces accelerate the decomposition. Store the solution in a refrigerator. Always use disposable gloves.

Operations with diazomethane must be performed in an efficient fume cupboard and behind a safety screen.

Wear a face mask.

2. Analysis of the investigation of degradability, toxicity, and accumulation

MATERIALS

In addition to the requirements in Section 2.4.1, the following reagents are also needed:

Reagents

- Isobutyl acetate, analytical grade
- dichloroacetic acid, 99 % pure
- methyl dichloroacetate, 99 % pure
- trichloroacetic acid, 99 % pure
- methyl trichloroacetate, 99 % pure
- 5 M sulphuric acid in water.
With stirring, pour 500 ml of 96 % sulphuric acid into 1600 ml of distilled water in a large beaker.
Work in a fume cupboard and wear gloves and protective goggles.
Keep the solution in a narrow-mouth 2.5-litre glass bottle with ground-in stopper.
Transfer part of the solution to a 1-litre glass bottle fitted with a 50-ml Kipp automatic dosing device.

PROCEDURE

The procedure described here is suitable for 500-ml samples and a range of concentrations from 0.1 to 10 $\mu\text{g.l}^{-1}$ (see Note 4).

- First read Section 2.4.1.
- Place 14 one-litre separating funnels in the rack
- Without using a funnel, transfer eight samples to eight separating funnels.
- Transfer the coding of the samples to the separating funnels.
- Using a measuring cylinder, transfer 500 ml of pure test medium into each of the other six separating funnels.
- Code these separating funnels as standard (x μl of y ppm added) and as an analytical blank + serial number.
- With a plunger pipette, add to five of these six separating funnels the required amounts of standard solutions of dichloro- and/or trichloroacetic acid in acetone.
- Choose five different concentrations for this purpose.
- Add no dichloro- and/or trichloroacetic acid to the water in separating funnel 14 but do add 200 μl of the acetone used to make up the standard solutions. This is the analytical blank.
- Then, by means of a Kipp automatic dosing device, add 50 ml of 5 M sulphuric acid to each of the separating funnels.
- Work in a fume cupboard as much as possible and wear gloves and safety goggles when handling the 5 M sulphuric acid.
- Check the pH of the blank with a stirring rod and pH paper ($\text{pH} \approx 0$).
- Wait till the sample has returned to room temperature.
- Using a Kipp automatic dosing device, add 10 ml of isobutyl acetate to each separating funnel.
- Close each separating funnel firmly with the ground-in stopper by placing this **wet** in the ground socket with a thrusting-turning motion.
- Shake briefly by hand and release the excess pressure through the tap.
- Place the separating funnels in the shaker.
- Shake for 60 min.

- Place fourteen 25-ml volumetric flasks under the separating funnel rack.
- Using a powder-dosing spoon, add 2 g of anhydrous sodium sulphate to each volumetric flask through a glass funnel (diameter 6 cm).
- Place a smaller glass funnel (diameter 3 cm) on each volumetric flask.
- Apply to the volumetric flasks the same coding as on the separating funnels.
- After an hour's shaking replace the separating funnels in the rack.
- Allow the contents of each separating funnel to run into a plastic bucket until about 1 cm of water still remains above the tap.
- Break any emulsions present.
- Then carefully allow the contents of each funnel to run into a 250-ml beaker until the interface of the two liquids is located in the bore of the tap.
- Dry the outlet tube of each separating funnel with a pipe-cleaner.
- Allow the ester layers to run into the 25-ml volumetric flasks.
- Rinse each separating funnel with ca. 5 ml of isobutyl acetate from a glass wash-bottle.
- Run this isobutyl acetate also into the volumetric flasks.
- Place the volumetric flasks in a wooden block with suitable holes.
- Do NOT fill the volumetric flasks up to the mark but close them well.
- With a suction pipette (in the fume cupboard) add 5 ml of a solution of diazomethane in ether (see Section 2.4.1, Appendix 7) to each volumetric flask.
- Allow the flask to stand for 30 min.
- If the solution has become decolourized, add another 5 ml of diazomethane and, if necessary, another such portion until the solution remains yellow.
- Blow off the excess of diazomethane with a current of nitrogen in a special apparatus in the fume cupboard until the contents of each flask become colourless. This causes the flasks to cool down.
- Replace the flasks in the wooden block.
- Allow the contents of the flasks to come to room temperature.
- Make up the volumes to the calibration mark with isobutyl acetate.
- Homogenize the contents of the volumetric flasks.
- Dry the extracts over the sodium sulphate for **at least** an hour.
- The extracts are now ready for gas-chromatographic analysis.

Set up the gas chromatograph as follows:

Column: 5 % of OV-210 on Chromosorb G-AW-DMCS, 80–100 mesh.

Glass; 220 cm $\times$ 3 mm internal diameter

Column temperature: 80°C.

Detector temperature: 300°C.

Carrier gas, auxiliary gas, and electrical detector variables: optimum for methyl dichloro- and/or trichloroacetate (see Section 2.1.1).

REPORTING OF THE RESULTS OF THE MEASUREMENTS

Give the concentrations in μg per litre of water.

Report any special features such as the appearance of unexpected peaks in the chromatogram.

NOTES

1. Pure methyl dichloro- and trichloroacetates are available commercially and are necessary to determine the retention times of these substances and for the optimization and checking of the sensitivity of the gas chromatograph.
2. The dichloro- and/or trichloroacetic acids can, if necessary, be purified by vacuum distillation.
3. When acidified, the samples can safely be kept for 1 week in the dark at room temperature.
4. When the volumes and concentrations differ, care must be taken that the amount of methyl dichloro- and/or trichloroacetates injected into the gas chromatograph is between 1 and 100 pg. Furthermore, the amounts of reagents must be suitably adapted.
5. Only small adaptations of this procedure are necessary for the determination of brominated, iodinated, and multiply-chlorinated acetic and propionic acids (e.g. Dalapon, 2,2-dichloropropionic acid).
The procedure is not suitable for monochloroacetic acid or for fluorinated carboxylic acids. With a suitable stationary phase a number of compounds can often be determined in the presence of one another.

2.4.4 Basic compounds

2.4.4.1 Gas-chromatographic determination of dichloroanilines in water

Subject

Gas-chromatographic determination of 2,4- and 3,4-dichloroanilines in water.

Field of application

The method is suitable for the gas-chromatographic determination of 2,4- and 3,4-dichloroanilines in fresh water, brackish water, and sea water used in biological test systems.

Range of concentrations: 0.1–30 µg.l⁻¹.

Sample size: 100–1000 ml.

When the samples sizes and/or concentrations differ, the procedure can be adapted (see Note 7).

After slight adaption, the procedure is also suitable for the determination of other chloroanilines (see Note 8).

Principle

The sample is acidified with hydrochloric acid to pH 0. After the addition of potassium bromide, potassium bromate, and 2,4,6-trimethylaniline as a catalyst, respectively 6-bromo-2,4-dichloroaniline and 2,6-dibromo-3,4-dichloroaniline are formed.

The excess of bromine is eliminated with sodium sulphite and the solution is made weakly alkaline with caustic soda. The brominated chloroanilines are then extracted with cyclohexane. When the extract has been dried, the brominated chloroanilines are determined by gas chromatography, using an electron capture detector.

EQUIPMENT AND MATERIALS

In addition to the requirements mentioned in Section 2.4.1, the following reagents, glassware, and auxiliary apparatus are needed:

Reagents

- 3,4-dichloroaniline, 99% pure
- 2,4-dichloroaniline, 99% pure
- 2,4,6-trimethylaniline (see Note 6)
- 2,6-dibromo-3,4-dichloroaniline (the synthesis is described in Appendix 1 of this procedure)

- sodium potassium tartrate, analytical grade
- sodium hydroxide pellets, analytical grade
- sodium sulphite, analytical grade
- concentrated sulphuric acid (96 %)
- 7M sodium hydroxide in water
Stir 1150 g of sodium hydroxide into 4 litres of distilled water in a large beaker. Store the solution in a narrow-neck glass bottle with a plastic screw-cap.
Avoid contact between the solution and the screw-cap as far as possible.
Transfer part of the solution to a 1-litre glass bottle forming part of a 25-ml Kipp automatic dosing device.
- Sodium potassium tartrate solution (ca. 20 %) in water
With stirring and gentle heating, dissolve 900 g of sodium potassium tartrate in 2.5 litres of distilled water in a large beaker. Store the solution in a narrow-neck glass bottle with a screw-cap.
Avoid contact between the solution and the screw-cap as far as possible.
Transfer part of the solution into a 500-ml conical flask fitted with a 10-ml Kipp automatic dosing device.
- Potassium bromate solution in water (ca. 12 g.l⁻¹)
With stirring, dissolve 12 g of potassium bromate in 1 litre of distilled water in a large beaker.
Store the solution in a 1-litre narrow-neck glass bottle with ground-in stopper.
Transfer part of this solution into a 250-ml conical flask fitted with a 2-ml Kipp automatic dosing device.
- 5M hydrochloric acid in water with 2,4,6-trimethylaniline as catalyst
On a top-weighing balance, weigh out 480 mg of 2,4,6-trimethylaniline by aspirating this with a 1-ml injection syringe from a volumetric flask with a well ground-in stopper and transferring it to a 1-gram weighing bottle.
With stirring, pour 800 ml of concentrated hydrochloric acid into 1200 ml of water in a large beaker.
Dissolve the trimethylaniline in 1 to 2 ml of acetone.
Transfer the trimethylaniline together with the weighing bottle into the beaker. Mix the solution by stirring.
Store the solution in a 2.5-litre narrow-neck dark glass bottle with ground-in stopper.
Store the solution in a refrigerator.
- 1M sulphuric acid
With a graduated pipette, add 10 ml of 96 % sulphuric acid to 350 ml of distilled water in a conical flask provided with a ground-in stopper. Mix the solution by stirring.
- Carbowax-pretreated Chromosorb W-AW
Chromosorb W-AW is deactivated by the treatment described by Aue *et al.* (1973).
6% of Carbowax 20M is applied to the Chromosorb by the usual gas-chromatographic procedure. The material is then packed into a tube and heated overnight in a tube oven at 280 °C under a slow current of nitrogen. It is then extracted with methanol for three days in a Soxhlet extractor.

Glassware

- Kipp automatic dosing device, 25-ml, with matching 1-litre glass bottle
- Kipp automatic dosing device, 2-ml, with matching 250-ml conical flask
- narrow-neck glass bottle, 1-litre, with ground-in stopper
- powder funnel (6 cm), fitting in a 250-ml separating funnel
- at least 14 Squibb conical 250-ml separating funnels with glass stoppers and glass taps.

2. Analysis of the investigation of degradability, toxicity, and accumulation

Auxiliary apparatus

- Separating-funnel rack for two rows of at least fourteen 250-ml separating funnels
- one 5-ml injection syringe.

PROCEDURE

The procedure described here is suitable for 100-ml samples in a range of concentrations from 0.3 to 30 $\mu\text{g.l}^{-1}$.

- First read Section 2.4.1.
- Place fourteen 250-ml separating funnels in the rack.
- Without using a funnel, transfer eight samples into eight separating funnels.
- Transfer the coding of the samples to the separating funnels.
- Using a measuring cylinder, transfer 100 ml of pure test medium into each of the six other separating funnels.
- Code the separating funnels as standards (x μl of y ppm added) and as analytical blank + serial number.
- With a plunger pipette, add the required amounts of standard solutions of dichloroaniline in 1M sulphuric acid to five of these six separating funnels.
- Choose five different concentrations for this purpose.
- Add no dichloroaniline to the water in separating funnel 14, but do add 200 μl of the 1M sulphuric acid that was used to prepare the standard solutions. This is the analytical blank.
- Then, with a Kipp automatic dosing device, add 25 ml of 5M hydrochloric acid containing 240 mg.l^{-1} of 2,4,6-trimethylaniline to each separating funnel.
- By means of a powder funnel (diameter 6 cm) and a powder-dosing spoon add 3 g of powdered potassium bromide to each separating funnel.
- Close each separating funnel firmly with the ground-in stopper by inserting this **wet** into the ground socket with a thrusting-turning motion.
- Shake by hand until all the potassium bromide has dissolved.
- Using a Kipp automatic dosing device, add 2 ml of the potassium bromate solution to each separating funnel.
- Close the separating funnels.
- Shake briefly by hand.
- Allow the separating funnels to stand in the rack for half an hour for bromination to take place.
- Open the separating funnels.
- Eliminate the excess of bromine by adding about 500 mg of sodium sulphite to each separating funnel by means of a powder funnel and a powder-dosing spoon.
- Swirl each separating funnel until the solution is just colourless.
- By means of a Kipp automatic dosing device, add successively to each separating funnel:
 - 10 ml of 20 % sodium potassium tartrate solution
 - 25 ml of 7M caustic soda
 - 5 ml of cyclohexane.
- Rinse the neck of each separating funnel clean with as little distilled water as possible from a glass wash-bottle.
- Close each separating funnel firmly with the ground-in stopper by inserting this **wet** into the ground socket with a thrusting-turning motion.
- Shake briefly and relieve the excess pressure through the tap.

- Place the separating funnels in the shaker.
- Shake for 30 min.
- Place fourteen 10-ml volumetric flasks under the separating funnel rack.
- Through a glass funnel (diameter 6 cm) add 1 g of anhydrous sodium sulphate to each volumetric flask by means of a powder-dosing spoon.
- On each volumetric flask place a smaller glass funnel (diameter 3 cm).
- Transfer the coding from the separating funnels to the volumetric flasks.
- After they have been shaken for half an hour, replace the separating funnels in the rack.
- Allow the contents of each separating funnel to run into a plastic bucket until there is still about 1 cm of water above the tap.
- Break any emulsions present.
- Carefully allow the contents of each funnel to run into a 250-ml beaker until the interface of the two liquids is in the bore of the tap.
- Dry the outflow tube of each separating funnel with a pipe-cleaner.
- Rinse each separating funnel with about 4 ml of cyclohexane from a glass wash-bottle.
- Run this cyclohexane also into the volumetric flasks.
- Place the volumetric flasks in a wooden block with suitable holes.
- Fill the volumetric flasks up to the mark with cyclohexane and close them.
- Homogenize the contents of each volumetric flask.
- Allow the extracts to stand over the sodium sulphate already present in the flasks for **at least 1 h**.
- The extracts are ready for gas-chromatographic analysis.

Set up the gas chromatograph as follows:

Column: 3 % of OV-225 on Chromosorb W-AW, 80–100 mesh, pre-treated with Carbowax (see 'Reagents')
Glass; 220 cm x 3 mm internal diameter.

Column temperature: for 2,6-dibromo-3,4-dichloroaniline: 180°C;
for 6-bromo-2,4-dichloroaniline: 160°C.

Detector temperature: 300°C.

Carrier gas, auxiliary gas, and electric detector variables: optimum for brominated chloroaniline (see Section 2.1.1).

REPORTING OF THE RESULTS OF THE MEASUREMENTS

Give the concentrations in μg per litre of water.

Report any special features such as the occurrence of colorations in the extracts (see Note 4) or the appearance of unexpected peaks in the chromatogram (see Note 6).

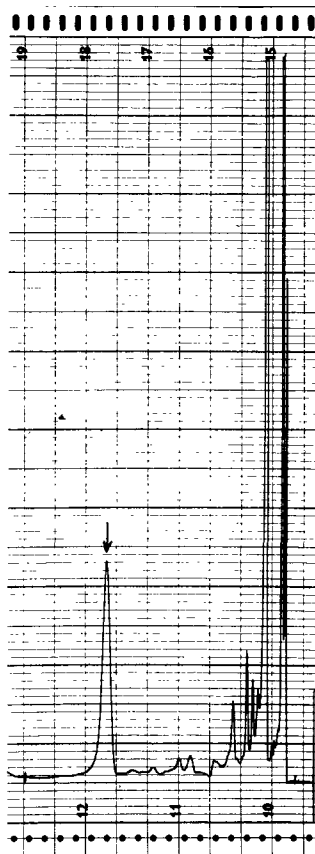
NOTES

1. Pure 2,6-dibromo-3,4-dichloroaniline and 6-bromo-2,4-dichloroaniline are necessary to determine the retention times of these substances and to optimize and check the sensitivity of the gas chromatograph.

The substances do not appear in the standard supply ranges of the chemicals manufacturers. The synthesis of 2,6-dibromo-3,4-dichloroaniline is described in Appendix 1 of this procedure.

2.4 Methods for the determination of individual test compounds in water

2. The 2,4- and 3,4-dichloroanilines must be purified by recrystallization from hexane.
3. According to our experience, the samples can be kept safely at room temperature in the dark for at least two weeks and the extracts for at least four weeks.
4. The extracts may be coloured pink as a result of a very small amount of impurity in the 2,4,6-trimethylaniline (even after purification by distillation). Additional peaks may appear in the chromatogram, which, however, do not interfere with the determination of the 2,6-dibromo-3,4-dichloroaniline.
5. Before use, the 2,4,6-trimethylaniline must be purified by distillation under dry nitrogen at reduced pressure through a small column filled with Wilson helices. After purification it must be stored in a volumetric flask in the dark in a refrigerator. We found the smallest amount of impurities in the Merck product.
6. There are very many additional peaks in the chromatogram. This is caused by the bromination process.
7. When the volumes and/or concentrations differ, care must be taken that the amount of brominated dichloroaniline injected into the gas chromatograph is between 1.5 and 150 pg. In addition, the amounts of the reagents must be adapted appropriately.
8. Only slight adaptations of the procedure are necessary to determine 4-chloroaniline, 3-chloroaniline, and 4-chloro-2-methylaniline (Kossmann, 1971). With a suitable stationary phase a number of these compounds can often be detected in the presence of one another.



2.4.4.1 Fig. 1 Example of a gas chromatogram obtained in the determination of 3,4-dichloroaniline as 2,6-dibromo-3,4-dichloroaniline in water. The peak represents 50 pg of 3,4-dichloroaniline derived from a water sample with a concentration of $10 \mu\text{g.l}^{-1}$

2.4.4.1 Appendix 1. Synthesis of 2,6-dibromo-3,4-dichloroaniline

Subject

Preparation of pure 2,6-dibromo-3,4-dichloroaniline.

Field of application

2,6-Dibromo-3,4-dichloroaniline is used in the gas-chromatographic determination of 3,4-dichloroaniline in water and in organisms. It serves as a standard in the gas-chromatographic analyses, as a reference substance for identification, and as an auxiliary for the determination of the extraction yield.

Principle

3,4-Dichloroaniline is dissolved in water acidified with hydrochloric acid. After the addition of KBr, KBrO_3 , and 2,4,6-trimethylaniline as a catalyst, 2,6-dibromo-3,4-dichloroaniline is formed. The excess of bromine is converted with sodium sulphite into bromide and finally the solution is made weakly alkaline with caustic soda. The compound which now precipitates is isolated, dried, and recrystallized.

EQUIPMENT AND MATERIALS

In addition to the requirements mentioned in Section 2.4.1, the following reagents and auxiliary glassware are needed:

Reagents

- n-Hexane, analytical grade
- potassium bromate, analytical grade
- potassium bromide, analytical grade, powdered.
Before use this is heated for 6 h at 300°C .
It is stored in a glass bottle with a ground-in stopper.
- sodium potassium tartrate, analytical grade
- sodium hydroxide pellets, analytical grade
- sodium sulphite, analytical grade.
Before use this is heated for 6 h to 300°C .
It is stored in a glass bottle with a ground-in stopper.
- 3,4-dichloroaniline, 99 % pure
- 2,4,6-trimethylaniline (see Note 6 in Section 2.4.4.1)
- 7M caustic soda solution (see Section 2.4.4.1)
- 6M hydrochloric acid in water (see Section 2.5.4.1).

Glassware

- 2 watchglasses, diameter 14 cm
- 1 beaker, 1 litre
- 1 funnel, diameter 10 cm.

PROCEDURE

- In a 1-litre beaker, with heating and stirring, dissolve 3.5 g of 3,4-dichloroaniline in 600 ml of distilled water to which 6M hydrochloric acid and ca. 3 mg of 2,4,6-trimethylaniline (ca. 3 μl) have been added (in the fume cupboard).
- To this solution at 40°C add a solution of 10 g of KBr in 10 ml of water.
- Then add a solution of 2.5 g of KBrO_3 in 10 ml of water. The mixture now becomes pale yellow.
- Allow to stand for 30 min with a watchglass covering the beaker.
- Add sodium sulphite until the mixture is white.

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- Now add dropwise 7M caustic soda until the mixture is alkaline. Check this with pH paper.
- Filter the precipitate with suction on a G3 sintered-glass filter and dry it in a vacuum desiccator at 50° C.
- Recrystallize the precipitate from 100 ml of warm n-hexane.
- Filter the hexane through warm, folded filter paper into a warm 250-ml beaker.
- Gently stir the solution in the beaker.
- Isolate the needle-like crystals that have appeared on cooling by filtration on a G3 sintered-glass filter and dry them as above.
- If necessary, repeat the above operations until the melting point of 2,6-dibromo-3,4-dichloroaniline (113–114° C) has been reached.
- Check the purity of the 2,6-dibromo-3,4-dichloroaniline by infrared spectrometry and gas chromatography.

2.4.4.2 Automated photometric determination of dichloroaniline

Subject

Determination of 3,4-dichloroaniline in fresh water, brackish water, and sea water.

Field of application

The method is directly applicable to the determination of 3,4-dichloroaniline in fresh water, brackish water, and sea water in the absence of interfering components. When interfering components are present, the method can be used after the application of extrapolation procedures.

Sample size: 1.6 ml minimum.

Range of concentrations: 200 µg per litre and higher.

Principle

The 3,4-dichloroaniline present in the sample is diazotized with sodium nitrite and is then coupled with N-(1-naphthyl)ethylenediamine dihydrochloride.

The optical density of the water sample treated in this way is then measured and compared against standard solutions treated under the same conditions.

EQUIPMENT AND MATERIALS

Glassware, general

- weighing bottle
- Pasteur pipette.

Glassware, calibrated

- volumetric flasks with calibration marks and ground-in stoppers, 250 ml
- volumetric flask, 1000 ml
- burette, 25 ml.

Various items of auxiliary apparatus, general

- refrigerator
- closed cupboard for storing samples and solutions in the dark
- felt-tip pen for marking glassware; the ink must be capable of being removed in a washing machine
- spatula.

Various items of auxiliary apparatus, calibrated

- analytical balance, accurate to 0.1 mg
- ruler
- millimetre paper.

Analyser

AutoAnalyzer type, manufactured by Technicon, consisting of the following modules:

- a sample-feed device, 10 samples per hour; aspiration of a sample takes place for 60 sec and rinsing for 300 sec
 - sample vessels, 2 ml
 - a proportioning pump provided with 11 pump tubes of various diameters
 - three glass mixing coils
 - a dialyser with a 'cuprophane' membrane
 - a heating bath, 45° C, with two coils
 - two time-delay coils
 - a spectrophotometer provided with interference filters having a maximum transmission at 555 nm and a flow-through cell with an optical path length of 50 mm.
- The scheme of analysis is given in the 'Procedure'.

Reagents and auxiliaries

- ethanol, analytical grade
- synthetic sea water or other pure test medium
- water, distilled or de-ionized
- 3,4-dichloroaniline, analytical grade
- solution of NaNO₂, analytical grade, in water, 20 g per litre
- solution of sulphamic acid, analytical grade, in water, 30 g per litre
- solution of N-(1-naphthyl)ethylenediamine dihydrochloride (NEDA), analytical grade, in ethanol, 5 g per litre
- hydrochloric acid, 1M, analytical grade
- standard solutions of 3,4-dichloroaniline.
Preparation of stock solutions: dissolve 50 mg of 3,4-dichloroaniline in 2 ml of ethanol in a 1000-ml volumetric flask. Make up to the mark with 1M hydrochloric acid. Report on the volumetric flask:
 - date of preparation of the solution
 - solute
 - solvent
 - concentration.

Store the stock solution in a refrigerator. From this stock solution in 250-ml volumetric flasks are prepared dilutions of 1M hydrochloric acid to concentrations necessary for the standard solutions to be used (see under 'Procedure').

These diluted solutions are also coded and can be kept in a refrigerator for 24 h. After this time they must be discarded.

PROCEDURE

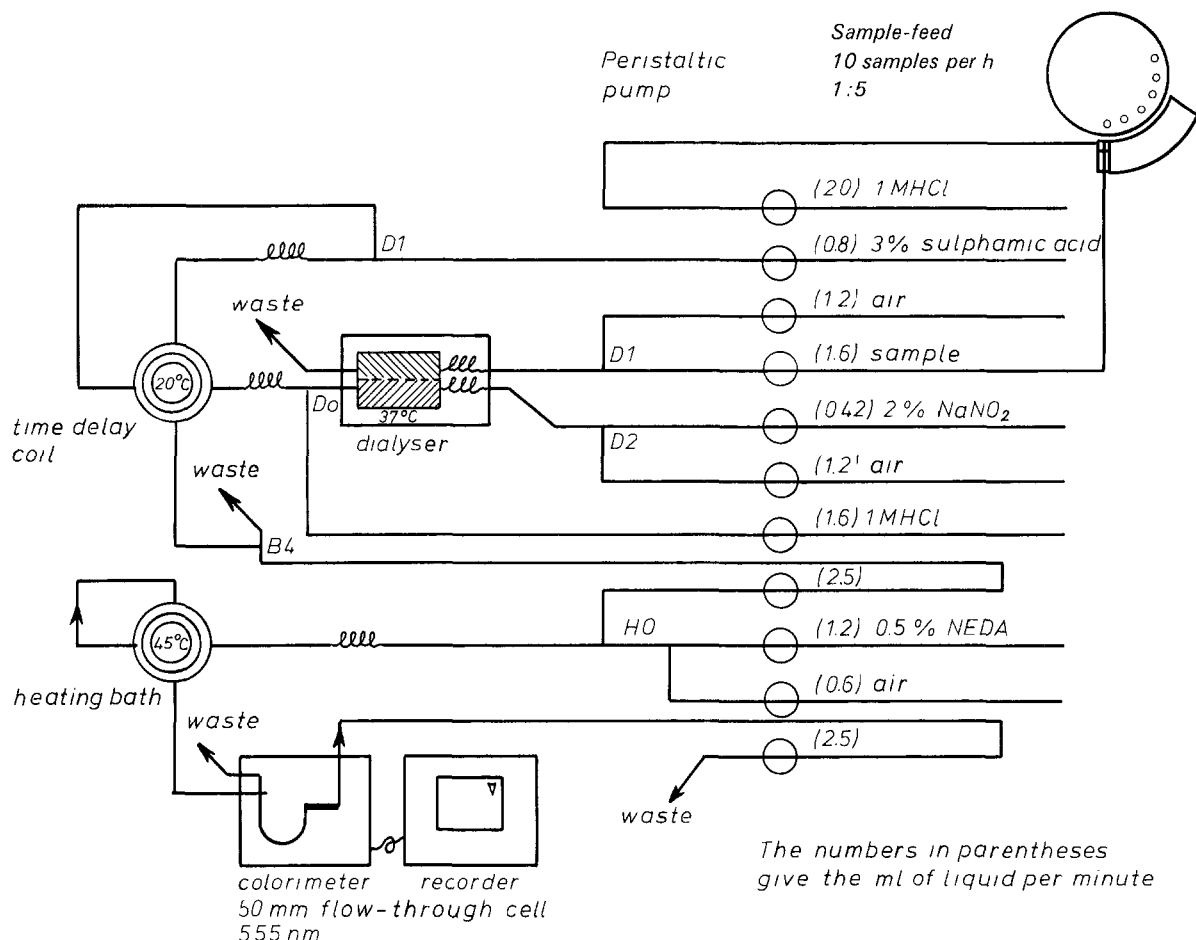
The procedure described here is suitable for water samples of at least 2 ml and a 3,4-dichloroaniline concentration between 200 and 5000 ppb.

If higher concentrations are present, the samples must be diluted by hand or, in the case of relatively large numbers, by means of a special provision in the AutoAnalyzer.

The scheme of analysis is shown in 2.4.4.2, Fig. 1.

Prepare standard solutions of 3,4-dichloroaniline as follows:

- Add 0, 1, 5, 12.5, and 25 ml of the stock solution of 3,4-dichloroaniline to separate 250-ml volumetric flasks and make up each



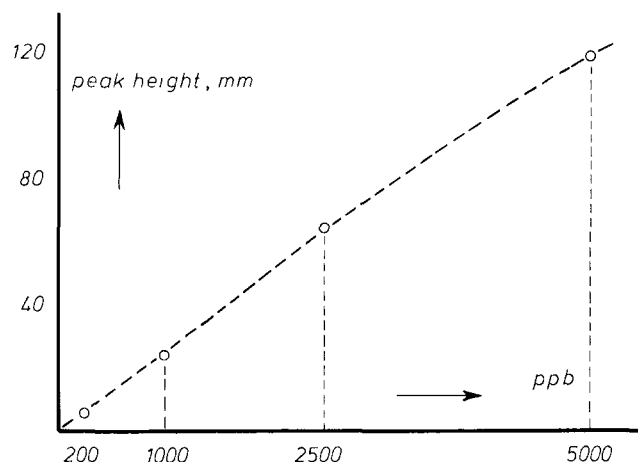
2.4.4.2 Fig. 1 Scheme of analysis for the AutoAnalyzer determination of 3,4-dichloroaniline in water

to 250 ml with 1M hydrochloric acid. These standard solutions contain respectively 0, 200, 1000, 2500, and 5000 ppb of 3,4-dichloroaniline.

- If the samples to be analysed have been stored in a refrigerator, allow them to warm up to room temperature on a laboratory bench.
- Then start the AutoAnalyzer, except for the sample-feed device. The sampling needle must be in the vessel with rinsing liquid.
- As soon as the baseline runs quite straight and parallel to the x-axis, place a series of standard solutions of 3,4-dichloroaniline with increasing concentrations in coded 2-ml vessels in the sample-feed device.
- Following this, place the samples to be analysed in the vessels coded for them in the sample-feed device.

After each group of approximately 30 samples another series of standard solutions is inserted, and after the last sample the group is completed with another series of standard solutions.

- Now start up the sample-feed device as well, so that all the standard solutions and samples are drawn in one after the other, which after some time (the running time), results in an equal number of peaks on the recorder chart. Code these peaks with the codes of the corresponding vessels.
- At the end of the analyses measure the heights of the peaks relating to each series of standard solutions and plot a calibration curve (see 2.4.4.2, Fig. 2).
- With the aid of this calibration curve read off the concentrations of 3,4-dichloroaniline in the samples.



2.4.4.2 Fig. 2 Calibration curve used in the AutoAnalyzer determination of 3,4-dichloroaniline in water

(Some modern pocket calculators have the capacity for linear regression analysis. Linear interpolations can then be carried out without drawing the curve).

REPORTING OF THE RESULTS OF THE MEASUREMENTS

Give the concentrations in μg of 3,4-dichloroaniline per litre and report any special features observed during or before the analysis.

2. Analysis of the investigation of degradability, toxicity, and accumulation

Check for errors of measurement due to interfering components

The most important sources of error are:

1. colour of the sample;
2. presence of substances that react with the reagents to form compounds also absorbing at 555 nm.

For checking errors of measurement, see the discussion in Section 2.1.2.4.

Naturally, in long series of closely related samples this check need not be repeated for each sample if measurement errors appear to be absent.

NOTES

1. At the end of the analyses the AutoAnalyzer system must be rinsed out with water. This is done by suspending all the feed tubes in water and continuing the pumping for about another 20 min.
2. After the switching off of the AutoAnalyzer, the pump must be opened and the bundle of tubes be relaxed.
3. If 3,4-dichloroaniline is present exclusively in a sample, its determination, with the aid of a calibration curve made with standard solutions of this compound, can be performed directly. In work with samples containing mixtures of anilines this is no longer quite the case. A number of anilines show molecular extinctions and maxima in the absorption spectrum whose values differ for the different compounds.

2.5 METHODS FOR THE DETERMINATION OF INDIVIDUAL TEST COMPOUNDS IN ORGANISMS

2.5.1 General part

Section 2.4.1, which constitutes the introduction to procedures for the determination of test compounds in water, also serves as a general introduction to the procedures for their determination in organisms.

A few brief supplementary observations shall be made.

Although the tables of requirements drawn up for the analysis of compounds in organisms are given here in Section 2.5.1, the explanation of these tables is in Section 2.4.1.

The organisms are homogenized in an Ultra-Turrax homogenizer, if necessary in the micro-model. Guppies and shrimps are homogenized whole, plaice are first cut into small pieces, and mussels are first removed from the shell (see Section 6.5.3).

In order to select the correct concentrations for the standards, it

is desirable that the first series of samples be analysed as rapidly as possible by means of a calibration curve over a very wide range of concentrations, for which purpose more than the usual number of five standards is taken and it is assumed that the accumulation is somewhere between 1 and 1000. In the following series the expected concentration can then be estimated within a much smaller range.

In general, 1 g of tissue is analysed. This is weighed to within 0.01 g and the weight is noted on the serial sheet (2.4.1, Fig. 3).

The concentrations in the samples, having been determined by means of the calibration curve made with each series, must be corrected for the actual sample weights. The concentrations in the standards are always calculated to precisely 1 gram of tissue, and to calculate the content in the organism the precise weight of the tissue sample must of course be included in the calculations.

2. Analysis of the investigation of degradability, toxicity, and accumulation

2.5.1 Appendix 1

Glassware, general • at least 12	Dieldrin 2.5.2.1	PCP 2.5.3.1	DCAN 2.5.4.1
Squibb separating funnel, 250 ml, provided with a glass tap and glass stopper (see 2.4.1, Fig. 8)		•	•
Centrifuge tube, 25 ml, with ground-in stopper	•	•	
Beaker, 5 litres		1	2
Beaker, 3 litres		2	2
Beaker, 250 ml		4	6
Conical flask, 25 ml, with ground-in stopper	4	4	4
Glass wash-bottle (see 2.4.1, Fig. 9)	4	6	6
Wide-neck glass bottle, 1 litre, with ground-in stopper	1	1	1
Wide-neck glass bottle, 50 ml, with ground-in stopper	2	2	2
Narrow-neck glass bottle, 2.5 litres, with ground-in stopper		1	1
Narrow-neck dark glass bottle, 2.5 litres, with plastic screw cap		2	3
Narrow-neck glass bottle, 1 litre, with ground-in stopper	2		2
Glass funnel, diameter 15 cm		3	3
Glass funnel, diameter 6 cm		3	5
Glass funnel, diameter 3 cm	20	40	40
1 gram weighing bottle	2	3	3
Pasteur pipettes, length 15 cm, in box	1		1
Porcelain dish, diameter 15 cm		2	2

2.5.1 Appendix 2

Glassware, calibrated • multiples of 12	Dieldrin 2.5.2.1	PCP 2.5.3.1	DCAN 2.5.4.1
Volumetric flasks with calibration marks and ground-in glass stoppers			
Volume: 100 ml	2	2	2
50 ml	4	4	4
25 ml	2	2	2
10 ml		•	•
Measuring cylinder, 1000 ml	1	1	1
Measuring cylinder, 500 ml	2	1	
Measuring cylinder, 100 ml	2	2	
Kipp automatic dosing device, 25 ml, with 1-litre bottle (2.4.1, Fig. 1)		1	1
Kipp automatic dosing device, 10 ml, with matching 500-ml conical flask	3	1	1
Kipp automatic dosing device, 5 ml, with matching 250-ml conical flask		1	1

2.5.1 Appendix 3

Auxiliary apparatus, general • at least 7	Dieldrin 2.5.2	PCP 2.5.3	DCAN 2.5.4
Laboratory glassware-washing machine	x	x	x
Drying cabinet, adjustable to 300° C	x	x	x
Bottle-rinsing apparatus with powerful water jet (2.4.1, Fig. 17)	x	x	x

Fume cupboard	x	x	x
Refrigerator	x	x	x
Deep-freeze cabinet	x	x	x
Closed cupboard for storing samples and standards in the dark	x	x	x
Device for shaking vessels in a water bath (2.4.1, Fig. 5)	x	x	
Water bath	x	x	
Rack for twelve 250-ml separating funnels that fits into the fume cupboard (2.4.1, Fig. 12)	2	2	2
Shaker for at least twelve 250-ml separating funnels (2.4.1, Fig. 13)	x	x	x
Wooden block with holes for at least twelve 10-ml volumetric flasks or twelve 25-ml centrifuge tubes (2.4.1, Fig. 14)	4	12	10
Combination of magnetic stirrer and heating plate	x	x	x
'Start-stop' time switch plug	2	1	1
Kitchen timer	1	1	1
Stainless-steel spoon-type spatula	3	4	4
Stainless-steel spatula	•	•	•
Plastic bucket, 10 litres	3	3	3
Thick blue felt-tip pen	2	2	2
Thick black felt-tip pen	2	2	2
Pipe cleaners, white, 4 mm x 15 cm in packs of 25		25	25
Disposable polythene gloves in packs of 100	5	5	5

2.5.1 Appendix 4

Auxiliary apparatus, calibrated	Dieldrin 2.5.2.1	PCP 2.5.3.1	DCAN 2.5.4.1
Analytical balance, accurate to 0.1 mg	x	x	x
Top-weighing balance, accurate to 0.01 mg	x	x	x
2-4 plunger pipettes with one or more fixed-adjustable metering volumes (e.g. 20, 50, 100, 200 µl) (2.4.1, Fig. 10)	x	x	x
500-1000 disposable tips for these pipettes	x	x	x
Powder-dosing spoon (2.4.1, Fig. 2)		x	x
Thermometer, 0-100° C	x	x	

2.5.1 Appendix 5

Reagents	Dieldrin 2.5.2.1	PCP 2.5.3.1	DCAN 2.5.4.1
Homogenized blank tissue identical with that of the sample to be determined	x	x	x
Distilled water	x	x	x
Acetone, analytical grade	x	x	x
Hexane, analytical grade	x	x	x
Isopropanol, analytical grade	x	x	x
Concentrated hydrochloric acid		x	x
Sodium sulphate, anhydrous	x	x	x
pH paper		x	x

2.5.2 Neutral compounds

2.5.2.1 Gas-chromatographic determination of non-volatile neutral compounds in aquatic organisms

Subject

Gas-chromatographic determination of dieldrin in aquatic organisms.

Field of application

The method is suitable for the determination of the dieldrin content in mussels, guppies, shrimps, and plaice.

Sample size: at least 3 grams of homogenized tissue or 1 gram of homogenized liver.

Range of concentrations: 50–3000 µg per kg wet weight.

After slight adaptation, the procedure is also used for the determination of other chlorine-containing pesticides (see Note 6).

Principle

Dieldrin is extracted from the homogenized tissue with an acetone/hexane mixture and then with a hexane/ether mixture. After saponification with alcoholic alkali, the hexane extract is suitable for gas-chromatographic analysis provided that an electron capture detector is used.

EQUIPMENT AND MATERIALS

In addition to the requirements mentioned in Section 2.4.1, the following reagents, glassware, and auxiliary apparatus are needed:

Reagents

- Dieldrin, 99 % pure
- potassium hydroxide pellets, analytical grade
- diethyl ether, analytical grade
- a mixture of acetone and hexane, 5:2 (v/v)
Mix 500 ml of acetone and 200 ml of hexane in a 1-litre bottle fitted with a 10-ml Kipp automatic dosing device
- a mixture of hexane and diethyl ether, 9:1 (v/v)
Mix 720 ml of hexane and 80 ml of diethyl ether in a 1-litre glass bottle fitted with a 10-ml Kipp automatic dosing device.

Glassware

- 1 measuring cylinder, 100 ml
- at least 12 centrifuge tubes, 10 ml, with ground-in stoppers
- sets of 12 centrifuge tubes, 5 ml, with ground-in stoppers.

Auxiliary apparatus

- 1 plunger pipette with an adjustable volume of 1–5 ml
- a large supply of disposable tips for this plunger pipette
- nitrogen cylinder
- evaporating apparatus in a water bath (see 2.5.2.1, Fig. 1)
- centrifuge for 12 centrifuge tubes taking both the 25-ml and the 10-ml tubes
- water bath; dimensions about 94 x 18 x 23 cm
- 2 test-tube racks with space for 48 centrifuge tubes (5 ml)
- 2 test-tube racks with space for 36 centrifuge tubes (10 ml)
- shaking block for at least twelve 25-ml centrifuge tubes
- 4 blocks with holes for at least twelve 100-ml pointed flasks.

PROCEDURE

- First read Sections 2.4.1 and 2.5.1.

- Place twelve 25-ml centrifuge tubes with ground-in stoppers in a wooden block.
- Code 6 centrifuge tubes as standards (x µl of y ppm added) and as analytical blank + serial number.
- Into each of the above-mentioned 6 centrifuge tubes weigh 1 gram of homogenized blank tissue of the same type as that of the sample.
- Choose as the standard concentrations the values expected in the sample, which can be estimated with the aid of the accumulation factors (see Note 1).
- For the standards, transfer the required amounts of standard solutions of dieldrin in acetone into the five coded centrifuge tubes with the aid of a plunger pipette.
- Transfer to the centrifuge tube of the blank 200 µl of the acetone used to prepare the standards.
- Close the tubes.
- Allow them to stand for half an hour.
- Transfer the coding of the samples to the other centrifuge tubes.
- Weigh about 1 gram of each of the 6 samples with an accuracy of 0.01 g into the corresponding coded centrifuge tubes.
- Record the weight of each sample on the serial sheet.
- Always use a clean stainless steel spatula for **each** sample.
- After the standards have stood for half an hour, add with a Kipp automatic dosing device 2 x 10 ml of the 5:2 acetone/hexane mixture to each of the centrifuge tubes.
- Shake the tubes for half an hour in the shaking block in the horizontal position.
- Centrifuge the tubes for 10 min at 2000 *g*.
- Place twelve 100-ml pointed flasks in a wooden block with suitable holes.
- Place this block in the fume cupboard (see Note 3).
- Transfer the coding from the centrifuge tubes to the pointed flasks.
- On each flask place a glass funnel with a diameter of 3 cm.
- Transfer the hexane layers from the centrifuge tubes into the pointed flasks.
- Then, by means of a Kipp automatic dosing device, add 20 ml of the 9:1 hexane/ether mixture to **each** centrifuge tube.
- Close them well.
- Shake the tubes first by hand until the contents are homogeneous and then for another 10 min in the shaking block.
- Centrifuge the tubes for 10 min at 2000 *g*.
- Transfer these hexane/ether layers also into the pointed flasks.
- By means of a Kipp automatic dosing device, transfer 10 ml of absolute ethanol to each pointed flask.
- Add a few glass beads to each flask.
- Suspend the flask in a water bath; for the apparatus, see 2.5.2.1, Fig. 1.
- Start the water bath at about 50°C and allow the temperature to increase to a maximum of 90°C.
- For the first 5–10 min take care that no delayed boiling takes place in the flask.
- Suspend in each flask a Pasteur pipette through which a slow current of nitrogen is being passed.
- Evaporate the contents of the flasks until no further appreciable change in the volume takes place.

2. Analysis of the investigation of degradability, toxicity, and accumulation

- Dissolve the residue in 10 ml of hexane added with the aid of a Kipp automatic dosing device.
- Keep this solution in hexane for **not longer** than 24 h.
- Pipette 2 ml of this solution into a 10-ml centrifuge tube with the aid of a plunger pipette.
- By means of a Kipp automatic dosing device, add to each tube 2 ml of 96 % ethanol.
- With the aid of a spoon-type spatula, add four KOH pellets to each tube.
- Place a few boiling stones in each tube.
- Close the tubes well.
- Shake the tubes in a water bath at 50°C for half an hour; for the apparatus, see 2.4.1, Fig. 5.
- Then, with the aid of a plunger pipette, add 4 ml of distilled water to each tube.
- Close the tubes well again.
- Shake them in the water bath for another 10 min.
- Centrifuge the tubes for 5 min at 2000 g. The liquid in the tubes now separates into two yellow layers.
- Place twelve 5-ml centrifuge tubes with ground-in stoppers in a test-tube rack.
- Transfer to these tubes the codings from the 10-ml centrifuge tubes.
- By means of a powder-dosing spoon add to each tube 1 g of anhydrous sodium sulphate.
- Now transfer the upper layers from the 10-ml centrifuge tubes into the 5-ml tubes.
This can be done in two ways:
 1. Freeze the lower layers in the tubes in solid carbon dioxide. Transfer the upper layers into the 5-ml centrifuge tubes. (We prefer this method).
 2. Transfer as much as possible of the upper layers into the 5-ml centrifuge tubes with the aid of Pasteur pipettes.
- Allow the extracts to stand for **at least** 1 h over the sodium sulphate already present in the tubes.
- The extracts are ready for gas-chromatographic analysis.

Set up the gas chromatograph as follows:

Column: 5 % of OV-101 on Chromosorb G-AW-DMCS,
80–100 mesh
Glass; 220 cm x 3 mm internal diameter

Column temperature: 200°C.

Detector temperature: 300°C.

Carrier gas, auxiliary gas, and electric detector variables: optimum for dieldrin (see Section 2.1.1).

REPORTING OF THE RESULTS OF THE MEASUREMENTS

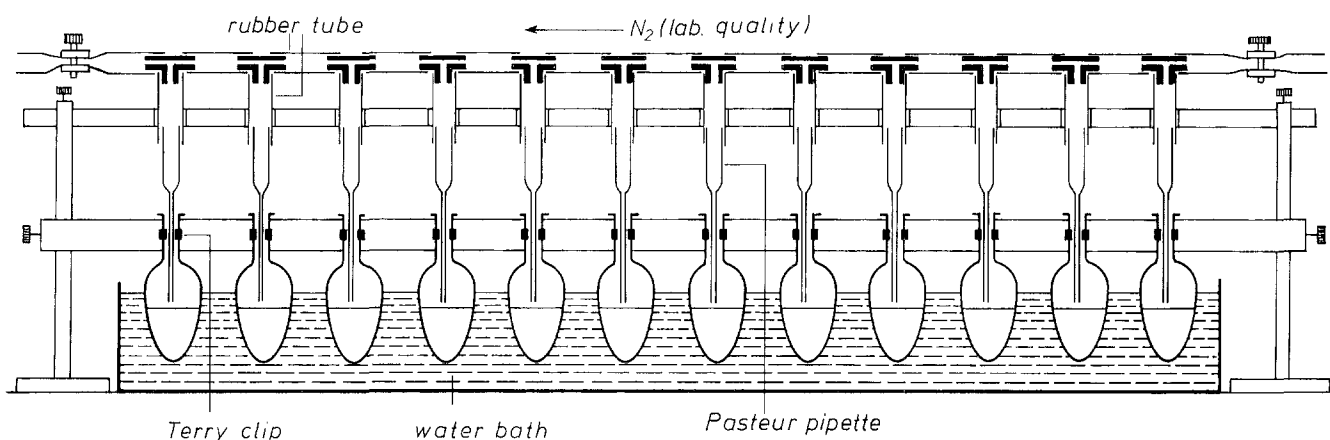
Give the concentrations in µg per kg wet weight. Take the weight of the sample into account (see Section 2.5.1). Report any special features, such as the appearance of unexpected peaks in the chromatogram.

NOTES

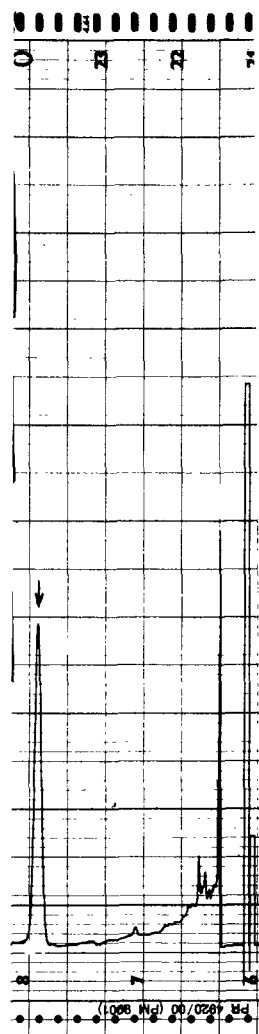
1. The estimated accumulation factors of dieldrin in living animals, after a residence time in water of 14 days, are as follows:

Animal	Concentration in water (µg.l ⁻¹)	Accumulation factor
Mussel	1–10	5000
Shrimp	0.1–1	8000
Guppy	0.1–1	1000
Plaice, muscle	0.4	1000
Plaice, liver	0.1–1	8000

2. After homogenization, the samples can be safely stored in the deep-freeze cabinet at –35°C.
3. In view of the volatility and fire-hazard of the solvents used, the work must be performed in a fume cupboard as far as possible.
4. According to our experience, the extracts can be stored safely in the dark for 1 week.
5. If necessary, dieldrin can be purified by recrystallization from ethanol.
6. After slight adaptation, the procedure is also suitable for the determination of other chlorinated pesticides (e.g. lindane, endrin, aldrin, Telodrin, heptachlor, endosulfan, pentachlorobenzene, hexachlorobenzene (HCB), pentachloronitrobenzene (PCNB), TDE, DDE, DDT), PCBs, octachlorostyrene, and hexachlorobutadiene. Here one must take into account whether saponification with alkali is admissible (see Jensen *et al.*, 1972). With a suitable stationary phase a number of these compounds can often be determined in the presence of one another.



2.5.2.1 Fig. 1 Apparatus for evaporation in a water bath



2.5.2.1 Fig. 2 Example of a gas chromatogram obtained in the determination of dieldrin in organisms. The peak represents 90 pg of dieldrin from a tissue sample with a concentration of 1780 µg/kg

2.5.3 Acidic compounds

2.5.3.1 Gas-chromatographic determination of highly electronegative phenols in aquatic organisms

Subject

Gas-chromatographic determination of pentachlorophenol in aquatic organisms.

Field of application

The method is suitable for the determination of the pentachlorophenol content in mussels, guppies, shrimps, and plaice.

Sample size: at least 3 grams of homogenized tissue or 1 gram of homogenized liver.

Range of concentrations: 50–3000 µg per kg wet weight.

After slight adaptation, the procedure is also suitable for the determination of tri- and tetrachlorophenols (see Note 7).

Principle

Pentachlorophenol is extracted from the homogenized tissue with 5M caustic soda at 50°C. After cooling, the highly alkaline extract is made weakly alkaline with hydrochloric acid. After the addition of borax and acetic anhydride, pentachlorophenyl acetate is formed, which is extracted with hexane.

After the hexane extract has been dried, the pentachlorophenyl acetate in it is determined by gas chromatography using an electron capture detector.

EQUIPMENT AND MATERIALS

In addition to the requirements mentioned in Section 2.4.1, the following reagents, glassware, and auxiliaries are needed:

Reagents

- pentachlorophenol, 99 % pure
- pentachlorophenyl acetate, 99 % pure (the synthesis is described in Appendix 1 of Section 2.4.3.1.)
- Methyl Green
- phenolphthalein
- acetic anhydride, analytical grade
- borax, analytical grade

Preparation:

In a 25-ml volumetric flask dissolve 25 mg of phenolphthalein in 25 ml of ethanol and make up precisely to 25 ml. Repeat this operation with Methyl Green.

Mix the solutions in a volume ratio of 1 part of phenolphthalein to 2 parts of Methyl Green in a dark-glass dropping bottle.

5M caustic soda in water

With stirring, dissolve 400 g of NaOH in 2 litres of water in a large beaker. Store the solution in a 2.5-litre glass bottle with a screw-cap. Avoid contact of the solution and the screw-cap as far as possible.

Transfer part of this solution into a 500-ml conical flask fitted with a 10-ml Kipp automatic dosing device.

0.1M sodium tetraborate solution in water

With stirring and gentle heating, dissolve 153 g of borax, $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$ (or 82 g of anhydrous sodium tetraborate) in 4 litres of distilled water in a large beaker.

Store the solution in glass bottles with screw-caps.

Transfer part of the solution into a 500-ml conical flask fitted with a 10-ml Kipp automatic dosing device.

2.3M hydrochloric acid

With stirring, add 370 ml of concentrated hydrochloric acid to 1630 ml of distilled water in a large beaker.

Store the solution in a glass bottle with ground-in stopper.

Transfer part of this 2.3M hydrochloric acid to a 1-litre glass bottle fitted with a 25-ml Kipp automatic dosing device, and another part into a glass wash-bottle.

Glassware

- 1 dark-brown dropping bottle.

Auxiliary apparatus

- 1 Vortex test-tube shaker.

PROCEDURE

- First read Sections 2.4.1 and 2.5.1.
- Place twelve 25-ml centrifuge tubes with ground-in stoppers in a wooden block.
- Code six centrifuge tubes as standards (x µl of y ppm added) and as analytical blank + serial number.
- Weigh 1 g of homogenized blank tissue of the same type as that of the samples into each of the six above-mentioned tubes.
- Choose as the standard concentrations the values expected in the samples, which can be estimated with the aid of the accumu-

2. Analysis of the investigation of degradability, toxicity, and accumulation

lation factors in Note 1.

- By means of a plunger pipette, add the desired amounts of standard solutions of pentachlorophenol in acetone to the individual coded centrifuge tubes for standards.
- Add to the centrifuge tube for the blank 200 µl of the acetone used for the preparation of the standards.
- Close the tubes.
- Allow them to stand for half an hour.
- Transfer the coding of the samples to the other centrifuge tubes.
- In the meantime, from each of the six samples weigh about 1 gram with an accuracy of 0.01 g into a 25-ml centrifuge tube.
- Note the weight of each sample on the serial sheet.
- Always use a clean stainless-steel spatula for **each** sample.
- After the standards have stood for half an hour, by means of a Kipp automatic dosing device add 10 ml of 5M caustic soda and a few glass beads to each of the centrifuge tubes.
- Rinse the neck of each tube with a small volume of water.
- Close each tube firmly with the ground-in stopper, inserting this **wet** into the ground socket with a thrusting-turning motion.
- Homogenize the contents of each centrifuge tube by placing it briefly on a Vortex test-tube shaker.
- Fix the tubes to the shaking device in the water bath (see Section 2.4.1) which has been brought to 50°C.
- Allow to shake gently for 1 h.
- Place twelve 250-ml separating funnels in the rack (see Section 2.4.1) in the fume cupboard.
- Transfer the coding of the samples and standards to the separating funnels.
- On each separating funnel place a glass funnel (diameter 3 cm).
- Transfer the contents of each centrifuge tube, after it has been removed from the water bath and cooled, through the funnel into the corresponding separating funnel.
- Rinse each centrifuge tube with distilled water from a glass wash-bottle.
- Transfer this rinsing water also to the separating funnel.
- Using a Kipp automatic dosing device, add 25 ml of 2.3M hydrochloric acid to each separating funnel.
- Add five drops of the mixed indicator to each separating funnel.
- Now add another few drops of 2.3M hydrochloric acid from a glass wash-bottle until the indicator changes colour (Note 6).
- Check the pH of the blank with a stirring rod and a strip of pH paper; the pH must be 8–9.
- If too much acid has been added, a white precipitate of fatty acids appears. If necessary, add a few drops of 5M caustic soda.
- By means of a Kipp automatic dosing device, add successively to each separating funnel:
 - 25 ml of a 0.1M solution of borax
 - 1 ml of acetic anhydride
 - 5 ml of hexane.
- Rinse the neck of each separating funnel with a few ml of water.
- Close each separating funnel firmly with the ground-in stopper by placing this **wet** in the ground socket with a thrusting-turning motion.
- Shake briefly by hand and relieve the excess pressure through the tap.

- Place the separating funnels in the shaking machine.
- Shake for 30 min.
- Place twelve 10-ml volumetric flasks under the separating-funnel rack.
- By means of a powder-dosing spoon, through a 6-cm glass funnel, add 1 gram of anhydrous sodium sulphate to each volumetric flask.
- Place a 3-cm funnel on each volumetric flask.
- Transfer to the volumetric flasks the same coding as was on the corresponding separating funnels.
- After they have been shaken for half an hour, replace the separating funnels in the rack.
- Allow the contents of each separating funnel to run into a plastic bucket until there is still about 1 cm of water above the tap.
- Break any emulsions present (see Section 2.4.1).
- Carefully allow the contents of each separating funnel to run into a 250-ml beaker until the interface of the two liquids is in in the bore of the tap
- Dry the outlet tube of each separating funnel with a pipe cleaner.
- Allow the hexane layer to run into the corresponding 10-ml volumetric flask.
- Rinse each separating funnel with 4 ml of hexane from a glass wash-bottle.
- Transfer this hexane also into the corresponding volumetric flask.
- Place the volumetric flask in a wooden block with suitable holes.
- Fill the volumetric flasks with hexane up to the calibration mark and close them.
- Allow the extracts to stand for **at least** 1 h over the sodium sulphate that was already present in the flasks.
- The extracts are now ready for gas-chromatographic analysis.

Set up the gas chromatograph as follows:

Column: 5% of OV 101 on Chromosorb G-AW-DMCS,
80–100 mesh

Glass; 220 cm x 3 mm internal diameter.

Column temperature: 180°C.

Detector temperature: 300°C.

Carrier gas, auxiliary gas, and electric detector variables: optimum for pentachlorophenyl acetate (see Section 2.1.1).

REPORTING OF THE RESULTS OF THE MEASUREMENTS

Give the concentrations in µg per kg wet weight.

Take the sample weight into account (see Section 2.5.1).

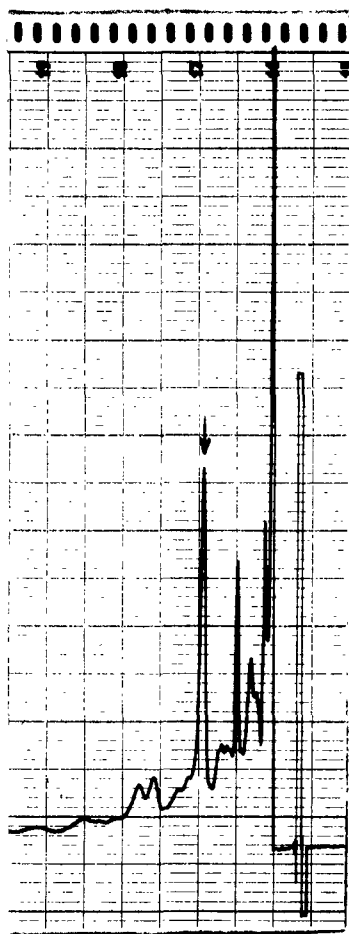
Report any special features such as the appearance of unexpected peaks in the chromatogram.

NOTES

1. The estimated accumulation factors of pentachlorophenol in living animals after a residence time of 14 days in water are as follows:

Animal	Concentration in water (µg.l ⁻¹)	Accumulation factor
Mussel	1–10	120
Shrimp	1–10	50
Guppy	1–10	100
Plaice, muscle	7.5	40
Plaice, liver	7.5	500

2. After homogenizing, the samples can be stored safely in the deep-freeze cabinet at -35°C for 3 weeks.
3. Pure pentachlorophenyl acetate is necessary to determine the retention time of this substance and to optimize and check the gas chromatograph. The substance is not offered in the standard ranges of chemicals manufacturers. The synthesis is described in Appendix 1 of Section 2.4.3.1.
4. Pentachlorophenol can be purified by recrystallization from toluene.
5. According to our experience, the extracts can be stored safely in the dark for 2 weeks.
6. The mixed indicator changes colour at pH 8.9. For acids the colour is green, for bases violet. The indicator is pale blue at pH 8.8 and violet at pH 9.0.
7. With a suitable stationary phase a number of polychlorinated phenols can often be determined in the presence of one another.



2.5.3.1 Fig. 1 Example of a gas chromatogram obtained in the determination of pentachlorophenol as pentachlorophenyl acetate in organisms. The peak represents 25 μg of pentachlorophenol derived from a tissue sample with a concentration of 500 $\mu\text{g}/\text{kg}$

2.5.4 Basic compounds

2.5.4.1 Gas-chromatographic determination of dichloroanilines in aquatic organisms

Subject

Gas-chromatographic determination of dichloroanilines in aquatic organisms.

Field of application

The method is suitable for the determination of the content of dichloroanilines in mussels, guppies, and shrimps.

Sample size: at least 3 grams of homogenized tissue.

Range of concentration: 50–3000 μg per kg wet weight.

After slight adaptation, the procedure is also suitable for the determination of other chloroanilines (see Note 7).

Principle

The dichloroanilines are extracted from the homogenized tissue by refluxing the sample for 1 h with 6M hydrochloric acid. Subsequently the sample is diluted until it is 1M in hydrochloric acid. After the addition of potassium bromide, potassium bromate, and 2,4,6-trimethylaniline as a catalyst, brominated dichloroanilines are formed. When the excess of bromine has been eliminated with sodium sulphite and the solution has been made weakly alkaline, the brominated dichloroanilines are extracted with hexane. After the extract has been dried, the brominated dichloroanilines can be determined gas-chromatographically using an electron capture detector.

EQUIPMENT AND MATERIALS

In addition to the requirements mentioned in Section 2.4.1, the following reagents, glassware, and auxiliary apparatus are needed:

- 3,4-dichloroaniline, 99 % pure
- 2,4-dichloroaniline, 99 % pure
- 2,6-dibromo-3,4-dichloroaniline (the synthesis is described in 2.4.4.1, Appendix 1)
- 2,4,6-trimethylaniline (see Note 5)
- sodium sulphite, analytical grade
- potassium bromate, analytical grade
- potassium bromide, analytical grade, powdered
Before use, this is heated for 6 h at 300°C . It is stored in a glass bottle with a ground-in stopper.
- 6M hydrochloric acid
With stirring, pour 950 ml of concentrated hydrochloric acid into 1050 ml of distilled water in a large beaker. Store the solution in a 2.5-litre glass bottle with a ground-in stopper. Transfer part of the solution into a 500-ml conical flask fitted with a Kipp automatic dosing device.
- 3.5M caustic soda
With stirring, dissolve 550 g of sodium hydroxide in 4 litres of distilled water in a large beaker. Store the solution in 2.5-litre glass bottles with screw-caps. Avoid contact between the solution and the screw-caps as far as possible. Transfer part of the solution into a 1-litre glass bottle fitted with a 25-ml Kipp automatic dosing device.
- Potassium bromate solution in water (ca. 12 $\text{g}\cdot\text{l}^{-1}$)
With stirring, dissolve 12 g of potassium bromate in 1 litre of distilled water in a large beaker. Store the solution in a 1-litre glass bottle with a ground-in stopper. Transfer part of the solution to a 250-ml conical flask fitted with a 2-ml Kipp automatic dosing device.
- 0.5M sulphuric acid
With stirring, add 56 ml of 100 % sulphuric acid to 2 litres of distilled water. Store the solution in a glass bottle with a screw-cap.
- ca. 20 % sodium potassium tartrate solution in water
With stirring and gentle heating, dissolve 900 g of sodium potassium tartrate in 2.5 litres of distilled water in a large beaker. Store the solution in a 2.5-litre glass bottle with a screw-cap.

2. Analysis of the investigation of degradability, toxicity, and accumulation

Avoid contact between the solution and the screw-cap as far as possible.

Transfer part of the solution to a 500-ml conical flask fitted with a 10-ml Kipp automatic dosing device.

Glassware

- one 50-ml Kipp automatic dosing device with matching 1-litre bottle
- one 2-ml Kipp automatic dosing device with matching 250-ml conical flask
- at least twelve 50-ml round-bottomed flasks with NS 29 ground joint
- narrow-neck 1-litre glass bottle with ground-in stopper
- one powder funnel (diameter 6 cm) fitting in a 250-ml separating funnel
- at least 12 coil condensers 20 cm long with NS 29 ground joints
- at least twelve 100-ml beakers.

Auxiliary apparatus

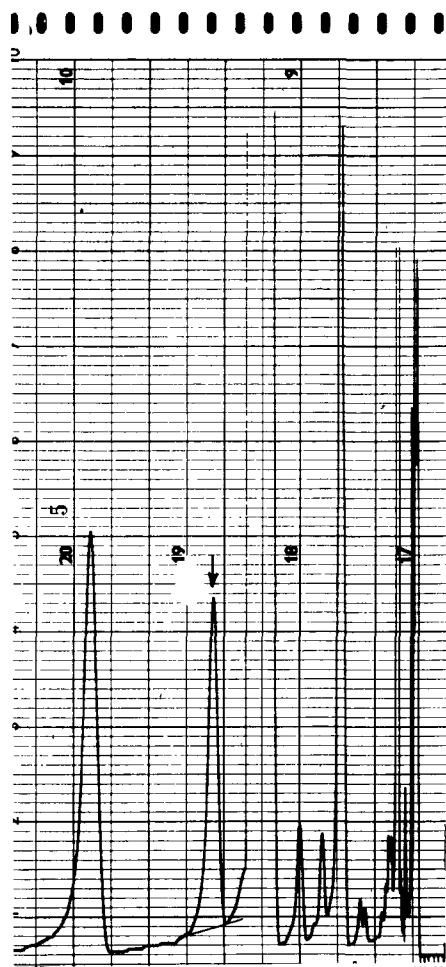
- at least 12 Suberit rings (diameter 8 cm)
- at least 12 heating mantles for 50-ml round-bottomed flasks
- one 5 µl injection syringe
- one box of black-band filter papers (diameter 5.5 cm).

PROCEDURE

- First read Sections 2.4.1 and 2.5.1.
- Place twelve 50-ml round-bottomed wide-neck flasks on 12 Suberit rings.
- Code six flasks as standards (x µl of y ppm added) and as analytical blank + serial number.
- Weigh into each of the above-mentioned six flasks 1 gram of homogenized blank tissue of the same type as that of the sample.
- Choose as the standard concentrations the values expected in the samples, which can be estimated by means of the accumulation factors (see Note 1).
- By means of a plunger pipette, transfer the required amounts of standard solutions of 3,4-dichloroaniline in 0.5M sulphuric acid into the coded round-bottomed flasks.
- Add to the centrifuge tube for the blank 200 µl of the sulphuric acid used for preparing the standards.
- Close the round-bottomed flasks.
- Allow them to stand for half an hour.
- Transfer the coding of the samples to the other six round-bottomed flasks.
- From each sample weigh out ca. 1 gram to within 0.01 g into a round-bottomed flask.
- Note the weight on the serial form (see Section 2.4.1).
- Always use a clean stainless-steel spatula for **each** sample.
- By means of a Kipp automatic dosing device, transfer 10 ml of 6M hydrochloric acid and a few glass beads to each flask.
- Mount a coil condenser on each round-bottomed flask.
- Place the flask in a heating mantle.
- Adjust the mantle temperature so that the contents of the flask boil gently for 1 h.
- In the meantime, place in front of each flask a 100-ml beaker onto which the coding of the corresponding sample has been transferred.
- By means of a Kipp automatic dosing device, add 50 ml of distilled water to each beaker.
- Place twelve 250-ml separating funnels in the rack in the fume cupboard (see Section 2.4.1).
- Transfer the coding from the round-bottomed flasks to these funnels.
- On each separating funnel place a glass funnel (diameter 3 cm) with black-band filter paper (diameter 5.5 cm) in it.
- After the hour's boiling, rinse each condenser with part of the 50 ml of water from the beaker.
- Transfer the contents of each round-bottomed flask through the corresponding filter into the corresponding separating funnel.
- Rinse each flask with the remainder of water in the beaker.
- By means of a 5-µl injection syringe transfer 3 µl of 2,4,6-trimethylaniline from the volumetric flask into each separating funnel.
- By means of a powder funnel (diameter 6 cm) and a powder-dosing spoon, add 1.5 g of powdered potassium bromide to each separating funnel.
- Close each separating funnel, shake until all the potassium bromide has dissolved, and relieve the excess pressure through the tap.
- Then, by means of a Kipp automatic dosing device, add 2 ml of the potassium bromate solution to each separating funnel.
- Reclose the separating funnel and shake briefly by hand.
- Allow the separating funnels to stand in the rack for half an hour for bromination to take place.
- After the half-hour, remove the excess of bromine in each separating funnel by adding 1 gram of sodium sulphite to each separating funnel with the aid of a powder funnel (diameter 6 cm) and a powder-dosing spoon.
- Swirl each separating funnel gently.
- By means of a Kipp automatic dosing device, add successively to each separating funnel:
 - 10 ml of 20 % sodium potassium tartrate solution
 - 25 ml of 3.5M caustic soda
 - 5 ml of hexane.
- Close each separating funnel firmly with the ground-in stopper by placing this **wet** in the ground socket with a thrusting-turning motion.
- Shake briefly by hand and relieve the excess pressure through the tap.
- Place the separating funnels in the shaker.
- Shake for 30 min.
- Place twelve 10-ml volumetric flasks below the separating funnel rack.
- To each separating funnel add 1 gram of anhydrous sodium sulphate through a glass funnel (diameter 6 cm).
- Place a small funnel (diameter 3 cm) on each volumetric flask.
- Apply to the volumetric flasks the same coding as on the corresponding separating funnels.
- Replace the separating funnels in the rack.
- Allow the contents of each separating funnel to run into a plastic bucket until about 1 cm of water still remains above the tap.
- Break any emulsions present (see Section 2.4.1).

2.5 Methods for the determination of individual test compounds in organisms

- Carefully allow the contents of each separating funnel to run into a 250-ml beaker until the interface of the two liquids is in the bore of the tap.
- Dry the outlet tube of each separating funnel with a pipe-cleaner.
- Allow the hexane layers to run into the 10-ml volumetric flasks.
- Rinse each separating funnel with about 4 ml of hexane from a glass wash-bottle.
- Transfer this hexane also into the corresponding volumetric flasks.
- Place these flasks in a wooden block with suitable holes.
- Fill the volumetric flasks with hexane up to the calibration mark and close them.
- Homogenize the contents of each flask.
- Allow the extracts to stand for **at least 1 h** over the sodium sulphate already present in the flask.
- The extracts are now ready for gas-chromatographic analysis.



2.5.4.1 Fig. 1 Example of a gas chromatogram obtained in the determination of 3,4-dichloroaniline as 2,6-dibromo-3,4-dichloroaniline in organisms. The peak represents 125 µg of 3,4-dichloroaniline derived from a tissue sample with a concentration of 5000 µg/kg

Adjust the gas chromatograph as follows:

Column: 3 % of OV-225 on Chromosorb W-AW pretreated with Carbowax (see Section 2.4.4.1).

Glass; 220 cm × 3 mm internal diameter.

Column temperature: 180° C.

Detector temperature: 300° C.

Carrier gas, auxiliary gas, and electric detector variables: optimum for brominated aniline (see Section 2.1.1).

REPORTING OF THE RESULTS OF THE MEASUREMENTS

Give the concentration in µg per kg wet weight.

Take the sample weight into account (see Section 2.5.1).

Record any special features such as the appearance of unexpected peaks in the chromatogram.

NOTES

1. The estimated accumulation factors of 3,4-dichloroaniline in living animals after a residence time of 14 days in water are as follows:

Animals	Concentration in water (µg.l ⁻¹)	Accumulation factor
Mussel	3–30	80
Guppy	3–30	300
Shrimp	3–30	50

2. After homogenization, the samples can be safely stored in the deep-freeze cabinet at –35° C for 3 weeks.
3. Pure 2,6-dibromo-3,4-dichloroaniline is necessary for determining the retention time of this substance and for the optimization and checking of the gas chromatograph. The substance does not appear in the standard ranges of chemicals manufacturers. The synthesis is described in Appendix 1 of Section 2.4.4.1.
4. According to our experience, the extracts can be safely stored in the dark for 4 weeks.
5. Before use, the 2,4,6-trimethylaniline must be purified by distillation under dry nitrogen at reduced pressure through a small column filled with Wilson helices. After purification, it must be stored in a volumetric flask under nitrogen in a refrigerator. We found the smallest amount of impurities in the Merck product.
6. The extracts may be coloured pink by a very small amount of impurity in the 2,4,6-trimethylaniline (even after distillation). Additional peaks may appear in the chromatogram which, however, do not interfere with the determination of 2,6-dibromo-3,4-dichloroaniline or of 6-bromo-2,4-dichloroaniline.
7. Only slight adaptations of the procedure are required for the determination of 4-chloroaniline, 3-chloroaniline, and 4-chloro-2-methylaniline (Kossmann, 1971). With a suitable stationary phase a number of these compounds can often be detected in the presence of one another.

2.6 THE USE OF LABELLED COMPOUNDS IN BIODEGRADATION RESEARCH

Subject

Determination of the degradation of ^{14}C -labelled substrates in die-away tests with the aid of radioactivity measurements.

Field of application

1. Die-away tests in which the concentration of the test compound is too low for the general analytical methods.
2. Monitoring complete mineralization.

Principle

After incubation, the medium is acidified. CO_2 is blown out and trapped in an absorbent. The amount of $^{14}\text{CO}_2$ is a measure of the mineralization of the substrate. After the removal of the $^{14}\text{CO}_2$, the radioactivity of the organisms is determined. This shows how much of the substrate the organisms incorporated during incubation.

EQUIPMENT AND MATERIALS

- scintillation counter
- scintillation vials, glass with screw-caps
- scintillation liquid
 - a. Aquasol (New England Nuclear Corp.) for aqueous solutions and membrane filters*
 - b. Toluene with 0.4 % of PPO (2,5-diphenyloxazole) and 0.01 % of POPOP (1,4-bis[2-(5-phenyloxazolyl)]benzene) for organic solvents
- CO_2 absorption liquid: a mixture of ethanolamine and methyl cellosolve (1:2) (Watson and Williams, 1970) (store in closed bottles)
- Porapak Q (Waters Assoc. Inc., Frammingham, Mass. USA)
- blowing-out apparatus for volatile compounds (see Section 2.6, Fig. 1)
- blowing-out apparatus for non-volatile compounds (see Section 2.6, Fig. 2)
- membrane vacuum pump
- plunger pipettes, 0.1, 1 and 5 ml, with disposable tips
- 2M H_2SO_4
- filter apparatus for membrane filters, dia. 25 mm
- membrane filters, dia. 25 mm, pore size 0.1 μ
- fume cupboard
- gas wash-bottle, 500 ml
- 20 % NaOH solution in water.

INTRODUCTION

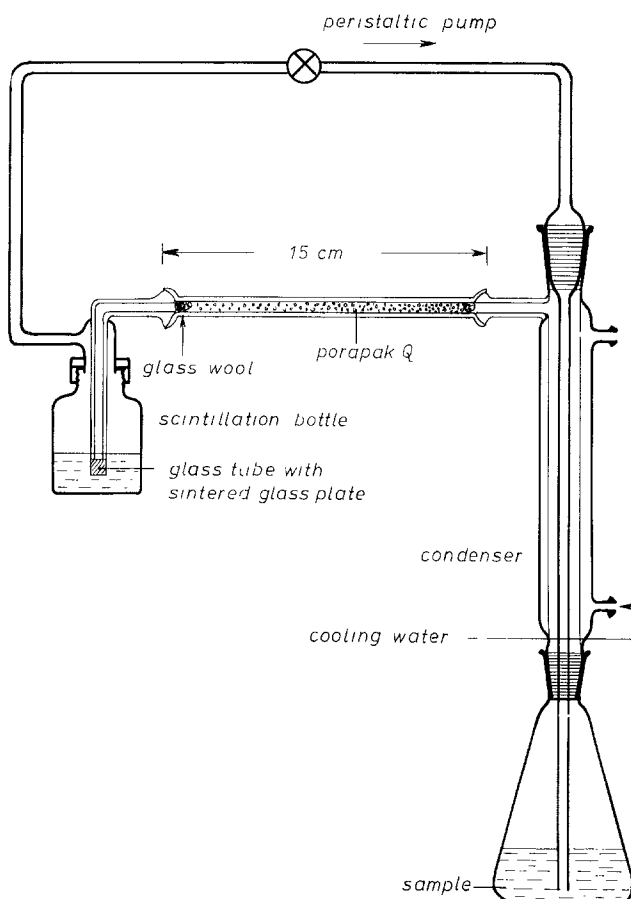
The procedure described in this section has been developed for a simple determination of the amount of $^{14}\text{CO}_2$ liberated in the biological degradation of a labelled compound. It is divided into two parts: the first part applies to non-volatile compounds and the second to volatile compounds, compounds that can easily co-distil with evaporating water, and compounds that give important amounts of volatile organic breakdown products.

This distinction is made because of procedural differences. In the first method (for non-volatile substances) several samples can be taken from one flask, while in the other method the whole contents

of the test flask must be used for the radioactivity measurement. CO_2 can be separated from a volatile test compound by means of Porapak Q.

It is assumed that the volatility of the test compound is known and that the analysts are familiar with the handling of radioactive materials.

Some general information on working with radioactive materials and on the acquisition of the necessary licences is given in Section 6.2.



2.6 Fig. 1 Blowing-out system for the separation of $^{14}\text{CO}_2$ and volatile compounds labelled with ^{14}C

PROCEDURE

Non-volatile compounds

- At the beginning of the test, directly after the addition of the labelled substrate, take two 1-ml samples and add each to 15 ml of Aquasol in scintillation vials, count A (Note 1).
- During the test, take 5-ml samples in duplicate, place these in scintillation vials, held in the blowing-out apparatus, in a fume cupboard, and assemble the blowing-out apparatus (2.6, Fig. 2).
- Add sufficient 2M H_2SO_4 to these samples through the inlets of the blowing-out apparatus to bring the pH to about 2.
- Aerate the samples for 10 min by engaging the membrane pump.
- Add 1 ml of the acidified sample to 15 ml of Aquasol in a scintillation vial, count B.

* Other brands of corresponding liquid may also be used.

- Filter the remainder (i.e. 4 ml) through a membrane filter and wash the scintillation vial and the filter with 5 ml of the test medium.
- Place the filter in 15 ml of Aquasol, count D (Note 2).
- Determine the activities in the two scintillation bottles and calculate the percentage degradation according to point 4 in this procedure.

Volatile compounds

- Assemble the blowing-out apparatus as shown in the drawing (Note 3).
- At the beginning of the test, directly after the addition of the labelled substrate, take two 1-ml samples and add each to 15 ml of Aquasol in scintillation vials, count A (Note 1).
- Attach the test-flask with the ground-joint to the condenser (moisten the ground joint with water or glycerol).
- Add 5 ml of the CO₂ absorption liquid to a scintillation vial and place it in the apparatus.
- Add sufficient 2M H₂SO₄ through the condenser to the test flask to bring the pH to about 2.
- Start the pump and flush air through for 1 min.
- Stop the pump and change the vessel containing the CO₂ absorbent.
- Start the pump and flush air through for another 4 min. Repeat this once more. On the last occasion, rinse the glass tube with 1 ml of methyl cellosolve with the aid of a Pasteur pipette and collect the cellosolve in the absorption vial.
- Add 10 ml of toluene scintillation liquid to the CO₂ absorption vial; count C.
- Remove the Porapak from the tube and add it to 15 ml of Aquasol in a scintillation vial; count E.
- A 1-ml sample of the acidified water is added to Aquasol, then 5 ml is filtered as described in para. 3.1 of this procedure; counts B and D.
- Calculate the degradation according to para. 4 of this procedure.

TREATMENT OF THE RESULTS

The counting and the measurement of radioactivity are carried out in such a way that the standard deviation of the results is below 3%.

All the counts are referred to 1 ml of the test medium and are recorded in a table.

The following quantities are then determined:

A: activity in the medium at the beginning of the test

B: activity in the medium after acidification and removal of ¹⁴CO₂

B₀: ditto, in the sterile blank (Section 3.5.2)

C: activity in the collected CO₂ fraction

C₀: ditto, for sterile blanks

D: activity in the microorganisms (on the filter)

E: activity in the Porapak

E₀: ditto, of the sterile blank.

The degradation can be calculated from these quantities. The activity in the Porapak can be used to check the blowing-out method, since in principle the following relationship must hold: A = B + C + E. However, this is not the case in practice, since then (A - B) > C because of the non-quantitative trapping and the counting method for ¹⁴CO₂. An efficiency factor can be found by carrying out tests with NaH¹⁴CO₃.

Tests with non-volatile compounds

The precise calculation depends on the behaviour of the sterile blank. If the procedure for non-volatile compounds is used, the degradation is measured as the difference between the measurements before and after acidification and blowing out. This difference also arises in the sterile blank, not because of chemical instability but as a consequence of adsorption on glass walls, etc. If this effect is considerable, a correction factor must be applied. If A - B₀ < 0.05 A, the degradation is calculated in the following way without the application of the correction factor.

Degradation:

$$a_1 = \frac{A - B}{A} \times 100\% \quad (1)$$

Uptake in microorganisms:

$$b = \frac{D}{A} \times 100\% \quad (2)$$

Total degradation caused by microorganisms:

$$c_1 = a_1 + b \quad (3)$$

For A - B₀ > 0.05 A, the degradation results must be corrected. For each concentration tested a sterile blank is also measured. The blank 'degradation' is calculated for each of these concentrations:

$$a_0 = \frac{A - B_0}{A} \times 100\% \quad (4)$$

By plotting this apparent degradation a₀ against the test concentration it can be established whether a₀ is concentration-dependent. If the result is affirmative, a blank test must be performed with a substrate concentration of 0.1 × the lowest concentration tested. If a₀ is independent of the concentration, the correction can be made by subtracting the percentage (a₀) of non-degraded fraction of the test compounds from c₁ in the following way:

corrected degradation:

$$c_2 = c_1 - (1 - 0.01 c_1) a_0 \quad (5)$$

If the blank degradation (a₀) is in fact concentration-dependent, the concentration x_t of the non-degraded test compound is calculated for each moment of sampling:

$$x_t = d(1 - 0.01 c_1) \quad (6)$$

where d is the concentration of the test compound at the beginning of the test. In this way, with the aid of the above-mentioned curve, it is possible to determine the percentage correction at each sample point. The correction is then performed in accordance with (5). If it is desired to determine an absolute concentration of the test compound, this can be calculated by means of the specific activity of the test compound.

The degradation results (a, b, and c) are plotted with the concentration or the percentage degradation along the y-axis and time along the x-axis. For further treatment, see Section 3.1.

Tests with volatile compounds

Degradation:

$$a_2 = \frac{C}{A} \times 100\% \quad (7)$$

Uptake by microorganisms:

$$b = \frac{D}{A} \times 100\% \quad (2)$$

Total degradation caused by the microorganism:

$$c_3 = a_2 + b \quad (8)$$

In this degradation test a certain systematic error arising from the determination of C is accepted. In the sterile blank C₀ must approximate to 0, otherwise the conditions of the blowing-out system are unsatisfactory.

NOTES

1. This sample serves together with the sterile blank as a basis for the calculation of the degradation.
2. When samples are added to the scintillation liquid, the mixture must always be well shaken and dirt or spilled liquid must be removed from the outside of the vial. A cellulose acetate or nitrate filter can be added to the scintillation liquid in the wet state and dissolves completely after some time. After this, the solution must be well shaken.
3. The blowing-out apparatus can be adapted to the selected test arrangement. The apparatus described here has been developed for tests with 1,1,2-trichloroethane (TCE). It does not appear to be usable for 1,2-dichloroethane (DCE), since DCE leaks through the Porapak.

It is therefore recommended that the conditions of this method be determined for each substance tested by carrying out trials with $\text{NaH}^{14}\text{CO}_3$ and the labelled test compound. The variables may be the rate and time of aeration, the amount of Porapak Q, the length of the column, and the column temperature. In addition, it is recommended that the apparatus be made of glass to the greatest possible extent, since many volatile organic compounds may dissolve in the plastic or rubber tubing.

The apparatus is constructed of normal glassware.

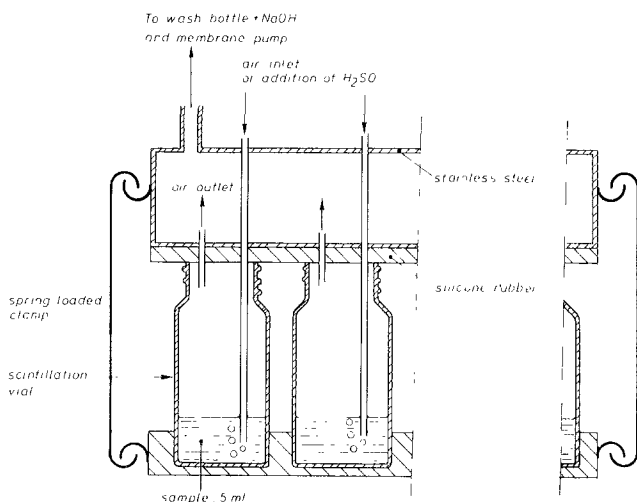
The two most important parts are a tube 15 cm long filled with 0.5 g of Porapak Q and the CO_2 -absorption vessel. The Porapak (sufficient for the absorption of 1000 μg of TCE) is kept in place with a plug of glass wool.

The CO_2 -absorption vessel is an ordinary scintillation vial with a screw-cap in which a hole has been drilled for a glass tube with a sintered glass disc. The screw-cap is provided with Teflon packing and the tube is firmly bonded with silicone cement. The pump may be a peristaltic pump capable of pumping air. Normally, the total volume of air must be able to circulate in the system in about 20 sec.

The absorption capacity of Porapak Q depends on the temperature.

The tube is easily cooled by wrapping it in a layer of cotton wool and dripping acetone onto it.

In principle, 5 ml of the CO_2 absorption liquid can absorb 118 mg of carbon in the form of CO_2 (Watson and Williams, 1970).



2.6 Fig. 2 Blowing-out system for the removal of $^{14}\text{CO}_2$ from a culture liquid with a non-volatile ^{14}C -labelled substrate

2.7 VARIOUS METHODS FOR MEASURING BIOLOGICAL ACTIVITY

2.7.1 Determination of oxygen uptake

2.7.1.1 Introduction

Respiration is one of the parameters that can be used as a measure of the biological activity of microorganisms.

In these paragraphs by respiration is meant the so-called aerobic respiration, which can be defined as:

an energy-yielding metabolic process in which either organic or reduced inorganic compounds act as electron donors and molecular oxygen as the ultimate electron acceptor (Stanier *et al.*, 1970).

Aerobic respiration can be measured by determining the uptake of oxygen. The suitability of these methods for degradation studies has been demonstrated by Wagner (1973) and by Fischer *et al.* (1974).

The uptake of oxygen is one of the methods of determining degradability in which no specific analysis of the substrate need be carried out. In addition to this, in view of the above-given definition, the oxygen uptake is also a very fundamental parameter for aerobic degradation.

The rate of the oxygen uptake in an adapted culture, after correction for the endogenous respiration, gives an idea of the degradability of a substrate (the rate at which it dies away).

The amount of oxygen consumed provides some information on the degree of mineralization of the substrate (Section 3.1.1).

In addition to the determination of degradation, the oxygen uptake can be used as a criterion for toxicity tests with microorganisms (Section 3.5.1.2).

In toxicity tests with algae the evolution of oxygen as a result of photosynthesis can be determined.

The following techniques are used to determine the oxygen uptake:

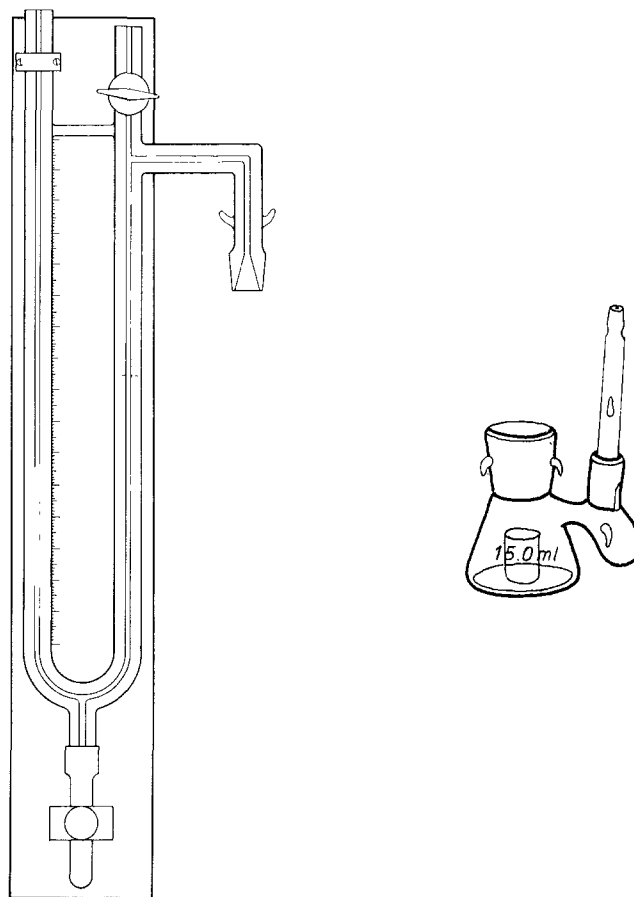
- manometric techniques (not suitable for measurements lasting longer than about 8 h) (1)
- compensation of the oxygen used by means of electrolysis (2)
- electrochemical determination of the oxygen concentration (3)
- chemical determination of the oxygen concentration (Winkler titration) (4)

In the **manometric methods** (1), the amount of oxygen taken up can be determined in three ways:

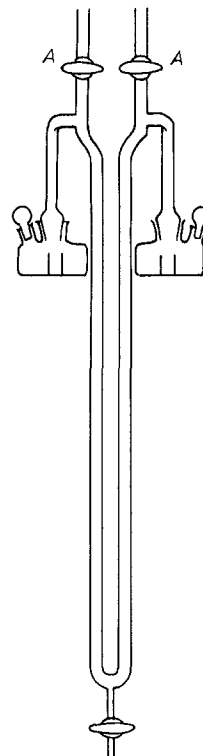
- In the Warburg method the oxygen uptake is calculated from the change of the pressure in the system, after the absorption of CO_2 , at constant volume. Although the calculation is not very complicated, the combined volume of the manometer and vessel (2.7.1.1, Fig. 1) must be accurately known in order to calculate the oxygen uptake on the basis of a change in pressure. Since one arm of the manometer is open, fluctuations in atmospheric pressure have a strong influence on the system. Corrections can be made for small fluctuations. According to our experience, the passage of a barometric depression makes a measurement unreliable.
- The differential manometer eliminates this problem, because such an apparatus is completely closed (2.7.1.1, Fig. 2).

Small variations in the laboratory temperature no longer have an influence on the measurement, but, just as in the Warburg method, the volumes of the vessels and the manometer must be known for the oxygen uptake to be calculated.

- The respirometer (2.7.1.1, Fig. 3) is a further improvement on the differential manometer. The respirometer works at a constant pressure. The volume of oxygen taken up is compensated by a rise of the mercury column in a calibrated tube or by a displacement of the piston. The oxygen uptake can be read off directly. The vessel volumes need no longer be known.



2.7.1.1 Fig. 1
Warburg manometer and vessel

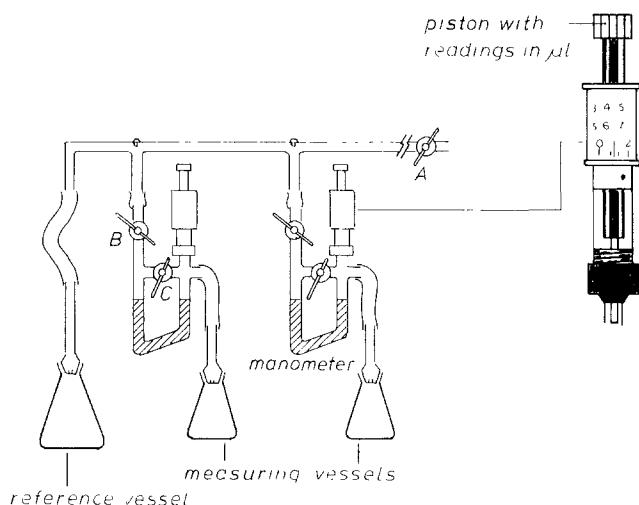


2.7.1.1 Fig. 2 A differential manometer

2. Analysis of the investigation of degradability, toxicity, and accumulation

The **manometric methods** (1) are intended for short-term determinations (up to about 8 h).

When these methods are used to determine, for example, the rates of enzymatic reactions, no growth must occur, since the reaction rate is proportional to the biomass. This implies that the duration of a measurement should be shorter than the generation time of the organism or that growth must be prevented by omitting an essential nutrient (e.g. nitrogen) from the medium.



2.7.1.1 Fig. 3 The basic scheme of the Gilson respirometer

- A. Tap isolating the system from the atmosphere
B. Tap isolating one unit from the rest of the system
C. Tap for short-circuiting the manometer

The oxygen used by microorganisms may also be accurately replaced by the **electrolysis of water** (2). Here the production of oxygen (in unit time) is directly proportional to the current intensity. By carefully stabilizing the electrolysis current the total time during which the current is switched on is a measure of the oxygen uptake. This allows a direct reading (analogue or digital) of the oxygen uptake.

This method can be used for long-term experiments in which an appreciable oxygen uptake may take place (up to several grams per litre). The rate of this process must not, however, exceed the production capacity of the electrolysis system.

The determination of the oxygen uptake with the **oxygen electrode** can be carried out in two ways:

- As an end-point determination. Here the amount of oxygen taken up at the end of a degradation test is measured in the aqueous phase.
- As a continuous measurement. Here the change in the amount of oxygen dissolved in the aqueous phase in short-time tests is monitored.

In both cases the determinations are performed in such a way that diffusion of atmospheric oxygen into the aqueous phase during the test (and the measurement) is prevented.

In the first case the determination with the electrode takes the place of the classical Winkler titration, as in the determination of the Biochemical Oxygen Demand (BOD). The tests are carried out in closed flasks completely filled with the medium. The substrate concentration is therefore low. The test duration may vary between a few days and a year.

In the second case the method is comparable with the manometric method. The sensitivity is approximately the same, but the test duration is only a few minutes. As in the manometric methods, the test should be started with an adapted culture.

The **Winkler titration** (4) is used in the determination of the BOD (Anon., 1971). The method is reliable and can be performed with standard laboratory glassware, though admittedly it is laborious. Since the Winkler titration is prescribed legally for the determination of the BOD in some countries, it is still much used for determinations in effluents. However, when it is used in sea water, interference may occur.

In addition to the uptake of oxygen, the liberation of carbon dioxide may be used as a measure of degradation or mineralization. According to Gledhill (1975), the carbon dioxide can be trapped in a barium hydroxide solution and be determined titrimetrically. In addition to the Winkler titration, the above-mentioned methods (1, 2, and 3) will be discussed in the following sections.

2.7.1.2 Determination of oxygen uptake with the aid of manometric techniques

Subject

Determination of the metabolic activity of microorganisms by measuring the uptake of oxygen.

Field of application

The respiration of microorganisms can be determined both in fresh water and in salt water as a criterion of aerobic degradation of organic compounds or of toxicity. In degradability tests the method is intended for work with an adapted culture in experiments that usually last no longer than about 8 h.

Principle

The uptake of oxygen by microorganisms is determined by measuring the change in pressure or volume in a closed system after the absorption of the carbon dioxide formed.

EQUIPMENT AND MATERIALS (Note 1)

- One of the following sets of equipment:
 - Warburg constant-volume respirometer
 - differential manometer apparatus
 - constant-pressure respirometer (Note 2)
- KOH solution (20/80, w/w, 0.2 ml per vessel)
- filter paper (ca. 2 × 2 cm), folded zigzag (1 per vessel)
- various pipettes (2, 1, 0.5, and 0.2 ml)
- RBS solution, 25 % (Hicol B.V., Rotterdam).

PROCEDURE

This procedure is applicable to the Warburg method, the method using differential manometers, and the constant-pressure respirometer. The differences between the methods are limited to the possibilities of use (Section 2.7.1.1) and calculation of the results.

- Add 2 ml of the bacterial suspension (10^6 – 10^8 cells per ml) to a buffered nutrient solution (Note 3) in a clean and dry Warburg flask provided with a central well.
- Introduce the substrate dissolved in a maximum of 0.5 ml of buffered nutrient solution in the side-arm of the vessel (Note 4).
- Grease the upper rim of the central well with silicone grease.
- Add 0.2 ml of KOH solution to the central well.
- With a pair of forceps, place a piece of filter paper in the central well (Note 5).

2.7 Various methods for measuring biological activity

- Grease the ground joints of the manometers, vessels, side-arms, and stoppers, and attach the vessels.
- Allow the vessels to come to equilibrium with their surroundings for 10–15 min by shaking in the water bath. Tap A (2.7.1.1, Fig. 1; 2.7.1.1, Fig. 2; 2.7.1.1, Fig. 3) remains open during this process.
- Prepare a 'thermobarometer' in a similar manner. This resembles the other vessels but contains no organisms (Note 6).
- Adjust the manometers to the reference points and close tap A.
- Add the contents of the side-arm to each flask by tipping it.
- Adjust the manometers at regular intervals and read them. The frequency depends on the rate of the oxygen uptake (Note 7).
- At the end of the test wash the flasks by boiling with RBS solution for an hour.
Rinse well with mains water and distilled water.
- Calculate the oxygen uptake (Note 8).

NOTES

1. This method is intended as a supplementation of other methods. The other requirements are mentioned in the descriptions of these methods.
2. At TNO Division of Technology for Society a Gilson constant-pressure respirometer is used in this method. The oxygen consumed (or produced in photosynthesis) is read off directly in microlitres from the displacement of a piston. It is therefore unnecessary to determine the volumes of the combination of vessel and manometer used.
3. All liquid volumes introduced into the vessels must be accurately known.
4. When the substrate is sparingly soluble in the medium, it may be placed in the vessel and the bacterial suspension in the side-arm. The bacterial suspension must then be a concentrated one, so that after dilution the correct concentration is obtained. In toxicity tests vessels with two side-arms may be used, one of the side-arms containing the substrate and the other the toxic substance.
5. The zigzag-folded filter paper must be about 5 mm longer than the height of the central well. The liquid contained therein (0.2 ml) must be sufficient to wet the whole piece of paper. When this is not the case, somewhat more KOH solution must be used (Note 3).
6. The thermobarometer enables the observations to be corrected for slight variations in temperature and atmospheric pressure, since the test is affected by such variations. The thermobarometer constitutes a blank.
In principle, a thermobarometer is not necessary in tests with differential manometers and constant-pressure respirometers, since these are completely closed systems. When working with a Gilson respirometer, however, it has been found that this is affected by transient thunderstorms and variations in ambient temperature.
The reason for this is presumably the fact that the connecting tubes and manometers are outside the water bath. It is therefore desirable to work in a temperature-conditioned room or in a room with only slight temperature fluctuations.
For short experiments the thermobarometer need not be sterile. Naturally, the number of microorganisms must be as low as possible.
7. The way in which the apparatus is set up varies in the different methods:
 - In the Warburg method, the liquid level in the closed arm of the manometer is always adjusted to the starting point with the screw below the manometer (2.7.1.1, Fig. 1). The difference between the liquid levels in the two arms of the manometer is read off and recorded.
 - In the case of the differential manometers the difference between the liquid levels in the two manometer arms is read off.
 - In the Gilson respirometer the oxygen uptake is read off directly from the displacement of the piston.
8. The results are calculated differently in the different methods:

Warburg method

The amount of gas taken up or given off, G , expressed in μl at 760 mm Hg and 0°C , is proportional to the change in height in the open arm of the manometer h and the vessel constant k :

$$G = k \times h \text{ (in } \mu\text{l)}.$$

The vessel constant must be calculated for each test from the formula:

$$k = \left\{ \frac{273}{T} V_g + \alpha V_l \right\} / P_0$$

where:

 - V_g is the total volume of gas in the system up to the calibration mark in μl
 - V_l is the total volume of liquid added in μl
 - T is the absolute temperature ($^\circ\text{K}$)
 - α is the solubility of the gas to be determined, in ml of gas per ml of liquid
 - P_0 is the standard pressure (760 mm for mercury or 10,000 mm for Kreb's solution when this is used as the manometer liquid).

The combined volume of each flask and manometer combination must be known. The vessel constant can then be calculated from the test data.
For the determination of α (for oxygen) the following table can be used in most cases. For salt concentrations of more than 1–2 M a correction must be made for this, since the solubility of oxygen then deviates too greatly from the values listed.

2.7.1.2 Table 1 The solubility of oxygen in water

α in $\frac{\text{ml O}_2 \text{ (STP)}}{\text{ml (H}_2\text{O) atm}}$	
Temperature, $^\circ\text{C}$	α
0	0.0487
10	0.0379
15	0.0344
20	0.0309
25	0.1282
30	0.0261

From: International Critical Tables (1928).

The composition of Kreb's solution is as follows:

NaBr, anhydrous	44 g
Triton X-100*	
(Rohm & Haas Co.)	0.3 g
Evans Blue	0.3 g
Distilled water	1000 ml.

* Any comparable non-ionic detergent may be used in place of Triton X-100.

2. Analysis of the investigation of degradability, toxicity, and accumulation

The density of Kreb's solution is 1.033 g.cm⁻³ at 20°C. The density must be checked, for example with a pycnometer (renew this solution regularly).

Before calculating the uptake of oxygen, the values of *h* for each vessel are first corrected by that of the thermobarometer.

Differential manometers

The oxygen uptake is calculated as in the Warburg method, using the formula:

$$G = k' \times h,$$

in which *k'* is the 'vessel constant' for the differential manometer. This is calculated from the following formula:

$$k' = \left\{ 1 + \frac{AP_o}{2V'_g} \right\} \left\{ \frac{1}{P_o} \left(\frac{273}{T} V_g + \alpha V_l \right) + \frac{1}{2} A \frac{273}{T} \right\}$$

Here:

V'_g is the total volume of gas in the compensation vessel and that side of the manometer

A is the cross-section area of the capillary in the glass tube of which the manometer arms are made. This area must be smaller than 1 mm².

The other symbols have the same meaning as in the formula for *k*.

Gilson respirometer

No calculations are necessary with this apparatus. The difference between two readings gives the consumption directly in micro-litres.

If required, a thermobarometer can always be used to check the influence of temperature fluctuations.

2.7.1.3 Determination of oxygen uptake by compensation of the consumed oxygen by means of electrolysis

Subject

Determination of the metabolic activity of microorganisms by measuring the uptake of oxygen.

Field of application

The method can be used in the determination of the degradability and toxicity of substances to microorganisms in fresh or salt water. Long-term experiments are possible.

Principle

A nutrient solution to which the substance under investigation has been added is placed in a Sapromat or similar system and is inoculated. As oxygen is consumed the pressure decreases, because the carbon dioxide formed is trapped. The pressure reduction activates an electrolysis cell which makes up the oxygen used. The flow of current is used as a measure of oxygen consumption.

EQUIPMENT (Note 1)

- A Sapromat or a comparable system (Montgomery, 1971)
- simple laboratory glassware:
 - a small number of conical flasks (1–3 litres)
 - a small number of measuring cylinders (250 ml).

PROCEDURE

- Set up and calibrate the apparatus in accordance with the manual or literature.
- Fill the flasks with the prescribed amount of liquid consisting of the medium, the substance to be investigated, and the micro-organisms.

- Place carbon dioxide absorbent in the wells provided for it.
- Carry out the remainder of the test in accordance with the instructions.
- The results are given in mg of oxygen consumed per litre.

NOTE

1. The method is intended as a supplement to other methods. The other requirements are given in descriptions of these methods.

2.7.1.4 Determination of oxygen uptake with the aid of an oxygen electrode

Subject

Determination of the metabolic activity of microorganisms by measuring the uptake of oxygen.

Field of application

By using oxygen electrodes long-term degradation experiments can be performed, in which the degradation can be calculated from the initial and final concentrations of the dissolved oxygen. In measurements lasting a few minutes in an adapted culture its activity in relation to a given substrate can be determined.

Principle

The change in the oxygen concentration (or the initial and final concentrations) in solution is measured with the aid of an oxygen electrode. The oxygen consumption is calculated from these measurements. Access of atmospheric oxygen is prevented as far as possible during the test and the measurements.

EQUIPMENT (Note 1)

- oxygen electrode with measuring instrument
- BOD bottles.

INTRODUCTION

In measurements of the oxygen concentration with the aid of electrodes, two types are employed:

- the polarographic (Clark) electrode (Clark, 1956)
- the galvanic (Mackereth) electrode (Mackereth, 1964).

The Clark electrode has a gold or platinum cathode and a silver anode, between which a polarization voltage is maintained with the aid of an external source.

The Mackereth electrode has a silver cathode and a lead anode. As the anode is consumed by the cell reactions, this electrode requires no polarization voltage.

The two electrodes are separated from the solution by a membrane. The oxygen is converted at the cathode, so that an electric current directly proportional to the oxygen supply flows through the cell. The concentration of oxygen in the cell can be supposed to be zero. The amount of oxygen diffusing through the membrane is then directly proportional to the concentration of oxygen in the solution. The current flowing through the electrode is therefore directly proportional to the oxygen concentration in the solution. In the mechanism outlined above there are several steps involving material transfer. The most important of these are the transfer of oxygen from the liquid phase to the membrane and its diffusion through the membrane.

The first factor is strongly affected by motion of the liquid along the membrane. This must always be turbulent. When the stirring is inadequate the electrode exhausts its surroundings by its own consumption of oxygen, so that a low value is obtained.

2.7 Various methods for measuring biological activity

The diffusion of oxygen through a membrane is strongly temperature-dependent. During the measurement the temperature must remain constant ($\pm 0.2^\circ\text{C}$). The electrode must always be calibrated at the measurement temperature.

Clark electrodes are used with an associated measuring instrument since they require a polarization voltage. The present-day meters are provided with temperature compensation, but this is reliable only over a moderate range of temperatures (5, sometimes 10°C). Since the Mackereth electrode itself generates a current, the measuring circuit consists of a resistance and a suitable voltmeter or recorder which shows the drop in voltage across the resistance. Temperature compensation is possible. For this purpose a temperature-sensitive resistance in combination with a voltage source provides the compensation voltage that eliminates the temperature dependence of the electrode (Morisi and Gualandi, 1966).

The most important differences between the two electrode systems are as follows:

- the faster response of the Clark electrode, which offers advantages particularly in measurements of concentration differentials, as in BOD measurements (Eye and Ritchie, 1966);
- the sterilizability of the Mackereth electrode;
- the simplicity of the external circuit of the Mackereth electrode, which makes it suitable for home-made construction (Brookman and Owen, 1968).

The oxygen electrodes are usually calibrated with air-saturated water or in air. For accurate measurements use can be made of calibration methods in which a known amount of oxygen is bound chemically (Misra and Fridovich, 1976).

2.7.1.4 Table 1 gives a review of the solubilities of oxygen in air-saturated water at an atmospheric pressure of 760 mm Hg for various temperatures and salt concentrations (Fox, 1907).

These values have been confirmed by more recent work (Montgomery *et al.*, 1964). Richards (1965) states that solubility measurements on gases are extremely difficult, but that the values given continue to be applicable.

Hart (1967) has given the relationship between temperature, air pressure, concentration, and percentage saturation in the form of a nomogram.

PROCEDURE

- Prepare and calibrate the electrode and the measuring instrument in accordance with the relevant instructions.
- Carry out the degradation experiments according to the following directions:
 - Fill BOD bottles according to Standard Methods (Anon., 1971). Use as many bottles as are necessary for the measurements (Note 2).
 - Determine the initial concentration of dissolved oxygen.
 - Determine the concentration of dissolved oxygen after, for example, 1, 2, 3, and 4 weeks.
 - Calculate the uptake of oxygen. (The difference between the concentrations at two measurements times the volume of the liquid).
- For activity measurements carry out the following operations:

- Add the test culture to a measuring cell (Note 3).
- Aerate the culture and measure the oxygen concentration in the solution.
- Shut the culture off from the external atmosphere (unless the re-aeration is known) and add the substance(s) to be investigated.
- Measure the change in the oxygen concentration until the latter has become constant or until it has fallen to about 20 % of the saturation value.
- The change in the oxygen concentration is a measure of the activity of the culture in question (Note 4).

NOTES

1. This method is intended as a supplementation of other methods. The other requirements are given in the descriptions of these methods.

2. The determination can be carried out in two ways.

- Several bottles may be used, a number of them being sacrificed at each measurement. This means that for a duplicate determination 10 bottles will be needed, two being sacrificed at the beginning and at each of four sampling times. When the bottles do not develop identically, the result is a scatter in the measurements.

- The various measurements can be carried out in the same bottle. For this purpose the liquid displaced from the bottle by the electrode during the measurement is made up by adding a few glass marbles to the bottle. The bottle can then be closed again without the enclosure of air bubbles. When the bottles are topped up with fresh medium errors are introduced, because the oxygen concentration in this medium will rarely be the same as in the bottle.

For these measurements it is best to use an electrode with its own stirrer, e.g. the Yellow Springs electrode (YSI 5420 A).

3. A number of vessels have been described for measuring the change in the oxygen concentration in a culture. Blok (1974) used a vessel* in which the culture was aerated. However, it can also be used without aeration.

The Yellow Springs Biological Oxygen Monitor has been developed specially for these measurements.

Shugarman and Appleman (1967) have designed a cell for measuring the production of oxygen in algal cultures.

However, this cell is also suitable for oxygen-uptake measurements.

Axt (1973) used a flow-through cell for toxicity measurements in which the inhibition of the respiration of an actively growing culture was taken as the criterion of toxicity.

4. Activity measurements by these methods have a basically relative nature, i.e. the fact that, for example, the degradation of a substance A is faster than that of a substance B if the maximum slope of the oxygen uptake curve is steeper in A than in B. Hill and Robinson (1974) give a method to calculate the maximum specific growth rate and the respiration rate from oxygen uptake measurements.

* The measuring apparatus is marketed by Messrs. Salm and Kipp.

2.7.1.4 Table 1 Solubilities of oxygen in air-saturated fresh and salt water at 760 mm Hg, in mg.l⁻¹ (Fox, 1907)

salinity ‰	0	1.8	3.6	5.4	7.2	9.0	10.8	12.6	14.4	16.2	18.0	19.8	21.6	23.4	25.2	27.0	28.8	30.6	32.4	34.2	36.0	
t °C chloride ‰	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	T °C
0	14.70	14.53	14.41	14.20	14.04	13.87	13.70	13.54	13.37	13.21	13.04	12.87	12.71	12.54	12.37	12.21	12.04	11.88	11.71	11.54	11.38	0
1	14.35	14.18	14.02	13.86	13.71	13.54	13.38	13.22	13.06	12.91	12.73	12.58	12.42	12.26	12.09	11.93	11.77	11.62	11.46	11.29	11.13	1
2	13.83	13.67	13.51	13.37	13.22	13.06	12.90	12.75	12.59	12.45	12.29	12.14	11.98	11.83	11.67	11.51	11.37	11.22	11.06	10.90	10.75	2
3	13.42	13.27	13.13	12.99	12.83	12.69	12.53	12.39	12.24	12.09	11.94	11.80	11.64	11.50	11.35	11.20	11.05	10.91	10.75	10.61	10.46	3
4	13.21	13.06	12.93	12.77	12.62	12.48	12.34	12.20	12.06	11.91	11.76	11.61	11.47	11.33	11.18	11.04	10.90	10.74	10.60	10.46	10.31	4
5	12.67	12.53	12.39	12.25	12.11	11.98	11.84	11.70	11.56	11.42	11.29	11.15	11.01	10.87	10.73	10.60	10.46	10.32	10.18	10.04	9.92	5
6	12.31	12.17	12.05	11.91	11.78	11.64	11.52	11.38	11.25	11.11	10.99	10.85	10.72	10.58	10.45	10.32	10.18	10.06	9.92	9.80	9.66	6
7	11.98	11.84	11.71	11.59	11.46	11.34	11.20	11.07	10.93	10.82	10.70	10.57	10.45	10.31	10.18	10.06	9.93	9.80	9.68	9.54	9.42	7
8	11.66	11.53	11.41	11.28	11.16	11.03	10.91	10.78	10.66	10.53	10.42	10.29	10.17	10.05	9.92	9.80	9.67	9.56	9.43	9.31	9.19	8
9	11.35	11.23	11.10	10.99	10.87	10.75	10.63	10.51	10.40	10.28	10.15	10.04	9.92	9.81	9.68	9.56	9.45	9.32	9.21	9.09	8.96	9
10	11.05	10.94	10.82	10.71	10.60	10.47	10.36	10.25	10.14	10.02	9.91	9.78	9.67	9.56	9.44	9.33	9.22	9.10	8.99	8.87	8.75	10
11	10.77	10.66	10.55	10.44	10.33	10.22	10.11	10.00	9.89	9.78	9.67	9.56	9.45	9.34	9.21	9.10	8.99	8.88	8.77	8.66	8.55	11
12	10.51	10.40	10.29	10.18	10.07	9.98	9.87	9.76	9.65	9.54	9.43	9.33	9.22	9.11	9.00	8.89	8.80	8.69	8.58	8.47	8.30	12
13	10.25	10.15	10.04	9.94	9.83	9.74	9.63	9.52	9.42	9.31	9.22	9.11	9.01	8.90	8.81	8.70	8.60	8.50	8.39	8.29	8.18	13
14	10.01	9.92	9.80	9.70	9.61	9.51	9.40	9.31	9.20	9.10	9.01	8.90	8.81	8.71	8.62	8.51	8.41	8.30	8.21	8.11	8.00	14
15	9.78	9.68	9.57	9.48	9.38	9.29	9.19	9.10	8.99	8.90	8.80	8.71	8.61	8.52	8.42	8.31	8.22	8.13	8.03	7.94	7.84	15
16	9.55	9.46	9.37	9.27	9.18	9.08	8.98	8.89	8.80	8.70	8.61	8.52	8.42	8.33	8.23	8.14	8.04	7.95	7.85	7.77	7.68	16
17	9.33	9.25	9.16	9.06	8.97	8.88	8.79	8.70	8.61	8.51	8.42	8.34	8.24	8.15	8.06	7.97	7.88	7.79	7.69	7.61	7.52	17
18	9.13	9.05	8.95	8.87	8.78	8.68	8.60	8.51	8.42	8.34	8.24	8.16	8.07	7.99	7.89	7.81	7.92	7.63	7.55	7.45	7.36	18
19	8.94	8.85	8.76	8.68	8.60	8.51	8.41	8.33	8.24	8.16	8.08	7.99	7.91	7.83	7.73	7.65	7.56	7.48	7.39	7.31	7.21	19
20	8.75	8.67	8.57	8.49	8.43	8.33	8.24	8.16	8.08	7.99	7.92	7.83	7.75	7.67	7.57	7.49	7.40	7.32	7.24	7.16	7.07	20
21	8.57	8.49	8.40	8.32	8.25	8.16	8.08	8.00	7.91	7.83	7.76	7.67	7.59	7.51	7.42	7.34	7.26	7.18	7.10	7.02	6.92	21
22	8.39	8.32	8.24	8.16	8.08	7.98	7.91	7.83	7.75	7.67	7.60	7.51	7.43	7.35	7.27	7.19	7.11	7.03	6.95	6.87	6.78	22
23	8.22	8.14	8.06	7.98	7.92	7.83	7.75	7.67	7.60	7.52	7.44	7.37	7.29	7.21	7.13	7.05	6.97	6.89	6.81	6.73	6.64	23
24	8.06	7.98	7.90	7.84	7.76	7.67	7.60	7.52	7.45	7.37	7.29	7.22	7.14	7.06	6.99	6.91	6.83	6.75	6.68	6.60	6.50	24
25	7.90	7.84	7.75	7.68	7.60	7.52	7.45	7.38	7.30	7.22	7.15	7.08	7.00	6.92	6.84	6.77	6.70	6.62	6.54	6.48	6.36	25
26	7.75	7.68	7.59	7.53	7.45	7.38	7.30	7.24	7.16	7.08	7.00	6.94	6.86	6.78	6.70	6.64	6.56	6.48	6.42	6.34	6.23	26
27	7.59	7.53	7.45	7.37	7.31	7.24	7.16	7.10	7.02	6.94	6.86	6.80	6.72	6.64	6.58	6.50	6.42	6.37	6.28	6.21	6.11	27
28	7.45	7.37	7.31	7.23	7.17	7.10	7.02	6.96	6.88	6.80	6.72	6.66	6.58	6.50	6.44	6.36	6.30	6.22	6.15	6.08	5.99	28
29	7.31	7.23	7.17	7.09	7.03	6.96	6.88	6.82	6.74	6.66	6.59	6.52	6.44	6.37	6.30	6.24	6.16	6.08	6.02	5.94	5.86	29
30	7.17	7.09	7.03	6.95	6.89	6.82	6.74	6.68	6.60	6.53	6.45	6.38	6.31	6.24	6.17	6.10	6.02	5.96	5.90	5.82	5.74	30

2.7 Various methods for measuring biological activity

2.7.2 Determination of adenosine triphosphate (ATP)

Subject

Measurement of the ATP concentration.

Field of application

The method can be used to determine the biomass and the activity of microorganisms. Within the framework of this handbook, it can be used in the determination of biological degradation (see Section 3.5).

Principles

ATP is extracted from the organism. The concentration of ATP in the extract is determined photometrically with the aid of the enzyme luciferase.

EQUIPMENT AND MATERIALS

- Tris(hydroxymethyl)aminomethane/HCl buffer, pH 7.75, 0.02M (Note 1)
- centrifuge tubes with graduations, to be closed with glass marbles
- volumetric flask, 50 ml, provided with a mark but not calibrated
- water bath (100°C)
- filtration equipment for membrane filters 25 mm in diameter
- membrane filters with pore diameters of 0.1 and 0.45 µm
- ATP-meter (DuPont 760 Biometer)*
- luciferin/luciferase
- ATP standard
- ice or cooling bath
- plunger pipette, 5 ml, with disposable tips.

INTRODUCTION

ATP (adenosine triphosphate) is present in all living cells. It has been found that the cellular ATP concentration is reasonably constant under various conditions. The measurement of the ATP content of a mixed microbial system (for example activated sludge) can thus provide information on the amount of living biomass in that system.

The measured ATP concentration can be expressed in terms of the number of cells, the weight of dry matter in mg, or the weight of carbon in mg. The ATP content varies according to the bacterial species. Holm-Hansen (1973) uses a factor of 250 as a general average for converting the ATP concentration into the amount of carbon in living biomass.

In their handbook for the ATP meter, DuPont give an average of 4.7×10^{-16} g ATP per bacterial cell. The concentration range given here is $2.2 - 10.3 \times 10^{-16}$ g ATP per cell. After the measurement of the ATP content, it is possible to calculate from these figures the amount of living biomass in the system under investigation (Note 2).

The method of determination consists of two steps:

1. Extraction of the ATP from the biological material;
2. Measurement of the ATP concentration with the aid of the enzyme luciferase and a photometer.

The first step is important for the quantitative determination of ATP; several methods have been described in the literature. In this procedure extraction with boiling Tris buffer is used.

A very sensitive determination of ATP is possible by measuring

the light that the luciferin/luciferase enzyme system emits in the presence of ATP. The light intensity is directly dependent on the ATP concentration. Special sensitive photometers have been developed for this measurement. The described method is based on the DuPont 760 Biometer.

In biodegradation research the method is used for two fields of application, namely to determine the microbial biomass in natural waters and to determine the biological activity of activated sludge.

PROCEDURE

Extraction methods

Extraction from natural water

The extraction method is based on that of Holm-Hansen and Booth (1966).

- Perform the determination in duplicate.
- Filter 10 ml of the water sample through a membrane filter (diameter 25 mm, pore diameter 0.1 µm).
- Immediately at the end of the filtration, transfer the filter to a graduated centrifuge tube that contains 5 ml of boiling Tris buffer and close the tube with a glass marble (Note 3).
- Incubate the tube for about 5 min in a boiling water bath.
- Shake the tube and make up the buffer volume to 5 ml with distilled water.
- Cool the extract in ice or in a cooling bath and measure the ATP content or store the extract at -20°C if the ATP measurement cannot be performed immediately (Note 4).

Extraction of activated sludge

The extraction method for ATP in activated sludge is the same as that described in Levin et al. (1975).

- Dilute the activated sludge 1:10 and the return sludge 1:20 with distilled water.
- Add 1 ml of the diluted sludge suspension to 35 ml of Tris buffer in a 50-ml volumetric flask placed in a boiling water bath (Note 5).
- Incubate the flask in the boiling water bath for about 5 min.
- Shake the flask well, cool it in ice, and make up the contents to 50 ml with the buffer.
- Filter the samples through a membrane filter (pore size 0.45 µm).
- Determine the concentration of ATP in the extract, or store the extract at -20°C if the ATP measurement cannot be carried out immediately (Note 4).

Measurement of the ATP concentration

The principle of the measurement is that the luciferin/luciferase enzyme system emits light when ATP is added. The intensity of the light is proportional to the ATP concentration (Notes 6 and 7). The ATP concentration can be measured with various instruments. This procedure relates to the DuPont 760 Biometer. For the use of the apparatus reference is made to the DuPont manual, but the following modifications have been introduced:

1. The apparatus is calibrated at the value of 2.10^8 instead of 1.10^8 ; this is faster and easier to perform.
2. The measurement procedure has been modified. According to the manual, 0.1 ml of the enzyme is placed in the cell and the ATP extract (10 µl) is injected. Instead of this, 10 µl of ATP extract is placed in the cell and 10 µl of the enzyme solution is injected. This gives more reproducible results and saves reagent.

Three determinations are carried out on each sample of ATP extract.

* Specification of a trade name implies no recommendation of the particular brand but is merely intended as a type reference.

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TREATMENT OF THE RESULTS

The measured values are displayed digitally in fg.ml⁻¹ of the extract (fg = femtograms = 10⁻¹⁵ g).

The results are given in µg.l⁻¹. Conversion factor:
1 fg.ml⁻¹ = 10⁻¹² g.l⁻¹ = 10⁻⁶ µg.l⁻¹. For sludge the results may also be expressed in µg.g⁻¹ dry weight of sludge, or in µg.g⁻¹ carbon.

The detection limit of the instrument is 0.2.10⁵ fg of ATP per ml, while the lower limit of the linear range is 1.10⁵ fg of ATP per ml. For three solutions of ATP we found the standard deviations reported in 2.7.2, Table 1.

2.7.2 Table 1 Standard deviation of measurements on three ATP solutions with different concentrations

Mean measured values from 10 measurements (fg.ml ⁻¹)			
x	1.77.10 ⁷	1.95.10 ⁶	4.5.10 ⁵
S	0.07.10 ⁷	0.11.10 ⁶	0.34.10 ⁵
V	4.0 %	5.4 %	7.6 %

It can thus be seen that the absolute scatter (S) of the results depends greatly on the measured ATP concentration (x), but the percentage error (V) is practically constant. If the whole extraction procedure is followed the accuracy may correspond to that in the above table. Rogue results occur fairly frequently; therefore, take the middle of the three measurements as the final measured value. Without rogue results the standard deviation of the middle value is about 0.6 × the standard deviation of one measurement.

NOTES

1. ATP-free water should be used as diluting water for the solutions. In our experience doubly distilled water is very satisfactory. The buffer solution is divided into portions and is stored in the deep-frozen state to prevent bacterial growth in the buffer.
2. 2.7.2. Table 2 gives a number of values for the ATP concentration in various microbiological systems. The values given have been taken from the literature; the table also demonstrates the variety of units that have been used.
3. It is frequently found in ATP extraction that the measured ATP concentration depends on the ratio between the buffer volume used and the amounts of biomass. In water with a high microbiological activity the method of extraction must be modified.
4. The ATP extract can be stored for several months at -20°C.
5. Activated sludge must be diluted; the best result is achieved at 0.1-1 mg of ATP.l⁻¹ of sludge.
6. The reaction of the luciferin/luciferase enzyme system is greatly inhibited by univalent and bivalent cations, especially by Ca²⁺. The reaction should be checked for such inhibition with the aid of the standard addition method. In this, four samples of ATP extract are prepared from each water sample and 10 µg ATP.l⁻¹ is added to two of the samples.
7. In biodegradation studies the test medium frequently contains toxic compounds that can also inhibit the enzyme luciferase. These substances can pass into the ATP extract via absorption in the filters and the biomass and thereby affect the ATP measurement.
A correction can be made for this effect by performing an extraction with a solution of the toxic substance in distilled water and adding 10 µg.l⁻¹ of ATP to this extract.

2.7.2 Table 2 Concentration of ATP in various micro-organisms (information from the literature)

The results were obtained by various extraction methods and are therefore not comparable in many cases.

Organism or system	ATP concn.	Units ^{d)}	Reference
Escherichia coli	1.64 ± 0.30	µg.mg ⁻¹ dry weight	Levin <i>et al.</i> (1975)
Zoogloea ramigera	7.71 ± 0.65	µg.mg ⁻¹ dry weight	Levin <i>et al.</i> (1975)
Bacillus sp.	2.9 ± 1.12	µg.mg ⁻¹ dry weight	Levin <i>et al.</i> (1975)
E. coli	1.4.10 ⁻¹⁵	g.cell ⁻¹	Karl and La Rock (1975)
Marine bacteria	0.07 – 0.22	percent.mg ⁻¹ dry weight	Holm-Hansen and Booth (1966)
Marine bacteria	0.3 – 1.1 (mean 0.4)	percent.mg ⁻¹ carbon	Hamilton and Holm-Hansen (1967)
13 species of gram-positive and gram-negative bacteria	2.1 ± 1.1	µg.mg ⁻¹ dry weight	D'Eustachio and Johnson (1968)
Activated sludge, batch	1.8 – 2.0	µg.g ⁻¹ VSS ^{a)}	Patterson <i>et al.</i> (1970)
Activated sludge, continuous	1.4 – 1.8	µg.g ⁻¹ VSS ^{a)}	Patterson <i>et al.</i> (1970)
Activated sludge, two days without nutrition	0.8	µg.g ⁻¹ VSS ^{a)}	Patterson <i>et al.</i> (1970)
Activated sludge	0.53 – 0.99	µg.mg ⁻¹ MLVSS ^{b)}	Upadhyaya and Eckingfelder (1975)
Activated sludge	0.6 – 1.4	mg.g ⁻¹ MLSS ^{c)}	Chin <i>et al.</i> (1973)

- a) VSS: volatile suspended solids
b) MLVSS: mixed liquor volatile suspended solids
c) MLSS: mixed liquor suspended solids
d) The units given here are as cited in the literature.

2.7.3 Measurement of primary production with the aid of ¹⁴C-labelled bicarbonate

Subject
Measurement of the rate of photosynthesis of cultures of unicellular algae and of natural phytoplankton for the purpose of toxicity tests.

Field of application
This method can be used in toxicity tests, except for substances that form precipitates with bicarbonate.

Principles
Radioactive bicarbonate is added to an algal culture or to a sample of natural water. After a certain incubation time the organisms are filtered off and their activity is determined using a scintillation counter. The difference between the activities in the samples with and without the addition of the toxic compound gives the toxic effect.

2.7 Various methods for measuring biological activity

EQUIPMENT

- Filtration equipment for membrane filters, diameter 25 mm
- membrane filters, cellulose nitrate, diameter 25 mm, pore size 0.45 μm
- pipettes, 5 ml
- scintillation vials
- scintillation liquid, Aquasol (New England Nuclear Corp.)^{*}
- scintillation counter
- forceps.

INTRODUCTION

In photosynthesis CO_2 is converted into organic carbon compounds. The biomass so produced is also sometimes called the primary production. Photosynthesis in natural phytoplankton communities can be measured by using ^{14}C as a tracer. This technique was introduced by Steeman-Nielsen (1951) and is usually called measurement of the primary production.

There is an extensive literature on the measurement of the primary production. For a corresponding procedure reference is made to handbooks such as that by Strickland and Parsons (1968) and by Vollenweider (1971).

This procedure includes only those methods that relate to toxicity tests with unicellular algae as described in Section 5.10.1.3. It is based on the assumption that the analyst is familiar with the handling of radioactive substances. Some general information for working with these and for obtaining the necessary licences is given in Section 6.2.

PROCEDURE

- Filter the contents of the test bottle.
- In this process do not use an excessive vacuum ($< \frac{1}{3}$ atmosphere).
- Wash the filter with 5 ml of non-radioactive test water (sea water, algal medium, etc.).
- Remove the filter from the filter holder with the aid of the forceps.
- Place the wet filter in a scintillation vial with scintillation liquid (Note 1).
- Determine the activity in the algae by means of a scintillation counter with an error of measurement $< 3\%$.

TREATMENT OF THE RESULTS

The results of the counts, expressed in dpm (disintegrations per minute), can be used directly for determining the concentration-effect relationship (Section 5.10.1.3).

These results can be transformed into the photosynthetic carbon fixation P , in mg of C per litre, by using the following formula:

$$P = \frac{A_a}{A_o} \times C \quad (1)$$

in which

A_o = total activity added to the test vessel at the beginning of the test, in dpm;

A_a = total activity in the algae at the end of the test, in dpm; and

C = total concentration of dissolved inorganic carbon (in the form of CO_2 , bicarbonate, and carbonate), in mg of C per litre.

P values determined in this way can also be used to calculate the toxic effect in accordance with Section 5.10.1.3.

NOTES

1. The filter and the algae dissolve in Aquasol. The mixture must be well shaken, and the vials must be allowed to stand for a few hours before counting, until the filter has completely dissolved. It has been found from tests that the total activity in the algae can in fact be counted in this way. If necessary, a dried filter can be counted in a toluene scintillator (Section 2.6).
2. Since phytoplankton and algae produce appreciable amounts of extracellular organic material, the photosynthetic production is generally underestimated. This can be corrected by not filtering but acidifying the contents of the test flask, blowing out the unconsumed carbon dioxide, and finally by determining the activity in the water (Schindler *et al.*, 1972).
In isolated cases inorganic carbon may also be deposited on the filters (McMahon, 1973). This can sometimes be avoided by further washing or by suspending the dry filter above concentrated hydrochloric acid before counting. Extracellular polymers may also cause erroneous measurements. Gieskes and Van Bennekom (1973) have discussed this problem and give indications for its correction.

2.7.4 Counting of microorganisms

2.7.4.1 Counting of viable bacteria

Subject

Determination of the total number of colony-forming units in fresh water and in sea water.

Field of application

This relates to an auxiliary method for:

- a. determining the degradation of organic compounds in die-away tests;
- b. characterizing the water to be used for degradation tests (see Note 5).

Principles

A number of dilutions of the water are made. A sample of each dilution is plated out on an agar nutrient medium. After incubation, the numbers of colonies are counted.

EQUIPMENT AND MATERIALS

- Agar nutrient medium

For sea water according to Oppenheimer and ZoBell (1952) (obtainable from Difco)

Bacto Peptone (Difco)	5 g
Bacto Yeast Extract (Difco)	1 g
FeCl_3	0.01 g
Bacto Agar (Difco)	15 g

Dissolve in 1 litre of natural or synthetic sea water with a salinity $> 27\%$.

For fresh water according to Jones (1970)

K_2HPO_4 , analytical grade	0.2 g

^{*} Other brands of corresponding liquids can also be used.

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MgSO ₄ · 7 H ₂ O, analytical grade	0.05 g
FeCl ₃ , analytical grade (4 drops of a 0.1 % solution)	trace
Bacto Peptone (Difco)	0.5 g
Soluble casein (BDH)	0.5 g
Glycerol, analytical grade	1.0 g
Ionagar No. 2 (Oxoid)	10.0 g

Dissolve in 1 litre of distilled water.

- plastic Petri dishes, diameter 10 cm
- graduated pipettes, 1 ml (sterile)
- plunger pipettes, 100 µl, with disposable tips
- culture tubes
- sterile sea water or sterile fresh water (see Section 5.2)
- autoclave
- incubation room for 25° C or 15° C
- colony counter
- glass filter equipment for membrane filters, diameter 50 mm
- Gelman cellulose acetate membrane filters, diameter 50mm, pore size 0.45 µm, with grids
- forceps
- bent glass rod
- ethanol, 96 %
- gas burner.

INTRODUCTION

In this procedure a method is described for the determination of the number of bacteria (per unit volume) in natural water that are capable of forming a colony on a given medium.

The number of microorganisms that form colonies may be only a fraction of the number that can be found by direct counting under the microscope.

There are three main reasons for this effect (Jannasch and Jones, 1959) :

1. The selective effect of the nutrient medium used. There is no universal medium promoting the growth of all bacterial species.
2. The presence of the bacteria in the water in fairly large aggregates which are then counted as one bacterium.
3. The presence of inactive or even dead bacteria. The usual direct counting methods make no distinction between living and dead bacteria.

In addition to these factors, an appreciable rise of the temperature during the bacterial counting procedure described in this chapter also affects the results (Klein and Wu, 1974). Plating out of bacteria on a solid agar medium is therefore preferred to the use of poured plates (Buck and Cleverdon, 1960; Jones, 1970).

It can thus be stated that counting of the bacteria that can form colonies is only a relative measure of the microbiological activity of natural waters. The results must therefore be interpreted with the necessary reservations.

PROCEDURE

Preparation of nutrient media

- For counting bacteria in sea water, use the nutrient agar according to Oppenheimer and ZoBell.
- For counting bacteria in fresh water, use the nutrient agar according to Jones.

- Sterilize the medium in an autoclave for 20 min at 121° C.
- Fill each Petri dish with 15 ml of the medium.

Counting by plating out on an agar medium

- From the water under investigation make two dilution series in steps of 1:10 in sterile water. The necessary number of dilutions depends on the number of bacteria in the water; in most cases the series 10⁻¹–10⁻⁶ is sufficient. The dilution series are made by pipetting 1 ml into a tube with 9 ml of sterile water, using a sterile pipette in each case, shaking well, pipetting 1 ml from this tube into the next tube, and so on (Note 1).
- Pipette 0.1 ml, with the plunger pipette, from the undiluted water and from each dilution onto four agar media. Begin with the highest dilution and end with the undiluted sample.
- Spread the drop of water out with the bent glass rod. Particular care is needed at this point. Between each plating the glass rod is flamed after being dipped in alcohol, and is then immediately cooled in the agar of the following plate.
- Incubate the agar plates upside down, preferably at the temperature of the sample water. If this cannot be done, then incubate at 15° C for samples of sea water and at 20° C for samples of fresh water (Note 2).
- Count the agar plates with the aid of one of the methods customary in microbiology (Postgate, 1969) (Note 3).

Counting with the aid of filtration

- Sterilize the filter apparatus (including the rubber protective cap), and the filters packed in paper in an autoclave for 20 min at 121° C.
- Place a filter aseptically in the filter-holder with the aid of sterile forceps.
- Pipette a few millilitres of sterile water onto the filter (for a good distribution of the sample).
- Filter a number of millilitres of the sample, as necessary.
- Remove the filter from the filter-holder with sterile forceps and place it on an agar plate.
- Now incubate the plates upside down and follow the procedure of 3.2 from incubation onwards.

TREATMENT OF THE RESULTS

The bacterial count, *n*, per millilitre of a water sample is calculated in the plating out method with the aid of the following formula (Note 4) :

$$n = a \cdot 10^{b+1}$$

where *a* = the average of the number of countable plates per dilution (countable plates contain 30 to 300 colonies)

b = the number of dilutions (undiluted: *b* = 0, 1:10 dilution, *b* = 1, and so on).

In the filtration method the bacterial count per ml of sample is found by dividing the average for four filters by the volume of the water sample per filter, in millilitres.

For all counting results the standard deviation *S* is estimated from:

$$S = \sqrt{\frac{1}{3} \sum_{i=1}^4 (n_i - \bar{n})^2} \quad (1)$$

where *n_i* = number of bacteria per ml of water sample in plate (filter), *i* (*i* = 1, 2, 3, or 4), and *n* = the average of the four plates (filters) :

$$n = \frac{1}{4} (n_1 + n_2 + n_3 + n_4)$$

To check on the significance of differences between the two duplicate series of four dishes or filters, use is made of Student's *t*-test.

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When the numbers of results of observation are the same, the value t is calculated from the formula:

$$t = \frac{(\bar{n}_1 - \bar{n}_2) \sqrt{m}}{\sqrt{S_1^2 + S_2^2}} \quad (2)$$

Here n_1 and n_2 are the two means, S_1 and S_2 are the two variances, and the (same) size of the random sample (m) is 4.

When $t > 1$ there is a significant difference between the parallel series. If $t < 1$ there is no difference, and all 8 plates can be used to calculate the mean and the standard deviation from the formula:

$$S = \sqrt{\frac{1}{7} \sum_{i=1}^8 (n_i - \bar{n})^2} \quad (3)$$

For the significance of n and i see the explanation attached to formula (1).

If there is a significant difference between the two dilution series, formula (3) cannot be used unless one of the two parallel series fits satisfactorily into a series of measurements with a definite trend.

NOTES

1. The sample to be counted must not deviate from the sampling temperature by more than 5°C throughout the procedure.
2. Depending on the incubation temperature, the time of incubation may range from a few days to two weeks. Counting is carried out twice: first after an incubation time of a few days and again after an incubation time of 2–3 weeks. During the incubation period, after a week or longer, a growth of slow-growing bacteria frequently takes place. The ratios of the counts in the different dilutions frequently do not agree at the time of the last count, and the scatter is often greater than at the first count. This is due to competition phenomena between bacterial species during growth on the agar medium. The highest possible dilution that still gives countable plates at the second count is then used to calculate the number of colony-forming units.
3. Optimum results are obtained with agar plates containing 40 to 60 colonies per plate, but plates with 30 to 300 colonies can also be used. If the number of colonies on plates with undiluted sea water is less than 20 (i.e. < 200 bacteria.ml⁻¹), the determination can be carried out after filtering the water sample through a membrane filter (see Section 3.3).
4. In the plating out of natural water, bacterial growth regularly occurs which rapidly spreads over the whole plate and interferes with the growth of other colonies. These plates are not included in the determinations of the results.
5. So far as concerns subsection a) of the field of application, the method is mainly applicable to substances that are in fact degraded but with which only the primary bio-degradation, i.e. the disappearance of a compound measured by a specific analysis, is measured. By this method one can investigate whether the growth of microorganisms is due to the degradation.

2.7.4.2 Counting of algae with a counting chamber

Subject

Determination of the number of cells in a culture of unicellular algae.

Field of application

Monitoring of the growth of unicellular algae in toxicity tests.

Principle

A drop of an algal suspension is placed in a counting chamber and the number of cells is counted with the aid of a microscope.

EQUIPMENT AND MATERIALS

- Counting chamber – improved Neubauer (depth 0.1 mm)
- Pasteur pipette
- tally counter
- microscope with a 10× or 25× objective
- formalin solution (35 > w/w)
- ether or acetone.

INTRODUCTION

Within the framework of this book the counting of algae is used to follow the growth of unicellular algae. Such growth measurements are necessary for toxicity tests with algae (Section 5.10.1.2). In this procedure only a very simple description is given. For the statistical treatment of the results and the use of the method for counting other organisms, reference is made to Oberzill (1967) and De Jonge (1963).

This method of counting is more laborious than the one using the Coulter Counter, but requires a lower capital investment.

PROCEDURE

- Algal suspensions that are too concentrated and therefore difficult to count are diluted with sufficient medium to allow satisfactory counting.
- Centrifuge an algal suspension that is too dilute and therefore difficult to count and suspend the deposit in a suitable amount of medium so that good counting becomes possible.
- Fix motile algae by adding 5 ml of formalin per litre of suspension.
- With the aid of a Pasteur pipette place a drop of the algal suspension in the counting chamber (Note 1).
- Place the counting chamber under the microscope and count the total number of cells in 20 small squares chosen at random. Do not count the cells that extend across the bottom or the right-hand side of the small squares but do count those that extend across the top or the left-hand side (Notes 2 and 3).
- Perform this whole process **three times** with a fresh drop each time.
- Use the middle value of the three counts for calculating the number of cells per 20 squares (0.005 mm²).

CALCULATIONS

- Multiply the middle value of the three counts by $2 \cdot 10^4$ to obtain the number of cells per millilitre.
- For calculating the accuracy and optimizing the counting procedure, reference is made to the literature (Oberzill, 1967; De Jonge, 1963).

NOTES

1. Flow of the suspension from under the cover slip or its penetration into the groove of the counting chamber should be avoided. The counting chamber has an area of 1 mm² and

2. Analysis of the investigation of degradability, toxicity, and accumulation

a depth of 0.1 mm. The chamber is divided into small squares, each of which has a surface area of $\frac{1}{400}$ mm².

- 20 squares or a total of 200–250 cells are counted.
- The counting chamber is washed in water and is then rinsed with ether and acetone.

2.7.5 Determination of chlorophyll

Subject

Determination of the chlorophyll concentration in sea water and fresh water.

Field of application

The chlorophyll content of natural water is the only reasonable measure of the content of plant material. In this manual the determination of chlorophyll is used to characterize the water used in degradation and toxicity tests.

Principles

The large zooplankton is removed by filtration through a plankton net. The phytoplankton is filtered off. The pigment is extracted from the phytoplankton collected in this way with 90 % acetone. The concentration in the extract is determined spectrophotometrically.

EQUIPMENT AND MATERIALS

- Plankton net, 0.3 or 0.15 mesh, depending on the type of water
- conical flask, 100 ml
- 300-ml polythene wash-bottle
- centrifuge tubes, 15 ml, graduated, with glass and polythene stoppers
- spectrophotometer with 1-cm and 10-cm cells (or fluorimeter, see procedure)
- filtration equipment for membrane filters, diameter 50 mm
- suitable glass-fibre filter (Schleicher und Schull, No. 6)* diameter 50 mm
- suitable membrane filters, diameter 50 mm, pore size 0.45 µm
- homogenizer with Teflon pestle; according to Potter
- acetone-water mixture, 90:10 (w/w) (so-called pure acetone frequently already contains much water)
- MgCO₃ solution; 1 g of MgCO₃ dissolved in 1 litre of double-distilled water
- HCl solution, 4M
- centrifuge

INTRODUCTION

The chlorophyll determination is carried out to establish the amount of vegetable material in a natural water. It is not an absolute determination, since the ratio between the pigment concentration and the biomass can vary widely (from 10⁻⁴ to 0.06; Round, 1965). Within the framework of this book this determination is used to characterize the water used in various investigations (degradation and toxicity tests). For this purpose the standard method described in the literature is given in this procedure.

Golterman's procedure (1971), as modified by Kool and Nijholt (1974), is followed here. This procedure was originally intended

* Other makes of glass-fibre filters of the same quality may also be used.

for fresh water, but it can also be used for sea water. It differs only in details from the more detailed procedure proposed by Strickland and Parsons (1968) and originally intended for sea water. The last-mentioned authors also give instructions relating to the size of the sample, sensitivity, accuracy, etc., which may apply equally well to determinations in fresh water.

PROCEDURE

- Filter 5 ml of 0.1 % MgCO₃ solution through a glass-fibre filter.
- Filter a known volume of the water to be investigated; 0.5 l will usually be sufficient (Note 1).
- Transfer the filter to a homogenizer containing 5 ml of acetone (90 %).
- Homogenize with the Teflon stirrer for a few minutes.
- Transfer the suspension to a conical flask and add to it 15–25 ml of acetone (90 %).
- Extract in the dark for 18–24 h, the whole being shaken gently if necessary.
- Filter the contents of the conical flask through a glass-fibre filter or centrifuge for 10 min at 3000 rpm.
- Measure the extinction of the filtrate in the spectrophotometer with a 1 cm cell at 665 and 750 nm.
- Add three drops of 4M HCl to the filtrate in the measuring cell and measure the extinction at the same wavelengths (Note 2).
- Calculate the chlorophyll content.

CALCULATION OF THE CHLOROPHYLL CONTENT

The chlorophyll concentration (in µg.l⁻¹) =

$$\frac{E_{chl}}{89} \times \frac{V_a}{V_w} \times 1000 \quad (1)$$

The phaeophytin concentration (in µg.l⁻¹) =

$$\frac{E_{pha}}{56} \times \frac{V_a}{V_w} \times 1000 \quad (2)$$

with

$$E_{chl} = 2.43 [(E_{665}^{n_1} - E_{750}^{n_1}) - (E_{665}^a - E_{750}^a)] \quad (3)$$

$$E_{pha} = E_{665}^a - E_{chl} \quad (4)$$

in which

V_a = the volume of the acetone extract in ml

V_w = the volume of the water sample in litres

E^n = the extinction of the filtrate at wavelength λ nm

E^a = the corresponding extinction of the acidified filtrate.

The numerical values in the above formulae have been taken from Strickland and Parsons (1968), who also describe the accuracy and the limitations of this method.

NOTES

- The volume of water that must be filtered depends on the chlorophyll content. On this point, see Strickland and Parsons (1968).
- For problems in the performance of the determination, for the determination of types of chlorophyll other than chlorophyll a, and for those cases in which the chlorophyll concentration is so low that it has to be determined fluorimetrically, reference is again made to Strickland and Parsons (1968).

2.7.6 The use of the Coulter Counter

Subject

The use of the Coulter Counter for counting unicellular algae.

Field of application

Monitoring the growth of unicellular algae in toxicity tests.

Principles

Counting with the Coulter Counter is based on the Coulter principle: two electrodes separated by a glass wall with a very small opening (aperture) are placed in an electrolyte solution. When a particle passes through the aperture, the resistance of the whole system changes temporarily. This electrical signal is measured. Under certain conditions the signal is proportional to the volume of the particle passing through the aperture. Particles with different volumes can be detected by varying the detection limit of the signal.

EQUIPMENT

- Electronic particle counter (Coulter Counter or other), including counting tubes with a 70 μm aperture and a special counting vessel.

INTRODUCTION

With the aid of an electronic counting apparatus (Coulter Counter) it is possible to determine the cell density in an algal culture very rapidly, easily, and with high accuracy. Moreover, with the aid of a multichannel analyser it is possible to determine rapidly the size distribution of the cells in the culture.

Within the framework of this book the growth of cultures of unicellular algae in toxicity tests is determined with this apparatus (Section 5.10.1).

For the theoretical and practical background of the Coulter Counter reference may be made, for example, to Kubitschek (1969) and to Sheldon and Parsons (1967).

There are a number of different instruments and models on the market, all working on the same principle but differing in constructional details, especially with regard to the method of automatic treatment of the results. Consequently, it is impossible to give here full instructions, and we shall content ourselves with a few practical hints supplementing the manuals of the available apparatus.

PROCEDURE

General remarks

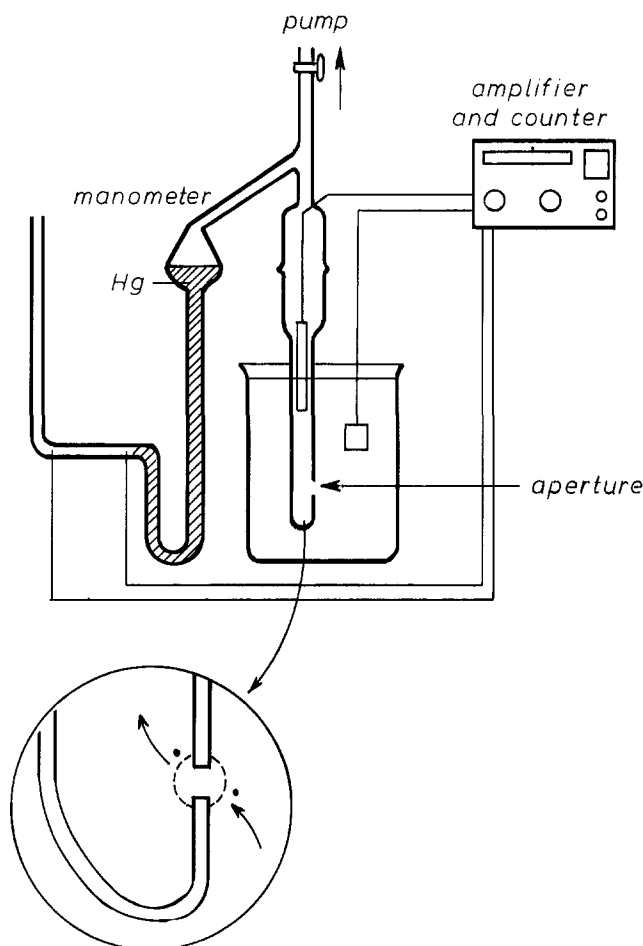
The principle underlying the Coulter Counter has already been given under Principles. The actual construction is shown schematically in 2.7.6, Fig. 1 (taken from Sheldon and Parsons, 1967).

By means of a pump, the mercury is raised in the right-hand arm of the U-tube. The tap to the pump is then closed. The mercury falls again and thereby draws the suspension through the aperture. The volume is determined by passage of the mercury past two successive contact points in the left arm of the U-tube.

In most measurements of the growth of algae a volume of 0.5 ml is counted. The volume measured depends on the particle concentration in the suspension. If the suspension is dilute a larger volume can be counted and if it is dense it is possible to use a smaller volume (for example 0.1 ml). The chances of blockage of the counting tube during counting are then also smaller.

A useful instrument is so constructed that the change in resistance occurring when a particle traverses the aperture is proportional to the particle volume.

An instrument can be adapted in various ways to count suspensions (see the manual supplied with the instruments). The most important variables are:



2.7.6 Fig. 1 Schematic representation of the Coulter Counter

1. The size of the aperture. A diameter of 2.5 to 50 $\times$ the diameter of the counted particle is desirable.
2. The sensitivity of the instrument. When the sensitivity is too low the apparatus does not detect the smallest particles, and when the sensitivity is too high, noise peaks are counted as particles.
3. The current intensity. The sensitivity of the instrument can be varied by changing the amplification (attenuation) of the signal or the current between the electrodes (aperture current).

The background level (noise) depends on the sensitivity of the instrument. **In order to keep the background count as low as possible, the electrolyte or the growth medium is filtered through 0.1 μm membrane filters. All tests are carried out in dust-free glassware.**

The size of the particles that are counted can be regulated with the aid of electronic filters known as threshold controls. Both the upper and the lower limit can be adjusted. The oscilloscope of the instrument gives an impression of the cell-size distribution. The electrical signals appear as peaks whose heights are proportional to the particle size. The effect of changing the sensitivity and the threshold can also be displayed on the oscilloscope.

2. Analysis of the investigation of degradability, toxicity, and accumulation

The aperture diameter sets a limit to the size of the particles that can be measured. The linear relationship between the strength of the electric signal and the particle volume does not apply to particles with a diameter greater than about 40 % of the aperture diameter, while on the other hand the signals of particles with a diameter smaller than 2 % of the aperture diameter cannot be distinguished from the noise.

For the algae used in toxicity tests (Section 5.10.1) a counting tube with an aperture of 70 μm is the most suitable. The adjustment of the instrument used in this work for various algae is given in Note 1.

The counting error depends on the number of particles that can be counted. For counts of about 200 particles/ml it is about 6 %. The accuracy increases with increasing number of cells.

In the counting of concentrated cell suspensions coincidences may occur, i.e. two or more particles may be counted as one.

Tables to correct for coincidences can be found in the manuals. It is also possible to count with a coincidence level of less than 10 %; the correction for coincidences can then be dispensed with.

The recommended maximum cell content for a 70 μm tube is 80,000 cells. ml^{-1} .

The usual electrolyte is NaCl, mostly 9 g per litre. The media for toxicity tests used in practice are sometimes sufficiently conducting. Particularly when counting in fresh water media, further NaCl must be added (Note 2).

Performance of the counting

- Start the instrument in accordance with the manual.
- Choose a measuring tube, for example, for *Chlorella* and *Chlamydomonas*, with a 70 μm aperture.
- Determine the correct adjustment of the attenuation (B) and the aperture current (D) by counting a sample of algae and adjusting the pulse height to half the height of the oscilloscope screen (see Note 1).

- Set the lower threshold (T) just above the background noise as seen on the oscilloscope (Note 1).
- If the counts are too high for the counting tube used, dilute the culture with filtered medium.
- Count the samples twice. Take care that non-motile algae do not settle.
- Take care that the counting takes place smoothly and that the counting tube does not become blocked.
- Finally, count a blank of filtered medium and determine the background.

NOTES

1. To perform standard counts of algae suspended in physiological saline (0.9 % NaCl in water) the following adjustments may be used. (Here B = attenuation, D = aperture current, and T = threshold; the nomenclature used for the Coulter Counter).

Adjustment Algal species	D	T	B
<i>Chlamydomonas</i>	32	10	1
<i>Dunaliella</i> sp.	32	10	1
<i>Phaeodactylum tricornutum</i>	32	7	0.707
<i>Monochrysis</i> sp.	32	12	0.707
<i>Chlorella ovalis</i>	64	8	1
<i>Chlorella pyrenoidosa</i>	4	8	0.5
<i>Scenedesmus quadricauda</i>	32	7	1

2. This procedure applies if the conductivity of the medium is sufficiently high.

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Residue analysis with the aid of gas chromatography

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They are, in order of importance:

Journal of the Association of Official Analytical Chemists and

Bulletin of Environmental Contamination and Toxicology.

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CHAPTER 3

Determination of degradability

by A. O. Hanstveit and J. F. de Kreuk



3.1 INTRODUCTION

This chapter deals with the methods for determining the chemical and biological degradability of organic compounds or their mixtures.

As an introduction, we shall first consider in turn the following subjects and their background:

- degradability
- the selected methods (Table 1)
- nutrient solutions (media)
- the microbiological inoculum
- the analyses required
- the calculation and evaluation of the results.

3.1.1 Degradability

A compound is regarded as **degradable** when it can be transformed in a given medium by microorganisms or under the influence of this medium (i.e. chemically). If the degradation end-products are exclusively inorganic (water, carbon dioxide, ammonia, etc.), the process is described as **mineralization** (Gilbert *et al.*, 1977). If no degradation takes place in the laboratory, the compound is called **recalcitrant** (Alexander, 1965) or **refractory**.

The customary term **persistence** indicates an extreme degree of recalcitrance. In this case the substance will not degrade under any environmental conditions. The demonstration of persistence demands a very wide investigation, and while the persistence of a compound can be demonstrated with a high probability, it can never be done with absolute certainty.

According to Alexander (1973), the five most important causes of the absence of a distinctly detectable biological degradation, in nature or in the laboratory, are as follows:

- No active degrading organism is present.
- The substance itself or its degradation products are toxic to the degrading organisms.
- Because of low solubility, absorption, or other factors, the substance is available in such low concentration, or is so poorly accessible to active microorganisms, that no appreciable degradation can occur.
- The substance does not induce the production of the necessary degrading enzymes. (This may also be a consequence of the above-mentioned causes).
- The substance can only be degraded completely (or mineralized) by several types of organisms that each carry out one or more successive reaction steps. The conditions required for these steps may be very different or even conflicting (for example, aerobic against anaerobic).

In the above cases the fundamental chemical structure need not exclude degradation, but the latter will not occur in laboratory or in practice. Finally, in some cases the chemical structure may in fact make degradation impossible under conditions such as temperature, etc. that occur in nature.

However, a recalcitrant compound can sometimes be degraded if a chemically related degradable compound is present. (This is called co-metabolism or co-oxidation). The substance itself is incapable of inducing the necessary degrading enzymes (see above), but it 'fits' to the enzymes induced by the degradable compound (Horvath, 1972; Skriabin *et al.*, 1974).

The investigation of degradability described in this chapter is based on the assumption that degradability is an intrinsic property of the substance. Here, in agreement with Alexander (1965), we shall reject the widely held view that all organic compounds can be biodegraded in one way or another.

However, in view of the above-cited remarks on recalcitrance, even

a degradable substance cannot be degraded under all circumstances. Consequently, much attention has been devoted in the literature to isolating and culturing an active organism (Section 3.2), and lengthy procedures have been used to bring about the adaptation of such organisms to the substance presented (the substrate). This adaptation may consist of a selection of organisms present in small numbers in the original material. Besides this, by mutation, 'new' organisms may arise, which maintain themselves under the new conditions.

Natural water is used (where possible) as a source of the microorganisms, because the investigations for which methods are given in this chapter are directed at the determination of degradability in the aquatic environment. The above-mentioned adaptation procedures are not used in this case, because it is very much a question whether the degradation performed by them will also occur in natural waters.

It must be mentioned that the relationship between the methods described in this chapter and 'practice' has not yet been investigated comparatively. In the choice of the test conditions, however, the practical situation has always been taken into account as far as possible.

3.1.2 Methods

A study of degradability can be divided into:

- determination of the chemical degradation;
- determination of the non-toxic concentration;
- determination of the biological degradation.

The first two parts provide information necessary for the subsequent degradability study proper.

An idea of the chemical stability of a substrate is necessary, so that the effects of chemical degradation can be taken into account in the biological and analytical procedures. The concentration at which no toxic effects are exerted on the degrading organism must be known. Naturally, the concentration used in the test must not exceed this level.

In the actual degradability test it is not always necessary to distinguish between chemical and biological effects, especially if the medium (the nutrient solution) has been obtained from the particular environment in which the degradation takes place. Only the total effect is of practical significance.

In the following sections the background of the methods selected will be explained. In 3.1, Table 1, a review is given of the biological methods and of their aspects in relation to apparatus, test duration, etc.

3.1.2.1 Determination of chemical degradation

Some compounds may undergo chemical (abiotic) changes (degradation) in the aquatic environment. The rate of these changes depends, inter alia, on the temperature, the oxygen content, and the pH. In fresh water and certainly in sea water these parameters vary within narrow limits. In the course of the study, however, it is often necessary to try wider variations, since these may occur during the biological and analytical procedures (for some examples see Section 3.4).

In order to distinguish chemical effects from biological ones, a sterile blank may be included in a degradability test. The circumstances are then similar to those in the actual test but the living organisms have been eliminated or killed.

3.1.2.2 The microbiological inoculum

A microbiological inoculum is a collection of microorganisms used in degradability tests to bring about the degradation of the compound in question.

3. Determination of degradability

As stated in Section 3.1.1, degradation can occur only if the right organisms are present and if these are capable of development under the test conditions.

Two approaches are now possible:

- When the test is used to determine degradability as a property of a substance, the inoculum must be as rich as possible in various types of organisms.
- When degradation must be considered in relation to a specific discharge, the inoculum must be taken from the medium in the vicinity of the point of discharge.

This principle is developed further in Section 3.2. In addition to a description of the inoculum preparation the possibilities described in the literature are reviewed, since particularly at this point the specification of fixed rules for obtaining inoculating material is very difficult.

It is worth mentioning that pure cultures may in fact be used for toxicity tests (see Section 3.1.2.3), but that they are unsuitable for degradability studies.

3.1.2.3 Determination of the non-toxic concentration

In the determination of the biological degradability of a substance, its toxicity with respect to the organisms responsible for the degradation must be known.

Three methods are given in this chapter (Sections 3.5.1.1, 3.5.1.2, and 3.5.1.3) for determining the toxicity, in which growth inhibition is used as the criterion.

The procedures are carried out with pure cultures or with mixed cultures. The growth of pure cultures is determined by measuring the turbidity of the culture liquid. In the case of mixed cultures the respiration (Section 2.7.1.) or the change in the ATP content (Section 2.7.2) is measured.

With pure cultures the toxic effect can be determined with high reproducibility. However, experience has shown that degradability studies should preferably be carried out at concentrations 10 to 100 times lower than the 'no-effect level' determined in the toxicity tests.

The determinations with mixed cultures, especially when these are based on a natural system, approximate more closely to the environment in which the degradability test is performed. Here again, however, at least a 10 times lower concentration should be used than the 'no-effect level' following from the toxicity tests.

The reproducibility of the results of tests with mixed cultures is lower than that with pure cultures.

3.1.2.4 Determination of biological degradation

The degradability tests can be divided into two groups:

- die-away tests in static systems;
- tests in flow-through systems.

In a die-away test the concentration of the substances to be tested (the substrate) is determined as a function of time. Here, during the test, the substance is present in a definite volume of the medium. In a flow-through system a constant stream of the medium with a given substrate concentration is added to a fully mixed vessel containing a constant amount of the medium. The degradation is calculated from the difference between substrate concentrations in the influent and the effluent (concentrations in entering and leaving streams of liquid).

The following tests are described in this manual:

- die-away test with shake flasks (Section 3.5.2)
- die-away test with 'trickling filters' (Section 3.5.3)
- determination of degradability in activated sludge flow-through systems (Section 3.5.4)
- determination of degradability in chemostats (Section 3.5.5).

The choice is determined by the following factors:

- the apparatus
- the substrate properties
- the concentration
- the test duration
- the adaptation
- the biomass.

In relation to these factors, the following remarks may be made:

- The **apparatus** for the various tests varies widely and its amount increases in the sequence of the four determinations mentioned above.

- The **substrate** is subject to very different requirements from method to method.

In die-away tests with shake flasks the substrate need not fulfil any special requirements. However, the flasks must be sealed if:

- the substrate included volatile components
- volatile degradation products are formed in addition to CO₂ and water
- a radioactively-labelled substrate is used.

Chemostats can be used to test substrates that dissolve in the nutrient solution or form a stable emulsion with it; the sterilization of such emulsions is difficult. For the other methods the substrates must always be soluble in water, and non-volatile, either alone or with steam (co-distillation). Co-distillation takes place, for example, with DDT and dieldrin (Mackay and Wolkoff, 1973; Oloffs *et al.*, 1972, 1973).

Although many substrates are adsorbed on plastics, or diffuse into or through plastics, the use of these materials can hardly be avoided (except in the die-away test with shake flasks). When plastics are used (e.g. in peristaltic pumps), it must always be borne in mind that substrate may be lost and that the plastics used may release plasticizers, stabilizers, etc. into the medium.

- The **substrate concentration** in die-away tests must be below the level at which toxic phenomena can occur (Section 3.1.2.3). Since in flow-through systems the substrate concentration in the vessel during degradation is much lower than that in the influent, the concentrations in that influent can be allowed to be higher. The test has to begin, however, with a non-toxic concentration, which is raised after adaptation of the culture. This is important if the analytical method used is marginally sensitive. The substrate in the influent and effluent can then reasonably be determined only after adaptation. A favourable aspect of flow-through systems is that the effluent can be used completely for the analysis if, immediately after collection, it is protected against further degradation.

- The **duration** of the die-away tests and of investigations with activated sludge in flow-through systems may run to 1–2 months. An experiment with a chemostat usually lasts 5 weeks, but in certain cases this can be reduced to 1 week (Section 3.1.2.8).

- The **adaptation**, i.e. the adjustment of the inoculum to the substrate, depends on the method followed. In a given test a mixed microflora from e.g. water, soil, or sludge is added. Each flora may contain microorganisms capable of carrying out the desired degradation, but usually their initial number is low. Under favourable conditions they multiply until they form an appreciable proportion of the microflora. A good example of such adaptation is the increase in the number of alkane-oxidizing bacteria in sea water after a discharge of oil. An inoculum is therefore, as it were, forced to adopt a different composition by a change in the external conditions. This force is known as selection pres-

sure. The selection pressure differs from test system to test system. It is greatest in the chemostat, because there all microorganisms not sufficiently adapted to the test conditions, and which therefore grow insufficiently rapidly, are washed out. All this also means that any variation of the test conditions in the chemostat (temperature, dilution rate, composition of the medium) may result in a re-adaptation of the culture in which also other organisms may become dominant.

In strong contrast to chemostats, in activated sludge systems the microflora is not washed out but is retained in the system as well as possible. Since, moreover, here an artificial effluent is used as a feed in place of an inorganic nutrient solution with the substrate as the only source of carbon, a much more varied flora arises, in which even the slower-growing microorganisms can develop. Account must be taken of the fact that the most readily degradable compounds are the first to be degraded, so that the degradation of difficultly degradable compounds may be retarded. On the other hand, the synthetic effluent may act as a source of energy when the substrate in itself yields insufficient energy for growth (a form of co-oxidation).

In static systems the selection pressure is much lower than in flow-through systems, and consequently the microflora is more varied (especially compared with chemostats). Furthermore, various species may gain the upper hand in succession, depending on the concentration of the original substrate and its degradation products. In a 'trickling filter' this may even occur at different heights in the column. In this respect the static systems are closer to practical conditions than the flow-through systems, although here too the adaptation process will differ from that in open water.

- The **biomass** depends greatly on the nature of the test. In activated sludge systems its amount is higher than in 'nature', but it is probably comparable with that in an aerobic purification plant although there are major differences of structure and species composition. The same applies to the die-away test with trickling filters.

At low substrate concentrations (< 10 mg of the substrate per litre), the biomass in the test with shake flasks and in chemostats will resemble that under practical conditions.

However, particularly in the chemostat, the species composition differs greatly from that in practice (see also above).

The various tests will be discussed in closer detail in the sections below.

3.1.2.5 The die-away test in shake flasks

This simple method, described by Hammerton (1955), is now the one used most often for the determination of degradability. Its origin is connected with problems concerning detergents and the prediction of their downstream behaviour when discharged into a river, and the test is therefore also called the 'river die-away test'. In the course of time the method has been standardized for the determination of the degradability of detergents (Anon. 1965, Anon. 1966, OECD 1971). These standard procedures have been discussed by Fischer *et al.* (1974), Wagner (1972), Wayman (1972), and Swisher (1970).

When a 'river die-away test' was carried out, the substrate was added to a conical flask containing river water and the change in its concentration was followed as a function of time. In the interpretation of the results it is assumed that the water in the conical flask undergoes the same development as a corresponding water compartment flowing down the river. For detergents, a requirement of 80% degradation in 20 days has been arrived at in this way, and for comparison a readily and a difficultly degradable detergent must be tested at the same time (OECD, 1971).

A more general test, not limited to detergents, has been described

by Payne *et al.* (1970). Halvorson *et al.* (1971) describe the test for pesticides.

The following factors important for carrying out degradability tests are described in this manual for the die-away test (see also Blok, 1975):

- physico-chemical properties of the substrate (Section 3.1.2.4)
- the concentration of the test compound (Section 3.1.2.4)
- the analytical method (Section 3.1.4)
- the medium; natural or artificial water (Section 3.1.3)
- the adaptation of microorganisms (Section 3.1.2.4)
- co-metabolism (Section 3.1.1)
- the temperature.

Most of these factors depend on the substrate. A choice is made of the factors that depend least on the substrate properties. In addition, the procedure is kept as flexible as possible.

In the case of volatile substrates or degradation products, the test must be carried out in closed flasks. The substrate concentration to be used is determined by the toxicity of the substrate, the detection limit of the analytical method, and physical properties of the substrate.

The choice of the analytical method depends on the substrate. It is preferable to use a general method such as COD (chemical oxygen demand) or DOC (dissolved organic carbon), to obtain a measure of the degree of mineralization of the substrate. These results must frequently be supplemented with an, often time-consuming, substance-specific analysis (such as gas chromatography). Particularly in the case of tests in a marine environment, natural sea water (preferably from the open sea) can be used as the medium. The disadvantage here is that the test is less reproducible because of variations in the water composition and in the microflora present. An advantage is the closer similarity to practical conditions. In this test adaptation takes place by the growth of the organisms that can degrade the substrate, while the other organisms die off. When a large amount of organic material is introduced with the inoculum, this additional source of carbon must be taken into account. The material may interfere with the analysis, promote the breakdown of the test substrate by co-metabolism, or appear to make degradation greater. This last case occurs when in the use of a general method of analysis (DOC, COD, etc.) this organic substance is also determined in the test. With the decomposition of this organic substance (the inoculum), therefore, a degradation is already detected when the original substrate itself has not (yet) been degraded.

A standard temperature can be adopted (for example 25°C). In addition, in the case of a waste water, work must also be carried out at the temperature of the point of discharge, since the degradability result is frequently highly dependent on temperature. Evans and David (1974) found that various polyethylene glycols in a river were well degraded in summer at a higher temperature and a slow water flow, but practically not at all in winter at a low temperature and a fast flow of water.

3.1.2.6 The die-away test with trickling filters

The die-away test with trickling filters was described by Jenkins *et al.* (1967). Just like the die-away test with shake flasks, it was originally designed for detergents.

The greatest difference between the two tests is that with the trickling filters the aim is to determine the degradability of a substance quickly as an intrinsic property, while the test with shake flasks is designed to give a picture of degradation in part of the environment. This is expressed particularly in the adaptation procedure used. The microflora in a trickling filter is cultured by renewing the medium every time it is exhausted. The organisms that grow under these conditions remain behind on the column packing. In the tests on detergents, a mixture of different detergents can be

3. Determination of degradability

used initially to create a diversified inoculum in the column. It should then be possible to determine the degradability of a detergent in about one day (Jenkins *et al.* 1967). As soon as the column has become adapted, it can in principle be used without limitation.

3.1.2.7 Determination of degradability with activated sludge flow-through systems

The degradability test with activated sludge is the most common after the die-away test with shake flasks. It too was designed for the determination of the degradability of anionic detergents (Bundesgesetzblatt, 1962). In this method the aim is to create a micro-biologically active and aggressive medium in which those processes take place that also occur in sewage-purification plants.

For this purpose the substrate, mixed with an artificial sewage, is added to the activated sludge system. This is in contrast to the other methods, in which the substrate is always used as the sole source of carbon.

In the OECD method (OECD, 1971) this test is prescribed whenever no degradation of a detergent can be demonstrated in the die-away test with shake flasks. In the flow-through systems degradation can be promoted by the high biomass (2.5–3 g of dry matter per litre) maintained in the system and by the occurrence of co-metabolism (Section 3.1.1), so that in the degradation of the organic material added with the artificial sewage the test substrate is co-oxidized. In addition to the similarities with a sewage-purification plant, certain differences must be pointed out as well.

- The structure of the floc differs greatly, as can be seen clearly from SEM photographs (3.1, Fig. 1, and 3.1, Fig. 2). The activated sludge from a sewage-purification plant fed exclusively with domestic sewage contains threads, dead bacterial material, and living bacteria (3.1, Fig. 1). The structure is also 'looser' than the activated sludge from a laboratory installation. It can be seen that this floc is formed fairly completely of living bacteria between which only a small amount of dead material is present (Section 3.1, Fig. 2).
- The species composition is more varied in activated sludge from sewage purification plants than in the laboratory (Janicke, 1969). It should be noted at this point that the inoculation of the laboratory system takes place via aerial infection and by 'infection' with unsterilized mains water (Hitz *et al.*, 1975). The species composition is also affected by the method of aeration. In the laboratory this is so vigorous that there is an almost uniform oxygen concentration throughout the system, close to the saturation value. In purification plants great differences occur in the oxygen concentration, so that frequently anaerobic zones may even be formed (e.g. in the activated-sludge settling tank).
- Values of up to 400 mg.l⁻¹ have been found for the biochemical oxygen demand (BOD) of the synthetic effluent with substrate. With an average residence time of 3 h and a sludge weight of 2.5–3 g of dry matter per litre, this means a sludge loading (K) of 1.1 to 1.3

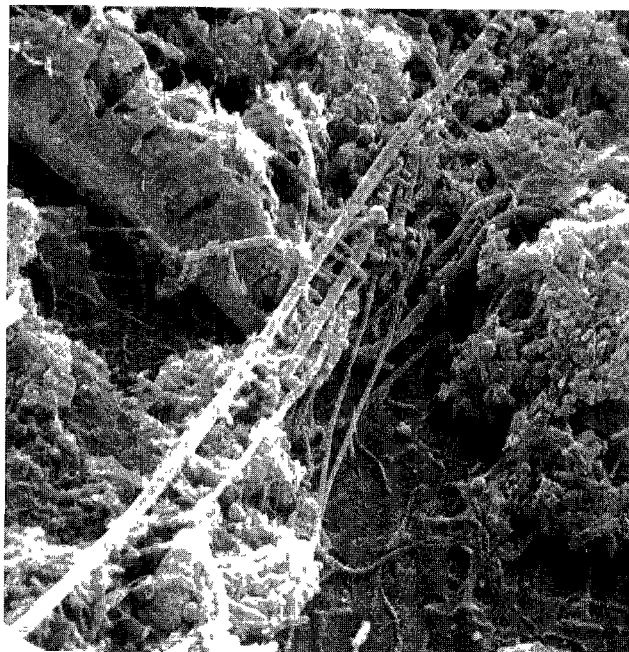
$$\left(K \equiv \frac{\text{g BOD per 24 h}}{\text{g dry matter of the activated sludge}} \right)$$

This sludge loading corresponds with that of a highly loaded purification plant (Koot, 1974) in which the sludge production (growth) is about 4 g of dry matter per day. Many purification plants work at a much lower loading.

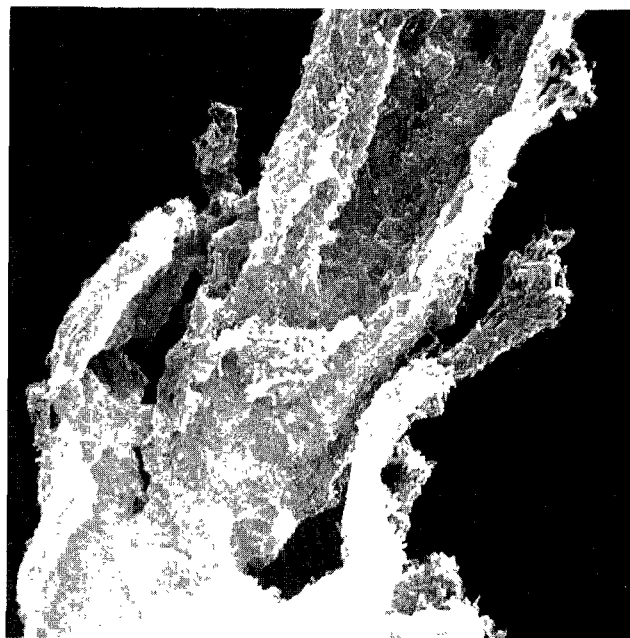
In tests with activated sludge a steady state must be maintained for a relatively long time (2–3 weeks). The sludge weight and the COD or the DOC of the effluent must remain constant during this time, a requirement frequently difficult to realize in practice.

Small disturbances in the functioning of the apparatus may have large effects on the system. An accurate metering of the influent, for example, is of great importance.

The time that the activated sludge requires to degrade a substrate is proportional to its recalcitrance. For a readily degradable substrate an average residence time of a few hours is sufficient. However, this may rise to several days for poorly degradable compounds.



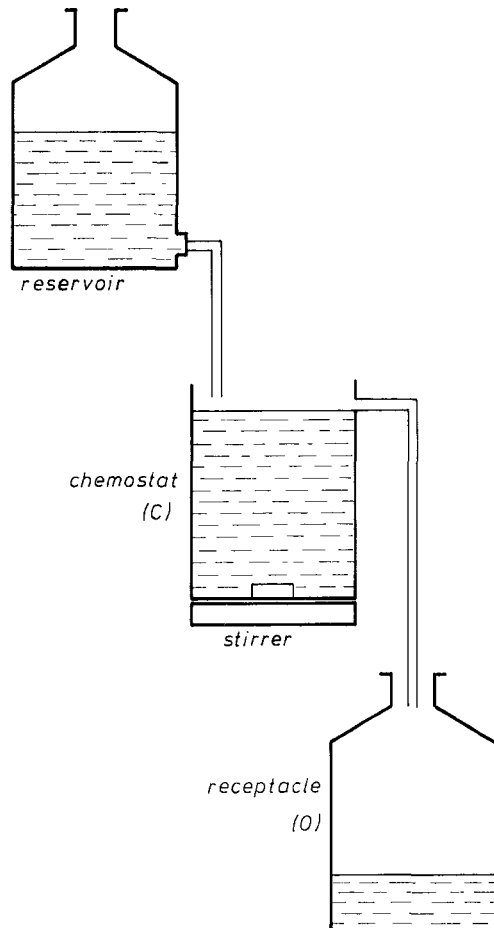
3.1 Fig. 1 Activated sludge from an oxidation ditch fed with domestic effluent (magnification 2200) (photograph from TNO Division of Technology for Society, Delft)



3.1. Fig. 2 Activated sludge from a laboratory flow-through system fed with artificial sewage (magnification 2200) (photograph from TNO Division of Technology for Society, Delft)

3.1.2.8 Determination of the degradability with a chemostat

The chemostat consists of a vessel through which medium is passed at constant rate and with continuous stirring. With the aid of e.g. an overflow the volume of liquid in the chemostat is kept constant (3.1, Fig. 3).



3.1 Fig. 3 Schematic illustration of a chemostat

A steady state is now established in which the growth rate of organisms selected by the system is in equilibrium with their rate of washing out and in which a certain (low) substrate concentration is maintained. Monod (1950) has published a theoretical consideration of the chemostat, on which the following argument is based.

A constant flow of nutrient solution passes from the reservoir to the chemostat C. The same amount runs over into receptacle O. It is now assumed that growth occurs only in C and ends directly for the microorganisms collected in O.

If x_c = bacterial mass in C

x_o = bacterial mass in O

x_t = total bacterial mass,

then:

$$x_t = x_c + x_o \quad (1)$$

On differentiation with respect to time, Eq. (1) gives:

$$\frac{dx_c}{dt} = \frac{dx_t}{dt} - \frac{dx_o}{dt} \quad (2)$$

On the basis that only the organisms in the chemostat multiply, for the increase in x_t due to growth we have:

$$\frac{dx_t}{dt} = \mu x_c \quad (3)$$

Here μ is the specific growth rate constant, which gives the relative increase in the amount of biomass in unit time.

The increase in the bacterial mass in O is proportional to that in C and to the rate of dilution of the medium D (D is the fraction of the contents of C replaced in unit time:

$$D = \frac{\text{flow rate of the medium (ml.h}^{-1}\text{)}}{\text{volume of liquid in the chemostat (ml)}}$$

This means that:

$$\frac{dx_o}{dt} = D x_c \quad (4)$$

and substitution of (3) and (4) into (2) gives:

$$\frac{dx_c}{dt} = (\mu - D) x_c \quad (5)$$

For the growth of microorganisms in a given amount of medium the following hyperbolic equation applies (Monod, 1950):

$$\mu = \mu_o \frac{S}{K_s + S} \quad (6)$$

where μ_o = the maximum specific growth rate constant

S = the substrate concentration

K_s = the affinity constant of the bacterial species for the substrate present ($K_s > 0$).

For each bacteria-substrate combination K_s has a specific value, equal to the substrate concentration at which the growth rate is half of its maximum.

A low value of K_s implies that the microorganism can still have a high growth rate at a low substrate concentration.

A high value of K_s indicates a low rate of growth under the same conditions.

The values of K_s for most substrates are between 10^{-2} and 10^{-6} g.mole.l $^{-1}$.

Monod (1950) also observed that there is a constant relationship between the consumption of a substrate and the biomass formed:

$$\frac{dx_t}{dt} = -Y \frac{dS}{dt} \quad (7)$$

Y is called the yield factor and can be defined as:

$$Y = \frac{\text{weight of bacteria formed}}{\text{weight of substrate consumed}}.$$

It has been found by later studies (Tempest and Herbert, 1965) that Y depends on the rate of dilution. Particularly at low growth rates, where e.g. $\mu < 0.1 \mu_o$, it may be found that a relatively large proportion of the substrate is necessary to maintain the cell ('maintenance energy'). At higher growth rates this proportion is relatively low.

A substrate balance now implies:

change in the concentration = inflow – outflow – consumption
or

$$\frac{dS}{dt} = DS_o - DS - \frac{1}{Y} \frac{dx_t}{dt} \quad (8)$$

3. Determination of degradability

S_0 is the substrate concentration in the medium fed in. After substitution of (3) and (6) into (8), we obtain:

$$\frac{dS}{dt} = D(S_0 - S) - \frac{x_c}{Y} \mu_c \frac{S}{K_s + S} \quad (9)$$

In the steady state in a chemostat the following conditions apply:

$$\frac{dS}{dt} = 0 \text{ and } \frac{dx_c}{dt} = 0.$$

With these conditions applied to (5), (6), and (9) it follows that:

$$(\mu - D) x_c = 0$$

or

$$\mu = \mu_0 \frac{\bar{S}}{K_s + \bar{S}} = D \quad (10)$$

and

$$D(S_0 - \bar{S}) = \frac{\bar{x}_c}{Y} \mu_0 \frac{\bar{S}}{K_s + \bar{S}} \quad (11)$$

The values for the substrate concentration and the biomass are fixed for each steady (equilibrium) state and are generally represented by $\bar{S}$ and $\bar{x}_c$ (Herbert *et al.*, 1956).

On the basis of this model the following observations can be made:

- It follows from Eq. (10) that D must be smaller than μ_0 , since

$$\frac{\bar{S}}{K_s + \bar{S}} < 1$$

When $D > \mu_0$, we have $\frac{dx_c}{dt} > 0$ (μ_0 is the maximum value of μ) and all organisms disappear from the chemostat (the following expression actually holds:

$$D > \frac{\mu_0}{1 + \frac{K_s}{S_0}}; \mu \text{ is maximum if } \bar{S} = S_0 \text{ (10)).}$$

- The equilibrium concentration of the substrate and of the biomass is maintained as follows:

When $S > \bar{S}$, then $\frac{dS}{dt} < 0$ (9) and the concentration of the substrate decreases. Conversely, when $S < \bar{S}$ then $\frac{dS}{dt} > 0$.

Here, the biomass (x_c) is $\bar{x}_c$.

When $x_c > \bar{x}_c$, then $\frac{dx_c}{dt} < 0$ (9), hence $S < \bar{S}$, and $\mu < D$ (10).

The biomass now decreases, since $\frac{dx_c}{dt} < 0$ (5). With similar reasoning, $\frac{dx_c}{dt} < 0$ when $x_c < \bar{x}_c$. Here the substrate concentration (S) is assumed to be equal to $\bar{S}$.

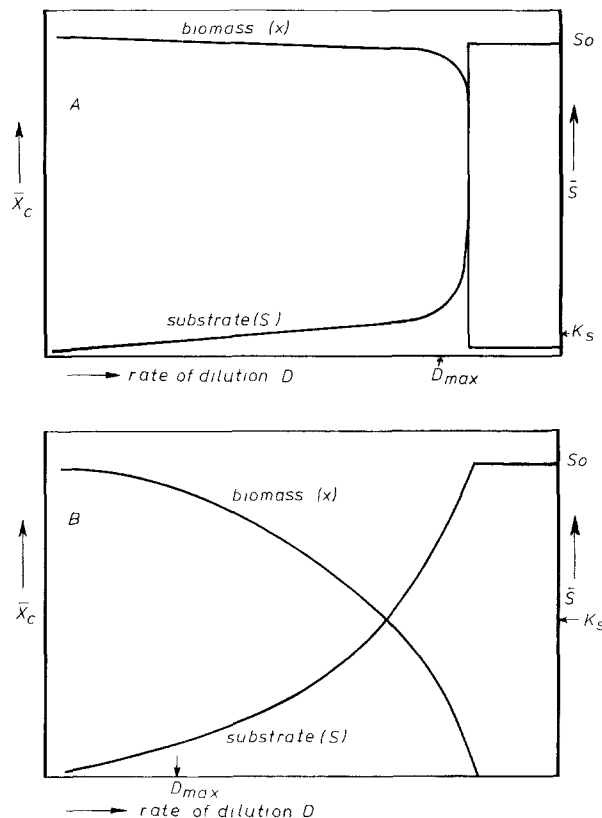
- For a given substrate-organism combination, the equilibrium concentration of the substrate $\bar{S}$ is determined exclusively by the dilution rate D (10), since μ_0 and K_s are constants.

The biomass $\bar{x}_c$ depends on the substrate concentration in the influent (S_0).

It follows from Eqs. (10) and (11) that:

$$\bar{x}_c = Y(S_0 - \bar{S}).$$

When K_s is small in relation to S_0 a plot of S_0 as a function of D has the form shown in 3.1, Fig. 4A. The form of the plot in 3.1, Fig. 4B occurs when K_s is large in comparison with S_0 (Tempest, 1970).



3.1 Fig. 4 Influence of the rate of dilution on the substrate concentration and on the biomass in the steady state
A when K_s is small in comparison with S_0 ;
B when K_s is large in comparison with S_0 .

- The theory described in this section applies to the behaviour of a pure culture in a chemostat. The determination of biological degradability, however, starts from an inoculum consisting of several species. What will happen is that the organism with the highest growth rate under the given conditions will predominate. The other organisms are washed out because of competition for the substrate. The theory of the chemostat is therefore also applicable to investigations with mixed cultures, since one or a few species will dominate. However, this washing out is usually not complete, so that with a change in the rate of dilution (and therefore in $\bar{S}$) another organism may displace the original one (for example, because it has a lower K_s).

- The maximum growth rate μ_0 of an organism is proportional to the degradability of a substrate provided that the substrate is the limiting factor for growth. Consequently, it is possible to define the degree of degradability as the maximum rate of dilution at which degradation still takes place.

In the case of curve A (3.1, Fig. 4) it is possible to determine D_{max} . However, K_s is frequently larger for poorly degradable compounds. In that case (curve B), D_{max} must be defined as the rate of dilution at which, for example, 90% degradation still takes place ($\bar{S} = 0.1 S_0$). The D_{max} can be determined, after degradation has set in, by increasing the rate of dilution stepwise until $\bar{S}$ rises. In this procedure different species of microorganisms may succeed one another as a result of this change in the conditions.

An opposite approximation may also be chosen for the test method. A definite rate of dilution (Da) is selected on the basis of the degradability determinations of substances whose behaviour (preferably also in the environment) is known. When no information is available, a value of $Da \geq 0.01 \text{ h}^{-1}$ may be chosen. The determinations are then performed at this rate of dilution. The substances degraded by more than 90% at this rate of dilu-

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tion are regarded as degradable ($\bar{s} \leq 0.1 S_o$), while substances which show no appreciable degradation ($\bar{s} \geq 0.8 S_o$) are recalcitrant. For those compounds whose degradation lies between these limits ($0.1 S_o < \bar{s} < 0.8 S_o$) the test may be repeated at a lower value of D (e.g. $\frac{1}{2} D$). If the substance still degrades less than 90 %, it is again regarded as recalcitrant. The constants μ_o and K_s can be calculated from the values of $\bar{s}$ at two values of D , but it must be investigated whether the degrading organism is the same in both cases.

- It follows from these considerations that the steady state in the chemostat is sensitive to changes in the test conditions. The rate of dilution (i.e. the rate of introduction of the medium), in particular, must be adjusted accurately.
- It has been found from experiments in this laboratory that sampling at times $\frac{n}{2} \cdot \tau_o$ gives adequate information (τ_o is the mean residence time of the liquid in the chemostat; $\tau_o = \frac{1}{D}$). In sampling directly from the vessel the smallest possible sample must be taken, since this temporarily reduces the volume in the chemostat. The dilution rate D therefore becomes greater, which may lead to changes in the population. Regular sampling may then make the system unstable.

3.1.3 Nutrient solutions

Nutrient solutions for degradation tests must contain all the essential elements. The carbon source (the substrate) is then the only limiting factor for the development of the organisms. When this condition is not satisfied the growth stops before the substrate has been consumed, so that the compound appears to be recalcitrant.

In general, the weight ratio of carbon to nitrogen in the feed solution should be 10:1 at the most and that of carbon to phosphorus 50:1 at the most.

The compositions of a number of nutrient solutions are given in Section 3.3.

3.1.4 Analysis

In a degradability test, the change in a substance concentration with time must be determined.

The choice of the method of analysis is determined by four factors:

- the information desired
- the lowest detection limit
- the permissible analytical error
- the test conditions.

Mineralization is the most desirable criterion of degradation (Wagner 1972), since on mineralization no harmful degradation products are formed and the breakdown products are taken back into natural circulation. General methods (not designed for the determination of any one compound) are most suitable for measuring mineralization.

General methods of determination involve:

- total organic carbon (TOC; Section 2.3.1)
- the chemical oxygen demand (COD, Netherlands Standard NEN 3235 5.3)
- the total oxygen demand (TOD, Section 2.3.1)
- the oxygen uptake (Section 2.7.1).

Specific analyses such as those based on spectrophotometric or chromatographic techniques (Section 2.4) are used where a direct determination of the mineralization by one of the above-mentioned methods is inadequate because mineralization proper is preceded

by a specific transformation and/or better detection is desired.

In such a general method of determination the **lowest detection limit** is of the order of a few milligrams of the substrate per litre. Consequently, if it is desired to demonstrate a degradation of 90 %, the initial concentration must be at least 20 to 50 mg per litre.

However, the solubility and the toxicity of the test substances set an upper limit to the substrate concentration. When a substance cannot be tested in the above range of concentrations, a substance-specific method must be used. The possible appearance of poorly degradable metabolites must then be borne in mind.

The **analytical error** of the determination need not be smaller than 10 %. This is because a small measured degradation of, for example, a few parts per cent still cannot be regarded as a proof of degradation, in view of the uncertainty of the degradation determinations themselves. In such cases 'degradation' is often caused by adsorption onto glass or plastic surfaces. Percentage degradations smaller than 10 % can, however, be determined with reasonable accuracy with radioactively-labelled compounds, if the purity of such compounds is beyond any doubt.

The **test conditions** contribute to determining the most suitable method of analysis. Thus, in the case of flow-through cultures larger samples can be taken, so that the detection limit for a specific analysis (by means of extraction) can be lowered.

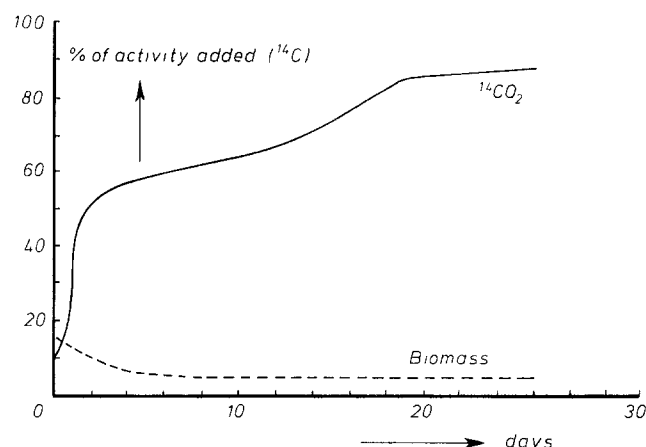
In flow-through cultures with activated sludge it is difficult to measure mineralization, since the medium contains organic substances other than the substrate alone.

In the determination of mineralization the suspended cell material can e.g. be centrifuged off.

However, the 'mineralization' deduced from the measurements includes in this case both the conversion of the substrate into inorganic material and its conversion into insoluble organic material.

In certain cases the cell material cannot be separated off without some loss of unconverted substrate, as for example in the testing of heterogeneous systems. A lower conversion is then measured (in a static test, especially at the beginning) than has actually occurred. This point is illustrated by the following example.

In a die-away test with ^{14}C -labelled *p*-chlorophenol the production of $^{14}\text{CO}_2$ was measured and at the same time the uptake of radioactivity into the cell material was determined. 3.1, Fig. 5 shows the result of this test.



3.1 Fig. 5 Degradation of ^{14}C -labelled *p*-chlorophenol in a die-away test with shake flasks

3.1.5 Calculation and reporting of the results

The results of degradation tests can be given in the form of percentage degradation (OECD, 1971; Bundesgesetzblatt, 1962).

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The following formula is used for the calculation:

$$\% \text{ degradation} = 100 \times \frac{C_{i0} - C_{it}}{C_{i0}}$$

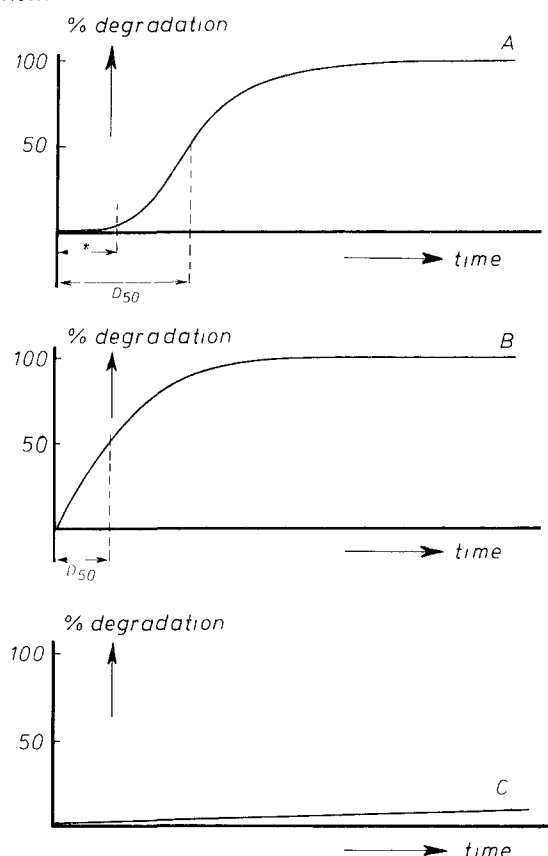
For the die-away tests in static systems and for tests in flow-through systems

C_{i0} is the substrate concentration at the start of the test and in the influent, respectively;

C_{it} is the substrate concentration at test time t and in the effluent, respectively.

With each result t is reported. In the case of flow-through systems the mean residence time (Section 3.4.1) must also be given.

To evaluate the results it is recommended to give the degradation (or the substrate concentration) graphically as a function of time. Several types of degradation curve are possible. 3.1, Fig 6 gives a review.



3.1 Fig. 6 Possible curves in degradability experiments (* adaptation period.)

Test A shows the most usual course of degradation. An adaptation period (Section 3.1.2.4) is followed by a period in which the culture grows exponentially (logarithmic phase). This can be represented as follows:

$$N_c = N_0 \cdot e^{\mu t}$$

where N_0 is the number of organisms at the beginning of the logarithmic phase, N_c is the number of organisms at time t , and μ is the specific growth rate constant.

When the substrate has been consumed or can be degraded no further, the growth stops (in the static systems) or, in the flow-through systems, becomes constant and proportional to the rate at which the substrate is fed in (Section 3.1.4).

Curve B shows a special course of the degradation process. There is no adaptation period, because the inoculum is already adapted to the substrate. Active sludge from a sewage purification plant fed exclusively with domestic sewage will thus degrade an anionic detergent or an acetate without an adaptation period, while the

degradation of glucose still usually requires a short adaptation period. The 'adaptation-less degradation' occurs in the tests with trickling filters (Section 3.1.2.6).

In the case of curve C the degradation is inhibited because it is not the substrate but another factor that limits the growth of the degrading organisms – for example, the absence of a source of nitrogen or phosphorus. Degradation then takes place exclusively via endogenous respiration.

A curve of type A or B may change into one of type C if, during the degradation process, the degradation is disturbed in the manner just described.

The temporary occurrence of different mechanisms indicates how difficult it may prove to compare degradation results of different substances with one another.

In the official instructions for the determination of the degradability of detergents (OECD, 1971; Bundesgesetzblatt, 1962), it is required only that a detergent is 80 % degraded under the prescribed conditions. In the OECD instructions for the die-away test with shake flasks a 'soft' and 'hard' standard are tested simultaneously as controls. Here the soft standard (dodecylbenzenesulphonate) must be degraded within 8 days. The hard standard (tetrapropylenebenzenesulphonate) degrades incompletely (this behaviour previously led to serious environmental problems; Weaver and Coughlin, 1964).

If a detergent does not undergo degradation in the last-mentioned static procedure, a test is carried out with an activated sludge flow-through system. The assumption is then that a substance completely degradable in one of the two tests is also degraded in nature. Although this thesis had proved to be tenable for detergents, such an attitude towards degradation results is inadmissible for other substances or for poorly defined effluents, simply because of the lack of standards.

Degradation according to curve A (3.1, Fig. 6) requires further discussion. Three phases must be distinguished, because the increase in the amount of biomass and the decrease in the amount of substrate take place side by side.

- In the first phase, after adaptation maximum growth takes place because the substrate concentration is 'high' in relation to the biomass. The rate of degradation is then proportional to the biomass formed.
- In the second phase the substrate concentration, which has now fallen, begins to be limiting for growth, so that the latter is retarded. The degradation rate is now proportional both to the biomass and to the substrate concentration.
- In the last phase, the degradation rate is determined exclusively by the substrate concentration.

The above-mentioned course of degradation agrees with a 2nd-order reaction, as Gaudy (1972) suggests for BOD determination. The maximum rate of degradation on the steepest part of curve A depends on the accessibility of the substrate and on the affinity of the microorganisms for the substrate. The accessibility governs the amount of biomass formed per unit weight of the substrate, and the affinity determines the moment at which the reduced substrate concentration begins to limit growth.

The maximum degradation rate can be used for a comparison of the degradability results. When degradation is calculated as a percentage, the substrate concentration is no longer used exclusively. At a given rate of degradation the slope of curve A does, however, depend on the initial concentration. A correct comparison is therefore possible only if the initial concentrations do not differ too greatly from one another (for example, by a factor of less than 5). It is also possible to use the time necessary for 50 % degradation as a measure of degradability. The length of this period is greatly affected by the adaptation time required and thus by the degree to which the inoculum is already adapted to the substrate. On the other hand, the adaptation period for poorly degradable compounds is longer than with readily degradable compounds, so that an impression of degradability can be obtained by using this factor.

3.1 Introduction

The same processes as described above for static systems take place in flow-through systems and similar degradation curves are obtained. Instead of a single addition of substrate at the beginning of the test, new substrate is fed in continuously and the degradation curve is the resultant of the degradation and supply of the substrate. The above-mentioned factors, rate of degradation and adaptation period, cannot be compared with one another for static and flow-through systems. However, this is more or less possible for the

maximum percentage degradations achieved when one bears in mind that the steady-state substrate concentration in flow-through systems is a function of D alone. When mixtures of compounds that differ in degradability are tested, there is frequently a degradation in phases, the readily degradable compounds being degraded first. In such cases a stepped curve is obtained, so that a clear indication of the degree of degradation is given only at the time at which the maximum degradation has been achieved.

3.1 Table 1 Review of methods for the determination of degradability

Aspects	Die-way tests in static systems		Die-away tests in flow-through systems	
	Shake-flasks § 3.1.2.5	Trickling filters § 3.1.2.6	Activated sludge § 3.1.2.7	Chemostat § 3.1.2.3
1. Comparability with actual environmental conditions	Point discharge into a river	None	Aerobic purification plants	Surface water and sea water
2. Adaptation mechanism	No special adaptation mechanism (growth and dying off)	Growth and dying off	Growth that need not take place exclusively on the substrate offered*	Satisfactory rapid growth, otherwise washing out. Very pronounced selection pressure for a degrading organism
3. Test duration	4–8 weeks	1st sample about 4 weeks, each subsequent one about 1 week	6–12 weeks	Approximately $8 \times$ the mean residence time (τ_o)
4. Media	No limitations	No limitations	Artificial sewage	No limitations
5. Substrate properties	No limitations	Soluble in the medium. Non-volatile	Soluble in the medium. Non-volatile	No solid matter**
6. Analysis	Mineralization measurements and specific analyses	Mineralization measurements and specific analyses	Specific analyses	Mineralization measurements. Specific analyses. Determination of residual toxicity
7. Apparatus	Simple glassware and shaker	Glassware and pump	Perspex or glass systems and an accurately adjustable pump	Chemostat including accurately adjustable pump. pH control
8. Oxygen	Can be performed aerobically and anaerobically***	Aerobic	Aerobic	Can be performed aerobically and anaerobically***
9. Temperature	25° C or temperature of the part of the environment receiving the waste	20–25° C	20–25° C	25° C or temperature of the part of the environment receiving the waste

* Through retention of microorganisms in the system, those organisms that grow only slowly (e.g. because of difficult accessibility of the substrate) can also maintain themselves and reproduce.

** Special provisions must be made for volatile substances and substances immiscible with the medium.

*** Only the aerobic version has been described.

3.2 THE MICROBIOLOGICAL INOCULUM

3.2.1 General discussion

The microbiological inoculum is a mixture of many species of microorganisms added to a test medium. In a degradability test an inoculum is used as a source of organisms that can degrade the substrate.

The inoculum is usually derived from 'natural' microbial ecosystems such as soil, mud, activated sludge, river water, sewage, etc.

The properties of an inoculum material may be difficult to define precisely, but the following description, taken from Gaudy and Gaudy (1972), gives a definition of a natural microbial ecosystem: "The terms 'mixed microbial populations', 'natural populations', and 'heterogeneous populations' have all been used to denote systems in which natural selection occurs and determines which organisms can survive and which will predominate in the ecosystem. From time to time, shifts in predominating species may occur, due to natural interactions between species or in response to an external stimulus. The external stimulus may be some change in the chemical, physical, or biological environment which affects the system and to which the population responds, but which is not brought about internally by the population itself. **Thus a natural microbial ecosystem is indeed an enrichment culture which tends to seek whichever balance or imbalance of species can evolve under the existing environmental or cultural conditions.** Such systems occur everywhere in nature, and they are also employed under somewhat controlled (or unnatural) conditions in various biological processes."

This means that if, for example, in a degradation test, a compound is added to a natural microbial ecosystem, an organism will develop that will degrade the substrate unless other factors are limiting for its development. The problem has been discussed exhaustively by Alexander (1973).

The literature gives little information on how an inoculum should be prepared for a given degradation test. Some authors work with thick suspensions of activated sludge, while others use very dilute suspensions. Moreover, many use selection and adaptation procedures to obtain an inoculum that under carefully chosen conditions degrades an otherwise refractory test compound.

Most of the published discussions concerning the use of inoculum material relate to the die-away test, since experience has shown that in this test the inoculum material has the greatest influence on the result. In the other tests (activated sludge and chemostat tests) the process is started from a natural water (with its own microflora) or from a fortuitous infection via air, as in activated sludge systems (Hitz *et al.*, 1975; OECD, 1971). Since the selection pressure is higher in these tests than in the die-away test, and since an adaptation period is allowed to precede the test proper, a microflora optimally adapted to the test compound is obtained. Blok (1975) has come to the conclusion that successful degradation tests with a highly adapted microflora are by no means always relevant to the solution of practical environmental problems. This view is supported by Fischer *et al.* (1974), who even state that one should not work with an adapted inoculum. The course of the adaptation process must be studied during the actual test. Only in this way is there a chance of obtaining results that can be utilized in practical environmental situations.

We shall now give a number of examples, taken from the literature, of the preparation of a microbiological inoculum.

• *Natural water*

Seki (1976), Oloffs *et al.* (1972, 1973), and Evans and David (1974) used natural water. Saeger and Tucker (1976) used decanted river water after it had settled for two days. Gledhill (1975a), and also others, used sewage, which contains more organisms and organic compounds than do natural waters.

• *Mineral medium inoculated with non-adapted flora*

A better reproducibility can be achieved with this inoculum than

in the test in natural water.

According to the OECD method (1971), 1 ml of soil extract is added to 1 litre of the test medium. Voets (Laboratorium voor Microbiologie, Ghent) has informed us that the reproducibility is improved further when duplicate experiments are performed starting with the same batch of air-dried soil. Fischer *et al.* (1974) use a similar method of inoculation.

According to the British standard test for detergents (Anon., 1966), 30 mg of air-dried activated sludge are suspended in 1 litre of the test medium. Geldhill (1975a) uses thick suspensions of activated sludge for degradability tests.

• *Mineral medium inoculated with adapted flora*

The simplest adapted inoculum is the enrichment culture. According to the SDA test (Anon., 1965), the seed is adapted by making two 72-h cultures. For final inoculation 1 ml of culture is used per 100 ml of fresh medium and the test is run for 8 days. Bunch and Chambers (1967) begin with 10 ml of settled sewage per 90 ml of the medium. Weekly subcultures are made in fresh medium for 3 consecutive weeks; the total time of the test is 28 days.

Paris *et al.* (1976) go still further in their adaptation procedure by adding the test compound and the glucose to river water. The amount of the test compound is gradually increased, and the amount of glucose is reduced, until a microflora remains that is adapted to a high concentration of the test compound. An elegant example of a complicated adaptation procedure is given by Gledhill (1975b).

Kirsch and Etzel (1973), who studied the degradability of pentachlorophenol, used a semi-continuous culture for their adaptation procedure. The adaptation procedure involved daily removal of a part of the settled culture liquor and replacing it with fresh medium. In addition to Nutrient Broth they added ever-increasing concentrations of pentachlorophenol. The initial inoculum was a soil sample obtained from the grounds of a wood-products manufacturer who used PCP as a preservative. We consider these procedures to be necessary if it is desired to show that a compound is in principle degradable, but their significance for practical environmental problems is not high.

• *Concentrated microflora from natural water*

Halvorson *et al.* (1971) have published a test procedure in which they wanted to use the microflora from natural water, but the concentration of biomass was too low. They remedied this by centrifuging the water.

3.2.2 Preparation of inoculum material

In the performance of a test the various conditions and also the inoculum must correspond as closely as possible to the specific environmental problem. Consequently, in all die-away tests natural water should be used so far as possible (see Section 3.5.2, 3.5.3, and 3.5.5).

In the activated sludge test and in control tests in the die-away test the following inoculum materials are used:

• *Soil extract according to OECD (1971)*

Suspend 100 g of organic garden soil in distilled water and make up to 1 litre. Allow the suspension to settle for 30 min. Filter the supernatant through coarse filter paper. Discard the first 0.2 litre of filtrate. The remainder is the inoculum material. Keep aerobic by aeration, use within 24 h, then discard it.

• *Standard inoculum*

Use this inoculum to ensure a rich and varied flora of microorganisms.

Centrifuge (20 min at 5000 g) 0.5 litre of effluent from a sewage purification plant and then 0.5 litre of effluent from a laboratory

3.2 The microbiological inoculum

activated-sludge installation. Collect the precipitates and add 5 g of garden soil and 5 g of sediment from a ditch or a river. Suspend the whole in 1 litre of mineral medium and, after vigorous stirring, filter through coarse filter paper (see Note 1).

• *Concentrated suspension of the microflora of natural water*

If tests in natural water yield no degradation because of toxic effects of the test compound (see Note 2), this can sometimes be avoided by raising the concentration of the bacteria.

Various techniques can be used for this purpose (Hobbie and Pomeroy, 1971/1972).

An **illustrative** example of the procedure is given below:

Take 100 litres of sea water and centrifuge continuously at 45,000 g. This provides 0.6 litre of concentrated and slightly turbid inoculum material (see Note 3). If the last-mentioned

suspension is used directly as the test medium, and if its total organic carbon content is then more than 10 mg per litre, add extra ammonium nitrate and phosphate.

3.2.3 Notes

1. The test compound may be added directly to this suspension or the suspension may be added to the medium (1 ml per 100 ml) as inoculum.
2. In this case, at these high concentrations of organic material, the possibility of co-metabolic processes must be taken into account in the interpretation of the experimental results.
3. It is also possible to use an ordinary centrifuge, but this is somewhat laborious.

3.3 NUTRIENT SOLUTIONS (MEDIA)

Here we shall mention the nutrient media suitable for degradability tests (see also 3.1.3). Both salt- and fresh-water media and their sterilization are described.

3.3.1 Sea-water media

The experiments can be performed in natural sea water or in artificial sea water prepared from commercial sea salt, e.g. H. Wiegandt Meeressalz. This type of sea water can be enriched with 100 mg of NH_4NO_3 and 20 mg of K_2HPO_4 per litre.

The salinity of the sea-water medium must be 29‰.

The natural sea water must be obtained from an unpolluted location, e.g. from the open sea. Whenever relevant, attempts may also be made to obtain water from the place where the waste in question is discharged or dumped.

Collect the water in well-washed glass, polythene, or polypropylene bottles. Rinse these first three times with 1N HCl and then with distilled water until no acid remains. Take the water from below the surface if possible, since there is always a higher concentration of organic material at the surface.

For each sample, record the following data:

temperature, state of the tide, wind force, and wind direction.

If it is to be used as inoculum, transport the water to the laboratory as quickly as possible. Keep the water cool and use it for the test within 24 h.

Determine the following parameters in the laboratory:

- salinity (Section 2.2.5)
- pH (Section 2.2.3)

and, if possible, also the following:

- TOC (Section 2.3.1)
- ATP (Section 2.7.2)
- bacterial count (Section 2.7.4.1)
- chlorophyll (Section 2.7.5)
- O_2 concentration (Section 2.7.1).

It has been found in our own tests that even water from the open sea contains sufficient phosphorus and nitrogen to permit the undisturbed degradation of substrates up to an organic carbon content (TOC) of 10 mg.l^{-1} .

After the addition of the substrate, the pH of the medium should be between 8.0 and 8.4.

The sea water may be sterilized by filtration (Section 3.3.3).

3.3.2 Fresh-water medium

For fresh water, in contrast to sea water, no 'unambiguous' source of 'natural' water can be found.

Fresh surface water may be used for environmental tests. In view of the great variability of fresh water, which is different in different places and at different times, the work must always be carried out with the water relevant to the investigation.

Many media have been described in the literature for control experiments and 'non-location-oriented' studies.

An extensive and useful review has been given by Aaronson (1970). The following media are quoted from it because of their general applicability:

- Mineral medium for the investigation of the degradability of aliphatic hydrocarbons

NH_4Cl	0.5 g
$\text{Na}_2\text{HPO}_4 \cdot \text{H}_2\text{O}$	0.5 g
KH_2PO_4	0.5 g
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.5 g
NaCl	4 g

Dissolve in distilled water and make up to 1 litre.

pH	7.0
substrate	1 g or less

If the medium is sterilized in an autoclave, the phosphates must be sterilized separately to prevent the formation of precipitates.

- Mineral medium for the investigation of the degradability of aromatic hydrocarbons

K_2HPO_4	0.8 g
KH_2PO_4	0.2 g
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.5 g
$\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$	0.05 g
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	0.01 g
$(\text{NH}_4)_2\text{SO}_4$	1 g

Dissolve in distilled water and make up to 1 litre.

substrate	1.5 g or less

See also the note regarding phosphates attached to the preceding medium.

- Mineral medium for the investigation of the degradability of phenols

K_2HPO_4	1.0 g
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.2 g
NaCl	0.1 g
CaCl_2	0.1 g
FeCl_3	0.02 g
$(\text{NH}_4)_2\text{SO}_4$	1 g

Dissolve in distilled water and make up to 1 litre.

substrate	1.5 g or less
pH	7.2–7.5

In the case of phenol-containing substrates, particular attention should be paid to possible toxic effects. In flow-through systems high concentrations may be used only if the actual concentration in the culture is low because of degradation.

In addition to the above-mentioned nutrient solutions, the two media described in the international method for the determination of the degradability of anionic detergents (OECD, 1971) are suitable for more general use.

The first is a mineral medium in which the substrate is added as the only source of carbon. The second, as artificial sewage, is intended for flow-through systems with activated sludge and is selected so that the C/N ratio is approximately the same as in domestic sewage. The composition of these media is as follows:

- Mineral medium for detergent degradability studies (BOD dilution water)

To 1 litre of distilled water add 1 ml of each of the following four stock solutions of analytical grade:

1. K_2HPO_4	21.75 g
KH_2PO_4	8.5 g
$\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$	33.4 g
NH_4Cl	1.7 g
2. $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	22.5 g
3. CaCl_2	27.5 g
4. $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$	0.25 g

The stock solutions are prepared in distilled water and each is made up to 1 litre. The substrate concentration in the medium must not be higher than about 5 mg.l^{-1} . The total medium must be prepared freshly from the solutions before each test.

- Artificial sewage for degradability tests in flow-through systems with activated sludge

peptone	160 mg.l^{-1}
meat extract	110 mg.l^{-1}
urea	30 mg.l^{-1}
NaCl	7 mg.l^{-1}
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	4 mg.l^{-1} *
CaCl_2	2 mg.l^{-1} *

3.3 Nutrient solutions (media)

Dissolve these ingredients in mains water and make up to 1 litre.

substrate ca. 20 mg.l⁻¹

Depending on the quality of the mains water, the substances marked with an asterisk may be omitted. The substrate concentration of 20 mg.l⁻¹ is based on the procedure for the investigation of detergents. Higher concentrations may be used when necessary of desirable (up to about 500 mg.l⁻¹).

3.3.3 Sterilization of the media

The nutrient solutions may be sterilized in the autoclave (120°C, 20 min).

Where natural water is used, or large amounts of the medium are sterilized, e.g. in the case of continuous cultures, sterilization by filtration is preferable. The apparatus used for this purpose is shown in Fig. 1.

To this end the medium is filled into the pressure vessel and forced through the filter. The filter is built up, in the direction of filtration, of a glass-fibre pre-filter, a membrane filter with a pore size of 0.45 µm, a second pre-filter, and a membrane filter with a pore size of 0.2 µm.

The whole pack rests on a supporting plate.

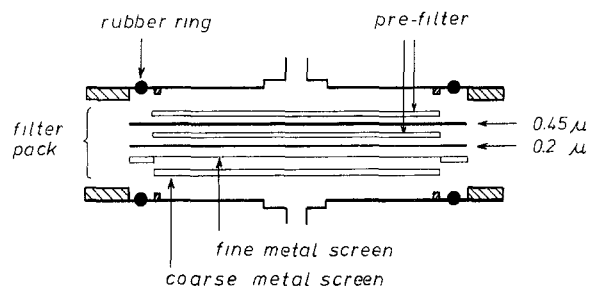
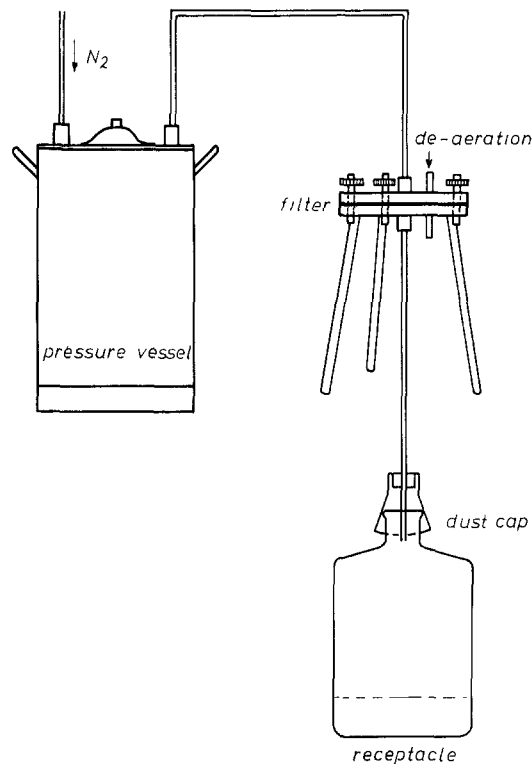
The filter holder is a sterilizable, stainless steel and Teflon-coated filter (e.g. Sartorius). It is connected to the pressure vessel by plastic pressure tubing with switch lock connections.

The dust cap over the outflow opening is essential and must also be sterilized. Without this cap the outflowing medium would again become infected (as the cap it is possible to use, for example, a glass powder funnel with a diameter of 20 cm).

The filtrate must be collected directly in a sterile storage bottle.

The test substance can be sterilized with the medium if it dissolves in it and is not absorbed in the filter.

When the substrate cannot be sterilized by filtration, heat sterilization (autoclave) must be used. If this too is impossible, the nutrient solution must be fed from a cooled storage bottle in the case of continuous cultures. The medium must then be prepared afresh every 2 to 5 days. It must be renewed daily if the stock is stored at room temperature. In the last two cases the storage bottle and the feed lines (tubes) must be sterilized at each renewal. If this is omitted, in principle a continuous culture already arises in this part of the system. Another answer to this problem is to meter a sterile nutrient solution into the culture and separately the substrate in a solution deficient in nutrients. Growth in the latter solution is thus inhibited.



3.3 Fig. 1 Sterilization apparatus

3.4 METHOD FOR THE DETERMINATION OF CHEMICAL DEGRADATION

Subject

Determination of the chemical transformations of a compound or a mixture of compounds in fresh water or sea water under environmental conditions.

Field of application

Chemical degradation can be investigated in fresh water and sea water under the conditions in which biological degradation is determined.

Principles

A substrate is dissolved in, or mixed with, a nutrient solution, a buffer, or water. Under sterile conditions, the liquid is then exposed to conditions that may be present in the environment or in laboratory experiments. Moreover, by varying these conditions over a greater range, transformations that normally take place very slowly or not at all can be accelerated. Changes in the substrate are determined with the aid of substance-specific analyses.

If a transformation takes place, an attempt may be made to identify the product formed.

The test is carried out under sterile conditions to distinguish chemical from (micro)biological transformations.

The sterility is checked at each sampling.

EQUIPMENT AND MATERIALS

- 78 volumetric flasks, 1 litre, each with a neck as narrow as possible, an accurately fitting stopper, and a volume of 10 ml **above** the mark on the neck (the volume of the flask depends on the size of the sample necessary for the analysis, which is usually 1 litre)

or

- 78 conical flasks, 2-litre (see 3.5.2, Fig. 1) (Note 1)
- working room at $30 \pm 1^\circ\text{C}$
- thermostatic bath adjustable to 80°C and to 100°C
- 10 round-bottomed flasks, 2-litre
- 10 reflux condensers
- light source(s): cooled immersion lamp(s), e.g. high-pressure mercury lamp with the reaction vessel (3.4, Fig. 1) or ultraviolet lamp(s) with heat filter(s)
- 8 quartz flasks, approximately 1½-litre
- buffer solutions at pH 2, 4, 6, 8, 10, and 12 (Rauen, 1956)
- for sterilizing glassware and solutions:
 - an autoclave
 - a pressure filter
- injection syringes with a filter device
- for checking sterility:
 - nutrient media (2.4.4.1)
 - 8 pipettes
 - 80 Petri dishes
- lighter-than-water extractant.

Depending on the nature of the substrate, the following analytical apparatus may, for example, be used (Note 2):

- gas chromatograph
- infrared spectrophotometer
- ultraviolet spectrophotometer
- combination of gas chromatograph and mass spectrometer.

INTRODUCTION

A compound that enters into the environment is exposed to one or more of the following influences:

- water (moisture)
- acid or alkali
- air
- light
- (elevated) temperature.

The influence of biological factors is left out of this list because this is discussed in Section 3.5. The conditions that can cause the substance to change chemically are usually gentle in the environment.

The pH will be between 6 and 8. In an aquatic medium the temperature will rarely exceed 30°C and the substance will frequently be exposed to little light in the active region (280–320 nm) because of the adsorption of light by humus-like materials. The test conditions for the method described here, at their extremes, go far beyond the above limits. This is because chemical transformations are often so slow under environmental conditions that they are only measurable under more extreme conditions. The method gives in the first instance an indication of the processes that may occur. The rates actually occurring in the environment can then be estimated from the values obtained.

The results of these experiments also indicate what risks are associated with certain (analytical) processes necessary for the determination of biological degradability.

The following two examples are given as an illustration:

- Trichloroacetic acid is stable in an aquatic medium under the conditions of a microbiological experiment. When nutrient solutions containing trichloroacetic acid are sterilized in an autoclave, this trichloroacetic acid decomposes with the formation of CO_2 and CHCl_3 . Both these components escape from the nutrient solution. The transformation is almost complete in 20 min at 120°C .
- Under the influence of alkali, 1,1,2-trichloroethane (TCE) is transformed into 1,1-dichloroethane (DCE). 3.4, Table 1 gives the results of a 6-day experiment in which the influence of the pH of a TCE solution (1000 mg.l^{-1}) on this transformation was investigated. The pH was adjusted with NaOH. The two components were analysed by gas chromatography. The figures in the table were estimated on the basis of the chromatograms.

3.4 Table 1 Influence of the pH of water on the chemical conversion of TCE into DCE

pH	Relative peak height of TCE	Relative peak height of DCE
6	1	n.d.*
9	1	n.d.*
11	0.5	about 0.3
12	trace	about 0.3
15	n.d.*	1

* n.d. = not detectable.

Since TCE is volatile, it is impossible to determine e.g. the respiration of microorganisms by manometric techniques (in such techniques a basic adsorbent is used for the carbon dioxide formed).

PROCEDURE

In view of the extent of this test, a preliminary test may first be performed. The procedure proper can then be started (Note 3). This procedure comprises the following parts:

- I. determination of chemical transformations caused by the nutrient solution or 'natural' water
- II. determination of chemical transformations due to acidic or alkaline media
- III. determination of chemical transformations due to an elevated temperature
- IV. determination of chemical transformations due to light
- V. determination of chemical transformations due to oxygen.

Preliminary experiments

- (1)
 - Sterilize 14 volumetric flasks in an autoclave (120°C, 30 min). The flasks and the stoppers are sterilized separately. Close each flask with a plug of cotton wool and pack each stopper separately in filter paper with aluminium foil around it. Also sterilize two quartz flasks if the irradiation is to be performed in daylight.
- (2)
 - Prepare the following buffered solutions: 0.1–1.0 mg substrate.l⁻¹ in nutrient solution, fresh water and sea water (Note 4):
 - a. 3.5 litres of solution with pH 2
 - b. 3.5 litres of solution with pH 4
 - c. 3.5 litres of solution with pH 6*
 - d. 3.5 litres of solution with pH 8*
 - e. 3.5 litres of solution with pH 10
 - f. 3.5 litres of solution with pH 12
 - g. 15 litres of solution buffered to the actual pH of the nutrient solution or the water.

* The pH closest to that of the nutrient solution or the water under investigation is adjusted to that value, e.g. if the pH of the water is 6.8, solution c is adjusted to 6.8 instead of 6.

- Sterilize the above solutions by filtration (Section 3.3.3) and collect the filtrate in the sterilized flasks. The solutions may be sterilized successively with the same filter, but then for each new solution the first litre of filtrate is discarded.

- (3)
 - With each of the solutions a to f, two 1-litre volumetric flasks are filled as close as possible to the mark; they are closed with stoppers. (In view of the sterility requirement, no corrections can be made later). The stoppers may only be handled by the upper edge. The operations must be carried out in a clean, draught-free, and preferably dust-free room not contaminated with micro-organisms derived from other biological tests (e.g. with activated sludge). After being closed, each flask is shaken once to moisten the ground joint.

- (4)
 - The flasks are stored in the dark at 25°C; after 2 weeks and 4 weeks for each pH value the contents of one flask are analysed. Its sterility and pH are checked. Directly after the flask has been opened, take 0.1 ml of the liquid with a sterile pipette and spread the liquid on one of the agar media (described in Section 2.4.4.1). Incubate the plates at 25°C for at least 1 week. If growth occurs, the test must be repeated.

- (5)
 - Perform an analysis of the contents of the flasks (4) (e.g. according to one of the methods given in Chapter 2). When an extraction is necessary for this purpose, the following procedure must be used:

For dissociable compounds, adjust the pH so that the compounds are in the undissociated form.

Add 10 ml of a suitable extraction liquid (lighter than water) to the volumetric flask, close the flask, and shake it for one hour in the horizontal position in a shaker.

Allow the layers to separate and take off the extract (with a pipette). This can now usually be introduced directly into the gas chromatograph.

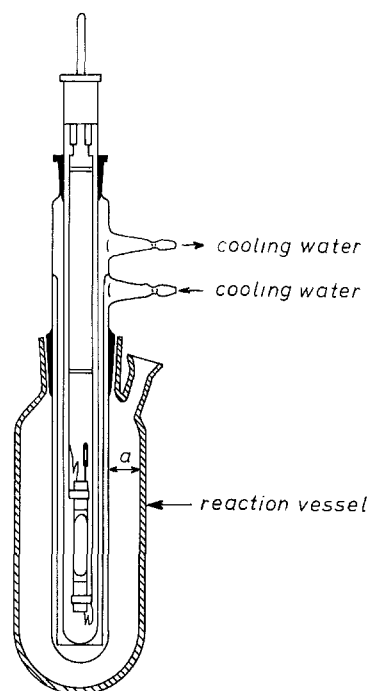
During the whole procedure keep the flask closed as much as possible to avoid loss of any volatile constituents from the substrate or volatile transformation products. It is recommended to add an internal standard to the extract in the neck of the flask before the analysis.

- (6)
 - The solutions and apparatus need not be sterilized for the determination of the influence of temperature on the substrate. In the following procedure the volatile compounds are lost (Note 5).

Fill four round-bottomed flasks and two conical flasks (capacity 2 litres) with 1 litre of solution g each. Fit the round-bottomed flasks with the reflux condensers and the conical flasks with cotton-wool plugs. Heat the round-bottomed flasks for 24 h, two at 80°C and two at 100°C.

Heat the conical flasks in an autoclave for 30 min at 120°C. Treat the liquids in accordance with the last-mentioned procedure (5).

- (7)
 - Add 1 litre of the sterile solution g. to each of the two sterile quartz flasks. Close the flasks with accurately-fitting stoppers. These flasks must now be irradiated in one of the following three ways:



3.4 Fig. 1 Irradiation apparatus with an immersion lamp. Cooling water or a filter solution can be passed through the feed pipes. The reaction vessel (hatched) can be made in various diameters. The layer thickness (a) may be selected according to the absorption of the test compound. Samples can be taken for analysis through the side-arm

3. Determination of degradability

Irradiation with artificial sunlight

The irradiation period depends on the nature of the artificial sunlight, but 72 h is usually sufficient. Place a heat filter in the path of the radiation. Determine the effective luminous flux.

Irradiation by an immersion lamp with a cooling jacket (3.4, Fig. 1)

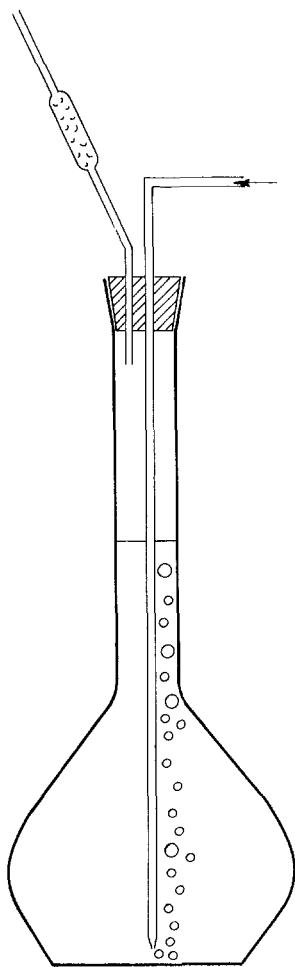
The solution is placed in the vessel belonging to the apparatus. The ultraviolet makes the usual sterilization superfluous. An irradiation time of 48 h is usually sufficient.

Daylight

This method of irradiation is used only if no lamp is available. The flasks are kept in daylight for one month.

After irradiation, the solution is worked up according to (5).

- (8)
- Fill two sterile 1-litre volumetric flasks with solution g (sterile). Saturate the solution with oxygen by passing through it a sterile stream of this gas (50 ml/min for 15 min). The necessary apparatus is shown in 3.4, Fig. 2.



3.4 Fig. 2 Apparatus for the oxygenation of solutions

- Close the flasks (3) and store them at 25°C in the dark for one month.
- Then analyse the contents of the flasks (5).
- Report the results as a % conversion in b days (3.1.5). The results of the analyses according to (5) provide information for the performance of parts I and II of the complete study.

The results of the analyses according to (6), (7), and (8) give information necessary for parts III, IV, and V, respectively.

The complete investigation (Note 3) Parts I and II

- Sterilize 60 volumetric flasks (1).
- (9)
- Prepare the following solutions of 0.1–1.0 mg substrate.l⁻¹ in nutrient solution and fresh and sea water, all buffered (Note 4):
 - a. 11 litres of solution with pH 2
 - b. 11 litres of solution with pH 4
 - c. 11 litres of solution with pH 6*
 - d. 11 litres of solution with pH 8*
 - e. 11 litres of solution with pH 10
 - f. 11 litres of solution with pH 12

* The pH closest to that of the nutrient solution or the water under investigation is adjusted to that value, e.g. if the pH of the water is 6.8, solution c is adjusted to 6.8 instead of 6.

- Sterilize solutions (9) by filtration (Section 3.3.3) and fill 10 sterilized flasks with each of the solutions. Use a new filter for each solution.
- Store the flasks in the dark at 25°C.
- After the following times, analyse the contents of one flask per solution: 1 h, 8 h, 24 h, 2 days, 4 days, 1 week, 2 weeks, 3 weeks, 4 weeks, and 8 weeks (5).

Part III

- Prepare 10 litres of solution g (2).
- Distribute these 10 litres over 10 round-bottomed flasks and heat five of them to 80°C and five to 100°C (Note 5). (The test at 120°C mentioned previously need not be performed again).
- Analyse the contents of one flask heated to 80°C and of one flask heated to 100°C after 1, 4, 8, 24, and 48 h (5).

Part IV

- For the test in daylight or with artificial sunlight sterilize 8 quartz flasks.
- Prepare 8 litres of solution g (2).
- Sterilize the solution and distribute it between the flasks (3).
- Irradiate the solutions (7).
- In the case of irradiation in daylight, analyse the contents of two flasks (5) in each case after 7, 14, 28, and 56 days.
- In the case of irradiation with artificial sunlight, analyse the contents of two flasks (5) after 1, 4, 7, and 14 days.
- Analyse the solutions irradiated with an immersion lamp after 1, 2, 4, and 8 days (5).

Part V

- Sterilize 18 volumetric flasks.
- Prepare 20 litres of solution g (2).
- Sterilize this solution by filtration (3) and fill the flasks.
- Displace the oxygen from 6 flasks with nitrogen. Saturate 6 flasks with air and 6 with oxygen (8).
- Analyse the contents of one flask from each group of six after 1, 4, 8, 16, 20, and 60 days (5).
- Report the results of the various parts as a % transformation in b days (Section 3.1.5).

3.4 Method for the determination of chemical degradation

NOTES

1. Conical flasks are used when, for example, the analytical method selected requires such high substrate concentrations that the substrate is no longer miscible with the aqueous phase. The conical flasks can be placed in a shaker so that good contact between the phases in the emulsion is maintained. When 'volumetric flasks' appears in the above instructions, this must in that case be read as 'conical flasks'.
2. Apart from instrumental methods, in some cases use can also be made of reactions for functional groups.
3. On the basis of the preliminary experiment it must be considered

whether other experiments need be performed. When, for example, effects are found only in an alkaline medium, only this part of the investigation should be pursued. When the results of the preliminary tests are clear enough, no further tests need be performed.

4. The substrate concentration is determined, among other factors, by the selected analytical method and its sensitivity. The limits given apply to the gas-chromatographic analysis.
5. If the substrate contains volatile compounds, the operations can also be carried out in a closed stainless-steel vessel or in a fusion-sealed well-screened glass ampoule.

3.5 METHODS FOR THE DETERMINATION OF BIOLOGICAL DEGRADATION

3.5.1 Determination of the non-toxic concentration

Degradation tests must be carried out with a concentration of the test substance that is not toxic to the degrading organism. The three toxicity tests described here are therefore used only to determine the permissible maximum concentration for the degradation test. Furthermore, the conditions of these toxicity tests should be as close as possible to the conditions of the degradation test that is being envisaged. Many toxicity tests are suitable for this purpose, but in practice at least one of the three toxicity tests described here will probably prove applicable.

These three tests may also be used in the actual investigation of toxicity (Chapter 5). This applies particularly to the tests with pure cultures (Section 3.5.5.1); the many variations of this test described in the literature have precisely this aim in view.

The choice between the three tests is determined by the nature of the test compound, by the microbiological system that brings about the degradation, and of course by the equipment available. For example, a compound that interferes with the bacterial growth test (Section 3.5.5.1) because of its strong light absorption must be tested by one of the other two methods.

If a natural water is used for the degradation test it is difficult to use the bacteria present in it for toxicity tests, and indirect parameters such as growth or oxygen uptake must be measured on a degradable substrate in the presence of the toxic compound. Since adaptation to the toxic compounds appears in this type of test, it is frequently necessary to use a lower concentration in the degradation test than a 'no-effect level' given by the toxicity test.

We believe that, of the three tests described here, the determination of ATP (Section 3.5.1.3) is generally the most relevant for natural water, while the influence on the growth of pure cultures (Section 3.5.1.1) is in this respect the least relevant test. However, it appears from our experience with tests on chlorinated phenols that in this case the results of the three methods of testing do not differ greatly.

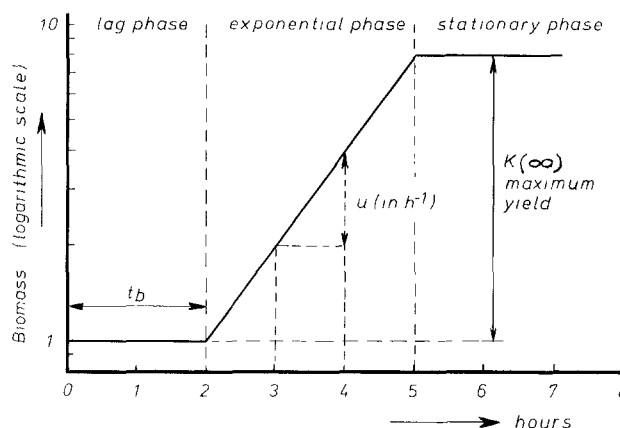
Before passing to a description of the procedures we shall first describe the three methods generally and in mutual relationship.

Inhibition of the growth of pure cultures as a test criterion

This procedure is based on the method of measuring bacterial growth described by Ellinghausen (1959) and its use in toxicity tests described by Breazeale and Camper (1972). These investigate the influence of toxic substances on the growth of pure bacterial cultures. The bacteria are cultured in a conical flask. The measurement of the growth is simplified by using a flask with a side-arm that fits into the cell housing of the spectrophotometer. In this way, the growth can be followed directly by measurement of the turbidity without the conical flask having to be opened to collect a sample.

The aim of the method described in this procedure is to determine the influence of toxic substances on the three parameters that characterize growth: the length of the lag phase, the rate of the exponential growth, and the total yield of cell material (3.5.1, Fig. 1). Various methods have been described (among others by Bringman and Meinck, 1964; Griffith, 1970; Schubert, 1973; and Breazeale and Camper, 1972), but only for the determination of one of the three parameters.

The procedure described here is applicable to each combination of bacterial species and nutrient solution that the investigator considers necessary. It is based on examples of *Pseudomonas fluorescens* as the test organism and the use of a mineral medium with glucose as the source of carbon. It should be noted once again that the method does not aim at predicting the effect of the test substances on the environment.



3.5.1 Fig. 1 Schematic representation of a growth curve of a microorganism with the three growth phases, and the three characteristic parameters each of which can be changed by the addition of toxic substances

Oxygen uptake as a test criterion

The oxygen uptake can be followed by means of a Sapromat, an apparatus for the determination of the biochemical oxygen demand (BOD) of waste water. The pressure of oxygen above a growing culture of microorganisms is kept constant, and the supply of oxygen necessary for this purpose is measured continuously (for a description see Section 2.7.1).

The Sapromat can also be used for the determination of the influence of toxic substances on the BOD of water samples (Deutsche Einheitsverfahren L12, 1971). It must be assumed that at a non-toxic concentration the course of the oxygen-uptake curve will not differ appreciably from that for the blank.

This test is much more reliable than, for example, an evaluation by means of a BOD₅ measurement, which gives only one point on the curve. This may be misleading because of the very different courses that the oxygen-uptake curve may follow.

Furthermore, much higher substrate concentrations can be used than in the classical BOD determination, because the oxygen concentration is kept constant during the measurement. In the determination of the influence of toxic substances on the growth of heterotrophic bacteria with the Sapromat, peptone is generally used as the nutrient substrate (Liebmann and Offhaus, 1966; Liebmann, 1967/68; Offhaus, 1969; Reimann, 1969; Verstraete *et al.*, 1974). The method in Section 3.5.1.2 uses a different nutrient.

The test with the Sapromat is considered more relevant for the aquatic environment than a toxicity test with a pure culture (Section 3.5.1.1). Where this is the case, natural water containing its naturally occurring microorganisms is used as the medium. A nutrient (carbon source) is added to the water. If it is desired to standardize the nutrient solution as well as possible, a synthetic mineral medium is used. This may be inoculated with, for example, a soil extract (see also Section 3.3).

The influence of an added amount of toxic material on the course of the oxygen-uptake curve is measured. The BOD curve determined in a Sapromat is due to the growth of microorganisms and has the same shape as the growth curve in 3.5.1, Fig. 1 (Gaudy, Jr., 1972).

When a substance toxic to some species in the microflora is added, selection of microorganisms resistant to this material may occur. The test in the Sapromat therefore usually gives higher effective inhibiting concentrations than the test with pure cultures, in which this type of selection is impossible.

The determination of ATP as a test criterion in 'natural' systems

The concentration of ATP is used increasingly as a measure of the amount of living biomass in microbial ecosystems (see Section 2.7.2).

However, the determination of ATP has so far been little used for the toxicity tests.

The only publications in this field derive from an investigation of new criteria for the control of sewage purification plants (Patterson *et al.*, 1970; Brezonik and Patterson, 1972). Clear concentration-effect relationships were found between the concentration of a toxic compound, e.g. a mercury salt, and the ATP content of activated sludge.

In a later publication, Brezonik *et al.* (1975) used the ATP concentration of algal cultures for the determination of toxicity. The fall in the concentration of ATP in a phytoplankton culture caused by the addition of mercury salts is then a measure of toxicity of the mercury salts at the concentration used.

The method described in this procedure has the aim of determining the toxic effect on the microflora in natural water. Little can be said about reproducibility, seasonal variation, and comparability with other toxicity tests in use, although in the case of chlorinated phenols as toxic substances approximately the same results were found as with the methods reported in Sections 3.5.1.1 and 3.5.1.2. It must also be borne in mind that the luciferin/luciferase enzyme system used to measure the ATP concentration may itself be very sensitive to toxic compounds. Consequently, in addition to the test with natural water a control test is performed in which the test compound is added to water that has passed through a bacterial filter. After the extraction of ATP from this control sample a known amount of ATP is added to the extract. In this way the influence of the toxic substance on the measurement of ATP with enzyme systems can be investigated as such.

3.5.1.1 Inhibition of the growth of pure bacterial cultures as a test criterion

Subject

Determination of the influence of toxic compounds on the growth of pure cultures of bacteria.

Field of application

a. General test for the determination of a toxic effect on bacteria.

b. Evaluation of the toxicity of compounds investigated in biodegradability tests.

The method is limited to substances or wastes that do not interfere with the test by imparting turbidity or colour to the medium.

Principle

Pure bacterial strains are grown in shake cultures in the presence of various concentrations of the toxic compounds. The bacterial concentration is calculated from the optical density of the culture measured in a spectrophotometer. The change in the concentration with time in relation to the blank provides a measure of the toxicity.

EQUIPMENT AND MATERIALS

Media

a. Mineral medium:

MgSO ₄ · 7H ₂ O	0.2 g
Citric acid · H ₂ O	2.0 g
L-Arginine	0.5 g
Glucose · H ₂ O	5.0 g
NH ₄ Cl	1.0 g
NaNH ₄ PO ₄ · H ₂ O	3.5 g
K ₂ HPO ₄	10.0 g

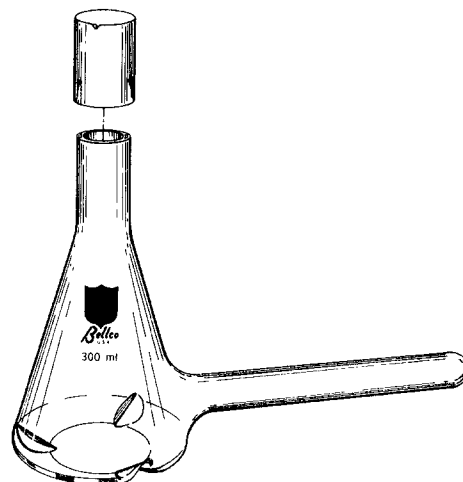
dissolved in 1 litre of distilled water.

b. Agar medium:

Mineral medium with 1.5 % agar.

The pH is adjusted to 7.2 and the medium is sterilized for 30 min at 110°C.

- culture tubes (150 × 180 mm)
- a culture of *Pseudomonas fluorescens* (ATCC 13525) or of another bacterium suitable for this investigation
- conical flasks, 300 ml
- conical flasks, 300 ml, each with ground-in stopper or screw-cap and side-arm (12 × 130 mm; 3.5.1.1, Fig. 1); fitting, for example, in a Bausch & Lomb Spectronic or a Vitatron spectrophotometer (Note 7)
- volumetric flasks, 100 ml and 1000 ml
- pH meter
- filtration equipment for sterile filtration, diameter 50 mm
- membrane filters, pore size 0.2 µm, diameter 50 mm
- 2N aqueous solutions of NaOH and HCl
- pentachlorophenol and sodium dichromate
- spectrophotometer (e.g. Bausch & Lomb Spectronic, Vitatron, etc.)
- orbital shaker (temperature range 20–50°C)
- autoclave
- sterile pipettes, 10 ml
- plunger pipette with disposable tips, 0.1 ml and 1 ml
- silicone rubber sponge caps
- dimethyl sulphoxide (DMSO, spectroscopic purity).



3.5.1.1 Fig. 1 Conical flask with a side-arm for the spectrometric monitoring of the growth of a bacterial culture

PROCEDURE

Preliminary preparations

Filling of the test flasks

- Carry out the tests in special flasks as in 3.5.1.1, Fig. 1.
- Fill the flasks with 40 ml of the medium, close them with silicone rubber sponge caps, and sterilize the flasks in an autoclave (30 min at 110°C).
- If volatile compounds or compounds that may co-distill with

3. Determination of degradability

water are being tested, close the flasks with sterile ground-glass stoppers (or screw-caps) (Note 1).

Preparation of solutions with the desired concentrations

- From substances readily soluble in water or from liquids readily miscible with water make sterile aqueous stock solutions 50 times as concentrated as the concentrations desired in the tests.
- From substances sparingly soluble in water, make stock solutions in DMSO 500 times as concentrated as the concentration required in the tests.
- Add 1 ml of the stock solution in water or 0.1 ml of the stock solution in DMSO to the test flasks shortly before the experiment begins (Note 2).
Check the pH and if necessary adjust it to 7.0 ± 0.2 by adding NaOH or HCl.
- Dilute waste waters with distilled water to the desired dilution. Prepare the medium to be used in the test with this dilution in place of the distilled water mentioned in the methods and sterilize these solutions by filtration.
- Choose the test concentrations or dilutions in a geometrical series (for example, 1; 3.2; 10; 32 and 100 mg.l^{-1}) and in such a way that the most concentrated solution gives a complete or at least a distinct inhibition of growth, and the least concentrated causes no measurable inhibition, compared to the blank (see Section 5.2.2).

Stock culture

- Maintain the bacterial strain used in culture tubes on agar nutrient medium having the same composition as that used in the test.
- Incubate for one or more days at the optimum temperature for the bacterium (for *P. fluorescens* this is 26°C).
- Use this culture to prepare the inoculum, which will be used to inoculate the pre-culture.

Pre-culture

- Inoculate a number of flasks (see 3.5.1.1, Fig. 1) with bacteria from the stock culture so that the medium becomes slightly turbid (Note 3).
- Incubate the flasks in a shaker at the optimum temperature for the bacteria and at 300 rpm. (The optimum temperature for *P. fluorescens* is 26°C).
- Measure the growth by determining the extinction E at 650 nm at intervals of an hour (Note 3).
- Stop the incubation at the end of the exponential phase. (For *P. fluorescens* this is at $E \approx 0.8$). (See Note 4).
- Use this bacterial suspension to inoculate the test flasks (see Note 5).

Performance of the test

- Carry out the test in duplicate.
- Carry out the test with pentachlorophenol and/or potassium dichromate as standard test substances.
- Inoculate a number of flasks with an amount of bacterial suspension that gives an initial concentration in the test of $E \approx 0.1$ (in the case of *P. fluorescens* this is 6 ml of a culture with $E \approx 0.80$).
- For each compound test at least 5 concentrations in the selected range, together with a blank to which no test compound has been added. The range of concentrations may be based on the results of an over-all toxicity test (see Section 5.2, Table 1).
- Incubate the flask at the optimal temperature for the test organisms in the shaker.

- Measure the absorbance until the growth in the blank flask has reached the stationary phase, preferably as long as is necessary to obtain a complete growth curve for the flasks containing the test compound.

The time between two measurements is usually three-quarters of an hour. It may be shorter with fast-growing bacteria and somewhat longer with slow-growing ones. The test duration is about 5 h.

Treatment of the results

- Tabulate the results and plot the growth curves on semi-logarithmic paper.
- Treat the results in accordance with the procedure given in Section 5.6.1.
- Where possible, calculate the concentration that gives 50 % inhibition (EC_{50}), the concentration that gives no appreciable inhibition, and the lowest concentration at which maximum inhibition occurs.
- Calculate the above three values, where possible, for t_b (the lag phase), V_m (the rate of growth), and K_∞ (the maximum yield of biomass) (3.5.1, Fig. 1 and 5.6.1, Fig. 1).
- From our own investigations, it appears that the growth rate in the exponential phase can be determined with a measurement error of $<5\%$, while the reproducibility (difference between the results of the same type of blank test performed on different days) is about 10 %.
- If it is desired to compare the effect of a compound on different bacterial species, the measurement of E must be correlated with the biomass (= dry weight) (see Note 6).
- The results of the tests with the standard substances are compared with the mean value of earlier results. If the results deviate by more than a factor of 2, the remaining results are discarded.

NOTES

1. Stoppering of the flask affects the growth curve since the amount of oxygen in the flask is limited.
2. It is assumed that the solubility in water and volatility are known (Sections 1.4.1 and 1.4.2).
If the test compound is added in the form of a stock solution in DMSO, care must be taken that the solubility limit of the compound in water is not exceeded. Furthermore, a corresponding amount of DMSO must be added to an extra blank flask.
3. The extinction E is measured at 600 nm. The absorbance A is defined as $A = \log_{10} (I_0/I)$, where I_0 and I are the intensities of the incident and the transmitted light respectively. The measurement is performed by placing the side-arm in the cell-holder of the spectrophotometer. Great care must be taken that the side-arm always adopts the same position, as small deviations here give large variations in the reading.
Since the cell-holder is opened during the measurement, the measurement can be disturbed by external light. The flask and the cell housing must therefore be protected with a dark cover during the measurement.
The nutrient solution without bacteria is used as the blank for the measurements, but it must be checked beforehand whether the test substance exhibits no interfering absorption at 650 nm.
4. There are three methods of preparing the inoculation material for the test. The choice depends on individual preference.
 - a. The growth curve is assumed to be known, and the test is planned in such a way that the pre-culture is in a suitable growth phase when the toxicity test is begun.
 - b. The growth curve is recorded the day before the actual toxicity test, and when the culture reaches the appropriate growth

3.5 Methods for the determination of biological degradation

phase the culture is stored at 4°C until the test can begin.

- c. If a bacterial species cannot grow fast enough in a given time to give sufficient inoculum, a suitable inoculum can be made by centrifuging under sterile conditions and producing a concentrated bacterial suspension.
5. In each test carried out an extra number of blank flasks may be inoculated. As soon as these are in the suitable growth phase they are stored at 4°C until the next day to serve as inoculum for the next test. They can reasonably be stored for at least 24 h.
6. The dry weight of bacteria is determined by filtering 10-ml samples of bacterial suspension through 0.1-µm membrane filters with a constant dry weight which have previously been dried for 1 h at 105°C and then weighed. After filtration, the filters are dried at 105°C for 24 h and are then re-weighed.
7. Flasks that fit a number of spectrophotometers are available commercially.* If necessary, they can be made by a glass blower.

3.5.1.2 The use of growth, measured as oxygen uptake, as a test criterion

Subject

Determination of the influence of toxic compounds on the growth of aerobic heterotrophic microorganisms that occur in natural water.

Field of application

Determination of the toxic concentration in relation to degradability tests.

This test can be used to determine the toxic effect on microorganisms from a natural environment. All substances that can be brought into aqueous solution or suspension and that do not consume or produce oxygen chemically can be tested.

Principles

The test is performed in a Sapromat or a similar system in which the oxygen uptake can be measured continuously over a long period. It is performed in a nutrient solution with an added carbon source that has been inoculated with a mixed microflora. The inhibition of the growth of this mixed microflora by a toxic substance is determined. The growth is measured as the oxygen uptake.

EQUIPMENT AND MATERIALS

- Sapromat, type B6 or B12 (from Voith GmbH, Heidenheim, Germany).
- nutrient solutions:
 - a. mineral medium according to Stanier (Verma and Martin, 1967)
(see also Section 3.3):

$(\text{NH}_4)_2\text{SO}_4$	1.0 g
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.2 g
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	0.01 g

trace element solution: 1 ml.
The salts are dissolved in 1 litre of phosphate buffer, pH 7.2, 0.02 M, prepared with distilled water. Glucose or another suitable substance is added as a source of carbon.
Trace element solution:

$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	0.5 g
$\text{MnSO}_4 \cdot 7\text{H}_2\text{O}$	0.5 g
CuSO_4	0.1 g
$\text{Co}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$	0.1 g
$\text{Na}_2\text{B}_4\text{O}_7$	0.1 g
$\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$	2.0 g
EDTA-Fe salt	2.5 g

dissolved in 1 litre of distilled water.

- b. natural water (sea water or fresh water) with the addition of 100 mg of NaNO_3 and 20 mg of K_2HPO_4 per litre
- inoculum (only in case a) (see Section 3.2 and Note 1)
 - plunger pipette with disposable tips, 0.1 ml and 5 ml
 - graduated glass cylinder, 250 ml
 - volumetric flasks, 100 ml and 1000 ml
 - glucose
 - glutamic acid
 - DMSO (spectroscopic purity).

PROCEDURE

Preparation of solutions of the test compound

- For the preparation of solutions of the test compound, reference is made in general to Sections 1.4.1, 1.4.2, 3.5.1.1, and 5.2.1.
- From readily water-soluble substances, make stock solutions in water that are 50 times as concentrated as the desired concentrations in the test, and from sparingly water-soluble substances make stock solutions in DMSO that are 2500 times as concentrated as the concentrations desired in the test (for volatile compounds, see Note 2).
- Dilute the waste water or effluents with distilled water to the desired dilution. With this, prepare the nutrient solution to be used in the test in place of the distilled water mentioned in para. 1 of this section (Note 3).
- Choose the concentrations or dilutions to be tested in a geometric series (for example, 1, 3.2, 10, etc.) (see Section 5.2.2) and in such a way that the most concentrated solution gives a complete or distinct inhibition, and the least concentrated solution causes no inhibition, compared to the blank.

Performance of the test

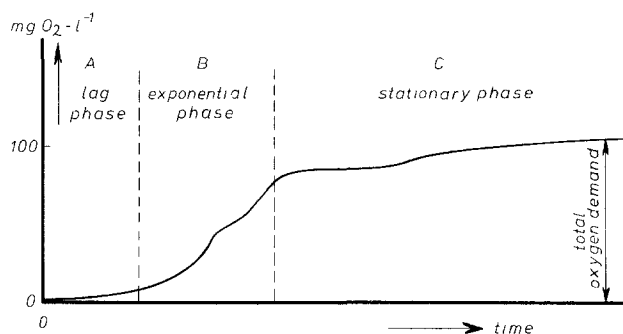
- Carry out the test in duplicate where possible (Note 4). The test arrangement is described here for a single test.
- Choose water (natural sea water or fresh water) relevant for the degradation test.
- For a standard test choose the mineral medium that is inoculated with 1 ml of soil extract (Note 1).
- Fill 5 flasks with 240 ml of water or waste water dilutions (Note 5) to which 150 mg of glucose and 150 mg of glutamic acid have been added per litre (Note 6).
- Fill 1 flask with 240 ml of the medium without the addition of glucose and glutamic acid (this serves as a **control** for the determination of the oxygen uptake or oxygen production due to the presence of the test compound).
- Prepare 4 different concentrations of the test compound by adding to 4 flasks containing the medium (+ glutamic acid and glucose) 5 ml of an aqueous stock solution of the test compound or 0.1 ml of a stock solution in DMSO.
- Add water or DMSO as the case may be to one flask with glucose and glutamic acid (the blank).
- Prepare a low concentration of the test compound or of the waste water in the flask without glucose and glutamic acid (control) (Note 7).
- If waste water is being tested or a standard test is being performed with mineral medium, inoculate with 1 ml of soil extract.
- Make up all the flasks to precisely 250 ml.
- Place the flasks in the incubation chamber of the Sapromat and incubate at 20°C for a week or longer (Note 8).

* Bellco Glass Inc., Vineland, New Jersey, U.S.A.

3. Determination of degradability

TREATMENT OF THE RESULTS

The growth curve usually has a form that, according to Gaudy (1972), can be divided into three phases (see 3.5.1.2, Fig. 1 and compare 3.5.1, Fig. 1 and Section 5.6.1).



3.5.1.2 Fig. 1 Example of an experiment in the Sapromat. Growth on glucose and glutamic acid without toxic compound

In phases A and B the bacteria grow on the two carbon sources. The slight inflection in B shows that the two carbon sources are used in succession. In phase C the growth of protozoa takes place. These use the bacteria as food. Phase C can begin with a more or less pronounced plateau.

A toxic substance can affect the growth in various ways:

1. by a change in the lag phase;
2. by changing the growth rate in the exponential phase;
3. by changing the total oxygen demand.

According to our own work, the exponential growth phase and the total oxygen demand are less affected than the lag phase. This is in agreement with the hypothesis that in the lag phase microorganisms adapted to the test conditions are being selected.

The influence on the lag phase can best be expressed as the time necessary for 50 % of the total oxygen uptake of the blank. This point can be read directly from the Sapromat graph or can be calculated with the aid of the method in Section 5.6.1.

If the influence on the exponential growth phase is to be established, the maximum growth rate r_m can be calculated with the aid of the logistic function in Section 5.6.1.

Information from our own work shows that the total oxygen demand does not easily lend itself to the accurate determination of an effect. It is found, in fact, that the oxygen demand in phase C (see 3.5.1.2, Fig. 1), which actually corresponds to the growth of protozoa, is frequently inhibited specifically by the toxic substance.

CALCULATIONS

The theoretical and practical hints for calculating the inhibition due to a toxic compound are given in Section 5.6.1. Two different calculations can be performed: one simple calculation if the test compound affects the lag phase alone and a complicated one if there is also an effect on other growth phases.

Influence on the lag phase alone

- Determine the oxygen uptake of the blank (Note 9).
- From the Sapromat graph read the time required to reach 50 % oxygen uptake for all the flasks.
- In accordance with Section 5.6.1, determine the no-effect level, the concentration that causes a doubling of the lag phase, and

the lowest concentration that causes the maximum inhibition; record these figures in a table.

Influence on other growth phases in addition to the influence on the lag phase

- Make a table with the oxygen uptake in $\text{mg} \cdot \text{l}^{-1}$ against time for all the flasks.
- From the tabulated figures and the formula in Section 5.6.1 calculate t_b (point of 50 % oxygen demand), r_m (the maximum growth rate), and N_∞ (the maximum oxygen demand).
- From this calculate the 'no-effect level', the EC_{50} , and the lowest concentration that causes maximum inhibition (Section 5.6.1).

No estimates have been made of the reproducibility of this determination. In a test under similar conditions a growth curve of reasonably comparative accuracy can be determined.

It is in principle impossible to reproduce exactly the results of these toxicity tests, because each inoculum is different from the one used previously.

There is also an impression that the spread between the duplicates in a test increases with increasing inhibitory effect.

NOTES

1. Preparation of soil extract as inoculum for toxicity tests in the Sapromat (according to OECD, 1971): see Section 3.2.2.
2. Volatile compounds can be tested in the Sapromat, although the Sapromat cannot be regarded as a completely closed system for all volatile compounds. The Sapromat flasks are always closed with PVC tubing and stoppers. Compounds that dissolve in this material and thus can diffuse through it cannot always be tested, unless the concentration in the flask can be monitored during the course of the test.
Care must also be taken to see whether the test compound reacts with the soda-lime in the CO_2 absorber.
For example, it follows from our own investigations with chlorinated alkanes that 1,2-dichloroethane and 1,1,1-trichloroethane remain in fairly constant concentration during the test, while 1,1,2-trichloroethane is rapidly decomposed by the soda-lime. Liquid test compounds may also be pipetted directly into the test medium.
3. If a complete inhibition of the growth of the microflora occurs at a concentration of 10 % of waste water or less, it may be added directly to the nutrient solution in the flasks.
4. The test can be carried out in duplicate only in a Sapromat B12. In a Sapromat B6 the test is carried out singly.
5. If dilutions of waste waters are tested, four flasks with different dilutions are used, while in the fifth flask the mineral medium with the addition of glucose and glutamic acid is used as a blank. In tests with waste waters the inoculation is always carried out with a soil extract.
6. A mixture of glucose and glutamic acid is recommended as a standard for BOD determinations. On this, see Standard Methods for the Examination of Waste and Waste Water (13th ed.), p. 493 (1971).
7. In the flask for checking the oxygen uptake by the test compound or the waste water a sufficient amount of the test compound is added for 20–100 $\text{mg} \cdot \text{l}^{-1}$ of carbon to be present. The concentration tested should preferably not be toxic.
8. The duration of the test depends on the water used and on the effect of the test compound. The test must always last long enough for the maximum uptake of oxygen to have been reached by the blank and by one or two of the flasks containing the test compound. With glucose and glutamic acid as nutrients at the concentrations used here, the oxygen uptake is about 200 $\text{mg} \cdot \text{l}^{-1}$. The duration can be reduced by incubation at 25 °C.

9. When waste waters are tested, the total oxygen uptake may be higher than that in the blank because of degradable components in the waste water. This together with the oxygen uptake by the test compound without the addition of a carbon source (control) provides information on the degradability of the test compound.

3.5.1.3 Use of the determination of ATP as a test criterion in 'natural' systems

Subject

Determination of the influence of the toxic compound on the ATP concentration in microorganisms occurring in natural water.

Field of application

Evaluation of the toxicity of compounds investigated in degradability tests.

Principle

Natural microbial ecosystems (water, activated sludge) are brought into contact with various concentrations of a toxic compound. The ATP concentration is measured, and the change in this concentration as a function of the concentration of the test compound gives a measure of the latter's toxicity.

EQUIPMENT AND MATERIALS

- Natural water or sludge
- conical flasks, 300 ml, with ground-in stoppers or screw-caps
- shaker, temperature adjustment from 0 to 50°C
- plunger pipettes with disposable tips for metering and sample collection, 0.2, 1, and 5 ml
- equipment for ATP analysis (see Section 2.7.2)
- adenosine triphosphate (ATP)
- graduated glass cylinders, 100 ml
- volumetric flasks, 100 ml and 1000 ml.

PROCEDURE

Preliminary preparations

- The water used in the test is taken from the place where the water for the degradation test is also obtained (Section 3.3).
- The water is treated in accordance with Section 3.5.2.
- Solutions of the toxic substances, or dilutions of waste waters, are prepared according to Section 3.5.1.1.
- The test is carried out in 300-ml conical flasks. The flasks are closed with glass stoppers if volatile compounds or compounds that may co-distill with water are being tested.

Performance of the test

- Perform the test in duplicate.
- Fill the flasks with 100 ml of water and add 1 ml (or more) of at least 5 concentrations of the toxic compounds to the various flasks. Flasks containing water without the addition of toxic compound are the blanks.
- Add 1 ml of the highest concentration of the test compound to flasks containing 100 ml of filtered water (control).
- Incubate the flasks on a shaker at 100 rpm and at the temperature of the place of origin of the water (Note 1).
- After incubation for 4 and 24 h take samples for ATP measurements (Note 2).

- Perform extractions of ATP in accordance with Section 2.7.2.
- Add 10 µg.l⁻¹ of ATP in filtered water to the extracts of the control.
- Measure the ATP contents in the extracts in accordance with Section 2.7.2.
- If the ATP measurement of the control gives a significantly lower value than that of the blank with the same added ATP concentrations, control tests must also be carried out with all the concentrations of the test compound.

TREATMENT OF THE RESULTS

- The results of the control test are calculated as percentage inhibition (x) in relation to the blank.
- The test results are corrected in accordance with the formula:

$$C = C' (1 + 0.01x) \quad (1)$$

where C is the corrected concentration, C' the measured ATP concentration, and x the percentage inhibition determined in the control test.

- The C values are plotted against the concentration of the test compound.
- From this graph read the lowest inhibitory concentration or the EC₅₀ and (where possible) the lowest concentration at which maximum inhibition occurs after 4 and 24 h (the ATP concentration is then equal to the detection limit of the method of determining ATP) (see Section 5.6.1).
- The measurement error of this method, i.e. the difference between the duplicates, is usually within the measurement error of the ATP-determination method itself. This last measurement error depends on the concentration of ATP in the water sample. No information is available on the reproducibility.

NOTES

1. If the temperature of the water at the sampling site cannot be used, the incubation is carried out at 25°C for fresh water and 15°C for sea water.
2. The times of 4 and 24 h have been chosen arbitrarily, and the time of incubation for each type of water must be based on a measurement of the change in the ATP concentration with time. The times given above were chosen so that the test can be performed in two days. The addition of the test compound and the sample collection must be organized in such a way that the incubation time is the same for all samples.

3.5.2 Die-away test with shake flasks

Subject

Determination of biological degradability with a natural microflora.

Field of application

The die-away test is a general test for the determination of degradability by a natural microflora. There are no limitations on the choice of the test compounds, analytical methods, and test medium, except that the test compound must be added in such a low concentration that its toxic effects on the microflora are negligible.

Principles

The test compound is added to a natural water or to a mineral medium inoculated with a mixed microflora. The concentration of the test compound is monitored during incubation.

3. Determination of degradability

EQUIPMENT AND MATERIALS

- Shaker (or shaker with a thermostat in which the temperature can be adjusted to 0°C)
- cupboard or other constant temperature room
- conical flasks (for standard tests, 300 ml, with ground-in stoppers or screw-caps)
For different volumes, see procedure
- special stoppers for sampling volatile compounds (3.5.2, Fig. 1)
- plunger pipettes with volumes of 0.2, 1, and 5 ml, with sterile disposable tips
- syringe with filter attachment (Millipore) for sterilizing small volumes (possibly radioactive) of the test compound
- sample bottles
- alkali, acid, or a deep-freeze cabinet (-20°C), depending on the method of analysis, for preserving the samples
- natural water (see Sections 3.2 and 3.3)
- mineral medium (see Section 3.3)
- microbiological inoculum (see Section 3.2)
- filter apparatus for membrane filters
- membrane filters, pore size $0.45\ \mu\text{m}$
- HgCl_2 solution, 10 %
- peptone
- bacterial media (see Section 2.7.4.1)
- equipment and materials for the analysis of water samples.

PROCEDURE

Preliminary preparations

- The Introduction (Section 3.1) is assumed to have been read.
- The solubility (Section 1.4.1), the volatility (Section 1.4.2), and other properties of the substance are assumed to be known.
- Perform toxicity tests with bacteria in accordance with one or more of the methods described in Section 3.5.1.
- Choose for the test at least three concentrations (e.g. 0.1, 1, and $10\ \text{mg.l}^{-1}$) 10 to 1000 times lower than the EC_{50} determined in the toxicity test. The purpose of testing different concentrations is to avoid toxic effects of the test compound that may still occur and, moreover, to check whether any limitation of nitrogen compounds and/or phosphate may also occur.
- Use preferably, and where possible, radioactively-labelled test compounds. If this is not possible, a general method of analysis is used (TOC, 2.3.2).
- In all the tests measure the biological activity (ATP: Section 2.7.2, or bacterial counts: Section 2.7.4.1).
- Choice of the test flasks
The test is usually carried out in 300-ml conical flasks containing 100 ml of water. This is sufficient for taking ten 5-ml samples. This sample volume is used in the case of radioactive substrates and in TOC measurements.
If the analytical method requires a larger sample volume than half the volume in the test flask, larger conical flasks must be used. The volume ratio between the liquid and the conical flask must be maintained at 1:3. A second possibility is to use a larger number of 300-ml conical flasks. The whole contents of one flask are then used for each analysis. The flasks are normally closed with a plug of cotton wool (Note 1).
When volatile substances are tested, suitable glass stoppers of

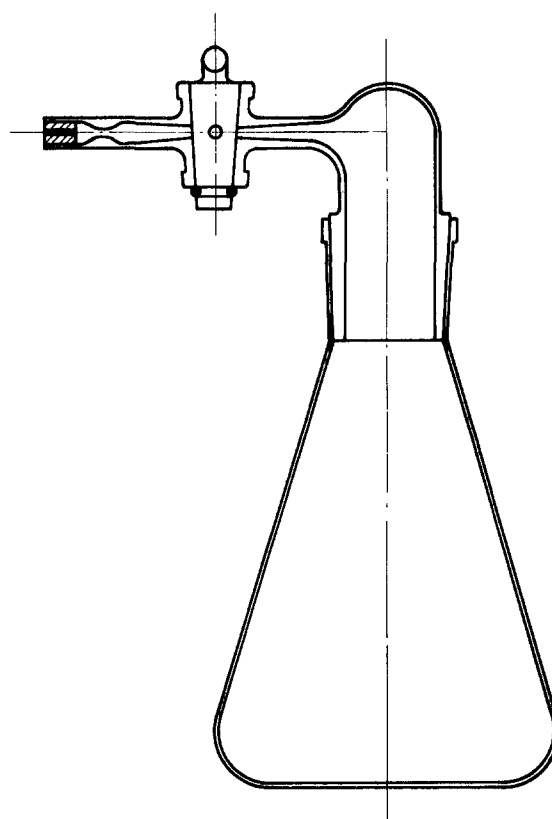
a special design are used, so that sampling with an injection syringe becomes possible (3.5.2, Fig. 1).

When radioactive substances are tested the same kind of stopper can be used, but if the whole contents of this flask are to be used for the analysis (e.g. volatile radioactive substances), a normal glass stopper or a flask with a screw-cap can be used.

- Preparation of stock solutions of the test compound. Prepare stock solutions in distilled water 100 times more concentrated than is necessary for the test. Add 1 ml of this solution per 100 ml of water.

If the test compound is sparingly soluble, stock solutions with lower concentrations may be prepared in the test medium or in filtered sea water or fresh water. Here the concentration in the stock solutions must be at least 10 times the concentration desired for the test.

The test compounds can also be added directly to the test medium.



3.5.2 Fig. 1 Conical flask with a special provision for determining the degradability of volatile substances. The stopper is provided with a side-tube carrying a tap and a rubber injection cap

Performance

- Perform the test in duplicate (Note 2).
- Test the compound in at least three concentrations in the natural water.
- In addition to the natural water, the following control tests are used:
 - sterile blank, highest test concentration (all concentrations if a radioactive test compound is used) (see 3.4)
 - a flask with natural water or inoculated medium without test compound to monitor the biological activity of the water
 - if the concentration of the test substance in the natural water is higher than $10\ \text{mg.l}^{-1}$ (sea water) one should include an

3.5 Methods for the determination of biological degradation

additional series in which the medium contains an additional 100 mg of NH_4NO_3 and 20 mg of K_2HPO_4 per litre.

- if the concentration of the substance to be investigated is more than 5 mg.l^{-1} , control flasks containing 0.05 % of peptone are used to check co-metabolism (see also the Introduction, Section 3.1).
- After observing the above, fill each of a number of sterile 300-ml conical flasks with 98 ml of water or medium and, where necessary, inoculate with 1 ml of inoculum per 100 ml of the test medium (see Section 3.2 and this procedure, point 2.1).
- After this, add 1 ml of a solution of the test compound (except to the control for biological activity), or dissolve a sufficient amount of the test compound directly in the medium.
- Sterilize the mineral medium of the sterile blank in an autoclave, or sterilize the natural water by filtration, and add 1 ml of the test compound with the aid of a sterile filter syringe (Note 4; Section 3.4).
- If a radioactively-labelled compound is used, 0.25 μCi of the active compound is added (in the case of the sterile blank, use the filter syringe; Note 5).
- After thorough mixing, take samples directly for chemical (radio-active) and biological analysis and close the flask with a plug of cotton wool or – if necessary – with a glass stopper (see Note 6 and point 2.1 of the **preparation** in this procedure).
- Incubate the flasks in two series on a shaker at 25°C and 8°C (Notes 7 and 8).
- Take two analytical samples on day 0, one on the 1st, 2nd, 3rd, 5th, and 7th days, and then on alternate days (Note 9).
- Check the sterility of the blank by streaking 0.1 ml on one of the media described in Section 2.7.4.1 and incubating at 25°C for at least 1 week.
- End the test after a maximum of 2 months.
- Give the percentage degradations (Section 3.1) as functions of time.

Treatment of the results

It is the aim of the die-away test to plot a complete degradation curve. This graph shows the percentage degradation as a function of time. The percentage degradation is calculated by means of the formula (OECD, 1971):

$$\% \text{ degradation} = \frac{C_{(0)} - C_{(t)}}{C_{(0)}} \times 100.$$

Here $C_{(0)}$ is the concentration at the beginning of the test and $C_{(t)}$ is the concentration at time t .

For further discussion of the significance of the degradation curve, reference is made to the Introduction (Section 3.1).

NOTES

1. Culture tube caps or silicone rubber sponge caps may be used in place of the cotton wool plugs. These cause less dust in the test medium than cotton wool. In addition, the silicone rubber sponge caps prevent evaporation. Evaporation is checked by weighing a flask containing 100 ml of the medium before and after incubation.
2. When, for example, non-homogeneous substrates are tested, or when it is found that an inoculum does not readily lead to degradation, the test must be performed in triplicate or quadruplicate.
3. The figure of 10 mg.l^{-1} is applicable to sea water along the Dutch coast. For other locations the concentration at which the test compound is not limiting for the rate of degradation may be different.

4. When sterilization cannot be performed by filtration or heating, it can be carried out by the addition of 1 ml of a 10 % solution of HgCl_2 per 100 ml of the test medium. In the analysis the presence of mercury must be borne in mind.
5. It must be checked that the test compound is not absorbed in the filter of the filter syringe.
6. Samples are taken with sterile pipettes or plunger pipettes (if the test compound does not dissolve in the plastic pipette tip). For radioactive compounds 5 ml is taken and is then treated as in Section 2.6. If the radioactive compound is volatile, the whole contents of the flask are used for the analysis. For TOC analysis 5 ml of the sample is taken and frozen, and for biological analysis 5 ml is used for the ATP determination.
7. The test is carried out at two temperatures for summer and winter conditions. It has been found from our own work that the temperature may affect both the rate of degradation and the amount degraded.
8. Normal laboratory illumination has little effect on the results of the degradation test unless the test compound is photochemically degradable (Section 3.4).
9. The frequency of the analyses can be adapted to the degradability of the test substance if inadequate information is obtained from the prescribed frequency. If the substrate is degraded within 1 week, samples must be taken at least once a day. The described scheme can be followed if the substance degrades in about 20 days. With a longer degradation period an analytical frequency of twice a week is sufficient.

3.5.3 Die-away test with trickling filters

Subject

Determination of biological degradability.

Field of application

The die-away test with trickling filters can be used for the investigation of series of samples composed of compounds from chemically related substances. The samples must be soluble in the medium and must not be volatile. The method can be used for both fresh water and sea water.

Principle

The substance is dissolved in an inorganic medium as the sole carbon source and is recycled by pumping through a gravel column. This medium is inoculated with microorganisms (Section 3.2). The substrate-degrading organisms will grow and settle mainly on the gravel.

Whenever the substrate concentration reaches a constant level, the medium with the substrate is renewed.

By using the trickling filter that has become biologically active in this manner, i.e. a filter that contains an adapted microflora, it is possible to determine the biological degradation of several substrates in a short time if the constituents of one substrate belong to the same chemical groups as those in the other. This is possible because the microflora that arose in the testing of the first substrate is capable of degrading the related compounds in the subsequent samples. It is therefore necessary to prepare an active filter only once. The duration of experiments with subsequent samples is also shortened because an adapted and concentrated inoculum is present from the very beginning (Jenkins *et al.*, 1967).

EQUIPMENT AND MATERIALS

- Trickling filter(s) (3.5.3, Fig. 1) consisting of:
 - a glass column; length about 70 cm; internal diameter about 3.5 cm
 - a sieve plate

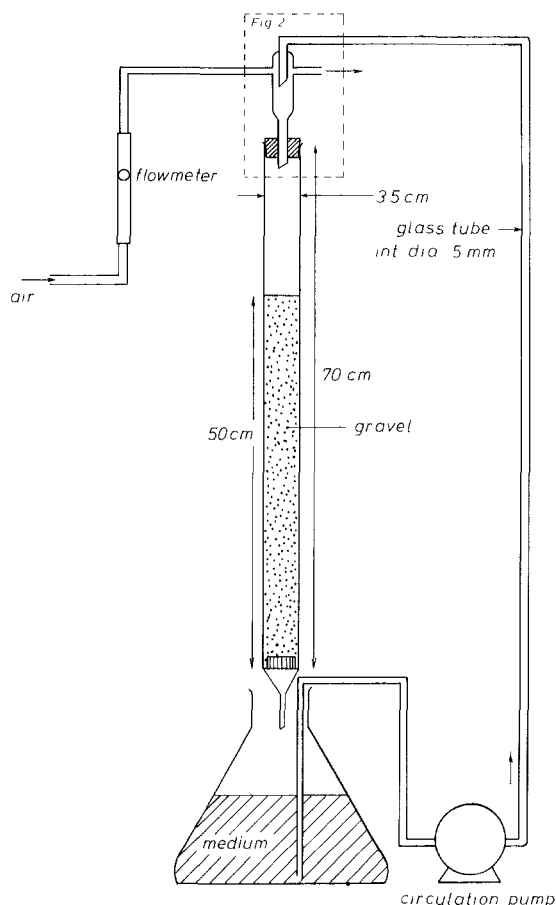
3. Determination of degradability

- a column head (3.5.3, Fig. 2)
- a circulating pump (for example a peristaltic pump with a capacity of about 750 ml.h^{-1})
- a conical flask, 2 litres
- 1.5 m glass tube, internal diameter about 5 mm
- gravel washed with mains water (5–8 mm dia.)
- flowmeter (capacity up to about 25 ml.min^{-1} , for air)
- compressed air and connecting tubing
- medium (see Section 3.3)
- pipette for sample collection (the volume depends on the analytical method selected)
- sample bottles
- for preserving the samples: alkali, acid, or a deep-freeze cabinet (-20°C), depending on the analytical method.

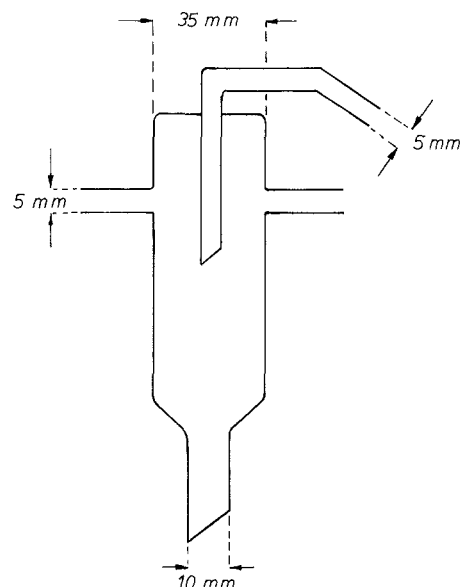
PROCEDURE

To perform the test the information given in the Introduction (3.1) is also required.

- Set up the apparatus as shown in 3.5.3, Fig. 1 and 2.
- Take care that no light penetrates into the apparatus (Note 1).
- Prepare 1 litre of nutrient solution (Section 3.3). Dissolve the substance in it. Transfer the whole to the conical flask.
- Prepare a suitable inoculum (Section 3.2) and add it.

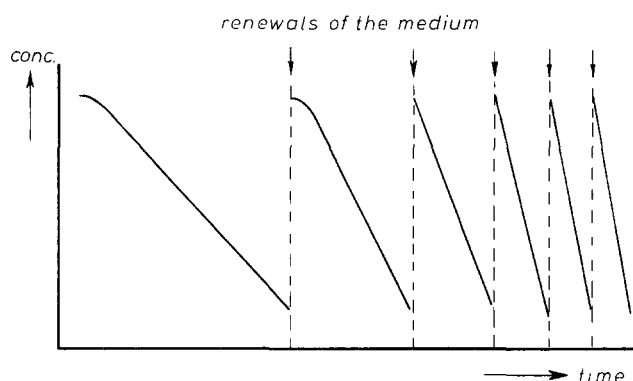


3.5.3 Fig. 1 Schematic drawing of a trickling filter



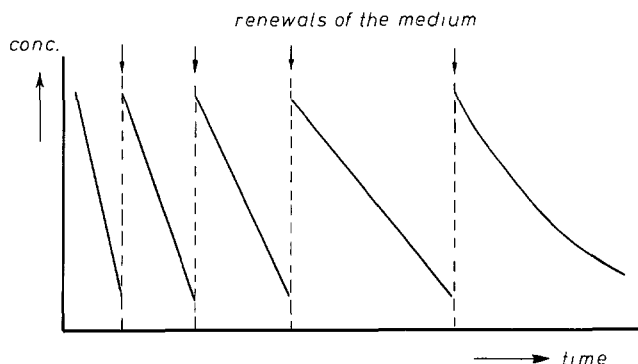
3.5.3 Fig. 2 Detail of a trickling filter: column head

- Adjust the flow of compressed air (see 3.5.3, Fig. 1) to approximately 10 ml.min^{-1} .
- Recirculate the nutrient solution about 18 times per day (in this case 750 ml.h^{-1}).
- Take one sample per day (Notes 2 and 3).
- Renew the nutrient solution (with substrate) whenever the substrate concentration has fallen by more than 90 %, when degradation does not proceed further, or when, because of the removal of samples, the amount of nutrient solution remaining is less than 0.5 litre (Note 3).
- End the test when the results of analyses of several successive renewals are identical.
- Plot the analytical results in a graph, as shown in 3.5.3, Fig. 3 and 3.5.3, Fig. 4.



3.5.3 Fig. 3 Change in the substrate concentration as a result of degradation

Since adsorption can easily take place on the filter surface, this must be distinguished from degradation. The change in the substrate concentration plotted against the time when only degradation takes place is shown in 3.5.3, Fig. 3. The rate of degradation increases with increasing biomass until a steady state sets in.



3.5.3 Fig. 4 Change in the substrate concentration as a result of absorption.

In the case of adsorption (3.5.3, Fig. 4) another situation arises, in which the 'rate of degradation' actually falls off because of saturation of the system.

- Report the degradation as a % in b days, where:
a = percentage degradation between two successive renewals (Section 3.1.5);
b = time between the two renewals.

NOTES

1. Degradability can be affected by light.
Since, because of their variability, natural conditions are difficult to simulate in the laboratory, an apparatus has been chosen in which light is excluded as far as possible.
2. The desired sampling frequency depends on the rate of degradation. In general, one should reckon with six measurement points between two renewals (see 3.5.3, Fig. 3).
3. The size of the sample depends on the analytical method. In the case of large samples the medium must be replaced whenever about 0.5 litre has been used, even when 90 % of the substrate has not yet been consumed.

3.5.4 Determination of degradability in activated sludge flow-through systems

Subject

Determination of biological degradability.

Field of application

The degradability test with activated sludge flow-through systems can be used for studies in fresh water (Note 1). The method is only suitable for testing non-volatile compounds that dissolve in the nutrient solution.

Principles

Synthetic 'domestic' sewage with the substrate to be investigated is fed as an influent to a laboratory apparatus for flow-through experiments with activated sludge. Activated sludge develops in the apparatus. This is separated off from the effluent by settling and is thus retained in the system.

By this procedure organisms that develop slowly and/or cannot grow on the substrate as the sole carbon source continue to exist in the activated sludge.

The degradation of the substrate is calculated from the difference in its concentrations determined in the influent and the effluent.

EQUIPMENT AND MATERIALS

Activated sludge flow-through apparatus (3.5.4, Fig. 1, Notes 2

and 3) consisting of:

- a Perspex 'system tank' (3.5.4, Fig. 2, Note 3)
- an adjustable medium pump (Note 4)
- two storage bottles, 20 litre
- one collecting bottle
- a glass coil (heat exchanger)
- connecting tubing and glass tubing (Note 5)
- a flowmeter (capacity up to about 1000 ml.min⁻¹)
- synthetic 'domestic' sewage (Section 3.3)
- sample bottles
- for preserving the samples, alkali, acid, or a deep-freeze cabinet (-20°C), depending on the analytical method
- for sterilizing the nutrient solution:
 - an autoclave
 - a pressure filter (see Section 3.3.3).

PROCEDURE

The information required for the choice of the test conditions is given in the Introduction (Section 3.1).

- Construct the apparatus in accordance with 3.5.4, Fig. 1 and 3.5.4, Fig. 2 (Note 4).
- Prepare the nutrient solution (Section 3.3).
- Sterilize the nutrient solution preferably by filtration (Section 3.3.3).

- Adjust the flow of influent to the desired value (see Section 3.1.2.7), depending on already known facts concerning the degradability of the substrate. When such information is not available, choose the flow of the influent in such a way that the residence time τ_o defined as

$$\tau_o = \frac{\text{apparatus volume}}{\text{flow rate of influent}}$$

is about 3 h. This residence time was found to be particularly applicable to anionic detergents.

When there is no degradation, tests can be carried out with a still larger value of τ_o (e.g. 30 h).

When the residence time is prolonged, the nutrient solution must be made proportionally more concentrated. In this way the sludge loading (K), expressed as grams of organic material (or BOD) per gram of sludge per day, is kept approximately constant (Section 3.1.2.7).

In general, the substrate concentration must not be increased since, when there is no degradation, the concentration in the system would then also rise.

In the case of toxic substrates this interferes with the test.

- Fill the system with the nutrient solution.
- Adjust the aeration so that good mixing is achieved and maintain it during the test.
- With the aid of the thermostat adjust the temperature to $25 \pm 1^\circ\text{C}$.
- Inoculate the system. Generally speaking, activated sludge will form without inoculation. When there is no degradation, renewed inoculation with a suitable inoculum (Section 3.2), after removal of the activated sludge from the system, may prove successful.
- Sample the influent and the effluent three times a week (for example on Mondays, Wednesdays, and Fridays).
- Check the activity of the activated sludge at these sampling times. For this purpose determine the chemical oxygen demand (Netherlands Standard NEN 3235 5.3) or the total organic

3. Determination of degradability

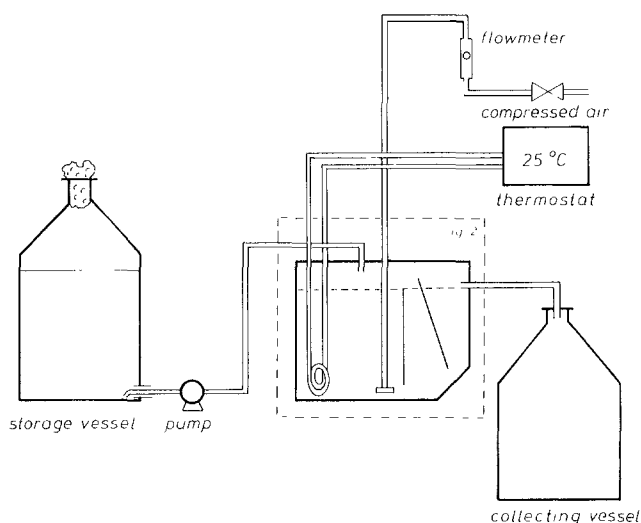
carbon (Section 2.3.2) of the nutrient solution and of the effluent.

- Determine the dry weight of the suspended activated sludge once a week. For this purpose dry a glass-fibre filter (dia. 45 mm) to constant weight at 105°C and weigh it (G_i). Place the filter in a filter holder. Then pipette 50 ml of liquid from the aeration chamber and filter off the solids. Next wash the sludge and the filter with about 25 ml of distilled water. Dry the filter again to constant weight and weigh it (G_s). Calculate the dry weight (D), accurate to 0.1 g per litre, by means of the formula:

$$D = 20 \times (G_s - G_i).$$

Remove the excess of the activated sludge whenever this is more than 2.5 g of dry matter per litre.

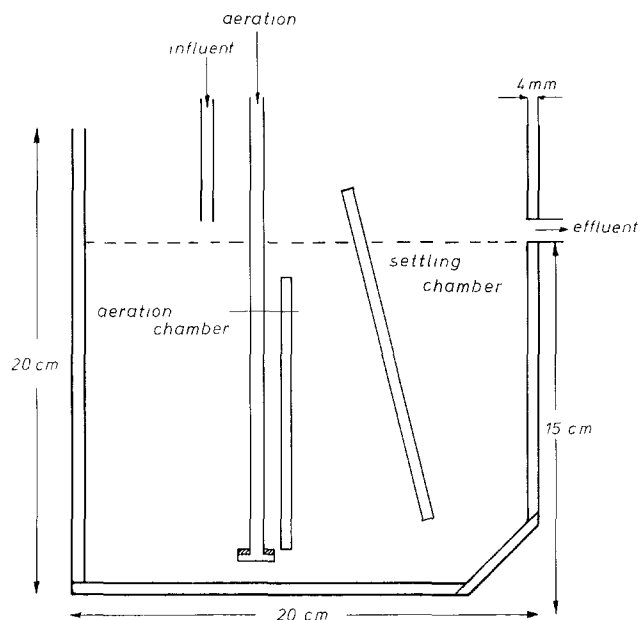
- Determine the substrate concentration in the samples (Section 3.1.4) and calculate the degradation (Section 3.1.5).
- Regard the experiment as ended when the degradation has remained constant during a disturbance-free period of 14 days (steady state). If no degradation had occurred the test may be stopped after 6 weeks if the activity of the sludge and its growth were adequate.
- Report the degradation as a% at an average residence time of b hours, where a is the mean percentage degradation at the steady state (Section 3.1.5).



3.5.4 Fig. 1 Activated sludge flow-through apparatus

NOTES

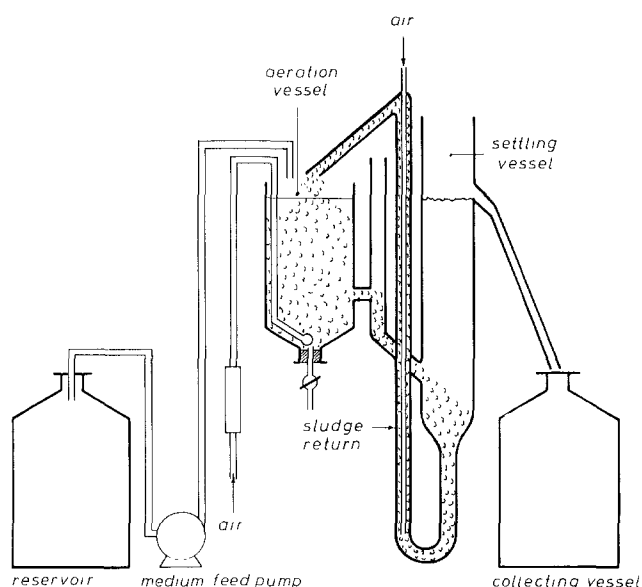
- Experiments with sea water are also possible. Here the artificial sewage is prepared with natural or artificial sea water. Problems occur in the tests because of bacterial growth on the walls of the tank and because of the greater density of sea water, which opposes the settling of the activated sludge.
- This apparatus was chosen because of its simplicity and small dimensions (Klee, 1970). The degradability test can also be performed with other apparatus. Some examples of these, which have been adopted mainly in detergent studies, are given below.
 - On the European continent much use is made of apparatus according to the German standard and the standard accepted by the OECD for the investigation of anionic detergents (3.5.4, Fig. 3).



3.5.4 Fig. 2 The 'system tank' (width 7 cm)

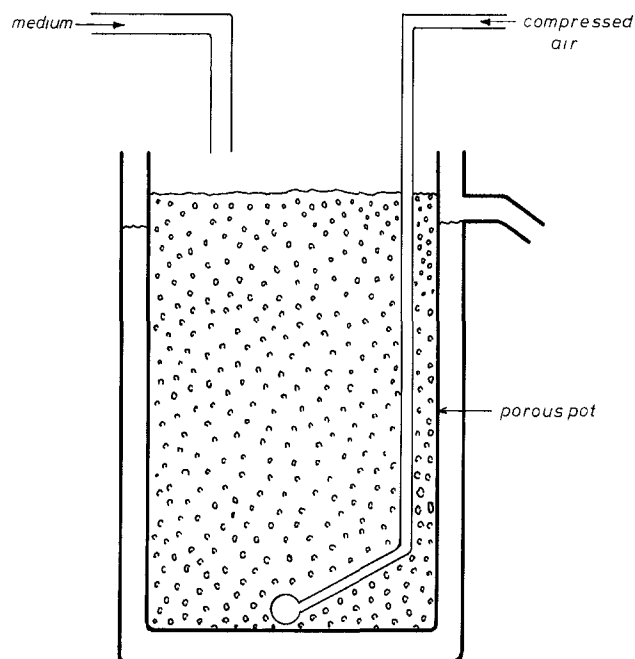
The apparatus consists of a storage vessel, a medium-metering pump, a 3-litre aeration vessel, a settling vessel, a sludge return, and a collecting vessel. The mean residence time in the aeration vessel is set at 3 h. For this purpose 1 litre of synthetic sewage is fed into the aeration vessel per hour. The mean residence time in the total system is, however, about 6 h (Bundesgesetzblatt, 1962; OECD, 1971).

- The so-called 'porous pot' system is in use in the United Kingdom (3.5.4, Fig. 4). The aeration vessel is made in the form of a porous pot so that there is no settling vessel. The nutrient solution is fed into the porous pot and the activated sludge produced is filtered out, after which the effluent flows from the space between the 'pot' and the impermeable outer wall via an overflow.



3.5.4 Fig. 3 Activated-sludge flow-through apparatus according to OECD (1971)

3.5 Methods for the determination of biological degradation

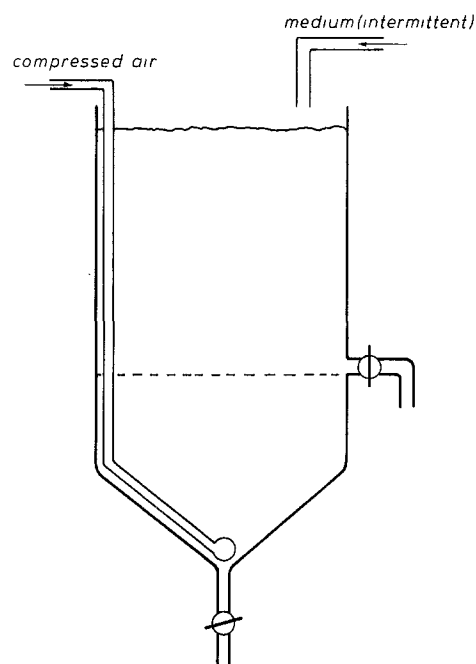


3.5.4 Fig. 4 Porous pot

The advantage of this system is that light sludge remains behind, while in the German system mentioned above it floats out of the settling vessel. A disadvantage is the clogging of the porous pot with sludge. When this occurs, it overflows and sludge is lost. This is prevented by taking a clean pot every week. When using this method and comparing it with others, it must be borne in mind that the activated sludge obtained is substantially different from that in other methods. This refers, for example, to the species composition and the number of bacteria not bound to flocs of sludge (Stennet and Eden, 1971).

- In the U.S.A. the so-called 'draw and fill' method is used widely (3.5.4, Fig. 5).

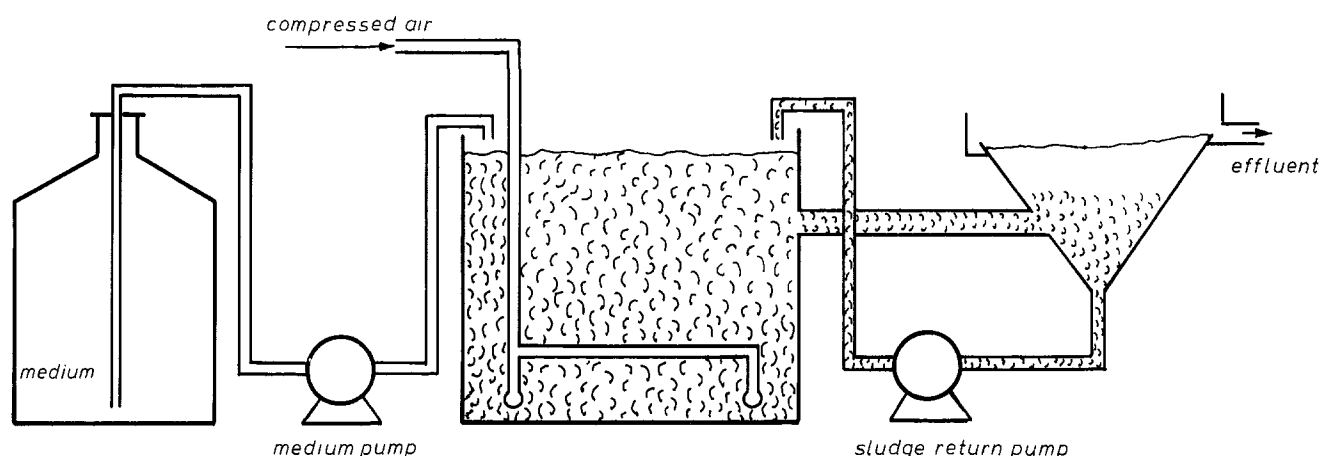
In this method, a vessel is filled with the medium. After 23 h of aeration the activated sludge is allowed to settle for 1 h and approximately $\frac{2}{3}$ of the medium is then run off. The aeration is then resumed and the contents are made up with fresh medium. Because of the great difference in the test conditions (intermittent as compared with continuous metering, and a mean residence time of 36 h as compared with the 3–6 h in the German method) the results obtained by this method can be compared with those of other methods only on the basis of 'degradable



3.5.4 Fig. 5 Apparatus for the 'draw and fill' method

or non-degradable' (Soap and Detergents Association of the United States, 1965; Symons *et al.*, 1960).

- In laboratory investigations on the biological purification of industrial effluents a larger system is often used (3.5.4, Fig. 6). This consists of a 100–200-litre aeration tank and a settling tank. The sludge is recycled by means of an adjustable pump. Because of its size and its possibilities of regulation, such apparatus is suitable for determining the process parameters for the final purification plant. The review given here is not complete. Many other forms are possible, and the choice is often determined partly by personal preference.
- 3. In the method described here the choice has fallen on a system in which the aeration space has a volume of 1.5 litres and the settling space a volume of 0.5 litre. This means that at a mean residence time of 3 h in the aeration chamber 0.5 litre of nutrient solution per hour is used. The advantage of this smaller system as compared, for example, with the OECD system (Note 2) is the saving in apparatus space and consumption of the medium. However, further reduction in size is not appropriate, since pro-



3.5.4 Fig. 6 Basic scheme of a large installation (pilot plant or semi-pilot plant scale)

3. Determination of degradability

blems then arise with the stability of the active sludge as a biological system while, conversely, problems with unstable systems can be solved by increasing the scale.

4. 3.5.4, Fig. 1 shows one feed pump. Since fairly large amounts of nutrient solution can be used (0.5 l.h^{-1}), the nutrient solution has to be prepared every day. Good results have been obtained by the use of two feed pumps. One pump feeds a 10 times concentrated nutrient solution and the other dilutes this with mains water to the desired concentration. In this way it is possible to prepare the nutrient solution only once a week.

5. In the use of tubes for the transport of nutrient solutions, it must be borne in mind that the tubes can absorb compounds from the test substrate. In addition, the tubes may release substances that interfere with the determination.

It is better to use glass as far as possible. The exclusive use of glass is practically impossible (consider, for example, peristaltic pumps). Flow-through systems are also frequently made of Perspex or of transparent rigid PVC. Serious attention must always be paid to the possibility that the use of plastics will affect the test.

3.5.5 Determination of the degradability in chemostats

Subject

Determination of biological degradability.

Field of application

Chemostats can be used for the investigation of degradability in both fresh water and sea water.

It is only possible to work with substrates that are soluble in the nutrient solution or form stable emulsions or dispersions with it.

The substrates may contain volatile components.

Chemostats are suitable to determine the behaviour of low concentrations of substrates in the presence of a small biomass.

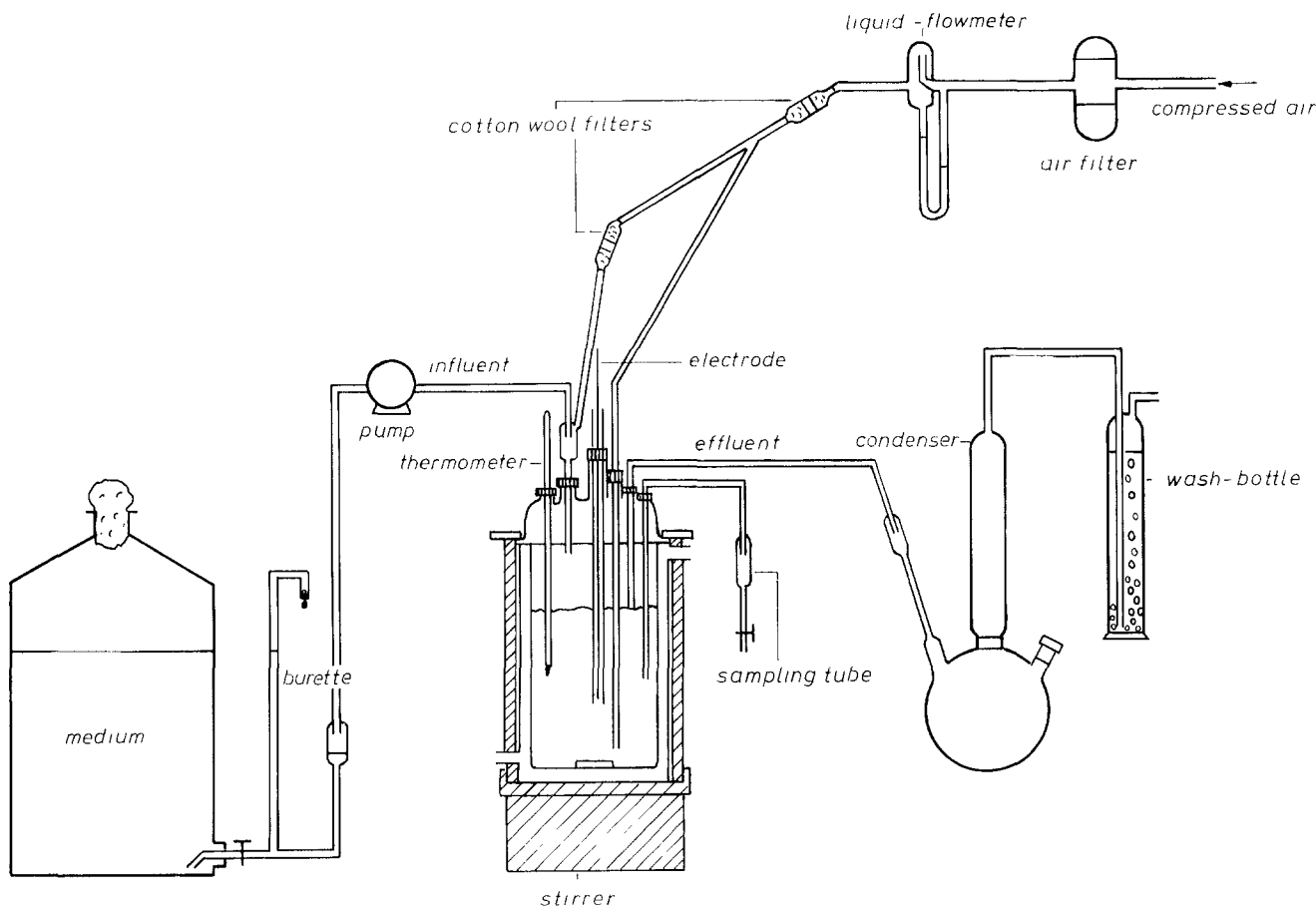
Principle

A nutrient solution that contains the substrate as the sole carbon source is fed at constant rate to a chemostat. Completely mixed liquid is removed from the chemostat at the same rate.

Of the microorganisms present in the chemostat, the species that have a lower growth rate than the rate at which the medium is renewed are washed out (Section 3.1.2.8). Of the others, the fastest-growing species will dominate. In the end a steady state is established. The selected organism then grows as fast as it is washed out. The concentration of the degradable part of the substrate is then low (Section 3.1.2.8) (Tempest, 1970).

EQUIPMENT AND MATERIALS

- A chemostat (3.5.5, Fig. 1, Note 1), consisting of:
 - a chemostat vessel (3.5.5, Fig. 2)
 - a magnetic stirrer (Note 2)
 - an adjustable metering pump
 - a thermostat
 - a flowmeter (capacity about $5.0 \text{ ml of air.min}^{-1}$)
 - a storage bottle with tap
 - a collecting bottle, if necessary with a reflux condenser
 - a burette for measuring the speed of the pump
 - connecting tubing and glass tube (Note 3)
- nutrient solution (see Section 3.3)
- microbial inoculum (see Section 3.2)
- sample bottles



3.5.5 Fig. 1 Apparatus for a chemostat test

3.5 Methods for the determination of biological degradation

- for preservation of the samples: alkali, acid, or a deep-freeze cabinet (-20°C), depending on the analytical method
- for the sterilization of the nutrient solution:
 - an autoclave
 - a pressure filter (see Section 3.3.3).

PROCEDURE

The necessary information concerning the choice of the test conditions has been given in the Introduction (3.1).

- Set up the apparatus in accordance with 3.5.5, Fig. 1 and 3.5.5, Fig. 2.
- Prepare the nutrient solution (Section 3.3).
- Sterilize the nutrient solution (Note 4).
- Adjust the feed pump to the desired rate (see Section 3.1.2.8). The pumping rate is checked by means of the burette in the following way.
By opening the tap in the storage vessel the burette is filled with the nutrient solution. **After** the tap has been closed the burette can be read, for example, every half-hour. The pump rate is calculated from the levels recorded and, if necessary,

is corrected on the basis of the results.

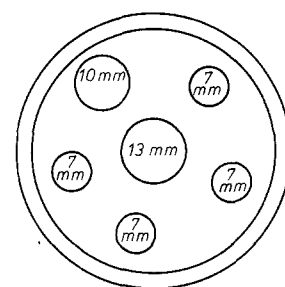
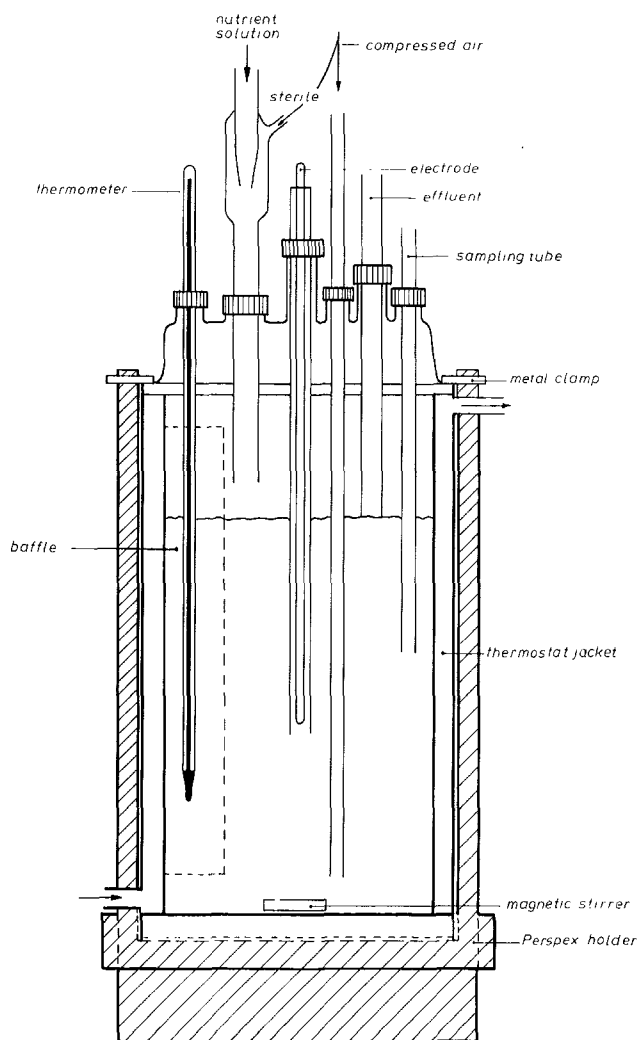
- Inoculate the chemostat (Section 3.2).
- With the aid of the thermostat adjust the temperature to $25 \pm 1^{\circ}\text{C}$ (Note 5).
- Aerate in such a way that no anaerobic conditions can arise. In general, a few ml per minute is sufficient.
- For a chemostat test we can define a characteristic time unit – the half-life τ_0 – as

$$\tau_0 = \frac{\text{volume of the chemostat}}{\text{pumping rate}}$$

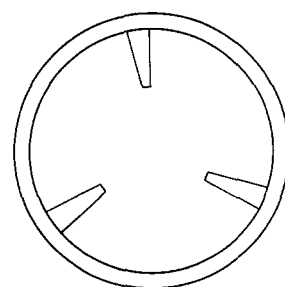
Collect the samples for analysis at times 0, $\frac{1}{2}\tau_0$, τ_0 , $\frac{3}{2}\tau_0$, etc. After 8 τ_0 -periods the time between the samplings can be made longer, since the condition can be regarded as having reached a plateau (steady state).

After the setting of new experimental conditions (pumping rate, temperature, pH), the above-described procedure must be repeated.

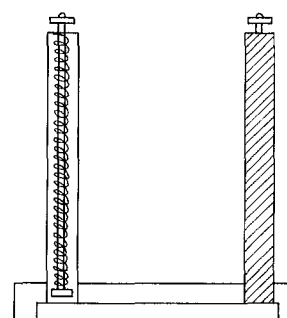
- Calculate the degradation from the difference between the substrate concentrations in the influent and effluent (see Sections 3.1.4 and 3.1.5). Report the results – preferably graphically – in the form of percentage degradation (y-axis) as a function of time (x-axis) and give also the time τ_0 .



top view of lid



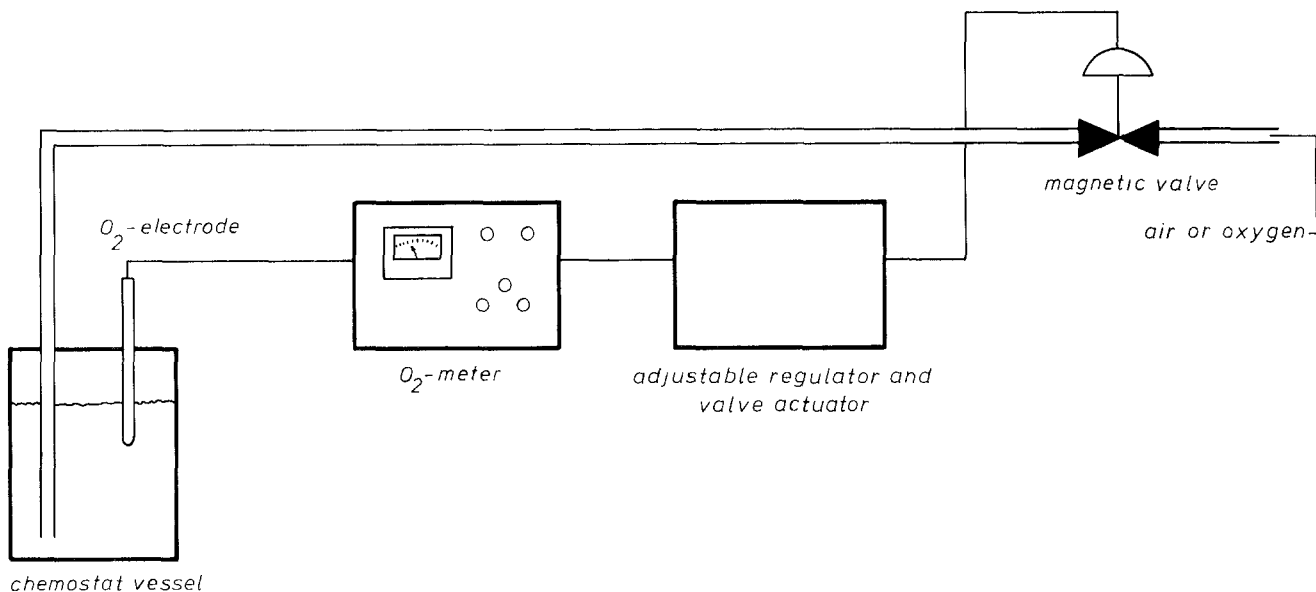
top view of vessel



clamp (internal)

3.5.5 Fig. 2 Chemostat vessel

3. Determination of degradability



3.5.5 Fig. 3 Principle of the scheme for oxygen control

NOTES

1. The procedure has described only some simple apparatus for the performance of chemostat tests. If necessary, some or all of the following additional apparatus can be used (Evans *et al.*, 1970).

• pH recording/adjustment

Recording of the pH provides additional information, because degradation may be accompanied by a change in the pH. A change in the pH by one unit or more must be prevented by buffering the nutrient solution or by a separate adjustment of the pH. A suitable electrode for this system is the Ingold 465-35 sterilizable combined pH electrode*.

• Measurement recording/adjustment of the oxygen concentration

The measurement and, possibly, recording of the oxygen concentration is desirable when, because of the presence of volatile compounds, the aeration must be kept to a minimum; this is possible by adjustment to requirements.

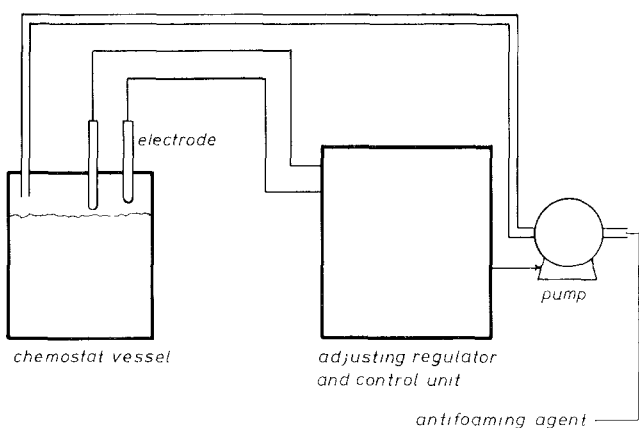
In the case of volatile compounds, pure oxygen can be used in the 'aeration'. In this way the flow of gas through the chemostat can be reduced still further.

An example of a simple installation is shown in 3.5.5, Fig. 3.

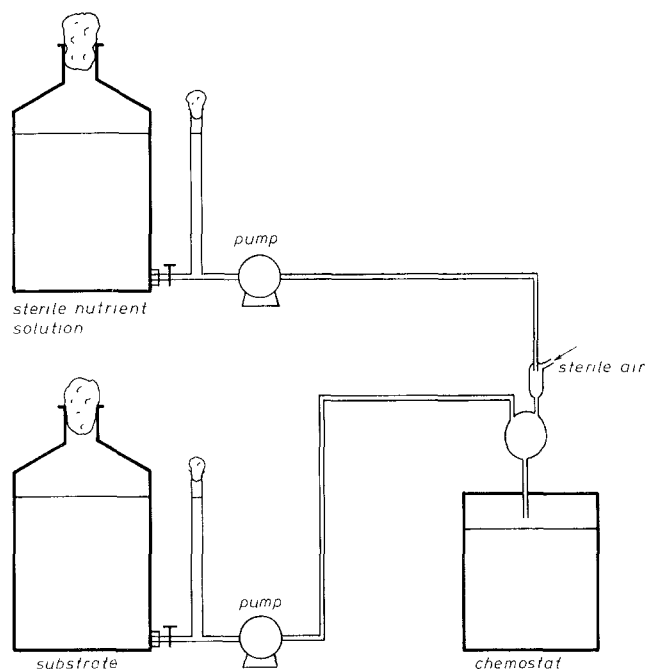
• Foam suppression

Foam problems occur rarely in degradability tests. When they nevertheless arise, it is best to use a controlled addition of an antifoaming agent (see 3.5.5, Fig. 4) (because of the glass vessel, mechanical suppression is impossible).

An antifoaming agent may also be added to the nutrient solution, but its possible influence on degradation must be taken into account.



3.5.5 Fig. 4 Principle of the scheme for foam suppression



3.5.5 Fig. 5 Separate feed of substrate and nutrient solution

* Applicon BV., Vlaardingen, Holland.

2. The magnetic stirrer must satisfy two requirements. It must have sufficient 'capacity' to obtain a good mixing of the chemostat vessel contents and it must not appreciably raise the chemostat temperature. The I.K.A. magnetic stirrer, combimag RCO type, satisfies these requirements.

3. When tubing is used for the transport of nutrient solutions, it must be borne in mind that it may absorb compounds from the substrate. In addition, the tubing may release substances that interfere with the test. It is better to use glass as far as possible (also for storage vessels). However, it is practically impossible to use glass exclusively (consider, for example, peristaltic pumps). Hence it must always be borne in mind that the material of the apparatus can affect the test.

4. Sterilization is better achieved by filtration (Section 3.3.3) than by heating, which can cause changes in the substrate. However, non-homogeneous nutrient solutions (emulsions or dispersions) cannot be filtered. In these cases the sterilization must either be carried out by heating or be omitted. Whenever no sterilization

is carried out, the medium must be prepared daily or be supplied from a cooled reservoir.

Mixing of the substrate (provided that it can be pumped) with the sterile nutrient solution is also possible (3.5.5, Fig. 5), but very low pumping rates (which are therefore difficult to adjust) are required. This easily leads to a discontinuous feed of the substrate, which can strongly affect the system in the chemostat.

5. It is also possible to work at a lower temperature, for example at the temperature of the environment receiving the waste material. The degradation then takes place more slowly. Since the relationship between the degradation rate in the laboratory and in practice is unknown, and since in this test biological degradability is determined as a property of a substance, it is possible to work at 25°C to shorten the test.

An exception can be made when psychrophilic organisms are to be used. The optimum temperature for these organisms is below 15°C.

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CHAPTER 4

Determination of eutrophication properties

by A. O. Hanstveit



4. Determination of eutrophication properties

4.1 DETERMINATION OF EUTROPHICATION PROPERTIES

In the Introduction to this manual (Part I) it has been stated that pollution may have not only an inhibitory but also a stimulating influence on an ecosystem. Eutrophication is mentioned as an example of such stimulating action, and implies the excessive growth of algae (plankton) that occurs when plant nutrients get into the water.

This aspect of pollution has not, however, been treated experimentally in the project that led to this manual. The problem of eutrophication is less important in sea water than in inland waters. Most inland waters in the Netherlands are already strongly eutrophic.

Nevertheless, it is a property of waste material that may be of importance in the over-all evaluation of the extent to which a given substance can be discharged at a certain site.

The most important macronutrients for plants are carbon dioxide, nitrate, phosphate, and – in sea water – also silicate. The growth of organisms is determined by the available amounts of the various nutrients and by the amount of light. According to Liebig's law, the nutrient for which the ratio between the actual concentration and the concentration required for a given organism is lowest will be rate-determining for the growth of that organism. One of the above-mentioned substances is usually the rate-determining nutrient, but it may also be others (such as Fe or K). The result of a supply of these nutrients is then an accelerated growth of algae.

It follows that the effect of waste materials on the receiving body of water depends on the composition of this water. A nutrient-poor water will be strongly affected by a supply of nutrients, while in an already eutrophic water the effect will be relatively slight. The determination of the eutrophication influence of waste on the receiving body of water has been described most completely in tests developed by the Environmental Protection Agency (EPA) (Anon., 1971 and 1974).

Determination of the eutrophication effect of a waste material, as a general property, requires another approach.

The first step in such an investigation is elementary analysis, to establish whether the known eutrophication elements (N, P, Fe, etc.) are present in the waste. Next, it is important to determine whether these elements are available to various organisms, possibly after biological degradation (Chapter 3).

This chapter does not describe any method for the determination of this eutrophication capacity on the basis of using defined media deficient in the nutrient to be investigated, since this has not been studied in the project that led to this manual.

However, one can use the test described in Section 5.10.1.2 with the alga *Scenedesmus quadricauda* in Rhode's medium. By leaving out various nutrients (phosphate, nitrate, etc.) from this medium it is possible to check on the consequences of discharging a given waste material. For more details of the eutrophication test, reference is made to the EPA procedures.

4.2 References

4.2 REFERENCES

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EPA-660/3-75-008.

CHAPTER 5

Determination of toxicity

by D. M. M. Adema

with contributions from

A. O. Hanstveit, R. N. Hoofman, G. J. Vink



5.1 INTRODUCTION

This chapter is concerned with methods used for the determination of toxicity of substances that pass into the aquatic environment. A toxic substance is an agent that in contact with a living organism causes damage to its structure or function. Toxicological studies are carried out to determine the nature and the extent of this damage.

For toxicological investigation with mammals the test substance is generally administered to the animal directly, e.g. intravenously, intramuscularly, or *per os*.

Although these methods could also be used in toxicity investigations with aquatic animals, subsequently called 'aquatic toxicology', it is in fact customary to add the test compound to the water (the external medium of the test animals). The relationship between the concentration in the **external** medium and the effects caused by it in the organism is determined. This relationship, represented as a concentration-response curve, shows a certain similarity to the dose-response curve in the toxicity tests on mammals, but there the dose is of course the amount of substance in the **interior** of the test animal. This basic difference must be remembered at all times.

In work on toxicity a distinction is made between acute and chronic tests. Likewise, subacute and subchronic phases are distinguished. In the case of mammals these terms are well defined. Although the greatest possible correspondence is sought, in the present manual these terms are defined somewhat differently, because of the special nature of aquatic toxicology.

By **acute toxicity tests** we shall understand those in which the duration of the exposure to the test compound and the observation period are both short in relation to the life cycle of the organism concerned.

By **chronic toxicity tests**, we shall understand tests over at least one life cycle, with an uninterrupted exposure to the test compound. If the tests are extended over several generations, these chronic investigations are also called reproduction tests.

Subchronic toxicity tests can also be distinguished. This distinction is not meaningful for tests with animals having a short life cycle, e.g. one month. Short-term tests with these animals can frequently be described as chronic.

For test animals with a longer life cycle it is appropriate to distinguish a subchronic phase in toxicity investigations.

Tests with animals that have a life cycle of 1 to 2 years and in which the test duration with continuous exposure to the test compound amounts to at least 10% of this period can be regarded as subchronic.

By subchronic toxicity tests we shall also understand those that cover several important developmental stages of the test animal.

The adjectives 'short-term' and 'long-term' are also used in aquatic toxicology, but they refer rather to the duration of the test itself.

The terms acute, subchronic, and chronic give more information on the nature of the investigations than the terms short-term and long-term. For example, a test lasting seven days is a short-term test; it is, however, acute for guppies but chronic for *Daphnia magna*.

In this chapter we shall consider only those methods with which concentration-response relationships can be determined. For this purpose the test compound is added to water, into which the animals are subsequently introduced. Several concentrations are tested simultaneously. These concentrations are kept as constant as possible in the course of the tests.

There are various ways in which toxicity tests as outlined above can be performed, the commonest of which will be described briefly below.

a. The static method

Here the concentrations of the substance are prepared in suitable test vessels, into which the animals are introduced. If necessary, the vessels are aerated. The required observations are then made during a specified time, without renewing the test medium.

In this arrangement the test concentrations actually present

depend greatly on the physico-chemical properties of the test substance and on the relative amount of biomass in the test medium.

For example, in the case of volatile and/or easily adsorbed substances the concentrations of the test compound in the test vessels can decline rapidly (Gillespie *et al.*, 1975). Moreover, with relatively long test periods some of the effects measured may not be caused by the test compound but by a deterioration in the water quality.

b. The semi-static method

Some of the shortcomings of static test methods can be obviated by a regular renewal of the medium. The more frequently the test medium is renewed, the more constant will the concentrations of the test compounds remain, and the concentrations of the excretion products will be lower than in the static method. In addition, the water remains better saturated with oxygen, though this is only important if aeration is impossible.

c. The flow-through method

In this a constant flow of water is passed into the test vessel while an equal volume of water is removed *via* an overflow. The incoming water is previously well aerated and contains the test compound in the desired concentration.

The flow rate is adjustable and is made adequate for the concentrations to remain constant and the oxygen demand of the test animals to be satisfied. The flow rate must not be so high that the animals experience any adverse effects.

The choice of the most suitable test arrangement depends on the physico-chemical properties of the test compound, on the test duration, and on the species of the test animal.

In addition to the methods given above, which entail a constant stressing of the animals with the test compound via the aqueous phase, some examples are given of methods based on other principles.

Examples of these are:

a. The so-called recovery tests

In this method the animals are exposed for a relatively short time (from a few seconds upwards) to – usually high – concentrations of the test compound. After each such contact period a statistically significant number of the test animals is kept in clean, preferably flowing, water for a relatively long time, during which the effect of the preceding exposure is determined. Recovery periods may also be added to standard acute toxicity tests.

No detailed procedural descriptions are given for such recovery tests; they can be performed without further instructions on the basis of those given for toxicity tests with constant stressing.

b. Feeding tests

Just as is customary in toxicity tests on mammals, the test compounds may also be administered to the animals with their food. The animals must then eat the food rapidly, since otherwise the test compound might diffuse from the food into the medium, and the test would then relate partly to an exposure *via* the aqueous phase. Thus, the 'saturation' of unicellular algae with a toxic substance followed by suspension of these algae in water as food for 'filter feeders', e.g. daphnids, is not very different from dosing the toxic substance directly into water and the addition of unpolluted unicellular algae to this water, provided that the absolute amounts of the toxic substance are the same.

An equilibrium distribution of the toxic substance between the unicellular algae and the water will be established. This equilibrium is reached very rapidly for most substances in view of the rate of uptake of the algal cells by the 'filter feeders'; in other words, when the equilibrium tends towards the algae, the substance is administered *via* the food, and when the equilibrium

5. Determination of toxicity

is towards the aqueous phase, the administration takes place *via* water, independently of whether the toxic substance was dosed *via* the water or *via* the algae.

c. Plankton model system test

Here in most cases a dose of the test compound is added to a plankton model system only once. Its effect on the ecosystem is then studied for a few weeks, even though after only a few days the compound may perhaps no longer be detectable in the aqueous phase.

In this chapter we shall consider only those methods in which the test compound is added to the water and its concentration is kept constant.

Sections 5.2 to 5.6 provide the background information necessary for the correct performance of such toxicity tests.

Section 5.7 describes various types of dilution water.

Section 5.8 describes the methods of breeding for a number of test animals.

Detailed descriptions of methods for acute, subchronic, and chronic toxicity tests are given in Sections 5.9 and 5.10.

These last sections are numbered in such a way that further test methods using the same test animal may be added at a later date. For more theoretically oriented discussions and literature reviews on toxicity tests reference is made to reviews such as those by Brown (1973; 1976), Brungs (1973), Hueck and Hueck-v.d. Plas (1976), and Sprague (1969, 1970, 1971). In addition, Lee (1973) has given a review of the possible reactions between the test compounds and the dilution water, and other chemical aspects that may affect the toxicity tests.

5.2 PREPARATION OF THE TEST SOLUTIONS

5.2.1 Types of water for the preparation of the test solutions

Depending on the test animal, a suitable type of fresh water or sea water or, in special cases, sea water having a low salinity (brackish water) is selected. Suitable types of water are given in the descriptions of the methods for acute and (sub)chronic tests (Sections 5.9 and 5.10).

In principle, both synthetically prepared water and natural water can be used. Although in the case of natural water one cannot be certain of the composition and the presence of impurities, the use of such water is generally the only practical possibility, especially if very large volumes are required (for example, more than 3000 litres per day).

Untreated tap water as a variety of fresh water can only be used if its quality satisfies the demands of the test animal concerned. It is also important that the composition should be constant and known.

The composition of synthetically prepared media is known when a good-quality distilled water and chemically pure substances are used. If, however, one starts with tap or spring water, which is often unavoidable for the preparation of large amounts, its initial quality may have an influence on the final composition. In the preparation of varieties of fresh water, corrections must be made for the amounts of non-toxic ions such as Na^+ , K^+ , Ca^{2+} , Mg^{2+} , Cl^- , and SO_4^{2-} already present; it is assumed that these are known. In the preparation of synthetic sea water starting from sea salt and tap water, no correction can be made for the ions present,

so that the calcium content in particular will be relatively high.

Heavy metals, organic compounds, free chlorine, etc., already present in tap water may present a problem. Free chlorine can be removed (see Section 2.2.1). Traces of heavy metals and organic compounds are removed best by filtration through active carbon. Chemical and biological checks will be necessary both for the media prepared synthetically from tap water and for natural water.

For formulations, composition, and the monitoring of various types of water, reference is made to Section 5.7.

Considerations concerning the preparation of synthetic water, the quality criteria of the initial water for it, and the quality criteria of natural water are also given in the EPA report, EPA-660/3-75-009 (1975).

A method for the preparation of large amounts of fresh or brackish water by mixing concentrated salt solutions with de-ionized water has been described by Abram (1970).

5.2.2 Choice of the concentrations of the test compound

Several, usually five, concentrations are generally tested simultaneously. These concentrations form a geometric series. Preliminary tests determine the order of magnitude of the effective concentrations. Table 1 gives a review of common concentration series. Other geometric series may also be used, but the use of non-geometric series such as 1-2-5-10-15-20 must be discouraged because of the greater probability of mistakes in the preparation of the solutions and the irregular distributions of points on graphical treatment in accordance with Section 5.6.

5.2 Table 1. Concentration series for toxicity tests

Concentration series*										Factor		Purpose
1.0									10 etc.	10^1	= 10	first impression about toxicity
1.0		3.2							10 etc.	$10^{0.5}$	= 3.2	over-all toxicity test
1.0	1.8	3.2							10 etc.	$10^{0.25}$	= 1.8	routine toxicity test
1.0	1.3	1.8	2.4	3.2	4.2	5.6	7.4		10 etc.	$10^{0.125}$	= 1.3	accurate toxicity test

* Only the ratios are given here. The true concentration range is selected for each test, e.g. 100-320-1000 mg.l^{-1} or 10-13-18-24-32 $\mu\text{l.l}^{-1}$, and so on.

Concentrations of the test compound that are greater than the maximum **solubility** in the type of water used at the prevailing temperature are as a rule not tested. This solubility is checked beforehand in accordance with Sections 1.3 and 1.5.

Before beginning the toxicity test proper, it may be useful to prepare a saturated solution and to see whether any measurable effect will take place in this during the selected test period.

If the solubility is high but the toxicity is relatively low, as a rule no higher concentrations are tested than 1000-3000 mg.l^{-1} (= 0.1 to 0.3 %). When testing still higher concentrations one must consider whether this in fact has any purpose, and whether one is not mainly testing the influence of an increased osmotic pressure.

5.2.3 Dosing of the test compound

The dosing of the test compound in flow-through systems is controlled automatically in some way or other; on this point, see Section 5.4.3.

When the tests are performed with (semi)static methods, the use of concentrated solutions of the test compound (stock solutions) for dosing is least labour-intensive.

In the case of readily water-soluble compounds, stock solutions forming a geometric series with the desired factor are prepared in distilled water, which are all 1000 times as concentrated as the test concentrations.

Example for the preparation of a series of test solutions: Concentrations to be tested: 10, 18, 32, 56, and 100 $\mu\text{g.l}^{-1}$.

Procedure:

Prepare 5 stock solutions containing respectively 10, 18, 32, 56, and 100 mg.l^{-1} .

Prepare the most concentrated stock solution (100 mg.l^{-1}) by weighing out, for example, 10 mg and dissolving this in 100 ml of distilled water (solution 1).

Prepare the next stock solution (56 mg.l^{-1}) by diluting 56 ml of solution 1 to 100 ml (solution 2).

Prepare the next stock solution (32 mg.l^{-1}) by diluting 56 ml of stock solution 2 to 100 ml (solution 3), and so on.

In this way the same amount of the previous stock solution is always diluted to the same final volume, which limits the number of mistakes in the preparation of the stock solutions.

To prepare the test solution, 1 ml of the stock solution is added per litre of the medium with the aid of plunger pipettes (or ordinary pipettes) so that the desired concentration is obtained.

Stock solutions of substances with acidic or basic properties are if necessary treated with alkali or acid to make the substances more soluble, but the pH and the formation of precipitates in the medium after dilution must be checked.

5. Determination of toxicity

Similarly, if the solubility demands this, it is possible to start from stock solutions that are only 100 times as concentrated as the test concentrations, but this is slower because of the larger volumes that have to be metered out.

If the solubility of the test compound in water is sufficient for the desired range of concentrations but too low for the preparation of a 100 times stock solution in water, the highest concentration is prepared directly in the test medium. Shaking or stirring is then carried out for several days. It is also possible to prepare saturated solutions by continuous percolation of the medium over the test compound, if necessary on an inert support (Chadwick and Kügemagi, 1968; Veith and Comstock, 1975). The first of these methods is suitable only if small amounts of the medium (e.g. 20 litres) are required per day.

Both methods are reliable only if the concentration achieved and the homogeneity of the test compound in the medium can be determined by chemical analysis.

The lower concentrations are prepared by dilution with the selected type of water.

If the test compound is readily soluble in water in the concentration range to be tested, but not sufficiently soluble for the use of stock solutions in water and the above-mentioned methods of dissolution are impossible or unnecessary, stock solutions in a suitable organic solvent are used.

The organic solvent at the final concentration must not be toxic to the test animals, must not affect the uptake of the test compound by the test animals, must not be rapidly degradable, and must be miscible with water. Efforts must be made at the addition of the smallest possible amount of this organic solvent. The maximum is usually 0.1 ml per litre.

Stock solutions in the selected organic solvent are prepared that are 10^4 times as concentrated as the concentrations desired in the test (in principle in the same manner as described for stock solutions in water), and 0.1 ml of this is added per litre of the medium. Plunger pipettes must be used for rapid and reproducible addition of 0.1 ml. A review of a number of organic solvents with properties relevant to this purpose is given in Table 2 of this section.

5.2 Table 2. Relevant properties of some organic solvents

Solvent	Solubility in water	Degrad- ability ¹⁾	Theoretical oxygen demand of 0.1 ml (mg)	96 h LC ₅₀ (%v/v)		Remarks
				<i>D.magna</i>	<i>C.marinus</i>	
methanol	∞	+++	120	2.0	1.4	
ethanol	∞	+++	166	1.8 ²⁾	1.3 ²⁾	
n-propanol	∞	+++	192	0.6	0.1	
isopropanol	∞	+	192	1.4	0.4	
n-butanol	7.9 g/100 ml	+++	210	0.2	0.08	
isobutanol	9.5 g/100 ml	+++	205	0.3	0.05	
tert.butanol	∞	—	208	0.6	0.1	sets at: 25°C
acetone	∞	++	174	1.6	0.5	
diethyl ketone	4.7 g/100 ml	++	214	0.2	0.4	
dimethyl sulphoxide	very good	—	180	1.6	1.3	
dimethylformamide	∞	++	227	1.7	3.2	

¹⁾ Determined under the conditions of the toxicity test, but without test animals.

²⁾ 48-h LC₅₀, (96-h LC₅₀ cannot be determined because of the excessive oxygen demand)

+++ gives rise to an immediate biological oxygen demand

++ gives rise to biological oxygen demand after some time

+ can give rise to biological demand (especially after bacterial adaptation)

— does not give rise to biological oxygen demand

When no growth of bacteria (and therefore uptake of oxygen) is to be feared since the test period is short (a few hours), or when adequate aeration can be provided, the usual choice is acetone. If the organic solvent must not undergo degradation, in view of the oxygen concentration of the test solution, tert.butanol

is one of the few usable solvents. If, however, solidification of the stock solutions in this solvent constitutes a problem, isopropanol may be selected. Its toxicity is as low as that of acetone, but it is somewhat less rapidly degradable in toxicity tests.

5.3 TEST ORGANISMS

5.3.1 Introduction

Toxicological investigations in water are usually performed with organisms representing different trophic levels. The significance of this is explained in Part I.

In the choice of the species availability plays a major part.

Test animals are obtained by:

- breeding in the laboratory
- purchase (exclusively in the case of fresh-water fish)
- collection in the field.

Breeding of the test organisms in the laboratory has the following advantages:

- Several developmental stages whose past history is known are available continuously for toxicity tests.
- With these bred animals both acute and chronic tests can be performed, provided that the life cycle of the animals is not too long (e.g. shorter than six months but preferably one or a few weeks).

However, only a small number of aquatic species are easy to breed.

Breeding in aquarium systems is usually labour-intensive; for some species another limiting factor is the space required. Various species, especially fresh-water fish, both from tropical and temperate regions, can be bought. These can be used for acute and sometimes subchronic toxicity tests. Information on the breeding conditions can frequently be obtained.

However, in the case of fish from temperate regions specimens of a definite, desired (usually small) size are available for only a limited part of the year; in the choice of these species their representativeness for the domestic fresh-water environments plays a part, which is naturally not the case for the tropical fish species obtained from ornamental fish breeders.

Collection of organisms in the field is particularly important for toxicity tests in sea water (Perkins, 1972 a and b). There are only a few native sea animals that can be bred in aquarium systems. Little can be ascertained of the past history of animals collected in the field; in the case of the adult animals usually only their length and the site of collection are reported. As a rule only acute toxicity tests are performed with organisms collected in the field; with some species there are possibilities for subchronic tests.

In addition to the 'availability' factor, the 'environmental relevance' plays an important role for the choice of the test species. This means that the selected organism must be representative for the environment threatened by the discharge or dumping of materials whose toxicity is to be determined.

Some test organisms much used in toxicity tests do not occur in temperate regions; these organisms are used simply because they are easy to breed in the laboratory (Mount, 1968). The results with these organisms are nevertheless valuable from the standpoint of comparative tests.

Theoretical considerations indicate that the aquatic toxicological investigation usually has a limited scope. It has already been mentioned that it is important to perform the toxicity tests with organisms corresponding to different trophic levels. In this connection it is recommended to carry out the acute toxicity tests on a given substance with at least two animal species; in general, the choice then falls on a representative fish and crustacean (for considerations relating to the choice of the test organisms see, *inter alia*, Sprague, 1970; EPA Report 660/3-75-009 (1975); and Doudoroff *et al.*, 1951).

In Section 5.3.2 brief characteristics are given of each test species that has been used regularly at our laboratory over a considerable time for acute, subchronic, and chronic toxicity tests in fresh and sea water. The choice of each organism is determined by the con-

siderations outlined briefly in this section. The list of test organisms is not intended to be definitive; some animal species are in fact less suitable for toxicity tests, but they are described because no suitable substitute has (so far) been found.

In Section 5.3.3 some observations are made concerning the collection, transport, and acclimatization of the test animals collected in the field or obtained from commercial breeders.

Section 5.3.4 gives some general rules relating to the number of test animals in the toxicity test and the amount of biomass used per test vessel or per test concentration of a substance.

5.3.2 Test organisms

The following brief characteristics of all test organisms currently in use at the TNO Division of Technology for Society for toxicity tests serve as a short introduction to a number of suitable test organisms. The organisms are always treated by species, with the exception of the unicellular algae and the pelagic marine copepods, each of which is described as a group.

For each organism discussed, a procedure for an acute toxicity test is described in Section 5.9 (except for algae).

For a number of these animals Section 5.10 gives a description of a chronic or a subchronic toxicity test method; Section 5.8 describes the breeding method in laboratory aquarium systems for the same animals (with the exception of the plaice, *Pleuronectes platessa*).

Further information on the organisms reported will be found in the relevant literature, such as is available in handbooks, books on taxonomy, etc., for the various groups of animals.

5.3.2 Table 1 gives a review of the test organisms whose characteristics are subsequently given in brief.

5.3.2.1 Unicellular algae

Algae, in the form of phytoplankton and phytobenthos, are the most important primary producers in the aquatic environment. They serve as food for other organisms (including zooplankton), and as such constitute the first biotic link in the aquatic ecosystem.

There is a large number of algal types and species, with differing properties. Some are found almost throughout the world, while others occur only in a specific habitat. Thus, there are 'groups' of algae that, for example, predominate in fresh water (*Chlorophyceae*, green algae), while they scarcely occur in sea water; the most important 'groups' of algae in the North Sea region are *Bacillariophyceae* (diatoms) and *Dinophyceae* (dinoflagellates) (see, for example, Prescott, 1968; Round, 1965; Levring *et al.*, 1969).

The division of algae into groups is based mainly on the type of their pigment (chlorophyll, phycobilin, carotenoids) and form of motion (flagellation). These properties are also decisive for the place where a certain group occurs in the environment; thus, red algae are those that occur deepest in the sea, since they can utilize the blue fraction of sunlight that penetrates deepest into the water. Algae are also characterized by a very wide morphological variation, from microscopic single cells to plants measuring tens of metres. Propagation may take place through simple cell division, but there are also very complicated life cycles.

Wastes can inhibit the growth of algae, but they can also stimulate it. Such eutrophication, usually caused by the addition of nutrient salts to the water, is treated briefly in Chapter 4.

For toxicity tests with algae the use of a natural water with the algal flora present in it would be ideal. An approximation to this is the use of microecosystems, which is referred to in Chapter 7. The use of algal monocultures that can be grown in the laboratory is almost always indicated for laboratory tests. Unicellular algae with simple nutritional demands are best; they can be cultured by the usual microbiological methods in more or less defined

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nutrient solutions. Many algal species that could be interesting test organisms often cannot be cultured in the laboratory in a simple manner. This applies particularly to the benthic species.

Hence the most important criterion that determines the choice of the test species is whether it can be cultured in the laboratory. At the same time, algae are selected from various important taxonomic groups and organisms that are important in different environments (sea water, fresh water). It is found from the literature that only a limited number of species (mainly green algae) are used in toxicity tests.

Some examples of algal species that are repeatedly mentioned in the literature in connection with toxicity tests (for the literature references see Section 5.10.1) are as follows:

Chlorophyceae (green algae)	<i>Chlorrella pyrenoidosa</i> <i>Scenedesmus obliquus</i> <i>Scenedesmus quadricauda</i> <i>Selenastrum capricornutum</i> <i>Dunaliella tertiolecta</i>

Cyanophyceae (blue-green algae)	<i>Microcystis aeruginosa</i> <i>Anabaena flos-aquae</i>
Bacillariophyceae (Diatomeae)	<i>Skeletonema costatum</i> <i>Thalassiosira pseudonana</i>
Chrysophyceae (yellow-green algae)	<i>Phaeodactylum tricornutum</i>

5.8.2 Table 1 gives a list of easily cultured species of algae. The choice is based mainly on the fact that the TNO Division of Technology for Society has had several years of experience with the use of these algae. Toxicity tests with unicellular algae are practically always chronic in nature, because of the short life cycle of the algae, and are therefore included in Section 5.10.

5.3.2 Table 1 Review of the test organisms used currently at the TNO Division of Technology for Society for the acute, subchronic, and chronic aquatic toxicological investigation of waste materials

Species/group of organisms	Order	Habitat fresh / brackish / salt	Laboratory breeding
Unicellular algae (various species)		fresh / brackish / salt	+
Mussel, <i>Mytilus edulis</i>	Bivalvia	salt	—
Slipper limpet, <i>Crepidula fornicata</i>	Gastropoda	salt	— (+)
Zebra mussel, <i>Dreissena polymorpha</i>	Bivalvia	fresh	—
Pond snail, <i>Lymnaea stagnalis</i>	Gastropoda	fresh	+
<i>Ophryotrocha diadema</i>	Polychaeta	salt	+
Pelagic marine copepods	Crustacea	salt	—
Brine shrimp, <i>Artemia salina</i>	Crustacea	salt	+
Shrimp, <i>Chaetogammarus marinus</i>	Crustacea	brackish / salt	+
Prawn, <i>Palaemonetes varians</i>	Crustacea	brackish / salt	— (+)
Brown shrimp, <i>Crangon crangon</i>	Crustacea	salt	—
Water flea, <i>Daphnia magna</i>	Crustacea	fresh	+
Common goby, <i>Pomatoschistus microps</i>	Pisces	brackish / salt	— (+)
Plaice, <i>Pleuronectes platessa</i>	Pisces	salt	—
Guppy, <i>Poecilia reticulata</i>	Pisces	fresh / brackish / salt	+
Golden orfe, <i>Leuciscus idus</i> var. <i>melanotus</i>	Pisces	fresh	—
Rainbow trout, <i>Salmo gairdneri</i>	Pisces	fresh	—

+ : can be bred (+) : good chances of breeding — : not bred, or not easily bred

5.3.2.2 The mussel, *Mytilus edulis* (Linnaeus, 1758) (Bivalvia, Anisomyaria)

The common mussel generally occurs in coastal regions within the temperate zone of the northern hemisphere. It is a prolific, sessile inhabitant of the tidal zone, in which the animals fall dry at ebb-tide, but mussels are also found in large numbers at greater depths (down to about 10 metres).

The reproduction of the mussel, which has different sexes, takes place in the spring. Enormous numbers of eggs (5 to 12 million per female mussel) and the sperm are expelled into the water. The larvae emerge after 1–2 weeks and live pelagically for another 2–3 weeks, after which, as small mussels, they deposit themselves on rocks, piles, seaweed, other mussels, etc., and also on buoys in the open sea. The mussels anchor themselves to these solid objects with attaching filaments produced by the byssus gland.

The mussel is very tolerant of variations in temperature and salinity (15–35‰ S). It gathers its food by pumping large amounts of sea water (up to several litres per hour, depending on its size) and filtering out the solid constituents; the mussel extracts the

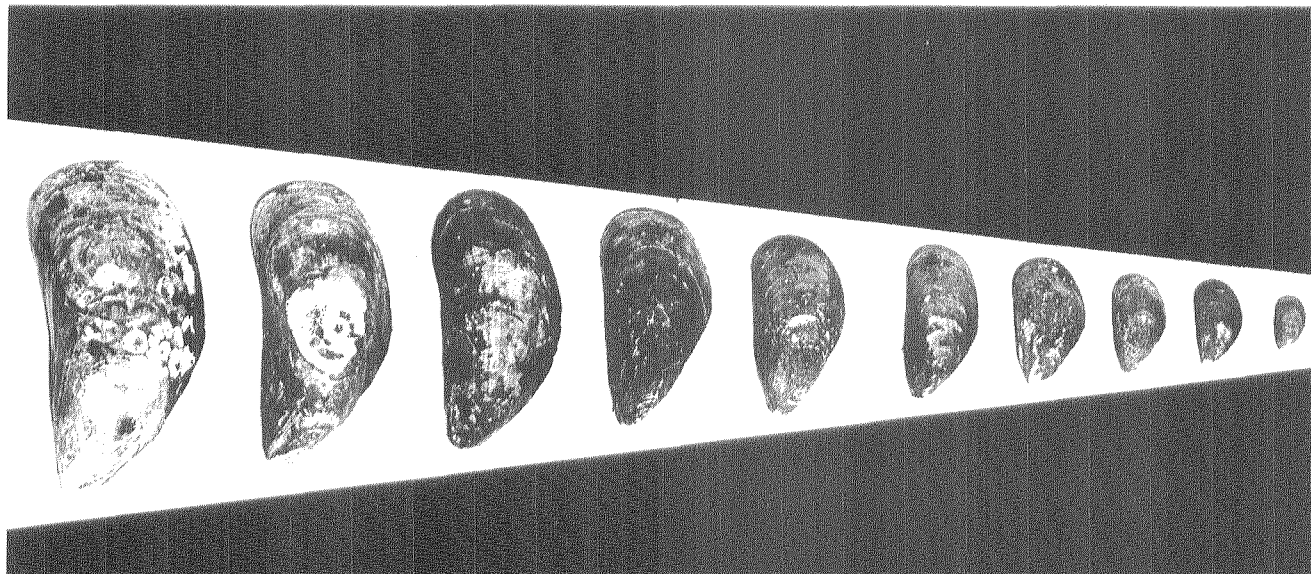
nutriment (phytoplankton) from it and expels the valueless material (Dral, 1967).

In this way, the mussel functions as a biological filter; animals that use this method to obtain food are called filter feeders.

Mussels can be collected throughout the year in all sizes and can easily be kept in aquaria. Mussel larvae are not easy to cultivate in aquaria. Consequently, for routine toxicity tests animals are used that have already changed to their sessile state. Because of the rapid rate of filtration and capacity for taking up all kinds of materials, and because of their sessility at a definite site, mussels are of great importance in field studies connected with biological monitoring; the animals are also much used in laboratory tests for the accumulation and the elimination of compounds.

In short-term toxicity tests the mussel is generally rather insensitive; by closing its shell, it can shut itself off for several days.

This makes a toxicity test on only a few days pointless, and therefore an acute toxicity test will usually last 2–4 weeks. If sufficient food (for example, the unicellular alga *Dunaliella* spec.) can be offered, which is not easy, subchronic tests can be performed by extending the test period.



5.3.2 Fig. 1 Mussel, *Mytilus edulis*. Natural size

5.3.2.3 Slipper limpet, *Crepidula fornicata* (Linnaeus, 1758) (Gastropoda, Mesogastropoda)

The shellfish *C. fornicata* originates from the east coast of North America. At the end of the last century this species was imported into Britain with American oysters and it spread rapidly along the European coasts. Because of its competing mode of life, *C. fornicata* can cause much damage to oyster beds and is therefore sometimes called the 'oyster pest'. In the Netherlands this species occurs on the muddy sea bottom just below the water surface in the environs of Yerseke.

The food of the animals consists of planktonic organisms, especially diatoms.

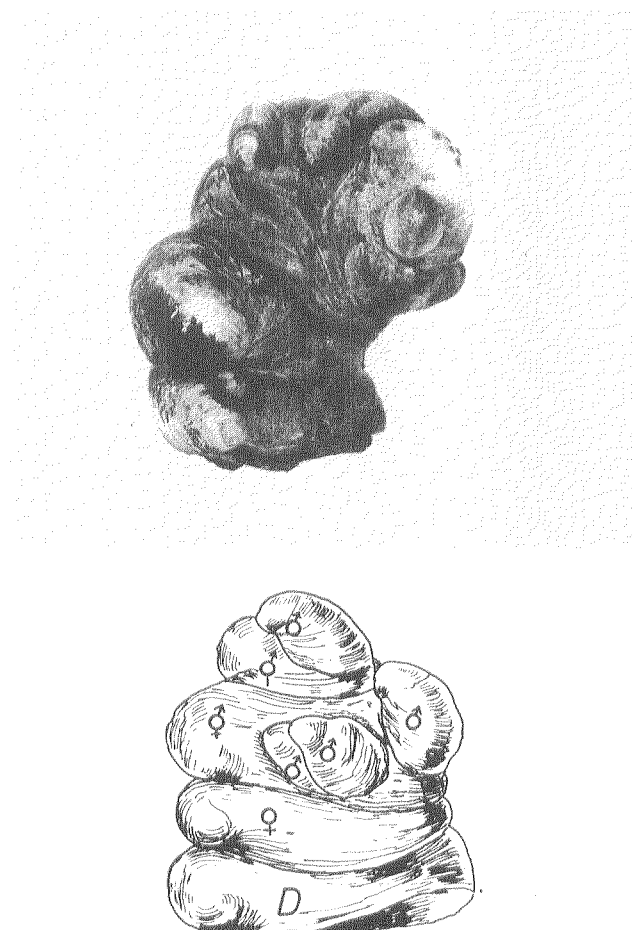
The younger animals are motile, but as they grow older they become attached to one another successively in spiral form, each successive individual becoming attached by suction to the shell of its predecessor (5.3.2, Fig. 2).

C. fornicata is protandrously hermaphroditic (Coe, 1936). Young animals are male, freely moving, and not yet functional. Older male animals are usually already attached to the chains. The male period is followed by a transitional phase; after this the animal goes into the female stage. Thus, in the chains the animals at the base are females and the animals at the top are males. *C. fornicata* produces egg masses in the period from March to September. The egg mass, deposited on a solid object, is covered by the parental animal. It consists of a cluster of about 40 capsules (egg sacs) which like grapes in a bunch are attached to the substrate by a common shaft. Each capsule may contain about 250 eggs. The eggs are about 0.18 mm in diameter. Their development takes place within the capsules. Fresh-laid eggs are light yellow. After about 4 weeks the egg mass becomes dark brown to black and the veligers are released (5.3.2, Fig. 3).

The larvae then possess a ciliated two-lobed velum (Werner, 1955). The head, the eyes, the heart, and the digestive system are already well developed. The extended velum is used as a propeller, enabling vertical or horizontal motion, and can be drawn in under unfavourable conditions.

After a pelagic period of about two weeks the larvae attach themselves to solid objects.

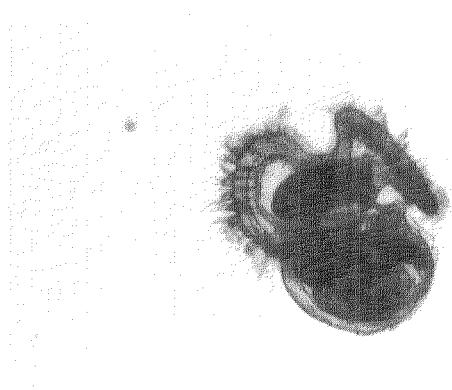
The animals can also be stimulated to lay eggs in the laboratory outside the natural breeding season, so that the continuous availability of eggs and veligers is assured (see Section 5.9.1). Insufficient attention has so far been paid to the possibility of breeding this animal in the laboratory and to the possibilities for toxicity tests.



5.3.2 Fig. 2 Slipper limpet, *Crepidula fornicata*

a. Photograph of a chain ($\times \frac{1}{2}$)

b. Sketch of a chain with at the base a female animal attached to a dead shell (D); above it there are two animals in the transitional phase and a male animal. There are also three small motile males on the chain (after Coe, 1936)



5.3.2 Fig. 3 Veliger of the slipper limpet *Crepidula fornicata* (magnification $\times 90$)

'The veliger's a lively tar, the liveliest afloat,
A whirling wheel on either side propels his little boat;
But when the danger signal warns his bustling submarine,
He stops the engine, shuts the port, and drops below unseen'.
(W. Garstang, 1951)

5.3.2.4 Zebra mussel, *Dreissena polymorpha* (Pallas, 1771) (Bivalvia, Heterodonta)

This fresh-water mussel occurs in nearly all surface waters of the Netherlands and in many other parts of Europe. The population densities may vary greatly from place to place.

The animals are located preferably some 20–50 cm below the water surface out of direct sunlight, e.g. on the underside of rocks.

The zebra mussel is fairly sensitive to the salt content of its environment, though up to 300 mg of chloride per litre is well tolerated. The animals are also tolerant of water contaminated with organic substances (α - or β -mesosaprobic), but a high silt content is not tolerated.

Like the common mussel along our coasts, the zebra mussel is a 'filter feeder'. The animals are of different sexes and the production of eggs is particularly high, as in the common mussel. The larvae live pelagically for 8 days, after which the young mussels attach themselves to a solid object. The motility of the animals is relatively high during the first three years. The older animals grow little, so that in older populations differences in length are slight. The most common length is 10–25 mm (Leentvaar, 1971, 1975).

Only the sessile animals in the post-larval stage are easy to maintain in aquaria and are suitable for acute toxicity tests; as in the case of the common mussel, if no nutriment is supplied minimum and maximum test times of 2 and 4 weeks can be recommended.

The 'bio-indicator' function that the common mussel fulfills in the marine environment may be assumed in the fresh-water environment by the zebra mussel. Investigations are in progress.

5.3.2.5 Pond snail, *Lymnaea stagnalis* (Linnaeus, 1758) (Gastropoda, Basomatophora)

This is a very common and widespread fresh-water snail.

The pond snail is a hermaphrodite and thus carries both male and female sex organs. However, self-fertilization takes place only when the animals are isolated. The eggs of the pond snail are deposited in oblong egg masses.

The embryonic development of the eggs is discussed in Section 5.8.3 in relation to toxicity tests with pond snail embryos.

The young snails emerge after 9 to 10 days at about 25°C; 3–4

months after the eggs are laid the snails are sexually mature and then produce 1 to 2 egg masses per week for about 5 months.

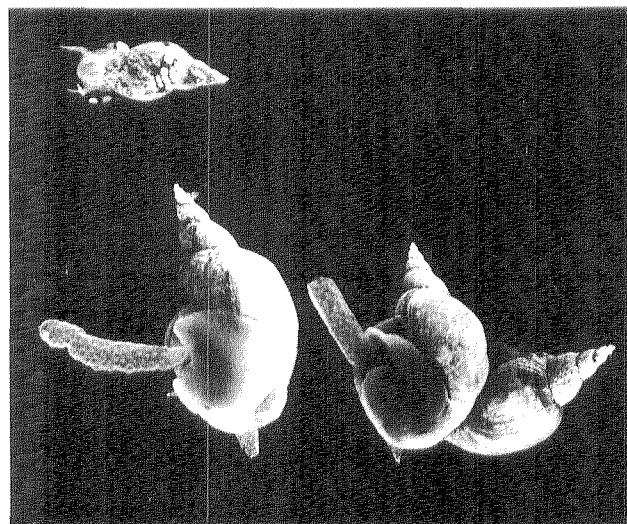
The pond snail is easily bred at room temperature and stimulated to lay eggs at any desired time. Extensive experience is available on the embryonic development of the pond snail and on the morphological and physiological deviations that may occur (Raven, 1964; Verdonk, 1965). The toxicity tests described are wholly directed at investigations with the embryos.

5.3.2.6 *Ophryotrocha diadema* (Åkesson, 1976) (Polychaeta)

The polychaete worms (so-called bristle worms) of the genus Oph-



5.3.2 Fig. 4 Zebra mussel, *Dreissena polymorpha*. Natural size



5.3.2 Fig. 5 Pond snail, *Lymnaea stagnalis*, engaged in deposition of egg masses. Natural size

ryotrocha are known to have a wide distribution area. They have been found along the Atlantic and Pacific coasts and in the region of the Mediterranean, but have never been found along the Dutch coast. *O. diadema*, which is used at our laboratory for toxicity studies, was obtained from the culture of Dr. B. Åkesson, Zoological Laboratory, University of Goteborg; this culture had originally been started with animals collected in the environs of Los Angeles (from mooring posts in harbours).

5.3 Test organisms

O. diadema is a hermaphrodite and therefore has both male and female sex organs. The female segments are posterior and the male ones anterior. Self-fertilization is not possible. The worms lay their eggs in transparent oval egg masses 3–4 mm in length.

An egg mass contains 10–35 eggs, depending on the age of the parent animal. Each sexually mature worm can produce a new egg mass about once a week.

The whole development of the egg takes place within the egg mass. At 22°C the larvae emerge from the egg mass after about 7 days. They are then about 1 mm long and have 4 segments. After about 3½ weeks the animals are sexually mature. The number of segments is now 15–17 and will usually be no greater than 20. The maximum length of the animal is 4.6 mm.

O. diadema is easy to breed in the laboratory, requiring little equipment and very little space. The worms have a high tolerance for variations in temperature (14–30°C) and salinity (18–42‰). The optimum conditions are 22°C and 33‰. The optimum conditions are 22°C and 33‰ S.

In a culture, finely chopped spinach may be offered as food. *O. diadema* is very suitable for chronic toxicity tests for which methods have been described: the tests are however, labour-intensive. Descriptions of *O. diadema* and other Ophryotrocha species, together with the possibilities of investigating toxicity with these organisms, have been given by Åkesson (1970, 1975, 1976).



5.3.2 Fig. 6 *Ophryotrocha diadema* (magnification $\times 8$)

5.3.2.7 Pelagic marine copepods (Crustacea, Calanoida)

The free-swimming marine copepods (length 1–4 mm) are the most important consumers of phytoplankton, the first link in the ecosystem.

In turn, the copepods are eaten by the larger planktonic animals including the larvae of fish such as herring, sole, and cod. Although for these reasons it is important to use these organisms as test animals in toxicity studies, they are in fact rarely used. The reason for this is that pelagic marine copepods are not easy to breed.

The pelagic copepods can be collected at sea by drawing a plankton net (mesh size 150–250 µm) slowly behind a ship about 1 m below

the surface. The net must end in an impermeable Perspex tube with a bottom, so that when it is hauled in the plankton does not dry out.

The plankton is transferred to large (e.g. 5 litre) Dewar vessels filled with sea water from the same site. The water is gently aerated en route to the laboratory.

The desired copepod species are then picked out.

In the case of highly mixed populations this is particularly time-consuming, and in addition a specialist knowledge of the species is required.

The desired species is kept in sea water for 24 h and can then be used for simple short-term toxicity tests (maximum 48 h). The animals must be kept before and during the tests in sea water collected where the copepods were trapped. It will be clear that the collection and selection of the animals with respect to variety implies much work in relation to the simple test.

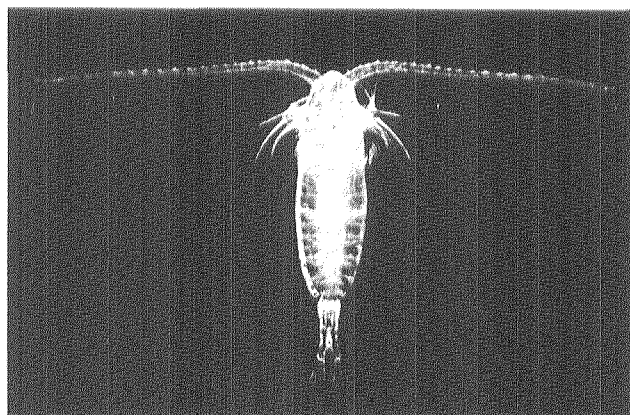
Various methods have been described for the breeding of pelagic copepods, but in our experience such methods are by no means always successful. The quality of the sea water available and of the food (unicellular algae) may be the stumbling block. Moreover, the breeding is always very labour-intensive.

Only on a few occasions have short-term toxicity tests been carried out at our laboratory with adult copepods collected at sea. Investigations have also been performed with cultures developed in micro-ecosystems (enclosed in large plastic bags – see Chapter 7), for example cultures of *Temora longicornis*. The latter is already a considerable improvement over collection at sea.

Collection in micro-ecosystems is simple and less dependent on weather conditions. Mechanical damage takes place less rapidly, and often one copepod species dominates in the plastic bags, so that selection with respect to species becomes simpler. For acute toxicity tests, the performance of which must not depend on the season or on fortuitous circumstances, and for chronic tests, however, the possibility of breeding pelagic copepods is essential. A clear description of the methods used for the breeding of various pelagic copepods, for the cultivation of algae necessary as their food, and for the performance of short-term toxicity tests is given by Gentile *et al.* (1974), who also cite the most important literature in this field.

According to these authors, large-scale uninterrupted breeding of *Acartia tonsa*, *Acartia clausi*, *Eurytemora affinis*, and *Pseudodiaptomus coronatus* is perfectly possible.

However, the method of breeding is fairly labour-intensive, especially because of the preparation of the copepods' food, which consists of four species of unicellular algae.



5.3.2 Fig. 7 Pelagic marine copepod (Crustacea, Calanoida)

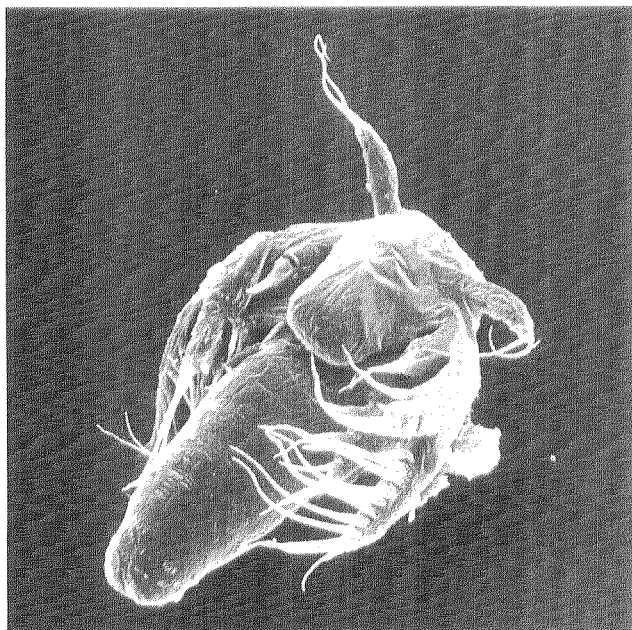
5.3.2.8 Brine shrimp. *Artemia salina* (Linnaeus, 1758) (Crustacea, Anostraca)

The brine shrimp does not inhabit seas or oceans but lives in either natural or artificial salt lakes.

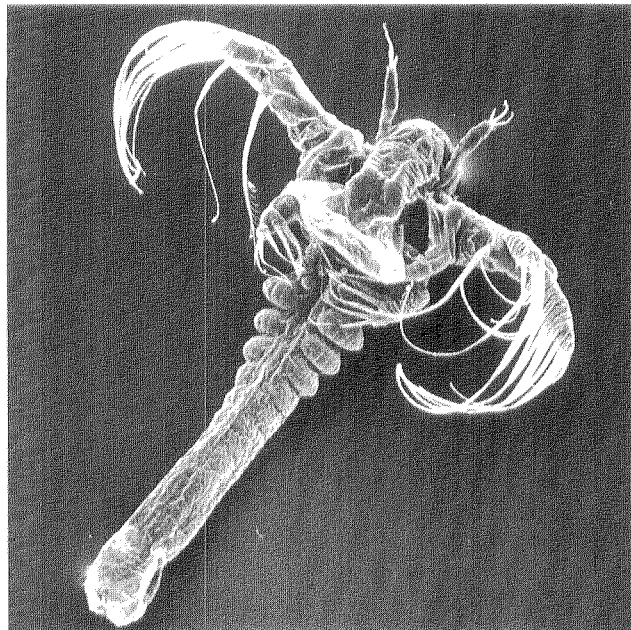
5. Determination of toxicity

A. salina can, however, be easily bred in normal sea water (30–35‰ S) and its absence in the sea is therefore presumably due to ecological rather than physiological reasons (Crogham, 1958). The brine shrimp has separate sexes. The female can produce two types of eggs, namely, eggs that are fertilized normally and emerge in the egg sac, and cysts with a hard shell that can withstand unfavourable conditions; these cysts arise parthenogenetically. The cysts are collected from salt lakes and are sold as food for ornamental fish. In sea water, larvae (nauplii) emerge from the cysts within a few days. The emergence and the rate of growth of the larvae are determined by the combination of temperature and salinity (Henting, 1971), and the rate of growth also by the nature

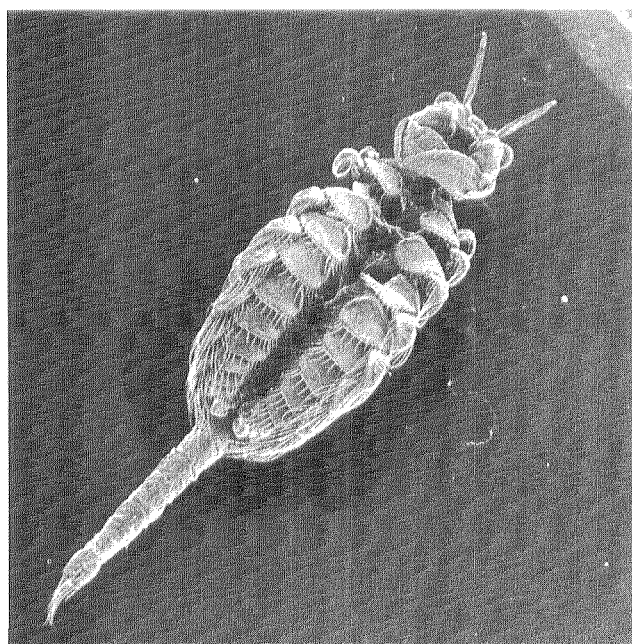
and the amount of food (Sorgeloos and Persoone, 1975 b). If the breeding of *A. salina* from the Great Salt Lake (Utah, U.S.A.) is carried out as described in Section 5.8.5, the first copulation may be expected about 10 days after the larvae have emerged and the first young emerge from the eggs after about 16 days. The length of the parent animals is then about 10 mm for the females and 8 mm for the males. After this, 100–200 larvae per female are laid for several weeks. Cysts appear at a later stage. Suitable foods for brine shrimp are unicellular algae, yeast cells, and bacteria; freeze-dried algae are also accepted (Sorgeloos and Persoone, 1975 a and b). *A. salina* is a filter feeder, which filters the food from the water with its thoracic phyllopodia.



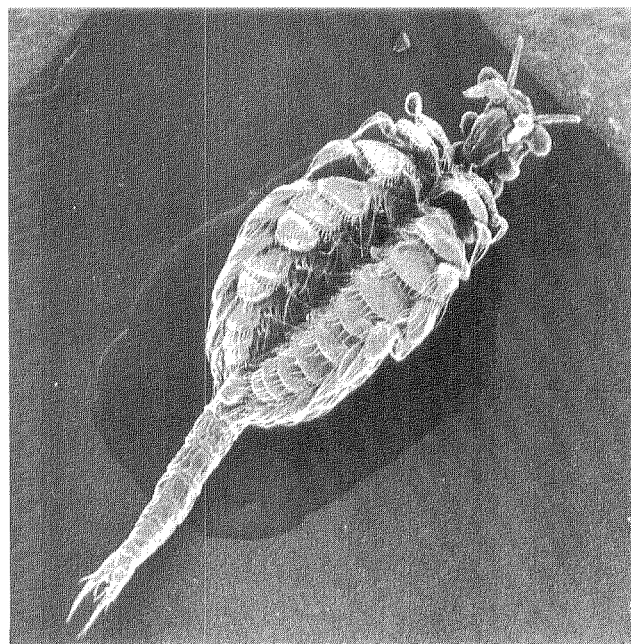
a. Larva, just after emergence, length 0,4 mm; magnification $\times 155$



b. Larva about 24 h old, length 0,8 mm; magnification $\times 90$



c. Male, about 9 days old, length 5.2 mm; magnification $\times 15$



d. Female, about 9 days old, length 5.4 mm; magnification $\times 15$

5.3.2 Fig. 8 Brine shrimp, *Artemia salina*, various stages of development (scanning electron micrographs)

5.3 Test organisms

Oxygen concentrations lower than 3.5 mg.l^{-1} have an unfavourable effect on the condition of the animals and on reproduction.

The brine shrimp is very tolerant of variations in temperature and salinity (Hentig, 1971). Since in toxicity tests *A. salina* is used as a substitute for native marine microcrustaceans, most of which are impossible or difficult to breed, normal sea water is used as the test medium. The temperature during breeding and the toxicity tests is kept at 24 to 28°C, at which the animals can develop well at the given salinity.

Acute toxicity tests can be carried out both with larvae from fertilized eggs (from own stock) or with larvae obtained from (purchased) cysts. Larvae 3 days old, well fed, and about 1.5 mm long are generally used for this purpose; younger larvae, e.g. 24 h old and 0.4 mm long, are more difficult to handle. Furthermore, second- and third-instar larvae are much more sensitive to toxic substances than first-instar larvae (Sorgeloos, in press). With the same exposure time, adult animals are distinctly less sensitive than third-instar larvae.

The maximum duration of acute toxicity tests during which the animals may be kept without food are as follows:

- 24 h for larvae younger than 24 h
- 48 h for larvae 3 days old
- 96 h for adult animals.

Although not at all representative of the North-Sea environment, *A. salina* is used very often as a test animal; this is because of the meagre possibilities of breeding native pelagic marine microcrustaceans. In comparison with various small marine organisms, the sensitivity of *A. salina* to toxic substances is low (Corner and Sparrow, 1956; Jung, 1975; Wiseley and Blick, 1967).

5.3.2.9 *Chaetogammarus (Marinogammarus) marinus*. (Leach, 1815) (Crustacea, Amphipoda)

C. marinus occurs widely in the tidal regions around the North Sea. At ebb tide it can easily be collected on sea walls overgrown with *Fucus* sp.

C. marinus is very tolerant of variations in salinity; the animal has been found at 7 to 30‰S (Den Hartog, 1964; Dorgelo, 1976). It is easy to cultivate in aquarium systems with *Fucus* sp. as food. Other organic material, such as the fish food 'Tetramin', is also accepted.

The life cycle of this crustacean is about 3 months. The females carry the eggs between their legs and the embryonic development is complete after about 10 days at a temperature of 15°C, which is the optimum (Vlasblom, 1969). Immediately after emergence, the young lead a benthic existence. The newly emerged larvae are then about 2 mm long and the length of the adult female and male animals is about 13 mm and 18 mm respectively.

(For the breeding of *C. marinus*, see Section 5.8.7).



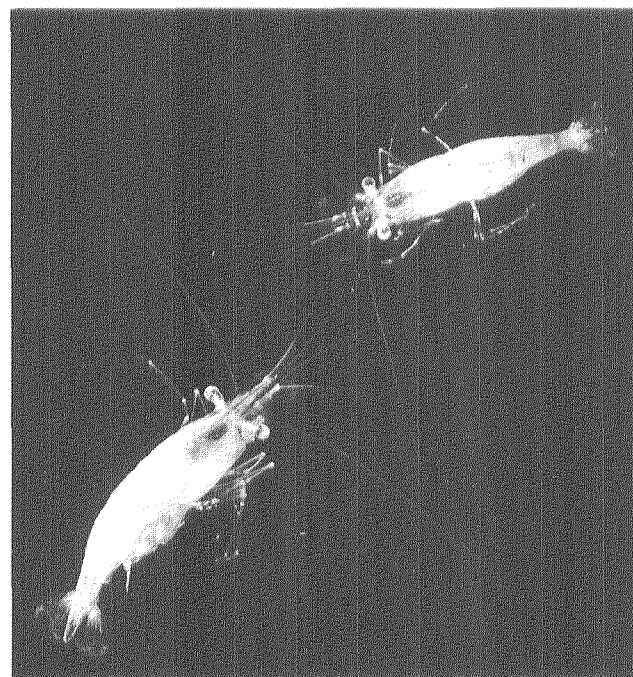
5.3.2 Fig. 9 *Chaetogammarus (Marinogammarus) marinus*. Adult specimen

C. marinus is suitable as a test animal for acute, subchronic, and chronic toxicity tests, but the long duration of the chronic test (the life cycle of *C. marinus* is 3 months) is a disadvantage. Acute toxicity tests with young animals (about 5 mm long) may last 4 days at the most if, as is customary in such tests, no food is being supplied. In comparison with other gammarid species (Den Hartog, 1964), *C. marinus* has little tendency towards cannibalism, though in the absence of food this may well occur.

5.3.2.10 Prawn, *Palaemonetes varians* (Leach, 1815) (Crustacea, Decapoda)

This prawn is widely distributed along the coasts of the North Sea, but especially in locations with brackish water. Together with the related shrimp, *Crangon crangon*, which has a totally different habitat, the prawn is one of the most abundant Decapoda natantia (swimming decapods) of the delta region in the south-west of the Netherlands.

P. varians is very tolerant to variation in salinity; it has been observed in almost fresh water and in normal sea water.



5.3.2 Fig. 10 Prawn, *Palaemonetes varians*. Adult specimens. Natural size

The prawn occurs in large numbers on the Zealand islands (Netherlands) in ditches inside the seawalls with up to 8‰S and more. Egg-carrying females are observed in these regions from April to September; larvae are present from July to September. At the age of 1 year the prawn is sexually mature and is then usually a few cm in length, but at least 7 mm (Heerebout, 1974).

The prawns, active swimmers, are easy to keep in aquaria. They readily accept all kinds of food, both dead and living organic material, such as pieces of mussel flesh, mosquito larvae, 'Tetramin', etc. Larvae emerging in the aquaria from egg-carrying females collected in the field can be bred up to the adult stage; however, this requires much care and the chances of survival still leave much to be desired.

In fact, the prawn is more attractive as a test animal for toxicity studies than the shrimp, among other things because it is not, or only slightly, cannibalistic and because of its greater tolerance to variations in salinity.

In addition, the possibilities for breeding the prawn in the laboratory offer favourable prospects, which is important for the performance of subchronic and possibly chronic toxicity tests. On the other

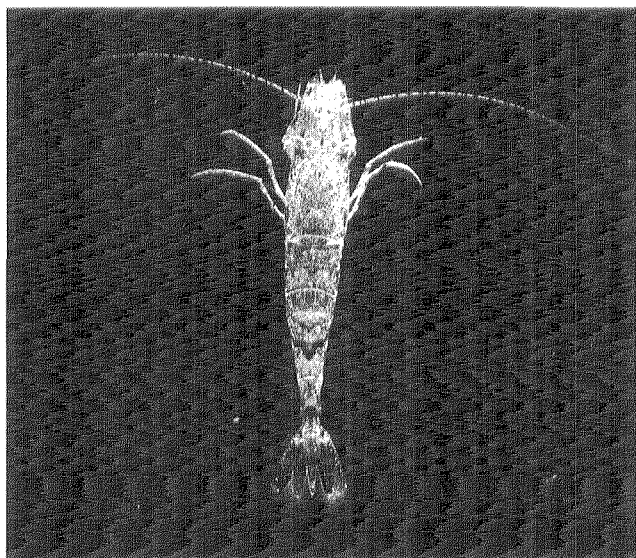
5. Determination of toxicity

hand, it must be realised that the prawn and the shrimp represent different habitats.

If no food is given, the prawns can be used in acute toxicity tests lasting about 2 weeks; longer tests are however possible, since the animals readily accept food.

5.3.2.11 Brown shrimp, *Crangon crangon* (Linnaeus, 1758) (Crustacea, Decapoda)

The brown shrimp, *Crangon crangon*, inhabits the west European coasts, the coast of the Baltic, and the western coast of the Mediterranean. It lives on sandy bottoms down to a water depth of 20 m, preferring shallow water in the summer. In the autumn the shrimp migrates to deeper waters and in the spring it returns (Boddeke, 1976).



5.3.2 Fig. 11 Brown shrimp, *Crangon crangon*. Adult specimen. Natural size

The female lays several thousand eggs 2–3 times a year, and carries them on the rear part of her body for 1–2 months. The larvae live pelagically to a length of 10 mm.

The shrimp is a hermaphrodite functioning for 1–2 years as a male and then as a female. The total lifespan is 3–4 years.

In the laboratory the animals can be kept satisfactorily at temperatures up to 18°C and a salinity of about 28‰. They readily accept food, both dead and living organic material, such as pieces of mussel.

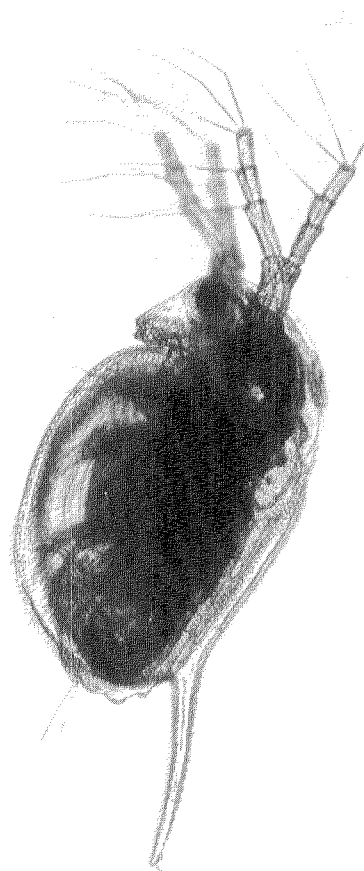
Larvae reared in the laboratory from egg-bearing females are extremely difficult to breed; the chances of survival are particularly low. (For a biological synopsis, see Tiews, 1967).

Shrimps form an important object for the coastal fishing industry. They are easy to catch in shallow inshore waters, even, on a small scale, with the aid of a small trawl.

Adult specimens can be used, without supplying them with food, in acute toxicity tests lasting about 2 weeks. Their tendency towards cannibalism is a serious drawback. In the toxicity tests each shrimp should be kept in a vessel separate from the others, e.g. by placing each animal separately in small wide-mesh rigid polythene cages.

5.3.2.12 Water flea, *Daphnia magna* (Straus, 1820) (Crustacea, Cladocera)

The water flea *D. magna* is an inhabitant of stagnant fresh water and occurs widely in the temperate climatic zone.



5.3.2 Fig. 12 Water flea, *Daphnia magna*

a. about 24 h old, magnification $\times 60$

b. about 7 days old (large photograph) and 24 h old (inset); both at a magnification of $\times 13\frac{1}{2}$

Under favourable conditions, such as the summer season, *D. magna* produces only females, and their eggs develop parthenogenetically, i.e. without fertilization, in the brood sac of the mother animal. *Daphnia* is capable of multiplying very rapidly during a favourable season.

During unfavourable periods, such as the winter season however, males appear and the females then produce two so-called winter eggs; these must be fertilized. After fertilization, they surround themselves with a hard shell against adverse circumstances (cold, drying out, absence of food).

Daphnids are filter feeders; they filter their food with the highly developed second antennae which, by their shape and rapid vibration, act as pumps and sieves and, in particular, are means of locomotion.

D. magna is easy to breed. Although in the older literature the use of natural water is said to be the only possibility for its breeding, several completely artificial media have been described in which the water fleas can be kept for years with no variation of the reproduction potential (Flückiger and Flück, 1949; Freeman and Fowler, 1953; Frear and Boyd, 1967).

The recommended hardness of these media is 100–200 mg.l⁻¹ expressed as CaCO₃; breeding has also been successful at hardnesses of up to 300 mg.l⁻¹. If the daphnids are bred in accordance with Section 5.8.6, the reproduction is exclusively parthenogenetic. When the mother animal is 7 days old and the length is then about 3.5 mm, the first young daphnids emerge from the brood sac, after which a new batch of young appears every 2–3 days; these are about 1 mm long.

The number of young per batch is 5–50, depending on the size of the female.

Under the above breeding conditions the life cycle is thus 7 days; the average lifespan is 2 months and the maximal length reaches about 5 mm.

Unfavourable breeding conditions, such as the absence of food, lead to the development of males and the production of winter eggs; this must be avoided.

Daphnids accept all types of foods, such as unicellular algae, even in the freeze-dried form (Sorgeloos and Persoone, 1975 a and b), yeast cells, and also finely-powdered trout food and grains of corn (Biesinger and Christensen, 1972).

D. magna is a very suitable experimental animal for acute (maximum test period 48 h) and chronic toxicity studies, on the one hand because it breeds easily and has a short life cycle and on the other hand because it is representative of the small native crustaceans that form an important link in fresh-water ecosystems. Moreover, *D. magna* is a sensitive test organism (Baudouin and Scoppa, 1974; McIntosh and Kevern, 1974; Morgan, 1972; Rawash *et al.*, 1975; Sanders *et al.*, 1973).

5.3.2.13 Common goby, *Pomatoschistus (Gobius) microps* (Krøyer, 1840) (Pisces, Perciformes)

The common goby occurs along the coasts of the North Sea and the Baltic, particularly in the shallow parts (0.2–2 m).

The fish has a maximum length of 5 cm and lives for about 2 years. The oval eggs, 1 mm in length, are deposited in the period from April to July at water temperatures of 12–16°C. The eggs, several thousand in number, are attached to substrates such as empty shells, rocks, and seaweeds and are watched over by the male fish.

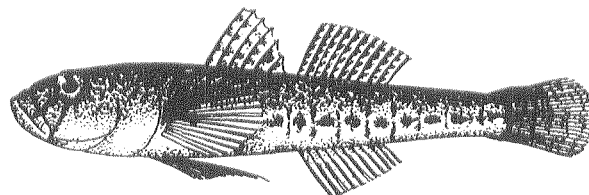
The larvae, about 3 mm long, which emerge after about 10 days, live pelagically until they are 11–12 mm long and 4–6 weeks old. The fish then change to a benthic mode of life and are sexually mature after 1 year.

P. microps is easy to catch with a beam trawl, easy to keep in an aquarium, and very tolerant to variations in temperature and salinity (down to 8‰ S).

The animals readily accept food such as mussel flesh, and they can reach the egg-depositing stage in an aquarium. The percentage of larvae that can be grown to maturity successfully is, however, very variable and is usually low.

Information concerning the biology and the occurrence of a couple of closely related fish species in the Wadden sea and some experience with their breeding in aquarium systems is given by Fonds (1970, 1973). Further studies on the breeding of *P. microps* for the purpose of toxicity studies are still necessary.

Because of its general occurrence along the Dutch coast, its small size, and the favourable prospects for breeding in aquarium systems, the common goby is a suitable subject for acute toxicity tests and also possibly in future for (sub)chronic toxicity tests.



5.3.2 Fig. 13 Common goby, *Pomatoschistus microps* ($\times 1.5$) (after Nyssen, 1968)

5.3.2.14 Plaice, *Pleuronectes platessa* (Linnaeus, 1758) (Pisces, Pleuronectiformes)

The plaice is the commonest flatfish in the North Sea. Its most important distribution area runs from the shallow coastal water (including the estuaries), where young plaice up to the age of about 2 years occur, to depths of about 40 m, where the older plaice live.

The plaice lays eggs during winter at a sea-water temperature of about 6°C and at depths of 20–40 m in spawning areas, several of which are located in the North Sea.

With their diameter of 1.6 mm, the fertilized eggs float to the surface and are there slowly transported towards the coast by the currents, the larvae emerging during this transport process 15–20 days after fertilization. The larvae, about 6 mm long, live pelagically and feed for the first month after absorption of the yolk sac on planktonic larvae and then on copepods. About two months after their emergence the larvae undergo metamorphosis; the left eye migrates over the head to the other side and the larvae grow greatly in 'width' (dorsoventrally), and thus acquire the typical flatfish shape. The young plaice (about 1.5 cm long) then swim with the left side turned downward, and the animals pass completely to bottom living and populate the shallow estuaries and coastal waters in great numbers (Zijlstra, 1972). In general, the plaice accepts food that it finds *in situ*. At the age of 2 years the plaice migrates to deeper water. The male plaice is sexually mature in the 3rd or 4th year of life, the female in her 6th year. For the laying of eggs and milt, the animals seek the spawning region where their lives began years ago.

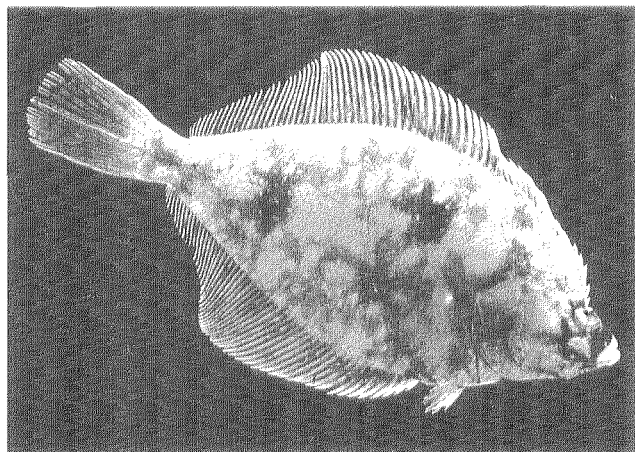
The plaice is a common test animal in marine biological laboratories and is used for investigations of all kinds. Success was achieved in its breeding some decades ago (Shelbourne 1963; Shelbourne *et al.*, 1963), and this gave the stimulus for the development of the breeding of marine fish of greater economic importance, such as the sole.

Because of the existing large body of knowledge concerning the plaice, the latter is a particularly attractive test animal for toxicological studies. However, its breeding for toxicological purposes cannot be justified. Young plaice up to the age of 2 years can be caught fairly easily with a beam-trawl; if healthy and undamaged, the animal can be kept satisfactorily in aquaria at 15°C and 28‰ S. (However, the tolerance to variations in temperature and salinity is very good). The caught fish need a considerable time to become accustomed to food such as mussel flesh; if they are used within 1–2 weeks for an acute toxicity test, feeding can be omitted. Because of the fairly fast growth of the young plaice (7–10 cm per year), fish of a given length are not available at every time

5. Determination of toxicity

of the year. The plaice is one of the few economically important North Sea fish whose eggs and larvae can be grown relatively easily in the laboratory, so that there is a possibility of carrying out subchronic toxicity studies with these young developmental stages.

However, plaice eggs are available only in the winter season, which is a serious disadvantage for toxicity studies.



5.3.2 Fig. 14 Plaice, *Pleuronectes platessa*

5.3.2.15 Guppy, *Poecilia reticulata* (Peters, 1859) (Pisces, Cyprinodontiformes)

The natural environment of the guppy is in the brackish river deltas in tropical regions. The fish is also well known to devotees of tropical fresh-water aquaria. In the guppy fertilization takes place internally. For this purpose the male guppies possess a gonopodium, a fusion of a number of rays of the anal fin to form an organ of copulation; this is how the male guppy can be distinguished from the female guppy. Moreover, the males are much more colourful than the females.

The embryonic development of the eggs and the hatching of the young guppies takes place within the body of the female. The guppy is viviparous. The female guppies can store sperm, so that 4 or 5 broods may arise from one mating. The first brood is always small (about 5 young) but with each brood the number of young increases up to the fourth, which is usually the largest (30–50 young); the number of young per brood then falls off again. 4–5 months after birth the guppies are sexually mature and can produce the first young about a month later. The guppy recognizes no definite spawning season; the production of young extends over the whole year.

The guppy is easily bred in fresh water, in brackish water, and in sea water (see Section 5.8.8). For this reason, and also because of the small size of the fish, guppies are suitable subjects for toxicological studies. It has been found that 3–4-week-old guppies are the most sensitive to toxic substances. In order to carry out toxicity tests with guppies of this age, a brood stock must be available.

Both an acute toxicity test (see Section 5.9.8.1) and a subchronic toxicity test (see Section 5.10.7.1) with such young guppies are described in this manual.

Although chronic toxicity tests are of course also possible with the guppy, no test method for this is described because experience so far has been insufficient.

Guppies are easily available commercially; it is recommended to buy them directly from an importer of ornamental fish. Ignorance of the age of the fish is sometimes a great disadvantage; an acute toxicity test with purchased guppies is described in this manual (see Section 5.9.9.1).

5.3.2.16 Golden orfe, *Leuciscus idus* var. *melanotus* (Linnaeus, 1758) (Pisces, Cypriniformes)

The golden orfe is a golden-yellow variety of the orfe, a fresh-water fish that occurs generally in the Netherlands and in the countries of central and northern Europe, but not in Britain, France, or south of the Alps.

In March to April the orfe swims up the rivers to spawn. Spawning takes place in shallow water at 7–8°C in places with a sandy and gravelly bottom. The somewhat sticky eggs are deposited on aquatic plants or stones, and the larvae emerge after 2–3 weeks and then seek calmer water.

The golden orfe variety is kept as an ornamental fish in ponds and aquaria. There are a number of golden orfe hatcheries in West Germany, where the golden orfe is frequently used as the test animal for acute toxicity tests. Only young specimens between 5 and 7 cm in length are used for the tests. Other conditions under which the test with the golden orfe must be performed have been described in detail (Anon., 1976).

5.3.2.17 Rainbow trout, *Salmo gairdneri* (Richardson 1836) (Pisces, Salmoniformes)

Various forms of this species were imported from North America around 1890 by European fish hatcheries and since then have been a fish farming species. Most attempts to build up a wild stock of the species by the introduction of bred fish have failed, although populations living in the wild were established for some time in central and south-eastern Europe.

In the Netherlands the rainbow trout is bred in a number of places for consumption and for introduction into surface waters for sport-fishing.

This trout spawns during winter. In the hatcheries the ripe eggs are then squeezed out of the female trout and fertilized artificially. The eggs, 1000–5000 per trout, are kept in oxygen-rich flowing water at the ambient temperatures (generally between 4 and 8°C). After about 1–2 months, depending on the temperature, the yolk-sac larvae hatch from the eggs; when after a few weeks the yolk sac has been absorbed, the young are brought on with a commercial, artificially prepared, protein-rich food.

The tolerance of this fish to variations in temperature and water hardness is fairly low, and the dissolved oxygen content must be high; suitable values are 12–15°C, ca. 50 mg.l⁻¹ expressed as CaCO₃, and 90–100% of the saturation value, respectively.

The rainbow trout is much used in toxicity tests because of its good availability (from hatcheries) and the extensive biological knowledge that exists concerning this fish, and also because of its relatively high sensitivity (in comparison with other fresh-water species) to toxic substances.

Specimens between 5 and 10 cm in length are generally used for acute toxicity tests. However, they are not available in this size throughout the year.

Chronic toxicity tests with the rainbow trout have also been described in the literature.

5.3.3 Collection, transportation, and acclimatization of the test animals

The animals used for toxicity studies must be in good health and condition at the start of the experiments. This implies that all operations associated with the collection, transportation, and keeping and/or acclimatization of the animals in the aquarium systems must be carried out with the greatest care and usually with many precautions.

The precautions include the preparation of aquaria in which the necessary number of animals can be contained without overcrowding; the physico-chemical and biological conditions of the aquarium environment should be adapted as closely as possible to the requirements of the organisms.

It is important that the capture and transportation of the animals

5.3 Test organisms

should cause no physical damage. However, this cannot always be ensured, and part of the catch may then be unsuitable for the tests.

Fishing gear for commercial purposes is frequently unsuitable for the collection of living animals.

The use of drag-nets behind motor boats may be the only way for certain test species, but the chances of damaging the animals are then very high and the collected material must be carefully sorted out. A slow, short 'trail' gives the best results.

It is preferred to catch pelagic and benthic organisms with nets operated by hand.

In tidal regions use can be made of the fact that many animal species, for example the plaice, the common goby, and the brown shrimp, withdraw at ebb-tide from the intertidal mudflats towards the slopes of the deeper gullies; at ebb-tide many benthic organisms are found in great numbers at these points.

The collection of sessile organisms such as mussels, slipper limpets, and zebra mussels presents no practical difficulties.

In general, from the brief characteristics given for a number of suitable test animals in Section 5.3.2 it is possible to determine what methods of collection will be most appropriate for a given animal; the local circumstances will often also contribute to the selection of the method.

After collection, do not expose the animals unnecessarily to air; sort the catch rapidly and transfer suitable specimens immediately into a tank with the same water as that in which they were caught. Transport them in this water to the laboratory. To keep the temperature of the water constant, it is convenient to transport the animals in water-tight plastic bags and to pack these in thermally insulated boxes. The use of a refrigerated vehicle may also be considered in this connection.

During the transportation sufficient oxygen must be provided. This can be done by connecting the containers with the animals to an air pump. Air-tight closed plastic bags, into which a substantial amount of pure oxygen has been introduced above the surface of the liquid, are very suitable for transporting the animals. (It must be mentioned, perhaps superfluously, that there must be no smoking when bags containing pure oxygen are opened). Under these conditions, the amount of water in which the animals are transported may be very small. Sessile organisms such as the above-mentioned shellfish can always be transported in very small amounts of water or even 'dry'. Transport only one animal species per container or plastic bag and avoid excessive numbers per container. Once in the laboratory, the animals must be placed immediately in aquaria filled with water of approximately the same composition (salinity, hardness, etc.) and approximately the same temperature as that in which they were caught. In any case, avoid shock effects.

Dead or severely damaged animals must be discarded at once. Keep not more than 4 g of biomass per litre in the aquarium system per 24 h, and preferably 1 g per litre. Filter the water continuously through active carbon if it is a recirculating aquarium system.

Use about 2 litres of active carbon per 100 litres of water and renew the carbon once a week to once a month.

In a recirculating system, with the above biomass loading, the water must be completely or 90 % renewed at least three times a week. Aerate the water continuously in such a way that the oxygen concentration remains above 80 % of the saturation value.

Acclimatize the animals to the laboratory conditions in this way for at least one week. If during this period the mortality is low ($\leq 10\%$), the animals can be used for the toxicity tests, provided that the physico-chemical conditions of the water in the experiments (apart from the toxic compound) are the same as those to which the animals have already been acclimatized. If the test conditions are different, e.g. different temperature, salinity, hardness, etc., the animals must first be acclimatized to the new conditions. This acclimatization period must also last at least one week, during which period the mortality must be low ($\leq 10\%$). If possible, during the entire acclimatization period the animals must be fed with suitable food (in the case of an acute toxicity test, they are fed up to 24 h before they are taken for the test).

Newly caught animals will sometimes refuse food in their new laboratory surroundings, but may occasionally be induced to eat by placing them with animals of the same species that do accept food. If the animals will not accept food, their condition deteriorates very rapidly and they can be used only in short-term experiments performed shortly after collection.

For literature on the subject of this section see, *inter alia*, EPA-660/3-75-009; Doudoroff *et al.*, 1951; Perkins, 1972 a and b.

5.3.4 Number of test animals and the amount of biomass per test vessel

For practical reasons, one would wish to keep the test vessels for the toxicity tests as small as possible. In order to satisfy the oxygen and the space requirements of the test animals the amount of biomass per vessel must then be limited to the greatest possible extent.

The number of test animals per concentration of the toxic test substance must of course be statistically appropriate. However, very little is known on this point. For the trout, Alabaster and Abram (1965) have indicated that with more than 10 individuals per test concentration the results are only slightly more accurate than with 10 individuals. For another test-animal, the fish *Rasbora heteromorpha*, the same authors also chose 10 individuals per vessel as the standard (see also Alabaster, 1970).

For 'minnows', Jensen (1972) indicates that by increasing the number of animals per test concentration the standard deviation of the LC_{50} falls appreciably until the number reaches 30. Doudoroff *et al.* (1951) have recommended a minimum of 10 fish per concentration. The EPA Report (EPA-660/3-75-009) has indicated a preference for numbers of 10 to 20.

In general, it can be stated that for subjects such as fish and macro-invertebrates it is customary to perform the toxicity tests with 10 individuals per test vessel; this means that 20 individuals per test concentration are used when the tests are carried out in duplicate. For small organisms (daphnids, copepods, pond snails, etc.) a larger number of individuals per test concentration is easily achievable. Numbers of 40 to 50 per concentration are then customary.

It is important that, as far as possible, the test animals should be of approximately the same size (and age) to minimize individual differences in sensitivity to the toxic substance.

However, this is not always possible with specimens collected in the field or obtained from commercial hatcheries. The animals must then be selected so that the largest and the smallest individuals of a group used in any one test series do not differ too greatly in size.

Doudoroff *et al.* (1951) give as the maximum permissible deviation that the largest fish may be 1.5 times the length of the smallest. In this connection, the above-mentioned EPA Report gives a factor of 2 for fish and macro-crustaceans. Some animal species, e.g. the mussel, can easily be collected in several sizes and it is easy to measure their length without causing damage. Stricter requirements can then be set for the length of the test-animals, e.g. 30–35 mm for mussels.

The total amount of biomass that may be present per unit volume depends mainly on the available amount of oxygen in the test solutions and on the extent to which the concentrations of the test compound will be reduced by the test animals (through accumulation).

In order to maintain lethal concentrations of an apolar material such as DDT, a ratio of 1 g of biomass/1 l/24 h appears to be sufficient (Prevost *et al.*, 1948).

Alabaster and Abram (1965) have indicated that in flow-through systems 1 g of biomass/10 l/24 h is necessary to maintain the oxygen concentration without special aeration of the water.

Other recommended biomass/water volume ratios are as follows:

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For 96-h static tests:

- ≤ 1 g/l (Am. Publ. Hlth. Ass., 1971)
- ≤ 0.4–0.8 g/l (EPA-660/3-75-009)
- ≤ 2 g/l and ≤ 1 individual/l (Doudoroff *et al.*, 1951).

For flow-through tests:

- per day ≤ 1 to 2 g/l and ≤ 10 to 20 g/l (absolute) (EPA-660/3-75-009).

In our laboratory the following biomass/water volume ratios are maintained:

For semi-static tests:

per day: < 1 g/2 l for tests with volatile substances (without aeration)

per day: < 1 g/1 l for tests with non-volatile substances (with aeration).

For flow-through tests:

per day: < 1 g/5 l for tests with volatile substances (without aeration)

per day: < 1 g/2 l for tests with non-volatile substances (with aeration).

The oxygen concentration in all the test vessels is always measured daily. If necessary, the amount of the test solution per gram of biomass can be adapted to the oxygen demand of the test animals.

5.4 TEST SYSTEMS AND THEIR USE

5.4.1 The static method

The test system for the static method consists of a series of vessels in which the animals are exposed to a series of increasing concentrations of the test compound in water; during the test this medium is not renewed. The performance of tests by the static method is basically simple. The problems involved have been discussed in various sections of Chapter 5.

The successive operations can be given briefly as follows:

- Place the necessary test vessels of suitable size and shape in a room or a water bath at a constant temperature optimal (or acceptable) for the test animal.
An illustration of suitable ratios of biomass and test volume is given in Section 5.3.4.
The influence of the physico-chemical properties of the test compound, such as its volatility and polarity, on the choice of the vessel dimensions is illustrated in Section 5.4.4.
- Code the test vessels and fill them with the medium having the correct concentration of the test compound or the correct dilution of the waste mixture (see Section 5.2).
- Check the pH, the oxygen concentration, and if necessary the salinity and adjust these to the desired (usually optimal) values (see Section 5.5.1).
- Collect samples of the test medium for chemical determination of the concentration of the test compound (if possible) (see Section 5.5.1).
- Aerate, if this does not unfavourably affect either the concentration of the test compound (see Section 5.4.4) or the condition of the test animals (see Sections 5.9 and 5.10).
- Place a statistically appropriate number of test animals in each test vessel (see Section 5.3.4).
- Regularly check the condition of the test animals: remove and count the dead specimens (see Section 5.5.2).
- Check the oxygen concentration daily and measure the pH and, if necessary, the salinity at least at the end of the test (see Section 5.5.1).
- If possible, determine the concentration of the test compound at the end of the test (see Section 5.5.1).
- End the test after 4 days at the latest (see Section 5.5.3).

The tests should preferably be done in duplicate; in order to determine the reproducibility, they are repeated at a different time. Each test is performed in comparison with a blank. If a solvent other than water is used for metering out the test compound, another blank including the solvent at the same concentration is tested.

5.4.2 The semi-static method

Semi-static tests differ from the static tests by the fact that the test medium (water with the correct concentration of the test compound) in the vessels is regularly renewed. The renewal frequency is selected on the basis of the oxygen demand of the test animals and the rate of die-away of the test compound. However, renewals must not be so frequent that the test animals are adversely affected. It is not practical to renew the medium with a regular frequency greater than once per 24 h. The usual times are once per 24 h, once per 48 h, or each Monday, Wednesday, and Friday. Renewal can be performed in two ways, namely:

- a. After the necessary checks have been made on the used test medium, about 90 % of the used medium is removed from the vessels without harming the animals. At the same time any solids

are removed from the bottom and the vessels are refilled with freshly prepared test solution on which the necessary checks have previously been carried out.

- b. By using two series of test vessels per test. One series contains the animals and the other is clean and empty. Just before renewal the test solution is freshly prepared in these latter vessels and the necessary checks are performed. The test animals are then transferred to them in such a way that they are not harmed. Subsequently the necessary checks are made in the used medium, after which these test vessels are emptied and cleaned out.
The coding on the vessels must be retained, so that on renewal the same concentrations of the test compound are always present in the same vessels. Traces of the test compound are not specially removed.

In method a. the fresh test solution is, as far as possible, prepared in a separate vessel and is then transferred to the appropriate test vessel. If for technical reasons (large volumes) this is impossible or impracticable, the animals are transferred to a clean vessel in a small volume of the test medium, after which the medium in the original vessel is renewed and the animals are replaced as rapidly as possible.

If the animals are harmed by these treatments, they are left in the original vessel in the remaining 10 % of the medium; this vessel is then three-quarters filled with water, the test compound is dosed in, and the vessel is rapidly filled to the final volume, care being taken that the contents are mixed rapidly and thoroughly. However, this can be done only if the pH does not need to be adjusted.

Method b. is preferred because the test vessels can be cleaned better, which prevents the consumption of oxygen and adsorption of the test compound by residues of dead organic material. However, the method is used mainly for small volumes of the medium (5 l) and therefore for tests with small test animals.

Determinations of the pH, the oxygen concentration, and the concentration of the test compound are carried out at least twice in the freshly prepared and twice in the used test solutions per test. Care is taken that the same solution that was checked in the freshly prepared state is also checked after use.

The test period can frequently be longer in semi-static tests than in static tests (see Section 5.5.3).

5.4.3 The flow-through method

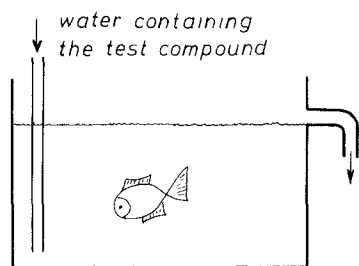
5.4.3.1 Introduction

The use of the flow-through method in place of the semi-static method offers advantages particularly in:

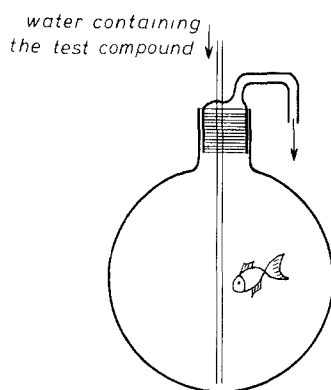
- a. Tests in which no aeration can be provided and in which the amount of the biomass per unit volume is too great for the amount of oxygen available per 24 h.
- b. The testing of substances that readily disappear from the aqueous phase.
- c. Long-term tests.
- re a. The degree of renewal achievable in semi-static tests amounts to 1 × the volume of the test vessel per 24 h. With flow-through systems the degree of renewal can be several times as large and is limited only by the available amount of water.
- re b. In these cases also a higher renewal frequency than once per 24 h is often necessary to keep the test concentration constant.
- re c. Toxicity tests with efficient permanent flow-through systems are less labour-intensive than the semi-static method in which repeated renewal must be carried out manually.

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The principle of tests in flow-through systems is shown schematically in 5.4, Fig. 1.



5.4 Fig. 1a Flow-through system for use only with non-volatile test compounds



5.4 Fig. 1b Flow-through system for all types of compounds

The test solution – dilution water with the correct concentration of the test compound – is directed continuously into the vessel with the test animals. An amount of solution equal to that supplied is discharged via an overflow. In some systems the feed is discontinuous, but so frequent in relation to the test duration, e.g. a certain amount every 5–10 min, that it can be regarded as continuous.

The dimensions of the vessels are determined by the species and number of the animals used. The degree of volatility of the test compound determines the method of closing the test vessels.

The desired flow rate through the system is then established. This depends on the physico-chemical properties of the compound and on the oxygen demand of the animals. The flow rate through the test vessels must be sufficient to maintain the concentration of the test compound throughout the test and, if no aeration is carried out, to maintain the oxygen concentration at more than 70 % of the saturation value. If the latter condition is satisfied, usually the former is also satisfied and at the same time the content of toxic excretion products of the animals (such as CO_2 and NH_3) is kept sufficiently low. For calculations of a suitable flow rate through the system, see Alabaster and Abram (1965a).

For the flow-through method the test solutions – dilution water with various concentrations of the test compound – must be made up continuously from a reservoir of dilution water and one or more stock solutions of the test compound, and be fed into the test vessels.

Various systems have been described for this purpose, which will be classified here according to their mode of operation:

- Systems utilizing hydrostatic pressure exclusively, e.g. Abram (1960); Grenier (1960); Mount and Warner (1965); Mount and Brungs (1967); Stark (1967); Chandler *et al.* (1974); Davis (1974); DeFoe (1975).
- Systems utilizing hydrostatic pressure but with pneumatically controlled valves, e.g. Abram (1973).
- Systems utilizing hydrostatic pressure but with electrically controlled valves, e.g. Alabaster and Abram (1965a and b); Alabaster (1970) (both the same system).
- Systems utilizing hydrostatic pressure but with electric metering pumps, e.g. Connor and Wilson (1972); Swedmark (1974).
- Systems controlled entirely with electric pumps and/or electrical switches.

The important considerations in the choice of one of these systems may be:

- a. the avoidance of electricity in the usually wet fish toxicology laboratories;
- b. the use of a system in which the metering of the test compound stops as soon as the metering of the dilution water fails.

5.4.3.2 Further illustration and short description of some common systems (review of the literature)

a. Systems working exclusively on hydrostatic pressure

A much-used system of this type is that according to Mount and Brungs (1967), in which flow rates of 50 to 500 litres per 24 h can be achieved.

For the described system the number of different concentrations that can be metered simultaneously is 5 plus a blank, each successive concentration being half to a quarter of the preceding one. (The system has also been constructed in our laboratory for 3 concentrations and one blank, with each successive concentration being one-third of the preceding one).

The system is particularly suitable for large test vessels and large amounts of biomass. The metering system is completely open, which is a disadvantage in the testing of volatile substances. The metering of the test compound stops as soon as the feed of dilution water fails.

The system was described by Mount and Brungs for metering stock solutions of the test compounds both in water and in organic solvents. In the former case the stock solution is diluted about 1:100 to prepare the most concentrated test solution. In the metering of substances dissolved in an organic solvent, the dilution is about $1:10^5$ (with a mechanically driven injection syringe).

On the basis of Mount and Brungs' system, DeFoe (1975) has described a system in which the metering of the test compound is modified in that concentrations can be selected independently of one another and the amount of the organic solvent in the different solutions is the same. Moreover, this system may be reasonably well sealed off from the metering point, for the purpose of retaining volatile materials.

Again basing themselves on the Mount and Brungs system, Chandler *et al.* (1974) described a system in which the metering of the test compound has been modified. In this the concentrations are dependent on one another and the system is completely open, but there are no moving parts.

b. System working on hydrostatic pressure and with electrically controlled valves

This system was described by Alabaster and Abram (1965 a and b) and by Alabaster (1970) for a flow rate of 10 ml/min (=14.4 litres per 24 h) and for completely closed vessels with a capacity of 0.5 litre.

The number of different concentrations that can be metered simultaneously is unlimited, and they can be chosen independently of one another. This system is suitable only for small amounts of biomass; it was described for 10 small fish with a total weight of about 1.4 g. Because of its closed construction, the system can also be used for testing volatile substances. Abram (1973) described a similar system in which, however, no electric power is necessary but pneumatic control is used.

This system was described, *inter alia*, for use with 40-l aquaria with metering.

c. Systems with electric metering pumps

The use of electric metering pumps makes the construction of continuous metering systems much simpler, but obviously also more vulnerable because of the possible power failure, malfunction of the pumps, etc., while attention must also be paid to electrical safety.

In these systems constantly adjustable streams of the dilution water and of the stock solution of the test compound are mixed before they enter the test vessel. Either both flow rates can be adjusted with metering pumps or only the flow rate of the stock solution, while the flow rate of the dilution water (which is usually much higher) can be controlled by an adjustable constant pressure difference.

Metering systems on the last-mentioned principle have been described for example by Connor and Wilson (1972) and by Swedmark (1974).

These can be constructed in various sizes and are suitable, with sufficient accuracy, for flow rates of 10 to 500 l per 24 h.

The number of different concentrations that can be metered simultaneously is unlimited, and the concentrations can be chosen independently of one another.

The principle is usable for large and small amounts of biomass, depending on the dimensions and the flow rate. By choosing the correct type of test vessel (see 5.4, Fig. 1) the system can be made suitable for various test compounds.

For smaller flow-through rates there is a number of other possibilities, for example:

- The flow rates of the dilution water and the stock solution are adjusted by peristaltic pumps or other suitable metering pumps.
- Stock solutions of the test compound in the dilution water are prepared at the desired concentration beforehand – sufficient for a few days – and are fed into the test vessels at a constant rate. The flow rate can be adjusted by a metering pump or a constant pressure, e.g. with a Mariotte bottle (Davis, 1974). This method can be used only if these stock solutions retain a constant composition.
- Commercially obtainable metering systems, e.g. the 'Programmable Dilution Apparatus' from Huntingdon Instruments. This system meters fully automatically, with electrical adjustment and in controllable fashion, five adjustable concentrations of the test compound plus a blank. The flow rate can be adjusted from 0.2 to 70 litres per 24 h. The apparatus is suitable only for soft fresh water. In the standard design the blank dilution water is subjected to slight contamination with the test compound.

The review given in this section is designed particularly to compare the construction and the properties of some systems frequently mentioned and described in the literature.

For a complete description of the systems, reference is made to the original literature.

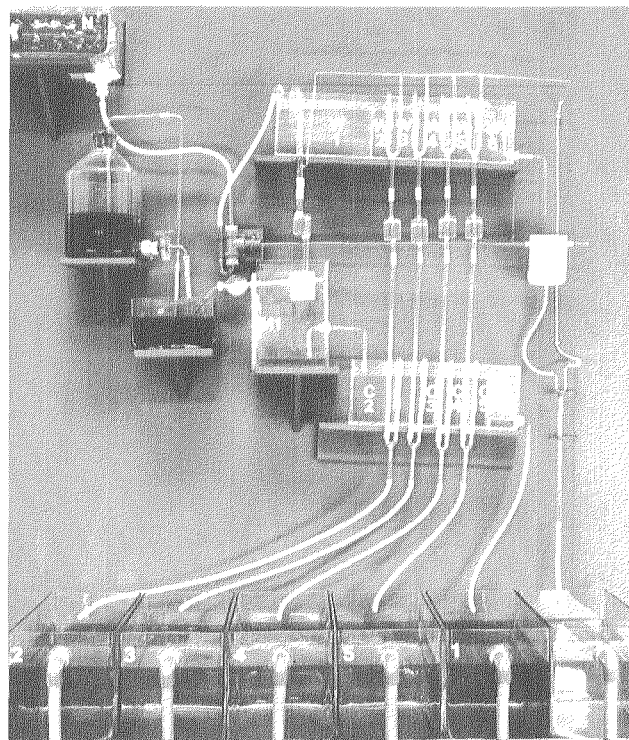
The systems with which our laboratory has had experience are described in the following section.

5.4.3.3 Description of some systems with which the TNO laboratory has had experience

a. The Mount and Brungs (1967) metering system

The photograph (5.4, Fig. 2) and the schematic drawings (5.4, Fig. 3) differ in a few inessential points because of slight modifications during construction.

Dilution water flows from the constant-level reservoir N (see 5.4, Fig. 2) via NV1 (see 5.4, Fig. 3a) into the cells W-1 to W-6 (the reservoir N is not shown in 5.4, Fig. 3a). As soon as W-6 is full the water flows via siphon WS-6 into the plastic bucket VIBu. As soon as this is also full, by its own weight it shuts off the



5.4 Fig. 2 Metering system with test vessels constructed according to Mount and Brungs (1967) and suitable for metering five concentrations from a stock solution in water plus a blank

water feed to W-1 via NV-1 (see 5.4, Fig. 3b). Via a siphon in the plastic container VIBu, this empties via tube VaV, so that an underpressure arises in the tube VaMa, and the cells W-1 to W-6 empty via siphons. At same time the water from W-6 renews the 'blank' test solution. The streams of water from W-2 to W-5 entrain the contents of C-2 to C-5, these cells all containing a 1:100 dilution of the stock solution of the test compound present in the reservoir and the constant-level vessel (see 5.4, Fig. 2; in 5.4 Fig. 3c, Mariotte bottle + funnel).

The mixed streams of water from W-2 + C-2, W-3 + C-3, etc., flow into the test vessels containing the animals (see 5.4, Fig. 2). The relative dimensions of the cells are chosen in such a way that the desired concentrations are formed. The test vessel with the most concentrated solution is renewed from C-1.

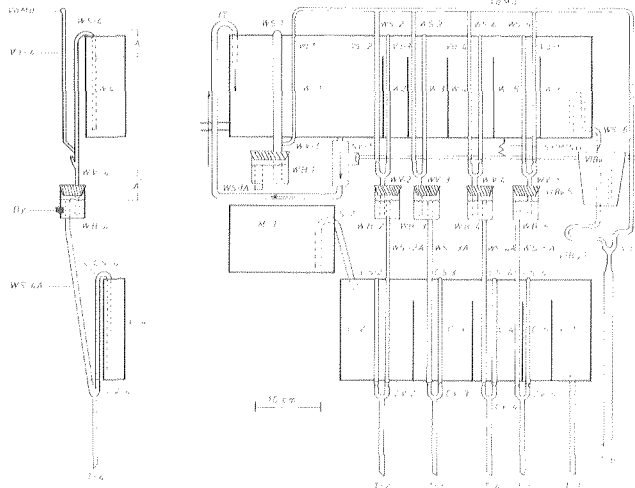
The cells C-1 to C-5 are filled each time with the 1:100 dilution of the stock solution because the water from cell W-1 via the plastic bucket with a drain hole makes the 'see-saw' tip, so that a measured amount of stock solution mixed with the water from W-1 passes into M-1 (see 5.4, Fig. 2 and 5.4, Fig. 3a and c) (measured amount of stock solution: volume of W-1 = 1:100). When M-1 has filled up to the siphon, C-2, C-3, C-4, C-5, and C-1 are filled from it.

As soon as the plastic bucket is completely empty (via the drain hole, 5.4, Fig. 3c), the 'see-saw' falls back due to the counterweight and is ready for the subsequent metering operation. The metering of the test compound is done from an aqueous solution. The metering system described, 5.4, Fig. 3c, is not suitable for the metering of poorly water-soluble substances.

Mount and Brungs (1967) have also described a system for metering solutions in an organic solvent. However, here different amounts of solvent are added to the test vessels.

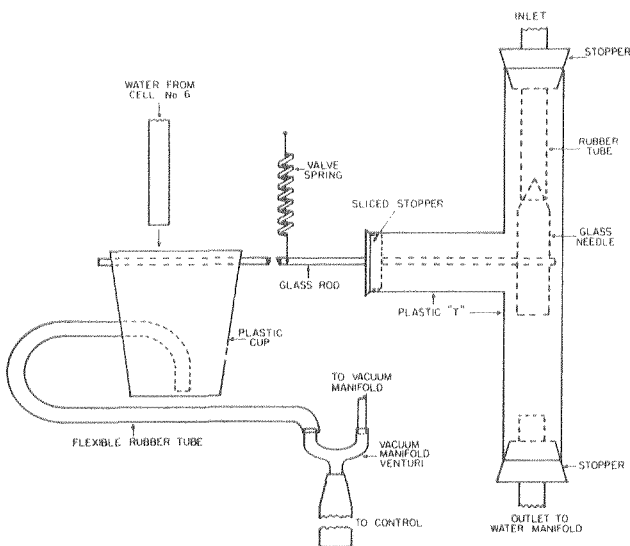
DeFoe (1975) has described a method in which the amounts of the organic solvent in each test vessel are the same and Veith and Comstock (1975) described a method of avoiding the use of organic solvents for compounds very sparingly soluble in water.

5. Determination of toxicity

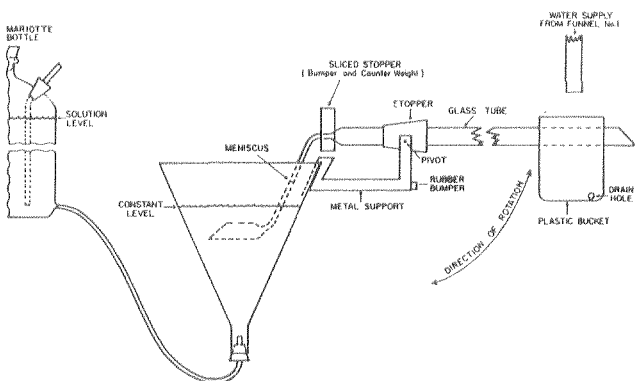


5.4 Fig. 3a Semi-schematic scale drawing of the diluter

Legend: B-block; Bu-bucket; By-bypass spout; C-chemical; I-influent; M-mixing; Ma-manifold; N-needle; S-siphon; T-tube; V-Venturi; Va-vacuum; VI-valve; W-water



5.4 Fig. 3b Needle valve and vacuum venturi detail

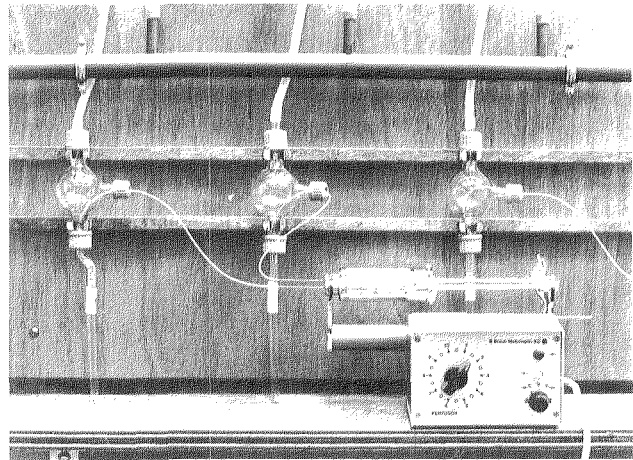


5.4 Fig. 3c Detail of chemical-metering apparatus

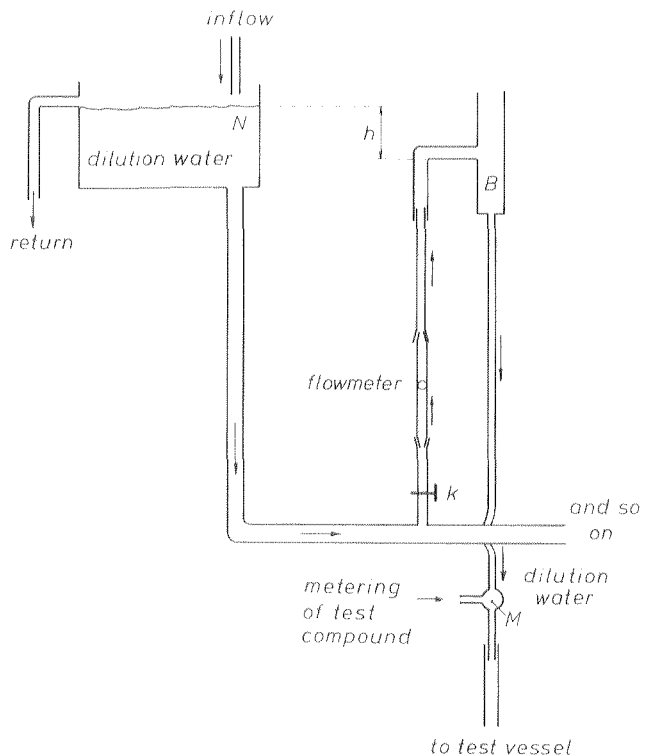
5.4 Fig. 3 Schematic drawings of the apparatus shown in 5.4, Fig. 2 and some of its details
(After Mount and Brungs (1967))

b. The Connor and Wilson (1972) metering system

Dilution water flows from the constant-level reservoir N (see 5.4, Fig. 6) to vessels B adjustable to various heights and from there to the mixing bulbs M. The distance h (see 5.4, Fig. 5) determines the rate of flow. A flowmeter can be placed in the position shown in the drawing and by means of this the flow rate can be monitored in a simple manner. Taps K are used to isolate the test vessels from the metering system. A stock solution of the test compound is also fed at a constant rate to the mixing bulb M with the aid of an accurately adjustable metering pump, e.g. a peristaltic pump or a 'perfusor' (see 5.4, Fig. 4). The mixture of the dilution water and the stock solution is fed in near the bottom of the test vessel.

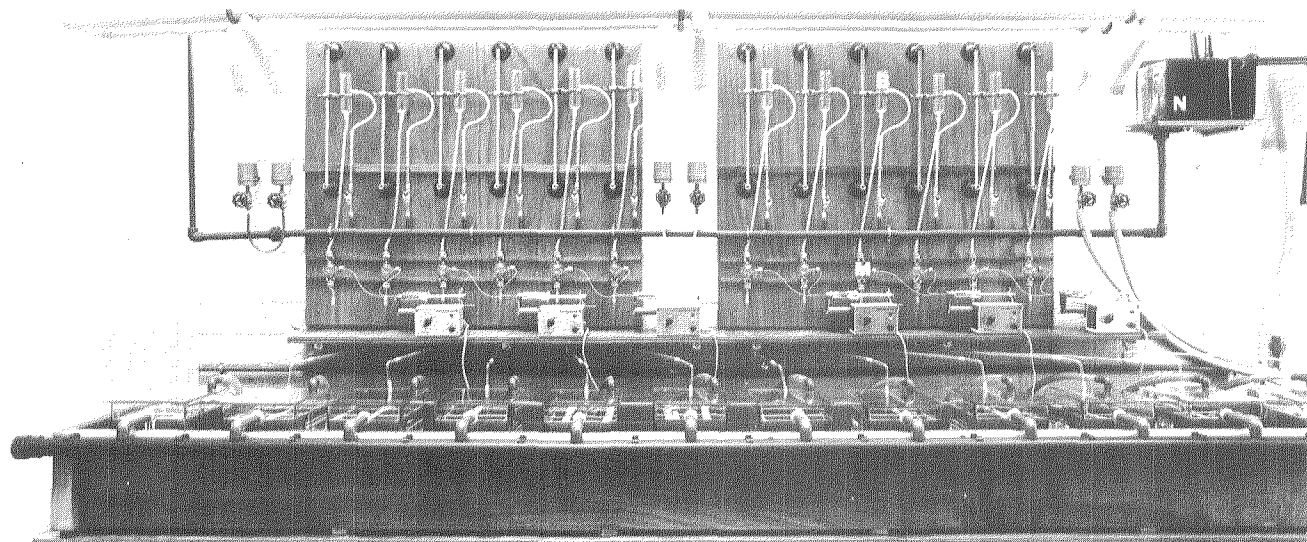


5.4 Fig. 4 Metering pump suitable for metering substances dissolved in an organic solvent



5.4 Fig. 5 Schematic drawing of 5.4, Fig. 6

There is no limit on the number of units that can be included, and the concentrations are independent of one another since the



5.4 Fig. 6 Metering system with test vessels constructed according to Connor and Wilson (1972) and suitable for metering five concentrations plus a blank in duplicate

stock solutions are each prepared and added separately. The apparatus shown in 5.4, Fig. 6 consists of 12 units and can, for example, meter five different concentrations plus a blank in duplicate or three concentrations plus a blank in triplicate. 'Perfusers' (see 5.4, Fig. 4) are suitable for metering a test compound dissolved in an organic solvent. The amounts of solution to be metered in unit time by these devices are extremely small and can be adjusted very accurately. However, the injection syringes must contain no material that is not resistant to the organic solvents. This can be achieved by simple modification of commercially available syringes. Accurate peristaltic pumps can also be used for metering aqueous solutions of test compounds.

5.4.3.4 Measurements during the tests with flow-through systems

Flow-through systems are calibrated each time before use, and are then allowed to run without test animals for at least 24 h. During this running-in period their operation is monitored. After 24 h the concentrations of the test compound in all the test vessels are determined without interrupting the flow. As soon as the concentrations have reached the desired value and have been found to be constant, the test animals are added. Tests in flow-through systems are accompanied by biological and non-biological measurements and observations in a similar manner to (semi)static tests:

- The temperature of the water in the test vessels, which is kept constant, is measured daily.
- The concentration of all the test solutions is measured daily.
- The pH of relevant test solutions is measured 2 to 3 times a week.
- The concentration of the test compound is, if possible, determined 2 to 3 times a week in the water of the test vessels and, if desired, also in the inflowing water.
- The condition of the test animals is observed once or twice a day; dead specimens are then counted and removed.

5.4.4 Investigations with stable 'pure' substances

'Pure' substances are pure and technical chemicals or known mixtures of these, but not waste mixtures of unknown composition.

As stated in the introduction (Section 5.1), we limit ourselves to toxicity tests in which the test compounds are added to the water in which the test animals live, the concentration during the test being kept as constant as possible.

The concentration of a stable compound can fall by volatilization, by co-distillation with evaporating water, and by adsorption on the vessel walls and on the organic material present (excreta, food, test animals). Moreover, the fall may be the consequence of uptake (bio-accumulation) in the test animals.

Partly in view of these causes of loss, it is desirable to divide test compounds into: a. apolar volatile compounds; b. apolar slightly volatile or non-volatile compounds; and c. polar compounds. The nature of the compound partly determines the performance of the toxicity study.

When **apolar volatile compounds** are present, the solutions cannot be aerated without loss and even the vapour phase in the closed test vessels must be limited to a minimum.

It is self-evident that the animals must be provided with sufficient oxygen during the test. In semi-static tests this can be achieved by a combination of a sufficiently large volume of water and a sufficiently short time between successive renewals. Since in practice it is often impossible to renew the water more than once every 24 h, the amount of biomass per unit volume must be chosen in such a way that sufficient oxygen is present for 24 h.

In flow-through tests the flow rate must be so high that the oxygen demand of the animals is satisfied. Other causes of loss of oxygen (e.g. oxidative degradation of the solvents) must be excluded.

Apolar slightly volatile compounds show a strong tendency towards adsorption. They may also co-distill with the evaporating water.

Adsorption losses may be difficult to prevent completely, but they can be minimized by the following means: a. by choosing test vessels with the smallest possible surface in relation to volume; b. by first rinsing out the system with the test solution; c. by the fastest possible removal of dead animals and waste; d. by frequent renewal of the solution in semi-static tests or fast flow in flow-through tests; and e. by a low biomass/volume ratio.

If the loss by co-distillation with the evaporating water is considerable in relation to the loss by adsorption, aeration is not recommended (the compound must then in fact be treated as if it were volatile).

These compounds frequently dissolve so poorly in water that the stock solutions must contain organic solvents.

5. Determination of toxicity

In general, **polar compounds** present few problems. Such substances are readily soluble in water, and they volatilize or are adsorbed to a relatively small extent, so that the concentrations in the aqueous phase generally remain satisfactorily constant in spite of aeration, the presence of food residues, etc.

The test vessels must be sufficiently large for the animals (swimming space, etc.), but part of the animals' oxygen demand can now be satisfied by gentle aeration of the solutions, unless the test animals do not tolerate this aeration.

The renewal frequency in semi-static tests or the flow rate in flow-through tests may therefore be smaller than if the whole oxygen demand of the test animal had to be covered by this renewal. The frequency is determined by the necessity to eliminate the excretion products and food residues and to compensate for the losses due to uptake by the test animals.

What has been said above is based on extreme cases. Not all compounds can be subdivided so sharply according to their physico-chemical properties, and moreover, the latter are not always (previously) known.

No fixed rules can be given for an optimum method of performing toxicity tests; they depend on the physico-chemical properties of the test compound and on the test animal used.

When the properties of the test compound are inadequately known, it is better not to aerate and to keep the possibilities of adsorption as small as possible. The requirements set by the test animal are usually well known and must be borne in mind when designing the experimental arrangement.

5.4.5 Investigations with effluent

By effluent is understood a dilute solution of one or more waste materials in water.

Suspended materials may also be present.

In general, the composition of effluents is not known, or is known only roughly.

Toxicity tests with effluents are in principle carried out as described for pure substances; problems relating to the dissolution of the test compound do not occur.

At the most concentrated solution the effluent is used undiluted. Lower concentrations are prepared by mixing the effluent in various proportions with a dilution water suitable for the particular test animal, in general with the same dilution factors as in 5.2, Table 1.

Problems can arise in the testing of effluents because of:

- a. the presence of degradable substances that may or may not contribute to the toxicity;
- b. the presence of toxic volatile substances in combination with degradable substances;
- c. a pH of the effluent that differs widely from 7–8;
- d. non-homogeneous nature of the effluent;
- e. availability of only limited amounts of slightly toxic effluent.
- f. unknown effluent composition in relation to 'non-toxic' inorganic substances, e.g. Na, K, Ca, Mg, Cl, SO₄.

re a.: The oxygen concentration of the test solution can fall very rapidly as a result of oxidative reactions.

If possible, before the toxicity test is performed the presence of rapidly oxidizable substances is checked, for example, in accordance with Section 3.5.1.2, but usually oxygen measurements on solutions without the test animals give a sufficient indication of this.

If degradable substances are present, an investigation is carried out, again without test animals, to see whether under normal aeration the oxygen concentration remains > 80 % of the saturation value at all dilutions.

If this is the case, toxicity tests can be carried out with aeration of the test solution.

The test animals are added only after the oxygen concentration of all test solutions is > 80 % of the saturation value.

Bear in mind that a mixture of unchanged and transformed compounds continually changing in composition is then being tested. If it is impossible to keep the oxygen concentration above 80 % of the saturation value by aeration, the effluent and/or its dilutions are allowed to oxidize beforehand, after which the toxicity of the end-products is tested.

Dilutions of effluents may degrade faster than the effluent itself, since in the undiluted form this may be toxic to the degrading bacteria.

In general, oxidation reduces toxicity.

Determination of the toxicity of an effluent in which rapidly degradable substances are still present without degradation occurring during the toxicity test is technically practically impossible to perform and rather pointless.

A toxicity test in which no degradation occurs can only be carried out with effluent in which the degradable substances are *highly toxic both to bacteria and to the test animals*. The test must be carried out in a system in which the effluent flows through rapidly, so that the degradation of the substances is prevented by the short residence time of the water in the test vessel (see Section 3.1.2.8). Because of the high toxicity of the effluent for the test animals, in this case only small amounts of effluent are necessary even at a high flow rate. If the effluent is *not toxic to bacteria (but is toxic to the test animals)*, degradation already takes place before the solutions have been fed into the test vessels. This degradation can only be prevented if the effluent or the stock solution prepared from it is kept at a low temperature up to the moment of its addition to the test vessels.

When the effluent is *practically non-toxic both to the bacteria and to the test animals*, large amounts of it would be necessary for a test in which no decomposition of degradable components would be allowed to take place. The accurate testing of such effluents is difficult.

Effluents are rarely *toxic to bacteria but not toxic to the test animals*, and this case will not be discussed here.

It will be clear from what has been said above that toxicity tests with effluent containing rapidly degradable substances that have not yet been degraded at the time of the test but would undergo degradation during the test are practically impossible.

The only answer is to allow the rapidly degradable substances of an effluent to be degraded by bacteria (oxidation) before the effluent is tested for toxicity.

Only effluent with acceptably slowly degrading components can be tested directly.

re b.: It follows from what has been said that effluents with volatile toxic compounds and also dissolved degradable substances cannot be tested in a simple manner with retention of the volatile substances in the test solutions. Because of the necessity of aeration, the volatile substances will be driven out of the solution.

re c.: The pH of effluents is often very low or high in relation to a value acceptable to the test animal.

It will be clear that testing effluents with a pH of 2 to 3 is pointless, since they are immediately lethal to the animals. Dilution with dilution water to pH 6 to 8 is possible, but in many cases what is then determined is which dilution is non-toxic exclusively on the basis of the pH acceptable to the test animals. However, it is reasonable to test effluents after neutralization, so that it is possible to determine the presence of toxic substances other than H⁺ or OH⁻ ions.

re d.: The effluent may contain suspended particles.

In static and semi-static tests, care is taken by thorough stirring to achieve sufficiently representative sampling. When flow-through systems are used the stock solution(s) must be stirred continuously. Here it is advisable to use only systems that do not easily become blocked.

5.4 Test systems and their use

Another problem is caused by the adverse effects that suspended particles may exert on test animals such as filter feeders.

It is not always desirable to test these more 'mechanical' disturbances together with the toxicity of the dissolved substances. Effluents can be filtered or centrifuged and the filtrate or the supernatant tested in comparison with untreated effluents.

re e.: In acute toxicity tests the effluent may be so weakly toxic that it needs to be diluted only slightly or not at all.

With static and semi-static systems this presents no difficulties. With flow-through systems it may be difficult, because of the amount of effluent required (the use of flow-through systems is particularly appropriate if a high degree of renewal is wanted) and because of the limited number of systems suitable for the preparation of low dilutions of the stock solution.

Another problem is that the composition of the dilution water that has been adapted to the test animal is now 'diluted' with the effluent to be tested.

In other words, with a mixture of 80 % of (fresh water) effluent with 20 % of sea water (as dilution water), no toxicity test can be carried out on marine organisms. Actually, this is an extreme example; in general, care must be taken that characteristics such as hardness, salinity, etc., of the mixtures of effluent and dilution water are adjusted in such a way that the solutions are suitable for the test animals, apart from the possible presence of toxic substances.

re f.: It is advisable to know the composition of effluents with respect to the content of Na, K, Mg, Cl, and SO_4 in order to choose suitable dilution water and suitable test animals.

Mortality of the test animals as a result of excessive salinity or unsuitable hardness of the medium brought about by addition of the effluent will need a different approach to mortality brought about by organic xenobiotic substances in the effluent.

Summarizing, it can be stated that for the testing of only slightly toxic effluents (4-day $\text{LC}_{50} > 10\%$) the possibilities are limited, particularly if the water contains degradable substances.

The testing of highly toxic effluents can be carried out in the same way as described for pure substances. The effluent is then as it were a stock solution of the toxic substances.

The range of toxicity is determined beforehand in a preliminary toxicity screening test.

5.4.6 Investigations with mixtures of waste substances

By a mixture of waste materials is understood here a **concentrated** solution or suspension of various substances in a solvent or a solid or liquid mixture of substances without a solvent.

The mixture may be non-homogeneous.

The composition of the waste mixture is generally unknown or is known only roughly.

Most of the problems described in Section 5.4.5 are also relevant here, but an additional difficulty appears in the dosing of the waste mixture into the dilution water. This must be done in such a way that all the substances present in the mixture are also present in the test solution, in the same proportions as in the mixture.

If the whole mixture is sufficiently soluble in water, there is no problem. The procedure is then as described in Section 5.2.

It must be checked whether there are any substances that can easily disappear. Indications on this point can be obtained, *inter alia*, from the origin of the mixture, from chemical analyses, from the oxygen consumption of the dilutions, or even from the results of semi-static toxicity tests as described in example 5.6.2, Fig. 10. If not all the components are equally soluble in water, it will still be desirable to test dilutions even if some components are dissolved and others not.

A mixture of this kind can be metered in the following way:

a. Establish whether there is a suitable solvent (see, for example, Section 5.2, Table 2) in which a concentrated homogeneous solution of the mixture can be prepared, and proceed as described for pure substances dissolved in an organic solvent (see Section 5.2.3);

or:

b. weigh out sufficient amounts of the mixture for **each** dilution, taking care that the sample is representative.

Both in case a. and in case b. it is appropriate to allow the mixture and the dilution water in the prepared ratios to come to equilibrium with one another, e.g. for 24 h prior to the test, so that the sparingly soluble substances also pass into the dilution water.

For testing complex mixtures the metering and the test system must be selected from case to case in such a way as to give optimum results.

In any event, dilution from non-homogeneous solutions must be avoided as far as possible, since it would be easy to dilute the various waste components in different ratios.

The testing of non-homogeneous waste mixtures with flow-through systems will frequently be impossible or will lead to less accurate results than a well-planned semi-static test.

For a far-reaching study of a toxic mixture it is advisable:

a. To separate the mixture into a solid and a liquid phase, if both are present, and to test these separately.

b. To determine the main component and to test this separately.

c. To determine whether small amounts of very toxic substances are present and to test these separately (see Section 1.8).

d. To analyse the composition as well as possible, so that the toxicity tests can be carried out in a better-programmed fashion (see Section 1.5).

5.4.7 Investigations with pure degradable substances

The testing of pure degradable substances for toxicity without degradation occurring during the toxicity test is technically difficult. Only if degradation can be largely prevented by a sufficiently short residence time in the test vessel is it possible to test the original substance.

If degradation cannot be prevented, then in fact a mixture of transformed and untransformed substances is being tested and problems will arise with maintenance of the oxygen concentration of the test solution. Aeration will usually be necessary, causing any volatile substances to be driven off. In the case of a high oxygen demand by the test solution, aeration can be avoided only by using complicated methods in which the amount of oxygen consumed is made up automatically.

In general, it is simpler and more sensible to test only the stable end-products of degradable substances for toxicity, especially when insight into long-term effects is required, e.g. on discharge after dilution into surface water. Testing of the compound undergoing degradation is appropriate when short-term effects must also be taken into account, such as in the introduction of an effluent into a purification plant where eventually a steady state will be achieved that lies between the initial state and the final steady state.

Thus, there will be substances that are readily degradable and even are mineralized, and whose toxicity should nevertheless be determined. Such tests must be accompanied by additional non-biological determinations (see Section 5.5.1). The samples taken for chemical analysis must be stored in such a way, or be analysed so rapidly, that no significant degradation takes place between the sampling and the analysis.

Here again, just as in tests with effluent and waste mixtures, the experimental arrangement must be determined from case to case.

5.4.8 Investigations with pure volatile liquids

Toxicity tests with volatile compounds at a constant concentration are difficult to perform. One possibility is to use closed vessels, but this can only be done with very small animals such as daphnids (Adema, 1978). In general, the oxygen demand of the test animals is so high that aeration of the test medium may even be necessary, which can result in volatile substances being almost totally lost.

A suitable method of maintaining the concentration of volatile liquids constant during both static and continuous flow tests is given by Benville (1974). In this system, air is bubbled through the volatile liquid (test compound) and this air + vapour of the test compound is led via an aeration stone into the dilution water, thus achieving a saturated solution.

By adding a second air stream (without the test compound), lower concentrations can be established. The flow rates of both the vapour stream of the test compound and the 'diluting' air stream must be accurately adjustable, kept constant, and be controlled by a flow meter.

For use in continuous flow tests the saturated solution (which is kept saturated by continuous bubbling of the vapour) is diluted with appropriate flows of clean dilution water.

In the author's experience this system worked well for benzene, toluene, xylene, and 1,2,4-trimethylbenzene.

The TNO Division of Technology for Society has no experience with this system. A problem seems to be the discharge of the excess of (very toxic) vapours and the avoidance of contamination of the laboratory. The whole system must be placed in a very well ventilated room or a fume cupboard. Furthermore, frequent analyses of the test compound are necessary.

5.4.9 Avoidance of contamination

Some hints

In the performance of static and semi-static tests always meter out the 'blank' solvent first and then the various test concentrations of the compound, going from low to high. Use a clean pipette for each test compound. Make as much use as possible of plunger pipettes with disposable tips or of disposable pipettes.

Place the test vessels in such a way that during the test no trace of the test compound can pass from one vessel to another.

If necessary, erect screens between test vessels with widely differing concentrations of the test compound or between vessels with different compounds. This may be necessary, for example, in arrangements with large test vessels that have to be vigorously aerated or have a fast flow of test solutions. When using flow-through systems, check particularly that the 'blank' vessel can in no way be contaminated by the test compound.

Always measure the oxygen concentration, pH, etc. first on the

'blank', with a thoroughly cleaned electrode, and subsequently on the various solutions of the test compound going from the lowest to the highest concentration. Rinse the electrodes thoroughly between the measurements on different test compounds. If necessary, perform the measurements on water samples taken from the test vessels and discard these samples after the measurements.

Take care that the equipment (glassware, etc.) as given in the detailed descriptions of the separate tests (see Sections 5.9 and 5.10) is thoroughly cleaned before use and, in particular, is free from traces of compounds that have been tested earlier. Where possible, use disposable equipment. This will usually be made of plastics (polythene, polystyrene); however, the use of such material is inadvisable for tests on organic compounds that can be adsorbed and dissolved in the plastic material to a certain extent. The use of disposable glassware is preferred in this case; in this connection, it may be observed that metal ions usually adsorb more on glass than on plastic.

The cleaning procedure for glassware must be reviewed specially for each test compound. In each case care must be taken that:

- no dangerous amounts of the test compound can come into a central washing room;
- after cleaning, the glassware is free from traces of the test compound.

Some guidelines, in sequence of performance, are as follows:

- Brush the glassware thoroughly in running water.
- If necessary, then rinse, depending on the substance used with:
 - acetone if the test compound was sparingly soluble in water;
 - 0.01M HCl if heavy metals or amines or other basic substances were used;
 - 0.01M NaOH if an acid, phenol, etc. was used.
- Then rinse with tap water until the apparatus is again free from acetone, acid, or alkali.
Steps a, b, and c must be carried out by the same persons who performed the test.
- Wash the glassware 'normally' with a non-toxic detergent, then rinse with tap water and distilled water.
- Heat at 300° C (for 6 h) if organic compounds were used, otherwise at 100° C (taking care that the air in the room with the drying ovens is renewed adequately), but heat only heat-resistant material.

A combination of some of these points is generally sufficient to obtain adequately clean glassware. For example, for most polar organic substances a, d, and e are sufficient.

Disposable glass or plastic must be used for substances that cannot be removed.

5.5 MEASUREMENTS AND OBSERVATIONS

5.5.1 Non-biological checks on the test solution before and during the tests

As a rule, regular checks are made on the temperature, the pH, the oxygen concentration, and, if possible, the concentration of the test compound in all or in some of the test vessels. The checks are based upon the assumption that the correct composition of dilution water has been determined beforehand on a large batch, at least sufficient for one test.

The temperature

The temperature of the water of a blank is checked at least once every 24 h, preferably with an alcohol thermometer.

The pH

The various types of dilution water have a stable pH if they are aerated. This is checked at least once for each batch, and 2 to 3 times a week if the batch is used over a long period.

Neutral compounds have no influence on the pH of the water, while small pH changes caused by other factors will have little influence on the toxicity of such compounds.

Acidic or basic test compounds may, however, change the pH of the medium. Test animals, food (residues), bacteria, etc., may also affect the pH, especially if there is no aeration and renewals are infrequent.

A change in the pH can have two different consequences: the toxicity of (weakly) acidic or basic substances is affected since the pH controls the degree of dissociation, and the condition of the test animals is affected when the pH extends outside the optimal range.

The testing of highly acidic or basic substances or effluents is therefore rather pointless at pH values that harm the test animals (see also Section 5.4.5).

However, it depends on the aim of the test whether in this case the solutions should be adjusted to the appropriate pH before the addition of the test animals. The testing of adjusted and of non-adjusted solutions can give an idea of the significance of a change in pH and also of the toxicity after neutralization.

When highly acidic solutions are neutralized, determine the final salt content and check its acceptability to the test animals.

The pH of dilution water is determined by its composition (if properly aerated) and cannot be adjusted to a stable different value in a simple manner, such as by additions of HCl or NaOH.

Since the pH can affect the result of the tests, it is recommended in all cases to **determine** the pH at relevant times and in relevant test solutions. These are:

- in static tests both at the beginning and at the end;
- in semi-static tests at the beginning of the test and also just before each renewal of the solution (i.e. the pH of the used solution) and just after renewal (i.e. the pH of the freshly prepared solution);
- in the case of flow-through systems, approximately 2 to 3 times a week (in the test vessel).

In all cases at least the pH of the blanks and of the most concentrated solutions is measured, since the latter will differ most from the blank. If an appreciable deviation (more than 0.3 unit) is obtained, as many concentrations are measured as are necessary to find one in which the pH is approximately the same as that of the blank (the pH is in this case measured from high to low concentrations in samples of the test solutions).

The oxygen concentration

The oxygen concentration of the test solutions exerts a very strong influence on the test results. Frequent measurements are therefore absolutely necessary.

An insufficient oxygen concentration can affect the results in two ways:

- by harming the test animals;
- by affecting the toxicity of the test compound even if there is no visible harm to the animals.

The two factors need not occur to the same extent for different test animals; see the example below (data of Adema).

5.5 Table 1 Influence of a lack of oxygen on the results of two toxicity tests

Test animal	Test compound	21-day LC ₅₀ (µg.l ⁻¹)		Blank mortality (%)	
		O ₂ > 70 % ¹)	O ₂ < 50 % ¹)	O ₂ > 70 % ¹)	O ₂ < 50 % ¹)
<i>Artemia salina</i>	Dieldrin	40	40	10	10
<i>Daphnia magna</i>	Dieldrin	> 180	90	10	10

¹) More than 70 % or less than 50 % of the saturation value at the prevailing temperature and pressure.

If it is not specially desired to test oxygen insufficiencies as a secondary stress factor, it is preferable to make it a rule to consider that for short-term tests the oxygen concentration must be > 80 % and for long-term tests > 70 % of the saturation value at the prevailing temperature.

A review of the saturation values for fresh water and sea water is given in Section 5.5, Table 2.

Oxygen measurements are carried out daily in all test vessels in the case of static tests and tests in flow-through apparatus, and in semi-static tests at least before and after each renewal of the solution, again in all test vessels.

The consumption of oxygen is due to:

- microbiological degradation or oxidation of the test compound;
- microbiological degradation of dead organic material (dead test animals, food residues, excreta);

c. microbiological degradation of any organic solvents used (see Section 5.2, Table 2);

d. respiration of the test animals.

The method of maintaining a sufficiently high oxygen concentration differs from case to case. Some general guidelines are listed below.

re a.: If the test compound takes up oxygen rapidly through chemical or microbiological reactions, toxicity tests are difficult to perform and are uninformative. It is then better to test the stable end products.

In the case of slowly degrading substances that are not volatile and do not co-distill with water, the oxygen concentration can be kept sufficiently high by aeration. However, interpretation of the results is difficult, since the concentration of the test compound falls and other compounds are formed (see also Sections 5.4.5 and 5.4.7).

5. Determination of toxicity

5.5 Table 2 Dissolved oxygen concentration in fresh water and sea water when saturated with air at 1 atm and various temperatures

Temperature, °C	O ₂ concentration on saturation (mg.l ⁻¹) fresh water	sea water
0	14.6	11.3
1	14.2	11.0
2	13.8	10.8
3	13.5	10.5
4	13.1	10.3
5	12.8	10.0
6	12.5	9.8
7	12.2	9.6
8	11.9	9.4
9	11.6	9.2
10	11.3	9.0
11	11.1	8.8
12	10.8	8.6
13	10.6	8.5
14	10.4	8.3
15	10.2	8.1
16	10.0	8.0
17	9.7	7.8
18	9.5	7.7
19	9.4	7.6
20	9.2	7.4
21	9.0	7.3
22	8.8	7.1
23	8.7	7.0
24	8.5	6.9
25	8.4	6.7
26	8.2	6.6
27	8.1	6.5
28	7.9	6.4
29	7.8	6.3
30	7.6	6.1

re *b.*: The presence of dead organic material can be largely prevented. For this purpose the dead test animals are removed from all the test vessels at least twice a day. Foodstuff residues, etc., are removed daily in the case of flow-through tests and at each renewal in semi-static tests. (The presence of organic waste also causes a decrease in the concentration of the test compound by adsorption, especially in the case of apolar substances).

re *c.*: If no aeration is used, even 0.1 ml of a degradable organic solvent such as acetone per litre of the medium can reduce the oxygen concentration within 24 h to $\leq 50\%$ of the saturation value. The use of degradable organic solvents in tests without aeration provides reliable information on toxicity only if a sufficiently high oxygen concentration is ensured. See also Section 5.2, Table 2.

re *d.*: If by adequate precautions the consumption of oxygen due to all the above-mentioned factors is avoided as far as possible, only the respiration of the test animals remains as a source of oxygen uptake. If there is no aeration, the amount of biomass per unit volume of the medium and the degree of renewal (in semi-static tests) or the flow rate (in flow-through tests) must be adapted to one another in such a way that the oxygen concentration does not fall below 70 to 80 % of the saturation value.

If there is no aeration, with a given number of test animals per unit volume of the medium static tests can therefore last only as long as sufficient oxygen is present.

The test compound

If chemical analysis of the test compound is possible, the amounts

actually present in the test vessel are determined during the test. Examples of this have been given in Chapter 2.

In static tests, samples of the test solutions are taken for analysis at least at the beginning and at the end of the test. In semi-static tests, samples of freshly prepared and used solutions are taken once or twice a week, but at least twice per test. In flow-through tests, samples of the water in the test vessels are taken once or twice a week but at least twice per test.

In static or semi-static tests the analysis sometimes requires a sample that removes an appreciable fraction (more than 10 %) of the test medium. In such cases an amount of test solution sufficient both for the test and for the analysis should be prepared before the start of the test.

5.5.2 Biological observations

The aim of the biological observations during toxicity tests is to determine the harmful effects of toxic compounds on the test animals.

Depending on the type of the toxicity study – acute, subchronic, or chronic – the observations are of different types.

In acute toxicity tests only the effects occurring within a short time can be determined. The primary evaluation criterion in these tests is death (mortality) of the test animals. Although in general there is no doubt whether a test animal is still alive or dead, in relation to the mortality of fish it is frequently stated that a fish is dead when it exhibits no further respiratory activity (movement of the gill cover) for 5 min. For the invertebrates no clear, visually detectable, characteristics of dead animals have been standardized, but there is usually no difficulty in judging the situation.

Where necessary, in the descriptions of the test methods in Sections 5.9 and 5.10 it is laid down how the death of the test-animal is determined. Effects of a non-lethal nature are also observed during acute toxicity tests, such as immobility, loss of equilibrium, disturbed swimming behaviour, discoloration, etc.

If other effects than death were observed in a test, these may be reported with the results; a clear description of the type of effect is then necessary. The best possible quantification of the effect must then be attempted.

During the last 10 years much work has been done on the development of quantitative methods and of apparatus for measuring changes in the behaviour and condition of test animals (see among others Warner, 1967 and Sprague, 1971). Much attention has also been devoted to physiological parameters such as respiratory activity (in fish the movement of the gill covers) (Heath, 1972), rate of food uptake, composition of the blood, etc. (Koeman and Strik, 1975; Sprague, 1971).

Sensitive toxicity criteria in the earliest possible phase of the test are required.

A number of the above-mentioned non-lethal parameters can be used in the acute toxicity tests. Others are better dealt with during subchronic toxicity tests. Apparatus for automatic recording of the effects has been developed for some methods.

The use of the above-mentioned non-lethal parameters in aquatic toxicity studies (acute or subchronic) is still problematic because of the relatively limited experience that has been gained with them, so that the interpretation of the results and the significance attached to the results is not always unambiguous.

For these reasons in the procedural descriptions in Sections 5.9 and 5.10 the measurements of one or more of the above-mentioned non-lethal toxicity criteria are not prescribed as obligatory. Nevertheless, some of the test methods described offer the possibility of making biological observations of this type, and the preferably quantitative results of these observations provide additional information concerning the toxicity of the compound tested.

In the subchronic toxicity methods described in Section 5.10, in addition to mortality the growth of the test animals (increase in length and weight) is used as a criterion. In agreement with the description in the 'Introduction' (Section 5.1), the study of the influence exerted by toxic compounds on the embryonic and larval

development of test animals is also regarded as subchronic in nature. In addition to death, the achievement or otherwise of certain phases of development and growth may be used as a criterion. At the same time, additional information may be available concerning morphological deviations of the embryos and larvae. Chronic toxicity studies are carried out with a view to tracing effects that can be expected only after stressing for a period covering at least one generation. The biological observations in these tests are directed primarily at the reproductive capacity. At the same time, observations are made in relation to mortality and, possibly, growth or development of the parent animals and the brood. Summarizing, in the various test methods described in Sections 5.9 and 5.10 the following main evaluation criteria are used:

Acute toxicity test	– mortality
Subchronic toxicity test	– mortality, growth, or development
Chronic toxicity test	– mortality, growth, or development, and reproductive capacity.

The method of biological observation and its frequency are specified as closely as possible in the description of each test method in Sections 5.9 and 5.10.

Additional possibilities for biological observation are also mentioned separately with each test method.

5.5.3 Duration of the test

The durations of the acute, subchronic, and chronic toxicity tests are laid down in the descriptions of these tests, as stated in the Introduction (Section 5.1). In relation to the description of subchronic and chronic toxicity tests, hardly any supplementary explanation is necessary in this connection. The duration of these tests is determined by the life cycle of the test animal.

The chronic test comprises at least one life cycle of the test animal used. This means that the duration of a chronic test with, for example, *Chaetogammarus marinus* is about 4 months, with *Daphnia magna* only 7–8 days, and with *Ophryotrocha diadema* about 4 weeks. The duration in the case of the method given in Section 5.10 for chronic tests is usually somewhat longer than the life cycle of the animals mentioned there, which offers the possibility of making further observations on such important parameters as the reproductive capacity.

It is clear that chronic toxicity studies can be carried out only with test animals whose reproduction in the laboratory is fully ensured. It is important that the test animals used for subchronic toxicity tests can also be bred in the laboratory. The duration of the various subchronic and chronic tests is given in the corresponding descriptions of the methods in Section 5.10.

The duration of the acute toxicity tests, which is described (Section 5.1) as 'short in relation to the life cycle', in particular, requires further explanation.

In contrast to the subchronic and chronic tests, the animals are as a rule not fed during acute toxicity tests. Young animals, in particular, die quickly if their food is withheld. Only by comparative studies can it be determined whether withholding food affects the animals' sensitivity to the toxic compounds. It will be clear that observations after, for example, 48 h in which the 'blank' mortality is less than 10 % will nevertheless be unreliable if after, say, 72 h the mortality in all the test vessels including the blank is 100 %.

The withholding of food is thus the limiting factor for the duration of the acute toxicity tests, especially in the young stages of development. A test time of 24 to 48 h is as a rule the maximum.

For adult test animals, especially for fish and macro-invertebrates such as the shrimp and the mussel, the test duration may be longer.

Although some animal species can live for several months without food, this will not constitute a measure for determining the duration of acute toxicity tests with these animals. An acute toxicity study is always directed at determining an acute effect, i.e. one that appears within a short time after the first exposure of the animals to the toxic compound.

Static tests with relatively large animals are usually terminated after 96 h, but for reasons other than lack of food (see Section 5.4.1). Semi-static tests and tests in flow-through systems are continued, with daily observations, until no further mortality among the test animals has occurred for 3 to 4 days, with a maximum test time of 14 days.

Mathematical treatment of the mortality results (see Section 5.6.2.3) at various concentrations of the test compound gives values expressed as $LC_{50(t)}$, i.e. of the test concentration that after a time t produces a mortality of 50 %. For increasing values of t the $LC_{50(t)}$ tends to a constant limit $LC_{50(t \rightarrow \infty)}$ called the lethal threshold value.

In acute toxicity tests most experience has been obtained with fish. Here it has been found that the lethal threshold value for many compounds and also for many types of effluent is reached in about 96 h, so that the test time of 7 to 8 days is usually sufficient to determine this value with certainty. If this is not the case, it is recommended to continue the test for 14 days. If the threshold value is not reached even in 14 days, further testing is necessary (see Section 5.6.2.6). However, in tests with a still longer test time without the supply of food the condition of the fish will be too greatly affected, even if the deterioration is not always directly observable.

The test times that have been found suitable for acute toxicity tests with fish are also used for tests with the larger fully grown crustaceans such as the shrimp.

The duration of tests with adult molluscs has been set at a minimum of 14 days, since acute mortality in these animals (such as the common mussel) frequently occurs only some days after the start of the test. After periods longer than 14 days without food, the animals' condition deteriorates. For this reason, and because of the frequently low sensitivity of fully-grown molluscs to toxic compounds, they are less suitable as subjects for acute toxicity tests.

The test duration recommended for each animal is given with the corresponding description of the method (see Section 5.9 for acute toxicity tests and Section 5.10 for the subchronic and chronic tests). There may be reasons for ending a test earlier than specified in the description of the test method, namely:

- when the mortality among the test animals in the 'blank' has risen to > 10 % in the acute tests or > 25 % in the (sub)chronic tests;
- when the oxygen concentration in one or more of the test vessels has fallen to below 50 % of the saturation value;
- when, because of the absence of food or withdrawal of food, the condition of the animals has deteriorated to such an extent that this affects the test results.

5.6 TREATMENT OF THE RESULTS

5.6.1 Treatment of the results of toxicity tests with microorganisms

5.6.1.1 General

The present section concerns the treatment of the results of toxicity tests with microorganisms. This relates to tests in which the growth of pure bacterial cultures (see Section 3.5.1.1.), the growth of natural bacterial populations (see Sections 3.5.1.2 and 3.5.1.3), the growth of pure algal cultures (see Sections 5.10.1.1. and 5.10.1.2) and the rate of photosynthesis of natural phytoplankton (see Section 5.10.1.3) are used. The results of all these tests can be treated largely in the same manner.

The treatment of the results is performed in various ways in the test methods reported in the literature. A common method is one in which the inhibiting influence on the total yield of organisms is determined (Bringman 1975, and Bringman and Kuhn, 1973), as is also done in the study of eutrophication (Anon., 1971 and 1974). In addition to this, or even exclusively, the rate of growth is used as a criterion (for example, Breazeale and Camper, 1972). The disadvantage of the different methods of treatment is that it is difficult to compare the results of different tests and experiments with one another, especially as the susceptibility of microorganisms to toxic compounds can be considerably different in different growth phases.

In this chapter a method will be described which, possibly, eliminates some of the above difficulties. This can be achieved by considering the influence on the various growth phases and taking the lowest value as the final effect. Some of these aspects have already been discussed in Section 3.5.1. A similar method has been used by Calder and Lader (1976).

5.6.1.2 General description of the growth of cultures of microorganisms

Populations of microorganisms, both in pure and in mixed cultures (see Sections 3.5.1.1 and 3.5.1.2) have a definite growth pattern with three phases when they are cultured in a limited volume of medium. The first phase is a lag phase, in which no growth is visible and the culture adapts itself to the prevailing conditions. This is followed by an exponential phase, in which the rate of growth is constant, and finally by a stationary phase, in which the rate of growth decreases and the maximum biomass concentration is reached. This division is shown schematically in Fig. 1 of Section 3.5.1. In batch cultures the growth rate in the exponential phase is usually defined as:

$$\frac{dN(t)}{dt} = r_m N(t) \quad (1)$$

or, after integration,

$$N(t) = N(0) e^{r_m t} \quad (2)$$

where $N(0)$ and $N(t)$ are the numbers of organisms (or the amounts of biomass) at times $t=0$ and $t=t$, and r_m is the growth coefficient (t^{-1}). The growth rate can be calculated via the natural logarithms of (2):

$$\ln \frac{N(t)}{N(0)} = r_m t \quad (3)$$

and in decadic logarithms:

$$r_m = \frac{\log \frac{N(t)}{N(0)}}{0.434 t} \quad (3a)$$

Here a distinction must be made between r_m (also denoted by μ) and the exponential growth coefficient k , which is based on the fact that under favourable conditions a population of microorga-

nisms doubles itself within a constant period:

$$N(t) = 2^{kt} N(0) \quad (4)$$

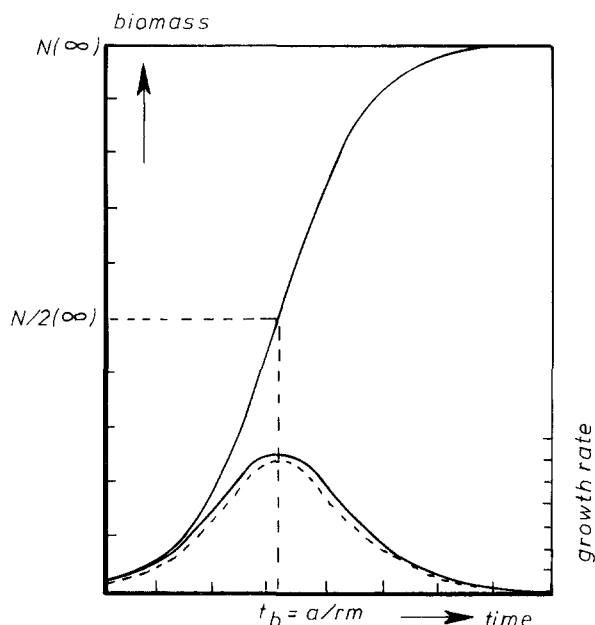
where k is the exponential growth constant, defined as the number of times the population doubles in unit time.

In decadic logarithms,

$$k = \frac{\log(t) - \log N(0)}{0.301 t} \quad (5)$$

The ratio r_m/k is 0.693.

These formulae apply only to the exponential phase, and therefore all measurements – even those at $t=0$ – must fall into this phase. The complete growth curve (number of cells or the amount of biomass on the y-axis, time on the x-axis) has a sigmoid shape (see 5.6.1, Fig. 1).



5.6.1 Fig. 1 Ideal growth curve (taken from La Motta, 1976). The continuous line is the cumulative growth and the broken line the growth rate. For symbols, see text. The point of inflection with the coordinates $N(t)/2$ and $t_b = a/r_m$ is shown

To an approximation, it can be described by the so-called logistic function (Andrewartha and Birch, 1954; Odum 1971). The simplest model is based on the exponential growth (1) with a limiting value $N(\infty)$ for $N(t)$ as $t \rightarrow \infty$.

In this case it must be taken that the growth rate increases slowly at the beginning (the establishment or positive acceleration phase) and then more rapidly (approaching the above-mentioned exponential phase); later the growth rate again falls off in consequence of unfavourable environmental conditions (the negative acceleration phase) until a stationary phase is reached.

In cultures of microorganisms the negative acceleration phase is mostly caused by the exhaustion of a nutrient. The simplest case occurs when the limiting factors are proportional to the population density.

$$\frac{dN(t)}{dt} = r_m \left(1 - \frac{N(t)}{N(\infty)}\right) N(t) \quad (6)$$

in which $r_m/N(\infty)$ is a measure of the growth-rate reduction with increasing population density.

At the maximum population density

$$\frac{dN}{dt} = 0 \text{ and } t = \infty$$

5.6 Treatment of the results

The solution of (6) is:

$$N(t) = \frac{N(\infty)}{1 + e^{a - r_m t}} \quad (7)$$

Here a is the integration constant, which is connected with the choice of the zero time.

Then:

$$a = \ln \frac{N(\infty)}{N(0)} - 1 \quad (8)$$

The constant r_m is the linear regression coefficient of

$$\ln \frac{N(\infty) - N(t)}{N(t)}$$

as a function of t ; r_m has a biological significance as the maximum growth coefficient (t^{-1}) under the given conditions. The curve has a point of inflection at $t_0 = a/r_m$ and $N(t_0) = N(\infty)/2$ (see 5.6.1, Fig. 1).

From this derivation we see that the growth curve of microorganisms is determined by three parameters, namely t_0 , the slope of the curve at the point of inflection ($= 1/4 r_m \cdot N(\infty)$), and the maximum yield of biomass ($= N(\infty)$). These three parameters can also be used to determine the toxic effect on the growth of a population. The model described for this purpose is very simple, but can often be used to describe the growth of microorganisms.

Naturally, $N(0)$ must be greater than zero, since without organisms no growth is possible. Furthermore, it is frequently found that after the adaptation of the parameters in formula (7) to measurements near the point of inflection, the value of $N(0)$ calculated in this way is in no way similar to the measured $N(0)$. This is caused by an additional acclimatization period of the organisms to their new (frequently toxic) environment. In practical tests this last situation is not uncommon. Although the model is not applicable to the whole course of the test, the time to reach $N(\infty)/2$ is still used as a parameter.

It should be pointed out that on the addition of toxic substances the course of the growth curve may also deviate greatly in other respects from the ideal case described by formula (7).

5.6.1.3 Principles of the determination of a toxic effect on the growth of microorganisms

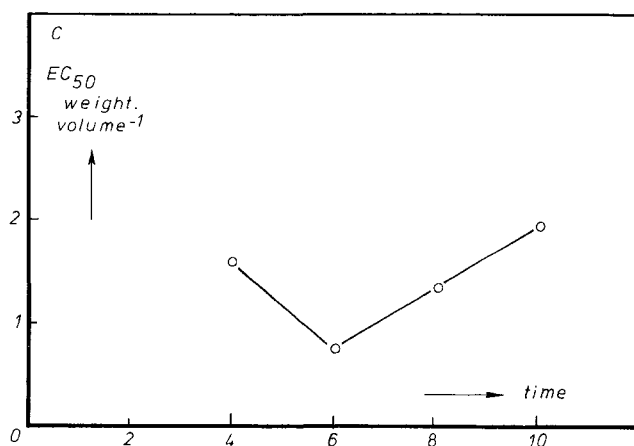
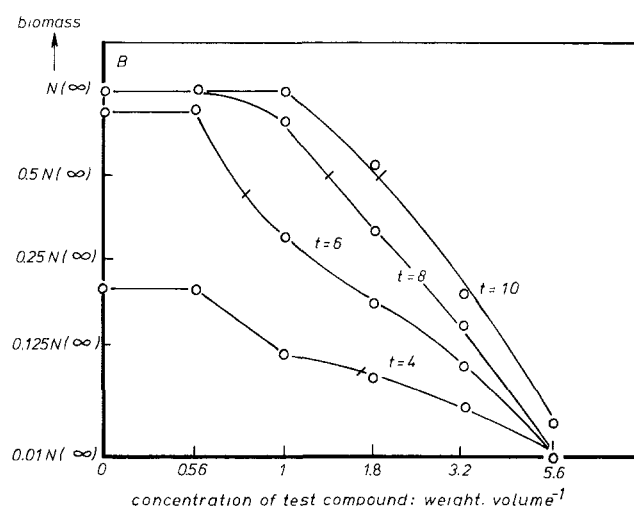
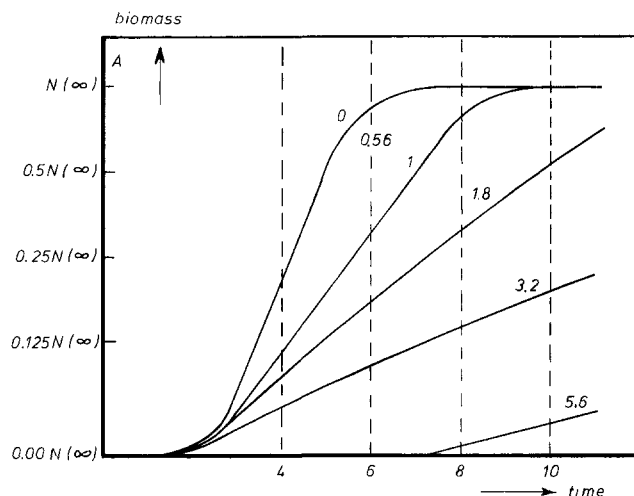
The aim of toxicity tests with microorganisms is the description of a concentration-effect relationship for a given organism and a given substance. It is customary to determine the EC_{50} (the effective concentration that gives 50% inhibition of the measured parameter), but the 'no-effect level' (the maximum concentration that gives no inhibition) and the minimum concentration that gives complete inhibition also provide information. This last value is used particularly to determine the required doses of antibiotics to be administered.

When microorganisms are used as the test organisms, one has to deal with the reaction of the whole population, i.e. with a prolongation of the lag phase (before growth begins), with a reduction in the rate of growth, and with a reduction in the total yield of biomass when a toxic substance is added.

When a growth curve is plotted, it is customary in many tests to use the yield of biomass at a given point of such a curve to determine the concentration-effect relationship (e.g. determination of the BOD or of eutrophication).

It should be noted that this mode of calculation gives a result that depends greatly on the phase in which growth is taking place. 5.6.1. Fig. 2 shows an example of this effect, in which it is demonstrated by means of a theoretical situation that the effect of a toxic compound calculated in this way is highly time-dependent.

If the test is continued, adaptation phenomena also set in, and the EC_{50} found will therefore steadily increase. This makes it difficult to compare the results of different tests. Consequently, for the tests described in this manual an attempt has been made to use, as far as possible, time-independent parameters to calculate the concentration-effect relationship.



5.6.1 Fig. 2 Theoretical example of a result of a toxicity test with microorganisms

A. The growth curve is plotted on a single logarithmic scale. The figures give the concentrations of the test compound in weight per volume. The broken lines show the times at which inhibition is calculated.

B. The total amount of biomass obtained at various times is plotted against the concentration.

C. For each curve the 50% point has been read off and plotted as EC_{50} against time

5. Determination of toxicity

The three parameters that determine a growth curve in accordance with formula (7) have been used for this purpose. The three parameters are:

- t_b = the time to reach 50 % of the total growth of the blank (point of inflection);
- r_m = the growth coefficient;
- $N(\infty)$ = the maximum yield of biomass.

The toxic compound may have different effects on these three different parameters; the most sensitive parameter is normally the length of the lag phase. For technical reasons, this approach cannot be used in the short-term tests described in Sections 3.5.1.3 and 5.10.1.3.

The parameters can be calculated with the aid of formula (7). For mathematical derivation and curve-fitting procedures reference is made to Pearl (1930). In most cases the calculation is unnecessary, and moreover it cannot always be used, since after the addition of toxic compounds the growth curve may deviate greatly from its normal course. Nevertheless, the above parameters will be used, and their derivation is discussed below.

The values of t_b and $N(\infty)$ can be read off directly from the graph, where r_m is the linear regression coefficient of $\ln(N(\infty)/N(t)-1)$ as a function of t . For the parameters $N(\infty)$ and r_m the percentage inhibition

$$100 \frac{P(c=0) - P(c)}{P(c=0)}$$

is calculated, in which $P = N(\infty)$ or r_m (see also Section 5.6.1.4) and is plotted as a function of the concentration (5.6.1, Fig. 5). The t_b is plotted directly as a function of the concentration (5.6.1, Fig. 4). The RC_{50} and the other data can then be read off. Some workers use probit analysis, but without achieving an appreciable improvement (for example Ewald et al., 1976). The accuracy is determined mainly by the interval between the concentration steps. In addition, it is impossible to estimate the minimum and maximum inhibiting concentrations, and the method is therefore not used in this chapter.

5.6.1.4 Treatment of the results

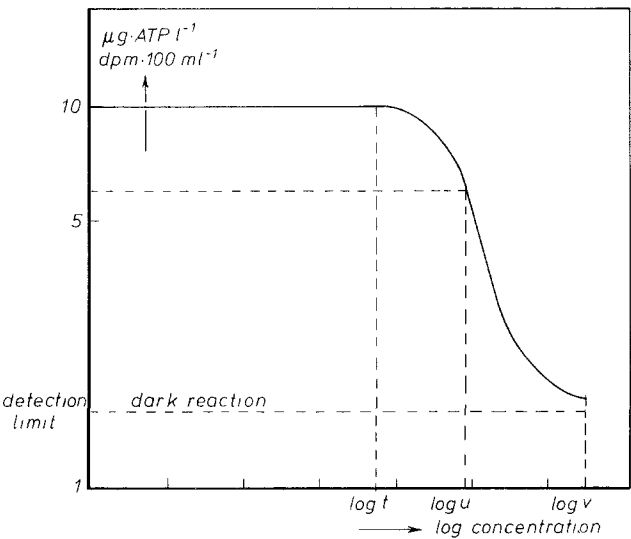
5.6.1.4.1 Simple screening test (Section 5.10.1.1)

- Tabulate the observations for the blanks and for all test concentrations for each time of observation.
- Note the highest concentration at which no difference in colour (growth) from the blank is detectable, the lowest concentration at which no further growth occurs (the minimum inhibitory concentration) and, if appropriate, the concentration at which a distinct inhibition has taken place.

5.6.1.4.2 Tests in which the growth is measured after a pre-determined time (Sections 3.5.1.3 and 5.10.1.3)

- Tabulate the results and construct a graph in which the measured values ($\mu\text{g ATP l}^{-1}$ or dpm per 100 ml) (see also Section 2.7.2) are plotted as a function of the logarithm of the concentration. Depending on the range measured, the graph is plotted on single-logarithmic or double-logarithmic paper. With it is given the detection limit (Section 3.5.1.3) or the dark reaction (Section 5.10.1.3). 5.6.1, Fig. 3 shows how the parameters are read off the graph

The point at which the measured values in the presence of the toxic substance reach 50 % of the blank (EC_{50}) is determined by subtracting the detection limit or the dark reaction from the blank and determining half the resulting difference (u in 5.6.1, Fig. 3); at the same time, the maximum concentration (t) that gives no inhibition and also the minimum concentration (v) that gives complete inhibition are stated. This last parameter is often difficult to determine.

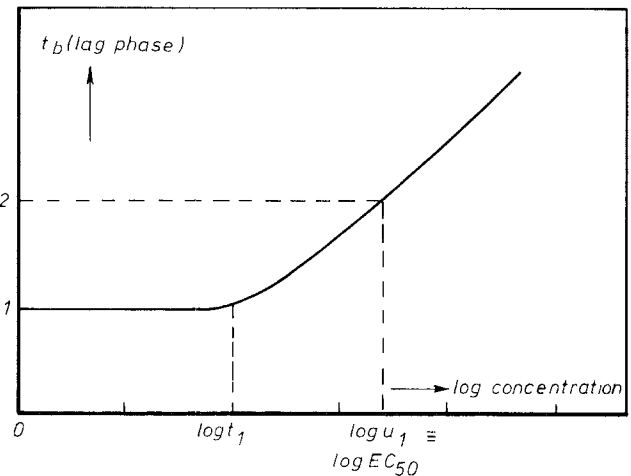


5.6.1 Fig. 3 Schematic representation of the concentration-effect curve of toxicity tests with a natural population of microorganisms (Sections 3.5.1.3 and 5.10.1.3). The ordinate may be linear or logarithmic, while the abscissa is always logarithmic. The parameters read off are as follows: t = the maximum concentration that gives no inhibition; $u = EC_{50}$; v = the minimum concentration that gives complete inhibition

5.6.1.4.3 Tests in which the complete growth curve is measured (Sections 3.5.1.1, 3.5.1.2, and 5.10.1.2)

The treatment of these tests is more complicated because of the three parameters that must be determined for each growth curve (see Section 5.6.1.2). The influence of the toxic substance on the three parameters is determined separately and the lowest value of EC_{50} is reported as the final result of the test.

The growth curve of the test with pure cultures of bacteria (Section 3.5.1.1) or algae (Section 5.10.1.2) is plotted on single-logarithmic paper. The results of the Sapromat test (Section 3.5.1.2) are obtained directly in the form of a graph.



5.6.1 Fig. 4 Schematic representation of the influence exerted by a toxic substance on the length of the lag phase of a growth curve. The following are given: t_1 = the maximum concentration that causes no inhibition, and $u_1 (EC_{50})$ = the concentration that leads to a doubling of the length of t_b

Influence on the lag phase (t_b)

The time at which 50 % of the total growth of the blank is reached is read off from the curve and noted in a table. The times are plotted in a graph as a function of concentration (5.6.1, Fig. 4). Here EC_{50} is defined as the concentration that causes a doubling of the length of t_b (lag phase), and is denoted by u_1 in 5.6.1, Fig. 4. This figure also shows the maximum concentration that produces no inhibition at t_1 . The minimum concentration that produces complete inhibition is defined as the lowest metered concentration at which no growth occurs, and which must be regarded as an approximation to the theoretical value.

Where the growth is so slow that in the test time used the total growth has not reached 50 % of the blank, a lower value can be used for calculating t_b (10 or 20 % growth in relation to the blank).

Influence on the growth coefficient (r_m)

By this is understood the influence of a toxic substance on the growth rate at the curve's point of inflection. This applies only if the results obtained fit the model.

The value of r_m can be determined from formula (3a).

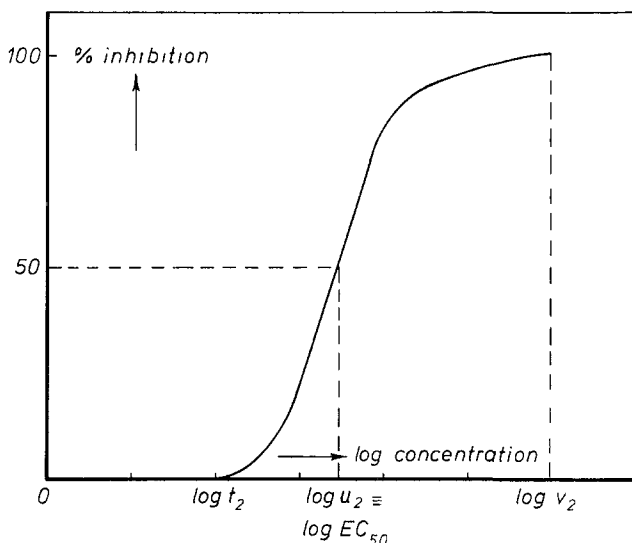
If the curve is drawn on single-logarithmic paper (3.5.1, Fig. 1), the constant k can be calculated by means of formula (5) and then r_m can be obtained from $r_m = 0.69 k$.

Where the course of the growth cannot be described by formula (7), the maximum value of k reached is used for the determination of the toxic effect. This applies particularly to tests in which the addition of a toxic compound leads to a deviating curve, and to tests with algae (Section 5.10.1.2) where a relatively small number of points per growth curve is frequently measured, and where the growth does not always reach the exponential phase.

For the Sapromat tests (Section 3.5.1.2) another approximation is necessary, since no numerical results are obtained. Here, the numerical results must be read off from the Sapromat graph and then r_m can be calculated with the aid of formula (7). It has been shown experimentally that the growth of microorganisms in Sapromat tests performed in accordance with the procedures given in this manual follows formula (7). The measured percentage inhibition is defined as follows:

$$\text{percentage inhibition} = 100 \frac{r_m(\text{bl}) - r_m(\text{tox})}{r_m(\text{bl})} \quad (9)$$

in which $r_m(\text{tox})$ and $r_m(\text{bl})$ are the values of r_m measured in the presence and in the absence of the toxic substance (0 % means that the substance is not appreciably toxic). These percentages



5.6.1 Fig. 5 Graph of the growth rate in the presence of a toxic substance. Percentage inhibition as a function of the logarithm of the concentration. t_2 =maximum concentration that produces no inhibition; u_2 =concentration that produces 50% inhibition (EC_{50}); v_2 =minimum inhibiting concentration that produces complete inhibition

are plotted as a function of the logarithms of the concentrations (5.6.1, Fig. 5).

At the same time the three factors that determine the concentration-effect relationship are given, namely: t_2 =maximum concentration that produces no inhibition, u_2 =concentration that produces 50 % inhibition (EC_{50}), and v_2 =lowest concentration that produces complete inhibition.

The last-mentioned factor cannot always be determined, since many compounds do not cause complete inhibition even in saturated solution.

Influence on the total yield of biomass ($N(\infty)$)

This parameter can reasonably be determined only if the growth curve reaches the stationary phase. If this is the case for most, and preferably all, of the test concentrations, useful conclusions can be drawn.

The value $N(\infty)$ of the total yield, expressed as the extinction (E), as mg dry weight/ml (Section 3.5.1.1), as the maximum oxygen uptake (Section 3.5.1.2), or as the maximum number of algal cells (Section 5.10.1.2), is read directly from the tabulated test results. However, the Sapromat tests (Section 3.5.1.2) require a separate treatment. As explained in Section 3.5.1.2, the stationary phase here is subdivided into a plateau where the bacterial growth stops, followed by a slow growth phase caused by protozoa. For the calculations in this connection the plateau value where bacterial growth stops is used. It must be reported separately whether the growth of protozoa is inhibited.

The yield of biomass in the presence of a toxic compound, $N(\infty, \text{tox})$ in relation to the blank $N(\infty, \text{bl})$ is calculated for all concentrations:

$$\text{percentage inhibition} = 100 \cdot \frac{N(\infty, \text{bl}) - N(\infty, \text{tox})}{N(\infty, \text{bl})} \quad (10)$$

The values are plotted graphically in the same manner as in 5.6.1, Fig. 5 and the inhibition is read off from the curve.

5.6.2 Treatment of the results of toxicity tests with higher organisms (metazoa)**5.6.2.1 Recording of the test results and of the measurements and observations**

It is recommended that the factors affecting the results of toxicity tests and which are important for the interpretation of the results should be recorded in standardized fashion, preferably on printed forms in the case of routine tests.

A number of such forms used in our laboratory have been attached to this section as examples.

Before the start of an investigation of a substance, the test animal and the test method must be selected. The necessary information is recorded as precisely as possible before the start of the test, e.g. as described in 5.6.2, Table 1.

The oxygen concentration of the test solutions, if necessary the pH and the temperature, and where possible the concentration of the test compound, are determined regularly during the tests.

In tests with mortality or other visually detectable changes as the criteria the condition of the test animals is observed regularly. These results are recorded, e.g. as illustrated in 5.6.2, Table 2. The forms can be used for most routine acute or subchronic tests. If, in chronic tests, the animals' reproduction is also evaluated, 5.6.2, Table 3 is also used. The method of recording and treating the results of (sub)chronic tests in which criteria other than death, visually detectable changes, and reproduction of the test animals are evaluated is so dependent on the type of the animal and on the criteria that it is impossible to give any general guidelines. Where necessary for these cases the treatment of the results is discussed in the test descriptions in Section 5.10.

5.6.2.2 Nomenclature

An expression much used in aquatic toxicity studies is the median lethal concentration or the LC_{50} .

5. Determination of toxicity

This is a special case of the EC_{50} , the median effective concentration: in this case the measured effect is death.

The EC_{50} is the concentration of the test compound that, in a given time, brings about the effect concerned in 50% of the test population.

The EC_{50} and LC_{50} values must be provided with an indication of the duration of the test to which they relate, e.g. 96-h LC_{50} .

The LC_{50} and EC_{50} coincide with the much-used terms LD_{50} and ED_{50} , the median lethal dose and median effective dose, used particularly in mammalian toxicology.

'Concentration' denotes the concentration of the test compound in the test solution, while 'dose' denotes the amount of the test compound administered directly into the test animal.

If neither the term 'concentration' nor the term 'dose' is appropriate, the terms EL_{50} and LL_{50} , respectively the median effective level and median lethal level, are used (e.g. if the effect of different temperatures is, being studied).

The definitions of LC_{50} , LD_{50} , ED_{50} , LL_{50} and EL_{50} agree with that of EC_{50} .

In place of LC_{50} and EC_{50} , the median tolerance limit TL_m is also used, particularly in the United States. TL_m is a general term by which both death and some other effect can be indicated.

When the EC_{50} and the TL_m are used, it is therefore necessary to specify not only the duration of the test but also the nature of the effect measured.

In addition to the EC_{50} and LC_{50} , EC_{10} and LC_{90} , etc., are also used. For example 48-h LC_{90} is the concentration lethal to 90% of the test population after 48 h exposure.

Another common expression is the ET_{50} , the median effective time, i.e. the time after which, at a given concentration, an effect has occurred in 50% of the test population.

The ET_{50} values must be supplemented by the concentration at which they were determined. The effect must also be indicated.

Similarly to EC_{50} and LC_{50} , the LT_{50} is the time lethal for 50% of the test population at a given concentration.

In addition to the above-mentioned parameters, the following are in use: the effective (or lethal) threshold concentration* and the effective (or lethal) threshold time. The former is particularly important in toxicity studies. For the significance of these two values, reference is made to Section 5.6.2.6.

The treatment of the results of toxicity tests, the terminology used, and the interpretation of the results are summarized by Brown (1973).

In reproduction tests the final result may, amongst others, be given as the reproduction coefficient or the reproduction inhibition. The **reproduction coefficient** is the number of young born per female during the test in a given test solution, divided by the corresponding number born in the blank solution. The **reproduction inhibition** is: $(1 - \text{reproduction coefficient}) \times 100$ (as an example, see 5.6.2, Table 3).

5.6.2.3 Calculation of LC_{50}

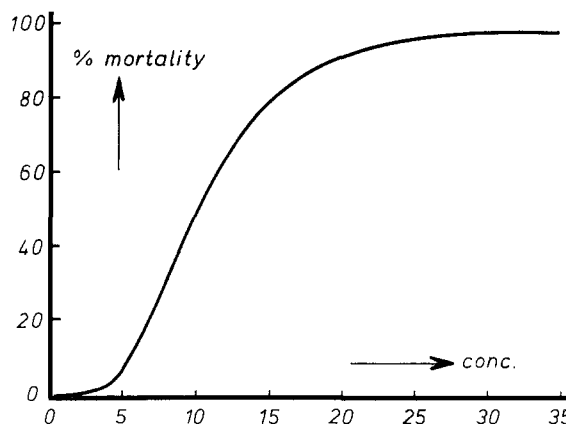
In toxicity tests with mortality as the criterion the number of dead animals in each test vessel is regularly counted. The simplest method of recording the results is then to draw up a table with the number of surviving (and/or dead) animals at the times of observation for the concentrations tested (see 5.6.2, Table 2).

The next obvious step is to calculate the percentage of dead (or surviving) animals in relation to the original number, corrected if necessary for the blank mortality (see Section 5.6.2.8, Note 1).

These results may be shown graphically, so that a curve is obtained for each time of observation.

When the percentage mortality is plotted against the concentration,

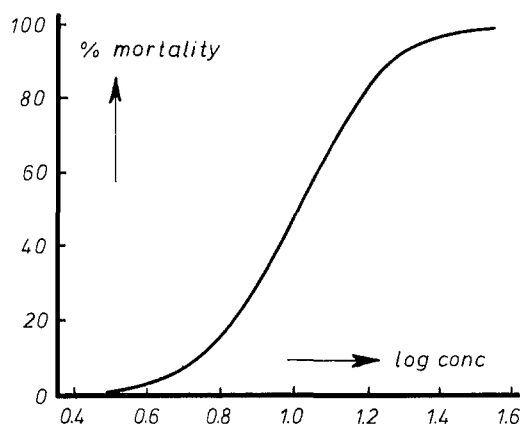
both on a linear scale, an asymmetric sigmoid curve is usually obtained (see 5.6.2, Fig. 1).



5.6.2 Fig. 1 Sigmoid curve of the relationship between % mortality and concentration

If, however, the concentration is plotted on a logarithmic scale, a symmetrical sigmoid curve is usually obtained (5.6.2, Fig. 2).

If, moreover, the % mortality (corrected, if necessary, for blank mortality) is converted into 'probits' (for this see 5.6.2, Table 4), then the sigmoid curve of Fig. 2 is as it were stretched out into a straight line (see 5.6.2, Fig. 3).



5.6.2 Fig. 2 Normal sigmoid curve of relationship between % mortality and log concentration

For further information reference is made to Finney's book (1952), from which 5.6.2, Figs. 1–3 and 5.6.2, Table 4 have been taken.

To establish the LC_{50} , for each time of observation the probits of the corrected percentage mortality and the logarithm of the corresponding concentration are determined. With these values the parameters a and b of the best-fitting regression line are calculated by the method of least squares.

For a regression equation we find $y = a + bx'$, with

y = probit value

x' = log concentration of x

b = slope of the regression line

a = intercept.

Since a probit value of 5 corresponds to 50% mortality, we have

$$\log x = \frac{5 - a}{b}$$

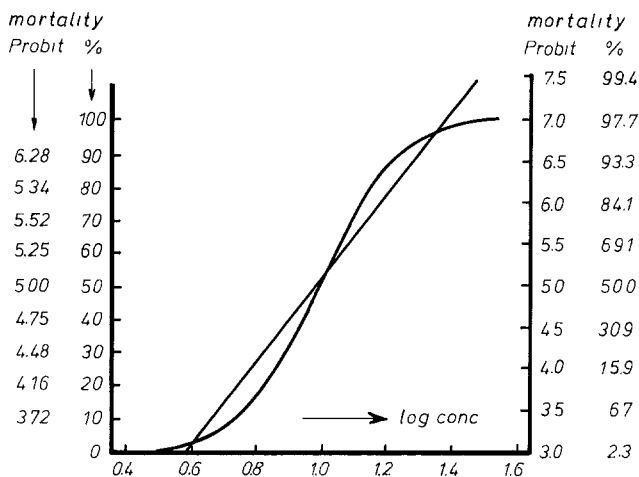
and

$$x = \text{antilog} \frac{5 - a}{b}$$

which gives the LC_{50} value.

* The following terms are also found in the literature: incipient lethal level, incipient LC_{50} , asymptotic LC_{50} , ultimate median tolerance limit, and incipient lethal threshold concentration. The German literature contains 'kritische Schwelle', 'Schwellenwert', and 'Totlichkeitsgrenze'.

5.6 Treatment of the results



5.6.2 Fig. 3 Effect of probit transformation. (The normal sigmoid curve of Fig. 2 is transformed into a straight line if the values on the ordinate are in a linear probit scale instead of percentages)

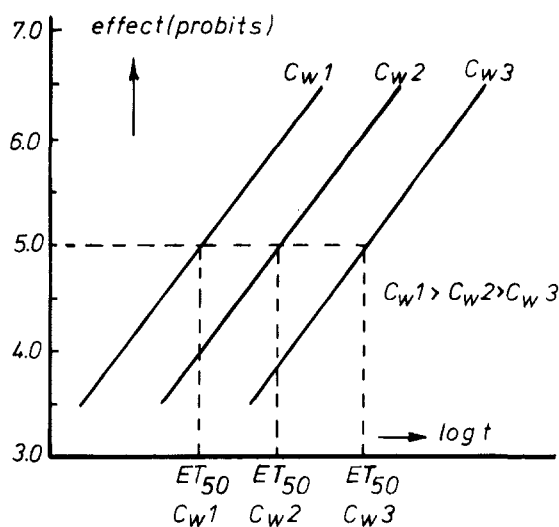
For each time of observation the concentration at which 50% mortality occurs is determined in this way (see 5.6.2, Fig. 5). The use of a good calculating machine with a memory capacity is recommended for speed of calculation.

A problem in calculating exact LC_{50} values can be the absence of a sufficient concentration of the test compound with partial mortality. The LC_{50} can then be only roughly estimated, and if necessary the test must be repeated with closer-spaced concentrations.

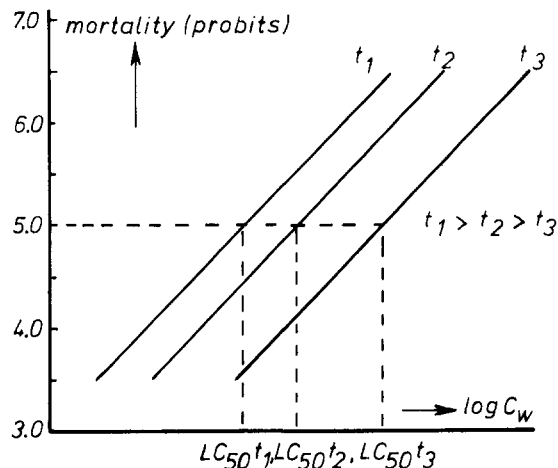
If necessary, the homogeneity of the results can be tested by means of the χ^2 -test. For this purpose the actual results are compared with those to be expected (which can be calculated from the regression line). If the results are heterogeneous according to the χ^2 -test, the calculated LC_{50} value has little significance.

For a further description of this test, see Finney (1952).

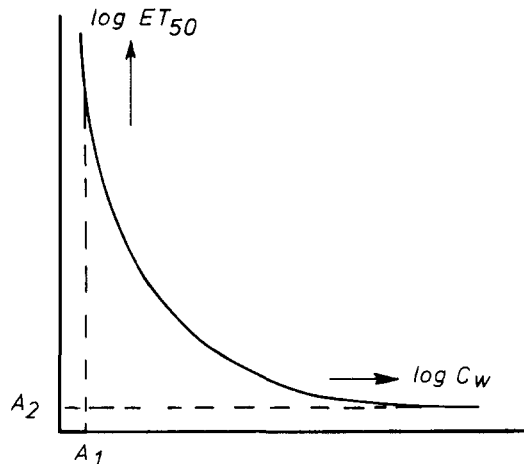
If one wishes to avoid the transformation into probits and logarithms and/or a high-speed calculator is not available, use can be made of the method described by Litchfield and Wilcoxon (1949). These authors have drawn up a series of ingenious nomograms with the aid of which the probit analysis can be carried out with a minimum of calculations. The method is more visual and in general no less accurate, and the statistical calculations are simpler.



5.6.2 Fig. 4 Relationship between the number of test animals showing a given effect and time, for various concentrations



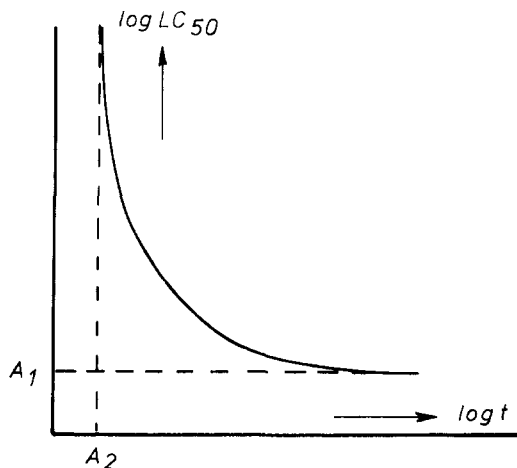
5.6.2 Fig. 5 Relationship between the number of dead test animals and the concentration, for various observation times



5.6.2 Fig. 6 Dependence of ET_{50} on C_w derived from Fig. 4

t = time

C_w = concentration of the test compound in water of the test vessels



5.6.2 Fig. 7 Dependence of LC_{50} on t derived from Fig. 5

A_1 = effective (Fig. 6) or lethal (Fig. 7) threshold concentration (at which 50% of the animals show the reaction)

A_2 = effective or lethal threshold time (minimum time at which 50% of the animals show the reaction)

5. Determination of toxicity

Some remarks

- LC_{50} or TL_m values can also be read off directly from the sigmoid curves of 5.6.2, Fig. 2 provided that these are drawn sufficiently smoothly and through a sufficient number of points and no statistical evaluation of the results is intended.
- When test animals that come from well-standardized laboratory cultures are used, it will often be found that only a few points are located between the 0 % and the 100 % effect, which in particular makes it difficult to calculate the statistical significance. This problem can be solved by selecting the concentrations in the critical region closer to one another and by increasing the accuracy of each individual observation, e.g. by using larger numbers of animals in the test. However, this can give problems for the analysis of the test compound and practical feasibility of the test. Then, however, the uncertainty of the test has in fact been reduced to an uncertainty (and accuracy) of the chemical analysis, which in further calculations determines the statistical treatment. The problem is due to the fact that the theory concerning the appearance of the sigmoid curve is based on the absence of homogeneity in the response of individuals in a population (Finney, 1952), while in fact attempts are made to prevent this lack of homogeneity by standardizing the laboratory culture as much as possible and by using test animals of the same age.
- When an effect other than mortality is determined, the EC_{50} values are calculated in the same way.
- In addition to the LC_{50} (or EC_{50}), it is sometimes useful to determine, for example, the LC_{10} , the LC_{90} , or other extreme percentage responses. These are by definition less accurate than the LC_{50} values, because of the chance distribution in the batch of test animals employed. The best estimate of such values is obtained by calculation, as for the LC_{50} , or by reading them off from the straight lines of 5.6.2 Fig. 5, since these are based on the results for the whole test animal population.
- The ET_{50} (or LT_{50}) values are calculated in a similar manner to the LC_{50} values. For this purpose the probit of the percentage mortality (or effect) for each test concentration is plotted against the logarithm of the time. A series of straight lines is again obtained (see 5.6.2 Fig. 4), from which, among others, one can read off in what time a certain percentage mortality (or effect) will occur at a given concentration. If sufficient concentrations and times of observations are used and the test follows a regular course, the results obtained by the two methods (% mortality against concentration at various times and % mortality against time at various concentrations) should be the same.

5.6.2.4 Interpretation and further treatment of the % mortality-concentration and % mortality-time relationships

As explained in Section 5.6.2.3, plotting of the % mortality in probits against the logarithm of the concentrations or the logarithm of the observation times yields series of curves. These curves are usually straight lines and have the same slope (see 5.6.2, Fig. 4, and 5.6.2, Fig. 5).

If the lines are not straight, or do not all have the same slope, this may indicate two or more different modes of action of the test compound (Sprague, 1969).

The LC_{50} or LT_{50} values determined from these lines are then plotted against the corresponding times or concentrations, both on a logarithmic scale, whereupon, in general, curves such as those shown in 5.6.2 Fig. 6, and 5.6.2 Fig. 7 are obtained.

5.6.2.5 Reliability of the LC_{50} values and further statistical treatment

Although this is by no means always done, and is not always possible, it is recommended to treat the test results statistically.

The following should be determined:

- the 95 % confidence limits of the calculated LC_{50} (LC_{10} , EC_{50} , ET_{50} , etc.) values
- the 95 % confidence limits of the slopes of the straight lines (5.6.2, Figs. 4 and 5)
- the heterogeneity of the observations
- the parallelism of the straight lines (5.6.2, Figs. 4 and 5).

These characteristics can be determined for each series of observations at a single time at given concentrations or at a single concentration for various times, i.e. for each test carried out **singly**, provided that a sufficient number of points has been found between 0 % and 100 % mortality (at least 3 but **preferably 5**).

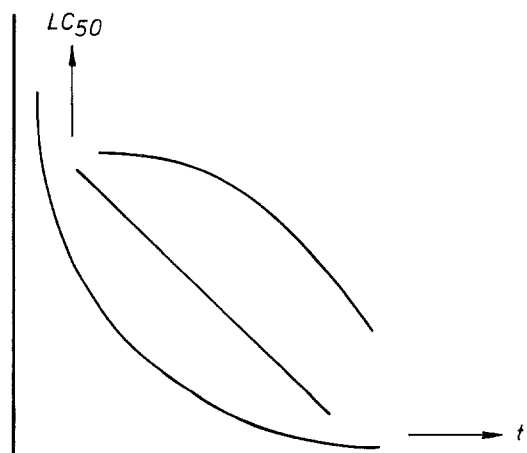
When the LC_{50} values and the slopes of the lines have been calculated by probit analysis, the confidence limits, the heterogeneity, and the parallelism can be calculated as described by Finney (1952). In this case, it is possible to write a computer program by which the calculations and the statistical treatments are carried out rapidly and automatically (Fink and Hund, 1965; Hermann, 1975). If probability-log graph paper is used for the calculations of the LC_{50} and the slope, the method described by Litchfield and Wilcoxon (1949) can be used for the statistical treatment of the results. In fact, the latter is simpler than Finney's method for this statistical treatment. The confidence limits found by Litchfield and Wilcoxon's method are generally somewhat wider than those calculated mathematically according to Finney.

5.6.2.6 Interpretation of the LC_{50} -time or ET_{50} -concentration curves

Hyperbolic curves

The curves of 5.6.2, Figs. 6 and 7, which otherwise contain the same information, represent 'normal' LC_{50} -time or ET_{50} -concentration curves (see Section 5.6.2.8, Note 2).

Two other values can be determined from the hyperbolic curves: the threshold concentration for 50 % mortality (lethal threshold value) and the threshold time for 50 % mortality (lethal threshold time). The lethal threshold concentration (A_1) is reached when no further mortality occurs upon prolongation of the test; the LC_{50} curves will then run parallel to the time axis (asymptotic LC_{50}). The lethal threshold (reaction) time (A_2) arises when a minimum time is necessary for (50 %) mortality to be reached, irrespective of the concentration. It is also possible to state after what time the threshold concentration has been reached. The lethal (or effective) threshold concentration is the most important parameter in toxicity studies. Each test must, if possible, be continued until this value has been reached, and if this is not the case, this must be stated in the report.



5.6.2 Fig. 8 Some forms of LC_{50} -time curves

5.6 Treatment of the results

In addition, one must not lose sight of the fact that this always relates to 50% mortality. The concentration that gives a result not statistically different from the blank should be regarded as the 'no-effect level'. If, however, the minimum time after which the threshold value of the LC_{50} is reached is known, then for this time the line that gives the relationship between % mortality (in probits) and the logarithm of the concentration can yield information on the concentration producing very low (e.g. 1%) mortality. Thus, a 'no-effect level' with respect to the mortality criterion can be approximated with any desired accuracy that is no longer dependent on the time of the test.

Instead of such hyperbolic LC_{50} -time (or ET_{50} -concentration) curves, straight lines or even convex curves are found (see 5.6.2, Fig. 8).

'Straight' LC_{50} -time curves

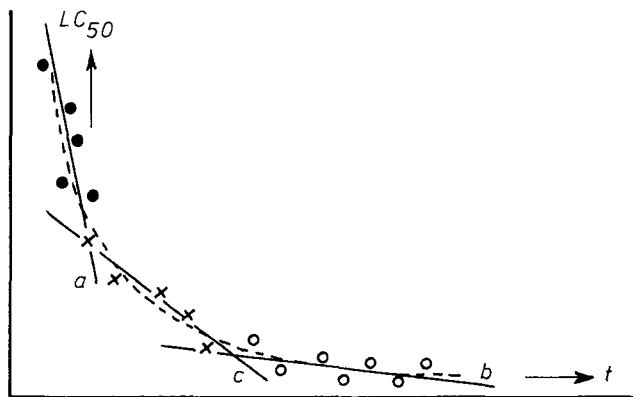
When the LC_{50} -time graph is a straight line (or approximates to one), a number of interpretations are possible (see 5.6.2, Fig. 9):

- The total time of observation was too short to find the remainder of the curve.
- Observations were started too late (see Note 3).
- The times of observation were somewhere between points a and c of the graph shown.

It can be seen from these examples that the interpretation of 'straight lines' must be carried out with some circumspection, bearing in mind the time factor in particular.

However, it is known that for many substances the lethal threshold values are reached in 4 to 7 days (Sprague, 1969).

If, therefore, **after a test time of about a week** the LC_{50} values are still falling (this usually occurs linearly on log scales), caution is necessary in the evaluation of the toxicity.



5.6.2 Fig. 9 Causes of apparently 'straight' LC_{50} -time graphs (a, b, or c, separately) which in reality are only a part of the total curve

- actual LC_{50} -time curve with a sufficient number of observations
- a . — . test time too short
- b o — o observations started too late
- c x — x observations started late and ended too early

In such cases one is probably dealing with substances that are slowly accumulated by the organism to continuously increasing concentrations or are slowly converted into the true toxic substance or substances whose effect is delayed in relation to the rate of absorption.

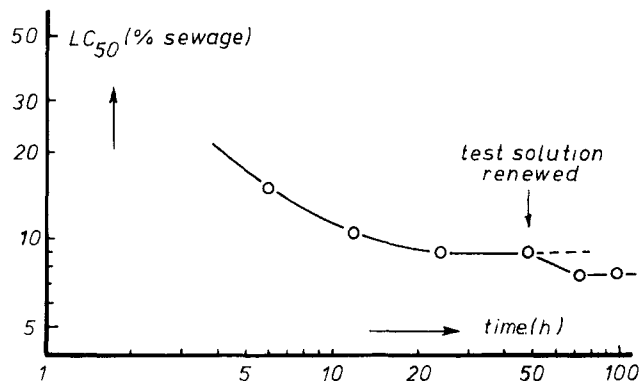
Such 'straight' LC_{50} -time curves are an important reason for further (sub)chronic studies and for the investigation of bio-accumulation. 'Straight' LC_{50} -time curves are found, for example, for heavy metals and pesticides such as DDT and dieldrin.

'Convex' LC_{50} -time curves

A convex LC_{50} -time curve (see 5.6.2, Fig. 8) indicates a complex situation. For example, the test sample may be a combination of a substance having a low toxicity but a rapid effect and a highly toxic substance with a slow effect. The compound may also be a substance of low toxicity that is converted into a more toxic compound in the course of the test. Such convex curves occur rarely.

'Irregularly falling' LC_{50} -time curves

Such curves (see 5.6.2, Fig. 10) are often found in semi-static tests. The possible reasons for this are explained here on the basis of an example in which, at the same time, the problems with effluents (see Section 5.4.5) are considered once again.



5.6.2 Fig. 10 Results of a toxicity test with an effluent

Example

A sample of effluent was tested on guppies in a semi-static test for 96 h with one renewal at $t=48$ h.

Since it had been found previously that the effluent consumed oxygen, it was decided to aerate all the solutions during the test so that the oxygen content remained above 80% of the saturation value. After an initially regular mortality, during the first 24 h, no further mortality was observed (48-h LC_{50} = 24-h LC_{50}). After renewal of the test solution (the effluent was kept at 4°C and, before renewal, was aerated until it contained sufficient oxygen), mortality of the remaining guppies occurred again during the first 24 h (72-h LC_{50} < 48-h LC_{50} , and 96-h LC_{50} = 72-h LC_{50}).

Conclusion: toxic substances were presumably present in the effluent, which disappeared **during the test**. Since consumption of oxygen took place, their disappearance by oxidation (e.g. by bacterial degradation) appeared an obvious assumption. The disappearance of volatile toxic substances possibly present because of aeration cannot, however, be excluded.

In addition to the four examples of the possible shapes of LC_{50} -time curves given here, others may be encountered (see, for example, Sprague, 1969, p. 806). Just as in the last example, such abnormal shapes frequently indicate the presence of concentrations of the test compound in the solution other than those expected.

5.6.2.7 Reproducibility of toxicity tests

When replicate toxicity tests are carried out at the same time with the same batch of test animals, the reproducibility of the LC_{50} values is generally very good. The coefficient of variation ($V = \frac{100s}{\bar{x}}$) is generally about 10; values of 25 are exceptional (s = standard deviation, $\bar{x}$ = mean LC_{50}).

If, however, a toxicity test is repeated at a different time, the reproducibility is less good. Experience shows that the LC_{50} values may then easily differ from one another by a factor of 2 to 3 (Canton

5. Determination of toxicity

and Adema, 1970). Brown (1968) gives a factor of 2.5 for fish obtained from the same stock and tested in the same laboratory. An important cause of this poor reproducibility is the condition of the test animals at the time of the test.

The reproducibility also depends on the test compound. Experience has shown that the results obtained with substances having a rapid effect are generally better reproducible than those obtained with substances having a slow effect.

NOTES

1. Correction for blank mortality

This is made in accordance with the formula:

$$p = \frac{p' - c}{100 - c} \times 100,$$

in which

p = corrected % mortality

p' = % mortality observed in the test

c = % blank mortality observed in the test.

2. Hyperbolic LC_{50} -time/ ET_{50} -concentration curves

The hyperbolic form occurs so frequently that a number of authors have attempted to describe it mathematically. The simplest form is $C^nT = K$ (in which C is the concentration of the test compound (C_w in 5.6.2, Figs. 4 to 7) and T is time); n and

K are constants characteristic for a given substance. The determination of such constants and their values is doubtful, in view of the many uncertainties already involved in the determination of the factors from which the required 'characteristic constants' are derived; we shall not go into this further here. For further information see Hynes (1960) and Ericksen Jones (1964).

3. Short reaction times

For lower chlorinated hydrocarbons, such as dichloroethane and trichloroethane, all deaths usually take place in the first 24 h.

5.6.2 Table 4 Transformation of percentages into probits

%	0	1	2	3	4	5	6	7	8	9
0	—	2.67	2.95	3.12	3.25	3.36	3.45	3.52	3.59	3.66
10	3.72	3.77	3.82	3.87	3.92	3.96	4.01	4.05	4.08	4.12
20	4.16	4.19	4.23	4.26	4.29	4.33	4.36	4.39	4.42	4.45
30	4.48	4.50	4.53	4.56	4.59	4.61	4.64	4.67	4.69	4.72
40	4.75	4.77	4.80	4.82	4.85	4.87	4.90	4.92	4.95	4.97
50	5.00	5.03	5.05	5.08	5.10	5.13	5.15	5.18	5.20	5.23
60	5.25	5.28	5.31	5.33	5.36	5.39	5.41	5.44	5.47	5.50
70	5.52	5.55	5.58	5.61	5.64	5.67	5.71	5.74	5.77	5.81
80	5.84	5.88	5.92	5.95	5.99	6.04	6.08	6.13	6.18	6.23
90	6.28	6.34	6.41	6.48	6.55	6.64	6.75	6.88	7.05	7.33
	0.0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
99	7.33	7.37	7.41	7.46	7.51	7.58	7.65	7.75	7.88	8.09

5.6 Treatment of the results

5.6.2 Table 1

BASIC INFORMATION

Commission No.: 00023
Code No.: ABC
Work done by: Jansen

Type of test : *acute toxicity test*
7-day LC₅₀

Test substance: *pentachlorophenol*
concentrations: 0 – 3.2 – 5.6 – 10 – 18 – 32 mg.l⁻¹

Test animal : species: *Crangon crangon*
age at the start of the test:
length at the start of the test: *average 4.8 cm*
weight at the start of the test: *average 0.72 g*

Origin lab. culture
collection site: : *Wadden Sea (Den Helder)* date: *10.9.1976*

acclimatization : time: *10 days* temp.: *15°C* food: *musse!*
other information :

Date of the start of the test: *20.9.1976*
Test duration : *7 days*
Temperature : *14.5°C*
Type of dilution water : *natural sea water*
hardness : *not applicable* ; pH: *8.1* ; ‰ Cl⁻: *15.6* ‰ S: *28.1*
Contents of test vessel : *12* litres of water and *10* test animals at the start of the test
food/no food; type? : ; how much? :
Renewal : ~~none; intermittent; 1 x per~~ continuous, flow rate: *750 ml.h⁻¹*
Type of test vessel : *glass aquaria* replication? *duplicate*
Oxygen/air : *passed through/passed over; high/low; continuous/intermittent; none*
Solvent for the test substance: *distilled water + NaOH* dosage: *0.660 ml.h⁻¹*
Analysis of the test substance performed? ~~yes/no~~; method: *gas chromatography (G.C.)*
Other information: *water samples diluted to a nominal concentration of 1–10 µg.l⁻¹*

5.6.2 Table 3 Test code PQR

5.6.2 Table 3 Sheet for reproduction tests

SHEET No. : 1

Date	day of test	nominal concn mg l ⁻¹	surviving animals	number ♀/♂	total no. of young	no. of young per ♀	Reproduction result			
							concn	Total no. of young during test		
								per ♀	average	as % of blank
19-4	14	0 a	16	10/6	336	34	0 a	126	150	100 (p.d.)
		0 b	18	9/9	475	54	0 b	175		
		0.32 a	19	7/12	137	20	0.32 a	59	55	37
		0.32 b	17	9/8	267	30	0.32 b	50		
		0.56 a	19	13/6	47	4	0.56 a	39	29	19
		0.56 b	19	11/8	0	0	0.56 b	19		
21-4	16	0 a	16	10/6	374	37				
		0 b	18	9/9	357	40				
		0.32 a	19	7/12	259	37				
		0.32 b	17	9/8	164	18				
		0.56 a	18	12/6	47	4				
		0.56 b	19	11/8	28	2				
23-4	18	0 a	16	10/6	106	11				
		0 b	18	9/9	309	34				
		0.32 a	19	7/12	8	1				
		0.32 b	17	9/8	21	2				
		0.56 a	18	12/6	52	4				
		0.56 b	18	11/7	141	13				
26-4	21	0 a	16	10/6	439	44				
		0 b	18	9/9	427	47				
		0.32 a	19	7/12	4	1				
		0.32 b	17	9/8	19	2				
		0.56 a	18	12/6	319	27				
		0.56 b	18	11/7	46	4				

Reproduction inhibition : 0.32 ppm : 63% (approx. 60%)

0.56 ppm : 81% (approx. 80%)

5.7 COMPOSITION AND MONITORING OF TYPES OF WATER

5.7.1 Introduction

Except in coastal regions, sea water has the same composition throughout the world. In most formulations for synthetic sea water, therefore, the composition is always approximately the same so far as the main components are concerned. Below we give two formulations, both of which are based on sea salt available commercially (Section 5.7.2, j and k). The salinity of the sea water can be selected by dissolving more or less of the salt. The quality of such synthetic water depends significantly on that of the water in which the sea salt has been dissolved. The two types of synthetic sea water are compared with each other and with natural sea water in 5.7, Table 1.

Since natural fresh water has no fixed composition, the many formulations available for synthetic fresh water differ greatly from one another. Below we give a few that have been selected solely because they can be used for the animals discussed in Section 5.3.

The compositions of the type of fresh water are compared in 5.7, Table 2. In addition to these more or less natural types of water two formulations are given, one for fresh water and one for sea water, with unnaturally high concentrations of nitrate and phosphate for the rapid cultivation of unicellular algae in large numbers. Apart from synthetic fresh water and sea water, it is also possible to use natural water. This is pumped from a suitable site and transported to the laboratory.

5.7.2 Formulations for some types of synthetic fresh water, sea water, and some other media

- a. Standard fresh water according to **Alabaster and Abram** with a hardness of 20 mg of calcium carbonate per litre ('soft A & A water')¹⁾.
The medium is prepared from three stock solutions in distilled water.

Stock solution 1:

Dissolve 320 g of $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$, 29 g of NaCl , and 9 g of NaNO_3 and make up to 1 litre.

Stock solution 2:

Dissolve 151 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 79 g of Na_2SO_4 and make up to 1 litre.

Stock solution 3:

Dissolve 27 g of NaHCO_3 and make up to 1 litre.

For each 100 litres of standard water to be prepared, 10 ml of stock solution 1, 10 ml of stock solution 2, and 100 ml of stock solution 3 are added to 100 litres of distilled water.

- b. Standard fresh water with a hardness of 200 mg of calcium carbonate per litre ('hard A & A water')²⁾.

For the stock solutions see medium a.

For each 100 litres of standard water to be prepared, 100 ml of stock solution 1, 100 ml of stock solution 2, and 1 litre of stock solution 3 are added to 100 litres of distilled water.

- c. Standard fresh water with a hardness of 200 mg of calcium carbonate per litre and a high salinity³⁾.

Formulation similar to b, but an additional 271 mg of sodium chloride is added per litre of standard water.

- d. Standard reference water⁴⁾.

The medium is prepared from six stock solutions in distilled water.

Stock solution 1:

Dissolve 71.0 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 6.5 g of K_2SO_4 , and 0.2 g of $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ and make up to 1 litre.

Stock solution 2:

Dissolve 18.6 g of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ and make up to 1 litre.

Stock solution 3:

Dissolve 25.0 g of NaHCO_3 , 3.0 g of NH_4NO_3 , and 1.1 g of $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$ and make up to 1 litre.

Stock solution 4:

Dissolve 32.2 g of CaO and make up to 1 litre. Make a suspension of CaCO_3 by passing pure CO_2 through this solution.

Stock solution 5:

Dissolve 62.6 g of $\text{Na}_2\text{SiO}_3 \cdot 9\text{H}_2\text{O}$ and make up to 1 litre.

Stock solution 6:

Dissolve 1.2 g of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ and make up to 1 litre.

To the desired amount of distilled water, add, per litre, 1-ml portions of stock solutions 1, 2, and 3. With the aid of an aeration stone, pass pure CO_2 through this solution for 15 min. The pH of the solution should then be about 4.3.

To this solution, per litre, add 1 ml of stock solution 4 and again pass in CO_2 until the solution is clear.

The pH of the solution should now be about 5.1.

Bring the pH to about 7.9 by allowing air to bubble through the solution for 25 min.

Then add, per litre, 1-ml portions of stock solutions 5 and 6 and aerate the solution for 60 min. After aeration, the final pH must remain constant at 7.9 ± 0.1 .

- e. Standard test medium (STM)⁵⁾.

The medium is prepared from three stock solutions in distilled water.

Stock solution 1:

Dissolve 100 g of NaHCO_3 and make up to 1 litre.

Stock solution 2:

Dissolve 112 g of CaCl_2 and make up to 1 litre.

Stock solution 3:

Dissolve 13 g of K_2SO_4 and make up to 1 litre.

For each 100 litres of STM to be prepared, add 200 ml of stock solution 1, 200 ml of stock solution 2, and 200 ml of stock solution 3 to 100 litres of distilled water with continuous stirring. The solution is then aerated overnight and stored for 24 h to reach equilibrium.

- f. Soft reconstituted fresh water (EPA-660/3-75-009)⁶⁾.

Dissolve per litre of distilled water or of de-ionized water filtered through activated carbon:

NaHCO_3	48 mg
$\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$	30 mg
MgSO_4	30 mg
KCl	2 mg

Also given in this report⁶⁾ are:

Very soft reconstituted fresh water (1/4 of the concentrations in medium f).

Hard reconstituted fresh water (4 times the concentrations in medium f).

- g. Dutch Standard Water⁷⁾:

Dissolve per litre of distilled or de-ionized water filtered through activated carbon:

NaHCO_3	100 mg
KHCO_3	20 mg

5. Determination of toxicity

CaCl₂·2H₂O 200 mg

MgSO₄·7H₂O 180 mg

Very hard reconstituted fresh water (8 times the concentrations in medium f).

h. Dilute sea water (for example, 1 %)⁸.

Add 1 litre of natural or synthetic sea water to 99 litres of distilled water.

i. Medium for rapid cultivation of unicellular green freshwater algae.

Per litre of distilled water, dissolve⁹:

KNO₃ 1.25 g

CaCl₂·2H₂O 0.01 g

MgSO₄·7H₂O 0.3 g

iron(III) citrate trihydrate 0.01 g

K₂HPO₄ 0.29 g

KH₂PO₄ 0.19 g

trace-element solution 0.5 ml

(agar agar 20 g)

The pH of the medium should be 6.5 to 6.7.

Prepare the trace element solution as follows:

H₃BO₃ 2.86 g

MnCl₂·4H₂O 1.81 g

ZnSO₄·7H₂O 0.22 g

(NH₄)₂MoO₄ 0.02 g

CuSO₄·5H₂O 0.079 g

Co(NO₃)₂·6H₂O 0.49 g

distilled water 1 litre

For the preparation of the **nutrient medium in culture tubes** add 20 g of agar agar per litre. The solution must be boiled until the agar has dissolved and then be distributed in the fluid state between the culture tubes (about 10 ml per tube). These must be closed with a plug of cotton wool and sterilized. Immediately after sterilization, place them in an inclined position so that the agar medium solidifies as a sloping layer.

j. Synthetic sea water according to H. Wiegandt (H.W. water). Dissolve a sufficient amount of H.W. salt per litre of water (distilled, spring, or mains water) to give the desired salinity¹⁰

k. Synthetic sea water according to Aquarium Systems Inc. Ohio ('Instant ocean')¹¹.

Dissolve the contents of a 33-pound pack of 'instant ocean synthetic sea salts' to a total volume of 100 gallons of water (this is 39.58 g/l). Then add the contents of the accompanying bottle containing the trace-element solution. The salinity is now about 3.32 % S, the chloride content 1.84 %, and the density 1.025 at 15°C.

l. Erdschreiber medium for the cultivation of marine algae. Pasteurize 1 litre of synthetic or natural sea water, cool, add aseptically 1 ml of a sterile NaNO₃ solution containing 0.1 g of NaNO₃ per ml, 1 ml of a sterile Na₂HPO₄ solution containing 0.02 g per Na₂HPO₄·12H₂O or 0.006 g of Na₂HPO₄ per ml, and 50 ml of sterile soil extract¹².

Instead of pasteurization, which may lead to a precipitate interfering with some tests, e.g. counting the algae in the Coulter Counter, the sea water may also be sterilized by filtration in the manner customary in microbiology.

In particular, if algae are used for toxicity or accumulation tests the necessary media should be sterilized by filtration.

References for water compositions and Notes

1. Literature:

Alabaster, J.S. and F.S.H. Abram (1965):

Estimating the toxicity of pesticides to fish.

Pest Articles and News Summaries Section C 1965, **11**, 91–97.

2. In the proposal of the United Kingdom delegation for a fish toxicity test on behalf of the Commission of European Communities, Directorate-General for Industry, Technology, and Science, Document III/1129/72-N, the following were recommended for fish tests.

as soft water:

medium a, in which all concentrations are 1.25 times as high and the hardness expressed as calcium carbonate is therefore 25 mg.l⁻¹

and as hard water:

medium a, in which all the concentrations are 12.5 times as high and the hardness is therefore 250 mg.l⁻¹. By analogy with this, in the present manual the composition of medium a, but with all concentrations 10 times as high, has likewise been chosen as hard standard fresh water (medium b).

3 For long-term cultivation of guppies the salinity of 'A & A water' appears to be too low. This medium c appears to have adequate salinity.

4. Literature:

Freeman, L. (1953):

A standardized method for determining toxicity of pure compounds to fish. *Sewage and Ind. Wastes* **25**, 845–848.

Freeman, L. and I. Fowler (1953):

Toxicity of combinations of certain inorganic compounds to *Daphnia magna* Straus.

Sewage and Ind. Wastes **25**, 1191–1195.

5. Literature:

Frear, D.E.H. and J.E. Boyd (1967):

Use of *Daphnia magna* for the microbioassay of pesticides.

I. Development of standardized techniques for rearing *Daphnia* and preparation of dosage – mortality curves for pesticides.

J. Econ. Entom. **60** (5), 1228–1236.

6. Literature:

EPA-660/3-75-009 (1975):

Methods for acute toxicity tests with fish, macroinvertebrates and amphibians.

7. In the Netherlands a simple medium was required, suitable for culturing most standard test animals and suitable as a dilution water in toxicity tests.

Media b, e, and f were considered; however, medium b contains no potassium, medium e contains no magnesium, and medium f contains more Mg than Ca.

All these conditions are not likely to occur in natural water.

DSW water contains all the natural macro-elements and has a hardness, salinity, and calcium-magnesium ratio average for Dutch surface waters.

8. This diluted sea water has approximately the same salinity as most standard types of fresh water, and moreover all biologically important elements are present.

9. This medium is used in particular for making concentrated suspensions of algal cells (e.g. for use as a food for filter feeders).

10. Some examples of the preparation of sea water:

39 g of 'HW salt' per litre gives a salinity of about 32.4‰ S and a chloride content of 18‰.

32.5 g of 'HW salt' per litre of water gives a salinity of about 27.0‰ S and a chloride content of 15‰.

5.7 Composition and monitoring of types of water

'HW salt' can be obtained from:

Spezial Grosshandel für Meeresaquaristik H. Wiegandt
415 Krefeld

Kölnerstrasse 514

Postfach 3135; telephone 37719.

The choice of the type of initial water depends on the final aim.

11. 'Instant ocean synthetic sea salts' can be obtained from: Aquarium Systems, Inc.

1450 East 289 Street, Wickliffe, Ohio, 44092.

The most important difference from 'HW sea water' is the addition of trace elements and the presence of EDTA (0.05 mg.l^{-1}) in this trace-element solution.

12. Many methods have been described for the preparation of soil extracts. The following prescription is from the Marine Laboratory in Plymouth, U.K.

Autoclave 1 kg of carefully selected leaf mould in 2 litres of distilled water for an hour at an excess pressure of $1/2 \text{ atm}$.

Allow to settle and cool, and filter or centrifuge.

Fill glass bottles with 50 ml of the clear solution, add 1 g of CaCO_3 per bottle, close with a plug of cotton wool, and sterilize for 20 min at 120°C .

Use the supernatant (not the CaCO_3 present on the bottom) for the Erdschreiber medium.

13. All types of water which are sterilized or pasteurized except those intended for the culture of algae can be prepared in large amounts. They retain a constant composition for a long time (at least a month) provided that they are well aerated with air saturated with water vapour.

Use glass bottles for the preparation and storage of small amounts, e.g. up to 50 litres, and polythene vessels for larger amounts.

14. As far as possible, synthetic water is prepared from good-quality distilled water whose conductivity, determined in accordance with Chapter 2, is smaller than $10^{-4} \text{ ohm}^{-1}.\text{cm}^{-1}$ (see also Section 2.2.5).

De-ionized water is not recommended, since it frequently contains toxic substances.

For the preparation of very large amounts, the use of spring or tap water cannot be avoided. This has little effect on the composition of the synthetic sea water apart from the calcium content. However, when using tap water, which may have been chlorinated, a check should be made whether active chlorine is still present in it (see Section 2.2.1). The following methods are available for removing the chlorine: aeration, irradiation with ultraviolet light, filtration through active carbon, and the addition of sodium thiosulphate ($\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$) (see also Section 2.2.1).

The preparation of synthetic fresh water from tap water is justifiable only if the composition of the mains tap is known and is taken into account.

5.7.3 Transport and storage of natural water

Small amounts of water can be transported in polythene vessels and, on arrival in the laboratory, filtered or centrifuged if necessary. The required checks are then performed (see Section 5.7.4).

Special provisions are required for large amounts. These can be illustrated on the basis of a specific example.

The water is pumped up and stored in a stainless-steel tank (e.g. a 10-ton road tanker). The tank has previously been thoroughly cleaned and then steamed out and may be used only for the transport of this water or, if this is impossible, for non-contaminating liquids such as dilute inorganic acids. On arrival at the laboratory the water is pumped into polyester/glass-fibre tanks lined with non-toxic PVC. The same material is also required for the piping system. The water is gently aerated for about 2 weeks so that suspended particles can settle. In general, it then becomes completely clear.

As soon as the checks (see Section 5.7.4) have been carried out and the quality has been found to be satisfactory, the water is pumped through a sand filter to remove any suspended particles still present, to a tank feeding, for example, a constant-level tank to which the working conduits are connected.

Sufficient well-examined water must always be kept in stock to bridge the time required for checking the next batch. If necessary, water can be freed more rapidly from silt particles by passing it through the sand filter at an earlier stage.

5.7.4 Quality checks

The quality checks described below are always performed before water collected outside the laboratory is used and before synthetically prepared water is used when the quality of the basic water is not assured.

5.7.4.1 Fresh water

- a. Determine the Na, K, Ca, Mg, Cl, and SO_4 contents and the alkalinity according to Section 2.2 and modify them if required by addition or dilution.

- b. Determine the pH and adjust it, if desired, by modifying the hardness or by passing in air with the CO_2 content found experimentally to be appropriate.

- c. If desired, determine the amounts of total inorganic P and total inorganic N by one of the methods customary in analytical chemistry.

- d. If desired, determine the contents of individual metals such as Cu, Zn, Hg, Cd, Pb, Cr, As by methods customary in analytical chemistry (e.g. by atomic absorption spectroscopy). Reject the water if these contents are unacceptably high.

- e. If desired, extract 1 litre of water with 5 ml of cyclohexane and obtain a gas chromatogram of the extract as described in Section 2.2.6.

Pay attention to chromatographic peaks that could indicate the presence of the known chlorine-containing pesticides.

Reject the water if these appear to be present in unacceptable concentrations (see Section 2.2.6).

- f. Carry out a biological quality check with *Daphnia magna*. For this purpose adjust the Na, K, Ca, Cl, Mg, and SO_4 contents and the pH to the values given for DSW or STM medium. With this water, in duplicate and in comparison with DSW or STM medium, carry out a 3-week chronic toxicity test with *Daphnia magna*. Renew the water every Monday, Wednesday, and Friday. The results must agree with the relevant requirements set for the blanks in description 5.10.5.

5.7.4.2 Sea water

- a. Determine the pH and the salinity according to Section 2.2. Reject natural sea water if these two values are too low. (This is because the sea water is then usually mixed with fresh water, which may be polluted). In the case of synthetic water also determine the Ca content.

- b. If desired, determine the contents of individual metals such as Cu, Zn, Hg, Cd, Pb, Cr, As by one of the methods customary in analytical chemistry (e.g. by atomic absorption spectroscopy). Reject the water if these contents are unacceptably high.

- c. If desired, extract 1 litre of water with 5 ml of cyclohexane and obtain a gas chromatogram of the extract as desired in Section 2.2.6.

5. Determination of toxicity

Pay attention to chromatographic peaks that could indicate the presence of the known chlorine-containing pesticides.
Reject the water if these appear to be present in unacceptable concentrations (see Section 2.2.6).

d. Carry out a biological quality check with *Artemia salina*.

With the sea water to be tested in duplicate and in comparison with 'HW water' prepared from distilled water perform a 3-week chronic toxicity test with *Artemia salina*.

Renew the water every Monday, Wednesday, and Friday.

The results must agree with the relevant requirements set for the blanks in description 5.10.4.

5.7 Table 1 Composition of some types of sea water

Component	Natural sea water ¹⁾		H.W. water ²⁾		Instant ocean ²⁾	
	mg.l ⁻¹	mmole.l ⁻¹	mg.l ⁻¹	mmole.l ⁻¹	mg.l ⁻¹	mmole.l ⁻¹
Na	10556	459.0	10752	467.5	10200	443.5
K	380	9.7	390	10.0	370	9.5
Mg	1272	53	1295	54.0	1200	50.0
Ca	400	10	416	10.4	370	9.3
Sr	8.5	0.1	13	0.15	8	0.09
Mn	0.001-0.01				1	
Li					0.2	
Rb					0.1	
Al					0.04	
Zn	0.005-0.014				0.02	
V	0.0002-0.007				0.02	
Co	0.0001-0.0005				0.01	
Fe	0.001-0.29				0.01	
Cu	0.01-0.024				0.003	
Cr	0.001-0.003					
Mo	0.0003-0.016					
Si	0.01-7.0					
Cl	18980	534.6	19346	545.0	18400	518.0
SO ₄	2649	27.6	2700	28.1	2500	26.0
HCO ₃ ³⁾	140	2.3	145	2.4	140	2.3
H ₃ BO ₃	26	0.42	27	0.44	25	0.40
Br	65	0.81	67	0.84	20	0.25
P	0.001-0.17		0.18		0.3	
NO ₃ +NO ₂ +NH ₄	0.001-0.7					
MoO ₄					0.7	
SiO ₃					0.4	
I			0.38		0.07	
F	1		1.3			
EDTA					0.05	

⁴⁾

¹⁾ Kinne, O. (Editor) 1970.

Marine Ecology, Vol. 1, Environmental Sectors Part 1, page 66.

Average composition of sea water of 34.325‰ S.

²⁾ According to information from the manufacturer.

³⁾ HCO₃ and CO₃.

⁴⁾ According to the manufacturer, the water also contains the following elements in amounts < 1 mg/l: Mn, Li, Rb, Al, Zn, V, Co, Fe, Cu, Mo, Sb, As, Ba, Be, Pb, Cd, Cs, Ce, Cr, Ga, Ge, Au, La, Ni, Nb, Hg, Rb, Sc, Se, Ag, Si, Th, Ti, U, Bi, W, Y, Sn.

5.7 Composition and monitoring of types of water

5.7 Table 2 Composition of some synthetic fresh waters

Component	Soft A & A water		Hard A & A water		Hard A & A water + extra NaCl		St. ref. water		STM		Sea water/ distilled water 1/100		Soft reconstituted fresh water		DSW	
	mg.l ⁻¹	mmole.l ⁻¹	mg.l ⁻¹	mmole.l ⁻¹	mg.l ⁻¹	mmole.l ⁻¹	mg.l ⁻¹	mmole.l ⁻¹	mg.l ⁻¹	mmole.l ⁻¹	mg.l ⁻¹	mmole.l ⁻¹	mg.l ⁻¹	mmole.l ⁻¹	mg.l ⁻¹	mmole.l ⁻¹
Na	11.3	0.49	113	4.9	220	9.6	17	0.74	54.74	2.38	107.5	4.68	13.14	0.571	27.4	1.19
K							3.4	0.084	11.7	0.30	3.9	0.10	1.05	0.027	7.8	0.20
NH ₄							0.68	0.038								
Ca	5.9	0.15	59	1.5	59	1.5	28.5	0.71	81.6	2.04	4.16	0.10	6.98	0.174	54.4	1.36
Mg	1.5	0.06	15	0.6	15	0.6	7.0	0.29			12.95	0.54	6.06	0.249	17.5	0.73
Sr											0.13	0.0015				
Mn							0.06	0.001								
Fe							0.22	0.004								
Cl	12.1	0.34	121	3.4	284	8.0	9.5	0.268	144.8	4.08	193.5	5.37	0.95	0.027	96.6	2.72
NO ₃	0.7	0.01	7	0.1	7	0.1	2.3	0.038								
SO ₄	11.2	0.12	112	1.2	112	1.2	31.4	0.328	14.4	0.15	27.0	0.28	40.61	0.423	70.1	0.73
HCO ₃	20.0	0.33	200	3.3	200	3.3	present		145.2	2.38	1.45	0.024	34.86	0.571	84.8	1.39
P							0.15	0.0048				0.0019	0.00006			
SiO ₃							17	0.22								
Br											0.67	0.008				
I											0.004	0.00003				
H ₃ BO ₃											0.27	0.0044				
Other characteristics																
pH	7.2±0.2		8.2±0.2		8.2±0.2		7.9±0.2		8.2±0.2		7.6±0.2		7.4±0.2		8.2±0.2	
Chloride in ‰	0.012		0.12		0.28		0.01		0.145		0.19		0.001		0.097	
Na/K (molar)	—		—		—		8.8		7.9		46.8		21.1		6.0	
Ca + Mg																
hardness																
in meq.l ⁻¹	0.42		4.2		4.2		2.0		4.0		1.28		0.84		4.2	
in mmole.l ⁻¹	0.21		2.1		2.1		1.0		2.0		0.64		0.42		2.1	
hardness as mg																
CaCO ₃ per litre	21		210		210		100		200		64		42		210	
hardness in																
German degrees																
of hardness	1.18		11.8		11.8		5.6		11.2		3.58		2.35		11.8	

5.8 BREEDING OF TEST ORGANISMS IN THE LABORATORY

5.8.1 Introduction

This section 5.8 gives the instructions only for the breeding of organisms that have been bred in our laboratory for several successive generations with very good yield, and which in addition are suitable for toxicity tests.

More or less fortuituous successes, such as the breeding of copepods over several generations (after which the culture failed for no apparent reason), the breeding of larvae from ripe eggs collected in nature, and breeding with a yield too low for use in toxicity tests, are therefore omitted.

Where the toxicity tests make use of animals that grow or develop during the test and that are not derived from laboratory populations, their development is described in detail with the descriptions of the tests themselves.

Since for both fresh water and for sea water we have to rely on synthetic water prepared in the laboratory or on the supply of natural water by road tankers, and therefore have to work under relatively difficult conditions, it should be possible to follow the breeding instructions in each laboratory regardless of the situation. It will often be possible to carry out the breeding in more or less modified form.

In addition to this, many other organisms suitable for toxicity tests can be bred in the laboratory.

Because of the special nature of studies with unicellular algae, Section 5.8.2 first gives some observations relating specially to the culturing of unicellular algae.

5.8.2 Unicellular algae

GENERAL OBSERVATIONS

The aim of culturing on the basis of the following procedure is to obtain algal cultures for toxicity tests. An attempt has been made to keep the methods as simple as possible, and they are therefore based on conventional microbiological procedures.

The algal cultures mentioned in 5.8.1, Table 1 were selected because of the ease with which they can be grown. The culturing procedure described in point 3 is of a general nature and is not adapted to any definite algal species.

The algal cultures may be infected with bacteria. In many cases infection with bacteria cannot be prevented, and some species grow poorly without bacteria. However, in the methods of culturing all operations are carried out under completely sterile conditions, in order to avoid contamination with bacteria and other algae in that stage.

The extent to which, in the culturing of the algae, use is made of the media that are also used for the maintenance or breeding of test animals, is referred to in Section 5.7.

However, the formulations of some media used exclusively for algae are given in Section 5.8.2, point 2, Equipment and Materials.

EQUIPMENT AND MATERIALS

- Axenic cultures or monocultures of unicellular algae suitable for toxicity tests (see 5.8.2, Table 1). A number of facts have been collected for algae to be used in the tests
- culture tubes
- culture bottles, 200 ml
- conical flasks, 200 ml, with ground-glass stoppers
- conical flasks, 3 litre

- incubators or climate rooms adjustable to 15 or 20°C, with fluorescent light (TL 33), approximately 5000 lux
- filter apparatus for membrane filters, 10 cm in dia.
- membrane filters, dia. 10 cm, with a pore size of 0.1 µm
- filtration flask, volume 5 litres
- crystallization basins
- silicone-sponge caps
- inoculating needle
- sterile graduated pipettes, 5 ml and 10 ml
- autoclave
- cell counter, such as the Coulter Counter (see Section 2.7.6) or counting chamber (see Section 2.7.4.2)
- nutrient solutions (media)
- pH meter.

a. Media based on sea water

- a.1 Erdschreiber medium (see Section 5.7.2 k)
- a.2 Erdschreiber medium with extra bicarbonate.
Prepare the Erdschreiber medium as described in Section 5.7.2 k and add 0.2 g of NaHCO₃ per litre. If this is to be added from a stock solution, the latter must be sterilized by filtration.
- a.3 Erdschreiber medium with extra silicate.
Prepare the Erdschreiber medium as described in Section 5.7.2 k and add 0.03 g of Na₂SiO₃·9H₂O per litre. This may be added as a sterile stock solution.

Soil extract is added exclusively to media intended for stock cultures, and not if they are intended for pre-cultures for the toxicity tests.

b. Fresh-water media

- b.1 'Chlorella' medium (see Section 5.7.2 h).
- b.2 Gorham's modified nutrient solution

NaNO ₃	0.5 g
NH ₄ NO ₃	0.33 g
K ₂ HPO ₄ ·3H ₂ O	0.052 g
MgSO ₄ ·7H ₂ O	0.075 g
CaCl ₂ ·2H ₂ O	0.035 g
Na ₂ CO ₃ ·10H ₂ O	0.054 g
Ferric citrate	0.006 g
Citric acid·H ₂ O	0.006 g
trace-element solution	1 ml
(see Section 5.7.2 h)	
Distilled water	1 l
pH	7.7 ± 0.3

c. Agar media

All the above-mentioned media can be used as agar media by the addition of 1.5–2 % agar.

5.8.2. Table 1 Review of algal species suitable for use in toxicity tests. Taxonomic classification, medium, and cultivation temperature

Algal species	Group	Medium	Temperature
<i>Chlamydomonas</i> sp.	Chlorophyceae	a.2	15°C
	Volvocales		
<i>Chlorella ovalis</i>	Chlorophyceae	a.2	15°C
	Chlorococcales		
<i>Dunaliella</i> sp.	Chlorophyceae	a.2	15°C
	Volvocales		
<i>Monochrysis</i> sp.	Chrysophyceae	a.2	15°C
<i>Phaeodactylum</i>			
<i>tricornutum</i>	Chrysophyceae	a.3	15°C
<i>Chlorella</i>	Chlorophyceae	b.1 or b.2	20°C
<i>pyrenoidosa</i>	Chlorococcales		
<i>Scenedesmus</i>	Chlorophyceae	b.2	20°C
<i>quadricauda</i>	Chlorococcales		
<i>Chlamydomonas</i>	Chlorophyceae	b.2	20°C
<i>reinhardtii</i>	Volvocales		

PROCEDURES FOR OBTAINING ALGAL CULTURES

Preparation of nutrient solutions (media)

In work with unicellular algae as the **test organisms**, the sea water and fresh water media are sterilized by filtration (see also **Pre-culture**) unless they contain agar.

The previously sterilized soil extract and the other required components are added aseptically.

Liquid and/or solid media are prepared for the **stock cultures**. Volumes of about 100 ml are generally used to maintain the cultures in nutrient solutions.

The necessary nutrient solutions are prepared in accordance with the formulae given above, sterilized by filtration, and distributed among sterile culture bottles or conical flasks.

To maintain cultures on **solid nutrient media**, the required media are prepared according to the instructions and are sterilized by heating in an autoclave at 120°C after the addition of 1.5 to 2% of agar, soil extract, etc. (see Section 5.7.2 h).

Only liquid media are used for **pre-cultures**. These are prepared as described above, but are divided into larger volumes (e.g. 2-l and 3-l conical flasks).

Stock culture

The stock cultures are small algal cultures that are regularly transferred to act as the initial test material.

If the cultures are not used regularly, many of the species (especially the Chlorophyceae) are streaked out on sloped agar tubes. These are transferred to fresh medium at least once every two months.

The other stock cultures are grown in culture bottles or conical flasks containing the appropriate mineral medium (volume about 100 ml).

When the algae are incubated at 15°C with periodic (day/night) illumination, it is sufficient to transfer the cultures once every two to three weeks; at higher temperatures and/or with continuous illumination, a weekly transfer is required.

During transfer, an amount of 'old' culture is transferred with sterile pipettes into a bottle of fresh medium, in such a way that with the fast-growing species the initial concentration is about 100 times, and with slow-growing species about 10 times, smaller than in the 'old' culture.

The growth rate of a species can be determined from the growth curve (see Sections 2.7.4.2 and 2.7.6). If this is known, it is possible to estimate at what density the culture should be transferred to the new medium. This must be done before the culture reaches the death phase.

Pre-culture

The pre-culture is intended to give an amount of algal culture in the exponential growth phase suitable for the inoculation of test cultures.

For this pre-culture use 2 litres of sterile nutrient solution in 3-litre conical flasks.

Add sufficient algal suspension from the stock culture to give a concentration of algae of approximately 10^4 cells.ml⁻¹.

Illuminate the flasks continuously with fluorescent light at 15 or 20°C, depending on the algal species (see 5.8.2, Table 1). Shake the cultures every day (this is particularly important for non-motile species; see Note 1).

Determine the cell concentration in the culture and for the toxicity tests use a culture in the exponential growth phase (see Note 2). See also Section 5.10.1.

NOTES

1. Other methods of culturing may also be used. Some algae (that can withstand it) can be grown rapidly in shake cultures. Aeration with air containing additional CO₂ also accelerates growth.
2. The incubation time depends on many factors, but with the described culturing method the pre-culture is optimal for inoculation in toxicity tests after 4–5 days in the case of the commonest algae. Depending on the species and on the condition of the stock culture, the incubation time may be longer.

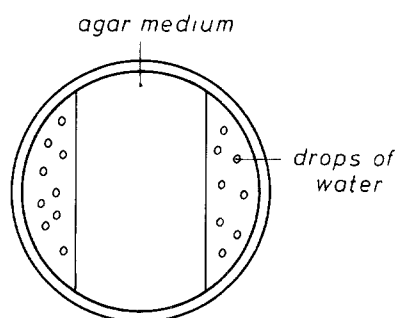
5.8.3 *Lymnaea stagnalis*

EQUIPMENT AND MATERIALS FOR BREEDING THE POND SNAIL *LYMNAEA STAGNALIS*

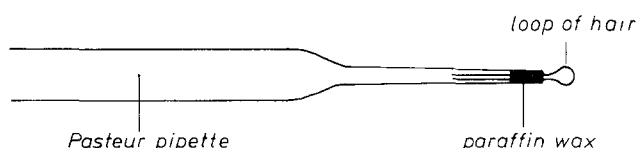
- Initial material: adult pond snails, collected in a ditch
- climate room, temperature $21 \pm 2^\circ\text{C}$, normal day/night rhythm of illumination
- incubator, temperatures $25 \pm 1^\circ\text{C}$
- all-glass aquaria, about 10 litres
- all-glass aquaria, about 2 litres
- aeration tubes of lengths fitting into the aquaria, diameter about 5 mm
- aeration apparatus
- sheet of filter paper
- Petri dishes, dia. 5 cm, with agar nutrient: prepare the agar nutrient by adding 2% of agar (agar Noble, Difco) to fresh water of standardized composition. Heat this mixture on a boiling water bath until the agar has dissolved. Pour the liquid agar solution into the Petri dishes to a layer thickness of 0.5 cm. After cooling, cut away two segments of the agar medium (see 5.8.3, Fig. 1) and add a few drops of water into the spaces so produced to prevent drying out of the agar
- embryo dishes
- knife with thin blade ending in a point
- Pasteur pipettes with cut-off tip, opening diameter ca. 2 mm, sharp edges rounded off and matching teats
- hairloop fixed to a Pasteur pipette (see 5.8.3, Fig. 2). Heat and pull out the thin part of a Pasteur pipette to form a capillary; break the capillary in the middle and round off the sharp edges in a flame. After cooling, insert a stiff human hair in the form of a loop (diameter 1 mm) into the opening and fix the loop by drawing a little liquid paraffin wax into the tip of the pipette

5. Determination of toxicity

- stereomicroscope with incident light illumination, magnification 4–40
- pH meter
- oxygen meter
- fresh water of standardized composition: standard reference water (see Section 5.7.2 d) or hard A & A water (see Section 5.7.2 b) or Dutch Standard water (see Section 5.7.2 g)
- food: washed lettuce leaves (preferably not treated with pesticides).



5.8.3 Fig. 1 Petri dish with agar medium



5.8.3 Fig. 2 Hairloop fixed to a Pasteur pipette

PROCEDURE

Familiarity with the course of development of the pond snail is assumed (see Section 5.3.2.5).

- Collect adult pond snails from a ditch (see Note 1).
- In the laboratory, transfer the snails to the 10-litre aquaria filled with fresh water (pH 8.0 ± 0.2 ; O_2 concentration $>80\%$ of the saturation value) and provide the aquaria with an air supply; two snails per litre of water; the water temperature should be $21 \pm 2^\circ\text{C}$.
- Feed the snails with lettuce leaves.
- On the following day (see Note 2) transfer the egg clusters deposited on the aquarium wall by hand to a Petri dish with a little water, check their quality under a microscope, and use only egg masses with not more than 10% of multipler (these and other terms are explained in Section 5.8.3.4).
- Free the suitable egg masses from their jelly, making a fine cut in the covering and then carefully rolling the capsules over moist filter paper; keep the egg masses separate from one another.
- Transfer the liberated capsules from one egg mass to the agar medium in a Petri dish with the aid of the knife blade.
- Spread the capsules with the hairloop over the agar (Note 3).
- Place the closed Petri dish with the eggs in the incubator, temperature $25 \pm 1^\circ\text{C}$.
- Take care that a few drops of water are always present in the agar-free segments of the Petri dishes to prevent drying out.
- On the 7th or 8th day after the eggs have been laid – i.e. with normal development shortly before emergence – check the quality of the egg masses under the microscope (see Note 4).
- Continue the culturing with egg masses of which at least 85% are normally developed snails.
- Transfer the snails that have emerged from the suitable egg masses to a 2-litre aquarium containing about 1 cm of water and place this aquarium in the breeding room at $21 \pm 2^\circ\text{C}$.
- Aerate this aquarium moderately.
- Feed the snails once every 2 to 3 days with lettuce leaves (remove the main veins) with amounts just sufficient for this period.
- Once a week add water to the water already present in the aquaria, so that the level rises by 1 cm per week (renewal of the water is unnecessary).
- Six weeks after their emergence, transfer the snails to the larger aquaria filled with 10 litres of water (50 to 60 snails per 10 litres of water).
- Aerate the water well in these aquaria.
- Feed the snails 2 to 3 times a week with washed lettuce leaves in amounts just sufficient for the period concerned; at the same time, remove the residues of old lettuce leaves.
- Renew the water in the aquaria once a week and clean the aquaria thoroughly during this process.
- Distribute the 3- to 4-month snails as soon as they start producing egg masses over a larger number of larger aquaria; 20 snails per 10 litres of water.
- If at a given time egg masses are required for toxicity tests (see Sections 5.9.2 and 5.10.3), stimulate the snails to lay eggs in the following way: 3–5 h before the time at which the egg masses are required, transfer a number of snails to a clean aquarium with fresh water (see Note 5).
If necessary, apply extra stimulation by:
 - a. raising the temperature of the fresh water from 20 to 25°C ;
 - b. subjecting the snails, which are normally bred under moderate illumination, to additional illumination (see Note 6).
- Repeat the whole breeding procedure starting from wild snails once every 6 months if it is necessary to have eggs continuously available for toxicity studies.

NOTES

1. Wild pond snails collected in ditches are usually infected with the larvae of parasitic flatworms. To prevent an infection of any pond snail culture already present in the laboratory, the wild snails must be kept separate from this culture. Transfer of the infection via hands must be prevented. The breeding procedure described in 'Procedure' is aimed at obtaining pond snails that are free from infection with flatworm larvae.
(Warning: allergy to free-swimming flatworm larvae is possible).
2. If no egg masses have been deposited on this day, the snails must be fed until egg-laying takes place. If necessary, renew the water once a week.
3. When the embryonic development takes place in the egg masses, the eggs at the edges develop faster than those located centrally. By dissecting the eggs out and placing them on an agar substrate a uniform development of the eggs is strongly promoted.
4. From the 5th day after egg laying, during the normal development of snail embryos, the following characteristics can be clearly recognized: the head with the eyes, the foot, and the intestinal

sac surrounded by a shell (see 5.8.3, Fig. 7). The heartbeat is detectable.

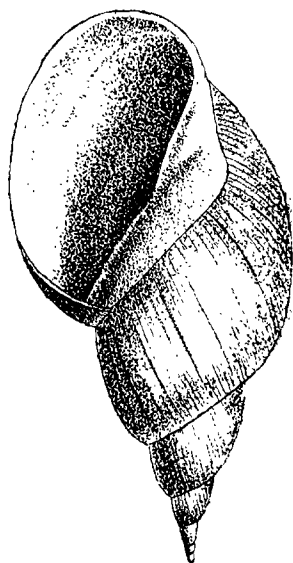
If the above developmental stage has not been reached, or other abnormalities of a morphological nature appear, they are regarded as not having developed normally.

5. Although ponds snails can lay egg masses every 3–4 days, it is better not to stimulate the same ones to egg-laying more often than once a week. If egg masses are required several days within one week, the stock of snails must be divided into batches so that a rotation system for stimulation can be arranged.
6. Additional stimulation measures may be necessary, particularly in winter. During this season the required stimulation period is frequently longer than in the summer.
In addition, during winter greater mortality frequently occurs among the cultured snails and the number of eggs per egg mass is often smaller than in the summer.

BRIEF DESCRIPTION OF THE DEVELOPMENT OF THE POND SNAIL *LYMNAEA STAGNALIS* L.

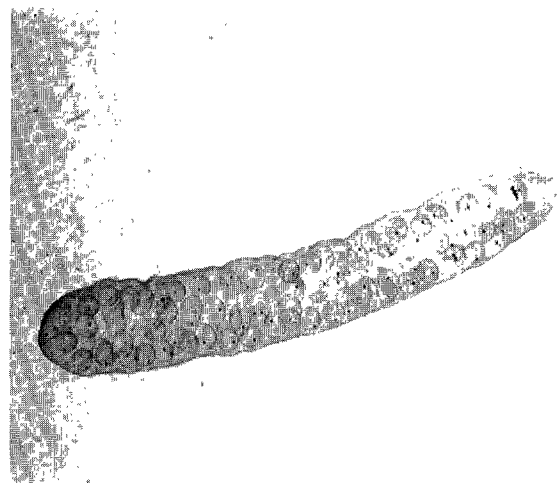
The pond snail (5.8.3, Fig. 3) is hermaphroditic and thus has male and female sex glands.

However, self-fertilization takes place mainly when individual snails are isolated. The eggs of the pond snail are held in transparent capsules surrounded by capsule fluid. These capsules are deposited in oblong, gelatinous egg masses. An egg mass may contain 75–130 eggs (see 5.8.3, Fig. 4).



5.8.3 Fig. 3 Shell of *Lymnaea stagnalis* (magnification 2.5)

An egg capsule contains an ovum surrounded by capsule fluid. Several ova per capsule do in fact occur, and are usually found at the end of the egg mass that has been deposited last (see 5.8.3, Fig. 4).



5.8.3 Fig. 4 Egg mass of *Lymnaea stagnalis* with egg capsules in which the ova, surrounded by capsule fluid, are located. At the tied-off end of the egg mass (on the right) there is a capsule containing several ova (magnification $\times 3$)

The multiplets are usually non-viable. In egg masses with numerous multiplets there is frequently also a high mortality among the capsules containing only one ovum each.

The fertilized ovum (the embryo) develops within the capsule. At a temperature of 25°C, this development takes place as follows (Raven, 1964; Verdonk, 1965): 10–70 min after oviposition (laying of the egg mass), the first polar body is expelled following the first maturing division. About 40 min after the first, following the second maturing division, the second polar body is expelled. The two polar bodies are expelled at the animal pole of the ovum, i.e. at the end of the cell's axis of symmetry on the side of the nucleus (5.8.3, Fig. 5a).

About 75 min after the expulsion of the second polar body the first cleavage takes place. The eggs in one egg mass do not all cleave simultaneously; the time difference between the first and the last cleavage is 10–45 min.

The first cleavage of the ovum begins with a flattening in the animal-vegetative direction and a constriction on the animal side (5.8.3, Fig. 5b) (in the toxicity test described in Section 5.9.3 this stage is used as a point of reference for the first cleavage and is called stage 1).

Completion of the first cleavage results in two identical halves or blastomeres (5.8.3, Fig. 5c).

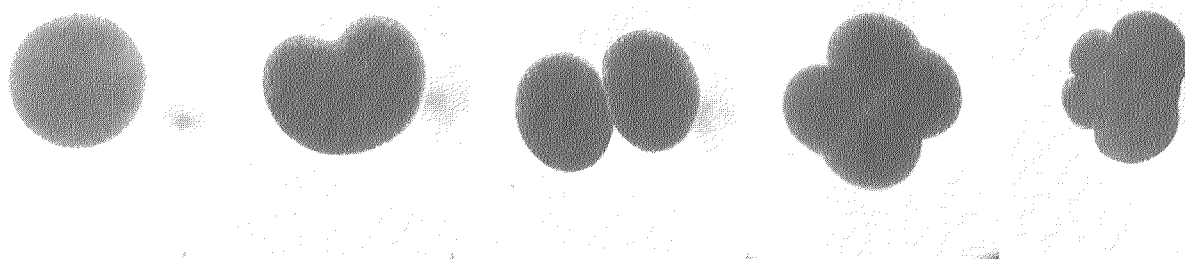
About 85 min after the beginning of the first cleavage the second cleavage takes place. Just like the first, it is holoblastic and equal. Four identical blastomeres have now been formed (5.8.3, Fig. 5d). The third cleavage takes place approximately 80 min after the second. It is a spiral cleavage, leading to 4 small cells (micromeres) and 4 larger ones (macromeres) (5.8.3, Fig. 5e).

The number of cells increases greatly, through further cell divisions, but the size of the cells decreases. 24 h after the first cleavage gastrulation takes place: the multicellular embryo (blastula) flattens in the animal-vegetative direction and directly after this invagination of cells at the vegetal pole takes place.

About 72 h after the first cleavage the trochophore stage is reached; this spherical larva swims around freely in the capsule fluid (5.8.3, Fig. 6).

Following the trochophore stage the veliger stage arises, already with a characteristic differentiation of head, foot, and intestinal sac. The late veliger stage is called the hippo stage. In this stage, which arises five days after the first cleavage, together with the embryonic characteristics adult characteristics are already present. The eyes can be recognized as dark spots in the head region; the intestinal sac is already surrounded by a shell and the heart is beating (5.8.3, Fig. 7).

5. Determination of toxicity



5.8.3 Fig. 5 Cleavage of the ovum of *Lymnaea stagnalis* (magnification 120)

5a. Uncleaved ovum with first and second polar bodies

5b. Stage 1, reference point for the first cleavage

5c. First cleavage completed. Two similar blastomeres have been produced

5d. Second cleavage, four blastomeres

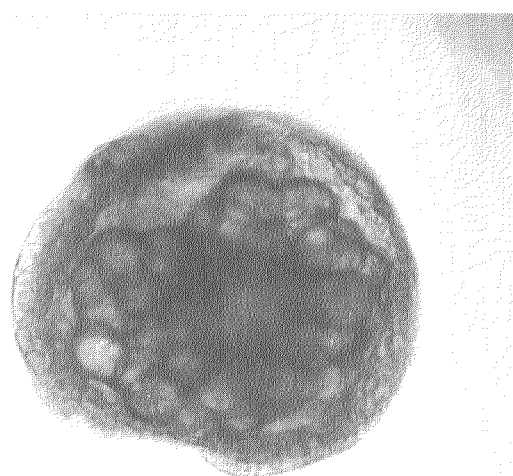
5e. Third cleavage, four macromeres and four micromeres.

On the 9th or 10th day after egg-laying the young snails emerge and then already have a shell of one to two turns with a height of about 0.8 mm. On further growth at room temperature the snails develop rapidly to form tall narrow shells and only later acquire the inflated final convolution. Even before this final stage, 3 months after egg laying (at 20°C), the animals are sexually mature and produce egg masses once or twice a week for about 5 months. 5.8.3, Table 1 shows the time-table of the pond snail's development.

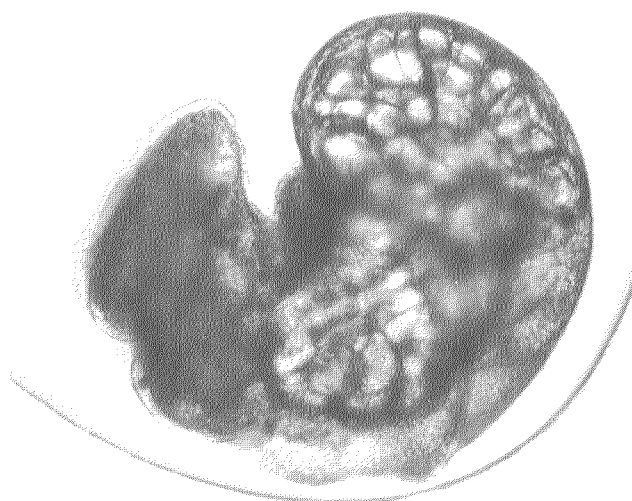
5.8.3 Table 1 Time course of the embryonic development of *Lymnaea stagnalis*.

The times between the successive stages are approximate (25±1°C)

Oviposition	
	10–70 min
1st polar body	
	40 min
2nd polar body	
	75 min
1st cleavage	
	85 min
2nd cleavage	
	80 min
3rd cleavage	
	24 h
gastrulation	
	2 days
trochophore stage	
	2 days
hippo stage	
	2 days
development of the embryo completed.	



5.8.3 Fig. 6 Trochophore larva swimming freely in the egg capsule, about four days after oviposition (temp. 25°C) (magnification 200)



5.8.3 Fig. 7 Young snail still within the egg capsule, about seven days after oviposition (temperature 25°C) (magnification ×100)

5.8.4 *Ophryotrocha diadema*

EQUIPMENT AND MATERIALS FOR BREEDING THE POLYCHAETE WORM *OPHRYOTROCHA DIADEMA*

- Worms obtained from an existing laboratory culture (Note 1)
- climate room, temperature $22 \pm 2^\circ\text{C}$ and $15 \pm 2^\circ\text{C}$, normal day/night rhythm of illumination (the illumination is not critical)
- refrigerator
- freezer
- breeding vessels; glass evaporating dishes, diameters 10 cm and 6 cm, with matching watchglasses as cover
- pH meter
- oxygen meter
- salinity meter
- stereomicroscope, with incident light, magnification 4–40
- Pasteur pipettes, with cut-off tips, opening diameter ca. 2 mm, sharp edges rounded off, and matching teats
- jars with tightly fitting lids, ca. 25 ml
- synthetic or natural sea water, salinity $32 \pm 2\text{‰}$ (see Section 5.7.2 j and k; Section 5.7.3; Section 5.7.4)
- food: liquidized spinach (see Note 2).

PROCEDURE

- Familiarity with the course of development of *Ophryotrocha diadema* is assumed to be known (see Section 5.3.2.6).
- Place the breeding vessels, evaporating dishes, diameter 10 cm, in the breeding room at $22 \pm 2^\circ\text{C}$.
- Fill the dishes with about 100 ml of sea water (pH 8.0 ± 0.2 , O_2 concentration over 80 % of the saturation value).
- Transfer about 40 worms from the stock to each breeding vessel. For this purpose, with a Pasteur pipette, draw the worms one by one from the stock and release them under the surface of the liquid in the breeding vessels.
- Add about 1 ml of liquidized spinach per cultivation vessel.
- Cover the breeding vessels with the watchglasses.
- Renew the water twice a week. For this purpose pour the water carefully from the breeding vessel (whereupon the worms remain on the glass wall) and rapidly add clean water to the dish.
- Feed after each renewal.
- Every week set up one or more new cultivation vessels with 100 larvae in each which, counted unilaterally, possess not more than five parapodia (see Note 3).
- Use the worms in the culture not longer than 6 weeks after the first egg laying.

If at any time worms of a given age are required for toxicity tests, these can be obtained by:

a. Isolation

Fill evaporation dishes, diameter 5 cm, with 5 ml of sea water. Add one sexually mature worm and one drop of liquidized spinach per dish. Place the fishes in the breeding room at $15 \pm 2^\circ\text{C}$. After about one week, transfer the worms to a breeding vessel (evaporating dish, diameter 10 cm) at $22 \pm 2^\circ\text{C}$. After 8 days collect the emerged larvae (see Note 4).

b. Selection

Select the newly emerged larvae from the brood stock. In both

cases a and b grow the larvae to the desired age and use them in the tests (see Note 5).

NOTES

1. Test animals

In addition to *Ophryotrocha diadema* there is a number of other species of polychaete worms suitable for toxicity tests, whose cultivation can be carried out in much the same manner.

Up to the present time all laboratory cultures of *Ophryotrocha diadema* and other *Ophryotrocha* species have been established with worms obtained, free from infection, from cultures of Dr. B. Åkesson of the Zoological Laboratory of the University of Goteborg, Sweden. Since newly collected worms are often infected with parasites which cause a marked decrease in reproduction, it is recommended to use existing infection-free cultures for toxicity tests.

2. Food

The food consists of deep-frozen spinach liquidized with a little sea water in a mixer. The liquidized spinach can be refrozen in tightly-stoppered jars. Opened pots of spinach must be stored in the refrigerator; after 4 days this spinach is no longer usable.

3. Losses during breeding

During the cultivation of larvae a number will be lost through mortality and owing to losses during the renewal of the medium, so that out of a cultivation vessel of 100 larvae only about 40–50 will become adults.

4. Synchronization of egg laying

After isolation for about a week at a temperature of about 15°C , sexually mature animals, after the end of isolation, will proceed to lay eggs within 24 h at a temperature of 22°C . It must be reckoned that at least twelve fully grown worms are necessary for 100 larvae.

5. Tests with 2- to 3-day larvae

The experiments with larvae must be performed with animals that are 2 to 3 days old and have begun to eat. The spinach is then clearly visible in the digestive tract. In this way they can be distinguished from the non-eating larvae that occur in many egg masses, live for only a few day, and are unusable.

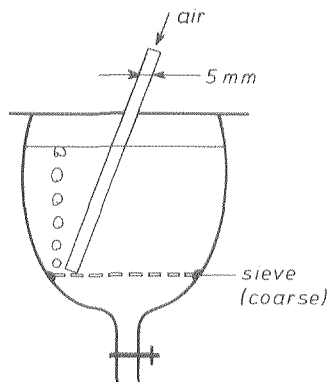
5.8.5 *Artemia salina*

EQUIPMENT AND MATERIALS FOR BREEDING THE BRINE SHRIMP *ARTEMIA SALINA*

- A few pairs of *Artemia salina* obtained from an existing laboratory culture or commercially obtainable cysts of this animal, both of known strain (see note)
- climate room, temperature $24 \pm 1^\circ\text{C}$, moderately illuminated for 8 h a day (the illumination intensity is not critical)
- all-glass aquaria, approximately 10-litre and/or all-glass 10-litre separating funnels provided with a nylon sieve plate with a mesh of approximately $1\text{ mm} \times 1\text{ mm}$ just above the run-off tap. (See 5.8.5, Fig. 1 and 5.8.5, Fig. 2)
- all-glass aquaria, approximately 2-litre, or plastic disposable vessels of approximately 2-litre capacity
- funnel-shaped sieves with nylon gauze sieve plates with a mesh of approximately $0.1 \times 0.1\text{ mm}$ and approximately $1 \times 1\text{ mm}$ (see 5.8.5, Fig. 3).
- aeration tubes with a length suitable for the breeding aquaria or funnels, about 5 mm in diameter
- aeration apparatus
- pH meter

5. Determination of toxicity

- oxygen meter
- salinity meter
- synthetic or natural sea water, salinity $30 \pm 2\text{‰}$ (see Section 5.7.2, j and k; Section 5.7.3; Section 5.7.4)
- suspension of *Dunaliella viridis* in sea water (see Section 5.8.5, point 3).



5.8.5 Fig. 1 Separating funnel provided with a nylon sieve

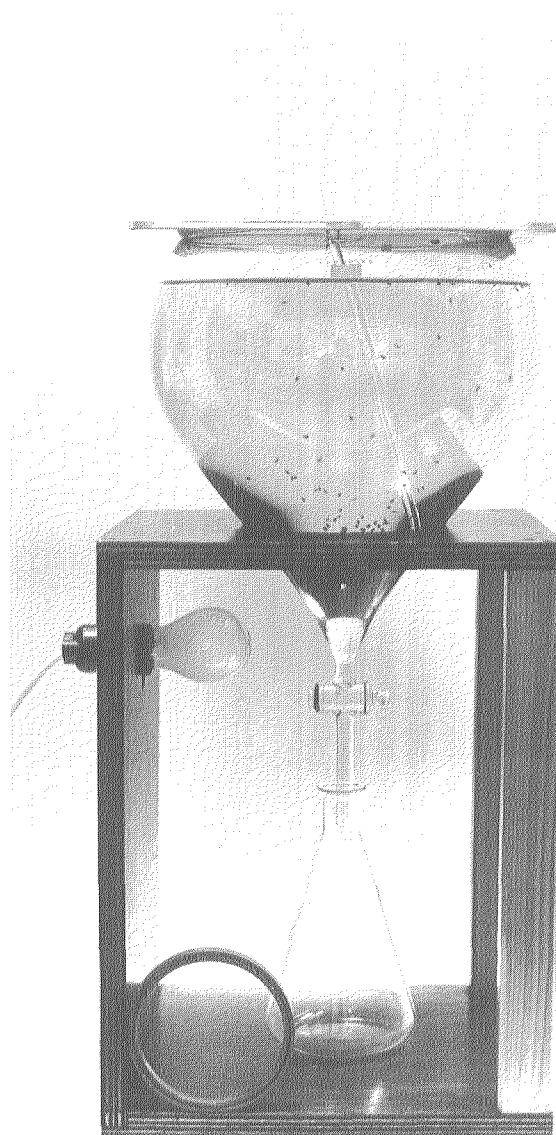
EQUIPMENT AND MATERIALS FOR CULTURING *DUNALIELLA VIRIDIS* AS A FOOD FOR *ARTEMIA SALINA*

- Pure or unialgal culture of *Dunaliella viridis*
- climate room, temperature $15 \pm 2^\circ\text{C}$, provided with a continuous illumination of about 4000 lux for the algal culture
- conical flasks, 300 ml, for the pre-cultures
- stock of pre-cultures prepared according to Section 5.8.5, point 3a
- conical flasks or other heat-resistant flasks (all-glass), 3- to 20-litre
- aeration stones
- sterile pipettes, 1 ml and 10 ml
- apparatus to supply air saturated with water vapour
- autoclave
- centrifuge
- pH meter
- salinity meter
- synthetic or natural sea water, salinity $30 \pm 2\text{‰}$ (see Section 5.7.2 j and k; Section 5.7.3; Section 5.7.4)
- sterile solution of 0.1 g of NaNO_3 per ml of distilled water
- sterile solution of 0.02 g of $\text{Na}_2\text{HPO}_4 \cdot 12 \text{H}_2\text{O}$ per ml of distilled water
- sterile soil extract.

PREPARATION OF A CONCENTRATED SUSPENSION OF *DUNALIELLA VIRIDIS* CELLS

a. Preparation of the pre-cultures

Autoclave a 300-ml conical flask closed with a cotton-wool plug, containing 100 ml of sea water, for 20 min at 120°C . Allow to cool and then add, aseptically, 0.1 ml of NaNO_3 solution, 0.1 ml of Na_2HPO_4 solution, and 5 ml of soil extract. Inoculate the contents of the flask aseptically with 1 ml of a previous culture about 12 days old.



5.8.5 Fig. 2 Separating funnel used for the breeding of *Artemia salina* and *Daphnia magna*

Place the conical flask in the climate room in the light (about 4000 lux) for 12 days. Shake the contents daily by hand. After approximately 12 days the culture contains about 4.10^6 cells per ml and can be used to start cultures intended for feeding and for inoculating new pre-cultures.

b. Preparation of cultures to be used as food for the brine shrimp

Pasteurize a conical or other flask of heat-resistant glass about two-thirds filled with seawater, provided with an aeration stone reaching to the bottom, and closed with a plug of cotton wool, for 2 h at 100°C .

Allow to cool and add aseptically, per litre of sea water: 1 ml of NaNO_3 solution, 1 ml of Na_2HPO_4 solution, and 50 ml of soil extract. Inoculate this solution aseptically with 100 ml of a pre-culture.

5.8 Breeding of test organisms in the laboratory

Place the flasks in the climate room in the light (about 4000 lux) for about 12 days. Pass in air saturated with water vapour continuously via the aeration stone. Shake the flasks daily by hand if necessary.

After about 12 days the culture density is approximately $4.5 \cdot 10^6$ cells per ml.

c. Preparation of the food suspension

Centrifuge the contents of the flasks for about 20 min at 2000 g, decant off the supernatant, and suspend the algae in sea water to a density of 3.10^8 cells per ml. Keep this suspension at about 4°C. The suspension can be stored for about 1 week without a deterioration in food value for the brine shrimps.

d. Observation

The cultures of *Dunaliella viridis* are seldom free from bacteria. This is no disadvantage for their use as food for *Artemia salina*, the cultures of which likewise contain bacteria. Nevertheless, work under aseptic conditions and with pasteurized solutions appears to promote the success of the cultures.

If a bacteria-free stock culture can be obtained, it is recommended to keep the pre-cultures free from bacteria.

PROCEDURE

Familiarity with the course of development of *Artemia salina* is assumed (see Section 5.3.2.8).

- Breed batches of brine shrimps synchronously in sea water.
- Start each batch with larvae approximately 24 h old and preferably originating from a laboratory culture. If necessary, larvae from commercially available cysts can be used. In both cases the strain should be known.
- Transfer about 350 larvae into 2 litres of sea water, e.g. in a small aquarium, in the climate room.
- Feed twice a day for about a week with approximately 5 ml of *Dunaliella viridis* suspension. The larvae are now about 4 mm long.
- Transfer 300 larvae into breeding vessels containing 10 litres of sea water.
- Aerate the water slowly, i.e. with large bubbles via glass tubes having a diameter of about 5 mm.
- Feed the brine shrimps twice a day with about 25 ml of algal suspension. The food must be consumed completely.
- Renew 50 % of the sea water twice a week.
- If necessary, clean the walls and the bottom of the test vessels.
- Remove all newborn larvae each day. If they are not required for tests or cultures, this can be done twice a week simultaneously with the renewal of the water.

The first copulations take place when the animals are about 10 days old (see note). The females and males are then respectively about 8.5 and 7.3 mm long. The larvae are born about 6 days after the first observed copulations.

Aquaria or 10-litre separating funnels can be used as the breeding vessels. The latter are preferably provided with a sieve plate with a 1 mm × 1 mm mesh, through which the water with the larvae can be removed while the original animals remain behind. This is easy both for renewal of the water and for the separation of larvae and parents.

- When a filter funnel is used, filter the whole contents through the sieve plate present and then through a sieve plate in a funnel with nylon gauze so fine that the larvae too are retained (see 5.8.5, Fig. 3).
- Return the water completely or, in the case of renewal, partially

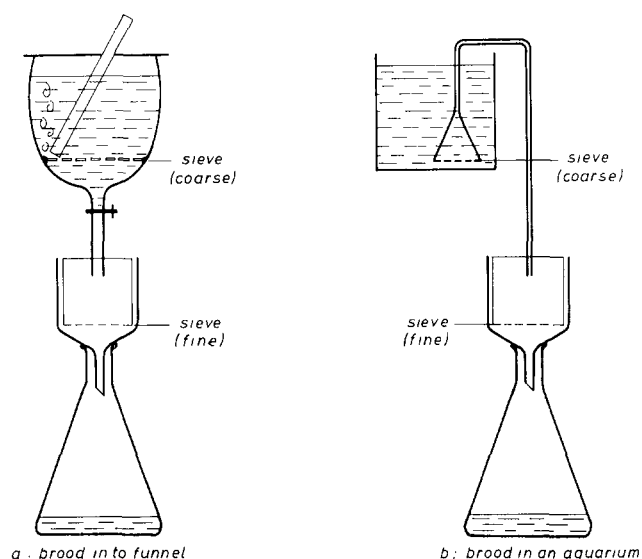
to the breeding separating funnel with the original animals as rapidly as possible.

- Place the larvae in sea water as rapidly as possible. These can be used to start new broods or for toxicity tests. In the latter case, one must be certain of the age (<24 h), so that a daily removal of all newborn larvae is necessary.

If aquaria or other simple vessels are used for breeding, the separation of the larvae and parents must be improvised; see for example 5.8.5, Fig. 3b.

The last small portion of water containing mainly the original animals must now be filtered through a separate coarse sieve (see 5.8.5, Fig. 4). In this way the youngest animals are removed. The original animals and the sea water are returned to the test vessel.

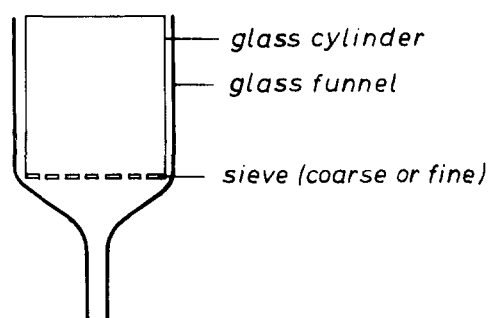
- Discard cultures older than four weeks.
- Start at least one culture each week so that larvae-producing cultures about three weeks old are always available.



5.8.5 Fig. 3 Filtration arrangement for the separation of large animals, small animals, and the medium

NOTE

The growth of brine shrimps depends not only on the culture conditions but also on their origin. The development pattern described here has been observed with animals originating from the Great Salt Lake, Utah, U.S.A., and bred in sea water as described in this section.



5.8.5 Fig. 4 Sieve for the removal of the youngest animals

5. Determination of toxicity

SUPPLEMENTARY REMARKS CONCERNING THE BREEDING OF *ARTEMIA SALINA*

The above description offers only one of the many possibilities for the breeding of brine shrimps and the food they require. Good results can also be obtained under quite different conditions, with different compositions of the media, at different temperatures, completely in the dark, with other food, in other culture vessels, etc. Here the following have been chosen:

- Sea water for the brine shrimps and enriched sea water (Erdschreiber medium) for *Dunaliella*, since it is mainly marine laboratories which work with brine shrimp and which have sea water available.
- A temperature optimum for growth at the selected salinity.
- A moderate illumination during the working period. Although growth in the dark is faster, in many laboratories the breeding chambers are in use during the day for several purposes. Very frequent alternations of light and dark (switching the light on and off) are unfavourable for the animals; screening of the *Artemia* cultures from light makes temperature regulation difficult.
- Living cells of *Dunaliella viridis* as food. This phytoflagellate is an excellent food for brine shrimps and, moreover, one of the easiest unicellular marine algae to culture in large numbers. The use of living material, in contrast to dried or freeze-dried food, facilitates the transition to food-chain investigation and the comparison with toxicity and accumulation tests with algae.
- Breeding vessels and other apparatus available in most biological laboratories.
The size of the cultures is adapted to the weekly production of larvae, per 10 litres of culture, in numbers sufficient for an amount of toxicity work that can be carried out by one person.

We shall not go here into large-scale production of larvae from cysts or into the large-scale breeding of brine shrimps, since this is not relevant for toxicity tests.

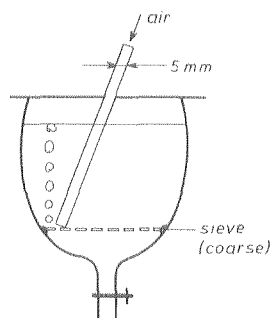
For detailed information on methods of obtaining emergence from cysts, large-scale breeding, and the influence of such factors as temperature, salinity, etc. on emergence from the cysts and the growth of the larvae, the reader is referred to Hentig (1971) and to Sorgeloos and Persoone (1975, a,b). These authors have also included exhaustive literature reviews relating to studies on *Artemia salina*.

5.8.6 *Daphnia magna*

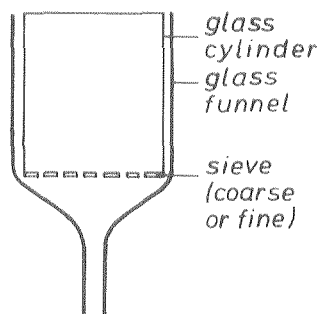
EQUIPMENT AND MATERIALS FOR BREEDING THE WATER FLEA *DAPHNIA MAGNA*

- A few specimens of *Daphnia magna* obtained from an existing laboratory culture or collected in the field and identified.
- climate room, temperature $19 \pm 1^\circ\text{C}$, illuminated moderately for 8 h per day (the illumination intensity is not critical)
- all-glass aquaria, approximately 10-litre, and/or all-glass 10-litre separating funnels provided with a nylon sieve plate with a mesh of approximately $1\text{ mm} \times 1\text{ mm}$ just above the run-off tap (see 5.8.6, Fig. 1 and 5.8.6, Fig. 2)
- funnel-shaped sieves with a nylon gauze sieve plate with a mesh of approximately $0.25 \times 0.25\text{ mm}$ and approximately $1 \times 1\text{ mm}$ (see Section 5.8.6, Fig. 3)
- aeration tubes of a length suitable for the breeding aquaria or funnels and with a diameter of about 5 mm
- aeration apparatus
- pH meter
- oxygen meter

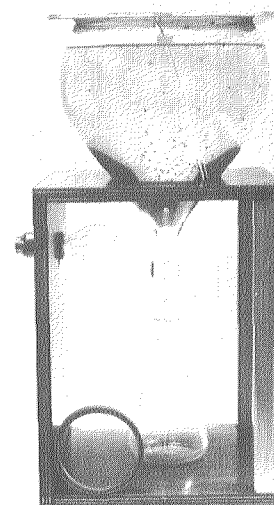
- fresh water of standardized composition: standard reference water, standard test medium, or Dutch Standard Water (see Section 5.7.2, d, e, and g) (see Note 1)
- suspension of a unicellular green alga, e.g. *Chlorella pyrenoidosa*, in the medium in which the daphnids are bred (see Section 5.8.6, point 3).



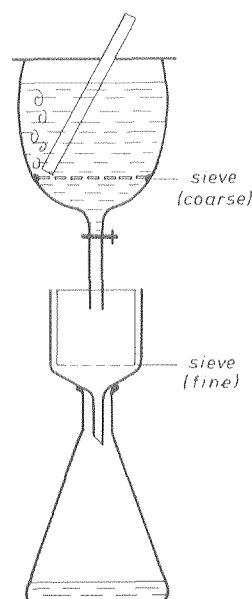
5.8.6 Fig. 1 Separating funnel provided with a nylon sieve



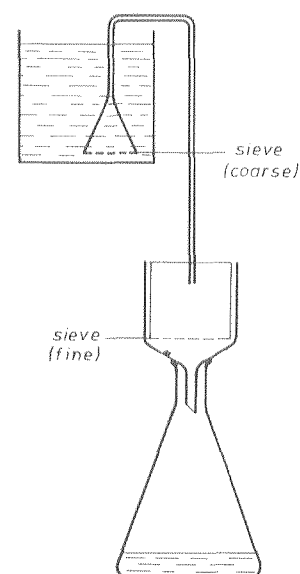
5.8.6 Fig. 3 Sieve for the removal of the youngest animals



5.8.6 Fig. 2 Separating funnel in use for the breeding of *Daphnia magna* and *Artemia salina*



a: brood in to funnel



b: brood in an aquarium

5.8.6 Fig. 4 Filtration arrangement for the separation of large animals, small animals, and the medium

EQUIPMENT AND MATERIALS FOR CULTURING *CHLORELLA PYRENOIDOSA* AS FOOD FOR *DAPHNIA MAGNA*

- Pure or unialgal culture of *Chlorella pyrenoidosa* or another suitable unicellular green alga
- climate room, temperature $22 \pm 3^\circ\text{C}$, provided with a continuous illumination of about 4000 lux for the algal cultures
- conical flasks, 300 ml, for the pre-cultures
- stock of pre-cultures prepared according to Section 5.8.6, 3a
- conical or other heat-resistant flasks (all-glass), 3 to 20 litre
- aeration stones
- sterile pipettes, 1 ml
- apparatus to supply air saturated with water vapour
- autoclave
- centrifuge
- pH meter
- standard reference water, standard test medium, or Dutch Standard Water (see Sections 5.7.2 d, e, and g)
- nutrient solution prepared according to Section 5.7.2 h.

PREPARATION OF A CONCENTRATED SUSPENSION OF *CHLORELLA PYRENOIDOSA* CELLS

a. Preparation of the pre-cultures

Autoclave a 300-ml conical flask closed with a cotton wool plug and containing 100 ml of nutrient solution prepared according to Section 5.7.2 i for 20 min at 120°C . Allow to cool. Inoculate this solution aseptically with 1 ml of a pre-culture about 9 days old. Place the conical flask in the climate room in the light (about 4000 lux) for 9 days.

Shake the contents daily by hand. After about 9 days, the culture contains $1.5 \cdot 10^7$ cells per ml and can be used to start the cultures intended for feeding and for inoculating new pre-cultures.

b. Preparation of cultures to be used as food for the daphnids

Autoclave for 20 min at 120°C a conical or other flask of heat-resistant glass about two-thirds filled with nutrient solution prepared according to Section 5.7.2 i, provided with an aeration stone reaching to the bottom, and closed with a plug of cotton wool.

Allow to cool and inoculate aseptically with 100 ml of a pre-culture.

Place the flasks in the climate room in light of about 4000 lux for about 9 days.

Pass in air saturated with water vapour continuously through the aeration stone. Shake the flasks daily by hand. After about 9 days the culture density is approximately $2 \cdot 10^7$ cells per ml.

c. Preparation of the food suspension

Centrifuge the flask contents for about 10 min at 2000 g. Decant off the supernatant and suspend the algae to a density of $5 \cdot 10^8$ cells per ml in the same medium as that in which the daphnids are being bred. Keep this suspension at about 4°C . The suspension can be stored for about 2 weeks without loss of food value for the daphnids.

d. Note

The food cultures of *Chlorella pyrenoidosa* are seldom free from bacteria. This is no disadvantage for *Daphnia magna* cultures which also contain bacteria. Nevertheless, work under aseptic conditions and with sterilized solutions appears to promote success of the cultures.

Chlorella pyrenoidosa is easy to obtain and to keep free from bacteria. It is then recommended to keep stock cultures in tubes with a solid nutrient medium by the methods customary in microbiology, and to use this regularly, e.g. once every 3 months, to start the pre-cultures.

A solid nutrient medium is prepared by adding 20 g of agar per litre (by conventional microbiological methods) to the nutrient solution, prepared according to Section 5.7.2 i.

PROCEDURE

The development of *Daphnia magna* is assumed to be known (see Section 5.3.2.12).

- Breed batches of daphnids synchronously in standard reference water, in standard test medium, or in Dutch Standard Water with a density of 25 animals per litre.
- Start each batch with newborn animals approximately 24 h old.
- Transfer 250 of these animals into 10 litres of the medium in an all-glass aquarium in the climate room.
- Aerate the water slowly, i.e. with large bubbles via a glass tube having a diameter of about 5 mm.
- Feed daphnids 1 to 2 days old twice a day with approximately 1 ml of *Chlorella pyrenoidosa* suspension.
- Double this every 2 days until an amount of about 10 ml (twice a day) per 250 nine-day old daphnids is reached. The food must be completely consumed.
- Renew 50% of the medium twice a week.
- If necessary, clean the walls and the bottom of the test vessels.
- Each day remove all animals that have been born in the meantime, so that the young present are always less than 24 h old.

The first young are born when the parents are 7 ± 1 days old.

One can use the 10-litre aquarium as the breeding vessel. In order to remove the young daily in a simple manner from the 7th day onwards it is better to use the separating funnels described. Each of these is provided with a sieve plate with $1 \text{ mm} \times 1 \text{ mm}$ mesh through which will pass the water and the newborn daphnids while the adult animals remain behind.

- Filter the whole contents through the sieve plate present in the funnel and then through a sieve placed in a funnel with nylon gauze that will retain the young (see 5.8.6, Fig. 4a). If a light is placed as shown in 5.8.6, Fig. 2, the young can be collected below the sieve plate by switching this light on for about an hour while the apparatus is placed in an otherwise dark room. It is then unnecessary to filter the whole contents.
- Return the medium without the animals wholly or, in the case of renewal, partly, to the breeding separating funnel with the adult animals.
- Place the young in the clean medium as rapidly as possible. These can be used to start new cultures or for toxicity tests.

If aquaria or other simple vessels are used for the breeding, the separation of the young and the parents must be improvised in a different manner, see for example 5.8.6, Fig. 4b.

The last small portion of water that contains mainly the original animals must now be filtered through a separate coarse sieve (see 5.8.6, Fig. 3). In this way the youngest animals are removed. The adult animals and the medium are then returned to the test vessel.

- Discard cultures older than 4 weeks.
- Start at least one culture each week, so that larvae-producing cultures about 3 weeks old are always available.

5. Determination of toxicity

NOTES

1. Standard reference water, standard test medium, and Dutch Standard Water have already been described in Sections 5.7.2 d, e, and g. They are media for the breeding of *Daphnia magna*. *Daphnia magna* was cultivated in standard reference water in our laboratory from 1966 to 1976. The standard test medium has been used successfully at the Netherlands Institute of Public Health (J.H. Canton, personal communication). Dutch Standard Water has been used in these two laboratories since 1977.
2. *Daphnia pulex* and *Daphnia cucullata* (and probably other *Daphnia* species) can be bred in the laboratory in the same way as described here for *Daphnia magna*.

5.8.7 *Chaetogammarus marinus*

EQUIPMENT AND MATERIALS FOR BREEDING THE SHRIMP *CHAETOGAMMARUS MARINUS*

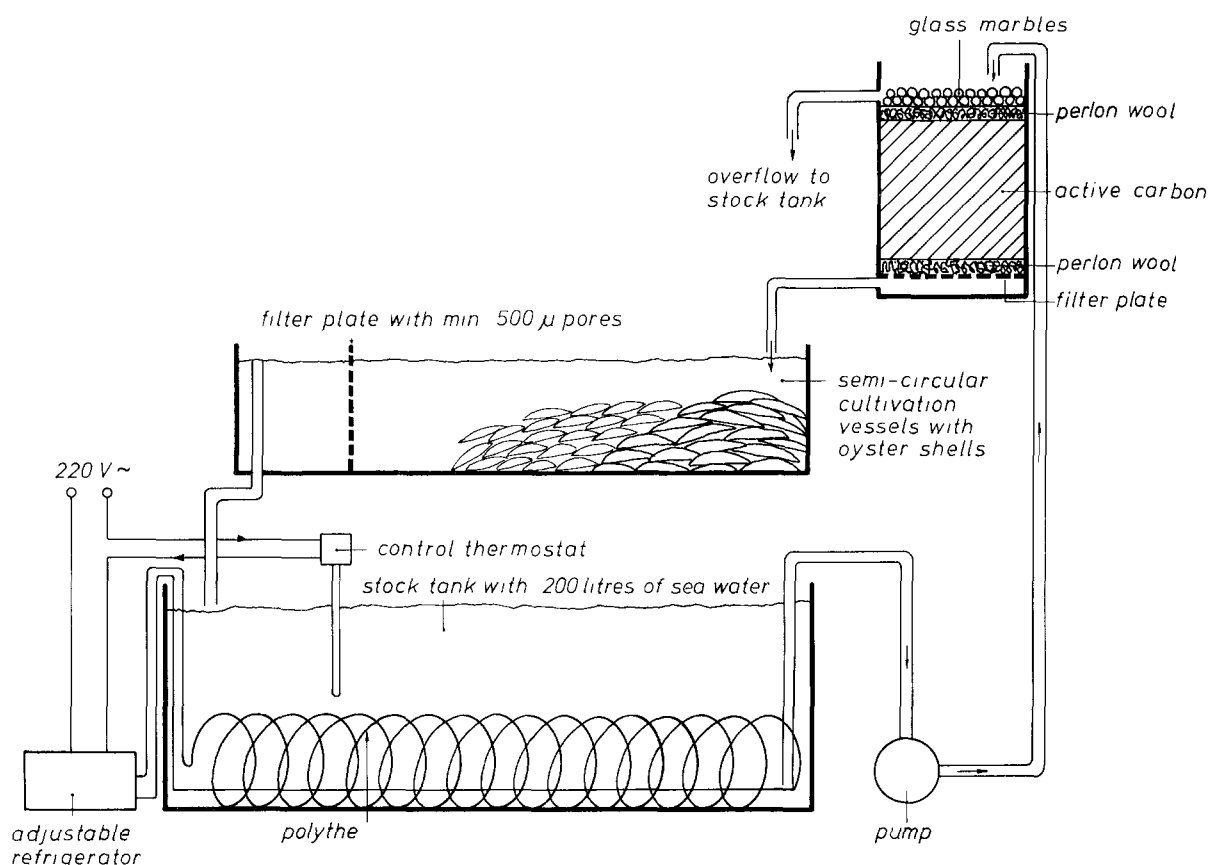
- A few pairs of *Chaetogammarus marinus* obtained from an existing laboratory culture or collected in the field and then identified
- apparatus made in accordance with 5.8.7, Fig. 1, the only materials used being Perspex, polythene, nylon, glass, silicone rubber, silicone glue, and PVC (the latter material as little as possible and then 'non-toxic')
- oyster shells
- thermometer
- pH meter

- salinity meter
- synthetic-fibre wool (e.g. Perlon wool and active carbon (Norite)) for packing the filter
- small fine-mesh scoop-nets
- synthetic or natural sea water, salinity $30 \pm 2\text{‰}$ (see Section 5.7.2 j and k, Section 5.7.3, and Section 5.7.4)
- *Fucus* sp. (a seaweed species, *Phaeophyceae*, *Rhodophyceae*).

PROCEDURE

The development of *Chaetogammarus marinus* is assumed to be known (see Section 5.3.2.9). A mixed population of *Chaetogammarus marinus* is maintained, from which animals of a given length are selected as required. These gammarids can be bred in high densities. In our laboratory this has been carried out in high yield without interruptions for 5 years in the apparatus described below (see 5.8.7, Fig. 1).

The breeding tank itself consists of a Perspex tube sawn lengthways through the middle, with a capacity of about 10 litres up to the outflow opening (the diameter and length are not critical). About two-thirds of the box length is filled with oyster shells between which the gammarids are located. A few cm in front of the outlet opening there is a nylon filter plate to retain the animals in the breeding tank. The gammarids are bred in sea water with a salinity of about 30‰. This sea water is continuously recirculated via a stock tank in which the temperature is brought back to 15 °C. From here it is pumped to a carbon filter placed above the breeding tank, into which it then flows. The flow rate is about 2 l.min⁻¹. In order to prevent overflowing, the filter is also connected directly to the stock tank.



5.8.7 Fig. 1 Apparatus for the breeding of *Chaetogammarus marinus*

The filter must be renewed as soon as its throughput decreases. The breeding tank is placed in a fairly dark place without direct daylight or direct illumination by artificial light.

At least once every two weeks the filter is cleaned, the water is replaced by clean water, and the stock tank, the cooling coil, and the breeding tank are cleaned mechanically.

The animals are fed daily with dried seaweed, as required. For this purpose a large stock of seaweed is collected from a 'clean' site, e.g. in the Wadden Sea or at the mouth of the East Schelde, Netherlands, and is well rinsed with mains water. Snails, gammarids, and other animals are removed as thoroughly as possible. The seaweed is then spread out to dry for one week, during which time any animals still remaining in the seaweed die off. When the dried seaweed is placed in sea water it swells up again.

If the quality of the seaweed is doubtful, a random sample can be taken from each batch to see whether the *Chaetogammarus marinus* eats it, grows on it, and remains alive.

5.8.8 *Poecilia reticulata*

EQUIPMENT AND MATERIALS FOR BREEDING THE GUPPY *POECILIA RETICULATA*

- At least 100 specimens of *Poecilia reticulata* (75 females and 25 males) aged 4 months to 18 months for breeding in fresh water:
obtain the fish from a good existing fresh-water brood or obtain them commercially
- for breeding in sea water:
obtain the fish from a good existing culture in sea water or acclimatize a batch from fresh to sea water (see Note 1)
- climate room, temperature $24 \pm 2^\circ\text{C}$, illuminated moderately for about 10 h a day (the intensity of the illumination is not critical)
- glass aquaria (bonded with silicone rubber), about 100-litre for quarantine and/or acclimatization of guppies from fresh to sea water
- glass breeding aquaria (bonded with silicone rubber) about 100 litre, provided with a vertical grid consisting of a frame and a net, both made of plastic; mesh width 2.5 to 3 mm. Place the grid so that the volume ratio between the two compartments is about 1:2.
- all-glass aquaria, approximately 10-litre
- filter pumps for 100-litre aquaria; capacity about $4 \text{ l} \cdot \text{min}^{-1}$
- filter vessels (external filters) for 10-litre aquaria
- synthetic-fibre wool (e.g. Perlon wool) and active carbon (Norite) for packing the filter
- washed fine gravel to cover the bottom of 100-litre aquaria
- aeration tubes with a length suitable for the breeding aquaria, about 5 mm in diameter
- apparatus to supply air saturated with water vapour
- pH meter
- oxygen meter
- salinity meter
- small fine-mesh fish net
- synthetic or natural sea water, salinity $28 \pm 2\text{‰}$ (see Section 5.7.2 j and k, Section 5.7.3, and 5.7.4)
fresh water of standardized composition: hard A & A water+NaCl, or Dutch Standard Water (see Section 5.7.2 c, g)
- fish food: Tetramin and Tetraphyl and, if possible, live water fleas from laboratory cultures (see Section 5.8.6).

PROCEDURE

- Transfer at least 100 guppies (75 females and 25 males) to quarantine aquaria filled with fresh water or sea water and provided with a supply of compressed air and filter pumps (Note 1).
 - Observe the initial batch in the quarantine aquaria daily for at least one week and remove any dead guppies and all guppies with visible external abnormalities such as deformation of the spinal column, haemorrhages, dark coloration, sluggish swimming, attack by fungi, or erosions.
 - After one week's observation and selection, transfer the remaining guppies to the large compartments of the breeding aquaria provided with compressed air, filter pumps, and a gravel bottom, and filled with fresh or sea water. Add a maximum of 100 guppies consisting of 75 females and 25 males to each 100-litre breeding aquarium.
 - Feed the guppies twice a day alternately with Tetramin and Tetraphyl, not much more than is necessary for one feeding period (in weight about 4 % of the total wet-fish weight per aquarium per day).
- If possible, replace the above-mentioned foods twice a week by live water fleas (see Section 5.8.6).
- Collect the newborn guppies (see Note 2) from the small compartments of the breeding aquaria every day and transfer them in age groups of one week together into 10-litre aquaria; a maximum of 200 young guppies per 10 litres of water. Provide these aquaria with compressed air and an external filter.
 - Give the aquaria with the young brood a batch number. Use guppies 3–4 weeks old for toxicity tests (see Section 5.9.8). These can also be used to continue the breeding (Note 3).
 - Feed the young guppies once a day with finely-ground Tetramin; not more than is necessary for one day.
 - Once or twice a week top up **all** the aquaria with distilled water to the original level. At the same time clean all the aquarium walls if necessary.
 - Renew the active carbon in the filter pumps once a week and that of the external filters once every two weeks.
 - Renew one-third of the water in the breeding aquaria every 3 months.
 - Renew the water in the 10-litre aquaria with the young brood completely once every 4 weeks.
 - Take care that an abundant supply of fresh water and/or sea water is always available at the correct temperature, pH, oxygen concentration, and salinity.
 - Check every day in all aquaria that the supply of compressed air and the filters are functioning properly.
 - Check the temperature daily.
 - Check the pH and the oxygen concentration in all aquaria once a week, and in breeding vessels with sea water check the salinity as well.
 - Examine all parent fish carefully at least twice a week; daily remove any dead ones (see Note 4) and those with the abnormalities mentioned above.

NOTES

1. *Medium*

If guppies from fresh water have to be acclimatized to sea water, proceed as follows: transfer the initial culture to a quarantine aquarium filled with fresh water and gradually raise the salinity

5. Determination of toxicity

over a period of 5 weeks by renewing the medium every week according to the schedule outlined below:

	fresh water, volume %	sea water, volume %
week 1	80	20
week 2	60	40
week 3	40	60
week 4	20	80
week 5	0	100

2. Reproduction

The production of young guppies depends on the age of the parents. At the beginning of the reproductive phase (age about 6 months) the number of young per litter per female is 5–10, while in a later phase (age 12–18 months) the production increases to 30–50 young per litter per female. A litter can be expected every 4–6 weeks.

Because of cannibalism (the older fish eat the young), the observed production of young in the cultivation tanks is lower than could be expected on the basis of the above figures, in spite of the use of a separating screen. When 100 guppies (75 females and 25 males) per breeding vessel are used, the observed production from about 6-month old parents is about 30 young

per breeding vessel per week increasing to about 100 young per breeding vessel per week when the parent fish are 12–18 months old.

Consequently, the number of breeding vessels to be set up must correspond to the number of young guppies required for tests. If, at a given time, the required number of young guppies is not obtained, individual highly productive females can be placed in small aquaria with 1 to 2 litres of water; the production of young is then increased because cannibalism is then often completely absent.

3. Continuation of the breeding

The animals born in any one month are collected and placed together in a 10-litre vessel with an external filter. As soon as they are 4 months old, they are placed in a breeding aquarium (75 females and 25 males). The animals born in one quarter may be combined.

4. Mortality

Although it is not easy to specify the percentage mortality in the cultivation of guppies, it must be borne in mind that regularly occurring deaths, e.g. daily or with intervals of a few days, are undesirable and indicate sub-optimal breeding conditions.

The mortality among young guppies should be zero one week after birth. Guppies older than 2 years are removed and killed.

5.9 DESCRIPTION OF ACUTE TOXICITY TEST METHODS

5.9.1 Test animal *Crepidula fornicata*

5.9.1.1 Test with mortality as the criterion

Subject

Determination of the acute toxicity to veliger larvae of the slipper limpet *Crepidula fornicata* (Linnaeus, 1758) (Gastropoda, Meso-gastropoda).

Field of application

This method is used in sea water, for pure substances, mixtures and effluents that do not give such coloration or turbidity at the dilutions used that the larvae become difficult to see.

Principle

The mortality of the veliger larvae of *Crepidula fornicata* is used as a measure of the toxicity of the test substance added to the water. For this purpose, the larvae are placed in sea water with various concentrations of the test substance or dilutions of the liquid to be investigated (e.g. effluent) and are kept there for 7 days. The mortality is compared with that in a blank test.

EQUIPMENT AND MATERIALS

- Free-swimming larvae of *C. fornicata* (see Section 5.9.1.1, point 5)
- climate room, temperature $15 \pm 1^\circ\text{C}$, illumination with normal day/night rhythm (the intensity of illumination is not critical)
- test vessels: glass evaporating dishes, diameter 10 cm, with suitable watchglasses as covers, or conical flasks with ground-glass stoppers, 100 ml
- plunger pipettes, 100 μl and 1 ml, with disposable tips
- volumetric flasks, 1000 ml
- graduated pipettes, 1 ml and 10 ml
- measuring cylinders, 100 ml and 1000 ml
- beakers, 100 ml
- Pasteur pipettes with cut-off tip, opening diameter ca. 2 mm, sharp edges rounded off, provided with teats
- stereomicroscope, incident light, magnification 4–40
- pH meter
- oxygen meter
- salinity meter
- synthetic or natural sea water, salinity $28 \pm 2\text{‰}$ (see Section 5.7.2 j and k, Section 5.7.3, and Section 5.7.4)
- food: suspension of *Dunaliella viridis*, density approximately $3 \cdot 10^8$ cells/ml (for the culturing of *Dunaliella*, see Section 5.8.5).

PROCEDURE

The procedure can be performed only if the instructions in Sections 5.1 to 5.6 are observed.

- Perform the test in duplicate.
- Select the test vessels in accordance with the volatility of the test substance (see Note 5).
- Place the test vessels in the climate room.
- Code the vessels according to the test substance and its concentration or dilution, denoting duplicates by a and b.

- Prepare the test solutions, 1 litre, in volumetric flasks according to the instructions in Section 5.2; mix well.
- Transfer approximately 100 ml of the blank and of the most concentrated solution to beakers and measure and record the pH, the oxygen concentration, and the salinity; if necessary, also measure those in the other concentrations.
- If necessary, bring the oxygen concentration up to the saturation value and, if desired, the pH to 8.0 ± 0.2 .
- When testing undiluted or slightly diluted effluent bring the salinity to about 28 ‰ with synthetic sea salt before dilution.
- Record the results of all measurements.
- Preferably, take samples of water from the volumetric flasks to determine the concentrations of the test compound.
- In each case, measure out 100 ml of the test solutions into measuring cylinders and transfer this amount to the test vessels.
- Transfer 25 veliger larvae to each test vessel.
For this purpose draw the larvae from the stock culture under the stereomicroscope, using a Pasteur pipette. Release them under water in the test vessel.
- To each test vessel add 1 drop (about 0.05 ml) of *Dunaliella* suspension.
- Close the test vessels.
- In tests on non-volatile compounds, renew the solutions in the test vessels at times $t=2$ and $t=4$ days. For this purpose transfer the larvae with a Pasteur pipette into clean test vessels containing freshly prepared solutions. Use the stereomicroscope.
- In tests on volatile compounds, renew once every 24 h (see Note 5).
- Feed at each renewal of the solution.
- During the test period check the pH, the oxygen concentration, and the salinity in the blanks and in the highest concentrations, both of the freshly prepared and of the used solutions (just before renewal) at least twice during the test period.
- During at least two renewals of the solution, determine the concentrations of the test compound in the solutions if the determination was also carried out at time $t=0$.
Just before the renewal:
Take samples from the test vessels and, if necessary, for each concentration combine the contents of two duplicates to form a single sample.
At $t=0$ and after renewals:
Take samples from the volumetric flasks with freshly prepared solutions.
- During the first 4 days count and remove any dead animals daily, and once again on the seventh day.
- Note all deviations from normal behaviour and quantify these as far as possible (see Note 4).
- If desired, repeat the whole test to determine the reproducibility.

TREATMENT OF THE RESULTS

The method of calculation is explained in Section 5.6.2.

- For each test unit separately and for each time of observation tabulate the number of living (and dead) animals and, as far as possible, the abnormalities observed.
- Record in the table the measured pH and the oxygen concentrations.

5. Determination of toxicity

- Record the concentrations of the test compound if the solutions have been analysed.
Then, if desired, use the mean concentrations obtained during the test for the calculations.
- From the observations calculate the LC_{50} for as many observation times as possible, if possible with the 95 % confidence limits for each test series separately. Record the calculation in the table, if desired with the averaged values.
- If effects other than mortality were observed, calculate the EC_{50} values in a similar manner, specifying the nature of the effect.
- Calculate and record, if desired, the ET_{50} (or LT_{50}) values for each test concentration.
- Plot, if possible on double-logarithmic graph paper, the LC_{50} (or EC_{50}) values against time or the LT_{50} (or ET_{50}) values against concentration.

NOTES

1. Conditions for the validity of the test

- The mortality in both blanks must not exceed 20 % during the test period.
- The LC_{50} values from one test series must be within the 95 % confidence interval of the other series, and vice versa, if it has been possible to calculate these separately.
- The oxygen concentration may not be less than 70 % of the saturation value during the entire test period in all the vessels.
- The pH and the salinity must be known at least for the blanks and for the most concentrated solutions and must be 8.0 ± 0.2 and $28 \pm 2\text{‰}$, respectively, for the whole test period.

2. Recommendation

It is recommended to include a reference substance in each test. The result obtained with this reference substance must be in reasonable agreement with previously obtained results.

3. Observation times

In tests on rapidly acting substances it may be desirable to choose observation times of, for example 1½, 3, 6, and 12 h.

4. Deviations from normal behaviour

Instead of mortality an abnormal behaviour may also be used as the test criterion. If such a criterion is quantified, an EC_{50} value can be calculated for it. An example of such abnormal behaviour is that the larvae no longer extend their velum and are immobilized on the bottom of the test vessel.

5. Choice of the test vessel

For non-volatile substances evaporating dishes covered with watchglasses may be used, and for volatile substances conical flasks with ground-glass stoppers. On renewal of the solutions the contents of the conical flasks must be emptied into an evaporating dish so that the larvae can be examined under the stereomicroscope.

APPENDIX

Production of *C. fornicata* veliger larvae.

Equipment and materials

- Initial material: *Crepidula* obtained in the field.
- climate room, temperature $15 \pm 2^\circ\text{C}$, normal day/night rhythm (the intensity of the illumination is not critical)
- glass aquaria, about 2 l
- aquaria, about 100 l

- filter pumps for 100 l aquaria, capacity about 4 l/min
- aeration apparatus
- pH meter
- oxygen meter
- salinity meter
- penknife
- tweezers
- evaporating dishes
- plastic bags, about 100×50 cm
- synthetic or natural sea water, salinity $28 \pm 2\text{‰}$ (see Section 5.7.2 j and k, Section 5.7.3, and Section 5.7.4)
- food: *Dunaliella viridis*, about $3 \cdot 10^8$ cells/ml (for the culturing of *Dunaliella*, see Section 5.8.5).

Procedure

- Familiarity with the course of development of *C. fornicata* is assumed (see Section 5.3.2.3).
- Collect *Crepidula* (see Note 1).
- Transport the molluscs in plastic bags to the laboratory (see Note 2).
- In the laboratory, transfer the molluscs to larger aquaria, of about 100 litres, filled with sea water ($\text{pH } 8.0 \pm 0.2$, saturated with oxygen, salinity $28 \pm 2\text{‰}$, temperature $14 \pm 2^\circ\text{C}$) and aerate well.
- Allow the molluscs to acclimatize for one week.

There are two methods of obtaining veliger larvae. The choice of the method depends on the season of the year (see Note 3).

Method 1, for the period from about March to September (see Note 4):

- Prise female *Crepidula* free with the penknife.
- Examine the animals for old dark egg masses (see Note 5).
- Transfer such an egg mass with tweezers to an evaporating dish.
- If the egg mass is in the right stage, use the larvae in the test (see Note 6).

Method 2, for the period from about October to February:

- Fill 2-litre aquaria and place them in the test chamber ($15 \pm 2^\circ\text{C}$).
- Aerate the water well.
- Select from the stock a number of males and females (Note 7).
- Place five females and five males in each tank.
- Feed the molluscs daily with 10 ml of *Dunaliella* suspension.
- Renew the sea water once per week.
- Check regularly whether eggs have been laid on the glass wall (see Note 8) and follow the development of the egg masses.
- Use for the tests the veliger larvae that emerge and swim to the surface.

Notes

1. At ebb-tide *Crepidula* can be picked up from shallow water. The best collecting sites are usually oyster beds.
2. The molluscs, with adherent water, can be transported in plastic bags for at least 6 h without any harmful effects.
3. The natural breeding season of *C. fornicata* runs from about March to September. The length of the season depends greatly on the weather conditions.

5.9 Description of acute toxicity test methods

4. During the summer months the molluscs can be kept in the laboratory without feeding for about 6–8 weeks and used for obtaining larvae.
5. During embryonic development there is a marked change in colour of the egg masses. Fresh-laid eggs are light yellow, subsequently there is a change in colour via light brown to dark brown and black when the larvae emerge.
6. The veligers are in the correct stage when the capsules with the larvae break up on being transferred into the evaporating dish and the veligers are released.
7. To select males and females, the molluscs must be carefully prised loose with a penknife. Males can clearly be distinguished from females by their smaller dimensions and long black penis.
8. In the laboratory, the molluscs are stimulated into egg-laying by feeding and a higher temperature as compared with the sea water in nature. The first egg mass is usually produced after about a week.

5.9.2 Test animal *Lymnaea stagnalis*

5.9.2.1 Test with the duration of the egg-cleavage cycle as the criterion

Subject

Determination of the acute toxicity to eggs of the pond snail *Lymnaea stagnalis* (Linnaeus, 1758) (Gastropoda, Basommatophora).

Field of application

The method is used in fresh water for pure substance, mixtures of substances, and effluents that are non-volatile and that do not give such coloration or turbidity at the dilution used that the eggs become difficult to see.

Principle

The delay in the first cleavages of the eggs of *Lymnaea stagnalis* is used as a measure of the toxicity of a substance added to water. For this purpose, the eggs are placed in fresh water with different concentrations of the test substance or different dilutions of the test liquid (e.g. effluent) and are kept in it until the first cleavage divisions, which together take about 165 min, have been completed. The time of the cleavage cycle is compared with that in a blank test.

INTRODUCTION

Whenever young cleavage stages of ova are exposed to substances added to the medium a delay in the cleavage may occur. It is also possible that no further cleavage will take place at all. Much research has been carried out with sea-urchin eggs (Kobayashi, 1971). The eggs of the pond snail can exhibit irregularities in their cleavage divisions as the result of damage to the DNA. Damage is known to result from exposure to short-wave ultraviolet radiation (Labordus, 1970) and from treatment with 8-methoxypsoralen followed by irradiation with long-wave UV (our own investigations). Treatment with caffeine, which at certain concentrations dissolves the spindle figure of the ovum, has a distinct effect (personal communication, Zoological Laboratory, Utrecht).

In this test it can be expected that a substance with a direct mutagenic action (i.e. without metabolic activation), in particular, will affect the duration of the cleavage cycle.

If the result is negative, one must reckon with the possibility of an impermeability of the membranes to certain substances. A vitelline membrane is found around pond snail ova. Furthermore, the ovum is surrounded by a capsular fluid which is in turn surrounded by a capsular membrane.

EQUIPMENT AND MATERIALS

- Undivided eggs of *L. stagnalis* (for breeding and stimulation to egg-laying, see Section 5.8.3)
- climate room, temperature $25 \pm 2^\circ\text{C}$
- test vessels: embryo dishes with suitable covers
- plunger pipettes, 100 μl and 1 ml, with disposable tips
- volumetric flasks, 1000 ml
- graduated pipettes, 1 ml and 10 ml
- measuring cylinders, 5 ml, 100 ml, 1000 ml
- conical flasks, 100 ml
- beakers, 100 ml
- Petri dishes, 5 cm in diameter
- knife with a thin blade tapering to a point
- Pasteur pipettes with cut-off tip, diameter ca. 2 mm, sharp edges rounded off, provided with teats
- hairloop fixed to a Pasteur pipette (see Section 5.8.3, point 1)
- filter paper
- stereomicroscope, incident light, magnification 4–40
- pH meter
- oxygen meter
- salinity meter
- fresh water of standardized composition, e.g. standard reference water, hard A & A water, or Dutch Standard Water (see Section 5.7.2 d, b, and g).

PROCEDURE

The procedure can be performed only when the instructions in Sections 5.1 to 5.6 are observed.

- Stimulate the pond snails to lay egg masses in good time (see Section 5.8.3).
- Prepare the test vessels (embryo dishes) in the climate room, taking 1 test vessel per concentration.
- Code these according to the test substance and concentration or dilution
- Prepare the test solution, 1 litre, in volumetric flasks according to the instructions given in Section 5.2. Mix well.
- Transfer 100 ml of the blank and of the most concentrated solution to beakers and measure and record the pH and the oxygen concentration; if necessary, also record these in the other concentrations.
- Bring the oxygen concentration of the test solution up to saturation value and, if desired, the pH to the value of the blank medium used.
- Record the results of all measurements.
- Preferably, take samples of water from the volumetric flasks to determine the concentrations of the test compound.
- Measure out 5 ml of the test solution into measuring cylinders and transfer this amount to the test vessels.
- Cover the dishes.
- By hand, transfer the egg masses that have been deposited on the aquarium wall to a Petri dish with a little medium, check the quality of the eggs under the stereomicroscope, and then use only egg masses with a maximum of 10% of multiplets (these and other terms are explained in Section 5.8.3 point 4).

5. Determination of toxicity

- Free the suitable egg masses from their jelly by making a cut through them with the knife and then carefully rolling the capsules over moist filter paper. Keep the egg masses separate from one another.
- Transfer the capsules to an embryo dish filled with a little medium.
- Check the eggs under the stereomicroscope.
- Separate out unsuitable eggs (multiplets and eggs that are already dead before the first cleavage) with the hairloop. Remove these eggs with a Pasteur pipette.
- Wait till the first eggs have reached stage 1, the reference point for the first cleavage (see Section 5.8.3, point 4).
- Separate these eggs with the hairloop.
- In this way collect a group of eggs that have reached a reference point of the first cleavage within 2 min.
- Note the time of the first cleavage.
- Draw up the group of eggs with a Pasteur pipette.
- Deposit the eggs on a moist piece of filter paper.
- Immediately transfer the eggs to a test vessel with a knife.
- Continue to collect groups of eggs as above until 10 eggs have been added per concentration.
- Check the eggs regularly up to the time of the second cleavage.
- At 5-min intervals note the number of eggs that have reached the second cleavage for each concentration.
- Do the same for the third cleavage.
- End the test when all the eggs have reached the third cleavage.
- Repeat the test at least twice.

TREATMENT OF THE RESULTS

- For each concentration calculate the average times of the first, second, and third cleavage.
- From the above results calculate the duration of the cleavage cycle (see Note 2).
- For each concentration calculate whether there has been a lengthening of the cleavage cycle.
- Record in a table for the various tests the mean delays of the second and third cleavage cycles.
- If possible, plot the mean delay against the average of the measured concentrations.
- If possible, record the concentration at which no further cleavage takes place.

NOTES

1. Conditions for the validity of the test

The percentage mortality in the blank must not exceed 10% during the test period. A lengthening of the cleavage cycle smaller than 8 min falls within the error of measurement.

2. Duration of the second and third cleavage cycles

The time between the first and the second cleavage divisions (second cleavage cycle) is about 85 min at 25°C. The time between the second and the third cleavage (third cleavage cycle) is 80 min.

3. Performance of the test

The test can be performed satisfactorily only after adequate experience and speed has been gained in working with the early cleavage stages.

5.9.3 Test animal *Ophryotrocha diadema*

5.9.3.1 Test with mortality as the criterion

Subject

Determination of the acute toxicity of substances to the polychaete worm *Ophryotrocha diadema*, Åkesson, 1976 (Polychaeta).

Field of application

The method is used in sea water, for pure substances, mixtures of substances, and effluents that do not give such coloration of turbidity at the dilution used that the worms become difficult to see.

For volatile compounds, the test can be performed only with adult worms (see Note 4).

Principle

The mortality of 2- to 3-day old larvae and of adult specimens of *Ophryotrocha diadema* is used as a measure of the toxicity of a substance added to water. For this purpose the worms are placed in sea water with various concentrations of the test substance or dilution of the test liquid (such as effluents) and are kept in them for 4 days. The mortality is compared with that in a blank test.

EQUIPMENT AND MATERIALS

- Two- to three-day old larvae or adult specimens of *O. diadema* (for breeding, see Section 5.8.4)
- climate room, temperature $22 \pm 2^\circ\text{C}$, illumination with normal day/night rhythm (the intensity of the illumination is not critical)
- test vessels: glass evaporating dishes, diameters 10 cm and 6 cm, with matching watchglasses to act as covers, or conical flasks, 100 ml, with ground-glass stoppers
- plunger pipettes, 100 μl and 1 ml, with disposable tips
- volumetric flasks, 1000 ml
- graduated pipettes, 1 ml and 10 ml
- measuring cylinder, 100 ml
- conical flasks, 100 ml
- beakers, 100 ml
- Pasteur pipettes with cut-off tips, opening diameter about 2 mm, sharp edges rounded off, provided with teats
- stereomicroscope, incident light, magnification 4–40
- pH meter
- oxygen meter
- salinity meter
- synthetic or natural sea water, salinity $32 \pm 2\text{‰}$ (see Section 5.7.2 j and k, Section 5.7.3, and Section 5.7.4)
- food: liquidized spinach (see Section 5.8.5).

PROCEDURE

The procedure is based on Åkesson's work (1970, 1975, 1976) and can be performed only if the instructions of Sections 5.1 to 5.6 are observed.

- Place the test vessels (see Note 4) in the climate room; use 4 test vessels per concentration or dilution (see Note 5); for two- to three-day larvae use evaporating dishes 6 cm in diameter; for adult animals use evaporating dishes 10 cm in diameter or conical flasks with ground-glass stoppers.

5.9 Description of acute toxicity test methods

- Code the test vessels according to the test substance and its concentration or dilution, and number the vessels.
- Prepare the test solutions, 1 litre in volumetric flasks, in accordance with the instructions given in Section 5.2; mix well.
- Transfer about 100 ml of the blank and of the most concentrated solution to beakers and measure the pH, the oxygen concentration, and the salinity; if necessary, also measure the pH and oxygen in the other concentrations.
- Adjust the oxygen concentration to the saturation value and, if desired, the pH to 8.0 ± 2 .
- In tests with slightly diluted or undiluted effluent, before the dilution adjust the salinity with synthetic sea salt to approximately 32‰ before dilution.
- Record the results of all measurements.
- Preferably, take samples of water from the volumetric flasks to determine the concentration of the test compound.
- Measure out the solutions to be tested in measuring cylinders and transfer the following amounts to the test vessels:
15 ml for two- to three-day old larvae,
80 ml for adult animals.
For this purpose, draw up the worms from the breeding vessel under the stereomicroscope with a Pasteur pipette fitted with a teat. Release them under water in the test vessel.
- Feed liquidized spinach (sufficient for one day),
for two- to three-day old worms:
one small drop per test vessel (about 0.03 ml), for adult worms:
one large drop per test vessel (about 0.1 ml).
- Close the test vessels.
- Renew the test solution daily. For this purpose, transfer the worms with a Pasteur pipette to clean test vessels with freshly prepared solutions. Feed at each renewal.
- During at least two renewals determine the concentration of the test compound before and after the renewal if it was in fact analysed at time $t=0$. For the determination before the renewal the contents of the four test vessels may if necessary be combined.
- During the test period check the pH, the oxygen concentration, and the salinity of the blank and of the most concentrated solutions at least twice, both for the freshly prepared and for the used solutions (just before renewal).
- Regularly count and remove any dead animals.
- Record all deviations from normal behaviour and quantify them if possible.
- End the test after 4 days.
- If desired, repeat the whole test to determine the reproducibility.
- Use the 96-h LC_{50} value to choose the concentration for the tests described in Sections 5.10.3.1 and 5.10.3.2.

TREATMENT OF THE RESULTS

The method of calculation is explained in Section 5.6.2.

- For each test series separately and for each time of observation tabulate the number of living (and dead) animals and, as far as possible, the abnormalities observed.
- Tabulate the measured pH and the oxygen concentrations.
- Record the concentrations of the test compound found if the solutions have been analysed for this.
Then, if desired, use the mean concentrations during the test.
- Calculate the LC_{50} values for as many observation times as pos-

sible, preferably with the 95 % confidence limits for each test series separately. Record the calculations in the table, if desired with the mean values.

- If effects other than mortality were observed, calculate and record the EC_{50} values in a similar manner, specifying the nature of the effect.
- If desired, also calculate and record the ET_{50} (or LT_{50}) values for each concentration tested.
- Plot, if possible on double-logarithmic graph paper, the LC_{50} (or EC_{50}) values against time or the LT_{50} (or ET_{50}) values against concentration.

NOTES

1. Conditions for the validity of the tests

- The mortality in the blank must not exceed 20 %.
- The oxygen concentration in all the test vessels must not be less than 70 % of the saturation value throughout the test.
- The pH and the salinity must be 8.0 ± 0.2 and 32 ± 2 ‰ respectively during the whole test period.

2. Recommendation

It is recommended to include a reference substance in each test. The result with the reference substance must be in reasonable agreement with previously obtained results.

3. Deviations from normal behaviour

Instead of mortality, abnormal behaviour may also be used as a test criterion. If such a criterion is quantified, an EC_{50} value for it can be calculated.

4. Tests of volatile compounds

In tests on volatile compounds conical flasks with ground-in stoppers must be used. The test is then not suitable for two- to three-day larvae because, on renewal, these remain attached to the glass wall of the conical flask and cannot be seen with the stereomicroscope.

5. Parallel test solutions

If the worms have to be counted and transferred under the stereomicroscope, larger test vessels cannot be used. The evaporating dishes used can contain 10 worms. Consequently, for each concentration the worms are divided into 4 groups of 10. For the calculations the results of the 4 test vessels per concentration or dilution are totalled.

6. Test animals

The test method can also be used with other Ophryotrocha species that are easy to breed. In the case of species that are not hermaphroditic, the females and the males must be tested separately, since for many substances a difference in sensitivity has been found.

5.9.4 Test animal *Artemia salina*

5.9.4.1 Test with mortality as the criterion

Subject

Determination of the acute toxicity of substances to the brine shrimp *Artemia salina* (Linnaeus, 1758) (Crustacea, Anostraca).

Field of application

The method is used in sea water, for pure substances, mixtures of substances, and effluents that do not give such coloration or turbidity at the dilutions used that the *Artemia* become difficult to see.

5. Determination of toxicity

Principle

The mortality of *Artemia salina* is used as a measure of the toxicity of the substance added to water.

For this purpose, the brine shrimps are kept for two days in solutions containing the test substance in various concentrations or the test liquid (e.g. effluent) in various dilutions. The mortality is compared with that in a blank test.

EQUIPMENT AND MATERIALS

- Three-day old larvae of *A. salina*, born from animals about three weeks old and grown to a length of about 2 mm (for breeding, see Section 5.8.5)
- climate room, temperature $25 \pm 2^\circ\text{C}$, illuminated moderately for about 10 h a day (the intensity of the illumination is not critical)
- beakers, 250 ml or 1000 ml, or glass conical flasks, 1000 ml, with ground-glass stoppers or with watchglasses
- volumetric pipettes or plunger pipettes with disposable tips, 1 ml
- plunger pipettes, 25 μl , 100 μl , and 250 μl , with disposable tips
- volumetric flasks, 100 ml, 250 ml, and 1000 ml
- graduated pipettes, 1 ml and 10 ml
- measuring cylinders, 25 ml, 100 ml, 250 ml, and 1000 ml.
- glass tubes, about 15 cm long and about 3 mm in diameter, provided with teats
- pH meter
- oxygen meter
- salinity meter
- synthetic or natural sea water, salinity $30 \pm 2\text{‰}$ (see Section 5.7.2 j and k, Section 5.7.3, and Section 5.7.4).

PROCEDURE

The procedure can be performed only if the instructions in Sections 5.1 to 5.6 are observed.

- Perform the test in duplicate.
- Choose test vessels depending in type and size on the physico-chemical properties of the test substance (see Section 5.4.4).
- Place the test vessels in the climate room.
- Code the vessels according to the test substance and its concentration or dilution, and denote duplicates by a and b.
- Prepare the test solutions, 250 ml or 1 litre, in the vessels in the selected type of sea water according to the instructions given in Section 5.2.
- Measure and record the pH and the oxygen concentration of at least the blanks and the most concentrated solutions, if necessary also of the other solutions.
- If necessary, adjust the oxygen concentration to the saturation value and, if desired, the pH to 8.0 ± 0.2 .
- In tests on effluents, measure and record the salinity of the undiluted sewage. In tests on undiluted or slightly diluted effluent, before dilution adjust the salinity to about 30‰ S with synthetic sea salt.
- Preferably take samples for the analysis of the test compound concentration in the prepared test solutions. Not more than 10% of the solution volume may be used for such samples.

- Place 25 artemias in each test vessel. Draw these up from a stock in convenient numbers with the aid of a glass tube having a diameter of about 3 mm and provided with a teat. Release them under the surface of the liquid in the test vessels. Avoid contamination.
- Do not aerate during the test.
- Do not feed during the test.
- Count and remove the dead artemias after (1½), 3, 6, (12), and 24 h, (see Note 3).
- Note any deviations from normal behaviour and quantify them as far as possible.
- After 48 h count the dead and the living artemias and check whether the total number tallies.
- After 48 h, check the pH and the oxygen concentration of the blanks and of the most concentrated solutions, if necessary also of the other solutions.
- After 48 h, analyse the test compound concentrations if such an analysis was also performed at time $t=0$.
- If desired, repeat the whole test to determine the reproducibility.

TREATMENT OF THE RESULTS

The method of calculation is explained in Section 5.6.2.

- For each test series separately and for each observation time tabulate the number of living (and dead) brine shrimps and, as far as possible, the abnormalities observed.
- Record the measured pH and the oxygen concentrations in the table.
- Record the test compound concentrations found if the solutions were analysed. Then, if desired, use the mean concentrations during the test for the calculations.
- Calculate the LC_{50} values for as many observation times as possible, preferably with the 95% confidence limits for each test series separately. Record the calculations in the table, if desired, with the mean values.
- If effects other than mortality were observed, calculate and record the EC_{50} values in a similar manner, specifying the nature of the effect.
- If desired, also calculate and record the ET_{50} (or LT_{50}) values for each concentration tested.
- Plot, if possible on double-logarithmic graph paper, the LC_{50} (or EC_{50}) values against time or the LT_{50} (or ET_{50}) values against concentration.

NOTES

1. Conditions for the validity of the test

- The mortality in both blanks at the end of the test must not exceed 10%.
- The 48-h LC_{50} (48-h EC_{50}) values of one test series must lie within the confidence limits of the other, and *vice versa*, if it has been possible to determine these separately.
- In all test vessels the oxygen concentration must have exceeded 80% of the saturation value throughout the test.
- In each case the pH for the blanks and for the most concentrated solutions must be known during the test, and if it was adjusted to 8.0 at time $t=0$ it must be 8.0 ± 0.3 after 48 h.

5.9 Description of acute toxicity test methods

2. Recommendation

It is recommended to include a reference substance in each test. The result with the reference substance must be in reasonable agreement with previously obtained results.

3. Observation times

The observation times of 1½ and 12 h are used only with fast-acting substances or if they are required for other reasons.

5.9.5 Test animal *Daphnia magna*

5.9.5.1 Test with mortality as the criterion

Subject

Determination of the acute toxicity of substances to the water flea *Daphnia magna* (Straus, 1820) (Crustacea, Cladocera).

Field of application

The method is used in fresh water, for pure substances, mixtures of substances, and effluents that do not give such coloration or turbidity at the dilutions used that the *Daphnia* become difficult to see.

Principle

The mortality of *Daphnia magna* is used as a measure of the toxicity of a substance added to water.

For this purpose the daphnids are kept for two days in solutions that contain the test substances in various concentrations or the test liquid (e.g. effluent) in various dilutions.

The mortality is compared with that in a blank test.

EQUIPMENT AND MATERIALS

- Daphnids less than 24 h old, born from animals about 3 weeks old (for breeding, see Section 5.8.6)
- climate room, temperature 19 ± 1 °C, illuminated moderately for about 10 h a day (the intensity of the illumination is not critical)
- beakers, 250 ml or 1000 ml, or glass conical flasks, 1000 ml, with ground-glass stoppers or watchglasses
- volumetric pipettes or plunger pipettes with disposable tips, 1 ml
- plunger pipettes, 25 µl, 100 µl, and 250 µl, with disposable tips
- volumetric flasks, 100 ml, 250 ml, and 1000 ml
- graduated pipettes, 1 ml and 10 ml
- measuring cylinders, 25 ml, 100 ml, 250 ml, and 1000 ml
- glass tubes, about 15 cm long and about 3 mm in diameter, provided with teats
- pH meter
- oxygen meter
- salinity meter
- water of standardized composition, standard reference water, standard test medium, or Dutch Standard Water (see Section 5.7.2 d, e, and g).

PROCEDURE

The procedure can be performed only if the instructions of Sections 5.1 to 5.6 are observed.

- Perform the test in duplicate.
- Choose test vessels depending in type and size on the physico-

chemical properties of the test substance (see Section 5.4.4).

- Place the test vessels in the climate room.
- Code the vessels according to the test substance and its concentration or dilution, and denote duplicates by a and b.
- Prepare the test solutions, 250 ml or 1 litre, in the vessels, in the selected synthetic standard water (see Note 4) according to the instructions given in Section 5.2.
- Measure and record the pH and the oxygen concentration of at least the blanks and the most concentrated solutions, if necessary also of the other solutions.
- If necessary, adjust the oxygen concentration to the saturation value and, if desired, the pH to the value of the blank medium used.
- In tests with effluents, measure the salt concentration and the concentrations of some other ions. Adjust these concentrations to be compatible with the requirements of *D. magna* (Note 4).
- Preferably take samples for the analysis of the test compound concentration in the prepared test solutions.
Not more than 10 % of the solution volume may be used for such samples.
- Transfer 25 daphnids to each test vessel.
Draw these up from a stock in convenient numbers with the aid of a glass tube about 3 mm in diameter provided with a teat. Release them under the surface of the liquid in the test vessels. Avoid contamination.
- Do not aerate during the test.
- Do not feed during the test.
- Count and remove the dead daphnids after (1½), 3, 6, (12), and 24 h (see Note 3).
- Note any deviations from normal behaviour and quantify these as far as possible (see Note 5).
- After 48 h count the dead and live daphnids and check whether the total number tallies.
- After 48 h check the pH and the oxygen concentration of the blanks and of the most concentrated solutions and, if necessary, also of other solutions.
- After 48 h analyse the test compound concentrations if such an analysis was also performed at time $t=0$.
- If desired, repeat the whole test to determine the reproducibility.

TREATMENT OF THE RESULTS

The method of calculation is explained in Section 5.6.2.

- For each test series separately and for each time of observation tabulate the number of live (and dead) test animals and, as far as possible, the abnormalities observed.
- Record the measured pH values and the oxygen concentrations in the table.
- Record the test compound concentrations found if the solutions were analysed.
Then, if desired, use the mean concentrations during the test for the calculations.
- Calculate the LC_{50} values for as many observation times as possible, preferably with the 95 % confidence limits for each test series separately. Record the calculation in the table, if desired, with the average values.
- If effects other than mortality were observed, calculate and record the EC_{50} values in a similar manner, specifying the nature of the effect.

5. Determination of toxicity

- If desired, also calculate and record the ET_{50} (or LT_{50}) values for each concentration tested.
- Plot, if possible on double-logarithmic paper, the LC_{50} (or EC_{50}) values against time or the LT_{50} (or ET_{50}) values against concentration.

NOTES

1. Conditions for the validity of the test

- The mortality in both blanks at the end of the test must not exceed 10 %.
- The 48-h LC_{50} (48-h EC_{50}) values of one test series must lie within the confidence limits of the other, and *vice versa*, if it has been possible to determine these separately.
- In all test vessels the oxygen concentration must have exceeded 80 % of the saturation value throughout the test.
- In each case the pH for the blanks and for the most concentrated solutions must be known during the test, and if it was adjusted at time $t=0$ it must be the same as the $t=0$ value within 0.3 units after 48 h.

2. Recommendation

It is recommended to include a reference substance in each test. The result with the reference substance must be in reasonable agreement with the results obtained previously.

3. Observation times

The observation times of 1½ and 12 h are only used with fast-acting substances or if they are desired for any other reason.

4. Use of synthetic standard water

In general, the tests are performed with standard reference water, standard test medium, or Dutch Standard Water as dilution water. Another type of standard water may be used for the test if it is established beforehand that the daphnids can develop normally in this water (according to Section 5.8.6) and they are then also taken from a culture in this water.

In tests on undiluted or slightly diluted effluent – with the synthetic standard water – it is desirable to determine the concentrations of relatively non-toxic ions such as Na, K, Ca, Mg, Cl, and SO_4 in this effluent.

For *Daphnia magna* to remain in good condition the hardness must be at least 0.5 mmole of Ca+Mg per litre.

The chloride content may not exceed 500 mg per litre.

5. Definition of mortality

Instead of mortality the 'immobilization' of daphnids is also used as a test criterion. The daphnids that lie on the bottom of the test vessel and are incapable of swimming freely are considered to be immobilized, though they may still move their antennae. The EC_{50} value, for example, can also be calculated for this criterion, if it has been quantified.

5.9.6 Test animal *Temora longicornis* or another pelagic copepod species

5.9.6.1 Test with mortality as the criterion

Subject

Determination of the acute toxicity of substances to pelagic copepods (Crustacea, Calanoida).

Field of application

The method is used in sea water for pure substances and mixtures of substances that do not give such coloration or turbidity at the dilutions used that the copepods become difficult to see.

Principle

The mortality of pelagic copepods is used as a measure of the toxicity of the substance added to water.

For this purpose the test animals are kept for two days in solutions that contain the test substances in various concentrations or the test liquid, e.g. effluent, in various dilutions.

The mortality is compared with that in a blank test.

EQUIPMENT AND MATERIALS

- About 300 adult, undamaged, calanoid copepods of one species, for example *Acartia clausi*, *Acartia tonsa*, *Eurytemora affinis*, or *Temora longicornis*
- conical flasks, 500 ml, wide-neck
- climate room, temperature 15 ± 1 °C, not illuminated
- plunger pipettes, 50 µl and 500 µl, with disposable tips
- volumetric flasks, 100 ml
- graduated pipettes, 1 ml and 10 ml
- glass tubes about 15 cm long and about 3 mm in diameter, provided with teats
- pH meter
- oxygen meter
- salinity meter
- natural sea water with a salinity equal to that at the place where the copepods were collected.

PROCEDURE

The procedure can only be performed if the instructions of Sections 5.1 to 5.6 are observed.

- Perform the test in quadruplicate.
- Place the conical flasks in the climate room.
- Code the conical flasks according to the test substance and its concentration and denote the four series by a, b, c, and d (see Note 4).
- Prepare 0.5 litre of the test solutions in the conical flasks in sea water following the instructions given in Section 5.2.
- Measure and record the pH and the oxygen concentration of at least the blanks and the most concentrated solutions, if necessary also of the other solutions.
- If necessary, adjust the oxygen concentration to the saturation value and, if desired, the pH to 8.0 ± 0.2 .
- Preferably take samples for the analysis of the test compound concentrations in the prepared solutions. Not more than 10 % of the solution volume may be used for such samples.
- Transfer 10 copepods to each test vessel. Draw these up from the selected stock with the aid of a glass tube having a diameter of about 3 mm, provided with a teat. Release them below the surface of the liquid in the test vessels. Avoid contamination.
- Do not aerate during the test.
- Do not feed during the test.
- Count and remove any dead copepods after (1½), 3, 6, (12), and 24 h (see Note 3).
- After 48 h count the dead and live copepods and check whether the total number tallies.

5.9 Description of acute toxicity test methods

- After 48 h check the pH and the oxygen concentration of the blanks and of the most concentrated solutions, if necessary, also of the other solutions.
- After 48 h analyse the concentration of the test compounds if such an analysis was also performed at time $t=0$.
- If desired, repeat the whole test to determine the reproducibility.

TREATMENT OF THE RESULTS

The method of calculation is explained in Section 5.6.2.

- For each test series separately and for each observation time tabulate the number of live (and dead) test animals and, as far as possible, the abnormalities observed.
- Record the measured pH and the oxygen concentrations in the table.
- Record the concentrations of the test compound determined if the solutions have been analysed.
- Then, if desired, use the mean concentrations during the test for the calculations.
- Calculate the LC_{50} values for as many observation times as possible, preferably with the 95 % confidence limits for each test series separately. Record the calculations in the table, if desired, with the average values.
- If effects other than mortality were observed, calculate and record the EC_{50} in a similar manner, specifying the nature of the effect.
- If desired, also calculate and record the ET_{50} (or LT_{50}) values for each concentration tested.
- Plot, if possible on double-logarithmic graph paper, the LC_{50} (or EC_{50}) values against time or the LT_{50} (or ET_{50}) values against concentrations.

NOTES

1. Conditions for the validity of the test

- The mortality in the blanks at the end of the test must not exceed 10 %.
- The 48-h LC_{50} (48-h EC_{50}) values of one test series must lie within the confidence limits of the others, and *vice versa*, if it has been possible to determine these separately.
- In all test vessels the oxygen concentration must have exceeded 80 % of the saturation value throughout the test.
- In each case for the blanks and for the most concentrated solutions the pH must be known during the test, and if it was adjusted to 8.0 at time $t=0$ it must be 8.0 ± 0.3 after 48 h.

2. Recommendation

It is recommended to include a reference substance in each test. The result with the reference substance must be in reasonable agreement with the results obtained previously.

3. Observation times

The observation times of $1\frac{1}{2}$ and 12 h are used only with fast-acting substances or if they are desired for any other reasons.

4. Parallel test solutions

It is intended that the test in duplicate should be carried out with 20 animals per litre. To make it easier to count the live and the dead animals without disturbing the test, these 20 are divided into two groups of 10 which, however, together form one unit.

It is decided beforehand which two solutions belong together (for example, a + b and c + d). The results are first summed and then treated as a normal duplicate test.

5.9.7 Test animal *Chaetogammarus marinus*

5.9.7.1 Test with mortality as the criterion

Subject

Determination of the acute toxicity of substances to *Chaetogammarus marinus* (Leach, 1815) (Crustacea, Amphipoda).

Field of application

The method is used in sea water, for pure substances, mixtures of substances, and effluent.

Principle

The mortality of *Chaetogammarus marinus* is used as a measure of the toxicity of a substance added to water. For this purpose the test animals are kept for 4 days in solutions that contain the test substance in various concentrations of the test liquid (e.g. effluent) in various dilutions. The mortality is compared with that in a blank test.

EQUIPMENT AND MATERIALS

- Young gammarids obtained from a laboratory culture, 5 ± 1 mm in length (for breeding, see Section 5.8.7)
- climate room, temperature 15 ± 1 °C, illuminated for about 10 h a day (the intensity of the illumination is not critical)
- beakers, 1 litre, or glass conical flasks, 1 litre, with ground-glass stoppers or with watchglasses
- volumetric pipettes or plunger pipettes with disposable tips, 1 ml
- plunger pipettes, 100 µl, with disposable tips
- volumetric flasks, 100 ml and 1000 ml
- graduated pipettes, 1 ml and 10 ml
- measuring cylinders, 100 ml and 1000 ml
- glass tubes about 15 cm long and about 3 mm in diameter, provided with teats
- aeration tubes, lengths fitting into the beakers, diameter about 3 mm
- aeration apparatus
- nylon tea-strainers
- pH meter
- oxygen meter
- salinity meter
- synthetic or natural sea water, salinity 30 ± 2 ‰ (see Section 5.7.2 j and k, Section 5.7.3 and Section 5.7.4).

PROCEDURE

The procedure can be performed only if the instructions in Sections 5.1 to 5.6 are observed.

- Perform the test in duplicate.
- Choose test vessels, 1-litre beakers or conical flasks, depending on the physico-chemical properties of the test substance.
- Place the test vessels in the climate room.
- Code the vessels according to the test substance and its concentration or dilution, and denote duplicate series by a and b.
- Prepare 1 litre of the test solutions in the vessels in the selected type of sea water in accordance with the instructions given in Section 5.2.

5. Determination of toxicity

- Measure and record the pH and the oxygen concentration of at least the blanks and the most concentrated solutions, if necessary also of the other solutions.
- If necessary, adjust the oxygen concentration to the saturation value and, if desired, the pH to 8.0 ± 0.2 .
- In tests on effluent, measure and record the salinity of the undiluted effluent. In tests on undiluted or only slightly diluted effluent before dilution adjust the salinity to about 30 ‰ with synthetic sea salt.
- Preferably, take samples for the analysis of the test compound concentrations in the prepared test solutions. Not more than 10 % of the solution volume may be used for such samples.
- Transfer 25 gammarids to each test vessel.
- Draw these up from a stock in convenient numbers with the aid of a glass tube having a diameter of about 3 mm, provided with a teat. Avoid contamination.
- Do not aerate during the test unless the oxygen concentration falls below 80 % of the saturation value.
- Do not feed during the test.
- Count and remove any dead gammarids after (1½, 3, 6, (12), 24, 48, and 72 h (see Note 3).
- Note any deviations from normal behaviour and quantify these as far as possible.
- Renew the solutions daily in tests on volatile or apolar substances and in this case do not aerate. For this purpose prepare and check the solutions in the same way as was done at the start of the test. Filter the solutions with the gammarids through the tea-strainer. Pour the used water away, count the live and dead gammarids, check whether the total number tallies, and place the live animals in the freshly prepared solution. Always use the same tea-strainer for the same solution and throw the strainer away at the end of the tests.
- If the test solutions were not renewed, count the dead and the live gammarids after 96 h and check whether the total number tallies.
- After 96 h check the pH and the oxygen concentration of the blanks and of the most concentrated solutions, if necessary also of the other solutions. If renewal was carried out, perform these checks on renewal, i.e. every 24 h.
- After 96 h analyse the test compound concentrations if such an analysis was also performed at time $t=0$. If the solutions were renewed, perform this analysis on the used water on renewal, i.e. after 24 h. In this case the analyses on the freshly prepared and the used water may, if desired, be performed more frequently.
- If desired, repeat the whole test to determine the reproducibility.

TREATMENT OF THE RESULTS

The method of calculation is explained in Section 5.6.2.

- For each test series separately and for each observation time tabulate the number of live (and dead) test animals and, as far as possible, the abnormalities observed.
- Record the measured pH and the oxygen concentrations in the table.
- Record the test compound concentrations found if the solutions were analysed. Then, if desired, use the average concentration during the test for the calculations.

- Calculate the LC_{50} values for as many observation times as possible, preferably with the 95 % confidence limits for each test series separately. Record the calculations in the table, if desired, with the average values.
- If effects other than mortality were observed, calculate and record the EC_{50} values in a similar manner, specifying the nature of the effect.
- If desired, calculate and record the ET_{50} (or LT_{50}) values for each concentration tested.
- Plot, if possible on double-logarithmic graph paper, the LC_{50} or EC_{50} values against time or the LT_{50} (or ET_{50}) values against concentration.

NOTES

1. Conditions for the validity of the test

- The mortality in both blanks must not exceed 10 % at the end of the test.
- The 96-h LC_{50} (96-h EC_{50}) value of one test series must lie within the confidence limits of the other, and *vice versa*, if it was possible to determine these separately.
- In all the test vessels the oxygen concentration must have exceeded 80 % of the saturation value throughout the test.
- The pH at least for the blanks and for the most concentrated solutions must be known during the test, and if it was adjusted to 8.0 at time $t=0$ it must be 8.0 ± 0.3 after 48 h.

2. Recommendation

It is recommended to include a reference substance in each test. The result of the reference substance must be in reasonable agreement with previously obtained results.

3. Observation times

The observation times of 1½ and 12 h are used only with fast-acting substances or if they are desired for any other reason.

5.9.8 Test animal *Poecilia reticulata*

5.9.8.1 Test with mortality as the criterion

Subject

Determination of the acute toxicity of substances to the guppy *Poecilia reticulata* (Peters, 1859) (Pisces, Cyprinodontiformes).

Field of application

The method is used in fresh water or sea water for pure substances, mixtures of substances, and effluent.

Principle

The mortality of *Poecilia reticulata* is used as a measure of the toxicity of a substance added to water.

For this purpose the guppies are kept for at least four days in water with various concentrations of the test substance or various dilutions of the test liquid (e.g. effluent).

The mortality and possibly other abnormalities are compared with those in a blank test.

EQUIPMENT AND MATERIALS

- Guppies, 3–4 weeks old (for breeding, see Section 5.8.8)
- climate room, temperature 24 ± 1 °C, illuminated moderately for about 10 h a day (the intensity of the illumination is not critical)
- all-glass aquaria, capacity about 1.5 litres, with glass cover-plates, or glass conical flasks, 3-litre, with ground-glass stoppers or with suitable watchglasses

5.9 Description of acute toxicity test methods

- volumetric pipettes, 1 ml and 3 ml, or plunger pipettes with disposable tips, 1 ml
- plunger pipettes, 100 µl and 300 µl, with disposable tips
- volumetric flasks, 100 ml, 250 ml, 1000 ml, and 2000 ml
- graduated pipettes, 1 ml and 10 ml
- measuring cylinders, 100 ml and 1000 ml
- aeration tubes, length appropriate to the aquaria, diameter about 3 mm
- aeration apparatus
- small fine-mesh scoop net
- pH meter
- oxygen meter
- salinity meter
- synthetic or natural sea water, salinity 28 ± 2 ‰ (see Section 5.7.2 j and k, Section 5.7.3, and Section 5.7.4) or fresh water of standardized composition: hard A & A water + NaCl or Dutch Standard Water (see Section 5.7.2 c and g).

PROCEDURE

The procedure can be carried out only if the instructions in Sections 5.1 to 5.6 are observed.

- Perform the test in duplicate.
 - Choose the test vessel (glass aquaria or conical flask) according to the volatility of the substance to be tested.
 - Place the test vessels in the climate room.
 - Code these vessels according to the test substance and its concentration or dilution, and denote duplicate series by a and b.
 - Prepare the test solutions in the vessels, 1 or 3 litres, according to the instructions given in Section 5.2, in the selected type of water (see Note 2).
 - Measure and record the pH and the oxygen concentration of at least the blanks and of the most concentrated solutions, if necessary, also of the other solutions.
 - If necessary, adjust the oxygen concentration to the saturation value and, if desired, the pH to the value of the blank medium used.
 - In tests on effluent in sea water, measure and record the salinity of the undiluted effluent. In tests on undiluted or only slightly diluted effluent, adjust the salinity before dilution to 28 ± 2 ‰ with synthetic sea salt.
 - Preferably, take samples for the analysis of the test compound concentrations in the prepared test solutions. Not more than 10 % of the solution volume may be used for such samples.
 - Transfer 10 guppies to each test vessel, avoiding contamination.
 - Aerate gently in tests on non-volatile compounds in the aquaria.
 - Do not aerate in tests on volatile compounds.
 - Do not feed the guppies.
 - Count and remove the dead guppies after 3, 6, 24, 48, and 96 h, and for a suitably longer period if the mortality is continuing (see Note 3).
 - Note all deviations from normal behaviour (see Note 4) and quantify these if possible.
 - In tests on non-volatile compounds, transfer all the fish to freshly prepared solutions once every 48 h.
 - In tests on volatile compounds, transfer all the fish into freshly prepared solutions once every 24 h.
- At each renewal check the pH and the oxygen concentration of the blank and of the highest concentration, if necessary also of the other concentrations.
 - During at least 2 renewals determine, both before and after the renewal, the concentrations of the test compound if it was analysed at time $t=0$.
 - End the test after 96 h or, if necessary, at a later time according to the recommendations given in Note 3.

TREATMENT OF THE RESULTS

The method of calculation is explained in Section 5.6.2.

- For each test series separately and for each observation time tabulate the number of live (and dead) fish and, as far as possible, the abnormalities observed.
- Record the measured pH and the oxygen concentrations in the table.
- Record the concentrations of the test compound found if the solutions were analysed. Then, if desired, use the mean concentrations during the test for the calculations.
- Calculate the LC_{50} values for as many observation times as possible, preferably with the 95 % confidence limits for each test series separately. Record the calculations in the table, if desired, with the average values.
- If effects other than mortality have been observed, calculate and record the EC_{50} in a similar manner, specifying the nature of the effect.
- If desired, also calculate and record the ET_{50} (or LT_{50}) values for each concentration tested. Plot, if possible on double-logarithmic graph paper, the LC_{50} (or EC_{50}) values against time or the LT_{50} (or ET_{50}) values against concentration.

NOTES

1. Conditions for the validity of the test

- The mortality in the blanks must be zero during at least 7 days and may not exceed 10 % in tests of longer duration.
- The LC_{50} (EC_{50}) values per observation time of one test series must lie within the confidence limits of the other, and *vice versa*, if it has been possible to calculate these separately.
- In all the test vessels the oxygen concentration must have exceeded 80 % of the saturation value throughout the test.
- The pH at least for the blank and for the most concentrated solutions must be known throughout the test, and if adjusted must have been the same as the $t=0$ value within 0.3 units throughout the test.

2. Medium

Poecilia reticulata can be bred satisfactorily both in fresh water and in sea water. If it is desired to perform the test in fresh water, the fish must be taken from a laboratory batch in fresh water of the same composition. If it is desired to carry out the test in sea water, the fish are taken from a laboratory culture in sea water.

3. Test duration

The most usual test duration is 96 h. If, on the basis of the observations at 72 h and 96 h, it may be expected that the mortality will continue, and the LC_{50} will therefore continue

5. Determination of toxicity

to decrease with time, it is recommended that the test time is extended to 14 days (maximum) or less if the 'threshold' value of the LC_{50} is reached earlier.

4. Other test criteria

Other abnormalities of the surviving guppies can sometimes be observed in a test.

In particular, this applies to the criterion of immobilization (guppies lying on their sides on the bottom of the test vessel; gill covers moving).

Other criteria:

- loss of equilibrium (swimming mostly on their sides)
- agitated swimming movements
- discoloration (generally to a darker colour)
- hyperventilation (accelerated movements of the gill covers).

It is desirable to quantify such non-lethal abnormalities and to calculate the corresponding EC_{50} values, specifying the nature of the effect.

5. Recommendation

It is recommended to include a reference substance in each test, the result of which must be in reasonable agreement with previously obtained results. Also, it is recommended to repeat the whole test to determine the reproducibility.

5.9.9 Various species as test animals

5.9.9.1 Test with mortality as the criterion

Subject

Determination of the acute toxicity of substances to various species collected in the field or obtained from commercial breeders

Field of application

The methods can be used in all types of water for which a suitable test animal is available, to test pure substances, mixtures of substances, and effluent.

Principle

Mortality is used as a measure of the toxicity of a substance added to water.

For this purpose the test animals are kept for 4 to 30 days, depending on the species, in solutions that contain the test substance in various concentrations or the test liquid (e.g. effluent) in various dilutions.

The mortality is compared with that in a blank test.

EQUIPMENT AND MATERIALS

- About 150 healthy animals of one species and approximately the same size; see also Section 5.9.9.1, point 5
- means of keeping the contents of the test vessels at the temperature required for the test animal (climate room or water bath)
- aquaria, 10-litre, 30-litre, and 100-litre (see Section 5.9.9.1, point 5)
- round-bottomed flasks, 10-litre
- plunger pipettes, 100 μ l, 300 μ l, and 1000 μ l
- graduated pipettes, 1 ml and 10 ml
- measuring cylinders, 100 to 1000 ml
- volumetric flasks, 100 to 1000 ml
- aeration tubes, length appropriate to the aquaria, diameter about 3 mm
- aeration apparatus
- nylon scoop nets
- if possible, a flow-through system to be connected to the test vessels that continuously meters water with the desired test concentration (see Section 5.4.3) in amounts adapted to the physico-chemical properties of the compound and to the weight of the animals.
- pH meter
- oxygen meter
- salinity meter
- water, free from harmful substances, with a composition suitable for the test animal used (see Section 5.9.9.1, point 5).

PROCEDURE

The procedure can be performed only if the instructions in Sections 5.1 to 5.6 are observed.

- Perform one test in duplicate or two single tests (see Note 4).
- Choose test vessels of a type and size depending on the physico-chemical properties of the test substance (see Section 5.4.4), the weight of the test animals (see Section 5.3.4), and the use of a flow-through system (see Section 5.4.3).
- Place the vessels at the selected constant temperature.
- Code the vessels according to the test substance and its concentration or dilution and with an indication of the units to which each belongs (see Note 4).
- Prepare the test solutions in the necessary amount in the selected type of water or connect the test vessels to a calibrated continuous metering system (flow-through system).
- Measure and record the pH and the oxygen concentration of at least the blank and the most concentrated solutions, if necessary also of the other solutions.
- If necessary, adjust the oxygen concentration to the saturation value and, if desired and the test is performed in the (semi-)static mode, adjust the pH to its required value in the water without the test substance (the blank).
- In tests on effluent, measure and record the concentrations of Na, K, Ca, Mg, SO_4 , and Cl in the undiluted sewage. In tests on undiluted or only slightly diluted sewage, before dilution adjust the ion concentrations, if they are too low, to the values required for the test animals.
- Preferably take samples for the analysis of the concentrations of the test compound in the prepared test solutions. Not more than 10 % of the solution volume may be used for such samples, unless flow-through systems are used.
- Transfer to each test vessel 10 test animals or as few as is necessary to ensure that the biomass/water volume ratios given in Section 5.3.2 are not exceeded (see Note 4). For this purpose, using the nylon scoop net, take already acclimatized animals selected for length and health from the stock (see Sections 5.3.3 and 5.3.4). Take care that the test animals are randomly distributed among the test vessels (see Note 5).
- Aerate, if this does not lead to a fall in the concentration of the test compound.
- Do not feed, unless the desired test time is too long (see Section 5.5.3).
- Choose the test animals in agreement with the instructions in Section 5.3.1.
- If flow-through systems are not used, and if the time of the

test is longer than two to four days, choose a renewal scheme (once per 24 h for volatile substances or every Monday, Wednesday, and Friday for substances that do not easily disappear from the water) (see Section 5.4.2).

- Count and remove any dead animals after (1½), 3, 6, (12), and 24 h and then twice a day for the duration of the test (see Note 3).
- Every day measure the oxygen concentrations in all the test vessels. In (semi-)static tests measure the oxygen concentrations of the used solution and of the freshly prepared solution; in flow-through tests measure the oxygen concentration in the test vessels.
- Occasionally measure the pH at least of the blank and of the most concentrated solution. In (semi-)static tests measure the pH in the used and in the freshly prepared solutions; in flow-through tests measure the pH in the test vessels.
- Analyse the concentrations of the test compound in the test vessels at least once per test or twice a week if such an analysis was also performed at time $t=0$.
- Note any deviations from normal behaviour and quantify these as far as possible.
- End the test according to the criteria given in Section 5.5.3.
- If desired, repeat the whole test to determine the reproducibility.

TREATMENT OF THE RESULTS

The method of calculation is explained in Section 5.6.2.

- For each test series separately and for each observation time tabulate the number of live (and dead) test animals and, as far as possible, the abnormalities observed.
- Record the measured pH and the oxygen concentrations in the table.
- Record the test compound concentrations found if the solutions have been analysed.
- Then, if desired, use the mean concentrations during the test for the calculations.
- Calculate the LC_{50} values for as many observation times as possible, preferably with the 95 % confidence limits for each test series separately. Record the calculations in the table, if desired, with the mean values.
- If effects other than mortality have been observed, calculate and record the EC_{50} values in a similar manner, specifying the nature of the effect.
- If desired, also calculate and record the ET_{50} (or LT_{50}) values for each concentration tested.
- Plot, if possible on double-logarithmic graph paper, the LC_{50} (or EC_{50}) values against time or the LT_{50} (or ET_{50}) values against concentration.

NOTES

1. Conditions for the validity of the test

- The mortality in both blanks at the end of the test must not exceed 10 %.
- The LC_{50} (or EC_{50}) value after a time t of one test series must lie within the confidence limits of the other, and *vice versa*, if it has been possible to calculate these separately.
- In all the test vessels the oxygen concentration must have exceeded 80 % of the saturation value throughout the test.
- The pH at least for the blanks and for the most concentrated solutions must be known throughout the test, and if it was adjusted to a certain value it must have remained constant to within 0.3 pH unit during the test.

2. Recommendation

It is recommended to include a reference substance in each test. The result of the reference substance must be in reasonable agreement with previously obtained results.

3. Observation times

The observation times of 1½ and 12 h are used only in the case of fast-acting substances or if they are required for any other reasons.

4. Number of test vessels per test unit

Especially when larger animals are used, test vessels sufficiently large for 10 specimens will not always be available if the limits set in Section 5.3.4 are taken into account. The 10 test animals can then be distributed between several vessels (these count as one vessel). When enough test vessels and space are available, as many vessels may be used as will permit 2×10 animals to be tested simultaneously (e.g. in 4 test vessels), so that the test is performed simultaneously in duplicate. It must first be decided which vessels taken together constitute a unit. The numbers of dead animals in the various vessels which, together, form such a unit are counted before calculating the results. If insufficient vessels or space are available for 2×10 animals simultaneously at each concentration, the test is first performed singly and the series is then repeated.

5. Random distribution of the test animals

The animals must be distributed between the test vessels at random. For this purpose, for example, first place one animal in each vessel, then place a second animal in each vessel, and continue in this way until the total number in each vessel has been reached. In this process the vessel sequence is selected on the basis of a table of random numbers. Avoid contamination during this distribution.

Better still, the animals required for each vessel can be chosen randomly beforehand in a suitable manner and placed in a separate vessel with blank medium, after which they are transferred to the appropriate test solutions.

For test animals such as the mussel, *M. edulis*, a random distribution can be achieved by numbering the individuals and using the tables of random numbers.

5. Determination of toxicity

INFORMATION ON VARIOUS ANIMAL SPECIES

This section contains information necessary for the performance of routine toxicity tests on a number of the most commonly used species.

Mytilus edulis (mussel)

size	— 1.5–3.5 cm; 0.05–1.5 g wet weight
medium	— sea water
temperature	— 15 °C
number and volume	— 10 specimens in 10 litres
minimum–maximum test duration	— 2 weeks–4 weeks
special features	— do not use the animals for the test when they are sexually active

Dreissena polymorpha (zebra mussel)

size	— 1–2 cm; 0.04–0.20 g wet weight
medium	— fresh water, hardness: 1.5 mmole Ca ²⁺ / l and 0.6 mmole Mg ²⁺ / l
temperature	— 15 °C
number and volume	— 10 specimens in 10 litres
minimum–maximum test duration	— 2 weeks–4 weeks
special features	— none

Palaemonetes varians (prawn)

size	— 3.0–4.0 cm; 0.15–0.45 g wet weight
medium	— brackish water or sea water (7–28‰ S)
temperature	— 15 °C
number and volume	— 10 specimens in 10 litres
minimum–maximum test duration	— 7 days–14 days
special features	— acclimatization to the salinity required in the test

Crangon crangon (shrimp)

size	— 4.5–5.5 cm; 0.6–1.2 g wet weight
medium	— sea water, 28‰ S
temperature	— 15 °C
number and volume	— 10 specimens in 10 litres
minimum–maximum test duration	— 7 days–14 days
special features	— place each shrimp separately in a wide-mesh polythene cage to prevent cannibalism

Poecilia reticulata (guppy) (if not bred in the laboratory)

size	— 3.5–4.5 cm; 0.4–0.6 g wet weight
medium	— fresh water, sea water of 28‰ S
temperature	— 24 °C
number and volume	— 10 specimens in 10 litres
minimum–maximum test duration	— 7 days–several months if fed with Tetramin in amounts of 1 to 2 % of the body weight per day
special features	— acclimatization to the salinity desired in the test

Pomatoschistus (Gobius) microps (goby)

size	— about 5 cm; about 1 g wet weight
medium	— sea water, brackish water
temperature	— 15 °C
number and volume	— 10 specimens in 10 litres
minimum–maximum test duration	— 4 days–7 days
special features	— aggressive during the spawning period

Pleuronectes platessa (plaice)

size	— 6–15 cm; 3.5–35 g wet weight
medium	— sea water
temperature	— 15 °C
number and volume	— 5 small specimens in 25 litres, 5 large specimens in 100 litres
minimum–maximum test duration	— 7 days–14 days
special features	— not obtainable in the same size throughout the year

Leuciscus idus var. *melanotus* (golden orfe)

size	— 4–7 cm; 1.2–1.8 g wet weight
medium	— fresh water, hardness: 2.14 mmole of Ca ²⁺ / l, 0.5 mmole of Mg ²⁺ / l, and 0.68–0.85 mmole of NaCO ₃ / l
temperature	— 20 °C
number and volume	— 10 specimens in 15 litres
minimum–maximum test duration	— 4 days–several weeks if fed with Tetramin in amounts of about 1 % of the body weight per day
special features	— not available in the same size throughout the year

Salmo gairdneri (rainbow trout)

size	— 3.5–15 cm; 0.5–35 g wet weight
medium	— soft fresh water, hardness: 0.15 mmole Ca ²⁺ / l, and 0.06 mmole Mg ²⁺ / l
temperature	— 15 °C
number and volume	— 10 small specimens in 5 litres; 5 large specimens in 100 litres
minimum–maximum test duration	— 4 days–7 days
special features	— not obtainable in the same size throughout the year

EXPLANATORY NOTES

• Size

The sizes easy to obtain in sufficient numbers are quoted. (N.B.: In the tests, however, animals must be used that are all of approximately the same size; the measurements given are therefore **not** intended to represent the minimum and maximum sizes of the animals in **one** test).

• Medium

The types of water suitable for toxicity tests with the animal concerned are given.

5.9 Description of acute toxicity test methods

- **Temperature**

For these acute tests the temperatures were standardized at 15, 20, and 24 °C. The most suitable temperature for each test animal was chosen.

- **Minimum and maximum test duration**

The quoted minimum test duration is one after which the lethal threshold value is reached for many compounds with the test animal concerned. The maximum test duration is one in which no mortality is expected in the blank.

- **Number and volume**

Only the ratio (number of test animals)/(volume of the medium) is given in view of the available space. The actually available amount of the medium per animal per 24 h is determined by the renewal frequency or the rate of through-flow. The required renewal frequency or rate of through-flow is determined mainly by the amount of biomass present and by whether aeration can be applied without loss of the test compound.

5.10 DESCRIPTION OF CHRONIC AND SUBCHRONIC TOXICITY TEST METHODS

5.10.1 Unicellular algae

The use of algae in toxicity tests on waste materials is discussed in Section 5.3, and the culturing procedures for the algae are given in Section 5.8.2.

There is an extensive literature on toxicity tests with algae (Meijer, 1974 a), and a number of standard tests have been published (Anon., 1971 and 1974; Bringman, 1975).

The procedures given in this manual are based on our experience over the last few years. We have aimed at simple tests that can be performed easily and reproducibly.

The algal tests are included in the chronic toxicity tests, since the toxic effect is tested on growing cultures that undergo a number of cell divisions (i.e. generations) during the test.

A number of methods are available to measure this growth, such as counting (counting chamber or Coulter Counter), determination of the rate of photosynthesis (uptake of $^{14}\text{CO}_2$ or O_2 production), or measurements of the biomass (dry weight) and of cell components (chlorophyll, protein, nucleic acids). We have chosen two methods of measurement, counting and uptake of $^{14}\text{CO}_2$, the former because of its simplicity and the speed of its performance and the latter because in this test it is possible to carry out tests using natural water and the results can be compared with tests performed by the same technique on pure algal cultures.

The method of culturing is the normal batch culture. The characteristic properties of the batch culture are determined by the limited volume of the culture and by the fact that changes take place within a limited period.

The cell density, the concentration of the nutrients, and often also the concentration of the toxic compound change during the test. The toxic compound is added only once, at the beginning.

For these reasons the batch culture is limited to giving an approximation of the lethal or sub-lethal concentration of the toxic compound.

Incidentally, it may be noted that methods eliminating some of the objections to the batch method have recently been developed. This relates to the 'turbidostat' (continuous culture controlled by keeping the cell density constant) (Kayser, 1976) and the dialysis culture (Jensen and Rystad, 1974). These methods deserve further study.

Three methods are included in this manual:

1. A simple screening test (Section 5.10.1.1), suitable for a rapid determination of the toxic concentrations of a large number of test compounds.

This method is based on a visual determination of the growth of algae in the presence of test compounds in relation to a blank. Several species of algae are generally used in the investigation (Section 5.8.2).

2. For a more accurate determination of the concentration-effect relationship, the growth of the organisms must be determined more accurately. In this test, which is described in Section 5.10.1.2, the growth is determined by counting the number of cells with a Coulter Counter or a counting chamber.

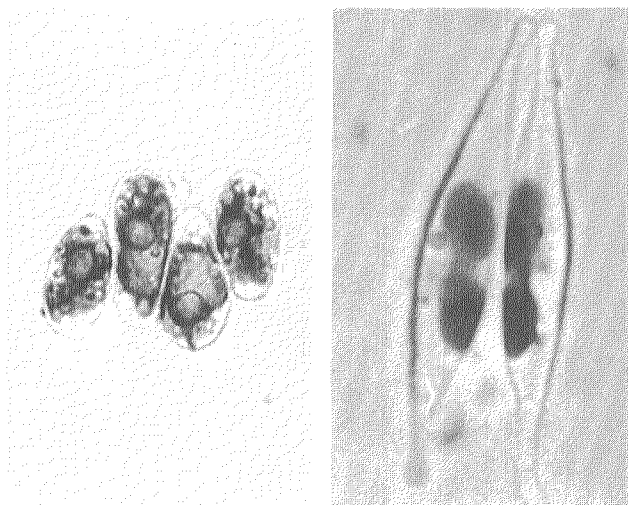
In this way, with the aid of three parameters — the length of the lag phase, the rate of growth, and the maximum production of cell material — it is possible to find the concentration-effect relationship expressed as an EC_{50} value (the EC_{50} is the concentration that gives 50% inhibition in relation to the blank), or the minimum concentration that gives complete inhibition (for further details, see Section 5.6.1).

Several organisms can be used in these tests, but usually a few standard ones are selected. In this case, the choice has fallen on *Phaeodactylum tricornutum* as the standard organism for sea water and *Scenedesmus quadricauda* as the standard organism for fresh water (5.10.1, Fig. 1).

3. Where it is desired to study the influence of toxic substances directly on natural water, one of the most obvious methods is to measure the influence on primary production (Sections 5.10.1.3 and 2.7.3). The principle is based on the measurement of the $^{14}\text{CO}_2$ uptake by phytoplankton. The method is often used for toxicity studies (see Meijer, 1974 a) and has the advantage of not being associated with specific algae. It can be carried out with naturally occurring combinations of algae.

The objection to this method is that in winter there is little phytoplankton in natural water, so that the primary production may be difficult to measure; if the natural phytoplankton is cultured under laboratory conditions at that time of the year, a non-representative collection of species is obtained.

For a short-term test, an incubation time of 4 h has been chosen. On the one hand this is a normal incubation time for measurements of primary production and, on the other hand, the growth of the phytoplankton is limited. The latter is an advantage in the treatment of the results, and the test acquires more the nature of an acute toxicity test. In general there is little point in performing this test with pure algal cultures, since the results then differ little from ones obtained by the laboratory method described in Section 5.10.1.2, except where results with pure cultures are required very rapidly and where a test compound includes a precipitate or particles that interfere with the counting of algae in a Coulter Counter.



5.10.1 Fig. 1 Photographs of the algae *Scenedesmus quadricauda* (left*) and *Phaeodactylum tricornutum* (right**) through a Wratten 48 blue filter

5.10.1.1 Screening test

Subject

Determination of the toxic effect on the growth of algae.

Field of application

The test is used to compare the toxicities of substances in a simple manner.

Principle

The toxic compound is added to an algal culture in a small tube. During the incubation period the growth is evaluated in relation to the blank.

* magnification 1600

** magnification 2560

EQUIPMENT AND MATERIALS

- Algal culture (see Section 5.8.2)
- medium (see Section 5.8.2)
- climate room, adjustable to about 15°C for sea-water test and to about 20°C for fresh water
- incubation apparatus with continuous illumination; for the description, see Meijer (1974 b) and Note 1
- filter holder for membrane filters (diameter 10 cm)
- membrane filters, diameter 10 cm, pore size 0.1 µm
- Buchner flask, 5 litres
- conical flask, 1 litre
- volumetric pipettes, 5 ml and 10 ml
- volumetric flasks, 50 ml and 100 ml
- ampoules or similar glassware with capacities of about 20 ml, previously sterilized at 160°C
- oven for sterilizing glassware at 160°C
- Coulter Counter (see Section 2.7.6)
- counting chamber (as Section 2.7.4.2)
- micropipette, 1 µl
- plunger pipette, 5 ml, with disposable tips
- gas burner
- dimethyl sulphoxide (DMSO), spectroscopically pure
- pH meter.

PROCEDURE

1. Preparation of solutions of the test compound

It is assumed that the solubility and the volatility of the test compounds are known.

- Filter the required medium through a membrane filter.
- In the case of readily water-soluble substances, prepare a stock solution in the test medium; the concentration of the substance must be twice as high as the highest concentration to be tested.
From this prepare a number of dilutions in the medium in a geometric series with a ratio of 10 or $\sqrt[3]{10}$ (Section 5.2.2), all of which are twice as high as the concentrations desired in the test.
- In the case of a sparingly water-soluble substance, make a stock solution in DMSO that is 10^4 times as concentrated as the highest concentration to be tested (Note 2).
- From this make dilutions in DMSO (Note 3).

2. Preparation of algal suspensions

- For testing readily water-soluble substances, prepare an algal suspension from the pre-culture (Section 5.8.2) in the medium, with a density of $2 \cdot 10^4$ cells·ml⁻¹.
- For substances sparingly soluble in water prepare an algal suspension from the pre-culture (Section 5.8.2) in the medium with a density of 10^4 cells·ml⁻¹.
- For substances that are dissolved directly in the test medium prepare an algal suspension of 10^6 cells·ml⁻¹, if necessary with the aid of centrifugation and resuspension.

3. Performance of the test

- Perform the test in duplicate.

- Choose for the test five concentrations such that the most concentrated solution completely inhibits growth or gives a distinct inhibition and the lowest concentration gives no deviation from the blank.

Readily water-soluble substances

- Measure out 5-ml portions of the algal suspension ($2 \cdot 10^4$ cells·ml⁻¹) with a plunger pipette into the required number of tubes.
- Add 5-ml portions of the dilutions of the test compound to the tubes, and 5 ml of the medium to two blank tubes.

Sparingly water-soluble substances

- With a pipette, measure out 10-ml portions of the algal suspension (10^4 cells·ml⁻¹) into the required number of tubes.
- Add 1 µl of the DMSO solutions with a micropipette to the tubes, and DMSO to two blank tubes (Note 4).

Compounds dissolved directly in the medium

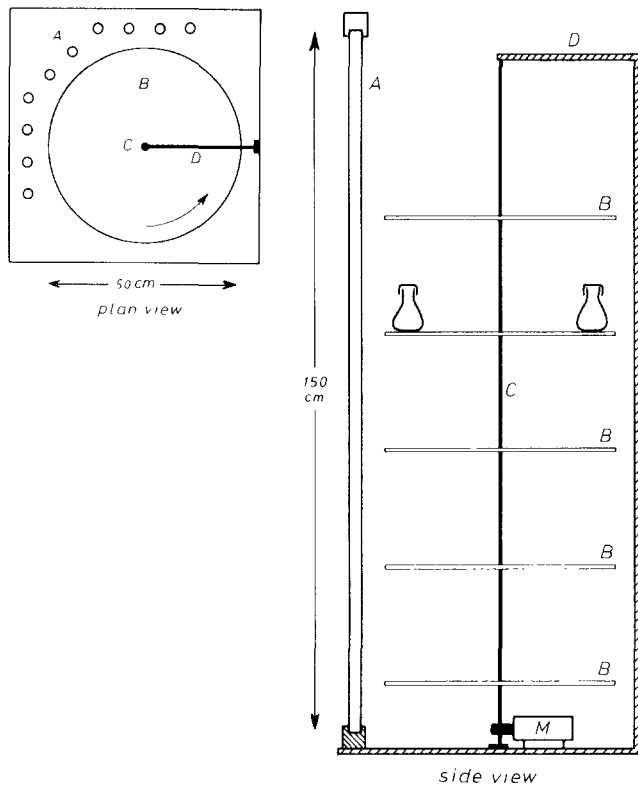
- With a pipette, add 9.9 ml of the test solutions to the tubes and 9.9 ml of the medium to two blank tubes.
- Add 0.1 ml of the algal suspension (10^6 cells·ml⁻¹) to all the tubes.
- If the test substance is volatile or easily distills with evaporating water, the ampoules must be fusion-sealed.
- Place the ampoules in the culturing apparatus, all at the same distance from the light source.
- Check daily whether the test liquid in the blank vessels has become coloured and note the difference in the vessels with test compounds from the blank growth in the following way:
+ : as coloured as the blank
± : less coloured than the blank
− : no colour.

CALCULATIONS (See Section 5.6.1.4.1.)

NOTES

1. The culturing apparatus consists of a vertical shaft on which circular plates (radius 25 cm) are fixed at various heights. The plates rotate at 40 revolutions per hour. Ten fluorescent tubes (60 W, length 1.5 m) provide the light. The tubes are placed at the sides, and therefore the direction and the intensity of the light change continuously. This also prevents the algae from growing on one side of the vessels. All test vessels placed on one plate at the same distance from the shaft pass through the same illumination cycle.
The apparatus is placed in a climate room. Because of the low speed of rotation, it is not necessary to fasten the test-vessels down. Culture flasks of different sizes can be used because the distance between the plates is adjustable.
2. The DMSO necessary to dissolve sparingly soluble substances must have no influence on the growth of the algae. If the effect is small, the blank with DMSO can be used in the calculation of the inhibitory effect, and if the effect is large (10% inhibition compared with the blank), a new test must be carried out with a smaller amount of DMSO.
3. The test compounds may also be dissolved directly in the test medium; this is necessary particularly if concentrations in the neighbourhood of the solubility limit are to be tested and it is preferred not to use DMSO.
4. If necessary, larger amounts of DMSO may be added.

5. Determination of toxicity



5.10.1.1 Fig. 1 Schematic drawing of the culturing apparatus according to Meijer (1974 b). A: fluorescent tubes; B: circular plates; C: vertical shaft; D: suspension; M: electric motor

5.10.1.2 Test with growth as the criterion

Subject

Determination of the toxic effect on the growth of algae.

Field of application

The test can be used for all compounds that do not interfere directly with the counting of algae in various media.

Principle

The toxic compound is added in various concentrations to growing algal cultures. The growth is followed with a Coulter Counter or with a counting chamber. The reduction of growth in relation to a blank is regarded as a measure of the toxicity.

EQUIPMENT AND MATERIALS

- algal culture (Section 5.8.2)
- medium (Section 5.8.2)
- culture flasks (Jena Duran glass 50, Jena No. 21.481.31), 180 ml
- conical flasks, 50 or 100 ml, with ground-glass stoppers
- conical flasks, 250 ml
- crystallizing basins, diameter 4 cm, or silicone-rubber sponge caps, to cover the culture flasks
- filter holder for membrane filters, diameter 10 cm
- membrane filters, diameter 10 cm, with 0.1 μm pores
- Buchner flask
- measuring cylinders, 50 ml and 100 ml
- volumetric pipettes, 5 ml and 10 ml

- volumetric flasks, 250 ml, 500 ml, and 1000 ml
- conical flasks, 1 litre
- climate rooms, adjustable to about 15°C for tests with sea water and to about 20°C for tests with fresh water
- culturing apparatus for algae with continuous illumination; for the description, see Section 5.10.1.1, Note 1
- micropipette, 10 μl
- oven for the sterilization of the culture flasks at 160°C
- autoclave
- Coulter Counter (see Section 2.7.6)
- counting chamber (see Section 2.7.4.2)
- dimethyl sulphoxide (spectroscopically pure)
- pH meter.

PROCEDURE

1. Preparation of solutions of the test compounds

See Section 5.10.1.1, Procedure, point 1.

2. Preparation of algal suspensions

See Section 5.10.1.1, Procedure, point 2.

3. Performance of the test

- Perform the test in duplicate.
- For the test compound choose five concentrations such that the highest completely inhibits growth (or at least gives a distinct inhibition) and the lowest shows no difference from the blank.
- If possible, choose the concentrations on the basis of the results obtained by the method described in Section 5.10.1.1.
- a. Readily water-soluble substances**
 - With a measuring cylinder, transfer 50 ml of algal suspension (containing $2 \cdot 10^4$ cells·ml⁻¹) to coded culture flasks.
 - After this, in the sequence of increasing concentrations, add 50 ml of the dilutions of the test substance.
 - Add 50 ml of the medium to the two blank bottles; two flasks containing 100 ml of the medium are used to determine the 'background' in the Coulter Counter measurements.
 - Cover each flask with an inverted crystallizing basin or a silicone-rubber sponge cap.
 - Shake the flasks and place them on the incubation apparatus in the climate room.
 - Take care that the flasks are placed at the same distance from the light source.
- b. Sparingly water-soluble substances**
 - With a measuring cylinder, transfer 100 ml of the algal suspension (containing 10^4 cells·ml⁻¹) to coded culture flasks.
 - With a micropipette, transfer 10 μl of the various dilutions of the test compound in DMSO to culture flasks, apart from the two that are acting as blanks.
 - Add 10 μl of DMSO to the two blanks.
 - Fill two flasks with 100 ml of the medium to determine the 'background' in counting with the Coulter Counter.
 - Cover each flask with a crystallizing basin or a silicone-rubber sponge cap.
 - Shake the flasks and place them on the incubation apparatus in the climate room.

- Place the flasks at the same distance from the light source.

c. Compounds that are volatile or distill with evaporating water

- Perform this test as described under a. or b., but use 50- to 100-ml conical flasks with ground-glass stoppers as the test vessels. These are filled completely with algal suspension containing the added test-compound solution.
- For each measurement time use a separate conical flask; the complete contents of a flask are used for each sampling time.

d. Test with compounds dissolved directly in the test medium

- Perform the test as described under b. and c., but now add 99 ml of the dilution of the test compound to the culture flasks and then 1 ml of the algal suspension (10^6 cells·ml⁻¹).

e. Measurements

- 24, 48, 72, and 96 h (or longer in the case of slow-growing algae) after the start of the test determine the algal concentration in a 10-ml sample from each test vessel (Note 1).
- Determine the number of cells twice with the aid of the Coulter Counter (Section 2.7.6) or three times with the aid of the counting chamber (Section 2.7.4.2) (Note 2).
- Calculate the number of cells per ml.

CALCULATIONS

Tabulate the results, plot the growth curves, and treat the results further as described in Section 5.6.1.

NOTES

1. If possible, the measurement should be continued until the blank culture has reached the stationary phase.
2. Where the Coulter Counter is used in tests with poorly conducting fresh-water media, it may be necessary to add a little extra NaCl to the sample to raise the conductivity to an acceptable level.
3. If an analytical method is available, it is recommended to determine the concentration of the test compound at the beginning and at the end of the test.
4. Under the influence of toxic substances deformation of the algal cells is possible. This should be checked by microscopic examination of samples of the culture during the test.
5. It is recommended that the results of the toxicity tests are compared with the results of a test with a standard substance; K₂Cr₂O₇ is recommended.

5.10.1.3 Test with the uptake of ¹⁴CO₂ as the criterion

Subject

Determination of the influence of toxic compounds on the uptake of ¹⁴CO₂ by phytoplankton and algal cultures.

Field of application

The test is used to determine the influence of toxic substances on natural water. It may also be used to determine the toxicity of compounds that are incompatible with the other two tests (Sections 5.10.1.1 and 5.10.1.2).

Principle

In addition to toxic substances, an amount of NaH¹⁴CO₃ is added to an algal culture. After incubation the algae are filtered off and their ¹⁴C activity is determined. The reduction of the uptake compared with that of a blank is a measure of the toxic effect.

EQUIPMENT AND MATERIALS

- Natural sea water or fresh water (for sampling and characterization, see Section 3.3)
- an algal culture, if required (see Section 5.8.2)
- medium (see Section 5.8.2)
- conical flasks, 100 ml, with ground-glass stoppers
- filter holder for membrane filters, diameter 25 mm
- membrane filters, diameter 25 mm, 0.45 µm pores
- equipment for the determination of ¹⁴C activity in the filters (see Section 2.7.3)
- measuring cylinders, 100 ml
- volumetric flasks, 250 ml, 500 ml, and 1000 ml
- plunger pipette, 1 ml, with disposable tips
- aluminium foil
- climate room with continuous illumination; temperature adjustable to about 15°C for sea-water tests or to 20°C for tests with fresh water
- incubation apparatus with continuous illumination (see Section 5.10.1.1, Note 1)
- solution of NaH¹⁴CO₃, 10 µCi in 8.5 ml with a bicarbonate concentration of 1 mg·ml⁻¹ and pH 9.5–10
- equipment for working with radioactively labelled compounds (Sections 2.6 and 2.7.3).

PROCEDURE

1. Preparation of solutions of the test compound

- Prepare a stock solution of readily water-soluble materials in filtered natural water or medium, at least 10 times — and best 100 times — as concentrated as the most concentrated solution used in the test.
- From this stock solution prepare dilutions as described in Section 5.10.1.1, Procedure, point 1.
- For sparingly water-soluble substances and for substances to be dissolved directly in the medium, see Section 5.10.1.1, Procedure, point 1.

2. Preparation of algal suspensions

- From a pre-culture, prepare algal suspensions of 10⁴ or 10⁶ cells·ml⁻¹. The suspensions are made in the medium.

3. Performance of the test

- Perform the test in duplicate.
- Select five concentrations for the test compound, such that the highest inhibits the ¹⁴CO₂ uptake completely (or at least gives a distinct inhibition) and the lowest shows no difference from the blank.
- Using the measuring cylinder, transfer 98 ml of natural water or algal suspension to each of the conical flasks.
- By means of a pipette, transfer 1 ml of the dilutions of the test compound into the test vessels, but not into the two blank vessels (Note 1).
- Immediately afterwards, with a plunger pipette, transfer 1 ml of the NaH¹⁴CO₃ solution to each vessel and place the glass stopper on it.
- Measure the dark reaction by wrapping one of the blanks in aluminium foil.
- Incubate the test vessels in the incubation apparatus for 4 h.

5. Determination of toxicity

- At the end of the test, take 1 ml of the flask contents and add it to 15 ml of scintillation liquid (Section 2.7.3) in scintillation bottles to determine the total radioactivity.
- Determine the radioactivity in the phytoplankton or the algae according to Section 2.7.3.

CALCULATIONS

The calculations are performed in accordance with Sections 2.7.3 and 5.6.1.

NOTE

1. If the test compound is dissolved in DMSO, a total of 10 µl of DMSO is added to the test flasks. Consequently, 10 µl of DMSO is also added to the blanks. If the compound is dissolved directly in the test medium, 1 ml of algal suspension (10^6 cells·ml⁻¹) is added.

5.10.2 Test animal *Lymnaea stagnalis*

5.10.2.1 Test with mortality, growth, and morphological abnormalities as the criteria

Subject

Determination of the subchronic toxicity of substances to embryos of the pond snail *Lymnaea stagnalis* (Linnaeus, 1758) (Gastropoda, Basommatophora).

Field of application

The method is used in fresh water for pure substances, mixtures of substances, and effluents that do not give such coloration or turbidity at the dilutions used that the eggs become difficult to see.

Principle

The mortality, the growth, and the morphological abnormalities of the snail embryos are used as a measure of the toxicity of a substance added to water.

Directly after egg-laying, capsules parted from the egg-mass are transferred into fresh water with various concentrations of the test substance or various dilutions of the test liquid (such as effluent) and are kept there for 3 weeks.

The mortality, growth, and morphological abnormalities during embryonic and juvenile development are compared with those in a blank test.

EQUIPMENT AND MATERIALS

- Just-fertilized eggs of the pond snail (for breeding the pond snail and its stimulation to egg-laying, see Section 5.8.3)
- incubator or climate room, temperature $25 \pm 2^\circ\text{C}$, illumination with normal day/night rhythm (the intensity of the illumination is not critical)
- test vessels: conical flasks with ground-glass stoppers, 25 ml
- plunger pipettes, 100 µl and 1000 µl, with disposable tips
- graduated pipettes, 1 ml and 10 ml
- measuring cylinders, 100 ml
- conical flasks, 100 ml
- beakers, 100 ml
- volumetric flasks, 1000 ml

- embryo dishes
- knife with a thin blade tapering to a point
- Pasteur pipettes, cut-off tip, diameter ca. 2 mm, sharp edges rounded off, and fitting teats
- hairloop fixed to a Pasteur pipette (see Section 5.8.3, point 1)
- filter paper
- Petri dishes, diameter 5 cm
- stereomicroscope, magnification 4–40
- pH meter
- oxygen meter
- salinity meter
- fresh water of standardized composition, e.g. standard reference water, hard A & A water, or Dutch Standard Water (see Sections 5.7.2 d, b, and g)
- streptomycin and penicillin
- food: washed lettuce.

PROCEDURE

The procedure can be performed only if the instructions of Sections 5.1 to 5.6 are observed.

- At the appropriate time stimulate pond snails to lay egg-masses (see Section 5.8.3).
- Place the test vessels – 25-ml conical flasks – in the climate room; use six flasks per concentration.
- Code the flasks for the test substance and its concentration or dilution; number the flasks.
- Prepare the test solutions in volumetric flasks according to the instructions given in Section 5.2; mix well.
- Add streptomycin/penicillin solution to the test solution.
Composition:
500 mg of streptomycin and 300 mg of penicillin dissolved in 20 ml of distilled water.
Dosage:
2 ml in 1 litre of the test solution.
- Transfer about 100 ml of the blank and of the most concentrated test solution to beakers and measure the pH and the oxygen concentrations. If necessary, do the same for the other concentrations (and, if necessary, also measure the salinity).
- If desired, adjust the pH of the test solutions to that of the blank medium used and the oxygen concentrations to the saturation value.
- Record the results of all measurements.
- Preferably, take water samples from the volumetric flasks to determine the concentrations of the test compound in the various solutions.
- Measure 20 ml of each test solution into a measuring cylinder and transfer this amount to the corresponding flask.
- Transfer by hand the egg-masses that have been deposited on the aquarium wall to a Petri dish containing water, check the quality under the microscope (see Note 3), and then use only egg-masses with not more than 10 % of multiplets. (These and other terms are explained in Section 5.8.3, under: Brief description of the development of the pond snail).
- Free the suitable egg-masses from their jelly by making a cut through them and then carefully rolling the capsules over moist filter paper; keep the egg-masses separate from one another.

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- Transfer the capsules to an embryo dish filled with a little medium.
- Check the eggs under the stereomicroscope; separate out unsuitable eggs (multiplets and eggs that are already dead before the first cleavage) with the hairloop. Remove these eggs with a Pasteur pipette.
- Wait until the first eggs have reached stage 1, the reference point for the first cleavage. Transfer these with a Pasteur pipette to the test vessel, so that one vessel for each concentration or dilution to be tested contains 10 eggs obtained from the same egg-mass. Avoid contamination.
- Continue in the same manner with subsequent egg-masses and always use eggs obtained from one egg-mass per vessel of one concentration series.
- Close the test vessels immediately after the introduction of the eggs.
- Place the test vessels in the incubator or climate room at 25°C.
- Renew the solutions to be tested three times a week at intervals of two or three days.
- For this purpose, first transfer the eggs, separately per concentration, to an embryo dish with a Pasteur pipette (see Note 4).
- Check the pH and the oxygen concentration of the blank and of the test mixture with the most concentrated solution, if necessary also of the other solutions, at least once a week; do this also for freshly prepared solutions and for the used solutions (just before renewal).
- At least once a week, both before and after removal, check the concentrations of the test compound in the solutions if this determination has also been performed at $t=0$.
Just before renewal:
Take samples from the test vessels; if necessary, combine the contents of the four vessels per concentration to form one sample.
After renewal:
Take samples from the volumetric flasks with the freshly prepared solutions.
- Observe the eggs daily during the first 4 to 5 days; then observe the eggs and/or the newly-emerged snails when the solutions are renewed.
- Count the number of dead embryos after gastrulation and before emergence.
- Count the number of dead snails after emergence.
- Count the number of normally developed embryos and/or snails.
- Count the numbers with the following abnormalities:
 - no differentiation after the gastrula stage into head, foot, and intestinal lumen (these have the appearance of trochophora larvae);
 - exogastrulas: no invagination, but folding of the primitive gut;
 - hydrobes: embryo swollen;
 - head abnormalities: for example, one or three eyes, two eyes present but abnormal positions, etc.
- Record all observations.
- Record the times at which the snails emerge (do not feed the emerged snails).
- End the test after 21 days; this is normally 7 to 12 days after the emergence of the first snails in the blank.

TREATMENT OF THE RESULTS

- Tabulate the average measured pH and oxygen concentrations and, if measured, the ion contents. Record the results of any special measurements.

- Record in the table, besides the nominal concentrations of the test compound, the average measured concentrations before and after renewal and, if possible, also the averages of these two values if the actual concentrations of the test compound have been determined in the solutions.
- Record the final results of the biological observations in a table as given below. For this purpose use the total number of different observations per test concentration (see Note 5).

Effects	Concentration					
	C ₀	C ₁	C ₂	C ₃	C ₄	C ₅
% mortality before gastrulation	•	•	•	•	•	•
% mortality after gastrulation	•	•	•	•	•	•
% mortality after emergence	•	•	•	•	•	•
% mortality, cumulative	•	•	•	•	•	•
time of emergence (average in days)	•	•	•	•	•	•
% of normally developed snails	•	•	•	•	•	•
% of snails with retarded growth	•	•	•	•	•	•
% of other abnormalities:						
no differentiation	•	•	•	•	•	•
exogastrulas	•	•	•	•	•	•
hydrobes	•	•	•	•	•	•
head abnormalities	•	•	•	•	•	•
• • • • •	•	•	•	•	•	•

- From the percentage mortalities calculate the LC₅₀ values for as many observation times as possible and plot these values, if possible, on double-logarithmic graph paper against time, or report them in a table.

NOTES

1. Conditions for the validity of the test

- The total mortality in the blank during the whole test must not exceed 20%.
- Although the oxygen demand of the embryos is low, the emerged snails take up atmospheric oxygen, so it is desirable that during the whole test the oxygen concentration in all vessels should be at least 70 % of the saturation value.
- The pH must be known at least for the blank and for the highest concentration and must be 8.0 ± 0.3 for the whole test period unless the influence of the pH is being determined simultaneously.

2. The medium

Water of standardized composition is preferably used to prepare the solutions. Another type of standard water, and also natural water, can be used if Section 5.7 is taken into account and if the pond snail develops normally in it (see Section 5.8.3, under: Brief description of the development of the pond snail).

3. Selection of test material

If in a given egg mass many eggs have died before the first cleavage, this egg-mass is not used for the test.

4. Handling of the capsules

The capsules lie on the vessel bottom. Hold the vessel slightly inclined and wait until the capsules slide into contact with one another; they can then be transferred to the embryo dish in one operation with a Pasteur pipette.

5. Parallel test solutions

If, because of the poor quality of an egg-mass, the mortality in the blank solution of a concentration series exceeds 30%,

5. Determination of toxicity

the results of this concentration series are not included in the calculation of the results.

5.10.3 Test animal *Ophryotrocha diadema*

5.10.3.1 Test with mortality, growth, and reproduction as the criteria

Subject

Determination of the chronic toxicity of substances to *Ophryotrocha diadema* (Åkesson, 1976, 1976) (Polychaeta).

Field of application

The method is used in sea water, for pure substances, mixtures of substances, and effluents that are not volatile and do not give such coloration or turbidity at the dilution used that the worms become difficult to see.

Principle

The mortality, the growth of the larvae, and the reproduction of adult specimens of *Ophryotrocha diadema* are used as a measure of the toxicity of a substance added to water. For this purpose larvae a few days old are placed in sea water with various concentrations of the test substance or various dilutions of the test liquid (such as effluent) and are kept there for a full life cycle of about 7 weeks. Mortality, growth, and reproduction are compared with those in a blank test.

EQUIPMENT AND MATERIALS

- Larvae of *O. diadema* two to three days old (for the breeding of *O. diadema*, see Section 5.8.4)
- climate room, temperature $22 \pm 2^\circ\text{C}$, illumination with normal day/night rhythm (the intensity of the illumination is not critical)
- glass evaporating dishes, diameters 10 cm and 6 cm, with suitable watchglasses or Petri dishes to serve as covers
- plunger pipettes, 100 μl and 1000 μl , with disposable tips
- graduated pipettes, 1 ml and 10 ml
- measuring cylinders, 25 ml and 100 ml
- volumetric flasks, 1000 ml
- conical flasks, 100 ml
- beakers, 100 ml
- pH meter
- oxygen meter
- salinity meter
- stereomicroscope, with incident light, magnification 4–40
- Pasteur pipettes with cut-off tip, opening diameter about 2 mm, sharp edges rounded off, and matching teats
- synthetic or natural sea water, salinity $32 \pm 2\text{‰}$ (see Section 5.7.2 j and k, Section 5.7.3, and Section 5.7.4)
- food: liquidized spinach (see Section 5.8.4).

PROCEDURE

The procedure is based on Åkesson's work (1970, 1975, 1976). It can be performed only if the instructions of Sections 5.1 to 5.6 are observed. In addition, the 96-h LC_{50} of the test substance is assumed to be known for *O. diadema* (for its determination, see Section 5.9.3.1).

- Place the test vessels, evaporating dishes 6 cm in diameter, in the climate room; use 4 dishes per concentration or dilution.
- Code the dishes according to the test substance, its concentration or dilution, and the number of dishes.
- Prepare the test solutions in volumetric flasks according to the instructions given in Section 5.2; mix well. Choose the concentration series between 1 and 80 % of the 96-h LC_{50} .
- Transfer about 100 ml of the blank and of the most concentrated solution to a beaker and measure the pH, the oxygen concentration, and the salinity; if necessary, do this also with the other concentrations.
- If desired, adjust the pH of the test solutions to 8.0 ± 0.2 and, if necessary, the oxygen concentration to the saturation value; if necessary, in tests on undiluted or slightly diluted effluent adjust the salinity to $32 \pm 2\text{‰}$ before the dilution.
- Record the results of all measurements.
- Preferably, take samples of water from the volumetric flasks to determine the test compound concentrations.
- Measure out the test solutions in measuring cylinders in amounts of 15 ml and transfer these amounts to the dishes.
- To each dish transfer 10 two- to three-day-old larvae. Using a stereomicroscope, draw the larvae one by one from the stock with a Pasteur pipette and transfer them to the dishes. Avoid contamination. Cover the dishes with suitable watchglasses or Petri dishes.
- Transfer the larvae to clean dishes with freshly prepared solutions three times a week (for example on Monday, Wednesday, and Friday) (always use the stereomicroscope and Pasteur pipettes for this purpose).
After a test duration of about 2 weeks transfer the larvae into the larger dishes (diameter 10 cm) containing freshly prepared solutions in amounts of 80 ml. During the renewal, again 3 times a week, do not transfer the larvae but carefully pour off all the solutions and re-fill each dish with 80 ml of fresh solution.
- At each renewal feed liquidized spinach, sufficient for 2 days. During the first and second weeks, one small drop (approx. 0.03 ml) per dish; from the third week: two small drops per dish.
- At least once a week check the pH, the oxygen concentration, and the salinity of the blank and of the most concentrated test solution, if necessary also of the other solutions, both for the freshly prepared solutions and for the used solutions (just before renewal).
- At least once a week, both before and after renewal, check the concentrations of the test compound in the solutions if the determination was also performed at $t=0$.

Just before renewal:

Take samples from the test vessels; if necessary combine the contents of the four dishes per concentration into a single sample.

After renewal:

Take samples from the volumetric flasks containing the freshly prepared solutions.

- Count and remove the dead animals at each renewal.
- Count the number of parapodia per animal twice a week (preferably during renewal, on Monday and Friday) during the first to fourth weeks of the test.
- Record all observations.
- Record the time of the first egg-laying and all subsequent ones at all test concentrations (the first egg-laying can be expected from the fourth week of the test onwards).
- Count and record the number of egg-masses per dish during

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the two weeks after the first egg-laying in the blank (fifth and sixth weeks).

- Count and record the number of eggs per egg-mass during this period.
- Number the position of the egg-masses on the outer wall of each dish with a felt-tip pen to avoid re-counting these egg-masses in later observations.
- Follow the development of the eggs in the egg-masses three times a week. Record the results for each egg-mass separately. In this way the number of successful egg-masses is determined at the same time, i.e. the number of egg-masses in which one or more eggs develop into larvae.
- Count, and not shortly before emergence, the number of larvae that have developed in each egg-mass.
(For this purpose larvae from eggs laid in the sixth week must still be observed in the seventh week of the test).
- End the test after seven weeks.

TREATMENT OF THE RESULTS

- Tabulate the average measured pH and oxygen concentrations and their standard deviations. Record any special features in the results of the measurements.

- Record in the table, together with the nominal concentrations of the test compound, the average measured concentrations before and after renewal and also their averages if the actual concentrations of the test compound have been determined in the solutions.
- In a graph, represent the growth of the larvae by plotting the average number of parapodia per day (per concentration) against time.
On each curve give the concentration of the test compound to which the curve relates.
- In order to calculate the end results in relation to the mortality and reproduction, fill in the **total** numbers of the various observations per test concentration or dilution (see Note 4) as in the following table:

concentration (c), mg.l ⁻¹ , dilution	c ₀	c ₁	c ₂	c ₃	c ₄	c ₅
number of larvae at the start (a)	a ₀	a ₁	a ₂	a ₃	a ₄	a ₅
number of surviving animals (b)	b ₀	b ₁	b ₂	b ₃	b ₄	b ₅
number of egg-masses (n)	n ₀	n ₁	n ₂	n ₃	n ₄	n ₅
number of eggs (e)	e ₀	e ₁	e ₂	e ₃	e ₄	e ₅
number of larvae (l)	l ₀	l ₁	l ₂	l ₃	l ₄	l ₅
number of egg-masses with larvae (p)	p ₀	p ₁	p ₂	p ₃	p ₄	p ₅

- Calculate the end results with respect to mortality and reproduction and record them in a table as shown below.

							Instructions for calculation (k = 0, 1, 2, 3, 4, 5)
concentration (c), mg.l ⁻¹ , dilution	c ₀	c ₁	c ₂	c ₃	c ₄	c ₅	
mortality among the parent animals (s), %	s ₀	s ₁	s ₂	s ₃	s ₄	s ₅	$s_k = 100 \frac{a_k - b_k}{a_k}$
time of the first egg-laying (t), days	t ₀	t ₁	t ₂	t ₃	t ₄	t ₅	
successful egg-masses (q), %	q ₀	q ₁	q ₂	q ₃	q ₄	q ₅	$q_k = 100 \frac{p_k}{n_k}$
mean number of egg-masses per animal (f)	f ₀	f ₁	f ₂	f ₃	f ₄	f ₅	$f_k = \frac{n_k}{b_k}$
mean number of eggs per egg-mass (g)	g ₀	g ₁	g ₂	g ₃	g ₄	g ₅	$g_k = \frac{e_k}{n_k}$
mean number of larvae per egg-mass (i)	i ₀	i ₁	i ₂	i ₃	i ₄	i ₅	$i_k = \frac{l_k}{n_k}$
mortality in egg-masses (m), %	m ₀	m ₁	m ₂	m ₃	m ₄	m ₅	$m_k = 100 \frac{e_k - l_k}{e_k}$
reproduction potential (r), % of control	r ₀	r ₁	r ₂	r ₃	r ₄	r ₅	$r_k = 100 \frac{l_k}{l_0}$

NOTES

1. Conditions for the validity of the test

- The mortality in the initial material of the blank during the whole test must not exceed 25 %. This mortality occurs mainly during the first two weeks of the test, i.e. in the early larval stage. Only incidental mortality should occur during the subsequent larval development and during the reproduction period.

- Although the oxygen demand of the worms is low, it is desirable that during the whole test the oxygen concentration in all the dishes should not fall below 70 % of the saturation value.
- Unless the influence of the pH and the salinity is also being determined in the test, their values should normally be 8.0 ± 0.2 and $32 \pm 2\%$, respectively, throughout the test.

5. Determination of toxicity

2. Test method and duration

The test period is set at seven weeks, divided roughly into a growth period (weeks 1–4) and a reproduction period (weeks 5–6), while in week 7 the development of the eggs laid in week 6 is still observed.

The period in which the reproductive capacity is determined constitutes only a part of the total reproduction period of the worms.

If more information is considered desirable on the reproductive capacity and/or the development of the deposited eggs and the larvae, it is possible to prolong the test period.

The growing larvae must, however, be transferred to separate test vessels to be able to distinguish them from the parent generation.

In Section 5.10.3.2 the reproductive capacity is used as a criterion right from the beginning of the test and is measured over a period of 30 days. In this test the initial material consists of adult worms.

3. Test animals

Although the test method in a more or less modified form can also be used with other polychaete worms of the genus *Ophryotrocha* that are easy to breed, the choice here has fallen on *Ophryotrocha diadema* on the basis of Åkesson's recommendations (1975, 1976).

4. Parallel test solutions

Since the worms must be counted under a stereomicroscope, it is impossible to use larger dishes. The evaporation dishes used can contain only 10 worms. For this reason the worms are divided into 4 groups of 10 for each concentration.

5.10.3.2 Test with mortality and reproduction as the criteria

Subject

Determination of the chronic toxicity of substances to *Ophryotrocha diadema* (Åkesson, 1976) (Polychaeta).

Field of application

The method is used in sea water, for pure substances, mixtures of substances, and effluents that are not volatile and do not give such coloration or turbidity at the dilutions used that the worms become difficult to see.

Principle

Mortality and reproduction are used as measures of the toxicity of a substance added to water. For this purpose adult worms 4 weeks old are placed in sea water with various concentrations of the test substance or various dilutions of the test liquid (e.g. effluent) and kept there for 30 days. The mortality and reproduction are compared with those in a blank test.

EQUIPMENT AND MATERIALS

- Adult specimens of *O. diadema*, four weeks old (for the breeding of *O. diadema* see Section 5.8.4)
- climate room, temperature $22 \pm 2^\circ\text{C}$, illumination with normal day/night rhythm (the intensity of the illumination is not critical)
- glass evaporating dishes, diameter 10 cm, with suitable watch-glasses to serve as covers.
- plunger pipettes, 100 μl and 1000 μl , with disposable tips
- graduated pipettes, 1 ml and 10 ml
- measuring cylinders, 100 ml
- volumetric flasks, 1000 ml
- conical flasks, 100 ml
- beakers, 100 ml
- pH meter
- oxygen meter
- salinity meter
- stereomicroscope, with incident light, magnification 4–40
- Pasteur pipettes with cut-off tip, opening diameter about 2 mm, sharp edges rounded off, and matching teats
- synthetic or natural sea water, salinity $32 \pm 2\text{‰}$ (see Section 5.7.2 j and k, Section 5.7.3, and Section 5.7.4)
- food: liquidized spinach (see Section 5.8.4).

PROCEDURE

The procedure is based on Åkesson's work (1970, 1975, 1976). The procedure can be carried out only if the instructions in Sections 5.1 to 5.6 are observed. Moreover, the 96-h LC_{50} value of the test substance for *O. diadema* is assumed to be known (for its determination, see Section 5.9.3.1).

- Place the test vessels, evaporating dishes 10 cm in diameter, in the climate room; use four dishes per concentration or dilution.
- Code the vessels according to the test substance, its concentration or dilution and number the dishes.
- Prepare the test solutions in volumetric flasks according to the instructions given in Section 5.2; mix well. Choose the concentration series between 1 and 80% of the 96-h LC_{50} .
- Transfer about 100 ml of the blank and of the most concentrated solution to beakers and measure the pH, the oxygen concentration, and the salinity; if necessary, also measure these for the other concentrations.
- Adjust the oxygen concentration of the test solution to the saturation value and, if desired, the pH to 8.0 ± 0.2 ; if necessary, in tests on undiluted or only slightly diluted effluent, adjust the salinity to $32 \pm 2\text{‰}$ before the dilution.
- Record the results of all measurements.
- Preferably, take water samples from the volumetric flasks to determine the concentration of the test compound in the various solutions.
- Measure the test solutions out in amounts of 80 ml in measuring cylinders and transfer these amounts to the test dishes.
- To each dish transfer 10 four-week-old worms. Using a stereomicroscope, draw up the larvae one by one with a Pasteur pipette and release them under water in the test dish. Avoid contamination. Cover the dishes with watchglasses.
- Renew the test solutions three times a week (e.g. every Monday, Wednesday, and Friday). To do this, carefully pour off the solutions and refill the test vessels with 80 ml of clean solution.
- At each renewal feed 2 drops of liquidized spinach (approx. 0.1 ml), which is sufficient for two days.
- At least once a week check the pH, the oxygen concentration, and the salinity of the blank and of the most concentrated solution and, if necessary, also of the other solutions, both for the freshly prepared and the used solutions (just before renewal).
- Determine the concentrations of the test compound in the solutions at least once a week, before and after renewal, if the determination was also performed at $t=0$. Just before renewal: take samples from the test dishes; if necessary, combine the contents of the 4 dishes per concentration to give a single sample.

5.10 Description of chronic and sub-chronic toxicity test methods

After renewal:

take samples from the measuring flasks containing the freshly prepared solutions.

- Count and remove the dead animals at each renewal.
- Record all observations.
- Count and record the number of egg-masses per dish three times a week for 30 days.
- Count and record the number of eggs per egg-mass during this period.
- Number the positions of the egg-masses on the outer wall of each dish with a felt-tip pen to avoid re-counting these egg-masses in later observations.
- Follow the development of the eggs in the egg-masses three times a week. Record the results for each egg-mass separately. In this way the number of successful egg-masses is determined at the same time, i.e. the number of egg-masses in which one or more eggs develop into larvae.
- Count and record shortly before emergence the number of larvae that have developed in each egg-mass (for this purpose larvae from eggs laid in the last week of the test period must be observed for another week after the 30-day period).
- End the test after 37 days.

TREATMENT OF THE RESULTS

- Tabulate the average measured pH and oxygen concentrations and their standard deviations. Record any special features in the results of the measurements.
- Record in the table, in addition to the nominal concentrations of the test compound, the average measured concentrations before and after renewal and also their averages if the two concentrations of the test compound were determined in the solutions.
- For the calculation of the final results in relation to mortality and reproduction, in the following review fill in the **total** numbers of the various observations per concentration or dilution (see Note 3):

concentration (c), mg.l ⁻¹ , dilution	C ₀	C ₁	C ₂	C ₃	C ₄	C ₅
number of worms at the beginning (a)	a ₀	a ₁	a ₂	a ₃	a ₄	a ₅
number of surviving animals (b)	b ₀	b ₁	b ₂	b ₃	b ₄	b ₅
number of egg-masses (n)	n ₀	n ₁	n ₂	n ₃	n ₄	n ₅
number of eggs (e)	e ₀	e ₁	e ₂	e ₃	e ₄	e ₅
number of larvae (l)	l ₀	l ₁	l ₂	l ₃	l ₄	l ₅
number of egg-masses with larvae (p)	p ₀	p ₁	p ₂	p ₃	p ₄	p ₅

- Calculate the final results on mortality and reproduction and record these in a table as given below.

							Instructions for calculation (k = 0, 1, 2, 3, 4, 5)
concentration (c), mg.l ⁻¹	C ₀	C ₁	C ₂	C ₃	C ₄	C ₅	
mortality among the parent animals (s), %	s ₀	s ₁	s ₂	s ₃	s ₄	s ₅	$s_k = 100 \frac{a_k - b_k}{a_k}$
successful egg-masses (q), %	q ₀	q ₁	q ₂	q ₃	q ₄	q ₅	$q_k = 100 \frac{p_k}{n_k}$
average number of egg-masses per animal (f)	f ₀	f ₁	f ₂	f ₃	f ₄	f ₅	$f_k = \frac{n_k}{b_k}$
average number of eggs per egg-mass (g)	g ₀	g ₁	g ₂	g ₃	g ₄	g ₅	$g_k = \frac{e_k}{n_k}$
average number of larvae per egg-mass (i)	i ₀	i ₁	i ₂	i ₃	i ₄	i ₅	$i_k = \frac{l_k}{n_k}$
mortality in egg-masses (m), %	m ₀	m ₁	m ₂	m ₃	m ₄	m ₅	$m_k = 100 \frac{e_k - l_k}{e_k}$
reproductive potential (r), % of control	r ₀	r ₁	r ₂	r ₃	r ₄	r ₅	$r_k = 100 \frac{l_k}{l_0}$

NOTES

1. Conditions for the validity of the test

- During the whole test the mortality of the initial material in the blank must not exceed 20 %.
- Although the oxygen demand of the worms is low, during the whole test the oxygen concentration in all the dishes should not fall below 70 % of the saturation value.
- Unless the effects of the pH and of the salinity are also determined, their values must normally be 8.0±0.2 and 32±2‰, respectively, throughout the test.

2. Test animals

Although the test method can also be used in a more or less modified form with other polychaete worms of the genus *Ophryotrocha* that are easy to breed, the choice here has fallen on *Ophryotrocha diadema* on the basis of Åkesson's recommendations (1975, 1976).

3. Parallel test solutions

Since the worms must be counted under a stereomicroscope, it is impossible to use larger dishes. The evaporation dishes used can contain only 10 worms. Consequently, the animals are divided into four groups of 10 for each concentration.

5. Determination of toxicity

5.10.4 Test animal *Artemia salina*

5.10.4.1 Test with mortality, growth, and reproduction as the criteria

Subject

Determination of the chronic toxicity of substances to the brine shrimp *Artemia salina* (Linnaeus, 1758) (Crustacea, Anostraca).

Field of application

The method is used in sea water, for pure substances, mixtures of substances, and effluents that do not give such coloration or turbidity at the dilutions used that the test animals become difficult to see or consume so much oxygen that intensive aeration is necessary.

Principle

The mortality, the growth, and the reproductive capacity of *Artemia salina* are used as measures of the toxicity of a substance added to water. For this purpose the shrimps are exposed for 3 weeks in solutions that contain the test substance in varying concentrations or the test liquid (e.g. effluent) in various dilutions.

The mortality, the growth, the time of the first production of young, and the number of young born are compared with the corresponding parameters in a blank test.

EQUIPMENT AND MATERIALS

- Artemias, three days old, born from parents about three weeks old and grown to a length of about 1.5 mm (for breeding, see Section 5.8.5)
- climate room, temperature 25 ± 1 °C, illuminated moderately for about 10 h a day (the intensity of the illumination is not critical)
- beakers, 2000 ml, with suitable watchglasses, or conical flasks, 2000 ml, with suitable watchglasses as covers or ground-glass stoppers
- volumetric pipettes or plunger pipettes with disposable tips, 2 ml
- plunger pipettes, 200 µl, with disposable tips
- volumetric flasks, 100 ml, 250 ml, and 1000 ml
- graduated pipettes 1 ml and 10 ml
- measuring cylinders, 100 ml, 250 ml, 1000 ml, and 2000 ml
- glass tubes, about 15 cm long, diameter about 3 mm and about 6 mm, each provided with a teat
- aeration tubes, lengths appropriate to the beakers, diameter about 3 mm
- aeration apparatus
- pH meter
- oxygen meter
- salinity meter
- synthetic or natural sea water, salinity 30 ± 2 ‰ (see Section 5.7.2 j and k, Section 5.7.3, and Section 5.7.4)
- suspension of *Dunaliella viridis* in sea water (see Section 5.8.5, point 3).

PROCEDURE

The procedure can be carried out only if the instructions in Sections 5.1 to 5.6 are observed. In addition, the 48-h LC_{50} of the test substance for *A. salina* is assumed to be known (for its determination, see Section 5.9.4.1).

- Perform the test in duplicate.
- Choose test vessels, conical flasks, or beakers depending on the physico-chemical properties of the test substance (see Section 5.4.4).
- Choose a renewal scheme depending on the physico-chemical properties and the stability of the test substance (see Sections 5.4.3 and 5.4.4) and the degradability of any organic solvent used for measuring out the substance (see Sections 5.2 and 5.4.4).
- Place the vessels in the climate room.
- Code the vessels according to the test compound and its concentration or dilution, and denote duplicate series by a and b.
- In these vessels, prepare 2 litres of the solution to be tested in the selected type of sea water according to the instructions given in Section 5.2. As the lowest concentration choose 1 % of the 48-h LC_{50} determined in accordance with Section 5.9.4, and as the highest concentration choose the 48-h LC_{50} value.
- Measure and record the pH and the oxygen concentration of at least the blanks and the most concentrated solutions, if necessary also those of the other solutions (see Note 3).
- If necessary, adjust the oxygen concentration to the saturation value and, if desired, the pH to 8.0 ± 0.1 .
- In tests on effluent, measure and record the salinity of the undiluted effluent. In tests on undiluted or only slightly diluted sewage, adjust the salinity to about 30‰ with synthetic sea salt before the dilution.
Preferably, take samples for the analysis of the concentrations of test compounds in the prepared test solutions.
Not more than 10 % of the solution volume may be used for such samples (see Note 3).
Add to each test vessel 0.7 ml of the algal suspension prepared according to Section 5.8.5.3, so that $2 \cdot 10^8$ algal cells are available per 20 artemias. Distribute the algal suspension as uniformly as possible through the test vessels.
- Transfer 20 artemias to each test vessel. Draw these up from a stock in convenient numbers with the aid of a glass tube having a diameter of about 3 mm and provided with a teat. Release them under the surface of the liquid in the test vessels. Avoid contamination.
- Renew the medium, standard water, and test compound once every 48 h (if desired, every Monday, Wednesday, and Friday) or daily if the nature of the test compound makes this necessary.
- Aerate the test beakers only if this is necessary, i.e. as soon as the oxygen concentration threatens to fall below 70 % of the saturation value. Do this only if it has been established that the test substance contains no volatile compounds and not before the artemias are about one week old.
- Feed daily with the required amount of algal cells (see Note 4).
- Check the oxygen concentration of all test solutions before each renewal. Record the results of these measurements.
- Check the pH at least of the blanks and of the most concentrated solutions at each renewal, and if necessary also of the other solutions. Record the results of these measurements (see Note 3).
- For the purposes of renewal, prepare a second set of beakers or conical flasks.
In these prepare the test solutions and check their pH and oxygen concentrations and, if desired, the concentrations of the test compound, all in the same way as at the start of the test (see Note 3). Add the necessary amount of food and distribute it as uniformly as possible.
- Count and remove any dead specimens of the original artemias (called the P generation).

5.10 Description of chronic and sub-chronic toxicity test methods

- Count the live specimens of the P generation of artemias and transfer these from the used test solutions into the freshly prepared solutions. For this purpose use the glass tubes (sufficiently wide not to damage the artemias) provided with a teat. Check whether the total number of artemias tallies.
- Record the results of the counts. Record, as soon as this is visually detectable, the numbers of female and male animals, the number of female animals with eggs, and the number of pairs. Also record the visually estimated condition and size of the P generation of artemias in relation to the blank.
- Count the newborn young (the F1 generation) and record their visually estimated condition. Young animals can be expected from about the twelfth day of the test.
After counting and examination, pour the young away unless they are to be used for a subsequent test (F1 generation). Check and record whether eggs from which no larvae have emerged are present on the bottom of the test vessel.
- Determine the concentration of the test compound in the used solutions if this was also analysed in the freshly prepared solutions (see Note 3).
- Then empty out the glassware, remove food residues, etc., rinse with distilled water, and keep this series coded for the following renewal so that each solution is put into the same vessel at each renewal. In each test series, therefore, two beakers or conical flasks are used alternately.
- The minimum and also the usual duration of the test is three weeks, so that when the renewals are carried out on Mondays, Wednesdays, and Fridays seven renewals per test are necessary.
- If desired, repeat the whole test to determine the reproducibility.

TREATMENT OF THE RESULTS

The method of calculation is explained in Section 5.6.2.

- Tabulate the number of live (and dead) test animals and, as far as possible, the abnormalities observed for each test unit separately and for each observation time.
Record the measured pH and the oxygen concentrations in the table.
- Record – if measured – the test compound concentrations in the solutions for all times at which analyses were performed in the freshly prepared and used solutions.
If desired, calculate the average concentrations that were present during the test and use these average concentrations for the calculations.
- Calculate at least the 48-h LC_{50} , 96-h LC_{50} , 7-day LC_{50} , 14-day LC_{50} , and 21-day LC_{50} values, if possible with the 95 % confidence limits, for each test series separately. Report the calculations in the table, if desired with the average values.
- If effects other than mortality were observed, calculate and record the EC_{50} in a similar manner, specifying the nature of the effect.
- If desired, also calculate and record the ET_{50} (or LT_{50}) values for each concentration tested.
- Plot, if possible on double-logarithmic graph paper, the LC_{50} (or ET_{50}) values against time or the LT_{50} (or ET_{50}) values against concentration.
- For each test unit (and also for the blanks) record on which day of the test the first young of the F1 generation were born. Calculate for each test unit the reproduction coefficient x summed over the test period:

$$x = \frac{\text{number of F1 animals}}{\text{number of P animals}}$$

and calculate $\bar{x} \pm 2s$ for each set of duplicates.

- Calculate the percentage reproduction inhibition in relation to

$$\text{the blanks} = \frac{\bar{x}}{\bar{x}(\text{blanks})} \times 100\%$$

NOTES

1. Conditions for the validity of the test

- The mortality in both blanks may not exceed 20 % after 3 weeks.
- The LC_{50} values of one test series must lie within the confidence limits of the corresponding values of the other test series, and vice versa, if it has been possible to calculate these limits separately for both series.
If it has not been possible to calculate the confidence limits for all LC_{50} values separately for each test series, the average LC_{50} and the standard deviations are calculated from each set of duplicates.
The coefficient of variation $\frac{100s}{LC_{50(av.)}}$ then must not exceed 20 % for any of the calculated LC_{50} .
- The oxygen concentration must have exceeded 70 % of the saturation value throughout the test.
- The pH at least for the blanks and for the most concentrated solutions must be known during the test and, if adjusted to 8.0 at time $t=0$, it must have been 8.0 ± 0.3 during the whole test.
- The first young must have been born in the blanks after 13 ± 1 days.
- The number of young per artemia in the blanks during the third week of the test must average at least 100 per week at a temperature of 25 °C.
- The coefficient of variation $100 s/\bar{x}$ for the ratio F1/P for the entire test period must not exceed 50.
- From the second week of the test the amount of food taken up by the animals in the blank solutions must be at least 90 %.

2. Recommendations

It is recommended:

- To include a reference substance in each test. The results with the reference substance must be in reasonable agreement with previously obtained results.
- To repeat the whole test with the second batch of F1 animals for all concentrations in which sufficient F1 animals were born. The object of this is to see whether these animals will grow normally in the same concentration of the test compound as that in which they were born and whether they are more or less sensitive than the P generation.
- To start the tests in such a way that 48 h, 96 h, 7 days, 14 days, and 21 days are exact observation times and therefore the LC_{50} values for these times do not have to be interpolated. (Thus, start on a Monday unless renewal is carried out daily; in the latter case it is possible to start on any day of the week).

3. Frequency of the non-biological measurements

The pH is measured at each renewal in each test solution for which this is considered necessary, both for the used and for the freshly prepared solutions.

The oxygen concentration is measured in **each** used test solution and in each freshly prepared test solution for which this is considered necessary.

If it is possible to determine the concentrations of the test compound in the test solutions, this is done at least once a week

5. Determination of toxicity

for all freshly prepared solutions and for the same solutions after use. It is desirable to perform the first analyses at the start of the test

4. Amount of food required

The amount of food is gradually increased from $2 \cdot 10^8$ algal cells per day for 20 three-day-old artemias to 10^9 algal cells per day for 20 fourteen-day-old and older artemias.

The artemias are fed daily even if renewal is not carried out on that day. If possible, give half the daily ration twice a day.

5.10.5 Test animal *Daphnia magna*

5.10.5.1 Test with mortality, growth, and reproduction as the criteria

Subject

Determination of the chronic toxicity of substances to the water flea *Daphnia magna* (Straus 1820) (Crustacea, Cladocera).

Field of application

The method is used in fresh water for pure substances, mixtures of substances, and effluents that do not give such coloration or turbidity at the dilution used that the test animals become difficult to see or consume so much oxygen that intensive aeration is necessary.

Principle

The mortality, the growth, and the reproductive capacity of *Daphnia magna* are used as measures of the toxicity of a substance added to water. For this purpose the test animals are exposed for 3 weeks in solutions containing the test substance in various concentrations or in the test liquid (e.g. effluent) in various dilutions.

The mortality, the growth, the time of the first production of young, and the number of young born are compared with the corresponding parameters in a blank test.

EQUIPMENT AND MATERIALS

- Daphnias less than 24 h old, born from animals approximately three weeks old (for breeding, see Section 5.8.6)
- climate room, temperature $19 \pm 1^\circ\text{C}$, illuminated moderately for about 10 h a day (the intensity of the illumination is not critical)
- beakers, 1000 ml, with suitable watchglasses as covers, or glass conical flasks, 2000 ml, with suitable watchglasses or ground-glass stoppers
- volumetric pipettes or plunger pipettes with disposable tips, 1 ml
- plunger pipettes, 100 μl and 200 μl , with disposable tips
- volumetric flasks, 100 ml and 1000 ml
- graduated pipettes, 1 ml and 10 ml
- measuring cylinders, 100 ml, 250 ml, 1000 ml, and 2000 ml
- glass tubes, about 15 cm long, diameter about 3 mm and about 6 mm, provided with a teat
- aeration tubes, lengths appropriate to the beakers, diameter about 3 mm
- aeration apparatus
- pH meter
- oxygen meter
- salinity meter
- water of standardized composition: standard reference water, standard test medium, or Dutch Standard Water (see Sections 5.7.2 d, e, and g)
- suspension of a unicellular green alga such as *Chlorella pyrenoidosa* in the medium in which the test is performed (see Section 5.8.6, point 3).

PROCEDURE

The procedure can be carried out only if the instructions in Sections 5.1 to 5.6 are observed. In addition, the 48-h LC_{50} of the test substance for *D. magna* is assumed to be known (for its determination, see Section 5.9.5.1).

- Perform the test in duplicate.
- Choose test vessels, conical flasks, or beakers, depending on the physico-chemical properties of the test substance (see Section 5.4.4).
- Choose a renewal scheme depending on the physico-chemical properties and the stability of the test substance (see Sections 5.4.3 and 5.4.4) and the degradability of any organic solvent used for measuring out the substance (see Sections 5.2 and 5.4.4).
- Place the vessels in the climate room.
- Code the vessels according to the test compound and its concentration or dilution, and denote duplicate series by a and b.
- In these vessels prepare the test solutions, 1 litre or 2 litres, with the selected synthetic standard water (see Note 3) according to the instructions given in Section 5.2. As the lowest concentration choose 1 % of the 48-h LC_{50} and as the highest concentration the 48-h LC_{50} value.
- Measure and record the pH and the oxygen concentration of at least the blanks and the most concentrated solutions, if necessary, also of the other solutions (see Note 4).
- If necessary, adjust the oxygen concentration to the saturation value and, if desired, adjust the pH to the pH of the standard water chosen, ± 0.2 .
- In tests on effluent, measure and record the salt concentrations and the concentrations of some other ions, such as Na, K, Ca, Mg, Cl, and SO_4 , in the undiluted effluent. Adjust these concentrations to agree with the requirements of *D. magna* (see Note 3). If necessary, add an extra test solution – undiluted or slightly diluted effluent – in which the Na, K, Ca, Cl, HCO_3 , and SO_4 concentrations have been made as similar as possible to those of the standard water used for the preparation of the dilutions.
- Preferably take samples for the analysis of the concentration of the test compound in the prepared test solutions. Not more than 100 % of the solution volume may be used for such samples (see Note 4).
- Add to each test vessel 0.2 ml of the algal suspension prepared according to Section 5.8.6, point 3, so that $1 \cdot 10^8$ algal cells are available per 25 daphnids. Distribute the algal suspension as uniformly as possible throughout the test vessels.
- Transfer 25 daphnids to each test vessel. Draw these from a stock in reasonable numbers with the aid of a glass tube having a diameter of about 3 mm and provided with a teat. Release them below the surface of the liquid in the test vessels. Avoid contamination.
- Renew the medium, standard water, and test compound once every 48 h (if desired, every Monday, Wednesday, and Friday) or daily if the nature of the test compound makes this necessary.
- Aerate the test beakers only if this is absolutely necessary, i.e. as soon as the oxygen concentration threatens to fall below 70 % of the saturation value. Do this only if it has been established that the test substance contains no volatile compounds and not before the daphnids are about 5 days old.

5.10 Description of chronic and sub-chronic toxicity test methods

- Feed daily with the required amount of algal cells (see Note 5).
- Check the oxygen concentration of all test solutions before each renewal. Record the results of these measurements.
- Check the pH at least of the blanks and of the most concentrated solutions at each renewal, and if necessary also of the other solutions. Record the results of the measurements (see Note 4).
- For purposes of renewal, prepare a second set of beakers or conical flasks.
In these prepare the solutions to be tested and check the pH and the oxygen concentrations, and if desired the concentrations of the test compound, all in the same way as at the start of the test.
Add the necessary amount of food and distribute it as uniformly as possible.
- Count and remove any dead specimens of the original daphnids (called the P generation).
- Count the live specimens of the P generation of daphnids and transfer these from the used test solutions into the freshly prepared solutions. For this purpose use glass tubes sufficiently wide not to damage the daphnids, provided with a teat.
Check whether the total number of daphnids tallies.
- Record the results of the counts.
- Record whether eggs are present in the brood pouch, whether males and winter eggs are present. Also record the visually estimated condition and size of the P generation of daphnids in relation to the blank.
- Count the newborn young (the F1 generation) and record their visually estimated condition. Young animals can be expected from about the sixth day of the test. After counting and examination, pour the young away unless they are to be used for a subsequent test (F1 generation).
Check and record whether eggs from which no larvae have emerged are present on the bottom of the test vessel.
- Determine the concentration of the test compound in the used solutions if this was also analysed in the freshly prepared solutions (see Note 4).
- Then empty out the glassware, remove food residues, etc., rinse with distilled water, and keep this series coded for the following renewal so that each solution is put into the same vessel at each renewal. Each test unit therefore has two beakers or conical flasks that are used alternately.
- The minimum (and also the usual) duration of the test is three weeks, so that when the renewals are carried out on Mondays, Wednesdays, and Fridays seven renewals per test are necessary.
- If desired, repeat the whole test to determine the reproducibility.

TREATMENT OF THE RESULTS

The method of calculation is explained in Section 5.6.2.

- Tabulate the number of live (and dead) test animals and, as far as possible, the abnormalities observed for each test unit, separately and for each observation time.
- Record the measured pH and the oxygen concentrations in the table.
- Record – if measured – the test compound concentration in the solutions for all times at which analyses were performed in the freshly prepared and the used solutions. If desired, calculate the average concentrations that were present during the test and use these average concentrations for the calculations.
- Calculate at least the 48-h LC_{50} , 96-h LC_{50} , 7-day LC_{50} , 14-day LC_{50} , and 21-day LC_{50} values, if possible with the 95 % confidence limits, for each test series separately.
Record the calculations in the table, if desired with the average values.
- If effects other than mortality were observed, calculate and record the EC_{50} in a similar manner, specifying the nature of the effect.
- If desired, also calculate and record the ET_{50} (or LT_{50}) values for each concentration tested.
- Plot, if possible on double-logarithmic graph paper, the LC_{50} (or ET_{50}) values against time or the LT_{50} (or ET_{50}) values against concentration.
- For each test unit (and also for the blanks) record on which day of the test the first young of the F1 generation were born. Calculate for each test unit the reproduction coefficient x summed over the test period:
$$x = \frac{\text{number of F1 animals}}{\text{number of P animals}}$$
and calculate $\bar{x} \pm 2s$ for each set of duplicates.
- Calculate the percentage reproduction in relation to the blanks
$$= \frac{\bar{x}}{\bar{x} \text{ (blanks)}} \times 100 \%$$

NOTES

1. Conditions for the validity of the test

- The mortality in both blanks may not exceed 20 % after three weeks.
- The LC_{50} values of one test series must lie within the confidence limits of the corresponding values of the other test series, and vice versa, if it has been possible to calculate these limits separately for both series.
If it has not been possible to calculate the confidence limits for all LC_{50} values separately, then for each test series mean LC_{50} values and the standard deviations are calculated from each set of duplicates.
The coefficient of variation $\frac{100 s}{LC_{50(av.)}}$ then must not exceed 10 % for any of the LC_{50} values calculated.
- The oxygen concentration must have exceeded 70 % of the saturation value throughout the test.
- The pH at least for the blanks and for the most concentrated solutions must be known during the test and, if it was adjusted, it must be the same as the $t=0$ value within 0.3 units throughout the test.
- The first young must have been born in the blanks after 7 ± 1 days.
- The number of young per daphnid in the blanks during the third week of the test must average at least 5 per day at a temperature of 25 °C.
- The coefficient of variation $100 s/\bar{x}$ for the ratio F1/P for the entire test period must not exceed 30.
- From the second week of the test the amount of food taken up by the animals in the blank solutions must be at least 90 %.

2. Recommendations

It is recommended:

- To include a reference substance in each test. The results with the reference substance must be in reasonable agreement with previously obtained results.
- To repeat the whole test with the third batch of F1 animals for all concentrations in which sufficient F1 animals were

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born. The point of this is to see whether these animals will grow normally in the same concentration of the test compound as that in which they were born and whether they are more or less sensitive than the P generation.

- To start the test in such a way that 48h, 96h, 7 days, 14 days, and 21 days are exact observation times and, therefore, the LC_{50} for these times do not have to be interpolated. (Thus, start on a Monday unless renewal is carried out daily; in the latter case the test can start on any day of the week).

3. Use of synthetic standard water

In general, the test is performed with standard reference water, standard test medium, or Dutch Standard Water as dilution water. Another type of standard water may be used if it has been established previously that daphnids can develop normally (according to Section 5.8.6) in this water. The daphnids must then also have been bred in this water.

In tests on undiluted effluent, or only slightly diluted effluent – with a synthetic standard water – it is desirable to determine the concentrations of relatively non-toxic ions such as Na, K, Ca, Mg, Cl, and SO_4 in the effluent.

For *Daphnia magna* to remain in good condition, the hardness must be at least 1 mmole of Ca+Mg per litre.

The chloride content may not exceed 500 mg per litre.

4. Frequency of the non-biological measurements

The pH is measured at each renewal in each test solution for which this is considered necessary – both that of the used and that of the freshly prepared solutions.

The oxygen concentration is measured in **each** used test solution and in each freshly prepared test solution for which this is considered necessary.

If it is possible to determine the concentrations of the test compound in the test solutions, this is done at least once a week for all the freshly prepared solutions and for the same solutions after use. It is desirable to perform the first analyses at the start of the test.

5. Amount of food required

The amount of food is increased gradually from 10^8 algal cells per day for 25 one-day-old daphnids to 10^9 algal cells per day for 25 nine-day-old and older daphnids.

The daphnids are fed daily even if renewal is not carried out on that day. If possible, give half the daily ration twice a day.

The amount of food is calculated for 25 daphnids in 1 litre of the medium. When 2-litre conical flasks are used, the cell density is halved. Although the absolute amount of food per daphnid is the same, it now takes the daphnids more energy to collect the food. With these amounts of food, however, this has no influence on the rate of growth.

5.10.6 Test animal *Chaetogammarus marinus*

5.10.6.1 Test with mortality and growth as the criteria

Subject

Determination of the (sub)chronic toxicity of substances to *Chaetogammarus marinus* (Leach, 1815) (Crustacea, Amphipoda).

Field of application

The method is used in sea water for pure substances that are not volatile (see Notes 1 and 5).

Principle

The mortality and the growth of *C. marinus* are used as measures of the toxicity of a substance added to water.

For this purpose the test animals are bred for 1 to 5 months (see Note 2) in solutions that contain the test substance at various concentrations.

The mortality, the growth, and (if the test time is longer than four months) the time of the first production of young are compared with the corresponding parameters in a blank test.

EQUIPMENT AND MATERIALS

- Young gammarids obtained from a laboratory culture, 4.5 ± 0.5 mm long (for breeding, see Section 5.8.7)
- climate room, temperature $15 \pm 1^\circ\text{C}$, illuminated moderately for about 10 h a day (the intensity of the illumination is not critical)
- beakers, 2 litres
- volumetric pipettes or plunger pipettes with disposable tips, 2 ml
- plunger pipettes, 200 μl , with disposable tips
- volumetric flasks, 100 ml, 250 ml, and 1000 ml
- graduated pipettes, 1 ml and 10 ml
- measuring cylinders, 100 ml, 250 ml, 1000 ml, and 2000 ml
- glass tubes, about 15 cm long, diameter about 3 mm and about 6 mm, provided with teats
- aeration tubes, length appropriate to the beakers, diameter about 3 mm
- aeration apparatus
- nylon tea strainers
- pH meter
- oxygen meter
- salinity meter
- synthetic or natural sea water, salinity $30 \pm 2\text{‰}$ (see Section 5.7.2 j and k, Section 5.7.3, and Section 5.7.4)
- *Fucus* sp. (a seaweed) (see Note 3).

PROCEDURE

The procedure can be carried out only if the instructions in Sections 5.1 to 5.6 are observed. Furthermore, the 96-h LC_{50} of the test substance to *C. marinus* is assumed to be known (for its determination see Section 5.9.7.1).

- Perform the test in duplicate.
- Place the beakers in the climate room.
- Code the vessels according to the test compound and its concentration or dilution, and denote duplicate series by a and b.
- Prepare in the beakers 2 litres of the test solutions in the selected sea water in accordance with the instructions given in Section 5.2. As the lowest concentration choose 1 % of the 96-h LC_{50} determined according to Section 5.9.7.1 and as the highest concentration this 96-h LC_{50} value.
- Measure and record the pH and the oxygen concentration of at least the blanks and the most concentrated solutions, if necessary also of the other solutions (see Note 4).
- If necessary, adjust the oxygen concentration to the saturation value, and if desired, the pH to 8.0 ± 0.2 .
- Preferably take samples for the analysis of the concentrations of the test compound in the prepared test solutions. Not more than 10 % of the solution volume may be used for such samples (see Note 4).
- Add a piece of fucus to each test vessel (see Note 3).
- Introduce aeration tubes into each test vessel and aerate the solutions slightly.

5.10 Description of chronic and sub-chronic toxicity test methods

- Transfer 25 gammarids to each test vessel.
- Renew the medium, sea water + test compound, once every 48 h or, if desired, every Monday, Wednesday, and Friday (see Note 5). Replace the fucus once a week (see Note 3).
- Check the oxygen concentrations of all test solutions before each renewal. Record the results of these measurements.
- At least once a week before renewal check the pH, at least of the blanks and of the most concentrated solutions, and if necessary also of the other solutions and possibly more frequently (see Note 4). Record the results of these measurements.
- Before renewal, take samples for the analysis of the concentrations of the test compound if the concentrations of the freshly prepared solutions were also analysed (see Note 4).
- For the purpose of renewal, prepare a second set of beakers. In these prepare the test solutions and, if desired, check the pH, the oxygen concentrations, and the concentrations of the test compound, all in the same way as at the start of the test (see Note 3).
- Filter the beaker contents through a sieve (use a separate sieve, e.g. a nylon tea strainer, for each test compound concentration throughout the test).
- Count the live and dead gammarids and check whether the total number tallies. Transfer the live specimens with the fucus into the freshly prepared solution. Record the results of these counts. Check whether the gammarids have eaten. Both the presence of excreta and disappearance of the fucus may give information on this point. Evaluate the growth visually in relation to that of the blanks.
- Clean the used beakers, rinse with distilled water, and keep this series coded for the subsequent renewal, so that the same concentration is put into the same vessel on each occasion. Each test unit therefore has two beakers that are used alternately.
- If the test is continued long enough young are born in the test solutions (see Note 2). Note the details of this (day of the test, number, test series.)
- At the end of the test, weigh and measure all the gammarids separately. Before weighing, dry the gammarids with filter paper.

TREATMENT OF THE RESULTS

The method of calculation is explained in Section 5.6.2.

- Tabulate the number of live (and dead) test animals and, if possible, any abnormalities observed for each test series separately and for each observation time.
- Record the measured pH and the oxygen concentrations in a table.
- Record – if measured – the test compound concentrations in the solutions for all times at which analyses were performed in the freshly prepared and used solutions.
If desired, calculate the average concentrations that were present during the test and use these average concentrations for the calculations.
- Calculate at least the 96-h, 1-week, 2-week, 3-week, etc. LC_{50} values, if possible with the 95 % confidence limits for each test series separately. Record the calculations in a table, if desired with the average values.
- If effects other than mortality were observed, calculate and record the EC_{50} in a similar manner, specifying the nature of the effect.
- If desired, also calculate and record the ET_{50} (or LT_{50}) values for each concentration tested.

- Plot, if possible on double-logarithmic graph paper, the LC_{50} (or EC_{50}) values against time or the LT_{50} (or ET_{50}) values against concentration.
- Calculate the mean weight (g) of the gammarids at the end of the test and the standard deviation s of the mean for each test unit separately, and if possible separately for the males and the females. Record $\bar{g} \pm 2s$ for each test unit.
- If applicable, record on which test day and in which test solutions pairs were observed.
- If applicable, record in which test solutions young were born, how many, and on which day of the test (see Note 2).

NOTES

1. Aeration of the test solution

The consumption of oxygen by gammarids is very high, particularly as they grow larger. Even if renewal is carried out daily, it is impossible to carry out acute toxicity tests on, for example 20 adult gammarids in 2 litres of unaerated sea water without depletion of the dissolved oxygen. In (sub)chronic tests these animals are, moreover, fed on dead organic material (see Note 3) that consumes oxygen due to microbiological decomposition. With a minimum number of 20 gammarids per test unit for a chronic test in vessels of a manageable dimension, and reasonable schemes of renewal or flow rates, therefore, aeration must be provided to maintain a sufficiently high oxygen concentration. Hence volatile or easily co-distilling substances cannot be tested in the way described here.

2. Test duration

In this test the test animals are supplied with food, the oxygen content is kept sufficiently high by aeration, and the concentration of the test compound is kept constant. In principle, the test duration is thus unlimited. For many compounds an impression of the subchronic toxicity with mortality as the criterion can be obtained in about one month. At the same time, it is then possible to evaluate visually whether the animals have grown. If the test is continued for 4 to 5 months it is also possible to determine whether reproduction takes place. However, the number of females and the number of young born will usually be too small for quantitative information, so that the conclusions concerning reproduction are limited to stating whether reproduction has taken place at all.

3. The food

A large supply of fucus (seaweed) is collected from a clean site and well rinsed with mains water; snails, gammarids, and other animals are removed as thoroughly as possible. It is then spread out to dry in air for a week, whereupon any remaining animals will die off. The amount required for the test is placed in clean sea water 24 h before use and is then well rinsed with sea water. Each test vessel is supplied with the same amount of fucus (previously weighed in the dry state). Once a week the piece of fucus in each vessel is replaced by a new one. The amount provided each week must be more than adequate as food, since the fucus also provides refuge for the gammarids. The amount required for one test is collected at one time, and the 'quality' of the batch is determined beforehand by taking a random sample and seeing whether *C. marinus* eats it, grows on it, and does not die from it.

As an alternative, a few shells can be placed on the vessel bottom to provide refuge and the gammarids can be fed with the commercial fish food Tetramin.

4. Frequency of the non-biological measurements

The pH is measured at each renewal in each test solution for which this is considered necessary.

5. Determination of toxicity

The oxygen concentration is measured in **each** used and in each freshly prepared test solution for which this is considered necessary.

If it is possible to determine the concentration of the test compound in the test solution, this is done at least once a week for all the freshly prepared test solutions and for the same solutions after use. It is desirable to perform the first analyses at the start of the test.

5. Renewal of the test solution

A semi-static test method for the (sub)chronic test has been described. However, with gammarids it is also quite possible to use flow-through systems.

The first two systems described in Section 5.4.3, point 2, for 'smaller flow-through rates' can be used. In particular, if it is intended to continue the test for a long time, the use of flow-through systems is preferable to the semi-static method. If the flow rate is made sufficiently high it is even possible to omit aeration (see Note 1), so that the method becomes applicable for a wider range of compounds.

6. Conditions for the validity of the test

- The mortality in both blanks during the test must not exceed 25 %.
- The LC_{50} values of one test series must lie within the confidence limits of the corresponding values of the other series, and vice versa, if it has been possible to calculate these intervals separately for the two series.
If it has not been possible to calculate the confidence limits for all LC_{50} values separately for each test series, the average LC_{50} and the standard deviation s are calculated from each set of duplicates. The coefficient of variation $\frac{100s}{LC_{50(av.)}}$ must not exceed 20 % of the calculated LC_{50} values.
- In all test vessels the oxygen concentration must have exceeded 70 % of the saturation value throughout the test.
- The pH at least of the blank and of the most concentrated solutions must always be known during the test and, if adjusted to 8.0 at time $t=0$, must have been 8.0 ± 0.3 throughout the test.

5.10.7 Test animal *Poecilia reticulata*

10.7.1 Test with mortality and growth as the criteria

Subject

Determination of the (sub)chronic toxicity of substances to the guppy, *Poecilia reticulata* (Peters, 1858) (Pisces, Cyprinodontiformes).

Field of application

The method is used in fresh water or in sea water for pure substances that are not volatile (see Notes 3 and 4).

Principle

The mortality and the growth of *P. reticulata* are used as measures of the toxicity of a substance added to water.

For this purpose the test animals are bred for 2 to 8 months (see Note 5) in solutions that contain the test substance at various concentrations.

The mortality, the growth, and (if the test time is longer than 4 months) the time of the first production of young are compared with the corresponding parameters in a blank test.

EQUIPMENT AND MATERIALS

- Guppies, 3–4 weeks old (for breeding, see Section 5.8.8)
- climate room, temperature $24 \pm 1^\circ\text{C}$, illuminated moderately for about 10 h a day (the intensity of the illumination is not critical)
- all-glass aquaria, capacity about 2, 5 and 10 litres, with glass cover-plates (see Note 6)
- volumetric pipettes, 2, 5, and 10 ml
- plunger pipettes, 200 μl , 500 μl , and 1000 μl , with disposable tips
- volumetric flasks, 100 ml, 250 ml, 1000 ml
- graduated pipettes, 1 ml and 10 ml
- measuring cylinders, 100 ml, 250 ml, 1000 ml, and 2000 ml
- aeration tubes, length appropriate to the aquaria, diameter about 3 mm
- aeration apparatus
- small fine-mesh scoop net
- pH meter
- oxygen meter
- salinity meter
- synthetic or natural sea water, salinity $28 \pm 2\text{‰}$ (see Section 5.7.2. j and k, Section 5.7.3, and Section 5.7.4) or fresh water of standardized composition: hard A & A water + NaCl or Dutch Standard Water (see Section 5.7.2. c and g)
- food: Tetramin and Tetraphyl.

PROCEDURE

The procedure can be carried out only if the instructions in Sections 5.1 to 5.6 are observed. Furthermore, the 96-h LC_{50} of the test substance to *P. reticulata* is assumed to be known (for its determination see Section 5.9.8.1).

- Perform the test in duplicate.
- Place the 2-l aquaria in the climate room.
- Code these aquaria according to the test substance and its concentration or dilution, and denote duplicate series by a and b.
- Prepare the test solutions in the aquaria, 2 litres, according to the instructions given in Section 5.2, in the selected type of water (see Note 2).
As the lowest concentration choose 1 % of the 96-h LC_{50} , determined according to Section 5.9.8.1, and as the highest concentration this 96-h LC_{50} value.
- Measure and record the pH and the oxygen concentrations of at least the blanks and of the most concentrated solutions, if necessary also of the other solutions (see Note 7).
- If necessary, adjust the oxygen concentration to the saturation value and, if desired, the pH to the value of the blank medium used.
- Preferably, take samples for the analysis of the test compound concentration in the prepared test solutions. Not more than 10 % of the solution volume may be used for such samples. Introduce aeration tubes into each test vessel and aerate the solutions slightly.
- Transfer 25 guppies to each aquarium, avoiding contamination.
- Feed the guppies twice a day with Tetramin and Tetraphyl (about 2 % on the weight of the wet fish).
- Count and remove the dead guppies twice a day.

5.10 Description of chronic and sub-chronic toxicity test methods

- Renew the medium (fresh water or sea water + test compound) once every 48 h or, if desired, every Monday, Wednesday, and Friday (see Note 4).
- Check the oxygen concentrations of all test solutions before each renewal.
- Record the results of these measurements.
- At least once a week before renewal check the pH, at least of the blanks and of the most concentrated solutions, and if necessary also of the other solutions and possibly more frequently (see Note 7).
- Record the results of these measurements.
- Before renewal, take samples for the analysis of the concentration of the test compound, if the concentrations of the freshly prepared solutions were also analysed (see Note 7).
- Siphon off about 90 % of the vessel contents; at the same time remove excreta and food residues.
- Check whether the guppies have eaten. Both the presence of excreta and a reduction in the amount of food give information on this point. Evaluate the growth visually with respect to the blanks. As soon as possible record how many females and how many males are present.
Make up immediately to the original volume with previously prepared and checked fresh medium (fresh water or sea water + test compound) (see also Note 6).
- If the test is continued for a sufficiently long time (longer than 4 months), young may be born in the test solutions (see Note 5).
- Record this information (day of test, number, test series, or vessel).
- At the end of the test, weigh and measure all the original guppies separately and record their sex. Dry the guppies with filter paper before weighing.

TREATMENT OF THE RESULTS

The method of calculation is explained in Section 5.6.2.

- For each test unit separately and for each observation time tabulate the number of live (and dead) guppies and, as far as possible, the abnormalities observed.
- Record the measured pH and the oxygen concentrations in a table.
- Record – if measured – the test compound concentrations in the solutions for all times at which analyses were performed on the freshly prepared and used solutions.
If desired, calculate the average concentrations that were present during the test and use these average concentrations for the calculations.
- Calculate at least the 96-h, 1-week, 2-week, 3-week, etc. LC_{50} values, if possible with the 95 % confidence limits, for each test series separately. Record the calculations in the table, if desired with the average values.
- If effects other than mortality have been observed, calculate and record the EC_{50} values in a similar manner, specifying the nature of the effect.
- If desired, also calculate and record the ET_{50} (or LT_{50}) values for each concentration tested.
- Plot, if possible on double-logarithmic graph paper, the LC_{50} (or EC_{50}) values against time or the LT_{50} (or ET_{50}) values against concentration.
- Calculate the mean weight (g) of the guppies at the end of the test and the standard deviation s of the mean for each test

unit separately and, if possible, separately for the males and females. Record $g \pm 2s$ for each test unit.

- If appropriate, record on which test day and in which test solutions males and females could be distinguished, with their numbers.
- If appropriate, record in which test solutions young were born, how many, and on which day of the test.

NOTES

1. Conditions for the validity of the test

- The mortality in both blanks must not exceed 25 % during the test.
- The LC_{50} values of one test series must lie within the confidence limits of the corresponding values of the other series, and vice versa, if it has been possible to calculate these limits separately for the two series.
If it has not been possible to calculate the confidence limits for all the LC_{50} values separately for each series, the mean LC_{50} and the standard deviation s are calculated from each set of duplicates. The coefficient of variation $\frac{100s}{LC_{50(av.)}}$ then must not exceed 20 % for any of the calculated LC_{50} values.
- In all test vessels the oxygen concentration must have exceeded 70 % of the saturation value throughout the test.
- The pH for the blanks and for the most concentrated solutions must be known during the test, and if adjusted must have been the same as the $t=0$ value within 0.3 units throughout the test.

2. Medium

Poecilia reticulata can be bred satisfactorily both in fresh water and in sea water. If it is intended to carry out the tests in fresh water, the fish must be obtained from a laboratory batch in fresh water of the same composition. If it is intended to carry out the test in sea water, the fish must be obtained from a laboratory batch in sea water.

3. Aeration of the test solutions

The consumption of oxygen by guppies is very high, particularly as they grow larger. Even if renewal is carried out daily, it is not possible to carry out acute toxicity tests on, for example, 20 young guppies in 2 litres of unaerated sea water without depletion of the dissolved oxygen. In (sub)chronic tests these fish are, moreover, fed on dead organic material (Tetramin, Tetraphyl) that consumes oxygen owing to microbiological decomposition. With a minimum number of 25 guppies per test unit for a chronic test, in vessels of manageable dimensions and with reasonable schemes of renewal or flow rates, aeration is necessary to maintain a sufficient oxygen concentration. Hence volatile or easily co-distilling substances cannot be tested in the manner described here.

4. Renewal of the test solutions

A semi-static method for this subchronic test has been described. However, with guppies it is also quite possible to use flow-through systems. Systems described in Section 5.4.3, point 2, for example, are applicable in principle.

In particular, if it is intended to continue the test for a long time the use of flow-through systems is preferable to the semi-static method. If the through-flow rate is made sufficiently high it is even possible to omit aeration (see Note 3), so that the method becomes applicable to a wider range of compounds.

5. Test duration

In this test the guppies are supplied with food, the oxygen concentration is kept sufficiently high by aeration, and the composition of the test medium is kept constant. In principle, the test

5. Determination of toxicity

duration is thus unlimited. For many test compounds, an impression of the subchronic toxicity with mortality as the test criterion can be obtained in about 2 months. At the same time it is then possible to evaluate visually whether the test animals have grown. If the test is continued to 4 to 6 months it is also possible to determine whether reproduction takes place. However, the number of females and the number of young born will usually be too small for quantitative information, so that the conclusions about reproduction are limited to stating whether reproduction takes place at all.

6. Size and maintenance of the test vessels

The test vessels must be regularly cleaned out. For this purpose the guppies are placed in a small volume of the test medium in another vessel, after which the original vessel is thoroughly cleaned. The guppies are returned as quickly as possible to the clean vessel refilled with freshly prepared medium.

If it is intended to continue the test for a long time, the guppies will grow appreciably. It is then also necessary to use larger test vessels after each renewal. For example, 5-litre aquaria may be used after 2 months and 10-litre aquaria after 4 months, with respectively 5 and 10 litres of the medium.

7. Frequency of the non-biological measurements

The pH is measured at each renewal in each test solution where this is considered necessary, both for the freshly prepared and the used solutions.

The oxygen concentration is measured in **each** used solution and in each freshly prepared solution for which this is considered necessary.

If it is possible to determine the concentration of the test compound in the test solutions, this is done at least once a week for all freshly prepared solutions and for the same solutions after use. It is desirable to perform the first analyses at the start of the test.

5.10.8 Test animal *Pleuronectes platessa*

5.10.8.1 Test with mortality, growth, and morphological abnormalities as the criteria

Subject

Determination of the sub-chronic toxicity with the eggs and larvae of the plaice *Pleuronectes platessa* (Linnaeus, 1758) (Pisces, Pleuronectiformes).

Field of application

The method is used in sea water, for pure substances, mixtures of substances, and effluents that do not give such coloration or turbidity at the dilution used that the eggs and the larvae become difficult to see.

Principle

The mortality, growth, and morphological abnormalities of plaice embryos and larvae are used as a measure of the toxicity of a substance added to water. For this purpose fertilized eggs are placed in sea water with various concentrations of the test substance or various dilutions of the test liquid (such as effluent) and are kept in it until some of the emerged larvae in a blank test have reached the stage of metamorphosis, which is after about 2½ months (at 6–8°C). The mortality, growth, and morphological abnormalities during this period are compared with a blank test.

EQUIPMENT AND MATERIALS

- Fertilized eggs of the plaice *P. platessa*, 0 to 2–3 days old (for the collection and fertilization of the eggs, see Note 2)

- climate room, temperature, adjustable between 5 and 10°C, illuminated moderately for about 10 h a day
- test vessels: all-glass aquaria, 2-litre, with suitable glass covers; all-glass aquaria, 5-litre, externally blackened (e.g. covered with self-adhesive black plastic sheets) (see Note 6); suitable glass covers;
or conical flasks, 3-litre, with ground-glass stoppers
- plunger pipettes, 100 µl and 1000 µl, with disposable tips
- measuring pipettes, 1 ml and 10 ml
- measuring cylinders, 100 ml, 1000 ml, and 2000 ml
- conical flasks, 100 ml and 1000 ml, with ground-glass stoppers
- volumetric flasks, 250 ml and 1000 ml
- beakers, 100 ml
- embryo dishes
- glass tubes, length about 15 cm, diameter about 5 mm, sharp edges rounded off, and matching teats
- nylon tea strainers
- aeration tubes, length appropriate to the aquaria, diameter about 3 mm
- aeration apparatus
- polyethylene vessels, 25-litre, for storage of the sea water in the climate chamber
- counting apparatus
- stopwatch
- stereomicroscope, magnification 4–40
- pH meter
- oxygen meter
- salinity meter
- synthetic or natural sea water, salinity $32 \pm 2\text{‰}$ (see Section 5.7.2 j and k, Section 5.7.3, and Section 5.7.4)
- streptomycin-penicillin solution
composition: 500 mg of streptomycin and 300 mg of penicillin dissolved in 20 ml of distilled water
dosage: 2 ml in 1 litre of the test solution
- food: nauplii of *Artemia salina*, one day old (see Note 6).

PROCEDURE

The procedure can be performed only if the instructions in Sections 5.1 to 5.6 are observed.

- Measure the salinity of the sea water in which the collected plaice eggs are fertilized.
- Bring the salinity of a batch of sea water, 100 to 200 litres, to the values just measured. The sea water must have been cooled previously in the climate room to $7 \pm 1^\circ\text{C}$.
Aerate this batch well.
Just before using the sea water check the temperature ($7 \pm 1^\circ\text{C}$), the pH (8.0 ± 0.2), and the oxygen concentration (saturation value).
Make further adjustments if necessary.
- Choose the test vessels (glass aquaria or conical flasks) according to the volatility of the test substance.
- Place the test vessels in the climate room.
- Code the vessels according to the test compound and its concentration or dilution.

5.10 Description of chronic and sub-chronic toxicity test methods

- Prepare the test solutions in 2-litre amounts according to the instructions given in Section 5.2; mix well.
- Pipette 4 ml of the streptomycin-penicillin solution per 2 litres of sea water into each test vessel; mix well.
- Measure and note the pH, the salinity, and the oxygen concentration of the blank and of the most concentrated solution, if necessary also of the other solutions; where necessary, adjust these to the values mentioned above.
- Preferably determine the concentration of the test compound in the prepared solutions.
Take care that the remaining amount of test solution in each test vessel is the same and is still at least 1.5 litres.
- Count out into each test vessel the same number of plaice eggs that were fertilized at the same time. Take 200, 300, or 400 eggs per vessel (see Note 3).
Draw up the eggs from the stock in suitable numbers with the aid of a glass tube provided with a teat. Count the eggs with the counting apparatus when they are present in the tube. Release the eggs under the surface of the liquid in the test vessels. Avoid contamination during the transfer of the eggs.
- Do not aerate.
- Three times a week (preferably on Mondays, Wednesdays, and Fridays) transfer the viable eggs to clean vessels with freshly prepared test solutions to which the streptomycin-penicillin solution mentioned above has also been added. When transferring the eggs, use the glass tube with teat and the nylon tea strainer.
- Check the oxygen concentration, and at least once a week the pH and the salinity, of the blank and of the most concentrated solution at least three times a week, if necessary also of the other concentrations, both for the freshly prepared solutions and for the used solutions.
- At least once a week, both before and after renewal, determine the concentrations of the test compound in the solutions, if the determination was also performed at $t=0$.
- Count and remove dead eggs daily (see Note 3).
- Observe daily about 25 eggs from each vessel in an embryo dish under the stereomicroscope and determine with the aid of 5.10.8, Fig. 1, the embryo's stage of development. Note the results (over-all indication). If in a large part of the observed eggs a striking morphological abnormality (see Note 4) is observed, estimate the percentage of abnormal eggs by counting 50 to 100 eggs.
- From the 8th to 9th day after fertilization to the emergence of the larvae count daily the embryos' heartbeat per minute. Perform the count on 25 eggs of the most concentrated test solution and of the blank and, if a distinct difference is found between the two, also of the other concentrations.
For this purpose transfer the eggs to an embryo dish and examine them under the microscope. Count the heartbeat with the aid of the stopwatch (see Note 5).
- In the period from the hatching of the larvae (on about the 14th–18th day after fertilization) count daily the number of hatched larvae in each vessel. Distinguish them as follows:
 - live, body stretched out
 - live, curled up
 - dead (see Note 3).Also count the number of eggs with living embryos and with dead embryos.
- As soon as about 50 % of the larvae in the blank have hatched, transfer the eggs and the larvae from each test vessel to the 5-litre vessels with freshly prepared solutions.
- Continue the renewal of the test solutions, the physical measurements, and the collection of samples for chemical determinations with the same frequency as reported above.
- Aerate each vessel gently unless the test substance is volatile.
- Count and remove the dead larvae (and eggs) on each occasion during the renewal of the test solutions (see Note 3).
- Always feed the larvae with one-day-old *Artemia* nauplii immediately after the renewal of the test solutions. Begin this at the moment when the yolk sac has been 3/4-absorbed (about seven days after hatching) (see Note 6).
Check every day whether there are sufficient nauplii in the test vessels; add more if necessary.
- Check the uptake of food by the larvae during 2–3 weeks from the beginning of feeding with the nauplii (see Note 6). Quantify the results if distinct differences in food uptake are observed between the larvae in the various test solutions.
- Determine the stage of development of all the larvae twice during the larval period. For this purpose use 5.10.8, Figs. 2–4. Carry this out the first time 3–4 weeks after hatching of the larvae in the blank and repeat the procedure at 6–7 weeks after hatching, i.e. just before the end of the test.
Note the stage of development reached for each larva in each solution. To this end transfer the larvae one by one with the glass tube (with teat) carefully to an embryo dish and observe them under the microscope.
- During the above procedure check whether the larvae show any morphological abnormalities and quantify these if possible (see Note 4).
- End the test when about 50 % of the larvae in the blank have started metamorphosing (5.10.8, Fig. 4, stage 4).

TREATMENT OF THE RESULTS

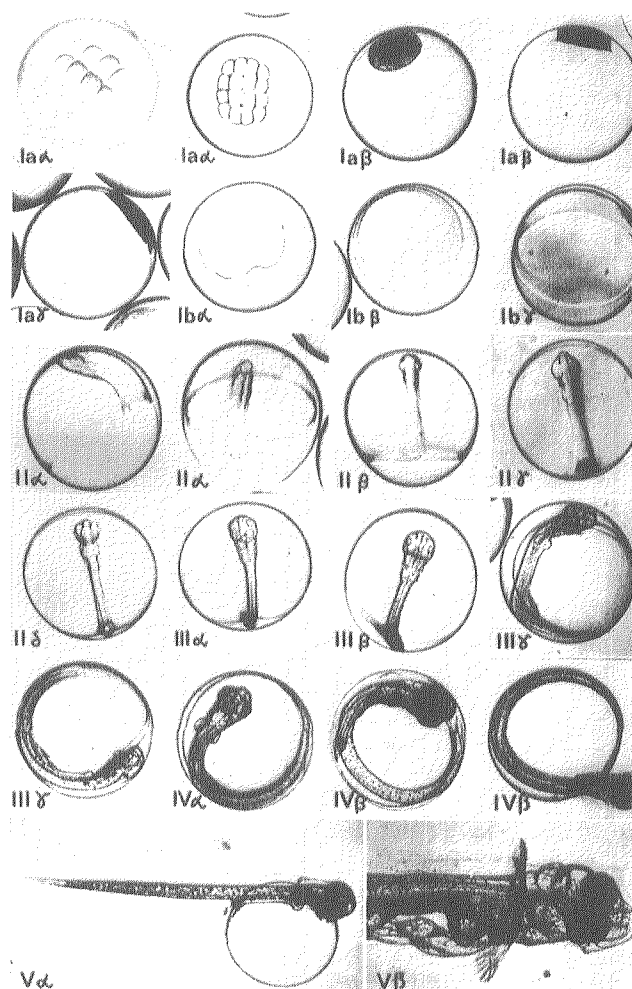
- Tabulate the mean measured pH values, oxygen concentrations, and salinities and their standard deviations. Record any special features in the measurement results.
- Record in a table, in addition to the calculated concentration of the test compound, the averages of the measured concentrations before and after renewal and also their averages if the actual concentrations of the test compound were determined in the solutions.
- Plot the percentage mortality of the eggs and larvae (for each concentration) against time. On the time axis (in days) also indicate the date of fertilization, periods of embryonic development, hatching of the larvae, yolk-sac absorption, and larval development. On each curve give the test concentration to which the curve relates.
- From the mortality results calculate the LC_{50} values for as many observation times as possible and plot these on double-logarithmic graph paper against time.
- Record any abnormalities in the embryos' heartbeat. Tabulate the results or plot the heartbeat per minute against time. On each curve give the corresponding concentration of the test compound.
- Record the results relating to the rate of development of the embryos in the various concentrations of the test compound. If possible, plot the stages of development (from 5.10.8, Fig. 1) against time and for each curve give the test concentration to which it refers.
- Record any morphological abnormalities in the embryos and the concentrations at which they were found.
Give a clear description of the abnormalities observed and, if necessary, illustrate them with a drawing or photograph. Quantify the results as far as possible.

5. Determination of toxicity

- Record the average time of hatching of the larvae for each test concentration separately.
- Record, if possible quantitatively, any observed differences in the uptake of food by the larvae.
- Record any observed differences in the rates of development of the larvae in the various test concentrations.
For both times (2–3 weeks after hatching of the larvae and 6–7 weeks after hatching of the larvae) give a tabular review of the developmental stages reached (according to 5.10.8, Figs. 2–4) and the percentage of larvae that have reached these stages, separately for each test concentration.
- Record, quantitatively if possible, any observed morphological abnormalities of the larvae and the concentrations at which these were found.

STAGES OF DEVELOPMENT OF EGGS AND LARVAE

- Ia α — Ia γ : formation of the embryonic disc
- Ib α — Ib γ : gastrulation
- II α — II δ : embryo covers 180° of the yolk sac
 II α : — primitive stage
 II β : — head and eye buds laid down
 II γ : — first somites, blastopore closed, 'Kupffer buds'
 II δ : — eye lens laid down, first black pigment
- III α — III δ : — embryo covers 180°–270° of the yolk sac
 III α : — eye lenses differentiated, yellow pigment, still no fin-fold
 III β : — pectoral fins laid down, black pigment on the head, caudal fin-fold visible, heart laid down, still not beating
 III γ : — intensified yellow pigment, first spontaneous movement of the embryo, first heartbeats
- IV α — IV β : embryo covers 270°–360° of the yolk sac
 IV α : — broad fin-fold, heartbeat still irregular, intestinal tract visible between the pectoral fins
 IV β : — first eye pigment, regular heartbeat, strong spontaneous movement of the embryo, first larvae hatch
- V α — V β : beginning of larval development (see further 5.10.8, Fig. 2)



5.10.8 Fig. 1 Development of a plaice egg. Time of development from fertilization to hatching of the larvae 15–20 days at 6–8°C (after H. von Westernhagen, 1970)

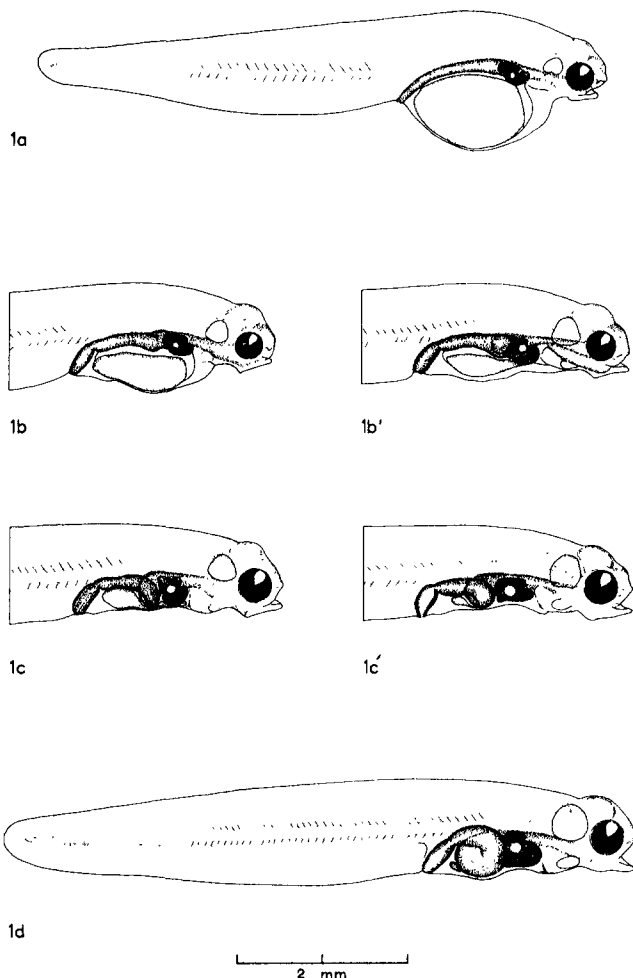
NOTES

1. Conditions for the validity of the test

- By the end of the test, when about 50% of the surviving larvae in the blank are in the stage of metamorphosis, the number of larvae in the blank must still be at least 10% of the original value, with a minimum number of 20 to 30.

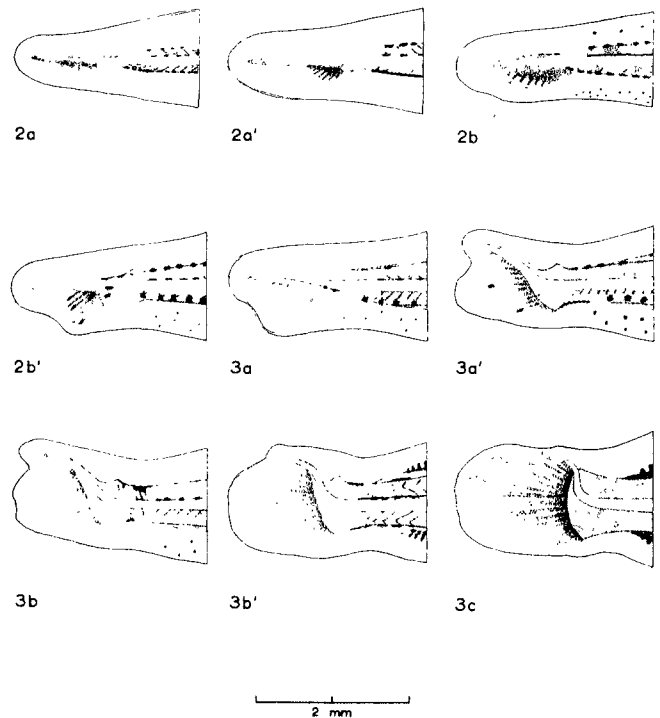
5.10 Description of chronic and sub-chronic toxicity test methods

- The oxygen concentration of the test solutions must have exceeded 70 % of the saturation value in all the test vessels throughout the test, unless the influence of the oxygen concentration is also being determined.
- The pH of the test solutions must have been 8.0 ± 0.2 in all the vessels throughout the test, unless the influence of the pH is also being determined.
- Unless the influence of the salinity is also being determined, the salinity at the time of the test in all the vessels must have been approximately the same (with variations of $\pm 2\%$) as that of the sea water of the spawning area from which the eggs were collected (usually about 33‰).
- The temperature of the test solutions during the egg stage must be $7 \pm 1^\circ\text{C}$ and during the larval stage $8 \pm 2^\circ\text{C}$, unless the influence of the temperature is also being determined. (N.B. the chances of survival of plaice eggs at temperatures higher than 12°C or lower than 2°C are very low).



5.10.8 Fig. 2 Plaice larva, stage 1
Absorption of the yolk sac
Time of development about 15 days at $6-8^\circ\text{C}$

- 1a extended larva, first day after hatching
- 1b-1d absorption of the yolk sac; appearance of a loop in the gut; development of the stomach; in stage 1d the larvae must be self-feeding
(after J.S. Ryland, 1966)



5.10.8 Fig. 3 Plaice larva, stages 2 and 3
Development of the caudal fin
Time of development for each stage separately about 15 days at $6-8^\circ\text{C}$

- Stage 2: 2a-2b' notochord straight, first development of fin rays, margin of fin-fold extending
- Stage 3: 3a-3c caudal extremity of the notochord turns up, development of fin rays; angle between the notochord and the body axis:
3a and 3a' : $< 45^\circ$
3b and 3b' : $> 45^\circ$, $< 90^\circ$
3c : approx. 90° , fin rays completely developed
(after J.S. Ryland, 1966)

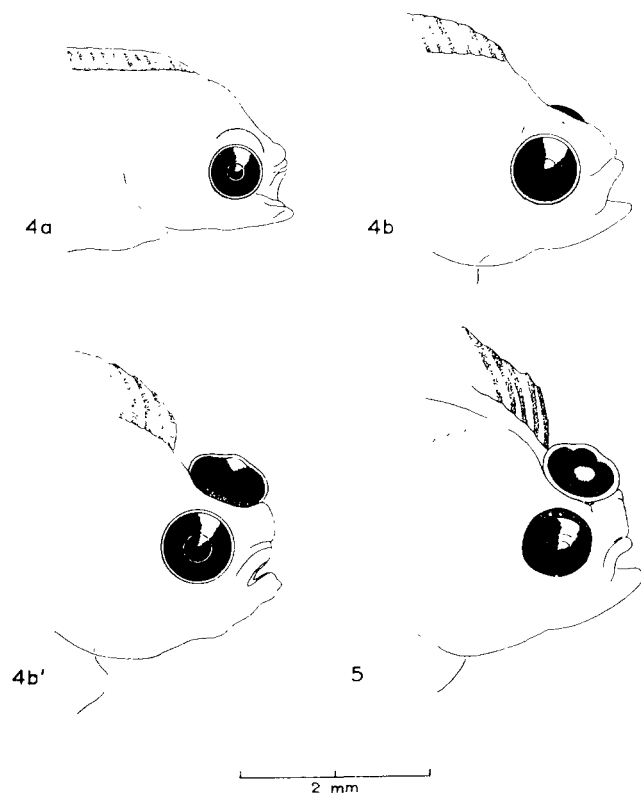
2. Collection and artificial fertilization of plaice eggs; determination of the percentage fertilization

The plaice spawns in the southern North Sea in winter at a water temperature of $4-8^\circ\text{C}$.

In order to have eggs that were all fertilized at the same time, sexually mature plaice must be caught and their eggs and sperm be stripped, after which artificial fertilization must take place immediately. The most suitable period for catching suitable plaice is between the middle of January and the end of February; North Sea plaice recognize a number of fairly limited spawning grounds and must be caught in these areas.

Immediately after the catch has been brought on board, it must be determined whether among the caught plaice there are some with eggs ripe for fertilization.

With the hand, exert a gentle pressure on the abdomen of the fish and stroke gently in the direction of the anal opening. If the eggs are ripe they will emerge under slight pressure and be light and transparent. Collect the eggs in a plastic dish. Next press out from one or more male specimens a few drops of milt and add this directly to the eggs. Add 10-20 ml of sea water and mix the eggs and sperm well (use sea water collected *in situ* and take care that its temperature does not differ too much from the prevailing sea temperature).



5.10.8 Fig. 4 Plaice larva, stages 4 and 5
Metamorphosis

Time of development for each stage separately about 15 days at 6–8°C

stage 4: 4a–4b' the position of the eyes becomes asymmetric: the left eye migrates over the head in the direction of the right eye; the typical flatfish shape appears

stage 5: 5 metamorphosis complete; dark pigmentation of the 'back'
(after J. S. Ryland, 1966)

After 5 min add a few hundred ml of sea water and filter the eggs off on a fine-mesh sieve (e.g. a tea strainer). Rinse the eggs three times in a large amount of sea water and filter them again to get rid of the sperm. Transfer the eggs to 1- to 2-litre glass bottles with perforated tops, filled with sea water. The eggs will float unless they have not been fertilized. Store the bottles with the eggs in a refrigerator, or on deck if the temperature there coincides with the prevailing sea temperature (usually 4–8°C). The eggs should be transported quickly to the laboratory, so that the toxicity test can begin at the earliest possible stage of embryonic development.

Although one female plaice can supply ten thousand eggs, it is advisable to strip the eggs from several specimens and to fertilize them. Keep the batches of eggs from different females separate from one another.

An impression of the quality of the egg batches collected can already be obtained on board by determining the percentage fertilization. Unfertilized eggs are opaque white and sink to the bottom, while fertilized eggs float on the surface (if the salinity of the water is about 33‰).

By counting out a small proportion, e.g. twice 100 to 200 eggs,

from a batch of eggs (derived from one plaice) immediately after fertilization in separate bottles, and evaluating each bottle a few hours after fertilization, the percentage fertilization can be calculated as follows:

$$F = \frac{A - B}{A} \cdot 100\%$$

in which

A = total number of eggs in the bottle

B = number of eggs that have sunk to the bottom.

Batches of eggs with a percentage fertilization smaller than 50% are (probably) less suitable for toxicity tests. If bottles with different concentrations of the test solution(s) are available (e.g. bottles prepared beforehand in the laboratory or on board), the effect of the test compound on fertilization can be determined in the same manner as that given above for unexposed eggs.

3. Mortality

The sensitive periods during the embryonic and larval development of the plaice are the pre-gastrula stage (preceding stage Iba in 5.10.8, Fig. 1), the period in which the larvae hatch (stage IVβ–Vβ in 5.10.8, Fig. 1); the period in which the larvae must become self-feeding (stages 1c and 1d and stages 2a and 2a' in 5.10.8, Figs. 2 and 3, respectively), and the period during which the larvae undergo metamorphosis (stages 4a, 4b, and 4b' in 5.10.8, Fig. 4). Mortality among eggs and larvae occurs particularly during the above-mentioned periods. During the period in which the larvae must become self-feeding, i.e. at the end of absorption of the yolk sac, percentage mortalities as high as 50–70% may occur in the blank.

For this reason the test must be started with a large number of eggs (at least 200 per test vessel), so that at the end of the test, during metamorphosis, sufficient larvae are still present to make the biological observations meaningful.

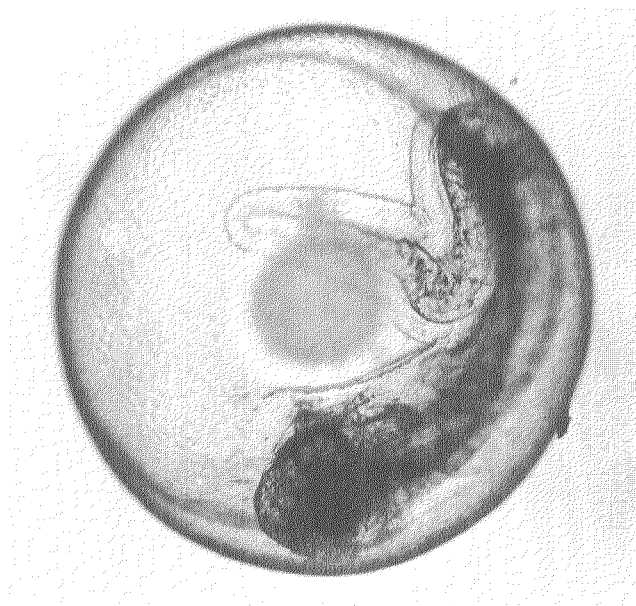
Dead eggs are easy to recognize since they become white and opaque and sink to the bottom, in contrast to the viable eggs, which float on the surface. Dead larvae also become white and opaque and sink to the bottom; one can also take immobility after stimulation as a characteristic of death for larvae. (N.B. during metamorphosis live larvae frequently stay on the bottom for some time).

4. Morphological abnormalities

The normal development of plaice embryos and larvae has been briefly outlined (5.10.8.1, point 4). Embryos with abnormal development can be detected in any batch of eggs of sufficient size. If abnormalities are found in embryos or larvae that have been exposed to a toxic compound, it is always necessary to investigate whether, and to what extent, this abnormality also occurs in unexposed embryos and larvae (blank).

Some morphological abnormalities that often occur, whether or not in consequence of exposure to a toxic compound, are, briefly:

- irregular cell division in the formation of blastomeres;
- the formation of blastomeres takes place normally, but the formation of the embryonic disc is irregular, with loose rounded cells;
- deformation of the spinal column in the caudal region; this frequently arises during the early development of the heartbeat (see 5.10.8, Fig. 5);
- swollen yolk sac (for these and the above-mentioned abnormalities, see von Westernhagen, 1970);
- abnormal absorption of the yolk sac, leading to curvature in the spinal column directly behind the head (axial bending) (Shelbourne, 1956).



5.10.8 Fig. 5 Embryo of *Pleuronectes platessa*
Deformation of the spinal column in the caudal region
(magnification $\times 40$)

In this connection it should be noted that the above-mentioned abnormalities may also occur as a result of stress factors other than the presence of a toxic compound, e.g. at a low oxygen content or a low salinity.

In general, it will be found that embryos and larvae with the above-mentioned morphological abnormalities are not viable. Depending on their type, the malformations may rapidly (but occasionally only after some time) lead to death. Irregularities in cell divisions in the early embryonic stage may cause the embryos to die in the pre-gastrula stage.

Deformations of the spinal column that arise in the formation of the caudal region may lead to failure of the larvae to hatch. If they do hatch, such larvae are generally not viable since they cannot swim normally and therefore, in a later stage, are partly or wholly incapable of catching food. Larvae with a swollen yolk sac have difficulties in leaving the egg; frequently the egg sac remains stuck in the chorion while the head and the tail project. Further abnormalities of morphological and histological nature during the growth of plaice larvae have been reported by Ehrlich *et al.* (1976).

The sub-lethal effects, including morphological abnormalities, that may occur during the embryonic and larval development of marine fish have been reviewed by Rosenthal and Alderdice (1976).

5. Development of the heartbeat

The heart is laid down in stage III β (see 5.10.8, Fig. 1), and the first, still very slow and irregular, heartbeat can be detected in stage III γ .

The heartbeat increases rapidly during further development, to reach 70 to 90 beats per minute just before the hatching of the larvae. Serious deviations from the normal development pattern of the heartbeat may be due to serious deformations of

morphological nature, such as those of the spinal column in the caudal region.

6. Uptake of food by the larvae

One-day nauplii of the brine shrimp *Artemia salina* are a suitable food for plaice larvae.

The number of nauplii consumed per plaice larva depends on the latter's stage of development.

The review given below, taken from Riley (1966) (see also Wyatt, 1972), gives an indication of the consumption of food (figures rounded off):

Stage of development	Nauplii/plaice larva day ^{*)}
1. 5.10.8 Fig. 2	0 $\rightarrow$ 10
2. 5.10.8 Fig. 3	10 $\rightarrow$ 100
3. 5.10.8 Fig. 3	100 $\rightarrow$ 200
4. 5.10.8 Fig. 4	200 $\rightarrow$ 50
5. 5.10.8 Fig. 5	50 $\rightarrow$ 200

^{*)} The food consumption increases during the given stage of development.

During the process of metamorphosis, however, the uptake of food decreases.

Shelborne *et al.* (1963) have shown that plaice larvae can see the nauplii better against a dark background, which stimulates the uptake of food. For this reason, it may be recommended to blacken the (external) walls of the aquaria e.g. by covering them with self-adhesive plastic sheets.

Plaice larvae may be fed with artificially prepared food particles, but in the present stage of research the chances of survival of the larvae are then lower than when the nauplii of *Artemia salina* are supplied.

During the 2 to 3 weeks immediately following the first feeding of the larvae with *Artemia* nauplii it is possible to obtain an impression of the food uptake (food acceptance) by counting daily, or at each renewal of the test solutions, the number of larvae in each vessel that have or have not taken up food. The gut of the larvae that have taken food is then red-brown, which can be seen with the naked eye.

Although one-day old nauplii of *Artemia salina* can if necessary be obtained from one's own culture (see Section 5.8.6), because of the large number required it is advisable to obtain artemia eggs and suitable breeding vessels commercially.

Since the quality of different egg batches is very variable, it is recommended to determine the quality of a purchased batch before it is used as food by investigating what percentage of the eggs hatch and what incubation time is necessary for this purpose.

7. Recommendation

It is recommended to test a reference substance at the same time. The result with the reference compound must then be in reasonable agreement with previously obtained results.

8. Applicability of the test

The application of the described test is limited because plaice eggs are available only for a short period; for the southern North Sea this period falls between the middle of January and the end of February.

It is advisable to collect a large batch of eggs (10^4 to 10^5), so that several compounds important from the aspect of marine pollution can be tested simultaneously for their toxicity to plaice eggs and larvae.

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CHAPTER 6

Determination of bio-accumulation and bio-elimination

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with contributions from

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6.1 INTRODUCTION

Accumulation and elimination studies in the aquatic environment are performed to obtain an idea of the distribution of xenobiotic substances between the aqueous phase, the particles suspended in it (including microorganisms), the plants and animals living in it, and any sediments that may be present.

Most of the research is carried out with animals (crustaceans, shellfish, and fish) since, apart from the health of the ecosystem, the concentration of xenobiotic substances in animals destined for human consumption is important for human health.

The uptake of substances by living organisms from, and the elimination into, the aqueous phase can be caused by:

- Passive adsorption, desorption, and diffusion processes, in which accumulation and elimination depend on the partition coefficients of the substance between the water and the biological tissues. This also takes place with non-living particles and dead organisms.
- Active transport and excretion processes, possibly combined with metabolism of the substance.
- Transport via food.

In practice these processes take place simultaneously and are not always easy to distinguish experimentally. For the final result, i.e. the determination of a certain amount of a substance in an organism or in a part of it, it is not of paramount importance how this amount has arisen. On the other hand, it is, of course, important for an understanding of the processes that have led to the presence of such definite amounts. In the case of microorganisms, accumulation and elimination are thought to be determined mainly by passive adsorption, desorption, and diffusion, since they have a large surface area in relation to volume. The time necessary for the establishment of equilibria between the aqueous phase and the microorganisms is generally very short, of the order of a few seconds or minutes, which confirms the above assumption.

In the case of higher animals, uptake from the water takes place mainly through the gills. Both passive diffusion and active uptake may occur. Transport to other tissues can take place via blood, lymph, or directly through membranes. It may also take place as the result of passive or active processes. Excretion can take place by diffusion to the surrounding water or by active transport, usually – in the case of apolar substances – after conversion of the original compound into a more polar compound and/or through conjugation reactions.

By far the greatest amount of work relating to active transport processes, metabolism, and excretion processes has been carried out with mammals. Little is so far known with certainty concerning its significance for aquatic organisms. A further discussion is given by Walker (1975).

In the case of aquatic animals the direct uptake of xenobiotic substances from the water is presumably by far the most important factor in accumulation, while for land animals these substances are taken up mainly with the food.

By **accumulation** we shall understand, in this context, the net increase in the concentration (C) of the test compound in the test organisms. This is the amount that is **taken up** minus the amount that is simultaneously **eliminated** at a constant concentration (C_w) of the test compound in the water surrounding the test organisms.

By **elimination** we shall understand the reduction of the concentration of the test compound in the test animals.

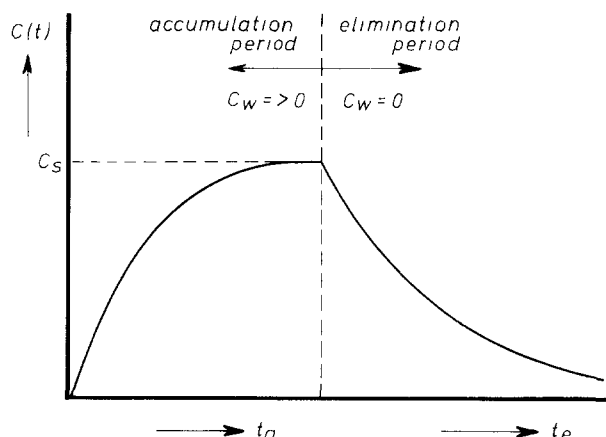
A schematic accumulation and elimination curve of a type that frequently occurs is given in 6.1, Fig. 1.

For the simplest conceivable case – that of the organism regarded as consisting of one compartment – we shall illustrate further how the shape of the accumulation – elimination curve is produced.

Theory of a one-compartment model (Moriarty, 1975)

If an organism is regarded as being made up of one compartment, e.g. as a sphere without differentiation into various tissues, and

it is assumed that no metabolism takes place, the accumulation and elimination process can be described mathematically in a simple manner. Although these assumptions are seldom valid in practice, a mathematical description of this case does give points of reference for the evaluation of accumulation and elimination curves encountered in practice.



6.1 Fig. 1 Schematic representation of an accumulation-elimination curve. $C(t)$ is the concentration of the test compound in the test animals at time t

The elimination process

Assumptions:

The organism consists of one compartment; it contains a certain amount Q of the test compound; it is placed in a relatively large volume of water not containing the test compound; it excretes the test compound without metabolism taking place; the rate of excretion (from the organism into the water) is directly proportional to Q .

Under these assumptions we therefore have:

$$\frac{dQ(t_e)}{dt_e} = -kQ(t_e) \quad (1)$$

in which t_e = elimination time, $Q(t_e)$ = the amount of test compound at time t_e , and k = rate constant of the excretion of the compound.

Integration of eq. (1) gives:

$$Q(t_e) = Q(0)e^{-kt_e} \quad (2)$$

in which $Q(0)$ is the test compound at time $t_e = 0$.

We can write eq. (2) in a more practical form as:

$$\log_{10} Q(t_e) = \log_{10} Q(0) - 0.4343 kt_e \quad (3)$$

If the above assumptions apply, the graph of $\log_{10} Q(t_e)$ against t_e will be a straight line with a slope of $-0.4343 k$.

Only in this case do we speak of a half-life $t_{e\frac{1}{2}} = 0.693/k$.

When the graph of $\log_{10} Q(t_e)$ against t_e is not linear, one of the assumptions is invalid and we can no longer speak of $t_{e\frac{1}{2}}$. Instead we speak of the empirical parameters t_{e50} and t_{e90} , times at which Q has fallen to respectively 50 % and 10 % (6.1, Table 1).

If we were sure that the change took place according to one power of e (see (2)), then from the change in Q over short periods it would be possible to **calculate** after how long Q would reach a given value. However, we must expressly warn against this procedure. Frequently the elimination takes place exponentially only during the first few days, after which it becomes slower, possibly following an exponential law with a different time constant, or sometimes it even stops completely at a certain concentration of the substance in the organism.

6. Determination of bio-accumulation and bio-elimination

There is a chance of exponential elimination curves in **higher organisms**, *inter alia* in the case of substances with such small molecules that the boundaries between compartments (e.g. cell membranes) present no hindrance, so that the molecules can move freely through the whole organism (e.g. lower chlorinated hydrocarbons).

The accumulation process

The accumulation can also be described by means of the simple one-compartment model and the other assumptions already made for the case of elimination.

We are now working with the accumulation time t_a and the associated amounts $Q(t_a)$.

We assume that $Q(0) = 0$, that the test compound enters at a constant rate R , and that the flow outwards is $kQ(t_a)$ (see (1)). In the steady state the two flows are equal ($R = kQ$). The concentration of the test compound is then called Q_s .

In the general case we have:

$$\frac{dQ(t_a)}{dt_a} = R - kQ(t_a) \quad (4)$$

from which it follows that:

$$Q(t_a) = \frac{R}{k} (1 - e^{-kt_a}) - Q(0)e^{-kt_a} \quad (5)$$

With $R/k = Q_s$ and $Q(0) = 0$ we can rewrite this as:

$$Q(t_a) = Q_s (1 - e^{-kt_a}) \quad (6)$$

After a long time we can neglect e^{-kt} in comparison with unity, and $Q(t_a)$ then approaches its stationary value Q_s . In a graph of $Q(t_a)$ against t_a the curve rises at small values of t_a with a slope R , after which it begins to bend over, finally reaching, at large values of t_a , the plateau value Q_s , where k is given by the relationship $k = R/Q_s$.

The above formulae apply only under a number of assumptions for a one-compartment model. When some assumptions are made wider, in certain cases the formulae can be used more easily while still retaining the model; for example, if $Q(0) \neq 0$.

Metabolic conversion may also be taken into account if the rate of conversion is proportional to $Q(t)$.

The most important cause of deviations is, however, invalidity of the one-compartment model.

Moriarty (1975) has also considered multi-compartmental models. A general problem in attempts to describe the experimental accumulation and elimination curves mathematically and to base conclusions on them results from the number of observations, which – in view of the wide scatter – is usually too small. Moreover, the test duration and the number of samples collected during the test are often too small to determine the plateau value with any certainty.

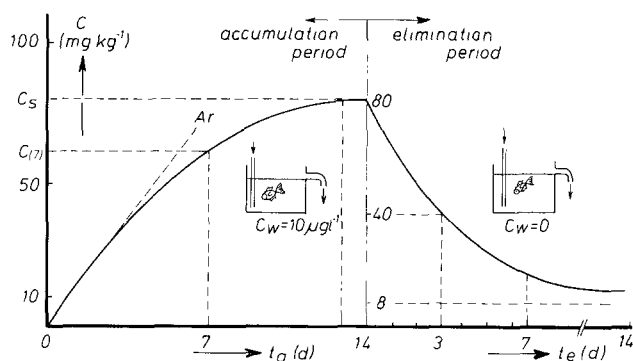
In many cases we must be satisfied with drawing curves fitting the experimental values as well as possible. To allow a mutual comparison of tests, a number of parameters that can be read off from these curves may be given in tabular form. These parameters, with the symbols used for them here and their meaning, are given in 6.1, Table 1; their use is illustrated in 6.1, Fig. 2.

6.1 Table 1 Symbols and units in accumulation and elimination studies

Symbols	Meaning	Unit (example)	Remarks
t	time	days	
t_a	accumulation time	days	
t_e	elimination time	days	
t_p	minimum time during the accumulation period necessary to approach the plateau value within the error of the test	days	
t_{e50}	elimination time necessary to reach the value $C(t_e) = \frac{1}{2}C(t_e = 0)$	days	
t_{e90}	elimination time necessary to reach the value $C(t_e) = 1/10 C(t_e \pm 0)$	days	
$t_{e\frac{1}{2}}$	half-life	days	only applicable if formula (3) is valid
C_w	concentration of the test compound in the water of the test vessel	$\mu\text{g} \cdot \text{l}^{-1}$	
$\bar{C}_w$	ditto, averaged over the test time	$\mu\text{g} \cdot \text{l}^{-1}$	
$C(t)$	concentration of the test compound in the organisms after a time t	$\mu\text{g} \cdot \text{kg}^{-1}$	dependent on t and $\bar{C}_w$
C_s	concentration of the test compound in the organisms at the plateau of the accumulation curve (steady state)	$\mu\text{g} \cdot \text{kg}^{-1}$	independent of t_a ; dependent on $\bar{C}_w$
A_r	initial rate of accumulation $\frac{dC}{dt} (t=0)$	$\mu\text{g} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$	dependent on $\bar{C}_w$
T_w	water transfer coefficient: $A_r / \bar{C}_w$	$\frac{\mu\text{g} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}}{\mu\text{g} \cdot \text{l}^{-1}}$	this is frequently found to be independent of C_w
$C_f(t_a)$	concentration factor after t_a days of accumulation. $C(t_a) / \bar{C}_w$	day^{-1}	dependent on t_a
A_f	accumulation factor at the plateau of the accumulation curve: $C_s / \bar{C}_w$		independent of t_a

6.1 Introduction

Because of the very specific nature of tests with microorganisms, these are described separately in Section 6.2. Section 6.3 contains the methods for animals with a wet weight of 0.5–5 g per individual. Modifications for working with larger or smaller animals are also explained briefly.



6.1 Fig. 2 Use of the parameters given in 6.1, Table 1

$$t_p = 13 \text{ days}$$

$$C_s = 80 \text{ mg kg}^{-1}$$

$$A_r = \frac{C_s}{C_w} = \frac{80 \text{ mg kg}^{-1}}{10 \mu\text{g l}^{-1}} = 8000$$

$$A_r = \left(\frac{dC}{dt} \right)_{t=0} = 10.7 \text{ mg kg}^{-1} \text{ day}^{-1}$$

$$C_7 = 62 \text{ mg kg}^{-1}$$

$$C_{f(7)} = \frac{62 \text{ mg kg}^{-1}}{10 \mu\text{g l}^{-1}} = 6200$$

$$T_w = \frac{A_r}{C_w} = 1070 \frac{\mu\text{g kg}^{-1} \text{ day}^{-1}}{\mu\text{g l}^{-1}}$$

$$t_{e50} = 3 \text{ days}$$

$$t_{e90} > 14 \text{ days}$$

$$t_{1/2} = \text{not relevant}$$

6.2 DETERMINATION OF THE ACCUMULATION AND ELIMINATION OF XENOBIOTIC SUBSTANCES BY MICROORGANISMS WITH THE AID OF ^{14}C -LABELLED TEST COMPOUNDS

6.2.1 General remarks on the test method

Accumulation and elimination of xenobiotic organic compounds in microorganisms are important for distribution of xenobiotic substances in the aquatic environment.

The xenobiotic substances accumulating in microorganisms can be transported via the microorganisms to the sediment or via 'filter feeders' through the food chain (Johnson and Kennedy, 1973; Paris and Lewis, 1976; and Section 6.1).

The principles of measuring the accumulation and elimination of xenobiotic substances in the laboratory are described in Section 6.1. Many of these principles can also be applied to microorganisms, but there is a number of differences in detail in the performance of the experiments with microorganisms:

- It is difficult to collect sufficient cell material from cultures to perform an extraction for an ordinary chemical analysis of the test compound. Consequently, radioactively labelled test compounds are used in the described test. The number of steps in the analytical procedure is minimized as far as possible to avoid losses of the test compound (see Note 1).
- Co-distillation with the evaporating water is very important in these tests and occurs frequently with the apolar, sparingly water-soluble, substances; yet these are precisely the ones of interest in accumulation studies. In tests with microorganisms this effect is more important, since the volume of microorganisms is relatively small in relation to the volume of water used in the test (Lichtenstein *et al.*, 1968; Mackay and Wolkoff, 1973; Oloffs *et al.*, 1973).
- The above considerations also determine the choice of methods for the separation of microorganisms and the test medium (filtration or centrifugation). Although filtration is easy (others have investigated this: Södergren, 1968 and 1971; Urey *et al.*, 1976; Wheeler, 1970), centrifugation is used in the procedure in this manual because the test solution is then less exposed to the air.
- The choice of the test organisms must be adapted to the problem in question. Since microorganisms show wide differences in their uptake capacity (Cox, 1970; Johnson and Kennedy, 1973; Kokke, 1970; Rice and Sikka, 1973; Trudgill and Bowering, 1972), no 'standard' organism can be specified. For algae, Lill Lund (1974) states that at least two organisms must be used, one of which has lipids as reserve material. The technique used to measure the accumulation, in contrast to the method of culture, is the same for all microorganisms.
- Both the accumulation and the elimination of apolar compounds usually take place very rapidly (Paris and Lewis, 1976; Rice and Sikka, 1973), so that short-term experiments are possible. The establishment of an equilibrium in less than 15 min has been reported. Publications that give a longer time for the establishment of equilibrium probably relate to methods with high losses of the test compound (Reinert, 1972). The equilibrium concentration reached in the organisms is usually proportional to the concentration in water. The equilibrium concentration may also depend on the amount of biomass in the test system (Cox, 1970; Johnson and Kennedy, 1973; Lill Lund and Wehrmann, 1974; Paris and Lewis, 1976; Rice and Sikka, 1973). The frequently passive nature of the uptake process is shown by the same or greater uptake in dead organisms as compared with live organisms (Johnson and Kennedy, 1973; Ko and Lockwood, 1968; Paris and Lewis, 1976; Chacko and Lockwood, 1967). Consequently, the study must be performed with different concentrations both of the biomass and of the test compound.

- In the described method the calculation of the accumulation factor for microorganisms is based on their dry weight. For a comparison with results obtained in experiments with higher organisms the wet weight is necessary (Lill Lund, 1974). The wet weight of microorganisms is difficult to determine; various methods are used for this purpose (Lill Lund and Wehrmann, 1974; Södergren, 1968 and 1971).

6.2.2 Description of the method

Subject

Determination of the uptake and the possible subsequent release of xenobiotic substances by microorganisms.

Field of application

The method can be used for the study of the accumulation and elimination of compounds available in radioactively labelled form.

Principle

An amount of the labelled test compound is added to a culture of microorganisms. After incubation, the microorganisms are separated from the test medium and their radioactivity is determined. The release of the substance is determined by transferring microorganisms previously incubated with the labelled test compound into clean medium and, after incubation, determining the residual radioactivity in the microorganisms.

EQUIPMENT AND MATERIALS

- A laboratory suitable for work with radioisotopes
- algal culture (see Section 5.8.2)
- algal media (see Section 5.8.2)
- incubation apparatus for algae (see Section 5.8.2)
- bacterial cultures and media (see Section 3.5.1.1)
- phosphate buffer, pH 7.0; 0.02 M
- sterile filtered synthetic sea water
- conical flasks, 300 ml, with glass stoppers
- measuring cylinder, 100 ml
- volumetric flasks, 250, 500, and 1000 ml
- centrifuge, preferably equipped with an enclosed head
- glass or steel centrifuge tubes, with covers, capacity 100 ml
- spatula
- absorbent ash-free filter paper
- acetone
- dimethyl sulphoxide (DMSO), spectroscopically pure
- scintillation liquid (Aquasol) (see Sections 2.6 and 2.7.3)
- scintillation counter (see Section 2.6, Note 4)
- combustion apparatus for radioactive samples
- fume cupboards
- plastic trough 50 × 50 cm
- rubber surgical gloves
- monitor to measure ^{14}C -activity
- scintillation vials.

PROCEDURE

- It is assumed that the volatility and solubility of the test compound are known (Sections 1.4.1 and 1.4.2).
- The work is performed with observation of the instructions for handling radioactive substances (Note 2).
- Carry out all operations with radioactive substances in a fume cupboard in a low plastic trough with absorbent paper.
- Preferably use surgical gloves and take care that radioactively contaminated waste (paper, pipette tips, scintillation liquid, radioactive solutions, etc.) and glassware are removed or washed in accordance with the rules laid down in the particular laboratory.
- Avoid contamination of equipment or test vessels by always using clean glassware or pipettes or by always working from low to high concentrations.
- Use a monitor to check the working premises and the test equipment for contamination (Note 3).

1. Preparation of the test solutions

- Dissolve the inactive test compound, preferably in distilled water or in DMSO, depending on its solubility in water.
- Choose as the highest test concentration one at which toxic effects just do not arise and prepare such dilutions that the concentrations form a geometric series with a factor of about 3.2.
- Prepare the dilutions of the inactive test compound as described in Sections 3.5.1.1 and 5.10.1.
- Dissolve the ^{14}C -labelled test compound in water or acetone, depending on its solubility in water. This stock solution is kept as concentrated as possible and is stored deep-frozen in a suitable place (Note 4).
- From this stock solution prepare a dilution of the ^{14}C -labelled test compound such that it contains about $1\ \mu\text{Ci}$ of ^{14}C -activity per ml in a volume of about 10 ml.
- Store this solution at -20°C until the moment of use.

2. Preparation of organism suspensions

- Choose the test organisms and methods to culture the algae according to Section 5.8.2 and for bacteria according to Section 3.5.1.1.
- Culture the microorganisms according to the instructions and use a culture in the exponential growth phase for the test.
- Centrifuge the suspension of organisms for 20 min at 6000 g ; suspend fresh-water algae and bacteria in a phosphate buffer (0.02 M; pH 7.0) and marine algae in filtered synthetic sea water (Section 5.2.1).
- Adjust algal suspensions to $3 \cdot 10^6$ cells $\cdot\text{ml}^{-1}$ and bacterial suspensions to an extinction $E=0.8$ (Note 5).
- Determine the dry weight of the suspension by filtering 2×10 ml through membrane filters of known weight.
- Determine the weights of the filters before filtration after an hour at 105°C and after filtration after drying for 24 h at 105°C and cooling in a desiccator.
- Prepare dilutions of the suspensions in buffer or sea water to algal concentrations of $3 \cdot 10^6$, 10^6 , $3 \cdot 10^5$, and 10^5 cells $\cdot\text{ml}^{-1}$, and bacterial suspensions with extinctions of 0.8, 0.4, 0.2, and 0.1.

3. Performance of the test

- Test four concentrations of the test compound in duplicate at each cell density.
- Place 100-ml portions of the suspensions of algae or bacteria

with different cell densities into 300-ml conical flasks (with glass stoppers).

- Add 1 ml of the aqueous solution of the inactive test compound or 0.1 ml of the DMSO solution to the flasks (Note 6).
- For the highest concentration of cells and test compound fill two extra flasks, which are used to study the elimination of the test compound.
- After this, immediately add to each flask $0.1\ \mu\text{Ci}$ (0.1 ml) of the above-mentioned solution of the radioactively-labelled test compound (Note 7).
- Insert glass stoppers (lubricated with water or glycerol).
- Incubate the conical flasks for 4 h at 20°C with slow shaking (Note 8).
- After incubation, take a 1-ml sample from each flask and add the sample to 15 ml of Aquasol in a scintillation bottle; determine the activity present. The total measured activity is denoted by t (dpm/100 ml).
- Pour the suspension into centrifuge tubes, close the tubes, and centrifuge for 20 min at 6000 g and 20°C .
- Pour off the supernatant and determine the radioactivity present in the supernatant (denoted by s).
- Resuspend in buffer or sea water and repeat the above operations.
- With a spatula and a piece of ash-free absorbent filter paper take the algae or the bacterial mass from the centrifuge tube and transfer them to scintillation bottles containing 15 ml of scintillation liquid or, if the amount of biomass is too large, burn the filter in an appropriate apparatus (Note 9).
- Determine the activity in the algae (denoted by p).
- Rinse the centrifuge tubes with 5 ml of acetone and determine its radioactivity (denoted by q) (Note 10).
- Treat the contents of the two extra flasks as described above. Immediately after the second centrifugation, suspend the organisms in 100 ml of buffer or sea water and incubate for 30 minutes.
- Centrifuge the suspensions, pour off the supernatant, and determine the radioactivities in the supernatant ($=r$) and in the algae ($=p_1$) as described above.

CALCULATION OF THE RESULTS

The counts are performed in such a way that the measurement error is below 3%. Tabulate the counts according to the following scheme (dpm/100 ml):

t = total measured activity

s = activity in the supernatant after centrifuging

p = activity in the microorganisms (p_1 after elimination)

q = activity in acetone after rinsing the tube

r = activity in the rinsing water in the elimination test.

In the ideal case $t - (s + p + q)$ should be zero. If the difference turns out to be positive, this means a loss of the test compound in the course of the test. Check the materials balance for all test flasks.

With the aid of the specific activity of the test compound, $\text{nCi}\cdot\text{ng}^{-1}$, the amount of this compound taken up per mg of dry weight of the microorganisms is calculated.

Plot the concentration in the organisms as a function of the concentration in the water and as a function of the amount of biomass.

An accumulation factor A_r can be calculated for each measurement (see Section 6.1):

6. Determination of bio-accumulation and bio-elimination

$$A_t = \frac{\text{ng of the test compound (mg biomass dry weight)}^{-1}}{\text{ng of the test compound (mg water)}^{-1}}$$

For an approximation of the wet weight of the biomass, the dry weight may be multiplied by a factor of 5 (after Lillelund and Wehrmann, 1974).

When the plot yields a reasonably straight line passing through the origin, its slope can be correlated with the accumulation factors mentioned.

NOTES

1. When a sufficiently accurate conventional method of chemical analysis is available, there is obviously no need to use radioactively labelled compounds. This applies particularly to the uptake of inorganic compounds (e.g. mercury and copper). When work is carried out with isotopes other than ^{14}C (e.g. metals), a suitable method of determination must naturally be available.
2. The instructions assume that the investigator is familiar with the handling of radioactive compounds.
In most countries, a licence is necessary for work with radioactive substances, which can be requested from the appropriate authorities.
The authorities also give information on the general rules and regulations that apply to this type of work.
A good review of the procedures for radioactivity determination, especially in relation to liquid scintillation counting in biological research, has been given by Price (1973a, b, c, d, e, f, g).
3. In these procedures it is assumed that without special provisions it is possible to work in laboratories only with $10\mu\text{Ci}$ of activity at a time.
4. Volatile or apolar test compounds are stored in sealed ampoules. Polar organic compounds are stored at such pH that the substance is present so far as possible in the dissociated form.
5. The cell density of the cultures is adjusted by determining the number of cells with a Coulter Counter (Section 2.7.6) or with a spectrophotometer (Section 3.5.1.1) and then diluting or concentrating to the required value.
6. DMSO must have no inhibiting effect on the culture of organisms (see Section 5.10.1.2).
7. In the performance of the tests one is restricted to a definite test concentration, and when a certain amount of radioactivity is to be added, this is best done by adding inactive and active substrates separately, which is possible only if the specific activity of the substrate is so high that the added amount of radioactive substrate scarcely affects the substrate concentration. If the specific activity of the substrate is too low, only radioactive substrate must be added and the amount must be calculated on the basis of the specific activity.
8. The time of incubation has been set at 4 h for practical reasons. If the rate of the uptake of a test compound is unknown, an uptake curve must be plotted at the highest concentration by measuring the concentration in the organisms and determining the time necessary to reach the equilibrium concentration. This is then used as the incubation time.
9. Direct counting in Aquasol can be done only if the amount of biomass is not too great. If it is too great, it is better to burn the sample, trap the CO_2 , and measure the $^{14}\text{CO}_2$ in it. *The combustion must be carried out in an appropriate apparatus (e.g. the Packard Sample Oxidizer).*
10. In the case of apolar test compounds acetone is used as the rinsing agent. Otherwise, the rinsing agent must be adapted to the chemical properties of the test compound.

6.3 DETERMINATION OF THE ACCUMULATION AND ELIMINATION OF XENOBIOTIC SUBSTANCES BY MOLLUSCS, CRUSTACEANS, AND FISH

6.3.1 Principle of the determination

The method described in Section 6.3.8 is intended for the determination of the accumulation of xenobiotic substances from the surrounding water and of their elimination back into this water by animals measuring a few cm. (For studies with microorganisms see Section 6.2, for studies with smaller animals see Section 6.3.9.1, and for studies with larger animals see Section 6.3.9.2).

In order to determine the accumulation, the test animals are exposed during the accumulation period to preferably more than one (e.g. three) concentrations of the test compound in the surrounding water (=the aqueous phase) that are constant during the time of the test. These concentrations ($\equiv C_w$) must not be so high that they lead to the death or visibly abnormal behaviour of the test animals during the test. The concentration in the test animals ($\equiv C$) is determined regularly in a representative sample. In general, this concentration C will after some time reach a constant value (see Section 6.1). The weight and the composition (protein content, fat content) of the test animals must remain approximately constant throughout the test.

Test animals with a high concentration of the test compound are used to determine the elimination, e.g. animals from an accumulation test. At the end of the accumulation period these animals are placed in clean water ($C_w=0$), after which the concentration C in the test animals is determined regularly in a representative sample. Elimination is preferably started when the accumulation plateau has been approached, but this is not necessary (see Section 6.3.9.3).

6.3.2 The duration of the test

The accumulation time necessary for a reasonable approach to the plateau and the time necessary for 90 % elimination are difficult to predict. These times vary greatly with the test animal and with the test compound. Ideally, the analyses in the animals during the test are performed so rapidly and so frequently that the course of the curve can be followed. At the same time, care must be taken to have a sufficient number of animals per test vessel to permit the test period to be extended if necessary. Since sometimes 'sub-plateaus' occur, the accumulation process must be followed for at least a few days when a plateau appears to have been reached. Another possibility is to choose the accumulation time, the elimination time, and the sampling times beforehand and to see what the shape of the curves will be. If nothing is known about the accumulation and elimination behaviour of a substance, suitable periods are 14 days of accumulation, followed by 14 days of elimination. The facilities should allow the latter period to be extended to 4 weeks.

In addition to this, preliminary tests may provide information on suitable accumulation and elimination times, but such preliminary work is expensive.

6.3.3 Test systems

To determine the course of the accumulation and elimination curves, both over 14 days, at least 10 samples of animal tissue must be analysed for the test compound, at least two of them being in duplicate (at $t_a=14$ days and at $t_e=14$ days). With a minimum number of 10 test animals per sample (See Section 6.3.7) and a wet weight of 0.5–5 g per animal, the biomass per test tank at the start of the test may therefore amount to a few hundred grams. The amount of water required per day is determined by the animals' oxygen demand and by the fact that the accumulation

of the test compound in the animals must not lead to an appreciable fall of its concentration in the aqueous phase.

It has been found that for toxicity tests 1 to 10 litres of water per gram of wet weight per 24 h are sufficient (see Section 5.3.4.). It appears reasonable to use at least similar amounts for accumulation tests. If the test compound is not volatile, the oxygen concentration may be kept sufficiently high by aeration. The required degree of renewal of the water is then determined exclusively by the accumulation of the compound in the test animals. Large amounts of water per day are still necessary for strongly accumulating substances.

Since in accumulation and elimination studies it is essential to keep the concentration of the test compound in the aqueous phase constant or as low as possible, it is our experience that such tests can be performed correctly only in flow-through systems, unless the amount of biomass is very low in relation to the volume of water (see Section 6.3.9.1).

For a review of flow-through systems, see Section 5.4.3. For accumulation and elimination tests with animals measuring a few cm, only flow-through systems with a large capacity (100–1000 litres per 24 h) can be considered.

Systems which simultaneously meter a number of different concentrations of the test compound, such as the Mount and Brungs system (1967), can be recommended only if the accumulation study is being performed at all these concentrations.

For example, the system according to Connor and Wilson (1972) can be constructed for any desired number of concentrations of the test compound and for any desired flow-through rate greater than 10 litres per 24 h. The concentrations can be selected independently of one another and, by means of a suitable pump, both solutions in water and solutions in an organic solvent can be metered. A disadvantage of this system is the electric drive of the metering pumps.

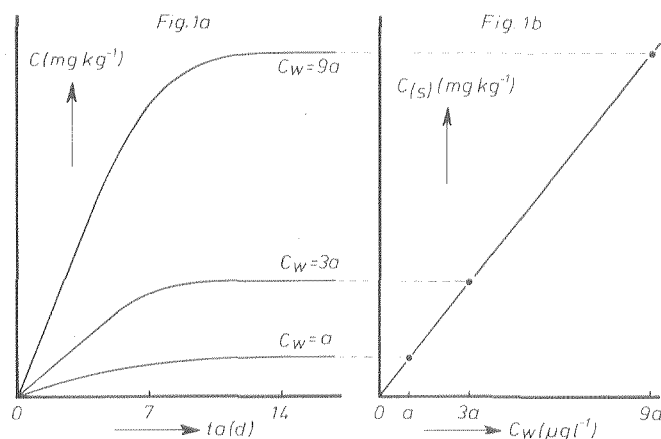
6.3.4 Choice of suitable concentrations of the test compound in the aqueous phase

The determination of the accumulation and elimination at only one test concentration in the aqueous phase ($\equiv C_w$) gives a reasonable idea of the accumulation and elimination in the animal species used. If a number of widely different concentrations are tested, however, the effect of C_w on the rate of uptake (A_r) and on the accumulation factor (A_f) can be determined simultaneously. If the saturation value of the test compound in the test organism has not yet been approached, and no active transport, metabolism, or excretion takes place, the steady-state concentration of the compound in the animal (C_s) is usually proportional to C_w (see 6.3, Fig. 1a and 1b).

The concentrations used in accumulation tests must satisfy the following conditions:

- they must be realizable in the type of water used in the accumulation test.
- during the whole test period they must not cause any mortality or visible deviations from normal behaviour in the test animals (order of magnitude $\leq 1\%$ of the 96-h LC_{50}), unless this is deliberately aimed at (see Section 6.3.9.4)
- they must be capable of being determined in the aqueous phase, i.e. be $\geq 3 \times$ the detection limit
- the concentrations expected in the animal tissues must be more than $3 \times$ the detection limit in animal tissue
- they must, if this is possible and desired, be relevant to practical conditions.

6. Determination of bio-accumulation and bio-elimination



6.3 Fig. 1a Schematic accumulation curves for three concentrations of the test compound in the aqueous phase

6.3 Fig. 1b Relationship between C_s and C_w after 14 days of accumulation

6.3.5 Measurements and observations

Accumulation and elimination tests are accompanied by regular determinations of the pH, the temperature, and the oxygen concentration of the water in the test vessels. The condition of the test animals is examined daily and dead specimens are removed; these are not analysed unless there is a special reason.

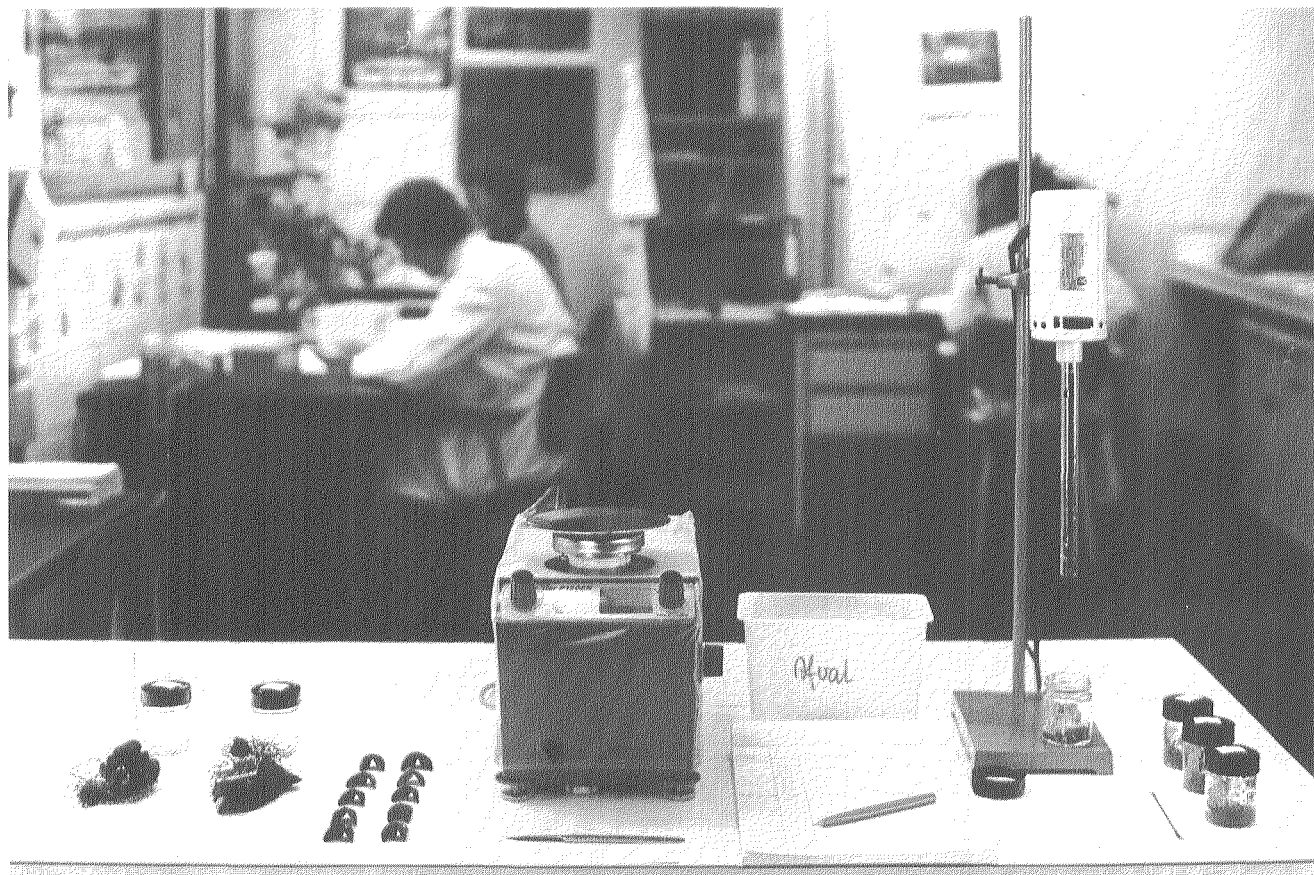
The concentration of the test compound in the water of the test vessels is determined daily or at least three times a week in all vessels, preferably in duplicate (for the analyses see Chapter 2).

The concentration of the test compound in the test animals or in certain organs is determined at regular intervals during the accumulation and elimination periods; for example, with a test period of 14 days, after 0, 1, 2, 4, 8, and 14 days, preferably in duplicate. Furthermore, it is recommended to take samples in duplicate at critical periods of the test, e.g. at the end of the accumulation period (which is generally also the beginning of the elimination period) and at the end of the elimination period.

By far the greatest number of problems in accumulation and elimination studies is of analytical nature. Determination of the test compound in the organisms, in particular, gives rise to many difficulties, especially in the case of ionizable compounds. In general, the standard deviations are between 10 and 30 %. This scatter is due to inaccuracies in the methods of analysis, to incomplete extraction from the organisms, and to non-homogeneity of the samples.

It must be realized that analyses in water and tissues can be performed only if the test compound, or in the case of mixtures the complete composition, is known and a reliable specific method of analysis is available (see Section 6.3.9.5).

Furthermore, analyses in tissues can be performed only if the test compound can be extracted quantitatively or with a known and constant yield from the tissues in question. Certainty about the yield of the extraction method can be achieved only with pure substances by loading living animals with this substance in radioactively labelled form.



6.3 Fig. 2 Preparation of homogenates. Each mussel is opened and placed upside down to drain off on filter paper for 1 min. The flesh is removed and weighed with an accuracy of 0.1 g for each mussel. On each occasion 10 mussels, constituting one sample, are homogenized together

The usual methods of analysis (e.g. extraction followed by gas-chromatographic analysis) are then followed step by step with analysis of the test compound by methods customary for radioactive isotopes. The concentration of the labelled compound is determined both in the extraction liquid and in the extracted tissues or solutions. By determining the amount of labelled substances that remains on extraction and comparing the final results obtained by the two methods, certainty can be achieved about the validity of the analytical methods used for the unlabelled substance.

However, this procedure can be adopted only if the test compound is not metabolized, since any conversion products that still contain the labelled atom are also determined by the isotope method but, for example, no longer by gas chromatography.

When the whole amount of tissue of one sample is not required for the analysis, analyses in biological tissue are carried out on a homogenate of the sample. The homogenate is prepared either from the whole test animal (customary for smaller animals) or from selected organs (customary for larger animals).

Each animal or organ is weighed separately and then those belonging to each sample are combined and homogenized. If sufficient material is available for the analysis, the animals or organs may, if desired, be analysed separately, but then this must be done on a sufficiently large number (preferably at least 10). In this way not only can mean concentrations be determined but also the inter-individual scatter. The procedure is very labour-intensive.

If organic compounds are to be analysed, a stainless steel homogenizer, such as an Ultra Turrax, may be used (see 6.3, Fig. 2). In the analysis of metals, especially chromium, nickel, and iron, the use of metal homogenizers is inadvisable.

The concentration of the test compound in the homogenate is preferably determined in duplicate. For most compounds this need not be done immediately. It is possible to perform these analyses equally well after keeping the samples deep-frozen for some time, but this should be checked beforehand. If desired, the following determinations are carried out simultaneously on the homogenate: the percentage of dry matter, ash, lipids, and protein (for this, see Section 2.3). These determinations are, however, performed immediately after sampling and homogenization.

6.3.6 Feeding of the test animals during accumulation and elimination studies

In the test described in this chapter the aim is to determine the accumulation and elimination of substances dissolved in the water in which the test animals live. If at all possible, the animals are fed to keep them in good condition (see Section 6.3.8, Note 3). They must consume the food completely in a short time, since otherwise there is a chance that the test compound will be administered partly via the food, which is not the aim of the test.

In the case of the filter feeders (e.g. *Daphnia*, *Artemia*, *Mytilus*, *Dreissena*) this is in principle impossible, for if such animals are fed, e.g. with unicellular algae, and particularly when the equilibrium between the test compound and the algae is achieved rapidly, the test compound will always be administered both via the aqueous phase and with the food. The reverse also applies here: if the animals are fed with algae containing a high concentration of the test compound, e.g. in food-web studies, the equilibrium between loaded algae and clean water will usually become established faster than the uptake of the algae by the animals.

In the case of animals such as fish, shrimps, etc., that are fed with food in granular form, worms, pieces of mussel flesh, and the like, it is quite possible to administer the test compound practically completely with the food, which is loaded with the test compound beforehand provided that the animals take up the food so rapidly that the liberation of the compound into the aqueous phase can be neglected.

Studies in which the test compound is administered with the food will not be described.

6.3.7 Test animals

6.3.7.1 Choice of species suitable for accumulation and elimination studies

As a first guideline, test animals for accumulation and elimination studies can be selected on the basis of the following primary and secondary considerations. Other considerations may apply for special purposes.

The factors of prime importance are as follows:

- potentially good properties for accumulation
- not too high a sensitivity to the test substance
- availability in large numbers throughout the year, easy to keep in the laboratory, capability of being used in tests over several weeks or months without greatly changing in weight and composition
- about 10 specimens taken together should supply sufficient material for the analysis envisaged.

In addition, the test animals should preferably:

- be important from the economic, consumer, or ecological points of view
- be characteristic for the biotope to be investigated, so that the laboratory accumulation and elimination studies can be compared with biological monitoring in the field
- be suitable for studies of active transport phenomena, metabolism, etc.

If possible, accumulation and elimination studies will be carried out with a number of organisms each of which occupies a different place in the food web. If, however, only a single species is used, it is recommended to choose one that satisfies as many as possible of the above-mentioned requirements. A good choice for sea water is then, for example, the mussel *Mytilus edulis* (see Section 5.3.2.2). The mussels have the following characteristics:

- very high rate of pumping (transport of water across the gills) (Dral, 1967)
- no particular sensitivity to variations in their environment (temperature, salinity) and to xenobiotic substances
- they are very easy to obtain throughout the year and can be kept satisfactorily in the laboratory
- they can be collected in many weight ranges
- they are used for human consumption
- they are frequently used in biological measuring networks and are widely distributed
- they have differentiated organs which, in principle, can be dissected out.
However, this is very time-consuming and the wet weight per organ is low, so that for most analytical methods large numbers of mussels per sample are necessary. In general a homogenate is made of the total soft parts of mussels.

Because of similar considerations, *Dreissena polymorpha* (see Section 5.3.2.4) is probably a suitable test animal for investigations in fresh water. However, little experience has so far been acquired with this species.

If there is a special interest in the distribution of the test compound between various organs and/or in the mode of its action, a species of fish is preferable as the test animal.

Young (1- to 2-year-old) plaice (see Section 5.3.2.14) or trout (see Section 5.3.2.17), obtained respectively from sea water and fresh water, are, for example, commercially important, easy to keep, and sufficiently large to be easily dissected and to supply sufficient weight per tissue. On the other hand, the determination of a com-

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plete accumulation and elimination curve with these fish requires very large test vessels and flow-through systems with a large capacity if, in addition to sufficient samples, a sufficient number of individuals per sample is to be analysed. These large fish are recommended only for special studies. See further Section 6.3.9.2.

If, however, it is desired to study accumulation and elimination with a test animal other than mussels, or if fish are preferred to mussels, small fish such as guppies (see Section 5.3.2.15) or common gobies (see Section 5.3.2.13) may be selected. Both species can be used in fresh water and in sea water. In general, fish have a high accumulation factor; they are, however, sensitive to many substances.

A complete accumulation and elimination curve determined with mussels or with a small fish species and supplementary information on the distribution of the substance between the various organs with a larger fish species generally give a good impression of the accumulation properties of the compound (see also Section 6.3.9.6). In addition to this, if desired, an investigation can be carried out with microorganisms (see Section 6.2).

Many other organisms may also be used. The species mentioned here were selected as examples because good experience has been acquired with them.

6.3.7.2 Collection, transportation, and acclimatization of the test animals

The animals used for accumulation and elimination studies must be in good health and condition at the start of the experiments. This implies that all operations associated with the collection, transportation, and keeping and/or acclimatization of the animals in the aquarium systems must be carried out with the greatest care and usually with many precautions.

The precautions include the preparation of aquaria in which the necessary number of animals can be contained without overcrowding; the physico-chemical and biological conditions of the aquarium environment should be adapted as closely as possible to the requirements of the organisms.

It is important that the capture and transportation of the animals should cause no physical damage. However, this cannot always be ensured, and part of the catch may be unsuitable for the test. Fishing gear for commercial purposes is frequently unsuitable for the collection of living animals.

The use of drag-nets behind motor boats may be the only way for certain test species, but the chances of damaging the animals are then very high and the collected material must be carefully sorted out. A slow, short 'trail' gives the best results.

It is preferred to catch pelagic and benthic organisms with nets operated by hand.

In tidal regions, such as the Wadden Sea, use can be made of the fact that many animal species, for example the plaice, the common goby, and the brown shrimp withdraw at ebb-tide from the intertidal mudflats towards the slopes of the deeper gullies; at ebb-tide many benthic organisms are found in great numbers at these points.

The collection of sessile organisms such as mussels, slipper limpets, and zebra mussels presents practically no difficulties.

In general, from the brief characteristics given for a number of suitable test animals outlined in Section 5.3.2 it is possible to determine what methods of collection will be most appropriate for a given animal; the local circumstances will often also contribute to the selection of the method.

After collection, do not expose the animals unnecessarily to air; sort the catch rapidly and transfer suitable specimens immediately into a tank with the same water as that in which they were caught. Transport them in this water to the laboratory. To keep the temperature of the water constant, it is convenient to transport the animals in water-tight plastic bags and to pack these in thermally insulated boxes. The use of a refrigerated vehicle may also be considered in this connection.

During the transportation sufficient oxygen must be provided. This can be done by connecting the containers with the animals to

an air pump. Air-tight closed plastic bags, into which a substantial amount of pure oxygen has been introduced above the surface of the liquid, are very suitable for transporting the animals. (It must be mentioned, perhaps superfluously, that there must be no smoking when bags containing pure oxygen are opened). Under these conditions, the amount of water in which the animals are transported may be very small. Sessile organisms such as the above-mentioned shellfish can always be transported in very small amounts of water or even 'dry'. Transport only one animal species per container or plastic bag and avoid excessive numbers per container. Once in the laboratory, the animals must be placed immediately in aquaria filled with water of approximately the same composition (salinity, hardness, etc.) and approximately the same temperature as that in which they were caught. In any case, avoid shock effects.

Dead or severely damaged animals must be discarded at once.

Keep not more than 4 g of biomass per litre in the aquarium system per 24 h, and preferably 1 g per litre. Filter the water continuously through active carbon if it is a recirculating aquarium system.

Use about 2 litres of active carbon per 100 litres of water and renew the carbon once a week to once a month.

In a recirculating system, with the above biomass loading, the water must be completely or 90 % renewed at least 3 times a week.

Aerate the water continuously in such a way that the oxygen concentration remains above 80 % of the saturation value.

Acclimatize the animals to the laboratory conditions in this way for at least one week. If during this period the mortality is low ($\leq 10\%$), the animals can be used for accumulation and elimination studies, provided that the physico-chemical conditions of the water in the experiments (apart from the toxic compound) are the same as those to which the animals have already been acclimatized. If the test conditions are different, e.g. different temperature, salinity, hardness, etc., the animals must first be acclimatized to the new conditions. This acclimatization period must also last for at least one week, during which period the mortality must be low ($\leq 10\%$).

If possible, during the entire acclimatization period the animals must be fed with a suitable food. Newly caught animals will sometimes refuse food in their new laboratory surroundings, but may occasionally be induced to eat by placing them with animals of the same species that do accept food. If the animals will not accept food, their condition deteriorates very rapidly and they can be used only in short-term experiments performed shortly after collection.

For literature on the subject of this section see *inter alia* EPA-660/3-75-009; Doudoroff *et al.*, 1951; and Perkins, 1972 a and b.

6.3.7.3 Number of test animals per sample and size distribution of individuals per test

At least 10 individuals are usually taken per sample, according to the considerations given in Section 5.3.4. In addition to this, in accumulation tests it is, however, necessary that the weight of one sample be sufficiently large for the selected method of chemical analysis in view of the expected concentration of the test compound in the test animals. This determines the minimum number of animals per sample, which with small animals will often run to more than 10; this has a favourable effect on the representativeness of the samples.

For the selection of the sizes of individuals per test, reference is made to Section 5.3.4.

6.3.8 Description of a method for a combined accumulation and elimination test with small animals

Subject

Determination of the accumulation and elimination of substances, both lasting 14 days, with animals having a wet weight of 0.5 to 5 g per individual.

Field of application

The method is used in fresh water and/or sea water for substances

that are soluble in it at the desired concentration and can be determined analytically both in water and in biological tissue.

Principle

Test animals are exposed to water with a known concentration of the test compound that is kept constant during the accumulation period. This concentration, which must be appreciably lower than the lethal concentration, is regularly determined. The concentration of the test compound in a representative sample of the animals or in some of their organs is also determined at regular intervals. If possible, the accumulation is continued until the concentration in the test animals is constant, but in practice 14 days is customary. After the accumulation period the remaining animals are placed in water not containing the test compound. During the subsequent elimination period the concentration of the test compound in the water is determined until it can no longer be detected, while the concentration in a representative sample of the animals or in some of the organs is determined at regular intervals. If possible, the elimination is continued until the compound can no longer be detected in the test animals.

INTRODUCTION

In the description of the method, the following starting points apply: one concentration of the test compound in the aqueous phase in comparison with a blank; an accumulation period of 14 days, followed by an elimination period of 14 days; the use of a flow-through system; the use of the mussel *Mytilus edulis*, length 30–35 mm, as the test animal; knowledge of the toxicity of the test compound for the mussel.

The test arrangement is, however, simple to adapt for other concentrations, other test times, or other test animals (see Notes 1, 2, and 3).

EQUIPMENT AND MATERIALS

- About 250 specimens of *M. edulis*, length between 30 and 35 mm (see 6.3, Fig. 3.)
- two test vessels of about 100 litres connected to a flow-through system with a capacity of about 100 to 1000 litres per 24 h
- apparatus for keeping the temperature of the water in the vessels constant at between 10°C and 15°C
- sufficient natural (or synthetic) sea water, salinity $28 \pm 2\text{‰}$ (see Section 5.7.2, j and k; Section 5.7.3), of such quality that *Artemia salina* reproduces normally in it (see Section 5.8.5)
- aeration apparatus (only necessary if the rate of through-flow is too low to keep the oxygen concentration sufficiently high (above 80 % of the saturation value) and only used if the test compound is not volatile)
- oxygen meter, pH meter, and salinity meter
- alcohol thermometer
- coarse-mesh net of very thin polyethylene threads (aperture diameters about 1 cm)
- homogenizer; such as an Ultra-Turrax
- balances accurate to 0.1 g and to 0.0001 g
- knife for opening the mussels
- glass bottles for keeping samples of water and tissue
- deep-freeze
- analytical facilities for the desired analyses.

PROCEDURE

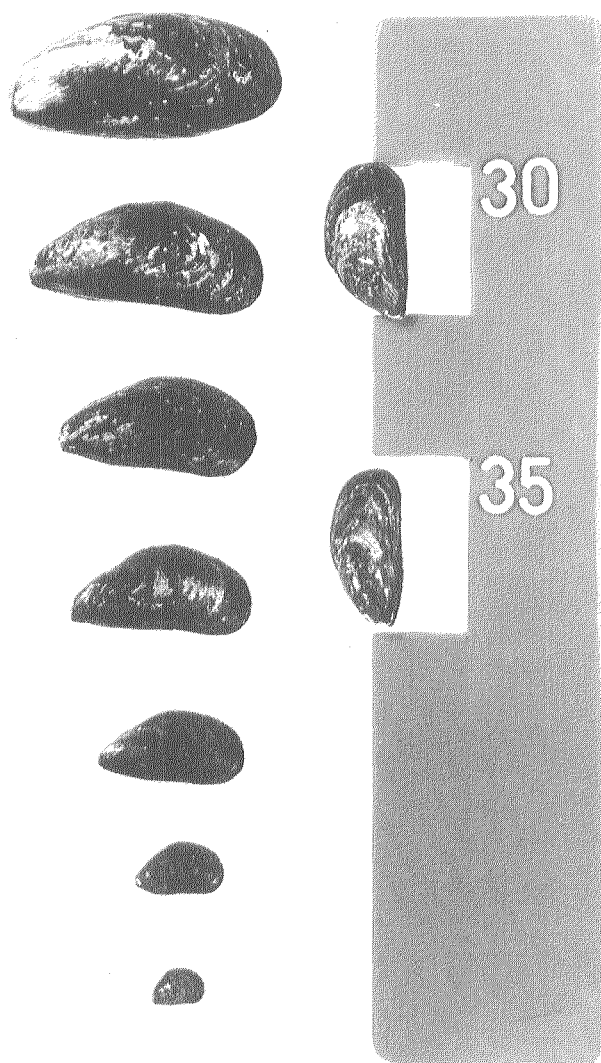
- Collect the mussels and select specimens with the desired length – on this, see 6.3, Fig. 3; remove other organisms from the shells.
Analyse the concentration of the test compound in two samples of 10 mussels each. This concentration must be very low, preferably 'not detectable.' Acclimatize the mussels for four days to the laboratory conditions. Check the mortality during this period; it must not amount to more than 10 %.
- Choose at random 18 batches of 10 mussels and place each batch in a coarse-meshed polyethylene net (for this purpose the mussels may be taken out of the water and placed on a clean table). Acclimatize these mussels for another three days and check mortality; it must be zero (for fish, see Note 4).
- Choose the concentration of the test compound, taking into account, so far as possible, the criteria given in Section 6.3.4.
- Place the two test vessels at a constant temperature, e.g. 12°C, and connect them to a flow-through system that meters sea water (also at 12°C) with the desired concentration of the test compound into one vessel and blank sea water into the other. The rate of through-flow at the start of the test must be about 300 litres per 24 h in the case of non-volatile test compounds, in which case aeration is applied, and about 900 litres per 24 h in the case of volatile test compounds, when aeration is not applied. These flow-through rates may, if desired, be adapted to the steadily decreasing number of animals per vessel (about 2 litres per g wet weight per 24 h is required with aeration and 5 litres per g wet weight per 24 h without aeration).
- Check the working of the system for 2 days and determine after the first day the concentration of the test compound in the water of both vessels.
- Start the test ($t=0$) as soon as it has been found that the concentration has the desired value and the flow-through apparatus is working satisfactorily.
- At $t=0$ place 16 batches of mussels in each vessel (see Notes 3 and 4 for other test species) and use the 17th and 18th batch for the analyses required at $t=0$ (in general, the same analyses as for the other test times).
- Measure and record daily the oxygen concentration, the temperature, and if necessary the pH and the salinity in both vessels.
- At least three times a week, but preferably daily, take duplicate samples of water from both vessels and determine the concentrations of the test compound in these samples.
- Take single samples of mussels from both vessels at accumulation times $t_a=1, 2$, and 8 days and duplicate samples at $t_a=4$ and 14 days.
- Within 2 days of collecting the samples, during which days the mussels must be stored at 4°C, cut the mussels open and leave the halves to drain upside down for one minute. Remove the flesh and transfer it to a previously weighed and coded glass container with an effective closure. Record the weight of the flesh per mussel. Make a homogenate of each sample of 10 animals (see 6.3, Fig. 2).

If desired (see Note 5), weigh out samples of homogenate directly for the determination of the content of dry matter, ash, fat, and protein (for these determinations see Section 2.3) and freeze the remainder immediately until the time of its analysis for the test compound (see Note 6).
- At $t_a=14$ days, after the taking of samples of water and tissue, empty the test vessel with the test compound and stop metering in the test compound.
- Now connect this vessel also to the clean sea water supply. If desired, lower the rate of through-flow; the vessels can now

6. Determination of bio-accumulation and bio-elimination

be aerated and there is now only half of the original number of test animals present (take care to achieve similar test conditions in the blank vessel and the test vessel).

- Analyse samples of water daily until the test compound can no longer be detected.
- Take samples of mussels from both test vessels, singly at elimination times $t_e = 1, 2,$ and 8 days and in duplicate at $t_e = 4$ and 14 days (see Note 7). Treat these samples as described for the accumulation period. The elimination can be ended as soon as the test compound can no longer be detected in the tissue at two successive sampling times.
- Determine the concentrations of the test compound either immediately after homogenization or after some time of deep-frozen storage (see Note 6). In the latter case, the sample is thawed out at 4°C and re-homogenized. After weighing out of the amount necessary for the analysis, the homogenate is immediately re-frozen so that the analyses can be repeated if necessary. The concentration is preferably determined in duplicate for each homogenate. If the difference between the two values is more than 20% of their average, the thawing, homogenization, and analysis are repeated.



6.3 Fig. 3 Simple method of selecting mussels with a maximum length between 30 and 35 mm. Each mussel that fits into a gap of 35 mm and does not fit into a gap of 30 mm has a greatest length between 30 and 35 mm

TREATMENT OF THE RESULTS

- Summarize the selected test conditions so far as possible in tabular form, e.g. as given in Example 6.3, Table 1.
- Calculate the mean concentration $\bar{C}_w$ of the test compound in the aqueous phase and its standard deviation.
- Calculate the mean temperature (t), oxygen concentration, pH, and salinity (S) and record whether, and if so when, high or low values of these values occurred.
- For each sample calculate the mean wet weight per animal and the standard deviation. Investigate whether there are significant differences between different samples and whether the wet weight decreases or increases regularly and significantly.
- Make a summary in tabular form; record for each sample separately:

The wet weight per individual with the standard deviation s .

The mean concentration of the test compound (C) per sample calculated for the wet weight, rogue results being discarded; the highest and lowest values included are recorded with the mean value, together with any rogue results.

The amounts of dry matter, ash, lipid, and protein, calculated for the wet weight for those samples for which these constituents were determined.

If desired, the mean concentration of test compound calculated to the dry ash-free weight and/or weight of lipid present in the test animals.

This table also gives $(\bar{C} \pm s)$, $\bar{t}$, $\overline{pH}$, $\bar{S}$, $\bar{O}_2$, and all serious deviations from the mean values that may have occurred.

- Plot the concentration of the test compound calculated for the wet weight versus time, both on a linear scale, and draw the accumulation and elimination curve best fitting the experimental points (for an example see 6.1, Fig. 2).
For the elimination period also plot a graph of $\log C$ against t and see whether this is a straight line (see Section 6.1).
- Calculate the parameters from 6.1, Table 1, to the extent that these are relevant and can be determined with sufficient accuracy (for an example, see 6.1, Fig. 2).
- Report all deviations or changes observed in the test animals during the accumulation and elimination periods, such as mortality, abnormal behaviour, gain or loss of weight, or changes in composition.

NOTES

1. Testing of several concentrations

More than one concentration of the test compound can be tested without difficulty if a sufficient number of vessels, sufficient amounts of water, and a flow-through system suitable for several concentrations together with a blank are available. It is advisable to choose concentrations differing from one another by a factor of at least 3 and to test at least 3 concentrations. All concentrations must satisfy the requirements set in Section 6.3.4.

2. Other test durations

When the animals are fed during the test, the total test period is limited only by their ageing. If the test animals are not fed, the test must not be continued so long that their condition deteriorates appreciably because of starvation. This can be seen from the analyses for fat and protein content, together with determinations of the wet and dry weight. For *M. edulis* a test must not last longer than 4–6 weeks.

If a shorter test time than 2×14 days is selected, the sampling times are adapted to this test time. The total number of samples is, however, kept the same.

3. Other test animals

For investigation in fresh water *Dreissena polymorpha* can be used instead of *M. edulis*.

If instead of mussels fish have to be used, these may for example be guppies (*Poecilia reticulata*), both in fresh water and in sea water.

As a rule, mussels are not fed during accumulation and/or elimination experiments, since their weight falls only slightly during the first 4 weeks and since the production of sufficient food (unicellular algae) for these animals is very time-consuming.

Guppies can easily be kept at a constant weight by feeding them daily, for example with Tetramin in amounts of 0.5 to 1 % of their body weight.

For suitable test conditions for animals other than *M. edulis* see the relevant sections in Chapter 5.

4. Small fish as test animals

If it is desired to use fish instead of mussels, either local species (e.g. *Pomatoschistus microps*) or commercially available species (e.g. *Poecilia reticulata*) can be used. They are collected or bought, fed, and acclimatized to the laboratory conditions as described in Section 6.3.7.2. *Poecilia reticulata* can also be obtained by breeding them in the laboratory (see Section 5.8.8). Fish are selected **visually** for length and health, rejecting specimens with visible abnormalities. The length of the largest fish must not exceed the length of the smallest by a factor of more than 1.5. However, the length is measured just after the taking of the sample at the same time as the determination of weight. The fish are distributed at random (see for example Section 5.9.9.1, Note 5) over the test vessels and **not** made up into batches before the start of the test. At each sampling time 10 arbitrarily chosen specimens are caught and, after measuring and weighing, are combined and homogenized.

5. Determination of the contents of dry matter, ash, lipid, and protein and of the wet weight per individual

The percentage of dry matter is reasonably constant for each species at a given age. Moreover, the dry weight can be determined more accurately than the wet weight. Thus by determining for each sample the ratio of dry to wet weight, an idea of the correctness of the wet weight can be obtained. If the dry weight is known, it is also possible to compare the results obtained with those of an investigation in which only the concentrations calculated on the dry weight are recorded.

The ash content is determined specially when using mussels to indicate a possible exceptionally high content of silt in these animals.

The wet weight per individual, the protein and lipid contents, and especially regular changes in these during the test give indications about the condition, such as emaciation, growth, and fattening of the test animals.

In tests on lipophilic substances the lipid content may also be important for an estimation of the potential accumulation properties (see Section 6.3.9.6).

In tests at several test concentrations and/or with several test periods, the time-consuming determinations of the ash, protein, and lipid contents are not always performed for all samples. A choice is then made of the types and numbers of determinations, depending on the further arrangement of the test.

6. Freezing of animal tissue homogenates

It is not always possible to perform the analyses for the test compound immediately after the sample collection. Furthermore, it often saves time to perform a number of analyses simultaneously and thus to save up the samples until there is a suitable number.

For this purpose the homogenates are frozen at -32°C immediately after their preparation, and are stored until the time of analysis if it has been established that freezing will not affect the amount of the test compound in the test animals or its analysis.

7. Extra samples

After the last sampling, at $t_e = 14$ days, 2 samples of mussels still remain per vessel. These were added as extras at $t = 0$ as reserve samples in case a sample was lost, e.g. in homogenization. If at $t_e = 14$ days they have not been used for this purpose and the elimination time is incomplete, the test may be continued to $t_e = 32$ days. However, particular attention must then be paid to possible emaciation of the test animals.

6.3.9 Adaptations of the general method described in Section 6.3.8 for special purposes

6.3.9.1 Studies with smaller animals (e.g. microcrustaceans)

Studies with smaller animals are in principle carried out as described in Section 6.3.8, but because of the low wet weight per individual (a fully grown *Daphnia magna* weighs about 3.5 mg and a fully grown *Artemia salina* about 5.5 mg) for many test compounds large numbers of individuals per sample are necessary to carry out the analyses.

The use of labelled test compounds can often simplify these tests. Because of the very low weight, the ratio of biomass to the volume of water is small, so that relatively small amounts of water need be loaded with the – labelled – test compound. Flow-through systems can be avoided by making the above-mentioned ratio so small that the fall in the concentration of the test compound in the aqueous phase can be neglected, or by keeping the concentration constant by further additions.

Since, even when isotopes are used, reasonable numbers of test animals per sample (e.g. 25 *Daphnias*) can or must be used, the sample in general is highly representative of the population, so that good duplicates are obtained. Because of the large surface/volume ratio in small animals, equilibria are often established quickly, which shortens the test duration; on the other hand, most of the test compound measured in the tissue samples may then be adsorbed on the surface of the test animal instead of being incorporated in the tissues.

6.3.9.2 Studies with larger animals (e.g. plaice, trout)

Studies with larger animals can in principle be carried out in the same way as described in Section 6.3.8, but large amounts of water are necessary. The test arrangement must therefore be limited so far as concerns the number of samples to be taken, which must always include 10 individuals per sample. Since the scatter in the concentrations per individual or per tissue of an individual may be very large, it is not advisable to limit the number of the test animals per sample. Moreover, with such test animals it is not meaningful, not necessary and often not possible, to homogenize them *in toto*.

Consequently, it is both more sensible from the point of view of the test technique and from the scientific point of view in the case of such animals to dissect out the various organs and to analyse flesh (muscle tissue), liver, brain, etc. separately for the test compound.

The various organs must be weighed separately so that, if desired, an average content per individual can be calculated for comparison with the contents found in homogenates of whole smaller animals. It may be recommended to analyse the organs of each test animal separately, so that the average content per sample **and its scatter** can be calculated.

6.3.9.3 Test with the emphasis on elimination

If one is interested exclusively or mainly in elimination, the test animals can be placed for a relatively short time (e.g. a few days) in a relatively high but still harmless concentration of the test compound, which need not be kept constant. Then, in general, a clearly increased concentration is achieved in the test animals, after which the elimination can be followed in the usual manner. If active trans-

port phenomena, metabolism, etc. do not take place, the course of the elimination will not be affected by the accumulation time. If, however, this is the case, one cannot distinguish whether it was the accumulation time or the concentration reached in this time that affected the course of the elimination.

6.3.9.4 Tests in which accumulation and/or elimination studies are performed simultaneously with the toxicity study

With sufficient capacity for the simultaneous testing of a large number of concentrations (e.g. 5–7) in the aqueous phase, accumulation and elimination studies can be performed simultaneously with toxicity studies.

The concentrations are then chosen so that lethal concentrations are tested in addition to the low concentrations customary for accumulation tests.

A partition is introduced into the vessels, each compartment containing the same number of test animals at $t=0$. One of these compartments is used for taking samples (live animals are chosen for this) for the accumulation study. At the non-lethal concentrations this compartment must be empty at the end of the accumulation period. The other compartment is used for the determination of the mortality during the accumulation period, from which LC_{50} values can be calculated, and for the elimination study with the remaining animals (as long as the stock lasts).

Dead animals are counted daily in both compartments and removed. If on a given day the number mounts up to almost 10 dead animals per vessel, these may, if desired, be analysed in comparison with the sample of live animals.

If the choice of the concentration in the aqueous phase was opportune, it is possible to determine:

- the accumulation and elimination at two or three non-lethal concentrations

- the accumulation and the beginning of elimination at one or two concentrations lethal to some of the animals during the accumulation period
- the accumulation at one or two concentrations lethal to the whole population during the accumulation period (during the time for which live specimens are still present.)

In addition, it is possible to study the condition (mortality) of the animals at a given **concentration of the test compound in the animals**; the A_t/C_w ratio even at extreme concentrations; the course of the accumulation and elimination at lethal concentrations in comparison with sublethal concentrations; and possibly the concentration of the test compound in a sample of dead animals in comparison with that in a corresponding sample of still living animals.

6.3.9.5 Tests with complex mixtures

In working with complex mixtures, only the accumulation and elimination behaviour of known components capable of being analysed can be determined. In general, the components will not be present in the same ratios in the aqueous phase and in the tissue.

6.3.9.6 The partition between n-octanol and water

For lipophilic substances it has been established that the lipid/water partition coefficient is indicative for the degree of accumulation in test animals. The lipid (e.g. liver oil)/water partition coefficient is however difficult to determine since the equilibrium lies far over on the side of the lipid in the case of lipophilic substances.

Since the n-octanol/water partition coefficient of a compound can be calculated from its molecular structure, and since it is much used in pharmacology to determine the lipophilicity of substances, this partition coefficient can be used to give an idea of the accumulation properties of organic compounds (see also Section 1.7).

6.3 Determination of the accumulation and elimination of xenobiotic substances by molluscs, crustaceans, and fish

6.3 Table 1

BASIC INFORMATION

Type of test	Commission No. :	
	Code No. :	
	Investigator :	
Test substance:		
concentrations:		
Test animal : species:		
age at the start of the test :		
length " " " " " " " :		
weight " " " " " " " :		
origin laboratory culture collection site: date: - -19		
acclimatization: time: temp:°C food:		
other information:		
Date of the start of the test: - -19		
Test duration :		
Temperature :°C		
Type of dilution water :		
Hardness :; pH:; ‰ Cl ⁻ :; ‰ S:		
Contents of test vessel : litres of water and test animals at the start of the test		
Food/no food; type? :; how much?		
Renewal : none; intermittent, once a; continuous, flow-through rate:		
Type of test vessel : replicates?		
Oxygen/air : passed through/over; high/low; continuous/intermittent; none		
Solvent for the test substance: dosage:		
Analysis of the test substance; method:		
Other information:		

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CHAPTER 7

The role of field work in environmental toxicological investigations

by H. J. Hueck and W. Chr. de Kock



7.1 INTRODUCTION

The evaluation of the effects of pollution on a natural living community must in the final account be based on the determination of the connection between the pollution level and its effect on the living community, i.e. on the dose-effect relationship.

For an accurate description of this relationship we must define in more detail the terms 'pollution level' and 'natural living community'. Some aspects of these two complex concepts have been treated in preceding chapters. In general terms, this concerned definite amounts of chemicals coming into contact with a single type of organism or, in the case of biodegradation, with a limited group of organisms. Moreover, the circumstances under which this contact takes place were usually defined as closely as possible. On the basis of a systematic ranking of partial information, such as that given in Chapter 1, it is then possible to attempt a construction of the picture of the total effect. However, it will be clear that, in addition to this, an integral approach to the problem is desirable from the point of view of all kinds of interactions and unforeseen complicating factors.

Examples of complicating factors:

- Concerning the dose (i.e. level of pollution):
Toxic substances can be supplied via the water and with the food. In addition, these agents may be distributed non-uniformly in the environmental compartment under consideration, e.g. by adsorption on sediments or suspended particles. This will also cause different organisms to be stressed differently. With respect to the food web, accumulation in the organisms that form part of the food is important. The relationship between the uptake with food and that via water is not always constant, which may also lead to differences in stress on individual species.
- Concerning the effect:
Under natural conditions we deal with systems that contain many species of organisms. In addition to the above-mentioned differences in stress on individual species, there may be great differences in susceptibility. This susceptibility may be determined for each species separately in a pure or axenic culture (bacteria, algae, invertebrates, fish, etc.).
In a system with several species, however, the changes in the mutual numerical ratios that may be due to this are less clear.

Ecologically, it is this very diversity that is of great importance. In addition to this, in systems with only one species certain ecologically important effects, such as the effects on behaviour, may be expressed less distinctly.

- Concerning the environmental conditions:
In a natural system the reaction conditions are changed continuously, e.g. by the day and night cycle, seasonal changes, effects of the weather, effects of currents, synergisms and antagonisms of additional stress-factors of variable intensity occurring simultaneously (e.g. thermal pollution, intermittent discharge of waste dumping). Although within certain limits this can be simulated in the laboratory, the actual situation as a whole is difficult to reproduce.

Direct determinations of the total dose-effect relationship in the natural environment are possible only exceptionally, e.g. in the case of disasters and already existing discharges. Frequently, however, experimentally necessary information, such as a knowledge of the 'blank' state (the base-line) or an accurate value of the dose, is lacking. Attempts have been made to design methods for field studies that meet the complex nature of the dose-effect relationship under natural conditions and that are experimentally feasible and reliable. In this situation it is unavoidable that some concessions must be made with respect to the representativeness of the test arrangement or treatment of the observations.

In general, two types of studies can be distinguished, namely a retrospective study, which primarily attempts to correlate the biologically observable effects with the degree of pollution, and a prospective study, which primarily starts out from the dose of the pollutant and attempts to determine causal relationships with the effects in biological systems of various degrees of complexity.

A well known example of the retrospective type of study is the so-called 'saprobic system' (Kolkwitz and Marsson, 1908), whereas various forms of aquarium tests in which pollutants are added deliberately are characteristic for the prospective studies. The development of such methods is still in progress and has formed no part of the systematic studies described in the earlier chapters of this manual. Some experience in this field, while making no claim to being a complete review, is reported in the following sections.

7.2 PROSPECTIVE ECOLOGICAL POLLUTION RESEARCH

7.2.1 Introduction

As already stated, in prospective research the emphasis is primarily on establishing the dose and on determining its effect afterwards. Here it is possible to start from a dose already present in the environment or from a selected and established dose.

In the first case, studies can be carried out:

- by the transfer (transplantation) of organisms within an existing gradient
- by measuring the effects after identifiable ecological disasters (e.g. the wrecking of an oil tanker).

In the second case, a distinction can be made between:

- addition of measured amounts to artificially partitioned sections of a natural system (basin tests)
- addition of measured amounts to artificial living communities (aquarium tests)
- addition of measured amounts to interconnected compartments each representing a different trophic level of an ecosystem (compartment tests).



7.2 Fig. 1 Basins set up on a mussel bank for environmental toxicology tests with heavy metals (De Wolf, De Kock and Stam, 1972) (Aerial photograph — Schiphol)

7.2.2 Gradient tests

In the case of already existing discharge sites of known effluents, a fairly stable concentration gradient will usually be present. This is very clear in the discharge of warm water, but it can also be demonstrated for chemical pollutants. Single organisms or natural

communities can be transferred from a relatively unpolluted part around the discharge site to a more polluted part, and the effects can then be observed (Dicks, 1976).

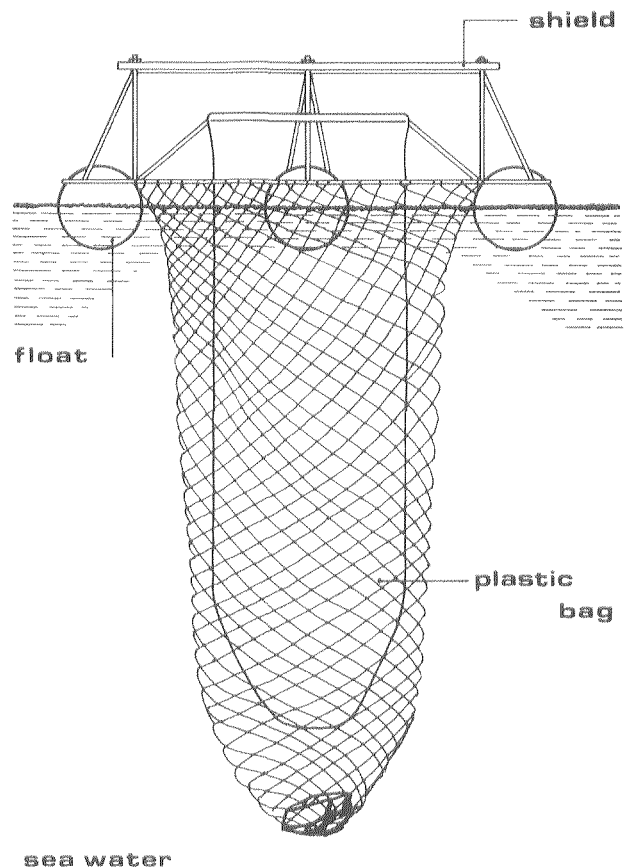
Studies of the natural population already present within the gradient can also provide valuable information (Koeman and Van Genderen, 1972). Finally, it is also possible to introduce laboratory-bred test animals into this gradient. A prerequisite for a satisfactory study is that the dose can be determined in various parts of the gradient by chemical analysis.

7.2.3 Disasters

A copy-book example of an unintentional experiment was the Torrey Canyon disaster (Smith, 1968). In particular, the observations of the oil on beaches and ones concerning the effects on entire communities and their recovery give a good picture of the possibilities in this field, though certain limitations are set by the need to improvise in such situations. In addition, the initial state is often insufficiently known.

7.2.4 Basin tests

Here one starts from spatially adequately isolated, and yet comparable systems. This can be achieved, for example, by enclosing a mussel bank (De Wolf *et al.*, 1972, see also 7.2, Fig. 1) or by screening off parts of a natural system in some other manner (for example Levell, 1976).

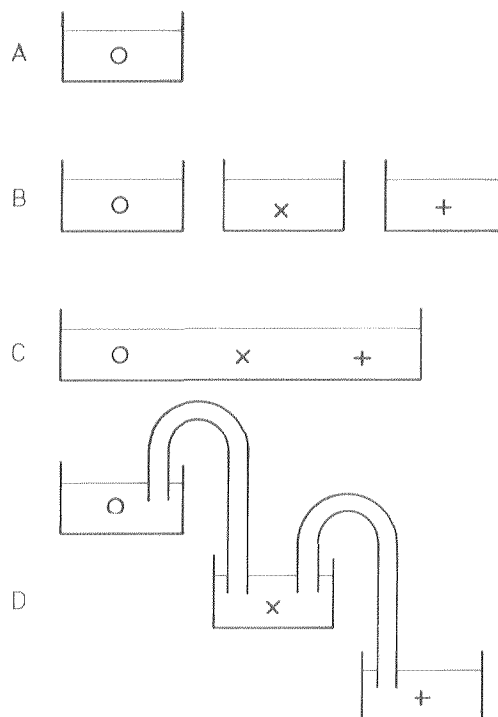


7.2 Fig. 2 Plastic bag used for environmental toxicology studies on a natural plankton population (after Kuiper, 1976)



7.2 Fig. 3 Plastic bags for environmental toxicology studies on plankton populations at TNO Applied Marine Research Laboratory, Den Helder, the Netherlands (part of TNO Division of Technology for Society)

The test substance can then be introduced into these natural basins in a series of concentrations. In studies on plankton populations plastic bags are often used for this isolation (Horstman, 1972; Reeve *et al.*, 1976; Kuiper, 1976). There is evidence that in these bags the development of the isolated living community remains comparable with the original situation for at least a month (Takabashi *et al.*, 1975). (An example of such a test arrangement used in our laboratory is given in 7.2, Fig. 2, and 7.2, Fig. 3). Several examples were described during the Ecosystem Research International Heligoland Symposium in 1976 (the report appeared in *Helgoländer Wiss. Meeresunters.* **30**, 1–735 (1977)).



7.2 Fig. 4 Comparison of some test systems
o ; x ; + = different test organisms

A = test with one organism

B = separate tests with different organisms

C = aquarium test with simultaneous testing of different organisms

D = compartment test with different spatially separated organisms

(After Hueck and Adema, 1968)

7.2.5 Aquarium tests

The starting point here is the establishment of an artificial community in an artificial or natural environment. For research purposes such an environment may assume very diverse forms, for example, an artificial brook, pond, glass or concrete tanks of various dimensions, etc. It is then possible to introduce species characteristic of a given ecosystem or to allow the latter to arise 'spontaneously' by regulating the conditions. In microbiology one then speaks of a selective accumulation culture guided by the adage that 'everything is everywhere but the environment selects'. Toxic agents can then also be added (Whitworth and Lane, 1969; Metcalf *et al.*, 1971; Saward *et al.*, 1975; Davis, 1976). According to Metcalf *et al.* (1971), by making use of labelled compounds it is also possible to study food-chain processes in such systems. The use of radioactive compounds is less feasible in the basin

tests described previously that take place in a natural environment.

7.2.6 Compartment tests

Compartment tests are different from ecosystem tests in that the test system consists of a number of compartments, each with its own organisms (see 7.2, Fig. 4). A simulation of a specific trophic level is generally attempted for each of the compartments. The compartments are connected with one another via the water phase, but the organisms of the various trophic levels remain spatially separated. Such a system has been put into practice by Ringelberg (1973, 1976) and by Kersting (1975). The system lends itself in particular to studies of the flows of energy and materials between the various trophic levels that may or may not be under stress.

7.3 RETROSPECTIVE STUDY OF ECOLOGICAL POLLUTION

7.3.1 Introduction

Living communities react in time to variation in the parameters of the environment when the latter is collectively stressed. The living part of the ecosystem can then be regarded as an integrating, information-yielding unit (Kaesler *et al.*, 1974). There is a causal relationship between the stressing dose and the effect, expressing itself in the composition of the living community.

In retrospective studies the starting point is primarily a qualitative and quantitative study of the species population and an attempt to correlate this with the parameters of the environment, one of which may be the degree of pollution. Thus, one uses bio-indicators for the degree of pollution, and it is possible to distinguish two directions of study:

- study directed at the whole population
- study directed at characteristic species (individual bio-indicators).

Bio-indicators lend themselves to 'monitoring' pollution and for the establishment of a pollution-measurement network.

7.3.2 The population as a whole

Qualitative and quantitative study of the species population in the long term can in principle provide much information about the 'health' of an ecosystem, particularly since it has been found that such a system in the undisturbed state shows a great diversity of species and a permanent existence of biomass components in time (stability).

When the variability of the structural and functional characteristics (e.g. number per species or primary production) of the unpolluted system or of its essential parts is known, the system will give a warning signal when through an unknown chemical stress it has been thrown so far out of balance that the natural variability is exceeded. Such ecosystem studies last a long time, and in addition it is often difficult to obtain a clear correlation with the cause of the changes. For fresh water, the classical 'saprobic system' of Kolkwitz and Marsson (1908) is an example of the systematic use of the species population as an indicator of pollution by bio-degradable organic substances. It also shows the limitation of the method. The system cannot be used for toxic pollution or in sea water. Even for fresh water it is still under discussion (see Pitwell's summary in Amavis and Smeets, 1975), although it has been shown here to be capable of application in various cases.

Some areas, such as estuaries, contain ecosystems with a low diversity index and a naturally unstable population. This is due to the great variability of environmental factors (light, temperature, salt content, etc.) in combination with an often short history on the evolutionary time scale. Against the background of this natural stress it is then difficult to use total ecological effects as a bio-indicator of pollution.

7.3.3 Individual bio-indicators

In certain communities characteristic species are found with a high fidelity to this community. In the sociology of plants, such characteristic species play a major role in the recognition of certain living communities. It is also possible to use such species for biological 'monitoring' of certain situations for general ecological purposes. According to Holdgate (1976), monitoring can be described as 'the repeated measurement of a variable so that a trend is defined that can be related to a goal or standard'. In addition to the analytical determination of waste substances, in this connection one can also use indicator organisms. Hueck (1975) has pointed out that in general three types of reactions to pollution may be envisaged:

- The organism is sensitive to pollution and will then decrease in number or quality.
- The organism tolerates the pollution within certain limits and will adapt itself to the polluted situation.
- The organism exploits pollution and thereby acquires an advantage over other organisms.

It is obvious that the positively or negatively reacting organisms can be used as pollution bio-indicators, but the organisms reacting neutrally in numerical terms may also be used as such. In many cases polluting substances will accumulate in these organisms, which they can tolerate up to a certain level. For example, the storage of such substances in the body fat may be regarded as an adaptation phenomenon. The determination of the degree of accumulation in these organisms can then be regarded as a measure of environmental pollution. Reasoning along similar lines, Baker (1976) came to the following classification of bio-indicators into four types.

- Detectors: naturally occurring species that show a measurable response to pollutants (for example, death or changes in behaviour, growth, or reproduction, etc.).
- Exploiters: naturally occurring species that profit from the presence of pollution. These species are frequently very numerous, since competing species have then been eliminated.
- Accumulators: naturally occurring species that take up waste substances and accumulate them in measurable amounts. Such species may be suitable for geographical monitoring studies.
- Sentinels: organisms the sensitivity of which is known and which are added to the medium as part of a 'physiological' warning system.

As follows from the above considerations, such bio-indicators can be brought into a 'passive' monitoring scheme by making use of species already present in the situation to be investigated. However, indicator organisms can also be introduced 'actively' and their reaction measured. This has the advantage that one is independent of the presence of sensitive species (by definition) and that, for example in the case of the tolerant accumulating species, a blank is available. We have had good experience with such active monitoring (De Kock, cited by Hueck, 1975).

7.3.4 Measurement network studies

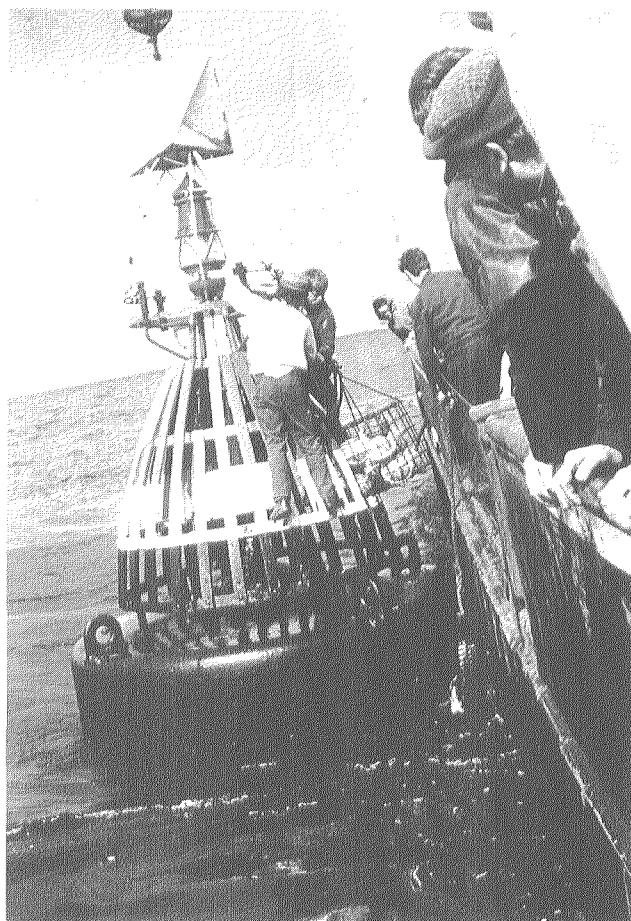
A specific use of accumulating bio-indicator organisms is that within the framework of a so-called measurement network. Naturally occurring organisms (cf. Pringle *et al.*, 1968; De Wolf and Lewis, 1972; Preston *et al.*, 1972; Butler, 1973) ('passive measurement network') or transplanted organisms (De Kock, cited by Hueck, 1975) ('active measurement network') may be used for this purpose.

Measurement of the polluting substances with the aid of accumulating organisms results in a certain integration in time. This is advantageous in comparison with intermittent measurements of waste substances in water samples where wide fluctuations occur frequently in the short term. The use of bio-accumulators in pollution studies to monitor the water quality may therefore mean a saving of costs, since the sampling needs to be less frequent than in direct chemical determinations in abiotic media. At the same time, direct information is obtained on the actual biotic stress, with which the analytically determined concentration present in the water is only in a remote relationship, e.g. because of binding to sediment, transformation, etc. To obtain an idea of the distribu-

tion of pollutants among all living organisms of a particular area it is necessary to choose a tolerant species that accumulates well and has a wide distribution. In view of its tolerance, the sensitivity of other species in the ecosystem will have to be determined in separate experiments and must be related to the accumulation level of the accumulator. Such laboratory studies are nevertheless already necessary to gain a knowledge of the accumulation and elimination characteristics of the tolerant bio-accumulator.

It has been found that the mussel *Mytilus edulis* is particularly suitable for such measurement networks. Its usefulness for indicating stress by metals has been demonstrated both in a passive measurement network (De Wolf, 1972, 1975) and in an active measurement network (De Kock, cited by Hueck, 1975). Goldberg (1975) has also recommended this organism. Moreover, it has been found that the active use of the mussel gives both more accurate and faster results than its passive use.

For the active measurement network mussels are harvested from a 'clean' location and placed in cages which are then set out for some weeks in the location where pollution is studied (see 7.3, Fig. 1). At the end of the exposure period the mussels are analysed to determine the accumulation of polluting compounds.



7.3 Fig. 1 Setting out mussels in cages for use in a biological pollution-measurement network

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PART III

A tabular review of the literature

by U. H. de Graaf



Standardized methods for the determination of the toxicity and degradability of chemicals in the aquatic environment

Tabular review of the literature

Since chemicals may pass into surface waters either directly or with effluents, in many countries laboratory tests have been described for the determination of the effects of such products in relation to their ecotoxicity and biodegradability.

Nationally and internationally accepted procedures have been developed for such tests, which can be adapted to individual substances. Most standard tests are static tests with fish, in which the acute toxicity is determined with death of the test organism as the evaluation criterion. The standard degradability tests were originally drawn up for detergents and are therefore more or less specific for this group of compounds.

Since studies on ecotoxicity and biodegradability in the laboratory with standard tests provide part of the information necessary for drawing up standards in this field, it appeared desirable to collect a number of relevant literature references and to arrange the corresponding standardized methods or ones accepted as standards according to their most important characteristics.

The test methods have been selected to the extent to which they have been standardized or have been accepted as standard. This means that the present review does not cover all the principles and procedures that have been proposed or used in this connection. *Methods are given in the order of bacteria, algae, representatives from the group of invertebrates, and certain species of fish.* Toxicity tests with fish based on sublethal criteria have been selected in relation to the possibility of automatic recording and their use in present-day environmental toxicology research.

The classification of the system is based primarily on the aim of the study (toxicity, degradability, or eutrophication) and then on the evaluation criterion (death or other aspects), the selected test mode (static and/or with a flow-through system), the principle, and the test organism.

Each method is described briefly, the following details being given in addition to the above-mentioned ones: procedure, test duration, medium, temperature, aeration/O₂ concentration, light, pH, number of organisms per unit volume at the start of the test, and others (treated separately as Remarks). Each number in the reference list corresponds to the number of the appropriate test in the scheme. The review was essentially terminated in 1976.



Bacteria: Determination of toxicity

Schubert, 1973

(1)

Criterion

Growth yield.

Principle

Counting the number of colonies.

Test organisms

Aeromonas hydrophila.

Procedure

Static, in 200–300-ml flasks. Inoculated with bacteria from a 24-h culture. After exposure, the bacteria are plated out and the number of colonies is counted after incubation for 24 h.

Test duration

Observation after 24 h and again after another 5 h and 24 h.

Medium

BOD dilution water.

Temperature

30° C.

Aeration

Not mentioned.

Light

Not mentioned.

pH

Not mentioned.

Number/vol.

50–200 colony-forming units in 0.1 ml.

Remarks

Starting from the same number of colony-forming units, the growth-yield of the test dishes is compared with that of control.

Association Française de Normalisation

(2)

Criterion

Growth yield.

Principle

Counting of the number of colonies.

Test organisms

Pseudomonas fluorescens.

Procedure

Static, in test-tubes. Inoculated with bacteria from a 24 h culture. Exposure: 24 h and 5 days. After exposure, the bacteria are incubated in Petri dishes, and after 5 days the number of colonies formed is counted. In duplicate.

Test duration

10 and 11 days. Observation after exposure for 24 h and 5 days.

Medium

Synthetic fresh water.

Temperature

25° C.

Aeration

None.

Light

Not mentioned.

pH

7.2.

Number/vol.

10⁶ microorganisms/ml.

Remarks

The concentrations at which 99.8% of the bacteria is killed after 24 h (C_1) and after 5 days (C_5) are determined.

Bacteria: Determination of toxicity

Abwasserabgabegesetz, 1974

(3)

Criterion

Growth yield.

Principle

Turbidimetric determination of the number of particles, with a spectrophotometer.

Test organisms

Pseudomonas fluorescens.

Procedure

Static, in closed 300-ml flasks. Inoculated with bacteria from a 24-h culture. In quadruplicate. In the stock culture the bacteria are transferred to fresh medium once a week.

Test duration

16 h. Observation before and after the test period.

Medium

Distilled water with added nutrients.

Temperature

25° C.

Aeration

None.

Light

Not mentioned.

pH

7.0.

Number/vol.

10 ml of bacterial suspension/100 ml.

Remarks

Cell multiplication is inhibited if the photometric absorbance of the test cultures is significantly lower than the absorbance in the control test.

Deutsche Einheitsverfahren, L 8, 1968

(4)

Criterion

Growth yield.

Principle

The conversion of glucose into organic acids is followed with a pH meter.

Test organisms

Pseudomonas fluorescens.

Procedure

Static, in open culture tubes. Inoculated with bacteria from a 96-h culture. At the start of the test the pH is adjusted to 7.5. The test is ended when the pH reaches 6 in the control sample. The bacteria of the stock culture are transferred to new medium once a month.

Test duration

At least 16 h. Observation every hour.

Medium

Undefined dilution water containing glucose.

Temperature

25° C.

Aeration

Not mentioned.

Light

Not mentioned.

pH

7.5.

Number/vol.

1 ml of bacterial suspension/10 ml.

Remarks

The conversion of glucose into organic acids is deemed to have been inhibited if the pH of the control is lower than that of the test solutions.

Bacteria : Determination of toxicity

Deutsche Einheitsverfahren, L 3, 1966

(5)

Criterion

Growth yield.

Principle

Photometric determination of the amount of reduced TTC (see Remarks).

Test organisms

Mixture of bacteria; activated sludge.

Procedure

Static, in centrifuge tubes. Culture of the activated sludge in synthetic sewage. 1 ml of TTC solution is added to the various test solutions.

Test duration

3 h.

Medium

Dechlorinated mains water; synthetic sewage.

Temperature

25° C.

Aeration

Not mentioned.

Light

None.

pH

Not mentioned.

Number/vol.

1 ml of activated sludge suspension/10 ml.

Remarks

2,3,5-Triphenyltetrazolium chloride (TTC) is reduced by the dehydrogenases of living cells to red 'formazan'.

Deutsche Einheitsverfahren, L 12, 1971

(6)

Criterion

Consumption of oxygen.

Principle

Titrimetric determination of the oxygen concentration.

Test organisms

Mixture of bacteria; sewage.

Procedure

Static, in oxygen flasks. The oxygen concentration of the control sample is determined directly at the start of the test. The initial oxygen concentration of the test solutions is about 5 ppm.

Test duration

24 h.

Medium

BOD dilution water; peptone solution.

Temperature

20–22° C.

Aeration

None.

Light

None.

pH

Not mentioned.

Number/vol.

Not mentioned.

Remarks

This test is a check to see how far biological self-purification is inhibited by the toxic substances present.

Bacteria: Determination of toxicity

Axt, 1973

(7)

Criterion

Consumption of oxygen.

Principle

Continuous measurement of the oxygen concentration with an oxygen electrode.

Test organisms

Pseudomonas fluorescens.

Procedure

Flow-through system consisting of a culture vessel with aeration and a measuring vessel. The flow rate is such that as many bacteria are washed out as are formed. This steady state is achieved after about 2 days. The mean residence time in the measuring vessel is 1 h.

Test duration

At least 30 min.

Medium

Undefined dilution water; nutrient solution.

Temperature

25° C.

Aeration

Pre-aeration; O₂ concentration < 0.5 ppm.

Light

Not mentioned.

pH

Not mentioned.

Number/vol.

2 × 10¹⁰ microorganisms/litre.

Remarks

With a decrease in the consumption of oxygen the electrode records a rise in the oxygen concentration. It is recommended to check this system with a standard substance.

Offhaus, 1969

(8)

Criterion

Consumption of oxygen.

Principle

Volumetric determination with the 'Sapromat'; 5-day BOD.

Test organisms

Mixture of bacteria.

Procedure

Static, in a closed system (volume 500 ml) with continuous compensation for the consumption of oxygen. The amount of oxygen necessary for this purpose is recorded on addition by an electrode. CO₂ is absorbed. In triplicate.

Test duration

At least 48 h. Observation every hour.

Medium

Standardized fresh water; peptone solution.

Temperature

20° C.

Aeration

None.

Light

None.

pH

6.5–8.

Number/vol.

Not mentioned.

Remarks

A decreased oxygen consumption is an indication of a toxic effect.

Bacteria: Determination of toxicity and degradability

Deutsche Einheitsverfahren, L 2, 1966

(9)

Criterion

Consumption of oxygen.

Principle

Manometric determination with the Warburg apparatus.

Test organisms

Mixture of bacteria: activated sludge.

Procedure

Measurement of the oxygen consumption as a pressure change in the Warburg apparatus; CO₂ is absorbed.

Test duration

3–5 days.

Medium

Standardized peptone-sodium chloride solution.

Temperature

20° C.

Aeration

Shaking.

Light

Not mentioned.

pH

Not mentioned.

Number/vol.

1 ml of activated sludge suspension/41 ml.

Remarks

A decreased oxygen consumption is an indication of a toxic effect; an increased oxygen consumption indicates degradation.

Ausgewählte Methoden, D, 1972

(10)

Criterion

Consumption of oxygen.

Principle

Manometric determination with the Warburg apparatus; BOD.

Test organisms

Mixture of bacteria; activated sludge.

Procedure

Measurement of the oxygen consumption as a pressure change in the Warburg apparatus; CO₂ is absorbed.

Test duration

At least 48 h.

Medium

BOD dilution water; peptone solution.

Temperature

20–25° C.

Aeration

Shaking.

Light

Not mentioned.

pH

Not mentioned.

Number/vol.

0.5 ml of bacterial suspension/20 ml.

Remarks

A decreased oxygen consumption is an indication of a toxic effect; an increased oxygen consumption indicates degradation.

Bacteria: Determination of degradability

American Society for Testing and Materials,
1970

(11)

Criterion

Degradation of the test compound.

Principle

5-day BOD with oxygen determination methods according to ASTM.

Test organisms

Mixture of bacteria.

Procedure

Static, in closed 250–300-ml flasks. The oxygen content is determined at the beginning and at the end of the test.

Test duration

5 days.

Medium

Standardized water according to ASTM.

Temperature

$20 \pm 1^\circ \text{C}$.

Aeration

None.

Light

None.

pH

7.0.

Number/vol.

Not mentioned.

Remarks

The activity of the bacteria is checked by measuring the degradation of a glucose-glutamic acid solution.

Japanese Environment Agency

(12)

Criterion

Degradation of the test compound.

Principle

Determination of the oxygen consumption, BOD. Determination of residual test substance and the TOC.

Test organisms

Mixture of bacteria; activated sludge.

Procedure

Static, closed system with oxygen-measuring apparatus. Semi-static culture of the bacteria in synthetic sewage.

Test duration

14 days.

Medium

Standard water according to the J.I.S.

Temperature

$25 \pm 2^\circ \text{C}$.

Aeration

Constant stirring.

Light

Not mentioned.

pH

7.0 ± 1.0 .

Number/vol.

0.03 ml of activated sludge suspension/litre.

Remarks

The activity of the bacteria is checked by measuring the degradation of aniline.

Bacteria : Determination of degradability

Gledhill, 1975

(13)

Criterion

Degradation of the test compound.

Principle

Titrimetric determination of the amount of CO₂ bound by baryta water.

Test organisms

Mixture of bacteria; activated sludge.

Procedure

Static, in 2-litre flasks on a shaker. Adaptation of the bacteria for 14 days.
Controls in duplicate.

Test duration

About 28 days.

Medium

Standardized nutrient solution.

Temperature

Room temperature.

Aeration

Yes, O₂ concentration >70 % of the saturation value.

Light

None.

pH

7.1.

Number/vol.

60 ml of activated sludge suspension/500 ml.

Remarks

The activity of the bacteria is checked by measuring the degradation of glucose.

Hammerton, 1955

(14)

Criterion

Degradation of the test compound.

Principle

Photometric determination of the MBAS* value.

Test organisms

Mixture of bacteria.

Procedure

Static, in flasks. The bacteria are naturally present in the river water used as the medium.

Test duration

24 h to 21 days. Observation after the test period.

Medium

River water.

Temperature

22° C.

Aeration

Not mentioned.

pH

Not mentioned.

Number/vol.

Not mentioned.

Remarks

The 'original' river die-away test.

*MBAS: Methylene-Blue-active substance.

Bacteria: Determination of degradability

Association Française de Normalisation, 1971

(15)

Criterion

Degradation of the test compound.

Principle

Photometric determination of the MBAS value.

Test organisms

Mixture of bacteria: sewage.

Procedure

Static, in 20-litre bottles. After incubation for 7 days, more test substance is added to the test solution.

Test duration

10 days. Observation after 7 and 10 days.

Medium

BOD dilution water; nutrient solution.

Temperature

$25 \pm 1^\circ \text{C}$.

Aeration

Yes.

Light

'Diffuse' daylight.

pH

7–7.2.

Number/vol.

10^6 to 10^7 microorganisms/ml.

Remarks

The activity of the bacteria is checked by measuring the degradation of sodium alkylbenzenesulphonate.

Japanese Industrial Standard, 1968

(16)

Criterion

Degradation of the test compound.

Principle

Photometric determination of the MBAS value.

Test organisms

Mixture of bacteria; sewage.

Procedure

Static, in 1-litre bottles on a shaker. Adaptation of the bacteria in which the bacteria are re-inoculated twice at 72-h intervals.

Test duration

8 days. Observation: 1st, 7th, and 8th days.

Medium

Undefined dilution water; nutrient solution.

Temperature

$25 \pm 3^\circ \text{C}$.

Aeration

Not mentioned.

Light

Not mentioned.

pH

Not mentioned.

Number/vol.

1 ml of bacterial suspension/100 ml.

Remarks

The activity of the bacteria is checked by measuring the degradation of n-dodecyl-sulphonate-1.

Bacteria: Determination of degradability

The Soap and Detergent Association, 1965

(17)

Criterion

Degradation of the test compound.

Principle

Photometric determination of the MBAS value.

Test organisms

Mixture of bacteria; activated sludge.

Procedure

A screening test; static, on a shaker. Adaptation: twice at 72-h intervals. If necessary, followed by a confirmatory test; semi-flow-through system with renewal every 24 h after aeration for 23 h.

Test duration

Screening: 8 days.
Observation on the 1st, 7th, and 8th days.
Confirmatory: at least 15 days.

Medium:

Screening: distilled water with nutrient solution.
Confirmatory: synthetic sewage.

Temperature

25 ± 3° C.

Aeration

Yes.

Light

Not mentioned.

pH

Not mentioned.

Number/vol.

1 ml of activated sludge suspension/100 ml.

Remarks

The activity of the bacteria in the system is checked by measuring the degradation of n-dodecyl-sulphonate-1.

Bundesministerium, 1962

(18)

Criterion

Degradation of the test compound.

Principle

Photometric determination of the MBAS value.

Test organisms

Mixture of bacteria; activated sludge.

Procedure

Flow-through system with special aeration chamber in which the activated sludge suspension is pumped round.
Retention time: 3 h.

Test duration

21 days.
Observation each day.

Medium

Synthetic sewage.

Temperature

Not mentioned.

Aeration

Yes, O₂ concentration > 2 ppm.

Light

Not mentioned.

pH

Not mentioned.

Number/vol.

Activated sludge suspension ≤ 3 g/litre.

Remarks

The system is checked by measuring the degradation of TBS.

Bacteria: Determination of degradability

Organisation for Economic Cooperation and Development, 1971

(19)

Criterion

Degradation of the test compound.

Principle

Photometric determination of the MBAS value.

Test organisms

Mixture of bacteria; activated sludge.

Procedure

A screening test: static, in 2-litre flasks on a shaker. If necessary, followed by a confirmatory test based on the German test (18). Screening in duplicate. The confirmatory test is ended as soon as the MBAS value has become constant.

Test duration

Screening: about 14 days.

Confirmatory: 21 days.

Observation: every day.

Medium

Screening: distilled water with a nutrient solution.

Temperature

$25 \pm 1^\circ \text{C}$.

Aeration

Yes, O_2 concentration > 2 ppm.

Light

Dark, screened from light.

pH

7.2.

Number/vol.

'Screening' inoculum 0.5 ml of filtered soil suspension.

'Confirmatory' biomass 2.5 g of activated sludge/l (dry weight).

Remarks

The system is checked by measuring the degradation of Marlon A and TBS (screening test).

Algae: Determination of toxicity

American Society for Testing and Materials,
1973

(20)

Criterion

Growth yield.

Principle

Counting of the number of cells under a microscope or with a hemacytometer, IL_m.

Test organisms

Nitzschia linearis, *Navicula seminulum*, *Navicula pelliculosa*.

Procedure

Static, in closed 125-ml flasks. Inoculated with algae in the logarithmic growth phase. In duplicate. The algal stock culture is transferred several times to fresh medium.

Test duration

7 days.
Observation: 1st and 7th days.

Medium

Standardized water according to ASTM or natural fresh water with nutrient solution.

Temperature

18–28°C.

Aeration

(Pre-)aeration or shaking.

Light

Artificial; illumination period 16 h.

pH

Not mentioned.

Number/vol.

1 ml of algal suspension/50 ml.

Remarks

It is assumed that the biologically safe concentration is equal to 0.3 times the concentration at which 50 % growth inhibition takes place.

Ausgewählte Methoden, D, 1972

(21)

Criterion

Growth yield.

Principle

Counting of the number of cells with a counting frame (Thoma chamber).

Test organisms

Ankistrodesmus falcatus.

Procedure

Static, in 300-ml flasks. Inoculated with algae from a 10- to 14-day-old culture.

The test is ended as soon as the control sample has a density of 2500–3000 cells/μl. The algal stock culture is transferred to fresh medium about 3 to 4 times.

Test duration

10–14 days.
Observation after the test period.

Medium

Mains water or distilled water with nutrients added.

Temperature

20°C.

Aeration

Shaking once a day.

Light

Artificial; illumination period 12 h.

pH

Not mentioned.

Number/vol.

2500 to 3000 cell/μl.

Remarks

The results are expressed as the concentration at which 50 % growth inhibition takes place.

Algae: Determination of toxicity

Bringmann & Kühn, 1974

(22)

Criterion

Growth yield.

Principle

Turbidimetric determination of the number of particles with a spectrophotometer.

Test organisms

Scenedesmus quadricauda.

Procedure

Static, in 10-ml culture tubes. Inoculation from a washed suspension having a transmission of 37%, from a 10-day-old culture. In quadruplicate. The algal stock culture is transferred to fresh medium every 10 days.

Test duration

8 days. Determination of the absorbance before and after the test period.

Medium

Double-distilled water with added nutrients.

Temperature

27°C.

Aeration

Shaking once a day.

Light

Artificial.

pH

7.0.

Number/vol.

1 ml of algal suspension/10 ml.

Remarks

Cell multiplication is deemed to have been inhibited if the absorbance of the test solution is lower than that in the control sample.

Gentile & Johnson, 1974

(23)

Criterion

Growth yield, growth rate.

Principle

Counting of the number of cells, preferably with an electronic particle counter; EC₅₀.

Test organisms

Skeletonema costatum, *Thalassiosira pseudonana*.

Procedure

Static, in 125-ml flasks. Inoculated with algae from a 1-week-old culture. The test is ended as soon as the growth in the control sample becomes stationary. In triplicate. The algal stock culture is transferred to fresh medium every 1–2 weeks. The algae must pass through at least two growth cycles in the medium to be used before starting the test.

Test duration

Observation : daily.

Medium

Natural or synthetic sea water; salt content 20 or 30‰. With nutrients.

Temperature

20°C or 8°C.

Aeration

Not mentioned.

Light

Artificial; illumination period 12 h.

pH

7.8–8.2.

Number/vol.

2500 cells/ml.

Remarks

It is recommended to use sodium dodecyl sulphate (SDS) simultaneously as a reference substance.

Algae: Determination of toxicity

Deutsche Einheitsverfahren, L 12, 1971

(24)

Criterion

Production of oxygen.

Principle

Titrimetric determination of the oxygen concentration.

Test organisms

Mixed culture of Protococcales.

Procedure

Static, in closed oxygen flasks. Inoculated with algae from a 10-day-old culture. Two flasks per test concentration in each case, one of which is inoculated with algae. Culture after spontaneous infection from the air.

Test duration

24 h.

Medium

BOD dilution water.

Temperature

20–22° C.

Aeration

None.

Light

Artificial.

pH

Not mentioned.

Number/vol.

Not mentioned.

Remarks

This method concerns the extent to which the biological self-purification process is inhibited by the toxic substances present.

Ausgewählte Methoden, C, 1972

(25)

Criterion

Production of oxygen.

Principle

Manometric determination with the Warburg apparatus.

Test organisms

Species of algae.

Procedure

Measurement of oxygen production as a pressure change in the Warburg apparatus. The partial pressure of CO₂ in the gas phase is kept constant by a buffer solution.

Test duration

Continuous.

Medium

Natural or synthetic sea water; salt content 30‰.

Temperature

15° C.

Aeration

Pre-aeration.

Light

Artificial: illumination period 8 h.

pH

8.

Number/vol.

Not mentioned.

Remarks

The method may also be used for the determination of the potential autotrophic activity of natural water.

Algae: Determination of toxicity

Plötz, 1975

(26)

Criterion

Production of oxygen.

Principle

Special measuring cell provided with an oxygen electrode.

Test organisms

Ulva lactuca.

Procedure

Static, in beakers or 250-ml flasks. Acclimatization period 3 days. In the measuring cell a thin bio-active layer is in direct contact with the membrane of an oxygen electrode.

Test duration

Continuous.

Medium

Natural or synthetic sea water; salt content 30‰.

Temperature

15°C.

Aeration

Pre-aeration.

Light

Artificial; illumination period 8 h.

pH

8.

Number/vol.

Not mentioned.

Remarks

Effects on algae and bacteria can be measured simultaneously.

Algae: Determination of eutrophication

Ausgewählte Methoden, C, 1972

(27)

Criterion

Growth yield.

Principle

Turbidimetric determination of the number of particles with a spectrophotometer.

Test organisms

Haematococcus pluvialis.

Procedure

Static, in closed culture tubes. Inoculated with algae from a phosphate-poor culture. The test is continued until a maximum amount of particles has been formed. In triplicate. The algal stock culture is transferred to fresh medium once a week.

Test duration

Observation : every 2 days.

Medium

Natural fresh water.

Temperature

22° C.

Aeration

Shaking; before and after the period of illumination.

Light

Artificial; illumination period 16 h.

pH

6–7.

Number/vol.

0.5–1.0 ml of algal suspension/8 ml.

Remarks

The method is used for the determination of the potential production and of the growth-limiting nutrients in natural water.

Norwegian Institute for Water Research, 1973

(28)

Criterion

Growth yield, growth rate.

Principle

Counting the number of cells with a Coulter Counter; determination of the dry weight

Test organisms

Selenastrum capricornutum, *Phaeodactylum tricornutum*, *Skeletonema costatum*.

Procedure

Static, in 100-ml flasks on a shaker. Inoculated with algae from a 1–2-week-old culture. The test is ended as soon as the growth yield in the control test reaches a maximum. The algal stock culture is transferred to fresh medium every 1 to 2 weeks.

Test duration

Observation : daily.

Medium

Nutrient solution; the last two species in sea water. Salt content 32‰.

Temperature

20 ± 1° C.

Aeration

Shaking.

Light

Artificial; illumination period 12 h.

pH

Not mentioned.

Number/vol.

0.5–15 × 10⁶ cells/litre.

Remarks

In addition to the potential production and the growth-limiting nutrients in natural water, toxic effects can be determined by this method.

Algae: Determination of eutrophication

U.S. Environmental Protection Agency, 1971

(29)

Criterion

Growth yield, growth rate.

Principle

Various methods for the determination of the biomass.

Test organisms

Selenastrum capricornutum, *Microcystis aeruginosa*,
Anabaena flos-aquae.

Procedure

Static, in flasks on a shaker. Inoculated with algae from a 1–2-week-old culture. The test is ended as soon as the increase in biomass falls below 5%. In triplicate. The algal stock culture is transferred to fresh medium once a week.

Test duration

5 days. Observation: daily.

Medium

Natural fresh water.

Temperature

$24 \pm 2^\circ \text{C}$.

Aeration

Shaking.

Light

Artificial.

pH

Below 8.5.

Number/vol.

$1\text{--}50 \times 10^6$ cells/litre.

Remarks

The method is used for the determination of the potential production and of the growth-limiting nutrients in natural water.

U.S. Environmental Protection Agency, 1974

(30)

Criterion

Growth yield, growth rate.

Principle

Various methods for the determination of the biomass.

Test organisms

Dunaliella tertiolecta, *Thalassiosira pseudonana*.

Procedure

Static, in flasks on a shaker. Inoculated with algae from a 5–7-day-old culture. The test is ended as soon as the increase in the biomass falls below 5%. The algal stock culture is transferred to fresh medium once a week. The procedure is based on the algal assay procedure (29).

Test duration

Observation: daily.

Medium

Natural sea water.

Temperature

$18 \pm 2^\circ \text{C}$.

Aeration

Shaking.

Light

Artificial.

pH

8.0.

Number/vol.

10^5 cells/litre.

Remarks

The method is used for the determination of the potential production and of the growth-limiting nutrients in natural sea water.

Protozoa : Determination of toxicity

Ausgewählte Methoden, D, 1972

(31)

Criterion

Death, abnormal behaviour.

Principle

Counting the dead animals and/or those behaving abnormally.

Test organisms

***Paramecium caudatum*.**

Procedure

Static, in Petri dishes. Paramecium is cultured together with algae and transferred to fresh medium every 2 weeks. A population approximately 10 days old is used for the test, which is performed in duplicate or triplicate.

Test duration

48 h. Observation every hour during the first 8 h.

Medium

Undefined fresh water.

Temperature

Not mentioned.

Aeration

Not mentioned.

Light

Natural.

pH

Not mentioned.

Number/vol.

At least 50/10 ml.

Remarks

Abnormal behaviour is determined from the movement, beating of cilia, and pulsation frequency of the vacuoles.

Deutsche Einheitsverfahren, L 10, 1968

(32)

Criterion

Inhibition of the food uptake.

Principle

Turbidimetric determination of the (bacterial) food suspension.

Test organisms

***Colpoda maupasi*.**

Procedure

Static, in closed 100-ml flasks. Inoculated with Colpoda from a 48-h culture. 1 ml of a bacterial suspension is added for each ml of Colpoda.

Test duration

At least 20 h.

Medium

Undefined fresh water.

Temperature

27°C.

Aeration

None.

Light

Not mentioned.

pH

7.5.

Number/vol.

1 ml of Colpoda suspension/10 ml.

Remarks

Tests are carried out at various concentrations with and without Colpoda as a control on the growth of the bacteria.

Hydrozoa: Determination of toxicity

Karbe, 1972

(33)

Criterion

Growth, resorption of the polyps.

Principle

Stereomicroscopic determination of growth, resorption, and morphological damage.

Test organisms

Eirene viridula.

Procedure

Semistatic, in 200-ml glass dishes. Renewal frequency: twice a week. The polyps are cultured in natural sea water and fed with the larvae of *Artemia salina*. After the period of exposure the polyps are placed in sea water not containing toxic substances. In duplicate.

Test duration

3 weeks to 3 months. Observation after 24 h and then every week.

Medium

Natural sea water; salt content 30‰.

Temperature

20°C.

Aeration

Not mentioned.

Light

Not mentioned.

pH

7.9–8.2.

Number/vol.

Not mentioned.

Remarks

Six different levels of toxicity are defined, a distinction being made between reversible and irreversible resorption (regeneration) of the polyps.

Mollusca: Determination of toxicity

Woelke, 1967

(34)

Criterion

Morphogenesis of eggs and larvae.

Principle

Microscopic determination of the number of eggs and larvae developing abnormally.

Test organisms

Crassostrea gigas.

Procedure

Static, in beakers. The time at which the eggs are laid can be determined beforehand. Eggs of the same age are used for the test. Samples of 150–250 larvae are counted and the percentage of abnormal larvae is calculated.

Test duration

48 h.

Medium

Undefined sea water

Temperature

20°C.

Aeration

None.

Light

Not mentioned.

pH

Not mentioned.

Number/vol.

Suspension of 20,000–30,000 eggs/litre.

Remarks

Under normal conditions fertilized eggs develop into free-swimming veligers during the first 48 h.

Annelida: Determination of toxicity

Ausgewählte Methoden, D, 1972

(35)

Criterion

Death, abnormal behaviour.

Principle

Counting the dead or abnormally behaving individuals; LC_{50} .

Test organisms

Tubifex spp. or *Limnodrilus* spp.

Procedure

Static, in crystallizing dishes. The worms are collected from the natural environment and are placed in a flow-through system for 2 days before the start of the test. In duplicate or triplicate.

Test duration

48 ± 2 h. Observation every hour during the first 8 h.

Medium

Undefined fresh water.

Temperature

Not mentioned.

Aeration

Not mentioned.

Light

Not mentioned.

pH

Not mentioned.

Number/vol.

10/100 ml.

Remarks

Effects of the respiration of Tubificidae can be measured with the Warburg apparatus at the same time.

Åkesson, 1970

(36)

Criterion

Death, growth, and reproduction.

Principle

Counting the dead individuals and the numbers of parapodia and of egg-packets; measurements of the body length.

Test organisms

Ophryotrocha labronica.

Procedure

Semi-static, in closable 15–80-ml crystallizing dishes. Renewal frequency: once or twice a week. *Ophryotrocha* is fed with spinach. The time at which the egg-packets are produced can be adjusted by varying the temperature. Larvae of the same age are used for the test. Size: ≤ 4.5 mm.

Test duration

One month.

Medium

Natural sea water; salt content 32–34‰.

Temperature

20°C.

Aeration

Not mentioned.

Light

Not mentioned.

pH

Not mentioned.

Number/vol.

About 75 larvae/15–80 ml.

Remarks

The reproductive potential of *Ophryotrocha* is high: an average of 120 eggs per female. At 20°C the whole life cycle is completed in one month.

Crustacea: Determination of toxicity

Deutsche Einheitsverfahren, L 11, 1968

(37)

Criterion

Motility.

Principle

Counting the number of motile individuals.

Test organisms

Daphnia magna.

Procedure

Static, in test tubes. The Daphnias are fed with algae, *Scenedesmus quadricauda*. The Daphnias are 24 h old.

Test duration

48 h.

Medium

Undefined fresh water.

Temperature

17° C.

Aeration

None.

Light

Artificial.

pH

7.5.

Number/vol.

10/10 ml.

Remarks

None.

Ausgewählte Methoden, D, 1972

(38)

Criterion

Death, abnormal behaviour.

Principle

Counting the dead or abnormally behaving individuals, LC₅₀.

Test organisms

Daphnia magna.

Procedure

Static, in 400-ml beakers. The Daphnias are fed with algae, *Chlorella* or *Scenedesmus*, and other microorganisms. In quintuplicate.

Test duration

24 h. Observation every hour during the first 8 h.

Medium

Undefined fresh water.

Temperature

25° C.

Aeration

Not mentioned.

Light

Natural.

pH

Not mentioned.

Number/vol.

10/400 ml.

Remarks

The mortality in the control sample must not exceed 10 %.

Crustacea: Determination of toxicity

Association Française de Normalisation, 1974

(39)

Criterion

Death.

Principle

Counting the number of live individuals; TL_m .

Test organisms

Daphnia magna.

Procedure

Static, in tubes covered with aluminium foil. The Daphnias are fed with microorganisms derived from soil extracts. No food during the test. In quadruplicate.

Test duration

24 h.

Medium

Double-distilled water with nutrients.

Temperature

 $20 \pm 2^\circ \text{C}$.

Aeration

 O_2 concentration $> 80\%$ of the saturation value.

Light

None.

pH

 8.0 ± 0.2 .

Number/vol.

5/10 ml.

Remarks

Only the third and later generations, bred in the laboratory, are used for the tests.

International Standards Organisation, 1975

(40)

Criterion

Death.

Principle

Counting the number of dead individuals: TL_m .

Test organisms

Daphnia magna.

Procedure

Static, in 250–300-ml vessels. The Daphnias are fed with algae, *Chlorella* or *Scenedesmus* sp. No food during the test. The Daphnias are of the same size and age. In triplicate.

Test duration

48 h.

Medium

Undefined fresh water; mains water.

Temperature

 $18\text{--}20^\circ \text{C}$.

Aeration

Pre-aeration.

Light

Not mentioned.

pH

Not mentioned.

Number/vol.

10/200 ml.

Remarks

During the cultivation, natural illumination for the growth of the algae. Food suspension density $1\text{--}1.5 \times 10^4$ algal cells/ml.

Crustacea: Determination of toxicity

Indian Standard, 1971

(41)

Criterion

Death.

Principle

Counting the living individuals; TL_m .

Test organisms

Daphnia magna.

Procedure

Static, in 150-ml flasks. The Daphnias are fed with a suspension of microorganisms. No food during the test. The Daphnias are 8 h old.

Test duration

24–48 h. Observation: every 12 h.

Medium

Undefined fresh water.

Temperature

Not mentioned.

Aeration

Not mentioned.

Light

Not mentioned.

pH

6.5–8.5.

Number/vol.

50; at least 10/150 ml.

Remarks

None.

Biesinger, 1972

(42)

Criterion

Death, reproduction.

Principle

Counting the live and newly hatched individuals.

Test organisms

Daphnia magna.

Procedure

Static, in 250-ml beakers or flow-through system in 2-litre tanks. Renewal frequency: 2–4 times the contents/24 h. Also continuous supply of food. Acclimatization period: 3 weeks. The Daphnias are 12–24 h old. In quadruplicate.

Test duration

3 weeks.

Medium

Undefined fresh water.

Temperature

$18 \pm 1^\circ \text{C}$.

Aeration

O_2 concentration 80 % of the saturation value.

Light

Artificial; illumination period 16 h.

pH

Not mentioned.

Number/vol.

5/200 ml.

Remarks

The percentage survival in the controls must be greater than 70 % after 3 weeks. Only the second and later generations raised in the laboratory are used for the tests.

Crustacea : Determination of toxicityZillioux *et al.*, 1973

(43)

Criterion

Death.

Principle

Counting the living individuals with the aid of the photometric reaction of Artemias.

*Test organisms**Artemia salina*.*Procedure*

Static, in 20-ml beakers. Artemia eggs are hatched. 24-h-old Artemias are used for the test. In duplicate.

Test duration

48 h.

Medium

Artificial sea water; salt content 34,5‰.

Temperature

20° C.

Aeration

Not mentioned.

Light

Artificial.

pH

8.0.

Number/vol.

20/15 ml.

Remarks

Sodium dodecyl sulphate is used simultaneously as a reference substance.

Jensen, 1975

(44)

Criterion

Hatchability of the eggs.

Principle

Counting the number of hatched eggs; C_mHR: median hatching rate concentration.

*Test organisms**Artemia salina*.*Procedure*

Static, in 50-ml flasks. A 'pinch' of eggs is added to the test solution.

Test duration

5 days.

Medium

Natural or synthetic sea water; salt content 30–35‰.

Temperature

20° C.

Aeration

Yes.

Light

Not mentioned.

pH

Not mentioned.

Number/vol.

Not mentioned.

Remarks

Artemia eggs can be stored in dry form for a long time. The percentage hatch remains high.

Crustacea: Determination of toxicity

Gentile *et al.*, 1974

(45)

Criterion

Death.

Principle

Counting the dead individuals, LC₁₀, LC₅₀, LC₁₀₀.

Test organisms

Acartia tonsa.

Procedure

Static, in 100-ml dishes. Acartia is fed with algae, *Skeletonema costatum*. It passes its full life cycle in the test medium before the start of the test. In quadruplicate.

Test duration

96 h. Observation: daily.

Medium

Natural or synthetic sea water; salt content 20‰.

Temperature

20°C.

Aeration

None.

Light

Artificial; illumination period 14 h.

pH

8.0 ± 0.2.

Number/vol.

15/100 ml.

Remarks

The mortality in the controls must not exceed 15%. Effects on growth and reproduction can be investigated at the same time.

Ausgewählte Methoden, D, 1972

(46)

Criterion

Mortality, abnormal behaviour.

Principle

Counting the dead or abnormally behaving individuals.

Test organisms

Asellus aquaticus.

Procedure

Static, in enclosed dishes. Asellus is bred in the laboratory and supplied with food up to 24 h before the start of the test. Size: 5–10 mm. In quintuplicate.

Test duration

48 h. Observation every hour during the first 8 h.

Medium

Undefined fresh water; mains water.

Temperature

Room temperature.

Aeration

None.

Light

Not mentioned.

pH

Not mentioned.

Number/vol.

10/50 ml.

Remarks

The establishment of a suitable Asellus culture in the laboratory requires 1–2 years.

Echinodermata: Determination of toxicity

Hägestrom & Lönning, 1973

(47)

Criterion

Morphogenesis of the eggs.

Principle

Cytological investigation of development with an electron microscope.

Test organisms

Paracentrotus lividus.

Procedure

The development of the fertilized eggs is stopped with sodium lauryl sulphate after predetermined periods. The test is carried out with eggs of the same egg population and of the same age.

Test duration

About 48 h. Observation: every 3 h.

Medium

Natural sea water.

Temperature

18–20° C.

Aeration

Not mentioned.

Light

Not mentioned.

pH

Not mentioned.

Number/vol.

Suspensions of 1000 eggs/ml and 10⁵ spermatozoa/ml.

Remarks

Each female yields 8 to 10 million eggs. Effects on chromosomes of the larvae can also be studied.

Pisces: Determination of toxicity

American Society for Testing and Materials, 1970

(48)

Criterion

Death.

Principle

Counting dead individuals (no longer showing respiratory movements); TL_m .

Test organisms

Centrarchidae, *Salmonidae*, *Cyprinidae*, or *Catostomidae*.

Procedure

Static or semi-static, in closed glass 20-litre (5-gallon) bottles. Acclimatization period at least 10 days. Food until 2 days before the start of the test. Size: 51–76 mm.

Test duration

96 h. Observation: every 24 h.

Medium

De-ionized fresh water; standardized water according to ASTM.

Temperature

20–25°C or 12–18°C.

Aeration

If necessary: O_2 concentration ≥ 4 –5 ppm.

Light

Not mentioned.

pH

7.9 $\pm$ 0.1.

Number/vol.

1 g/litre.

Remarks

The mortality during the acclimatization period must be below 10 %. If effluent is tested, a species that occurs naturally in the receiving water must be selected.

American Public Health Association, 1971

(49)

Criterion

Death, abnormal behaviour.

Principle

Counting the number of dead individuals (no longer showing respiratory movements); TL_m .

Test organisms

Centrarchidae, *Salmonidae*, or *Cyprinidae*.

Procedure

Static or semi-static, in 20-litre glass tanks or flow-through systems; renewal frequency: 4 times the contents/24 h. Acclimatization period 10 to 30 days. Food up to 2 days before the start of the test. Size: ≤ 75 mm. In duplicate or triplicate.

Test duration

48 h or 96 h. Observation: every 24 h.

Medium

Undefined fresh water

Temperature

25 $\pm$ 2°C or 15 $\pm$ 2°C.

Aeration

If necessary: O_2 concentration ≥ 4 –5 ppm.

Light

Not mentioned.

pH

Not mentioned.

Number/vol.

10 fish; 1 g/litre.

Remarks

The mortality during the acclimatization period must be below 5 %; mortality in the control sample < 10 %. If effluent is tested, a species must be chosen that occurs naturally in the receiving water.

Pisces: Determination of toxicity

Ministry of Housing and Local Government,
U.K., 1969

(50)

Criterion

Death.

Principle

Counting the dead individuals; LC_{50} .

Test organisms

Salmo gairdneri.

Procedure

Static, in 20-litre aquaria. Acclimatization period: 2 days. Size: 60–150 mm.

Test duration

48 h.

Medium

Standardized fresh water; hardness: 25 or 250 mg/l.

Temperature

$15 \pm 1^\circ \text{C}$.

Aeration

Yes; O_2 concentration >5 ppm.

Light

Not mentioned.

pH

Not mentioned.

Number/vol.

10/20 litres.

Remarks

None.

Eidgenössisches Ministerium des Innern, 1971

(51)

Criterion

Death.

Principle

Counting the dead individuals, LC_{50} .

Test organisms

Salmo gairdneri, *Salmo trutta*, *Leuciscus cephalus*, *Phoxinus phoxinus*.

Procedure

Static, in glass 5–10-litre aquaria. Acclimatization period: 1–2 weeks. Size: 60–150 mm.

Test duration

At least 24 h.

Medium

Undefined fresh water.

Temperature

$16 \pm 2^\circ \text{C}$.

Aeration

If necessary: O_2 concentration $>90\%$ of the saturation value.

Light

Not mentioned.

pH

7.5.

Number/vol.

2–5 in the preliminary test, then 10.

Remarks

The mortality during acclimatization must not exceed 10%.

Pisces: Determination of toxicity

Bundesministerium des Innern, 1972

(52)

Criterion

Death.

Principle

Counting the dead individuals (no longer showing respiratory movements); LC₅₀.

Test organisms

Idus idus melanotus, *Leuciscus idus melanotus*.

Procedure

Static, in glass 12–15-litre tanks. Acclimatization period: at least 3 days. Food up to 48 h before the start of the test. Size 50–70 mm.

Test duration

48 h. Observation: after 2, 4, 24, and 48 h.

Medium

Dechlorinated mains water or standardized fresh water.

Temperature

20°C.

Aeration

Yes; O₂ concentration $\geq$ 5 ppm.

Light

Not mentioned.

pH

7.

Number/vol.

10/10 litres.

Remarks

The mortality of the stock population must not exceed 1 %.

Ausgewählte Methoden, D, 1972

(53)

Criterion

Death, abnormal behaviour.

Principle

Counting the dead individuals or ones behaving abnormally; LC₅₀.

Test organisms

Poecilia (Lebistes) reticulata.

Procedure

Static, in 5-litre glass aquaria. During the breeding process the young animals must be separated from the parents, since the adult fish frequently eat their young. No food during the test. Size: average 20 mm. In duplicate or triplicate.

Test duration

3–4 days. Observation: every hour for the first day and after this 3 or 4 times a day.

Medium

Undefined fresh water.

Temperature

20°C.

Aeration

None; O₂ concentration > 5 ppm.

Light

Indirect, natural.

pH

Not mentioned.

Number/vol.

5–10/5 litres.

Remarks

The mortality of the stock population must not exceed 5 %; the mortality in the controls must be zero.

Pisces: Determination of toxicity

Tunstall Laboratory, 1973

(54)

Criterion

Death, abnormal behaviour.

Principle

Counting the dead individuals or ones behaving abnormally.

Test organisms

Rasbora spp. and *Poecilia* spp.

Procedure

Static, in 2-litre beakers. 5 Rasboras and 5 Poecilias are placed in each test concentration. Acclimatization period: 2–3 weeks. Food up to 24 h before the start of the test. Size: fish of the same length.

Test duration

96 h. Observation: every 24 h.

Medium

Standardized hard and soft fresh water.

Temperature

20–24°C.

Aeration

Yes; O₂ concentration > 5 ppm.

Light

Artificial, with day-and-night rhythm.

pH

Not mentioned.

Number/vol.

5 Rasboras and 5 Poecilias/2 litres.

Remarks

The mortality in the control sample must be zero.

Ministry of Agriculture and Forestry of Japan, 1974

(55)

Criterion

Death, abnormal behaviour.

Principle

Counting the dead individuals, TL_m.

Test organisms

Cyprinus carpio, *Pseudorasbora parva*, or *Oryzias latipes*.

Procedure

Static, in 10-litre glass tanks. Acclimatization period: at least 1 week. Food until 2 days before the start of the test. Size of the carp: 50 mm.

Test duration

72 h. Observation: every 24 h.

Medium

Undefined fresh water.

Temperature

20–28±2°C.

Aeration

None.

Light

Not mentioned.

pH

Not mentioned.

Number/vol.

5–10/10 litres; 1 g/litre.

Remarks

The mortality in the controls must not exceed 10%. It is recommended to test sodium pentachlorophenoxide at the same time. Official toxicity categories are given. Accumulation is also tested by a standardized method.

Pisces: Determination of toxicity

Norwegian Institute for Water Research, 1973

(56)

Criterion

Death.

Principle

Counting the surviving individuals

Test organisms

Salmo salar.

Procedure

Semi-static, in 5-litre glass aquaria. Fertilized eggs are hatched in the laboratory and grown on the test medium. No food during the test. Size: 30–50 mm.

Test duration

3–6 days.

Medium

Undefined fresh water; soft mains water.

Temperature

10°C.

Aeration

Yes.

Light

Not mentioned.

pH

6.3.

Number/vol.

3–5/5 litres

Remarks

Salmon and trout are also tested in a flow-through system.

Environmental Protection Service, Canada, 1972

(57)

Criterion

Death.

Principle.

Counting the dead individuals; LC₅₀.

Test organisms

Salmo gairdneri.

Procedure

Semi-static or flow-through system. Renewal frequency; 2.0 litres/gram of fish/24 h. Food up to 2 days before the start of the test. Size: 2–10 g, the largest being less than twice the size of the smallest. Semi-static test in triplicate.

Test duration

96 h.

Medium

Undefined fresh water or sea water.

Temperature

15±1°C

Aeration

Yes: O₂ concentration ≥ 8 ppm.

Light

Not mentioned.

pH

Not mentioned.

Number/vol.

10; 1 g/200 ml.

Remarks

The mortality in the controls must be zero.

Pisces: Determination of toxicity

Davis and Mason, 1973

(58)

Criterion

Death.

Principle

Counting the dead individuals (no longer showing respiratory movements); LC_{50} .

Test organisms

Oncorhynchus kisutch, *Oncorhynchus nerka*, and *Oncorhynchus gorbuscha*.

Procedure

Static, in glass aquaria or in a flow-through system. Rate of flow: 5.5 litres/h. The fish are hatched and raised in the laboratory. No food during the test. Size: 40–110 mm.

Test duration

96 h. Observation: every 24 h.

Medium

Undefined fresh water; hardness: 15–20 ppm.

Temperature

10–13 ± 5° C.

Aeration

Yes.

Light

Not mentioned.

pH

Not mentioned.

Number/vol.

1 g /2.5 litres.

Remarks

Nets are hung in the aquaria so that the fish can be lifted out and inspected at close quarters.

Ministry of Agriculture, Fisheries and Food, U.K., 1969

(59)

Criterion

Death.

Principle

Counting the dead individuals; LC_{50} .

Test organisms

Rasbora heteromorpha.

Procedure

Flow-through system in 500-ml or 1000-ml bottles. Rate of flow: 100 ml/10 min. Acclimatization period: at least 1 week. Food until the day on which the test begins. Size: 13–30 mm.

Test duration

48 h. Observation: every 24 h.

Medium

Standardized fresh water; hardness: 20 mg $CaCO_3$ /litre.

Temperature

20 ± 0.5° C.

Aeration

Pre-operative; O_2 concentration >90% of the saturation value.

Light

Not mentioned.

pH

Not mentioned.

Number/vol.

10/500 ml.

Remarks

The mortality in the controls must be zero.

Pisces: Determination of toxicity

International Standards Organisation, 1975

(60)

Criterion

Death.

Principle

Counting the dead individuals; LC_{50} .

Test organisms

Brachydanio rerio.

Procedure

Flow-through system in 1-litre bottles. Flow rate: 25 litres/24 h. Acclimatization period: at least 2 weeks.

Test duration

96 h. Observation after 3, 6, 12, 24, and 96 h.

Medium

Standardized fresh water; hardness: 100 mg $CaCO_3$ /l

Temperature

$23.5 \pm 1^\circ C$.

Aeration

None; O_2 concentration > 70 % of the saturation value.

Light

Not mentioned.

pH

7.8 ± 0.2 .

Number/vol.

10/litre.

Remarks

The mortality of the stock population must be below 10%; the mortality of the controls must not exceed 10%.

U.S. Environmental Protection Agency, 1972

(61)

Criterion

Death, growth, and reproduction.

Principle

Counting the number of dead – TL_{50} – and young individuals. The growth is determined photographically.

Test organisms

Salvelinus fontinalis, *Lepomis macrochirus*, *Pimephales promelas*, *Jordanella floridae*.

Procedure

Flow-through systems for the various life stages. Renewal frequency: 6–10 times the contents/24 h. Acclimatization period: at least 1 month. A description of the food necessary for the various life stages is given. In duplicate.

Test duration

96 h to 3 months. Observation: every 24 h.

Medium

Undefined fresh water.

Temperature

$25-28^\circ C$ or $9-15^\circ C$.

Aeration

Yes; O_2 concentration > 60 % of the saturation value.

Light

Artificial.

pH

Not mentioned.

Number/vol.

6 fish.

Remarks

Effects on reproduction and on the development of eggs and larvae can be studied at the same time.

Pisces : Determination of toxicity from sublethal effects

Juhnke and Besch, 1971

(62)

Criterion

Disturbance of positive rheotaxis.

Principle

Automatic recording of the mechanical pressure against a framework exerted by the fish in flowing water.

Test organisms

Cyprinus carpio.

Procedure

Elliptical flow-through system in which two flow directions are possible. Rate of flow : 20 cm/sec. Acclimatization period : 5 days. Size : 80–120 mm. A disturbance of positive rheotaxis is defined when 25% of the fish remain pressed against the framework for at least 30 sec.

Test duration

Unlimited.

Medium

Undefined fresh water.

Temperature

10–17°C.

Aeration

O₂ concentration > 80% of the saturation value.

Light

Not mentioned.

pH

Not mentioned.

Number/vol.

1 fish/2.2 litres.

Remarks

The method can be used exclusively for acute toxicity tests. A test of the same type has been proposed as a standard test in France, Switzerland, and the Netherlands.

Spoor *et al.*, 1971

(63)

Criterion

Respiratory and other movements.

Principle

An electrode chamber in which any movement of the water changes the potential difference between the electrodes.

Test organisms

Lepomus macrochirus.

Procedure

Flow-through system consisting of an electrode chamber with an electrode at each end. Rate of flow : 200–400 ml/min. The chamber is somewhat narrower than the length of the fish; the fish remains oriented along the long axis. Acclimatization period : a few days. Size : 100 mm. In quadruplicate.

Test duration

Unlimited. Observation : 2 min every hour.

Medium

Undefined fresh water.

Temperature

20°C.

Aeration

Not mentioned.

Light

Not mentioned.

pH

Not mentioned.

Number/vol.

One fish per test chamber.

Remarks

The potential difference is also affected by factors such as temperature, O₂ concentration, pH, and salt content. However, the sensitivity of the system is not decreased if these factors are kept constant.

Pisces: Determination of toxicity from sublethal effects

Lindahl, 1971

(64)

Criterion

Capacity to compensate the torsion of a rotating body of water.

Principle

Determination of the critical rate of rotation.

Test organisms

Leuciscus rutilus.

Procedure

Flow-through system consisting of a narrow horizontal tube rotating around its axis. Before the fish are placed separately in the rotary flow they are exposed to the test medium for 5–16 days. No food during the test. Size: 100–150 mm.

Test duration

Unlimited.

Medium

Undefined fresh water, mains water

Temperature

8° C.

Aeration

Yes: O₂ concentration > 8.3 ppm.

Light

Not mentioned.

pH

Not mentioned.

Number/vol.

During exposure: 10/30 litres.

Remarks

None.

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