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Toxicological investigations bearing on pollution problems in the North Sea

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Summary

In the introduction it is suggested that toxicological investigations concerning pollution problems in the marine environment should aim at the following points:

a. *long-term experiments, preferably covering one or more generations of the test organisms;*
b. *investigation of transport and accumulation of toxic agents within and between species, preferably linked in a food chain;*
c. *investigation of the influence of secondary stress (e.g. starvation, 0_2- deficiency etc.), on toxicity levels.*

The experimental part describes the set up of laboratory test system covering the first two mentioned items. In principle it is an artificial food chain consisting of unicellular algae, microcrustaceans and fishes in which each member is investigated in continuous-flow experiments. Some results obtained in experiments with algae and daphniae, using copper as the toxic agent, are given. In a comparison of static cultures, intermittently renewed cultures and continuous-flow experiments of Dahpnia magna *it is shown that continuous-flow experiments are the more discriminating and sensitive tools. As parameters for the assessment of the results, both mortality and reproductive capacity (generation size) were used. As might be expected, it is found that reproduction is the more sensitive measure for toxicity.*
The alga used (Chlorella pyrenoidosa) *accumulates copper in its cells. Whilst the inhibiting concentration for growth of* Daphnia magna *was about 56 p.p.b., it was found that* Chlorella pyrenoidosa *could stand concentrations as high as 1000 p.p.b.*
However, it was found that Daphnia magna, *when fed with algae cultured in copper containing media, only died when fed with algae which had been grown in a medium containing 560 p.p.b.*
It appears, therefore, that even with a concentration of copper in these algae, poisoning by way of food is less efficient than direct poisoning by way of the medium. The amount and the rate of feeding should be taken into account.

Samenvatting

Uiteengezet wordt in de inleiding, dat toxicologisch onderzoek betreffende mariene afvalproblemen de volgende doelen dient na te streven:

a. *langdurige experimenten die bij voorkeur een of meer generaties van de toetsorganismen omvatten;*
b. *onderzoek van transport en ophoping van toxische stoffen in en tussen soorten welke bij voorkeur in een voedselketen verbonden dienen te zijn;*
c. *onderzoek naar de invloed van secundaire belasting (bijv. ondervoeding, tekort aan zuurstof, etc.) op het niveau van de giftigheid.*

Het experimentele deel beschrijft een proefopzet in het laboratorium die de eerstgenoemde twee punten betreft. In principe is het een kunstmatige voedselketen die uit eencellige algen, microcrustaceeën en vissen bestaat en waarbij iedere deelnemende soort in doorstroomexperimenten wordt onderzocht. Enige proefresultaten verkregen met algen en daphnia's en met koper als toxisch agens worden beschreven. Uit een vergelijking van statische cultures met tussentijds vernieuwde cultures en doorstroomexperimenten met Daphnia magna *wordt aangetoond, dat doorstroomexperimenten gevoeligere en beter discriminerende onderzoekmethodes zijn. Als parameters voor het beoordelen van de resultaten werden zowel mortaliteit als voortplantingscapaciteit (generatiegrootte) gebruikt.*
Zoals verwacht mocht worden, werd gevonden dat de voortplanting een gevoeliger maat is voor de toxiciteit dan de mortaliteit.
De gebruikte alg (Chlorella pyrenoidosa) *hoopt koper in zijn cellen op. Terwijl de remmende concentratie voor de toename van* Daphnia magna *bij ongeveer 56 p.p.b. was gelegen werd voor* Chlorella pyrenoidosa *gevonden dat het concentraties van 1000 p.p.b. kon verdragen. Wanneer echter* Daphnia magna *gevoed werd met algen gekweekt in koperbevattend medium, dan gingen ze pas dood met algen die gekweekt werden in een medium dat 560 p.p.b. bevatte. Blijkbaar is zelfs met de waargenomen ophoping van koper in deze algen de vergiftiging door middel van voedsel minder efficiënt dan die welke direkt via het medium tot stand komt. Klaarblijkelijk spelen omvang en snelheid van voedselopname een belangrijke rol.*

1. Introduction

Already some time ago, problems connected with the increasing need for disposal of industrial and domestic waste into the North Sea have drawn the attention of scientists in the Netherlands (cf de Wolf, 1965). As a result of a tentative distribution of work among several laboratories and institutes concerned, the Central Laboratory TNO undertook to study the toxicological aspects of marine water pollutants. For this purpose, we wish to establish a laboratory system in which we may introduce, under more or less defined conditions, the different pollutants coming under investigation. It appears furthermore to be desirable to check the toxic effects of pollutants, either in field experi-

ments or under semi-natural conditions when field experiments are impractical.
In this paper we shall discuss the guiding principles adopted for the laboratory system, together with some preliminary results which we have obtained with copper as a toxic agent.

2. Aims of the investigation

In toxicology, a sharp distinction should be made between acute toxicity and chronic toxicity. It is inherent to pollution problems that the chronic toxicity due to low concentrations of the pollutant is of great importance. This calls for tests of long duration, preferably covering one or more generations of the test organisms.
In the marine environment, as in most other environments, the interrelations between different species, as in food chains, are very important. A toxicological testing system, therefore, should cover a number of species, that are representatives of different large taxonomic groups and, if possible, it should take into account inter-specific relations. Studies on the fate of radio-active waste in the marine environment (cf Polikarpov, 1966), and recent experiences with non-degradable pesticides and surfactants, have shown the importance of transport and accumulation of toxic substances in and between organisms (Robinson et al, 1967). A test system, therefore, should allow one to take these phenomena into account, if possible. Last but not least we must mention, as a special feature of the problem we are facing, that pollutants are hardly ever pure substances, readily definable in chemical terms.
We generally have to do with mixtures of toxic substances, which, moreover, may be accompanied by substances or conditions (e.g. organic waste, leading to a lowering of oxygen tension; starvation; heat; radio-activity; acid or basic substances) which, though not in themselves directly toxic or lethal, may provide a secondary stress to the organisms in question. Taking these considerations into account, we have set ourselves as a goal the development of a test system that has the following features:

1. the system should allow for long-term experiments, involving at least one generation of the test organism;
2. the system should involve several species of test organisms, preferably linked in a food chain;
3. the system should allow for the application of secondary stress.

So far our efforts have been mainly concentrated on the first two items, and they will be discussed here further.

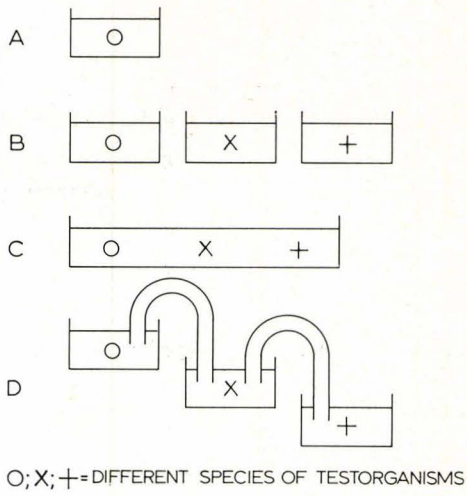


Fig. 1. Possible schemes of toxicity tests with aquatic animals. Further explanation in text.

Figure 1 A shows the simple situation of an aquarium experiment with one organism only. If more organisms are to be investigated, one could think of a simple multiplication of this design as shown in B. In that case, however, the inter-specific relations, as e.g. prevalent in a food chain, are not taken into account. A design without this drawback is given in C. Such a setup (the „microcosm" technique), however, is hardly conducive to an accurate analysis of what is happening, both chemically and biologically, as is necessary in toxicological tests. A way-out is shown in design D, where each organism has its own compartment but is connected by overflow, or similar devices, with its predecessors in the food chain.
Such a device calls for continuous culture or, at any rate, for continuous-flow experiments. Such a set up would also be favourable to the first requirement, viz. the necessity of carrying out long-term experiments. In acute toxicity experiments of short duration, static tests are generally used. Such tests are useful to supply a specified dose to a test organism. Our problem, however, is to investigate the exposure of test organisms to a certain environmental concentration of the toxic agent (Mount & Warner, 1965). In static tests, the concentration of the toxic agent rapidly decreases through absorption by the test organism and the apparatus, through breakdown, evaporation, precipitation, etc.
To maintain controlled conditions, especially the level of the toxic agent, use of continuous-flow tests appears to be highly desirable (cf Hueck & Adema, 1967). Accordingly, we have chosen to work with continuous-flow cultures using as test organisms unicellular algae, micro-crustaceans and fishes in the experimental set-up of design D in Fig. 1. To establish the feasibility of such a system, we are working for the moment with freshwater species in order to avoid the difficulty of the (continuous) cultivation of marine organisms. It is our intention to switch to truly marine organisms in 1968, when better facilities for this work will be available. It will be appreciated that for a com-

[1]) Shortened version of a progress report read at the „International Symposium on Biological and Hydrographical Problems of Water Pollution in the North Sea and Adjacent Waters" held in Helgoland, September 1967.
The full text will be published in „Helgoländer Wissenschaftliche Meeresuntersuchungen".

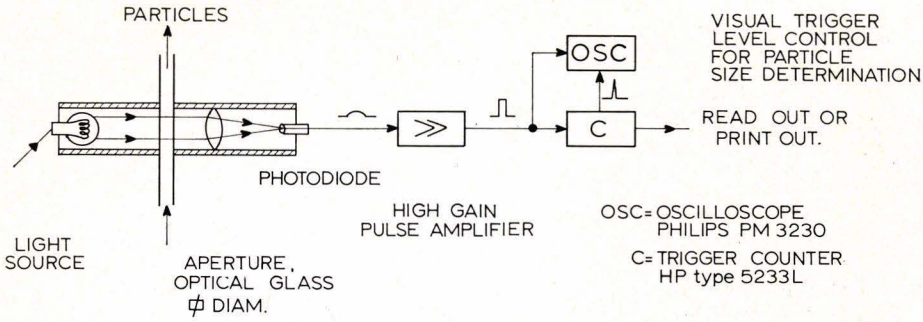


Fig. 2. Photometric counting of particles (Daphniae).

parison of the merits of static and continuous-flow experiments and the importance of inter-specific relations, as will be reported in the experimental part of this paper, use of fresh-water test organisms is convenient and permissible.

3. Materials and methods

In the following we will give some information on toxicity tests with an unicellular alga (*Chlorella pyrenoidosa,* Chick, University of Wisconsin, strain 2005) and a branchiopod (*Daphnia magna,* Straus, own isolation): *Chlorella* was cultured in a synthetic medium (Hueck & Adema, 1967), and *Daphnia magna* in a medium according to Freeman & Fowler (1953).

The main criterion used in our evaluation was the assessment of numbers of these organisms. For algae this was done with microscopical counting chambers and, later, with a Coulter-counter. The daphniae were counted by catching them individually with a pipette. Later an apparatus was developed in which daphniae, passing with a flow of water through a counting cell, interrupted a beam of light. The interruptions were counted electronically[2]) (Fig. 2). The apparatus is still under development, but has already given some promising results.

As a toxic agent, copper was used as $CuSO_4$. The organisms were maintained in aquaria of different sizes. The flow of water was maintained by peristaltic pumps (LKB type 4912 A).

Most experiments were carried out in a conditioned room at 22°C, in normal day light. The distilled water used for making up the media was that taken from the laboratory main and filtered over carbon (Norit PKX 0.5 - 1 mm).

This filtration step was found to be necessary, as media made up with untreated distilled water proved to be unsatisfactory.

Estimation of copper was carried out colorimetrically with diethyldithiocarbamate (cf Strickland & Parsons, 1965).

2) The apparatus was developed by Ir. G. A. Schwippert of our institute.

4. Experimental part

4.1. *Introduction*

From the many experiments in our investigation, a few are chosen here to illustrate the following points:

a. a comparison of the results of static and continuous-flow toxicity tests with daphniae;
b. food relations between algae and daphniae under influence of copper.

Our investigation as a whole aims at drawing up a budget of copper present in the organisms linked in the artificial food chain, under the influence of different levels of this toxic agent in the environment. Apart from the fact that we have not yet completed the picture, the scope of this paper does not allow for an overall discussion of this broad problem.

4.2. *A comparison of static and continuous-flow toxicity tests with Daphnia magna*

Toxicity tests with daphniae are generally carried out in two different ways:

1. 30 young daphniae are put in small vessels (300 ml) and kept until they produce a second generation. From the second generation a sample is drawn, with which the experiment is continued.
2. Five adult females, which are on the verge of spawning, are introduced into a large experimental vessel (10 liters).
   The offspring produced remains in the experimental vessel, where they produce after some time a second generation. The population is left undisturbed, except for countings at distinct intervals.

As parameters both mortality (complementary to survival) and the production of offspring are used in experiments of type 1. In the other experiments the total development of the population was used as a measure for toxicity.

The daphniae in the following experiments are fed with algae grown in non-toxic media. In the expe-

Table 1

Effect of copper in static cultures and intermittently renewed cultures of Daphnia magna

type of culture	concentration copper (p.p.b.)	P = parent generation		F_1 = first generation offspring	
		survival %	reproduction coefficient	survival %	reproduction coefficient
static	0	100	13	89	8
culture	10	100	4–10	91	7
	18	100	11	86	9
	32	95	9	30	3
	56	27	0	–	–
	100	1	0	–	–
	180	0	–	–	–
	320	0	–	–	–
culture	0	99	10	93	8
medium	10	99	7	46–91	9
renewed	18	100	5	75	5
every 3	32	100	1	63	0
days	56	43	0	0	–
	100	0	–	–	–
	180	0	–	–	–
	320	0	–	–	–

riment summarized in Table 1, the influence of copper in a static test of the design 1 is given: one series is run without any renewal of the test medium while simultaneously in another series the test medium is renewed with intervals of 3 days. The data given are means of duplicate tests. In cases of severe discrepancies between parallels, both data are given. The survival in a certain period was calculated as a „percentage" according to:

$$\text{percentage survival} = \frac{100 \cdot \int_0^{t_f} N_t \cdot dt}{N_0 t_f}$$

where N_t = number of daphniae present at time t_f = time of finishing the experiment.

A crude coefficient of reproduction was calculated by dividing the offspring after a certain period by the number of parents present at the data of spawning. The duration of the experiment was about three weeks.

It will be seen from this table that in this experiment the F_1 generation shows a more sensitive reaction towards copper than the P generation.

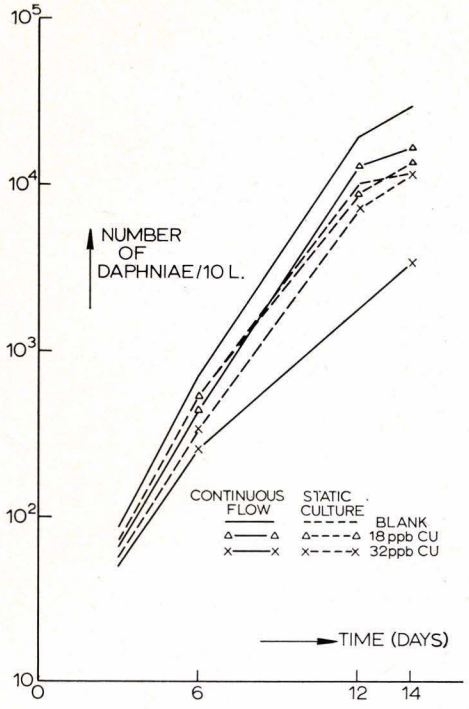
Furthermore, we may note that reproduction shows a tendency towards greater sensitivity to copper. To investigate the effect of renewal further we carried out an experiment comparing continuous-flow at a rate of 5 l/day in aquaria of 10 l contents, with static cultures (design 2). The result is shown in Graph 1. Though the result is less detailed than that in the previous experiment, it shows clearly that in static culture there is only some initial retardation in the development of the population due to copper but that this levels out in the long run.

The final size of the population is apparently determined by the size of our aquaria. This may be explained by exhaustion of the limited amount of copper available through absorption by the organisms. Continuous-flow, on the other hand, allows a denser population to develop in the experimental vessel and gives rise in this experiment to a clear-cut differentiation of population size under influence of copper. As may be expected from Table 1, no appreciable development of the population occurs on the level of 56 p.p.b. copper; reproduction at that level is fully impaired, though mortality is not quite 100%.

Even with the flow-rate maintained in this experiment, ehxaustion of copper occurs. Improvement is looked for. At any rate we may conclude that continuous-flow experiments, preferably over two or more generations, provide a more realistic picture of actual toxicity of copper than static experiments.

4.3. *Food relations between daphniae and algae under influence of copper*

Chlorella pyrenoidosa can stand much higher concentrations of copper than daphniae; this can be seen from Graph 2, which shows the result of the development of this alga in static culture under influence of copper. Continuous culture tests are being carried out, but have not yet been comple-



Graph. 1. Toxicity of copper against Daphnia magna.

ted. We may note again an initial retardation of growth with lower concentrations of copper, but this too levels off in the long run so that the final density reached is the same over the range from 0 - 560 p.p.b. copper.

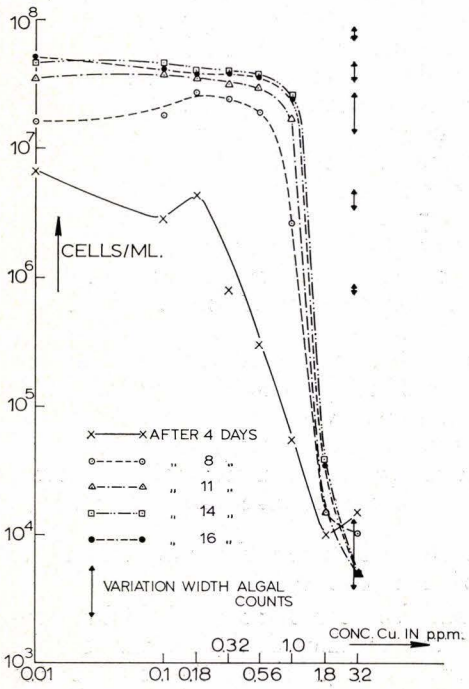
Over the range 560 p.p.b. - 3000 p.p.b. copper (= 3 p.p.m.) production steeply drops to zero.

In these cultures, *C. pyrenoidosa* accumulates considerable quantities of copper. Polikarpov (1966, page 78) quotes concentration factors of 100 - 2000 x in data from radio-ecological studies. Our findings point to the same concentration levels, or even higher.

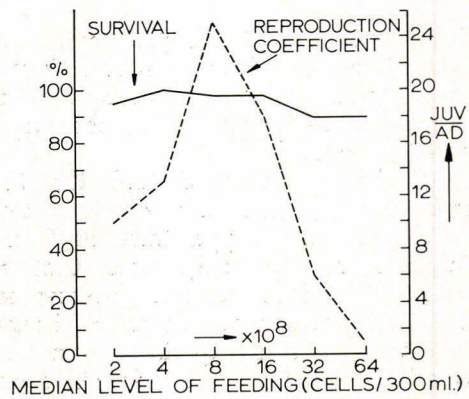
We wondered what would be the effect if we fed our sensitive daphniae with algae cultured in media containing different concentrations of copper. We first tried to establish the amount of algae consumed by the daphniae. For the purpose we provided 6 levels of algal food to 10 daphniae in 300 ml beakers. As the daphniae grow during the experiment and small daphniae cannot be held in too dense algal suspensions, we had to increase the amount of algae fed every two days. Thus we started with levels of 2.5 x 10^7, 5 x 10^7, 10 x 10^7 etc. algal cells/300 ml on the first day and we ended with levels after 8 days of 40 x 10^7, 80 x 10^7, 160 x 10^7 etc. algal cells/300 ml. Generations were counted separately. The result is shown in Graph 3, in which the median level of feeding (after 6 days) is taken as a parameter representing the level of feeding.

It will be noted that mortality (survival) is hardly affected. On the other hand, the reproductive capacity shows a clear-cut optimum. There was a tendency for the generation time to be optimum too in the same region. Furthermore, we noted the appearance of white bodies, giving fat reactions, in the daphniae at the three higher levels of feeding. Because of settling of algae in the experimental vessels, we could not determine exact percentages of algae consumed at the higher levels of feeding.

At the lower levels, the algae were consumed practically quantitatively. It could not be decided,



Graph. 2. Development of Chlorella pyrenoidosa in static culture in different concentrations copper.



Graph. 3. Influence of different levels of feeding with algae on development of daphniae (10 d/300 ml.)

Table 2

Influence of copper on daphniae by way of food and directly dissolved in the medium.

type of experiment	Initial copper concentration in algal medium in ppb	Initial copper concentration in daphniae medium in ppb	total amount copper available in 6 days per Daphnia in μ gram				P = parent generation		F_1 = first generation offspring	
			present		consumed		Survival %	reproduction coefficient $\left(\frac{\text{Juv}}{\text{Ad}}\right)$	Survival %	reproduction coefficient $\left(\frac{\text{Juv}}{\text{Ad}}\right)$
			P	F_1	P	F_1				
toxic food	0	0	0	0	0	0	90	12	100	14
	100	0	0.45	0.39	0.43	0.39	87	5–10	100	11
	180	0	1.2	1.1	1.1	0.9	75	(4)	97	13
	320	0	2.2	2.2	<1.6	<1.7	87	12	90	2– 9
	560	0	3.3	3.5	<1.5	<3.0	90	2	98	2
	1000	0	7.7	–	<1.5	–	20	0	–	–
			present							
toxic medium	0	0	0				100	10	98	6–10
	0	10	0.20				100	5-8	72	9–13
	0	18	0.36				100	3-7	82	2– 8
	0	32	0.64				100	1-7	80	0
	0	56	1.12				43	1	3	0
	0	100	2.00				0	–	–	–
	0	180	3.60				0	–	–	–
	0	320	6.40				0	–	–	–

Juv = juveniles
Ad = adults

in this experiment, whether the unfavourable effect of high levels of feeding was due to overeating or crowding of the experimental vessels with algae. We chose to continue our experiments with feeding the daphniae at a median level of 8.10^8 cells/300 ml (= level 3).

In a last experiment to be reported here, we cultured algae in media containing different concentrations of copper. The algae thus obtained were fed at level 3 to *D. magna* in a non-toxic medium. The result is shown in Table 2, and compared with the influence of copper dissolved directly in the daphnia medium and with intermittent renewal of the medium concurrent with the feeding of algae.

In this table the results for survival etc. are not directly comparable with those in Table 1, as we had to finish the experiment at an earlier date. It will be noted, however, that the trend in both experiments is about the same. An inconsistency occurred in the feeding experiment, parent generation, at 180 p.p.b. From earlier experiments, not recorded here, we had noted a consistent drop at 560 p.p.b. so that we suspect an experimental error to have occurred, which does not affect our main conclusion. Though the experiment is not perfect, it shows clearly that poisoning daphniae by way of food is not nearly as efficient as direct poisoning by copper in the medium, even with a suggestive high concentration factor in *Chlorella*. We tried to calculate, on the basis of the volume of algal cells consumed and an assumed concentration factor of 4500[3]), how much copper was really available to each daphnia. We next compared this calculated amount with the direct poisoning, assuming in that case a total exhaustion of copper in the medium.

The outcome of this provisional calculation is given in column 4 of Table 2. In carrying out this calculation, we found that the interpretation of its results is far more complicated than we expected when first starting the experiment. We wish to reserve further comment for future occasions, and for the present use the data only to show in which way we expect to proceed. In the long run, a complete budget of copper in the organisms and the environment should be reached.

Acknowledgement

The experimental work has been performed by Mrs. D. van Drongelen, Miss Th. A. M. van Zijl, Miss J. Zuidweg, Miss C. W. M. van Holsteijn and Miss S. S. Yu at the Department of Biology of the Central Laboratory TNO.

3) This factor was found by us in the only experiment available at the time. We wish to corroborate this figure in further experiments.

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