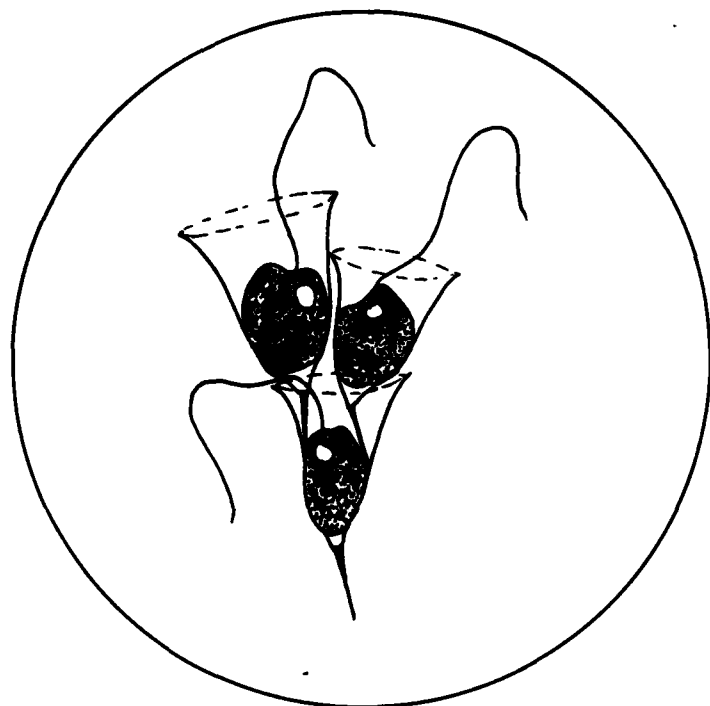
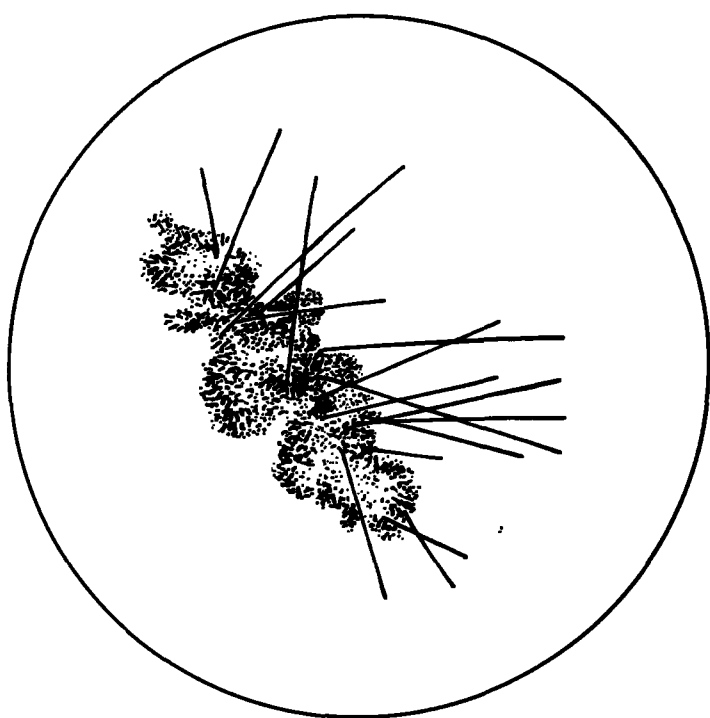
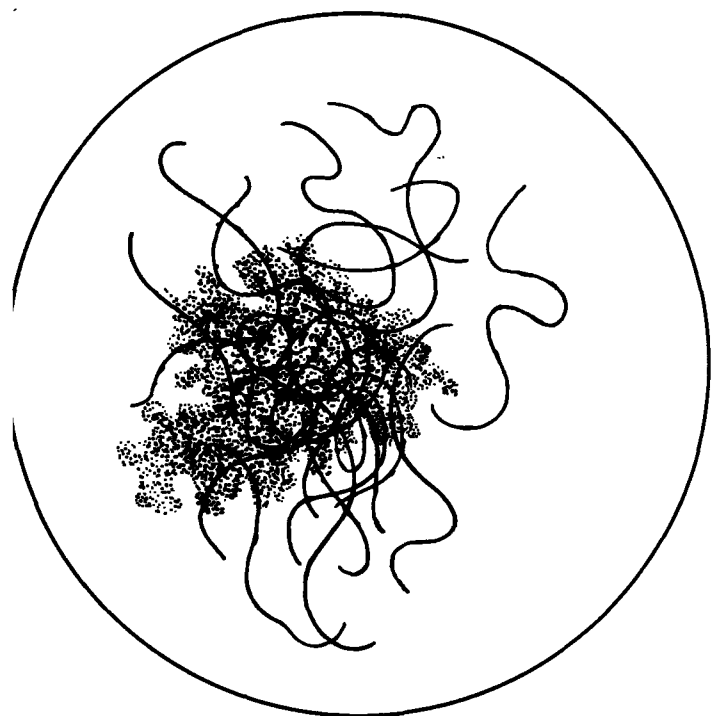


MICROSCOPIC SLUDGE INVESTIGATION MANUAL



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TNO RESEARCH INSTITUTE FOR ENVIRONMENTAL HYGIENE

Microscopic sludge investigation manual

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PREFACE

In treatment plants based on the activated sludge principle, the sludge flocs themselves form the most important link in the chain of processes that contribute successively to the removal of the influent waste products.

A poor quality final effluent is very often caused directly by the less than optimum condition of the sludge flocs in the plant.

The standard investigative procedure used most often nowadays for checking the course of the treatment process and providing information for adjustment if necessary, involves mainly chemical and physical analysis of influent and effluent. However, the results of these analyses give little direct information to plant operators about the actual quality of the activated sludge floc in the aeration tank. As a result it is often not possible to indicate the cause of a disturbance in the treatment process and, consequently, to initiate corrective action in order to improve plant operation.

Microscopic investigation of activated sludge is a simple analysis which gives information about the form and structure of flocs, the presence of filamentous microorganisms, the number of protozoa etc. Regular microscopic investigation of sludge therefore contributes to a better insight into the composition and structure of the sludge floc and through this into the functioning of the activated sludge process.

Within the research programme of the Water and Soil Department of the TNO Research Institute for Environmental Hygiene much attention is given to the microbiology and biochemistry of activated sludge. In particular much progress has been made during the last ten years in understanding the problem of bulking sludge. Based on this a guide for microscopic sludge investigation was compiled for, and with the aid of, the Foundation for Applied Research for the Treatment of Wastewater (STORA) in order to make microscopic quality control possible and to encourage its application in full-scale treatment plant control. The guide, which also appeared as a STORA report, forms the content of this English language manual. Compared to the Dutch edition, a few additions were made in order to take into account some of the latest developments.

We are very much indebted to Mr. L.T.A.M. Miezenbeek and Dr. M.J.D. White (Water Research Centre, Stevenage) for their work on the translation of this manual.

PREFACE TO THE SECOND EDITION

We are pleased to find that the interest for the biology of the activated sludge process has so much increased the last few years. No doubt an important role is played by the fact that, due to a poor floc quality, many plants do not meet the design effluent standards.

The method described in this manual is now applied by many plant operators etc. - viz. almost all Dutch Water Authorities - for checking regularly the course of the treatment process in full-scale plants.

In this second edition, if necessary, the text has been corrected and revised. Besides, some extra identification keys have been included.

Delft, March 1983

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1. INTRODUCTION

For the treatment of waste water the activated sludge system is used on a large scale. Although this system is applied in many different ways the basic principles of all the applications, as they are found in practice, are comparable. The activated sludge flocs are the hub around which the whole process turns. Many operational problems are caused directly by the fact that these flocs do not have an optimal quality. Knowledge of the quality of the floc is therefore almost indispensable to enable a correct interpretation of poor plant performance and thus to be able to improve this in a direct way. Thus for instance a turbid effluent may not be caused only by poor settling of the flocs in the final clarifier (due to a massive growth of filamentous microorganisms) but also by flocs that are too small, the absence of protozoa or by dispersed growth of bacteria. Although the final effect of all these failures, viz. a turbid effluent, are comparable, there are different causes. It is evident that in the final choice of corrective action to improve plant performance the real nature of the problem must be known. For this a microscopic examination of the sludge is usually indispensable, because most often in this way information about the actual quality of the floc may be obtained. Moreover, regular microscopic examination of sludge is necessary to check the course of the treatment process accurately and allows in this way a deterioration of the sludge quality to be detected before problems occur on a large scale.

Obtaining a microscopic view of sludge is however not usually part of the standard program of analyses at treatment plants. There are several reasons for this. That much of the information available on the subject is not easily accessible to people responsible for plant operation is undoubtedly an important factor. Besides, in several publications on this subject the matter is not dealt with as a whole but only a certain aspect, for instance the protozoa in sludge, is discussed.

The above mentioned reflections have led to the decision to compile a sludge investigation manual which will contain 'all' available information concerning the quality control of sludge flocs with the

aid of microscope. This manual does not completely review all that is known about the biology of the activated sludge process. This was not aimed for by the authors.

Only that information which is indispensable in order to interpret a microscopic view correctly is inserted in this report. It has been assumed here that those who are going to perform the microscopic sludge analyses in practice have often had a chemical and/or a sanitary engineering background and know little of the biology of the activated sludge process.

On page 79 of this manual the interested reader, who wishes to learn more about the biology of the activated sludge process, will find a number of titles of books and some references to literature on this subject.

2. ACTIVATED SLUDGE: AN ECOSYSTEM UNDER ARTIFICIAL CIRCUMSTANCES

In our environment there is a constant production of organic compounds. Many processes of life result in the synthesis of carbohydrates, proteins, lipids etc. The solar energy is the driving force in these synthesis processes. Plants (including algae) can make use of this energy for the production of cell material. The minerals needed for this (C, H, N, O, P, S etc.) are taken from the environment. The cell material produced may then serve as food for other animals etc; who in their turn are sometimes consumed by other organisms. Plants and animals will, however, die one day after which the bacteria begin to contribute to the cycle by decomposing the whole gamut of organic compounds again. The inorganic components formed in this mineralization process are subsequently used by the photosynthetic organisms. With this the cycle is complete.

So the microorganisms (especially bacteria and fungi) are by nature actively involved in the decomposition of organic material. They do this because they can make good use of the dead organic material. The available organic compounds are partly decomposed. The energy released in this process is used to transform the remaining part to new cell material.

There are not enough microorganisms in waste water to allow the decomposition of organic compounds to proceed in a rapid and efficient way. The activated sludge system does away with this objection.

2.1. The activated sludge process

When waste water is aerated, flocs of bacteria will be formed spontaneously after some time. When the aeration is stopped the flocs will settle. The supernatant liquor, the degree of pollution of which has decreased significantly can be discharged. The flocs may be used to treat a new portion of waste water. The activated sludge process is based on this discovery.

What has happened in the aerated waste water? Oxygen has been added to the sewage in which by nature many bacteria are present. The different bacteria grow fast and regularly divide themselves. The total biomass ($=M$) increases until the available nutrients have been used. The concentration of the nutrients that are present ($=F$) diminishes during the aeration because the organic compounds are partly decomposed and the remaining part is transformed into new cell material. So, during the aeration phase the F/M

ratio continually decreases. At the starting point there were at the most a few milligrams of biomass per liter present in the waste water, while the nutrient concentration was 500-1000 mg per liter. So when starting the F/M ratio is high. The bacteria are then still dispersed in the liquid. During their growth the F/M ratio changes rapidly. Both the increase of M and the decrease of F are logarithmic. As soon as the F/M ratio has reached a sufficiently low level, the cells start forming agglomerates; the so-called activated sludge flocs. In treatment plants these flocs are subsequently separated from the final effluent by means of a settlement process. The settled biomass is partly returned to the aeration tank, thus maintaining a low F/M ratio in this basin. This is not only beneficial to the forming of flocs but also to the substrate removal rate. The overproduction of bacterial cells is removed as excess sludge.

During the treatment process new cell material is continually formed. A large part, sometimes even 60-70% of the organic compounds present in the waste water, is not decomposed but transformed into cell material. This does not only concern new cells but also the production of foodreserves by the bacteria.

Together with this, inorganic compounds are also incorporated in bacterial cells.

For synthesis of its cell material the bacterium needs certain quantities of C,N,P,H,O,S, etc. In normal domestic waste water the amount of carbon available is the limiting factor of growth. In consequence, carbon compounds present in the waste water are almost completely removed in the treatment process. The other compounds, which are present in excess, are found to a large extent in the effluent

2.2. Composition and function of the activated sludge floc

In an activated sludge plant the sludge flocs form the hub around which the treatment process turns. What then are these composed of? This is not so easy to answer. Roughly speaking it may be stated that in a floc we may find:

- living microorganisms; mainly bacteria,
- dead cells,
- undigested large organic fragments, trapped in the floc,
- an inorganic fraction; among other things the grit present in the waste water. The size of this fraction may vary from 10-50 percent in weight.

The way in which the floc is composed in certain treatment plants depends on the situation on the spot. The quality of the influent waste water of course is an important factor. Are all the necessary nutrients sufficiently available? In some industrial waste water plants for example there is too little N and/or P present. This inhibits the growth of many bacteria. The treatment becomes less complete and sludge containing slime is produced, causing a more difficult dewatering and an increase of the sludge volume index.

The presence of a grit-chamber and/or a primary sedimentation tank determines the number large (in)organic particles that end up in the aeration tank.

In addition, the extent to which the treatment plant is loaded has a very important influence on the composition of the floc. The sludge age is directly related to the load of the plant. To maintain oneself in the floc population the generation time for a specific bacterial species should equal at least the sludge age.

In highly loaded treatment plants the percentage of living cells in the sludge will be greater than in sludges originating from oxidation ditches, with low loading.

When waste water is aerated flocs are formed spontaneously. A low F/M-ratio is the most important condition which must be fulfilled. The reason why flocs are formed is however still a partly inexplicable phenomenon. It is known that a number of factors play a part in the formation of activated sludge flocs, viz:

- many bacteria form slime capsules. The slime, which is built up mainly of polymeric compounds, glues the cells together as it were;
- bacteria have a negative charge. Positively charged ions contribute to the joining of the cells;
- some bacteria form a network of extremely thin filaments (fibrils) around the cell. These are mostly composed of cellulose or another polysaccharide. This network can also contribute to the joining of cells and the trapping of other bacteria.

The activated sludge process is in principle an aerobic treatment process. This means that the microorganisms need oxygen for their metabolism. The sludge water mixture is aerated in order to provide for the necessary oxygen. The bacteria at the edge of the floc can absorb the oxygen directly from the water phase. On the other hand the organisms in the centre of the floc must depend on the oxygen which is provided through diffusion processes from the water phase. Especially in somewhat bigger flocs the amount of oxygen provided by diffusion is insufficient to maintain an aerobic centre of the floc. Consequently "anaerobic" processes will occur here. In this way a part of the available nitrates are converted to N_2 gas (denitrification).

The greater part of the compounds which are supplied can not directly be taken up by the cells of the floc. In principle only dissolved non-polymeric molecules can pass through the cell wall. The other organic compounds must first be reduced in size outside the cell by means of enzymes given off by the organisms. This takes some time. The removal of the compounds which are present in the waste water proceeds faster than the consumption by the bacteria, however. This is because sorption processes play an important part. Most of the organic compounds available are in the first instance attached to the floc. Subsequently they are reduced in size and taken up by the cells.

2.3. Ecology of the activated sludge

In a treatment plant a large number of different (in)organic compounds are brought into contact with a possibly just as large variety of microorganisms. It is not true that certain compounds can be assimilated just like that by every organism. There are big differences. Some micro-organisms are typically omnivorous while other bacteria are a lot more specialized. The quality of the influent is indeed of great importance to the sludge population. In treatment plants with a dairy influent, for example, we are concerned with different organisms than in treatment plants fed with a domestic waste water.

In general it may be said that there is keen competition between the different microorganisms for the available nutrients. The composition of

the floc depends on the result of this competition. Apart from the quality of the influent, the actual growth rate of the different microorganisms mainly determines the degree in which the different types are represented in the floc population. The growth rates of different species are usually not the same but on the contrary show quite big differences. Besides, the growth rate of a certain bacterium is not always constant. The speed of cell division depends on the environmental circumstances in which the organism finds itself.

The oxygen supply, the pH value, the quality and quantity of the available nutrients, the temperature etc, all have an influence on the growth rate. Only under optimum conditions does the growth rate of a certain bacterium reach its maximum. For most bacteria in a treatment plant the actual growth rate is considerably lower than the maximum rate of cell division.

In an activated sludge system we have a bacterial population apparently in equilibrium but where in reality there are continuous changes. Looking at the total treatment this is usually not discernable, which is only logical since the changes in the sludge population will be directed towards making maximum use of the supplied nutrients; in other words: the removal of the substances present in the waste water. The relatively stable character of the activated sludge process is directly related to this. The population will continually adjust to changes in operational procedures, the quality of the waste water, seasonal influences etc, although naturally, there are certain limits.

In a treatment plant, the mass of sludge is kept at a certain level by removing the increase in sludge; the so-called surplus sludge. This clearly means a rather large disturbance to the processes which maintain the steady state. Consequently the organisms with a high growth rate have an advantage over the slow growing organisms. All species of which the doubling time exceeds the sludge age will be removed in the surplus sludge. In highly loaded treatment plants the diversity of organisms in the floc is clearly smaller than in, for example, oxidation ditches. To what extent this affects the degree of treatment is not altogether clear. Some compounds are probably not or not entirely decomposed in highly loaded systems because the organisms that should perform this cannot hold their own in the system.

The absence of a nitrifying flora which perform the oxidation of ammonia, in systems where the sludge-age is less than 3-5 days is an example for this.

The bacteria and fungi, although the latter do not play a big part in the treatment process, grow on the organic compounds which are presented to them. However, in their turn they may be consumed by higher organisms. In this context the Protozoa, and to a lesser extent, Rotifers and Nematodes must be mentioned. These organisms can make a substantial contribution to the treatment process because they consume many dispersed (not attached to the floc) bacterial cells. Their number is generally subject to some fluctuations.

2.4. Some applications of the activated sludge principle

In the Netherlands the best known modifications of the activated sludge process are the oxidation ditch and the so-called conventional activated sludge systems (=AS). With the latter we may again distinguish two modifications. Table 1 gives an outline on the most important differences between these systems.

Table 1. Outline of the most important differences between three modifications of the activated sludge process.

	Oxidation Ditch	Conventional Activated sludge	
		low load	high load
Primary settlement tank	absent	present	present
BOD-load ¹⁾	0.05	0.1 - 0.4	> 0.5
Aeration period (hours)	60 - 72	5 - 8	< 4
Nitrification	> 90%	almost ²⁾ complete	absent
Sludge age	4 - 12 weeks	> 5 days	< 3 - 5 days
Sludge stabilization	aerobic	anaerobic	anaerobic

1) kg BOD/kg dry matter . day

2) in winter frequently incomplete.

With exception of the presence of a primary settlement tank all other differences are caused directly by differences in load (= F/M ratio). An oxidation ditch has a very low load. As a result the amount of available nutrients will be insufficient to allow a high growth rate. The sludge age is 4-12 weeks. Hence, slow growing organisms, e.g. nitrifying bacteria, hold their own in this treatment system. At this low F/M-ratio the flocs have a firm structure. The amount of dispersed cells usually is very low. The sludge is stabilized aerobically. By this is meant that a large part of the compounds formed and stored (reserve materials; contents of dead cells etc.) are decomposed during the aeration period. The respiration level of the sludge is very low because of this lack of sufficient nutrients.

In an A.S. a higher F/M-ratio is applied. This results in a higher growth rate; the sludge also contains more living cells. In a low loaded A.S. nitrification may occur but as soon as the sludge age is less than 3-5 days the oxidation of ammonia stops. The nitrifying bacteria grow so slowly that they can only maintain their position in treatment plants with a higher sludge age. Especially in highly loaded A.S. many dispersed cells may be found in the liquid between the flocs. There are obviously still enough nutrients present in the liquid to make this growth possible.

Moreover, rather weak flocs are frequently formed at high loadings

→ mechanical forces, due to aeration of the activated sludge, may also result in many dispersed bacterial cells. The respiration level of sludge from an A.S. is high because the cells still contain many compounds that may be rapidly decomposed. This is the reason why sludge from an A.S. must be separately stabilized. This is mostly done anaerobically in a digester, in admixture with primary sludge.

3. MICROSCOPY

The human eye is not capable of clearly distinguishing objects with a diameter of less than 0.1 mm. Bacteria have a diameter of approximately 0.001 mm (= 1 μm = 1 micrometer) and can therefore only be perceived with the aid of a microscope.

3.1. Basic principles

The maximum magnification that can be reached with a certain type of microscope depends in the first place on the resolving power. By this is meant the capacity to distinguish two adjacent points as distinct and separate. To put it differently: the smallest diameter that can be observed sharply.

The resolving power of a microscope is determined by the wavelength of the applied rays (e.g. light) and the numerical aperture (NA) of the objective; this relation can be expressed in the following formula:

$$\text{resolving power} = \frac{\text{wavelength}}{2 \times \text{NA}}$$

In the numerical aperture among other things the refractive index and the thickness of the layer between the slide and the objective are incorporated. The wavelength of visible light lies between 0.4 and 0.7 μm . When we have for example a wavelength of 0.5 μm and a NA of 1.25 (maximum realisable value) it can be calculated that: resolving power = $\frac{0.5}{2 \times 1.25} = 0.2 \mu\text{m}$. So,

with an ordinary light-microscope we can only observe particles or parts of these when they have a diameter of more than approximately 0.2 μm .

An electron-microscope does not use visible light but an electron beam.

The wavelength of these rays are much shorter than those of visible light.

The resolving power of an electron microscope is consequently much greater; approximately 0.001 μm . In this manual we will however leave this type of microscope out of consideration.

An ordinary light-microscope, see Figure 1, consists of:

- a microscope stand,
- a specimen stage; on this lies the slide,
- a nose piece; this is the objective holder,

- a number of objectives,
- one or two eyepieces (oculars); one ocular equipped with a focusable micrometer scale,
- a condenser; used to converge the rays from the illumination in order to concentrate them on the slide,
- an illuminator; sometimes this is mounted in the foot of the microscope. With other types a separate source of light is used in combination with a mirror.

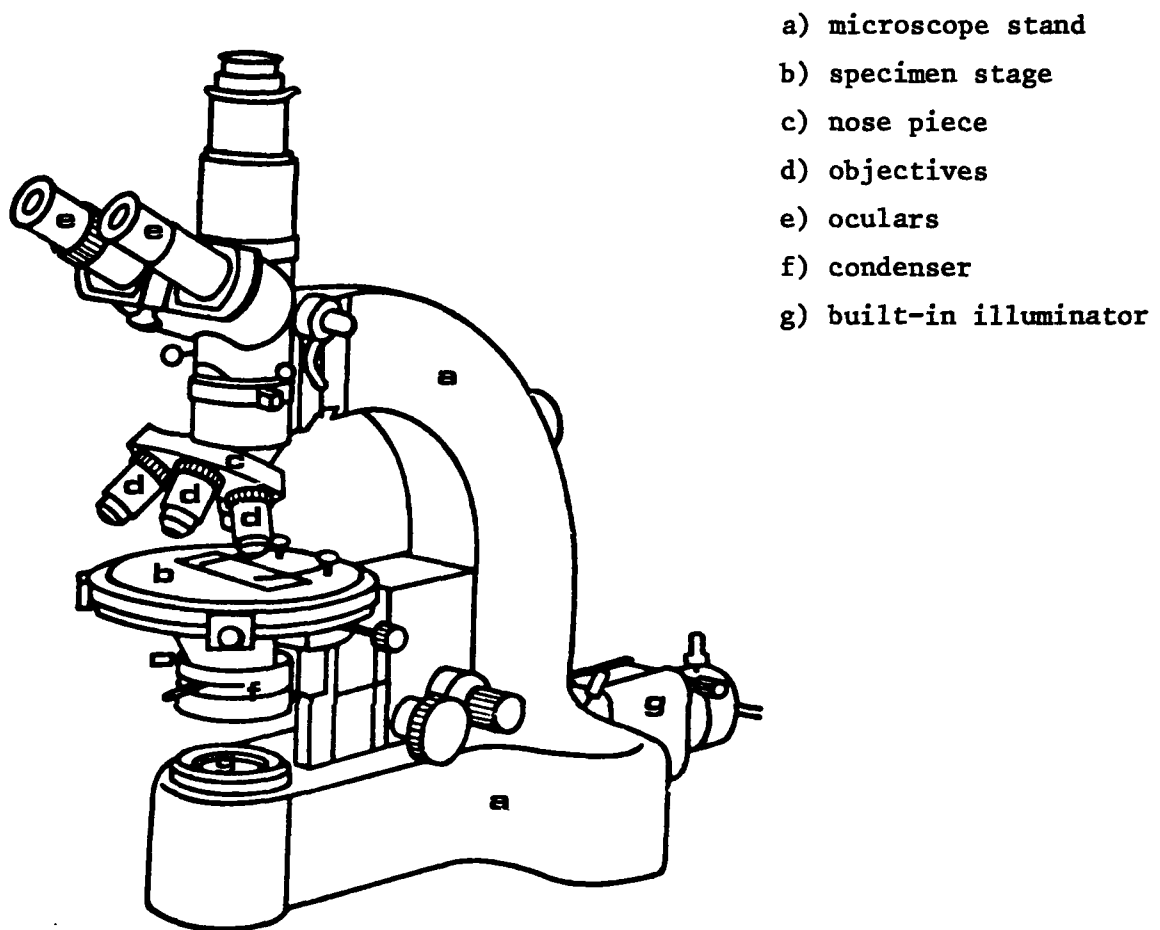


Fig. 1 Diagram of a microscope

This type of microscope is a compound microscope. The magnification of the object is performed by a pair of lens systems, viz. an objective and the ocular lens. The final magnification equals the product of the magnification

of each separate lens. In most cases a 10 x ocular is used; sometimes however also lenses with a magnification of 6, 15 or 20. Most laboratory microscopes are equipped with 3-4 objectives. Although other objectives can be obtained, objectives that give a magnification of 10, 40 or 50 and 100 times are usually applied. So this results in a total magnification (when a 10 x ocular lens is used) of 100, 400 or 500 and 1000 times respectively. The choice of the kind of objective depends on the size of the object we want to study (see also chap.4.1).

With higher magnifications (the 100 x and sometimes also the 50 x objective) the space between the front lens of the objective and the coverglass must be filled up with immersion oil. This oil has the same refractive index (viz. 1.52) as the glass of the lenses and the microscope slide. In this manner the rays of light on the borderline glass/oil are not diverted but may pass through freely.

3.2. Bright-field and phase-contrast

When light passes through a medium which is not of a uniform composition, e.g. a drop of water containing small sludge flocs, part of the rays of light will be more diverted or dispersed than the other rays. This is caused by the difference between the refractive index of the water phase and that of the floc particles. Small differences in refractive indices can however hardly be observed by means of a bright-field microscope. The consequence of this is that bright field is less suitable for observing preparations with little contrast. Nowadays this optical system (by this is meant: objective + condenser) is mainly used to study microorganisms that are rather contrasty in themselves; e.g. organisms with a very clear structure, a characteristic colour, stained smears etc. However, for the study of organisms from activated sludge flocs, this bright-field optical system is not ideal. A microscope with a phase-contrast equipment is clearly preferable for this purpose. The phase-contrast optical system translates relatively small differences in refractive indices into clearly observable differences in brightness. Because of this, this equipment is pre-eminently suitable to study organisms such as bacteria that are not very contrasty in themselves. Also the structure in the cells of microorganisms can be observed much more clearly with the aid of phase-contrast.

3.3. Preparing microscopic slides

When making a microscopic slide we can choose from several possibilities; those that are important in the scope of this manual will be discussed successively.

Wet mount

A drop of activated sludge is placed on a glass slide. This slide must be clean and free of fat. Subsequently a clean cover glass is placed on the drop. The entrapment of airbubbles must be avoided as much as possible. The preparation is now ready and may be studied. If an oil immersion objective is used, a drop of immersion oil must first be placed on the cover glass.

It is important that the drop of water is not too big but also not too small. With the latter there will be insufficient liquid to completely fill up the space under the cover glass allowing airbubbles to be entrapped. The preparation will then dry up very quickly as a result of evaporation along the sides of the cover glass. This causes a strong flow of liquid under the cover glass which makes it difficult to study the preparation. On the other hand, if the drop of water is too big the layer of water between the slide and the cover glass becomes too thick. Not only will we then have problems with the depth of focus, but what is more, the cover glass will as it were float on the drop of water. When using the oil immersion lens this will cause problems because the cover glass will show a tendency to shift when the slide is moved to another position. This is not only very tiring to the eyes but also disturbs the preparation. Placing a drop of water of the right size can best be done by means of a pipette with a very small opening (a Pasteur pipette) or with a so-called inoculating needle (the opening having a diameter of approximately 3-4 mm). Surplus liquid can be removed by holding a piece of filterpaper against the edges of the cover glass.

The layer of water between the cover glass and the slide must be of the same thickness everywhere on the slide.

However, sludge flocs may sometimes contain grains of sand with a diameter larger than the desired thickness of the water layer. The cover glass will then "rest" on these grains. This appears from that it will be not possible to fill up the space between the slide and the cover glass with water.

On one side air will remain present. When this occurs it will be better to make a new slide.

Fixed smear

When staining etc. usually so-called fixed smears are applied. A drop of the sludge suspension is placed on a slide and spread out on a surface of approximately 1 cm². Subsequently the slide is dried in the air and fixed by moving the bottom of the slide shortly through the pilot-flame of a Bunsen burner. Now the final treatment, e.g. staining, can be performed.

Counting chamber

Sometimes we want to know how many specimens of a certain organism are present in a sludge sample. If it concerns a microorganism with clearly observable characteristics, e.g. protozoa, then it is possible to determine this number by counting them in a microscopic slide. In this case, however, the volume of liquid between slide and cover glass must be known. In a normal wet mount the area of the slide is known but not the height of the column of water between the cover glass and the specimen slide. A counting chamber is constructed in such a way that the height remains constant. Thus the volume of the chamber is known.

3.4. Adjustment and use of a microscope

The quality of a microscopic image is largely determined by whether the microscope has been adjusted correctly. Especially when using a phase-contrast optical system the greatest care is required. Without going into this matter too deeply - the way in which a microscope must be adjusted depends largely on the make and can therefore be best explained by the salesman - a number of *mistakes that occur frequently* will be mentioned successively. Apart from this it is doubtless wise to have a microscope serviced annually.

The condenser is meant to concentrate the light on the slide. If the condenser has not been adjusted correctly the result is not only a low light intensity but also a qualitatively less sharp image. The condenser

adjustment must therefore be regularly checked. This is a very simple action. After almost closing the diaphragm that condenser adjustment is sought by changing the height which produces the illuminated circle, as it is seen through the microscope, with the smallest possible diameter. Also the phase adjustment must be checked regularly. Not only must it be checked by means of the adjustment eyepiece, whether the phase rings are still concentric with each other, but it must also be verified whether the position of the condenser corresponds with the objective lens used at that moment.

With some microscopes the source of light is not mounted in the foot of the microscope but a separate one is used. In order to obtain an optimum image it is necessary that the light is concentrated on the mirror in the foot of the microscope. A separate illuminator obliges us to check regularly if the microscope and the lamp have not moved with regard to each other.

Objective lenses and oculars must be well cleaned regularly. The use of lens paper, made especially for this purpose, is preferable to the use of tissues because the latter leave bits of fluff.

There may be no air bubbles present in the drop of immersion oil on the coverglass. Such an air bubble has a different refractive index than the oil and thus disturbs the path of light and hence the brightness and sharpness of the image.

Finding a microscopic image is often a problem to people who do not work with a microscope daily. This occurs mainly when working with large magnifications. The space between the front lens of the objective and the surface of the cover glass (the so-called working distance) is then very small.

With a 100 x objective this will be approximately 0.15 mm. The risk of moving the objective too far down, when searching for the image is quite real. The objective then touches the surface of the slide and can thus be damaged. This may be prevented by not looking through the tube in the first place, when moving the objective towards the slide, but instead by bringing them together as close as possible and closely observing the distance from one side. Only then do we look through the tube and by moving

the slide we look for something we can recognize (e.g. a sludge floc) after which by means of the fine adjustment the image can be focused.

Stained smears must be observed with brightfield and not with phase-contrast.

4. MICROSCOPIC INVESTIGATION OF SLUDGE

A microscope is a very useful aid when gathering information about the quality of the biomass in a treatment plant. It is however no more than that. The microscopic image mainly gives information on a number of visually observable characteristics of the sludge concerned. The activity of the biomass must be determined in different ways.

When analysing a microscopic image we must, in principle, work with sludge that is as fresh as possible. Some of the sludge characteristics change quite rapidly during storage of the samples, in particular when they originate from highly loaded treatment systems. Activated sludge samples which cannot be analysed directly must be stored at 4-7 degrees centigrade (bottles filled with sludge up to one third of the volume). Freezing is undesirable because this affects the structure of the floc. Sludge from highly loaded plants can be stored for 2-3 days before the floc characteristics begin to change very much. For sludge from oxidation ditches a storage period of approximately one week is allowed.

Storing of samples, even in a refrigerator, may affect the population of Protozoa, as certain species rapidly die.

The observations are performed on sludge from the aeration tank without there being a question of thickening or any other kind of treatment. It is wise always to take the samples in the same spot; e.g. near the inlet to the final settlement tank.

4.1. The microscopic image

To an untrained eye every activated sludge floc looks like a conglomerate almost without structure, highly heterogeneously composed and irregularly shaped. Here and there we may observe particles that move about; while between the flocs filamentous structures are sometimes present. The colour of the floc components may vary from yellowish-grey to brownish-black. A more experienced observer will however note that not all flocs look the same but that we may speak of clearly observable differences. These, visually observable, differences are then related to:

- firmness, shape, structure and size of the floc;
- composition of the floc: Is there a clearly observable variation of microorganisms; are characteristic groups of bacteria present or absent; are there many (in)organic nonbacterial particles in the floc?
- filamentous microorganisms: are these organisms present in the sludge; what kinds can be distinguished; does the population change in time?
- dispersed growth of bacteria: are many free bacterial cells present between the flocs?
- "higher" organisms: which protozoa etc. are present in the sludge and how many?
- are many Spirochetes and/or Spirils present?

Information may be obtained on all these sludge characteristics with the aid of a normal phasecontrast microscope. The total analysis can be performed rapidly and on the spot; an experienced observer requires no more than about 15 minutes for this. An important point in this matter is the magnification which is used. The latter must be adjusted to the size of the object we want to study. It is quite useless to look at a particle with a diameter of approximately 1 μm using a magnification of 100 x, because then it will merely be 0.1 mm thick and thus hardly observable. The combinations given below are a guideline in choosing the correct magnification.

<u>magnification</u>	<u>object</u>
100 - 200 x	- shape, size and structure of activated sludge flocs; - identification of Protozoa etc. (excluding of Flagellates); - the extent of filamentous growth; - shape of filamentous bacteria; - characteristic bacterial colonies (Zoogloea etc.); - (in)organic particles
400 - 500 x	- composition and internal structure of sludge flocs; - firmness of the flocs; - dispersed growth of bacteria; - presence Spirochetes and/or Spirils;

	- identification of filamentous bacteria (diameter $>1\ \mu\text{m}$) and Flagellates.
1000 x	- identification of filamentous organisms (diameter $<1\ \mu\text{m}$); - diversity

The recording of the observations forms an essential part of microscopic investigation. With this, however, a number of problems occur. The result of a chemical analysis may be expressed in figures; e.g. the concentration of a certain compound. In this way a certain situation is adequately recorded and may also be understood by others. In general however, the results of a microscopic sludge analysis cannot simply be translated into precise figures. In theory it is possible to record every thing we observe by counting and measuring, but this procedure is almost impracticable taking into account the amount of time needed. So the quantification of the mainly visual observations must be done in a different way. We must avoid subjective measures making it impossible to compare the results of observations performed by different people. A certain floc structure must not be described as "compact" by one observer and as "open" by another.

These kinds of problems may be prevented by creating a kind of reference image with which different observers can compare a specific microscopic sludge image. On the basis of these comparisons a certain floc structure etc. could be characterized as good or bad. Thus we will establish a uniform way of handling standards with respect to sludge quality parameters which makes it possible to compare the results of sludge analyses that have been performed by different people.

The reference images exist as photographs of several sludge quality parameters. In this manual it will be shown by means of photographic material, what is meant by, for example, a compact sludge structure or a lot of dispersed cell material. In addition it will be shown how observations of some sludge characteristics may be quantified.

The microscopic view of a certain activated sludge must always be based on a number of observations. By this is meant that a large number of flocs must be observed in one preparation, before anything can be said about the *average* quality of the floc in the sludge with which we are concerned

because the biomass does not consist of uniform particles. There are often big morphological differences between the flocs in the same sludge. So, it is completely useless to describe every floc exactly; a general impression suffices.

For the recording of the results the form inserted at the end of this manual can be used (Appendix 1).

4.2. Interpretation of the observations

Very often the interpretation of the microscopic image is not so easy. Or, in other words: what do the observations, made by means of a microscope, mean? Is it possible to use this information for a better control of the treatment plant? In what way can the microscopic observations be translated into technological terms and/or measures?

Microscopic sludge investigation gives information about a number of visually observable characteristics of the floc and the sludge population. In this way information concerning the size of the floc, filamentous bacteria, Protozoa etc. is obtained. The way in which a treatment plant functions is determined largely by the quality of the biomass in the system. Many operational problems are caused directly by the fact that the sludge floc is not in an optimum condition. Microscopic sludge investigation is a good aid for determining this. It is indispensable for making a proper diagnosis. This is the principal function of a microscopic sludge investigation. This does not mean, as is sometimes thought, that it will be immediately known what the real cause of an abnormal floc quality is. It is certainly not true that one has only to look through a microscope to see how plant operation must be changed in order to overcome certain problems. Knowledge about the subject is still insufficient, because up to now very little systematic research has been performed in this field. Another function of this manual is to enable more of this kind of systematic research to be performed.

5. MORPHOLOGY OF THE FLOC

In this chapter a number of morphological (morphos = shape) characteristics of the floc will be discussed in detail. The bulking phenomenon and the protozoa will be discussed in separate chapters. Unless otherwise stated, a magnification of 100-200 x is used.

5.1. Shape, structure and firmness of the floc

A sludge floc may vary from a somewhat globular and compact unit to a very open and irregularly shaped structure. Making a correct description of the flocs in a certain sludge is the most difficult part of microscopic sludge examination.

The flocs are characterized on the basis of:

- *Shape: somewhat rounded/irregularly shaped*

Truly spherical flocs do not exist in activated sludge. Consequently, all flocs which have a more or less spherical shape (see for example Fig.2) are characterized as "rounded". Only if the shape differs strongly from the globular one they are called "irregular" (fig.3); in this case large parts stick out of the floc on different sides.

The presence of filamentous bacteria may result in an irregularly shaped floc. The filaments form a kind of backbone around which the floc is constructed.

Increasing the load of a plant results sometimes also in irregularly shaped flocs.

- *Firmness: weak/firm*

The difference between these two characteristics is difficult to describe. In a weak floc the cohesion between the bacterial cells is rather low, so a firm centre of the floc is absent. The structure of the floc can be easily destroyed by pressing gently on the cover glass of the slide. The borderline between floc and liquid is usually not sharply marked off, because at the edges of the flocs there are many cells of which it is not clear whether they are free in the liquid or part of the floc (fig.5, upper part of the floc). With a weak floc, a lot of dispersed

cell material is usually evident (magnification 500-1000 x).

The firmness of the flocs is determined largely by the loading of the treatment plant. When a very low sludge load is applied (BOD load $<0.025 \text{ g BOD/g MLSS.day}^{-1}$), the floc has the tendency to disintegrate into smaller particles. With this low F/M-ratio there are obviously insufficient nutrients available to keep the floc-forming organisms alive. This does not play a part at a high loading (BOD load $>0.4-0.6 \text{ g BOD/g MLSS.day}^{-1}$).

Nevertheless in this loading range a weak floc is often found of which small particles can easily be detached. At this loading the bacteria are less inclined to form flocs (see also paragraph 5.4). During the intermediate loading range mainly rather firm flocs are formed (Fig.2). If in spite of this a large number of free cells or small agglomerates of bacteria are observed, these are nearly always associated with a certain disturbance in the treatment process or a non-optimum influent quality.

- *Structure: compact/open*

Within compact flocs there are only few open spaces (Fig.2). In an open floc, on the contrary, parts of the floc are clearly separated from each other by open spaces (Fig.4). An open floc structure is often, but not always, caused by the presence of filamentous organisms.

The agglomerates (Fig.6) represent a separate group of open flocs. These consist of a network of filamentous bacteria, containing small compact firm flocs. These agglomerates are often found in sludges from oxidation ditches.

The morphology of the floc in a particular activated sludge must usually be described by means of a combination of the above-mentioned features; for example a firm, irregular and compact sludge floc. After characterizing the different flocs in a specific sludge in this way, the proportion of the various floc types in the total MLSS content is grossly estimated by eye.

The shape of the flocs influences the sedimentation rate in the final settlement tank. From the view of settling characteristics a floc which is as round and compact as possible would be preferable. Mostly however this shape is not ideal. In the final settlement tank we aim at a best

possible separation of sludge and effluent. The suspended matter in an effluent mainly consists of small sludge flocs which cannot settle fast enough by themselves. However, most of these kind of small flocs present in the sludge do nevertheless settle because they are trapped in the sludge blanket. This trapping is influenced by the shape of the floc. Usually entrapment progresses better when the flocs are not too globular.

5.2. Dimensions of the floc

When we speak of the dimensions of a sludge floc we do not mean the size of the microscopic sludge flocs which are formed as soon as the sludge settles; as for instance can be clearly observed in a measuring cylinder. These agglomerates with a diameter of approximately 10 mm hardly show any cohesion and as a result of this easily fall apart again. Agglomerates of this type consist of a great number of much smaller particles of which the diameter is almost always less than 1 mm. When we speak of activated sludge flocs we refer to the latter particles. Their size depends largely on the loading of the treatment plant, the quality of the sewage and the turbulence in the aeration tank. With the aid of a calibrated eyepiece micrometer the approximate diameter of the flocs can usually be quickly determined. This is performed by measuring the two points which are farthest apart; not taking into account, however, the filamentous organisms protruding from the floc.

Three groups (Figures 7 up to 9) may be distinguished namely:

- | | |
|-----------------------|--|
| 1) large flocs | ; diameter >500 μm |
| 2) middle-sized flocs | ; 150 μm <diameter <500 μm |
| 3) small flocs | ; diameter <150 μm |

The proportion of a certain type of floc in the total MLSS content is again estimated by eye and recorded on the analysis form.

A floc which is too small usually causes a turbid effluent. Small flocs may be the result of very low sludge loading, a too violent turbulence in the aeration tank, poisoning of the floc population, the presence of high concentrations of complexing compounds etc.

5.3. Composition of the floc

The variation in the composition of the floc population (the diversity) and the presence of characteristic groups of bacteria is directly connected with the quality and the quantity of the available nutrients.

A sludge floc usually consists of a whole range of microorganisms. It is this great diversity which makes an activated sludge system so flexible and enables a large number of different compounds to be simultaneously metabolized. With the aid of a microscope (magnification 500-1000x) we can usually get a quick and fairly good impression of the large number of species in the floc. In doing this we only have to note the differences in the shapes and the dimensions of the bacteria which form the floc. Sometimes, however, the floc population is composed of only a few bacterial species. Figure 10 is an example of a sludge floc with a very low diversity of the floc population. A low diversity is recorded with a minus (-) on the analysis form.

If a sludge floc consists of only a few species, there is usually a very high sludge load (load of more than approx. 1 g BOD/g Dry.Matter.Day⁻¹) and/or an influent with a limited concentration of some nutrient (e.g. N or P). A small diversity makes a plant rather vulnerable because the process depends on the functioning of only a few types of bacteria. If anything happens to them the entire treatment process will immediately be disturbed.

In an activated sludge conglomerates are sometimes observed that seem to consist of only one type of cells (fig.11). In that case we are usually concerned with one bacterial species of which the cells are surrounded by a layer of slime. This capsule glues the cells together. When this kind of conglomerates are present in activated sludge the bacterium is usually *Zoogloea ramigera*. This bacterium forms very characteristic outgrowths from the floc (Fig.12). The whole thing looks very much like a hand with fingers. A massive growth of *Zoogloea* fingers is usually the result of high sludge loading and/or an influent containing an insufficient amount of certain nutrients. Sludge containing a large number of *Zoogloea* colonies usually settles badly. The voluminous *Zoogloea* colonies do not only cause a considerable increase in volume but also bind a lot of water. A high

sludge volume index is the result of this.

An activated sludge floc consist largely of living and dead bacterial cells. Together with these in almost any sludge macromolecular (in)organic particles can be observed which clearly do not originate from bacteria. These are particles which were present in the influent and were then enclosed by the sludge flocs. Apart from their size the organic particles can be mostly recognized by their somewhat fibrous structure (Fig.13). The inorganic particles, mostly grains of sand etc., have a higher refractive index than the rest of the floc material. They therefore stand out because of their relative brightness (Fig.14). The extent to which these particles are present is mainly determined by the presence or absence of a grit chamber and/or a primary sedimentation tank.

The extent to which Zoogloea colonies and (in)organic particles are present is recorded as follows:

- = absent
- + = incidentally observed
- + = regularly observed (5-10 colonies or particles in one preparation)
- ++ = frequently observed (more than 10-15 colonies or particles in one preparation)

5.4. Dispersed growth of bacteria

In a well-functioning activated sludge system almost all cell material occurs as activated sludge flocs. The growth of the biomass mainly proceeds as an increase of the flocs. The sparse non-attached cell material is mainly consumed by the protozoa in the sludge. As a result a clear liquid remains after settling of the flocs. Sometimes however the effluent of a treatment plant is not clear but turbid, to a greater or lesser extent. This may be caused, among other things, by the presence of a lot of non-attached cell material. This phenomenon mostly occurs when there is a high BOD sludge loading level and/or an oxygen limitation in the aeration tank. With higher loading the flocs becomes weaker which easily causes deflocculation of the biomass. Besides, with high loading there are also enough nutrients available outside the floc for the bacteria. This stimulates a dispersed growth of the

sludge population. In extreme cases this can lead to a sludge in which hardly any flocs are present.

Oxygen limitation during the aeration phase has a comparable effect. But then we do not only see growth of (facultative) anaerobic bacteria in the liquid between the flocs but also sludge flocs that are partly disintegrating caused by the dying of the strictly aerobic organisms in the floc. We may speak of a kind of poisoning of the aerobic sludge population. The same thing happens when the biomass is really poisoned by for instance a discharge of certain metals etc. This is frequently attended by a strong increase of the amount of dispersed cell material (isolated cells and minuscule flocs). The difference between living and dead cells, by the way, can hardly be seen by means of phase-contrast or bright-field microscopy. If we want to know anything about the activity of the biomass other methods must be applied. The extent to which dispersed growth occurs can be reasonably well quantified visually (magnification 400-500x):

- : almost absent
- + : some tens per field of view
- ++ : hundreds per field of view

Figures 2 and 3 are examples of sludges with little and a lot of dispersed cell material respectively. All the small dark spots in Figure 3 are bacterial cells.

In activated sludge extremely motile bacteria may be observed of which the shape of the cell resembles a spiral or a corkscrew. These are Spirochetes. These bacteria have a very characteristic shape which is why they so easily attract attention (Figure 15). It is not known whether their presence or absence in the sludge population has a special meaning. Spirochetes are mainly found in activated sludge in the summer period and then again mainly in low loaded treatment plants.

Spirochetes slightly resemble Spirils. The latter bacteria do not consist of a double spiral. Besides, Spirils move faster, whilst their diameter is at least about twice as big as the Spirochetes. They are found in plants where the oxygen concentration in the aeration tank is low. The extent to which Spirochetes and Spirils are present (magnification 400-500x) is quantified and recorded as follows:

- = absent
 + = incidentally observed
 + = 5-10 specimens in one preparation
 ++ = more than 10 - 15 specimens in one preparation

To illustrate the way in which the various floc characteristics discussed in this chapter must be recorded on the analysis form (see Appendix 1) two examples are given.

Example 1: A characteristic oxidation ditch sludge (extended aeration plant; moderate filamentous growth; no primary sedimentation tank)

MORPHOLOGY OF THE SLUDGE FLOC						VARIOUS FEATURES	
Firmness and shape of the floc	structure		size			Diversity	+
	open	compact	small	middle	large		
Firm, somewhat rounded		x	+	+		Free cells ³⁾	-
Firm, irregular shape						Zoogloea's ²⁾	-
Weak, somewhat rounded						Spirochetes ²⁾	+
Weak, irregular shape						Spirils ²⁾	-
Agglomerates	x			++	+	Organic fibres ²⁾	+

2) : - = absent; + = incidentally; + = some (5-10) cells or particles per preparation; ++ = many cells or particles per preparation.

3) : - = few; + = some tens per field of view; ++ = hundreds per field of view.

Example 2: Main characteristics of a high-loaded activated sludge
(no nitrification; small numbers of filamentous bacteria;
with primary sedimentation).

MORPHOLOGY OF THE SLUDGE FLOC						VARIOUS FEATURES	
Firmness and shape of the floc	structure		size			Diversity	+
	open	compact	small	middle	large		
Firm, somewhat rounded						Free cells ³⁾	++
Firm, irregular shape						Zoogloea's ²⁾	±
Weak, somewhat rounded		x	±	+	+	Spirochetes ²⁾	-
Weak, irregular shape	x			+	+	Spirils ²⁾	±
Agglomerates						Organic fibres ²⁾	-

2) : - = absent; ± = incidentally; + = some (5-10) cells or particles per preparation; ++ = many cells or particles per preparation.

3) : - = few; + = some tens per field of view; ++ = hundreds per field of view.

6. BULKING SLUDGE

6.1. Introduction

The last phase of the treatment process consists of a separation of the activated sludge flocs and the effluent. In the operation of a treatment plant the principal aim is always the production of a clear effluent. This cannot always be achieved. The presence of suspended matter in the final effluent may have different causes. The following may be mentioned:

1. Dispersed growth of bacteria (high sludge load, absence of Protozoa etc.) results in small particles that do not settle in the final settlement tank.
2. Deflocculation of sludge flocs caused by excessive turbulence in the aeration tank, poisoning of the sludge etc.
3. Incomplete trapping of small flocs by the sludge blanket in the final settlement tank. These 'pin-point' flocs are then discharged together with the effluent.
4. Hydraulic overloading of the final sedimentation tank sometimes causes loss of sludge, while the sludge has good settling characteristics.
5. Incorrect construction of the final settlement tank, where the weir loading is not uniform around the tank.
6. Rising sludge caused by denitrification processes in the final settlement tank, the presence of small granules of fat in the sludge floc, an excessive growth of the bacterial genus *Nocardia*, the trapping of air-bubbles by the floc etc.
7. When the retention time of the sludge on the bottom of the final settlement tank is too long, e.g. because the sludge scraper does not function correctly, the sludge will become anaerobic. This results in gas production, and may often be observed from clumps of sludge floating on the surface of the clarifier.
8. The sludge has bad settling characteristics because of a massive growth of filamentous microorganisms and/or *Zoogloea* bacteria.

We only speak of bulking sludge when the situation mentioned under 8 is the case. The use of the term "bulking sludge" for the other ways of losing sludge is wrong and confusing.

Bulking sludge may be defined as follows: *Sludge which only settles slowly and compacts poorly because of an excessive growth of filamentous and/or Zoogloea organisms.*

The sludge volume index (the volume of 1 g of sludge) is a measure for the settling properties of the sludge. When the latter is more than 150 ml/g (determined according to the dilution method) we speak of bulking sludge. The 150 ml/g level is an arbitrary one. If this level is exceeded, it will often cause problems; e.g. loss of sludge with the final effluent. However, it does not mean that this will never happen if the index is less than 150 ml/g. It is closely related to the hydraulic load of the plant, the construction of the final settlement tank etc. Only when the index is below 100 ml/g we can really speak of sludge which settles well. So the range of 100-150 ml/g is a critical one.

The settling characteristics of the floc are mainly determined by:

1. The extent to which filamentous microorganisms are present.
2. The amount of slime etc. This kind of polymeric compound, produced by microorganisms, usually contains a lot of water. The slime substances, mainly bacterial capsules, thus cause voluminous flocs to originate.

So two different factors may contribute to the originating of bulking sludge. This is why in literature there is a distinction on this subject between filamentous and Zoogloea bulking sludge. This distinction is not quite correct because the presence of filamentous microorganisms does not necessarily mean that bacteria of the Zoogloea type are absent. Especially in conventional activated sludge systems settling problems are frequently caused by the presence of a large number of both filamentous and Zoogloea organisms. A sludge which settles badly and in which there are hardly any filamentous organisms, the real Zoogloea sludge, is only found incidentally and even then mainly in highly loaded plants. The settling problems in oxydation ditches, carousels etc. (so in low-loaded systems) are caused practically always by filamentous organisms. Under these circumstances the Zoogloea bacteria hardly play a significant role.

6.2. Zoogloea sludge

In chapter 5.3. and in the introduction of this chapter something has already been said about the influence of the presence of Zoogloea

bacteria on the settling properties of the sludge. The presence of this type of bacterium causes the voluminous character of the sludge floc. The large amount of water bound by the slime capsules etc. prevents a rapid settling of the flocs. The bad settling characteristics of this type of sludge can, however, hardly be predicted on the basis of microscopic observations, because of the difficulty of distinguishing the Zoogloea bacteria from the other microorganisms in the sludge floc. The specific finger-like outgrowths (see Fig.12) are practically only observed at the edge of a sludge floc. Their number is mostly not so large.

6.3. Filamentous sludge

In almost every kind of activated sludge filamentous microorganisms are present. They actually belong to the normal sludge population. Their presence may even contribute to a better sludge quality; among other things this is often apparent from the very good final effluent quality of treatment plants where filamentous bacteria are found in the sludge. As long as the number of filamentous bacteria remains within reasonable bounds nothing is the matter. Real problems only arise as soon as we can speak of a massive growth of filamentous microorganisms. From several investigations in for example Germany, Great Britain and the Netherlands it appears that 40-50% of all treatment plants suffer from this kind of bulking sludge problems. At some plants bulking sludge is present almost continuously, at others, on the other hand, the number of filamentous bacteria fluctuates strongly.

This is especially dependent on the sludge age; if this is high then of course the sludge quality will in general not change very fast.

6.3.1. Filamentous microorganisms

Bulking sludge is caused by a massive growth of filamentous microorganisms. Although fungi may also incidentally cause settling problems, filamentous bacteria are almost always involved. A bacterium is a spherical or rodshaped, unicellular organism which multiplies through cell division. In this way two new cells originate which subsequently usually separate from each other. This separation does not take place

with filamentous bacteria. Consequently such a filament consists of a chain of cells. In these cells a normal cell division takes place after which the two cells stay together. Sometimes they are unable to separate because the cells are surrounded by a hollow structure, a so-called sheath (see Fig.16). With a normal light-microscope the cross-walls between the cells in a filament can usually be observed without difficulty (see e.g. Fig.33). However, in some filamentous bacteria the crosswalls can hardly be seen in this way (Fig.36). This does not mean that they are absent; because with an electron microscope they can be observed (Fig.17).

Growing as a filament is characteristic for certain bacteria. This means that these bacteria form filaments under practically all circumstances. The supposition which has sometimes been suggested, that most filamentous bacteria form normal separate cells in other circumstances - growing in the shape of a filament would be considered abnormal - is absolutely wrong.

In the past *Sphaerotilus natans* was held responsible for almost all bulking sludge problems. Recent research has shown that about 30 filamentous bacteria may cause bulking sludge. However, these organisms do not all occur with the same frequency. Most of these filamentous bacteria were totally unknown until recently. Consequently they have not yet been named. This is why these unknown types are indicated by a number.

So we may speak of a great variety of filamentous microorganisms in activated sludge. Some types form long stout filaments, others short thin filaments. Certain filamentous bacteria are mainly found in the floc while other types usually occur on a massive scale in the liquid phase between the flocs. It will be clear that the various filamentous organisms do not all influence the settling characteristics of the floc in the same way. It is even true that certain filamentous organisms hardly influence these settling characteristics in a negative way. Therefore it is important to make a regular check on the population of filamentous bacteria in a treatment plant in order to be able to note the development of certain unwanted types in good time.

6.3.2. Quantification of the extent of filamentous growth

In some activated sludges filamentous bacteria are almost completely absent, in other sludges their presence is so massive that it completely dominates the microscopic view. Between these 2 extremes a large number of gradations are possible. It is mostly desirable to quantify the number of filamentous bacteria in a sludge in a certain way when analysing a microscopic image. This will make it possible as time goes by to record changes in the sludge population that are, for example, the result of a certain method of bulking sludge control.

In theory it is possible to determine quite accurately the number of filaments in a certain sludge by means of counting and measuring. However this is not only very time consuming but moreover it can only be done when we are concerned with more or less straight filaments. Highly bent filaments, that often form tangles and run right through parts of the floc cannot be measured in this way. For the routine investigation of a large number of sludge samples this method is not very suitable. A much simpler and much faster method consist of a gross estimation by eye of the number of filamentous organisms which are present in a certain sludge sample. The number of filaments are thus quantified visually. This does not seem to be very accurate but it has been proved in practice that it may not only be performed rapidly but that it also produces useful information. On the basis of visual interpretation five categories may be distinguished, viz:

- category 0 : hardly any filamentous microorganisms (m.o.) present (Fig.18)
- category 1 : small numbers of filamentous m.o. present (Fig.19)
- category 2 : moderate numbers of filamentous m.o. present (Fig.20 and 21)
- category 3 : large numbers of filamentous m.o. present (Fig.22 and 23)
- category 4 : excessive numbers of filamentous m.o. present (Fig.24 and 25).

In ascertaining the category of a certain sludge the microscopic view is compared with the serie of reference images of the different categories (Figs.18-25). The sludge is given the category number of the photograph which best resembles the microscopic image. In this way, the extent of filament formation is more or less quantified. Application of this system offers many advantages. Subjective descriptions such as 'little', or 'many', which may cause much confusion, are replaced by a number. This is also understood by others, provided they use the same reference images. Moreover, variations occurring in the course of time can be recorded simply using this method.

This method is only meaningful if an increase in the category number actually involves a deterioration in the settling properties of the sludge. Usually, there is such a relationship. A general rule for this cannot be given, however, because the settling properties of activated sludge not only depend on the extent of filamentous growth, but also on the kind of filaments present (large filaments cause more problems than thin, twisted ones), the specific gravity of the floc and the bound water in de floc. A shift of the floc population into filamentous bulking is often observed more clearly, and consequently at an earlier stage, from an increase in the category number than from an increase in the Sludge Volume Index (SVI). If the SVI only is recorded bulking sludge is not identified until the SVI increases significantly. Then the sludge usually contains many filaments already. Category 2-3 often forms a kind of critical range. The sludge still settles quite well but a small increase of the number of filaments results in real bulking problems.

Using 5 different groups is of course an arbitrary choice but it has proved to be sufficient to be able to classify correctly the different sludge qualities. A smaller number of categories has the disadvantage that it is more difficult to record changes in the number of filamentous bacteria because the difference between 2 and 3 for example becomes too big. A larger number of categories complicates the system unnecessarily because the differences between the various categories become so small that classification in the right category will be a problem.

One extra category (4⁺ or 5) can be applied for recording an extremely filamentous growth (when the microscopic field of view is almost completely filled with filaments).

Most of the time it will prove to be sufficient for the determination of the correct category, to study one preparation thoroughly. Sometimes it will be necessary to study more than one slide, because filamentous bacteria are not always spread regularly across the various flocs and the water phase between the flocs. In the same sludge there may be flocs that are completely free of filaments while other flocs are practically surrounded by filamentous bacteria. This phenomenon however only occurs in the lower categories. In these cases an average is taken.

The classification in categories is in the first place based on observations made with a magnification of 100 - 200 x. Sometimes however, it will prove necessary to change this classification when the sludge is studied with a higher magnification, because thinner filaments are sometimes overlooked with a low magnification.

6.3.3. The terms: Dominating and Secondary

In a certain activated sludge very often different filamentous micro-organisms are present simultaneously. This of course rather complicates the identification of these organisms. However, the problem is less complicated than it seems at first sight, because different types usually do not occur in the same numbers. Especially in oxidation ditches and carousels the population of filamentous bacteria is usually composed in such a way that one, at the most two types, occur in much larger numbers than the rest of the filamentous organisms. This is why we must distinguish between dominating and secondary filamentous bacteria in activated sludge. As a rule of thumb we call a certain filament 'secondary' when its number is 10 to 20 times smaller than the number of the dominating form(s). In principle this distinction is based on observations made with the magnification which is also used to identify the filamentous organisms; viz. 500 - 1000 x.

It should be noted that it is risky to simply go by the observations made with wet mount preparations. Some filamentous bacteria (*Nostocoida limicola* and Type 0092) are often largely surrounded by parts of the floc and can thus easily be overlooked. A correct impression of the number of filaments of these types can only be obtained after studying stained smears (see chapter 7.2).

It has already been said that in low-loaded activated sludge systems we usually have no more than 2 dominating types. A population which is very often observed in oxydation ditches consists of:

Microthrix parvicella	: dominating
Type 0092	: secondary
Haliscomenobacter hydrossis	: "
Type 0041	: "

In the more conventional treatment plants the microscopic image is often more complicated, especially when there is a relatively large amount of non-domestic wastewater. In these sludges very often 3 to 4 different dominating types of filamentous bacteria occur at the same time.

The distinction dominating/secondary has, for the greater part, nothing to do with the classification in categories as discussed in the previous section. This means that with every category we must distinguish between dominating and secondary filamentous organisms. Only with the lower categories (1, sometimes 2) it frequently occurs that we do not have a clearly dominating form. In this case all types are called secondary.

One may wonder in how far it is useful to identify filamentous organisms whose numbers are so few that we must qualify them as secondary. Or, in other words, why should we worry about filamentous organisms that, because of their low number in a certain sludge, hardly influence the settling characteristics of the sludge. In itself this is a reasonable question. We must however realise that the biomass in a treatment plant is subject to constant changes. It is a dynamic system in which shifts in population constantly take place; especially in systems with a low sludge age. Not all filamentous organisms however cause serious bulking problems. This is related to their shape (stout filaments usually have a greater influence on the settling characteristics than thin, bent filaments) and the fact that it appears that certain types are capable of developing in a more massive way than other filamentous bacteria. It is most certainly important always to describe the population of filamentous organisms as accurately as possible. This is

the only way to obtain a correct picture of changes that take place in the population as time passes. We are then prepared in time if a certain filamentous bacterium, of which it is known (from literature or personal experience) that it may cause serious settling problems, starts to develop. The reverse is of course also the case. In addition, the presence or absence of certain types may some times inform us about the way the treatment plant works. In this way some filamentous organisms may act as indicator organisms. The presence of Thiotrix and/or Beggiatoa bacteria will more or less always be the result of an oxygen limitation in the aeration tank or of a stale sewage.

7. IDENTIFICATION OF FILAMENTOUS MICROORGANISMS

In this chapter it will be shown how to distinguish the various filamentous microorganisms from each other. In paragraph 7.4 some identification keys are given. While compiling these keys, which has been thoroughly tested, it has been taken into account that the people who perform microscopic sludge analysis in practice, do not have expensive equipment, like an electron microscope for example, at their disposal. Consequently, only those characteristics have been inserted in the determination keys which can be observed with the aid of a normal phase-contrast microscope.

7.1. Morphological characteristics

When bacteria are studied through a microscope we look at the length and diameter of the cells, inclusions, crosswalls, motility etc.; that is; mainly characteristics that have to do with the morphology of the cell. Now, in succession these characteristics that are important in the scope of this manual will be discussed. Some characteristics will be explained by means of drawings (Figs. 26 and 26a; p.41 and 42). The only correct way to observe most of the characteristics mentioned is by using a magnification of 500 - 1000 x. The sequence corresponds to that on the analysis form (Appendix 2).

Branching: The filaments of some filamentous organisms are sometimes branched. This may occur in 2 ways viz.:

- a. The cells are branched. This is called real branching (Figs.37 and 41).
- b. Branching which originates with sheath forming bacteria when a free cell attaches itself to the sheath and subsequently grows into a new filament. More or less comparable is branching which originates when a sheath is damaged and an outward opening is formed. The cell near this opening in the sheath gets the opportunity to grow further and thus to form an offshoot. This is called false branching (Fig.42).

Real branching mainly occurs with fungi and the *Nocardia* bacteria. *Spaerotilus natans* often forms false branches.

Motility: Only a few of the filamentous organisms are motile by means of a movement known by the name of 'gliding movement'. It may be compared more or less with the way in which a worm moves forwards. If it is thought that

a certain filament is motile, one must make sure that there is no flowing of liquid in the preparation caused by evaporation of the water.

Inclusions: In the cells of some filamentous organisms we sometimes see dark or even bright granules. These consist of food reserves stored by the cell. These granules are usually of a dark hue as long as they are small. Large volutin granules (see par.7.2.2.), which can be observed sometimes at a high magnification, are dark as well, but most of the larger globules are very refractive and contrast highly with the rest of the cell (see Fig.47). Usually the granules consist of sulphur. PHB (=poly- β -hydroxybutyrate) granules are only incidentally present. The difference between these two kinds of granules is hard to see. To demonstrate which form is present one must place a drop of ethanol against one side of the cover glass of the preparation, and a piece of filter paper against the other side. The ethanol is thus drawn across the preparation. The S-granules will dissolve while the PHB-granules remain intact, which can be controlled microscopically (500 - 1000 x).

Septa or crosswalls: The walls between adjoining cells. It must be mentioned that in some filamentous bacteria crosswalls cannot be seen through a normal light-microscope.

Shape of the filaments: The shape of the filaments of various filamentous organisms is an important aid when characterizing. We may distinguish between:

- straight filaments,
- bent filaments,
- twisted or coiled filaments

It must, however, be said that straight filaments are usually not dead straight. Although dead straight filaments do occur (those of *H. hydrossis*) straight filaments are usually somewhat bent in sludge. This is especially the case with relatively long filaments.

Attached growth: The surface (the outerwall) of filamentous organisms is usually 'smooth'. Sometimes however other bacterial cells or small flocs are attached to the surface of the filaments. In the manual we call this 'attached growth'. The attached growth phenomenon mainly occurs with bacteria that are surrounded by a sheath. We only speak of attached growth

if quite a lot of unicellular organisms are attached to the surface of the filament.

Constrictions: Some filamentous bacteria have a continuous outerwall. The position of the septa is not emphasized by a constriction of this wall. With other bacteria this does occur.

Shape of the cell: With unicellular bacteria the following cellshapes may be distinguished:

- spherical or coccus: The cells are more or less round.
- rod-shaped : The length of the rather straight cells clearly exceeds the celldiameter.
- spiral-shaped : The length of the cells, twisted in the form of a spiral, is always much bigger than the celldiameter.
- vibrio-shaped : Cells are somewhat bent.

Filamentous microorganisms consist of coccus or rod-shaped or cells with are derived from these forms. By this is meant that the cells in a filament, because there are sometimes no constrictions of the outerwall of the filament in the position where a crosswall is found, are often more or less square or rectangular.

Sheath: This is the name for the cylinder-shaped structure in which the cells of some bacterial genera are wrapped. It is usually difficult or impossible to observe the sheath with a light-microscope. Only the empty sheaths can be observed in this manner. In stained smears the sheaths can sometimes be observed more clearly.

Rosettes: In sludge, filaments that are connected to eachother at their base may incidentally be observed. This is called a rosette of filaments. This is caused by the fact that these bacteria excrete certain compounds at the base of the filament with which they can stick together. As far as known only *Thiothrix* and Type 021N sometimes show this feature. It is not inserted in the identification key.

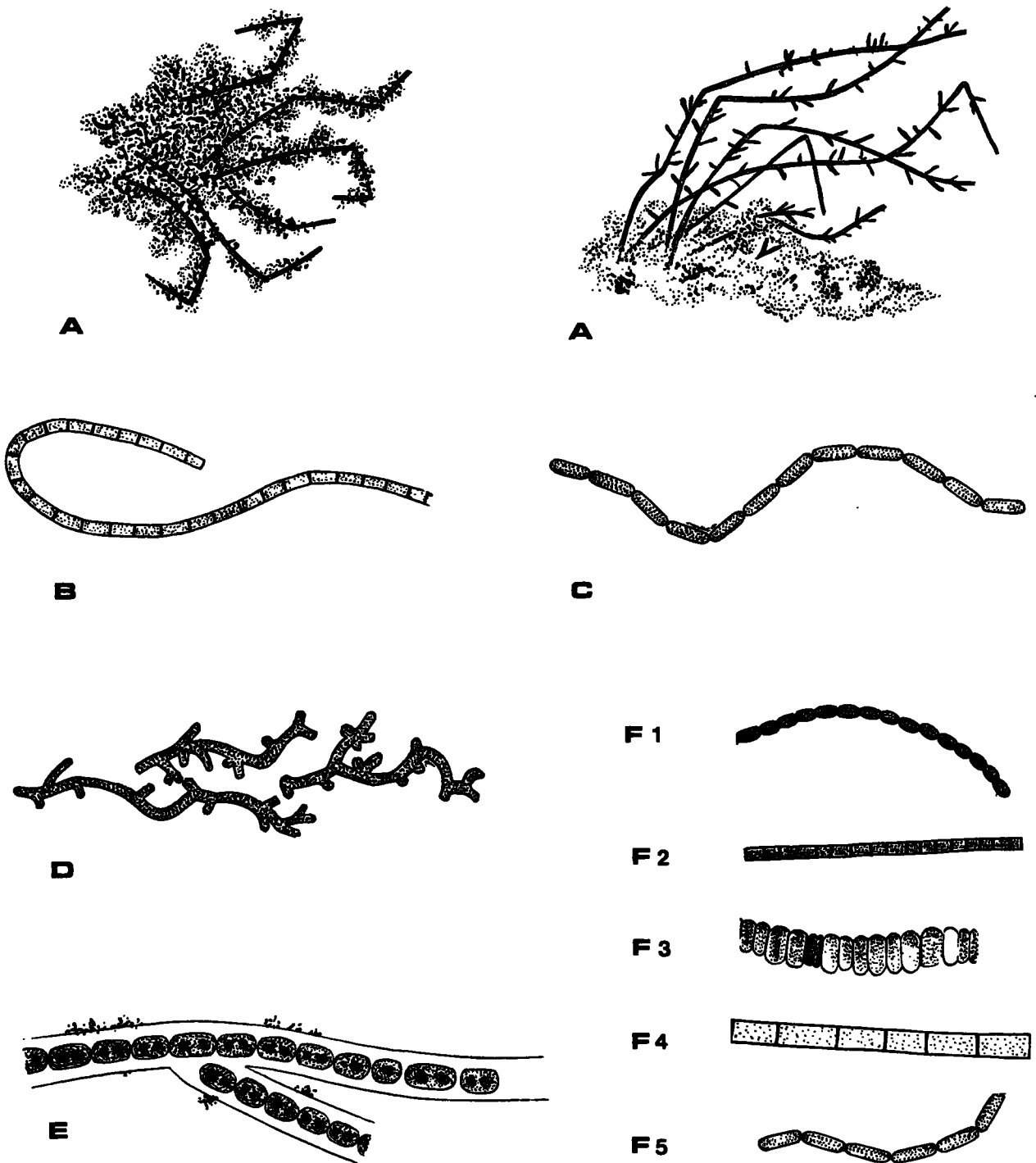


Fig.26: Several morphological characteristics

A: Attached growth to the filaments
 B: No constrictions of the outerwall
 C: Clear constrictions

D: Real branching
 E: False branching; the cells are surrounded by a sheath
 F: Cellshape; (1) coccus, (2) square, (3) disc-shaped (4) rectangular, (5) rod-shaped

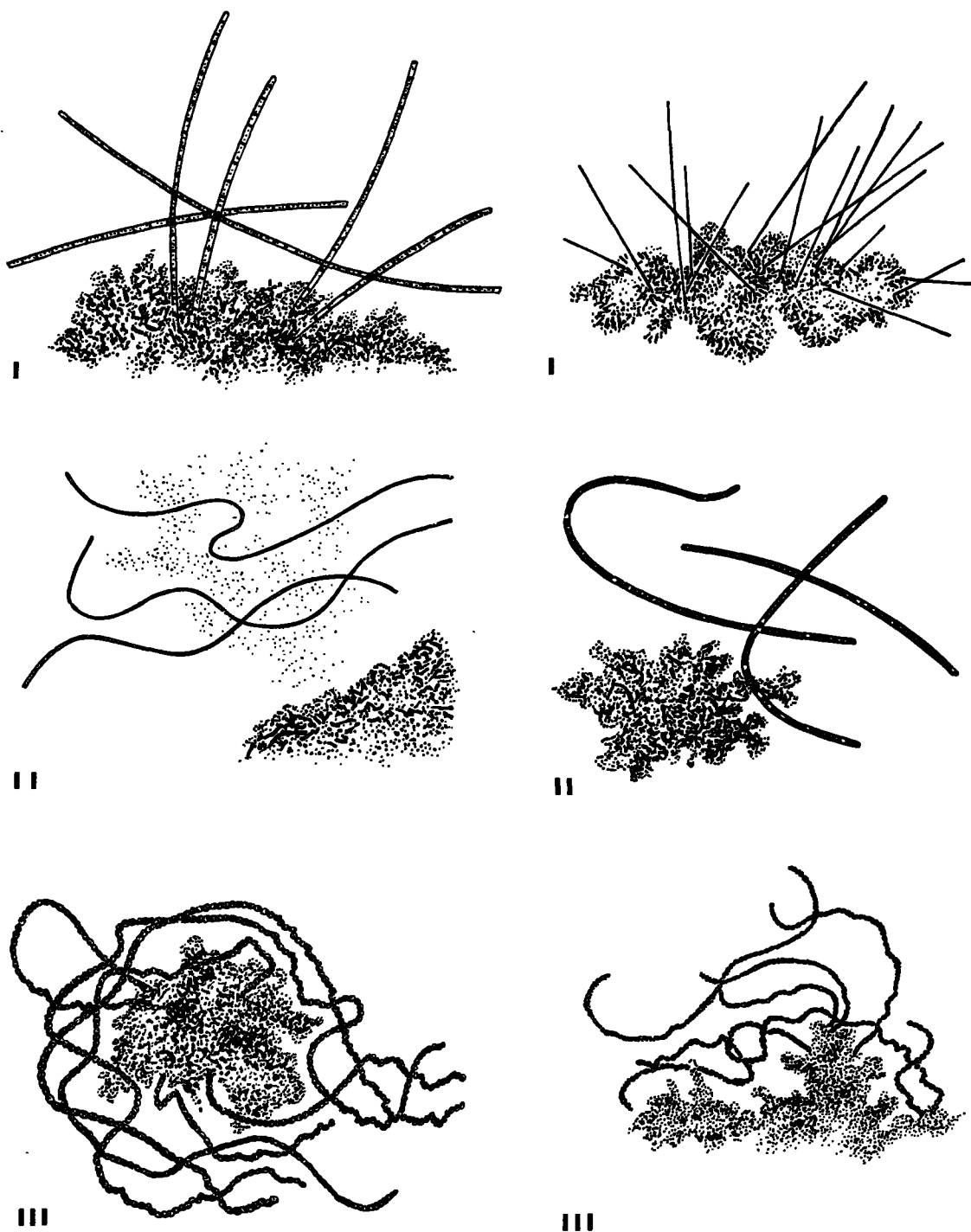


Fig. 26a: Shape of the filaments

- I: Straight filaments
- II: Curved or bent filaments
- III: Coiled filaments

7.2. Staining techniques

It is possible to get a better view of various parts of the cell by means of specific staining. In this manual the staining methods according to Gram and Neisser will be dealt with. The principle of almost all staining methods is that a certain part of the cell is linked more tightly to the pigment than other parts of the cell. The sulphur storage test will also be discussed. This is not meant for staining but to test the ability of the cell with regard to sulphur storage.

7.2.1. Staining according to Gram

Gram staining is an essential aid when identifying bacteria. With this method the bacteria are first stained blue by means of carbolgentianviolet. Subsequently the cells are rinsed with an alcohol solution. During this treatment the cells of some bacterial genera release the dye. This is why these bacteria are called Gram negative. The Gram positive bacteria do not release the absorbed carbolgentianviolet when rinsed with alcohol. The colourless Gram negative bacteria are subsequently counter-stained with safranine. This gives them a light-red colour.

Required solutions

- A. Carbolgentianviolet solution: Dilute 10 ml of the stock solution with 90 ml of a 5% phenol solution.
- | | | | |
|----------------|---|---------------------|--------|
| Stock solution | : | Carbolgentianviolet | 10 g. |
| | | alcohol (96%) | 90 ml. |
- B. Lugol's iodine solution : Dissolve 3 g of KI in a few ml of distilled water, add 1 g of I₂ and dilute to 300 ml with distilled water.
- C. Alcohol solution : Dilute 7 ml of the stock solution with 1000 ml of alcohol (96%).
- | | | | |
|----------------|---|-----------------|---------|
| Stock solution | : | I ₂ | 100 g |
| | | KI | 40 g |
| | | ethanol (96%) | 1250 ml |
| | | distilled water | 100 ml |
- D. Safranine solution : Dissolve 0.25 g of safranine in 10 ml ethanol (96%) and dilute with 100 ml of distilled water.

Staining procedure

- 1) Prepare a so-called fixed smear (see chapter 3.3).
- 2) Apply solution A, period of contact 60 sec; subsequently allow the dye to flow from the slide.
- 3) Apply solution B; period of contact 60 sec; subsequently allow the dye to flow from the slide.
- 4) Immerse the slide in solution C for 30 sec.
Move the slide gently to and for during this procedure.
- 5) Rinse the slide with tap water; allow the water to flow gently down the back of the slide.
- 6) Apply solution D; period of contact 120 sec, subsequently rinse the slide again with tap water.
- 7) Allow the slide to dry and study it through a bright-field objective lens (400-600 x). A blue filter improves the contrast.

Results

Gram negative bacteria are stained light-red and Gram positive bacteria dark-blue (Fig.27-29). The activated sludge population consists for the greater part of Gram negative bacteria. With some of the filamentous bacteria, especially Type 0041 sometimes not all the parts of a filament have the same colour after staining. This is mainly caused by attached growth of other bacteria.

This growth masks the filament to a certain extent which is why the blue dye cannot penetrate sufficiently. In this case the results of the staining are mainly judged through observation of the protruding ends of the filaments.

Comments

- 1) Most of the solutions can be bought ready made.
- 2) In literature on this subject there are tens of different recipes for Gram staining. The recipe given above appears to give the best results with filamentous organisms; it produces the best possible contrast.
- 3) There are no storage restrictions for most solutions. Solution C (not the stock solution) must be freshly prepared every month.
- 4) The slides must be well scoured (fat free).
- 5) With certain objectives of an inferior quality the difference between red and blue is difficult to observe.

7.2.2. Neisser staining

In chapter 7.1. something has already been said about the storing of food reserves in the cell. Some bacteria form granules in the cell which mainly consist of polyphosphates. These granules cannot clearly be observed with a light-microscope without staining. By means of the Neisser staining their colour turns to blue-black. The polyphosphate granules can however be clearly observed by means of an electron microscope (see Fig.17).

Two bacterial species, *Nostocoida limicola* and Type 0092, turn completely grey-blue with this method. It is not known how this is brought about. It is however a good aid for the identification of these two types.

Required solutions

A. Methylene blue	0.1 g
Acetic Acid, glacial	5 ml
Ethanol (96%)	5 ml
Distilled water	100 ml
B. Crystal violet 10% in ethanol (96%)	3.3 ml
Ethanol (96%)	6.7 ml
Distilled water	100 ml
C. Chrysoidin Y, 1% aqueous solution	33.3 ml
Distilled water	66.7 ml

Staining procedure

1. Prepare a fixed smear.
2. Apply a freshly prepared solution consisting of 2 parts of A and one part of B; period of contact 10-15 sec. Subsequently allow the excess of the dye to flow from the slide.
- 3) Apply solution C; period of contact 45 sec.
- 4) Rinse the slide with tap water; allow the water to flow down the back of the slide.
- 5) Allow the slide to dry and study it through a bright-field objective lens (400-600 x).

Results

Neisser negative filaments stain light-brown to yellowish (Fig.30) and are often hardly visible! Neisser positive filaments contain dark granules (fig.31) or are stained entirely grey-blue (Fig.32).

Comments

- There is almost no storage limit for the solutions A, B and C. They can be bought ready made.
- The size of the polyphosphate granules depends on the amount of nutrients available to the bacteria. In the filaments originating from highly loaded treatment plants these granules are mostly smaller than in sludge from oxidation ditches.
- If granules are present they almost always indicate the presence of *M.parvicella*. As far as known, *N.limicola* and Type 0092 are the only filamentous bacteria which stain entirely grey-blue. Consequently, Neisser positive bacteria can be easily identified.
- Some unicellular species also store polyphosphate in their cells, viz. *Acinetobacter* sp. etc. (biological P-removal). Dark colonies of these bacteria may be observed in the sludge floc after Neisser staining (if the cells actually contain polyphosphate).

7.2.3. Sulphur storage test

The bacteria *Beggiatoa*, *Thiothrix* and Type 0914 are able to store sulphur granules in their cells provided they grow in an environment with enough reduced sulphur compounds. In activated sludge representatives of these three genera may be found. In *Thiothrix* however, the sulphur granules are not always present. This makes a correct identification more difficult. By treating *Thiothrix* with Na_2S it is possible to make this filamentous bacterium store a large number of S-granules in a very short time.

Procedure

Mix a certain volume of activated sludge together with a same volume of a Na_2S -solution (200 mg of $\text{Na}_2\text{S} \cdot 7 \text{ H}_2\text{O}$ per 100 ml). Allow the mixture to rest for 10-15 min. (Shake now and then to keep the sludge in suspension). Now check if there are indeed S-granules stored in the cells. The S-granules can be clearly observed with a magnification of approximately 500 x.

Results

Thiotrix cells store many, large, highly refractive granules (see Fig.47). In the cells of the other filamentous bacteria only small S-granules are formed or none at all.

7.3. Description of the filamentous microorganisms

In this chapter a description will be given of 29 filamentous organisms which up to now have been observed in activated sludge. The organisms which have not been inserted in the identification key, because they are only incidentally observed in activated sludge, have been marked with an *. Some highly infrequent types, which on the basis of morphological characteristics can hardly be distinguished from other filamentous organisms are not dealt with.

The order of discussion is alphabetical respectively numerical for the organisms with a number. The length of the filaments mentioned in these descriptions are maximum values. This means that shorter specimens do occur quite frequently.

1. *Beggiatoa*

Curved, somewhat short filaments (length <200 μm) which clearly have a movement of their own. Septa are not visible in filaments containing a large number of sulphur globules. The filaments are found isolated in the liquid between the flocs. In the sludge sometimes different types are observed which may vary as to cell shape, diameter etc. The filaments contain S-granules and they are both Gram and Neisser negative.

2. '*Cyanophyceae*' (Fig.33)

Straight, stout, non-motile filaments; length 300-1000 μm . The cells, (diameter 2.5-3.0 μm) are almost square to rectangular. Septa are clearly visible; hardly any constrictions. Usually no sheath. Branching and attached growth are hardly ever found. No food-reserve granules in the cells. Both Gram and Neisser negative; sometimes slightly Gram positive. The S-test is also negative (incidentally some small S-granules).

Remark: It is not certain whether these are indeed bluegreen algae. Morphologically they look very much like them but they are non-motile and have no distinct colour.

The filaments of these organisms very much resemble those of Type 021N. With this last type however we usually have constrictions and the filaments are also less stout.

3. *Flexibacter*

Curved, somewhat short filaments (length 200 μm) which clearly have a movement of their own. Several representatives of this bacterial family are found incidentally in sludge; among themselves they differ as to cell shape, diameter of filaments, etc. The filaments which are almost always free-floating in the liquid do not contain S-granules and also show a negative S-test. They are both Gram and Neisser negative.

4. *Fungi* (Fig.41)

Long, non-motile, stout, real branched filaments; length 200-600 μm . Septa clearly visible. Hardly any constrictions. The rectangular cells have a diameter which may vary from 2.0 to approximately 5.0 μm ; this depends on the type of fungus. There is neither a sheath nor attached growth. The cells often show a clear structure. Fungi slightly react to Gram staining (negative). They are Neisser negative. S-test negative.

5. *Haliscomenobacter hydrossis* (Fig.34)

Short (usually <100 μm) non-motile, often dead straight filaments that protrude from the flocs like needles. The cells are surrounded by a sheath. Septa and/or constrictions not visible. No branching or food-reserve granules. Cell diameter approximately 0.3 μm . Only incidental attached growth. Gram and Neisser negative. No storage of S-granules.

6. *Microthrix parvicella* (Figs.35 and 36)

Highly bent, often twisted filaments that sometimes form tangles around and through the flocs. The non-motile filaments mostly have a length of 200 - 400 μm and a diameter of approximately 0.5 μm . Septa and/or constrictions are hardly observable with a light microscope. No sheath present. Branching hardly occurs. Some attached growth. No clearly visible granules of foodreserves in the filaments. With certain microscopes the polyphosphate granules are somewhat visible and should not be mistaken for septa! Neisser positive (dark polyphosphate granules); sometimes negative. Gram positive. S-test negative.

M.parvicella resembles Type 0581 and *N.limicola* I. Type 0581 is however Gram negative while *N.limicola* does not store polyphosphate granules in the cell, but stains entirely grey-blue with the Neisser stain.

7. *Nocardia* (Fig.37)

Short (length <100 μm) branched filaments which are mostly surrounded by parts of the floc. The non-motile filaments have a diameter of approximately 0.5 μm . Neither septa nor constrictions can be seen with a light-microscope. There is no sheath. No attached growth; also no feed reserve granules. Gram positive, Neisser negative. S-test negative.

8. *Nostocoida limicola* I (Fig.38)

Bent, non-motile filaments; length varies from 100-300 μm . In the filaments, mainly free-floating in the liquid between the flocs, the septa are hardly observable. The cells are more or less spherical with a diameter of 0.6-0.7 μm . Constrictions are difficult to see. There is no sheath and no branching. No attached growth. Foodreserves in the shape of visible granules are not found. Gram positive. S-test negative. Neisser positive (the filaments are completely stained grey-blue).

N.limicola I resembles *M.parvicella* and can only be distinguished definitely from this bacterium by means of the Neisser staining.

9. *Nostocoida limicola* II (Fig.39)

See *N.limicola* I. However, the filaments are somewhat thicker (cell diameter 1.0-1.2 μm), Septa and constrictions are both reasonably visible. Spherical to disc-shaped cells.

10. *Nostocoida limicola* III (Fig.40)

Twisted, non-motile filaments which often form tangles around which a floc is built. It is usually impossible to determine the length of the filaments. Septa and constrictions are clearly visible. The disc-shaped cells (diameter approximately 1.5 μm) are not surrounded by a sheath. Branching hardly ever occurs. Gram positive. S-test negative. Neisser positive (filaments are completely stained grey-blue).

Remark: Sometimes filamentous bacteria are observed, especially in industrial plants, which closely resemble *N. limicola* but which are Gram and Neisser negative.

11. *Sphaerotilus natans* (Fig.42 and 43)

This bacterium forms long (500-1000 μm) non-motile, slightly bent filaments which usually protrude from the floc.

The rod-shaped to rectangular cells (length 1.5-5.0 μm ; diameter 1.2-2.0 μm) are surrounded by a sheath and sometimes contain PHB-granules. The constrictions are usually clearly visible especially at the end of the filaments. False branching occurs frequently. Sometimes the sheath has attached growth. The bacterium is both Gram and Neisser negative. The S-test does not result in large S-granules.

Non-branched filaments may be mistaken for the filaments of Type 021 N. The longer filaments of the latter are usually more curved than those of *S. natans*. Besides, they differ in the shape of their cells. Thiotrix II also resembles *S. natans*. However, Thiotrix shows a clearly positive S-test.

12*. *Streptococcus* (Fig.44)

Bent, non-motile, short filaments (length <100 μm) which occur free-floating in the liquid between the flocs. The coccus-shaped cells (diameter 0.7-0.8 μm) are not surrounded by a sheath. Neither attached growth nor branching occur. Visible foodreserve granules are not formed. The S-test is negative. The cells are Gram positive and Neisser negative.

Remark: Morphologically this type very much resembles a *Streptococcus*. It is however not completely certain whether this name is correct. This type can easily be mistaken for Type 1863. However the latter bacterium is clearly Gram negative.

13. *Thiotrix I* (Figs. 45-47)

Slightly bent, non-motile filaments which mainly protrude from the flocs in a radial way. The length of the filaments is highly variable (50-500 μm). The diameter of the rectangular cells may vary from 0.4 to 1.5 μm (the younger cells are thinner than the older ones). Tapering

of the filaments can often be clearly observed in one and the same filament. There is no sheath. No branching or attached growth. The filaments often contain S-granules. There is a clearly positive reaction to the S-test. Both Gram and Neisser negative.

Remark: In some sludges this bacterium only occurs in the shape of short (length up to 100 μm) thin filaments. In these filaments the septa are hard to see. There is however still a clearly positive reaction to the S-test.

14. *Thiotrix* II (Figs. 48 and 49)

Slightly bent non-motile filaments which mainly protrude from the flocs in a radial way. The length of the filaments may vary from 200 to 800 μm . The cells (diameter 0.8-1.5 μm) are rectangular or rod-shaped. Constrictions are usually only seen at the end of the filaments. The septa and the shape of the cells can only be observed after removal of the S-granules which are present in the cells. There is neither attached growth nor branching. The cells are surrounded by a sheath. The cells often contain S-granules. The reaction to the S-test is definitely positive. Both Gram and Neisser negative.

These filaments very much resemble *S. natans*. However, the latter bacterium does not store S-granules.

15. *Type 0041* (Figs. 50 and 51)

Straight or slightly bent, non-motile filaments; length 200-300 μm . The filaments are not only found isolated in the liquid but also surrounded by the floc. Square to rectangular cells (diameter 1.0-1.4 μm , length 0.7-2.3 μm), which are surrounded by a sheath. Incidental branching. Frequently substantial attached growth; in some industrial plants however this does not occur. Septa visible, but sometimes masked by attached growth. The cells incidentally contain small S-granules, a slight positive reaction to the S-test. Neisser negative. Gram positive; parts with a lot of attached growth are negative or only slightly positive.

Filaments with no attached growth resemble the filaments of the Type 021 N. The latter is however Gram negative.

16. *Type 0092* (Fig.52)

Short (length <100 μm), rather bent, non-motile filaments which are usually found surrounded by floc particles. Only in treatment plants that receive influent mostly from industrial sources the filaments of this type also occur massively in the liquid between the flocs. The cells, with a diameter of 0.5-0.7 μm , are rectangular. The septa however are very hard to see. There are no constrictions. Neither branching nor attached growth. No storage of S-granules. The reaction to the S-test is also negative. Gram negative. Neisser positive (grey-blue filaments)
Remark: The filaments in non-stained slides are easily overlooked, mostly because they tend to occur inside the flocs. Not until Neisser staining has been performed, do we get a good impression of their number.

H.Hydrossis can easily be mistaken for Type 0092. The former however is Neisser negative.

17* *Type 0211* (Fig.53)

Bent and twisted non-motile filaments (length <100 μm) which mainly occur free-floating in the liquid between the flocs. The rod-shaped cells - the constrictions are fairly well visible - have a diameter of 0.2-0.3 μm . There is no sheath. Branching does not occur. There is no attached growth. S- and PHB-granules are not formed. S-test negative. The cells are both Gram and Neisser negative.

18. *Type 021 N* (Figs. 54 and 55)

Long (500-1000 μm) somewhat bent, non-motile filaments. Septa clearly visible. The shape of the cells varies strongly. The latter may vary from short (length 0.4-0.7 μm ; diameter 1.8-2.2 μm), disc-shaped to long rod-shaped cells (length 2.0-3.0 μm ; diameter 0.6-0.8 μm).

In principle all intermediate forms are possible, but almost square cells occur most often. Sometimes some highly refractive cells are found in the filaments. Constrictions are not always clearly visible. No sheath or branching. Incidental rosettes. Attached growth does not occur very

often. Small S-granules are sometimes found in the cells. Gram negative. Sometimes parts of the filaments are somewhat positive. Neisser negative. S-test mostly somewhat positive.

Type 021 N may be mistaken for Type 0041, *S.natans*, *Thiotrix*, *Leucothrix* and Type 0961. Type 0041 is however Gram positive. *S.natans* has a different cell shape whereas *Thiotrix* clearly reacts in a positive way to the S-test. Finally, the filaments of Type 0961 consist of almost transparent, truly rectangular cells.

The morphological differences between Type 021N and *Leucothrix* are very small. It is not possible to distinguish them from each other by microscopic techniques. However, *Leucothrix* is hardly ever present in activated sludge, as far as known.

19* *Type 0411* (Fig.56)

Bent or twisted, short (50-150 μm), non-motile filaments which are mainly found near the edges of the flocs. The long, rod-shaped cells - the constrictions are clearly visible - have a diameter of 0.5-0.7 μm . There is neither a sheath nor branching nor attached growth. This bacterium does not form S- or PHB-granules. The cells are both Gram and Neisser negative and the S-test is also negative.

20 *Type 0581* (Fig.57)

Bent or twisted filaments, usually free-floating in the water phase, which sometimes form tangled balls. The non-motile filaments have a length of 100-300 μm and a diameter of 0.3-0.4 μm . Septa and constrictions cannot be seen with a light-microscope. No attached growth. No sheath. No branching of filaments. No foodreserve granules. Gram negative. Neisser staining negative or slightly positive. So test negative.

The Type 0581 very much resembles *M.parvicella*. However, the latter is Gram positive and its filaments are somewhat thicker.

21* *Type 0675* (Fig.58)

This microorganism closely resembles Type 0041, but it is clearly thinner (diameter of the cells 0.5-0.7 μm)

22 *Type 0803* (Figs. 59 and 60)

Straight or bent, non-motile filaments (length 100-300 μm), mainly found in the liquid between the flocs. The cells (diameter 0.7-0.8 μm) are square to rectangular. No constrictions. The septa are sometimes difficult to see because they are more or less masked by small dark granules (probably polyphosphate). Often 3 to 4 filaments are attached to a piece of highly refractive inorganic material (see Fig.60). The filaments are neither branched nor surrounded by a sheath. No attached growth. There are no S-or PHB-granules in the cells. Negative reaction to the S-test. Both Gram and Neisser negative; incidentally Neisser positive (granules).

Remark: In some plants sometimes a filamentous bacterium is found which very much resembles Type 0803 but which is Gram positive.

23 *Type 0914* (Figs. 61 and 62)

Straight or somewhat bent, non-motile filaments which mainly occur isolated in the liquid between the flocs. The length of the filaments may vary from 100-200 μm . The cells have a diameter of 0.7-0.9 μm . The septa (Fig.62) are only then clearly observable, when the S-granules have been removed (see chapter 7.1). The cells are square to rectangular (so no constrictions). There is no sheath. Neither branching nor attached growth. There are S-foodreserves in the cells, however mostly not in the shape of nice sperical globules but as granular material. This bacterium, surprisingly enough, shows a negative S-test. Gram positive, Neisser negative.

24 *Type 0961* (Fig.63)

Straight, non-motile, rather long filaments (300-500 μm), which usually protrude from the flocs. The truly rectangular cells (diameter 1.1-1.5 μm) seem to be almost transparent. Neither constrictions nor branching have been observed. There is no sheath. No attached growth. Neither S- nor PHB-granules are formed. This bacterium is both Gram and Neisser negative, S-test also negative.

25 *Type 1701* (Figs. 64 and 65)

The length of the filaments of this type is usually 100-200 μm . They are non-motile and somewhat bent (if inside the floc, then often twisted filaments). The rod-shaped cells have a length of 2.5-3.5 μm and a diameter of 0.7-0.9 μm and are surrounded by a sheath. They sometimes contain small dark PHB-granules. Septa and constrictions are clearly visible (especially at the end of the filaments). Branching (false) only occurs incidentally. There is usually a lot of attached growth. Both Gram and Neisser negative. No storage of S-granules.

26* *Type 1702* (Fig.65)

Short (length 50-150 μm), straight, non-motile filaments which protrude from the floc. The rectangular cells have a diameter of approximately 0.6 μm . Both septa and/or constrictions are usually not clearly visible. The cells are surrounded by a sheath. There is neither branching nor attached growth. This bacterium does not form visible foodreserve granules. The S-test is negative. The cells are both Gram and Neisser negative.

This type may easily be mistaken for the H.hydrossis type. However the latter bacterium forms thinner filaments.

27 *Type 1851* (Figs. 66 and 67)

Straight or somewhat bent, non-motile filaments; length 200-400 μm . The longer filaments are sometimes cracked. Sometimes the filaments are found in bundles. Rectangular cells (diameter 0.5-0.7 μm , length 1.7-3.5 μm) surrounded by a sheath. Septa difficult to see. No branching or storage of foodreserve granules. Often attached growth, hardly ever excessive, but usually perpendicular on the outerwall of the filaments. Gram positive (colour however not very dark-blue); Neisser negative. S-test negative.

28* *Type 1852* (Fig.68)

Straight or somewhat bent, non-motile filaments which protrude from the floc. The rectangular cells have a diameter of 0.6-0.8 μm . There are no constrictions. The cells are somewhat transparent just like those of Type 0961.

There is no branching. Although the cells are surrounded by a sheath, no attached growth has ever been observed. Neither S- nor PHB-granules are formed. The S-test is negative. The cells are both Gram and Neisser negative.

Type 1852 resembles Type 0961. However, the filaments of the latter are far more stout.

29 Type 1863 (Fig.69)

Short (length <150 μm), non-motile, bent filaments which mainly occur free-floating in the liquid phase. The filaments consist of coccus or rod-shaped cells. Cell diameter approximately 0.8 μm . Septa and constrictions are clearly visible. Neither a sheath nor branching nor attached growth. S- and/or PHB-granules are not formed. The cells are both Gram and Neisser negative and the reaction to the S-test is also negative.

Type 1863 shows some resemblance to a Streptococcus. The latter however is Gram positive.

7.4. The identification keys

In the identification keys 22 filamentous organisms have been inserted. Apart from organisms that are regularly found in sludge, a number of filamentous bacteria which may be easily identified on the basis of some very characteristic properties have also been inserted. In order to ensure that these keys does not become too complicated, six organisms which are only incidentally found, have not been inserted. This considerably lessens the chance of reaching wrong conclusions.

The principal aim of the various keys is to enable plant operators and people involved in wastewater treatment research to distinguish the filamentous microorganisms from each other in the mixed activated sludge population. A correct taxonomic classification was not the authors object. Thus, when two microorganisms are inserted closely together in a key this does not mean that these bacterial species are related to each other.

In the original edition of this manual only one key was inserted (Nr.I, p 61)

However, by arranging the various features in different ways modified versions of this key are possible. An example proving to be useful during courses on this subject is key nr.II. Of course, in the latter key a correct interpretation of the Gram and Neisser stains is very important.

The actual population of filamentous organisms in an activated sludge plant depends, among other things, on the sludge load applied. It is possible to compile identification keys in which only those organisms are inserted which at least are observed regularly at a specific sludge load. This simplifies considerably the identification because the number of organisms which is important at a certain sludge load is rather restricted. In the tables 2-4 some information is given concerning the frequency of occurrence of the various filamentous organisms in three different modifications of the activated sludge process. For each sludge load a key is given (nrs.III-V). It should be emphasized that by application of these keys the chance of reaching wrong conclusions is increased considerably if the results are not checked carefully (see identification procedure). Otherwise infrequent species will be overlooked completely or will be identified falsely. Moreover, the frequency of occurrence is based on research in Dutch treatment plants. There is some evidence that in other countries slight differences in frequency of occurrence may exist. *Nocardia*, for example, the main cause of scum formation, is hardly ever found in Dutch treatment plants.

In identifying filamentous microorganisms it is important to work systematically. By this is meant that primarily a survey of the different morphological characteristics of the organisms in a certain activated sludge must be made as accurately as possible. When determining the morphological characteristics of a certain bacterium several filaments of the same type must be observed accurately, because the chance of making a wrong identification becomes considerably bigger if only one filament of a certain type is observed. This is because the morphological characteristics are influenced by the condition of the filamentous organisms. Subsequently the two stainings and the S-test are performed. The analysis form, inserted in this manual as Appendix 2, may be used for the recording of all these data. Not until all the required information has been gathered can the correct name or number of a certain organism be determined with the aid of

an identification key. Subsequently it is ascertained (as a kind of final check) whether the observed characteristics do indeed belong to the organism concerned. For this, Table 5 may be used, in which the characteristics of the different filamentous organisms are summed up. Besides, the microscopic image must be compared with the figures of the various filamentous organisms.

If after checking it appears that the observed characteristics do not correspond at all with those of the identified organism a mistake has most probably been made. In that case the characteristics that have been recorded must once more be critically observed in order to detect the cause of the wrong identification. Very often it will appear that a certain characteristic cannot be ascertained with certainty. For example a different interpretation of the term 'clearly visible or not clearly visible' where septa are concerned, often presents problems.

Following the identification key again will usually lead to a correct identification. Sometimes however, it will not be possible to succeed in identifying a certain filamentous bacterium in this manner. In that case we usually have to do with a bacterium which has not been inserted in the identification key.

The *identification procedure* can be summarized in the following way:

1. Start with 'easily' observing the sludge at a low (100-200 x) magnification. Try to find out how many different species are present.
2. Distinguish between dominating and secondary types.
3. Record the shape of the filaments.
4. Switch over to a magnification of 500-1000 x. Check observations 1-3 and correct them if necessary.
5. Record the characteristics observed on the analysis form. (Appendix 2).
6. Perform the Gram and Neisser staining and the sulphur storage test.
7. Record the results.

Remarks: 1. To find again in the stained smears the different types which have been observed in the wet mount is often difficult. Consider features like shape and size of the filaments, attached growth and whether the filaments protrude from the flocs etc.

2. Sometimes in the stained slides filaments are observed which have been overlooked in the wet mount.

8. Follow the identification key → names or numbers.
9. Verify the conclusions obtained by comparing the microscopic image with the figures in the manual. Besides, the recorded characteristics should also agree with those mentioned in Table 5.
10. Critically judge the recorded characteristics whether these do not correspond at all with those of the identified organism(s).
11. Record the results obtained on the analysis form for a microscopic view of activated sludge (Appendix 1).

7.5. Some mistakes which are frequently made.

In performing microscopic sludge analysis it is very important that the microscope is well adjusted. A poor image quality is often caused by derangement of the microscope. This makes correct observation of features like cross walls, shape of the cells and the presence of inclusions impossible. So, it is important to check regularly the position of the condensor and the phase-contrast adjustment.

Stained smears must be observed with bright-field and not with phase-contrast. Otherwise the results of Gram and Neisser staining will be interpreted wrongly. Do not forget to change the condensor to the bright-field position!

A certain feature will not be recorded on the analysis form (Appendix 2) before it is observed regularly. So, several filaments of a certain species must be observed before the features can be recorded.

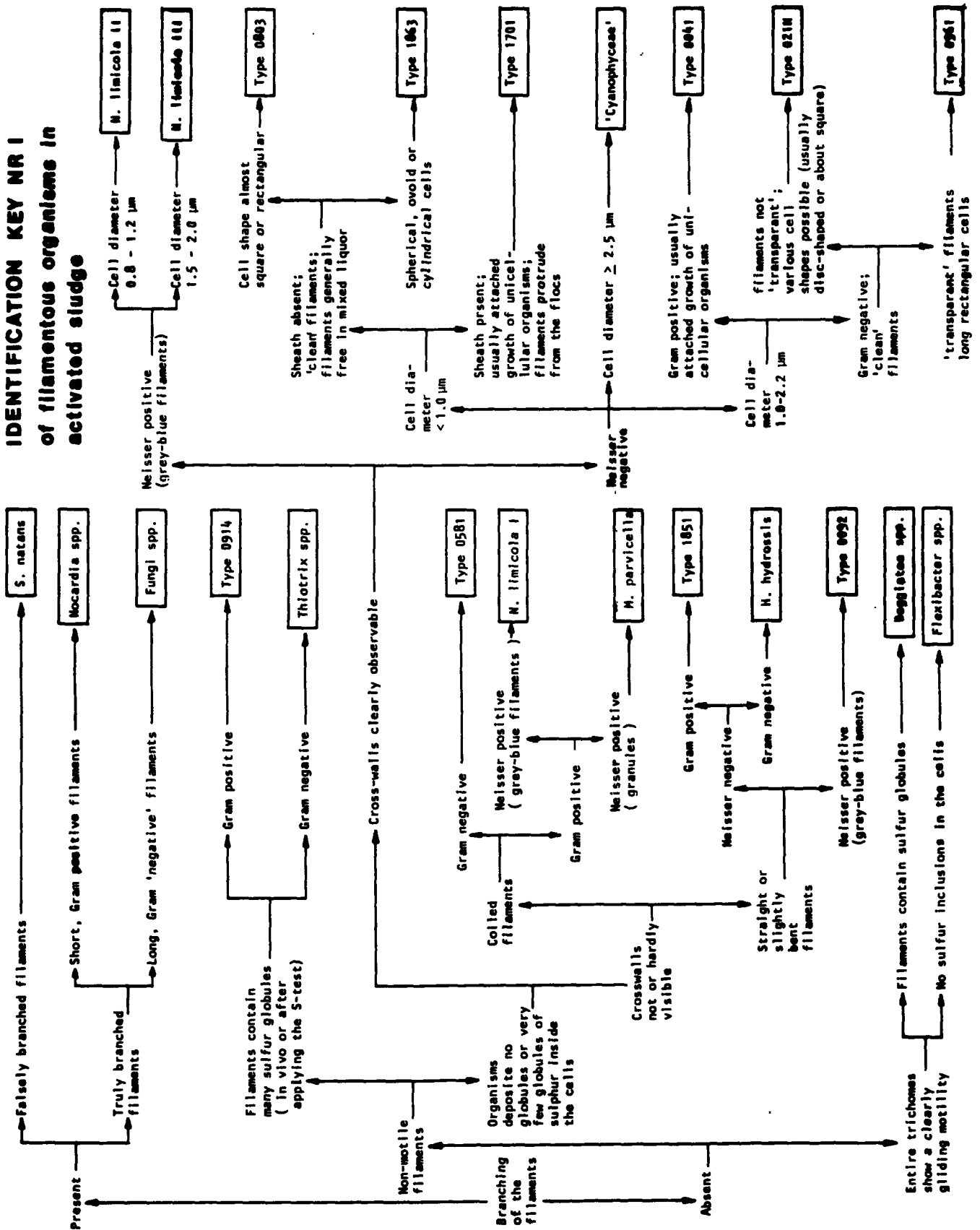
Sometimes, the polyphosphate granules in *M. parvicella* can be observed vaguely at a high magnification without staining (Neisser) and thus can be mistaken for cell septa.

Only *Thiothrix* filaments contain many large and bright S-granules after the sulphur test. Nearly always, the sulphur granules in the Types 0041 and 021N are much smaller and not so bright as those in *Thiothrix*. This feature is only recorded "positive" on the analysis form if many large granules are present.

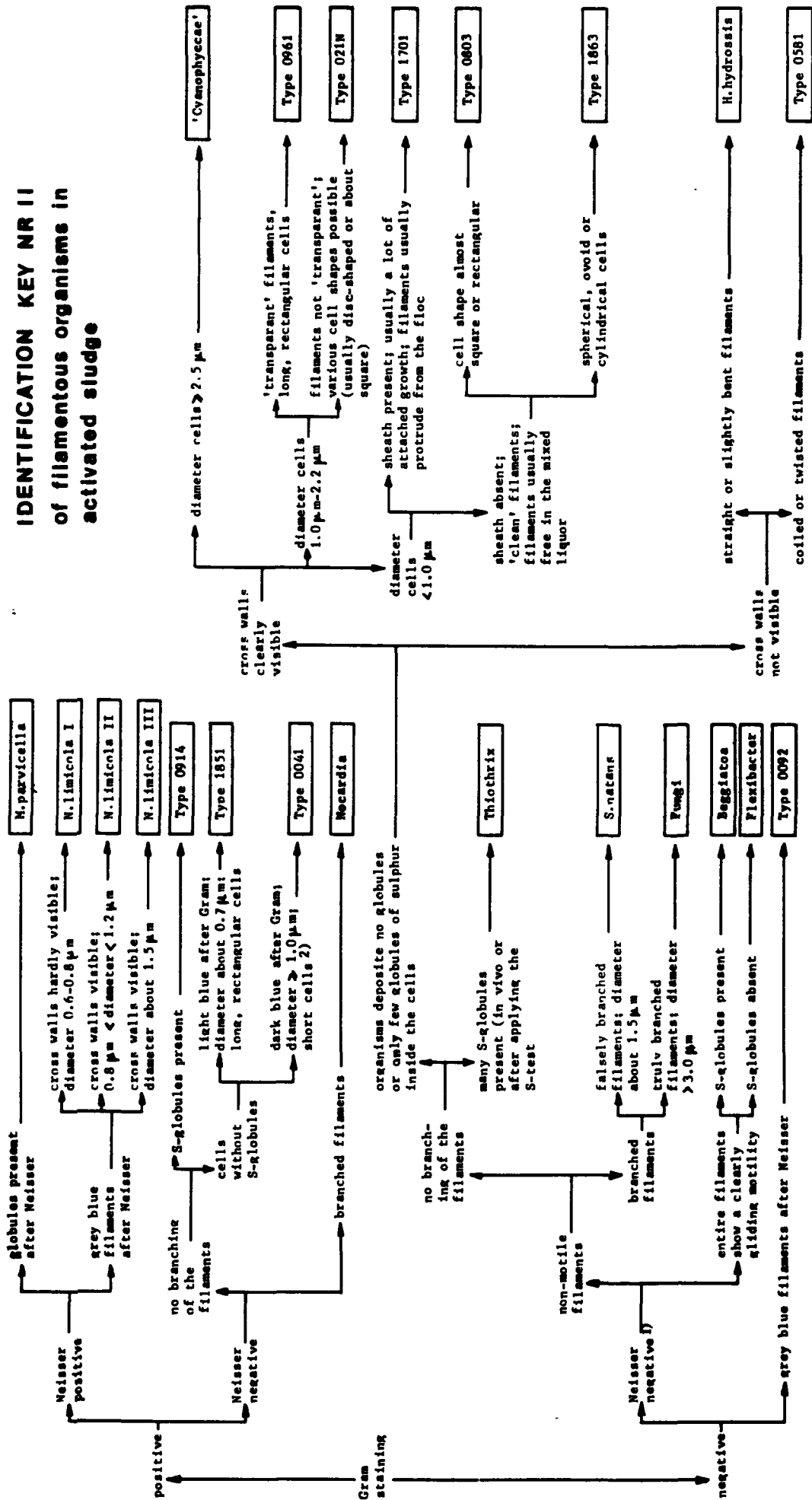
Very short, transparent ends of filaments can be mistaken for empty sheaths. In most cases, a short transparent end will concern pretty surely an empty cell. Usually, it is not possible to ascertain the presence of a sheath definitely with a light microscope unless rather long (length $>5-10\ \mu\text{m}$), completely transparent structures can be observed at the end of the filaments. Substantial attached growth indicates the presence of a sheath, however.

Attached growth of unicellular bacteria can be mistaken by an unexperienced observer for branching of the filaments. So it is important to compare the microscopic image carefully with the figures 37 and 41-43, if something looking like branching of the filaments is observed.

IDENTIFICATION KEY NR 1 of filamentous organisms in activated sludge



IDENTIFICATION KEY NR II of filamentous organisms in activated sludge



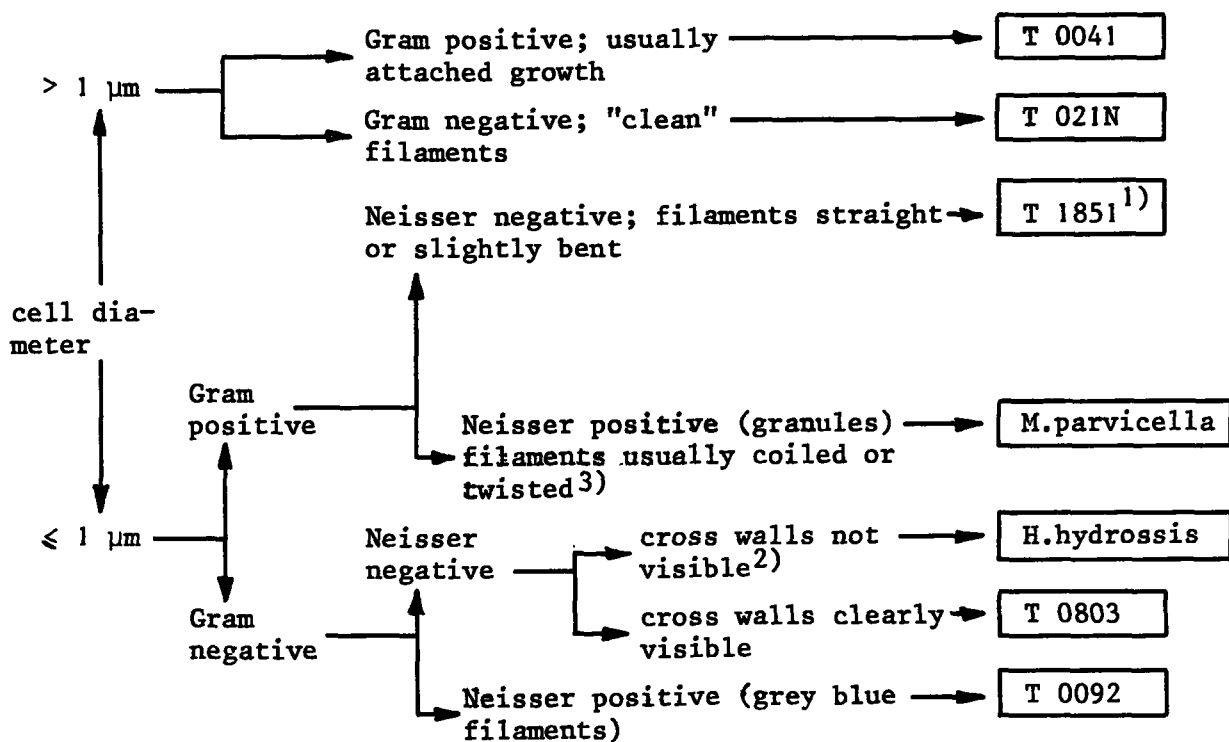
1) : Incidentally some dark granules

2) : diameter 0.6-0.8 μm → Type 0675

Table 2. OCCURRENCE OF FILAMENTOUS BACTERIA IN LOW LOADED TREATMENT PLANTS (EXTENDED AERATION PLANTS, OXIDATION DITCHES ETC.). INFLUENT MAINLY OF DOMESTIC ORIGIN.

Species	Filament abundance
M. parvicella	frequently dominant, in particular in winter
Type 0092	in summer sometimes dominant; frequently secondary
Type 1851 Type 0803	incidentally dominant, regularly secondary
H. hydrossis Type 0041/Type 0675 Type 021 N Type 0581 N. limicola	hardly ever dominant; regularly secondary

IDENTIFICATION KEY NR III



1) presumably T 0041 or T 0675 if cell length < cell diameter

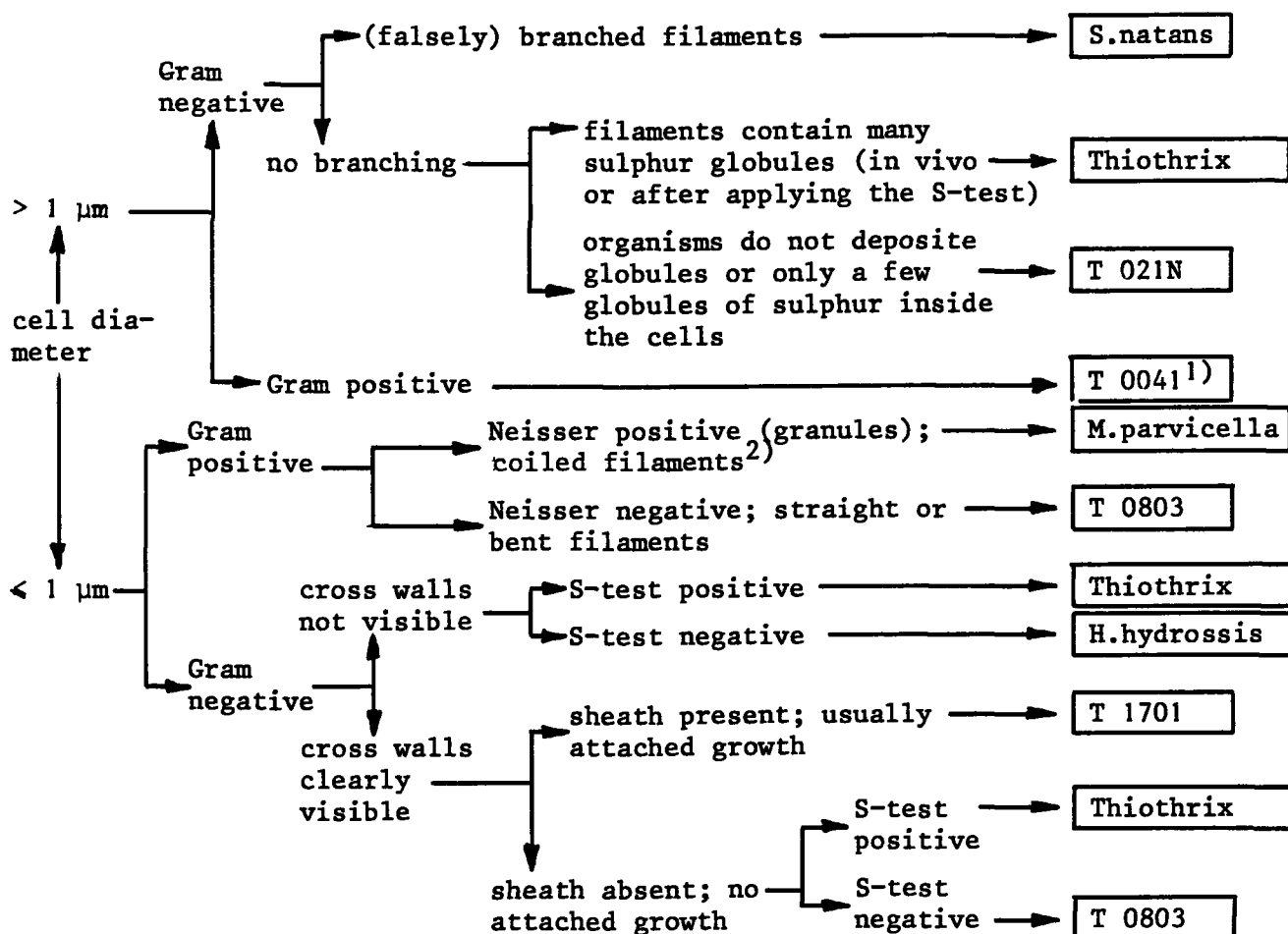
2) filaments are coiled or twisted → Type 0581

3) filaments are grey blue with Neisser → N. limicola

Table 3. OCCURRENCE OF FILAMENTOUS BACTERIA IN CONVENTIONAL ACTIVATED SLUDGE PLANTS WITH COMPLETE OR PARTIAL NITRIFICATION. INFLUENT MAINLY OF DOMESTIC ORIGIN.

Species	Filament abundance
Type 021N M.parvicella	frequently dominant
Type 1701 Type 0803 H.hydrossis	regularly dominant; regularly secondary
S.natans Thiothrix Type 0041 N.limicola	hardly ever dominant; regularly secondary

IDENTIFICATION KEY NR IV



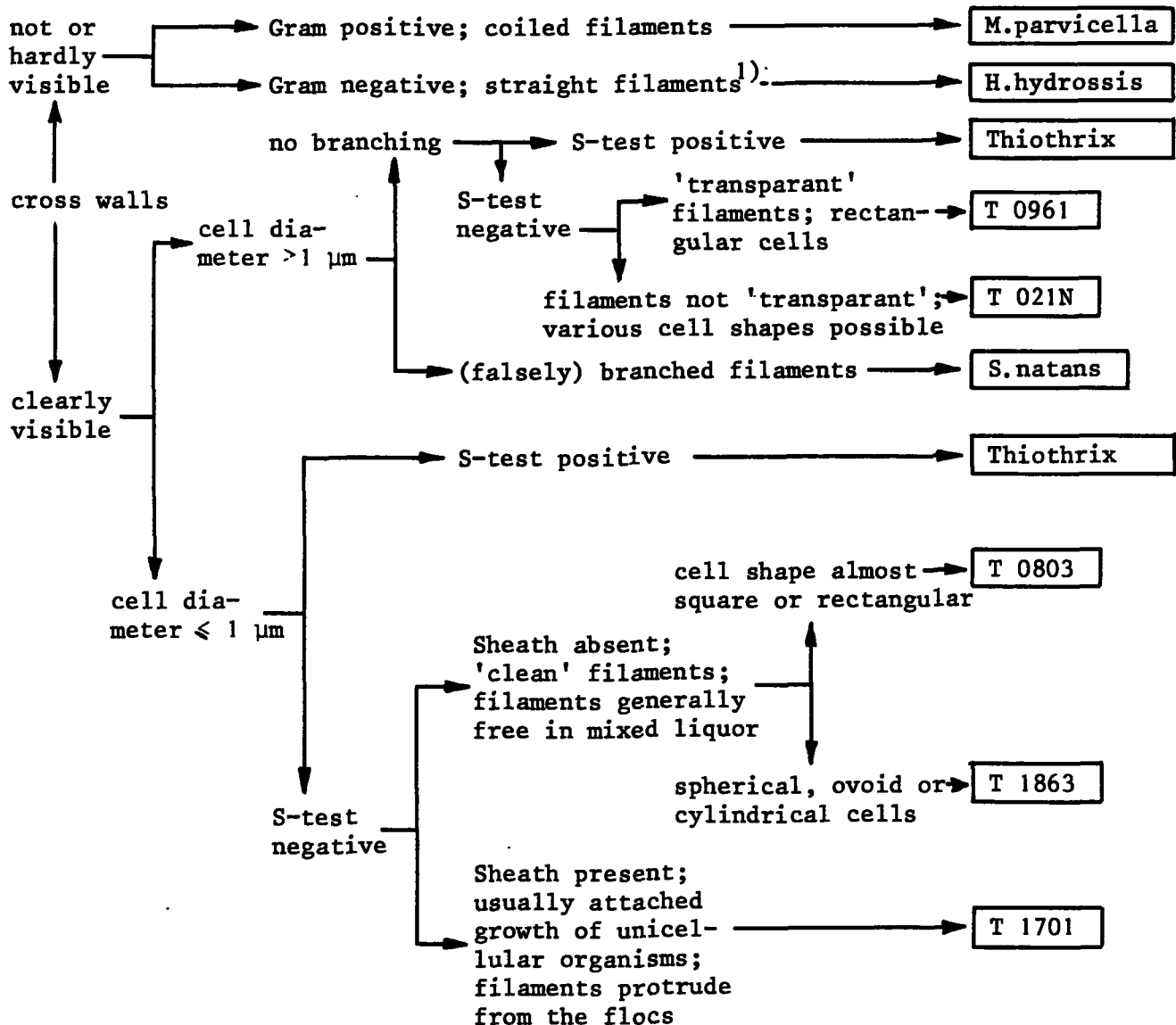
1) Gram positive and Neisser positive → N.limicola

2) Neisser positive (grey blue filaments) → N.limicola

Table 4. OCCURRENCE OF FILAMENTOUS BACTERIA IN CONVENTIONAL ACTIVATED SLUDGE PLANTS WITHOUT NITRIFICATION. INFLUENT MAINLY OF DOMESTIC ORIGIN

Species	Filament abundance
Type 021 N	frequently dominant
Type 1701 H.hydrossis	regularly dominant; frequently secondary
S.natans	regularly (?) dominant; frequently secondary
M.parvicella	incidentally dominant; sometimes secondary
Type 1863	sometimes secondary
Thiotrix Type 0803 Type 0961	frequency of occurrence of these three species in high loaded plants not known

IDENTIFICATION KEY NR V



1) S-test clearly positive → Thiotrix

Table 5. SURVEY OF THE CHARACTERISTICS OF THE FILAMENTOUS MICROORGANISMS INSERTED IN THE IDENTIFICATION KEYS

	PHASE CONTRAST MICROSCOPE; 700-1000 x																BRIGHT FIELD				
	branching	motility	straight or slightly bent	shape of filaments	coiled or twisted	length filaments <200 μm	attached growth of unicellular organisms	sheath present	crosswalls clearly visible	< 1.0 μm	1.0-2.2 μm	> 2.5 μm	diameter cells	rectangular or square cells	cells rounded (ovoid, spherical, disc-shaped or rod-shaped)	sulfur deposited in the cells	in vivo	after applying the S-test	PHB ⁹) granules present	Gram stain	Neisser stain
Beggiatoa		+	+		+	+			+3)	+	+	+	+	+3)		+	+			+	+
"Cyanophyceae"		+	+					?	+			+	+	+						+	+
Flexibacter		+	+		+	+			+	+	+		+	+	+					+	+
Fungi	+		+								+	+		+						4)	4)
H.hydrossis			+			+	+	+		+				8	8					+	+
M.parvicella					+	+	+			+				8	8	8				+	+6)
Nocardia	+				+	+				+				8	8	8				+	+
N.limicola I			+		+	+	+			+					+					+	+7)
N.limicola II					+	+	+		+	+	+				+					+	+7)
N.limicola III					+	+	+		+		+				+					+	+7)
S.natans	+1)		+				+	+	+		+				+				+	+	+
Thiothrix	2)		+			+	+	+	+3)	+	+		+	+3)	+3)	+	+			+	+
Type 0041			+			+	+	+	+	+	+		+	+			+8)			+	+
Type 0092			+			+				+				8	8					+	+7)
Type 021N	2)		+			+			+	+	+		+	+	+		+8)			+	+
Type 0581					+	+				+				8	8					+	+
Type 0803			+			+			+	+				+						+	+6)
Type 0914			+			+			+3)	+				+3)		+				+	+
Type 0961			+					?	+		+			+						+	+
Type 1701	+1)		+		+	+	+	+	+	+					+				+	+	+
Type 1851			+		+	+	+	+	+	+				8	8					+5)	+
Type 1863			+		+	+			+	+					+					+	+

+ = sometimes; ? = unknown; ∞ = cell shape usually not visible by phase contrast microscopy;

1) falsely branched; 2) sometimes formation of rosettes; 3) only visible after removal of the sulfur inclusions

4) Fungi do not stain by the Gram stain; 5) faintly; 6) granules; 7) filaments grey-blue; 8) small granules;

9) poly-β-hydroxy butyric acid

8. PROTOZOA, ROTIFERS AND NEMATODES IN ACTIVATED SLUDGE

In most activated sludges several higher organisms may be found together with the bacteria that form the flocs. These organisms mainly feed on bacterial cells that are free in the liquid and at the edges of the flocs. In this way they remove many loose bacterial cells and thus contribute to a better quality of the effluent. Their presence is regarded as a positive contribution to the treatment process.

The presence of certain species also says something about the performance of the process. In general it may be said that ciliates and rotifers are mainly found in plants of which the loading level is not too high. Besides, there must be enough oxygen. In highly loaded treatment plants and also in plants with an oxygen level which is low or too low, flagellates and Rhizopoda (amoebae etc.) occur relatively more often. For more information the reader is referred to literature on this subject.

The protozoa, rotifers and nematodes are generally much bigger than bacteria. Their size may vary from 10 to approximately 1000 μm . Besides, they usually have a characteristic shape. This makes them very conspicuous in a microscopic preparation. Hundreds of different types have been observed in activated sludge. It is however quite a difficult task to identify these organisms correctly and a thorough knowledge of this matter is required. This is not the objective of this manual. Therefore only a short description of a number of types which frequently occur in activated sludge will be given. Along with the descriptions of the different types, drawings and photographs (if available) have been inserted. Very characteristic features are underlined in the descriptions.

A short glossary is given (p.78) which explains some terms in simple language.

If one wishes to record the number of cells of a certain type in activated sludge, this can be done by counting. For this a counting chamber may be used.

8.1. Protozoa

The protozoa are subdivided in Ciliates, Flagellates, Rhizopoda and Actinopoda. These subgroups will be discussed in this order in the following chapters.

8.1.1. Ciliates

The ciliates are characterised by the fact that the surface of their cells is completely or partly covered with cilia. Around the opening of the "mouth" the cilia are often grouped in a certain way. This enables the ciliates to cause the surrounding liquid to flow. They "fan" their food (bacteria, large organic particles) into their direction. The cilia also play a part in the movement of the cell.

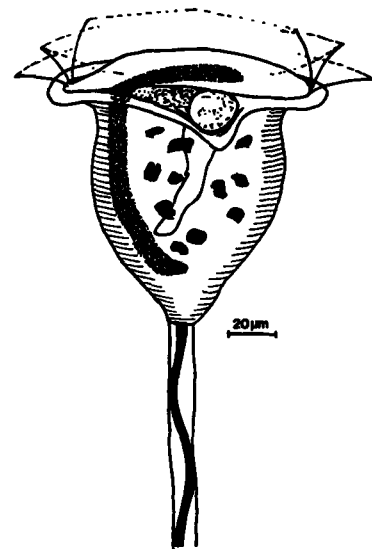
This group is subdivided into sessile, creeping (over the flocs) and free (in the liquid) ciliates. With the sessile ciliates the cell is situated on a stalk which is usually attached to a sludge floc. Therefore the sessile ciliates cannot move about.

I SESSILE CILIATES

Vorticella sp. (Figs. 70 and 71)

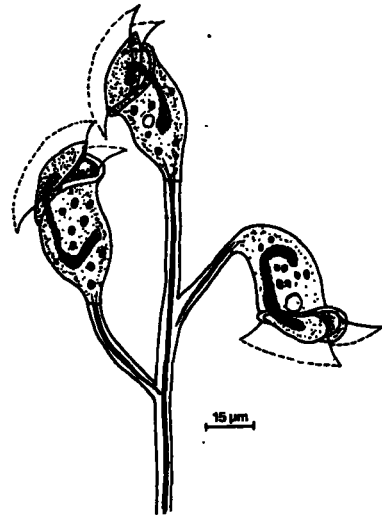
The body, diameter 50-150 μm , is bell-shaped and mostly situated on a stalk. The opening of the mouth is surrounded by rings of cilia; there are usually no other cilia. However, when they are in the free swimming stage aboral cilia can sometimes be observed. There is a myoneme in the stalk allowing the organism to contract. In contracted position the stalk resembles a corkscrew (Fig. 71).

The Vorticellas are solitary organisms; the stalks are not branched.



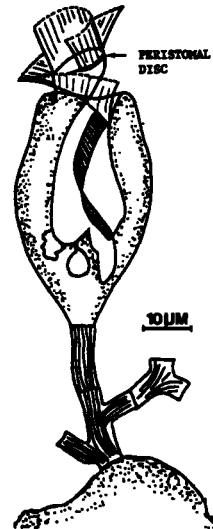
Carchesium sp. (Fig.72)

A bell-shaped body with a wide opening on the anterior side. Diameter 100-125 μm , and again rings of cilia around the opening of the mouth. The rest of the body is 'bare'. The stalk contains a myoneme allowing the organism to contract. The stalks are usually branched, so colonies do exist. There is however a break in the myoneme near every branch.



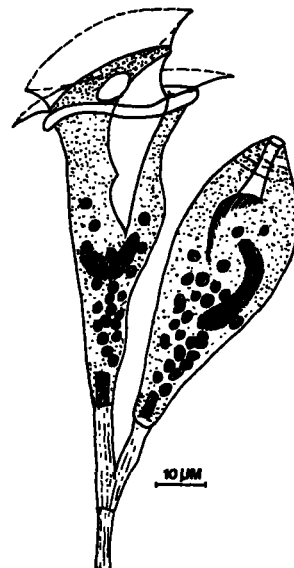
Opercularia sp.

This organism very much resembles the representatives of the former family. The stalks are also branched, consequently colonies do grow. There is however no myoneme in the stalk, so that this organism cannot contract. The cells have a diameter of approximately 140 μm . A peristomal disc is usually present. The diameter of this very characteristic disc is about one-fifth of the bell-opening width.



Epistylis sp. (Fig.73)

This ciliate has a trumpet or vase-shaped body; diameter 70-100 μm . The branched stalk does not contain a muscle and is sometimes segmented.



These four sessile species have a great many features in common. They can be distinguished from each other by closely observing the following characteristics:

	muscle in the stalk	stalk branched	peristomal disc	shape of the body
Vorticella	+	-	large	bell
Carchesium	+	+	large	bell
Opercularia	-	+	small	bell
Epistylis	-	+	large	vase

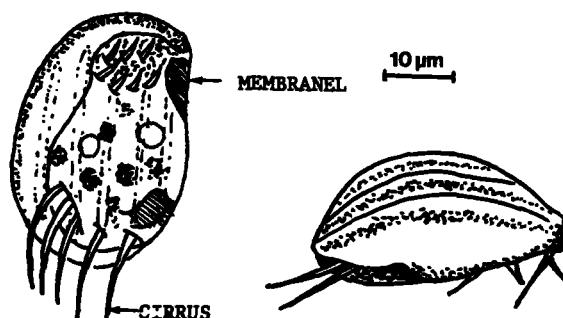
II CREEPING CILIATES

Aspidisca sp.

Body ovoid, inflexible and convex dorsally. Length of the body 25-55 μm . On the back we find 3-6 ridges. The cilia occur in the shape of cirri and so-called membranels. Seven cirri fronto-ventral; 5-12 anal. On the right side of the ventral surface we find a membranel. There is no clear mouth.

Aspidisca usually creeps with great speed across the surface of the flocs.

In sludge four types may be observed. Of these *A. costata* (body length about 30 μm) is extremely common and may be found in almost any sludge examined.



Trachelophylum pusillum

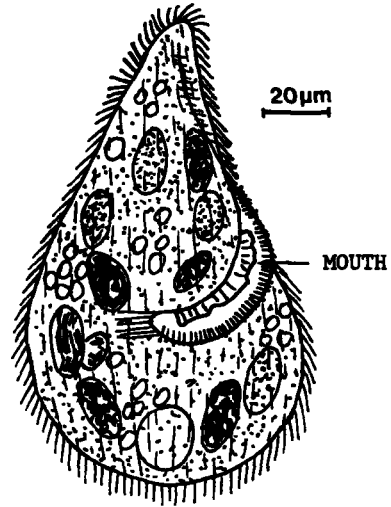
A flat, flexible, long body (length approximately 70 μm) which is completely covered in cilia. The round mouth is fairly visible and is surrounded by a number of rather large cilia, which stand upright. This protozoa slides slowly through and all over the sludge floc. The body contains two macro-nuclei and a terminal contractile vacuole. This species is very common in activated sludge. It can be mistaken for small *Lionotus* specimens.



III FREE-SWIMMING CILIATES

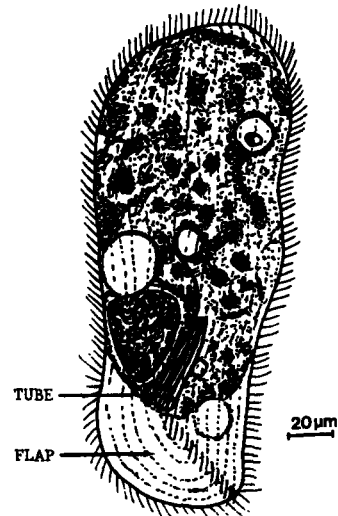
Blepharisma sp.

An elongate, hardly flexible organism which moves slowly through the liquid. Its body, length 80-400 μm , is completely covered in cilia. Near the mouth they occur in the shape of membranels. The mouth is somewhat submerged. The cells of this organism very often have a light-pink hue.



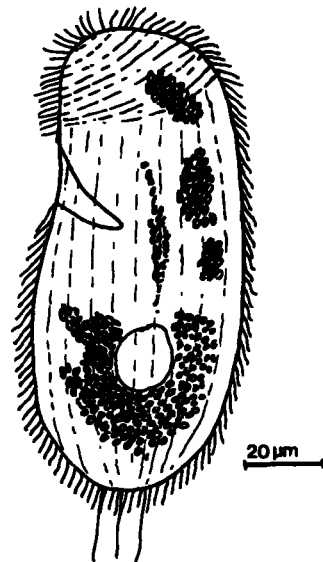
Chilodonella cuculatus (Fig.75)

A flat somewhat ovoid-shaped body, dorsal surface convex, ventral surface flat, length 40-125 μm . The cilia on the ventral surface are often hard to see. There are no dorsal cilia. The mouth has a bulge around it in the shape of a short tube. Chilodonella glides rather slowly across the surface of the sludge flocs. The flap on the frontside curls up when Chilodonella "mounts" a sludge floc.



Colpidium colpodem

The cells of this organism are kidney- or bean-shaped; the mouth and the area around it are clearly submerged in the flattened ventral side. The length of the cell is approximately 50-120 μm . The organism is covered in cilia. C.colpodem usually moves rapidly through the liquid.



Euplotes sp.

This organism forms ovoid-shaped cells; its length varies from 80-120 μm .

On the dorsal side 6 ridges are present. The ventral surface is flattened. Separate cilia do not occur; there are some cirri on the surface of the cell. These cirri (9 in front and 5 in the back) are usually clearly visible. Well developed membranels in the peristomal area.

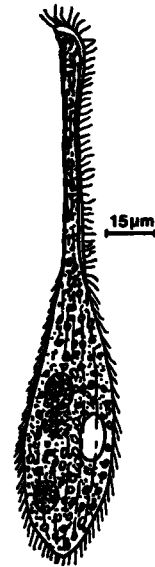
Euplotes resembles *Aspidisca*, but the latter ciliate is clearly smaller than *Euplotes*. Besides, *Euplotes* moves freely in the liquid between the flocs, where as *Aspidisca* creeps over the surface of the flocs.



10 μm

Lionotus sp. (Fig.74)

The cells of this organism are bottle-shaped. The neck and the remaining part of the cells are of the same length; viz 50 μm . The presence of cilia is restricted to the dorsal side. The stout neck region bends towards the dorsal side.

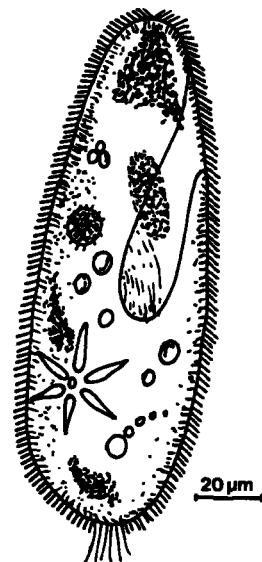


15 μm

Paramecium *candatum*

The cells of this organism have the shape of a cigar. Cilia occur all around the cell. The nucleus is large and is usually fairly visible. The star-shaped, contractile vacuole is very characteristic. The length of the cell is 120-300 μm .

Consequently, in comparison with other protozoa, this is a large organism.



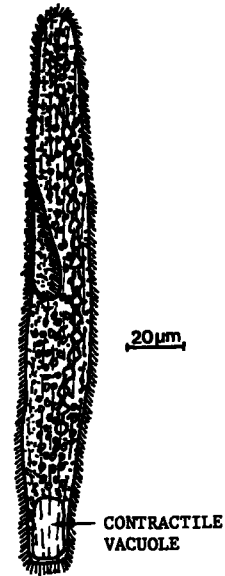
20 μm

Spirostomum sp.

A very long, fast moving ciliate.

The body is completely covered in cilia.

At the posterior end a big, characteristic vacuole is present. The cells can grow to a length of 500-900 μm . Spirostomum is the largest ciliate which is frequently found in activated sludge.



8.1.2. Flagellates

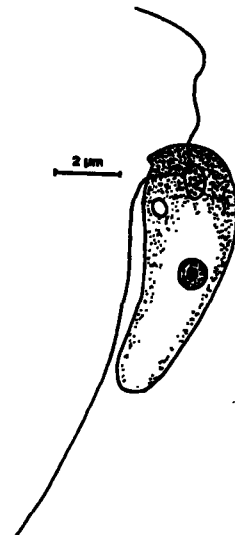
The flagellates derive their name from having one or more (usually no more than 8) flagella. These flagella are clearly bigger and stouter than cilia and therefore they can hardly be mistaken. The flagellates need these flagella for moving around; they propel the cell through the liquid. Some types have a mouth through which food is taken in. Other types completely depend on a food-intake through the wall of the cell. Flagellates are usually much smaller than ciliates.

Bodo sp.

Shape of the cell more or less ovoid.

Two flagella of which one directed anteriorly and the other posteriorly and trailing.

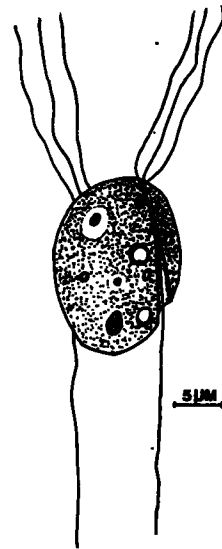
The flagella are not planted in the surface but originate in a more inward position. Bodo, length of the cell 10-25 μm can easily be recognised by its 'jerking' movement.



Hexamitus sp.

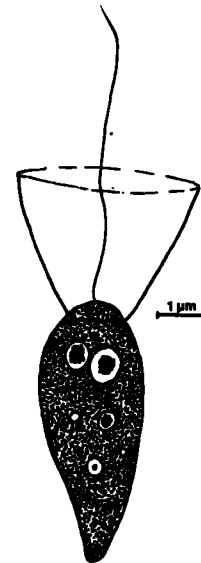
Six (2x3) anterior and two trailing flagella. The flat body is more or less circular. The length of the cell is approximately 20 μm .

Hexamitus moves about by means of a rapid swimming movement. The flagella cannot be seen while the organism is swimming.



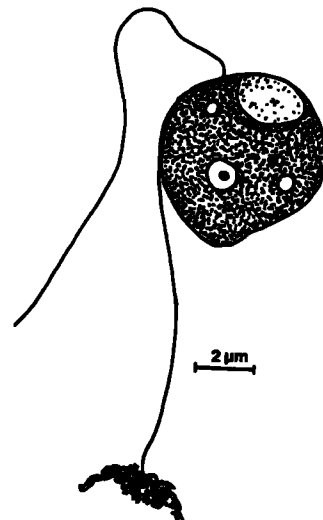
Monosiga sp.

The shape of the cell is more or less ovoid. At the top there is a thin lorica through which the flagellum protrude. Monosiga is usually attached to a sludge floc. The length of the cells varies from 10-15 μm .



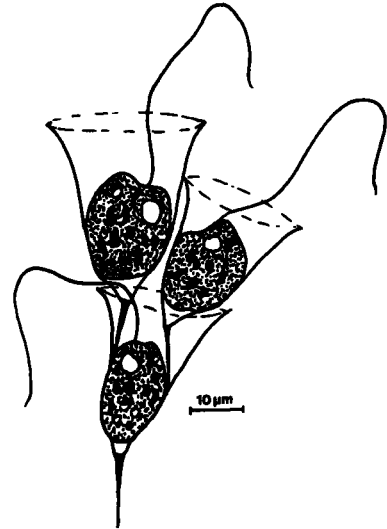
Pleuromonas sp.

A small, circular flagellate; the diameter varies from 6-20 μm . Near the place where the two flagella are planted, the wall of the cell is somewhat dented. The longest flagellum is often attached to the sludge floc. Pleuromonas can easily be recognized by the fast and jumpy way it moves about.



Poteriodendron sp. (Fig.76)

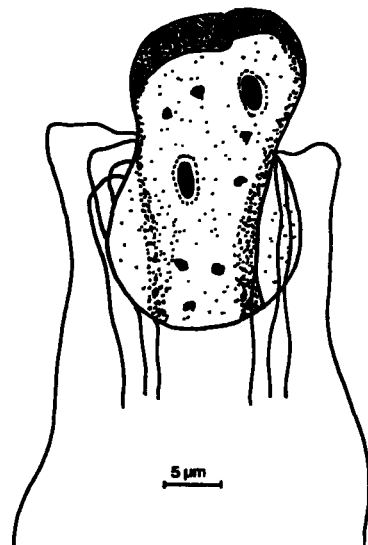
The cells, length approximately 20 μm , are ovoid-shaped and surrounded by a vase-shaped lorica. Underneath the lorica there is a stalk with which the complete organism is attached to another cell. In this way a colony of *Poteriodendron* cells is formed. The cells are equipped with one flagellum by means of which they move about in the lorica. However, they cannot leave the lorica.



Trepomonas sp.

Seen from above the cell is ovoid-shaped; from aside it is flattened. The cells have a length of approximately 30 μm . The organism has 2x4 flagella which are implanted on its sides. Along with two long ones (20 μm) there are always also six short ones (8 μm).

Rotating swimming movements.



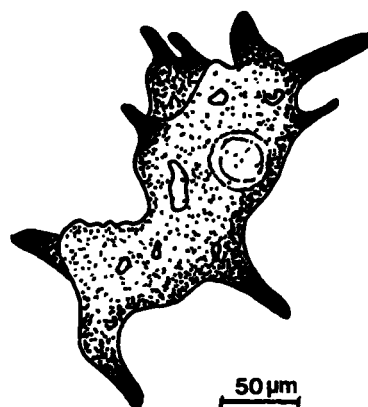
8.1.3. Rhizopoda

This group of organisms is characterised by their ability to move about by means of pseudopodia. These pseudopodia are temporary bulges of the contents of the cell which do not have a rigid cell wall. The speed with which these cells move about is usually so low that it often seems that they do not move at all.

Amoebae (Figs. 77 and 78)

Amoebae are unicellular organisms which do not have a firm outer-wall. This is why their shape may vary. They absorb their food by more or less enclosing it. The sizes of the different types vary from 50-400 μm .

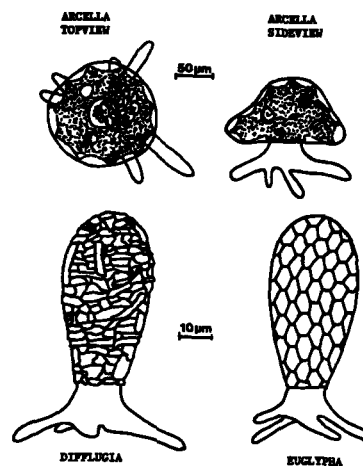
The sizes of *A.proteus*, *A.verrucosa* and *A.limax* may equal that of a sludge floc. Because of their granulated structure they can be mistaken for flocs by an unexperienced observer. *A.limax* may move 'fast', but only in one direction. Of the smaller amoebae *A.radiosa* may frequently be observed.



Thecamoebae (Fig.79)

With this group of amoebae the cell is surrounded by a kind of shell. In this shell there is an opening from which the pseudopodia protrude. The shape of the shell (globular, bell-shaped etc.) depends on the type of amoebae. Their size may vary from 30-200 μm .

Arcella, Centropyxis, Diffugia and Euglypha specimens are often observed in sludge. They can be distinguished from each other by observing the features mentioned in the subjoining table.

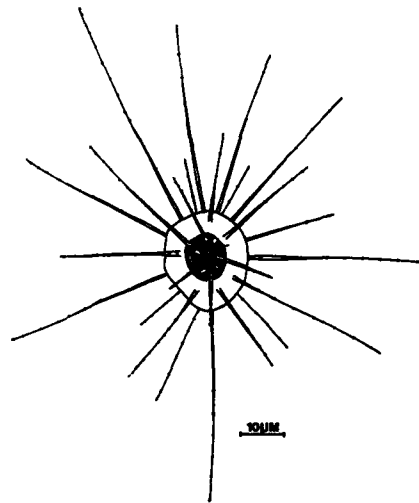


	shape	shell transparent	shell with a texture	coloured shell ¹⁾	opening central
Arcella	globular	-	+	+	+
Centropyxis	globular	-	-	+	-
Diffugia	bell	-	-	-	+
Euglypha	bell	+	+	-	+

1) Older Arcella and Centropyxis specimens are usually coloured yellowish-brown.

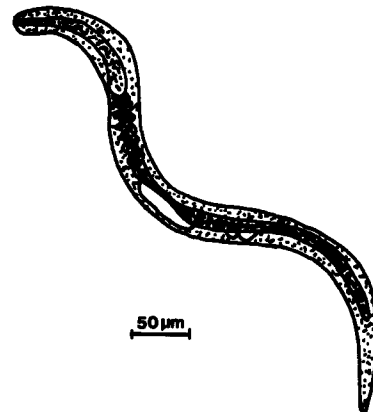
8.1.4. Actinopoda

In this group the Heliozoae are found. These organisms form globular cells that are surrounded by pseudopodia which are as thin as needles and dead straight. The latter are contractile. They are not used for the movement of the cell but for catching algae etc. Organisms that are touched by these pseudopodia are paralysed and can subsequently be consumed. Its diameter may vary from 40-100 μm .



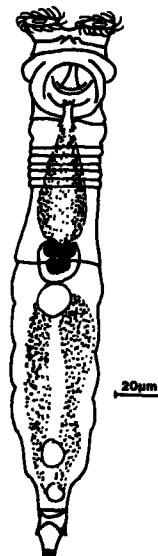
8.2. Nematodes

The nematodes (Fig.80) are characterized by their long, cylinder-shaped body. The ends of the multicellular body are thinner than the central part. Their length may vary from 500-3000 μm . Consequently, they are much bigger than the protozoa and that is why they are studied at a low magnification. However, because they are extremely lively it is often a problem to keep them focussed.



8.3. Rotifers

Rotifers (Fig.81) are large, lively, somewhat stretched multicellular organisms. Their length may vary from 100-500 μm . The body is surrounded by a kind of shield in which the 'head and tail' may be retracted. The organism has several bundles of cilia on its head. The tail is mostly branched in a very characteristic way and also plays a very important part in



the movements of the cell. With it the organism first secures itself after which the body 'stretches'. Subsequently the tail lets go, the head remains in the same position and the 'abdomen' is taken in. This way of moving very much resembles the movements of a snail.

8.4. Glossary

<i>Aboral</i>	: the region of the cell opposite to the mouth.
<i>Cilium (pl.cilia)</i>	: tiny thread projecting from the surface of the cell along with many others. Used for locomotion and feeding.
<i>Cirri</i>	: Compound cilia; tufts of cilia bound together.
<i>Dorsal</i>	: the back.
<i>Lorica</i>	: a membranous shell (a kind of collar).
<i>Membrane</i>	: short transverse rows of cilia fused together.
<i>Myoneme</i>	: contractile fibre or muscle strand found in the stalk of certain protozoa.
<i>Nucleus (pl.nuclei)</i>	: distinct part of a cell which contains the genetic code.
<i>Peristome</i>	: the area around the mouth
<i>Peristomal disc</i>	: the disc filling most of the bell-opening in the peristome.
<i>Sessile</i>	: attached (to the sludge floc for example).
<i>Unicellular</i>	: composed of one cell.
<i>Vacuole</i>	: a clear space in the cell.

9. GUIDE TO FURTHER READING

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Appendix 1: ANALYSIS FORM FOR A MICROSCOPIC SLUDGE
INVESTIGATION. THE OBSERVATIONS RECORDED
CONCERN A BULKING OXIDATION DITCH SLUDGE

FILAMENTOUS MICRO-ORGANISMS ¹⁾				CATEGORY: 4	
"Cyanophyceae"		S.natans		Type 0914	
H.hydrossis	0	Thiothrix		Type 0961	
M.parvicella	X	Type 0041	0	Type 1701	
N.limicola I		Type 0092	0	Type 1851	0
N.limicola II		Type 021 N			
N.limicola III		Type 0581			
Nocardia		Type 0803		Various species	

PROTOZOA - ROTIFERS - NEMATODES - AMOEBAE ²⁾					
<i>Ciliates</i>	+	Lionotus		Monosiga	
Carchesium		Paramecium		Pleuromonas	
Epistylis		Spirostomum		Poterialodendron	
Opercularia	+	Trachelophylum		Trepomonas	
Vorticella	+				
Aspidisca				Amoebae	-
Blepharisma				Thecamoebae	++
Chilodonella		Flagellates	-	Heliozoa	-
Colpidium		Bodo		Rotifers	+
Euplotes	+	Hexamitus		Nematodes	-

MORPHOLOGY OF THE SLUDGE FLOC						VARIOUS FEATURES	
Firmness and shape of the floc	structure		size			Diversity	+
	open	compact	small	middle	large		
Firm, somewhat rounded		x	+			Free cells ³⁾	+
Firm, irregular shape						Zoogloea's ²⁾	-
Weak, somewhat rounded						Spirochetes ²⁾	++
Weak, irregular shape						Spirils ²⁾	-
Agglomerates	x			++	++	Organic fibres ²⁾	+

1) : x = Dominant; 0 = Secondary

2) : - = absent; + = incidentally; + = some (5-10) cells or particles per preparation;
++ = many cells or particles per preparation.

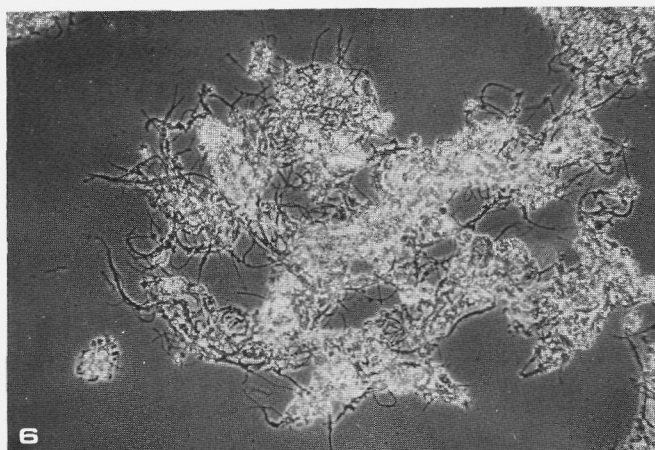
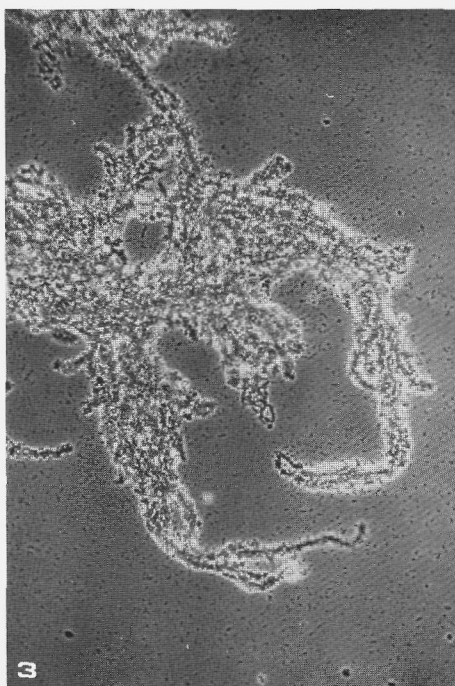
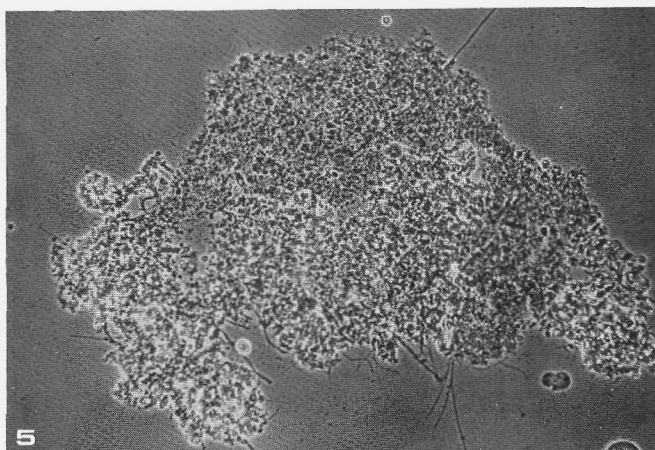
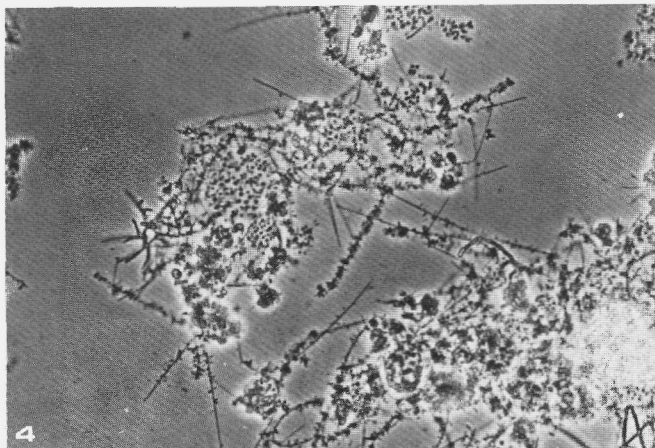
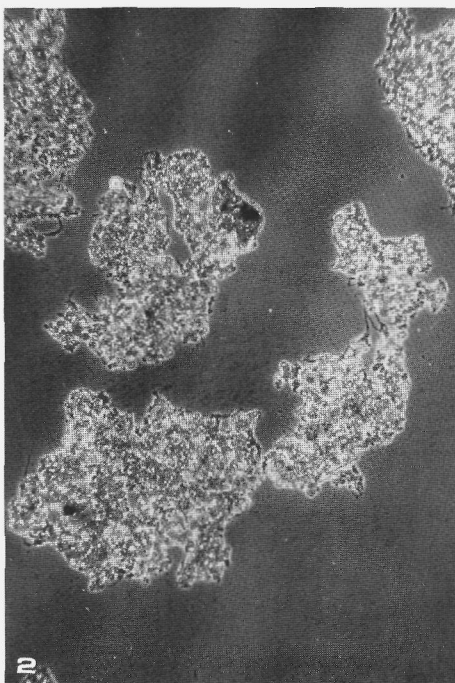
3) : - = few; + = some tens per view; ++ = hundreds per view

Remarks:

Appendix 2: ANALYSIS FORM FOR THE IDENTIFICATION OF FILAMENTOUS MICROORGANISMS IN ACTIVATED SLUDGE.

Name plant	Date	Filamentous organisms						Remarks
		1	2	3	4	5	6	
Dominating								
Secondary								
Branching	absent							
	false branching							
	real branching							
Motility								
many S-granules	present in vivo							
	present after the s-test							
Septa	clearly visible							
	not/hardly visible							
Shape filaments	straight							
	bent							
	coiled/twisted							
Neisser staining * = positive	granules*							
	cells grey-blue*							
	negative							
Gram staining	positive (blue)							
	negative (red)							
Diameter filaments	< 1.0 µm							
	1.0 - 2.2 µm							
	> 2.5 µm							
Attached growth	substantial							
	absent/scanty							
Constrictions	clearly observable							
Shape cells	disc-shaped							
	spherical/coccus							
	rod-shaped							
	square							
	rectangular							
sheath	present							

Conclusions: 1= _____
 2= _____
 3= _____
 4= _____
 5= _____
 6= _____

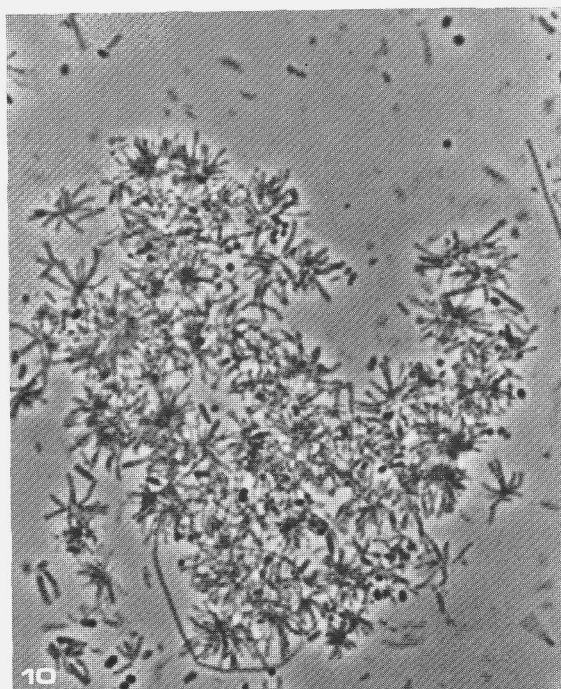
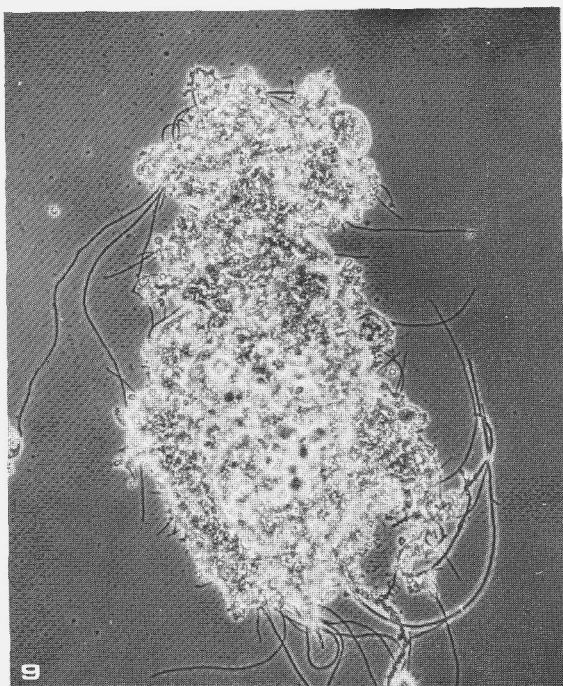
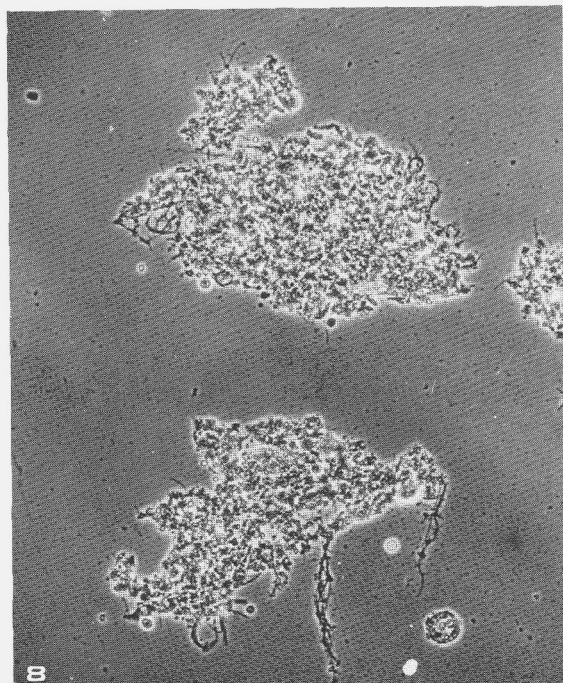
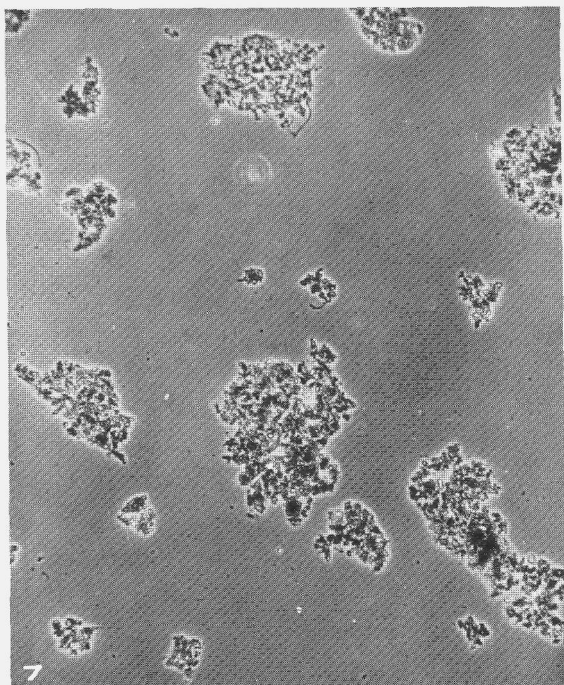


Plaat A. Vorm en structuur van actief-slibvlokken.

- Fig. 2. Stevige, compacte vlokken. x 90.
- Fig. 3. Onregelmatig gevormde vlok; veel losse bacteriecellen. x 90.
- Fig. 4. Slibvlok met een open structuur. x 180.
- Fig. 5. Grote, losse slibvlok; veel losse bacteriecellen. x 90.
- Fig. 6. Agglomeraat van draden en kleine vlokjes. x 90.

Plate A. Shape and structure of activated sludge flocs.

- Fig. 2. Strong, compact flocs. x 90.
- Fig. 3. Irregularly shaped floc; many free bacteria cells. x 90.
- Fig. 4. Sludge floc with an open structure. x 180.
- Fig. 5. Large, weak sludge floc; many free bacteria cells. x 90.
- Fig. 6. Agglomerate of filaments and small floc particles. x 90.

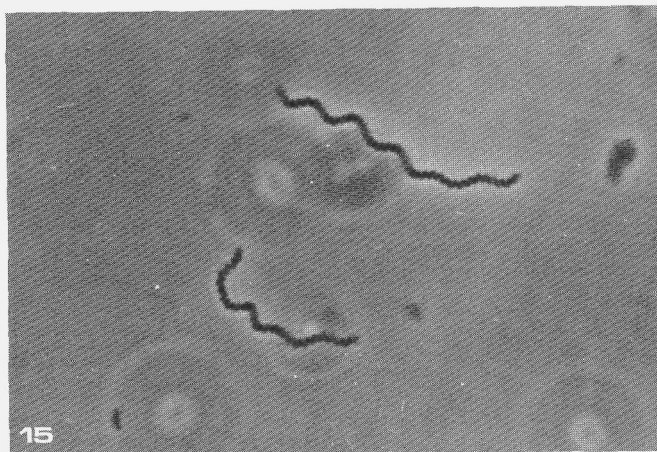
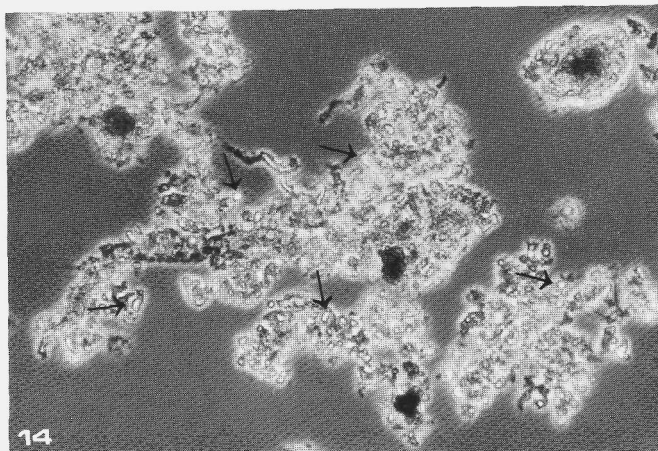
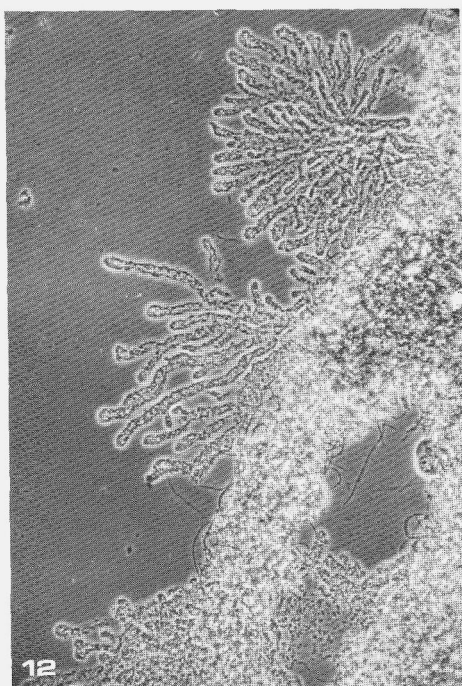
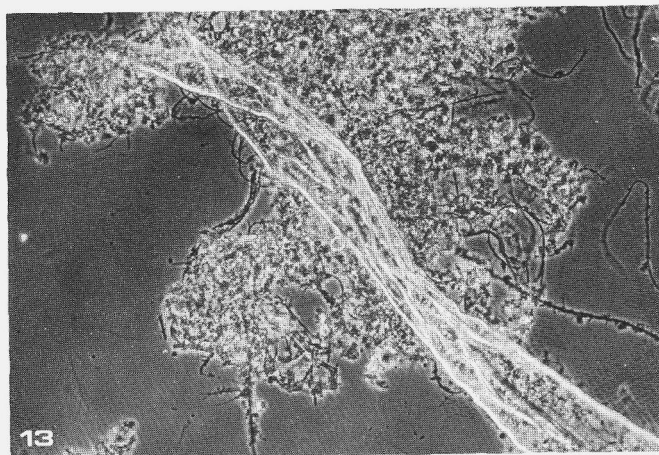
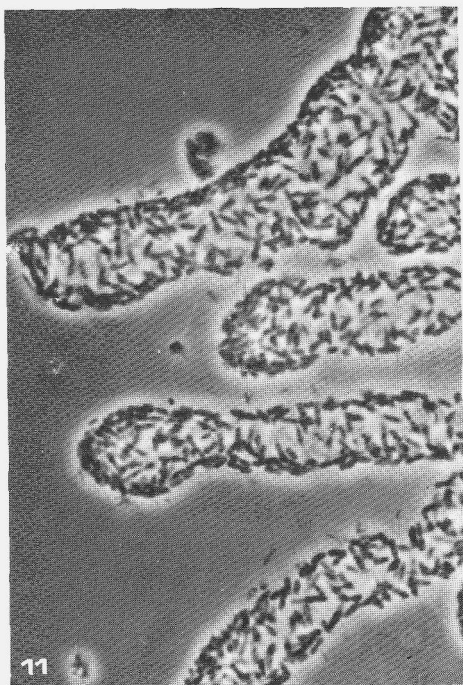


Plaat B. Afmetingen en diversiteit van slibvlokken.

- Fig. 7. Kleine vlokjes; diameter $< 150 \mu\text{m}$. x 115.
 Fig. 8. Middelgrote vlokken; $150 \mu\text{m} < \text{diameter} < 500 \mu\text{m}$. x 115.
 Fig. 9. Grote vlok; diameter $> 500 \mu\text{m}$. x 115.
 Fig. 10. Vlok met een lage diversiteit. x 1150.

Plate B. Size and diversity of sludge flocs.

- Fig. 7. Small flocs; diameter $< 150 \mu\text{m}$. x 115.
 Fig. 8. Medium-sized flocs; $150 \mu\text{m} < \text{diameter} < 500 \mu\text{m}$. x 115.
 Fig. 9. Large flocs; diameter $> 500 \mu\text{m}$. x 115.
 Fig. 10. Sludge floc with a low diversity. x 1150.

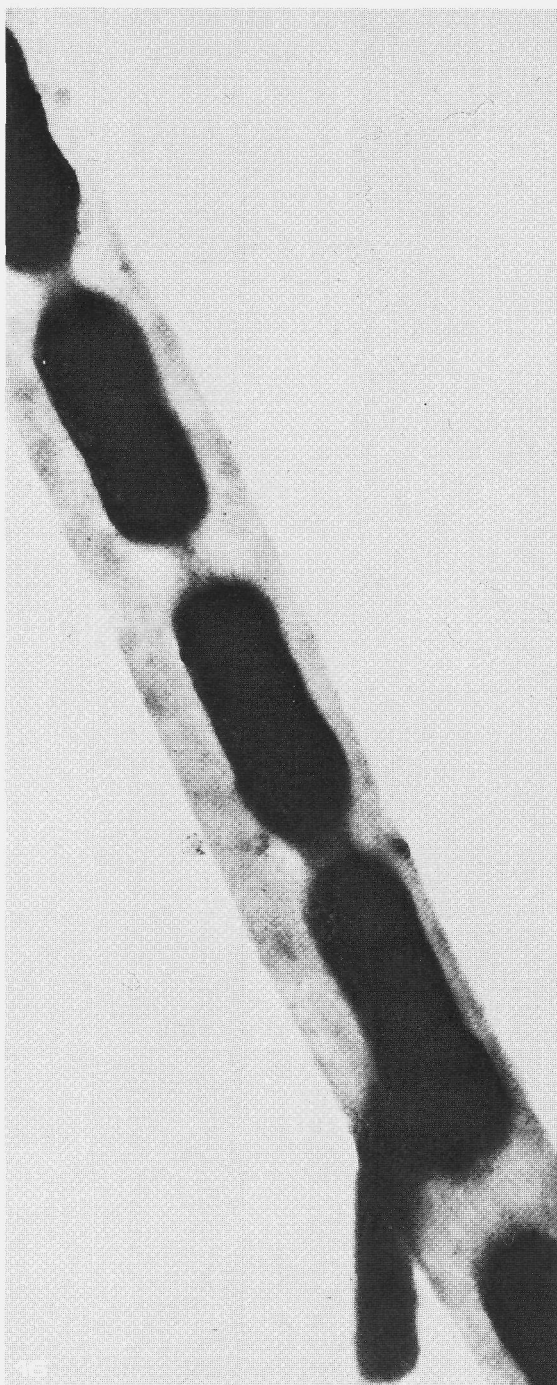


Plaat C. Samenstelling van slibvlokken.

- Fig. 11. Zogenaamde Zoogloea-vingers. De staafvormige cellen liggen ingebed in een slijm matrix. x 890.
 Fig. 12. Zoogloea kolonies in actief-slib. x 180.
 Fig. 13. Een grote, onverteerde, organische vezel. x 180.
 Fig. 14. Slibvlokken waarin veel anorganische deeltjes (zie pijlen) aanwezig zijn. x 180.
 Fig. 15. Spirocheten. x 1335.

Plate C. Composition of sludge flocs.

- Fig. 11. So-called Zoogloea-fingers. The rod-shaped cells are embedded in a slime matrix. x 890.
 Fig. 12. Zoogloea colonies in activated sludge. x 180.
 Fig. 13. A large, non digested organic fibre. x 180.
 Fig. 14. Sludge flocs containing a large number of small inorganic particles (see arrows). x 180.
 Fig. 15. Spirochetes. x 1335.



Plaat D. Elektronenmicroscopie van draadvormige bacteriën.

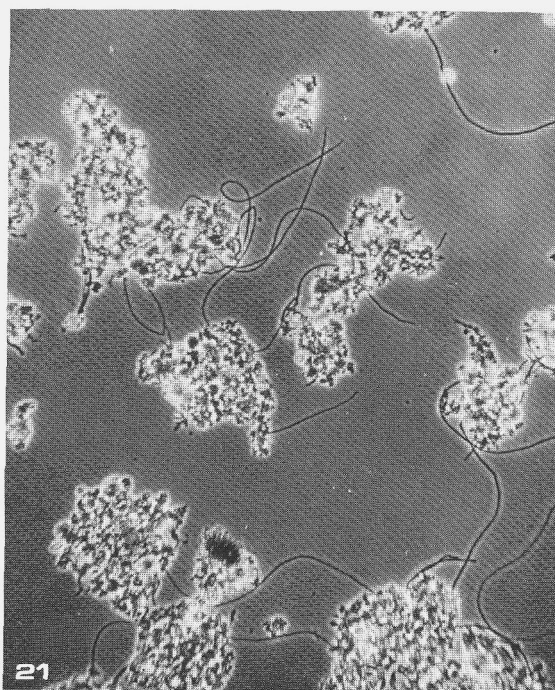
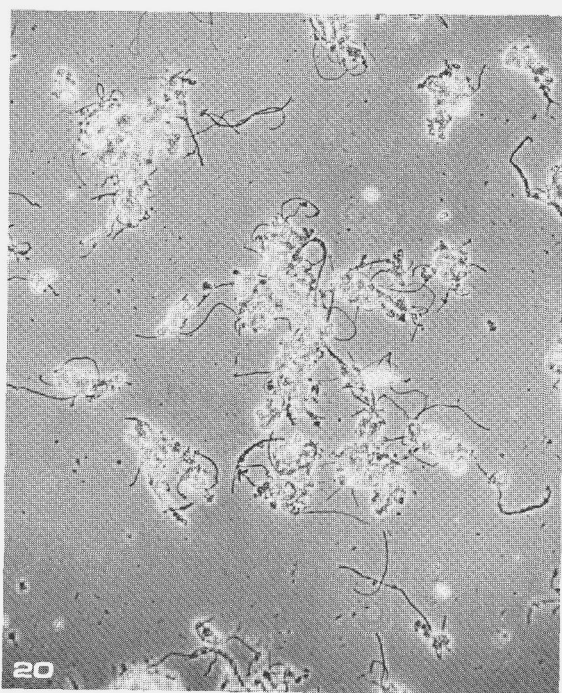
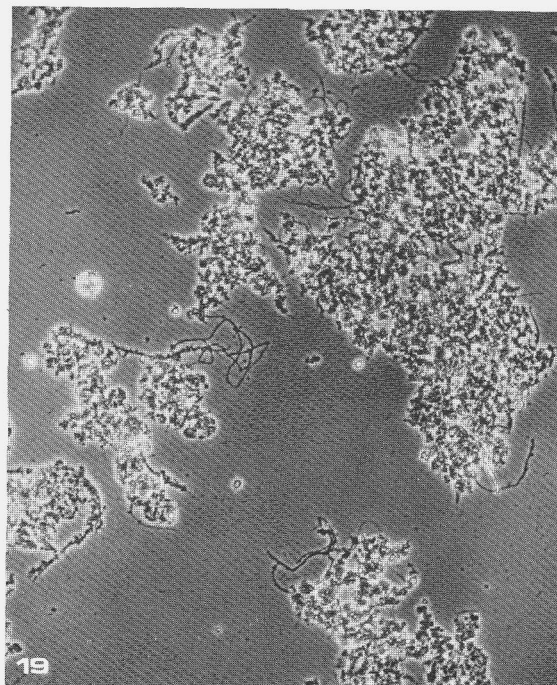
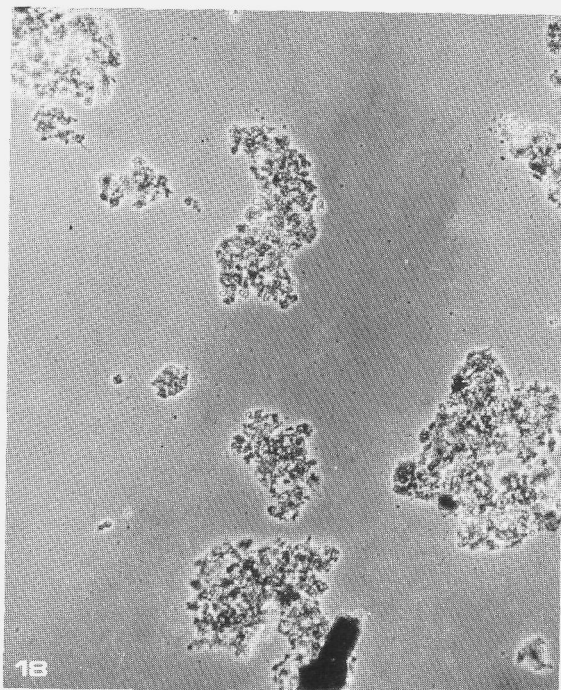
Fig. 16. Type 1701. De cellen zijn omgeven door een schede. x 22000.

Fig. 17. *Microthrix parvicella*. De septa zijn bij deze vergroting wel duidelijk zichtbaar. De zwarte balletjes bestaan uit polyfosfaten en worden gekleurd met het Neisser-reagens. x 26300.

Plate D. Electronmicroscopy of filamentous bacteria.

Fig. 16. Type 1701. The cells are surrounded by a sheath. x 22000.

Fig. 17. *Microthrix parvicella*. Crosswalls are clearly visible at this magnification. The black granules consist of polyphosphates and are stained with the Neisser reagent. x 26300.



Plaat E. Indeling van actief-slibben op basis van een visuele beoordeling van de mate van draadvorming. x 115.

Fig. 18. Categorie 0: Draadvormige organismen vrijwel afwezig.

Fig. 19. Categorie 1: Weinig draadvormige organismen.

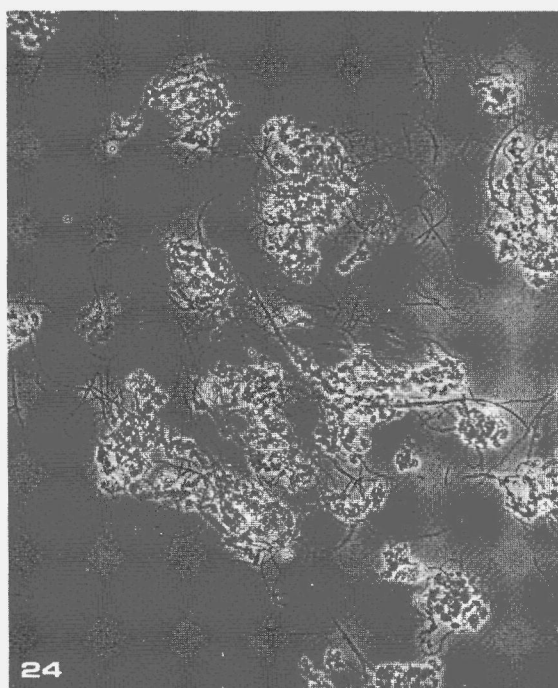
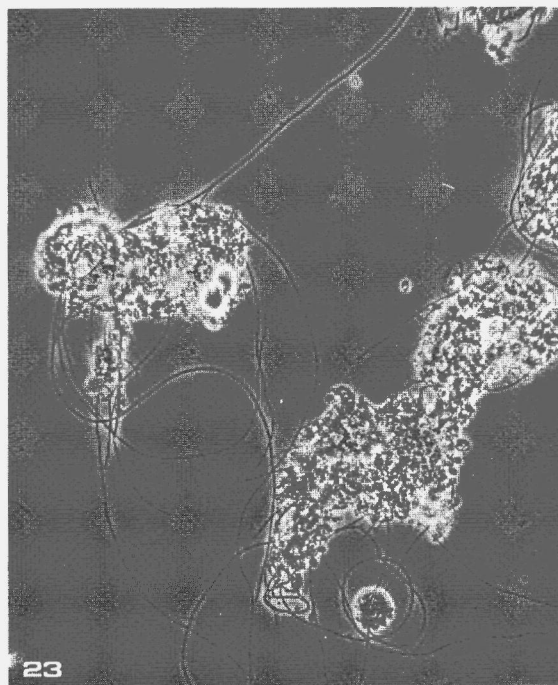
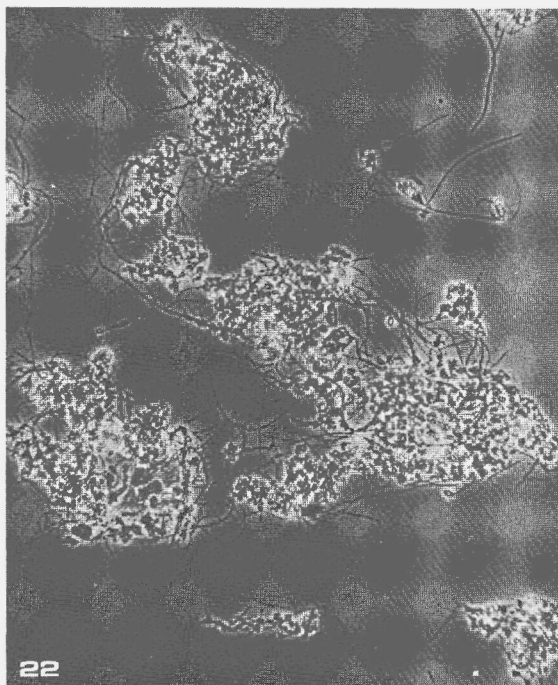
Fig. 20 en 21. Categorie 2: Vrij veel draadvormige organismen. Fig. 20. *M. parvicella* dominerend. Fig. 21. Type 021 N dominerend.

Plate E. Classification of activated sludges based upon a visual estimation of the extent of filamentous growth. x 115.

Fig. 18. Category 0: Filaments almost absent.

Fig. 19. Category 1: Small numbers of filaments.

Fig. 20 and 21. Category 2: Moderate numbers of filaments. Fig. 20. *M. parvicella* predominating. Fig. 21. Type 021 N predominating.

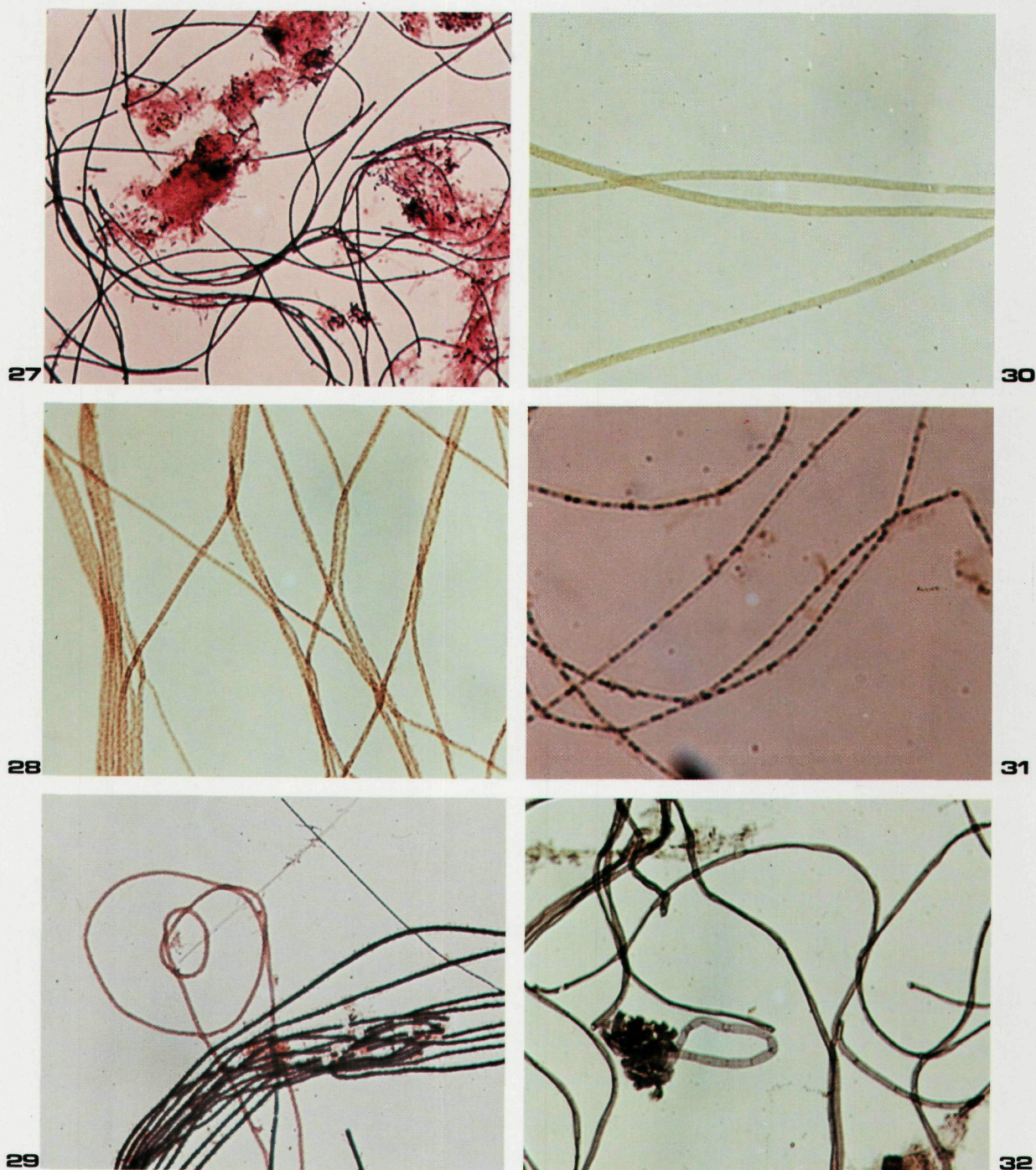


Plaat F. Indeling van actief-slibben op basis van een visuele beoordeling van de mate van draadvorming. x 115.

- Fig. 22 en 23. Categorie 3: Veel draadvormige organismen.
 Fig. 24 en 25. Categorie 4: Erg veel draadvormige organismen.
 Fig. 22 en 24. *M. parvicella* dominerend.
 Fig. 23 en 25. Type 021 N dominerend.

Plate F. Classification of activated sludges based upon a visual estimation of the extent of filamentous growth. x 115.

- Fig. 22 and 23. Category 3: Large numbers of filaments.
 Fig. 24 and 25. Category 4: Excessive numbers of filaments.
 Fig. 22 and 24. *M. parvicella* predominating.
 Fig. 23 and 25. Type 021 N predominating.



Plaat G. Kleuringen van actief-slib.

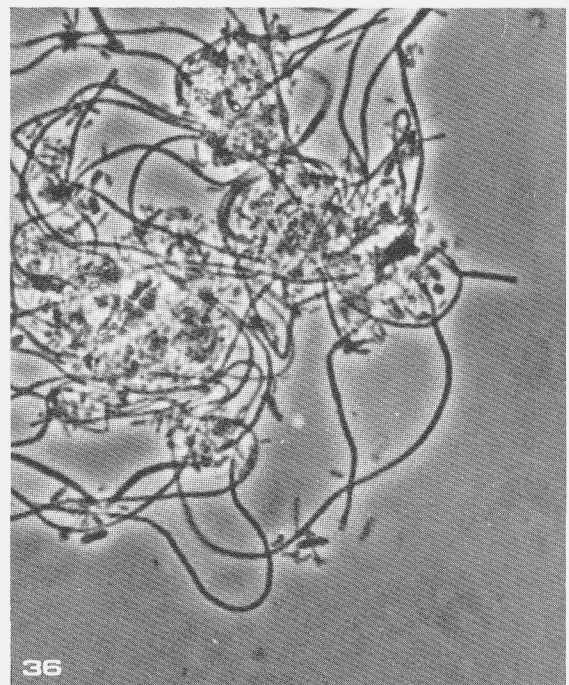
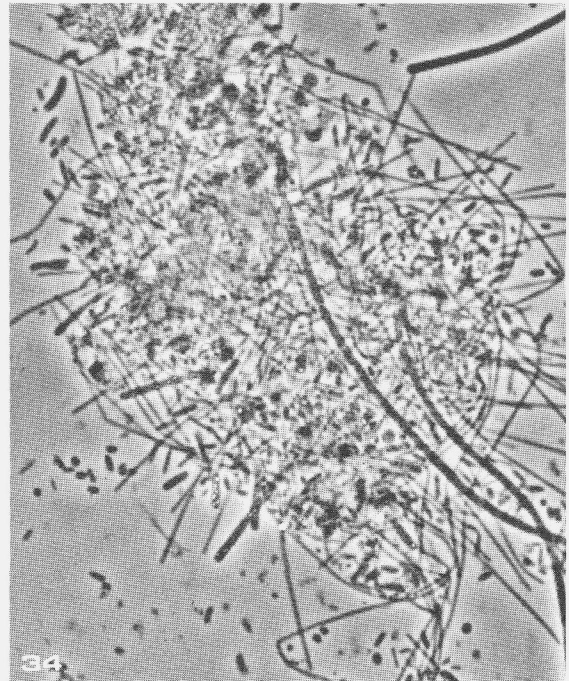
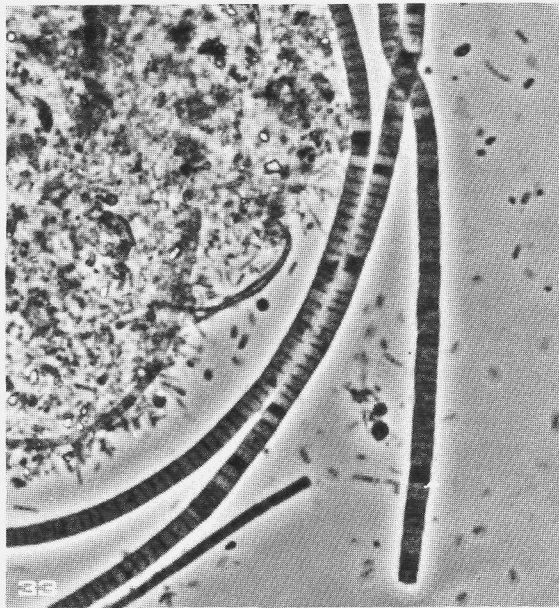
Fig. 27-29. Gram-kleuring. Gram-positieve draden kleuren blauw; Gram-negatieve draden rood.

Fig. 30-32. Neisser-kleuring. Neisser-negatieve draden kleuren lichtbruin (figuur 30). Neisser positieve draden bevatten donkere granulen (figuur 31) of worden grijs-blauw gekleurd (figuur 32).

Plate G. Staining of activated sludge.

Fig. 27-29. Gram staining. Gram positive filaments stain blue; Gram negative filaments red.

Fig. 30-32. Neisser staining. Neisser negative filaments stain light brown (figure 30). Neisser positive filaments contain dark granules (figure 31) or are stained greyish-blue (figure 32).

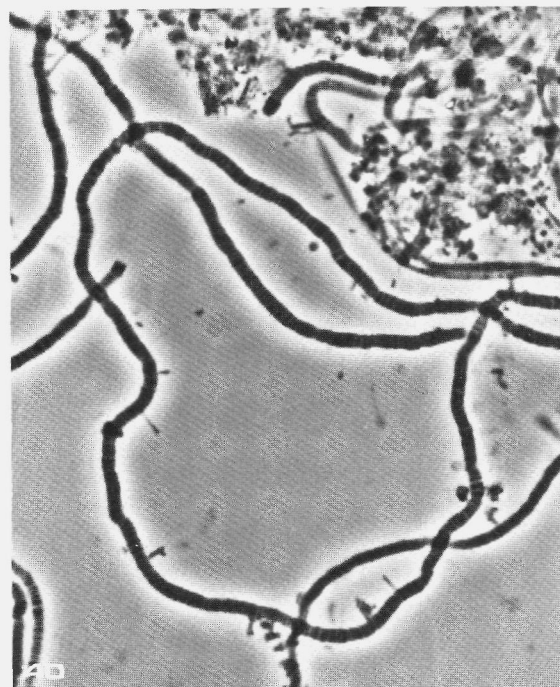
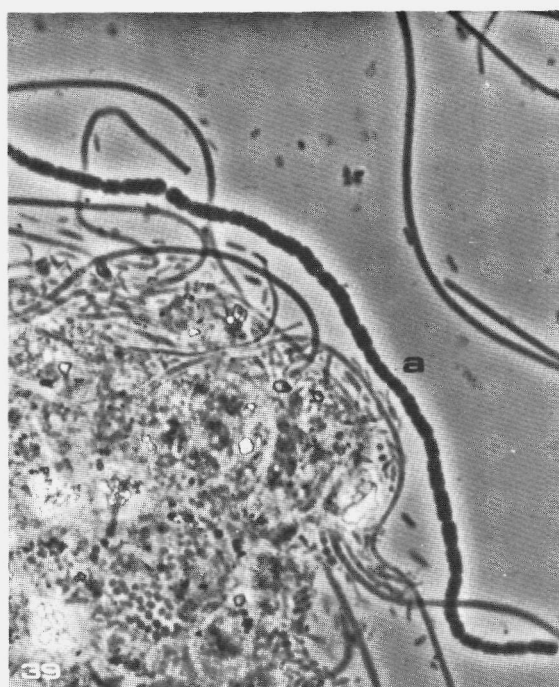
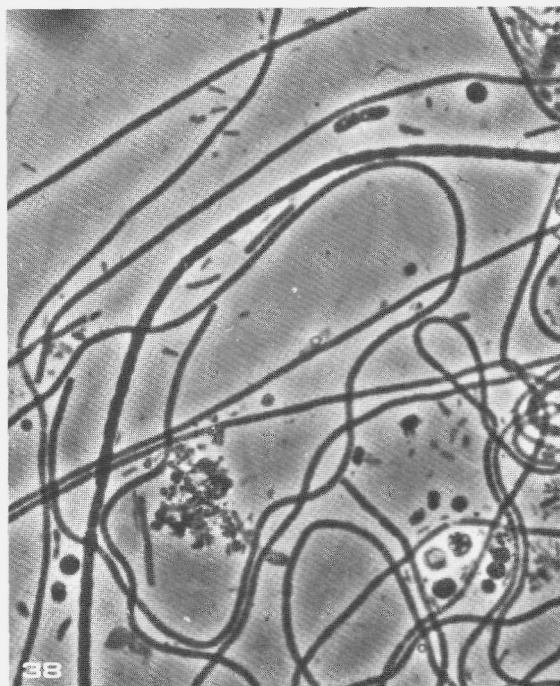
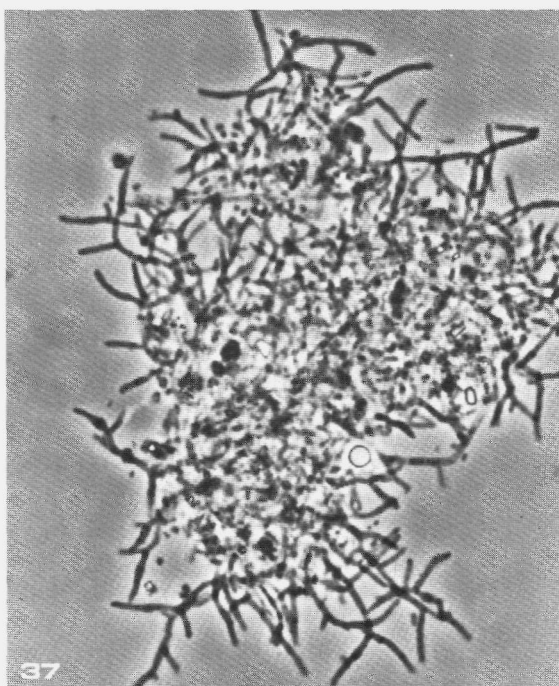


Plaat H. Draadvormige bacteriën in actief-slib.

- Fig. 33. "Blauwwieren". Robuuste draden. x 1150.
 Fig. 34. *Haliscomenobacter hydrossis*. Rechte draden. x 1150.
 Fig. 35. *Microthrix parvicella*; een kluwen draden. x 460.
 Fig. 36. *M. parvicella*; slibvlokjes omgeven door draden. x 1150.

Plate H. Filamentous bacteria in activated sludge.

- Fig. 33. "Cyanophyceae". Robust filaments. x 1150.
 Fig. 34. *Haliscomenobacter hydrossis*. Needle-like filaments. x 1150.
 Fig. 35. *Microthrix parvicella*; a clew of filaments. x 460.
 Fig. 36. *M. parvicella*; floc particles surrounded by filaments. x 1150.

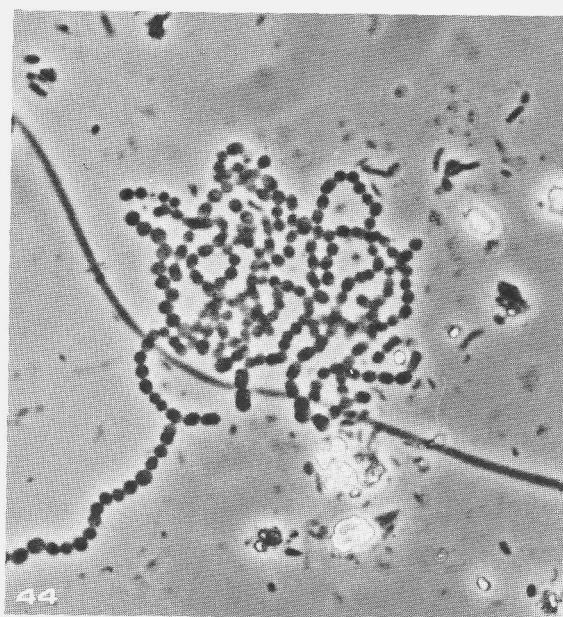
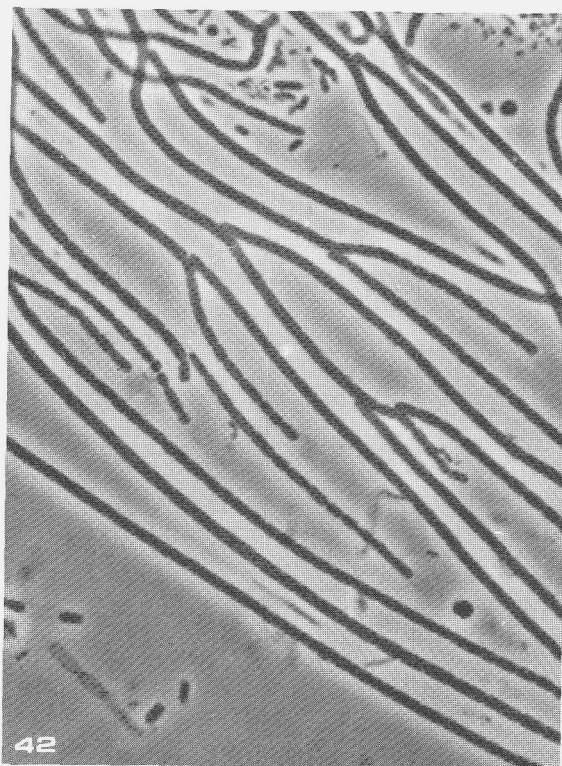
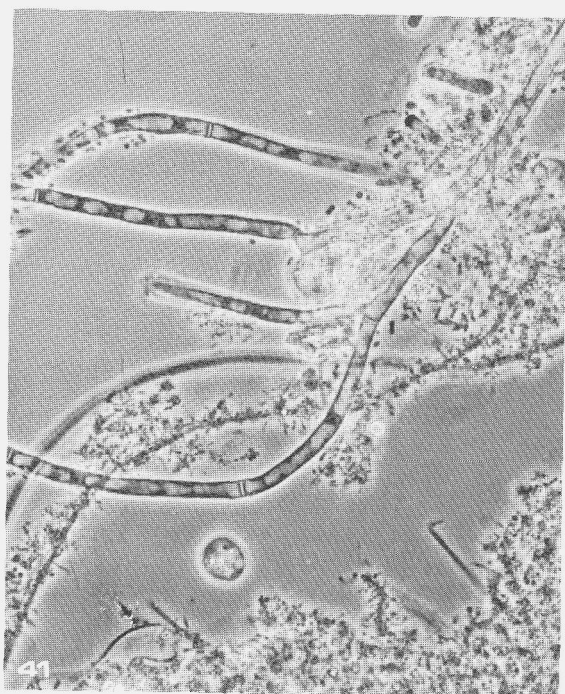


Plaat I. Draadvormige bacteriën in actief-slib. x 1150.

- Fig. 37. *Nocardia*. Vertakte filamenten.
 Fig. 38. *Nostocoida limicola* I. Septa vrijwel niet te zien.
 Fig. 39. *N. limicola* II(a). Septa duidelijk zichtbaar.
 Fig. 40. *Nostocoida limicola* III. Kronkelige draden.

Plate I. Filamentous bacteria in activated sludge. x 1150.

- Fig. 37. *Nocardia*. Branched filaments.
 Fig. 38. *Nostocoida limicola* I. Crosswalls hardly observable.
 Fig. 39. *N. limicola* II(a). Crosswalls clearly observable.
 Fig. 40. *Nostocoida limicola* III. Curling filaments.

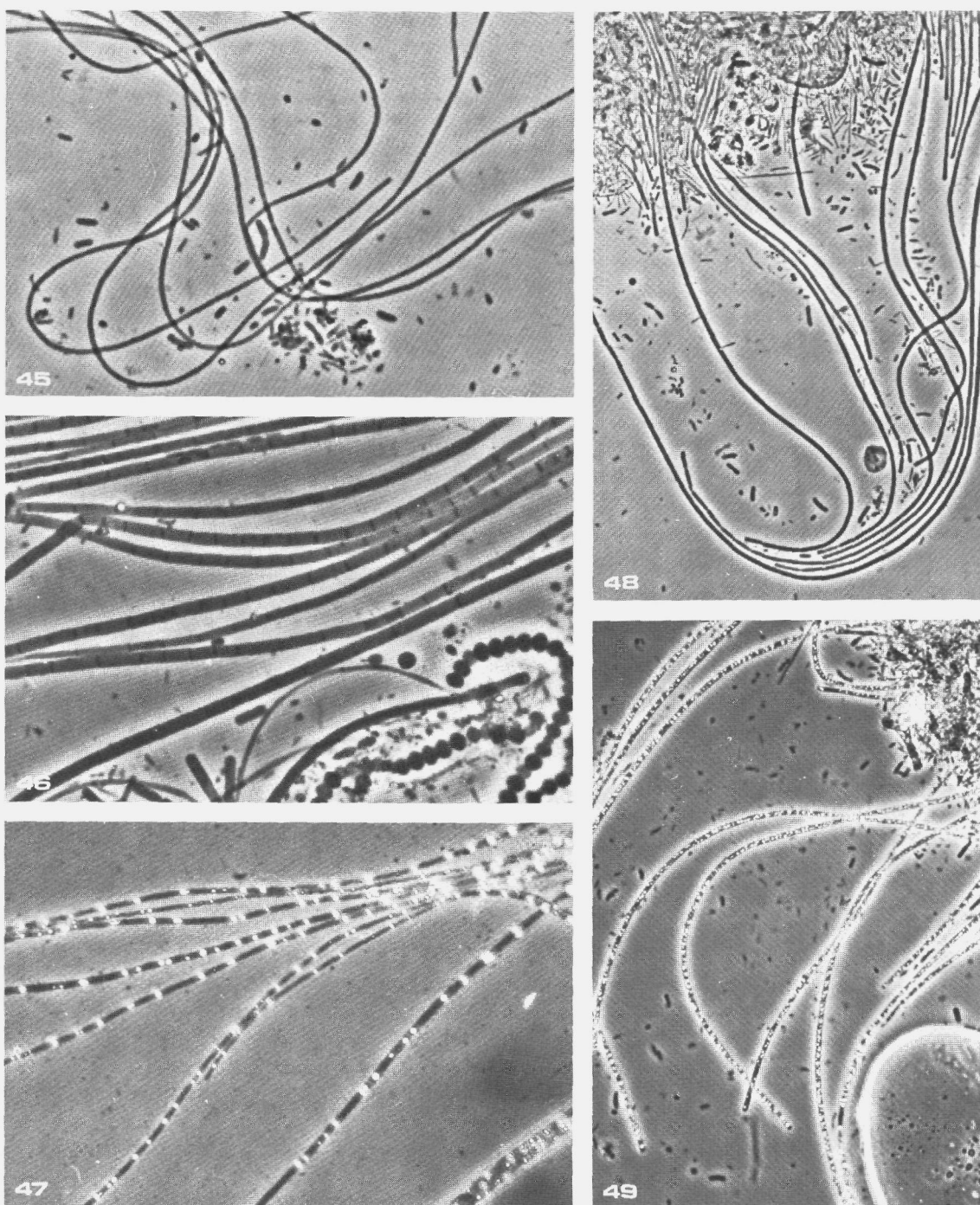


Plaat J. Draadvormige bacteriën in actief-slib.

- Fig. 41. Schimmels. De draden zijn vertakt. x 1150.
 Fig. 42. *Sphaerotilus natans*. Valse vertakkingen. x 1150.
 Fig. 43. *S. natans*. De draden bestaan uit staafvormige cellen. x 600.
 Fig. 44. "Streptococcus". Keten van coccusvormige cellen. x 1150.

Plate J. Filamentous bacteria in activated sludge.

- Fig. 41. Fungi. Branched filaments. x 1150.
 Fig. 42. *Sphaerotilus natans*. False branching. x 1150.
 Fig. 43. *S. natans*. The filaments consist of rod-shaped cells. x 600.
 Fig. 44. "Streptococcus". Chains of coccus-shaped cells. x 1150.

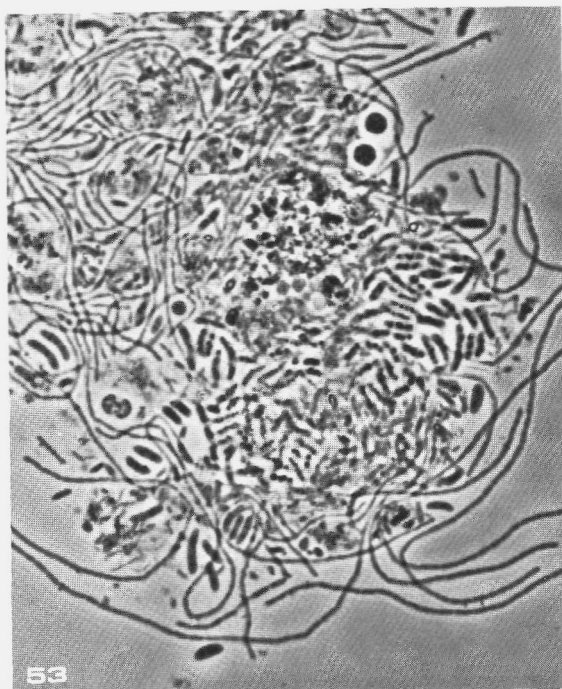
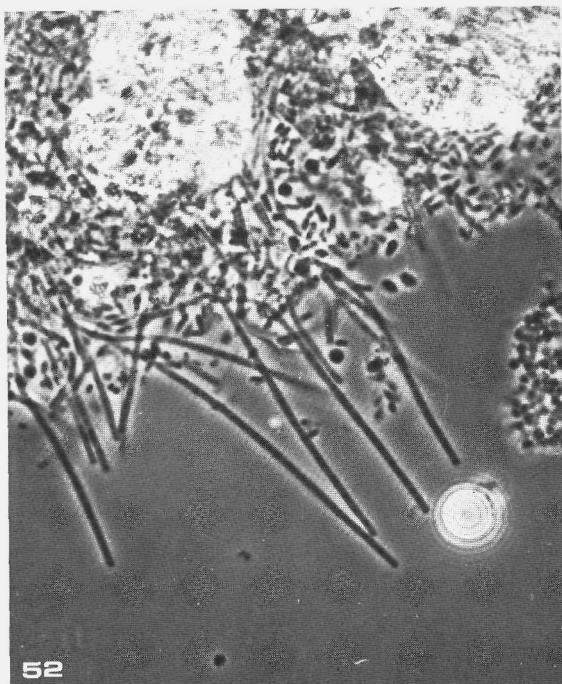
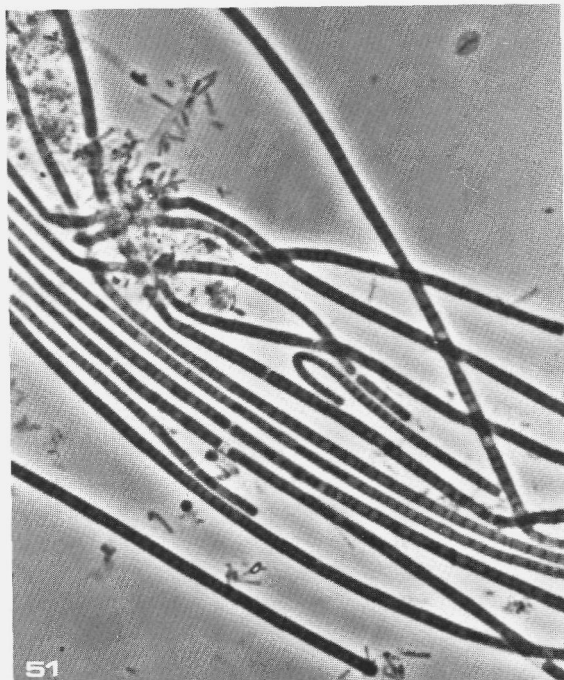
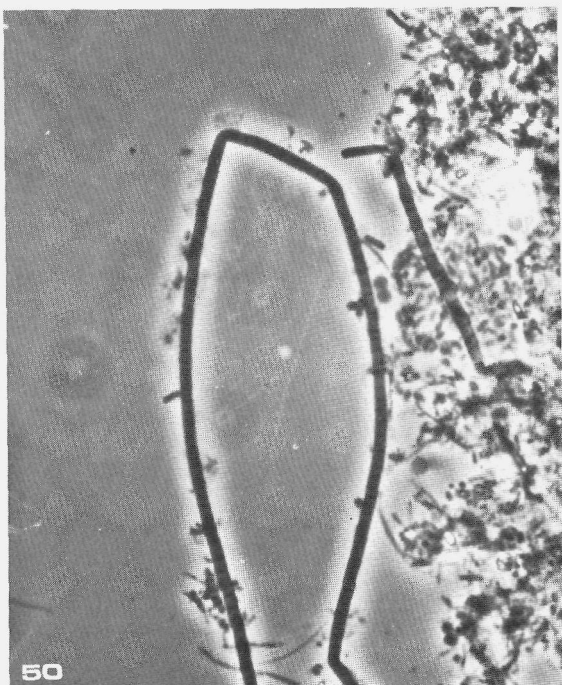


Plaat K. Draadvormige bacteriën in actief-slib.

- Fig. 45. Thiotrix I. Dunne uiteinden van de filamenten. x 1150.
 Fig. 46. Thiotrix I. Septa zijn midden in de draden duidelijk zichtbaar. x 1150.
 Fig. 47. Thiotrix I. S-granulen na de zwaveltest. x 1150.
 Fig. 48. Thiotrix II. Lange gebogen draden. x 575.
 Fig. 49. Thiotrix II. Draden vol met zwavel. x 575.

Plate K. Filamentous bacteria in activated sludge.

- Fig. 45. Thiotrix I. Slender ends of filaments. x 1150.
 Fig. 46. Thiotrix I. Central part of filaments; crosswalls clearly visible. x 1150.
 Fig. 47. Thiotrix I. S-granules after the sulphur test. x 1150.
 Fig. 48. Thiotrix II. Long, bent filaments. x 575.
 Fig. 49. Thiotrix II. Filaments filled up with sulphur granules. x 575.

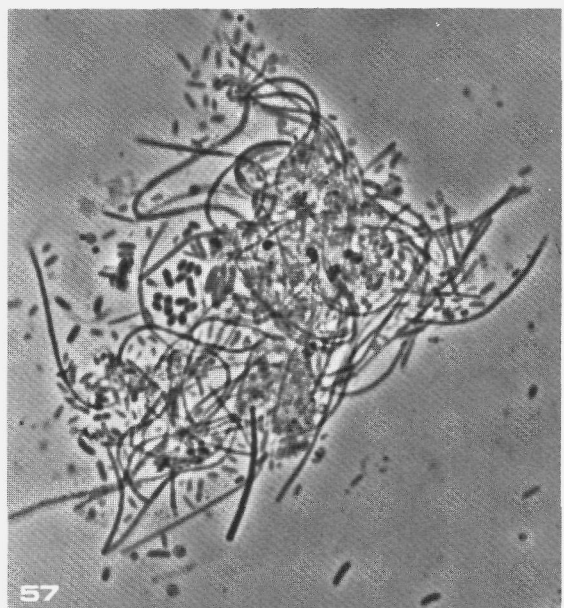
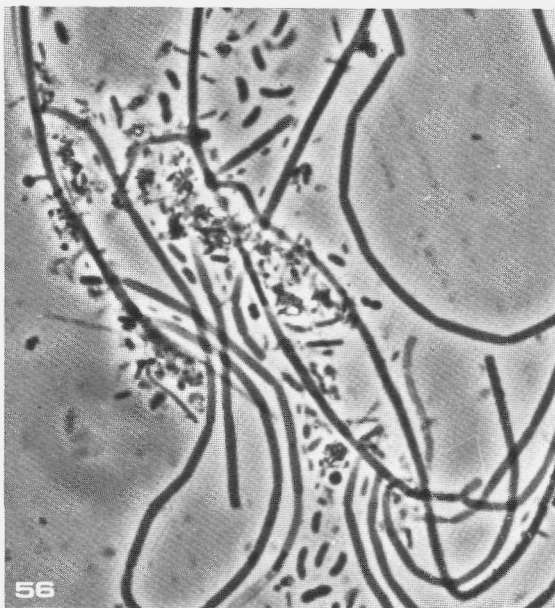
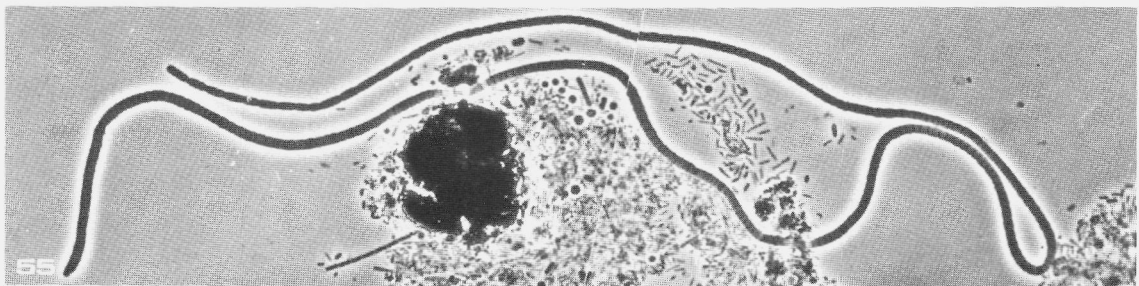
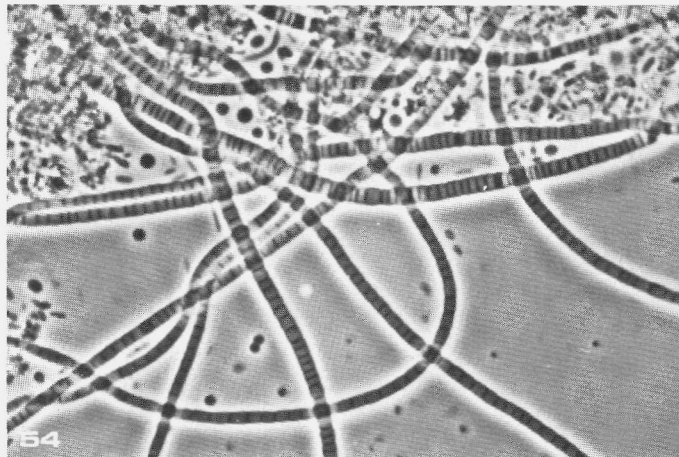


Plaat L. Draadvormige bacteriën in actief-slib. x 1150.

- Fig. 50. Type 0041. Aangroei van de draden.
 Fig. 51. Type 0041. Septa duidelijk zichtbaar.
 Fig. 52. Type 0092. Septa nauwelijks zichtbaar.
 Fig. 53. Type 0211. Dunne, gebogen draden.

Plate L. Filamentous bacteria in activated sludge. x 1150.

- Fig. 50. Type 0041. Attached growth to the filaments.
 Fig. 51. Type 0041. Crosswalls clearly observable.
 Fig. 52. Type 0092. Crosswalls hardly visible.
 Fig. 53. Type 0211. Thin, bent filaments.

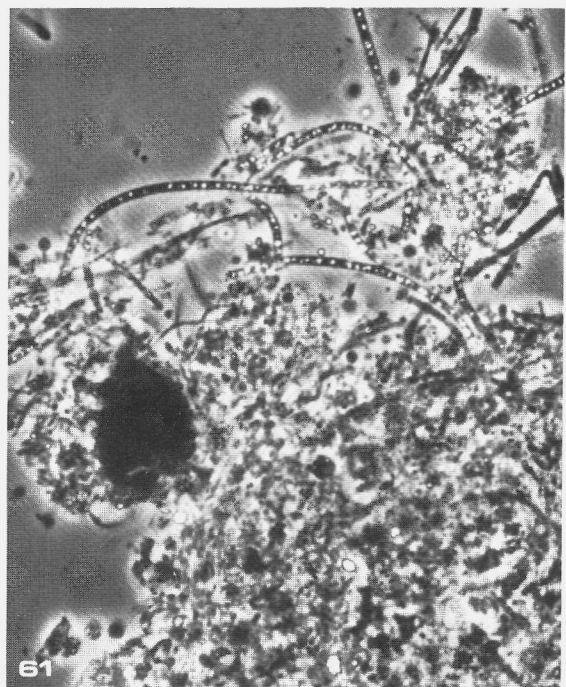
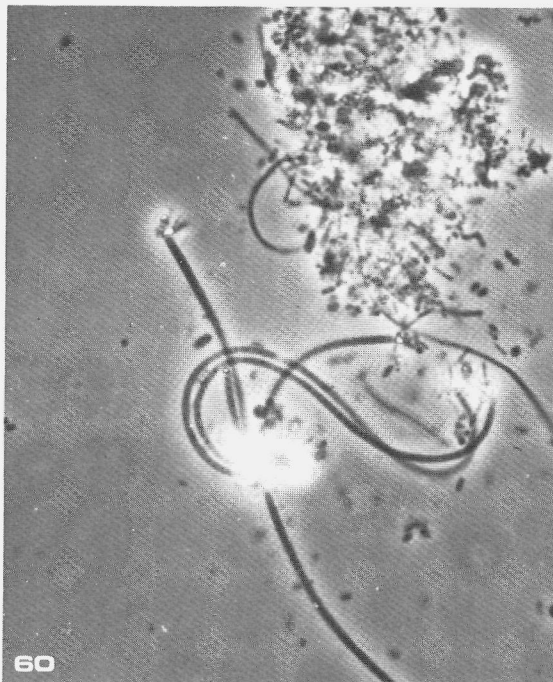
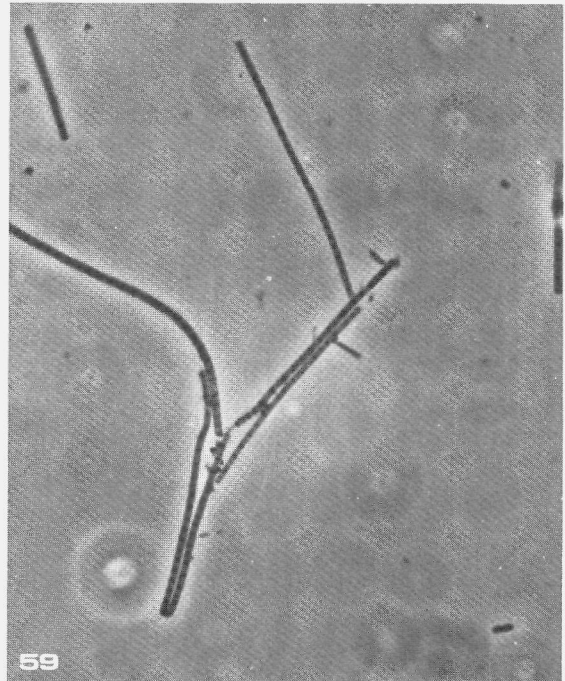


Plaat M. Draadvormige organismen in actief-slib.

- Fig. 54. Type 021 N. Draden in actief slib. x 1150.
 Fig. 55. Type 021 N. Verschillende celvormen in één filament. x 650.
 Fig. 56. Type 0411. Lange, staafvormige cellen. x 1150.
 Fig. 57. Type 0581. Dunne, gebogen draden. x 1150.

Plate M. Filamentous organisms in activated sludge.

- Fig. 54. Type 021 N. Filaments in activated sludge. x 1150.
 Fig. 55. Type 021 N. Different cell shapes in one filament. x 650.
 Fig. 56. Type 0411. Long, rod-shaped cells. x 1150.
 Fig. 57. Type 0581. Thin, bent filaments. x 1150.

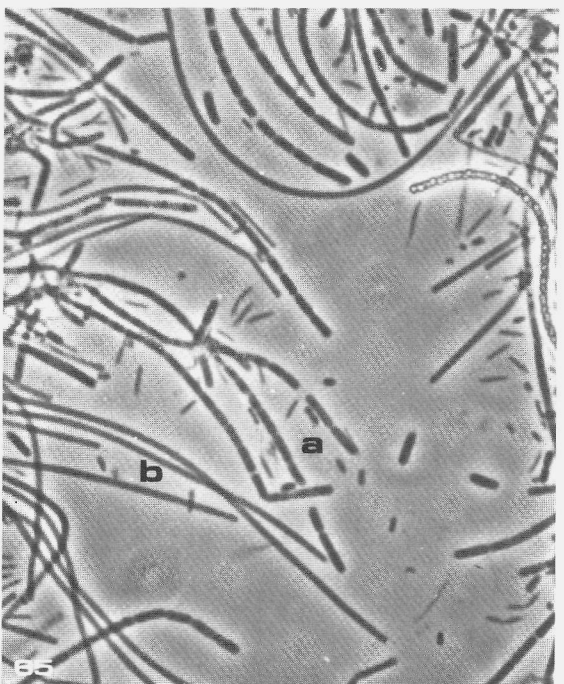
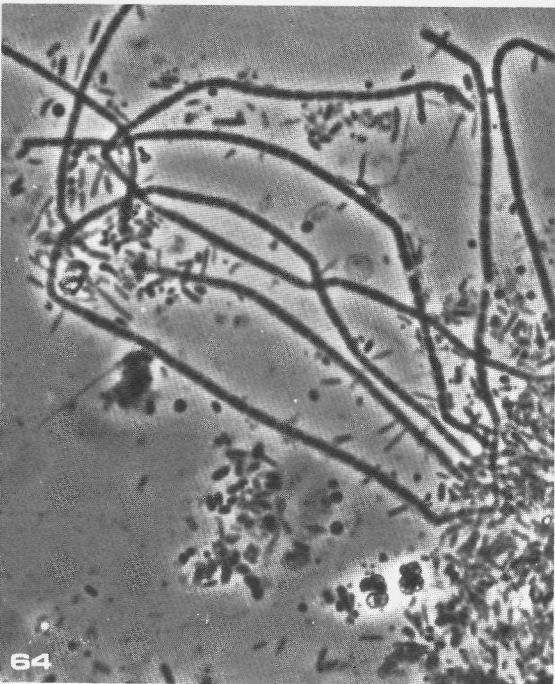
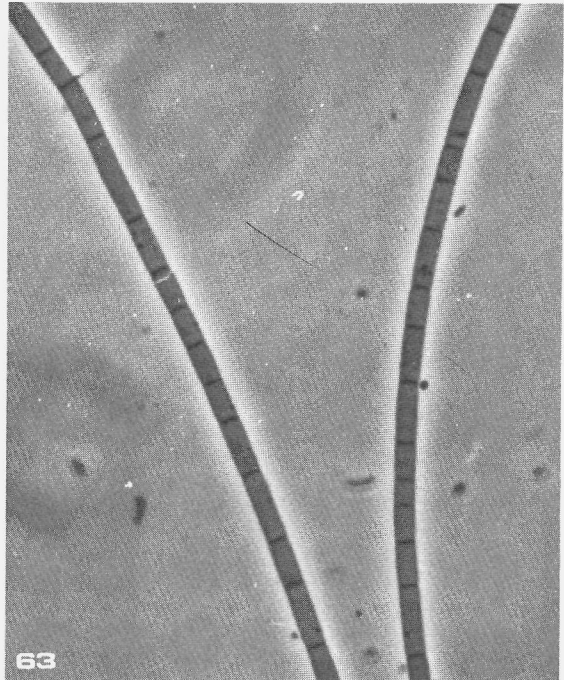
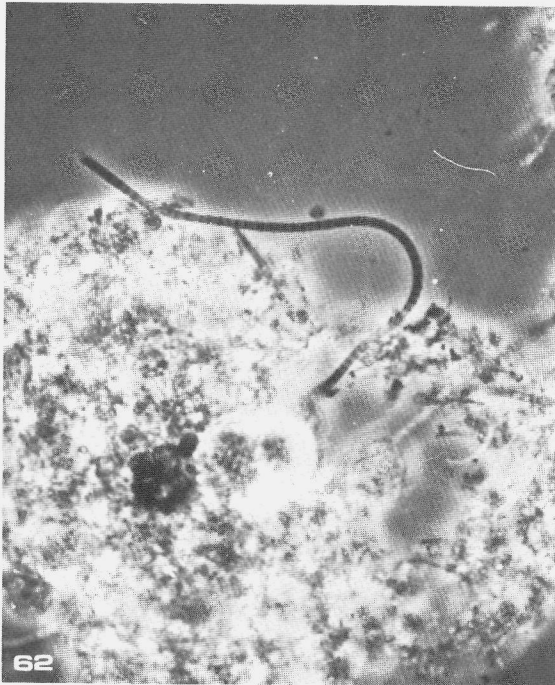


Plaat N. Draadvormige bacteriën in actief-slib. x 1150.

- Fig. 58. Type 0675. Sterke aangroei van de draden.
 Fig. 59. Type 0803. Septa zichtbaar.
 Fig. 60. Type 0803. De draden hebben zich vastgezet op een brokje anorganisch materiaal.
 Fig. 61. Type 0914. De draden bevatten S-bolletjes.

Plate N. Filamentous bacteria in activated sludge. x 1150.

- Fig. 58. Type 0675. Massive attached growth to the filaments.
 Fig. 59. Type 0803. Crosswalls clearly observable.
 Fig. 60. Type 0803. Filaments are attached to a piece of inorganic material.
 Fig. 61. Type 0914. Filaments containing S-granules.



Plaat O. Draadvormige organismen in actief-slib. x 1150.

Fig. 62. Type 0914. Een draad zonder zwavelbolletjes.

Fig. 63. Type 0961. De cellen lijken transparant.

Fig. 64. Type 1701. Aangroei van draden.

Fig. 65. Typen 1701 (a) en 1702 (b).

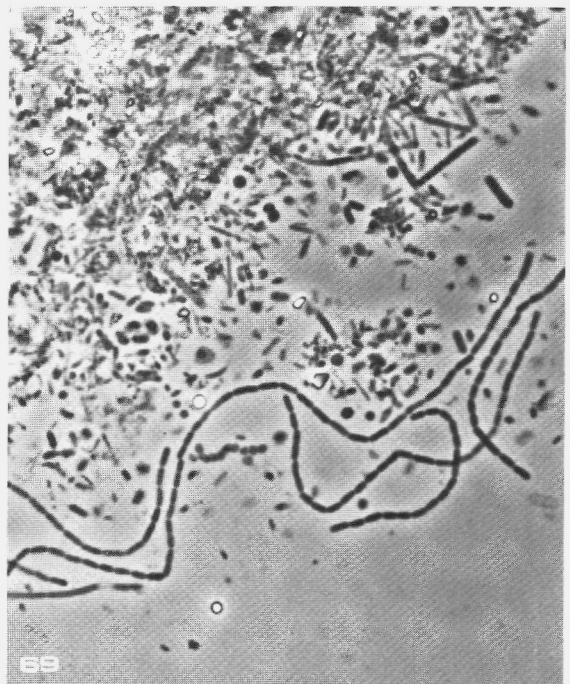
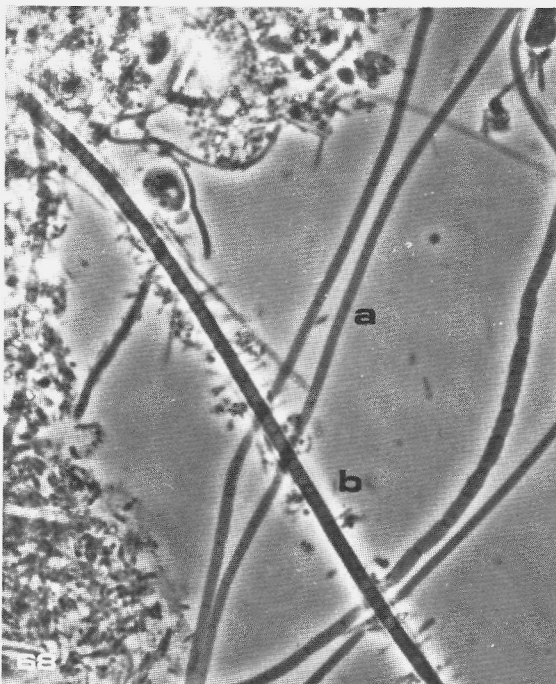
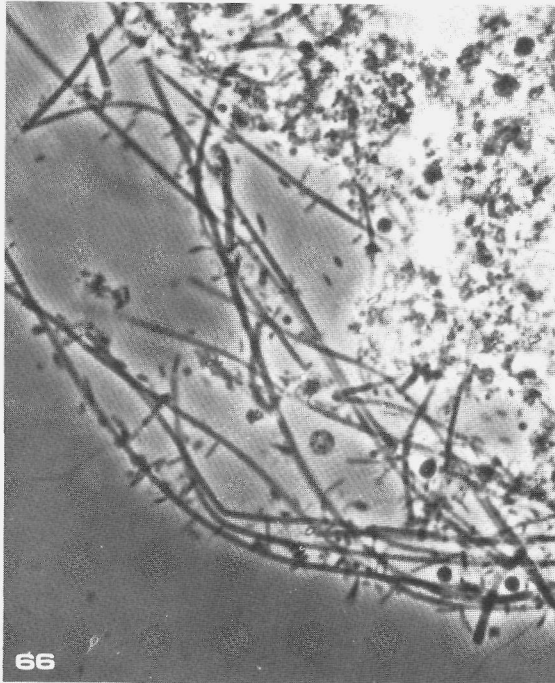
Plate O. Filamentous organisms in activated sludge. x 1150.

Fig. 62. Type 0914. A filament without sulphur granules.

Fig. 63. Type 0961. The cells seem to be transparent.

Fig. 64. Type 1701. Attached growth to the filaments.

Fig. 65. Types 1701 (a) and 1702 (b).

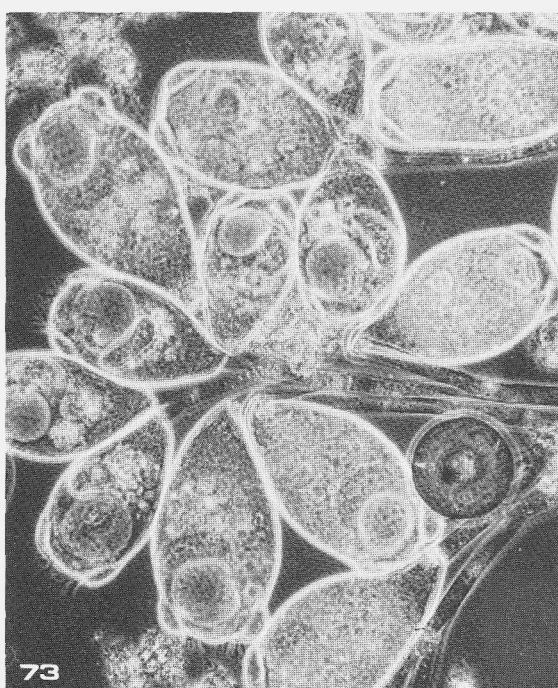
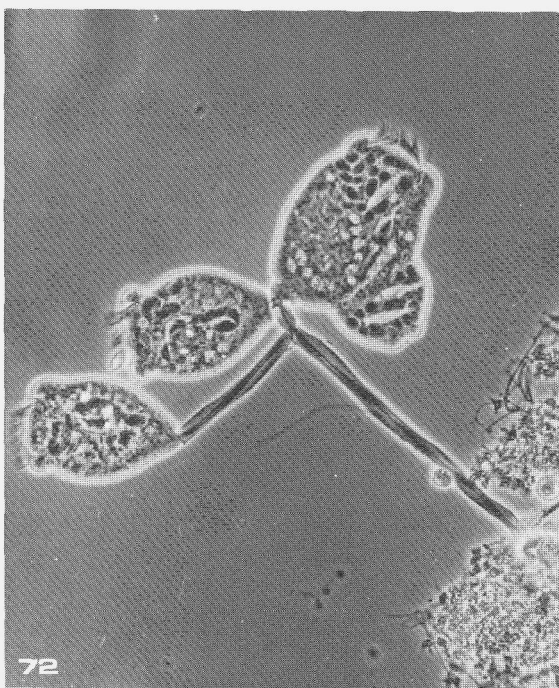
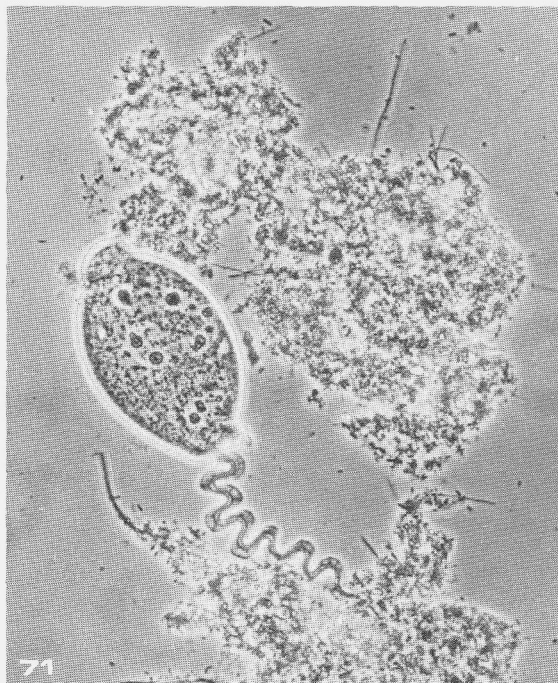
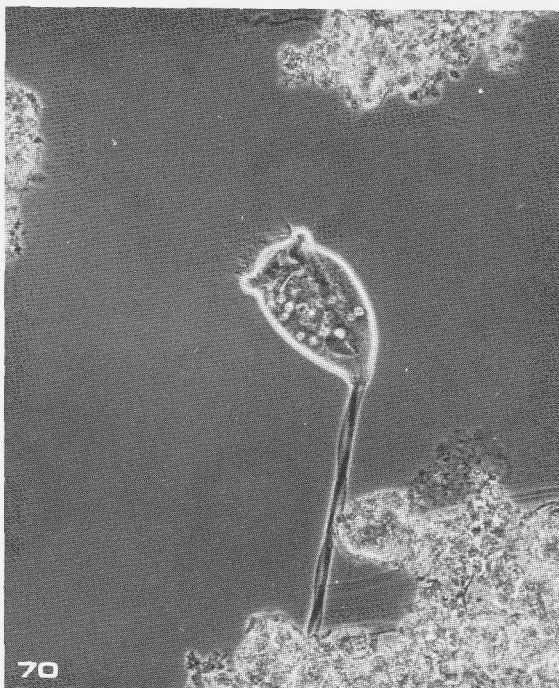


Plaat P. Draadvormige organismen in actief-slib. x 1150.

- Fig. 66. Type 1851. Aangroei van de draden.
 Fig. 67. Type 1851. Een bundel draden.
 Fig. 68. Typen 1852 (a) en 0041 (b). Septa zijn duidelijk zichtbaar.
 Fig. 69. Type 1863. Gebogen draden.

Plate P. Filamentous organisms in activated sludge. x 1150.

- Fig. 66. Type 1851. Attached growth to the filaments.
 Fig. 67. Type 1851. A bundle of filaments.
 Fig. 68. Types 1852 (a) en 0041 (b). Crosswalls clearly observable.
 Fig. 69. Type 1863. Curling filaments.

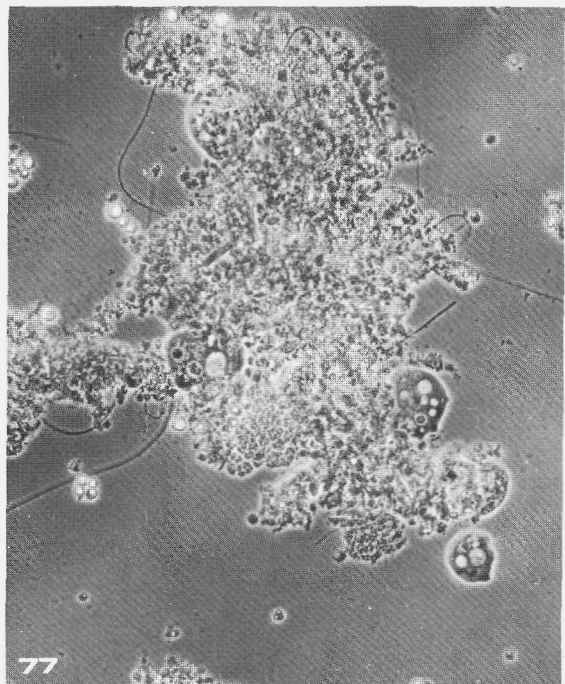
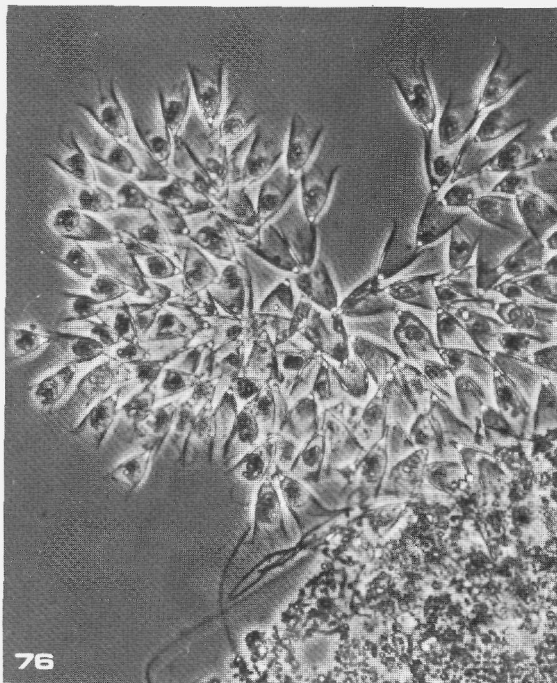
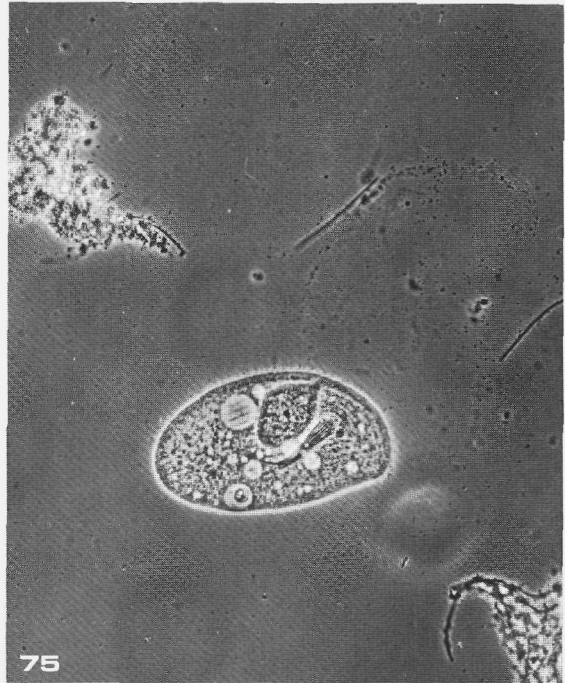
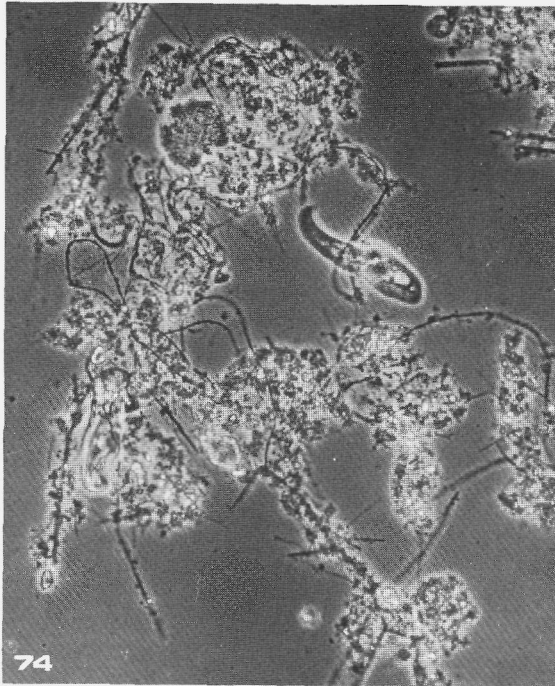


Plaat Q. Sessiele Ciliata in actief-slib.

- Fig. 70. Vorticella sp. De steel bevat een spier. x 230.
 Fig. 71. Vorticella sp. De steel is samengetrokken. x 345.
 Fig. 72. Carchesium sp. Kolonie van cellen. De steelspier is niet vertakt. x 345.
 Fig. 73. Epistylis sp. Kolonie van cellen. De steel bevat geen spier. x 230.

Plate Q. Sessile Ciliata in activated sludge.

- Fig. 70. Vorticella sp. The stalk contains a muscle. x 230.
 Fig. 71. Vorticella sp. Contracted stalk. x 345.
 Fig. 72. Carchesium sp. Colony of cells. The stalkmuscle is not branched. x 345.
 Fig. 73. Epistylis sp. Colony of cells. The stalk does not contain a muscle. x 230.



Plaat R. Enkele protozoa welke in actief-slib kunnen worden waargenomen.

Fig. 74. Lionotus. x 345.

Fig. 75. Chilodonella sp. x 345.

Fig. 76. Poteriodendron sp. x 460.

Fig. 77. Amoeba sp. x 230.

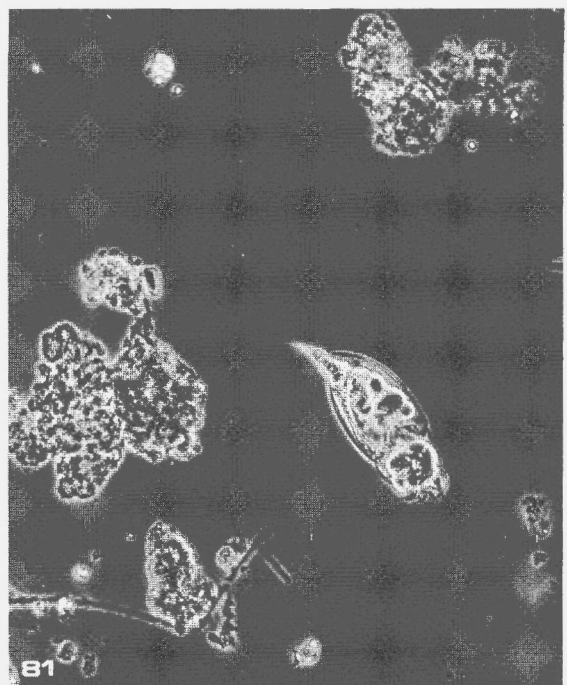
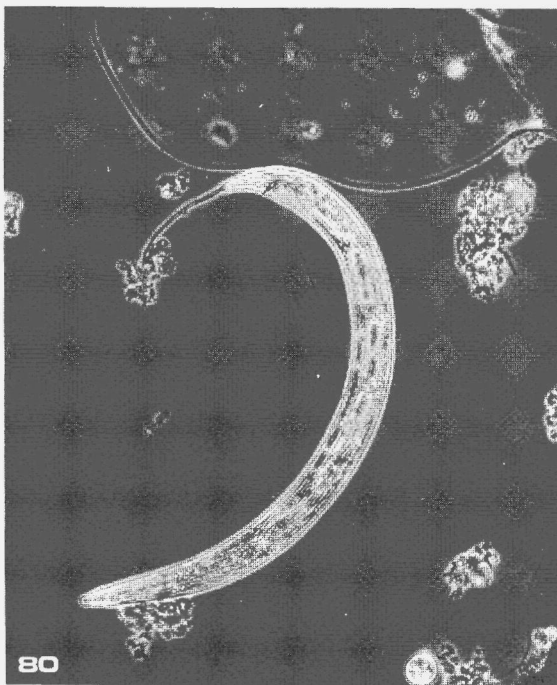
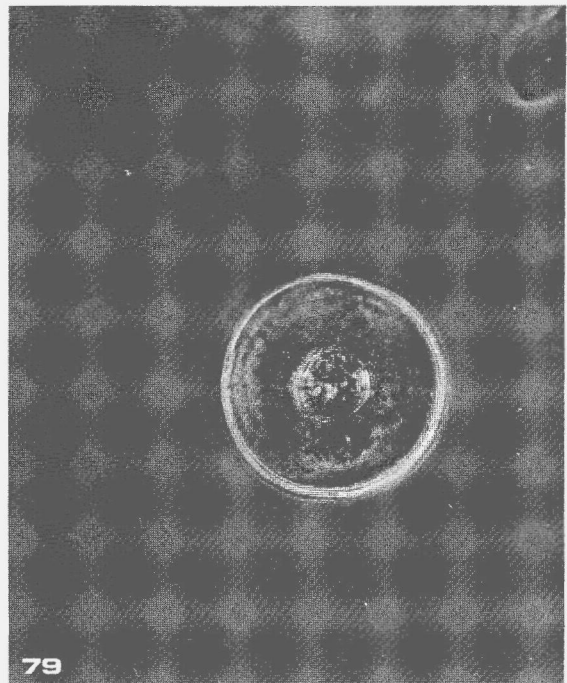
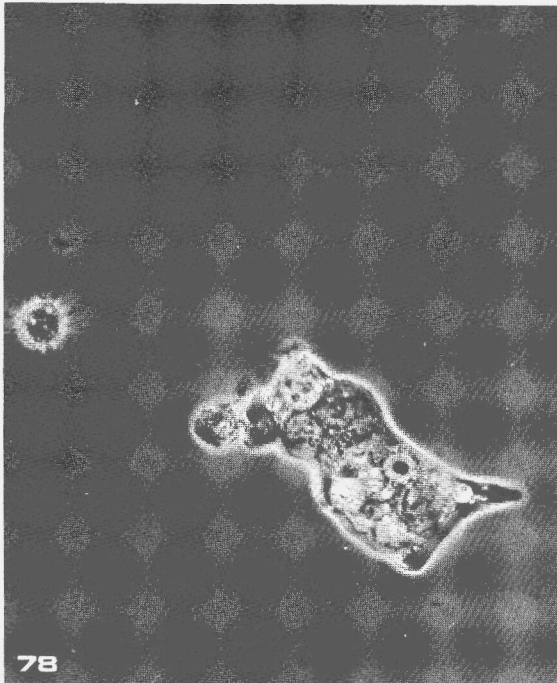
Plate R. Some protozoa which may be observed in activated sludge.

Fig. 74. Lionotus. x 345.

Fig. 75. Chilodonella sp. x 345.

Fig. 76. Poteriodendron sp. x 460.

Fig. 77. Amoeba sp. x 230.



Plaat S. Organismen in actief-slib.

- Fig. 78. Amoeba sp. x 312.
 Fig. 79. Schaalamoeba sp. Bovenanzicht. x 416.
 Fig. 80. Nematoda sp. x 104.
 Fig. 81. Rotifera sp. x 208.

Plate S. Organisms in activated sludge.

- Fig. 78. Amoeba sp. x 312.
 Fig. 79. Shellamoeba sp. Front view. x 416.
 Fig. 80. Nematoda sp. x 104.
 Fig. 81. Rotifera sp. x 208.