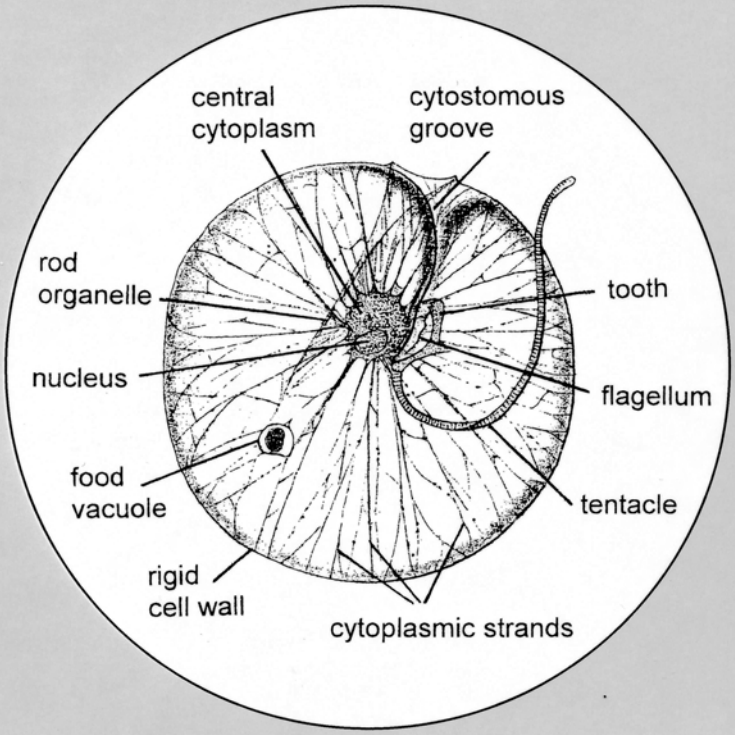
PROTOZOOLOGICAL MONOGRAPHS

Volume 4, issued January 2009

Rudolf Röttger, Robert Knight and Wilhelm Foissner (Eds.)

A Course in Protozoology



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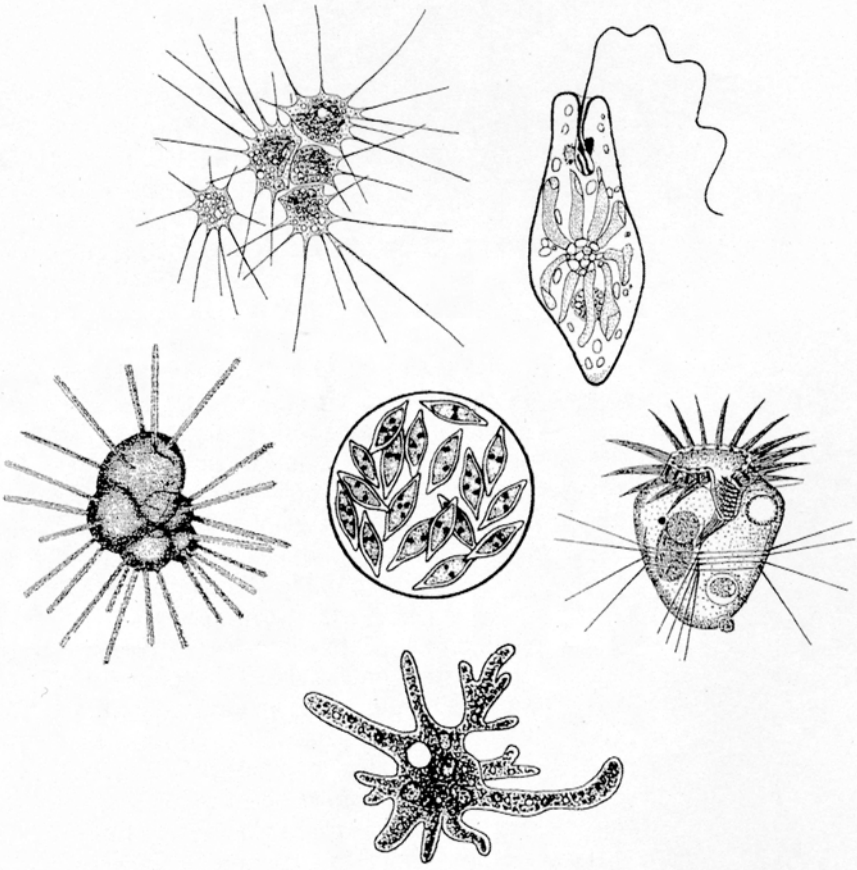
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Cover: *Noctiluca scintillans*, a bioluminescent marine dinoflagellate up to 1 mm in diameter (see p. 43 of this book)



Chrysamoeba sp., *Euglena viridis,*
Hastigerina pelagica, Monocystis sp., *Halteria grandinella,*
Amoeba proteus

Protozoological Monographs

Wilhelm Foissner (Editor)

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A Course in Protozoology

Rudolf Röttger, Robert Knight and Wilhelm Foissner (Eds.)

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From the Preface to the First Edition 1995

Since the publication in 1928 of the 5th edition of Max Hartmann´s "Praktikum der Protozoologie" there have been no further works in this field. The present book has been written to make good this deficiency and to help the student to appreciate the wide variety of forms within the protozoa – surely one of the most striking manifestations of life – and to relate these forms to their varied way of life. Some subjects lend themselves to field trips in which students are shown both how to collect free-living protozoa and to relate different types of protozoa to their habitats. In some cases as little as an hour may be long enough for a walk to a forest, pond, bog, river bank or to the sea shore. Here the lecturer might explain the special features of each biotope. From a first hand view of the habitat and the living individual isolated from it a deeper knowledge develops than from the study of the organism derived from a laboratory culture.

The present work is designed to show how protozoa are collected, isolated and processed prior to microscopical examination, and how the methods vary depending on the types of protozoa. While a living specimen offers many insights into its biology, staining or impregnation is needed in order to see many of the often taxonomically important structures.

A preparation is best understood by drawing it; and protozoa are easier to draw than many other biological entities. Often just a contour line and some internal structures are sufficient to bring to light the distinguishing features of a preparation. The object of drawing a specimen is to improve the depth of the observation as well as to provide training in quickly recognising and relating observed forms and structures. Thus a course in protozoology may well become a first step towards scientific drawing and, indeed, scientific work in general.

The choice of topics follows systematic and, where possible, ecological viewpoints. Thus, as well as monographic presentations of systematic units, the protozoa are also treated in an ecological context as exemplified by free-living naked rhizopods, testate amoebae in mosses and forest soils, soil ciliates, ciliates of freshwater biofilms and symbiotic flagellates in termites.

The book contains units for half-day or one-day practical sessions. However, many subjects are best studied over several days if time is available.

Preface to the Second Edition

This translated and updated second edition of the German "Praktikum der Protozoologie" (Gustav Fischer Verlag, Stuttgart, Jena, New York, 1995) is the first course in protozoology for English speaking students. Most of the authors from the first edition have contributed to the second edition. However, seven chapters have been dropped and six new ones added.

The most important prerequisite for running any practical class is the supply of material. The first edition claimed to be based on protozoa easily accessible from habitats of central Europe. However, many examples that were portrayed in the sections "Genera and Species" occur world-wide and this is particularly true for marine protozoa and parasites.

Drawing is unquestionably the very best way to learn about the structure of an organism (see the preface to the first edition of this book). The fact that digital cameras are now commonplace means that they can sometimes be used as an adjunct to drawing. A trinocular microscope is by no means essential and, with some digital cameras, perfectly adequate shots can be obtained simply by holding the lens of the camera as close as possible to the eyepiece. Moreover, many digital cameras are able to make short video recordings. These can be of very great use when transitory events are recorded and later viewed, perhaps several times, to elucidate a sequence of events. Digital recordings, resulting in still or moving pictures, have other significant advantages, namely that the results can be seen immediately (there is no need to wait for developing and printing) and the availability of the ubiquitous computer leads to both a significant reduction in cost and the ability to share images at no cost.

We retained the term "protozoa" because the organisms treated are predominantly unicellular heterotrophic eukaryotic microorganisms with the ability to phagocytose, as well as coloured euglenids and dinoflagellates which, according to the theory of endosymbiosis, evolved from phagocytosing protozoan hosts with algal endosymbionts. A chapter on marine planktonic diatoms is now included because diatoms occur in most plankton samples. All of the organisms treated here are also protists (see definition below). However, the user of a textbook entitled "A course in protistology" would expect to find more unicellular algae and lower fungi included.

Most of our authors have used the system given in the second edition of the Illustrated Guide to the Protozoa (Lee et al. 2000), while others have preferred the system of Cavalier-Smith (1998, 2003). For algae, the textbook of van den Hoek, Mann & Jahns (1995) has been followed.

A bibliography at the end of each chapter contains important original papers, review articles, monographs, available literature for identification, and scientific films. Textbooks are listed at the end of the book in front of the index.

To see a World in a grain of sand,
And a Heaven in a wild flower,
Hold infinity in the palm of your hand,
And Eternity in an hour.

William Blake (1757 – 1827)

Die Welt des Kleinen auch
ist wunderbar und groß,
und aus dem Kleinen
bauen sich die Welten.

The world of tiny creatures
Is wondrous to behold,
Yet from such small beginnings
Our living world evolved.

Christian Gottfried Ehrenberg (1795 – 1876)

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Introduction: Protozoa, Protophyta, Protists

Rudolf Röttger

Protozoa, protophyta and protists are three words with the same basic meaning. They were given in the 19th century to unicellular eukaryotes that were considered to be primitive, or prototypes, on the basis of their (at that time certainly insufficiently investigated) structure. The object of studying them was to find model organisms that would make understandable the evolution of multicellular organisms.

The polyphyletic kingdom Protozoa (the term was first used in 1845 for "unicellular animals") predominantly consists of unicellular phagotrophic, non-photosynthetic eukaryotes, mainly diplomonads, parabasalids, amoebae in the widest sense (including Radiolaria, Heliozoea and Foraminifera), kinetoplastids, apicomplexans and ciliates. Phagotrophy is their most important ancestral feature. The photosynthetic euglenids and dinoflagellates are protozoa with three-membrane chloroplast envelopes, and they arose through the phagocytic uptake of a unicellular eukaryotic alga that transformed from a symbiont to a chloroplast (Fig.1, 2). The ability for phagotrophy was lost after this event (theory of endosymbiosis).The euglenids are allied to the kinetoplastids (both are united as Euglenozoa), while the dinoflagellates are related to the ciliates and apicomplexans (the three united as Alveolata).

Like the phototrophic euglenids and dinoflagellates, chrysophytes are also evolutionary chimaeras between a heterotrophic host and an algal endosymbiont. Although they are not protozoa but chromists (kingdom Chromista), they are included here because they are well suited to the demonstration of protozoan organization, and often occur in plankton tows.

Protozoan cells possess many unique structural and physiological features that do not occur in the cells of animal tissues: a wide variety of cell investments including organic and inorganic tests and skeletons; the ability to form cysts, vegetative quiescent stages; specialized polymorphic ciliated organelles for food gathering and movement; contractile fibres for changing body shape; a large number of types of flagella and kinds of flagellation and ciliation; pulsing vacuoles; many types of extrusomes; complicated life cycles connected with change of structure and physiological ability related to change of habitat; the occurrence of diplonts with gametic meiosis and of haplonts with zygotic meiosis, and heterophasic alternation of generations.

In recognition of all of these features as fundamental differences in structure and body plan, the frequently used term "unicellular animals" for protozoa is no longer justified. Animals are monophyletic multicellular diploid organisms with tissue- and organ-forming cells. They develop from a large egg cell fertilized by a small sperm (Fig. 3). Their zygote develops to a blastula (Fig. 4) and an ancestrally triploblastic (with three germ layers) embryo.

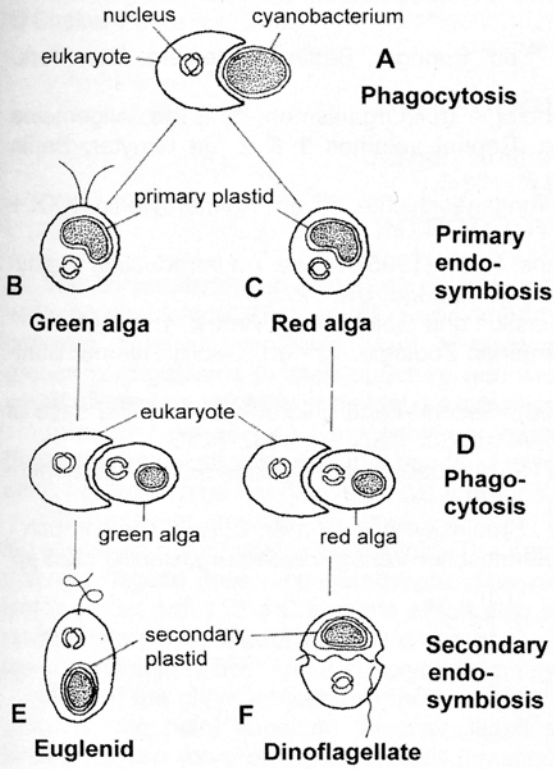
A comparison between heterotrophic protozoa and unicellular plants stimulated Ernst Haeckel (1889, p. 420) to introduce the term protophyta for the latter. His protophyta were unicellular non-tissue plants, that is algae. This term is still used today for unicellular algae (Sitte et al., 2002, p. 209), comprising Euglenophyta and Dino-

phyta (belonging to the kingdom Protozoa), Chrysophyta or golden algae and Bacillariophyceae or diatoms (belonging to the kingdom Chromista) and Chlorophyta or green algae like *Chlamydomonas, Dunaliella, Chlorella* and *Cosmarium* (belonging to the kingdom Plantae). Thus algae do not form a taxon but denote a level of organization characterized as non-embryophytes with chloroplasts.

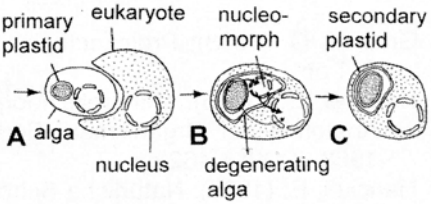
Haeckel, like other scientists of his time, recognized the special feeding habits of many unicellular algae that included combinations of phototrophy and phagotrophy as well as phototrophy and saprotrophy and that did not allow an unequivocal classification within either protozoa or protophyta. He therefore coined the term protist (Haeckel, 1866, p. 203) comprising these mixotrophic organisms (Fig. 5, 6) and their definite phototrophic and definite heterotrophic relatives. Today all protozoa and the above listed protophyta regardless of both their chloroplast content and their feeding habit and also lower unicellullar fungi are classified as protists.

The term protoctist, not used by authors of this book, embraces not only the protists but also the algae with leaf-like and filamentous thalli (because of their relationship to unicellular algae), the parasitic and saprophytic oomycetes and chytridiomycetes (with chitin as cell wall component) and other taxa. Thus, all algae are included into the protoctists. The kingdom Plantae only comprises the embryophytes (Bryophyta, Pteridophyta, Spermatophyta).

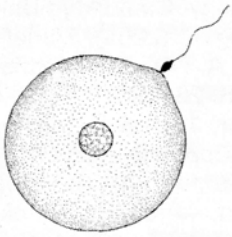
Fig. 1. A brief history of euglenids and dinoflagellates (theory of endosymbiosis). **A:** A unicellular heterotrophic eukaryote phagocytosed a photosynthetic cyanobacterium but failed to digest it. The cyanobacterium lost many genes and transferred many others to the host nucleus, eventually becoming a specialized organelle, called primary plastid (Fig. **B, C**). This is bound by two membranes. From this primary endosymbiosis green algae and their close relatives, the land plants and red algae evolved. All three form the kingdom Plantae. Plantae are characterized by plastids with double envelopes, starch as a product of photosynthesis and without phagocytosis. **D:** When a primary alga is engulfed by a heterotrophic eukaryote and degenerates into a photosynthetic organelle, a secondary plastid originates by secondary endosymbiosis (**E, F**). A characteristic of secondary plastids is the original presence of four membranes around the plastids. In euglenids and dinoflagellates one of these membranes has been lost. **Fig. 2.** Secondary endosymbiosis, transformation of a primary alga into secondary plastid. **A:** A primary (eukaryotic) alga is incorporated by a heterotrophic eukaryote. **B:** Stage of degeneration of the alga with a relict nucleus (the nucleomorph) between the second and the third membranes. Arrows show gene transfer. **C:** Final stage with the secondary plastid surrounded by four membranes. **Fig. 3.** Schematic representation of the fertilization in animals, initial stage. A tiny sperm cell has reached the large egg cell. **Fig. 4.** Scheme of the total and equal cleavage of a fertilized animal egg (sea urchin), resulting in a blastula. Invagination and gastrulation will follow. **Fig. 5.** Fine structure of a mixotrophic protist. Note the double membrane of the chloroplast envelope and the invagination of the cell membrane during phagocytosis. **Fig. 6.** Mixotrophic protists. **A:** *Ochromonas mutabilis* (Class Chrysophyceae) (1030 µm), a cell able to change shape (metaboly), with two chloroplasts and a food vacuole demonstrating phagocytosis. **B:** *Chromulina ovalis* (Class Chrysophyceae) (9–14 µm), another species exhibiting metaboly with one chloroplast and a food vacuole. **C:** *Cryptochrysis gigas* (Class Cryptophyceae) with two chloroplasts and a phagocytosed *Cryptomonas* cell. (Fig. 1 from Keeling 2004, modified; Fig. 2 from Palmer & Delwiche 1996; Fig. 3, 4 from Kühn 1955; Fig. 5 from Grell 1968, modified; Fig. 6 from Pascher 1917)



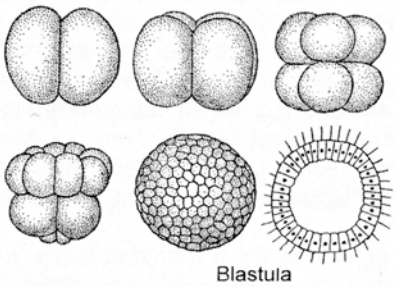
1 History of euglenids and dinoflagellates



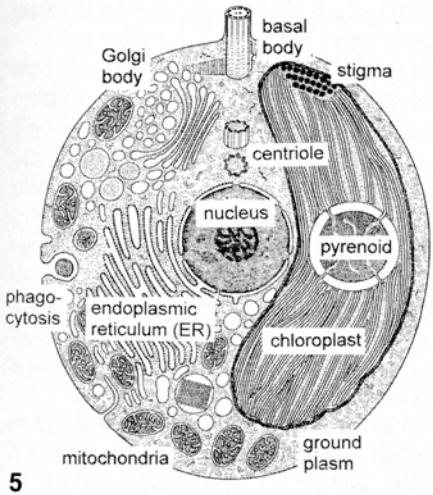
2 Secondary endosymbiosis



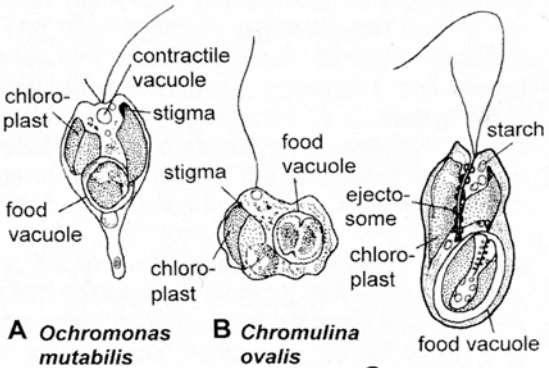
3 Fertilization of an animal egg



4 Cleavage of an animal egg



5 Fine structure of a mixotrophic protist



6 Mixotrophic protists

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Chrysophytes

Jørgen Kristiansen

INTRODUCTION

The chrysophytes or golden algae include about 1200 species mainly in freshwater phytoplankton. They are of special importance for the study of the relationships between algae and protozoa. Many of them combine features from both of these groups of organisms in their structure and mode of nutrition, mainly a membrane which allows phagotrophy and plastid acquisition.

Thus, as algal classes Chrysophyceae and Synurophyceae, they find their position in the botanical system within the division Heterokontophyta, which also includes diatoms, brown algae and several minor groups.

Chrysophytes are, indeed, algae, according to the definition "a polyphyletic grouping of non-embryophytes with plastids". The name Heterokontophyta refers to the two different flagella (see next paragraph). The Heterokontophyta are part of a much larger protist group, the Chromista which also contain the divisions Cryptophyta and Haptophyta (Prymnesiophyta). All chromistan algae are evolutionary chimaeras between a eukaryotic host and a eukaryotic algal symbiont (see p. 1 and p. 3, Fig. 1, 2).

Most of the chrysophytes are motile, unicellular flagellates. The cell has two flagella, a long hairy flagellum (flimmer flagellum, pleuronematic flagellum) characterized by two rows of tripartite hairs (mastigonemes, stramenopiles) and a short smooth flagellum (Fig. 1a, b). Beside the flagellate forms, there are also amoeboid and immobile, even filamentous and branched forms, and many species occur as several of these life forms during their life history.

The nucleus lies in the centre of the cell (Fig. 1a) while behind it there is a large storage vacuole containing the carbohydrate chrysolaminaran.The outer of the two nuclear membranes is continuous with the outer of the four chloroplast membranes. There is one bilobate chloroplast that often has a red eyespot (stigma) adjacent to a photoreceptor on the short flagellum. The photosynthetic pigments are the chlorophylls *a* and *c*. Their green colour is hidden by the brown pigment fucoxanthin; this is the reason for the yellowish-brown colour of these algae. Freshwater forms always have contractile vacuoles that maintain the osmotic equilibrium. A characteristic of the chrysophytes is their endogenous silicified resting stage, the stomatocyst, which distinguishes them from all other groups of algae. Most of the taxonomically important features of the chrysophytes can only be examined by means of electron microscopy.

The chrysophytes are mostly solitary, but in some genera the cells are arranged in spherical (*Uroglena, Synura*) or branched colonies (*Dinobryon*). Many genera have naked cells, delimited only by their plasma membrane. Other genera have different types of envelopes, either as urn- or vase-shaped loricae or as an armour of individual minute silica scales. Under the electron microscope such silica scales

show complicated patterns of ornamentation which are characteristic of each species. Reproduction is most often asexual by longitudinal division of the cell. However, sexual reproduction occurs in some species by isogamous or anisogamous fusion of gametes that often cannot be distinguished from vegetative cells. The fusion results in a zygote which forms a siliceous wall just like a vegetative stomatocyst. At the end of the vegetation period the cells encyst. The stomatocyst wall is deposited endogenously within the cell, and it has a porus which finally will be closed by a stopper. The cyst wall is smooth or spiny. The mature cysts sink to the bottom where they germinate in the next or a following year. At germination, the stopper is dissolved and a juvenile cell escapes through the porus.

We can trace how different colourless groups can be derived from pigmented chrysophytes. In the colourless genus *Paraphysomonas* the reduction of the chloroplast and photoreceptor system can be followed. Several colourless amoebae show that they are reduced descendants of chrysophytes, because they are still able to form stomatocysts.

Most chrysophytes live in freshwater. They prefer ponds and small lakes, often with brownish water and with a low content of nutrients. Very nutrient-rich waters with mass development of cyanobacteria and green algae are avoided. However, the species have wide ranging ecological requirements. Some prefer oligotrophic, others more eutrophic localities, and many show distinct pH preferences. These can be used as indicator species. This is especially the case for the silica-scale bearing species, because the scales can easily be identified to species and are preserved in the bottom sediments. They can therefore be used in investigations on lake history, as markers of eutrophication or acidification.

The seasonal occurrence of the chrysophytes shows characteristic features. Most species and the highest cell numbers are found in spring, typically shortly after ice-break. A few species are less dependent on temperature and occur during the whole year. Species of *Synura* and *Uroglena* can, by mass development, give the water an unpleasant smell and taste. Thus they may cause problems in drinking water reservoirs.

PRACTICAL TECHNIQUES

As most chrysophytes are planktonic, sampling is often done by means of a plankton net with 20 µm mesh. Often a 10 µm net may be useful for the still smaller organism, but it is difficult to use because it easily clogs. If we want to examine the total plankton, including the nanoplankton, the samples must be taken by means of a bottle or by a vertical water sampler. In the laboratory, the sample can then be concentrated by filtration, centrifugation or sedimentation.

All samples must be kept cool, in a thermos flask or in thermo bags. In the laboratory they must be kept in a fridge or in a cold culture room. Many species, especially cold-water forms, but also *Uroglena* and *Dinobryon* from summer samples, are extremely delicate and must be examined under the microscope as soon as possible. Parallel samples should always be fixed at once in acidic Lugol´s iodine (see p. 236). Drops should be added until the sample gets a slightly brownish colour.

Crude cultures can be established by adding a few drops of nutrients. For pure cultures, media containing soil extract are often used as they contain the necessary

trace elements. Methods and recipes are given by Stein (1973).

For examination of flagella, envelopes and silica scales and bristles, phase contrast is necessary. The silica structures are best studied in dry mounts. A drop of the sample is simply dried on a coverslip, and the coverslip with the sample downwards is then mounted on a slide by means of Canada balsam or nail varnish in the corners, so that the structures then can be viewed in air which has a refractive index very different from that of silica. Phase contrast microscopy is then used, with objectives x 40 or x 100 (oil immersion).

The scales of *Synura* and *Mallomonas* are best examined by electron microscopy. The preparation is very simple. Lugol-fixed material is rinsed in distilled water in a centrifuge. By means of a micropipette a drop of the centrifugate is placed on a formvar coated grid and dried. The material is then ready for examination in the transmission electron microscope. Better contrast and more information can be obtained after shadow casting with, for example, gold-palladium. Scanning electron microscopy shows the three-dimensional structure of the surface of the object. The material must be mounted on the specimen holder and then covered with a thin conductive layer of gold in a vacuum evaporator, before it can be inserted in the SEM specimen chamber. Electron microscopy is necessary for identification for most of the scale-covered species, because the very delicate ornamentation of the scales is used as a taxonomic character.

Many genera without scales require examination in the living state for identification.The most recent general identification work for chrysophytes is the volume by Starmach (1985). In many respects, however, this is outdated and must be supplemented by the chrysophyte chapters in "The freshwater algal flora of the British Isles" (Kristiansen 2002) and in the appropriate chapter "Fresh water algae of North America" (Nicholls & Wujek 2003; Siver 2003). Some other useful works are mentioned in the literature list at the end of this paper.

GENERA AND SPECIES

Ochromonas Cl. Chrysophyceae, O. Chromulinales, Fam. Chromulinaceae (Fig.1, 2)

This very large genus of mainly freshwater flagellates contains about 100 species, most of which are very difficult to identify, as many of the taxonomic characters are vague and difficult to define.

The cell is oblong to pear-shaped. Many species show metaboly and can also include amoeboid stages in their life history. The cell has two visible flagella anteriorly, the long hairy flagellum and the short smooth flagellum. The latter has near its base a swelling, a photoreceptor, which together with the stigma in the adjacent chloroplast lobe acts as the photokinetic system. The cell surface is generallly naked, but in some species it is covered with a woolly layer. A system of microtubules below the plasmalemma maintains the specific form of the cell. By colchicine treatment these microtubules are destroyed and the cell becomes globular.

Even though *Ochromonas* has a well developed chloroplast, it has a mixotrophic mode of nutrition: it combines photosynthesis (only vitamin B is necessary) with

uptake of dissolved organic matter (osmotrophy) and phagotrophy (uptake of other organisms such as bacteria and small algae). A cytoplasmic basket supported by flagellar roots catches the prey and ingests it into the cell. Digestion takes place in a special vacuole in the rear of the cell. In darkness, the chloroplast is reduced and the two latter methods of nutrition take over.

0chromonas can be seen as the model for the chrysophyte cell, and most other chrysophytes can be thought to be derived from it. In the field, it is only seldom found in great quantities. For use in the laboratory it can be obtained from culture collections (for addresses see p. 30). *Ochromonas* and the closely related *Poterioochromonas* have served as test organisms for vitamins and for heavy metals, and also for many general cytological and physiological purposes.

Uroglena Cl. Chrysophyceae, O. Chromulinales, Fam. Chromulinaceae (Fig. 5)

About 20 species. *Uroglena* has large spherical colonies formed by *Ochromonas*-like cells, which are held on radiating stalks and kept together by mucilage. It occurs in ponds and small lakes, and by mass development it can give the water an unpleasant fishy smell.

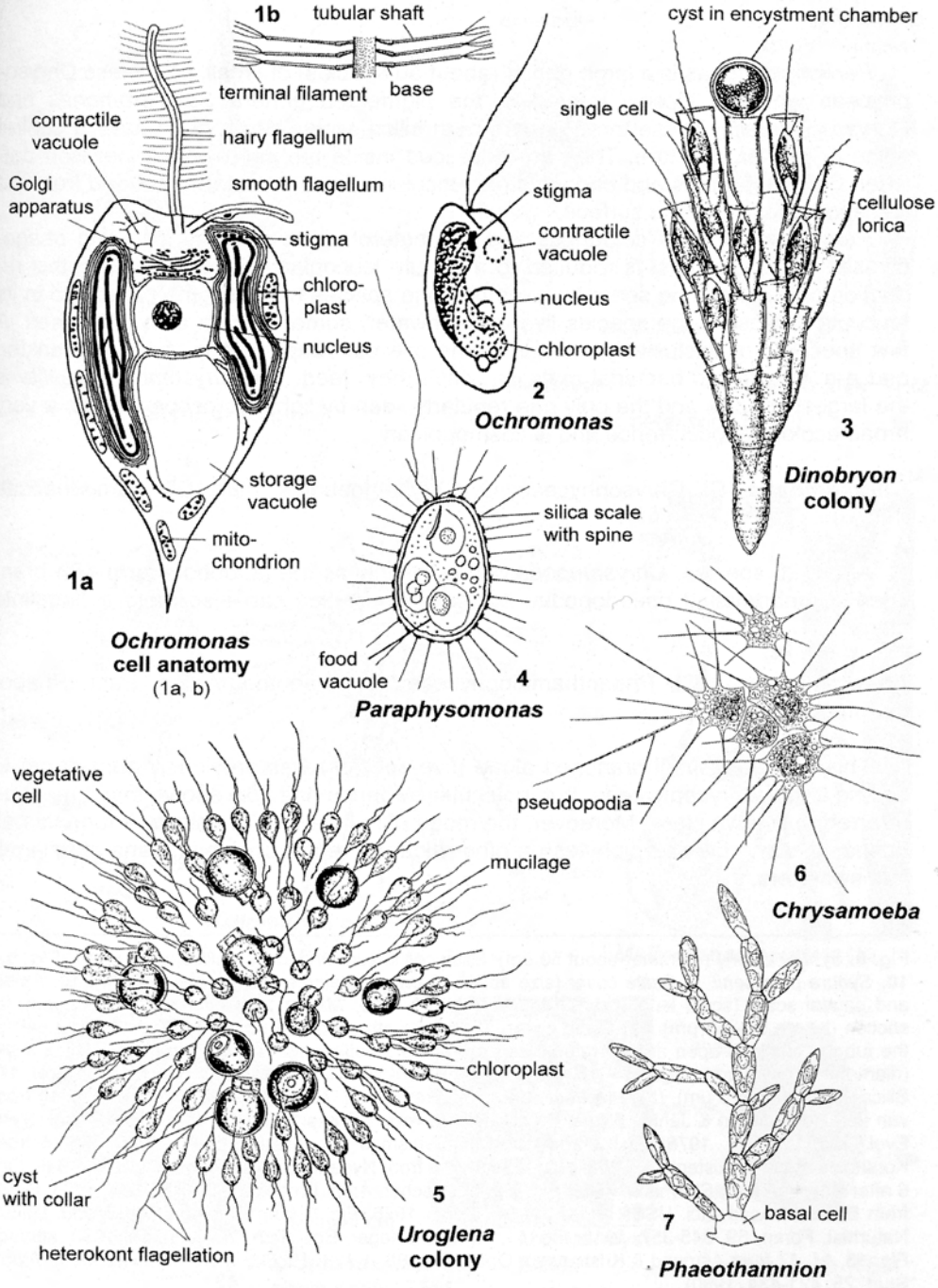
Dinobryon Cl. Chrysophyceae, O. Chromulinales, Fam. Dinobryaceae (Fig. 3)

About 40 species. The *Dinobryon* cell is of the typical *Ochromonas* construction and is attached in a vase-shaped or conical envelope, a lorica. During rotation the cell constructs its lorica by secretion of fine cellulosic fibrils. After cell division, one of the daughter cells moves to the edge of the parent lorica and produces its own lorica. This is the origin of the branched colonies that are typical of many *Dinobryon* species, although several are solitary. The shape of the lorica and the architecture of the colony serve as taxonomic characters.

Sexuality is rarely observed and was discovered only a few years ago. A motile cell from a male colony fuses with a cell in a female colony and they form a zygote. Before encystment, the zygote moves to the top of the lorica and constructs a special encystment chamber, where the stomatocyst is formed. This is globular, more or less smooth, and in some species provided with a long, curved neck.

Some *Dinobryon* species are very important components of the phytoplankton, especially in clear-water lakes, where they occur mainly in spring and autumn. Although the *Dinobryon* cells have well developed chloroplasts, they are also phagotrophic, and they ingest bacteria in such great quantities that they play an important role in the carbon budget of the food web in the lake. Bacteria have a better uptake of phosphorus than algae, and thus the bacterial diet gives *Dinobryon* an advantage over other algae in waters poor in phosphate.

Fig.1a. *Ochromonas*, structural plan. Membrane systems simplified. **Fig. 1b.** Detail from the hairy flagellum of a chrysophyte. **Fig. 2.** *Ochromonas* (3–4 µm). **Fig. 3.** *Dinobryon* colony (length about 150 µm). One cell has formed a cyst. **Fig. 4.** *Paraphysomonas* (20 µm). **Fig. 5.** *Uroglena* colony (diameter about 200 µm). Some of the cells have formed a cyst with the typical collar. **Fig. 6.** *Chrysamoeba* (cell size 10–15 µm), a chrysophyte with pseudopodia. **Fig. 7.** *Phaeothamnion*.(size 100–150 µm), a chrysophyte with filiform, branched thallus.


1b
tubular shaft
terminal filament
base
contractile vacuole
hairy flagellum
Golgi apparatus
smooth flagellum
stigma
chloro-plast
nucleus
storage vacuole
mito-chondrion
1a
Ochromonas cell anatomy (1a, b)
stigma
contractile vacuole
nucleus
chloroplast
2
Ochromonas
cyst in encystment chamber
single cell
cellulose lorica
3
Dinobryon colony
silica scale with spine
food vacuole
4
Paraphysomonas
pseudopodia
6
Chrysamoeba
vegetative cell
mucilage
chloroplast
cyst with collar
heterokont flagellation
5
Uroglena colony
basal cell
7
Phaeothamnion

Paraphysomonas Cl. Chrysophyceae, O. Chromulinales, Fam. Paraphysomonadaceae (Fig. 4)

Paraphysomonas is a large genus (about 50 species) of small, colourless Chrysophyceae which is closely related to the pigmented genera *Spiniferomonas* and *Chrysosphaerella*. These three genera bear silica scales which often have a central spine or similar structure. They are produced inside the cell by a cooperation between Golgi apparatus and endoplasmic reticulum. Finally they are extruded from the cell and deposited on its surface.

Paraphysomonas is colourless and has heterotrophic nutrition, including phagocytosis. The chloroplast is reduced to a minute leucoplast. Several steps in this reduction process can be seen: for example, one species has a stigma preserved in its leucoplast. Most of the species live in freshwater, some of them also in the sea. A few species are exclusively marine. Some are very important in the nanoplankton and are attached to bacterial mats on which they feed. *Paraphysomonas vestita* is the largest species and the only one regularly seen by light microscopy. It has a very broad ecological occurrence and is cosmopolitan.

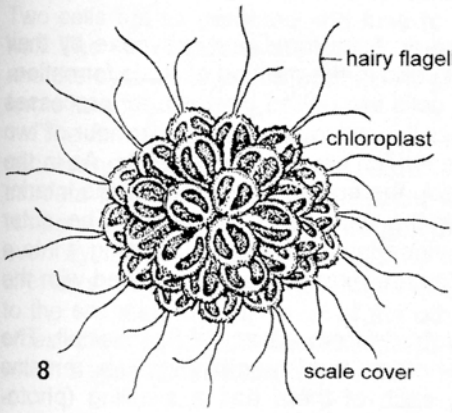
Chrysamoeba Cl. Chrysophyceae, O. Chrysamoebales, Fam. Chrysamoebaceae (Fig. 6)

About 20 species. *Chrysamoeba* occurs mainly as the amoeboid form with branched or unbranched pseudopodia, but several species can also have a flagellate stage.

Phaeothamnion Cl. Phaeothamniophyceae, O. Phaeothamniales, Fam. Phaeothamniaceae (Fig. 7)

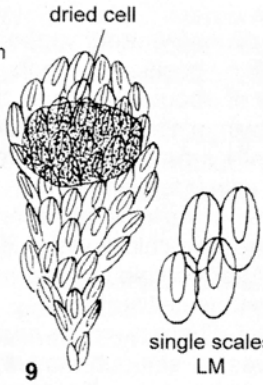
This genus of small branched algae (five species) was previously considered to belong to the Chrysophyceae, but molecular evidence has now shown that it must be referred to its own class. Moreover, the mode of cell division is unique. It forms small bushes which grow as epiphytes on other algae. It reproduces by means of biflagellate swarmers.

Fig. 8. *Synura* colony (diameter about 50 µm). Each cell is surrounded by a silica scale cover. **Fig. 9 – 10.** *Synura petersenii.* **9:** Scale cover (size about 25 µm) and single scales (about 4 µm). **10:** Apical and caudal scale (scale length about 4 µm). **Fig. 11 – 15.** *Mallomonas caudata.* **11:** Longitudinal section (length 50– 60 µm). **12:** Scale cover. **13:** Bristle attached to a scale. **14:** The terminal part of the tubular bristle is open and serrated along one edge (bristle length up to 80 µm). **15:** Silica scale (diameter about 9 µm). **Fig. 16 – 17.** *Mallomonas acaroides.* **16:** Stomatocyst (diameter 15 µm). **17:** Silica scale (length 6 µm). (Fig. 1a from Gibbs, Int. Rev. Cytol. 72, 49-99, 1981, altered; Fig. 1b from van den Hoek, Mann & Jahns, Algae, Cambridge University Press 1995; Fig. 2 from Ettl, Plant. Syst. Evol. 129, 221-241, 1978; Fig. 3 from Stein, Organismus der Infusionsthiere, 1870; Fig. 4 from Korshikov, Arch. Protistenk. 67, 253-290, 1929; Fig. 5 from Nygaard, Dansk Planteplankton, 1945; Fig. 6 after Pascher, from Starmach, 1985; Fig. 7 from Pascher, Arch. Protistenk. 52, 489-564, 1925; Fig. 8 from Balonov, Acad. Sci. USSR Proc. 31, 34, 61-81, 1976; Fig. 9 from Petersen, Vid. Medd. Dansk Naturhist. Foren. 69, 345-357, 1918; Fig. 11, 12 from Krieger, Bot. Arch. 29, 345-357, 1930, altered; Fig. 13, 14, 17 from Asmund & Kristiansen, Opera Bot. 85, 1-128, 1986; Fig. 16 from Cronberg, Algol. Stud. 25, 421-434, 1980)



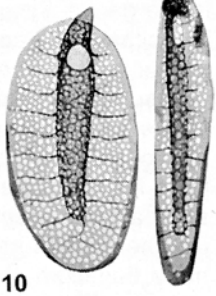
8

***Synura* colony**



9

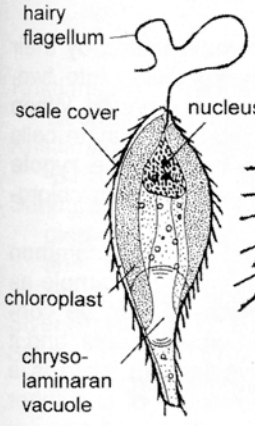
scale cover



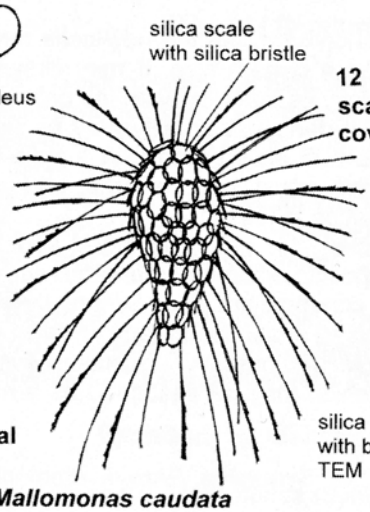
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single scales TEM

Synura petersenii (9, 10)

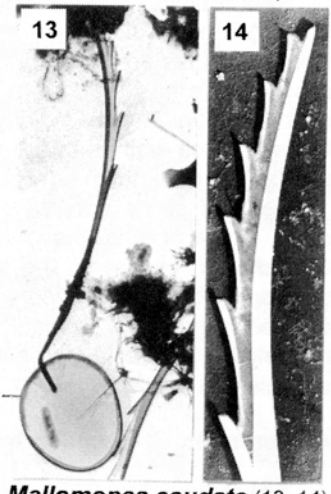


11 longitudinal section

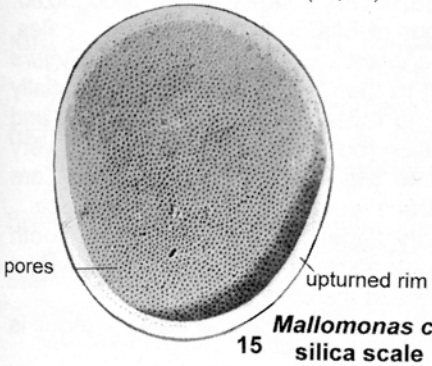


12 scale cover

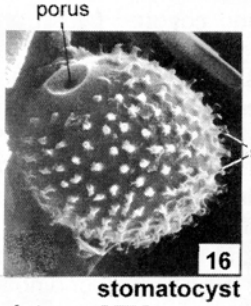
Mallomonas caudata
(11, 12)



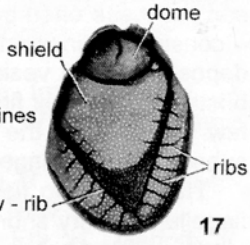
Mallomonas caudata (13, 14)



15 *Mallomonas caudata* silica scale TEM



stomatocyst SEM



17

silica scale TEM

Mallomonas acaroides (16, 17)

Synura Cl. Synurophyceae, O. Synurales, Fam. Synuraceae (Fig. 8 – 10)

Members of the class Synurophyceae deviate from the Chrysophyceae by their chlorophyll composition, position of the flagella and in the method of scale formation. In the genus *Synura* of about 20 species the cells are united by posterior processes into spherical, free-swimming colonies. Each cell is surrounded by an armour of two to four types of spirally arranged silica scales, which are species specific. As in the Chrysophyceae (for example *Paraphysomonas*), the scales are formed in the interior of the cell, but here in vesicles attached to the chloroplast envelope. The outer chloroplast membrane (the chloroplast ER) bulges into this vesicle, shaping it into a mold for the scale. After completion, the scales are extruded and combined with the scaly armour covering the cell surface.

The flagella are of different length and protrude from an apical flagellar pit. The hairy flagellum moves in sinuous waves, whereas the smooth flagellum remains almost stiff when beating. Near their basis, each of them has a swelling (photoreceptor), but there is no corresponding stigma in the chloroplast. However, a dense *Synura* population shows distinct phototaxis. The two chloroplasts are of brownish-yellow colour.

Reproduction is by longitudinal division, but the new cells remain united by their tails. When a colony has reached a certain size, it may likewise divide into two. Resting stages are smooth stomatocysts that remain in the colony for a period. When a population has reached a certain density, sexuality may occur. Small single cells liberated from a male colony fuse with cells sitting in a female colony. The zygote forms a silica wall as in the vegetative cyst, but it is larger and contains four chloroplasts. It is not known how germination takes place.

Besides some very rare species, the genus includes about six very common ones. Some of these have very specific environmental requirements, for example as regards pH. However, the most common species, *Synura petersenii*, can be considered ubiquitous. It occurs during all seasons in many types of water bodies, and it can be found from tropical to polar regions. During mass development there is a remarkable characteristic smell, which may be described as a mixture of cucumber and pickled herring.

Mallomonas Cl. Synurophyceae, O. Synurales, Fam. Mallomonadaceae (Fig. 11 – 17)

About 130 species plus many varieties and forms.This genus is easily recognized. The cells are solitary and covered by an armour of bilaterally symmetrical scales, each of these often bearing a silica bristle. These silica structures are – as in *Synura* – constructed inside the cell in vesicles adjacent to the chloroplast. They are initially deposited in flat vesicles which then are bent and rolled up so that the scales and bristles obtain their final shapes. Only after extrusion (but this is only known from very few species) do the bristles become attached to the scales, and the scales are subsequently arranged in an armour of spiral or transverse rows on the cell surface.

There are two flagella, but only the long hairy flagellum is visible. The smooth flagellum is very short and hardly projects from the flagellar pit. It is provided with a photoreceptor, but there is no corresponding stigma in the chloroplast.

Asexual reproduction is by longitudinal division, after which the scale armour is

quickly reestablished. In a few cases sexual reproduction has also been observed. Two cells act as gametes and fuse to a zygote, which surrounds itself with a silica wall. The stomatocysts are initially covered with silica scales, but these soon fall off and the cyst sinks to the bottom. At germination, a naked cell escapes which soon forms its scaly armour.

The fine structure of the silica scales serves as the taxonomic markers that are necessary for description and identification of the individual species. *Mallomonas caudata* is one of the most frequent species in slightly humic lakes and ponds in temperate regions. It occurs throughout the whole year and it is easy to recognize – even by light microscopy (best by phase contrast) – by its large size, its almost circular structureless scales and its long, curved and serrated bristles that are glued to the scale surface. In most of the other species, for example in *Mallomonas acaroides,* the scales have a complicated ornamentation and the bristles are attached within a special structure, the dome.

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Marine Planktonic Diatoms

Mona Hoppenrath

INTRODUCTION

Diatoms have their place in the botanical system, as algal class Bacillariophyceae within the division Heterokontophyta. The Heterokontophyta are part of a much larger group of protists, the Chromista. According to the theory of endosymbiosis these are chimaeras with plastids bound originally by four membranes (see p. 1 and p. 3, Fig. 1, 2). There are about 10,000 or 100,000 living diatom species (different estimates for all habitats) within over 250 genera. Approximately 1400 to 1800 species are from marine plankton and their size varies between 5 and 2000 µm.

The coccoid (non-flagellate) diatom cell is characterized by its unique type of silica cell wall, also named frustule (Latin, frustulum, little part). The frustule is formed like a Petri dish consisting of two halves, the epitheca (upper part) and the hypotheca (lower part) (Fig. 1, 2). Each theca consists of two parts, a more or less shallow dish-like part or valve, and a ring-like side wall, the girdle or cingulum. The classic distinction between basically radially symmetrical forms, the centric diatoms, and the bilaterally symmetric ones, the pennate diatoms, has now been shown by molecular studies to be false. For practical reasons the classification in centric and pennate diatoms is still used. Many of the bilateral genera have longitudinal slits (raphe) down the middle of the valve. Most diatoms with this system can move but those that lack the structure like all the radially symmetric species cannot but with one or two exceptions. Some diatoms are heterovalvate, the two valves of their frustule being morphologically dissimilar.

The chloroplasts are brown, because of the accessory pigment fucoxanthin, which masks the chlorophylls *a* and *c*, and they have pyrenoids. Their ultrastructure is typically Heterokontophyte-like, with four chloroplast membranes, lamellae with three thylakoids and a girdle lamella. Centric diatoms generally contain numerous small discoid chloroplasts, whereas pennate diatoms often have two plate-like, parital (located near the frustule wall) chloroplasts. The large nucleus often lies centrally in the cell in a cytoplasm bridge. Mitochondria are of the tubular type. Gametes of centric diatoms possess one pleuronematic (hairy) flagellum. Chrysolaminaran and oil drops are the storage products that sometimes can be seen in the cells of centric and pennate diatoms.

Depending on the aspect from which it is viewed, the cell can have several appearances, valve, girdle and apical view (Fig. 1, 2). Some taxa can form different types of chain-like colonies (Fig. 4 – 10). The linking between cells can be formed by mucilage threads (*Thalassiosira*, Fig. 10), by mucus pads between cell corners (*Thalassionema*, Fig. 7) or by spines (*Stephanopyxis*, Fig. 9).

For vegetative, binary cell division the protoplast swells and divides after mitosis in the valvar plane. The synthesis of one new theca (always a hypotheca) for the

daughter cells starts immediately. This special type of theca formation usually results in one daughter cell somewhat smaller than the mother cell and therefore the average size of cells in a population decreases. This simplified description is not true for all species, but for those that do comply, the size may reach a critical small size, at which a sexual phase may be triggered that results in the formation of auxospores. Because these have a stage with an elastic cell wall, the original maximum size for the species can be restored and normal cell divisions continues. Although usually the result of sexual processes, auxospores can be formed asexually.

Sexual reproduction is different between centric and pennate diatoms. In centric diatoms it is an oogamy. Meiosis of a diploid mother cell (diatoms are diplonts) provides an egg cell or motile gametes with a single flagellum. In pennate diatoms, however, sexual reproduction is an isogamy in which morphologically identical non-motile gametes are produced. After fusion of the protoplasts of compatible gametes in both cases, the young zygote grows into an auxospore. This is surrounded by an elastic wall within which the two valves of the first (initial) cell are formed.

Resting spore formation is a reaction to environmental stress (for example nutrient depletion). Planktonic resting spores fall to the bottom, until restored to the surface during more favourable growing conditions. The spores show a special morphology with thick walls. Examples are *Stephanopyxis turris* (Fig. 38, 39) and *Chaetoceros diadema* (Fig. 16, 17).

Resting cells (physiological resting stages) do not have a special morphology; they look like vegetative cells but perhaps with a little thicker cell wall, but are prepared to resume metabolism as soon as more favourable conditions return as for example in *Odontella aurita* (Fig. 30).

Pennate diatoms like *Pseudo-nitzschia* species can glide over surfaces (Fig. 36). *Bacillaria paxillifer* (Fig. 42) forms special motile colonies in which adjacent cells are linked in such a way that they are able to glide along one another (as *Bacillaria paradoxa*). These colonies expand and contract and this movement also occurs in the water co-lumn, independent of a solid surface.

Diatoms colonize diverse habitats. They occur in marine and freshwater plankton and benthos, on damp rocks, on soil, in and on sea ice. They also grow on various plants (epiphytic), on animals (epizoic) or they are endocytosymbionts (for example in the larger foraminifera, see p. 124). They are a major component of the marine phytoplankton, together with dinoflagellates and some other flagellates such as the Haptophyta. As the main component of the microplankton (20–200 µm) diatoms are a basis for almost all other marine life, which is directly or indirectly dependent on their primary production. They reach the greatest numbers in spring (diatom spring-bloom), and in a second smaller peak in the autumn.

PRACTICAL TECHNIQUES

Collection of Material

Planktonic diatoms can be sampled by taking a bottle sample or better by using a plankton net (20 or 80 µm mesh size) towed slowly through the water for a short time. The bottle sample can be used for quantification. Near-surface net samples are useful for morphological and taxonomic studies, because they provide larger numbers of

specimens. The samples can be transported in white plastic bottles and should not be stored directly in the sun. Back in the laboratory, the bottles should be opened. Such living samples will stay in good condition for a few hours only, depending on the biomass concentration and zooplankton content. To save some living material for the next day, smaller subsamples can be stored in Petri dishes in an illuminated culture cabinet at about 15° C with light, or at a place near a window without direct sunlight. The appropriate temperature depends on the temperature of the sampling site. Samples with high cell densities should be diluted with fine-filtered seawater from the sampling site or with a culture medium such as f/2 (Guillard & Ryther 1962). Subcultures with a small inoculum supplied with medium and stored in culture cabinets with light can be investigated for some days and can provide species that occurred only in low numbers in the natural sample.

Examination and Measurements

The samples and cultures in small Petri dishes can be easily studied under a stereomicroscope or an inverted microscope. Sea water immersion objectives of light microscopes are very useful for observation of Petri dish samples. During microscopic observation, specimens can be easily turned from valve view in girdle view by slightly shaking the Petri dish or by manipulation with a dissecting needle. For more detailed observations with higher magnification, cells should be isolated by micropipetting, placed on a microscope slide and preferably studied with differential interference contrast. Organic threads and delicate setae of chain forming species are best visible with a phase contrast microscope. Gently pressing or tapping the coverslip may cause the diatom cell to turn and reveal the other view of the cell.

To identify diatom genera and species from living material, one should look at cell shape and size first. Both views – valve view and girdle view – are important and the investigator must pay attention to size variability in diatoms in connection with the life cycle. Lengths (apical axis) and widths (transapical axis) are usually given as range that can include the maximum dimensions if auxospore formation has been observed. Usually the width of a valve is maximal in the centre, but there are exceptions, especially in heteropolar diatoms. The cell depth is the distance between the valve faces of a cell and can be measured only in the girdle view. The student must pay attention to colony type and morphological details of the valves like raphe, spines, setae, costae, septa, striae and alveolar pattern. Chloroplast number, position and shape and other distinctive cellular inclusions are also useful for identification. Some species are attached to fine sediment particles and broken frustules of larger diatoms which may be found floating in the water column.

Preparation

It is preferable to examine living samples wherever possible. If not, samples may be fixed with a few drops of neutral Lugol´s iodine (see p. 236), giving the sample a weak brown colour. Adding sufficient fixative will retain the position and shape of the chloroplasts. These samples can be investigated directly under the light microscope or, after washing with distilled water, dehydration through an ethanol series and mounting (with gentle warming) in Pleurax (Cox 1996; www.diatoms.co.uk). Colony

form, valve details and some cellular contents can still be examined. Another preparation method is to clean the frustules of their organic components and to mount them in an embedding medium such as Naphrax (Barber & Haworth 1981; www.biologie-bedarf.de; www.brunelmicroscopes.co.uk). All cytological and colony details are lost but species can be identified using micro-morphological features of the frustules.

For chemical cleaning of diatom frustules (removing all organic material) one can use oxidizing agents such as inorganic acids (for example nitric acid). One cleaning method is as follows: transfer culture material or a mixed sample (not too dense) to a glass beaker, so that the beaker is filled only to one third. Decant as much water as possible without losing too many diatoms. *Use a fume hood and acid-proof gloves for the next steps. Add carefully an equal amount of concentrated nitric acid – the acid will react with the water, generating some heat!* Then bring the sample to the boil (on a hotplate) and prevent it from boiling over. Remove the beaker (not by hand !) from the plate, wait until bubbling stops and bring again to the boil three to five times, depending on the size and coarseness of the specimens in the sample. Leave the sample standing undisturbed for five hours or more. When the suspension has finally settled, carefully remove two thirds to three quarters of the top acidified seawater with a pipette, without bringing the settled frustules into suspension again. Refill the beaker with distilled water, bring the frustules up and allow them to settle again. This process should be repeated at least six times, after which the frustules should be free of acid and salt water. The use of a centrifuge (after the first washing step) will speed up the sedimentation process. A subsample of these cleaned diatom cell walls is then ready to be dried and prepared for the scanning electron microscope or for mounting on permanent slides. The liquid sample can be stored in small glass tubes with airtight caps. Other methods for chemical cleaning of diatom frustules are in common use (Cox 1996, Barber & Haworth 1981, Hasle & Syvertsen 1996). The following species have been selected to show the range of different cell and colony morphologies, which is most likely to be observed and identified in student courses.

GENERA AND SPECIES

The systematics follow Round et al. (1990). For more detailed species descriptions and identification see Hasle & Syvertsen (1996), Throndsen et al. (2003), Horner (2002), Hustedt (1961), Drebes (1974), and Cupp (1943).

Centric Diatoms

Actinoptychus senarius (Syn. *Actinoptychus undulatus*) Fam. Heliopeltaceae (Fig. 11 – 13)

The disc-shaped cells are conspicuous in valve view, looking like the sign "radiation warning". The valve is divided into (usually) six, coarsely areolated sectors, which are alternately depressed and raised and a hexagonal, hyaline centre. One labiate process (an opening/tube through the silica wall with an internal tube or slit) is situated centrally at the periphery of each raised sector. Cells are 20–150 µm in diameter. Initial cells of *A. senarius* are morphologically different and do not show sectors. They

have been identified as species of a different genus, *Debya*, in the past. Changes in the typical morphology occur during life cycle occur which often makes identification difficult or impossible. This species is primarily benthic but may be recorded throughout the year in plankton samples.

Chaetoceros densus Fam. Chaetocerotaceae (Fig. 14)

Chaetoceros densus is a coarse species with straight chains that can be slightly twisted around their axis. Cells are rectangular in girdle view and broad elliptical to circular in valve view and their apical axis is 10–40 µm. The apertures (open spaces between adjacent cells in the chain) are nearly closed, narrowly lanceolate. Cells and setae contain numerous small chloroplasts. Setae cross over at the chain corner and lie almost perpendicular to the chain axis or they are directed slightly towards the chain ends. The setae diverge from the apical plane at equal angles. Terminal setae are not distinctly differentiated. In the North Sea, *Chaetoceros densus* occurs throuthout the year but with a higher abundance in summer.

Chaetoceros diadema Fam. Chaetocerotaceae (Fig. 6, 15 –17)

Chaetoceros diadema is a more delicate species with distinct apertures and chains that are not twisted. Cells are compressed (radial axis short), therefore much broader than long, with an apical axis of 11–60 µm. Cells contain one girdle-positioned chloroplast and the centrally positioned nucleus can be seen easily. The intercalary setae (setae between two cells) fuse near the margin of the chain of cells and are directed perpendicular to the chain axis or bent towards the ends of the chain, regularly occur (not shown in the figures). Terminal setae (setae at the end of the chain) are normally thicker and form a broad U-shaped curve. In the North Sea in winter this species occurs nearly exclusively, but infrequently, as a 2-cell colony (not shown) with thick intercalary and thin terminal setae. It is common in spring. Resting spores with 4 to12 dichotomously branched protrusions on the epivalve and smooth hypovalve (Fig. 16, 17) are conspicuous.

Coscinodiscus wailesii Fam. Coscinodiscaceae (Fig. 18 – 20)

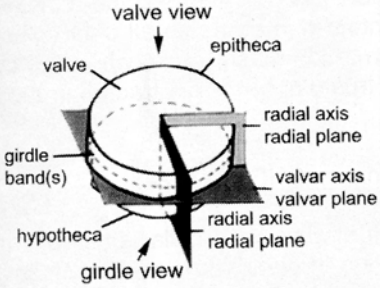
The large species *Coscinodiscus wailesii*, 280–500 µm in diameter, is cylindrical and can be as high as wide. The valves are flat, and in girdle view the cells appear rectangular (focussing through!). Areolation and the distinct central hyaline area can be seen in valve view.

Ditylum brightwellii Fam. Lithodesmiaceae (Fig. 21, 22)

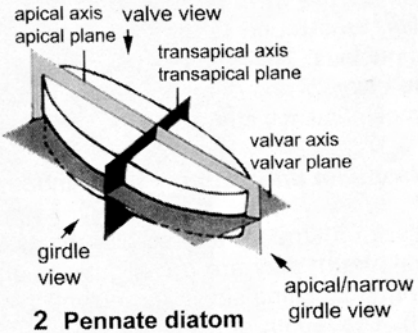
The long, prismatic cells are solitary. They are rectangular in girdle view and triangular in valve view. The length of the valve side is 25–100 µm. A long central, tubular labiate process is easily visible in the light microscope and is a characteristic

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Fig. 1 – 10. Morphology and types of colony of diatoms.

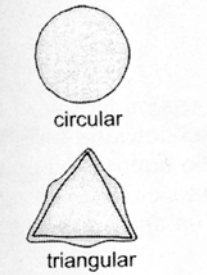


1 Centric diatom

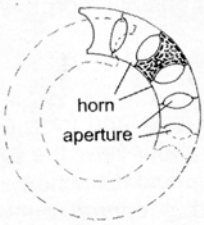


2 Pennate diatom

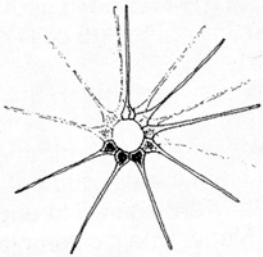
Axes, planes and gross morphology of a diatom frustule



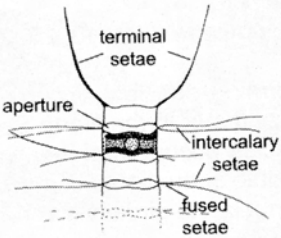
3 Cell / valve shapes



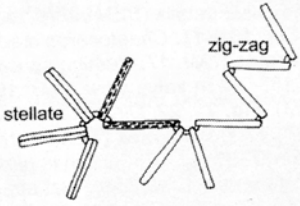
4 *Eucampia zodiacus*
curved to helical chain



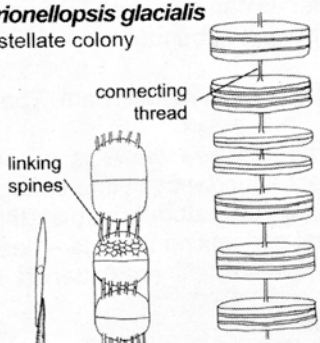
5 *Asterionellopsis glacialis*
stellate colony



6 *Chaetoceros diadema*
straight chain
cells linked by setae of the valve faces



7 *Thalassionema nitzschioides*
zig-zag and/or stellate
cells linked by mucus pads



10 *Thalassiosira rotula* chain
cells linked by
mucilaginous threads

9 *Stephanopyxis turris*
straight chain
cells linked by spines of the
valve faces

8 *Pseudo-nitzschia pungens*
stepped chain
lanceolate cells linked at their ends

Colony types (4 – 10)

feature of this species. This conspicuous thin tube at both sides of the cell arises at the centre of the valve face. A ridge, which consists of a dense spine-chaplet or collar with slits, runs round the margin. Cells contain numerous small chloroplasts and a central nucleus. This species often shows irritation-plasmolysis, when the cytoplasm with the chloroplasts does not fill the whole frustule, but is contracted in the cell centre with cytoplasmic strands to the margin.

Lithodesmium undulatum Fam. Lithodesmiaceae (Fig. 23, 24)

Cells form straight and stable chains with "windows" (collar-shaped transparent marginal ridge); they are rectangular to square in girdle view and triangular in valve view, with undulating sides and elevations at the valve corners (focussing through all planes!). The length of the valve side is 37–93 µm. A central tubular labiate process can be seen through the "windows". Cells contain numerous small chloroplasts. This species should be studied in girdle and valve view, to give an accurate concept of the 3-dimensional shape of the cells and their special morphology.

Eucampia zodiacus Fam. Hemiaulaceae (Fig. 4, 25)

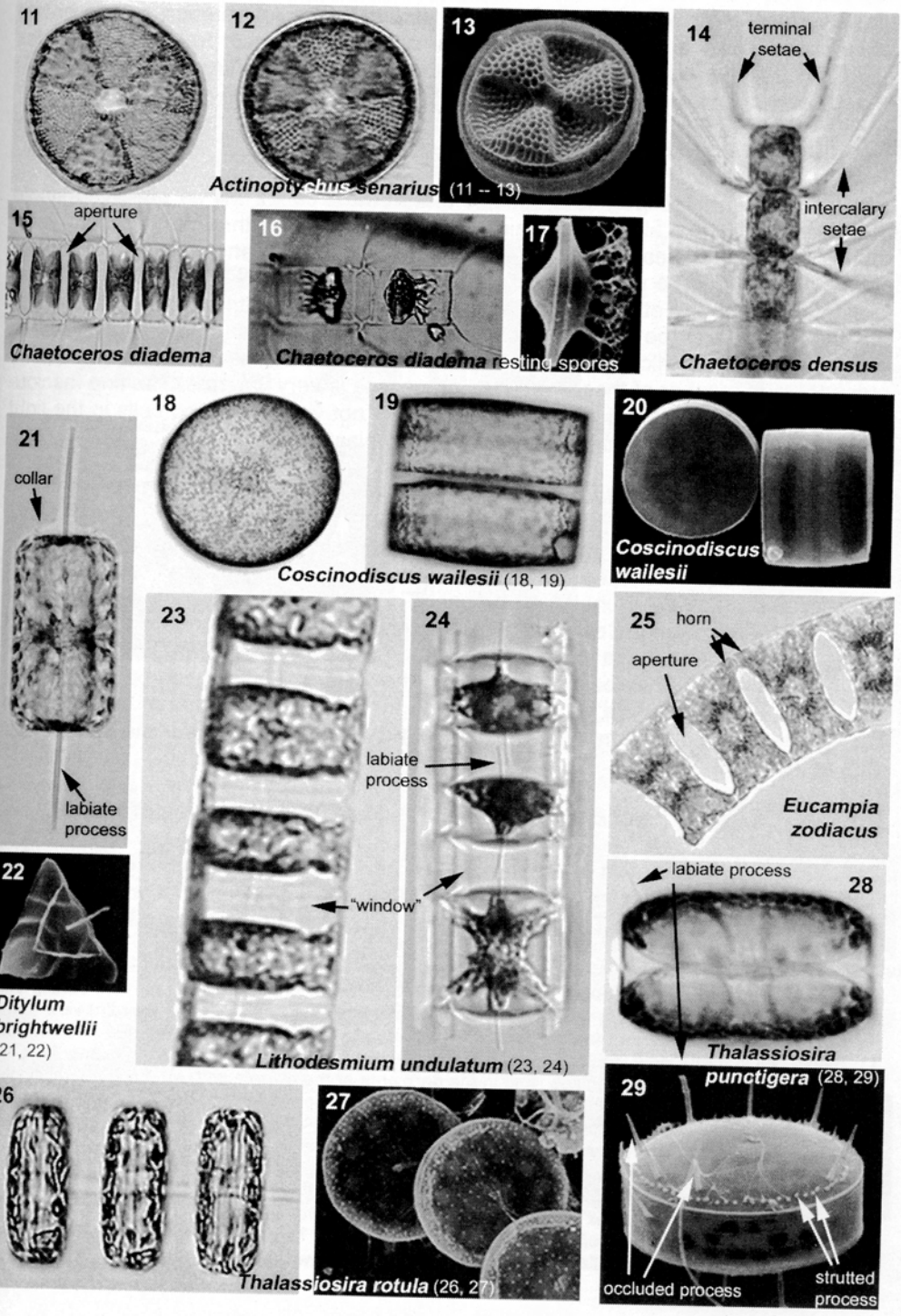
Cells are flat, 8–100 µm in the apical axis, and form curved chains that are helically coiled when long enough. In broad girdle view the apertures between cells are visible and they are square to angular elliptical. Low horns are the connecting points of the cells. Many small chloroplasts and a central nucleus are visible. *Eucampia zodiacus* can be observed from April until October in the North Sea and sometimes it can be quite abundant.

Thalassiosira rotula Fam. Thalassiosiraceae (Fig. 10, 26, 27)

Thalassiosira rotula is a chain-forming species, occurring all year around with highest abundance in late spring and summer in the North Sea. Cells are 8–61 µm in diameter. In natural samples the species occurs in chains with relative short, thick, central connecting threads – easily visible in the light microscope as one thick thread. The discoid cells are flattened, rectangular in girdle view. When focussing onto the

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Fig. 11 – 13. *Actinoptychus senarius* (diameter 20–150 µm). **11:** Slightly angular cell in valve view (LM). **12:** Round cell in valve view (LM). **13:** Valve details (SEM). **Fig. 14.** *Chaetoceros densus* (apical axis 10–40 µm), chain in girdle view (LM). **Fig. 15 – 17.** *Chaetoceros diadema* (apical axis 11–60 µm). **15:** Chain in girdle view (LM). **16:** Resting spores (LM). **17:** Resting spore (SEM). **Fig. 18 – 20.** *Coscinodiscus wailesii* (diameter 280–500 µm). **18:** Cell in valve view (LM). **19:** Dividing cell in girdle view (LM). **20:** Cell in valve and girdle view (SEM). **Fig. 21, 22.** *Ditylum brightwellii* (length of the valve side 25–100 µm). **21:** Cell in girdle view (LM). **22:** Single valve (SEM, valve view). **Fig. 23, 24.** *Lithodesmium undulatum* (length of the valve side 37–93 µm). **23:** Chain in girdle view with cells completely filled with cytoplasma (LM). **24:** Chain in girdle view with two cells just after division and one cell showing plasmolysis (cytoplasm contraction) (LM). **Fig. 25.** *Eucampia zodiacus* (8–100 µm in the apical axis), curved chain in girdle view (LM). **Fig. 26, 27.** *Thalassiosira rotula* (diameter 8–61 µm). **26:** Chain in girdle view with cells connected by mucilaginous threads (LM). **27:** Cells in valve view (SEM). **Fig. 28, 29.** *Thalassiosira punctigera* (diameter 40–186 µm). **28:** Dividing cell in girdle view (LM). **29:** Cell in oblique girdle view showing valve details (SEM).


11
12
13
Actinoptychus senarius (11 -- 13)
14
terminal setae
intercalary setae
Chaetoceros densus
15
aperture
Chaetoceros diadema
16
Chaetoceros diadema resting spores
17
18
19
Coscinodiscus wailesii (18, 19)
20
Coscinodiscus wailesii
21
collar
labiate process
22
Ditylum brightwellii (21, 22)
23
24
labiate process
"window"
Lithodesmium undulatum (23, 24)
25
horn
aperture
Eucampia zodiacus
labiate process
28
Thalassiosira punctigera (28, 29)
26
27
Thalassiosira rotula (26, 27)
29
occluded process
strutted process

girdle surface, the uneven thickness of the girdle bands can be seen as one or two white lines. Cleaned valves show a cluster of central strutted processes (complex open process penetrating the silica wall through which an organic thread is extruded). Cells contain numerous small chloroplasts.

Thalassiosira punctigera Fam. Thalassiosiraceae (Fig. 28, 29)

Thalassiosira punctigera is a species that in most cases will be observed single celled but which can also form chains with thin connecting threads. It occurs year-round in the North Sea. Cells are 40–186 µm in diameter and have convex, disc-shaped valves in valve view. A good feature for identification is one very long, tubular labiate process, lying at the valve margin and easily visible with the light microscope. In addition there can be a ring of occluded marginal processes (hollow external tubes not penetrating the silica wall) of nearly the same size as the labiate process. The presence and number of these occluded processes is very variable. The fine fasciculated areolation and ribbed margin of the valve is not visible in living cells in the light microscope. Cells contain numerous small chloroplasts.

Odontella aurita (Syn. *Biddulphia aurita*) Fam. Triceratiaceae (Fig. 30, 31)

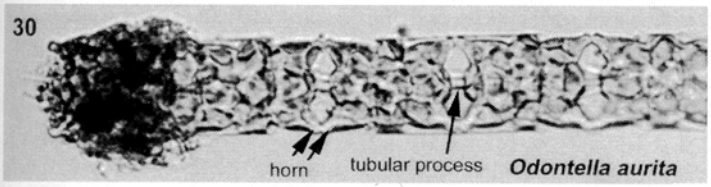
Cells are connected in long, straight or zig-zag chains. The apical axis is 10–97 µm. The valve face between the horns is strong and evenly inflated, with two centrally located spine-like, tubular processes. The horns are thick, obtuse, inflated at the base and diverging. Cells contain numerous small chloroplasts lying at the cell periphery. *Odontella aurita* is a tychopelagic species, meaning that it is primarily a benthic species that may be brought up into the water column. It can be found regularly in the plankton in late winter and spring from February until April (especially in the Wadden Sea areas where it may bloom). For the rest of the year the species occurs at the seafloor.

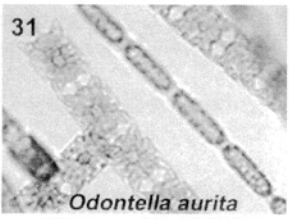
Odontella sinensis (Syn. *Biddulphia sinensis*) Fam. Triceratiaceae (Fig 32, 33)

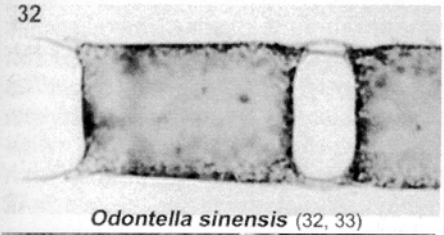
Cells occur singly or in short straight chains. This conspicuous large species is pillow-shaped with an apical axis of 80–440 µm. The valve face between the processes is flat or concave.The long, spine-like, tubular processes are located close

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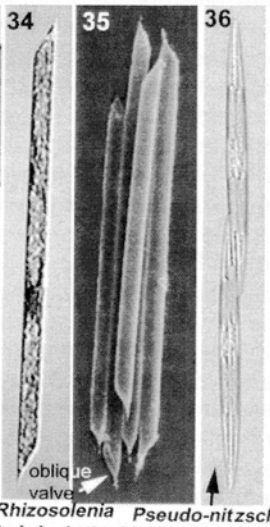
Fig. 30, 31. *Odontella aurita* (apical axis 10–97 µm). **30:** A new chain growing out of a resting cell surrounded by fine detritus (LM). **31:** Chains of a bloom in girdle and apical (narrow girdle) view (LM). **Fig. 32, 33.** *Odontella sinensis* (apical axis 80–440 µm). **32:** First cell in a chain in girdle view (LM). **33:** Single cell in girdle view (SEM). **Fig. 34, 35.** *Rhizosolenia imbricata* (diameter 3–57 µm). **34:** Single cell in girdle view with the nucleus lying in the dark area of the cytoplasm (LM). **35:** Several single cells in girdle view (SEM). **Fig. 36.** *Pseudo-nitzschia pungens* (apical axis 74–142 µm, pervalvar axis up to 8 µm), two-cell-chain with cells in valve view (LM). **Fig. 37 – 39.** *Stephanopyxis turris* (diameter 10–115 µm). **37:** Chain in girdle view with two cell pairs just after cell division and separation (LM). **38:** Two resting spores in girdle view (LM). **39:** Resting spore in girdle view (SEM). **Fig. 40, 41.** *Asterionellopsis glacialis* (apical axis 30–150 µm). **40:** Stellate, helical chain (LM) in side view. **41:** Stellate chain in top view, looking "into the helix" (LM). **Fig. 42.** *Bacillaria paxillifer* (apical axis of a cell 70–150 µm), band-like colony with "mobile" cells in girdle view (LM). **Fig. 43.** *Thalassionema nitzschioides* (apical axis of the cells 10–110 µm), stellate colony with cells in girdle view (LM).

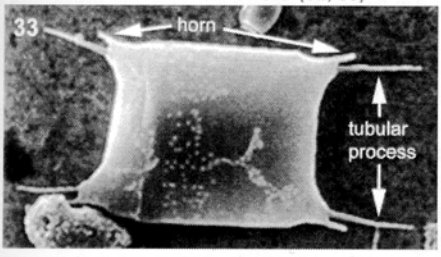


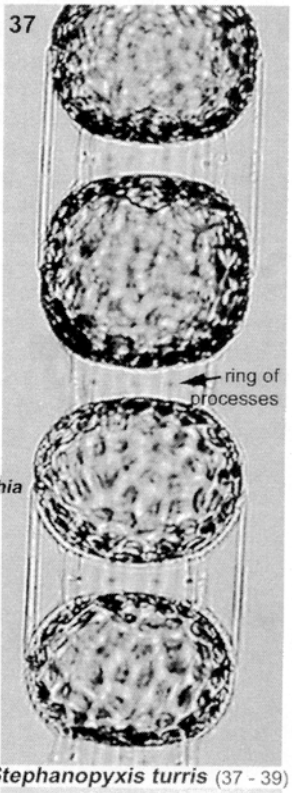


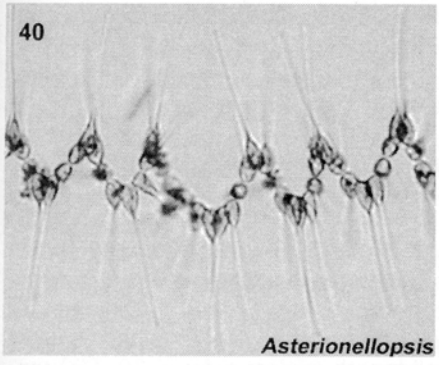


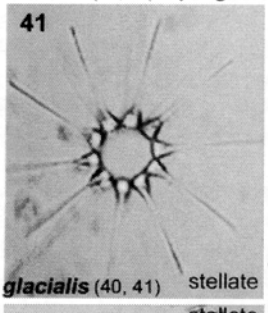
Odontella sinensis (32, 33)

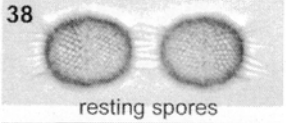


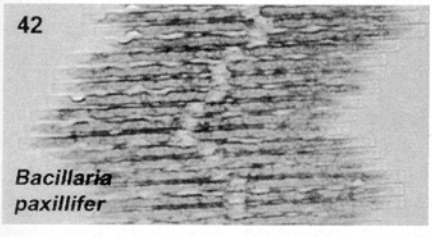


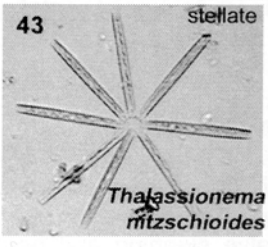


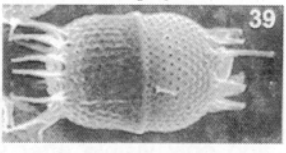












to the slender horns, which diverge only slightly. Cells contain numerous small chloroplasts lying at the cell periphery. The nucleus is located centrally in a pocket of cytoplasm. This species can be found in the North Sea all year with a greater abundance in summer.

Rhizosolenia imbricata (Syn. *Rhizosolenia shrubsolei*) Fam. Rhizosoleniaceae (Fig. 34, 35)

Rhizosolenia imbricata has obliquely conical valves. The basally swollen, short process has a narrow distal tube and a small otarium (one of a pair of membranous wings that occur at the base of the external process) extending along the swollen part. The cells are rod-shaped and are solitary or closely connected in chains. The diameter is 3–57 µm. The girdle segments occur in two lateral columns. Cells contain many small chloroplasts in their peripheral cytoplasm and at high magnification, cytoplasm movement can be observed (chloroplasts drifting on "streets" next to each other in opposite directions). The nucleus lies in a cytoplasmic bridge in the middle of the cell. This species occurs from spring to autumn and can be very abundant in early summer in the German Bight.

Stephanopyxis turris Fam. Stephanopyxidaceae (Fig. 9, 37 – 39)

The cells, 10–115 µm in diameter, are connected in straight chains by their long external processes. These processes are positioned in a ring on the valve face. The valve is curved and the girdle is high. Large hexagonal areolae can be seen on the valve surface and these areolae are of the same size on the entire valve. *Stephanopyxis turris* forms characteristic resting spores (Fig. 38, 39). The species occurs sporadically from late spring to early winter in the German Bight.

Pennate Diatoms

Asterionellopsis glacialis (Syn. *Asterionella glacialis, Asterionella japonica*) Fam. Fragilariaceae (Fig. 5, 40, 41)

The asymmetrical, heteropolar cells (cells with dissimilar ends) are connected at their expanded basal poles, forming helical chains that can have a star-like appearance. The expanded basal cell pole contains the chloroplast(s) and is nearly triangular in girdle view, which is the most likely view when observing the chains. The rest of the cell is long and tapers to a point. Total cell length (apical axis) is 30–150 µm. The species occurs all year in the German Bight but with greater abundance in spring.

Bacillaria paxillifer (Syn. *Bacillaria paradoxa*) Fam. Bacillariaceae (Fig. 42)

This species is a raphid pennate diatom and has a central raphe (one or two longitudinal slits through the silica wall). However, the raphe is not a striking structure and only visible after valve cleaning and permanent mounting. *Bacillaria paxillifer* forms band-like colonies and is easy to identify when live by its unique type of

motility. Adjacent cells are linked at their valve faces so that they are able to slide along one another – the colonies expand and contract. Cells are rectangular in girdle view, which is the cell view when observing the colonies, with an apical axis of 70–150 µm. They contain two long, plate like chloroplasts. The species occurs sporadically in plankton samples, especially after storm conditions.

Pseudo-nitzschia pungens Fam. Bacillariaceae (Fig. 8, 36)

Pseudo-nitzschia pungens cells are linear to fusiform with pointed ends. Cell overlap in chains is one third or more of the cell length. The apical axis is about 74 to 142 µm and the pervalvar axis (the axis between the valve faces) can be up to 8 µm. Striae (rows of pores) on the valves are visible under the light microscope. The species can be observed especially in spring (when it can bloom, although the timing differs from year to year) but occurs from April until November in the North Sea.

Thalassionema nitzschioides (Syn. *Synedra nitzschioides, Thalassiothrix nitzschioides)* Fam. Thalassionemataceae (Fig. 7, 43)

The more or less long, narrow, rectangular (girdle view) cells form stellate or zigzag colonies (frustules are joined at their bases). The total cell length (apical axis) is 10–110 µm. Cells are lanceolate in valve view with a marginal row of areolae that appears as a rib-like structure in the light microscope (not easy to observe). Numerous small chloroplasts.

GLOSSARY

areola = the regularly repeated perforation through the basal siliceous layer normally occluded by a velum, a thin perforate layer of silica.

labiate process = a tube (Fig. 28) or opening through the valve wall with an internal flattened tube or longitudinal slit often surrounded by two lips (internal structure only visible with the SEM); widespread in diatom taxa.

occluded process = hollow external tube occluded at one end, not penetrating the silica wall.

seta = hollow outgrowth of the valve (Fig. 6) projecting outside the valve margin with a different structure from the valve (*Chaetoceros*).

strutted process = a tube through the valve (Fig. 29) surrounded by 2–5 chambers or satellite pores (only visible with the SEM), through which a thread of organic material is extruded; found in the Thalassiosiraceae.

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Drebes G. (1969 a): Asexual reproduction of the diatom *Stephanopyxis turris* (Centrales). – Film C 982 IWF Göttingen, 7½ min.
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More details and additional films can be found at the IWF Wissen und Medien GmbH homepage www.iwf.de.

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Marine Dinoflagellates

Malte Elbrächter and Rudolf Röttger

INTRODUCTION

In the kingdom Protozoa dinoflagellates are included as Dinoflagellata. Photosynthetic dinoflagellates, which contain chloroplasts with a triple membrane, arose through the phagocytic uptake of a unicellular eukaryotic alga by a heterotrophic protozoan host (theory of endosymbiosis, see p. 1 and p. 3, Fig. 1, 2). On the basis of this evolutionary event, dinoflagellates are also called protozoan algae, namely protozoa with algal features. Thus, in the botanical system, dinoflagellates are classified as algal class Dinophyceae. Within the Protozoa the dinoflagellates (like ciliates and apicomplexans) belong to the Alveolata on account of their special cell cortex (termed amphiesma) containing alveolae (amphiesmal vesicles) that may or may not encompass cellulosic thecal plates (Fig. 1). About half of the 2000 extant species are non-photosynthetic and probably derive from photosynthetic ones by secondary loss of chloroplasts.

Some dinoflagellates include in their life cycles an encysted stage which is an integral part of the sexual reproductive cycle. In marine environments cysts are not a means of surviving harsh conditions. The cyst is formed inside the cellulosic outer covering, the theca. Dinoflagellate cysts, or dinocysts, which may be composed of an extremely resistant and complex organic wall, are abundant fossils in Mesozoic and Cenozoic (Tertiary, Quaternary) strata. Many of the microfossils now called acritarchs may actually be dinoflagellate cysts.

MORPHOLOGY

In most dinoflagellates the cell is divided into the anterior episome and the posterior hyposome separated by an equatorial groove called the girdle or cingulum (Fig. 1, 10). The characteristic helical movement of dinoflagellates results from two flagella. In a typical dinoflagellate a ribbonlike flagellum, the transverse one (Fig. 1, 2, 10), winds around the cell, invariably to the cell´s left, causing it to turn as well as providing forward thrust. The second flagellum, the longitudinal one, beats posteriorly and both provides some forward propulsion and steers the cell. The two flagella arise from the side designated as ventral. The transverse flagellum lies in the girdle, the longitudinal one in the sulcus, a second depression roughly perpendicular to the girdle and containing the flagellar pores through which the flagella emerge.The sulcus divides the cell into left and right halves (Fig. 10, 24, 25). Many dinoflagellates have a theca constructed of thin cellulose plates that fit closely together along linear sutures (Fig. 3). The patterns formed by the plates (tabulation) are of critical importance in taxonomy. The wall of the episome is called the epitheca, that of the hyposome the

hypotheca. The amphiesma (Fig. 1) is composed of the cell membrane and a single layer of vesicles, followed by microtubuli. Within these amphiesmal (cortical, thecal) polygonal flat vesicles the plates are formed (thecate species); in athecate species the vesicles are devoid of plates. The entire structural complex, regardless of the presence or absence of plates, is called the amphiesma.

The dinoflagellate nucleus, the dinokaryon, has some special features including unusual high amounts of DNA per cell. In the dinokaryon the chromosomes remain continously condensed and visible both with light and electron microscopy during interphase and mitosis. The DNA is not associated with histones, and, consequently, nucleosomes are lacking. In those species investigated, there is an unusual substitution (12–68%) of the base thymine by 5-hydroxymethyluracil.

Typical dinoflagellate chloroplasts are characterizid by triple-membraned envelopes, similar to those of euglenids. Within the chloroplasts the thylakoids are united in stacks (lamellae) of three (sometimes two or four). Girdle lamellae are generally absent; they are characteristic of the division Heterokontophyta (chrysophytes, diatoms, see p. 5, 14) and are peripheral stacks of thylakoids which lie just beneath the chloroplast envelope. The most important chlorophyll is chlorophyll *a* with chlorophyll c_2 also present. The chloroplasts are usually brown, since the green chloroplasts are masked by yellow and brown accessory pigments (β-carotene and several xanthophylls), of which the most important is peridinin.

Several dinoflagellates have been found to be the hosts of algal endosymbionts. The brackish water species *Durinskia baltica* (*Peridinium balticum*) and a few other species harbour members of the Heterokontophyta coloured brown by fucoxanthin, their principal accessory pigment. These dinoflagellates possess two different nuclei, a dinokaryon and the normal Heterokontophyte nucleus. Another dinoflagellate harbours a vestigial alga containing chlorophyll *a* and *b* within its chloroplasts which are bounded by two membranes as in the Chlorophyta.

Dinoflagellate cells usually contain pusules (Fig. 1), complex systems of vesicles and tubes of unknown function, that are invaginations of the outer cell membrane. It opens to the exterior near the bases of the flagella. The pusule is particularly well seen as a hyaline vacuole in *Protoperidinium* (Fig. 39, 43, 45).

Rod-shaped extrusomes (trichocysts) lie in the amphiesma perpendicular to the cell membrane each one beneath a hole in the theca. During extrusion, the paracristalline, proteinaceous rod elongates eight times or more. Another and much more elaborate type of extrusome is found in *Polykrikos, Nematodinium* and *Proterythropsis.* These nematocysts (Fig. 15) are named for their resemblance to the stinging cells of coelenterates, which are also called nematocysts (or cnidocysts).

FEEDING

The usual method of feeding in heterotrophic dinoflagellates is a special kind of phagotrophy called myzocytosis. This is performed in many cases by means of a tube-shaped extensible pseudopod for capture and ingestion of prey, the so-called peduncle (Fig. 6). Pallium feeding is another feeding method (Fig. 7, 8). A sheet-like pseudopodium, the pallium, is deployed from a sulcal pore. It engulfs the prey, which is often much larger than the dinoflagellate itself. The prey is digested within a food vacuole outside the predator (for feeding of *Noctiluca scintillans* see p. 40).

ECOLOGY

Dinoflagellates are considered second only to diatoms in their importance as primary producers among the plankton. The greatest concentrations occur in temperate coastal waters. Many dinoflagellates like *Ceratium, Gonyaulax polyedra, Prorocentrum* and *Protoperidinium* perform daily vertical migrations of several metres. At night the cells remain in the deeper nutrient-rich waters, and during the day they make use of the light for photosynthesis.This migration follows an endogenous circadian rhythm (circadian meaning about a 24 hour periodicity) between negative and positive geotaxis, the natural alternation between day and night playing only a subsidiary, modifying role.

Photoautotrophic members of the genera *Gymnodinium, Gonyaulax, Ceratium, Dinophysis* and *Prorocentrum* are the cause of red tides, namely accumulations of the carotinoid pigments by high specimen densities of these dinoflagellates. Red tides are primarily a phenomenon of warm water areas, but they also occur in the temperate zone in late spring or early summer. Several of the coastal bloom formers kill fish and lamellibranchs or cause lamellibranchs or fish to be lethally toxic to humans.

Many marine invertebrates (most reef-building corals, some medusae and sea anemones, the large tropical lamellibranch *Tridacna*) and protozoa (foraminifera, radiolaria) harbour non-flagellate (coccoid) dinoflagellates, called zooxanthellae, within their tissues and cells. These hosts are transformed from being purely heterotrophic into partly phototrophic (mixotrophic) organisms. The photosynthesis enhances the calcification of the reef corals.

PRACTICAL TECHNIQUES

Obtaining Specimens

Planktonic dinoflagellates are collected using 20, 50, 75 or 100 µm pore-size nets from a pier or from a gently drifting boat. Empty the contents into glass bottles to be opened in the laboratory. Specimens will stay alive for several hours if room temperature does not differ too much from the temperature of the sampling site. Do not use plastic bottles in which some species will die. Check the plankton sample in a Petri dish with a stereomicroscope to learn about species composition and abundance. Isolate specimens with a pipette on a normal or a depression slide for observation with a light microscope. If the original sample is undisturbed for a while most specimens will concentrate on the bottom of the vessel, while some will gather at the water surface where they can be easily collected for fixation. A well-tried fixative is neutral Lugol´s iodine (see p. 236). Fixed samples have to be refixed regularly because the brownish colour bleaches. Lugol´s iodine is particularly good for the fixation of flagella.

Some species can be ordered from culture collections and cultivated in the laboratory over longer periods of time. The institutions send species lists upon request.

Sammlung von Algenkulturen, Pflanzenphysiologisches Institut der Universität, Nikolausberger Weg 18, 37073 GÖTTINGEN, GERMANY.

Culture Collection of Algae and Protozoa, Freshwater Biological Association, The Ferry House, ABLESIDE, Cumbria, LA22OLP, UK.

CCMP, Bigelow Laboratory for Ocean Sciences, WEST BOOTHBAY HARBOR, ME 04575, USA.

Fixed material from the plankton of Helgoland is provided by Alfred Wegener-Institut für Polar- und Meeresforschung, 27498 HELGOLAND, GERMANY. These samples mainly contain *Ceratium* and *Protoperidinium* species.

Preparation for Light Microscopy and Staining Methods

Visualisation of the Nucleus

A quick and easy way of seeing the nucleus is by using iron-carmine-acetic acid (ICA). Method: 1–2 g ground carmine are gently heated for about 30 min with 100 ml 45% aqueous acetic acid in a 500 ml Erlenmeyer flask. Use a reflux condensor! When the mixture has been cooled and filtered a few drops of a solution of iron hydroxide in 50% acetic acid are added. The solution can be used immediately or can be stored for about a month in ground-glass stoppered flasks. Pipette the fixed samples onto microscope slides, add a drop of ICA, cover with a coverslip and heat gently to about 40° C on a hot plate or with a Bunsen burner. The nuclei become coloured rose to red. The sample becomes overcoloured after about one hour. This is a quick check for the size and location of the nucleus and also of nuclear division.

Thecal Plates

Locate a cell under the microscope and then add a drop of household bleach (sodium hypochlorite solution, NaOCl) diluted 1:1 with water to the margin of the coverslip. Watch the cell continously. At first the bleach makes the cell swell and then destroys the protoplast, finally making the thecal plates separate from one another. The process is speeded up by gentle pressure on the coverslip.

Sutures and Thecal Plates

Onto a coverslip preparation of a living sample put 1 drop of a 0.1% solution of Fluorescent Brightener (sigma F 3543, www.sigmaaldrich.com) and observe the sutures for a minute or two under UV light. The fluorescence is bleached during this time but the slide can be restained. If a sample fixed with Lugol´s iodine (see p. 236) is used it will take some hours for it to bleach after which you should refix with formalin (final concentration 3–4%), then add the Fluorescent Brightener and again examine under UV light. The pattern of thecal plates can now be observed even if the cells still retain their contents. UV light is potentially dangerous, so for safety´s sake read the operating instructions of the microscope manufacturer.

Nuclear Staining with DAPI

Using either a living or a fixed preparation place a drop of a 0.01% solution of DAPI (4, 6-diamidino-2-phenylindole) under the coverslip and allow it to react for about 10 min. The DNA of the nucleus (and of the chloroplasts and of engulfed bacteria) will fluoresce intensively blue-white under excitation with UV light. Use the same filter combination as with the Fluorescent Brightener.

GENERA AND SPECIES

Prorocentrum micans O. Prorocentrales (Fig. 11, 12). Almond-shaped cell of two opposing valves, one designated left and the other right. Without girdle groove and sulcus; 36–52 µm without spine. Desmokont flagellar insertion (Fig. 9). Anterior periflagellar area of several small plates one of which has a conspicuous spine. Chloroplasts present. Neritic, estuarine and oceanic. Worldwide in cold temperate to tropical waters, North Sea and western Baltic Sea. Culture collections.

Prorocentrum minimum O. Prorocentrales (Fig.13, 14). Conspicuously smaller than *Prorocentrum micans*, about 20 µm. Oval to triangular in valve view, flattened as *Prorocentum micans.* Short apical spine sometimes observable. Valves covered with short, broad-based spines that can appear as rounded papillae. Chloroplasts present. May form toxic blooms with densities of several million cells per litre. Worldwide in cold temperate to tropical and also brackish waters (western Baltic Sea, to which it immigrated in the 1980´s).

Dinophysis O. Dinophysiales. *Dinophysis norvegica, Dinophysis acuta, Dinophysis acuminata* (Fig. 16 – 22). Laterally flattened, almond-shaped cells with a dinokont flagellar orientation.The girdle has shifted to the anterior cell pole. Girdle and sulcus are bordered by lists (lists are flanges arising from the margin of plates and spines; they also occur in *Protoperidinium* species). *Dinophysis norvegica, D. acuta* and *D. acuminata*, unlike other dinoflagellates, have a reddish colour caused by phycobilins that only otherwise occur in cyanobacteria, cryptophytes and rhodophytes. Their chloroplasts light up in the epifluorescence microscope not deep red but bright orange. Their chloroplasts are surrounded by only two membranes as in the chloroplast envelopes of green and red algae. If these chloroplasts have been derived from endosymbiotic chryptophytes, these must, in the course of evolution have lost the plasmalemma and all organelles. *Dinophysis acuta* can be easily confused with *D. norvegica*. The distinction between the two species can be made by the larger width of the valve in valve view which in *D. norvegica* is about midway between the lower cingular list and the antapex; in *D. acuta* it is about two-thirds of this distance, that is nearer to the antapex. *Dinophysis norvegica* is a cold water species, *D. acuta* occurs worldwide in cold temperate waters while *D. acuminata* occurs worldwide in both cold and warm temperate waters.

Akashiwo sanguinea (Syn. *Gymnodinium sanguineum*) O. Gymnodiniales (Fig. 23). This phototrophic species occurs in coastal waters of the North Sea where it sometimes causes red tides. It can easily be cultivated at temperatures of 15–18° C. It is well suited for the study of cell anatomy and movement. Watch the helical movement in a Petri dish with a stereomicroscope and continue to study the anatomy (magnification x 200 and x 400 on a slide with coverslip): apical, antapical, ventral, dorsal; location of girdle and sulcus and insertion of the flagella. The longitudinal groove (sulcus) divides the hyposome, thus providing the typical bilobed cell shape.The dinokaryon with its chromosomes can be located in the middle of the cell as this area is devoid of chloroplasts. In order to fix the flagella, put a drop of Lugol´s iodine on the margin of the coverslip of a live preparation.Transverse flagella will then detach from

the girdle groove in some specimens, allowing their typical helical structure to be seen. Nuclear staining can be performed with iron carmine acetic acid (ICA) or DAPI.

Polykrikos kofoidii O. Gymnodiniales (Fig. 15). Athecate, pseudocolonial. The external morphology resembles that of a chain of 2, 4 or 8 specimens, each unit (zooid) possessing paired flagella and a girdle. The sulci form a continuous longitudinal furrow. The zooids are fused internally, and the number of nuclei is half the number of zooids. Individuals with 8 zooids are 100–150 µm long. Divides transversely at the centre. No chloroplasts, feeds on other flagellates as well as on diatoms. Several or many nematocysts. Worldwide in temperate to tropical waters. In *Polykrikos schwartzii* the girdles are almost equatorial. It lacks the longitudinal ridges of the hypocones of *P. kofoidii* which are, however, not always visible and not shown in Fig. 15.

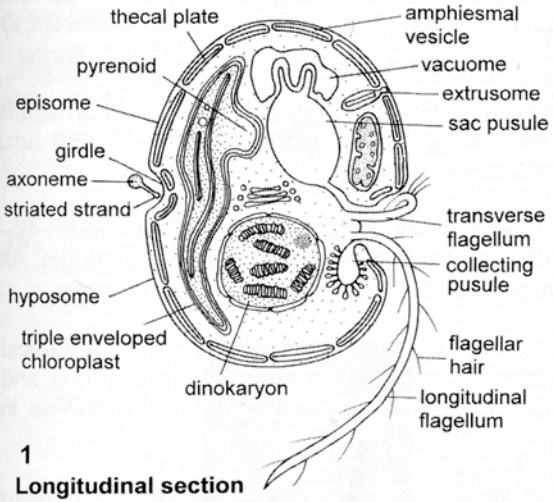
Ceratium O. Gonyaulacales

Thecate; dinokont cell type (Fig. 10). Body with mostly three open or closed hollow horns (Fig. 29). Central body dorsoventrally compressed. The ventral side has a depressed area referred to as the ventral area. Many yellow-brown chloroplasts present; cells sometimes with food vacuoles due to phagocytic activity. The identification of selected species is possible with Fig. 28 – 38.

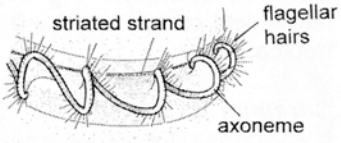
Ceratium fusus (Fig. 28). Apical horn and left antapical horn greatly extended, right antapical horn reduced to a little tooth-like process. Cell length 300–600 µm. Worldwide in cold temperate to tropical waters.

Ceratium tripos (Fig. 29, 30). Conspicuous strongly dorsoventrally compressed body. Antapical horns pointed and closed, the right one somewhat shorter and weaker. Girdle width 60–90 µm. Worldwide in cold temperate to tropical waters.

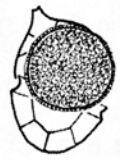
Fig. 1. Morphological features of a dinoflagellate. Within the chloroplast each lamella is composed of three thylacoids. **Fig. 2.** Detail from a transverse flagellum. Diameter of flagellum 0.28 µm. **Fig. 3.** The thecal pattern (tabulation) for a peridinoid or gonyaulacoid taxon. Members of the order Gonyaulacales are also placed in the Peridiniales. The numbering of plates, a tool of taxonomy, is not given in this book. **Fig. 4, 5.** Resting cyst of *Scrippsiella trochoidea* (20 µm). **4:** Young resting cyst filled with chromatophores escaping from its mother cell. **5:** Completed resting cyst covered by calcite processes. **Fig. 6.** Myzocytosis, a kind of phagocytosis. The dinoflagellate (*Paulsenella chaetoceratis*, 30 µm) sucking out the contents of a prey organism by means of a sucking tube, the peduncle. **Fig. 7, 8.** Pallium feeding. **7:** A dinoflagellate (*Protoperidinium conicum*, 70–85 µm) enclosing its prey by a pouch-like pseudopodium, the pallium. The digestion occurs within an extrasomal food vacuole. **8:** The dinoflagellate *Protoperidinium spinulosum* (cell length 90 µm) feeding on a chain-like colony of the centric diatom *Chaetoceros curvatus*. **Fig. 9.** Flagellar arrangement of the desmokont cell type.The theca is prorocentroid: it consists of two large plates, a right and a left valve. **Fig. 10.** Flagellar arrangement of the dinokont cell type, ventral side. The theca is peridinoid: it consists of five latitudinal series of plates (see Fig. 3). **Fig. 11, 12.** *Prorocentrum micans*, valve views. **11:** Schematic line drawing (length 50 µm without spine). **12:** SEM photograph (42 µm). **Fig. 13, 14.** *Prorocentrum minimum* (valve views). **13:** Schematic line drawing. **14:** *Prorocentum minimum* var. *triangulatum*, SEM photograph (length 18 µm). **Fig. 15.** *Polykrikos kofoidii*, schematic line drawing (length 70 µm). Pseudocolonial binucleate cell of four zooids, each zooid with its own girdle but all sharing a common longitudinal sulcus.



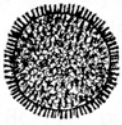
1
Longitudinal section through a generalized dinoflagellate



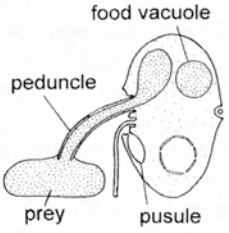
2
Detail from a transverse flagellum



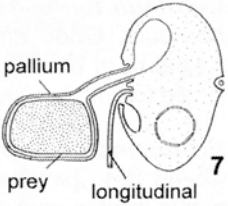
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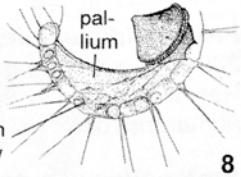
5
Resting cyst
(4, 5)



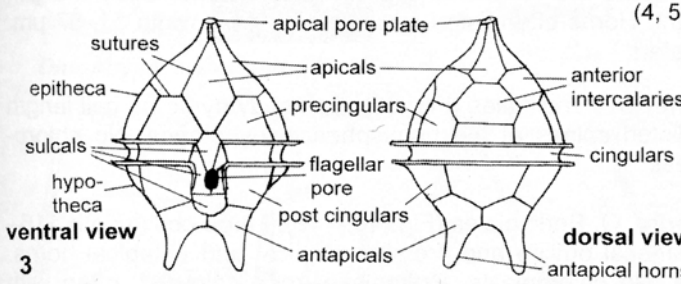
6 Myzocytosis



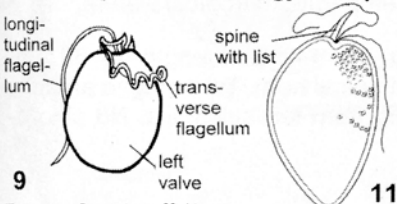
7



8
Pallium feeding (7, 8)

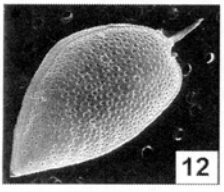


3
Thecal plate terminology for a peridinoid dinoflagellate

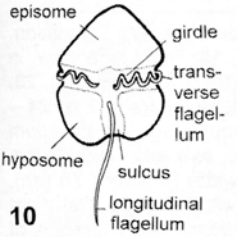


9
Desmokont cell type

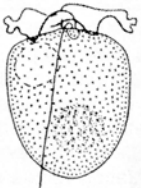
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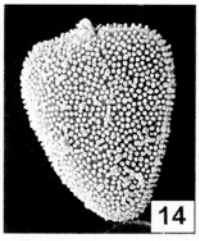
12
Prorocentrum micans (11, 12)



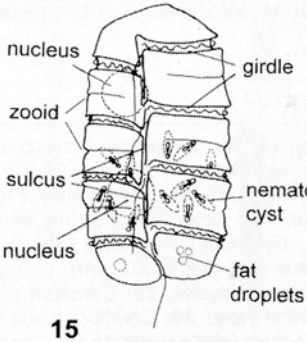
10
Dinokont cell type



13



14
Prorocentrum minimum (13, 14)



15
Polykrikos kofoidii

Ceratium lineatum (Fig. 31). Apical horn formed from triangular epitheca. Antapical horns with closed ends, left horn longer. Cell length 70–180 µm. Cold temperate to tropical waters.

Ceratium furca (Fig. 34). Parallel or slightly divergent serrated antapical horns, left horn longer. Epitheca gradually tapers into apical horn. Cell length 210–380 µm. Worldwide in cold temperate to tropical waters.

Ceratium horridum (Fig. 32, 33). Cell body small, with long horns. Girdle width 42–57 µm. Proximal ends of antapical horns level with straight posterior body margin. All horns open. Surface with linear markings. Worldwide in cold to temperate waters.

Ceratium macroceros (Fig. 35, 36). Extremely long open ended horns. Antapical serrated horns extend beyond straight posterior body margin before bending and continuing almost parallel to the apical horn. Girdle width 45–57 µm. Worldwide in cold temperate to tropical waters.

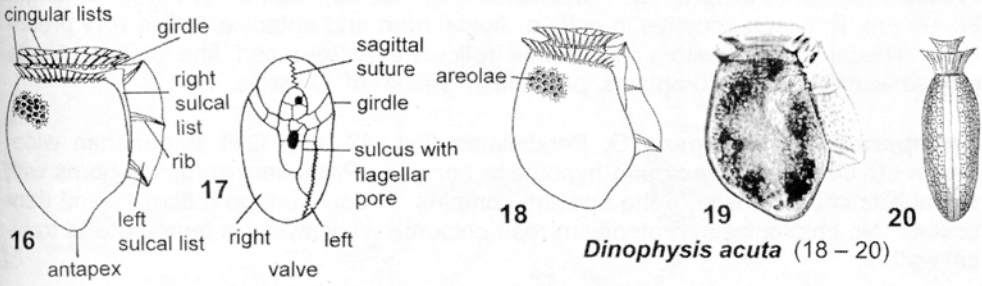
Ceratium longipes (Fig. 37, 38). This variable species is very similar to *Ceratium horridum*. Open antapical horns are level with the straight posterior body margin. Apical horn bent to the right. Horns often listed or serrated. Girdle width 51–57 µm. Arctic to cold temperate waters.

Protoperidinium pyriforme O. Peridiniales (Fig. 39). Cell body pyriform, cell length 42–86 µm. Two broadly listed spines at the hemispherical hypotheca. No chloroplasts, protoplasm colourless.

Protoperidinium depressum O. Peridiniales (Fig. 40 – 42). Large cell (length 116–200 µm) with concave epithecal outline and prominent apical and antapical horns. Reticulated thecal surface. No chloroplasts. Protoplasm rose coloured, often with numerous rose coloured oil droplets. Worldwide in temperate to tropical waters.

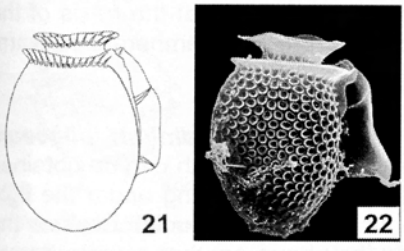
Protoperidinium pellucidum O. Peridiniales (Fig. 43, 44). Cell length 40–68 µm. Circular in cross section. Broadly pyriform with short apical horn. Two winged antapical spines and one curved winged spine originating from left sulcal list. No chloroplasts. Worldwide in temperate to tropical waters.

Fig. 16, 17. *Dinophysis norvegica*, cell organization (56–70 µm). **16:** Right side view. **17:** Tabulation, top ventral view. **Fig. 18 – 20.** *Dinophysis acuta*. **18:** Right side view (80 µm). **19:** Right side view, in vivo (84 µm). **20:** Dorsal view. **Fig. 21, 22.** *Dinophysis acuminata* (40 µm), right side views. **Fig. 23.** *Akashiwo sanguinea* (60 µm), ventral view. The cell is strongly compressed dorsoventrally. **Fig. 24 – 27.** *Gymnodinium gracile* (about 140 µm), ventral views, variation in cell shape. **Fig. 28.** *Ceratium fusus* (cell length 370 µm), ventral view, in vivo. **Fig. 29, 30.** *Ceratium tripos*, two examples of this variable species. **29:** *Ceratium tripos* var. *balticum*, cell organization (girdle width (marked) 70 µm), ventral view. **30:** *Ceratium tripos* var. *subsalsum* (girdle width 75 µm), ventral view. **Fig. 31.** *Ceratium lineatum* (girdle width 45 µm), ventral view, in vivo. **Fig. 32, 33.** *Ceratium horridum*, two examples of this variable species. **32:** Dorsal view, in vivo (girdle width 40 µm). **33:** Ventral view (girdle width 55 µm). **Fig. 34.** *Ceratium furca*, dorsal view (cell length 240 µm).

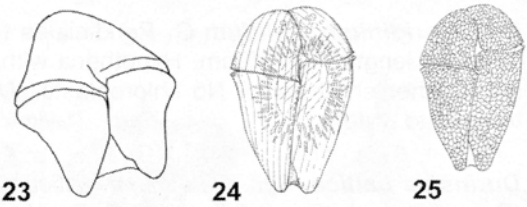


Dinophysis norvegica (16, 17)

Dinophysis acuta (18 – 20)

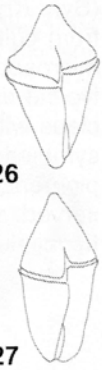


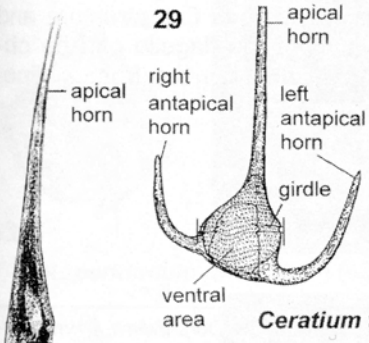
Dinophysis acuminata (21, 22)



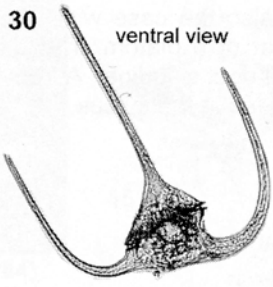
Akashiwo sanguinea

Gymnodinium gracile (24 – 27)





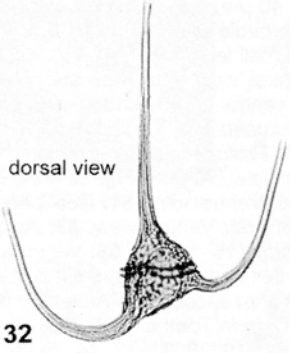
Ceratium tripos (29, 30)

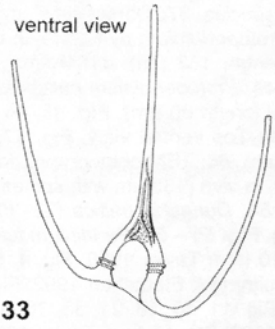




Ceratium lineatum

Ceratium fusus





Ceratium horridum (32, 33)



Ceratium furca

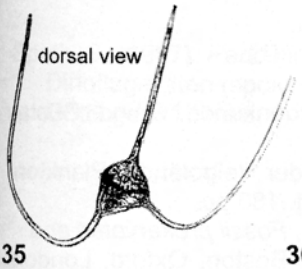
Protoperidinium curtipes O. Peridiniales (Fig. 45, 46). Cell short but wide, length 80–95 µm. Epitheca concave in outline. Apical horn and antapical horns very prominent. Thecal surface with conspicuous reticulate pattern and fine spines. Girdle groove with lists. No chloroplasts, protoplasm yellowish. Oceanic.

Protoperidinium divergens O. Peridiniales (Fig. 47, 48). Cell longer than wide, length 80–84 µm. Epitheca and hypotheca concave. Prominent antapical horns with sulcal lists characteristic of the species complex. Thecal surface reticulate and denticulate. No chloroplasts, protoplasm rose coloured. Worldwide in temperate to tropical waters.

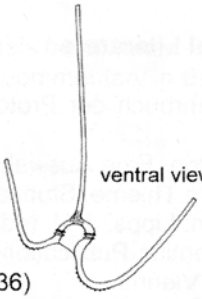
Protoperidinium pallidum O. Peridiniales (Fig. 49 – 51). Cell dorsoventrally compressed, length 70–100 µm. Hypotheca with two winged spines, at the basis of the left another short spine. No chloroplasts. Worldwide from cold temperate to warm temperate waters.

Durinskia baltica (Syn. *Peridinium balticum*) (Fig. 52 – 54), ***Peridinium foliaceum*** (Syn. *Kryptoperidinium foliaceum*) O. Peridiniales (Fig. 55, 56). Both can be obtained from culture collections. Observe cell shape and mode of swimming under the light microscope. Nuclear staining: both species harbour two kinds of nuclei. Besides the typical dinokaryon with condensed chromosomes they have another eukaryotic nucleus with the interphasic chromosomes not visible. This latter belongs to an endosymbiotic diatom which is also the case with the chloroplast; its fine structure and pigment, fucoxanthin, is that of a diatom. The structure of the flagella can be observed after staining with Lugol´s iodine. A red stigma that derives from a dinoflagellate chloroplast, is located in the sulcus.

Fig. 35, 36. *Ceratium macroceros*, two examples of this variable species. **35:** Dorsal view, in vivo (girdle width 55 µm). **36:** Ventral view (girdle width 40 µm). **Fig. 37, 38.** *Ceratium longipes*, two examples of this variable species. **37:** Dorsal view, in vivo (girdle width 60 µm). **38:** Ventral view (girdle width 80 µm). **Fig. 39.** *Protoperidinium pyriforme*, in vivo (cell length 60 µm). **Fig. 40 – 42.** *Protoperidinium depressum* (cell length 133 µm). **41:** Bottom ventral view. **42:** Top ventral view. Main thecal components. **Fig. 43, 44.** *Protoperidinium pellucidum*, ventral views. **43:** In vivo (cell length 50 µm). **44:** SEM photograph (cell length 60 µm). **Fig. 45, 46.** *Protoperidinium curtipes*, in vivo (cell length 93 µm). **45:** Ventral view. **46:** Top ventral view. **Fig. 47, 48.** *Protoperidinium divergens*. **47:** Ventral view, in vivo (cell length 78 µm). **48:** TEM photograph, dorsal view (73 µm). **Fig. 49 – 51.** *Protoperidinium pallidum*. **49:** Dorsal view, in vivo (130 µm with spines). **50:** Ventral view. **51:** SEM photograph, lateral view (112 µm). **Fig. 52 – 54.** *Durinskia baltica* (22–30 µm). **52:** Ventral view. **53:** Apical view. **54:** Nuclei after nuclear staining. **Fig. 55 – 56.** *Peridinium foliaceum* (15–50 µm). **55:** Ventral view. **56:** Lateral view.
(Fig. 1 – 3, 9, 10 from Taylor 1990; Fig. 4, 5, after Wall, Guillard, Swift & Watabe from Drebes 1974; Fig. 6, 7 from Schnepf & Elbrächter 1992; Fig. 8 after Jacobsen & Anderson from van den Hoek, Mann & Jahns 1995; Fig. 11, 16, 18, 21, 33, 36, 38, 42 from Tomas 1995; Fig. 12 – 14, 22 – 27, 48, 50, 51 from Thomsen 1992; Fig. 17 from Taylor 1987; Fig. 19, 28, 31, 32, 34, 35, 37, 39 – 41, 43, 45 – 47, 49 from Drebes 1974; Fig. 20 after Schütt from Doflein & Reichenow 1953; Fig. 29 from Peters 1930)

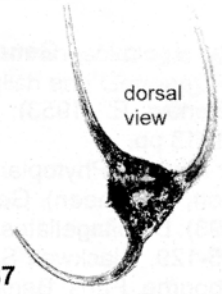


35

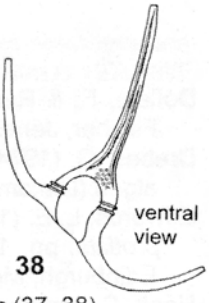


36

Ceratium macroceros (35, 36)

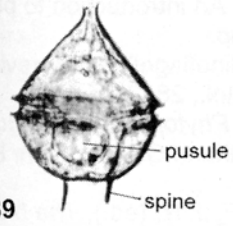


37



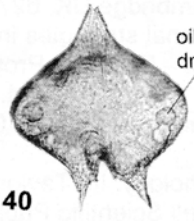
38

Ceratium longipes (37, 38)

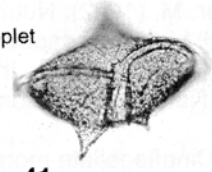


39

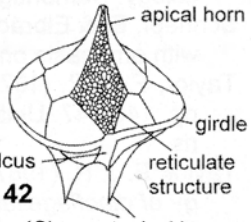
Protoperidinium pyriforme



40

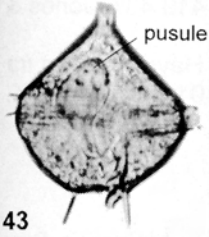


41

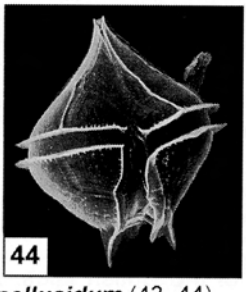


42

Protoperidinium depressum (40 – 42)

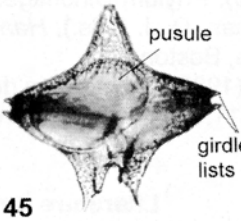


43



44

Protoperidinium pellucidum (43, 44)



45

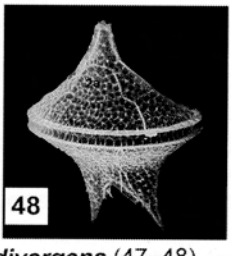


46

Protoperidinium curtipes (45, 46)

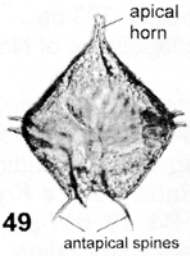


47

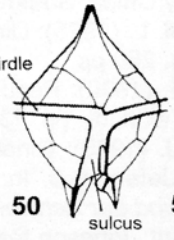


48

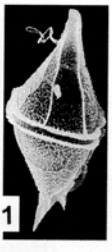
Protoperidinium divergens (47, 48)



49

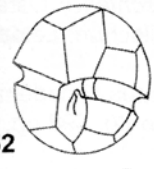


50

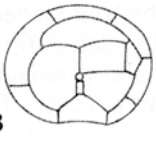


51

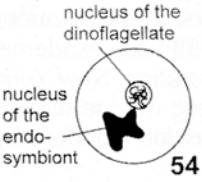
Protoperidinium pallidum (49 – 51)



52



53



54

Durinskia baltica (52 – 54)



55



56

Peridinium foliaceum (55, 56)

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Noctiluca scintillans

Malte Elbrächter and Gotram Uhlig

INTRODUCTION

Noctiluca occurs worldwide in the coastal waters of all the oceans. It is often present in dense aggregations, brought about by both physical and biological processes. This dinoflagellate is one of the largest of the flagellates, the inflated, unarmoured cell reaching more than 1 mm in diameter and making it easily recognized with the naked eye. Although several species have been described, all of them are currently regarded as *Noctiluca scintillans* Macartney, 1810. As well as causing spectacular red tides during bloom conditions, *Noctiluca* is noteworthy also because of its bioluminescence, a feature noted in the species name. Biologists are also attracted by several additional features, namely (1) the unusual morphology of the highly vacuolated cell, (2) the unresolved life cycle, (3) its variable buoyancy, (4) the different feeding strategies shown by *Noctiluca*, including social feeding, and (5) its population dynamics leading to the formation of red tides.

MORPHOLOGY

Vegetative cells are spherical, subspherical or kidney-shaped, varying in size from 200 µm to 1.2 mm. The most obvious features are illustrated in Figs. 1 – 3. The cell is greatly expanded by one fluid vacuole. A net of cytoplasmic strands radiates from an eccentrically located cytoplasmic mass (the "central cytoplasm" containing the nucleus) through the vacuole towards a cytoplasmic network underlying the rigid cell wall. *Noctiluca*, in contrast to most other dinoflagellates, has a permanent cytostome, used for both food uptake and defaecation. It is located within a distinct ventral groove, the cytostomous groove or peristome, which is marked at one end by the rod organelle and at its other end by a transversely striated tentacle. The very extensible slit-shaped cytostome continues towards the interior of the cell as a funnel-shaped membrane serving for the transport of food particles.The groove also contains a short flagellum and a tooth organelle, a reinforced structure of the cell wall. The rod organelle, a strengthening of the cell wall, stretches with a reducing width to the dorsal side.The tentacle containing myonemes beats slowly and is easily recognized. Neither the tentacle nor the flagellum is used for effective locomotion. Most cells have a transparent cytoplasm in which food particles and reserve material may be present. A slightly orange colour is confined to the cell wall.

FOOD UPTAKE

Noctiluca is a voracious polyphage without any specificity. It feeds on nearly the entire microzooplankton and microphytoplankton including crustacean and polychaete

larvae and fish eggs. *Noctiluca* has two different feeding strategies (Fig. 5 – 7). (1) Prey is engulfed by contact with the cytostome region. Large prey organisms may distort the shape of *Noctiluca* cells. Oil droplets present after oil spills may also be ingested. The tentacle plays a function in the collection and transport of microalgae like *Dunaliella* and *Chlamydomonas* that are trapped and glued together by slime filaments secreted by the tentacle. Waving slowly back and forth, the tentacle transports these clumps towards the cytostome. The tooth organelle serves to separate the particles from the tentacle and so facilitates their transport to the cytostome. (2) In particular during calm weather conditions, *Noctiluca* cells aggregate and produce a three dimensional slime net. They then sink together through the water column and catch all the small particles in their path including bacteria (Fig. 7).The slime net, together with the trapped particles, is eventually phagocytosed.

LIFE CYCLE

Noctiluca shows two different division modes. Normal vegetative reproduction is by binary fission (Fig. 4) starting with the resorption of all the organelles, the tentacle, flagellum, rod organelle and cytostome, all of which will be eventually regenerated by the daughter cells after completion of cell division. During cell division, no food uptake takes place. As in many other dinoflagellates, cell division shows a circadian rhythm with peaks of cell division occurring every day at about midnight. The meaning of the second cell division mode is so far not well understood (Fig. 8 – 10). At its beginning the nucleus is transported to the cell surface by protoplasmic streaming. Then ten multiple nuclear divisions provide successively more than a thousand daughter nuclei from which bud-shaped extensions of the cell surface, the primordia of the swarmers, develop (Fig. 8, 9). Eventually about 1000 or even 2000 small swarmers are released, but frequently also groups of not fully developed swarmers detach earlier from the mother cell, so that cells with 2 or even 4 longitudinal flagella can be observed. While mature swarmers have only one emergent flagellum (Fig. 10), a second one is concealed (Höhlfeld & Melkonian 1995). These swarmers have been interpreted as haploid isogametes in a sexual life cycle, in which *Noctiluca* is regarded as a diploid organism (Zingmark 1970). This has been disputed by Schnepf and Drebes (1993) who postulate that *Noctiluca* has an oogamic sexual life cycle, in which the swarmers are the microgametes.

POPULATION DYNAMICS AND RED TIDES

Cell division, one of the main factors of population dynamics, is mainly governed by one biological factor, food availability, and two abiotic factors, temperature and salinity. Experiments have revealed that vegetative reproduction (binary fission) is optimal at temperatures of about 22° to 24° C and a salinity of about 22 to 25‰, conditions that occur in shallow nearshore estuaries in temperate regions.

In the southern North Sea, increase in cell numbers starts in March, mainly in coastal zones, and reaches its maximum in July and August (Fig. 11). In spring certain areas of the Wadden Sea of the German Bight have been shown to be centres of a high rate of cell division that decreases towards the open sea. In warm summer

months near the island of Helgoland up to 2000 cells per litre may be counted in the surface water. Every year this summer population is overcome by a sudden cessation of food uptake. Undigested food remains are discharged resulting in a reduction of the specific weight. The irreversibly damaged cells uplift to the surface where, under calm weather conditions, they form a special type of red tide: streaks and patches of highly concentrated, autolyzing cells. Red tides are often observed at physical fronts in the water. They can be observed even by satellite images but also in small embayments or in harbours.

In most years there is a second but significantly lower population increase in September and October. A very low proportion of these survives the winter as inactive individuals, but those that do, form the starting population for the next spring bloom. In contrast, populations harbouring the phototrophic symbiont *Pedinomonas*, bloom over months in waters with temperatures higher than 24° C and apparently at higher salinities.

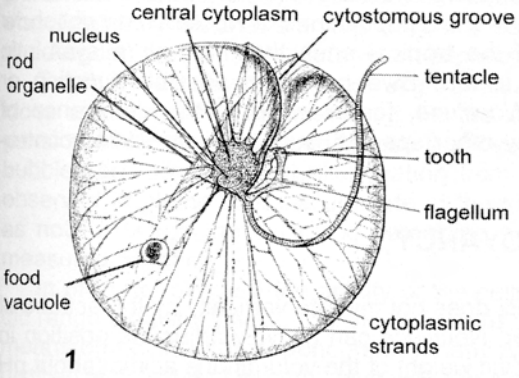
BIOLUMINESCENCE

It was Ehrenberg who first recognized as early as 1834 that *Noctiluca* exhibits bioluminescence. Bioluminescence is the production of light by living organisms. As in other bioluminescent species, the light is produced as a result of the reaction of an enzyme, a luciferase, with its substrate, a luciferin. In the natural world there are very many different luciferases, each with its own chemically specific luciferin. *Noctiluca* produces, in common with other dinoflagellates (some species of the phototrophic genera *Ceratium, Gonyaulax, Protogonyaulax, Pyrocystis* and *Pyrodinium* and the heterotrophic genus *Protoperidinium*) these compounds themselves, in contrast to many other bioluminescent organisms in which symbiotic bacteria produce both the luciferin and the luciferase. All of the named dinoflagellates cause marine bioluminescence observable at any time in the darkness as well as in the daytime. Biolumines-

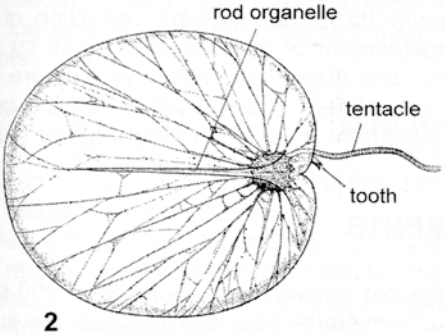
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All figures refer to *Noctiluca scintillans*

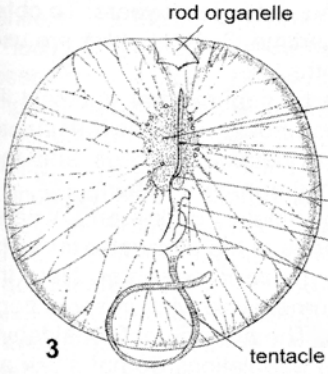
Fig. 1 – 3. Cell construction of living specimens (larger diameter 600 µm).**1:** View of the tentacle from posterior-ventral. Below the tooth a short flagellum originates which is considered to mark the ventral side as in armoured dinoflagellates. The nucleus lies within the eccentrally located cytoplasm. From this "central cytoplasm" many branching and anastomosing cytoplasmic strands radiate towards the cytoplasmic network underlying the cell wall. The rod organelle of the dorsal side is visible as a line through the semitransparent cell body. **2:** View of the side of the rod organelle (dorsal side). This organelle originates at the anterior end of the cytostomial groove and attenuates towards the posterior end of the cell. **3:** View of the ventral side with the slit-shaped cytostome at the bottom of the cytostomial groove. **Fig. 4.** Late stage of binary fission (larger diameter 840 µm). **Fig. 5 – 7.** Food uptake. **5, 6:** Food uptake in cultivated specimens. The rod-, spiral- and sickle-shaped structures are cell aggregates of the green alga *Chlamydomonas* that were engulfed as large lumps (size of *Noctiluca* specimens c. 500 µm). **7:** Collective food gathering and feeding. Diatoms (*Sceletonema*) are gathered by a three-dimensional net of slime filaments secreted by thousands of *Noctiluca* specimens. The tiny opaque dots are single *Noctiluca* cells with their food vacuoles filled with algae. **Fig. 8 – 10.** Formation of swarmers. **8:** During multiple nuclear divisions, daughter nuclei gather in bud-shaped elevations of the cell surface where they continue to divide forming sickle-shaped promordial swarmers. **9:** Early (**a**) and late (**b**) stage of swarmer formation (size of mother cell 500 µm). **10:** Uniflagellate swarmers seen ventrally and from the side (length of cell body 20 µm). **Fig. 11.** Six annual cycles of abundance near Helgoland, German Bight (1970–1975). (Fig. 1 – 4, 9, 10 from Pratje 1921; Fig. 5, 6 from Gross 1934; Fig. 8 from Doflein & Reichenow 1953)



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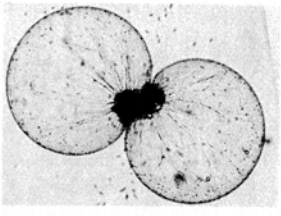


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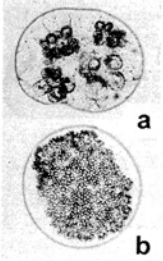


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Cell anatomy (1 – 3)

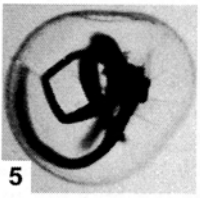


4 Binary fission



9

Swarmer formation on the surface of mother cells

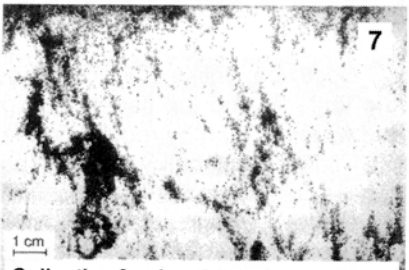


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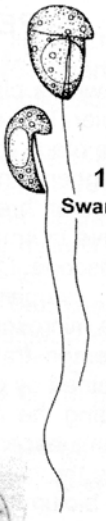
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Specimens with food vacuoles

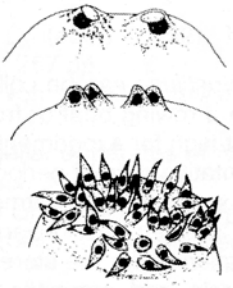


Collective food gathering and feeding

Food uptake (5 – 7)



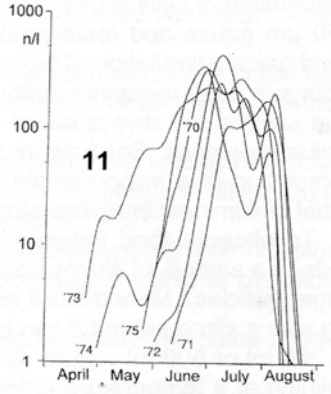
10 Swarmers



8

Formation of bud-shaped primordia

Formation of swarmers (8 –10)



11

Six annual cycles of abundance

cence can be induced mechanically by shaking or stirring, or by chemical means: the addition of formaldehyde. Several of the world's populations of *Noctiluca* do not show bioluminescence, in particular most of the tropical ones that harbour a symbiotic chlorophyte, *Pedinomonas noctilucae* (Subr.) Sweeney. The possible function or functions of the bioluminescence of *Noctiluca*, for example in the deterrence of predators, is unknown, as it is for many other species, but still gives rise to controversy.

BUOYANCY

Noctiluca, unlike other dinoflagellates, does not migrate vertically by the actions of the tentacle and the flagellum. However, *Noctiluca* can actively adjust its position in the water column by regulating the specific weight of the voluminous acidic (about pH 3.5) fluid of the cell vacuole. There are mainly three mechanisms: reduction of all heavy divalent ions, in particular SO_4^{2-} ; replacement of the heavier K^+ ions by the lighter Na^+ ion and concentration of light NH_4^+ ions (Kesseler 1966). Furthermore the nutritional status also influences buoyancy, as well-fed cells sink while starving cells accumulate at the surface. The horizontal movement of cells is brought about solely by ocean currents and winds.

EXPERIMENTS

Noctiluca can be collected with a plankton net (mesh size about 100 to 250 µm) from a rowing boat or from a pier. In the southern North Sea, cell densities are sufficient high for experiments to be made from May until August in most years. To obtain quantative data at periods of higher densities in summertime, 3–10 l bottles are used and sampling is performed in two or three different depths.

Cells are best observed alive in small Petri dishes by brightfield or darkfield illumination under the stereomicroscope. Details of cell morphology such as the striated tentacle, rod organelle, tooth, cytoplasmic strands, cytostome, nucleus and food vacuoles can be observed on a microscope slide at a higher magnification.

Bioluminescence can be demonstrated only in a completely dark room. A dense suspension of cells is best obtained by pouring a few litres of the sea water through a 250 µm gauze and resuspending the residue on the gauze in seawater. Note that mechanical stimulation of bioluminescence shows a diurnal cycle with, in some populations, a peak response during the hours of darkness. The addition of formaldehyde to a sample will always cause bioluminescence. A few populations do not show any bioluminescence. See how frequently you can obtain bioluminescence by mechanical stimulation of a living sample by measuring the time needed for recovery once the initial bioluminescence has stopped.

To observe food uptake under the stereomicroscope, add large phytoplankton cells to a sample of *Noctiluca*. Does the tentacle play an active part in food uptake of large particles? More difficult and time consuming is the demonstration of social feeding with a slime net but it can be done. Concentrate *Noctiluca* cells and transfer them to 200 ml or 500 ml culture dishes with slightly diluted filtered sea water (about 28‰ salinity) at a temperature between about 20° and 24° C. After time for adaptation to

Dunaliella (a green alga) or *Skeletonema* (a diatom) until the culture medium shows discoloration. Note that starving *Noctiluca* cells from dense populations (mainly July and August) are not suitable for such experiments.

To observe buoyancy, make a graduation mark on a 1 m long glass tube and close one end with a suitable bung. Fill the tube to the very top with a seawater suspension of *Noctiluca* cells and close it above also with a second bung so that no air bubbles are trapped. With illumination from the side, *Noctiluca* cells can be easily observed. If all the cells accumulate at the top, turn the tube slowly and carefully so as not to disturb the cells and determine how long it takes the cells to migrate a measured distance.

In order to measure the acidity of the cellular fluid, squeeze single cells onto acidic range indicator paper, say pH 3–5. More accurate measurements are possible by means of a microprobe. Concentrate many *Noctiluca* cells on a little sieve, rinse with distilled water and place the sieve with the *Noctiluca* cells and probe onto filter paper. As the cells gradually dry, the cell walls rupture and the fluid is set free from the vacuole.

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Euglenida (Euglenophyta)

Angelika Preisfeld

INTRODUCTION

Euglenids form an assemblage of phototrophic and heterotrophic flagellates. The best-known species is *Euglena gracilis* because of its availability from culture collections and easy cultivation. Hence it will serve to demonstrate most of the cytological characters (Fig. 1). Most euglenids have two flagella that arise from the base of a bottle-like invagination (reservoir) at the anterior end of the cell and protrude through an apical or subapical canal. Both flagella can emerge or one can be reduced to a stub and remain within the reservoir. The anterior flagellum can be held stiffly or can beat with a sweeping or undulating motion. If the posterior flagellum is emergent, it can trail behind the moving cell or lie closely attached to the cell body, sometimes hidden in a groove (*Anisonema, Peranema,* Fig. 17, 19). The cell is confined by a helically or longitudinally structured pellicle that consists of the cell membrane as the outmost layer with underlying repeating proteinaceous units (strips) followed by a number of subtending microtubules and tubular vacuoles of endoplasmic reticulum (Fig. 1d). The strips fuse into one another at each end of the cell. Close to the reservoir, a single contractile vacuole for osmotic regulation is surrounded by small vesicles. The main vacuole discharges into the reservoir at regular intervals. Another distinctive feature of euglenids is their unique movement: they move not only by swimming, gliding or squirming, but mostly also by performing a typical cell rotation or gyration. Many species exhibit a squirming that is called "euglenoid movement" (metaboly) caused by sliding movements between adjacent pellicle strips, and this leads to rapid changes of body shape (Fig. 1a). Muciferous bodies underlying the pellicle release mucus to cover the cell.

Some species endure suboptimal living conditions such as deficiency of light, water or nutrients by forming cysts or palmellae. In both cell forms, flagella are reduced, the cell becomes rounded and several layers of mucus are released to form a sturdy cyst wall. Cell divisions occur within the mucilaginous envelope of the palmella stage. Both, cysts and palmellae release flagellates after adequate living conditions have been restored. Euglenids reproduce asexually by longitudinal fission beginning at the anterior end of the cell. During mitosis the nuclear membrane remains intact. Condensed chromosomes and prominent nucleoli are visible during the interphase. Sexual reproduction has not been reported in euglenids.

In most euglenids, a unique storage product called paramylon can be seen as bright starch-like granules, needles or rings surrounded by a membrane. The eyespot (stigma) is a feature of phototrophic and some secondarily osmotrophic euglenids (see nutrition modes). It consists of orange-red droplets of carotenoids, and it is independent of chloroplasts closely attached to the reservoir. Together with the photoreceptor, recognizable as a delicate paraflagellar swelling at the base of the emer-

system as Euglenida. Previous morphologically based assumptions of a close relationship to kinetoplastids and diplonemids have been recently confirmed by molecular data sets: together with kinetoplastids, diplonemids and the enigmatic genus *Postgaardi* the euglenids belong to the supertaxon Euglenozoa.

PRACTICAL TECHNIQUES

Equipment for field trips: buckets with lids, sieve, jars, plankton net (60 µm mesh), big spoon, tubes, plastic trays and vessels, pocket knife, pipettes, microscope slides. Make sure also to take some bottles for water from the collection site for culture work.

Euglenids can be collected by several methods according to their preferred habitat.

1. Collection of plankton: Planktonic phototrophic and many osmotrophic forms, such as *Menoidium* and *Distigma*, are collected by drawing the net several times through the euphotic zone of still waters or against the stream of rivers. Many of the common photosynthetic forms can also be collected in large numbers by scooping up green-coloured films and foam from the surface of puddles, ditches, ponds, lakes, river banks or marine habitats. Osmotrophic forms occur only in freshwater habitats.
2. Collection from natural biofilm sources: Benthic phototrophic and phagotrophic forms are collected by scraping off greenish or grayish layers from sediments, plants, rocks or decaying plant material.
3. Collection from artificial biofilm sources: In order to capture some of the benthic euglenids of a certain biotope, microscope glass slides can be fixed onto a staining tray and kept submersed preferably close to the sediment, to stones or to aquatic plants for at least one week. After gathering the slides they should be kept submersed until examination. Subsequent to cleaning the under surface of the slide and placing a large coverslip on top of the other side, a huge assortment of phototrophic and phagotrophic euglenids is caught in the liquid between the slide and the coverslip and can be observed in detail.
4. Accumulation of phagotrophs: Since numerous phagotrophs glide or creep on and in the sediment, they are collected best from mud surface layers of high organic contamination to a depth of about 1 cm. To remove unwanted animal and plant materials, all samples should be sieved at the collection site into a bucket with a sufficient amount of water to prevent the sample from drying. Back at the laboratory, sieve the samples and place the sediment in trays in a layer about 1 cm deep. Allow the sediment to settle in the trays for an hour and remove excess water with a pipette prior to covering the sediment with a sheet of lens tissue (for elimination of unwanted large protozoa) and several coverslips. From this moment on the sample should not be moved to avoid dirt spoiling the coverslip surface. Lids should be loosely closed to reduce further evaporation. The developing microclimate between the glass surface of the coverslips and the tissue will attract gliding and creeping phagotrophs. After at least 2 hours (best results after 24–48 hours) at room temperature the coverslips can be removed, carefully placed onto slides with the wet side down and studied.
5. Collection of cysts and palmellae: A specific technique to provoke encystment is to starve a sample containing euglenids for a few days under dim light without additional food sources, but allowing for air exchange. Under these conditions most eugle-

nids will throw off their flagella and form palmelloid forms or cysts. The advantage of this preparation is that cell division and mitosis happen quickly after applying fresh culture medium to the encysted cells enabling intense examination of various stages. As a culture medium, sterile filtered water from the collection site in addition to one pea, rice or wheat grain per tube may be used.
6. Separation of green and colourless forms: Photosynthetic euglenids can be separated from heterotrophic forms by means of their positive phototactic response: placed on a north-facing window sill or under a modest light source for 1 to 2 hours with only a small spot of the tube exposed to the light, photosynthetic forms will accumulate and can easily be removed using a pipette.

Observations with Light Microscopes

Most euglenids move quickly. In order to retain them within the field of view when they are being examined with a light microscope, it is convenient to slow them down by increasing the viscosity of the medium in which they are being observed. A viscous liquid is produced by soaking quince seeds (*Cydonia oblonga*, Fam. Rosaceae, a small deciduous tree native from southwestern Asia and naturalized in Europe) in water for a few minutes. A 3% solution of gelatine cooled to about 40° C and added to the preparation will also work. The speed of the protists will also be significantly reduced if the preparation contains fibres of cotton wool to restrict their movements. Finally, if there is no debris on the slide, you can remove liquid by placing a piece of tissue near the edge of the coverslip and continue this until the coverslip presses gently on the organisms to stop them moving.

Staining Procedures

Certain cytological structures are best seen after specific stains have been applied. Staining of the cells is accomplished by gently drawing a tiny drop of stain under the coverslip by a piece of filter paper held on the opposite side. After a few minutes the stain is replaced with water by the same means.
Flagellar structures: Neutral Lugol's iodine (see p. 236), Indian ink.
Mucilage and stalks: Indian ink.
Mucilage, muciferous bodies and pellicle structures: Neutral red solution (2 % weight per volume), methylene blue (0.1 % w/v), alcian blue (0.2 % w/v). All solutions are prepared with distilled water as the solvent.
Mucilage and nuclei: Prepare a solution of 1 g alcian blue in 100 ml 3 % acetic acid and adjust to pH 2.5 with acetic acid. Prepare another solution of 0.1 g nuclear fast red and 2.5 g aluminium sulphate in 100 ml distilled water. Stain cells for 5 min with a drop of alcian blue stain, replace the stain with water. Stain with nuclear fast red for 5 min and rinse again with water. Mucilage will be stained bright blue, nuclei red.

GENERA AND SPECIES

1. Phototrophic Euglenids

Phototrophics are the most abundant euglenids in most water samples. They are

characterized by chloroplasts and belong to two suborders Eutreptiina and Euglenina (Marin et al. 2003). Members of the Eutreptiina have two or, rarely, four emergent flagella, whereas only one emergent flagellum exists in members of the Euglenina. This section refers to some of the more common species. For a comprehensive list of species, see Huber-Pestalozzi 1955 and Leedale 1967.

Eutreptiina (**Eutreptiales** according to International Code of Botanical Nomenclature, ICBN)

The Eutreptiina contain only three genera, *Eutreptia* (2 equally long flagella), *Eutreptiella* (2 unequally long flagella), and *Tetreutreptia* (four flagella). They occur mostly in marine or brackish conditions. The pellicle is faintly striated. Cells are not rigid, but usually show enhanced metaboly. Movement occurs by rotation along the longitudinal axis. Chloroplasts are without pyrenoids.

Eutreptia viridis (Fig. 4) is 50 to 70 µm long, of spindle-like shape, with an elongated posterior end and with active metaboly. The flagella are of equal length and highly mobile. The numerous discoid chloroplasts are without pyrenoids. Paramylon granules appear as small rings or grains. Occurs in green ponds and sometimes produces large algal blooms.

Euglenina (**Euglenales** according to ICBN)

The Euglenina are distinguished from the Eutreptiina by the reduction of the posterior flagellum to a stub that remains inside the reservoir, and most only have a single highly mobile anterior flagellum. Some species exist with two reduced, non-emergent flagella. Green and colourless forms (secondarily osmotrophic) are known. This suborder comprises primarily freshwater genera.

Euglena

One emergent flagellum, without a rigid pellicle or lorica, showing pronounced metaboly, paramylon abundant.

Euglena gracilis (Fig. 1) is up to 65 µm long and 15 µm wide, tapering cigar-shaped and not flattened and can also be recognized by its varying cell shape (metaboly) and its rapid swimming The highly flexible pellicle is delicately striated. A bright eyespot is visible close to the contractile vacuole. The number of shield-shaped chloroplasts varies between 8 and 12. A single pyrenoid is located centrally on top of each chloroplast and it is covered by a paramylon cap. Since *E. gracilis* strains can be purchased at culture collections, it is relatively easy to perform physiological observations even if dense cultures are needed. In order to observe the positive phototactic response of *E. gracilis,* add a droplet of a dense suspension of *E. gracilis* to a microscope slide, cover first with a coverslip and then with a template of black paper with a small hole or other stencilled forms or letters. Expose to bright light for 10 minutes and then gently remove the black template to see that the cells have accumulated in the areas exposed to light. Light sources too intense will cause the cells to disperse.

Euglena ehrenbergii (Fig. 5) is one of the largest euglenids (100 to 400 µm long) and can be found in small eutrophic freshwater habitats. Cells, showing distinct metaboly, are elongated with rounded ends and sometimes twisted, and have a delicately striped pellicle. Swimming cells are flattened, have no beating flagella and creep on surfaces. The short emergent flagellum is directed posteriorly. Cytoplasmic streaming is very obvious. Numerous small shield-shaped chloroplasts occur distributed all over the cell. Paramylon grains are small, flattened or ovoid, but are sometimes larger and rod shaped. No mucilaginous sheath is visible.

Euglena viridis (Fig. 6) is 50 to 60 µm in length and a common species in rather polluted habitats, where it sometimes produces dense green clouds directly under the water surface. Cells are spindle-shaped, with the posterior end pointed. Pronounced metaboly leads to visible contortions in the middle of the cell. The most distinguishing features are the ribbon-like chloroplasts arranged in a stellate manner with paramylon granules in the centre. Cysts and palmellae occur.

Colacium

Stalked freshwater organisms that form dendroid colonies, often attached to copepods or rotifers. Stalked cells in colonies without flagellum, a single flagellum in free-swimming stages, numerous discoidal chloroplasts with pyrenoids.

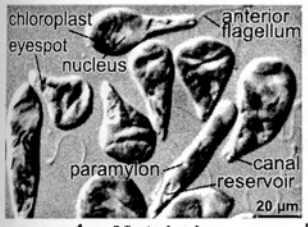
Colacium vesiculosum (Fig. 7) cells are about 20 to 30 µm long. These phototrophic freshwater organisms are enclosed in mucilaginous envelopes and attached to the substrate by mucilaginous stalks at the anterior end of the cells. Cell division occurs to produce bunches, sheets, or dendroid colonies of a few to hundreds of cells attached to algae, aquatic plants, *Cyclops, Daphnia* and other animals. Settled cells shed the emergent part of their anterior flagellum, but any individual can regrow this flagellum, escape from the colony, settle elsewhere and secrete a new stalk and sheath of mucilage. The paraflagellar swelling is reduced in size in attached cells. Seven to 10 chloroplasts are distributed peripherally.

Lepocinclis

Ovoidal to ellipsoidal cell body in freshwater habitats often with a short caudal spinous projection. One emergent single flagellum. Rigid periplast with spirally striated pellicle. Discoidal chloroplasts numerous, paramylum grains large and mostly ring-shaped.

Lepocinclis spirogyroides (Fig. 8) was formerly known as *Euglena spirogyra*, but has recently been transferred to the genus *Lepocinclis* because molecular and morphological characters reveal greater similarities with *Lepocinclis* than with *Euglena*

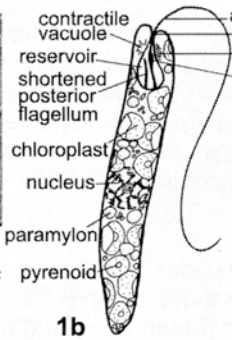
Fig. 1. *Euglena gracilis.* **a:** Metaboly. **b**: Morphological features. **c**: Anterior part of cell, showing opening of the canal, emerging anterior flagellum and pellicular strips. (cs: see Fig. 2). **d:** Fine structure of pellicular strips. **Fig. 2.** *Distigma proteus,* cross section of reservoir. Location of cross section is indicated in Fig. 1c. **Fig. 3.** *Entosiphon sulcatum*, morphological features, cs: cross section of feeding apparatus shown in figures 3b and c. **Fig. 4 – 9**. Examples of phototrophic euglenids.



1a Metaboly

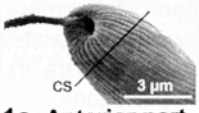
1 *Euglena gracilis*

(1a – 1d)

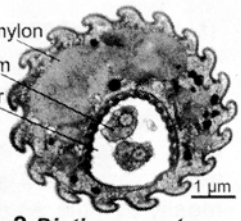


1b Morphological features

50 µm

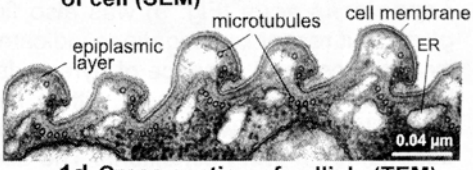


1c Anterior part of cell (SEM)

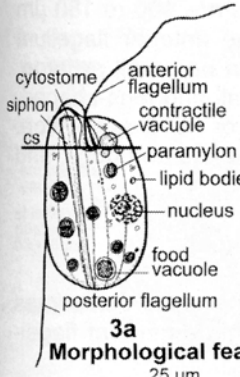


2 *Distigma proteus*

Cross section reservoir



1d Cross section of pellicle (TEM)

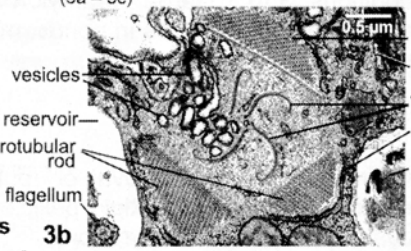


3a Morphological features

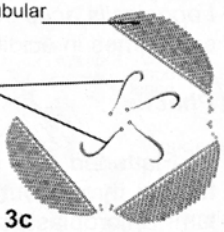
25 µm

3 *Entosiphon sulcatum*

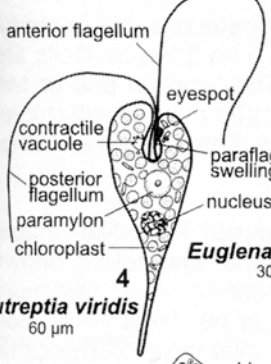
(3a – 3c)



3b Cross section (cs) feeding apparatus (TEM)



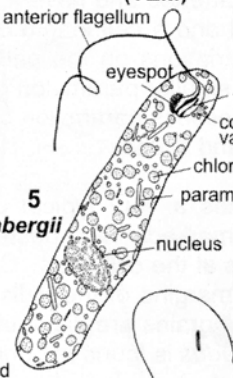
3c Cross section schematic



4

Eutreptia viridis

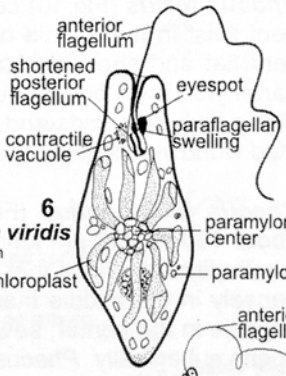
60 µm



5

Euglena ehrenbergii

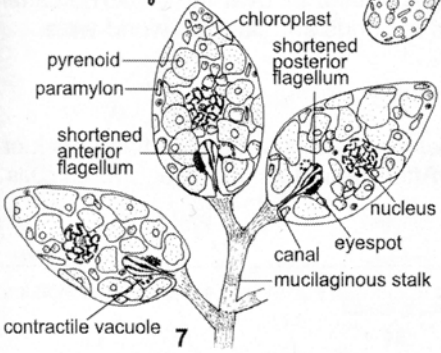
300 µm



6

Euglena viridis

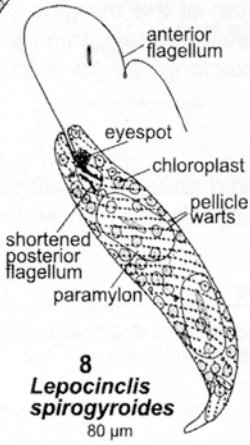
60 µm



7

Colacium vesiculosum

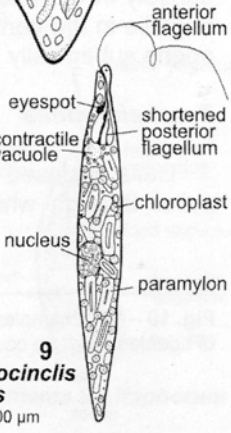
60 µm



8

Lepocinclis spirogyroides

80 µm



9

Lepocinclis acus

100 µm

(Marin et al. 2003). The most prominent character of the very size-variable species (from 45 to 250 µm length) is the visibly striated pellicle with spiral rows of yellow-brownish warts. Cells are almost completely rigid, of cylindrical shape, and contain many lens-shaped chloroplasts without pyrenoids. A posterior spine is often present. Cells are slightly flattened and twisted, usually with two large paramylon rings. This widely distributed species prefers iron-containing pools, ditches and ponds and is mostly benthic, seldom planktonic.

Lepocinclis acus (Fig. 9) was also first described as a member of the genus *Euglena*, but recent investigations indicate that it belongs to *Lepocinclis* due to the rigidity of the cell and absence of pyrenoids (Marin et al. 2003). Cells are 100 to 180 µm long and sometimes curved with a hyaline posterior spine. The anterior flagellum spins like a lasso while the cells circle constantly. Eyespots can be small or large, depending on living conditions. The several chloroplasts are small and lens-shaped. Dependent on nutrient conditions, 3 to 10 large paramylon rods or needles develop. *Lepocinclis acus* occurs as a planktonic species in ponds, marshlands and bogs, and sometimes in acidic waters.

Phacus

Flattened and leaf-like freshwater cells of oval or rounded shape, sometimes twisted throughout the cell or only posteriorly. Rigid pellicle, single emergent flagellum. Chloroplasts numerous and small without pyrenoids.

Phacus tortus (Fig.10) cells are flattened and have a short posterior spine, a prominent twist in the middle of the cell and a cell length of less than 100 µm. Cells are very flat and show conspicuous striations on the pellicle. They contain one or two paramylon rings beside numerous smaller paramylon grains. The many small chloroplasts lack pyrenoids and do not possess paramylon sheaths. The species is distributed worldwide in ponds, puddles and ditches.

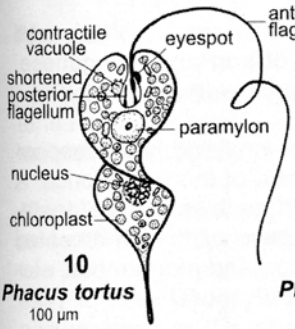
Phacus longicaudus (Fig. 11) has a very typical compressed leave-like shape, about 100 to 150 µm long with a markedly long posterior spine. The pellicle shows longitudinal striations that converge at the cell ends. Chloroplasts are gathered more densely in the middle than at the margins of the cells. One or two large paramylon rings lie in the center; several small grains are distributed all over the cell. The canal opens subapically. *Phacus longicaudus* is found in ponds and puddles world-wide.

Trachelomonas

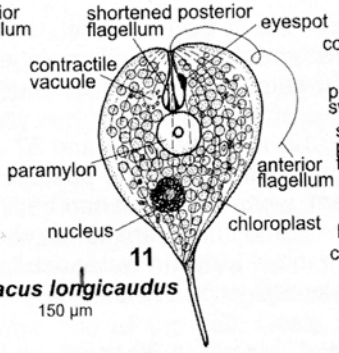
Cells enclosed in a rigid envelope or lorica which often has a well-defined neck or collar through which the flagellum emerges. After reproduction, one or both cells

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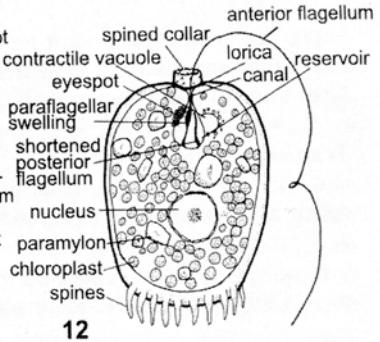
Fig. 10 – 19. Examples of phototrophic and heterotrophic euglenids. For explanations see descriptions of species. (Fig. 3b courtesy of Almut Vollmer)



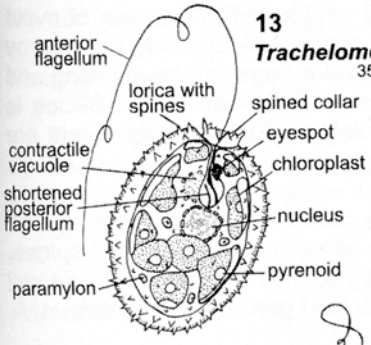
10
Phacus tortus
100 µm



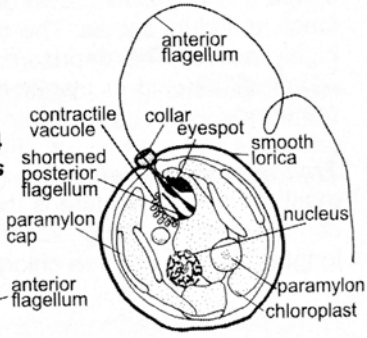
11
Phacus longicaudus
150 µm



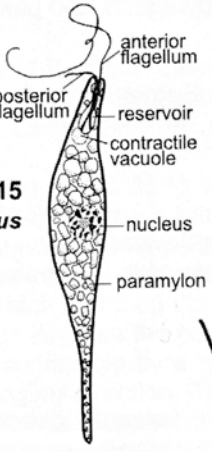
12
Trachelomonas armata
30 µm



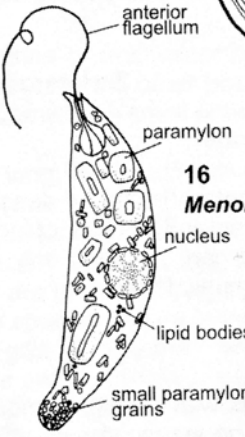
13
Trachelomonas hispida
35 µm



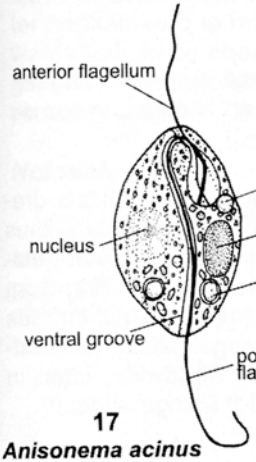
14
Trachelomonas volvocina
30 µm



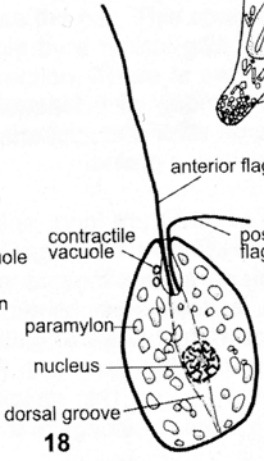
15
Distigma proteus
70 µm



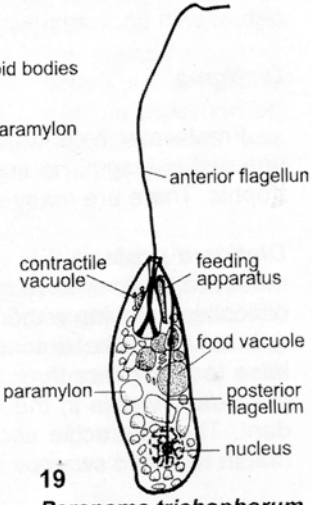
16
Menoidium cultellus
50 µm



17
Anisonema acinus
30 µm



18
Notosolenus apocamptus
10 µm



19
Peranema trichophorum
70 µm

leave the envelope and a new one is built after cell division by means of released mucilaginous material that becomes rigid by incorporation of iron and manganese. The lorica is often brown and ornamented with deposits of ferric hydroxide.

Trachelomonas armata (Fig. 12) is between 29 and 37 µm long and has "weapon-like" spines of 1 to 9 µm length positioned at the posterior end of the ovoid lorica. A collar can be present or absent: when present it can be lined by rows of small tooth-like protrusions. Eyespots are sometimes hard to see because of the ornamented and coloured lorica. Species habitually live in water rich in iron and manganese, elements that are needed for the construction of the lorica.

Trachelomonas hispida (Fig. 13) is about 30 to 40 µm long. The lorica is of ovoid shape and of yellow-brown or red-brown colouration and usually densely covered by small and blunt spines. The collar is fairly flat. The anterior flagellum is very long and highly active. The approximately 10 chloroplasts have pyrenoids. This species is widely distributed in plankton of ponds and smaller lakes. Ferric environments are preferred.

Trachelomonas volvocina (Fig. 14) measures only about 30 µm in diameter. The most remarkable feature is the spherical lorica with a smooth surface without spines, combined with a flat or absent collar. The anterior flagellum is at least twice the cell length. The two or three chloroplasts contain pyrenoids and paramylon sheaths.

2. Heterotrophic Euglenids

Primary osmotrophs

All primary osmotrophs can be described best by the lack of chloroplasts, stigma, paraflagellar swelling and the lack of ingestion devices for phagocytosis. Osmotrophs are colourless and can be found only in freshwater. They are usually collected together with phototrophic forms.

Distigma

Freshwater cells with strong metaboly, two forwardly directed emergent flagella unequal in length, no ingestion organelle, colourless; no eyespot. Nutrition is osmotrophic. There are many small paramylon grains.

Distigma proteus (Fig. 15) is 40 to 120 µm long, spindle-shaped and exhibits dramatic metaboly even (a rare feature) when swimming. Cells are highly mobile and thus difficult to examine without slowing down their movements by quince mucilage or gelatine solution. The anterior flagellum lashes about wildly, with waves travelling from base to tip. Two or three very large Golgi bodies and numerous mitochondrial threads are clearly visible in the anterior half of the living cell. Paramylon granules are abundant. The contractile vacuole is very large. This species occurs worldwide, often in marsh land and swamps. *Distigma proteus* belongs to the suborder Distigmatina.

Menoidium

Rigid freshwater organisms with one emergent flagellum, cell body strongly flattened and elongated, slightly curved, colourless, no ingestion organelle, nutrition osmotrophic, no eyespot.

Menoidium cultellus (Fig. 16) is about 50 µm long, flattened with a bean-like profile and with a slightly slanted and elongated anterior end. There is one emergent flagellum and a rigid pellicle. Cells swim rapidly by gyration when the flattening of cells is easy to see. Usually with large paramylon rings, while small paramylon grains are accumulated in the posterior end of the cell. Cysts and palmellae are unknown. *Menoidium cultellus* belongs to the class Aphageae, suborder Rhabdomonadina.

Phagotrophs

The most distinctive feature of phagotrophic euglenids is the ingestion apparatus needed to take up prokaryotic and/or eukaryotic prey. Although all phagotrophic euglenids studied so far contain such a device of some kind, it might not be identifiable by light microscopy. Food vacuoles occur as larger vesicles filled with organic material.

Anisonema

Asymmetrically ovoid flattened cells of marine or freshwater habitats, colourless with two flagella, one of which is directed anteriorly, the other posteriorly. Cells rigid or with light metaboly. Longitudinal furrow. Paramylon in small grains.

Anisonema acinus (Fig. 17) is 20 to 40 µm long, flattened, with a rigid pellicle with dotted striations. The ingestion apparatus (when visible) is wedge-shaped. Two emergent flagella. The posterior flagellum is thicker than the anteriorly directed flagellum, 2 to 3 times the cell length and located in a deep longitudinally orientated groove. Paramylon grains and food vacuoles are present. Preferred food is probably diatoms, sometimes as large as the cell. This species occurs in marine and freshwater habitats and is recognizable by a typical gliding movement with a sudden backwards jerk while changing direction. There is some dispute about the ingestion organelle as the species was first described without an ingestion organelle (which was probably missed). The phylogenetic position of *Anisonema acinus* is still unresolved.

Notosolenus

Rigid, oval and colourless, usually strongly flattened phagotrophic cell, freshwater and marine habitats. Ingestion organelle mostly not visible, 2 unequal emergent flagella (distinction from *Petalomonas* species), the longer directed anteriorly during swimming, the shorter trailing. Ventral surface convex, dorsal surface with broadlongitudinal groove.

Notosolenus apocamptus (Fig. 18) is small (about 10 µm long), ovoid and much compressed, unusual in being flattened dorsoventrally (most euglenids are flattened laterally). The anterior flagellum is straight during swimming with minute waves at the tip only. The posterior flagellum is very shcrt and trails backwards. Paramylon granules and food vacuoles are present. An ingestion device is not visible. Occurs in bog pools and mud. This species belongs to the suborder Sphenomonadina.

Entosiphon

Rigid freshwater organism with two flagella, the anterior having a waving action, the posterior or recurrent one trailing under moving cells. Colourless phagotrophic cells with strongly developed ingestion organelle containing a moveable siphon. Paramylon present.

Entosiphon sulcatum (Fig. 3) is about 20 to 25 µm long, ovoid and slightly flattened. Swims with slow gyration and is able to glide with trembling motions. Twelve helical grooves proceed along the cell body (the rigid pellicle composed of 12 articulating strips). The two flagella emerge from a deep apical depression: the anteriorly directed flagellum is about as long as the cell and beats with a sweeping motion. The thicker and longer posterior flagellum trails behind the cell. The noticeable ingestion organelle is situated next to the canal and composed of 3 prominent rods stretching along the whole cell length (Fig. 3b, c). Food uptake occurs by the use of the protruding feeding apparatus. Food vacuoles and paramylon granules are present. This common species is quite often found in freshwater habitats.

Peranema

Sac-shaped phagotrophic euglenid with two emergent flagella, anterior flagellum thick with only the distal tip beating. Cells highly plastic, delicate pellicle with fine striation. Contractile vacuole visible close to the reservoir.

Peranema trichophorum (Fig. 19) is 35 to 80 µm long. The posterior end is truncated or slightly indented. The anterior flagellum is extremely thick. The very thin posterior flagellum lies in a narrow longitudinal groove. The pellicle is highly flexible resulting in violent metaboly. Movement by swimming and gliding. The ingestion organelle consists of two rods close to the reservoir. Paramylon, in addition to large food vacuoles, can be so abundant as to make examination of organelles difficult. The paramylon can be reduced by starving the cells prior to examination. The species is common in freshwater and marine habitats. *Peranema trichophorum* belongs to the suborder Heteronematina.

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Parasitic Kinetoplastids

Justus Schottelius and Mansour El-Matbouli

INTRODUCTION

Flagellates of the order Kinetoplastea together with the order Euglenida form a monophyletic taxon, the Euglenozoa. They are characterized by: one or two flagella arising from a pocket-like invagination; a protein structure paralleling the axoneme of the flagellum, the paraxonemal rod; cortical microtubules and mitochondria with discoid cristae. The order Kinetoplastea is subdivided into two suborders, Trypanosomatina and Bodonina, each containing a single family: Trypanosomatidae and Bodonidae respectively. The Bodonidae are biflagellate and include the parasitic genera *Cryptobia* and *Trypanoplasma* (Leedale & Vickerman 2000).

The family Trypanosomatidae contains the homoxenous (the life cycle is confined to one host) genera *Crithidia, Herpetomonas*, *Leptomonas* and *Blastocrithidia,* which live in the gut of insects, as well as the heteroxenous genera *Leishmania, Trypanosoma, Endotrypanum, Phytomonas* and *Sauroleishmania,* which are carried by insects, leeches and mammals. Trypanosomatidae have only one flagellum that is either free or can give rise to an undulating membrane with the pellicle. During their developmental cycle there is a visible dislocation of the complex of kinetoplast and basal body of the flagellum (the nucleus does not change its position), giving rise to several distinct morphological stages or forms: amastigote (cryptomastigote), promastigote, epimastigote, trypomastigote and opisthomastigote (Fig. 1 – 5). The amastigote stages (Fig. 1) have a length of 2–3 µm and are rounded or oblong. They have a very short flagellum that remains in the flagellar pocket without emerging from the surface. The nucleus and kinetoplast (often bacillary in size) lie closely together. The promastigote cell (Fig. 2) is stretched, the kinetoplast lies at a distance anterior to the nucleus, and the flagellum, which arises in front of the kinetoplast, leaves the cell at its anterior pole. The opisthomastigote stage (Fig. 3) is stretched: the kinetoplast lies at the caudal end, and the flagellum originates in front of the kinetoplast and leaves the cell anteriorly. The epimastigote cell (Fig. 4) is stretched, the kinetoplast lies immediately anterior to, or beside, the nucleus, and the flagellum, which originates in front of the kinetoplast, leaves the cell laterally and continues along the cell surface where it can give rise to a flimsy undulating membrane. In the trypomastigote stage (Fig. 5) the kinetoplast lies at the caudal end of the often slender s-shaped or c-shaped cell and the flagellum, which originates in front of the kinetoplast, leaves the cell laterally to give rise to an undulating membrane with a free end anteriorly.

The genus *Trypanosoma,* of which two representatives will be discussed here, contains parasitic flagellates that infest the blood of all vertebrate classes. Species of *Trypanosoma* live in the intestinal tract of their vectors, insects and leeches. The trypanosomes of mammals are subdivided into two groups: Salivaria (Latin, saliva) and Stercoraria (Latin, stercus: faeces). Salivaria trypanosomes finish their developmen-

tal cycle in the salivary glands of the vector, with transmission occurring upon inoculation of saliva into a vertebrate. *Trypanosoma gambiense* and *T. rhodesiense* (causing sleeping sickness) and *T. congolense* (causing nagana in cattle) are of major importance in Africa and their vector, tsetse flies of the genus *Glossina,* equally so. Sleeping sickness has killed hundreds of thousands of people living around Lake Victoria and elsewhere.The Stercoraria finish their developmental cycle in the hindgut of the vector, with metacyclic (infectious) stages in the faeces. Infection results from faecal contamination of the host´s stab wound or its mucous membranes (eye, mouth, *Trypanosoma cruzi)* or by ingestion of the vector itself by the host (*Trypanosoma melophagium). Trypanosoma cruzi* is a pathogenic stercorarian and causes Chagas´ disease in Latin America. In Central Europe four stercorarian *Trypanosoma* species are known, none of which is pathogenic. *Trypanosoma lewisi* lives in rats with rat fleas as vectors. *Trypanosoma musculi* lives in the blood of the common house mouse, is also transmitted by fleas, and has a worldwide distribution. *Trypanosoma theileri* lives in cattle with Tabanidae as vectors.*Trypanosoma melophagium* (Fig. 6a, b) is found in the blood of sheep.

PRACTICAL TECHNIQUES

Trypanosoma melophagium

Melophagus ovinus (sheep-ked) are collected from the fleece and can be kept together with some wool and a few drops of water in a closed box kept near a source of warmth. After 24 hours about 50% of the ked will still be alive. Equipment required for making preparations: very fine scissors, forceps, dissecting needles, stereomicroscope, small glass plates, microscope slides, coverslips, mounting medium PBS buffer pH 7.4 (dissolve 8 g NaCl + 0.2 g KCl + 1.87 g $Na_2HPO_4 \cdot 2H_2O$ + 0.2 g KH_2PO_4 in 1000 ml of boiled distilled water, and adjust pH to 7.4 with 0.1 M NaOH or HCl); buffered water (dissolve 0.49 g KH_2PO_4 and 1.47 g $Na_2HPO_4 \cdot 2H_2O$ in 1000 ml of boiled distilled water and adjust pH to 7.2), methanol, immersion oil, glass cuvette, graduated glass cylinder, Giemsa solution. Before preparation, observe the anatomical characteristics of the sheep-ked under a stereomicroscope or a magnifying glass: winglessness, plain and bendable chitin cuticula, claws. Both the sheep-ked (O. Diptera, Fam. Hippoboscidae) and the tsetse fly (O. Diptera, Fam. Muscidae) give birth to larvae almost ready to pupate (they are pupiparous),which is why these insects are named Pupipara. When dissecting sheep-keds, one can frequently find these larvae in the abdomen.

To dissect specimens: place the sheep-ked on its back on a microscope slide and hold it with a dissecting needle stuck through the thorax. The caudal part of the abdomen is then abscised with scissors (that is cut at right angle to the longitudinal axis of the body); after this the abdomen is sliced mid ventral up to the thorax, then separated from the thorax and placed on a new microscope slide in a drop of PBS (so it does not dry out). The abdomen is then opened very carefully and its contents (gut, genitalia, fat body, windpipes) are removed without damaging the gut, and placed on a new microscope slide with another drop of PBS. The gut is then laid out so its components can be identified (Fig. 8). To observe living flagellates, millimetre-long sections are cut off the gut segments, picked to pieces with dissecting needles in a

small amount of PBS, before being covered with a coverslip. The blood-filled part of the midgut does not contain any trypanosomes. In the caudal part of the midgut, long epimastigote flagellates can be found in large numbers (Fig. 6a). In the colon, which starts behind the Malpighian tubules, even shorter epimastigote and trypomastigote-metacyclic flagellates can be found (Fig. 6b).

Flagellate stages are identified by staining with Giemsa solution: very small pieces of the midgut and colon are picked to pieces on a microscope slide in some PBS, smeared immediately, and left to air dry. Subsequently these are fixed in 100 % methanol for 5 min (cuvette, 100 ml) and then air-dried again. After that they are stained with Giemsa solution (Giemsa dye plus buffered water 1 + 3).

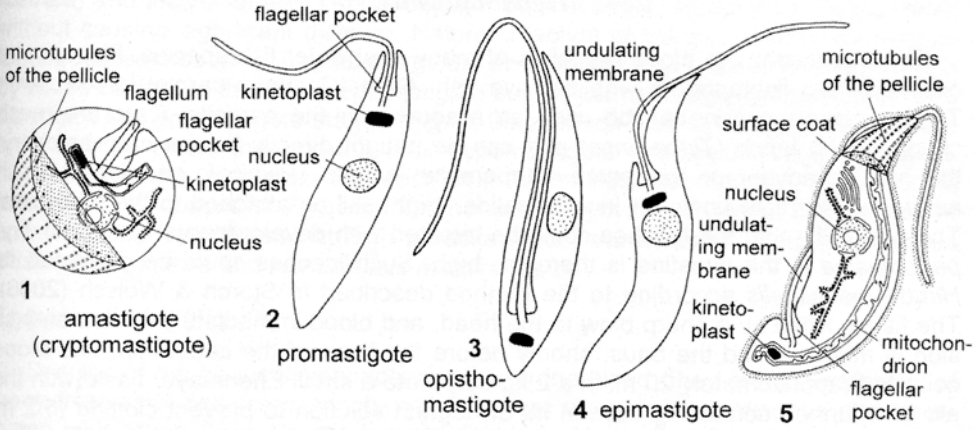
If only single slides are to be stained, prepare diluted stain in a graduated glass cylinder by taking about 3 ml Giemsa solution and adding about 10 ml of buffered water. Slides are put on a horizontal staining bridge (two parallel glass tubes or wood sticks) in a sink, then each slide is covered with up to 3 ml of the diluted staining solution. After 45 minutes the staining solution is slowly washed away with tap water and the slides left to dry in air after which a drop of mounting medium is added followed by a cover slip. The next day the flagellates can be examined at high magnification using immersion oil. It is also possible to examine non-covered slides under immersion oil to see flagellates; the oil can be subsequently removed by carefully dabbing with a xylol-impregnated swab.

For routine staining, 200–250 ml of Giemsa staining solution can be made up in a cuvette and used for one week. Place slides into an empty staining cuvette (100 ml), cover with 100 % methanol for 5 min, pour it off and allow the slides to air dry. Add diluted Giemsa solution (1 + 3, or lower concentrations down to 1 + 10 as described above) to the cuvette. After 45 minutes remove the slides from the cuvette, rinse off excess stain with tap water and air dry (for Giemsa solution see also p. 67).

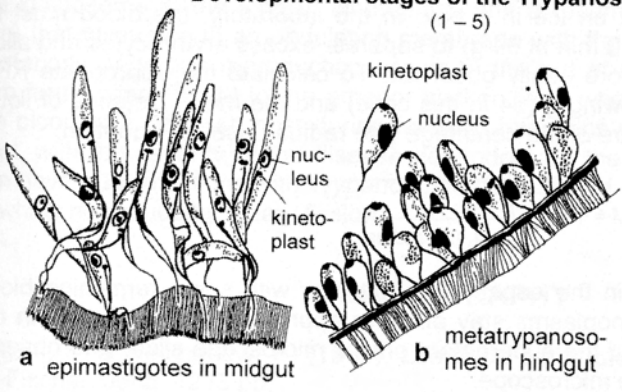
Trypanosoma granulosum

The eel is anaesthetised (3–5 ml ethyleneglycolmonophenylether in 10 litres of water) and placed on its back so that it is stretched out straight but relaxed. Ethyleneglycolmonophenylether can be obtained from www.vwr.de (article no. 807291). A syringe with a fine needle is inserted vertically, approximately 1 cm behind the anus to intersect the caudal vein (which lies along the axis of the eel). Blood is then drawn into the syringe by gently pulling the plunger. One drop of blood is placed on a microscope slide, covered by a coverslip and immediately examined for trypanosomes with a microscope (Fig. 9a, b).

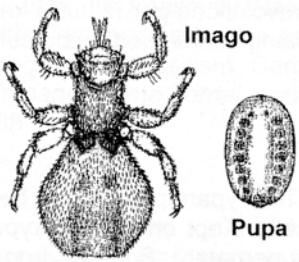
Fig. 1 - 5. Developmental stages of the Trypanosomatidae. **Fig. 6.** *Trypanosoma melophagium.* **a:** Epimastigote forms adhere via the flagellum to the midgut of the sheep-ked (length without flagellum 13–22 µm). **b:**Trypomastigote-metacyclic stages adhere via the flagellum to the hindgut of the sheep-ked (length 4–6 µm). **Fig. 7.** Sheep-ked (size 6 mm) and its pupa. **Fig. 8.** Alimentary tract of the sheep-ked. **Fig. 9.** *Trypanosoma granulosum.* **a, b:** Trypomastigotes in eel blood with two nucleate erythrocytes (size of the erythrocytes 18 µm). **c:** Epimastigote form from the gaster of a leech. **d, e:** Promastigote stages from the posterior gut of the leech. **Fig. 10.** *Trypanoplasma* sp. (length of the larger individual 50 µm). (Fig. 1 – 5 from Vickerman, 1976; Fig. 6 – 8 from Hoare, 1972; Fig. 9 after Brumpt from Kükenthal & Matthes, 1953; Fig. 10 after Martin from Doflein & Reichenow, 1953)

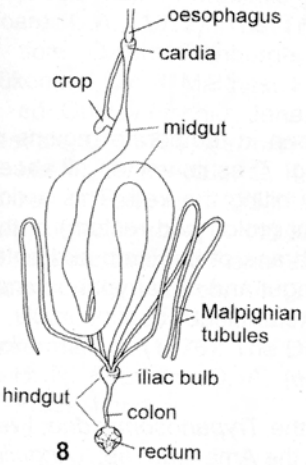


Developmental stages of the Trypanosomatidae
(1 – 5)

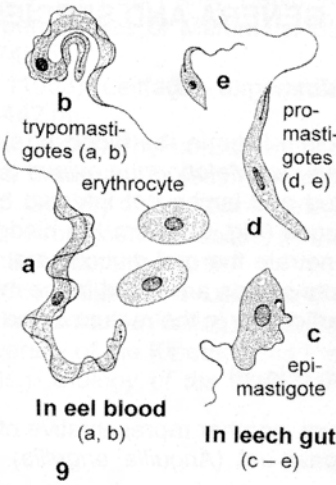


6 *Trypanosoma melophagium* in sheep-ked´s gut

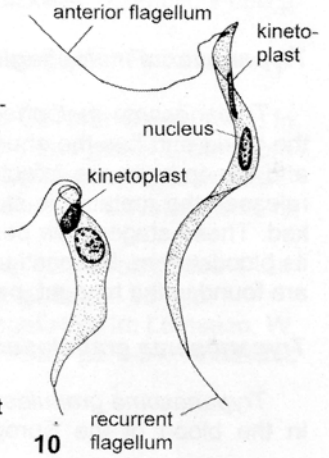




Alimentary tract of sheep-ked



Trypanosoma granulosum



Trypanoplasma

Trypanoplasma

Trypanoplasms are blood parasites of many freshwater fish species. Fish can be obtained from fishmongers who sell live fish. Carp (*Cyprinus carpio)* infected with *Trypanoplasma cyprini* can be used as a source for the parasite. Carp are much cheaper than tench (*Tinca tinca)* and can be bought directly from a carp hatchery; this has the advantage that some ectoparasitic leeches (*Piscicola geometra)*, which act as intermediate hosts for kinetoplastids, might still be attached to the epidermis. The possibility that the leeches could be infected with developmental stages of *Trypanoplasma* in the intestine is therefore high. Such leeches could be dissected as *Hirudo medicinalis* according to the method described in Storch & Welsch (2006). The fish is killed by a sharp blow to the head, and blood immediately drawn: an incision is made behind the anus, shortly before the base of the caudal fin, and blood collected (approximately 20 ml in a 2 kg carp) into a small Erlenmeyer flask (with the aid of a funnel) containing several ml of heparin solution to prevent clotting (5.2 ml heparin solution in 10 ml PBS buffer, final concentration heparin solution: blood approximately 1 :10) and kept on ice in a box. In the laboratory, the blood must be centrifuged (approximately 10 min at 64 g) to separate excess erythrocytes and allow any trypanoplasms to be more easily observed. To calculate the appropriate RPM (revolutions per minute) knowing g (64 in this case) and the mean distance of liquid being centrifuged to the centre of the centrifuge (the radius), use the equation:

$$\text{RPM} = 1000 \sqrt{\frac{g}{1.12 \cdot \text{radius}}}$$

The trypanoplasms will be in the supernatant together with some remaining blood cells. Kept on ice the trypanoplasms stay alive for approximately three days in the supernatant. Put one drop of the supernatant onto a microscope slide, and observe the trypanoplasms under the microscope.

GENERA AND SPECIES

Trypanosoma melophagium (Fig. 6 – 8)

Trypanosoma melophagium lives in the blood of sheep in temperate regions of the world and has the sheep-ked *Melophagus ovinus* (Fig. 7) as its vector; all sheep and sheep-keds are infected and lambs get infected by biting the ked. This action releases the metacyclic stages (Fig. 6b) from the hindgut (colon and rectum) of the ked. These stages now penetrate the oral mucous membrane of the lamb and enter its bloodstream. Epimastigote stages are found in the midgut and metatrypanosomes are found in the hindgut, particularly in the rectum of the ked.

Trypanosoma granulosum (Fig. 9)

Trypanosoma granulosum, another representative of the *Trypanosomatidae*, lives in the blood of the European eel (*Anguilla anguilla*), the American eel (*Anguilla*

rostrata) and the Asian eels (*Anguilla anguilla, A. rostrata, A. japonica, A. australis*), without causing significant disease. Natural infection of the eels results via haematophagous leeches (*Hemiclepsis marginata* and *Piscicola geometra*). Reproduction is by binary fission. In the leech, epimastigote stages journey to the hindgut where they transform into promastigote stages (Fig. 2, 9d,e) which then travel to the leech's anterior sucker, where they exist in their trypomastigote (metacyclic) form until transmission to a new host when the leech next feeds. Eels also harbour *Trypanosoma danilewskyi* (synonym for *Trypanosoma carassii;* length 20 µm; length of flagellum 14 µm) which causes disease at high parasite loads; transmission is via fish leeches (*Piscicola geometra* and *Hemiclepsis marginata*).

Trypanoplasma (Fig. 10)

Species of the genus *Trypanoplasma* live in the blood (they are haematozoic) of many freshwater fish (carp, tench, eel); they are normally heteroxenous and transmitted by leeches. They have a forward-directed anterior flagellum that originates in the kinetosome at the longish kinetoplast, and a recurrent flagellum (trailing flagellum) that gives rise to an undulating membrane with the pellicle and with a free end posteriorly. Vigorous reproduction occurs in the gut of the leech, giving rise to the slim forms which travel to the anterior sucker, from where they are transmitted into the blood of a new fish host during feeding. Infections with *Trypanoplasma* can be fatal, with the severity of the disease dependent on the degree of parasitism. Carp can have dual infections with *Trypanosoma carassii* and *Trypanoplasma borreli*, both of which reproduce by binary fission.

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Intestinal Flagellates of Mice (Trichomonadida, Diplomonadida)

Heinz Mehlhorn

INTRODUCTION

Three species of parasitic zooflagellates can colonize the intestines of mice and other small laboratory animals: *Giardia muris*, *Hexamita muris* (Order Diplomonadida) and *Tritrichomonas muris* (Order Trichomonadida). The pathogenicity of all three species is rather low, so that often infections – even with high numbers of individuals – do not introduce severe signs of disease and thus may remain undiagnosed. Infectious stages are regularly transmitted from the mother to the juveniles. It is, therefore, generally the case that all members of a population within a laboratory are infected, thus offering a good opportunity to obtain fresh material for teaching courses with a diagnostic background. Similar or even the same species, although this is not fully confirmed, iive in the intestines of other rodents such as guinea pigs, golden hamsters, rats, jirds (a group of tiny rodents of the genus *Meriones*).

PRACTICAL TECHNIQUES

Examination of Faeces

Diplomonadids (*Giardia*, *Hexamita*) – in contrast to trichomonadids – produce cysts that can be isolated from the faeces of mice, enabling the easy selection of animals for teaching purposes. Cysts can be obtained from the (not necessarily fresh) faeces by using a flotation method. This is in general use for the collection or diagnosis of coccidian oocysts, several types of tapeworm and digenean eggs, as well as for the eggs of nematodes.

This method is based on the buoyancy of light particles in a dense solution. As a flotation medium a saturated solution consisting of 200 g ZnCl, and 200 g NaCl and 800 ml H_20 may be used. About 2–5 g of mouse faeces are mixed with 100 ml of the flotation fluid and squeezed through a net with a 1 mm mesh. The suspension is then centrifuged for 3–5 minutes at 200 g. For the conversion from g to RPM (revolutions per minute) see p. 64. The uppermost layer of the flotation medium is now used for microscopic analysis.

Obtaining Trophozoites

Since the members of the Trichomonadida do not form cysts, the following method must be used to isolate *Tritrichomonas muris,* as well as the flagellate trophozoites of the diplomonadids. A mouse is killed and its intestine is removed and

opened by a longitudinal cut. Using a scalpel, small portions of the mucous layer of the duodenum, the caecum and the colon are scraped away and suspended with faeces from the caecum in a warm (38° C) physiological solution of 0.85 % NaCl. It is very important to make sure that in all cases the cells of the mucous layer of the intestinal wall are included, because the trophozoites of *Giardia muris* are attached exclusively to them, while the trophozoites of the Trichomonadida are found in the caecal contents. The suspension is then filtered through a 100 µm mesh and centrifuged for 10 minutes at 200 g. The supernatant is carefuly removed and the sediment resuspended in a small volume of 0.85 % NaCl. This faecal suspension can now be studied by light microscopy.

Preparations

To study microscopically the trophozoites of all three genera and the cysts of *Giardia* and *Hexamita,* the following preparations can be used:

Fresh preparations
A drop of the faecal suspension is placed on a glass slide, covered with a coverslip and studied without further fixation or staining using phase contrast. This method facilitates the study of living specimens, their movements and the activity of their flagella. In order to see the disposition and size of the flagella, a drop of neutral Lugol´s iodine (see page 236) is very helpful.

Permanent preparation
For fixation, 5–10 drops of an 2% aqueous solution of OsO_4 are added to 5 ml of a suspension containing parasites (see above). *The vapour of osmium tetroxide is dangerous and this solution must only be made in a fully functioning fume cupboard.* After 30 minutes the suspension is centrifuged at 200 g for 10 minutes and the supernatant is discarded. The sediment is suspended in 5 ml phosphate buffer at pH 7.2. and a small volume of the suspension carefully spread onto a microscope slide. After drying, the preparation is postfixed for 3 minutes with methyl alcohol and finally stained for 20 minutes with Giemsa solution. Giemsa solution should only be used freshly prepared: 0.3 ml stock solution (Giemsa is Merck article no. 9204, www.merck-chemicals.com) are added to 10 ml Weise buffer of pH 7.2; Weise buffer: 0.40 g KH_2PO_4, 1.14 g Na_2HPO_4 made up to 1 litre with double distilled water.

GENERA AND SPECIES

Tritrichomonas muris (Syn. *Tritrichomonas criceti*) O. Trichomonadida (Fig. 1, 2)

This species is found regularly in the caecum and colon of rats, but only rarely in the small intestine of rats (for example *Rattus norvegicus*), mice, golden hamsters and several wild rodents. However, it is not yet clear whether the observed and described trophozoites all belong to the same species. The trophozoites reach a size of 16–26 µm x 10–14 µm (depending on their nutritional condition). This species is characterized by three free apical flagella in addition to a broader recurrent flagellum which stretches to the posterior end of the cell body. As with other Trichomonadida,

T. muris also has a central, longitudinally stretching axostyle, which consists of a bundle of microtubules that can only be distinguished by means of electron microscopy. This structure stabilizes the body shape of the trophozoite. At the apical pole the spherical nucleus, a structure of microtubules called pelta, parabasal bodies and the costa are situated. In the cytoplasm small pale vacuoles, probably food vacuoles, are visible in addition to opaque spherical bodies that probably represent the hydrogenosomes that replace the mitochondria. As the Trichomonadida do not possess cytostomes, phagocytosis may occur at many places on their surface. *T. muris* ingests mainly bacteria as do the other Trichomonadida that live in body cavities.

Reproduction of the Trichomonadida occurs in all cases by longitudinal binary fission. Initially the basal bodies become duplicated giving rise to stages with two sets of the flagella (Fig. 2, stage in the middle). Then one set of flagella moves away from the other and may reach the opposite side of the cell. Only then does the duplication of the axostyle and the other cell organelles occur, followed by the nuclear and cell division. Because this process of division occurs constantly, many dividing stages are generally found in fresh as well as in fixed preparations. By addition of colchicine to a culture, the division process of the different trophozoites may become synchronized; this method can be used to demonstrate the spindle apparatus and its microtubules, which in the case of trichomonadids are situated extranuclearly in a groove of the nuclear membrane. The latter remains visible during the steps of nuclear division. Several electron microscope studies have shown that the fine structure of the trichomonads is characteristic and rather uniform, so that it can be used as a generic characteristic.

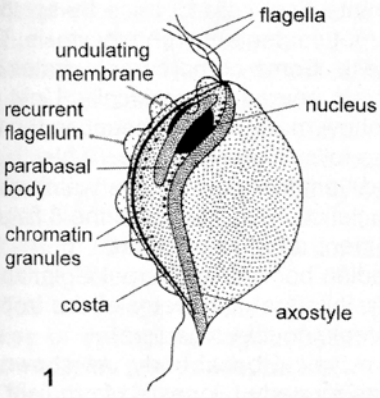
Transmission of the trichomonadids between animals occurs without the intermediate stage of cysts and is brought about by intimate body contact. In the case of trichomonadids the transmission apparently occurs by constant licking of one another and by oral uptake of trophozoites from fresh and warm faeces.

The pathogenicity of trichomonadids is low and often not convincingly shown. In combination with other agents of disease, however, infections with trichomonads may lead to severe signs of disease. In the case of *Tritrichomonas muris* infections, severe diarrhoea may occur if a double infection with bacteria occurs. This may lead to a life-threatening dehydration. However, in most cases *T. muris* does not show signs of disease.

Giardia muris (Syn. *Lamblia muris*). O. Diplomonadida (Fig. 3 – 5)

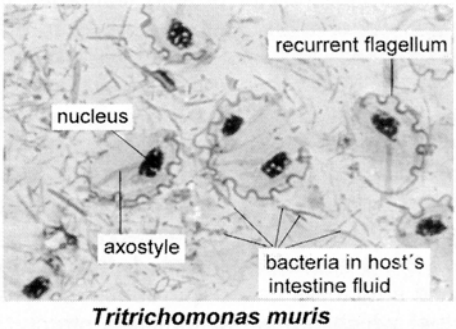
This species is found mainly in the upper intestine of the house mouse, of rats, of golden hamsters and of several other rodents (including many laboratory animals). As is the case with *Tritrichomonas muris* it is debatable, whether the trophozoites found in these various host organisms belong to one and the same species. The guinea pig, however, has a separate species, *Giardia caviae*. The trophozoites of *Giardia muris,* which have a watch-glass shape, measure 7–13 µm x 5–8 µm. The cysts are ovoid and reach a diameter of 10–12 µm at the maximum. The trophozoites

Fig. 1 – 6. Morphological features of trophozoites of trichomonadids and diplomonadids and of the cyst of the diplomonadid *Giardia muris.*



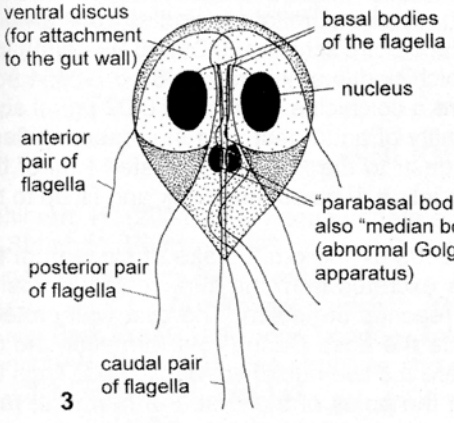
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Tritrichomonas muris
Morphological features of the trophozoite (10 µm)
(after Wenrich 1921)



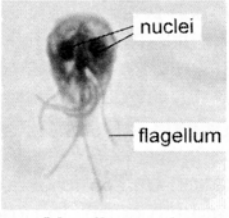
2

Tritrichomonas muris
Fixed and stained smear preparation of trophozoites (20 µm) from the caecum of a mouse with binucleate stage in binary fission (center)



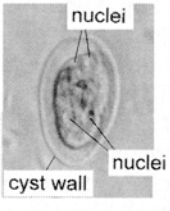
3

Giardia muris
Morphological features of the trophozoite (10 µm)
(ventral side)



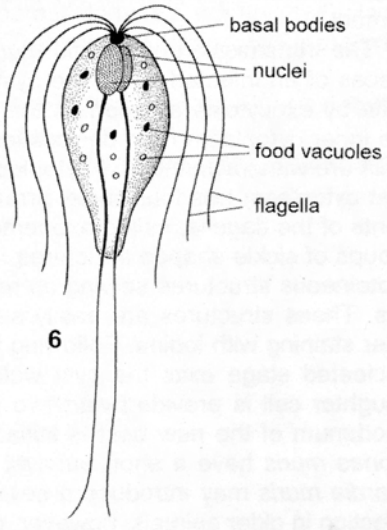
4

Giardia muris
Photomicrograph of a trophozoite (13 µm)



5

Giardia muris
Photomicrograph of a living quadrinucleate cyst (10 µm)



6

Hexamita muris
Morphological features of the trophozoite (9 µm)

are attached to the surface of intestinal cells by their ventral attachment system, the discus, consisting of protein filaments. In order to obtain live specimens for microscopic observation, it is necessary to obtain the intestinal cells of mice by scraping the intestinal wall. The trophozoites take up food (gut contents) by phagocytosis. This occurs at the dorsal side of the attached trophozoite. Some of the carbohydrates are stored as glycogen granules and are used to obtain energy anaerobically. Light microscopic investigations of *Giardia muris* do not show many details, because the trophozoites and cysts are very small. Thus, only the following details are visible: 1. the drop-like shape of the trophozoites in dorsal and ventral view and the watch glass shape in profile view, 2. the two similarly-sized nuclei of 2.5 x 1.5 µm, 3. the 8 flagella in a 2 x 4 set arrangement, 4. the ventral attachment apparatus (discus), 5. the two ovoid structures called "parabasal bodies" or "median bodies" (abnormal Golgi apparatus) which represent fortifying structures, 6. the bilateral symmetry of the trophozoite, for which the Diplomonadida are named (Greek, double organism).

Each of the 8 flagella originates from its own typical basal body, as shown by electron microscopic studies, and they do not – as formerly suggested from light microscopy – derive from only 4 "basal grains". The reproduction of the trophozoites occurs rather quickly as a longitudinal binary fission, so that in most fresh and fixed preparations no dividing stages are seen. However, the addition of colchicine to fresh preparations of *Giardia* is helpful in obtaining mitotic spindles and dividing stages: add a tiny amont of an aqueous solution of colchicine (0.01 – 0.02 µg/ml) to a fresh preparation on a microscope slide (colchicine is article no. 64-86-8 G9754 from www.sigma-aldrich.com). In order to prepare a colchicine solution of 0.02 µg/ml aqua dest., add 20 mg colchicine to a small quantity of aqua dest. in a 1l measuring flask, whirl to dissolve while filling up with aqua dest. to the 1l-mark. Transfer 1 ml of this solution with a 1 ml volumetric pipette again into a 1l measuring flask and fill up to the 1l mark.

The transmission of *Giardia muris* proceeds by the oral uptake of cysts from the faeces of an infected host. The cyst wall is excreted from the surface of the trophozoite by exocytosis as soon as this stage reaches the colon. The cyst wall protects the inner cytoplasm from desiccation outside the host. During cyst formation the flagella are withdrawn into the cytoplasm, where the two nuclei are duplicated. Thus the cyst cytoplasm has four nuclei arranged at the poles of the cyst. Furthermore, remnants of the flagella, called axonemes, are seen inside the cytoplasm as well as two groups of sickle-shaped structures. The latter are, as shown by electron microscopy, proteineous structures serving as reinforcements at the inner side of the ventral discus. These structures are easily seen by light microscopy using phase contrast or after staining with iodine. Following the oral uptake of a cyst by a new host, the four-nucleated stage exits the cyst wall and performs cell division, during which each daughter cell is provided with two of the four nuclei. Thus, the colonization of the duodenum of the new host is initiated. Trophozoites of *Giardia muris* and *Tritrichomonas muris* have a short survival time in excreted faeces. A heavy infection with *Giardia muris* may introduce a severe diarrhoea, especially in younger animals. An infection in older animals, however, often remains cryptic.

Hexamita muris (Syn. *Octomitus muris)* O. Diplomonadida (Fig. 6)

Trophozoites of *Hexamita muris* are found in the mucus and in the crypts of the jejunum and ileum as well as in the caecum of rats, mice, golden hamsters as well as in other rodents. The trophozoites are pear-shaped reaching a size of 7–9 µm x 2–3 µm and are somewhat flattened dorso-ventrally. As with most other genera of the diplomonadids (for example *Giardia*, *Octomitus*) *Hexamita* trophozoites possess free flagella. Six are situated at the apical pole, while 2 stretch to the posterior pole, where they become free. In the cytoplasm the axonemes are lined by fortifying microtubules, so that axostyle-like structures appear (giving rise in *Octomitus* to a posterior protrusion). Since this protrusion is lacking in *Hexamita* this genus was separated from the genus *Octomitus*.

The two ovoid nuclei are about 1 µm in size and are situated just below the basal bodies of the apical flagella. Additional inclusions are found inside the cytoplasm: food vacuoles and dark spherical inclusions of unknown contents.

The transmission from host to host occurs on the oral-fecal route by uptake of cysts which are formed in the colon in the same way as in *Giardia*. These cysts may keep their infectious potential for at least one year. Inside the cyst no cell divisions occur, however the nuclei become duplicated so that the cysts are quadrinucleate.

The *Hexamita muris* infection may remain symptomless. Depending on the immune system of the host, however, some infections may also introduce severe diarrhoea and even death in some animals.

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Symbiotic Flagellates in the Gut of Lower Termites

Renate Radek and Klaus Hausmann

INTRODUCTION

Besides thousands of free-living flagellates that have a diverse systematic affiliation and are found in all conceivable biotopes, there are numerous parasitic species which infect invertebrates and vertebrates. Symbiotic flagellates occur in only two flagellate taxa, namely Parabasalia and Oxymonadida, and occur exclusively in termites and some cockroaches.

The approximately 2800 species of termites belong to the insect order Isoptera; they are closely related to the cockroaches. Only about a quarter of the termite species, the lower termites and here especially the families Kalotermitidae, Termopsidae and Rhinotermitidae, feed primarily on wood. They do substantial damage to wood and other cellulose-containing materials. Nests are built within tree trunks and beams (Kalotermitidae, Termopsidae) or in the ground (Hodotermitidae, Rhinotermitidae). The symbiotic flagellates are especially involved in the digestion of cellulose.

The majority of termite species (higher termites, family Termitidae) are not specialized on wood but feed on diverse plant materials. Termitidae do not contain symbiotic protozoa in their hindgut, but do have symbiotic bacteria and yeasts. Some commensal/parasitic amoebae, ciliates, gregarines and flagellates may also occur. Higher termites construct conspicuous hill-shaped nests.

Termites live in complex communities with morphologically and functionally different individuals belonging to special castes. Soldiers defend the nest with strong mandibles and/or aggressive secretions. Workers maintain the nest, gather food and feed larvae and all the other inhabitants of the nest. Workers and soldiers are sterile members of both sexes. The lower termites, including the species *Kalotermes flavicollis* that is a subject of this chapter, do not demonstrate a true worker caste. Worker functions are taken over by older larvae (pseudergates). King and queen produce the offspring. When conditions are suitable sexual individuals develop that are alate, pigmented and equipped with functional eyes. These swarm and found new colonies.

In lower termites the anterior portion of the hindgut forms a wide paunch offering a suitable habitat for the numerous anaerobic, symbiotic and commensal zooflagellates and bacteria. During the molt of the termites, most flagellates get lost because the ectodermal paunch is equipped with an intima that more or less envelops the contents of the paunch. The molted termites are generally refaunated by proctodeal feeding (mouth-anal transmission). Thereby, the termite which lost its symbionts takes in a drop of content from the paunch which has been extruded by another termite. During the last molt of these hemimetabolous insects leading to an imago, more flagellates may be held back in the gut and these often show morphological changes as well as sexual processes. True cysts have never been found in termite flagellates. Such resistant stages do not seem to be necessary since transmission is

ensured by proctodeal feeding.

Lower termites are specialized on cellulose-containing food, such as wood. Their symbiotic flagellates are indispensable for the digestion of cellulose and thereby for supplying energy. If the symbionts are killed, for example by elevating oxygen pressure or temperature, the termites will die within two to four weeks despite continued feeding.

The flagellates phagocytose the wood particles that are ingested by the termites with the help of their mandibles and the particles are further ground in the gizzard. Cellulose is broken down to glucose in several steps. A mixture of enzymes, generally referred to as cellulases, enzymatically hydrolyses the cellulose as follows: Endoglucanases cleave internal glycolytic bonds along the polyglucan chain of amorphous cellulose and water-soluble derivatives, releasing glucose, cellobiose, cellotriose, and other higher oligomeres. Exoglucanases attack the terminus of a polyglucan chain including highly crystalline cellulose, liberating cellobiose or glucose from the non-reducing end. Cellobiases hydrolyze cellobiose and water-soluble cellodextrins to glucose. While some cellulase components which partially attack cellulose are synthesized in the salivary glands and the midgut of lower termites, the flagellates are the major agents of wood cellulose hydrolysis. Bacteria living in association with the flagellates as endobionts or ectobionts might also contribute to cellulose degradation.

Most of the released glucose is glycolytically transformed to pyruvate. Two molecules of ATP are generated for each molecule of glucose by this chain reaction. The pyruvate is used for further energy production by special organelles, the hydrogenosomes. They produce another 2 ATP by an anaerobic substrate-level phosphorylation reaction, releasing CO_2, H_2 and acetate. Acetate is absorbed by the wall of the paunch and used as the major oxidizable energy source of the termites, as well as constituting an important biosynthesis precursor. Because wood is poor in usable nitrogen compounds, additional nitrogen sources are necessary: For example, there are heterotrophic, N_2-fixing bacteria and others that recycle nitrogen from uric acid in the paunch; part of the symbionts may be digested during the gut passage following proctodeal feeding, and exuvia may be ingested which consist of the N-containing chitin. The association with diverse gut bacteria is absolutely vital for the maintenance of a functioning symbiotic system. For example, some of them use the oxygen diffusing into the gut and thus maintain the anoxic status of the internal gut regions. Acetogenic and methanogenic bacteria remove the CO_2 and H_2 that is released by the flagellates.

PRACTICAL TECHNIQUES

Termites may be purchased from biological supply companies, for example from the Federal Institute of Materials Research and Testing (Bundesanstalt für Materialforschung, Labor Zoologie und Materialbeständigkeit, Unter den Eichen 87, 12203 BERLIN, GERMANY). The termite *Kalotermes flavicollis* is host to several small flagellate species and one impressive, large parabasalid, *Joenia annectens*. *K. flavicollis* is indigenous to the Mediterranean area. Termites may also be collected from the wild (rotten tree trunks, roots and branches) and may be found within human environments such as in wooden buildings and fences, paper items, etc.

The termites can be maintained in plastic jars with a few pieces of wood. Termites from the wild should be left in the pieces of wood in which they are nesting. The addition of moistened Vermiculite (a natural basaltic mineral expanded with heat) or filter paper guarantees a high humidity. However, an excess of water leads to mould growth and in the worst case to the death of the colony. Keep the jar at room temperature and preferably in darkness. When experiments are planned, the termites can be removed from the pieces of wood by careful chopping with a knife and subsequently stored for several days in a Petri dish with moistened filter paper.

To obtain flagellates, the head of a termite is held with a forceps and the end of the abdomen is secured with a second, fine forceps. Upon pulling, the fine chitin cuticle will tear so that the hindgut is extracted. The paunch is then opened in a drop of saline, for example 0.6% NaCl solution, on a slide. A longer observation time may be possible by more complex solutions (for example 117 mg NaCl, 75 mg KCl, 11 mg $CaCl_2$, 84 mg $NaHCO_3$, 69 mg NaH_2PO_4, 294 mg Na citrate · 2 H_2O in 100 ml distilled water). Another method of isolation is to fix the termite at its thorax with a forceps and simply press the contents of the gut out of the abdomen with a dissecting needle. The sample should be quickly covered with a coverslip, after a sufficient depth of liquid has been guaranteed by supporting Vaseline, plasticine, or glass splinters. A thin surrounding border of Vaseline or paraffin oil hinders oxygen from diffusing under the coverslip. Despite these precautions, some of the flagellates will die soon due to exposure to oxygen during the preparation and suboptimal ionic milieu. However, many flagellates will live for up to two hours.

Besides observation under bright field or phase contrast conditions, differential interference contrast is particularly suited to the studies. The form of the cells and their internal structures such as axostyle and nucleus can be readily seen. Ingested wood particles are easily recognized due to their birefringence under polarized light.

Observation of live samples can be supplemented by studying mounts stained with hematoxylin. To prepare such a mount of the gut, the paunch is opened in only a small drop of saline on a slide to ensure that the sticky properties of the gut fluid are not lost. Otherwise the flagellates might come off when solutions are changed. The sample is distributed with a needle or by dabbing of the opened gut. The smears are then consecutively transferred into staining jars filled with the different solutions without allowing them to dry (wet mount). It is also possible to use Petri dishes for small amounts of samples.

Iron Hematoxylin Staining after Heidenhain

The wet mounts intended for iron hematoxylin staining are fixed in sublimate alcohol according to Schaudinn for 1–5 minutes. Then they are transferred into 70% ethanol (3 min) and afterwards into distilled water (3 min). Subsequently, the mounts are tanned in 2% aqueous iron alum for 1 hour, then rinsed in distilled water for about a minute. Differentiation (partial destaining) is performed in a 0.5% solution of iron alum until the inner structures of the flagellates become apparent. This process should be controlled under a microscope. Then rinse with tap water for 15 minutes. Dehydrate with ethanol (70% 3 min, 96% 3 min, 100% 2 x 5 min), clear with xylene (2 times 5 min) and mount with a resinous medium such as Entellan new (order

number Merck: 107960 or 107961, www.merck-chemicals.com) or Euparal (order number Roth: 7356.1, www.carl-roth.de).

Preparation of Stock Solutions

Sublimate Alcohol according to Schaudinn (Mayer, 1966, p. 45)
Dissolve 3.5 g sublimate ($HgCl_2$) in 50 ml of hot distilled water and, after cooling the solution, add 25 ml 96% ethanol. *Metal tools must not come into contact with this solution. Sublimate is very poisonous*. Prepare only small amounts.

Iron Alum (Mayer, 1966, p. 50)
Dissolve 10 g violet iron alum crystals ($NH_4Fe(SO_4)_2 \cdot 12\ H_2O$) without warming in 100 ml distilled water.

Hematoxylin (Mayer, 1966, p. 50)
Dissolve 0.5 g hematoxylin crystals ($C_{16}H_{14}O_6$) in 5 ml 96% ethanol and dilute with 45 ml distilled water. Ageing is done by oxidation in the air for 14 days or, using chemical oxidation, by adding 0.1 g sodium iodate ($NaIO_3$). Before use, the staining solution is diluted with distilled water 1:1 and filtered.

GENERA AND SPECIES

The composition of flagellate species is typical of each of the about 800 species of lower termites. The symbionts generally belong to the two taxa Parabasalia and Oxymonadida.

Parabasalia

The classical split of the Parabasalia into the small, simple trichomonads and the large, multiflagellate, more complex hypermastigids is no longer phylogenetically valid. Ultrastructural and molecular phylogenetic investigations have shown that they belong to four major subgroups: trichomonadids, cristamonads, trichonymphids, and spirotrichonymphids. Only trichomonadids and cristamonads occur in *Kalotermes flavicollis*. All parabasalids possess axostyles composed of rolled-up microtubular ribbons and a microtubular pelta which partially surrounds the flagellated area. Microfibrillar, cross-striated roots (parabasal filaments) have their origin at the basal bodies and are associated with dictyosomes (parabasal bodies). Hydrogenosomes, substitutes for mitochondria, produce ATP by an anaerobic substrate-level phosphorylation reaction. Nuclear division is a pleuromitosis (closed mitosis with extranuclear half-spindles oriented at a certain angle).

Trichomonadids

This subgroup comprises small trichomonads which usually possess four flagella and which may or may not have an undulating membrane (lamellar fold of cell body with attached recurrent flagellum) and a costa (subtending cross-striated root). Basal body number 2 is the most important; it bears the sigmoid fibres and the two striated

parabasal roots. A rod-like axostyle protrudes posteriorly. Genera: *Tricercomitus, Hexamastix, Tetratrichomonas*, *Pentatrichomonoides*, *Trichomitopsis*, *Pseudotrypanosoma.*

The termite *Kalotermes flavicollis* usually contains two species of trichomonads. *Tricercomitus divergens* (Fig. 3) is only 4–8 µm long and has four, seldom three, apical flagella. One of these flagella is a long, thickened recurrent flagellum which is attached to the cell body. *Tricercomitus* feeds by resorption of dissolved organic nutrients. *Hexamastix termitis* (Fig. 4) has a length of 10–30 µm and possesses six flagella of equal length and thickness, one being recurrent. *Hexamastix termitis* phagocytoses wood and bacteria.

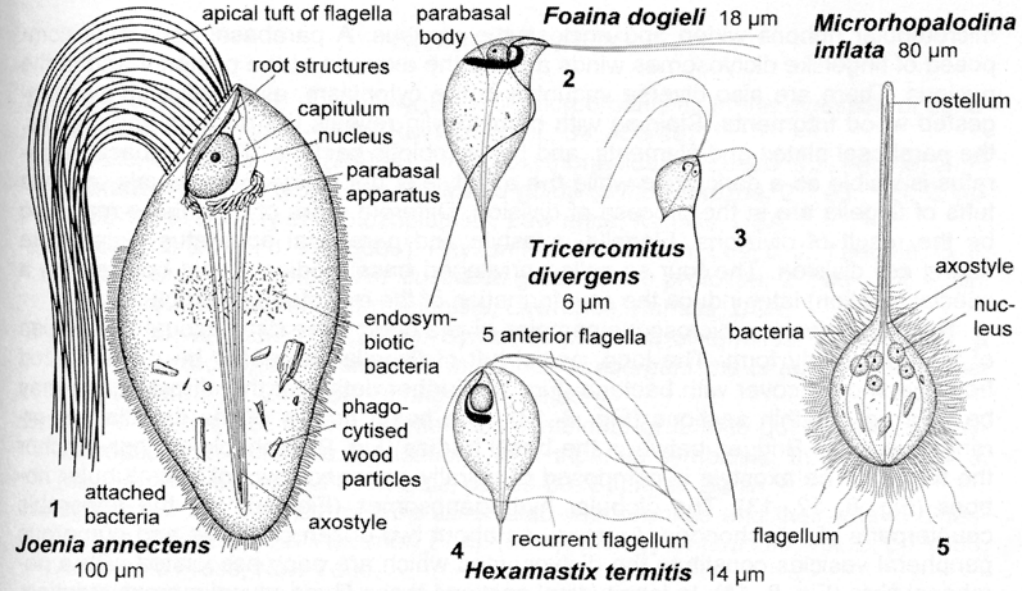
Cristamonads

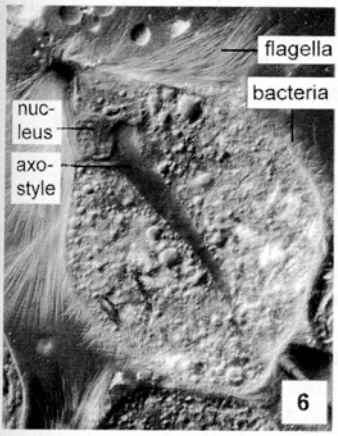
There is no costa and undulating membrane, but a microfibrillar structure named a cresta in front of the recurrent flagellum. The recurrent flagellum has a cord-like or ribbon-like structure. Devescovinids possess four flagella; *Projoenia* and the joeniids possess numerous flagella with the basic four basal bodies becoming so-called privileged basal bodies. The multinucleated calonymphids are polymastigote devescovinids. The flagella of the multiflagellate species may be arranged in tufts or in a dome-shaped area. At division the flagella disappear and are reformed by the daughter cells. A long parabasal filament is frequently twisted around the axostyle. Genera: for example *Deltotrichonympha, Foaina, Joenia, Lophomonas, Rhizonympha.*

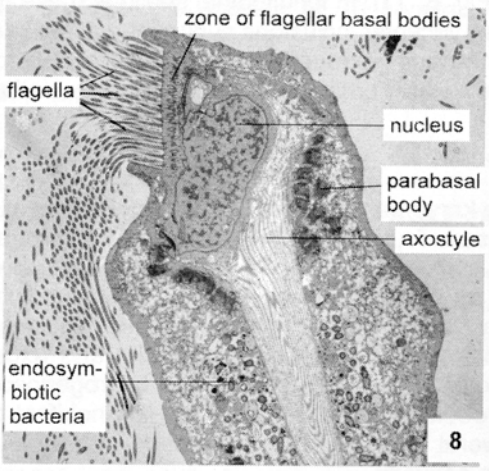
Present in *Kalotermes flavicollis*: *Joenia annectens*, *Foaina dogieli, Foaina grassii. Foaina dogieli* (Fig. 2) measures about 12–30 µm and possesses three short anterior and one long, thickened recurrent flagella. The recurrent flagellum is attached to the cell body. The cresta is long and thin. *Foaina grassii* is somewhat smaller, measuring 10–20 µm, and the thick recurrent flagellum is nonadherent. The anterior cell pole is slightly elongated into a movable papilla. The cresta is small and sickle-shaped. *Foaina* species phagocytose wood particles.

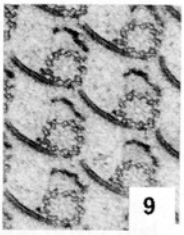
Joenia annectens is the most conspicuous and largest flagellate in the gut of the dry wood termite *Kalotermes flavicollis*, measuring 70–180 x 30–45 µm (Fig. 1, 6, 7). It possesses one tuft of long flagella at one side of the anterior cell pole. Under the cover glass, the beating tuft lets the organisms move in close circular paths or they rotate. Attached rod-like bacteria and occasional spirochetes cover the cell. Several internal structures may be clearly recognized using light microscopy. A tapering axostyle runs longitudinally through the cell body. Anteriorly, its spirally arranged

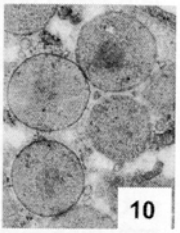
Fig. 1 – 13. The most abundant flagellates in *Kalotermes flavicollis* are *Joenia annectens* (**1**), *Foaina dogieli* (**2**), *Tricercomitus divergens* (**3**), *Hexamastix termitis* (**4**) and *Microrhopalodina inflata (5*). **Fig. 1, 6 – 13.** *Joenia annectens*. **6:** *Joenia* in differential interference contrast (175 µm), showing the apical tuft of flagella, bacteria attached to the cell surface, axostyle, nucleus, parabasal bodies and vacuoles. **7:** Scanning electron micrograph (120 µm). *Joenia annectens* bears long flagella and a dense cover of rod-like bacteria. **8, 9:** The anterior body part shows the flagella and their basal bodies, the nucleus (largest diameter 10 µm), microtubular ribbons of the axostyle in oblique longitudinal section, parabasal bodies and endobiotic bacteria. **10:** Hydrogenosomes (width about 1.1 µm) with paracrystalline inclusions. **11:** Dictyosomes (width about 1.3 µm) associated with cross-sectioned parabasal fibres. **12:** Cross-section of the axostyle (4.2 µm). The microtubular ribbons are arranged in spirals. **13:** Detail of Fig. 12 showing microtubular ribbons at higher magnification. Single microtubules (24 nm across) are seen. (Fig. 2 – 5 after Grassé, Traité de Zoologie, tome 1, 1952)

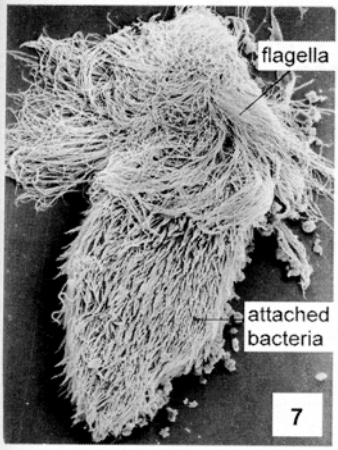

apical tuft of flagella
root structures
capitulum
nucleus
parabasal apparatus
endosymbiotic bacteria
phagocytised wood particles
attached bacteria
axostyle
1
Joenia annectens
100 µm
parabasal body
Foaina dogieli 18 µm
2
Tricercomitus divergens
6 µm
3
5 anterior flagella
recurrent flagellum
4
Hexamastix termitis 14 µm
Microrhopalodina inflata 80 µm
rostellum
axostyle
nucleus
bacteria
flagellum
5

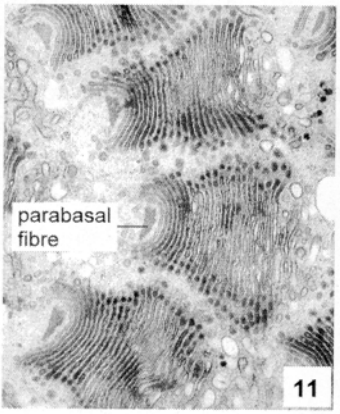

flagella
bacteria
nucleus
axostyle
6

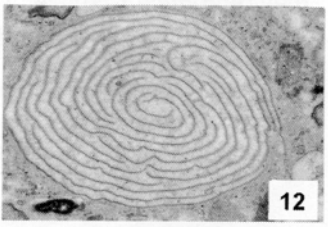

zone of flagellar basal bodies
flagella
nucleus
parabasal body
axostyle
endosymbiotic bacteria
8

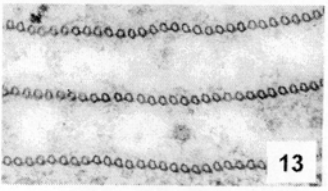

9


10


flagella
attached bacteria
7


parabasal fibre
11


12


13

microtubular ribbons widen and enclose the nucleus. A parabasal apparatus composed of fingerlike dictyosomes winds around the axostyle at the posterior end of the nucleus. There are also diverse vacuoles in the cytoplasm, especially such with ingested wood fragments. Staining with hematoxylin reveals the nucleus, the flagella, the parabasal plates and filaments, and the ectobiotic bacteria. The parabasal apparatus is visible as a dark circle while the axostyle is not stained. Individuals with two tufts of flagella are in the process of division. Different sizes of organisms may also be the result of divisions. Flagella, axostyle and parabasal apparatus disintegrate during cell division. The four so-called privileged basal bodies (basal bodies with a special function) later induce the new formation of the microtubular structures.

Scanning electron microscope samples give a good three-dimensional impression of the outer body form. The long, apical tuft of flagella can easily be differentiated from the shorter cover with bacteria (Fig. 7). Further details of the ultrastructure may be seen in ultrathin sections (Fig. 8–13). The basal bodies of the flagella are arranged in rows. Bridges between the basal bodies (Fig. 9) probably strongly anchor the flagella. The axostyle is composed of spirally arranged rows of microtubular ribbons (Fig. 8, 12, 13). The globular hydrogenosomes (Fig. 10) are the anaerobic counterparts of mitochondria. As many as about two dozen cisternae and numerous peripheral vesicles constitute the dictyosomes which are each associated with a parabasal fibre (Fig. 8, 11). In longitudinal sections these fibres reveal a cross-striation. Besides diverse vacuolar inclusions such as food vacuoles containing wood particles, hydrogenosomes and endogenous bacteria are also found in the inner cytoplasm (Fig. 8). The ectoplasm contains no larger structures other than cytotic canals.

Oxymonadida

Most of the karyomastigonts (nucleus-flagella-axostyle-associations) of this flagellate taxon possess four flagella that are typically arranged in two pairs. The pairs are connected by a paracrystalline structure called a preaxostyle from which the staggered rows of axostyle microtubules arise. The microtubular ribbon of the pelta covers the anterior part of the cell and the nucleus. Species with multiple mastigont systems have corresponding numbers of nuclei and axostyles. In some species the axostyle is motile. Parabasal bodies and hydrogenosomes are always missing. A long, apical protrusion called the rostellum is a holdfast that aids in attachment to the gut wall in several species. Mitosis is closed with an internal spindle. Some species may reproduce sexually.

The oxymonad *Microrhopalodina inflata* (Fig. 5) may occur in *Kalotermes flavicollis*. Its cell body measures 13–90 µm and the long non-contractile rostellum an additional 20–100 µm. *M. inflata* has several karyomastigonts, consisting of one nucleus, two pairs of flagella and one axostyle, each. Ectobiotic bacteria densely cover the cell surface. Wood and bacteria are ingested as food.

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Free-living Naked Rhizopods (Amoebozoa)

Norbert Hülsmann

INTRODUCTION

The scientific naming of the taxon Rhizopoda by Carl Theodor von Siebold in 1845 has its origin in the disclosure of the protoplasmic nature of the cell body in foraminifera by Felix Dujardin (1835). Until Dujardin's discovery, the foraminifera were considered to be small cephalopods. Dujardin named the living, streaming substance which emerges from the foraminiferan test "sarcode", a term which was later replaced by "protoplasm" or "cytoplasm" and therefore passed out of use. The similarity of the foraminiferan type of protoplasm to that of the other amoeboid microorganisms led to the assumption that not only the protoplasmic substance but also the changeability of body forms and the ability to form pseudopodia are common homologous characteristics of all rhizopods and that therefore all these protozoans form a natural group.

After the creation of the cell theory, it became attractive to think that all the pseudopodia formed by the corresponding taxa might serve, together with the possession of shells, as direct morphological indicators for systematic purposes. This supposition has been held to be true until the present time. Nowadays, however. the atestate condition is no longer considered to be an indication of a primitive status, and the type of pseudopodium is no longer used for systematic purposes. With the exception of the granuloreticulopodium that is characterized by a unique bidirectional streaming and serves as an apomorphic character in the foraminifera, all the other pseudopodial sub-types emerged several times in the course of evolution. Filopodia – the filiform pseudopodia – arose in the testate Monadofilosa (*Euglypha*, *Trinema*, *Paulinella*) a subgroup of Cercozoa, but also in the atestate Aconchulina (*Gobiella*, *Vampyrella*), a subgroup of the Amoebozoa; in protostelids (also a sub-group of Amoebozoa) and – beside some other types of pseudopodia – in the polyphyletic radiolarians and heliozoans.The ability to form lobose pseudopodia occurs in the Amoebozoa (*Amoeba*, *Vannella*), in some Cercozoa (*Cercomonas*), in the Heterolobosa (*Naegleria*, *Vahlkampfia*), and in some body cells of animals. Therefore, we can state that amoeboid life has a great diversity, and that this complexity can be understood only by its long period of evolution.

In spite of this diversity, the modern representatives of amoeboid life are organized according to comparable principles: they behave very similarly and form an ecological entity that allows us to speak of them as a group (amoebae, rhizopods). Besides the numerous slime moulds, about 250 naked lobose and about 150 naked filose "true" species are known from the different terrestrial, freshwater and marine environments. Nevertheless it is to be expected that several hundred additional species names exist in the literature, and that some thousands of species still await discovery.

Naked amoebae exist in all aquatic and terrestrial habitats. They are mainly

bound to the substrate and creep during locomotion; they may enter the water column by drifting after assuming the floating radiosa form (Fig. 5).They live mainly among vegetation, where they compete with predatory flagellates, ciliates and rotifers. Despite their slowness, they are successful hunters and trappers and are masters of phagocytosis.They often occur in great numbers, particularly in surface scum. Some feeding specialists may regulate the abundance of certain autotrophic and heterotrophic prey populations.

PRACTICAL TECHNIQUES

Because research institutes at universities do not necessarily maintain permanent collections of amoebae, and because commercial sources may be quite expensive, it is best (and sometimes necessary) to obtain living material from natural sources. The isolation of specimens from natural habitats has important advantages in that it provides insights into the ecology of the protists. Besides the simple catching and transport devices, a good stereomicroscope with brightfield illumination together with disposable Petri dishes are the most important pieces of equipment needed. However, it often takes several weeks or even months for cultures to become fully established, if no well-tried and flourishing sources are known.

Referring to their feeding strategies, the usually strictly heterotrophic naked amoebae may be subdivided into three ecological types independent of their origin in freshwater or marine sources. All small species – those ranging in size from less than 5 µm to about 90 µm – occur in and on biofilms and thrive on small particles and other food organisms such as bacteria and, to a lesser extent, on yeasts, small unicellular algae and nanoflagellates (bactivorous fraction, type 1). In the larger forms – from 15 µm to more than 500 µm – the spectrum of prey also includes larger organisms, namely filamentous and unicellular algae (algivorous fraction, type 2: for example the vampyrellid amoebae) or other protozoa and small metazoa (carnivorous or zootrophic fraction, type 3). It may, therefore, be possible to decide in advance whether a sample of water and sediment meets the requirements for the occurrence of amoeboid predators: if we can recognize potential food organisms we may well find amoebae, perhaps after a short delay. Enrichment procedures may then be restricted to promoting the development of phototrophic and heterotrophic microorganisms as prey.

Pelomyxa palustris

The few species of the larger representatives of naked amoebae are not rare, but quite hard to detect. This is especially true for the giant amoeba *Pelomyxa palustris*, a species which, as yet, has not been cultivated, but is unparalleled in its appropriateness for the demonstration of amoeboid motion. The best sites for this algivorous or herbivorous species are submersed leaf litter of forest swamps and pools with low oxygen and high hydrogen sulphide content (especially during summer and autumn). Further, they can be found in decaying algal mats floating in ponds and reservoirs as well as in the putrefying mud of stagnant waters (during spring and summer). Having knowledge of such superb resources would be of great advantage, as one can

expect sufficient investigation material without losing a lot of time for enrichment. For demonstration of specimens, a sample of about 1 litre of vegetation remnants and water is collected from the habitat the day before the investigation and carefully (with a minimum of disturbance) transported to the laboratory. It is then distributed between several Petri dishes of 15 cm or more in diameter. Once the sediment particles have settled and partially clumped, which may take several hours, the giant amoebae are easy to detect in the translucent regions of the bottom, normally under moderate magnification of the stereomicroscope. In some cases, or with a little experience, the often up to 2.5 mm sized specimens become visible to the naked eye. The isolation and transfer to coverslip preparations is best done with aid of blunt polypropylene pipettes with correspondingly wide diameters; the use of sharp-edged glass pipettes may lead to injuries. It is necessary to support the coverslip with wax moulding clay (Plasticine, Plastilin) or fragments of glass needles to protect the thick amoebae from too high a pressure. During the preparation, any contact of the delicate surface of amoebae with the phase border between the liquid and gaseous phase must be avoided because of the risk of the cells bursting.

Under similar but slightly more hygienic conditions, one can find the large amoebae of the so-called *proteus-chaos* group in pools and ponds as well as on the banks of lakes and slow running creeks (sometimes even in aquaria). The breeding of these predatory amoebae, which occur normally on the surface of submerged vegetation (periphyton) or in the organic mud of sediments, is usually easier if the initial culture is started with a sufficient quantity of potential prey organisms (mainly ciliates such as *Paramecium*, *Spirostomum*, *Stentor, Tetrahymena*). Such prey organisms may be part of the original sample, or they can be added from laboratory stocks. The cultivation takes place in shallow Petri dishes (diameter 9 cm) after the addition of one or two boiled rice or wheat grains which must be replaced with fresh ones every three weeks. Under such conditions, a food chain from bacteria via bacterivorous ciliates to predatory amoebae develops. Larger predators such as snails and some oligochaetes or turbellarians, which may feed on amoebae, must be removed. For the study of the locomotion and feeding behaviour, only amoebae which have been kept individually in Petri dishes and starved for at least 24 hours should be used. The starved amoebae are then transferred (together with prey organisms) to coverslip preparations for observation, which takes place under moderate magnifications using x 10 and x 40 objectives. A similar method of cultivation, based on a defined culture medium is described on p. 92.

Thecamoeba

When the material has been sampled from less productive waters (classified as oligotrophic or mesotrophic habitats) containing cyanobacteria or single-celled green algae, it is sometimes possible to detect the naked cells of the genus *Thecamoeba* (which does not belong to the lobose testate amoebae as the name suggests). However, it is not possible to obtain the quantities of specimens of this species necessary for teaching purposes without several months of crude cultivation. To enhance the growth, occasional addition of filamentous cyanobacteria (*Oscillatoria, Lyngbya*) from separate stock cultures is necessary.

Smaller Amoebae

If the students are pressed for time, or if no suitable habitats have been found, the enrichment methods for smaller amoebae remain the only solution. Overfeeding of normal scratching samples (that is material that was scratched from naturel surfaces), which may be achieved by adding four wheat grains or pinhead-sized pieces of dried swede (rutabaga, *Brassica napus napobrassica*) to the Petri dishes, leads to the formation of a surface scum within a few days. Such a scum contains rich populations of bacteria, yeasts, nanoflagellates, ciliates and so-called *limax*-amoebae. The slug-like amoebae and some others that are more spatulate in outline (*Vannella*) may be picked up from the surface with a coverglass and transferred directly to the slide. Another method to enrich specifically *Vannella* species is based on agar techniques. About 10 days before use, some microlitres of pond water or seawater with old decaying plant material are dropped onto the surface of thin layers of 1% agar in Petri dishes. The introduced amoebae multiply rapidly on the agar surface, and from the incubation site they grow out in a circular growth pattern ("fairy rings"). Resuspended in water and transferred with a micropipette to the coverslip preparation, or dipped away from the agar with the coverslip, they should be analysed with stronger magnifications (x 40, x 63 or x 100 oil immersion) and phase contrast optics; or, best of all, with Nomarski differential interference contrast.

Amoeboid Motion

When studying amoeboid motion under the light microscope, it may be advantageous to provoke the formation of pseudopodia artificially by slightly modifying the adjustment rules for proper Köhler illumination. A minimal opening size of the field diaphragm is a quite useful tool, when the investigator shifts the body of the amoeba (via the stage control) to the border of the illuminated field so that the central parts of the specimen are in the illuminated area and one peripheral part in the shadowed area. Because of their preference for darkness, that is negative phototaxis, all amoebae tend to move spontaneously into the darker regions of the field of vision, a process that can be accelerated or slowed down by changing the voltage of the illumination source. Such tricks are necessary in order to see the typical locomotory forms depicted in the identification guides, especially when using phase contrast optics. The opposite behaviour – the retraction of pseudopodia – is inducible by a more intensive illumination of the tip of an expanding pseudopodium.The time span needed for the experimental induction of streaming reversal and retraction in a formerly expanding pseudopodium corresponds with the period necessary for assembling a functioning actin network in the ectoplasmic wall. Such a time interval – about eight seconds in the case of *Amoeba proteus* – can be determined with the aid of a stop clock or, more impressively, by video documentation. Experimental actions of this kind are very helpful in obtaining a clear picture of the streaming and transformation processes; unfortunately, however, some of the freshly isolated strains (in contrast to established cultures) may be quite insensitive to changes in illumination.

An initial determination of the different kinds of food acquisition and engulfment is achieved by the stereomicroscopic inspection of those Petri dishes used for enrichment and cultivation of amoebae of the *proteus-chaos* group, especially at such

places where the amoebae surround the cereal grains that are deposited at the bottom. If the sediment materials remain undisturbed, observation under low magnification (and moderate illumination) will reveal how quickly pseudopodia may be formed and how effectively they engulf the prey.

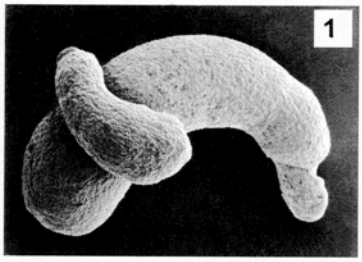
Phagocytotic activities cannot be analysed in detail without using higher magnifications. Normally, one needs many prey organisms and amoebae in the coverslip preparation. If there are enough, one has a good chance of seeing the entire process of phagocytosis, especially after enhancing differenciation with contrast methods. However, it is necessary to search at the beginning only with low magnification and under very moderate light intensity. It is important to recognize that three pseudopodia are formed simultaneously in most cases, one each on the left, right and upper side of the prey specimen, and that their tips fuse as the enclosure of the prey is completed. It is remarkable (and easy to study) that the short-lasting contact with a fast-swimming *Paramecium* cell might already be a tactile stimulus strong enough to start the pseudopodial formation and the process of engulfment.

Vampyrellids

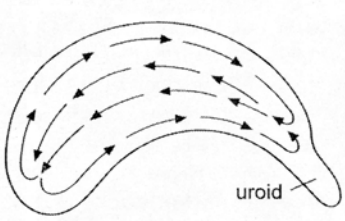
Filopodial amoebae such as the vampyrellids *Hyalodiscus*, *Vampyrella*, *Gobiella* and *Leptophrys* are mainly predators of or grazers on algae; they occur, therefore, particularly in algal mats and in the biofilms of aquatic plants, submersed branches, stones, and so forth, in fact in all freshwater habitats and in marine rock pools. They are detectable during the whole year, even under ice, but mostly from spring to early autumn. The isolated material is stored in Petri dishes at low densities and must be inspected every second or third day. It is essential that the samples contain abundant diatoms or filamentous green algae (conjugates such as *Spirogyra*, *Mougeotia* or *Zygnema*, Class Zygnematophyceae) or *Oedogonium* and *Bulbochaete* (Class Ulvophyceae). Freshwater species of the filamentous green algae belonging to the genus *Cladophora* (Class Ulvophyceae) are not suitable, only those isolated from marine habitats. If a sample is infected, the enrichment of amoebae can be started immediately. Microscopic investigation can be performed when the infection is well underway or reaches its climax, a phenomenon that is accompanied by the appearance of brown-red spots within the otherwise greenish algal wads. This moment will be reached after about eight to ten days in culture. For performing analyses of attacks of vampyrellids against unicellular or filamentous algae, it is essential that a sufficient quantity of freely moving amoeboid 'swarmers' are available. In the course of the life cycle (Fig. 11), such motile stages appear after hatching out of digestive cysts and division cysts; if enough healthy algal filaments are present, vampyrellids immediately start the attacking procedures. A good chance of success exists when freshly hatched amoebae are caught with micropipettes and transferred together with

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Fig. 1, 2. *Pelomyxa palustris*. **1**: Size of larger specimen about 1 mm. **2**: Scheme of protoplasmic streaming. **Fig. 3, 4.** *Amoeba proteus* (size about 600 µm), changes of cell shape. **Fig. 5 – 8.** *Vannella simplex* (50 µm). **Fig. 9.** *Thecamoeba sphaeronucleolus* (120 µm) with numerous food vacuoles in the posterior part of the granular cytoplasm; hyaline marginal ectoplasm with longitudinal folds. **Fig. 10.** *Thecamoeba striata* (80 µm) with typical longitudinal stripes of the pellicle. **Fig. 11.** Life cycle of the vampyrellids.

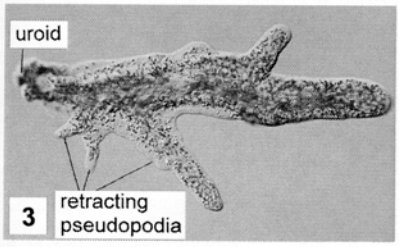


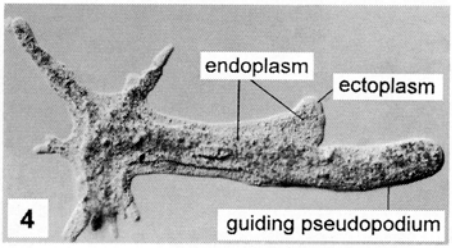
Two specimens (SEM)



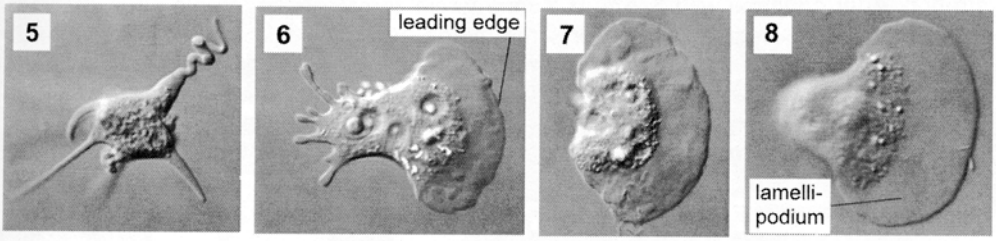
Fountain streaming of the protoplasm

Pelomyxa palustris (1, 2)

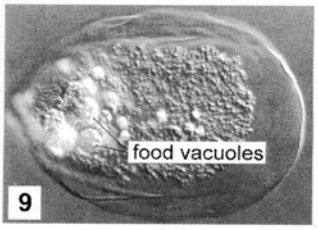




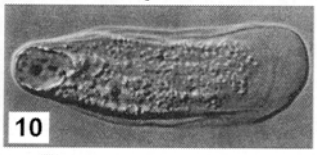
Amoeba proteus (3, 4)



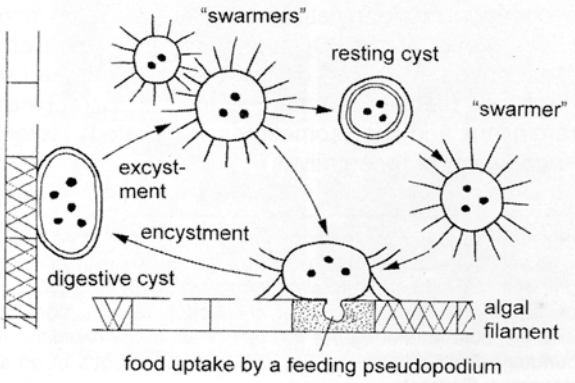
Vannella simplex, **transition from the floating form (radiosa form) to the normal creeping form** (5 – 8)



Thecamoeba sphaeronucleolus



Thecamoeba striata



11 Life cycle of the vampyrellids

healthy algae to the coverslip preparation. In well-infected cultures in which empty algal cells and swarming parasites are in the majority, it may be sufficient to investigate a piece of the brown-red areas directly. One should bear in mind that the highly aggressive cells of *Vampyrella lateritia* may already finish the first attack during the short time span necessary for the preparation. For the other species, however, the process of food acquisition requires about 20 to 30 minutes, depending on the size and starvation status of the vampyrellids. Large specimens (especially of *Gobiella*) may feed for two or more hours and are able to attack more than thirty algal cells. The early phases of the attacks are very sensitive to illumination, that is reduction of the lamp voltage to a minimum is essential. After getting in close touch with the algal cells, however, the vampyrellids become more insensitive to light. From then on, the light-consuming optical contrast enhancement may be brought into action without being detrimental to the spectacular events occuring during ingestion. The activity of filopodia is best documented when specimens are moving along algal filaments. In profile view (Fig. 17), the formation, prolongation and bending of frontal filopodia as well as the contraction and collapse of caudal filopodia may be easily followed.

Terrestrial Amoebae

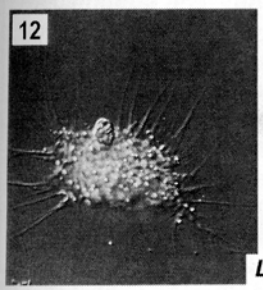
Regarding the enrichment procedures for terrestrial amoebae, the reader is referred to the agar methods: pea-sized crumbs of garden soil or compost soil or of leaf litter are brought onto the surface of agar plates about five to six days before demonstration. Agar enriched with 3–5% of a commercial tomato or vegetable juice will also exhibit amoeboid stages of acellular slime molds. Some organisms that are too sensitive and unsuitable for the agar method may be collected by washing out moss pads (*Thecamoeba terricola*).

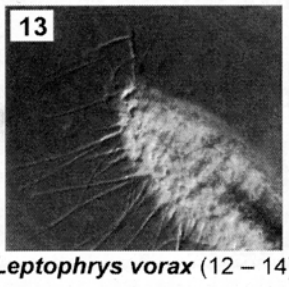
When dealing with naked rhizopods, studies of locomotion and of the different kinds of food uptake might be given top priority.

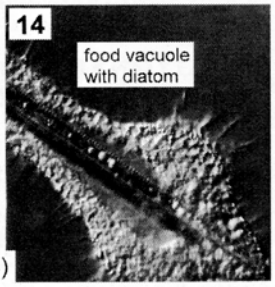
GENERA AND SPECIES

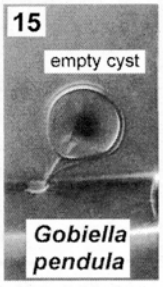
The microorganisms treated in this chapter can ecologically and functionally be described as amoebae or rhizopods. With respect to their phylogeny they are part of a common monophyletic taxon (Amoebozoa) and are presently believed to be the sister group of the Opisthokonta, the so-called Fungi-Animalia supergroup. The Amoebozoa, however, do not contain all those taxa that may be considered also as rhizopods (sensu lato), namely the Cercozoa (with the filopodial testaceans), the Foraminifera and the (sometimes flagellated) Heterolobosa (with *Naegleria* and *Vahlkampfia* as representatives).

Fig. 12 – 14. *Leptophrys vorax*. **12:** Size without filopodia 35 µm. **13, 14:** Details of the cell. **14:** Large food vacuole with enclosed diatom. **Fig. 15.** *Gobiella (Vampyrella) pendula,* diameter of empty cyst 30 µm. **Fig. 16 – 18.** *Vampyrella lateritia.* **16:** Top view (45 µm). **17:** Profile view (40 µm). **Fig. 19.** *Gobiella borealis* during attack on an *Oedogonium* filament. **Fig. 20, 21.** *Halodiscus pedatus* (65 µm). The filopodia anchoring the cell to the substrate are difficult to recognize. **Fig. 22.** *Hyalodiscus rubicundus* (45 µm), top view. **Fig. 23 – 28.** Six stages of an attack by *Hyalodiscus* on a cell of an *Oedogonium* filament.

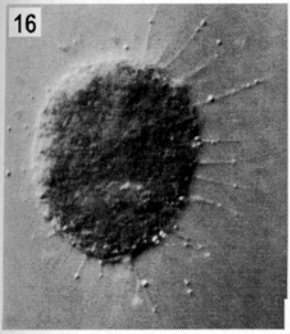


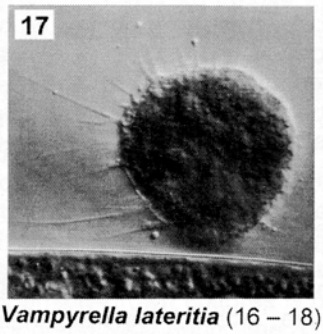


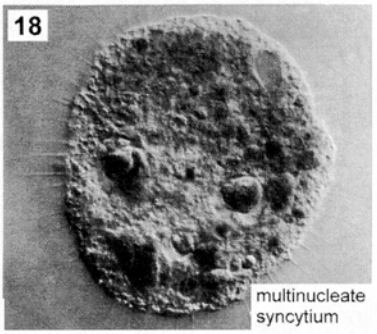




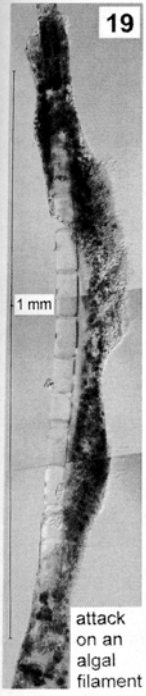
Leptophrys vorax (12 – 14)



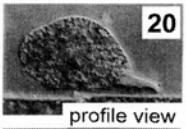


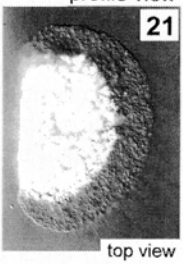


Vampyrella lateritia (16 – 18)

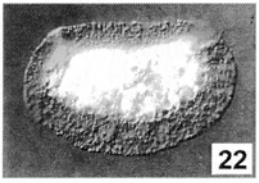


Gobiella borealis

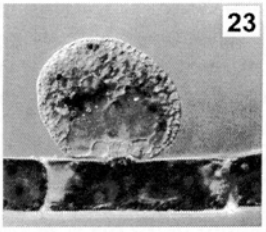


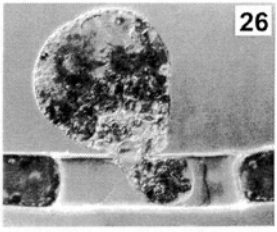


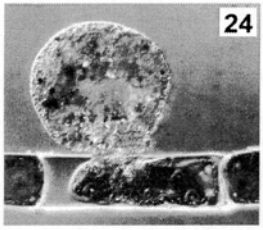
Hyalodiscus pedatus (20, 21)

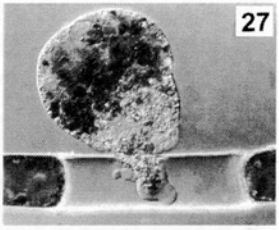


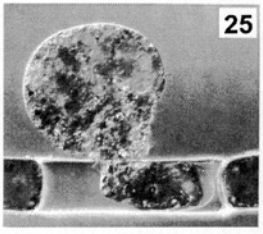
Hyalodiscus rubicundus

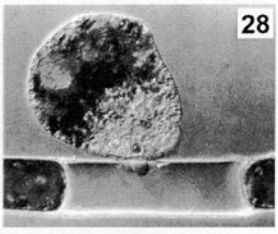












Attack on an *Oedogonium* cell by *Hyalodiscus* (23 – 28)

AMOEBOZOA

LOBOSA
GYMNAMOEBEA
TESTACEALOBOSEA
CONOSA
ARCHAMOEBA
MYCETOZOA
"Slime Moulds" (Protostelea, Myxogastrea, Dictyostelea)
ACONCHULINA

Current opinion has the taxon Amoebozoa containing two large subtaxa: the Lobosa (with the naked Gymnamoebea and the testate Testacealobosea) and the Conosa. The Conosa are subdivided into the Archamoeba (*Pelomyxa palustris)* and the Mycetozoa (with the classical true slime moulds and the filopodial Aconchulina).

In the following, only members of the subtaxa in bold letters of the above box are considered.

Pelomyxa palustris (Archamoeba) (Fig. 1, 2)

The taxon Archamoeba contains only a few species. Due to the possession of non-functioning flagella and some 'archaic' features, these species were formerly believed to represent a very primitive status in the evolution of protozoa. Nowadays these features (lack of mitochondria, dictyosomes and endoplasmic reticulum) are seen as the results of a secondary simplification. The slug-like cell body of *Pelomyxa* contains several types of endosymbiotic bacteria that surround the numerous nuclei and obviously function as mitochondria. In a coverslip preparation fountain-like streaming (fountain movement) with onward streaming of endoplasm and retrograde gliding of the ectoplasm can be observed. The transformation zone, in which the conversion of peripheral gel-like protoplasm (ectoplasm) into centrally streaming protoplasm (endoplasm) occurs, is located in the posterior region, the uroid. The opposite process at the anterior end is called endoplasm-ectoplasm-transformation (Fig. 2).

Amoeba proteus and ***Chaos*** sp. (Gymnamoebea, Amoebidae) (Fig. 3, 4)

For over a century, the predatory species belonging to the so-called *proteus-chaos* group serve as model organisms for the study of amoeboid movement and have played a central role in the study of contractility. This importance is based on the presence of actomyosins and related proteins in the pseudopodia. *Amoeba proteus* is treated in a special chapter.

Most representatives of the genus *Chaos* are multinucleate and appear as polypodial cells. Due to their large size (1–2 mm is quite common), two or three leading pseudopodia may be formed temporarily. In feeding experiments, giant *Spirostomum* cells may be engulfed within a few minutes.

Thecamoeba and ***Vannella*** (Gymnamoebea, Thecamoebidae, Vannellidae) (Fig. 5 – 10)

The longish to transverse-oval and uninucleate or binucleate representatives of the Thecamoebidae and Vannellidae are easy to identify by their weakly developed capability to change their body form as long as the amoebae are creeping. The Thecamoebidae possess a comparatively rigid pellicle, very often with longitudinal folds leading to a striated appearance (Fig. 10). Typical of both families is the strong contact between the ventral cell surface and the substrate. This fastening leads to a kind of movement that is called «rolling motion».

This kind of motion is demonstrable by foreign particles adhering to the cell surface. As indicated by the particles, the dorsal (free) surface glides at twice the velocity of the cell itself to the frontal edge, where it turns down to the substrate and becomes stationary. In *Thecamoeba*, it is best, in order to see the process, to add some yeast cells or latex particles to the medium; in *Vannella*, the many bacteria of the culture medium may play a similar role as marker particles.

The observation of food uptake in *Thecamoeba* is exciting but difficult to realize. One has to offer filamentous cyanobacteria; these are slowly bent by the amoeba, rolled up within an invagination of the cell surface and then incorporated. However, the individual phases of the phagocytotic process become easily discernible when a surplus of amoebae from densely-grown cultures is brought into action. The food uptake in *Vannella* occurs spontaneously during the rolling motion when the cells overrun and ingest those bacteria that are affixed to the substrate (glass). The phagocytotic events take about one minute; during this time the food vacuoles form and separate from the ventral surface.

Gobiella, Hyalodiscus, Leptophrys and ***Vampyrella*** (Aconchulina, Vampyrellidae) (Fig. 11 – 28)

All members of the Vampyrellidae show a life cycle that is marked by the succession of freely-moving and encysted stages (Fig. 11). The encysted stage lasts one or two days; it starts immediately after food uptake and ends with the hatching of the swarming filose trophozoites ('swarmers'). These uninucleate or multinucleate cells are distinguished by unusual kinds of food uptake: they feed on the living cell contents of algal filaments (for example *Spirogyra*, *Mougeotia* or *Oedogonium*) or unicells (for example *Closterium* or *Cosmarium*) that are captured with the aid of a special bell-shaped ingestion pseudopodium (*Gobiella*, *Hyalodiscus*, *Vampyrella*), or they feed on algae that are surrounded by feeding pseudopodia and completely ingested (*Leptophrys*).

The uninucleate or multinucleate representatives of *Leptophrys vorax* (Fig. 12–14) reach a length of 30–500 µm or more. The actual size (and number of nuclei) depends on the life history of individuals which may fuse into large syncytia. Normally, they creep, strongly flattened onto the substrate, and the frontal filopodia pull the cell forward. They encompass the prey (diatoms, filamentous algae, sometimes also nematodes) during locomotion and then transform into the encysted stage. When hatching, they leave the undigested prey remnants in the empty cyst.

Most of the about 70 named species, subsumed under the genera *Gobiella*, *Hyalodiscus* and *Vampyrella* (Fig. 16 – 28), differ from other amoebae by their bright reddish (orange-red, brick-red) colour and are therefore easy to recognize. This hue stems from undigested carotenes and other pigments taken up with the algal diet. The trophozoites or "swarmers" are mostly radially symmetrical and superficially resemble heliozoans, from which they are distinguished by the ultrastructure of their pseudopodia. Regarding the spectrum of prey or host organisms, the individual species have realized nearly all imaginable ecological niches ("walled organisms" from green algae, red algae and diatoms to animal eggs and protozoan cysts). A widespread species in Europe and North America is *Vampyrella lateritia*, which attacks the filaments of Zygnematophyceae. Similarly frequent is *Gobiella closterii* (= *Vampyrella*) and *Gobiella borealis* (Fig. 19) with unicellular *Closterium* cells and *Oedogonium* filaments as prey organisms. *Gobiella pendula* (= *Vampyrella*) forms a stemmed cyst anchored within an emptied *Oedogonium* or *Bulbochaete* cell (Fig. 15).

The first step of food uptake is the penetration by puncture of the cell wall, mainly either by focal disintegration (*Vampyrella*, *Hyalodiscus*) or by cutting a round operculum (= fenestration, *Gobiella*). In both cases, short pseudopodia crumble away the cellulose layers of the wall. After perforation, the algal cell content normally bursts out due to its hydrostatic pressure, and fluid and compact cytoplasmic components stream into the amoeba. After a short phase of collapsing, one or two bell-shaped pseudopodia are formed at the puncture: each prolongs into the lumen, turns over the cytoplasmic algal remnants, retracts and is reabsorbed by the amoeba. The edges of such pseudopodia frequently do not reach a breadth of more than 1 µm and are therefore difficult to recognize under the light microscope; in the past, the question of their participation in the process of food acquisition remained unsolved and led to the wrong assumption that sucking phenomena must play an essential role in food uptake (in the genus *Vampyrella*).

The mainly uninucleate cells of *Hyalodiscus* look like *Vannella* in that they show a separation into a posterior or central hump and a frontal lamellipodium. However, they do not move directly on the substrate but have short filopodia on the lower side that mediate the contact (Fig. 20). The filopodial stumps and myelin-like bodies (membranosomes) on the cell surface give a dotted appearance (Fig. 21, 22), a character not only useful for separation and identification of the genus, but also for analysing its characteristic rolling motion. The spectrum of prey is the largest within the vampyrellids: it comprises (in corresponding species from freshwater and marine habitats) algal filaments (Chlorophyceae, Streptophyceae and Rhodophyceae), solitary algal cells (Chlorophyceae, Streptophyceae and diatoms), rotifer eggs and ciliate cysts. The mode of penetration of the protective coverings resembles that of *Vampyrella*.

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FILMS (spoken commentary in German)

Grell, K. G. (1967): Shape and locomotion of free-living amoebae. – Film C 942 IWF Göttingen, 10 ½ min.

Grell, K.G. (1967): Feeding and reproduction of free-living amoebae. – Film C 943 IWF Göttingen, 11 ½ min.

Hülsmann, N. (1983): Development and mode of nutrition in *Vampyrella lateritia* (Rhizopoda). – Film C 1522 IWF Göttingen, 12 ½ min.

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Amoeba proteus

Renate Radek, Klaus Hausmann and (the late) Wilhelm Stockem

Because of its size and ease of handling, *Amoeba proteus* is well suited for light microscopic observations. Since it is not always possible to find the species in foul, bacteria-rich pools and ponds, for example on the underside of water lily leaves (*Nymphaea alba, Nuphar lutea),* the use of laboratory cultures is recommended. The advantages of obtaining living specimens from natural habitats are described on page 81 – 82 as well as the practical techniques for their isolation.

Possible sources of laboratory cultures are Ward's Natural Science, 5100 West Henrietta Road, P.O. Box 92912, ROCHESTER, NEW YORK 14692-9012, USA, toll-free phone in USA 001-800-962-2660, internet http//wardsci.com; Dr. W. Hölters, Am Grünen Weg 24, 50259 PULHEIM, GERMANY, phone 00 49-2234-986200, fax 00 49-2234-986201.

CULTIVATION

Amoeba proteus may be cultured in Petri dishes in the (slightly modified) medium of Chalkley. To make a ten times concentrated solution, dissolve 800 mg of NaCl, 40 mg $NaHCO_3$, 40 mg KCl, 54 mg $CaCl_2 \cdot H_2O$ and 16 mg $Ca(H_2PO_4) \cdot H_2O$ in 1 litre of distilled water. Dilute this in the ratio 1 part of stock solution to 9 parts of distilled water before use. Permanent cultures should be supplied with boiled wheat or rice grains to support the growth of the bacteria that will be used as food by the amoebae. Densely clustered amoebae will gather around the grains, because they will predominantly stay and divide where they find a good source of food. Addition of larger food organisms such as ciliates of the genera *Tetrahymena*, *Paramecium* or *Colpidium* is preferred when larger numbers of amoebae are required. Feeding should take place once or twice a week with a small amount of concentrated ciliate culture. When the amoebae cultures become too dense, some individuals can be transferred to a new dish by pipetting. The culture vessels should be kept in at least partial darkness, because intense light is a stress factor for colourless organisms. The pH should be slightly acidic (6.8–6.5) and the temperature about 20 to 22° C (room temperature). It is important to realise that the readiness to move and feed is optimal when the amoebae have been previously starved for 4-5 days in pure culture fluid.

PRACTICAL TECHNIQUES

By means of a stereomicroscope, some amoebae are collected from the starved cultures with a pipette, put onto a microscope slide and covered with a coverslip. The corners of the coverslip will have been previously dabbed with small amounts of wax or Vaseline to avoid too much pressure on the large organisms. Fibres of cotton or slivers of coverslips may be used instead. Due to the disturbance, the amoebae

will remain in a resting form for some time before cytoplasmic streaming and locomotion recover. Avoid too much warming from the microscope light and mechanical vibrations. Heterotrophic protists such as amoebae generally try to escape from illuminated regions. By closing the field diaphragm, portions of an amoeba may be specifically illuminated, and as a result the amoeba moves away from that region.This process is described in more detail on page 83.

OBSERVATIONS

Morphology, Locomotion and Membranes

Amoeba proteus has a size of 300–600 µm and an irregular form (Fig. 1a, 2). Cell organelles visible in the light microscope include the disc-like nucleus (Fig. 1c, 2) with its numerous nucleoli and the contractile vacuole (Fig. 1d, 2). There are also food vacuoles of different sizes and contents and crystal-bearing vacuoles with end products of digestion as triuret, a derivate of uric acid. Mitochondria, lipid droplets and small vesicles are part of the compact granules of the endoplasm.

The cell shape changes constantly. Rigid skeletal elements supporting the plasma membrane, such as the microtubules in the cortex of ciliates, are absent from amoebae. Instead, an irregular network of actin filaments supports the plasma membrane. On the one hand, this network gives the cell some stability and, together with the plasma membrane, is instrumental in maintaining the high internal hydrostatic pressure. On the other hand, the actin filaments (via interaction with myosin) allow active dynamic processes, such as the change of shape, motility and ingestion of food. Such active processes can occur because of the ability of the actomyosin systems to undergo rapid changes in conformation. The actomyosin network is followed by the transparent gel-like ectoplasm known as the hyaloplasm (Fig. 1e). The border between the ectoplasm and the last layer, the endoplasm, is formed by a second layer of actomyosin that functions like a filter. Fluid, hyaline cytoplasm flows through the meshes, but larger cell organelles are held back. The sol-like endoplasm is opaque under the light microscope by virtue of its numerous organelles ("granules") and is called granuloplasm. As in most protozoa, the cell surface (the plasma membrane) is covered by a surface coat, the glycocalyx.

During movement, broad pseudopodia (lobopodia), which are not only used for locomotion but also for food capture, are formed (Fig. 1a, b, 2, 3). Pseudopodia of other amoebae may be long and slender filopodia, as in the Vampyrellidae (p. 84) and in testate amoebae of the order Euglyphida (p. 107) or like a network, the reticulopodia of foraminifera (p.112). *Amoeba proteus* individuals floating in the medium have long pseudopodia radiating in all directions; creeping forms, however, are flattened and possess a clear anterior and posterior region (uroid; Fig. 2). Polarity is only a temporary physiological condition. A posterior to anterior cytoplasmic streaming leads to the formation of new pseudopodia in the direction of movement, whereas at the uroid the pseudopodia retract. The surface of the retracting pseudopodia is irregularly folded, that of the expanding ones is smooth. Important for active movement is the formation of focal contacts with the substrate. These small cell extensions anchor the cell to uneven patches and thus serve as a lever for locomotion. New focal contacts are formed and old one detached.

The membrane necessary for the formation of new cell extensions is mainly supplied by shifting the complete plasma membrane from posterior to anterior. Membrane growth by fusion with exocytotic vesicles and membrane synthesis is of lesser importance. The membrane shift can be visualized by incubating the amoeba in a medium containing particles such as latex or charcoal which attach to the surface. However, single particles are seen to move in a posterior direction simultaneously to the anteriorly directed membrane shift. Via the surface coat, these particles are tightly fixed to membrane components. Some minutes after such marked amoebae have been transferred to fresh medium free of particles, the particles aggregate in several areas and begin to concentrate at the uroid. Now, the cell surface is cleaned by permanent pinocytosis in these areas and by release of the material to the underlying substrate. A track of particles several hundred micrometers long remains on the substrate.

Ingestion of Food by Flow of Membranes

The ingestion of particulate food (phagocytosis) can be distinguished from ingestion of fluid or suspended nutrients (pinocytosis) with the light microscope. The term pinocytosis goes back to the Greek and means "drinking of the cell", phagocytosis means "ingestion of particulate food by the cell". Normally, moving amoebae have a permanent pinocytotic activity at the uroid. The addition of certain substances such as albumin, ferritin pH 3–4.5, or alcian blue at pH 6.0 to resting amoebae induces pinocytosis. In both processes, phagocytosis and induced pinocytosis, positively charged substances or prey organisms first adhere to the plasma membrane. For ingestion, they are then enclosed by pseudopodia or directly pinched off in vacuoles. Later on, fusion with lysosomes provides enzymes for digestion. Indigestible residues are released via fusion of the defecation vacuoles with the plasma membrane.

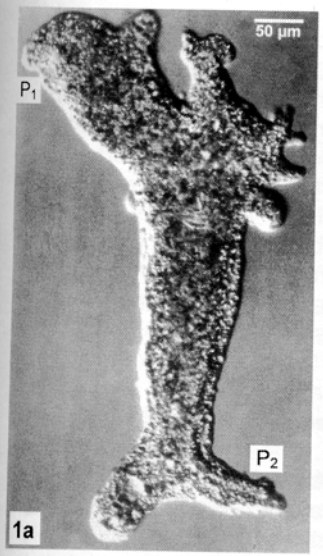
For observation of phagocytosis, a medium with numerous food ciliates (for example *Tetrahymena, Colpidium, Paramecium*) is added to one side of the coverslip after the amoebae have started their normal movement under the microscope. The amoebae mostly beome rounded as a first reaction and then begin to form small, hyaline pseudopodia.

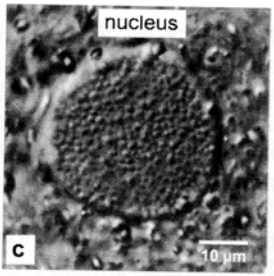
By careful observation, the way in which the pseudopodia flow around and ingest the prey will be understood (Fig. 3). Often, the prey organisms stop their locomotion after they contact the surface of the amoeba, and only become active again when they are completely enclosed in a phagocytotic cavity. The shape and structure of the prey completely disintegrate during digestion.

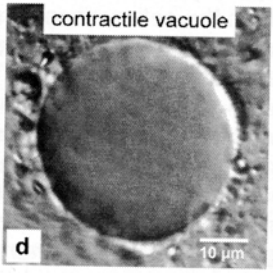
For observation of induced pinocytosis, fresh egg white diluted 1:10 with distilled water may be used. Some amoebae are transferred to the microscope slide, the

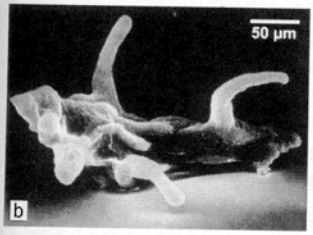
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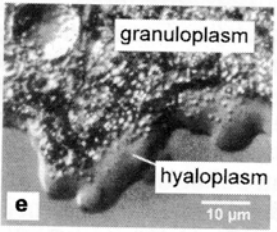
Fig. 1. *Amoeba proteus.* **a:** Polypodial amoeba with expanding (P_1) and retracting (P_2) pseudopodia. **b:** Scanning electron micrograph showing pseudopodia. **c:** Nucleus with numerous nucleoli. **d:** Contractile vacuole in filled state (diastole). **e:** Division between hyaloplasm and granuloplasm. **Fig. 2.** Scheme of *Amoeba proteus* with pseudopodia and cell organelles. Long arrow: direction of movement; small arrows: direction of cytoplasmic streaming in pseudopodia. **Fig. 3.** Phagocytosis. Several short pseudopodia are formed and enclose the prey in a cavity. Fat arrow: prey particle; slender arrows: direction of cytoplasmic flow. **Fig. 4.** Pinocytosis induced with egg albumin. Initial phases (**a, b**) and pinocytotic vesicles pinching off at the base of a canal (**c, d**).

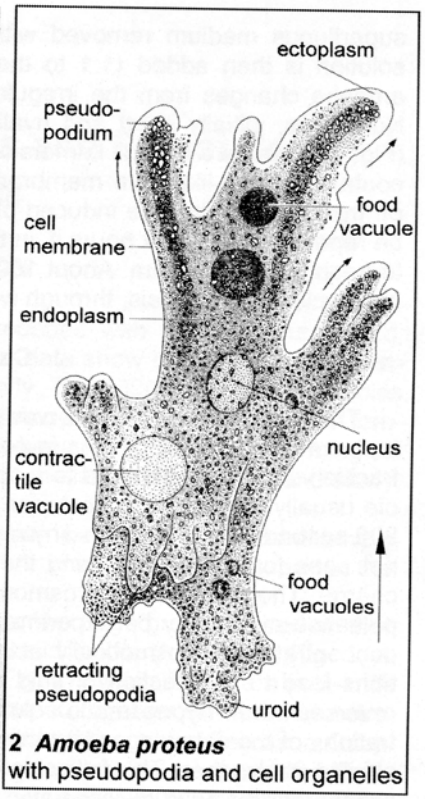




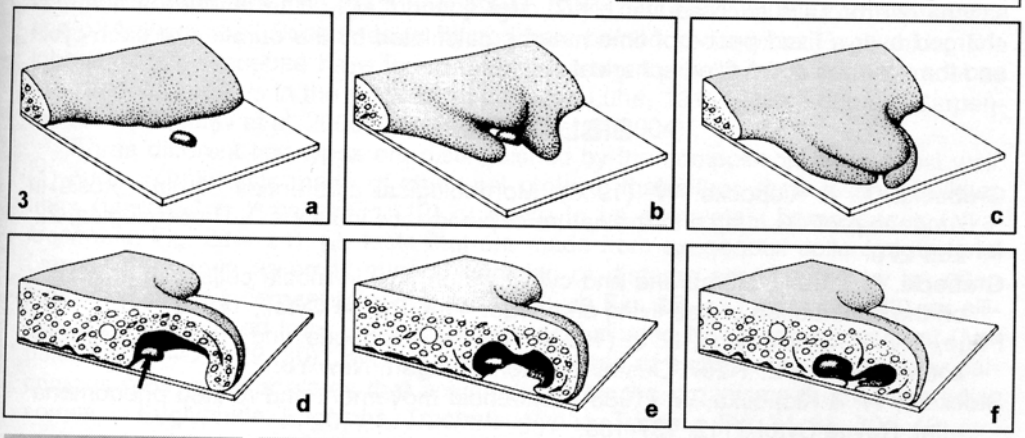


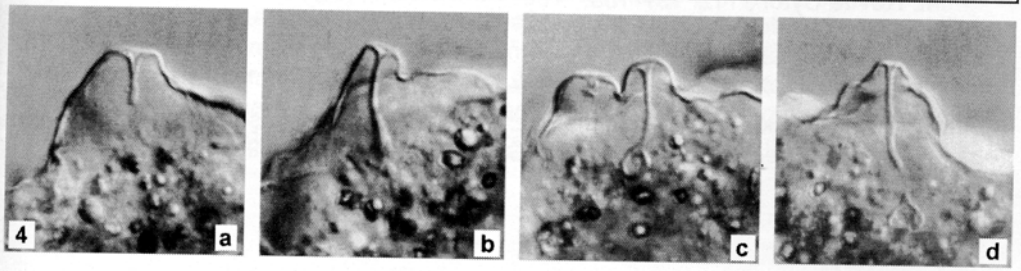






2 *Amoeba proteus*
with pseudopodia and cell organelles





superfluous medium removed with a piece of filter paper, and a drop of induction solution is then added (1:1 to the rest of the medium). A few minutes later, the amoeba changes from the irregular locomotory form to the spherical resting form. Numerous, initially short and hyaline peudopodia are formed at the cell periphery (Fig. 4). Using a x 100 oil immersion objective, it can be seen that most pseudopodia contain a canal-like, thin membrane invagination. Endocytotic vesicles are pinched off from their ends. The induced pinocytosis takes about 10 to 15 minutes and may be repeated every four hours if, in the meantime, the amoebae have been transferred to fresh culture medium. About 100 pinocytotic canals are formed per amoeba during one cycle of pinocytosis, through which the cell surface is reduced to about one half.

Contractile Vacuole

The contractile vacuole serves mainly for osmoregulation. It floats freely in the cytoplasm of amoebae. This is in contrast to the situation in ciliates, in which the contractile vacuole is anchored to a special pore complex. In *Amoeba proteus*, the vacuole usually opens in the posterior region. The contents are discharged every 150 to 800 seconds by fusion with any region of the plasma membrane. After expulsion it is not seen for a short time and then is seen to grow continuously until the next discharge. The mechanism of osmoregulation has not been completely elucidated. The pulsation cycle may be experimentally changed by addition of media with different concentrations of osmotically active substances: Concentrated, hyperosmotic solutions lead to a reduction of fluid expulsion by the vacuole, whereas the reaction is reversed when hypoosmotic solutions are used. However, not only are the concentrations of the substances important, but also their chemical nature and their relationship to each other. The following substances may be used for experiments: 30 mM saccharose or salts such as NaCl, KCl, $MgCl_2$ and $CaCl_2$. The amount of fluid discharged over a fixed period of time may be calculated by the duration of each cycle and the volumes of the filled spherical vacuole.

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FILM

Klein, H. P. & Stockem, W. (2002): Pinocytose und Phagocytose bei *Amoeba proteus* (spoken commentary in English and German). – Film C 7059 IWF Göttingen, 15 min.

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Testate Amoebae in Mosses and Forest Soils

Ralf Meisterfeld

INTRODUCTION

Under the name testate amoebae various amoebae with a single chambered, rigid or flexible test (shell) are combined. Molecular data show that the ability to generate such tests evolved several times independently. The 1600 or so known species therefore do not form a natural group of closely related organisms with a single common ancestor. However, for practical reasons, because of their morphological similarity, their identical habitats and the similarity of methods of collection and study, they are still treated as one large group.

In bogs and acidic forest soils testate amoebae belong to the quantitatively largest group of heterotrophic organisms and thus play an important part in the energy flow and nutrient cycles of these ecosystems.

Testate amoebae are traditionally subdivided into two large groups based on their pseudopodia: those with lobed or finger-shaped pseudopodia (lobopodia, Fig. 1, 4, 12, 17) and those with thin filament-like filopodia (Fig. 2), which fold like a jack-knife when retracted. The filose testate amoebae are grouped together with the heterotrophic flagellates Cercomonadida as Monadofilosa within the phylum Cercozoa Cavalier-Smith 1998 (Cavalier-Smith & Chao 2003). Molecular studies show that filose testate amoebae are also not monophyletic (Wylezich et al. 2002). With the exception of *Archerella* all filose species figured here belong to the order Euglyphida. The lobose testate amoebae have found their place together with large naked amoebae like *Amoeba proteus* in the phylum Amoebozoa Lühe, 1913, class Lobosea Carpenter 1861 (Nikolaev et al. 2005, Cavalier-Smith et al. 2004).

Three different test types are distinguished by the composition of their test wall: (1) pure organic tests made of structural proteins that either show distinct building units ("areoles" in *Arcella*, Fig. 12) or seem to be completely homogenous (*Hyalosphenia*, Fig. 22 – 24); (2) tests that are made from exogenous collected material (xenosomes) such as small mineral particles or diatoms agglutinated by an often structured organic cement (*Centropyxis, Difflugia* Fig. 18 – 21, 25 – 27, 33), or siliceous platelets taken from Euglyphida also agglutinated by an organic cement (*Nebela,* Fig 25 – 27, 29, 30) ; and (3) tests, composed of endogenous siliceous or calcareous rods, nails or plates that are produced by the organisms themselves (idiosomes, in Euglyphida: *Euglypha*, *Tracheleuglypha, Trinema,* Fig. 34, 35, 39 – 43).

Testate amoebae show a wide range of morphological adaptations especially to the physical structure of their habitats (thin water films, gap systems of various pore sizes in soils, sediments and moss) as well as changing water content in semi-aquatic and terrestrial biotopes. These adaptations are expressed by size, shape and position of the pseudostome and by the size, shape and building material of the test.

This results in various test types, four of which are shown here (Fig. 3). (1) acrostome tests: with a terminal pseudostome and a round (*Difflugia bacillifera*) or compressed wedge-shaped cross section of the test (*Hyalosphenia, Nebela*); (2) tests with radial (= axial) symmetry and a ventral sole (*Arcella, Cyclopyxis*); (3) plagiostome tests: with an eccentric pseudostome and a flat ventral sole (*Centropyxis aerophila*); (4) cryptostome tests with a ventral sole and a slit-like pseudostome that is hidden under the dorsal or ventral pseudostome lip (*Bullinularia, Plagiopyxis*).

The size of testate amoebae varies between 10 and 500 µm, the majority ranging between 20 and 150 µm. The largest species live in aquatic biotopes like lake sediments whereas species in mosses and soils are usually smaller. Testate amoebae are predominantly fresh water organisms; only about 100 species live in marine sands. According to the quantity and changing presence of water one can distinguish between three groups of habitats, each colonized by characteristic communities: (a) aquatic biotopes, like the periphyton and sediments in lakes, ponds, pools and running water; (b) semiaquatic biotopes like the water-filled interstice of *Sphagnum* (bogs); (c) terrestrial biotopes like moss, humus and soils. Here the water forms only a thin layer and does not even constantly cover the substrate. Thus, the most important factor for the distribution of testate amoebae is the availability of water. The less constantly wet the substratum, the more frequently those species with morphological adaptations (visor, cryptostomy, flat ventral sole and reduced size) occur. Their specialized test morphology prevents evaporation and makes locomotion and food uptake possible even in capillary water films. Most species of the semiaquatic habitats and all terrestrial forms are able to survive unfavourable periods as cysts (Fig. 28, 31). Besides typical cysts, precysts are formed in which tests are only sealed temporarily by a thin membrane. In aquatic and semiaquatic biotopes one can also find species forming a symbiosis with green algae (zoochlorellae). Their vertical distribution is limited by the availability of light.

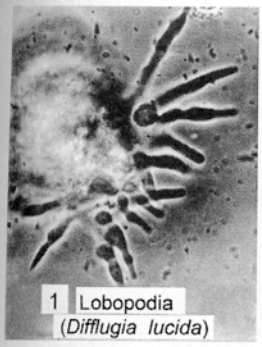
Testate amoebae usually reproduce by binary fission, in which one generation cycle lasts between hours and days, depending on temperature. All species with rigid tests form a new test immediately prior to cell division and one of the daughter cells keeps the old test. Species with idiosomes have synthesized them during the cell cycle and stored them often in a regularly stacked way, within the cytoplasm. Sexual reproduction (autogamy) has been proved for *Arcella*.

While the majority of testate amoebae have a single nucleus, almost all *Arcella* have two and only a few species have numerous nuclei (Fig. 14). Species with a vesicular nucleus have one large nucleolus (Fig. 10) and those with an ovular nucleus have several to many smaller nucleoli (Fig. 4, 5).

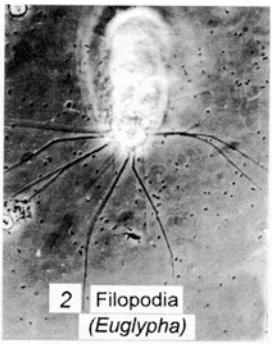
Often one can find an eye-catching zone of a particularly dense rough endoplasmic reticulum surrounding the nucleus which is also visible in phase contrast and differential interference contrast (DIC) or after staining with basic dyes like borax carmine (the "chromidium") (Fig. 4, 28).

Food such as bacteria, fungi, algae and protozoa, is taken up by phagocytosis. Many species selectively feed on specific food organisms. The larger *Nebela* species are predacious and ingest small Euglyphidae (*Corythion, Euglypha, Trinema*) utilizing the prey's idiosomes as building material (kleptoidiosomes).

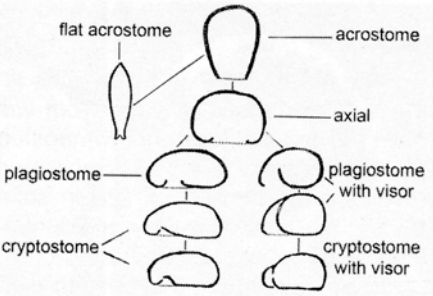
Fig. 1 – 44. Structural features of testate amoebae; these are explained by captions and in the text.



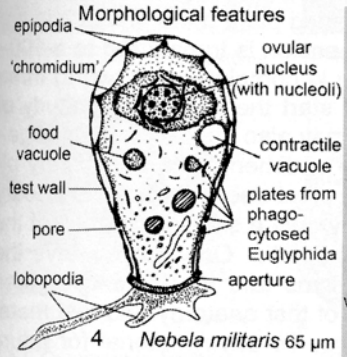
1 Lobopodia
(*Difflugia lucida*)



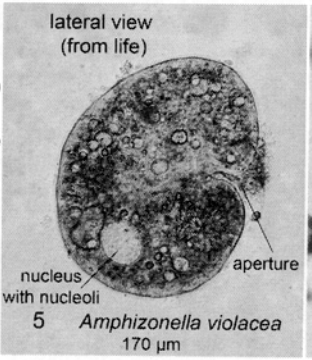
2 Filopodia
(*Euglypha*)



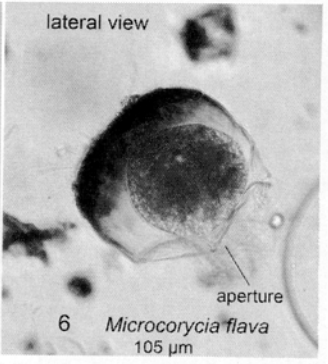
3 Main test types (after Bonnet)



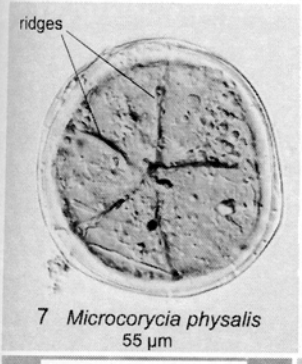
4 *Nebela militaris* 65 µm



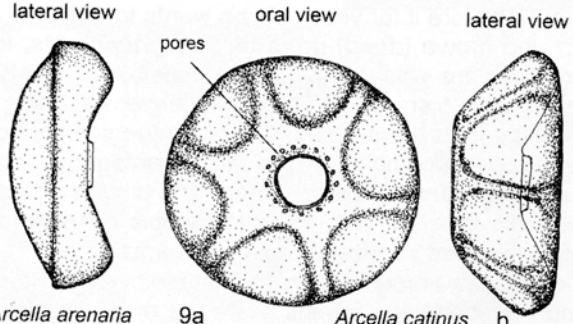
5 *Amphizonella violacea*
170 µm



6 *Microcorycia flava*
105 µm

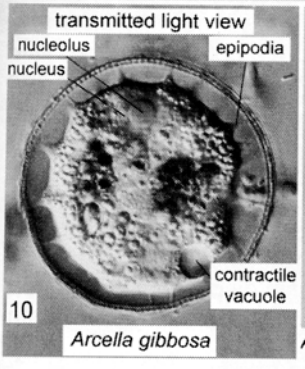


7 *Microcorycia physalis*
55 µm

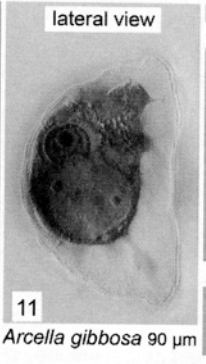


8 *Arcella arenaria*
80 µm

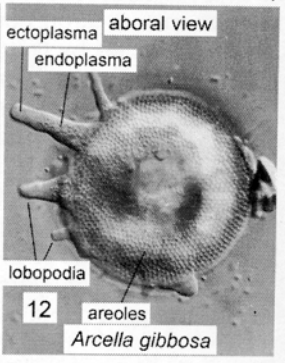
9a *Arcella catinus* b
100 µm



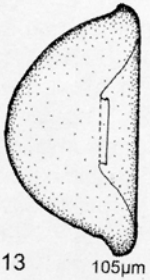
10 *Arcella gibbosa*



11 *Arcella gibbosa* 90 µm



12 *Arcella gibbosa*



13 105µm
Arcella vulgaris

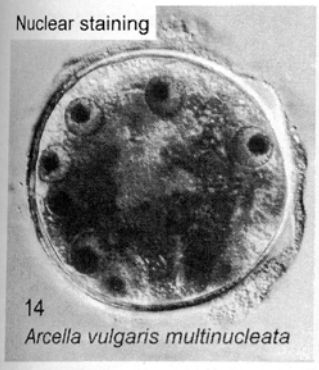
PRACTICAL TECHNIQUES

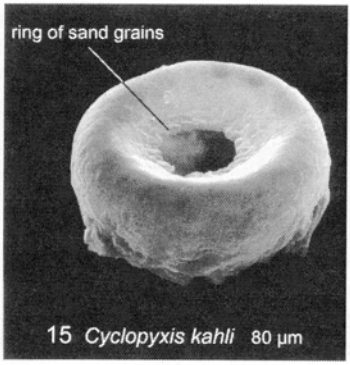
The object of these techniques is to get to know typical testate amoebae from dry moss from roofs and walls, from wet *Sphagnum* and from forest soils, and to compare the architecture and composition of the various tests. Species with acrostome, flattened tests dominate in *Sphagnum* (*Nebela, Hyalosphenia*) while plagiostome and cryptostome forms dominate in soils. The examination of the vertical distribution of testate amoebae can be considered as an additional task. The methods used for extraction of specimens depend on the substrate to be examined.

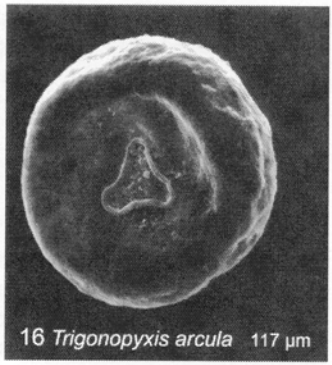
From *Sphagnum* it is easy to isolate a community rich in often diverse individuals. For that purpose a handful of moss has to be washed with 150 ml (depending on the amount of moss) filtered water from the habitat. Interfering plant parts and debris are extracted by sieving (mesh size 0.5–1 mm). The suspension is transferred to a 100–250 ml beaker or funnel-shaped glass. Sedimentation is complete after two to three hours. However, after about fifteen minutes one can start the microscopic study of the living sample that is rich in testate amoebae, and may also contain various flagellates and unicellular algae. Take a small drop of the sediment with an ordinary pipette and put it on a microscope slide. Because of the plant debris it is not necessary to support the coverslip. Live testate amoebae (usually only a small proportion of the sample) can be recognized by their protruding pseudopodia. One can observe the cytoplasmic streaming, the movements of the organisms and the symbiotic green algae (zoochlorellae) in *Hyalosphenia papilio.* Details of test anatomy and test material can also be studied in dead and fixed specimens. To save material for future studies, decant the supernatant and fix the sediment with formalin (2% final concentration) and store it for years. If one wants to examine the different associations of the green and brown (dead) parts of *Sphagnum* mats, the parts need to be separated before they are washed. It is also possible to wash and analyze short *Sphagnum* stem sections in small Petri dishes or in glass staining blocks.

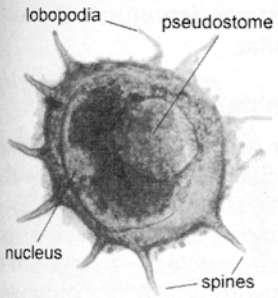
Mosses from trees, roofs, walls and the soil surface are treated similarly. In case the mosses are completely dry, it is advantageous to moisten them for several hours or days to enhance the excystment and growth of active testate amoebae. Adherent humus can interfere with the microscopic observation and should therefore be removed or treated separately as a soil sample.

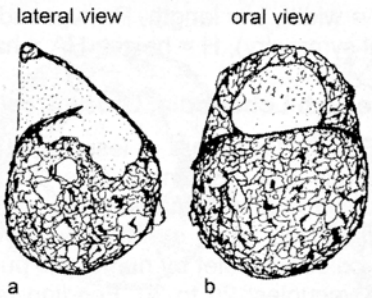
Soil testate amoebae are easily obtained from partly decomposed litter from deciduous or coniferous forests. Here the number of individuals is greater than in mineral soils with a low content of organic matter. The litter is washed and sieved in the same way as a moss sample. A large number of empty tests without any disturbing mineral particles can be extracted by flotation. For this purpose c. 50 g air-dried litter or humus-rich soil are suspended in water and sieved through a screen (mesh size about 0.5 mm) to remove fragments of leaves and small wood particles. The filtrate is left in a tall beaker for approximately fifteen minutes until the heavier soil particles settle. The air-filled tests of testate amoebae accumulate together with other light weight components (pollen, wood particles, etc.) on the surface as a ring along the wall of the beaker. From there they can be collected with a pipette or a fine brush and gathered in ethyl alcohol (96% plus 2 drops detergent) in a small screw cap container. After 30-60 minutes, or better overnight, the air bubbles disperse and the tests sink to the bottom in the closed container. Permanent mounts are easily made with

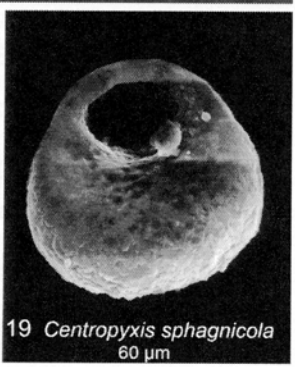

Nuclear staining
14
Arcella vulgaris multinucleata


ring of sand grains
15 Cyclopyxis kahli 80 µm

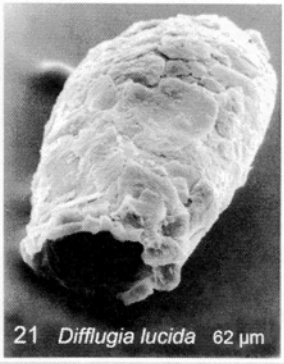

16 Trigonopyxis arcula 117 µm

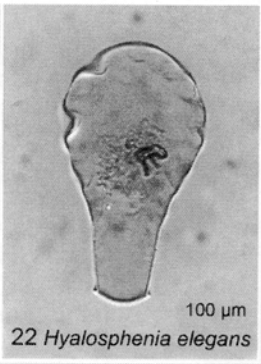

lobopodia
pseudostome
nucleus
spines
17 Centropyxis aculeata
105 µm

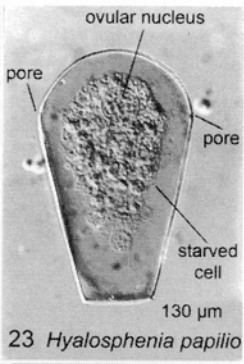

lateral view
oral view
a
b
18 Centropyxis aerophila 75 µm


19 Centropyxis sphagnicola
60 µm

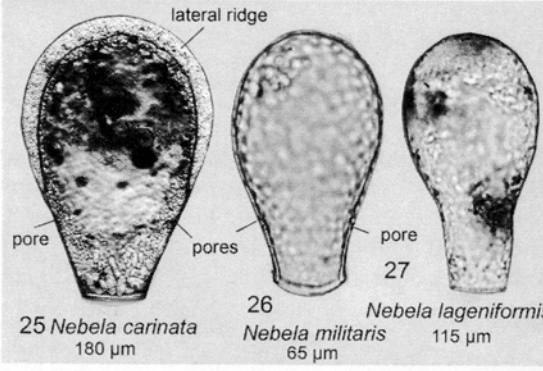

20
140 µm
Difflugia bacillifera

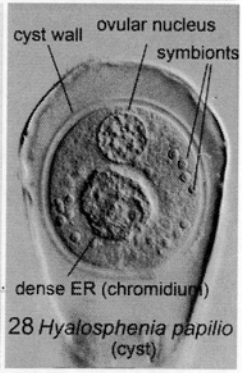

21 Difflugia lucida 62 µm


100 µm
22 Hyalosphenia elegans


ovular nucleus
pore
pore
starved cell
130 µm
23 Hyalosphenia papilio


24 Hyalosphenia subflava


lateral ridge
pore
pores
pore
27
26
Nebela lageniformis
25 Nebela carinata
180 µm
Nebela militaris 115 µm
65 µm


cyst wall
ovular nucleus
symbionts
dense ER (chromidium)
28 Hyalosphenia papilio
(cyst)

the mounting medium Euparal (p. 75) to which the tests are transferred from ethyl alcohol (96%) with a micropipette. If no phase contrast or DIC microscope is available the colourless siliceous platelets of the Euglyphida can be examined best in a high resolution diatom mounting medium (Pleurax or Naphrax, p. 16 – 17).

GENERA AND SPECIES

The genera covered here are often very rich in species, therefore only few typical or common forms can be presented. For the determination to species characters of the test are mainly used, sometimes the kind of nucleus, the presence of symbionts or the colour of the plasma may be important.

Abbreviations used: W = width, L = length, Ps = pseudostome (aperture), D = diameter (for tests with radial symmetry), H = height, HA = habitat.

Testate Amoebae with Lobopodia. Order Arcellinida Kent, 1880

Amphizonella violacea (Fig. 5). Test more or less round or oval; bilayered, sac-like pellicle; outer layer gelatinous 8–2 µm; inner layer thin chitinoid, undulates with internal movements; pseudostome invaginated with variable shape. Cell size 125–200 µm. Pseudopodia: cylindrical, finely granular, with rounded ends. Movements sluggish. Cytoplasm clear, coloured violet by numerous purple vesicles (~1 µm). One ovular nucleus. Contractile vacuoles: 20 to 30. Feeding: algivorous. HA dry mosses (on roofs, walls).

Microcorycia

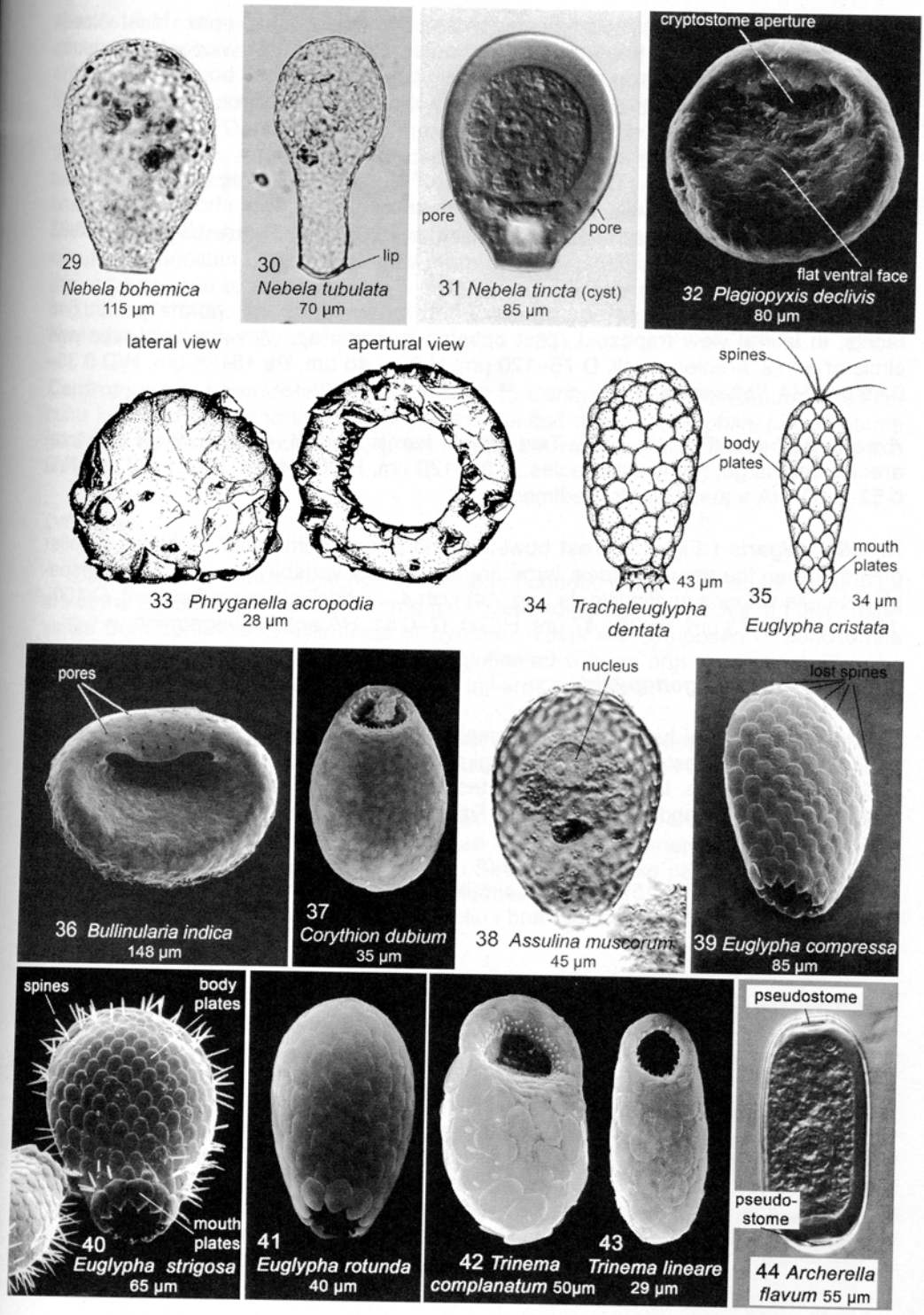
Test flexible, bowl-shaped, extends as membranous transparent border towards the aperture, capable of completely closing the opening. Aboral part brownish, less flexible, in lateral view like a dome, sometimes with concentric ridges or small horns, usually covered with small debris or mineral particles. Shape of the aperture variable, pseudopodia lobate; endoplasm granular, yellowish to reddish, some species with glycogen spheres (2–5 µm). One ovular (*M. flava*) or vesicular nucleus. Cyst external. Feeding herbivorous. HA dry mosses.

Microcorycia flava (Fig. 6). Aboral part dome-shaped, covered with small mineral particles and debris. D = 70–100 µm.

***Microcorycia* physalis** (Fig. 7). Aboral dome with 5 – 6 ridges. D = 50–70 µm.

Arcella

Arcella species are frequently found in aquatic habitats, a few (for example *A. arenaria*) also in dry mosses and litter. The completely organic (proteinaceous) tests are of the axial type (Fig. 3), aperture invaginated, in many species surrounded by a short tube and a circle of pores. The fine areolar test surface (Fig. 12) originates from single-layered box-like building units. Young tests are colourless, older ones become


29
Nebela bohemica
115 µm
30
Nebela tubulata
70 µm
lip
31 Nebela tincta (cyst)
85 µm
pore
pore
cryptostome aperture
flat ventral face
32 Plagiopyxis declivis
80 µm
lateral view
apertural view
33 Phryganella acropodia
28 µm
spines
body plates
mouth plates
43 µm
34 Tracheleuglypha dentata
35
34 µm
Euglypha cristata
pores
36 Bullinularia indica
148 µm
37
Corythion dubium
35 µm
nucleus
38 Assulina muscorum
45 µm
lost spines
39 Euglypha compressa
85 µm
spines
body plates
mouth plates
40
Euglypha strigosa
65 µm
41
Euglypha rotunda
40 µm
42 Trinema complanatum 50µm
43
Trinema lineare
29 µm
pseudostome
pseudo-stome
44 Archerella flavum 55 µm

brown due to iron and manganese deposition into the building units. Most *Arcella* have two nuclei with one large, central nucleolus each (Fig. 10) (vesicular nucleus), while some such as *A. megastoma* may have up to 200. The cell body is fixed to the test wall with contractile epipodia. Peripherally located contractile vacuoles empty at intervals of 1–2 minutes.

Arcella arenaria (Fig. 8). The test consists of a flat, aboral, somewhat undulated "bowl" attached to an equally sized dish. Oral cavity distinct, with short oral tube and circle of pores, areoles very fine, D 70–95 µm, H 25–38 µm, Ps 14–18 µm, H/D 0.33 –0.41, HA dry mosses and litter.

Arcella catinus (Fig. 9). In aboral view often irregularly round, normally with 6–8 facets, in lateral view trapezoid. Test opening invaginated, with apertural tube and circle of pores, areoles small, D 75–120 µm, H 30 – 46 µm, Ps 18–26 µm, H/D 0.35–0.46 µm, HA *Sphagnum*, moss.

Arcella gibbosa (Fig. 10 – 12). Test almost hemispherical, surface often undulate, areoles very large, many subspecies, D 80–120 µm, H 50–74 µm, Ps 21–32 µm, H/D 0.53–0.69, HA aquatic plants, sediments.

Arcella vulgaris (Fig. 13). Test bowl-like, the aboral dome with a slightly smaller diameter than the apertural face, large areoles; a very variable species! The subspecies *Arcella vulgaris multinucleata* (Fig. 14) with 4 – 9 nuclei is more frequent, D 100–145 µm, H 52–73 µm, Ps 30– 47 µm, H/D 0.37–0.51, HA aquatic vegetation.

Cyclopyxis and Trigonopyxis

Tests of the axial type: mostly hemispherical with a central, always invaginated pseudostome. The tests are made of agglutinated external mineral particles, diatoms and also fine detritus. In *Cyclopyxis* the test opening is always round, often surrounded by a crown of sand grains whilst in *Trigonopyxis* triangular or polygonal pseudostomes are typical.

Cyclopyxis kahli (Fig. 15). Aperture circular, bordered with small sand grains, D 80 –10 µm, H 55–60 µm, HA mosses and soils (from Wanner, Arch. Protistenk. 136: 97-106, 1988).

Trigonopyxis arcula (Fig. 16). The pseudostome is triangular or irregular with an organic rim, D 110–140 µm, H 60– 80 µm, HA dry *Sphagnum*, mosses and humus layer.

Centropyxis

Species of *Centropyxis* are bilaterally symmetrical and have an eccentric, often round or oval test opening (plagiostome test type). The lips of the pseudostome can be bent inwards and can connect the oral test wall with the dorsal face by bridge-like structures. The test is made of an organic cement in which small mineral particles (small grains of sand, fragments of diatoms) may be incorporated. Some aquatic

species (for example *Centropyxis aculeata*) are decorated with hollow spines. The ovular nucleus is located in the posterior half of the cell and contains many nucleoli.

Centropyxis aculeata (Fig. 17). Very variable collective species with spines at the fringe of the test. Dorsoventraly compressed, test brownish, with a changing proportion of xenosomes (grains of sand, diatoms) according to the habitat, D 90–150 µm, HA aquatic vegetation, sediment.

Centropyxis aerophila (Fig. 18). Test without spines, in oral view oval with a semicircular pseudostome. In lateral view more or less spherical, tapers sharply towards the aperture, oral side flat , L 53–85 µm, HA mosses and soils (Fig. 18 from Bonnet et Thomas (1960): Thécamoebiens du sol. In: *Faune terrestre et d'eau douce des Pyrénées-Orientales,* 5, Vie et Milieu, Suppl. 1 -103).

Centropyxis sphagnicola (Fig. 19). Similar to *Centropyxis aerophila* but almost circular in dorsal view and in lateral view less vaulted than *C. aerophila*, pseudostome bordered by two convex arches, L 49–70 µm, HA mosses, also dry *Sphagnum* and soils.

Difflugia

Difflugia is the genus most rich in species of aquatic testate amoebae. Their tests are of the acrostome or flat acrostome type. Depending on the species the test shape varies from spherical to cylindrical or pyriform. Tests are composed of xenosomes (mineral particles or diatoms) that are agglutinated with an organic cement. The rim of the terminal pseudostome is never bent inwards or slit-like.

Difflugia bacillifera (Fig. 20). The longish pyriform test is predominantly covered with diatom frustules, nucleus with many nucleoli, 120– 80 µm, HA wet *Sphagnum.*

Difflugia lucida (Fig. 21). Test compressed, relatively transparent, surface covered with plane mineral particles. The amoebae accumulate gathered building material outside the test opening prior to divisions. Several finger-like pseudopodia. Nucleus with numerous nucleoli, L 45–85 µm, HA mosses and soils.

Hyalosphenia

The tests are laterally compressed, with wedge-, flask- or egg-shaped outline.They are organic and yellowish-transparent and without any foreign material.The cytoplasm is attached to the test with epipodia. There is a single ovular nucleus.

Hyalosphenia elegans (Fig. 22). Test flask-shaped, laterally notched, with a long neck; aperture with a thickened curved lip. Surface with hemispherical dents, at the neck's base two pores, L 68–130 µm, B 40–60 µm, HA common in wet *Sphagnum.*

Hyalosphenia papilio (Fig. 23, 28). Test wedge-shaped, cytoplasm coloured green by symbiotic algae (zoochlorellae), L 90–175 µm, B 60–115 µm, HA wet *Sphagnum*;

due to the symbionts only in the green parts of wet *Sphagnum*; excellent indicator in paleoecology.

Hyalosphenia subflava (Fig. 24). Test egg-shaped, L 45–78 µm, B 30–35 µm, HA dry mosses, *Sphagnum*, raw humus and acidic soils.

Nebela

Tests transparent with siliceous body plates taken from phagocytosed Euglyphida (*Corythion, Euglypha, Tracheleuglypha, Trinema*) or of diatom frustules in a non-structured organic cement. Tests mostly compressed, often with pores, some species with a lateral ridge. One single ovular nucleus in the posterior part of the test.

Nebela carinata (Fig. 25). Test longish-pyriform, highly compressed, broad lateral ridge, L 151–240 µm, B 140–180 µm, HA wet or submersed *Sphagnum* in bogs.

Nebela bohemica (Fig. 29). Pear-shaped with a short neck, L 98–130 µm, HA common in moist *Sphagnum*, also in forest soils. *Nebela collaris*: The rim of the pseudostome is convex and the test narrower.

Nebela tincta (Fig. 31). Similar *to Nebela bohemica* but smaller, L 76–97 µm, HA frequent in *Sphagnum*, dry mosses and acidic forest soils.

Nebela lageniformis (Fig. 27). Flask-shaped, pseudostome elliptical, often with a small lip, L 95–140 µm, HA moist mosses and humus.

Nebela tubulata (Fig. 30). Smaller and slimmer than *N. lageniformis*, L 55–77 µm, HA *Sphagnum*.

Nebela militaris (Fig. 4, 26). Test oblong pyriform, aperture somewhat wider than the neck, with a distinct lip, L 50–80 µm, B 25–38 µm, HA frequent in mosses and acidic forest soils.

Plagiopyxis declivis (Fig. 32).Test of the cryptostome type, hemispherical, smooth, flat ventral side. Pseudostome eccentric, an elongate slit, perpendicular to long axis of the test. Anterior apertural lip is somewhat incurved. The posterior lip is an elongation of the evenly depressed ventral side and ends underneath the anterior lip hiding the opening. The slit-shaped pseudostome is therefore hard to identify with a light microscope. The agglutinated test consists of flat mineral particles; on the aboral side even bigger sand grains are embedded, D 65–90 µm, HA widely distributed, characteristic soil species.

Bullinularia indica (Fig. 36). Large species. Test of the cryptostome type. Outline elliptical, oral side flattened and invaginated; the eccentric pseudostome as in *Plagiopyxis declivis* but the front lip as well as the back lip is rounded. Under the light microscope easily visible pores. Test dark brown with xenosomes, L 120–180 µm, B 150–220 µm, HA mosses and soils.

Phryganella acropodia (Fig. 33). Test hemispherical, of the axial type. Pseudostome large (approximately half the test diameter), not or only slightly invaginated. Test composed of mineral particles of variable size embedded in an organic matrix which in older specimens can become dark brown due to manganese and iron deposition. Pseudopodia hyaline, occasionally tapered, with the ability to form anastomoses (reticulolobopodia), D 30–50 µm, H 25–40 µm, HA frequent in mosses and soils (from Lüftenegger et al., Arch. Protistenk. 136: 153-189, 1988).

Testate Amoebae with Filose Pseudopodia

With the exception of *Amphitrema and Archerella* all genera discussed here build their tests of siliceous platelets that are synthesized in the posterior part of the cell and stacked around the nucleus prior to the formation of the daughter test.

Order Euglyphida Copeland, 1956

Assulina muscorum (Fig. 38).Test of the flat acrostome type, broad egg-shaped, flattened, biconvex in cross-section, in most cases brownish but also colorless, built from overlapping elliptical scales. Pseudostome narrow-oval, with crenulated rim of organic cement. Never denticulate mouth plates! One nucleus with a single nucleolus, L 33–60 µm, B 28 45 µm, HA dry mosses and soils, frequent. *Assulina seminulum*: very similar test shape but 60–90 µm long, nucleus with few nucleoli.

Corythion dubium (Fig. 37).Test egg-shaped, in longitudinal and cross section biconvex, flattened. Pseudostome subterminal, oval, spoon-like elongated to the front end, dorsal lip never invaginated. Test built from small, colourless, usually rounded rectangular or oval body plates, mouth plates with one median tooth, L 30–60 µm, B 18–20 µm, HA dry mosses, *Sphagnum* and acidic soils.

Euglypha

Test of acrostome type, transparent, assembled from overlapping siliceous scales which are arranged in alternating longitudinal rows. Test surface smooth or often covered with spines. Pseudostome terminal, always with denticulate mouth plates. Cell body usually divided into a highly vacuolar anterior region rich in food vacuoles, a dark band (pigment zone) with cement vesicles and excretion crystals, and a dense compact posterior region, which contains the nucleus surrounded by dense endoplasmic reticulum. Important diagnostic characters are the cross section of the test, the shape of the pseudostome and of the mouth and body plates as well as the presence of spines. It is also important whether these spines are separate or appendices to body plates.

Euglypha compressa (Fig. 39). Test and pseudostome highly compressed; long, in the middle often reinforced spines, located only laterally. They frequently break off in empty tests and then possibly lead to confusion with the so-called '*glabra*-forms'. Body plates large, easily visible, mouth plates oval with one larger, middle tooth and

three smaller ones on each side, L 70–130 µm, B 40–80 µm, HA *Sphagnum* and also acid humus.

Euglypha cristata (Fig. 35).Test longish, cylindrical, with slightly constricted neck and terminal tuft of diverging spines, L 35–70 µm, B 12–23 µm, HA mosses, *Sphagnum* and soil (Fig. 35 from Lüftenegger et al., Arch. Protistenk. 136: 153-189, 1988).

Euglypha strigosa (Fig. 40). Pear-shaped, oval in cross section, aperture circular with reinforced mouth plates with seven teeth each, rear rim of the mouth plates more or less straight, spined, L 55–90 µm, B 30–50 µm, HA mosses and soils.

Euglypha rotunda (Fig. 41).Test longish-egg shaped, small, compressed, no spines, aperture circular, one nucleolus! L 25–50 µm, HA widely spread in mosses and soils. This species stands representatively for a number of similar species which can only be determined by means of a scanning electron microscope or DIC light microscope.

Tracheleuglypha dentata (Fig. 34). Test and aperture round in cross section, scales large, round to broad-oval, aperture without denticulate mouth plates but sometimes with dentate, organic collar, which is produced during fission to hold the opposed tests together, L 35–60 µm, B 20–33 µm, HA mosses and humus-rich soils. In soil the dentate collar is reduced or missing!

Trinema

Test outline longish-elliptical, conspicuous ventral side with always invaginated pseudostome. Siliceous idiosomes mostly round, multiple size groups in one test. The smaller ones are found around the pseudostome and between the larger scales. Mouth plates with a median tooth.

Trinema complanatum (Fig. 42). Test broad-oval or rounded rectangular, in lateral view sharply tapering towards the pseudostome. Test height very variable. Pseudostome small, invaginated, round or oval, L 25–75 µm, B 15–45 µm, HA *Sphagnum*, mosses and soils.

Trinema lineare (Fig. 43). Slender elliptical in outline, aperture round. The tests vary highly in shape and size depending on the population. One nucleus with one central nucleolus, L 18–32 µm, HA widely spread in all aquatic and terrestrial habitats. *Trinema enchelys* is similar but larger. Habitat like *Trinema lineare*, L 32–60 µm.

Order Tectofilosida Cavalier-Smith, 2003

Archerella flavum (Syn. *Amphitrema flavum)* (Fig. 44). Test thick, rigid, compressed, with parallel sides and rounded ends. Wall organic, three-layered, without xenosomes, brown due to iron accumulation. Two elliptical pseudostomes at opposite ends of the test with slightly thickened rim and very thin, short collar (at the limits of the resolution of the light microscope). Cell body nearly fills test, green from zoochlorellae. Vesicular nucleus with a single nucleolus. Filopodia very thin and

rarely branching, emerging at both pseudostomes from an internal ectoplasmic stalk. L 45–77 µm, B 23–45 µm, HA: *A. flavum* is an important character species of upper green parts of constantly wet *Sphagnum*. In undisturbed bogs abundant, after drainage rare or absent.

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Benthic Foraminifera

Rudolf Röttger and Gunnar Lehmann

INTRODUCTION

Foraminifera (rhizopods of the Class Foraminifera) are marine protozoa characterized by tests (shells), granular reticulopodia and an alternation of generations. One of the smallest species is *Rotaliella roscoffensis* with a diameter of 40 µm, while the largest are *Cycloclypeus carpenteri* and *Acervulina* with diameters of up to 12 cm and 20 cm respectively. Most of the approximately 10,000 extant species are between 200 and 500 µm in size. *Reticulomyxa filosa* is the one known exception to the marine habitat in that it has no test and lives in fresh water and wet leaf litter.

Foraminifera are classified into three groups according to the composition of the test wall. These are (1) organic: glucosaminoglycan, (2) agglutinated (arenaceous): detrital particles held together by organic or calcareous cements (Fig. 1a) or (3) calcareous: made of secreted mixed crystals of calcium carbonate (calcite) in which calcium is replaced by a lower or higher proportion of magnesium.Traces of strontium also occur.

Foraminifera with organic tests are single chambered (unilocular, monothalamous) and placed in the order Allogromiida. Most of the foraminifera with calcareous walls have multichambered (plurilocular, polythalamous) tests and are subdivided on the basis of the crystalline microstructure of the walls. The order Miliolida is characterized by three-layered calcitic walls (with a proportion of 10–17 mole percent magnesium) (Fig. 1b, 13): an outer and an inner layer of crystals oriented parallel to the test surface enclose a median layer of rodlike, randomly oriented crystals. No pores penetrate the test wall. This arrangement reflects the light and produces a milky white opacity when seen in reflected light (porcelaneous-walled or calcareous imperforate foraminifera). In the order Rotaliida (Fig. 2, 3, 5, 15 – 20) and some other orders the test walls may consist of one layer of large calcite crystals (with a proportion of 3–10 mole percent magnesium), that are oriented perpendicular to the test surface. These radial hyaline walls are glassy, translucent and penetrated by fine pores (Fig.1c, 3) (hyaline or calcareous perforate foraminifera).

The pore canals of 1–4 µm diameter pass through the entire wall; their proximal end is closed by an organic membrane with micropores. While pores do not allow the passage of cytoplasm between test lumen and seawater, they do allow the transport of carbon dioxide, oxygen and nutrient salts in those species with endosymbiotic algae. Many hyaline foraminifera have a wall composed of several lamellae. In the bilamellar type (Fig. 2) the wall of the youngest chamber is composed of two layers. The outer layer also covers all other chambers of the last whorl of the spiral. Thus the number of layers (lamellae) increases from the youngest to the oldest chamber. A precise terminology is used to describe the foraminiferal test (Fig. 3).

The great diversity in the appearance of foraminiferal tests is a product of the

differing shapes and arrangements of the chambers of the tests. Single chambered tests may be spherical, flask-shaped, tubular, stellate or arborescent. Multichambered tests pass through an ontogeny lasting weeks or months as new chambers are added to the test, a process unique amongst the protozoa. The process starts with the formation of the initial chamber, the proloculus.

In uniserial tests, the simplest form of multichambered tests, the chambers are arranged in a single column. The walls that separate adjacent chambers are called septa and the opening of the youngest chamber to the outside, the opening through which pseudopodia protrude, is called the aperture. When a new chamber is added it is formed over the previous aperture, which, now lying within the test, is called a foramen (Latin, window) from which the class Foraminifera is named. Thus each chamber in a multichambered test has a foramen, apart from the most recently added chamber which has an aperture.

The chambers of biserial tests are arranged in two columns alternating left and right. In planispiral tests the chambers are coiled in a plane (Fig. 5, 11, 15, 16, 18 – 20). In all of these figured examples later chambers cover earlier ones so that only the chambers of the last whorl are visible (involute test). In trochospiral tests the chambers are coiled in a low or high helicoid spiral (Fig. 4, 8, 17). This results in a test in which all the chambers of the spiral are visible on one side only (the evolute or spiral side). The other side is known as the involute or umbilical side. In the high trochospiral test few (≥3) chambers form a whorl (Fig. 8).

Winding growth with only two chambers of elongate tubular shape to the whorl (with the apertures alternately at one end of the test and then to the other) is called milioline (Fig. 9, 13). For more detailed information on the milioline test see the captions of figures 9 and 13. The milioline type of chamber arrangement occurs only in many porcelaneous and some agglutinated genera, whereas the other types of chamber arrangement occur in foraminifera with hyaline or agglutinated walls. For other types of chamber arrangement in porcelaneous foraminifera see the descriptions of *Peneroplis* and *Sorites variabilis* (p. 130).

The main function of the test is the protection of the protoplasm of the cell. Foraminifera pass through the intestine of sediment feeders and predators without being digested. Only the protoplasm outside the test, which includes the pseudopodia, is lost. Thus foraminifera, though occurring in high specimen densities, do not really provide much usable biomass.

Benthic foraminifera colonize all substrata of marine habitats from tropical shallow waters to the deep-sea floor and brackish water environments (while planktonic foraminifera occupy other parts of the water column). Benthic foraminifera live both on the surface and within the uppermost centimetres of the sediment through which they actively move up and down in search of food, or they live epiphytically on algal thalli and seagrass blades. They extrude a sticky three-dimensional pseudopodial network (reticulopodia) through the aperture (Fig. 5). These collect food particles: (1) detritus of planktonic diatoms and dinoflagellates, so-called phytodetritus (deposit feeding), (2) benthic diatoms and bacteria (grazing), (3) various protists, small invertebrates and organic detritus, gathered by pseudopodia extended from elevated apertures into the water column (suspension feeding).

PRACTICAL TECHNIQUES

Two habitats are easy accessible, namely coastal salt marshes and the adjacent intertidal mud flats. Typical coastal salt marshes border parts of the Wadden Sea of the German Bight and the Netherlands, as well as coasts around the British Isles and the Atlantic coast of North America. Small salt marshes also border sheltered bays of the western Baltic Sea. Sediment samples from sublittoral environments are taken by a grab or corer on board a ship.

Lower salt marsh zones contain halophytes of the genera *Salicornia, Spartina* and *Bolboschoenus*, median salt marsh zones are dominated by sea poa *Puccinellia,* and higher salt marsh zones by *Festuca*, *Juncus* and *Plantago.* The median and the higher salt marshes are irregularly flooded at high water by spring tides and wind-driven tides. The organisms of these habitats are well adapted to changes in salinity, water content of the sediment and temperature. Within the felt-like root system of *Puccinellia,* agglutinated foraminifera like *Miliammina fusca* (Fig. 9) and *Trochammina inflata* (Fig. 4) and the porcelaneous walled *Triloculina oblonga* (Fig. 13) are common.

In order to obtain these foraminifera, it is necessary to wash root samples with tap water (in the laboratory, the foraminifera will die) or with sea water (at the sampling location, when the foraminifera will stay alive). A few handfuls of immersed roots must be kneaded in a bucket half filled with water until all sediment and plant particles including the foraminifera are separated from the roots. Inorganic mud and organic debris are put into suspension by strong stirring and, after a break of a few seconds, half the volume of liquid (containing smaller and lighter clay particles and some organic debris) is slowly poured off. The foraminifera, together with heavy mineral particles such as sand, will have settled faster than smaller and lighter particles and will have gathered on the bottom of the bucket. After repeated additions of water, whirling and decanting, the water will have become clearer. The final residue will now consist only of small quantities of quartz grains and the foraminifera, visible with the naked eye as brownish points.This residue is flushed by a squeeze bottle containing seawater into a jar and split into many subsamples in the laboratory for study in small Petri dishes.To observe living specimens the Petri dish is left undisturbed for a quarter of an hour or more. Live specimens (usually only a small proportion of the sample) will protrude pseudopodia visible in transmitted or oblique transmitted light under a stereomicroscope. A living specimen is transferred by an artist´s brush (0 or 1) to a microscope slide in a drop of seawater. This kind of preparation allows a detailed microscopical study of the pseudopodial movements in transmitted light or darkfield illumination. It is important to support the coverslip by plasticine or coverslip splinters, or to use an excavated slide, so that the foraminifer is not squashed. If a small quantity of detritus is present it is possible to watch the foraminifer accumulate particles in front of its aperture by means of its pseudopodia.

To collect foraminifera from intertidal mud flats or other soft-bottom areas, the uppermost millimetres or centimetres of the sediment surface are sampled with a spoon. A mud volume of 50–100 ml is sufficient. The foraminifera are isolated by washing the sediment through stainless steel sieves or home-made sieves of plankton gauze of 200, 500 and 1000 µm mesh size, placing one sieve on top of the other (smallest mesh on the bottom). In this way the material is simultaneously washed

and divided into different size fractions. Washing of samples with seawater provides specimens for live observation while washing with tap water or distilled water provides specimens for permanent preparations. In the latter case, the residues are flushed out of the sieves into porcelain dishes and left to dry in the air or in an oven.

The easiest way to separate the foraminiferal tests from accompanying mineral particles in dry samples is to heat the sample and then to drop it into cold water. Tests whose empty chambers are filled with hot air will float. Another flotation method utilizing differences in specific gravity is more efficient. If the dry product of the sieving process is gently strewn, in small amounts, onto the surface of a liquid of high density such as carbon tetrachloride (CCl_4, specific gravity 1.59) in a 400 ml beaker under a fume-hood (*do not inhale the poisonous vapours!*) sand grains will sink to the bottom while most of the air-filled foraminiferal tests will float on the surface or adhere to the glass wall at the surface. With the help of an artist's brush both adherent and surface foraminifera are transferred, together with some of the carbon tetrachloride, to a pleated filter paper in a glass funnel. The filter with its foraminifera and some organic particles are then left to dry – *in the fume cupboard. The remainder of the carbon tetrachloride in the beaker must be recovered (in the fume-hood) for further use. Because of its poisonous nature it must not be discarded.*

Another treatment of the residue from the bottom of the beaker with carbon tetrachloride will increase the yield. The extraction of foraminifera by carbon tetrachloride is not practicable in more landward parts of salt marshes where the proportion of plant particles is very high. In this case a modified method must be employed (Lehmann & Röttger 1997).

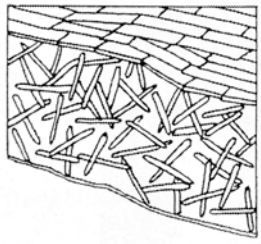
Sampling of soft bottom material from a ship with a grab is performed in the same way as sediment from the Wadden Sea. It is advantageous to use a windowed grab (van Veen) or a Reineck box-corer to ensure that the surface of the sediment is minimally disturbed. When the window of the grab has been opened the remaining water is carefully sucked off and the uppermost layer of the sediment is removed using a spoon as described above.

Separation and mounting of specimens from dried residues are described on p. 125.

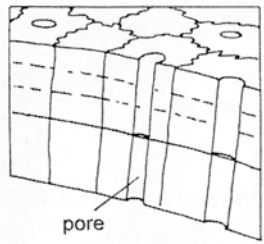
Fig. 1. Test wall structures. **a:** Agglutinated, surface view; in this case grains strongly differ in size. **b:** Porcelaneous, outer surface layer of laths over a jumble of calcite needles. **c:** Block view of a calcareous hyaline wall showing radial crystal arrangement, pores and organic layers within the wall. **Fig. 2.** Equatorial section of a planispiral test of a rotaliid (member of the Order Rotaliida) with a bilamellar wall. The last whorl consists of seven chambers. **Fig. 3.** Diagram to show general morphological features of a rotaliid test. **Fig. 4.** *Trochammina inflata.* **a:** Top view of a low helicoid spiral. **b:** Life cycle. The upper part depicts the well documented dimorphic cycle of many foraminifera consisting of a regular alternation between a haploid megalospheric gamont (megalospheric: with large proloculus) (A_2) with its gametes and a zygote, and a diploid microspheric agamont (microspheric: with small proloculus) (B) with its offspring produced by multiple fission. In *Trochammina inflata,* as in several other foraminifera, a third generation, a megalospheric schizont (A_1) reproducing by cyclic schizogony occurs. According to the hypothesis of trimorphism, it occurs between agamont and gamont (lower part of cycle). **Fig. 5.** *Elphidium williamsoni.* **a:** Lateral view of a live specimen with reticulopodia protruding from the aperture. **b:** Reticulopodia at higher magnification.



a agglutinated

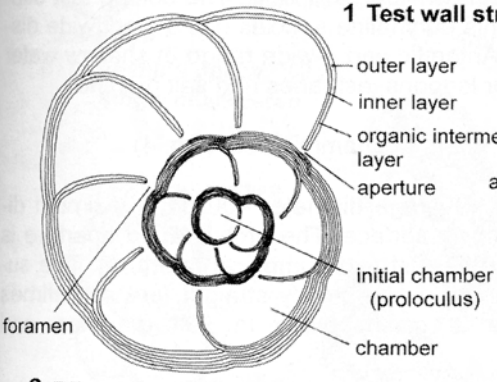


b porcelaneous

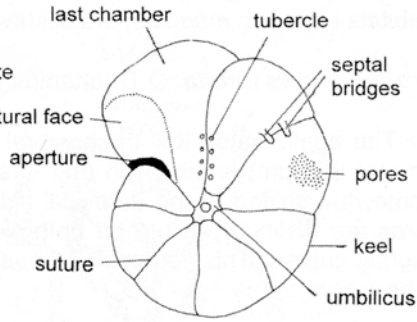


c calcareous hyaline

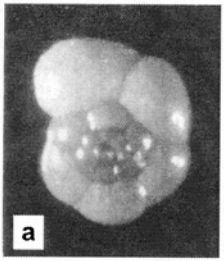
1 Test wall structures



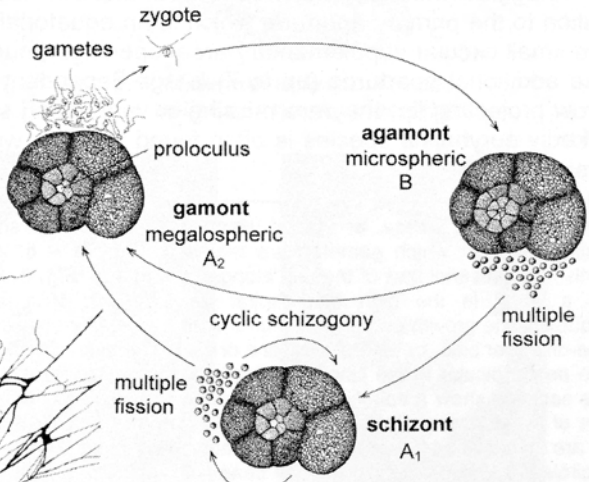
2 Bilamellar wall structure of a planispiral rotaliid equatorial section



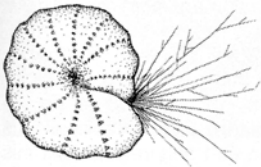
3 Morphological features of a foraminifer lateral view



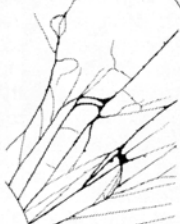
4 **a** ***Trochammina inflata*** 486 µm **spiral side**



4b ***Trochammina inflata*** **Life cycle**



a Living specimen



b Reticulopodia

5 ***Elphidium williamsoni*** 525 µm

GENERA AND SPECIES

Many of the species described below from salt marshes, salt flats and other shore areas from the German Bight and the western Baltic Sea have a worldwide distribution.

Miliammina fusca O. Lituolida, Fam. Rzehakinidae (Fig. 9)

The chambers of this agglutinated foraminifer are arranged in a milioline-quinqueloculine manner with five chambers being visible externally. The oblong test consists of agglutinated fine quartz grains. This euryhaline species has a worldwide distribution extending from the Arctic to the Antarctic and a wide range of shallow water habitats including mudflats, brackish water lagoons, estuaries and salt marshes.

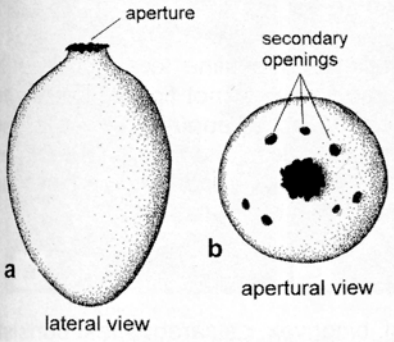
Trochammina inflata O.Trochamminida, Fam.Trochamminidae (Fig. 4)

The agglutinated low trochospiral test (average diameter 400 µm, maximum diameter 950 µm) is brownish and has a shiny surface. The arch-shaped aperture is somewhat shifted to the umbilical side of the test (extraumbilical aperture). The sutures are distinctly visible on both sides and, while mostly straight, are sometimes slightly curved. This euryhaline species is cosmopolitan in salt marshes and other shore areas.

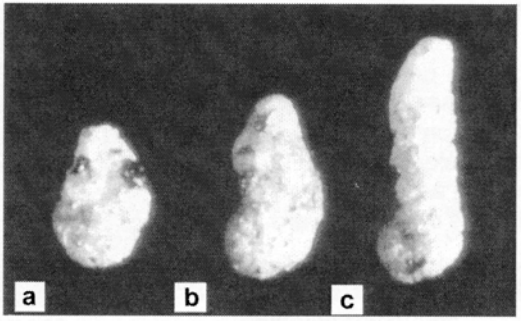
Jadammina macrescens O.Trochamminida, Fam. Trochamminidae (Fig. 10)

The agglutinated very low trochospiral and thin test has no distinct umbilicus. In addition to the primary aperture, which is an equatorial reniform slit, there are one or more small circular supplementary areal openings (multiple aperture). The number of these additional apertures (up to 7) is age-dependent and each is surrounded by a narrow projecting lip; they are missing in very young specimens. This cosmopolitan, markedly euryhaline species is often found together with *Trochammina inflata* in salt marshes.

Fig. 6. *Ovammina opaca.* **a:** Lateral view. **b:** Apertural view showing the aperture and secondary openings through which gametes are released. **Fig. 7a – c.** *Ammotium salsum,* three stages of growth. The uniserial part of the test elongates with age. **Fig. 8.** *Eggerelloides scabrus*, ≥3 chambers form a whorl in the high trochospiral test. **Fig. 9.** *Miliammina fusca,* example of milioline-quinqueloculine growth pattern. **a:** Four-chamber side, line showing direction of transverse section. **b:** Three-chamber side. **c:** Lateral view of **a** or **b. d:** Transverse section (its location depicted in **a**). This is made perpendicular to the longitudinal axis of the elongated test and through the proloculus. Transverse sections show a spiral chamber arrangement. This is not to be confused with the basic coiling mode of two chambers to the turn (**a, b, 13a, b**). In the spiral revealed by transverse section, chambers are added at 144° to each other. By adding chambers in a 144° succession, five chambers make two circles (5 x 144° = 720°) with five chambers visible externally in a 72° succession. Measurements of angles in sectioned specimens, however, show a great range of variation and only rarely meet this arithmetical pattern. **1, 2, 3:** The last three chambers to have been added, **1** being the most recently added. **Fig. 10.** *Jadammina macrescens.* **a:** At the spiral side of the low trochospiral test all chambers are visible (evolute side). **b:** At the umbilical side only the chambers of the last whorl are visible (involute side). **Fig. 11.** *Haplophragmoides manilaensis,* lateral view of the planispiral involute test.



6 ***Ovammina opaca*** 350 µm
single chambered

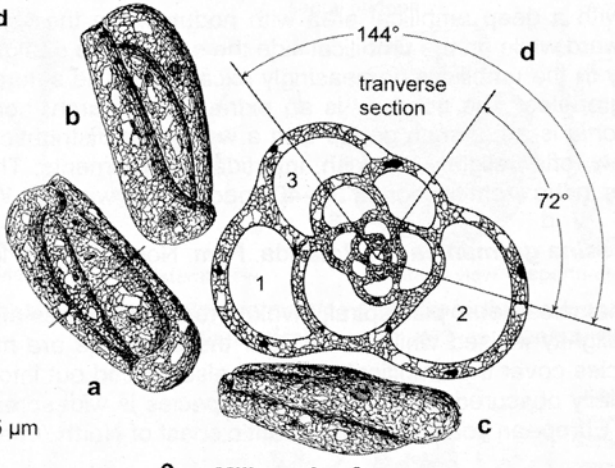


7 ***Ammotium salsum*** 356 µm, 463 µm and 602 µm

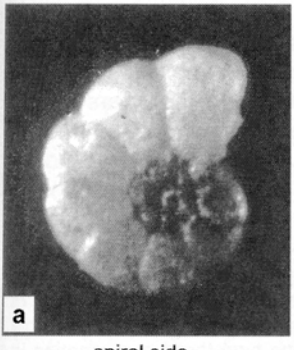


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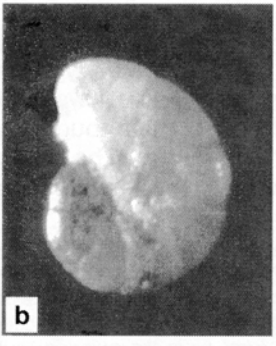
Eggerelloides scabrus 625 µm
high trochospiral



9 ***Miliammina fusca*** 478 µm
milioline - quinqueloculine

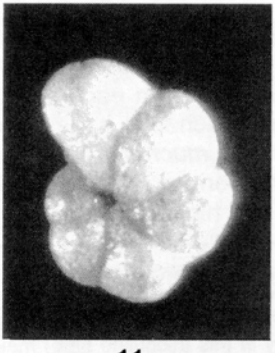


spiral side



umbilical side

10 ***Jadammina macrescens*** 461 µm
low trochospiral



11 ***Haplophragmoides manilaensis*** 634 µm
planispiral

Triloculina oblonga O. Miliolida, Fam. Haueriniidae (Fig. 13)

This porcelaneous-walled foraminifer has a milioline-triloculine test, that is only three chambers are visible externally and transverse sections (not figured, for plane of section see Fig. 9a) reveal a spiral of chambers added at about 120°. As in the quinqueloculine type there is a variation in the size of angles. The test is three times as long as it is wide and has a round aperture with a short, bifid tooth. It is a common species of the seaward parts of salt marshes, lagoons and mudflats.

Ammonia sp. O. Rotaliida, Fam. Rotaliidae (Fig. 17)

Members of this genus have a low trochospiral, biconvex, calcareous test consisting of a coil with 3 to 4 whorls. The spiral side is evolute and the umbilical side involute with a deep umbilical area with nodules. On the spiral side the sutures curve backward while on the umbilical side the sutures are straighter and, from the test periphery to the umbilicus, increasingly excavated. The sutures are bordered by numerous granules. The aperture is an extraumbilical arch, bordered by a protruding lip. *Ammonia* is a common genus with a worldwide distribution and occurs in sheltered, shallow, often slightly brackish, intertidal environments. There are considerable difficulties in the identification of 30–40 species (Hayward, B. W. et al. 2004).

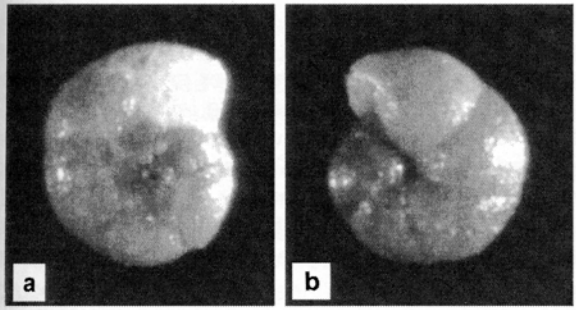
Haynesina germanica O. Rotaliida, Fam. Nonionidae (Fig. 15)

The calcareous, planispiral, involute test has sutures at the test periphery that are only slightly incised while those near the umbilicus are more deeply incised. Small tubercles cover the umbilical area and also spread out into the sutures. The aperture is usually obscured by tubercles. The species is widespread in mudflats and estuaries of European coasts and the Atlantic coast of North America.

Haplophragmoides manilaensis (Syn. *Haplophragmoides bonplandi)* O. Lituolida, Fam. Haplophragmoididae (Fig. 11)

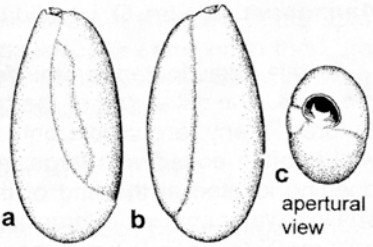
The agglutinated, planispiral, involute test generally has 5 to 6 chambers in the last turn. The chambers are inflated and the periphery of the test is lobate. There is a small umbilicus on each side of the test. The test wall is thin and the coarse sand particles attached to it make the chamber surface rougher and less shiny than that of *Jadammina macrescens* with its finer particles. The aperture is a short but mostly broad reniform or archlike slit in the equatorial plane. It is a cosmopolitan species of middle and high marsh areas.

Fig. 12a, b. *Arenoparrella mexicana,* spiral and umbilical side of the agglutinated planispiral test. **Fig. 13.** *Triloculina oblonga.* Transverse sections (not figured, for plane see Fig. 9a) reveal a spiral of chambers added at about 120°. By adding chambers in a 120° succession, three chambers (visible externally in this triloculine type) make one turn. As in the quinqueloculine type there is a variation in the size of angles. **Fig. 14 – 20.** Examples of foraminifera abundant in shelf and littoral habitats of the southern North Sea and the Baltic Sea. (Fig. 1 and 3 from Murray 1979; Fig. 2 from Haynes 1981; Fig. 4b from Lehmann, Röttger & Hohenegger 2006; Fig. 8 from Höglund 1947; Fig. 9 combined from Hofker 1977 and Haynes 1981; Fig. 16 – 19 from Hofker 1977)



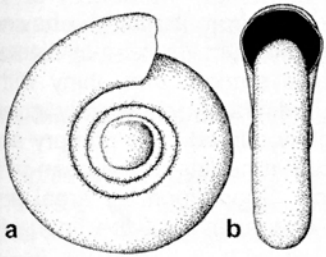
spiral side umbilical side

12 *Arenoparrella mexicana* 333 µm



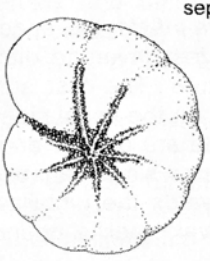
three-chamber side two-chamber side

13 *Triloculina oblonga* 422 µm
milioline-triloculine



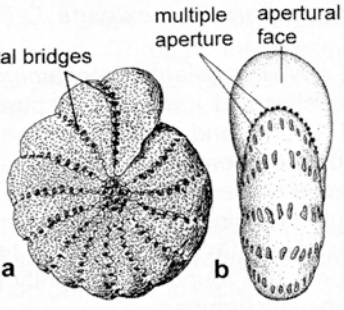
lateral view apertural view

14 *Cornuspira involvens*
200 µm **planispiral**



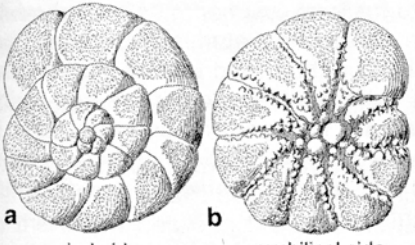
lateral view

15 *Haynesina germanica*
575 µm **planispiral**



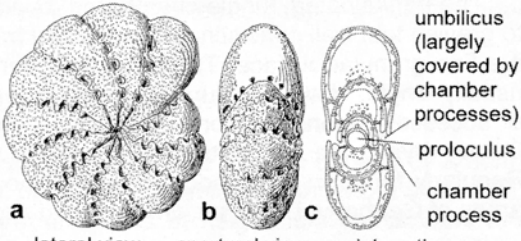
lateral view apertural view

16 *Elphidium williamsoni*
463 µm **planispiral**



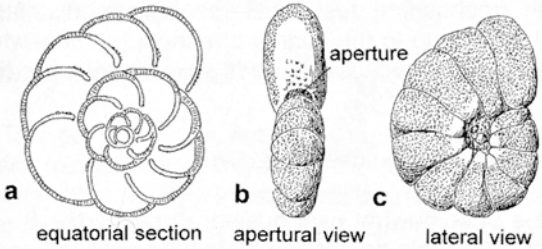
spiral side umbilical side

17 *Ammonia* sp. 583 µm
low trochospiral



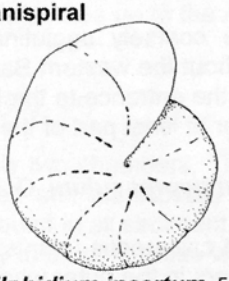
lateral view apertural view axial section

18 *Elphidium excavatum* 533 µm
planispiral



equatorial section apertural view lateral view

19 *Nonion depressulum* 411 µm **planispiral**



20 *Elphidium incertum* 512 µm
planispiral

Ammotium salsum O. Lituolida, Fam. Lituolidae (Fig. 7)

In this agglutinated foraminifer the juvenile differs in chamber arrangement from the adult. The initial part of the test is planispiral and evolute, the later part uniserial. Sutures, if any, are visible only in the uniserial part. The coarsely agglutinated test wall is often dotted with large mica and feldspar grains. The aperture is a rounded opening located at the end of the often tapering final chamber. It is a widespread, shallow water species of brackish water such as small bights, estuaries, lagoons. It is only rarely found in low marshes and prefers sediment types with a higher proportion of coarser sand particles.

Arenoparrella mexicana O.Trochamminida, Fam. Trochamminidae (Fig. 12)

While similar to *Trochammina inflata*, this species differs from it mainly in having a smaller and lower trochospiral test (average diameter 300 µm, maximum diameter 470 µm) and a multiple aperture. The test surface is smooth and shiny with a rounded periphery. The sutures on the spiral side are radial and only slightly incised, while those on the umbilical side are deeper and slightly curved. The primary aperture is a straight to comma-shaped slit of variable width. It begins at the base of the apertural face and extends towards the umbilical side. Supplementary areal apertures are rounded or slightly oval porelike openings at the apex of the final, often whitish chamber.

Ovammina opaca O. Astrorhizida, Fam. Saccamminidae (Fig. 6)

The agglutinated, single chambered, ovoid to fusiform test has a soft, often white to greyish test wall consisting of very small mica plates and diatom frustules that give it a fine granular surface. The aperture is terminal on the broad end of the test and usually single. However, during gametogenesis the main aperture closes and a ring of accessory openings, from which the gametes are released, is formed around it. During drying, the soft tests often lose their shape and shrink to amorphous lumps. Regularly found in the Wadden Flats, lagoons and other low energy, muddy shore areas of Germany, Denmark, Sweden and Argentina.

Eggerelloides scabrus O.Textulariida, Fam. Eggerellidae (Fig. 8)

The coarsely agglutinated high trochospiral test of *E. scabrus* is distributed throughout the western Baltic Sea but only up to the Darss threshold (a shoal which marks the entrance to the Baltic Sea proper) because it needs salinities of more than 20‰ for at least part of the year.

Nonion depressulum O. Rotaliida, Fam. Nonionidae (Fig. 19)

The calcareous, planispiral test is of somewhat oval outline. Because the 8 – 12 chambers in the outer whorl in adult individuals do not completely enclose the chambers of the penultimate whorl, both sides of the test are somewhat evolute and the umbilicus is wide (Fig. 19 c). The umbilical area is covered by a grainy coating that

may spread out into the sutures. The aperture is a small slit at the base of the apertural face. *Nonion depressulum* is a cosmopolitan species. It is common in the European Wadden Sea between high and low tide marks, in estuaries and other nearshore intertidal areas, independent of sediment quality and salinity.

Elphidium williamsoni O. Rotaliida, Fam. Elphidiidae (Fig. 5, 16)

The calcareous, planispiral and involute test is characterized by the large number of chambers (10–14) of the last whorl and many long septal bridges (retral processes: 9 or 10 between adjacent chambers on each side of the test) that span the deeply incised, gently curved sutures. The typical *Elphidium* aperture is a single row of porelike openings at the base of the apertural face. Often this multiple aperture is hidden by numerous tubercles and tapered pillars. Through the glassy-transparent test the green colouration caused by endosymbiotic chloroplasts of diatoms is visible in living individuals and even in dried tests.

Elphidium excavatum O. Rotaliida, Fam. Elphidiidae (Fig. 18)

Elphidium excavatum is distinguished from *Elphidium williamsoni* by the reduced numbers of chambers (6 or 7) in the last whorl and of septal bridges. Moreover, the septal bridges are shorter than in *E. williamsoni* and often only incompletely span the sutures. It is common in the inner shelf areas of the North Atlantic and characteristic of the uppermost but still flooded zone of low water movement of the Wadden Sea, particularly in the region of *Salicornia,* where the foraminifer lies free on the sand surface, often on top of a basement of sand grains. One of the morphological variants is *Elphidium excavatum clavatum,* which is restricted to deeper areas of higher salinity of the Baltic Sea.

Elphidium incertum O. Rotaliida, Fam. Elphidiidae (Fig. 20)

The sutures of this calcareous, planispiral and involute test are defined by a number of characteristic delicate slits (septal openings) variable in both length and width. In the areas between the slits the lateral test walls of neighbouring chambers are continuous without forming the typical septal bridges of the genus. It has a multiple aperture and occurs off the Atlantic and Pacific shores of North America, the Gulf of Mexico and Taiwan and in deeper regions of the western Baltic Sea up to the Arkona Basin.

Cornuspira involvens O. Miliolida, Fam. Cornuspiridae (Fig. 14)

This porcelaneous, flat disc-shaped test consists of only two chambers. The globular proloculus is followed by the long, tubular, undivided and planispirally coiled second chamber. It is a cosmopolitan, nearshore species found in all sediment types. There are numerous records from European coasts, mostly in mudflat areas where it is found in low abundance.

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Larger Foraminifera

Rudolf Röttger

INTRODUCTION

About 40 of the 10,000 extant species of foraminifera are grouped as "larger foraminifera". They attain sizes ranging from 1 mm to 3,5 cm (the soritid *Marginopora vertebralis)* and even to 12 cm (the nummulitid *Cycloclypeus carpenteri).* These large, multichambered, calcareous foraminifera have complex morphologies and have evolved convergently in several phylogenetic lineages, namely the porcelaneous walled families Alveolinidae, Peneroplidae and Soritidae (Order Miliolida) and the hyaline perforate families Amphisteginidae, Calcarinidae and Nummulitidae (Order Rotaliida). They are the remains of genera rich in species from the geological past. Their tests often constitute a major part of sedimentary rocks, for example the fusulinids of the Carboniferous and the nummulitids of the lower Tertiary (Fig. 2). Today, larger foraminifera occur in high densities in the nutrient-deficient shallow seas of the tropics and subtropics where they are important producers of carbonate sediments. Common to all is the presence of endosymbiotic unicellular algae, high water temperatures and a uniform all year round light. These factors limit them to a depth of about 100 m where the clear water of the euphotic zone ends. The symbionts are diatoms and dinoflagellates (both also called zooxanthellae), green algae (also called zoochlorellae) and red algae, by which the foraminifera obtain their yellow-green to brown, green or red coloration. Many species are mixotrophic, feeding on particulate food in addition to hosting algal endosymbionts, while others live photoautotrophically by means of their algae. Nitrate uptake by these foraminifera has been demonstrated, while micronutrients may be supplied by epibiotic bacteria. The algae provide energy from photosynthesis to the foraminifer, enhance calcification and also take up nitrogen- and phosphorus-containing host metabolites (recycling) that otherwise would be excreted into the sea. Algal symbiosis provides the host-symbiont system with a high energetic advantage over non-symbiotic competitors in nutrient-deficient environments. Endosymbiosis is supported by structural characteristics of the large tests. The transparent hyaline test walls, almost paper thin in the deepest dwelling members, can transmit most of the available light energy (in nummulitids). The lamellar wall structure (see p.111), including an enveloping canal system, reduces light penetration in shallow, brightly lit tropical surface waters and provides anchoring and stability for the calcarinids in high-energy reef-flat environments.

PRACTICAL TECHNIQUES

Sand rich in larger foraminifera (from Hawaii, Ryukyu Islands, Belau; Fig. 1) can be provided for student courses by the author of this chapter. These sands will have

been prepared as follows. Thalli of brown, red and green algae to which the foraminifera are attached, often in large numbers, are collected in the sublittoral zone of sheltered rocky shores, in rock pools and tidal pools or in shallow pools on reef flats at low tide. The collection is then vigorously stirred in a bucket of seawater. The algae are taken out of the water by hand and algal fragments are removed using a coarse-meshed sieve (3–5 mm). By repeated additions of water, stirring and decanting, the remaining sediment is washed several times. The foraminifera are not lost during this procedure: because of their density they always sink to the bottom immediately. For more quantitative results, small quantities of seaweeds can be processed for a few seconds in a blender, a process in which the foraminifera are not damaged. The algal fragments are then removed by means of a coarse-meshed sieve. The sediment is transferred to a large Petri dish or divided into subsamples. If this live sample is covered by a few millimetres of sea water and examined under a stereomicroscope it is seen to consist of a coarse biogenic sand together with a rich fauna of undamaged, millimetre-sized invertebrates. The sample will also contain shells and skeletons, whole and fragmentary, of gastropods, bivalves, echinoderms, stony corals, polychaetes, sponges, crustaceans, calcareous red and green algae and many foraminifera, including larger foraminifera. After live observation, the sample may be washed several times in tap water and dried. The dried sample is subdivided according to the number of students and stored in vials. To mount dried specimens you will need small Petri dishes (say 80 mm), artist's brushes (size 0 or 1), 10 ml vials containing distilled water and wallpaper glue respectively and micropalaeontological slides (Fig. 3). The sample is tapped gently out of the vial into a Petri dish so that the grains are evenly spread and not on top of one another. By means of a stereomicroscope the sample can be examined systematically. Each selected foraminifer is picked up by a moistened artist's brush and transferred to the slide where it is put onto a tiny drop of glue. Wallpaper glue is transparent and can be redissolved at any time with the wet brush should one want to examine the other side of the foraminifer. In this way the external morphology of each species from the collection can be studied. Micropalaeontological slides can be ordered from Dr. F. Krantz, Fraunhoferstr. 7, 53121 BONN, GERMANY.

GENERA AND SPECIES

Heterostegina depressa O. Rotaliida, Fam. Nummulitidae (Fig. 4)

The 40–50 chambers of the adult gamont are coiled in a plane, that is they form a planispiral test. There is no morphological difference between upper and lower sides. Only the last whorl of the spiral is visible and consists, in the specimen of Fig. 4, of 17 chambers that have overgrown the older whorls that are discernable as the shaded area (involute test, see definition on p.112). Each chamber is separated from its neighbours (the older and the younger one) by a concentric wall (chamber wall or septum) standing perpendicular to the paper plane. Many radially oriented secondary septa divide each chamber into chamberlets. The chamberlets are connected radially and tangentially with neighbouring chamberlets by canal-like openings, the stolons. Thus all chambers form a continuum for the cytoplasm that contains but one nucleus.

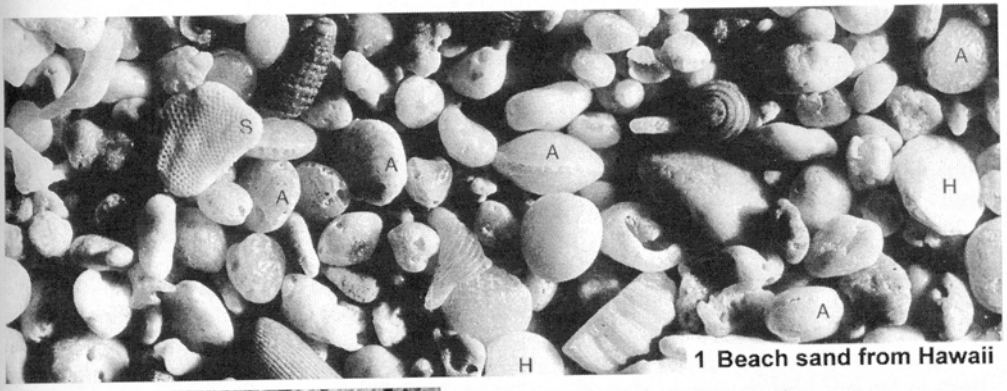
A second cavity consists of a canal system located within the septa, the secondary septa and the keel (or marginal cord) which surrounds the test at its periphery. The canals are connected both with the interior of the chambers and with the exterior. This compartmentalisation is also reflected in the cytoplasm: the 'chamber cytoplasm' contains the symbiotic algae while the 'canal cytoplasm" forms pseudopodia that protrude from the marginal cord and serve locomotion, transport of digestive waste to the exterior and the process of chamber formation. The canals are passageways both for excretions and gametes. During multiple fission, in both the agamont and the schizont (see below), the entire cytoplasm, including the symbionts, is evacuated through the canals and, once outside the test, divides into the daughter cells. In live individuals the symbiotic diatoms are visible through the hyaline lateral test wall through which light passes for photosynthesis by the symbionts. *H. depressa* lives photoautotrophically by means of its symbiotic diatoms. Its lateral test walls are penetrated by a second kind of openings, innumerable pore canals, through which pass respiratory gases and micronutrients, but not cytoplasm. The canal system of the keel and its openings as well as the pores can be studied at a magnification of x100 in transmitted light. This larger foraminifer has a trimorphic life cycle: besides typical alternation between a 3 mm gamont and an agamont as large as 2 cm, a 2 mm schizont may occur which produces hundreds of daughter cells by multiple fission (see p.115, Fig. 4b). *H. depressa* is one of the most widespread of all larger foraminiferal species throughout the tropical and subtropical Indian and Pacific Oceans.

Amphistegina O. Rotaliida, Fam. Amphisteginidae (Fig. 5 – 9)

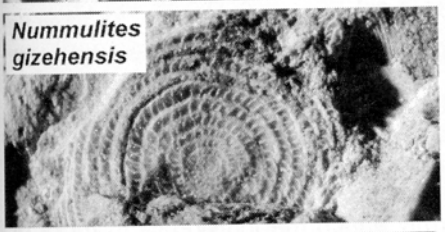
The spiral (upper) side and the umbilical (lower) side of the trochospiral test differ considerably from each other in their pattern of sutures. Sutures are faint linear elevations or depressions on the lateral surfaces of the test and mark the places where septa meet the upper and the lower test wall. Intersepta are incomplete septa which

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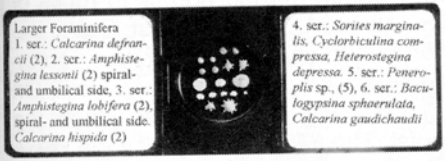
Fig. 1. Beach sand from Hawaii (size of the grains 1–3 mm). This biogenic, calcareous sand contains a high proportion of larger foraminifera that have been polished by the surf. Also present are prosobranch shells and part of a sea urchin spine. **A:** *Amphistegina sp.,* **H:** *Heterostegina depressa,* **S:** A soritid. **Fig. 2**. *Nummulites gizehensis* (10 mm) in nummulitic limestone of the Eocene (lower Tertiary) from Giza (Egypt). **Fig. 3**. Micropalaeontological slide (75 x 25 mm) with mounted larger foraminifera. **Fig. 4**. *Heterostegina depressa* (3950 µm). The structures on the exterior surface of the lateral test labelled "septum" and "secondary septum" are the faint linear elevations or depressions called sutures. They mark the places where septa and secondary septa, which stand perpendicular to the plane of the paper and subdivide the test lumen, meet the interior surface of the lateral test wall. **Fig. 5 – 7.** *Amphistegina lessonii* (875 µm). **Fig. 8, 9**. *Amphistegina lobifera* (1125 µm). The structures labelled "septum", "interseptum" and "toothplate" are sutures of the respective septa. They are similar to the corresponding structures in *Heterostegina depressa* (see Fig. 4). In comparison to the simply curved sutures of *Amphistegina lessonii, A. lobifera* has winding sutures. Furthermore, *A. lessonii* is characterized by the star-shaped pattern formed by its supplementary chambers. As each new chamber is formed (as in *Heterostegina* and *Calcarina),* the entire test is enveloped by a calcareous lamella (see p.115, Fig. 2). Thus, as the number of covering layers increases with age, the visibility of older sutures decreases. **Fig. 10**. *Peneroplis pertusus* showing the test lumen.



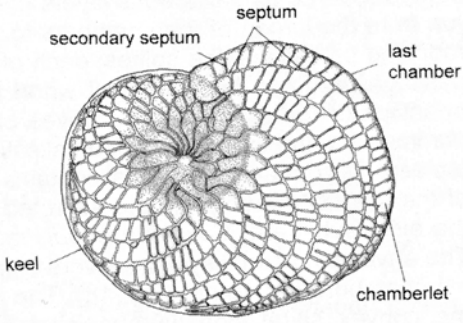
1 Beach sand from Hawaii



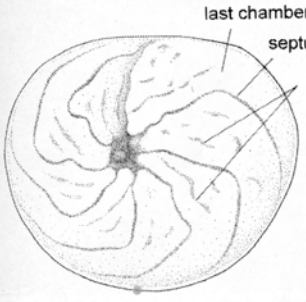
2



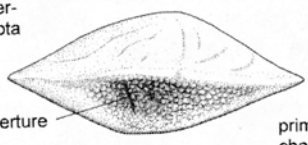
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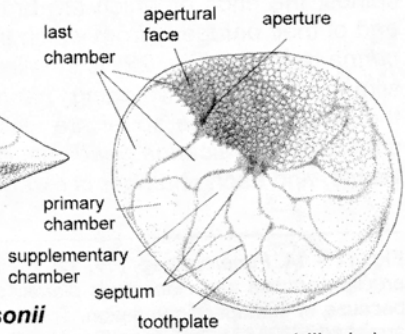
4 Heterostegina depressa



5 spiral view

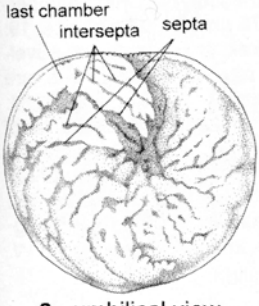


6 profile view

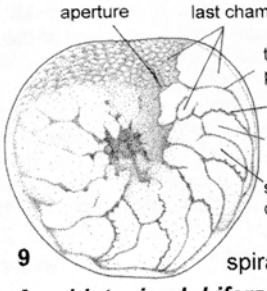


7 umbilical view

Amphistegina lessonii
(5 – 7)

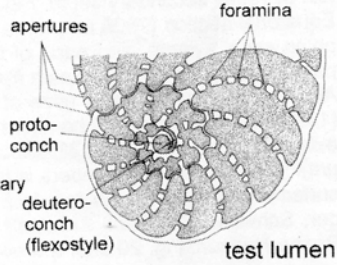


8 umbilical view



9 spiral view

Amphistegina lobifera (8, 9)



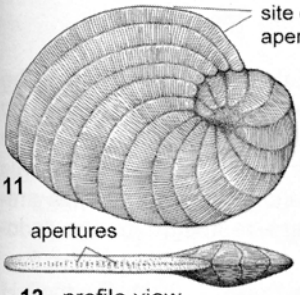
10 ***Peneroplis pertusus***

originate at the 'ceiling' of the chamber but which do not reach the floor. Their sutures are thus confined to the spiral side. Toothplates are perforated septa which originate on the chamber 'floor' and connect two septa but do not continue to the 'ceiling'. They thus form sutures only on the umbilical side. A tooth plate divides a chamber into a primary chamber and a supplementary chamber. *Amphistegina lessonii* and *A. lobifera* host endosymbiotic diatoms that provide individuals with a brownish coloration that is still visible in dried specimens. They occur abundantly at depths of less than 30 m in the Indo-Pacific.

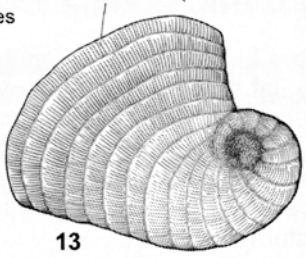
Calcarina O. Rotaliida, Fam. Calcarinidae (Fig. 18 – 23)

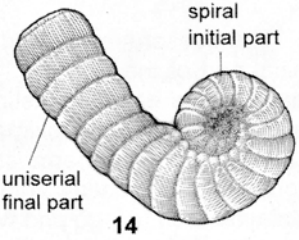
Species of the genus *Calcarina* have low trochospiral tests. The spiral of chambers is covered by calcareous layers containing a canal system (Fig. 19). The canals run from the lumen of the chambers to the surface of the test. In addition, there are radiating processes, the spines, each of which contains a bundle of canals forming a passageway from the innermost whorl to far beyond the periphery of the test. The openings of the canals lie in grooves on the entire surface of the spines. As in the nummulitids, the test compartmentalisation corresponds to a differentiation of the cytoplasm. The 'chamber plasma' contains the endosymbiotic diatoms. At the distal end of the spines, elastic pads are secreted that firmly attach the individuals to rocks or the algal substrate. *Calcarina gaudichaudii* (Figs. 18 – 21) is the largest *Calcarina*. The enveloping canal system covers a spiral of up to 100 chambers. Only the youngest chambers are visible (Fig. 18). The test resembles a cushion in lateral view. On the convex spiral side lie roundish tubercles, 'pillars' (Fig. 18), between which the openings of the canal system are visible as dots. The rare agamonts have more spines, the ends of which are branched. They form a bubble brood chamber at the end of their ontogeny from which the young gamonts escape (viviparity, Fig. 20). *Calcarina defrancii* (Fig. 22) is smaller than *C. gaudichaudii* and has a larger number of slim spines. The enveloping canal system is more delicate so that all chambers of the last turn of the spiral are visible at the umbilical side. *Calcarina calcar* is also smaller than *Calcarina gaudichaudii.* The entire spiral of up to 30 chambers is clearly visible, and each chamber is extended into a short spine containing canals.

Fig. 11 – 14. *Peneroplis* sp. (**11, 12**: 1525 µm; **13**: 1325 µm; **14**: 1625 µm). Three individuals of *Peneroplis pertusus* and *Peneroplis planatus*. It is often difficult to differentiate between these species because of intraspecific variation. The profile view (apertural view) (Fig. 12) shows the many serially arranged apertures (compare Fig. 10). **Fig. 15 – 17**. *Sorites variabilis* (**15, 16**: 2350 µm). The chambers of the discoid test are ring-shaped (annular discoid test structure) and subdivided into chamberlets by radial secondary septa. **Fig. 18 – 21**. *Calcarina gaudichaudii*. **18**: 3175 µm including spines. **19**: Equatorial section (2800 µm) of an araldite cast showing one turn of the spiral of chambers, the enveloping canal system, and parts of the spines. The spines, each consisting of a bundle of canals, are passageways for the cytoplasm from the innermost part of the test to the surrounding sea water. **20**: Alternation of generations (size of agamont 4000 µm including spines). **Fig. 22**. *Calcarina defrancii* (1950 µm). The spiral of chambers is only visible from the umbilical side. Each chamber is pointed or extends into a spine. **Fig. 23**. *Calcarina calcar* (1425 µm). **Fig. 24**. *Baculogypsina sphaerulata* (2200 µm). The network of chambers is interrupted by a network of pillars oriented perpendicular to the test surface. (Fig. 10 from Winter, Arch. Protistenk., 10: 1-113, 1907; Fig. 19 from Hottinger & Leutenegger, Schweiz. Paläontol. Abhandl., 101, 115-151, 1980; Fig. 4 – 18, 21 – 24 after drawings by W. Roehe-Hansen; Fig. 20 after a drawing by R. Krüger)

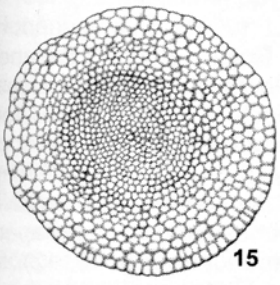


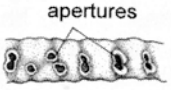
12 profile view





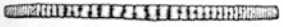
***Peneroplis* sp.** (11 – 14)



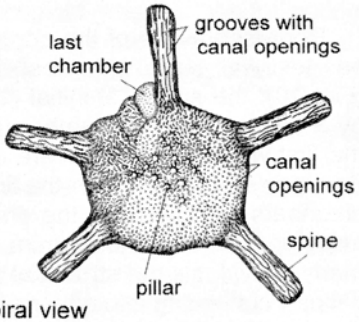


profile view detail

Sorites variabilis
(15 – 17)

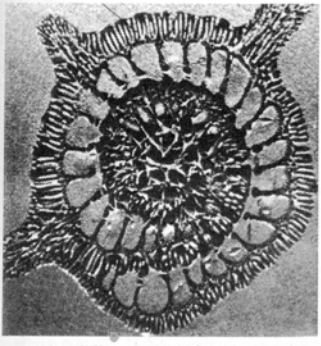


16 profile view of last chamber

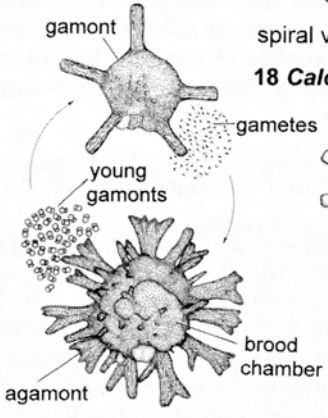


spiral view

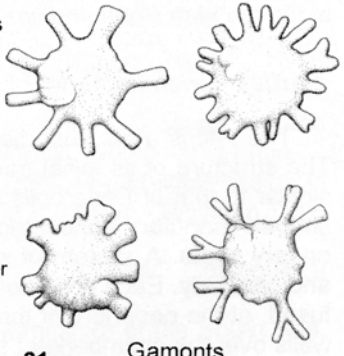
18 *Calcarina gaudichaudii* gamont



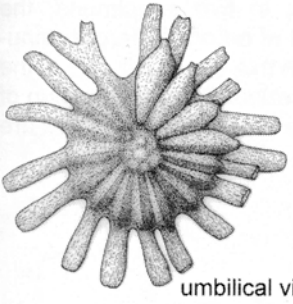
19 Araldite cast of test cavity



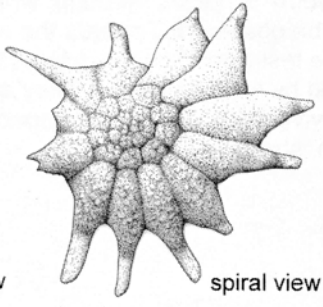
20 Alternation of generations
Calcarina gaudichaudii
(19 – 21)



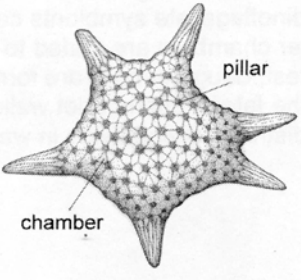
21 Gamonts
Variation in test shape



22 *Calcarina defrancii*



23 *Calcarina calcar*



24 *Baculogypsina sphaerulata*

Baculogypsina sphaerulata O. Rotaliida, Fam. Calcarinidae (Fig. 24)

The subspherical test differs in its structural plan from the other calcarinids. The initial part of the test is a tiny flat spiral of less than two turns. This is enveloped by many layers each consisting of a net of chambers connected to each other. These layers conceal the proximal parts of the spines with their central bundle of canals running from the innermost spiral to the periphery. The Calcarinidae, which live predominantly in shallow, high energy reef environments, are restricted to the Indo-Malayan archipelago and western Pacific.

Peneroplis O. Miliolida, Fam. Peneroplidae (Fig. 10 – 14)

Representatives of the porcelaneous Peneroplidae and Soritidae (the porcelaneous test wall strucure is described on p. 111) have a distinct initial part of the test (Fig. 10): the spherical initial chamber (proloculus, protoconch) is partly surrounded by a tubular second chamber (deuteroconch, flexostyle). The following chambers of the first whorl of the spiral are connected one to another by a single tubular opening (foramen). However, after the first whorl, the number of foramina between successive chambers increases as the chambers increase in size. Consequently, many apertures open to the exterior from the last chamber (multiple aperture), (Fig. 10, 12). In many individuals the structural plan changes during ontogeny with the planispiral initial part continuing into either a narrow or a wide uniserial final part (Fig. 13, 14). Because of this intraspecific variation in the final form it may be difficult to differentiate between several species. Living *Peneroplis* are coloured red by the unicellular symbiotic red alga *Porphyridium.*

Sorites variabilis O. Miliolida, Fam. Soritidae (Fig. 15 – 17)

The test is a flat, thin disc and its mode of coiling is known as "annular discoid". The structure of its initial part, which is no longer discernable in adult specimens, is similar to that of *Peneroplis* (peneroplid structure, Fig. 10). Later chambers are ring-shaped (annular), concentric and divided into numerous chamberlets by radial secondary septa. A system of stolons connects neighbouring chamberlets both radially and circularly. Each chamberlet of the last chamber has two apertures, which may be fused, at the periphery of the test. (Fig. 17). The lateral walls of the chamberlets (the walls over the chamberlets) are particularly thin and the calcite needles of the median layer (see p. 115, Fig. 1b) are oriented perpendicular to the surface. They do not scatter the light and thus form windows through which, in live specimens, the dinoflagellate symbionts can be observed. Towards the end of ontogeny larger annular chambers are added to the test (brood chambers). Within this youngest part of the test, daughter cells are formed by multiple fission. They are released by dissolution of the lateral chamberlet walls (viviparity). The several species of the genus *Sorites* are distributed worldwide in warm, shallow seas.

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Röttger, R., Spindler, M., Schmaljohann, R., Richwien, M. & Fladung, M. (1984): Functions of the canal system in the rotaliid foraminifer *Heterostegina depressa*.– Nature, 309: 789-791. Erratum note: Nature, 315: 77 (1985).

FILMS (spoken commentary in English and German)

Röttger, R. (1982): The larger foraminifer *Heterostegina depressa.* Organization and growth of the megalospheric generation. – Film C 1451 IWF Göttingen, 11 min.

Röttger, R. (1983): The larger foraminifer *Heterostegina depressa.* Multiple fission in microspheric and megalospheric generation. – Film C 1506 IWF Göttingen, 10½ min.

Röttger, R. (1983): Ecology of larger foraminifera. – Film C 1497 IWF Göttingen, 9 min.

Röttger, R. & Krüger, R. (1989): Calcarinidae – larger foraminifera of the South Sea. – Film C 1672 IWF Göttingen, 9 min.

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Planktonic Foraminifera

Michael Spindler

INTRODUCTION

Planktonic foraminifera (rhizopods of the Class Foraminifera, Order Globigerinida) are widely distributed in the world´s oceans. They are truly open ocean organisms and are only rarely found in shelf seas such as the North Sea. All species have multichambered low-magnesium calcite tests (calcite with a proportion of 3–10 mole percent magnesium).Their overall sizes range between 100 µm in species such as *Tenuitella parkerae* up to 5000 µm in *Hastigerina digitata*. Spinose species with intact spines attain overall sizes of more than 8000 µm. The walls are primarily bilamellar and the chambers are either coiled planispiral or in a trochospire. In trochospiral tests the direction of coiling may be dextral (clockwise) or sinistral (anticlockwise) when viewed from the spiral side. For further explanations of chamber arrangements and wall structure see p. 111 – 112. The tests are perforated by numerous pores that are sealed off from the inside by an organic layer. This layer is permeable to gases (O_2 and CO_2) and low-molecular weight inorganic nutrients. Some species are non-spinose, whereas other species are covered with calcareous, but very flexible, spines of various types (Fig. 2, 3, 15a), at times exceeding 3500 µm in length. These spines are lost or broken off in specimens collected from sediment samples (Fig. 1, 4, 5, 6, 8, 12, 14, 15 b, c). The cytoplasm inside the test cannot penetrate the pores. However, the pseudopodial network together with the cytoplasm covering the surface of the test and the spines are connected with the inner cytoplasm through the primary and (if present) the secondary apertures.

An alternation of generations (see p. 115, Fig. 4b) has never been proved to occur in planktonic foraminifera. So far, only sexual reproduction has been observed, during which 300,000 – 400,000 biflagellated gametes are released. In several species reproduction is linked to the lunar cycle, resulting in a generation time of 29 days (for example *Hastigerina pelagica*) or 14 days (for example *Globigerinoides ruber*). In these species the gametes are regularly released a few days after the full moon. For some non-spinose species, reproductive cycles can be much longer, and it is speculated that some cycles may last for up to a year.

There are two distinct feeding strategies observed in planktonic foraminifera. Spinose species are mainly carnivorous and their extended spines are covered with sticky cytoplasm to which zooplankton such as other protozoa (tintinnids, radiolaria), invertebrate larvae and planktonic copepods adhere.The prey is transported towards the cell body where it is digested both outside and inside the test. Non-spinose species are not able to catch living zooplankton and are herbivorous, feeding mainly on diatoms, dinoflagellates, haptophytes and chrysophytes. The prey is ingested and empty frustules or thecae are discarded when digestion has been completed.

While no organisms are known to feed on planktonic foraminifera, parasitic

dinoflagellates and sporozoa have been (rarely) observed in tests. Several species, however, are associated with symbiotic algae (for example *Globigerinoides sacculifer, G. ruber, Globigerinella siphonifera, Orbulina universa*). The symbionts are mainly dinoflagellates but in some species chrysophytes are found as the obligatory symbionts.

Planktonic foraminifera dwell in the water column mainly within the photic zone, and only a few species live at greater water depths. One species, *Neogloboquadrina pachyderma*, lives within the brine channel system of Antarctic sea ice. There, it attains densities of more than 100 individuals per litre of melted sea ice, whereas generally the maximum densities of this species in surface waters only rarely exceeds 100 individuals per m^3.

The tests of planktonic foraminifera that have reproduced sink as the globigerine ´snowfall´ down the water column to the deep ocean floor. Here they accumulate, though slowly, and form a blanket of ´*Globigerina* Ooze´, which covers almost half of the abyssal plain beyond the continental shelves. It is the dominant sea floor deposit from approximately 600 m down to the Calcite Compensation Depth (CCD) at about 4500 m (see p. 141). Planktonic foraminifera show continous rapid evolutionary development from Cretaceous times making them excellent index fossils (indicators of geological age). By means of their appearance in the geological record and the short vertical time ranges of particular species and species groups, planktonic foraminiferal biozones can be distinguished. In oil exploration the basic technology is the drilling of holes on land and on the sea floor in order to discern and map biozones for the identification of the relative age of sedimentary rocks. It is because of their small size that species of foraminifera can be readily identified from the drillings returned to the surface. Furthermore, palaeoenvironmental conditions such as temperature and salinity, mainly of the Pleistocene, can be identified by analysing stable oxygen isotopes (the ratio of $^{18}O/^{16}O$) and stable carbon isotopes (the ratio of $^{13}C/^{12}C$) stored in the calcium carbonate tests of both fossil planktonic and benthic foraminifera.

PRACTICAL TECHNIQUES

Tows with plankton nets (mesh sizes between 10 and 300 µm) are usually deployed to collect foraminifera from the water column. Nets can be towed vertically, horizontally or obliquely. Nets should be towed at low ship speed (1 knot or less) and for short times (5 to 10 min), if specimens are to be used for live observations. Foraminifera should be separated from the rest of the sample by using a pipette as soon as possible and thereafter allowed to recover for about 24 hours in Petri dishes or similar small containers.

An extremely gentle collection method is the capture of individual specimens in glass jars by SCUBA divers since at least the spinose species are recognizable by the naked eye. Individuals captured with this method are completely undisturbed and in perfect condition. Sediment samples from the sea floor, or from suspended traps, can be used to obtain foraminiferal tests. They should be recovered from above the calcite compensation depth (CCD). Sediments can be retrieved by grabs (for example van Veen Grab) or coring devices (for example Multi-Corer). Samples from the very top of sea floor surfaces are sifted and dried as described on p. 113 –114. Material for student courses can be obtained from the author on request. Picking of

specimens and mounting on micropalaeontological slides is described on p. 125.

GENERA AND SPECIES

The specimens of the sample provided by the author mainly consist of the representatives of two families, Globigerinidae and Globorotaliidae. The Globigerinidae are characteristically spinose, their chambers are spherical or ovate and secondary apertures are present. The Globorotaliidae are characteristically non-spinose, their chambers are angular, ovate, spherical or compressed and the tests may have a keel. The sample was collected with a bottom grab at 580 m depth about 7 km (4 miles) West of the Caribbean Island of Barbados (West Indies). It was rinsed through a sieve with a mesh size of 50 µm to remove fine-grained silt and clay. The species are introduced and illustrated according to their abundance within the sample (Figs. 4 – 17): *Globigerinoides sacculifer*, *G. ruber*, *G. conglobatus* and *Neogloboquadrina dutertrei* are very abundant, *Orbulina universa*, *Globorotalia truncatulinoides*, *G. menardii* and *G. tumida* are abundant, *Sphaeroidinella dehiscens* and *Pulleniatina obliquiloculata* are rare and *Globigerinella siphonifera*, *Hastigerina pelagica*, *Globorotalia inflata* and *Candeina nitida* are very rare.

Globigerinoides sacculifer (Syn. *G. trilobus, G. quadrilobatus*) Fam. Globigerinidae (Fig. 4)

In this species the chambers are arranged in a trochospiral mode and the coiling direction is to the left or right. Unique for this species is the honeycomb structure of the chamber surfaces. The aperture is situated slightly extraumbilically, extends over three previous chambers and may be bordered by a pronounced rim. One secondary aperture per chamber is found on the spiral side. The last chamber to be built is often shaped in a sac-like fashion (Fig. 4b). *G. sacculifer* is the dominant species in tropical surface waters but also extends to subtropical areas.The maximum test size may exceed 1000 µm without spines.

Globigerinoides ruber Fam. Globigerinidae (Fig. 5)

Several characteristics make this species easily recognizable. First of all, older parts of the test are red in colour. Additional features such as two secondary apertures per chamber and a more or less roundish terminal chamber just over the two previously built chambers are good diagnostic features. The primary aperture is pronounced, slightly rimmed and in an umbilically centered position. There is no preferred coiling direction in *G. ruber*. A form lacking the red colour (forma *alba*) is less abundant and also attains a smaller maximum size than the *rosacea* form which may grow up to 600 µm in diameter. *G. ruber* is a typical tropical to subtropical surface water dweller.

Globigerinoides conglobatus Fam. Globigerinidae (Fig. 6)

In contrast to the aforementioned species, the last chamber formed in *Globigerinoides conglobatus* is situated above three previous chambers and likewise spans

the primary aperture over them. The outline of the last chamber is somewhat depressed and not that round when compared to *Globigerinoides ruber*. Two secondary apertures per chamber are present on the spiral side of the test. This species is coarsely spinose and occurs in surface waters of the tropics and subtropics. Adult specimens may migrate to deeper waters and, after secretion of additional calcite layers, will become difficult to recognize. Test size may reach 1000 µm.

Neogloboquadrina dutertrei (Syn . *Globoquadrina dutertrei, Globigerina eggeri*) Fam. Globorotaliidae (Fig. 7)

This is a non-spinose species with a trochospiral test, mostly right coiling (max. length up to 700 µm). The number of chambers within the last whorl and their arrangement is a characteristic diagnostic feature. The last chamber arches over 4 to 5 chambers and there is a very distinct umbilicus. Very often this last formed chamber is smaller than the previously formed one (kummerform). The sole aperture may possess a lip. The chambers appear very regular when viewed from the spiral side. *Neogloboquadrina dutertrei* is a common species in surface and near surface waters of the tropics and subtropics.

Orbulina universa Fam. Globigerinidae (Fig. 3, 8)

Adult specimens form a unique spherical last chamber encompassing a trochospiral test that is only weakly calcified. The trochospiral juvenile test is either resorbed prior to reproduction or dissolved during settlement to the seafloor, and is therefore not present within bottom sediment samples. The trochospiral stage possesses additional secondary apertures, while in the terminal, spherical stage true apertures are missing. Some authors regard as apertures the larger pores (diameters larger than 20 µm) that are irregularly spaced between the normal pores (diameters less than 5 µm). Experiments have shown that under very favourable conditions (for example larger amounts of food) a second or part of a second spherical chamber can be formed, thereby leading to a so-called Biorbulina. Test diameters of *Orbulina universa* may attain 1000 µm. This species is widely spread from the tropics to even subpolar regions.

Globorotalia truncatulinoides Fam. Globorotaliidae (Fig. 9)

This is a non-spinose species with a high trochospiral test in which both coiling directions are found. The periphery has an obvious keel. The aperture is in the form of a slit that is covered with a lip and extends from the umbilicus to the periphery. Viewed from the flat spiral side, all chambers are visible in younger specimens. Older specimens often show pustule formation on older chambers, thus obscuring the sutures between adjacent chambers. *G. truncatulinoides* is regarded as a subtropical species but right-coiling individuals preferentially extend into tropical regions, while more left-coiling ones extend to cold temperate areas. Maximum test diameters of up to 900 µm are recorded.

Globorotalia menardii (Syn. *G. cultrata*) Fam. Globorotaliidae (Fig. 10)

This species has very compressed umbilical and spiral sides, resulting in a flat side view (Fig. 10b). The chambers are arranged in a low trochospiral mode, bordered by a prominent keel. The preferred coiling direction is to the left. The aperture is elongate and reaches from the umbilicus to the periphery and shows a distinct broad lip. The outline of the test is more or less round and differs from *Globorotalia tumida* by also having a flatter and thinner test. An additional differentiating characteristic is the shape of the last-formed chamber, which is wider than its height. *Globorotalia menardii* is a non-spinose species of the tropics and subtropics and can reach 1500 µm as a maximum test size. The preferred water depth is within the lower photic zone between 200 and 400 m.

Globorotalia tumida Fam. Globorotaliidae (Fig. 11)

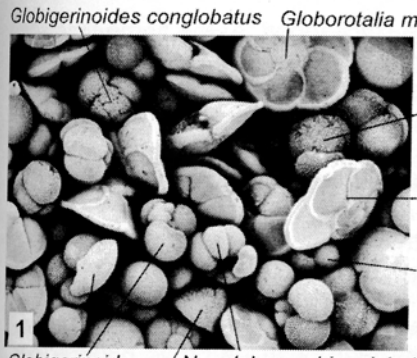
This species resembles *Globorotalia menardii*, having a prominent keel, a low trochospiral arrangement of chambers and an elongated aperture. Features that distinguish between the two species include the arrangement and size of the chambers. In side view (Fig 11b), *G. tumida* is less flat and the overall shape, when seen from the spiral or umbilical side, is elongate oval. The last-formed chamber, when measured from the umbilical side, is about as wide as its height.The maximum test size may reach up to 1400 µm. The coiling direction in this tropical to subtropical non-spinose species is mostly to the left. *G. tumida* also lives in similar depths to *G. menardii*.

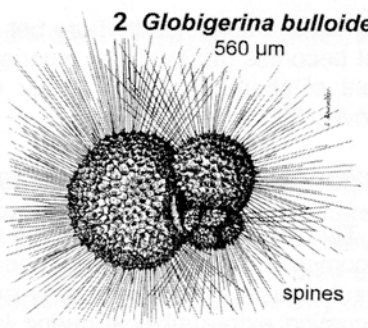
Sphaeroidinella dehiscens Fam. Globigerinidae (Fig. 12)

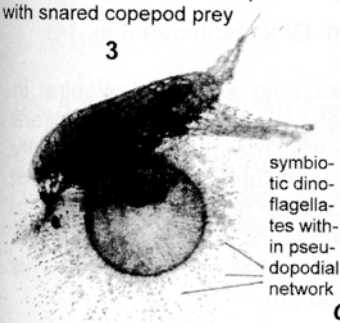
Sphaeroidinella dehiscens is a deep water species occuring well below 500 m in tropical and subtropical areas of the world´s oceans. In the juvenile stage it possesses spines that are shed later during ontogeny so that the adult stage is spineless. In this later stage the whole test morphology is obscured by a thick calcite crust leaving

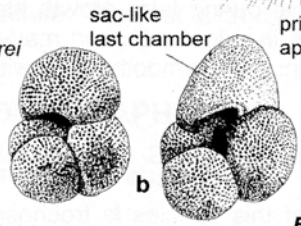
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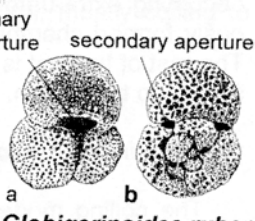
Fig. 1. Scanning electron micrograph of a tropical sediment sample collected from 580 m depth 4 miles west of Barbados. **Fig. 2.** *Globigerina bulloides* (Family Globigerinidae), a spinose species of subpolar and cold temperate water. Undamaged specimen. **Fig. 4 – 17.** Species from a sediment sample collected off Barbados.The spines of spinose specimens are broken off. **Fig. 4:** *Globigerinoides sacculifer*. **a:** Umbilical view of "normal" type specimen (670 µm). **b**: Specimen with sac-like last chamber (920 µm). **Fig. 5:** *Globigerinoides ruber* (350 µm). **a:** Umbilical view. **b**: Spiral view. **Fig. 6:** *Globigerinoides conglobatus*. **a:** Umbilical view (760 µm). **b**: Spiral view (610 µm). **Fig. 7:** *Neogloboquadrina dutertrei* (410 µm). **a:** Umbilical view. **b**: Spiral view. **Fig. 8:** *Orbulina universa* (820 µm). The spherical last chamber was intentionally broken to reveal the preadult spiral stage. **Fig. 9:** *Globorotalia truncatulinoides* (740 µm). **a:** Umbilical view. **b**: Spiral view. **c:** Side view. **Fig. 10:** *Globorotalia menardii* (850 µm). **a**: Spiral view. **b:** Side view. **Fig. 11:** *Globorotalia tumida* (990 µm). **a**: Umbilical view. **b:** Side view. **Fig. 12:** *Sphaeroidinella dehiscens* (610 µm). **Fig. 13:** *Pulleniatina obliquiloculata* (460 µm). **Fig. 14:** *Globigerinella siphonifera*. **a**: Side view (640 µm). **b:** Frontal view (840 µm). **Fig. 15:** *Hastigerina pelagica* (780 µm). **a**: Undamaged specimen. **b:** Side view. **c:** Frontal view. **Fig. 16:** *Globorotalia inflata* (500 µm). **a:** Umbilical view. **b:** Side view. **Fig. 17:** *Candeina nitida*, umbilical view (290 µm). (Fig. 2, 9b, 15a after Rhumbler 1901; Fig. 4, 5, 6a, 7, 8, 9, 10a, 11a, 12, 13, 14b, 15b, 16a, and 17 after Hemleben et al. 1989; Fig. 6b, 10b, 11b, 14a, 15c, and 16b after Bé 1967)

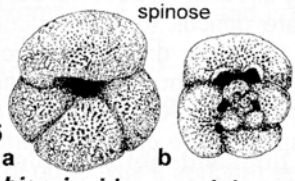

Globigerinoides conglobatus
Globorotalia menardii
Globigerinoides conglobatus
Globorotalia menardii
Globigerinoides ruber
1
Globigerinoides sacculifer
Neogloboquadrina dutertrei
Globorotalia truncatulinoides

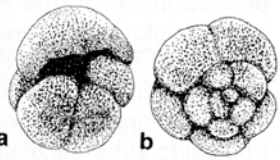

2 Globigerina bulloides
560 µm
spines

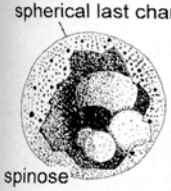

Orbulina universa 535 µm
with snared copepod prey
3
symbiotic dinoflagellates within pseudopodial network

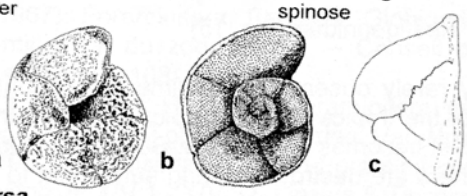

sac-like last chamber
a
b
4 Globigerinoides sacculifer

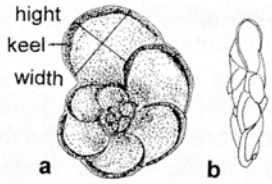

primary aperture
secondary aperture
a
b
5 Globigerinoides ruber

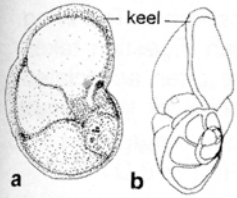

spinose
6
a
b
Globigerinoides conglobatus

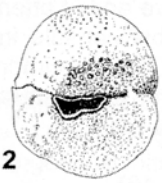

spinose
7
a
b
Neogloboquadrina dutertrei

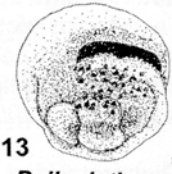

spherical last chamber
spinose
8 Orbulina universa

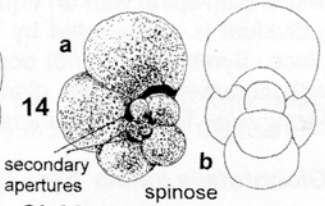

a
b
spinose
c
9 Globorotalia truncatulinoides

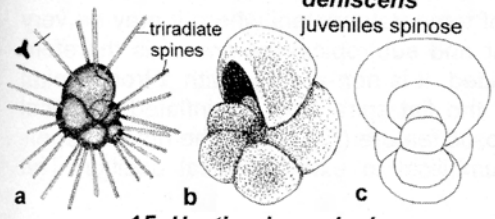

hight
keel
width
a
b
10 Globorotalia menardii

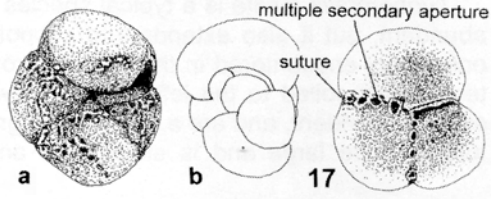

keel
a
b
11 Globorotalia tumida


12
Sphaeroidinella dehiscens


13
Pulleniatina obliquiloculata


a
14
secondary apertures
b
spinose
Globigerinella siphonifera


triradiate spines
juveniles spinose
a
b
c
15 Hastigerina pelagica


multiple secondary aperture
suture
a
b
16 Globorotalia inflata
17
Candeina nitida

only slit-like openings that are not apertures in the strict sense. At the same time the test becomes more or less sperical in shape and the primarily trochospiral arrangement of chambers is no longer discernible. Maximum test diameters are slightly above 1000 µm.

Pulleniatina obliquiloculata Fam. Pulleniatinidae (Fig. 13)

This is a non-spinose species with a trochospiral chamber arrangement that later becomes streptospiral (coiling becomes irregular). The coiling direction of this tropical to subtropical species is mostly to the right. The aperture is in an umbilical position becoming extra-umbilical during later growth stages. It spans several of the previously formed chambers in a low arch and may develop pustules on its outer edge. The rest of the test is unusually smooth, often with a glossy touch. The maximum test size is up to 800 µm.

Globigerinella siphonifera (Syn. *G. aequilateralis)* Fam. Globigerinidae (Fig. 14)

The coiling mode of this species is trochospiral becoming almost planispiral in older individuals. The coiling direction is both right and left. *Globigerinella siphonifera* occurrs in two forms that are difficult to differentiate. One "morphotype" is slightly more involute compared to the other one. In addition, each form harbours different species of symbiotic chrysophytes. Thus they may actually be regarded as separate species, but are treated here as one. *Globigerinella siphonifera* is a spinose species with very long and flexible spines. The primary aperture is visible as a low arch. This tropical to subtropical species reaches a size of up to 1000 µm.

Hastigerina pelagica Fam. Hastigerinidae (Fig. 15)

This species is only very rarely observed in sediments although it is relatively abundant in surface waters of the tropics and subtropics. During gametogenesis, all septa are resorbed, and the test becomes very fragile. Some tests already break during gamete release, while others are destroyed during settlement to the sea floor or within the sediments. The spines are relatively thick and triradiate throughout. The test is planispiral with an equatorial large aperture and is often transparent. The living individual is surrounded by a cytoplasmic bubble capsule in which digestion takes place. Symbionts do not occurr in this species, but specimens are often associated with commensal larger dinoflagellates belonging to different species of the genus *Pyrocystis*. Test diameters reach up to 1200 µm.

Globorotalia inflata Fam. Globorotaliidae (Fig. 16)

Globorotalia inflata is a typical species of temperate waters where it may be very abundant, but it also extends into subpolar and subtropical regions. It is therefore only rarely encountered in the sample provided. It is non-spinose with a trochospiral test mostly coiled to the left. In side view, the flat spiral and the inflated umbilical sides are evident, and are a valuable diagnostic feature (Fig. 16b). The rimmed aperture is quite large and is situated in an umbilical to extra-umbilical position. The

maximum test size is 700 µm. Mature specimens are encrusted by low pustules that eventually coalesce to create a uniform calcite crust.

Candeina nitida Fam. Candeinidae (Fig. 17)

Candeina nitida is a non-spinose, microperforate species (pore size less than 1 µm) of the tropics, where it occurs in very low abundance. The primary aperture is present in juveniles and may still be seen in adults in an umbilical position. However, the unique characteristics of this species are the multiple secondary apertures situated along the sutures of the chambers. These apertures are not found in any other species of recent planktonic foraminifera. The test is trochospiral, mostly coiling to the right and attains a size of 800 µm.

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Radiolaria

Roger O. Anderson

INTRODUCTION

Radiolaria encompass a large group of open-ocean dwelling protozoa with elegant siliceous skeletons, largely found in "blue water" at locations with great depth. However, open ocean currents also may carry them closer to land, especially near islands or peninsulas.

Radiolaria are characterized by a rather elaborate cytoplasmic organization (Fig. 1 – 6). The central mass of cytoplasm known as the central capsule, endoplasm or intracapsulum contains the nucleus or nuclei and other cytoplasmic organelles. It is the main site of food storage, metabolism and reproduction. A porous central capsular wall encloses the endoplasm. The central capsule is surrounded by a frothy ectoplasm or extracapsulum with digestive vacuoles and, when present, algal and/or cyanobacterial endosymbionts. Axopodia emerge through the pores in the central capsular wall and provide cytoplasmic continuity between endoplasm and ectoplasm. The ectoplasm is the main site of digestion. Prey becomes entangled within the web-like sticky pseudopodia supported on stiffened radiating axopodia. Some species of radiolaria are herbivores, consuming only algal prey. Others are omnivorous and consume algae, smaller protozoa, and small invertebrates such as crustacea. Some are probably exclusively carnivorous.The ectoplasm flows around the prey and encloses digestible material within food vacuoles.

When algal endosymbionts are present, they are enclosed in vacuoles throughout the nearly transparent, gelatinous and rhizopodial ectoplasm, a habitat to which they are well adapted as it allows them both to obtain energy from sunlight and to be protected from predation. The algal symbionts of radiolaria include dinoflagellates and prasinophytes. Symbiotic cyanobacteria occasionally occur together with algal symbionts, though they are segregated in separate specialized symbiosome vacuoles. They give the cytoplasm of the host a distinctive greenish hue. The distribution of algal symbionts within the radiolarian rhizopodial network is under dynamic control of the radiolarian host. In the evening, the symbionts are gathered in toward the central capsules by cytoplasmic streaming of the rhizopodia. In the morning, the symbionts are distributed outward into the rhizopodial network where they can maximally capture sunlight. Algal symbionts contribute a substantial amount of food to the host by secreting photosynthetically produced organic compounds into the host cytoplasm. In colonial species, the exudates from the symbionts provide sustenance for all of the radiolarian cells within the colony.

A skeleton, when present, is composed of amorphous silica (biogenic opal) and varies between species in shape and complexity. In the simplest case, the skeleton consists of scattered spicules embedded within the ectoplasm. The shape of the spicules is species-specific and can vary from smooth curved needles, tapered to a

point at each end, to elaborate triradiate structures consisting of a central shaft with three, thorn-like spines at each end. More complex skeletons consist of porous or latticed shells. The shells may be single or multiple and arranged around, and/or within, the central capsule and with a concentric arrangement in Spumellaria.

There are two major groups of radiolaria: Polycystinea and Phaeodarea (Table 1). The Polycystinea have solid opal skeletons and consist of two subgroups: the Spumellaria and the Nassellaria. The Spumellaria typically have spherical central capsules with circular pores uniformly distributed over the surface of the capsular wall. Skeletons, when present, may vary from spicules to elaborate latticed shells. Solitary and colonial species occur in the Spumellaria. The Nassellaria have an ovoid central capsule with the pore field restricted to one pole. The nassellarian skeleton, when present, is either a simple tripod, or a sagittal ring without a tripod, or a variety of helmet-shaped porous shells enclosing the ovoid central capsule. The Phaeodaria have siliceous skeletons that often contain organic matter, and the central capsular wall is distinctively provided with two kinds of pores, a large opening known as the astropyle and two laterally placed smaller openings known as parapyles. The most commonly encountered radiolaria are Polycystinea, especially in microfossil preparations of skeletons from rocks and sediments.

Solitary species of radiolaria vary in size from large species several millimetres in diameter to smaller species in the range of 50 to 100 µm. Each colony (within the Spumellaria) contains hundreds of central capsules interconnected by a network of rhizopodial strands enclosed by a clear gelatinous sheath. Fine axopodia radiate outward from the gelatinous sheath and capture prey. Colonial species vary in size from a centimetre, or several centimetres, up to very long cylindrical colonies that are several mm in diameter and a metre in length.

Prior to reproduction, the nucleus undergoes repeated division and the cytoplasm is completely converted into swarmers, leaving behind only an empty, ruptured capsular wall. There is no convincing evidence that the flagellated swarmers are gametes. Colonies reproduce by fission into two or more daughter colonies. Some solitary species reproduce by complicated processes of binary fission. Daughter cells of skeleton-bearing species develop into new mature individuals by formation of new shells. The lifespan of some radiolaria has been estimated to be at least one month.

Radiolaria occur throughout the water column at all depths. Some species dwell largely in the sunlit surface layers and these often have algal symbionts, while others are adapted to life in the dark, colder waters at great depths. Many species found at great depth also occur in colder surface waters in polar regions. Some species are restricted in geographic range and inhabit specific oceanic regimes and are thus good water mass indicators. Radiolaria appear to be particularly sensitive to water quality and seldom survive long in near coastal water. However, radiolaria can be collected in plankton nets in clear water locations a mile or so from land, for example in upwelling locations near the Arabian coast, in subtropical water near open ocean islands (for example Bermuda and Caribbean Islands), or in more temperate water such as near the Canary Islands, or near Florida, and in the Gulf of Mexico, and even in colder water at higher latitudes such as the fjords of Norway.

Radiolarian skeletons are abundant in some oceanic sediments (red clays and so-called siliceous oozes) especially where calcareous microfossils are absent due to dissolution caused by water pressure below the calcite compensation depth (CCD).

Table 1. Synopsis of Major Radiolarian Taxa

Class: Polycystinea. Radiolaria with a thin-walled central capsular membrane containing round pores of equal size, skeletons when present composed of solid amorphous silica.

Order: Spumellaria. Central capsule round, or nearly so, with uniformly distributed pores over the entire surface of the capsular wall.

Suborder: Collodaria. Solitary and colonial species, lacking a skeleton, or with only scattered spicules distributed in the ectoplasm.

Suborder: Sphaerellaria. Mainly solitary, but some colonial species, skeleton a porous or latticed shell.

Order: Nassellaria. Central capsule ovoid, pores situated at one pole of the long axis of the central capsule forming a pore field. Skeleton a simple tripod, a sagittal ring lacking a tripod, or a helmet-shaped cephalis of varying complexity among species.

Suborder: Plectellaria. Skeleton lacking, or if present never a conical or helmet shaped cephalis.

Suborder: Cyrtellaria. Skeleton a simple unsegmented cephalis, or constricted forming septa at places to produce a segmented shell. The basal opening is either simple or ornamented with feet or various extensions.

Class: Phaeodarea. Capsular wall thickened with a double layer, pores of two kinds, a large astropylum and two, usually laterally placed, parapylae. Skeletons composed of amorphous silica containing organic matter, sometimes forming hollow tubes.

Order: Phaeocystina: Central capsule suspended in the center of the extracapsular cytoplasmic network. Skeleton absent or incomplete, composed of numerous solitary, scattered pieces or spicules without organic connections.

Order: Phaeosphaeria: Central capsule located in the centre of a simple or double spherical lattice-shell, not bivalve, with a simple shell mouth, lacking feet or teeth.

Order: Phaeogromia: Central capsule located excentrically, aborally, in a simple latticed shell typically provided with a large shell-mouth surrounded by peculiar elongate extensions known as feet or teeth, sometimes with elaborate branches.

Order: Phaeoconchia: Central capsule enclosed within a bivalve latticed shell composed of a dorsal and a ventral boat-shaped valve that are completely separated, rarely connected by a ligament on the aboral pole.

PRACTICAL TECHNIQUES

Collecting Living Radiolaria

Living radiolaria can be collected conveniently only at certain locations near open ocean water. If you have access to an appropriate sea sampling vessel, you can collect radiolaria in surface water using a plankton net. Individual specimens can also be captured by SCUBA divers within small, hand-held jars (Anderson 1996). For smaller species, a 75 µm pore-size net is preferable with a fine screen in the cod end. Larger

species can be collected with a 220 µm pore-size net. It is important to deploy the net from a gently drifting boat, or one that is moving very slowly. Otherwise, too much plankton will be collected and the radiolaria will become ensnared with other organisms. Allow the net to collect for several minutes. When the cod end is brought on board, immediately empty the contents gently into a large container such as a 3 litre plastic jar containing ample freshly collected seawater. This will dilute the plankton catch and prevent the radiolaria from becoming entangled with larger plankton during the trip back to your home base. Upon return to the laboratory, examine the jar of water with the plankton. Some larger radiolaria may be floating in the water and can be seen with the unaided eye.They can be withdrawn by placing a rubber bulb on the tapered end of a 10 ml pipette and, using the large end, gently drawing the floating radiolarian up into the pipette using suction from the rubber bulb.To sample the settled radiolaria, the overlying water in the jar can be gently poured off and any settled plankton dispersed into 9 mm culture dishes or deep Petri dishes. Observe the dispersed plankton with a stereomicroscope and gently withdraw clean, living radiolaria using a small pipette. If the suspension of plankton is sufficiently diluted, the living radiolaria will cleanse themselves of debris by cytoplasmic streaming over a period of several hours. Place the living radiolaria in a depression slide and observe under high power.

To observe the skeletons, a clean sample can be prepared as follows: place the plankton sample in a tall container such as a graduated cylinder and allow it to settle. Withdraw the overlying water and place the sediment in a tall beaker (be certain that the beaker is only about 1/4 filled with suspended sample to prevent boiling over in the next step). Add 20% hydrogen peroxide and warm the preparation on a hot plate to digest away as much organic matter as possible. This may have to be repeated several times by resuspending the settled preparation in fresh hydrogen peroxide. CAUTION: *Use care in warming the hydrogen peroxide solution and protect your eyes.* Consult a safety officer about proper laboratory procedures.The hydrogen peroxide-digested preparation can be rid of calcium carbonate by adding a few drops of concentrated hydrochloric acid until all calcareous debris has been dissolved or dissipated. Collect the remaining sample on a fine sieve (about 45 to 75 µm mesh) or use a plankton mesh net that has been prepared as a sieve, and rinse the sample with water. Collect the sieved and washed sample into a small beaker or watch glass with sufficient water to make a slurry. Deposit some of the washed particles on a glass slide and prepare a wet mount covered by a cover glass. You should be able to observe the radiolarian skeletons using a moderate power objective lens. If you prefer, the skeletons can be dried, spread on a clean slide, dispersed in a drop of xylene and mounted in synthetic resin to prepare a permanent slide (see the procedure described below in the protocol for preparing samples from rocks). If you do not have access to living samples, some biological supply houses serving educational institutions offer samples of radiolarian oozes or strews that can be used for the preparation of slides of the skeletons.

Preparing Samples from Ocean Sediments or Rocks

If you are in communication with an oceanic or geological institute, you may be able to request a sample of radiolarian-rich sediments such as core tops from sedi-

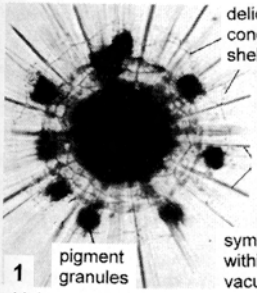
ment cores or radiolarian oozes. The institute may also provide radiolarian plankton samples. If the sample has not been cleaned prior to being sent, purify the sample using a 75 µm or smaller pore-size sieve to remove small sediment particles. Back wash the sample into a beaker and add drops of concentrated hydrochloric acid until all calcareous material has been dissolved. Sieve the sample again and wash it with water. Collect the sample into a small beaker or watch glass and prepare it for light microscopic examination as explained above for cleaned plankton samples.

Some fossil radiolaria can be separated from silica-rich unconsolidated rock. The preparation involves a more elaborate protocol as follows: boil a sample in water until the rock has disintegrated. Sieve the sample over a 45 µm screen to remove most of the fine fraction and organic matter. Boil the residue again, add hydrogen peroxide to the water (the samples, having been sieved, will not boil over). Sieve the sample over a 45 µm screen. Add dilute hydrochloric acid to dissolve the calcareous matter. Mount the residue on microscope slides, let dry, add a drop of xylene, embed the skeletons in a resinous medium (see p. 74/75), and add a coverslip, taking care to exclude air bubbles. If unwanted bubbles are trapped under the coverslip, heat gently on a hotplate until the bubbles dissipate, and put the slide on a cold surface such as a tile or metal plate. For more details go to the Quaternary section of the website www.radiolaria.org (authors Kjell R. Bjørklund and Giuseppe Cortese).

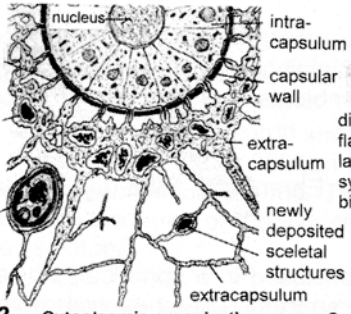
GENERA AND SPECIES

There are numerous genera of radiolaria, and current estimates place the number of living species between 700 and 1000. It is therefore not possible to provide a truly representative description of most major living radiolaria within a short synopsis. However, the examples given illustrate the remarkable diversity of this morphologically elegant group of single-celled organisms. The genera are presented according to the classification scheme in Table 1 with a focus on the Polycystinea.

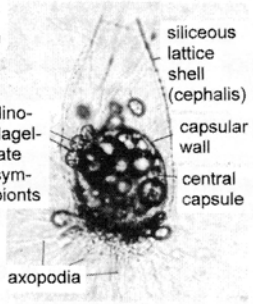
Fig. 1 – 6. Basic radiolarian morphology. **1:** Size of shell 200 µm. Dark pigmented granules become aggregated into masses along the axopodia. **2:** The intracapsulum, containing the nucleus, is surrounded by a porous capsular wall with cytoplasmic strands (fusules) connecting to the frothy extracapsulum. Size of central capsule 20 µm (it can be as large as several hundred microns or even several millimetres in larger species). **3:** The delicate, helmet-shaped, skeletal shell (cephalis) encloses the central capsule (length 85 µm). Rhizopodia contain algal symbionts. **4:** The nucleus is situated at the apical pole of the ovoid central capsule. A cone of microtubules (podoconus) provides a cytoskeleton for the axopodia protruding through the mouth of the cephalis (length of cephalis 150 µm). **5:** An elaborate geodesic latticed shell (larger diameter 1540 µm) encloses the central capsule with a dense mass of partially digested debris known as the phaeodium. Inset shows the details of the central capsule. **6:** The intracapsular cytoplasm is enclosed by a capsular wall. The large opening, astropyle (approximate diameter 30 µm), is the cytostome. From the smaller openings, the parapylae, emerge cytoplasmic strands, fusules, containing microtubule central shafts. **Fig. 7 – 20.** Polycystine radiolaria. **7:** Diameter of disc-shaped colony 4 cm. **8:** Length of colony 6 cm. **9:** 1522 µm. **10:** 170 µm. **11:** Size of spherical skeleton 55 µm. **12:** 140 µm. **13:** 255 µm. **14:** 270 µm. **15:** 80 µm. **16:** 400 µm. **17:** 125 µm. **18:** 145/100 µm. **19:** 120 µm. **20:** 190 µm. (All figures with the exception of Fig. 4 adapted from Anderson 1983 and Anderson et al. 2000; Fig. 4 from Kühn, Morphologie der Tiere in Bildern, 2. Heft, Protozoen: 2. Teil: Rhizopoden, Gebr. Bornträger, Berlin 1926)



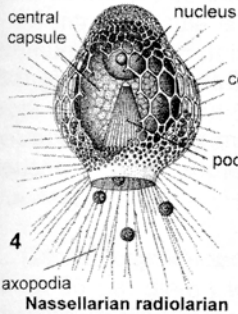
1 **Living spumellarian radiolarian with concentric latticed shells**



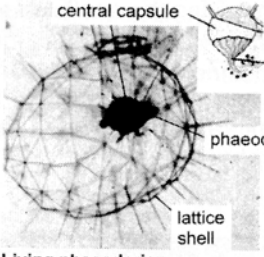
2 **Cytoplasmic organization of a spumellarian radiolarian**



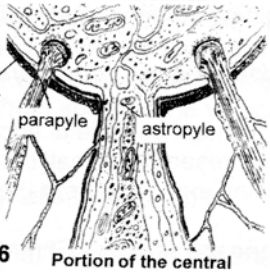
3 **Living juvenile nassellarian radiolarian**



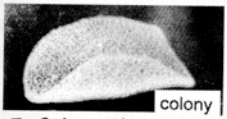
4 **Nassellarian radiolarian**



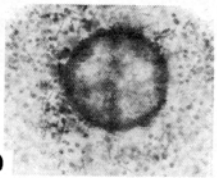
5 **Living phaeodarian radiolarian**



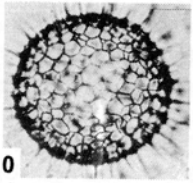
6 **Portion of the central capsule of a phaeodarian**



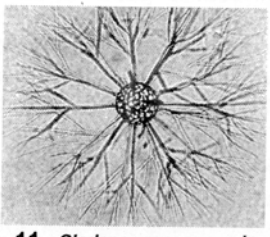
7 ***Solenosphaera* sp.**



9 ***Lampoxanthium pandora***



10 ***Acanthosphaera tunis***



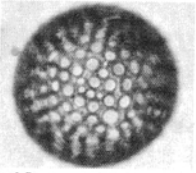
11 ***Cladococcus scoparius***



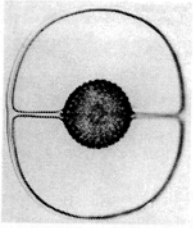
8 ***Collozoum caudatum***



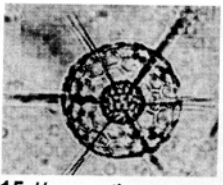
14 ***Didymocyrtis tetrathalamus***



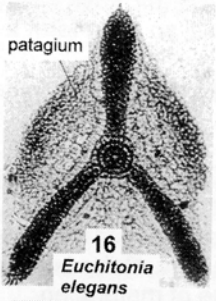
12 ***Cenosphaera* sp.**



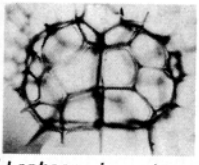
13 ***Saturnalis circularis***



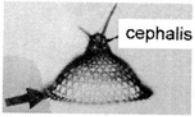
15 ***Hexacontium axotrias***



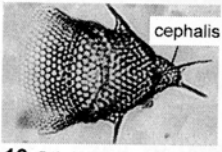
16 ***Euchitonia elegans***



17 ***Lophospyris pentagona***



18 ***Eucecryphalus* sp.**



19 ***Stichopilium bicorne***



20 ***Eucyrtidium hexagonatum***

Lampoxanthium pandora Haeckel, 1887 O. Spumellaria, S. O. Collodaria (Fig. 9)

Solitary, with a large (about 1 mm), opalescent central capsule surrounded by a thick extracapsulum containing large alveoli, but none within the central capsule, and numerous algal endosymbionts intermingled with siliceous spicules that are simple or branched.

Acanthosphaera tunis Ehrenberg, 1858 O. Spumellaria, S. O. Sphaerellaria (Fig. 10)

Central capsule is enclosed in a spherical, latticed shell, with fairly regular pore size, arrangement and spacing. The shell wall of varying thickness has prominent, primary, unbranched spines extending radially from the vertices of the lattice bars yielding an appearance of bristles covering the shell surface.

Cladococcus scoparius Müller, 1857 O. Spumellaria, S. O. Sphaerellaria (Fig. 11)

Central capsule is enclosed within a small (about 50 µm diam.), spherical, latticed shell resembling that of *Acanthosphaera tunis*, but with numerous stout, branched spines extending from the surface.

Cenosphaera sp. Ehrenberg, 1854 O. Spumellaria, S. O. Sphaerellaria (Fig. 12)

Central capsule is enclosed by a latticed primary shell (about 200 µm diam.) with pore shape and distribution as in *Acanthosphaera*, but pore size may vary (5–20 µm diam.) within a shell. The shell lacks surface spines, thus clearly separating it from *Acanthosphaera* and *Cladococcus*.

Saturnalis circularis Haeckel, 1881 O. Spumellaria, S. O. Sphaerellaria (Fig. 13)

Central capsule is enclosed within a spherical central shell surrounded by a latticed larger shell (about 80 µm diam.) with two robust primary (polar) spines joined distally so as to form a characteristic outer ring, about 200 µm diameter.

Didymocyrtis tetrathalamus Haeckel, 1881 O. Spumellaria, S. O. Sphaerellaria (Fig. 14)

Central capsule is enclosed by multiple, concentric latticed shells. The innermost shells are spherical to ellipsoidal while the outermost shells are ellipsoidal with an equatorial constriction producing an hour-glass shape, about 150–300 µm on the long axis. Polar latticed caps are frequently observed and may be a more advanced stage of maturation.

Hexacontium axotrias Haeckel, 1881 O. Spumellaria, S. O. Sphaerellaria (Fig. 15)

Central capsule enclosed by three concentric latticed shells (two medullary and one cortical, about 80 µm) bearing six primary spines arranged symmetrically and

extending radially from the central shell beyond the outermost shell as prominent, unbranched surface spines.

Euchitonia elegans Ehrenberg, 1860 O. Spumellaria, S. O. Sphaerellaria (Fig. 16)

Central capsule is enclosed within a discoidal central shell (about 100 µm diam.) with three spongiose Y-shaped arms (about 200 to 300 µm long) forming a trigonal pattern. A spongy, veil-like patagium connects the arms. The patagium, a veil-like spongy meshwork deposited between the arms of the skeleton, produces a flange-like extension around the mature skeleton.

Lophospyris pentagona Haeckel, 1887 O. Nassellaria, S. O. Plectellaria (Fig. 17)

Central capsule is suspended within a cytoplasmic network anchored to the surrounding skeleton. It is composed of two intersecting, bilaterally symmetrical, sagittal hemispheres (about 150 µm maximum dimension) with an open pentagonal meshwork of pores (about 50 µm). Short, pointed spines project from some of the vertices of the pentagonally arranged lattice bars.

Eucecryphalus sp. Haeckel, 1860 O. Nassellaria, S. O. Cyrtellaria (Fig. 18)

Central capsule enclosed by a simple cephalis (small apical segment of the shell, about 25 µm diam.) with one additional segment (thorax) that is widely conical resembling a Chinese peasant hat (dau-li). A skirt-like, rudimentary, flared abdomen appearing as a thin, crenated brim (arrow), extends around the perimeter of the thoracic opening. The apex of the cephalis bears branched horns.

Stichopilium bicorne Haeckel, 1881 O. Nassellaria, S. O. Cyrtellaria (Fig. 19)

Central capsule enclosed by a spherical small unilocular cephalis (about 25 µm diam.), with two apical horns (hence *bicorne*) bearing more than two basal flared segments, the last with a broad opening. Radial apophyses (wings) are attached to the thorax (segment nearest to the cephalis).

Eucyrtidium hexagonatum Ehrenberg, 1847 O. Nassellaria, S. O. Cyrtellaria (Fig. 20)

Central capsule is enclosed by a spherical unilocular cephalis with one apical horn. Several basal segments, the last with a wide opening (about 100 µm diam.), extend from the cephalis, producing a rather spindle-shaped shell with a total length of 200 µm. There are no apophyses (wings) on the thorax, and the shell is uniformly smooth with regularly arranged pores.

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Rolf Entzeroth

INTRODUCTION

Gregarines belong to the phylum Apicomplexa, previously named Sporozoa, which is part of the Alveolata within the kingdom Protozoa. The term Apicomplexa is based on the ultrastructure of the apical region of sporozoites and merozoites visible under the electron microscope. The zoites of gregarines lack some of the typical structures (conoid, rhoptries, micronemes).The phylum Apicomplexa contains more then 4000 known species. Two classes are recognized: Perkinsozoa, parasites of oysters, and Sporozoa. The latter are divided into Gregarinia and Coccidia. The gregarines are mostly extracellular parasites of the body cavity or intestine of annelids, insects and other invertebrates. According to their developmental cycles and cell structure three orders have been established. The Archigregarinida (also named Schizogregarinida) undergo schizogony, gamogony and sporogony. The gamont cells have no subdivision (they are aseptate). This group of parasites lives exclusively in marine invertebrates: annelids, sipunculids, hemichordates and ascidians, and is considered to be more primitive than the other orders. Schizogony (presumably acquired secondarily) as well as gamogony and sporogony also occur in the life cycle of the Neogregarinida (*Lipocystis*, *Mattesia)*, which are found only in insects. Members of this order are believed to have evolved from the third order Eugregarinida in that in some species Eugregarinida-like structures such as division into a protomerite and a deutomerite are present. Archigregarines and neogregarines are not discussed further.

The Eugregarinida are extracellular parasites of annelids and arthropods. They perform gamogony and sporogony and are considered to have lost schizogony while arising from the archigregarinids. Representatives of two suborders are presented here. The gamonts of the Aseptatina (Acephalina) have no cellular subdivision and they are globular, ovoid or elongate (*Monocystis,* Fig. 4 – 6, *Rhynchocystis,* Fig. 14 – 16). The gamonts of the Septatina (Cephalina) are subdivided by a septum into protomerite and deutomerite (*Gregarina,* Fig. 1, 17 – 20). The Septatina, with a size of up to 14 mm, are among the largest of the protozoa. The cells are cylindrical, spindle-shaped or egg-shaped, globular or stretched to a worm-like form. The subdivision of the cell is visible from the exterior as a constriction and a ring of hyaline cytoplasm, while the interior contains a 0.1 µm thick fibrillar septum. This septum, however, does not hinder completely circulation of the cytoplasm between protomerite and deutomerite. The epimerite is a differentiation of the anterior pole of the protomerite in young gamonts (all individuals called "gregarines" are more or less developed gamonts), that occurs in contact with an epithelial cell of the host tissue. The epimerite is either shed at some time or resorbed during development.

The pellicle of gregarines consists, as in sporozoites and merozoites, of three

membranes, a characteristic of the Alveolata. In Septatina the pellicle is put into narrow longitudinal folds (Fig.1 – 3). The epimerite lacks the inner membrane complex. The outer (single) membrane forms microvilli and folds. These may serve as a feeding site for nutrients excreted by the host cell into the periparasitic space, a cap-shaped zone between host cell and epimerite membrane. The host cell membrane is not perforated; the parasites remain extracellular. Species with or without reduced epimerite may take up nutrients through micropores that are distributed over the entire surface, mostly between the pellicular folds.

The endocytosed material is synthesised into polysaccharides and protein granules that give the gregarines an opaque appearance. The richness in glycogen granules in many species is in parallel with a poverty of mitochondria, indicating an anaerobic metabolism.The structural basis for the motility are antagonistically acting, longitudinally arranged myofibrils, and supporting dense fibrils of the pellicle (Fig. 3). Ring-shaped myonemes under the bases of the folds enclose the endoplasm. The coordinated undulation of the pellicular folds may move liquid at the cell surface to give rise to a swimming or gliding motility. The hypothesis of motiliy mediated by secretion of mucus lacks a morphological basis: no slime-producing organelles have been found. It is most likely that the motility of gregarines is based on an actomyosin complex located within the pellicle as has been shown in other Apicomplexa.

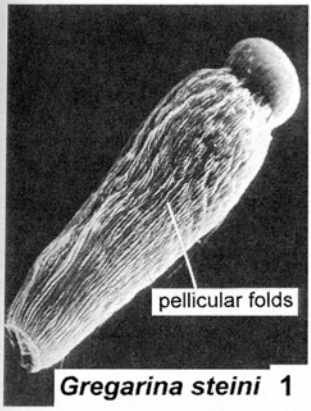
Numerous gregarines are strictly host specific: they are always found in one and the same host. Most species, however, seem to be only genus-specific. Some gregarines occur in several hosts but there are also hosts of one genus which contain in their intestine two, three or four different gregarines simultaneously. In phylogenetic older hosts a greater number of specific forms is found: host and parasites evolved together.

PRACTICAL TECHNIQUES

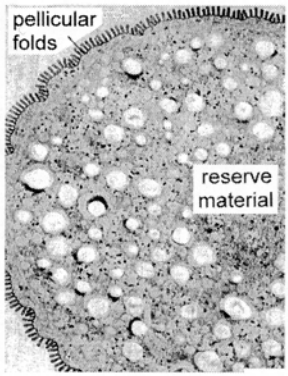
Gregarines of the genus *Monocystis* (SO. Aseptatina) are found in the seminal vesicles of earthworms (*Lumbricus terrestris*). Earthworms are collected from garden soil. They are anaesthetised in 1–5% isopropyl alcohol or 1% chloroform in tap water. The body cavity is opened from the dorsal surface by a longitudinal section of segment 8–14 in a dissecting dish filled with tap water to prevent drying and for better visibility (preparation according to Storch & Welsch 2006). The emerging white seminal vesicles are lifted by forceps and cut off by scissors at their narrow basis. The vesicles are opened by scissors in 0.6% NaCl-solution in a cubical (embryo) cup. The appearing milky suspension often contains few or many gamont cysts filled with spores and less often free gamonts and gamont cysts with 2 gamonts each. Under the stereomicroscope the gamonts ("gregarines") are transferred with a pipette to a slide for microscopic examination in transmitted light or dark field illumination. In order not

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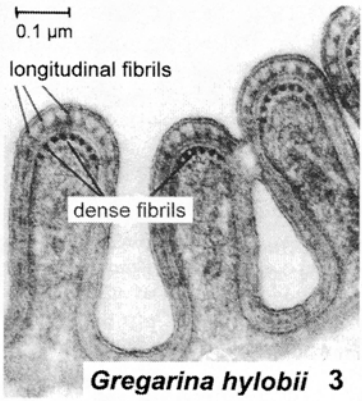
Fig.1. *Gregarina steini* (50 µm), with prominent pellicular folds, SEM micrograph. **Fig 2.** *Gregarina cuneata*, cross section of a gamont (total diameter 45 µm) showing pellicular folds (height 0.5 µm) and numerous polysaccharide and protein inclusions. **Fig. 3.** *Gregarina hylobii.* Enlarged pellicular folds (height 0.5 µm) with longitudinal fibrils under the cell membrane and dense fibrils under the median and inner membrane. **Fig. 4.** Life cycle of the gregarine *Monocystis* sp. in a seminal vesicle of the common earthworm (*Lumbricus terrestris*). The developmental stages are not to scale.



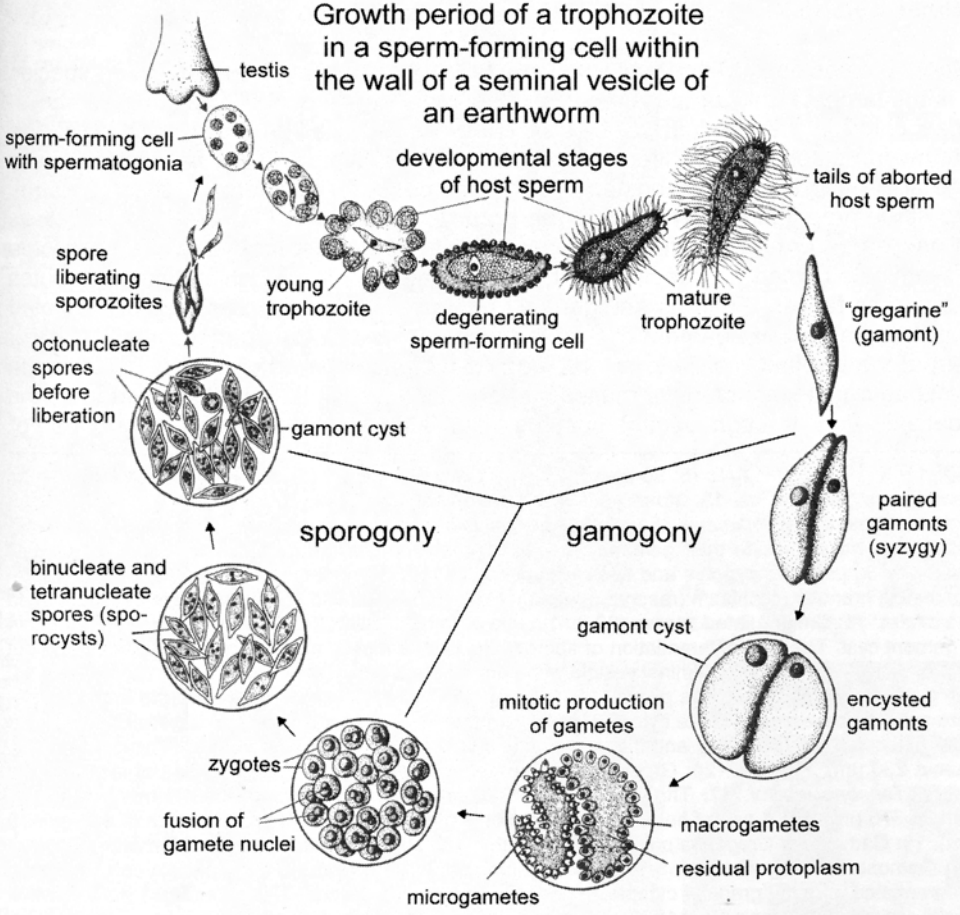
***Gregarina steini* 1**



***Gregarina cuneata* 2**



***Gregarina hylobii* 3**



4 Life cycle of the gregarine *Monocystis*, a parasite of the earthworm seminal vesicles

to damage the cells, pieces of a coverslip are placed between slide and coverslip. For the preparation of a total mount, the contents or part of the contents of a seminal vesicle are transferred to a slide, air-dried, fixed in methanol and stained with Giemsa, as with smears of *Trypanosoma melophagium* (see p. 62).

Gregarines of the genus *Gregarina* (SO. Septatina) live in the intestine of the mealworm (the larva of the beetle *Tenebrio molitor*), which can be obtained in animal supply shops. The larvae are decapitated and the most posterior segment is cut off. The intestine, brown in colour and surrounded by fat body, is pulled out from the chitinous cuticle by means of a forceps and placed into a small Petri dish containing 0.85% NaCl solution. The intestine is torn into pieces with dissecting needles. The cigar-shaped gregarines become free and can be transferred with a pipette to a slide for microscopic examination.

GENERA AND SPECIES

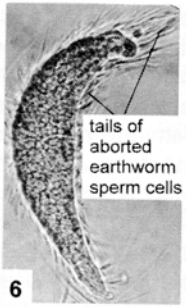
Monocystis sp. O. Eugregarinida, SO. Aseptatina, Fam. Monocystidae (Fig. 4 – 13)

Monocystis belongs to the Monocystidae; with 27 genera and 188 described species it is the largest family of the Aseptatina. The majority of species within the Monocystidae colonise the seminal vesicles of earthworms while the rest are found in the earthworm´s coelom. The life cycle of *Monocystis* is typical of the family Monocystidae and the order Eugregarinida (Fig. 4). Gamont cysts of different sizes in one and the same preparation may indicate the occurrence of several species of *Monocystis* in one earthworm. The infective stages are the spores (sporocysts). They become disseminated when an infected earthworm is eaten by a bird which later distributes them in its faeces. There is another way however in which spores may be liberated from the earthworm: gamont cysts filled with spores are transported from the lower part of the seminal vesicle to the coelom. After being covered with amoebocytes, the cysts aggregate into globular masses (often called 'balls') that are transported by the coelomic fluid through ventral openings of the dissepiments to the posterior part of

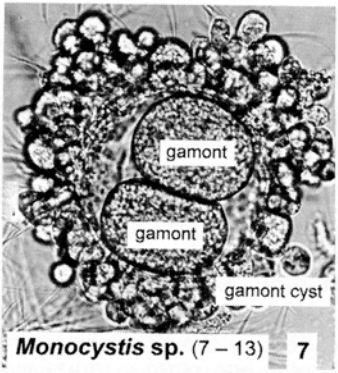
Fig. 5 – 6. *Monocystis agilis* (5: 90 µm, 6: 75 µm). Gamonts without (5) and with attached earthworm sperm cells (6). **Fig. 7 – 13.** *Monocystis* sp., developmental stages. **7:** A pair of gamonts (syzygy) within a gamont cyst (diameter 75 µm). **8:** After nuclear divisions the gamonts have formed isogametes, which are still inside their gamont cell wall (diameter of gamont cyst 120 µm). **9:** Gamont cyst (diameter 80 µm) with zygotes and two residual bodies. **10:** Gamont cyst with spores (length 20 µm) containing granular cytoplasm (reserve material). Residual bodies and residual gametes are located in the centre. **11:** Unsporulated spores (20 µm) within a gamont cyst. **12:** Partly sporulated spores within a gamont cyst. The final differentiation of sporozoites occurs mostly after spores have passed the gut of a bird. **13:** Contents of a seminal vesicle with gamont cysts (80–100 µm) containing spores. **Fig. 14, 15.** Gamonts of *Rhynchocystis pilosa* (14: 115 µm, 15: 200 µm) with nucleus near the anterior pole. The pellicle possesses cilia-like cytopilia, which are thinner and shorter than the flagella of earthworm spermatozoa. **Fig. 16.** A long and thin and in this case also constricted gamont of *Rhynchocystis* sp. (about 230 µm). **Fig. 17 – 20:** Gregarines of the genus *Gregarina* from the intestine of larvae of the beetle *Tenebrio molitor*. **17:** Three gamonts of *Gregarina cuneata*, two of them forming a syzygy (length 375 µm). **18:** A pair of heteromorphic gamonts of *Gregarina polymorpha* (length of syzygy 470 µm). **19:** Gamonts of *Gregarina polymorpha* (length about 200 µm).The roundish protomerite is typical. **20:** Gamonts of *Gregarina polymorpha* with epimerites. In dark field the thick pellicle can be clearly differentiated from the granular cytoplasm (length of the central gamont 270 µm). (Fig. 1 from Vávra & Small 1969; Fig. 2, 3 from Rühl 1976; Fig. 4 from Storch & Welsch 2006, modified)



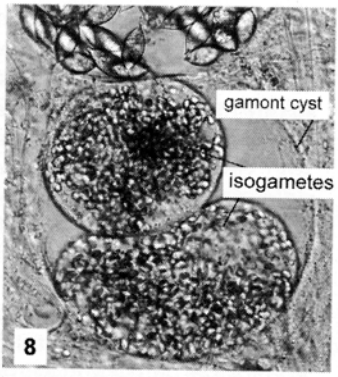
Monoystis agilis gamonts (5, 6)



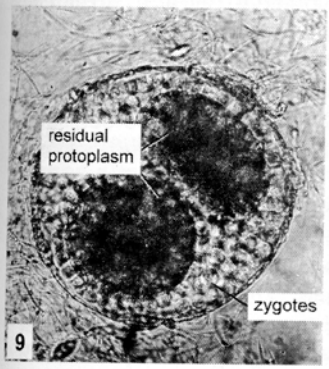
5 6



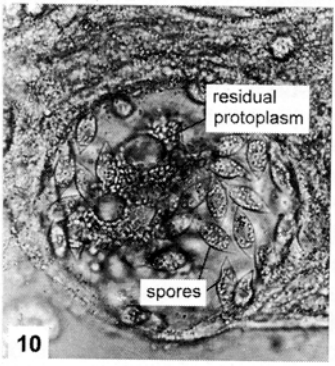
***Monocystis* sp.** (7 – 13) 7



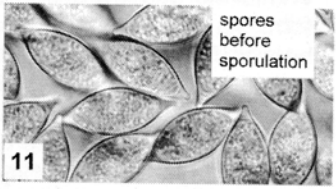
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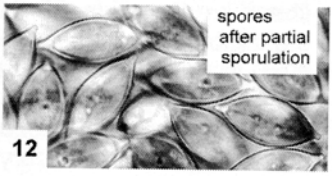
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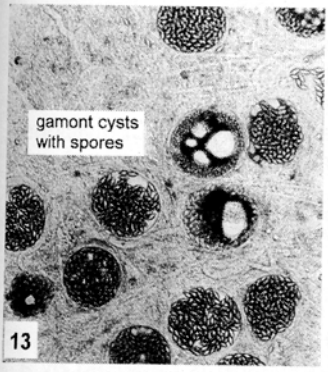
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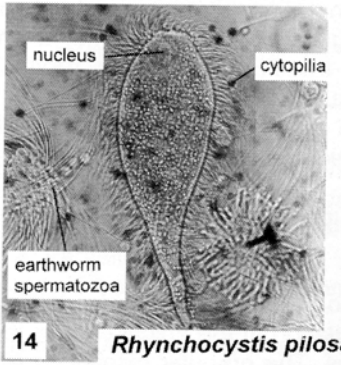
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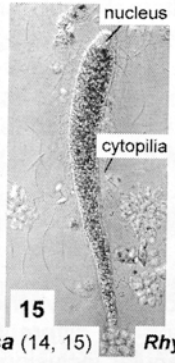
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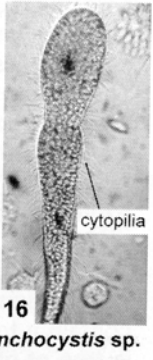
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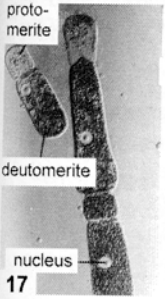


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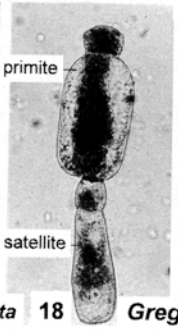


16

Rhynchocystis pilosa (14, 15) ***Rhynchocystis* sp.**

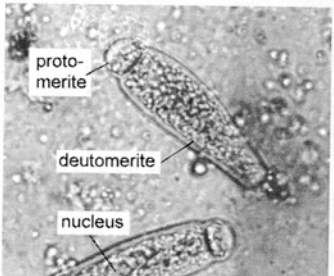


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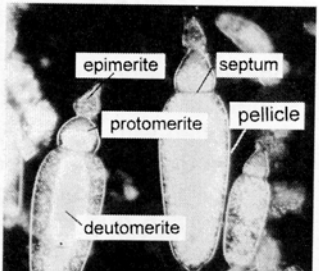


18

Gregarina cuneata ***Gregarina polymorpha*** (18, 19)



19



20 ***Gregarina polymorpha***

the earthworm. Here they may be set free through dorsal pores. But there are also within the seminal fluid conglomerations of gamont cysts that appear as brownish globular masses and contain earthworm chaetae and nematode larvae. Spores are taken up with soil by feeding earthworms. The route of the liberated sporozoites to the seminal vesicles is still unknown. Seminal vesicles of adult earthworms are filled with various stages of developing sperm cells within sperm-forming cells as well as differentiated sperm cells. The sporozoites of *Monocystis* invade sperm-forming cells and grow as trophozoites to gamonts. Differentiated gamonts escape from the residues of sperm-forming cells and unite in pairs. This pairing is called gamontogamy and results in the syzygy (Greek, paired). In *Monocystis* and *Gregarina* adult gamonts form pairs while in other genera young gamonts associate and grow up in pairs. The syzygy is surrounded by a cyst wall and thus becomes a gamont cyst. Within the gamont cyst the gamonts undergo numerous nuclear divisions to finally form many gametes. Gametes from the two gamonts fuse with thier opposite numbers to form zygotes. Residual protoplasm as well as gametes that did not fuse remain within a dark residual body. With the formation of zygotes, gamogony, the part of the life cycle containing sexual processes, is finished. The zygotes surround themselves with a dense wall and transform to spindle shaped spores (sporocysts). The spore is thus an encysted zygote and homologous to a coccidian oocyst. The liberation of the spores from the gamont cyst and the formation of the spores from the zygote (the latter process called sporogony) often only occurs during passage through a bird´s gut. The generation of sporozoites is formed from the zygote by a succession of nuclear divisions and the following cytokinesis. The sporozoites gradually transform via trophozoites into young gamonts within the sperm-forming cells of the earthworm. The life cycle of *Monocystis* (and all other Eugregarinida) does not contain an alternation of generations. It consists of only one (haploid) generation, the gamont, which goes through an ontogenesis with a prominent growth phase and conspicuous morphological transformations (sporozoite, trophozoite, adult gamont). The zygote is a stage of survival and propagation, not an individual with an ontogenesis and therefore not a generation. Each zygote undergoes a series of divisions leading to the formation of eight sporozoites (the infective stages).

Rhyncocystis pilosa O. Eugregarinida, SO. Aseptatina, Fam. Monocystidae (Fig. 14 – 16)

The gamonts of *Rhynchocystis pilosa* are oblong, oval or cylindrical in shape and contain a trunk-like differentiation at the apical end. In the apical part the endoplasm is free of granular inclusions. The granular cytoplasm (endoplasm) is enclosed by a pellicle, which forms numerous thread-like, up to 40 µm long, extensions, named cytopilia. Cytopilia can be mistaken for the attached earthworm spermatozoa that are common in *Monocystis*. In comparison to the flagella of spermatozoa, cytopilia are somewhat smaller and do not have the headlike swellings of spermatozoa. The nucleus, often with easily discerned nucleolus, is usually situated in the posterior hyaline endoplasm. The shape of gamonts, the structure of the apical end and the length of cytopilia undergo changes during development. That is why club-shaped (Fig 14), long and thin (Fig.15) and long and thin constricted gamonts (Fig.16) are found, which are 50–300 µm in length and 18–35 µm in width. The gamonts become more

numerous towards the end of the year at the walls of the seminal vesicles. *Rhynchocystis porrecta* (115–500 µm) is readily differentiated from numerous other species of *Rhynchocystis* by virtue of its serpentine appearance and its swollen anterior end. Species of *Nematocystis* (Greek, nemat-, a thread), as the name implies, are long (up to 5 mm) and thin and also colonise the seminal vesicles of earthworms.

Gregarina cuneata O. Eugregarinida, SO. Septatina (Cephalina), Fam. Gregarinidae (Fig 2, 17)

This gregarine (150–300 x 55–100 µm) is the most abundant of the three species (*Gregarina cuneata, G. polymorpha, G. steini*) in the intestine of larvae of *Tenebrio molitor*. The body of the gamonts is divided into protomerite and deutomerite. Gregarines of this genus are parasites of insects and colonise the intestine. Here, however, only gamonts and sometimes gamont cysts are found. Gamont cysts are discharged with the faeces and the formation of spores occurs outside the host.The protomerite of *G. cuneata* is differentiated from the deutomerite by a constriction (Fig.17). The nucleus with prominent nucleolus is generally situated in the upper third of the deutomerite. In contrast to the gamonts of *Monocystis*, the gamonts of *Gregarina* attach one behind the other. The front partner is called primite, the rear partner is called satellite. *Gregarina polymorpha* (130–250 x 55–80 µm) can be differentiated from *G. cuneata* by a less prominent, roundish protomerite (Fig. 18 – 20). The deutomerite often attenuates somewhat towards the posterior end. However, both size and shape of the gamonts are variable (Fig. 18). The deutomerite is often topped with an epimerite which anchors the parasite to the intestinal wall of the host, but also acts as an attachment organelle during syzygy formation.

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Ciliates of Freshwater Biofilms and their Staining by Silver Impregnation

Weibo Song and Norbert Wilbert

INTRODUCTION

The organisms comprising biofilms (biofilm~periphyton~aufwuchs) live on substrates that may be living, dead or inanimate. They consist of both sessile and vagile organisms of which the latter feed on the former, or live in the gaps between them. There are also sessile, suspension feeding and predatory ciliates that feed on motile microorganisms. Biofilm communities include bacteria, fungi, protozoa and algae and play a major role in substrate modification, for example in the transformation (mineralisation) of organic material. They are also a significant part of the total population of benthic organisms, and hence are extremely important in recycling in both freshwater and marine ecosystems.

Depending on the degree to which light penetrates the water, biofilm communities will be populated mainly by autotrophic or by heterotrophic organisms. When decomposable organic matter is abundant, the dominant species are bacteria, cyanobacteria and the lower fungi, which in turn form the basic food supply for many protozoa. Of the latter, the ciliates are most in evidence. From an ecological viewpoint, most of the ciliates feed on bacteria and hence are directly dependent on the amount of bacterially decomposable matter present in the water. This relationship is clearly reflected in ciliate species richness: when the organic load is slight to intermediate, as is probably the case for most of our bodies of water, a freshwater biofilm can be expected to contain about 150 different ciliate species.

PRACTICAL TECHNIQUES

Collection

Various methods have been developed for investigating periphytic and benthic protozoan communities. We describe two of the more common methods that also provide sufficient material for preparations.

The Coverslip Method (Berger and Foissner 2003)

This is a very simple and effective technique. For sampling, wide-mouthed (about 8 cm), 500 ml plastic jars are used but only three quarters filled in order to keep an air reserve. This is especially important for oxygen-demanding, heavily polluted material. It is usual to sample the biofilm, the mud and the macrophytes separately.

Brush or scrape off the biofilm into a 500 ml jar. The sample should consist of about one third of biofilm material and two thirds of site water. The aerobic mud (especially the surface of organic mud) is collected with a spoon to a depth of up to 3 cm and again the sample should consist of one third mud and two thirds site water. Anaerobic mud, which is poisonous to aerobic organisms, must be collected separately. The third jar is filled with about 50% each of site water and submerged macrophytes, mosses, and filamentous algal mats. To avoid the potential release of substances toxic to ciliates this material must not be squashed. Samples should be transported under cool conditions (about 5° C) and investigated within 12 hours.

Two 40 x 20 mm coverslips are now floated on the surface of the water in the jar. One of these is removed with forceps or with thumb and forefinger after 30 min. The second coverslip is removed for examination when the first has been studied or, alternatively, is used for a permanent preparation. The coverslip is placed, wet side down, on a microscope slide and the numbers and kinds of ciliates present are noted. Ciliates accumulate on the coverslip due to oxygen depletion in the deeper zones of the bottle and because of their lifestyle: many are biofilm dwellers and will thus attach to the solid surface of the coverslip. The periphytic and benthic ciliate community obtained in this way is very clean and rich. Do not pour the collected material into a large Petri dish! This would slow down oxygen depletion and subsequent attachment of the ciliates to the coverslip.

The Microscope Slide Method

Microscope slides provide an ideal, artificial substrate for ciliates, but construction of a good slide holder (Fig. 1) is rather time-consuming. Instructions are found in Friedrich (1973) and Wilbert (1975). In all types of holder the slides are inserted in pairs, back-to-back. This is to ensure that ciliates settle only on one side of each slide.

Comparisons between the colonization of a natural substrate (such as a stone) permanently submerged in water and freshly exposed slides have revealed that a fresh, unoccupied substrate undergoes a primary, followed by a long-term colonization, the latter depending on the season of the year and the conditions in the environment. In the case of eutrophic standing and flowing bodies of water, exposure of the slides for two weeks is ideal.

Staining boxes of the Hellendahl type have proved useful for transporting the slides. The boxes are first filled with water from the investigation site and, using forceps or fingers, while maintaining the back to back arrangement, the slides are transferred from the holders into the transport containers. The containers should not be closed, so that air can enter. If they are kept in insulated bags the specimens will remain undamaged during transport. However, the subsequent investigation should take place as soon as possible.

For visual examination, an ordinarily equipped light microscope suffices. Workplaces should be provided with extra slides, coverslips of thickness 0 (various sizes), pipettes and a small glass beaker to contain water from the collecting site.

The pairs of slides are separated and investigated individually. The unoccupied surface is first patted dry with absorbent paper such as filter paper or kitchen towel and the slide is then placed on another slide with the colonized surface toward the

observer; this prevents water from contacting the microscope stage. During the examination (with or without a coverslip) care should be taken to keep the organisms from drying out by using water collected from the site. When necessary, a drop of the water is transferred to the slide with a pipette. Depending on the needs of the investigation, the slide can also be put into a Petri dish filled with water from the site and observed there with a stereomicroscope.

The organisms on a slide or coverslip are equivalent to a raw culture. For laboratory culture the slides or converslips should be placed in a Petri dish filled with water from the site and several grains of crushed rice added. Ciliates that feed on bacteria will be nourished by the bacteria that are feeding on the rice.

SILVER IMPREGNATION METHODS

Silver impregnation methods have acquired major significance in present day studies of ciliates. The two most common methods, Chatton-Lwoff and protargol, are described.

Data to supplement the structures seen during a study of the living organisms are best obtained by the protargol impregnation method. With only a few exceptions it is suitable for all ciliates and is both easy to learn and simple to perform. The cells appear transparent and organelles such as the nuclear apparatus and the infraciliature, as well as cortical and cytoplasmic fibres and extrusomes, are generally evenly stained.

The method employing silver nitrate impregnation is more complicated with regards to both equipment and preparation. While the cells retain their form, their cytoplasm is stained and not transparent. The stained structures are mainly cortical ones, namely basal bodies (kinetosomes), the silverline system (argyrome), the cytopyge and the pores of contractile vacuoles.

Ciliates such as scuticociliates (Fig. 19, 20), peritrichs (Fig. 26) and euplotid ciliates (Fig. 35) that are identifiable by cortical characteristics typical of their genus or species must be prepared by this method.

Finally, it should be noted that a species diagnosis must be based on an investigation and careful description of living creatures. The identification of fixed ciliates is in principle impossible.

The Wet (Chatton-Lwoff) Silver Method

The equipment consists of a refrigerator, a stereomicroscope, a heating plate, a UV lamp, square (embryo) cups with lids, micropipettes, needles or inoculation rings, degreased microscope slides (cleaned with an alcohol-soaked cloth), hot gelatine, and a large Petri dish that has been filled with water and allowed to freeze in the refrigerator. The following reagents and solutions must be precooled to about 2° C in the refrigerator: a 1% solution of $AgN0_3$ in a brown bottle with dropping pipette, distilled water in a wash bottle, staining cuvette with 70% ethanol.

Washing: To change the reagents after sedimentation of the fixed material the supernatant liquid is removed by suction with a micropipette under continuous visual control before the next solution is applied. In this process sedimented organisms will inevitably be swirled up from the bottom. It is for this reason that 2 cups should be

processed in parallel: during sedimentation of the cells in one cup, the other one is under the stereomicroscope undergoing the next stage of preparation, and so on.

Pipettes: For handling individual cells, whether alive or fixed, very fine pipettes are used with diameters only slightly larger than the size of the ciliates. These are pulled from a glass tube (Fig. 3). In order to heat the glass tube evenly use both hands to rotate it while it is being held in the flame. As soon as the glass is soft enough to be pulled, withdraw it from the flame and pull evenly with both hands. The faster the tube is pulled, the finer is the bore of the finished product. After cooling cut or break the capillary tube. In case you get sealed pipettes (the pointed end closed) you can proceed in the way shown in Fig. 3, but drawing must again be performed outside the flame.

Reagents: a) Champy fixative: 7 volumes 1% aqueous chromium trioxide solution (CrO_3), 7 volumes 3% aqueous potassium bichromate solution ($K_2Cr_2O_7$), and 4 volumes of 2% aqueous osmium tetroxide solution (OsO_4) are mixed together just before use. *The vapour of osmium tetroxide is dangerous and this solution must be only made in a fully functioning fume cupboard.* Use a dropping pipette to add the fixative directly to the embryo cup, drop by drop and use the same, sufficiently precise, method for the other mixtures. b) Da Fano mixture: 90 ml distilled water, 10 ml formalin (40%), 1 g cobalt nitrate ($Co(NO_3)_2$), 1 g sodium chloride (NaCl). The Da Fano mixture will keep indefinitely so a relatively large amount can be prepared if it is likely to be required in the future. c) Salted gelatine: 1 g pulverized gelatine, 5 mg sodium chloride (NaCl), 10 ml distilled water. Heat the gelatine with the water in a small conical flask in a water bath (or in a microwave oven set to low power) to dissolve it. Now add the salt, sterilize the solution by bringing it gently to the boil, cool and store in the refrigerator. The cooled and solidified gelatine solution must be clear. It remains usable until fungi or bacteria invade it and make it cloudy. d) Silver nitrate solution: dissolve 1 g $AgNO_3$ in 100 ml distilled water. The solution can be kept in a brown bottle for an unlimited period.

Protocol

1. Into each of two embryo cups place about 1 ml freshly prepared Champy fixative, and with the micropipette drop the ciliates directly into the fixative. Swirl the liquid in the cup to concentrate the cells in the middle. The fixation time should not exceed 10 minutes to avoid the cells becoming black.

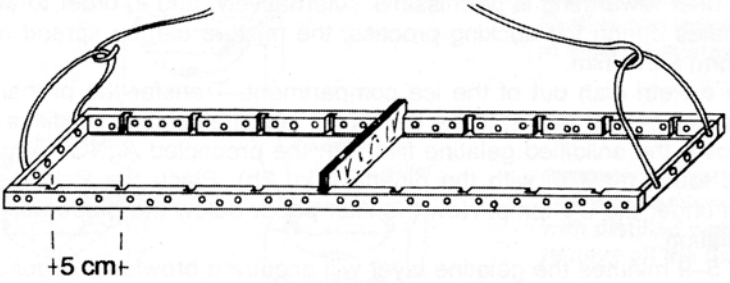
2. After 7 minutes replace the fixative with Da Fano solution. This removes the fixative from the cells and produces an immediate postfixation. At this stage the procedure can be interrupted for any length of time as the ciliates are stable in this solution. However, keep the cells in this solution for at least 5 minutes. During this time place several degreased slides and the lid of a small Petri dish on a heating plate that has been warmed to 40–50° C. Heat the gelatine to 40–50° C in the water bath.

3.Take the lid of the Petri dish off the heating plate and place the slide on the top of the lid. Place the smallest possible drop of the material to be examined in the middle of the slide (Fig. 2a). Observe the level of the liquid in the pipette and then add

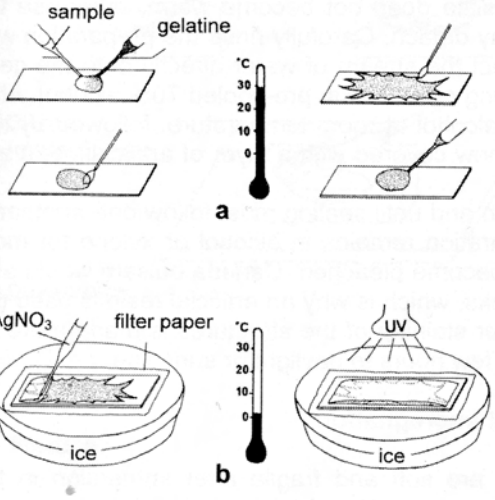
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Fig. 1. Microscope slide holder for the investigation of biofilms. **Fig. 2.** Wet (Chatton-Lwoff) silver nitrate impregnation (for details see text). **Fig. 3.** Preparing pipettes (for details see text).

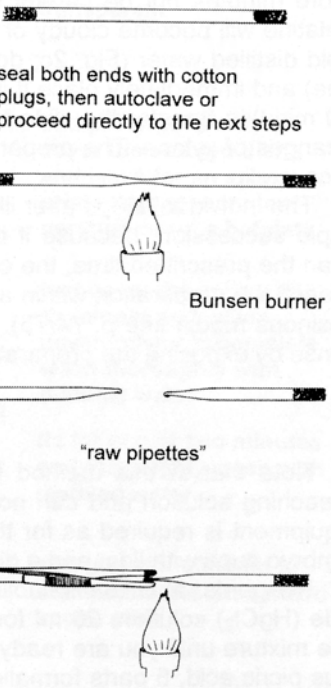
1 Microscope slide holder for the investigation of biofilms



2 Preparation of wet (Chatton-Lwoff) silver impregnation



3 Preparing pipettes



the same amount of heated gelatine and, under magnification, mix thoroughly using an inoculation ring or dissecting needle. With the micropipette suck away the excess water-gelatine mixture to such an extent that the cells are just barely embedded. This step must be performed quite rapidly, so that the gelatine mixture does not solidify. If necessary, brief rewarming is permissible. Alternatively, and in order to avoid losses of small ciliates during the sucking process, the mixture can be spread out with the needle to form a thin film.

4. Take a Petri dish out of the ice compartment. Transfer the preparation, supported by a strip of filter paper, to the dish. Wait until the gelatine solidifies (but is not frozen!). Cover the solidified gelatine film with the precooled $AgNO_3$ solution, taking care not to touch the film with the pipette (Fig. 2b). Place the Petri dish with the preparation under the UV lamp. Now the filter paper below the preparation will intensify the radiation.

5. After 5–9 minutes the gelatine layer will acquire a brownish, cognac coloration as the reduction of the silver nitrate is completed. Check the preparation under the microscope. The impregnated structures (basal bodies of cilia, silverline pattern) should now stand out from their surroundings with their dark-brown to black appearance. If the impregnation is still too weak, illuminate the preparation for a few more minutes but be careful that the slide does not become warm, otherwise the gelatine will become cloudy or even may detach. Carefully rinse the preparation with cold distilled water (Fig. 2c; do not direct the stream of water directly onto the gelatine) and immediately place it in a staining cuvette with pre-cooled 70% alcohol. After 10 minutes transfer the slide to 100% alcohol at room temperature, followed by two changes of xylene. The preparation is now covered with a layer of artificial resin and a coverslip.

The individual steps after illumination and until sealing must follow one another in rapid succession, because if the preparation remains in alcohol or xylene for more than the prescribed time, the cells will become bleached. Canada balsam would also bleach the preparation within a few weeks, which is why an artificial resin is used (for resinous media see p. 74/75). The silver staining of the structures is made more intense by exposing the preparation for a few hours to daylight or sunshine.

Protargol Impregnation

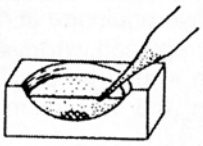
Note that in this method the cells are soft and fragile after immersion in the bleaching solution and can no longer be concentrated by centrifugation. The same equipment is required as for the previous method: stereomicroscope, micropipettes, embryo cups with lids, and a dissecting needle or an inoculation ring.

Reagents: a) Stieve's sublimate fixative: 76 ml saturated aqueous mercuric chloride ($HgCl_2$) solution, 20 ml formalin (40%), 4 ml glacial acetic acid. Do not prepare the mixture until you are ready to use it. b) Bouin's fixative: 15 parts saturated aqueous picric acid, 5 parts formalin (40%), 1 part glacial acetic acid. Prepare just before use. c) Glycerol-albumin: put 1 part egg white and 1 part glycerol into a small beaker, mix well and filter. The glycerol-albumin mixture should be stored in a bottle that can be closed tightly and is protected against bacterial decomposition by the addition of a

Fig. 4. Protargol impregnation (for details see text).

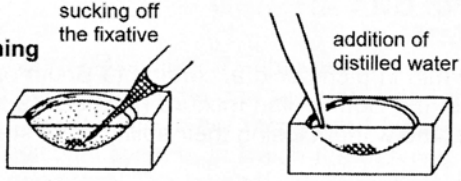
4 Protargol impregnation

a
(1) Fixation



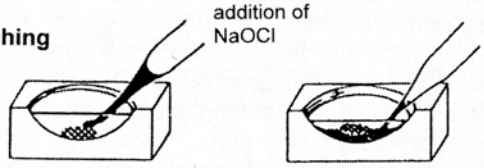
add the ciliates to the fixative and leave for at least 10 minutes

b
(2) Washing



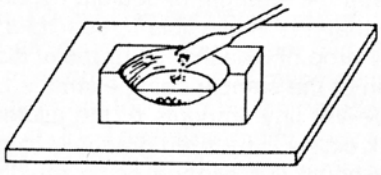
remove the fixative and wash several times with distilled water to remove all the fixative

c
(3) Bleaching



add the suitably diluted bleach solution and leave until the cells become transparent

d
(4) Silver impregnation



add protargol powder, cover the embryo dish, and leave for an appropriate time at room temperature or on a hotplate

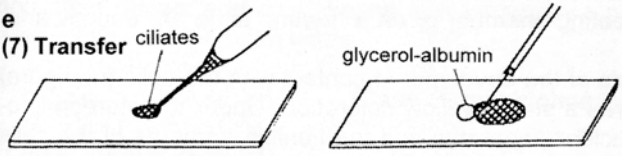
(5) Developing

just cover the cells with developer and, when development is complete, wash thoroughly with distilled water

(6) Fixation

fix for one or two minutes and thoroughly wash with distilled water

e
(7) Transfer



add glycerol-albumin to stained ciliates on a microscope slide

(8) Dehydration

remove excess liquid, dehydrate and add xylene

(9) Permanent preparation

mount in resin under a coverslip

small crystal of thymol. d) Bleaching solution: prepare sodium hypochlorite solution (NaOCl, household bleach) 1:30 with distilled water. e) Silver proteinate (Protargol), this is Fluka article no. 05495 – 5 (DEOrders@europe.sial.com). f) Developer: dissolve 5 g sodium sulphite (Na_2SO_3) and 1 g hydroquinone in 100 ml distilled water. This solution is kept in the refrigerator and replaced when it becomes brownish. Since a single drop suffices for a preparation it may be prepared in much smaller amounts. g) Fixative after silver impregnation: 0.5 % sodium thiosulphate solution ($Na_2S_2O_3$) in distilled water.

Protocol (Wilbert 1975)

(1) Fix the ciliates for at least 10 min in picric acid according to Bouin or in sublimate according to Stieve (Fig. 4a). In the Bouin-Allen mixture (Romeis 1968, § 309) the cells can be kept for quite a long time without losing their ability to be stained with silver.

(2) Wash with distilled water (Fig. 4b). Allow the cells to sediment and remove the water above them using a micropipette. Monitor the process using a stereomicroscope. Exchange the water several times until the supernatant is colourless.

(3) Bleach the cells with the solution of sodium hypochlorite (Fig. 4c). This step is the most difficult in the whole process because if bleaching is excessive the cells can no longer be impregnated or they may actually disintegrate. However, if the cells are not made bright enough, the basal bodies will not be stained later on. The main problem encountered in this process is that the strength of sodium hypochlorite in household bleach decreases with age, so that it is impossible to specify a precise concentration. Start with a dilution of 1:30 (1 drop of bleach + 30 drops of distilled water) and use this as a trial with a small portion of the sample being examined. Use the stereomicroscope and a micropipette to deliver tiny amounts of the diluted bleach around the fixed and washed cells. The dark cells will be seen gradually to become light and transparent over several seconds. Should this change occur rapidly then a second trial with a more dilute solution of bleach should be made. Immediately after the reaction carefully remove most of the bleach from the bottom of the embryo dish and wash the cells several times with distilled water to remove any traces of bleach that would otherwise precipitate the protargol used in the next step.

(4) Impregnation with protargol (Fig. 4d). Structures with an affinity for silver will be stained by very low concentrations of silver ions, so a small spatula tip of protargol powder scattered onto the water in the embryo dish is adequate. The impregnation is time-dependent and temperature-dependent. Twelve hours at room temperature or 30–45 min at 40–50° C in a heating chamber or on a heating plate are equally adequate.

(5) Place very small amounts of the developer in contact with the cells (no figure) to give the argyrophilic structures a strong yellow coloration. Under the stereomicroscope the basal bodies, the nuclear apparatus and the fibrillar elements of the cells will now be visible. Wash thoroughly with distilled water.

(6) Cover the impregnated cells (no figure) with the sodium thiosulphate solution for 1–2 min and then completely remove the fixative by washing with distilled water.

(7) Transfer by pipette the stained cells in a tiny drop of water to a degreased microscope slide (Fig. 4e), add a drop of glycerol-albumin solution and mix it well with the water. Remove excess liquid and allow the preparation to dry in the air or on the

heating plate.

(8) Dehydrate the stained ciliates with the series 70% alcohol, 100% alcohol, xylene (x2) (no figure). Use the dried slide for examination under oil immersion. For a permanent preparation:

(9) Add a drop of synthetic resin after the second xylene stage and seal the preparation with a coverslip (no figure).

GENERA AND SPECIES

The modern classification within the phylum Ciliophora is based on the organization and ontogenesis of the oral apparatus and the fine structure of the cortex. However, this classification is not recognized by all ciliatologists. Hence it is justified to use the traditional system, in which the defining characteristics are the oral and somatic ciliary pattern. This has also the practical and didactic advantage that many of the features can be seen with the light microscope in the living ciliate.

As examples, figures 5 – 35 show representatives of the motile and sessile ciliate community that can be found among the biofilm of a eutrophic body of water. The first colonizers are grazers, feeding on the initial coating of bacteria and coccal algae: *Chilodontopsis planicauda* (Fig. 16), *Pseudochilodonopsis algivora* (Fig. 17), *Chilodonella uncinata* (Fig. 18). Sessile filter feeders are also represented by many species in this first and the subsequent early colonization phases. They can occupy the fresh substrate until algal filaments cut them off from a supply of food from the surrounding water: *Calyptotricha lanuginosum* (Fig. 19), *Tintinnidium emarginatum* (Fig. 33), *Chaetospira muelleri* (Fig. 31), and many peritrichs (Fig. 25 – 29).

These first settlers are followed by their predators. The holotrichous ciliates *Songophrya armata* (Fig. 5), *Trachelius ovum* (Fig. 13) and *Amphileptus procerus* (Fig. 14) feed on peritrichs. Other predators include *Dileptus jonesi* (Fig. 7), *Lacrymaria vaginifera* (Fig. 8), *Litonotus obtusus* (Fig. 9), *Acineria punctata* (Fig. 11), *Loxophyllum carinatum* (Fig. 12), *Amphileptus fusidens* (Fig. 15). Finally the suctorians *Heliophrya rotunda* (Fig. 23) and *Acineta tuberosa* (Fig. 24) appear. They prey on both the grazers and the ciliates that swim freely within the biofilm. Some representatives of other motile species are also illustrated: *Urotricha discolor* (Fig. 6), *Nassula ornata* (Fig. 22), *Halteria grandinella* (Fig. 30), *Oxytricha balladyna* (Fig. 32), and *Euplotes woodruffi* (Fig. 35). These have a mixed diet, feeding on algae and bacteria. One species, *Coleps hirtus* (Fig. 10), deserves special mention as it utilizes as food dead rotifers and crustaceans which had been part of the biofilm itself or had fallen into it from the open water.

Class Heterotrichea

Stentor igneus (Fig. 34)

Length 200–400 µm and trumpet-shaped when fully extended; the species name "*igneus*" (Latin, burning, flaming red) refers to the strikingly reddish colour of this ciliate. Uniform somatic ciliation; an adoral zone of membranelles extends spirally around the anterior end and into the oral funnel. Single spherical macronucleus near mid-body; contractile vacuole near oral funnel. Attached to stones, plants, sometimes

free-swimming. Feeds an bacteria, algae, flagellates, and diatoms.

Class Spirotrichea

Euplotes woodruffi (Fig. 35)

Length 110–180 µm; body dorso-ventrally flattened, elliptical when viewed from ventral side, inflexible. Ventral side with characteristic set of somatic cirri: 9 fronto-ventral, 5 transverse, 4 caudal; dorsal side with short cilia in 8 rows. Adoral zone of membranelles along anterior and left body margin, peristomal field broadly triangular, about 2/3 of body length. Macronucleus T- or Y-shaped; contractile vacuole right of transverse cirri. Crawls on various substrates and feeds on small algae, flagellates, and bacteria.

Tintinnidium emarginatum (Fig. 33)

Lorica 40–350 µm long, usually tubular with posterior end closed; its wall slimy, thin and covered by foreign material (for example diatom frustules). Sometimes several individuals form a pseudocolony (b). Body about 40 µm long, more or less trumpet-shaped and fixed to bottom of lorica by a contractile stalk. Adoral membranelles prominent, around apical end; somatic ciliation restricted to rows of bristle-like cilia. Single macronucleus and contractile vacuole. Planktonic and attached to substrates; feeds on flagellates, diatoms, and bacteria.

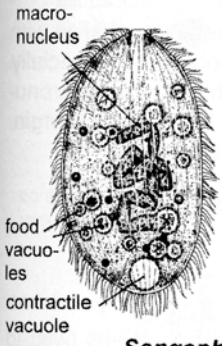
Halteria grandinella (Fig. 30)

Length 20–50 µm; spherical. About 15 membranelles in a circle anteriorly; lateral body surface with 8 clusters of long, spine-like cilia. Macronucleus and contractile vacuole near mid-body. Performs conspicuous jumps. Planktonic and benthic, feeding on bacteria and small flagellates.

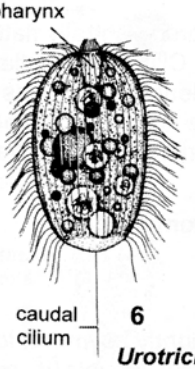
Chaetospira muelleri (Fig. 31)

Length 200–500 µm when extended, strongly contractile, lives in a tubular, smooth lorica 300–1300 µm long; body elongate and in a loose helix, especially the long, flexible proboscis; somatic cirri in three rows; adoral zone of membranelles extends along proboscis. Four ellipsoidal macronuclear nodules; single contractile vacuole posterior to the mid-body. Attached to various substrates; feeds on bacteria, flagellates, and diatoms.

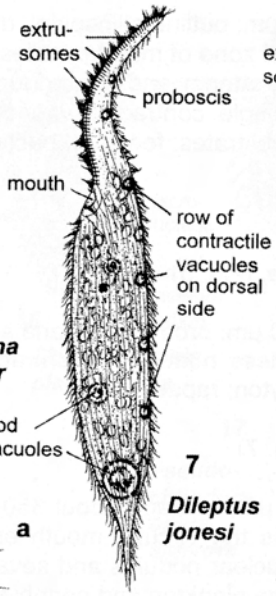
Fig. 5. *Songophrya armata* (100–200 µm in length). **Fig. 6.** *Urotricha discolor* (30–40 µm). **Fig. 7.** *Dileptus jonesi* (300–500 µm). **8a.** *Lacrymaria vaginifera* (100–200 µm), an individual in its lorica. **b.** Fully extended specimen. **Fig 9.** *Litonotus obtusus* (70–110 µm). **10a.** *Coleps hirtus* (30–50 µm). **b.** Calcium carbonate plate. **Fig. 11.** *Acineria punctata* (80–180 µm). **Fig. 12a.** *Loxophyllum carinatum* (120–200 µm). **b.** The distribution of extrusomes in a contracted specimen. **Fig. 13a.** *Trachelius ovum* (200–400 µm). **b.** A specimen full of food vacuoles.



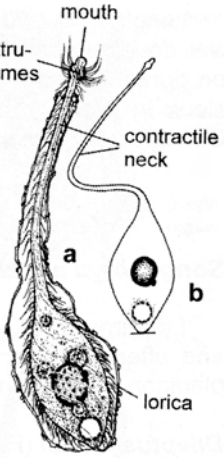
5 ***Songophrya armata***



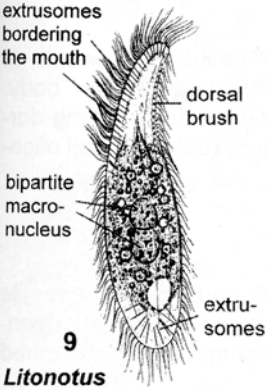
6 ***Urotricha discolor***



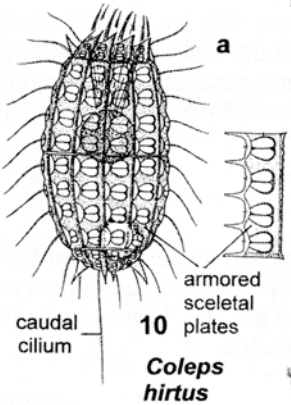
7 ***Dileptus jonesi***



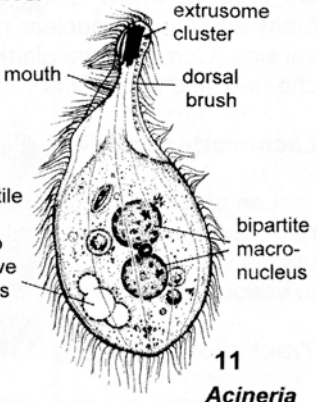
8 ***Lacrymaria vaginifera***



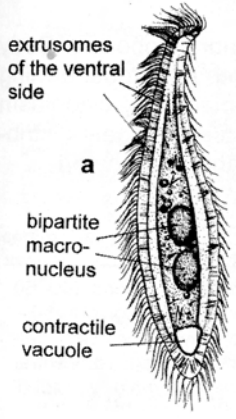
9 ***Litonotus obtusus***

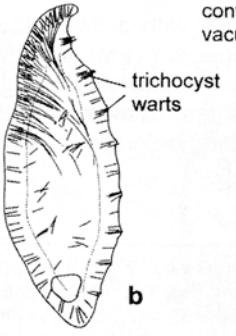


10 ***Coleps hirtus***

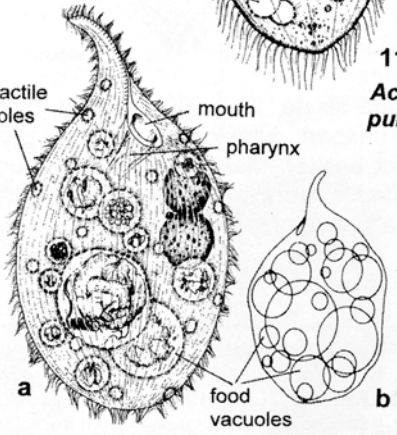


11 ***Acineria punctata***





12 ***Loxophyllum carinatum***



13 ***Trachelius ovum***

Oxytricha balladyna (Fig. 32)

Length about 50 µm; outline ellipsoidal, dorso-ventrally flattened. Buccal area with well developed adoral zone of membranelles. Ciliary organelles prominent, especially on dorsal side and posterior end. Macronucleus in two parts with a single micronucleus in between; single contractile vacuole in mid-body near to the left margin. Crawls on various substrates; feeds on bacteria, algae, and flagellates.

Class Litostomatea

Songophrya armata (Fig. 5)

Length about 150 µm; broadly oval and slightly asymmetric. Macronucleus twisted and often more or less nodulated; contractile vacuole in rear end. Occurs in the plankton and periphyton; rapacious.

Dileptus jonesi (Fig. 7)

Length 300–500 µm, average about 350 µm. Slender with neck-like proboscis, at the base of which is the circular mouth entrance. Uniform ciliation all over body. Many small macronuclear nodules and several small contractile vacuoles along dorsal side. Occurs in the plankton and periphyton; feeds on ciliates, rotifers, small oligochaetes, and planarians.

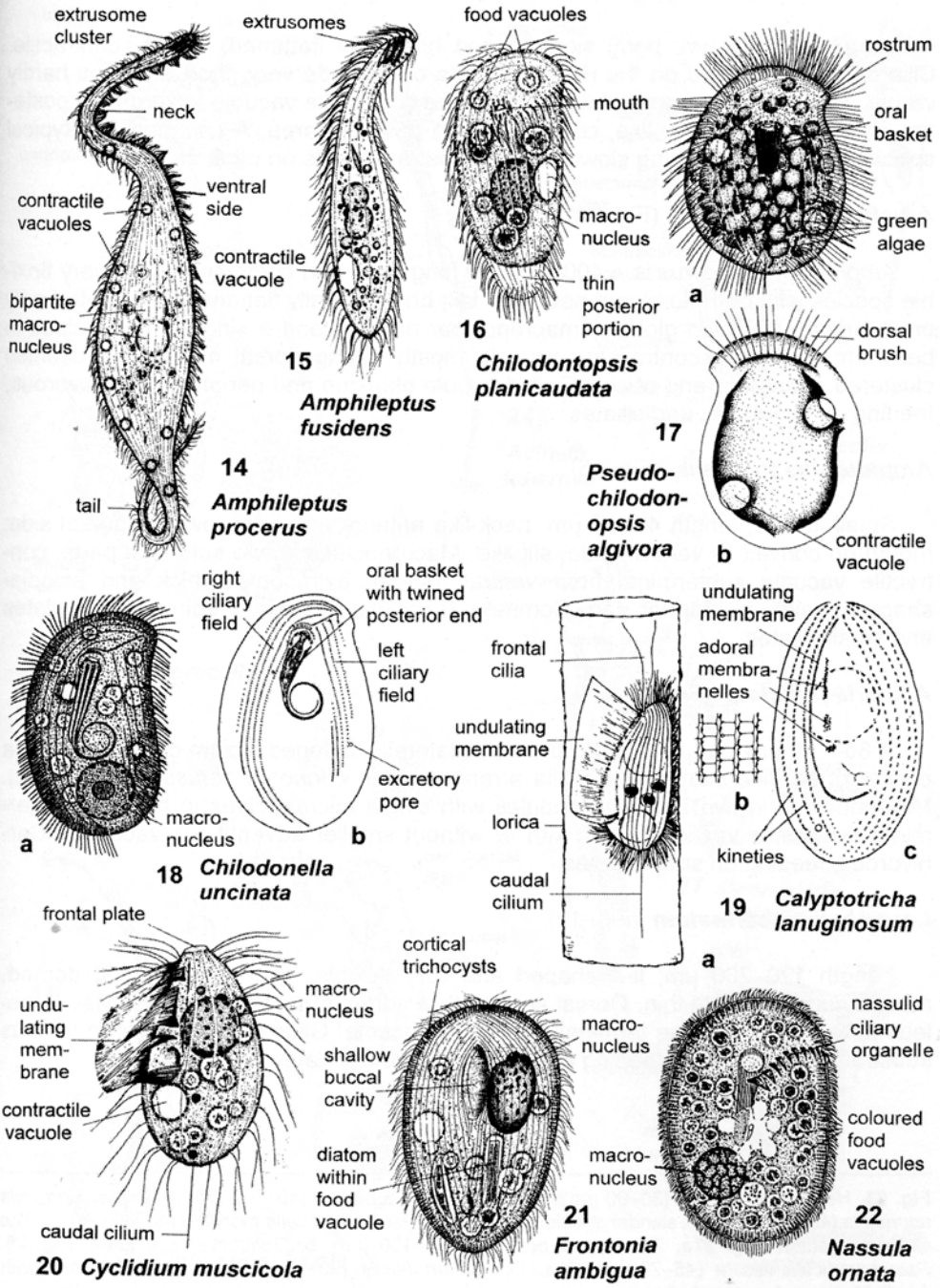
Lacrymaria vaginifera (Fig. 8)

Length 100–200 µm; body divided into two parts: oval trunk and highly contractile neck, twice as long as trunk when fully extended (b). Single macronucleus and a contractile vacuole in the posterior end of the cell. Lives in a flask-shaped lorica attached to various substrates (a). Feeds on ciliates.

Trachelius ovum (Fig. 13)

A large ciliate, 200–400 µm long; globular with a distinct, short proboscis. Body ciliation uniform, cytostome at base of proboscis; cytopharynx with long rods forming a distinct basket. Many contractile vacuoles scattered throughout body; cytoplasm vacuolated. Carnivorous, feeding on flagellates, ciliates, and rotifers. Widely distributed in all types of standing and flowing waters, belongs to the vagile periphyton.

Fig. 14. *Amphileptus procerus* (400–800 µm), right side view. **Fig. 15.** *Amphileptus fusidens* (40–60 µm). **Fig. 16.** *Chilodontopsis planicaudata* (25–40 µm). **Fig. 17a.** *Pseudochilodonopsis algivora* (50–60 µm). **b.** Dorsal view. **Fig. 18a.** *Chilodonella uncinata* (30–40 µm). **b.** Ventral view of the ciliary pattern. **Fig. 19a.** *Calyptotricha lanuginosum* from life (40 µm). **b.** Detail of silverline pattern. **c.** Ventral view of ciliary pattern. **Fig. 20.** *Cyclidium muscicola* (20–30 µm). **Fig. 21.** *Frontonia ambigua* (about 100 µm). **Fig. 22.** *Nassula ornata* (about 200 µm).


extrusome cluster
neck
ventral side
contractile vacuoles
bipartite macro-nucleus
tail
14
Amphileptus procerus
extrusomes
contractile vacuole
15
Amphileptus fusidens
food vacuoles
mouth
macro-nucleus
thin posterior portion
16
Chilodontopsis planicaudata
rostrum
oral basket
green algae
a
dorsal brush
17
Pseudo-chilodon-opsis algivora
contractile vacuole
b
right ciliary field
oral basket with twined posterior end
left ciliary field
excretory pore
macro-nucleus
a
b
18
Chilodonella uncinata
frontal cilia
undulating membrane
lorica
caudal cilium
a
undulating membrane
adoral membra-nelles
b
kineties
c
19
Calyptotricha lanuginosum
frontal plate
undu-lating mem-brane
contractile vacuole
caudal cilium
macro-nucleus
20
Cyclidium muscicola
cortical trichocysts
shallow buccal cavity
diatom within food vacuole
macro-nucleus
macro-nucleus
21
Frontonia ambigua
nassulid ciliary organelle
coloured food vacuoles
22
Nassula ornata

Litonotus obtusus (Fig. 9)

Length 70–110 µm; body sigmoid and bilaterally flattened, slightly contractile. Cilia densely arranged on the right side, cilia on left side very short and thus hardly visible. Two macronuclear nodules and a large contractile vacuole towards the posterior end, extrusomes bar-like, conspicuous in posterior area. A common and typical species of biofilms crawling slowly on the substrate; feeds on other ciliates.

Amphileptus procerus (Fig. 14)

Amphileptus procerus is a 400–800 µm long giant and commonly seen, very flexible species with both dominant neck and tail; body laterally flattened and ciliated only on the right side. Two globular macronuclear nodules and a single micronucleus in between; numerous contractile vacuoles mostlyl along dorsal margin; extrusomes clustered at anterior end of cell. Found in both plankton and periphyton; carnivorous, feeding on flagellates and ciliates.

Amphileptus fusidens (Fig. 15)

Small in size, length 40–60 µm; neck-like anterior end bent towards dorsal side; mouth on convex (= ventral) side, slit-like. Macronucleus in two spherical parts; contractile vacuole subterminal near ventral margin; extrusomes thick and spindle-shaped, mainly in anterior end. Common in various biofilms, feeding on flagellates and small ciliates.

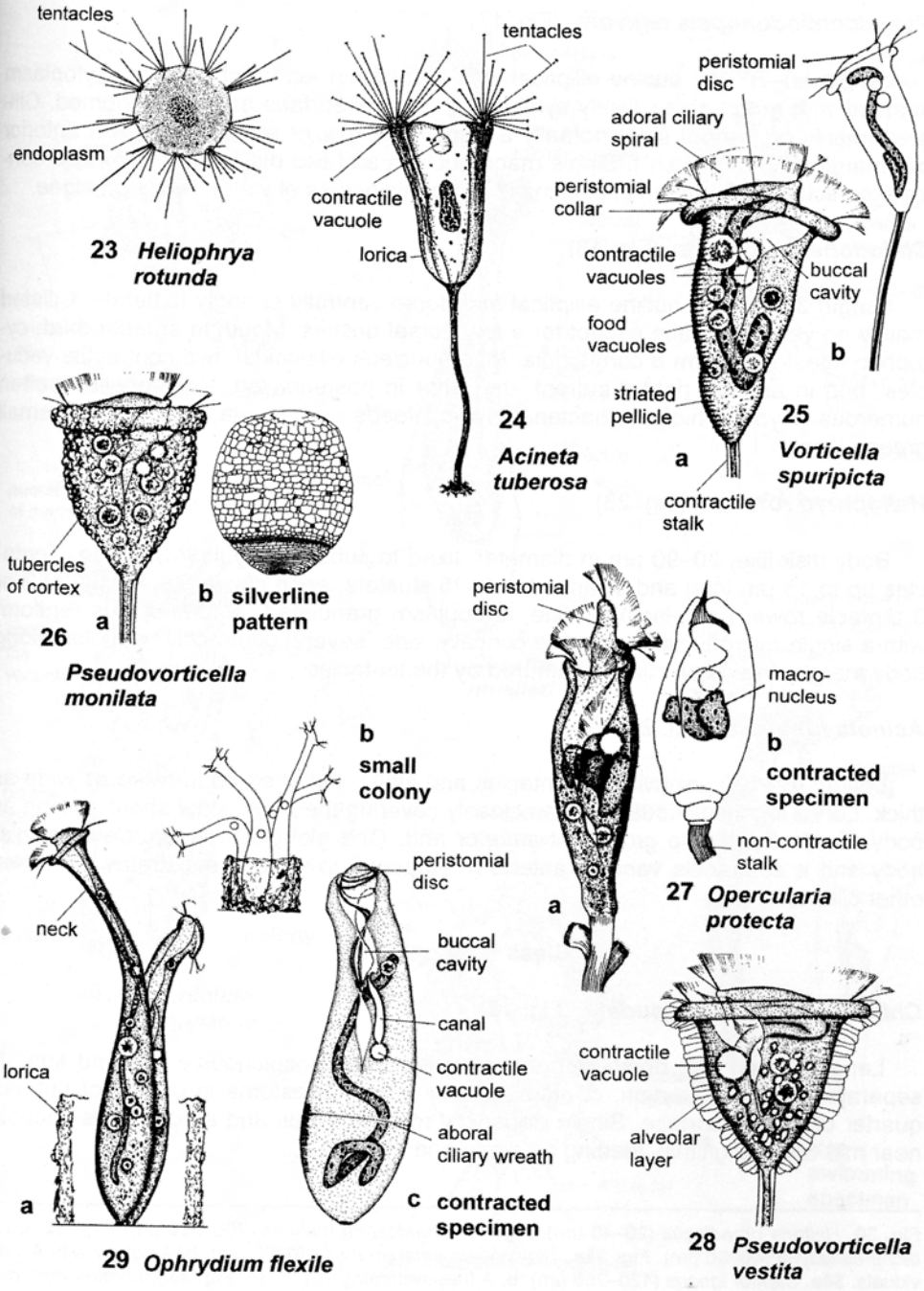
Acineria punctata (Fig. 11)

A 80–180 µm long, highly flexible and bilaterally flattened biofilm ciliate. Apically a cluster of bar-like extrusomes. Cilia arranged as in *Litonotus obtusus* (see above). Macronucleus in two spherical nodules with single micronucleus in between; a terminal contractile vacuole either with or without smaller adventitious vacuoles. Carnivorous, feeding on small ciliates.

Loxophyllum carinatum (Fig. 12)

Length 120–200 µm; leaf-shaped and very flexible, right side flat, left domed, margin conspicuously thin. Dorsal edge with a series of trichocyst warts. Macronucleus in two parts; a large terminal contractile vacuole. Glides majestically on various substrates; carnivorous, feeding on rotifers and other ciliates.

Fig. 23. *Heliophrya rotunda* (30–90 µm). **Fig. 24.** *Acineta tuberosa* (40–100 µm). **Fig. 25a.** *Vorticella spuripicta* (40–50 µm). **b.** A slender specimen. **Fig. 26a.** *Pseudovorticella monilata* (40–60 µm). **b.** The silverline pattern. **Fig. 27a.** *Opercularia protecta* (60–100 µm). **b.** Contracted specimen. **Fig. 28.** *Pseudovorticella vestita* (45–70 µm). **29a.** *Ophrydium flexile* (90–160 µm). **b.** A small colony with 4 individuals. **c.** A contracted specimen.


tentacles
endoplasm
23 Heliophrya rotunda
tentacles
contractile vacuole
lorica
24 Acineta tuberosa
peristomial disc
adoral ciliary spiral
peristomial collar
contractile vacuoles
buccal cavity
food vacuoles
striated pellicle
contractile stalk
a
b
25 Vorticella spuripicta
tubercles of cortex
a
b silverline pattern
26 Pseudovorticella monilata
peristomial disc
macro-nucleus
b contracted specimen
non-contractile stalk
a
27 Opercularia protecta
b small colony
neck
lorica
a
peristomial disc
buccal cavity
canal
contractile vacuole
aboral ciliary wreath
c contracted specimen
29 Ophrydium flexile
contractile vacuole
alveolar layer
28 Pseudovorticella vestita

Pseudochilodonopsis algivora (Fig. 17)

Length 50–70 µm; outline elliptical with an anterior left-hand rostrum. Cytoplasm studded with green algae (likely symbionts). Ventral surface flat, dorsal domed. Ciliated mainly on ventral side; dorsally a transverse row of stiff cilia beneath anterior cell margin ("dorsal brush"). Single macronucleus and two diagonally positioned contractile vacuoles. Common in biofilms of eutrophic bodies of water; feeds on algae.

Chilodonella uncinata (Fig. 18)

Length 30–40 µm; outline elliptical and dorso-ventrally strongly flattened. Ciliated mainly on ventral surface except for a few dorsal bristles. Mouth in anterior third, cytopharyngeal rods form a cornucopia. Macronucleus ellipsoidal; two contractile vacuoles, one in anterior right quadrant, the other in posterior left. Cosmopolitan, often numerous in young biofims (bacterial layers); feeds on bacteria, diatoms, and small green algae.

Heliophrya rotunda (Fig. 23)

Body disk-like, 30–90 µm in diameter; fixed to substrate by its lower side. Tentacles up to 15 µm long and arranged in 8–15 clusters, each cluster composed of 2 or 3 tentacle rows. Ectoplasm hyaline, endoplasm granulated. Macronucleus reniform with a single micronucleus near the concave side; several contractile vacuoles along body margin. Feeds on ciliates captured by the tentacles.

Acineta tuberosa (Fig. 24)

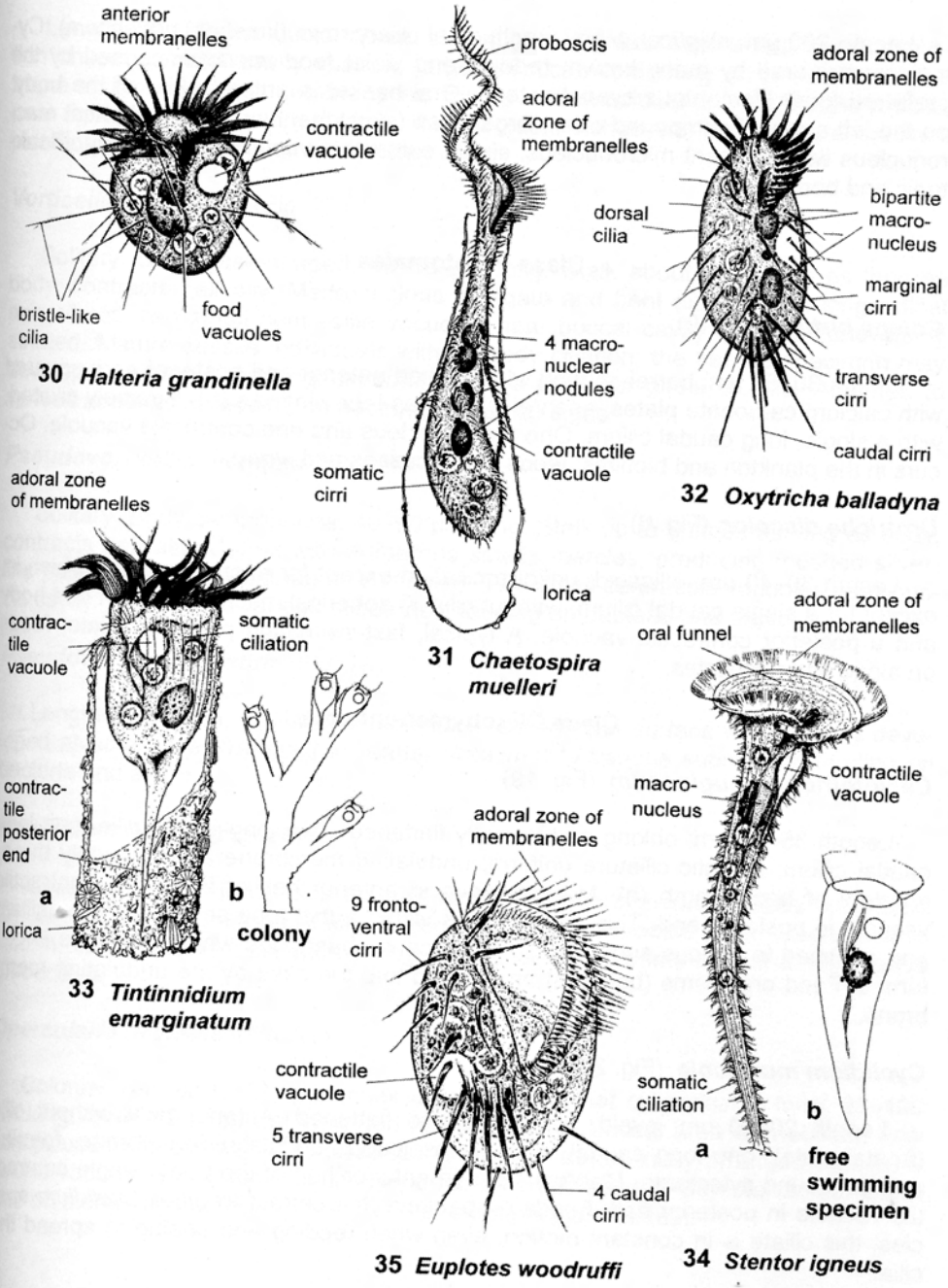
Length 40–100 µm (without tentacles and stalk), flattened up to twice as wide as thick. Lorica inconspicuous because closely covering the body; stalk about as long as body. Tentacles in two groups at anterior end. One globular macronucleus in mid-body and a contractile vacuole anteriorly. Attached to various substrates; feeds on other ciliates.

Class Nassophorea

Chilodontopsis planicaudata (Fig. 16)

Length 25–40 µm; dorso-ventrally flattened with conspicuously thin and sharply separated posterior portion; ciliated on both sides. Cytostome in middle of anterior quarter of ventral surface. Single ellipsoidal macronucleus and a contractile vacuole near mid-body. Biofilmic, feeding on algae and bacteria.

Fig. 30. *Halteria grandinella* (20–40 µm). **Fig. 31.** *Chaetospira muelleri* (200–300 µm). **Fig. 32.** *Oxytricha balladyna* (40–60 µm). **Fig. 33a.** *Tintinnidium emarginatum* (30–50 µm). **b.** A colony with 4 individuals. **34a.** *Stentor igneus* (120–250 µm). **b.** A free-swimming specimen. **Fig. 35.** *Euplotes woodruffi* (110–180 µm). (Fig. 6, 18, 22 – 24 from Foissner et al. 1995)


anterior membranelles
contractile vacuole
bristle-like cilia
food vacuoles
30 Halteria grandinella
proboscis
adoral zone of membranelles
4 macronuclear nodules
contractile vacuole
somatic cirri
lorica
31 Chaetospira muelleri
adoral zone of membranelles
dorsal cilia
bipartite macronucleus
marginal cirri
transverse cirri
caudal cirri
32 Oxytricha balladyna
adoral zone of membranelles
contractile vacuole
somatic ciliation
contractile posterior end
a
lorica
b
colony
33 Tintinnidium emarginatum
adoral zone of membranelles
9 frontoventral cirri
contractile vacuole
5 transverse cirri
4 caudal cirri
35 Euplotes woodruffi
oral funnel
adoral zone of membranelles
macronucleus
contractile vacuole
somatic ciliation
a
b
free swimming specimen
34 Stentor igneus

Nassula ornata (Fig. 22)

Length 200 µm; elliptical. Many longitudinal ciliary rows (kineties, not shown). Cytoplasm coloured by many brown, reddish and violet food vacuoles caused by the preferred food, filamentous cyanobacteria. Oral basket in anterior third of the body; on the left a row of compound ciliary organelles (synhymenium). One spherical macronucleus with adjacent micronucleus; single contractile vacuole in mid-body. Planktonic and benthic.

Class Prostomatea

Coleps hirtus (Fig. 10)

Length 30–50 µm; barrel-shaped with toothed anterior and posterior end; covered with calcium carbonate plates, each of which has four windows (b). Sparsely ciliated, with a single, long caudal cilium. One macronucleus and one contractile vacuole. Occurs in the plankton and biofilms; feeds on carcasses and algae.

Urotricha discolor (Fig. 6)

Length 30–40 µm; ellipsoid; uniform ciliation except for posterior region which is, except for a single caudal cilium, without cilia. A spherical macronucleus in mid-body and a posterior contractile vacuole. A typical, fast-swimming plankton ciliate; feeds on algae and flagellates.

Class Oligohymenophorea

Calyptotricha lanuginosum (Fig. 19)

Length 35–40 µm; oblong and dorsally flattened. Two long frontal cilia, one long caudal cilium; somatic ciliature uniform; undulating membrane approximately three-quarters of body length (a). Macronucleus in anterior half of the body; contractile vacuole in posterior end. This species lives within a thin tube secreted by the ciliate and attached to various substrates. The lorica is abandoned when the ciliate is disturbed. Food organisms (bacteria) are whirled into the case by the undulating membrane.

Cyclidium muscicola (Fig. 20)

Length 20–30 µm; ovoid, ventral surface flattened. Anterior pole without cilia (frontal plate), one long caudal cilium; conspicuous undulating membrane, forming pocket around cytostome. Macronucleus in anterior half of the body; single contractile vacuole in posterior half. Feeds on bacteria. In contrast to other *Cyclidium* species, this ciliate is in constant motion, even when feeding and unable to spread the cilia.

Frontonia ambigua (Fig. 21)

Length 80–100 µm; ovoid, dorsal side slightly convex, ventral flat. Somatic ciliation uniform; buccal cavity shallow, large, elliptical, containing an undulating membrane and three adoral membranelles. Single macronucleus; one contractile vacuole near right body margin in mid-body; many cortical trichocysts. In biofilms, sometimes planktonic; feeds on diatoms and other algae.

Vorticella spuripicta (Fig. 25)

Solitary. Body vase-shaped, 40–50 µm long. Stalk about 2–3 times as long as body, contracts helically. Macronucleus elongate and bent extending in longitudinal axis of cell; two small contractile vacuoles near buccal cavity; pellicle transversely striated. Mature sessile individuals without body ciliation; the sessile specimen may develop to å free-swimming "teletroch" with a posterior girdle of cilia. Attached to various substrates, feeding on bacteria and small algae.

Pseudovorticella monilata (Fig. 26)

Solitary. Body campanulate, 40–60 µm long. Stalk up to 3 times as long as body, contracts helically. Cortex with numerous small tubercles, producing meshed silverline pattern (transverse striae in *Vorticella*). Two small contractile vacuoles near buccal cavity. Attached to various substrates, feeding on bacteria and algae.

Pseudovorticella vestita (Fig. 28)

Length 50–70 µm; similar to *P. monilata* (above), but surface with a highly developed alveolar layer (rather than warts). Attached to various substrates, feeding on bacteria and algae.

Ophrydium flexile (Fig. 29)

Usually, 24 individuals form a small, slimy colony attached to various substrates (b). Zooids 90–60 µm long; flask-shaped with long, contractile neck. Macronucleus filiform; contractile vacuole with a long canal that empties into buccal cavity. Feeds on bacteria.

Opercularia protecta (Fig. 27)

Colonies with up to 12 specimens. Zooids somewhat cylindrical in form, 80–100 µm long. Stalk 50 –150 µm long and not contractile. Buccal area with oblique disk conspicuously raised (a). Macronucleus C-shaped, transversely arranged; one contractile vacuole near buccal cavity. Reported as an epizoon of *Gammarus*, but found also on stones and artificial substrates (for example glass slides).

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Peritrichia, Chonotrichia and Suctoria on Gammarids

Horst Schödel

INTRODUCTION

Like most aquatic macroinvertebrates, species of the genus *Gammarus* are colonized by various peritrichous ciliates. Gammarids are widespread and abundant in rivers and brooks. Some 80 different peritrich species as well as about 10 suctorians and chonotrichs have been reported from freshwater gammarids worldwide (Schödel 1987). In Central Europe, about 25 species can be expected, of which the 16 most common were selected for this chapter.

The peritrichs (Cl. Oligohymenophorea, Subcl. Peritrichia) are, due to specific traits, a distinct systematic unit easily distinguishable from other ciliates. A feature common to all peritrichs is the reduction of the ciliature to an adoral ciliary spiral, which extends to the cytostome by the counter-clockwise rotation of a double ciliary row (Fig.1). Further, most peritrichs are attached to the substrate with a stalk, which is secreted by the scopula in the posterior centre of the cell. The stalk can be contracted by a myoneme (spasmoneme) as in the genera *Carchesium* and *Zoothamnium*. The epistylidids lack a stalk myoneme and thus can contract only the zooid. The adoral ciliary spiral generates a strong water current that drives food particles (bacteria, debris, algae) through the oral cavity to the cytostome, where they are enclosed in a food vacuole. The ciliary spiral encircles the peristomial disc in one and a quarter and one and a half circuits and then plunges into the vestibulum (buccal cavity, oral cavity). The bell-shaped or barrel-shaped zooid is usually widened into a peristomial collar anteriorly, that is, at level of the peristomial disc. If zooids are disturbed mechanically or chemically, they are able to retract the oral disc and close the peristome. Indigestible materials are excreted through the cytopyge, which opens into the oral cavity. Osmoregulation occurs, as usual, by a contractile vacuole (in rare cases two), the fluid contents of which are also released into the oral cavity. The macronucleus is spherical or oblong, and in many species it surrounds the cytopharynx in the shape of a horseshoe. The micronucleus is near the macronucleus.

Due to the sessile mode of life, binary fission of peritrichs is longitudinal. The daughter cell subsequently forms a swarmer in solitary species, or secretes a stalk in colony-forming species. Sexual reproduction of peritrichs takes place by total conjugation. By means of unequal division, small microconjugants are formed and attach to the larger, immobile macroconjugants, which resorb the microconjugants. The partners do not reciprocally exchange nuclei, as other ciliates do, rather the microconjugant, after meiosis, transfers one of its haploid nuclei to the macroconjugant's nucleus (one-way-fertilization).

Under unfavourable conditions peritrichs form free-swimming swarmers, also called telotroch larvae, as a result of which new habitats are colonized. The peristomial disc is retracted and the zooid forms an aboral ciliary wreath (telotroch) in the

rear half of the body (Fig. 2). After detachment from the stalk, the swarmer swims off by means of this ciliary wreath. The swarmer then fastens onto a suitable location with the scopula and produces a new stalk.

Chonotrichs (Cl. Phyllopharyngea, Subcl. Chonotrichia) are sessile, mostly vase-shaped ciliates with a complicated, cone-shaped perioral region. In this region, which leads to the cytostome, short cilia are arranged in spiral rows. Ciliated swarmers are generated by unequal division of the cell or by exogenous budding, as in *Spirochona gemmipara*. Most of the approximately 130 known species live as epizoa on marine crustaceans. *Spirochona gemmipara* is the only freshwater species in Europe and lives on *Gammarus* (Fig. 3).

Mature suctorians (Cl. Phyllopharyngea, Subcl. Suctoria) are non-ciliated, and most species have a stalk attached to various substrates. Few live in the plankton of lakes and rivers, and several loricate species are epibionts of aquatic insects, such as dytiscids, hydrophilids and corixids. The outstanding feature of the suctorians is the contractile tentacles that may be arranged in fascicles or spread over the surface. Each tentacle ends in a knob, which contains haptocysts to immobilize and hold the ciliate prey, from which the cytoplasm is "sucked" into the suctorian body. For reproduction, ciliated swarmers without tentacles are generated by endogenous or exogenous budding.

PRACTICAL TECHNIQUES

Epizoic peritrichs are commonly, and worldwide, found on *Gammarus* (Fig. 5). The richest epizoic ciliate community occurs in moderately or critically polluted rivers and brooks. By turning over stones or stripping over aquatic plants with a dip-net, one can capture plenty of gammarids.

For observation, large, dark (not recently moulted) specimens should be used. They should be killed in a watch-glass by pinching off the head with two sharp forceps. Legs, mouth parts, coxal plates and gills are separated from each other using two dissecting needles. These procedures must be done in water from the sampling site and the water should be changed several times because most epizoans do not like water containing too much debris from the host. For microscopic examination, freshly prepared parts of *Gammarus* must be used. A stereomicroscope is necessary for the preparation and subsequent transfer of the isolated body parts onto microscope slides. A stereomicroscope also helps to recognize large colonies of *Epistylis anastatica* or *Zoothamnium gammari* on the host's back. When preparing slides, one should make sure that the ciliates are oriented toward the coverslip. The nucleus can be stained with methyl-green-pyronin (p. 196) after live observation. To locate the ciliates, a magnification of about x 100 is sufficient; for more precise observations a magnification of x 500 is necessary.

GENERA AND SPECIES

Epistylis *spp.* Subcl. Peritrichia, O. Sessilida, Fam. Epistylididae (Fig. 6 – 8)

Epistylis forms dichotomously branched colonies which cannot contract because the stalks lack myonemes. The zooids are often barrel-shaped and have a thickened

peristomial collar. Three species of *Epistylis* live on gammarids. *E. kolbi* forms colonies with 5–10, rarely up to 40 zooids (Fig. 6). The stalk of this species always shows distinct transverse folds. While *E. kolbi* lives mainly on the leg bristles, *E. sommerae* colonizes the leg surface (Fig. 7). Colonies of *E. sommerae* contain only up to eight zooids, and the secondary stalks are very short. *Epistylis anastatica* is attached to the outer surface of the coxal plates and on the back of *Gammarus* (Fig. 8). This species forms large colonies with smooth stalks.

Opercularia protecta Subcl. Peritrichia, O. Sessilida, Fam. Epistylididae (Fig. 9)

In contrast to the genus *Epistylis*, *Opercularia* does not have a thickened peristomial collar. *O. protecta* is the only representative of this genus found on gammarids. It colonizes the leg bristles and usually forms colonies with 2–12 zooids tapering proximally. The peristomial margin is crenulate and the buccal cavity conspicuously wide.

Carchesium dipneumon Subcl. Peritrichia, O. Sessilida, Fam. Vorticellidae (Fig. 10)

All species of *Carchesium* form colonies and have a helically contracting myoneme interrupted at the branching sites; thus, each zooid can contract individually. *Carchesium dipneumon* is found between the antennae, on the inside of the coxal plates, and also at the base of the gills and legs of *Gammarus*. Depending on the site, the shape of the zooids and stalks can vary considerably; lone zooids are frequent and can be mistaken for a *Vorticella*. A long nucleus reaching the peristomial disc and a short, transverse oral cavity underneath of which two contractile vacuoles alternately pulse are common to all of these ecotypes. The two contractile vacuoles are important for identification, but are difficult to recognize.

Intranstylum rhabdostyla Subcl. Peritrichia, O. Sessilida, Fam. Zoothamniidae (Fig.11)

In contrast to the non-contractile epistylidids and the helically contracting vorticellids, the zoothamniids have a myoneme which contracts in a zigzag. However, the ability to contract is reduced in many epizoic species and often restricted to a nodding of the zooid or stalk. This is typical for *Intranstylum rhabdostyla* which, with a zooid length of 30–40 µm, is the smallest peritrich species found on *Gammarus*. One usually finds *I. rhabdostyla* on the bristles of the extremities, especially those of the gnathopods. Apart from the small size, the doubled peristomial collar is the most characteristic feature of this species (Fig. 11, arrowheads).

Pseudocarchesium steini Subcl. Peritrichia, O. Sessilida, Fam. Zoothamniidae (Fig. 12)

This is the most common species found on *Gammarus*, where it colonizes only the gills.The colonies are usually composed of two to eight zooids. In polluted waters, larger colonies, which also occupy other body parts (legs, coxal plates), have been observed. The myoneme corresponds to that of *Carchesium*, namely, it is interrupted

at the branching sites, but the stalk does not contract. Frequently, the stalk is overgrown with bacteria, making the myoneme unrecognizable. A further *Pseudocarchesium* species, *P. ovatum* colonizes the basal peduncles of the pleopods, between the two flexible rami.

Zoothamnium *spp.* Subcl. Peritrichia, O. Sessilida, Fam. Zoothamniidae (Fig.1, 13)

The *Zoothamnium* stalk contracts zigzag-like. In contrast to *Pseudocarchesium*, the myoneme is not interrupted at the branching sites (Fig.1). *Z. affine* lives on the legs of *Gammarus* and, like *Epistylis kolbi*, prefers the bristles. The colonies can have up to 30 zooids. A particularly striking trait of this species is the large contractile vacuole in the discus (Fig.13, arrow). The zooids have a length of 50–70 µm and the entire colony can be as long as 200 µm.

A further species, *Zoothamnium gammari* colonizes the outside of the coxal plates and the back of *Gammarus*. This species is identified by its doubled peristomial collar (Fig.1). *Z. gammari* can form very large colonies (up to 1.3 mm) visible to the naked eye as a mould-like coat. The same is true for *Epistylis anastatica* which, however, can be easily distinguished from *Z. gammari* by the lack of a stalk myoneme.

Ruthiella gammari Subcl. Peritrichia, O. Sessilida, Fam. Rovinjellidae (Fig.14)

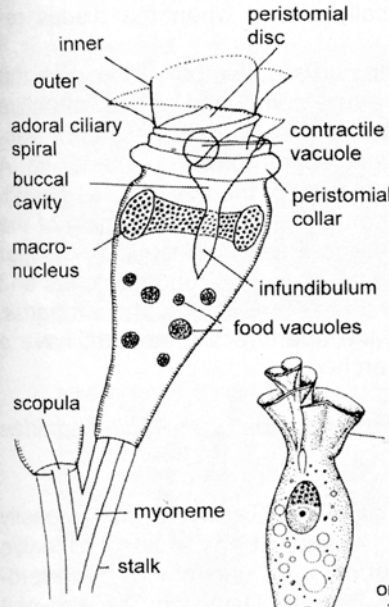
With regard to site specificity, *Ruthiella gammari* is the most specialized peritrich found on *Gammarus*. It colonizes only the underside of the labium (lower lip) and is characterized by the accordion-like contracting myoneme and the lorica. Up to 20 zooids live in a cone-shaped, very hyaline lorica, the stalk of which is bent in such a way that the ciliary spirals of the zooids face the frontal edge of the labium. Each zooid possesses a short, isolated myoneme with which it can withdraw into the lorica. Contrary to the spirally contracting myonemes of the Vorticellidae and the zigzag-like ones of the Zoothamniidae, the myoneme of the Rovinjellidae contracts accordion-like, that is, it shortens in a linear manner, producing transverse folds on the stalk, which remains straight. The peristome is opened only slightly during feeding. The short and curved nucleus is in the rear half of the zooid (Fig.14, arrows).

Lagenophrys *spp.* Subcl. Peritrichia, O. Sessilida, Fam. Lagenophryidae (Fig. 15, 16)

Lagenophrys differs greatly from other peritrichs because it is stalkless and lives in a flattened lorica that is attached directly to the substrate. At the apical end of the lorica is a short tube, consisting of two lips, through which the discus extends during

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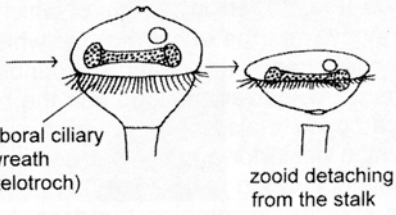
Fig. 1. *Zoothamnium gammari*, length of zooid 85 µm. Oral apparatus with doubled peristomial collar. **Fig. 2.** Formation of a swarmer. **Fig. 3.** *Spirochona gemmipara*, length approximately 100 µm. **Fig. 4.** *Dendrocometes paradoxus*, height of cell 50 µm. **Fig. 5.** Organization of *Gammarus* and locations of epizoic ciliates. P1 – P7: Pereiopods; P1 – P2: Subchelate gnathopods; P3 – P4: Walking legs, directed forward; P5 – P7: Walking legs, directed backward; Pp1 – Pp3: Swimming legs (pleopods), each consisting of a basal peduncle and two flexible rami; Up1 – Up3: Uropods.



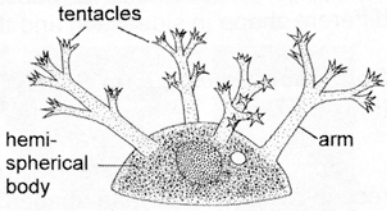
1
Organization of a peritrichous ciliate
(***Zoothamnium gammari***)



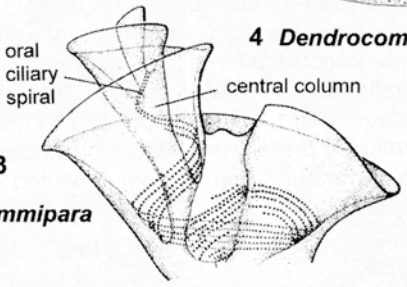
2 Formation of a swarmer

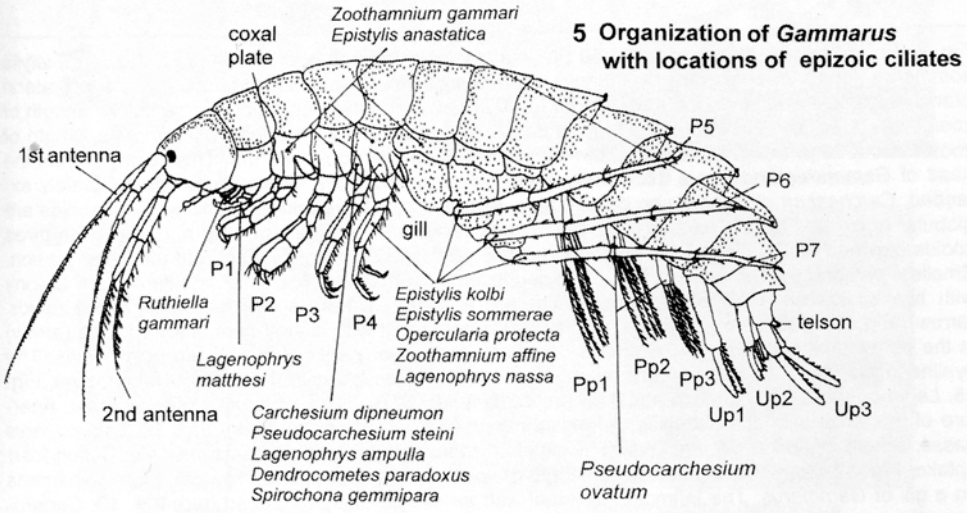


3
Spirochona gemmipara



4 *Dendrocometes paradoxus*





5 Organization of *Gammarus* with locations of epizoic ciliates

feeding. This double-lipped opening is automatically closed when the discus retracts.

Three *Lagenophrys* species are found on *Gammarus: L. ampulla* occupies the faces of the gills. The lorica is circular and the aperture composed of five refractive bands (Fig. 15, arrowheads), of which the three shorter ones form the posterior (facing away from the substrate) lip, while the two longer bands form the anterior lip. A crescent-shaped thickening surrounds the aperture in front of the lips (Fig.15, arrow). *L. nassa* colonizes the legs and the back of *Gammarus* (Fig.16). The opening of the lorica consists also of an anterior and posterior lip. The anterior lip is slightly longer than the posterior and finely crenulate, while the posterior lip has rough notches and a distinct incision at its edge. *L. matthesi* lives only on the maxillipeds of *Gammarus*. This species resembles *L. nassa*, but the lips of the aperture are smooth, have a different shape in side-view, and the lorica is more arched.

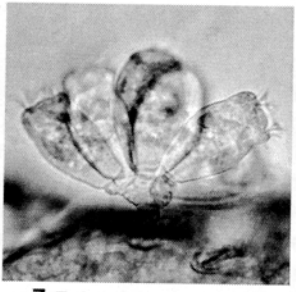
Spirochona gemmipara Subcl. Chonotrichia, O. Exogemmida, Fam. Spirochonidae (Fig. 3, 17)

Spirochona gemmipara lives on the margin of the gills of *Gammarus* and is easily recognizable by the vase-shaped body about 100 µm long. It has a small adhesive disc, but lacks a contractile vacuole. The macronucleus of chonotrichs is heteromerous, which means composed of two parts (DNA-rich and DNA-poor respectively) with different staining capacities. Members of the ciliate order Cyrtophorida have the same type of nucleus, and are thus supposed to be the closest relatives of the Chonotrichia. *S. gemmipara* prefers unpolluted or moderately polluted waters and is thus usually absent on gammarids from strongly polluted sites.

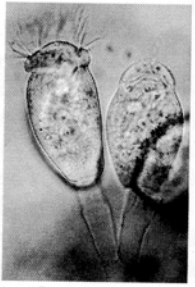
Fig. 6. *Epistylis kolbi*, length of zooid 50 µm. Colony on a leg bristle of *Gammarus*. **Fig. 7.** *Epistylis sommerae*, length of zooid 50 µm. Colony with a short main stalk, which produces very short lateral branches; zooids always densely crowded. **Fig. 8.** *Epistylis anastatica*, part of a large colony, length of zooids about 75 µm. **Fig. 9.** *Opercularia protecta*, part of a colony on a leg of *Gammarus*, length of zooids about 90 µm. **Fig. 10.** *Carchesium dipneumon*, length of zooids 80 µm. This colony is from the head of *Gammarus* and has a thick stalk and barrel-shaped zooids. The stalk is not completely extended. *Carchesium dipneumon* on coxal plates, legs and gills have thinner stalks and the zooids are globular or ovoid. **Fig. 11.** *Intranstylum rhabdostyla*, length of zooids about 40 µm. Colony with three zooids between the bristles of the gnathopods. The peristomial collar is widened in the lower portion. Smallest peritrich on *Gammarus*. **Fig. 12.** *Pseudocarchesium steini*, length of zooids 45 µm. Colony with two zooids on a gill of *Gammarus*. The aboral ciliary wreath is near the middle of the zooids (arrow). **Fig. 13.** *Zoothamnium affine*, single 60 µm long zooid with dilated contractile vacuole (arrow) in the peristomial disc. **Fig. 14.** *Ruthiella gammari*, large colony with about 50 µm long zooids. The hyaline lorica cannot be seen on this micrograph. Note macronucleus in rear half of cell (arrows). **Fig. 15.** *Lagenophrys ampulla*, lorica about 65 µm in diameter. Dorsal view of a lorica of a swarmer. Aperture of the lorica with characteristic, refractive bands (arrow and arrowheads). **Fig. 16.** *Lagenophrys nassa*, length of lorica 75 µm. Lateral view of a zooid with extended peristomial disc during food uptake. **Fig. 17.** *Spirochona gemmipara*, length of specimens approximately 100 µm. Eight specimens on a gill of *Gammarus*. The leftmost individual with an exogenously produced bud. **Fig. 18.** *Dendrocometes paradoxus*, height of hemisperical body 50 µm. This specimen has two arborescent extensions with finger-like tentacles. (Fig. 3 from Grell 1968)



6 *Epistylis kolbi*



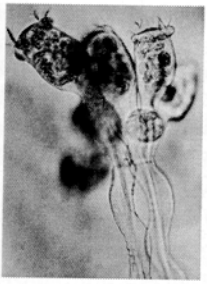
7 *Epistylis sommerae*



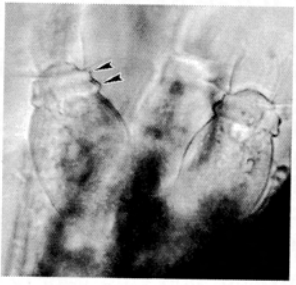
8 *Epistylis anastatica*



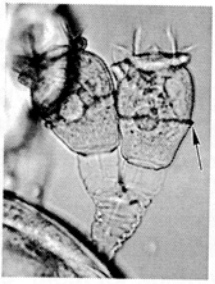
9 *Opercularia protecta*



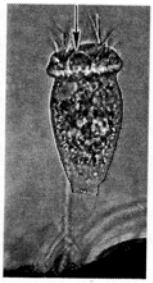
10 *Carchesium dipneumon*



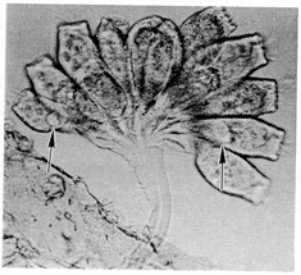
11 *Intranstylum rhabdostyla*



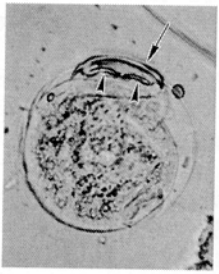
12 *Pseudocarchesium steini*



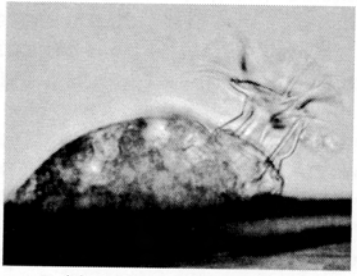
13 *Zoothamnium affine*



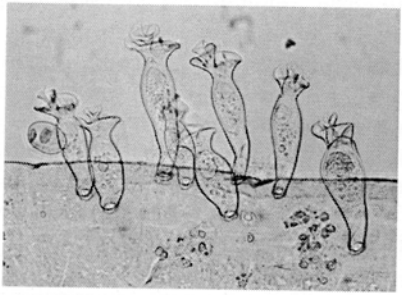
14 *Ruthiella gammari*



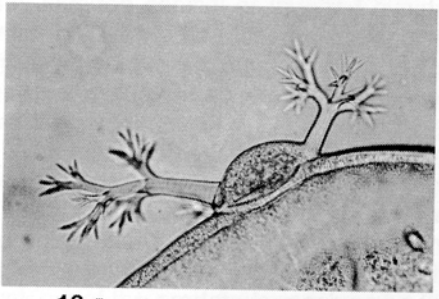
15 *Lagenophrys ampulla*



16 *Lagenophrys nassa*



17 *Spirochona gemmipara*



18 *Dendrocometes paradoxus*

Dendrocometes paradoxus Subcl. Suctoria, O. Evaginogenida, Fam. Dendrocometidae (Fig. 4, 18)

On gammarids, species of the genera *Acineta*, *Tokophrya* and *Podophrya* may occasionally sit on the bristles of the legs. But the most abundant and characteristic species is *Dendrocometes paradoxus*, which colonizes the gill plates (Fig.18). This species differs greatly from the typical suctorian appearance in that it is hemispherical and lacks stalk and ordinary tentacles, which are modified to branched, arm-like extensions of the body and used for prey capture and ingestion. *Dendrocometes* feeds on mobile ciliates and swarmers, which are immobilized by the haptocysts in the tips of the arms.

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Matthes, D. (1982): Sesshafte Wimpertiere. Peritricha, Suctoria, Chonotricha. Neue Brehm-Bücherei. A. Ziemsen, Wittenberg – Lutherstadt. 120 pp.

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Oligotrich Planktonic Ciliates

Sabine Agatha

INTRODUCTION

The plankton of lakes, oceans, and large rivers comprises a wide variety of microorganisms that cannot counteract water currents. The bacterioplankton utilizes dissolved organic matter produced by the phytoplankton (mainly diatoms) and is itself ingested by nanoplanktonic (2–20 µm in size), heterotrophic flagellates such as bodonids, choanoflagellates, cryptophytes, chrysophytes, and dinoflagellates. Autotrophic and heterotrophic nanoflagellates are in turn preyed upon by the microzooplankton (20–200 µm in size), including copepod nauplii, heterotrophic dinoflagellates and different kinds of oligotrichs, many of which also feed directly on bacteria. All these organisms belong to the so-called "microbial loop" that supplies some energy to the conventional phytoplankton-based planktonic food web, because they are consumed by metazoan plankton such as the omnivorous copepods and fish larvae (Azam et al. 1983). Besides the heterotrophic nutrition mode, several planktonic ciliates retain the plastids of their prey and "cultivate" them for some weeks in their cytoplasm: such species are called mixotrophs. The dominating and most characteristic planktonic ciliates belong to the Superclass Spirotricha of the Phylum Ciliophora, Class Oligotrichea ("oligotrichs") which comprises the orders Halteriida ("halteriids"; Fig. 3, 4), Oligotrichida (Fig. 5, 6) and Choreotrichida ("choreotrichs"), with the "house-building" (loricate) tintinnids (Fig. 11 – 17) and the naked (aloricate) strobilidiids (Fig. 7 – 10).

Most members of the class (about 95%) occur in marine environments, but some are also common in freshwater lakes. They occur mainly from spring to autumn when food is most abundant (r-strategy). The highest densities are found in estuaries with maximum numbers of about 2×10^4 tintinnids and 1×10^6 other (aloricate) oligotrichs per litre. Additionally, the high growth rates, with up to four divisions per day, contribute to the importance of the oligotrichs in the energy flux.

The oligotrichs are globular, ellipsoidal, inverted conical or inverted ovoidal and cover an enormous size range from 5 µm to 1000 µm. The most striking species are the loricate tintinnids (Order Choreotrichida, Suborder Tintinnina). They are represented by about 15 extant families, 75 genera, and 1200 species in marine waters, while only about six species occur in freshwater (Fig. 11, 13 – 17). The main feature of the tintinnids is the 20 µm to 1000 µm long lorica which is vase-shaped or tubular and has a hyaline (clear), reticulated, or agglomerated (covered by environmental particles) wall. Due to the robustness of the lorica, the tintinnids left fossils, ranging from the Ordovician period (400–450 million years ago) into the Pleistocene (1 million years ago). It is supposed that the lorica provides some protection against predation: (i) its weight might cause the tintinnid to sink out of the predator's reach when the cell stops swimming and/or (ii) the tintinnid might abandon the lorica which is eaten or

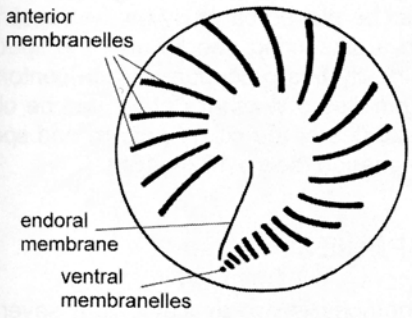
rejected by the predator, while the cell escapes. The systematics of the tintinnids is based solely on features of the lorica, as the characteristics of the cell are usually unknown. Unfortunately, the structure and shape of the lorica varies greatly according to both the environmental conditions and the stage of the life cycle (Fig. 12). Hence, the number of actual species is probably much lower than the 1200 taxa mentioned above.

A conspicuous, apical adoral zone of membranelles used for locomotion and food collection (suspension feeding) is typical of the oligotrichs; each membranelle originates from several rows of basal bodies, forming a so-called polykinetid.The arrangement of the adoral membranelles is used to separate the Halteriida and Oligotrichida which have a C-shaped zone (Fig. 1) from the Choreotrichida with a circular zone (Fig. 2). In Halteriida and Oligotrichida the zone of membranelles is clearly divided between an anterior and a ventral region, while a circle of external membranelles and some internal ones occurs in the Choreotrichida. The endoral (undulating) membrane is inconspicuous and extends across the peristomial field (oral area) in choreotrichs or along the inner side of the buccal lip in the other oligotrichs. The oral cavity opens apically in choreotrichs or ventrally in the other taxa and contains the internal (choreotrichs) or ventral membranelles (other taxa). The somatic ciliature is reduced, especially in the Oligotrichida and Halteriida. While species of the former group possess only two ciliary rows (kineties: girdle row and ventral row) with stubby cilia (Fig. 5, 6), the halteriids have conspicuous cilia that form long bristles (Fig. 3, 4). Although water is very viscose for a ciliate, halteriids and many aloricate choreotrichs are able to perform enormous jumps of up to 18-fold body length with a velocity of about 4 mm s^{-1}.

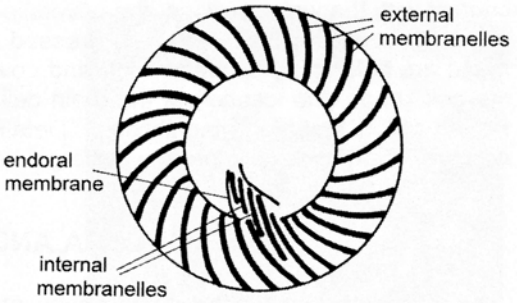
PRACTICAL TECHNIQUES

Fresh field material is usually investigated because only *Halteria* can easily be cultivated; however, the loricae of the tintinnids can also be studied in fixed material. A 20 µm-plankton net is recommended for sampling, as it provides a sufficient abundance of loricate and large aloricate species for study. The samples are taken from a pier or a boat and subsequently kept cool, preferably at the actual water temperature, as most species are sensitive to rapid temperature changes. In the laboratory the samples should be distributed between Petri dishes in which many species will accumulate near the water surface. It is strongly recommended that the fresh material should be investigated soon after collection. The behaviour of the ciliates is initially studied in the Petri dish under a stereomicroscope. Then one or more specimens are picked up with a fine pipette and put on a microscope slide. For the following steps, a microscope with an oil immersion objective and, preferably, interference contrast optics should be used. At low magnification (×100), size, shape, and general organization are investigated. Now four minute dabs of vaseline are arranged around

Fig. 1, 2. Top views showing the shapes of the adoral zone of membranelles in the Halteriida and Oligotrichida (1) and in the Choreotrichida (2). Each curved line represents the densely arranged basal bodies of the cilia constituting a membranelle.The endoral membrane extends into the buccal cavity and consists of a single row of ciliated basal bodies. **Fig. 3, 4.** *Halteria grandinella* (20–40 µm), ventral views, from life and in the scanning electron microscope. **Fig. 5, 6.** *Strombidium capitatum* (35–85 × 35–70 µm), ventral views, after silver impregnation and in the scanning electron microscope.

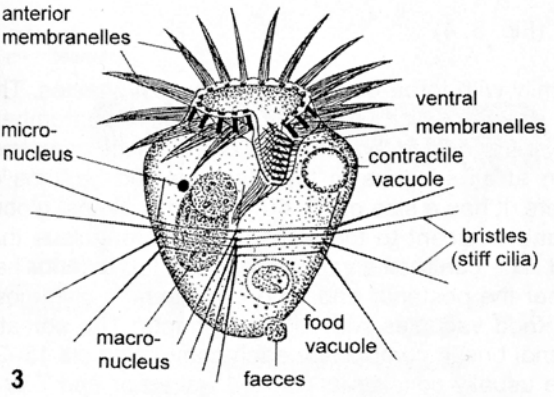


1 Halteriida and Oligotrichida



2 Choreotrichida

Shapes of adoral zone of membranelles
(1, 2)

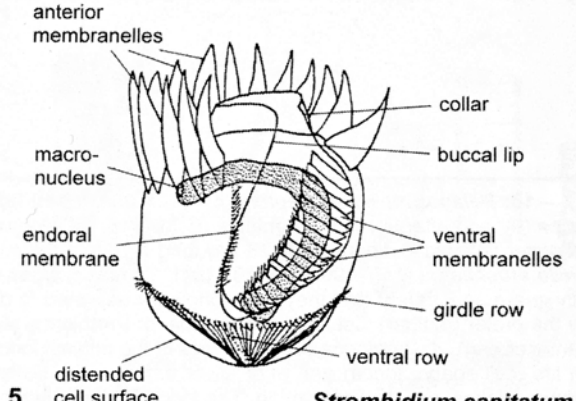


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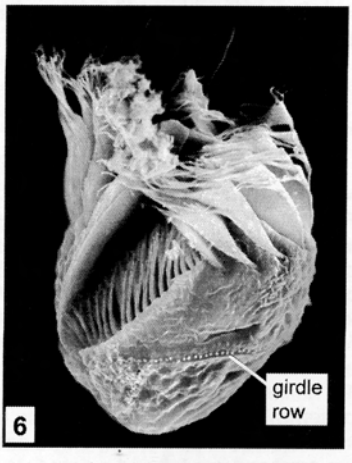


4

Halteria grandinella
(3, 4)



5



6

Strombidium capitatum
(5, 6)

the drop. Since aloricate specimens are very fragile and are easily damaged, even by contact with the water surface, the coverslip must be placed carefully on the vaseline dabs. The coverslip is now gently pressed with a dissecting needle until the specimens are held firmly between slide and coverslip. By this procedure, which contorts the cell shape, the location of the main cell organelles and other details can be observed at low and high magnification. Detailed studies of the ciliary pattern and species identification usually require protargol impregnation (see p.162 – 165).

GENERA AND SPECIES

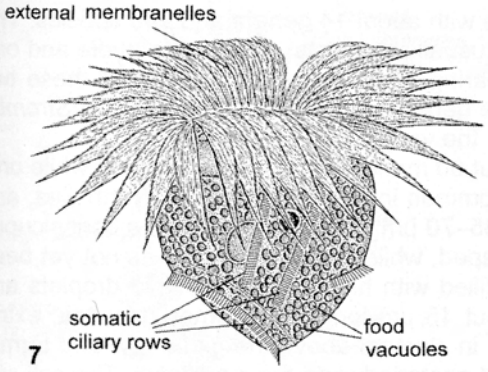
A complete key for the identification of oligotrichs is not available, but several monographs are recommended: Foissner et al. (1999) for planktonic ciliates from freshwater; Kahl (1932), Maeda & Carey (1985), and Maeda (1986) for aloricate oligotrichs; and Kofoid & Campbell (1929) for tintinnids. Unfortunately, none of these includes the more recent data.

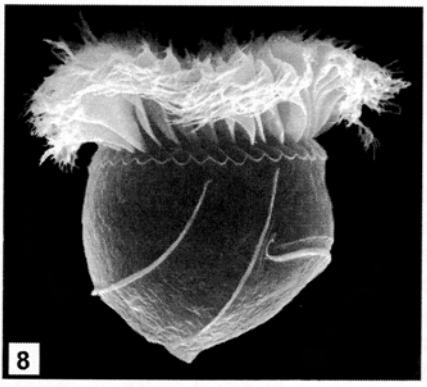
Halteria grandinella O. Halteriida (Fig. 3, 4)

The Halteriida comprise one family with three genera and about 20 species. The genus *Halteria* contains about nine species which are easily confused with members of the genus *Pelagohalteria*, as they differ only in the structure of the bristle complex.

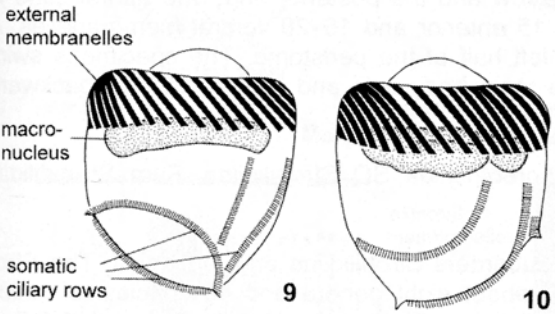
Halteria grandinella is common at all seasons in the plankton and benthos of ponds, reservoirs, and running waters. It has a size of 20–40 µm and is almost globular. The micronucleus is globular and adjacent to the ellipsoidal macronucleus that frequently shows a replication band. The contractile vacuole is in the left anterior half of the cell, while the cytopyge is near the posterior end. The cytoplasm is colourless but occasionally contains coloured food vacuoles with algal remnants. The somatic cilia are arranged in 9 or 10 equatorial bristle complexes, each comprising six 15–25 µm long, stiff cilia. The adoral zone usually consists of 15 or 16 anterior and 7 or 8 ventral membranelles.The specimens swim fast along a helical path and their course is interrupted by wide jumps. They frequently stand still or rotate on the spot for a few seconds.

Fig. 7 – 13. Strobilidiids and tintinnids. **7 – 10:** *Pelagostrobilidium neptuni* (45–60 × 45–55 µm) from life (7), in the scanning electron microscope (8), and after silver impregnation (9, 10). **11:** *Tintinnopsis cylindrata*. The empty lorica of this freshwater tintinnid is about 90 × 25 µm long and covered with particles from the environment. **12:** *Favella ehrenbergii* (145–400 x 55–135 µm). The main types of lorica in the life cycle of this marine tintinnid are so different that they were originally assigned to different families. **a, b:** Protoloricae built by the proter (mother) just after cell division. **c:** Protolorica plus epilorica (secondarily added part at the anterior end). **d:** Paralorica built after loss of the original lorica. **13:***Tintinnidium fluviatile* combined from life (cell shape, lorica) and after silver impregnation (ciliary pattern). This tintinnid is common in freshwater lakes, especially in spring. The lorica is highly variable in size (95–170 × 40–50 µm), and has mineral particles and diatom frustules attached. The ciliary rows are arranged in a left and a right field, and two special rows occur, namely the ventral organelles.

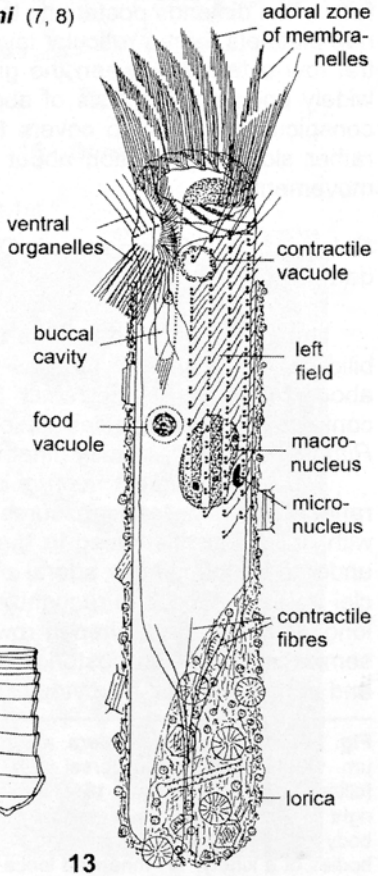




Pelagostrobilidium neptuni (7, 8)



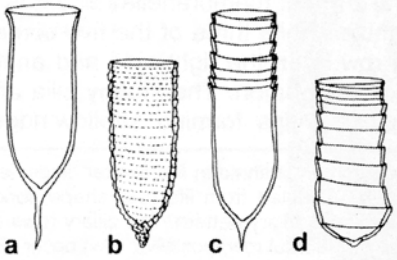
Pelagostrobilidium neptuni
after silver impregnation (9,10)



Tintinnidium fluviatile
Morphological features
13



11
Tintinnopsis cylindrata
Lorica



12 ***Favella ehrenbergii***
Types of lorica

Strombidium capitatum O. Oligotrichida, Fam. Strombidiidae (Fig. 5, 6)

The Oligotrichida contain four families with about 14 genera and 140 species. The somatic ciliature is much reduced and usually consists of only one girdle and one ventral row with paired basal bodies bearing stubby cilia. The pattern of these two ciliary rows is a diagnostic feature for the genera within the Oligotrichida. In *Strombidium*, the girdle row extends horizontally, the ventral row longitudinally.

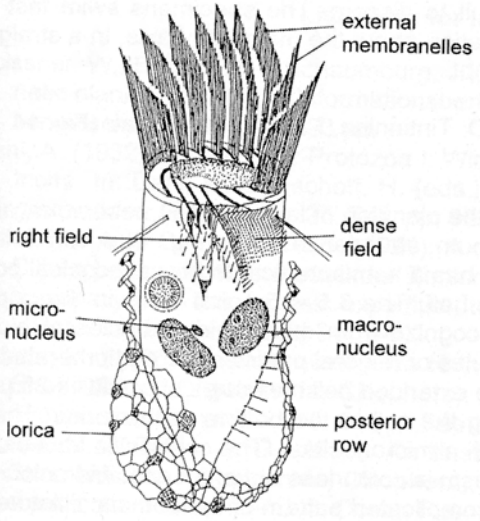
Strombidium capitatum is one of about 60 marine species of the genus, while only one species occurs in freshwater. It is common in the North Atlantic, North Sea, and Skagerrak. The cell measures 35–85 × 35–70 µm, is ovoidal, and has a conspicuous apical collar. The macronucleus is C-shaped, while a micronucleus has not yet been observed. The colourless cytoplasm is filled with highly refractive lipid droplets and food vacuoles. The needle-shaped, about 15 µm long, extrusomes (trichites, extrusive organelles, not shown) are inserted in clusters above the girdle row and form a distinct funnel by extending obliquely and posteriorly into the cytoplasm. The cell surface often distends posteriorly to the girdle row, where minute polygonal polysaccharide platelets form a reticular layer. The girdle row is below mid-body. The short ventral row extends between the girdle row and the posterior end. The adoral zone is widely open and consists of about 15 anterior and 16–20 ventral membranelles. A conspicuous buccal lip covers the left half of the peristome. The specimens swim rather slowly by rotation about the main body axis and escape by fast backward movements.

Pelagostrobilidium neptuni O. Choreotrichida, SO. Strobilidiina, Fam. Strobdilidiidae (Fig. 7 –10)

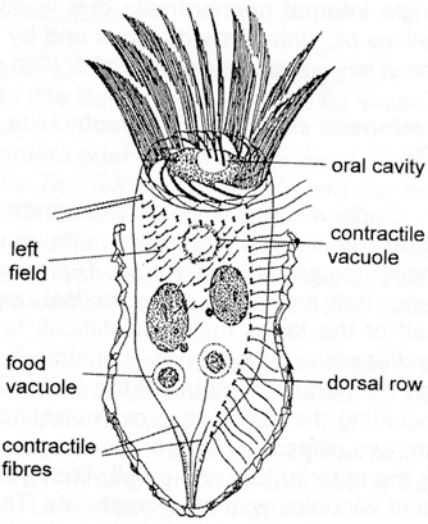
The Choreotrichida include the suborders Strobilidiina and Tintinnina. The Strobilidiina comprise four families with about eight genera and 45 species, of which about 24 belong to the family Strobilidiidae. The marine genus *Pelagostrobilidium* contains only six species which are easily confused with members of the genus *Rimostrombidium*, as they differ only in the course of the somatic ciliary rows.

Pelagostrobilidium neptuni is common in the North Sea, Irish Sea, and Mediterranean Sea. The cell measures 45–60 × 45–55 µm and has a subspherical shape with an oblique flattening in the left posterior half. The C-shaped macronucleus is underneath the circular adoral zone of membranelles and has attached two micronuclei that are difficult to recognize. While three of the five somatic ciliary rows extend longitudinally, one L-shaped row is on the right side and another row extends in a semicircle in the left posterior cell portion. The stubby cilia are very closely spaced and bent leftwards by cytoplasmic lips forming shallow ridges along the rows. The

Fig. 14 – 17. *Codonella cratera*, a common tintinnid in freshwater lakes; length of the lorica about 50 µm. **14, 15:** Ventral and dorsal view combined from life (cell shape, lorica) and silver-impregnated (ciliary pattern) specimens. **16:** Diagram of ciliary pattern. The ciliary rows are arranged in a left and a right field, and special rows (ventral row, dorsal row, posterior row) occur. Each dot represents a basal body, each dot with dash symbolizes a ciliated basal body, and the longitudinal lines connect the basal bodies of a kinety. **17:** Inhabited lorica (scanning electron microscope). Only the membranelles of the contracted cell are recognizable. Mineral particles and diatoms are attached to the lorica. (Fig. 1, 2 after Fauré-Fremiet 1970; Fig. 3, 4, 11, 13 – 17 after Foissner et al. 1999; Fig. 5 after Montagnes et al. 1988; Fig. 12 after Laval-Peuto & Brownlee 1986)

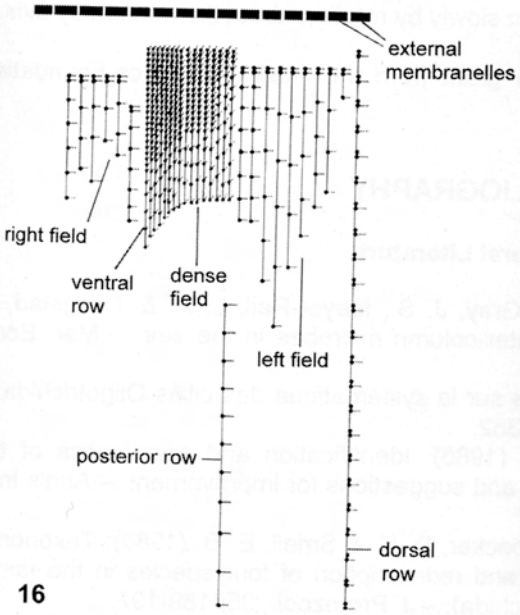


14 Ventral view



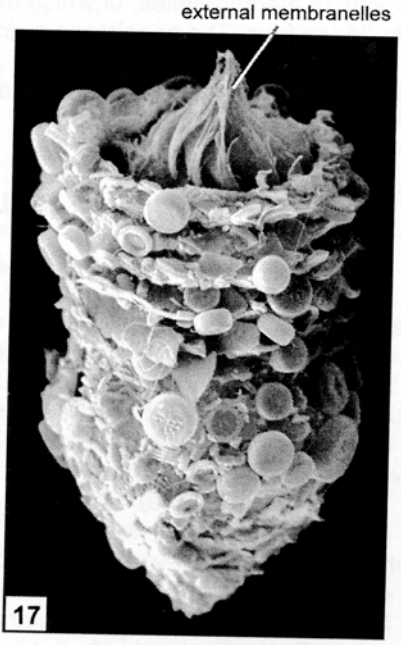
15 Dorsal view

Codonella cratera
Morphological features (14,15)



16

Diagram of ciliary pattern



Specimen within lorica

Codonella cratera (16,17)

zone usually consists of 36 external membranelles with cilia up to 30 µm long and a single internal membranelle that is difficult to discern. The specimens swim fast in helices or, over some distance and by rotation about the main body axis, in a straight line. They escape by means of a wide jump.

Codonella cratera O. Choreotrichida, SO. Tintinnina, Fam. Codonellidae (Fig. 14 – 17)

Codonella cratera is very common in the plankton of lakes, ponds, reservoirs, and large, slowly running rivers with maximum abundance in spring. The stiff lorica measures about 50 × 40 µm. Typically, it has a hemispherical to broadly conical posterior half and a tubular or helical upper half. The 3.5–4.5 spiral turns in the upper half of the lorica might be difficult to recognize from outside, as particles of biotic and/or abiotic origin such as diatom frustules or mineral particles are agglomerated to the membranous matrix of the lorica. The extended cell measures about 70 × 25 µm, including the stalk-like process anchoring the cell to the bottom of the lorica. There are two ellipsoidal macronuclei, each with a micronucleus. The contractile vacuole is in the anterior half of the cell. The cytoplasm is colourless but often contains coloured food vacuoles with algal remnants. The complicated pattern of the somatic ciliature is typical of a wide variety of marine tintinnids. There are 29–32 ciliary rows arranged in three fields (right field, left field, and dense field) and three special rows (ventral row, dorsal row, and posterior row), as shown in figure 16. The adoral zone is comprised of about 15 membranelles, of which three extend into the oral cavity; the oral cilia are up to 35 µm long. The specimens swim slowly by rotation about the main body axis.

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Paramecium caudatum

Klaus Hausmann

INTRODUCTION

Paramecium caudatum is a "classic" in teaching and protozoological research due to the ease with which it is cultivated and handled (Fig. 1). Basic facts about ciliates, including the nuclear events during mitosis and conjugation, the ultrastructure of the cortex, ciliary movement, and the processes of food ingestion and digestion were first described in *Paramecium*. Moreover, *Paramecium* has served as a model organism in cell biological research for elucidating general phenomena such as endocytosis, induced exocytosis, membrane fusion, cell to cell and prokaryote to eukaryote interactions, to mention only a few facets.

The genus *Paramecium* belongs to the subphylum Intramacronucleata (division spindle within nucleus) of the phylum Ciliophora, and herein to the class Oligohymenophorea, subclass Peniculia (Lynn & Small 2000). Formerly, *Paramecium* was classified in the now outdated Holotricha because it is completely ciliated, a feature which is especially evident in the scanning electron microscope (Fig. 2).

CULTURE AND PREPARATIONS

Natural, reliable sources of *Paramecium* are the mud of permanent ponds and aggregations of *Sphaerotilus*, a filamentous bacterium which forms chains of cells living within an extracellular, slimy sheath. *Sphaerotilus* is easily found in heavily contaminated rivers. According to current knowledge, *Paramecium* is unable to form cysts; consequently, it cannot be found in hay infusions set up with tap water, in spite of frequent references in the literature to the contrary.

Cultures can be established as follows: A large Petri dish is filled with 50 ml tap water supplemented with 2–3 crushed wheat grains. The wheat grains serve as a nutrient source for bacteria, the preferred food of *Paramecium*. One teaspoon of pond mud or *Sphaerotilus* aggregation are added. After a few days, a mass of different protists will occur, including some *Paramecium* specimens. From this raw culture, some ciliates are isolated under a stereomicroscope using fine pipettes, and then transferred to a 10 cm Petri dish that has been prepared with wheat grains as described above. A dense population of paramecia should develop after a week or two.

The usually fast-swimming *Paramecium* cells can be slowed down for easier microscopical observation by adding to the microscope slide some particles of mud, or cellulose fibres from cotton or filter paper. The ciliates then swim more slowly due to the numerous obstacles and tend to rest thigmotactically among the filaments. The use of highly viscous media, such as methyl cellulose or Protoslow to retard the ciliates should be avoided because the cells become deformed, resulting in distorted swimming patterns.

ANATOMY

The vernacular name "slipper animalcule" refers to the shape of *Paramecium caudatum*. The typical shape is best seen when it is not compressed by a coverslip (Fig. 1). At low magnification the two transparent contractile vacuoles with their radially arranged ampullae and pulsating contractions are obvious (Fig. 3, 9). The contractile vacuoles have an osmoregulatory function. They slowly fill with water (diastole) which is periodically expelled into the environment through rapid contraction of the vacuole (systole). Water flows continuously into the cell due to the osmotic gradient between the cytoplasm and the surrounding medium. In the absence of contractile vacuoles the cell would burst due to the continuous influx of water.

The contractile vacuole complex (CVC) of *Paramecium* is composed of the central contractile vacuole, a pore and ampullae with collecting canals (Fig. 9, 10). The latter are surrounded by a tubular network, called spongiome, that is not visible under the light microscope. The periphery of the spongiome is connected to bundles of stiff, hexagonally packed tubules which are coated with mushroom-shaped, helically arranged proton pumps (V-ATPases) on their cytoplasmic face (only visible in TEM peparation). It is assumed that these are the elements by which the water is actively segregated from the cytoplasm in a process which is still not completely understood. The CVC is supported and held in position by microtubule bundles that originate in the wall of the excretory pore. The microtubules extend along the ampullae and pass to the very end of the collecting canals (Fig. 10).

The cycle of the CVC of *Paramecium* starts with the contraction of the spherical vacuole (systole) at the periphery of the cell and the expulsion of its contents through the pore and into the surrounding medium. At this moment, the contractile vacuole is not visible under the light microscope (Fig. 11). It reverts into one or a few continuous masses of interconnected tubules visible only with the electron microscope. When the ampullae are filled during diastole the contractile vacuole becomes visible again (Fig. 12 – 14). The pulsation cycle lasts about eight seconds in *Paramecium caudatum*. As far as is currently known, the force for fluid expulsion is generated by internal tensions of the involved membranes of the CVC, and not by one of the better-known force generation systems such as actin-myosin complexes.The macronucleus – also visible with bright field illumination – is in the middle of the cell and appears (particularly after staining with methyl green) as a reniform structure. The ellipsoidal micronucleus, which lies in a depression of the macronucleus, is difficult to recognize without staining.

Paramecium has a characteristic shape, as have all ciliates. The morphological basis for this is the complex cortex. Although most of the details of the cortex can be seen only with an electron microscope (Fig. 5), certain staining procedures (see below) allow some of these structures to be recognized even with the light microscope (Fig. 4, 6).

The preoral part of the ventral side has a rather distinct indentation, called the peristomial groove. In this area, the cilia are arranged in such a way that food particles are swirled into the buccal cavity, which is a deep, funnel-shaped depression. At the end of this cavity is the cytostome, where food vacuoles with a diameter of about 15 µm are pinched off. The ventral side of a protist is defined by the location of its oral apparatus (Fig. 3).

The somatic ciliature becomes visible only at higher magnifications. The cilia are arranged very regularly in parallel longitudinal rows, the so-called kineties (Fig. 2, 4). The movement of the cilia is coordinated in a metachronal pattern. Details of the somatic as well as of the oral ciliature can be studied only after applying special staining procedures such as silver impregnation (see below).

Also visible only at higher magnifications are numerous spindle-shaped, 5–7 µm long organelles attached to the cortex (Fig. 3). These are resting trichocysts, that is, organelles which are membrane-bound and can be exocytotically released in a split second. This type of organelle, common to many protozoa, is generally called an extrusome. The different types of extrusive organelles have different morphologies and may differ in their function. However, they all share one feature: a wide variety of stimuli causes their more or less rapid extrusion. During this process, the size and structure of the extrusomes changes in a predictable and characteristic manner; in the case of *Paramecium* from a spindle-shaped structure to a hair-like rod eight times longer than the resting stage.

The best-known extrusomes are indeed the spindle trichocysts of *Paramecium*. Depending on body size, every *Paramecium* cell possesses 5000 to 8000 of these organelles which protect them from predators.

A massive discharge of spindle trichocysts can be induced by adding a minute drop of a saturated solution of picric acid in water to a drop of a *Paramecium* culture. Although the cells are killed almost instantly, there is still sufficient time to extrude, and thus make visible, almost all their trichocysts (Fig. 7, 8). The specimens should be air-dried to ensure that they are not washed away from the surrounding ring of trichocysts.

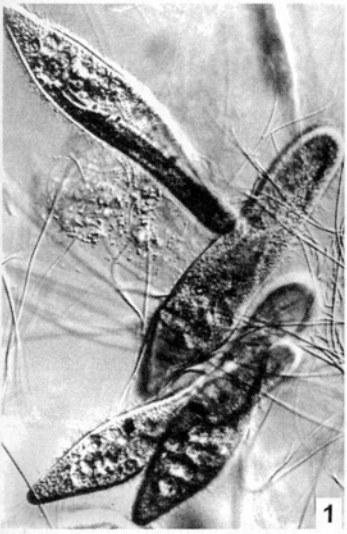
STAINING PROCEDURES

A solution of methyl green pyronin (0.1 g methyl green pyronin is dissolved in 100 ml distilled water and 1 ml glacial acetic acid added) can be used as a simple but reliable nuclear stain for *Paramecium* cells. Fixation of the cells and staining of their nuclei occurs simultaneously when a drop of this solution is added to a drop of a dense culture (methyl-green-pyronin is Chroma article no. 1A 560, www.chroma.de).

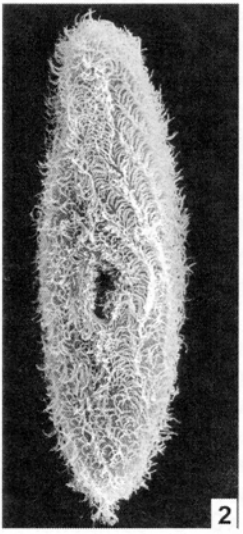
Opal blue is useful for studying the surface anatomy of *Paramecium*. A small spatula tip of opal blue powder is dissolved in 2 to 3 ml water. A minute drop of a dense *Paramecium* culture and a very small drop of opal blue solution are mixed on a microscope slide and spread to a thin film which should appear in a royal blue colouration. After air drying and embedding in immersion oil or artificial resin, the preparation can be observed with brightfield illumination.

The surface of a *Paramecium* cell can be best compared with the surface of a waffle which is subdivided by longitudinal and transverse elevations.The staining effect is due to the impregnation of very regularly arranged indentations of the cortex

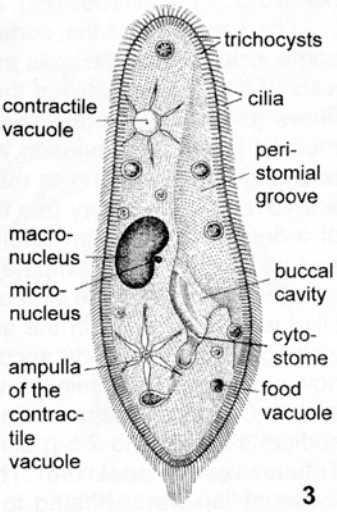
Fig. 1 – 8. Appearance of *Paramecium caudatum* (size of live specimens 180–300 µm). **1**: Overview in bright field microscopy. **2**: Ciliature of ventral side in scanning electron microscopy. **3**: Anatomy. **4**: Silverline pattern after dry silver nitrate impregnation. **5**: Three-dimensional reconstruction of cortex. **6**: Waffle-like surface pattern after opal blue staining. **7**: Massive discharge of trichocysts after addition of saturated picric acid. **8**: Trichocyst tips in scanning electron microscopy. (Fig. 1, 2, 4, 8 courtesy of W. Foissner; Fig. 3 after Grell 1973)



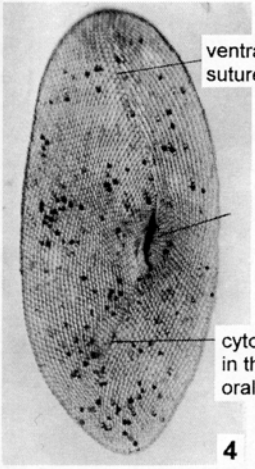
Appearance in bright field illumination



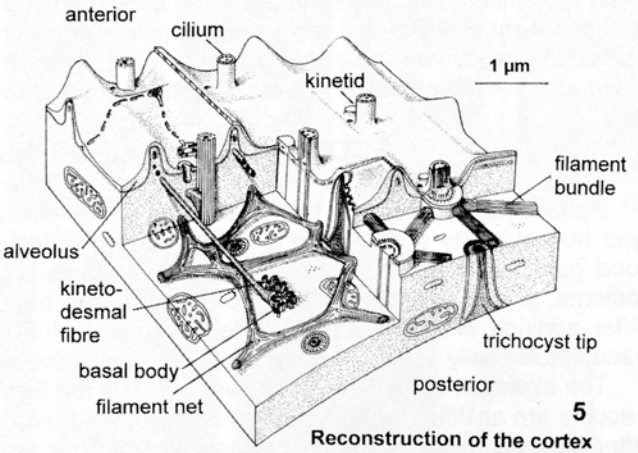
Ciliature (SEM)



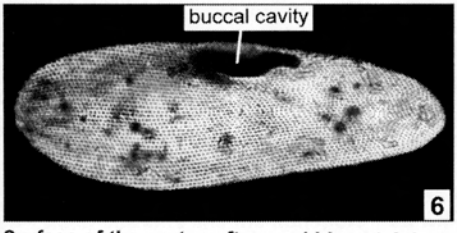
Scheme showing the organelles visible by light microscopy



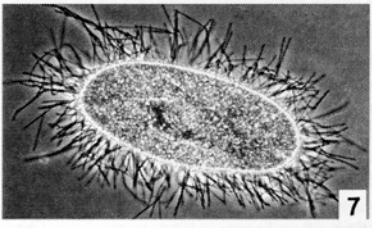
Silverline pattern



Reconstruction of the cortex



Surface of the cortex after opal blue staining





Extruded trichocysts, phase contrast (7), SEM (8)

located between the elevations.The indentations correspond to the ciliary organelles (kinetids) of the kineties (Fig. 5, 6).

The elements of the cortex are collectively called the infraciliature and they become visible when the cells are treated with silver compounds. The infraciliature consists of the basal bodies of the cilia, the cortical microfilaments and the kinetodesmal fibres as well as certain microtubules and microtubule ribbons. A routine staining method is the impregnation with silver nitrate, revealing the so-called silverline pattern (Fig. 4). According to the protocol of this procedure, several microscope slides are covered with a very thin film of egg white (applied with a fingertip). Then, a drop of a dense *Paramecium* culture is put on each prepared slide, spread with a needle, and air dried at room temperature (dry silver impregnation!). The dried slides are now covered for one minute with a 1 % aqueous solution of silver nitrate, rinsed with distilled water and dried in the air at room temperature. They are now exposed to a 40 watt light bulb for 30–60 seconds at a distance of about 5 cm. The preparations are now covered for one minute, with a reduction mixture composed of components A, B and C. Component A consists of 10 g boric acid, 10 g borax, 5 g hydroquinone, 100 g sodium sulphite and 2.5 g ultrafin liquid (a photographic developer, manufactured by Tetenal: www.tetenal.com). These substances are dissolved in the given sequence in 1 litre of tap water heated to 40° C. Component B is a highly concentrated photographic developer of normal commercial usage (such as Rodinal, Agfa or R 09, Calbe Chemie: www.calbe-chemie.de). Component C is a 10 % caustic soda solution. Immediately before use, these three components are mixed in a ratio of 20 ml A + 0.5–1.0 ml B + 0.5–1.0 ml C. After application of this reduction solution for the specified one minute the slides are briefly but vigorously rinsed with tap water, air-dried again and embedded in a resin such as Euparal (www.carl-roth.de).

FOOD UPTAKE AND DIGESTION

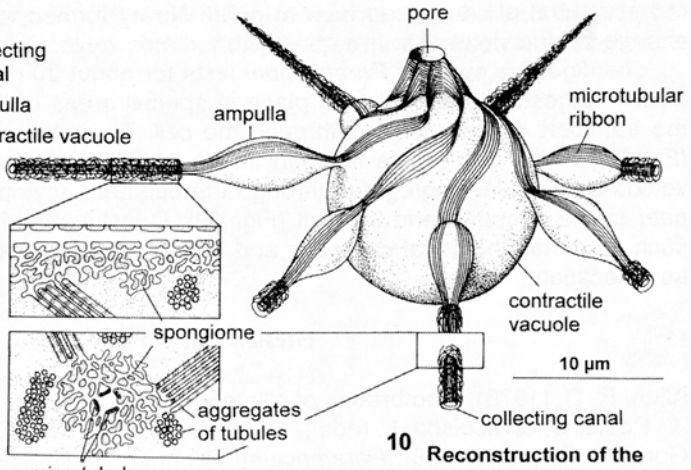
Paramecium is a typical filter feeder, using the ciliature of the peristomial groove and buccal cavity to generate a strong current of water which transports potential food particles to the buccal cavity (Fig. 17). At its proximal region, called the cytostome, a food vacuole of 15 µm diameter is pinched off about every 45 seconds. After addition of highly diluted, detergent-free (!) Indian ink, the formation of food vacuoles is easily observed due to their jet-black colouration (Fig. 18).

The digestion of food occurs in two steps. In the first step the contents of the food vacuole are acidified when so-called acidosomes fuse with the vacuoles immediately after the vacuoles have been pinched off from the cytostome. In the second step, lysosomes containing hydrolytic enzymes also fuse with the vacuole, and digestion begins in what is now called the digestive vacuole. Acidification of the vacuoles can be demonstrated by feeding the ciliates with yeast cells that have been stained with

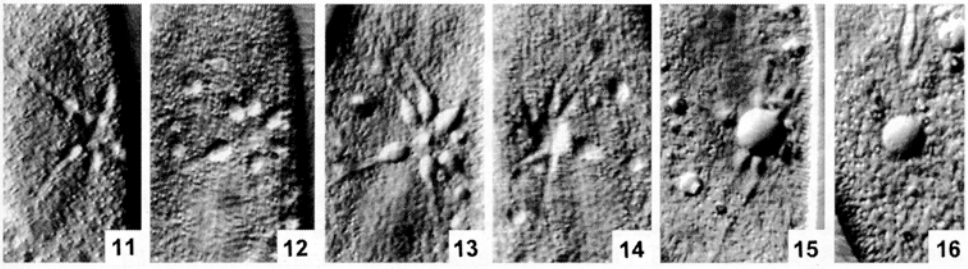
Fig. 9 – 16. Contractile vacuole complex (CVC) of *Paramecium*. **9**: Contractile vacuole in interference contrast microscopy (diameter about 15 µm). **10**: Three-dimensional reconstruction of the CVC. **11 – 16**: Cycle of CVC. **Fig. 17 – 20.** Food uptake and digestion. **17**: Long-time exposure dark field micrograph of filtering paramecia (longish white structures); the water currents produced by the oral cilia become visible by the food particles added. **18**: Food vacuoles are black after feeding with Indian ink. **19**: Defecation after feeding with Indian ink. **20**: Scheme of cyclosis of food vacuoles (arrows). (Fig. 20 after Sikora 1981)



Contractile vacuole in differential interference contrast



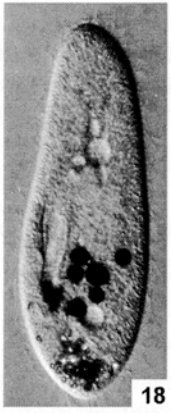
10 Reconstruction of the contractile vacuole complex



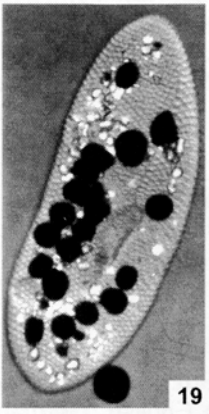
Contractile vacuole in differential interference contrast



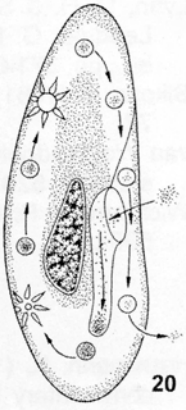
Long time exposure showing the water currents and the transport of food particles by the oral ciliature



Food vacuoles filled with Indian ink



Defecation



Cyclosis of food vacuoles

the indicator dye, Congo red (0.1 g Congo red is dissolved in 100 ml of distilled water, 1.0 g of baker's yeast is added and the suspension is briefly boiled). Congo red is red at neutral pH and deep blue at pH 3. Newly formed food vacuoles are red but change to blue-violet after five minutes.

The digestive cycle of *Paramecium* lasts for about 30 minutes under natural conditions. Digestion does not take place in special areas of the cell, but rather during the transport of the vacuoles through the cell. The latter process is called cyclosis (Fig. 20). If the ciliates are fed with Indian ink, the indigestible carbon particles are voided as globular aggregates through the cell anus (cytoproct, cytopyge), which is near to the posterior end the cell (Fig. 19). Constituents that are difficult to digest, such as certain bacterial cell walls and spores, are also expelled, a process known as defecation.

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The Rumen Ciliates

André-Denis Girard Wright

INTRODUCTION

Ciliates are the most abundant protozoa found in the rumen of both domesticated and wild ruminants, their numbers ranging from 10^4 to 10^6 per ml of rumen contents. Collectively known as rumen ciliates, they were first discovered over 160 years ago (Gruby & Delafond 1843). This agriculturally important group of ciliates is involved in their host's metabolism and the digestion of plant material (Williams & Coleman 1992). Having eaten some of the ruminants food , the ciliates themselves are digested further along the digestive tract, and they thus become food for the ruminant.

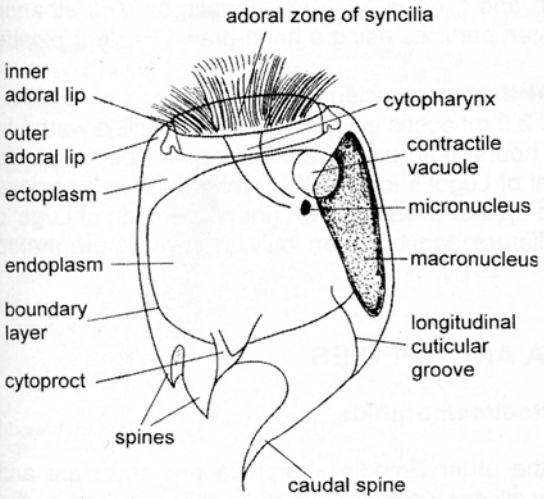
Rumen ciliates are closely related to other ciliates which inhabit the forestomach and large intestine of a variety of non-ruminant vertebrate animals, including birds, camels, elephants, fish, gorillas, hippopotamuses, horses, humans, kangaroos, rhinoceroses, rodents, and warthogs. While only ciliates occurring in ruminant animals should be called rumen ciliates, it should be noted that a few exceptions have been observed; for example rumen ciliates have been found in the hindgut of the capybara (Dehority 1987). Conversely, so-called hindgut ciliates and non-ruminant foregut ciliates have sometimes been observed in the rumen (Dehority 1986).

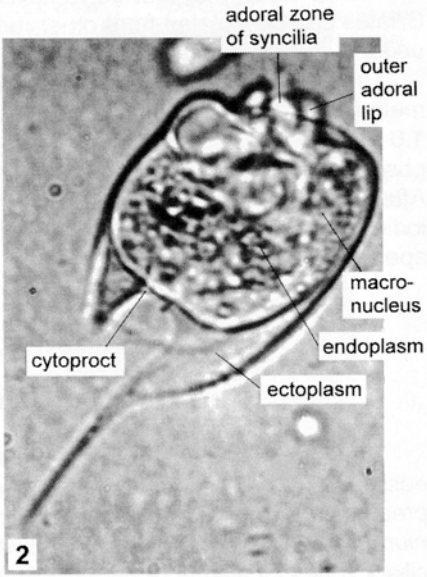
Rumen ciliates range in size from 15–250 µm in length and 10–200 µm in width (Dehority 2003). They are characterized by generally having: (1) a somatic kinetid that is typically made up of one kinetosome (basal body) with usually two transverse microtubular ribbons, both evident only during kinetosomal replication, (2) microtubular bundles (nematodesmata) that extend into the cytoplasm from the bases of kinetids that surround the cytostome, and (3) unspecialized oral ciliature. Based upon these ultrastructural features, two orders of rumen ciliates, the Entodiniomorphida and the Vestibuliferida, are recognized (Lynn & Small 2000) within the subclass Trichostomatia of the class Litostomatea, and this status is supported by molecular data (Wright & Lynn 1997; Cameron & O'Donoghue 2004).

PRACTICAL TECHNIQUES

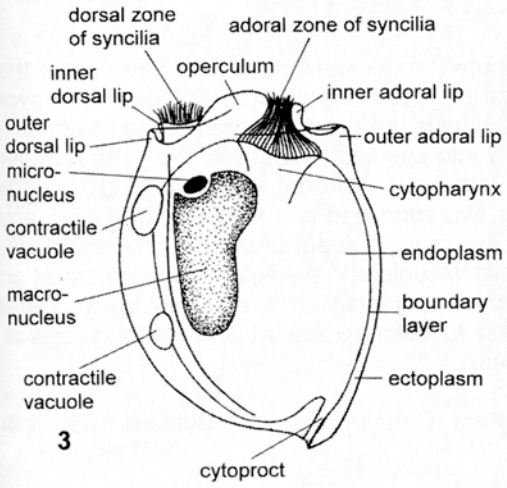
Rumen fluid is collected using a stomach pump or obtained from an abbattoir. If the temperature is kept near to 30° C in a thermos flask, living ciliates can be observed directly in the rumen fluid. Preserved fluid is obtained by the following procedure. Approximately 10–20 ml of rumen fluid is collected, filtered through 1–2 layers of cheese cloth to remove plant and feed material, and then put into a separating funnel

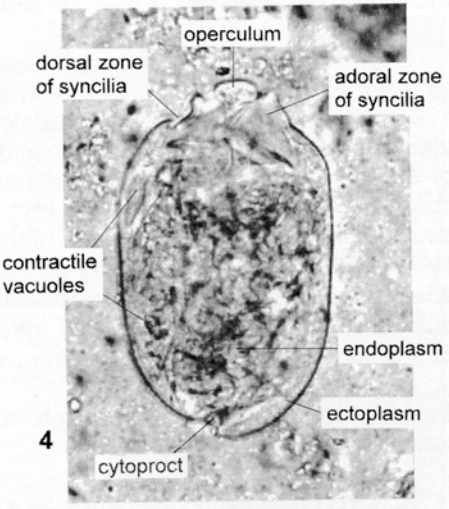
Fig. 1 – 16. Structural features of rumen ciliates. For explantions see both the captions and the text.





Entodinium caudatum 40 µm (1, 2)





Diplodinium sp. 100 µm (3, 4)

for 1 h at 39° C to allow the ciliates o settle. The sedimented ciliates will form a noticeable white layer, that can be removed and fixed in 15–20% formalin or 70% ethanol. Ciliates can be isolated from other rumen particles using a hand-drawn Pasteur pipette under a stereomicroscope.

To observe the macronucleus and the micronucleus, add 1–2 drops of acidified methylene blue (0.5 g methylene blue, 2.0 ml acetic acid, and 98.0 ml distilled water) to 1.0 ml of the fixed sample. After 2–4 hours, both nuclei should appear deep blue. To observe the skeletal plates, add 0.5 ml of Lugol´s iodine to 1.0 ml of the fixed sample. After 30 min, the skeletal plates should appear a deep brown (for preparation of Lugol´s iodine see p. 236). Details of the ciliature can be seen only in silver-impregnated specimens (for methods see p.159).

GENERA AND SPECIES

The Entodiniomorphids

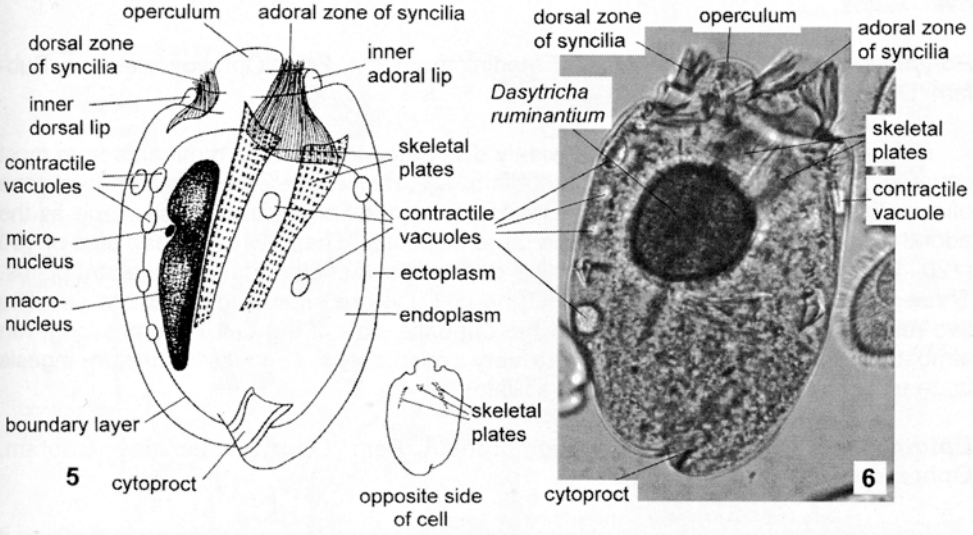
The rumen ciliates belonging to the order Entodiniomorphida are abundant and easily distinguishable from other rumen ciliates.They are generally characterized by the presence of a rigid pellicle, spines, skeletal plates (except for *Entodinium* and *Diplodinium*), and one or two tufts, or bands, of ciliature confined to the adoral and dorsal ciliary zones located at or near the anterior end. Within this order, the Ophryoscolecidae is the largest family of rumen ciliates.

Entodinium caudatum O. Entodiniomorphida, Fam. Ophryoscolecidae, Subfam. Entodiniinae (Fig. 1, 2)

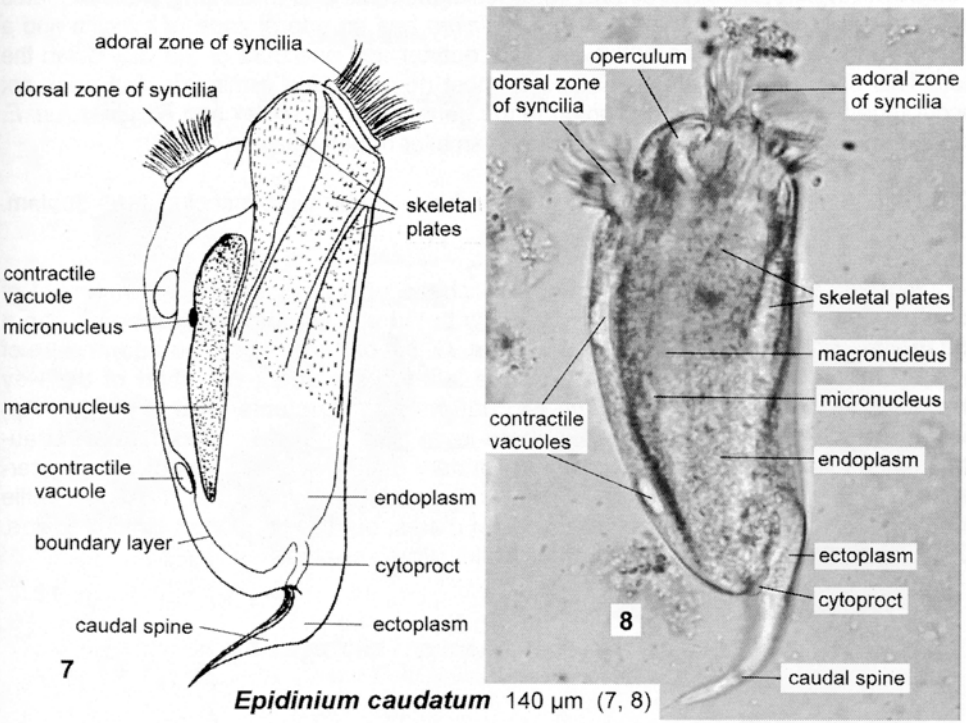
Entodinium caudatum is widely distributed in domestic ruminants and one of the smallest rumen ciliates, with a mean size of 35(28–70) x 28(25–50) µm. With over 120 presumed species, the genus *Entodinium* is the most common of the rumen ciliates and the most troublesome to classify into species because of the large number of similar forms. *Entodinium caudatum* can have different forms ranging from its characteristic three caudal spines (that is two short and one long) to not having any caudal spines.The shape and size of the macronucleus are of taxonomic importance. Members of the genus have one contractile vacuole and a single tuft of cilia called an adoral zone of syncilia, but no skeletal plates. *Entodinium* is probably an ancestral ophryoscolecid and also probably the first to colonize the rumen. Some species of *Entodinium* ingest bacteria and starch grains.

***Diplodinium* sp**. O. Entodiniomorphida, Fam. Ophryoscolecidae, Subfam. Diplodiniinae (Fig. 3, 4)

Species belonging to the genus *Diplodinium* have an adoral zone of syncilia and an additional tuft of cilia, called the dorsal zone of syncilia, located on the same transverse plane as the adoral zone of syncilia. However, the species of *Diplodinium* are the only members of the subfamily Diplodiniinae that do not have skeletal plates. Species of *Diplodinium* are small to large ellipsoidal organisms ranging in size from 30–118 µm



Polyplastron multivesiculatum 160 µm (5, 6)
with an ingested *Dasytricha ruminantium* (6)



Epidinium caudatum 140 µm (7, 8)

x 20–77 µm. *Diplodinium* ingests bacteria, starch grains, plant fragments and other rumen ciliates.

Polyplastron multivesiculatum O. Entodiniomorphida, Fam. Ophryoscolecidae, Subfam. Diplodiniinae (Fig. 5, 6)

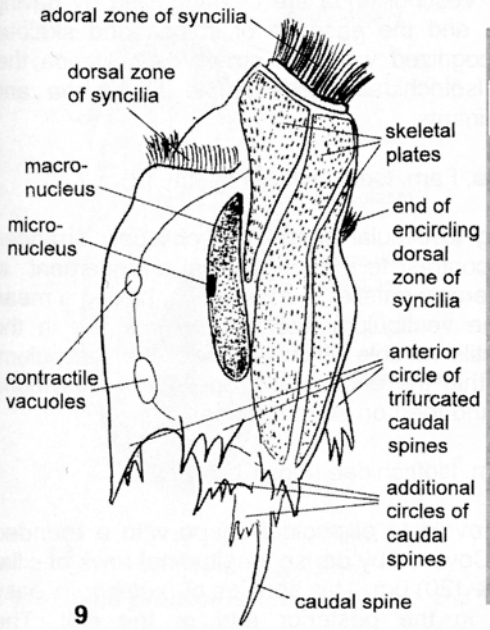
Polyplastron multivesiculatum is widely distributed in domestic ruminants from most countries. Like other members of this subfamily, *P. multivesiculatum* has an adoral zone of syncilia and a dorsal zone of syncilia located on the same transverse plane as the adoral zone. *P. multivesiculatum* has a large ellipsoidal shape with a mean size of 160 (120–190) x 95(78–140) µm. It has nine contractile vacuoles and five skeletal plates. Three of the plates are on one side of the cell and are short and difficult to see.The two remaining skeletal plates are on the opposite side of the cell.They are long, run almost parallel to each other, and are very conspicuous. *P. multivesiculatum* ingests large plant fragments and other rumen ciliates.

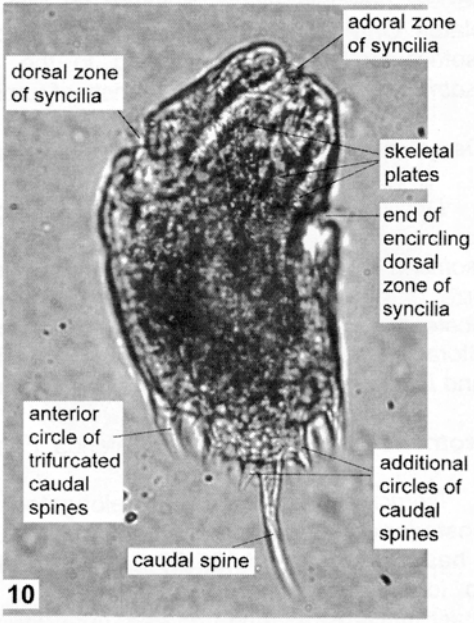
Epidinium caudatum O. Entodiniomorphida, Fam. Ophryoscolecidae, Subfam. Ophryoscolecinae (Fig. 7, 8)

Epidinium caudatum has an oblong shape and a tube-like cytopharynx that allows it to ingest large plant fragments from the contents of the rumen. It has a mean size of 107(85–140) x 44(37–54) µm. Two contractile vacuoles and three long skeletal plates are key characteristics of the genus. *Epidinium* has an adoral zone of syncilia and a dorsal zone of syncilia that is between one quarter and one third of the way down the cell. This species is widely distributed in most domesticated ruminants, but does not occur together with species belonging to the genera *Ophryoscolex* and *Polyplastron*. *E. caudatum* ingests smaller starch grains and smaller rumen ciliates.

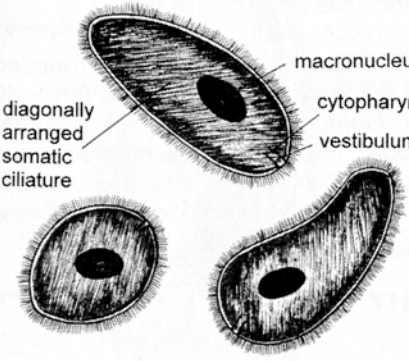
Ophryoscolex caudatus O. Entodiniomorphida, Fam. Ophryoscolecidae, Subfam. Ophryoscolecinae (Fig. 9,10)

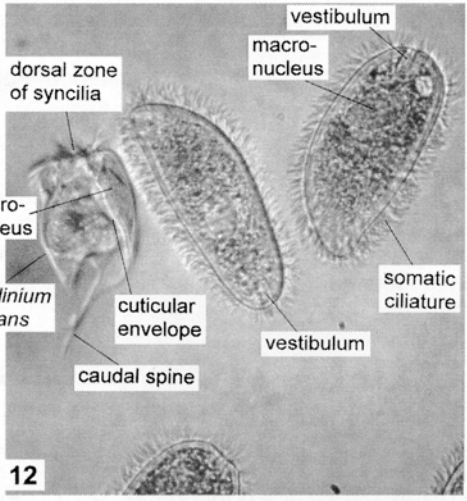
Ophryoscolex caudatus has an oblong shape with an imposing arrangement of caudal spines. This is the most widely distributed species of the genus and has a mean size of 150(137–162) x 90(80–98) µm. *O. caudatus* has both an adoral zone of syncilia and a dorsal zone of syncilia, the latter being about one third of the way down the cell and encircling about three quarters of the circumference of the cell. Approximately two-thirds down the cell, there is an anterior circle of six trifurcated caudal spines followed by three circles of secondary caudal spines.Two other characteristics of this species are the 47–60 µm long caudal spine and the nine contractile vacuoles. There are also three long skeletal plates, but they are often difficult to see. *O. caudatus* ingests starch grains and smaller rumen ciliates.





Ophryoscolex caudatus 150 µm (9, 10)





Dasytricha ruminantium 60 µm (11, 12)
and *Entodinium simulans* (12)

The Vestibuliferids

Rumen ciliates belonging to the order Vestibuliferida are characterized by having flexible pellicles entirely covered by cilia, and the absence of spines and skeletal plates. Only one rumen ciliate family is recognized within the order Vestibuliferida: the Isotrichidae. Species belonging to the Isotrichidae (for example *Dasytricha* and *Isotricha*) are found in a wide variety of ruminants.

Dasytricha ruminantium O. Vestibuliferida, Fam. Isotrichidae (Fig. 11, 12)

Dasytricha ruminantium has an oblong to circular shape and obliquely arranged rows of cilia winding around the cell, in contrast to the longitudinal arrangement in *Isotricha* species. *D. ruminantium* is considerably smaller than *Isotricha*, having a mean size of 58(46–100) x 27(22–50) µm. The vestibulum and cytopharynx are in the posterior end, and there is only one contractile vacuole which is close to the vestibulum. Storage polysaccharides are abundant within the endoplasm. Species of *Dasytricha* and *Isotricha* are generally found together and feed on starch grains.

Isotricha prostoma O. Vestibuliferida, Fam. Isotrichidae (Fig. 13, 14)

Isotricha prostoma has an elongated ovoid to ellipsoidal shape with a rounded posterior end and a pointed anterior end. Covered by dense longitudinal rows of cilia, it has a mean size of 135(80–200) x 70(50–120) µm. This species of *Isotricha* is easy to identify because the vestibulum is in the posterior end of the cell. The macronucleus is reniform and near the vestibulum. Both species of *Isotricha* have 6–12 contractile vacuoles, numerous food vacuoles containing ingested starch grains, and abundant storage polysaccharides within the endoplasm.

Isotricha intestinalis O. Vestibuliferida, Fam. Isotrichidae (Fig. 15, 16)

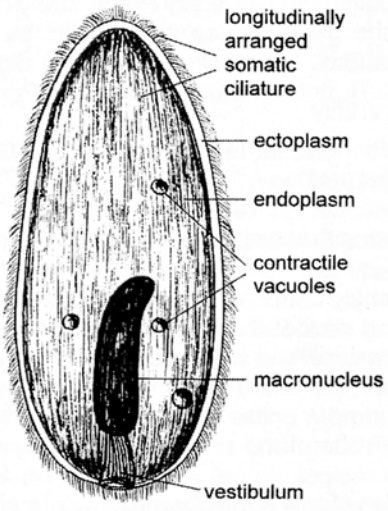
Isotricha intestinalis has an elongated ovoid to ellipsoidal shape with a rounded posterior end and a pointed anterior end. It has a mean size of 110(90–200) x 60 (45–150) µm. It is easy to differentiate from *Isotricha prostoma* because the vestibular opening is about two thirds of the way down the cell, and the macronucleus is usually spherical to ovate and near the mid-body. *I. prostoma* and *I. intestinalis* may occur together in the rumen and are widely distributed in wild and domesticated ruminants.

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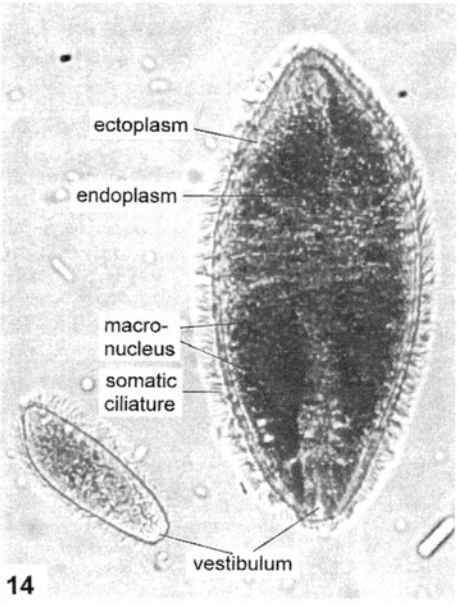
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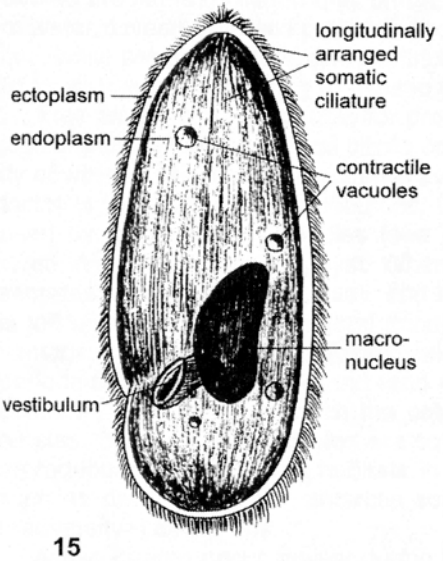


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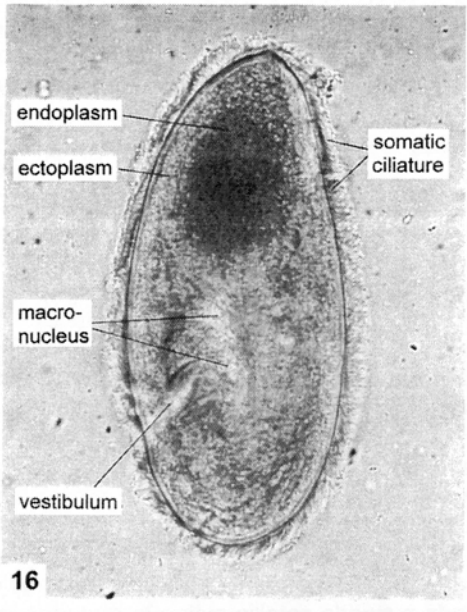


14

Isotricha prostoma 160 µm (13, 14) and the smaller ***Dasytricha ruminantium*** (14)



15



16

Isotricha intestinalis 140 µm (15, 16)

The schematic drawings Fig. 1, 3, 5, 7, 9, 11, 13 and 15 are from Dehority 1993

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Soil Ciliates

Wilhelm Foissner

INTRODUCTION

Of the 10,000 ciliate species described, about 1000 are known to occur in terrestrial habitats, such as mosses, leaf litter, soil, and the mixture of mud and soil at the bottom of puddles. However, the real number of terrestrial ciliates is much higher because new species are continuously being described. The earlier view that the same ciliate species occur in soil and freshwater is outdated. We now know that most soil ciliates are autochthones, that is they occur only, or preferably, in terrestrial habitats.

Soil ciliates have interesting morphological and functional adaptations for the optimal use of the resources of the soil. These adaptations demonstrate important ecological principles. The morphological peculiarities are caused by the spatial constraints of the narrow soil pores and the film-like distribution of the water. Thus, most soil ciliates are small, their average length, width, and biomass being significantly smaller than in limnetic communities. The diminution is reached either by a general size reduction (Fig. 1, 2, 7, 9, 10, 12, 13) or by the reduction of body width, causing the vermiform shape typical for many soil organisms (Fig. 3 – 6, 8, 11). Usually, soil ciliates are flat and ciliated only on the ventral (oral) surface and exploit the thin films of water covering the soil particles and walls of soil pores. Most soil ciliates are mobile, while sessile Suctoria and Peritricha are frequent in limnetic biotopes. The ability to move freely is necessary to escape food depletion in the soil pores.

Free water is indispensable for protozoan life. Thus, most functional and physiological peculiarities of the soil ciliate community are related to the changing availability of water in the habitat; most soils even dry out from time to time. The second main factor is the special food resources, for example, the abundance of fungi that are used by mycophagous species (see *Grossglockneria acuta*). Dry periods are survived in a so-called resting cyst. When encysting, the ciliate rounds up, loses water, secretes a thick, waterproof wall, and then strongly reduces its metabolism. The cyst is left when good environmental conditions are available again. Obviously, it is advantageous to have a wide ecological range and fast reproduction to use the short periods of optimal moisture and food supply. Such organisms are called r- (<reproduction>) strategists, of which the colpodid ciliates described below are typical examples. Some of these ciliates are so moderate in their requirements that they can reproduce in very extreme habitats in which water is present for only a few hours, such as dew drops and antarctic soils.Today, such specialists are also called a-(<adversity>) strategists.

Active ciliates occur mainly during the very early stages of soil formation, especially in the fresh leaf litter. Thus, vertical distribution of active ciliates shows a sharp break (Table 1): the abundance abruptly decreases to almost zero in 3–9 cm soil depth, i. e., in the fermentation layer (= zone with intense decomposition processes).

This phenomenon is called "ciliatostasis". It has been supposed that certain substances, for example antibiotics, accumulate in evolved (older) soils and arrest excystment and reproduction of ciliates, as they are known to do for fungi (fungistasis). This inhibition can be partially released, for example, by drying the soil. When dry soil is rewetted, great numbers and intense reproduction of ciliates occur, at least in the laboratory, because many specimens, which were encysted for years, excyst and commence to reproduce (see the first four habitats in Table 1). The following method uses this phenomenon for obtaining rich raw cultures of ciliates and other protists.

Table 1: Number of active ciliates (per g dry mass of soil) in various soils.

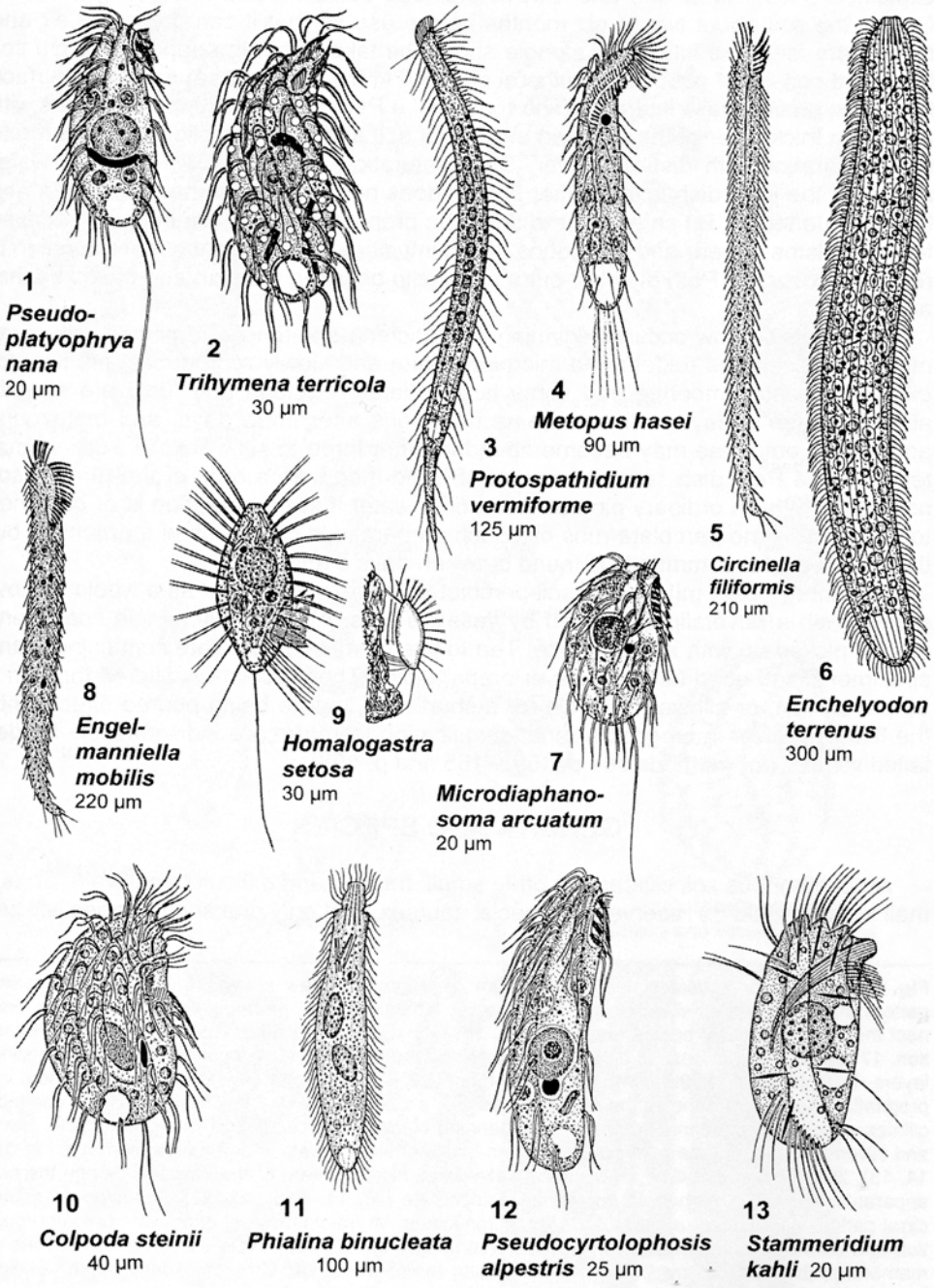
Habitat	fresh soil	dried, rewetted soil
Field	1	379
Meadow	3	520
Needle litter	468	17,390
Leaf litter	3326	104,340
Needle litter		
O_L 0 – 1 cm	350	not investigated
O_L 1 – 3 cm	109	not investigated
O_F 3 – 9 cm	14	not investigated

PRACTICAL TECHNIQUES

Soil ciliates can be collected 24–48 h after rain by rinsing some wet leaves and/or needles with a few ml of rain water or tap water. A drop of the resulting suspension can be investigated either as a whole, or single specimens can be picked out with a fine pipette under the stereomicroscope or other microscope at a magnification of x 40. The investigation of fresh, wet forest litter is strongly recommended for an introductory demonstration; it is impressive over and over again to see how rich natural forest litter is populated with bacteria, fungi, protists, and small metazoans.

Pure cultures of protists are often both difficult and time-consuming to obtain. However, the species discussed here are rather easily cultivated. The methods are given as the individual species are described. Material from water-saturated soil cultures usually suffice, and show diversity much better than a series of pure cultures.

Fig. 1 – 13. Small size or vermiform shape as adaptations to the soil habitat. **1:** *Pseudoplatyophrya nana* (20 µm), a mycophagous, flattened colpodid. **2:** *Trihymena terricola* (30 µm), a bacterivorous colpodid. **3:** *Protospathidium vermiforme* (125 µm), a raptorious holotrich. **4:** *Metopus hasei* (90 µm), a bacterivorous spirotrich indicating oxygen depletion. **5:** *Circinella filiformis* (210 µm), a bacterivorous, very slender hypotrich. **6:** *Enchelyodon terrenus* (300 µm), a large, holotrichous predator. **7:** *Microdiaphanosoma arcuatum* (20 µm), a bacterivorous, flattened colpodid. **8:** *Engelmanniella mobilis* (220 µm), a bacterivorous, slender hypotrich. **9:** *Homalogastra setosa* (30 µm), a hymenostome ciliate; at right, a specimen attached to soil particles and feeding on bacteria. **10:** *Colpoda steinii* (40 µm), a bacterivorous colpodid (compare Fig. 20). **11:** *Phialina binucleata* (100 µm), a holotrichous predator. **12:** *Pseudocyrtolophosis alpestris* (25 µm), a bacterivorous, flattened colpodid. **13:** *Stammeridium kahli* (20 µm), a bacterivorous, strongly flattened, microthoracid ciliate.


1
Pseudo-
platyophrya
nana
20 µm
2
Trihymena terricola
30 µm
3
Protospathidium
vermiforme
125 µm
4
Metopus hasei
90 µm
5
Circinella
filiformis
210 µm
6
Enchelyodon
terrenus
300 µm
7
Microdiaphano-
soma arcuatum
20 µm
8
Engel-
manniella
mobilis
220 µm
9
Homalogastra
setosa
30 µm
10
Colpoda steinii
40 µm
11
Phialina binucleata
100 µm
12
Pseudocyrtolophosis
alpestris 25 µm
13
Stammeridium
kahli 20 µm

This so-called "non-flooded Petri dish method", which uses the release of ciliatostasis explained above, is simple and always provides sufficient and interesting material. Collect the soil about two to six months before use so that it can dry in the air and remain dry for some time. The sample should be taken from the upper 0–10 cm soil layer and consist of about 70% mineral soil (soil in the strict sense) and 30% surface litter (tree and/or grass litter) and fine roots. Fill a Petri dish (10–20 cm diameter) with a 1–3 cm thick layer of the air-dried mixture of soil and litter and slightly oversaturate the preparation with distilled water. Supersaturation is reached when some water runs off if the Petri dish is tilted after 5h; if it does not then add some more water and test again after 1h. Be careful not to flood the preparation as for an infusion because few organisms appear and reproduce in soil infusions. When slight supersaturation is reached, cover the Petri dish but put a paper clip between the dish and the lid so that air can enter.

A succession now occurs beginning with bacteria and fungi and ending with small metazoans, such as rotifers and microturbellaria and slowly reproducing protists, for example testate amoebae and some large ciliates. Bacteria and fungi are already abundant after a day, protists become numerous after three days, and metazoans and testate amoebae may become abundant after three to six weeks. To collect material, tilt the Petri dish several times at 45° and then take a drop of the run off (soil percolate) with an ordinary pipette. Add some water if the preparation is or becomes too dry, that is, no percolate runs off. Such preparations can be used for months, but the richest protist community is found between days 5 and 20.

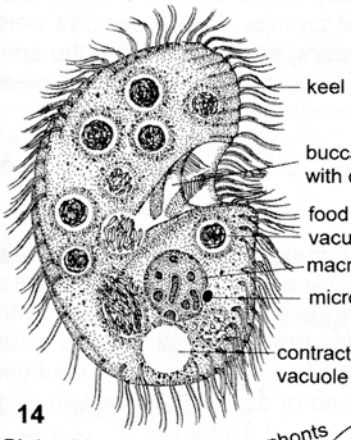
A minute (~ 0.01 ml) drop of soil percolate can be investigated as a whole by covering it with a coverslip supported by vaseline tabs. Alternatively, single specimens can be picked up with a fine pipette. Ten to twenty ml soil percolate containing many specimens is needed for some silver preparations. The percolate is filtered through a 50 µm screen, or allowed to settle for a short time, before being poured directly into the fixative. Silver impregnation and identification literature are indispensable for detailed studies (for methods see p. 159 – 165 and p. 198).

GENERA AND SPECIES

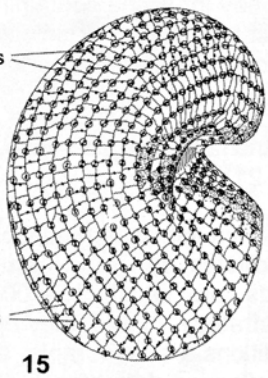
Autochthonous soil ciliates are often small, fragile, and difficult to cultivate. Thus, their study should be reserved for special courses, and only one such species will be

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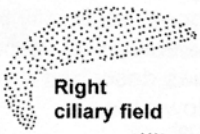
Fig. 14 – 19. *Colpoda cucullus*, length 60–80 µm. **14:** Right side view in vivo. **15:** Ciliary and silverline pattern of right side after silver nitrate impregnation. The silverlines, probably some sort of fibre, connect the individual basal bodies and their cilia. **16:** Oral ciliary fields after silver carbonate impregnation. **17:** Life cycle. **18:** Resting cyst, living, diameter 50 µm. The cyst wall consists of several distinct layers. The cell proper is the compact, central mass. **19:** Nuclear apparatus after silver carbonate impregnation. The arrow denotes the micronucleus with a size of 3 x 2 µm. **Fig. 20 – 24.** Some common ciliates after silver impregnation and in the scanning electron microscope. **20:** *Colpoda steinii*, ciliary and silverline pattern of the right side after Klein-Foissner silver nitrate impregnation (compare Fig. 10, 14, 15). **21:** *Colpoda cucullus*, macronucleus (MA) and ciliary pattern of the ventral side and the oral apparatus after silver carbonate impregnation (compare Fig. 14 – 16). **22, 23:** *Gonostomum affine*, cirral pattern and nuclear apparatus (MA, macronucleus; MI, micronucleus) after protargol impregnation and in the scanning electron microscope (compare Fig. 30 – 32). Note the large adoral zone of membranelles (AZM). Arrows mark the undulating membranes. **24:** *Grossglockneria acuta*, feeding tube (arrow) in the scanning electron microscope (compare Fig. 25 – 27).



14
Right side view
in vivo



15
Ciliary and silver-
line pattern

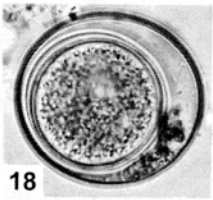


Right
ciliary field

16



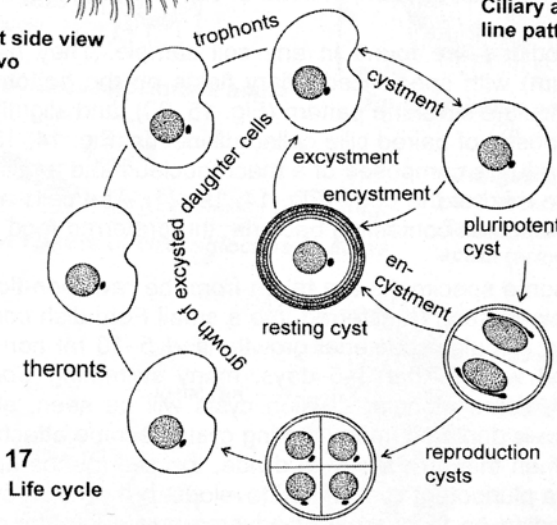
Left
ciliary field



18
Resting cyst



19
Nuclear apparatus

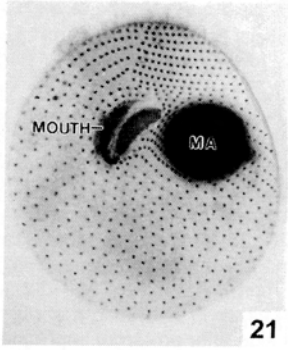


17
Life cycle

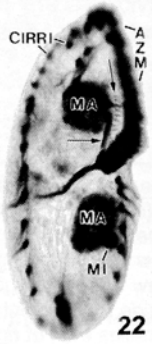
Colpoda cucullus (14 – 19, 21)



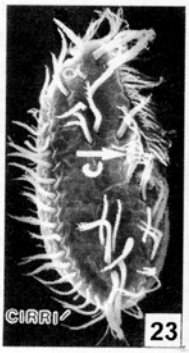
20
Colpoda steinii
Ciliary and silverline pattern



21



22



23



24

described here, namely the obligate mycophage *Grossglockneria acuta*. The other species chosen are more robust and may occasionally occur also in limnetic biotopes, especially in hay infusions containing some soil crumbs. These species were selected with respect to survival (r/k selection) and feeding strategies. All of the species described are common in non-flooded Petri dish cultures of forest and/or meadow soils.

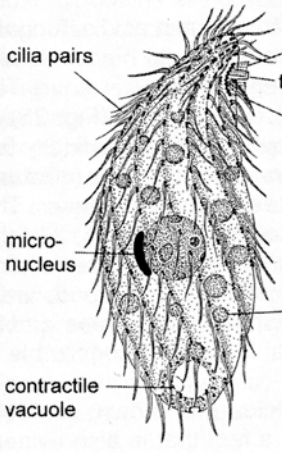
Colpoda spp. Holotricha in a broad sense, Cl. Colpodea, O. Colpodida, Fam. Colpodidae (Fig. 10, 14 – 21)

The soil ciliate community is termed Colpodetea (in analogy to plant communities) because colpodid ciliates are very frequent and numerous in terrestrial habitats. In general, colpodids are small (30–100 µm), moderately flattened ciliates with r-selected survival strategy, that is, they emerge quickly from the resting cysts when environmental conditions are favourable, and then soon become numerous because they generate four offspring during each division, instead of the more usual two (Fig. 17).

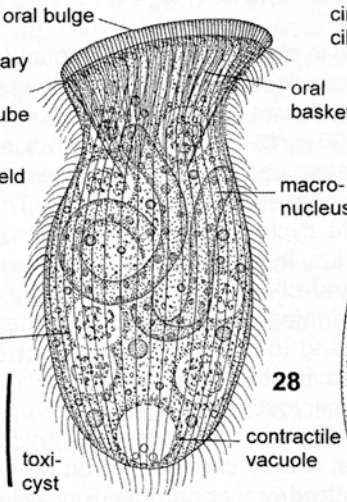
Species of the family Colpodidae are found in any soil sample. They have a subapical oral funnel (vestibulum) with specialized ciliary fields on the bottom and roof (Fig. 14 – 16, 20, 21), a reticulate silverline pattern (Fig. 15, 20), and slightly spirally extending ciliary rows composed of paired cilia called dikinetids (Fig. 14, 15, 20, 21). As usual, the nuclear apparatus is composed of a macronucleus and a micronucleus, both located in the middle third of the body (Fig. 14, 19, 21). The cells are often studded with globular food vacuoles containing bacteria, the preferred food (Fig. 14).

For studying the life cycle, some specimens are taken from the raw (non-flooded Petri dish) culture with a micropipette and transferred into a small Petri dish containing a squashed wheat grain (to stimulate bacterial growth) and 5–10 ml non-chlorinated tap water or still bottled water. After 3–5 days, many swimming *Colpoda* specimens and, on the bottom, some globular division cysts will be seen; after 2 weeks, when the culture medium is depleted, many resting cysts become attached to the bottom of the Petri dish. When they are ready to divide, the cell rounds up and secretes a thin wall, producing a pluripotent cyst which develops to a division cyst or thick-walled resting cyst, depending on favourable or adverse environmental conditions (Fig. 17, 18). The first division within the cyst produces two offspring, which then divide once more. Thus, four individuals are produced in each cycle (Fig. 17). Occasionally, the offspring divide several times generating 8 or 16 individuals.

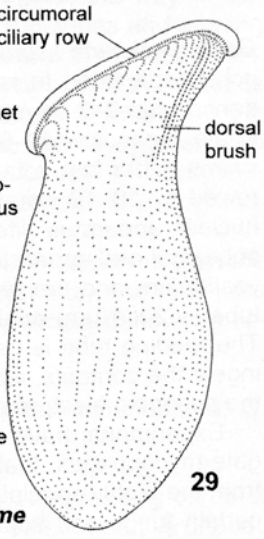
Fig. 25 – 27. Morphology and cultivation of the mycophagous *Grossglockneria acuta*. **25:** Right side view in vivo, length 40–80 µm. **26:** Cultivation (see text). **27:** A fungal hypha with a small hole (arrow) produced by the feeding tube of *Grossglockneria acuta*. **Fig. 28, 29.** Morphology of the raptorious *Epispathidium amphoriforme*. **28:** Left side view from life, length 130 µm; left a toxicyst from the oral bulge at higher magnification. **29:** Left side view after protargol impregnation. **Fig. 30 – 32.** Morphology of *Gonostomum affine*. **30** and **31:** Right side (about ventral) views in vivo, length about 100 µm. **32:** Infraciliature (cirral pattern, adoral membranelles etc.) of right side after protargol impregnation.



25 ***Grossglockneria acuta***

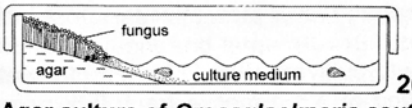


28



29

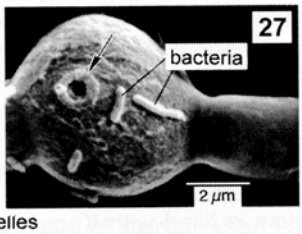
Epispathidium amphoriforme (28, 29)

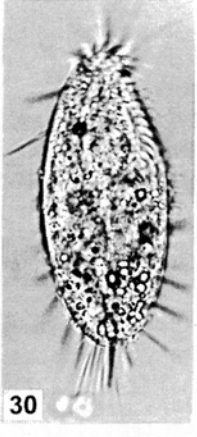


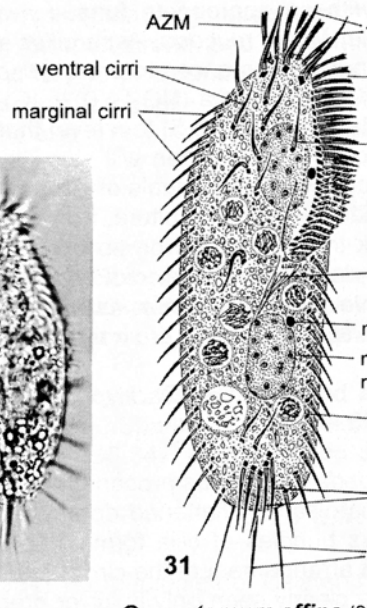
26

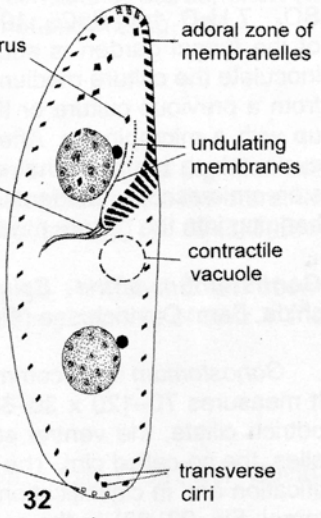
Agar culture of *Grossglockneria acuta*

A fungus hypha perforated by *Grossglockneria acuta* (arrow)









Gonostomum affine (30 – 32)

Grossglockneria acuta Holotricha in a broad sense, Cl. Colpodea, O. Grossglockneriida, Fam. Grossglockneriidae (Fig. 24 – 27)

Grossglockneria acuta is also a colpodid ciliate and is very frequent in acidic, fungal-rich needle litter. In non-flooded Petri dish cultures, it develops to its highest abundance after 2–3 days, showing the r-selected survival strategy described above. The ciliate measures 40–60 x 15–35 µm and shows an acute anterior end (Fig. 25). It swims rather fast, rotating about its main body axis. The pellicle is often distinctly furrowed by 10–12 spirally extending ciliary rows. The general organization (ciliature, nuclear apparatus, life cycle etc.) matches that of *Colpoda* described above. The subapical oral apparatus, in contrast, has a unique organization (Fig. 24, 25, 27): the vestibulum and the oral ciliary fields are strongly reduced, while a minute feeding tube (~ 2 x 1 µm) originates from the centre of the very small (3–5 µm) mouth area. The feeding tube is used to penetrate the wall of fungal hyphae and spores and to ingest the contents, leaving behind a characteristic hole that is easily recognizable in the scanning electron microscope (Fig. 24, 27).

Experiments have shown that grossglockneriids (7 species are known) are obligate mycophages, that is they cannot feed on other items, a fact that is also evident from the highly specialized oral apparatus. Several species of the family feed only on certain fungi, and some feed on plant pathogens, for example, *Ustilago maydis*, a very damaging pathogen of maize. Thus, mycophagous ciliates offer the possibility for biological control of soil-borne fungal diseases.

Grossglockneria acuta is comparatively easy to cultivate (Fig. 26). Half of a Petri dish is obliquely filled with nutrient agar consisting of 3.5 g malt extract, 0.5 g peptone, 3 g agar, and 93 ml tap water (sterilized by steaming for 3 x 20 min). During solidification, the agar is inoculated with an appropriate fungus available in most microbiological laboratories, for example, *Mucor mucedo*, *Aspergillus* spp., or *Absidia orchidis*. After some days, when the fungus commences to produce spores, the Petri dish is filled with Knop´s solution (1g KNO_3, 0.1g Ca $(NO_3)_2$, 0.2g K_2HPO_4, 0.1g Mg $SO_4 \cdot 7\ H_2O$, 1mg $FeCl_3$, 1000 ml distilled water, pH 6.5) to a level that only the edge of the fungal garden is submerged (flooding the garden will stop its growth!). Now inoculate the culture medium with one or several individuals of *Grossglockneria acuta* from a previous culture or the non-flooded Petri dish culture, from which it is picked up with a micropipette. After one week the ciliates will be so abundant that the life cycle, which matches that of *Colpoda* described above, can be studied. Under the stereomicroscope, students can observe *Grossglockneria acuta* feeding on hyphae hanging into the culture medium and on spores lying on the bottom of the Petri dish.

Gonostomum affine Spirotricha in a broad sense, Cl. Hypotrichea, O. Stichotrichida, Fam. Oxytrichidae (Fig. 22, 23, 30 – 32)

Gonostomum is as common as *Colpoda* and is thus present in most soil samples. It measures 70–120 x 30–60 µm and is distinctly flattened dorsoventrally. As a hypotrich ciliate, the ventral surface bears bundles of cilia forming compound organelles, the so-called cirri. The very varied arrangement of the cirri is important for identification and in classification but can be clearly seen only in silver preparations (protargol; Fig. 22, 32) or the scanning electron microscope (Fig. 23). On the dorsal sur-

face, there are three ciliary rows each composed of 3 µm long, inconspicuous bristles. The lack of a functional dorsal ciliature is associated with the way of life: most hypotrichs are grazers "walking" with the cirri on various substrates and swirling food, often bacteria, into the mouth with the help of the adoral zone of membranelles (AZM), which is usually the most prominent feature in hypotrichs. The AZM extends along the left anterior half of the body and is composed of about 27 ciliary plates, each consisting of four rows of cilia. The right border of the buccal cavity bears two inconspicuous undulating membranes. The nuclear apparatus consists of two ellipsoidal macronucleus nodules with a micronucleus each (Fig. 22, 31, 32). The contractile vacuole is in mid-body near the left body margin.

Most hypotrichs are k-(capacity) strategists, that is, have other ecologies than the r-selected colpodids discussed above. For example, they excyst later and reach the abundance maximum more slowly because they reproduce by simple transverse fission producing only two daughter cells.

Spathidium sensu lato. Holotricha in a broad sense. Cl. Kinetofragminophora, O. Spathidiida, Fam. Spathidiidae (Fig. 28, 29)

The Spathidiidae are holotrichous (completely ciliated) predators feeding on other ciliates. There are several genera and over 200 species which differ in details of body shape, nuclear apparatus, and ciliary pattern (Fig. 3, 28). The species shown here, is rather frequent and large: 90–150 x 35–65 µm. *Epispathidium amphoriforme* has an amphoriform body with the entire anterior margin modified to a mouth bulge. This bulge contains hundreds of 7 µm long toxicysts that kill the prey, which is transported into the cell along the conical oral basket (Fig. 28, 29). The basket is made of many fine rods (nematodesmata) originating from the circumoral ciliary row. The macronucleus is a long, tortuous strand, and the contractile vacuole is in the rear of the body (Fig. 28). The ciliary rows extend meridionally and three dorsal rows are anteriorly modified to a special organelle, the dorsal brush, which consists of paired, short (≤ 5µm), bristle-like cilia. The function of the dorsal brush is not known (Fig. 29).

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Foissner, W. (2004): Protozoa. In: Hillel, D., Rosenzweig, C., Powlson, D. S., Scow, K. M., Singer, M. J., Sparks, D. L., Hatfield, J. (eds.), *Encyclopedia of soils in the environment*, pp. 336-347. Elsevier, Oxford.

FILM

Grell, K. G. (1964): Morphologie der Ciliaten I – Holotricha. – Film C 881 IWF Göttingen (spoken commentary in German), 9 ½ min.

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Microfauna of Activated Sludge

Helmut Berger

INTRODUCTION

Activated sludge reactors are the most common type of sewage treatment plants. The purification of the waste water follows the same biological processes as self-purification in natural waters. The activated sludge treatment is a truly aquatic process in which the sewage and the organisms are aerated together in tanks for several hours. The organisms form flocculent growths, that is, activated sludge, which may then be easily separated from the fluid phase in settling tanks. Bacteria account for about 96% of the total biomass (predominantly in the form of sludge flocs) in the aeration tank where the organic wastes are mainly degraded. The flocs are irregularly formed masses, usually 50–300 µm in size, and composed of inorganic and organic material. They consist of (1) carbonates and phosphates, iron and aluminium hydroxide and organic substance including fibrous material and starch grains and (2) the biochemically active agglutinated bacteria embedded in a gelatinous matrix. In the interstices of the flocs are suspended bacteria (mainly intestinal bacteria such as *Escherichia coli)* that form the major food source for the eukaryotic activated sludge organisms, including flagellates, naked and testate amoebae, ciliates, rotifers and nematodes (Bernerth 1978; Curds 1992; Ettl 2000; Foissner 1991).

Many of the protozoa found in polluted rivers also occur in sewage treatment plants. However, the diversity is usually much lower in a given sample of sludge, there being hardly more than 15 protozoan and three metazoan species. The activated sludge microfauna can be used as an indicator of plant performance and sludge quality.

The species composition and the dominance depend on the type of plant (Table 1). High-rate plants usually degrade only carbon compounds and are characterized by a low sludge age of less than four days (the average floc age of the sludge in the system) together with a high sludge loading, that is, a high concentration of easily degradable material. This process is often used for the partial treatment of used water with the objective of reducing the loading of pollutants. By contrast, conventional plants have a low sludge loading and a high sludge age (10–30 days). Thus, they harbour not only organisms with a short generation time (bacteria, flagellates), but also taxa with a longer generation time, such as ciliates, testate amoebae, rotifers, nematodes and sometimes even small oligochaete worms. Conventional plants not only degrade carbon compounds, but also reduce the concentration of ammonia (nitrification), limit the oxidized nitrogen (denitrification), and – in some cases – even remove phosphorus (for reviews see Hawkes 1983; Stier et al. 2003). The degradation and mineralisation of the pollutants is mainly done by the floc bacteria. The protozoans and, to a smaller extent the micrometazoans, have three major functions in the activated sludge process (Curds 1992; Foissner 1991): (1) Various protozoan species, for example the ciliates *Aspidisca* and *Chilodonella,* graze on the surface of

the flocs which then become compact and easier to separate from the purified water. This is important because a high abundance of filamentous bacteria causes the phenomenon known as "bulking", in which the sludge becomes difficult to settle and as a result may be discharged in the effluent. (2) Hundreds of species of intestinal bacteria, including pathogens which are not incorporated in flocs would make even purified waste water turbid. Usually, however, these dispersed bacteria are ingested by filter feeding protozoa, for example, *Vorticella* and *Epistylis*. They make the effluent clear! (3) The grazing impact of the microfauna increases the turnover of the system by stimulation of bacterial growth.

Table 1. Comparison of some biological features of high-rate waste water treatment plants and conventional activated sludge plants.

Parameter	high-rate plants	conventional plants
Dominant bacteria	non-flocculating	flocculating
Dominant protozoa	flagellates	ciliates
Rotifers	usually lacking	few to many
Nematodes	usually lacking	few

PRACTICAL TECHNIQUES

Since activated sludge always contains pathogens, safety has to be a primary concern!

Activated sludge plants are now present in most larger villages. Contact the operator before sampling. Use fresh activated sludge, which is taken from the aeration tank with a plastic beaker on a long handle. Put about 300 ml sludge into a 500 ml wide-necked, screw-capped plastic bottle and take it to the laboratory under cool conditions.

For investigation, shake the bottle, take a drop with an ordinary pipette, put it on a microscope slide, and cover the preparation with a coverslip. If three replicates are investigated, reliable data on the species present are obtained. Put parts of the sample into Petri dishes or aerate the sample to avoid fouling when the sludge is kept for a longer period.

In practice, a semiquantitative investigation with a rating scale will be sufficient. However, quantitative investigation is also possible and easily performed with the method by Aescht & Foissner (1992). Sludge quality can be assessed with the sludge biotic index (SBI) of Madoni (1994) or the method by Grossmann et al. (1999).

COMMON ORGANISMS IN ACTIVATED SLUDGE

Almost 300 protozoan species have been recorded from activated sludge plants (Curds 1992; Ganner et al. 2002). All species are freshwater inhabitants usually occurring in moderately (betamesosaprobic) to strongly (polysaprobic) polluted rivers. Thus, most protozoan taxa can be identified with the guides given in the bibliography of this chapter. Activated sludge bacteria can be identified with the guide of Eikelboom & Buijsen (1999). The metazoan fauna of the activated sludge usually consists

of rotifers and nematodes and only rarely tardigrads (*Thulinia*) and annelids such as *Aelosoma.*

Heterotrophic Flagellates

Heterotrophic flagellates ("zooflagellates", "Zoomastigophora") do not form a phylogenetically coherent group. Thus they are defined operationally as free-living protists moving and/or feeding by the use of flagella and feeding exclusively by heterotrophic means or, if with plastids, then also capable of ingesting particles (mixotrophy). Heterotrophic flagellates occur in all activated sludge samples, but a high abundance indicates poor plant performance.

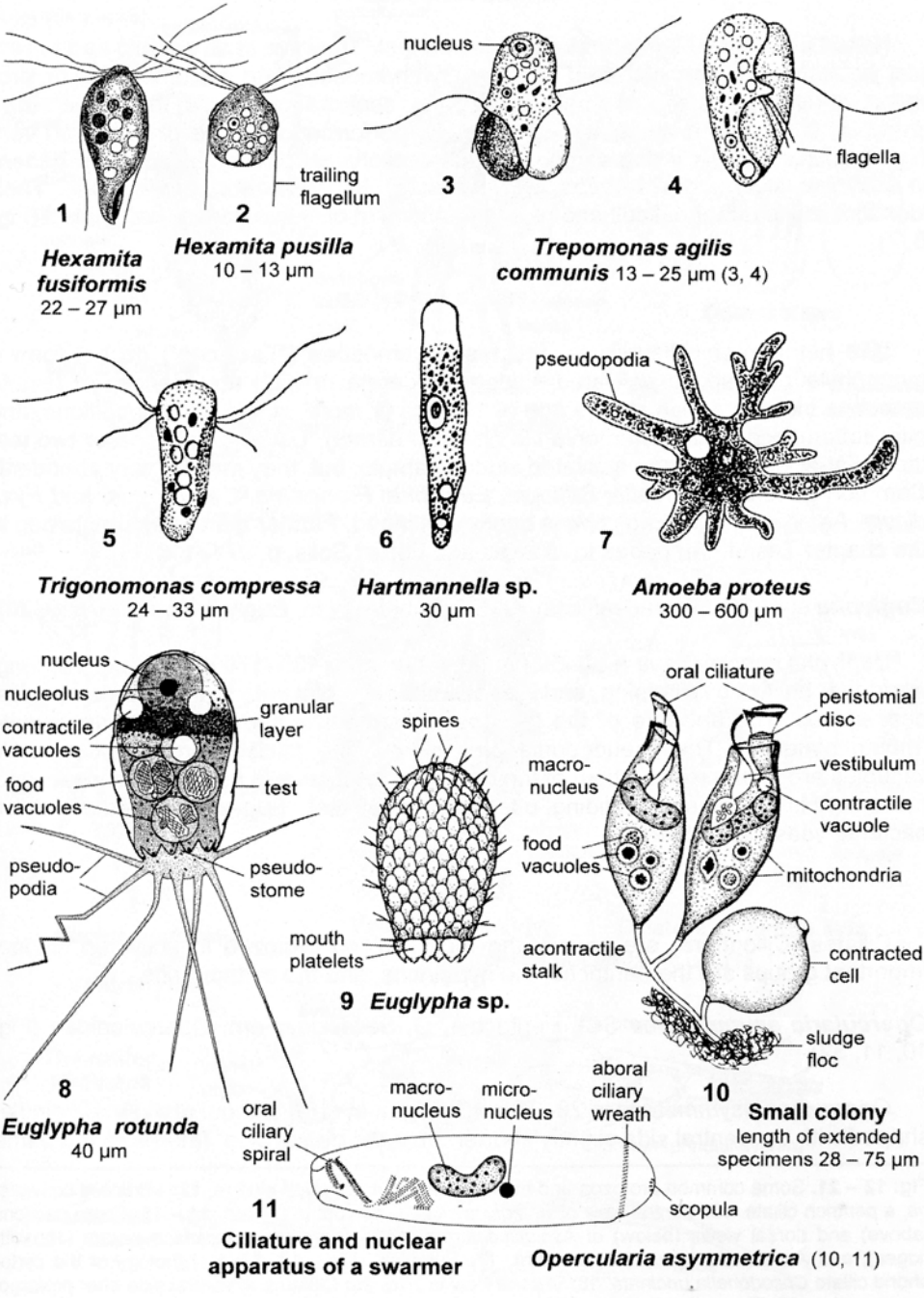
Hexamita spp. O. Diplomonadida, Fam. Hexamitidae (Fig. 1, 2, 22, 23)

Hexamita species are 10–13 × 8–10 µm (*H. pusilla*) to 24–35 × 14–18 µm (*H. crassa*) in size. The body is soft, ellipsoidal, and of twofold rotational symmetry, that is, all organelles (nuclei, flagellar system, cytostome) are doubled. The nuclei are in the anterior part of the cell between the two flagellar (mastigont) systems. One or two, rarely more contractile vacuoles circulate in the cytoplasm which often contains small, highly refractive inclusions. Eight long flagella are arranged in two subapical bundles. The cytostomes are about in mid-body. The movement of *Hexamita* species is creeping, rotating, or shooting like a flash. Species of *Hexamita* and of other genera of the Diplomonadida (*Trepomonas*, Fig. 3, 4, 24, 25; *Trigonomonas*, Fig. 5) lack mitochodria and dictyosomes, and thus have an anaerobic metabolism, that is, they normally occur at sites with very little or no oxygen. They therefore indicate insufficient aeration and/or high loading of the activated sludge.

Bodo saltans O. Kinetoplastea, Fam. Bodonidae (Fig. 26, 27)

Bodo saltans is a bean-shaped, slightly flexible organism with a usual body length of 5–9 µm. The nucleus is slightly behind the mid-body, the contractile vacuole subapical. Behind the flagellar pocket is the so-called kinetoplast, that is a widened, DNA-rich part of the single, tubular mitochodrion. The two flagella emerge from the anterior of the body; the anterior (swimming) flagellum is of about body length, while the posterior (trailing) flagellum is two to three times as long. The subapical oral apparatus is a tiny gap recognizable in the microscope at a magnification of × 1000. *Bodo saltans* is easily recognizable by its dancing-springing movement while it is attached to the substrate by the trailing flagellum. *Bodo saltans* feeds on bacteria and is therefore common in activated sludge, where it is usually attached to the flocs (Fig. 27). However, it is often rare in fresh sewage and highly loaded plants.

Fig. 1 – 11. Some common protozoa in activated sludge. **1:** *Hexamita fusiformis*, a diplomonadid flagellate. **2:** *Hexamita pusilla.* **3, 4:** *Trepomonas agilis communis.* Broad side (3) and narrow side view (4) showing the twofold rotational symmetry. **5:** *Trigonomonas compressa. Trigonomonas* has only 3 flagella per bundle. **6:** *Hartmannella* sp., a naked amoeba. **7:** *Amoeba proteus.* The cytoplasm is usually dark due to many refractive inclusions. **8:** *Euglypha rotunda*, a testate amoeba. **9:** *Euglypha* sp., a testate amoeba with spiny test. **10, 11:** Morphology of the peritrich ciliate *Opercularia asymmetrica.* **10:** Small colony with two extended and one contracted specimens. **11:** Ciliature and nuclear apparatus of a swarmer (50 µm) after protargol impregnation.


trailing flagellum
1
2
Hexamita fusiformis
22 – 27 µm
Hexamita pusilla
10 – 13 µm
nucleus
3
4
flagella
Trepomonas agilis communis 13 – 25 µm (3, 4)
5
6
7
pseudopodia
Trigonomonas compressa
24 – 33 µm
Hartmannella sp.
30 µm
Amoeba proteus
300 – 600 µm
nucleus
nucleolus
contractile vacuoles
food vacuoles
granular layer
test
pseudo-podia
pseudo-stome
8
Euglypha rotunda
40 µm
spines
mouth platelets
9 Euglypha sp.
oral ciliature
peristomial disc
vestibulum
contractile vacuole
macro-nucleus
food vacuoles
mitochondria
acontractile stalk
contracted cell
sludge floc
10
Small colony
length of extended specimens 28 – 75 µm
oral ciliary spiral
macro-nucleus
micro-nucleus
aboral ciliary wreath
scopula
11
Ciliature and nuclear apparatus of a swarmer
Opercularia asymmetrica (10, 11)

Naked Amoebae

Naked amoebae ("Gymnamoebae") lack a test. They live in all aquatic and terrestrial habitats including activated sludge. Gymnamoebae are bacteriovores or predators of other protists and small metazoans such as rotifers. An increased abundance of small naked amoebas indicates low performance of the plant due to very high loading, not easily degradable wastes, or technical problems. Common genera in activated sludge are *Amoeba*, *Hartmannella*, *Naegleria* and *Vahlkampfia*. Their identification is rather difficult and representations of only two genera are figured (Fig. 6, 7).

Testate Amoebae

Like heterotrophic flagellates, the testate amoebae ("Testacea") do not form a monophyletic group. In activated sludge, moderate or high abundances of testate amoebae indicate a high sludge age of 10 days or more, stable plant conditions, and nitrification (oxidation of ammonia via nitrite to nitrate). Usually, only one or two testacean species occur in an activated sludge sample, but they may be very abundant. Common genera are *Arcella*, *Difflugia*, *Euglypha*, *Frenzelina*, *Centropyxis*, and *Pyxidicula*. As an example, *Euglypha* is briefly described. Further genera are contained in the chapter Testate Amoebae in Mosses and Forest Soils, p. 97 – 110.

Euglypha spp. SCl. Testaceafilosia, O. Euglyphida, Fam. Euglyphidae (Fig. 8, 9, 28)

Euglypha species have a 20–50 µm (*E. rotunda*) to 135–170 µm (*E. aspera*) long, ovoid test built of overlapping, endogenous siliceous platelets that, in some species, bear spines. The aperture of the test (pseudostome) is surrounded by denticulate "mouth platelets". The pseudopodia are filiform. The nucleus and the contractile vacuoles are in the rear portion of the cell. The cytoplasm is usually colourless, and movement is an amoeboid gliding, often with erect test. Euglyphids feed mainly on bacteria, yeast, and algae.

Ciliates

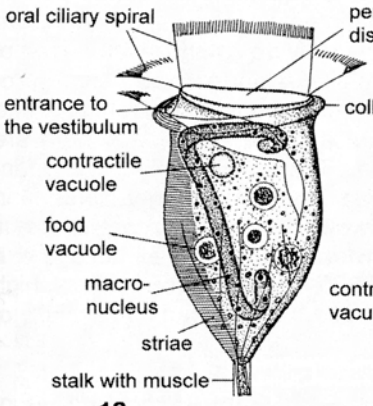
Ciliates (Ciliophora) are usually the most striking protozoa in activated sludge. Important groups are the peritrichs, the hypotrichs, and the cyrtophorids.

Opercularia asymmetrica SCl. Peritrichia, O. Sessilida, Fam. Operculariidae (Fig. 10, 11, 32)

Opercularia asymmetrica is 28–75 × 13–40 µm in size (without stalk) and spindle-shaped with the ventral side slightly shorter than the dorsal side (therefore the name

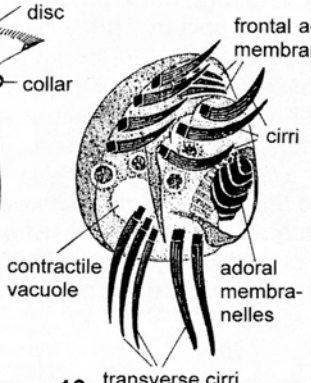
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Fig. 12 – 21. Some common protozoa and micro-metazoa in activated sludge. **12:** *Vorticella convallaria*, a peritrich ciliate. **13:** Ventral view of *Aspidisca cicada*, a hypotrich ciliate. **14 – 16:** Cross sections (above) and dorsal views (below) of *Aspidisca turrita* (14; with spines), *Aspidisca cicada* (15; with ridges), and *Aspidisca lynceus* (16; smooth). **17:** *Euplotes affinis*. **18, 19:** Morphology of the cyrtophorid ciliate *Chilodonella uncinata*. **18:** Ventral view in vivo. **19:** Ciliature of ventral side after protargol impregnation. **20:** The rotifer *Philodina*. **21:** A nematode.



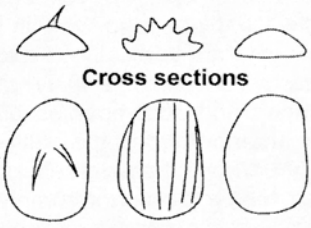
12

Vorticella convallaria
60 – 80 µm



13

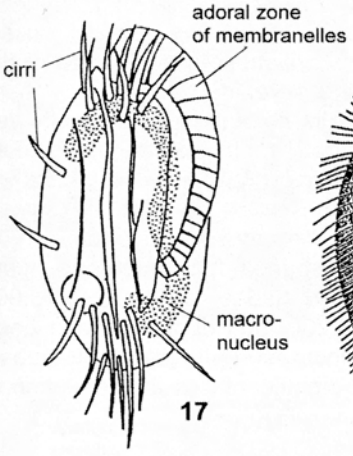
Aspidisca cicada
30 µm



14 15 16

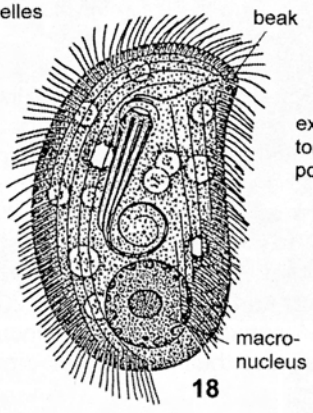
Aspidisca turrita ***Aspidisca cicada*** ***Aspidisca lynceus***

Comparative morphology of *Aspidisca* species



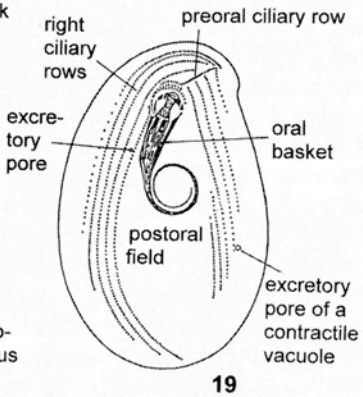
17

Euplotes affinis
40 – 70 µm
Ventral view



18

Ventral view in vivo

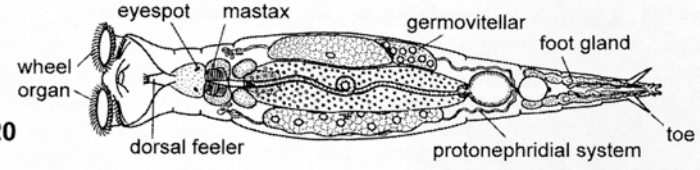


19

Ciliature of ventral side

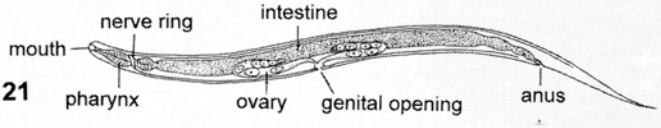
Chilodonella uncinata 40 µm (18, 19)

The rotifer *Philodina*
400 µm



20

A nematode
100 – 1000 µm



21

asymmetrica). Contracted cells are globular. The cells (zooids) are frequently without a stalk but, if one is present, it is thin (about 2 µm in diameter), short and usually unbranched. Thus the zooids are often solitary and only rarely do colonies composed of between 2 and 14 specimens occur. The peristomial disc is narrow and usually projects slightly beyond the thin peristomial collar. The vestibulum is comparatively large and extends vertically to mid-body. The oral ciliature and the silverline pattern are clearly recognizable only after silver impregnation. The macronucleus is bean-shaped and accompanied by a 3–4 µm micronucleus. The contractile vacuole is in the anterior third of the cell on the dorsal vestibular wall. The cell surface (pellicle) is very narrowly transversely striated. *Opercularia asymmetrica* is, like all peritrichs, a filter feeder devouring mainly suspended bacteria (2000–3000 per hour). Thus, high numbers of peritrichs distinctly reduce the number of free bacteria and the turbidity of the purified waste water.

Vorticella convallaria group SCI. Peritrichia, O. Sessilida, Fam. Vorticellidae (Fig. 12, 33)

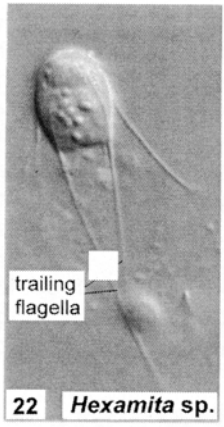
Extended cells are usually 70 × 40 µm in size (without stalk) and bell-shaped when extended. They are globular when contracted. The peristomial collar projects slightly beyond the body proper. The peristomial disc is slightly convex and raised, while the vestibulum is moderately large and extends obliquely to the centre of the body in feeding specimens. The oral ciliature and the silverline pattern are clearly defined only after silver impregnation. The cytoplasm is colourless to distinctly yellowish (*Vorticella citrina*). The macronucleus is J-shaped and the contractile vacuole lies on the ventral wall of the vestibulum. The cell surface (pellicle) is very narrowly transversely striated. The stalk is unbranched, 4–7 µm across, 50–800 µm (usually about 150 µm) long, and contracts helically due to a distinct muscle (myoneme). *Vorticella convallaria* is attached to sludge flocs; when abundant, it may form pseudocolonies. The species is common in healthy activated sludge where up to 18,000 specimens/ml occur; in stabilization ponds up to 19,200 individuals/ml have been counted. It is very likely that *V. convallaria* indicates increased nitrification. It ingests up to 14,000 bacteria per day, and thus contributes significantly to the reduction of floating bacteria.

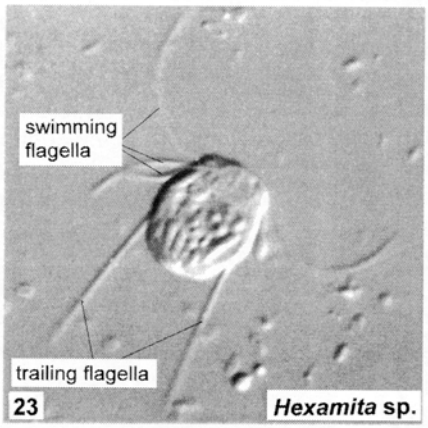
Aspidisca spp. Cl. Spirotrichea, O. Euplotida, Fam. Aspidiscidae (Fig. 13 – 16, 29 – 31)

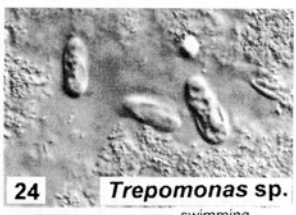
The size of both *Aspidisca cicada* and *Aspidisa turrita* is 25–40 × 20–40 µm, while *Aspidisca lynceus* is slightly larger (35–50 × 30–45 µm). The body is rigid and shows

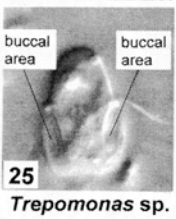
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Fig. 22 – 32. Light (22–27, 32) and scanning electron (28–31) micrographs of some activated sludge protozoa. **22, 23:** *Hexamita* species, a diplomonad flagellate (length about 20 µm). **24:** Specimens of *Trepomonas* sp. among bacterial flocs (length about 15 µm). **25:** A *Trepomonas* species (length about 15 µm) with conspicuous, concave buccal areas. **26, 27:** *Bodo saltans* (length about 7 µm), a common heterotrophic flagellate in activated sludge, has a swimming and a trailing flagellum. Many specimens are often seen attached to a sludge floc. **28:** Test of *Euglypha rotunda* (length 40 µm). Note the serrated mouth platelets. **29, 30, 31:** The dorsal side of the hypotrich ciliate *Aspidisca. A. cicada* has ridges (29), while that of *A. turrita* has a conspicuous horn (30); by contrast, the dorsal side of *A. lynceus* (31) is smooth. **32:** Three solitary specimens of the peritrich ciliate *Opercularia asymmetrica* attached to a bacteria floc.

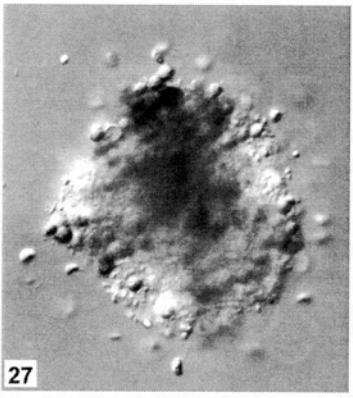




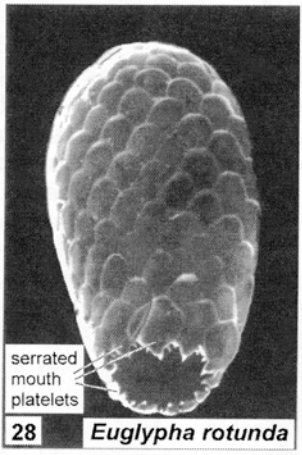


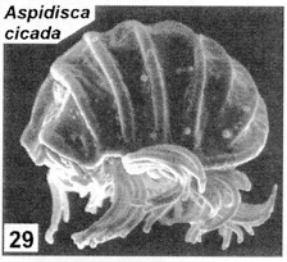


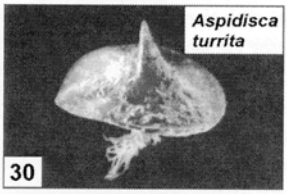


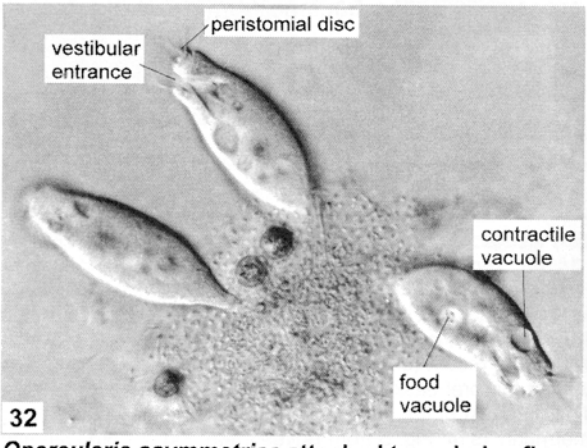


Bodo saltans, many specimens attached to a sludge floc

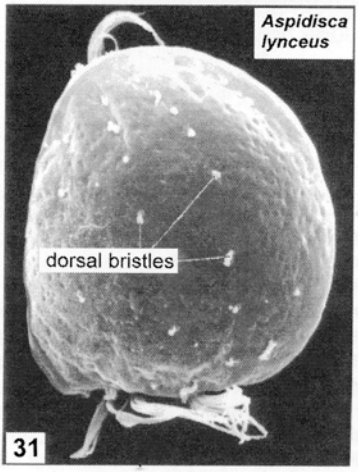








***Opercularia asymmetrica* attached to a sludge floc**



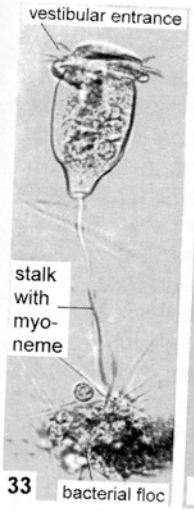
a roundish-triangular outline, that is, the right cell margin is convex, the left almost straight, the anterior is rather broadly rounded, and the rear transversely truncated. The ventral side is plane, the dorsal slightly to distinctly vaulted in *A. lynceus*. By contrast, *A. cicada* has 6–8 distinct dorsal ridges, and *A. turrita* has a dorsal horn. The macronucleus is horseshoe-shaped, the contractile vacuole is in the right posterior part of the body. The ciliature of *Aspidisca* is very conspicuous being composed of thick cirri arranged as shown in figure 13. By contrast, the dorsal side bears 5–6 longitudinal rows of very short bristles. The oral apparatus is bipartite: anteriorly are three small frontal membranelles; in the left posterior part of the body is a bowl-shaped buccal cavity with about 11 adoral membranelles.

Aspidisca species belong to the most common and often most abundant ciliates (up to 40,000 specimens/ml) in activated sludge. They indicate low to medium sludge loading and sufficient oxygen supply. Like the cyrtophorids (see below), *Aspidisca* species graze on the sludge flocs making them compact.

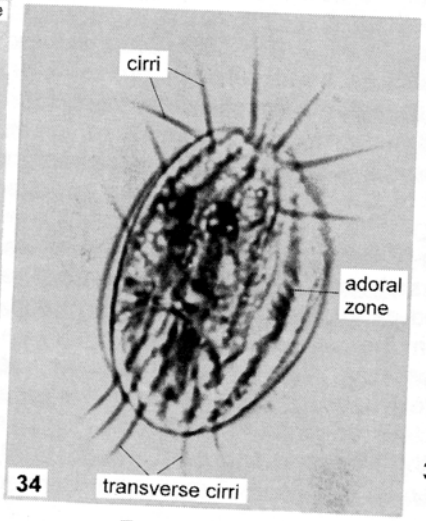
Euplotes spp. Cl. Spirotrichea, O. Euplotida, Fam. Euplotidae (Fig. 17, 34, 35)

The size of the common *Euplotes* species ranges from 40–70 × 25–45 µm (*E. affinis*) to 140–230 × 95–35 µm (*E. eurystomus*). The body is rigid and has an elliptical outline. The ventral side is roughly plane, while the dorsal side is vaulted and more or less distinctly longitudinally ribbed. The macronucleus is C- to 3-shaped and the contractile vacuole is in the right posterior part of the body. The ciliature of *Euplotes* spp. is conspicuous and consists of nine (*Euplotes affinis*, *E. aediculatus*, *E. eurystomus*) or 10 (*E. moebiusi*) thick frontoventral cirri and five distinct transverse cirri and 3–4 caudal cirri near the posterior end of the cell. On the dorsal side are 6–11 longitudinal rows of very short bristles. The oral apparatus is huge: it commences at the anterior end and extends along much of the left body margin. *Euplotes* species are common inhabitants of sewage treatment plants, showing abundances of up to 10,000 individuals/ml. *E. affinis* (Fig. 17, 34) is characteristic of activated sludge plants with sludge stabilization, and indicates a good effluent quality. Like *Aspidisca* and cyrtophorids, *Euplotes* species are grazers, ingesting bacteria from the surface of the sludge flocs.

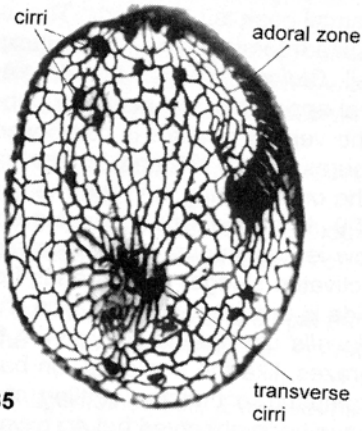
Fig. 33 – 38. Common ciliates in activated sludge. **33:** *Vorticella convallaria*, a peritrichous ciliate attached to a bacterial floc, length 70 µm. **34, 35:** Ventral view of the hypotrichous ciliate *Euplotes affinis* (34) and silverline pattern of *Euplotes moebiusi* (35). **36 – 38:** The cyrtophorid ciliate *Chilodonella uncinata* (length about 40 µm) is a common inhabitant of activated sludge which grazes bacteria from the surface of the sludge flocs. **36:** Ventral view showing, inter alia, the excretory pores of the contractile vacuoles and the non-ciliated field behind the mouth (oral basket). **37:** Lateral view after protargol impregnation, showing the macronucleus and the oral basket whose rear end is conspicuously rolled up. **38:** Ciliature and silverline pattern (fine meshes) of the ventral side after dry silver nitrate impregnation. (Fig. 1 – 5 from Pascher & Lemmermann 1914; Fig. 6 from Bovee 1985; Fig. 7 from Liebmann 1962; Fig. 20 from Bunke & Schmidt 1976, *Philodina citrina* (Rotatoria) – Film E 2332 IWF Göttingen; Fig. 21 from Schwab 1995; Fig. 32 from Aescht & Foissner 1992)



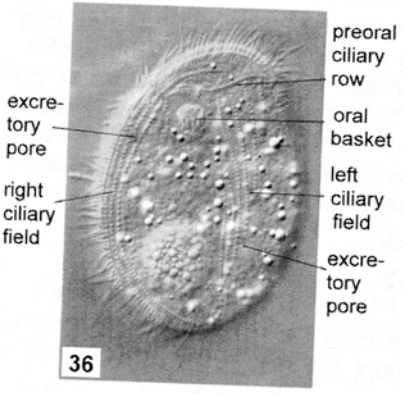
Vorticella convallaria



Euplotes affinis
Ventral view



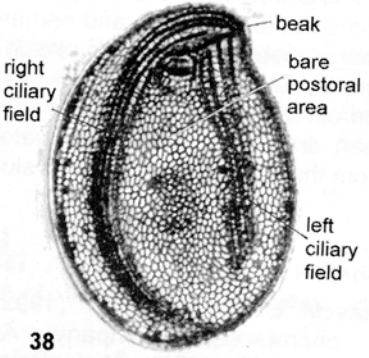
Euplotes moebiusi
Silverline pattern



Ventral view in vivo



Lateral view, protargol impregnation



Ventral side, ciliature and silverline pattern

Chilodonella uncinata (36 – 38)

Chilodonella uncinata Cl. Phyllopharyngea, O. Cyrtophorida, Fam. Chilodonellidae (Fig. 18, 19, 36 – 38)

Chilodonella uncinata has a usual size of 45 × 30 µm and ellipsoidal outline with a small beak left anteriorly. The cell is flattened ventrally and more or less distinctly vaulted dorsally. The macronucleus is globular and usually near the rear end of the cell. *Chilodonella* has two contractile vacuoles, the anterior immediately behind the oral apparatus, the posterior subequatorially near the left margin of the ventral side. The ventral ciliature is composed of 10–12 longitudinal rows arranged as shown in figures 18, 19, 36 and 38, that is, in two groups that leave blank the postoral area. The oral opening is in the median of the anterior quarter; the oral basket, consisting of 9–11 strong rods extending dorsally, has its rear end rolled up. The preoral ciliary row is continuous (not fragmented as in *Pseudochilodonopsis* which also occurs in activated sludge) and extends from the oral opening to the anterior beak. The dorsal side is unciliated, except for a short, subapical row, the so-called dorsal brush. *Chilodonella uncinata* is common in freshwater habitats and in activated sludge where it grazes, like the euplotids, on bacteria adhering to the flocs which therefore become compact so that the settling of the sludge is improved. Up to 1200 specimens/ml have been observed but an oxygen content below 1 mg/l is hardly tolerated.

Micro-Metazoa

The abundance of metazoa is usually very low in activated sludge. Often only some rotifers (Fig. 20) and nematodes (Fig. 21) are present. They indicate – like testate amoebae – high sludge age. Occasionally one can find other metazoa, for example, *Aeolosoma hemprichi* which is easy to recognize by the red "oil-droplets" in the epidermis. This minute (~ 1 mm) oligochaete can reach up 180 individuals/ml and then drastically reduces activated sludge biomass. Metazoans can be eliminated from the system by increasing sludge loading and so reducing sludge age.

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Ecological Methods for the Study of Heterotrophic Nano- and Microplankton of Fresh and Marine Waters

Thomas Weisse, Jens Boenigk and Helga Müller

INTRODUCTION

Planktonic organisms ranging in size from 2–20 µm are termed nanoplankton, while those in the next size class from 20–200 µm, are called microplankton. These size classes are operationally defined: 20 µm is the lower limit of common plankton nets and 2 µm is, with a few exceptions, the lower size limit of pelagic eukaryotes. The nanoplankton and microplankton are composed of both autotrophic and heterotrophic protists. The heterotrophic nanoplankton in the free water column (pelagial) of lakes and oceans consists mainly of colourless flagellates. Even smaller 'picoflagellates' (<2 µm) occur regularly, in particular in nutrient-rich freshwater bodies, but they are also numerous in the ocean. Their contribution to the total protist biomass is, however, negligible. Heterotrophic flagellates larger than 15 µm are considerably less abundant than smaller forms and require a different method of quantification (Arndt et al. 2000). Small ciliates between 7 and 30 µm form another part of the heterotrophic nanoplankton and microplankton. Species composition, abundance and biomass as well as estimates of grazing, growth and loss rates are the most frequently investigated factors in ecological studies (Weisse 2004).

Pelagic nanoprotists are of tremendous importance for the processing and cycling of matter in all aquatic ecosystems. **Heterotrophic nanoflagellates (HNF)** are the most abundant eukaryotic organisms in the pelagial of fresh and marine waters, and they are the primary consumers of bacteria. Their selective grazing affects the composition of bacterial communities, in terms of their size distribution, taxonomic composition and biogeochemical pathways. By their feeding and excretion, flagellates remineralise nutrients (N, P), and flagellate grazing thus stimulates bacterial and phytoplankton growth. The HNF are a functional unit and comprise several groups of organisms that are only distantly related. The abundance of HNF in different pelagic habitats can vary from approximately 20 to more than 20,000 HNF ml^{-1}, but usually ranges from 100 to 10,000 HNF ml^{-1}. Rather similar taxonomic groups form the major part of the flagellate community in very different environments (Arndt et al. 2000). Heterokont taxa (mainly bicosoecids and chrysomonads such as *Spumella*, Fig. 1 – 4) are numerically dominant and contribute 20–50% to the average HNF biomass, followed by choanoflagellates (5–40%) and kathablepharids (10–>25%). Though always present, kinetoplastids, small dinoflagellates, thaumatomonads, apusomonads, colourless cryptomonads and euglenids usually form only a minor part of the HNF biomass.

The majority of planktonic **ciliates** fall into the microplankton size class (20–200 µm). Small nanoplanktonic species are common within the subclasses Oligotrichia

and Choreotrichia (Class Spirotrichea, Fig. 5), the subclass Scuticociliatia (Class Oligohymenophorea) and the order Prostomatida (Class Prostomatea, Fig. 6). As with their bacterial, HNF and phytoplankton prey, the total abundance of pelagic ciliates is related to the trophic state of the habitat. Total cell numbers in lakes and in the ocean usually range from 0.1 to 200 cells ml^{-1}, with the low end typical of oligotrophic waters. High numbers are found in hypertrophic ponds and polluted coastal areas. Most planktonic ciliates (Fig. 5) prefer small algae and HNF as food and thus contribute substantially to their total grazing loss. These small and numerous ciliates are also an important food source for larger predators, such as copepods and daphnids.

The great majority of the nanoplanktonic and microplanktonic **dinoflagellates** live in the ocean, where some colourless species can be highly abundant. Many dinoflagellates are mixotrophic. Little is known about the significance and ecological role of other protozoan taxa, such as naked and testate **amoebae** and the **heliozoa**. The former range from about five to several hundred µm in size and are common in marine and freshwater plankton. Naked nanoamoebae (5–20 µm) possibly prevail in lakes. Evidence based mainly on direct counts of live samples suggests that they occur in abundances of up to >100 cells ml^{-1}. Large populations of heliozoans with peak abundances exceeding 50 cells ml^{-1} have been recorded from mesotrophic and eutrophic freshwater lakes.

PRACTICAL TECHNIQUES

Sampling

Planktonic protists can be obtained from virtually any water body at any time. Water samples are taken with closing sampling bottles (for example, Ruttner or Niskin bottles) from various depths or as bucket samples from the surface. Net samples are inadequate for quantitative sampling of the nanoplankton because a major part of the tiny cells will be squeezed through the meshes of the net. In temperate, small inland water bodies, which are thermally stratified during the growing season and which may become anaerobic close to the bottom in summer, it is recommended to take a vertical profile of several samples.

Live Observation and Raw Cultures

Live nanoprotists can be observed within several hours or even days after sampling, provided that massive changes in water temperature are avoided. However, the community composition may change even within hours after sampling because some taxa may be very sensitive to sampling and handling, while others may take advantage of the removal of larger predators, thus rapidly increasing their cell numbers. If larger nanoprotists and microprotists are the target and metazoan predators such as copepods or daphnids are present, the sample should be filtered gently through a 200 µm mesh gauze to avoid loss of cells due to predation. A smaller mesh size (10–15 µm) should be used for the study of HNF, which will be preyed upon by many metazoa and ciliates. Inverse filtration, that is, the sieve is gently pressed into the water sample and the filtrate removed by a large pipette, is recommended in both cases. In ponds and trenches rich in nutrients, and also in coastal areas and rock

pools with high numbers of flagellates and nanociliates, it is sufficient to pipette one or a few drops of the sample onto a slide. The protists can be observed under a stereomicroscope or, preferably, with phase contrast at magnifications ranging from x 100 to x 1000. Chloroplast-bearing species can be differentiated from colourless taxa by their colour.

Live observation may be facilitated by simple enrichment cultures. In the case of bactivorous taxa, growth of the bacterial food must be promoted. This can be achieved by inoculating a small volume, say 10–50 ml, of an adequate bacterial medium with a few ml of the sample. Reagent tubes, microculture tissue plates, small Erlenmeyer or culture flasks and small Petri dishes can be used for bacterial and algal enrichment cultures. If the culture is kept at temperatures ranging from 10–25° C, bacterial numbers usually increase within the first one or two days.The bacterial peak

Table 1. MWC medium (Modified Woods Hole Medium) for the culture of autotrophic and heterotrophic freshwater protists.

Stock solutions		gl^{-1}
(1)	$CaCl_2 \cdot 2H_2O$	36.76
(2)	$MgSO_4 \cdot 7H_2O$	36.97
(3)	$NaHCO_3$	31.50
(4)	$K_2HPO_4 \cdot 3H_2O$	4.35
(5)	$NaNO_3$	42.50
(6)	$Na_2Si0_3 \cdot 9H_2O$	21.20
(7)	$FeCl_3 \cdot 6H_2O$	3.15
(8)	Combined trace elements:	
	Na_2EDTA	4.36
	$CuSO_4 \cdot 5H_2O$	0.01
	$ZnSO_4 \cdot 7H_2O$	0.022
	$CoCl_2 \cdot 6H_2O$	0.01
	$MnCl_2 \cdot 4H_2O$	0.18
	$Na_2MoO_4 \cdot 2H_2O$	0.006
	H_3BO_3	0.13
(9)	Vitamin mix:	
	Thiamine HCl (Vit. B1)	0.1
	Biotin (Vit. H)	0.0005
	Cyanocobalamin (Vit. B12)	0.0005
(10)	TES (= buffer; add dry when making up medium)	0.115

Medium

Stock solutions (1 – 9)	1.0 ml each
Dry buffer (10)	0.115 g

Combine stock solutions 1–8 and the dry buffer (to yield pH 8.0, with 4 drops of 1 M NaOH) and make up to 1 litre with deionized water. Autoclave for 20 minutes at 121° C. Add 1 ml of Vitamin mix (9) under sterile conditions. If an analytical balance with high precision is not available, a larger amount of the various ingredients can be weighed and then diluted to the respective concentration. TES buffer denotes N-[Tris(hydroxymethyl) methyl]-2-aminoethanesulphonic acid. All chemicals can be purchased from various biochemical manufacturers.

is followed by a dense population of HNF. Higher temperatures usually enhance the bacterial and flagellate growth. An alternative approach is to add a cooked and then

autoclaved wheat grain to a few ml of the natural sample. The wheat grain serves as the complex substrate for the various bacteria that will support the growth of HNF.

Algivorous flagellates and ciliates can be obtained from algal enrichment cultures. An algal medium (Table 1) is inoculated with an adequate food alga. Small (<10 µm in length) cryptophytes of the genera *Cryptomonas* and *Rhodomonas* are the preferred food of many freshwater and marine protists (Fig. 7). They can be easily obtained from several culture collections, such as the Culture Collection of Algae and Protozoa (CCAP) at Oban, Scotland (www.ccap.ac.uk). The culture collections also provide information on adequate media. Routinely, we use the MWC medium (Table 1) for many freshwater protist species. When the algal culture has reached high cell numbers (10^4 to 10^5 ml^{-1}), several ml of the original water sample with the natural protist community may be added. While bacterial enrichment cultures can be kept in the dark, algal enrichment cultures must be reared in dim light; note that direct sunlight causes photodamage in many protist species. Such bacterial and algal enrichment cultures may be kept for several weeks at room temperature.

Quantitative Investigations

Live counting

Quantitative live counting of the most numerous HNF can be achieved by analysing minute droplets (5–10 µl) of the original sample on slides using a light microscope; note that counting must be performed within a few hours after sampling at the ambient temperature. This technique offers the advantage that some morphological and, primarily, behavioural features can be used for taxonomic identification. Behavioural aspects such as swimming or feeding can be analyzed in more detail with the aid of video microscopy. Live counting is impossible for most of the larger and rarer flagellates, such as dinoflagellates and most ciliate species. Live counting of heliozoans can be performed in small (3–5 ml) Petri dishes or small settling chambers after carefully concentrating one to several litres of the sample with a 10 µm mesh gauze. Due to species-specific sensitivity to various laboratory conditions, live observation after enrichment can not be used for quantitative investigations of the absolute and relative abundance of individual taxa.

Fixation and preservation

Accurate estimates of the abundance of nanoplanktonic and microplanktonic protists entail concentration by filtration, centrifugation or sedimentation, procedures usually only feasible with fixed cells. Natural water samples must be fixed immediately after sampling for quantitative studies. Irrespective of the fixative used, it is advisable to add a larger volume of sample to a small volume of concentrated fixative that has been measured into the sample bottle prior to sampling. Fixation with buffered or unbuffered formalin (37% formaldehyde) or glutaraldehyde (final concentration 1–2% vol/vol) is widely used for flagellates. If the fixed samples are kept cool and dark, they may yield realistic estimates of the abundance of autotrophic and heterotrophic nanoprotists for weeks or even months, although rather fast specific cell loss has been reported in fixed samples. The most common fixative for phytoplank-

ton and ciliates is (acidic) Lugol´s iodine (1–2 % vol/vol) which is available in several modifications. To prepare a Lugol´s stock solution, dissolve10 g potassium iodide in 100 ml of distilled water and slowly add 5 g iodine crystals, while shaking. Thereafter, filter and store the solution in a tightly stoppered brown bottle in the dark. To acidify Lugol´s iodine, glacial acetic acid is added in a ratio of 1:9, that is 10 ml glacial acetic acid are added to 90 ml Lugol´s. Acid Lugol´s usually preserves protists longer than the neutral version. As with aldehyde fixation, Lugol-fixed samples can be stored in a cool, dark place for weeks, but extended storage over months may cause loss of cells. The iodine increases the specific weight of the protists upon fixation, thus enhancing the sinking of cells. Algae and ciliates can therefore be concentrated in settling chambers and counted by inverted microscopy (see below). Lugol´s not only fixes and preserves the cells but also provides them with a light to dark brown colour.

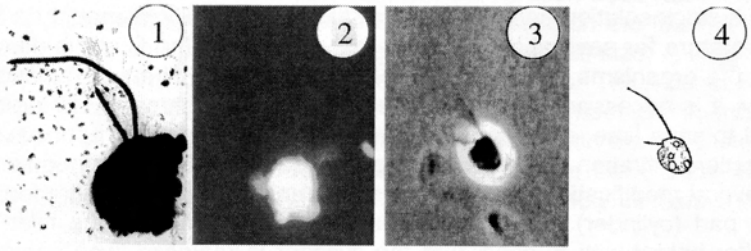
Counting of HNF by epifluorescence microscopy

Epifluorescence microscopy is the currently most widely used method to count natural assemblages of HNF. The method requires several steps. Samples are fixed immediately upon sampling, and then stained with a fluorochrome and filtered in the laboratory; the filter is then mounted onto a slide and investigated under the epifluorescence microscope.

A small volume (2–10 ml, depending on total protist abundance) of the fixed sample is gently vacuum-filtered (at <20 kPa) onto polycarbonate or similar filters of defined pore size (1, 2 or 5 µm) and 25 mm diameter (Fig. 8). Prestaining of the polycarbonate filters by Sudan black or Irgalan black or the use of the respective commercially available prestained filters such as nuclepore black enhances the contrast and simplifies identification by epifluorescence microscopy. Staining solutions are easy to make. Make a stock solution by dissolving 1.0 mg Sudan black in 100 ml

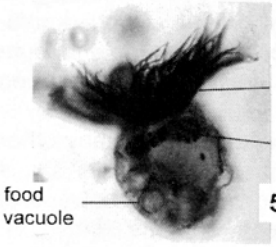
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1 – 4. *Spumella* sp., a chrysomonad flagellate about 5 µm in length. **1:** Scanning electron microscopical photograph of an osmium tetroxide-fixed cell. **2:** Fluorescence microscopical image of a DAPI stained cell after formaldehyde fixation. **3:** Phase contrast image of a live cell. **4:** Schematic line drawing. **Fig. 5.** *Rimostrombidium* sp. (25 µm), a common planktonic oligotrich ciliate after protargol impregnation. Note food vacuoles with ingested diatoms *(Stephanodiscus hantzschii).* **Fig. 6.** *Urotricha furcata* (15 µm), a common prostome freshwater ciliate after protargol impregnation. **Fig. 7.** Laboratory experiment with the common alga *Cryptomonas* sp. as food and the ciliate *Urotricha farcta* as predator. Cell numbers of prey (circles, left Y-axis) and predators (triangles, right Y-axis) were measured each day. Open circles denote *Cryptomonas* sp. in controls without ciliates, filled circles show *Cryptomonas* sp. in experimental containers with ciliates. Symbols denote mean values of two experimnental replicates, error bars 1 standard deviation of the mean. **Fig. 8.** Filtration apparatus consisting of a cylinder, a filter holder with clamp, a vacuum flask as trap for droplets, and a vacuum pump (right).The small parts (cylinder,filter holder, glas funnel and clamp) are arranged in front. **Fig. 9.** Counting procedure using a settling chamber and inverted microscopy. If a chamber with separate upper and lower parts (cylinder and slide) is being used (**9a**), the cylinder will be moved and replaced by the coverslip once the cells have settled (**9b**). The bottom part of the chamber can then be examined with an inverted microscope, and the cells counted according to their abundance (**9c – e**) in fields (**c**), in two transects at right angles (**d**), or in transects across the whole of the chamber bottom (**e**). With compound settling chambers, the whole chamber is placed on the stage of the inverted microscope. (Fig. 1 Copyright The Natural History Museum/G. Novarino)

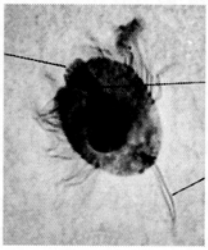


SEM photograph | Epifluorescence image | Live cell in phase contrast | Schematic line drawing

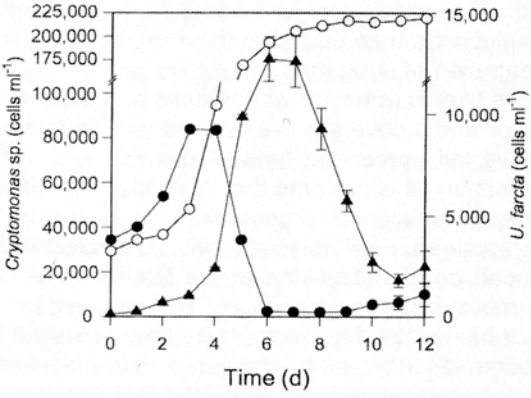
Spumella (1 – 4)



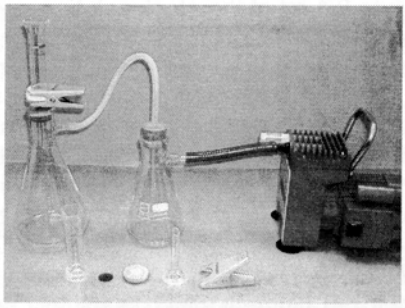
5 *Rimostrombidium*
Protargol impregnation



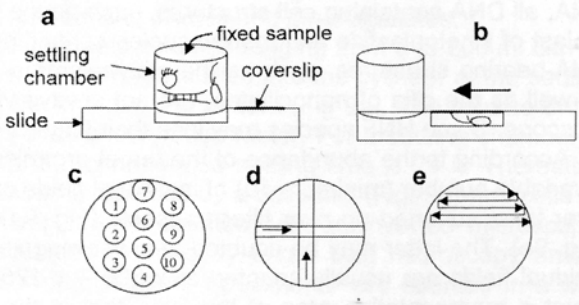
6 *Urotricha furcata*
Protargol impregnation



7 Laboratory experiment with *Cryptomonas* as food and *Urotricha farcta* as predator



8 Vacuum filtration apparatus



9 Counting procedure using settling chambers and the inverted microscope

absolute ethanol (or industrial methylated spirits). To 90 ml of absolute ethanol add 10 ml of this stock solution and 100 ml of distilled water. Filters should be stained at room temperature for several hours. Note that staining of the filters is different from staining of the organisms. To detect the organisms on the filters by epifluorescence microscopy, it is necessary to stain the cells with a fluorochrome. For practical purposes, and to save time, staining is commonly done in a filtration apparatus, directly prior to filtration. Filtration devices can be purchased from laboratory equipment suppliers in several modifications, either as a single unit (Fig. 8) or as filtration manifold. The upper part (cylinder) of the filtration apparatus is removed, the filter is placed onto the filter holder with forceps, and the cylinder is replaced. The sample is then poured or pipetted into the cylinder and the fluorochome is added by pipette. The staining time in the filter holder should range from 1–5 minutes. It is important to adjust the filtration volume to the abundance of the target organisms and the other cells present in the sample. If the sample volume is too small, it is difficult to obtain sufficient cells for an accurate estimate of their natural abundance. If the sample volume is too large, the filter pores may clog and some delicate cells may be disrupted upon filtration.

A large array of fluorochromes specific for proteins, nucleic acids and many other cellular constituents is now available. The most widely used bacterial and HNF dye is the A-T-selective DNA stain DAPI (4',6-diamidino-2-phenylindole). A stock solution of 5 mg DAPI ml^{-1} is prepared by dissolving 10 mg DAPI powder in 2 ml distilled water. This stock solution will keep for months if it is deep frozen. For the working solution, dilute 0.1 ml of the stock solution in 46.3 ml distilled water and add 2.7 ml formalin to prevent bacterial growth. The working solution is then added to the sample in a ratio of 1:9 vol/vol to yield a final DAPI concentration of 1 µg ml^{-1}.

Immediately after vacuum filtration, the filter is removed with forceps and mounted on a slide. A small droplet of immersion oil and a coverslip are then added. To examine the cells with high resolution, that is, oil immersion lenses of x 60 or x 100 magnification, another droplet of oil immersion oil is put onto the coverslip. The filter is now ready for investigation under the epifluorescence microscope.

As with bacteria, counting of DAPI-stained HNF is made with UV excitation (Zeiss filter set 48 77 02). Autotrophs can be identified due to the fluorescence of their red pigment which is usually not masked by the white-blue DAPI-induced fluorescence. Switching to additional blue or blue-violet filter sets (Zeiss filter sets 48 77 09 and 48 77 06) allows for unequivocal discrimination between autotrophic and heterotrophic HNF, if the chlorophyll autofluorescence is weak. Since DAPI binds to DNA, all DNA-containing cell structures, such as the nucleus, mitochondria, the kinetoplast of kinetoplastids and food vacuoles containing prey appear brighter than non DNA-bearing structures, such as the plasmalemma and flagella (Fig. 2). The latter, as well as the cilia of nanociliates, are not always visible in the epifluorescence microscope. Some HNF species may lose their flagella during fixation or filtration.

According to the abundance of the target organisms, cell numbers are counted in a variable number (minimum 10) of individual fields of view (Fig. 9c) or, for larger and rarer taxa, summed up over filter transects (Fig. 9d) or even the entire filtered area (Fig. 9e). The latter may be counted at lower magnification (x 200 – x 400), while individual fields are usually counted at x 600 – x 1250. In any case it is essential to count a representative area of the filter, that is the counting area must be spread

evenly across the filter. This is important because filtration does not always result in the ideal of a completely random distribution of the cells on the filter. An ocular grid facilitates counting and permits a crude estimate of the cell size. A calibrated ocular micrometer is needed for more accurate measurements. The abundance of HNF (cells ml^{-1}) is obtained by (1) dividing the average cell number per counting area (n) by the filtered volume (vol) and (2) multiplying the result with the ratio filtered area to counting area (F); the result needs (3) to be corrected for the dilution effect due to fixation (f; original sample volume divided by sample volume plus fixative added):

$$\text{HNF cell number } (ml^{-1}) = (n/vol) \times (F/f) \qquad \text{(Eq. 1)}$$

A problem inherent to almost any fixative applied is cell shrinkage; only rarely do specimens become inflated upon fixation. If the goal is to arrive at a realistic description of the diversity of the HNF community and its biomass, we recommend combining live observation with epifluorescence counts of fixed material and more detailed investigations with cells originating from enrichment cultures.

Only the most numerous larger flagellates and nanociliates, reaching several tens of cells per ml, may be counted accurately by epifluorescence microscopy. Larger dinoflagellates and ciliates are counted by the Utermöhl technique outlined below. We recommend using a filter pore size between 3 and 5 µm for epifluorescence counting of nanociliates to get rid of most bacteria. The macronucleus and micronucleus of ciliates appear bright white-blue after DAPI staining and UV excitation, while the rest of the cell appears dull blue. The size and shape of the cell, the location, size and shape of the nuclei, and the visible part of the ciliature are the main features used for the taxonomic identification of nanociliates under epifluorescence microscopy.

Counting of nanoplankton and microplankton by the Utermöhl technique

The sampling technique for ciliates, dinoflagellates, amoebae and heliozoans is identical to the one described above for HNF. In fact, the same water sample may be split to fix and count HNF and the other protists separately. Epifluorescence microscopy is inadequate for most pelagic ciliates and dinoflagellates which occur in typical numbers of less than 0.1 ml^{-1} up to 10 ml^{-1}; their cell numbers are comparable to those of many algal species. Accordingly, the Utermöhl (1958) method, where the fixed cells are collected in a settling chamber and investigated under an inverted microscope, has been widely used for counting ciliates and dinoflagellates.

Calibrated settling chambers for sample volumes ranging from 5 to 200 ml can be purchased from several suppliers. The sample is filled into the cylinder of the settling chamber, and the fixed cells will sediment to the bottom (Fig 9a). The volume of the cylinder (5 to 200 ml) determines the concentration factor. For a settling chamber of 50 ml volume and 10 cm height the recommended settling time is 24 h. Thereafter, the cylinder of the sediment chamber is replaced by a coverslip (Fig. 9b) and cells on the bottom of the settling chamber can be counted with an inverted microscope. Counting of ciliates and dinoflagellates is done by bright field microscopy and/or phase contrast at x 100 to x 400 magnification. Specimens are counted in a way analogous to counting HNF by epifluorescence microscopy, that is, the magnification

increases and the counting area decreases in proportion to the abundance of the target organisms (Fig. 9c – e). Protist abundance in the sample can be calculated by dividing mean cell numbers counted by the sampling volume and multiplying the result with the ratio bottom area to counting area. Again, the results need to be corrected for the dilution due to the addition of the fixative (Lugol´s iodine) to the sample.

Unfortunately, there is no single method available that reveals all cell structures needed for a proper taxonomic identification of a ciliate. Likewise, dinoflagellates, amoebae and heliozoans are difficult to identify in Lugol-fixed samples. The method most widely used in ecological investigations is silver staining with protargol (Fig. 5, 6). Protargol stains well the nuclei and the ciliary pattern. Protargol staining can be performed with Lugol-fixed samples after postfixation with concentrated Bouin´s (5 % vol/vol final concentration). Protargol impregnation is described in chapter 18. For a combination of quantitative and qualitative investigations of ciliate communities, special protargol methods (QPS) have been developed.

Estimating Growth and Feeding Rates

Growth and feeding rates of nanoprotists can be measured by several methods, but there is no protocol available that could be applied universally. Predator and prey cycles of bacteria and HNF, or of algae and ciliates, can be measured easily in a) enrichment cultures (described above), b) in nutrient-rich natural samples following differential filtration or c) in laboratory experiments with selected prey and predator species (Fig. 7). An inherent assumption in this approach is that predation controls the numbers of the prey organisms. In the experimental containers with the predators, the numbers of prey organisms vary in response to their growth and grazing rates, so that what is measured is the net change in their abundance. The prey population increases if growth rate is higher than grazing loss and decreases if predation exceeds growth.

Selective filtration

If predators are removed by selective filtration and growth is not limited by other factors, such as substrate or nutrient supply, prey numbers will increase exponentially until the carrying capacity of the system is reached. During the exponential phase, growth approaches gross growth rates. Selective filtration should be performed by inverse filtration described above or by gravity filtration. With the latter technique, 100 to 200 ml of the sample are prefiltered with 50–200 µm mesh size to remove metazoan predators; the sample is then poured into a filtration apparatus similar to the one shown in Fig. 8, but with a larger diameter (>40 mm) and filters with an adequate pore size avoiding pressure or vacuum. Filtration is achieved by gravity of the water column in the filter holder. The pore size of the filters should remove potential predators of the target organisms without damaging the latter. In the example shown in Fig. 7, numbers of the small cryptophyte *Cryptomonas* sp. (prey) and the ciliate *Urotricha farcta* (predator) were monitored over two weeks at 20° C. In such experiments, subsamples are taken every 2 –24 hours, depending on the abundance and growth of the organisms.

Calculating growth and grazing rates

Cell numbers are determined as described above. Growth rates (μ) of prey and predators can be calculated from changes in their numbers (N), assuming exponential growth over the experimental period according to

$$\mu = \ln (N_t / N_0) / t \qquad \text{(Eq. 2)}$$

with N_t denoting numbers at experimental time t, N_o initial numbers, and t time (in h or d).

In the experiment shown in Fig. 7, the population growth rate of *Cryptomonas* sp. in the experimental containers (filled circles) observed during the first three days were positive, because initial ciliate numbers were too low to contol algal cell numbers. During the onset of the ciliate peak, their grazing pressure increased until algal loss rates were higher than their growth rates. This phase (from approximately 4–6 day after the beginning of the experiment) yielded a ciliate gross growth rate of 0.83 d^{-1} (μ =ln (12.724 cells ml^{-1}/2397 cells ml^{-1})/2) because there were no cililiate predators present and ciliate growth was not food limited. If a control experiment is run without ciliates, grazing loss rates of the algae and ingestion rates of the ciliates can be calculated from a comparison of the algal net and gross growth rates in experiments without (controls, open circles in Fig. 7) and with ciliates (Fig. 7, filled circles): The difference between gross growth rates (in controls, μ_g = 0.64 d^{-1}) and net growth rates (in ciliate treatments, μ_n = -1.80 d^{-1}) yields the grazing coefficient (g, unit per time). Multiplication of the grazing coeffizient with the geometric mean algal cell numbers during the experimental period (P_m) yields population loss rate. If the product is divided by the mean cell number of the predators (R_m), their per capita ingestion rate (I, prey cells per ciliate and time) will be obtained:

$$I = \frac{(P_m \times g)}{R_m} \qquad \text{(Eq. 3)}$$

In the example shown in Fig. 7, each ciliate ingested 26.9 algal cells d^{-1} on average between experimental day 4 and 5 (P_m = 53.654 cells ml^{-1}, g = 2.44 d^{-1}, R_m = 4861 cells ml^{-1}).

Note that all experimental containers should be run in duplicate or triplicate to measure variation between parallel treatments and to obtain results as mean values with the respective standard deviation.

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Entries in **bold** refer to pages where a genus, a species, a structure, a developmental stage or technique is depicted. Underlined vowels indicate the pronunciation of the names of genera, species and families.

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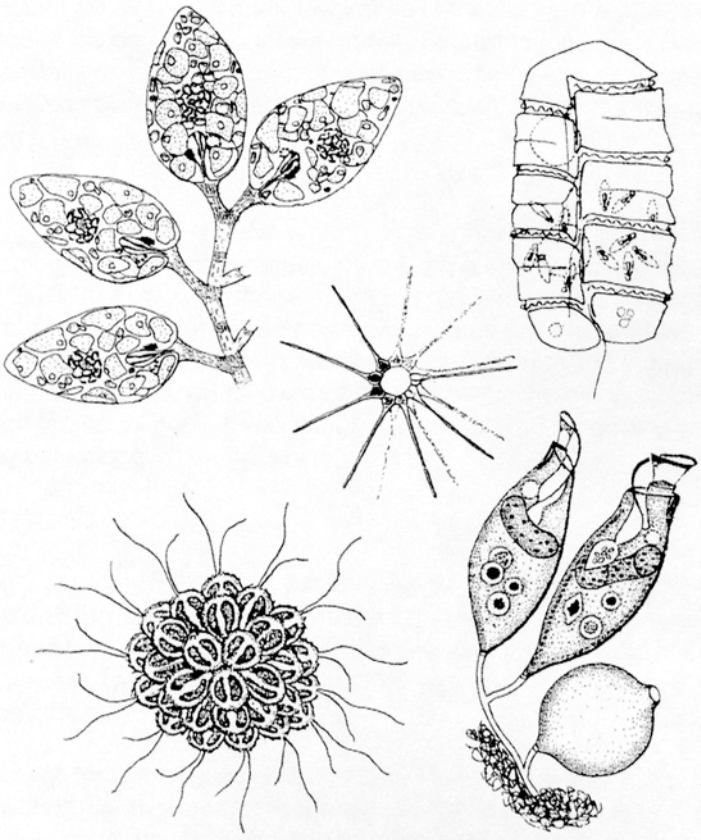
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