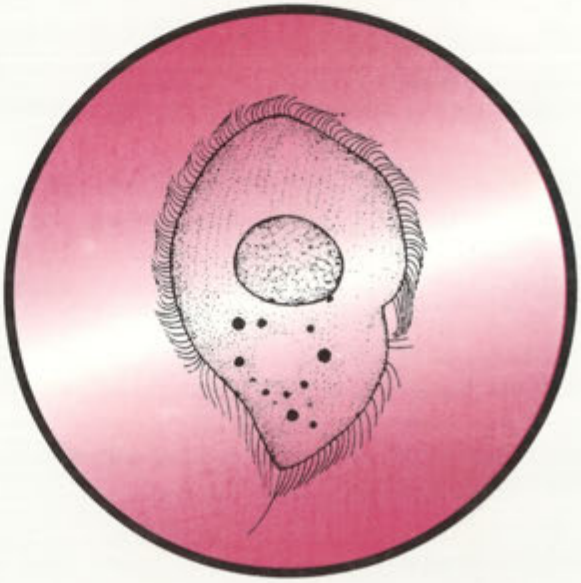
# ACTA PROTOZOOLOGICA



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# The Interface Between Taxonomy and Ecology in Modern Studies on the Protists

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**Summary.** Taxonomy and ecology, extensive fields of research in themselves, are not antagonistic in their relationship to one another. They are complementary areas of inquiry. Both are indispensable in modern biological studies, and they possess an interface of mutual benefit to one another. Protist **ecologists** working in the field – fresh-water, marine, or soil habitats – must strive to carry out more complete investigations, including better descriptions of species, improved statistical analyses, and more accurate distributional records. Codes of Nomenclature should be adhered to with care. On the other hand, protist **taxonomists**, specialists on specific groups, should produce identification keys more useful to field workers and publish more helpful descriptions of both new and old species. Works in the literature should not be ignored or overlooked simply because they appear in non-major journals or in languages other than English. "Model" publications of half a dozen current protistologists who seem to have bridged the gap between ecology and taxonomy are cited as examples of ideal ways to satisfy the requirements of both fields. The interfacing of the areas becomes clear in examination of such papers, works that are often monographic in nature.

**Key words.** Field ecology, taxonomy, algae, protozoa, protists.

## INTRODUCTION

In past years, field ecologists often tended to view taxonomy, in the sense of identification of their organisms, as solely a handmaiden to ecology. Perhaps on the other extreme, in the E. O. Wilsonian view of taxonomy (or, better, systematics) – that it embraces practically all biological disciplines – ecology may be seen merely as supplying one more set of data useful in systematic and evolutionary studies. The truth is that both ecology and taxonomy *sensu lato* are well-defined, if broad, fields of biological inquiry, albeit with areas of overlap. The principal emphasis of the present overview paper is on the interfacing, to mutual advantage, of these two great areas of research with respect to studies on protozoan (or, preferably, **protistan**: see Corliss 1986, 1991b, and references therein) communities in nature.

Biological diversity is a subject of growing interest around the world today. Recall, for example, the theme of the meeting of the 24th General Assembly of the International Union of Biological Sciences convened in September 1991 in Amsterdam, The Netherlands: "Biological Diversity and Global Change." As I emphasized in a special Workshop on Biodiversity of Microorganisms which followed that IUBS meeting,

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ecology and taxonomy both play major roles in studies on such a topic. And the mostly unicellular eukaryotic microorganisms (algae, protozoa, and plasmodial plus zoosporic fungi) are significant factors, too, although many "metazoologists" and "metaphytologists" continue to ignore their far from negligible presence in the biosphere. Indeed, protists are being widely used today in research on population dynamics, biomass, food chains, nutrient recycling, carbon sinks, biocontrol, community structure, colonization, toxicology, symbiosis and mutualism, coevolution, ecosystem functioning, competition, molecular phylogeny, adaptive etho-ecology, and biomonitoring, to mention major areas of interest. The results of such studies in which, in effect, algae and protozoa are being employed as "the experimental organism," are of considerable importance in advancing our understanding of biological phenomena at all levels, and from both applied and theoretical points of view.

An especially appropriate lament that may be made here is one with respect to the many studies made by field microbiologists of fresh-water lake, river, and stream systems that are all too often ignored by (other) ecologists. Furthermore, it is unconscionable if the reason for such neglect is that the work has appeared in a non-major but quite respectable journal and/or in a language other than English.

## TAXONOMY AND FIELD ECOLOGY

In turning to the specific topic of the interrelationship of taxonomy *sensu stricto* to field ecological studies on protists, it must be noted that some of the same problems that have plagued progress in the area for scores of years still remain with us. I reiterated two of these at the last International Congress of Zoology, convened in Monaco some 20 years ago, in brief review of the then current status of protozoan ecology (Corliss 1973a): "(1) the absolute necessity for proper taxonomic identification of the particular species (one or more) that may be involved; and (2) the similar need for more highly refined methods of investigation, including controlled laboratory conditions, approaches that should complement the data-gathering activities in the field." But genuine progress **has** been made with respect to such major difficulties or roadblocks, as will become more apparent in subsequent pages of the present review.

### Inaccurate or Incomplete Identifications

First, we should note briefly why the taxonomic conclusions often reached by field ecologists carrying out surveys or other work on fresh-water, marine, or soil protists are not always satisfactory, making their results considerably less useful than they might be. Generally unable to carry out refined or detailed microscopical observations in the field or to employ rather sophisticated fixing and staining procedures out there, the ecologist must rely on good eyesight, low power light microscopy, and the keys or drawings of earlier workers. And often he or she must work rapidly with their samples. Although in Hungary the superb cytological/taxonomic observations of biologists like the late Professor József Gelei are available in the research literature (see papers in Bereczky 1986), most workers everywhere, even today, are still heavily dependent on the now quite outdated monographs of still earlier workers such as Alfred Kahl, who published on ciliates in the late 1920s and early 1930s (e.g., see Kahl 1926, 1930-1935). Kahl's monographs have been indispensable for many years and are still to be greatly admired today; yet the number of species (of ciliates) has more than doubled since 1935 and the cytological techniques of study have vastly improved. His keys, understandably, are also now out of date.

In many older works, specific names are no longer valid; or, even more often, a species complex may later have been split into several separately identifiable species. The reverse of this last condition is also occasionally true: the outstanding example of all time in the ciliate literature concerns that ubiquitous genus *Tetrahymena* which was known in the past under some 15 different generic names (Corliss 1973b, Corliss and Dougherty 1967), especially *Leucophrys*, *Colpidium*, and *Glaucoma*. Unfortunately, some modern ecologists (and other biologists as well) persist in using those three names as the generic vehicle for certain freeliving *Tetrahymena* species. *Colpidium* and *Glaucoma* **are** perfectly good genera, but only in their own right; thus they are **not** usable as synonyms of *Tetrahymena*, an action that only confounds the situation unnecessarily.

At any rate, the disadvantages of using inaccurate names are obvious; and it is difficult if not impossible to compare results among several studies (contemporary or with some of earlier vintage) if the true identities of the organisms listed in different papers by different authors remain unclear.

On the encouraging side, in recent years some fresh, and more extensive, keys to the "lower" eukaryotes are appearing in the literature. And many field ecologists are finding ways to perform more precise cytological examination of their materials (for a very recent example, consider Bereczky 1991). In some instances, samples are brought back to the laboratory and established in culture, providing opportunities for careful identification over a period of time. It is not surprising that even in survey work new species are quite frequently being described today, attesting to the patience and thoroughness of the investigator: some examples of such studies are cited later (below). Nonetheless, as Foissner (see citations to his works in the bibliography of this paper) has repeatedly pointed out, while **taxonomists** must provide ecologists with better keys, **ecologists** must provide taxonomists with better identifications and improved distributional records. Much valuable information on species distribution is unfortunately being lost because of the difficulties in knowing the proper identity of the species involved.

### Use of Generic Name plus "sp."

A word of advice to workers who simply are not able to make the rather complete morphological, genetic, or physiological investigations so often desirable: do **not** hesitate to use the designation "sp." (meaning exact specific identity unknown or unclear) after the probable **generic** name of the observed organism. It is misleading to be positive about your identification when/if several species may be involved. Perhaps your organism is even **new** to science; but to draw such a conclusion may involve quite a detailed investigation, both of the organism and of the possibly widely scattered relevant taxonomic literature in non-English as well as English outlets and published over a long period of time. The special cases of the popular and nearly ubiquitous species of the ciliate genera *Paramecium* and *Tetrahymena* might be singled out here, especially because of the continuing confusion in some of the ecological literature over the proper nomenclature to employ in each case (despite such efforts at clarification as that published by Corliss and Daggett 1983). It is now 15 years ago since geneticists D. L. Nanney (Nanney and McCoy 1976) and the late T. M. Sonneborn (1975), working respectively on *Tetrahymena* and *Paramecium* species, seized the bull by the horn, bit the bullet, or whatever, and entered the awesome arena of protozoan taxonomy. The results, **very** briefly, were the rather sudden appearance of some 14 sibling (but nomenclaturally valid and separate) species of a *Paramecium aurelia* complex (the number has now risen to 15), and at least 17 separate species of a *Tetrahymena pyriformis* complex.

Unless a field biologist can carry out extensive electrophoretic studies (e.g., yielding esterase zymograms of comparative value), or the necessary cross-breeding genetic experiments (requiring acquisition of pedigreed mating types, etc.), or certain sophisticated molecular approaches (e.g., determining DNA base ratios), he or she is advised to designate organisms that "look like" the old (although the **name** "aurelia" is now unavailable) *Paramecium aurelia* or the (now strictly amicronucleate) *Tetrahymena pyriformis* as simply/safely "*Paramecium* sp." or "*Tetrahymena* sp." It might be wise, in addition, to include a footnote mentioning the complexes of (sibling but taxonomically valid) species involved in each case (again, see Corliss and Daggett 1984, for more detailed advice).

### Frustrations of and Advances in Field Ecology

Fortunately, most species – be they flagellates, amoebae, or ciliates – encountered in fresh-water, marine, or soil habitats do not have the complex taxonomic background described above for *Tetrahymena* and *Paramecium*. Nevertheless, for most protists identification is never easy; and the modern investigator must keep abreast of the latest taxonomic literature on his or her group. I can appreciate the frustration to the field worker when so-called "key" characters separating a new form from other species of a given genus may involve **ultrastructural** features hardly visible to an investigator to whom an electron microscope is not available. On the other hand, there is less excuse, today, for not using fixation fluids directly in the field on (adequate) samples collected there that will permit use, back in the laboratory, of one of several cytological methods yielding excellent preparations for comparative taxonomic study. For ciliates, in modern times, the outstanding example is any one of the several major techniques of silver impregnation, which are being more widely employed with every passing year since their popularization commencing some three to four decades ago (see history in Corliss 1979). Incidentally, many taxonomic ciliatologists today practically **insist** on the usage of silver methods by **everyone**, although admit-

tedly there are occasionally times when it is not possible for a worker to heed such advice.

Not-to-be-overlooked improvements introduced by field ecologists themselves in recent years include better design of their experiments or of their sampling or collecting methods and greater usage of mathematically more sophisticated statistical techniques. As in the case of more precise species identifications, more reliable analyses have permitted more meaningful comparisons of similar field studies carried out by different workers.

## Kinds of Taxonomy

Our concern here is primarily with **alpha** taxonomy: that is, field ecologists generally need to identify only the species that they are observing or collecting. **Beta** taxonomy deals more often with subspecies or genetic strains, mating types, etc., groups of organisms useful also in studies on evolution; and molecular biology may supply the most appropriate techniques in such research. **Gamma** taxonomy is concerned with high-level groups, and with the phylogenetic and evolutionary interrelationships of the members of such taxa. Improvements in – or, at least, **changes** in! – the names and rankings of high-level protist assemblages need be of little concern to the practicing ecologist who is often working on applied problems. Nevertheless, I should like to put in a word of encouragement here with respect to the preferred usage of "protists" over "protozoa" or "algae" since many members of those last two traditionally entrenched groups are so intermingled phylogenetically – and thus taxonomically – that it is unwise to separate them by kingdom or phylum barriers (see Corliss 1984, 1987, 1990, 1991b, and references therein).

Interestingly enough, in recent years, more or less in revival of an older European idea, some American ecologists (e.g., Sieburth and Estep 1985) have proposed that high-level groupings of conventional algal and protozoan supraordinal taxa be separated from other groups on the basis of a synthesis of trophic and taxonomic information. These workers include some prokaryotic (bacterial) assemblages, too, in their "ecological" classification schemes. Such **functional** systems, however, cannot – or should not – replace **taxonomic** classifications, in my opinion (Corliss 1990).

I have concentrated our attention on the service of taxonomy to ecology; but, of course, a mutualistic relationship exists. Certain ecological habits, adaptations, distributions, and other characteristics of a given organism are also often important in the identification or classification of the species concerned. Subsequently useful "key" characters may refer to the protist's predilection for certain food materials, its position in the water column (and other hydrobiological factors), its association with other organisms, or its mode of attachment to the available substrate. Such features or preferences may serve as invaluable and quick guides to later precise identifications by other workers. And they are certainly more "handy" for use by field ecologists than are data, for example, on the ultrastructure of a cilioprotist's buccal organelles!

## Troubles with Codes of Nomenclature

Another aspect of taxonomic-ecological research that cannot be ignored is **purely** nomenclatural in nature. I am referring here specifically to the International Codes of Nomenclature, both botanical and zoological, and their application to problems mainly of the proper specific names to be used when identifying or even just listing organisms that are involved in any given investigation. In defense of the Codes, one should reiterate that their goals – to help maintain universality of and a reasonable stability in taxonomic names of organisms – are highly laudable ones. Chaos would result without such internationally approved guidelines.

Unfortunately, in the unique case of the protists, the two major Codes are sometimes in conflict with one another because of the **dual coverage** of the rather numerous species simultaneously considered to be "animals" by protozoologists and "plants" by phycologists. Particularly affected are species belonging to the taxa containing many photosynthetic forms, such as the dinoflagellates, the euglenids, the chrysomonads, and the like. There is a growing literature on how to resolve the conflicts (real or potential) of this Codes-caused dilemma. At the meetings of the 1st International Conference of Hungary on Protozoology, which were convened in Budapest in 1985, Patterson (1986a) introduced the term "ambiregnal taxonomy" to describe the predicament. Since then, he and I and others (see Corliss 1986, 1990, 1991a, and references therein; and see also Patterson and Larsen 1991, Ride 1982, Ride and Younès 1986, Taylor et al. 1986) have proposed possible solutions to the problems involved. But universal agreement has yet to be reached on many facets of this highly complex matter.

## WORKERS WHO ARE SPECIALISTS IN BOTH TAXONOMY AND ECOLOGY

In bridging what has often been a gap between the protist ecologist and the protist taxonomist, it is ideal to have a single worker who is simultaneously an expert or specialist in both areas of research. Fortunately for progress in protistology, a number of such persons can be found today, investigators clearly aware of the combined problems and determined to contribute to their solutions. Time and space do not permit citation of a full list and discussion of such individuals; but, below, I should like to present, albeit highly selectively, half a dozen current workers whose publications are outstanding in demonstrating these dual qualities, or even **triple** if one includes abilities in the field of cytology as well. Many of their papers neatly illustrate the interface between ecology and taxonomy.

First, let me emphasize that it is not only in modern times that we are blessed with such individuals. In the area of ciliatology (cilioprotistology) alone, one has no difficulty in recalling "greats" or "giants" of the past who were remarkable in their demonstration of these combined talents. Let me mention, for example, the names of several readily recognized past leaders coming immediately to mind: E. Chatton and E. Fauré-Fremiet of France, E. Penard of Switzerland, W. A. Dogiel of the USSR., A. Kahl of Germany, L. E. Noland and C. A. Kofoid of America, and, J. Gelei of Hungary. Still other "oldtimers" could easily be added to the list: see numerous examples in the brief biographies of protistologists included in the historical sketches that I published several years ago (Corliss 1978-1979).

### Outstanding Contemporary Workers

While many laboratories around the world are active today in **either** taxonomy **or** field ecology of protists, rather fewer contain workers who have earned solid reputations in **both** ecological studies **and** taxonomic/cytological investigations. In this paper, I am limiting consideration to just six contemporary workers who are broadly trained persons successful, as **individuals**, in helping to bridge the gap between the major fields of protistological ecology and protistological systematics, the theme of this paper. Several works of these investigators (Drs. Fenchel, Foissner, Lee, Lynn, Page, Patterson: see below) are cited as examples of model publications in their particular areas of coverage. At the same time, I am well aware that still **other** protozoologists (and some of **their** publications) could be used equally well to illustrate the points that I am trying to make. For two good examples of this, let me mention very briefly the productivity of Jean Dragesco, of France and Africa, and that of Anatol Jankowski, of the USSR., men both carrying out exemplary research in ciliatology for some years now. References to most of their papers and monographs may be found in Corliss (1979). But also see Dragesco (1960, 1970, 1984), Dragesco and Dragesco-Kernéïs (1986), and Jankowski (1964, 1973, 1979, 1980). These several citations are representative of the past and continuing major contributions of significance that Messrs Dragesco and Jankowski are making to protozoan ecology and systematics.

Now to the "favored" half dozen.

### Tom Fenchel of Helsingør, Denmark

Dr. Fenchel's interests in protist ecology have been wideranging over the past 25 years. A convenient summary of many of his ideas appears in his well received recent book (Fenchel 1987) entitled "Ecology of Protozoa: the Biology of Free-living Phagotrophic Protists." See also these excellent overview papers: Fenchel (1986a, b). His monographs on the ecology of the marine microbenthos (for a major example, see Fenchel 1969) opened up an entire area of research on protists and helped bring deserved attention to the importance of ciliate communities in such habitats, including the "sulfide system" discovered underlying the oxidized layer of marine sand bottoms (see the seminal paper by Fenchel and Riedl 1970). One of his earliest taxonomic monographs (Fenchel 1965) is still often cited. In these and all of his studies, the Danish worker has not hesitated to describe with accuracy, and often as new species, many of the unique forms of ciliates and heterotrophic microflagellates that he has found. Fenchel has also contributed to theoretical aspects of such phenomena as suspension- and filter-feeding, respiration, and niche differentiation (e.g., see Fenchel 1978, 1980, 1986a,b; Fenchel and Finlay 1983; Finlay and Fenchel 1986). He has deservedly earned an outstanding worldwide reputation, and is currently without doubt **the** leader in modern eukaryotic microbial ecology.

**Wilhelm Foissner of Salzburg, Austria**

Dr. Foissner has produced a large number of papers on topics ranging from the systematics and evolution of diverse taxa of protists (not limited to ciliates) to field ecology of the same or other groups; for example, see such selected quite recent monographic productions as Foissner (1979, 1982, 1984, 1985, 1988, 1989), Foissner and Foissner (1988), Foissner et al. (1988), Foissner and O'Donoghue (1990). Foissner's cytological techniques have included various methods of silver impregnation and scanning and transmission electron-microscopy as well. He has described scores of new species and many new higher taxa, too. One of his most significant monographs to date, not cited above, is his masterful review (Foissner 1987) on progress in the neglected science of soil protozoology. His own field work, involving amoebae and various flagellates as well as diverse ciliate groups, has ranged across many kinds of habitats, terrestrial as well as fresh-water and marine; and he has included precise comparative statistical analyses, in such papers, of his data and of the data of others. His youthful enthusiasm serves as an inspiration to many.

**John J. Lee of New York City, USA**

Dr. Lee, of the American Museum of Natural History and the City University of New York system, has long been interested in the trophic dynamics, community structure, and algal symbioses of selected foraminiferans, as well as the overall systematics of the group (see Bock et al. 1985; Lee 1974, 1980, 1983, 1990; and references within). These marine "sarcodinid" protists represent one of the major taxa in the kingdom Protista, from the point of view of numbers of species and their practical value: many of the fossil forams abundant in the earth's crust are useful (e.g., to the petroleum industry) in determining the age as well as the depositional environment of sedimentary strata. Lee has studied the physiology and ecology – and occasionally taxonomy – of salt marsh communities and has investigated the complex relationship between certain so-called "larger forams" and their indispensable algal endosymbionts. The last-mentioned studies (e.g., see Lee et al. 1985, and references therein; also see Cavalier-Smith and Lee 1985) reveal that such systems may serve as models for investigating both **intercellular** and **intracellular** integration and adaptation, with implications even for the evolutionary origins of such cell organelles as mitochondria and chloroplasts. Workers on living forams are not numerous, and Lee is one of the most respected leaders in the field.

**Denis H. Lynn of Guelph, Canada**

Dr. Lynn, of Guelph, Ontario, started out as an evolutionary protistologist and cell biologist, giving special attention to the ultrastructural features of the kinetidal system of the ciliated protozoa (Lynn 1981, Lynn and Small 1981, and references therein). But at an early date in his career, he also became involved in research on the systematic position of various taxonomically enigmatic ciliates, principally (but not exclusively) members of the class Colpodea (e.g., see Lynn 1976a,b). Along with colleagues E. B. Small and J. O. Corliss, he has proposed novel classification systems for the whole phylum Ciliophora (Lynn and Corliss 1991; Lynn and Small 1988, 1990; Small and Lynn 1981, 1985). In very recent years, Lynn and his students (e.g., see Lynn and Montagnes 1991) have commenced a series of studies on the ecology and taxonomy of species of certain thought-to-be-well-known oligotrich ciliates that dominate the marine microzooplankton. He has also become involved in sophisticated molecular biological studies (e.g., Lynn and Sogin 1988) on the phylogenetic interrelationships of various ciliate groups. Lynn is an authority, too, on protist cytoterminology (Lynn 1988). Today, his ciliatological laboratory is one of the most active on the North American continent.

**Frederick C. Page of Cambridge, UK**

Dr. Page, trained at the University of Wisconsin by the great American protist ecologist L. E. Noland (himself a student of Birge and Juday), is a well known, highly respected authority on the so-called rhizopod sarcodinid amoebae: on their morphology, using living and stained material under the light microscope and fixed specimens under the electronmicroscope; on their systematics, describing new and redescribing old species from fresh data obtained by the techniques just mentioned; on their physiology and laboratory culturability; and on their general ecology (see such major contributions as Page 1967, 1971, 1974, 1976a, b, 1977, 1981, 1983, 1986, 1987, 1988; Page and Siemensma 1991; and other references therein). He has collected and

studied forms from fresh-water, marine, and soil habitats, often bringing samples back to his laboratory to establish thriving populations there. He has provided keys and excellent species descriptions invaluable to protist field ecologists everywhere. A meticulous researcher himself, Page has set high standards for others also working in the difficult field of "sarcodinid" systematics and ecology.

## David J. Patterson of Bristol, UK

Dr. "Paddy" Patterson, of Bristol University, U. K. (but soon to move to Sydney, Australia), has worked in diverse areas of protistology: physiology, ultrastructure, systematics, phylogeny, ecology, and even nomenclature, as mentioned earlier in the present paper. Many of his publications also include heuristic hypotheses and new concepts. Both a "bench" and a "field" worker, his attention most recently has been concentrated on the great and largely unexplored diversity of the heterotrophic microflagellates of marine habitats. Another of his latest research passions centers on solving high-level phylogenetic/evolutionary problems of protist interrelationships by application of the molecular technique of rRNA sequencing, while not neglecting the continuing impact of ultrastructural studies on such fascinating enigmas. The depth, breadth, and diversity of Patterson's protistological interests can be appreciated by scanning the titles alone of the following selected works, some of monographic proportions, cited in the bibliography of the present paper: Brugerolle and Patterson (1990), Larsen and Patterson (1990), Patterson (1978, 1980, 1985, 1986, 1989), Patterson and Brugerolle (1988), Patterson and Delvinquier (1990), Patterson and Fenchel (1985), Patterson and Larsen (1991), Patterson et al. (1989), Smith and Patterson (1986).

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# The Role of Protozoan Communities in Freshwater and Soil Ecosystems

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**Summary.** A selection of production values for testacean and ciliate communities in lakes, streams, mosses and soils is quoted. Two energy budgets for protozoan communities in the field are constructed using ecological efficiences. For the given habitats, protozoan production is compared with that of metazoa. In many ecosystems, protozoa are second only to bacteria in terms of biomass turnover. The main factor controlling production is the distribution of mortality over a growth period, with a number of morphological and behavioural strategies also being important.

Nutrients accumulated by protozoa are released soon after their death, making then available again for the bacteria. Decomposition times of the shells of shelled protozoa depend on environmental factors and can be used as an indicator of microbial activity.

**Key words.** Protozoan communities, freshwater, soil, production biology.

## INTRODUCTION

In this paper, the role of protozoa in natural ecosystems is considered in terms of their contribution to energy flow. The many other functions of protozoa in ecosystems are not considered here. The production of biomass is a process fundamental to the flow of energy and the cycling of nutrients in ecosystems. Thus, information on the production of protozoan communities is necessary. This paper is restricted to testate amoebae and ciliates in freshwater and soil habitats. A selection of annual production values for protozoan communities in the above-mentioned habitats is given in Table 1.

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## PRODUCTION AND ENERGY BUDGETS OF TESTATE AMOEBAE AND CILIATES

In running waters, testate amoebae seem to have only an insignificant role. In soils, however, they are a dominant group, contributing to the cycling of nutrients. This applies in particular to acid humus, less to mull (mild humus) and least of all to arable soils.

In mosses, testate amoebae seem to have a low turnover of biomass, but a high production of individuals. This may be a consequence of their flat and small shells in this habitat, which are adapted to the thin films of water on moss leaves. For protozoa, the P/B quotient is extremely low, but a quotient based on the production of individuals, P/N, yields a value of 85. This demonstrates the influence of habitat structure and phylogenetic adaptation on production biology. Unfortunately, there is still too little information of the production biology of protozoa in this interesting habitat. This also applies to *Sphagnum*, the most important habitat for testate

Table 1

Annual production values of protozoan communities

| Habitat | (i)$m^2a^{-1}$ | kJ $m^2a^{-1}$ | P/B(a) | Author |
|---|---|---|---|---|
| | | *Testacea* | | |
| River (Saale) | $5 \cdot 10^6$ | 2.6 | 17 | Schönborn 1981 b |
| Small river (Ilm) | $262 \cdot 10^3$ | 0.16 | N.D. | Schönborn 1982 c |
| Moss | $145 \cdot 10^6$ | 0.2 | 8.1 (P/N = 85) | Schönborn 1977 |
| Soil (Mull) | $940 \cdot 10^6$ | 50.0 | 44 | Schönborn 1982 b |
| Soil (Mull) | $3.6 \cdot 10^9$ | 258 | 43 | Meisterfeld 1986 |
| Soil (Moder-Raw humus) | $91 \cdot 10^9$ | 397 | 285 | Lousier-Parkinson 1984 |
| Soil (Raw humus) | $13.2 \cdot 10^9$ | 610 | 125 | Schönborn 1986 a |
| Soil (Alpine mat on pseudogley) | $118 \cdot 10^6$ | 10.4 | 5.0 | Foissner & Adam 1981 |
| | | *Ciliophora* | | |
| Shallow eutrophic lake | N.D. | $3.4 - 400 \cdot 10^3$ | N.D. | Finlay 1978 |
| River Saale | $615 \cdot 10^6$ | 215 | 207 | Schönborn 1982 a |
| Brook | $11.1 \cdot 10^6$ | 18.5 | 195 | Schönborn 1981 a |
| | | *Protozoa, total* | | |
| Soil (Tundra) | N.D. | 26.2 | N.D. | Ryan 1977 |
| | | *Gymnamoebia* (*Amoeba* s.p.) | | |
| Sphagnum pool | N.D. | 49.7 | N.D. | Rogerson 1982 |

N. D. – Not determined

amoebae. The only production value available is for a species of naked amoeba, which had a very high value in a *Sphagnum* pool.

Contrary to the testate amoebae, ciliates are rare in soils, and occur frequently in running waters, especially polluted rivers. Their high species diversity and abundance enable ciliates to be used as pollution indicators in rivers. However, they also have an important role in the turnover of biomass.

Ciliate production can also be high in pools and lakes. The highest-known production of ciliate communities was recorded in a shallow, eutrophic lake (Table 1).

Production should be estimated under natural conditions, encompassing an entire year. There are only a few reliable estimates for the production of protozoan communities.

Production values estimated using data for ecological efficiencies obtained from laboratory populations can be used to construct energy budgets for protozoan communities in the field. Ecological efficiencies used in these budgets are average values from all the known data, obtained under various condition: (Table 2). Fig. 1 shows the energy budget for testate amoebae in a raw humus soil (spruce forest), subdivided into litter and humus horizons. A comparison with the most important soil metazoa shows that the production of testate amoebae reaches 73 % of the metazoan production. The additional metazoan data are derived from investigations of beech wood soils.

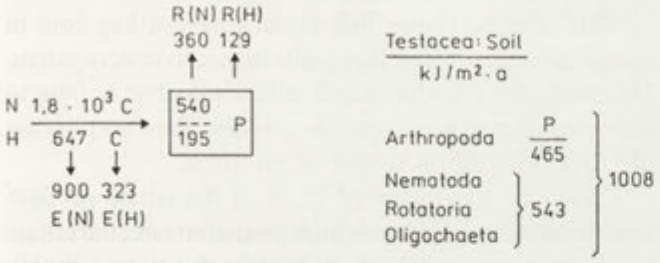


Fig. 1. Energy budget for a testacean community in a spruce forest soil. N = needle horizon, H = humus horizon. Further explanations are given in Tab. 2 (Data from Grimm 1983 and Schönborn 1986 a)

The importance of testate amoebae decreases in mull and arable soils, particularly where there are high densities of lumbricid earthworms.

The diagram also indicates the difference in production between these two horizons. Biomass production in the litter, or needle, horizon is, on average, 2.8 times higher than in the underlying horizon. This is caused by the high surface area of needles in the upper horizon, as well as a high mortality rate in the underlying humus.

Adaptations in shell morphology are apparent in both these horizons (Fig. 2). In the needle horizon, shells are frequently minute and/or flattened, or else they are wedge-shaped with an adhesive ring.

These shapes enable the shells to fit into the thin films of water on the needles. When these films dry up, the shells adhere to the needle surface and the adhesive ring closes the pseudostome. Most of the shells possess idiosomes. Further, the shells have a diaphragm to close the pseudostome, which also protects the cytoplasm from desiccation. In the humus horizon, large and/or spherical forms dominate. These feature a rounded cross section and a reduced pseudostome. These forms are adapted to the lack of moisture in the pores of humus soil. The spherical shape is optimal for minimizing evaporative loss. Most shells in the humus horizon possess xenosomes. Nevertheless, despite the excellent adaptation to dry conditions in the humus horizon, mortality rates are still high. Average mortality in the humus horizon was 11.0% /d, as opposed to 8.8% /d in the needle layer, with this difference being statistically significant (Schönborn 1986a). Production of individuals in the litter horizon in the above-mentioned example (Fig. 1) was 16 times higher than in the underlying horizon. This is significantly higher than the production of biomass, and is due to the large and spherical shell shapes reducing the difference between biomass production in these horizons.

This information of the biology of both soil horizons shows that they belong to two different production regimes, and that habitat structure and phylogenetic adaptation are important factors influencing production. Future research should combine the investigation of production biology and evolution biology.

In the mull, moder, raw humus series (from mild to acid humus), species diversity, abundance and production of testate amoebae generally increase. This also has a phylogenetic explanation. The mull supports an rich invertebrate fauna, whereas acid humus is an environment without strong competition in which the testate amoebae could adapt and subsequently develop.

However, some results may be difficult to interpret. Trends in the above series may also be influenced by the thinness of the mull layer, especially if production values are quoted in planar units. There are also differences in production between mull soils.

Fig. 3 shows the energy budget for ciliates in the polluted River Saale (Germany). Ciliates account for 8 % of the entire invertebrate annual production. In an shallow English lake, this value was higher than 16 %. However, it should be noted that heterotrophic flagellates and naked amoebae were not included in these investigations of protozoan productivity. In rivers, these groups probably have a high turnover of biomass. Thus, protozoan productivity in polluted rivers can be decisive in the cycling of nutrients. In Fig. 3, consumption was determined experimentally and by calculation. The experimental value is higher than the calculated value, which is surprising, and the actual value may lie somewhere between the two.

Table 2

Mean ecological efficiences of rhizopods and ciliates

| | |
|---|---|
| P = 0.25 C (rhizopods) | P = 0.40 C (ciliates) |
| R = 0.20 C (rhizopods and ciliates) | E = 0.50 C (rhizopods and ciliates) |

(P = production; C = consumption; R = respiration; E = egestion)

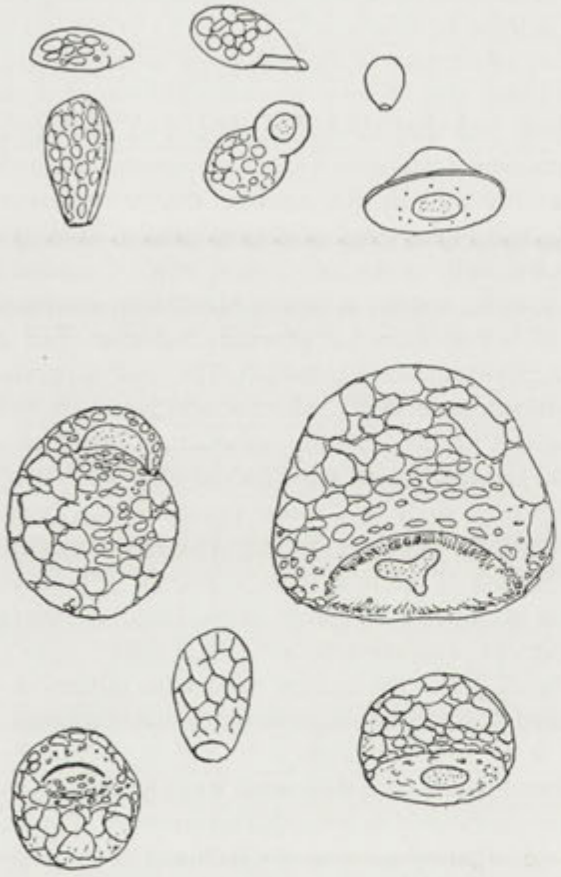

Fig. 2. Adaptation of testacean shells in the needle (litter, above) and the humus (below) horizons

## PROTOZOA AS PART OF THE ENERGY FLOW

As with lumbricid earthworms in eutrophic soils (mull), the leech *Erpobdella octoculata* shifts the turnover of biomass in the direction of metazoan invertebrates. This can be demonstrated by considering the transfer of energy through the River Saale (Germany) (Fig. 4).

There are three main flows of energy:

(1) From bacteria and microalgae to ciliates, to *Chaetogaster diastrophus* (a naidid oligochaete), to *Erpobdella octoculata*. *Chaetogaster diastrophus* is the main predator of ciliates in the River Saale.

(2) From detritus (included bacteria) to *Nais* (a detritovorous naidid oligochaete), to tubificids, to chironomids, to *Erpobdella* and fish.

(3) From diatoms (and other microbenthic organisms as well as detritus) to *Hydropsyche angustipennis* (a larva of *Trichoptera, Insecta*), to fish.

This scheme shows that ciliates have a key role in one of the main energy flowpaths in this river ecosystem. Heterotrophic flagellates and naked amoebae belong to the same functional group as ciliates, thus amplifying the first flowpath in favour of protozoa.

*Chaetogaster* consumed 72 % of the ciliate production. However, despite this high predation rate, the ciliate production remained high, indicating this to be a highly productive system. Predation reduced the density of ciliates by the extent to which their production was stimulated. In the Lake Dalnee (Kamchatka), about 85 % of the protozoa produced in the pelagial were consumed by rotifers and copepods (Sorokin & Paveljeva 1972). If protozoan production is compared with that of bacteria, their role in a number of ecosystems becomes clearer.

In Lake Dalnee, the production of planktonic ciliates amounted to 44 % of the bacterial production (Sorokin & Paveljeva 1972). According to data from Brock (1985), the annual production of bacterial may be only 1-40 times higher than previously – known benthic ciliate production, and only about 0.5-8 times higher than ciliate consumption.

In soils, however, bacterial production seems to be significantly higher than in water bodies. This is because soils have a particularly high concentration of organic matter. Research by Foissner (1987) showed that respiration of protozoa in soils was only 6 % of that of bacteria. However, protozoan respiration contributed 69 % of the entire invertebrate respiration in soils.

These examples demonstrate that, in many ecosystems, protozoa are second only to bacterial in terms of biomass turnover.

## FACTORS INFLUENCING PROTOZOAN PRODUCTION

The main factor steering production is mortality. Decisive is not only the degree of mortality, but, above all, its distribution over a growth period. In the following model, it is clear that the generation time is less important than mortality (Fig. 5). In I, mortality is distributed over the whole growth phase, in II it occurs at the end of this phase. The observation period was 5 days. In I, the

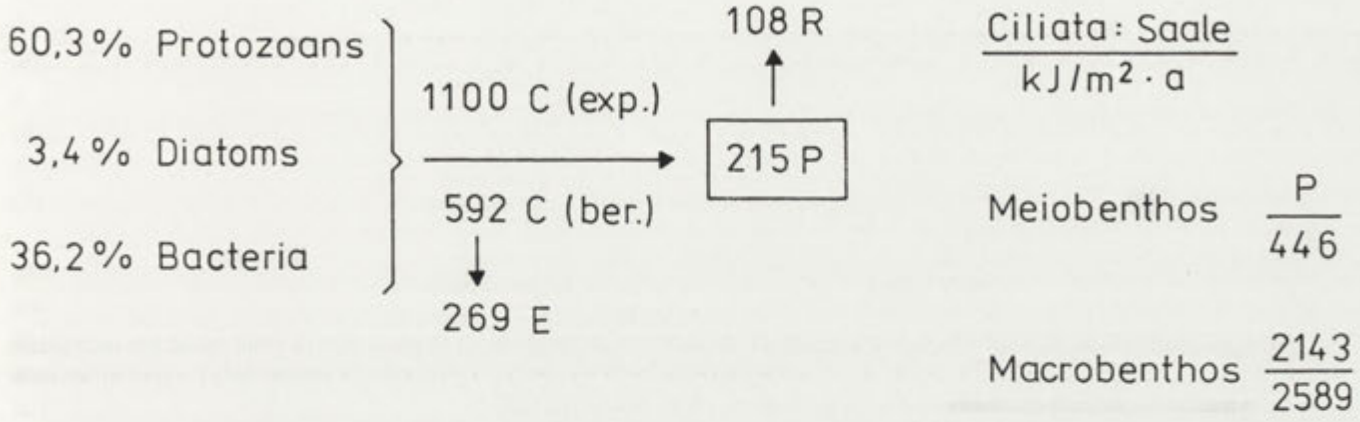


Fig. 3. Energy budget for a ciliate community in the River Saale (Germany). exp = experimentally determined, ber = by calculation. Further explanations are given in Tab. 2. (Data from Schönborn 1982 a)

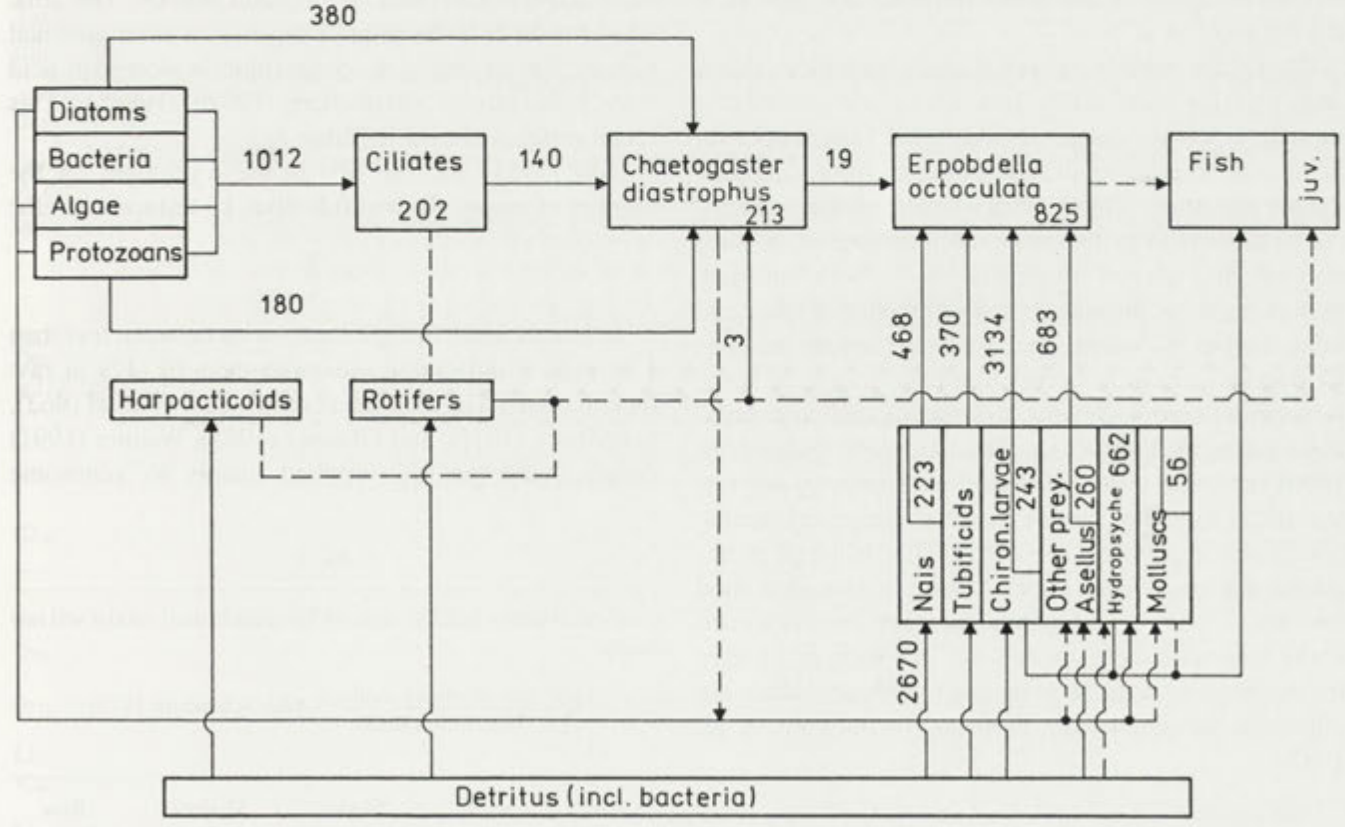


Fig. 4. Energy transfer through the middle reach of the River Saale (Germany). All values in $kJm^{-2}\ yr^{-1}$. Numbers in boxes show the annual production, numbers on arrows show transmission to the next consumer. Arrows with data are main energy flow paths. All values refer to planar habitats (exposed slides). (After Schönborn 1987)

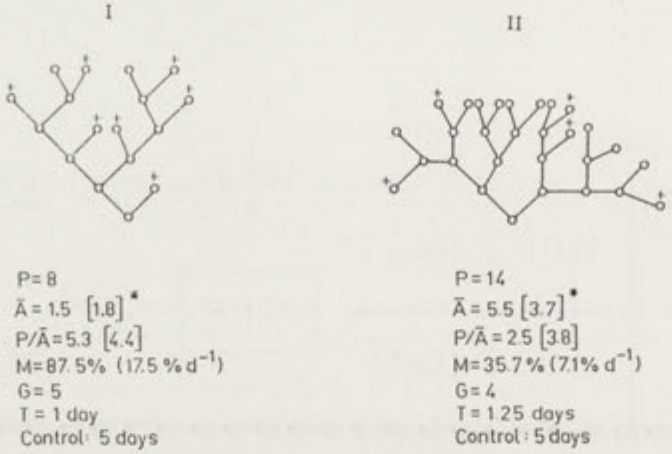


Fig. 5. Graphic models to explain the influence of generation times and mortality distribution on production (o = full shells, live individuals; o+ = empty shells, dead individuals). P = production; $\bar{A}$ = mean abundance; M = mortality; G = number of generations; T = generations time; []* = $\bar{A}$ as total density of shell divided by number of generations. (After Schönborn 1986 b)

generation time is shorter and the number of generations greater than in II, but production is lower. Conversely, mortality is higher in I than II. Applied to soils, type I is more frequent in the lower horizon, and type II in the litter horizon.

There are various morphological and behavioural strategies for maximizing production and minimizing mortality. Many species exhibit size polymorphism, being able to develop different morphs for a variety of habitat structures. These species have a greater ecological maneuverability than monomorphic species. In soils, polymorphic species frequently live in both litter and humus horizons. In wide-pored sediments of lakes and other bodies of water, the dominant testate amoeba *Centropyxis aculeata* occurs in a broad range of sizes; in narrow-pored sediments, only small-shelled populations occur (Schönborn & Peschke 1988; Schönborn 1990). In soils, a typical behavioural strategy for testaceans is to gather humus material during favourable conditions (e.g., high moisture). This material is accumulated around the shell aperture in so-called food bundles. These food bundles can then be consumed under less-favourable conditions. This strategy enables the feeding radius to be extended to habitats which are otherwise unsuitable for protozoa (Schönborn et al. 1987).

## PROTOZOA AS BIOINDICATORS OF SOILS

Protozoa are short-lived organisms. When they die, their accumulated nutrients (P and N) are recycled rapidly, making then available for the bacteria. This appears to be an other important role of protozoa in ecosystems. The shells of shelled protozoa decompose more slowly than their protoplasm bodies. The time taken for shells to decompose depends on environmental factors. For example, decomposition is slower in acid than mild humus (Schönborn 1982b, 1986b). This relationship is shown in Table 3.

Volz (1951) was the first to use a quotient for the number of empty (E) and full (live, L) testacean shells:

$$Q = \frac{E}{L}$$

In humus soils, this quotient varies between less than 1 in mull (mild humus) to more then 10 (17) in raw (acid humus). This has been confirmed by Rosa (1962), Schönborn (1973), and Foissner (1985). Wanner (1991) demonstrated that this applied mainly to xenosome

Table 3

Quotient of empty and full shells of Testacea in mull, moder and raw humus:

$$Q = \frac{\text{Nos. of empty shells}}{\text{Nos. of full shells}}$$ (After Schönborn 1973)

| Kind of humus | Mull | Moder | Moder - Raw humus | Raw humus |
|---|---|---|---|---|
| C/N | 19.0 | 24.8 | 25.0 | 30.1 |
| Q | 1.9 | 3.8 | 12.3 | 17.2 |

shells. Idiosome shells decompose quickly (Table 4). The quotient is influenced by accumulation of empty shells in the underlying horizons, as well as differing relationships between production, mortality, and abundance. In the stationary phase, a lack of cell divisions and the consequent longevity of individuals can lead to a decrease in mortality. In other cases, the stationary phase can result from a balance between production and mortality, along with a high number of empty shells (Schönborn 1983).

Nevertheless, these relationships cannot affect the general trend of the shell quotient. Meisterfeld (1980) and Wanner (1991) proposed using the shell quotient to indicate microbial activity in soils (and other habitats). Increasing quotients indicate a decrease in microbial activity. The current data suggest low microbial activity when the shell quotient is generally higher than 10.

Experimental verification can be carried out by manipulating natural plots of soils. This research is still in its infancy and so there are still problems with the interpretation of results. Some results are shown in Table 4. Irrigation reduced the quotient, suggesting that microbial activity increased.

Liming had no effect on the quotient. It is probable that the death of many species confined to acid humus was compensated for by a higher rate of decomposition. A decrease in the density of acidophilous species can be caused directly by an increase in pH, or indirectly by an increase in competition from other invertebrates. This may show that the shell quotient is not formally applicable.

The application of Lindane produced an interesting effect. This insecticide caused a significant decrease in the shell quotient for over twenty months after its application. Lindane does not inhibit bacteria, and is frequently stimulatory. An elevated soil respiration can be detected for months after its application, revealing high microbial activity. The low shell quotient may show that this effect was long-lived.

The field ecologist should view the entire spectrum of ecological factors as a characteristic of the system. He cannot analyze the individual factors. In nature, many factors induce conflicting effects for protozoa. For example, temperature increases in soils also lead to increasing dryness, while in water bodies they can result in a qualitative change in the bacterial food resource.

The laboratory ecologist, investigating the role of individual factors, generally cannot apply his results to the field because of the complexity of interacting factors. Thus, if the system is investigated, the role of individual factors has to be neglected, whereas if individual factors are analyzed, an overview of the system is lost. The combination of both types of research is desirable but not yet possible. Both types of research are legitimate, but neither can be expected to reveal the entire picture.

Table 4

Decomposition of testacean shells in raw humus (Spruce forest soils at Ulm, Germany). Untreated and treated plots

$$Q = \frac{\text{Nos. of empty shells}}{\text{Nos. of full shells}}$$

(Data after Wanner 1991)

| | Q : xenosome shells | idiosome shells |
|---|---|---|
| Untreated | 11.9 ± 7.9 | 3.0 ± 1.5 |
| Irrigation | 7.3 ± 5.3 | 3.4 ± 2.9 |
| Liming (20 kg 95% $CaCO_3$/100 $m^2$) | 14.1 ± 9.7 | 3.8 ± 2.8 |
| Lindane (2.4 mg/2l = 120 g "Nexit stark"/ha) | 5.1 ± 2.2 | 2.8 ± 1.3 |
| NaCL (225 g/l) | 9.5 ± 7.2 | 2.2 ± 1.4 |

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# Etho-ecology of Ciliates: a Reappraisal of Their Adaptive Biology, an Insight in Their Environmental Constraints

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**Summary**. The etho-ecological approach to the biology of Ciliates is proposed, discussed, exemplified. The aim of this review article is to undertake the study of an old problem (namely the adaptive biology of ciliates) in a completely new perspective: the attempt of widening the knowledge and of deepening the understanding of the evolutionary strategies (definitely peculiar and quite antiintuitive for us, large mammals) which guided protozoa to adapt to different microhabitats using a completely brand new tool, namely the ethogram (*sensu* Eibl-Eibesfeldt 1967). The examples briefly described and discussed strongly support the relevance of this kind of investigation, not only because of its interdisciplinary nature but also for its high level integration and, consequently, for its high level significance.

**Key words**. Ciliates, adaptive biology, ecology, ethology, ethogram, micropatches.

The story of Ecology, understood generally as meaning "the study of the environment", is actually the story of many different "chapters", concerning either a particular technical or cultural approach to environmental problems or a particular group of organisms used to understand and to monitor new environmental elements, phenomena or relationships (Fenchel 1987a). The present article constitutes an attempt at reconsidering the interaction between Ecology and Protozoa over the years, in the light of a very recent technical breakthrough, namely the successful qualitative and quantitative analysis of the behaviour of Ciliated Protozoa (Ricci 1989a).

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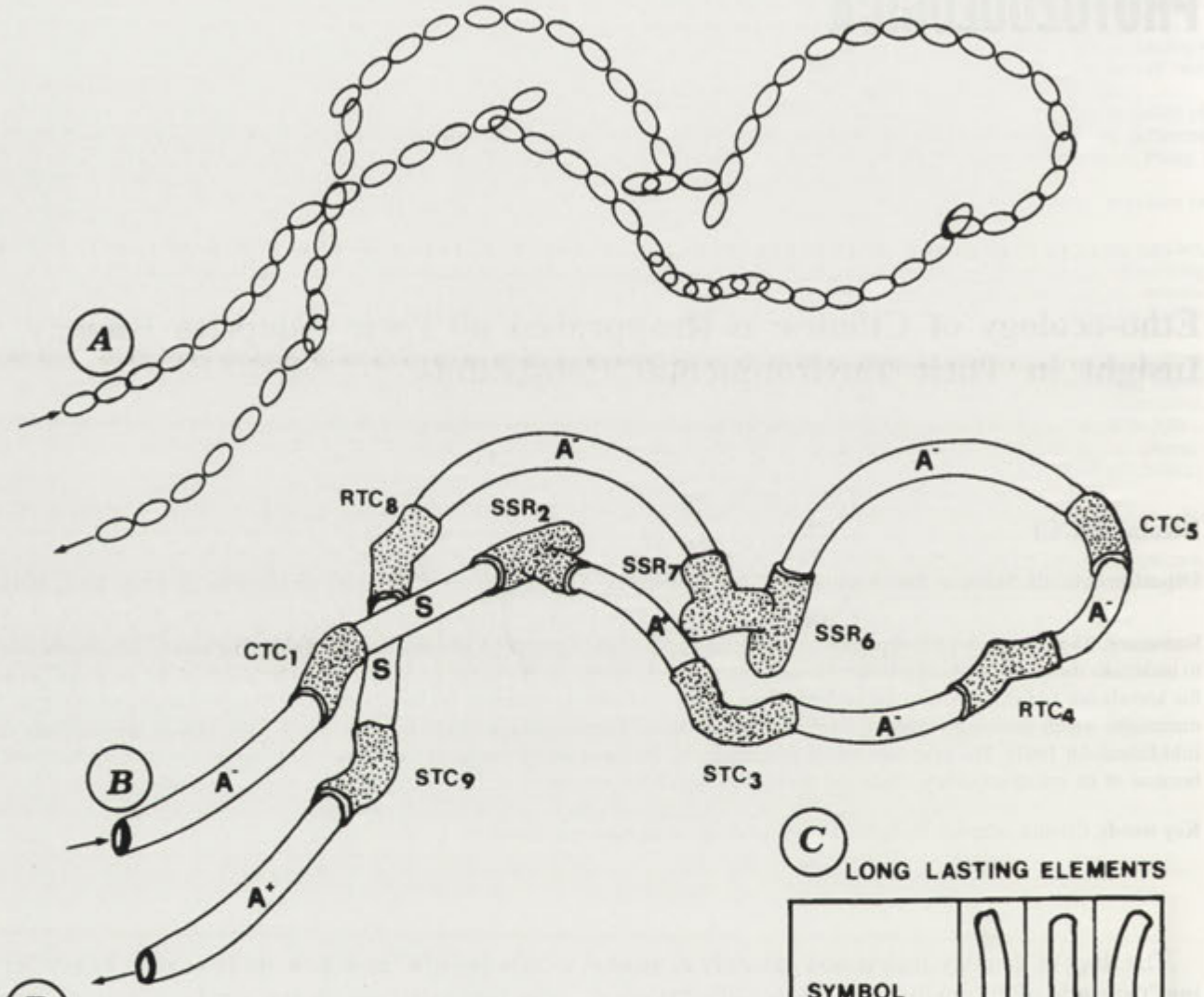
During the seventies the scientific trend led researchers to attempt the study and description of ciliates as organisms capable of revealing different physical, chemical and biological characteristics of various environments: studies, now classics in this respect, are the papers of J. Cairns' group (for example: Cairns and Henebry 1982) and of H. Bick's group (for example: Bick 1972). The high point in this trend was achieved by Madoni and Ghetti (1981), who succeeded in using the relative quality and quantity of ciliate populations to monitor the functioning of sewage water treatment plants. In the wake of these enthusiastic, interesting and stimulating papers an increasingly large number of scientists was persuaded to explore the ecology of protozoa biology, from widely differing angles. To mention only the most original and/or classic works, let us cite Bamforth (1973), Corliss (1973), Foissner (1982), Lee (1983), Schoenborn (1977), Sorokin (1978), Taylor (1978), Bereczky (1991) and last but not least the very




C LONG LASTING ELEMENTS

| SYMBOL | | | |
|---|---|---|---|
| ELEMENT | $A^-$ | S | $A^+$ |
| RADIUS | $r$ | – | $r$ |
| LENGTH | $\ell$ | $\ell$ | $\ell$ |
| ANGLE | $\beta°$ | – | $\beta°$ |
| FREQUENCY | % | % | % |

D SHORT LASTING ELEMENTS

| SYMBOL | | | | |
|---|---|---|---|---|
| ELEMENT | CTC | STC | RTC | SSR |
| CORRECTION ANGLE | $\alpha°$ | $\alpha°$ | $\alpha°$ | $\alpha°$ |
| BACKWARD MOTION | – | – | – | $\mu m$ |
| FREQUENCY | % | % | % | % |

Fig. 1. The analysis of the track of a creeping *Euplotes crassus*, taken as an example; A: the track as recorded at the TV monitor; B: its interpretation in term of a random succession of Long Lasting Elements (LLE) and Short Lasting Elements (SLE); C: the parameters describing the LLE and (D) those of SLE

recent ecotoxicological research of Piccinni (1989) and Nilsson (1991).

The present article, however, owes a far greater debt to the papers published by Finlay and Fenchel in the eighties: their approach to the biology of protozoa in connection with environmental problems was in many respects more complex and differentiated than that of the others, due to their clear-cut consciousness of the close ecological relationships between cell physiology, ecogenetic strategies, adaptive biology and so on. From this point of view Fenchel's masterpiece (1987b) cannot but be taken as a landmark in its field, and doubly so: on the one hand, in some ways it marks the end of an era and perfectly resumes what had already been done; on the other, it points the way towards a new approach to the problem where, unless the basic concepts of the adaptive biology of the studied organisms is already known and adequately understood, it becomes almost meaningless to study the physical and chemical quality of the environment, or albeit to describe perfectly the quality of the protozoan populations found here and there.

In this perspective, interdisciplinary interaction and cultural feed-back play a central role; one should expect for instance to understand in full a peculiar cell differentiation of a certain protozoan species only by an environmental interpretation of as many factors as possible of its biology (Ricci et al. 1989a).

## The eco-ethology of ciliates: a new attitude, a new tool

It is widely accepted in modern science that most breakthroughs in any field depend upon the availability of new, reliable tools and techniques, capable of supporting new attitudes and ideas. Now, in recent years a new branch of biology has developed characterized by the attempt to interpret much of animal adaptive biology by combining knowledge of their ethology and ecology (Krebs and Davies 1981). The aim of the present article is to propose the use of a new analytical tool (the ethogram of a species) to penetrate an only apparently straight forward problem: what is the real influence of the environment on the adaptive biology of Protozoa and viceversa. Before discussing a few examples, I shall try to explain the three concepts at the basis of the proposal itself: (a) the ethogram; (b) the environment; (c) the protozoa.

The term "ethogram" was introduced by Eibl-Eibesfeldt in 1967, when he defined the concept of the catalogue, as precise and complete as possible, of all the behavioural patterns of a species. While for metazoa this concept proved to be not as useful as expected in the actual practice of ethology, due to their complex structure, function and, consequently, behaviour, for ciliates it turned out to be a precious cultural tool for their behaviour analysis. Five species were in fact studied, five ethograms were drawn (*Oxytricha bifaria*, Ricci 1981, 1982; *Euplotes crassus*, Ricci et al. 1987a, b; *Litonotus lamella*, Ricci and Verni 1988; *Blepharisma japonicum*, Ricci and Tortorella 1990; *Aspidisca* sp., Ricci et al. 1991) and a comparative study made of the outcome: the main general result was that these five ethograms all consist of the same qualitative elements, while they differ from each other mainly in the quantitative aspects of the parameters describing them (Ricci 1990). The creeping tracks of the five species so far studied from this viewpoint, consist of 3 Long Lasting Elements (LLE), namely leftward arcs (A-), linear segments (S) and rightward arcs (A+), joined to each other randomly by one of the 4 Short Lasting Elements (SLE), namely the peculiar reactions which mediate the passage from one LLE to the next; the LLE and the SLE are described in Fig. 1, together with the elements one can measure by precise units. As shown in Fig. 2, the new-generation ethogram (such as that of *B. japonicum* for instance) is formed by 43 measurements, each describing a different parameter of the different locomotory elements forming the complex behaviour of a ciliate: the ethogram is an incredibly versatile, sensitive and reliable instrument to investigate the biological reactions of a population under experimental conditions namely, to study how the physiology of an organism is specifically affected by any one environmental condition and/or experimental treatment. From the experimental point of view it must be said that the locomotion of ciliates can be studied and analyzed very simply and fairly cheaply by instruments such as T.V. cameras coupled to optical microscopes, videorecorders, photographic cameras, personal computers and simple software (Russo et al. 1988).

The second basic concept to be considered is the "environment": although water is quite a simple and almost trivial one for organisms of our dimensions, it becomes completely antiintuitive and very difficult to understand and to handle conceptually when organisms the size of ciliates are considered. The size alone of Protozoa, indeed, implies a series of critical consequences at least at two levels of their ecology. The first is the spatial and temporal discontinuity in their en-

vironment: being such small organisms, ciliates experience one single habitat of "our" world (the shores of a creek, for instance) as a myriad of different microcosms (defined and individuated by their only apparently slight physical and chemical differences) which actually oblige us to consider them as completely different pieces of that environmental puzzle which protozoa have to adapt to in their real world. The second problem is the nature itself of water as experienced by a ciliate creeping or swimming through it and as perfectly described by the Reynolds number. This adimensional number ($Re = l \times \vec{v} \times \rho/\eta$, where $l$ = the length of the organism and $\vec{v}$ = its velocity; $\rho$ = the density of the fluid and h its viscosity) indicates that for "large" animals (body size of the order of magnitude of cm or more) the movements are predominantly guided by inertial forces (the swimming of a fish, for instance), while small organisms (body size smaller than hundreds of mm) swim and creep under predominantly viscoelastic conditions. In other words, a ciliate swimming through water could be compared to a man swimming in something like molasses or honey !! A more detailed, very clear and suggestive discussion of this aspect was made by Purcell (1977). The obvious conclusion cannot but be that it is quite meaningless to study the environment and/or the behaviour of ciliates without taking into account their size thus disregarding the relative consequences.

The final concept to be recalled and discussed, albeit briefly, as basic to any eco-ethological approach to the biology of protozoa, is the nature itself of these animals; many things could be said on this topic, but the most relevant and important one for our purposes is their double-sided nature, which makes them so precious for ecological investigations: each single protozoon, indeed, is at the same time a functional unit, namely a perfect and very complex eukaryotic cell, and a selective unit, namely a complete, self-sufficient organism, which has to face the environmental challenges met at any time and in any microhabitat! This characteristic must make us aware of the critical fact that, while it is a quite possible to study Protozoa as eukaryotic cells by means of all the incredible tools and techniques evolved by cell biologists, it is totally incorrect to consider them "simply" as cells, because, in so doing, one grossly underestimates (not to say, ignores completely) the complex meaning of a particular trait of their morphology and physiology, a meaning which can be understood only in the more general light of the complete adaptive

| | | | | |
|---|---|---|---|---|
| | (A) | SWIMMING | | |
| | | pitch | µm | 1 |
| | | | RU | 2 |
| | | radius | µm | 3 |
| | | | RU | 4 |
| | | stop reor. reaction | | 5 |
| | | translation velocity | µm/sec | 6 |
| | | | RU/sec | 7 |
| | | real velocity | µm/sec | 8 |
| | | | RU/sec | 9 |
| | (B) | CREEPING | | |
| Long lasting elements | $A^+$ | frequency | % | 10 |
| | | length | µm | 11 |
| | | | RU | 12 |
| | | radius | µm | 13 |
| | | | RU | 14 |
| | | central.. | β | |
| | | velocity | µm/sec | 16 |
| | | | RU/sec | 17 |
| | S | frequency | % | 18 |
| | | length | µm | 19 |
| | | | RU | 20 |
| | | velocity | mm/sec | 21 |
| | | | RU/sec | 22 |
| | $A^-$ | frequency | % | 23 |
| | | length | µm | 24 |
| | | | RU | 25 |
| | | radius | µm | 26 |
| | | | RU | 27 |
| | | central angle | β | 28 |
| | | velocity | µm/sec | 29 |
| | | | RU/sec | 30 |
| Short lasting elements | CTC | frequency correction | % | 31 |
| | | angle | | 32 |
| | STC | frequency correction | % | 33 |
| | | angle | | 34 |
| | RTC | frequency correction | % | 35 |
| | | angle | | 36 |
| | SSR | frequency correction | % | 37 |
| | | angle | | 38 |
| | | backward motion | µm | 39 |
| | | | RU | 40 |
| | ΔT | | SEC | 41 |
| | | velocity | µm/sec | 42 |

Fig. 2. The qualitative elements and the relative quantitative parameters forming the "new generation" standard ethogram, as exemplified by that of *Blepharisma japonicum* (Ricci and Tortorella 1990)

biology of whole organisms. Fortunately this new attitude is today steadily gaining ground among protozoologists, so much so that a whole volume has been devoted to this topic: "Ciliates, cells as organisms" (Hausmann in press).

At this point we are now able to introduce, although very simply, several examples of what has been done so far in the line of this new ethoecological attention to protozoa.

**A - Theoretical developments of the eco-ethologic approach**

The already mentioned analysis which enables us to interpret the behaviour of ciliates thoroughly, also lends itself as an almost perfectly to the creation of special software capable of guiding an IBM Personal Computer to generate simulated tracks of ciliates. In collaboration with Prof. F. Oliveira-Pinto of King's College, London, a program (called PISACILI) has been written, capable of producing simulated creeping tracks: the basic steps of the program are indicated in Fig. 3, left side, while an example of its real outcome is shown in the upper right hand scheme, as reproduced by the computer monitor. One hundred and fifty simulated tracks have been experimentally analyzed by the same techniques as those used for the "real" tracks: it has been shown that no difference at all ever occur between real and simulated tracks (Pinto et al., in prep.). The latter figure, in particular, shows how powerful the PISACILI program is. It enables us not only to change the scale of the simulation (the white bar indicates 300 µm in both the schemes on the right of Fig. 3), but also to generate experimentally perturbed tracks: in the upper, right scheme, the track ending at the level of the empty arrowhead is a "perturbed" one, namely at the level of the full arrowhead) with only one ethographical parameter changed: the width of the correction angle of the Side Stepping Reaction which has been doubled by the operator. The lower, right panel of Fig. 3 shows the same effects at a very low magnification: it is quite evident that while a normal ciliate tends to stay in the same area (on the left), a wide SSR performing ciliate tends to disperse (on the right) far much faster than the normal ones. Far from representing technicalities, interesting to the computer scientist alone, the possibility of simulating both a "normal" and a single perturbed track makes it possible to evaluate, theoretically at least, the relative effect of each single parameter of the ethogram in affecting that critical parameter of the biology of anyone species (its spatial dispersal), namely the relative "weight" of that single element in controlling such a critical parameter of the biology of a species. If one considers the very complex mathematics involved in the problem (cleverly reviewed and discussed by Okubo 1980), the relevance of our simulation program will become quite evident not only in solving the problem at least theoretically, but also in indicating the most important locomotory parameters for the dispersal of the species, thus simplifying the work of those researchers dealing with the real experimental study of the problem.

**B - Practical developments of the eco-ethological approach**

The first attempt at using an ethological approach to the comprehension of the evolutionary strategies of protozoa dealt with the study of two related species of hypotrichs (*O. bifaria* and *E. crassus*), well known for their markedly clear-cut thigmotaxis. Several clues indicated that there had to be some profound differences between the two species: the cross sections indeed of the conjugating pairs (Fig. 4, upper panel, B vs E) are completely different, and the same is true for the way the migrant pronuclei follow to unite to the different stationary ones (Fig. 4, upper panel, C vs F).

The conclusive observation was an eminently behavioural one: while *O. bifaria* follows a mating dance which relies upon a flat, smooth substrate at least at 3 successive steps (Fig. 4, lower panel, A, B, C), the *E. crassus* mating dance never depends upon a two dimensional substrate (Fig. 4, lower panel, D, E, F). The sum of these observations seemed to indicate that while *O. bifaria* had evolved adapting to a two dimensional, flat substrate (pair morphology, mating dance), *E. crassus* had followed a different path, adapting to a tridimensional, articulated substrate such as that found in the narrow channels among the granules of a sandy substrate (pair morphology, mating dance). By the simple experimental set-up shown in Fig. 5, upper part, it was actually demonstrated that both single and paired oxytrichas distribute on the bottom of the experimental wells, while both single and paired euplotes unfailingly live amid the sand granules, if only their granulometry allows it (Ricci 1989b).

The general conclusion to be drawn is that at the spatial scale of *O. bifaria* and *E. crassus*, sunken leaves and sandy bottoms represent completely different

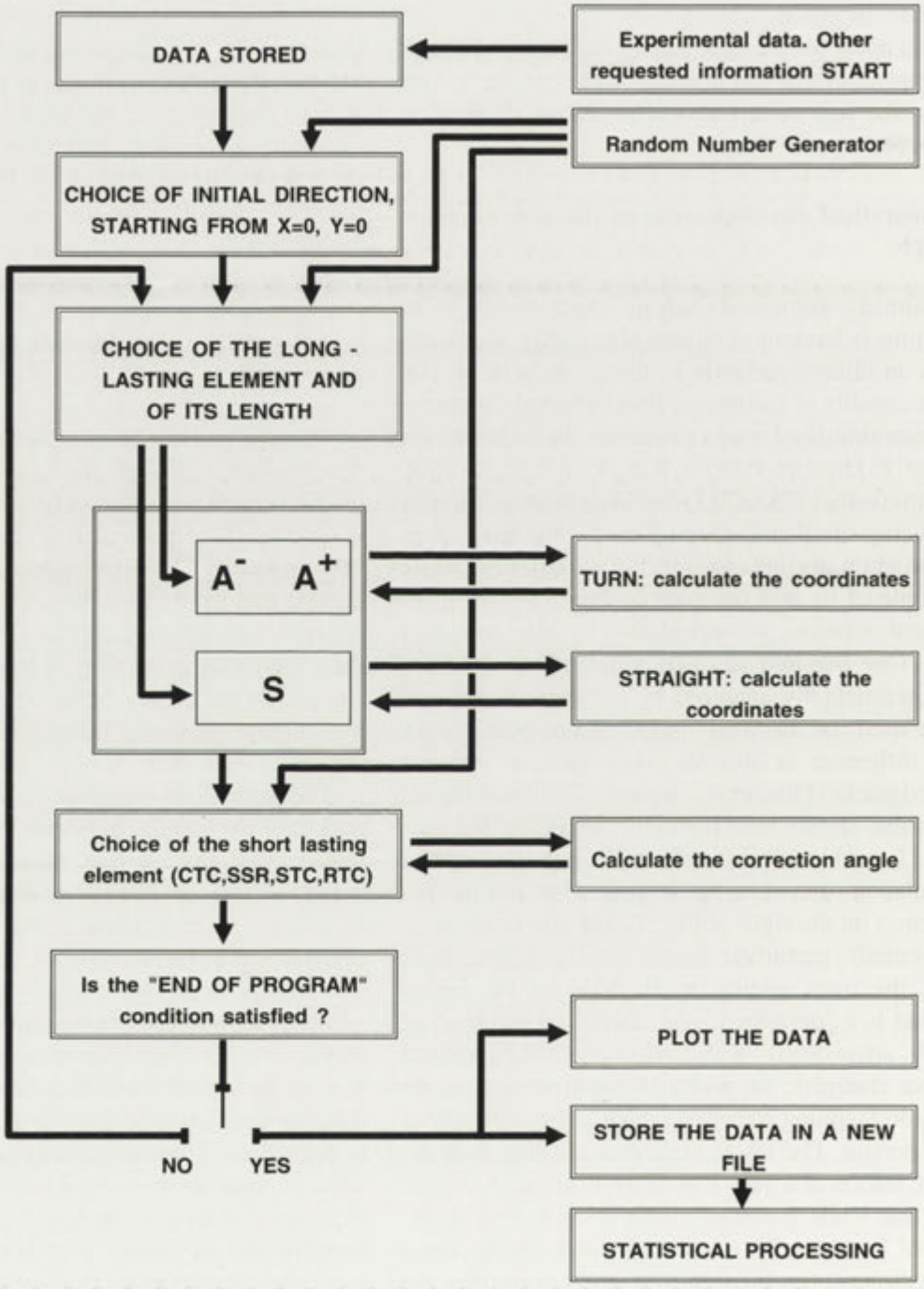
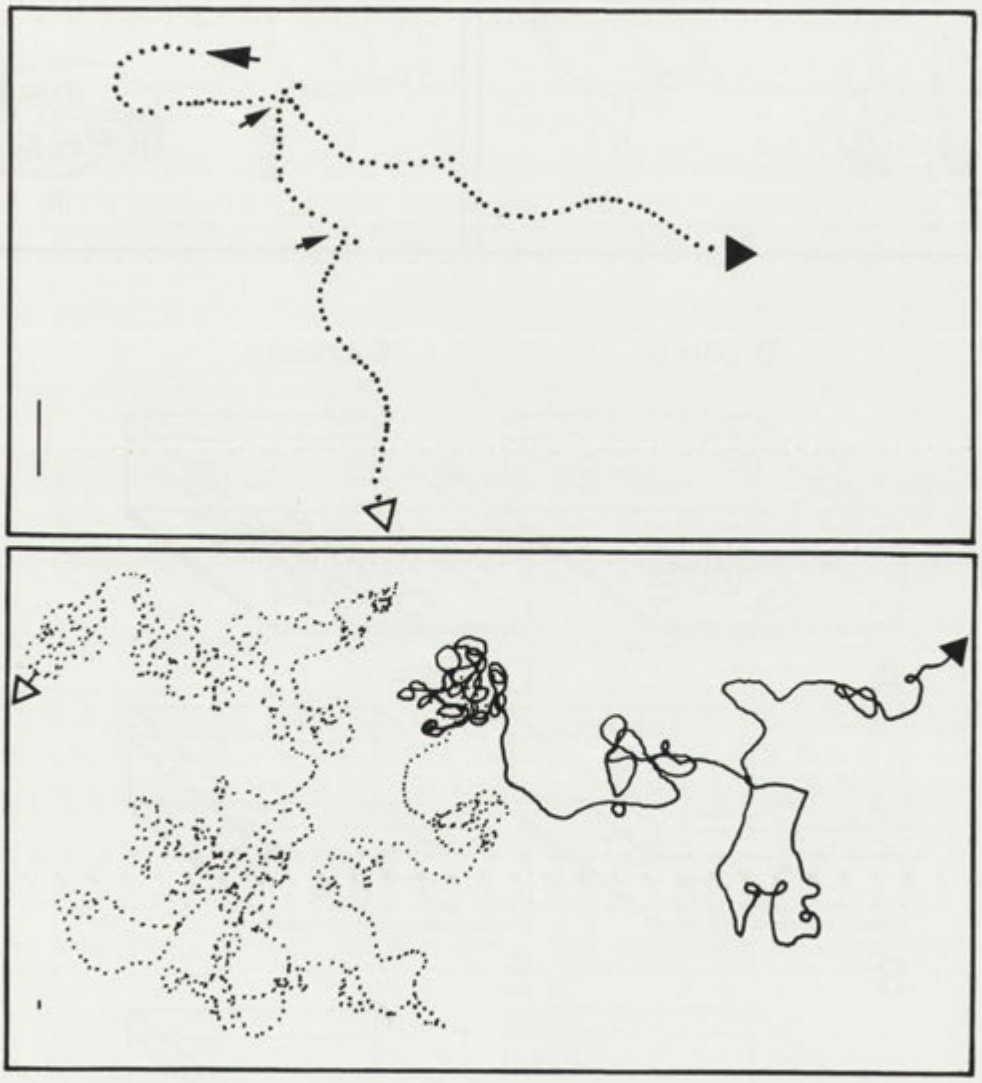



Fig. 3. The structure (on the left) and the outcome (on the right) of PISACILI; the upper right part of the figure describes a simulated track of *Aspidisca* sp. (from the arrow to the full arrowhead) and the simulated, perturbed one (from the arrow to the empty arrowhead); the perturba-



tion (indicated by the two small arrows) consists of a doubling of the correction angle ($\alpha^{\circ}$) of the SSR. The lower panel shows a simulated track (dotted line) and its perturbation (continuous line). The bars indicate 300 µm

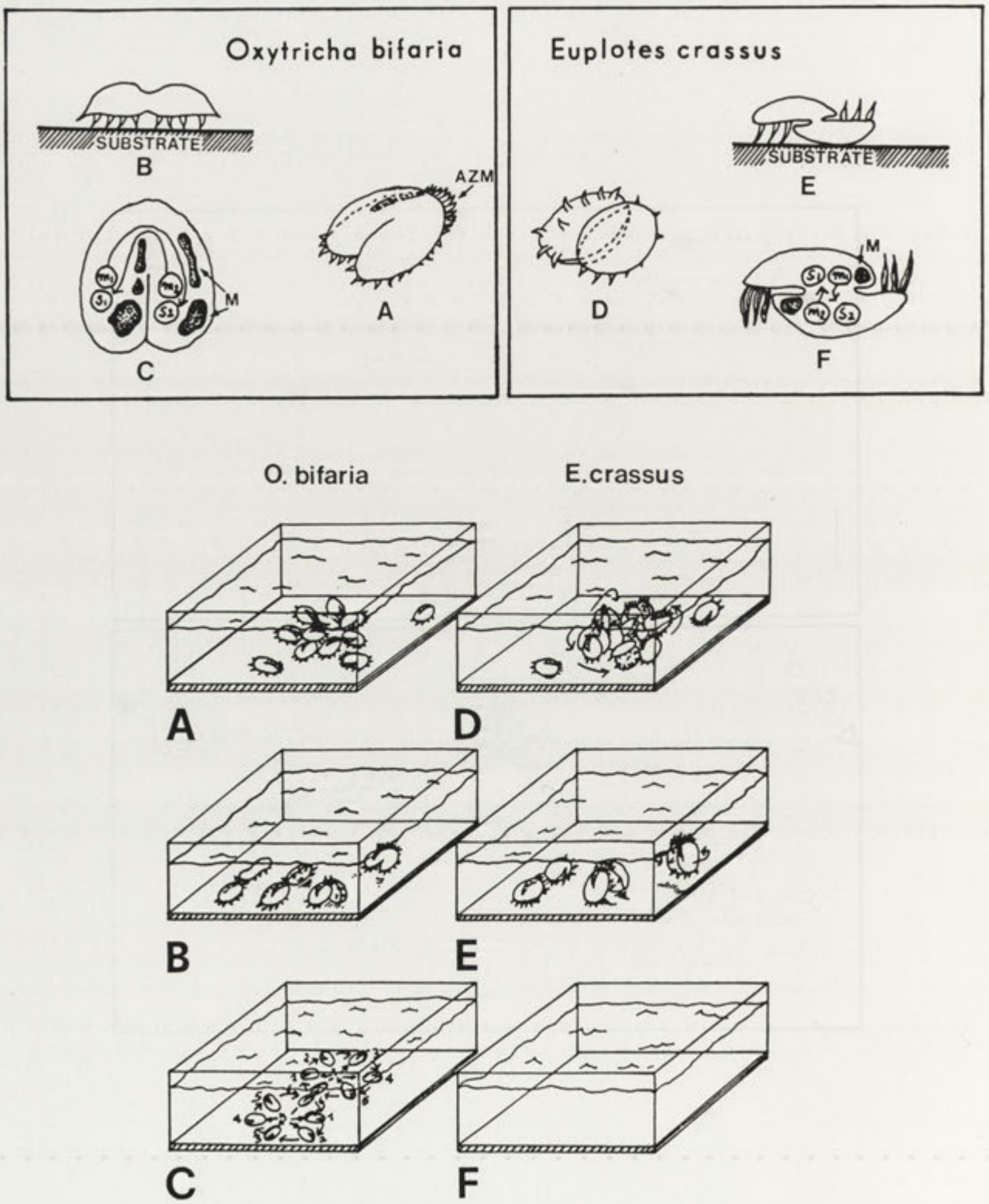



Fig. 4. The pair morphology, its cross section, the way of exchanging migrant pronuclei of *O. bifaria* (A, B, C) and of *E. crassus* (D, E, F), respectively, are given in the upper panel, while the lower one describes the behavioural pre-mating behaviour of the two species

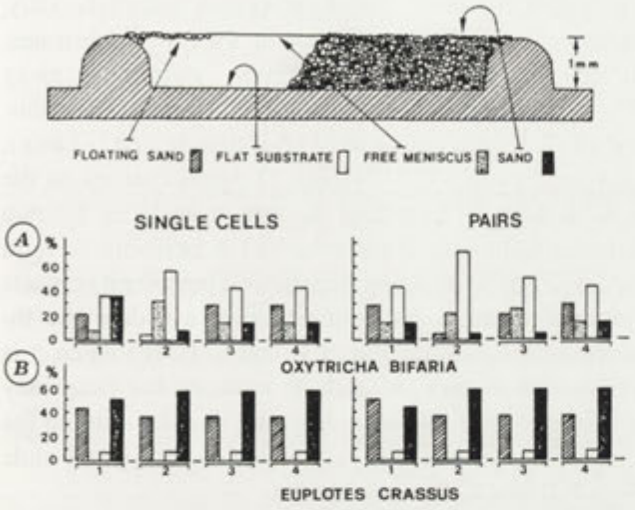
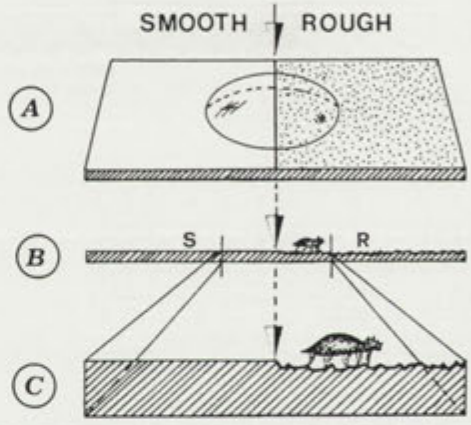



Fig. 5. In the upper part, the experimental apparatus used to study the substrate "preference" of *O. bifaria* and *E. crassus* is shown together with the 4 experimental microhabitats created in it: the graphic indication is the same as that used in the histograms below; A: the relative spatial distribution of *O. bifaria* in the 4 microhabitats; B: the same for *E. crassus*

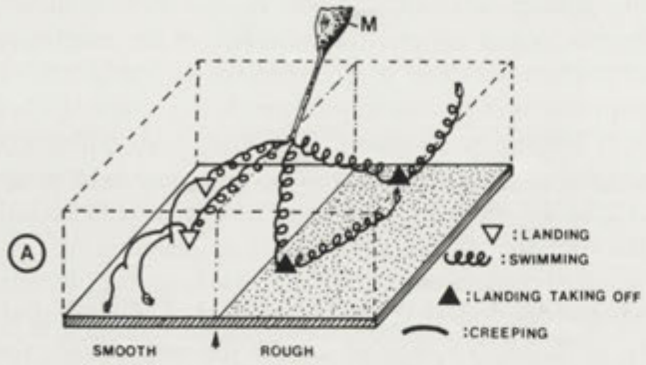
worlds, to which the two species differentially, specifically adapted, by profoundly different morphologic and ethologic adaptive answers. From a more general ecological point of view, this finding indicates that even a single decaying leaf on a sandy substrate on the bottom of a canal, of a pond or suchlike allows the settling of a new microfauna completely different from that colonizing the sand: this conclusion in turn suggests that the choice of a sampling point (station) and the analysis of the samples themselves must be made with the greatest attention, with total awareness of the risk potential for making clamorous mistakes in the interpretation of the results of environmental investigations, when the world at the protozoa's scale is considered.

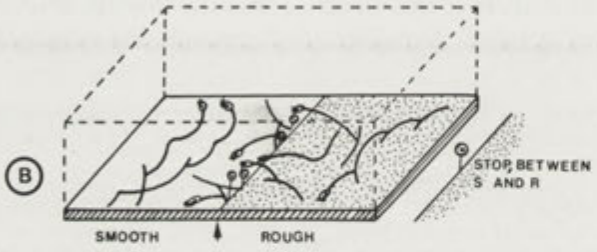
A further difficulty, unfailingly encountered whenever the environment of Protozoa is studied, is represented by the kinds of real stimuli the environment itself can exert on these organisms: although obviously we are conscious that light, temperature, pH, dissolved oxygen, redox potential and other basic parameters such as these cannot but be taken into account when the environment has to be studied, we still have a certain degree of difficulty in figuring out the real scale of the quantitative differences of these parameters perceived by protozoan populations and therefore capable of inducing adaptive answers such as changes in spatial dislocation, behaviour and so on. In an attempt to find the answer, at least for one of the most common environmental factors, the influence of the roughness of a glass substrate in determining the settlement of populations of *O. bifaria* was specifically studied (Ricci et al. 1989b). As shown in Fig. 6 A, B, C, six differently roughened glass surfaces were prepared and the average size of the superficial scratches and ornamentations of the six experimental substrates was measured by randomized pictures of these slides at the Scanning Electron Microscope: these superficial obstacles were found to be of 2, 2.8, 3.5, 6.5, 17 and 25 µm respectively, for the six experimental slides. The experimental set up shown in Fig. 6 A enables us to measure the temporal




| SLIDE / ΔT | 25 | 17 | 6.5 | 3.5 | 2.8 | 2 | C |
|---|---|---|---|---|---|---|---|
| 3' | 1.5 ● ○ ○ | 1.7 ● ● ● | 1.7 ● ○ ○ | 1.7 ● ● ○ | 1.5 ● ○ ○ | 0.89 ○ ○ ○ | 1.06 ○ ○ ○ |
| 30' | 1.6 ● ○ ○ | 2.5 ● ● ● | 1.8 ● ● ○ | 1.4 ○ ○ ○ | 1.3 ○ ○ ○ | 1.3 ○ ○ ○ | 0.9 ○ ○ ○ |
| 16h | 1.4 ● ○ ○ | 2.1 ● ● ● | 1.4 ○ ○ ○ | 1.3 ○ ○ ○ | 1.2 ○ ○ ○ | 1.3 ○ ○ ○ | 1.0 ○ ○ ○ |
| 42h | 1.5 ● ● ○ | 1.3 ● ○ ○ | 1.1 ○ ○ ○ | 1.2 ○ ○ ○ | 1.3 ○ ○ ○ | 0.8 ○ ○ ○ | 1.01 ○ ○ ○ |

Fig. 6. The simple experimental set up used to study the spatial distribution and the behaviour of populations of *O. bifaria*. A: a water droplet with the experimental populations is placed on the border between smooth and rough glass surfaces; B: the cross section of (A) and (C) the same at a higher magnification. The lower panel shows the spatial distribution (number of cells per surface unit on smooth substrate divided by the number of cells per surface unit on rough substrate); in the time (Δt) when the different substrates are considered; the experimental slide indicated by the size of its scratches. The significance of the numerical differences between smooth-rough substrates are given by the following code: ○○○ : $p > 0.05$; ●○○ : $0.05 > p > 0.01$. ●●○ : $p \leq 0.0l$; ●●● : $p << 0.001$




C

| EFFECTS \ SLIDE | C | 2.8 | 3.5 | 6.5 | 1 7 | 2 5 |
|---|---|---|---|---|---|---|
| I % SSR (S→R) | 100 | 110 | 140 | 160 | 195 | 180 |
| II n° SSR/sec. (ON R) | 0.3 | 0.3 | 0.5 | 0.6 | 1.1 | 0.8 |

Fig. 7. The behaviour accounts for the spatial distribution of *O. bifaria* when differently rough substrates are available. (A) short term strategy: after contacting a rough surface *O. bifaria* swims away instantaneously. (B) long term strategy: *O. bifaria* avoids entering the rough surface by performing several successive Side Stepping Reactions. (C) *O. bifaria* preferentially distributes on smooth surfaces by means of 2 mechanisms. I: it performs an SSR whenever it passes from smooth to rough surfaces; II: it enhances the frequency of the spontaneous occurrence of the SSR themselves

trend of the populations of *O. bifaria* settling on smooth vs rough surfaces: the results (Fig. 6, lower panel) demonstrated that (a) each substrate is perceived as different (even the "slight" difference between the slide 2 μm and the slide 2.8 μm induces a different distribution), (b) the most avoided substrate is the rough 17 μm slide (and this might be the consequence of the spatial distribution of the cirri on the ventral surface of the oxytrichas according to Ammermann 1985), (c) as time passes, the differential distribution tends to become less evident, very likely due to a bacterial film gradually covering the rough surfaces.

The behavioural analysis led us to draw the ethograms of *O. bifaria* creeping on the different substrates and it was found that (a) *O. bifaria* perceives instantaneously, namely as soon as it contacts them, the rough substrates, so that it avoids them by simply swimming away (Fig. 7 A) and this accounts for the differential distribution of *O. bifaria* over a very brief time scale (1-3 min); after longer times (15-20 min), *O. bifaria* settles on the glass substrates, avoiding the rough surfaces by two different behavioural patterns: (b) it performs several successive Side Stepping Reactions whenever it contacts the smooth-rough discontinuity, thus avoiding the inconvenient substrate (Fig. 7 B and C I); (c) when it is on a rough surface, it tends to enhance the frequency of occurrence of the same reaction, thus facilitating the return onto the smooth part of the experimental slide (Fig. 7 B and C II).

The general outcome of these studies is that an appropriate eco-ethological approach to the biology of a species leads to a far deeper and wider comprehension of their biology: *O. bifaria* not only tends to settle on smooth flat substrates, but is also able to avoid the rough ones, by proper behavioural patterns, being capable of perceiving very subtle differences among rough substrates. This general conclusion once more suggests that our attitude towards the protozoan environment must be an extremely cautious, and must not discount the importance of, for example, one certain rough substrate differing from another even by merely 1 μm, in the dimensions of its superficial ornamentations.

The third example I should like to discuss deals with brand-new result (Ricci et al. in press) obtained in research into the behaviour of *O. bifaria* "creeping" underneath the water/air interface (WAI). The position assumed by *O. bifaria* when contacting the WAI (Fig. 9, A I) indicates that it considers such an interface as a good "substrate" to creep on: the number of cells actually settling on the WAI, however, is a very small percentage of the entire population (Fig. 8, A). To solve this apparent contradiction, the ethograms of cells creeping on the substrate (Fig. 9, B I: controls 1), on a floating glass coverslip (Fig. 9, B II: controls 2) and underneath the WAI (Fig. 9, B III) were drawn and compared. After showing that no difference at all occurs between I and II population (the relative position in space does not of itself affect the creeping of *O. bifaria*), the behaviour of the III population was analyzed and found to be quite peculiar; the most relevant peculiarity is represented by the quite evident slipping *O. bifaria* shows when creeping underneath the meniscus (Fig. 9, D, right panel vs left panel). When the ethographic elements (Fig. 8, B) and the general geometry of the tracks are considered

(A)

| Time | 0h | 1h | 3h | 5h | 20h | 24h |
|---|---|---|---|---|---|---|
| number of cells | | | | | | |
| Absolute TOTAL | 245 | 239 | 257 | 273 | 316 | 327 |
| n/mm² | 9.7 | 9.4 | 10.1 | 10.7 | 12.2 | 12.7 |
| Absolute BOTTOM | 230 | 212 | 223 | 226 | 180 | 193 |
| n/mm² | 9.1 | 8.4 | 8.8 | 8.9 | 7.1 | 7.6 |
| Absolute MENISCUS | 15 | 27 | 34 | 47 | 136 | 134 |
| n/mm² | 0.6 | 1 | 1.3 | 1.8 | 5.1 | 5.1 |
| RATIO B:M | 15.3 | 8.03 | 6.56 | 4.8 | 1.3 | 1.4 |

(B)

| | | | Geometric elements | | | |
|---|---|---|---|---|---|---|
| | Arcs | N | r (μm) | β° | l (μm) | v (μm.s-1) |
| 0' to 20' | R | 66 | 247 ± 132 | 141 ± 47 | 550 ± 139 | 220 ± 37 |
| 180' to 200' | R | 29 | 846 ± 298 | 93 ± 42 | 621 ± 321 | 354 ± 78 |
| | L | 55 | 815 ± 269 | 109 ± 44 | 561 ± 498 | 423 ± 116 |
| 300' to 320' | R | 26 | 1,571 ± 952 | 58 ± 37 | 1,045 ± 440 | 572 ± 205 |
| | L | 28 | 1,362 ± 1,187 | 79 ± 53 | 1,268 ± 616 | 520 ± 130 |
| | LS | 20 | – | – | 932 ± 517 | 558 ± 185 |
| Controls | L | 132 | 894 ± 522 | 51 ± 33 | 582 ± 309 | 545 ± 199 |
| | LS | 24 | – | – | 274 ± 192 | 400 ± 227 |

Fig. 8. A) The temporal trend of the spatial distribution of *O. bifaria* on the WAI in comparison with the bottom is expressed by the ratio between the number of cells per square millimeter on the bottom (B) and that underneath the WAI (M)

(Fig. 9, C) it can be easily seen that *O. bifaria* behaves more and more normally as time passes. According to this observation, it was found that the "normalization" of *O. bifaria*'s behaviour perfectly parallels the development of a superficial bacterial film: this film is quite a good substrate for *Oxytricha* to move on, once more confirming that fact that the species has adapted to smooth, flat substrates (as already found). The next step in this research was very logical and simple: we reasoned that if what we had found was true, then *O. bifaria* should settle at the level of the WAI trapped among the floating leaves and in the felt of stems emerging from the shallow waters of marshes, ponds and river banks. *Ad hoc* samples were collected in the freshwater canals near Pisa, where *O. bifaria* is found most commonly: the water volumes to be studied were collected paying great experimental and technical attention to the problem of the relative spatial differences in sampling points. Six different kinds of stations were chosen (Fig. 10, A) and the analysis of their different populations definitely demonstrated that *O. bifaria* tends to settle underneath the bacterized WAI with even greater frequency than on its already known specific substrate, namely that formed by the rotting leaves on the bottom rivers, creeks, ponds and lakes. Beyond demonstrating the real occurrence of environmental micropatches in *O. bifaria*'s biology this research led to a very clear and relevant conclusion: the bacterized WAI, which covers most of

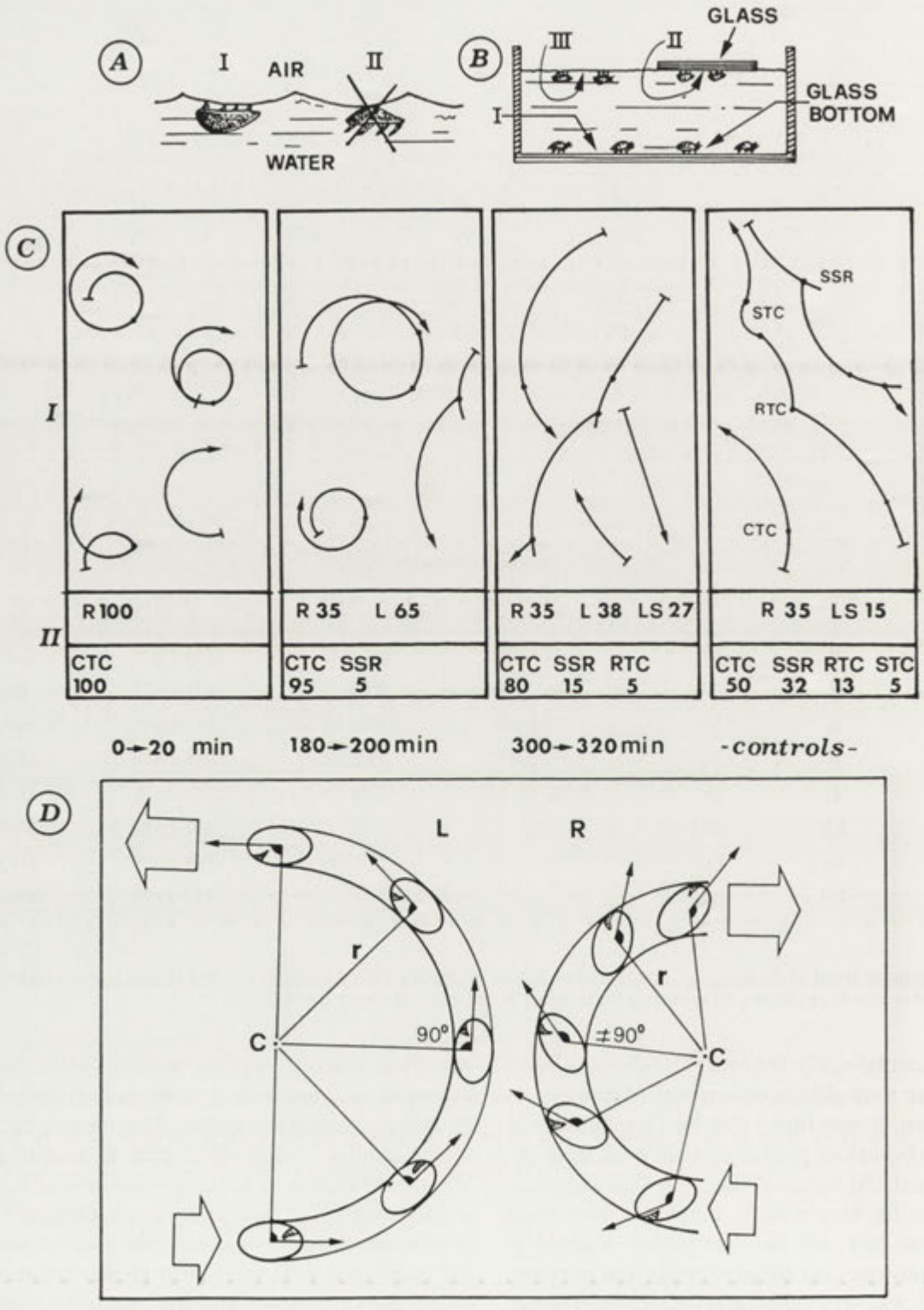
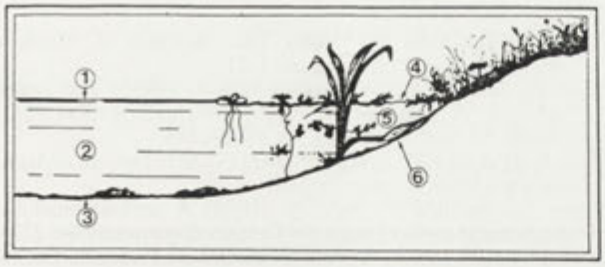



Fig. 9. A: A schematic drawing of how *O. bifaria* contacts the WAI: II type never occurs. B: The ethograms of *O. bifaria* on the substrate, (I), on the similar substrate placed upside down (II) and underneath the WAI were studied. C: The main results of the ethographical analysis: as time passes the behaviour of an *O. bifaria* creeping underneath the WAI tends to become normal, according to a temporal trend perfectly mirroring the development of a bacterial film. D: While on a solid substrate *O. bifaria* creeps along leftward arcs and tangentially to them, underneath the WAI it slips along rightward arcs, at obtuse angles with respect to the radius

(A)




(B)

| | STUDIED HABITATS | | | | | |
|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 |
| Hypotrichs | + | + | + | + + + + | + | + + + |
| other ciliates | | + + | + + | + | + + | + |

Fig. 10. The natural microhabitats of *O. bifaria* in natural conditions. A) Six stations were chosen to study spatial distribution in *O. bifaria*. B) The quantitative results of this round of sampling experiments, given by a conventional code: (+) = 5-10 organisms; (++) = 10-50; (+++) = 50-100; (++++) = 100-500.3

the marshes (or equivalent environments) which represent about 17% of the whole surface of our world, is actually a peculiar habitat specifically colonized by *O. bifaria* as an all-purpose habitat, where the species settles, creeps, feeds, reproduces, mates, undergoes cell differentiation and struggles for survival! The fact that an entire family of Diptera Insects, the Dixidae, have larvae feeding only on ciliates living at the WAI level, strongly supports the idea that even at the level of this peculiar microhabitat ciliates mediate the flow of both organic materials and energy from the microbial to the macrobial loop.

## C - Conclusive remarks

To conclude, I should like to recall the thesis initially proposed as the central core of this article: an appropriate and conscious use of the techniques available today for an analytical study of behaviour of ciliates, coupled to a naturalistic and inquisitive attitude towards their biology, may indeed represent a neo-holistic approach to the matter, a sort of foundation stone for a new comprehension of aquatic environments in general and of adaptive answers opportunistically given by protozoa living in them, as well. In this light two general aspects of Biology quit being just two different fields of scientific interest to become, rather, the two faces of the same coin, both necessary for its definition, both furthering an understanding of the coin itself (and positively interacting with each other in this), namely of its shape, of its function, or, even better, of its basic nature.

Only the future will demonstrate the truth of this proposal, but in my opinion the results, obtained by this initial research specifically planned and conducted, seem to support seriously this way of thinking.

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# Contribution to the Taxonomy of the Family *Paranophryidae* Jankowski in Small & Lynn, 1985

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**Summary**: The scuticociliate family *Paranophryidae* Jankowski in Small & Lynn, 1985 is characterized and the genera *Paranophrys* and *Anophryoides* are described. Because of its silverline system, infraciliature and mode of stomatogenesis, the species *Paranophrys carnivora* Czapik & Wilbert, 1986 is transferred to the genus *Anophryoides*.

**Key words**: *Anophryoides, Paranophryidae*, taxonomy.

## INTRODUCTION

The family *Paranophryidae* (*Scuticociliatida, Philasterina*) is comprised of five - mostly marine - genera: *Anophryoides* De Puytorac & Grolière, 1979; *Mesanophrys* Small & Lynn, 1985; *Metanophrys* De Puytorac et al., 1974; *Mugardia* Small & Lynn, 1985; and *Paranophrys* Thompson & Berger, 1965. The genera *Paranophrys* and *Metanophrys* formerly were considered to belong to either the family *Philasteridae* (because of their ciliferous adoral membranelles 1-3, Corliss 1979) or the family *Uronematidae* (because of their mode of stomatogenesis, Didier & Wilbert 1976a, 1976b, Thompson & Berger 1965). De Puytorac & Grolière (1979) described a new genus *Anophryoides* and suggested for it a family between the *Philasteridae* and the *Uronematidae*. This was realized with the creation of the family *Paranophryidae* by Jankowski in Small & Lynn, 1985. Up to now there has been no diagnosis of this family. We compared the species of the *Paranophryidae*, as described in the literature and in this way we worked out the characteristics of the family. During our investigation it became apparent, that the species *Paranophrys carnivora* should be rightfully transferred to the genus *Anophryoides*.

## RESULTS

Diagnosis of the family *Paranophryidae*:

The ciliates have an ovoid to pyriform shape, with an unciliated apical pole and a single caudal cilium. The buccal cavity is one quarter to one half of the body length, with 3 adoral membranelles on the left, a paroral membrane on the right and a postoral

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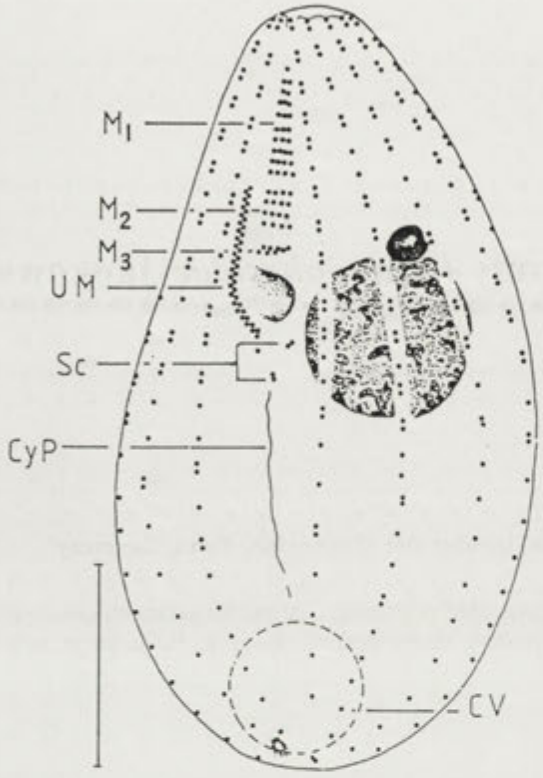


Fig. 1. *Paranophrys thompsoni* (from Song & Wilbert 1989), ventral infraciliature. CV - contractile vacuole; Cyp - cytoproct; M1-M3 - adoral membranelle 1-3; Sc - scuticus; UM - undulating (= paroral) membrane. Scale 10 µm

scuticus. The cytoproct lies in the posterior half of the cell between the first and the last somatic kinety. One contractile vacuole is more or less terminally located; it opens with one pore, usually at the end of the second somatic kinety. The macronucleus is spherical to ovoid and is centrally located; there is one adjacent micronucleus. The kinetosomes of the somatic kineties are paired anterior and are single posterior.

The genus *Paranophrys* is comprised of two marine and one fresh-water species (Fig. 1). Its body shape is pyriform, the body is 25-75 µm long and has 10 to 21 somatic kineties. The buccal cavity is one half of the body length. M1 is more or less triangular in shape and is clearly longer than the roughly rectangular M2 (8 µm and 3 µm, respectively). M3 is very small and triangular. The paroral membrane starts at the anterior end of M2 and curves slightly around the cytostome. The scuticus consists of several kinetosomes, arranged in two groups.

Caudal cilium, cytoproct, contractile vacuole and its pore, macronucleus and micronucleus are just as described for the family. (Borror 1972, Curds et al. 1983, Didier & Wilbert 1976a, 1976b, Song & Wilbert 1989, Thompson & Berger 1965, Wilbert 1980).

The genus *Anophryoides* was created by De Puytorac & Grolière in 1979 for a species, which was formerly described by Mugard (1949) as *Anophrys salmacida* (Fig. 2). The body is roughly ovoid and is 55-85 µm long, with 21 or 22 somatic kineties. The buccal cavity is about one half of the body length. M1 is almost as long as M2 (8 µm and 9 µm, respectively). Left of the latter, there is a row of closely packed kinetosomes. M3 is small and triangular. The paroral membrane starts at the anterior end

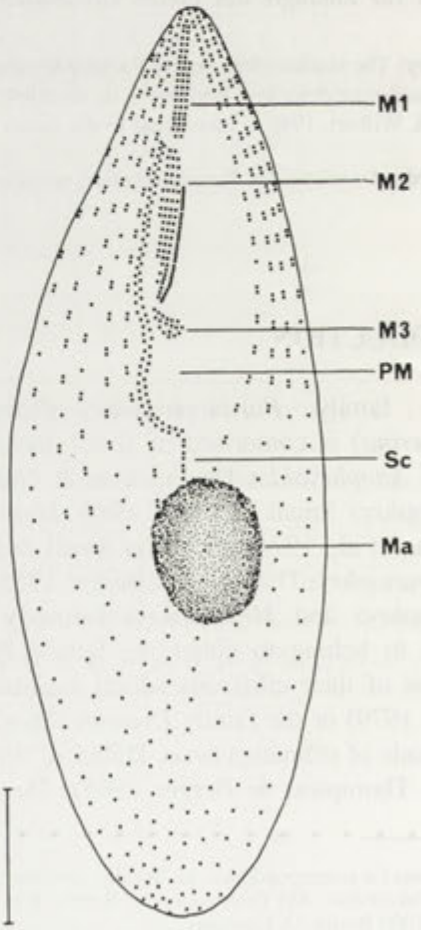


Fig. 2. *Anophryoides salmacida* (after De Puytorac & Grolière 1979), ventral infraciliature. M1-M3 - adoral membranelle 1-3; Ma - macronucleus; PM - paroral membrane; Sc - scuticus. Scale 10 µm

of M2, but it describes two or three curves along the right side of the buccal cavity. There is a scuticokinety which is made up of paired and/or single kinetosomes. Cytoproct, contractile vacuole and its pore, macronucleus and micronucleus are the same as described for the family.

In 1986, Czapik & Wilbert described a marine and histophagous species: *Paranophrys carnivora* (Fig. 3). This species is transferred to the genus *Anophryoides* because there are more similarities with this genus than with *Paranophrys*. These similarities are:

1) The oral structures of *Anophryoides carnivora* (Fig. 4) show nearly the same arrangement as those of *Anophryoides salmacida*. M1 is almost as long as M2 (8 µm and 9 µm, respectively) but not longer, as

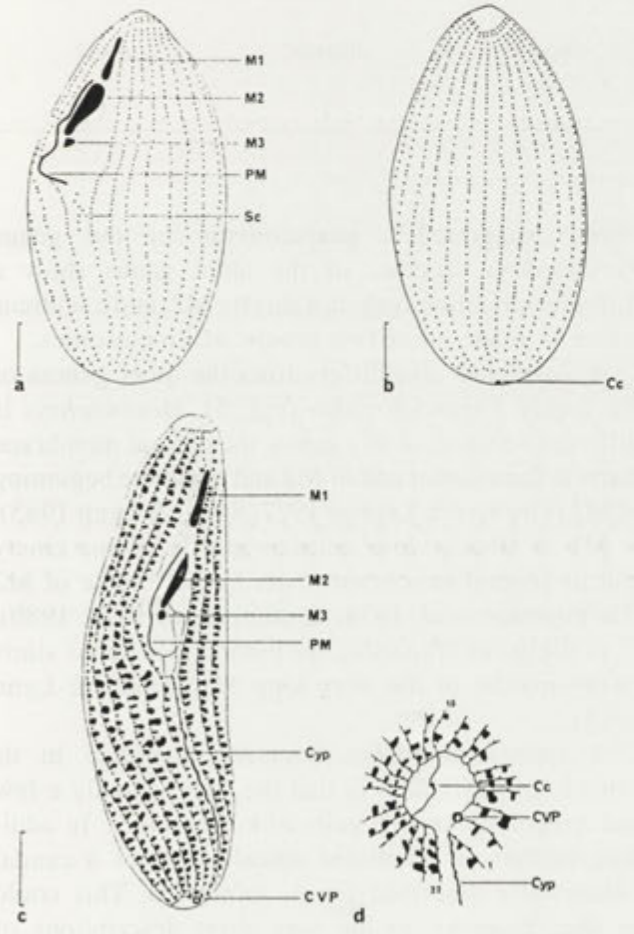


Fig. 3. *Anophryoides carnivora* (from Czapik & Wilbert 1986), a: ventral, b: dorsal infraciliature. c: ventral silverline system; d: caudal pole. Cc - caudal cilium; CVP - porus of the contractile vacuole; Cyp - cytoproct; M1-M3 - adoral membranelle 1-3; PM - paroral membrane; Sc - scuticus. Scale 10 µm

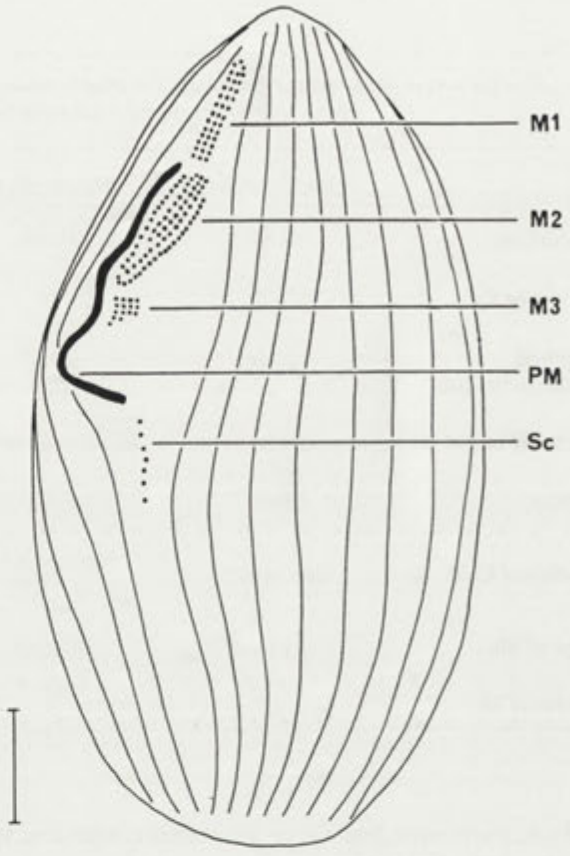


Fig. 4. *Anophryoides carnivora*, infraciliature of some buccal structures. M1-M3 - adoral membranelle 1-3; PM - paroral membrane; Sc - scuticus. Scale 10 µm

it is characteristic of the genus *Paranophrys*. Left of M2, there is a row of closely packed kinetosomes. M3 is very small. The paroral membrane runs irregularly in two or three curves along the right side of the buccal cavity, and there is a postoral scuticokinety with single kinetosomes.

2) *A. carnivora* has a silverline system that has broad projections to the left of the primary silverlines (Fig. 3c, d). The same pattern is shown for *A. salmacida* in the drawings of Mugard (1949). All described *Paranophrys* species have only simple primary silverlines, which connect the kinetosomes within each somatic kinety.

3) During stomatogenesis M3 of the opisthe is derived from the parental scuticus and the other buccal structures are derived from the parental paroral membrane. This is true for both *A. carnivora* and *A. salmacida*. In *Paranophrys*, however, the parental scuticus gives origin to M3 and M2 of the opisthe.

Table 1

Comparison of the genera of the family *Paranophryidae*. CVP - porus of the contractile vacuole; K1, K2, K3 - kinety number; Ma - macronucleus; Mi - micronucleus; PM - paroral membrane; SK - somatic kinety

| | *Anophryoides* | *Mesanophrys* | *Metanophrys* | *Mugardia* | *Paranophrys* |
|---|---|---|---|---|---|
| Length (µm) | 55-85 | 35-55 | 50-120 | ca. 65 | 25-75 |
| Number of SK | 21-22 | 11 | 11-20 | ? | 10-21 |
| Length of buccal cavity (µm) | 29-30 | 12-18 | 15-18 | ca. 30 | 11-24 |
| Beginning of PM | anterior end of M2 | anterior end of M3 | middle of M2 | middle of M1 | anterior end of M2 |
| Scuticus | kinety | 2 groups | kinety or 2 groups | kinety | 2 groups |
| Location of CVP | end of K2 | between K1 and K2 | end of K2 and K3 | end of K2 | end of K2 |
| Shape of Ma | spherical | ellipsoid | ellipsoid | ellipsoid | ovoid |
| Number of Mi | 1 | 1 | 1 | 2 | 1 |

4) *A. carnivora* has 21 or 22 somatic kineties, with mostly single kinetosomes.

5) *A. carnivora* shows a dimorphic life cycle with a fusiform, longer trophont and an ovoid, smaller theront. The same was described by Mugard (1949) for *A. salmacida*, but is not known for *Paranophrys*.

Caudal cilium, cytoproct, contractile vacuole and its pore, macronucleus and micronucleus are typical of the family. Kahan et al. (1987) described the feeding behaviour of this species.

## DISCUSSION

Because of its body shape, the unciliated apical pole, the single caudal cilium, the cytoproct in the posterior half of the cell, the terminal lying contractile vacuole, with its one pore at the end of the second somatic kinety, and the centrally located macronucleus, *A. carnivora* is a typical member of the family *Paranophryidae*.

The oral structures, the pattern of the silverline system, the mode of stomatogenesis, and the dimorphic life cycle, however, are characteristic of the genus *Anophryoides* and are not - as Czapik & Wilbert (1986) suggested - characteristic of the genus *Paranophrys*. Species of the latter genus show a different silverline system, a shorter M2, and a scuticus which is made up of two groups of kinetosomes.

*A. carnivora* also differs from the other genera of the family *Paranophryidae* (Fig. 5). *Mesanophrys* is different because, in this genus, the paroral membrane starts at the anterior end of M3 and not at the beginning of M2 (Grolière & Leglise 1977, Small & Lynn 1985).

M1 of *Metanophrys* consists mostly of one kinety and its paroral membrane starts in the middle of M2 (De Puytorac et al. 1974, Grolière et al. 1978, 1980).

In the genus *Mugardia*, the paroral membrane starts in the middle of the very long M1 (Small & Lynn 1985).

*A. carnivora* differs from *A. salmacida* in its somatic infraciliature, in that the first has only a few and irregularly spaced pairs of kinetosomes. In addition, neither an unciliated apical pole nor a caudal cilium were described for *A. salmacida*. This could be due, however, to the very short descriptions of Mugard (1949) and De Puytorac & Grolière (1979), dealing mainly with oral structures and stomatogenesis.

The silverline systems of *Mugardia* and *Mesanophrys* haven't been described yet. Therefore,

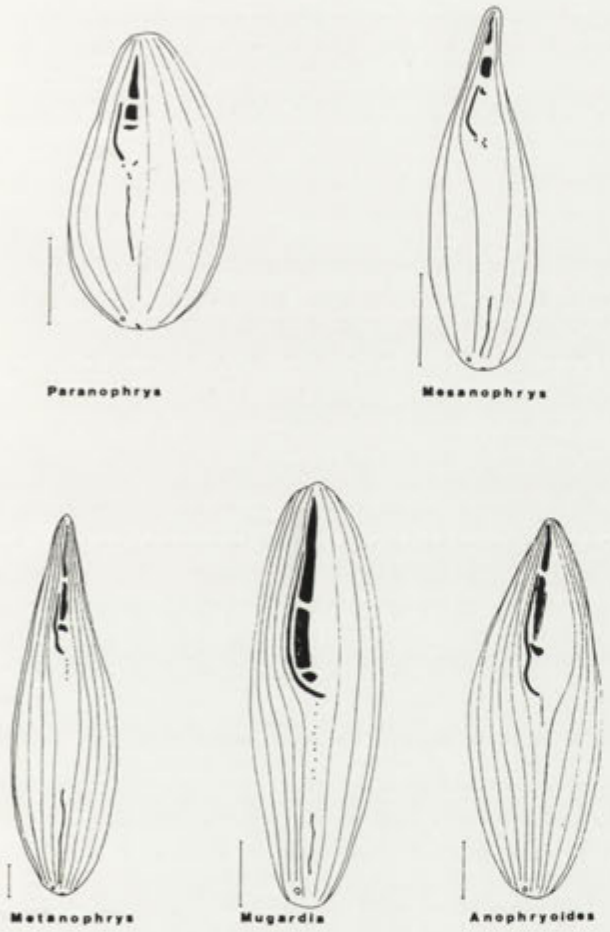


Fig. 5. Schematic drawings of the genera of the family *Paranophryidae*, Scale 10 µm

it remains to be seen, whether the genus *Anophryoides* is the only member of the family *Paranophryidae* that shows a tetrahymenid silverline system or not.

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# Morpho-physiological Characteristics of *Tetrahymena rostrata* (Ciliata). I. Changes During Establishment of Laboratory Strains

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**Summary**. The morpho-physiological variability of *Tetrahymena rostrata* after change of habitat from parasitic to free-living form was studied. It has been found that during 13 months of cultivation of ciliates in axenic medium, the size and shape differ from the parasitic form. The morphological data suggest that *T. rostrata* are smaller than the wild strain. On the other hand, the conditions of cultivation (e. g. composition of medium) affect cell morphology. The findings show that the phagocytosis rates in the ciliates after the change are very low and depend on phases of cultural growth. Probably, the low rate of phagocytosis produced the lengthening of generation times of the cells and the non-typical course of phases of cultural growth.

**Key words**. *Tetrahymena rostrata*, parasitic form, free-living form.

## INTRODUCTION

*Tetrahymena rostrata* Kahl, 1926 (Corliss, 1952) is found as a free-living form in edaphic habitats in nature (moss, sphagnum and so on). It is also frequently discovered as a facultative parasite (or sometimes histophagous scavenger) in such widely diverse hosts as enchytraeid oligochaetes, rotifers, snails and slugs (Corliss 1960, 1973; Kazubski and Szablewski 1978). Although a facultative parasite, it is truly pathogenic. The kidney or renal organ is a primary site of infection in slugs. Other tissues and organs may be entered. In laboratory, the organism is grown on bacteria or in pure culture on proteose-peptone.

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There are only few data on morphology and physiology of this species. It is known that it is a polymorphic species and that body form and size depend on the stage or phase in the life history (Corliss 1973). The species has considerable potential as an experimental organism in several areas of cellular research.

The investigations were aimed at morpho-physiological characteristic of *T. rostrata* after change from parasitic to free-living form.

## MATERIALS AND METHODS

### Isolation of axenic strains of *T. rostrata*

The ciliates were isolated from renal organ of *Zonitoides nitidus* (Mollusca, Gastropoda) in 1 mM buffer Tris/HCl, pH=7.4 on Petri dishes. After isolation, the cells were transferred into buffer Tris/HCl with renal organ of snail as food, and then, after 7 days, to nutrient medium (1.5% proteose-peptone + 0.1% yeast

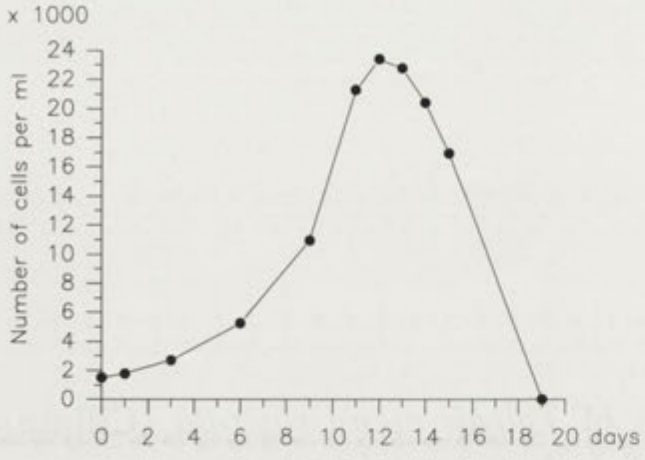


Fig. 1. Cell density versus time of culture growth of *T. rostrata*

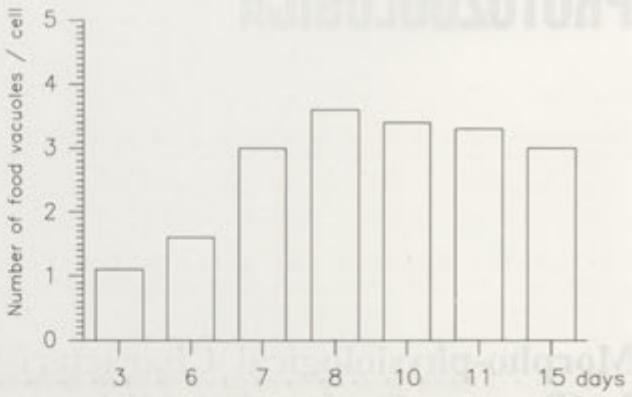


Fig. 2. Average number of food vacuoles per cell related to the exposure time

extract) with anibiotics (500 µg/ml of penicillin G and streptomycin and 200 units/ml of nystatin) (Kozloff 1956). After the next 24 hours, the ciliates were transferred again into a fresh medium and after 4 weeks - into medium without antibiotics. The stock cultures were inoculated every 10 days by transfer of 0.5 ml of cell suspension to 5 ml of medium. To ensure optimal thermal conditions the cultures were placed in an incubator at 20°C (Szablewski - unpublished data).

## Multiplication rates of *T. rostrata*

The experimental cultures were maintained in 200 ml flasks containing 50 ml of the medium, its composition and thermal conditions being the same as in stocks. The culture density, measured by means of an electronic cell counter, was then ca.1500 cells per ml. The samples were taken every 24 hours. The experiments were conducted on ciliates 6 to 12 months after isolation from renal organ.

## Course of phagocytosis

The number of food vacuoles formed in the suspension of India ink (Rotring) diluted with distilled water (1:100) was counted. The duration of feeding was established experimentally as 60 min (Tab.1). One ml of cell suspension was transferred to goblets at the appropriate time and 0.1 ml of India ink suspension was added. After 60 min. the cells were fixed with 0.1 ml 10% formalin solution. Subsequently the number of food vacuoles was counted in 100 cells. During experiments, the cups were placed in an incubator at 20°C.

Table 1

| | Duration of ink suspension uptake (in min.) | | | | |
|---|---|---|---|---|---|
| | 15 | 30 | 40 | 60 | 70 |
| food vac. | 0.0 | 0.74 | 0.75 | 1.14 | 1.11 |

Average number of food vacuoles per cell measured by India ink uptake by *Tetrahymena rostrata*

## The morphological study

Specimens for the morphological measurements were prepared using a Chatton-Lwoff procedure modified by Frankel and Heckmann (1968). The measurements were performed according to the method proposed by Taylor et al. (1976). The investigated sample contained ca. 50 cells. The observations were performed using a light microscope with immersion oil, the total magnification being 1600 x.

The following media were used: 1) 1.5% proteose-peptone + 0.1% yeast extract (PPY) and 2) 1.5% proteose-peptone + 0.1% yeast extract + salts (PPYS). The salt-containing medium was prepared according to the method by Plesner et al.(1964).

The samples were taken 7 days, 8 and 13 months after the isolation of ciliates from snails.

# RESULTS

## Growth of *T. rostrata*

In the first two days after inoculation of cells, only a slight increase in their number was observed (Fig. 1).

Subsequently the number of cells grew with time, doubling within approximately 4 days after inoculation. Another doubling of culture density was observed approximately 7 days. During next 4 days (up to the 11th day after inoculation) intense increase of cells' number was observed. From 11th to 12th day of experiment, cells' number gradually decreased. After this point, rapid decrease in the culture density was observed. This stage of growth of *T. rostrata* lasts for 7 days (to 19th day after beginning of experiment).

The phases of culture growth were: lag phase lasts during the first 2 days of experiment, acceleration phase from 2nd to 6th day, exponential phase from 6th to 12th day, decline phase takes place one day, and logarithmic decline - during next 6 days (Fig. 1).

**Course of phagocytosis**

The present experiment was concerned merely with the rate of endocytosis and did not deal with the process of egestions. The results obtained in experiments indicate that the rate of food vacuole formation is very low (Tab. 1). The time necessary to form one food vacuole per cell was longer than 40 minutes.

The study demonstrated that there was a connection between the rate of phagocytosis in *T. rostrata* and the phase of culture growth (Fig. 2). During the first three days of experiment, the average number of food vacuoles was 1.1. From 4-th day, the value gradually increased to about 3.6 of food vacuoles per cell (8-th day). Subsequently the number of food vacuoles gradually decreased. But the decline of rate of food vacuole formation is very low (from 3.6 of food vacuoles per cell at 8-th day to about 3.0 during the next 7 days).

**Morphological study**

The changes of morphological and morphometric parameters of *T. rostrata* during the cultivation in axenic medium are shown in Fig. 3.

During the first 7 days of incubation in buffer with antibiotics, the size of the ciliates decreased. The size also changed in the group of these cells as compared to *T. rostrata* just after isolation from *Z. nitidus*. After the 8 months of incubation of *T. rostrata* in PPY or PPYS, the characteristic cell shape changed and they become more round. The continuation of cultivation of the cells in PPY or PPYS had effects on morphological parameters. In both cases the size was reduced in comparison to *T. rostrata* from culture after 8 months of cultivation in axenic medium. The shape of these ciliates was similar to the parasitic forms of *T. rostrata*. But the cells were smaller than the parasitic forms. The average length of the parasitic for form, free-living *T.rostrata* in PPY and in PPYS (13 months after isolation) was: 59.8 µm; 51.4 µm; 48.7 µm, respectively. For the average width these values were: 40.9 µm; 38.4 µm and 35.4 µm, respectively.

Composition of medium affected morphological parameters, these effects, however, are small.

The length of cytostome in the cells incubated in buffer with antibiotics and in PPY or PPYS was smaller than that of the parasitic form. The value mentioned above decreased from 9.15 µm (parasitic form) to 7.81 µm for *T. rostrata* incubated during 13 months in PPYS.

Incubation of the cells in axenic medium also leads to an important decrease of the number of kineties (from an average 21.16 on one side of the cell in *T.rostrata* from *Z. nitidus* to 13.98 for ciliates after 13 months of incubation in PPY) Fig. 3.

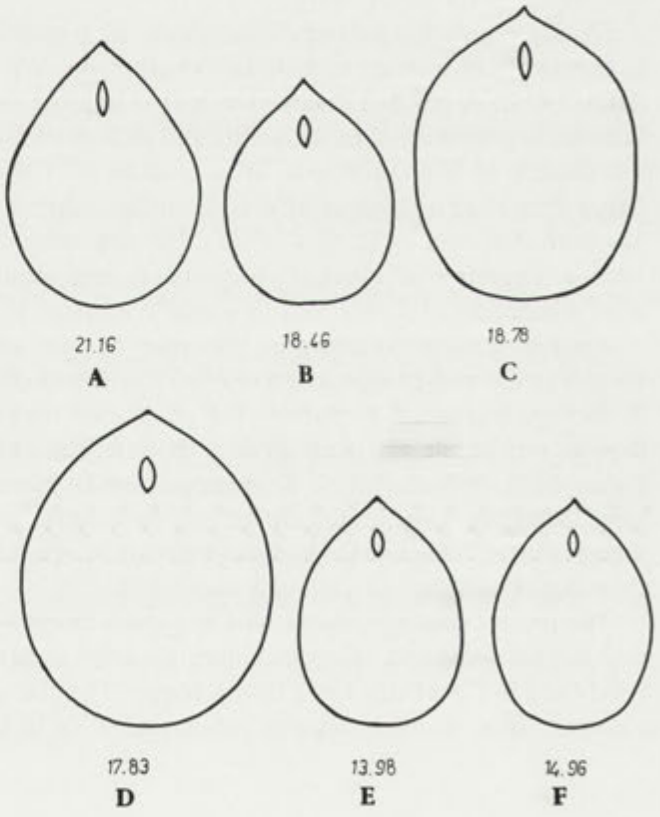


Fig. 3. Morphological variability of *T. rostrata* related to the time of cultivation in axenic medium and conditions of cultvation. *T. rostrata* directly from *Z. nitidus* (A), after 7 days of incubation in buffer Tris/HCl + antibiotics (B), after 8 months of incubation in PPY (C), and PPYS (D), after 13 months of incubation in PPY (E), and in PPYS (F). The value under the outline - the average number of kineties on one side of *T. rostrata*

## DISCUSSION

The obtained results show that change of habitat of *T. rostrata* has a strong influence on the investigated morpho-physiological parameters. These parameters, however, depend on the conditions of cultivation. The complementary experiments on effect of temperature show a slight influence on investigated parameters. The stronger effect was observed in dependence on the time of cultivation of *T. rostrata* in axenic medium and on composition of medium. Morphological variability during cultivation of the cells depends on composition of medium, but the effect is not clear and it is difficult to explain. The presence of salts affects the size of the ciliates. At one time the cells are greater in presence of these compounds (8 months after isolation), but after 13 months of cultivation in axenic medium the cells are smaller in PPYS than in PPY.

During regulation of body dimensions, *T. rostrata* is regulating of number of kineties (Frankel and Williams 1974). A general conclusion is that number of kineties is proportional to the width. But in *T. rostrata* the change of life conditions from parasitic to free-living form had a stronger effect. A similar response has been observed by Corliss (1973). The dependence "width - number of kineties" is probably important after adaptation of *T. rostrata* to a new environment.

Another change is seen in the case of rate of phagocytosis and phases of growth in *T. rostrata*. In *T. thermophila* and *T. pyriformis* the generation times depend on the rate of phagocytosis (Rasmussen and Orias 1975, Nilsson 1979, Szablewski 1984). Here long doubling times are related to low rates of phagocytosis. If the rates of phagocytosis are increased then doubling times of cells are decreased.

The present findings suggest that important morpho-physiological changes take place during a shift in life conditions in *T. rostrata*. Long time is required for these changes. How is it in natural environment? Which changes occur during the change from a free-living to a parasitic life style? More investigation in the area of morphogenesis and physiology of *T. rostrata* in nature and in laboratory will be necessary to answer these questions.

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# Morpho-physiological Characteristics of *Tetrahymena rostrata* (Ciliata). II. Effects of Long-term Incubation in Axenic Medium

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**Summary**. After a few years of cultivation of *Tetrahymena rostrata* in axenic medium, the cells differ from their "mother" form, which is parasitic. The rates of phagocytosis and the doubling times of the cells are like in *T. pyriformis*. For long-lasting cultivation in laboratory, the process of autogamy (in cysts) is necessary. After five years after isolation from *Zonitoides nitidus*, *T. rostrata* is still capable of infecting snails.

**Key words**. *Tetrahymena rostrata*, laboratory strain, morpho-physiology.

## INTRODUCTION

*Tetrahymena rostrata* has a considerable potential as an experimental organism in several areas of cellular research. But it is unfortunate that there are few studies on this species. There are data on the geographical distribution and morphology (Corliss 1973, Kazubski and Szablewski 1978) and on cysts (Corliss 1965, Dobra et al. 1980, McArdle et al. 1980). Cytogenetic and biochemical studies have been started.

There are few studies on morpho-physiological variability of *T. rostrata* produced by change of life conditions. The observations suggest that in this case, the process of morpho-physiological stabilization is long-lasting (Oleszczak and Szablewski 1992). Even as late as 13 months after the cells were isolated from the snails, their morphology had not been stabilized.

Parasitic *T. rostrata* are bigger than the free-living forms. The generation times in the first case are probably longer than in the latter. These are only general conclusions. In the parasitic phase, there are 26 to 46 ciliary rows, although generally between 32 and 41. In its free-living phase or in laboratory culture, the meridional range is between 24 and 32 (Corliss 1973). These values and other morphological parameters of *T. rostrata* were greatly influenced by the species of the host (Kazubski and Szablewski 1978). If laboratory populations of this species are established from originally parasitic populations, the species of the host may be important. We have undertaken to describe of morphology and general physiology of laboratory populations of *T. rostrata*, established from parasitic form.

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## MATERIAL AND METHODS

### Organisms

These experiments were carried out on laboratory *Tetrahymena rostrata*, 6 to 8 years since they had been established from parasitic populations. The laboratory population was obtained from land snails, *Zonitoides nitidus* (Oleszczak and Szablewski 1992). After isolation from the snail, the cells were cultivated in tubes containing 5 ml of medium (1.5% proteose-peptone + 0.1% yeast extract + salts) - PPYS. The salt-containing medium was prepared according to the method of Plesner et al. (1964). The cultures were placed in an incubator at 20°C.

### Rates of growth

The experimental cultures were maintained in 200 ml flasks containing 50 ml of medium. Nutrient composition and thermal conditions were the same as in the stocks. The culture density was measured by means of an electronic cell counter. At the moment of inoculation (0 h), the culture density was ca. 1000 cells/ml. During the first 7 hours of the experiment, the samples were taken every hour, during the next 43 hours - every 4 hours, and in the remaining 122 hours - every 12 hours.

### Capability of ciliates to infect snails

The experiments were carried out on four species of snails: *Cochlicopa lubrica*, *Succinea putris*, *Zenobiella rubiginosa* and *Zonitoides nitidus*. These species are known as hosts of *T. rostrata* (Kazubski and Szablewski 1978). The snails were collected at the places where *T. rostrata* was not found. Moreover, a few specimens from each species of snails were sectioned for examination of natural infection by *T. rostrata*. In all cases the results were negative; the snails were *Tetrahymena*-free.

The snails and ciliates were placed in Petri dishes containing 1.5% agar + 500 µg/ml of penicillin and streptomycin + 200 units/ml of nystatin. 48 hours before contact with snails, the ciliates were washed with Dryl solution and starved. Just before transfer into Petri dishes, the snails were washed, or not, with antibiotics (1000 µg/ml of penicillin and streptomycin + 400 units/ml of nystatin). The duration of contact was 8 to 37 days. The death rate of the snails under these conditions was about 80%. After this time, the snails were transferred twice every 48 h into new, dried Petri dishes. This procedure was necessary to exclude the presence of tetrahymenas on the snails (body, shell). After this procedure, the snails were sectioned and investigated with a stereomicroscope. The isolated ciliates ("secondary" parasitic form) were used in the morphological study. In the complementary experiments, cysts of *T. rostrata* were used.

### Morphology

The observations were performed on *T. rostrata* incubated 5 years in PPYS and isolated from snails ("secondary" parasitic form) and compared to free-living and "primary" parasitic form. The free-living form of *T. rostrata* was isolated from a streamlet near Warsaw and incubated axenically in PPYS. The slides from "secondary" parasitic form were due after 7 days of incubation of tetrahymenas in PPYS. The number of these ciliates was very low.

Specimens for observation were prepared using a Chatton-Lwoff procedure modified by Frankel and Heckmann (1968). The measurements were taken in accordance with the system and nomenclature proposed by Taylor et al. (1976). The investigated sample contained ca. 50 tetrahymenas.

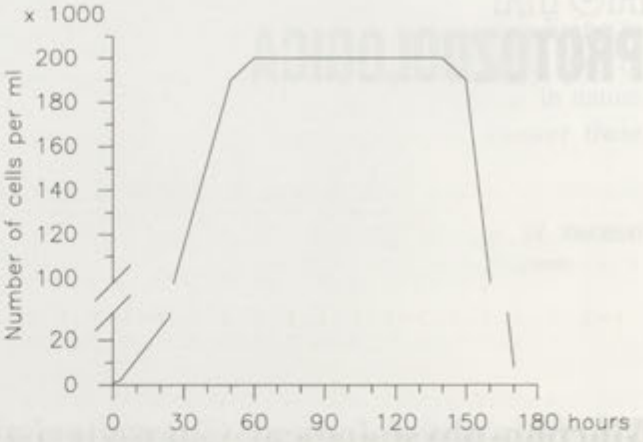


Fig. 1. Number of *Tetrahymena rostrata* cells versus time

### Production of encysting organisms

The formation of cysts was induced by the partially modified procedure proposed by McArdle et al. (1980). The trophozoites from axenic cultures of *T. rostrata* were washed 3 times by centrifugation (800 rpm for 4 min) with PBS-buffer and transferred into Petri dishes with sterile PBS-buffer at 25°C. The ciliates remained in this condition for 2 weeks. After this time, PPYS was added. Microscopical observations were made every day.

## RESULTS

### Rate of growth

During the first hour after inoculation of *T. rostrata* cells, only a slight increase in number was observed (Fig. 1). Subsequently the number of cells grew with time, doubling within approximately 3 hours after inoculation. During the next 21 hours, an intense increase in number was observed. Between 24 and 50 h after inoculation the rate gradually decreased and after approximately 60 h, no changes were found. 150 h after inoculation, a rapid decrease of cell density was observed.

The results show that the lag phase lasted for the first hour of the experiment, the acceleration phase from the second to the fourth hour, the exponential phase continued to the 24 h after inoculation, the decelerating phase to 60 h, stationary phase to 140 hours. The declining phase lasted then 10 hours and the logarithmic decline - the next 12 hours.

**Capability of ciliates to infection of snails**

The results obtained in this experiment suggest that laboratory strains of *T. rostrata* can infect snails. The positive results were obtained only with trophozoites. If cysts were used, not all snails were infected. It is noteworthy that only *Z. nitidus* was infected, however other potential hosts were also used. *Tetrahymena* were found only in renal organ. The extensity and intensity of invasion were lower than in the natural environment (about 30% and about 10 specimens, respectively). The time of contact of ciliates with snails had a little effect on these.

**Morphology**

The investigations performed demonstrated that long-lasting cultivation in PPYS and "secondary" parasitic life changed the morphology of *T. rostrata*. As a control for investigations of effects of long-lasting cultivation of tetrahymenas, ciliates from *Z. nitidus* ("primary" parasitic form) were used (Fig. 2; B and C). During the period of time (5 years), cell dimensions and their proportion changed in the group of investigated cells (Fig. 2; compare C and D). Small differences depending on the time of cultivation in PPYS were noted in the case of e. g. WLEN, %WLN. These changes, however, may be too small to be the basis upon which conclusions can be drawn about the mechanism of action of long-lasting cultivation in laboratory of *T. rostrata*. Length, cytostome, A-M1, A-UM become slightly reduced.

Cultivation in PPYS also leads to a decrease of the number of kineties, but to an increase of width, %BRD, %BD1 and %BDM1.

Changes in morphology of "secondary" parasitic forms were observed (Fig. 2; F). The controls were cells after long-lasting cultivation in PPYS (Fig. 2; D). The length and A-UM of the "secondary" parasitic form were the same as in controls. The cytostome and WLEN were elongated, the values of %PWI, %AUM, %WLN and %BUC were increased. Other dimensions and values were reduced. Thus the characteristic shape of *T. rostrata* cells changes and the cells become more elongated. This is due to the decrease of value of width, BDM1 and BDUM. The size and shape of these cells were comparable to the free-living form, and to the starved cells (Fig. 2; A and E, respectively).

**Production of encysting organism**

After transfer of trophozoites to starvation medium, the cells were kept without food until the organisms ceased their random swimming and began to aggregate before encystment. This aggregation was generally observed within several hours, then the ciliates became rounded. Two days after transfer of ciliates, dividing cells were not observed. This shows that the number of trophozoites slowly declined. After two weeks, free-swimming trophozoites were not observed. Cysts remained in this stage for 3 days and then PPYS (in

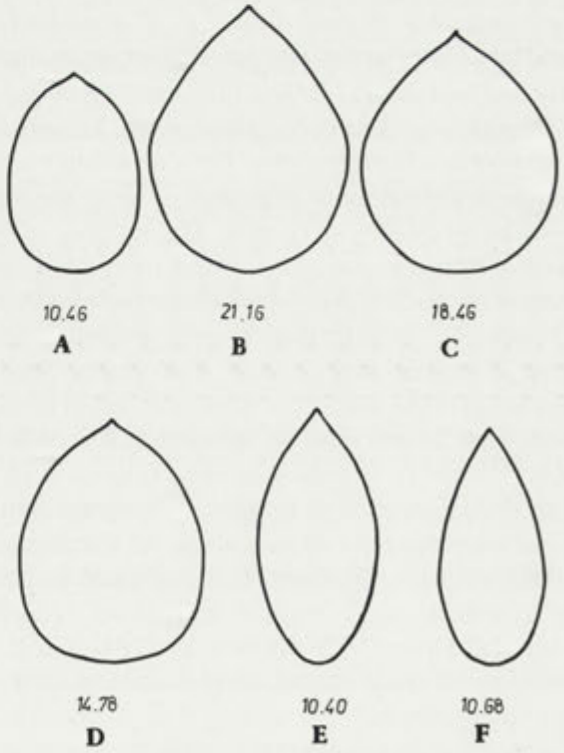


Fig. 2. Morphological variability of *Tetrahymena rostrata* in relation to the conditions of life. A-Free-living form, B-*T. rostrata* from *Z. nitidus*, C-*T. rostrata* after 7 days of incubation in PPYS, D-Laboratory population in PPYS, E-Starved laboratory population, F-"Secondary" parasitic form. The value under the outline - the average number of kineties on one side of *Tetrahymena rostrata*

the same volume as starvation medium) was added. Twenty four hours later only free-swimming trophozoites were observed. About 30% of the ciliates were at a stage of cytokinesis and the number of cells grew with time.

If the cysts remained at this condition more than 10 days, or were dried, trophozoites were not observed after addition of PPYS.

## DISCUSSION

*T. rostrata* is a polymorphic species. Morpho-physiological functions depend on the stage or phase in the life history (Corliss 1973). The investigations performed demonstrated that the change of life conditions altered the rate of growth of *T. rostrata*. During the few months after transfer of ciliates from snails to PPYS, long generation time of cells was observed. The first doubling of cell number was observed approximately 4 days after inoculation (Oleszczak and Szablewski 1992). Six years after a laboratory strain had been established from parasitic population, the rates of growth of *T. rostrata* were comparable to these values in *T. pyriformis* - a typical laboratory strain. The generation time in optimal conditions was about 3 h. The first doubling of population density was observed approximately 3 hours after inoculation to a fresh medium. The shortening in doubling time in the culture was probably due by the higher rate of phagocytosis in ciliates. The number of food vacuoles formed in the cells derived from a laboratory culture, was much higher (approximately more than 10 food vacuoles per cell) than in cells derived from the culture 13 months after isolation from snails. The time necessary to make one food vacuole per cell in the latter ciliates was longer than 40 minutes (Oleszczak and Szablewski 1992). In cells cultivated 6 years in PPYS, during the 15 minutes of feeding, the average number of food vacuoles per cell was about 10 (Szablewski - unpublished data). This was been confirmed by results of other authors on *T. pyriformis* (Nilsson 1979, Rasmussen and Orias 1975, Szablewski 1984). Great differences in maximal culture density between these two forms were observed.

Despite the long-lasting cultivation of *T. rostrata* in PPYS, the ciliates kept their capacity for infection of snails. But it is interesting that only specimens of *Z. nitidus* were infected. This result suggests that these cells have a high specificity for the host species. The same observations were made in natural environment. Different species of snails (hosts of *T. rostrata*) were collected from one place, for example, from under a piece of wood. But only *Z. nitidus* was infected (Szablewski - unpublished data). Other findings indicate that there are important morphological differences between tetrahymenas collected from different species of hosts (Corliss 1973, Kazubski and Szablewski 1978). Our observations presented in this paper suggest a high specificity of *T. rostrata* to one species of host. Maybe it is one species (*T. rostrata*) but with different forms (subspecies ?, varieties ?). It will be necessary to do more precise observations in the laboratory and in the natural environment.

The results obtained by other authors (Corliss 1965, 1973, McArdle - personal com.) indicate that production of resting cysts is necessary in *T. rostrata*. Our findings confirm these observations, but the cyst stage may last a short time.

The tomite is a stage in the life cycle of *T. rostrata*, which does not divide. The presented findings indicate that *T. rostrata* may go through a tomite stage in a short time. After excystment of the ciliates, the dividing cells were observed. The duration of tomite-stage is probably determined by the composition of medium.

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# Differential Responses to Neutral Red Uptake of Mating Type I and Mating Type II Cells in *Paramecium primaurelia*. I. Cell Size Variation

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**Summary**. In *Paramecium primaurelia* the mating type I and mating type II cells react in a different way to the action of neutral red: the mating type II cells increase their size when stained with this vital dye; on the contrary, the mating type I cells show no variation in this respect. Since the neutral red, at a low extracellular concentration and for short exposure times, accumulates in lysosomes, it can be assumed that the mating type II cells possess a higher number of food vacuoles and also have more cytoplasmic binding sites for dye storage. A larger accumulation of neutral red inside the cell might cause the osmotic influx of water and the cell size increase observed in mating type II cells.

**Key words**. *Paramecium primaurelia*, mating type, neutral red uptake, cell size variation.

## INTRODUCTION

Studies carried out by Crippa Franceschi (1981, 1987) on the determination and expression of the mating type in *Paramecium primaurelia* demonstrated that mating type I caryonides have different rates of growth as compared to those of mating type II. Moreover, it was pointed out that mating type II populations show, at mating reactivity, a higher cell density than mating type I populations (Crippa Franceschi 1987, Ramoino 1988). These results let us assume that the different rates of growth can lead, at mating reactivity, to different cell conditions so as to allow mating and that, therefore, the populations of the two mating types can differ in morphological and/or physiological aspects (Crippa Franceschi 1985, 1987; Crippa Franceschi et al. 1984). On the other hand, Delmonte Corrado (1987) had shown that in *P. primaurelia* mating type I cells have a higher macronuclear DNA content than mating type II cells, according to the different number of fissions effected. Differences between mating types V and VI have been reported in *P. caudatum* as regards the stability of mating reactivity to formalin (Hiwatashi 1950), the adhesion of paramecia to cation-exchange resins (Hiwatashi 1969), the action of triton X-100 on the mating reactivity of cilia (Watanabe 1977) and the thickness of the surface coat when stained with ruthenium red (Watanabe 1981).

As to the search for differences between mating type I and mating type II cells of *P. primaurelia*, it was assumed that mating type II cells, having on the average a shorter cell cycle and a lower macronuclear DNA content, could also differ in cell size as compared to mating type I cells. Since the differences in size between the two mating type cells could be minimized owing to the possible presence, within the population, of unreactive cells, the partners of split pairs were measured, as the pairs formed, in order to test certainly reactive cells. These pairs were obtained by mixing populations of a given mating type with previously stained complemen-

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tary mating type cells. For this purpose neutral red was used, a weak basic dye, which diffuses rapidly through the plasma membrane and is employed for staining in vivo the cells (Hiwatashi 1951).

It has long been known that neutral red accumulates in granules distributed in a punctated pattern throughout the cell and around certain food vacuoles (Mast 1947). A part of these granules (lysosomes) contains acid phosphatase reaction products (Rosenbaum and Wittner 1962, Allen and Fok 1983) and is involved in the intracellular digestion of ingested material (Koehring 1930, Kitching 1956). Another part of the granules (acidosomes) is devoid of acid phosphatase reaction products: it remains near the newly formed vacuoles and represents the mechanism responsible for food vacuole acidification (Allen and Fok 1983). Neutral red binds also to RNA-coated vesicles and to free ribosomes (Bulychev et al. 1978). The storage of dye into the cell, by giving rise to a passive influx of water (de Duve et al. 1974, 1978), might cause an increased cell size. Temporary passive changes in the cell volume were observed in *Tetrahymena* and in peritrich ciliates, consequent to variation in the external environmental conditions (Kitching 1967, Dunham and Kropp 1973, Jones 1974).

Another purpose of this study is to verify whether neutral red can variously affect mating type I and mating type II cells depending on the different number of dye binding sites, i.e. on the difference in their metabolic activities.

## MATERIAL AND METHODS

*Paramecium primaurelia* stock 90 cells were grown at 25°C in a lettuce infusion (pH 6.8) bacterized with *Klebsiella pneumoniae* one day before use (Sonneborn 1970).

Conjugating pairs were isolated after mixing cells of complementary mating types. The exconjugants and the caryonides were isolated and transferred into a fresh culture medium. The caryonides were cultured in test-tubes except for the period of a few fissions immediately after conjugation, during which the cultures were maintained in depression slides. The test-tube cultures were obtained by inoculating the cells into test-tubes containing 3 ml of fresh culture medium and by adding, two days later, 15 ml of fresh culture medium. The starved cells were used to test their mating reactivity by mixing them with tester cells. The caryonides of mating types I and II were in this way identified. The cultures were maintained under sexual reactive conditions for 8 days since the mating type expression (Sonneborn 1950).

At different time (day) intervals from the mating type expression, cells of mating type I were stained in vivo for 10 min with 0.0005% neutral red and mixed with unstained cells of mating type II, and v.v. As soon as the pairs formed, they were split and the stained and unstained cells of both mating types were measured by a micrometer ocular (1 eye - piece division = 4.17 μm). The maximum length and width of the body were measured in a given number of cells. The food vacuole number was determined in stained and unstained cells examined using phase contrast microscopy.

The means and standard deviations were calculated; the differences between the samples were evidenced by using Student's t-test.

## RESULTS

Mating type I and mating type II cells do not differ in size, both when the analysis is carried out on populations which include, besides reactive cells, a small part of unreactive cells too, and when the analysis is made on certainly reactive cells, i.e. on cells which formed some clumps with stained cells of the opposite mating type (Table 1). So, when the reactivity starting time is equal, the cells of the two mating types do not differ significantly in size. On the contrary, differences are found between cells stained with neutral red: the mating type II cells have a larger size than mating type I cells. By comparing neutral red stained cells with unstained cells, it appears that, at the beginning of the mating reactivity and on the 2nd, 3rd and 4th day of sexual reactivity maintenance, the mating type II stained cells increase their size with respect to unstained cells, whereas the mating type I cells show no variation. On the 8th day of reactivity maintenance, the mating type II cells, too, do not swell.

As most neutral red enters the cells by simple permeation through the plasma membrane, it should not induce the formation of new food vacuoles. Actually, no significant differences in food vacuole number between unstained and neutral red stained cells of both mating types are found at the beginning and during the mating reactivity maintenance except for the case of mating type II cells on the 8th day (Table 2).

Table 2 also shows that in the unstained cells the number of food vacuoles decreases during reactivity maintenance. Furthermore, mating type II cells, which have at the beginning a higher number of food vacuoles, feed and starve more rapidly thus showing on the 8th day of reactivity maintenance, a lower number of food vacuoles than mating type I cells.

## DISCUSSION

The results of this study show that in *Paramecium primaurelia* the mating type I and mating type II cells do not differ in size during mating reactivity, whereas

they respond in a different way to the action of neutral red. Therefore, the question is how the dye can cause such different consequences in the cells.

Neutral red is a weak basic dye; it is taken up by cells and localized in subcellular structures where it accumulates in large quantities. It was demonstrated that at low extracellular concentrations and for short exposure times, i.e. when the amount of neutral red taken in is relatively small, the bulk of intracellular dye is largely associated to lysosomal hydrolases (Rosenbaum and Wittner 1962, Koening 1963, Allison and Young 1964, Bulychev et al. 1978). Accumulation of weak basic dye in lysosomes derives from the rapid diffusion of unprotonated base through the plasma and lysosomal membranes and, because of the acidity inside the lysosomes, it is trapped there by protonation, thus increasing intralysosomal concentration. When the concentration within the lysosomes is sufficiently high (approaching isotonicity), an osmotic influx of water causes the lysosomes to swell (de Duve et al. 1974, 1978, Ohkuma and Poole 1981, Poole and Ohkuma 1981). It was demonstrated that, besides accumulating in lysosomes, neutral red can be also stored in acidosomes (Allen and Fok 1983) or in RNA-coated vesicles, free ribosomes, membranes and soluble proteins (Bulychev et al. 1978).

Therefore, it can be assumed that mating type II cells can accumulate a larger amount of neutral red inside themselves as compared to mating type I cells, because of the different permeability of their membranes to the protonated form of the base and/or because of the different number of the cytoplasmic binding sites for this dye. The accumulation of the material would cause the osmotic influx of water and, consequently, a cell size increase.

The assumption that mating type II cells have a larger number of cytoplasmic binding sites for dye storage is supported by the fact that they have, at mating reactivity,

Table 1

Length and width of the mating type I and matting type II cells at the beginning (1st day) and during mating reactivity maintenance (days 2 - 8). Cells are split without and after exposure to neutral red

| Time in days | Synclone | m.t. | Mean length (μm) ± s.d. | | Mean width (μm) ± s.d. | |
|---|---|---|---|---|---|---|
| | | | without exposure to neutral red | after exposure to neutral red | without exposure to neutral red | after exposure to neutral red |
| 1 | A | I | 134.79 ± 11.49 | | 60.96 ± 5.24 | |
| | | II | 135.45 ± 11.01 | | 59.17 ± 4.38 | |
| 1 | Z | I | 130.78 ± 13.06 | 128.90 ± 8.36 | 60.12 ± 8.58 | 61.02 ± 7.00 |
| | | II | 127.51 ± 9.66 | 134.80 ± 11.02 | 62.11 ± 4.15 | 65.94 ± 4.38 |
| 2 | V | I | 124.68 ± 7.07 | 125.49 ± 11.30 | 57.45 ± 5.62 | 55.18 ± 5.73 |
| | | II | 126.19 ± 13.20 | 140.81 ± 13.62 | 58.82 ± 5.23 | 67.17 ± 5.37 |
| | A | I | 129.65 ± 9.53 | 128.92 ± 10.87 | 57.08 ± 4.50 | 57.17 ± 5.62 |
| | | II | 125.84 ± 8.58 | 142.25 ± 6.54 | 56.18 ± 3.61 | 61.44 ± 2.83 |
| 4 | Z | I | 116.48 ± 5.51 | 119.79 ± 7.93 | 55.93 ± 4.85 | 57.34 ± 6.69 |
| | | II | 118.32 ± 9.07 | 126.59 ± 11.37 | 55.73 ± 4.76 | 60.99 ± 2.69 |
| 8 | Q | I | 127.67 ± 8.85 | 124.04 ± 7.79 | 52.13 ± 4.34 | 54.40 ± 3.29 |
| | | II | 132.30 ± 11.55 | 133.14 ± 8.37 | 55.54 ± 3.64 | 55.10 ± 2.55 |

(*) Length and widht in non-split cell populations. The sample of split cells was ranging between 20 and 30 cells; that of non-split cells was 60 cells. The mean lengths and widths underlined by the same line were significantly different from each other using Student's t-test

Table 2

Food vacuoles in the mating type I and mating type II cells at the beginning (1st day) and during mating reactivity maintenance (days 3-8). Cells are split without and after exposure to neutral red

| Time in days | Synclone | m.t. | Mean number of food vacuoles ± s. d. | | t | P(t) |
|---|---|---|---|---|---|---|
| | | | without exposure to neutral red | after exposure to neutral red | | |
| 1 | Z | I | 7.27 ± 1.42 | 6.88 ± 1.54 | 0.82 | > 0.70 |
| | | II | 8.93 ± 2.31 | 9.31 ± 3.12 | 0.36 | > 0.70 |
| 3 | A | I | 3.09 ± 1.45 | 2.93 ± 2.64 | 0.60 | > 0.70 |
| | | II | 5.00 ± 3.37 | 5.83 ± 3.71 | 0.60 | > 0.70 |
| 4 | Z | I | 4.33 ± 1.30 | 4.85 ± 1.99 | 0.75 | > 0.70 |
| | | II | 5.00 ± 1.31 | 5.28 ± 0.35 | 0.35 | > 0.70 |
| 8 | Q | I | 1.46 ± 1.45 | 1.33 ± 2.06 | 0.18 | > 0.70 |
| | | II | 0.73 ± 1.10 | 2.43 ± 2.28 | 2.27 | 0.05 - 0.02 |

The sample size was ranging between 20 and 30 cells

a larger number of food vacuoles than mating type I cells. Moreover, the mating type II cells form a higher amount of food vacuoles than mating type I cells not only at mating reactivity (Ramoino 1989), but also during the log growth phase (Ramoino and Crippa Franceschi 1990). A different number of food vacuoles in cells of complementary mating types could represent a different amount of active phosphatase, ribosomes and membrane, i.e. a different number of binding sites for neutral red and, therefore, a different accumulation of dye inside the cells. It was demonstrated that the hydrolase activity is related to digestive processes (Estève 1970, Müller 1972, Fok and Paeste 1982) and that the cellular metabolism is reduced when food vacuole formation is inhibited (Skriver and Nilsson 1978).

Furthermore, since starvation increases during sexual reactivity maintenance and the cells of both mating types show very few or no vacuoles, it is supposed that the binding sites for neutral red decrease. In this case the amount of dye inside the cells does not reach a concentration causing the entrance of an amount of water necessary for a significant increase in size. Even though the number of food vacuoles increased in mating type II cells stained with neutral red, no size increase caused by an increased active acid phosphatase occurred, as it could be expected. In fact, a more complete activity of this enzyme, caused by the induction of endocytosis in starved cells, occurs after 1h and reaches its peak after 2h (Ricketts 1970); moreover, this adaptive response only occurs after digestion of digestible material (Ricketts and Rappitt 1975), which indicates that a new synthesis is involved.

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# Differential Responses to Neutral Red Uptake of Mating Type I and Mating Type II Cells in *Paramecium primaurelia*. II. Change in the Macronuclear DNA Content

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**Summary.** The cytofluorometric measurements of the macronuclear DNA content of reactive split cells stained with neutral red solution reveal that the dye affects differently the mating type I (stocks 90 and P) and the mating type II (stock 90) cells of *Paramecium primaurelia*. In fact, unlike mating type I populations, the mating type II stained cells show a macronuclear Feulgen-DNA content significantly higher than that of the unstained population. It can be assumed that the differential effect of neutral red is related to a different storage of dye in the two mating types cells. These results give further evidence that the expression of the two mating types, related to the mode of clonal growth, is linked to with cell conditions which are found to be different.

**Key words.** *Paramecium primaurelia*, mating type, neutral red uptake, macronuclear DNA content.

## INTRODUCTION

The studies which consider the mating type expression in *Paramecium primaurelia* due to differential cell surface conditions related to the mode of clonal growth from cell reorganization up to reactivity (Crippa Franceschi 1981, 1985, 1987; Crippa Franceschi et al. 1984, Morchio and Crippa Franceschi 1986, 1989), have shown that the mating type I and mating type II reactive populations in stock 90 are characterized by a higher and a lower mean macronuclear DNA content, respectively. This difference persists between the populations kept under sexually reactive conditions and it is not compensated up to at least 15 days after the onset of mating type expression (Delmonte Corrado 1985, 1987). Because one of the paired members was marked by a vital dye, the split of pairs made it possible to measure the macronuclear Feulgen-DNA content of mating type I and mating type II unstained populations consisting of reactive cells only.

This paper deals with the macronuclear DNA content of split stained partners of both mating types. Staining method employed neutral red at low concentration and for short exposure periods. It is known that under these conditions, a weak basic dye such as neutral red is stored up in the cells by binding to several cytoplasmic components (Bulychev et al. 1978). We have verified whether neutral red uptake does affect the macronuclear DNA content of *Paramecium primaurelia* complementary mating type populations obtained by the split pair method.

Previous researches evidenced a series of differential responses of the two mating types under sexual reactivity

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conditions. In particular, the time of mating inhibition after trypsin treatment (Crippa Franceschi et al. 1987), the time of autogamy onset (Crippa Franceschi et al. 1989b), the electromigration rate (Crippa Franceschi et al. 1989a), and the rate of food vacuole formation (Ramoino 1989) differed. In this connection, a different effect of the dye on the macronuclear Feulgen-DNA content revealed a differential response of the mating types.

## MATERIAL AND METHODS

*Paramecium primaurelia* stock 90 (mating types I and II) and stock P (mating type I) were grown at 25°C in a lettuce infusion (pH=6.8) bacterized with *Klebsiella pneumoniae* the day before use (Sonneborn 1970). Stock 90 and stock P caryonides were grown to obtain mating type expression. Based upon their mating reaction with tester caryonides of stock P or of stock 90 mating type II, the stock 90 mating type II and mating type I caryonides were identified, respectively. Then the cultures were kept under sexual reactivity conditions for at least 8 days after the onset of mating type expression (Sonneborn 1950). Both mating type I (stocks 90 and P) and mating type II (stock 90) cells were stained for 10 min with neutral red solution at the final 0.0005% concentration (Bleyman 1967) and mixed with the unstained cells of the complementary mating type. The pairs were split as soon as they formed, and the partners were transferred upon the slide and allowed to dry. Thus, as the split mates were identifiable, neutral red stained reactive cells and unstained reactive cells (controls) of stock 90 both mating types and of stock P mating type I were available. The samples were then fixed in an ethanol-acetic acid mixture (3:1) for 20 min, hydrolyzed all together for 20 min in 1 N HCl at 40°C, stained in 0.1% Schiff-pararosaniline for 1 h, bleached in sulfured water, dehydrated and mounted.

The macronuclear Feulgen-positive content was determined by fluorescence cytophotometry (Böhm and Sprenger 1968) by means of a Leitz MPV II microphotometer (E. Leitz, Wetzlar, Germany) fitted with an incident light fluorescence system (Ploem 1967). Individual macronuclei were focused in a phase-contrast system by a 54 x oil immersion objective. The specific optical arrangement used for fluorescence measurements consisted of a high-pressure XBO 75W/2 Xenon lamp (Osram, Munich, Germany), a 2 mm heat-protecting filter, a 4 mm BG 38 red adsorbing filter, a S546 interference excitation filter, a TK580 dichroic beam-splitting mirror and a K665 barrier filter. The fluorescence intensities were recorded by a photomultiplier S20 type 9558AQ and the fluorescence intensity values (fluorescence units) were read directly on a digital voltmeter display.

## RESULTS AND DISCUSSION

The results indicate that mating type I and mating type II split cell responses to uptake of a vital dye (neutral red) clearly differ as far as their macronuclear DNA contents are concerned. In fact, no significant difference in the Feulgen-positive contents appears between the unstained and neutral red stained cells of mating type I of both stocks 90 and P; however, a significant increase is found between the unstained and stained mating type II populations (Table 1). These results are confirmed by the measurements carried out in populations kept under sexual reactivity conditions for the same time period (eight days) after the onset of mating type expression (Table 2). In this case too, the only significant difference is referred to the macro-

Table 1

Macronuclear Feulgen-DNA content of the mating type I and mating type II split cells without and after exposure to neutral red

| Stock | m.t. | Mean ± S.E. | |
|---|---|---|---|
| | | without exposure to neutral red | after exposure to neutral red |
| P | I | 32.20 ± 0.72 | 31.38 ± 0.97 |
| 90 | I | 29.33 ± 0.62 | 29.11 ± 0.63 |
| | II | 24.25 ± 0.56 | 29.56 ± 1.14 |

Means in arbitrary fluorescence units ± standard error. Student's t-test was used to compare the differences between the split cells without and after exposure to neutral red. Only means underlined by the continuous line are significantly different from each other ($P<0.001$). The sample size was between 25 and 65 cells

Table 2

Macronuclear Feulgen-DNA content of the mating type I and mating type II cells, maintained under sexual reactivity conditions, measured at the 8th day following mating type expression and split without and after exposure to neutral red

| Stock | m.t. | Mean ± S.E. | |
|---|---|---|---|
| | | without exposure to neutral red | after exposure to neutral red |
| P | I | 30.18 ± 1.20 | 32.25 ± 1.68 |
| 90 | I | 27.96 ± 1.40 | 28.19 ± 1.03 |
| | II | 24.70 ± 0.81 | 30.95 ± 1.47 |

Means in arbitrary fluorescence units ± standard error. Student's t-test was used to compare the differences between the split cells without and after exposure to neutral red. Only means underlined by the continuous line are significantly different from each other ($P<0.01$). The sample size was comprised between 11 and 26 cells

nuclear Feulgen-positive content of the mating type II stained cells characterized by a greater mean value than that of the unstained mating type II population.

Two main subjects of discussion derive from the above mentioned results: a) the uptake of neutral red reveals its effect in the increased fluorescence emitted by the macronuclei of the mating type II cells in comparison with their controls of the same mating type; b) the cells of both mating types are differently affected by neutral red uptake regarding their macronuclear Feulgen-DNA content.

As for the first assertion, it can be assumed that the uptake of a weak basic dye, like neutral red, which enters cells by simple permeation and accumulates in intracellular binding sites by a reversible process (Allen and Fok 1983, Bulychev et al. 1978), determines an increased concentration of the solute in the cytoplasm and consequently the increase of the intracellular pH value. In regard to this, Gillies and Deamer (1979) showed that two alkaline shifts of the intracellular pH observed during the cell cycle of synchronized cultures of *Tetrahymena pyriformis* are associated with DNA replication, thus suggesting that the intracellular pH might also act as a signal or regulatory factor in controlling DNA replication; moreover, the alkaline shift of intracellular pH following fertilization of sea urchin eggs was supposed to serve as a stimulus for derepression of DNA synthesis (Shen and Steinhardt 1978). Therefore, it can be assumed that in *Paramecium primaurelia* too, the variation of the intracellular pH value following neutral red uptake is related to the real increase of the macronuclear DNA amount. In this case, the increased fluorescence emitted by the macronuclei of the mating type II stained cells in comparison with their controls of the same mating type, would indicate that the effect of neutral red consists in stimulating DNA synthesis.

On the other hand, the extent of the increased DNA value related to mating type II cells after neutral red uptake (20% more than in controls) suggests that the greater fluorescence emitted by the macronuclei could also be due to a change in the functional state of their chromatin in addition to a real increase of the DNA content. In fact, 10-20% cytophotometric absorbance differences are described between compact nuclei and nuclei with more dispersed chromatin, but characterized by the same karyotype (Noeske 1971, Duijndam and Van Duijn 1975). Moreover, an increased Feulgen-DNA value without increase of the DNA content in nuclei of cells stimulated by hormones or by other means is found to depend on a change in the state of their chromatin which appears loosened at the optical and electron microscope inspection (Noeske 1971). These aberrations could be explained by the relationship between the degree of chromatin compactness and the depurination rate evidenced in a study on swollen and condensed nuclei, where lower absorbance maxima are encountered in compact forms of chromatin (Duijndam and Van Duijn 1975). These results lead to the suggestion that neutral red uptake could also affect the state of macronuclear chromatin of the mating type II cells, thus increasing the emitted fluorescence.

Referring to the second point of discussion, the differential response of the mating type I and mating type II cells to vital dye uptake suggests some considerations. It can be assumed that neutral red is accumulated in a different amount by the two mating types. The reasons of this quantitative differential uptake could be found both in the different permeability of the cell membrane and in the different amount of the cytoplasmic binding sites of the dye. Taking into consideration that in reactive *P. caudatum* cells the two ciliary surfaces involved in the mating are hydrophobic (Kitamura 1982), it has been assumed and then shown that a different hydrophobicity characterizes the two reactive mating types in *P. primaurelia* (Crippa Franceschi 1985, 1987; Crippa Franceschi et al. 1991). Therefore, it can be suggested that the dye enters cells in different quantities according to the cell surface properties of their mating type. The presence of a large number of cytoplasmic binding sites for neutral red in mating type II would be consistent with the presence of a larger number of food vacuoles observed in reactive mating type II cells with respect to reactive mating type I cells (Ramoino and Delmonte Corrado 1992). It has been shown that at low concentration rates and for short exposure periods, the neutral red also binds with various cytoplasmic components such as ribosomes, membranes and soluble proteins in addition to lysosomal hydrolases (Bulychev et al. 1978), the activity of which is closely related to digestive processes (Estève 1970). Consequently, the decrease in the food vacuole number observed during the maintenance of reactivity (Ramoino and Delmonte Corrado 1992), would imply the decrease of lysosomal binding sites for neutral red. Nevertheless, it can be supposed that in mating type II reactive cells, unlike in mating type I, the number of cytoplasmic binding sites, above lysosomes, is still large enough to accumulate a neutral red amount such as to stimulate macronuclear DNA synthesis. Therefore, the effect of neutral red on the DNA content would be significant

only in case of mating type II cells, where dye accumulation would result higher than in mating type I.

Following the above considerations, it can be concluded that the effect of neutral red uptake on the macronuclear Feulgen-DNA content is related to the cell functional state and that this state differs in mating type I and in mating type II reactive cells, also when examined after the same time period from the onset of sexual reactivity expression. Summing up connection, the results of the present paper provide further evidence supporting the statement that the different mating types expression is related to cell conditions which prove to be different.

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