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Patterns of growth in coccoid, aggregate forming cyanobacteria

Katarzyna A. Palińska & Wolfgang E. Krumbein

Palińska, K. A. & Krumbein, W. E., Carl von Ossietzky University, ICBM, Geomicrobiology, P.O. Box 2503, D-26111 Oldenburg, Germany

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Several *Merismopedia*-like forms were observed and collected from the microbial mats at Mellum and Norderney Islands. The formation of giant cells and giant cell aggregates in otherwise smaller *Merismopedia* cultures was often observed in all cultures. In the course of this study it was possible to show, that accelerated and delayed division occurs in Chroococcales not unlike the multiple fission pattern in Pleurocapsales. On the basis of the existence of the terms baeocyte and nanocyte for cell size decrease in one clone and our observation of delayed division in *Merismopedia* isolates we suggest the following terms: (1) *baeocyte* for rapid, multiple fission resulting in very small individual cells (motile and non-motile), (2) *nanocyte* for accelerated division resulting in considerably smaller cell sizes, and (3) *megacyte* for considerably enlarged cell sizes upon delayed division. Nanocyte formation and megacyte formation when occurring under stable environmental conditions may actually have been and still be misinterpreted as separate species in field samples and herbarium materials.

Key words: baeocyte, cyanobacteria, *Merismopedia*, nanocyte, taxonomy

INTRODUCTION

The cyanobacteria constitute one of the largest sub-groups of Gram-negative prokaryotes. As a result of their traditional assignment to the algae, the classification of these organisms was developed by phycologists, working under the rules of the botanical code. This consideration is supported by their functional position in nature since cyanobacteria as phototrophic organisms are important primary producers in almost all biotopes on the Earth.

According to Geitler (1932), considering their types of reproduction, non-filamentous species are classified in 3 orders: Chroococcales, Chamaesiphonales and Pleurocapsales. Komarek and Anagnostidis (1986) classified all coccoid "cyanophytes" in a simple order Chroococcales with 7 definable families (Microcystaceae, Chroococcaceae, Entophysalidaceae, Chamaesiphonaceae, Dermocarpellaceae, Xenococcaceae and Hydrococcaceae). Their arguments to do so were based on the type of division, which is basically the same in all coccoid cyanobacteria. The bacteriologists — Rippka *et al*. (1979) and Bergey's Manual (Castenholz 1989a, 1989b, Waterbury 1989, Waterbury & Rippka 1989,) — divided coccoid or non-filamentous cyanobacteria into two orders:

Chroococcales (reproduction by binary fission in one, two or three planes or by budding), and Pleurocapsales (multiple fission or combination of multiple fission and binary fission).

The differences between "botanists" (Geitler, Komarek and Anagnostidis) and "bacteriologists" (Rippka, Castenholz, Waterbury) are that the former used the keys and descriptions of cyanobacteria in the field and herbaria, and the latter accept only pure cultures. According to Bergey's Manual and the traditional botanical literature, the presence or absence of cell aggregates and their characteristics, the number and regularity of the planes of division, as well as statistically stabilized cell size clusters are the major characteristics used to determine genera and groups of coccoid cyanobacteria. The presence of extracellular sheath layers in several coccoid taxa (*Gloeobacter*, *Gloeothece* and *Gloeocapsa*) in culture has proven to be a stable feature as well. However, *Synechocystis*, *Merismopedia*, *Microcystis* and *Eucapsis* (in botanical sense) occur in cell aggregates in nature but rarely in culture. Additionally, in practice, it is often difficult to determine the number of successive planes of division, and the presence or absence of a glycocalyx.

Thus it seemed worthwhile to study the morphological, physiological, biochemical and molecular aspects of coccoid cyanobacteria without baeocytes (lacking rapid multiple fission), in the field and laboratory. For this particular group of cyanobacteria much less is known in terms of behaviour in culture, and their taxonomic position is still very confused.

MATERIAL AND METHODS

Origin, growth and culture conditions of the strains

Field sampling was carried out in June 1991 on the Island of Mellum; and from 1991 to 1995, each year in October, on the Norderney Island. Both sites are located in the German Bight of the North Sea.

For isolation, a small sample of a microbial mat material was used as an inoculum on a liquid medium. Enrichment was done in 100 ml Erlenmeyer flasks containing 50 ml of medium MN (Waterbury & Stanier 1978) of North Sea salinity, and 50 µg ml^{-1} cycloheximide (Serva Heidelberg). The flasks were placed in an illuminated incubator at very low shaking speed (Gallenkamp, UK), at 800 lux and 20°C. Coccoid cyanobacteria from the genera *Synechocystis*, *Synechococcus*, *Merismopedia* and *Eucapsis* appeared homogeneously suspended in the medium, while the filamentous cyanobacteria showed, in general, heavy wall growth.

A second isolation method was the direct single cell or cell aggregate transfer into the liquid medium with the help of inverted microscope (Zeiss Axiovert 10, Germany) equipped with a micromanipulator (Zeiss, Germany). The Cooper Dish technique (Waterbury & Stanier 1978) gave very good results as well. In this case, small aggregates were placed on agar plates and observed microscopically until the next transfer was advisable.

The isolates were grown in MN, half-concentrated MN (0.5 × MN), double-concentrated MN (2 × MN) liquid media prepared according to Waterbury and Stanier (modified) (1978), and BG 11 according to Rippka *et al*. (1979). Cultures were kept at room temperature in Erlenmeyer flasks without further aeration and were illuminated with natural daylight or with Osram tungsten light tubes of 200 lux. All isolates showed very good growth on a solid substrate such as ∅0.5 mm glass beads (B. Braun Melsungen AG, Germany), sea sand (Merck, Germany), and glass fibber filters (Whatman 934-AH). Sterile glass beads or sea sand were used as an adhesion substrate for the isolated strains and were simply spread on agar plates. Glass fibber filters were prepared by packing five ∅90 mm filters into the bottom of a glass Petri dish (100 mm × 20 mm), covering the plate, and then sterilizing the plate by heating at 260°C for 30 min. After cooling, the filters were saturated with 20 ml of sterile liquid medium.

Microscopical investigations

Colony, aggregate, and cell morphology were examined using a Zeiss photomicroscope III. Pictures were taken on Kodak EPY64 tungsten films. Cell and aggregate dimensions were measured with an ocular and an objective micrometer.

For scanning electron microscopy, the cell suspensions or sediment grains with the attached cyanobacteria were spread onto poly-l-lysine coated glass slides (Tsutsui *et al*. 1976) while small mat pieces were wrapped in thin blotting-paper (lens paper, neoLab Heidelberg), fixed in 4% glutaraldehyde (Fluka Chemie, Switzerland) in 0.1M sodium phosphate buffer (pH 7.2), and washed two times in this buffer. They were then dehydrated in a series of ethanol-water solutions starting with 10% of ethanol, then proceeding through seven steps to pure ethanol, with each step lasting 30 minutes. The dehydration series ended with two washes in pure ethanol. Afterwards, the samples were critical-point dried in an Balzers Union (CPD 010) apparatus, before gold sputtering (Balzers Union, SCD 030). The objects were examined with a Zeiss DSM 940 or a Hitachi S-450 scanning electron microscope operated at 10 or 20 kV and with working distances of 7 to 9 mm. Pictures were taken on Agfapan APX25 or Ilford FP4 films.

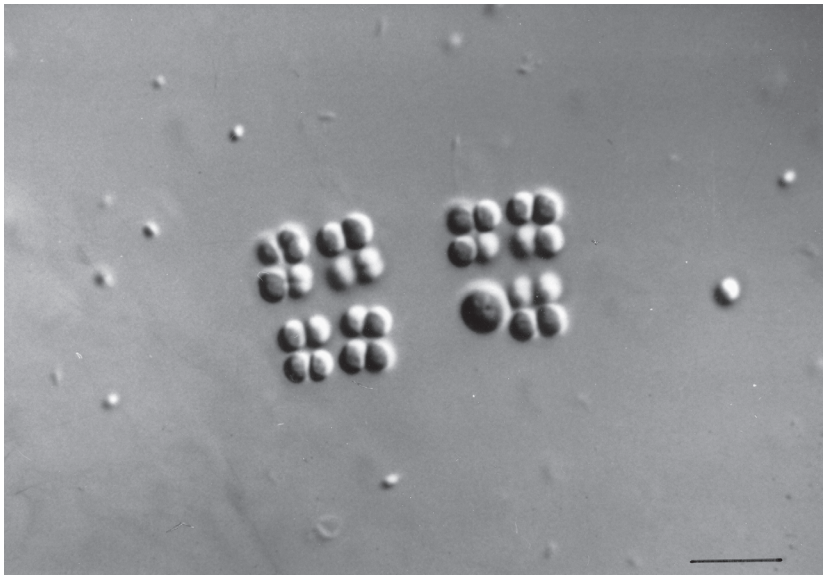

Fig. 1. Irregular division pattern with formation of megacyte, frequently observed in culture. [Bar = 25 µm].

RESULTS

Several *Merismopedia*-like forms were observed in the field in the Mellum microbial mats. Several of these forms have been isolated in uni-cyanobacterial culture (Stal & Krumbein 1985, Krumbein 1987). One of the original isolates of 1984 is still maintained in Oldenburg under the strain assignment Ol 86. On the basis of morphological features, it was attributed to the species *Synechocystis* sp. (Geitler name: *Merismopedia punctata*). To study the phenomenon of the considerable differences between the phenotype in the laboratory culture, and the phenotype (or ecophene) in its proper ecological niche, more intense, new observations and isolations were carried out in 1991.

Two types of *Merismopedia*-like forms were isolated in these isolation attempts (Mellum 1991). One larger type (OL 202) with cell sizes between 4.0–6.7 µm, and a smaller one (OL 201) with cell sizes varying between 1.9 and 3.8 µm. After several transfers in medium BG 11, strain Ol 201 lost completely the potential for aggregate formation, while the original cell cluster transferred continuously in medium MN maintained this capacity. The strain assignment Ol 201a was given to the isolate that now had to be regarded as *Synechocystis* sp. (no aggregate formation).

Strain OL 201, according to the classical assignments of Geitler (1932), could be attributed to the species *Merismopedia punctata*. Strain OL 202 exhibits features known for *M. elegans* (*sensu* Geitler), and was grown on the BG 11 medium with sediment extract from the Wadden Sea (30 ml in 1 litre). The ability to grow in aggregates did not change much under these laboratory conditions.

Seven *Merismopedia*-like cyanobacteria were collected and isolated from the microbial mats at Norderney Island. After several subculturing steps, five strains that are still kept as uni-cyanobacterial cultures in our laboratory have remained. These strains could represented *M. glauca, M. punctata* and *M. elegans sensu* Geitler (1932).

The formation of giant cells (Figs. 1 and 2), and giant cell aggregates (Figs. 3 and 4) was frequently observed in all cultures isolated from Mellum (1984 and 1991), and from Norderney (1991–1995), especially in the first 8 transfers upon isolation. These abnormal cell sizes (2–3 times bigger than common cells) are most probably results of irregular (possibly delayed) division in one or two planes. The occurrence of giant cells and cell aggregates in otherwise smaller *Merismopedia* cultures further hint to the possibility that new, different conditions in the culture influence the morphology of the isolates. This, together with field observations and molecular analyses ultimately points to the necessity of combining field observation, culture observation, and molecular techniques for identification of growth characteristics of individual cyanobacteria.

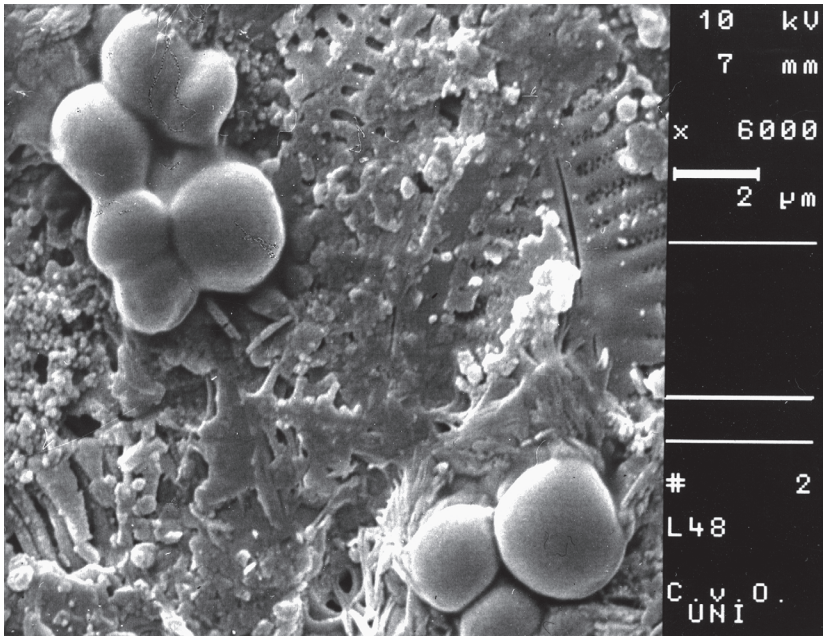

Fig. 2. SEM-photomicrograph of a subculture of *Merismopedia*-like isolate, exhibiting delayed division with megacyte production.

DISCUSSION

The composition and development of microbial mats in the intertidal sediments of the Wadden Sea in the southern North Sea have already been studied for several years by members of the Geomicrobiology Division of ICBM at the University of Oldenburg (Stal 1985, Stal & Krumbein 1985, Gerdes *et al*. 1987, Krumbein *et al*. 1994, Palińska *et al.* 1996).

In the early stages of the microbial mat development, coccoid cyanobacteria such as *Gloeocapsa* sp., *Synechocystis* sp., *Synechococcus* sp. and *Merismopedia* sp. were frequently observed. Their ecological position and importance in the mat as well as their generic assignment is not yet understood. In order to get new isolates, we decided to use the classical sites, namely the microbial mats described by Oerstedt (1841) for the Baltic, and by Schulz (1936) for the North Sea.

The genera *Eucapsis*, *Merismopedia*, *Synechocystis* and *Synechococcus* are usually expected to comprise coccoid cyanobacteria reproducing by binary fission (or regular division in contrast to e.g., multiple fission in the Pleurocapsaleans). They have been subdivided on the basis of the cell size, the division pattern and aggregate formation. In the classical nomenclature all the four genera belong to the order Chroococcales. Geitler (1932) classified them into four different genera within one family and order, while Komarek and Anagnostidis (1986) put them together in one family Microcystaceae instead of Chroococcaceae maintaining, however, the Geitler's order name. They included into this order all coccoid cyanobacteria and introduced new families or family divisions by separating those coccoid cyanobacteria that have division planes perpendicular to each other from those with irregular division planes, a separation not applied so strictly by the Geitlerian system.

The approach of Komarek and Anagnostidis (1986) to the taxonomy of this complex group is a significant departure from the Geitlerian system. The most important difference between the two approaches is the departure from classifying organisms into different systematic groups only on the basis of their ability of forming or maintaining cell aggregates. The presence of cell aggregates seems to be an unreliable taxonomic characteristic. They, however, stressed the importance of other features, namely a type of reproduction (binary or multiple fission, budding) or the orientation of the division planes (perpendicular or irregular). All these features, however, seem to be variable when cultures of the group are studied. Holtkamp (1985) for example showed that the division planes of *Aphanothece halophytica* are dis-

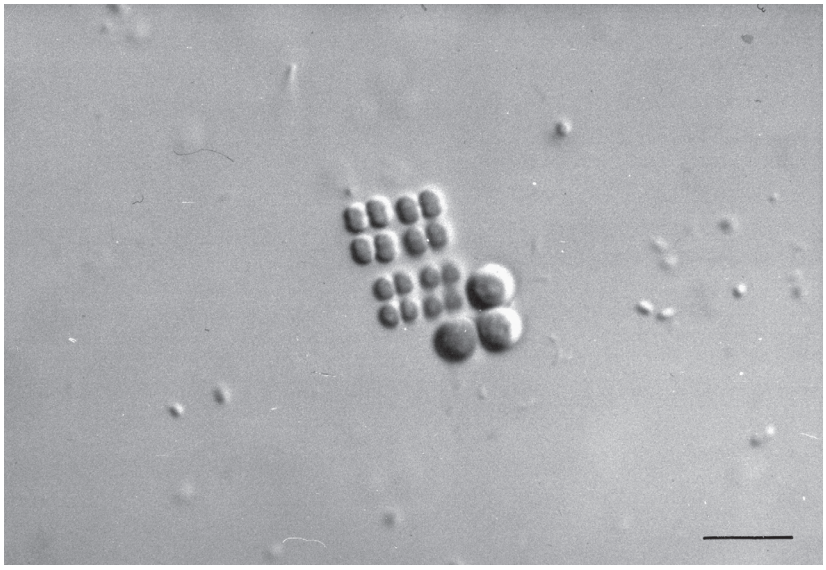

Fig. 3. An aggregate of originally 4 cells (right corner). One of the cells divided properly into 4 new cells giving rise to an aggregate of 16 cells; the upper right package of 8 showing the fourth division while three cells of the original package of four remained undivided but formed giant cells. [Bar = 25 μm]

torted considerably, and even to rich filamentous morphologies under positive or negative osmotic stress. Golubic *et al*. (1996) showed highly variable cell forms and sizes in field material and cultures of *Solentia sanguinea*. Komarek and Lund (1990) and Palińska *et al*. (1996) found that the *Spirulina*/*Arthrospira* complex may also show considerable morphological variations through environmental stress and other cell wall and division related factors (e.g. pore size and thickness of cell walls in the punctured portions of the cell wall). The slime production in a culture is also affected by the growth phase of the cyanobacteria, by the conditions under which they were grown, and by the degree of purity of the culture (Becker 1992).

In addition, to our knowledge, practically no extended physiological work has been done and reported on isolates going back to *Merismopedia* or *Eucapsis* field "types" although in several cyanobacteria collections benthic and planktonic forms are maintained.

The few data collected so far on the division pattern, slime formation, cell sizes, and aggregate build-up of this complex group of coccoid cyanobacteria dividing in one, two or three regular or irregular planes do not allow far-ranging conclusions. Physiological and cultural experiments on isolates of the group seem to lack completely (Geitler 1932, Niell & Anadon 1978, Rippka *et al*. 1979, Stal & Krumbein 1985).

It has been shown in the cultural experiments with three different isolates that irregular colony patterns may emerge from regular ones within one clone. Further, it has been shown that an originally aggregate forming clone can transform irreversibly into a culture that forms irregular aggregates or single cell homogeneously growing forms which according to classical taxonomy should be named *Synechocystis* sp. or *Synechococcus* sp.

Cell division in coccoid cyanobacteria exhibits (generally speaking) "normal" binary cell division or production of so called "spores" or "cytes". "Endospores" or "nanocytes" ("nannocystes" in Bourrelly 1970) arise by rapid successive division of a single cell into several small daughter cells. Waterbury and Stanier (1978) and others use the term "baeocytes" instead of "endospores", because the latter term already has a different sense in bacteriology. Chadefaud (*see* Bourelly 1970) proposed the term "coccospore" instead of "endospore". Fritsch (1945) called daughter cells which grow to the original cell size and are liberated singly "planococci". Bourelly (1970) defines the "planococci" ("planocoques") as motile "coccospores". Kaas (1983) proposed the term "monospore" for similar reproductive cells, when non-motile, analogous to such in the Rhodophyceae.

The differences in cell diameters which have already been frequently observed before (Palińska & Krumbein 1994) are not necessarily good taxo-

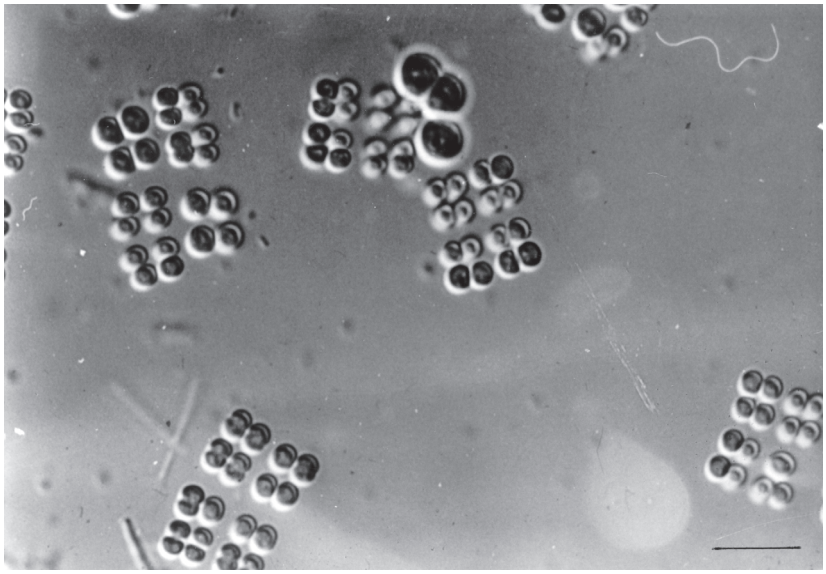

Fig. 4. Pattern of delayed division in the aggregate forming *Merismopedia punctata*. Giant cell aggregates are clearly visible. [Bar = 25 μm].

nomic characters. The formation of giant cells and giant cell aggregates as an irregular division pattern was frequently observed especially in the first eight transfers upon isolation. The formation of giant cells or abnormal, small cells or cell aggregates is certainly affected by an irregular division pattern caused by a delayed division or faster, accelerated division.

In the course of these studies, it was possible to show that accelerated and delayed divisions occur in this Chroococcales cyanobacteria group as opposed to the multiple fission pattern in Pleurocapsalean cyanobacteria. Because of the great differences and two different terms used by Waterbury and Stanier (1978, baeocyte), and Komarek and Anagnostidis (1986, nanocyte), a new classification of cells reflecting not only their sizes, but also their division pattern is proposed. It is suggested to use nanocyte for cases of accelerated division and a new term *megacyte* for cases of delayed division in cell size differentiation of *Merismopedia punctata*, whereas baeocyte should be restricted to more or less rapid, multiple fission followed by a release of motile or non-motile "propagules" from the glycocalyx of the precursor cell, in the group of Pleurocapsales. This in turn, further differentiates the principles and development lines of the coccoid group of *Merismopedia*-like strains from the Pleurocapsalean group, in which usually only the single cell unit is motile.

The giant cells — in contrast to dwarf cells called nanocytes by Komarek and Anagnostidis (1986) — are, thus, called megacytes while the term baeocyte introduced by Waterbury and Stanier (1978) points rather to developmental cycles in the reproduction. The occurrence of nanocytes and megacytes may be a consequence of adaptation to different field conditions and seasonal or annual limitations. Microbial mats of the "Farbstreifen-Sandwatt" type, which the Mellum and Norderney mats are, are a complex, dynamic system, and that is, why one will always witness the establishment of several ecotypes or ecophenes of the same species type in terms of molecular and physiological characteristics.

The division between the two major groups of coccoid cyanobacteria, however, can still be maintained. Further, since the time of Cohn (1853) and Hansgirg from Prague (1892) it has still been discussed if "swarmed" ("Schwärmerzellen") cells (e.g., baeocytes *sensu* Waterbury) are to be found in Section I (Rippka *et al*. 1979), especially in the "*Synechocystis-Merismopedia"* complex. Finally, it depends on the mode and time of separation of a single nanocyte or megacyte whether the process can be called budding as well. Thereby, a junction with *Chamaesiphon*, where usually only the terminal cells divide could be made.

In short, there is a remarkable difference between megacyte and nanocyte as compared to an endospore or baeocyte formation and a normal binary fission. We agree with Komarek and Anag-

nostidis (1986) who classified coccoid cyanobacteria in a single, big order Chroococcales. We propose, however, to consider ephemeral motility of daughter cells derived from a rapid multiple fission as a fundamental taxonomic character, whereas motility of grown-up mature vegetative cells, cell aggregates and trichomes seems to be widespread and complex in character.

Finally the data presented point to the possibility that, in terms of the embracing taxonomy, cyanobacterial genera and species could be kept much more flexible in the strain histories. The molecular taxonomy, thus, does not not make the identification easier or replacie the older systems. It is adding a new tool and dimension to the questions of taxonomy and taxonomy related physiological and ecological characteristics of the living world.

All the three genera *Synechocystis*, *Merismopedia* and *Eucapsis* are well distinguishable in nature: *Synechocystis* lives as solitary cells, *Merismopedia* forms rectangular flat, and *Eucapsis* cubic colonies. All the three genera are clearly different in the reproduction type as well. Transitions were not found in natural populations, and this is the reason why all these three genera are still classified separately by classical taxonomists, although the situation in culture is entirely different. The slime layers very often disappear and the resulting strains grow as solitary cells. It happens often in *Merismopedia* strains but also in *Eucapsis*, *Microcystis* and various *Aphanocapsa* isolates. Thus, if one obtains a strain of the "*Synechocystis*"-type where the cells grow solitary, it is very difficult, if not impossible, to identify the generic classification without the knowledge of the original material from the field i.e. the "strain history". The *Synechocystis* and *Eucapsis* strains (populations) unlike *Synechocystis/Aphanocapsa/Merismopedia* -strains, can be easily distinguished by the study of division types in special chambers or on agar plates (Kovacik 1983). Maybe, the close genotypic relatedness (or identity?) of these three genera will be proved with the help of 16S rDNA analysis.

The intrageneric taxonomy of *Merismopedia* is still very foggy. According to traditional approaches (e.g., Geitler 1932), the various species differ practically only in cell dimensions. Modern studies on *Merismopedia* sp. are lacking completely. As it was shown in Results, the cell size variability is evidently wider than it is known in the literature. Populations with more or less stable cell sizes, but also other ones that by changes in cell diameter cover the range of several species, exist. From all these results another question arises: how to classify types, constantly different in their various environments, but being identical according to 16S rDNA sequences (Palińska *et al*. 1996)? They evidently exist in nature, in different biotopes. In their environment they are morphologically well distinguishable, and play a different role in the biotope.

The stability and existence of various different phenotypes in one uniform biotope (which really exists) must be explained at first. The molecular methods are certainly to be used parallel or as supplement to the phenotypic characterisation but never to replace it. Otherwise, the more precise and perhaps more reliable molecular data would produce confusing results concerning the occurrence of cyanobacterial genera and species in nature. This holds true not only for the phenomenological aspects (relatively stable and partially largely different morphotypes in the field) but also for the processes these taxa regulate in a natural environment (e.g., capability of different types of physiology, motility, fertility and genetic exchange).

The molecular taxonomy thus, in the sense and stage we have it now, is unfortunately not making the identification easier or replacing the older systems. It is adding a new tool and dimension to the questions of taxonomy, and taxonomy related physiological and ecological characteristics of the living world.

The concept of species (and/or genera, strains, phenotypes etc.) in cyanobacteria, especially in coccoid ones, must be changed in the future, but there are yet not enough data for this evaluation, and the modern molecular tools are not yet sufficiently developed. In some cases, we simply need to acknowledge the limits to our actual taxonomic power, as methods and approaches in any scientific inquiry are not perfect.

It is very artificial to divide the cyanobacterial realm into morphological, ecological, bacteriological, molecular or other genera or even species. Exactly this, however, is presently and unfortunately, a fact. The current cyanobacterial tax-

onomy must be changed, and it is obvious that only the combined, polyphasic molecular — cytomorphological — ecological approach can be applied.

CONCLUSION

Several *Merismopedia*-like forms were observed in the field in the microbial mats of the East Fresian Islands. The formation of giant cells and giant cell aggregates was frequently observed in all isolated cultures. The formation of giant cells or abnormal, small cells or cell aggregates is affected by an irregular division pattern, as a result of a delayed or accelerated division. One should consider as well the effectiveness of cyanophages as a possible factor causing changes in the division pattern. Unfortunately, there are still few studies on viral infections done in natural populations of cyanobacteria.

In the course of these studies, it was possible to show that accelerated and delayed divisions occur in this cyanobacteria group as opposed to the multiple fission pattern in Pleurocapsalean cyanobacteria. Because of the great differences, and two different terms used by Waterbury and Stanier (1978; baeocyte) and Komarek and Anagnostidis (1986; nanocyte), a new classification of reflecting not only cells sizes, but also their division patterns is proposed. It is suggested to use nanocyte for cases of accelerated division, and a new term megacyte for cases of delayed division in cell size differentiation of *Merismopedia punctata*, whereas baeocyte should be restricted to more or less rapid, multiple fission followed by release of motile or non-motile "propagules" from the glycocalyx of the precursor cell, in the group of Pleurocapsales.

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