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1 Gregarious Cyanobacteria

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8
9 Abstract

10 Huber and collaborators reported in this issue of *Environmental Microbiology* about
11 freshwater picocyanobacteria that showed phenotypic plasticity in the sense that they
12 appeared as single cells as well as in aggregates. The authors suggested that aggregation
13 might be an inducible defense as a response to the presence of grazers. This has been
14 described for eukaryotic phytoplankton and for the cyanobacterium *Microcystis* but thus far
15 not for picocyanobacteria. Although inducible defense as an explanation is an attractive
16 possibility, it is also problematic. Aggregation is common among cyanobacteria and it offers
17 many advantages as compared to a free-living lifestyle. Here these advantages are
18 highlighted and the possibility of inducible defense is critically assessed.

19
20
21 Huber *et al.* (2017) investigated the phenotypic plasticity of freshwater picocyanobacteria,
22 *i.e.* on the one hand their appearance as free-living single cells and on the other hand as
23 more or less structured colonies and aggregates. Picocyanobacteria (by definition unicellular
24 cyanobacteria smaller than 2 μm) have often been considered to occur and grow as free-
25 living single cells (Pcy). However, it has become clear that these organisms have also been
26 recorded as 'microcolonies' (<50 cells), 'short (pseudo)trichomes' (<10 cells), and 'large
27 colonies' (up to several hundreds of cells) (CPcy) (Fig. 1a). The latter have frequently been
28 observed in warm, shallow and nutrient-rich lakes, while Pcy are more abundant in deep
29 oligo- to mesotrophic lakes (Callieri *et al.*, 2012).

30
31 Single cells may produce sticky extracellular polymeric substances (EPS) causing the cells to

32 stay together after division. This EPS formation is usually the result of unbalanced growth
33 caused by nutrient limitation (Crosbie *et al.*, 2003). While the small size of picoplankton and
34 the consequent large surface-to-volume ratio render these organisms a high affinity for
35 nutrients and is considered to be an adaptation for growth under oligotrophic conditions,
36 aggregation seems to undo this advantage. In shallow nutrient-rich waters with a high
37 standing stock of Pcy, the chance that cells encounter each other and stick together must be
38 greater than in deep lakes with more mixing. In shallow waters, the formation of aggregates
39 may also increase the transparency of the water column, while the colony protects the cells
40 inside from excess irradiation. In deep water, aggregates would sink leaving relatively more
41 Pcy in the euphotic zone. Aggregates may also have an important advantage as they
42 establish a self-sustaining microcosm in which nutrients are effectively recycled and provide
43 for instance a habitat for O₂-sensitive diazotrophic (N₂-fixing) microorganisms (Klut and
44 Stockner, 1991). The EPS matrix may absorb and retain metabolites as well as immobilize
45 nutrients and toxins. The mucilage in which the cells in the aggregate are embedded may
46 also be difficult to digest and certainly represents an inferior food source because it is
47 composed mostly of carbohydrates and is low in other nutrients. Hence, there is a plethora
48 of reasons why living in aggregates is preferred above a life as free-living single cells.

49

50 Colony morphology has been thought to be species-specific. Although there is no doubt that
51 this is to some extent the case, Huber *et al.* detects >30 phylotypes but recognize only 6
52 major colony morphologies. These CPcy may have characteristic morphologies that have
53 been given taxonomic value and depending on the arrangement of the cells in the colony
54 they are assigned as 'Cyanodictyon' (irregularly shaped spherical colonies with cells
55 irregularly arranged), 'Aphanocapsa' (regular spherical colonies with cells regularly
56 arranged), and 'Eucapsis' (colonies cubic with cells arranged in groups of 4). Many taxa of
57 unicellular cyanobacteria grow as multicellular organisms in well-organized and structured
58 colonies that have been given taxonomic value. This may be problematic because the
59 colonial growth form is sometimes lost in culture (Komarek *et al.*, 2011). The question
60 addressed by Huber *et al.* was whether Pcy and CPcy may in fact belong to the same
61 genotype that show a phenotypic plasticity. And if this would be the case, which factors
62 would trigger these organisms to change from one morphotype into another.

63

64 Huber *et al.* investigated the temperate lake Chascomús in the Pampa region of Argentina to
65 answer these questions. Lake Chascomús is a shallow (1.9 m) and hypertrophic, turbid and
66 fully mixed lake. The most common CPcy morphotype that was present in the plankton was
67 'Cyanodictyon' and the occurrence of this morphotype correlated with Pcy, including the
68 microcolonies and short trichomes. 16S rRNA and ITS sequence analysis of flow-cytometric
69 fluorescent-activated cell sorting (FACS) of Pcy and CPcy (which were only of the
70 'Cyanodictyon' type and also included the microcolonies and short trichomes) revealed no
71 indication of genotypic difference between the two sorted groups. They were all related to
72 *Cyanobium* and *Synechococcus* and belong to the '*Cyanobium + Anathece*' cluster and
73 matched the sequences that were obtained from clone libraries and DGGE from samples of
74 the lake. Hence, when Pcy and CPcy are belonging to the same organism, what causes them
75 to take either of the two morphologies? Huber *et al.* investigated the possibility that the
76 CPcy morphology protects from grazing. Although they observed only weak correlations of
77 the distribution of Pcy and CPcy with grazers, there was one exception: cladocerans. Pcy
78 were negatively correlated with small cladocerans while the percentage of cells in colonies
79 was positively correlated. This observation was experimentally tested by adding the
80 cladoceran *Bosmina* sp. to lake water from which grazers were removed. While no changes
81 were observed in the control, Pcy were less abundant and the number of cells in CPcy
82 increased when *Bosmina* sp. was present. The CPcy, which was mostly of the '*Cyanodictyon*'
83 morphotype, doubled in size during the 6 days that the experiment lasted. While this is all
84 pretty convincing and exciting, it leaves us with many unanswered questions about the
85 mechanisms behind this behavior. What causes (pico)cyanobacteria to form aggregates?
86 When indeed the formation of the colony is a response on the presence of a grazer, how is
87 this grazer sensed? Are there infochemicals involved and if so, what is their nature, who
88 produces them, and how are they released and sensed? The authors suggest that the colony
89 grows at the expense of Pcy (single cells). Hence, the single cell joins the CPcy, rather that
90 selective grazing diminishes Pcy. Do CPcy fall apart and take back the Pcy morphology when
91 the danger has passed? These questions remain unanswered in this paper and would require
92 further research. In the grazing experiment, there seems to be no statistical significant
93 difference between the number of cells per milliliter between day 0 and at day 6 with or
94 without *Bosmina* sp. Does this mean that there is no growth and grazing or are growth and
95 grazing in equilibrium and in the control without the grazer would growth and lysis cancel

96 out? Or is the presence of *Bosmina* sp. alone enough to cause the shift from Pcy to CPcy and
97 hence lowers the chance of grazing the lower abundance of Pcy? Why would not all Pcy
98 aggregate in the CPcy? Or is just getting the abundance of Pcy below a certain threshold
99 enough to prevent effective grazing? There seems indeed to be an 'ideal free distribution'
100 for the ratio of single cells to colonies that represents the optimum for growth under
101 herbivory (DeAngelis *et al.*, 2007). Moreover, although the increase in size by aggregation of
102 cells could help to avoid being grazed by a certain organism, it could become the preferred
103 food for other, bigger predators (Van Donk *et al.*, 2011). Among zooplankters, the rotifers in
104 particular seemed to feed preferentially on the CPcy (Callieri *et al.*, 2012). And if aggregation
105 would be beneficial with regard to grazing, it has to outweigh the negative effect of sinking
106 (Van Donk *et al.*, 2011). Hence, there are many questions that demand an answer in order to
107 understand this proposed strategy to avoid grazing pressure.

108

109 The response of eukaryotic phytoplankton towards herbivores is well-known and described.
110 The green alga *Scenedesmus* induces aggregate formation when exposed to grazer-released
111 infochemicals in a dose-response manner (Verschoor *et al.*, 2004). However, much is still
112 unclear about the nature of these infochemicals, how they are released and sensed. About
113 the role that infochemicals may have for inducing aggregation in cyanobacteria even less is
114 known.

115

116 For instance, the unicellular cyanobacterium *Microcystis aeruginosa* produces aggregates
117 when treated with spent medium of *Daphnia* or by disrupted *Microcystis* cells (Becker *et al.*,
118 2010). The toxin microcystin produced by *M. aeruginosa* has been shown to induce
119 aggregate formation (Sedmak and Elerseck, 2006) although also other infochemicals may
120 have been released from the disrupted cells. *M. aeruginosa* induced colony formation when
121 grazed by the flagellate *Ochromonas* (Yang *et al.*, 2006). However, these authors also
122 indicated that the colony-inducing effect was weak, which they attributed to the fact that
123 they used an axenic culture in their experiment, suggesting that associated (heterotrophic)
124 bacteria also play a role. Yang *et al.* (2006) also admitted that other, abiotic, factors
125 contribute to the formation of larger colonies found under natural conditions. Moreover, the
126 cost of the increased sinking rates and the decreased effective quantum yield of

127 photosystem II of herbivory-induced colonial *M. aeruginosa* should be taken into account to
128 judge the effectiveness of the strategy (Yang *et al.*, 2009).

129

130 The formation of aggregates and, hence, the increase in size has advantages for
131 phytoplankton. Many planktonic cyanobacteria (filamentous and unicellular) produce gas
132 vesicles that provide these organisms with buoyancy. When these organisms are mixed deep
133 in the water column, the speed with which they float to the surface is proportional with the
134 square of the radius (Stokes' Equation). Hence, the bigger size of a cell aggregate helps to
135 get back quickly to the illuminated water surface after a deep mixing event, where a single
136 cell would need many days and would not survive. This is the well-documented strategy of
137 the unicellular *Microcystis* (Fig. 1b) as well as of the heterocystous cyanobacterium
138 *Aphanizomenon* (Fig. 1c), and the non-heterocystous *Trichodesmium* (and many others),
139 which all form aggregates.

140

141 Aggregates of cyanobacteria that do not contain gas vesicles such as the picocyanobacteria
142 discussed in Huber *et al.* also obey Stokes' equation by sinking out more rapidly than single
143 cells. The sinking rate of single cells of picocyanobacteria is negligible. While staying in the
144 illuminated surface water they deplete the nutrients (nitrogen and phosphorus) locally. This
145 leads to the production of transparent extracellular polymers (TEP) (by overflow
146 metabolism), that cause aggregation of the Pcy. These aggregates of picocyanobacteria
147 (CPcy) may be >1 mm and sink 400 m d^{-1} (Deng *et al.*, 2016). This represents an important
148 carbon export, but would it also be a selective advantage for these picocyanobacteria? I
149 think it probably would. Removing a large part of the picocyanobacterial biomass from the
150 euphotic zone would increase the transparency of the water column and increase the
151 relative availability of nutrients, which would increase the survival of the Pcy. Also,
152 regeneration of cyanobacterial biomass in the euphotic zone would allow heterotrophic
153 bacteria to scavenge the nutrients. Finally, on a larger scale, carbon export (the carbon
154 pump) is essential for balancing the global carbon budget.

155

156 There are other, bigger, unicellular cyanobacteria that form more or less structured
157 aggregates. *Gloeotheca* forms irregular groups of sheathed cells. This genus is known to fix
158 N_2 aerobically in the light. The aggregates with their multilayered gelatinous envelopes may

159 be an adaptation to allow aerobic N₂ fixation in the light (Ortega-Calvo and Stal, 1991). Only
160 when a certain size is achieved, it would be possible to establish O₂ gradients such that the
161 oxygen-sensitive nitrogenase can be active. Moreover, such aggregates might also allow
162 some differentiation between cells. Some cells in the aggregate may cease oxygenic
163 photosynthesis and fix N₂, while exchanging fixed nitrogen and electrons with the oxygenic
164 cells. This still needs to be demonstrated but it would explain the differences of *Gloeotheca*
165 with other unicellular N₂-fixing cyanobacteria such as *Cyanotheca* and *Crocospaera*, which
166 do not form such aggregates and fix N₂ during the dark (Compaoré and Stal, 2009).

167

168 *Merismopedia* is a genus of unicellular cyanobacteria that is characterized by rectangular
169 colonies embedded in a mucilaginous matrix (Fig. 1d). Their colonies are regular shaped. The
170 cells divide in two dimensions that produce a flat plate with a grid-like pattern, which is
171 often lost when isolated and cultured in the laboratory. This has made the taxonomy of this
172 organism difficult because when grown as single cells they would be assigned to
173 *Synechocystis* (Palinska and Krumbein, 1998). However, the typical colony shape can be
174 restored when silicate is added to the growth medium (as for diatom media). Hence, silicate
175 seems to be a requirement to form this aggregate (Stal and Wollenzien, unpublished
176 observations). *Merismopedia* is often found in intertidal microbial mats on fine-sandy
177 sediments. The aggregates are motile (Stal, unpublished observations). Individual small cells
178 released from *Merismopedia* aggregates have been reported to be motile (Palinska and
179 Krumbein, 1998), but motility of the whole aggregate in a coordinated manner has hitherto
180 not been reported. It appears that the whole single cell layer plate may be either oriented
181 towards the light, so that all cells receive maximum light, or that the plate tumbles with the
182 single cell side of the plate directed towards the light, resulting in the lowest light harvesting
183 for the plate. Hence, this may be an aggregate behavior to acclimate to light.

184

185 The benthic cyanobacterium *Microcoleus chthonoplastes* (re-named *Coleofasciculus*
186 *chthonoplastes*) is known to form microbial mats and bio-crusts. These cyanobacteria
187 produce bundles of a few to many tens of trichomes enclosed by well-structured
188 polysaccharide sheaths, a property that is usually lost upon isolation and culturing in the
189 laboratory (Stal, 2012). The function of this growth form is not well understood. It has been
190 suggested that it protects against desiccation which would come handy in intertidal

191 microbial mats that are often exposed or in terrestrial bio-crusts. The bundle could also
192 serve to maintain a consortium of microorganisms that provide this microcosm with
193 necessary growth factors. The sheath that surrounds the bundle of trichomes could protect
194 against external factors such as heavy metals and other (toxic) compounds, which may be
195 bound and immobilized by the polysaccharides. The sheath might even prevent grazing.

196

197 Microbial mats that are formed by cyanobacteria are multicellular aggregates that behave as
198 macroscopic living entities (Fig. 1e). The consortia of a plethora of different functional
199 groups of microorganisms perform ecosystem level functions that would be impossible at
200 the level of single organisms (Paerl and Pinckney, 1996). The activities of single organisms
201 are coordinated through biological clock and other rhythmic processes and by signaling
202 through info-chemicals and quorum-sensing systems, most of which are largely unknown.
203 Microbial mats are among the oldest systems known from the fossil record (stromatolites)
204 and therefore this form of aggregation has been proven very successful. Pcy are not
205 exclusively pelagic and are found in microbial mats and periphytic biofilms, but little is
206 known about these organisms. They may differ from their pelagic counterparts and be
207 typical benthic organisms, but it is also possible that they originate from the water column
208 and deposited in these benthic ecosystems.

209

210 It is likely that cyanobacteria evolved from a unicellular type that gave rise to filamentous
211 forms (Schirrmeister *et al.*, 2011). Filamentous cyanobacteria have the advantage to
212 exchange metabolites and information directly between the cells in a trichome, which led to
213 the development of gliding motility and the orientation of the trichome in physical and
214 chemical gradients. This allowed the cells in a trichome to 'collaborate' and get the best of
215 both, often incompatible, worlds. To add to this, the multicellular filamentous cyanobacteria
216 also developed a distribution of tasks by cell differentiation, which is common among
217 Eukarya, but unique among Bacteria (and Archaea). Some cyanobacteria develop
218 'heterocysts', cells that have lost photosystem-I, do not fix CO₂ and produce a thick glycolipid
219 cell wall, and that serve as the N₂-fixing factories of the organism. Many heterocystous
220 cyanobacteria also differentiate a second cell type, the akinete, which serves the survival of
221 the organism. The formation of aggregates by unicellular cyanobacteria may represent a
222 similar evolutionary development. While the evolution of cyanobacteria from 'simple' single

223 cells to more complex filamentous organisms can be considered as the typical direction
224 evolution takes, it has also become clear that the taxa of unicellular cyanobacteria are not
225 monophyletic and that evolution has taken the opposite direction from filamentous forms
226 back to unicellular organisms (Schirrneister *et al.*, 2011). Obviously, the cost of
227 multicellularity does not always pay off.

228

229 What I tried to highlight here is that free-living microorganisms in general, and
230 cyanobacteria in particular, seem to be rather an exception than the rule and that most
231 microorganisms prefer to live as a consortium in benthic environments, attached to a
232 substratum, or as aggregates. There are many advantages to live in such consortia compared
233 to a free-living lifestyle. In the latter, the cell is surrounded by a diffusive boundary layer that
234 is nutrient depleted, although this boundary layer decreases with smaller cell size. The small
235 cell size may make picocyanobacteria more attractive to grazers and filter-feeders, but
236 aggregates offer more benefits than only preventing grazing. Aggregation of
237 picocyanobacteria as a response to the presence of grazers is a possibility but there are
238 certainly many other factors that trigger the formation of colonies as is the case in larger
239 unicellular- and filamentous cyanobacteria. Cyanobacteria are gregarious organisms.

240

241

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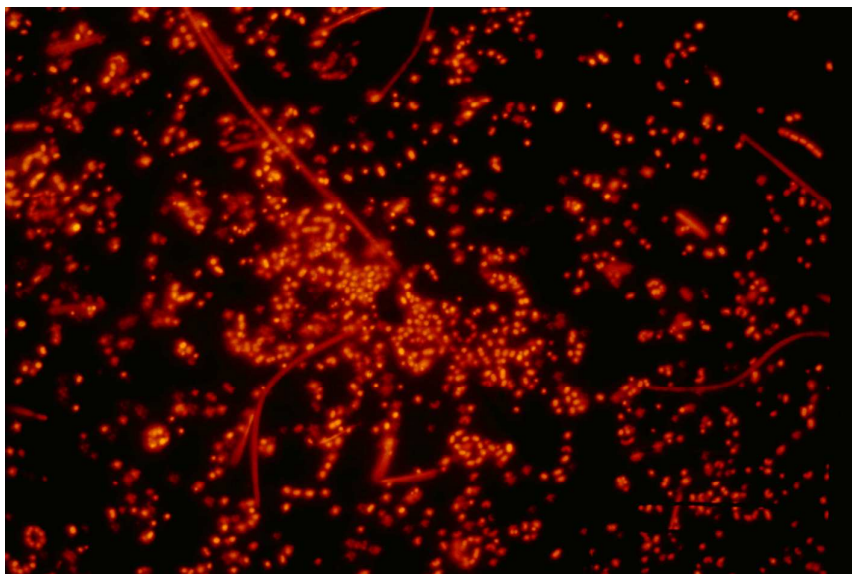
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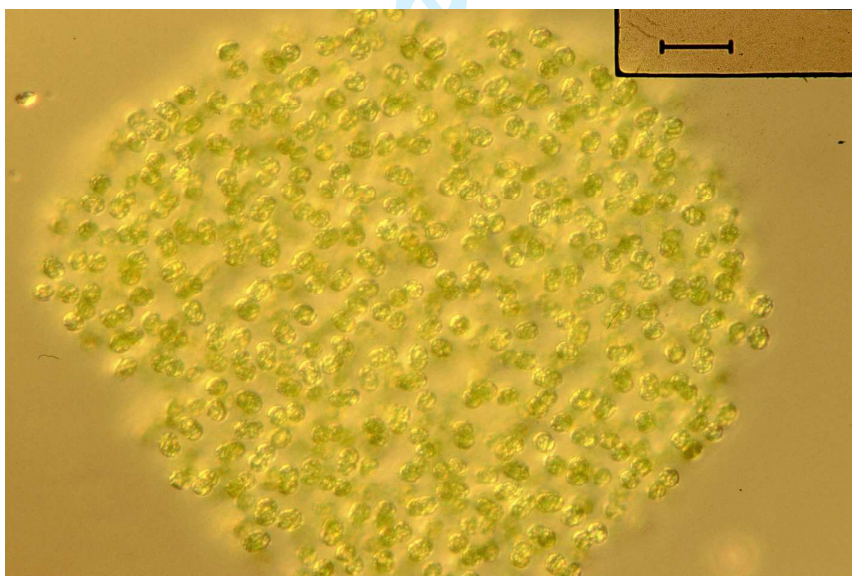
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312

313 Fig. 1a. Aggregates and single cells of picocyanobacteria in the Baltic Sea

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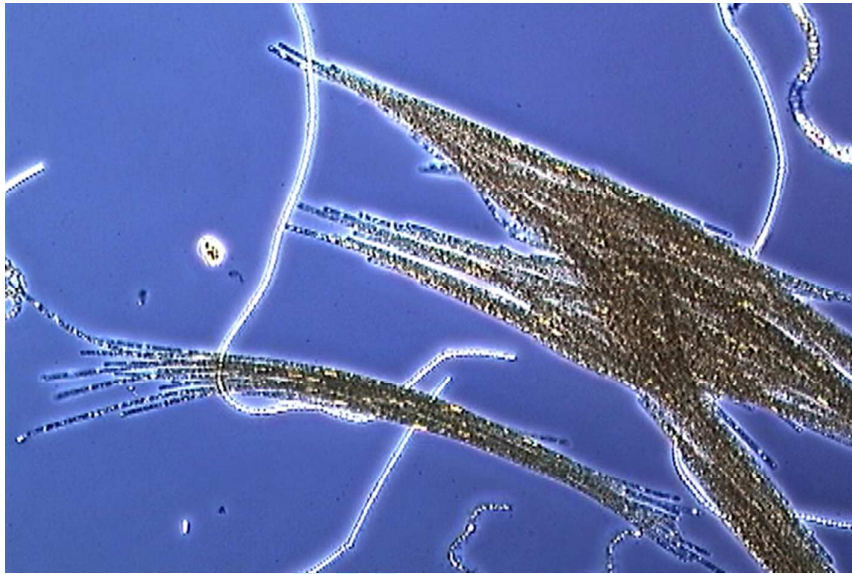


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316

317 Fig. 1b. Aggregate of the gas vacuolated unicellular cyanobacterium *Microcystis aeruginosa*

318 (Culture Collection Yerseke, CCY, ccy.nioz.nl).

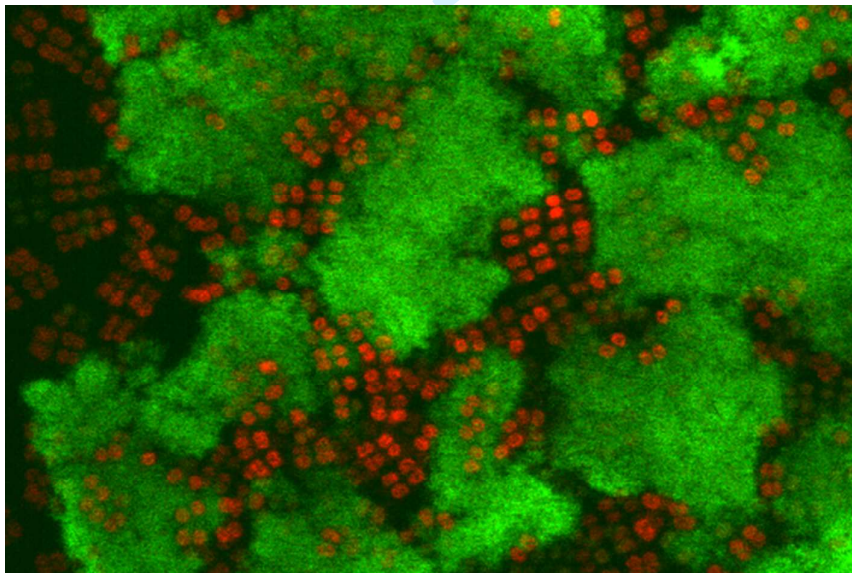


319

320 Fig. 1c. Colonies of the filamentous heterocystous and gas-vacuolated cyanobacterium

321 *Aphanizomenon flos-aquae* in the Baltic Sea.

322



323

324 Fig. 1d. *Merismopedia* sp. (cells fluoresce red) embedded in EPS (fluoresces green) (Culture

325 Collection Yerseke, CCY, ccy.nioz.nl).

326



327

328 Fig. 1e. An intertidal microbial mat; another form of aggregation of cyanobacteria.