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EXPERIMENTAL ARTICLES

Experimental Study of *Microcystis*-Associated and Free-Living Bacteria

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Abstract—In the experiment with water from the hypereutrophic Lake Frederiksborg Slotso (Denmark) sampled during the autumn peak of *Microcystis* growth, the quantity and production of free-living and cyanobacteria-associated heterotrophic bacteria were determined, as well as the extracellular enzymatic (aminopeptidase) activity. The functional diversities of associated and free-living bacterial communities were additionally compared using BIOLOG GN microplates to reveal the possible export of *Microcystis*-attached bacteria into ambient water. It has been shown that the cell size, production values, and growth rates of associated bacteria were less than those of free-living bacteria. At the same time, the potential aminopeptidase activity of associated bacteria was always higher than that of free-living bacteria. The experimental results have shown significant compositional differences in the structure of bacterial communities from different habitats.

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Key words: associated bacteria, free-living bacteria, production, aminopeptidase activity.

In the study of organic carbon transformation, the emphasis is generally placed on free-living bacteria due to their predominant quantities in most water ecosystems. Although microparticles are often intensively colonized by bacteria, associated bacteria usually constitute less than 10% of the total quantity of pelagic bacteria [1]. The flow of organic matter from primary producers to heterotrophic bacteria can be achieved through the attachment of bacteria to algal and cyanobacterial cells [2, 3]. For example, heterotrophic bacteria colonize the cells of *Microcystis* cyanobacteria, which are widespread in eutrophic waters and generally dominate in summer and autumn phytoplankton. Single studies assess the relative contribution of *Microcystis*-associated bacteria in the period of intensive growth of *Microcystis* as 6–40% of the total quantity and 7–55% of the production of free-living bacteria [4, 5]. Thus, the dynamics of organic carbon can be considerably affected by intensive growth of these cyanobacteria. The results of previous studies on Frederiksborg Slotso Lake (Denmark) do not exclude significant export of *Microcystis*-associated heterotrophic bacteria into the ambient water, where they make up a significant part of the pelagic bacterial products [5].

The goal of this research was to compare the dynamics of the quantity and activity of *Microcystis*-associated and free-living bacteria and to determine the possibility of significant export of *Microcystis*-associated bacteria into ambient water.

MATERIALS AND METHODS

The water from the hypereutrophic Lake Frederiksborg slotso (Denmark) was taken for the experiment on October 3, 1997, in the end of autumn bulk growth of cyanobacteria ("blooming"). To prepare experimental variants, the *Microcystis* colonies with associated bacteria were introduced into sterile (filtered through a 0.2-μm filter) lake water (2 l).

The *Microcystis* colonies with associated bacteria (MB) were harvested by filtration of 2 l of lake water (after prefiltration through 60-μm mill gauze) through 20-μm mill gauze for maximal removal of free-living bacteria (FB), washed with sterile water, and then washed off the gauze into experimental vials with 2 l of sterile lake water. The whole procedure was repeated four times. Two vials were used as experimental MB variants. The water from two other (control) variants was immediately filtered through mill gauze (20 μm) to remove *Microcystis* and associated bacteria. The washing of the mill gauze with *Microcystis* colonies with sterile water removed most of the free-living bacteria present in the MB fraction. The remaining free-living bacteria were defined as the initial FB concentration in experimental and control variants. The vials of the experimental and control variants were maintained at 15°C and exposed to natural (semi-basement) and artificial (130 μmole quanta m^{-2} s^{-1}) continuous lighting to avoid the possible repression of *Microcystis* growth due to lack of light. The duration of the experiment was three days.

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The initial quantity of bacteria, bacterial production, and extracellular aminopeptidase activity of MB and FB were measured within 3–4 h after water sampling. The total quantity and biomass of bacteria were determined daily. The bacterial production and aminopeptidase activity of *Microcystis*-associated and free-living bacteria were determined on the first and third days of the experiment.

Chlorophyll *a* concentrations were determined in the total phytoplankton and in the *Microcystis* fraction. After phytoplankton filtration through Whatman GF/C filters, chlorophyll was extracted with 96% ethanol. Chlorophyll concentration was determined spectrophotometrically [6].

The MB and FB quantities were determined in water samples fractionated by filtration through 20-μm mill gauze as described above. All samples were fixed by glutaraldehyde (final concentration 1.2%). Associated bacteria were detached from cyanobacteria by the destruction of *Microcystis* colonies with ultrasound (Branson Sonifier 250, 25 Wt, amplitude 306 μm, 2 min) [7]. The quantity of bacteria was determined by the epifluorescence method using DAPI (4,6-diamidine-2-phenylindole) fluorochrome (final concentration 2 μg ml^{-1}) [8]. No *Microcystis* colonies were revealed by the counting of bacteria on the filters, which indicated their effective destruction by sonication. MB and FB cell volumes were calculated on the basis of the length/width or diameter (cocci) of bacterial cells as measured on magnified microphotographs. The calculated bacterial biomass was expressed in carbon units (coefficient 105 fg C μm^{-3} [9]).

The bacterial production in the MB and FB fractions was assessed by the rate of [^{3}H]-thymidine inclusion into bacterial DNA [10]. Production values were calculated from the rate of consumption of labeled thymidine (conversion factor 2×10^{18} cells per mole of thymidine) and then converted into carbon units (coefficient 75 fg C $cells^{-1}$ [11]). Growth rates (day^{-1}) were calculated as a ratio of relevant production and biomass values.

Extracellular aminopeptidase activity was assessed by the intensity of fluorescence induced by enzymatic hydrolysis of the nonfluorescent substrate Leu-MCA (leucine-4-methylcumarinyl-7-amide) with the formation of a strongly fluorescent reaction product: Leu-AMC (leucine-7-amine-4-methylcumarine) [12]. The enzymatic activity was determined in the filtrates from the vials with MB or FB, with the addition of 5, 10, 25, 100, and 200 μM Leu-MCA as a substrate. Measurements were performed separately for MB and FB exposed to natural illumination. Fluorescence was determined with a Shimadzu RF-5002PC spectrofluorimeter at 364 nm (excitation) and 445 nm (emission). The initial fluorescence in all samples was determined immediately after substrate addition; the measurements were repeated after three hours of incubation in the dark at room temperature. The enzymatic activity was expressed in μmole Leu-MCA l^{-1} h^{-1}. The resulting curves were used to calculate the dependence of aminopeptidase activity on substrate concentration, the maximal reaction rate (MRR), and the specific activity (AC = MCP/quantity of bacteria).

The functional diversity (structure) of MB and FB communities was estimated by utilization of individual carbon substrates by bacteria on BIOLOG GN microplates [13]. Each microplate had 96 wells; 95 contained different organic substrates and one well (control) lacked organic compounds. The substrates were of the types widespread in water: carbohydrates, ethers, polymers, carbonic acids, ketones, alcohols, amides, amino acids, and aromatic hydrocarbons. Tetrazolium violet was added to the substrates. Its reduction due to bacterial metabolic activity generated coloration of different intensity in the wells. Each sample was diluted fivefold with sterile water to avoid intensive bacterial growth in the control cell resulting from high concentrations of biogenic elements in the lake water. The aliquots (150 μl) were introduced into 96 wells of two microplates (2 repeats). Then the microplates were incubated for 24, 48, 72, and 96 h at 15–17°C. Color intensity in the wells was determined spectrophotometrically (ASYS HITECH spectrophotometer for microplates, 595 nm). The values of light absorption in the wells, corrected against the level of absorption in the control well, were the measure of utilization of different substrates by bacterial communities. Thus, the comparison of color intensity in the wells with the same substrates on microplates inoculated with different samples is a gauge of similarity or difference in the structure of bacterial communities. Since no changes in color were observed after 72-h of incubation, the results of the 96-h BIOLOG GN exposure were used.

RESULTS AND DISCUSSION

In early October (water temperature 12°C), the chlorophyll *a* concentration in the Frederiksborg Slotso Lake was 36.8 μg l^{-1}, with more than 70% chlorophyll in the 20-μm (*Microcystis*) fraction.

There was a significant increase of the quantity of free-living bacteria from the beginning to the end of experiments (about 13-fold, Table 1), by far greater than the increase of the quantity of associated bacteria, although the initial content of FB was much less as compared with MB. The reasons for such intensive FB reproduction were probably the abundance of available organic matter and the absence of zooplankton in experimental vials, because grazing is one of the main factors that control the quantity of bacteria in water ecosystems [14]. The drastic increase of FB quantity was accompanied by a significant (2- to 3-fold) increase of cell volume. For MB, the increase of cell volume was observed only in the variants with artificial illumination. Thus, the associated bacteria were smaller than the free-living ones. In literature, the opinion that associated bacteria are usually bigger than free-living bacteria predominates; however, there is evidence to

Table 1. The quantity, cell size, biomass, production, and growth rate of MB and FB

Date	Quantity of bacteria, 10^9 cells l^{-1}	Cell volume, μm^{-3}	Bacterial biomass, $\mu g\ C\ l^{-1}$	Production of bacteria, $\mu g\ C\ l^{-1}\ h^{-1}$	Specific production of bacteria, 10^{-12} nM cells^{-1} h^{-1}	Bacterial growth rate, day^{-1}
Microcystis-associated bacteria (MB), natural illumunation						
3.10.97	1.83	0.22 ± 0.04	192.9	0.93	2.4	0.12
4.10.97	1.99	0.27 ± 0.03	229.9			
5.10.97	2.24	0.24 ± 0.03	244.8	26.3	53.8	2.60
Microcystis-associated bacteria (MB), artificial illumunation						
3.10.97	1.82	0.21 ± 0.06	184.6	0.77	2.1	0.10
4.10.97	2.18	0.41 ± 0.10	321.5			
5.10.97	2.50	0.62 ± 0.09	464.8	38.30	41.3	2.00
Free-living bacteria (FB), natural illumunation						
3.10.97	0.26	0.22 ± 0.03	26.7	0.25	4.7	0.22
4.10.97	1.25	0.43 ± 0.08	188.1			
5.10.97	2.88	0.57 ± 0.12	510.8	120.80	118.2	5.70
Free-living bacteria (FB), artificial illumunation						
3.10.97	0.45	0.23 ± 0.06	48.0	0.02	0.3	0.01
4.10.97	1.77	0.47 ± 0.09	281.8			
5.10.97	5.90	0.69 ± 0.11	1164.7	302.20	129.8	6.20
Control (FB)						
3.10.97	0.27	0.23 ± 0.05	28.8	0.32	5.5	0.27
4.10.97	1.28	0.42 ± 0.07	191.4			
5.10.97	3.55	0.67 ± 0.13	686.5	433.00	316.0	6.3

the contrary as well [1, 15, 16]. The obtained difference in the cell size of bacteria from two different communities points to more rapid growth of free-living bacteria [1]. This fact is supported by the vastly more significant increase in the growth rate and production of free-living bacteria by the end of the experiment, as compared with associated ones.

The production and growth rate of both FB and MB considerably increased by the end of the experiment (Table 1), suggesting the inflow of organic substrate available for bacteria at the end of autumn "blooming." The same enhancement of bacterial activity at the end of spring water "blooming" was observed previously in Lake Frederiksborg Slotso[3], as well as in some other lakes (e.g., Lake Constance) [15]. The production and growth rate of MB remained much lower as compared with FB. The works of some authors show that the production, growth rate, and quantity of associated bacteria are generally higher than those of free-living bacteria. This particularly concerns data for natural reservoirs obtained in the summer period [1, 5, 15]. Nevertheless, there is also evidence that the production and growth rate of associated bacteria in hypereutrophic lakes (in particular, in Lake Frederiksborg Slotso) in spring, late summer, and autumn may be less than these values for free-living bacteria [3, 4]. In the case of similar MB and FB cell sizes, the production and the reproduction rate of MB may be less than those of FB, because associated bacteria must spend energy on more intensive production of extracellular polymers [1].

The variants exposed to light around the clock showed no significant enhancement of the quantity and production of MB, although their cell size increased about 2.5-fold. Under these conditions, MB growth rate and specific production were even lower than under natural illumination. Thus, our data demonstrate no repression of *Microcystis* growth and, accordingly, no repression of the activity of associated bacteria due to insufficient illumination. In contrast to associated bacteria, the quantity and production of FB exposed to artificial illumination significantly increased (2- and 2.5-fold, respectively) with the simultaneous increase of cell size (0.57 μm^{-3} in the semi-basement and 0.69 μm^{-3} in the light) as compared with FB growing at daylight. The growth rates of free-living bacteria in these two variants were very high and close to each other (5.7 and 6.2 $days^{-1}$). It is known that the rate of excretion of dissolved organic carbon by phytoplankton is higher in the light than in the dark [17]. It may be suggested that the

additional illumination of *Microcystis* increases the content of extracellular low-molecular organic substances (peptides, carbohydrates), which stimulates the growth of FB to a greater extent than of MB.

The results of measurement of FB and MB extracellular enzymatic (aminopeptidase) activities depending on the time of incubation in the experiments are given in Table 2. The obtained aminopeptidase activity values are comparable with those for other eutrophic shallow-water reservoirs [18]. The aminopeptidase activity of both FB and MB increased by day 3 of the experiment, resulting in a higher maximal reaction rate (MRR); in addition, the MRR was higher for *Microcystis*-associated bacteria than for FB (Table 2). The specific aminopeptidase activity of *Microcystis*-associated bacteria was also much higher as compared with free-living bacteria. Taking into account the higher extracellular enzymatic activity of associated bacteria as compared to free-living bacteria, it may be assumed that MB destroy high-molecular organic compounds of the *Microcystis* mucous membrane, thus increasing the substrate availability for themselves and supplying it to FB. Indeed, many researchers emphasize the important role of associated bacteria in the hydrolysis of polymers to low-molecular compounds: they consume negligible amounts of hydrolysis products, which for the most part diffuse into water and support the growth of free-living bacteria [3, 19].

In this work, BIOLOG GN microplates were used to compare (1) the structures of two different bacterial communities (MB and FB) in experimental vials and (2) the structures of communities of free-living bacteria from experimental (with *Microcystis*) and control vials; they were also used to verify the assumption of the export of *Microcystis*-associated bacteria into the ambient water.

The results showed that the samples from experimental vials with FB and MB were characterized by different values of light absorption in the wells with the same substrate (without any significant correlation between FB and BM communities); this finding indicates the different structure of these communities (Fig. 1). On the contrary, the samples from the control (without *Microcystis*) and experimental (with *Microcystis*) vials containing only free-living bacteria demonstrated close values of light absorption in the same wells of microplates ($r = 0.84$, $P < 0.01$), which is evidence of the similar structure of the communities (Fig. 2). Moreover, the quantity of *Microcystis*-associated bacteria in the <20 μm (FB) fraction cannot be significant. Our data are in agreement with the results of phylogenetic analysis of ribosomal RNA sequences of the bacteria [20], which has shown that the communities of associated and free-living bacteria have different species structures. Associated bacteria belong mainly to *Planctomyces, Cytophaga*, and the group of γ-*Proteobacteria*, whereas free-living bacteria belong to α-*Proteobacteria*.

Table 2. Aminopeptidase activity of associated (MB) and free-living (FB) bacteria

		MRR	AC
First day (reference time)	Control (FB)	0.057	1111
	MB	0.779	1956
	FB	0.087	1500
Third day	Control (FB)	1.030	1789
	MB	1.200	2750
	FB	0.756	1497

Note: MRR, maximal reaction rate, μmole Leu-MCA l^{-1} h^{-1}; AC, specific aminopeptidase activity, amole Leu-MCA $cell^{-1}$ h^{-1}.

Thus, in contrast to free-living bacteria, the quantity of MB varied insignificantly during the experiment, while their cell size, production and growth rate were less than those parameters for FB. However, the aminopeptidase activity of associated bacteria was higher than that of free-living bacteria. Taking into account the slower growth of *Microcystis*-associated bacteria as well as significant compositional differences in the structure of the two bacterial communities, the intensive export of *Microcystis*-associated bacteria into ambient water is improbable. Taking into account the higher extracellular enzymatic activity of MB as compared with FB, one may consider the bacterial associates with *Microcystis* as the zones of more intensive polymer hydrolysis of to low-molecular compounds

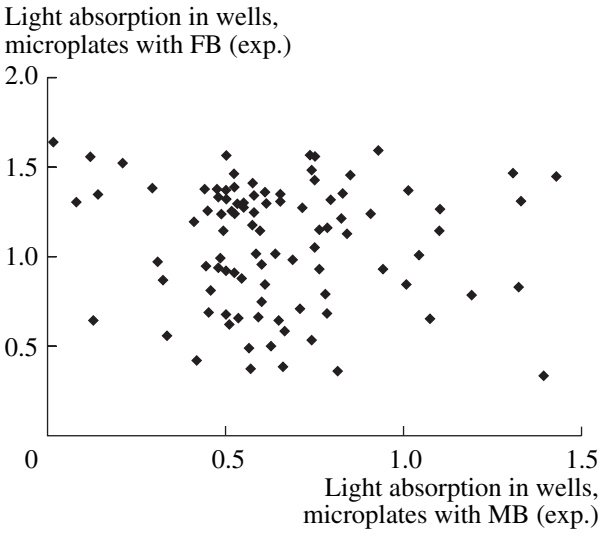

Fig. 1. Comparison of the functional diversity of MB and FB. Different color intensities in the wells with the same substrate and the absence of their correlation indicate the different structures of MB and FB communities. Light absorption values are plotted along the axes. The *X* axis, absorption for MB; the *Y* axis, absorption for FB in the experiment.

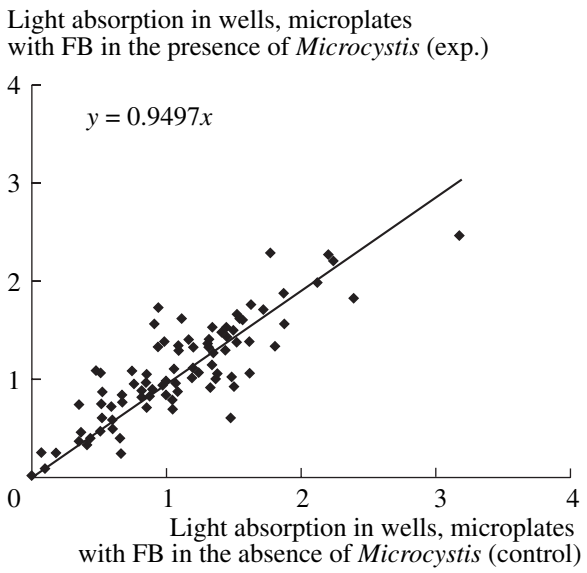

Fig. 2. Comparison of the functional diversity of FB communities from experimental vials in the presence and absence (control) of *Microcystis*. Close color intensities in the wells with the same substrate and significant correlation ($r = 0.84$, $P < 0.01$) indicate similar structures of FB communities in experimental and control vials.

than in the ambient water; these compounds support the growth of free-living bacteria.

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