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Morphological and physiological changes in *Microcystis aeruginosa* as a result of interactions with heterotrophic bacteria

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SUMMARY

1. To reveal the role of aquatic heterotrophic bacteria in the process of development of *Microcystis* blooms in natural waters, we cocultured unicellular *Microcystis aeruginosa* with a natural *Microcystis*-associated heterotrophic bacterial community.
2. Unicellular *M. aeruginosa* at different initial cell densities aggregated into colonies in the presence of heterotrophic bacteria, while axenic *Microcystis* continued to grow as single cells. The specific growth rate, the chl *a* content, the maximum electron transport rate (ETR_{max}) and the synthesis and secretion of extracellular polysaccharide (EPS) were higher in non-axenic *M. aeruginosa* than in axenic *M. aeruginosa* after cell aggregation, whereas axenic and non-axenic *M. aeruginosa* displayed the same physiological characteristic before aggregation.
3. Heterotrophic bacterial community composition was analysed by PCR–denaturing gradient gel electrophoresis (PCR–DGGE) fingerprinting. The biomass of heterotrophic bacteria strongly increased in the coinoculated cultures, but the DGGE banding patterns in coinoculated cultures were distinctly dissimilar to those in control cultures with only heterotrophic bacteria. Sequencing of DGGE bands suggested that *Porphyrobacter*, *Flavobacteriaceae* and one uncultured bacterium could be specialist bacteria responsible for the aggregation of *M. aeruginosa*.
4. The production of EPS in non-axenic *M. aeruginosa* created microenvironments that probably served to link both cyanobacterial cells and their associated bacterial cells into mutually beneficial colonies. *Microcystis* colony formation facilitates the maintenance of high biomass for a long time, and the growth of heterotrophic bacteria was enhanced by EPS secretion from *M. aeruginosa*.
5. The results from our study suggest that natural heterotrophic bacterial communities have a role in the development of *Microcystis* blooms in natural waters. The mechanisms behind the changes of the bacterial community and interaction between cyanobacteria and heterotrophic bacteria need further investigations.

Keywords: aggregation, community composition, extracellular polysaccharide, heterotrophic bacteria, *Microcystis aeruginosa*

Introduction

The phytoplankton in eutrophied lakes is usually dominated by large, colony-forming species of cyanobacteria (Dokulil & Teubner, 2000). Species of the

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genus *Microcystis* frequently achieve large populations and form dense toxic surface blooms, which can create serious water quality problems (Sivonen, 1996; Jochimsen *et al*., 1998; Tyagi *et al*., 1999). To achieve and maintain such dominance, *Microcystis* possesses a broad array of specific morphological and physiological adaptive mechanisms. In freshwaters, *Microcystis* commonly forms large mucilaginous colonies with numerous heterotrophic bacteria embedded in the mucilage (Whitton, 1973; Brunberg, 1999). The formation of colonies and aggregates is of decisive importance for the physiology and behaviour of cyanobacteria (Dokulil & Teubner, 2000). *Microcystis* occurs mainly as colonial morphs under natural conditions (Reynolds *et al*., 1981), but as single cells in laboratory cultures. Previous laboratory studies (Shen & Song, 2007; Wu *et al*., 2007) indicated that unicellular and colonial *Microcystis* display different physiological characteristics, especially in terms of their responses to environmental stress. Formation of colonies or aggregates could be induced by flagellate grazing (Burkert *et al*., 2001; Yang *et al*., 2008) and by extracellular microcystins (Sedmak & Eleršek, 2005).

The typical development and decline of *Microcystis* blooms includes aggregation of cells, formation of colonies and disaggregation of colonies and has been studied extensively (Worm & Søndergaard, 1998; Jacoby *et al*., 2000; Wilson, Wilson & Hay, 2006; Yoshida *et al*., 2007). In this regard, the role of aquatic bacteria has received increasing attention. Doucette (1995) suggested that interactions between bacteria and harmful algal species are important for understanding the dynamics of harmful algal blooms. Grossart, Czub & Simon (2006) showed that heterotrophic bacteria play an important role in the control of the development and aggregation of a diatom. In aquatic ecosystems, bacteria and algae are the numerically dominant organisms in the plankton, and their metabolism largely controls energy flow and nutrient cycling (Cole, 1982). Each lake has its own distinct bacterioplankton community, suggesting that differences are partly related to the equilibrium state of the lake (Gucht *et al*., 2005). Different phylogenetic groups of bacteria, with specialised exoenzymes to metabolise specific types of organic matter, are associated with the initiation, maintenance and termination phases of algal blooms (Smith *et al*., 1995; Riemann, Steward & Azam, 2000). On the other hand, the quantitative and qualitative differences in phytoplankton species composition may lead to pronounced differences in the bacterioplankton species composition (Pinhassi *et al*., 2004). Some bacteria may promote the growth of algae or live in symbiosis with them (Ferrier, Martin & Rooney-Varga, 2002; Uribe & Espejo, 2003), while others inhibit the growth of algae or exert algicidal activities (Lovejoy, Bowman & Hallegraeff, 1998; Lee *et al*., 2000).

A considerable number of studies have focused on the interactions between *Microcystis* species and its associated bacteria. These studies include the following: (i) competition or exchange of nutrients (Steppe *et al*., 1996; Fuks *et al*., 2005; Jiang *et al*., 2007), (ii) lysis of *Microcystis* cells directly or indirectly (Manage, Kawabata & Nakano, 2001; Ozaki *et al*., 2008) and (iii) degradation of microcystin (Maruyama *et al*., 2003; Lemes *et al*., 2008). In natural freshwaters, the relationship between *Microcystis* and bacteria is probably as complex as any other kind of interaction. Results from field experiments (Worm & Søndergaard, 1998; Kapustina, 2006) have shown significant differences in the quantity, production and activity of *Microcystis*-associated bacteria and free-living bacteria. In addition, growth and physiological traits of *Microcystis* may change during the development of blooms because cyanobacteria may play a role both as primary producers and prey in the microbial loop (Fuks *et al*., 2005).

Despite the large number of field and experimental studies that have been carried out, understanding of the interaction between *Microcystis* and its associated bacteria remains unclear. The above-mentioned results are from laboratory studies of *Microcystis*-bacteria interactions obtained with monoclonal bacterial strains. It is therefore quite difficult to extrapolate these results to natural conditions and to understand the complexity of the interaction since most (>99%) aquatic bacteria cannot be purified and cultured. To understand how bacteria influence the development of *Microcystis* blooms in natural waters, it is necessary to test the impact of natural bacterial communities and not just the impact of monoclonal strains. *Microcystis* populations mainly consist of colonies under natural conditions (Reynolds *et al*., 1981), and we hypothesised that heterotrophic bacterial communities might be crucial for the aggregation of *Microcystis* cells and through morphological changes of *Microcystis* lead to physiological changes, which facilitate maintenance of a high biomass. The aim of this study

was to simulate the natural environment in a surface cyanobacterial bloom to study the interactions between *Microcystis aeruginosa* and a natural bacterial community. The morphological and physiological characteristics of *M. aeruginosa* and changes of heterotrophic bacterial community composition were investigated in the course of coculture of *M. aeruginosa* and heterotrophic bacterial community. Our results contribute to understanding the specific role of heterotrophic bacteria in the dynamics of *Microcystis* bloom in natural water bodies.

Methods

Strains, cultivation and experimental design

A unicellular toxic *M. aeruginosa* strain was obtained from the Culture Collections of Freshwater Algae of the Institute of Hydrobiology, Wuhan, China. This strain was isolated from Lake Taihu (a shallow eutrophic lake in the east China) in 1997. An axenic isolate was established by streak plate isolation on BG-11 agar medium (Stanier *et al.*, 1971), and then grown in batch culture in BG-11 medium at 25 ± 1 °C and illuminated in a 12 : 12 h light : dark cycle at irradiance of 50 μE m^{-2} s^{-1}. The axenic cultures were transferred weekly to fresh medium and were maintained in the exponential phase. Regular inspection using DAPI staining, in conjunction with epifluorescence microscope, showed that cultures were axenic at the beginning of the experiment and that the biomass of bacteria in the axenic cultures never exceeded 1% of *Microcystis* biomass during the experiment.

The natural bacterial community used in this study was isolated from the mucilage of *Microcystis* colonies. Lake water was taken from the surface layer (0.5 m) of Lake Taihu (31°24′N, 120°14′E) in June 2008. At the time of sampling, a large part of the lake surface was covered with dense *Microcystis* blooms, consisting mainly of *M. aeruginosa*. The *Microcystis* colonies were collected by filtration of 1 L of lake water through a 20-μm mesh plankton net. According to Worm & Søndergaard (1998) and Kapustina (2006), we operationally defined that bacteria attached to *Microcystis* dominated the >20-μm size fraction in abundance and activity. The collected *Microcystis* samples were washed in sterile deionised water (≥16 MΩ) to disaggregate the colonies into single cells and to dissolve the *Microcystis* mucilage (Plude *et al.*, 1991). The suspension was centrifuged at 10 000 ***g*** for 10 min at 4 °C, and the supernatant was transferred to a new flask. The remaining pellet was washed and centrifuged three times. The supernatant, containing the natural bacterial community from the mucilage of *Microcystis*, was filtered through GF/C filter papers (pore size 1.2 μm, Whatman, UK) before use to rule out contamination by naturally occurring *Microcystis* cells. Each step of the isolation procedure was carried out under sterile conditions. The experiment was started immediately after isolation.

The axenic *M. aeruginosa* was harvested by centrifugation, washed and centrifuged three times with sterile distilled water and thereafter transferred to 1-L Erlenmeyer flasks containing 500-mL autoclaved BG-11 medium. Seven different treatments were established (see Table 1): three axenic treatments (treatment 1, 3 and 5) with initial cyanobacterial cell concentrations of 1, 10 and 100×10^5 cells mL^{-1}; three different non-axenic treatments (treatment 2, 4 and 6) with initial cyanobacterial cell concentrations of 1, 10 and 100×10^5 cells mL^{-1}, all three inoculated with 25 mL of the natural heterotrophic bacterial community with initial concentration of 10×10^5 cells mL^{-1}; and one treatment with a pure culture of heterotrophic bacteria (treatment 7) with an initial cell concentration of 10×10^5 cells mL^{-1}. Each treatment was prepared in triplicate, and all the cultures were incubated under the conditions described above. The whole experiment lasted 34 days, and the cultures were harvested every third day to monitor changes in growth and physiological parameters. The harvested culture volume was replaced with fresh medium to sustain growth.

Microcystis cell count and cell morphology

After staining with Lugol's Iodine solution, *Microcystis* cells were enumerated in a haemocytometer using an Olympus BX50 (Tokyo, Japan) microscope at 600× magnification. The specific growth rate was calculated using the method given by Pirt (1975). Cell size was determined with an object micrometre at 600× magnification. Cyanobacterial cells and colonies in at least 10 randomly fields of view were measured. The areas of cyanobacterial cells and colonies were calculated by measuring diameter and width (Montagnes *et al.*, 1994).

Table 1 The initial concentrations of *Microcystis aeruginosa* and heterotrophic bacteria in different treatments at the beginning of the experiment and the growth characteristics of *M. aeruginosa* and heterotrophic bacteria in different treatments

Treatment	1	2	3	4	5	6	7
Microcystis aeruginosa ($\times 10^5$ cells mL^{-1})	1	1	10	10	100	100	0
Heterotrophic bacteria ($\times 10^5$ cells mL^{-1})	0	10	0	10	0	10	10
Microcystis growth							
Exponential phase	6–30 d	6–18 d	6–24 d	6–12 d	6–15 d	3–12 d	
Stationary phase	>30 d	>18 d	>24 d	>12 d	15–30 d	12–18 d	
Declining phase	–	–	–	–	>30 d	>18 d	
Microcystis aggregation							
Began to form aggregates	–	24 d	–	18 d	–	12 d	
Bacteria growth							
Exponential phase		>12 d		>12 d		>12 d	3–9 d
Stationary phase		–		–		–	>9 d
Declining phase		–		–		–	–

–, not determined; d, day.

DGGE analyses of bacterial community composition

The composition of the bacterial community was determined by DGGE of the PCR-amplified fragments of 16S rDNA gene, followed by cloning and sequencing of all visible bands.

A 10-mL sample of culture was sonicated to detach associated bacteria and pre-filtered with 3-μm filters to remove *M. aeruginosa* (Xing & Kong, 2007). The filtrate was then filtered through a 0.2-μm pore size polycarbonate filter (Whatman) to capture bacteria. Filters were frozen at −80 °C immediately and kept frozen until use. For DNA extraction, the filters containing microbes were cut into small pieces (1–2 mm^2) with a sterile scalpel, and then total DNA was extracted using a bacterial DNA Kit (Omega, Bio-tek, Doraville, GA, USA) according to the manufacturer's recommendations. The variable V3 region of 16S rRNA fragments was amplified by PCR using a universal primer 518R (5′-ATTACCGCGGCTGCTGG-3′) and a bacterial primer 357F (5′-CCTACGGGAGGCAGCAG-3′) with a 40-bp GC clamp attached to its 5′ end (Muyzer, Waal & Uitterlinden, 1993). PCRs of 50 μL contained 1 × PCR buffer, 1.5 mM $MgCl_2$, 200 μM of each dNTP, 0.2 μM of each primer, 2.5 units of Taq DNA polymerase (Takara, Shuzo, Japan), 50 ng of template DNA and DNase- and RNase-free water. Touchdown PCR was conducted according to the modified method of Riemann *et al.* (1999). With 5-min initial denaturation at 94 °C followed by a thermal cycling programme as follows: 1-min denaturation at 94 °C; 1-min primer annealing at an initial 65 °C, decreasing 1 °C every cycle to a final of 55 °C; 3-min primer extension at 72 °C. Thirty cycles were run followed by a final 7-min incubation at 72 °C. A negative control, in which the template was replaced by an equivalent volume of sterile deionised water, was included in the PCRs. After PCR, the size of the products was confirmed on a 1.5% agarose gel.

About 20 μL of PCR products was loaded on a 8% (w/v) polyacrylamide gel (37.5 : 1 acrylamide: bisacrylamide) with a denaturing gradient that ranged from 40 to 60%, where 100% denaturant was defined as 7 M urea plus 40% deionised formamide. DGGE was performed with a Dcode system (Bio-Rad Laboratories, Hercules, CA, USA) using 1 × TAE running buffer (20 mM Tris, 10 mM acetic acid, 0.5 mM EDTA, pH 8.0) at 60 °C for 7 h at 150 V. The gel was stained in 1 : 10000 diluted GelRed (Biotium, Hayward, CA, USA) nucleic acid staining solution for 30 min and photographed using a Bio Image System (Gene Com. Ltd., Hong Kong) under UV light.

All visible bands in the DGGE gel were carefully excised from the gel, and DNA was eluted overnight at 4 °C in 40 μL of sterile deionised water. One microlitre of eluted DNA was used as a template for PCR amplification with the same primers and programme as described above. The reamplicons were electrophoresed again on a DGGE gel to check the positions of the original band and then purified by a Gel Recovery Purification Kit (AxyPrep™, Union City, CA, USA) and ligated into pMD18-T plasmid vector system (Takara) and transformed into *Escherichia coli* DH5α-competent cells. Positive clones were cultured overnight and then submitted for sequencing using M13 primers and an automated

ABI DNA sequencer at the Genomics Company (Wuhan, China).

Cluster analyses of DGGE profiles were performed with the NTSYS program version 2.10e (Exeter software, Setauket, NY, USA). A binary matrix was constructed by scoring presence or absence of DGGE bands. Pairwise similarities between gel banding patterns were quantified using the Dice coefficient as: $S_D = (2N_{AB})/(N_A + N_B)$, where N_{AB} is the number of bands common to the samples A and B, and N_A and N_B are the number of bands in samples A and B, respectively. The unweighted pair group method with arithmetic average (UPGMA) was used to investigate differences among bacterial community composition according to the method of Xing & Kong (2007). Bacterial sequences were compared to known sequences in the GenBank database by the BLAST algorithm to identify sequences with a high degree of similarity. Sequences were aligned using CLUSTAL W and corrected manually to delete ambiguous and non-informative bases, and phylogenetic tree was constructed using a neighbour-joining algorithm with MEGA 4. The 16S rRNA gene sequences from this study were deposited in the NCBI GenBank database under accession numbers GU362868–362879.

Bacterial abundance

Samples for determination of bacterial abundance were preserved with 4% (v/v) formaldehyde. The fixed sample of 0.5–2 mL was stained with 4′6′ diamidino-2-phenolindole [DAPI; Sigma (St Louis, MO, USA), final concentration 1 μg mL^{-1}] for 10–15 min (Porter & Feig, 1980). Afterwards, the sample was gently filtered onto a 0.2-μm pore size black polycarbonate filter (Whatman, Maidstone, UK). Total bacterial cell numbers were counted using epifluorescence microscope (Zeiss Axioskop 20, Oberkochen, Germany). A minimum of 10 replicates was counted for each sample.

Determination of chlorophyll a content in Microcystis

To determine the content of chlorophyll *a* (Chl *a*) in *M. aeruginosa*, the cyanobacterial cells were extracted overnight in 95% ethanol in the dark. Debris was then removed by centrifugation, and the supernatant was analysed spectrophotometrically (UV-1601; Shimadzu, Kyoto, Japan) at 665 nm and 649 nm against 95% ethanol as blank. The concentration of Chl *a* was calculated according to Wellburn (1994).

Determination of chlorophyll fluorescence parameters

A pulse-amplitude-modulated fluorescence monitoring system (PAM; Walz, Effeltrich, Germany) was used to measure the *in vivo* chlorophyll fluorescence at room temperature. The rapid light curves (RLCs) based on measurement of the relative *ETRs* were derived from estimates of $\Delta F_v/F_{m'}$ (the operational quantum yield of photosynthetic system II (PSII), Φ_{PSIIe}). The numerical values of chlorophyll fluorescence of samples exposed to 12 intensities of actinic light increasing from 0 to 1265 μmol photons PAR m^{-2} s^{-1} were recorded during a 3-min time series. The photosynthetic *ETR* was calculated using the following formula: $ETR = ((F_{m'} - F_t)/F_{m'} \times 0.84 \times 0.5 \times \text{PAR}$ (m^{-2} s^{-1})), where $F_{m'}$ and F_t denote the maximum and steady state fluorescence in light, respectively. The approximate amount of incident light absorbed by the algae was 0.84, of which approximately 0.5 was transferred to PSII (Shen & Song, 2007).

Determination of extracellular polysaccharides

Actively growing cultures of *M. aeruginosa* colonies were harvested for staining of bound extracellular polysaccharides (EPS) by low-speed centrifugation and resuspended in approximately 0.5 mL of distilled water. Two drops of the cell suspension were mixed with five drops of stain (0.1% w/v Alcian Blue 8GX in 0.5 N acetic acid) in a small test tube, mixed and allowed to stand at room temperature for 5 min (Crayton, 1982). Samples were observed and photographed using a microscope (Olympus BX50) under bright field illumination and at a magnification of 600×. The concentrations of the total dissolved EPS in the medium of all five treatments were determined by the phenol-H_2SO_4 method (Dubois *et al.*, 1956) using a standard of D-galactose.

Statistics and data processing

Results are expressed as means ± standard deviation (SD). The statistical analysis was carried out using one-way ANOVA followed by a Tukey *post hoc* test to elucidate any significant differences between

treatment groups and control groups. Probability values of <0.05 were accepted. Regression analysis was used to test the relationship between specific growth rate and *M. aeruginosa* initial concentration and followed by Student's *t*-test to test the significance of the correlation coefficient. Statistics and graphs were obtained using the software Microcal™ Origin 7.0 (Microcal Software Inc., Northampton, MA, USA).

Results

Cell aggregation

Axenic and non-axenic cultures of unicellular *M. aeruginosa* displayed distinct morphological differences during the experiment. Axenic *M. aeruginosa* maintained its single-celled form (Fig. 1a) throughout the observation period with cells evenly spread in the counting chamber. In contrast, *M. aeruginosa* in the non-axenic treatments 2, 4 and 6 aggregated into colonies (Fig. 1b). The colonies consisted of multicellular structures that were surrounded by a polysaccharide substance so that the cells remained in tight arrangements after shaking. When aggregates of *M. aeruginosa* in the non-axenic treatments were stained with Alcian Blue, the cells surface turned blue (Fig. 1c), which is cytochemical evidence for the presence of acidic polysaccharides in the colonies. However, the axenic *M. aeruginosa* cells failed to stain with Alcian blue.

Aggregation of *M. aeruginosa* cells was faster in treatments with a high initial concentration of cyanobacterial cells compared with treatments with lower initial cell concentrations (Fig. 2). The first aggregates of *M. aeruginosa* occurred after 12 days of incubation at an initial cell concentration of 100×10^5 cell mL^{-1}. The average aggregate area in each treatment increased with incubation time and reached their maximal area (approximately 800–1000 μm^2) from day 27 to 33. There was no significant difference ($P > 0.05$) in maximal area among treatments.

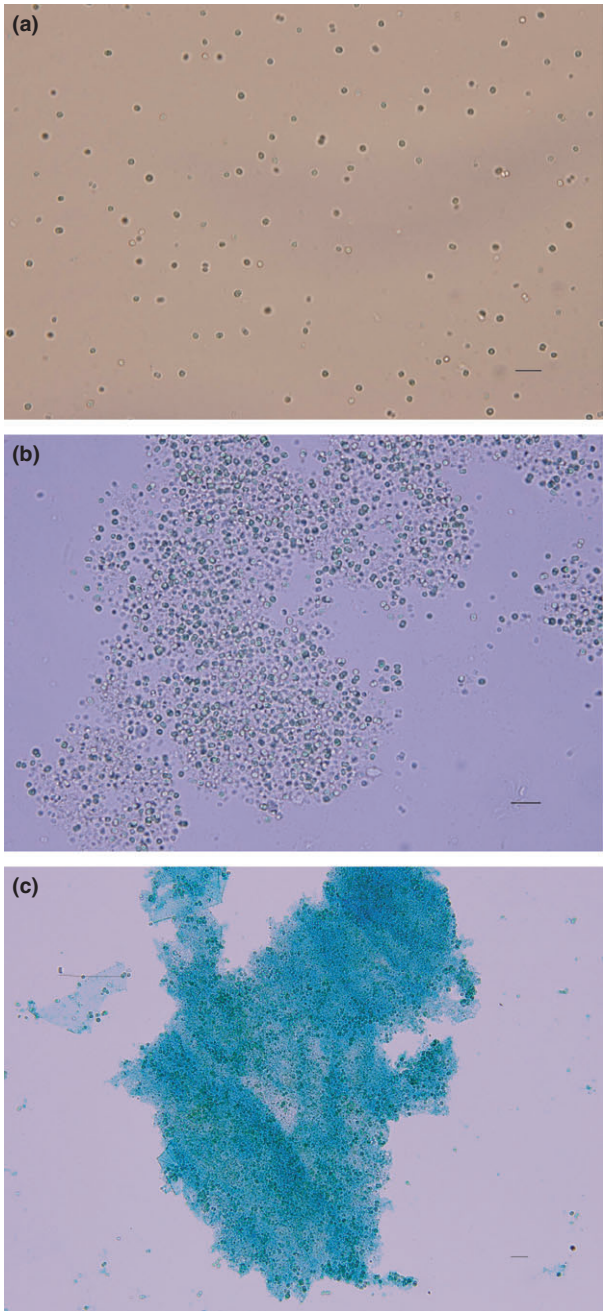

Fig. 1 Micrographs of axenic and non-axenic cultures of unicellular *Microcystis aeruginosa* grown for 24 days. Scale bar equals 10 μm. (a) axenic culture of *M. aeruginosa* (treatment 3); (b) non-axenic culture of *M. aeruginosa* with an initial concentration of 10×10^5 cells mL^{-1} (treatment 4); (c) non-axenic culture of *M. aeruginosa* cells stained with Alcian Blue (treatment 4).

Growth of M. aeruginosa and heterotrophic bacteria

The growth characteristics of *M. aeruginosa* and heterotrophic bacteria in axenic and non-axenic cultures showed pronounced differences between the exponential and stationary phase during the experiment (Table 1 & Fig. 3a). The non-axenic *M. aeruginosa* (treatment 4) grew faster than axenic *M. aeruginosa* (treatment 3) during the exponential phase from day 6

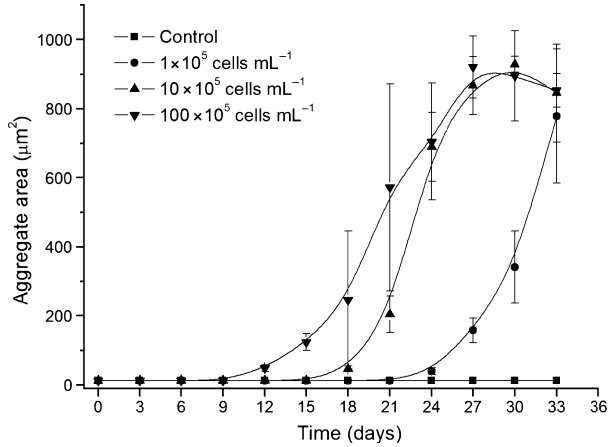

Fig. 2 The influence of heterotrophic bacteria on the average aggregate area of unicellular *Microcystis aeruginosa* with different initial cell concentration of 1×10^5 cell mL^{-1}, 10×10^5 cell mL^{-1},100×10^5 cell mL^{-1}, respectively. Values are means ± SD.

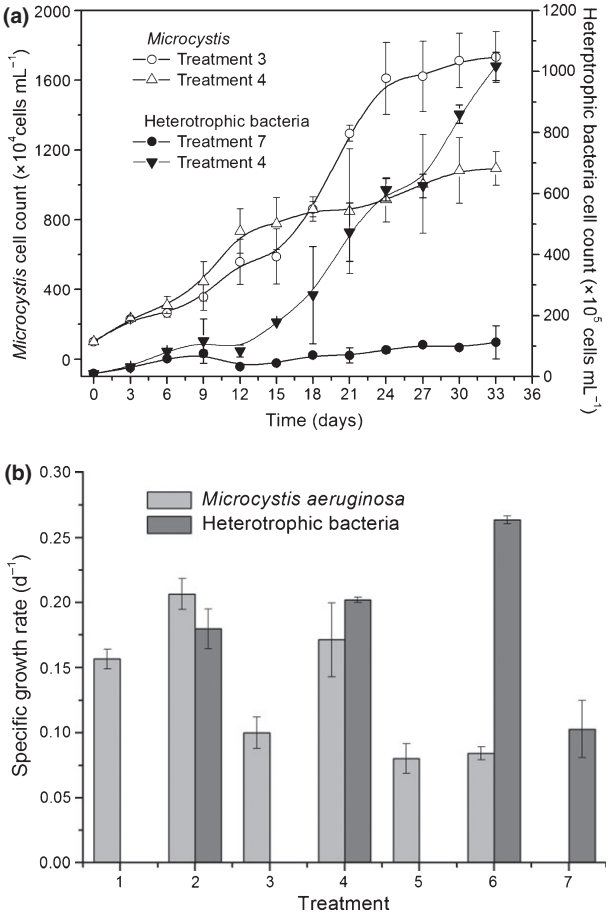

Fig. 3 Growth characteristics of *Microcystis aeruginosa* and heterotrophic bacteria in various treatments. Treatment 1, 3, 5: axenic culture of *M. aeruginosa*, initial cell density 1×10^5, 10×10^5 and 100×10^5 cell mL^{-1}, respectively; Treatment 2, 4, 6: coculture, initial heterotrophic bacterial cell density all 10×10^5 cell mL^{-1} and initial cyanobacterial cell density 1×10^5, 10×10^5 and 100×10^5 cell mL^{-1}, respectively; Treatment 7: pure culture of heterotrophic bacteria, initial cell density 10×10^5 cells mL^{-1}. Values represent means and error bars represent SD. (a) Cell density changes of *M. aeruginosa* and heterotrophic bacteria in treatment 3, 4 and 7. Open symbols represent cyanobacteria growth; Filled symbols represent heterotrophic bacteria growth. (b) The specific growth rates of *M. aeruginosa* and bacteria in the exponential phase.

to 18. However, the exponential phase of non-axenic *M. aeruginosa* (treatment 4) lasted shorter period than that of axenic *M. aeruginosa* (treatment 3). During the stationary phase, the cell density of axenic *M. aeruginosa* (treatment 3) was higher than that of non-axenic *M. aeruginosa* (treatment 4) (Fig. 3a). Similarly, the growth of heterotrophic bacteria in non-axenic *M. aeruginosa* (treatment 4) and in pure culture (treatment 7) also showed significant differences (Fig. 3a). Cell densities of heterotrophic bacteria in the pure culture (treatment 7) reached a maximum on day 9 and remained almost constant thereafter. In contrast, cell densities of heterotrophic bacteria cocultured with *M. aeruginosa* (treatment 4) were higher than that in the pure culture throughout the whole experiment period. In the non-axenic cultures (treatment 4), the growth of *M. aeruginosa* stabilised and the cells entered the stationary phase when the density of heterotrophic bacteria increased dramatically after day 12. A similar phenomenon was also observed in treatment 2 and 6 (data not shown).

The specific growth rate of non-axenic *M. aeruginosa* correlated negatively ($r = -0.948$, $n = 9$, $P < 0.001$) to the initial cyanobacterial density (Fig. 3b), and the specific growth rates were higher in the two non-axenic *M. aeruginosa* treatments with initial inoculation concentrations of 1×10^5 ($F = 35.7071$, $P = 0.00938$) and 10×10^5 cell mL^{-1} ($F = 19.311$, $P = 0.02184$) than that in the axenic *M. aeruginosa* (treatment 1 and 3), whereas the growth rate in treatment 6 (initial cell concentration of 100×10^5) compared to axenic *M. aeruginosa* (treatment 5) was slightly higher ($P > 0.05$) (Fig. 3b). On the other hand, *M. aeruginosa* in higher concentrations promoted heterotrophic bacterial growth. The growth rate of heterotrophic bacteria in coculture with *M. aeruginosa* (treatment 2, 4 and 6) compared to those in pure

culture (treatment 7) was significantly stimulated ($F_3 = 47.8632$, $P = 0.00136$) by *M. aeruginosa*, which were correlated positively ($r = 0.998$, $n = 9$, $P < 0.001$) with the *M. aeruginosa* initial concentration.

Heterotrophic bacteria community composition

The DGGE gel showed pronounced changes in relative brightness and position of DGGE bands of PCR-amplified community DNA in the non-axenic culture of *M. aeruginosa* (treatment 4) during the course of the experiment, especially after day 9 (Fig. 4a). The heterotrophic bacterial community consisted of 15 distinguishable DGGE bands on day 0, but had decreased to five by day 9. After day 9, the numbers of detected 16S rDNA bands remained about the same. UPGMA clustering based on the position and intensity of the bands indicated that the bacterial community on day 0 was different from that on other days (Fig. 4b). However, DGGE banding patterns of the pure culture of heterotrophic bacteria (treatment 7) from different days were very similar (Fig. 5a); only

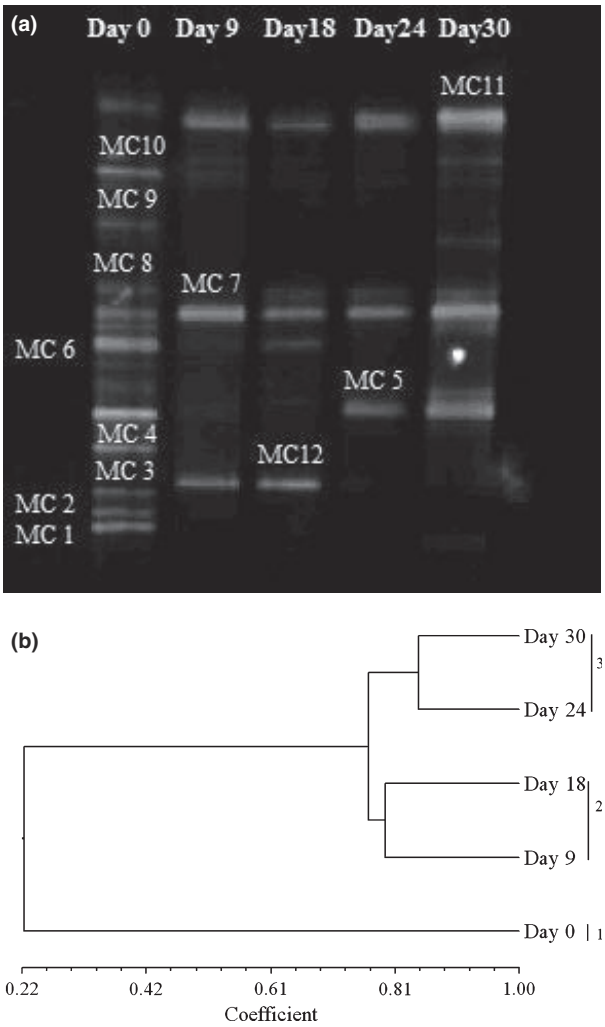

Fig. 4 Denaturing gradient gel electrophoresis (DGGE) profiles (a) of heterotrophic bacterial community composition over time in treatment 4 (non-axenic culture and initial cyanobcterial cell density of 10×10^5 cell mL^{-1}) and unweighted pair group method with arithmetic average analysis dendrograms (b) of bacterial community of DGGE profiles. Each excised, cloned and sequenced band is labelled in (a). Samples are grouped into three major clusters defined as 1, 2 and 3 in (b).

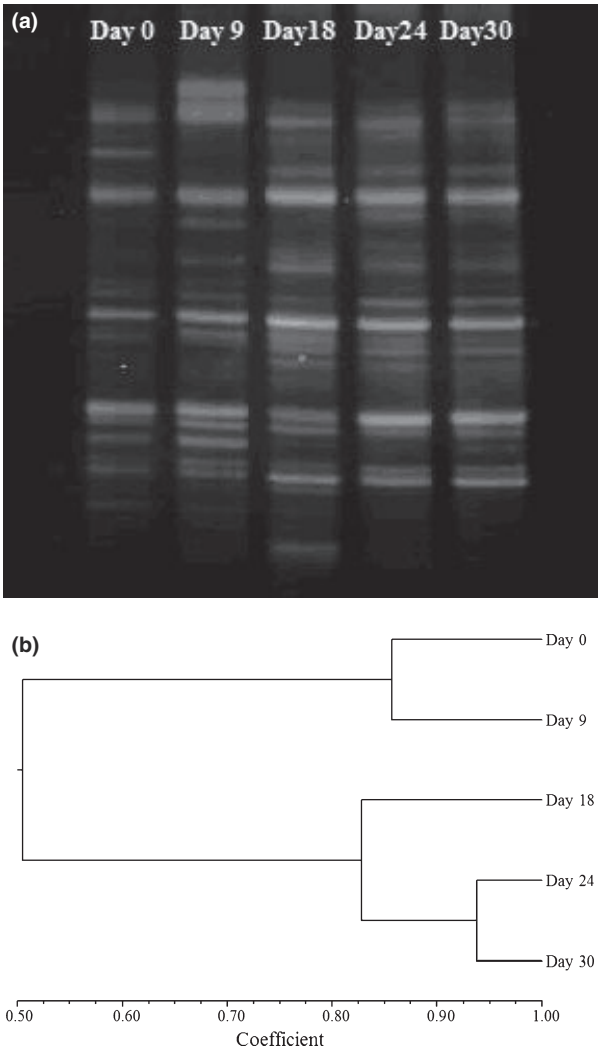

Fig. 5 Denaturing gradient gel electrophoresis (DGGE) profiles (a) of the heterotrophic bacterial community composition over time in treatment 7 (pure culture of heterotrophic bacteria and initial cell density of 10×10^5 cell mL^{-1}) and unweighted pair group method with arithmetic average analysis dendrograms (b) of heterotrophic bacterial community of DGGE profiles.

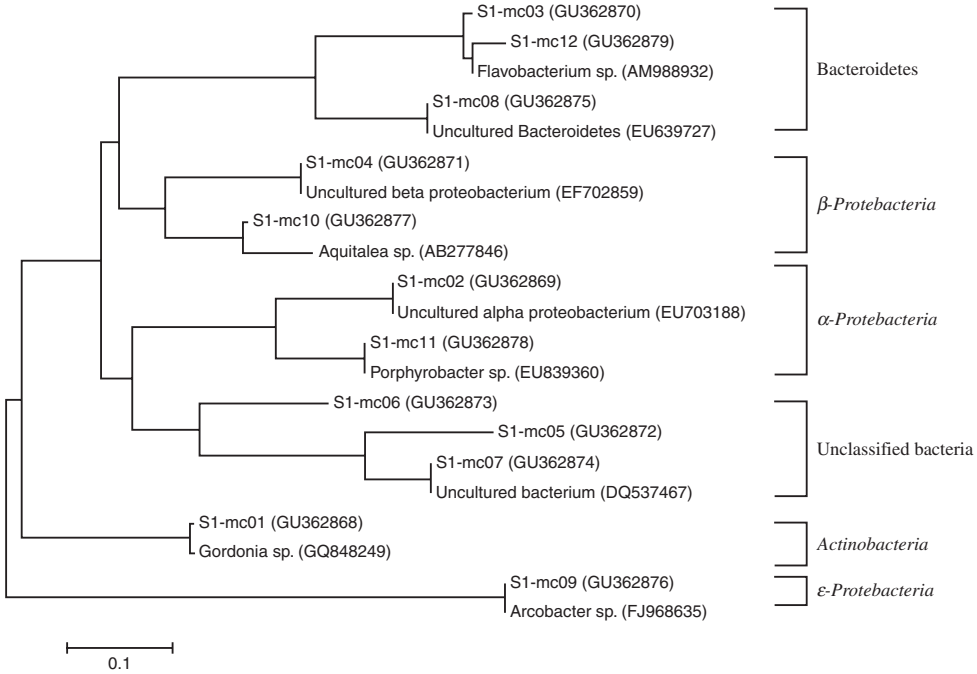

Fig. 6 A neighbour-joining tree showing phylogenic relationships of the sequences among the dominant denaturing gradient gel electrophoresis bands.

minor differences among days were observed (Fig. 5b). Bands from different days with identical vertical positions in the gel were assumed to have identical sequences (Riemann *et al*., 1999). A total of 12 bands in treatment 4 were excised and successfully sequenced (Fig. 4a). The majority of the bacterial community in our study could be divided into six groups: *Bacteroidetes*, *α-Proteobacteria*, *β-Proteobacteria*, *ϵ-Proteobacteria*, *Actinobacteria* and unclassified bacteria (Fig. 6), all of which appeared at the beginning of the experiment (Fig. 4a). Three unclassified bacteria (band MC5, MC6 and MC7) clustered close to or inside *α-Proteobacteria*. Of all these bacteria, *α-Proteobacteria* (band MC11) and two unclassified bacteria (band MC5 and MC7) were most prominent towards the end of the experiment, while other bands decreased in brightness or disappeared after day 9.

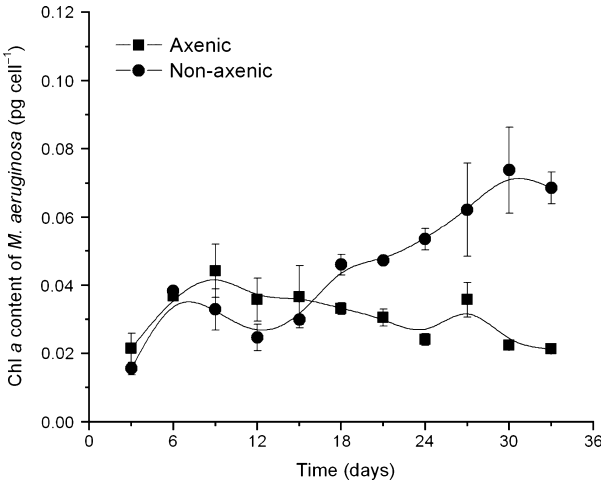

Fig. 7 The content of chlorophyll *a* per cell of *Microcystis aeruginosa* in the axenic (treatment 3) and non-axenic (treatment 4) cultures both at an initial inoculation concentration of 10×10^5 cell mL^{-1}. Points represent means and error bars represent SE.

Chlorophyll a content

The changes in cell content of chl *a* in the axenic and non-axenic *M. aeruginosa* (treatment 3 and 4) were similar between treatments until day 12 (Fig. 7). Subsequently, the chl *a* content per cell in the axenic *M. aeruginosa* (treatment 3) decreased until the end of experiment, whereas it increased markedly in the non-axenic treatment (treatment 4). When chl *a* content was related to *M. aeruginosa* growth stage, the divergence in chl *a* content between two treatments occurred in stationary phase.

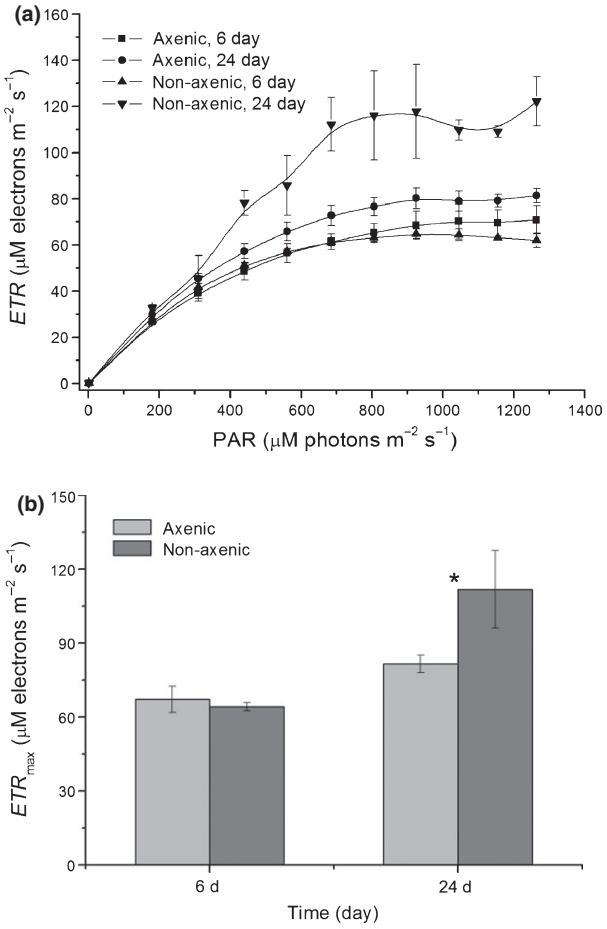

Fig. 8 Electron transport rate (*ETR*) curves (a) and maximal electron transport rate (ETR_{max}) (b) of axenic and non-axenic *Microcystis aeruginosa* (treatment 3 and 4) grown for 6 and 24 days. Points in the curves represent means and error bars represent SD. Columns represent means and error bars represent SD. *$P < 0.05$ indicates significant differences between axenic and non-axenic *M. aeruginosa*.

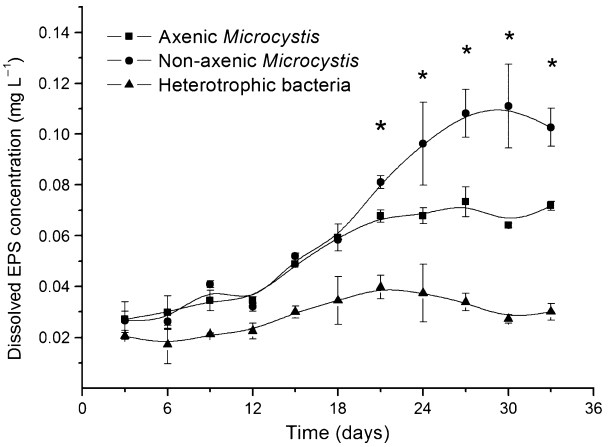

Fig. 9 The concentration of extracellular polysaccharides in the medium of *M. aeruginosa* in treatment 3 (axenic treatment), treatment 4 (non-axenic treatment) and treatment 7 (pure culture of heterotrophic bacteria). The initial density of cyanobacteria and heterotrophic bacteria was both approximately 10×10^5 cells mL^{-1}. Points represent means and error bars represent SD. *$P < 0.05$, indicate significant differences between axenic and non-axenic *M. aeruginosa*.

Chlorophyll fluorescence parameters

The light response of ETR was measured in the exponential and stationary stages of *M. aeruginosa* in treatments 3 and 4 (Fig. 8a). On day 6 of the experiment, the light response curves of axenic and non-axenic *M. aeruginosa* did not show any significant difference. However, on day 24, non-axenic *M. aeruginosa* was the colonial morph and the initial slope of the light response curve (α) of colonial *M. aeruginosa* increased sharply. In contrast, those of axenic *M. aeruginosa* only slightly increased ($P > 0.05$) compared to day 6. An identical phenomenon was also observed for ETR_{max} (Fig. 8b).

Dissolved extracellular polysaccharides

As mentioned above, the presence of acidic polysaccharides in the colonies was confirmed by the staining of the cell surface of non-axenic *M. aeruginosa* with Alcian Blue (Fig. 1c). Dissolved EPS was determined in all treatments and constantly increased throughout the experiment (Fig. 9). However, the concentration of dissolved EPS in the non-axenic *M. aeruginosa* (treatment 4) was higher ($F = 30.8668$, $P = 0.0005$) than in the axenic treatment (treatment 3) from day 21 to the end of incubation, whereas no significant differences ($P > 0.05$) were observed between these two treatments before day 21. The dissolved EPS was also detected in the pure culture of heterotrophic bacteria (treatment 7), but its concentration was significantly lower than that in the axenic ($F = 16.8538$, $P = 0.0005$) and non-axenic ($F = 13.4048$, $P = 0.0016$) *M. aeruginosa* (treatment 3 and 4) throughout the experiment.

Discussion

Cell aggregation

The cyanobacterium *M. aeruginosa* occurs mainly as single cells in laboratory cultures but in the colonial morph under natural conditions (Reynolds *et al.*, 1981), and the reasons for this morphological change

seem to be very complicated. Several laboratory experiments have reported that the formation of colonies or aggregates might be a response by *Microcystis* to various environmental stresses. Burkert *et al.* (2001) and Yang *et al.* (2008) showed induction of colonies in axenic *M. aeruginosa* in the presence of the predator *Ochromonas*. Sedmak & Eleršek (2005) reported that unicellular *M. aeruginosa* strains aggregated in the presence of commercial microcystins. The present study, however, clearly revealed that heterotrophic bacteria affected the aggregation of *M. aeruginosa*, leading not only to aggregation but also to mucilage exudation by *M. aeruginosa*. Our results indicate that aggregations of *M. aeruginosa* cells are not merely a defensive strategy against heterotrophic bacteria but also an interaction with heterotrophic bacteria. A similar phenomenon was reported for natural blooms where mucilaginous glue is present on the surface of *Microcystis* cells (Tien *et al.*, 2002; Gao & Yang, 2008).

In our experiment, when heterotrophic bacteria was present, *M. aeruginosa* with various initial cell concentrations all formed colonies but differed in time to aggregation, indicating that formation of aggregates is related to the cell density of *M. aeruginosa*. This result is in accordance with those of Sedmak & Eleršek (2005) who showed that when *Microcystis* populations reach plateau densities in the presence of microcystins, they start to combine into aggregates. Grossart *et al.* (2006) found that an exponentially growing diatom *Thalassiosira rotula* readily formed aggregates, but diatom cells in the stationary growth phase showed signs of bacterial decomposition. The different aggregation behaviours of *M. aeruginosa* in different growth phases were related to the succession of the associated heterotrophic bacterial community during the various growth phases. Some algae-associated bacteria may play a protective role (Armstrong *et al.*, 2001) while others may have an algicidal role (Caiola & Pellegrini, 1984; Middelboe *et al.*, 1995).

Interactions between Microcystis and heterotrophic bacterial community

The results of the present study clearly show that the interactions between *M. aeruginosa* and heterotrophic bacteria affected the growth of both *M. aeruginosa* and heterotrophic bacteria, and such interactions also varied with the growth phase. *M. aeruginosa* influence on heterotrophic bacterial growth was very limited during the exponential phase of the experiment but strongly increased heterotrophic bacterial growth during the stationary phase. A similar bacterial growth characteristic was found in coculture of a dinoflagellate and bacteria (Simon *et al.*, 2002), which suggested that heterotrophic bacterial growth was supported by organic matter derived from algal or cyanobacterial cells (Cole, 1982). The stimulation of *M. aeruginosa* growth by heterotrophic bacteria took place at the initiation of the exponential phase when heterotrophic bacteria grew slowly and bacterial diversity was high. Cyanobacterial growth may be stimulated by heterotrophic bacteria in ways that produce some stimulatory products (e.g. vitamins) (Cole, 1982). However, the growth rates of *M. aeruginosa* were negatively correlated to the initial incubation concentrations, since higher concentration of cyanobacteria enhanced the growth of heterotrophic bacteria. In turn, increased numbers of heterotrophic bacteria resulted in a decrease in the bacterial diversity and the cyanobacterial growth rate; furthermore, the aggregation of *Microcystis* cells was stimulated. Our results imply that the interactions between *M. aeruginosa* and associated heterotrophic bacterial communities can induce the morphological changes in *M. aeruginosa*, which were characterised by changes in composition of the heterotrophic bacterial community. Mayali & Doucette (2002) suggested that significant competition occurs within the microbial community in the various phases, and the growth of algae could be inhibited if algicidal bacteria outcompeted its inhibitors. Our results also demonstrated that bacterial community structure changed with the growth of cyanobacteria. Moreover, Fuks *et al.* (2005) found that mucilage events can substantially modify the relationships between cyanobacteria and heterotrophic bacteria in the natural water column. *Microcystis* provides a suitable microenvironment for attached bacteria (Brunberg, 1999), which contributes importantly to the carbon metabolism of heterotrophic bacteria (Worm & Søndergaard, 1998).

The DGGE profiles indicated that a few of the original heterotrophic bacterial species had started to dominate the community at the exponential growth phase of *M. aeruginosa*, and this change of composition in bacterial community preceded the change of morphology in *M. aeruginosa*. Therefore, the heterotrophic bacteria are more sensitive than *Microcystis* to

the ambient environment. These results imply that predominantly heterotrophic bacteria could coexist well with *Microcystis*. The mucus of some cyanobacteria is a highly specialised microenvironment for certain bacteria (Cole, 1982); in addition, we suggest that certain heterotrophic bacteria play an important role in the formation and maintenance of the mucus of some cyanobacteria. Bands MC7, MC11 and MC12 are seen as bright bands prior to the cell aggregation and probably represent specialist heterotrophic bacteria responsible for the aggregation of *M. aeruginosa*. *Porphyrobacter* species (band MC11) (Berg *et al*., 2009; Hube, Heyduck-Söller & Fische, 2009; Shi *et al*., 2009) and *Flavobacteriaceae* species (band MC12) (Eiler & Bertilsson, 2004; Mueller-Spitz, Goetz & McLellan, 2009) have previously been detected in association with *Microcystis* or other cyanobacterial blooms, but the uncultured bacterium S1-mc07 (band MC7) had never been reported in cyanobacterial blooms. Band MC12 disappeared and band MC5 increased in brightness after colonial *M. aeruginosa* formation, indicating that pronounced competition among these dominating heterotrophic bacteria and uncultured bacterium S1-mc05 (band MC5) perhaps related to maintenance of colonies of *M. aeruginosa* with other dominant heterotrophic bacteria (bacterium S1-mc07 and S1-mc11). Although the same bacterial divisions have been reported in association with cyanobacteria-dominated lakes all over the world, the relationship among these heterotrophic bacteria and cyanobacteria is unclear.

Chlorophyll a content and chlorophyll fluorescence parameters

Chlorophyll *a* is the essential photosynthetic pigment in cyanobacteria, because only chlorophyll *a* can utilise the absorbed light energy for the synthesis of chemical energy ATP. In the current experiment, the high chl *a* content in the aggregated *M. aeruginosa* confirmed that heterotrophic bacteria led to simultaneous morphological and physiological changes in *M. aeruginosa*. A similar result was reported by Sedmak & Eleršek (2005) who found that three microcystin variants led to cell aggregation, increase in cell volume and overproduction of photosynthetic pigments. They considered the three effects to be related to each other but were not necessarily caused by the same mechanism. The ETR can provide supplementary information about the status of the photosynthetic apparatus at the level of PSII-dependent electron transport (Masojídek *et al*., 2001). The increased value of ETR_{max} in non-axenic *M. aeruginosa* demonstrates that heterotrophic bacteria impart positive effect on the structure of PSII in *M. aeruginosa*. Therefore, *M. aeruginosa* possessed higher photosynthetic activity after cells became aggregated, which supported the view that the colonial *Microcystis* had higher photosynthetic parameters than those in unicellular *Microcystis* (Shen & Song, 2007); this physiological characteristic might facilitate *Microcystis* maintaining predominance in natural water columns. On the other hand, aggregation of *Microcystis* enhances the degree of self-shading of their own cells. Cell size and relative Chl *a* concentration may be factors influencing photosynthesis because of self-shading (Taguchi, 1976).

Extracellular polysaccharides

In lakes, *Microcystis* is the major phytoplankton genus accounting for cell-associated mucilage production (Tien *et al*., 2002). In our experiment, dissolved EPS was present in all treatments but at higher concentrations in colonial cells, implying that the heterotrophic bacterial community plays a crucial role in the aggregation of cells and formation of mucilage. The ability to secrete EPS helps bacteria to colonise *Microcystis* cells. Decho (1990) showed that secretions of polysaccharides from bacteria serve many functions that enhance the survival and competitive success of microbial cells under natural conditions. However, the ability of heterotrophic bacteria to secrete EPS was limited compared with cyanobacteria. The presence of heterotrophic bacteria can stimulate the EPS release by *M. aeruginosa* since increasing EPS production may be a defensive response of *Microcystis* to environmental stresses. Yang *et al*. (2008) found that synthesis and secretion of EPS by *M. aeruginosa* cells increased under flagellate grazing pressure. The morphological change in non-axenic *M. aeruginosa* was another important reason for the increased EPS release. *M. aeruginosa* in colonies have higher photosynthetic activity, thereby imparting synthesis of relatively high amounts of photosynthetic intermediates (carbohydrates), which is in agreement with the results of Fogg (1983) that actively growing phytoplankton release a considerable proportion of their

photoassimilated carbon into the aquatic environment.

The mucilage exudation is crucial for the formation of aggregates of *Microcystis*, which may relate to certain ecological processes. Reynolds (2007) reviewed the role and functions of the mucilage provision in phytoplankton and found that it included density reduction, dynamic streamlining, sequestration and storage of nutrients, defence against oxygen, metal poisoning and grazing. In the present study, the production of EPS in non-axenic *M. aeruginosa* created microenvironments that serve to link both cyanobacterial cells and their associated bacterial cells into mutually beneficial colonies. This result from the laboratory helps explain field observations (Mayali & Doucette, 2002) that the number of bacteria was low as long as the algal cells were in low concentrations as a result of the minimal amount of algal-derived dissolved organic matter in the water. However, bacteria populations increased when, or soon after, the phytoplankton biomass reached it maximum. Giroldo, Ortoland & Vieira (2007) also found that phytoplanktonic extracellular organic compounds, including carbohydrates, supported dense bacterial populations, even at very low concentrations. Dissolved EPS released from phytoplankton is a major component of dissolved organic carbon (DOC) in freshwater and is beneficial for bacterial growth (Sundh, 1992). On the other hand, bacterial activity strongly influenced aggregation behaviour of algae because of biodegradability of DOC by the bacterial community (Grossart *et al*., 2006). Hence, the roles of EPS in the *Microcystis*-heterotrophic bacteria interaction are very significant and need to be further studied in future work.

Conclusion

To date, no study has addressed the changes in morphology of *Microcystis* induced by heterotrophic bacteria. Our study indicates that the *Microcystis*-heterotrophic bacteria community interaction resulted in substantial physiological changes to *M. aeruginosa* including morphology, growth, pigment content, ETR and EPS; meanwhile, the composition of the heterotrophic bacterial community was also changed. The general process can be described as follows: the growth of *M. aeruginosa* was promoted by heterotrophic bacteria when the cyanobacteria were at low concentration. With the increase in cyanobacterial concentration, the bacterial community increased markedly in biomass and the predominance of heterotrophic bacteria changed, inducing cyanobacterial cells to form colonies. The increase of EPS originating from *M. aeruginosa* played an important role in the aggregation of cells and formation of mucilage. The results from our study can partly explain the role of natural bacterial communities in the development of *Microcystis* blooms in natural waters, although the mechanisms behind the changes in the bacterial community and the interaction between cyanobacteria and heterotrophic bacteria need further investigations.

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