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Induction and Resuscitation of the Viable but Nonculturable State in a Cyanobacteria-Lysing Bacterium Isolated from Cyanobacterial Bloom

Huirong Chen · Lixian Fu · Lingxi Luo · Jun Lu · W. Lindsey White · Zhangli Hu

Abstract The viable but nonculturable (VBNC) state has been found to be a growth strategy used by many aquatic pathogens; however, few studies have focused on VBNC state on other aquatic bacterial groups. The purpose of this study was to explore the VBNC state of cyanobacteria-lysing bacteria and the conditions that regulate their VBNC state transformation. Three cyanobacteria-lysing heterotrophic bacterial strains (F1, F2 and F3) were isolated with liquid infection method from a lake that has experienced a cyanobacterial bloom. According to their morphological, physiological and biochemical characteristics and results of 16SrDNA sequence analysis, F1, F2 and F3 were identified as strains of *Staphylococcus* sp., *Stappia* sp. and *Microbacterium* sp., respectively. After being co-cultured with the axenic cyanobacterium, *Microcystis aeruginosa* 905, for 7 days, strains F1, F2 and F3 exhibited an inhibition effect on cyanobacterial growth, which was expressed as a reduction in chlorophyll concentration of 96.0%, 94.9% and 84.8%, respectively. Both autoclaved and filtered bacterial cultures still showed lytic effects on cyanobacterial cells while centrifuged pellets were less efficient than other fractions. This indicated that lytic factors were extracelluar and heat-resistant. The environmental conditions that could induce the VBNC state of strain F1 were also studied. Under low temperature (4°C), distilled deionized water (DDW) induced almost 100% of F1 cells to the VBNC state after 6 days while different salinities (1%, 3% and 5% of NaCl solution) and lake water required 18 days. A solution of the cyanobacterial toxin microcystin-LR (MC-LR) crude extract also induced F1 to the VBNC state, and the effect was stronger than DDW. Even the lowest MC-LR concentration (10 μg L^{-1}) could induce 69.7% of F1 cells into VBNC state after 24 h. On the other hand, addition of *Microcystis aeruginosa* cells caused resuscitation of VBNC state F1 cells within 1 day, expressed as an increase of viable cell number and a decrease of VBNC ratio. Both VBNC state and culturable state F1 cells showed lytic effects on cyanobacteria, with their VBNC ratio varying during co-culturing with cyanobacteria. The findings indicated that VBNC state transformation of cyanobacteria-lysing bacteria could be regulated by cyanobacterial cells or their toxin, and the transformation may play an important role in cyanobacterial termination.

Introduction

Eutrophication of fresh water bodies can cause the overgrowth of cyanobacteria in surface waters, a process that is called cyanobacterial bloom formation [30]. Cyanobacterial blooms not only reduce the recreational value of water bodies, but also threaten public health as several kinds of toxins can be released by some cyanobacteria. Many environmental factors have been reported to impact on the occurrence of cyanobacterial blooms [30], but currently there is no single method to efficiently prevent and control the blooms.

H. Chen · L. Fu · L. Luo · J. Lu · Z. Hu (✉)
Shenzhen Key Laboratory of Marine Bioresource
and Eco-environmental Science, College of Life Science,
Shenzhen University,
Shenzhen 518060, China
e-mail: huzl@szu.edu.cn

J. Lu · W. L. White
Department of Interdisciplinary Studies,
Faculty of Health and Envinronmental Sciences,
Auckland University of Technology,
Auckland, New Zealand

Most studies have focused on the factors that trigger the onset of a cyanobacterial bloom; few have attempted to elucidate the mechanism of bloom decline. The bloom termination process normally takes only 1 or 2 days, and it is difficult to imagine environmental factors changing markedly over such a short time. A reasonable speculation is that a minor change in some environmental factor(s) after the bloom formation upsets a delicate balance between the bloom and the environment. Exceeding this "tipping point" starts a process that quickly reduces cyanobacterial cell density. This process could be induced by biological factors, such as lytic bacteria.

The lytic activity of various bacteria to cyanobacteria has been reported for decades [9, 10]. More recent studies have shown that population dynamics of these lytic bacteria is closely related with cyanobacterial density (increasing during a bloom and peaking after bloom disappearance) [12, 17, 18, 26]. This suggests that lytic bacteria may play an important role in the termination of cyanobacterial blooms and could be used as a biocontrol agent [16, 17, 26]. However, it remains unclear how lytic bacterial cell numbers change so quickly during the bloom termination. We suspect that a transformation of dormant and active states of lytic bacterial cells occurred during this process.

The phenomenon of viable but nonculturable (VBNC) bacteria refers to a special physiological state that some nonendospore forming bacteria enter under stressful conditions, wherein they cannot regenerate and form a colony on standard laboratory media but still maintain some biochemical activity [21]. When transferred to a favorable environment, they can resuscitate into culturable state and form colonies on growth media [4, 7]. Several environmental factors have been associated with the VBNC state, including low temperature, starvation, salinity, visible and UV light and air pressure [22]. Due to the low concentration of nutrients in freshwater, most bacteria are starving and it is estimated that over 90% of freshwater bacteria are VBNC state [27]. Low temperatures (e.g. 4°C), starvation and salinity are the most common conditions used in the laboratory to induce the VBNC state bacteria [1, 30]. Although VBNC state is common in all groups of aquatic bacteria, most studies have focused on human pathogens, including *Escherichia coli*, *Legionella pneumophila*, and species of *Vibrio* and *Salmonella* [22]. Very few studies have reported the VBNC state of bacteria in relation to cyanobacteria, with the exception of the finding that cyanobacteria can serve as a long-term reservoir for VBNC state pathogens [19, 20].

In this study, we collected surface water from a natural water body that has experienced cyanobacterial blooms and used liquid infection method to isolate bacterial strains that could lyse cyanobacterium *M. aeruginosa*. Cells of these bacterial strains were then successfully induced into VBNC state under laboratory conditions. The cyanobacteria-lysing effects of culturable and VBNC state bacterial cells were also compared. We found that the lytic effect of VBNC state bacterial cells were lower than that of culturable cells and the existence of host cyanobacterial cells could affect the VBNC state transformation of lytic bacteria.

Methods

Isolation and Identification of Cyanobacteria-Lysing Bacteria in Lake Water

Water sample (15 cm below the surface) from Wenshan Lake on the campus of Shenzhen University was used as the source of lytic bacteria. The shallow Wenshan Lake (mean depth 1.5 m) experiences cyanobacterial (mainly *M. aeruginosa*) blooms frequently during the summer months. Surface water samples were collected with sterile sample bottles after the peak of a cyanobacterial bloom.

For lytic bacteria isolation, 200 mL water sample was first filtered through a 0.45 μm membrane filter to remove cyanobacterial cells, and then passed through a 0.22 μm membrane filter to retain lytic bacteria on the filter. The 0.22 μm filters were placed in 50 mL log phase *M. aeruginosa* 905 culture (Institute of Hydrobiology, Chinese Academy of Sciences) and co-cultured in an incubator for a week to check the lytic activity. The temperature in the incubator was set to $26 \pm 2°C$, with fluorescent illumination at 20.3–27.1 μmol m^{-2} s^{-1} under 14L:10D cycle. Decolorized groups (compared with the axenic control culture) were chosen for lytic bacteria isolation. Bacteria–cyanobacteria mixed cultures were then diluted, streaked on LB agar plates and incubated for 24–48 h at 37°C. Single colonies were isolated and purified three times by repeated streaking and co-cultured with *M. aeruginosa* to confirm the lysing effect.

Several identification and characterization tests were performed on the lytic bacterial isolates, including colony morphology, Gram stain, biochemical properties and antibiotic sensitivities. Detailed methods can be found in Shen et al. [28].

After isolation, genomic DNA of each lytic bacterial strain was extracted with a Wizard genomic DNA purification kit (Promega, Inc.) prior to polymerase chain reaction (PCR) amplification of the bacterial 16SrDNA gene and sequencing. PCR analysis was performed with a total volume of 50 μL containing 50–100 ng template DNA, 25 μL PCR Premix, 0.5 μL each of forward and reverse primers (16SrDNA Bacterial Identification PCR Kit, Takara Co., Shanghai) and 16S-free H$_2$O. Reactions were performed in a PTC-100 Thermo Cycler (Bio-Rad Laboratories, Inc.) with denaturation at 94°C for 5 min, followed by

94°C for 1 min, 50–55°C for 1 min, 72°C for 1.5 min for 30 cycles, followed by final elongation at 72°C for 5 min. The PCR products (1,500–2,000 bp) were sent to TaKaRa Co. (Shanghai, China) for sequencing with Ladderman™ Sequencing primers RV-M/M13-47 and compared with the sequences available from the GenBank nucleotide sequence databases (http://www.ncbi.nlm.nih.gov/blast/blast.cgi) using the BLAST search system.

Lytic Activity of Bacterial Isolates against *M. aeruginosa*

In order to compare the lytic activity among bacterial isolates and different fractions of culture, isolated lytic bacterial strains grown in liquid LB medium were treated under four different conditions before adding to cyanobacterial cultures:

(1) Untreated;
(2) Autoclaved at 121°C, for 20 min;
(3) Filtered through a 0.22 μm sterile syringe filter and filtrate retained; or
(4) Centrifuged at 8,228×g for 4 min, pellet rinsed with distilled deionized water (DDW) and retained.

For each group, 5 mL log phase bacterial cultures (10^8 CFU mL^{-1}) after treatments were added to 45 mL axenic *M. aeruginosa* 905 culture and incubated for 1 week. This was to ensure enough predator–prey ratio to be reached for lysis to process. Based on our preliminary experiments (unpublished data), we set the ratio to 40:1 (bacteria:cyanobacteria). Cyanobacterial culture with no bacterial culture acted as controls. Each treatment was sampled every 24 h by aseptically removing 1 mL of broth. The chlorophyll concentration was measured with a Water-Pam Chlorophyll Fluorometer (Heinz Walz GmbH Co., Germany). The lytic activity of bacteria was expressed as the chlorophyll change rate (R) calculated as below:

$$R(\%) = (C_0 - Ce)/C_0 \times 100. \tag{1}$$

As C_0 (mg L^{-1}) is the chlorophyll concentration of control cyanobacterial culture, Ce (mg L^{-1}) is the chlorophyll concentration of treatment culture.

VBNC State Transformation of Lytic Bacteria and their Lytic Activity

We tested the ability of isolated lytic bacteria to be induced into VBNC state under a range of conditions. These conditions included low temperature and NaCl concentration that have been commonly used to induce VBNC state bacteria, and also different concentrations of microcystin-LR (MC-LR), a toxin produced by *M. aeruginosa*.

Part 1: Induce Culturable Lytic Bacteria into VBNC with Different Solutions (DDW, Lake Water, NaCl and MC-LR Solutions) under 4°C

Nine treatments were tested to induce lytic bacteria into a VBNC state at 4°C. Treatment solutions were prepared as follows. For the DDW treatment (negative control), 100 mL of DDW was autoclaved at 121°C for 20 min. For the bacteria-free lake water treatment, 100 mL of Wenshan Lake water was passed through 0.22 μm sterilized filters. Filtrate was streaked on LB agar plates to ensure all bacterial cells were removed by filtration. For the NaCl treatments, 100 mL of three NaCl solutions (1%, 3% and 5%, W/V) were autoclaved at 121°C for 20 min. Finally, 100 mL of four MC-LR solutions was prepared (10 μg L^{-1}, 20 μg L^{-1}, 50 μg L^{-1} and 100 μg L^{-1}) by serial dilution of a 100 mg L^{-1} stock solution made from a crude extract of *M. aeruginosa* 905 (purity greater than 95%). Stock solution aliquots were stored in 1.5 mL LEP tubes at −80°C.

Log phase bacterial culture in LB liquid medium (cell density 10^8 CFU mL^{-1}) was resuspended in 0.9% NaCl solution and centrifuged (8,228×g, 4 min) to collect bacterial cells. The pellet was rinsed with DDW three to four times to remove medium and then resuspended in DDW, added to the nine treatment solutions to a final concentration of 10^6–10^7 CFU mL^{-1}. Cells were incubated with treatment solutions for 24 days at 4°C (3 days for MC-LR solutions). Cell counts were performed every 3 days, with the exception of the groups with MC-LR solutions, which were counted every 12 h.

The number of total viable cells was determined with LIVE/DEAD baclight bacterial viability kit (Molecular Probes, Inc.). In brief, the lytic bacterial treatments above were mixed with two stains (SYTO 9 and propidium iodide) and examined with an Olympus IX-71 fluorescent microscope. SYTO 9 penetrates all bacterial membranes and stains the cells green, while propidium iodide only penetrates cells with damaged membranes. The combination of the two stains produces red fluorescing cells. Therefore, all cells fluoresce red + green and viable cells only fluoresce green.

The number of culturable cells was assessed by the standard plate count method. Serially diluted bacterial cultures were streaked on LB agar plates and grown at 37°C for 24 h. The culturable cell concentration was calculated from the number of colonies and the dilution and expressed as CFU (colony-forming units) per mL.

The VBNC concentration and ratio of lytic bacterial cultures were calculated from the equations:

$$VBNC = V - C \tag{2}$$

$$VBNC \ ratio \ (\%) = (VBNC/V) \times 100 \tag{3}$$

where V is the cell density (cell mL^{-1}) of viable cells determined under a microscope and C (CFU mL^{-1}) is the concentration of culturable cells measured with plate counting method.

Part 2: Stability of VBNC State Lytic Bacteria under Different Conditions

A characteristic of VBNC state cells is their ability to resuscitate into culturable state. Researchers have reported resuscitation of VBNC bacteria with temperature increase, nutrient addition or growth with host cells [22]. These conditions were also tested on VBNC lytic bacterial cells isolated from Wenshan Lake.

VBNC state cells were induced by growing medium-free lytic bacterial cells in DDW at 4°C for 6 days. The viable cell and culturable cell numbers were checked (see above) to ensure that the VBNC ratio was close to 100%. Three groups were set up to grow VBNC state bacterial cells in incubator with:

(1) Log phase axenic *M. aeruginosa* 905 culture (bacteria: cyanobacteria cell ratio of 40:1);
(2) BG-11 medium; or
(3) DDW.

The volume of the VBNC bacterial solution was calculated to provide enough bacterial cells for 45 mL cyanobacterial culture with the ratio of 40:1. This volume of VBNC bacterial solution was added to 45 mL treating solution (cyanobacterial culture, BG-11, or DDW) and grown for 3 days. Samples were taken everyday to check the total viable and culturable bacterial cell number.

Part 3: Lytic Activity of VBNC State Lytic Bacteria against *M. aeruginosa*

Both VBNC state and culturable state lytic bacterial cells were added into *M. aeruginosa* culture to compare their lytic activity on cyanobacterial cells and to check the VBNC state transformation.

Culturable state lytic bacterial cells were obtained by centrifuging log phase lytic bacteria and resuspending in DDW to remove growth medium, as described above. VBNC state cells were induced by growing medium-free lytic bacterial cells in DDW at 4°C for 6 days. Culturable or VBNC state lytic bacterial cells (DDW) were added into 45 mL log phase axenic *M. aeruginosa* 905 culture to obtain a bacteria:cyanobacteria cell ratio of 40:1. The control group was a pure *M. aeruginosa* 905 culture without bacterial cells. The control group and two experimental groups were grown for 12 days at 26±2°C with fluorescent illumination (20.3–27.1 μmol m^{-2} s^{-1}, 14L:10D cycle) and sampled every 3 days to check the chlorophyll concentration and the total viable and culturable bacterial cell number.

Statistics

For all the experiments above, unless mentioned, means and standard deviation (SD) are presented for triplicates. Paired *t*-test analyses and one-way analysis of variance (ANOVA) were performed to compare results between two groups or among several groups. Differences were considered significant if $P<0.05$. Statistical analyses were conducted with SPSS 13.0 for Windows.

Results

Isolation and Classification of Cyanobacteria Lytic Bacteria

Three bacterial strains that could lyse cyanobacterial cells were isolated from lake water and named F1, F2 and F3. Co-cultures of the three strains of lytic bacteria and cyanobacteria turned yellowish and dead cell precipitates were visible on the flask within 5 days. On LB agar plates, colonies for all three strains appear round and convex with smooth edges. More morphological and biochemical characteristics of the three bacterial strains are listed in Table 1. The generation times (doubling time) for F1, F2 and F3 on LB medium were 0.62 h, 0.74 h and 0.79 h, respectively. The 16SrDNA sequencing results suggests that F1 is *Staphylococcus* sp. in the class *Bacillales*, F2 is putatively *Stappia* sp. in the class *Alphaproteobacteria* while F3 is *Microbacterium* sp. in the class *Actinobacteridae* (Table 2).

Lytic Activity of Bacterial Isolates against *M. aeruginosa*

Cultures of the three lytic bacterial strains were treated with several methods (filtered, autoclaved or centrifuged) before co-culturing with cyanobacteria. After 7 days, the control culture and the culture amended with centrifuged bacterial culture remained dark green, while the groups added with whole, autoclaved and filtered bacterial culture had become decolorized. Compared with the control, all the treatments showed lytic effect with decreased chlorophyll concentrations ($P<0.001$) (Fig. 1). The whole culture (group A) showed the strongest lytic effect with the R values as high as 96.0±0.04%, 94.9±0.13% and 84.9±0.27% for F1, F2 and F3, respectively; significantly higher than groups B, C and D ($P<0.02$) (Fig. 1). Strong lytic effects of autoclaved and filtered bacterial cultures (groups B and C) indicated that most lytic substances were in the culture medium and stable under high temperatures. There were no significant differences of R values among the three strains.

Table 1 Morphology and biochemical characteristics of isolated algae-lysing strains. +++, resistant to antibiotic up to 200 μg mL^{-1}; ++, resistant to antibiotic up to 100 μg mL^{-1}; +, resistant to antibiotic up to 50 μg mL^{-1}; −, sensitive to antibiotic

	F1	F2	F3
Colony morphology	Round and convex, cream colored	Round and convex, transparent light yellow	Round and convex, cream colored in early growth stages and turn orange later
Colony size (diameter)	1–2 mm	2–3 mm	2–4 mm
Cell morphology	Cocci	Bacilli	Bacilli
Gram's stain	G$^+$	G$^+$	G$^-$
Endospore production	No	Yes	No
Exoenzyme	Lipase,urease, gelatinase	Urease, gelatinase	Urease, amylase
Glucose digestion	Yes	Yes	Yes
Ampicillin	−	+++	−
Kanamacin	+++	++	+
Gentamicin	−	++	−
Chloromycetin	+	−	−

Induction and Resuscitation of VBNC State Lytic Bacteria

F1 was chosen to perform the VBNC experiments as it showed the strongest lytic effect and could not produce endospores. Under 4°C, DDW could induce 96.2% F1 cells into VBNC state within 3 days without a significant change of total viable cells (Fig. 2). For cultures treated with lake water or three NaCl concentrations, all of them showed an increase in the VBNC ratio over time, with a marked decrease of total viable cells (Fig. 2). The 1% NaCl treatment was more effective than other solutions ($P<0.05$), although still slower than DDW treatment (Fig. 2).

Compared with DDW, lake water and NaCl solutions, MC-LR solutions were the most effective reagents to induce VBNC state F1 cells. All of the four tested MC-LR solutions produced a VBNC ratio higher than 70% within 36 h (Fig. 3). The group treated with the lowest concentration (10 μg L^{-1}) showed a lower VBNC ratio than other groups ($P<0.05$), but still reached 89.4% by 72 h (Fig. 3). No significant difference was found among the total viable cell number in groups treated with different MC-LR solutions.

The VBNC state F1 cells induced by DDW and low temperature (4°C) were put into an incubator (26±2°C) with the addition of M. aeruginosa, or BG-11 medium, or DDW to test their ability to resuscitate. Treatment with M. aeruginosa showed an increase of total viable number and decreased VBNC ratio on day 1, which was not shown in treatments of BG-11 or DDW (Fig. 4). During the tested 3 days, the group with M. aeruginosa showed higher viable number than those added with BG-11($P=0.002$) or DDW ($P<0.001$), although the VBNC ratio was similar within all the three treatments ($P=0.243$) (Fig. 4). Resuscitation occurred in all the groups (potentially caused by temperature increase), but only M. aeruginosa produced significant regeneration.

The co-culture experiment showed that both VBNC state and culturable state F1 cells lysed M. aeruginosa cells, as shown in lower chlorophyll concentration compared with control ($P=0.007$) (Fig. 5). However, total viable cell number was higher in the group with VBNC F1 than the group with culturable F1 ($P<0.001$) (Fig. 6). With different initial VBNC ratios, both groups maintained the VBNC

Table 2 Genetic identification of cyanobacteria-lysing bacterial strains isolated from Wenshan Lake

Strain no.	Accession no.	Closest matching organism in Genbank	Closest matching accession no.	Sequence rDNA similarity (%)	Sequence length (bp)
F1	JF899874	Firmicutes Bacillales Staphylococcus sp. R-25050	AM084016.1	100%	1,532
F2	JF899875	Proteobacteria Alphaproteobacteria Rhodobacteraceae Stappia sp.	EU726271.1	99%	1,448
F3	JF899876	Actinobacteria Actinobacteridae Microbacteriaceae Microbacterium sp.	AB167383.1	99%	1,445

Figure 1 Change of chlorophyll concentration (mg L^{-1}) in cyanobacterial cultures 7 days after adding different portions of bacterial cultures. *Control*: no bacteria; **a**: whole culture added; **b**: autoclaved culture added; **c**: filtered culture added; **d**: centrifuged culture added. *Error bars* represent the standard deviation of the mean of triplicates
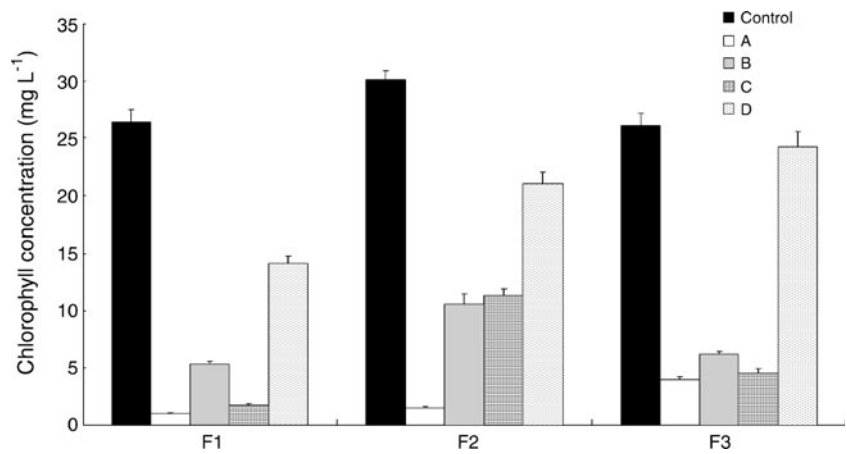
level between day 3 to day 9, and then increased to 79.3% (VBNC treatment) and 87.4% (culturable treatment) on day 12 (Fig. 6).

Discussion

In this study, three strains of lytic bacteria were isolated from a lake that has experienced cyanobacterial (*M. aeruginosa*) blooms. Strains F1, F2 and F3 were identified as *Staphylococcus* sp., with the closest record *Staphylococcus* R-25050 (Phylum Firmicutes), *Stappia* sp. (Phylum Proteobacteria) and *Microbacterium* sp. (Phylum Actinobacteria), respec-

tively. Members of the genus *Staphylococcus* have been reported to produce algal lytic chemicals [6, 25], but no study has previously reported algal lytic activity by *Stappia* or *Microbacterium*. On the contrary, some strains in *Microbacterium* enhanced the growth of *Microcystis* in a study of European water bodies [5]. All the three strains showed lytic effect on *M. aeruginosa* 905, although they need a minimum cell density of 10^8 mL^{-1} and a predator–prey ratio of 40:1 to lyse the cyanobacterial cells (unpublished data), not as potent as some lytic bacteria reported before [14]. Moreover, they may not be the main cyanobacteria-lysing bacteria in the field for two reasons. First, only strains that could form colonies on LB agar plates were isolated and purified.

Figure 2 The total viable cell number (*top*) and VBNC ratio (%) (*bottom*) of cyanobacteria-lysing bacteria F1 culture under different stresses
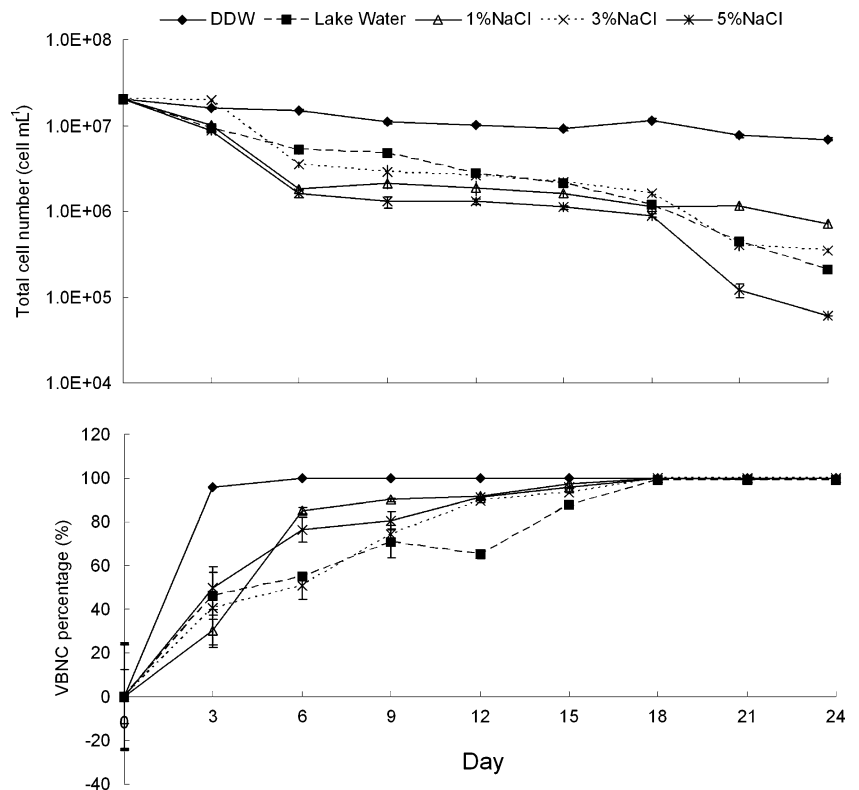
Figure 3 The total viable cell number (*top*) and VBNC ratio (*bottom*) of F1 cells under different concentrations of microcystin-LR incubated at 4°C. Control is cultures treated with DDW
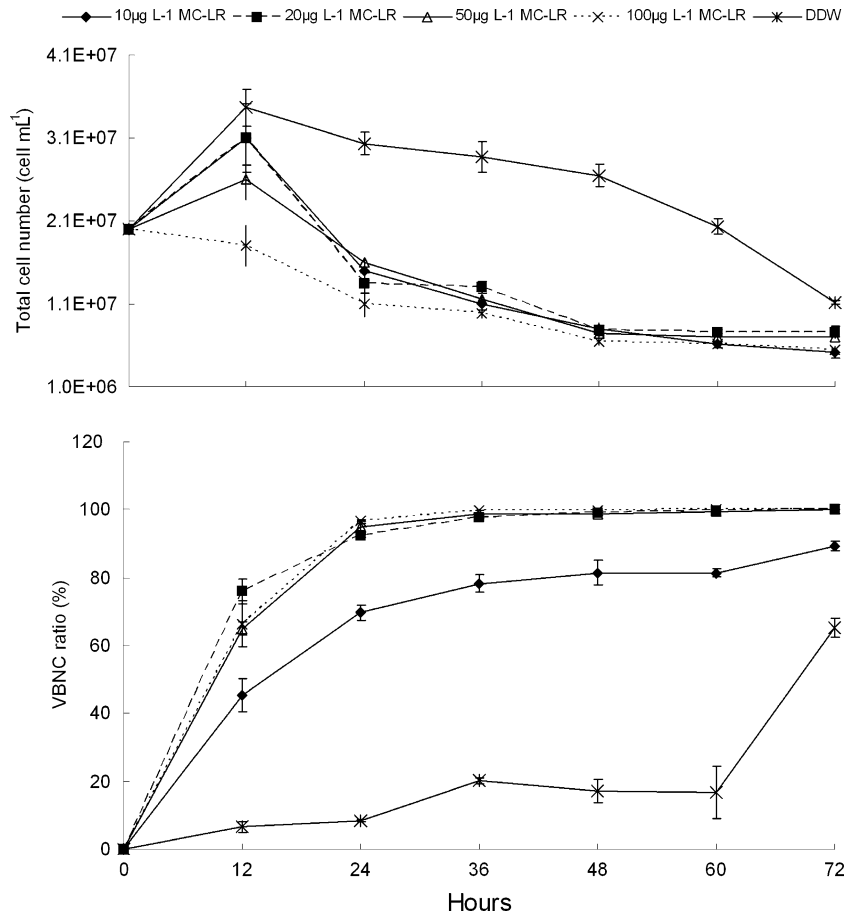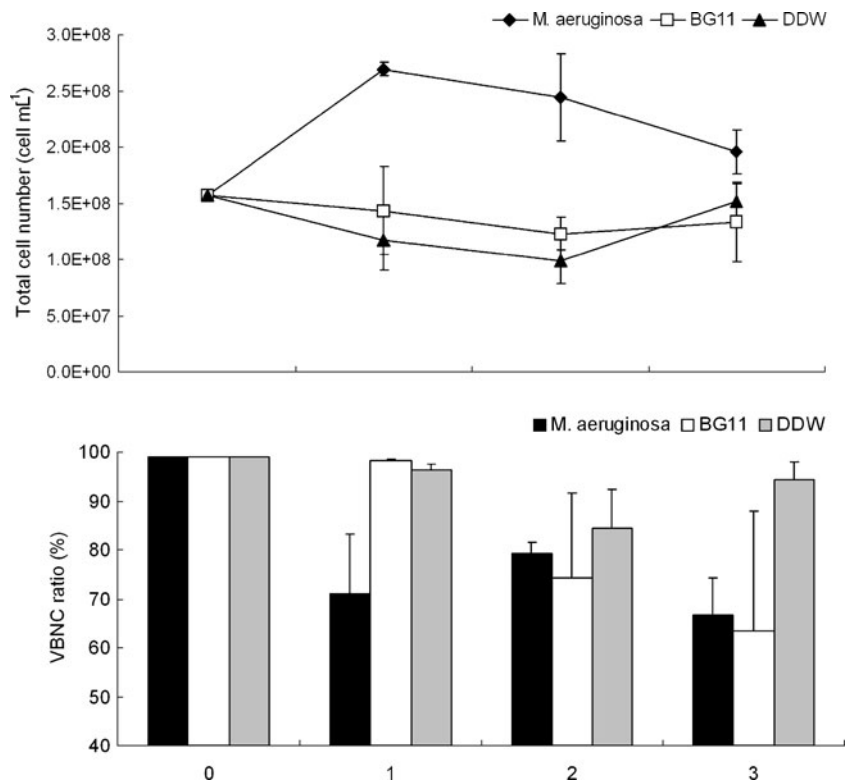

Figure 4 The total viable cell number (*top*) and VBNC ratio (*bottom*) of VBNC state F1 cells treated with different solutions at 26°C
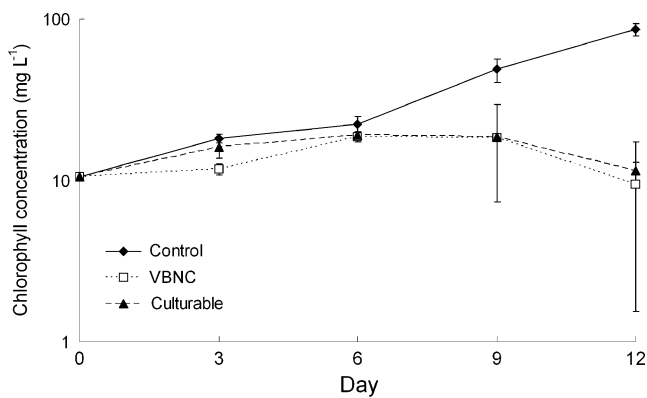
Figure 5 Change of chlorophyll concentration in *M. aeruginosa* cultures treated with VBNC or culturable state cyanobacteria-lysing bacterium F1 cells

Second, the cyanobacterium *M. aeruginosa* 905 was an axenic strain isolated from another freshwater lake. However, these strains provided us a good chance to study their lysing mechanism and VBNC transformation when co-cultured with cyanobacteria.

In contrast to our results that showed reduced lytic activity in autoclaved cultures, some researchers have reported an increase of lytic activity after heat treatment, which they attributed to heat-induced rupturing of bacterial cell walls and release of lytic substances into the medium [29]. However, in a field study in a Canadian lake [26], no diffusible lytic substances from lytic bacteria were found and electron micrographs showed that direct contact was necessary for bacteria to lyse cyanobacteria. Various lysing mechanisms could exist for different bacteria, including soluble secreted factors or direct cell to cell contact. Even for a single bacterial strain, the lysing activity could be changed at different growth phases or with changes in environmental conditions.

With the addition of DDW, most F1 cells could be induced into VBNC state under low temperature after 3 days, faster than those added with different NaCl solutions. However, the MC-LR solution induced F1 cells into VBNC state even faster indicating that for bacterium F1 isolated from a subtropical freshwater lake, MC-LR is a better inducer than starving or NaCl solution. It is still uncertain how MC-LR could affect the VBNC state of bacteria. Degradation of MC-LR by some bacteria has been reported before [8], while reports on the allelopathic effect of MC-LR on bacteria are scarce. As the MC-LR solution used in this study was crude extract of *M. aeruginosa* 905 cells, there is the possibility that other chemicals in the extract caused inhibitive effect on F1 cells and induced the VBNC state. This could also explain why there was no linear relationship between MC-LR dosage and number of F1 cells entering a VBNC state. Interestingly, our preliminary study showed a relationship between the toxin MC-LR with the VBNC state bacterial dynamics in natural environment [15] and the existence of crude MC extracts encourage VBNC state *Aeromonas sobria* back into the culturable state [24]. More work need to be carried out to elucidate the exact effect of MC-LR on bacterial cells.

Several environmental conditions were tested to resuscitate VBNC state F1 cells. Host cyanobacterial cells could resuscitate the most cells (Fig. 4), which is consistent with resuscitation experiments carried out on pathogens [22]. Resuscitated VBNC state F1 cells regained their lytic activity on cyanobacteria but were less efficient than culturable cells as shown on the similar chlorophyll concentration (Fig. 5) and higher total viable cell number (Fig. 6). However, host cyanobacterial cells not only resuscitate VBNC state F1 cells, but also induce culturable F1 cells into VBNC state: a VBNC ratio of 60% seemed to be the most stable state for co-cultured *M. aeruginosa* 905 and F1. This VBNC ratio of 60% (equal to 40% culturable) was comparable with the ratio of total and active bacteria in water bodies with similar eutrophic level, such as 28–34% in Lake Taihu, China [11]. Thus, we propose that in the natural environment, there is a

Figure 6 Change of total viable cell number (*bar graph*) and VBNC ratio (*line graph*) in cyanobacteria-lysing bacterium F1 cells grown with *M. aeruginosa*. Black bar and continuous line: culturable F1 cells; grey bar and dashed line: VBNC state F1 cells
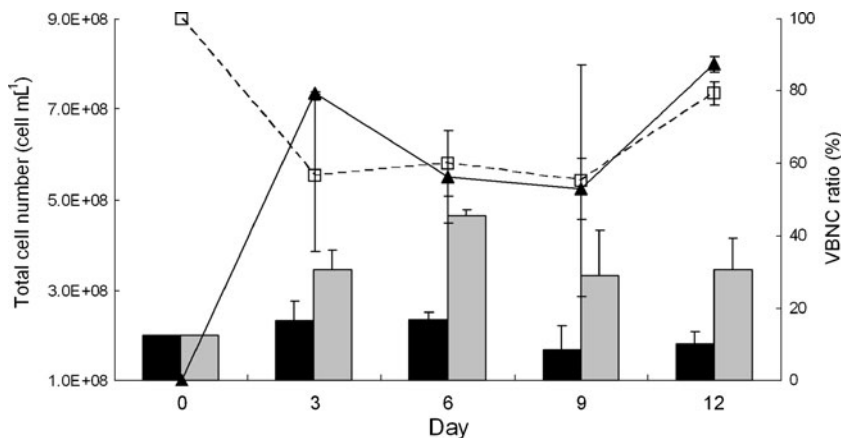
balance between cyanobacteria and lytic bacteria partly in VBNC state throughout the cyanobacterial bloom, until environmental factors resuscitate more VBNC bacteria and cause the lysis and decline of the cyanobacterial bloom. The environmental factors that could trigger the resuscitation, include temperature, nutrient concentrations, salinity and UV light intensity, all of which have been proven to affect bacterial VBNC transformation in the lab [1, 2, 13, 23]. In addition, cyanobacterial toxin and other chemicals released by cyanobacterial cells could play a role as shown in this study.

The actual situation in the natural environment is probably far more complicated than speculated above. For example, a study carried out on the toxic dinoflagellate *Ostreopsis lenticularis* showed that thermal stress could regulate the spectrum of culturable and nonculturable symbiotic bacterial species, which consequently affected the toxin production [3]. When researching the environment–bacteria–cyanobacteria relationships, cause and effect is hard to determine, and the complexity of bacterial communities should be considered.

Overall, our data showed transformation of VBNC state cyanobacteria-lysing bacterium F1 when grown with the host cyanobacteria. Further work is required both to exploit the potential application of this finding in bloom control and to assess how this phenomenon is widespread among other cyanobacteria-lysing bacteria.

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