Detection of *Microcystis* in lake sediment using molecular genetic techniques

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Summary

Microcystis, which are toxic microcystin-producing cyanobacteria, normally bloom in summer and drop in numbers during the winter season in Senba Lake, Japan. Recently, this lake has been treated by ultrasonic radiation and jet circulation which were integrated with flushing with river water. This treatment was most likely sufficient for the destruction of cyanobacterial gas vacuoles. In order to confirm whether *Microcystis viridis* was still present, a molecular genetic monitoring technique on the basis of DNA direct extraction from the sediment was applied. Three primer sets were used for polymerase chain reaction (PCR) based on rRNA intergenic spacer analysis (RISA), the DNA dependent RNA polymerase (*rpo*C1) and a *Microcystis* sp.-specific *rpo*C1 fragment. The results from each primer were demonstrated on the basis of single strand conformation polymorphisms (SSCP). Using the RISA primer showed different results from the *rpo*C1 and *Microcystis* sp.-specific *rpo*C1 fragment; meanwhile, the *rpo*C1 *Microcystis* sp.-specific fragment was more specific than the RISA primer. Therefore, the *Microcystis* sp.-specific *rpo*C1 fragment was further analysed by denaturing gradient gel electrophoresis (DGGE). The DNA pattern representing *M. viridis* could not be detected in any of the sediment samples. However, the results were confirmed with another technique, terminal restriction fragment length polymorphisms (T-RFLP). Although T-RFLP patterns of 16S rDNA in sediment at 91 bp and 477 bp lengths were matched with the T-RFLP of *M. viridis* (*Hha*I and *Msp*I endonuclease digestion, respectively), the T-RFLP pattern of 75 bp length was not matched with *M. viridis* (both of HhaI and *Msp*I endonuclease digestion) which were the major T-RFLP pattern of *M. viridis*. Therefore, the results most likely indicated that *M. viridis* seems to have disappeared because of the addition of the ultrasonic radiation and jet circulation to the flushing treatment.

Introduction

Microcystis spp., cyanobacteria that frequently occur as noxious blooms in eutrophic freshwater, are of major concern because many strains produce cyclic heptapeptide toxins called microcystins (Carmicheal 1994). The microcystins are secondary metabolites, which are hazardous to humans, live-stock and wildlife. Moreover, water blooms dominated by cyanobacteria (especially *Microcystis*), damage the natural scenery of the water as in Lake Senba. Lake Senba is a small recreational lake in the city of Mito, Japan, which many people visit for relaxation. This lake has two seasons for *Microcystis*, the blooming season (BS) from May to October and the non-blooming season (NS) from November to April. Thus there was increasing pressure from Mito citizens for drastic measures to control these blooms. The municipal government of Mito initially used flushing of the lake with river water for water bloom control. However, the flushing rate was not sufficient because of the limitation of available river water. Therefore, ultrasonic radiation and water jet circulation were integrated with the existing flushing process (Nakano *et al.* 2001). Ultrasonic radiation of 3 s was sufficient for the destruction of gas vacuoles and the buoyant ability of cyanobacteria to find optimum levels of illumination in the water column. Damage was also inflicted on the photosynthetic machinery. Consequently, the damage would delay growth recovery and slow the growth rate (Fogg *et al.* 1973). Therefore, the washout of sonicated cyanobacteria with a slower growth rate would be possible at the existing flushing rate, and water bloom occurrence would be controlled despite eutrophic lake conditions. The performance of the integrated treatment system was evaluated by monitoring the water and sediment quality of the lake for 2 years. The results

showed that the water quality was improved such as for chemical oxygen demand (COD) and phosphorus, and the operation of an ultrasonic irradiation system might reduce floating cyanobacteria; therefore, a higher transparency per chlorophyll *a* ratio was observed during the blooming season. Although the changes in the sediment quality were monitored at different distances from the ultrasonic radiation system, it is not yet clear whether the cyanobacteria which have lost their buoyancy ability by this method will accumulate on the lake bottom or not.

Therefore, this research aims to confirm whether there is a persistence of *Microcystis* in the lake sediment after treatment with the integrated system on the basis of conventional enumeration and molecular genetic techniques. This research was started by using rRNA intergenic spacer analysis (RISA) (Borneman & Triplett 1997) and *rpo*C1 (Wilson *et al.* 2000) as gene primers which were amplified by PCR. Then a specific primer was designed based on *rpo*C1, the DNA-dependent RNA polymerase gene. In this study, microcystin synthetase gene *mcyB* analysis was also conducted for the detection. In addition, both DGGE and T-RFLP techniques with PCR-amplified gene fragment coding for the specific primer base were applied as a detection system for the persistence of *Microcystis* in the sediment.

Materials and methods

Cyanobacterial strains and culture condition

Nostoc linckia was obtained from the Tsukuba Algal Collections, National Science Museum, Tsukuba, Japan. *Microcystis viridis*, *M. aeruginosa* and *Phormidium* sp. were previously isolated from Lake Senba, Mito, Japan. *Anabaena* sp., *Hapalosiphon* sp. DASH 05101 and *Scytonema* sp. were obtained from the Department of Soil Science, Faculty of Agriculture, Chiangmai University, Thailand.

M. viridis, *M. aeruginosa* and *Phormidium* sp. were grown in MA medium (Ichimura 1979), whereas *N. linckia*, *Anabaena* sp., *Hapalosiphon* sp. DASH 05101 and *Scytonema* sp. were cultured in BG11 medium (Richmond 1986) under continuous aeration at 25 ± 1 °C with a 12 h/12 h light/dark cycle with an average light irradiance of 400 μE/s/m^2 for 3–4 weeks.

Sampling and DNA extraction

The samples of sediment from Lake Senba were collected during 1999–2000. The lake has a surface area of 33 ha and a liquid volume of 365,000 m^3. It is a very shallow lake, with a mean depth of 1.0 m, and the study site is depicted in Figure 1. The sample collection was conducted during both blooming season (BS) from May to October and the non-blooming season (NS) from November to April.

DNA direct extraction from the sediment samples was developed in this study. One gram dry weight of sediment sample was washed in 0.1 M phosphate buffer (pH 7.0). After centrifugation, the pellet was resuspended with lysozyme solution and incubated at 37 °C for 30 min. The aliquot was added with 1 ml of 10% (w/v) SDS and incubated at 50 °C for 30 min. Then 20 μl of proteinase-K solution (20 mg/ml) was applied in the sample before being incubated at 65 °C for 1 h. The mixture, after the addition of 1 ml of 0.2 M NaOH, was shaken at room temperature for 15 min. Three cycles of freezing in liquid nitrogen and thawing at 70 °C were carried out. An equal volume of water-saturated phenol solution was added and the phases were mixed by intermittent vortexing and then separated by centrifugation at 8500 × *g* for 10 min. The nucleic acid in aqueous phase was precipitated with 2 volumes of isopropanol at −20 °C, washed with 70% (v/v) ethanol, dried and dissolved in TE buffer containing 1/10 RNase A, and then incubated at 55 °C for 10 min and stored at 4 °C for further analysis.

Crude DNA from the sediment was purified by application through a MicroSpin Sephacryl S-300 column (Edgcomb *et al.* 1999) twice, before being applied onto 1% (w/v) agarose gel electrophoresis at 80 V for 2 h. The purified DNA was extracted from the agarose gel by using a Quantum Prep Freeze N Squeeze Spin Column and applied through a Sephacryl S-300 Microspin column again.

The DNA extraction method for cyanobacterial culture was followed by Teaumroong *et al.* (2002).

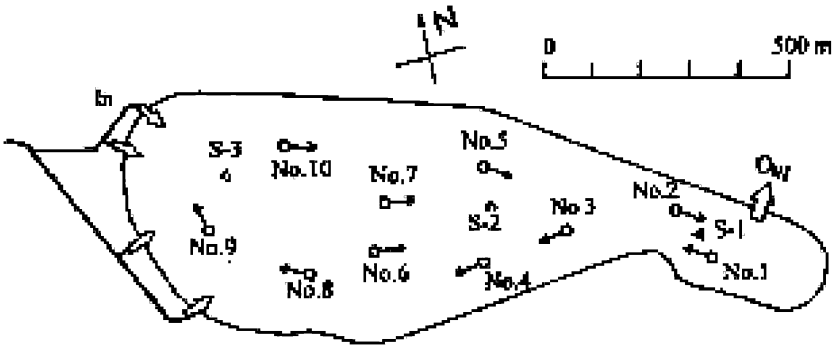

Figure 1. Sampling sites and the location of the ten USRS units arranged in Lake Senba, symbols: O; USRS, Δ; sampling sites. Arrows show the direction of the water jet current generated by USRS (Nakano *et al.* 2001).

PCR analysis

The DNA primers used were rRNA intergenic spacer analysis (RISA) (Borneman & Triplett 1997), *rpo*C1 (Wilson *et al*. 2000) and microcystin synthetase gene *(mcyB)* (Tiquia *et al*. 2002). The sequences of primers were summarised in Table 1.

The reactions were run in a Thermal cycler (GeneAmp®PCR System 9700, Applied Biosystems). The PCR was done in a 50 μl reaction mixture using 2.5 U of Taq polymerase (Promega, USA), the buffer supplied by Promega, 1.5 mM $MgCl_2$, 0.2 mM dNTPs and 500 nM (each) primer. For RISA, all reagents were combined and reaction was carried out in accordance with Borneman & Triplett's (1997) condition. For *rpo*C1, the mixture was run according to Wilson *et al*. (2000). For *mcy*B, the reactions were heated at 94 °C for 5 min, then PCR was performed with 35 cycles at 94 °C for 30 s, 60 °C for 60 s and 72 °C for 120 s, followed by elongation at 72 °C for 10 min. The results were detected on 1% agarose gel electrophoresis staining with 10 mg/ml of ethidium bromide.

Designed Microcystis *sp.-specific rpoC1 fragment*

The *rpoC*1 PCR product of *M. viridis* was sequenced directly by a DNA sequencer (Applied Biosystems, USA) and aligned with the amino acid sequence of the other cyanobacteria obtained from the GenBank database to design the specific primer. The specific primers used were MV1-f and MV1-r (Table 1). The PCR product was used in a final PCR with MV1-f and MV1-r to give a 419 bp. Fifty microlitre PCR mixture contained 10–50 ng of sediment DNA, 500 nM (each) primer, 200 μM dNTPs, 1.5 mM $MgCl_2$ and 2.5 U Taq polymerase (Promega, USA) in reaction buffer (20 mM Tris–HCl (pH 8.0), 100 mM KCl, 0.1 mM EDTA, 1 mM DTT, 50% glycerol, 0.5% Tween®20 and 0.5% Nonidet®-P40). The thermal-cycling conditions were run with one cycle at 95 °C for 3 min, then 35 cycles at 92 °C for 60 s, 45 °C for 60 s and 72 °C for 60 s, followed by elongation at 72 °C for 10 min. The results were detected on 1% (w/v) agarose gel and 6% polyacrylamide–Tris–borate–EDTA gel containing 7 M urea for the single strand conformation polymorphism (SSCP) technique.

Analysis of PCR product by SSCP

The PCR products were denatured by heating at 95 °C for 5 min. The DNA was applied with a loading buffer on to 6% polyacrylamide–Tris–borate–EDTA gel containing 7 M urea for the SSCP technique with silver staining. Small pieces of selected SSCP bands were punched from the gel. The PCR products of the cutouts were reamplified based on 16S rDNA by PCR (Table 1). Before being sequenced, the PCR products were purified with a QIAquick Spin PCR Purification kit (Qiagen, Germany). The purified PCR products were sequenced directly by using an ABI model 310 automated DNA sequencer (Applied Biosystems) and a BigDye terminator cycle sequencing kit (Applied Biosystems). All the sequences were compared with similar sequences of reference organisms by a BLAST search.

Analysis of PCR product by DGGE

The MV1-f primer which is specific for *M. viridis* contains at its 5′ end a 40-base GC clamp (Table 1) to

Table 1 Summary of primers used in this study

Gene region and Primers	Sequences	References
RISA		
1406F	5′-TGYACACACCGCCCGT-3′	Borneman & Triplett 1987
23SR	5′-GGGTTBCCCCATTCRG-3′	
rpoC1		
rpoc1-1	5′-GAGCTCYAWNACCATCCAYTCNGG-3′	Wilson *et al*. 2000
rpoc1-T	5′-GGTACCNAAYGGNSARRTNGTTGG-3′	
Microcystis sp. specific rpoC1 fragment		
MV1-f	5′-GATGGGAATAGCGAGACTAAAGCC-3′	This study
MV1-f-GC-clamp[a] (DGGE)	5′-GC –clamp-GATGGGAATAGCGAGACTAAAGCC-3′	
MV1-r	5′-AAGCTCCAAGAATCTTTAGGAGGA-3′	
mycB		
tox2$^+$	5′-AGGAACAAGTTGCACAGAATCCGCA-3′	Kaebernick *et al*. 2000
tox2$^-$	5′-ACTAATCCCTATCTAAACACAGTAACTCA-3′	
16rDNA		
27f	5′AGAGTTTGATCCTGGCTCAG-3′	Martin-Laurent et al. 2001
1392r	5′-ACGGGCGGTGTGTACA-3′	Amann *et al*. 1995

[a]GC –clamp : 5′(-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG

stabilise the melting behaviour of the DNA fragment. The MV1-r primer was used as a reverse primer (Table 1). The PCR was performed with a Thermal cycler (Applied Biosystems, USA). The PCR mixture contained 10–50 ng of genomic DNA of the bacterial isolates or 1 μl of DNA preparations from sediment samples, 500 nM of each primer, 800 μM dNTPs, 2.5 mM $MgCl_2$, 0.1% (v/v) BSA and a PCR buffer (Promega, USA). The sample was first incubated at 95 °C for 10 min to denature the DNA, then 5 U of Taq DNA polymerase (Promega, USA) was added. PCR conditions were 35 cycles at 92 °C for 120 s, 45 °C for 60 s and 72 °C for 120 s, followed by elongation at 72 °C for 10 min.

The PCR product obtained from the genomic DNA of pure culture and the extracted DNA from the sediment were used for separation in a denaturing gradient gel. 300 μl of the PCR product were pooled, precipitated and resuspended in 30 μl of TE buffer. Before loading on to the DGGE gel, the PCR products were incubated at 95 °C for 5 min and gradually cooled to 4 °C to avoid non-complementary annealing of DNA. Gels for DGGE were 6% polyacrylamide gel (6%-acrylamide and *N, N*-methylenebisacrylamide solution (37.5:1, v/v), 40% (v/v) formamide, 7 M urea and 1 × TAE) containing a linear gradient of the denaturant concentration ranging from 25% to 60%. The denaturing gradient gel was run for 300 min at 60 °C and 250V by the Dcode system (Bio-Rad, USA). After completion of electrophoresis, the gels were stained in an ethidium bromide solution (0.5 μg/ml) and documented on Gel documentation and analysis (Ultra Violet Product, USA).

Analysis of PCR product by T-RFLP

For the T-RFLP analysis, primer 27f end-labelled with 6-FAM (5-[6]-carboxyfluorescein, Operon Technology, Alameda, CA, USA) and unlabeled 1392r primers were used to an amplified extracted DNA sample from the samples of the sediment (Table 1). The PCR mixture contained 10–50 ng of genomic DNA of the bacterial isolates or 1 μl of DNA preparations from the sediment samples, 500 nM of each primer, 800 μM dNTPs, 2.5 mM $MgCl_2$, 0.1% BSA and PCR buffer (Promega, USA). The sample was first incubated at 94 °C for 3 min to denature the DNA, then 5 U of Taq DNA polymerase (Promega, USA) was added. PCR conditions were 35 cycles at 94 °C for 30 s, 59 °C for 15 s and 72 °C for 60 s, followed by elongation at 72 °C for 15 min. The PCR products were purified using a Qiaquick® PCR purification kit (Qiagen). The purified PCR products (10–50 ng) were digested with 1 U of the *Msp*I and *Hha*I restriction endonuclease (Promega, USA) for 1 h at 37 °C. The digested samples were analysed on an ABI PRISM™ 310 Genetic Analyzer (USA). The sizes of fragments were compared with internal standards and determined by the GeneScan software (Applied Biosystems, USA).

Results

PCR analyses

The cyanobacteria from the sediment were enumerated by the plate count method. These results suggested that the cyanobacterial population in the sediment from the blooming season was higher than the non-blooming season, about 360 cells (g dry weight of sediment)$^{-1}$ and 150 cells (g dry weight of sediment)$^{-1}$, respectively. Whereas the unicellular cell population in the blooming season sediment was lower than the non-blooming season sediment, about 120 cells (g dry weight of sediment)$^{-1}$ and 90 cells (g dry weight of sediment)$^{-1}$, respectively. The results indicated that the unicellular cyanobacteria might precipitate in the sediment during the non-blooming season. The unicellular cells showed a colony type in the *Synechocystis*-group, expected to be a *Microcystis* cluster. The cells are spherical to oval and vary from 3 to 8 μm in diameter; some cells occur singly or in pairs with light refractile gas vacuoles (data not shown) that are characteristic of a natural population of *Microcystis* (Stanier *et al.* 1971). The results from a conventional plate count were further confirmed using the molecular genetic techniques. The microbial community DNA from the sediment at Lake Senba in each season was directly extracted, and the yield was about 0.3 μg (g dry weight of sediment)$^{-1}$. Enzymatic amplification of rRNA intergenic spacer and *rpo*C1 was performed on DNA extracted from the winter, spring and summer sediment samples. Application of the RISA analysis in the PCR from the samples of the sediment and reference strains yielded multiple distinct DNA products ranging in size from approximately 400 to 1500 bp. In the case of the *rpo*C1 analysis, *Scytonema* sp. showed two band products with sizes about 650 and 900 bp. *N. linckia* showed four band products with sizes about 400, 650, 900 and 1400 bp. *M. aeruginosa* showed three band products with sizes about 400, 650 and 900 bp. *Phormidium* sp. showed a one band product with a size about 700 bp. *Hapalosiphon* sp. DASH 05101 showed two band products with sizes about 650 and 1400 bp, and *M. viridis* showed four band products with sizes about 500, 600, 800 and 1300 bp. However, the excised bands of sediment samples on 1% agarose gel electrophoresis could not be clearly compared with the reference strains even though they yielded distinct DNA products ranging in size from approximately 250 to 1500 bp (data not shown). Thus the PCR products were again distinguished on the basis of the single strand conformation polymorphism technique (SSCP). Four signal bands of the PCR product based on the rRNA intergenic spacer were similar with the band product of *M. aeruginosa* with sizes about 500, 650, 720 and 1000 bp (Figure 2). Those band products were sequenced and compared with the database in the GenBank. The results were not an identical correlation with *M. aeruginosa* (data not shown). In contrast, the RISA analyses were different from the *rpo*C1-PCR

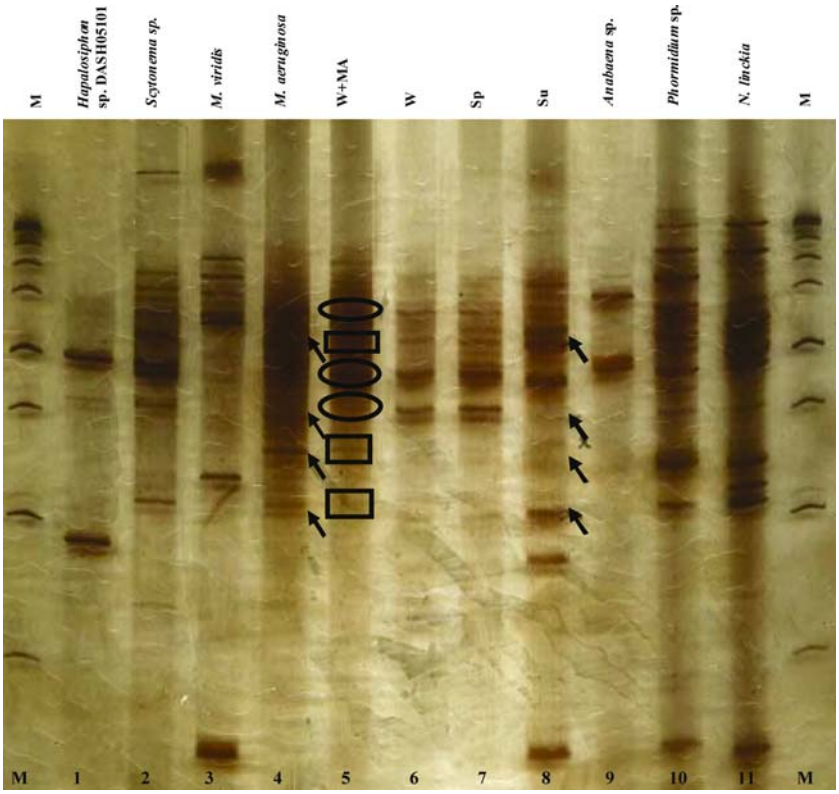

Figure 2. rRNA intergenic spacer pattern on SSCP from sediment samples (RISA). Lane M; 1 kb ladder marker, lanes 1 to 4 and lanes 9 to 11; reference strains, lane 5; sediment sample combined with reference strains (the combination with lane 4 and lane 6 in boxes and oval circle, respectively) and lanes 6 to 7; sediment samples (W + MA; sediment from winter + *M. aeruginosa*, W; sediment from winter, Sp; sediment from spring and Su; sediment from summer).

analysis because there were no signal bands at the same size when compared with the reference strains (lanes 1 to 5 in Figure 3). Nevertheless, the results indicated that the sediment samples from the spring season showed higher conformation than the sediment samples from the summer and winter seasons, respectively.

Use of the primers based on rRNA intergenic spacer and *rpo*C1 gene could not clearly distinguish *Microcystis* sp. from the other microorganisms in the sediment samples. Therefore, a specific primer was designed for detecting *Microcystis* sp. The PCR product based on *rpo*C1 was sequenced and aligned with amino acid of the other cyanobacteria. There was sufficient difference between the *rpo*C1 amino acid alignment of *Microcystis* sp. and the other cyanobacteria at the positions 256 to 333. Primers MV1-f and MV1-r were used to amplify a 419 bp diagnostic PCR product from the *rpo*C1 gene of *Microcystis* sp. Then extracted DNA was amplified and the results were illustrated in Figure 4. Although DNA from every reference strain could be amplified on the specific primer, the major band of *Microcystis* sp. appeared at about 400 bp (lanes 3 and 6, Figure 4). Furthermore, the *Microcystis* sp. specific *rpo*C1 fragment was also used to analyse DNA from the sediment samples. The results showed no signal band that correlated with *Microcystis* sp. (data not shown).

To confirm the results of the PCR analyses, *mcy*B was also performed with DNA from the sediment samples of both the blooming and non-blooming seasons. There were 3 bands of PCR products from *M. viridis* in size about 400, 750 and 1000 bp, on the other hand, no signal bands on all sediment samples were found (data not shown).

DGGE analyses of PCR products based on Microcystis *sp.-specific rpoC1 fragment*

To enhance the resolution of the detection system, the DGGE technique was employed. Figure 5 shows a DGGE analysis pattern of the PCR products obtained after amplification based on a *Microcystis* sp. specific *rpo*C1 fragment of four DNA aliquots from sediment in the non-blooming season (lanes 2 to 5), four DNA aliquots from sediment in the blooming season (lanes 6 to 9) and one from the purified *M. viridis* (lane 1). There were 6 fragments found from the *M. viridis* pure culture. Several fragments appeared in three lanes (lanes 3 to 5) of the sediment from the non-blooming season; however, none of the fragments were of equal size with *M. viridis*. Each band in the electrophoresis pattern presumably originates from one bacterial species present in the original material. In contrast, each lane of sediment from the blooming season showed no fragments. The complex patterns of the DGGE fragment were derived from the bacterial populations and their nucleic

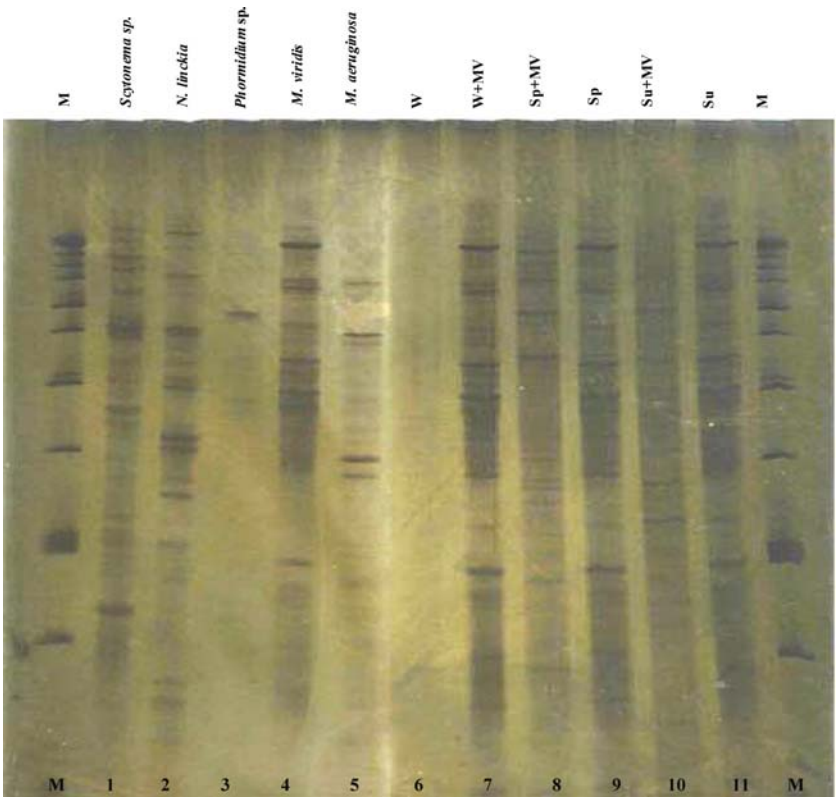

Figure 3. *rpo*C1 analysis pattern on SSCP from sediment samples. Lane M; 1 kb ladder marker, lanes 1 to 5; reference strains, lanes 7, 8 and 10; sediment sample combined with reference strains and lanes 6, 9 and 11; sediment samples (W + MA; sediment from winter + *M*. *aeruginosa*, W + MV; sediment from winter + *M*. *viridis*, W; sediment from winter, Sp + MV; sediment from spring + *M*. *viridis*, Sp; sediment from spring, Su + MV; sediment from summer + *M*. *viridis* and Su; sediment from summer).

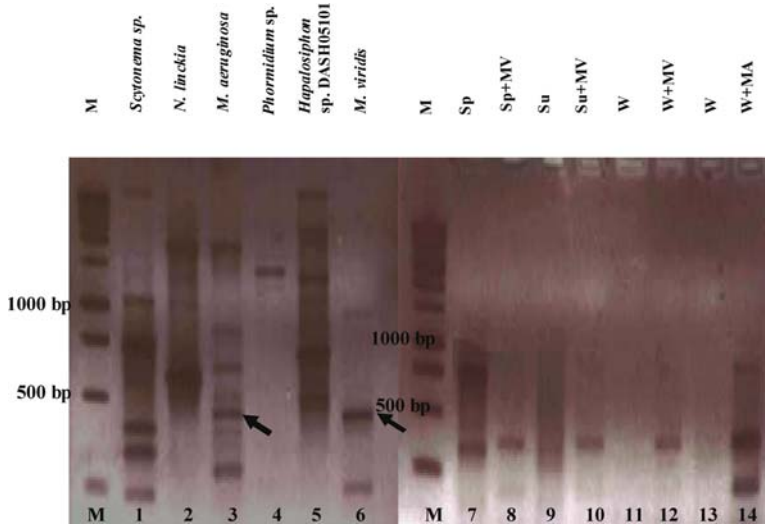

Figure 4. *Microcystis* sp.-specific *rpo*C1 fragment analysis from sediment samples. Lane M; 1 kb ladder marker, lanes 1 to 6; reference strains, lanes 8, 10, 12 and 14; sediment sample combined with reference strain and lanes 7, 9, 11 and 13; sediment samples (Sp; sediment from spring, Sp + MV; sediment from spring + *M*. *viridis*, Su; sediment from summer, Su + MV; sediment from summer + *M*. *viridis*, W; sediment from winter, W + MV; sediment from winter + *M*. *viridis* and W + MA; sediment from winter + *M*. *aeruginosa*).

acid. Thus the results indicated that there was a larger community of bacteria from the sediment samples in the non-blooming season than from the sediment in the blooming season.

T-RFLP analyses of PCR products based on 16S rDNA

The diversity of 16S rDNA in two different bacterial sediment samples was examined with a T-RFLP

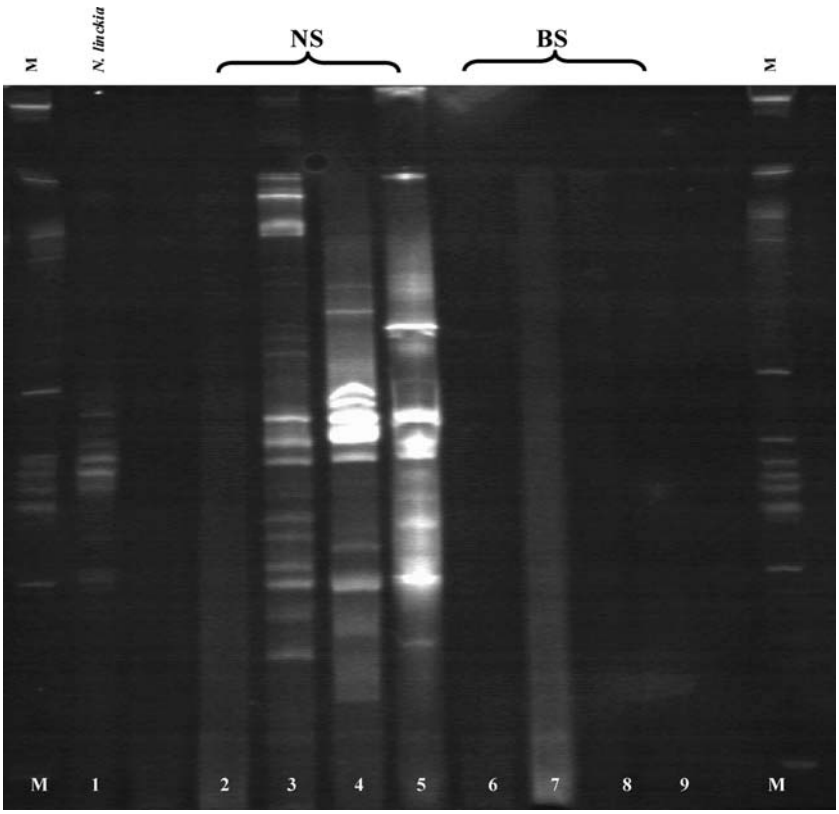

Figure 5. Ethidium bromide-stained DGGE pattern of PCR product derived from *Microcystis* sp.-specific *rpo*C1 fragment. Lane M; 1 kb ladder marker, lane 1; MV, lanes 2 to 5; NS, lanes 6 to 9; BS and lane M. (MV; *M. viridis*, NS; sediment from non-blooming season, BS; sediment from blooming season, lane 2 to lane 9 represent the collection time; January to August).

analysis. The T-RFLP analysis detected relatively few peaks in the sediment samples which were digested with both restriction enzymes *Hha*I and *Msp*I. The T-RFLP pattern of the sediment samples showed at least three major T-RFLP patterns of 84 bp, 91 bp, and 208 bp lengths based on restriction enzymes *Hha*I (Figure 6a). The T-RFLP pattern of 91 bp length was matched with the T-RFLP of *M. viridis*. While the T-RFLP pattern obtained from the sediment samples which were digested with *Msp*I, the results indicated that only one major T-RFs 477 bp length matched with T-RFLP of *M. viridis* (Figure 6b). Even though the T-RFLP technique based on 16S rDNA showed that the 91 bp length and 477 bp length were matched with *M. viridis* by digestion of *Hha*I and *Msp*I, respectively, the T-RFLP with *Hha*I endonuclease digestion patterns of *M. viridis* were 35, 75, 91, 119 and 373 bp lengths. While the T-RFLP with *Msp*1 endonuclease digestion patterns of *M. viridis* were 35, 75, 119 and 477 bp lengths, the T-RFLP pattern of the sediment samples that seem to be *M. viridis* should have a peak size of 75 bp length. Therefore, the results indicated that *M. viridis* might not remain in the Lake Senba sediment. Furthermore, the T-RFLP patterns also indicated more diversity of microorganisms in the non-blooming season than the blooming season.

Discussion

Since the detection and analysis of the cyanobacterial morphology from the sediment samples performed by the conventional plate count method was inconclusive, the analysis had to be combined with molecular approaches. The rRNA intergenic spacer analysis (RISA) fingerprinting technique was used because it is easy to perform, allows the rapid examination of the composition of complex bacterial communities, and can also be performed without the use of specific and expensive equipment. Different fingerprints and consequently different genetic structures were observed between the unfractionated soil and the microenvironments, and also among the various microenvironments, giving evidence that some populations were specific to a given location in addition to the common populations of all the microenvironments (Ranjard *et al*. 2000a). In this case, the rRNA internal spacer analysis was represented because this spacer was more variable in size than the 16S rRNA gene (Martinez *et al*. 1999). The results on single strand conformation polymorphism (SSCP) had better resolution than agarose gel electrophoresis and showed different community structures in Lake Senba in each season. A possible reason for the formation of more than one product from a pure culture is that the

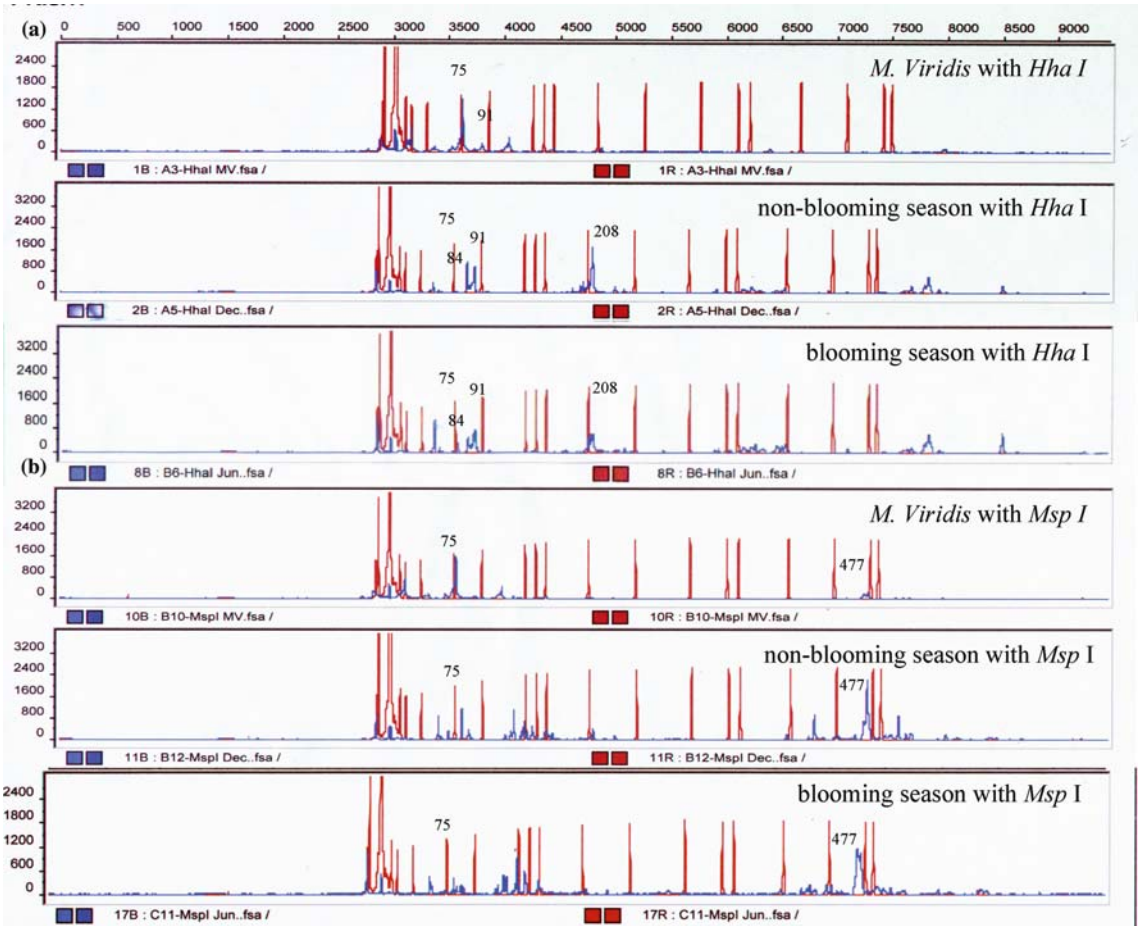

Figure 6. T-RFLP analysis of 16S rDNA in sediment samples: (a) T-RFLP patterns with the *Hha*I endonuclease digestion and (b) T-RFLP patterns with the *Msp*I endonuclease digestion. (red; size standard GENESCAN-500 pattern and blue; T-RFLP pattern of sediment samples).

universal primers amplified more than one operon. It is widely recognised that several bacterial species contain more than one 16S rRNA gene in their genomes. Another reason for detecting more than one fragment from pure culture by PCR-SSCP was the formation of metastable conformers, i.e., where the same molecule folds into more than one conformation with different electrophoretic mobilities (Clapp 1995). Nevertheless, four signal bands were found of the same size with *M. aeruginosa* (Figure 2). Since distinct and distantly related organisms had intergenic spacer sequences (IGSs) of different sequences but with similar sizes (Ranjard *et al*. 2000b), the bands of interest which were sequenced and compared with similar sequences of the reference organism by a BLAST search were shown not to be identical with *M. aeruginosa*.

The *rpo*C1 gene analysis had been shown to be more discriminatory than using 16S rRNA analysis, especially for chlorophyll-containing prokaryotes (Palenik & Haselkorn 1992). PCR primers designed from conserved regions of the cyanobacterial *rpo*C1 gene were used to analyse for *Microcystis* sp. in the sediment samples. In a previous study, these primers were used in PCRs for strain-level identification of a number of taxonomic groups to study the diversity of the cyanobacterial genus *Synechcoccus* (Toledo & Palenik 1997). In addition, the primers have been used to examine the phylogenetic relationship of prochlorophytes to each other and to the green chloroplasts (Rippka & Herdman 1992) and to analyse *Cylindrospermopsis raciborskii* isolates (Wilson *et al*. 2000). Thus, the *rpo*C1 gene might differentiate strains of *Microcystis* sp. isolated from the sediment of Lake Senba. However, larger community structures were found in the summer and spring seasons because the conditions such as transparency, temperature and suspended solids in Lake Senba were suitable for growth. A PCR test was developed to amplify a 419-bp *Microcystis* sp.-specific *rpo*C1 fragment from both the laboratory and environmental samples. Although all of the reference strains could be amplified based on the *Microcystis* sp.-specific *rpo*C1 fragment, there was a major band at about 400 bp only for the *Microcystis* sp. Nevertheless, the sediment samples were not found to have DNA products resembling a *Microcystis* sp.

Microcystin is produced nonribosomally via a multi-functional enzyme complex, consisting of both peptide synthetase and polyketide modules which are encoded by the *mcy* gene cluster (Teske *et al*. 1996). *mcy*B, the number of microcystin synthetase gene clusters was observed in the sediment samples, and were also not

found in the PCR products in this case. The morphology of *Microcystis* also related to a *mcy*B genotype, for example, the *Microcystis* population of Lake Wannsee (Germany) consists mainly of the morphospecies *M. aeruginosa*/*M. flos-aquae* and *M. ichthyoblabe*. Those morphospecies also differ significantly in the percentage of microcystin genotype; i.e. 73% of colonies assigned to *M. aeruginosa* contain *mcy*B, while only 16% of colonies assigned to *M. ichthyoblabe* contain it (Kurmayer *et al.* 2003). *M. aeruginosa* has frequently been reported to form large and firm colonies, while the colonies of *M. ichthyoblabe* are typically small and fragile (Watanabe 1996). Kurmayer & Kutzenberger (2003) reported that large colonies of *Microcystis* (>100 μM) had a high mcyB proportion. However, *Microcystis* normally has a size about 3–8 μM (Rippka *et al.* 1979) as in this study, thus the results mentioned above tended to indicate the disappearance of *Microcystis* sp. in the Lake Senba sediment.

The PCR-DGGE pattern showed a large community of bacteria in the non-blooming season. The finding of radiations of highly similar *Microcystis* sp.-specific *rpo*C1 fragment sequences contained in the DGGE bands also has an interesting implication for the population dynamics and ecological functions of sediment samples. The appearance and disappearance of a DGGE band reflects the increase and decrease of the corresponding bacterial population, respectively. Intensities of different DGGE bands derived from different bacterial species, do not allow quantitative conclusions about the abundance of the different bacteria, because of a possible unknown PCR basis in the amplification of different templates (Teske *et al.* 1996).

Corresponding with the T-RFLP pattern, T-RFLP fingerprints of the 16S rRNA gene from sediment samples were surprisingly similar in each season. While significant differences in the T-RFLP-based diversity indices (Shannon diversity index and equitability index) were not observed, the T-RFLP patterns showed quite a larger community of a bacterial population during the non-blooming season than during the blooming season. When *Microcystis* sp. blooms cover the surface area of the lake and decrease the light intensity and O_2 for microorganisms, the community of microorganisms decreases in the blooming season. The results also showed no excised band product similar to *Microcystis* sp. Nevertheless, supposing a constant PCR amplification bias for (or against) a specific bacterial *Microcystis* sp.-specific *rpo*C1 fragment, T-RFLP patterns clearly demonstrated that there was no major band which indicated specificity with the *M. viridis* fragment; therefore, the results suggested that *M. viridis* might not remain in the Lake Senba sediment after algal bloom had been controlled by the addition of ultrasonic radiation and jet circulation to the flushing treatment.

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