Microbial ecological study on the degradation process of cyanobacterial hepatotoxin (Microcystin) in natural eutrophic lake

THESIS

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富栄養湖における藍藻肝臓毒素(microcystin)の分解機構に関する微生物生態学的研究

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Microbial Ecological Study on the Degradation Process of Cyanobacterial Hepatotoxin (Microcystin) in Natural Eutrophic Lake

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CONTENTS

I Summary

Cyanobacterial Hepatotoxin, microcystin (Mcyst), which was produced by *Microcystis* and other several cyanobacteria, is detected in eutrophic and temperate lakes and ponds. This toxin is not easily degraded by either chemical or enzymatic treatments so far studied toxicologically. Little is known about the degradation procedure of Mcyst by bacteria under the natural environment. We succeeded in isolation of microcystin-degrading bacteria (MCD-bacterium), strain Y2, from Lake Suwa during the blooming period of *Microcystis*. According to 16S rRNA gene analysis, strain Y2 is elucidated to be a new species of new genus. To reveal the process of degradation of hepatotoxic microcystin during the bloom of *Microcystis*, I employed fluorescence *in situ* hybridization (FISH) to analyze the population dynamics of microcystin-degrading bacteria (MCD-bacterium) both in *Microcystis* mucilage and in water. Based on the 16S rRNA gene sequence of this strain, we designed an oligonucleotide probe which is specific to this MCD-bacterium.

In both the 1998 and 1999 tests, FISH clearly showed that MCD-bacteria existed in the mucilage and that, when a high concentration of cell-bound microcystin was detected, MCD-bacteria exceeded 10% of the sum of bacteria hybridized with group-specific probes. The concentration of MCD-bacteria was highest in summer 1998, when a toxic species, *M.*

viridis, was dominant. There was a high correlation between the number of MCD-bacteria in the mucilage and the concentration of cell-bound microcystin in the lake. My results suggest that MCD-bacteria responded to change in concentration of microcystin and degraded microcystin when it was released from *Microcystis* cells. I also analyzed changes of bacterial community structure associated with the *Microcystis* colonies by using domain- and group-specific oligonucleotide probes. Changes in the concentrations of the *Cytophaga/Flavobacterium* group and δ-Proteobacteria, which can degrade macromolecules derived from *Microcystis* cells, were synchronized with changes in the concentration of *Microcystis*. The results suggest not only the key role of MCD-bacteria in detoxification, but also kept a possible sequence in degradation from *Microcystis* cells to microcystin maintained in the cell, which is carried out by bacterial consortia in the mucilage.

Although free-living MCD-bacteria couldn't be detected by direct-FISH method in 1998, they were successfully visualized by direct viable count coupled with FISH method (DVC-FISH) due to the increasing rRNA content in 1999. the highest concentration of bacteria was detected when the concentration of dissolved Mcyst was the highest and at a high *Microcystis* concentration. I studied degradation of microcystin (Mcyst) using three samples of free-living bacteria collected from different periods of the bloom in 2000. Free-living bacteria in all samples completely degraded Mcyst-LR and 6(Z)-Adda-Mcyst-LR within 28

days and lag times varied in different free-living bacterial populations. Free-living bacteria in mid-bloom of *Microcystis* had a lag time of 3 days but degradation was complete within 7 days. Free-living MCD-bacteria detected by direct-FISH method increased markedly just after Mcyst degradation. Highest degradation activity of MCD-bacteria was estimated in mid-bloom of *Microcystis*. Other members of the bacterial community didn't change significantly except δ-Proteobacteria. These findings reveal the coexistence of MCD-bacteria in various bacterial consortia in water and degrade Mcysts released from *Microcystis*, adapting the production of degradation enzyme to the bloom of *Microcystis*. Thus, I conclude that in situ Mcyst produced by *Microcystis* was effectively degraded in various bacterial consortia by combination of 3 actors: MCD-bacteria in mucilage of *Microcystis*, MCD-bacteria in water, and the other coexistences contribution to degrade intermediate products

II Background of the study

(i) An overview

Toxic cyanobacteria and livestock suffered from cyanobacterial toxins were first reported by Francis in 1878 [10]. Numerous researches have elucidated that toxic cyanobacteria distributed ubiquitously [31] (Figure 2-1). It is known that cyanobacterial toxins, which consisted of neurotoxin and hepatotoxin, are produced by algae with several genera: *Anabaena. Aphanizomenon, Microcystis, Nostoc, Nodularia* and *Oscillatoria* [31].

Microcystis is known to major producer of hepatotoxic microcystin (Mcyst) and blooms widely from South Africa and Australia in the South sphere to Japan, USA, and Finland in the North sphere [24, 31](Figure 2-2). In addition, other genus, *Anabaena, Nostoc, Oscillatoria, Anabaenopsis* and *Haphalosiphon*, are candidate for Mcyst producer [31]. Mcyst is not easily degraded by chemical and enzymatic treatments so far studied toxicologically [11, 32, 33]. In 1996, 54 out of 130 patients at a dialysis center in Caruaru, Brazil, died from water for dialysis contaminated by Mcysts [13].

Mcyst is a cyclic heptapeptide consisting of 7 amino acids, which consisted of two unusual amino acids: 3-amino-9-methoxy-10-phenyl-2, 6, 8,-trimethyldeca-4, 6-dienomic acid (Adda) and N-methyldehydroalanine (Mdha), three D-amino acids: alanine (Ala), D-

erythro-b-methylaspartic acid (D-MeAsp) and r-linked glutamic acid (Glu) and two variable L-amino acids(X, Y) (Figure 2-3).

Sixty structural variants of this toxin have been characterized [27]. The name of Mcysts depends on the structural variations in all amino acid, particularly the variation of two L-amino acids, e.g., leucine and arginine (Mcyst-LR), arginine and arginine (Mcyst-RR), tyrosine and arginine (Mcyst-YR) (Figure 2-3). These three Mcysts have been detected in Japan. The structures of Mcyst affect the strength of toxicity, which is expressed by lethal dose 50 value (LD_{50}) (Table 2-1). Acute toxicity of Mcyst-LR is higher than that of cholera toxin produced by *Vibrio cholerae* and less than tetrodotoxin produced by globefish (Table 2-1). The toxicity of Mcyst-RR is less than cholera toxin. Mcyst is known to inhibit protein phosphatases 1 and 2A (Figure 2-4) [35]. This inhibition results in the promotion of tumours [21] even if concentration of Mcyst is low. World Health Organization (WHO) decided the guideline in which the values for drinking water quality must be less than 1 ug L^{-1} in 1999.

Mcyst is produced and maintained mainly within healthy cyanobacterial cells, though approximately 10% to 20% of intracellular Mcyst is released from healthy cyanobacterial cells in culture [29, 31]. When *Microcystis* cells are decayed, the concentration of dissolved Mcyst may increase. However, dissolved Mcyst concentrations remain at very low

concentration in lake water even during the decay period of the bloom of *Microcystis* [20, 25].

To date, only two isolates which could degrade Mcyst completely were reported: Jones et al. [15] isolated a single strain of Mcyst-degrading bacterium, strain MJ-PV, from drainage in Australia. This strain was a gram-negative rod, 2-5um in length and 0.5um in width and identified as *Sphingomonas* sp. by 16S rDNA analysis. In 1996, our group isolated a strain of MCD-bacterium, Y2, from eutrophic shallow lake, Lake Suwa in Japan [26]. The lake has a surface area of 13.3 km^2, a maximum depth of 6.4 m, and an average depth of 5 m. *Microcystis* bloom was first observed in this lake in 1948. Then, nutrient concentration increased owing to the inflow of wastewater from industries, agricultural area and houses, and then eutrophication was accelerated markedly in this lake throughout 60's and 70's [22]. *Microcystis* blooms nowadays annually in summer in this lake. We identified MCD-bacterium, Y2, and elucidated the characteristics of this strain in relation to Mcyst degrading activity.

(ii) Isolation and degradation activity of strain Y2

Ten strains were isolated from Lake Suwa by nutrient agar plate containing 0.05% beef extract, 0.1% peptone, 0.05% NaCl and 1% agar during the bloom of *Microcystis*. Among

them, only one, strain Y2, degraded Mcyst–LR, -RR and –YR in nutrient broth containing 0.05% beef extract, 0.1% peptone, 0.05% NaCl with different degradation rate. During the degradation, two new peaks appeared in high performance liquid chromatography (HPLC) analysis have very similar to UV spectra of Adda (238nm) but they were observed on different retention times. These degradation products were degraded.

Strain Y2 degraded Mcysts in nutrient broth in temperature regime from 5℃ to 30℃ (Figure 2-5), corresponding to water temperature in all seasons in Lake Suwa. The highest degradation rate was observed at 30°C, the lowest degradation rate was observed at 5°C but the toxins were degraded. These results suggest that strain Y2 can degrade Mcyst exuded from *Microcystis* cell in all seasons.

(iii) Identification of Mcyst-degrading bacteria strain Y2

The strain Y2 was strictly aerobic, chemoorganotrophic, and gram-negative. The cell was rod-shaped, 0.79±0.23 um in length and 0.49±0.08 um in width(Figure 2-6). It was catalase and oxidase positive and produced yellow-colored colonies on nutrient agar media. The results of phenotypic tests with a ID-Test-NF18 kit (Code No. 527400) showed that this isolate could be classified as *Sphingomonas paucimobilis*, although the probability was very low 0.17%. According to the 16S rDNA sequence analysis, the strain Y2 was most

similar to the sequence of *Rhizomonas subrifaciens* with 94.6% similarity among the sequences of the established species. Evolutionary distances were calculated for a dataset that consisted of the sequence of strain Y2 and other sequences of the α-4 group of the α-Proteobacteria with *Rhodospirillum rubrum* as an outgroup. A neighbor-joining phylogenetic tree was reconstructed on the basis of the distance matrix data thus obtained (Figure 2-5). Strain Y2 branched deeply from a major cluster, which consisted of *Rhizomonas subrifaciens, Blastomonas natatoria*, and some members of the genus *Sphingomonas* including the other MCD-bacteria, strain MJ-PV. To date, because the genus *Sphingomonas* were phylogenetically diverse, the genus was proposed to classify into four new genera: *Sphingomonas, Sphingobium, Novosphingobium* and *Sphingopyxis* [34]. Thus, it is more appropriate to classify strain Y2 as a new genus and species.

(iv) The aim of this study

From 90's, some researchers focused on the degradation of Mcyst in natural water. Kenefick et al. [17] and Jones and Orr [14] reported that Mcyst was degraded in water, when Mcyst was released from *Microcystis* cell after treating with copper sulfate as an algicide. Rapala et al. [28] and Cousins et al. [7] reported that Mcyst-LR was degraded in

water samples collected from the bloom of *Microcystis* in a reservoir. These studies lead an idea that Mcyst was degraded in natural water, leaving us the other question; "How was Mcyst degraded in water?" Several studies elucidated the degradation process of MCD-bacteria carrying out the Mcyst degradation experiment in vitro [4, 15, 26]. Thus, these studies confirmed that Mcyst could be degraded in natural water and that a strain of MCD-bacteria exists in it. However, the relation between MCD-bacteria and degradation process of Mcyst in natural environment still remain to be elucidated. Mcyst degradation is probably carried out in various functional bacteria. However, approximately 99% of total bacteria in the natural environment are not elucidated by the conventional culture-dependent method [6], and bacterial community structure and function markedly differ between free-living bacteria and aggregate/attached bacteria [1, 2, 9, 16].

To reveal bacterial function throughout in situ degradation of Mcyst directly, we applied the fluorescence *in situ* hybridization method (FISH, [3, 8]), which was recently developed to identify individual cells independent of culturability at levels ranging from species to domain by using oligonucleotide probes specific to the 16S or 23S rRNA sequence. Modified FISH methods [5, 12, 18, 19, 23] have also been developed to visualize the metabolic function of a specific bacterial group.

The aim of this study is to elucidate the process of the degradation of Mcyst in situ

focusing on the interaction between MCD-bacteria and the other coexistences elucidating the ongoing microbial activities in a given system. Based on the FISH method, we designed an oligonucleotide probe specific to the 16S rRNA sequence of strain Y2, and applied several probes specific to phylogenetic groups [20]. I tried to reveal that

1) The population dynamics of associated MCD-bacteria and structural changes in the associated bacterial community with *Microcystis* colonies during the periods of a *Microcystis* bloom and its decaying.

2) The population dynamics of free-living MCD-bacteria and structural changes in the free-living bacterial community during the periods of a *Microcystis* bloom and its decaying.

Results from these studies will give useful information to the researchers of environmental health and water treatment especially in some regions where people need not only the low cost but also high quality water treatment.

(v) References

1. Acinas SG, Anton J, Valera FR. (1999) Diversity of free-living bacteria and attached bacteria in offshore western mediterranean waters as depicted by analysis of genes encoding 16SrRNA. Appl Environ Microbiol 65: 514-522.

2. Acinas SG, Rodoriguez-Valera F, Pedros-Alio C. (1997) Spatial and temporal variation in marine bacterioplankton diversity as shown by RFLP fingerprinting of PCR amplified 16SrDNA. FEMS Microbiology Ecology,. 24: 27-40.

3. Amann RI, Ledwig W, Schleifer K-H. (1995) Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol Rev 59:143–169

4. Bourne DG, Jones GJ, Blakeley RL, Jones A, Negri AP, Riddles P. (1996) Enzymatic pathway for the bacterial degradation of the cyanobacterial cyclic peptide toxin microcystin LR. Appl Environ Microbiol 62:4086–4094

5. Cottrell MT, Kirchman DL. (2000) Natural assemblages of marine proteobacteria and members of the *Cytophaga-Flavobacter* cluster consuming low- and high-molecular weight dissolved organic matter. Appl Environ Microbiol 66:1692–1697

6. Colwell RR, Grims DJ. (2000) Semantics and strategies. p. 3. In R. R. Colwell, and D. J. Grimes (eds), Nonculturable Microorganisms in the Environment. ASM Press, Washington

7. Cousins IT, Bealing DJ, James HA, Sutton A. (1996) Biodegradation of microcystin-LR by

indigenous mixed bacterial populations. Wat Res 30: 481-48.

8. Delong, EF, Wickham GS, Pace N. (1989) Phylogenetic stains: ribosomal RNA-based probes for the identification of single cells. Science 243:1360–1363

9. Delong EF, Franks DG, Alldredge AL. (1993) Phylogenetic diversity of aggregate-attached vs. free-living marine bacterial assemblages. Limnol Oceanogr 38: 924-934.

10. Francis G. (1878) Poisonous Australian Lake. Nature18: 11-12

11. Harada K-I, Tsuji K, Watanabe MF, Kondo F. (1996) Stability of microcystins from cyanobacteria-III Effect of pH and temperature. Phycologia (Supplment) 35: 83-88.

12. Hodson RE, Dustman WA, Garg RP, Moran MA. (1995) In situ PCR for visualization of microscale distribution of specific genes and gene products in prokaryotic communities. Appl Environ Microbiol 61:4074–4082

13. Jochimsen EM, Carmichael WW, An J, Cardo DM, Cookson ST, Holmes CEM, Antunes BC, de Melo Filho DA, Lyra TM, Barreto VST, Azevedo AMFO, Jarvis WR. (1998) Liver failure and death after exposure to microcystins at a hemodialysis center in Brazil. N Eng J Med 338:873–878

14. Jones GJ, Orr PT (1994) Release and degradation of microcystin following algicide treatment of a *Microcystis aeruginosa* bloom in a recreational lake, as determined by HPLC and protein phosphatase inhibition assay. Wat Res 28: 871-876.

15. Jones GJ, Bourne DG, Blakeley RL, Doelle H. (1994) Degradation of the cyanobacterial hepatotoxin microcystin by aquatic bacteria. Natural Toxins 2:228–235

16. Kato, K. (1989) Planktonic bacterial strategy in aquatic ecosystem: free-living vs. attached. pp. 373-377. In Hattori T, Ishida Y, Maruyama Y, Morita R, Uchida A (eds) Recent Advances in Microbial Ecology. Jpn. Sci. Soc. Press.

17. Kenefick SL, Hrudey SE, Peterson HG, Prepas EE. (1993) Toxin release from *Microcystis aeruginosa* after chemical treatment. Wat Sei Tech 27: 433-440

18. Lange M, Tolker-Nielsen T, Molin S, Ahring BK. (2000) In situ reverse transcription-PCR for monitoring gene expression in individual *Methanosarcina mazei* S-6 cells. Appl Environ Microbiol 66:1796–1800

19. Lee N, Nielsen PH, Andereasen KH, Luretschko S, Nielsen JL, Schleifer K-H, Wagner M. (1999) Combination of fluorescent in situ hybridization and microautoradiography—new tool for structure-function analysis in microbial ecology. Appl Environ Microbiol 65:1289–1297

20. Maruyama T, Kato K, Yokoyama A, Tanaka T, A Hiraishi A, Park H-D, Dynamics of microcystin-degrading bacteria in mucilage of *Microcystis*. Microbial Ecology (in press)

21. Nishiwaki- Mastushima R, Ohta T, Nishiwaki S, Suganuma M, Kohyama K, Ishikawa T, Carmichael WW, Fujiki H. (1992) Liver tumor promotion by the cyanobacterial cyclic peptide toxin microcystin-LR, J Cancer Res Clin Oncol 118:420-424

22. Okino T, Kato K. (1987) Lake Suwa - Eutrophication and its partial recent recovery. GeoJournal 14: 373-375

23. Ouverney CC, Fuhrman JA. (1999) Combined microautography-16S rRNA probe technique for determination of radioisotope uptake by specific microbial cell types in situ. Appl Environ Microbiol 65:1746–1752

24. Park, HD, Watanabe MF. (1996) Toxic *Microcystis* in eutrophic lake. pp. 57-77. In Watanabe MF, Harada K-I, Carmichael WW, Fujiki H (eds) Toxic *Microcystis*. CRC Press.

25. Park H-D, Iwami C, Watanabe MF, Harada K-I, Okino T, Hayashi H. (1998) Temporal variabilities of the concentrations of intra- and extracellular microcystin and toxic *Microcystis* species in a hypertrophic lake, Lake Suwa, Japan (1991-1994). Environ Toxicol Water Qual 1: 61-72.

26. Park H-D, Sasaki Y, Maruyama T, Yanagisawa E, Hiraishi A, Kato K. (2001) Degradation of the cyanobacterial hepatotoxin microcystin by a new bacterium isolated from a hypertrophic lake. Environ Toxicol 16:337–343

27. Park H-D, Namikoshi M, Brittain SM, Carmichael WW, Murphy T. (2001) [D-Leu^{1}] microcystin –LR, a new microcystin isolated from waterbloom in a Canadian prairie lake. Toxicon 39:855–862.

28. Rapala J, Lahti K, Sivonen K and Niemelä SI. (1994) Biodegradability and adsorption on

lake sediments of cyanobcterial hepatotoxin and anatoxin-a. Letters in Applied Microbiology, 19: 423-428.

29. Rapala J, Sivonen K, Lyra C, Niemelä, SI. (1997) Variation of microcystins, cyanobacterial hepatotoxins, in *Anabaena* spp. as a function of growth stimuli. Appl Environ Microbiol 63:2206–2212

30. Sivonen K. (1990) Effect of light, temperature, nitrate orthophosphate, and bacteria on growth of and hepatotoxin production by *Oscillatoria agardhii* strains. Appl Environ Microbiol 56 :2658–2666

31. Sivonen K, Jones G. (1999): Cyanobacterial toxins, pp. 41-112 in Chorus I, Bartram J. (eds), Toxic Cyanobacteria in water. World Health Organization.

32. Takenaka S, Watanabe MF. (1997) Microcystin-LR degradation by *Pseudomonas aeruginosa* alkaline protease. Chemoshere 34: 749-757.

33. Takenaka S. (1998) Formation of 3-amino-2, 6, 8,-trimethyl-10-phenyldeca-4E, 6E-dienoic acid from microcystin-LR by the treatment various proteases, and its detection in mouse liver. Chemosphere 36: 2277-2282.

34. Takeuchi M, Hamana K, Hiraishi A. (2001) Proporsal of thye genus *Sphingomonas sensu strico* and three new genera, *Sphingobium, Novosphingobium* and *Sphingopyxis,* on the basis of phylogenetic and chemotaxonomic analysis. International Journal of Systematic and

evolutionary Microbiology 51: 1405-1417

35. Yoshizawa S, Matsushima R, Watanabe MF, Harada, K-I, Ichihara A, Carmichael WW, H. Fujiki. (1990) Inihibition of protein phosphatases by microcystin and nodularin associated with hepatotoxicity. Cancer Res Clin Oncol 116: 609-614

(vi) Figure legends

Figure 2 - 1.

The geographical distribution of toxic cyanobacteria

Figure 2 - 2.

The bloom of *Microcystis* and four species of the genera, which is commonly observed in Japan.

Figure 2 - 3.

Structures of microcystins which are constituted by 7 amino acids. Two unusual amino acids: 3-amino-9-methoxy-10-phenyl-2, 6, 8,-trimethyldeca-4, 6-dienomic acid (Adda) and N-methyldehydroalanine (Mdha); three D-amino acids: alanine (Ala), D-erythro-b-methylaspartic acid (D-MeAsp) and r-linked glutamic acid (Glu); two variable L-amino acids (X, Y).

Figure 2 - 4. Toxic mechanism of microcystin in hepatocyte.

Figure 2 - 5.

Degradation of microcystin-LR and -RR by MCD-bacteria, strain Y2, at 5, 10, 20, and 30℃ in the dark. Initial concentration of microcystins in 10^{-1} nutrient broth medium was adjusted to 20 mg L^{-1}.

Figure 2 - 6.

Microcystin-degrading bacteria (MCD-bacteria), strain Y2, stained by DAPI.

Figure 2 - 7.

Distance matrix tree showing phylogenetic relationship between strain Y2 and other members of the alpha-4 group of Proteobacteria. The sequence of *Rhodospirillum rubrum* was used as an outgroup to root the tree. Bootstrap values with 1000 trials are shown at branching points of interest. Scale bar=1% nucleotide substitution.

Table 2 - 1. A variety of biotoxins

Toxins	LD50(μg/kg)	Organisms
Botulinum toxin	0.00003	*Clostridium botulinum*
Tetrodotoxin	8	globefish, goby
cholera toxin	250	*Vibrio cholerae*
Mcyst-LR	50	*Microcystis, Anabaena, Nostoc, Oscillatoria, Anabenopis, Haphalosiphon*
Mcyst-RR	600	
6(Z)Adda* Mcyst-LR	>1200	

* stereoisomer of Adda at the Δ6 double bond.

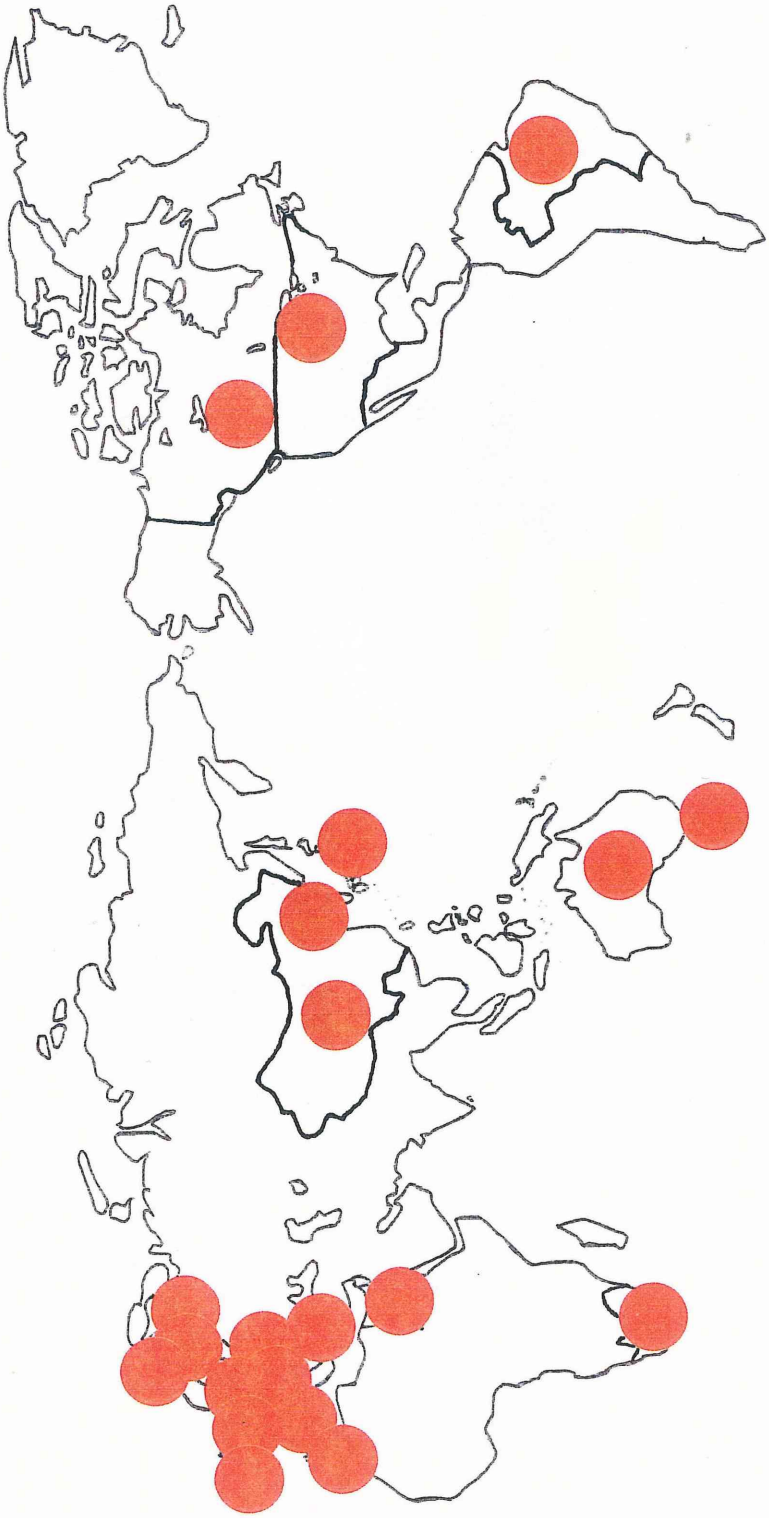
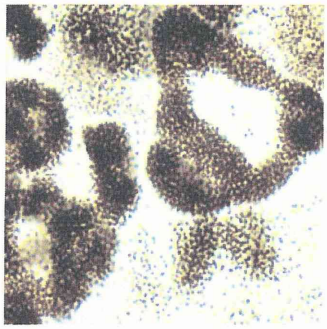
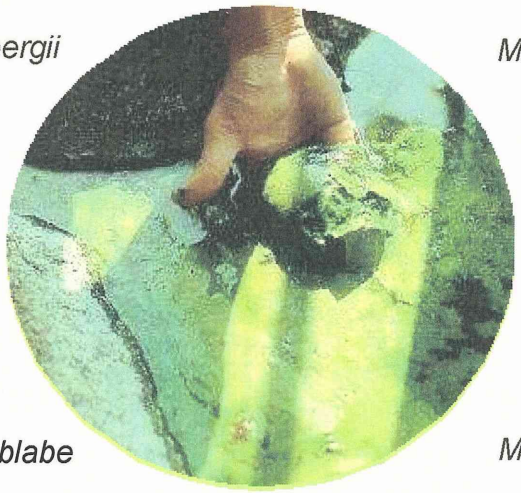


Figure 2-1



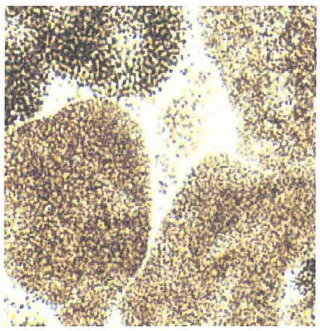
Microcystis wesenbergii

Microcystis aeruginosa



Microcystis ichthyoblabe

Microcystis viridis



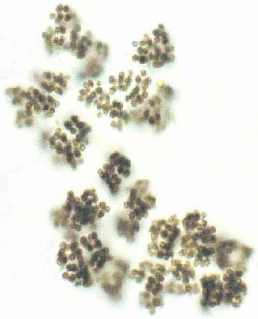
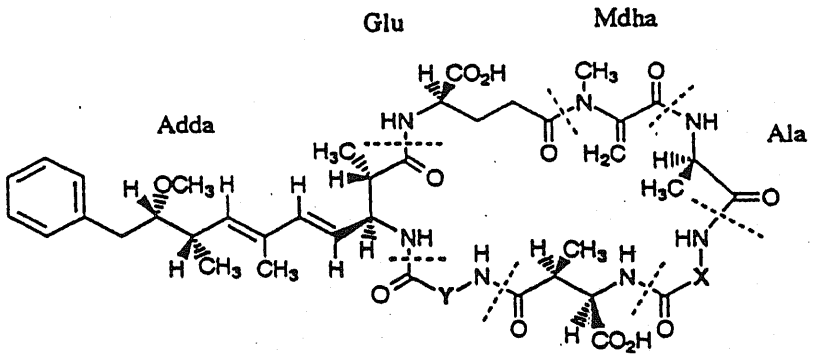
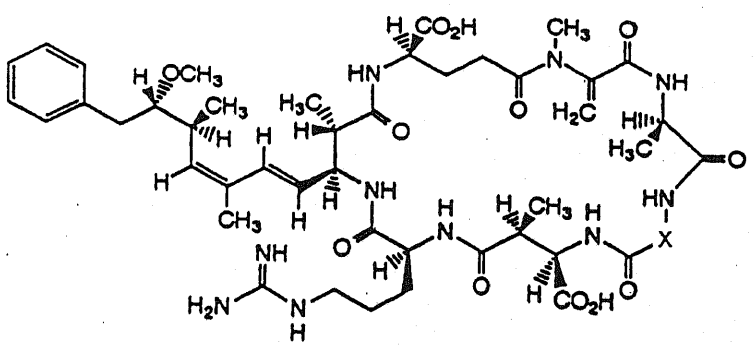


Figure 2 - 2



	X	Y
microcystin-LR	Leu	Arg
microcystin-RR	Arg	Arg
microcystin-YR	Tyr	Arg



		X
6(Z)-Adda	microcystin-LR	Leu
6(Z)-Adda	microcystin-RR	Arg

Figure 2-3

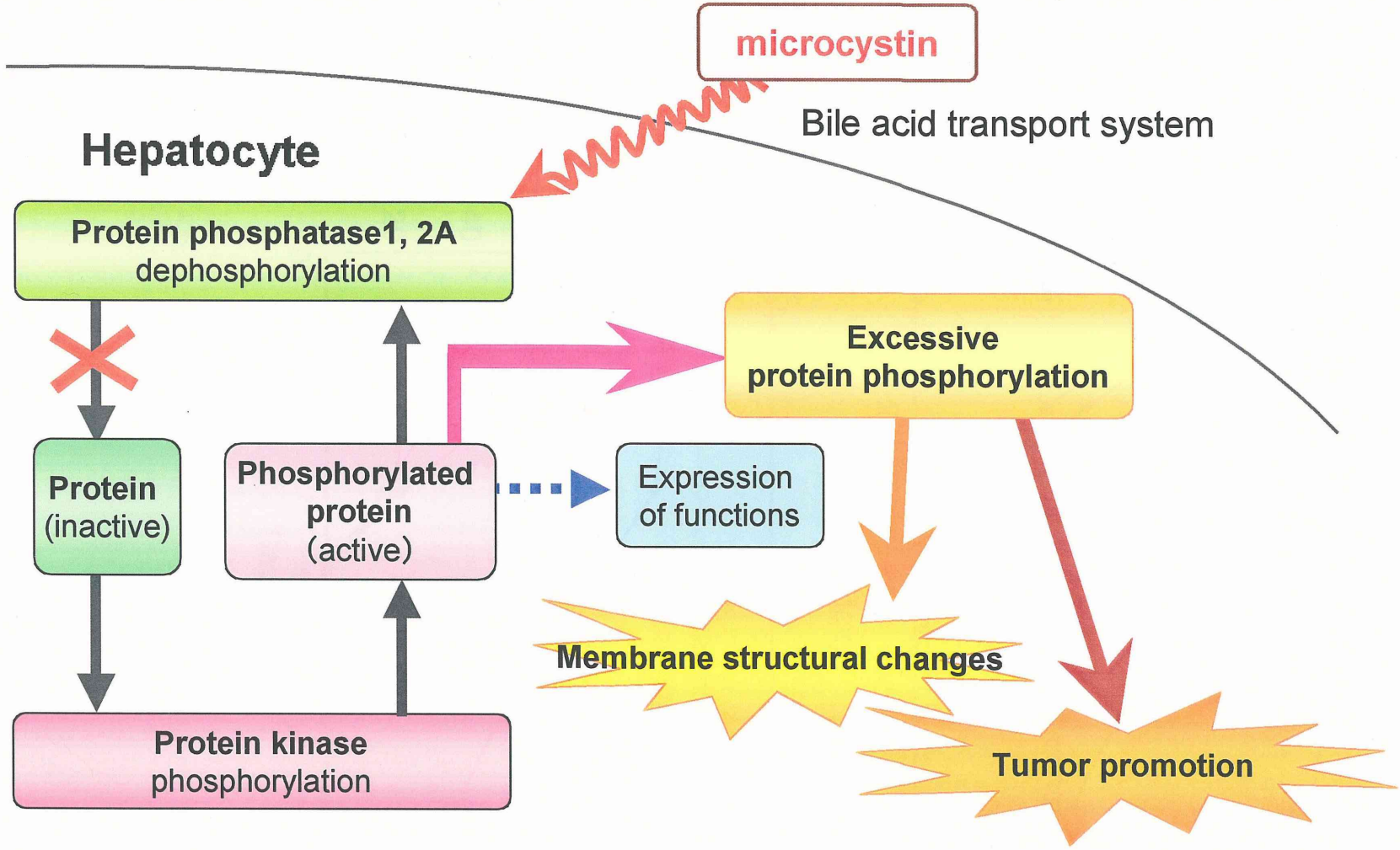

microcystin
Bile acid transport system
Hepatocyte
Protein phosphatase1, 2A
dephosphorylation
Excessive
protein phosphorylation
Protein
(inactive)
Phosphorylated
protein
(active)
Expression
of functions
Membrane structural changes
Tumor promotion
Protein kinase
phosphorylation

Figure 2-4.

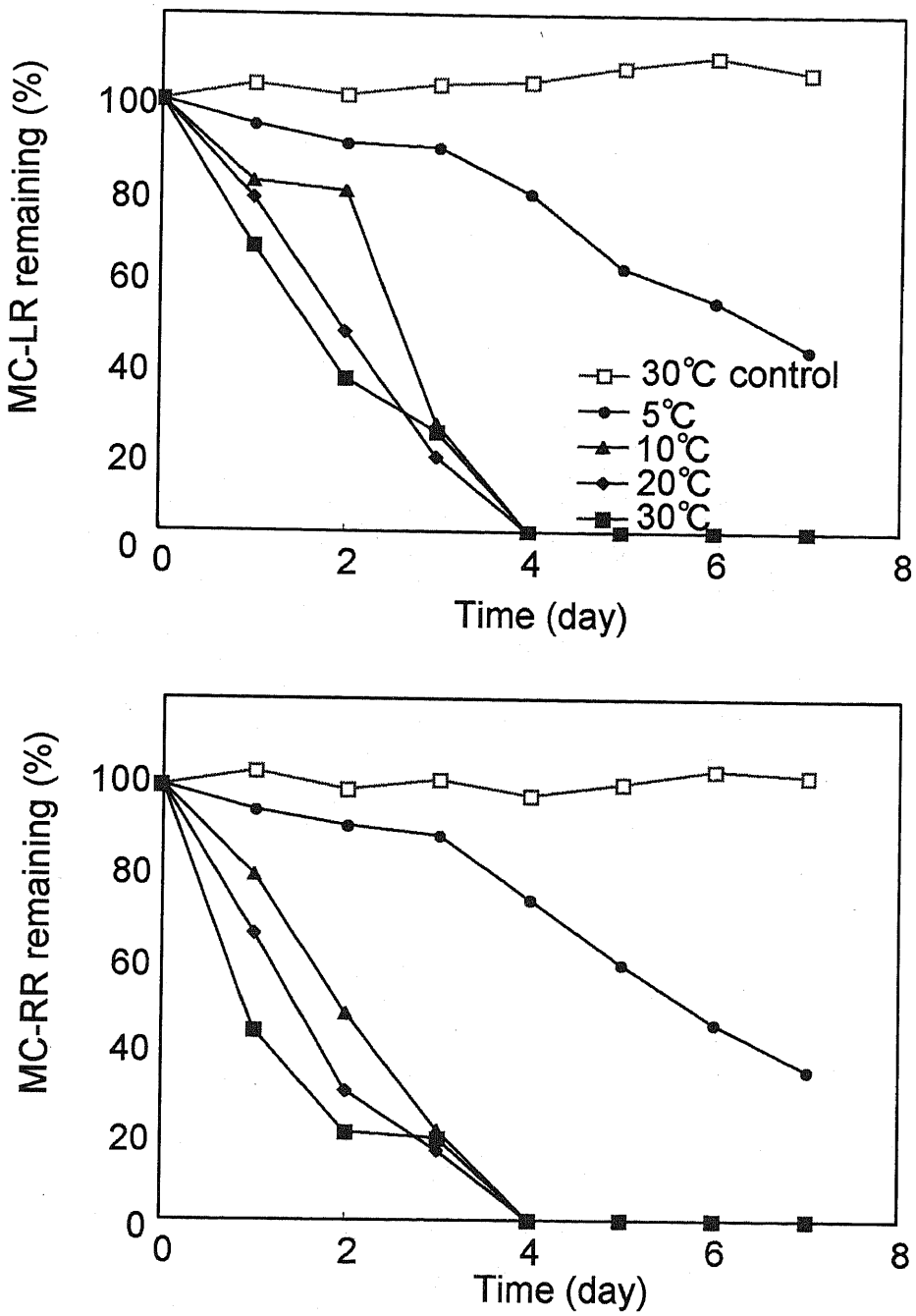

MC-LR remaining (%)
100
80
60
40
20
0
0
2
4
6
8
Time (day)
30℃ control
5℃
10℃
20℃
30℃
MC-RR remaining (%)
100
80
60
40
20
0
0
2
4
6
8
Time (day)

Figure 2 - 5

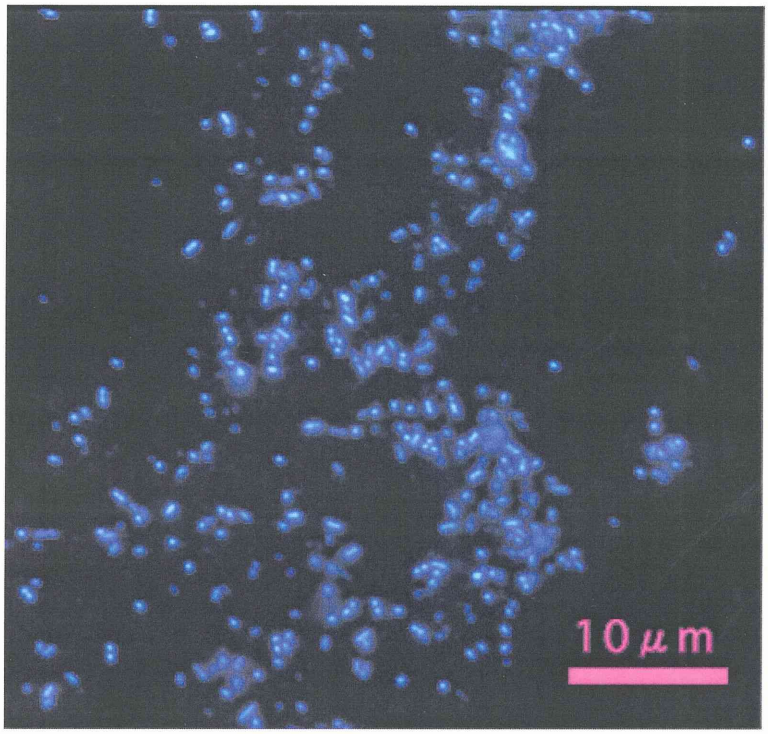


Figure 2-6

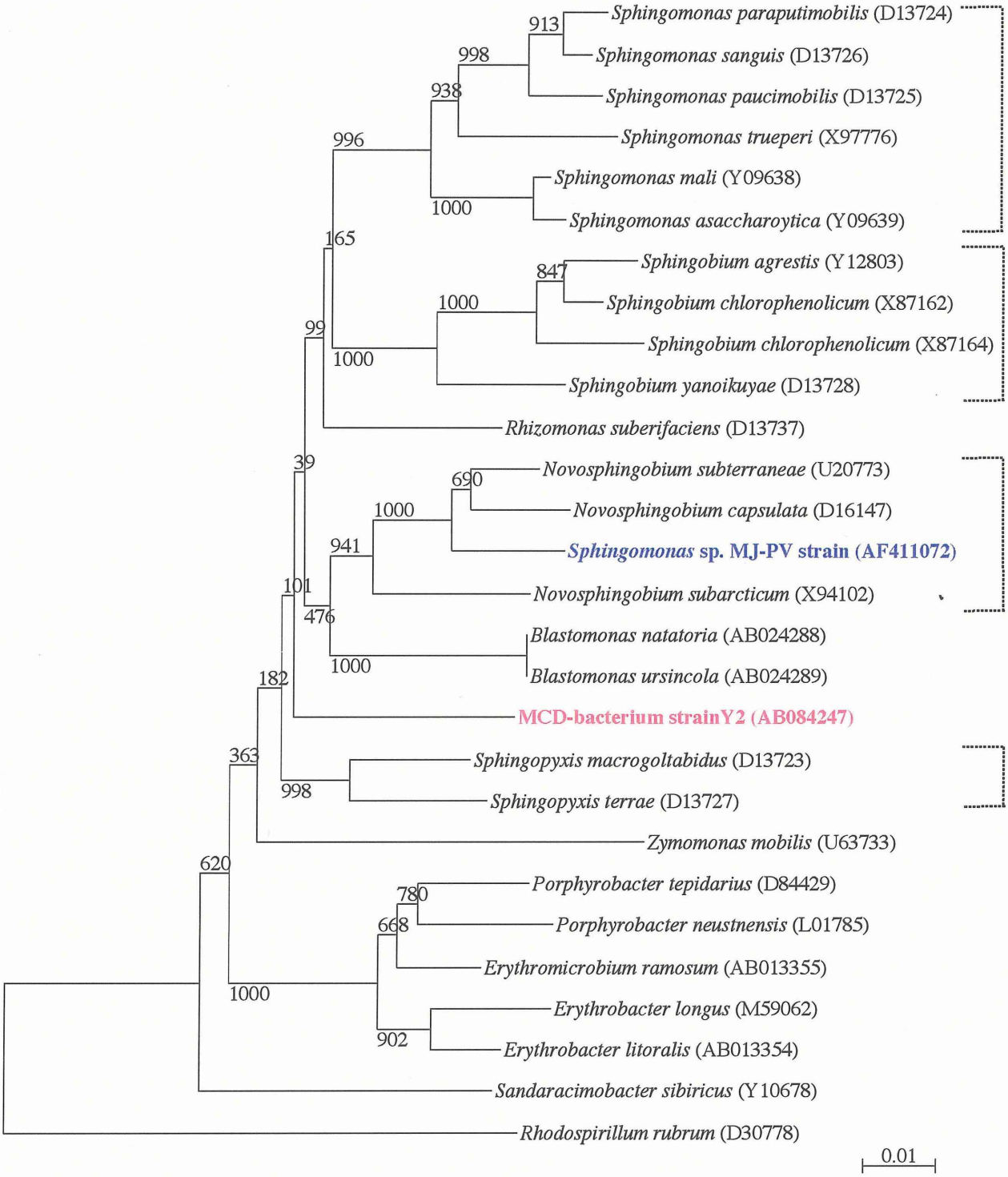

Sphingomonas paraputimobilis (D13724)
913
998
Sphingomonas sanguis (D13726)
938
Sphingomonas paucimobilis (D13725)
996
Sphingomonas trueperi (X97776)
Sphingomonas mali (Y09638)
1000
Sphingomonas asaccharoytica (Y09639)
165
Sphingobium agrestis (Y12803)
847
1000
Sphingobium chlorophenolicum (X87162)
99
Sphingobium chlorophenolicum (X87164)
1000
Sphingobium yanoikuyae (D13728)
Rhizomonas suberifaciens (D13737)
39
Novosphingobium subterraneae (U20773)
690
1000
Novosphingobium capsulata (D16147)
941
Sphingomonas sp. MJ-PV strain (AF411072)
101
Novosphingobium subarcticum (X94102)
476
Blastomonas natatoria (AB024288)
1000
Blastomonas ursincola (AB024289)
182
MCD-bacterium strainY2 (AB084247)
363
Sphingopyxis macrogoltabidus (D13723)
998
Sphingopyxis terrae (D13727)
Zymomonas mobilis (U63733)
620
Porphyrobacter tepidarius (D84429)
780
668
Porphyrobacter neustnensis (L01785)
Erythromicrobium ramosum (AB013355)
1000
Erythrobacter longus (M59062)
902
Erythrobacter litoralis (AB013354)
Sandaracimobacter sibiricus (Y10678)
Rhodospirillum rubrum (D30778)
0.01

Figure 2-7

III Dynamics of microcystin-degrading bacteria in mucilage of *Microcystis*

Degradation of Mcyst produced in *Microcystis* occurs during the 2-fold decomposition process of *Microcystis*: (a) the cell decays by autolysis, grazing by protozoa, zooplankton and fish [9], or viral [16] or bacterial activity [10, 31], and (b) the cellular products are then degraded by bacterial consortia. Because these findings concerning cell decomposition of *Microcystis* were obtained by culture-dependent techniques, our knowledge is limited to the function of culturable bacteria.

In this chapter, I attempt to elucidate the process of the degradation of Mcyst by MCD-bacteria under the possible coexistence with various other bacteria with varying functions during the bloom of *Microcystis* in Lake Suwa. I found that the numerous bacteria exist in mucilage of *Microcystis* in the degrading stage of *Microcystis* (Figure 3-1). I examine population dynamics of MCD-bacteria and structural changes in the associated bacterial community with *Microcystis* colonies during the periods of a *Microcystis* bloom and its decline applying FISH method with a designed probe specific to strain Y2 and several probes specific to and phylogenetic groups.

(i) Materials and methods

Sampling of heterotrophic bacteria associated with Microcystis colony

Lake Suwa, located in Nagano Prefecture in Japan, is a eutrophic, temperate, shallow lake. The lake has a surface area of 13.3 km^2, a maximum depth of 6.4 m, and an average depth of 5 m.

Samples of heterotrophic bacteria associated with *Microcystis* colonies were collected from the surface water of the center of the lake during *Microcystis* blooms on 26 August, 9 September, and 7 October 1998, and on 11 August, 25 August, 8 September, and 6 October 1999. As the biomass of *Microcystis* was significantly lower in 1999, we slightly changed the collection procedure. In 1999, water samples were prescreened with a nylon net with a mesh size of 40 μm and were kept in a glass bottle for a few hours. The floating *Microcystis* colonies and associated bacteria were then collected using a pipette. The isolated *Microcystis* colonies were filtered through a 3-μm-pore-size Nuclepore filter, and washed 3 to 5 times with phosphate-buffered saline (pH 7.2, Dulbecco's PBS(–), Nissui, Japan) to eliminate any free-living bacteria. The washed colonies were sonicated 3 times (BRANSONIC B1200, 45 kHz, 30 W, 3 s mL^{-1}) to disperse the heterotrophic bacterial cells and *Microcystis* cells.

Fixation of heterotrophic bacteria for total count and fluorescence in situ hybridization

Heterotrophic bacterial samples for total bacterial count and FISH were fixed in paraformaldehyde solution (pH 7.4, final concentration 3%) for up to 24 h at 4 °C. To avoid destruction of the *Microcystis* colonies in 1998 or the *Microcystis* cells and bacterial cells in 1999, fixed samples for FISH were filtered gently on a 0.22-μm Nuclepore filter (25 mm diameter) [14]. Cells on the filter were rinsed 3 times with PBS and dehydrated in 1 mL of 50%, 80%, and 99% ethanol for 3 min each, and the filter was then air-dried. Filters were stored at –20 °C until hybridization.

Total count of associated bacteria

Fixed samples were stained with 4′,6′-diamidino-2-phenylindole (DAPI, final concentration 0.01 μg/mL [24]) and filtered gently on a 0.22-μm Nuclepore filter (25 mm diameter). A total of more than 1000 bacterial cells were enumerated by epifluorescence microscopy (universal epifluorescence microscopic system BX50-FLA, Olympus, Japan).

Whole-cell in situ hybridization

The 16S rRNA targeted oligonucleotide probes used in this study are shown in Table 3-1. They comprised a domain-specific probe for Bacteria (EUB338), phylogenetic-group-

specific probes for α-, β-, γ-, and δ-Proteobacteria (ALF1b, BET42a, GAM42a, DEL) and the *Cytophaga/Flavobacterium* group (CF319a), and an MCD-probe designed specifically for MCD-bacteria ("strain Y2" in this study). These probes were labeled with rhodamine obtained from Takara Biotechnology (DALIAN), Japan.

Hybridization stringency was adjusted by varying the concentration of formamide in the hybridization buffer and NaCl in the washing solution. Hybridizations were performed at 46 °C for 90 min on filters placed on slides coated with gelatin, with hybridization buffer containing 0.9 M NaCl, 20 mM Tris•HCl (pH 7.4), 0.01% SDS, formamide (20% for EUB338, ALF1b, CF319a, and MCD, 35% for BET42a, GAM42a and DEL), and 5 ng μL^{-1} of the respective labeled probe. Each filter was washed at 48 °C for 15 min in pre-warmed washing buffer containing NaCl (0.225 M for EUB338, ALF1b, and MCD, 0.080 M for BET42a, GAM42a, DEL and CF319a), 20 mM Tris•HCl (pH 7.4), 5 mM EDTA, and 0.01% SDS, rinsed with distilled water, and air-dried. The preparations, which were counterstained with 0.1 μg mL^{-1} DAPI [30] on glass slides for 5 min, were observed under a universal epifluorescence microscopic system, BX50-FLA with a 3CCD camera (C5810, Hamamatsu Photonics, Japan) with an image analysis system (SP500F, Olympus). More than 500 DAPI-stained bacterial cells were counted to determine the proportion of the probe-specific-labeled cells among the total of the associated bacteria.

The accuracy of the MCD-probe had previously been examined by using sequences of the 16S rRNA gene obtained from a GenBank: the MCD-probe was found to contain 1 mismatch for the 2 other known sequences of the 16S rRNA gene in the database; both sequences had been isolated from marine oligotrophic bacteria (AB021704, AB022713). These bacteria, which were obtained courtesy of Dr. I. Yoshinaga of Kyoto University, were not succeeded in hybridization of the designed MCD-probe with various condition of hybridization adjusted by concentration of formamide and temperature. There exist some arguments about the binding strength between the sequences of the designed probe and the target position of 16S rRNA. According to the reports of Fuchs et al. [11], the 16S rRNA position of the MCD-probe was not easily hybridized. However, we confirmed that Y2 strain was successfully visualized by using MCD-probe (Figure 3-2).

Determination of concentrations of Microcystis cells, chlorophyll a, and total cell-bound and dissolved microcystin in the lake

To determine the concentrations of *Microcystis*, chlorophyll *a*, and cell-bound and dissolved Mcyst, surface water samples were collected from the center of the lake once two weeks between April and December in 1998 and 1999. Samples for cell counting of *Microcystis* were fixed in formaldehyde solution (final concentration, 1.5 % w/v). The

concentration of *Microcystis* cells was estimated by using a Fuchs-Rosenthal hemocytometer (Kayagaki works, Japan) under a microscope (BH-2, Olympus).

To measure the chlorophyll *a* concentration, water samples were filtered through a glass fiber filter (GF/C, Whatman, UK), which was then soaked in 10 mL of methanol for 24 h at 4℃. After that, the residue was centrifuged at 3000 rpm for 15 min. Measurement of the chlorophyll *a* concentration from the supernatant was quantified spectrophotometrically by the method of Maker et al. [20].

Measurements of Mcyst concentration and the clean-up of Mcyst in preparation for high-performance liquid chromatography (HPLC) were carried out according to Park et al. [24]. For measurement of cell-bound Mcyst, *Microcystis* cells were concentrated on a GF/C filter. The filter was then homogenized and extracted with 5% aqueous acetic acid, and, after centrifugation at 4000 rpm for 15 min, the supernatant was poured into an ODS cartridge (Bakerbond spe Octadecyl [C_{18}] 3 mL, USA). Mcyst extracted from the cartridge with 0.1% trifluoroacetic acid (TFA) – methanol was applied to the HPLC system (LC-9A S-I, Shimadzu, Japan), which was equipped with an ODS column (Cosmosil 5C18-AR 4.6×150 mm, Nacalai, Japan). The conditions of HPLC for analysis of Mcyst were as follows: absorbance at 238 nm; methanol: 0.05 M phosphate buffer (58:42; pH 3.0) in the mobile phase; and a 1-mL-min^{-1} flow rate.

Dissolved Mcyst was measured by 2 methods using a GF/C filtrate. In 1998, the GF/C filtrate sample was poured into an ODS cartridge (5 g, Chromatorex ODS, 100–200 mesh, Fuji Silysia Chemical, Kasugai, Japan). The cartridge was then rinsed with water and then with 20% methanol. Dissolved Mcyst was eluted from the cartridge with 90% methanol and evaporated to dryness. After a silica gel cartridge (2 g, SepPak) had been preconditioned with methanol, the residue was dissolved in methanol and applied to the cartridge. After the cartridge was rinsed with methanol, dissolved Mcyst was eluted with 70% methanol. The eluate was evaporated to dryness, and the residue was re-dissolved in methanol. The methanol solution was then analyzed by HPLC under the same conditions as above.

In 1999, dissolved Mcyst was analyzed by enzyme-linked immunosorbent assay (ELISA). Nagata et al. [22] produced a monoclonal antibody, M8H5, against Mcysts. ELISA analysis with this antibody determined the total concentration of Mcysts without discriminating Mcyst derivatives. Nagata et al. [22] showed very similar estimates from ELISA and a liquid chromatographic method for the analysis of Mcyst, although the sensitivity of the ELISA method was higher. Thus, we used the ELISA method after Nagata et al. [21, 22].

(ii) Results

Concentrations of Microcystis cells, chlorophyll a, and cell-bound and dissolved-microcystin during Microcystis blooms

Changes in the concentrations of *Microcystis*, chlorophyll *a*, and cell-bound Mcyst in water are given in Figure 3-3. In 1998 *Microcystis* appeared in the middle of June and bloomed, the dominant species being *M. ichthyoblabe* in July, and *M. viridis* from August to October. Relative proportion of *Microcystis* biomass formed more than 99% in total phytoplankton biomass during the blooming period of *Microcystis*. The concentration of *Microcystis* cells had 2 peaks: 2.4 $\times$ 10^9 cells L^{-1} on 29 July, and over 7 $\times$ 10^8 cells L^{-1} on 9 and 25 September, after which dates it decreased (Figure 3-3A-1). The chlorophyll *a* concentration also showed 2 large peaks: 662 μg L^{-1} on 29 July and over 600 μg L^{-1} on 9 and 25 September. By 7 October it had decreased markedly to 180 μg L^{-1} (Figure 3-3A-2). The cell-bound Mcyst concentration increased exponentially in September up to 100 μg L^{-1}, then decreased to 50 μg L^{-1} or less in October (Figure 3-3A-2). Although the changes in *Microcystis* cell concentration were paralleled by those of chlorophyll *a* and cell-bound Mcyst, nonparametric Spearman statistical analysis showed significance only between the concentration of *Microcystis* cells and chlorophyll *a* (r = 0.95, $P < 0.01$, n = 12). The concentration of cell-bound Mcyst fluctuated in parallel with that of chlorophyll *a* (r = 0.81,

$P < 0.01$, n = 13). The dissolved Mcyst concentration in the water was less than 0.5 μg L^{-1} throughout the 1998 study period (Table 3-2) – similar to the findings of Park et al. [24]. The concentration of dissolved Mcyst was the highest on 29 July; by 3 December it had decreased to below the detection limit. Although its fluctuation was similar in pattern to that of the *Microcystis* cell concentration, no significant relationship between the two was detected by nonparametric Spearman statistical analysis.

In contrast to 1998, a minor bloom of *Microcystis* was observed in September 1999, showing that relative proportion of *Microcystis* biomass formed more than 99% in total phytoplankton biomass. It consisted of *M. ichthyoblabe*, *M. aeruginosa*, *M. novacekii*, and *M. wesenbergii*. However, none of those strains dominated strikingly. The abundance of *Microcystis* was 1 order of magnitude lower than that in 1998, ranging from 4.3×10^6 to 6.8×10^7 cells L^{-1} (Figure 3-3B-1). The concentrations of chlorophyll *a* and cell-bound Mcyst from 11 August to 6 October in 1999 ranged from 16 to 48 μg L^{-1} and from 0.54 to 4.49 μg L^{-1}, respectively (Figure 3-3B-2). Cell-bound Mcyst was not analyzed between April and July, as the biomass of *Microcystis* was insignificant. The total concentration of dissolved Mcyst in the water was less than 0.2 μg L^{-1} throughout the observation period (Table 3-2). Although the changes in the concentrations of cell-bound Mcyst and dissolved Mcyst appeared to parallel changes in the *Microcystis* cell concentration, nonparametric

Spearman statistical analysis failed to show any significant relationships.

Population dynamics of bacteria associated with Microcystis

The density of bacteria associated with the *Microcystis* colonies ranged from 7.2×10^6 to 8.5×10^7 cells mL^{-1} of lake water in 1998 and from 1.1×10^4 to 3.2×10^5 cells mL^{-1} in 1999 (Table 3-3). The number of bacterial cells associated with each *Microcystis* cell increased from 25 on 26 August to 118 on 9 September 1998, but did not change markedly in 1999, ranging from 2.6 to 4.7 cells. The highest concentration of associated bacteria in the lake water was found on 9 September 1998 and on 8 September 1999, reaching 8.5×10^7 and 3.2×10^5 cells mL^{-1}, respectively. Whereas associated bacteria constituted 95% of the total bacteria in the water on 9 September 1998, associated bacteria in the 1999 study period constituted only 0.2% to 3.3% of the total bacteria. The difference in the concentration of associated bacteria between 1998 and 1999 was ascribed to the difference in the species composition of *Microcystis*.

Changes in community structure of associated bacteria

The community structure of bacteria associated with *Microcystis* was expressed on the basis of the number of each bacterial species, as determined by rRNA-targeted

oligonucleotide probes, per 10^2 cells of *Microcystis* (Figure 3-4), because an average colony of *Microcystis* in Lake Suwa consisted of at least 10^2 cells, although it fluctuated ranging from 127 to 529 cells. In 1998, the concentration of domain Bacteria visualized with the probe EUB338 ranged from 1.4×10^3 to 7.9×10^3 cells per 10^2 *Microcystis* cells, and the Bacteria were composed of between 56% and 69% DAPI-stained particles (data not shown). α-Proteobacteria were the second most common after β-Proteobacteria on 9 September and 7 October, increasing from 2.6×10^2 (26 August) to 2.0×10^3 (7 October) cells per 10^2 *Microcystis* cells (18% to 26% of the sum of the bacteria hybridized by the oligonucleotide probes specific to α-, β-, γ-, and δ-Proteobacteria and the *Cytophaga/Flavobacterium* group) (Figure 3-4A-1). β-Proteobacteria were dominant during the blooming of *Microcystis,* ranging from 5.1×10^2 to 2.7×10^3 cells per 10^2 *Microcystis* cells (28% to 36%). γ-Proteobacteria increased from 3.8×10^2 (26 August) to 1.3×10^3 (7 October) cells per 10^2 *Microcystis* cells (17% to 27%). δ-Proteobacteria made up the lowest percentage of the sum of bacteria hybridized with group-specific probes on 26 August, 9 September, and 7 October (5.3% to 13%). However, this group increased from only 75 (26 August) to 9.9×10^2 (9 September) cells per 10^2 *Microcystis* cells. This increase was about 2- to 4-fold higher than that of other groups in the period from August to September. The concentration of the *Cytophaga/Flavobacterium* group increased from 1.9

$\times$ 10^2 (26 August) to 1.5 $\times$ 10^3 (7 October) cells per 10^2 *Microcystis* cells (13% to 20%).

In 1999, the concentrations of domain Bacteria visualized with EUB338 ranged from 3.1 $\times$ 10^2 to 3.9 $\times$ 10^2 cells per 10^2 *Microcystis* cells, and the Bacteria were composed of between 78% and 94% DAPI-stained particles (data not shown). These numbers were higher than those in 1998, although the density of associated bacteria in 1999 was about 100 times less than in 1998. α-Proteobacteria existed at concentrations of between 1.2 $\times$ 10^2 cells and 1.4 $\times$ 10^2 cells per 10^2 *Microcystis* cells throughout the study period (Figure 3-4B-1), and predominated in all samples, accounting for at least 30% of the sum of bacteria hybridized with group-specific probes. β-Proteobacteria also remained nearly constant at 1.0 $\times$ 10^2 cells per 10^2 *Microcystis* cells during the study period, constituting from 26% to 31% of the sum of bacteria hybridized with group-specific probes – a similar percentage to that in 1998. γ-Proteobacteria ranged from 49 to 57 cells per 10^2 *Microcystis* cells (13% to 18%). The densities of these 3 phylogenetic groups were similar to each other during the 1999 observations. δ-Proteobacteria and the *Cytophaga/Flavobacterium* group showed similar fluctuations with regard to relative abundance and cell density: both parameters for both groups had increased by 8 September and decreased by 6 October. The number of δ-Proteobacteria ranged from 11 to 50 cells per 10^2 *Microcystis* cells, reaching a peak on 8 September. This group made up only 3.5% to 13% of the sum of bacteria hybridized with

group-specific probes. The *Cytophaga/Flavobacterium* group ranged from 13 to 69 cells per 10^2 *Microcystis* cells (4.0% to 18%), reaching a maximum on 8 September.

MCD-bacteria had increased remarkably in both relative abundance and density of associated bacteria per 10^2 *Microcystis* cells by 9 September 1998 (Figure 3-4A-2). MCD-bacteria increased remarkably from 93 cells to 1.3×10^3 cells per 10^2 *Microcystis* cells between 26 August and 9 September, and by 7 October had decreased to 7.8×10^2 cells per 10^2 *Microcystis* cells, thus accounting for 6.6% to 17% of the total bacteria hybridized with group-specific probes. Their relative abundance among α-Proteobacteria was also high, ranging from 36% to 76% (data not shown). In 1999, MCD-bacteria ranged from 20 to 41 cells per 10^2 *Microcystis* cells and showed a tendency to increase in numbers in September, accounting for 6.1% to 11% of the sum of bacteria hybridized with group-specific probes (Figure 3-4B-2). The relative abundance of MCD-bacteria among α-Proteobacteria was not as high as in 1998, ranging from 17% to 34%. The highest proportions of MCD-bacteria, in relation to both the sum of bacteria hybridized with group-specific probes and α-Proteobacteria, were observed on 8 September. These results from 1998 and 1999 indicated that the number of MCD-bacteria associated with 10^2 *Microcystis* cells increased in September and then decreased in October, closely paralleling changes in the Mcyst concentration in both the water ($r = 1$, $P < 0.01$, $n = 6$) and *Microcystis* cells ($r = 0.89$, $P <$

0.05, n = 6).

(iii) Discussion

Strains of several species of the genus *Microcystis* produce 60 variants of hepatotoxic Mcyst [26]. *M. viridis* is one such toxic species in Lake Suwa [23, 24]. The highest concentration of cell-bound Mcyst was observed when *M. viridis* was dominant in September 1998.

Microcystis is surrounded by mucilage, which consists mainly of polysaccharide [2, 4] composed of glucose, mannose, fucose, xylose, galactose, and rhamnose. The thickness and solubility of the mucilage vary among *Microcystis* species [3, 15]: the mucilage of *M. viridis* is harder to dissolve in water than those of the other species [3]. During blooms, numerous bacteria are known to exist in the mucilage [7]. Furthermore, the abundance and community structure of the embedded bacteria might differ according to the *Microcystis* species.

When Mcyst is released from a cell of *Microcystis,* it is diffused in the mucilage because of the mucilage's high viscosity. To reveal the process of degradation of Mcyst, we therefore focused on the function of the bacteria embedded in the mucilage and tried to describe the population dynamics of the MCD-bacteria there. Our results revealed that the number of MCD-bacteria in the mucilage increased in September of both years and correlated with the

concentration of cell-bound Mcyst, and the highest concentration of MCD-bacteria existed in 1998 when *M. viridis* was dominant. It is remarkable in natural systems that 1 gene specific clone of MCD-bacteria detected by FISH made up 1/10 of the whole bacterial community in September 1998 and 1999. These results suggest that MCD-bacteria responded to changes in the concentration of Mcyst, and that MCD-bacteria were active in the mucilage of *Microcystis* when produced Mcyst was present there. Jones et al. [13] reported that Mcyst-degrading isolates require a lag time in the degradation of Mcyst when they have not been exposed to Mcyst *a priori* under experimental conditions. However, we assumed that MCD-bacteria in the mucilage were on 'stand-by' until the degradation of Mcyst occurred: they could be directly exposed to any Mcyst released from cells in the *Microcystis* colony. This suggests that MCD-bacteria could thus initiate the degradation of Mcyst in the mucilage within 2 weeks so far we examined. These findings can explain why a bacterial species becomes predominant in a given system if it exerts a very specific function to degrade a specific compound, such as Mcyst.

During the bloom of *Microcystis*, the concentrations of the *Cytophaga/Flavobacterium* group and δ-Proteobacteria were apparently synchronized with that of the *Microcystis* cells, with $r = 0.89$ ($P < 0.05$, n = 6) and $r = 0.94$ ($P < 0.05$, n = 6), respectively. To our knowledge, members of the *Cytophaga/Flavobacterium* group are able to degrade not only

macromolecular compounds [8, 29], but also *Microcystis* cells [31]. Van Hannenn [29] suggested that Cytophagales, the related 16S rRNA sequence of which appeared in denaturing gradient gel electrophoresis after the lysis of cyanobacteria, could contribute to degradation of dissolved organic matter (DOM) released from this lysis. Recently, Cottrell and Kirchman [8] suggested from fluorescence *in situ* hybridization (MICRO-FISH) studies that the mode of bacterial utilization of DOM differs among phylogenetic groups: the *Cytophaga/Flavobacterium* group tends to prefer high-molecular-weight DOM such as proteins and chitin. Yamamoto et al. [31] showed by a culture-dependent method that some *Microcystis* were lysed specifically by some strains of this group isolated from the surface waters of Lake Suwa. These findings suggest that the *Cytophaga/Flavobacterium* group contributes to the lysis of *Microcystis* and degrades DOM derived from intracellular products of *Microcystis* in the mucilage. Grilli Caiola et al. [12] reported that *Bdellovibrio*-like bacteria, constituents of the δ-Proteobacteria, infect *Microcystis* cells and degrade peptidoglycan and the cell wall, although *Bdellovibrio* is known to be a bacterial predator [5]. This suggests that δ-Proteobacteria might contribute to the lysis of *Microcystis*.

α-Proteobacteria and β-Proteobacteria tended to dominate in the mucilage of *Microcystis* during the bloom of *Microcystis*. Of the α-Proteobacteria, *Caulobacter* can attach to cyanobacteria and take up exudates of photosynthetic products [28]. *Alcaligenes* and

Pseudomonas, which are β-Proteobacteria, are known to lyse *Microcystis* cells by attaching to them [17, 31].

We studied the process of degradation of Mcyst in the light of changes in bacterial community structure in a natural environment, focusing particularly on strain Y2 of MCD-bacteria, which belongs to an undescribed genus [25]. We found that MCD-bacteria existed in a restricted space of the mucilage of *Microcystis*, and that the change in concentration of these bacteria was synchronized with the increase in the concentration of cell-bound Mcyst. This suggests that MCD-bacteria in the mucilage responded to changes in the concentration of cell-bound Mcyst; the Mcyst was exuded from the cell of toxic *Microcystis* and degraded by the bacteria. The *Cytophaga/Flavobacterium* group and δ-Proteobacteria also changed their population densities in the mucilage, suggesting that they contributed to the degradation of *Microcystis* cells. Mucilage is revealed not only as a compound that binds *Microcystis* cells together, but also as a habitat for bacteria that exert their specific function to utilize and thus degrade *Microcystis* cellular materials.

(iv) References

1. Amann RI, Binder BJ, Olson RJ, Chrisholm SW, Devereux R, Stahl DA. (1990) Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. Appl Environ Microbiol 56:1919–1925
2. Amemiya Y, Nakamura O. (1986) Some properties of the sheath material. Rep Fac Engineer, Yamanashi Univ 37:70–75
3. Amemiya Y, Kato K, Nakayama O. (1988) Extracellular products of *Microcystis* species: Formation of slime layer and DOC pool in surrounding waters. Verh Int Ver Limnol 23:1886–1892
4. Amemiya Y, Kato K, Okino T, Nakayama O. (1990) Changes in the chemical composition of carbohydrates and proteins in surface water during a bloom of *Microcystis* in Lake Suwa. Ecol Res 5:153–162
5. Brock TD (1970) Prokaryoic diversity: bacteria. In: Michael TM, John MM, Jack P. (eds), The Biology of Microorganisms, 8th ed: Prentice Hall, New Jersey, p 684
6. Brosius J, Dull TL, Sleeter DD, Noller HF. (1981) Gene organization and primary structure of a ribosomal RNA operon from *Escherichia coli*. J Molec Biol 148:107–127
7. Brunberg A-K. (1999) Contribution of bacteria in the mucilage of *Microcystis* spp. (Cyanobacteria) to benthic and pelagic bacterial production in a hypereutrophic lake.

FEMS Microbiol Ecol 29:13–22

8. Cottrell MT, Kirchman DL. (2000) Natural assemblages of marine proteobacteria and members of the *Cytophaga-Flavobacter* cluster consuming low- and high-molecular weight dissolved organic matter. Appl Environ Microbiol 66:1692–1697

9. Christoffersen K. (1996) Ecological implications of cyanobacterial toxins in aquatic food webs. Phycologia 35: 42-50

10. Daft MJ, McCord SB, Stewart WDP. (1975) Ecological studies on algal-lysing bacteria in fresh waters. Freshwat Biol 5:577–596

11. Fuchs BM, Wallner G, Beisker W, Schwippl I, Ludwig W, Amann R. 1998 Flow cytometric analysis of the in situ accessibility of *Escherichia coli* 16S rRNA for fluorescently labeled oligonucleotide probes. Appl Environ Microbiol 62:1998–2005

12. Grilli Caiola M, Pellegrini S, Geora FM, Ribaldone A. (1991) *Bdellovibrio*-like bacteria in *Microcytsis aeruginosa.* Arch Hydrobiol Suppl Algol Stud 64:369

13. Jones GJ, Bourne DG, Blakeley RL, Doelle H. (1994) Degradation of the cyanobacterial hepatotoxin microcystin by aquatic bacteria. Natural Toxins 2:228–235

14. Kato K, Sakamoto M. (1983) The function of free-living bacterial fraction in the organic matter metabolism of mesotrophic lake. Arch Hydrobiol 97:287–302

15. Komárek J. (1991) A review of water-bloom forming *Microcystis* species, with regard to

populations from Japan. Arch Hydrobiol Suppl Algol Stud 64:115–127

16. Manage PM, Kawabata Z, Nakano S-I. (1999) Seasonal changes in densities of cyanophage infectious to *Microcystis aeruginosa* in a hypereutrophic pond. Hydrobiologia 411:221–216

17. Manage PM, Kawabata Z, Nakano S-I. (2000) Algicidal effect of the bacterium *Alcaligenes denitrificans* on *Microcystis* spp. Aquat Microb Ecol 22:111–117

18. Manz W, Amann R, Ludwig W, Wagner M, Schleifer K-H. (1992) Phylogenetic oligooxynucleotide probes for the major subclasses of proteobacteria; problems and solutions. Syst Appl Microbiol 15:593–600

19. Manz W, Amann R, Ludwig W, Vancanneyt M, Schleifer K-H. (1996) Application of a suite of 16SrRNA-specific oligonucleotide probes designed to investigate bacteria of the phylum cytophaga-flavobacter-bacterioides in natural environment. Microbiology 142:1097–1106

20. Marker AFH, Nusch EA, Rai H, Riemann B. (1980). The measurement of photosynthetic pigments in fresh waters and standardization of methods: conclusions and recommendations. Arch Hydrobiol Beih 14:91–106

21. Nagata S, Soutome H, Tsutsumi T, Hasegawa A, Sekijima M, Sugamata, M, Harada K-I, Suganuma M, Ueno Y. (1995) Novel monoclonal antibodies against microcystin and

their protective activity for hepatotoxicity. Natural Toxins 3:78–86

22. Nagata S, Tsutsumi T, Hasegawa A., Yoshida F, Ueno Y. (1997) Enzyme immunoassay for direct determination of microcystins in environmental water. J AOAC Int 80:408–417

23. Park H-D, Watanabe MF, Harada K–I, Suzuki M, Hayashi H, Okino T. (1993) Seasonal variations of *Microcystis* species and toxic heptapeptide microcystins in Lake Suwa. Environ Toxicol Water Qual 8:425–435

24. Park H-D, Iwami C, Watanabe MF, Harada K–I, Okino T, and Hayashi H (1998) Temporal variabilities of the concentrations of intra- and extracellular microcystin and toxic *Microcystis* species in a hypertrophic lake, Lake Suwa, Japan (1991–1994). Environ Toxicol Water Qual 13: 61–72

25. Park H-D, Sasaki Y, Maruyama T, Yanagisawa E, Hiraishi A, Kato K. (2001) Degradation of the cyanobacterial hepatotoxin microcystin by a new bacterium isolated from a hypertrophic lake. Environ Toxicol 16:337–343

26. Park H-D, Namikoshi M, Brittain SM, Carmichael WW, Murphy T. (2001) [D-Leu1] microcystin –LR, a new microcystin isolated from waterbloom in a Canadian prairie lake. Toxicon 39:855–862.

27. Poter KG, Feig YS. (1980) The use of DAPI for identifying and counting aquatic

microflora. Limnol Oceanogr 25:943–948

28. Stanier RY. (1976) Gram-negative bacteria: aerobic chemoheterotrophs. In: Stanier RY, Adelberg EA, Ingraham JL (eds), The Microbial World, 4th ed: Prentice-Hall, New Jersey, pp 598–602

29. Van Hannen EJ, Zwart G, van Agterveld MP, Gons HJ, Ebert J, Laanbroek HJ. (1999) Changes in bacterial and eukaryotic community structure after mass lysis of filamentous cyanobacteria associated with viruses. Appl Environ Microbiol 65:795–801

30. Weiss P, Schweitzer B, Amman R, Simon M. (1996) Identification in situ and dynamics of bacteria on limnetic organic aggregates (Lake Snow). Appl Environ Microbiol 62:1998–2005

31. Yamamoto Y, Nizuma S, Kuroda N, Sakamoto M. (1993) Occurrence of heterotrophic bacteria causing lysis of cyanobacteria in a eutrophic lake. Jpn J Phycol 41:215–220

(v) Figure Legends

Figure 3 – 1.

Associated bacteria with (A) growing stage of *Microcystis* and (B) degrading stage of *Microcystis*, which are stained by DAPI. The cell size of *Microcystis* is about 5um in diameter.

Figure 3 - 2

In situ hybridization of bacteria associated with colonies of *Microcystis*, viewed by epifluorescence microscopy. Bacteria in the colony of *Microcystis* stained by DAPI (A), and hybridized with a rhodamine-labeled probe specific to microcystin-degrading bacteria (B). Particles about 5 μm in diameter are cells of *Microcystis*.

Figure 3 – 3.

Seasonal changes in the concentrations of *Microcystis* cells, chlorophyll *a*, and cell-bound microcystin at the center of Lake Suwa in 1998 (A) and 1999(B). A-1 and B-1 show the concentrations of *Microcystis* cells (triangles). A-2 and B-2 show the concentrations of chlorophyll *a* (open circles) and cell-bound microcystin (closed circles).

Figure 3 – 4.

The composition of the bacterial assemblages in *Microcystis* colonies, as detected by rRNA-targeted oligonucleotide probes specific for α-, β-, γ-, and δ-Proteobacteria, the *Cytophaga/Flavobacterium* group, and microcystin-degrading bacteria. Samples were collected from the surface waters of Lake Suwa on 26 August, 9 September, and 7 October 1998 (A), and on 25 August, 8 September, and 6 October 1999 (B), when *Microcystis* was in bloom.

Table 3 - 1. Probe sequences and target sites

Probe name	Target organism	Sequence	Target site [a] rRNA Position	Reference
EUB338 :	domain Bacteria	5'-GCTGCCTCCCGTAGGAGT-3',	16S, 338-355	1
ALF1b :	α-Proteobacteria	5'-CGTTCG(C/T)TCTGAGCCAG-3'	16S, 19-35	18
BET42a :	β-Proteobacteria	5'-GCCTCCCCACTTCGTTT-3'	23S, 1027-1043	18
GAM42a :	γ-Proteobacteria	5'-GCCTCCCCACATCGTTT-3'	23S, 1027-1043	18
DEL :	δ-Proteobacteria	5'-CGGCGTCGCTGCGTCAGG-3'	16S, 385-402	1
CF319a :	*Cytophaga/Flavobacterium* group	5'-TGGTCCGTGTCTCAGTAC-3'	16S, 319-336	19
MCD :	MCD-bacteria	5'-CGCCACCAAAGCCTAAAAGG-3'	16S, 839-858	This study

[a] *Escherichia coli* numbering. [6]

Table 3 - 2. Concentration of dissolved microcystin in Lake Suwa, 1998 and 1999

Year	Date	dissolved microcystin concentration (μ g L^{-1})
1998[a]	Jun. 17	0.05
	Jul.29	0.47
	Aug. 26	0.24
	Sept. 25	0.17
	Oct. 21	0.09
	Dec. 3	N.D.[c]
1999[b]	May.20	0.09
	Jul. 28	0.03
	Aug. 11	0.04
	Aug. 25	0.10
	Sept. 8	0.12
	Sept. 22	0.08
	Oct. 6	0.06

[a]Microcystin determined with HPLC. Sum of microcystin-LR and -RR.

[b]Microcystin determined with ELISA.

[c]N.D., microcystin not detected.

Table 3 - 3. Number of *Microcystis* cells, free-living bacteria and bacteria associated with colonies of *Microcystis*

	Concentration of *Microcystis* cells (cells mL^{-1})	Associated bacteria (cells mL^{-1})[a]	Associated bacteria (cells *Microcystis* $cell^{-1}$)	Free-living bacteria (cells mL^{-1})	Ratio of associated bacteria to total bacteria (%)
1998. Aug. 26	2.9×10^5	$7.2\times10^6 \pm 1.1\times10^7$	25.1 ± 36.8	$4.6\times10^6 \pm 1.1\times10^6$	61
Sept. 9	7.4×10^5	$8.5\times10^7 \pm 1.8\times10^8$	116 ± 248	$4.3\times10^6 \pm 1.1\times10^6$	95
Oct. 7	2.2×10^5	$2.6\times10^7 \pm 4.2\times10^7$	118 ± 193	$3.9\times10^6 \pm 9.2\times10^5$	87
1999. Aug. 11	4.3×10^3	$1.1\times10^4 \pm 3.0\times10^3$	2.6 ± 0.7	$7.5\times10^6 \pm 1.5\times10^6$	0.2
Aug. 25	3.9×10^4	$1.5\times10^5 \pm 7.7\times10^4$	4.0 ± 2.0	$7.8\times10^6 \pm 2.0\times10^6$	1.9
Sept. 8	6.8×10^4	$3.2\times10^5 \pm 1.0\times10^5$	4.7 ± 1.5	$9.4\times10^6 \pm 1.5\times10^6$	3.3
Oct. 6	1.1×10^4	$3.8\times10^4 \pm 1.2\times10^4$	3.5 ± 1.1	$5.7\times10^6 \pm 1.2\times10^6$	0.7

[a] (*Microcystis* cells per mL of lake water) × (bacteria associated with *Microcystis* per *Microcystis* cell)

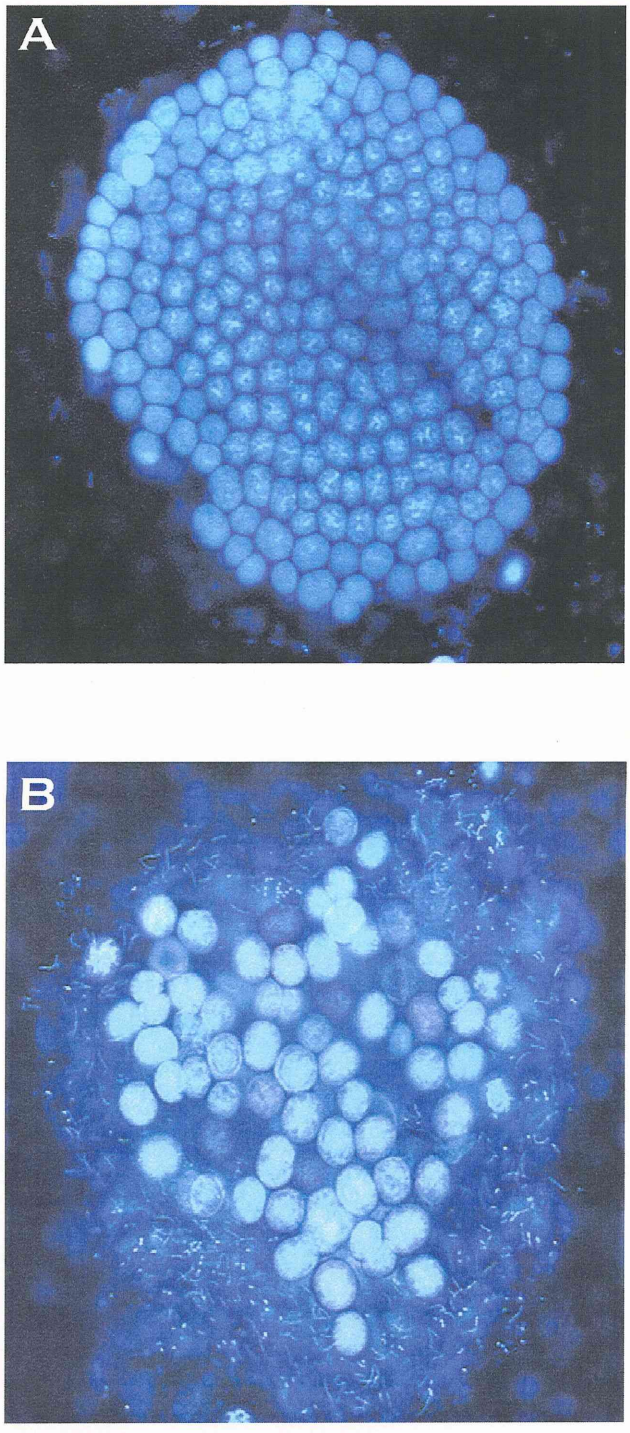


Figure 3-1

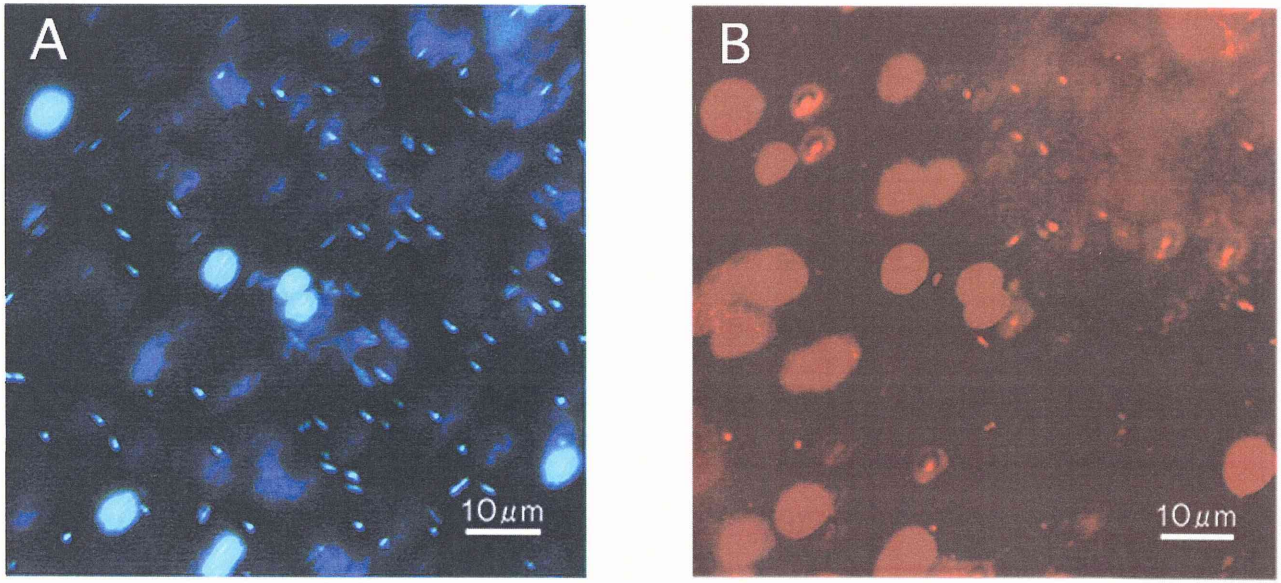


Figure 3-2

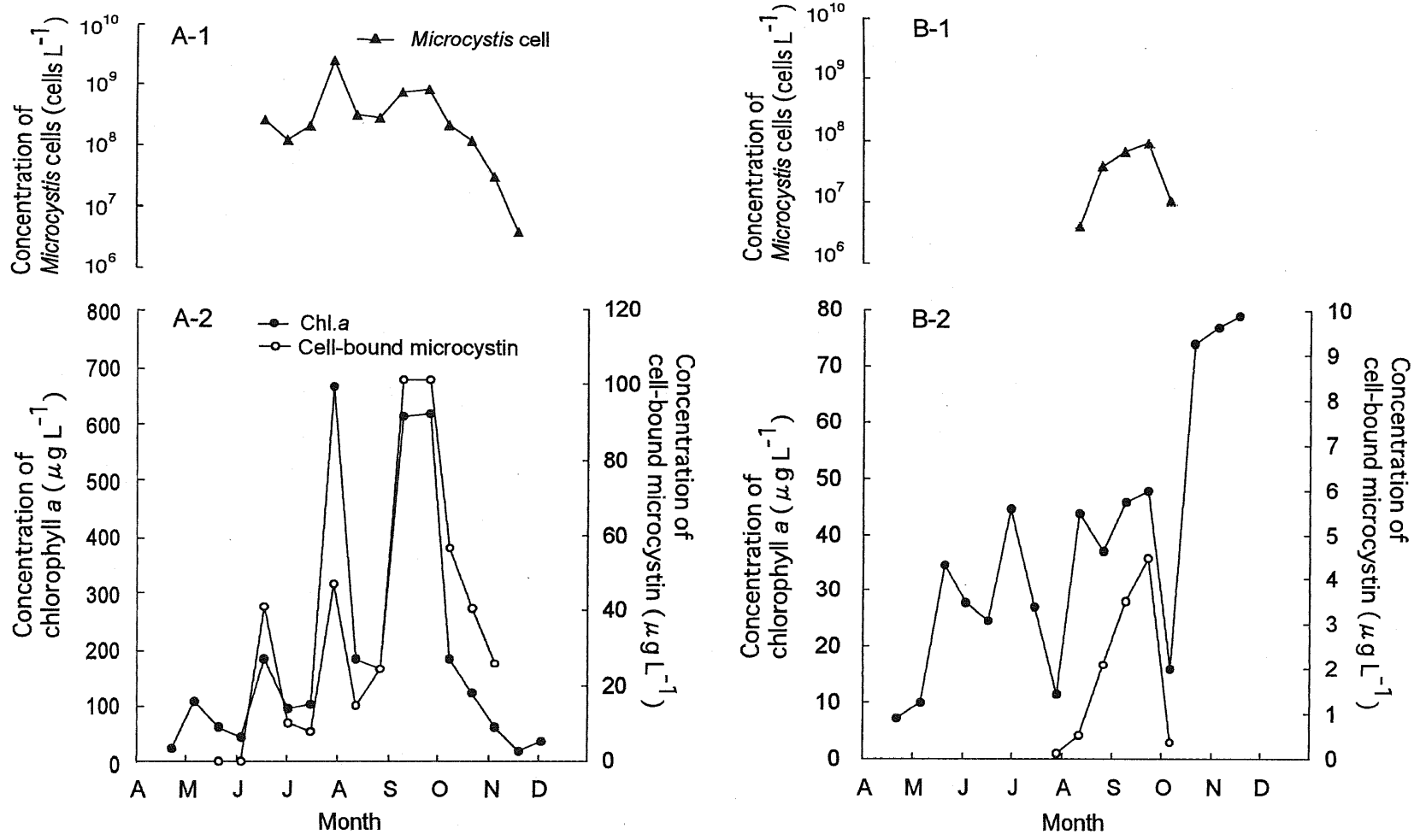

A-1
Microcystis cell
Concentration of Microcystis cells (cells L-1)
B-1
A-2
Chl.a
Cell-bound microcystin
Concentration of chlorophyll a (μg L-1)
Concentration of cell-bound microcystin (μg L-1)
B-2
A M J J A S O N D
Month

Figure 3 - 3

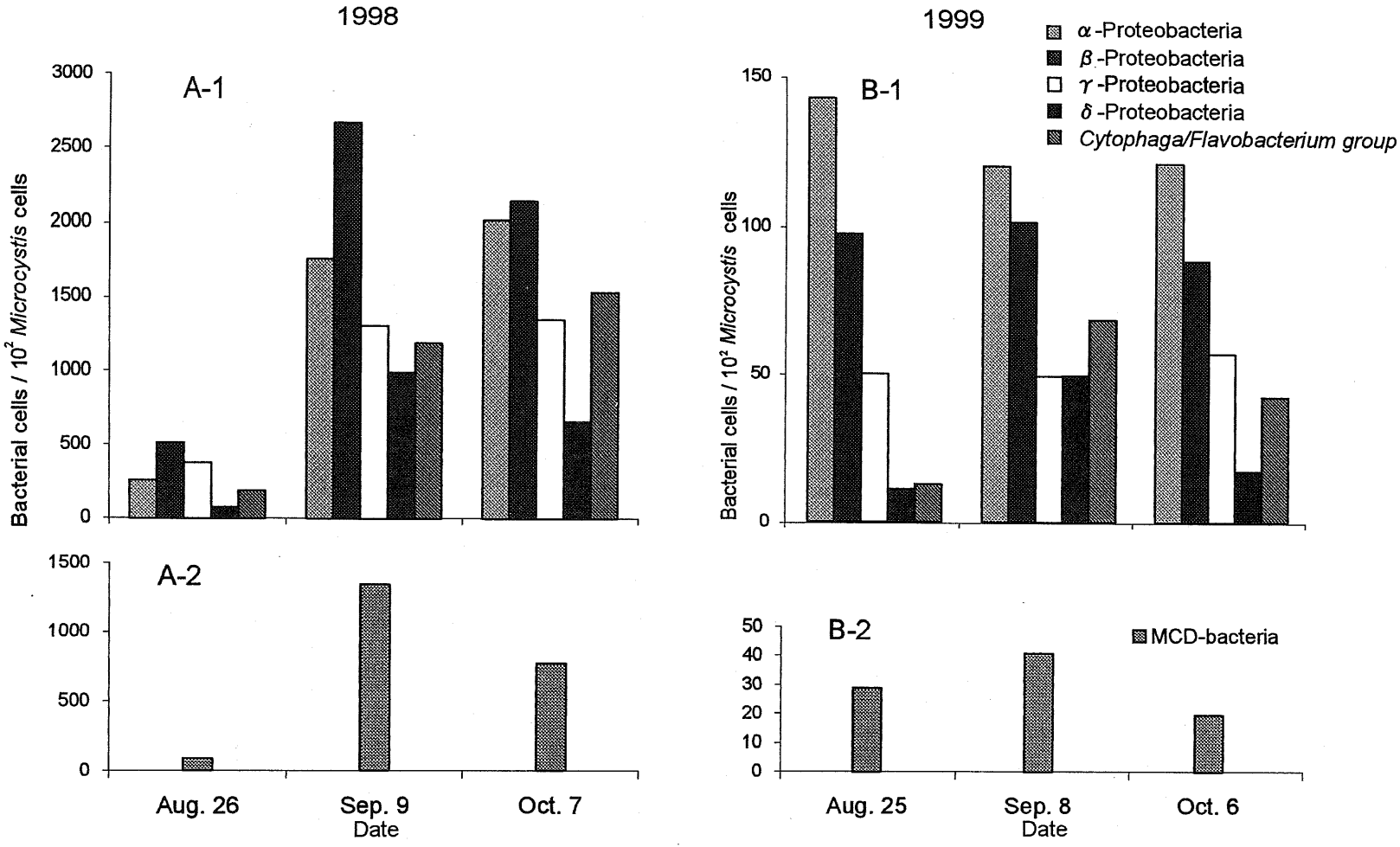

1998
1999
A-1
B-1
A-2
B-2
α-Proteobacteria
β-Proteobacteria
γ-Proteobacteria
δ-Proteobacteria
Cytophaga/Flavobacterium group
MCD-bacteria
Bacterial cells / 10² Microcystis cells
3000
2500
2000
1500
1000
500
0
150
100
50
40
30
20
10
Aug. 26
Sep. 9
Oct. 7
Aug. 25
Sep. 8
Oct. 6
Date

Figure 3-4

IV Free-living bacterial degradation of microcystin in an eutrophic lake: revealed with direct-FISH and DVC-FISH

In the chapter III, I reported first describing the population dynamics of MCD-bacteria and bacterial community structure in the mucilage of *Microcystis* during the bloom of *Microcystis*, revealing existence of MCD-bacteria in the mucilage of *Microcystis* [15]. The fluorescence in situ hybridization (FISH) method revealed a significant correlation between concentration of MCD-bacteria and the concentration of cell-bound Mcyst. Thus I suggested that MCD-bacteria associated with *Microcystis* colony responded to the changing of the concentration of cell-bound Mcyst, which was released from lysed *Microcystis* cells. The degradation process of Mcyst in situ was suggested in the previous study, but it remains uncertain whether Mcyst is completely degraded in the mucilage. The concentration of dissolved Mcyst fluctuated.

To understand the problem, I focused on free-living bacterial community structure in relation to Mcyst degradation and possible coexistence with other bacteria in an eutrophic lake. Previous studies indicate that free-living bacteria markedly differ from non free-living bacteria such as aggregate and attached bacteria in many ways [1, 2, 6]. They differ by their inability to degrade various macromolecules as non free-living counterparts. They also have smaller cell size and slower growth rate when compared to non free-living bacteria.

In this study, I attempted to characterize:

1) The population dynamics of free-living MCD-bacteria *in situ* .

2) The degradation process of Mcyst and the population dynamics of in situ free-living MCD-bacteria in the presence of Mcyst during different periods of *Microcystis* bloom.

I tried to employ the direct viable count (DVC) method [14] combined with the FISH analysis (DVC-FISH) [16] to enhance the detectability of specific bacteria.

(i) Materials and methods

Sampling of free-living bacteria

Samples of free-living bacteria were collected from the surface water of the center of Lake Suwa on May 20, August 26, September 9, October 7, October 21 in 1998, on May 20, August 11, August 25, September 8, October 6 in 1999, and on August 2, August 30, September 27 in 2000. Water samples were prescreened with a 3 μ m pore size Nuclepore filter (Whatman Ltd., UK) to collect free-living bacteria. The concentration of Chl.*a*, cell-bound Mcyst, dissolved Mcyst, *Microcystis* cells and associated bacteria with colony of *Microcystis* in 1998 and 1999 have been already reported in the chapter III [15].

Method of fixation for total count, direct viable count and fluorescence in situ hybridization

Bacterial samples for total free-living bacterial count, direct viable count and FISH were fixed by using 3% paraformaldehyde solution (pH 7.4) for 24 hours at 4℃. Fixed samples for FISH were filtered gently on a 0.22 μ m pore size Nuclepore filter (25 mm ϕ) [11]. Cells fixed on filters were rinsed 3 times with PBS, dehydrated by 1 mL of 50%, 80% and 99% ethanol each for 3 minutes, and filters were air-dried. Filters were stored at –20℃ until hybridization.

Total free-living bacterial count

Fixed samples were stained with 4', 6'-diamidino-2-phenylindole (DAPI, final concentration, 0.01 μ g mL^{-1}) and more than 500 bacterial cells were enumerated by epifluorescence microscopy (a universal epifluorescence microscopic system BX50-FLA, Olympus Co., Ltd., Japan).

Whole cell in situ hybridization

The 16S rRNA targeted oligonucleotide probes used in this study are shown in Table 3-1. They are comprised of a domain specific probe for Bacteria (EUB338), a group specific probe for α-, β-, γ-, and δ-Proteobacteria (ALF1b, BET42a, GAM42a, DEL), the *Cytophaga/Flavobacterium* group (CF319a), and an MCD-probe designed specifically for Mcyst-degrading bacteria, strain Y2 [15]. These probes were labeled with rhodamine (Takara Biotechnology (DALIAN) Co., Ltd., Japan).

Hybridizations were performed on filters put on slides coated with gelatin at 46℃ for 90 min with hybridization buffer, containing 0.9 M NaCl, 20 mM Tris•HCl (pH 7.4), 0.01% SDS, 20% formamide and 5 ng μ L^{-1} of the respective labeled probe. Each filter was washed in pre-warmed washing buffer at 48℃ for 15 min, containing 0.225 M NaCl, 20 mM Tris•HCl (pH 7.4), 5 mM EDTA, and 0.01% SDS, rinsed with distilled water and air-dried. The preparations, which were counterstained by 0.1 μ g mL^{-1} DAPI [18] on glass slides for 5 min, were observed using a

universal epifluorescence microscopic system BX50-FLA (Olympus Co., Ltd., Japan) with 3CCD camera (C5810, Hamamatsu photonics, Co., Ltd., Japan) with an image analysis system (SP500F, Olympus Co., Ltd., Japan). More than 500 DAPI-stained bacterial cells were counted to determine the contribution of the probe specific-labeled cells to the total of associated bacteria.

DVC – FISH method

An attempt was made to combine the DVC method [14] with FISH [16] and apply the technique to study population dynamics of free-living bacteria by determining concentrations of nalidixic acid and yeast extract over incubation time. A water sample was collected on May 20, 1999 and pre-filtered through a 3 μ m pore size Nuclepore filter to collect free-living bacteria. These samples (100mL) were incubated with 0.001%, 0.002% and 0.003% nalidixic acid containing 0.0025% or 0.025% yeast extract in the dark at 20℃. Samples were withdrawn at 0, 6, 12, 15, 18 and 21hours and then fixed with parafolmaldehyde solution as described above. To determine the effect of nalidixic acid on cell division, total bacterial count was estimated by staining fixed samples with acridine orange, AO (final concentration of 0.01 μ g mL^{-1}). More than 500 bacterial cells were enumerated by epifluorescence microscopy (BH2, Olympus Co., Ltd., Japan). The concentration of free-living bacteria defined by the cell number appeared to be inhibited by 0.003% nalidixic acid until 15hr(data not shown). In order to prolong incubation

period for DVC method until 16hr, Kalmbach et al. [10] raised the concentration of pipemidic acid to 0.003% to prevent cell division. The total bacterial cell number in samples receiving 0.003% nalidixic acid was not significantly different from those receiving 0.025% and 0.0025% yeast extract. Hence, the DVC method in combination with 0.003% nalidixic acid and 0.025% yeast extract was applied at 20℃ for 15hr.

Determination of concentration of Microcystis cells

Water samples collected from June to October were fixed with 1.5% formaldehyde solution. Abundance of *Microcystis* cells was estimated using an EKDS Haemacytometer (J.H.S., Bunkyo, Tokyo, Japan) under a microscope (BH-2, Olympus, Japan).

Purification and analysis of microcystin

Microcystis ichthyoblabe TAC95 (Tsukuba algal collection no. 95), which produces Mcyst-LR and 6(Z)-Adda-Mcyst-LR, was lyophilized and stored at −30℃ until purification of Mcyst was performed. Purification of Mcyst was carried out according to the method of Harada et al. [7] Lyophilized cells were extracted with 5% aqueous acetic acid and then supernatant was applied to an ODS silica gel cartridge (5g, Chromotorex ODS 100-200 mesh, packed into a polypropylene cartridge). The 90% of methanol-extracted elute from cartridge was then concentrated by rotary

evaporation at 30°C. The residue was used in the Mcyst degradation experiments. The concentrations of Mcyst were measured by the High performance liquid chromatography system (LC-9A S-I, Shimadzu, Japan) equipped with an ODS column (Cosmosil 5C18-AR 4.6×150 mm, Nacalai, Japan). The conditions of HPLC for analysis of Mcyst were: absorbance at 238 nm, a methanol: 0.05 M phosphate buffer (pH 3.0, 58:42) of moving phase, and a 1 mL min^{-1} flow rate.

Determination of microcystin degrading activity of free-living microcystin-degrading bacteria

Water samples were collected from Lake Suwa during the bloom of *Microcystis* on August 2, August 30, and September 27, 2000. Samples for free-living bacteria were collected by the procedure described above. Mcyst-LR was spiked into each free-living bacterial sample to a final concentration of 5 μ g mL^{-1}, incubated at 20°C with rotary shaking in the dark. Samples were withdrawn at various times for the analysis of Mcyst (100 μ L) and for total bacterial count and for fluorescence in situ hybridization (3mL).

Statistical analysis

Statistical analysis was performed by using the F-test, Welch's *t*-test and Peason's correlation coefficient test.

(ii) Results

Changes in concentration of free-living microcystin-degrading bacteria

Population dynamics of free-living bacteria in 1998 (Figure 4-1A) was investigated by direct-FISH method. Concentration of domain Bacteria increased from 4.9×10^5 cells mL^{-1} on May 20 to 2.4×10^6 cells mL^{-1} on October 21, 1998. Relative abundance of domain Bacteria increased from 30% of total free-living bacteria on May 20 to 54% of total free-living bacteria on October 7. Concentration of α -Proteobacteria, which included MCD-bacteria, increased from 1.7×10^5 cells mL^{-1} on May 20 to 1.0×10^6 cells mL^{-1} on October 7, and decreased to 7.4×10^5 cells mL^{-1} on October 21. MCD-bacteria were never detected by the direct-FISH method in 1998.

Since low rRNA concentration is the most ascribable reason for low sensitivity in the conventional FISH method as indicated by Amann et al. [3], I tried to increase rRNA concentration through incubation with yeast extract and nalidixic acid that prevents cell division. Thus, in 1999, I succeeded in detecting MCD-bacteria that existed in lake water by DVC-FISH method (Figure 4-1B). Concentration of domain Bacteria increased from 5.8×10^5 cells mL^{-1} on May 20 to 5.6×10^6 cells mL^{-1} on September 8, and decreased to 3.8×10^6 cells mL^{-1} on October 6, which range from 48% to 66% of the total free-living bacteria, respectively. Concentration of α -Proteobacteria increased from 1.1×10^5 cells mL^{-1} on May 20 to 1.3×10^6 cells mL^{-1} on August 11, and they ranged from 1.2 to 1.5×10^6 cells mL^{-1} from August 25 to October 6. Concentration

of MCD-bacteria increased markedly from 9.5×10^4 cells mL^{-1} on May 20 to 1.1×10^6 cells mL^{-1} on September 8, and the number dropped to 6.4×10^5 cells mL^{-1} on October 6. The relative abundance of MCD-bacteria to total bacteria ranged from 11% to 17% during the bloom of *Microcystis*. That ratio to α-Proteobacteria was high during the bloom, varying from 70% to 86% except August 11.

Degradation experiment using in situ free-living bacteria from early- to late-bloom of Microcystis

Microcystis appeared in the middle of June in 2000 and it's concentration increased from 5.1×10^4 cells L^{-1} on July 19 to 1.2×10^6 cells L^{-1} on August 15. The cell concentration dropped significantly from 3.8×10^5 cells L^{-1} on September 13 to 2.1×10^4 cells L^{-1} on September 27 (Figure 4-2). Free-living bacteria were collected from the lake during early-bloom of *Microcystis* (August 2): early-free-living bacteria (early-FL), mid-bloom of *Microcystis* (August 30): mid-FL, and late- bloom of *Microcystis* (September 27): late-FL. The concentration of cell-bound Mcyst on August 2, August 30 and September 27 in 2000 was 4.99, 1.43 and 3.25 μ g L^{-1}, respectively (Figure 4-2). Degradation of Mcysts in these samples is shown in Figure 4-3 A (early-FL), 4-3 B (mid-FL) and 4-3 C (late-FL). Mcyst-LR and 6(Z)-Adda-Mcyst-LR in all samples were degraded below the detection limits of HPLC. Both Mcysts in early-FL were not degraded at least until day 7, which is regarded as the lag time, and were degraded within 28 days (Figure 4-3 A). Analysis

of Mcyst was not done between day 7 and day 28. In mid-FL, both Mcysts were not degraded for 3 days and but the degradation was complete within 7 days (Figure 4-3 B). The degradation rate of MC-LR from day 3 to day 7 was 1.34 ug mL^{-1} day^{-1}, which is the highest number from three experiments. In late-FL, Mcyst-LR was not degraded for 14 days, but it degraded to 20 % of its initial concentration on day 21, with rapid degradation within 26 days (Figure 4-3 C). The degradation rate was 0.75 ug mL^{-1} day^{-1} between day 21and day 26. (Z)-Adda-Mcyst-LR in late-FL was gradually degraded until day 21, and then it degraded rapidly before day 23. The degradation rates were 0.08 ug mL^{-1} day^{-1} between the initial day and day 21 and 1.29 ug mL^{-1} day^{-1} between day 21 and day 23. 6(Z)-Adda-Mcyst-LR was degraded faster than Mcyst-LR. Mcysts were not degraded in filter-sterilized lake water.

Behavior of microcystin-degrading bacteria in early-, mid-, and late-bloom of Microcystis

The degradation activity of free-living MCD-bacteria differed in early-, mid- and late-bloom of *Microcystis*. I employed both direct-FISH and DVC-FISH to detect MCD-bacteria and succeeded in elucidating their population dynamics by the former technique. Concentration of total free-living bacteria, domain Bacteria and MCD-bacteria in Mcysts-added free-living bacteria in 2000 was shown in Figure 4-3 D, E, F. Total bacteria of early-FL increased from 1.2×10^7 cells mL^{-1} on the initial day to 5.7×10^7 cells mL^{-1} on day 8 and then decreased to 3.4×10^7 cells mL^{-1} on day 28

(Figure 4-3 D). Domain Bacteria in early-FL increased from 1.8×10^6 cells mL^{-1} to 4.0×10^7 cells mL^{-1} on day 8 and then decreased to 2.6×10^7 cells mL^{-1} on day 28. MCD-bacteria in early-FL increased from 1.6×10^5 cells mL^{-1} to 2.4×10^5 cells mL^{-1} on day 8.

Total bacteria of mid-FL gradually increased from 4.1×10^6 cells mL^{-1} to 4.3×10^7 cells mL^{-1} on day 7 (Figure 4-3 E). Domain Bacteria in mid-FL increased from 1.4×10^6 cells mL^{-1} to 3.0×10^7 cells mL^{-1} on day 7. MCD-bacteria didn't increase within 3 days fluctuating at around $1\text{-}2\times10^4$ cells mL^{-1}, which represents lag phase for degradation of Mcyst. This was followed by a rapid increase up to 2.0×10^5 cells mL^{-1} up to day 7 when Mcysts had already degraded. This increasing is significant ($P>0.01$). The doubling time for the period was 1.0 day.

Total bacteria of late-FL gradually increased from 8.9×10^6 cells mL^{-1} to 2.3×10^8 cells mL^{-1} on day 28 (Figure 4-3 F). Domain Bacteria in late-FL increased from 8.0×10^6 cells mL^{-1} to 2.2×10^8 cells mL^{-1} on day 28. MCD-bacteria in late-FL gradually increased from 7.5×10^4 cells mL^{-1} to 1.7×10^5 cells mL^{-1} on day 14 ($P>0.01$) during the lag time for Mcyst degradation and then they increased significantly to 4.9×10^6 cells mL^{-1} on day 28 ($P>0.01$) when Mcysts had already degraded. Doubling times from the initial day to 14 day and from day 14 to day 28 were 11.8 days and 2.7 days, respectively.

Concentration of α-, β-, and γ-Proteobacteria and *Cytophaga/Flavobacterium* group in the bacterial community structure between mid- and late-bloom was elucidated by direct-FISH

analysis (Figure 4-4). α-, β-, and γ-Proteobacteria and *Cytophaga/Flavobacterium* group except δ-Proteobacteria increased constantly during Mcyst degradation experiments and didn't exhibit the marked change derived from the addition of Mcysts.

(iii) Discussion

I recently elucidated the population dynamics of MCD-bacteria and their degradation activity of Mcyst in the mucilage of *Microcystis* [Chapter III, 15]. The MCD-bacteria in mucilage of *Microcystis* were the most abundant when concentrations of *Microcystis* and cell-bound Mcyst were the highest. This suggests that the degradation of Mcyst in mucilage of *Microcystis* occur through bacterial function. The concentration of dissolved Mcyst started to decrease before the decaying of *Microcystis* bloom, when Mcyst leaked from lysing *Microcystis*. This decreasing of Mcyst in water, thus, might be ascribable to degradation of Mcyst by biological activity. Free-living bacteria degraded Mcyst once it was released from *Microcystis* to surrounding water. In this study, I tried to elucidate whether free-living MCD-bacteria existed in water and whether in situ free-living MCD-bacteria degraded Mcyst in water.

Concentration of the free-living MCD-bacteria found in water increased markedly when the highest concentration of dissolved Mcyst and high concentration of *Microcystis* were observed with MCD-bacteria exceeding 10% of the total free-living bacteria. It is remarkable that in natural systems, as revealed by the FISH method, one gene specific clone of MCD-bacteria made up 1/10 of total free-living bacteria. Then, the concentration of dissolved Mcyst started to decrease before the decaying of *Microcystis* bloom [Chapter III, 15]. These facts suggest that free-living MCD-bacteria degraded Mcyst in water. It was known that microcystin is produced and maintained

within healthy cyanobacterial cells, but approximately 10 to 20% of Mcyst is released from healthy cyanobacterial cells to surrounding water [20, 21]. Mcyst was thus supplied to free-living MCD-bacteria even before *Microcystis* was decaying and bacteria could utilize it for their growth producing Mcyst-degrading enzyme.

The Mcyst degradation experiments using in situ free-living bacteria clearly showed that Mcyst was degraded completely within 28 days and lag time differed in free-living bacterial populations collected from the different blooming periods of *Microcystis*. Previous studies have shown that Mcyst was degraded in water but the degradation pattern of Mcyst varied with variable degradation rates and lag times before the degradation [4, 5, 9, 19]. Jones and Orr [9] reported that Mcyst-LR degraded rapidly within 3 days (95-95%) when *Microcystis* was treated by algicide ($CuSO_4$) and high concentration of Mcyst with 1.8 μ g mL^{-1} was kept for 9 days in water. Rapala et al. [19], Cousins et al. [5], and Christffersen et al. [4] carried out Mcyst degradation experiments using low concentrations of Mcyst (<0.2 μ g mL^{-1}) and showed that the degradation periods of Mcyst varied from 4 days to 2 weeks. These findings indicated the fact that both high and low concentration of Mcyst could be degraded in natural water with variable degradation periods although they could not reveal what degraded Mcyst in water.

Rapala et al. [19] and Cousins et al. [5] suggested that the indigenous microflora which exposed to cyanobacterial blooms degraded Mcyst faster than when they did not expose to it. Jones et al.

[8] also reported that Mcyst-degrading isolate could degrade Mcyst without lag time, if it was exposed to Mcyst before the experiment. Cousins et al. [5] suggested that the period of Mcyst degradation depended on temperature and community structure. Kiviranta et al. [13] reported that biodegradation of [D-Asp^3]-Mcyst-RR did not occur in the water sample which was not exposed to the bloom. These data suggest that differences in lag time and degradation period of free-living bacteria in different periods of bloom was affected by both temperature and exposure to Mcyst. Two free-living bacterial populations taken from a similar temperature regime: 27.0 ℃ (early-FL) and 26.1℃ (mid-FL) had differing lag times; with latter population showing more rapid degradation due to exposure of causal bacteria to Mcyst longer than the early phase of bloom.

Our results did not show the remarkable changes in free-living bacterial community structure during the Mcyst degrading experiments. Cell number of each phylogenetic bacterial group except δ -Proteobacteria increased during the experiments. Decrease in δ -Proteobacteria during degradation periods suggests that this group did not related to the degradation of Mcysts and even their growth was suppressed by Mcysts. Christffersen et al. [4] tried to elucidate the changes of indigenous bacterial community structure in the Mcyst-degrading experiments using DGGE, and showed that the diversities of bacteria increased during the Mcyst-degrading experiments responding to *Microcystis* lysates. However, the community structure was not discussed phylogenetically. Van Hannenn [22] showed that the 16S rRNA sequence related to Cytophagales,

which could contribute to degrading dissolved organic matter (DOM) derived from cyanobacteria, appeared in DGGE after the lysis of cyanobacteria. Together with these findings, we previously reported that two characteristic peaks of Mcyst intermediate degradation products appeared during the Mcyst degradation experiments using the isolate [17]. However, these products were not detected during all three degradation experiments in this study. The degradation of Mcyst is consisted of several steps [17], thus the process might be carried out not only by MCD-bacteria but also other free-living bacteria, which degrade the intermediate products.

However, these findings do not deny the fact that the MCD-bacteria are the major population responsible for Mcyst degradation and at least the initiation of Mcyst degradation. Specific Mcyst degradation activity of MCD-bacteria was estimated by using the amount of degraded Mcyst and the number of free-living MCD-bacteria measured by direct enumeration. The specific activity of Mcyst degradation per MCD-bacterial cell in early-, mid- and late-bloom of *Microcystis* was 5.89 $\times 10^{-8}$, 9.59×10^{-7} and 8.01×10^{-9} μ g cell^{-1} hr^{-1}, respectively. Converting the concentration of Mcyst into the carbon, the concentration of carbon uptake per MCD-bacterial cell in each sample was 3.46×10^{-8}, 5.56×10^{-7} and 4.64×10^{-9} μ g C cell^{-1} hr^{-1}, respectively. If compared these numbers with those of glucose uptake measured in a mesotrophic lake, Lake Constance [12], the specific activity of Mycst degradation was significantly high. The highest specific activity of free-living MCD-bacteria was calculated in mid-bloom of *Microcystis* when high concentration of

Microcystis was observed. Thus, I suggest that free-living MCD-bacteria degraded Mcyst actively in water and their degradation activity was caused by the exposure to Mcyst.

(iv) References

1. Acinas SG, Anton J, Valera FR. (1999) Diversity of free-living bacteria and attached bacteria in offshore western mediterranean waters as depicted by analysis of genes encoding 16SrRNA. Appl Environ Microbiol 65: 514-522.

2. Acinas SG, Rodoriguez-Valera F, Pedros-Alio C. (1997) Spatial and temporal variation in marine bacterioplankton diversity as shown by RFLP fingerprinting of PCR amplified 16SrDNA. FEMS Microbiol Ecol 24: 27-40.

3. Amann RI, Ledwig W, Schleifer K-H. (1995) Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol Rev 59: 143-169.

4. Christoffersen K, Lyck S, Winding A. (2002) Microbial activity and bacterial community structure during degradation of microcystins. Aquat Microb Ecol, 27: 125-136.

5. Cousins IT, Bealing DJ, James HA, Sutton A. (1996) Biodegradation of microcystin-LR by indigenous mixed bacterial populations. Wat Res 30: 481-48.

6. Delong EF, Franks DG, Alldredge AL. (1993) Phylogenetic diversity of aggregate-attached vs. free-living marine bacterial assemblages. Limnol Oceanogr 38: 924-934.

7. Harada K-I, Matsuura K, Suzuki M, Oka H, Watanabe MF, Oishi S, Dahlem AM, Beasley VR, Carmichael WW. (1988) Chemical analysis of toxic peptides produced by cyanobacteria. J Chromatogr 448: 275-283.

8. Jones GJ, Bourne DG, Blakeley RL, Doelle H. (1994) Degradation of the cyanobacterial hepatotoxin microcystin by aquatic bacteria. Natural Toxins 2: 228-235.

9. Jones GJ, Orr PT. (1994) Release and degradation of microcystin following algicide treatment of a *Microcystis* aeruginosa bloom in a recreational lake, as determined by HPLC and protein phosphatase inhibition assay. Wat Res 28: 871-876.

10. Kalmbach S, Manz W, Szewzyk U. (1997) Isolation of new bacterial species from drinking water biofilms and proof of their in situ dominance with highly specific 16S rRNA probes. Appl Environ Microbiol 63: 4164-4170.

11. Kato K, Sakamoto M. (1983) The function of free-living bacterial fraction in the organic matter metabolism of mesotrophic lake. Arch Hydrobiol 97: 287-302.

12. Kato K, seasonal observation of heterotrophic activity and active bacterial number in lake Constance Arch Hydrobiol suppl, 1985. 66: 309-319.

13. Kiviranta J, Sivonen K, Lahti K, Luukkainen R, Niemelä SI. (1991) Production and biodegradation of cyanobacterial toxins: a laboratory study. Arch Hydrobiol 121: 281-294.

14. Kogure K, Simidu U, Taga N. (1979) A tentative direct microscopic method for counting living marine bacteria. Can J Microbiol 25: 415-420.

15. Maruyama T, Kato K, Yokoyama A, Tanaka T, Hiraishi A, Park H-D, Dynamics of microcystin-degrading bacteria in mucilage of *Microcystis*. Microb Ecol (in press).

16. Nishimura M, Kogure K, Tsukamoto KK, Ohwada K. (1995) Detection and direct count of specific bacteria in natural seawater using 16S rRNA oligonucleotide probe. Bull Jap Soc of Microbiol Ecol 10: 109-113.

17. Park H-D, Iwami C, Watanabe MF, Harada K-I, Okino T, Hayashi H. (1998) Temporal variabilities of the concentrations of intra- and extracellular microcystin and toxic *Microcystis* species in a hypertrophic lake, Lake Suwa, Japan (1991-1994). Environ Toxicol Water Qual 1: 61-72.

18. Poter KG, Feig YS. (1980) The use of DAPI for identifying and counting aquatic microflora. Limnol Oceanogr 25: 943-948.

19. Rapala J, Lahti K, Sivonen K and Niemelä SI. (1994) Biodegradability and adsorption on lake sediments of cyanobcterial hepatotoxin and anatoxin-a. Letters in Applied Microbiology, 19: 423-428.

20. Rapala J, Sivonen K, Lyra C, Niemelä SI (1997) Variation of microcystins, cyanobacterial hepatotoxins, in *Anabaena* spp. as a function of growth stimuli. Appl Environ Microbiol 63: 2206-2212.

21. Sivonen, K. (1990) Effect of light, temperature, nitrate orthophosphate, and bacteria on growth of and hepatotoxin production by *Oscillatoria agardhii* strains. Appl Environ Microbiol 56: 2658-2666.

22. Van Hannen EJ, Zwart G, van Agterveld MP, Gons HJ, Ebert J, Laanbroek HJ. (1999) Changes in bacterial and eukaryotic community structure after mass lysis of filamentous cyanobacteria associated with viruses. Appl Environ Microbiol 65: 795-801.

(v) Figure Legends

Figure 4 - 1

Number of free-living bacteria detected by direct- and DVC-FISH with probes specific for domain Bacteria, α-Proteobacteria and microcystin degradation bacteria (MCD). The samples collected from surface waters of Lake Suwa on 20 May, August, 8 September, 6 October 1999.

Figure 4 - 2

Changes in the concentrations of *Microcystis* cells (closed circles) and cell-bound microcystin (solid bars) at the center of Lake Suwa in 2000.

Figure 4 - 3

Degradation of microcystins in filtered water using 3 μm pore size Nuclepore filter. Water samples were collected from Lake Suwa on 2 August (A, D), 30 August (B, E), 27 September (C, F) 2000. A, B, C indicate the changes in the concentration of Mcyst-LR (circles) and its isomer (squares) in filtered lake water during incubation. Black and open points indicate with and without free-living bacteria, respectively. E, F, G show the changes in the number of total bacteria (open circles), domain Bacteria(open squares) and MCD-bacteria (solid bars)during incubation.

Figure 4 - 4.

Dynamics of free-living bacterial community structure estimated by rRNA-targetted oligonucleotide probes specific for α -, β -, γ -, δ -Proteobacteria and *Cytophaga/Flavobacterium* group during microcystin degradation. Water samples were collected from Lake Suwa on August 30 (A) and on September 27(B), 2000. Black and open circles indicate total free-living bacteria and domain Bacteria, respectively. Asterisks indicate not detected.

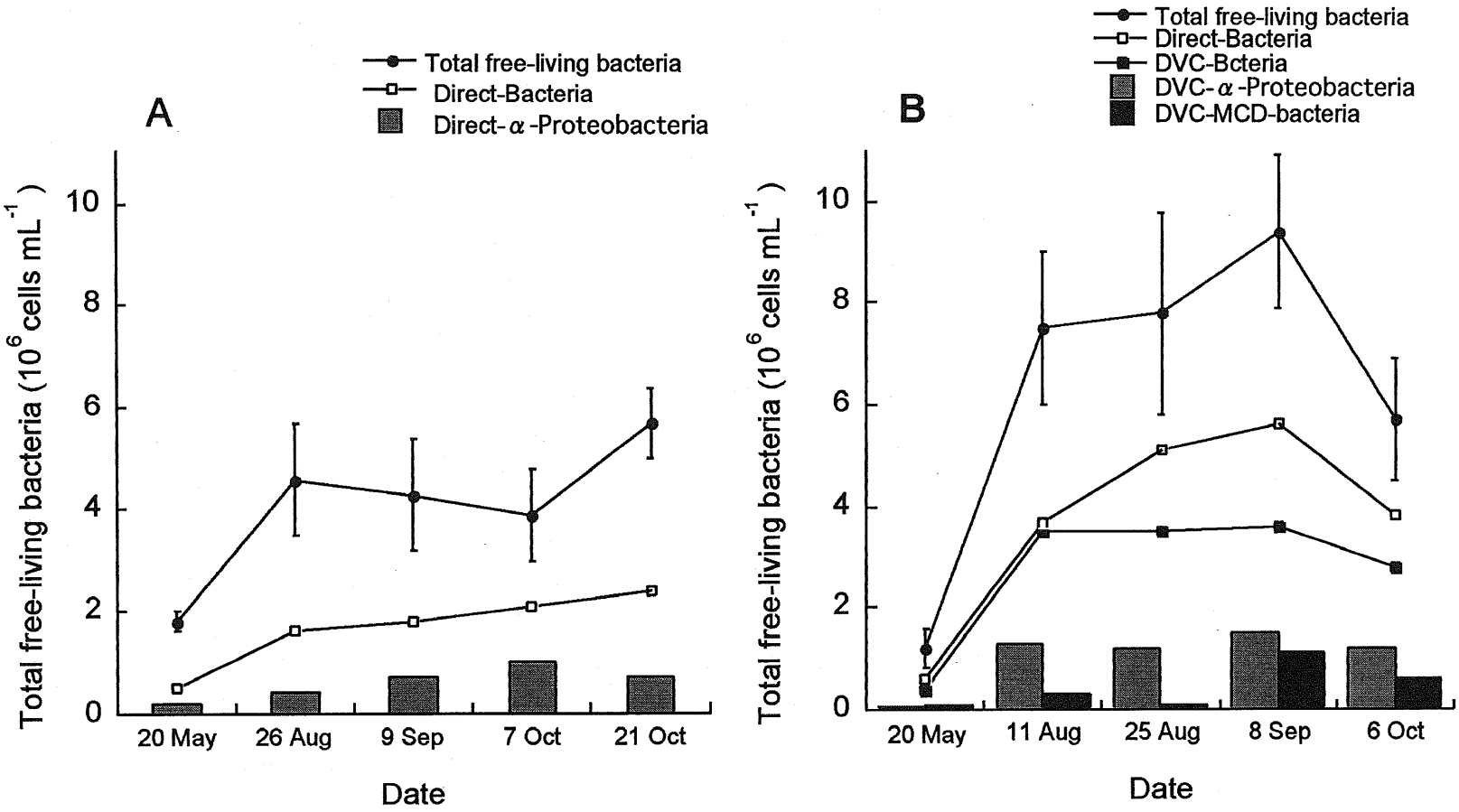

A
Total free-living bacteria
Direct-Bacteria
Direct-α-Proteobacteria
Total free-living bacteria (10^6 cells mL^-1)
0
2
4
6
8
10
20 May
26 Aug
9 Sep
7 Oct
21 Oct
Date
B
Total free-living bacteria
Direct-Bacteria
DVC-Bcteria
DVC-α-Proteobacteria
DVC-MCD-bacteria
Total free-living bacteria (10^6 cells mL^-1)
0
2
4
6
8
10
20 May
11 Aug
25 Aug
8 Sep
6 Oct
Date

Figure 4 - 1

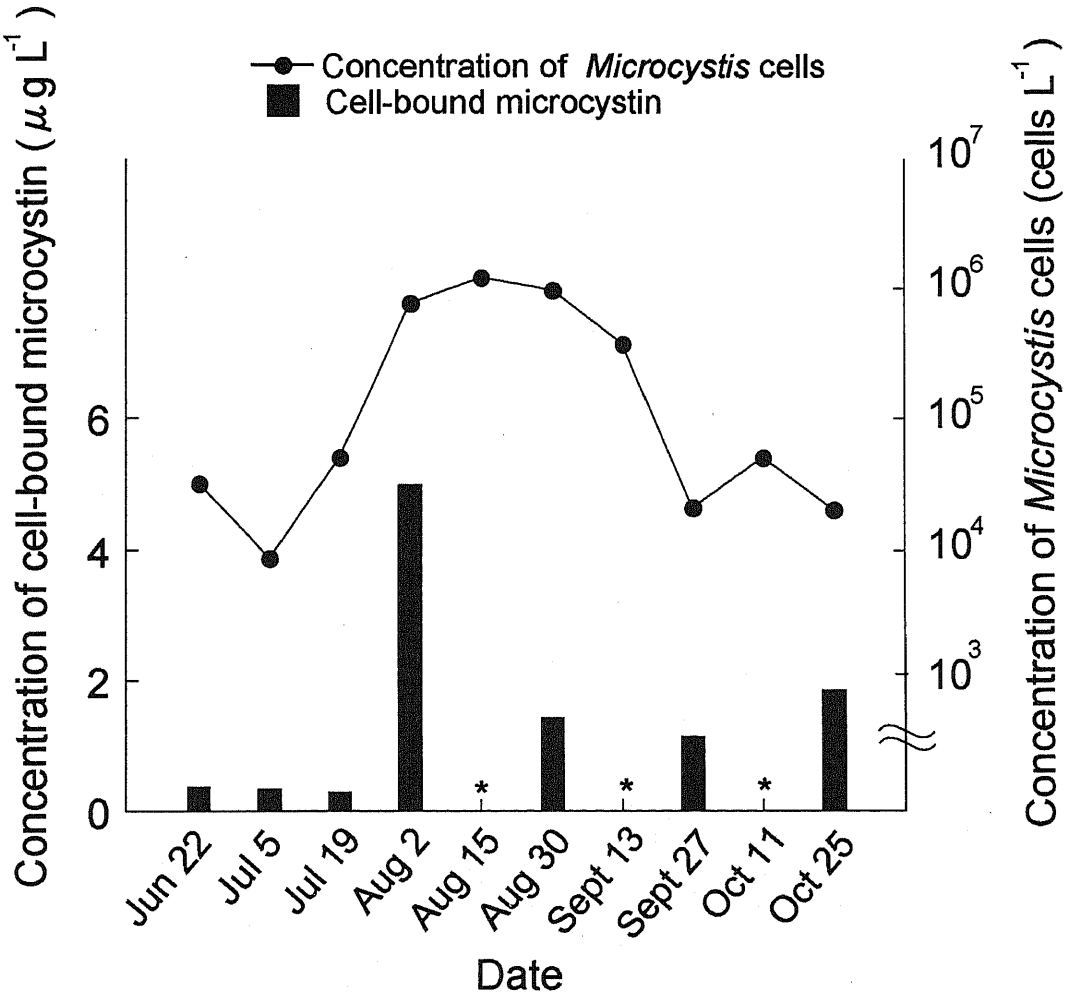

Concentration of *Microcystis* cells
Cell-bound microcystin
Concentration of cell-bound microcystin (μg L^{-1})
Concentration of *Microcystis* cells (cells L^{-1})
0
2
4
6
10^3
10^4
10^5
10^6
10^7
*
Jun 22
Jul 5
Jul 19
Aug 2
Aug 15
Aug 30
Sept 13
Sept 27
Oct 11
Oct 25
Date

Figure 4 - 2

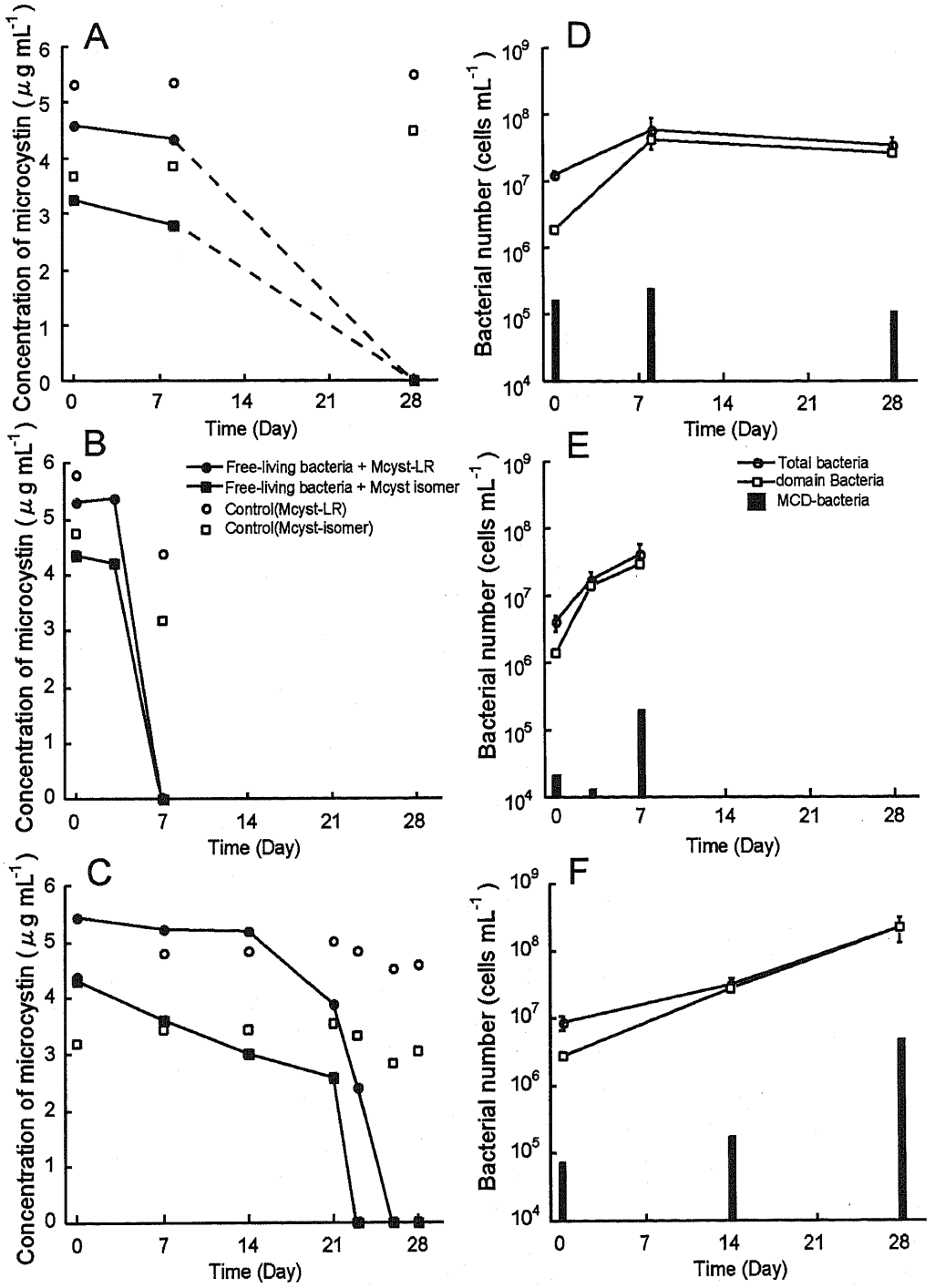

A
B
C
D
E
F
Concentration of microcystin (μg mL^{-1})
Bacterial number (cells mL^{-1})
Time (Day)
Free-living bacteria + Mcyst-LR
Free-living bacteria + Mcyst isomer
Control(Mcyst-LR)
Control(Mcyst-isomer)
Total bacteria
domain Bacteria
MCD-bacteria

Figure 4 - 3

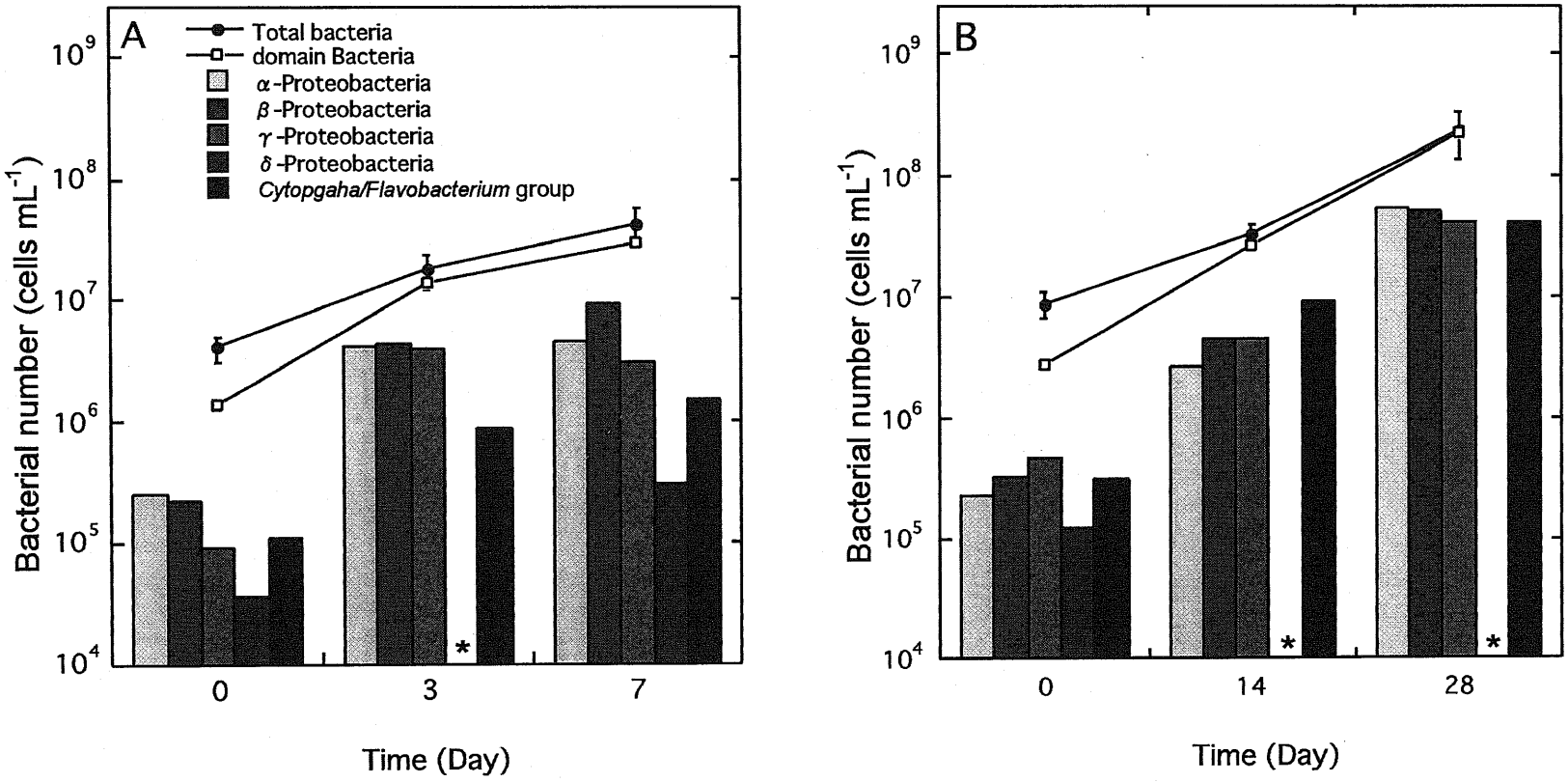

A
Total bacteria
domain Bacteria
α-Proteobacteria
β-Proteobacteria
γ-Proteobacteria
δ-Proteobacteria
Cytopgaha/Flavobacterium group
Bacterial number (cells mL-1)
10^9
10^8
10^7
10^6
10^5
10^4
0
3
7
*
Time (Day)
B
Bacterial number (cells mL-1)
0
14
28
*
Time (Day)

Figure 4 - 4

V Concluding remarks

Little is known about the degradation procedure of Mcyst by bacteria under the natural environment. To elucidate this, I focused population dynamics of cyanobacterial hepatotoxic microcystin-degrading bacteria (MCD-bacterium) in a eutrophic lake, Lake Suwa, where *Microcystis* blooms annually. The findings shown in part III and IV can be summarized as follows:

- Lake Suwa is a eutrophic, temperate and shallow lake with a maximum depth of 6.4 m and an average depth of 5m. The maximum concentration of total nitrogen and phosphorus ca. 2000 ug L^{-1} and 200 ug L^{-1}. Water temperature ranged from 20 to 27 ℃ through out the blooming period of *Microcystis*.
- We succeeded in isolation of MCD-bacterium, strain Y2, from Lake Suwa during the blooming period of *Microcystis*.
- According to 16S rRNA gene analysis, strain Y2 is elucidated to be a new species of new genus. Based on the 16S rRNA gene sequence of this strain, we designed an oligonucleotide probe which is specific to this MCD-bacterium.
- Fluorescence in situ hybridization (FISH) analysis using the designed probe clearly showed that MCD-bacteria existed in the mucilage of *Microcystis* as well as in water

during the blooming period of *Microcystis*.

- Direct viable count method combined with FISH method showed high potential to detect free-living MCD-bacteria if their rRNA content is low.
- When a high concentration of cell-bound Mcyst was detected, MCD-bacteria existed in the mucilage exceeding 10% of the sum of bacteria detected by hybridization with group-specific probes.
- There exists a high correlation between the number of MCD-bacteria in the mucilage and the concentration of cell-bound Mcyst.
- *Cytophaga/Flavobacterium* group and δ-Proteobacteria in the mucilage were synchronized in population dynamics according to the changes in the concentration of *Microcystis*.
- The highest concentration of free-living MCD-bacteria was found when the both concentrations of dissolved Mcyst and *Microcystis* were high.
- In the time a high concentration of dissolved Mcyst was detected, MCD-bacteria existed exceeding 10% of total bacteria.
- Free-living bacteria completely degraded Mcyst-LR and 6(Z)-Adda-Mcyst-LR. Lag times for the degradation differed apparently among free-living bacterial populations taken from early-, mid-, late bloom of *Microcystis*. Mcyst were degraded the fastest in

mid-bloom.

- The highest degradation activity of MCD-bacteria was estimated in mid-bloom of *Microcystis*.
- Free-living MCD-bacteria increased remarkably just after the Mcyst degradation.
- Free-living bacteria other than MCD-bacteria increased during the degradation of Mcyst but their community structure didn't change apparently except δ -Proteobacteria.

Mcyst, which was produced by *Microcystis* and the other several cyanobacteria, is detected in eutrophic and temperate lakes and ponds. This is the first study describing the in situ bacterial Mcyst degradation focusing on population dynamics of MCD-bacteria in natural environment. It is extremely high that one gene specific clone (MCD-bacteria) exceeded 10% of sum of bacteria hybridized with probes. The results that a high correlation between the concentration of MCD-bacteria in mucilage and cell-bound Mcyst suggest that MCD-bacteria in mucilage produced the degradation enzyme responding to changes in the concentration of Mcyst exuded from *Microcystis* cells to mucilage. Since the fluorescence intensity of the hybridized cell derived from probes labeled with fluorescence depends on cellular rRNA content, the successful detection of MCD-bacteria in mucilage explains that they contained sufficient amount of rRNA which leads that they are active. On the other side,

FISH analysis revealed that concentration of MCD-bacteria was high in water in relation to the concentration of Mcyst in water. These findings reveal that MCD-bacteria in water also carried out degradation of the toxin released from *Microcystis* into surrounding water. The results that the number of MCD-bacteria increased just after the degradation with high degradation activity strongly suggest MCD-bacteria degrade Mcyst to utilize for their growth. Thus, I suggest that MCD-bacteria are the major player of Mcyst degradation both in mucilage and in water in natural environment with various bacteria.

The role of the coexistent bacteria concerning the Mcyst degradation was also elucidated from FISH analysis that *Cytophaga* group and δ-Proteobacteria in the mucilage probably contribute to the degradation of *Microcystis* cells. The findings suggest not only the key role of MCD-bacteria in detoxification, but also a possible sequence in degradation of *Microcystis* cells and microcystin kept in the cell, which is carried out by bacterial consortia in the mucilage. The intermediate products of degradation were not detected during the Mcyst degradation experiments using free-living bacteria, though they appeared when Mcyst was degraded by strain Y2. The finding suggests that the other coexistent bacteria at least in water probably contribute to the degradation of the intermediate products. Thus, I conclude that in situ Mcyst degradation was carried out in various bacterial consortia with combination of 3 actors: MCD-bacteria in mucilage of *Microcystis*, MCD-bacteria in water, and the other

coexistent bacteria contribution to degrade intermediate products (Figure 5-1).

The biodegradation of Mcyst occur in eutrophic lakes where toxic cyanobacteria producing Mcyst blooms. To initiate the production of degradation enzyme specific to Mcyst, the exposure to Mcyst is the most important inducer for MCD-bacteria. The fact that Mcyst degradation period varied during the bloom of *Microcystis* revealed that MCD-bacteria both in the mucilage and in water sensitively respond to changes in the concentration of the toxin leaked from *Microcystis* and that they adjust production of the specific degradation enzyme.

The condition of water temperature is variable in lakes and ponds where Mcyst producing toxic cyanobacteria blooms, since toxic cyanobacteria widely distribute from temperate to cold region, such as Finland, Denmark and Norway (Figure 2-1). The Mcyst degradation experiments using strain Y2 revealed that the degradation rate apparently depends on the temperature (Figure 2-5). Thus, the degradation rate and period may vary depending on in situ water temperature even if toxic cyanobacteria could bloom even under the low temperature there and in situ MCD-bacteria are exposed by the toxin.

MCD-bacteria degraded variable structure of Mcysts such as Mcyst-LR, -RR, -YR [Chapter II, 5] and 6(Z)-Adda-Mcyst-LR with variable degradation rate. 6(Z)-Adda-Mcyst-LR is produced not only by *Microcystis* species but also by isomarization of Mcyst-LR derived from photolysis [7]. The results from Mcyst degradation experiments showed that

6(Z)-Adda-Mcyst-LR was degraded faster than Mcyst-LR in water. Thus, the isomarization of 6(Z)-Adda-Mcyst-LR may accelerate biodegradation in natural water.

Two isolates of MCD-bacteria, strain Y2 and strain MJ-PV, belonging to different genus but phylogenetically related, belong to the group of *Sphingomonas* and the relatives of α-Proteobacteria. They appeared under the existence of Mcyst as once prior candidates of these bacteria were exposed to Mcyst, and then they became to produce the specific degradation enzyme to degrade Mcyst. To elucidate the evolutional relationship between occurrence s of Mcyst and MCD-bacteria, I attempt to analyse the vertical distribution of MCD-bacteria and concentration of Mcyst in sediment. The history of the bloom of *Microcystis* caused by eutrophication of the lake as well as the occurrence of MCD-bacteria was kept in sediment. Little is known about the in situ Mcyst biodegradation in sediment focusing on functional bacterial population dynamics [1, 4, 6]. I already revealed that MCD-bacteria exist in sediment of Lake Suwa with FISH analysis[3]. The combination of FISH analysis with specific probe for MCD-bacteria and quantitative analysis of Mcyst should elucidate the Mcyst biodegradation activity and its history in sediments.

(i) References

1. Cousins IT, Bealing DJ, James HA, Sutton A. (1996) Biodegradation of microcystin-LR by indigenous mixed bacterial populations. Wat Res 30: 481-48.

2. Maruyama T, Kato K, Yokoyama A, Tanaka T, Hiraishi A, Park H-D, Dynamics of microcystin-degrading bacteria in mucilage of *Microcystis*. Microb Ecol (in press).

3. Morris RJ, Williams DE, Luu HA, Holmes CHB, Andersen RJ, Calvert SE. (2000) The adsorption of microcystin-LR by natural clay particles. Toxicon 38: 303-308

4. Park H-D, Sasaki Y, Maruyama T, Yanagisawa E, Hiraishi A, Kato K. (2001) Degradation of the cyanobacterial hepatotoxin microcystin by a new bacterium isolated from a hypertrophic lake. Environ Toxicol 16: 337-343.

5. Rapala J, Lahti K, Sivonen K and Niemelä SI. (1994) Biodegradability and adsorption on lake sediments of cyanobcterial hepatotoxin and anatoxin-a. Letters in Applied Microbiol 19: 423-428.

6. Tsuji K, Naito S, Kondo F, Ishikawa N, Watanabe MF, Suzuki M, Harada K-I. (1994) Stability of microcystins from cyanobacteria: effect of light on decomposition and isomerization. Environ Sci Technol 28: 173-177.

(ii) Figure legend

Figure 5-1. Bacterial degradation process of microcystin in natural water

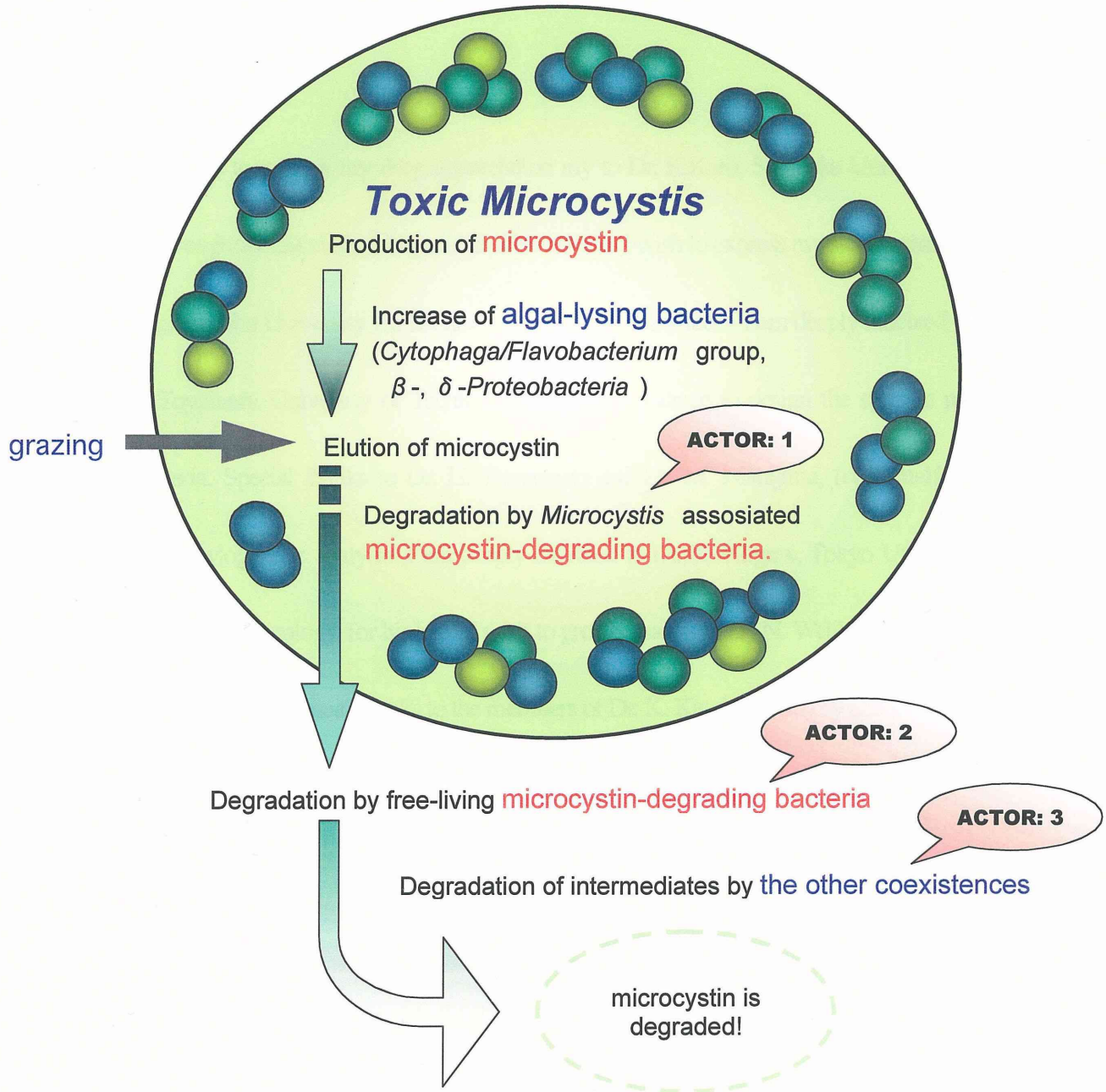

Toxic Microcystis
Production of microcystin
Increase of algal-lysing bacteria
(Cytophaga/Flavobacterium group,
β-, δ-Proteobacteria)
grazing
Elution of microcystin
ACTOR: 1
Degradation by Microcystis assosiated
microcystin-degrading bacteria.
ACTOR: 2
Degradation by free-living microcystin-degrading bacteria
ACTOR: 3
Degradation of intermediates by the other coexistences
microcystin is
degraded!

Figure 5-1

VI. Acknowledgement

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