

Seasonal changes in the abundance of biological agents killing *Microcystis aeruginosa* in a hypereutrophic pond

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Abstract

Seasonal changes in abundance of the heterotrophic flagellates *Polytomella* sp., rotifers *Cephalodella* sp., *Brachionus caliciflorus* and the testate amoeba *Penardochlamys* sp. as grazers, cyanophages and algicidal bacteria as infectious agents on mortality of cyanobacterium, *Microcystis aeruginosa* was intensively studied in a hypereutrophic pond from September to November 2000. Abundance of the rotifers *Cephalodella* sp., *B. caliciflorus* and the *Penardochlamys* sp. were relatively high with large fluctuations. The cell density of *M. aeruginosa* ranged between 3.0×10^5 to 1.9×10^7 cells ml^{-1} , where those of algicidal bacteria were between 0.3×10^2 to 3.7×10^2 PFU ml^{-1} and cyanophages were between 0.9×10^3 to 7.1×10^3 PFU ml^{-1} . Algicidal bacteria were relatively high with fluctuation between 0.3×10^4 to 3.7×10^4 PFU ml^{-1} and bacterial peaks were followed with *M. aeruginosa* peaks. Occasional cyanophages peaks on 2 and 20 October were followed by sudden collapse of *M. aeruginosa* bloom. Percentages of *M. aeruginosa* cells in food vacuole of *Penardochlamys* sp. was high (>77%) during September following increase of the *M. aeruginosa* cell density. The flagellate *Polytomella* sp. was increased to a peak (2.5×10^5 indi. m^{-1}) from 10 to 17 November with decrease of *M. aeruginosa* cell density. The percentage of *B. caliciflorus* individuals ingesting colonies of *M. aeruginosa* during the bloom period

was significantly high (t-test, $P < 0.01$). Percentage of testate amoebae *Penardichlamys* sp. ingested *M. aeruginosa* cells was very high (>77%) during the bloom period. The food vacuoles of *Penardichlamys* sp. contained only *Microcystis* indicating its specific preference of *Microcystis*. The overall results of the study suggest the grazers' protozoa; *Polytomella* sp., and the testate amoeba *Penardochlamys* sp., zooplankton; *Cephalodella* sp. and *Brachionus caliciflorus*, cyanophages and algicidal bacteria successively involved in suppressing *M. aeruginosa* bloom in a freshwater environment.

Key words : *Microcystis aeruginosa*, cyanophages, algicidal bacteria, *Penardochlamys* sp., *Cephalodella* sp. and *Brachionus caliciflorus*,

Introduction

Cyanobacterial blooms have been reported in many eutrophic and hypertrophic water bodies, most commonly as *Microcystis aeruginosa* (Olli, 1996, Takamura & Watanabe, 1988, Zohar & Roberts, 1989). Massive accumulations of bloom of *Anabaena* sp., *Microcystis* sp. can have wide range of social, economic and environmental problems such as fish kills (Bury et al. 1996), accumulation of toxin in aquatic animals (Lykke & Kirsten, 1999 -*Daphnia magna*), death of animals (Galey et al. 1987 -Cattle); Matsunaga et al. 1999 (bird), foul odours (Silvey & Roach, 1975) and potential harmful effect on human health (Carmichael, 1988; Gorham & Carmichael, 1988; Falconer, 1989; Song et al., 1998). The physiology and ecological characteristics of *M. aeruginosa* have been extensively studied, and the blooming mechanism has been clarified (Zohary & Roberts, 1989). However, little has so far been examined on the decomposition processes of cyanobacterial bloom. Previous studies have reported that some microbial processes were responsible for decomposition of cyanobacterial blooms. Despite of their large biomass, the cyanobacteria are inedible prey for many planktonic organisms because of their toxic effects (Fulton & Pearl, 1987a; Christoffersen, 1996) large colony size (Fulton & Pearl, 1987a,b) and low nutritional values (Hanazato & Yasuno, 1987). Some species of protists can be important consumers of bloom-forming cyanobacteria. Graze on *M. aeruginosa* by flagellates (Klaveness, 1995, Nishibe et al. 2002, Zhang et. al 1996), ciliates (Zhang et al. 1996), Sarcodin (Dryden & Wright 1987; Canter

et al., 1990) testate amoeba (Nishibe et al. 2004) rotifers (Fulton & pearl, 1987a) crustaceans (Fulton 1988) have been reported both *insitu* and the laboratory experiments. Further, cyanobacteria infection by chytrids fungi (Canter, 1972), cyanophages, (Safferman & Morris, 1964; Manage et al.1999, 2001;) and bacteria (Daft et al., 1975; Manage et al.2000, 2001) have been recorded as important algal lysing agents in many water bodies and are known to play an important role in decomposing cyanobacterial blooms.

However, most of previous studies have separately examined the above heterotrophs and cyanophages as biocontrol agents in terms of their isolation, morphology and biology in the laboratory experiments. Little is known about the abundance of protists grazing, algicidal bacteria and cyanophages infection on *M. aeruginosa* in natural freshwater environments. Thus, the present study reports that the seasonal changes in the abundance of biological agents on regulation of *M. aeruginosa* in a hypereutrophic pond.

Material and Methods

Study area

Furuike Pond (33° 49' N, 132° 48' E) in Sancho, Matsuyama City, Ehime Prefecture, Japan (is hypereutrophic due to anthropogenic loading from the watershed. The cyanobacterium, *M. aeruginosa* is dominant in the phytoplankton every year from May to November and forms heavy bloom and algal scum over the surface of the pond during September to November. The pond has a surface area of ca. 7400 m² with an average depth of 0.97 ± 0.24m during summer and 0.38 ± 0.13m during winter (Manage et al., 1999).

Sample collection and chlorophyll-a measurement

Surface water samples were collected with a 15 L plastic bucket at the near-shore station (Fig. 1), between 9:30-10:30 am, twice a week during heavy *M. aeruginosa* bloom from September to November 2000. Water temperature and pH were measured simultaneously using a thermistor pH meter (TOA Electronics Co. Ltd. Tokyo, Japan)

To determine chlorophyll-*a* concentration a measured portion of water sample was filtered through a Nuclepore filter with pore size of 0.2 µm to retain seston. The filter was then placed into a covered glass test tube

(10ml) and 6 ml of N, N -dimethy formamide (DMF) was added to extract chlorophyll-*a* and the concentration was determined by the fluorometric method (Rami & Porath, 1980).

Enumeration of organisms

Phytoplankton and Protists Immediately after collection of surface water samples, a 100 ml portion of the water sample was fixed with acidified Lugol's solution at a final concentration of 1% and the fixed sample was concentrated by natural sedimentation. Protists and phytoplankton cells were enumerated for each species in a Burkner-Turk type haematocytometer under a microscope at a magnification of x 400 at least 3 times. *Microcystis* species and other phytoplankton species were identified according to their morphology. *Microcystis* was identified based on cell size, colony form and sheath characteristics. After enumeration of phytoplankton, each sample was slightly sonicated (20 kHz, 2-3min) to break up colonies of *M. aeruginosa*, and the cyanobacteria cells were counted as described above. Because of fragility of some protists by fixation, an unfixed portion of the water sample was used for identification of dominant protists species within 2 hours of sample collection using 10 % methylcellulose solution to slow down swimming protists.

Zooplankton: For counting small and large zooplankton, 1 to 5 liter portions of water samples were respectively filtered through 20 μ m and 71 μ m mesh plankton nets to concentrate rotifer and crustaceans which were then fixed with acid Lugol's solution at a final concentration of 1%. The fixed samples were concentrated by natural sedimentation in a glass cylinder for more than 24 hours. A 0.2 to 1.0 ml aliquot of the concentrated sample was taken using a pipette and all the zooplankton were counted at least three aliquots, on a scaled Sedgwick Rafter counting glass slide.

Food vacuole content of heterotrophs: Immediately after collection, a measured volume of water sample was filtered through 20 μ m mesh plankton net for concentration of the sample. The concentrated sample was then fixed with 4% buffered glutaraldehyde at a final concentration of 2% to reduce the digestion of gut contents. The temperature of the fixative was adjusted to be the same as *in situ* water temperature as a

preliminary experiment had shown that cell breakage due to fixation was minimal with fixation using 4% temperature adjusted, buffered glutaraldehyde compared to use of 4% ice-cold buffered glutaraldehyde. For analysis of food vacuole contents of testate amoebae *Penardichlamys* sp. fixed sample was concentrated by natural sedimentation and a 0.05ml aliquot of the concentrated sample was mounted on a glass slide and food vacuole contents were identified and counted under a microscope at x 400 magnification. We used unfixed sample to observe the food vacuole contents of flagellate *Polytomella* sp. About 0.05ml of unfixed sample was mounted on a glass slide and mixed with 10% methylcellulose to slow down swimming flagellates. The food vacuole contents of *Polytomella* sp. was identified and counted under a microscope at x 400 magnification within 1 to 4 hours after sampling. For large ciliates and metazoan zooplankton, 0.1 to 0.5 ml of the fixed sample was mounted on a glass slide and observed their gut contents under an epifluorescence microscope. We counted number of *Penardichlamys* sp. (testate amoebae) cells and *Brachionus calyciflorus* individuals both of which ingested *Microcystis* cells.

Algicidal bacteria and Cyanophages: Bacteria and cyanophages both of which were infectious to *M. aeruginosa* was determined by a slightly modified version of the plaque assay method (Manage et al., 1999, 2000). *M. aeruginosa* algal lawns were prepared on the MA agar medium in petri dishes, using the axenic culture of *M. aeruginosa* (NIE-298) obtained from the Global Environmental Forum, Japan. Water samples were slightly sonicated and respectively filtered through 0.2 and 0.8 μm Nuclepore filters. Then, chloroform was added to the former filtrate to kill bacteria (Safferman & Morris, 1964). Thus, the chloroform-added filtrate contained cyanophages as *Microcystis* killers, while the 0.8 μm filtrate did bacteria and cyanophages. A portion of 0.2 μm filtrate was autoclaved and used as a control. We prepared serial 10-fold dilutions of these filtrates, and 1 ml of each dilution solution was spread on the *M. aeruginosa* lawn in triplicate. The *M. aeruginosa* lawns thus treated were incubated at 25 °C, $(48.8\text{-}58.6) \mu\text{E m}^{-2} \text{s}^{-1}$ with 12h light: 12h dark photo-cycle, for 10 or 12 days. After the incubation, plaques appeared on the algal lawns were counted as plaque formation unit (PFU/ml). The PFU of the bacteria infectious to *M. aeruginosa* was

determined by subtracting PFU of the chloroform-added 0.2 μm filtrate from that of the 0.8 μm filtrate.

Results

Fluctuations of surface water temperature were observed range between 27.2°C and 23.5°C from September to 27 October. This further decreased from 30 October, to 30 November from 18 °C to 7°C. The pH values varied with minor fluctuations resulting from 8.5 to 10.4 from September 1 to 30 November.

M. aeruginosa predominant in the phytoplankton community and bloom with algal scum was observed during the study period. Several peaks in the cell density of *M. aeruginosa* fluctuated between 3.0×10^5 and 1.2×10^6 cells ml^{-1} from 1 September to 6 October, reaching the maximum (1.9×10^7 cells ml^{-1}) on 16 October, followed by a sharp decline to 20 October (4.7×10^6 cells ml^{-1}), thereafter decline onwards (Fig. 1A). *M. aeruginosa* contributed on average 95% of the total cell density of phytoplankton during the bloom period. The chlorophyll a concentration reached a maximum ($1230 \mu\text{g l}^{-1}$) on 16 October, followed by a sharp decline to 20 October. The pattern of changes in chlorophyll-a was similar to that of the fluctuation of cell densities of *M. aeruginosa* (Fig. A) shows that the population of *M. aeruginosa* that preliminary determined the concentration of chlorophyll-a throughout the study period.

Abundances of the rotifers; *Brachionus calyciflorous*, *Cephalodella* sp. and the testate amoebae *Penardichlamys* sp. were relatively high during September and early October (Fig. 1 B & C). Individual densities of *B. calyciflorus* largely fluctuated during the period, while *Cephalodella* sp. and the *Penardichlamys* had several peaks on 15 September (1.30×10^4 inds. l^{-1}), 9 October (0.6×10^4 cells l^{-1}), and on 2 October (2.9×10^6 cells l^{-1}), respectively. By contrast, the heterotropic flagellate *Polytomella* sp. was abundant with a peak (2.5×10^5 cells l^{-1}) on 17

November (Fig. 1 C). The ingestion of *M. aeruginosa* cells by the flagellate was frequently detected from microscopic observation. It was observed that *Polytomella* sp. graze not only on *Microcystis* sp. but also on the other phytoplankton (diatoms). These results showed that the *Polytomella* sp. also is one of the important consumers of *Microcystis* and it dominated when rotifer and *Cephaladell* sp. was at low densities or undetectable level (Fig. 1C). Cell density of the large ciliates largely fluctuated during the study period, and we could not find the seasonal trends (data not shown).

Density of algicidal bacteria (Fig.1D) were relatively high with large fluctuation between 0.3×10^4 and 3.7×10^4 PFU ml⁻¹ throughout the study period, and relatively high densities were recorded on 8 September and 02 October, there after fluctuated considerably from 6 October to 13 November and then decreased rapidly to 20 November onwards. It was detected that the high cell density of infectious bacteria followed by the high cell density of *M. aeruginosa* (Fig. 1A and 1D).

Densities of the cyanophages remained were low ($< 0.9 \times 10^3$ PFU ml⁻¹) during September and an occasional peak (6.4×10^3 PFU ml⁻¹) was detected on 2 October, thereafter undetectable until 16 October and then had a sudden peak on 20 October (7.10×10^3 PFU l⁻¹). Cyanophage became undetectable from 27 October onwards (Fig. 1D). The densities of cyanophages increased when cell densities of *M. aeruginosa* reached their maximum and at least two sharp peaks of cyanophages were associated with drastic declines of *M. aeruginosa* during September to October period (Fig.1A &D).

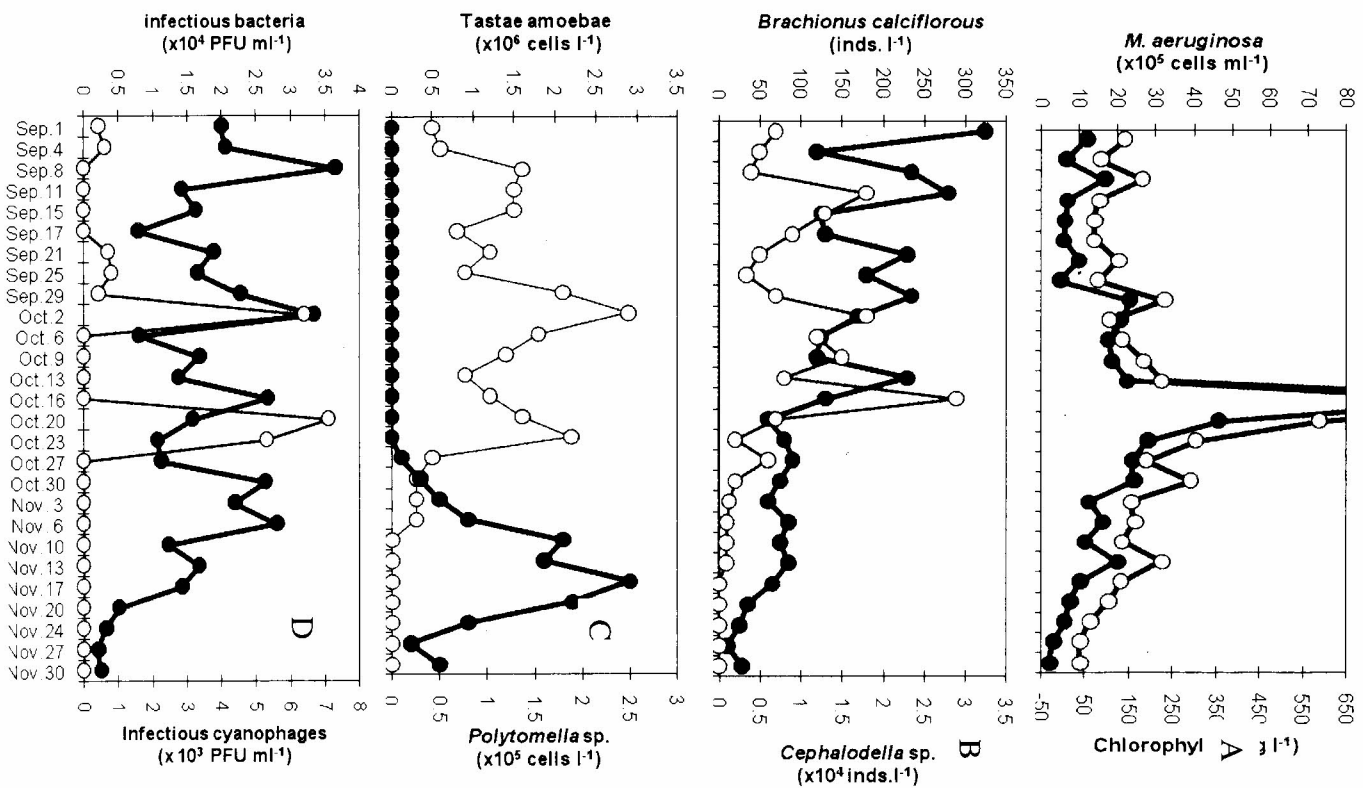


Figure 1. Seasonal changes in cell densities of *Microcystis aeruginosa* (A, closed circles), concentrations of chlorophyll-a (A, open circles), individual densities of rotifer *Brachionus calyciflorus* sp. (B, closed circles), those of the rotifer *Cephalodella* sp. (B, open circles), cell densities of the *Penardichlamys* sp. (testae amoebae) (C, opened circles), those of the flagellate *Polytomella* sp. (C, closed circles), plaque forming units (PFU/ml) of infectious bacteria (D, closed circles) and those of infectious cyanophages (D, open circles) during the study period in Furuike Pond.

Percentages of *B. calyciflorus* individuals ingesting colonies of *M. aeruginosa* from 1 September to 6 October were significantly higher (t-test, $p < 0.01$) than those from 9 October except 20 October on wards (Fig. 2). Percentages of *Penardichlamys* sp which ingested *M. aeruginosa* cells were very high ($>77\%$) from 1 to 25 September and thereafter rapidly decreased (Fig. 2). We examined 1392 cells of *Penardochlamys* sp. for food vacuole contents, and 96.3% of cells consisted of only *Microcystis* and other phytoplankton were not detected.

The testate amoeba was found attached only to colonies of *M. aeruginosa* but not to other substrates. More amoebae were found attached to *Microcystis* colonies than were found freely suspended (Student's t test, $p < 0.005$). Freely suspended amoeba cells were always found when *Penardochlamys* sp (testate amoebae) were present which suggests that the *Penardochlamys* sp has selective grazing on *Microcystis*.

Discussion

There is still limited information concerning ecological control of bloom of *M. aeruginosa in situ*. In the present study, we could detect the rotifer *Brachionus calyciflorus*, *Cephalodella* sp., testate amoebae *Penardichlamys* sp., large ($>50\ \mu\text{m}$) ciliates and the flagellate *Polytomella* sp. as *Microcystis* sp., grazers, and bacteria and cyanophages as infectious algicidal agents.

Within aquatic ecosystem protozoa have an important role in the reduction of phytoplankton population by grazing (Canter et al. 1990). Cyanobacteria in particular provide a suitable food source for several genera of protozoa, including the ciliate *Nassula* (Canter et al. 1990), the flagellate *Ochromonas* (Cole and Wynne, 1974) and amoebae *Acanthamoeba*, *Mayoella* and *Nuclearia* (Yamamoto, 1981). In this study, we detected *Polytammella* sp. grazing on *Microcystis* sp. and the other phytoplankton diatom and green algae. We did not examine size selectivity of flagellates but judging from the food vacuole contents, the protists and zooplankton grazed not only unicellular *M. aeruginosa* but also its colonial form that consisted of 2 to 40 cells. Noshibe et al. (2004) also noted that flagellate *Collododictyon triciliaum* is capable of grazing various size of phytoplankton, and thus, flagellates have a wide range of edible prey. In Furuike pond, another important consumer of *M. aeruginosa* is the rotifer *Brachionus calyciflorus*, which was abundant and fluctuated during September (Fig. 1B) and percentages of *B. calyciflorus* individuals ingesting colonies of *M. aeruginosa* during the bloom period were significantly high (t-test, $p < 0.01$). Grazing effect of the *B. calyciflorus* on *M. aeruginosa* has been documented (Fulton and Pearl, 1987a). The microscopic observation of living *Cephalodella* sp. showed that they fed on colony of *M. aeruginosa* and packed at the cyanobacterial cells. Percentages of testate amoebae *Penardichlamys* sp. which ingested *M. aeruginosa* cells were very high (>77%) during the bloom period. The food vacuoles of *Penardichlamys* sp. contained only *Microcystis* cells and that no amoeba cells attached to particles other than *Microcystis* colonies, suggests its specific preference for *Microcystis* species. Cook et al. (1974) also reported that some rizopodes collected from natural waters seemed to have strong feeding selectivity on specific prey. Unfortunately, the data in the present study cannot confirm this fact because *Microcystis* presented at very high densities ($> 10^6$ cells ml^{-1}) and predominated markedly in the phytoplankton community (average, 95%) during the bloom period in the pond. Hence, it is possible that the amoeba only utilized *Microcystis* because the latter was the most accessible and available prey or attachment substrate. Cook et al., (1974) reported that the abundance of a mayorellid-like amoeba rapidly increased following the bloom of *A. planktonica* and the grazing of the amoeba is a major cause of termination in the cyanobacterial bloom in Lake Sidney Lainer. However, information on rotifer *B. calyciflorus* and *Cephalodella* sp.,

large ciliate and flagellate *Polytomella* sp. as biological agents are limited. Thus, the present work may provide information for evaluation of the effectiveness of *B. calyciflorous* sp., *Cephalodella* sp. and the flagellate *Polytomella* sp. in addition to the genus *Penardichlamys* sp. grazing for the biological control of cyanobacteria blooms. Therefore, we suggests that the use of those heterotrophes as biological control agents in lakes and reservoir management should be regarded as just one of a number of available control measures that for part of an integrated management policy.

Bacteria interaction with harmful algal species has been reviewed (Doucette et al. 1998). The present study it was found a relationship found between algicidal bacteria and ($r = 0.81$, $n = 27$, $p < 0.01$, data not shown) and *M. aeruginosa* cell density. During the study period we isolated algicidal bacteria strains and the bacterium *Alcaligenes denitrificans* (Manage et al. 2000; Manage et al. 2001), showed significant growth inhibitory or algicidal effect on *M. aeruginosa*. Thus, we suggest that algicidal bacteria contribute to regulate the abundance of *M. aeruginosa* in the lake. Even we observed many bacteria cells attached to *M. aeruginosa* colonies during the bloom period, we could not detect a significant increase of algicidal bacteria in our enumeration method. Thus, we underestimated the abundance of algicidal bacteria, since filtration through $0.8\mu\text{m}$ Nuclepore filters could eliminate *Microcystis*-bacteria consortia from the water samples because only unattached bacteria would pass through the filter. Similar result were reported using red tide organisms (Imai et al., 1998) and *Microcystis* spp. (Manage et al., 2001)

Cyanophage infection is expected to be the most prominent during algal blooms, where the host is abundant (Waterbury & Valois, 1993, Fuhrman, 1999; Manage et al 1999). In this study cyanophages were below the detection limit when the densities of *M. aeruginosa* were low during early September and late November. Density of cyanophages was increased when cell density of *M. aeruginosa* rose to their maximum (Fig. 1D). Fuhrman (1999) reported that viral infection is thought to be both density dependent and species specific because virus must diffuse randomly from host to host, and rare hosts are less susceptible to the spread of infection than more common host. Lytic viruses can only increase in abundance when the average time it takes

to diffuse from host to host is shorter than the average time the viruses remain infections. Thus, at least two sharp peaks of cyanophages were associated with drastic declines of *M. aeruginosa* during September to November period (Fig. 1 A & D) and undetectable when the abundance of *M. aeruginosa* was low. Thus, the results suggest that cyanophages infection on *M. aeruginosa* can only control the algal population temporarily but do substantially reduce or terminate the *M. aeruginosa* bloom.

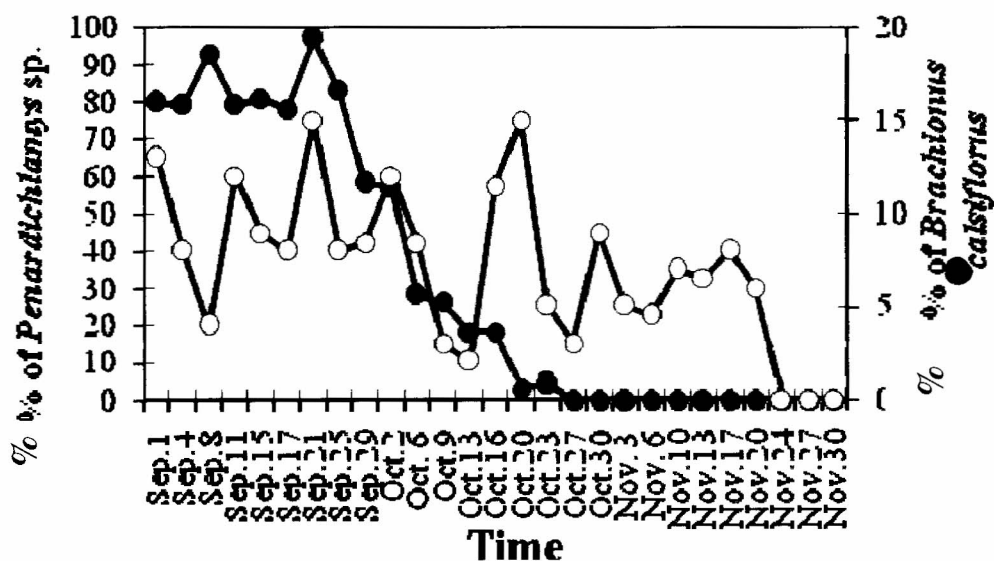


Figure 2. Seasonal changes on percentages of rotifer *Brachionus calyciflorus* (open circles) and *Penardichlamys* sp. (tastae amecobae) (closed circles) both of which ingested *Microcystis* cells during the study period in Furuike Pond.

The overall results of the study demonstrated the biological control of *M. aeruginosa* in the hypereutrophic Furuike Pond. Together with succession of protozoan and zooplankton, this could graze on *M. aeruginosa*, and infection of cyanophages and algicidal bacteria efficiently suppress massive growth of *M. aeruginosa*. We were not a position to explain the reason for the seasonal succession of the killing consortia of *Microcystis* from comparisons between the succession and

the seasonal changes in some environmental variables such as water temperature and pH, and this should be elucidated in further studies. Also, it was well known that *Microcystis* blooms suddenly disappear in many lakes, though the mechanisms are still unclear. We suggest the grazing effect of protists, zooplankton and infection of bacteria and cyanophage on *M. aeruginosa* may causes the collapse of cyanobacteria blooms (Fig.1 A & D).

If the biological control of *Microcystis* biomass using the above heterotrophs and cyanophages is feasible with the aid of some artificial regulation, this may be useful for the better management of Lake eutrophication. Further studies of the biology and physiology of these bio control agents are required, and their effect on, and relationship to the host organisms should be understood in order to the role of these heterotrophs and cyanophages on *M. aeruginosa* mortality.

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