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RESEARCH PAPER

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Abstract The dynamics of cyanophage-like particles and algicidal bacteria that infect the bloom-forming cyanobacterium *Microcystis aeruginosa* was followed in a hypereutrophic pond from September 1998 to August 1999. The densities of *M. aeruginosa* ranged between 4.0×10^5 and 1.9×10^7 cells ml^{-1}, whereas those of algicidal bacteria were between 4.0 and 5.1×10^2 plaque-forming units (PFU) ml^{-1} and those of cyanophage-like particles were between $<5.0 \times 10^2$ and 7.1×10^3 PFU ml^{-1}. A significant relationship was found between the densities of algicidal bacteria and *M. aeruginosa* ($r = 0.81$, $n = 69$, $P < 0.001$), suggesting that the dynamics of the algicidal bacteria may regulate the abundance of *M. aeruginosa.* Occasional peaks of density of cyanophage-like particles were detected in October, June, and August, when sharp declines in *M. aeruginosa* cell densities were also observed. The densities of cyanophage-like particles became undetectable when the abundance of *M. aeruginosa* was low, suggesting the density-dependent infection of *M. aeruginosa* by cyanophage-like particles. Thus, we suggest that infections of both algicidal bacteria and cyanophage-like particles are important biological agents that decompose blooms of *M. aeruginosa* in freshwater environments.

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Key words *Microcystis aeruginosa* · Algal bloom · Cyanophage-like particles · Algicidal bacteria · Algicidal effect

Introduction

Massive accumulations of cyanobacteria (known as blooms) can cause a wide range of social, economic, and environmental problems, such as deterioration of water quality, toxicity, and decrease in aesthetic value of the affected water (Angeline et al. 1994; Carmichael et al. 1985; Watanabe et al. 1996). *Microcystis* (Cyanophyceae) is well known all over the world as one of the most common bloom-forming cyanobacteria, which has harmful effects on animals and, potentially, on human beings (Carmichael 1988; Gorham and Carmichael 1988; Skulberg et al. 1984; Song et al. 1998). The physiology and ecology of *M. aeruginosa* have been extensively studied, and the blooming mechanism has been explained (Pearl 1988; Reynolds 1984; Zohary and Roberts 1989), but little work has so far been done on the decomposition processes of *M. aeruginosa* blooms.

Previous studies reported that bacteria (Caiola and Pellegrini 1984; Yamamoto and Suzuki 1977) and cyanophages (Kenneth and Haselkorn 1973; Robert et al. 1976; Safferman and Morris 1963; Yvonne et al. 1981) are important algal lysing agents in many lakes. A number of algicidal bacteria have been isolated, and reports have been published of their algicidal effects on *Anabaena cylindrica* (Gromov et al. 1973; Yamamoto and Suzuki 1977); *Synechococcus cedorum* and *Nostoc* sp. (Gromov et al. 1973; Shilo 1970); *Phormidium tadzschicicum* and *P. luridum* (Gromov et al. 1973); *A. flosa-aquae*, *Aphanizomenon flosa-aquae*, *A. circinalis*, and *Nostoc ellipsosporum* (Daft and Stewart 1973); and *Oscillatoria* spp. and *Microcystis* sp. (Daft et al. 1975; Yamamoto and Suzuki 1977).

Recently, viral mortality has also been highlighted as an important factor in the termination of algal blooms (Bratbak et al. 1993; Manage et al. 1999; Nagasaki et al. 1994a; Waterbury and Valois 1993). Cyanophage- or virus-

like particles have been isolated and identified from some bloom-forming cyanobacteria, such as *Synechococcus* sp. (Leach et al. 1980); *Lyngbya* sp., *Plectonema* sp., and *Phormidium* sp. (Daft et al. 1970; Safferman and Morris 1963, 1964); and *M. aeruginosa* (Fox et al. 1976).

These previous studies have discussed separately the algicidal effects of bacteria (Daft et al. 1975; Yamamoto and Suzuki 1977) and cyanophages (Bratbak et al. 1996; Daft et al. 1970; Safferman and Morris 1963, 1964) on phytoplankters. The present study was undertaken to investigate the seasonal occurrence and effects of both algicidal bacteria and cyanophage-like particles on *M. aeruginosa.* The ecological significance of both algicidal agents for *M. aeruginosa* mortality in a hypereutrophic pond is discussed. As far as we are aware, this is the first report of infection of *M. aeruginosa* by both algicidal bacteria and cyanophage-like particles in a natural freshwater environment.

Materials and methods

Furuike Pond in Sancho, Matsuyama City, Ehime Prefecture, Japan (Fig. 1) is hypereutrophic due to anthropogenic loading from the watershed. The cyanobacterium *M. aeruginosa* is dominant in the phytoplankton every year from May to October. The pond has a surface area of ca. 7400 m^2, with an average depth of 0.97 ± 0.24 m during summer and 0.38 ± 0.13 m during winter (Nakano et al. 1998).

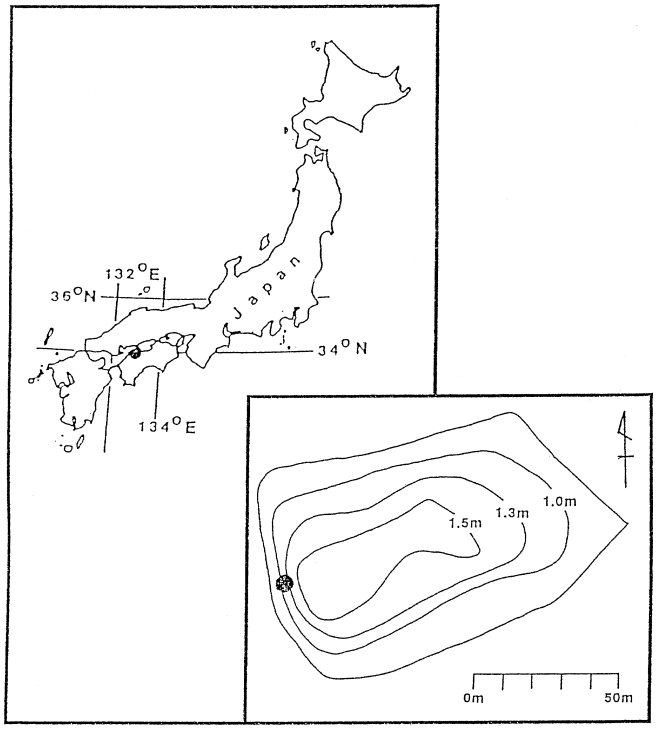

Fig. 1. Map of Furuike Pond showing the location of the sampling station (*closed circle*)

Surface water samples were collected with a 15-l bucket, between 09:30 and 10:30 a.m., at the station shown in Fig. 1, twice a week from September to November 1998 and once a week from December 1998 to August 1999. Water temperature and pH were measured simultaneously using a thermistor pH meter (TOA Electronics, Tokyo, Japan).

The concentration of chlorophyll *a* was determined by the method of Rami and Porath (1980). To enumerate the cell density of the phytoplankton, 100 ml of water sample was fixed with acidified Lugol's solution at a final concentration of 1%, followed by natural sedimentation for 24 h to concentrate the sample. Cells were counted by using a haematocytometer under a microscope. Colonies of *M. aeruginosa* were then dispersed by slight sonication (50 kw, 60 s), and their cells were enumerated.

The plaque count method (Safferman and Morris 1964) was used, with slight modification, for enumeration of algicidal bacteria and cyanophages. Algal lawns were prepared on MA agar medium (Manage et al. 1999) in petri dishes, using an axenic culture of *M. aeruginosa* (NIES-298) provided by the National Institute of Environmental Studies, Japan. Two 10-ml portions of the pond water sample were slightly sonicated (50 kw, 30 s), and then one was filtered through a sterilized 0.2-μm and the other through a 0.8-μm Nuclepore membrane filter (Millipore, Massachusetts, USA). Five milliliters of the 0.2-μm filtrate was treated with chloroform (Crosse and Hingorani 1958; Safferman and Morris 1964) to kill bacteria. Thus, this 0.2-μm filtrate served as the cyanophage fraction, while the 0.8-μm filtrate served as the cyanophage plus bacteria fraction. Another portion of the 0.2-μm filtrate was autoclaved and used as a control. One milliliter of each filtrate was serially diluted 10-fold, and 1 ml of each dilution was then spread on triplicate algal lawns. The *M. aeruginosa* lawns thus treated were incubated at 25 ± 2°C, under a light intensity of 48.8 to 58.6 $\mu E\,m^{-2}s^{-1}$ with a 12 h light, 12 h dark photocycle for 10 to 12 days. We counted the number of plaques that appeared on the algal lawn, assuming that each plaque originated from a single microbial agent, either cyanophage or bacterium. The plaque-forming units (PFU) of the bacteria infectious to *M. aeruginosa* were determined by subtracting the PFU of the 0.2-μm filtrate to which chloroform had been added from that of the 0.8-μm filtrate.

Results

The surface water temperature ranged between 26.8 and 23.0°C from 1 September to 23 October, decreased from 27 October (17.6°C) to 13 January (4.0°C), and gradually increased to 28.8°C on 25 August (Fig. 2). The pH values ranged between 8.1 and 10.3 from September 1 to 24 November, between 6.5 and 7.7 from 27 November to 25 February, and thereafter increased with fluctuations (Fig. 2).

There were two pronounced peaks in chlorophyll *a* concentration in October (1230 $\mu g\,l^{-1}$) and May (1620 $\mu g\,l^{-1}$), which corresponded with the highest abundances of *M.*

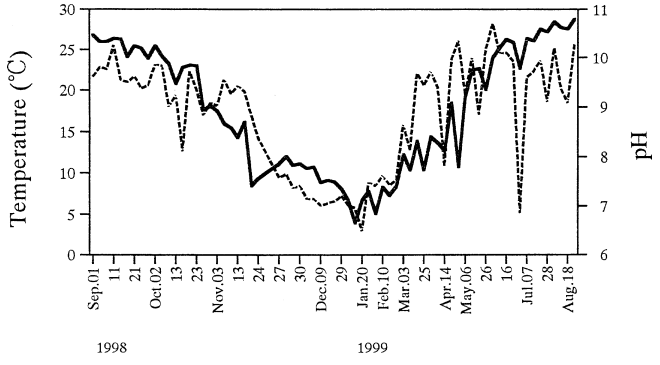

Fig. 2. Changes in water temperature (*thick line*) and pH (*broken line*) in Furuike Pond during the study period

aeruginosa that were detected (Fig. 3). It is clear from Fig. 3 that it was the population of *M. aeruginosa* that primarily determined the concentration of chlorophyll *a* throughout the sampling period. Of the other algae that were dominant at some period, only *Synedra* sp. and *Anabaena* sp. occurred in any abundance during the autumn at the same time as *M. aeruginosa.* We also detected considerable densities of the algae belonging to Chlorophyceae (*Scenedesmus* sp. and *Pediastrum* sp.) and Bacillariophyceae (*Tabellaria* sp.) when the density of algicidal bacteria was high. Major peaks of the other two species illustrated occurred only in spring, when the abundance of *M. aeruginosa* was low.

The cell densities of *M. aeruginosa* fluctuated between 6.0×10^5 and 1.2×10^6 cells ml^{-1} from 1 September to 6 October (Fig. 3B), 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}). The cell density of *M. aeruginosa* was low during winter and then increased again gradually and reached two sharp peaks on 26 May (1.3×10^7 cells ml^{-1}) and 21 July (4.5×10^6 cells ml^{-1}), followed by drastic decline.

The densities of algicidal bacteria (Fig. 4A) were relatively high, with large fluctuations between 0.7×10^2 and 3.7×10^2 PFU ml^{-1} from September to November, and decreased to 14 April (0.5×10^2 PFU ml^{-1}). From May to 25 August, they tended to increase from 0.4×10^2 to 5.1×10^2 PFU ml^{-1}. There was a significant correlation between algicidal bacteria and *M. aeruginosa* ($r = 0.81$, $n = 69$, $P < 0.001$) (Fig. 5).

The density of cyanophage-like particles (Fig. 4B) was low ($<1.0 \times 10^3$ PFU ml^{-1}) during September, high early in October (6.4×10^3 and 7.1×10^3 PFU ml^{-1}), and became undetectable from 27 October until April. Cyanophage-like particles were detectable again from 6 May (0.9×10^3 PFU ml^{-1}) and tended to increase to peaks on 2 June (5.9×10^3 PFU ml^{-1}) and 18 August (4.5×10^3 PFU ml^{-1}) before decreasing again.

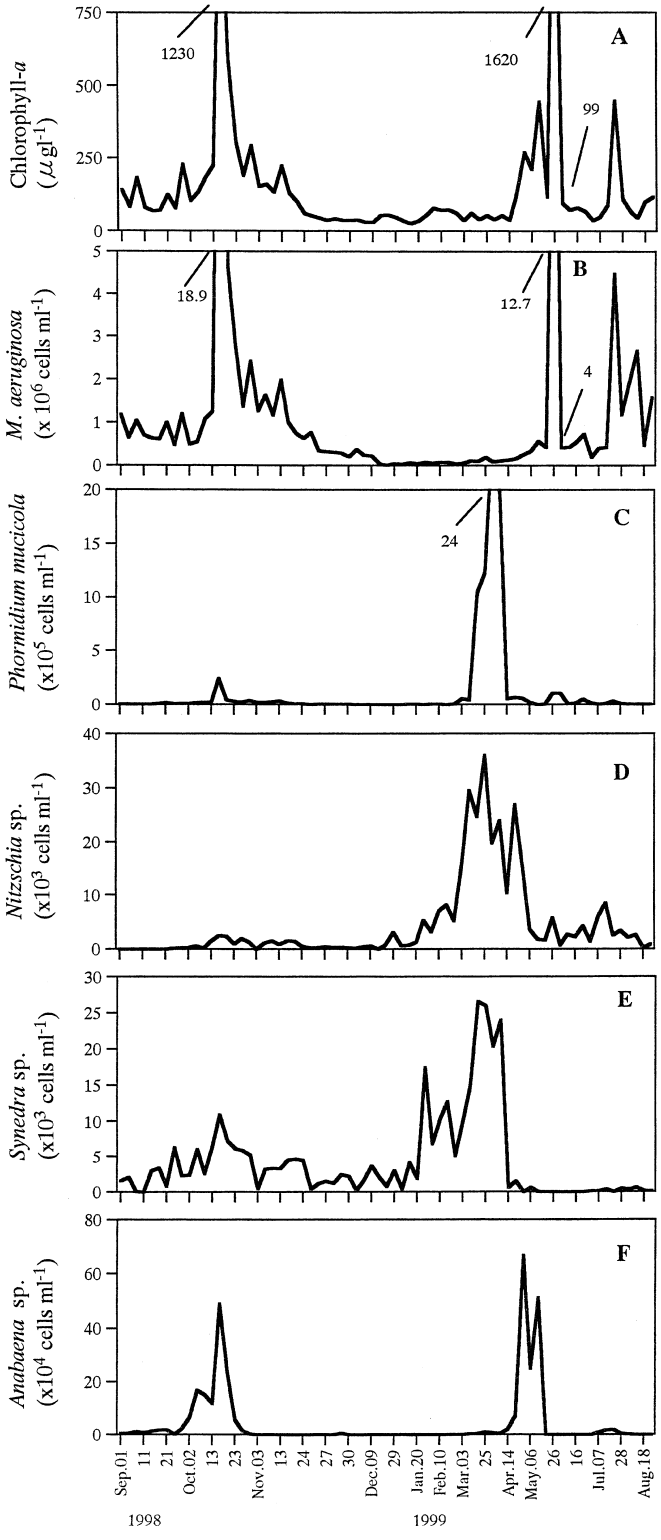

Fig. 3. Seasonal changes in chlorophyll *a* concentration (**A**) and in cell densities of *M. aeruginosa* (**B**), *Phormidium mucicola* (**C**), *Nitzschia* sp. (**D**), *Synedra* sp. (**E**), and *Anabaena* sp. (**F**) in Furuike Pond during the study period

Discussion

Daft et al. (1975) reported that algal lysis by bacteria was rapid at water temperatures of 25° to 37°C and pH values of 8.0 to 9.5 in eight freshwaters in Scotland, England, and Wales. The temperature in Furuike Pond was in excess of 25°C in September, at the beginning of the study period, reaching a maximum of 29°C in August with high pH values

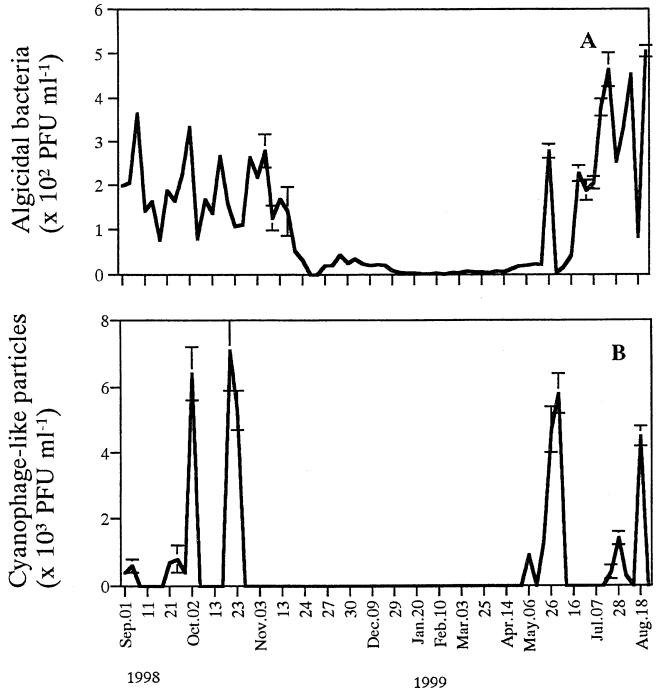

Fig. 4. Seasonal changes in abundance of algicidal bacteria (**A**) and cyanophage-like particles (**B**) in Furuike Pond during the study period. *Vertical bars* indicate standard deviations

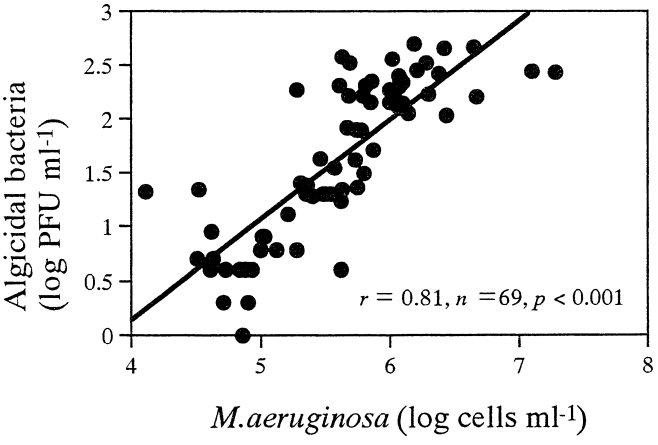

Fig. 5. Logarithmic relationships between algicidal bacteria and *M. aeruginosa. Solid line* is functional regression

(9.0 to 10.0). It only fell below 15°C and pH 7.5 in November, almost exactly coinciding with the virtual disappearance of algicidal bacteria (Figs. 2 and 4A). Thus, we found a significant relationship between the number of algicidal bacteria, water temperature, and pH ($r = 0.70$, $n = 69$, $P < 0.001$; $r = 0.51$, $n = 69$, $P < 0.001$; data not shown), suggesting that the most rapid lysis of *M. aeruginosa* in the pond was likely to occur during the summer months.

Bacterial interaction with harmful algal bloom species has been reviewed recently by Doucette et al. (1998). In the present study, we found a significant relationship between algicidal bacteria and *M. aeruginosa* ($r = 0.80$, $n = 69$, $P < 0.001$) (Fig. 5). We have isolated algicidal bacteria strains, and the bacterium *Alcaligenes denitrificans* was identified as an agent that has significant growth-inhibitory or algicidal effects on *Microcystis aeruginosa* (Manage et al. 2000). Thus, we suggest that algicidal bacteria regulate the abundance of *M. aeruginosa* in Furuike Pond. We also observed many bacterial cells attached to *Microcystis* colonies during the bloom period. Although the host-pathogen relationship between *M. aeruginosa* and algicidal bacteria is clearly substantial in the pond, we may have underestimated the abundance of algicidal bacteria, since filtration through 0.8-μm Nuclepore filters could eliminate *Microcystis* bacteria consortia from the water samples because only unattached bacteria would pass through the filter. Imai et al. (1998) reported similar results using red tide organisms.

The densities of algicidal bacteria were low when high densities of the other phytoplankters except *Synedra* sp. and *Anabaena* sp. were detected (Figs. 3C–F and 4A). This suggests that the some of the other algal species detected in the present study were not susceptible to bacterial attack in the pond. However, from September to November, the densities of *Synedra* sp. and *Anabaena* sp. were relatively high at the same time as those of algicidal bacteria, suggesting that there may be some interactions between those phytoplankters and algicidal bacteria. In our previous paper (Manage et al. 2000), we reported that some of the tested cyanophyceae were susceptible to attack by algicidal bacterial (*Alcaligenes denitrificans*), suggesting that it has a wide host range. Thus, infection by the algicidal bacteria examined in the present study may not be host-specific.

Infection by cyanophage-like particles is expected to be most prominent during algal blooms, when the host is abundant (Fuhrman 1999; Waterbury and Valois 1993). Previous studies have also reported that such infection by cyanophages is not strictly species-specific (Daft et al. 1970; Hennes et al. 1985; Leach et al. 1980; Safferman and Morris 1963, 1964). However, in the present study, the densities of cyanophage-like particles were below the detection limit when the densities of *M. aeruginosa* were low during winter (Figs. 3B and 4B). During the period of low *M. aeruginosa* abundance, other phytoplankters, such as *Phormidium mucicola*, *Nitzschia* sp., and *Synedra* sp., were gradually increasing (Fig. 3C–E). Although this may suggest species-specific infection of *M. aeruginosa*, chlorophyll *a* concentrations during winter were low, indicating low abundance of any hosts available for cyanophages. If chlorophyll *a* concentrations had been high in winter, the densities of cyanophage-like particles might have also been high. Thus, we cannot say that coupled oscillations between the abundance of *M. aeruginosa* and cyanophage-like particles were due to species-specific infection by cyanophage-like particles.

However, Fuhrman (1999), Suttle et al. (1990), and Suttle and Chan (1994) recently reported that viral infection is thought to be both density dependent and species specific. Because viruses must diffuse randomly from host to host, rare hosts are less susceptible to the spread of infection than more common hosts. Lytic viruses can only increase in abundance when the average time it take to diffuse from

host to host is shorter than the average time the virus remains infectious. Thus, when the population of a particular algal species becomes more dense, it is more susceptible to infection. Bratbak et al. (1993, 1996) recorded a negative virus–host relationship during the collapse of *Emiliania huxeyi* bloom, and Imai et al. (1998) and Nagasaki et al. (1994a, b) recorded virus-like particles inside the cells of *Heterosigma akashiwo* in the final stage of a red tide bloom. Waterbury and Valois (1993) noted that the *Synechococcus* phage titers began to increase about a month after the onset of the *Synechococcus* spring bloom, and the phage titers oscillated between 10^3 and 10^4 phage ml^{-1} during summer months and decreased drastically coincident with the decline of *Synechococcus* spp. Phage titers fell to 100 phage ml^{-1} and were undetectable during the winter months with low density of *Synechococcus* spp. Similar oscillation patterns of *M. aeruginosa* and cyanophage-like particles were detected in the present study. The densities of cyanophage-like particles increased when the cell densities of *M. aeruginosa* were rising to their maximum, and at least two sharp peaks of cyanophage-like particles were associated with drastic declines of *M. aeruginosa* during the fall and summer months. Cyanophage-like particles were undetectable during winter, when the abundance of *M. aeruginosa* was low. Thus, we suggest that cyanophage-like particles infecting *M. aeruginosa* can only control the population temporarily but do substantially reduce or terminate the *M. aeruginosa* bloom. Our data and the previous studies (Manage et al. 1999; Waterbury and Valois 1993; Bratbak et al. 1990, 1993, 1996; Imai et al. 1998) support the view that the cyanophage-like particles may among the important biological agents that contribute to substantial reduction of *M. aeruginosa* blooms, although we did not directly observe infection of *M. aeruginosa* by cyanophage, because we did not conduct an experiment to observe cyanophage in the samples under the electron microscope.

The densities of algicidal bacteria were also high during the *Microcystis* bloom. Since some algicidal bacteria attached to *Microcystis* colonies, these bacteria could avoid being eaten by protists. By contrast, the pattern of change in the cyanophage population showed drastic increases and decreases of cyanophage abundance within a very short period. Since the host available for the cyanophage-like particles was plentiful during the *Microcystis* bloom, the cyanophage was not limited by "food." This suggests that loss processes are very important for the changes in densities of cyanophage in Furuike Pond. Bratbak et al. (1990), Suttle and Chan (1994), and Fuhrman (1999) reported that pronounced fluctuations in virus abundance over time indicated synchronized host-cell lysis and rapid degradation of a large proportion of the progeny viruses.

The present study strongly suggested that cyanophage-like particles and algicidal bacteria decomposed the *M. aeruginosa* bloom in the natural freshwater environment of Furuike Pond. However, the relative contributions of algicidal bacteria and cyanophage-like particles to the decomposition of *M. aeruginosa* in Furuike Pond are still unclear. Further studies of the ecology and physiology of these algicidal bacteria and cyanophages are required, and their effect on, and relationships to, the host organisms should be understood in order to elucidate the role of these algicidal agents in *M. aeruginosa* mortality.

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