

Nutrient enrichment and selective predation by zooplankton promote *Microcystis* (Cyanobacteria) bloom formation

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An experiment was conducted with a natural freshwater phytoplankton community from a eutrophic pond to investigate the combined effects of phytoplankton community competitive interactions, nutrient enrichment and zooplankton on *Microcystis* bloom formation. The pond water initially had a very low concentration of *Microcystis*, but the total phytoplankton biomass as chlorophyll *a* reached $\sim 110 \mu\text{g L}^{-1}$ in summer. The pond water was incubated outdoors at natural temperature and light with a 2×2 factorial manipulation of nutrient (nutrient additions versus no additions) with and without zooplankton. The interaction of a two-phase nutrient addition (a net increase in concentrations of $250.0 \mu\text{M N}$ and $16.1 \mu\text{M P}$ each time) and the presence of zooplankton significantly altered phytoplankton community composition. When the water initially contained zooplankton, nitrogen and phosphorus enrichment promoted a surface *Microcystis* bloom. However, when the zooplankton was removed from the water at the start of the experiment, no surface *Microcystis* bloom formed, regardless of nutrient additions. Chlorophyta dominated in the absence of zooplankton, when the same nutrient was provided. Our results demonstrate that *Microcystis* bloom formation in this eutrophic water body at a mean temperature of about 36°C at 14:00 h was closely related to the initial presence of zooplankton and a sufficient supply of nitrogen and phosphorus. We believe this is one of the first demonstrations of zooplankton controlling *Microcystis* bloom formation in a water body previously free of surface cyanobacterial blooms.

KEYWORDS: nutrient; zooplankton; selective predation; *Microcystis* bloom

INTRODUCTION

Harmful cyanobacterial blooms, especially those of the toxic, non-nitrogen fixing, colony-forming cyanobacterial genus *Microcystis*, are symptomatic of advanced eutrophication world-wide (Paerl, 1988; Oliver and

Ganf, 2000; Prepas and Charette, 2003). Despite their world-wide distribution, the mechanisms underlying the formation of surface cyanobacterial blooms in specific water bodies remain elusive and poorly understood.

One of the greatest limitations to understanding and predicting cyanobacterial bloom formation is our inability to create experimentally controlled bloom conditions. For example, laboratory incubations cannot adequately simulate colony formation of *Microcystis*. Even when colony formation can be induced (Burkert *et al.*, 2001; Yang *et al.*, 2006), the colonies are much smaller than those observed in natural blooms. Typically, *Microcystis* reverts to single-cell populations under experimentally enclosed conditions. Moreover, *Microcystis* can often form surface blooms simultaneously with other cyanobacteria genera (Oliver and Ganf, 2000; Paerl *et al.*, 2001), and the bloom composition in nature is not always constant (Fukushima *et al.*, 1999), all of which add to the difficulty in understanding the mechanisms controlling bloom formation.

The formation of cyanobacterial blooms is dependent on the complex interactions of several key factors (Paerl, 1988; Oliver and Ganf, 2000; Paerl *et al.*, 2001). Paerl *et al.* (Paerl *et al.*, 2001) noted that phytoplankton population dynamics in natural freshwater ecosystems are regulated by complex interactions between phytoplankton and many other factors such as hydrodynamics (freshwater discharge, flushing and residence time), nutrient cycling and presence of grazers. Lagus *et al.* (Lagus *et al.*, 2007) found that the bottom sediment plays an important role in structuring phytoplankton communities. Bloom formation can also be affected by the presence/absence of fish (Fukushima *et al.*, 1999).

Nutrients are important abiotic factors affecting cyanobacterial bloom formation (Sommer, 1985; Olsen *et al.*, 1989; Steinberg and Hartmann, 1992; Oliver and Ganf, 2000; Prepas and Charette, 2003). However, eutrophic conditions do not necessarily ensure cyanobacterial bloom formation (Tilman *et al.*, 1982; Steinberg and Hartmann, 1992; Jensen *et al.*, 1994; Nydick *et al.*, 2004; Heisler *et al.*, 2008). In many eutrophic water bodies in China, there can be high concentrations of phytoplankton, but not necessarily cyanobacterial blooms (Wang *et al.*, 2004).

Zooplankton is an important biotic factor affecting phytoplankton competition. Numerous feeding experiments have been undertaken to determine the relationships between phytoplankton and zooplankton (Burns, 1987; Haney, 1987; de Bernardi and Giussani, 1990; Gliwicz, 1990). Field observations have shown that the presence of cyanobacterial blooms is usually related to the decreased abundance of large-sized cladocerans and increased abundance of rotifers, copepods and smaller-sized cladocerans (Allan, 1977; Edmondson and Litt, 1982). Toxins contained in some *Microcystis* species may also inhibit potential competitors and affect the competition between *Microcystis* and the other phytoplankton

(Sedmak and Kosi, 1998). The colonial nature of *Microcystis* has been considered as a self-defense against grazing by zooplankton (Nizan *et al.*, 1986; Fulton and Paerl, 1987; Paerl *et al.*, 2001).

The trade-offs phytoplankton exhibit in their competitive ability to utilize resources versus their susceptibility to predation by zooplankton have also been studied (Vanni, 1987; Vrede *et al.*, 1999; Steiner, 2003). These trade-offs may be due to their different resource utilization properties, differences in morphology and nutritional quality (de Bernardi and Giussani, 1990) and grazing characteristics of various consumers. Steiner (Steiner, 2003) offered support for a size-based keystone trade-off (the keystone predator effect, with the underlying trade-off among prey termed the “keystone trade-off”) in a laboratory-grown algal assemblage. A predator–prey model of an adaptive trade-off between resistance to consumption and resource-saturated population growth rate among phytoplankton species was constructed by Agrawal (Agrawal, 1998). These studies have provided examples of the trade-offs affecting the phytoplankton community under different environmental conditions. However, none of these experiments directly addressed *Microcystis* bloom dynamics.

Climatic conditions also affect cyanobacterial bloom potential. Blooms most frequently occur during warm seasons, i.e. summer–fall, and ambient temperature plays a key regulatory role (Moss, 1996; Jacoby *et al.*, 2000; Jöhnk *et al.*, 2008; Paerl and Huisman, 2008), and in shallow lakes and ponds, blooms may occur for even longer periods. Some studies have investigated the combined effects of nutrient enrichment and zooplankton community structure on natural phytoplankton communities during summer (Pérez-Martínez and Cruz-Pizarro, 1995; Cottingham, 1999).

Based on the known presence of a *Microcystis* bloom in summer prior to their experiments, Hua and Zong (Hua and Zong, 1994) were able to successfully maintain a surface *Microcystis* bloom in enclosures with nutrient additions in Yanghe reservoir of China. Ghadouani *et al.* (Ghadouani *et al.*, 2003) also observed heavy cyanobacterial blooms of mixed-species, including *Microcystis*, in *in situ* shallow enclosures enriched with dissolved inorganic nitrogen (DIN) and phosphorus (DIP) in the presence of zooplankton and discussed the effects of toxin producing cyanobacteria on zooplankton (*Daphnia pulicaria*). However, we are not aware of a case in which summer *Microcystis* blooms have been experimentally stimulated under natural conditions in water bodies initially free of blooms.

Based on the information obtained from previous experiments, we have been able to repeatedly induce surface *Microcystis* blooms from eutrophic freshwater of a natural algal assemblage previously free of cyanobacteria

blooms (Wang *et al.*, unpublished results). In this method, eutrophic water with a chlorophyll *a* content $\geq 100 \mu\text{g L}^{-1}$ is transferred to glass containers, with subsequent additions of DIN and DIP. The containers are incubated outdoors in summer under natural temperature and light conditions. Interactions with fish, wind and sediments have been eliminated in the study design, thereby enabling us to solely examine the combined effects of zooplankton and nutrient additions. This allowed us to further address the following questions as well as the effects of nutrient additions: (i) do zooplankton play a critical role in bloom formation and (ii) if so, what is their role?

Here, we tried to simulate the process of colonial *Microcystis* bloom formation from a water body previously free of such blooms, in order to clarify the mechanisms triggering and sustaining blooms. We examined the effects of inorganic N and P addition in the presence or absence of the naturally occurring zooplankton on a phytoplankton community free of cyanobacterial blooms from a pond.

The experimental design is similar to several previous studies (Pérez-Martínez and Cruz-Pizarro, 1995; Steiner, 2003). The main difference between this and previous studies is that this experiment was carried out in small glass jars incubated under natural irradiance and temperature conditions, with a relatively high initial chlorophyll *a* content ($\sim 100 \mu\text{g L}^{-1}$) reflecting the pond's trophic state.

METHOD

Water source

Water was collected from a 300 m² eutrophic pond located at the Nanjing Institute of Geography and Limnology, Chinese Academy of Sciences, in subtropical Nanjing, China, which contained abundant phytoplankton, and was stocked with golden carp (*Carassius* sp.) and water lilies (*Nymphaea* sp.). No surface *Microcystis* blooms have been observed in the pond since its construction in 2003.

No special feeding was provided for the golden carp, and the food web in the pond was mainly composed of phytoplankton, zooplankton and golden carp. The depth of the pond was around 60 cm, with a Secchi depth of ~ 25 cm. The bottom of the pond was pebbled. The pond was surrounded by two rows of deciduous trees of ~ 25 m in height, with one row of *Metasequoia* sp. and the other row of *Platanus orientalis* sp. Many leaves fell into the pond during later autumn and early winter.

At the beginning of the experiment, the chlorophyll *a* content of the pond water was around $110 \mu\text{g L}^{-1}$. The

following phytoplankton genera were present: Chlorophyta: *Ankistrodesmus*, *Chlamydomonas*, *Crucigenia*, *Kirchneriella*, *Scenedesmus*, *Selenastrum*, *Tetraedron* and *Ulothrix*; Cyanobacteria: *Aphanocapsa*, *Chroococcus*, *Merismopedia*, *Microcystis*, *Planktothrix* and *Synechocystis*; Bacillariophyta: *Fragilaria* and *Synedra*. The five dominant genera by wet weight biomass were *Ankistrodesmus*, *Crucigenia*, *Planktothrix*, *Scenedesmus* and *Synedra* (Fig. 1).

Experimental design and monitoring

We used a bioassay approach, similar to that of Paerl and Bowles (Paerl and Bowles, 1987), but not in *in situ*. The experiment was conducted outdoors in early summer 10–22 May 2008, with 10 May as day 0 and 22 May as day 12. On the morning of 10 May, 50 L of pond water was collected and gently mixed to ensure the even distribution of phytoplankton. The containers were transparent borosilicate glass jars of 2.5 L each (14.0 cm diameter, 28.0 cm high), which were filled and placed on a cement platform near the pond. The jars received full direct sunlight from about 08:00 h to about 14:30 h, and the direct sunlight was sheltered by nearby buildings after 14:30 h.

There were three treatments and a control, with three replicates each. Details of the treatments are provided in Table I. Hereafter, +Z and -Z mean presence and absence of the initial naturally occurring zooplankton, while +N and -N indicates either nutrient additions or no nutrient additions, respectively. Nutrient was added twice during the course of the experiment: at the start (day 0) and at day 7. Water samples were collected for analysis before the nutrient additions.

The +Z + N and -Z + N treatments were supplemented with a combination of nitrogen (N, as KNO_3) and phosphorus (P, as Na_2HPO_4 and NaH_2PO_4 , in a 61:39 Na_2HPO_4 : NaH_2PO_4 molar ratio), whose N:P ratio (molar ratio) was 15.5:1, both on day 0 (10 May) and day 7 (17 May), to produce a net increase in concentrations of $250.0 \mu\text{M N}$ and $16.1 \mu\text{M P}$ each day. In the nutrient addition treatments, the total N and P concentrations were increased by a factor of 1.6 and 3.2, respectively, in comparison with the initial pond water. The nutrient additions were made from a stock solution at pH 7.0 with 250.0 mM N in a 15.5:1 N:P molar ratio. The experiment was initiated at $\sim 13:00$ on 10 May 2008.

In order to remove as much zooplankton (Z) and as little phytoplankton as possible, a $125 \mu\text{m}$ nylon mesh was used to separate the zooplankton following the technique of Vanni and Temte (Vanni and Temte, 1990). Thirty liters of pond water were passed through the mesh to retain zooplankton. The retained zooplankton comprised 48 individuals (ind.) of copepods, 8

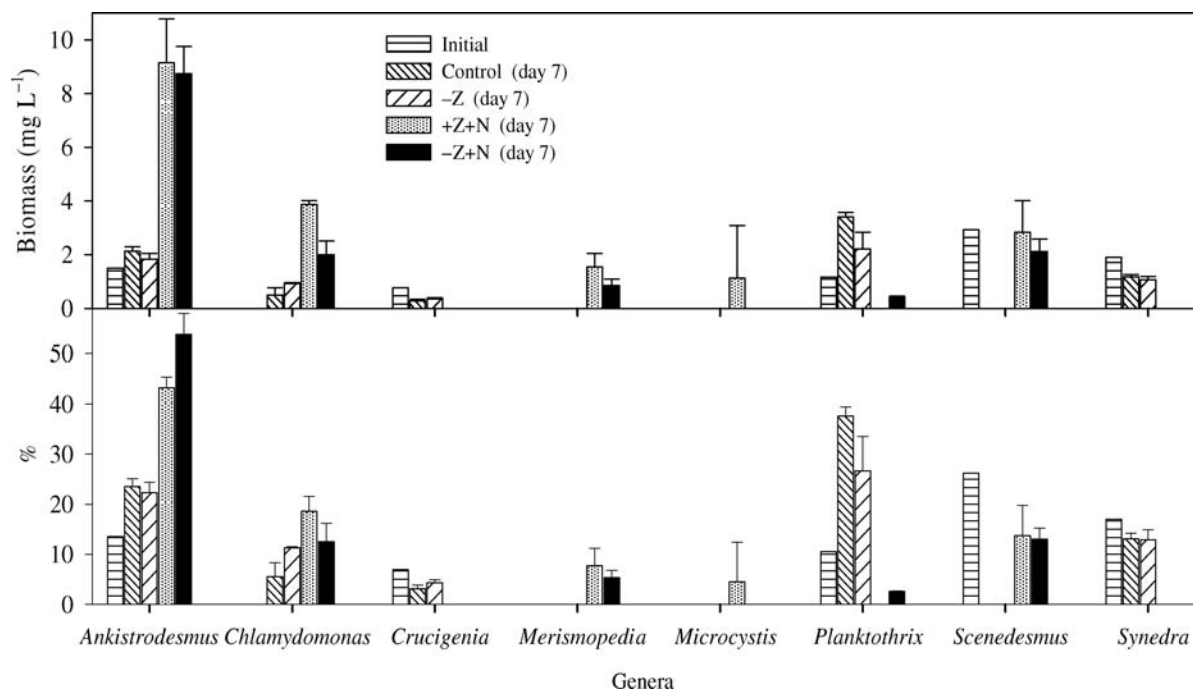


Fig. 1. The variation of the biomass (mg L^{-1}) and percentage of the five dominant genera in the control and three treatments in the initial and on day 7. The error bars represent the standard deviation of triplicate jars.

rotifers, 11 cladocerans and a few protozoans, with a density of approximately 2 ind. L^{-1} . The retained phytoplankton included 11 colonies of *Microcystis flos-aquae* comprising of tens to hundreds of cells each and a few *Ankistrodesmus* sp., *Synedra* sp., *Pediastrum* spp. and *Scenedesmus* spp. This filtration step removed $<1\%$ of the total *Microcystis* biomass and $<0.1\%$ of the total biomass of *Ankistrodesmus* sp., *Synedra* sp., *Pediastrum* spp. and *Scenedesmus* spp. (based on wet weight biomass).

At the beginning of the experiment, $125 \mu\text{m}$ mesh nylon screens were fixed on the top of each jar to deter insects from entering the jars. During the experiment, the contents of the jars were gently mixed at 08:00 h and 14:00 h daily. Ultrapure water was used to replenish that lost by evaporation every 3 days. During periods of rainfall, the jars were covered.

Sub-samples were collected from the jars on 17 and 22 May, at 08:00–09:00 h after gently mixing the jars.

Table I: Experimental treatment of the pond water

| Treatment | Filtered through $125 \mu\text{m}$ mesh | N and P added on days 0 and 7 |
|--------------|---|-------------------------------|
| Control (+Z) | – | – |
| –Z | + | – |
| +Z + N | – | + |
| –Z + N | + | + |

+, present; –, absent.

Care was taken to not disturb the algae that had settled to the bottom when sampling. There was no detectable surface *Microcystis* bloom on 17 May in any of the treatments, while there were noticeable surface *Microcystis* blooms on 22 May in treatment +Z + N after the second nutrient addition. Five nutrient variables were determined: total nitrogen (TN), total phosphorus (TP), dissolved TN (DTN), dissolved TP (DTP) and soluble reactive phosphorus (SRP).

Weather conditions were recorded daily, and water temperature was measured at 08:00 h and 14:00 h daily after gently mixing from day 1 to day 12. The water temperature on day 0 was measured at 14:00 h. During the course of the experiment, the jars with nutrient additions became greener than those receiving no nutrient. Therefore, the water temperatures of these two contrasting sets of jars were measured separately. The air temperatures were measured at a nearby weather station ZSNJ (58238) in Nanjing, China.

The phytoplankton biomass was expressed as wet weight biomass. For determining phytoplankton density, 50 mL water samples were preserved with 1% Lugols solution and stored in darkness until identification. If the density of phytoplankton was too low for counting, the sample was concentrated after settling. For enumeration, two replicate aliquots were placed in 0.1 mL plankton counting chambers that were modified from the Palmer and Maloney design (Palmer and Maloney, 1954).

Most cells were observed at 400 \times magnification by light microscopy, while large algal cells were observed at 100 \times magnification. They were mainly identified to the genus level as referenced by morphologies (Hu and Wei, 2006). Algal volumes were calculated from cell density and cell size measurements. Cell volumes were estimated by approximation to the nearest simple geometric solid after measurement of at least 40 algal units. Conversion to wet weight biomass assumed that 1 mm³ of volume was equivalent to 1 mg of wet weight biomass.

Water temperature was measured using a mercury thermometer held at \sim 5 cm depth. Water for analysis of DTN, DTP and SRP was filtered through GF/C filters (1.2 μ m pore size, Whatman, Maidstone, UK), which were rinsed with deionized water before using. Measurements of TN, TP, DTN and DTP were according to the methods of Gross and Boyd (Gross and Boyd, 1998), while SRP was determined by molybdenum–antimony–ascorbic acid colorimetry (Eaton *et al.*, 1995).

Statistical analyses

Responses of the phytoplankton biomass (total biomass, the biomass of the main phyla and *Microcystis*) and the percentage of total biomass accounted for by *Microcystis* or the main phyla between different treatments on days 7 and 12 were analyzed by two-way ANOVA using repeated measures (nutrient \times zooplankton \times time). The effect of nutrient enrichment on the total biomass was also analyzed by one-way ANOVA both on days 7 and 12. Before comparison, the percentage of total biomass accounted for by *Microcystis* or the main phyla were arcsine square-root-transformed, and the other data were log-transformed to increase homogeneity of variances (Underwood, 1997). Analyses were carried out using SPSS15.0. All data were shown as mean \pm SD. The differences are reported as significant if $P < 0.05$, and the Least-significant difference (LSD) test was chosen for pairwise comparisons.

RESULTS

Total phytoplankton biomass responses to nutrient

Since there was no steady mixing to keep the algae suspended in the jars, sedimentation was allowed to proceed, with obvious algal layers forming at the bottom of all jars from day 3 onward, especially that in the jars of nutrient additions was thick. Phytoplankton generally did not attach to the walls of the jars.

The changes in nutrient conditions in the jars are shown in Fig. 2a–g. The concentrations and ratios of nutrient of +Z + N and –Z + N treatments on days 0 and 7 were determined prior to nutrient additions. Overall, the concentrations of SRP, DTN, DTP, TN and TP in +Z + N and –Z + N treatments were higher than those of the control and –Z treatment both on days 7 and 12. This confirmed that the addition of nutrient could be observed as an increase in their concentrations.

With respect to TN and TP concentrations (Fig. 2d and e), the differences of the measured values between the treatments of nutrient addition and that of no nutrient addition were much smaller than 250.0 μ M N and 16.1 μ M P, respectively, both on days 7 and 12. Furthermore, there was very little increase in the TN and TP concentrations from day 0 to day 7, or from day 7 to day 12 for the +Z + N and –Z + N treatments. High phytoplankton sedimentation losses may have been the reason for this result.

The DTN:DTP (molar ratio) and TN:TP (molar ratio) in +Z + N and –Z + N treatments were similar (Fig. 2f and g), while those in the control and –Z treatment were also similar (Fig. 2f and g). The DTN:DTP (molar ratio) in +Z + N and –Z + N treatments was all around 99 on day 7, and they were reduced to about 64 on day 12. The DTN:DTP (molar ratio) in the control and –Z treatment was about 76 on day 7, and they increased to around 130 on day 12. The TN:TP (molar ratio) in the control and –Z treatment was about 40–50 both on days 7 and 12, and in +Z + N and –Z + N treatments, it was around 30 both on days 7 and 12.

The temporal variation of total phytoplankton biomass is shown in Fig. 2h. The analysis of repeated-measures two-way ANOVA indicated that nutrient enrichment, zooplankton presence and nutrient–zooplankton interaction did not affect the total biomass (Table II). A one-way analysis showed the total biomass of –Z + N and +Z + N treatments on day 7 was significantly higher than that of the control and of –Z treatment on day 7 (one-way ANOVA, $P < 0.01$) and that of +Z + N treatment was significantly higher than that of –Z + N treatment on day 7 (one-way ANOVA, $P < 0.01$). Other comparisons were not significantly different on day 7. On day 12, the total biomass of almost all the treatments decreased from day 7, and there were no significant differences between any of the treatments and the control.

Biomass of dominant phytoplankton genera

Figures 1 and 3 showed the biomass of the five dominant genera in the control and three treatments. On day 0, the five dominant genera were *Ankistrodesmus*, *Crucigenia*, *Planktothrix*, *Scenedesmus* and *Synedra* with

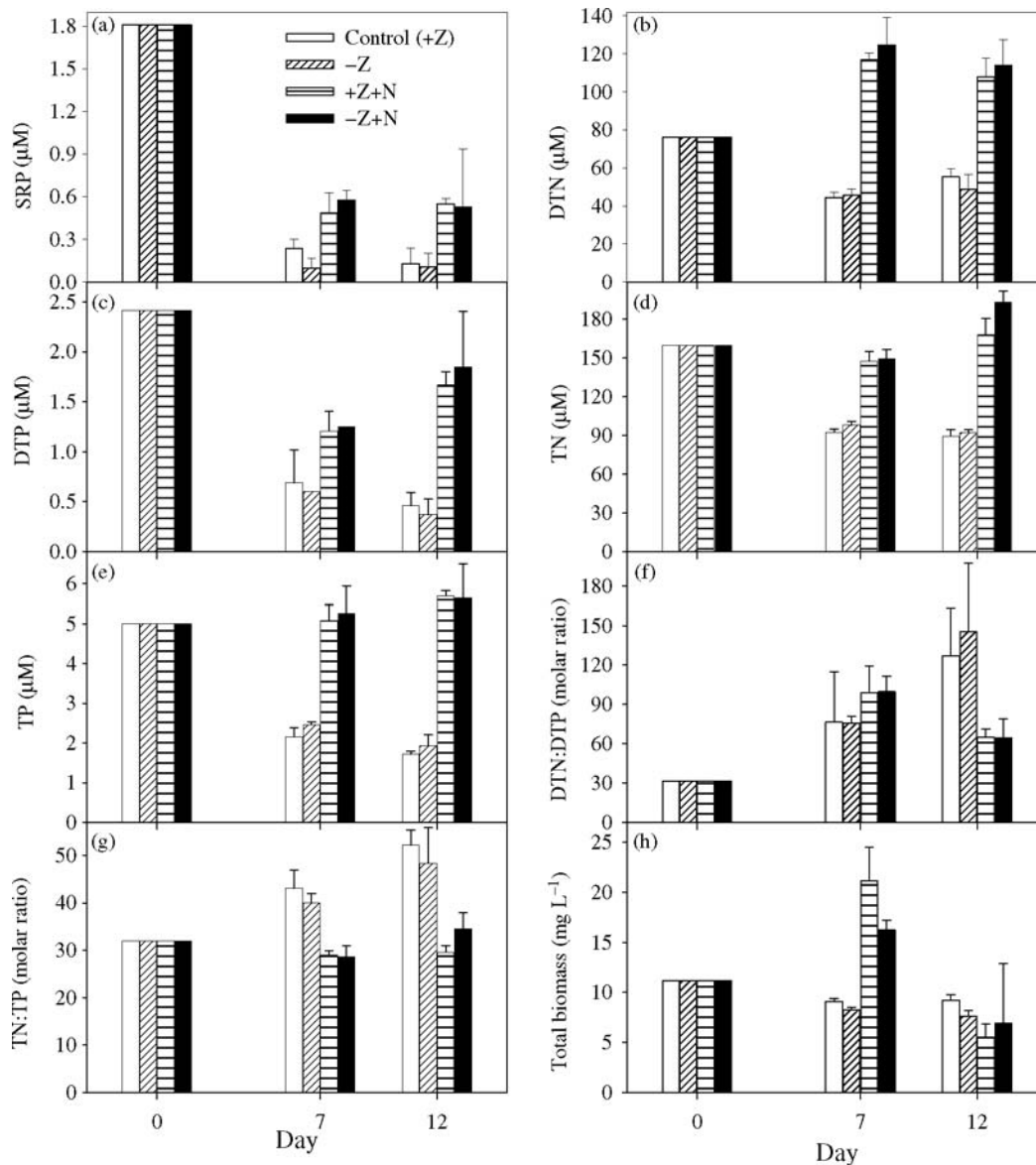


Fig. 2. Temporal changes of the concentrations of (a) SRP, (b) DTN, (c) DTP, (d) TN, (e) TP, (f) DTN to DTP ratio (DTN:DTP, molar ratio), (g) TN to TP ratio (TN:TP, molar ratio) and (h) the total wet weight biomass of phytoplankton of the three treatments and the control on sampling days, and the values of +Z + N and -Z + N treatments on days 0 and 7 were before nutrient addition. The net increase was 250.0 μM N and 16.1 μM P both on day 0 (10 May) and day 7 (17 May) for +Z + N and -Z + N treatments. The error bars represent the standard deviation of triplicate jars.

biomass of 1.504, 0.775, 1.171, 2.926 and 1.902 mg L^{-1} , respectively, showing that the most dominant genera were mainly green algae.

On day 7, the dominant genera in the control and -Z treatment were *Ankistrodesmus*, *Chlamydomonas*, *Planktothrix* and *Synedra*, and those in +Z + N and -Z + N treatments were *Ankistrodesmus*, *Chlamydomonas*, *Merismopedia* and *Scenedesmus* (Fig. 1).

On day 12, there were some differences among the five dominant genera between different treatments (Fig. 3). *Ankistrodesmus*, *Chlamydomonas* and *Planktothrix*

were most dominant in the control (+Z) and -Z treatment. *Chroococcus*, *Microcystis* and *Pediastrum* were most dominant in +Z + N treatment. *Chlamydomonas*, *Pediastrum* and *Scenedesmus* were most dominant in treatment -Z + N. They showed that the presence/absence of zooplankton changed the dominant genera in nutrient addition more easily than that of no nutrient addition. The total number of the five dominant genera for all the treatments increased to 12 from 9 of day 7.

The most dominant genus in +Z + N treatment was *Microcystis* (Cyanobacteria) on day 12, with biomass

Table II: Results of the tests of between subjects effects of repeated-measures ANOVA for comparison of phytoplankton on days 7 and day 12

| | Day 7 versus day 12 | |
|----------------------------------|---------------------|----------|
| | F | P |
| Total biomass | | |
| Nutrients | 1.08 | 0.330 |
| Zooplankton | 1.03 | 0.339 |
| Nutrients × zooplankton | 0.01 | 0.907 |
| Cyanobacteria | | |
| Nutrients | 72.67 | 0.000*** |
| Zooplankton | 98.53 | 0.000*** |
| Nutrients × zooplankton | 38.88 | 0.000*** |
| Percentage of Cyanobacteria | | |
| Nutrients | 14.70 | 0.005** |
| Zooplankton | 21.60 | 0.002** |
| Nutrients × zooplankton | 10.44 | 0.012* |
| Chlorophyta | | |
| Nutrients | 7.45 | 0.026* |
| Zooplankton | 2.05 | 0.190 |
| Nutrients × zooplankton | 1.89 | 0.206 |
| Percentage of Chlorophyta | | |
| Nutrients | 31.68 | 0.000*** |
| Zooplankton | 20.17 | 0.002** |
| Nutrients × zooplankton | 11.99 | 0.009** |
| Bacillariophyta | | |
| Nutrients | 274.51 | 0.000*** |
| Zooplankton | 0.05 | 0.837 |
| Nutrients × zooplankton | 0.98 | 0.351 |
| Percentage of Bacillariophyta | | |
| Nutrients | 378.66 | 0.000*** |
| Zooplankton | 0.72 | 0.421 |
| Nutrients × zooplankton | 1.87 | 0.209 |
| <i>Microcystis</i> | | |
| Nutrients | 7.26 | 0.027* |
| Zooplankton | 3.39 | 0.103 |
| Nutrients × zooplankton | 14.54 | 0.005** |
| Percentage of <i>Microcystis</i> | | |
| Nutrients | 5.94 | 0.041* |
| Zooplankton | 0.50 | 0.501 |
| Nutrients × zooplankton | 11.97 | 0.009** |

Nutrient (d.f. = 1), zooplankton (d.f. = 1) and their interaction (d.f. = 1) effects were tested. Asterisk, double asterisk and triple asterisk indicate the significant differences with $P < 0.05$, 0.01 and 0.001 , respectively.

increasing to $4.482 \pm 1.468 \text{ mg L}^{-1}$ from $1.131 \pm 1.958 \text{ mg L}^{-1}$ of day 7 (Figs 2 and 3). In the $-Z + N$ treatment *Scenedesmus* (Chlorophyta) was dominant on day 12, with biomass increasing to $2.461 \pm 3.058 \text{ mg L}^{-1}$ from $2.119 \pm 0.469 \text{ mg L}^{-1}$ of day 7 (Figs 2 and 3). This indicated that the presence of zooplankton led to changes in the most dominant genus by day 12.

Biomass of *Microcystis*

The biomass of *Microcystis* and percentage of *Microcystis* were significantly increased by nutrient enrichment alone, but the zooplankton alone had no effect. There

was a significant nutrient and zooplankton interactive stimulation of *Microcystis* biomass and percentage of *Microcystis* (Table II). This showed that *Microcystis* bloom formation was promoted by the simultaneous presence of zooplankton and nutrient enrichment.

From day 9 (19 May) until the end of the experiment (22 May), there was an observable surface *Microcystis* bloom every morning before gently mixing in the $+Z + N$ treatment (without removal of initial zooplankton, nutrient added). The bloom dispersed into the water column after gentle mixing. The dominant *Microcystis* species was *M. flos-aquae* (Wittr.) Kirchner, with individual *Microcystis* colonies contained tens to hundreds of cells.

Phytoplankton community biomass

At the beginning of the experiment (day 0), three algal groups were present: Chlorophyta, Cyanobacteria and Bacillariophyta (Figs 4 and 5). On day 7, the number of phyla among all the treatments was 7, with Cyanobacteria and Chlorophyta being the most dominant in the control and $-Z$ treatment, while Chlorophyta became the most dominant in the $+Z + N$ and $-Z + N$ treatments (Figs 4 and 5). On day 12, the total number of phyla observed among all the treatments and controls was 5, with Cyanobacteria and Chlorophyta being dominant in the control and $-Z$ treatment, while Cyanobacteria dominated in $+Z + N$ treatment and Chlorophyta dominated in $-Z + N$ treatment (Figs 4 and 5).

The presence of zooplankton alone significantly increased the biomass of Cyanobacteria and the percentage of Cyanobacteria, while nutrient enrichments alone significantly reduced both of them (Table II), indicating that the effects of zooplankton and nutrient enrichments on the Cyanobacteria were different from each other. Furthermore, the nutrient plus zooplankton interaction significantly enhanced the biomass of Cyanobacteria and the percentage of Cyanobacteria (Table II), showing that Cyanobacteria were promoted by zooplankton and nutrient additions simultaneously.

Nutrient enrichments increased the biomass of Chlorophyta and the percentage of Chlorophyta significantly, and neither zooplankton nor nutrient plus zooplankton interactions affected the biomass of Chlorophyta (Table II). Meanwhile, when present, zooplankton significantly decreased the percentage of Chlorophyta and there was a significant nutrient plus zooplankton interaction that decreased it (Table II). Nutrient enrichments significantly decreased the biomass of Bacillariophyta and percentage of Bacillariophyta, and neither zooplankton nor nutrient

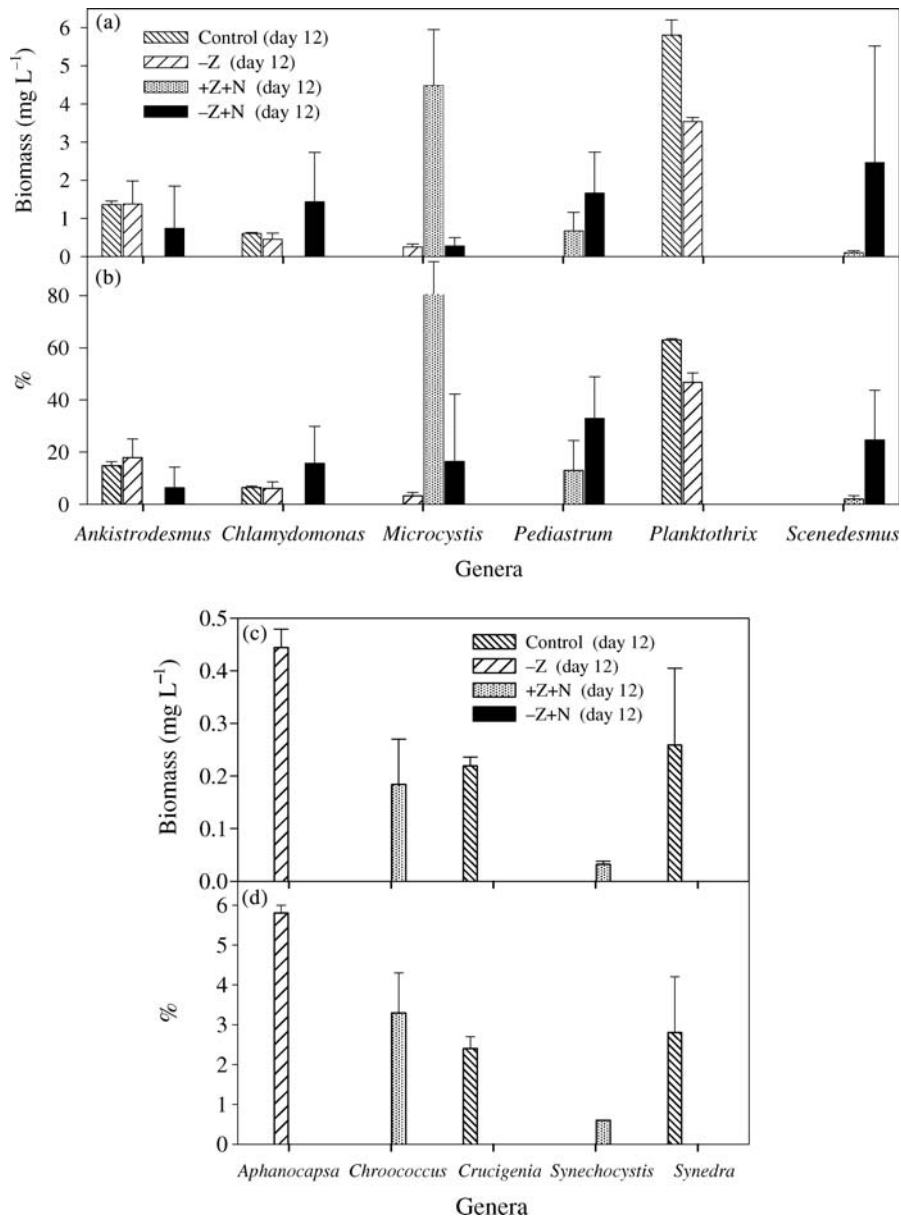


Fig. 3. The variations of the biomass (mg L⁻¹) and percentage of the five dominant genera in the control and three treatments on day 12. (a) and (b) are for the genera with relatively higher content of biomass, while (c) and (d) are for the less dominant genera. The error bars represent the standard deviation of triplicate jars.

plus zooplankton affected the biomass of Bacillariophyta and the percentage of Bacillariophyta (Table II).

Weather conditions and water temperature

The weather during the experimental period was generally sunny, except for 18 May (day 8) which was cloudy and rainy. There was no significant thermal stratification in the jars. The mean ± SD of water temperature in the jars containing nutrient additions at 08:00 h and

14:00 h was 21.9 ± 2.5°C and 36.6 ± 5.4°C, respectively, and those of no nutrient addition at 08:00 h and 14:00 h was 21.8 ± 2.4°C and 36.1 ± 5.3°C, respectively (Fig. 6). The lowest temperature was 18.6°C in the no nutrient addition treatments at 08:00 h on day 3, while the highest was 44.1°C at 14:00 h in the nutrient enrichment treatments on day 12.

The air temperatures are shown in Fig. 6, where the mean ± SD of daily maximum temperature (DMAT) and daily minimum temperature (DMIT) was 27.6 ± 4.2°C

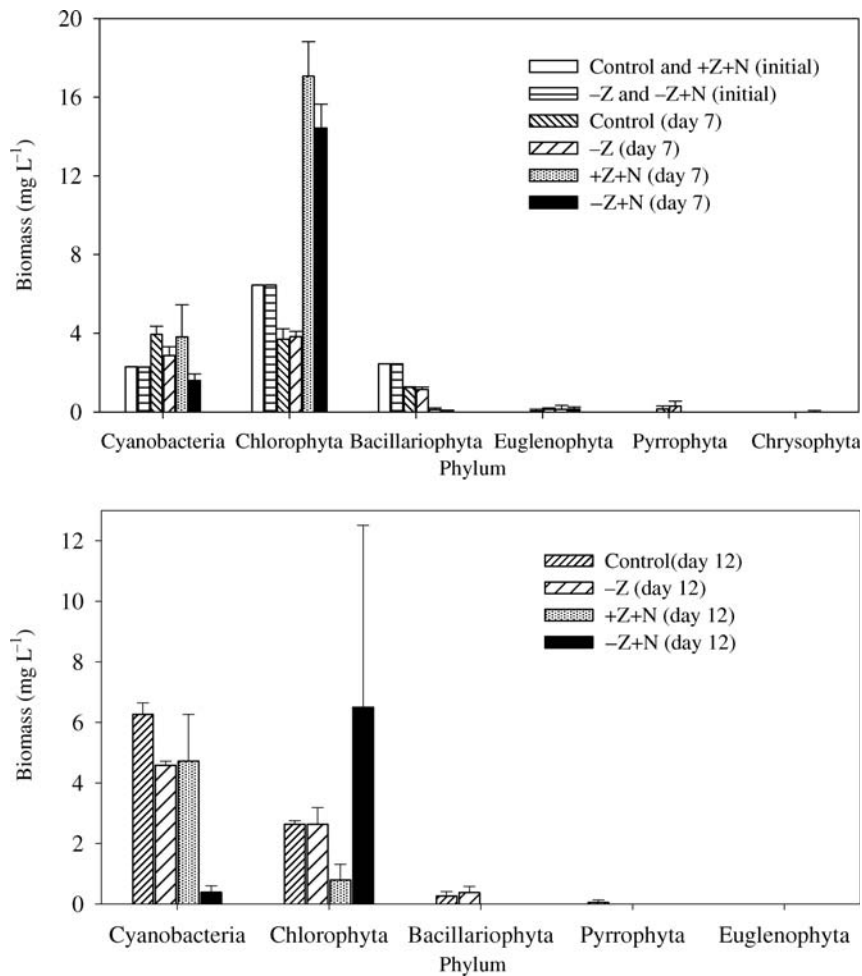


Fig. 4. The variation of the mean biomass (mg L^{-1}) of the algal composition in the control and three treatments in the initial and on days 7 and 12. The initial differences between before and after the filtering by $125 \mu\text{m}$ mesh are shown. The error bars represent the standard deviation of triplicate jars.

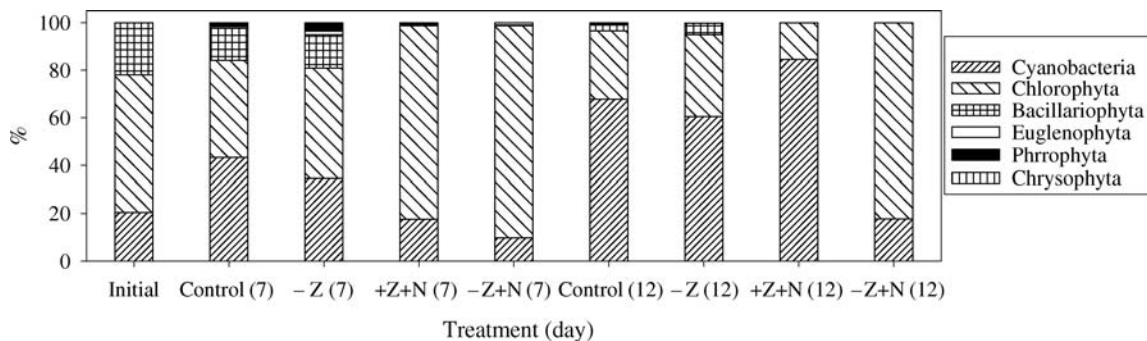


Fig. 5. The variations of percentage of the algal groups in the control and three treatments in the initial and on days 7 and 12. The number in parenthesis after the treatment of the horizontal axis is the sampling days. The values are the means of the three replicates of each treatment.

and $15.3 \pm 2.8^\circ\text{C}$, respectively (Fig. 6). There was a general upward trend in the morning and afternoon temperature during the study, with a marked decline on day 8 as conditions became cloudy and rainy.

Although the water temperature of the pond was not measured during the experiment, it was obvious that the pond water temperature was much more stable and lower than that of the jars, as the water temperature in

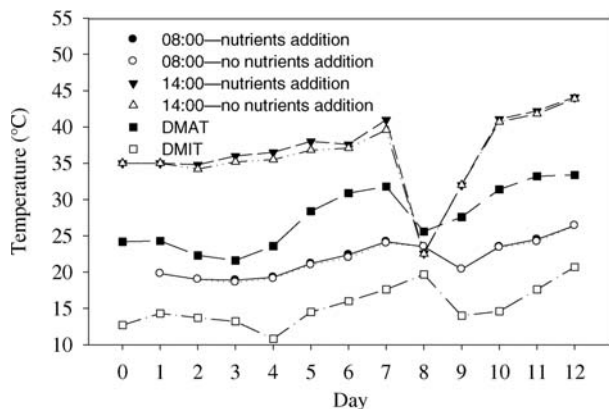


Fig. 6. The daily variations of water temperature at 08:00 and 14:00 h with or without nutrient addition, and the DMAT and DMIT of the air measured at a nearby weather station ZSNJ (58 238) in Nanjing, China.

the jars of small-scale incubation fluctuated with time of the day and weather changes.

DISCUSSION

Nutrient addition effects on phytoplankton community composition

The nutrient additions (Fig. 2a–g) significantly increased total phytoplankton biomass regardless of the presence or absence of the zooplankton on day 7 (Fig. 2h), similar to former observations (Pérez-Martínez and Cruz-Pizarro, 1995; Steiner, 2003). Nutrient additions and nutrient plus zooplankton interactions caused the phytoplankton community composition to change, with Cyanobacteria and Chlorophyta becoming the most dominant phyla, especially on day 12 (Table II, Figs 4 and 5). This is similar to the long-term succession of phytoplankton phyla observed in eutrophic ponds and lakes in summer (Schelske and Stoermer, 1971; DeNoyelles and O'Brien, 1978; Chen *et al.*, 2003).

Studies on the effects of nutrients on phytoplankton composition have examined the roles of nutrient ratios as well as nutrient concentrations. Smith (Smith, 1983) indicated that low N to P ratios favor dominance by cyanobacteria in lake phytoplankton, while other studies (Elser *et al.*, 1990; Jensen *et al.*, 1994) found that N to P ratios were not necessarily strong indicators of cyanobacterial dominance. The results of this experiment (Figs 1–5, Table II) showed that the N to P ratios between +Z + N and –Z + N treatments were very similar, while responses of the phytoplankton genera differed. Thus, specifically, N and P enrichment and the

interaction of these nutrient enrichments with zooplankton, rather than the N to P ratios, were main factors affecting phytoplankton composition in this experiment.

Our experiment also showed that *Microcystis* does not necessarily bloom in response to nutrient enrichment and that green algae dominate in response to nutrient enrichment when zooplankton is removed. This supports earlier work, showing that there was not a consistent relationship between the degree of eutrophication and *Microcystis* bloom formation (Tilman *et al.*, 1982; Steinberg and Hartmann, 1992; Nydick *et al.*, 2004). Our results differ from those observed in laboratory studies, in which competition between *Scenedesmus* (Chlorophyta) and *Microcystis* was affected by modes of nutrient supply (Olsen *et al.*, 1989; Watanabe and Miyazaki, 1996). However, Watanabe and Miyazaki (Watanabe and Miyazaki, 1996) also showed that *Microcystis* did not necessarily out-compete *Scenedesmus* at high inorganic nutrient supply rates, which was probably due to the larger maximum cellular uptake rates (V_{max}) for ammonium of *Scenedesmus* as opposed to *Microcystis*.

Zooplankton effects on *Microcystis* bloom formation

The presence of zooplankton and nutrient additions facilitated shifts in dominance from *Scenedesmus* and *Pediastrum* (Chlorophyta) to *Microcystis* (Cyanobacteria) (Figs 1 and 3), demonstrating that *Microcystis* bloom formation was related to interactive effects of nutrient enrichment and the presence of zooplankton. The preference of zooplankton for the more nutritious phytoplankton of *Scenedesmus* to *Microcystis* is similar to earlier findings that chlorophytes (e.g. *Scenedesmus*, *Chlorella*) were more readily grazed than cyanobacteria (e.g. *Microcystis*) (Arnold, 1971; de Bernardi and Giussani, 1990). Similarly, *Microcystis* blooms in lakes always coexist with a high abundance of smaller-sized zooplankton (Allan, 1977; Edmondson and Litt, 1982; Hanazato, 1991). This would also suggest that zooplankton grazing is not always effective in reducing *Microcystis* bloom occurrence and magnitude.

Bloom formation has been examined in the presence of zooplankton. The response of phytoplankton to zooplankton is a net result of grazing loss and of growth, and some colonial species showed increased growth rates in the presence of grazers (Lehman and Sandgren, 1985). Sellner *et al.* (Sellner *et al.* 1993) found that the majority of cyanobacterial production (including *Microcystis*) remained ungrazed by some rotifers and a cyclopid copepod. In addition, Porter (Porter, 1973) found that the growth of algal species resistant to digestion was enhanced rather than reduced by heavy

grazing. Similarly, Sarnelle (Sarnelle, 1993) and Grover (Grover, 1995) concluded that grazer-resistant algae should bloom and dominate under heavy grazing regimes. By reviewing the evidence concerning the role of grazer-resistant algae in aquatic trophic cascades, Agrawal (Agrawal, 1998) considered that both defense theory and aquatic trophic cascade theory suggested that grazer-resistant algae should be favored in an environment with heavy grazing, while edible algae should be favored in the absence of grazing.

Microcystis appeared to defend itself against zooplankton grazing in this experiment. Thus, a trade-off behavior of different phytoplankton in response to zooplankton grazing and nutrient enrichment was exhibited here. A size-based keystone trade-off explanation was offered by Steiner (Steiner, 2003), and Ghadouani *et al.* (Ghadouani *et al.*, 2003) suggested that there are at least three major aspects which may render cyanobacteria unsuitable as food for zooplankton. In this experiment, the cell numbers of each *Microcystis* colony always ranged between tens and hundreds, which was much lower than observed among colonies during blooms in nearby Lake Taihu, China. In our experiments, the *Microcystis* colony size did not appear to be large enough to create mechanical interference for zooplankton grazing. We therefore deduce that *Microcystis* was grazed but not easily digested and survived grazing to support additional blooms.

The zooplankton community size structure and taxonomic composition affect their selective grazing (Bergquist *et al.*, 1985; Cyr and Curtis, 1999). Zooplankton grazing by both large crustacea and protozoa on phytoplankton appears selective (Pace and Orcutt, 1981; Finlay *et al.*, 1988; Finlay and Esteban, 1998). Several investigations have shown that zooplankton–phytoplankton interactions are complex, with algal responses to zooplankton frequently being species-specific (Lehman and Sandgren, 1985; Elser *et al.*, 1987; Pérez-Martínez and Cruz-Pizarro, 1995). Meanwhile, large cladocerans, especially large-bodied *Daphnia* were considered as an important biomanipulation approach for controlling algal blooms (Gliwicz, 1990). Thus, the effects of zooplankton on the phytoplankton depend on the composition of both.

Since changes in the zooplankton composition during this experiment were not examined, the types of the initial zooplankton responsible for the selective grazing could not be determined. Clearly, there is a need to consider zooplankton composition in further studies. Additional experiments with a design similar to those conducted here using the same pond water during different months showed that *Microcystis* bloom formation was related to the survival of naturally occurring zooplankton and nutrient additions.

The roles of zooplankton were also observed in laboratory incubations of *Microcystis*. Laboratory monocultures of *Microcystis* in a medium with high nutrient concentrations (BG-11 medium) did not produce *Microcystis* blooms (colonies dominate at the water surface) even when *Microcystis* colonies (cells assembled together) formed. After introducing zooplankton to the culture, Yang *et al.* (Yang *et al.*, 2006) found that the grazing of protozoan flagellates caused *Microcystis aeruginosa* to form colonies, but the roles of cladocerans, copepods and rotifers actually could not be evaluated because they died during the experiment.

Weather and effects of other factors on *Microcystis* bloom formation

It is interesting that no *Microcystis* blooms formed in the pond, even in potentially favorable summer months. The reasons may be that the relationship between the zooplankton and phytoplankton is affected by multiple factors. It may partly be due to the fact that the golden carp grazed both the zooplankton and phytoplankton, hence affecting their balance. Also, algal sedimentation was significant in the experimental jars, which reduced the algal density in suspension, and was beneficial for dominance by *Microcystis*, and enhancing bloom potential. It is also possible that the temperature of the pond water was not high enough to allow *Microcystis* to dominate, although this genus is known to bloom in numerous nearby water bodies at much lower temperatures (Qin *et al.*, in press).

This experiment was conducted in early summer when sunny conditions and high water temperatures prevailed. The mean water temperature in the containers at 14:00 h reached about 36°C (Fig. 6), which may be much higher than the usual temperatures of the pond and those used in laboratory incubations. Commonly, 25°C is the temperature chosen for laboratory incubations. Most phytoplankton cellular processes are temperature dependent, their rates accelerating exponentially with increasing temperature, with maximal values occurring between 25°C and 40°C (Reynolds, 1984). Thus, the high water temperature promoted the competition for shared nutrient and improved the proliferation of some competitive species, and numerous studies have shown that high temperature in summer favors cyanobacterial blooms, including *Microcystis* blooms (Jacoby *et al.*, 2000; Jöhnk *et al.*, 2008; Paerl and Huisman, 2008).

Mechanisms of *Microcystis* bloom formation

The means by which only *Microcystis* becomes dominant and accumulates as surface blooms reflects the fact that

Microcystis out-competes other cyanobacterial and eukaryotic genera in this experiment. Reasons for this may include the fact that *Microcystis* has several unique characteristics promoting bloom formation, including gas vacuoles (buoyancy), efficient light capturing capabilities, light-protective pigments and exogenous factors (Paerl *et al.*, 1985; Paerl, 1988; Oliver and Ganf, 2000; Paerl *et al.*, 2001), as well as the benefits of zooplankton grazing, extremely high water temperature and irradiance. Thus, the development of *Microcystis* blooms from the eutrophic freshwater pond having initially low *Microcystis* density under summer conditions can be attributed to the combined effect of selective grazing of zooplankton on other algae, especially chlorophytes, accompanied by nutrient being supplied to support the exponential growth of *Microcystis* cells that were not grazed or survived from the grazing.

Mitra and Flynn (Mitra and Flynn, 2006) proposed a related mechanism to explain harmful algal bloom dynamics using a multi-nutrient predator–prey model, which demonstrated that these blooms can develop through the self-propagating failure of normal predator–prey activity, resulting in the transfer of nutrients into bloom growth at the expense of competing algal species. It should be stressed that the proper N and P nutrient additions and the natural zooplankton community structure were factors that facilitated the *Microcystis* bloom in this experiment.

CONCLUSIONS

This experiment provided an approach for simulating and examining mechanisms of *Microcystis* bloom formation in natural pond water, which initially was eutrophic ($110 \mu\text{g L}^{-1}$ chlorophyll *a*) but showed no bloom activity. *Microcystis* bloom formation from these naturally eutrophic water samples was dependent on sufficient N and P additions (net increase of $250.0 \mu\text{M}$ N and $16.1 \mu\text{M}$ P was provided twice in this experiment) and selective grazing of the naturally occurring zooplankton. Additional experimental work is needed to clarify the roles specific zooplankton taxa play in stimulating *Microcystis* bloom formation in controlled systems.

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