Grazing on *Microcystis* (Cyanophyceae) by testate amoebae with special reference to cyanobacterial abundance and physiological state

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Abstract

We examined the growth of testate amoebae preying on *Microcystis* whose physiological states were different in laboratory experiments and a hypertrophic pond. We prepared three experimental systems using water samples dominated by *Microcystis aeruginosa*: light incubation (control), dark incubation (dark), and light incubation with addition of nitrogen and phosphorus (+NP). In all the systems, colony density of *M. aeruginosa* decreased slightly during incubation. Physiological activity of phytoplankton as determined by chlorophyll fluorescence was high and almost constant in the control and +NP systems, whereas that decreased in the dark system. Cell densities of testate amoebae increased in the control and +NP systems, whereas in the dark system they remained low. Thus, growth of the amoebae was low in the systems where physiological activity of *Microcystis* was low. In a hypertrophic pond, cell density of testate amoebae increased and remained high when *M. aeruginosa* predominated. Cell density of testate amoebae increased remarkably, simultaneously with the increases in *M. aeruginosa* colony density and phytoplankton physiological activity. We also found a significant correlation between densities of *M. aeruginosa* colonies and testate amoebae. We suggested that the physiological activity of *Microcystis* is one important factor affecting the growth of testate amoebae grazing on *Microcystis*.

Keywords: *Microcystis*, testate amoebae, grazing, physiological state

Introduction

Blooms of cyanobacteria are notorious symptoms of eutrophication in freshwaters all over the world, deteriorating water quality as well as the health of human and natural resources. The genus *Microcystis* is the most frequently found in cyanobacterial blooms. We already have numerous reports on the physiological and ecological characteristics of *Microcystis*, and their bloom-forming mechanisms have been clarified (Reynolds et al. 1981; Oliver and Ganf 2000; Nakano et al. 2001*a*). However, loss processes of *Microcystis* populations are not yet fully understood.

Microcystis abundance is influenced by the usual biological interactions such as competition, grazing and infection, of which grazing may be the most important loss process controlling *Microcystis* abundance. Previous studies have reported as possible grazers of *Microcystis*: protists (Cole and Wynne 1974; Dryden and Wright 1987), rotifers (Snell 1980; Fulton and Pearl 1987), crustacean zooplankton (Hanazato and Yasuno 1984; Jarvis et al. 1987), fish (Moriarty 1973, Kawanabe and Mizuno 1989; Miura 1990). There are only a limited number of rotifers, crustaceans and fish which graze on *Microcystis* but various protistan species have been shown to do so (Zhang et

al. 1996; Nishibe et al. 2002 and 2004; Kim et al. 2006; Wilken et al. 2010). Indeed, grazing on *Microcystis* by protists occasionally dominates in the collapse of *Microcystis* blooms (Dryden and Wright 1987). Thus, it is possible that the wax and wane of a *Microcystis* bloom is dependent on grazing by protists.

Among such protistan grazers, rhizopods, including both naked and testate amoebae, are frequently found to be abundant when significant decreases in *Microcystis* abundance are detected in lakes, and grazing on *Microcystis* by some rhizopod species has been demonstrated in laboratory experiments (Yamamoto 1981; Yamamoto and Suzuki 1984; Nishibe et al. 2004) and field observation (Whitton 1973; Nishibe et al. 2004). Unfortunately, we still have limited eco-physiological information about the rhizopods which graze on *Microcystis*. Rodriguez-Zaragoza (1994) has reported that excessive nutrients and elevated water temperatures may be beneficial to common rhizopod species because such environmental conditions favor bacterial growth, which in turn feed rhizopods. Nishibe et al. (2004) reported that the abundance of the testate amoebae *Penardochlamys* sp. which grazes on *Microcystis* was high when *Microcystis* was abundant in a hypereutrophic pond. High rhizopod abundance with high *Microcystis* abundance may be reasonable, since the relationship of consumption by a grazer on various densities of prey follows the Michaelis-Menten equation. However,

not only quantity but also the quality of prey is also important for growth of the grazer. For the Excavata amoebae, Liu et al. (2006) examined the food selection mechanism and the digestion process of a Vahlkampfiid amoebae *Naegleria* sp. using several cyanobacterial strains and found that *Microcystis* was inappropriate food for the amoebae even when the cyanobacteria were heat-killed. By contrast, we still do not have any information about the effects of prey quality on the growth of testate amoeba (the Amoebozoa, Unikonts). In addition, no studies have so far examined the importance of the physiological state of *Microcystis* for the growth of rhizopods until now.

In the present study, we hypothesized that the rhizopods which grazed on healthy *Microcystis* would grow actively. To examine this hypothesis, we conducted laboratory experiments in which we fed testate amoebae using *Microcystis* with different physiological states. We also conducted field monitoring in a hypereutrophic pond to collect information about seasonal changes in abundance of testate amoebae together with the abundance and physiological state of the *Microcystis*. This is the first study which reports the importance of physiological state of *Microcystis* for growth of rhizopods.

Materials and methods

Laboratory experiment

We conducted laboratory experiments using twelve liters of water collected on 30 June and 27 September 2005, from Furuike Pond (33°49'N, 132°48'E), Matsuyama City, Ehime Prefecture, Japan using a water column sampler to collect an integrated water sample from the whole water column. The pond is hypertrophic due to anthropogenic loading from the watershed, and its physical and chemical characteristics have been described in our previous studies (Nakano et al. 1998 and 2001b; Manage et al. 1999 and 2001; Nishii et al. 2001). *Microcystis* species usually become dominant in this pond from May to October (Nakano et al. 1998; Manage et al. 2001; Nishii et al. 2001).

We prepared three experimental systems, each in duplicate. A 1.5-liter portion of the mixed water sample was poured into a 3-liter flask, and KH_2PO_4 and KNO_3 were added at 5 μmol P L^{-1} and 80 μmol N L^{-1} respectively (+NP system). For the control system, a 1.5 liter portion of the water sample was poured into a 3 liter flask. These two systems were then incubated at 25 ºC at a photon flux density of 60 μmol m^{-2} s^{-1} under a 12:12 hour light:dark cycle with daily shaking. It is likely that the light intensity used in the present study was appropriate, since most species of phytoplankton have the light

intensity of saturation in the range of 60 and 100 μE m^{-2} s^{-1} (Harris 1978). The remaining 1.5 liters of the water sample were poured into a 3 liter flask and incubated in the dark at 25 ºC with daily shaking. We took a 100 ml subsample from each system every day and followed changes in density of *Microcystis* colonies, cell density of amoebae and physiological activity of the phytoplankton.

For enumeration of *Microcystis* colonies and amoeba cells, a 50 ml portion of the water sample was fixed with acidified Lugol's solution at a final concentration of 1% and concentrated by natural sedimentation. *Microcystis* colonies and amoeba cells were counted in a haematocytometer (Burker-Turk) under a light microscope at a magnification of ×400 at least 3 times.

A 50 ml portion of the water sample was used to measure the physiological activity of the phytoplankton using a Water-PAM Chlorophyll Fluorometer (Heinz-Walz). PAM fluorescence measurements are based on the determination of the ground fluorescence; F_0 which is measured in weak, constant irradiation of a dark-adapted sample (all reaction centers in the open state). The maximal fluorescence, F_m, is measured in a saturation pulse light (all reaction centers in the close state). The variable fluorescence, F_v, is calculated as the difference between F_0 and F_m. The efficiency of photochemistry of open reaction centers of photosystem II (F_v/F_m) was

calculated as follows:

$$F_v / F_m = (F_m - F_0) \times F_m^{-1}$$

Immediately after taking the water samples, a 20 ml subsample was placed in the dark for 20 min and then we measured the minimum (F_0) and maximum (F_m) fluorescence yield.

Field monitoring

Weekly field monitoring was conducted from 16 May to 23 November 2006 in Furuike Pond. Surface water temperature was determined using a thermistor (ABT-1, ALEC Electronics Co. Ltd.). Water samples were taken as described previously.

To determine chlorophyll *a* concentration, 10 ml of each water sample were filtered through a 0.2 μm Nuclepore filter (25 mm in diameter, CORNING Nuclepore) under negative pressure at 0.05 MPa to retain seston. The filter was then transferred into a glass tube containing 8 ml of *N*,*N*-dimethylformamide to extract chlorophyll *a* and kept in a freezer at -20 ºC. The amount of chlorophyll *a* was determined using a fluorometer (Turner Designs, 10-AU) (Moran and Porath 1980).

For enumeration of phytoplankton and testate amoebae, 300 ml of the water sample was fixed with acid Lugol's solution at a final concentration of 1%.

Enumeration of cells of phytoplankton and amoebae were conducted as explained above.

Physiological activity of phytoplankton was determined using another 50 ml portion of the water sample as described previously.

Results

Laboratory experiment

The experiments started from 30 June and 27 September had high reproducibility. During the experimental period, densities of *Microcystis* colonies decreased in all the systems (Figs. 1A). Almost no difference was found between *Microcystis* colony densities in the controls and the +NP systems, although those in the dark system were the lowest (Figs. 1A). *Microcystis aeruginosa* predominated throughout the experimental period in the control, +NP and dark systems.

Physiological activity of the phytoplankton was almost constant in the control and +NP systems (Figs. 1B). By contrast, physiological activity in the dark system gradually decreased (Figs. 1B).

The dominant amoeba found in the present study was a testate amoeba which belonged to the genus *Penardochlamys*. We also counted naked amoebae, but their

densities were very low compared with those of the testate amoebae (data not shown). Cell densities of the testate amoeba gradually increased in the control and +NP systems (Figs. 1C) from 550 cells ml^{-1} (0 day) to 3.2×10^4 cells ml^{-1} (9 day) and from 330 cells ml^{-1} (0 day) to 3.8×10^4 cells ml^{-1} (9 day), respectively. The testate amoeba grew in the dark system (Fig. 1C). However, its growth was negligible, ranging between 660 cells ml^{-1} (0 day) and 9500 cells ml^{-1} (6 day) (Fig. 1C).

Field monitoring

Chlorophyll *a* concentration in Furuike Pond increased from May to June, reaching their maximum (723 $\mu g\ l^{-1}$) on 13 June, remained relatively high in July and August with fluctuations and then gradually decreased in September and October (Fig. 2A). Physiological activity of the phytoplankton showed cyclic oscillations from May to October, ranging between 0.194 and 0.528 (Fig. 2B). Dominant phytoplankton species during the study period were *Microcystis aeruginosa* and *M. wesenbergii*.

Densities of *M. aeruginosa* colonies fluctuated widely between May and June, remained low in July and August (Fig. 2C), then increased to high densities recorded in September and October, followed by a decrease in November (Fig. 2C). *M. wesenbergii* colony density increased from 16 May to 24 July and became almost stable from 31

July onwards, although a relatively high density (15.4×10^3 colonies ml^{-1}) was found on 17 November (Fig. 2C).

The increase in cell density of testate amoebae was slow between 16 May (37 cells ml^{-1}) and 21 August (222 cells ml^{-1}), followed by a rapid increase to 30 August (1037 cells ml^{-1}) (Fig. 3A). The maximum density was recorded on 21 September (1593 cells ml^{-1}), and then the cell density decreased from 27 September onwards (Fig. 3A).

The percentage of testate amoebae attached to *Microcystis* colonies was relatively high between June and July, and between September and October, but in August was negligible (Fig. 3B). Relatively high densities of testate amoebae were found on *M. wesenbergii* colonies between June and July, whereas the densities of amoebae attached to *M. aeruginosa* colonies between August and October were higher than those on *M. wesenbergii* colonies (Fig. 3C).

Pearson Correlation Analysis showed that there was no significant relationship between cell density of testate amoebae and concentration of chlorophyll *a*, or physiological activity of the phytoplankton (Table 1). We did find a significant correlation between the densities of testate amoebae and *M. aeruginosa* ($r = 0.7664$, $n = 25$, $p<0.001$), but the correlation between the densities of testate amoebae and *M. wesenbergii* ($r = 0.1562$, $n = 25$) was insignificant (Table 1).

Discussion

Laboratory experiment

During the present study, colony density of *Microcystis* decreased in all our experimental systems, despite the fact that we added large amounts of N and P to the +NP systems (see Materials and methods). The patterns of decrease in *Microcystis* colony density in the +NP systems were similar to those in the control systems (Fig. 1A), indicating that the *Microcystis* in the present study was not subjected to N or P limitation. Thus, it is likely that another element(s) was responsible for the decrease in *Microcystis* colony density, since light was available in the +NP and the control systems. All experiments in the present study were conducted in batch cultures where no additional nutrients were supplied after the beginning of the experiment. This was not the case for CO_2, because CO_2 would be supplied by mixing in each system when we took subsamples. However, we did not bubble-mix the systems, and CO_2 supply in our experiments might have been insufficient for *Microcystis* growth. Thus, we think that the decrease in *Microcystis* colony density in our experimental systems was due to carbon limitation. Even if carbon was limiting for *Microcystis*, the decrease in colony density was small in the +NP and control systems, and we had a variety of physiological

activities in our systems. We therefore believe that carbon limitation on *Microcystis* in the present study does not affect our interpretation of the results.

The dominant amoeba found in the present study belonged to the testate amoeba *Penardochlamys*. It has been reported that *Microcystis* is the only prey for *Penardochlamys* (Nishibe et al. 2004), suggesting that the abundance of amoebae depend on that of the cyanobacteria. However, in the dark system, growth of the testate amoeba was low (Figs. 1C) in spite of high *Microcystis* colony densities (Fig. 1A). Thus, the low growth of the testate amoeba could not be explained by *Microcystis* abundance. In the dark system, physiological activity of the phytoplankton predominated by *Microcystis* decreased (Figs. 1B), although the *Microcystis* colony density was high (Figs. 1A). Thus, growth of the testate amoebae was low in the systems where the physiological activity of *Microcystis* was low which suggests that the physiological activity of *Microcystis* is responsible for changes in growth of the testate amoebae.

Field monitoring

In our field monitoring we found a significant logarithmic correlation between chlorophyll *a* concentration and *Microcystis* colony density (*M. aeruginosa* plus *M. wesenbergii*) ($n = 25$, $r = 0.464$, $p<0.05$). Thus, the phytoplankton physiological activity

shown in Fig. 2B can be regard as that of *Microcystis*. However, we did not find any significant correlations between concentration of chlorophyll *a* and cell density of testate amoebae, or between physiological activity of phytoplankton and cell density of testate amoebae (Table 1). These results suggest the importance of prey species for growth of testate amoebae. There was a clear succession of dominant *Microcystis* species (Fig. 2C), and *M. aeruginosa* predominated during September (Fig. 2C) when cell density of testate amoebae increased and remained high (Fig. 3A). In addition, a significant correlation between densities of *M. aeruginosa* colonies and testate amoebae (Table 1) and higher densities of testate amoebae attached to *M. aeruginosa* colonies (Fig. 3C) suggest that prey availability is important for the growth of this testate amoebae, and that the food linkage between *M. aeruginosa* and testate amoebae in the present study is to some extent species-specific.

However, this is contrary to the results of our previous study (Nishibe et al. 2004) where the food linkage between *Microcystis* and testate amoebae was not species-specific. Indeed, also in the present study, percentages of amoebae attached to *M. wesenbergii* colonies (Fig. 3C) were high relative to those on *M. aeruginosa* colonies during the dominance of *M. wesenbergii* (Fig. 2C), although overall cell density of testate amoebae was low (Fig. 3A). The dominant testate amoebae species described in

Nishibe et al. (2004) was *Penardochlamys* sp., and this might be the case in the present study. Some previous studies have reported that some rhizopods collected from natural waters seemed to have strong feeding selectivity on specific prey (Cook et al. 1974; Becares and Romo 1994), but others have shown that some rhizopods have a wide range of prey within cyanobacterial species (Ho and Alexander 1974; Yamamoto and Suzuki 1984; Laybourn-Parry et al. 1987). We need to collect more information about abundance and composition of amoebae attached to *Microcystis* colonies In order to understand which amoebae species are important as grazers of the cyanobacteria.

In early and mid-August, colony density and physiological activity of both *M. aeruginosa* and *M. wesenbergii* remained low (Fig. 2), and cell density of testate amoebae was also low (Fig. 3A). Moreover, almost no amoebae were attached to *Microcystis* colonies during that period (Fig. 3B). Cell density of testate amoebae markedly increased from late August (Fig. 3A), simultaneously with the increases in density of *M. aeruginosa* colonies (Fig. 2C) and phytoplankton physiological activity (Fig. 2B). Thus, our field monitoring suggested that not only prey availability but also prey quality are important for growth of testate amoebae grazing on *Microcystis*, although we did not find any significant correlation between physiological activity of phytoplankton and cell density of testate amoebae (Table 1).

Conclusions

Rhizopods have long been considered to be of minor importance in the food webs of freshwater and marine systems. However, food linkage between rhizopods and *Microcystis* may provide another important role for amoebae in aquatic food webs. From the results obtained in the present study, we concluded that physiological activity of *Microcystis* is another important factor which affects the growth of testate amoebae that graze on *Microcystis*. The chemical composition of *Microcystis* probably varies, depending on the physiological state of these cyanobacteria. Thus, changes in growth of testate amoebae may be affected by the chemical composition or nutritional value of their prey. Unfortunately, we did not examine the chemical composition of the *Microcystis* in the present study. Further studies are required to elucidate changes in the growth of amoebae in relation to changes in the chemical composition or nutritional value of their prey. In addition, not only are biological but also physico-chemical variables most likely to be responsible for growth of amoebae, and further studies to examine which environmental factors affect the abundance and composition of amoebae are needed to elucidate their ecology.

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Table 1 Pearson Correlation Analysis between testate amoebae density and phytoplankton variables.

	r	Significance	Relationship
Chlorophyll *a* concentration	0.116	Not significant	Negative
Fv / Fm	0.076	Not significant	Positive
Colony density of *M. aeruginosa*	0.766	$p<0.001$	Positive
Colony density of *M. wesenbergii*	0.156	Not significant	Negative

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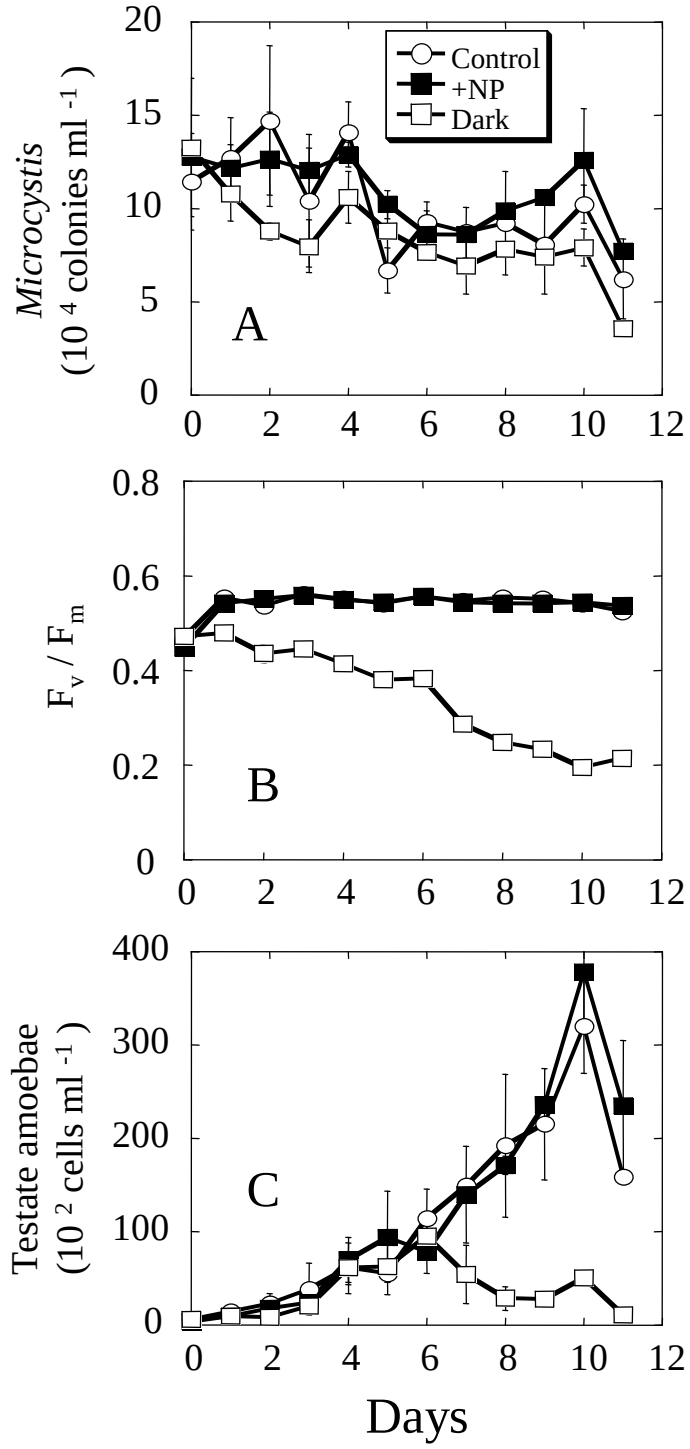
Figure captions

Fig. 1 Changes in colony density of *Microcystis aeruginosa* (A), physiological activity of *M. aeruginosa* (B) and cell density of testate amoebae (C) in the experiment started on 30 June 2005. Vertical bars that indicate differences between duplicates are shown when they exceeded the size of the symbol.

Fig. 2 Seasonal changes in chlorophyll *a* concentration (A), phytoplankton physiological activity (B) and colony density of *Microcystis aeruginosa* and *M. wesenbergii* (C) in Furuike Pond between May and November 2006.

Fig. 3 Seasonal changes in cell density of testate amoebae (A), the percentage of testate amoebae attached to *Microcystis* colonies (B) and the percentage of testate amoebae attached to *Microcystis aeruginosa* colonies (white part) or to *M. wesenbergii* colonies (gray part) (C) in Furuike Pond between May and November 2006.



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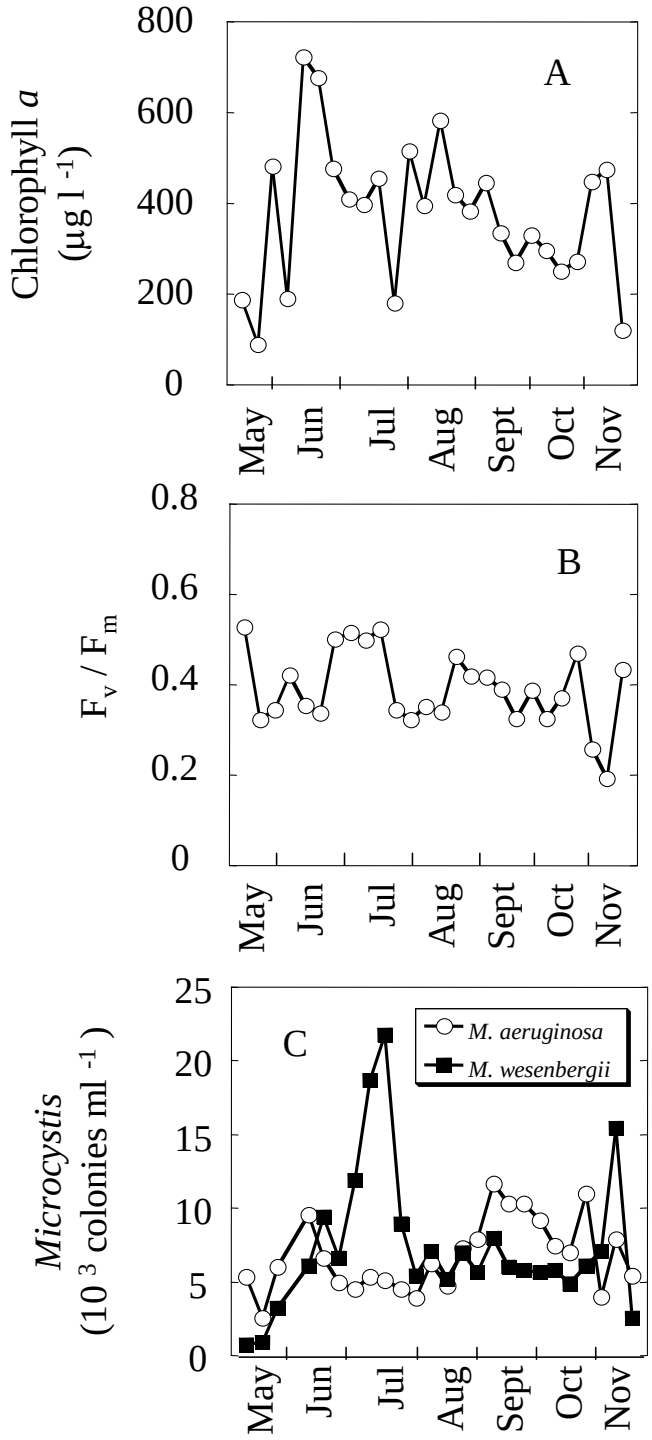

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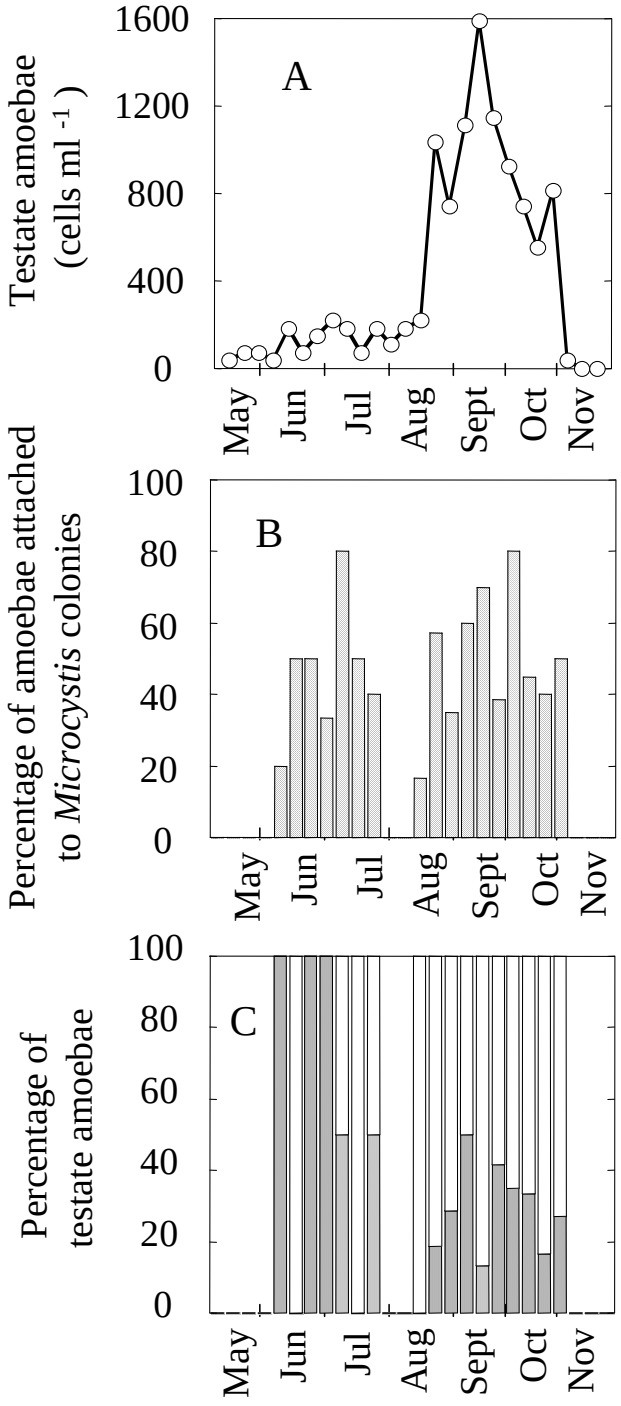

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