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The effect of a mixotrophic chrysophyte on toxic and colony-forming cyanobacteria

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SUMMARY

1. In order to test the effect of *Ochromonas* sp., a mixotrophic chrysophyte, on cyanobacteria, grazing experiments were performed under controlled conditions. We studied grazing on three *Microcystis aeruginosa* strains, varying in toxicity and morphology, as well as on one filamentous cyanobacterium, *Pseudanabaena* sp. Furthermore, we analysed the co-occurrence of *Ochromonas* and *Microcystis* in natural systems in relation to various environmental parameters (TP, TN, DOC, temperature, pH), using data from 460 Norwegian lakes.
2. *Ochromonas* was able to feed on all four cyanobacterial strains tested, and grew quickly on all of them. The chrysophyte caused net growth reductions in all three *Microcystis* strains (the very toxic single-celled strain PCC 7806; the less toxic colony-forming Bear AC and the less toxic single-celled Spring CJ). The effect of *Ochromonas* was strongest on the Spring CJ strain. Although the effect of *Ochromonas* grazing on the growth of *Pseudanabaena* was relatively smaller, it also reduced the net growth of this cyanobacterium significantly.
3. After 4 days of incubation with *Ochromonas* the total amount of cyanotoxins in the three *Microcystis* strains was reduced by 91.1–98.7% compared with the controls.
4. *Ochromonas* occurred in similar densities across all 460 Norwegian lakes. *Microcystis* occurred only at higher TN, TP, temperature and pH values, although its density was often several orders of magnitude higher than that of *Ochromonas*. *Ochromonas* co-occurred in 94% of the samples in which *Microcystis* was present.
5. From our study it is not clear whether *Ochromonas* could control *Microcystis* blooms in natural lakes. However, our study does demonstrate that *Ochromonas* usually occurs in lakes with *Microcystis*, and our small scale experiments show that *Ochromonas* can strongly reduce the biomass of *Microcystis* and its toxin content.

Keywords: cyanobacterial blooms, microcystin degradation, *Microcystis aeruginosa*, mixotrophy, *Ochromonas*

Introduction

Cyanobacteria have increasingly become a nuisance in aquatic ecosystems due to bloom formation and toxin production (Chorus & Bartram, 1999). They dominate many aquatic ecosystems due to increases in nutrient loading, water temperatures, duration of summer stratification, increased water residence time and salinisation (Paerl & Huisman, 2008). Cyanobacteria may cause mass mortality of aquatic organisms in lakes (e.g. Krienitz *et al*., 2003) and also human illness and even death have been linked to toxic cyanobacteria (Carmichael *et al*., 2001). One of the most notorious freshwater cyanobacteria that may form dense surface scums in temperate regions during late

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summer is the colony-forming species *Microcystis aeruginosa*.

The first and most crucial step in mitigating cyanobacterial blooms is to decrease the nutrient load to the ecosystems. An additional measure may be to promote the growth of filter-feeders by food-web manipulation, a strategy known as biomanipulation (Shapiro & Wright, 1984; Gulati & Van Donk, 2002). In earlier studies, the emphasis of biomanipulation lay on taking measures that lead to an increase of the biomass of large cladocerans like daphniids (Shapiro & Wright, 1984). However, cyanobacteria may be difficult for cladocerans to ingest, since their size and shape interfere with the filtering system of cladocerans (Lampert, 1987). Also the production of microcystins or other cyanotoxins by cyanobacteria contributes to their inedibility (Rohrlack *et al*., 1999; Ghadouani *et al*., 2004; Hansson *et al*., 2007). Furthermore, cyanobacteria may lack long-chained polyunsaturated fatty acids, which are essential for cladocerans (Müller-Navarra *et al*., 2000; Wacker & Von Elert, 2001).

Cole & Wynne (1974) were among the first to report the feeding of *Ochromonas danica* Pringsheim, a mixotrophic chrysophyte, on single-celled *Microcystis aeruginosa* Kuetz., the toxic content of which was not measured. They suggested that *Ochromonas* might serve as a possible biological control agent against *Microcystis* blooms. Recently, we observed a negative effect of *Ochromonas* sp. on a bloom of colony-forming *M. aeruginosa*. *Ochromonas* invaded a preliminary experiment in which we were studying the scum-layer formation of *M. aeruginosa* in large-scale laboratory mesocosms.

Although mixotrophy among planktonic nanoflagellates has been well documented in the literature (Sanders, 1991; Isaksson, 1998; Jones, 2000), most studies have been conducted in relation to heterotrophic bacteria. Despite several studies that showed the ability of mixotrophic chrysophytes to graze on various phytoplankton species (Cole & Wynne, 1974; Zhang, Watanabe & Inouye, 1996; Zhang & Watanabe, 2001; Zhang *et al*., 2008), very little attention has been given to the ecological importance of these organisms as primary consumers in aquatic systems (but see: Tittel *et al*., 2003; Ptacnik *et al*., 2004).

The genus *Ochromonas* has been reported from a broad range of habitats in both marine and fresh waters (Sanders, 1991). It has been successfully cultured under autotrophic, mixotrophic and heterotrophic conditions (Rothhaupt, 1996; Sanders *et al*., 2001). This flexibility in metabolic modes has been considered adaptive under stressful situations, such as low nutrient concentrations (Stibor & Sommer, 2003) or low light conditions (e.g. Holen, 1999; Jones, 2000; Flöder, Hansen & Ptacnik, 2006). Under such conditions, mixotrophy may offer a competitive advantage over specialist autotrophy on the one hand, and specialist heterotrophy on the other (Katechakis & Stibor, 2006).

Based on these studies, and our own mesocosm observations, we conducted laboratory experiments to analyse the effect of *Ochromonas* feeding on different strains of cyanobacteria, varying in toxicity and the degree of colony formation. Further, very little is known about the natural occurrence of *Ochromonas* in eutrophic lakes, its effects on cyanobacterial blooms and the mechanisms controlling its abundance and grazing rates. To see whether there is a possibility for the interaction between these two organisms to play a role in natural systems, we also analysed data from 460 lakes in Norway. *Microcystis* and *Ochromonas* are both common in Scandinavian lakes, making the latter suitable field sites for the study of the coexistence of *Ochromonas* and *Microcystis* under a wide range of environmental conditions.

Methods

Grazing experiment

Phytoplankton pre-cultures. Four cyanobacterial strains were used in the grazing experiment (Table 1). We selected three *Microcystis aeruginosa* strains that varied in toxicity and morphology; (i) the very toxic single-celled strain PCC 7806; (ii) the less toxic colony-forming Bear AC and (iii) the less toxic single-celled Spring CJ. We also used in the grazing experiment the filamentous cyanobacterium, *Pseudanabaena* sp. CCY 9704, which has relatively long filaments and a low toxin content. The mixotrophic chrysophyte *Ochromonas* sp. (probably *Ochromonas globosa* Skuja, determined by Dr R. Bijkerk, Koeman & Bijkerk B.V., Ecological Research and Advice, Haren, The Netherlands) has been isolated from the mesocosms, in which it occurs as a contaminant, using glass pipettes. Stock cultures were maintained autotrophically in COMBO-medium using ammonia

Table 1 Cyanobacteria strains used in the grazing experiments. The mean particle volumes (MPV) of the strains are given in μm^3, filament length of *Pseudanabaena* is in μm (average filament diameter 1.2 μm). The microcystin content of the species is given in amount per biovolume (μg μm^{-3})

	MPV (μm^3)		Microcystin content (μg μm^{-3})	
Phytoplankton species	Mean	SD	Mean	SD
Microcystis aeruginosa PCC 7806*	8.48	6.87	4.12×10^{-6}	9.81×10^{-7}
Microcystis aeruginosa Spring CJ†	153	59.9	6.06×10^{-7}	6.09×10^{-8}
Microcystis aeruginosa Bear AC†	810	425	9.81×10^{-7}	5.58×10^{-7}
	Filament length (μm)			
Pseudanabaena sp. CCY 9704‡	78.1	98.0	7.89×10^{-8}	9.11×10^{-9}

*Pasteur Culture Collection, Paris, France.
†Strain provided by Prof. Dr Alan Wilson (Auburn University, Auburn, U.S.A.).
‡Culture Collection Yerseke, NIOO-Centre for Estuarine and Marine Ecology, Yerseke, The Netherlands.

as a nitrogen-source. All species were pre-cultured under experimental conditions in Erlenmeyer flasks containing 200 mL of COMBO medium.

Experimental conditions. The grazing experiment was performed in 250 mL Erlenmeyer flasks filled with 50 mL of cyanobacteria, growing exponentially on COMBO medium. The *Ochromonas* treatment flasks were then inoculated with 50 mL of *Ochromonas* culture, while the controls received another 50 mL of COMBO medium. Initial nutrient concentrations in both the controls and treatments were high, supporting the growth of cyanobacteria without nutrient limitation.

The initial biovolume of the cyanobacteria was ca. 1.5×10^7 μm^3 mL^{-1} (*M. aeruginosa* PCC 7806: ca. 1.77×10^6 cells mL^{-1}; *M. aeruginosa* Bear AC: ca. 1.86×10^4 colonies mL^{-1}; *M. aeruginosa* Spring: ca. 9.82×10^4 cells mL^{-1}; *Pseudanabaena* sp. CCY 9704: ca. 1.53×10^4 filaments mL^{-1}) and of *Ochromonas* ca. 2.4×10^6 μm^3 mL^{-1} (ca. 2.4×10^4 cells mL^{-1}). Both the treatments and controls were performed in triplicates. The flasks were placed in an incubator on a rotating table (50 rpm) for 4 days with a 14 h : 10 h light: dark cycle, a light intensity of 125 μmol quanta [PAR] s^{-1} m^{-2} and a temperature of 25 °C. Samples were fixed with glutaraldehyde and formaldehyde, final concentrations being 0.025 mass % and 0.0037 mass % respectively. The changes in cell numbers and biovolumes of the cyanobacteria and *Ochromonas* were determined daily using a Flow Cytometer (MoFlo™ XDP Cell Sorter; Beckman Coulter, Inc., Fullerton, CA, U.S.A.). From these measurements, we calculated the growth rates of the cyanobacteria and *Ochromonas* in the controls and the treatment flasks over a period of 4 days. Daily specific growth rates (μ) for the 4-day period were calculated for each flask as:

$$\mu = \frac{\ln(N_t) - \ln(N_0)}{t}$$

where N_0 and N_t are the cell densities at the start and the end. The net growth reductions of the cyanobacteria, due to the presence of *Ochromonas*, were calculated from the differences in growth rates between controls and *Ochromonas* treatments. Changes in size distribution of the cyanobacteria, due to grazing by *Ochromonas*, were studied using an image analyser connected to an inverted microscope measuring the greatest linear dimension of 100 cells or colonies in both control and treatment flasks at the end of the grazing experiment.

To investigate further whether *Ochromonas* could have an impact on *Microcystis* cell yield and microcystin concentrations at densities typical of Norwegian lakes, we did a second grazing experiment with *Ochromonas* grazing on strain PCC 7806. PCC 7806 was selected for this experiment because this very toxic strain was found to be less edible. We inoculated a lower initial biovolume of 6.2×10^6 μm^3 mL^{-1} of *Microcystis* (ca. 2.7×10^5 cells mL^{-1}) and 3.2×10^4 μm^3 mL^{-1} (ca. 3.2×10^2 cells mL^{-1}) of *Ochromonas* into 500 mL Erlenmeyer flasks filled with 300 mL COMBO-medium and a control with *Microcystis* only. Both treatments were done in four replicates. The flasks were incubated at 23 °C under 14 h : 10 h light: dark cycle with a light intensity of 90 μmol quanta [PAR] s^{-1} m^{-2}. Cell

densities were determined using a CytoSense flow cytometer (CytoBuoy BV, Woerden, The Netherlands). Sampling and fixation of samples were done as described for the previous experiment.

Microcystin analyses

The effect of *Ochromonas* on the microcystin concentrations (both dissolved and particulate microcystin) in the treatment as well as in the control flasks was measured at the end of the grazing experiments (day 4). For measuring the microcystin content of the four different cyanobacteria strains, samples of the cultures were filtered through glass fibre filters (Whatman GF/F; pressure 10 kPa) to separate the cyanobacteria cells from the medium. The remaining filtrate was used for analysis of extracellular microcystins. The GF/F filters were freeze-dried and cell-bound microcystin on these filters was extracted in 75% methanol, using three extraction rounds as described in Fastner, Flieger & Neumann (1998), with an extra step for grinding of the filters with 0.5 mm silica beads in a Mini Bead beater (BioSpec Products Inc., Bartlesville, OK, U.S.A.) (Tonk *et al*., 2005). The extracts were blow-dried with N_2 at 40 °C and then re-dissolved in 50% methanol for analysis of microcystin, using an HPLC with photodiode array detection (Kontron Instruments Ltd., Bletchley, U.K.). Successively, the extracts were separated using a LiChrospher 100 ODS 5 μm LiChorCART 250-4 cartridge system (Merck, Darmstadt, Germany) and a 30–70% gradient of acetonitrile with 0.05% trifluoroacetic acid at a flow rate of 1 mL min^{-1}. The different microcystin variants were identified on the basis of their characteristic UV spectra and were quantified by means of microcystin-LR and microcystin-RR gravimetric standards (provided by Prof. G.A. Codd, University of Dundee, U.K.). The concentration of extracellular microcystins and the microcystin concentration in the second grazing experiment, with densities of *Ochromonas* as found in the Norwegian lakes, were detected and quantified using an enzyme-linked immunosorbent assay (ELISA), according to the protocol of a Microcystin Plate Kit (SDI EnviroGard®, Portland, ME, U.S.A.). To extract the cellular microcystins for ELISA-quantification, cells were subjected to three freeze-and-thaw cycles each followed by sonification (Gustafsson, Rengefors & Hansson, 2005).

Statistical analyses

Most statistical analyses were carried out with the STATISTICA data analysis software system (StatSoft, Inc., 2007, version 8.0. http://www.statsoft.com). Data were first tested for homoscedasticity (Levene's test for ANOVA). Net growth rates reductions and growth rates of *Ochromonas* on different cyanobacteria were compared by one-way ANOVA, followed by *post hoc* comparisons (Tukey HSD test). Because of their heteroscedasticity, the data of the final biovolumes and cyanobacteria sizes were analysed using the nonparametric Mann–Whitney *U*-test. The data on microcystin concentrations (particulate, dissolved and total) were also heteroscedastic, even after transformation. Therefore, the interactive effects of strain identity and *Ochromonas* treatment on microcystin concentrations were analysed manually using a spreadsheet to perform the Scheirer-Ray-Hare extension of the Kruskal–Wallis test (Sokal & Rohlf, 1995), followed by pair-wise comparisons within strains using the Mann–Whitney *U*-test.

Field data analysis

For the analysis of field data, we used data from a total of 1395 phytoplankton summer samples collected in 460 Norwegian lakes between 1988 and 2000 (Moe, Dudley & Ptacnik, 2008). Phytoplankton composition and biovolume were analysed by light microscopy and physical and chemical parameters by standard methods (Moe *et al*., 2008). *Microcystis* and *Ochromonas* were identified to genus (identification to species was not possible in the preserved lake samples). Data on water temperature were not available, thus we derived estimates of local air temperature from a grid of long-year averages (Mitchell & Jones, 2005). We compared the biovolume concentrations of *Microcystis* and *Ochromonas* along several environmental gradients: total phosphorus (TP), total nitrogen (TN), TN: TP ratio, temperature, dissolved organic carbon (DOC: colour mg Pt L^{-1} was used as measure for the DOC concentration), pH and total phytoplankton biovolume concentration.

Results

Grazing experiment

Ochromonas caused a net biovolume reduction within all strains (all strains: Mann–Whitney *U*-test: $Z_{adj} = 1.964$, $P = 0.0495$, $n = 2 \times 3$: Fig. 1a). Within the different *Microcystis* strains this reduction was strongest for the less toxic single-celled strain (Spring CJ: 99.8%), whereas the influence on the other two strains of *Microcystis* (the more toxic PCC 7806; and the one building colonies, Bear AC) was less strong (98.6% and 96.5% respectively). The effect of *Ochromonas* on the biovolume of *Pseudanabaena* was relatively weaker, although still substantial (92.2%).

The net growth reductions were significantly different among the different strains (one-way ANOVA, $F_{2,9} = 29.397$, $P = 0.0001$) (Fig. 1b). The highest net growth reduction was found for the less toxic single-celled strain *Microcystis aeruginosa* Spring CJ ($\Delta\mu = 1.71 \pm 0.22$ day^{-1}), followed by the very toxic single-celled *Microcystis* PCC 7806 ($\Delta\mu = 1.07 \pm 0.11$ day^{-1}), the colony-forming *Microcystis* Bear AC ($\Delta\mu = 0.834 \pm 0.11$ day^{-1}) and, finally, *Pseudanabaena* ($\Delta\mu = 0.648 \pm 0.12$ day^{-1}). The growth rates of *Ochromonas*, feeding on different cyanobacterial prey strains, varied between 0.19 ± 0.025 and 0.47 ± 0.012 day^{-1}. The growth rate was significantly lower on *Pseudanabaena* (one-way ANOVA, $F_{2,9} = 10.218$, $P = 0.0041$) (Fig. 1c).

The size distribution of the small *Microcystis* PCC7806 (2.35 μm mean cell diameter; Fig. 2) did not differ significantly between treatments. However, for the larger *Microcystis* strains and for *Pseudanabaena*, the mean greatest linear dimensions of the cells and colonies increased in the *Ochromonas* treatment (Fig. 2). In the presence of *Ochromonas*, for the average-sized *Microcystis* Spring CJ, the mean particle diameter changed from 6.7 to 7.4 μm (Mann–Whitney *U*-test: $U = 8736.5$, $P = 0.0008$, $n = 2 \times 150$), and for the larger *Microcystis* Bear AC, from 12.7 to 13.2 μm ($U = 16862.5$, $P = 0.0026$, $n = 2 \times 202$). For *Pseudanabaena* the difference was the greatest, with the mean filament length increasing from 78.1 to 192.8 μm in the presence of *Ochromonas* ($U = 26574.5$, $P < 0.0001$, $n = 2 \times 300$). Visual observations showed that *Ochromonas* seemed to prefer the more readily ingestible smaller filaments, even though it also attacked the ends of larger filaments.

For all *Microcystis* strains, we observed a dramatic decrease in total microcystin content in the presence of *Ochromonas* (Fig. 3). We did not find differences between strains, but did find a significant *Ochromonas* treatment effect on total microcystin content (Scheirer-Ray-Hare extension of Kruskal–Wallis ANOVA, ($H_{1,24} = 12.40$, $P < 0.001$). This was mainly due to the cell-bound (particulate) microcystins, which decreased significantly in concentration due

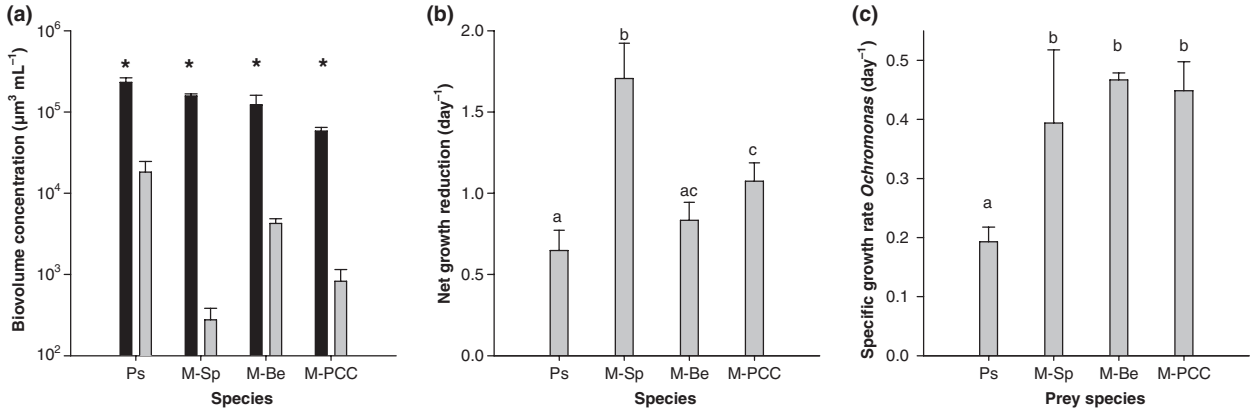

Fig. 1 Growth parameters of the phytoplankton species used in the grazing experiments: (a) biovolume concentrations of the different cyanobacteria on day 4 in the absence (black bars) or presence (grey bars) of *Ochromonas*. Asterisks indicate significant differences between *Ochromonas* treatments and controls within the different strains. Note the logarithmic ordinate; (b) net growth reduction of the different cyanobacteria due to the presence of *Ochromonas*; (c) growth rate of *Ochromonas* when cultured with different cyanobacteria. Letters above columns (a,b,c) indicate homogeneous groups, i.e. groups that do not differ significantly after nonparametric multiple comparisons. Cyanobacteria strain codes: Ps = *Pseudanabaena* sp. CCY 9704; M-Sp = *Microcystis aeruginosa*, Spring CJ; M-Be = *Microcystis aeruginosa*, Bear AC; M-PCC = *Microcystis aeruginosa*, PCC 7806. Error bars indicate ± 1SD.

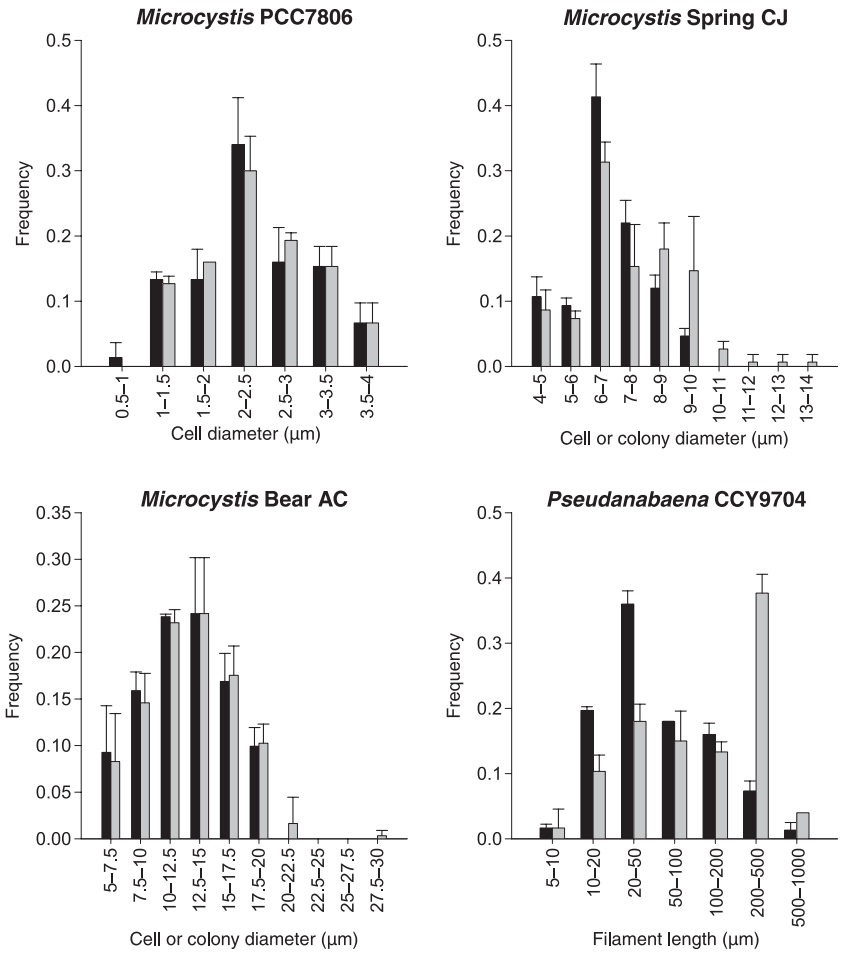

Fig. 2 Effect of grazing by *Ochromonas* on the size distributions of the different cyanobacteria used in the grazing experiment. To compare the different shapes, we used the greatest linear dimension of each species, i.e. the greatest cell or colony diameter for *Microcystis*, and *Pseudanabaena*. Black bars depict the fraction of the control population in a particular size class on day 4, whereas the grey bars depict the fraction of the population exposed to *Ochromonas* in that particular size class on day 4. Bars give averages (±1SD) of three replicates.

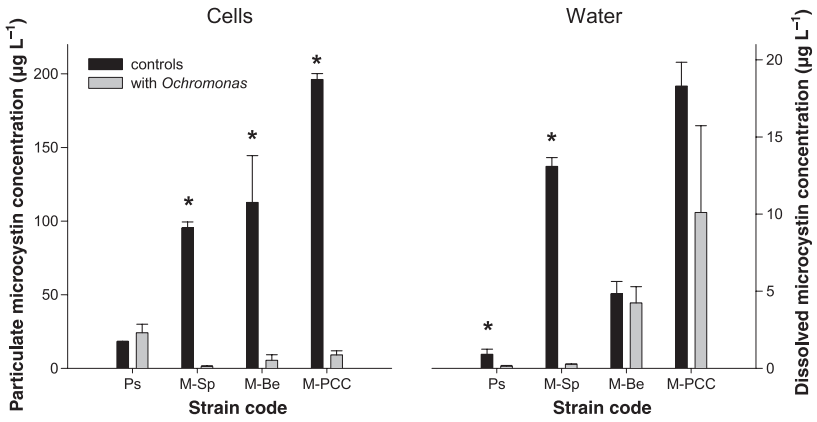

Fig. 3 Average (±1SE) total microcystin concentrations measured in treatments without (black bars) and with *Ochromonas* (grey bars). Left panel: particulate microcystin concentrations; right panel: dissolved microcystin concentrations. Asterisks indicate significance within-strain differences. Strain codes are as in Fig. 2. Note the difference in scales.

to *Ochromonas* grazing ($H_{1,24} = 14.52$, $P < 0.001$). Total and particulate microcystin concentration in the treatments with the weakly toxic *Pseudanabaena* did not decrease significantly. However, for all *Microcystis* strains, microcystin content decreased significantly due to *Ochromonas* grazing (all *Microcystis* strains: Mann–Whitney *U*-test: $Z_{adj} = 1.964$, $P = 0.0495$, $n = 2 \times 3$). For the dissolved microcystins we also found a significant strain effect ($H_{3,24} = 13.07$, $P = 0.0045$) and a significant treatment effect ($H_{1,24} = 4.81$, $P = 0.028$), but no significant interaction.

In the second grazing experiment, with abundances of *Ochromonas* as found in the Norwegian lakes, there remained a significant negative effect of *Ochromonas* on *Microcystis* biovolume concentration on day 4 (t = 26.53, d.f. = 6, $P < 0.0001$) and microcystin concentration (t = 8.42, d.f. = 6, $P < 0.001$). While the net growth reduction of *Microcystis* due to *Ochromonas* was 0.28 day^{-1} (66%), the total microcystin concentration had been reduced by 42% compared to that in the control on day 4 (Fig. 4).

Field data analysis

Ochromonas occurred in 1377 out of 1395 lake samples (99%). *Microcystis* was found in 97 samples (7%), of which 91 also contained *Ochromonas* (94% of the *Microcystis* locations). *Ochromonas* occurs in almost all Norwegian lakes, while the distribution of *Microcystis* is restricted to lowland lakes in the more densely populated southern Norway, where nutrients, temperature and pH are generally higher than in northern and central Norway (Fig. 5). *Ochromonas* had more or less similar biovolume concentrations (within one order of magnitude) across a wide range of nutrient concentrations (phosphorus, nitrogen) and ratios (N : P), temperature, dissolved organic carbon and pH (Fig. 5). In contrast, *Microcystis* biovolumes varied over five orders of magnitude, having higher abundance at higher nutrient concentrations, and temperatures, and at neutral to alkaline pH (Fig. 5). The fact that *Microcystis* and *Ochromonas* were identified only to genus may explain the wide distribution of both in relation to the environmental variables. Because of the relatively similar biovolume concentration of *Ochromonas* over a wide range of nutrient concentrations, and the increasing total phytoplankton concentration with increasing nutrient concentration, the relative abundance of *Ochromonas* decreased with increasing total phytoplankton biovolume concentration (Fig. 6).

Discussion

Effect of Ochromonas on growth rates of cyanobacteria

An important observation of this study is that *Ochromonas* can feed on all *Microcystis* strains tested, including the very toxic *Microcystis* PCC 7806 and the colony-forming *Microcystis* Bear AC. Moreover, *Ochromonas* achieved similar growth rates in the presence of all the *Microcystis* strains. The reduction in net growth rate caused by *Ochromonas* in *Microcystis* was strongest for the less toxic strain Spring CJ of *Microcystis*, while even the impact on the other two *Microcystis* strains was also high. Even if *Ochromonas* was inoculated in concentrations more comparable to natural concentrations, it caused a significant reduction in net growth of the toxic strain PCC 7806. Although the effect of *Ochromonas* on *Pseudanabaena* was less than on *Microcystis*, the effects on biovolume and growth were still substantial (Fig. 1a,b).

Since we did not have a treatment with only the filtrate of an *Ochromonas* culture added, we can not completely rule out a negative effect of chemicals released by *Ochromonas*. However, in experiments performed recently to study the impact of substances excreted by *Ochromonas*, we observed no significant effects of filtrates from *Ochromonas* cultures on the growth and toxin production of these cyanobacteria

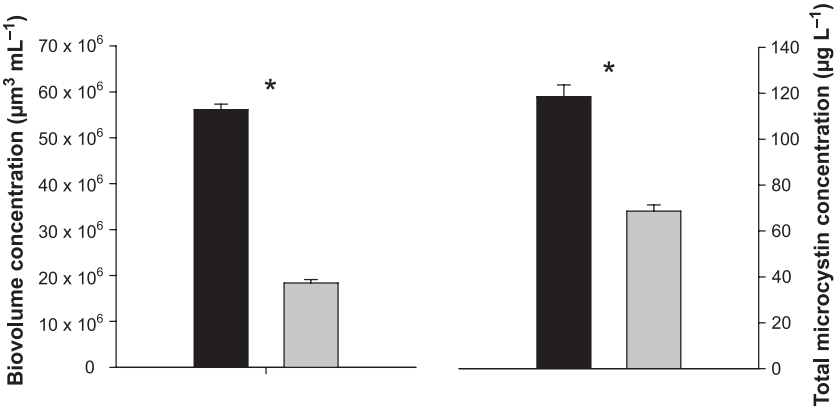

Fig. 4 Results of the grazing experiment with densities of *Ochromonas* similar to those found in Norwegian lakes. Black bars show controls and grey bars *Ochromonas* treatments. Left panel: biovolume concentrations of *Microcystis* PCC 7806 on day 4; right panel: total microcystin concentration on day 4. Asterisks indicate significant differences between treatments. Error bars indicate ±1SD.

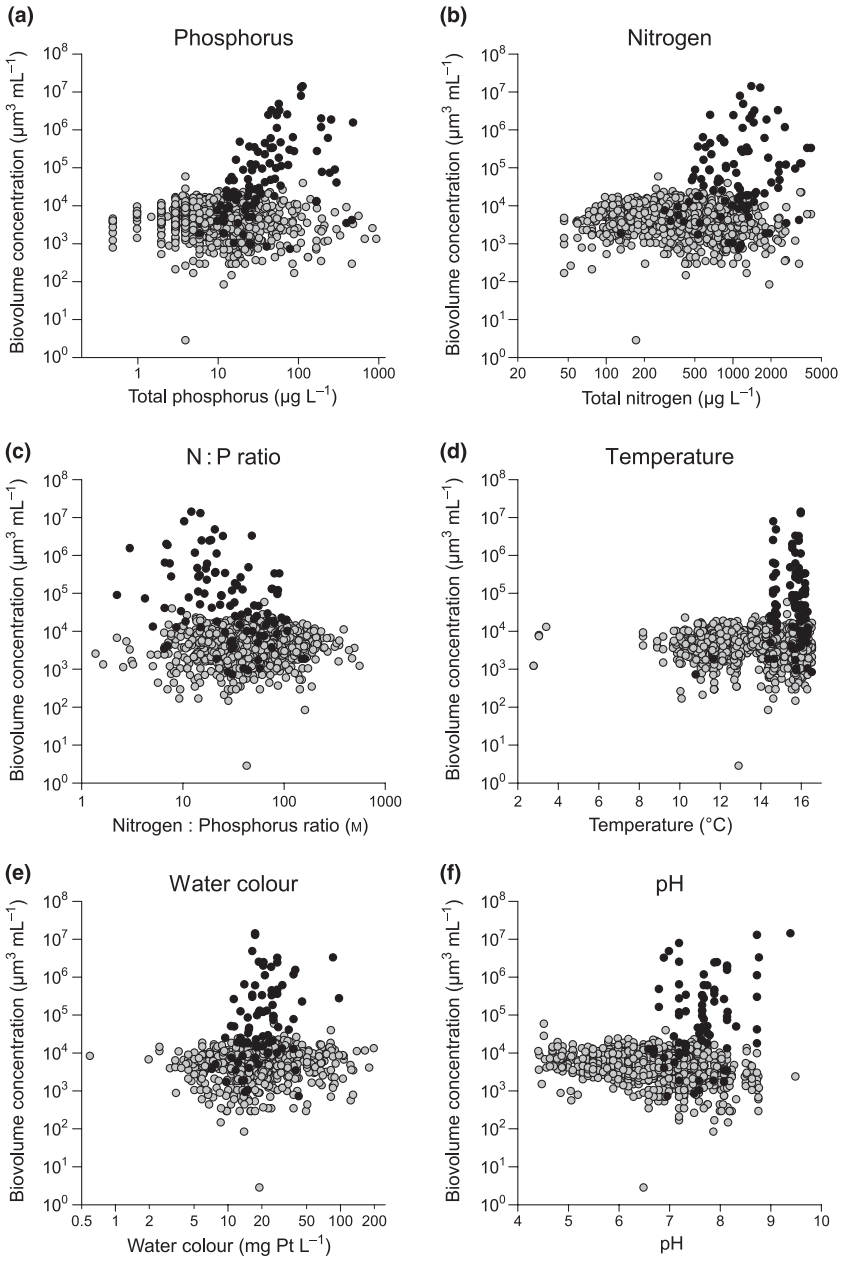

Fig. 5 Biovolume concentrations of *Microcystis* (black) and *Ochromonas* (grey) in 460 Norwegian lakes (1395 summer samples in the period from 1988–2000) in relation to total phosphorus concentration (a), total nitrogen concentration (b), the total nitrogen: total phosphorus ratio (c), temperature (d), water colour (e) and pH (f).

(Wilken, pers. comm). The *per capita* clearance rates of *Ochromonas* ranged from 23 ± 3.1 nL cell^{-1} day^{-1} for *M. aeruginosa* Bear AC as prey, to 47 ± 12 nL cell^{-1} day^{-1} for *M. aeruginosa* Spring. These rates are similar to those of 29–418 nL cell^{-1} day^{-1} for *Ochromonas* sp. grazing on *Synechococcus* (Boenigk *et al*., 2001), and 24 nL cell^{-1} day^{-1} for *O. minima* in a Norwegian Fjord (Nygaard & Hessen, 1990). It seems likely, therefore, that the negative effect of *Ochromonas* on cyanobacterial growth in our experiment is mainly due to grazing and not by other interactions like allelopathy.

Effect of Ochromonas on microcystin concentrations

After 4 days of incubation with *Ochromonas*, the total microcystin content in the cultures with *Microcystis* was reduced by 91.1–98.7% compared with those in the controls (Fig. 3). These reductions can mainly be attributed to the decrease in particulate microcystin, which declined by 95.4–98.8% compared with the controls and is within the same range as the reduction in biovolume. The decrease seems to be caused by both the degradation of ingested microcystins by

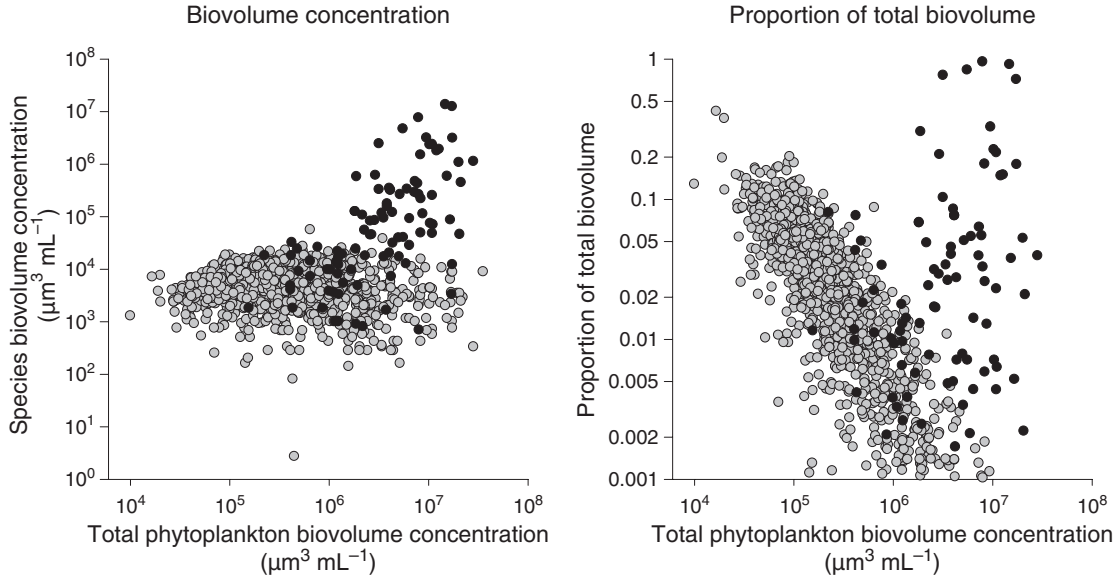

Fig. 6 Abundance of *Ochromonas* (grey) and *Microcystis* (black) in Norwegian lakes in relation to the total phytoplankton biovolume. Left panel: biovolume concentrations of the two species; right panel: proportion of total phytoplankton biovolume of the two species.

Ochromonas and the reduction in net growth of *Microcystis*. In the second grazing experiment, with abundances of *Ochromonas* similar to those found in the Norwegian lakes, the net growth reduction of *Microcystis* due to *Ochromonas* was 66% and the total microcystin concentration had been reduced by 42% compared to that in the control. Only for *Pseudanabaena* was the particulate microcystin concentration not reduced significantly, probably because *Ochromonas* grazes less efficiently on this species. Even though the calculation of microcystins per unit biomass resulted in slightly higher values at the end than at the start of the experiment (data not shown), we cannot draw any conclusion about an increased intracellular concentration of microcystins in the cyanobacteria, because we did not distinguish between microcystins in the cyanaobacteria and microcystins in *Ochromonas* .

Degradation of microcystin from ingested single-celled *Microcystis* PCC 7806 has recently also been found for the closely related chrysophyte *Poterioochromonas* sp. (Zhang *et al*., 2008). In contrast to the results of Zhang *et al*. (2008), we also found a reduction in dissolved microcystin concentrations in the grazing treatments. This again could have been caused indirectly by the reduction in biovolume of the cyanobacteria, resulting in a lower amount of cyanobacterial cells that may release microcystins due to lysis. Moreover, the reduction could have partly been caused by the uptake and degradation of dissolved microcystins by *Ochromonas*, as the degradation of dissolved microcystins has been described in *Poterioochromonas* (Ou *et al*., 2005). Regardless of which mechanism is more important in reducing microcystin concentrations, we show that the amount of total microcystins present can be reduced by *Ochromonas*, even when *Ochromonas* is present in low densities. By detoxifying the ingested microcystins, it might even make the cyanobacterial carbon available to other grazers, which are not able to graze directly on cyanobacteria that produce microcystin.

Size of particles ingested

The mean colony volume of the largest *Microcystis* strain (*Microcystis* Bear AC) was around 810 μm^3 (Table 1) and these colonies could apparently still be ingested easily. These results agree with those of Zhang *et al.* (1996), who found that the volume of particles ingested by a closely related mixotrophic chrysophyte *Poterioochromonas malhamensis* Pringsheim, to range from about 0.52 μm^3 (bacteria) to 3178 μm^3 [the chlorophyte *Carteria inversa* (Korshikov) Bourrelly]. The wide range of particle sizes ingested by *Ochromonas* in our experiments might be explained by the fact that it encloses its prey by pseudopodia (Boenigk & Arndt, 2000), rather than by engulfing food particles by invagination of its cell surface, as do many other phagotrophs. Even though *Ochromonas*

grazed efficiently on all *Microcystis* strains tested, it caused a small but significant shift in size distribution to larger sizes for the strains Spring CJ and Bear AC, whereas this did not occur for the smaller strain PCC7806 (Fig. 2). Thus *Ochromonas* seems to select the smaller particles over the larger ones. This agrees with the results of Pfandl, Posch & Boenigk (2004), who showed a food-size preference in *Ochromonas* sp. for particles between 0.9 and 1.2 μm. Although *Ochromonas* grazed on the filamentous cyanobacteria *Pseudanabaena*, it grew more slowly on this alga than on *Microcystis*. *Ochromonas* fed mainly on the *Pseudanabaena* filaments <50 μm long, thereby skewing the filament length distribution of *Pseudanabaena* towards longer filaments. Similar shifts in length distribution as those shown for *Pseudanabaena* have been reported by Wu, Boenigk & Hahn (2004) for *Ochromonas* grazing on the filamentous bacterium *Spirochaeta aurantia* WQM4 ranging from 5 to 30 μm in filament length. Because *Pseudanabaena* has a smaller mean particle volume than, for example, the readily ingestible *Microcystis* Spring CJ, it seems that the greatest linear dimension of a particle, rather than its volume, determines whether a particle can still be ingested. The mean diameter of the *Microcystis* colonies used in our study lies within the range of filament lengths that can be ingested by *Ochromonas* [Correction added after online publication 7 April 2009: *Pseudanabaena* corrected to *Ochromonas*].

Food-web relationships

Mixotrophic nanoflagellates are important members of planktonic food webs in both marine and freshwater systems (Sanders, 1991; Isaksson, 1998; Tittel *et al*., 2003). Phagotrophy by predominantly autotrophic mixotrophs is an important mechanism for obtaining energy and nutrients, and it gives mixotrophs a competitive advantage over autotrophs during nutrient-limiting conditions. Similarly, predominantly heterotrophic mixotrophs have an advantage over purely heterotrophic organisms because they can use light as an energy source when prey abundance is low (Jones, 2000). In both cases, mixotrophs will be favoured by oligotrophic conditions and they are traditionally thought to be inferior competitors in eutrophic systems, because of the higher energy costs of maintaining both nutritional metabolisms (Raven, 1997). However, the predominantly mixotrophic genus *Poterioochromonas* has been shown to achieve growth rates comparable with those of heterotrophic nanoflagellates (Pålsson & Daniel, 2004), and should therefore be able to compete with the flagellates even under eutrophic conditions. Although mixotrophs in general are important grazers in oligotrophic lakes (Bergström *et al*., 2003), and can also dominate the plankton community during the winter (Wiedner & Nixdorf, 1998), their role in eutrophic lakes during summer is largely unknown (Tittel *et al*., 2003). This does not necessarily mean that they are not important in those systems, as many investigations distinguish only between pigmented and non-pigmented flagellates, and mixotrophs are likely to be mistaken for autotrophs in those studies. Therefore we tested data from 460 Norwegian lakes for the co-occurrence of *Microcystis* and *Ochromonas*. The data show that *Ochromonas* occurred in 94% of the samples in which *Microcystis* was present, and the interaction of both species might therefore be important in natural systems. *Ochromonas* is abundant over a broad range of phosphorus and nitrogen concentrations in Norwegian lakes, including the higher concentrations under which also *Microcystis* becomes more important. While the total abundance of *Ochromonas* is generally low, and seemed to be unaffected by the environmental factors tested, its relative contribution to the plankton community decreased with increasing nutrient load and pH. Our data confirm that *Ochromonas* is a good competitor, especially under nutrient poor conditions, and that it may tolerate low pH, but it also shows that *Ochromonas* occurs over a wide gradient of environmental conditions, including eutrophic lakes (Figs 5 & 6). Under meso- to eutrophic conditions, *Ochromonas* could potentially be controlled by grazers, e.g. daphniids. Arvola & Salonen (2001) showed that the abundance of *Ochromonas* and other flagellates increases after removal of daphniids from a natural zooplankton community. On the other hand the closely related *Poterioochromonas* has been shown to be toxic to rotifers and daphniids in laboratory experiments (Boxhorn, Holen & Boraas, 1998; Boenigk & Stadler, 2004). Even though competition with specialists and grazing on *Ochromonas* might play a role in reducing the abundance of *Ochromonas* in eutrophic lakes, this chrysophyte can maintain population densities similar to those seen under oligotrophic conditions (Fig. 5). Due to its high grazing rates, *Ochromonas* may have a strong impact on the

phytoplankton composition and abundance even when its density is lower than other grazer species.

Because mixotrophs can sustain relatively high abundances by photosynthesis, they do not rely soley on the abundance of their prey and are therefore less prone to strong fluctuations in population densities. Hammer & Pitchford (2005) showed theoretically that a system in which a small fraction of the herbivorous plankton is capable of photosynthesis, as for e.g. *Ochromonas*, is less likely to show strong oscillations or to exhibit phytoplankton blooms. By this mechanism the presence of *Ochromonas* might decrease the chance of a *Microcystis* bloom formation.

From our study it is not clear whether *Ochromonas* can control *Microcystis* blooms in natural lakes. Yet our study does demonstrate that *Ochromonas* can strongly reduce the *Microcystis* biomass and toxins in small-scale experiments. This study also shows that *Ochromonas* occurs in lakes where *Microcystis* is present. However, more work is needed on the natural occurrence of *Ochromonas* and its trophic relationships in eutrophic lakes. Light intensity, nutrients and organic matter concentration, bacterial and picophytoplankton abundance, as well as grazing on *Ochromonas* are potentially important factors regulating its natural abundance. More field work is therefore needed to clarify which of these factors drives the distribution and seasonal dynamics of *Ochromonas* in nature.

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References

Arvola L. & Salonen K. (2001) Plankton community of a polyhumic lake with and without *Daphnia longispina* (Cladocera). *Hydrobiologia*, **445**, 141–150.

Bergström A.K., Jansson M., Drakare S. & Blomqvist P. (2003) Occurrence of mixotrophic flagellates in relation to bacterioplankton production, light regime and availability of inorganic nutrients in unproductive lakes with differing humic contents. *Freshwater Biology*, **48**, 868–877.

Boenigk J. & Arndt H. (2000) Particle handling during interception feeding by four species of heterotrophic nanoflagellates. *Journal of Eukaryotic Microbiology*, **47**, 350–358.

Boenigk J. & Stadler P. (2004) Potential toxicity of chrysophytes affiliated with Poterioochromonas and related 'Spumella-like' flagellates. *Journal of Plankton Research*, **26**, 1507–1514.

Boenigk J., Matz C., Jurgens K. & Arndt H. (2001) The influence of preculture conditions and food quality on the ingestion and digestion process of three species of heterotrophic nanoflagellates. *Microbial Ecology*, **42**, 168–176.

Boxhorn J.E., Holen D.A. & Boraas M.E. (1998) Toxicity of the Chrysophyte flagellate *Poterioochromonas malhamensis* to the rotifer *Brachionus angularis*. *Hydrobiologia*, **388**, 283–287.

Carmichael W.W., Azevedo S., An J.S., Molica R.J.R., Jochimsen E.M., Lau S., Rinehart K.L., Shaw G.R. & Eaglesham G.K. (2001) Human fatalities from cyanobacteria: chemical and biological evidence for cyanotoxins. *Environmental Health Perspectives*, **109**, 663–668.

Chorus I. & Bartram J. (1999) *Toxic Cyanobacteria in Water: A Guide to Their Public Health Consequences, Monitoring and Management*. E and FN Spon, London.

Cole G.T. & Wynne M.J. (1974) Endocytosis of *Microcystis aeruginosa* by *Ochromonas danica*. *Journal of Phycology*, **10**, 397–410.

Fastner J., Flieger I. & Neumann U. (1998) Optimised extraction of microcystins from field samples – a comparison of different solvents and procedures. *Water Research*, **32**, 3177–3181.

Flöder S., Hansen T. & Ptacnik R. (2006) Energy-dependent bacterivory in *Ochromonas minima* – a strategy promoting the use of substitutable resources and survival at insufficient light supply. *Protist*, **157**, 291–302.

Ghadouani A., Pinel-Alloul B., Plath K., Codd G.A. & Lampert W. (2004) Effects of *Microcystis aeruginosa* and purified microcystin-LR on the feeding behavior of *Daphnia pulicaria*. *Limnology and Oceanography*, **49**, 666–679.

Gulati R.D. & Van Donk E. (2002) Lakes in the Netherlands, their origin, eutrophication and restoration: state-of-the-art review. *Hydrobiologia*, **478**, 73–106.

Gustafsson S., Rengefors K. & Hansson L.A. (2005) Increased consumer fitness following transfer of toxin tolerance to offspring via maternal effects. *Ecology*, **86**, 2561–2567.

Hammer A.C. & Pitchford J.W. (2005) The role of mixotrophy in plankton bloom dynamics, and the consequences for productivity. *ICES Journal of Marine Science*, **62**, 833–840.

Hansson L.A., Gustafsson S., Rengefors K. & Bomark L. (2007) Cyanobacterial chemical warfare affects zooplankton community composition. *Freshwater Biology*, **52**, 1290–1301.

Holen D.A. (1999) Effects of prey abundance and light intensity on the mixotrophic chrysophyte *Poterioochromonas malhamensis* from a mesotrophic lake. *Freshwater Biology*, **42**, 445–455.

Isaksson A. (1998) Phagotrophic phytoflagellates in lakes – a literature review. *Archiv für Hydrobiologie. Special Issues. Advances in Limnology*, **51**, 63–90.

Jones R.I. (2000) Mixotrophy in planktonic protists: an overview. *Freshwater Biology*, **45**, 219–226.

Katechakis A. & Stibor H. (2006) The mixotroph *Ochromonas tuberculata* may invade and suppress specialist phago- and phototroph plankton communities depending on nutrient conditions. *Oecologia*, **148**, 692–701.

Krienitz L., Ballot A., Kotut K., Wiegand C., Putz S., Metcalf J.S., Codd G.A. & Pflugmacher S. (2003) Contribution of hot spring cyanobacteria to the mysterious deaths of Lesser Flamingos at Lake Bogoria, Kenya. *FEMS Microbiology Ecology*, **43**, 141–148.

Lampert W. (1987) Laboratory studies on zooplankton-cyanobacteria interactions. *New Zealand Journal of Marine and Freshwater Research*, **21**, 483–490.

Mitchell T.D. & Jones P.D. (2005) An improved method of constructing a database of monthly climate observations and associated high-resolution grids. *International Journal of Climatology*, **25**, 693–712.

Moe S.J., Dudley B. & Ptacnik R. (2008) REBECCA databases: experiences from compilation and analyses of monitoring data from 5,000 lakes in 20 European countries. *Aquatic Ecology*, **42**, 183–201.

Müller-Navarra D.C., Brett M.T., Liston A.M. & Goldman C.R. (2000) A highly unsaturated fatty acid predicts carbon transfer between primary producers and consumers. *Nature*, **403**, 74–77.

Nygaard K. & Hessen D.O. (1990) Use of C-14 protein-labelled bacteria for estimating clearance rates by heterotrophic and mixotrophic flagellates. *Marine Ecology-Progress Series*, **68**, 7–14.

Ou D.Y., Song L.R., Gan N.Q. & Chen W. (2005) Effects of microcystins on and toxin degradation by *Poterioochromonas* sp. *Environmental Toxicology*, **20**, 373–380.

Paerl H.W. & Huisman J. (2008) Blooms like it hot. *Science*, **320**, 57–58.

Pålsson C. & Daniel C. (2004) Effects of prey abundance and light intensity on nutrition of a mixotrophic flagellate and its competitive relationship with an obligate heterotroph. *Aquatic Microbial Ecology*, **36**, 247–256.

Pfandl K., Posch T. & Boenigk J. (2004) Unexpected effects of prey dimensions and morphologies on the size selective feeding by two bacterivorous flagellates (*Ochromonas* sp and *Spumella* sp.). *Journal of Eukaryotic Microbiology*, **51**, 626–633.

Ptacnik R., Sommer U., Hansen T. & Martens V. (2004) Effects of microzooplankton and mixotrophy in an experimental planktonic food web. *Limnology and Oceanography*, **49**, 1435–1445.

Raven J.A. (1997) Phagotrophy in phototrophs. *Limnology and Oceanography*, **42**, 198–205.

Rohrlack T., Dittmann E., Henning M., Borner T. & Kohl J.G. (1999) Role of microcystins in poisoning and food ingestion inhibition of *Daphnia galeata* caused by the cyanobacterium *Microcystis aeruginosa*. *Applied and Environmental Microbiology*, **65**, 737–739.

Rothhaupt K.O. (1996) Utilization of substitutable carbon and phosphorus sources by the mixotrophic chrysophyte *Ochromonas* sp. *Ecology*, **77**, 706–715.

Sanders R.W. (1991) Mixotrophic protists in marine and freshwater ecosystems. *Journal of Protozoology*, **38**, 76–81.

Sanders R.W., Caron D.A., Davidson J.M., Dennett M.R. & Moran D.M. (2001) Nutrient acquisition and population growth of a mixotrophic alga in axenic and bacterized cultures. *Microbial Ecology*, **42**, 513–523.

Shapiro J. & Wright D.I. (1984) Lake restoration by biomanipulation: Round Lake, Minnesota, the first two years. *Freshwater Biology*, **14**, 371–383.

Sokal R.R. & Rohlf F.J. (1995) *Biometry: The Principles and Practice of Statistics in Biological Research*. W.H. Freeman and Company, New York.

Stibor H. & Sommer U. (2003) Mixotrophy of a photosynthetic flagellate viewed from an optimal foraging perspective. *Protist*, **154**, 91–98.

Tittel J., Bissinger V., Zippel B., Gaedke U., Bell E., Lorke A. & Kamjunke N. (2003) Mixotrophs combine resource use to outcompete specialists: implications for aquatic food webs. *Proceedings of the National Academy of Sciences of the United States of America*, **100**, 12776–12781.

Tonk L., Visser P.M., Christiansen G., Dittmann E., Snelder E., Wiedner C., Mur L.R. & Huisman J. (2005) The microcystin composition of the cyanobacterium *Planktothrix agardhii* changes toward a more toxic variant with increasing light intensity. *Applied and Environmental Microbiology*, **71**, 5177–5181.

Wacker A. & Von Elert E. (2001) Polyunsaturated fatty acids: evidence for non-substitutable biochemical resources in *Daphnia galeata*. *Ecology*, **82**, 2507–2520.

Wiedner C. & Nixdorf B. (1998) Success of chrysophytes, cryptophytes and dinoflagellates over blue-greens (cyanobacteria) during an extreme winter (1995/96) in eutrophic shallow lakes. *Hydrobiologia*, **370**, 229–235.

Wu Q.L., Boenigk J. & Hahn M.W. (2004) Successful predation of filamentous bacteria by a nanoflagellate challenges current models of flagellate bacterivory. *Applied and Environmental Microbiology*, **70**, 332–339.

Zhang X.M. & Watanabe M.M. (2001) Grazing and growth of the mixotrophic chrysomonad *Poterioochromonas malhamensis* (Chrysophyceae) feeding on algae. *Journal of Phycology*, **37**, 738–743.

Zhang X.M., Watanabe M.M. & Inouye I. (1996) Light and electron microscopy of grazing by *Poterioochromonas malhamensis* (Chrysophyceae) on a range of phytoplankton taxa. *Journal of Phycology*, **32**, 37–46.

Zhang X., Hu H.Y., Hong Y. & Yang J. (2008) Isolation of a *Poterioochromonas* capable of feeding on *Microcystis aeruginosa* and degrading microcystin-LR. *FEMS Microbiology Letters*, **288**, 241–246.

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