



Microcystins do not provide anti-herbivore defence against mixotrophic flagellates

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ABSTRACT: While most experiments investigating zooplankton grazing on harmful cyanobacteria have been carried out with metazoan plankton, several protozoa can also feed efficiently on cyanobacteria. We investigated grazing by the mixotrophic flagellate *Ochromonas* sp. on the toxic cyanobacterium *Microcystis aeruginosa*. *Ochromonas* sp. grew rapidly on *M. aeruginosa* and had a strong impact on the population density of its prey. However, specific growth rates of *Ochromonas* sp. decreased over time, possibly indicating a negative impact on *Ochromonas* sp. mediated by *M. aeruginosa*. Grazing did not have any effect on the intracellular microcystin content of *M. aeruginosa*, and the ingested microcystins did not accumulate within *Ochromonas* sp. We studied the functional and numerical response of *Ochromonas* sp. grazing on the microcystin-producing strain *M. aeruginosa* PCC 7806 and its microcystin-deficient mutant. *Ochromonas* sp. showed a Type 3 functional response of very similar shape on both the toxic and non-toxic *M. aeruginosa* strain. Ingestion rates of *Ochromonas* sp. were even slightly higher on the toxic *M. aeruginosa* strain. We therefore found no indication of microcystins acting as a defence against mixotrophic flagellates.

KEY WORDS: *Microcystis* · Microcystins · Mixotrophic flagellates · *Ochromonas* sp. · Grazing

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INTRODUCTION

Cyanobacterial blooms have increasingly become a nuisance in many freshwater and brackish ecosystems (Chorus & Bartram 1999, Carmichael 2001, Huisman et al. 2005). They are facilitated by high water temperatures and nutrient load and therefore benefit from anthropogenic influences like eutrophication and climate warming (Codd 2000, Jöhnk et al. 2008, Paerl & Huisman 2008). Microcystin-producing cyanobacteria of the *Microcystis* genus are widely distributed bloom-forming species. The hepatotoxic microcystins inhibit protein phosphatases in eukaryotes (MacKintosh et al. 1990) and are therefore toxic to many potential grazers of *Microcystis* (Fulton & Paerl 1987b, DeMott et al. 1991). Other factors contributing to the low edibility of *Microcystis* are the formation of colonies leading to interference with the feeding behaviour of many zooplankton species (Fulton & Paerl 1987a, Yang et al.

2006) and the low content of long-chain polyunsaturated fatty acids and sterols, which are essential for many herbivores (Wacker & von Elert 2001, von Elert et al. 2003).

While most experiments investigating effects of toxic cyanobacteria on pelagic grazers have been done with metazoans, several protozoa are able to maintain high growth rates when feeding on cyanobacteria including *Microcystis* (Cole & Wynne 1974, Nishibe et al. 2002, Kim et al. 2006). Especially mixotrophic chrysophytes, such as *Ochromonas* spp. and *Poterioochromonas* spp., can graze efficiently on *Microcystis* and even degrade the cyanotoxins (Ou et al. 2005, Zhang et al. 2008, Van Donk et al. 2009). The genus *Ochromonas* is abundant in freshwaters and has been reported to frequently co-occur with *Microcystis* (Van Donk et al. 2009). *Ochromonas* has therefore been proposed as a potential biological control agent against *Microcystis* blooms (Cole & Wynne 1974, Zhang et al. 2008), although little is

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thus far known about its interaction with toxic cyanobacteria. As mixotrophs, these organisms differ considerably from purely heterotrophic grazers by their photosynthetic machinery and the presence of several biochemical pathways typical of autotrophic organisms. For instance, in contrast to many heterotrophs, *Ochromonas* is able to synthesize long-chain polyunsaturated fatty acids (Boëchat et al. 2007) and thus does not depend on the presence of these substances in its prey. However, *Ochromonas* might be susceptible to the other 2 mechanisms of grazer deterrence in *Microcystis*, e.g. colony formation and microcystin production. Colony formation in *Microcystis* can be induced by the presence of *Ochromonas* sp. (Burkert et al. 2001, Yang et al. 2006). Even though *Ochromonas* is able to ingest surprisingly large prey items, there is certainly an upper limit of suitable prey size, and inducible colony formation is likely to be an efficient way of defence against grazing by flagellates.

In contrast to colony formation, the role of microcystin production as a defence against flagellate grazers is less clear. The original function of microcystins is still under debate, and its role as a defence against grazers has been questioned, because the genes for microcystin biosynthesis seem to be older than metazoan grazers such as copepods and cladocerans (Rantala et al. 2004). However, since the earliest protozoa probably already preyed upon cyanobacteria long before the metazoans evolved, the early evolution of microcystins does not necessarily exclude its original role as a defence against protozoa. Furthermore, the function of these secondary metabolites might have changed during their evolution, and regardless of the original function of microcystins they are currently toxic for many species. *Ochromonas* could pay a metabolic cost for its ability to grow on toxic cyanobacteria and to degrade the toxins, which might be reflected in lower growth rates compared to growth on non-toxic food. Ou et al. (2005) described an initially stimulating effect of dissolved microcystins on the growth of *Potriochromonas* sp., but decreased growth rates after prolonged exposure to the toxins. This suggests that microcystins might indeed act as a defence against grazing by protozoan flagellates. Grazer-induced defences are well known for several phytoplankton taxa, such as the induction of colony formation in the green alga *Scenedesmus* (Lürling & Van Donk 1997). Jang et al. (2003) reported an increased microcystin content in *Microcystis* as a response to *Daphnia* sp. grazing, suggesting the microcystin production of cyanobacteria to be induced by info-chemicals released

by the grazer. Whether the microcystin production of *Microcystis* responds to flagellate grazing is not known yet, however.

Here, we investigate the functional and numerical response of the mixotrophic flagellate *Ochromonas* sp. grazing on the microcystin-producing cyanobacterium *Microcystis aeruginosa* PCC 7806 and its microcystin-deficient mutant. Conversely, we also investigate whether flagellate grazing induces an enhanced microcystin production by *M. aeruginosa*.

MATERIALS AND METHODS

Phytoplankton strains. We investigated the microcystin-producing cyanobacterium *Microcystis aeruginosa* PCC 7806, its microcystin-deficient mutant (Dittmann et al. 1997) and the microcystin-producing strain *M. aeruginosa* HUB 524. All 3 *M. aeruginosa* strains were single celled and did not show any colony formation in the stock cultures. Our *Ochromonas* sp. strain was detected as contamination in large-scale mesocosm experiments with *M. aeruginosa*. It was isolated by micro-needle techniques. Stock cultures of all strains were maintained on COMBO-medium (Kilham et al. 1998). For *Ochromonas* sp., ammonium was used as the nitrogen source in the stock cultures, because the strain grew rather poorly on nitrate. All cultures were unialgal, but not axenic. Abundances of heterotrophic bacteria were low, however, and never exceeded 1% of the total biovolume. Cellular microcystin contents and cell volumes of the strains are summarized in Table 1.

Effect of grazing on toxin production. We investigated effects of grazing on the microcystin production of *Microcystis aeruginosa* PCC 7806 and *M. aeruginosa* HUB 524. The experiments were done in batch cultures using 500 ml Erlenmeyer flasks filled with 300 ml COMBO-medium (Kilham et al. 1998). We applied 4 experimental treatments to each *M. aeruginosa* strain: (1) monocultures of *M. aeruginosa* that served as controls, (2) monocultures of *M. aeruginosa*

Table 1. *Microcystis aeruginosa* and *Ochromonas* sp. Microcystin content (n = 16) and biovolume (n = 3) of the *M. aeruginosa* strains and *Ochromonas* sp. used in the experiments. Standard deviations are given in parentheses. Measurements reported in this table were made on Day 0 of the grazing experiment

Strain	Microcystin content (fg cell ⁻¹)	Biovolume (µm ³ cell ⁻¹)
<i>M. aeruginosa</i> PCC 7806 wildtype	53.32 (±6.66)	25.02 (±1.64)
<i>M. aeruginosa</i> PCC 7806 mutant	–	28.10 (±3.07)
<i>M. aeruginosa</i> HUB 524	27.16 (±3.00)	32.08 (±0.14)
<i>Ochromonas</i> sp.	–	146.57 (±30.91)

to which we added the filtrate of an *Ochromonas* sp. culture pre-fed with the same *M. aeruginosa* strain, (3) monocultures of *M. aeruginosa* to which we added the filtrate of an *Ochromonas* sp. culture pre-fed with a different prey species, the Eustigmatophyte *Nannochloropsis limnetica*, and (4) mixed cultures of *M. aeruginosa* and *Ochromonas* sp. that served as grazing treatment. All 4 treatments were performed in 4 replicates. The experiments were incubated at 23°C with a low shaking frequency of 20 rpm and an incident light intensity of 90 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ under a 14 h light:10 h dark photoperiod.

In all treatments, we inoculated *Microcystis aeruginosa* to a final population density of 3×10^5 cells ml^{-1} . In the species mixture of the fourth treatment, *Ochromonas* sp. was added to a final population density of 500 cells ml^{-1} . These rather low population densities were chosen to prevent *M. aeruginosa* from reaching the stationary phase in the control while it was still growing exponentially in the grazing treatment. Such a situation might lead to differences in microcystin concentration caused by differences in growth rates, nutrient status (Long et al. 2001), or light intensity (Wiedner et al. 2003) between the grazing treatment and the control that would not be directly related to the grazing itself. Our inoculation densities were lower than *M. aeruginosa* abundances in dense surface blooms, but comparable to the abundances of both species in natural waters during non-bloom conditions (Van Donk et al. 2009). The filtrates originated from rather dense cultures of *Ochromonas* sp. pre-fed with *Nannochloropsis limnetica* and *M. aeruginosa*. The filtrates were added daily in volumes corresponding to the density of *Ochromonas* sp. in the grazing treatment, adding up to 25% of the culture volume over the course of the experiment. Equal volumes of fresh medium were added to the control and grazing treatments each time. Based on preliminary experiments, samples for population densities and microcystin analysis were taken every 2 to 3 d over a period of 9 d.

In the first set of experiments, with *Microcystis aeruginosa* PCC 7806, population densities were measured directly after sampling using a CytoSense flow cytometer (CytoBuoy b.v.). In the second set of experiments, with *M. aeruginosa* HUB 524, samples were fixed with a mixture of glutaraldehyde and formaldehyde (final concentrations of 0.025 and 0.0037 percent by mass, respectively) and measured at a flow cytometer (MoFlo XDP Cell Sorter, Beckman Coulter) after a few days of storage at 4°C. Fresh samples were regularly checked for colony formation by light microscopy, but *M. aeruginosa* remained single celled in both experiments.

Microcystin concentrations were measured using an enzyme-linked immunosorbent assay (ELISA) (Quantiplate Kit for Microcystins; EnviroLogix). For measure-

ment of particulate microcystins, the samples were centrifuged and the pellet was resuspended in MilliQ water. Samples were diluted to a final cell density of about 5×10^4 cells ml^{-1} to ensure that the microcystin concentration fell within the measurable range of the ELISA. Microcystins were extracted from the cells by 3 freeze and thaw cycles, each of which was followed by sonification for 15 min using a sonic bath (Gustafsson et al. 2005). For the measurement of dissolved microcystins, samples were filtered through glass fibre filters (Whatman GF/C), and the filtrate was analysed for microcystins. To distinguish between microcystins in *Microcystis aeruginosa* and *Ochromonas* sp. cells, we sorted 2.5×10^5 *M. aeruginosa* cells from the mixed cultures using a flow cytometer (MoFlo XDP Cell Sorter, Beckman Coulter). The sorted fraction containing only *M. aeruginosa* cells was diluted, and population density and microcystin content were measured using the same methods as for the other samples (CytoSense for the first experiment and MoFlo for the second experiment). Because population densities of *Ochromonas* sp. were too low for flow cytometer sorting throughout most of the experiment, it could only be sorted at the last day.

When variances were homogenous, the data were tested for a treatment effect by repeated-measures ANOVA, followed by a Tukey test as a post hoc comparison. When variances remained heterogeneous even after logarithmic transformation (microcystin data of PCC 7806), the non-parametric Kruskal-Wallis ANOVA was used instead. All statistical analyses were done using STATISTICA 8 software.

Functional and numerical response experiment. To investigate effects of microcystins on *Ochromonas* sp., the functional and numerical response of *Ochromonas* sp. grazing on the toxic strain *Microcystis aeruginosa* PCC 7806 and its microcystin-deficient mutant were compared. *Ochromonas* sp. was pre-cultured for 1 wk on the 2 strains at prey concentrations used in the experiment. During pre-culturing, prey concentrations were measured daily using a CytoSense flow cytometer and readjusted to the desired concentrations by either dilution or addition of more prey. Stock cultures were harvested by centrifugation and resuspended in fresh COMBO-medium to prevent any potential effect of 'spent' medium. To assess the functional and numerical response of *Ochromonas* sp., we used 8 different concentrations of *M. aeruginosa* (Table 2). This included a treatment without any *M. aeruginosa* as food, to estimate the specific growth rate of *Ochromonas* sp. when grown photo-autotrophically. At each *M. aeruginosa* concentration, we had a treatment with *Ochromonas* sp. grazing and a control for *M. aeruginosa* growth without *Ochromonas* sp. All treatments were run in triplicate. The experiment was performed

Table 2. *Microcystis aeruginosa* and *Ochromonas* sp. Initial population densities used in the functional and numerical response experiment

Treatment no.	<i>M. aeruginosa</i> (cells ml ⁻¹)	<i>Ochromonas</i> sp. (cells ml ⁻¹)
1	0	3.9 × 10 ⁴
2	2.6 × 10 ³	5.0 × 10 ²
3	8.0 × 10 ³	8.4 × 10 ²
4	2.4 × 10 ⁴	2.9 × 10 ³
5	8.0 × 10 ⁴	7.3 × 10 ³
6	2.5 × 10 ⁵	2.5 × 10 ⁴
7	8.2 × 10 ⁵	7.3 × 10 ⁴
8	2.7 × 10 ⁶	1.2 × 10 ⁵

in 100 ml Erlenmeyer flasks under continuous illumination of 90 μmol photons m⁻² s⁻¹. Cultures were incubated at 23°C for 3 d, and samples were taken at 0, 3, 6, 12, 24, 48 and 72 h after inoculation, fixed with a mixture of glutaraldehyde and formaldehyde as described in 'Effect of grazing on toxin production' and stored at 4°C until measurement with a flow cytometer (MoFlo XDP Cell Sorter, Beckman Coulter).

Because *Ochromonas* sp. might ingest heterotrophic bacteria as well, and our cultures were not axenic, we performed bacterial counts on several samples. We picked samples from Treatments 3, 5 and 7 (Table 2) taken at 0 and 12 h after inoculation, for both the wild-type and the mutant of *Microcystis aeruginosa* PCC 7806. Bacteria were stained with SYBR Green (Invitrogen) prior to counting with the MoFlo flow cytometer.

Population densities were plotted against time on a semi-log scale. During periods in which a linear relation was observed, the specific growth rate, μ , was calculated as:

$$\mu = \frac{\ln(N_2) - \ln(N_1)}{t_2 - t_1} \quad (1)$$

where N_1 and N_2 denote population densities at time t_1 and t_2 , respectively. Clearance rates were calculated over the first 3 h of incubation, except for the lowest 2 prey concentrations for which changes in concentrations were low enough to use a longer time span. The following formula was used (Heinbokel 1978):

$$CI = \frac{\mu_{Mc} - \mu_{Mt}}{N_O} \quad (2)$$

where CI is the clearance rate, μ_{Mc} and μ_{Mt} are the specific growth rates of *Microcystis aeruginosa* in the control and the grazing treatments, respectively, and N_O is the mean *Ochromonas* sp. abundance over the period used for calculation. Ingestion rates are then given by:

$$I = N_M CI \quad (3)$$

where I denotes the ingestion rate and N_M denotes the mean *M. aeruginosa* abundance.

Ochromonas sp. showed a slightly sigmoid increase of the ingestion rate and an initial increase of the clearance rate with increasing *Microcystis aeruginosa* abundance. This is indicative of a Type 3 functional response, where *Ochromonas* sp. increases its feeding rate with increasing prey density (Holling 1959, Kiørboe 2008). Therefore, we fitted a Type 3 functional response model to the ingestion rate data (Real 1977):

$$I = \frac{I_{\max} N_M^x}{K^x + N_M^x} \quad (4)$$

where I_{\max} is the maximum ingestion rate reached at saturating prey concentrations, the power x describes the curvature of the sigmoid functional response (we note that, if $x = 1$, the model simplifies to a Type 2 functional response) and K is the half-saturation constant, representing the prey density at which half the maximum ingestion rate is reached. The model parameters were estimated by non-linear regression, using the iterative least-squares method of STATISTICA 8. The ingestion-rate data were log-transformed prior to the model fit to improve the homogeneity of variance.

RESULTS

Effect of grazing on toxin production

Both *Microcystis aeruginosa* strains showed positive growth rates in all treatments, reaching population densities of 4 to 10 × 10⁶ cells ml⁻¹ at the end of the experiments (Fig. 1). Nevertheless, the presence of *Ochromonas* sp. had a strong effect on *M. aeruginosa* densities, reducing *M. aeruginosa* PCC 7806 by 44 % and *M. aeruginosa* HUB 524 by 39 % relative to the controls (repeated-measures ANOVA, for PCC 7806: $F_{3,12} = 36.37$, $p < 0.001$; for HUB 524: $F_{3,12} = 48.08$, $p < 0.001$). The addition of *Ochromonas* sp. filtrates did not show any effect on *M. aeruginosa* abundances. *Ochromonas* sp. grew rapidly on both strains, increasing its population density by almost 1000-fold over the course of the experiments (Fig. 1C,D). It reached its highest specific growth rates of 2.1 d⁻¹ (on PCC 7806) and 1.5 d⁻¹ (on HUB 524) at the beginning of the experiment, followed by a decrease of its specific growth rates to 0.21 d⁻¹ (on PCC 7806) and 0.36 d⁻¹ (on HUB 524) by the end, even though prey densities increased (Fig. 2).

The total particulate microcystin concentrations showed a similar increase as the population densities of *Microcystis aeruginosa*, reaching concentrations of 344 μg l⁻¹ (PCC 7806) and 175 μg l⁻¹ (HUB 524) by the last day in the controls, and about 32 % (PCC 7806) and 18 % (HUB 524) lower values in the grazing treatment with *Ochromonas* sp. (Fig. 3A,B). The treatment effect was significant for *M. aeruginosa* HUB 524 (repeated-

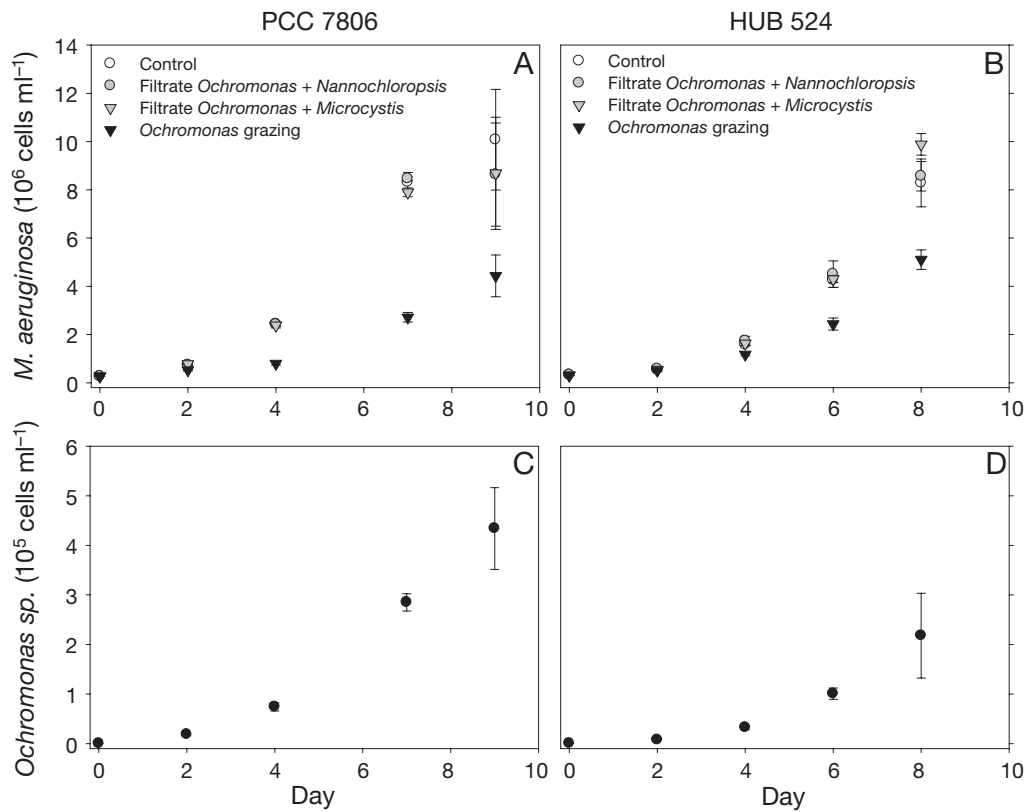


Fig. 1. (A,B) *Microcystis aeruginosa* and (C,D) *Ochromonas* sp. Population densities over the course of the grazing experiments with *Ochromonas* sp. as predator and (A,C) *M. aeruginosa* PCC 7806 and (B,D) *M. aeruginosa* HUB 524 as prey. Error bars indicate standard deviation

measures ANOVA, $F_{3,12} = 8.03$, $p < 0.01$) and for *M. aeruginosa* PCC 7806 on Days 4 and 7 (Kruskal-Wallis ANOVA, $H_3 = 9.53$, $p < 0.01$ and $H_3 = 8.49$, $p < 0.05$, respectively). The intracellular microcystin content of *M. aeruginosa* PCC 7806 was about twice as high as

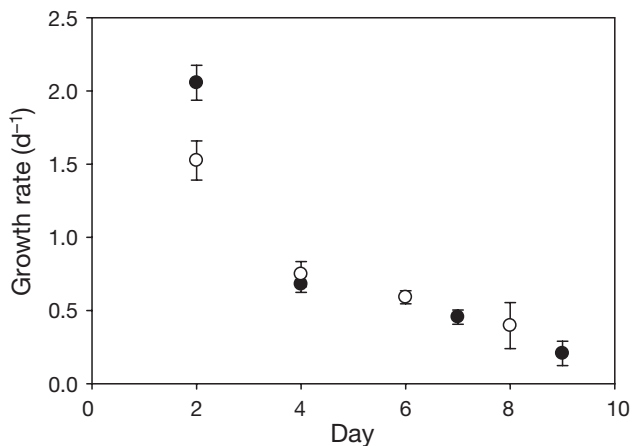


Fig. 2. *Ochromonas* sp. Specific growth rates of *Ochromonas* sp. on *Microcystis aeruginosa* PCC 7806 (filled circles) and *M. aeruginosa* HUB 524 (open circles) over the course of the grazing experiment. Error bars indicate standard deviation

that of *M. aeruginosa* HUB 524 (Fig. 3C,D). The intracellular microcystin contents of both strains remained constant across all treatments (Fig. 3C), except at the end of the experiment with *M. aeruginosa* PCC 7806, where it fell to significantly lower values in the grazing treatment by Day 9 (Kruskal-Wallis ANOVA, $H_3 = 8.74$, $p < 0.05$). Dissolved microcystin concentrations were low compared to the particulate concentrations, exceeding 5% of the total microcystins only for *M. aeruginosa* PCC 7806 on Day 9. Dissolved microcystin concentrations increased with increasing population densities of *M. aeruginosa*, and reached considerably higher concentrations for *M. aeruginosa* PCC 7806 than for *M. aeruginosa* HUB 524 (Fig. 3E,F). From Day 4 onwards, the dissolved microcystin concentrations were significantly higher in the presence than in the absence of *Ochromonas* sp. (repeated-measures ANOVA, for PCC 7806: $F_{3,12} = 16.90$, $p < 0.001$; for HUB 524: $F_{3,12} = 61.78$, $p < 0.001$). The intracellular microcystin content of *Ochromonas* sp. could be measured only at the end of the experiments, when population densities of *Ochromonas* sp. were high enough to be sorted by the flow cytometer. *Ochromonas* sp. contained $3.91 (\pm 3.19 \text{ SD}) \text{ fg microcystin cell}^{-1}$ after 9 d

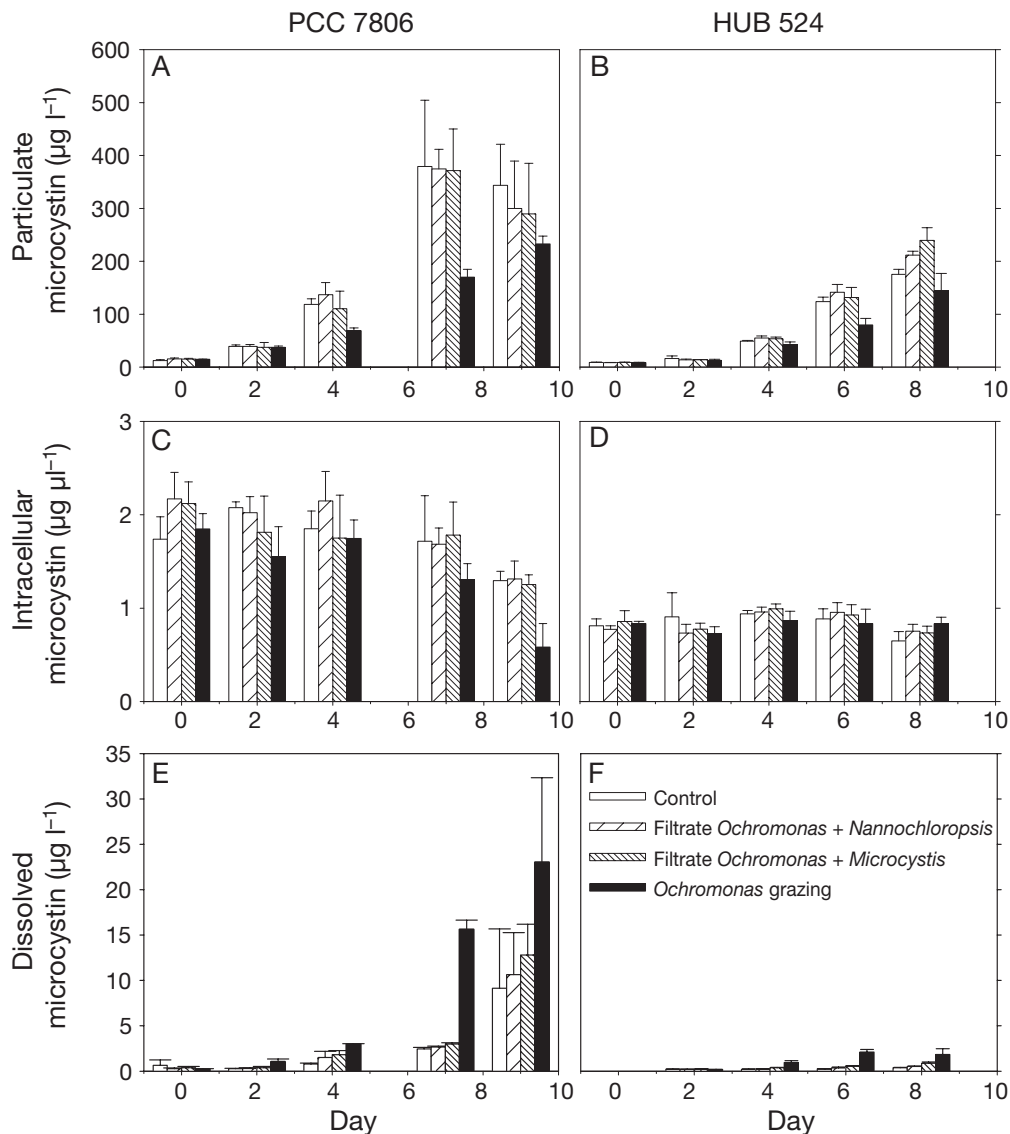


Fig. 3. *Microcystis aeruginosa*. Changes in (A,B) particulate microcystin concentration, (C,D) intracellular microcystin content and (E,F) dissolved microcystin concentration over the course of the grazing experiments with *Ochromonas* sp. as predator and *M. aeruginosa* PCC 7806 (left panels) and *M. aeruginosa* HUB 524 (right panels) as prey. Error bars indicate standard deviation

of grazing on *M. aeruginosa* PCC 7806 and $5.23 (\pm 1.18 \text{ SD}) \text{ fg microcystin cell}^{-1}$ after 8 d of grazing on *M. aeruginosa* HUB 524. Hence, the intracellular microcystin contents of *Ochromonas* sp. were about one order of magnitude lower than the intracellular microcystin contents of the *M. aeruginosa* cells upon which they fed (Table 1).

Functional and numerical response

Ochromonas sp. showed a very similar functional response irrespective of whether it was feeding on the microcystin-producing wildtype or on the microcystin-de-

ficient mutant of *Microcystis aeruginosa* PCC 7806 (Fig. 4A). The ingestion rate of *Ochromonas* sp. was well described by the Type 3 functional response model of Eq. (4). We note that the sigmoid shape of the Type 3 functional response is not visible in Fig. 4A, because both axes are plotted on logarithmic scales. The estimated model parameters are summarised in Table 3. The power x had a similar value on both *M. aeruginosa* strains and was significantly > 1 , confirming the Type 3-like shape of the functional response of *Ochromonas* sp. The maximum ingestion rate I_{max} was slightly but significantly higher on the microcystin-producing wildtype than on the microcystin-deficient mutant (Table 3). The initial increase of the clearance rate with the *M.*

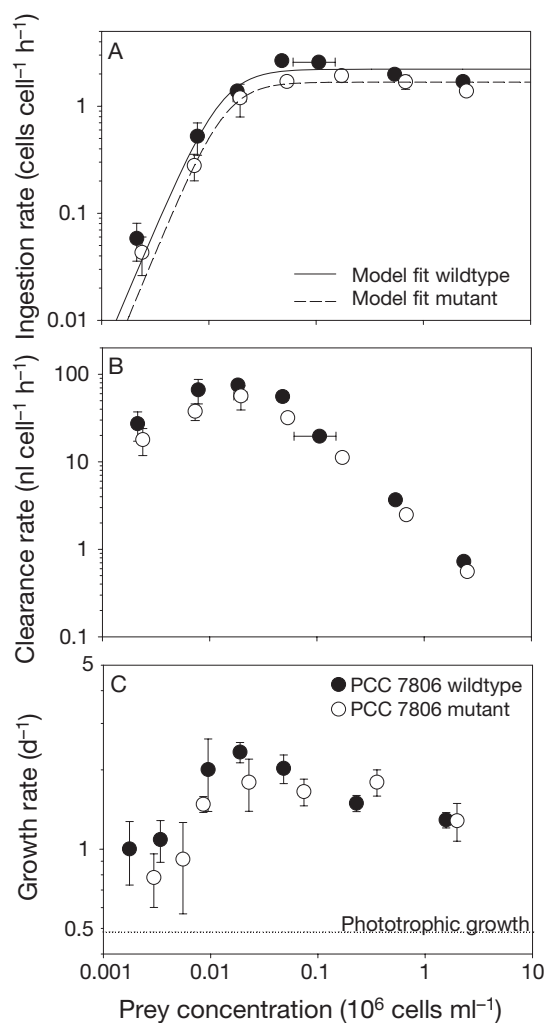


Fig. 4. *Ochromonas* sp. (A) Ingestion rates, (B) clearance rates and (C) specific growth rates of *Ochromonas* sp. grazing on the microcystin-producing wildtype and microcystin-deficient mutant of *Microcystis aeruginosa* PCC 7806. Error bars indicate standard deviation. (A) Lines show the fit of the Type 3 functional response model to the ingestion rate data. (C) Dotted horizontal line shows the specific growth rate of *Ochromonas* sp. when grown photoautotrophically (i.e. without *M. aeruginosa*)

Table 3. *Microcystis aeruginosa* and *Ochromonas* sp. Estimated parameter values for the Type 3 functional response model fitted to the ingestion rate data of *Ochromonas* sp. grazing on the microcystin-producing wildtype and the microcystin-deficient mutant of *M. aeruginosa* PCC 7806. I_{\max} : maximum ingestion rate of *Ochromonas* sp.; K : half-saturation constant; x : curvature of the sigmoid functional response (Eq. 4). 95% confidence intervals are given in parentheses

<i>M. aeruginosa</i> strain	I_{\max} (cells cell ⁻¹ h ⁻¹)	K (10 ⁶ cells ml ⁻¹)	x
Wildtype	2.21 (±0.25)	0.013 (±0.025)	2.37 (±0.97)
Mutant	1.68 (±0.14)	0.014 (±0.025)	2.43 (±0.85)

aeruginosa concentration is typical for a Type 3-like functional response (Fig. 4B). The specific growth rate of *Ochromonas* sp. showed a very similar dependence on *M. aeruginosa* abundance irrespective of whether it was feeding on the microcystin-producing wildtype or the microcystin-deficient mutant (Fig. 4C). The specific growth rate of *Ochromonas* sp. was much lower when grown photo-autotrophically than when feeding on *M. aeruginosa* (Fig. 4C).

Abundances of heterotrophic bacteria remained below 1% of the total biovolume and did not decrease in the grazing treatments relative to the control. The contribution of heterotrophic bacteria to the nutrition of *Ochromonas* sp. was therefore regarded as negligible in our experiments.

DISCUSSION

Genetic evidence has shown that microcystins have a long evolutionary history, indicating that cyanobacteria could probably produce microcystins before metazoan grazers such as copepods and cladocerans entered the evolutionary record (Rantala et al. 2004). This makes it unlikely that microcystins have evolved as a defence against metazoans. However, protozoa arrived at the scene much earlier, and microcystins could therefore have evolved as an anti-herbivore defence against protozoan grazers. Our laboratory experiments confirm the strong impact of protozoan grazing by the flagellate *Ochromonas* sp. on the cyanobacterium *Microcystis aeruginosa*. The population of *Ochromonas* sp. grew quickly to large densities and suppressed the abundance of *M. aeruginosa* substantially. This implies that *Ochromonas* has the potential to impact *M. aeruginosa* populations in natural waters, where *Ochromonas* is often present at relatively low background densities, but occasionally dominates the phytoplankton (Ollrik & Nauwerck 1993, Van Donk et al. 2009).

Grazing by *Ochromonas* sp. did not cause an increase in intracellular microcystin content. Therefore, microcystins do not seem to act as an inducible defence against flagellate grazing as has been reported for daphnids (Jang et al. 2003). Since we did not observe any colony formation in the presence of grazers, we cannot totally exclude the possibility that the population densities of *Ochromonas* sp. in our experiment were too low to cause a response in *Microcystis aeruginosa*. Colony formation in *M. aeruginosa* can be induced by *Ochromonas* grazing (Burkert et al. 2001, Yang et al. 2006). However, these studies used different strains of *M. aeruginosa*, and we could not ascertain that the strains used in our experiments had the ability to form colonies. Our inoculation densities

were comparable to population densities observed in natural waters (Van Donk et al. 2009), and *Ochromonas* sp. grew rapidly to higher densities over the course of the experiment. Therefore, if these population densities were too low to induce higher intracellular microcystin contents in our experiments, *Ochromonas* can not be expected to induce higher intracellular microcystin in natural waters either. Dissolved microcystin concentrations increased significantly due to grazing by *Ochromonas* sp., probably due to lysis of *M. aeruginosa* cells or excretion of consumed microcystin by *Ochromonas* sp.

While *Ochromonas* sp. grew with very high specific growth rates at the start of the experiment, and increased population densities 500- to 1000-fold, specific growth rates decreased markedly during the course of the experiment (Fig. 2), even though prey densities remained high. This deceleration of the growth rate was not observed in earlier studies using *Ochromonas* or *Poterioochromonas* (Cole & Wynne 1974, Zhang et al. 2008), because these earlier studies used very high inoculation densities of the flagellates, resulting in the disappearance of *Microcystis aeruginosa* within short periods. What might have caused the reduction of the specific growth rate cannot be answered from our experiments. One possible reason might be the excretion of allelopathic substances by *Microcystis* (e.g. Sukenik et al. 2002). Another explanation might be the toxic effect of microcystins ingested by *Ochromonas* sp. This possibility has been further investigated in the functional response experiment.

We used the microcystin-producing wildtype and microcystin-deficient mutant of *Microcystis aeruginosa* PCC 7806 to investigate the effect of microcystins on the functional and numerical response of *Ochromonas* sp., since the only difference between these 2 strains is their ability to produce microcystins. The content of other potentially toxic peptides like cyanopeptolins and microviridins (Jungmann 1992, Tonk et al. 2009) should be the same in both of them, while strains of different origin would probably have differed in their contents of many peptides, fatty acids and other cellular constituents.

The ingestion and clearance rates of *Ochromonas* sp. showed a Type 3 functional response. This shape is explained by an increased foraging effort with increasing prey concentration. In the mixotroph *Ochromonas*, such a pattern is most likely caused by a trade-off between phototrophic and heterotrophic growth. At low prey densities, mixotrophs will mainly benefit from investments in phototrophic growth, while foraging efforts on prey will contribute less to their total carbon metabolism. Conversely, at high prey densities, the rewards of heterotrophic growth will be high and photosynthesis will therefore become less important. The

growth rate achieved under purely autotrophic conditions was much lower than the maximum growth rate of *Ochromonas* sp. (Fig. 4C), indicating that saturating densities of *Microcystis aeruginosa* covered at least 80% of the carbon demand of *Ochromonas* sp.

Quantitatively, our estimates of the ingestion rates compare well to those of $1.7 \text{ cells cell}^{-1} \text{ h}^{-1}$ reported for *Ochromonas danica* grazing on *Microcystis aeruginosa* (Cole & Wynne 1974). The same is true for the clearance rates measured at high prey concentrations. However, the maximum clearance rates of 55 to 75 $\text{nl cell}^{-1} \text{ h}^{-1}$ measured in the present study at prey concentrations of about $3 \times 10^4 \text{ cells ml}^{-1}$ are much higher than those of 0.6 to 1.9 $\text{nl cell}^{-1} \text{ h}^{-1}$ reported for *Poterioochromonas* sp. (Zhang et al. 2008) or of 0.7 to 4.1 $\text{nl cell}^{-1} \text{ h}^{-1}$ for the heterotrophic flagellate *Colloidietyon triciliatum* grazing on *M. aeruginosa* (Nishibe et al. 2002). The lower clearance rates found in the literature can be explained by the higher prey concentrations used in most other studies and the strong decline of clearance rates with increasing prey concentrations (Fig. 4B).

The intracellular microcystins did not have a negative effect on *Ochromonas* sp. On the contrary, *Ochromonas* sp. even showed slightly higher maximum ingestion rates on the microcystin-containing wildtype than on the microcystin-deficient mutant. Whether this is due to a stimulating effect of microcystins (Ou et al. 2005) or to the slightly smaller size of the wildtype strain (Table 1) cannot be answered with certainty. In any case, differences in ingestion rates between the wildtype and its microcystin-deficient mutant were small, and intracellular microcystin, therefore, does not seem to act as a defence against these mixotrophic flagellates.

In addition to mixotrophic flagellates such as *Ochromonas*, several heterotrophic flagellates have been reported to graze on *Microcystis* (Nishibe et al. 2002, Park et al. 2003, Kim et al. 2006). Microcystins inhibit the eukaryotic protein phosphatases Types 1 and 2A (MacKintosh et al. 1990) and are therefore toxic to most eukaryotes. However, since the protein phosphatases are located in the cytoplasm, microcystins have to be transported into the cell to cause a toxic effect. Active transport into mammalian hepatocytes has been shown (Dawson 1998), while most other organs remain unaffected, presumably because they do not take up microcystins. A possible explanation for the absence of microcystin toxicity in *Ochromonas* might therefore be the lack of an uptake system from the lysosomes into the cytoplasm. Furthermore, *Ochromonas* and *Poterioochromonas* are both able to degrade microcystins (Zhang et al. 2008, Van Donk et al. 2009), and we did not find any bioaccumulation of microcystins within *Ochromonas* sp. A rapid degrada-

tion of the toxins is important in preventing a negative effect on growth. Due to degradation, only a small fraction of the microcystins ingested by *Ochromonas* sp. was released into the dissolved pool, which is contrary to what would be expected if microcystins were egested directly. Because our cultures were not axenic, there might have been some degradation of dissolved microcystins by bacteria as well.

While our results show that the mixotrophic flagellate *Ochromonas* sp. is not affected by microcystins, other studies indicate that several species of heterotrophic flagellates are susceptible to microcystins (Christoffersen 1996, Moustaka-Gouni et al. 2006). These contrasting results suggest great variability in the response of different protozoa to microcystin-producing cyanobacteria. In addition to microcystin production, colony formation in *Microcystis* can also act as a defence against grazers and has been shown to affect *Ochromonas* clearance rates (Yang et al. 2009). Because *Microcystis* often forms large colonies, the impact of *Ochromonas* grazing on single-celled *Microcystis* strains in our small-scale laboratory experiments cannot be easily extrapolated to natural waters. Further experiments are needed to elucidate the role of *Ochromonas* grazing on colonial *Microcystis* populations.

In conclusion, protozoa such as the mixotrophic flagellate *Ochromonas* can be very effective grazers suppressing the population development of *Microcystis aeruginosa*. *Ochromonas* is not negatively affected by the ingested microcystins, nor is microcystin production by *M. aeruginosa* enhanced by *Ochromonas* grazing. While microcystins are a very powerful defence against many metazoan grazers (DeMott et al. 1991, Lürling 2003), they seem to be less useful against mixotrophic flagellates, and colony formation might be the more effective protection against these very small protozoan grazers.

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