



Cell lysis of competitors and osmotrophy enhance growth of the bloom-forming alga *Gonyostomum semen*

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ABSTRACT: The nuisance alga *Gonyostomum semen* (Raphidophyceae) is known to form dense blooms in many freshwater bodies, and has increased its distribution and abundance. However, information on the mechanisms behind bloom formation and maintenance is scarce. Field observations indicate that *G. semen* may be favored in humic lakes. In the present study, we performed controlled laboratory experiments on cultures to test whether *G. semen* growth is enhanced by humic acids. In addition, we tested the mixotrophic capability of *G. semen* by providing it with *Rhodomonas lacustris* as prey. *G. semen* increased its growth rate in the presence of humic substances due to either acquisition of carbon or some unknown growth factor. Moreover, growth rate was enhanced in the presence of *R. lacustris*, which in turn was negatively influenced when cultured together with *G. semen*. We found no evidence of phagotrophy, but observed lysis of *R. lacustris* cells exposed to *G. semen* pre-grown with *R. lacustris*. Thus, our experiments suggest that cell lysis (probably via trichocyst action) and osmotrophy could be 2 mechanisms involved in establishing and maintaining dominance of *G. semen* in humic lakes.

KEY WORDS: Cell lysis · Osmotrophy · Mixotrophy · Humic acids · *Gonyostomum semen* · Raphidophyceae · Nuisance bloom

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INTRODUCTION

During the last 2 decades, the freshwater raphidophyte *Gonyostomum semen* Ehrenberg (hereafter referred to as *Gonyostomum*) has received increased scientific attention due to its propensity to form blooms and thereby negatively influence the recreational value of lakes. Originally, *Gonyostomum* was found mainly in small lakes and ponds with a high humic content (Eloranta & Råike 1995, Pithart et al. 1997). During recent decades, however, there has been a trend toward a wider distribution in more nutrient-rich and non-humic environments (Cronberg 2005). In addition, there has been an increased frequency of blooms (Lepistö et al. 1994, Findlay et al. 2005) with cell concentrations up to $1.2 \times 10^6 \text{ l}^{-1}$ (Hehmann et al. 2001). Although *Gonyostomum* has a worldwide distribution (Europe, Asia, North and South America,

Africa), blooms have been recorded primarily in the Fenno-Scandinavian countries (Eloranta & Råike 1995). In the lakes of this region, *Gonyostomum* forms blooms that usually dominate the phytoplankton community completely, in particular during late summer (Salonen & Rosenberg 2000). During such events, *Gonyostomum* often comprises up to 95% of the phytoplankton biomass (Cronberg et al. 1988, Hongve et al. 1988, Havens 1989, LeCohu et al. 1989, Pithart et al. 1997, Salonen et al. 2002, Willén 2003, Findlay et al. 2005). Its ability to form high density blooms (in combination with the discharge of mucilaginous strands when stimulated by physical contact) makes *Gonyostomum* a nuisance species that can cause skin irritation in swimmers and clog filters in water treatment plants (Cronberg et al. 1988, Hongve et al. 1988).

Several hypotheses have been proposed to explain the recent spreading of *Gonyostomum* and its ability to form

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blooms. There is a positive correlation between total phosphorus and *Gonyostomum* abundance (Hehmann et al. 2001, Findlay et al. 2005), and increasing eutrophication has been suggested as a possible cause (Hongve et al. 1988, Lepistö & Saura 1998). However, the onset of blooms appears to be dependent on multiple factors. *Gonyostomum* occurs mostly in humic lakes with high concentrations of dissolved organic carbon (DOC), low pH and low light penetration. Hence, its ability to tolerate acidic conditions and low light levels may give it a competitive advantage over many other algae (Cronberg et al. 1988, Eloranta & Råike 1995). Another important factor influencing bloom formation is the ability of *Gonyostomum* to form benthic resting cysts (Cronberg 2005, Figueroa & Rengefors 2006). When environmental conditions become favorable, the resting cysts can act as a 'seed' bank for recruitment, and cyst germination may precede formation of high density swimming populations (Figueroa & Rengefors 2006). *Gonyostomum* is also capable of detecting chemical cues released by grazers (*Daphnia*) and adjusts its recruitment rate from resting cysts in the sediment accordingly (Hansson 1996, 2000). As grazing by *Daphnia species* may be an important regulatory factor (Findlay et al. 2005), germination at low predator abundance might further increase the likelihood of *Gonyostomum* becoming dominant (Hansson 1996). *Gonyostomum* may also reduce grazing and metabolic losses by extensive vertical migrations (Salonen & Rosenberg 2000). Furthermore, when migrating downward at night, *Gonyostomum* may access nutrients in the hypolimnion and thus overcome epilimnetic nutrient depletion (Salonen & Rosenberg 2000). Vertical migration also allows the alga to adjust its position in the water column to that of optimal photosynthetic light conditions (Eloranta & Råike 1995). However, information about *Gonyostomum*'s growth mode is still rather limited and there have been few experimental studies with this alga.

The aim of the present study was to investigate whether *Gonyostomum* is mixotrophic, i.e. does it have the ability to combine phototrophy and heterotrophy, either by utilizing humic substances or by preying on bacteria and/or other algae. We propose that *Gonyostomum*'s dominance in brownwater lakes is influenced by its ability to utilize humic substances and competing algae as nutrient sources. We tested this proposal by exploring the growth of *Gonyostomum* in controlled laboratory settings.

MATERIALS AND METHODS

Cultures. Two *Gonyostomum semen* (hereafter *Gonyostomum*) cultures (GSBO2 and GSTVB3) were established from single cell isolates from the humic

lakes Bokesjön (September 2004) in southern Sweden and Tvigölen (September 2006) in central Sweden, respectively. Culture GSBO2 was used in Expts 1 to 3, while GSTVB3 was used for Expt 4 (since GSBO2 had died). Stock cultures were maintained in artificial freshwater MWC medium buffered at pH 7 (modified Woods Hole medium, Guillard & Lorenzen 1972) at a photon flux of $30 \mu\text{mol m}^{-2} \text{s}^{-1}$. However, *Gonyostomum* was grown at a low light intensity ($10 \mu\text{mol m}^{-2} \text{s}^{-1}$) in all experiments (in order not to inhibit potential mixotrophy by light over-saturation); this low photon flux approximates natural ambient light level. For example, in a Finnish humic lake *Gonyostomum* aggregated at $\leq 10 \mu\text{mol m}^{-2} \text{s}^{-1}$ (Eloranta & Råike 1995). Maintenance cultures and experiments were kept in a temperature-controlled walk-in incubator at $20 \pm 1^\circ\text{C}$ under a light:dark cycle of 14:10 h.

Expt 1: Mixotrophy. In order to determine whether *Gonyostomum* grew better when provided with humic acids or with a prey, 3 treatments were set up: 'Control' with MWC medium only; 'Fulvic acids (FA)' with MWC medium and an addition of humic substances at 12 mg l^{-1} (equivalent to 5.2 mg C l^{-1}); and 'Rhodomonas' with MWC medium plus the addition of a potential algal prey (*Rhodomonas lacustris*, Cryptophyceae). Prior to the start of the experiment, *Gonyostomum* (GSBO2) was acclimated to the prevailing light regime for approximately 1 mo. The FA were obtained from Fredriks Research Products, and had been extracted from Laurential soils. The partial elemental composition of the FA (according to the supplier) was C (42.3%), H (4%), N (0.83%), Na (0.27%), Fe (0.013%), and ash (<1%). Kritzberg et al. (2006) showed that this particular FA was utilized as a DOC source by a natural bacterial lake community. All treatments were replicated 6 times; however, 1 replicate of the FA treatment failed to grow after inoculation and the results presented from this treatment are based on 5 replicates.

At the start of the experiment, 109 ml of medium was added to 250 ml culture flasks (Nunclon™), which were inoculated to give an initial *Gonyostomum* concentration of 75 cells ml^{-1} (corresponding to a pre-bloom level). In the *Rhodomonas* treatment, *Rhodomonas lacustris* (NIVA 8/82) (hereafter *Rhodomonas*) cells were added at an initial density of $140 \text{ cells ml}^{-1}$. Initial samples were immediately removed from the flasks, and subsequent samples were removed after 4, 8, 12, 20, 28, 36 and 40 d. Samples (total 9 ml) were split into 2 equal halves, one preserved with Lugol's acid solution and another with sterile-filtered glutaraldehyde (2.5% final concentration). On each sampling occasion, the volume removed was replaced with fresh MWC medium. Thus, the volume in the culture bottles was kept

constant at 100 ml. In the FA treatment, the replacement medium was enriched with FA.

Density counts of bacteria and *Rhodomonas* were obtained from the glutaraldehyde-preserved samples using a FACSort (Becton Dickinson) flowcytometer. Bacteria were stained with SYTO-13 according to del Giorgio et al. (1996), while *Rhodomonas* was identified from the fluorescence of chlorophyll and phycoerythrin. *Gonyostomum* was enumerated in settling chambers using an inverted microscope (Nikon Eclipse TS 100). Specific growth rates (d^{-1}) of *Gonyostomum* and *Rhodomonas* were determined by linear regressions of the natural log of cell densities against time, and were corrected for dilution effects.

On Day 40, samples were also collected for chlorophyll *a* (chl *a*) and mineral nutrient analysis. For chl *a*, a known volume from each replicate was filtered onto precombusted (2 h, 450°C) GF/F filters (Whatman). The filters were stored frozen and chl *a* was extracted in ethanol overnight (Jespersen & Christoffersen 1987). Chl *a* absorbance was measured on a Beckman DU 650 spectrophotometer. *Gonyostomum* cellular chl *a* concentrations for the Control and FA treatments were calculated by dividing total chl *a* by *Gonyostomum* density. Due to the presence of *Rhodomonas* cells in the *Rhodomonas* treatment, *Gonyostomum* cellular chl *a* could not be calculated. The cell-free filtrate was collected for nutrient analyses and stored frozen in acid-washed plastic vials. Concentrations of PO_4^{3-} and NO_3^- were measured in randomly selected duplicates from each treatment using a Technicon Autoanalyzer II according to Technicon protocols and Swedish standard methods (SS028126 and SS028133; Bydén et al. 1996).

Expt 2: Growth by humic acid concentration. In this experiment, *Gonyostomum* was cultured in MWC medium with varying concentrations of humic substances. As before, maintenance cultivation and experiments were done in temperature-controlled walk-in incubators at $20 \pm 1^\circ C$ under a light:dark cycle of 14:10 h and a photon flux of $10 \mu mol m^{-2} s^{-1}$. *Gonyostomum* was grown with (1) 2 mg FA l^{-1} (0.9 mg C l^{-1} , Low treatment), (2) 9 mg FA l^{-1} (3.9 mg C l^{-1} , Medium treatment), and (3) 22 mg FA l^{-1} (9.5 mg C l^{-1} , High treatment). All treatments were run in 6 replicate flasks (Nunclon™) with starting densities of approximately 90 *Gonyostomum* cells ml^{-1} . Sampling was performed as described in Expt 1 except that samples were removed at 8 d intervals (apart from the last sampling, which was done after 76 d following a 4 d interval). Fresh medium was added on each sampling occasion. Densities of *Gonyostomum* were obtained by visual counts of Lugol's preserved samples (Nikon Eclipse TS 100) from alternate sampling dates. Again, growth rates were determined by linear regressions of the

natural log of cell densities against time. Density counts of bacteria were obtained by flow cytometry of the glutaraldehyde-preserved samples from each sampling occasion as described above.

When the incubations were terminated on Day 76, samples were taken for chl *a* and mineral nutrient analysis as described for Expt 1. Primary production rates were measured by the ^{14}C incorporation method (Wetzel & Likens 1991) using one 23 ml glass vial for each replicate and a control consisting of equal volumes of culture from the 6 replicates. $H^{14}CO_3$ (Amersham CFA3, specific activity 58 mCi (2.15 GBq $mmol^{-1}$) was added (1.76 μCi final activity) and the vials were incubated for approximately 4 h. During incubation, all vials except the control (incubated in darkness) were exposed to the same light and temperature conditions as the original cultures. Cell-specific primary production rates were calculated for each treatment by dividing the production rate h^{-1} by cell density. Cell-specific primary production rates normalized to chl *a* were calculated by dividing the production rates by cellular concentrations of chl *a*.

Bacterial abundance and production were measured to determine whether they were correlates of *Gonyostomum* growth rate and/or density. Bacterial production (Day 76 only) was measured by the incorporation of leucine (Amersham TRK 510, specific activity 22.5 Ci $mmol^{-1}$, final concentration 100 nmol l^{-1}) according to Smith & Azam (1992). The incubations (approximately 60 min) were performed with samples (1.7 ml) from each replicate plus one trichloroacetic acid (TCA)-killed control. The incubations were terminated by the addition of TCA (5% final concentration) and bacteria were concentrated to pellets by centrifugation at $15\,000 \times g$ for 10 min. The pellets were rinsed with 5% TCA and 80% ethanol. Finally, 0.5 ml of scintillation cocktail (Ecoscint A, Kimberly Research) were added to each and 3H activity was measured on a Beckman LS 6500 Scintillation counter. Leucine incorporation rates were transformed into bacterial carbon production rates according to Smith & Azam (1992). All statistical analyses were performed using SPSS 10 software. Data was tested for variance homogeneity and normality prior to running ANOVAs.

Expt 3: *Rhodomonas* disappearance. In order to determine whether *Gonyostomum* preyed upon *Rhodomonas*, a prey disappearance experiment was conducted after terminating Expt 1. The design included a treatment with *Rhodomonas* exposed to *Gonyostomum* cells, while the control consisted of *Rhodomonas* with cell-free *Gonyostomum* culture medium. The cell-free *Gonyostomum* medium was used (instead of MWC medium only) to rule out any differences resulting from potential water-released allelochemicals. The mixed cultures from Expt 1 were thus used in order to

include *Gonyostomum* that was acclimated to prey. For the *Gonyostomum* treatment, 25 ml were transferred to each of 3 culture flasks (Nunclon™). The *Gonyostomum*:*Rhodomonas* abundance ratio in this treatment was set to approximately 1:24 by adding more *Rhodomonas* cells. For the Control treatment, the remaining volume of mixed culture was gravity filtered consecutively through 25 and 10 µm nets in order to remove *Gonyostomum*. Due to its trichocysts, *Gonyostomum* clogs filters rapidly, and sequential filtration is needed to remove all cells. The filtrate was transferred to 3 replicate flasks with a final volume of 25 ml each. The removal of *Gonyostomum* from the Control was successful and no *Gonyostomum* cells were found in this treatment. In the treatment with *Gonyostomum*, densities were 536 ± 96 cells ml⁻¹ (mean ± SD). Filtration also removed some *Rhodomonas* and starting densities were $13.1 \pm 0.94 \times 10^3$ and $10.7 \pm 0.70 \times 10^3$ cells ml⁻¹ in the *Gonyostomum* and Control treatments, respectively. Samples (5 ml preserved with Lugol's acid solution and sterile filtered glutaraldehyde, 2.5% final concentration) were removed immediately to estimate starting densities of *Gonyostomum* and *Rhodomonas*. Additional samples (5 ml) were removed after 8 and 29 h. In between sample retrievals, the culture bottles were exposed to the same light and temperature conditions as those in Expt 1.

Densities of *Rhodomonas* were counted by flow cytometry as described above, and *Rhodomonas* specific growth rates (d⁻¹) were determined by linear regressions of the natural log of cell densities against time. Disappearance rates were determined from the reduction in *Rhodomonas* densities in the treatment with *Gonyostomum* compared to the Control, assuming exponential growth (following Skovgaard 1996):

$$\frac{dX}{dt} = \mu_X X - UY \quad (1)$$

and

$$\frac{dY}{dt} = \mu_Y Y \quad (2)$$

where X is prey concentration, Y is *Gonyostomum* concentration, μ is specific growth rate, U is per capita ingestion rate, and UY is the mortality of prey.

In order to further investigate the potential phagotrophy of *Gonyostomum* on *Rhodomonas*, we examined *Gonyostomum* for food vacuoles in a parallel experiment. The remaining 3 replicates from treatment P were mixed and *Rhodomonas* was added to a 1:24 *Gonyostomum*:*Rhodomonas* ratio, as described above. Sub-samples were removed from each of 3 replicates and live samples were observed continually in Sedgewick-Rafter chambers under light microscopy over the first few hours, and then hourly for up to 8 h. In addition, samples (8 ml) from each replicate were

collected at the start and after 8 h incubation at 20°C and 10 µmol photons m⁻² s⁻¹. The samples were preserved with ice-cold glutaraldehyde (2.5% final concentration) and were filtered onto 25 mm black 5 µm pore size polycarbonate membrane filters (Osmonics). Cells were stained with DAPI or CYTOX according to Sherr & Sherr (1993) and *Gonyostomum* was examined for food vacuoles using epifluorescence microscopy (Nikon Labophot-2) at 1000× magnification.

Expt 4: Lysis experiment. A follow-up experiment to Expt 3 was performed in order to test whether lysis (due to cell contact or an exuded chemical) was the mechanism that led to the disappearance of *Rhodomonas*. To do this, we ran a 72 h experiment with *Rhodomonas* cells exposed to live *Gonyostomum* cells or cell-free filtrates. By testing cell-free filtrates, any effect due to chemical mediated lysis (allelopathy) could be separated from cell-contact mediated lysis. *Rhodomonas* cells were exposed to 4 different treatments: MWC medium only (Control); *Gonyostomum* cells grown in unialgal culture (Single); 'induced' *Gonyostomum* pre-grown in a mixed culture with *Rhodomonas* for 9 d (Induced); and *Gonyostomum*-free filtrate from *Gonyostomum* pre-grown with *Rhodomonas* (Filtrate). As the GSBO2 culture had died, GSTVB3 was used. Treatments were set up in 24-well Nunc plates (Nunclon), with 4 replicate wells per treatment, in 5 parallel sets for 5 sampling occasions. The initial *Rhodomonas* cell concentration was 8860 ml⁻¹ in all treatments. The cell concentration of *Gonyostomum* was 370 ml⁻¹ in the Single treatment, thereby reaching the 1:24 *Rhodomonas*:*Gonyostomum* cell ratio used in the prey disappearance experiment. For the Control treatment, *Rhodomonas* was added from a culture of *Rhodomonas* grown unialgally to a reach a final concentration of 8860 ml⁻¹. For the Induced treatment, most *Rhodomonas* cells were removed by filtering through a 10 µm Nyltex net, and the *Gonyostomum* cells were re-suspended with fresh medium. Unialgal *Rhodomonas* cultures were added to reach a final concentration of 8860 ml⁻¹. The filtrate from the mixed *Gonyostomum* and *Rhodomonas* culture was utilized for the Filtrate treatment. The 10 µm filtrate was passed through a 2.0 µm filter to remove remaining *Rhodomonas* cells. This filtrate nevertheless contained 9847 *Rhodomonas* cells ml⁻¹ and was used for the filtrate treatment. Since each well contained 1 ml filtrate and 950 µl of MWC medium, additional *Rhodomonas* cells were added to reach a final concentration of 8860 ml⁻¹ in the wells. A smaller pore filter was not used, as these become clogged with *Gonyostomum* trichocysts. To minimize any affect of nutrient limitations, at least 0.5 ml of replete MWC medium was added to all treatments. The total volume was 2 ml in all wells.

The plates were incubated at 20°C, 10 µmol photons m² s⁻¹ on a 14:10 light:dark cycle. A set of samples was

harvested after 4, 8, 24, 48 and 72 h by adding 15 μl of Lugol's solution. Samples were counted at 40 \times magnification directly in the wells.

RESULTS

Expt 1: Mixotrophy

The highest densities of *Gonyostomum* were obtained in the *Rhodomonas* treatment followed by the FA and Control (pure MWC) treatments (Fig. 1) (repeated measures ANOVA: $F = 12.75$, $df = 2$, $p < 0.001$). The mean *Gonyostomum* specific growth rate ($0.158 \pm 0.010 \text{ d}^{-1}$ based on 40 d of incubation, mean \pm SD) obtained in the *Rhodomonas* treatment was significantly higher than in the FA treatment, which was followed by the Control (0.140 ± 0.004 and $0.125 \pm 0.008 \text{ d}^{-1}$, respectively; ANOVA: $F = 24.42$, $df = 2$, Tukey's HSD, $p < 0.001$) (Table 1). Moreover, bacterial densities were highest in the *Rhodomonas* treatment throughout the experiment ($19.8 \times 10^6 \pm 5 \times 10^6$ bacteria ml^{-1} on Day 40; Table 1, repeated measures ANOVA: $F = 50.64$, $df = 2$, $p < 0.001$) compared to the FA and the Control treatments. There were no significant differences in *Gonyostomum* cell-specific concentrations of chl *a* among the Control and FA treatments (t -test, $p > 0.05$, Table 1). Concentrations of nitrate and phosphate on Day 40 were similar in the 3 treatments and ranged between 11.9–13.6 $\text{mg NO}_3^- \text{ l}^{-1}$ and 1.5–1.9 $\text{mg PO}_4^{3-} \text{ l}^{-1}$.

In the *Rhodomonas* treatment, mean *Rhodomonas* cell density was $11.7 \times 10^3 \text{ ml}^{-1}$ on Day 40, with a large variation between the replicates ($SD = 2.7 \times 10^3 \text{ cells ml}^{-1}$). A major part of this variation can be explained by the abundance of *Gonyostomum* because densities of *Rhodomonas* were negatively correlated with densities of *Gonyostomum* during the last 2 wk of incubation (when the treatments had entered a faster growth phase and started diverging, see Fig. 1, linear regression, $p < 0.01$, $r^2 = 0.336$; Fig. 2). When the entire growth period was included in the regression, no significant negative correlation was found.

Expt 2: Growth by humic acid concentration

In the growth by humic acid concentration experiment, the highest densities of *Gonyostomum* were reached in the treatment with the lowest FA concentration, followed by the Medium and High treatments (repeated measures ANOVA: $F = 88.73$, $df = 2$, $p <$

0.001) (Fig. 3). When *Gonyostomum* average growth rates were determined for the entire incubation period (76 d), they differed considerably among treatments (ANOVA, $p < 0.001$); the highest rate was obtained in the Low treatment ($0.2074 \pm 0.0010 \text{ d}^{-1}$) followed by the Medium ($0.2034 \pm 0.0022 \text{ d}^{-1}$) and High ($0.1965 \pm$

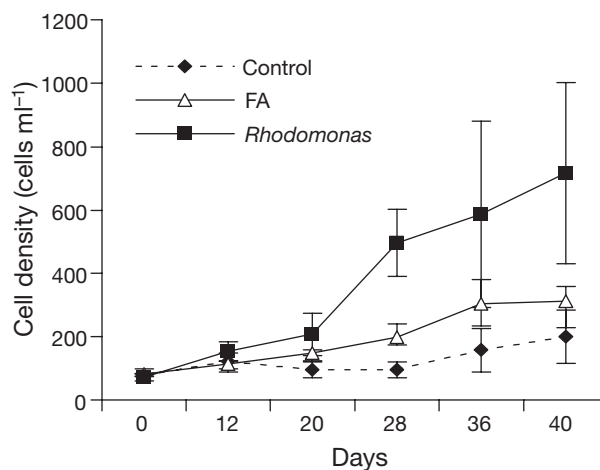


Fig. 1. *Gonyostomum semen*. Expt 1: Cell densities (means \pm SD) after 40 d at low light intensity ($10 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$) in pure MWC medium (Control, $n = 6$), MWC enriched with fulvic acids (FA) (12 mg l^{-1} ; $n = 5$), and MWC with *Rhodomonas lacustris* (*Rhodomonas*, $n = 6$)

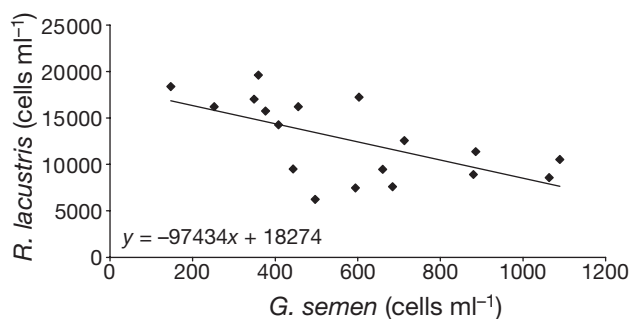


Fig. 2. *Gonyostomum semen* and *Rhodomonas lacustris*. Expt 1: Relationship between cell densities (cells ml^{-1}) in mixed cultures during the last 12 d of incubation (linear regression, $p < 0.01$, adj. $r^2 = 0.336$)

Table 1. *Gonyostomum semen*. Expt 1: Mean (SD) growth rate, bacterial abundances (BA) and *Gonyostomum* cellular chl *a* concentrations on Day 40 in cultures at low light intensity ($10 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$) and grown in (1) pure MWC medium (Control), (2) MWC enriched with fulvic acids (FA), and (3) mixed culture with *Rhodomonas lacustris* (*Rhodomonas*). $n = 6$ (for treatment FA, $n = 5$); -: not measured/applicable

Treatment/parameter	Control	FA	<i>Rhodomonas</i>
Specific growth rate (d^{-1})	0.12 (0.008)	0.14 (0.003)	0.16 (0.01)
BA ($\times 10^6 \text{ cells ml}^{-1}$)	7.0 (0.7)	9.2 (0.7)	19.8 (5.0)
Chl <i>a</i> (pg cell^{-1})	258 (27)	296 (31)	-

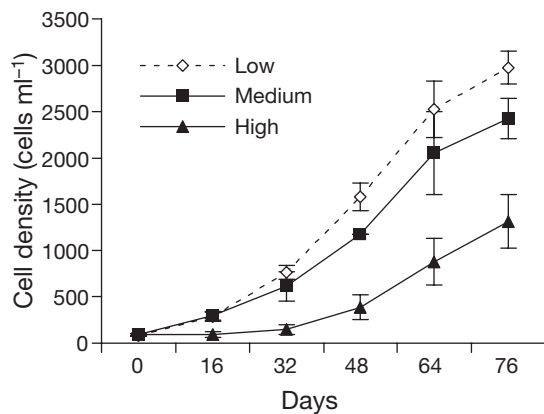


Fig. 3. *Gonyostomum semen*. Expt 2: Cell densities at a low light intensity ($10 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) in MWC medium enriched with fulvic acids at 2 mg l^{-1} (Low), 9 mg l^{-1} (Medium) and 22 mg l^{-1} (High). Means \pm SD, $n = 6$

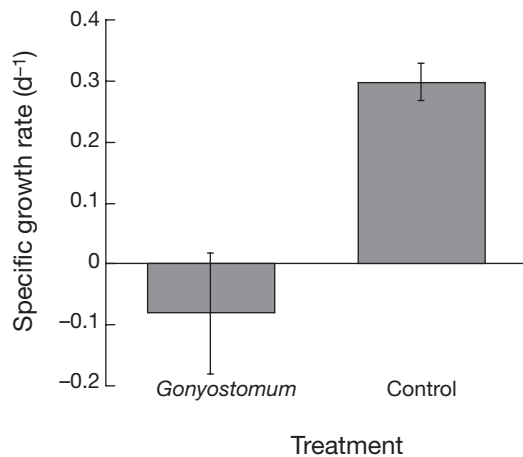


Fig. 4. *Rhodomonas lacustris*. Expt 3: Specific growth rates in cultures with *Gonyostomum semen* (*Gonyostomum*) or from which *Gonyostomum* had been removed (Control). Means \pm SD, $n = 3$

Table 2. Expt 3: Mean (SD) bacterial abundances (BA), bacterial production (BP), cellular concentrations of chl *a* in *Gonyostomum*, cellular production rates (PP) and production rates normalized to chl *a* on Day 76 in *Gonyostomum* cultures grown in MWC medium enriched with fulvic acids at 2 mg l^{-1} (Low), 9 mg l^{-1} (Medium) and 22 mg l^{-1} (High) at a low light intensity ($10 \mu\text{mol photons m}^{-2} \text{s}^{-1}$). $n = 6$

Treatment/Parameter	Low	Medium	High
BA ($\times 10^6 \text{ cells ml}^{-1}$)	19.9 (3.5)	18.6 (3.7)	16.7 (4.0)
BP ($\mu\text{g C l}^{-1} \text{ h}^{-1}$)	10.6 (0.8)	8.7 (0.7)	10.1 (2.3)
Chl <i>a</i> (pg cell^{-1})	376 (26)	360 (33)	333 (29)
PP (pg C cell^{-1})	39.7 (4.4)	43.6 (5.9)	75.5 (5.8)
PP chl <i>a</i> ⁻¹ ($\text{pg C pg chl a}^{-1}$)	0.11 (0.01)	0.12 (0.02)	0.23 (0.03)

0.0035 d^{-1}) treatments. By Day 76, growth rates had leveled out in the Low and Medium treatments, but not in the High treatment. Bacterial densities continued to increase throughout the experiment, reaching approximately $20 \times 10^6 \text{ cells ml}^{-1}$ on Day 76 (Table 2). No differences in bacterial abundance (BA) and production rate (BP) were observed among the treatments on Day 76 (ANOVA: $F_{BA} = 1.1$, $F_{BP} = 2.66$, $df = 2$, $p > 0.050$) (Table 2).

Gonyostomum cell-specific concentrations of chl *a* ranged between 333 and 376 $\text{pg chl a cell}^{-1}$ and were not affected by the differing additions of FA (Table 2, ANOVA: $F = 3.34$, $df = 2$, $p > 0.050$). A higher rate of cell-specific primary production was measured on Day 76 in the High treatment ($75.5 \text{ pg C cell}^{-1} \text{ h}^{-1}$) than in the Low and Medium treatments (39.7 and 43.6 $\text{pg C cell}^{-1} \text{ h}^{-1}$, respectively; ANOVA: $F = 79.68$, $df = 2$, $p < 0.001$). In addition, primary production per unit of chl *a* varied significantly among treatments (ANOVA: $F = 63.34$, $df = 2$, $p < 0.001$), and production in the High treatment was double that in the other 2 treatments (Table 2). The concentrations of nitrate and phosphate were similar in all treatments and ranged from 12.0 to 13.0 and 2.9 to 3.7 mg l^{-1} , respectively.

Expt 3: *Rhodomonas* disappearance

In the disappearance experiment, *Rhodomonas* growth rates were significantly lower in the *Gonyostomum* treatment ($-0.081 \pm 0.099 \text{ d}^{-1}$) than in the Control ($0.298 \pm 0.031 \text{ d}^{-1}$, *t*-test, $p < 0.01$, Fig. 4). This difference was equivalent to a disappearance rate of approximately 0.7 *Rhodomonas* cells per *Gonyostomum* cell h^{-1} , or 20 *Rhodomonas* cells per *Gonyostomum* cell over the course of the experiment.

No food vacuoles were observed in *Gonyostomum* cells in either stained (DAPI and CYTOX) or live cells (epifluorescent microscopy). More than 400 cells were checked. The abundant trichocysts and their tendency to discharge during handling made it difficult to determine whether or not *Rhodomonas* cells observed in filter samples were truly ingested. However, in live samples it was clear that *Gonyostomum* did not contain food vacuoles. Moreover, when examining live samples, no behavior indicating phagotrophic ingestion was apparent. The observations, however, revealed that close contact between the 2 species often resulted in lysis of *Rhodomonas*, which led to execution of Expt 4.

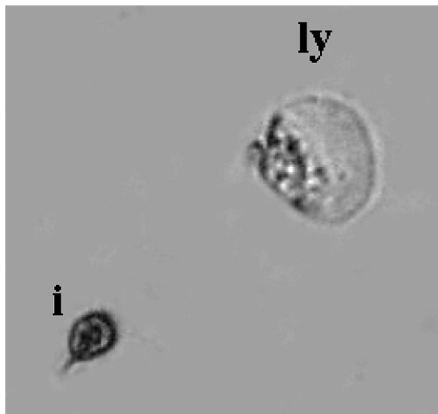


Fig. 5. *Rhodomonas lacustris* (length = 10 μm). Expt 4: Light micrograph of intact (i) and lysing (pre-lysed) (ly) cells in the treatment that included a *Gonyostomum* culture pre-grown with *Rhodomonas* cells. Cultures were not axenic

Expt 4: Lysis experiment

Rhodomonas exposed to induced *Gonyostomum* cells, i.e. *Gonyostomum* cells that had been pre-grown with *Rhodomonas* for 9 d (Induced treatment), were deformed and swollen, and appeared to be lysing (Fig. 5). These 'pre-lysed' cells were first observed after 4 h and increased to 12% of the total cell number after 72 h (Fig. 6A). The number of pre-lysed cells in the Induced treatment was significantly higher than the other treatments (repeated measures ANOVA: $F = 25.39$, $df = 3$, Bonferroni pairwise comparison, $p < 0.001$). In the Filtrate treatment, some pre-lysed cells appeared after 72 h and amounted to 3%. However, there was no decline in intact cells among the 4 treatments during this time period (Fig. 6B). In the Filtrate treatment, *Rhodomonas* cell number was slightly and significantly higher (repeated measures ANOVA: $F = 25.39$, $df = 3$, Bonferroni pairwise comparison, $p < 0.001$)

DISCUSSION

The occurrence of *Gonyostomum* blooms has increased in Fenno-Scandian freshwaters during recent decades, and several specific features have been suggested as explanations for its success (e.g. Lepistö et al. 1994, Salonen & Rosenberg 2000). We performed controlled laboratory experiments to examine some nutritional strategies of *Gonyostomum* that may influence bloom formation and maintenance. Growth of *Gonyostomum* was stimulated by the presence of FA, which may explain its preference for humic lakes. In addition, we found that *Gonyostomum*

enhanced its own growth when cultured with another alga (*Rhodomonas* that was eliminated by cell lysis). We propose that these are mechanisms by which *Gonyostomum* attains high biomass, and achieves and maintains dominance.

In our experiments, growth of *Gonyostomum* was positively influenced both by the presence of a small alga and by the addition of humic substances (FA). The experiments were conducted under controlled environmental conditions with fixed temperature and low light, using a culture medium with high nutrient concentrations and a neutral pH. We suggest that the observed growth enhancement resulted from heterotrophic nutrition, specifically osmotrophy. In the mixotrophy experiment (Expt 1), the highest growth rates of *Gonyostomum* were obtained when it was cultured together with the cryptophyte *Rhodomonas lacustris*, which is a common lake phytoplankter. In contrast, growth of *Rhodomonas* appeared to be inhibited by the presence of *Gonyostomum*, as indicated by the negative relationship between densities of the 2 species and the results from the disappearance experi-

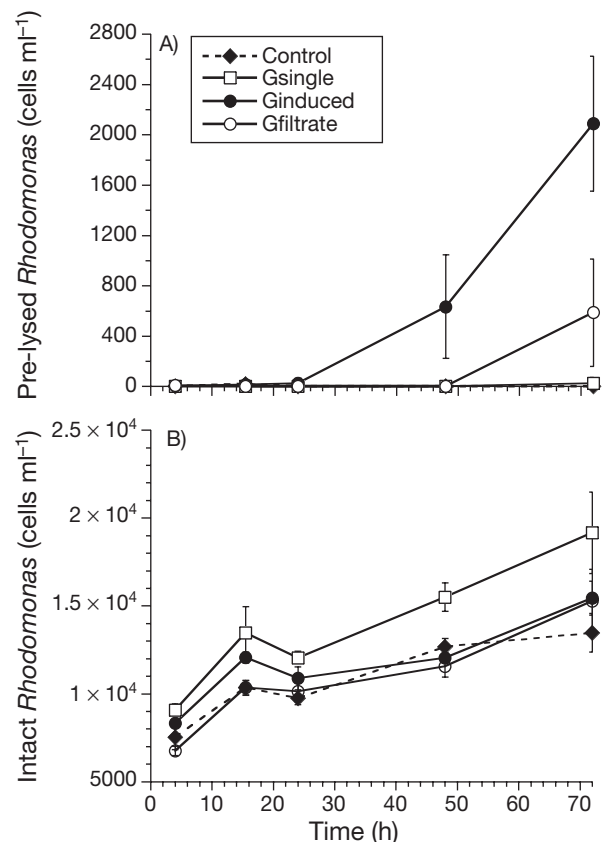


Fig. 6. *Rhodomonas lacustris*. Expt 4. (A) Abundance of pre-lysed cells. (B) Abundance of intact cells. Means \pm SD ($n = 4$) are shown. Gsingle: *Gonyostomum* grown in uni-algal culture; Ginduced: *Gonyostomum* pre-grown with *Rhodomonas*; Gfiltrate: filtrate from induced treatment; Control: MWC medium only

ment (Expt 3). The disappearance and lysis experiments (Expts 3 and 4) indicated that the suppression of *Rhodomonas* was due to cell lysis and not phagotrophy. Because of the high concentrations of nutrients in the culture medium, it is unlikely that *Gonyostomum* was favored by release of nutrients following lysis of its competitor. Rather, the higher growth rates were likely the result of a heterotrophic uptake of carbon (mixotrophy).

Mixotrophic nutrition (phagotrophy or osmotrophy) may be a means by which phytoplankton obtain carbon to supplement or even substitute the photosynthetic supply when light availability is too low to meet the metabolic demands of the cell (Jones 2000). *Gonyostomum* is often found in low light environments (Eloranta & Raike 1995, Pithart et al. 1997, Findlay et al. 2005) and has also been reported to remain in the dark hypolimnion during daytime (Salonen & Rosenberg 2000). In field studies, high abundances of *Gonyostomum* have correlated with increased DOC concentrations (humic substances) (Pithart et al. 1997, Lepisto & Saura 1998, Hehmann et al. 2001, Findlay et al. 2005). This response has been explained primarily by *Gonyostomum's* ability to tolerate acidification and reduced light climate in brown-water humic lakes. However, the results from Expt 1 suggest carbon assimilation by mixotrophic nutrition as an explanation for the correlation between *Gonyostomum* and DOC/ water color. Growth rates were significantly higher than controls in the *Rhodomonas* treatment and in the treatment with added FA, while light climate, culture medium, and pH were constant. An alternative hypothesis is that the humic substances and lysed *Rhodomonas* cells contain some growth factor or trace element(s) not found in the MWC medium (which contains trace metals and vitamins).

Other phytoplankton species are also known to be stimulated by high DOC availability. For instance, growth of the green alga *Chlamydomonas* sp. is enhanced by high DOC availability even when photosynthesis is saturated and inorganic nutrients are available (Tittel & Kamjunke 2004). In *Chlamydomonas*, the high DOC assimilation relative to total algal production (23%) indicates the considerable importance of heterotrophic carbon uptake, which is probably even higher at lower light intensities (Tittel & Kamjunke 2004). Likewise, *Ochromonas* growth is enhanced by the organic MES buffer in its medium (Sanders et al. 2001). The marine dinoflagellate *Alexandrium catenella* can take up high molecular weight dextrans (carbohydrate macromolecules), which provides evidence that large molecules such as humic substances can be taken up by mixotrophic algae (Legrand & Carlsson 1998).

In the humic acid growth experiment (Expt 2), we compared the effects of different FA concentrations on *Gonyostomum* growth. The highest mean densities and growth rates were obtained in the treatment with the lowest concentration of humic acids (2 mg FA l⁻¹) and growth could not be further enhanced by increased FA concentration. It should be noted that the FA level used in the initial mixotrophy experiment was 12 mg FA l⁻¹, i.e. in the range of the Medium and High treatments. Cell densities and growth rates for all treatments reached higher levels than in the initial mixotrophy experiment. This finding can be explained by the less frequent sampling, and thereby diminished disturbance of *Gonyostomum* cells. *Gonyostomum* cells are fragile and easily explode during handling. There was no indication that there were differences in the light climate among the different treatments (due to different concentrations of humic acids), as indicated by the similar cellular chl *a* content in all treatments. By the end of the experiment, growth rate appeared to level off and proceed into stationary phase in the Low and Medium treatments, in which cell concentrations had reached approximately 3000 ml⁻¹. Typically, our cultures reached between 3500 and 4000 cells ml⁻¹ before collapsing (K. Rengefors unpubl. data). No leveling off was noted in the High treatment, where cell densities had reached only 1200 ml⁻¹ by the termination of the experiment. These findings were corroborated by the high primary production in the High treatment on Day 76, which was almost twice as high as in the other treatments.

We have argued that the observed growth enhancement of *Gonyostomum* in response to FA was likely due to osmotrophic assimilation of DOC. However, another possibility is that *Gonyostomum* is capable of bacterial ingestion (phagotrophy). Although attempts were made to feed *Gonyostomum* with fluorescently labeled bacteria, we could neither demonstrate nor rule out their inclusion into food vacuoles (due to methodological problems caused by trichocyst expulsion). In many flagellates, bacterial ingestion rates and hence growth rates increase with increasing bacterial densities (Sanders et al. 1990). Thus, bacterial ingestion may potentially explain high *Gonyostomum* growth rates in the *Rhodomonas* treatment in Expt 1 since bacterial abundance was high in this treatment. However, the observed differences in *Gonyostomum* growth rates in Expt 2 cannot be explained by discrepancies in bacterial abundance and production rate. Thus, we conclude that osmotrophy is more likely than bacterial ingestion.

The observed mortality of *Rhodomonas* by *Gonyostomum* resulted from cell lysis and not from phagotrophy, as we proposed originally. We observed no cells feeding and no food vacuoles in either stained or

live samples. In the *Rhodomonas* disappearance experiment (Expt 3), 20 *Rhodomonas* cells disappeared for each *Gonyostomum* cell present during the the experiment. Among the hundreds of cells counted, food vacuoles or feeding events would have been detected had they occurred. Hence, we rule out phagotrophy and argue that disappearance of *Rhodomonas* and increased growth rate of *Gonyostomum* in its presence were due to cell lysis of *Rhodomonas*.

Expt 3 indicated that the negative effect of *Gonyostomum* on *Rhodomonas* took place primarily when cell-cell contact occurred, whereas positive growth occurred in the cell-free filtrate treatment. Furthermore, in the Induced treatment of the lysis experiment (Expt 4), deformed swollen *Rhodomonas* cells that were most likely a first stage of lysis were present. These cells also occurred in Expts 1 and 3, but were not quantified since counts were made by flow cytometry. No such lysis effect was seen in the control or in the treatment with *Gonyostomum* that had been raised in unialgal culture. A small percentage of pre-lysed cells was found in the filtrate treatment on the final sampling occasion. However, the number of lysing cells was low and they were observed only once; we consider this insufficient evidence to suggest that an allelochemical is produced. Instead, our data indicate that *Gonyostomum* lyses *Rhodomonas* by cell contact, most likely through the trichocyst firing, either mechanically or through a toxin connected with the trichocyst expulsion. Furthermore, results from both Expts 1 and 4 show and that the lysis effect on *Rhodomonas* is induced when the species are grown together. Microscope observations (not quantified) suggest that induced *Gonyostomum* cells have a higher number of trichocysts than *Gonyostomum* grown unialgally.

Trichocyst extrusion has been proposed to be a protective mechanism that acts mechanically as a grazer deterrent in a marine raphidophyte (Tillmann & Reckermann 2002). As trichocyst extrusion is known to occur upon stimulation by physical contact, it may occur upon contact with another alga. Uchida et al. (1995, 1999) also observed cell lysis after cell contact. The growth of the dinoflagellates *Gyrodinium instriatum* and *Gymnodinium mikimotoi* is strongly suppressed when they are cultured together with the dinoflagellate *Heterocapsa* sp. (a result of cell immobilization and subsequent lysis). Uchida et al. (1999) proposed that inhibition of a competitor after cell contact would be energetically more efficient than inhibition through allelopathy, as the latter is dependent on a constant secretion of a secondary metabolite into the ambient water.

The production of a secondary metabolite such as a toxin has not been documented in *Gonyostomum*.

However, several closely related marine raphidophytes such as *Chattonella* spp., *Heterosigma akashiwo* and *Fibrocapsa japonica* (Figueroa & Rengefors 2006) have been responsible for mass mortalities of fish in many areas of the world (Clough & Strom 2005, Handy et al. 2005). The toxins produced by the marine raphidophytes appear to be neurotoxins similar to the potent brevetoxin (Tillmann & Reckermann 2002). In addition, marine raphidophytes produce toxic reactive oxygen species such as superoxide, hydrogen peroxide and hydroxyl radicals (Kuroda et al. 2005). Raphidophyte toxins are known to affect herbivorous predators negatively (Clough & Strom 2005), and the toxin produced by *Heterosigma akashiwo* significantly inhibits the diatom *Skeletonema costatum* (Pratt 1966). Recently, it was shown that *H. akashiwo* filtrate had an inhibitory effect on several other marine phytoplankton species (Yamasaki et al. 2007). For *Gonyostomum*, however, we have no strong evidence of an allelochemical effect, but rather of a cell-contact mediated effect.

In Expt 4, in contrast to Expt 3, we found no decrease in total *Rhodomonas* cell number. This finding may have 2 causes. First, *Gonyostomum* was pre-grown with *Rhodomonas* for only 9 d, in contrast to 40 d in the disappearance experiment. Presumably, it takes time to induce and build trichocysts. Secondly, a different, perhaps less potent strain was used for the lysis experiment. Surprisingly, we also found a slight stimulatory effect on the total number of *Rhodomonas* intact cells in the *Gonyostomum* single treatment in the lysis experiment. Intact cell numbers were significantly higher than the other treatments after 48 h. Apparently, *Gonyostomum* has some stimulatory effect before its trichocysts are induced.

To summarize, growth of *Gonyostomum* was stimulated when cells were cultured with another alga and when cells were raised in media enriched with FA. In both cases, growth stimulation may have been the result of either osmotrophically obtained carbon, or by acquisition of a growth factor originating from the FA or released upon cell lysis of *Rhodomonas*. When cultured together with *Gonyostomum*, *Rhodomonas* was severely inhibited, but not grazed upon. We suggest that the mechanism of inhibition is cell lysis, either by mechanical damage due to trichocyst expulsion, or trichocysts combined with a toxin. Moreover, we showed that this cell-contact mediated inhibition was induced by pre-growing *Gonyostomum* with its competitor. We propose that lysis of competitors may be a strategy by which *Gonyostomum* remains dominant in the phytoplankton community. We also argue that utilization of humic substances may contribute to the ability of *Gonyostomum* to become dominant and maintain blooms.

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