

# Phytoplankton growth inhibited by the toxic and bacterivorous ciliate *Uronema marinum* (Protozoa, Ciliophora)

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**ABSTRACT:** The ubiquitous marine ciliate *Uronema marinum* is mainly bacterivorous. It was therefore surprising that in a ciliate-contaminated experiment the growth rate of the phytoplankton species *Emiliana huxleyi* was significantly reduced. As *U. marinum* does not ingest *E. huxleyi* cells, their growth inhibition was probably caused by a toxin secreted by the ciliate, presumably a novel type of chemical interaction between ciliates and phytoplankton. A possible function of toxin secretion is to lyse algal cells that are too large for *U. marinum* to ingest, to increase dissolved organic matter (DOM) concentrations and hence the growth of heterotrophic bacteria, the main food source of *U. marinum*. To test this hypothesis *U. marinum* or the filtrate of *U. marinum* cultures was added to cultures of phytoplankton with different cell sizes. The presence of *U. marinum* or the filtrate of *U. marinum* cultures showed an inhibiting growth effect and a negative effect on the physiology of all species tested although both effects were variable between species. Diatoms appeared less sensitive than non-diatom species. *U. marinum* acclimatization to phytoplankton led to stronger inhibiting effects, presumably from increased toxin production. Bacterial DGGE analysis of *U. marinum* cultures did not reveal known toxic bacteria that might account for the observed negative effects on the phytoplankton. Bacterial growth rates in an *E. huxleyi* culture increased when *U. marinum* filtrate had been added. In mixed cultures of bacteria, *E. huxleyi* and *U. marinum*, bacterial abundance first increased, then decreased due to ciliate predation. These findings support the hypothesis that toxin secretion by *U. marinum* increases non-prey phytoplankton-derived DOM and stimulates the growth of the bacterial prey.

**KEY WORDS:** *Uronema marinum* · Toxin · Phytoplankton · Bacteria · Growth rate · Physiology · Flow cytometry · FDA · Epifluorescence microscopy

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## INTRODUCTION

Ciliates play an important role in aquatic ecosystems. They are usually heterotrophs that feed on bacteria, algae or other planktonic species (Lynn & Small 1990). As grazers, they are an important part of the decomposition food chain and a link in carbon and nutrient cycling (Lynn & Small 1990). Some ciliate species are very specific in their diet, preying only on bacteria or algae, just on a few bacterial species or on bacteria with a certain shape (Capriulo 1990). Hence,

they are important regulators of aquatic algal and bacterial biomass and species composition (Strom & Morello 1998).

One of the marine ciliates, *Uronema marinum* Du Jardin (Oligohymenophorea, Scuticociliatia), is a globally distributed species that can be found in Antarctic, tropical as well as temperate waters (Coppellotti 1990, Warren & Scott 2010). Marine ciliates can grow and reproduce in a wide range of temperatures (6 to 30°C) and salinities (10 to 43 g kg<sup>-1</sup>) (Hamilton & Preslan 1969, Jee et al. 2001). Some *Uronema* spp. can

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even grow (albeit slowly) in water with a salinity  $<10 \text{ g kg}^{-1}$  (Hamilton & Preslan 1969). *Uronema* spp. can be found residing in sediments and are abundant in coastal waters (Anderson et al. 2009). They are free-living ciliates and can be fast swimmers (Pan et al. 2010). Variations in *U. marinum* growth under similar culture conditions can be explained as genetic variability within species as a result of habitat or geographical isolation (Pérez-Uz 1995). The highest growth rates of *U. marinum* ( $0.25 \pm 0.03 \text{ h}^{-1}$ ) were found in isolates from an estuarine environment (Pérez-Uz 1995).

*Uronema marinum* is mainly bacterivorous and can easily be cultured on bacteria. Bacterial genera known to be utilized by *U. marinum* are *Chromobacterium*, *Serratia*, *Vibrio*, *Pseudomonas* and *Micrococcus* (Plunket & Hidu 1978). *U. marinum* can feed on small phytoplankton as well (Rubin & Lee 1976). Strom & Morello (1998) cultured *U. marinum* on relatively small phytoplankton species such as *Isochrysis galbana*, *Pyrenomonas salina*, *Mantoniella squamata* and the cyanobacterium *Synechococcus* sp. (Strom & Morello 1998). Presumably, the buccal cavity of *U. marinum* is unfit to feed on relatively large phytoplankton. A presumed parasitic variant of *U. marinum* is also histophagous, feeding on fish tissue (Jee et al. 2001, Ahn et al. 2007). Ciliates are eaten by larger zooplankton such as copepods, juvenile fish and foraminiferans (Capriulo et al. 1986).

Recently, the presence of *Uronema marinum* in a mesocosm experiment led to the decline of *Emiliania huxleyi* cell numbers. This decline was not caused by grazing of *U. marinum*, but apparently by a toxin secreted by the ciliate (Peperzak et al. unpubl.). So far, toxin secretion by *U. marinum* and the inhibition of growth of other unpreyed, non-competitive planktonic species have not been studied and can possibly play an important role in the life cycle of the ciliate and its place in the microbial ecosystem.

Ciliates in general, including *Uronema marinum*, are known to secrete various substances. Most ciliates have extrusomes (Pan et al. 2010), which are membrane-bound structures with contents that are discharged outside the cell under certain conditions. Some extrusomes have acid phosphatase activity that aid in the external predigestion of food (Lynn & Small 2000). Toxicysts, which are another type of extrusomes, contain proteolytic or paralytic toxins that are used by predatory ciliates to capture and ingest prey (Lynn & Small 2000). A chemical released by protists that inhibits the growth of co-occurring species that are not direct prey or predator is only known when it concerns competitors for, e.g., food sources. Chemi-

cals exchanged via cell-to-cell contact, by ciliates of the genus *Euplotes* for instance, inhibit the growth of competitive ciliates (Guella et al. 2010). The freshwater ciliate *Spirostomonum ambiguum* uses toxins as a chemical defence, but it is suggested that the chemicals are also used to limit the presence of competitors (Buonanno et al. 2012).

A possible function of the presumed *Uronema marinum* toxin is to kill algal or perhaps other planktonic cells in order to release dissolved organic matter (DOM) in the water, which can then serve as a substrate for heterotrophic bacteria. More DOM means more bacteria and because *U. marinum* is assumed to be mainly bacterivorous, an increase in bacterial abundance would be beneficial for the ciliate. A diagram of this hypothetical cycle is presented in Fig. 1.

In the present study, several experiments have been performed to investigate the interaction between bacteria, phytoplankton and *Uronema marinum*. To facilitate rapid cell counting, methods for ciliate enumeration by flow cytometry were investigated first. As turbulence can have an effect on protozoan grazing (Shimeta et al. 1995) the effect of turbulence on the growth of *U. marinum* was examined. Next, the effect of the presumed toxin secreted by *U. marinum* on various small and large algae, including potentially edible species, was examined. Furthermore, the effect of *U. marinum* on bacterial composition and bacterial growth was investigated. In a final experiment the growth dynamics of bacteria and phytoplankton were examined in the presence of either *U. marinum* cells or the presumed *U. marinum* toxin.

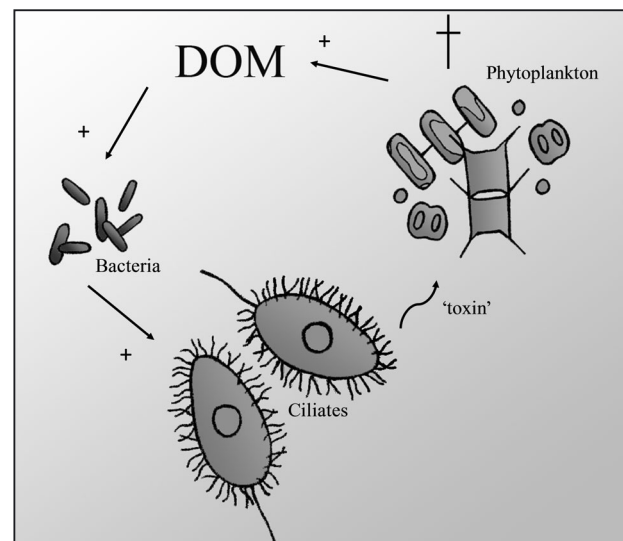


Fig. 1. Hypothesis for the role of 'toxin' secretion by the bacterivorous ciliate *Uronema marinum*. Phytoplankton cells that are too large to ingest are killed, release dissolved organic matter (DOM) and stimulate bacterial growth

## MATERIALS AND METHODS

### Cultures

*Uronema marinum* and co-occurring bacteria were cultured on autoclaved C3+ medium made of sea water,  $\text{KH}_2\text{PO}_4$  (3  $\mu\text{M}$ ),  $\text{NH}_4\text{Cl}$  (30  $\mu\text{M}$ ) and Na-pyruvate (1.5 M). In some experiments, *U. marinum* was grown in cultures of phytoplankton species of various sizes (Table 1), obtained from the NIOZ culture collection, in f/2 medium (Guillard & Ryther 1962) with trace metals added in accordance with Keller et al. (1987). For the diatoms, silicate (150  $\mu\text{M}$ ) was added to the f/2 medium. The salinity of all cultures, ciliate as well as phytoplankton, was between 35 and 36.5  $\text{g kg}^{-1}$ . Cultures were incubated at 15°C in a 16:8 h light:dark cycle (60  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ). In a preliminary experiment it was found that the filtrate of a *U. marinum* culture in exponential growth phase did have a negative effect on *Emiliana huxleyi* growth

rate, while the filtrate of a culture in stationary growth phase did not. Therefore, in all experiments *U. marinum* and phytoplankton cultures were in exponential growth phase. To ensure exponential growth, *U. marinum* and phytoplankton were inoculated in fresh culture medium 3 to 4 and 2 to 3 d, respectively, prior to the experiments. Because *Micromonas pusilla* and *Chaetoceros calcitrans* were fast growing, they were inoculated in fresh f/2 medium 1 d prior to the experiments. Filtrates of *U. marinum* and phytoplankton cultures were obtained with 0.2  $\mu\text{m}$  Acrodisc™ filters (Pall). Controls were also filtered using Acrodisc filters to ensure that treatments were the same.

### Photosynthetic efficiency

Quantum efficiency of photosystem II ( $F_v/F_m$ ) was used as a proxy for phytoplankton physiology and was measured by pulse amplitude modulation (PAM) fluorimetry using a WATER-ED PAM instrument (Waltz). Samples were dark adapted for 15 min prior to the measurement.

### Cell enumeration

Phytoplankton and bacteria were enumerated with a FACSCanto II™ flow cytometer (Becton Dickinson) equipped with a 20 mW 488 nm (Blue) solid state laser providing 9 × 65  $\mu\text{m}$  elliptical spots in a 180 × 430  $\mu\text{m}$  quartz flow cell. The flow cytometer was calibrated daily with CST™ (scatter and fluorescence intensities) and TruCount™ (flow rate) beads (Becton Dickinson). Flow cytometer and bi-plot axes in which cell clusters were counted are listed in Table 2. Bacteria were enumerated with the same flow cytometer 15 min after adding to 400  $\mu\text{l}$  of TE-buffer: 10  $\mu\text{l}$  TX-100 (40× diluted), 100  $\mu\text{l}$  sample and 10  $\mu\text{l}$  PicoGreen™ (Invitrogen, 500× diluted) (Veldhuis et al. 1997). Phytoplankton was measured using chlorophyll autofluorescence as trigger (Table 2). *Uronema marinum* cells were initially counted using a 1 ml Sedgewick Rafter chamber

Table 1. Cyanobacteria and phytoplankton species used in experiments

Class	Species	Size ( $\mu\text{m}$ )	Strain
Cyanophyceae	<i>Synechococcus</i>	1	543
Prasinophyceae	<i>Micromonas pusilla</i>	2	Mp1545
Prymnesiophyceae	<i>Phaeocystis globosa</i>	4–6	Pg22
Prymnesiophyceae	<i>Emiliana huxleyi</i>	~5	1516
Bacillariophyceae	<i>Chaetoceros calcitrans</i>	6–8	CCMP 1315
Bacillariophyceae	<i>Thalassiosira rotula</i>	8–55 <sup>a</sup>	RCC766

<sup>a</sup>Diatoms reproduce by binary fission, where each daughter cell receives one parent cell theca as epitheca (outer layer of the cell wall). This leads to a size reduction of the offspring, where variation can be 8 to 10 times the diameter, dependent on the species (Carmelo 1996)

Table 2. Flow cytometer settings. Non-fluorescent *Uronema marinum* and bacterial cells were stained with fluorescein diacetate (FDA) or PicoGreen, respectively. Auto-fluorescent phytoplankton species were not stained. Listed are triggers and thresholds, and bi-plot axes in which the cells were counted. FSC = forward scatter, SSC = side scatter, FBG = green fluorescence emission measured at blue excitation (488 → 530 nm), FBR1 = red auto-fluorescence emission measured at blue excitation (488 → 660 nm), FBR2 = far red auto-fluorescence emission measured at blue excitation (488 → >780 nm) and FBO = orange fluorescence emission measured at blue excitation (405 → 510 nm)

Species	Stain	Trigger & Threshold	Bi-plot axes
<i>Uronema marinum</i>	None	FSC 5000	SSC × FBG
<i>Uronema marinum</i>	FDA	FBG 200	FBG × FBR2
<i>Micromonas pusilla</i>	None	FBR1 5000	SSC × FBR1
<i>Phaeocystis globosa</i>	None	FBR1 5000	FBR1 × FBO
<i>Chaetoceros calcitrans</i>	None	FBR1 1000	SSC × FBR1
<i>Thalassiosira rotula</i>	None	FBR1 500	FSC × FBR1
<i>Emiliana huxleyi</i>	None	FBR1 2000	SSC × FBR1
Bacteria	PicoGreen	FBG 1000	SSC × FBG

(Graticules) under a Zeiss Axioplan 2 microscope. A number of techniques for counting *U. marinum* were used. A 2 ml live sample for flow cytometry was incubated for 1 h with 10  $\mu$ l of fluorescein diacetate (FDA, Invitrogen, 20 mM). FDA is converted in live cells into green fluorescent fluorescein after the cleavage of acetates by intracellular esterases (Peperzak & Brussaard 2011). For microscope counts, samples were live or fixed with Lugol's iodine (10  $\mu$ l + 2 ml sample). A 1 ml sample was counted live to distinguish between live (moving) and presumed dead (non-moving) cells for a comparison with flow cytometry counts.

### Effect of turbulence on *Uronema marinum* growth

Growth curves were made to establish the effect of turbulence on *Uronema marinum* growth. Samples from this experiment were used to test different ciliate counting techniques. Four cultures were made by inoculating 1.5 ml of a *U. marinum* culture in 4 flasks with 150 ml C3+ medium. Duplicate cultures were shaken constantly at 120 rpm on an orbital shaker (Labotech HS500), duplicate controls were not shaken. Instead, they were swirled 3 times daily before sampling.

### Predation

*Uronema marinum* was added to the cultures listed in Table 1 to investigate if the phytoplankton species were preyed upon. Fluorescently labelled bacteria (FLB) (Sherr & Sherr 1993) and *Synechococcus*, which is known to be preyed upon by *U. marinum* (Christaki et al. 1999), were used as a control. Slides for epifluorescence microscopy (Zeiss Axioscope 2) were made at regular intervals by filtering 5 to 10 ml of sample on Sudan-Black stained 0.2  $\mu$ m polycarbonate Poretics™ filters. The filters were stained for 24 h in a solution of 8 mg Sudan-Black dissolved in 67 ml of ethanol and diluted with 676 ml of Milli-Q (Millipore™).

### *Uronema marinum* effect on phytoplankton

To investigate the effect of the presumed *Uronema marinum* toxin on phytoplankton growth and physiology, *U. marinum* culture or culture filtrate was added to the species listed in Table 1 (except *Synechococcus* sp.). C3+ medium (control), *U. marinum* in

C3+ culture or culture filtrate was added in equal volumes to phytoplankton cultures. Phytoplankton cell numbers and physiology were measured daily with flow cytometry and PAM, respectively.

In a second experiment, *Uronema marinum* was not grown in C3+ but pre-cultured with the same phytoplankton species to which its filtrate was later added (Table 1, except *Synechococcus* sp.). Sterile sea water (control) or *U. marinum* filtrates were added 1:1 to each phytoplankton culture. Extra nutrients were added at f/2 concentrations to prevent nutrient limitation. Phytoplankton cell numbers and physiology were measured daily.

### Bacterial species composition

Bacteria in the *Uronema marinum* cultures served as a food source for the ciliate, but certain species are known to be toxic to phytoplankton and as such can be mistaken for *U. marinum* toxins. To investigate which bacterial and possibly toxic species were present in the *U. marinum* cultures, a DGGE analysis was performed in 6 *U. marinum* C3+ cultures and 4 bacterial cultures which were isolated from a *U. marinum* culture by dilution series. The oldest *U. marinum* culture was 3 mo old, the youngest was re-inoculated a week before the analysis. The oldest cultures had been re-inoculated 2 or 3 times in C3+ medium. The isolated bacteria were cultured on C3+ and on C6 (15 mM glucose instead of Na-pyruvate). DNA extraction was performed on a 20 ml sample, concentrated on a 0.2  $\mu$ m polycarbonate filter, with the MOBIO Powersoil™ DNA isolation kit in accordance with the manufacturer's 'maximum yield' protocol. The 16S rRNA encoding gene fragment of bacteria was amplified using PCR with primers 341F (forward + GC-clamp), 907RA (reverse) and 907RC (reverse) in 20 (1 min, 94°C; 1 min, 65°C; 3 min, 72°C) and 15 (1 min, 94°C; 1 min, 55°C; 3 min, 72°C) cycles. DGGE was performed in accordance with Schäfer & Muyzer (2001). A DGGE band extract (2  $\mu$ l) was used as template in 50  $\mu$ l PCR reactions using forward primer 341F and reverse primers 907RA and 907RC in 20 (1 min, 94°C; 1 min, 55°C; 3 min, 72°C) and 11 (1 min, 94°C; 1 min, 55°C; 3 min, 72°C) cycles. Sequencing was performed by MacroGen Europe.

### Bacterial growth in lysed phytoplankton culture

In one of the previous experiments the difference in bacterial cell abundance between *Uronema mar-*

*inum* cultured in C3+ and in an *Emiliana huxleyi* culture was a factor of 10. Therefore, bacterial growth was measured in a lysed phytoplankton culture with C3+ as a control. An *E. huxleyi* culture was heated to 60°C for 20 min to destroy algal cells, then filtered. The filtrate was diluted 0×, 2.5× and 6.75× with sterile sea water and used as growth medium. To 10 ml of these different dilutions and to 10 ml C3+ (control), 1 ml *U. marinum* culture was added that had been concentrated 3× by centrifugation (2000 × g, 20 min). *U. marinum* and bacteria were counted daily by microscopy and flow cytometry, respectively. The inorganic nutrients were analysed at the beginning and the end of the experiment, according to Peperzak et al. (2011), but were not limiting.

### Bacterial growth in phytoplankton-*Uronema* culture

To investigate the hypothesis that phytoplankton lysis by *Uronema marinum* toxins enhances bacterial growth, either *U. marinum* or *U. marinum* filtrate was added to a phytoplankton culture. Sterile sea water (control), *U. marinum* cultured on *Emiliana huxleyi* and the filtrate of the latter culture were added in equal volumes to *E. huxleyi* cultures. Nutrients were added at f/2 concentrations to prevent *E. huxleyi* nutrient limitation. *U. marinum*, phytoplankton and bacteria were counted daily by flow cytometry.

Table 3. Summary of the effect of *Uronema marinum* and its filtrate on phytoplankton growth rate and physiology. Two experiments were done, one in which *U. marinum* was pre-cultured in C3+ (I) and one in which *U. marinum* was pre-cultured in phytoplankton cultures (II). I: +*Uronema* = *U. marinum* cells grown in C3+ were added. I: +Filtrate = the filtrate of *U. marinum* grown in C3+ culture was added. II: +Filtrate = the filtrate of *U. marinum* grown in phytoplankton cultures was added. Differences in cell abundance on Day 7 between the treatment and the control are ↓ ≤ 10×, ↓↓ ≤ 100×, ↓↓↓ ≤ 1000×, ↓↓↓↓ > 1000×, differences in photosynthetic efficiency ( $F_v/F_m$ ) are ↓ ≤ 0.1, ↓↓ ≤ 0.2, ↓↓↓ ≤ 0.5, ↓↓↓↓ > 0.5. Statistical difference in growth over 7 d between the treatment and the control: \*p < 0.10, \*\*p < 0.05, \*\*\*p < 0.001. See Table 2 for full species names

Treatment	Grazed <i>M. pusilla</i>	Not grazed			
		<i>P. globosa</i>	<i>C. calcitrans</i>	<i>T. rotula</i>	<i>E. huxleyi</i>
<b>Cell abundance</b>					
I + <i>Uronema</i>	↓↓↓↓***	–	↓**	↓*	↓***
I + Filtrate	–	–	↓	↓**	↓
II + Filtrate	↓↓***	↓↓↓↓*	↓	↓**	↓↓*
<b><math>F_v/F_m</math></b>					
I + <i>Uronema</i>	↓↓↓↓***	↓	↓↓	–	↓↓↓↓***
I + Filtrate	↓	↓	↓	–	↓
II + Filtrate	↓↓↓**	↓↓↓↓***	↑***	↓**	↓↓↓***

### Statistics

Linear regression was performed in Excel™ version 2007. Repeated measures ANOVA and Tukey's post hoc test (Statistica™ 10) was used to test the null hypothesis of no effect between different treatments in growth rate and in the temporal development of cell abundances and PSII efficiencies. The null hypothesis was rejected, i.e. a significant difference between treatments was obtained at p < 0.05. In the results of the phytoplankton growth experiments a statistical difference at p < 0.10 was also shown to reveal trends (Table 3).

## RESULTS

### *Uronema marinum* enumeration

Microscopy and flow cytometry were compared for the reliable enumeration of live *Uronema marinum* cells. Linear regression showed that the abundance of unfixed, moving and non-moving ciliate cells counted by microscopy was a factor 2 higher than counts in Lugol-fixed samples (unfixed cell numbers = 2.15 × Lugol cell numbers + 184,  $r^2 = 0.87$ ). Apparently, swimming cells under a microscope were counted more than once. The abundance of live *U. marinum*, i.e. cells that were stained with FDA, enumerated by flow cytometry was slightly lower than the abundance in Lugol-fixed samples enumerated by microscopy, which was also confirmed by the comparison (FDA cell numbers = 0.89 × Lugol cell numbers + 65,  $r^2 = 0.87$ ). It was therefore assumed that FDA-stained cell concentrations obtained from flow cytometer counts are an accurate representation of the abundance of live *U. marinum* cells.

### Effect of turbulence on *Uronema marinum*

Ciliate growth rate in exponential growth phase (first 3 d) was not significantly different between cultures with and without turbulence (Fig. 2). However, in both cultures with turbulence cell numbers declined after 7 d, while the cell numbers in non-turbulent cultures remained stable. This was also found in previous research on other cil-

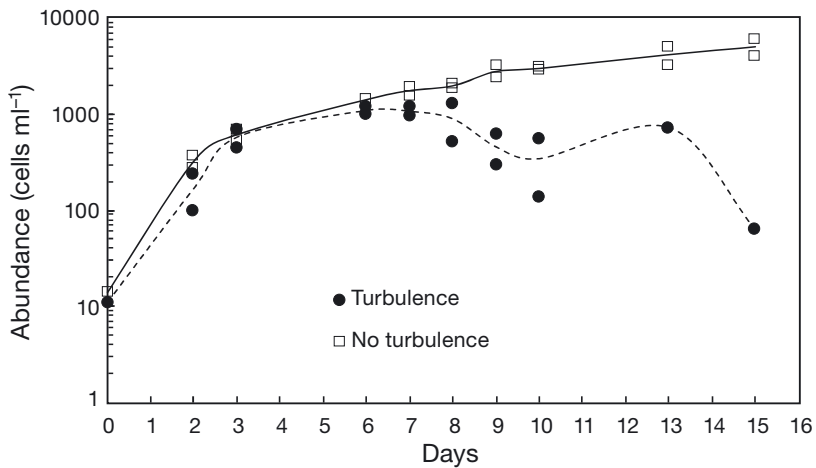


Fig. 2. Comparison of *Uronema marinum* growth in turbulent and non-turbulent conditions. *U. marinum* abundance was measured by counting ciliates unstained and unfixed using microscopy. Ciliates numbers measured by microscopy after Lugol fixation or by flow cytometry after addition of fluorescein diacetate (FDA) yielded similar growth curves

iate species, possibly caused by a reduced ingestion due a change in swimming speed or pattern (Dolan et al. 2003). In all following experiments *U. marinum* was cultured without continuous turbulence.

### Predation

Observations using epifluorescence microscopy demonstrated that *Synechococcus* and *Micromonas pusilla* (Fig. 3A,B) and FLB (result not shown) were ingested by *Uronema marinum*, while *Phaeocystis globosa*, *Thalassiosira rotula* (Fig. 3C,D), *Chaetoceros calcitrans* and *Emiliana huxleyi* (results not shown) were not.

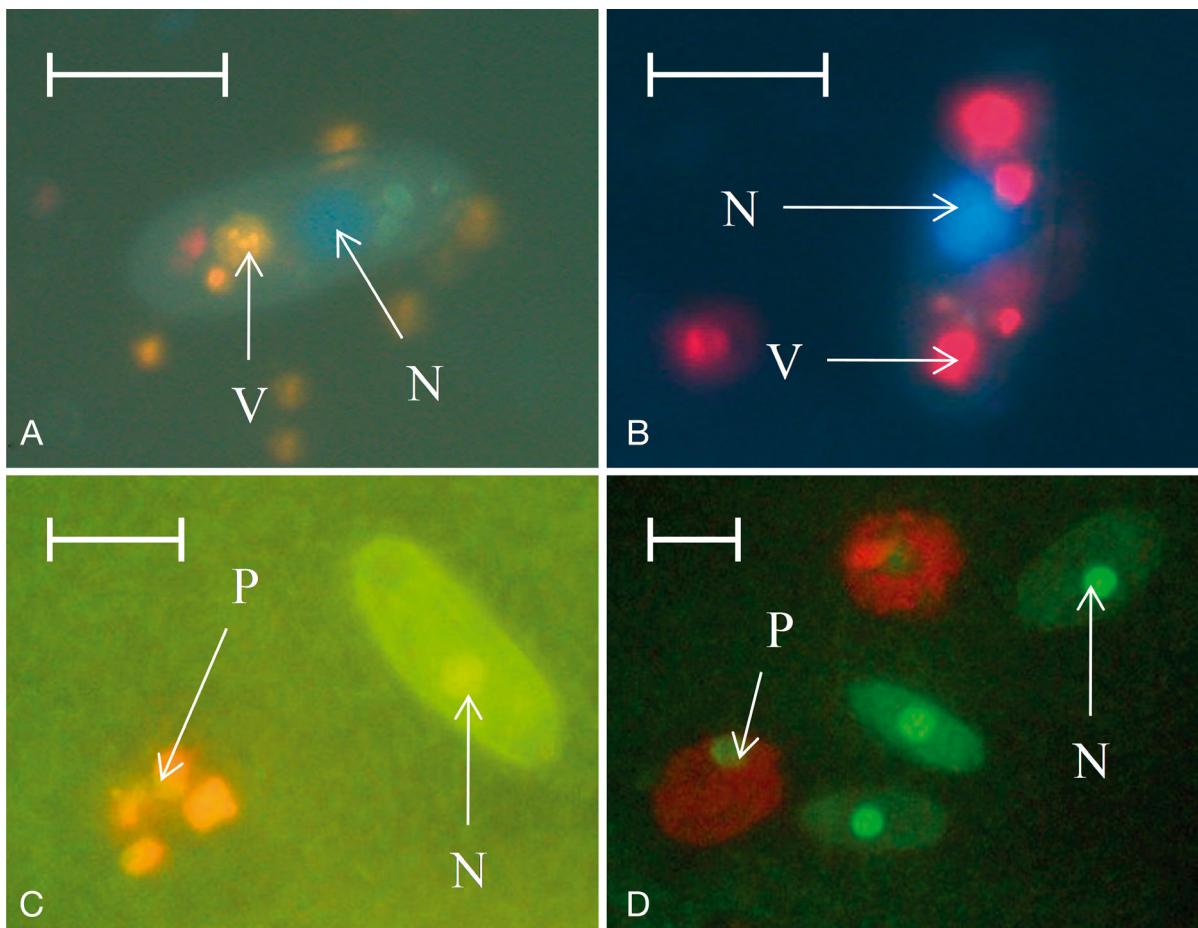


Fig. 3. Images showing ingestion by epifluorescence. (A–D) Examples of *Uronema marinum* added to different auto-fluorescent plankton species: (A) *Synechococcus*, (B) *Micromonas pusilla*, (C) *Phaeocystis globosa*, (D) *Thalassiosira rotula*. N = *Uronema marinum* cell nucleus stained with DAPI; V = vacuole in *U. marinum* cell with ingested fluorescent cell particles in A & B. C & D were not ingested by *U. marinum*. P = phytoplankton cells. Scale bars represent 10  $\mu\text{m}$

### Effect of *Uronema marinum* on phytoplankton

Growth of all phytoplankton species tested was negatively affected by the addition of *Uronema marinum* or *U. marinum* filtrate at least once in both phytoplankton growth experiments (Figs. 4 & 5, Table 3). This also holds for PSII efficiency, with just one exception (*C. calcitrans* pre-cultured with phytoplankton, Table 3). The effects on different species are however variable within and between experiments. In the filtrate experiments where *U. marinum* had been pre-cultured in the presence of phytoplankton, the negative effects were stronger than when the ciliate had been pre-cultured in C3+ (Table 3). As expected, the decline in *M. pusilla* abundance was higher in the experiment with *U. marinum* cells, which is due to predation. In most species that were not grazed cell abundance or PSII efficiency declined more in the presence of *U. marinum* compared to its filtrate, probably due to the secretion of the presumed toxin by living ciliates. Interestingly, the diatoms appeared less sensitive than the prasinophyte and the prymnesiophytes. The 'flagellates' showed a decline in growth (up to 1000×) or PSII efficiency (>0.5) in both *U. marinum* filtrates, while the effects of filtrates on diatoms were much less severe (Table 3). For instance, the flagellate decline in PSII efficiency after 1 to 2 d was dramatic (Fig. 5B,D,J).

### Bacterial species composition

DDGE analysis showed that bacteria present in all *Uronema marinum* cultures belonged to 3 genera: *Fluviicola* sp. (Cryomorphaceae), *Oceanobacter* sp. (Oceanospirillaceae) and *Nisaea* sp. (Rhodospirillaceae). There were no differences in species composition between young and old cultures, or between C3+ and C6 media.

### Bacterial growth in lysed phytoplankton culture

C3+ culture medium contains only 1 carbon source for bacterial growth. It was, therefore, not surprising that the growth rate of *Uronema marinum* was higher in an undiluted medium of artificially lysed algal cells (Table 4). Even when this medium was diluted, the bacterial and *U. marinum* cell numbers after 3 d were higher compared to C3+ (Fig. 6). Bacterial growth rates in the diluted media of lysed algal cells appeared relatively low compared to C3+ medium due to grazing by *U. marinum* (Table 4).

### Bacterial growth in phytoplankton-*Uronema* culture

The abundance of bacteria in untreated *Emiliana huxleyi* cultures was relatively low (Fig. 7A). The addition of *Uronema marinum* led to a decrease in *E. huxleyi* cells, most probably by lysis due to the ciliate's toxin (Fig. 7B). Phytoplankton lysis provided a substrate for the bacteria and bacterial growth rate increased (Table 5). In addition, bacterial abundance declined again on Day 3 due to the increased predation by *U. marinum*. The filtrate of *U. marinum* also caused a decline of *E. huxleyi* abundance and a concomitant increase in bacterial growth rate and abundance (Fig. 7C). The photosynthetic efficiency decreased directly in the presence of *U. marinum* and after 3 d in the presence of *U. marinum* filtrate. In the control the photosynthetic efficiency remained stable (results not shown).

## DISCUSSION

### Effect of *Uronema marinum* on phytoplankton and bacterial growth

*Uronema marinum* grazes on bacteria, cyanobacteria and small phytoplankton species (<4 µm). Apart from *Micromonas pusilla*, the algal species used in this study were not grazed by *U. marinum*. Larger phytoplankton species are probably too large for the ciliate's buccal cavity. Strom & Morello (1998) found that *U. marinum* growth was also supported by the cryptophyte *Pyrenomonas salina*, which has a length of approximately 10 µm. However, this does not mean that *P. salina* was preyed upon by *U. marinum* as experiments in the present study showed that *U. marinum* growth can be enhanced by the presence of unpreyed phytoplankton. The decrease in the *P. salina* cell numbers in Strom & Morello (1998) could be caused by the *Uronema*-toxin.

Algal abundances and photosynthetic efficiencies decreased in the presence of *Uronema marinum* cells and after the addition of the filtrate of an *U. marinum* culture. It is likely that the filterable compound, the presumed toxin, caused lysis of the algal cells. Algal lysis was not the result of toxic bacteria because the species present in the *U. marinum* cultures are not known to be algicidal. Apparently there is no effect of the toxin on bacterial species, which could be due to fundamental differences in cell wall composition between eukaryotes and prokaryotes. Differences in cell wall composition between 'flagellates' and dia-

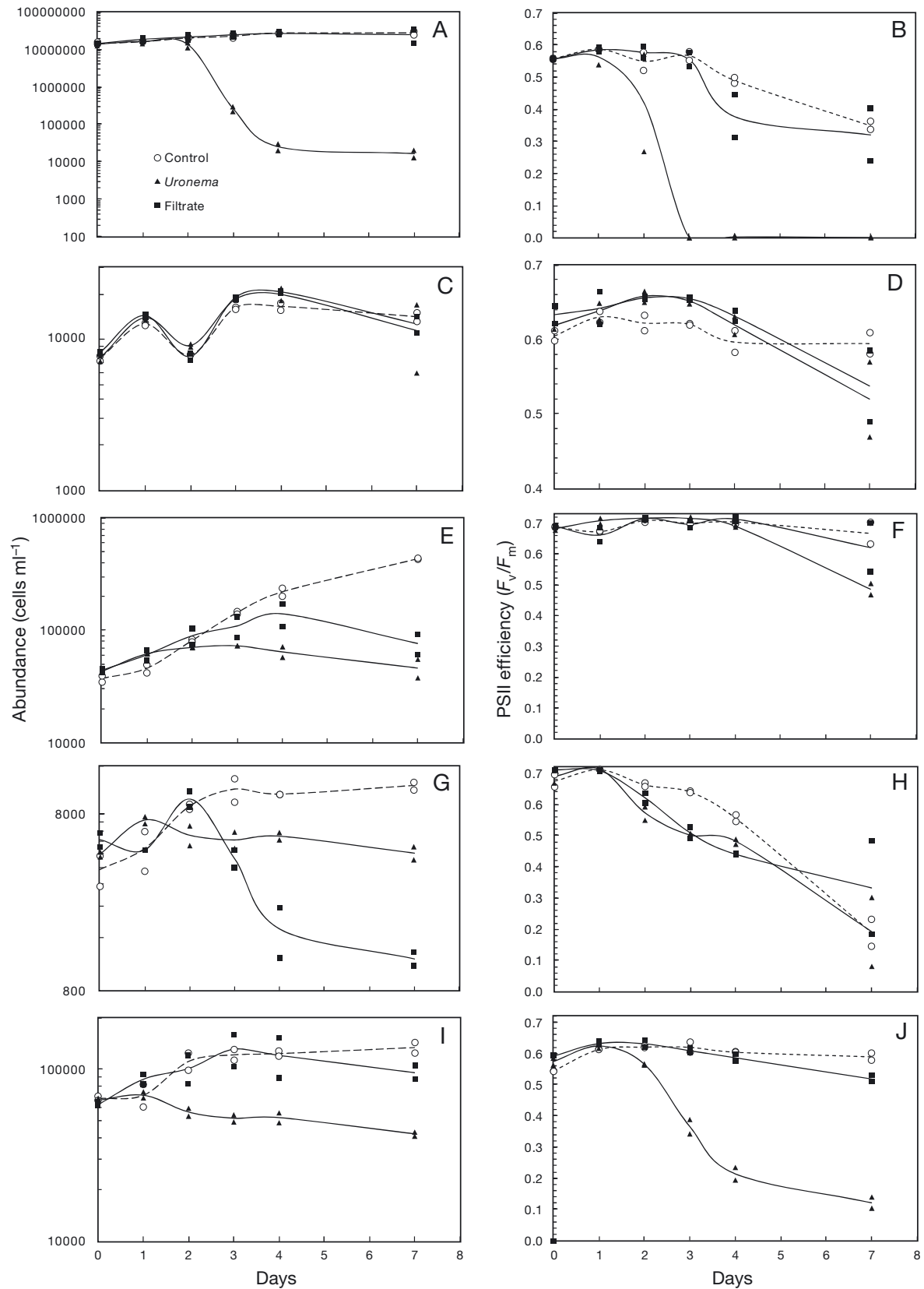


Fig. 4. Effect of *Uronema marinum* and its filtrate on phytoplankton growth and physiology. C3+ medium, the medium used to grow *U. marinum* (Control), *U. marinum* cultured in C3+ (*Uronema*) or the filtrate of *U. marinum* cultured in C3+ (Filtrate) was added to phytoplankton cultures. (A,C,E,G,I) Abundance and (B,D,F,H,J) PSII efficiency were measured. (A,B) *Micromonas pusilla*; (C,D) *Phaeocystis globosa*; (E,F) *Chaetoceros calcitrans*; (G,H) *Thalassiosira rotula*; (I,J) *Emiliana huxleyi*



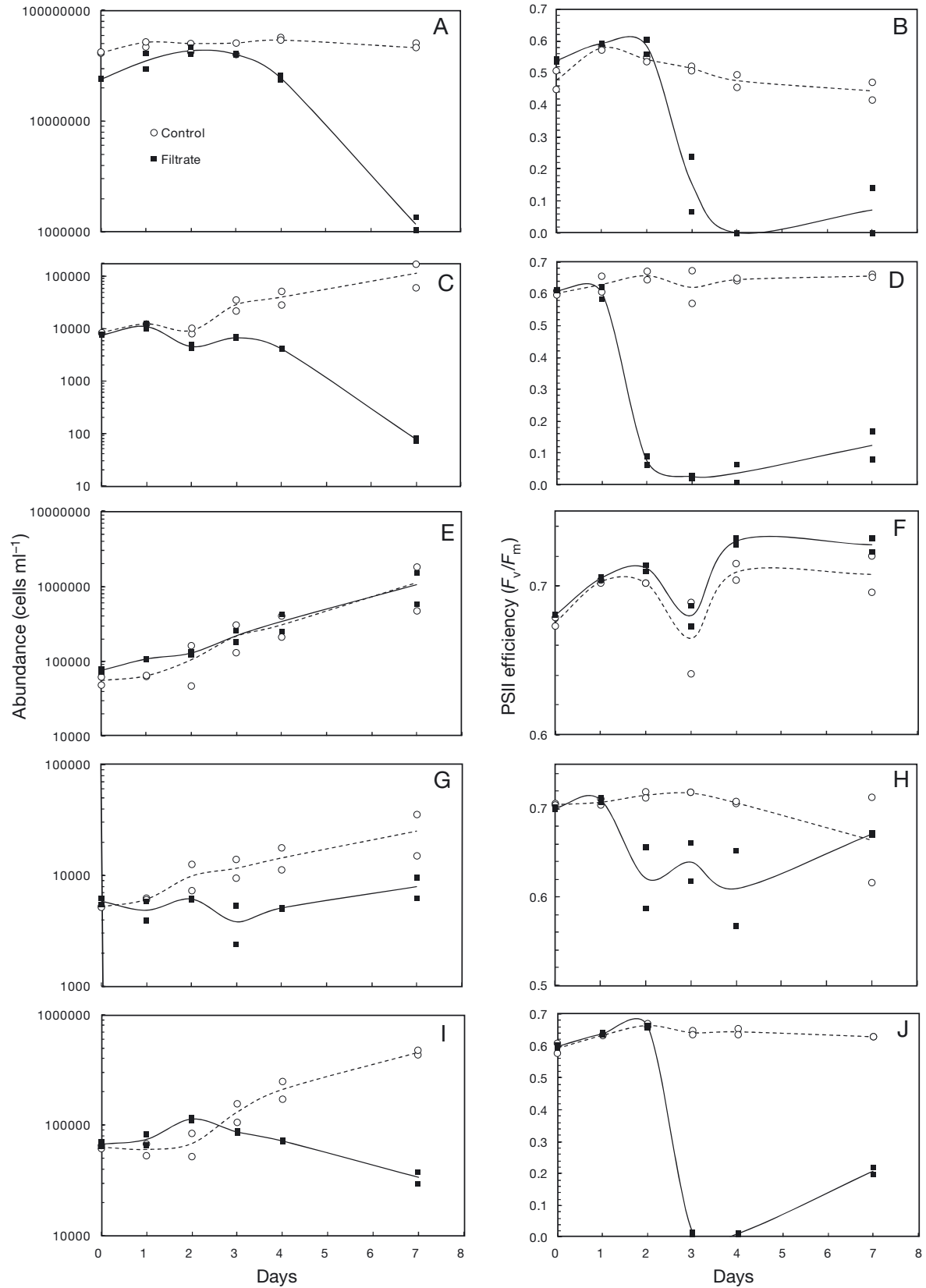


Fig. 5. Effect of *Uronema marinum* and its filtrate on phytoplankton growth rate and physiology. Sterile sea water (Control) and the filtrate of *Uronema marinum* cultured in the presence of phytoplankton (Filtrate) was added to phytoplankton cultures. (A,C,E,G,I) Abundance and (B,D,F,H,J) PSII efficiency were measured. (A,B) *Micromonas pusilla*; (C,D) *Phaeocystis globosa*; (E,F) *Chaetoceros calcitrans*; (G,H) *Thalassiosira rotula*; (I,J) *Emiliania huxleyi*

Table 4. *Uronema marinum* and bacterial growth rate per day in different culture media. Ciliates and bacteria were grown in a dilution series of a medium of artificially lysed *Emiliana huxleyi* cells and in C3+

Species	Medium of artificially lysed algal cells			C3+
	0× diluted	2.5× diluted	6.75× diluted	
Bacteria	0.60	0.35	0.02	0.48
<i>U. marinum</i>	1.58	1.68	1.52	1.13

toms (Madigan & Martinko 2006) may also underlie their different susceptibility to the *Uronema* toxin.

Bacterial abundance in *Emiliana huxleyi* cultures increased dramatically in the presence of *Uronema marinum* cells or *U. marinum* filtrate. This can be explained as a result of phytoplankton cell lysis and an increase of DOM, a good substrate for bacterial growth (Romaní & Sabater 2000), and is in accordance with the hypothetical *Uronema*–phytoplankton–bacteria cycle (Fig. 1).

**Toxin secretion**

*Uronema marinum* grew better on a phytoplankton-bacteria mixture than on a mixture of bacteria in the synthetic C3+ culture medium. When *U. marinum* was pre-cultured on C3+, there was sometimes a negative effect on the phytoplankton by the presence of the ciliates but not of the *U. marinum* filtrate. This could be because growing ciliates in the experi-

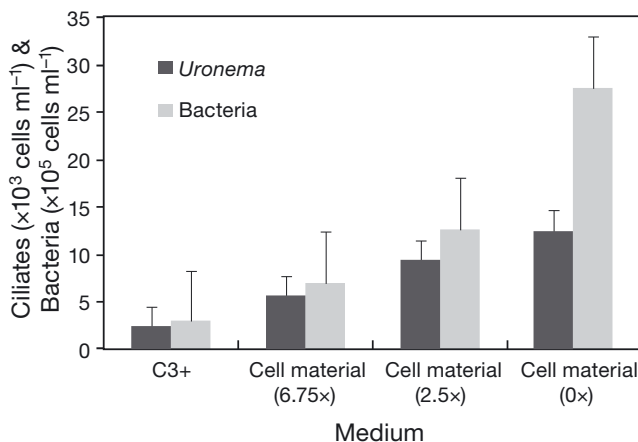


Fig. 6. *Uronema marinum* and bacterial cell numbers after 3 d growth in different culture media. Ciliates and bacteria were grown in a medium of artificial lysed *Emiliana huxleyi* cells which was diluted 0×, 2.5× and 6.75×. Cell numbers were compared with ciliates and bacteria grown in C3+. Error bars represent +SE

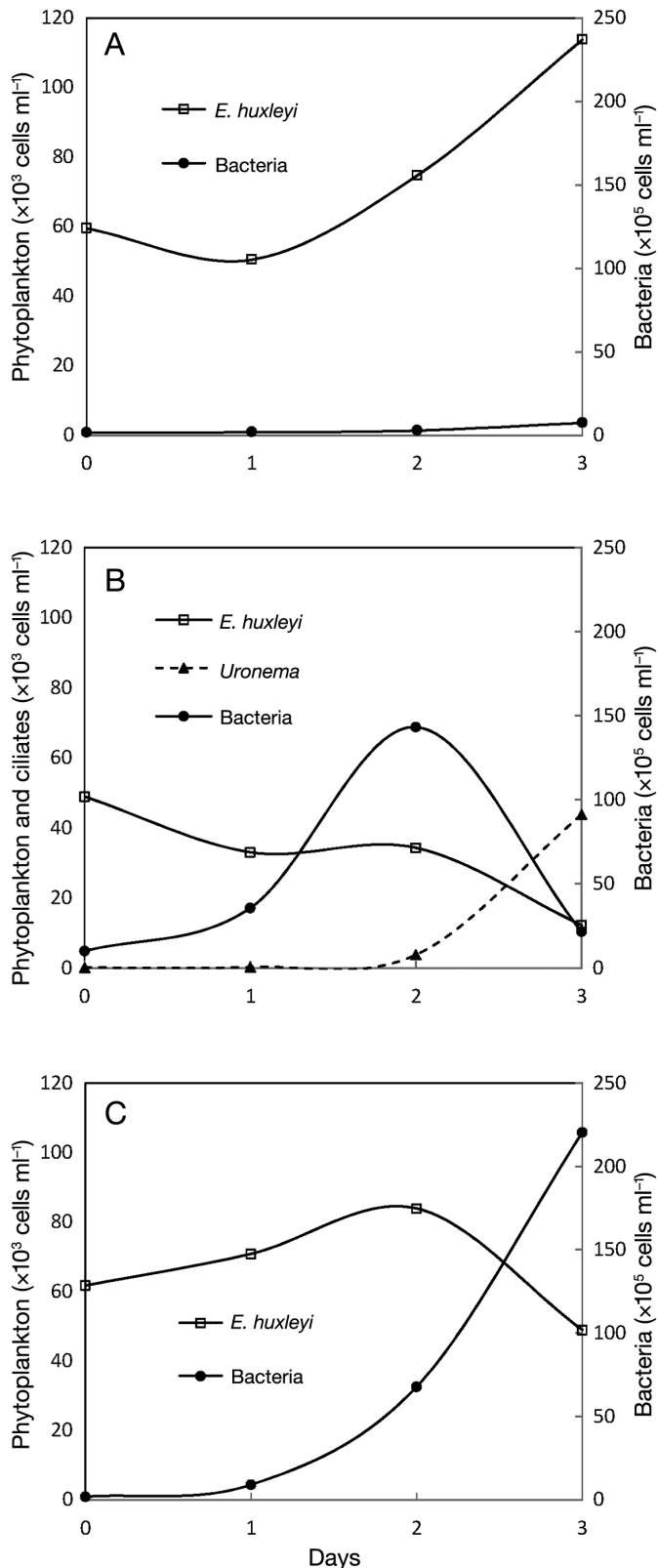


Fig. 7. Bacterial growth in the presence of (A) *Emiliana huxleyi* (Control), (B) *E. huxleyi* and *Uronema marinum* or (C) *E. huxleyi* and *U. marinum* filtrate

Table 5. Initial (2 d) growth rates ( $d^{-1}$ ) of *Emiliania huxleyi*, *Uronema marinum* and bacteria, under different culture conditions. Sterile sea water (control), *U. marinum* cultured on *E. huxleyi* and the filtrate of the latter culture were added to *E. huxleyi*/bacteria cultures

	Control	+ <i>U. marinum</i> cultured in <i>E. huxleyi</i>	+ <i>U. marinum</i> filtrate
Phytoplankton	0.11	-0.18	0.15
Bacteria	0.29	1.33	1.82
<i>U. marinum</i>		2.22	

ments increased the total toxin concentration. This does not explain the effect on the growth rate of *Thalassiosira rotula* on which the *U. marinum* filtrate had a greater negative effect after 2 d incubation.

Negative effects were stronger when *Uronema marinum* was pre-cultured on phytoplankton. A possibility is that when *U. marinum* was cultured with phytoplankton, acclimatization to phytoplankton triggered toxin production or toxin release. So far, such an interaction has only been studied with toxins released by prey in defence of predators. Assuming that defence mechanisms are costly, activating toxin production only in the presence of a predator can reduce these costs (Van Donk et al. 2011). In the case of *U. marinum*, the toxin would only be produced when a potential prey is present.

#### Known secretion by *Uronema marinum*

At this point, it is unclear whether the presumed toxin is an enzyme, which are known to be secreted by *Uronema marinum*, or some other chemical compound. Some ciliates expel chemicals that are involved in predator-prey interactions (Wolfe 2000, Tillmann 2004, Hartz et al. 2008, Roberts et al. 2011). Phagocytic protists can recognize their prey by differences in cell surface composition. There are also chemical cues released by prey which cause a motile response in protists (Roberts et al. 2011). Some phytoplankton species can recognize and react on chemicals released by predators by growing grazer resistant morphologies (Lürding & Von Elert 2001, Long et al. 2007). It is unknown if chemicals released by ciliates can also have an effect on co-occurring species that are not their prey, predator or competitor. There are however studies where, e.g., the toxin euplotin C, produced by the ciliate *Euplotes crassus*, inhibits the growth or even kills non-aquatic eukaryotic organisms such as pathogenic protozoa, yeasts,

bacteria and even human tumor cells (Savoia et al. 2004, Cervia et al. 2006). Human tumor cells are also killed by climacostol produced by the freshwater ciliate *Climacostomum virens* (Buonanno et al. 2008).

There are several enzymes (proteases and phosphatases) known to be secreted by *Uronema marinum*. The presumed parasitic variant of *U. marinum* causes scuticociliatosis in fish, a disease caused by invading ciliates that destroy fish tissue. In parasitic diseases proteases play an important role in pathogenicity (Lee et al. 2003) for instance by facilitating host tissue invasion (Kwon et al. 2002). *U. marinum* is known to secrete a metalloprotease. In some parasitic protozoans, metalloprotease is important for invasion and degradation of host tissues and therefore essential for the progress of the disease (Lee et al. 2003). Besides a metalloprotease, *U. marinum* also secretes cathepsin L-like cysteine protease. In parasitic organisms, this protease functions in the degradation of intracellular proteins, host-parasite attachment, immuno-evasion, excystment/encystment and the processing of proteins (Ahn et al. 2007). It could be possible that the presumed *U. marinum* toxin that inhibited the growth of *E. huxleyi* is a phosphatase or a protease.

In a recent study, Shimeta et al. (2012) discovered that biofilm associated ciliates had a variety of effects on the settlement of marine invertebrate larvae. For instance, the settlement of the tube worm *Galeolaria caespitosa* and the blue mussel *Mytilus galloprovincialis* was significantly reduced by the presence of bacteria and ciliates compared to the presence of a pure bacterial biofilm (Shimeta et al. 2012). *Uronema marinum* was part of the biofilm assemblage and it can be speculated that its toxins produced the negative effects on larval settlement and post-settlement mortality.

#### Implications

To further understand the mechanism of the *Uronema*-phytoplankton-bacteria cycle it is necessary to isolate and identify the toxic compound secreted by *U. marinum*. Additionally, it would be interesting to investigate the cellular localization of the extrusomes containing the toxin and the physiological mechanism of their discharge. Comparable studies have been performed on the chemical defence of heterotrich ciliates (Miyake et al. 2003, Buonanno et al. 2012). Toxin concentrations as well as fluxes of carbon from phytoplankton to bacteria and *Uronema* should be quantified. It would be helpful to study the

range of environmental conditions under which the toxin has an effect on phytoplankton in order to develop a standard method for further research. To better understand the ecological implications the effects of *U. marinum* toxin on taxonomically different phytoplankton groups should be examined further. Also, the effect on other planktonic taxa such as other ciliates and on ciliate predators should be investigated. By comparison, toxic phytoplankton species can be harmful to different groups of organisms. The alga *Prymnesium parvum* for example, is toxic to bacteria, other phytoplankton and ciliates (Fistarol et al. 2003).

The natural abundance of ciliated protozoa varies from  $10^2$  to  $>10^6$  cells  $\text{ml}^{-1}$ , with highest densities found in the sediment surface or at the oxic/anoxic interface (Glud & Fenchel 1999, Dupuy et al. 2011). In a study of a large biofilm of sulphur bacteria, *Uronema* spp. cell densities reached  $9 \times 10^4$  cells  $\text{ml}^{-1}$  (Glud & Fenchel 1999). In the present study, highest *U. marinum* cell densities ( $>10^4$  cells  $\text{ml}^{-1}$ ) were obtained when the ciliate was cultured on a medium of lysed algal cells. *In situ* growth rates of ciliates have been estimated or calculated using frequency of cell division (Gilron & Lynn 1989, Carrick et al. 1992). Carrick et al. (1992) estimated ciliate growth rates of 0 to  $2.28 \text{ d}^{-1}$ . For tintinnine ciliate species *in situ* growth rates of  $-2.55 \text{ d}^{-1}$  have been found (Gilron & Lynn 1989).

Bacterial concentrations  $>10^5$  cells  $\text{ml}^{-1}$  can sustain *Uronema marinum* growth, although growth parameters differ depending on bacterial species composition and the preference of the ciliates for certain bacterial prey (Pérez-Uz 1996). Bacterial abundances in sea water generally lie between  $10^5$  and  $10^7$  cells  $\text{ml}^{-1}$ , which is similar to bacterial concentrations reached in this study. Therefore, in the present study, the abundances of *U. marinum* and bacteria, as well as the ciliate's growth rate, were comparable to data obtained in field studies.

When *Uronema marinum* grow in biofilms, dispersion of its toxin may be relatively low compared to a pelagic environment (Shimeta et al. 2012). This means that in future ecological laboratory studies of *U. marinum* and its toxin a distinction should be made between pelagic and benthic or biofilm habitats.

Predator–prey interactions in general are very complex and remain difficult to understand (Olson & Lessard 2010). Many studies focussed on phytoplankton defence mechanisms and protist prey selection (Wolfe 2000, Granéli & Johansson 2003, Strom et al. 2003, Tillmann 2004, Roberts et al. 2011). The

present study on *Uronema marinum* adds a new dimension to predator–prey interactions, where chemical compounds have an effect on co-occurring species that are not directly preyed upon or are direct competitors. The role of ciliates in phytoplankton community composition and nutrient cycling (Lynn & Small 1990, Strom & Morello 1998) may be different then hitherto assumed, increasing the complexity of already dynamic and complex aquatic food webs.

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