

Allelopathic effect on a nutrient-limited phytoplankton species

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ABSTRACT: For aquatic systems, studies on allelopathic interactions among phytoplankton have increased over recent years, with the main focus on the role of the donor organism. In this study, we report on the response of a target organism to allelochemicals and whether this response was affected by stress conditions (nutrient limitation). We exposed the diatom *Thalassiosira weissflogii*, grown under different nitrogen (N) and phosphorus (P) conditions (NP, -N, or -P), to single or daily additions of a cell-free filtrate of *Prymnesium parvum* (grown with no nutrient limitation). When we exposed *T. weissflogii* to a single addition of filtrate, all 3 treatments were inhibited by *P. parvum*. However, *T. weissflogii* NP was the most resistant, while *T. weissflogii* -N showed the highest sensitivity to *P. parvum* filtrate, followed by *T. weissflogii* -P. When *T. weissflogii* was exposed to daily additions of *P. parvum*, the degree of inhibition of all *T. weissflogii* treatments was higher than when only 1 initial addition was made. In this case, even the treatment that had the highest resistance (*T. weissflogii* NP) was not only inhibited by the filtrate, but also showed a decrease in cell numbers. Nevertheless, *T. weissflogii* -N was still more sensitive than the other treatments. Therefore, nutrient-limiting conditions may increase allelopathic effects, by making the target more susceptible to allelopathic compounds. Under these conditions, allelopathy may play a strong role in phytoplankton competition, especially in natural environments where the allelochemicals are continuously released and, thus, the target species do not have time to recover.

KEY WORDS: Allelopathy · Phytoplankton · Target organism · Nutrient limitation · *Thalassiosira weissflogii* · *Prymnesium parvum*

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INTRODUCTION

Allelopathy is the study of 'any direct or indirect, harmful or beneficial effect of plants, protists (e.g. microalgae, ciliates), bacteria, or viruses on another through production of chemical compounds that are released into the environment' (modified from Rice 1984). It has been widely studied in terrestrial ecology, due to its economical importance for agriculture (Rizvi et al. 1992, Einhellig 1995). The fact that aquatic plants and algae probably have allelopathic properties has been proposed sporadically since Akehurst suggested the existence of non-nutritional interactions between phytoplankton mediated by organic compounds in 1931 (Maestrini & Bonin 1981). However, it is only

recently that more effort has been put into the study of allelopathy among phytoplankton in aquatic environments (Legrand et al. 2003), with most emphasis placed on understanding the role of the donor organisms and their effects. Little is known about the factors influencing the response of the target organisms.

However, because of the characteristics of the aquatic environment, both donor and target organism are under the influence of the same stress factors (e.g. nutrient limitation). Stress factors affect the donor species by, e.g., increasing the production of allelochemicals (Tang et al. 1995, Granéli & Johansson 2003). It has also been suggested that stress could affect the target organism by making it more sensitive to allelochemicals (Einhellig 1995, Tang et al. 1995, Reigosa et

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al. 1999), although few studies have really tested this hypothesis (Einhellig 1996) and those that did only concentrated on higher plants.

Allelopathy is a form of interference competition, and, together with resource exploitation, it is used to explain patterns in plant competition dynamics (Rice 1984). Thus, factors that enhance the allelopathic effect may change the competitive balance towards allelopathic organisms. Since all plants and algae experience some kind of stress during growth, it is probable that the stress factors interact with allelopathy, thus changing the outcome of competition.

In the present study, we investigated if the response of target phytoplankton species, which were exposed to allelochemicals, is affected by nutrient limitation. The fact that the allelopathic effect may be lost some time after exposure due to the degradation of the causative compounds was taken into consideration. Therefore, we also tested the response of nutrient-limited target species when exposed to a single addition of filtrate or to daily additions, and compared the results. Daily filtrate additions may better mimic what occurs in the environment, since they simulate a continuous release of allelochemicals (Suikkanen et al. 2004). The diatom *Thalassiosira weissflogii* (KAC 32, Kalmar Algal Collection) was chosen as the target species, and the prymnesiophyte *Prymnesium parvum* (KAC 39, Kalmar Algal Collection) as the donor. Neither of the cultures was axenic, and both algae were isolated from the Baltic Sea, where *Thalassiosira* spp. usually precede *P. parvum* in annual succession (Edler 1979, Lindholm & Virtanen 1992). Besides using species from the same environment, we chose these 2 species because it has been demonstrated that the algal allelopathic effect is usually more significant on groups preceding the allelopathic algae, since allelopathy can be used to achieve dominance over the predecessor group (Keating 1977, Fistarol et al. 2003).

MATERIALS AND METHODS

Algal cultures and growth conditions. *Thalassiosira weissflogii* cultures were grown in a basic *f/10* medium (Guillard 1975): all components, except nitrate and phosphate, were adjusted to *f/10* concentrations; nitrate and phosphate concentrations were adjusted to obtain nutrient limitation. The medium was made with filtered Baltic Sea water (GF/C glass microfiber filters), with salinity adjusted to 10 psu. The cultures were kept in a controlled culture room at 16°C, 100 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ and a 16:8 h light:dark cycle. To obtain limitation by nitrate or phosphate, *T. weissflogii* was grown in semi-continuous cultures (500 ml culture), and 3 sets (with 3 replicates each) with different nutrient condi-

tions (N:P ratios) were made: (1) *T. weissflogii* diluted with medium at N:P = 16:1 ($\text{NO}_3^- = 116 \mu\text{M}$, $\text{PO}_4^{3-} = 7.2 \mu\text{M}$) (*T. weissflogii* NP); (2) *T. weissflogii* diluted with medium at N:P = 3.2:1 ($\text{NO}_3^- = 23.2 \mu\text{M}$, $\text{PO}_4^{3-} = 7.2 \mu\text{M}$), which was considered to be nitrogen limited (*T. weissflogii* -N); and (3) *T. weissflogii* diluted with medium at N:P = 80:1 ($\text{NO}_3^- = 116 \mu\text{M}$, $\text{PO}_4^{3-} = 1.45 \mu\text{M}$), which was considered to be phosphorus limited (*T. weissflogii* -P). These cultures were grown as batch cultures for 7 d prior to dilution procedures. Dilutions were made daily and lasted for 43 d. The dilution rates used were chosen according to the growth rate of each treatment during the batch growth, as is indicated to establish a semi-continuous culture. Thus, *T. weissflogii* NP was diluted 35% d^{-1} , *T. weissflogii* -N was diluted 16% d^{-1} and *T. weissflogii* -P was diluted 20% d^{-1} .

The *Thalassiosira weissflogii* cultures were used in the experiment during steady-state growth (after Day 30) (Fig. 1), when *T. weissflogii* -N and -P were nitrogen and phosphorus limited, respectively, as shown by cellular nutrient contents and C:N, C:P and N:P ratios (Table 1). During the steady-state period, growth rates were calculated according to the steady-state growth rate calculations for semi-continuous cultures:

$$\mu = \frac{\ln\left(\frac{V_{\text{tot}}}{V_{\text{tot}} - V_{\text{rep}}}\right)}{t} \quad (1)$$

where V_{tot} is the total culture volume, V_{rep} is the volume replaced (volume of medium used in the dilutions) and t is the time between dilutions. Thus, the growth rate (μ) during the steady-state period was $\mu = 0.43 \text{ d}^{-1}$ for *T. weissflogii* NP, $\mu = 0.17 \text{ d}^{-1}$ for *T. weissflogii* -N and $\mu = 0.22 \text{ d}^{-1}$ for *T. weissflogii* -P.

A semi-continuous culture of *Prymnesium parvum* was established in parallel to the *Thalassiosira weissflogii*

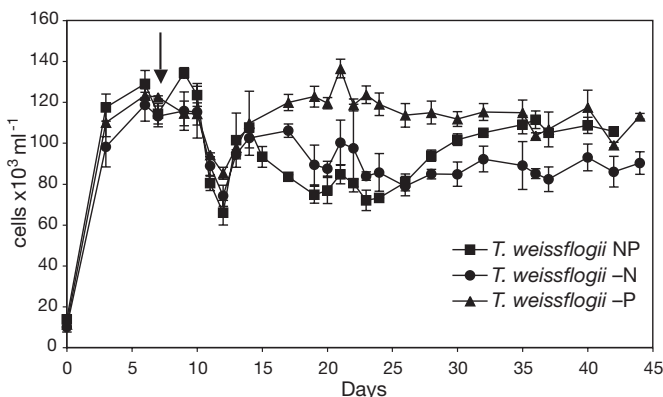


Fig. 1. *Thalassiosira weissflogii*. Growth curve of cultures grown with no nutrient limitation (*T. weissflogii* NP), with N limitation (*T. weissflogii* -N) and with P limitation (*T. weissflogii* -P). Arrow indicates when the daily dilutions started

Table 1. Cellular content (pg C/N/P cell⁻¹) and molar ratios (C:N, C:P, and N:P) for the 3 *Thalassiosira weissflogii* treatments at the beginning (Day 31), and at the end (Day 43) of the steady state period (n = 3, mean ± SD). The cellular content (pg C/N/P cell⁻¹) and molar ratios (C:N, C:P, and N:P) for *Prymnesium parvum* culture during steady-state are also shown (Day 41)

Treatment	pg C cell ⁻¹	pg N cell ⁻¹	pg P cell ⁻¹	C:N	C:P	N:P
Day 31						
<i>T. weissflogii</i> NP	52.9 ± 4.3	10.9 ± 0.8	2.2 ± 0.01	5.6	63.0	11.2
<i>T. weissflogii</i> -N	55.3 ± 5.7	4.7 ± 0.6	1.6 ± 0.2	13.6	86.5	6.3
<i>T. weissflogii</i> -P	50.5 ± 6.5	7.1 ± 3.4	0.5 ± 0.2	10.8	347.0	32.1
Day 43						
<i>T. weissflogii</i> NP	55.8 ± 1.1	11.5 ± 0.2	2.2 ± 0.1	5.7	65.0	11.5
<i>T. weissflogii</i> -N	47.5 ± 2.7	5.8 ± 0.5	1.4 ± 0.1	9.6	89.1	9.3
<i>T. weissflogii</i> -P	68.4 ± 2.8	10.4 ± 0.2	0.5 ± 0.01	7.7	353.9	46.1
Day 41						
<i>P. parvum</i>	28.6 ± 1.1	2.7 ± 0.1	0.4 ± 0.1	12.3	199.2	16.2

cultures. This culture was maintained in *f/10* medium (Guillard 1975) with N:P = 16:1. The media used to dilute *P. parvum* culture had the same NO₃⁻ and PO₄³⁻ concentrations as that used for dilution in the *T. weissflogii* NP treatment. The other components of the culture medium contained the concentrations of *f/10* medium (Guillard 1975). The cellular contents and the C:N, C:P and N:P ratios for *P. parvum* are shown in Table 1.

Experimental set-up. All experiments were performed by exposing *Thalassiosira weissflogii* to *Prymnesium parvum* cell-free filtrate. The filtrate was obtained by gentle filtration (a pressure lower than -2 kPa was used to create the initial vacuum for the filtration) of samples taken from the semi-continuous cultures of *P. parvum* through GF/F glass fiber filters. Two experiments were set, varying the frequency of filtrate additions.

Expt 1: We exposed *Thalassiosira weissflogii* grown under different nutrient conditions to a single addition of cell-free filtrate from *Prymnesium parvum*. In this test, the only factor varying was the nutrient state of *T. weissflogii*. The tests were performed in 21 ml scintillation vials (total of 9 vials, 3 vials for each *T. weissflogii* treatment) containing a total of 20 ml (12 ml were *P. parvum* cell-free filtrate, which corresponded to a culture that would contain 60 × 10³ cells ml⁻¹ of *P. parvum*, and 8 ml were filled with aliquots from one of the *T. weissflogii* treatments, i.e. *T. weissflogii* NP, -N, or -P). The final concentration of *T. weissflogii* in the test tubes was 15 × 10³ cells ml⁻¹ (medium corresponding to the respective *T. weissflogii* treatment was added to complete the volume, if necessary). One control (in triplicate) was made for each *T. weissflogii* (NP, -N, -P) treatment by adding, instead of filtrate, 12 ml of *f/10* medium with the same nutrient concentration as that used to dilute the *P. parvum* NP culture. At the beginning of the experiment, the cell concentration of *T. weissflogii* in the test tubes was the same for all

treatments and as in the controls. Aliquots (2 ml) were sampled daily for direct cell counts (i.e. the samples were not fixed and were analyzed immediately). The experiment lasted 4 d, and the allelopathic effect was observed by measuring differences in the cell numbers of the *T. weissflogii* treatments that received *P. parvum* filtrate compared to their respective controls (i.e. a control that was made by adding medium to a *T. weissflogii* culture that was grown under NP, -N, or -P conditions); this eliminated the effect of nutrient limitation on growth, since the control was under the same growth conditions. Thus, any decrease in growth due to nutrient limitation was reflected in the control and discounted from the allelopathic effect.

Expt 2: We exposed *Thalassiosira weissflogii* to repeated additions of *Prymnesium parvum* cell-free filtrate. The first addition of filtrate and the experimental set-up were as described in Expt 1. After that, a new addition was made every day, over 4 d, by removing 3 ml of test volume, used for the cell counts, and replacing it with an equal volume of fresh filtrate or control medium.

Analytical procedures. Cell counts of non-fixed *Thalassiosira weissflogii* and *Prymnesium parvum* were made using a flow cytometer (FACScalibur, Becton Dickinson). Inorganic nutrients (NO₃⁻ and PO₄³⁻) were analyzed according to Valderrama (1995). Cellular contents (C, N, and P) were measured in cells retained on 25 mm pre-combusted (450°C, 2 h) GF/C filters. The filters were dried overnight at 65°C. Particulate organic carbon (POC) and particulate organic nitrogen (PON) were analyzed with a CHN elemental analyzer (FISONS Instruments, NA 1500 NC), and particulate organic phosphorus (POP) was analyzed following the method of Solórzano & Sharp (1980). In addition, the pH values of *T. weissflogii* and *P. parvum* cultures were measured at the beginning and at the end of the steady state with a pH Meter 691 (Metrohm).

Statistical analysis. Statistical analyses were performed using the software SPSS 10 for Macintosh. To verify if each *Thalassiosira weissflogii* treatment was affected by *Prymnesium parvum* NP filtrate, we compared the growth rate in the treatments that received filtrate with their respective controls (e.g. we compared the 3 *T. weissflogii* –P that received *P. parvum* filtrate with the 3 *T. weissflogii* –P that received control medium). The difference in the growth rate between each *T. weissflogii* treatment and its control was tested using a Student's *t*-test ($n = 3$, for each treatment). We also compared *T. weissflogii* grown under different nutrient conditions (e.g. in Expt 1, we compared the response of the 3 *T. weissflogii* treatments, NP, –N, P) to observe if there was a difference in sensitivity to allelochemicals. To assess the difference in sensitivity between the treatments, we performed 2 analyses: (1) testing differences in the growth rates of each treatment (which shows if the effect in one treatment was stronger than in the other) and (2) comparing the percentage of cells alive in each treatment (to observe if there was a difference and when the treatments started to differ). Both these tests were made using the GLM (general linear model) univariate analysis of SPSS, which corresponds to an ANOVA (analysis of variance). These analyses were made for both Expts 1 and 2.

We used Student's *t*-test to compare growth rates of the corresponding *Thalassiosira weissflogii* treatments from Expts 1 and 2 (e.g. *T. weissflogii* NP single addition with *T. weissflogii* NP daily additions, and so on, to assess if there was a difference in the effect when adding the filtrate once or daily).

RESULTS

Thalassiosira weissflogii –N and –P semi-continuous cultures (Fig. 1) were N and P limited, respectively, as shown by the cellular nutrient contents and molar ratios (C:N, C:P and N:P) (Table 1). The pH values of *T. weissflogii* (NP, –N and –P) and *Prymnesium parvum* cultures were approximately the same at the beginning and at the end of the steady state (Table 2), and they were higher in the *T. weissflogii* cultures (~9.3) than in the *P. parvum* cultures (~8.2).

All 3 *Thalassiosira weissflogii* cultures (NP, –N and –P) were affected by a single addition of *Prymnesium parvum* filtrate (Fig. 2A). A single addition of *P. parvum* filtrate caused a decrease in the cell numbers of *T. weissflogii* –N and –P 1 d after exposure. However, by the second day, *T. weissflogii* –N and –P started to grow again, though less so than in the control. In addition, with the single filtrate addition, *T. weissflogii* NP retained positive growth, which was, however, lower

Table 2. pH in *Thalassiosira weissflogii* (NP, –N, and –P) and *Prymnesium parvum* semi-continuous cultures at the beginning (Day 30) and at the end (Day 42) of the steady state ($n = 3$, mean \pm SD)

Treatment	<i>T. weissflogii</i>		<i>P. parvum</i>	
	Day 30	Day 42	Day 30	Day 42
NP	9.2 \pm 0.1	9.5 \pm 0	8.2 \pm 0	8.2 \pm 0
–N	9.1 \pm 0.1	9.1 \pm 0	–	–
–P	9.4 \pm 0.1	9.6 \pm 0	–	–

than in the control. All 3 treatments showed lower growth rates than their respective controls ($n = 3$, mean \pm SD) — (1) *T. weissflogii* NP: filtrate treatment $\mu = 0.38$ d⁻¹ \pm 0.03, control $\mu = 0.52$ d⁻¹ \pm 0.02 (*t*-test, $p = 0.003$); (2) *T. weissflogii* –N: filtrate treatment $\mu = 0.19$ d⁻¹ \pm 0.02, control $\mu = 0.32$ d⁻¹ \pm 0.02 (*t*-test, $p = 0.002$); and (3) *T. weissflogii* –P: filtrate treatment $\mu = 0.32$ d⁻¹ \pm 0.02, control $\mu = 0.44$ d⁻¹ \pm 0.01 (*t*-test, $p = 0.001$).

We compared the response between the 3 *Thalassiosira weissflogii* treatments that received a single filtrate addition to see if the nutrient state of *T. weissflogii* influenced its sensitivity to allelochemicals (Fig. 2B). *T. weissflogii* –N had a lower growth rate than the other 2 treatments (ANOVA, *T. weissflogii* –N \neq *T. weissflogii* NP, $p = 0.0001$; *T. weissflogii* –N \neq *T. weissflogii* –P, $p = 0.002$). *T. weissflogii* –P had a lower growth rate than *T. weissflogii* NP, though the difference was only significant at the 90% level (ANOVA, *T. weissflogii* –P \neq *T. weissflogii* NP, $p = 0.056$). By looking at the percentage of cells in each filtrate treatment in relation to those in the respective control, we observed that *T. weissflogii* –N was more affected than the other 2 treatments (Fig. 2B). By Day 1, *T. weissflogii* –N already differed from the *T. weissflogii* NP treatment (ANOVA, $p = 0.001$), by Day 2 it differed both from the *T. weissflogii* NP (ANOVA, $p = 0.008$) and from the *T. weissflogii* –P treatment (ANOVA, $p = 0.006$). However, thereafter, *T. weissflogii* cells started to recover: by Day 3 *T. weissflogii* –N differed only from *T. weissflogii* –P (ANOVA, $p = 0.006$) and on Day 4 there was no difference between the treatments.

Repeated additions of *Prymnesium parvum* NP filtrate caused a decrease in the cell numbers of *Thalassiosira weissflogii* in all treatments (Fig. 3A–C). Furthermore, all treatments showed a negative growth rate, in contrast to Expt 1, where only a decrease in the growth rate was observed. The growth rates of the 3 filtrate treatments were significantly different from the positive growth of the controls ($n = 3$, mean \pm SD) — (1) *T. weissflogii* NP: filtrate treatment $\mu = -0.08$ d⁻¹ \pm 0.04, control $\mu = 0.37$ d⁻¹ \pm 0.01 (*t*-test, $p = 0.0001$); (2) *T. weissflogii* –N: filtrate treatment $\mu = -0.63$ d⁻¹ \pm 0.08, control $\mu = 0.20$ d⁻¹ \pm 0.02 (*t*-test, $p = 0.0001$); and (3) *T.*

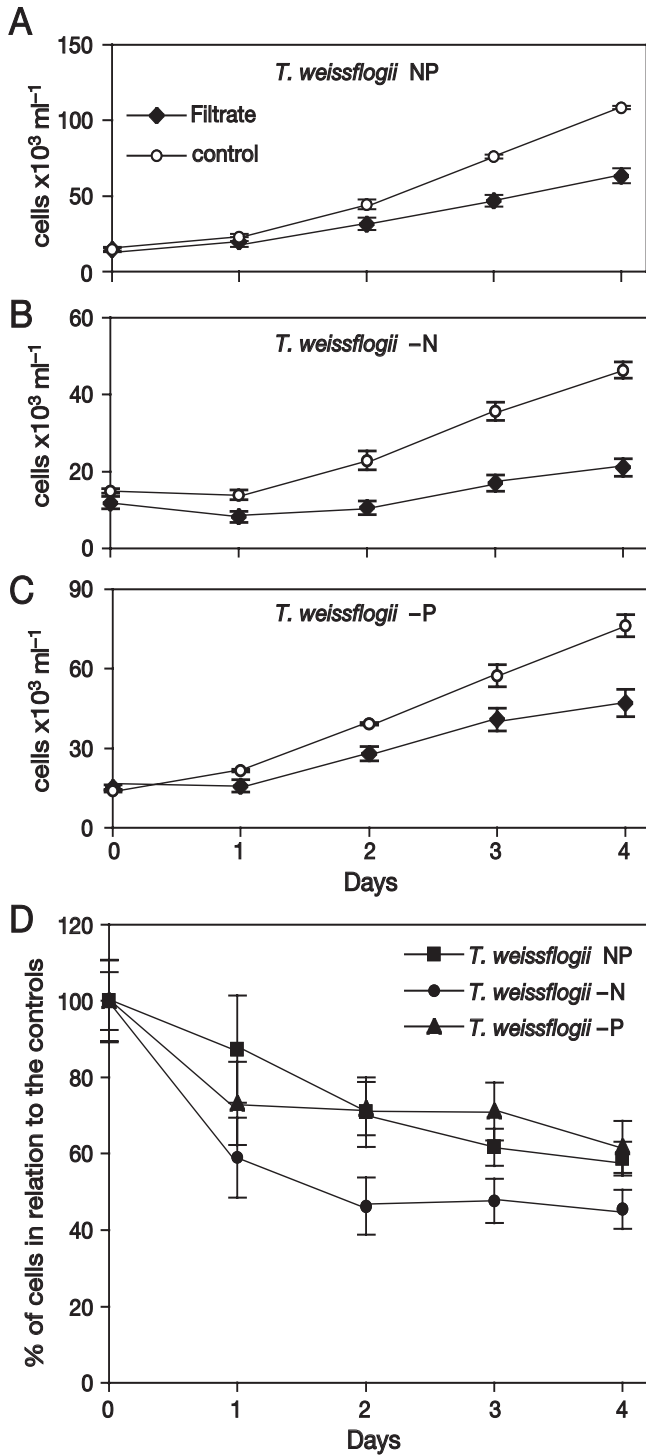


Fig. 2. *Thalassiosira weissflogii*. (A–C) Growth curves of 3 *T. weissflogii* treatments when exposed to a single addition of *Prymnesium parvum* filtrate and of their respective controls (A: *T. weissflogii* NP + filtrate and *T. weissflogii* NP + control medium; B: *T. weissflogii* -N + filtrate and *T. weissflogii* -N + control medium; C: *T. weissflogii* -P + filtrate and *T. weissflogii* -P + control medium). (D) Comparison between the sensitivity of the 3 *T. weissflogii* treatments, shown as percentage of cells in the filtrate treatment relative to the respective control (n = 3, mean ± SD)

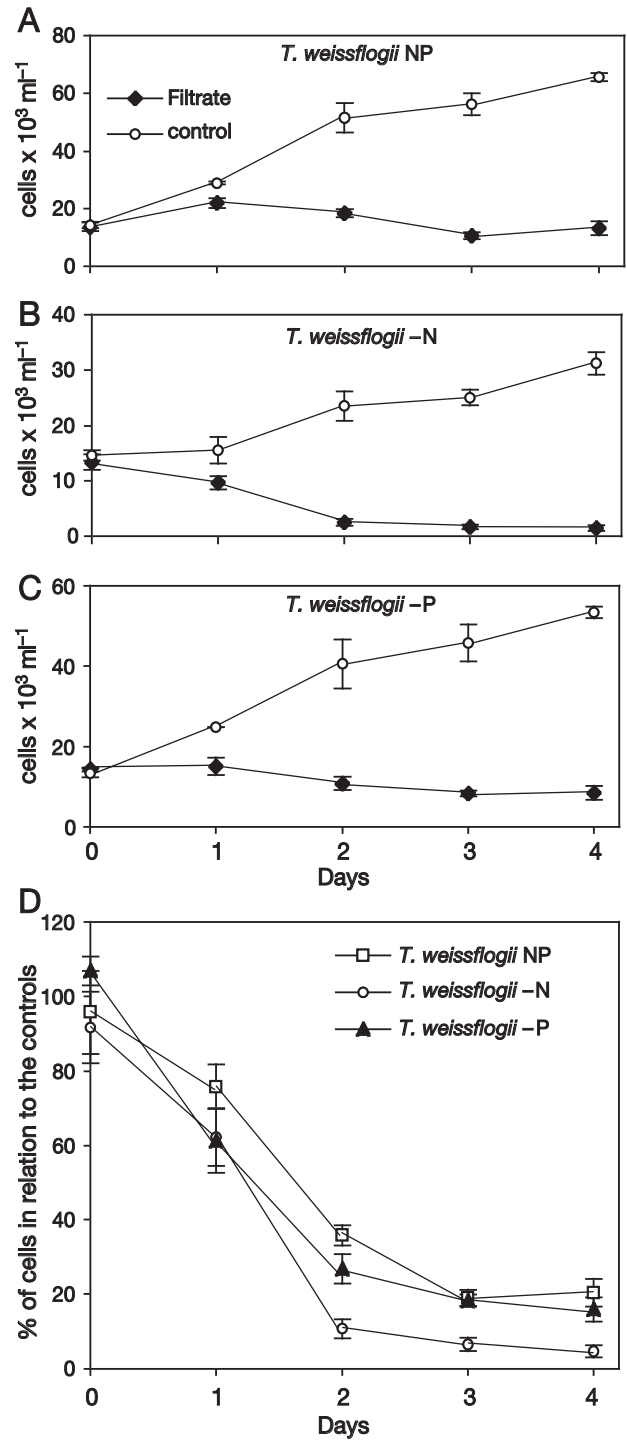


Fig. 3. *Thalassiosira weissflogii*. (A–C) Growth curves of 3 *T. weissflogii* treatments when exposed to daily additions of *Prymnesium parvum* filtrate and of their respective controls (A: *T. weissflogii* NP + filtrate and *T. weissflogii* NP + control medium; B: *T. weissflogii* -N + filtrate and *T. weissflogii* -N + control medium; C: *T. weissflogii* -P + filtrate and *T. weissflogii* -P + control medium). (D) Comparison between the sensitivity of the 3 *T. weissflogii* treatments, shown as percentage of cells in the filtrate treatment relative to the respective control (n = 3, mean ± SD)

weissflogii -P: filtrate treatment $\mu = -0.17 \text{ d}^{-1} \pm 0.04$, control $\mu = 0.44 \text{ d}^{-1} \pm 0.01$ (*t*-test, $p = 0.01$) (Fig. 3A–C). *T. weissflogii* -N was again the most affected treatment, with a growth rate lower than that of the other 2 treatments (ANOVA, *T. weissflogii* -N \neq *T. weissflogii* NP, $p = 0.0001$; *T. weissflogii* -N \neq *T. weissflogii* -P, $p = 0.0001$). A difference in the percentage of cells between *T. weissflogii* -N and the other 2 treatments could already be observed on Day 1 (ANOVA, *T. weissflogii* -N \neq *T. weissflogii* NP, $p = 0.0001$; *T. weissflogii* -N \neq *T. weissflogii* -P, $p = 0.004$), and this difference was maintained until the end of the experiment (ANOVA, *T. weissflogii* -N \neq *T. weissflogii* NP, $p = 0.0001$; *T. weissflogii* -N \neq *T. weissflogii* -P, $p = 0.0002$). A significant difference between *T. weissflogii* -P and *T. weissflogii* NP could be observed on Days 1 and 2 (ANOVA, Day 1, $p = 0.0002$; Day 2, $p = 0.00016$). However, the daily additions of filtrate also caused a decrease in the *T. weissflogii* NP treatment, and by Day 3 there was no difference between *T. weissflogii* -P and *T. weissflogii* NP (ANOVA, $p = 0.80$) (Fig. 3D).

When we compared the effect of a single addition of *Prymnesium parvum* filtrate with the effect of daily additions, we observed that all 3 *Thalassiosira weissflogii* were more affected by repeated filtrate additions than by a single addition of *P. parvum* NP filtrate (1 filtrate addition \neq repeated filtrate additions, *t*-test, *T. weissflogii* NP, $p = 0.0001$; *T. weissflogii* -N, $p = 0.0001$; *T. weissflogii* -P, $p = 0.0001$) (Fig. 4).

DISCUSSION

We showed that *Thalassiosira weissflogii* is affected by allelochemicals produced by *Prymnesium parvum* and that the effect varied depending on the nutrient state of *T. weissflogii*. *T. weissflogii* -N showed the highest sensitivity to allelochemical attack, *T. weissflogii* -P had an intermediate response and *T. weissflogii* NP was the most resistant. The role the donor organism plays in allelopathic interactions has already been investigated. *P. parvum* has been shown to affect both autotrophic and heterotrophic microorganisms in culture (Granéli & Johansson 2003, Skovgaard & Hansen 2003, Skovgaard et al. 2003, Tillmann 2003) and also natural plankton communities (Fistarol et al. 2003). However, in the present study, the results show the importance of the physiological state of the target organism in the outcome of the allelopathic interaction.

Plant competition interactions are usually explained by resource exploitation and allelopathy (Rice 1984). Both mechanisms can often occur simultaneously, and it is very difficult to separate the effect of resource exploitation from allelopathy in natural systems. It is important to evaluate the relative contribution of each

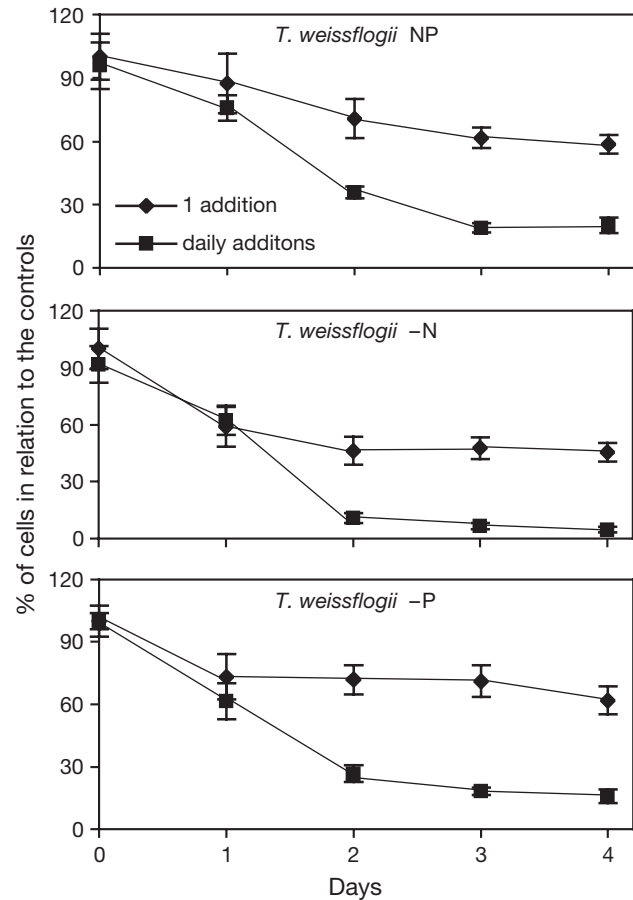


Fig. 4. *Thalassiosira weissflogii*. Comparison between the effect of a single addition and daily additions of *Prymnesium parvum* filtrate to each *T. weissflogii* treatment (NP, -N, -P), expressed as percentage of cells in the filtrate treatment relative to the respective control ($n = 3$, mean \pm SD)

mechanism during competitive interactions (Inderjit & del Moral 1997). Furthermore, stress has been considered to be an important factor influencing allelopathic interactions. As pointed out by Inderjit & del Moral (1997), it is unclear how stress reduces the importance of resource competition and makes allelopathy a major force structuring plant communities. One possibility is that stress increases the production of allelochemicals (Tang et al. 1995). In the present study, we demonstrated that nutrient stress also enhanced the impact of allelopathy on a microalgal species by increasing the sensitivity of the target organisms.

All our experiments were performed by exposing *Thalassiosira weissflogii* to *Prymnesium parvum* cell-free filtrate, ensuring that the affecting compounds had been excreted to the medium, which is necessary to characterize allelopathy (see Willis 1985). This procedure also excluded the effects of competition, which could occur if *P. parvum* cells were added to the *T. weissflogii* cultures, as well as the mixotrophic effect of

P. parvum. Both donor and target species were ecologically relevant, since they were isolated from the same environment and were at cell concentration levels realistic in nature (blooms of *P. parvum* can reach concentrations of 50 to 100×10^6 cells l^{-1} , and even up to 10^9 cells l^{-1} ; Edvardsen & Paasche 1998). pH is also a factor that can influence the outcome of allelopathic interactions. It has been shown that the allelopathic effect of *Chrysochromulina polylepis* on *Heterocapsa triquetra* increases if the pH is raised, with the highest effect being observed at a pH between 8 and 9, depending on the cell concentration of *C. polylepis* (Schmidt & Hansen 2001). In our experiment, the pH in *P. parvum* cultures was lower than that in *T. weissflogii* cultures; therefore, we argue that the pH did not increase the allelopathic effects. The algal cultures used in our experiment were not axenic. Bacteria could influence allelopathy by degrading allelochemicals or by metabolizing them into new compounds. The first case would decrease the allelopathic effect, while the latter could either increase or decrease it, depending on the activity of the new compound. Bacteria may also produce their own allelochemicals. However, some studies (Tillmann & John 2002, Suikkanen et al. 2004) seem to indicate that bacteria present in algal cultures are not responsible for the allelopathic effect of the microalgae. Because bacteria may interfere in the allelopathic effect, their presence represents a more natural situation (Maestrini & Bonin 1981). Nevertheless, since the removal of bacteria from the filtrates used to test allelopathy did not change the effect (Suikkanen et al. 2004), the role of bacteria may be minor.

Effect of nutrient limitation and enhancement of the allelopathic effect

Both nitrogen and phosphorus limitation occur in the Baltic Sea (nitrogen is usually the limiting nutrient in coastal areas of the Baltic proper; phosphorus can be found limiting production during spring and summer) (Granéli et al. 1990). Unbalanced nutrient conditions are a problem in several coastal areas due to eutrophication (Paerl 1995, Skei et al. 2000). Thus, it is important to understand how nutrient limitation will affect species interactions. The resultant allelopathic effect of *Prymnesium parvum* on *Thalassiosira weissflogii* was enhanced by having the target algae limited by nutrients. Both nitrogen and phosphorous limitation increased the sensitivity of *T. weissflogii* to allelochemicals, though nitrogen limitation had a stronger influence. This indicates that allelopathy, and consequently resource competition, is affected, not only by nutrient limitation in general, but also according to the limiting nutrient.

Though some studies indicate that nutrient limitation can increase the toxicity of *Prymnesium parvum* (Granéli & Johansson 2003, Barreiro et al. 2005), it has not yet been tested if toxin production would decrease if nutrients were added to a limited *P. parvum* culture. It has also not been tested if addition of nutrients would alleviate the sensitivity of a nutrient-limited target. We hypothesize that, given enough time for the target to recover, i.e. the cells would have sufficient time to incorporate the nutrient added before being killed by the allelochemicals, the nutrient-replete cells would probably show a lower sensitivity, similar to that in the *Thalassiosira weissflogii* NP treatment.

Although it has been proposed that stress increases the sensitivity of target organisms to allelochemicals (Einhellig 1996, Reigosa et al. 1999), the biochemical mechanisms that cause this increase have not been demonstrated. However, the general physiological responses of plants and microorganisms to nutrient limitation are the accumulation of carbon in the cells, first as carbohydrate and then as lipid, while protein/ amino acid cellular contents decrease as does cell division (Healey 1973). Due to the extremely important roles that proteins (including enzymes) play in regulating all cell functions, it is understandable that a decrease in protein content would affect cell resistance. To affect the physiology of a target cell, allelochemicals need to penetrate the cell membrane, which acts as a barrier against toxic compounds. Since cell membranes are formed by a thin film of a lipid bilayer and protein molecules, held together mainly by non-covalent interactions (Alberts et al. 1994), changes in the protein and lipid concentrations and in their production are bound to have consequences for the plasma membrane. Furthermore, proteins are responsible for most membrane functions, e.g. transporting specific molecules, or catalyzing reactions such as ATP synthesis (Alberts et al. 1994). Thus, if there is a decrease in the protein content of the cell due to nutrient limitation, it will not only have a structural effect on the membrane, but many functions will also be compromised.

An example of the effect of nutrient limitation on the membrane is given by Ferenci (1999) and Liu & Ferenci (2001). They showed that bacteria respond to nutrient limitation by altering membrane permeability. Limitation elicits a complex gene regulation system that makes the membrane more permeable to nutrients, e.g. glucose. If nutrient stress were to trigger this kind of response in algae so that they can increase nutrient uptake, it would, in fact, cause a disadvantage if the algae were also under allelochemical attack.

Thus, N and P limitation of *Thalassiosira weissflogii* might have caused an increase in the sensitivity of this alga by increasing membrane permeability and also by reducing protein, nucleic acids and phospholipids,

which may compromise the physiology of the cell, membrane functions and structure.

Besides the physiological state of the target algae, daily additions of filtrate also enhanced the allelopathic effect of *Prymnesium parvum* on *Thalassiosira weissflogii*. Allelochemicals seem to have a short lifespan, as shown in our study by the recovery of organisms exposed to a single addition of filtrate some time after exposure. *T. weissflogii* –P, which had an intermediate response, showed some resistance when exposed to a single addition of *P. parvum* filtrate, but it was not resistant when continuously exposed to filtrate. When the exposure to allelochemicals was prolonged, not even *T. weissflogii* NP survived; it was not only inhibited, but also killed by the filtrate, as was *T. weissflogii* –N and –P. Allelochemicals may be removed from the system by degradation (e.g. by light reactions or bacteria degradation), as occurs for some microalgal toxins (Reich & Parnas 1962, Christoffersen et al. 2002), or by, for example, binding to cell membranes (Tillmann 2003). Nevertheless, in nature, allelochemicals are probably constantly released from the donor species. Thus, applying repeated filtrate additions better mimics natural environmental conditions.

Consequences for the outcome of allelopathic interactions

It has been shown that some microalgae can cause allelopathy under non-limiting nutrient conditions (Arzul et al. 1999, Schmidt & Hansen 2001, Tillmann & John 2002, Fistarol et al. 2003, 2004, Suikkanen et al. 2004). This means that it is not only a strategy used under nutrient-limited conditions. However, the allelopathic effect may be enhanced under certain conditions. Under limiting nutrient conditions, the allelopathic effect is higher due to the increased sensitivity of the target. The allelopathic effect is also higher when the allelochemicals are continuously released by the donor, as occurs under natural conditions. In these situations, the competitive balance turns towards the allelopathic species. Reigosa et al. (2002) proposed that allelopathy would only become important in special situations, when plants are under stress, i.e. the stress hypothesis. Thus, nutrient limitation may be an example of when allelopathy becomes more important than resource competition in competitive interactions.

In conclusion, our work has shown that under nutrient limitation allelopathic interactions can play a major role in phytoplankton competition, especially if we consider that in natural environments allelochemicals are continuously released. Further studies should try to reveal the exact biochemical pathway that renders the target algae more sensitive when nutrient limited.

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