Allelopathy in *Alexandrium* spp.: effect on a natural plankton community and on algal monocultures

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ABSTRACT: We studied allelopathy in the dinoflagellate genus *Alexandrium* by testing the effect of *A. tamarense* on a natural plankton community from Hopavågen Bay, Trondheimsfjord, Norway, and the effect of toxic and non-toxic strains of *A. tamarense* and a toxic strain of *A. minutum* on algal monocultures. Also, a possible relation between the allelopathic effect and the production of paralytic shellfish poison (PSP) toxin was investigated. *A. tamarense* affected the whole phytoplankton community by decreasing the growth rate and changing the community structure (relative abundance of each species, dominant species). A negative effect of *A. tamarense* was also observed on ciliates, but not on bacteria numbers. In the bioassay with algal monocultures, the diatom *Thalassiosira weissflogii* and the cryptophyte *Rhodomonas* sp. were exposed to the filtrate of *Alexandrium* spp. All tested *Alexandrium* strains negatively affected *T. weissflogii* and *Rhodomonas* sp. cultures, independent of whether PSP toxins were produced. The compounds released by *Alexandrium* caused lysis of natural and cultured algal cells, suggesting that the allelopathic effect may be connected with previously described ichthyotoxic and haemolytic properties of *Alexandrium*. Furthermore, the observation that several components of the plankton community were affected by compounds released by *A. tamarense* emphasizes the importance of allelopathy for the ecology of this species.

KEY WORDS: *Alexandrium* · Allelopathy · Natural community · Algal monocultures · Cysts · Paralytic shellfish poison · PSP method · PSP toxins

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INTRODUCTION

The dinoflagellate genus *Alexandrium* includes the largest number of toxic species (at least 8 species) associated with harmful algal blooms (HABs) (Anderson 1998). The toxic *Alexandrium* species (e.g. *A. tamarense* and *A. minutum*) produce a suite of toxins associated with paralytic shellfish poisoning (PSP), an illness that can affect humans, and causes a series of neurological symptoms (Hallegraeff 1995). *Alexandrium* species also cause environmental problems due to the effect on co-occurring aquatic organisms. Toxic effects of *Alexandrium* spp. have been observed on heterotrophic dinoflagellates, ciliates, zooplankton and fishes (Mortensen 1985, Ogata & Kodama 1986, Hansen 1989, Hansen et al. 1992, Lush & Hallegraeff

1996, Frangópulos et al. 2000, Tillmann & John 2002). These negative effects of *Alexandrium* spp. on aquatic organisms are not always related to PSP toxins (Ogata & Kodama 1986, Lush & Hallegraeff 1996, Tillmann & John 2002), but seem to be caused by lytic compounds, and related to the haemolytic activity of this genus (Simonsen et al. 1995).

Besides toxic effects on organisms from higher trophic levels, some *Alexandrium* species (*A. lusitanicum*, *A. minutum*, *A. tamarense*, *A. catenella*) have been reported to cause allelopathic effects on other phototrophic phytoplankton species (Blanco & Campos 1988, Arzul et al. 1999). Allelopathy refers to any process involving secondary metabolites (in this case, called allelochemicals) released by plants, micro-organisms, viruses and fungi that affect competing

organisms (International Allelopathic Society 1996; available at www-ias.uca.es/bylaws.htm#CONSTI). The release of allelopathic compounds is an intriguing concept, since these substances would interfere with species interactions. Allelochemicals have been suggested to play an important role in plankton succession, competition and bloom formation (Pratt 1966, Keating 1977, Rice 1984 and references therein, Wolfe 2000, Rengefors & Legrand 2001, Vardi et al. 2002). Although there is evidence that allelochemicals can indeed affect plankton succession (Pratt 1966, Keating 1977, Vardi et al. 2002), allelopathy among phytoplankton has not yet received the attention it deserves.

Since *Alexandrium* spp. can affect other aquatic organisms, from other phytoplankton to fishes, it is important to understand how chemicals released by *Alexandrium* affect planktonic interactions such as competition, allelopathy and species succession. To improve the understanding on the mechanisms of allelopathy, future works should include identification and isolation of the causative allelochemicals, which would provide additional insights into the mode of action of such compounds (Seigler 1996, Einhellig 2002). Use of bioassays is the first step in identifying which fraction or which group of compounds is responsible for a certain toxic effect, in this case, allelopathy.

The aims of our study were to investigate (1) whether *Alexandrium tamarense* had an allelopathic effect on a natural plankton community, (2) the effect of *A. tamarense* allelochemicals on different plankton groups, community biomass and species dominance, (3) the allelopathic effect of *Alexandrium* on algal monocultures, and (4) whether the allelopathic effect is related to the production of PSP toxins.

Since allelochemicals must be excreted to the environment to characterize an allelopathic interaction (Willis 1985), all allelopathic assays in this study were performed by exposing the plankton community and the algal cultures to cell-free filtrate of the different *Alexandrium* species.

MATERIALS AND METHODS

Allelopathic effect of *Alexandrium tamarense* on a plankton community. A natural plankton community was collected in Hopavågen Bay, at the entrance of the Trondheimsfjord, Norway, in September 2001. The plankton samples were collected from 2 to 6 m depth using a 3 l Niskin bottle. Water temperature and salinity during sampling were 13.6°C and 31.8 psu, respectively. The samples were filtered through a 150 μm mesh-size nylon net to remove the effect of grazing by mesozooplankton.

Alexandrium tamarense KAC 02 (Kalmar Culture Collection, University of Kalmar) was grown in f/2 medium (Guillard 1975) at 20°C, 32 psu, in a light:dark cycle of 16:8 h, 80 μmol photons m^{-2} s^{-1}, under cool white fluorescent light. To test whether *A. tamarense* filtrate would have different effects on the plankton community if this alga was grown at a different temperature, a subculture of *A. tamarense* was also grown at 14°C (approximately the same temperature as in the bay). Both *A. tamarense* cultures (at 20 and 14°C) were used to test its allelopathic effect and the results were compared. The cultures used in the experiment were in exponential growth, and had 113.3×10^3 cells l^{-1} at that time. The cultures were not axenic.

Cell-free filtrates used in the experiments were obtained by gentle filtration (a pressure lower than –2 kPa was used to create initial vacuum for the filtration) of *Alexandrium tamarense* cultures through GF/F glass-fibre filters. Freshly prepared filtrate (150 ml) was added to triplicate tissue culture flasks (750 ml) containing 350 ml of the plankton community. This filtrate corresponded to a final concentration of 34×10^3 cells l^{-1} of *A. tamarense*. Controls (also in triplicate) were made by adding 150 ml of f/2 medium instead of filtrate. Nitrate and phosphate concentrations in the control medium were adjusted to the same levels as in the *A. tamarense* filtrate (328.2 μM N-NO_3^- and 20.4 μM P-PO_4^{3-}) to avoid discrepancies due to different nutrient conditions. *A. tamarense* filtrate and control medium were added daily to the plankton community over a period of 3 d. Each day, 150 ml was removed from the test bottles, and replaced with either fresh filtrate or control medium. The bottles were incubated at 14°C.

The allelopathic effect of *Alexandrium tamarense* on the plankton community was assessed by daily chlorophyll *a* concentration measurements, and plankton cell enumeration at the beginning and the end of the experiment. To estimate the allelopathic effect of *A. tamarense* on co-occurring plankton species, we only considered the dominant species and those that allowed reliable cell counts. At least 100 cells of each group were counted per sample, except for *Ceratium* spp., *Chaetoceros* spp., and the ciliates *Mesodinium rubrum*, *Lohmaniella oviformis*, and tintinnids, from which at least 20 cells of each species/group were counted per sample. A total of at least 600 cells were counted per sample. Besides counting the plankton species cell numbers during the experiment with the plankton community, we observed formation of temporary cysts of *Scrippsiella trochoidea*. Thus, we also counted the number of cysts (100 to 250 cysts were counted per sample). The effect of *A. tamarense* filtrate on the bacterial community was examined by monitoring the bacterial numbers daily.

Toxicity of *Alexandrium tamarense* (KAC 02) cells suspension and filtrate was estimated on 1 occasion using *Artemia* sp. (Vanhaecke et al. 1981) and *Heterocapsa triquetra* bioassays (Schmidt & Hansen 2001).

Allelopathic effect of *Alexandrium* spp. on algal monocultures. The allelopathic effect of 5 non-axenic strains of *A. tamarense* (KAC 02, CCAP 1119/4, CCAP 1119/1, CCAP 1119/8, CCAP 1119/9) and 1 strain of *A. minutum* (AM89BM) was investigated on monocultures of the diatom *Thalassiosira weissflogii* (KAC 32) and the cryptophyte *Rhodomonas* sp. (KAC 30).

All algal cultures were grown in f/2 medium prepared with 28 psu seawater, at 20°C, and a light:dark cycle of 12:12 h, 70 µmol photons m^{-2} s^{-1}, under cool white fluorescent light. Allelopathic experiments were performed under the same conditions. The cultures used in these experiments were in exponential growth when used in the tests (cell numbers ranged between 18.2 and 30.6×10^3 cells ml^{-1}).

Thalassiosira weissflogii and *Rhodomonas* sp. were exposed for 48 h to cell-free filtrate from each *Alexandrium* strain. The test was made in triplicate 20 ml scintillation glass vials. *Alexandrium* filtrate and the target species cells were combined to give a final concentration corresponding to 10.5×10^3 *Alexandrium*:42×10^3 target cells ml^{-1}, 1:4 ratio. This was the initial concentration in the test bottles. Controls were made as previously described. The allelopathic effect on the target cells was measured by comparing the cell numbers in the filtrate treatments with the controls after 24 and 48 h. The difference between the cell numbers in the controls (Ctn) and in the filtrate treatments (Ftn) for the same sampling occasion, normalized by the cell numbers in the control, and expressed as percentage (Eq. 1), was called the allelopathic effect (AE). It represents the percentage of decrease or increase of cells in the filtrate relative to the control.

$$AE = \left[\frac{(Ctn - Ftn)}{Ctn} \right] \times 100 \qquad (1)$$

Relation between toxicity and allelopathy. By comparing the effect of the toxic and non-toxic species/strains of *Alexandrium* on *Thalassiosira weissflogii* and *Rhodomonas* sp., we tested whether the allelopathic effect of *Alexandrium* spp. was associated with the production of PSP toxins. PSP toxins were analyzed using HPLC, to identify the toxic and non-toxic *Alexandrium.* Samples for the toxin analysis (50 ml) were taken at the beginning of the experiment with the algal monocultures.

Analytical procedures. Samples for cell counts of *Alexandrium* spp. and natural plankton species were preserved with acidic Lugol's solution, and counted with an inverted microscope according to Utermöhl (1958). Samples for cell counts of target algae were preserved by freezing with liquid-N (*Thalassiosira weissflogii*), and with glutaraldehyde 0.5% (*Rhodomonas* sp.). Counts of *T. weissflogii* and *Rhodomonas* sp. were done using a flow cytometer (FACScalibur, Becton Dickinson). Bacterial abundance was estimated by flow cytometry according to the method of del Giorgio et al. (1996).

Chlorophyll *a* concentrations were determined by fluorometry using ethanol extracts of cells retained on GF/C filters (Jespersen & Christoffersen 1987). Specific growth rates of the phytoplankton community were calculated based on the chlorophyll *a* values, according to Eq. (2):

$$\mu \ (d^{-1}) = \frac{(\ln N_1 - \ln N_0)}{t} \qquad (2)$$

where N_1 and N_0 are the chlorophyll *a* concentrations at t_1 and t_0, and t is the time, in days, between sampling occasions.

The *Artemia* sp. bioassay used to test the toxicity of *Alexandrium tamarense* was based on Vanhaecke et al. (1981). *Artemia* sp. eggs were hatched in filtered seawater (31.5 psu), at 20°C under continuous aeration and light. After 24 h, hatched nauplii were transferred to freshly filtered seawater and kept for an additional 24 h. The test was performed in multiwell plates (Falcon, 12 wells), and approximately 30 *Artemia* sp. were added per well. The toxic effect of both cell suspension and filtrate of *A. tamarense* on *Artemia* sp. was tested. Cells and filtrate were added to each well, corresponding to 34×10^3 cells l^{-1} of *A. tamarense.* f/2 medium was used as a control. Mortality of *Artemia* sp. nauplii was examined after 24 h by counting, with a stereomicroscope, the number of dead nauplii and comparing with the total number (percentage mortality).

A bioassay with the dinoflagellate *Heterocapsa triquetra*, based on Schmidt & Hansen (2001), was performed by exposing *H. triquetra* cells to cell suspension and filtrate of *Alexandrium tamarense* and observing the number of non-motile cells after 4 h. Cell suspension and filtrates of *A. tamarense* (2 ml, final concentration 34×10^3 cells l^{-1}) were mixed with *H. triquetra* culture (50 µl, final conc. 500×10^3 cells l^{-1}) in a multiwell plate (Falcon, 12 wells). Control experiments were carried out using f/2 medium instead of the *A. tamarense* culture. All treatments were carried out in triplicate. After 4 h of incubation, the number of non-motile *H. triquetra* was counted in each well, then 20 µl of Lugol's solution was added to the wells and the total number of *H. triquetra* cells was counted. The results were expressed as percentage of non-motile *H. triquetra* relative to total cell numbers.
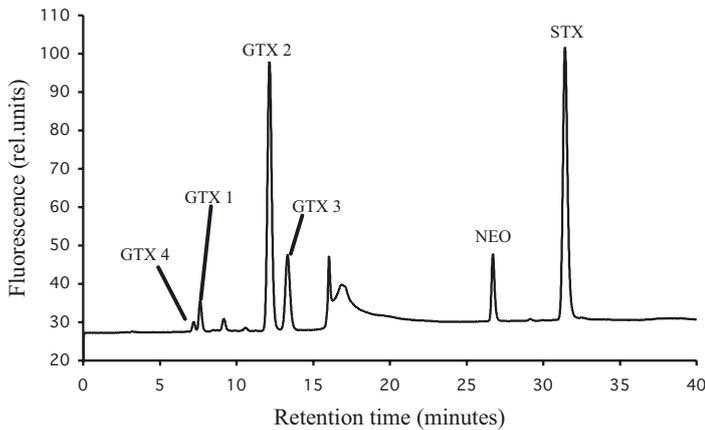
Fig. 1. Chromatogram of paralytic shellfish poison (PSP) standard mixture obtained using method described in 'Materials and methods'. GTX: gonyautoxin; STX: saxitoxin; NEO: neosaxitoxin; rel. units: relative units

PSP toxins analysis. PSP toxins were measured after separation by ion-pair chromatography and fluorometric detection after post-column oxidation. The separation was done using only 1 injection run to analyze gonyautoxins (GTXs 1-4), saxitoxins (STX), and neosaxitoxin (NEO) (Fig. 1). Algal cells were collected on GF/F filters and stored at –20°C until analysis. The cells were extracted in 0.03 M acetic acid, in an ice bath by sonication (3 times for 1 min, Ultrasonic Liquid Processor XL, Heat Systems). For determination of N-sulfocarbamoyl toxins, 150 µl of the acetic acid extract was mixed with 37 µl of 1.0 N hydrochloric acid and heated for 15 min at 90°C. After cooling down to room temperature the mixture was neutralized with 75 µl of 1.0 M sodium acetate. Samples were centrifuged for 10 min (20 800 g, Eppendorf Centrifuge 5417C) and the supernatant was filtered through a 0.45 µm PTFE (Scantec Lab, No. F2504-3) filter before injection into the HPLC system. Standards for saxitoxin (STX), neosaxitoxin (NEO), and gonyautoxins (GTX1, 2, 3, and 4) were purchased from the National Research Council, Marine Analytical Chemistry Standards Program (NRC-PSP-1B), Halifax, Nova Scotia, Canada. The standard run contained a mixture of STX (1.453 µM), NEO (1.404 µM), GTX1 (1.118 µM), GTX2 (0.961 µM), GTX3 (0.233 µM), and GTX4 (0.490 µM) (Fig. 1). All chemicals used were analytical grade. The reversed-phase HPLC system consisted of a Merck-Hitachi 7000 series equipment, with a fluorescence detector (Merck-Hitachi L7480) and a Phenomenex prodigy ODS(3) (5 µm) 250–4.6 mm column. The method used 3 aqueous eluents (Table 1); the gradients are presented in Table 2. The oxidizing reagent used for the post-column derivatization consisted of 10 mM periodic acid and 550 mM NH_3

solution (flow: 0.3 ml min^{-1}), and the acidifying reagent of 1 M acetic acid (flow: 0.4 ml min^{-1}). The post-column reaction took place at 50°C using a 10 m PEAK tubing inner diameter 0.25 mm. Fluorescence detection was employed with excitation and emission wavelengths of 330 and 395 nm respectively. N-sulfocarbamoyl toxins concentrations were determined by the difference in the concentrations of these readings from those obtained by acetic acid extraction.

Statistical analyses. Statistical analyses were performed using the software SPSS 10 for Macintosh. A Student's t-test was used to compare the toxic effect of *Alexandrium tamarense* grown at 14 and 20°C on the plankton community (in terms of growth rate), on the dinoflagellate *Scrippsiella trochoidea* (number of cells that died and cysts formed), on the dinoflagellate *Heterocapsa triquetra* (number of non-motile/dead cells), and on the brine shrimp *Artemia* sp. (mortality). A Student's t-test was also used to compare whether growth rates of the phytoplankton community, and the bacterial numbers in the treatment that received *A.*

Table 1. Eluents used in liquid chromatography

Eluent	Method
Eluent 1	
97.5%	11 mM octanesulfonic acid (sodium salt) and 40 mM phosphoric acid, adjust pH to 6.9 with NH_3
2.5%	Tetrahydrofurane
Eluent 2	
82.5%	11 mM octanesulfonic acid (sodium salt) and 40 mM phosphoric acid, adjust pH to 6.9 with NH_3
15%	Acetonitrile
2.5%	Tetrahydrofurane
Eluent 3	
97.5%	40 mM phosphoric acid, adjust pH to 6.9 with NH_3
2.5%	Tetrahydrofurane

Table 2. Eluent gradients used for chromatography. Injection: 10 µl; flow: 1.0 ml min^{-1}; column temperature: room temperature

Time (min)	Eluent 1 (%)	Eluent 2 (%)	Eluent 3 (%)
0	50	0	50
10	50	0	50
12	0	100	0
35	0	100	0
36	100	0	0
47	100	0	0
48	50	0	50
57	50	0	50

tamarense filtrates, differed from the controls on specific experimental days. ANOVA and Tukey's post hoc test were used to test whether the final plankton community in the control differed from that in the filtrate treatment. A linear regression was used to correlate the allelopathic effect of the different strains of *A. tamarense* and *A. minutum* on *Thalassiosira weissflogii* and *Rhodomonas* sp. with their PSP toxin content. ANOVA was used to test if the regression found was significant. The data were tested for normality and homogeneity of variance. Arcsine of the square root was used to transform the *Rhodomonas* sp. data to meet these assumptions.

RESULTS

Effect of *Alexandrium tamarense* on natural plankton community

There was no difference between the effect of filtrate obtained from *Alexandrium tamarense* grown at 14 and 20°C, for any of the parameters compared, i.e.

Table 3. Plankton species/groups composition in Hopavågen Bay, Trondheim Fjord, Norway. Underlined species/groups are those used to estimate allelopathic effect of *Alexandrium tamarense*. Initial cell concentration for the dominant species/group is shown (n = 3, mean ± SD)

| Group/species	Cells 10^2 l^{-1}
Dinoflagellates	
<u>Scrippsiella trochoidea</u>	95.7 ± 8.5
Ceratium fusus	
Ceratium furca	
<u>Ceratium</u> spp.	0.6 ± 0.3
Gonyaulax spp.	
Dinophysis spp.	
<u>Small dinoflagellates</u> (<30 μm)	60 ± 0,1
Flagellates	
<u>Nanoflagellates</u> (<20 μm)	503 ± 53
Dictyocha speculum	
Diatoms	
<u>Leptocylindrus</u> spp.	341 ± 34
<u>Chaetoceros</u> spp.	2.13 ± 0.7
<u>Skeletonema costatum</u>	35.2 ± 2.2
Eucampia spp.	
Guinardia spp.	
Rhizosolenia spp.	
Pseudo-nitzschia spp.	
Euglenoides	
Ciliates	
<u>Mesodinium rubrum.</u>	7.9 ± 1.4
<u>Tintinnids</u>	5.1 ± 0.7
Tontonia gracillima	
Strobilidium spiralis	
<u>Lohmaniella oviformis</u>	2.8 ± 0.3
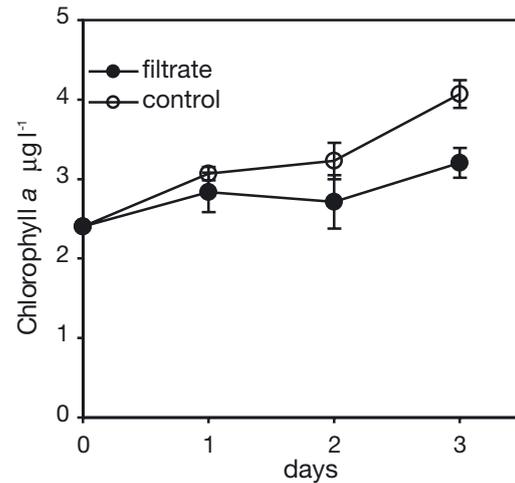
Fig. 2. Growth curve of phytoplankton community (expressed as chlorophyll *a*) in the treatment with *Alexandrium tamarense* filtrate and control (filtrate n = 6, control n = 3, mean ± SD)

phytoplankton community growth rates, number of dead *Scrippsiella trochoidea* cells and temporary cysts formed, bacterial numbers, effect on *Heterocapsa triquetra*, and on the mortality of *Artemia* sp. (*t*-test, p > 0.05 for each comparison). Since there was no difference in the effect of these 2 filtrate treatments, these data were pooled and presented as 1 data set.

The plankton community was composed of different species of dinoflagellates, nanoflagellates, diatoms, euglenoids and ciliates (Table 3). At the start of the experiment, the dominant groups/species were the dinoflagellate *Scrippsiella trochoidea*, nanoflagellates, and the diatoms *Leptocylindrus* spp. (Table 3).

The growth of the plankton community was inhibited after exposure to the compounds released by *Alexandrium tamarense*. After 3 d exposure, the chlorophyll *a* concentration was lower in the treatment with *A. tamarense* filtrate than in the control (Fig. 2). Consequently, the growth rate of the plankton community was significantly lower (*t*-test, p < 0.05) in the filtrate treatment (μ = 0.08 d^{-1}) than in the controls (μ = 0.16 d^{-1}).

Alexandrium tamarense filtrate had an inhibitory effect on ciliates and on all phytoplankton organisms except the small dinoflagellates (<30 μm), which increased in the filtrate treatment (Fig. 3). The increase in the cell numbers of small dinoflagellates was not significant at the 5% level (*t*-test, p > 0.05), but only at the 10% level (*t*-test, p < 0.1). The degree of inhibition was different for each group/species, causing a change in the species abundance and dominance during the experiment. Ciliates, nanoflagellates and the dinoflagellate *Scrippsiella trochoidea* were the most affected groups/species (Fig. 3).
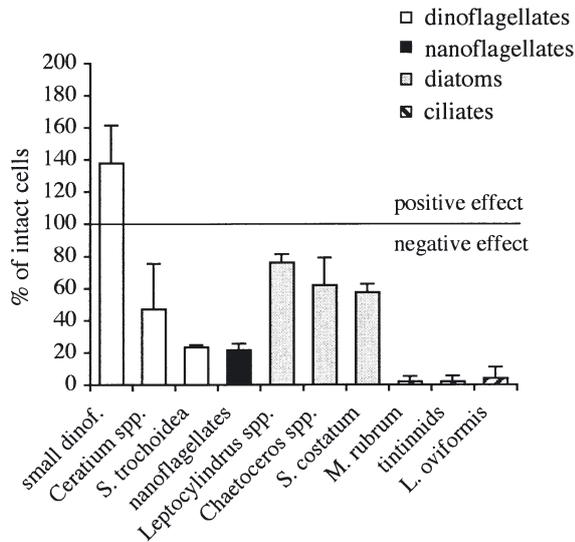
Fig. 3. Percentage of intact cells for each species/group of the plankton community in the treatment with *Alexandrium tamarense* filtrate relative to cell numbers in the control on Day 3 (n = 3, mean ± SD). Values >100% indicate that growth was higher in the filtrate treatment than in the control (positive effect). Full specific names as in Table 3

The relative contribution of each group/species to the total community abundance and the species dominance changed during the experiment in the control and in the filtrate treatment (Fig. 4). However, the changes in the control were different from those in the filtrate treatment, i.e. the resulting plankton community in the filtrate was different from that in the control (ANOVA, p < 0.05), indicating that the additions of filtrate interfered in the algal species interactions and altered the plankton community composition. Thus, the cell numbers of most of the species/groups (*Scrippsiella trochoidea*, nanoflagellates, *Leptocylindrus* spp., *Skeletonema costatum*, and the ciliates *Mesodinium rubrum*, *Lohmaniella oviformis*, and tintinnids) in the final plankton community in the filtrate differed significantly from those

in the controls (Tukey's post hoc, p < 0.01). The cell numbers of *Ceratium* spp. in the filtrate differed from the control at p < 0.075, and small dinoflagellates at p < 0.10. Only *Chaetoceros* spp. showed no difference between the filtrate and the control at any significance level (p > 0.05). The allelopathic effect of *Alexandrium tamarense* on different organisms of the plankton community enabled species that were less affected by the filtrate, such as *Leptocylindrus* spp., to achieve dominance over highly affected species, such as nanoflagellates. In the controls on the other hand, nanoflagellates still dominated the community at the end of the experiment, as they had at the beginning.

Microscopical observation revealed that the filtrate of *Alexandrium tamarense* caused cell blistering, lysis, and loss of pigmentation in all phytoplankton groups/species, and empty diatom frustules. Besides causing a decrease in the cell number of *Scrippsiella trochoidea*, the filtrate also induced ecdysis and the formation of temporary cysts in this species. A preliminary test showed that these effects could already be observed after 24 h, although the number of damaged and dead cells and temporary cysts increased with prolonged exposure.

After the first addition of *Alexandrium tamarense* filtrate, bacterial numbers increased considerably in this treatment. At the end of the experiment, bacteria had a significantly higher biomass (*t*-test, p < 0.05) in the filtrate treatments (9×10^6 bacteria ml^{-1}) than in the control (1.2×10^6 bacteria ml^{-1}) (Fig. 5).

Alexandrium tamarense filtrate had no effect on *Artemia* sp., while exposure to cell suspensions of *A. tamarense* caused 40% of mortality in *Artemia* sp. (Fig. 6A). Both *A. tamarense* filtrate and cell suspension caused the immobilization and death of the dinoflagellate *Heterocapsa triquetra*, which were significantly different from those in the control (*t*-test, p < 0.01 and p < 0.05, respectively) after 4 h incubation (Fig. 6B). Prolongation of the test (>10 h), resulted in death (blistering and lysis) of the immobilised cells.
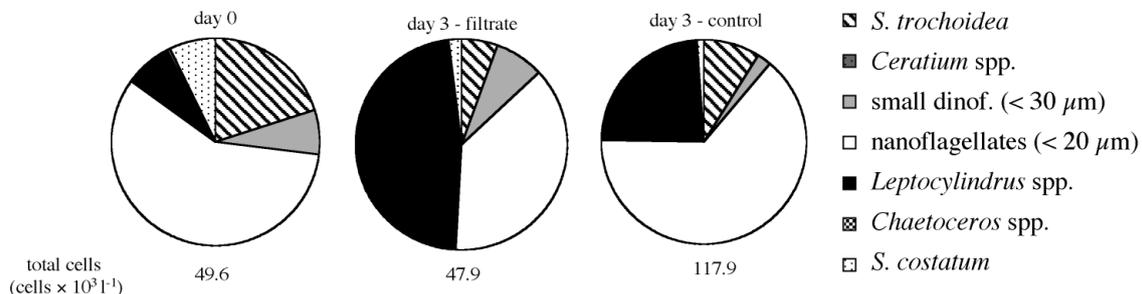

Fig. 4. Relative contribution of different plankton groups/species to total plankton community abundance: initially (Day 0), and after 3 d in the treatment with *Alexandrium tamarense* filtrate and in the control. Full specific names as in Table 3
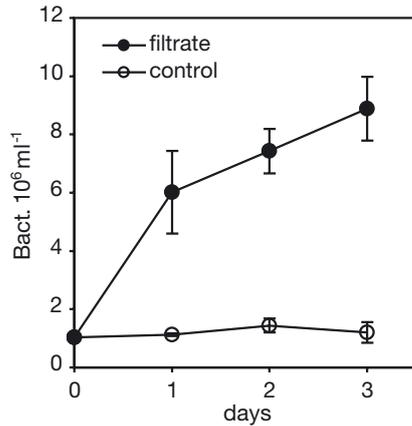
Fig. 5. Growth curve of bacteria in the treatment with *Alexandrium tamarense* filtrate and in the control (filtrate n = 6, control n = 3, mean ± SD)

Allelopathic experiments with algal cultures

Alexandrium minutum and 3 strains of *A. tamarense* were toxic, containing PSP toxins with different contents and profiles (Table 4). No PSP toxins were detected for the Strains CCAP 1119/9, and CCAP 1119/1 (*A. tamarense*).

All *Alexandrium* strains used in the experiment, independent of whether they produce PSP toxins or not, had an allelopathic effect on *Thalassiosira weissflogii* and *Rhodomonas* sp. (Fig. 7). Accordingly, no correlation was found between PSP toxin content and allelopathic effect (ANOVA, p > 0.05 for both species) (Fig. 8). In all filtrate treatments but 1 (*A. tamarense* CCAP 1119/4), *Rhodomonas* sp. was more affected than *T. weissflogii.*

Fig. 6. Effect of *Alexandrium tamarense* cells and filtrate on *Artemia* sp. and on *Heterocapsa triquetra.* (A) Percentage of dead *Artemia* sp. after 24 h exposure; (B) percentage of total immobilized (dead and alive) *H. triquetra* after 4 h exposure. No mortality of *Artemia* sp. was observed in control
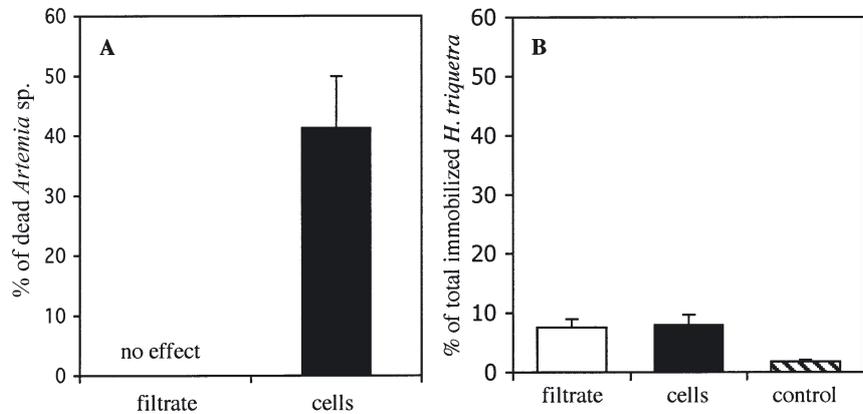

DISCUSSION

Blooms of *Alexandrium* spp. have been reported worldwide (Therriault et al. 1985, Hallegraeff et al. 1991, Shimada et al. 1996, Sorokin et al. 1996, Anderson 1998), usually associated with shallow salt ponds, coastal bays and open coastal waters or large estuaries (Anderson 1998). These blooms can reach densities of 2 to 4×10^6 cells l^{-1} (Sorokin et al. 1996) and even up to 10^8 cells l^{-1} (Cannon 1990). *Alexandrium* spp. has also been reported to occur in Hopavågen Bay (Chuaychan 1998). Therefore *Alexandrium* is a relevant local dinoflagellate genus, and our experiment showed a significant allelopathic effect of *A. tamarense* on a plankton community at realistic natural densities (34×10^3 cells l^{-1}).

Although the *Alexandrium* spp. cultures were not axenic, the possible effect of bacteria present in algal cultures has been tested and proved to be null (Suikkanen et al. in press). Also, Tillmann & John (2002) tested whether the allelopathic effect of different strains of *Alexandrium* on heterotrophic dinoflagellates could have been caused by the bacteria present in the cultures. They found no involvement of the bacteria in the effects observed.

Reports on the effect of temperature on the production of allelochemicals in phytoplankton are scarce in the literature. For the cyanobacteria *Oscillatoria angustissima* and *Calothrix parietina*, the accumulation of antibiotic substances in the medium is temperature-dependent (Issa 1999). However, our results
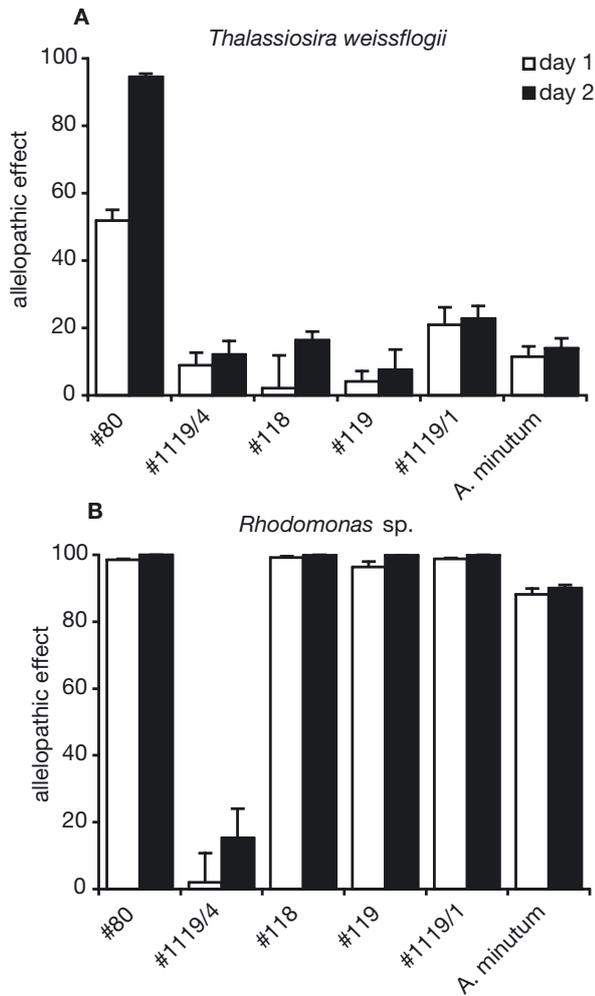
Table 4. *Alexandrium* spp. Paralytic shellfish poisoning (PSP) toxin profile and cellular toxin content (fmol cell^{-1}) in *A. tamarense* Strains KAC 02, CCAP 1119/4, CCAP 1119/8, and *A. minutum* AM89BM. nd = below analytical detection

Strains	STX	NEO	GTX-1	GTX-2	GTX-3	GTX-4	B1	C-1	C-2	C-3	C-4	Total
KAC 02	8.5	3.83	nd	0.33	nd	nd	0.39	nd	0.28	nd	0.39	13.7
CCAP1119/4	0.1	1.15	nd	nd	nd	nd	nd	0.02	nd	nd	nd	1.27
CCAP1119/8	0.05	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	0.05
AM89BM	0.25	22.5	nd	0.64	1.01	nd	nd	12.7	3.78	nd	nd	40.9

Fig. 7. *Alexandrium* spp. Allelopathic effect of *A. tamarense* Strains 80, 1119/4, 118, 119 and 119/1, and *A. minutum* Strain AM89BM on (A) *Thalassiosira weissflogii* and (B) *Rhodomonas* sp. after 24 h (Day 1) and 48 h (Day 2) of exposure to cell-free filtrate (n = 3, mean ± SD)
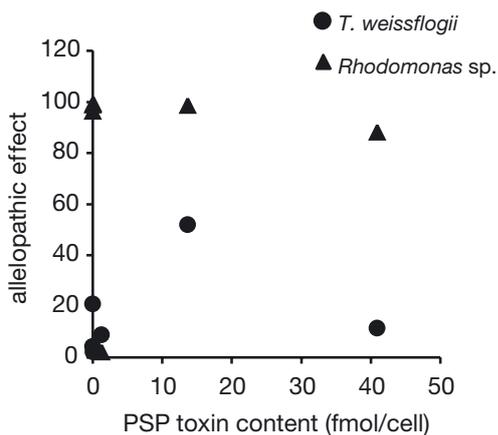

Fig. 8. *Alexandrium* spp. Relation between allelopathic effect on *Thalassiosira weissflogii* and *Rhodomonas* sp. and toxin content of each *Alexandrium* strain/species

showed that filtrate, whether from *Alexandrium tamarense* grown at 14 or at 20°C, had the same effects on the natural plankton community. This suggests that, at least within this range, temperature has no effect on the production and/or release of allelochemicals by *A. tamarense*. However, there is need for detailed examination of the effect of temperature on the production and accumulation of allelochemicals.

Effect of *Alexandrium* spp. on different plankton organisms

Effect on phytoplankton

The similar results obtained in the experiments with a natural community and algal monocultures (nanoflagellates and *Rhodomonas* sp. being more affected than diatoms and *Thalassiosira weissflogii*, respectively) show that the use of laboratory cultures may provide a representative result of the situation in the environment. The higher sensitivity of *Rhodomonas* sp. to the allelopathic compounds might be due to its weaker cell wall compared (e.g.) to the silica frustules of diatoms. *Rhodomonas* sp. has also been reported to be sensitive to cyanobacterial allelochemicals (Vance 1965). On the other hand, diatoms were one of the groups most affected by *Prymnesium parvum* allelochemicals (Fistarol et al. 2003). These reports suggest that the allelopathic effect may be dependent on specific group characteristics of the target algae, and also on the organisms producing the allelochemicals, which may produce compounds with different characteristics.

The effect of *Alexandrium tamarense* on the phytoplankton from the natural community can be divided into 3 types: (1) strong negative effect, causing a decrease in the biomass of the affected organism (e.g. *Scrippsiella trochoidea* and nanoflagellates); (2) inhibitory/sublethal effect, whereby the affected organism maintained a positive growth rate, although smaller than that of the control (e.g. *Leptocylindrus* spp.); (3) positive effect, whereby the target organism had a higher growth than that of the control (e.g. small dinoflagellates). The intensity of the allelopathic effects varied depending on the target organism, as has been observed for other phytoplankton species (Gross et al. 1991, Schmidt & Hansen 2001, Mulderij et al. 2003). The consequence of the positive growth of some species in our study was that the biomass of the community (chlorophyll *a*) increased during the experiment, although the increase in chlorophyll *a* and the growth rate were significantly lower than in the control, demonstrating a general inhibitory effect of *A. tamarense* allelochemicals. In cases where the allelo-

pathic algae cause very strong negative effects, such as *Prymnesium parvum*, most phytoplankton organisms die causing a decline in the plankton community, which is unable to maintain a positive growth rate (Fistarol et al. 2003).

It has been proposed that the differential effect of allelochemicals due to differential sensitivity of target species might influence the composition and biomass of phytoplankton communities (Mulderij et al. 2003). Allelochemicals can be beneficial to the releaser species by decreasing the number of competitors. However, because these compounds are released into the water, they may also benefit any species that is not so sensitive to the compounds. Therefore, more resistant species may achieve dominance over species more severely affected, altering the plankton community, as observed in our experiment. This is an example of distributed benefits, whereby certain organisms may indirectly benefit from compounds released by other species, while avoiding the metabolic costs of producing the allelochemicals (Huntley et al. 1986, Lewis 1986).

Although the increase in small dinoflagellate cell numbers was not strongly significant, we cannot say that they were negatively affected. This suggests that this group might have some resistance to allelochemicals released by *Alexandrium tamarense*, and raises the question if *A. tamarense* can be stimulatory to related organisms. The stimulatory effect may be due to a direct stimulatory effect of the compounds released by *A. tamarense*, to tolerance mechanisms of related species, or to an indirect effect related to a decrease in competition (phytoplankton) and grazing (ciliates).

Effect on potential predators

Alexandrium tamarense had a strong negative effect on ciliates, causing the death of most of the cells (98 %). Although some aquatic microorganisms can respond to chemical signals through movement (Wolfe 2000), the ciliates in our experiment could not escape the effect of *A. tamarense* exudates. However, the strong effect of *A. tamarense* filtrate on ciliates indicates that these compounds would cause the death of these organisms rather than act as a chemical signal triggering an escape mechanism. Hansen (1989) and Hansen et al. (1992) found that the toxic effect on ciliates becomes evident at high concentrations of *A. tamarense* (3000 cells ml^{-1}) and *A. ostenfeldii* (2000 cells ml^{-1}). The toxic effect on potential predators, especially at high concentrations, can in part explain why blooms of *Alexandrium* species can develop and persist for months (Hansen 1989).

Cell suspension of *Alexandrium tamarense* caused mortality of *Artemia* sp., while *A. tamarense* cell-free filtrate did, in contrast to the results of Lush & Hallegraeff (1996), who showed that *Alexandrium minutum* filtrate can be toxic to *Artemia* sp. These authors used a filtrate corresponding to approximately 6×10^5 *A. minutum* l^{-1}, whereas we used a much lower cell density (3.4×10^3 *A. tamarense* l^{-1}). Although *A. minutum* has a biovolume approximately 3 times less than *A. tamarense*, the cell concentration used by Lush & Hallegraeff (1996) was 100 times higher than that we used. Probably the higher cell concentration used by Lush & Hallegraeff (1996) is the reason why the filtrate was toxic in their experiment. It is interesting to note that the filtrate we used (at a concentration of 3.4×10^3 *A. tamarense* l^{-1}) was already sufficient to have a negative effect on phytoplankton.

Effect on bacteria

Filtrate from *Alexandrium tamarense* caused an increase in bacterial numbers in a similar way to allelochemicals released by the ichthyotoxic *Prymnesium parvum*, although the latter caused a decrease in bacterial production (Fistarol et al. 2003). In the present study, whether the increase in bacteria numbers was a direct stimulatory effect of *A. tamarense* allelochemicals or a response to the increase in organic compounds released by lysing plankton cells is unclear. The increase in bacterial numbers could also be related to the number of nanoflagellates (e.g. mixotrophic and heterotrophic flagellates), which were in high abundance in the control and decreased in the filtrate treatment. Consequently, the grazing pressure on bacteria was lower in the latter bottles, probably contributing to the high bacteria numbers in the filtrate bottles, as has been reported earlier (Shiah & Ducklow 1994, Carlsson & Caron 2001).

Consequences of allelopathic effect for the ecology of *Alexandrium*

Multiple-purpose compounds

Alexandrium tamarense affected more than 1 level of the plankton community, i.e. potential competitors (phytoplankton) and grazers (ciliates). *A. tamarense* exudates may, therefore, be used as an allelopathic strategy (competition) and for grazer deterrence. Although mesozooplankton was not present in the natural community, it has been shown that *Alexandrium* spp. can negatively affect *Artemia* sp. (Lush & Hallegraeff 1996, our study), and also other zooplankton

groups (Bagoien et al. 1996). Many secondary meta-bolites appear to have multiple functions such as anti-herbivory, antiparasitic, antifungal, allelochemical, and internal regulation (Seigler 1996). The release of a substance with multiple purposes gives a further advantage to the producing species and is an efficient strategy (Wolfe 2000). A good example of the advantage of producing multiple-purpose compounds was observed for the prymnesiophyte *Chrysochromulina polylepis*. During the height of a bloom of *C. polylepis* in Scandinavian waters (Kattegat/Skagerrak area, 1988), no potential grazers (heterotrophic flagellates, ciliates, copepods) were present in the subsurface bloom, and bacteria concentration and production were extremely low (Nielsen et al. 1990). Maestrini & Granéli (1991) suggested that a deleterious effect on all components of the food chain most likely contributed significantly to the bloom formation. Later, Schmidt & Hansen (2001) confirmed the deleterious effect of *C. polylepis* on several phytoplankton groups (dinoflagellates, diatoms, silicoflagellates, raphido-phytes, euglenophytes, cryptophytes, and prasino-phytes) in laboratory experiments. Schmidt & Hansen (2001) also suggested that the release of chemical compounds by *C. polylepis* could indeed explain a bloom like the one in 1988, although other factors such as pH and perhaps CO_2 affect the toxicity of *C. polylepis*, and must be taken into consideration.

Changing plankton community structure

Allelochemicals have been suggested as influencing planktonic species competition, succession, and bloom formation or maintenance (Pratt 1966, Keating 1977, Rice 1984 and references therein, Lewis 1986, Wolfe 2000, Rengefors & Legrand 2001, Vardi et al. 2002, Legrand et al. 2003). Regarding competition, since *Alexandrium tamarense* cells were not present in the test bottles (the allelopathic effect must be caused by released compounds), we do not know if *A. tamarense* would have achieved dominance in the plankton community. However, our results showed that the number of competitors decreased, which would be favourable to allelopathic species. Also, small dinoflagellates were positively affected, indicating that *A. tamarense* would probably beneficiate from the release of allelochemicals.

The changes in the plankton community structure observed in our study support other evidence that allelochemicals can interfere with species interactions and change the plankton community structure. Keating (1977) reported that cell-free filtrates of dominant cyanobacteria from a freshwater lake inhibited the growth of predecessor species, offering evidence for a probable involvement of allelopathy in algal succession in the lake and suggesting the implication of algal allelopathy as a controlling factor in bloom sequence determination. Allelopathy can also be used by algae to achieve dominance, as shown for *Olisthodiscus luteus* by Pratt (1966) in Narragansett Bay. In the Sea of Galilee, the alternating dominance by the dinofla-gellate *Peridinium gatunense* and the cyanobacterium *Microcystis* sp. may be caused by mutual density-dependent allelopathic interactions (Vardi et al. 2002).

The primary effect of allelochemicals is on the organisms of the plankton community, causing (e.g.) death and decreasing growth rate. This changes the species dominance and the community structure. Changes in the community structure have indirect effects, because a new community arises, with different dominant species and different competitive requirements (whereby the best competitor will be that most resistant to the allelochemicals). The species interactions in this new community will lead to a new and different species succession than that which would occur without interference by the allelochemicals. Whether directly or indirectly, allelochemicals are the initial trigger factor causing changes and affecting the plankton environment.

Allelochemicals and PSP toxins

The lack of correlation between PSP toxin production and allelopathic effect shown by our results has also been suggested by other studies. Tillmann & John (2002) reported no correlation between *Alexandrium* spp. PSP toxin content and their effect on heterotrophic dinofla-gellates. Based on indirect evidence, Arzul et al. (1999) also suggested that substances other than PSP toxins affected phytoplankton. It has also been suggested that it is not the PSP toxins that have toxic effects on zooplank-ton (Huntley et al. 1986, Lush & Hallegraeff 1996), fishes (Ogata & Kodama 1986), and haemolysis (Simonsen et al. 1995, Arzul et al. 1999). All these effects are comparable and similar to those of lytic compounds, as indicated by the microscopic observation of cell lysis of phytoplank-ton, ciliates (this study), and heterotrophic dinoflagel-lates (Tillmann & John 2002). Other ichthyotoxic algae have also been found to have allelopathic properties, such as *Prymnesium parvum* (Fistarol et al. 2003, Granéli & Johansson 2003, Skovgaard & Hansen 2003), *Karenia mikimotoi* (*Gyrodinium* cf. *aureolum*) (Arzul et al. 1993), and *Chrysochromulina polylepis* (Edvardsen & Paasche 1998, Schmidt & Hansen 2001), reinforcing the idea that ichthyotoxicity, allelopathy, and haemolysis may be connected. Arzul et al. (1999) also suggested that allelopathic activity of *Alexandrium* spp. is caused by a complex of chemicals rather than by a single substance.

In conclusion, the results presented here showed that compounds produced and released by *Alexandrium tamarense* are likely to have multiple ecological functions, affecting more than one level of the plankton community and altering the plankton community structure. The fact that our experiment used natural concentrations of *A. tamarense* and the effect was tested on a plankton community in which *A. tamarense* has been reported, indicates that allelopathy is a relevant part of the ecology of this species, interfering in species interactions and, perhaps, in bloom formation.

PSP toxins were not responsible for the allelopathic effect observed. The independence of *Alexandrium* spp. allelopathy from PSP toxin production shows that this genus produces other active compounds that may be more important to the success of *Alexandrium* than PSP toxins, since the allelochemicals affect species which directly interact with *Alexandrium*.

Based on observations of phytoplankton succession in Linsley Pond, Hutchinson (1944) pointed out that some events could not be explained on the basis of abiotic factors and that allelochemicals were the most plausible explanation. We do not want to underrate the significance of abiotic factors on phytoplankton interactions, but to call attention to the fact that allelochemicals also may influence these interactions.

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