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Allelopathic effects of *Alexandrium tamarense* on other algae: evidence from mixed growth experiments

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ABSTRACT: The effect of 2 strains (Alex2 and Alex5) of the marine red tide dinoflagellate *Alexandrium tamarense* on 10 other planktonic algal target species common in temperate waters was studied in mixed growth experiments under nutrient-rich conditions. In a comparative approach, the 2 strains of *A. tamarense*, similar in their cellular paralytic shellfish toxin (PST) content, were selected because of their fundamentally different lytic potencies. The Alex2 strain clearly affected all target algae while the Alex5 strain had no negative effect on the growth of any of the target species during the study period, even though cell concentrations of Alex5 became very high (2×10^4 cells ml^{-1}). As both strains contained comparable amounts of PST, this confirmed previous suggestions that so far unidentified compounds are causing the negative effects on other algae. Sensitivity of the tested algae to Alex2 differed considerably. The growth of some species was affected at very low Alex2 cell concentrations (<10^2 cells ml^{-1}), while the growth of other algae was not affected until cell concentrations exceeded 10^3 cells ml^{-1}. While a complete dieoff was the ultimate fate for almost all target species when grown in mixed culture with Alex2, *Scrippsiella trochoidea* formed temporary cysts, the number of which remained constant during the course of the experiment. The pH in the mixed cultures increased as the cultures grew dense. This had a substantial effect on Alex5 in the mixed cultures, in which Alex5 eventually died off because the target species have a higher tolerance to high pH. pH values did not determine the outcome of the experiments with Alex2 because the adverse effects of Alex2 on the growth of the other algae was evident before pH values became too high. Lytic extracellular compounds, which are produced by the large majority of *A. tamarense* strains tested so far, clearly have the potential to benefit this dinoflagellate by reducing competitor growth rates.

KEY WORDS: *Alexandrium* · PST · Paralytic shellfish toxin · Allelopathy · Growth · Algae · pH

INTRODUCTION

Species of the dinoflagellate genus *Alexandrium* are widely distributed in temperate, subtropical and tropical coastal waters. They have received much attention over the past 50 yr because they are toxic. Their toxicity has been shown to affect an extraordinarily wide spectrum of organisms including humans as consumers of contaminated seafood, large multicellular organisms like fish, mussels, and marine mammals, and competitors (other microalgae) as well as protistan and metazoan grazers. This broad range of target organisms is explained by the fact that *Alexandrium* species are able to produce a whole suite of different toxins. Best known are the paralytic shellfish toxins (PSTs), of which 24 naturally occurring derivatives have now been described (Shimizu 1996). These are potent neurotoxins with sodium channel blocking activity, and they are responsible for the reported toxic effects of *Alexandrium* spp. on marine mammals and humans (Cembella 2003).

For a long time, PSTs were also suspected to be responsible for the effects of *Alexandrium* spp. on metazoan and protistan grazers and algal competitors (Sykes & Huntley 1987, Hansen 1989, Hansen et al.

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1992, Fulco 2007). Turner et al. (1998) compiled data on the effects of *Alexandrium* spp. on copepods and reached the conclusion that PSTs most likely were not involved in the effects observed on copepods. With respect to protistan targets, it is now quite clear that PSTs are not involved in the negative observed effects (Tillmann & John 2002, Tillmann et al. 2008a). Thus, it is evident that *Alexandrium* spp. produce some as yet unknown compounds with lytic capabilities in addition to the neurotoxic PSTs, and that these unknown compounds are responsible for the effects on algae and protistan grazers. Chemically mediated effects of planktonic algae on competitors and/or micrograzers have recently gained much interest and have been covered in a number of comprehensive reviews (Cembella 2003, Legrand et al. 2003, Granéli & Hansen 2006, Tillmann et al. 2008b).

Six species of *Alexandrium* have so far been shown to have the ability to kill other algae and heterotrophic protists (Tillmann et al. 2008a). Among the initial sublethal effects on tintinnids are alterations of swimming speed and pattern, including backwards swimming (Hansen 1989, Hansen et al. 1992, Fulco 2007). After a few minutes of exposure, cells tend to swell and subsequently lyse. The notion that the unknown *Alexandrium* spp. lytic compounds may primarily act on cell membranes suggests that they may also cause the death of fish by destroying their gills (Mortensen 1985, Cembella et al. 2002).

In contrast to the PSTs, which are mainly stored inside the cells, the toxicity of *Alexandrium* spp. for other protists is caused by lytic compounds that are excreted into the medium (Hansen 1989, Tillmann & John 2002, Fistarol et al. 2004b, Tillmann et al. 2008b). These extracellular compounds can directly affect heterotrophic protists without *Alexandrium* spp. being ingested. In fact, heterotrophic protists may grow just as well on PST-containing *Alexandrium* spp. cells as on other prey organisms (Hansen 1989, Hansen et al. 1992, Kamiyama & Suzuki 2006) without any apparent effects of the incorporated PSTs.

A number of factors determine how *Alexandrium* spp. affects other protists. For the lytic species and strains studied so far, the cell concentration of *Alexandrium* spp. is very important. Not surprisingly, the negative effect is always most pronounced at high *Alexandrium* spp. cell concentrations. However, considerable variation in toxicity towards target cells occurs among species/strains. Some species or strains are apparently lytic at very low cell concentrations (EC_{50} <10 to 40 cells ml^{-1}), while others need to be present in much higher concentrations (>7000 cells ml^{-1}) to kill target cells (Hansen 1989, Hansen et al. 1992, Tillmann et al. 2008a). Almost all *Alexandrium* species and strains tested so far have been shown to negatively affect other protists. However, while most target cells that have been tested are killed by *Alexandrium* spp., a few species/strains seem insensitive to *Alexandrium* lytic compounds, even at very high cell concentrations (Tillmann et al. 2007).

Nearly all previous experiments were performed over short exposure periods lasting from minutes up to 24 h. It has been shown that the length of the incubation time may lead to considerable differences in the response of target species to *Alexandrium* spp. (Hansen 1989). Thus, even though *Alexandrium* spp. can kill target cells in minutes if cell concentrations of the dinoflagellate are very high, sub-lethal cell concentrations may take several days to affect target cells negatively, leading to a reduced growth rate of the target cells. Thus, there is clearly a need for studies focussing on long-term exposure of different target cells to *Alexandrium* spp. This can be achieved by carrying out mixed growth experiments of *Alexandrium* spp. and selected target algae.

Such experiments are not trivial, because a number of factors may influence their outcome (see Schmidt & Hansen 2001), making interpretations difficult. The main difficulty is to unambiguously separate effects caused by allelochemicals from other effects such as direct competition for nutrients or other interspecific interactions like mixotrophy or predation. The potentially most confounding problem arises from changing pH levels in mixed batch cultures in combination with species-specific pH limits of growth (Hansen 2002). Some of these problems might be avoided by exposing target cells to culture filtrate of the donor species. However, this approach has limited value because some toxins cannot be quantitatively filtered (Ulitzur 1973, Tillmann et al. 2008b) or are labile, so toxic effects may cease after some time because compounds are not exuded continuously as in the case of mixed cultures (Granéli & Hansen 2006).

An elegant way to overcome all these problems is a comparative approach using different strains of the same algal species that are virtually identical in all aspects except toxicity. In a detailed study to simultaneously investigate genotypic diversity and phenotypic variation among clonal isolates from a single *Alexandrium tamarense* population (Alpermann et al. 2009), a total of 67 clonal cultures were screened for their allelochemical potency and found to be very variable in their expression of lytic properties. Only 2 of these 67 clonal isolates did not cause severe damage to *Rhodomonas salina* target cells in a short-term bioassay, indicating that the absence of measurable lytic activity within different strains of *A. tamarense* is a rare exception. The non-lytic clonal *A. tamarense* strains, together with well characterised lytic strains now available (Tillmann et al. 2009), represent perfect tools

to analyze various aspects of the ecological effects and consequences of being lytic in a comparative approach.

The aim of the present study was to investigate the growth response of *Alexandrium tamarense* and other competing microalgae when grown in mixed cultures. By comparing the effects of a lytic with an apparently non-lytic strain of *A. tamarense* in mixed cultures, we were able to unambiguously attribute the observed effects to allelochemical interactions.

MATERIALS AND METHODS

Algal cultures. Two clonal strains of *Alexandrium tamarense*, Alex2 and Alex5, were used in the experiments. Both were isolated from one plankton net haul from coastal waters of the Scottish North Sea coast by micro-capillary isolation of single cells (for details see Alpermann et al. 2009). These 2 strains were selected out of about 100 clonal strains (all originating from the same net haul) based on their different lytic capacity as quantified by a *Rhodomonas* bioassay (Tillmann et al. 2009). Ten different target species, all common in temperate waters and representing different systematic affiliations were obtained from various culture collections. The dimensions of the target species were measured on live cells under the microscope (n = 20 to 30 cells), and cell volumes were estimated using simple volumetric formulae (Table 1).

All stock cultures were grown non-axenically in K medium (Keller et al. 1987), supplemented with selenite (Dahl et al. 1989) prepared from 0.2 µm sterile-filtered (VacuCap, Pall Life Sciences) natural North Sea water (salinity 32, pH adjusted to 8.0) in 500 ml Erlenmeyer flasks under controlled conditions at 15°C with artificial light at a photon flux density of 100 µmol m^{-2} s^{-1} (provided by cool white fluorescent lamps) on a 16:8 h light:dark cycle. Stock cultures were diluted regularly and were in exponential growth at the start of the experiment.

Experimental design. Cell concentrations in all stock cultures were estimated by microscopic cell counts. Triplicate mixtures (100 ml mixture in 100 ml Erlenmeyer flasks) of each *Alexandrium tamarense* strain and each target species were prepared to initial concentrations of about 100 cells ml^{-1} for each species, with the exception of *Ceratium lineatum*, for which an initial concentration of 500 cells ml^{-1} was chosen. In addition, triplicate monocultures for each species and *A tamarense* strain were set up, resulting in 96 flasks in total. After mixing, 2 ml samples were taken to measure initial algal cell concentrations.

Flasks were placed randomly on an illuminated bench (100 µmol m^{-2} s^{-1}, 16:8 h light:dark cycle) in a temperature-controlled culture room at 15°C. To avoid small differences in light exposure, positions of the flasks were randomized every day. At the beginning, daily samples (2 ml) for cell counts were taken from each flask. As soon as a target species in mixtures with *Alexandrium tamarense* strain Alex2 was no longer detectable, sampling frequency was reduced to every 2 d. Every second day, pH was measured directly in the experimental flasks using an EcoScan pH5 (Eutech Instruments) pH-meter. Sampling was terminated when a target species reached stationary growth phase.

Cell counts and growth rate calculation. All counts of donor and target cell concentrations were performed on samples fixed with Lugol's (final concentration 2%) and using an inverted microscope (Zeiss Axiovert 40 C). Depending on the cell concentration, the volume for cell counts varied from 0.1 to 2 ml. Whole chambers or representative sub-areas were counted. The total number of cells counted per species was always >400 cells

Table 1. Target species cultures, their approximate cell volume and origin. CCMP: Provasoli-Guillard National Center for Culture of Marine Phytoplankton; KAC: Kalmar Culture Collection; SAG: Culture Collection of Algae at the University of Göttingen; AWI: Alfred Wegener Institute for Polar and Marine Research; SCCAP: Scandinavian Culture Collection of Algae and Protozoa; MBL: Marine Biological Laboratory

Species	Strain no.	Approx. size (cell volume, $µm^3$)	Collection/origin (if known)
Rhodomonas salina (Cryptophyceae)	KAC30	250	KAC
Dunaliella salina (Chlorophyceae)	–	590	AWI
Skeletonema costatum (Bacillariophyceae)	SAG 19.99	200	SAG
Thalassiosira weisflogii (Bacillariophyceae)	–	900	AWI
Ceratium lineatum (Dinophyceae)	–	8100	MBL/The Sound, Denmark, 1995
Prorocentrum minimum (Dinophyceae)	SCCAP K-0295	1040	SCCAP Kattegat, Denmark, 1989
Scrippsiella trochoidea (Dinophyceae)	–	1500	AWI/North Sea, 2001
Heterocapsa triquetra (Dinophyceae)	SCCAP K-0481	1040	SCCAP The Sound, Denmark, 1988
Chrysochromulina ericina (Prymnesiophyceae)	CCMP 281	600	CCMP
Heterosigma akashiwo (Raphidophyceae)	CCMP 2274	1900	CCMP

per sample, except for the first few days when cell concentrations were below 200 cells ml^{-1} and in the case of target species that were drastically reduced in abundance over the course of the experiment.

The exponential growth rate μ (d^{-1}) was calculated separately for each replicate by linear regression of log (ln)-transformed cell number versus time for a time period indicated in Table 2. For replicate cultures, covariance analysis of regression lines using Statistica software (StatSoft) showed that exponential growth in replicate cultures did not differ significantly ($p > 0.1$); therefore, the exponential growth rate in the treatments was calculated using mean cell numbers of the 3 replicates, and treatments were compared using covariance analysis. The growth rate for 2 consecutive sampling dates was calculated as:

$$\ln(N_{t_1}) - \ln(N_{t_0})/t_1 - t_0$$

where N_{t_1} is the cell concentration at time t_1, and N_{t_0} is the initial cell number.

In this case, the growth rate was calculated for each replicate, and mono- and mixed cultures were compared using Student's *t*-tests.

Measurement of PSTs. After 25 d of growth at the early stationary phase, cells of the 2 *A. tamarense* strains (Alex2 and Alex5) grown in monoculture were harvested for PST analysis. A 14 ml sample of each monoculture was centrifuged (3220 × *g*, 10 min at 10°C). Cell pellets were suspended in 1.0 ml of 0.03 M acetic acid and subsequently transferred into a FastPrep tube containing 0.9 g of lysing matrix D. The samples were homogenized by reciprocal shaking at maximum speed (6.5 m s^{-1}) for 45 s in a Bio101 FastPrep instrument (Thermo Savant). After homogenization, samples were centrifuged (Eppendorf 5415 R) at 16 100 × *g* at 4°C for 15 min. The supernatant (400 µl) was transferred to a spin-filter (pore-size 0.45 µm, Millipore Ultrafree) and centrifuged for 30 s at 800 × *g*. The filtrate was transferred into an LC vial and analyzed by LC–FLD (liquid chromatography–fluorescence detection) with post-column derivatisation as described in Tillmann et al. (2009).

RESULTS

Paralytic shellfish toxins in *Alexandrium tamarense* strains

Both clonal strains of *Alexandrium tamarense* produced PSTs (Fig. 1). Cell quota of total PST were higher for Alex5 (43 fmol $cell^{-1}$) than for Alex 2 (27 fmol $cell^{-1}$). PST profiles were quite similar for both strains, with saxitoxin (STX), neosaxitoxin (NEO) and N-sulphocarbamoyl C toxins being most prominent.

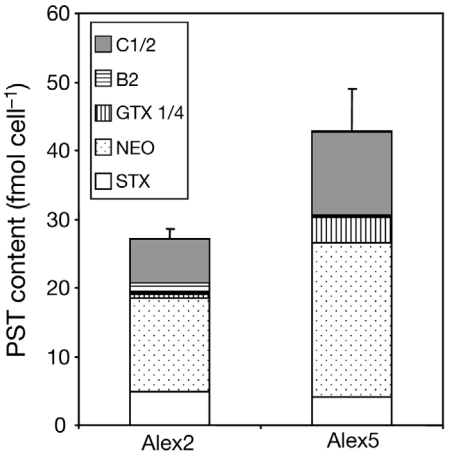

Fig. 1. *Alexandrium tamarense*. Paralytic shellfish toxin (PST) cell quota and composition of the 2 *A. tamarense* clones, Alex2 and Alex5. STX: saxitoxin; NEO: neosaxitoxin; GTX 1/4: gonyautoxin-1/4; B2: N-sulphocarbamoyl B2; C1/2: N-sulphocarbamoyl C1 and C2. Minor compounds (<0.05 fmol $cell^{-1}$) are not indicated in the key. Data are mean (+SD) of n = 3 cultures

Mixed growth experiments — growth of target species

Growth curves of each target species in monoculture and in mixtures with *Alexandrium tamarense* strain Alex2 or Alex5, as well as pH of the cultures, are summarised in Fig. 2. Growth curves of all target species were similar in monoculture and in mixed culture with Alex5. The exponential growth rate of the target species (Table 2) ranged from 0.36 d^{-1} (*Prorocentrum minimum*) to 1.48 d^{-1} (*Skeletonema costatum*). For all target species, exponential growth rates did not differ significantly between monocultures and mixed cultures with Alex5 (co-variance analysis, $p > 0.21$). In contrast, growth of the target species was drastically affected when cultured with Alex2. *Dunaliella salina* and *Rhodomonas salina* completely disappeared after 1 d of incubation. Cell counts of *S. costatum* and vegetative cells of *Scrippsiella trochoidea* immediately started to decline and became undetectable after 4 to 6 d. Cell numbers of other target species initially increased but, after a few days, growth stopped, followed by a rapid population decline and complete die off after 10 to 15 d. For unknown reasons, *Ceratium lineatum* (Fig. 2J) grew poorly both in monoculture and when mixed with Alex5. Nevertheless, when mixed with Alex2, cell numbers drastically declined and all cells disappeared after 6 d.

In the case of *Scrippsiella trochoidea* (Fig. 2C), the number of vegetative cells sharply declined and temporary cysts appeared in cultures with Alex2. The number of cysts remained relatively stable at 52 ± 14 cysts ml^{-1} (mean ± SD) over the course of the exper-

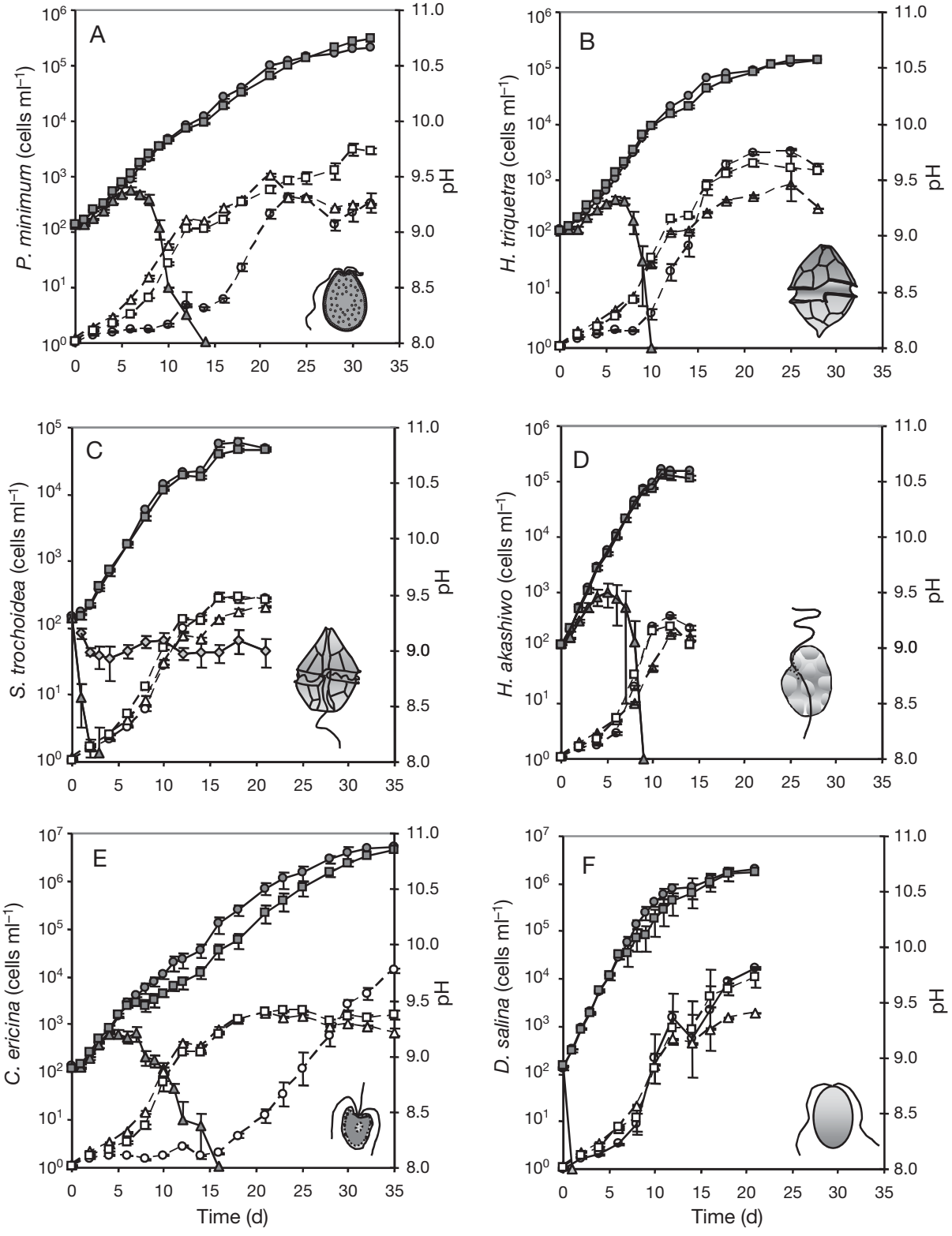

Fig. 2. (above and following page). Effect of *Alexandrium tamarense* on growth and survival of 10 different algal target species (see Table 1). (A–J) Average cell concentration (filled symbols) of individual algal species in monoculture (●), in mixed culture with Alex5 (■) and in mixed culture with Alex2 (▲) as well as pH (open symbols) in monoculture (○), mixed culture with Alex5 (□) and mixed with Alex2 (△). Data are treatment means (± 1SD), n = 3. In Fig. 2C (*Scrippsiella trochoidea*), (▲) refer to the abundance of vegetative cells and (◆) refer to the abundance of temporary cysts

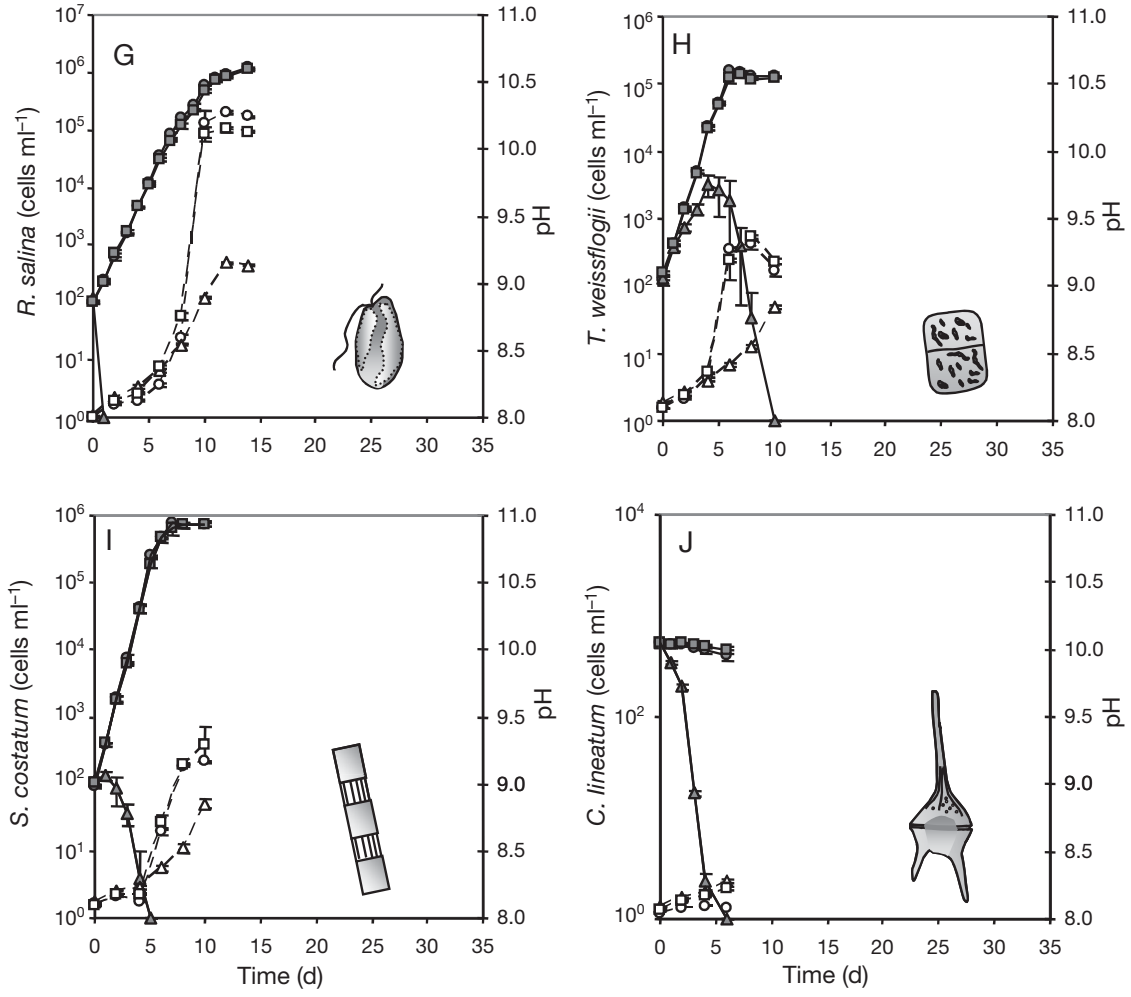

Fig. 2 (continued)

iment, indicating that roughly 1/3 of the initial population successfully formed temporary cysts. In all cultures, pH increased with increasing cell densities from 8.0 to values well above 9. It is important to note that a decline of target species in mixtures with Alex2 occurred at pH values well below that reached in the monocultures (>9).

Population dynamics of those target species that initially started to grow in mixed culture with Alex2 are shown in detail in Fig. 3. The exponential growth rate

Table 2. Growth rate μ (d^{-1}) (95% CI) of target species in monoculture and with *Alexandrium tamarense* clones Alex5 and Alex2, calculated for the indicated time period

Species	Period (d)	Monoculture	Alex5	Alex2
Prorocentrum minimum	1–8	0.36 (0.34–0.39)	0.39 (0.37–0.40)	0.18 (0.07–0.29)
Heterocapsa triquetra	1–8	0.44 (0.40–0.48)	0.46 (0.44–0.47)	0.13 (–0.03–0.29)
Heterosigma akashiwo	0–6	0.79 (0.75–0.83)	0.76 (0.72–0.80)	0.38 (0.22–0.53)
Chrysochromulina ericina	1–6	0.61 (0.55–0.67)	0.58 (0.55–0.61)	0.32 (0.11–0.54)
Thalassiosira weissflogii	0–6	1.21 (1.13–1.30)	1.16 (1.06–1.26)	0.47 (0.20–0.75)
Scrippsiella trochoidea	1–6	0.49 (0.38–0.60)	0.51 (0.45–0.57)	<<0
Skeletonema costatum	0–6	1.48 (1.35–1.62)	1.45 (1.34–1.56)	<<0
Dunaliella salina	0–6	0.90 (0.87–0.93)	0.91 (0.85–0.96)	<<0
Rhodomonas salina	0–6	0.98 (0.95–1.01)	0.95 (0.92–0.98)	<<0
Ceratium lineatum	0–6	–0.05 [(–0.07)–(–0.03)]	–0.03 [(–0.02)–(–0.04)]	<<0

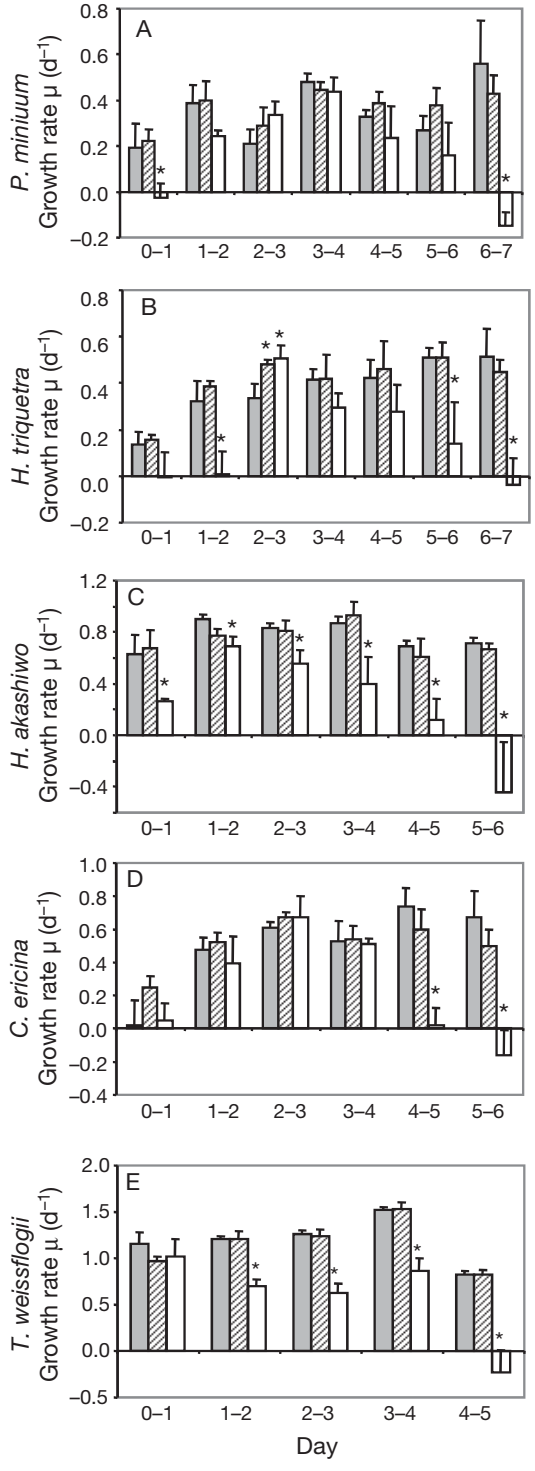

Fig. 3. Detailed initial dynamics of data shown in Fig. 2 for (A) *Prorocentrum minium*, (B) *Heterocapsa triquetra*, (C) *Heterosigma akashiwo*, (D) *Chrysochromulina ericina*; and (E) *Thalassiosira weissflogii*. Growth rate μ (d^{-1}), calculated for each consecutive sampling day. Grey bars: monoculture; hatched bars: mixed culture with Alex5; open bars: mixed culture with Alex2. Data are treatment means (±1 SD), n = 3. *above bars indicate significant ($p < 0.05$) difference from monoculture

of all species grown with Alex2 during the first 6 to 8 d was significantly ($p < 0.05$) reduced (Table 2). However, calculating the growth rate for this time period is largely affected by the final decrease in cell number. Therefore, growth rate was calculated for each consecutive sampling day and here, species-specific differences became evident (Fig. 3).

Prorocentrum minimum, mixed with Alex2, showed a significant (compared to the monoculture) lag-phase of 1 d. Thereafter, daily growth rates did not differ significantly from the control up to Day 6, when cell numbers started to decline. Likewise, *Heterocapsa triquetra* mixed with Alex2 showed a lag-phase of 2 d, but resumed growth at a rate not significantly lower than in monocultures until Day 5. Following this, a positive but significantly reduced growth rate was observed for 1 d and, subsequently, cell numbers declined. *Heterosigma akashiwo* grown with Alex2 also showed a pronounced 1 d lag-phase, after which positive, but significantly reduced growth rates occurred until Day 5. In contrast, *Chrysochromulina ericina* and *Thalassiosira weissflogii* did not show a prolonged lag-phase compared to monocultures. *C. ericina* in mixture with Alex2 grew as fast as in monoculture until Day 4, when growth stopped and subsequently became negative. *T. weissflogii* grew with positive but significantly reduced rates until Day 4, after which the growth rate became negative.

Cell concentrations of Alex2 in mixed cultures at the time growth rates of the target species became negative are listed in Table 3. For *Scrippsiella trochoidea*, *Dunaliella salina*, *Rhodomonas salina* and *Ceratium lineatum*, mortality (decreasing cell numbers) had already occurred at the starting concentration of about 140 cells ml^{-1}. After 1 d, *Skeletonema costatum* started to decline at a concentration of 170 Alex2 ml^{-1}. For all other targets, Alex2 concentrations causing population decline ranged from 700 to 1500 Alex2 ml^{-1}.

Table 3. Concentration of *Alexandrium tamarense* clone Alex2 at which a population decline of the respective target species was observed. <<140: population decline already occurred at the Alex2 start-concentration of 140 cells ml^{-1}

Target species	Day of decline	Alex2 concentration (cells ml^{-1})
Prorocentrum minimum	6	1544
Heterocapsa triquetra	6	1221
Heterosigma akashiwo	5	967
Chrysochromulina ericina	4	737
Thalassiosira weissflogii	4	762
Skeletonema costatum	1	170
Scrippsiella trochoidea	0	<<140
Dunaliella salina	0	<<140
Rhodomonas salina	0	<<140
Ceratium lineatum	0	<<140

Mixed growth experiments — growth of *Alexandrium tamarense*

In both mono- and mixed cultures, growth curves of Alex2 (Fig. 4A) and the corresponding pH dynamics (Fig 4B) were quite similar. The exponential growth rate of Alex2, calculated for the period Day 1 to 6, ranged from 0.43 to 0.48 d^{-1} (Table 4) and was not significantly different among treatments (co-variance analysis, $p = 0.14$).

Exponential growth rates for Alex5, again calculated for Day 1 to 6, was slightly lower compared to Alex2, ranging from 0.34 to 0.44 d^{-1} (Table 4). Despite this higher variability, co-variance analysis showed no significant differences among treatments ($p = 0.078$). Although the initial exponential growth rate of Alex5 was similar for all treatments, full growth curves (Fig. 5A) as well as pH dynamics (Fig. 5B) differed substantially. In monoculture, fast exponential growth obviously ceased around Day 10 to 12, when the pH reached values of >9. Nevertheless, cell numbers continuously increased up to 28×10^3 ml^{-1} at the end of the experiment, together with a slow increase in pH up to 9.4. In contrast to the monoculture, rapid exponential growth of Alex5 in mixed cultures stopped earlier, and maximum cell numbers were lower for all treatments, except in mixed culture with *Chrysochromulina ericina*. Moreover, mortality of Alex5, indicated by declining cell numbers, was observed in several mixed cultures. A close inspection of pH dynamics indicate that (1) an increase in pH in the mixed cultures to values >9 coincides with the time when growth of Alex5 stopped, and (2) mortality of Alex5 occurred when pH in the mixed cultures reached values >9.4.

Table 4. *Alexandrium tamarense*. Growth rate μ (95% CI) of Alex2 and Alex5 clones in mixed cultures for the period Day 1 to 6

Cultured with	Growth rate μ (d^{-1})	
	Alex2	Alex5
Monoculture	0.48 (0.43–0.52)	0.39 (0.34–0.44)
Prorocentrum minimum	0.47 (0.42–0.51)	0.41 (0.33–0.49)
Heterocapsa triquetra	0.43 (0.37–0.48)	0.41 (0.37–0.46)
Scrippsiella trochoidea	0.49 (0.46–0.52)	0.44 (0.39–0.50)
Heterosigma akashiwo	0.44 (0.39–0.49)	0.38 (0.33–0.43)
Chrysochromulina ericina	0.47 (0.42–0.52)	0.38 (0.32–0.44)
Thalassiosira weissflogii	0.48 (0.46–0.50)	0.34 (0.30–0.38)
Skeletonema costatum	0.45 (0.39–0.50)	0.38 (0.36–0.43)
Dunaliella salina	0.46 (0.38–0.53)	0.41 (0.37–0.45)
Rhodomonas salina	0.45 (0.42–0.47)	0.40 (0.34–0.47)
Ceratium lineatum	0.43 (0.39–0.46)	0.37 (0.35–0.38)

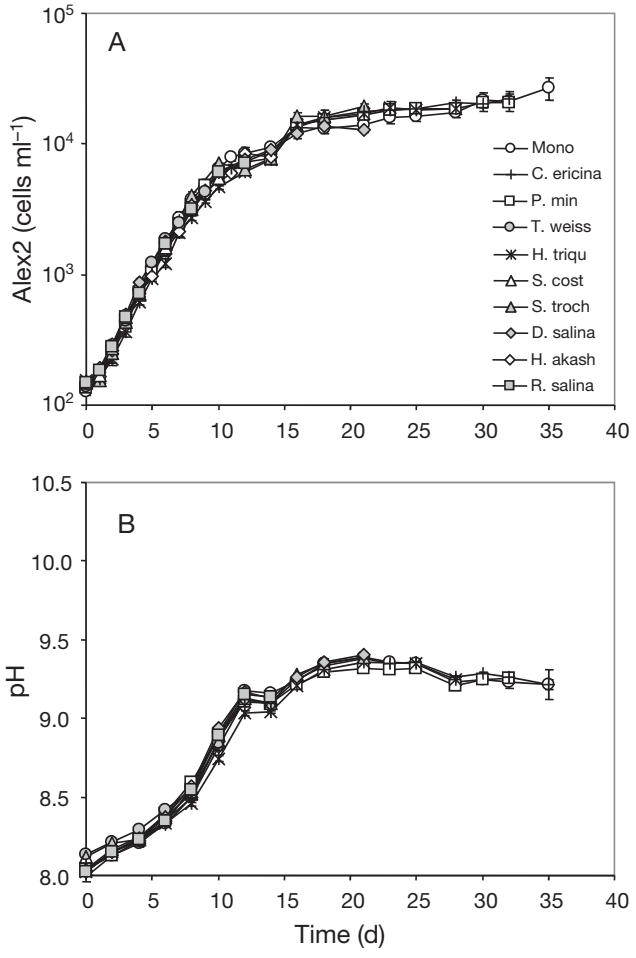

Fig. 4. (A) Growth of *Alexandrium tamarense* clone Alex2 and (B) pH in the monoculture (Mono) and in all mixed cultures. P. min: *Prorocentrum minimum*; H. triqu: *Heterocapsa triquetra*; S. troch: *Scrippsiella trochoidea*; H. akash: *Heterosigma akashiwo*; C. ericina: *Chrysochromulina ericina*; T. weiss: *Thalassiosira weissflogii*; S. cost: *Skeletonema costatum*; D. salina: *Dunaliella salina*; R. salina: *Rhodomonas salina*

DISCUSSION

We studied the effect of *Alexandrium tamarense* on 10 other planktonic algal species in mixed growth experiments. To separate effects caused by allelochemicals from other effects like direct competition for nutrients or other interspecific interactions like mixotrophy or predation, our experiment was designed as a comparative approach using 2 different *A. tamarense* clonal strains that have been shown to differ in their potential to produce lytic extracellular compounds (Tillmann et al. 2009).

The 2 clones, Alex2 and Alex5, were deliberately selected out of 67 clonal strains originating from one

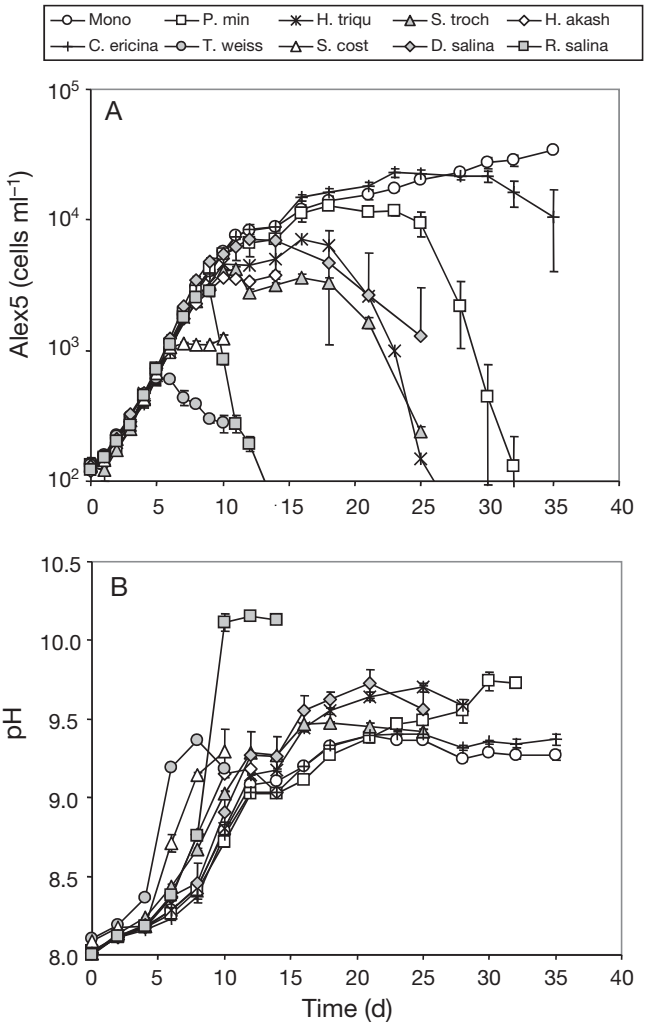

Fig. 5. *Alexandrium tamarense*. (A) Growth of Alex5 and (B) pH in monocultures (Mono) and in all mixture experiments. Species abbreviations as in Fig. 4

population, which were screened with respect to lytic potency by Alpermann et al. (Alpermann et al. in press). Among these 67 clones, only 2 isolates did not cause severe damage to *Rhodomonas* target cells, so the absence of measurable lytic activity in strains of *Alexandrium tamarense* seems to be a rare exception. The basic difference between Alex2 and Alex5 in allelochemical potency (Tillmann et al. 2009) is fully supported by the results from the present study: all target algae, which are common species in temperate waters and thus potentially co-occur and compete with *A. tamarense* in the North Sea, were clearly affected by the Alex2 strain. In contrast, the Alex5 strain had no negative effect on any of the target species during the study period, even though cell concentrations became very high (2×10^4 Alex5 ml^{-1}). As both strains contain comparable amounts of PSTs, this also supports earlier reports suggesting that PSTs are not involved in the harmful effects of *Alexandrium* spp. on other algae (Tillmann & John 2002, Fistarol et al. 2004b).

Based on the comparable growth rates of the 2 *Alexandrium tamarense* strains (Table 4), it can be assumed that they do not differ much with respect to their primary metabolism. Thus, their observed effects on target species in mixed culture can be attributed to allelochemical interactions caused by lytic secondary metabolites. pH can have a clear effect in mixed growth experiments and has to be taken into account in the interpretation of our results. This is because algae have different tolerances to high pH (Schmidt & Hansen 2001, Hansen 2002), which evolves in non-aerated batch cultures like the ones used in the present study. However, in the experiments with Alex2, high pH could not explain the outcome. Here, the target algae were killed at a pH considerably lower than that which would affect their growth (Table 5). However, it is obvious that some of the target algae grew so dense and pH increased so much that it did kill the mixed cultures with Alex5 (Table 5, Fig. 5).

While all target species were obviously negatively affected by alleochemicals of strain Alex2, the growth of some species (*Scrippsiella trochoidea*, *Dunaliella salina*, *Rhodomonas salina*, *Ceratium lineatum*, *Skeletonema costatum*) was already affected at very low cell concentrations of Alex2. The influence of Alex2 on the growth of these species was detectable at the first sampling occasion (24 h) at an *Alexandrium tamarense* concentration of just above 100 Alex2 ml^{-1} (Fig.1). Mixed growth experiments with *Alexandrium* spp. and other

Table 5. Maximum pH in monocultures and pH in mixed cultures with Alex2 and Alex5 at which growth of target species stopped. –: not measured

Target species	pH		
	Mono-cultures	Mixed culture (Alex2)	Mixed culture (Alex5)
Ceratium lineatum	8.7[a]	8.2	–
Prorocentrum minimum	9.75	8.4	9.75
Heterocapsa triquetra	9.6	8.3	9.6
Scrippsiella trochoidea	9.5	8.1	9.5
Heterosigma akashiwo	9.3	8.3	9.3
Chrysochromulina ericina	9.75	8.3	9.75
Thalassiosira weissflogii	9.4	8.2	9.4
Skeletonema costatum	9.4	8.1	9.4
Dunaliella salina	9.75	8.1	9.75
Rhodomonas salina	10.3	8.1	10.3

[a]pH limit for growth for *C. lineatum* derived from Hansen (2002)

species have not been carried out before, but acute lytic effects on other algae have been reported to occur within hours at this very low cell concentration. In short-term bioassays, the EC_{50} (*Alexandrium* spp. concentration causing lysis of 50% of the target population) of 10 clonal strains of *A. tamarense* for *Rhodomonas* as the target ranged from 80 to 640 cells ml^{-1} (Tillmann et al. 2009). For the same target, EC_{50} values of 230 to 520 cells ml^{-1} were estimated for *A. ostenfeldii*, *A. catenella* and *A. minutum* (Tillmann et al. 2008a). Cell concentrations of only 20 to 170 cells ml^{-1} of these species also lysed 50% of a population of the chlorophyte *D. salina* within 24 h (Tillmann et al. 2008a), which is similar to our findings in the present study. Our results show that the growth of other species was not affected by Alex2 until the concentration reached ca. 600 Alex2 ml^{-1} (*Heterocapsa triquetra*, *Prorocentrum minimum* and *Chrysochromulina ericina*), and a decline in the cell concentration of these species was not achieved before Alex2 had reached a cell concentration of ~700 to 1500 Alex2 ml^{-1} (Table 3). Thus, it appears that some species are far more resistant to the allelochemicals produced by *Alexandrium* spp. than others. However, for a direct comparison of target sensitivity it has to be kept in mind that targets differ in various aspects probably relevant to lytic effects, like cell size or surface area (Tillmann et al. 2009). Nevertheless, structural properties probably also play an important role in susceptibility to extracellular allelochemicals, as the smallest target (*C. ericina*) was among the most resistant species and the largest target (*C. lineatum*) among the most susceptible ones (Fig. 2). Likewise, without any relationship to size, a few target species were found to be relatively refractory or even unaffected by extracellular compounds of *A. ostenfeldii* (Tillmann et al. 2007).

In the present study, complete die-off was the ultimate fate for most target species. The remarkable exception was *Scrippsiella trochoidea*: when mixed with Alex2, roughly 1/3 of the initial population rapidly formed temporary cysts, the number of which remained constant during the course of the experiment. This observation is in agreement with a number of recent findings showing that temporary cyst formation may represent an inducible defence mechanism in response to deleterious chemical cues (Fistarol et al. 2004a, Toth et al. 2004, Tillmann et al. 2007). Temporary cysts of *S. trochoidea* are able to readily revert to a motile stage after a time-dependent degradation or inactivation of allelochemicals produced by *Alexandrium ostenfeldii* (Tillmann et al. 2007).

Thus, both quantitative (sensitivity) and qualitative (temporary cyst formation) responses to *Alexandrium tamarense* allelochemicals seem to be highly species-specific and, consequently, have the potential to cause temporal shifts in plankton community composition and succession (Fistarol et al. 2004b).

While a rapid population decrease of target species due to cell lysis seems to be the ultimate effect at sufficiently high *Alexandrium tamarense* concentrations, the data also show that, for some targets, a gradual decrease in growth rate of the target population can be observed at medium concentrations (Fig. 2). This may be either due to lysis of a certain percentage of the population while the rest is dividing at the same rate or because the compounds really slow down the growth rate of all individuals of the population. In any case, even a small reduction in the growth rate of competing species should benefit *A. tamarense* in gaining dominance in plankton communities.

The question remains as to whether our results can be generalised. In other words, will *Alexandrium tamarense*, by the production of allelochemicals, always win in competition with other algae? The answer to this question is clearly no. (1) *A. tamarense* clonal and strain differences in the amount of lytic compounds produced may be large (Alpermann et al. 2009). (2) There are species-specific differences in the sensitivity of the target species, and the question of potential strain variability in sensitivity of the target species has not yet been addressed experimentally. (3) *Alexandrium* spp. could be outcompeted by other, more pH tolerant species (see Hansen 2002) in eutrophic coastal waters, where pH values may, due to high primary production rates, increase to as much as 9.5.

Lysis of target species is obviously dependent on the concentration of *Alexandrium* spp. cells. However, it also depends on the cell concentration of the targeted species (Tillmann 2003, Tillmann et al. 2007), which is probably due to compounds being removed by binding to the target membrane. This shows that the actual density of other protists (or, more generally, the density of all absorbing particles) may influence the effectiveness of extracellular allelochemicals. Abundance of the target and, more importantly, the abundance ratio of donor:target species are largely determined by the initial (as well as modified) growth rates of both target and donor species which, in turn, are a function of the given environmental growth conditions. Finally, there are probably additional and poorly known factors that determine the actual and effective concentration of extracellular allelochemicals. Physico-chemical degradation and adsorption of compounds, for example, is likely to be very important for the determination of whether and how compounds may accumulate during growth in batch culture. The interplay of factors and processes that finally decide whether *Alexandrium* will make it to a concentration representing a 'point of no return' for other species is very complex.

Consequently, the outcome of these mixed growth experiments may be different for other clones/strains/species, for different environmental conditions or even for other donor:target density ratios. Once cell density of a fast-growing target becomes high enough to either change the pH to unfavourably high values for *Alexandrium* spp. and/or to 'absorb' lytic compounds without acute cell lysis, this competitor might 'win' the mixed growth experiment in spite of its potential sensitivity to the allelochemicals produced by *Alexandrium* spp.

In any case, it is clear that lytic compounds have the potential to benefit *Alexandrium tamarense* by reducing competitor population growth. The potential relief of *A. tamarense* from competition for nutrients etc., together with the benefit of reducing grazing loss by protistan grazers (Hansen 1989, Tillmann & John 2002, Fistarol et al. 2004b), is likely to be important for *A. tamarense* bloom development. Other advantages of lytic compounds are also conceivable: very recently, mixotrophy in *A. tamarense* has been described (Jeong et al. 2005). For a number of other mixotrophic harmful algal bloom (HAB) species, there is good evidence that toxic compounds are involved in prey immobilization and prey capture (*Prymnesium*: Skovgaard & Hansen 2003, Tillmann 2003; *Karlodinium*: Adolf et al. 2007). However, in the present investigation, careful light microscopy inspection of many *A. tamarense* cells for the presence of food vacuoles yielded no indication of mixotrophy, and the growth rate of Alex2 in mixed cultures was not enhanced, either compared to the control or compared to the non-lytic strain Alex5. Experiments under limiting conditions (nutrient, light) monitoring the growth of *A. tamarense* in the presence of potential prey are needed to determine whether this species is capable of allelochemically enhanced mixotrophy.

Even though the chemical structure of the lytic compounds still remains to be determined, the ecological potency of these compounds, which may negatively affect both competitors and micrograzers, has been sufficiently demonstrated in laboratory experiments. Field studies on the ecological significance and importance of allelochemical interactions in natural populations, preferably at different stages of bloom formation of *Alexandrium tamarense*, now have to follow.

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LITERATURE CITED

Adolf J, Krupatkina D, Bachvaroff TR, Place AR (2007) Karlotoxin mediates grazing by *Oxyrrhis marina* on strains of *Karlodinium veneficum.* Harmful Algae 6:400–412

Alpermann T, Beszteri B, John U, Tillmann U, Cembella AD (2009) Implications of life history transitions on the population genetic structure of the toxigenic marine dinoflagellate *Alexandrium tamarense.* Mol Ecol 18:2122–2133

Alpermann T, Tillmann U, Beszteri B, Cembella AD, John U (in press) Phaenotypic variation and genotypic diversity in a planktonic population of the toxigenic marine dinoflagellate *Alexandrium tamarense.* J Phycol

Cembella AD (2003) Chemical ecology of eukaryotic microalgae in marine ecosystems. Phycologia 42:420–447

Cembella AD, Quilliam MA, Lewis NI, Bauder AG and others (2002) The toxigenic marine dinoflagellate *Alexandrium tamarense* as the probable cause of mortality of caged salmon in Nova Scotia. Harmful Algae 1:313–325

Dahl E, Lindahl O, Paasche E, Throndsen J (1989) The *Chrysochromulina polylepis* bloom in Scandinavian waters during spring 1988. In: Cosper EM, Bricelj VM, Carpenter EJ (eds) Novel phytoplankton blooms: causes and impacts of recurrent brown tides and other unusual blooms. Springer Verlag, Berlin, p 383–405

Fistarol GO, Legrand C, Rengefors K, Granéli E (2004a) Temporary cysts formation in phytoplankton: a response to allelopathic competitors? Environ Microbiol 6:791–798

Fistarol GO, Legrand C, Selander E, Hummert C, Stolte W, Granéli E (2004b) Allelopathy in *Alexandrium* spp.: effect on natural plankton community and on algal monocultures. Aquat Microb Ecol 35:45–56

Fulco VK (2007) Harmful effects of the toxic dinoflagellate *Alexandrium tamarense* in the tintinnids *Favella taraikaensis* and *Eutintinnus* sp. J Mar Biol Assoc UK 87: 1085–1088

Granéli E, Hansen PJ (2006) Allelopathy in harmful algae: a mechanism to compete for resources? In: Granéli E, Turner JT (eds) Ecology of harmful algae. Springer, Berlin, p 189–201

Hansen PJ (1989) The red tide dinoflagellate *Alexandrium tamarense*: effects on behaviour and growth of a tintinnid ciliate. Mar Ecol Prog Ser 53:105–116

Hansen PJ (2002) Effect of high pH on the growth and survival of marine phytoplankton: implications for species succession. Aquat Microb Ecol 28:279–288

Hansen PJ, Cembella AD, Moestrup Ø (1992) The marine dinoflagellate *Alexandrium ostenfeldii*: paralytic shellfish toxin concentration, composition, and toxicity to a tintinnid ciliate. J Phycol 28:597–603

Jeong HJ, Yoo YD, Park JY, Song JY and others (2005) Feeding by phototrophic red-tide dinoflagellates: 5 species newly revealed and 6 species previously known to be mixotrophic. Aquat Microb Ecol 40:133–150

Kamiyama T, Suzuki T (2006) Lack of accumulation of paralytic shellfish poisoning (PSP) toxins in the tintinnid ciliate *Favella taraikaensis* feeding on the toxic dinoflagellate *Alexandrium tamarense.* Mar Ecol Prog Ser 317:57–65

Keller MD, Selvin RC, Claus W, Guillard RRL (1987) Media for the culture of oceanic ultraphytoplankton. J Phycol 23: 633–638

Legrand C, Rengefors K, Fistarol GO, Granéli E (2003) Allelopathy in phytoplankton — biochemical, ecological and evolutionary aspects. Phycologia 42:406–419

Mortensen AM (1985) Massive fish mortalities in the Faroe Islands caused by a *Gonyaulax excavata* red tide. In: Anderson DM, White AW, Baden DG (eds) Toxic dinoflagellates. Elsevier, Amsterdam, p 165–170

Schmidt LE, Hansen PJ (2001) Allelopathy in the prymnesiophyte *Chrysochromulina polylepis*: effect of cell concentration, growth phase and pH. Mar Ecol Prog Ser 216: 67–81

Shimizu Y (1996) Microalgal metabolites: a new perspective.

Annu Rev Microbiol 50:431–465

Skovgaard A, Hansen PJ (2003) Food uptake in the harmful alga *Prymnesium parvum* mediated by excreted toxins. Limnol Oceanogr 48:1161–1166

Sykes PF, Huntley ME (1987) Acute physiological reactions of *Calanus pacificus* to selected dinoflagellates: direct observations. Mar Biol 94:19–24

Tillmann U (2003) Kill and eat your predator: a winning strategy of the planktonic flagellate *Prymnesium parvum.* Aquat Microb Ecol 32:73–84

Tillmann U, John U (2002) Toxic effects of *Alexandrium* spp. on heterotrophic dinoflagellates: an allelochemical defence mechanism independent of PSP toxins. Mar Ecol Prog Ser 230:47–58

Tillmann U, John U, Cembella AD (2007) On the allelochemical potency of the marine dinoflagellate *Alexandrium ostenfeldii* against heterotrophic and autotrophic protists. J Plankton Res 29:527–543

Tillmann U, Alpermann T, John U, Cembella A (2008a) Allelochemical interactions and short-term effects of the dinoflagellate *Alexandrium* on selected photoautotrophic and heterotrophic protists. Harmful Algae 7:52–64

Tillmann U, John U, Krock B, Cembella A (2008b) Allelopathic effects of bioactive compounds produced by harmful algae. In: Moestrup O (ed) Proceedings of the 12th International Conference on Harmful Algae. International Society for the Study of Harmful Algae and Intergovernmental Oceanographic Commission of UNESCO, 2008, Kopenhagen, p 12–18

Tillmann U, Alpermann T, Purificacao R, Krock B, Cembella A (2009) Intra-population clonal variability in allelochemical potency of the toxigenic dinoflagellate *Alexandrium tamarense.* Harmful Algae 8:759–769

Toth GB, Norén F, Selander E, Pavia H (2004) Marine dinoflagellates show induced life-history shifts to escape parasite infection in response to water-borne signals. Proc R Soc Lond B 271:733–738

Turner JT, Tester PA, Hansen PJ (1998) Interactions between toxic marine phytoplankton and metazoan and protistan grazers. In: Anderson DA, Cembella AD, Hallegraeff GM (eds) Physiological ecology of harmful algal blooms. Springer, Berlin, p 453–474

Ulitzur S (1973) The amphiphatic nature of *Prymnesium parvum* hemolysin. Biochim Biophys Acta 298:673–679

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