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Allelopathic potential of the dinoflagellate *Alexandrium tamarens* on marine microbial communities

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ABSTRACT

The impacts of two strains of the dinoflagellate *Alexandrium tamarens*, differing in lytic activity, on the abundance and the composition of microbial communities ($<150\ \mu\text{m}$) were studied in North Sea water during spring with *Phaeocystis globosa* as a dominant species. Cell-free suspensions (supernatant) of exponentially growing lytic and non-lytic *Alexandrium* culture were added at different concentrations to natural microbial communities under nutrient rich conditions. The non-lytic strain had a positive impact on diatoms whereas the lytic strain suppressed phytoplankton growth in comparison to the control. *P. globosa*, present as single cells in the initial community, increased in abundance and formed colonies in all treatments. However, total abundance and number of colonies was low with lytic *Alexandrium* additions, whereas shape of the colonies, but not abundance of cells, was affected by non-lytic *Alexandrium* additions. During the 4-day experiment, bacterial abundance was constantly higher with high lytic additions (highest concentration equivalent to $1000\ \text{cells ml}^{-1}$) whereas nanoflagellate abundance in the same treatments was found to be lower at the end of the experiment. Initial bacterial community composition differed significantly among lytic *Alexandrium*, non-lytic *Alexandrium* and North Sea water. However, neither bacterial activity nor composition was significantly affected by the supernatants after 96 h. Our results indicated that *Alexandrium* allelochemicals do not inhibit growth and production of bacteria in seawater collected during spring in the North Sea.

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1. Introduction

The adverse effects of harmful algal blooms (HABs) in marine ecosystems have focused attention on defining the role of factors that determine their abundance and distribution. Some marine dinoflagellates of the genus *Alexandrium* Halim (Balech) produce neurotoxins, which cause Paralytic Shellfish Poisoning (PSP), resulting in life-threatening human illness due to the consumption of contaminated seafood (Anderson, 1997). Blooms of toxic *Alexandrium* spp. occur worldwide, usually associated with embayments, estuaries or open coastal waters. *Alexandrium* spp. neurotoxins can also be transferred through the marine food web to higher trophic levels where they may affect zooplankton, fish, birds and marine mammals. *Alexandrium* species also release lytic compounds suppressing competitors (Fistarol et al., 2004; Tillmann et al., 2007) and heterotrophic micrograzers (Tillmann and John, 2002). Allelopathy is a known phenomenon in a number of phytoplankton groups (e.g. Cyanobacteria, Haptophytes, Dinoflagellates) and has been hypothesized to be one of the forces structuring phytoplankton communities (Legrand et al., 2003) and

the formation of harmful algal blooms (Smayda, 1997). Chemical interactions among *A. tamarens* and other marine protists are an important biological factor for the dominance of *Alexandrium*, not solely restricted to the competition for nutrients (Fistarol et al., 2004; Tillmann et al., 2008). Although bacteria and phytoplankton are closely linked in coastal systems, little is known about their interactions in shaping the pelagic microbial communities (Riemann et al., 2000; Hay and Kubanek, 2002; Mayali and Azam, 2004). A few studies have shown that bioactive compounds produced by phytoplankton can inhibit bacteria (Ribalet et al., 2008; Doucette, 1995). However, experiments with natural communities showed higher bacterial abundances in presence of allelochemicals (Fistarol et al., 2004; Uronen et al., 2007). The flagellate *Prymnesium parvum* produces compounds that can lyse competitor cells and alter the functioning of the planktonic food web by increasing carbon transfer through the microbial loop (Uronen et al., 2007). Hence, phytoplankton may select for a specific phylogenetic and/or phenotypic composition of microbial communities in its surrounding environment. Many studies report the phylogenetic composition of bacterioplankton during *Alexandrium* blooms (Green et al., 2004; Wichels et al., 2004; Jasti et al., 2005; Sala et al., 2005). However, the mechanisms creating a specific type of phytoplankton–bacteria assemblage are unknown. The aim of this study was to examine the allelopathic effect of the

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dinoflagellate *Alexandrium* on bacterial and natural plankton communities using field samples from the North Sea and cultures of *A. tamarens*. Here, we compare the response of a plankton community to extracellular compounds produced by two strains of *A. tamarens* (lytic and non-lytic). This comparative approach was chosen to distinguish between the impact of the added *A. tamarens* suspension per se and the impact of lytic compounds, produced by *A. tamarens*, which can not be separated from other algal exudates today.

2. Materials and methods

2.1. Algal cultures

Alexandrium tamarens strains (Alex2 and Alex5) were isolated from the east coast of Scotland (North Sea) in 2004 (Alpermann et al., 2009) and grown non-axenic in K-medium (Keller et al., 1987). These two clones were selected based on lytic capacity quantified by a *Rhodomonas* bioassay (Tillmann et al., 2009). Alex5 was found to have no lytic impact on *Rhodomonas* and will further be referred to as non-lytic *Alexandrium*. Alex2 had a high lytic capacity and is therefore called lytic *Alexandrium* in the further text.

Both *A. tamarens* and *Rhodomonas salina* (Kalmr Algal Collection, KAC 30) were grown in batch cultures in K-medium (salinity 32), prepared from sterile-filtered (VacuCap 0.2 µm Pall Life Sciences) North Sea water in 2000 ml Erlenmeyer flasks. Cultures were maintained under controlled conditions at 15 °C under cool-white fluorescent light (65 µmol m⁻² s⁻¹) on a 16 h light:8 h dark cycle. Prior to sampling, culture flasks were shaken gently by hand to allow for a homogenous cell distribution.

To quantify the lytic activity of *A. tamarens* on natural plankton communities, cell-free supernatant of *A. tamarens* cultures was used since filtration can remove up to 90% of the allelopathic activity probably due to absorption of the bioactive compounds on the filter (Tillmann et al., 2008). Aliquots (18 × 50 ml) of exponentially growing cultures of *A. tamarens* (lytic and non-lytic *Alexandrium*, 5000 cells ml⁻¹) were centrifuged for 15 min at 12,000 rpm. Aliquots of supernatant (30 ml) were stored in a glass bottle at 4 °C in the dark and used within 5 days. The lytic activity of the supernatant was checked prior the experiment using a *Rhodomonas* bioassay (bioassay modified after Tillmann et al., 2009). Additionally, bacterial abundance and community composition in lytic and non-lytic *Alexandrium* supernatant and the North Sea water were measured.

2.2. Field sampling

A natural plankton community was collected during spring in May 2007 in the southern Helgoland Bight (North Sea) from the surface sampled with a bucket. Seawater was filtered through 150 µm nylon nets to remove zooplankton and large detritus. Samples for bacterial abundance (in triplicates, 1.5 ml each) and production, chl a concentration (in triplicates, 300 ml each), phytoplankton (50 ml) and bacterial community composition (500 ml) were immediately taken. Nitrate (580 µM final concentration), phosphate (36 µM final concentration) and peptone (2 mg Cl⁻¹) were added to ensure nutrient saturated conditions for both, phytoplankton and bacteria. Enriched seawater (400–495 ml) was placed in 1 l glass bottles and incubated at 15 °C overnight prior to the experiment start.

2.3. Allelopathic effect of *Alexandrium* supernatant on microbial communities

Lytic and non-lytic *Alexandrium* supernatant were added, respectively, in three treatments (each in triplicates) corresponding

to the lytic activity of 200 (low continuous addition), 1000 (high continuous addition) and 1000 (single addition) cells ml⁻¹, respectively.

The single addition corresponding to 1000 cells ml⁻¹ was added in 100 ml supernatant at *T*₀ to a final volume of 500 ml. Continuous additions corresponding to 300 (high continuous addition) and 50 (low continuous addition) cells ml⁻¹ were added daily in 30 and 5 ml of supernatant (*T*₀, *T*₂₄, *T*₄₈, *T*₇₂) to a final volume of 515 and 590 ml, respectively. We added K-medium to the control to obtain comparable nutrient conditions in control as well as *Alexandrium* treatments. Due to the different amounts of added supernatant, overall nutrient concentrations between treatments and controls may have slightly differed. However, since *A. tamarens* was cultivated in full K-medium, and the supernatant was produced during exponential growth, we assume that the amount of nutrients added to both the *Alexandrium* treatments as well as the controls exceeded the amount of nutrient that could possibly be used by phytoplankton and bacteria during the 96 h of incubation. 30 ml of K-medium were added daily (*T*₀, *T*₂₄, *T*₄₈, *T*₇₂) to triplicate control bottles to a final volume of 590 ml. All bottles were incubated for 96 h under similar conditions as the algal cultures. Samples for bacterial abundance were taken daily. After 96 h (Day 4), samples for bacterial abundance and production, chl a, phytoplankton and bacterial community composition were collected in treatments and controls. From *T*₂₄ and *T*₉₆, bacterial volumes in the treatments were calculated. Samples for bacteria counts were fixed in 2% formalin and placed at 4 °C. Whole water samples (20 ml) were filtered on glass fiber filters (Gelman A/E) and frozen at –20 °C prior to chl a analysis. Samples for identification and quantification of phytoplankton were preserved with Lugol's solution. For extraction of bacterial community DNA, microbial biomass from 50 to 400 ml seawater was collected onto 0.2 µm-pore-size Supor 200 membrane filters by vacuum filtration at <150 mmHg. Filters were stored frozen at –20 °C in TE buffer (10 mM Tris, 1 mM EDTA).

2.4. Analytical procedures

2.4.1. *Rhodomonas* bioassay

A dilution series was made using different amounts of *Alexandrium* supernatant and whole cell culture corresponding to the following *Alexandrium* cell densities: 50, 75, 100, 150, 250, 375, 635, 937, 1250, 2500 cells ml⁻¹ in a total bioassay volume of 4 ml. K-medium (triplicate) served as control. *R. salina* was spiked to all samples to a final density of 12,500 *R. salina* cells ml⁻¹. After an incubation period of 3 h, samples were fixed with Lugol's solution (2% final concentration) and counted with an inverted microscope (Zeiss Axiovert 35). A sub-area corresponding to at least 500 cells in the control was counted. Values of EC₅₀, defined as the *Alexandrium* cell concentration causing lysis of 50% of target cells, were calculated by using a sigmoidal curve fit function (Tillmann et al., 2009) in the mathematical programme Origin.

2.4.2. Chlorophyll a and enumeration of phytoplankton and nanoflagellates

Chlorophyll a concentration was measured fluorometrically (Turner AU-10 fluorometer) after 8 h extraction in the dark with 96% ethanol (Jespersen and Christoffersen, 1987). For each treatment, phytoplankton growth day⁻¹ was calculated by using the following Eq. (1):

$$\mu = (LN(Chl_{96}) - LN(Chl_0)) \frac{1}{4} \quad (1)$$

Except *Phaeocystis globosa*, phytoplankton was counted using a Zeiss Axiovert inverted microscope and, when possible, identified at the species or genus level. The volume sedimented for the counts (5–50 ml) varied between different treatments. The total number of intact cells counted was always >200 per sample, except for the highest lytic *Alexandrium* treatments with >100 total counts per sample. *P. globosa* colonies were observed and counted in fresh samples fixed with Lugol's solution a few hours after sampling. *P. globosa* total cells and nanoflagellates were counted at 1000× using an Olympus BX50 inverted microscope and blue light epifluorescence. Samples fixed with Lugol's solution were bleached with sodium iodide and stained for 2 min with proflavine (Haas, 1982). Stained samples were filtered onto 0.8 µm black polycarbonate filters. At least 300 *P. globosa* cells per filter were counted, except in the initial community and lytic *Alexandrium* single addition treatments, where approximately 100 *P. globosa* cells per filter were counted. Nanoflagellates <10 µm and >10 µm were counted in the same way to estimate bacterial grazing pressure. At least 250 nanoflagellates per filter were counted, except in the initial community where 100 nanoflagellates per filter were counted.

2.4.3. Bacterial abundance and production

Heterotrophic bacteria were counted using flow cytometry (BD FACs Calibur) after staining with Syto13 DNA dye (Gasol and Del Giorgio, 2000). Bacterial production was measured as carbon production by the uptake of ³H-leucine (Smith and Azam, 1992). For each sample, triplicate aliquots (1.2 ml) and a trichloroacetic acid killed control were incubated with 40 nM [³H]Leucine (final concentration) for 1–2 h at 15 °C. For the natural community and the *Alexandrium* supernatant, four aliquots and two controls were incubated with 40 nM [³H]Leucine to measure bacterial production. [³H]Leucine concentration was measured in dpm by using a scintillation counter and converted to bacterial carbon production (µg C l⁻¹ h⁻¹) using the conversion factor of 0.86 (Simon and Azam, 1989).

2.4.4. Bacterial biomass

To calculate bacterial biomass, at least 100 bacteria in treatments containing K-medium and single (1000 cells ml⁻¹) and high continuous (1000 cells ml⁻¹) additions of lytic and non-lytic *Alexandrium* supernatant were measured using epifluorescence microscopy photographs (1000 magnification) and Gimp Software. The volume of bacteria with the largest side smaller than 0.5 µm was calculated as a sphere, whereas all bacteria with the largest side larger than 0.5 µm were calculated as a cylinder with an average thickness of 0.36 µm. Bacterial volume was converted into bacterial carbon biomass by using the conversion factor of 7.2×10^{-13} g C µm⁻³ (Kroer, 1994).

2.4.5. DNA extraction, PCR amplification and denaturing gradient gel electrophoresis (DGGE)

DNA was extracted directly from filters as previously described (Boström et al., 2004). Briefly, cells were treated with lysozyme, proteinase K and sodium dodecyl sulphate followed by phenol–chloroform–isoamyl alcohol extraction. The 16S rRNA gene fragment (approximately 550 bp long) was amplified by PCR, using the bacteria specific primer GM5 GC (corresponding to placers 341–358 of the *Escherichia coli* 16S rRNA) and the universal primer 907 RM (corresponding to positions 907–927 of the *E. coli* 16S rRNA). The reaction mixture volumes were 100 µl, containing 10–50 ng DNA template, 200 µM of both primers, 48 µl PCR water and 50 µl Master mix (including 5U/ml Taq DNA polymerase, 400 µM dNTP's, 3 µM MgCl₂). The template DNA was denatured in a thermal cycler for 5 min at 95 °C followed

by a touchdown PCR as described by Muyzer and Smalla (1998), to increase the specificity of amplification. PCR products were visualized by agarose gel electrophoresis with a molecular size standard in the gel. Approximately 80 µl of the template was mixed with 20 µl 6× loading buffer and the gel was loaded on a 6% polyacrylamide gel with a denaturing gradient that ranged from 20 to 80% (where 100% is defined as 7 M urea and 40% deionized formamide). The gel was run at 200 V for 3.5 h in 1×TAE running buffer (40 mM Tris (pH 7.4), 20 mM sodium acetate, 1 mM EDTA). The gel was stained with ethidium bromide for 30 min and visualized by UV luminescence.

2.4.6. PCR and transverse restriction fragment length polymorphism (TRFLP)

The genetic fingerprinting technique terminal restriction fragment length polymorphism is based on the restriction digestion of fluorescently labelled PCR products. A 16S rRNA gene fragment (approximately 550 bp long) was amplified by PCR, using the bacteria specific primer 21 F and the universal primer 907 RM. The reaction mixture volumes were 50 µl, containing 10–50 ng DNA template, 100 µM of both primers, 22 µl PCR water and 25 µl Master mix (including 5U/ml Taq DNA polymerase, 400 µM dNTP's, 3 µM MgCl₂). The template DNA was denatured in a thermal cycler for 5 min at 95 °C followed by PCR. PCR products were visualized by agarose gel electrophoresis with a molecular size standard in the gel. The PCR product was cleaned by using Sephadex and digested with the restriction enzyme Alu 1. After an additional cleaning, the digested product was marked with the Map Marker 1000 and denatured. The following step, the capillary electrophoresis, separated the labelled fragments after their length. The data was visualised and analysed by using the applied biosystems software Gene mapper (applied biosystems). A statistical analysis in Past (Hammer et al., 2001) was performed to compare the different treatments.

2.4.7. Statistics

Statistic tests were accomplished using Statistica software (Stat Soft Inc.). To test for significant differences between control and treatments or between *A. tamarensis* non-lytic and lytic supernatant additions of the same concentration, independent samples *t*-tests (2 different groups) were used. Since data from control treatments were repeatedly analysed, the level of significance was reduced to 0.008 (Bonferroni adjustment) instead of 0.05 for *t*-tests comparing controls to various treatments. To test for significant differences between all groups, One Way ANOVA was used. Data was tested for normal distribution (Kolmogorov–Smirnov test) (*t*-test, One Way ANOVA) and homogeneity of variances (Cochran's test) (One Way ANOVA). If the assumptions for parametric tests could not be fulfilled, data were LOG or SQRT transformed. Differences between treatments were significant at $p < 0.05$.

2.4.8. Analysis of DGGE gels

DGGE gel band patterns were subjected to computer analysis to compare the bacterial community structure using the program Quantity One (Bio Rad). On basis of the volume and band pattern in the gel, a dendrogram (Algorithm: UPGMA) of the samples was prepared.

2.4.9. Analysis of TRFLP patterns

For the TRFLP analysis, non-metric multi dimensional scaling analysis was conducted to assess for the relationship between the different treatments. Non-metric multi dimensional scaling was performed with PAST software package (Hammer et al., 2001).

3. Results

3.1. Characterisation of *A. tamarensis* cell-free supernatants

Lytic *Alexandrium* caused cell lysis of *R. salina* in a dose-dependent manner with an EC_{50} of 110 ± 50 *A. tamarensis* cells ml^{-1} . Cell-free supernatant was slightly less effective ($EC_{50} = 180 \pm 4$ *A. tamarensis* cells ml^{-1}). Non-lytic *Alexandrium* supernatant or culture did not lyse *R. salina* at any concentration. Bacterial abundance was 4 times higher in lytic *Alexandrium* cultures (3.7×10^6 bacteria ml^{-1}) compared to non-lytic cultures (0.9×10^6 bacteria ml^{-1}). However, bacteria were on average twice as large in non-lytic *Alexandrium* culture ($0.15 \mu m^3$) compared to the lytic culture ($0.07 \mu m^3$) because the non-lytic culture contained large filamentous bacteria that were not observed in the lytic *Alexandrium* culture. Thus, bacterial biomass in the lytic *Alexandrium* culture ($188 \mu g C l^{-1}$) was twice as high as in the non-lytic *Alexandrium* culture ($95 \mu g C l^{-1}$). Bacterial production was significantly higher in lytic *Alexandrium* compared to non-lytic *Alexandrium* culture ($228 \pm 32 d^{-1}$ and $183 \pm 12 d^{-1}$, respectively). However, production biomass $^{-1}$ was lower in lytic *Alexandrium* compared to non-lytic *Alexandrium* culture ($1.2 \pm 0.2 d^{-1}$ and $1.9 \pm 0.1 d^{-1}$, respectively). Centrifugation resulted in 90% (lytic *Alexandrium*) and 85% (non-lytic *Alexandrium*) reduction in bacterial numbers in the supernatant. Bacterial production in lytic and non-lytic *Alexandrium* supernatant was approximately 75% lower as in the corresponding cultures. Production biomass $^{-1}$ was higher in *Alexandrium* supernatant compared to the *Alexandrium* cultures and equal in lytic and non-lytic *Alexandrium* supernatant.

3.2. Effect of *A. tamarensis* on North Sea phytoplankton and nanoflagellates

Seawater was collected during the spring at a moderate chl *a* concentration of $3.7 \mu g Chl a l^{-1}$. In 2007, the spring phytoplankton was dominated by the colony forming *P. globosa* (Haptophyte) and cryptophytes (Table 1).

Table 1
Plankton composition of the initial North Sea water.

Group	Genus/species	Cells l^{-1}
Dinoflagellates	<i>Gyrodinium spirale</i>	7079
	<i>Scrippsiella</i> spp.	6262
	<i>Protoperdinium</i> spp.	4901
	<i>Dinophysis acuminata</i>	4084
	<i>Pyrophacus horrologium</i>	2178
	<i>Prorocentrum micans</i>	545
	<i>Alexandrium</i> spp.	240
	<i>Polykrikos schwartzii/kofoiidii</i>	140
	<i>Ceratium lineatum</i>	40
Cryptophytes	<i>Teleaulax</i> spp.	43224
	<i>Leucocryptos</i> spp.	6372
Haptophytes	<i>Phaeocystis globosa</i>	971307
Diatoms	<i>Pseudonitzschia</i> spp.	7351
	<i>Leptocylindrus danicus</i>	2723
	<i>Chaetoceros</i> spp.	1260
	<i>Rhizosolenia</i> spp.	420
	<i>Ditylum brightwellii</i>	320
	<i>Guinardia delicatula</i>	50
	<i>Coscinodiscus</i> spp.	40
Ciliates	<i>Laboea strobila</i>	2040
Unidentified nanoflagellates <10 μm		150720
Unidentified nanoflagellates >10 μm		795467

Phytoplankton biomass, measured as chl *a*, increased during the experiment in all treatments at very different rates. The growth rate of the phytoplankton community, calculated from chl *a* measured initially and at the end of the experiment, was significantly lower in high lytic *Alexandrium* additions (*t*-test, $p < 0.008$; $\mu = 0.32 d^{-1}$ (single addition) and $\mu = 0.48 d^{-1}$ (high continuous addition)) compared to control treatments ($\mu = 0.72 d^{-1}$). All other treatments did not differ significantly from the control (*t*-test, $p > 0.008$).

The plankton community composition changed over the course of the experiment; cryptophytes disappeared and both diatoms and *P. globosa* became dominant in all treatments (Fig. 1 and Table 1).

Below, plankton community composition of the different treatments will be compared to the control treatments. Low continuous additions of *Alexandrium* supernatant did not affect phytoplankton and nanoflagellate groups. High continuous additions of lytic supernatant inhibited the abundance of *P. globosa* and large flagellates compared to the control. *Chaetoceros* spp. benefitted from high continuous additions of non-lytic *Alexandrium* supernatant. In single additions of lytic *Alexandrium* supernatant, *P. globosa* and *Chaetoceros* spp. > 10 μm were inhibited in comparison to the control treatments. Single additions of non-lytic *Alexandrium* supernatant increased the abundance of *Chaetoceros* spp. < 10 μm and pennate diatoms (independent samples *t*-test, $p < 0.008$).

When the treatments lytic and non-lytic *Alexandrium* were compared according to the amount of added supernatant, in low continuous additions the amount of nanoflagellates < 10 μm was higher in lytic *Alexandrium* treatments. In high continuous additions, abundances of *Chaetoceros* spp., *P. globosa* and small and large flagellates were elevated in non-lytic *Alexandrium* treatments whereas dinoflagellates were more abundant in lytic *Alexandrium* treatments. *P. globosa*, *Chaetoceros* spp., large flagellates and pennate diatoms were suppressed in single additions of lytic *Alexandrium* compared to non-lytic *Alexandrium* (independent samples *t*-test, $p < 0.05$).

3.3. Bacterial abundance

Abundance of heterotrophic bacteria ranged in between 0.6 and 0.7×10^6 bacteria ml^{-1} at the experimental start. Bacteria grew rapidly during the first 24 h of the experiment in all treatments (Fig. 2). Bacterial abundances in lytic *Alexandrium* supernatant high continuous and single additions were significantly higher than in any other treatments (multivariate test after repeated ANOVA, $p < 0.05$ Tukey HSD Post Hoc). The number of bacteria declined between 48 h and 76 h in all treatments. The magnitude of this reduction differed between the three groups: lytic *Alexandrium* supernatant high continuous and single additions ($29 \pm 3\%$ loss); controls and low continuous lytic and non-lytic *Alexandrium* supernatant ($45 \pm 6\%$ loss of bacteria) and non-lytic *Alexandrium* high continuous and single additions ($62 \pm 10\%$ loss) (Tukey HSD Post Hoc test after One Way ANOVA, $p < 0.05$).

3.4. Bacterial morphology and production

The mean volume per bacterium was similar in all treatments after 24 h of incubation (One Way ANOVA, $p = 0.221$, data not shown). After 96 h, bacterial volume had decreased in the high continuous and single lytic *Alexandrium* supernatant treatments (from 0.056 to $0.043 \mu m^3$) whereas it increased in the other treatments (from 0.056 to $0.069 \mu m^3$).

Bacterial biomass after 96 h was significantly lower in low continuous non-lytic *Alexandrium* additions and high continuous lytic *Alexandrium* additions compared to the control (Fig. 3). Bacterial production rates reached $44 \mu g C l^{-1} d^{-1}$ in the initial

Fig. 1. Chl *a* concentrations and abundance of the dominant plankton groups, *Chaetoceros* spp. (<10 μm and >10 μm), Pennate diatoms, *Ditylum brightwellii*, dinoflagellates, nanoflagellates <10 μm and >10 μm and *Phaeocystis globosa* in initial North Sea water (May 2007), and after 96 h of incubation with different *Alexandrium* supernatant concentrations (lytic *Alexandrium*, non-lytic *Alexandrium*). Controls were incubated with K-medium: Mean ($n=3$; $\pm$ S.D.). Treatments significantly different from control are marked with a star. Lytic *Alexandrium* supernatant treatments significantly different from corresponding non-lytic *Alexandrium* supernatant treatments are marked with a triangle.

Fig. 2. Bacterial abundance in treatments containing single and continuous high and low concentrated additions of lytic and non-lytic *Alexandrium* supernatant during 96 h. Controls were incubated with K-medium. Results expressed as triplicate mean $\pm$ 1S.D.

North Sea water. Bacterial production at the end of the experiment was elevated to approximately $100 \mu\text{g C l}^{-1} \text{d}^{-1}$. Bacterial production was significantly lower from the control in single non-lytic *Alexandrium* additions.

However, production bacteria $^{-1}$ and production biomass $^{-1}$ did not differ from the control with K-medium in any of the treatments (Fig. 3).

3.5. Bacterial community composition

For initial North Sea water, initial lytic and non-lytic *Alexandrium* supernatant and control and high single lytic and non-lytic *Alexandrium* additions ($1000 \text{ cells ml}^{-1}$) after 96 h, bacterial community composition was analyzed using DGGE profiles of 16S rDNA fragments of duplicate samples. The genetic similarity analysis showed that the bacterial communities in initial North Sea water and in both lytic and non-lytic *Alexandrium* supernatant were unique and different (Fig. 4). After four days of incubation, profiles of the 16S rDNA fragments recovered from bacteria from all treatments were similar, and more similar to the initial North Sea community composition than either of the *A. tamarens* supernatant. However, control and non-lytic *Alexandrium* treatments showed a higher similarity to each other than with lytic *Alexandrium* treatments (Euclidean distance similarity in UPGMA).

Comparative analysis of TRFLP fingerprints was done on the basis of one replicate from the initial North Sea water and both initial *A. tamarens* supernatants, and all experimental treatments 96 h after incubation. Two replicates were analyzed in control treatments and high lytic *Alexandrium* treatments. Lytic and non-lytic *Alexandrium* supernatants differed from each other and the initial bacterial community (Fig. 5). Bacterial community composition changed in all treatments during the experiment, but still showed high similarities among the different treatments. However, treatments with high continuous additions of lytic and non-lytic *Alexandrium* differed more from the control than treatments with single additions of lytic and non-lytic *Alexandrium* (Fig. 5).

4. Discussion

Our results confirmed that allelopathic substances can shape the natural plankton community without the presence of the allelopathic cells, and also impact bacteria. The lytic *Alexandrium* supernatant inhibited biomass of phytoplankton and large nanoflagellates but benefitted bacteria, whereas the non-lytic *Alexandrium* supernatant provoked higher final abundances in several plankton species and benefitted nanoflagellates and bacteria.

Allelopathy is the impact of secondary metabolites, produced by phytoplankton, on growth and functions of other microbes (Cembella, 2003; Legrand et al., 2003; Gross, 2003; Graneli and Hansen, 2006; Ianora et al., 2006). According to this definition, both *A. tamarens* strains, lytic and non-lytic *Alexandrium*, exhibited allelopathy. However, adding a supernatant of any algal culture probably will show some “effects” on a community when compared to adding medium. Some of these observations may result from feedback mechanisms within the microbial community (i.e. an increase in bacterial biomass can explain an increase in heterotrophic nanoflagellate biomass). The beneficial effects of the non-lytic *Alexandrium* supernatant on *Chaetoceros* spp. may be explained by metal complexing properties of extracellular organic material, derived from phytoplankton, which can affect trace metal availability as well as seawater toxicity (Croot et al., 2000; Vasconcelos et al., 2002; Rijkenberg et al., 2008). In a laboratory study, growth of the diatom *Phaeodactylum tricornutum* was enhanced by algal exudates of different macro- and microalgae. The exudates supported nutrient uptake but also changed the intensity of production and nature of *P. tricornutum* own exudates (Vasconcelos and Leal, 2008).

Previous comparisons of both *Alexandrium* strains showed that only the lytic strain (Alex2) affected a whole range of different target species whereas the non-lytic strain (Alex 5) showed no negative effect (Tillmann and Hansen, 2009; Tillmann et al., 2009). Therefore, we interpret all changes caused by the lytic *Alexandrium* that differ from changes caused by the non-lytic strain in this study as due to lytic compounds.

High lytic *Alexandrium* supernatant additions inhibited the growth of *P. globosa*, whereas diatom abundance was not significantly negatively affected in comparison to the control (except *Chaetoceros* $>10 \mu\text{m}$ in the single addition). However, compared to non-lytic supernatant, lytic *Alexandrium* supernatant had a significantly negative effect on diatoms. In previous field experiments, several diatom species were only slightly inhibited by *A. tamarens* allelochemicals (Fistarol et al., 2004). When compared to other target species, *Thalassiosira weissflogii* was relatively resistant to lytic substances released from different *Alexandrium* species (Tillmann et al., 2008) supporting the hypothesis that diatoms are more resistant to the allelochemical substances produced by toxic dinoflagellates than ciliates and flagellates. However, recent co-incubation experiments of *A. tamarens* and 10 different phytoplankton species showed that both diatom species (*Skeletonema costatum*, *T. weissflogii*) clearly were affected, with *S. costatum* being amongst the most sensitive species (Tillmann and Hansen, 2009). Species-specific consequences of allelopathy are known from other allelopathic dinoflagellates such as *Karlodinium micrum* and *Karenia brevis* (Adolf et al., 2006; Kubanek et al., 2005). When exudates of the red-tide dinoflagellate *K. brevis* were tested on 12 different phytoplankton target species, only six of them were inhibited compared to the control (Kubanek et al., 2005). Among the diatoms tested by these authors, *S. costatum* and *T. weissflogii*, were also among the most sensitive target species.

During the first 24 h, bacteria grew in all treatments due to the labile carbon added in form of peptone. Since PO_4 and NO_3 were

Fig. 3. Bacterial abundance, biomass, production, production bacteria⁻¹ and production biomass⁻¹ ratios in initial North Sea water (May 2007), lytic and non-lytic *Alexandrium* supernatant, and after 96 h of incubation with addition of different *Alexandrium* supernatant concentrations. Controls were incubated with K-medium. Mean ($n = 3$; $\pm$ 1 S.D.). Treatments significantly different from control are marked with a star. Lytic *Alexandrium* supernatant treatments significantly different from corresponding non-lytic *Alexandrium* supernatant treatments are marked with a triangle.

added in excess, bacteria were probably carbon limited when bacterial abundance stabilized after 24 h. Bacteria reached higher growth rates and bacterial yields in treatments containing high additions of supernatant. Highest bacterial yields were reached in lytic *Alexandrium* single additions. The release of DOM is usually an important source of labile dissolved organic carbon (DOC) for bacteria (Valiela, 1995). Dense algal cultures contain DOM due to phytoplankton exudates. The addition of supernatant provided an additional source for DOC, and therefore supported higher bacterial yields in both lytic and non-lytic *Alexandrium* supernatant treatments, compared to control treatments. However, DOM deriving from *A. tamarensis* cultures was found to be essentially bacteria resistant (Chen and Wangersky, 1996). Additionally, lysis of plankton in lytic *Alexandrium* supernatant treatments may have

increased the DOM pool even further. Mixed culture trials of *R. salina* and the allelopathic haptophyte *P. parvum* lead to a significant increase of DOC concentration after 30 min and an increase in bacterial biomass after 6–12 h (Uronen et al., 2007). We assume that DOC released from lysing cells was available to bacteria in lytic *Alexandrium* supernatant treatments.

Bacterial growth usually results in a slightly delayed growth of bacterivores; those have to adapt to the new nutrient conditions, but can reach high growth rates (4–6 h doubling time) (Weisse and Scheffelmöser, 1991; Jurgens et al., 2000). When bacterial grazers become abundant, bacteria decrease rapidly. We could observe this rapid decrease in all treatments after 48 h of incubation. However, bacterial decrease (a proxy for bacterivory) was lower in single and high continuous additions of lytic *Alexandrium*

Fig. 4. DGGE profiles of 16S rRNA gene fragments from (A) lytic *Alexandrium* culture, (B) non-lytic *Alexandrium* culture, (C) initial North Seawater, and treatments (two replicates each) with high single additions of: (D, E) lytic *Alexandrium* supernatant; (F, G) non-lytic *Alexandrium* supernatant. (H, I) K-medium control after 96 h of incubation. Cluster analysis (UPGMA) showing the similarity of the lanes is shown below the gel image. Values give Euclidean distance similarity between the lanes.

supernatant (30%) compared to non-lytic additions (60%), suggesting that predation pressure was lower in treatments containing lytic compounds.

Nanoflagellate grazing accounts for most of the protistan bacterivory in plankton (Jürgens and Massana, 2008). Previous studies have shown that the presence of allelopathic substances

could eliminate up to 80% of nanoflagellates over 3–4 days, and therefore contribute to a high bacterial abundance in algal filtrate treatments (Fistarol et al., 2003, 2004). Our study confirms these results, since the abundance of flagellates after 96 h of incubation was suppressed in lytic *Alexandrium* compared to non-lytic *Alexandrium* treatments.

A strong top down control in nutrient rich conditions may lead to a high production per bacterium but a low bacterial abundance (Ducklow et al., 2001). The larger size of bacteria and the relatively higher production per bacterium in non-lytic *Alexandrium* treatments could be explained by better growth caused by nutrient recirculation, a side effect of nanoflagellate grazing due to sloppy feeding. However, production biomass⁻¹ did not differ between lytic *Alexandrium* and all other treatments, indicating that bacterial production after continuous and single addition was not affected by *A. tamarens* supernatant after 96 h.

The effect of allelochemicals on bacterial abundance and production has been reported in a few studies (Nielsen et al., 1990; Fistarol et al., 2004; Uronen et al., 2007). In this study, the effect of allelochemicals on bacterial composition in natural communities is reported for the first time. Studies on *Alexandrium* spp. blooms revealed a high abundance of bacteria that were repeatedly found in association to phytoplankton blooms (Wichels et al., 2004). Bacteria associated with diatom cultures revealed specific bacterial “satellite” communities (Schäfer et al., 2002; Grossart et al., 2005; Kaczmarek et al., 2005). In *Alexandrium* cultures, the presence of specific bacterial consortia may be explained by symbiotic relationships between bacteria and the dinoflagellate (Amaro et al., 2005; Jasti et al., 2005). Additionally, cell wall components, exudates, pigment composition and life cycle features of the algae may also select for specific bacterial consortia. However, bacterial communities associated to algae are not necessarily species specific, and strains of the same algae can harbour different bacterial communities (Sapp et al., 2007).

In this study, the initial composition of each bacterial “satellite” community was very different among the two *Alexandrium* supernatants and the North Sea water. After 96 h of incubation, the bacterial community shared a high similarity in all treatments and was closer to the initial North Sea Water than to any *Alexandrium* supernatant. The development of the bacterial community towards a similar composition among the treatments may be due to the addition of nutrients, especially peptone, and the selection for copiotrophic bacteria (Lauro et al., 2009). If bacterial “satellite” communities can be explained by their adaptation to

Fig. 5. TRFLP bacterial community composition data analysed by non-metric multidimensional scaling, showing the relative similarities between the treatments based on Euclidean distribution.

algal released DOM (Sapp et al., 2007), an easily converted carbon source such as peptone may have made it irrelevant for bacteria to take up algal released DOM, and therefore, no strong bacterial selection could be observed.

In nature, allelochemicals are assumed to be continuously released into the surrounding seawater but not much is known about degradation of the compound or adaptation of target organisms. Allelochemicals of *A. tamarensis* have been shown to be quite stable at room temperature and not degraded by the bacteria consortium present in the culture (Ma et al., 2009). Our results showed that addition of one high pulse of lytic *A. tamarensis* supernatant affected the natural plankton community more than adding almost the same quantity of supernatant semi-continuously. Experimental observations of cell blistering and lysis in target species indicate that the compounds primarily seem to act upon the outer membrane of the target organisms (Tillmann et al., 2007). This binding probably leads to a removal of compounds from solution, as lysis of target species is not only depending on the concentration of donor cells but, interestingly, also depends on concentration of the cells of the targeted species (Tillmann, 2003; Tillmann et al., 2007). In addition, initial characterizations have shown a general “stickiness” of the compounds to all kind of surfaces, e.g. for filter material and for plastic containers compared to glass (Ma et al., 2009). Consequently, lytic substances binding to different matrices like growing *P. globosa* colonies, diatoms and organic surfaces may explain the lower efficiency of the continuous additions. Hence, as the amount of matrices increased over time, the impact of lytic substances did not accumulate but weakened in the microcosms. Furthermore, the phytoplankton community in treatments with continuous additions of *A. tamarensis* supernatant might have adapted to the exposure of allelochemicals or might have metabolized or degraded allelopathic compounds.

Recently, allelopathy has been questioned as to its involvement in algal bloom formation (Jonsson et al., 2009). A meta-analysis of data from several experiments regarding allelopathy showed that donor organisms provoked inhibitory effects towards other species only at Chl *a* concentrations $>5 \mu\text{g l}^{-1}$ (bloom conditions). On the other hand, studies with low concentrations of donor organisms are scarce. In our study, the lower tested concentrations of lytic *Alexandrium* supernatant did not obviously impact the natural plankton community. However, motile phytoplankton, such as *A. tamarensis*, reach highest densities in calm weather conditions and accumulate in horizontal layers, along thermoclines or the water surface (MacIntyre et al., 1997; Mouritsen and Richardson, 2003; Ryan et al., 2008). High concentrations of cells, and thus, secondary metabolites, similar to those in this experiment, can accompany *A. tamarensis* cells and impact the phytoplankton community around these layers. There is increasing interest in exploring microbial interactions at the nanometer to millimeter scale (Azam and Malfatti, 2007). Microorganisms possess complex and sophisticated sensory and behavioral adaptations to their environment (Jennings, 1906). Diverse populations of marine microbes have been shown to markedly modify their foraging behavior when navigating a patchy habitat (Seymour et al., 2009) and may avoid allelopathic compounds via negative chemotaxis. Our results indicate that allelochemicals have no direct negative impact on bacteria. Layers of lytic dinoflagellates could therefore be bacterivore free, nutrient rich microhabitats for bacteria. However, the interplay between allelopathy and bacterivory needs to be further investigated.

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