Intraspecific facilitation by allelochemical mediated grazing protection within a toxigenic dinoflagellate population

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Living in a cloud: intraspecific facilitation by allelochemical mediated grazing protection

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Abstract

Dinoflagellates are a major cause of harmful algal blooms, with consequences for coastal marine ecosystem functioning and services. *Alexandrium tamarense* is one of the most abundant and widespread toxigenic species in the temperate northern and southern hemisphere, and produces paralytic shellfish poisoning toxins as well as lytic allelochemical substances. These bioactive compounds may support the success of *A. tamarense* and its ability to form blooms. Here we investigate grazing of highly (Alex2) and moderately (Alex4) allelochemical active *A. tamarense* strains and a non-allelochemical active conspecific (Alex5) by the heterotrophic dinoflagellate *Polykrikos kofoidii* in monoclonal and mixed experimental set-ups. While Alex4 and particularly Alex5 were strongly grazed by *P. kofoidii* in monoclonal set-ups, both strains did grow well in the mixed assemblages (Alex4+Alex5 and Alex2+Alex5). Hence, the allelochemical active strains facilitate the non-active strain by protecting the population against grazing. This allelochemical mediated facilitation resembles associational resistance observed in various macroalgal and plant communities. Occurring intraspecifically it may partly explain the high genotypic and phenotypic diversity of *Alexandrium* populations. It is furthermore conceivable that these diverse populations comprise multiple cooperative traits that may support mutual intraspecific facilitation, which in turn will promote the success of this notorious harmful algal bloom species.

Key words: Allelochemicals; facilitation; *Alexandrium tamarense*; species interaction; grazing; harmful algal blooms; phenotypic diversity; allele specific quantitative PCR (asqPCR); associational resistance.

Introduction

Marine phytoplankton account for approximately half of the global annual net primary production (Falkowski et al. 2004). Their high biomass turnover rate and conversion of light energy, CO_2 and inorganic nutrients into organic material drive the marine pelagic ecosystem. Like many aquatic microorganisms, phytoplankton can have large population sizes, and typically have high rates of predominantly asexual reproduction. Furthermore, phytoplankton live in a rather open and seemingly homogenous pelagic habitat. Yet, communities may also exhibit a patchy distribution even on small geographic scales, may show horizontal organization in thin layers and seem to be, at least partly, organized as metapopulations (Montagnes et al. 1999, Rynearson et al. 2009, Durham and Stockert 2012, Menden-Deuer 2012). Molecular data show that plankton populations are genetically diverse, and that they are temporally and spatially distributed based on historical, ecological and local oceanographic conditions (Foissner 2006, Alpermann et al. 2009, Nagai et al. 2007, Tahvanainen et al. 2012). Distinct phytoplankton populations typically comprise a variety of genotypes and phenotypes (Alpermann et al. 2009, Bachvaroff et al. 2009, Alpermann et al. 2010). Such genotypic variability and phenotypic diversity suggest high adaptive capabilities of populations, particularly advantageous in highly dynamic environments like coastal waters (Sultan and Spencer 2002). High adaptive capabilities, in turn, may support species and populations to thrive and form dense algal blooms under favourable environmental conditions.

Some phytoplankton species have the ability to produce toxins, and their proliferation leads to so-called harmful algal blooms (HABs). Such HABs can have major implications for marine ecosystems, causing mortality of whales, fish, and other marine life, and threatening human

health through accumulation of toxins in the food chain (Hallegraeff 2003, Anderson et al. 2012). Dinoflagellates are notorious HAB formers and can produce a broad variety of potent bioactive substances from which paralytic shellfish poisoning toxins (PSTs) are most common (Anderson et al. 2012). Additionally, many dinoflagellates have the ability to produce allelochemical compounds of poorly characterized chemical nature (Tillmann and John 2002, Cembella 2003, Ma et al. 2011). PSTs and allelochemicals have been shown to provide cells with protection against gazers (Tillmann and John 2002, Selander et al. 2006, Tillmann et al. 2009, Wohlrab et al. 2010) and competitors (Tillmann and Hansen 2009). Since grazing can remove up to 50% of gross biomass production (Landry and Calbet 2004), production and release of bioactive compounds may add to the success of dinoflagellates (Smayda 2002,Cembella 2003, Granéli and Hansen 2006). During HABs, phytoplankton population densities are high and allelochemicals likely provide protection against grazers, as well as competitors. At the onset of HABs, overall population densities are typically considered to be too low to effectively use extracellular allelochemicals to suppress competitors (Jonsson et al. 2009). It has been observed, however, that some phytoplankton species form dense clouds and thin layers where allelochemicals may become effective. These patches may be formed by behavioural processes (swimming, growth, and grazing) as well as by physical aggregation (e.g. Durham and Stockert 2012, Menden-Deuer 2012 and references therein). It is in these clouds with locally high population densities where concentrations of bioactive compounds can become concentrated enough for population protection, even when densities across the water column are relatively low.

Understanding the evolution of variation in phenotypic traits such as production of allelochemicals in genotypically diverse populations of HAB species is a challenge, as some

92 individuals carry potential costs, whereas the benefits are shared within the whole population,
93 supporting non-producers as well (i.e. cheaters, Lewis 1986). Within a population with closely
94 related individuals, however, cooperative traits can be favoured as a public good and facilitate
95 the success of the entire population (Hamilton 1964, Perkins and Swain 2009, Xavier 2011). This
96 principle of facilitation (Bruno 2003, West et al. 2006), has been shown in populations of various
97 organisms including bacteria (Lee et al. 2010), toxigenic cyanobacteria (van Gremberghe et al.
98 2009), amoeba (Mehdiabadi et al. 2006), and yeast (MacLean et al. 2010). Yet, to the best of our
99 knowledge, no study so far has experimentally investigated intraspecific facilitation in planktonic
100 protist populations such as marine dinoflagellates.

101 Here, we assess whether facilitation may occur within a population of the common HAB
102 species *Alexandrium tamarense*. Can production of allelochemical substances by some
103 individuals in an experimental population facilitate non-allelochemical producing individuals
104 and thereby support the success of multiple strains? To answer this question, we investigated
105 whether *Alexandrium* strains with intermediate and high allelochemical activity can protect a
106 non-allelochemical conspecific against the heterotrophic protist *Polykrikos kofoidii*. We show
107 that the allelochemically non-active strain is protected by the active strains. In fact, all strains
108 show enhanced growth in mixed assemblages. Our results clearly demonstrate intraspecific
109 facilitation, which may partly explain the high genotypic and phenotypic diversity often
110 observed in marine dinoflagellate populations.

111
112

Materials and methods

Algal cultures

Three clonal strains of *Alexandrium tamarense* (Group I) were isolated in May 2004 from the North Sea coast off Scotland (Tillmann et al. 2009) and grown in sterile filtered North Sea water (salinity 33 %) enriched according to K-medium (Keller et al. 1987) at 15°C, with an incident light intensity of 150 µmol photons m^{-2} s^{-1}, provided by cool white fluorescent lamps at 14h:10h light:dark cycles. All three strains Alex2, Alex4, and Alex5 produce PSTs. Alex2 and Alex4 are further characterized by producing allelochemical active, unknown lytic compound(s) (Tillmann et al. 2009). Alex2 was highly allelochemical active (lytic), Alex4 was moderately active (lytic), and Alex5 was non-active (non-lytic). The culture of *Polykrikos kofoidii* was established in 2009 also from coastal waters off Scotland (Tillmann and Hoppenrath 2013). The culture was routinely held in 63 mL culture flasks on a slow rotating plankton wheel (1 rpm) at 15°C and low light (10-20 µmol photons m^{-2} s^{-1}) and fed with *Lingulodinium polyedrum* (CCMP 1738) or the non-lytic strain Alex5. Prior to the experiments, *Polykrikos* was regularly (about once a week) fed *L. polyedrum* for a period of three months. A dense subculture used for experiment inoculation was starved for ~ 1 day so that no food algae were present, but *Polykrikos* did also not yet start massive gamete formation (Tillmann and Hoppenrath 2013).

Experimental design and set-up

Before starting the experiment, each strain was treated with antibiotics in order to reduce the bacterial background flora (Tillmann and Hansen 2009). Monoclonal cultures were grown for up-scaling from 500 mL to final 5,000 mL in serial batch cultures in order to guarantee equal physiological status of the strains in the experiment. Cells in exponential growth phase were

tested for their allelochemical properties with the *Rhodomonas salina* bioassay (see below) and then washed three times with sterile filtered K-medium over 10 µm pore size sieve in order to remove extracellular allelochemical compounds. This was also confirmed with the *Rhodomonas salina* bioassay. Thus, any observed allelochemical mediated effect is a result of the accumulated allelochemicals during the course of the experiments. The experiment was performed in a temperature and light controlled culture room on a slowly rotating plankton wheel with a speed of 1 rpm allowing homogenous mixing, but with minimal turbulence. The three strains of *Alexandrium* were grown in monoclonal cultures with starting cell densities of 500 cells mL^{-1}. Additionally, Alex5 was grown in two-strain mixtures with Alex2 and with Alex4 to a final concentration of 1,000 cells mL^{-1} (i.e. 500 cells mL^{-1} per strain). In order to control for the higher cell densities in the mixed cultures, additional experiments with monoclonal cultures of Alex5 were performed with initial cell densities of 1,000 cells mL^{-1}. All three strains and their mixtures were grown in triplicate with and without adding *Polykrikos* cells (20 cells mL^{-1}). The experiment started in completely filled 1,000 mL Schott flasks, and 500 mL was harvested after 24 h. The rest was further incubated in 500 mL flasks and 250 mL was harvested at day 2. Again, the remaining culture was incubated in 250 ml flasks and 125 mL was harvested at day 3. The remaining culture incubated in 125 mL flasks was harvested on day 4. The harvested samples were divided for cell counts of *Alexandrium* and *Polykrikos*, for allelochemical activity measurements using the *Rhodomonas salina* bioassay, and for DNA extraction and subsequent allele-specific quantitative PCR (asqPCR).

Counting procedure

Lugol's fixed (1 % final concentration) *Alexandrium* cells were counted after sedimentation of 3 x 1 mL aliquots using an inverted microscope. All or at least 300 cells in each 1 mL aliquot were counted. For counting *Polykrikos*, 10 ml samples were fixed with a mixture of formalin (1 % final, and Lugol's iodine solution (0.3 % final) and settled in 10 mL settling chambers. Whole chambers were counted. For each sample from experimental set-ups (see below), *Polykrikos* was scored as either containing food particles in their food vacuoles or without visible food vacuoles, in order to estimate the proportion of active grazers

Rhodomonas salina bioassay

A bioassay was performed with *Rhodomonas salina* strain KAC 30 as a measure of extracellular allelochemical toxicity of different monoclonal and mixed culture set-ups, i.e. the three strains Alex2, Alex4, and Alex5 growing monoclonally with initial cell densities of 500 cells mL^{-1} and the mixed assemblages of Alex2+Alex5 and Alex4+Alex5. Allelochemical activity towards *R. salina* was assessed at the start (day 0) and end (day 4) of each experiment. Bioassays were performed with *A. tamarense* cell-free supernatant gained after centrifugation of 50 mL sample. Supernatant was stored in 50 mL glass bottles at -20°C until used. For bioassays, 4 mL of a mixture of *A. tamarense* supernatant in up to five different dilutions (each dilution in triplicate) and *R. salina* (final cell concentration 1.0×10^4 cells mL^{-1}) were incubated in glass scintillation vials at 15°C for 24 h in darkness. Incubation was stopped after 24 h by fixing samples with Lugol's iodine solution (2 % final concentration). The concentration of surviving *R. salina* (cells with a normal cell shape) was estimated with an inverted microscope and compared to the

control (filtered seawater) to calculate percentage of survival. For all samples, a sub-area corresponding to >800 *Rhodomonas salina* cells in the control was counted.

Allele-specific quantitative PCR

As the three haploid *Alexandrium* strains used in this study were characterized by differently sized alleles at certain microsatellite loci, the strain specific amplicons derived by PCR from a mixed DNA template – such as those that were derived from mixed culture experiments – could be distinguished and relatively quantified by allele-specific quantitative PCR (asqPCR) (Meyer et al. 2006). In order to perform accurate ratio analyses with asqPCR, DNA extraction, alleles and corresponding primer and PCR conditions have been adjusted. A volume of ~50-75 x 10^3 cells per sample was taken from each culture at every sample time. The genomic DNA extractions were performed with a DNeasy plant mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. In order to ensure a complete cell lyses the protocol was optimized as described in the following. To carry out a successful DNA extraction, cells were harvested in a 50 mL reaction tube, centrifuged at 3000 g for 5 min and then further pelletized in a 2 mL microcentrifuge tube (8000 g, 5 min). A mixture of ~300 µL of 1 mm and 0.3-0.6 mm sized glass beads was added to the microcentrifuge tube. 400 µL of Buffer AP1 was added to every tube and mixed by vortexing. The mixture was incubated for 15 min at 95°C in a ThermoMixer (Eppendorf, Hamburg, Germany), while constantly mixing the samples with a speed of 1,200 rpm. After a short cooling step on ice, the remaining intact cells were disrupted in the TissueLyser (Qiagen) for 2 times 1 min at 20 Hz. After two disruptions, 4 µL of RNaseA stock solution (100 mg/mL, Qiagen) was added and incubated at 65°C for another 15 min, while mixing in the ThermoMixer with a speed of 1,200 rpm. The following steps in DNA purification

have been performed according to the manufacturer's instructions.The purity and quantity of the resulting DNA was analysed by UV-spectroscopy with a NanoDrop ND-1000 (Peqlab, Erlangen, Germany) and the integrity of DNA fragments of a molecular weight of about 20 kb was verified on 0.8 % agarose gel.

PCR reactions were carried out with a Type-it Microsatellite PCR Kit (Qiagen, Hilden, Germany) as 25 µL reactions containing 1 µL (10 ng) template DNA, 12.5 µL 2 x Type-it Multiplex PCR Master Mix (including Taq polymerase and reaction buffer), and 0.2 µL (0.2 µMol, final conc.) of each primer per reaction (Atama15; Fwd: CCACATGCTCAACATTCACGTATACAG, Rev: GTATTTGCTCATATGGCTTGG (Nagai et al. 2004). For better resolution in subsequent fragment analysis, 2.5 µL of Q-Solution (5x) were added to the reaction mix. After the initial denaturation (95°C, 5 min), 35 cycles of denaturation at 95°C for 30 sec, annealing at 60°C for 1 min and elongation at 70°C for 30 sec were carried out, followed by a final extension at 60°C for 30 min in a Gradient Mastercycler (Eppendorf, Hamburg, Germany). For fragment analysis purposes, the forward primers were labelled with a fluorescent dye 6-FAM. Sizing of amplified microsatellite alleles was carried out with GeneMapper version 3.7/4.0 (Applied Biosystems, Darmstadt, Germany) after capillary electrophoresis on a 3130xl Genetic Analyzer (Applied Biosystems).

Cell numbers of the different *Alexandrium* strains in mixed cultures were calculated using the peak area under the specific allele peak, i.e. the sum of fluorescence signal from a strain specific allele. Total peak-area was calculated for each sample as the sum of the peak area values of the two differently sized microsatellite alleles, each representative for a specific strain. The estimate of the relative abundance of a strain was then calculated as the proportion of the peak area of the specific allele of a strain from the total peak area (i.e., the sum of peak areas of alleles

from both strains). This relative abundance estimate for both strains in the mixed assemblages was then converted to cell numbers by multiplication of strain specific relative abundance estimates with total cell numbers obtained by Utermöhl counts from the respective sample. The asqPCR assays were validated with standard curves derived from mixed-matrices with relative contributions to the mixed population from 0, 20, 40, 50, 60, 80 and 100% of one of the two strains. The linear regression estimated for these mixed populations of the combinations Alex2+Alex5 and Alex4+Alex5 showed that the relative contribution of the peak area of the allele of one strain was directly proportional to the actual proportion of cells of the respective allele in the mixture. The slope and the regression coefficient of strain Alex5 was 1.057 and R^2=0.96 and 1.001 and R^2=0.995 for mixed populations with Alex4 and Alex2, respectively. Cell numbers of strains from experimental samples are presented as mean values of triplicate cultures and their standard deviation.

Statistical analysis

The population growth in replicated culture set-ups was calculated from day 1 to day 4 in all cases but for the set-up with Alex5 with grazer, for which the experiment lasted only until day 3 when all cells were grazed. Growth rate (μ) was calculated by fitting an exponential function though all replicate cell counts in the respective time periods according to:

$$A = A_1 e^{\mu t} \qquad A=A_1 e^{\mu t}$$

where A refers to the cell density, A_1 to cell density at the day 1, and t to the time of the experiment.

Differences in *Alexandrium* growth between treatments were tested using one-way ANOVA, followed by post-hoc comparison of the means using Tukey's HSD (Sokal and Rohlf

1995). Differences of *Polykrikos* growth was either tested with an ANOVA as stated above or using a t-test (Sokal and Rohlf 1995). Normality was tested according to Shapiro-Wilk (Sokal and Rohlf 1995). Data were log-transformed if this improved the homogeneity of variances, as evaluated by Levene's test. All statistical analyses were carried out in Sigmaplot version 12 (Systat Software, Erkrath, Germany).

Results

Allelochemical activity

Rhodomonas salina bioassays were performed after washing the cultures prior to each experiment. These assays demonstrated that at the start of each experiment, cultures were not lytic (Table 1). During the course of the experiment, strain Alex2 (monoclonal set-up), Alex2+Alex5, and Alex4+Alex5 showed increased allelochemical lytic activity after four days (ANOVA, $p < 0.05$). For cultures with only Alex5 or Alex4 no allelochemical activity could be detected throughout the experiment.

Growth in monoclonal and mixed cultures without grazer

All *Alexandrium* cultures showed a one day lag phase, after which the exponential phase started (Fig. 1). Growth rates of all three strains in the monoclonal cultures (Table 2) were comparable. Because monoclonal cultures started with 500 cells mL^{-1} and mixed cultures with 2 x 500 cells mL^{-1}, we tested whether this difference in initial population densities may have had an effect on growth of an individual strain, Alex5. We could confirm that growth of Alex5 cultures starting

with 500 or with 1,000 cells mL^{-1} was comparable with 0.41 ± 0.06 d^{-1} and 0.37 ± 0.02 d^{-1}, respectively.

Grazing impact on Alexandrium

Net population growth of both Alex4 and Alex5 became negative after addition of *Polykrikos*, whereas growth of Alex2 remained unaffected compared to the control (ANOVA, $p<0.05$; Fig. 1A; Table 2). When *Alexandrium* strains were grown in the presence of *Polykrikos*, both Alex2 and Alex5 showed increased growth rates as compared to the mixed assemblage of both strains without grazer, whereas Alex4 mixed with Alex5 showed a lower or similar growth rate as compared to their respective monoclonal cultures (ANOVA $p<0.05$; Fig. 1B; Table 2)

Alexandrium *impact on the grazer*

In all experimental set-ups where *Polykrikos* was added, initial grazing was indicated by a general positive growth (Fig. 2A, B) and a high number of *Polykrikos* cells that contained at least one visible food particle at day 1 (Fig. 2C, D). This confirms that washing of the *Alexandrium* cultures successfully prevented addition of allelochemicals produced prior to the experiment (Table 1). After one day all *Polykrikos* were found with ingested *Alexandrium* cells when fed with Alex5, but when fed with Alex4 and Alex2 a substantial proportion of *Polykrikos* was found without visible food inside (Fig. 2C).

Initial growth rates of *Polykrikos* (i.e. until day 1) were positive, except when grown on Alex5 mixed with Alex2 which showed no change in cell density (Fig. 2A, B). Highest growth rates of *Polykrikos* were found when fed on monoclonal cultures of Alex5, intermediate growth rates when fed on Alex4, and negative growth rates when fed on Alex2. This corresponds to the

relative lytic activity of the different strains based on the *Rhodomonas salina* bioassay. When Alex5 was provided in a mixture, growth of *Polykrikos* was strongly reduced (with Alex4) or even became negative (with Alex2; Fig. 2B; see also Table 2). After one day, 60-95% of *Polykrikos* cells had grazed on *Alexandrium* cells, however with significanty lower numbers when grown with the allelochemical active strains Alex2 and Alex4 as food compared to the non-lytic strain Alex5 (Fig. 2C, D). The number of *Polykrikos* cells with visible food vacuoles subsequently declined when fed on monocultures of Alex2 and Alex4, and a mixture of Alex2 with Alex5, reaching 0-30% by the end of the experiment (Fig. 2C, D). When grown on a mixture of Alex4 and Alex5, a majority of *Polykrikos* cells exhibited food vacuoles until the end of the experiment, whereas when grown on Alex5, *Polykrikos* became fully starved at the end of the experiment when all food was grazed away (Fig. 2C, D).

Discussion

All three *Alexandrium* strains exhibited comparable growth rates when grown in monoclonal cultures (Table 2). Furthermore, in mixed cultures growth rates remained unaltered, indicating that the allelochemicals from the lytic strains did not affect growth of the non-lytic strain. While net population growth of the most lytic Alex2 was not affected by *Polykrikos*, growth of both Alex4 and Alex5 decreased as result of grazing by *Polykrikos*. Interestingly, when grown in mixtures and with *Polykrikos* added, net population growth in all three strains was positive (Fig. 1). Furthermore, the lytic strain Alex2 grew significantly better (ANOVA $p<0.05$) in the mixed assemblage as compared to its monoclonal culture when in presence of *Polykrikos*, indicating a benefit not only for the non-lytic Alex5 but also for the lytic strain Alex2. Thus, our results

317 demonstrate that allelochemical active strains of *Alexandrium* facilitate growth of a strain not
318 producing allelochemicals by protecting it from grazing.
319 *Polykrikos* could grow well on monocultures of Alex4 and Alex5. Yet, a mixture of both
320 strains did reduce its growth with about 33% as compared to growth on Alex5 alone. This can be
321 explained by the higher population densities in the mixed assemblages of 1000 vs. 500 cells mL^{-1},
322 which may have reduced the direct grazing pressure of *Polykrikos* on Alex4. As a
323 consequence, Alex4 could reach sufficiently high cell densities in order to produce enough
324 allelochemicals to be an effective protection against *Polykrikos*. When Alex5 was provided
325 together with Alex2, growth of *Polykrikos* was affected stronger due to the high allelochemical
326 potency of Alex2, and all *Polykrikos* cells died before the third day of the experiment (Fig. 2).
327 Our results clearly demonstrate that allelochemicals can protect both the producer as well
328 as non-producing conspecifics against grazing. Other studies have shown that the same
329 allelochemicals strongly affect growth of many other phytoplankton species that are potential
330 resource competitors (Fistarol et al. 2004, Tillmann et al. 2008, Tillmann and Hansen 2009,
331 Weissbach et al. 2011). The view that allelochemicals play an important ecological role is widely
332 appreciated (see e.g., reviews by Smayda 2002, Cembella 2003, Tillmann et al. 2008). The
333 question remains, however, how allelochemical traits that benefit the producer as well as the
334 population as a whole can have evolved, and how these traits are maintained in the population. In
335 general, selection for a public good will take place when costs versus benefits for the producer
336 are relatively low as compared to the relatedness between producer and non-producer (Hamilton
337 1964, MacLean et al. 2010, Xavier 2011). In other words, if the allelochemical active
338 *Alexandrium* strains are closely related enough to the non-active strains, production of
339 allelochemicals will still be under selection and can "tolerate" non-producers, providing a benefit

for the survival success of the entire population.The *Alexandrium* strains used in our study were collected from the same population, and thus evolution of traits (allelochemical production) that serve the public good (protection) is likely supported due to their close relatedness. This form of group selection for allelochemicals can only apply, however, when the relative abundance of producers is high enough to support the entire population. Indeed, in a natural population of *A. tamarense*, only two out of 88 clonal isolates were non-producers, whereas all others were allelochemically active (Alpermann et al. 2010). Yet, there seems to be certain constraints on the expression of allelochemical properties and on the production of allelochemicals as intermediate lytic phenotypes were in the majority and the trait was normally distributed within the strains sampled from a natural population (Alpermann et al. 2010).

Allelochemical properties in *Alexandrium* spp. not only show quantitative differences, but also show qualitative differences in their target spectrum (Tillmann et al. 2008). Intraspecifically, however, no such qualitative differences could be shown, indicating a similar cocktail of allelochemical compounds within a species of *Alexandrium* (Tillmann et al. 2009). A high variability in phenotypic traits may allow intraspecific facilitation, and thereby may promote the overall success of *Alexandrium*. Besides acting as a grazer deterrent, it has been shown that allelochemicals also negatively affect various phytoplankton species, thus protecting the entire population against potential resource competitors (Fistarol et al. 2004, Tillmann and Hansen 2009). The protection of non-allelochemical producers by allelochemical producers will also enable the survival of a larger diversity of genotypes. It is conceivable that with a higher diversity, a population contains more cooperative traits, providing additional benefits for the entire population. Such alternative traits may include for instance chain formation, swimming speed, nutrient uptake capabilities, and intrinsic growth rate. In our experiment, the non-lytic

strain Alex5 contained the highest amount of PSTs, a trait that potentially allows protection against grazing by copepods (Selander et al. 2006, Wohlrab et al. 2010, Selander et al. 2011). Under calm environmental conditions, relatively few *Alexandrium* strains may aggregate and form a cloud in which allelochemical concentrations can become high enough to protect the population from grazing by protists, and from potential resource competitors (Fig. 3A.B). Production of high amounts of PSTs by some genotypes may protect the population against grazing by larger zooplankton species such as copepods (Fig. 3C). Thus, *Alexandrium* populations may exhibit mutual intraspecific facilitation by multiple traits. The variability in selection acting on those traits may, at least partly, explain the high genotypic and phenotypic diversity observed in natural populations.

Associational resistance describes the facilitation of a plant by its neighbor by providing protection against grazing by an herbivore (Tahvanainen and Root 1972, Barbosa et al. 2009). It has been shown to occur within terrestrial plant communities (Atsatt and O'Dowd 1976, Hambäck et al. 2000, Hambäck et al. 2003), as well as between macroalgal species and their epiphytes (Wahl and Hay 1995, Karez et al. 2000, Smith et al. 2010). For associational resistance to be effective, species should be in close proximity. This is evident in neighboring plants in terrestrial ecosystems, as well as for macroalgae and their epiphytes. Here we demonstrate that associational resistance may also occur in pelagic microalgae populations, when exhibiting the ability to form dense aggregates such as clouds (Fig. 3). Furthermore, besides occurring between species, we show that associational resistance may also occur within a species, and thereby further promotes the intraspecific diversity of a population.

We have experimentally shown that intraspecific facilitation within *Alexandrium* populations may benefit the success of multiple strains. But can these findings be extrapolated to

386 the natural environment? A high genotypic diversity will support a high population fitness under
387 diverse environmental conditions (Sultan and Spencer 2002). Thus, for phytoplankton
388 populations, genotypic diversity may be sustained by group selection. Yet, group selection is
389 often not considered to occur in marine phytoplankton populations due to their seemingly
390 homogeneous or ephemeral spatial distribution pattern and typical low population densities that
391 may prevent beneficial interactions. Dispersal of phytoplankton cells in the water column via
392 turbulence may indeed prevent cell aggregation, and in particular may separate daughter cells
393 after cell division, which in turn will prevent accumulation of certain traits and corresponding
394 alleles. A recent meta-analysis concluded that there is no evidence for an inhibitory effect of
395 allelochemicals during the initiation phase of planktonic microalgal blooms, derived from a
396 diffusion model that assumes turbulent mixing (Jonsson et al. 2009). Cell accumulation and
397 bloom formation, however, typically occur at low mixing and water column stratification
398 (Smayda 2002), and a model assuming turbulent mixing may not represent very well those
399 conditions under which dinoflagellate blooms typically are formed. In fact, plankton populations
400 are often not homogenously distributed, but rather show a spatially structured distribution, for
401 instance as clouds or thin layers (Montagnes et al. 1999, Durham and Stockert 2012, Menden-
402 Deuer 2012). Accumulation of cells in patches is also supporting the life cycle of dinoflagellates.
403 *Alexandrium* species, for instance, have a prominent vegetative phase, but sexual reproduction
404 plays a crucial role in the life cycle (Anderson et al. 2012). Obviously, *Alexandrium* gametes find
405 their corresponding mating type in a three dimensional spatial matrix at a needed high encounter
406 rate, even when bulk cell concentrations are too low and would only allow for few casual
407 encounter (Wyatt and Jenkinson 1997). The functioning of allelochemical mediated facilitation
408 will therefore depend on the degree of species dispersal, i.e. the local accumulation of a

409 population, as well as on the rate at which extracellular allelochemicals are produced and the rate
410 of their diffusion in the three dimensional spatial matrix.
411 All in all, we show that allelochemical active strains of *Alexandrium tamarense* protect a
412 non-lytic conspecific from grazing by *Polykrikos kofoidii*. This allelochemical mediated
413 intraspecific facilitation may partly explain the high genotypic and phenotypic diversity of
414 *Alexandrium* populations. Obviously, beside traits related to defense and competition more traits
415 exist in phytoplankton populations that may serve the entire population. Consequently, multiple
416 traits within an *Alexandrium* population potentially lead to mutual facilitation, which may further
417 promote the success of this notorious harmful algal bloom species.
418

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564

Table 1. Overview of the allelochemical potency in terms of percentage *Rhodomonas* lyses with respect to culture volume at the start and end of each experiment. Superscript letters indicate significant differences between the experimental set-ups (ANOVA, $p<0.05$). Values show mean ±SD (n=3).

Allelochemical potency	
Set-up	% lysed *Rhodomonas*
Experiment start (day 0): Alex2	0.2 ± 4.2^{A}
Experiment start (day 0): Alex4	3.4 ± 6.5^{A}
Experiment start (day 0): Alex5	0.2 ± 1.4^{A}
Experiment end (day 4): Alex2	58.7 ± 10.3^{B}
Experiment end (day 4): Alex4	2.2 ± 2^{A}
Experiment end (day 4): Mix Alex2+Alex5	72.9 ± 7^{B}
Experiment end (day 4): Mix Alex4+Alex5	16.7 ± 1.5^{C}

572
573 **Table 2.** Overview of the population growth of different experimental set-ups in this study. Superscript letters indicate significant differences
574 between the experimental set-ups (ANOVA $p<0.05$) or t-test in the case of *Polykrikos* + clonal prey versus *Polykrikos* + mix prey ($p=0.05$).
575 Capital superscript letter indicate significant different groups in a column and small superscript letter indicate significant different groups in the
576 rows. Values show mean ±SD (*n*=3).
577

Set-up	Growth (μ)				
	Alex2	Alex4	Alex5	Total in mixed set-up	*Polykrikos*
Monoclonal	$0.40\pm0.04^{A,a}$	$0.31 \pm 0.03^{A,a}$	$0.41 \pm 0.06^{A,a}$	-	-
Mix Alex2+Alex5	$0.32 \pm 0.01^{A,a}$		$0.34 \pm 0.01^{A,a}$	$0.33 \pm 0.01^{A,a}$	-
Mix Alex4+Alex5		$0.34 \pm 0.04^{A,a}$	$0.36 \pm 0.04^{A,a}$	$0.35 \pm 0.04^{A,a}$	-
Monoclonal + Grazer	$0.31\pm0.04^{A,a}$	$-0.91 \pm 0.31^{B,b}$	$-1.61 \pm 0.10^{B,c}$		A2: -0.57 ± 0.06^{A} A4: 0.27 ± 0.07^{BC} A5: 0.39 ± 0.04^{B}
Mix Alex2+Alex5 + Grazer	$0.57 \pm 0.03^{B,a}$		$0.46 \pm 0.03^{A,b}$	$0.51 \pm 0.03^{B,ab}$	-1.27 ± 0.90^{A}
Mix Alex4+Alex5 + Grazer		$0.18 \pm 0.04^{C,a}$	$0.42 \pm 0.08^{A,b}$	$0.35 \pm 0.09^{A,ab}$	0.26 ± 0.06^{C}

578 **Figure legends**

579

580 **Figure 1.** Growth of *Alexandrium tamarense* strains when exposed to *Polykrikos kofoidii*. (A)
581 Growth rates in the monoclonal set-up with the lytic Alex2 (black circle), the moderate-lytic
582 Alex4 (grey square) and the non-lytic Alex5 (white triangle). (B) Growth rates in the mixed
583 assemblages containingAlex2+Alex5 and Alex4+Alex5, with fraction of Alex2 (black circle)
584 and fraction of Alex4 (grey square), and the fraction of Alex5 in the Alex2+Alex5 mix (white
585 triangle) and in the Alex4 + Alex5 mix (dark grey triangle). Error bars indicate the standard
586 error of the mean (n = 3).

587

588 **Figure 2.** Growth and grazing of *Polykrikos kofoidii* in monoclonal and mixed assemblages
589 of *Alexandrium tamarense.*(A) Growth of *Polykrikos kofidii* grazing on *Alexandrium*
590 *tamarense* strains in monoclonal set-up with the lytic Alex2 (black circle), the moderate-lytic
591 Alex4 (grey square), and the non-lytic A5 (white triangle). (B) Growth of *Polykrikos kofoidii*
592 grazing on *Alexandrium tamarense* mixed assemblages containing Alex2+Alex5 (white
593 circle) and Alex4 + Alex5 (white square). (C) Percentage of *Polykrikos kofoidii* with a least
594 one visible food particle when fed *Alexandrium tamarense* strains in monoclonal set–ups of
595 the lytic Alex2 (black circle), moderate-lytic Alex4 (grey square), and the non-lytic Alex5
596 (white triangle). (D) Percentage of *Polykrikos kofoidii* with at least one visible food particle
597 when fed *Alexandrium tamarense* strains in mixed assemblages with either Alex2+Alex5
598 (white circle) or Alex4 + Alex5 (white square). Error bars indicate the standard error of the
599 mean (n = 3).

600

601 **Figure 3.** Conceptual diagram of intraspecific allelochemical mediated grazing protection in
602 *Alexandrium tamarense*. (A) Non-allelochemical active strains can be grazed by

603 heterotrophic protists. (B) Allelochemical active strains protect the non-active strains in a
604 high cell density patch (i.e. cloud). (C) Multi-strain populations may comprise multiple
605 cooperative traits including competition avoidance and protection from grazing by various
606 grazers.
607