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

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Dinoflagellates are a major cause of harmful algal blooms (HABs), with consequences for coastal marine ecosystem functioning and services. *Alexandrium fundyense* (previously *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. fundyense* and its ability to form blooms. Here we investigate the impact of grazing on monoclonal and mixed set-ups of highly (Alex2) and moderately (Alex4) allelochemically active *A. fundyense* strains and a non-allelochemically active conspecific (Alex5) by the heterotrophic dinoflagellate *Polykrikos kofoidii*. While Alex4 and particularly Alex5 were strongly grazed by *P. kofoidii* when offered alone, both strains grew well in the mixed assemblages (Alex4 + Alex5 and Alex2 + Alex5). Hence, the allelochemical active strains facilitated growth of the non-active strain by protecting the population as a whole against grazing. Based on our results, we argue that facilitation among clonal lineages within a species may partly explain the high genotypic and phenotypic diversity of *Alexandrium* populations. Populations of *Alexandrium* may comprise multiple cooperative traits that act in concert with intraspecific facilitation, and hence promote the success of this notorious HAB species.

## 1. Introduction

Marine phytoplankton account for approximately half of the global annual net primary production [1]. 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 homogeneous pelagic habitat. Yet, communities may also exhibit a patchy distribution even on small geographical scales, may show horizontal organization in thin layers and seem to be, at least partly, organized as metapopulations [2–5]. Molecular data show that populations of plankton species are temporally and spatially distributed based on historical, ecological and local oceanographic conditions [6–9]. Furthermore, distinct phytoplankton populations have high genetic diversity [9–13] and typically comprise a variety of genotypes and phenotypes [7,14,15].

Some phytoplankton species have the ability to produce toxins, and their proliferation leads to harmful algal blooms (HABs). These HABs can have major implications for marine ecosystems, causing mortality of fishes and other marine life, and threaten human health through accumulation of toxins in the food chain [16,17]. Among the notorious HAB formers, some dinoflagellates are found to produce a variety of potent bioactive substances of which paralytic shellfish poisoning toxins (PSTs) are most common [17]. Additionally, many dinoflagellates have the

ability to produce and release allelochemical compounds of poorly characterized chemical nature that exert negative effects on a range of unicellular eukaryotic organisms [18–20]. Intracellular PSTs and the extracellular allelochemicals have been shown to provide cells with protection against grazers [19,21–23] and competitors under certain conditions [24]. Since grazing can remove up to 50% of gross biomass production [25], production and release of bioactive compounds may add to the ecological success of dinoflagellates [20,26,27].

Understanding the evolution of phenotypic traits, such as the production of PSTs or allelochemicals, and their variation in genotypically diverse dinoflagellate populations [7,14] is a challenge, as only some individuals carry the costs, whereas the benefits are shared within the whole population. Thus, the production of toxins and allelochemicals provide an advantage to non-producers (i.e. cheaters), particularly in spatially unstructured populations [28]. Within a structured population of closely related individuals, however, cooperative traits can be favoured as a public good and facilitate the success of the entire population [29–32]. This principle of facilitation [33,34] has been shown in populations of various organisms, including bacteria [35], toxigenic cyanobacteria [36], amoeba [37] and yeast [38]. Little is yet known about intraspecific facilitation in populations of planktonic algae. Among flagellated algae many are known to produce extracellular toxins [20,27,39–41], and, depending on the environmental conditions, these toxins might serve as a direct benefit at the cell level or as an exploitable public good for the whole population [42].

Here, we assess whether facilitation may occur within phenotypically diverse populations of the common toxigenic bloom forming dinoflagellate *Alexandrium fundyense* (previously *Alexandrium tamarense* [43]) [7,14], and whether production of allelochemical substances by some individuals in an experimental population provides benefits to non-allelochemical producing individuals and thereby facilitate the success of multiple strains. To answer this question, we investigated whether *Alexandrium* strains with intermediate and high allelochemical activity can protect a non-allelochemical producing conspecific against the heterotrophic protist *Polykrikos kofoidii*. In this study, we applied for the first time, to our knowledge, allele-specific quantitative PCR (asqPCR) in a marine microalgae. The method proved to be highly reliable in mixtures of known cell ratios of clonal assemblies and thus allows assessing the relative cell numbers with high precision in time course experiments to obtain insights into intraspecific processes. We show that the allelochemically non-active strain is protected by the active strains. Our results thereby demonstrate intraspecific facilitation, which may partly explain the high genotypic and phenotypic diversity that is often observed in marine dinoflagellate populations. Hence, intraspecific facilitation might be another, yet often overseen mechanism that can explain the occurrence of exploitable public goods in genotypically diverse populations.

## 2. Material and methods

### (a) Algal cultures

Three clonal strains of *A. fundyense* (group I) were isolated in May 2004 from the North Sea coast of Scotland [21] and grown in K-medium [44] prepared with sterile filtered north seawater (salinity 33), pH adjusted to 8.0 at 15°C, with an incident light intensity of 150 µmol photons $m^{-2} s^{-1}$, provided by cool white fluorescent lamps at a 14 L : 10 D photocycle. All three strains, Alex2, Alex4 and Alex5 are morphologically indistinguishable, have a similar cell size and produce PSTs. Shortly after their isolation, Alex2 and Alex4 were shown to produce allelochemically active compounds. Alex2 was highly allelochemically active (lytic), Alex4 was moderately active (lytic), and Alex5 was non-active (non-lytic) [21]. The culture of *P. kofoidii* was established in 2009 also from coastal waters off Scotland [45]. The culture was routinely held in 63 ml culture flasks on a slow rotating plankton wheel (1 r.p.m.) at 15°C and low light (10–20 µmol photons $m^{-2} s^{-1}$) and fed with *Lingulodinium polyedrum* (CCMP 1738). A dense subculture used for experiment inoculation was starved for approximately 1 day so that no food algae were present (i.e. *Polykrikos* cells contained no visible food vacuoles, see the electronic supplementary material, figure S1), and *Polykrikos* had also not yet started massive gamete formation [44].

### (b) Experimental design and set-up

Before starting the experiment, each *Alexandrium* strain was treated by a multi-antibiotic cocktail (50 µg $ml^{-1}$ ampicillin, 3.3 µg $ml^{-1}$ gentamycin, 25 µg $ml^{-1}$ streptomycin, 1 µg $ml^{-1}$ chloramphenicol and 10 µg $ml^{-1}$ ciprofloxacin) for 5 days in order to reduce background bacterial numbers [46], without affecting the dinoflagellate nor its allelochemical properties. Small sub-samples were stained with acridine orange and checked by epifluorescence microscopy, confirming the removal of bacterial contamination [46]. After a 1 : 10 dilution in new antibiotic free media, all three strains grew in the exponential phase for 5 days. Thereafter, monoclonal cultures were grown for scaling up from 500 ml to a final 5000 ml in serial batch cultures and kept in exponential growth to ensure a similar physiological status of the strains in the experiment. Cells in exponential growth phase were washed three times with sterile filtered K-medium over a 10 µm pore size sieve in order to remove extracellular allelochemical compounds. Thus, any observed allelochemical mediated effect is assumed to be a result of the allelochemicals accumulated during the course of the experiment.

The experiment was performed in a temperature- and light-controlled culture room on a slowly rotating plankton wheel with a speed of 1 r.p.m. allowing homogeneous 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 distinct two-strain mixtures with Alex2 (i.e. Alex2 + Alex5) and with Alex4 (i.e. Alex4 + Alex5) to a final concentration of 1000 cells $ml^{-1}$ (i.e. 500 cells $ml^{-1}$ per strain). To test for a potential impact of the higher cell densities in the mixed cultures on the ability of *Polykrikos* to exert control over the *Alexandrium* population compared to the monoclonal culture experiments, additional experiments with monoclonal cultures of Alex5 with 1000 cells $ml^{-1}$ were performed (see the electronic supplementary material, figure S2). All experimental *Alexandrium* cultures were grown in triplicate with and without adding *Polykrikos* cells (20 cells $ml^{-1}$). The experiment started in completely filled 1000 ml Schott flasks, and 500 ml was harvested after 24 h. The remaining cultures were 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 cultures incubated in 125 ml flasks was harvested on day 4. The harvested samples were divided for cell counts of *Alexandrium* and *Polykrikos*, and for DNA extraction and subsequent asqPCR.

### (c) Counting procedure

Lugol's fixed (1% final concentration) *Alexandrium* cells were counted after sedimentation of 3 × 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 concentration), and Lugol's

iodine solution (0.3% final concentration) and settled in 10 ml settling chambers. Whole chambers were counted. For each sample, *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 (see the electronic supplementary material, figure S1).

### (d) Allele-specific quantitative PCR

The three haploid *Alexandrium* strains used in this study were genotyped at 18 microsatellite loci [47,48] (see also the electronic supplementary material, table S1) and were found to carry differently sized alleles at certain microsatellite loci. Hence, 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 asqPCR [49]. Genomic DNA extractions were performed with a DNeasy plant mini Kit (Qiagen, Hilden, Germany) with slight modifications of the manufacturer's instructions. Cells (approx. $50–75 \times 10^3$) from experimental cultures were harvested in a 50 ml reaction tube and centrifuged at 3000*g* for 5 min. Cells were resuspended after addition of 400 µl buffer AP1 and the suspension was transferred to a microcentrifuge tube, into which a mixture of approximately 300 µl of 1 mm and 0.3–0.6 mm-sized glass beads was added beforehand. Contents of the tube were then mixed by vortexing and incubated for 15 min at 95°C (ThermoMixer, Eppendorf, Hamburg, Germany). Remaining intact cells were disrupted in a TissueLyser (Qiagen) for $2 \times 1$ min at 20 Hz. Afterwards, 4 µl RNaseA stock solution (100 mg $ml^{-1}$, Qiagen) was added and the sample was incubated at 65°C for 15 min. The following steps for DNA purification have been performed according to the manufacturer's instructions (Qiagen).

PCR reactions were carried out with a Type-it Microsatellite PCR Kit (Qiagen, Hilden, Germany) with 25 µl reaction containing 1 µl (10 ng) template DNA, 12.5 µl 2 × 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 [48]. For fragment analysis purposes, the forward primer was labelled with the fluorescent dye 6-FAM. For better resolution in subsequent fragment analysis, 2.5 µl of Q-Solution (5×) were added to the reaction mix. After the initial denaturation (95°C, 5 min), 35 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 1 min and elongation at 70°C for 30 s were carried out, followed by a final extension at 60°C for 30 min in a Gradient Mastercycler (Eppendorf, Hamburg, Germany). Sizing of amplified microsatellite alleles was carried out with GeneMapper v. 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 from 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 estimation 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 mircoscopical cell counts from the respective sample. The asqPCR assays were validated with standard curves derived from DNA mixes; i.e. from mixed samples with relative contributions of one of the two strains to the mixed population from 0, 20, 40, 50, 60, 80 and 100%. 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 were 1.057 and $r^2 = 0.96$ and 1.001 and $r^2 = 0.995$ for mixed populations with Alex2 and Alex4, respectively (see the electronic supplementary material, figure S3). Cell numbers of strains from experimental samples are presented as mean values of triplicate cultures and their standard deviation.

### (e) Statistical analysis

The population growth in replicated culture set-ups was calculated from day 1 to day 4 in all cases but 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 through all replicate cell counts in the respective time periods according to

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

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 three-way ANOVA with strain and mix versus mono culture and/or grazing treatment as fixed factors, followed by post-hoc comparison of the means using Tukey's HSD [49] in Statistica v.6 (StatSoft, Hamburg, Germany). Differences of *Polykrikos* growth and grazing was either tested with a one-way ANOVA followed by post-hoc comparison of the means using Tukey's HSD, or using a *t*-test [49] and were carried out in SigmaPlot v. 12 (Systat Software, Erkrath, Germany). Normality was tested according to Shapiro–Wilk [50].

## 3. Results

### (a) Growth in monoclonal and mixed cultures without grazer

All *Alexandrium* cultures started with exponential growth after day 1. The attained growth rates of all three strains in the monoclonal cultures were not significantly different and were not significantly different within the mix culture set-ups (table 1 and figure 1*a*). Growth rates of Alex5 in cultures starting with 500 or with 1000 cells $ml^{-1}$ were not significantly different, with $0.41 \pm 0.06$ $d^{-1}$ and $0.37 \pm 0.02$ $d^{-1}$, respectively (*t*-test, $t_4 = 1.169$; $p = 0.307$; see also the electronic supplementary material, figure S2).

### (b) Grazing impact on *Alexandrium* in monoclonal and mixed cultures

In the monoclonal cultures, net population growth of both Alex4 and Alex5 became negative after addition of *Polykrikos*, whereas growth of Alex2 remained unaffected compared to the control (table 1 and figures 1*b* and 2*a*,*b*). All strains showed a higher growth rate in the mixed cultures Alex4 + Alex5 and Alex2 + Alex5 as compared to their respective monoclonal cultures under grazing pressure (table 1 and figure 1*b*). Growth rates of Alex5 and Alex2 in the mixed culture Alex2 + Alex5 together with *Polykrikos* appear to be slightly increased, although this was statistically not significant compared with the growth rates in the mixed culture without grazer. Within the other

**Table 1.** Summary of three-factorial ANOVA results on the effect of the treatments (grazing, *Alexandrium* strain identity and culture form) on clonal growth rates. (d.f., degrees of freedom; MQ, mean square; *F*, test statistic; *p*, level of significance.)

| | d.f. | MQ | *F* | *p* |
|---|---|---|---|---|
| grazing (yes/no) | 1 | 2.366040 | 271.1199 | <0.000001 |
| strain (Alex2, Alex4, Alex5) | 2 | 0.918121 | 105.2057 | <0.000001 |
| culture form (monoclonal culture/mixed culture) | 1 | 2.648118 | 303.4428 | <0.000001 |
| grazing × strain | 2 | 0.956716 | 109.6282 | <0.000001 |
| grazing × culture form | 1 | 3.086409 | 353.6656 | <0.000001 |
| strain × culture form | 2 | 0.718774 | 82.3630 | <0.000001 |
| grazing × strain × culture form | 2 | 0.728015 | 83.4219 | <0.000001 |
| | 30 | 0.008727 | | |

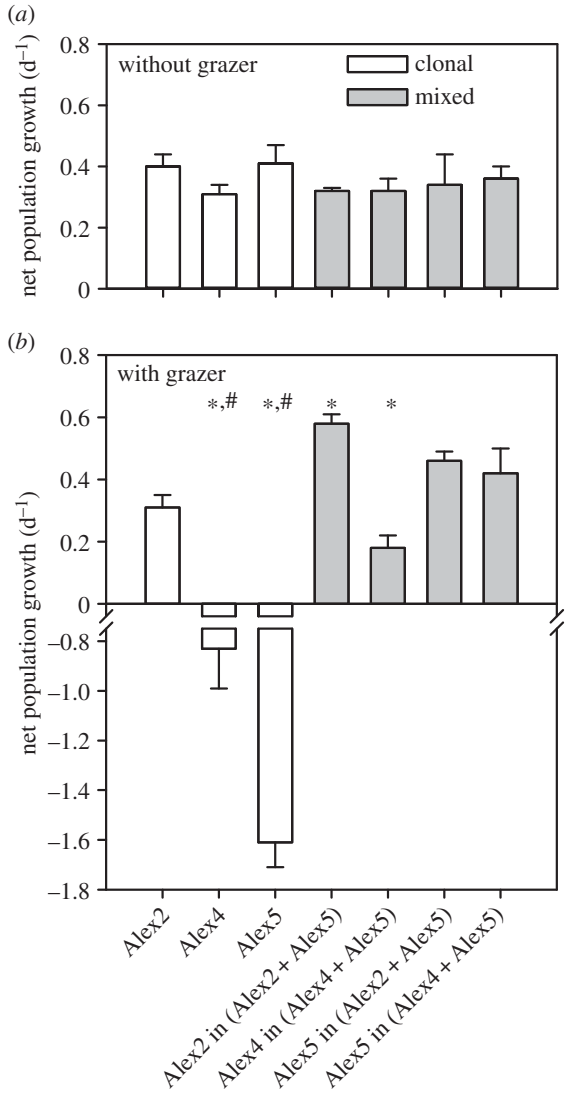


**Figure 1.** Overview of *Alexandrium fundyense* growth rates. (*a*) Monoclonal and mixed assemblages without *Polykrikos kofoidii*. (*b*) Monoclonal and mixed assemblages with *P. kofoidii* grazer. Values for mixed assemblages show growth rates of individual clones with the mixtures Alex2 + Alex5, and Alex4 + Alex5. Asterisks indicate significant differences between clonal and mixed assemblages, and hashtags indicate significant differences between set-ups without and with grazers (Tukey HSD post-hoc test, $p < 0.05$). Error bars indicate standard deviation ($n = 3$).

mixed culture with *Polykrikos* (i.e. Alex4 + Alex5), Alex5 showed no change in growth rate, while growth of Alex4 seemed to have reduced (table 1 and figure 1).

### (c) *Alexandrium* impact on the grazer

In all experimental set-ups where *Polykrikos* was added, initial grazing was documented and in more than 60% *Polykrikos* cells at least one visible food particle at day 1 was observed (figure 3*c*,*d*). After day 1, 60–95% of *Polykrikos* cells had grazed on *Alexandrium* in all cultures. In monoclonal cultures, however, the number of *Polykrikos* cells with visible food vacuoles on day 1 was significantly lower when fed on the lytic strains Alex2 and Alex4 when compared with the non-lytic strain Alex5 (figure 3*c*,*d*). After day 1, the number of *Polykrikos* cells with visible food vacuoles declined when fed on monoclonal cultures of Alex2 and Alex4, as well as on the mixture Alex2 + Alex5, reaching 0–30% by the end of the experiment (figure 3*c*,*d*). When grown on the mixture Alex4 + Alex5, a majority of *Polykrikos* cells possessed food vacuoles until the end of the experiment. When *Polykrikos* was fed on different Alex5 concentrations, its growth rate was higher when more food was provided (*t*-test, $t_4 = 6.707$, $p = 0.003$), with $\mu = 0.39 \pm 0.04$ and $\mu = 0.58 \pm 0.03$ for 500 and 1000 cell ml$^{-1}$, respectively. *Polykrikos* grazed down all cells of the Alex5 cultures and became fully starved at the end of the experiment (figure 3*c*,*d*), independent of the initial Alex5 cell densities (data not shown).

Initial growth of *Polykrikos* (i.e. from day 0 to day 1) was positive, except when fed on the mixed culture Alex2 + Alex5 (figure 3*a*,*b*). Subsequent growth rates of *Polykrikos* depended on the *Alexandrium* strain being fed with (one-way ANOVA, $F_{2,6} = 245$, $p < 0.001$), showing highest growth rates when fed on monoclonal cultures of Alex5 ($\mu = 0.39 \pm 0.04$), intermediate growth rates when fed on Alex4 ($\mu = 0.27 \pm 0.07$), and negative growth rates when fed on Alex2 ($\mu = -0.57 \pm 0.06$). When *Alexandrium* clones were provided in a mixture, growth of *Polykrikos*, when compared to the monoclonal culture of Alex5 (1000 cells ml$^{-1}$; $\mu = 0.58 \pm 0.03$), was reduced on Alex4 + Alex5 as a food source ($\mu = 0.26 \pm 0.06$, *t*-test, $t_4 = 8.904$, $p < 0.001$) and even became negative when offered Alex2 + Alex5 ($\mu = -1.27 \pm 0.09$, *t*-test, $t_4 = 8.205$, $p = 0.001$).

## 4. Discussion

In our experiments, all three *Alexandrium* strains exhibited comparable growth rates when grown in monoclonal cultures without a grazer. The strains differ significantly in their lytic activity under the chosen culture conditions, which according to our current understanding, is best explained by distinct

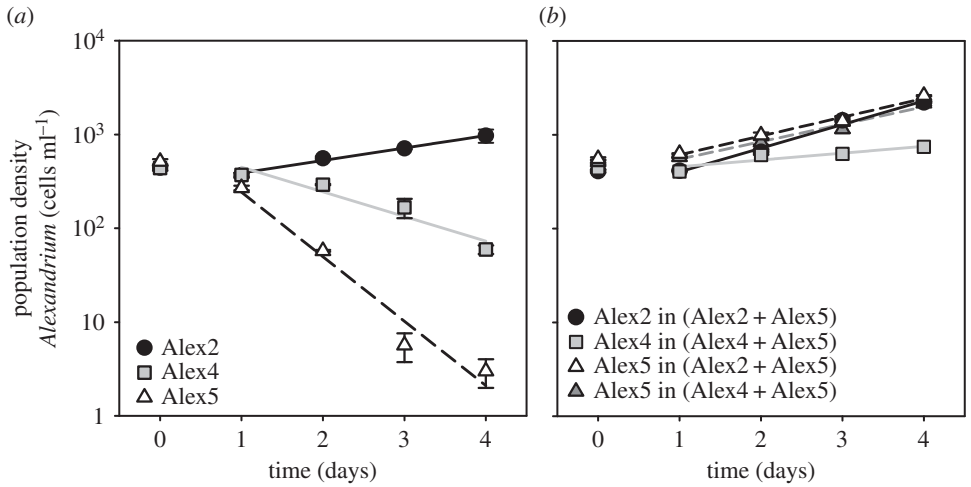


**Figure 2.** Growth of *Alexandrium fundyense* strains when exposed to *Polykrikos kofoidii*. (*a*) Growth rates in the monoclonal set-up with the lytic Alex2, the moderate-lytic Alex4 and the non-lytic Alex5. (*b*) Growth rates of individual strains in the mixed assemblages. Error bars indicate standard deviation ($n = 3$).

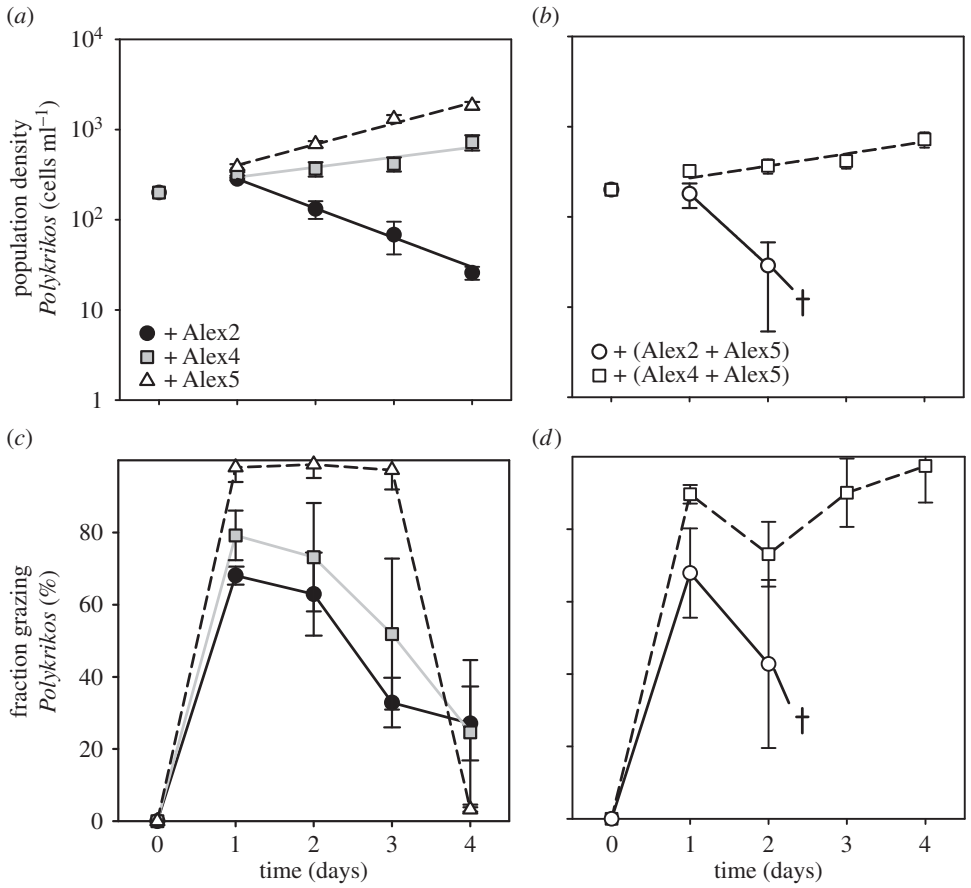


**Figure 3.** Growth and grazing of *Polykrikos kofoidii* in monoclonal and mixed assemblages of *Alexandrium fundyense*. (*a*) Growth of *P. kofoidii* grazing on monoclonal set-ups with lytic Alex2, the moderate-lytic Alex4 and the non-lytic Alex5. (*b*) Growth of *P. kofoidii* grazing on mixed assemblages. (*c*) Percentage of *P. kofoidii* cells with food particles when fed on monoclonal cultures, or (*d*) mixed assemblages. Error bars indicate standard deviation ($n = 3$).

levels of production and release of allelochemical active substances [21]. This shows that for the three strains used herein, potential cost of allelochemical production does not lead to a reduced growth rate. Furthermore, growth rates of all strains remained unaltered in mixed cultures, proving that allelochemicals produced by the lytic strains did not have intraspecific inhibitory (or supportive) effects as they do not affect growth of the non-lytic strain. While in monoclonal cultures the net population growth of the most lytic strain, Alex2, was not affected by *Polykrikos*, growth of both Alex4 and Alex5 decreased as a result of grazing by *Polykrikos* (figures 1*b* and 2*a*). Interestingly, net population growth of all three strains was positive when grown with the other *Alexandrium* strain in mixed cultures (i.e. Alex2 + Alex5 and Alex4 + Alex5) with *Polykrikos* (figures 1 and 2*b*). The slightly higher growth rate of the lytic strain Alex2 in the presence of *Polykrikos* and Alex5 indicates a benefit not only for the non-lytic Alex5 but perhaps also for the lytic strain Alex2. Whereas the beneficial effect for Alex5 can be explained by reduction of grazing owing to effects of allelochemicals produced by Alex2, a beneficial effect for Alex2 is more difficult to explain and needs to be confirmed in additional

experiments. Increased growth solely related to mixotrophy is unlikely, as Alex2 does not benefit in terms of growth from lysed grazers in the monoclonal grazing set-up, indicating that other mutual processes might be involved in the mixed cultures. An additional hint for synergistic effects in the mixed set-ups is that *Polykrikos* was slightly more affected in the mixed culture Alex2 + Alex5, indicated by the extinction of all *Polykrikos* cells before the third day of the experiment (cf. figure 3*c*,*d*).

The increase in growth rate of Alex4 in the mixed culture Alex4 + Alex5 with grazers when compared with that in the monoculture with grazers seems to be an indirect positive effect of the presence of Alex5. The grazing pressure of *Polykrikos* on the lytic Alex4 in the mixed culture could be lower simply because Alex5 was available as alternative food source. As a consequence, population densities of Alex4 could remain relatively high allowing a sufficient production of allelochemicals for protection against *Polykrikos*, although *Polykrikos* did not show a reduced growth in the Alex4 + Alex5 mixed culture compared to the monoclonal culture of Alex4 (figure 2). Yet, growth of *Polykrikos* in mixed culture with Alex4 is reduced compared with the growth when Alex5 is provided as food alone, when its growth was highest, demonstrating the effect of the allelochemical substances as a grazer deterrent (figure 3*a*).

*Polykrikos* grew well on monoclonal cultures of Alex5. The mixture Alex2 + Alex5 reduced its growth when compared with growth on Alex5 alone. Although the mixed assemblage started at a higher cell density (with 1000 versus 500 cells $ml^{-1}$ in the monoclonal cultures), it is unlikely that this initial density difference negatively affected *Polykrikos* growth, as *Polykrikos* grew better on a higher Alex5 population density (see the electronic supplementary material, figure S2). Hence, a detrimental effect of allelochemicals produced by Alex2 and Alex4 on *Polykrikos* is the most likely cause for its reduced growth in the presence of allelochemically active *Alexandrium*.

The view that allelochemicals play an important ecological role is widely shared (see reviews by [20,26,40,41,51]). It may be argued that other competing species might also benefit from reducing grazer fitness [52]. However, it has been shown that the same allelochemicals strongly affect growth of many phytoplankton species that are potential resource competitors [24,53–55]. Therefore, any indirect beneficial effects for competitors (e.g. through release from grazing pressure) might be reduced or excluded as long as their growth is also affected by the allelochemicals. Our results clearly demonstrate that allelochemicals can protect both the producers as well as a non-producing conspecific against grazing. The observed facilitation by grazing protection might resemble associational resistance [56,57], described for terrestrial plant communities [58–60], as well as among macroalgal species and their epiphytes [61–63]. Such associational resistance may also occur in pelagic microalgae populations even at the intraspecific level, within assemblages of sufficiently high population densities. Our results indicate that facilitation plays a role in phytoplankton populations and we show that benefits are shared between producer and non-producer strains.

It is as challenging to understand what drives the evolution of a trait such as production and release of allelochemical substances that benefit the producer as well as the population as a whole, as to understand how these traits are maintained in the population [64]. What is the selective advantage of allelochemical substances for the producer, i.e. how can this trait evolve and how can it be maintained in phenotypically diverse populations under natural selection? According to theory, selection for a public good might take place when costs versus benefits for the producer are relatively low and the relatedness between producer and non-producer is relatively high [31,38]. Positive population effects of allelochemicals might result at the cellular level (private good) and higher structural levels (populations) could benefit indirectly [65]. Before allelochemicals start serving as a public good, a high relative abundance of producers is required in order to support the entire population. Consequently, the allelopathic phenotype as a trait might be maintained by frequency-dependent selection. Indeed, in a natural population of *A. fundyense*, only two out of 88 clonal isolates were non-producers, whereas all others were allelochemically active, though the allelochemical potency was normally distributed and varied widely [14].

In general, intraspecific genetic and phenotypic diversity is discussed to have an important impact on evolutionary and ecological process and hence the population's dynamics and success [66–68]. A high variability in phenotypic traits involved in interactions among individuals of different clonal linages within a population may allow mutualistic intraspecific facilitation in various ways, and thereby promote the overall success of *Alexandrium*. If cooperative traits governing intraspecific interactions are common in mixed *Alexandrium* populations, the high phenotypic and genotypic diversity of these populations may be explained for example, by compatibility among these beneficial phenotypic traits in different strains. Such alternative traits may include for instance, chain formation, swimming speed, nutrient uptake capabilities, intrinsic growth rate and PST content [22,23,69–71]. Indeed, the non-lytic strain Alex5 in our experiment contained the highest amount of PSTs (data not shown), a trait that potentially allows protection against grazing by copepods [22,23,69]. It is conceivable that with a higher genotypic diversity, more cooperative traits can be provided that benefit the entire population.

The observed high genotypic diversity of phytoplankton populations [9–13] may be sustained by mutualistic interactions of cooperative traits. Yet, with respect to the functioning of extracellular allelochemical substances, the seemingly homogeneous or ephemeral spatial distribution pattern of marine phytoplankton populations and their typical low population densities may contradict with (or limit) the effectiveness of beneficial interactions derived from laboratory experiments with high cell concentration [64]. The functioning of allelochemical mediated facilitation in natural populations will thus depend on the degree of spatial dispersal, i.e. the local accumulation of a population, as well as on the rate at which extracellular allelochemicals are produced and excreted, and on the rate of their diffusion and degradation [51,72]. In dinoflagellates, bloom formation typically occurs at low mixing and water column stratification [26]. Under such conditions, plankton populations are often not homogeneously distributed, but rather show a spatially structured distribution, for instance as patches or thin layers [2–4,72]. Flagellar movement may favour accumulation in patches [72], which is presumably also required for sexual reproduction in the life cycle of *Alexandrium* [17], as increased encounter rates in patches may allow *Alexandrium* gametes to find their corresponding mating type [73]. The fine-scale analysis of the spatial distribution of dinoflagellates in the water column indicates that they tend to accumulate. Cooperative traits such as allelochemicals might facilitate the population success within patches of high cell densities.

The conditions in our culture experiment obviously represent a simplification of the natural environment, where *Alexandrium* populations are composed of a much higher diversity in genotypes and phenotypic traits. We partially accounted for this natural diversity by selecting three strains with distinct allelochemical activities, i.e. one highly active (Alex2), one intermediately active (Alex4) and one non-active (Alex5). Such a range of allelochemical activities is also found in the natural environment, and it is therefore very likely that the observed facilitation by allelochemicals occurs during natural *Alexandrium* blooms. In our experiment, we worked with cell densities reflecting dense *Alexandrium* bloom conditions rather than pre-bloom conditions [74,75]. Obviously, production of allelochemicals becomes favourable when sufficient cells are present, and these compounds may thus play a crucial role in the prolongation of HABs [64]. Especially, if phenotypic traits in allelopathic microalgae serve multiple purposes over the course of a bloom, for example by functioning as private versus public good during low and high population densities, respectively [42]. It is conceivable that the transient nature of a selective advantage by allelochemical production leads to an increase in the phenotypic diversity, and its underlying genetic diversity, of natural populations, because the selective advantage of various phenotypes at different stages of population development will be balanced over time.

In this study, by adopting the approach of asqPCR for *A. fundyense*, we were able to follow the strain-specific responses to grazing in mixed culture set-ups, and we showed that allelochemical active *A. fundyense* strains can protect a non-lytic conspecific from grazing by *P. kofoidii*. Our findings are in line with the view that a multitude of hitherto not well-recognized cooperative traits, including allelochemical mediated intraspecific facilitation, may contribute to the high genotypic and phenotypic diversity of *Alexandrium* populations. Multiple traits potentially lead to mutual facilitation among phenotypically diverse clonal lineages within an *Alexandrium* population, and thereby further promote the success of these notorious HAB species.

Data accessibility. Additional experimental data are available at http://dx.doi.org/10.1594/PANGAEA.836236

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