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

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

2

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23 **Abstract**

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

41

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

45

46 **Introduction**

47

48 Marine phytoplankton account for approximately half of the global annual net primary  
49 production (Falkowski et al. 2004). Their high biomass turnover rate and conversion of light  
50 energy, CO<sub>2</sub> and inorganic nutrients into organic material drive the marine pelagic ecosystem.  
51 Like many aquatic microorganisms, phytoplankton can have large population sizes, and typically  
52 have high rates of predominantly asexual reproduction. Furthermore, phytoplankton live in a  
53 rather open and seemingly homogenous pelagic habitat. Yet, communities may also exhibit a  
54 patchy distribution even on small geographic scales, may show horizontal organization in thin  
55 layers and seem to be, at least partly, organized as metapopulations (Montagnes et al. 1999,  
56 Ryneerson et al. 2009, Durham and Stockert 2012, Menden-Deuer 2012). Molecular data show  
57 that plankton populations are genetically diverse, and that they are temporally and spatially  
58 distributed based on historical, ecological and local oceanographic conditions (Foissner 2006,  
59 Alpermann et al. 2009, Nagai et al. 2007, Tahvanainen et al. 2012). Distinct phytoplankton  
60 populations typically comprise a variety of genotypes and phenotypes (Alpermann et al. 2009,  
61 Bachvaroff et al. 2009, Alpermann et al. 2010). Such genotypic variability and phenotypic  
62 diversity suggest high adaptive capabilities of populations, particularly advantageous in highly  
63 dynamic environments like coastal waters (Sultan and Spencer 2002). High adaptive capabilities,  
64 in turn, may support species and populations to thrive and form dense algal blooms under  
65 favourable environmental conditions.

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

69 health through accumulation of toxins in the food chain (Hallegraeff 2003, Anderson et al.  
70 2012). Dinoflagellates are notorious HAB formers and can produce a broad variety of potent  
71 bioactive substances from which paralytic shellfish poisoning toxins (PSTs) are most common  
72 (Anderson et al. 2012). Additionally, many dinoflagellates have the ability to produce  
73 allelochemical compounds of poorly characterized chemical nature (Tillmann and John 2002,  
74 Cembella 2003, Ma et al. 2011). PSTs and allelochemicals have been shown to provide cells  
75 with protection against grazers (Tillmann and John 2002, Selander et al. 2006, Tillmann et al.  
76 2009, Wohlrab et al. 2010) and competitors (Tillmann and Hansen 2009). Since grazing can  
77 remove up to 50% of gross biomass production (Landry and Calbet 2004), production and  
78 release of bioactive compounds may add to the success of dinoflagellates (Smayda  
79 2002, Cembella 2003, Granéli and Hansen 2006). During HABs, phytoplankton population  
80 densities are high and allelochemicals likely provide protection against grazers, as well as  
81 competitors. At the onset of HABs, overall population densities are typically considered to be too  
82 low to effectively use extracellular allelochemicals to suppress competitors (Jonsson et al. 2009).  
83 It has been observed, however, that some phytoplankton species form dense clouds and thin  
84 layers where allelochemicals may become effective. These patches may be formed by  
85 behavioural processes (swimming, growth, and grazing) as well as by physical aggregation (e.g.  
86 Durham and Stockert 2012, Menden-Deuer 2012 and references therein). It is in these clouds  
87 with locally high population densities where concentrations of bioactive compounds can become  
88 concentrated enough for population protection, even when densities across the water column are  
89 relatively low.

90         Understanding the evolution of variation in phenotypic traits such as production of  
91 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

113

## 114 **Materials and methods**

### 115 *Algal cultures*

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

### 131 *Experimental design and set-up*

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

136 tested for their allelochemical properties with the *Rhodomonas salina* bioassay (see below) and  
137 then washed three times with sterile filtered K-medium over 10 µm pore size sieve in order to  
138 remove extracellular allelochemical compounds. This was also confirmed with the *Rhodomonas*  
139 *salina* bioassay. Thus, any observed allelochemical mediated effect is a result of the accumulated  
140 allelochemicals during the course of the experiments. The experiment was performed in a  
141 temperature and light controlled culture room on a slowly rotating plankton wheel with a speed  
142 of 1 rpm allowing homogenous mixing, but with minimal turbulence. The three strains of  
143 *Alexandrium* were grown in monoclonal cultures with starting cell densities of 500 cells mL<sup>-1</sup>.  
144 Additionally, Alex5 was grown in two-strain mixtures with Alex2 and with Alex4 to a final  
145 concentration of 1,000 cells mL<sup>-1</sup> (i.e. 500 cells mL<sup>-1</sup> per strain). In order to control for the higher  
146 cell densities in the mixed cultures, additional experiments with monoclonal cultures of Alex5  
147 were performed with initial cell densities of 1,000 cells mL<sup>-1</sup>. All three strains and their mixtures  
148 were grown in triplicate with and without adding *Polykrikos* cells (20 cells mL<sup>-1</sup>). The  
149 experiment started in completely filled 1,000 mL Schott flasks, and 500 mL was harvested after  
150 24 h. The rest was further incubated in 500 mL flasks and 250 mL was harvested at day 2. Again,  
151 the remaining culture was incubated in 250 mL flasks and 125 mL was harvested at day 3. The  
152 remaining culture incubated in 125 mL flasks was harvested on day 4. The harvested samples  
153 were divided for cell counts of *Alexandrium* and *Polykrikos*, for allelochemical activity  
154 measurements using the *Rhodomonas salina* bioassay, and for DNA extraction and subsequent  
155 allele-specific quantitative PCR (asqPCR).

156

157

158



159 *Counting procedure*

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

167

168 *Rhodomonas salina* bioassay

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

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

183

#### 184 *Allele-specific quantitative PCR*

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

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

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

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

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

239

#### 240 *Statistical analysis*

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

$$245 \quad A=A_1e^{\mu t}$$

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

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

250 1995). Differences of *Polykrikos* growth was either tested with an ANOVA as stated above or  
251 using a t-test (Sokal and Rohlf 1995). Normality was tested according to Shapiro-Wilk (Sokal  
252 and Rohlf 1995). Data were log-transformed if this improved the homogeneity of variances, as  
253 evaluated by Levene's test. All statistical analyses were carried out in Sigmaplot version 12  
254 (Systat Software, Erkrath, Germany).

255

## 256 **Results**

257

### 258 *Allelochemical activity*

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

265

### 266 *Growth in monoclonal and mixed cultures without grazer*

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

272 with 500 or with 1,000 cells mL<sup>-1</sup> was comparable with  $0.41 \pm 0.06 \text{ d}^{-1}$  and  $0.37 \pm 0.02 \text{ d}^{-1}$ ,  
273 respectively.

274

#### 275 *Grazing impact on Alexandrium*

276 Net population growth of both Alex4 and Alex5 became negative after addition of *Polykrikos*,  
277 whereas growth of Alex2 remained unaffected compared to the control (ANOVA,  $p < 0.05$ ; Fig.  
278 1A; Table 2). When *Alexandrium* strains were grown in the presence of *Polykrikos*, both Alex2  
279 and Alex5 showed increased growth rates as compared to the mixed assemblage of both strains  
280 without grazer, whereas Alex4 mixed with Alex5 showed a lower or similar growth rate as  
281 compared to their respective monoclonal cultures (ANOVA  $p < 0.05$ ; Fig. 1B; Table 2)

282

#### 283 *Alexandrium impact on the grazer*

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

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

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

306

## 307 **Discussion**

308 All three *Alexandrium* strains exhibited comparable growth rates when grown in monoclonal  
309 cultures (Table 2). Furthermore, in mixed cultures growth rates remained unaltered, indicating  
310 that the allelochemicals from the lytic strains did not affect growth of the non-lytic strain. While  
311 net population growth of the most lytic Alex2 was not affected by *Polykrikos*, growth of both  
312 Alex4 and Alex5 decreased as result of grazing by *Polykrikos*. Interestingly, when grown in  
313 mixtures and with *Polykrikos* added, net population growth in all three strains was positive (Fig.  
314 1). Furthermore, the lytic strain Alex2 grew significantly better (ANOVA  $p < 0.05$ ) in the mixed  
315 assemblage as compared to its monoclonal culture when in presence of *Polykrikos*, indicating a  
316 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<sup>-1</sup>,  
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



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

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

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

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

384         We have experimentally shown that intraspecific facilitation within *Alexandrium*  
385 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 homogeneously 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 Deur 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

419

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563

564

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

568

Allelochemical potency	
Set-up	% lysed <i>Rhodomonas</i>
Experiment start (day 0): Alex2	$0.2 \pm 4.2^A$
Experiment start (day 0): Alex4	$3.4 \pm 6.5^A$
Experiment start (day 0): Alex5	$0.2 \pm 1.4^A$
Experiment end (day 4): Alex2	$58.7 \pm 10.3^B$
Experiment end (day 4): Alex4	$2.2 \pm 2^A$
Experiment end (day 4): Mix Alex2+Alex5	$72.9 \pm 7^B$
Experiment end (day 4): Mix Alex4+Alex5	$16.7 \pm 1.5^C$

569

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572

573 **Table 2.** Overview of the population growth of different experimental set-ups in this study. Superscript letters indicate significant differences574 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  $\pm$ SD ( $n = 3$ ).

577

Set-up	Growth ( $\mu$ )				<i>Polykrikos</i>
	Alex2	Alex4	Alex5	Total in mixed set-up	
Monoclonal	$0.40 \pm 0.04^{A,a}$	$0.31 \pm 0.03^{A,a}$	$0.41 \pm 0.06^{A,a}$	-	-
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	$0.31 \pm 0.04^{A,a}$	$-0.91 \pm 0.31^{B,b}$	$-1.61 \pm 0.10^{B,c}$		A2: $-0.57 \pm 0.06^A$ A4: $0.27 \pm 0.07^{BC}$ A5: $0.39 \pm 0.04^B$
Mix Alex2+Alex5 + Grazer	$0.57 \pm 0.03^{B,a}$		$0.46 \pm 0.03^{A,b}$	$0.51 \pm 0.03^{B,ab}$	$-1.27 \pm 0.90^A$
Mix Alex4+Alex5 + Grazer		$0.18 \pm 0.04^{C,a}$	$0.42 \pm 0.08^{A,b}$	$0.35 \pm 0.09^{A,ab}$	$0.26 \pm 0.06^C$

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 containing Alex2+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 kofoidii* 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



Figure 1

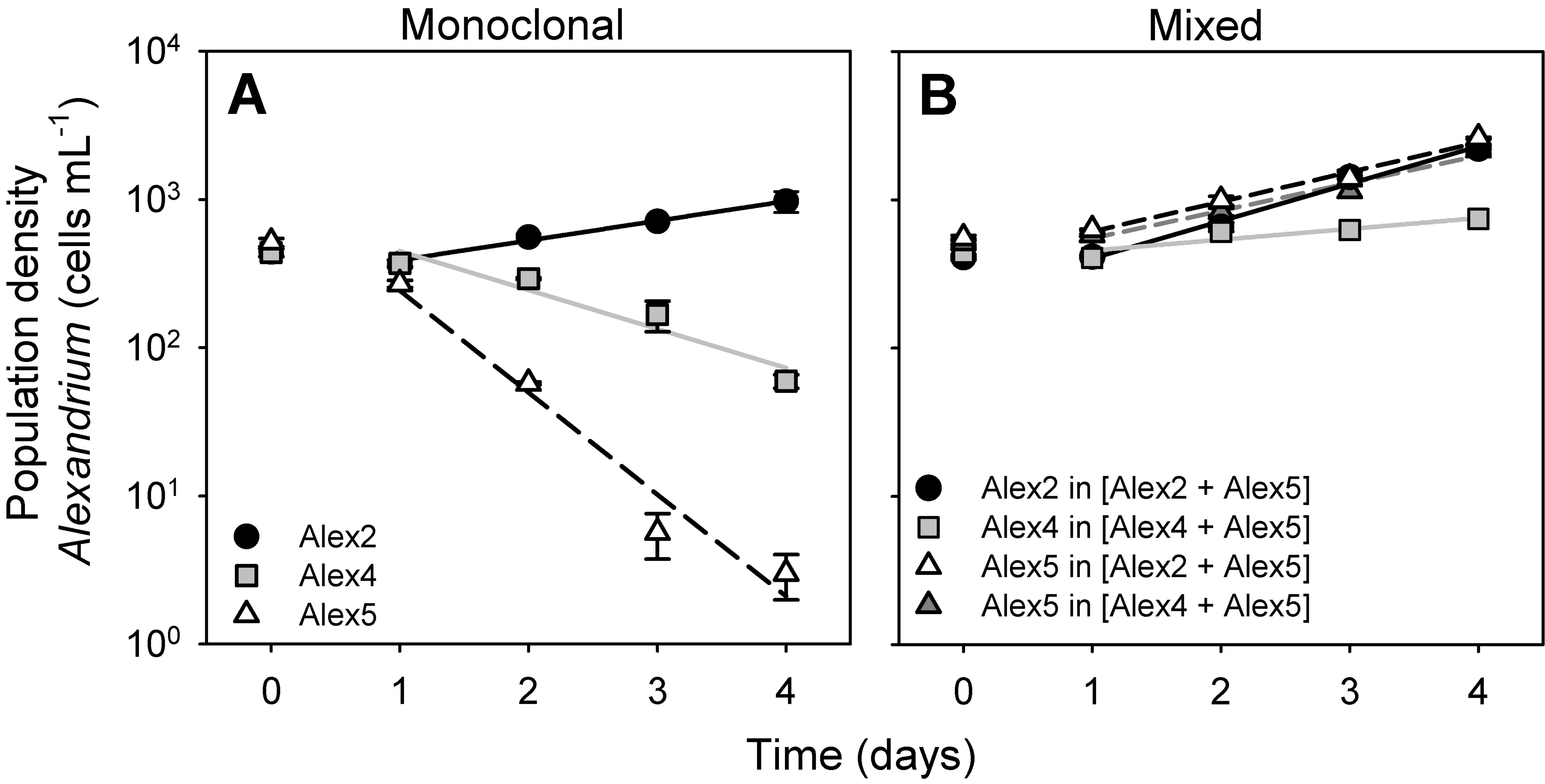


Figure 2

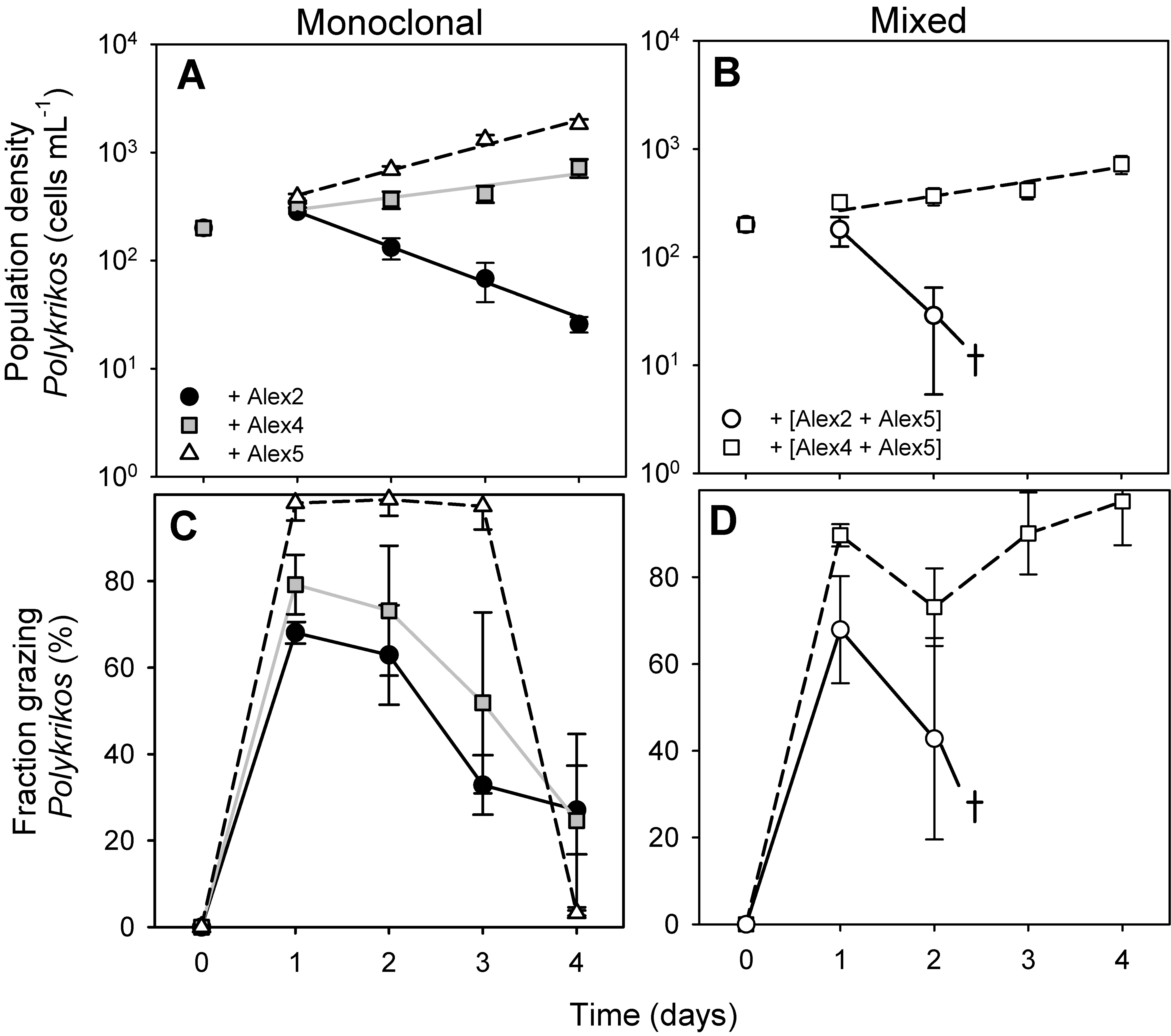


Figure 3

