

1 Impact of elevated pCO<sub>2</sub> on paralytic shellfish poisoning toxin content and  
2 composition in *Alexandrium tamarense*

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21 **Abstract**

22 Ocean acidification is considered a major threat to marine ecosystems and may particularly  
23 affect primary producers. Here we investigated the impact of elevated pCO<sub>2</sub> on paralytic  
24 shellfish poisoning toxin (PST) content and composition in two strains of *Alexandrium*  
25 *tamarense*, Alex5 and Alex2. Experiments were carried out as dilute batch to keep carbonate  
26 chemistry unaltered over time. We observed only minor changes with respect to growth and  
27 elemental composition in response to elevated pCO<sub>2</sub>. For both strains, the cellular PST  
28 content, and in particular the associated cellular toxicity, was lower in the high CO<sub>2</sub>  
29 treatments. In addition, Alex5 showed a shift in its PST composition from a non-sulfated  
30 analogue towards less toxic sulfated analogues with increasing pCO<sub>2</sub>. Transcriptomic  
31 analyses suggest that the ability of *A. tamarense* to maintain cellular homeostasis is  
32 predominantly regulated on the post-translational level rather than on the transcriptomic level.  
33 Furthermore, genes associated to secondary metabolite and amino acid metabolism in Alex5  
34 were down-regulated in the high CO<sub>2</sub> treatment, which may explain the lower PST content.  
35 Elevated pCO<sub>2</sub> also induced up-regulation of a putative sulfotransferase *sxtN* homologue and  
36 a substantial down-regulation of several sulfatases. Such changes in sulfur metabolism may  
37 explain the shift in PST composition towards more sulfated analogues. All in all, our results  
38 indicate that elevated pCO<sub>2</sub> will have minor consequences for growth and elemental  
39 composition, but may potentially reduce the cellular toxicity of *A. tamarense*.

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42 Key words: Ocean acidification, dinoflagellates, Harmful Algal Blooms, PSP toxins,  
43 saxitoxin, gene regulation, sulfur metabolism

44

## 45 **1. Introduction**

46 Since the industrial revolution, atmospheric CO<sub>2</sub> levels are rising at an unprecedented rate  
47 (Solomon et al., 2007). This increase in atmospheric pCO<sub>2</sub> affects the carbonate chemistry of  
48 ocean waters, which shifts towards higher concentrations of CO<sub>2</sub> and bicarbonate (HCO<sub>3</sub><sup>-</sup>),  
49 lower concentrations of carbonate (CO<sub>3</sub><sup>2-</sup>), and a reduction in pH, i.e. ocean acidification  
50 (Caldeira and Wickett, 2003; Wolf-Gladrow et al., 1999). Such changes in ocean carbonate  
51 chemistry will have implications for phytoplankton that convert inorganic carbon into organic  
52 biomass. For instance, increasing concentrations of CO<sub>2</sub> and HCO<sub>3</sub><sup>-</sup> were shown to promote  
53 phytoplankton growth and photosynthesis (Hein and SandJensen, 1997; Tortell et al., 2008),  
54 whereas decreasing pH and concentrations of CO<sub>3</sub><sup>2-</sup> were hold responsible for the adverse  
55 effects observed in calcification (Beaufort et al., 2011; Riebesell et al., 2000).

56 Our current knowledge about the sensitivity of phytoplankton towards ocean  
57 acidification is almost entirely based on the work with diatoms, coccolithophores, and  
58 cyanobacteria (see Riebesell and Tortell, 2011). Relatively little is yet known about the  
59 responses of autotrophic dinoflagellates (Brading et al., 2011; Burkhardt et al., 1999; Van de  
60 Waal et al., 2013), in particular of toxin producing species (Fu et al., 2010; Hallegraeff, 2010;  
61 Kremp et al., 2012). This is surprising because, among all eukaryotic phytoplankton species,  
62 dinoflagellates feature the primary carboxylating enzyme ribulose 1,5-bisphosphate  
63 carboxylase/oxygenase (RubisCO) with lowest affinities for its substrate CO<sub>2</sub> (Badger et al.,  
64 1998). Thus, the predicted increase of CO<sub>2</sub> concentrations in the future ocean may particularly  
65 favour this group.

66 Some toxic dinoflagellate species can proliferate under favourable environmental  
67 conditions, thereby producing Harmful Algal Blooms (HABs) that may cause mass  
68 mortalities of fish, illness and death of marine mammals, seabirds, and humans (Anderson et  
69 al., 2012b; Granéli and Turner, 2006). Of all the dinoflagellate HAB species, the genus  
70 *Alexandrium* is among the most prominent with respect to the diversity and distribution of its

71 globally widespread blooms (Anderson et al., 2012a). Moreover, *Alexandrium* blooms are  
72 often responsible for the outbreak of paralytic shellfish poisoning (PSP), which is caused by  
73 the neurotoxin saxitoxin (STX) and its analogues, from which neosaxitoxin (NEO),  
74 gonyautoxins (GTX1, 2, 3 and 4), C1 and C2 are often most abundant. Although non-sulfated  
75 STX and NEO are highly toxic (LD<sub>50</sub> i.p. mice ~8 µg kg<sup>-1</sup>), the addition of one sulfate group  
76 in GTXs reduces this toxicity by ~40%, and subsequent incorporation of a sulfonyl group in  
77 C1+C2 reduces its toxicity by up to 99% (Wiese et al., 2010). Thus, the cellular toxicity of  
78 these PST producing HAB species is not only determined by their PST content, but also by  
79 the relative composition of the different PST analogues.

80         Toxin production by HAB species is strongly affected by changes in resource  
81 availabilities, such as light and nutrients (Cembella, 1998; Granéli and Flynn, 2006; Neilan et  
82 al., 2013; Sivonen and Jones, 1999; Van de Waal et al., In press). Current changes in ocean  
83 carbonate chemistry may have consequences as well. For instance, elevated pCO<sub>2</sub> was found  
84 to cause an increase or a decrease in the production of domoic acid by the diatom *Pseudo-*  
85 *nitzschia multiseriis* (Sun et al., 2011; Trimborn et al., 2008). Elevated pCO<sub>2</sub> was also shown  
86 to affect the composition of karlotoxins in the dinoflagellates *Karlodinium veneficum*, which  
87 shifted towards a more toxic analogue (Fu et al., 2010). Furthermore, PST content and  
88 composition differentially changed in various *Alexandrium ostenfeldii* strains (Kremp et al.,  
89 2012). The observed responses in these studies have been attributed to CO<sub>2</sub>-induced changes  
90 in growth and energy allocation, even though the mechanisms underlying the production and  
91 composition of these toxins remain unclear.

92         Here we investigated the impact of elevated pCO<sub>2</sub> on PST content and composition in  
93 two strains of *Alexandrium tamarense* (Alex5 and Alex2), which were isolated from the same  
94 population at the Scottish east coast of the North Sea (Alpermann et al., 2009; Tillmann et al.,  
95 2009). Both *A. tamarense* strains differ in their growth rate, PST content as well as in their  
96 PST composition, comprising distinct contributions of STX, NEO, C1+C2, GTXs and

97 decarbamoylated dcSTX (Tillmann et al. 2009). Earlier experiments with these strains  
98 illustrated that their PST production as well as the regulation of their genome can be affected  
99 by abiotic and biotic factors such as nutrient availability and grazing (Van de Waal et al., In  
100 press; Wohlrab et al., 2010). Little is yet known about the regulation of genes involved in PST  
101 synthesis by dinoflagellates (Hackett et al., 2013), and no study thus far investigated the  
102 impact of elevated pCO<sub>2</sub> on toxin production and gene regulation in *A. tamarense*.

## 103 **2. Materials and Methods**

### 104 **2.1. Culture conditions**

105 Cultures of *Alexandrium tamarense* Alex5 and Alex2 (Alpermann et al., 2009; Tillmann et  
106 al., 2009) were grown as dilute batch in 2.4 L air-tight borosilicate bottles. Filtered natural  
107 seawater (0.2  $\mu\text{m}$  pore size; Satorius, Goettingen, Germany) was enriched with metals and  
108 vitamins according to the recipe of f/2-medium (Guillard and Ryther, 1962), except for  $\text{FeCl}_3$   
109 ( $1.9 \mu\text{mol L}^{-1}$ ),  $\text{H}_2\text{SeO}_3$  ( $10 \text{ nmol L}^{-1}$ ), and  $\text{NiCl}_2$  ( $6.3 \text{ nmol L}^{-1}$ ). The added concentrations of  
110  $\text{NO}_3^-$  and  $\text{PO}_4^{3-}$  were  $100 \mu\text{mol L}^{-1}$  and  $6.25 \mu\text{mol L}^{-1}$ , respectively. Cultures were grown at a  
111 light:dark cycle of 16:8 h and an incident light intensity of  $250 \pm 25 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$   
112 provided by daylight lamps (Lumilux HO 54W/965, Osram, München, Germany). Bottles  
113 were kept at  $15^\circ\text{C}$  and placed on a roller table to avoid sedimentation. Prior to inoculation, the  
114 culture medium was equilibrated with air containing a  $\text{pCO}_2$  of  $180 \mu\text{atm}$  (~Last Glacial  
115 Maximum),  $380 \mu\text{atm}$  (~present-day),  $800 \mu\text{atm}$  (~2100 scenario), and  $1200 \mu\text{atm}$  (>2100  
116 scenario). Each treatment was performed in triplicate.

117

### 118 **2.2 Carbonate chemistry**

119 Carbonate chemistry was assessed by total alkalinity (TA), dissolved inorganic carbon (DIC),  
120 and  $\text{pH}_{\text{NBS}}$ . For TA analyses, 25 mL of culture suspension was filtered over glass-fibre filters  
121 (GF/F,  $\sim 0.6 \mu\text{m}$  pore size; Whatman, Maidstone, UK) and stored in gas-tight borosilicate  
122 bottles at  $3^\circ\text{C}$ . Duplicate samples were analysed by means of potentiometric titrations using  
123 an automated TitroLine burette system (SI Analytics, Mainz, Germany). For DIC analyses, 4  
124 mL culture suspension was filtered over cellulose-acetate filters ( $0.2 \mu\text{m}$  pore size; Thermo  
125 Fisher Scientific Inc. Waltham, USA), and stored headspace free in gas-tight borosilicate  
126 bottles at  $3^\circ\text{C}$ . Duplicate samples for DIC were analysed colorimetrically with a QuAAtro  
127 autoanalyser (Seal Analytical, Mequon, USA). pH was measured immediately after sampling  
128 with a pH electrode (Schott Instruments, Mainz, Germany), applying a two-point calibration

129 on the NBS scale prior to each measurement. Calculations of the carbonate system were based  
130 on TA and pH and performed with the program CO2sys (Pierrot et al., 2006). An average  
131 phosphate concentration of  $6.4 \mu\text{mol L}^{-1}$  was assumed, the dissociation constant of carbonic  
132 acid was based on Mehrbach et al. (1973), refit by Dickson and Millero (1987), and the  
133 dissociation constant of sulfuric acid was taken from Dickson (1990).

134

### 135 **2.3. Population densities and growth rate**

136 In order to ensure dilute batch conditions, cell densities were kept below  $<400 \text{ cells mL}^{-1}$ .  
137 Prior to the experiments, cells were acclimated to the respective  $\text{CO}_2$  concentrations for at  
138 least 7 cell divisions. During the experiments, cell growth was followed for 8 days, a period  
139 comprising at least 4 cell divisions. Cell densities were determined daily or every other day by  
140 means of single or duplicate cell counts with an inverted light microscope (Axiovert 40C,  
141 Zeiss, Germany), using 1-18 mL culture suspension fixed with Lugol's solution (2% final  
142 concentration). Triplicate cell counts were performed on the last day of each experiment, i.e.  
143 on day 8. Growth rates were estimated from each biological replicate by means of an  
144 exponential function fitted through all cell counts over time, according to  $N_t = N_0 \exp^{\mu t}$ , where  
145  $N_t$  refers to the population density at time  $t$ ,  $N_0$  to the population density at the start of the  
146 experiment, and  $\mu$  to the growth rate (Fig. A.1 and A.2).

147

### 148 **2.4 Organic carbon and nitrogen**

149 For organic carbon and nitrogen analyses, 250-500 mL cell suspension was filtered over  
150 precombusted GF/F filters (12 h,  $500^\circ\text{C}$ ) and stored at  $-25^\circ\text{C}$  in precombusted Petri dishes.  
151 Prior to measurements, 200  $\mu\text{L}$  of 0.2 N HCl (analytical grade) was added to the filters to  
152 remove all inorganic carbon, and filters were dried overnight. Filters were analysed on carbon  
153 and nitrogen content in duplicate on an Automated Nitrogen Carbon Analyser mass

154 spectrometer (ANCA-SL 20-20, SerCon Ltd., Crewe, UK). Carbon production rates were  
155 estimated by multiplication of the organic carbon content with  $\mu$ .

156

## 157 **2.5 PST analogues**

158 Different analogues of PSTs were analysed and included the non-sulfated STX, NEO and  
159 dcSTX, the mono-sulfated GTX1+4 and GTX2+3, and the di-sulfated C1+C2. The cellular  
160 toxicity was estimated based on the cellular PST content and the relative toxicity of each PST  
161 analogue (Wiese et al., 2010). For analyses of the different PST analogues, 200-500 mL of  
162 culture suspension was filtered over polycarbonate filters (0.8  $\mu\text{m}$  pore size; Whatman) and  
163 stored in Eppendorf tubes at  $-25^{\circ}\text{C}$ . Prior to analyses, cells were re-suspended in 1.2 mL  $0.03$   
164  $\text{mol L}^{-1}$  acetic acid and lysed for 1 min with a Sonifier 250 ultrasonic probe (Branson  
165 Ultrasonics, Danbury, CT, USA). Subsequently, samples were transferred into new 1.5 mL  
166 reaction vials and centrifuged for 15 min at 10,000 g at  $4^{\circ}\text{C}$ . The supernatant was transferred  
167 into an LC vial and analysed by liquid chromatography via fluorescence detection (LC-FD)  
168 with post-column derivatization (Krock et al., 2007).

169

## 170 **2.5 RNA extraction**

171 For RNA extraction, 500 mL of culture suspension was concentrated to 50 mL with a 10  $\mu\text{m}$   
172 mesh sized sieve, and subsequently centrifuged at  $15^{\circ}\text{C}$  for 15 min at 4000 g. Cell pellets  
173 were immediately mixed with 1 mL  $60^{\circ}\text{C}$  TriReagent (Sigma-Aldrich, Steinheim, Germany),  
174 frozen with liquid nitrogen and stored at  $-80^{\circ}\text{C}$ . Subsequently, cell suspensions were  
175 transferred to a 2 mL cryovial containing acid washed glass beads. Cells were lysed using a  
176 BIO101 FastPrep instrument (Thermo Savant, Illkirch, France) at maximum speed ( $6.5 \text{ m s}^{-1}$ )  
177 for  $2 \times 30 \text{ s}$ , with an additional incubation of 5 min at  $60^{\circ}\text{C}$  in between. For RNA isolation,  
178 200  $\mu\text{L}$  chloroform was added to each vial, vortexed for 20 s and incubated for 10 min at  
179 room temperature. The samples were subsequently centrifuged for 15 min at  $4^{\circ}\text{C}$  with 12,000



180 g. The upper aqueous phase was transferred to a new vial and 2  $\mu\text{L}$  5 M linear acrylamide, 10  
181 % volume fraction of 3  $\mu\text{mol L}^{-1}$  sodium acetate, and an equal volume of 100 % isopropanol  
182 were added. Mixtures were vortexed and subsequently incubated overnight at  $-20^{\circ}\text{C}$  in order  
183 to precipitate the RNA. The RNA pellet was collected by 20 min centrifugation at  $4^{\circ}\text{C}$  and  
184 12,000 g. The pellet was washed twice, first with 70 % ethanol and afterwards with 96 %  
185 ethanol, air-dried and dissolved with 100  $\mu\text{L}$  RNase free water (Qiagen, Hilden, Germany).  
186 The RNA sample was further cleaned with the RNeasy Kit (Qiagen) according to  
187 manufacturer's protocol for RNA clean-up including on-column DNA digestion. RNA quality  
188 check was performed using a NanoDrop ND-100 spectrometer (PeqLab, Erlangen, Germany)  
189 for purity, and the RNA Nano Chip Assay with a 2100 Bioanalyzer (Agilent Technologies,  
190 Böblingen, Germany) was performed in order to examine the integrity of the extracted RNA.  
191 Only high quality RNAs ( $\text{OD}_{260}/\text{OD}_{280} > 1.8$  and  $\text{OD}_{260}/\text{OD}_{230} > 1.8$ ) as well as RNA with  
192 intact ribosomal peaks (obtained from the Bioanalyzer readings) were used for microarrays.

193

## 194 **2.7 Microarray hybridizations**

195 For microarray hybridizations, RNA Spike-In Mix (Agilent, p/n 5188-5279) was added to the  
196 RNA samples prior to the labelling substances as an internal standard for hybridization  
197 performance (Agilent RNA Spike-In Kit protocol). 200 ng total RNA from samples was  
198 reversely transcribed, and the resulting cDNA was linear amplified into labelled cRNA (two-  
199 colour Low Input Quick Amp Labeling kit, p/n 5190-2306). Incorporation of labelled cytidine  
200 5'-triphosphate (Perkin Elmer, Waltham, USA) into the cRNA from the 180, 380 and 800  
201  $\mu\text{atm CO}_2$  treatments (Cy-3) as well as for the pooled reference cRNA (380  $\mu\text{atm CO}_2$ , Cy-5)  
202 was verified photometrically using the NanoDrop ND1000 (PeqLab). Labelling efficiencies  
203 were calculated as  $\text{pmol dye (ng cRNA)}^{-1}$ . Microarray hybridizations of the 180, 380 and 800  
204  $\mu\text{atm CO}_2$  treatments were carried out in biological triplicates against the reference pool using  
205 SureHyb hybridization chambers (Agilent, p/n G2534A). 300 ng of each Cy-3 and Cy-5

206 labelled cRNA was hybridized to  $8 \times 60K$  custom-built microarrays (Agilent). Probe design  
207 was done using Agilent's eArray online platform. Following the Two-Color Microarray-based  
208 Gene Expression Analysis protocol (Agilent, p/n 5188-5242), hybridization was performed in  
209 a hybridization oven at  $65^{\circ}\text{C}$  for 17 h at an agitation of 6 rpm. After hybridization,  
210 microarrays were disassembled in Wash Buffer 1 (Agilent, p/n 5188-5325), washed with  
211 Wash Buffer 1, Wash Buffer 2 (Agilent, p/n 5188-5326), acetonitrile (VWR, Darmstadt,  
212 Germany) and 'Stabilization and Drying Solution' (Agilent, p/n 5185-5979) according to  
213 manufacturer's instructions. Stabilization and Drying Solution, an ozone scavenger, protects  
214 the Cy-dye signal from degradation. Arrays were immediately scanned with a G2505C  
215 microarray scanner (Agilent) using standard photomultiplier tube (PMT) settings and 3  $\mu\text{m}$   
216 scan resolution.

217

## 218 **2.8. Data analysis**

219 Normality and equality of variances of growth, elemental composition, and PST data were  
220 confirmed using the Shapiro-Wilk and Levene's test, respectively. Variables were log-  
221 transformed if this improved normality and the homogeneity of variances. Significant  
222 differences between strains and treatments were tested using a mixed effect two-way  
223 ANOVA, with  $\text{CO}_2$  treatment as fixed factor and strain as random factor, and followed by  
224 post hoc comparison of the means using Tukey's HSD test ( $\alpha=0.05$ ; Quinn and Keough,  
225 2002). For PST composition, significant differences between treatments were tested using a  
226 one-way ANOVA followed by post hoc comparison of the means using Tukey's HSD test.

227 Microarray raw data of Alex5 was extracted with Feature Extraction Software version  
228 9.0 (Agilent), incorporating the GE2\_105\_Dec08 protocol. Array quality was monitored using  
229 the QC Tool v1.0 (Agilent) with the metric set GE2\_QCMT\_Feb07. Analysis was performed  
230 using GeneSpring 12 (Agilent). Raw data including LOWESS-normalized data were  
231 submitted to the MIAMEexpress database hosted by the European Bioinformatics Institute

232 (accession code E-MEXP-3946). Differential gene expression was evaluated using the  
233 GeneSpring GX software platform version 12 (Agilent). After combining biological  
234 replicates, the 180 and 800  $\mu\text{atm}$   $\text{CO}_2$  treatments were tested against the 380  $\mu\text{atm}$   $\text{CO}_2$   
235 treatment using a one-way ANOVA. Genes were considered to be differential expressed when  
236  $P < 0.05$  and fold changes  $> 1.5$ . Obtained differential expressed genes were categorized  
237 according to KOG with the batch web CD-search tool (Marchler-Bauer et al., 2011) and an e-  
238 value cut-off of  $e^{-7}$ .

## 239 **3. Results**

### 240 **3.1. Carbonate chemistry**

241 The drift in carbonate chemistry as result of biomass built-up remained below 4 % with  
242 respect to alkalinity and DIC in all incubations (Table B.1). The applied CO<sub>2</sub> treatments  
243 clearly differed from each other with respect to pCO<sub>2</sub> and pH throughout the experiment. In  
244 the incubations with Alex5, pCO<sub>2</sub> values ranged from 162 ± 24 μatm to 995 ± 248 μatm,  
245 accompanied by a pH of 8.50 ± 0.06 and 7.83 ± 0.12, respectively. For Alex2, pCO<sub>2</sub> values  
246 ranged from 151 ± 9 μatm to 1167 ± 112 μatm, which was accompanied by a pH of 8.51 ±  
247 0.02 to 7.75 ± 0.04, respectively (Table B.1).

248

### 249 **3.2. Growth and elemental composition**

250 Alex2 clearly exhibited a lower growth rate (two-way ANOVA,  $F_{1,16}=287,0$ ,  $P<0.001$ ) and  
251 organic carbon production rate (two-way ANOVA,  $F_{1,16}=102,5$ ,  $P=0.002$ ) as compared to  
252 Alex5 (Table 1). Growth rates of Alex2 decreased by up to 25% (two-way ANOVA,  
253  $F_{3,16}=7.2$ ,  $P=0.003$ ) and carbon production rates by up to 35% (two-way ANOVA,  $F_{3,16}=13.7$ ,  
254  $P<0.001$ ) from the lowest to the higher pCO<sub>2</sub> treatments. Growth and carbon production rates  
255 of Alex5 remained largely unaffected in response to elevated pCO<sub>2</sub>. The carbon content in  
256 both strains was comparable at the lowest CO<sub>2</sub> treatment and increased with elevated pCO<sub>2</sub> in  
257 Alex5. As a consequence, Alex5 contained more carbon in all but the lowest CO<sub>2</sub> treatment  
258 (two-way ANOVA,  $F_{3,16}=5.0$ ,  $P=0.012$ ). In both strains, the nitrogen content remained  
259 unaltered by the applied changes in pCO<sub>2</sub>. The cellular nitrogen content of Alex5 was up to  
260 79% higher as compared to Alex2 (two-way ANOVA,  $F_{1,16}=100.9$ ,  $P=0.002$ ), resulting in up  
261 to 33% lower C:N ratios (two-way ANOVA,  $F_{1,16}=51.9$ ,  $P=0.006$ ).

262

### 263 **3.3. PST content and composition**

264 Alex5 showed a substantially higher cellular PST content (two-way ANOVA,  $F_{1,16}=1041$ ,  
265  $P<0.001$ ) and toxicity (two-way ANOVA,  $F_{1,16}=1035$ ,  $P<0.001$ ) as compared to Alex2 (Fig.  
266 1). In both strains, cellular PST content and toxicity were significantly affected by elevated  
267  $p\text{CO}_2$  (Fig. 1), decreasing by up to 21% in Alex5 (two-way ANOVA,  $F_{3,16}=7.0$ ,  $P=0.004$ ) and  
268 26% in Alex2 (two-way ANOVA,  $F_{3,16}=5.0$ ,  $P=0.009$ ). The most abundant PST analogues  
269 produced by both strains were C1+C2, STX and NEO, contributing up to 74% and 98% of the  
270 total PSTs in Alex5 and Alex2, respectively (Fig. 2). Cellular contents of GTXs were lower,  
271 particularly in Alex2, and trace amounts of dcSTX ( $<0.3\%$ ) were found in Alex5 (data not  
272 shown). In Alex2, the change in toxicity is caused by a reduction in toxin content, while its  
273 PST composition did not change (Fig. 2B,D,F). In Alex5, the PST composition was strongly  
274 affected by elevated  $p\text{CO}_2$  (Fig. 2A,C,E). Specifically, the cellular contribution of non-  
275 sulfated STX decreased by 54% (one-way ANOVA,  $F_{3,8}=225.6$ ,  $P<0.001$ ), the mono-sulfated  
276 GTX1+4 increased by 159% (one-way ANOVA,  $F_{3,8}=109.4$ ,  $P<0.001$ ), and di-sulfated  
277 analogues C1+C2 increased by 8% from the lowest to the highest  $\text{CO}_2$  treatment (one-way  
278 ANOVA,  $F_{3,8}=98.4$ ,  $P<0.001$ ). This shift in PST composition towards less toxic analogues  
279 explains about half of the overall reduction in cellular toxicity of Alex5.

280

### 281 **3.4. Gene expression profiles**

282 A substantial number of genes in Alex5 were differentially expressed as compared to the  
283 control treatment (i.e. 380  $\mu\text{atm}$ ), with a total of 894 genes in the 180  $\mu\text{atm}$   $\text{CO}_2$  and 1238  
284 genes in the 800  $\mu\text{atm}$   $\text{CO}_2$  treatment (Fig. 3A). Part of the regulated genes could be annotated  
285 and were grouped according to KOG categories representing various cellular functions (Fig.  
286 3B and A.3; Appendix C). In both treatments, the highest number of regulated genes was  
287 related to the KOG categories ‘Signal transduction mechanisms [T]’ and ‘Posttranslational  
288 modification, protein turnover, chaperones [O]’, together representing about 35% of the total  
289 number of annotated genes. Another 31% of the total number of annotated genes was

290 associated to transport and metabolism of inorganic ions, carbohydrates, lipids, secondary  
291 metabolites and amino acids (Fig. 3B). Generally, the number of genes being differentially  
292 expressed was higher in the 800  $\mu\text{atm CO}_2$  as compared to the 180  $\mu\text{atm CO}_2$  treatment,  
293 particularly those genes related to posttranslational modification and the transport and  
294 metabolism of carbohydrates (Fig. 3B). In only a few KOG categories, the number of  
295 regulated genes clearly differed between the high and low  $\text{CO}_2$  treatment. Specifically, the  
296 number of down-regulated genes associated to the categories ‘Secondary metabolites  
297 biosynthesis, transport and catabolism [Q]’ and ‘Amino acid transport and metabolism [E]’  
298 was higher in the 800  $\mu\text{atm CO}_2$  as compared to the 180  $\mu\text{atm CO}_2$  treatment (Fig. 3 and A.3).

299         In view of the pronounced changes in toxin composition of Alex5 from non-sulfated  
300 to sulfated PST analogues, we analyzed the genes involved in sulfur metabolism more  
301 closely. A total of 17 genes within the sulfur metabolism were found to be regulated in the  
302 180 and 800  $\mu\text{atm CO}_2$  treatment compared to the 380  $\mu\text{atm CO}_2$  treatment (Table 2). In  
303 response to elevated  $\text{pCO}_2$ , we also observed a significant up-regulation of a gene with strong  
304 resemblance to a sulfotransferase in the cyanobacterium *Cylindrospermopsis*, which may thus  
305 be a putative *sxtN* homologue involved the synthesis of sulfated PST analogues (Hackett et  
306 al., 2013; Moustafa et al., 2009; Soto-Liebe et al., 2010; Stucken et al., 2010). However, the  
307 relative change was small (i.e. 1.3 fold; Tables 2 and B.2) and we did not observe differential  
308 expression of other putative homologues of STX genes.

309

## 310 **4. Discussion**

311 While growth and carbon production remained largely unaffected with elevated pCO<sub>2</sub> (Table  
312 1), the cellular PST content and associated toxicity were clearly reduced under high pCO<sub>2</sub> in  
313 both strains (Fig. 1). Furthermore, PST composition in Alex5 shifted towards more sulfated  
314 analogues with increasing pCO<sub>2</sub>, whereas PST composition in Alex2 remained unaltered (Fig.  
315 2). The observed decrease in PST content of Alex5 may be explained by a decrease in the  
316 relative expression of genes involved in amino acid metabolism (Fig. 3), presumably  
317 including arginine which is an important precursor in PST biosynthesis. The shift in PST  
318 composition towards more sulfated analogues is in line with CO<sub>2</sub>-dependent changes in the  
319 expression of genes involved in sulfur metabolism, notably a putative sulfotransferase *sxtN*  
320 homologue associated to PST synthesis (Table 2; Hackett et al., 2013; Moustafa et al., 2009;  
321 Soto-Liebe et al., 2010). The largest number of genes being differentially expressed compared  
322 to the control was related to signal transduction and post-translational modification. This  
323 suggests that the ability of Alex5 to maintain cellular homeostasis with respect to growth and  
324 elemental composition is predominantly achieved by post-translational modification of  
325 general physiological processes rather than a specific transcriptomic regulation.

326

### 327 **4.1 CO<sub>2</sub> effects on growth and PST contents**

328 The minor changes of growth and carbon production rates in response to elevated pCO<sub>2</sub> are  
329 somewhat surprising, as dinoflagellates were expected to be sensitive to changes in CO<sub>2</sub> due  
330 to their low affine RubisCO. This suggests that both *A. tamarensis* strains do not rely on  
331 diffusive CO<sub>2</sub> supply alone and instead operate a carbon concentrating mechanism (CCM), as  
332 has been shown for several red-tide dinoflagellate species (Rost et al., 2006). Such a CCM  
333 may be regulated as a function of CO<sub>2</sub> supply, enabling the cell to keep its growth relatively  
334 unaffected over the tested CO<sub>2</sub> range. Regarding the gene expression in Alex5, we observed a  
335 significant down-regulation of a gene homologous to carbonic anhydrase (CA) with elevated

336 pCO<sub>2</sub> (Appendix C). As CA accelerates the interconversion between HCO<sub>3</sub><sup>-</sup> and CO<sub>2</sub>, it often  
337 plays an important role in the functioning of CCMs (Giordano et al., 2005a; Reinfelder,  
338 2011). The lowered expression of CA homologues under elevated pCO<sub>2</sub> is in line with the  
339 often observed down-regulation of CA activities (Sultemeyer et al., 1989; Trimborn et al.,  
340 2013), and may therefore be taken as an indication for a down-regulation of the CCM. In  
341 Alex5, we further observed a larger number of genes associated to carbohydrate transport and  
342 metabolism being regulated in the high CO<sub>2</sub> treatment (Fig. 3B), which points towards re-  
343 constellation of internal carbon fluxes helping to maintain homeostasis in terms of growth and  
344 elemental composition.

345         In Alex2, highest growth rate as well as PST content was observed in the lowest CO<sub>2</sub>  
346 treatment, whereas in all other treatments the growth rate and toxin content were consistently  
347 lower. Furthermore, PST contents in the faster growing Alex5 were much higher than in  
348 slower growing Alex2, hinting toward a general relationship between growth and toxin  
349 content. When nutrients are in ample supply, cellular PST content has indeed been dependent  
350 on the growth cycle and tend to increase with growth rate (Cembella, 1998; Taroncher-  
351 Oldenburg et al., 1997). The growth rates of Alex5 were in line with earlier reported values in  
352 conventional batch cultures, while growth rates of Alex2 were lower (Zhu and Tillmann,  
353 2012). This may be the result of differences in experimental conditions, suggesting that Alex2  
354 may not have been growing optimally under the imposed conditions. Our findings seem to  
355 confirm that growth rate plays an important role in determining the PST content in *A.*  
356 *tamarensis*.

357         Cells of Alex5 have lower C:N ratios than Alex2 and hence contain relatively more  
358 nitrogen. This nitrogen can be allocated to nitrogen containing metabolites, such as amino  
359 acids and PSTs, which would be in line with the observed differences in PST content between  
360 both strains of *A. tamarensis*. Transcriptomic analyses in Alex5 furthermore reveal a down-  
361 regulation of amino acid transport and metabolism with elevated pCO<sub>2</sub> (Fig. 3). This down-



362 regulation may also affect the availability of arginine, an important precursor of PSTs  
363 (Shimizu, 1996), and thus may explain the decreased PST content with elevated pCO<sub>2</sub>.  
364 Additional studies including both amino acid composition and transcriptome analyses should  
365 further elucidate the role of amino acid metabolism in the synthesis of PSTs.

366

#### 367 **4.2 CO<sub>2</sub> effects on PST composition**

368 PST composition in Alex5 shifted from a non-sulfated towards sulfated PST analogues with  
369 increasing pCO<sub>2</sub> (Fig. 2A,C,E), which implies that sulfation of PSTs is enhanced under these  
370 conditions. Sulfotransferases play a key role in sulfation of PST analogues and their activity is  
371 therefore important in determining the PST composition (Moustafa et al., 2009; Sako et al.,  
372 2001; Soto-Liebe et al., 2010). We observed a small, yet significant up-regulation in the  
373 expression of a putative sulfotransferase *sxtN* homologue in our high CO<sub>2</sub> treatment.  
374 Furthermore, we found substantial regulation in the expression of genes coding for sulfatases,  
375 which catalyse the hydrolysis of sulfate esters, and thereby possibly affect the PST  
376 composition (Fig. 4; Taroncher-Oldenburg et al., 1997). In the high CO<sub>2</sub> treatment, expression  
377 of sulfatase significantly decreased, whereas it increased in the low CO<sub>2</sub> treatment (Table 2).  
378 In other words, the transformation of di-sulfated and mono-sulfated PST analogues to mono-  
379 sulfated and non-sulfated PST analogues, respectively, is less likely to occur at elevated  
380 pCO<sub>2</sub>, whereas these reactions are potentially more frequent at low pCO<sub>2</sub> (Fig. 4). We also  
381 observed a significant decrease in the expression of genes involved in sulfite reduction in the  
382 high CO<sub>2</sub> treatment, whereas it increased in the low CO<sub>2</sub> treatment. Sulfite reductase plays a  
383 role in the assimilation of sulfur into amino acids, starting with the production of cysteine  
384 (Fig. 4; Giordano et al., 2005b; Shibagaki and Grossman, 2008). Our data thus suggests that  
385 with elevated pCO<sub>2</sub>, more sulfur is allocated in sulfated PST analogues, while less is  
386 assimilated to cysteine (Fig. 4). Although the exact processes remain to be elucidated, our

387 results clearly demonstrate that elevated pCO<sub>2</sub> can affect sulfur metabolism in *A. tamarensis*  
388 and thereby causes a shift in PST composition towards more sulfated analogues.

389 PST composition in Alex2 was not affected by changes in pCO<sub>2</sub>. Given that the same  
390 treatments have been applied to both strains, the observed differences in CO<sub>2</sub>-sensitivity are  
391 presumably strain-specific (Alpermann et al., 2010; Tillmann et al., 2009). There have been  
392 earlier attempts to assess strain-specific differences in the response of PST composition  
393 towards elevated pCO<sub>2</sub> in *Alexandrium ostenfeldii* (Kremp et al., 2012). The observed trends  
394 in the latter study remain inconclusive, however, due to the large shifts in carbonate chemistry  
395 that occurred during the experiments. Hence, further experiments are needed to test whether  
396 elevated pCO<sub>2</sub> indeed alters sulfur metabolism and subsequently the production of sulfated  
397 PST analogues in *Alexandrium* as well as other PST producers.

398

### 399 **4.3 Ecological implications**

400 What will be the impact of elevated pCO<sub>2</sub> on PST production in natural occurring blooms of  
401 *A. tamarensis*? Obviously, the natural system is far more complex than our dilute batch  
402 experiments, and blooms of *A. tamarensis* comprise many more genotypes with different  
403 growth rates and PST characteristics than can be tested in laboratory studies (Alpermann et  
404 al., 2009; Alpermann et al., 2010; Tillmann et al., 2009; Yoshida et al., 2001). Being aware of  
405 this, we chose the two strains with different properties, for instance in terms of growth rate,  
406 elemental composition, PST characteristics, and most notably their allelopathic properties  
407 (Tillmann et al., 2009). We also worked with low cell densities in our experiments (i.e. <400  
408 cells mL<sup>-1</sup>) in order to be comparable with population densities of *Alexandrium* that may be  
409 reached during blooms (Wyatt and Jenkinson, 1997). Hence, natural blooms will likely be  
410 exposed to similar CO<sub>2</sub> levels as tested here, which may lead to the conclusion that with a  
411 decrease in the cellular toxicity of *A. tamarensis*, future blooms may become less toxic.

412 *A. tamarense* blooms with population densities exceeding those reached in our  
413 experiments can shift seawater carbonate chemistry towards lower CO<sub>2</sub> concentrations and a  
414 higher pH. Ultimately, this may cause phytoplankton to become limited by CO<sub>2</sub>, or to become  
415 negatively affected by the high pH (Hansen, 2002; Hansen et al., 2007; Tillmann and Hansen,  
416 2009). Such a change in carbonate chemistry may have contrasting consequences for the  
417 toxicity of *A. tamarense* as compared to elevated pCO<sub>2</sub>, i.e. the cellular toxicity could increase  
418 (Fig. 1). On top of that, elevated pCO<sub>2</sub> may promote higher population densities as cells can  
419 presumably sustain growth for a longer period before becoming CO<sub>2</sub> limited, or before  
420 reaching their pH limit. Hence, even if their cellular toxicity is not affected, higher population  
421 densities will increase the toxicity of an *A. tamarense* bloom. Experiments with natural  
422 occurring blooms of *A. tamarense* are required in order to understand the complex  
423 interactions that exist between carbonate chemistry, phytoplankton growth, and the toxicity of  
424 HABs.

425 Ecological consequences of changes in PST contents and composition will depend on  
426 the role of these compounds. It has been suggested that PSTs play a role in grazer defense  
427 (Selander et al., 2012; Selander et al., 2006; Wohlrab et al., 2010), act as pheromones (Wyatt  
428 and Jenkinson, 1997), and may have physiological functions as well (Cembella, 2003). Recent  
429 findings have further suggested that PST may play a role in the maintenance of cellular ion  
430 homeostasis (Pomati et al., 2004; Soto-Liebe et al., 2012), and this putative function seems to  
431 become more pronounced under elevated pH (Pomati et al., 2004). In our study, the highest  
432 PST content was indeed associated to the treatments with highest pH, i.e. the treatment with  
433 the lowest pCO<sub>2</sub> (Table B.1). It remains to be determined, however, whether the observed  
434 changes in PST content are primarily due to shifts in pH or by changes in CO<sub>2</sub> availability.

435

#### 436 **4.4. Conclusions**

437 Here we show that growth and elemental composition in *A. tamarensis* remain largely  
438 unaltered in response to elevated pCO<sub>2</sub>. This ability to maintain cellular homeostasis under  
439 substantial changes in carbonate chemistry appears to be achieved primarily by post-  
440 translational regulation. In both *A. tamarensis* strains, cellular PST content and particularly the  
441 associated toxicity were lower in the highest CO<sub>2</sub> treatments. In Alex5, PST composition  
442 further shifted towards more sulfated analogues under these conditions. These CO<sub>2</sub>-dependent  
443 changes in PST content and composition are accompanied by substantial regulation of  
444 multiple genes, including those associated to secondary metabolite and amino acid  
445 metabolism. Notably, we found that elevated pCO<sub>2</sub> caused an opposing regulation of  
446 sulfotransferase and sulfatase, leading to an enhanced production of less toxic sulfated PST  
447 analogues. All in all, our findings suggest that elevated pCO<sub>2</sub> may have minor consequences  
448 for growth and elemental composition of *A. tamarensis*, yet may potentially cause a decrease  
449 in its cellular toxicity.

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459

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631

632 **Figure legends**

633 Fig. 1. PST content and cellular toxicity of Alex5 (A,C) and Alex2 (B,D) at the different CO<sub>2</sub>  
634 treatments. Error bars denote standard deviation ( $n=3$ ). Letters above bars indicate significant  
635 differences between treatments (two-way ANOVA,  $P<0.05$ ).

636

637 Fig. 2. Relative composition of PST analogues grouped as non-sulfated (A,B), mono-sulfated  
638 (C,D) and di-sulfated (E,F), in Alex5 (A,C,E) and Alex2 (B,D,F) at the different CO<sub>2</sub>  
639 treatments. Error bars denote standard deviation ( $n=3$ ). Letters above bars indicate significant  
640 differences between treatments of grouped toxins, as well as STX (A) and GTX1+4 (C) (one-  
641 way ANOVA,  $P<0.05$ ).

642

643 Fig. 3. Venn diagram showing the total and annotated number (between brackets) of  
644 differentially expressed genes in Alex 5 (A), and the regulation of annotated genes grouped  
645 by most represented KOG categories (B). Values indicate the actual number of genes being  
646 up-regulated ( $\uparrow$ ) and down-regulated ( $\downarrow$ ) in the 180  $\mu\text{atm}$  CO<sub>2</sub> (A, left circle; B, black bar) and  
647 800  $\mu\text{atm}$  CO<sub>2</sub> (A, right circle; B, white bar) treatments in comparison to the control treatment  
648 of 380  $\mu\text{atm}$  CO<sub>2</sub>. Short KOG category names refer to ‘Posttranslational modification, protein  
649 turnover and chaperones [O]’, ‘Signal transduction mechanisms [T]’, ‘Inorganic ion transport  
650 and metabolism [P]’, ‘Carbohydrate transport and metabolism [G]’, ‘Lipid transport and  
651 metabolism [I]’, ‘Secondary metabolite biosynthesis, transport and catabolism [Q]’ and  
652 ‘Amino acid transport and metabolism [E]’.

653

654 Fig. 4. Schematic diagram of observed CO<sub>2</sub> effects on sulfate (SO<sub>4</sub><sup>2-</sup>) assimilation in Alex5.  
655 Vertical arrows next to enzymes indicate up-regulation ( $\uparrow$ ) or down-regulation ( $\downarrow$ ) in relevant  
656 genes under elevated CO<sub>2</sub>. After taken up by the cell, SO<sub>4</sub><sup>2-</sup> is transported to the plastid and  
657 activated to 5'-adenylsulfate (APS). This APS can be reduced to sulfite (SO<sub>3</sub><sup>2-</sup>) and

658 subsequently to sulfide ( $S^{2-}$ ). The latter reaction is catalyzed by  $SO_3^{2-}$  reductase. The resulting  
659 free  $S^{2-}$  is immediately incorporated into cysteine, the first stable sulfur-containing organic  
660 biochemical. APS can also undergo a second phosphorylation, yielding 3'-phosphoadenosine  
661 5'-phosphosulfate (PAPS), which can be used by sulfotransferases to catalyze sulfation of  
662 various metabolites, including PSTs. Transformation of di-sulfated and mono-sulfated PST  
663 analogues to mono-sulfated and non-sulfated PST analogues is catalysed by sulfatases.