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Impact of elevated pCO_2 on paralytic shellfish poisoning toxin content and composition in *Alexandrium tamarense*

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Abstract

Ocean acidification is considered a major threat to marine ecosystems and may particularly affect primary producers. Here we investigated the impact of elevated pCO_2 on paralytic shellfish poisoning toxin (PST) content and composition in two strains of *Alexandrium tamarense*, Alex5 and Alex2. Experiments were carried out as dilute batch to keep carbonate chemistry unaltered over time. We observed only minor changes with respect to growth and elemental composition in response to elevated pCO_2. For both strains, the cellular PST content, and in particular the associated cellular toxicity, was lower in the high CO_2 treatments. In addition, Alex5 showed a shift in its PST composition from a non-sulfated analogue towards less toxic sulfated analogues with increasing pCO_2. Transcriptomic analyses suggest that the ability of *A. tamarense* to maintain cellular homeostasis is predominantly regulated on the post-translational level rather than on the transcriptomic level. Furthermore, genes associated to secondary metabolite and amino acid metabolism in Alex5 were down-regulated in the high CO_2 treatment, which may explain the lower PST content. Elevated pCO_2 also induced up-regulation of a putative sulfotransferase s*xtN* homologue and a substantial down-regulation of several sulfatases. Such changes in sulfur metabolism may explain the shift in PST composition towards more sulfated analogues. All in all, our results indicate that elevated pCO_2 will have minor consequences for growth and elemental composition, but may potentially reduce the cellular toxicity of *A. tamarense*.

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

1. Introduction

Since the industrial revolution, atmospheric CO_2 levels are rising at an unprecedented rate (Solomon et al., 2007). This increase in atmospheric pCO_2 affects the carbonate chemistry of ocean waters, which shifts towards higher concentrations of CO_2 and bicarbonate (HCO_3^-), lower concentrations of carbonate (CO_3^{2-}), and a reduction in pH, i.e. ocean acidification (Caldeira and Wickett, 2003; Wolf-Gladrow et al., 1999). Such changes in ocean carbonate chemistry will have implications for phytoplankton that convert inorganic carbon into organic biomass. For instance, increasing concentrations of CO_2 and HCO_3^- were shown to promote phytoplankton growth and photosynthesis (Hein and SandJensen, 1997; Tortell et al., 2008), whereas decreasing pH and concentrations of CO_3^{2-} were hold responsible for the adverse effects observed in calcification (Beaufort et al., 2011; Riebesell et al., 2000).

Our current knowledge about the sensitivity of phytoplankton towards ocean acidification is almost entirely based on the work with diatoms, coccolithophores, and cyanobacteria (see Riebesell and Tortell, 2011). Relatively little is yet known about the responses of autotrophic dinoflagellates (Brading et al., 2011; Burkhardt et al., 1999; Van de Waal et al., 2013), in particular of toxin producing species (Fu et al., 2010; Hallegraeff, 2010; Kremp et al., 2012). This is surprising because, among all eukaryotic phytoplankton species, dinoflagellates feature the primary carboxylating enzyme ribulose 1,5-bisphosphate carboxylase/oxygenase (RubisCO) with lowest affinities for its substrate CO_2 (Badger et al., 1998). Thus, the predicted increase of CO_2 concentrations in the future ocean may particularly favour this group.

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

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

Toxin production by HAB species is strongly affected by changes in resource availabilities, such as light and nutrients (Cembella, 1998; Granéli and Flynn, 2006; Neilan et al., 2013; Sivonen and Jones, 1999; Van de Waal et al., In press). Current changes in ocean carbonate chemistry may have consequences as well. For instance, elevated pCO_2 was found to cause an increase or a decrease in the production of domoic acid by the diatom *Pseudo-nitzschia multiseries* (Sun et al., 2011; Trimborn et al., 2008). Elevated pCO_2 was also shown to affect the composition of karlotoxins in the dinoflagellates *Karlodinium veneficum*, which shifted towards a more toxic analogue (Fu et al., 2010). Furthermore, PST content and composition differentially changed in various *Alexandrium ostenfeldii* strains (Kremp et al., 2012). The observed responses in these studies have been attributed to CO_2-induced changes in growth and energy allocation, even though the mechanisms underlying the production and composition of these toxins remain unclear.

Here we investigated the impact of elevated pCO_2 on PST content and composition in two strains of *Alexandrium tamarense* (Alex5 and Alex2), which were isolated from the same population at the Scottish east coast of the North Sea (Alpermann et al., 2009; Tillmann et al., 2009). Both *A. tamarense* strains differ in their growth rate, PST content as well as in their 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_2 on toxin production and gene regulation in *A. tamarense*.

2. Materials and Methods

2.1. Culture conditions

Cultures of *Alexandrium tamarense* Alex5 and Alex2 (Alpermann et al., 2009; Tillmann et al., 2009) were grown as dilute batch in 2.4 L air-tight borosilicate bottles. Filtered natural seawater (0.2 µm pore size; Satorius, Goettingen, Germany) was enriched with metals and vitamins according to the recipe of f/2-medium (Guillard and Ryther, 1962), except for $FeCl_3$ (1.9 µmol L^{-1}), H_2SeO_3 (10 nmol L^{-1}), and $NiCl_2$ (6.3 nmol L^{-1}). The added concentrations of NO_3^- and PO_4^{3-} were 100 µmol L^{-1} and 6.25 µmol L^{-1}, respectively. Cultures were grown at a light:dark cycle of 16:8 h and an incident light intensity of 250 ± 25 µmol photons m^{-2} s^{-1} provided by daylight lamps (Lumilux HO 54W/965, Osram, München, Germany). Bottles were kept at 15°C and placed on a roller table to avoid sedimentation. Prior to inoculation, the culture medium was equilibrated with air containing a pCO_2 of 180 µatm (~Last Glacial Maximum), 380 µatm (~present-day), 800 µatm (~2100 scenario), and 1200 µatm (>2100 scenario). Each treatment was performed in triplicate.

2.2 Carbonate chemistry

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

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

2.3. Population densities and growth rate

In order to ensure dilute batch conditions, cell densities were kept below <400 cells mL^{-1}. Prior to the experiments, cells were acclimated to the respective CO_2 concentrations for at least 7 cell divisions. During the experiments, cell growth was followed for 8 days, a period comprising at least 4 cell divisions. Cell densities were determined daily or every other day by means of single or duplicate cell counts with an inverted light microscope (Axiovert 40C, Zeiss, Germany), using 1-18 mL culture suspension fixed with Lugol's solution (2% final concentration). Triplicate cell counts were performed on the last day of each experiment, i.e. on day 8. Growth rates were estimated from each biological replicate by means of an exponential function fitted through all cell counts over time, according to $N_t = N_0 \exp^{\mu t}$, where N_t refers to the population density at time t, N_0 to the population density at the start of the experiment, and μ to the growth rate (Fig. A.1 and A.2).

2.4 Organic carbon and nitrogen

For organic carbon and nitrogen analyses, 250-500 mL cell suspension was filtered over precombusted GF/F filters (12 h, 500°C) and stored at -25°C in precombusted Petri dishes. Prior to measurements, 200 μL of 0.2 N HCl (analytical grade) was added to the filters to remove all inorganic carbon, and filters were dried overnight. Filters were analysed on carbon and nitrogen content in duplicate on an Automated Nitrogen Carbon Analyser mass

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

2.5 PST analogues

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

2.5 RNA extraction

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

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

2.7 Microarray hybridizations

For microarray hybridizations, RNA Spike-In Mix (Agilent, p/n 5188-5279) was added to the RNA samples prior to the labelling substances as an internal standard for hybridization performance (Agilent RNA Spike-In Kit protocol). 200 ng total RNA from samples was reversely transcribed, and the resulting cDNA was linear amplified into labelled cRNA (two-colour Low Input Quick Amp Labeling kit, p/n 5190-2306). Incorporation of labelled cytidine 5'-triphosphate (Perkin Elmer, Waltham, USA) into the cRNA from the 180, 380 and 800 µatm CO_2 treatments (Cy-3) as well as for the pooled reference cRNA (380 µatm CO_2, Cy-5) was verified photometrically using the NanoDrop ND1000 (PeqLab). Labelling efficiencies were calculated as pmol dye (ng cRNA)$^{-1}$. Microarray hybridizations of the 180, 380 and 800 µatm CO_2 treatments were carried out in biological triplicates against the reference pool using SureHyb hybridization chambers (Agilent, p/n G2534A). 300 ng of each Cy-3 and Cy-5

206 labelled cRNA was hybridized to 8 × 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°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 µm
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 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 (α=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 MIAMExpress database hosted by the European Bioinformatics Institute

(accession code E-MEXP-3946). Differential gene expression was evaluated using the GeneSpring GX software platform version 12 (Agilent). After combining biological replicates, the 180 and 800 µatm CO_2 treatments were tested against the 380 µatm CO_2 treatment using a one-way ANOVA. Genes were considered to be differential expressed when $P<0.05$ and fold changes>1.5. Obtained differential expressed genes were categorized according to KOG with the batch web CD-search tool (Marchler-Bauer et al., 2011) and an e-value cut-off of e^{-7}.

3. Results

3.1. Carbonate chemistry

The drift in carbonate chemistry as result of biomass built-up remained below 4 % with respect to alkalinity and DIC in all incubations (Table B.1). The applied CO_2 treatments clearly differed from each other with respect to pCO_2 and pH throughout the experiment. In the incubations with Alex5, pCO_2 values ranged from 162 ± 24 µatm to 995 ± 248 µatm, accompanied by a pH of 8.50 ± 0.06 and 7.83 ± 0.12, respectively. For Alex2, pCO_2 values ranged from 151 ± 9 µatm to 1167 ± 112 µatm, which was accompanied by a pH of 8.51 ± 0.02 to 7.75 ± 0.04, respectively (Table B.1).

3.2. Growth and elemental composition

Alex2 clearly exhibited a lower growth rate (two-way ANOVA, $F_{1,16}$=287,0, P<0.001) and organic carbon production rate (two-way ANOVA, $F_{1,16}$=102,5, P=0.002) as compared to Alex5 (Table 1). Growth rates of Alex2 decreased by up to 25% (two-way ANOVA, $F_{3,16}$=7.2, P=0.003) and carbon production rates by up to 35% (two-way ANOVA, $F_{3,16}$=13.7, P<0.001) from the lowest to the higher pCO_2 treatments. Growth and carbon production rates of Alex5 remained largely unaffected in response to elevated pCO_2. The carbon content in both strains was comparable at the lowest CO_2 treatment and increased with elevated pCO_2 in Alex5. As a consequence, Alex5 contained more carbon in all but the lowest CO_2 treatment (two-way ANOVA, $F_{3,16}$=5.0, P=0.012). In both strains, the nitrogen content remained unaltered by the applied changes in pCO_2. The cellular nitrogen content of Alex5 was up to 79% higher as compared to Alex2 (two-way ANOVA, $F_{1,16}$=100.9, P=0.002), resulting in up to 33% lower C:N ratios (two-way ANOVA, $F_{1,16}$=51.9, P=0.006).

3.3. PST content and composition

Alex5 showed a substantially higher cellular PST content (two-way ANOVA, $F_{1,16}$=1041, P<0.001) and toxicity (two-way ANOVA, $F_{1,16}$=1035, P<0.001) as compared to Alex2 (Fig. 1). In both strains, cellular PST content and toxicity were significantly affected by elevated pCO_2 (Fig. 1), decreasing by up to 21% in Alex5 (two-way ANOVA, $F_{3,16}$=7.0, P=0.004) and 26% in Alex2 (two-way ANOVA, $F_{3,16}$=5.0, P=0.009). The most abundant PST analogues produced by both strains were C1+C2, STX and NEO, contributing up to 74% and 98% of the total PSTs in Alex5 and Alex2, respectively (Fig. 2). Cellular contents of GTXs were lower, particularly in Alex2, and trace amounts of dcSTX (<0.3%) were found in Alex5 (data not shown). In Alex2, the change in toxicity is caused by a reduction in toxin content, while its PST composition did not change (Fig. 2B,D,F). In Alex5, the PST composition was strongly affected by elevated pCO_2 (Fig. 2A,C,E). Specifically, the cellular contribution of non-sulfated STX decreased by 54% (one-way ANOVA, $F_{3,8}$ =225.6, P<0.001), the mono-sulfated GTX1+4 increased by 159% (one-way ANOVA, $F_{3,8}$=109.4, P<0.001), and di-sulfated analogues C1+C2 increased by 8% from the lowest to the highest CO_2 treatment (one-way ANOVA, $F_{3,8}$=98.4, P<0.001). This shift in PST composition towards less toxic analogues explains about half of the overall reduction in cellular toxicity of Alex5.

3.4. Gene expression profiles

A substantial number of genes in Alex5 were differentially expressed as compared to the control treatment (i.e. 380 µatm), with a total of 894 genes in the 180 µatm CO_2 and 1238 genes in the 800 µatm CO_2 treatment (Fig. 3A). Part of the regulated genes could be annotated and were grouped according to KOG categories representing various cellular functions (Fig. 3B and A.3; Appendix C). In both treatments, the highest number of regulated genes was related to the KOG categories ‘Signal transduction mechanisms [T]’ and ‘Posttranslational modification, protein turnover, chaperones [O]’, together representing about 35% of the total number of annotated genes. Another 31% of the total number of annotated genes was

associated to transport and metabolism of inorganic ions, carbohydrates, lipids, secondary metabolites and amino acids (Fig. 3B). Generally, the number of genes being differentially expressed was higher in the 800 µatm CO_2 as compared to the 180 µatm CO_2 treatment, particularly those genes related to posttranslational modification and the transport and metabolism of carbohydrates (Fig. 3B). In only a few KOG categories, the number of regulated genes clearly differed between the high and low CO_2 treatment. Specifically, the number of down-regulated genes associated to the categories 'Secondary metabolites biosynthesis, transport and catabolism [Q]' and 'Amino acid transport and metabolism [E]' was higher in the 800 µatm CO_2 as compared to the 180 µatm CO_2 treatment (Fig. 3 and A.3).

In view of the pronounced changes in toxin composition of Alex5 from non-sulfated to sulfated PST analogues, we analyzed the genes involved in sulfur metabolism more closely. A total of 17 genes within the sulfur metabolism were found to be regulated in the 180 and 800 µatm CO_2 treatment compared to the 380 µatm CO_2 treatment (Table 2). In response to elevated pCO_2, we also observed a significant up-regulation of a gene with strong resemblance to a sulfotransferase in the cyanobacterium *Cylindrospermopsis*, which may thus be a putative *sxtN* homologue involved the synthesis of sulfated PST analogues (Hackett et al., 2013; Moustafa et al., 2009; Soto-Liebe et al., 2010; Stucken et al., 2010). However, the relative change was small (i.e. 1.3 fold; Tables 2 and B.2) and we did not observe differential expression of other putative homologues of STX genes.

4. Discussion

While growth and carbon production remained largely unaffected with elevated pCO_2 (Table 1), the cellular PST content and associated toxicity were clearly reduced under high pCO_2 in both strains (Fig. 1). Furthermore, PST composition in Alex5 shifted towards more sulfated analogues with increasing pCO_2, whereas PST composition in Alex2 remained unaltered (Fig. 2). The observed decrease in PST content of Alex5 may be explained by a decrease in the relative expression of genes involved in amino acid metabolism (Fig. 3), presumably including arginine which is an important precursor in PST biosynthesis. The shift in PST composition towards more sulfated analogues is in line with CO_2-dependent changes in the expression of genes involved in sulfur metabolism, notably a putative sulfotransferase s*xtN* homologue associated to PST synthesis (Table 2; Hackett et al., 2013; Moustafa et al., 2009; Soto-Liebe et al., 2010). The largest number of genes being differentially expressed compared to the control was related to signal transduction and post-translational modification. This suggests that the ability of Alex5 to maintain cellular homeostasis with respect to growth and elemental composition is predominantly achieved by post-translational modification of general physiological processes rather than a specific transcriptomic regulation.

4.1 CO_2 effects on growth and PST contents

The minor changes of growth and carbon production rates in response to elevated pCO_2 are somewhat surprising, as dinoflagellates were expected to be sensitive to changes in CO_2 due to their low affine RubisCO. This suggests that both *A. tamarense* strains do not rely on diffusive CO_2 supply alone and instead operate a carbon concentrating mechanism (CCM), as has been shown for several red-tide dinoflagellate species (Rost et al., 2006). Such a CCM may be regulated as a function of CO_2 supply, enabling the cell to keep its growth relatively unaffected over the tested CO_2 range. Regarding the gene expression in Alex5, we observed a significant down-regulation of a gene homologous to carbonic anhydrase (CA) with elevated

336 pCO_2 (Appendix C). As CA accelerates the interconversion between HCO_3^- and CO_2, 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_2 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_2 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_2
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 *tamarense*.

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. tamarense*. Transcriptomic analyses in Alex5 furthermore reveal a down-
361 regulation of amino acid transport and metabolism with elevated pCO_2 (Fig. 3). This down-

regulation may also affect the availability of arginine, an important precursor of PSTs (Shimizu, 1996), and thus may explain the decreased PST content with elevated pCO_2. Additional studies including both amino acid composition and transcriptome analyses should further elucidate the role of amino acid metabolism in the synthesis of PSTs.

4.2 CO_2 effects on PST composition

PST composition in Alex5 shifted from a non-sulfated towards sulfated PST analogues with increasing pCO_2 (Fig. 2A,C,E), which implies that sulfation of PSTs is enhanced under these conditions. Sulfotransferases play a key role in sulfation of PST analogues and their activity is therefore important in determining the PST composition (Moustafa et al., 2009; Sako et al., 2001; Soto-Liebe et al., 2010). We observed a small, yet significant up-regulation in the expression of a putative sulfotransferase s*xtN* homologue in our high CO_2 treatment. Furthermore, we found substantial regulation in the expression of genes coding for sulfatases, which catalyse the hydrolysis of sulfate esters, and thereby possibly affect the PST composition (Fig. 4; Taroncher-Oldenburg et al., 1997). In the high CO_2 treatment, expression of sulfatase significantly decreased, whereas it increased in the low CO_2 treatment (Table 2). In other words, the transformation of di-sulfated and mono-sulfated PST analogues to mono-sulfated and non-sulfated PST analogues, respectively, is less likely to occur at elevated pCO_2, whereas these reactions are potentially more frequent at low pCO_2 (Fig. 4). We also observed a significant decrease in the expression of genes involved in sulfite reduction in the high CO_2 treatment, whereas it increased in the low CO_2 treatment. Sulfite reductase plays a role in the assimilation of sulfur into amino acids, starting with the production of cysteine (Fig. 4; Giordano et al., 2005b; Shibagaki and Grossman, 2008). Our data thus suggests that with elevated pCO_2, more sulfur is allocated in sulfated PST analogues, while less is assimilated to cysteine (Fig. 4). Although the exact processes remain to be elucidated, our

results clearly demonstrate that elevated pCO_2 can affect sulfur metabolism in *A. tamarense* and thereby causes a shift in PST composition towards more sulfated analogues.

PST composition in Alex2 was not affected by changes in pCO_2. Given that the same treatments have been applied to both strains, the observed differences in CO_2-sensitivity are presumably strain-specific (Alpermann et al., 2010; Tillmann et al., 2009). There have been earlier attempts to assess strain-specific differences in the response of PST composition towards elevated pCO_2 in *Alexandrium ostenfeldii* (Kremp et al., 2012). The observed trends in the latter study remain inconclusive, however, due to the large shifts in carbonate chemistry that occurred during the experiments. Hence, further experiments are needed to test whether elevated pCO_2 indeed alters sulfur metabolism and subsequently the production of sulfated PST analogues in *Alexandrium* as well as other PST producers.

4.3 Ecological implications

What will be the impact of elevated pCO_2 on PST production in natural occurring blooms of *A. tamarense*? Obviously, the natural system is far more complex than our dilute batch experiments, and blooms of *A. tamarense* comprise many more genotypes with different growth rates and PST characteristics than can be tested in laboratory studies (Alpermann et al., 2009; Alpermann et al., 2010; Tillmann et al., 2009; Yoshida et al., 2001). Being aware of this, we chose the two strains with different properties, for instance in terms of growth rate, elemental composition, PST characteristics, and most notably their allelopathic properties (Tillmann et al., 2009). We also worked with low cell densities in our experiments (i.e. <400 cells mL^{-1}) in order to be comparable with population densities of *Alexandrium* that may be reached during blooms (Wyatt and Jenkinson, 1997). Hence, natural blooms will likely be exposed to similar CO_2 levels as tested here, which may lead to the conclusion that with a decrease in the cellular toxicity of *A. tamarense*, future blooms may become less toxic.

A. tamarense blooms with population densities exceeding those reached in our experiments can shift seawater carbonate chemistry towards lower CO_2 concentrations and a higher pH. Ultimately, this may cause phytoplankton to become limited by CO_2, or to become negatively affected by the high pH (Hansen, 2002; Hansen et al., 2007; Tillmann and Hansen, 2009). Such a change in carbonate chemistry may have contrasting consequences for the toxicity of *A. tamarense* as compared to elevated pCO_2, i.e. the cellular toxicity could increase (Fig. 1). On top of that, elevated pCO_2 may promote higher population densities as cells can presumably sustain growth for a longer period before becoming CO_2 limited, or before reaching their pH limit. Hence, even if their cellular toxicity is not affected, higher population densities will increase the toxicity of an *A. tamarense* bloom. Experiments with natural occurring blooms of *A. tamarense* are required in order to understand the complex interactions that exist between carbonate chemistry, phytoplankton growth, and the toxicity of HABs.

Ecological consequences of changes in PST contents and composition will depend on the role of these compounds. It has been suggested that PSTs play a role in grazer defense (Selander et al., 2012; Selander et al., 2006; Wohlrab et al., 2010), act as pheromones (Wyatt and Jenkinson, 1997), and may have physiological functions as well (Cembella, 2003). Recent findings have further suggested that PST may play a role in the maintenance of cellular ion homeostasis (Pomati et al., 2004; Soto-Liebe et al., 2012), and this putative function seems to become more pronounced under elevated pH (Pomati et al., 2004). In our study, the highest PST content was indeed associated to the treatments with highest pH, i.e. the treatment with the lowest pCO_2 (Table B.1). It remains to be determined, however, whether the observed changes in PST content are primarily due to shifts in pH or by changes in CO_2 availability.

4.4. Conclusions

Here we show that growth and elemental composition in *A. tamarense* remain largely unaltered in response to elevated pCO_2. This ability to maintain cellular homeostasis under substantial changes in carbonate chemistry appears to be achieved primarily by post-translational regulation. In both *A. tamarense* strains, cellular PST content and particularly the associated toxicity were lower in the highest CO_2 treatments. In Alex5, PST composition further shifted towards more sulfated analogues under these conditions. These CO_2-dependent changes in PST content and composition are accompanied by substantial regulation of multiple genes, including those associated to secondary metabolite and amino acid metabolism. Notably, we found that elevated pCO_2 caused an opposing regulation of sulfotransferase and sulfatase, leading to an enhanced production of less toxic sulfated PST analogues. All in all, our findings suggest that elevated pCO_2 may have minor consequences for growth and elemental composition of *A. tamarense,* yet may potentially cause a decrease in its cellular toxicity.

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Figure legends

Fig. 1. PST content and cellular toxicity of Alex5 (A,C) and Alex2 (B,D) at the different CO_2 treatments. Error bars denote standard deviation (*n*=3). Letters above bars indicate significant differences between treatments (two-way ANOVA, P<0.05).

Fig. 2. Relative composition of PST analogues grouped as non-sulfated (A,B), mono-sulfated (C,D) and di-sulfated (E,F), in Alex5 (A,C,E) and Alex2 (B,D,F) at the different CO_2 treatments. Error bars denote standard deviation (*n*=3). Letters above bars indicate significant differences between treatments of grouped toxins, as well as STX (A) and GTX1+4 (C) (one-way ANOVA, P<0.05).

Fig. 3. Venn diagram showing the total and annotated number (between brackets) of differentially expressed genes in Alex 5 (A), and the regulation of annotated genes grouped by most represented KOG categories (B). Values indicate the actual number of genes being up-regulated (↑) and down-regulated (↓) in the 180 µatm CO_2 (A, left circle; B, black bar) and 800 µatm CO_2 (A, right circle; B, white bar) treatments in comparison to the control treatment of 380 µatm CO_2. Short KOG category names refer to 'Posttranslational modification, protein turnover and chaperones [O]', 'Signal transduction mechanisms [T]', 'Inorganic ion transport and metabolism [P]', 'Carbohydrate transport and metabolism [G]', 'Lipid transport and metabolism [I]', 'Secondary metabolite biosynthesis, transport and catabolism [Q]' and 'Amino acid transport and metabolism [E]'.

Fig. 4. Schematic diagram of observed CO_2 effects on sulfate (SO_4^{2-}) assimilation in Alex5. Vertical arrows next to enzymes indicate up-regulation (↑) or down-regulation (↓) in relevant genes under elevated CO_2. After taken up by the cell, SO_4^{2-} is transported to the plastid and activated to 5'-adenylsulfate (APS). This APS can be reduced to sulfite (SO_3^{2-}) 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.