

Effects of nitrate and phosphate on grazer-induced toxin production in *Alexandrium minutum*

Erik Selander,¹ Gunnar Cervin, and Henrik Pavia

Department of Marine Ecology, Göteborg University, Tjärnö Marine Biological Laboratory, SE 452 96 Strömstad, Sweden

Abstract

Two strains of *Alexandrium minutum* were exposed to waterborne cues from copepod grazers in factorial combinations of nitrate and phosphate concentrations to evaluate the importance of grazer-induced paralytic shellfish toxin (PST) production under different nutrient regimes. In nitrate-rich treatments, the presence of waterborne cues from grazers resulted in significantly increased cell-specific PST content. In low nitrate treatments, however, waterborne cues from grazers did not result in any detectable increase in cell-specific PST content. The grazer-induced increase in cell-specific PST content in nitrate-rich treatments was comparable in size to the effects of nitrate in the absence of grazers. Effects of phosphate limitation were less consistent, resulting in increased PST content in nitrate-rich treatments in one of the *A. minutum* strains, but had no significant effect in the other. The ability of dinoflagellates to sense and respond to the presence of grazers by increased PST production may be one of the most important factors affecting cell-specific PST content under nitrogen replete conditions.

Paralytic shellfish toxins (PSTs) are a group of highly potent neurotoxic alkaloids. In the marine environment, PSTs are produced by the dinoflagellates *Gymnodinium catenatum* (Mee et al. 1986), *Pyrodinium bahamense* (Harada et al. 1982), and several species of the genus *Alexandrium* (Hansen et al. 2003). *Alexandrium* spp. are important global producers of PSTs and are routinely monitored to provide early warnings of PST contamination of shellfish. The cell-specific PST content in *Alexandrium* spp. is, however, highly variable and may differ from not detectable up to a few percent of the dry weight, both among and within species (John and Flynn 2002). Moreover, the amount of PSTs produced by a single toxic isolate may differ by an order of magnitude depending on the environmental factors it experiences (Anderson et al. 1990). Contrary to the total cell specific toxicity, the relative composition of different toxins, or toxin profile, is more stable with time (e.g., Franco et al. 1994). Several studies have shown that PST production is decreased under nitrogen-limited conditions (e.g., Leong et al. 2004). Phosphate seems to have an opposite effect to that of nitrogen, as phosphate-limited conditions generally result in high cell-specific PST content (John and Flynn 2002). Other abiotic environmental factors that affect cell-specific toxicity include irradiance (Lim et al. 2006), temperature (Anderson et al. 1990), salinity (Grzebyk et al. 2003), and shear stress (Juhl et al. 2001). In general, growth-limiting

conditions caused by factors other than nitrogen depletion or low irradiance seem to result in higher cell-specific toxicity of *Alexandrium* spp.

In a recent study, it was shown that the cell-specific PST content can also be affected by an increased risk of predation (Selander et al. 2006). In an experiment where *Alexandrium minutum* was exposed to waterborne cues from the copepod grazer *Acartia tonsa*, cell-specific PST content increased significantly compared to controls that did not receive waterborne cues from grazers. The presence of grazer-induced toxin production in *A. minutum* indicates that not only resource availability and physical stress, but also biological interactions, may be important in determining the toxicity of *Alexandrium* spp. A grazer-induced increase in production of toxic metabolites is a widespread phenomenon in primary producers (Karban and Baldwin 1997; Toth and Pavia 2007). In vascular plants, as well as in seaweeds, the allocation of resources to defensive metabolites is interactively affected by abiotic and biotic factors (Karban and Baldwin 1997; Svensson et al. 2007). It is reasonable to expect that toxin production in marine phytoplankton will show similar patterns in resource allocation. The relative importance of grazer-induced toxin production in *A. minutum* and the possible interactive effects of grazing and other extrinsic factors on toxin production are, however, still unknown. So far, grazer-induced toxin production has only been shown in one strain of *A. minutum* under nutrient replete conditions (Selander et al. 2006). Here, we present results from a factorial experiment where both nitrate and phosphate availability were manipulated, in the presence as well as absence of waterborne cues from copepod grazers, to elucidate the possible interactive effects of these factors on the cell-specific PST content of two different *A. minutum* strains. More specifically, we hypothesized that grazer-induced PST production would be higher under nitrate replete compared to nitrate limited conditions, but lower under phosphate replete compared to phosphate limited conditions.

¹ Corresponding author (Erik.Selander@marecol.gu.se).

Acknowledgments

We thank Maria Grazia Giacobbe for providing the CNR AMIA5 strain of *A. minutum*, Josephine Karlfeldt for technical assistance, Gunilla Toth, and two anonymous referees for comments on the manuscript.

Financial support was provided by the Swedish Research Council Formas through contract 21.0/2003-1122, by MARICE (Marine Chemical Ecology, an interdisciplinary research platform at the Faculty of Sciences, Göteborg University, Sweden), and by the foundations of Wählström, Carl Trygger, and Colliander.

Material and methods

Organisms—Two different strains of *A. minutum* were used for the PST induction experiment. *A. minutum* 83, synonymous with CCMP113, AI, and ALIV, isolated from Ria de Vigo on the northwest coast of Spain, was obtained from the Göteborg University Algal Culture Collection (GUMACC). *A. minutum* CNR AMI A5 (hereafter referred to as *A. minutum* A5), isolated from the Mediterranean Sea near Sicily, was provided by Dr. Maria-Grazia Giacobbe (Institute for Coastal Marine Environment, Messina, Italy). *A. minutum* 83 cells were slightly larger (equivalent spherical diameter [ESD] $19.9 \pm 1.7 \mu\text{m}$ mean \pm SD) than *A. minutum* A5 cells (ESD $16.2 \pm 2.3 \mu\text{m}$). Cultures were reared on K medium (Leftley et al. 1987) at $150 \mu\text{mol m}^{-2} \text{s}^{-1}$ fluorescent light (14 : 10 light : dark [LD] cycles), 18°C , and reinoculated every second week.

The calanoid copepod *Acartia tonsa*, previously shown to induce PST production in *A. minutum* (Selander et al. 2006), was used as the grazer in the induction experiments. The *A. tonsa* culture was originally provided by the National Environmental Research Institute (NERI) in Denmark.

Nutrient treatment—To be able to manipulate the nutrient concentration, we carried out the experiments in low nutrient filtered seawater (LNSW) collected in the offshore northeast Atlantic Ocean (Ocean Scientific International Ltd). To reduce nutrient carryover, the *A. minutum* cultures were washed by reducing the culture volume (>50%) through a $10\text{-}\mu\text{m}$ plankton mesh and replacing it with LNSW. The procedure was repeated six times. The washed cultures were diluted with LNSW to a final concentration of 3,800 cells mL^{-1} and 4,300 cells mL^{-1} in *A. minutum* 83 and A5, respectively, determined with a particle counter (Elzone 180 XY). Each of the *A. minutum* cultures were divided between four bottles and supplemented with nitrate and phosphate corresponding to the following four nutrient regimes: Low nitrate ($0.5 \mu\text{mol L}^{-1}$) and low phosphate ($0.03 \mu\text{mol L}^{-1}$), nitrate to phosphate ratio (N:P) = 16; low nitrate ($0.5 \mu\text{mol L}^{-1}$) and high phosphate ($6.3 \mu\text{mol L}^{-1}$) N:P < 0.1; high nitrate ($100 \mu\text{mol L}^{-1}$) and low phosphate ($0.03 \mu\text{mol L}^{-1}$) N:P > 3,000; and high nitrate ($100 \mu\text{mol L}^{-1}$) and high phosphate ($6.3 \mu\text{mol L}^{-1}$) N:P = 16. All experiments received trace metals and vitamins corresponding to K medium (Leftley et al. 1987) diluted ten times. The high nutrient treatment corresponds to the upper extreme of naturally occurring nutrient concentrations, but is below the levels that cause experimental artifacts (John and Flynn 2002). To account for any nutrient carryover from cultures, start samples were taken from each treatment, filtered, and stored frozen until analysis.

Waterborne cues from grazers—Each of the four nutrient regimes with the same concentration of *A. minutum* cells was divided between eight identical experimental beakers, each containing a cage made from polypropylene tubes with a heat-sealed nylon-mesh bottom (mesh size $10 \mu\text{m}$). The beakers received 70 mL of the culture, and the cage received 30 mL. Two copepods (adult female *A. tonsa*) were added to

half of the cages (i.e., $n = 4$). With the experimental setup used, *A. minutum* cells kept outside the cages received waterborne cues from the contents inside the cages (Selander et al. 2006), whereas both copepods and dinoflagellates were restricted to their original compartment by the mesh in the bottom of the cages.

All flasks were then incubated in a light- and temperature-controlled incubator at $150 \mu\text{mol m}^{-2} \text{s}^{-1}$ fluorescent light (14 : 10 LD cycles) at 18°C . The experiment with *A. minutum* A5 was terminated after 3 days, and the experiment with *A. minutum* 83 terminated after 5 days. At the termination of the experiments, cages were removed from the glass bottles and the content of each compartment (outside and inside the cages) was transferred through careful rinsing with filtered seawater to clean beakers. The volume and algal cell concentration of the sample were determined, and the number of cells in each compartment was calculated. The number of cells in each compartment was divided by the original volume of the compartment (70-mL outside cages, and 30-mL inside cages) to get cell abundance in cells mL^{-1} . Five milliliters of each sample from outside the cages (i.e., the cultures receiving waterborne cues) were removed, filtered ($0.45 \mu\text{m}$), and frozen for analysis of dissolved inorganic nutrients (see Chemical analyses section). A known number of cells was filtered onto 25-mm glass fiber filters (GF/A) for toxin analysis. Filters were lyophilized and stored at -80°C until toxin extraction was performed (see Chemical analyses section).

Analysis—Chemical analyses: The concentration of ammonium, nitrate, and phosphate was quantified with a continuous-flow automated system (TRAACS 800, Bran-Luebbe) according to standard colorimetric procedures (Strickland and Parsons 1972). PST samples were extracted with 1.5 mL 0.05 mol L^{-1} acetic acid (aq) through four consecutive freeze-thaw cycles. The extract was filtered (GF/A) and frozen (-80°C) in glass vials until analysis with high-performance liquid chromatography (HPLC) with fluorescence detection (Asp et al. 2004). HPLC analyses were done on a Hitachi-7000 system equipped with a RP8 Column (Supelco Discovery C8, $5 \mu\text{m}$, 150 mm, inner = 4.6 mm). PST standards (saxitoxin, decarbamoylsaxitoxin, neosaxitoxin, and gonyautoxin [GTX] 1–4) were obtained from the certified reference material program of the National Research Council Canada, Halifax, Canada. *A. minutum* 83 is only known to produce GTX 1–4 (Selander et al. 2006). To exclude the possible presence of N-sulfocarbamoyl toxins (C-toxins), a subset of the toxin samples was hydrolyzed and analyzed for changes in corresponding carbamate toxin concentrations. Because only GTX 1–4 were detected, an isocratic elution with 50 mmol L^{-1} ammonium-phosphate buffer (pH 7.1) and 2 mmol L^{-1} sodiumheptanesulfonate at 0.8 mL min^{-1} was used to separate the toxins. After the separation, toxins were oxidized with 7 mmol L^{-1} periodic acid in 50 mmol L^{-1} sodium phosphate buffer (pH 9.0, 0.4 mL min^{-1}) in a polyetheretherketone (PEEK) capillary (10 m, inner 0.25 mm , 80°C). The oxidation was terminated with 0.5 mol L^{-1} acetic acid (0.4 mL min^{-1}) before fluorescent detection at $\lambda_{\text{ex}} = 330 \text{ nm}$, $\lambda_{\text{em}} = 390 \text{ nm}$.

Table 1. Concentration of dissolved nitrate, ammonium, and phosphate at the start and end of the experiments with *A. minutum* 83 and *A. minutum* A5 presented as mean values of four replicates \pm standard error of mean.

Strain ID	Nutrient enrichment	Grazing	NO ₂ +NO ₃ ($\mu\text{mol L}^{-1}$)	NH ₄ ($\mu\text{mol L}^{-1}$)	PO ₄ ($\mu\text{mol L}^{-1}$)
83	control	start	2.91 \pm 0.17	1.27 \pm 0.05	0.25 \pm 0.02
		control	0.10 \pm 0.10	2.44 \pm 1.44	0.08 \pm 0.05
		grazed	0.03 \pm 0.02	1.25 \pm 0.06	0.05 \pm 0.01
	phosphate	start	2.21 \pm 0.48	0.87 \pm 0.21	5.20 \pm 0.47
		control	0.02 \pm 0.03	2.05 \pm 1.12	4.51 \pm 0.19
		grazed	0.04 \pm 0.04	1.16 \pm 0.01	4.47 \pm 0.34
	nitrate	start	112.8 \pm 5.45	1.02 \pm 0.01	0.17 \pm 0.01
		control	2.98 \pm 4.69	2.05 \pm 1.13	0.06 \pm 0.06
		grazed	3.51 \pm 2.26	1.19 \pm 0.12	0.02 \pm 0.02
	nitrate and phosphate	start	86.79 \pm 15.14	1.22 \pm 0.29	5.62 \pm 0.38
		control	0.93 \pm 0.02	1.43 \pm 0.10	1.54 \pm 0.23
		grazed	1.10 \pm 0.10	2.54 \pm 1.48	1.52 \pm 0.63
A5	control	start	4.04 \pm 0.06	1.15 \pm 0.03	0.35 \pm 0.00
		control	0.16 \pm 0.02	1.56 \pm 1.90	0.12 \pm 0.02
		grazed	0.03 \pm 0.03	1.44 \pm 1.20	0.09 \pm 0.02
	phosphate	start	3.27 \pm 0.5	0.95 \pm 0.2	5.28 \pm 0.11
		control	0.32 \pm 0.33	1.96 \pm 1.80	0.36 \pm 0.20
		grazed	0.04 \pm 0.06	1.14 \pm 1.19	0.16 \pm 0.04
	nitrate	start	114.5 \pm 1.38	0.82 \pm 0.11	0.28 \pm 0.02
		control	46.57 \pm 15.78	0.60 \pm 0.38	0.17 \pm 0.08
		grazed	64.93 \pm 6.83	2.45 \pm 1.66	0.14 \pm 0.05
	nitrate and phosphate	start	99.65 \pm 5.86	1.86 \pm 0.47	6.00 \pm 0.47
		control	5.93 \pm 4.13	1.75 \pm 1.94	0.83 \pm 0.22
		grazed	12.23 \pm 7.47	2.05 \pm 1.74	0.32 \pm 0.02

Statistical analysis: The effect of phosphate, nitrate, and waterborne cues from grazers on cell-specific toxin content and cell abundance at the end of the experiments was tested with a three-factor analysis of variance (ANOVA) (Underwood 1997). A Student Neuman Keul (SNK) post hoc procedure was conducted to detect significant differences between means. Cochran's C test was performed on all data before analysis. Heterogeneous variances ($p < 0.01$) were detected only in cell abundance data from the outside cage compartment for experiments with *A. minutum* A5 (Cochran's C = 0.61, C_{crit} 8,3 = 0.52). ANOVA performed on cell abundance data from *A. minutum* A5, however, did not produce any significant differences between treatments; hence, the risk for making type 1 errors was not increased and data were not transformed before analysis (Underwood 1997).

Results

Nutrients—Dissolved inorganic nutrients at the end of the experiment (Table 1) reflected the initial treatments, with higher nitrate levels in nitrate-enriched treatments and higher phosphate concentrations in phosphate-enriched treatments. Substantially more nitrate remained in nitrate-enriched treatments in the shorter experiment with *A. minutum* A5 as compared to the experiment with *A. minutum* 83. On the contrary, phosphate enrichment was more marked at the end of the longer experiment with *A. minutum* 83 (Table 1). Despite the fact that copepods are partly ammonotelic (Miller and Glibert 1998), the ammonium concentration was not significantly higher in grazed treatments.

Cell abundance—In the experiment with *A. minutum* A5, the different treatments did not result in significantly different growth rates outside cages (Table 2; Fig. 1). In the experiment with *A. minutum* 83, however, there was an interaction between the factors phosphate and nitrate (ANOVA $F_{1,24} = 34.5$, $p < 0.001$). Post hoc comparisons of means revealed that all combinations of nitrate and phosphate resulted in significantly (SNK $p < 0.05$) different cell abundance at the end of the experiment. Most cells formed in the combined nitrate and phosphate enrichment, followed by the nitrate enrichment, controls and finally phosphate enrichment treatment which produced the lowest cell abundance. The presence of copepods did not have any significant effect on the growth rate of *A. minutum* outside cages (Table 2). The significant effects of nutrient treatments on growth rate in *A. minutum* 83 indicate that nutrient limitation was more pronounced for this strain compared to *A. minutum* A5.

Inside the cages, cell abundance was generally lower in cages that contained copepod grazers (Table 2, Fig. 1). In the experiment with *A. minutum* 83, however, copepod grazers only had a significant negative effect on cell abundance in cages from high nitrate treatments (SNK $p < 0.05$), whereas the cell abundance was not significantly different in low nitrate treatments with or without copepods (SNK $p < 0.71$; Fig. 1). Thus, grazing was more extensive in the nitrate-rich treatments in the experiment with *A. minutum* 83. In contrast, there were no detectable interactive effects between the presence of grazers and nutrient regime on cell abundance in *A. minutum* A5; instead, grazing had a general negative effect on cell abundance (Table 2; Fig. 1) in this experiment.

Table 2. Results from the ANOVA analysis of cell abundance (cells mL⁻¹) outside and inside cages at the end of the experiment with *A. minutum* 83 and *A. minutum* A5. Significant results are indicated in bold. Factor abbreviations as follows: G, Grazer presence; N, nitrate concentration; and P, phosphate concentration. Cochran's C is shown for each ANOVA, and *F* and *p* values based on data with heterogeneous variance are indicated in italics.

Strain	Source	Outside cages		Inside cages	
		<i>F</i> _{1,24}	<i>p</i>	<i>F</i> _{1,24}	<i>p</i>
83	G	0.6	0.461	4.1	0.053
	N	101.7	>0.001	10.1	0.004
	P	6.5	0.018	1.3	0.264
	G*N	2.2	0.15	4.3	0.049
	G*P	0.2	0.626	0.4	0.543
	N*P	34.5	>0.001	2.7	0.111
	G*N*P	0.9	0.363	0.041	0.842
	Cochran C (<i>C</i> _{crit} = 0.52)	0.24		0.26	
A5	G	4	0.058	21.4	>0.001
	N	0.3	0.572	<0.001	0.976
	P	0.2	0.664	0.4	0.541
	G*N	0	0.947	0.2	0.696
	G*P	0	0.887	3.6	0.070
	N*P	0.3	0.584	1.3	0.262
	G*N*P	1.7	0.199	0.4	0.533
	Cochran C (<i>C</i> _{crit} = 0.52)	0.60		0.24	

Cell-specific toxicity—The most obvious results on cell-specific toxicity for both *A. minutum* strains were the 5–15 times higher cell-specific PST content under nitrate replete compared to nitrate limited conditions and the strong PST-

enhancing effects of waterborne grazer cues under nitrate replete conditions (Fig. 2). ANOVA analysis, however, revealed a significant three-way interactive effect between nitrate, phosphate, and waterborne cues from grazers in the

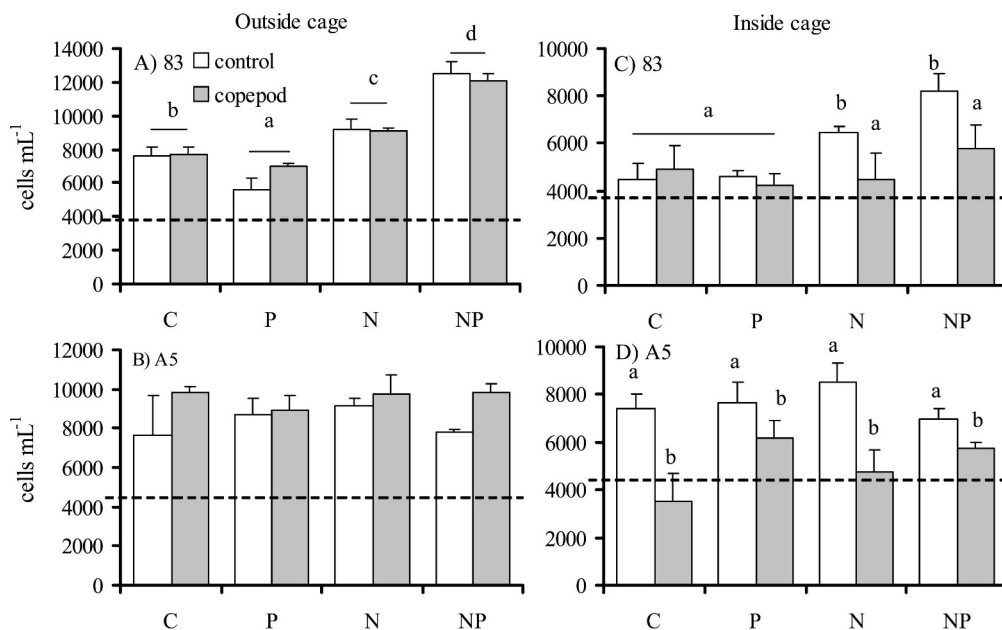


Fig. 1. (A, B) Cell abundance (cells mL⁻¹) at the end of the experiment outside cages and (C, D) inside cages. Panels A and C show the experiment with *A. minutum* 83; Panels B and D show the experiment with *A. minutum* A5. Grey bars indicate treatments with grazers and white bars treatments without grazers. Category axis labels represent nutrient treatments: C corresponds to controls with low phosphate and nitrate P to phosphate-enriched treatments, N to nitrate-enriched treatments, and NP to nitrate- and phosphate-enriched treatments. The hatched horizontal lines show the cell concentration at the start of the experiment: 3,800 cells mL⁻¹ for *A. minutum* 83 and 4,300 cells mL⁻¹ for *A. minutum* A5. Letters above bars indicate statistically homogenous subsets based on the SNK post hoc procedure, except for panel B where no significant differences were detected. Bars denote mean values of four replicates. Error bars show standard error of mean.

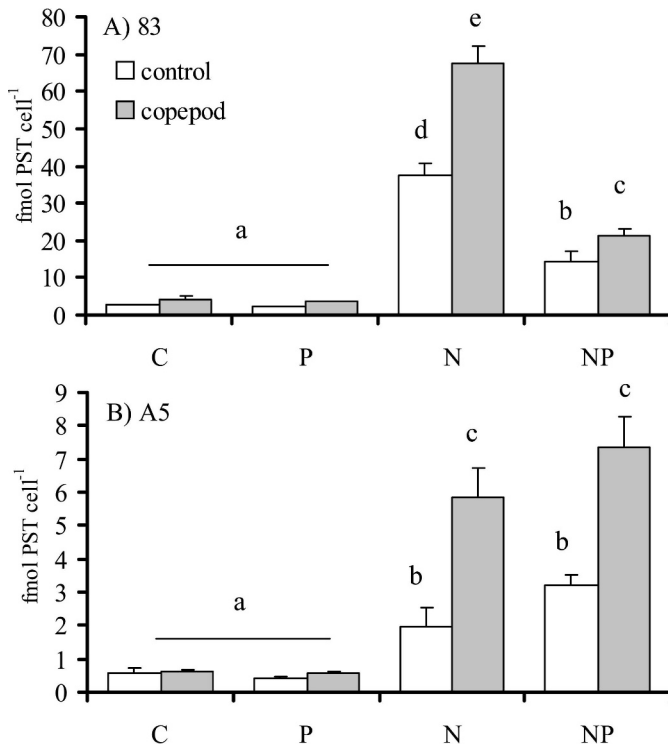


Fig. 2. Cell-specific PST content (fmol cell⁻¹) outside cages from the experiments with (A) *A. minutum* 83 and (B) *A. minutum* A5, respectively. Grey bars indicate treatments that received waterborne cues from grazers and white bars treatments without waterborne cues from grazers. Category axis labels represent nutrient treatments: C corresponds to controls with low phosphate and nitrate P to phosphate-enriched treatments, N to nitrate-enriched treatments, and NP to nitrate- and phosphate-enriched treatments. Letters above bars indicate statistically homogenous subsets based on the SNK post hoc procedure. Bars denote mean values of four replicates. Error bars show standard error of mean.

experiment with *A. minutum* 83 and a significant interactive effect between nitrate and waterborne cues from grazers in the experiment with *A. minutum* A5 (Table 3). Post hoc comparisons of means showed that in addition to the significantly higher cell-specific PST content in nitrate-rich treatments as compared to nitrate-depleted treatments in both experiments ($p < 0.05$), waterborne cues from grazers consistently induced significantly increased cell-specific PST content in nitrate-rich treatments ($p < 0.05$), but not in nitrate-depleted treatments in any of the strains (*A. minutum* 83 $p = 0.93$, *A. minutum* A5 $p = 0.85$). Phosphate limitation further increased the cell-specific toxin content in nitrate-rich treatments in the experiment with *A. minutum* 83 ($p < 0.05$), but had no significant effect on toxicity in the experiment with *A. minutum* A5 (Table 3).

The toxin profile was similar in both experiments and was dominated (>94%) by GTX 4 in all treatments. Small amounts (<6%) of GTX 1 were present in all samples, whereas GTX 2 and 3 contributed a minor part (<1%) of the total toxin content.

Table 3. Results from ANOVA analysis of cell-specific PST content in *A. minutum* 83 and *A. minutum* A5 outside cages. Factor abbreviations as follows: G, waterborne cues from grazers; N, nitrate concentration; and P, phosphate concentration. Significant results are indicated in bold. Cochran's C is shown for each ANOVA table and indicates homogenous variances ($C_{crit} = 0.52$).

Strain	Source	$F_{1,24}$	p
83	G	34.9	>0.001
	N	356.9	>0.001
	P	107.3	>0.001
	G*N	25.5	>0.001
	G*P	11.1	0.003
	N*P	100.8	>0.001
	G*N*P	11.1	0.003
	Cochran C	0.51	
A5	G	33.9	>0.001
	N	130.8	>0.001
	P	3.2	0.084
	G*N	30.7	>0.001
	G*P	0.1	0.808
	N*P	4.1	0.054
	G*N*P	<0.01	0.917
	Cochran C	0.42	

Discussion

The present study demonstrates strong interactive effects of nitrate availability and waterborne cues from grazers on the cell-specific toxicity of *A. minutum* and implies that the magnitude of grazer-induced PST production in *A. minutum* is directly proportional to the degree of nitrogen availability. Waterborne cues from grazers resulted in a significant and substantial increase in cell-specific PST content in the high, but not in the low, nitrate treatments in both *A. minutum* strains tested (83 and A5). Nitrogen availability has repeatedly been shown to be one of the most significant extrinsic factors affecting the cell-specific PST content of *Alexandrium* spp. For example, some earlier studies report that low nitrogen availability results in a low cell-specific PST content in *Alexandrium fundyense* (John and Flynn 2000) as well as *Alexandrium tamarense* (Leong et al. 2004), whereas nitrate addition increases PST production in nitrate deprived *A. minutum* (Flynn et al. 1994). The results of this study support the notion that nitrogen availability is a crucial factor for PST production rate in dinoflagellates, and further suggests that waterborne cues from grazers can be as important as nitrogen availability in determining the cell-specific toxicity of *A. minutum*. Because *A. tamarense* cells cultured with ammonium have been reported to produce significantly more PST than *A. tamarense* cultured with nitrate (Leong et al. 2004), the ammonium exudates from copepods could potentially explain the difference in increased toxicity in copepod treatments. This is, however, not supported by nutrient data, which showed no elevated ammonium concentrations in copepod treatments (Table 1).

The effects of phosphate were less consistent in comparison to the effects of nitrate availability on PST levels. The inverse relationship between cell-specific toxicity

and phosphate concentration previously reported for *A. minutum* (Guisande et al. 2002; Frangopulos et al. 2004), *A. tamarensis* (Taroncher-Oldenburg et al. 1999), and *A. fundyense* (Anderson et al. 1990), was confirmed in the experiments with *A. minutum* 83, but not with *A. minutum* A5. It should, however, be noted that phosphate limitation was more pronounced in the somewhat longer experiment with *A. minutum* 83, as indicated by the decreased growth rate and phosphate concentration at the end of the experiment. Taroncher-Oldenburg and coworkers (1999) showed that PST toxins are formed in the G1 phase of the cell cycle, and they attributed increased cell-specific PST content in phosphate- and temperature-limited conditions to the prolonged G1 phase. In this study, the prolonged G1 phase was probably only present in the experiment with *A. minutum* 83, where phosphate limitation resulted in decreased growth. This may explain the different effect of phosphate availability on toxin concentration in *A. minutum* 83 as compared to A5. The lack of growth reduction that was observed with *A. minutum* A5 despite the low phosphate concentration indicates that the affinity constant for phosphate uptake may be lower than the 1.16 mol L^{-1} previously suggested for *A. minutum* (Frangopulos et al. 2004). The cellular content of C:N:P was, however, not measured, and it is therefore possible that the phosphate exhaustion of the medium had not yet resulted in a corresponding depletion of cellular nutrient content.

Two influential models that have been proposed to explain and predict spatial and temporal variation in the concentration of secondary metabolites in terrestrial plants are the carbon nutrient balance model (CNBM) and the optimal defense model (ODM), (see Stamp 2003). The CNBM is a resource- or supply-based model stating that allocation of resources to the production of secondary metabolites is determined by the relative availability of carbon and nutrients (Bryant et al. 1983). In contrast to the CNBM, the ODM and its corollary induced defense model (IDM) are demand-based models, which state that the amount of resources invested in defense metabolites will be directly proportional to the probability of attack (Rhoades 1979). These models have been explicitly addressed in several studies on marine macroalgae (Cronin and Hay 1996; Pavia et al. 2002; Toth et al. 2005), but only rarely tested or discussed in experiments on marine microalgae or toxic algal blooms. The strong correlation between nitrogen availability and cell-specific PST content observed in this study is in accordance with what would be predicted for nitrogen-rich secondary metabolites by CNBM (Bryant et al. 1983). PSTs are alkaloids that are rich in nitrogen (C:N \approx 1.4) compared to the average C:N ratio of marine phytoplankton (6.6) (Redfield 1958). Thus, it is reasonable to assume that nitrogen rather than carbon is limiting for production of PSTs in *A. minutum*. Furthermore, the rapid and substantial increase in PST levels of both *A. minutum* strains in response to waterborne grazer cues suggests that the secondary metabolism of dinoflagellates is not only dependent on resource availability, as predicted by the CNBM, but also on the risk of predation. Therefore, the results of the present study suggest that both the ODM and the CNBM are relevant, in an interactive manner, to

explain phenotypic variation in the PST content of *A. minutum* cells.

Two important assumptions behind the ODM are that secondary metabolites function as chemical defenses and that their production is costly in terms of producer fitness (Rhoades 1979). A common and potentially useful method to assess the costs of defenses is to correlate the levels of defensive traits with a fitness estimate, commonly growth rate (Karban and Baldwin 1997). In this study, the growth rate of the more toxic *A. minutum* cells outside cages with grazing copepods was not significantly reduced compared to *A. minutum* cells not induced by copepods (Fig. 1), which is in agreement with previous attempts to evaluate the cost of PST formation (John and Flynn 2002). This result suggests that the cost inflicted by grazer-induced toxin production in *A. minutum* is low, at least in terms of growth rate under the experimental nutrient regimes. The costs of inducible defenses are, however, not necessarily reflected in decreased growth rate in short-term laboratory experiments (Tollrian and Harvell 1999). Furthermore, the expression of defensive traits may be associated with other costs, e.g., ecological costs arising from conflicting selective pressures, than direct resource allocation costs that are reflected in decreased growth. For example, the freshwater protozoan genus *Scenedesmus* spp. forms colonies in response to waterborne cues from grazers (Hessen and Van Donk 1993), and the colonies appear to be protected from grazing by their larger size (Lurling and Van Donk 1996). No direct cost in terms of decreased growth was found to be associated with colony formation in laboratory batch cultures, but colonies had higher settling velocities compared to single cells, and the higher settling velocity was suggested to inflict a cost in nature because colonies will sink faster to deeper water and thereby experience lower irradiance and temperature as compared to that of single cells (Lurling and Van Donk 2000). In a similar way, it is possible that the grazer-induced increased production of PST in *A. minutum* is associated with more subtle costs that are not detectable in laboratory batch cultures.

There was an overall negative effect of grazer presence on the number of *A. minutum* A5 cells inside the cages, indicating that the copepods were feeding on this strain irrespective of the nutrient regime under which it was grown. Furthermore, there was a significant negative effect of grazer presence on the number of *A. minutum* strain 83 in the high nitrate treatments. Assuming that PST production was induced also in the *A. minutum* cells directly grazed by copepods inside the cages, no significant negative correlation between PST content and grazing was found. This is contrary to what the hypothesized role of PSTs as an inducible defense would predict. However, the grazing experiment in the present study was conducted as a no-choice experiment (i.e., the grazers were only offered one type of food), which does not provide relevant data on the feeding preferences of grazers. In more natural experiments, where grazers were offered alternative prey, copepods tended to discriminate against PST containing prey (Teegarden 1999; Guisande et al. 2002; Selander et al. 2006; but see Colin and Dam 2003). The low grazing rates in nitrate-limited treatments could potentially explain the

absence of induction in these treatments. However, in a previous study it was shown that scents from both feeding and starving copepods induce PST formation in *A. minutum* (Selander et al. 2006), and it is therefore not likely that the different ingestion rates contributed significantly to the outcome of the present experiment. Guisande and co-workers (2002) suggested that PSTs may be produced in phosphate-limited conditions in order to redirect the grazing pressure toward alternative non-toxic competitors, compensating PST-producing species for being poor competitors for available phosphate. The current investigation shows that this mechanism, if operational, is not necessarily restricted to phosphate-limited conditions. If resistance to grazers is correlated to cell-specific PST content in *A. minutum*, the level of defense is probably more dependent on the concentration of available nitrogen and the presence of selective grazers (Teegarden et al. 2001). It should, however, be pointed out that the putative grazer deterrent role of PSTs has not been unequivocally tied to the PSTs. Other traits of dinoflagellate cells, which correlate to the PST content, could potentially confound results of feeding experiments (Selander et al. 2006).

In summary, we have shown that both nitrate and waterborne cues from grazing copepods increase the cell-specific PST content of *A. minutum* and that there is a strong interactive effect of these factors on toxin production. Consequently, grazer-induced PST production will be most significant when *A. minutum* with high intrinsic capacity for toxin production grow in a nitrate-rich environment, whereas the effect of waterborne cues from grazers on cell-specific toxicity will probably be small or absent in nitrogen-limited environments. The presence of strong grazer-induced PST production in both *A. minutum* 83 and *A. minutum* A5, together with previous data on the deterrent effect of PST containing food (Teegarden 2001; Guisande 2002; Selander et al. 2006), indicate that these toxins can function as an inducible chemical defense against grazing. Based on the existing information from laboratory experiments, it is reasonable to hypothesize that grazer-induced PST production can contribute to the formation of harmful algal blooms by redirecting grazing pressure to less toxic species under nitrogen replete conditions, although this remains to be tested under natural conditions.

References

- ANDERSON, D. M., D. M. KULIS, J. J. SULLIVAN, S. HALL, AND C. LEE. 1990. Dynamics and physiology of saxitoxin production by the dinoflagellates *Alexandrium* spp. *Mar. Biol.* **104**: 511–524.
- ASP, T. N., S. LARSEN, AND T. AUNE. 2004. Analysis of psp toxins in Norwegian mussels by a post-column derivatization HPLC method. *Toxicon* **43**: 319–327.
- BRYANT, J. P., F. S. CHAPIN, AND D. R. KLEIN. 1983. Carbon/nutrient balance of boreal plants in relation to vertebrate herbivory. *Oikos* **40**: 357–368.
- COLIN, S. P., AND H. G. DAM. 2003. Effects of the toxic dinoflagellate *Alexandrium fundyense* on the copepod *Acartia hudsonica*: A test of the mechanisms that reduce ingestion rates. *Mar. Ecol.-Prog. Ser.* **248**: 55–65.
- CRONIN, G., AND M. E. HAY. 1996. Within plant variation in seaweed palatability and chemical defenses: Optimal defense theory versus the growth differentiation balance hypothesis. *Oecologia* **105**: 361–368.
- FLYNN, K., J. M. FRANCO, P. FERNANDEZ, B. REGUERA, M. ZAPATA, G. WOOD, AND K. J. FLYNN. 1994. Changes in toxin content, biomass and pigments of the dinoflagellate *Alexandrium minutum* during nitrogen refeeding and growth into nitrogen or phosphorus stress. *Mar. Ecol.-Prog. Ser.* **111**: 99–109.
- FRANCO, J. M., P. FERNANDEZ, AND B. REGUERA. 1994. Toxin profiles of natural populations and cultures of *Alexandrium minutum* from galician (Spain) coastal waters. *J. Appl. Phycol.* **6**: 275–279.
- FRANGOPULOS, M., C. GUISANDE, E. DEBLAS, AND I. MANEIRO. 2004. Toxin production and competitive abilities under phosphorus limitation of *Alexandrium* species. *Harmful Algae* **3**: 131–139.
- GRZEBYK, D., C. BECHEMIN, C. J. WARD, C. VERITE, G. A. CODD, AND S. Y. MAESTRINI. 2003. Effects of salinity and two coastal waters on the growth and toxin content of the dinoflagellate *Alexandrium minutum*. *J. Plankton Res.* **25**: 1185–1199.
- GUISANDE, C., M. FRANGOPULOS, I. MANEIRO, A. R. VERGARA, AND I. RIVEIRO. 2002. Ecological advantages of toxin production by the dinoflagellate *Alexandrium minutum* under phosphorus limitation. *Mar. Ecol.-Prog. Ser.* **225**: 169–176.
- HANSEN, G., N. DAUGBJERG, AND J. M. FRANCO. 2003. Morphology, toxin composition and LSU rDNA phylogeny of *Alexandrium minutum* (dinophyceae) from Denmark, with some morphological observations on other European strains. *Harmful Algae* **2**: 317–335.
- HARADA, T., Y. OSHIMA, H. KAMIYA, AND T. YASUMOTO. 1982. Confirmation of paralytic shellfish toxins in the dinoflagellate *Pyrodinium bahamense* var. *compressa* and bivalves in palau. *Bull. Jap. Soc. Sci. Fish.* **48**: 821–825.
- HESSEN, D. O., AND E. VAN DONK. 1993. Morphological-changes in *Scenedesmus* induced by substances released from *Daphnia*. *Archiv Fur Hydrobiologie* **127**: 129–140.
- JOHN, E. H. 2002. Modelling changes in paralytic shellfish toxin content of dinoflagellates in response to nitrogen and phosphorus supply. *Mar. Ecol.-Prog. Ser.* **225**: 147–160.
- , AND K. J. FLYNN. 2000. Growth dynamics and toxicity of *Alexandrium fundyense* (dinophyceae): The effect of changing N:P supply ratios on internal toxin and nutrient levels. *Eur. J. Phycol.* **35**: 11–23.
- JUHL, A. R., V. L. TRAINER, AND M. I. LATZ. 2001. Effect of fluid shear and irradiance on population growth and cellular toxin content of the dinoflagellate *Alexandrium fundyense*. *Limnol. Oceanogr.* **46**: 758–764.
- KARBAN, R., AND I. T. BALDWIN. 1997. Induced responses to herbivory. Univ. Chicago Press.
- LEFTLEY, J. W., D. K. KELLER, R. C. SELVIN, W. CLAUS, AND R. R. L. GUILLARD. 1987. Media for the culture of oceanic ultraphytoplankton. *J. Phycol.* **23**: 633–638.
- LEONG, S. C. Y., A. MURATA, Y. NAGASHIMA, AND S. TAGUCHI. 2004. Variability in toxicity of the dinoflagellate *Alexandrium tamarense* in response to different nitrogen sources and concentrations. *Toxicon* **43**: 407–415.
- LIM, P. T., C. P. LEAW, G. USUP, A. KOBIYAMA, K. KOIKE, AND T. OGATA. 2006. Effects of light and temperature on growth, nitrate uptake, and toxin production of two tropical dinoflagellates: *Alexandrium tamiyavanichii* and *Alexandrium minutum* (dinophyceae). *J. Phycol.* **42**: 786–799.
- LURLING, M., AND E. VAN DONK. 1996. Zooplankton-induced unicell-colony transformation in *Scenedesmus acutus* and its effect on growth of herbivore daphnia. *Oecologia* **108**: 432–437.

- , AND ———. 2000. Grazer-induced colony formation in *Scenedesmus*: Are there costs to being colonial? *Oikos* **88**: 111–118.
- MEE, L. D., M. ESPINOSA, AND G. DIAZ. 1986. Paralytic shellfish poisoning with a *Gymnodinium catenatum* red tide on the pacific coast of Mexico. *Mar. Env. Res.* **19**: 77–92.
- MILLER, C. A., AND P. M. GLIBERT. 1998. Nitrogen excretion by the calanoid copepod *Acartia tonsa*: Results of mesocosm experiments. *J. Plankton Res.* **20**: 1767–1780.
- PAVIA, H., G. B. TOTH, AND P. ABERG. 2002. Optimal defense theory: Elasticity analysis as a tool to predict intraplant variation in defenses. *Ecology* **83**: 891–897.
- REDFIELD, A. C. 1958. The biological control of chemical factors in the environment. *American Scientist* **46**: 205–221.
- RHOADES, D. F. 1979. Evolution of plant chemical defense against herbivores. Academic Press.
- SELANDER, E., P. THOR, G. B. TOTH, AND H. PAVIA. 2006. Copepods induce paralytic shellfish toxin production in marine dinoflagellates. *Proc. R. Soc. Lond. Ser. B-Biol. Sci.* **273**: 1673–1680.
- STAMP, N. 2003. Out of the quagmire of plant defense hypotheses. *Q. Rev. Biol.* **78**: 23–55.
- STRICKLAND, J. D. H., AND T. R. PARSONS. 1972. A practical handbook for seawater analysis. 2nd edition. Fish. Res. Bd. Canada.
- SVENSSON, C. J., H. PAVIA, AND G. TOTH. 2007. Do plant density, nutrient availability, and herbivore grazing interact to affect phlorotannin plasticity in the brown seaweed *Ascophyllum nodosum*. *Mar. Biol.* **151**: 2177.
- TARONCHER-OLDENBURG, G., D. M. KULIS, AND D. M. ANDERSON. 1999. Coupling of saxitoxin biosynthesis to the g(1) phase of the cell cycle in the dinoflagellate *Alexandrium fundyense*: Temperature and nutrient effects. *Natural Toxins* **7**: 207–219.
- TEEGARDEN, G. J. 1999. Copepod grazing selection and particle discrimination on the basis of psp toxin content. *Mar. Ecol.-Prog. Ser.* **181**: 163–176.
- , R. G. CAMPBELL, AND E. G. DURBIN. 2001. Zooplankton feeding behavior and particle selection in natural plankton assemblages containing toxic *Alexandrium* spp. *Mar. Ecol.-Prog. Ser.* **218**: 213–226.
- TOLLRIAN, R., AND C. D. HARVELL. 1999. The ecology and evolution of inducible defences. Princeton Univ. Press.
- TOTH, G. B., O. LANGHAMER, AND H. PAVIA. 2005. Inducible and constitutive defenses of valuable seaweed tissues: Consequences for herbivore fitness. *Ecology* **86**: 612–618.
- , AND H. PAVIA. 2007. Induced herbivore resistance in seaweeds: A meta-analysis. *J. Ecol.*, **95**: 425–434.
- UNDERWOOD, A. J. 1997. Experiments in ecology: Their logical design and interpretation using analysis of variance. Cambridge Univ. Press.

Received: 20 February 2007

Accepted: 1 September 2007

Amended: 30 October 2007