Limnol. Oceanogr., 56(3), 2011, 829–840

doi:10.4319/lo.2011.56.3.0829

Effects of changing pCO_2 and phosphate availability on domoic acid production and physiology of the marine harmful bloom diatom *Pseudo-nitzschia multiseries*

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Abstract

Some members of the diatom genus *Pseudo-nitzschia* produce the toxin domoic acid (DA), which through trophic transfer causes mass mortalities of wildlife, shellfish harvesting closures, and risks to human health. Nutrient and micronutrient limitation have been shown to regulate DA production. This study tested the hypothesis that changing partial pressure of CO_2 (pCO_2) can interact with nutrient limitation to help determine cellular DA levels, an environmentally relevant issue in light of current increases in atmospheric pCO_2. Cultures of the toxic species *Pseudo-nitzschia multiseries* were incubated using semicontinuous methods under a matrix of three pCO_2 conditions: ~ 22 Pa (220 ppm), ~ 41 Pa (400 ppm), and ~ 74 Pa (730 ppm), and two phosphate concentrations: 20 μmol L^{-1}, P-replete; and 0.5 μmol L^{-1}, P-limited. DA production was regulated by both pCO_2 and phosphate availability. DA concentrations were 30–50 times higher in P-limited cultures compared to P-replete ones, at the same pCO_2 levels. Increasing CO_2 levels stimulated DA production under both nutrient conditions, but especially in P-limited cultures, where DA levels increased approximately four times over the pCO_2 range examined. Growth rates, primary productivity, photosynthesis vs. irradiance parameters, and cellular elemental ratios also responded interactively to the availability of both CO_2 and phosphate. Our results raise the possibility that growth rates and toxicity of the diatom *Pseudo-nitzschia multiseries* could increase substantially in the future high-CO_2 ocean, suggesting a potentially escalating negative effect of this harmful algal bloom species on the future marine environment.

Harmful algal blooms have major negative effects on human and ecosystem health and on coastal economies. Worldwide, thousands of people are sickened or killed each year as the result of ingesting algal toxins, mostly through consumption of contaminated seafood (Anderson et al. 2002). Some species of the planktonic diatom genus *Pseudo-nitzschia* produce the neurotoxin domoic acid (DA), which causes amnesiac shellfish poisoning (ASP) in humans. This toxin is a glutamate analog that causes serious human and wildlife intoxication events (Colman et al. 2005). DA is transferred through food-web interactions, and can affect the ecosystem over large spatial and temporal scales. DA has been detected in marine consumers ranging from copepods to mollusks, fish, and mammals (Doucette et al. 2006; Caron et al. 2010), and ASP toxicity events have greatly increased in both frequency and intensity over the last decade along the U.S. West Coast (Scholin et al. 2000; Trainer et al. 2000). In California waters, outbreaks of DA poisoning from toxic *Pseudo-nitzschia* spp. blooms have been responsible for thousands of sea lion and seabird deaths, and recently these blooms appear to have intensified significantly in areas such as the San Pedro Channel and Los Angeles harbor (Schnetzer et al. 2007).

Although many factors affect the trophic transfer of toxins, the environmental health risk posed by these blooms is first of all a function of the levels of DA produced by the cells. High cellular toxin content poses a risk of human and animal exposure through potentially high accumulations of DA through the food chain. Consequently, a number of studies have attempted to determine the factors that cause or stimulate DA production by toxic *Pseudo-nitzschia*. Circumstantial evidence points to natural mechanisms of aggregation (due to currents and tides) and enrichment of coastal waters with nutrients as possible contributing factors (Bates et al. 1998). Experiments with various *Pseudo-nitzschia* species have indicated that DA production can be stimulated when growth is limited by the major nutrients phosphate or silicate (Bates et al. 1991; Pan et al. 1998), and by the micronutrient iron (Maldonado et al. 2002; Trick et al. 2010), or by shifts between different nitrogen sources (Howard et al. 2007).

Today, we recognize that fossil fuel combustion is modifying both the seawater chemistry and climate of the coastal environment where *Pseudo-nitzschia* blooms occur, through CO_2-induced ocean acidification and greenhouse warming. Current levels of atmospheric CO_2 are predicted to more than double by 2100 (Intergovernmental Panel on Climate Change [IPCC] 2007). Enrichment with CO_2 in general accelerates the carbon fixation rates of some phytoplankton groups (Riebesell 2004). CO_2 acquisition can be limiting for some algae at present atmospheric CO_2 concentrations, depending on the efficiency of their inorganic carbon concentrating mechanisms (CCMs). Consequently, some algal groups including harmful algal bloom (HAB) species can potentially be carbon dioxide–limited, and may preferentially benefit from future higher CO_2 levels in seawater (Fu et al. 2008*b*; Hutchins et al. 2009). Thus, it appears that future 'CO_2 fertilization' of the ocean and associated potential hydrogen ion concentration (pH) changes could result in more frequent or more intense toxic algal blooms. The concentration of CO_2 in seawater and associated pH changes could, therefore, directly influence *Pseudo-*

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nitzschia growth and physiology, and so possibly its cellular toxicity. Two recent culture studies have shown that elevated seawater pH (lowered pCO_2) inhibited growth and also induced DA production by two strains of *Pseudo-nitzschia multiseries* (Lundholm et al. 2004; Trimborn et al. 2008).

In addition to ocean acidification, modeling work also predicts that warming and surface-seawater freshening from melting ice and increased precipitation will intensify oceanic stratification and, thus, reduce vertical nutrient supply to the euphotic zone. These changes in nutrient supplies, in concert with changing pCO_2 and temperature, could have a strong effect on the physiology of marine phytoplankton, including coastal HABs (Hutchins et al. 2009; Boyd et al. 2010).

Expanded research effort is now being directed toward understanding global change effects on marine phytoplankton. For instance, interactive effects have been documented between changing pCO_2 and various permutations of nutrients, iron, light, and temperature on the physiologies of coccolithophorids such as *Emiliania huxleyi* (Feng et al. 2008) and the cyanobacteria *Trichodesmium* (Hutchins et al. 2007), *Crocosphaera*, and *Synechococcus* (Fu et al. 2007, 2008*a*). However, relatively few studies have so far addressed harmful algal species (Moore et al. 2008). Recent work has shown that the physiological characteristics of particular dinoflagellate and raphidophyte HAB species may cause them to be particularly favored by increasing seawater CO_2 and temperature (Fu et al. 2008*b*). Other work has also suggested that bloom-forming dinoflagellates could benefit from higher pCO_2, due in part to their relatively inefficient carbon-fixing enzyme, a Type II Rubisco (Rost et al. 2003).

Previous studies have suggested that both nutrient availability and pH can affect toxin production by HAB species, but questions remain about how toxin production may be influenced by the combination of changing pCO_2 levels with altered nutrient availability. Phycotoxin biosynthesis is directly linked to the autotrophic metabolism of most HAB algae, so it is perhaps not entirely surprising to find that CO_2 and nutrient availability can interactively affect cellular toxicity. One recent study showed that the dinoflagellate *Karlodinium veneficum* responds to the combination of increased CO_2 and reduced P availability with greatly increased cellular toxicity (Fu et al. 2010).

Here we present the results of culture experiments examining the combined effects of CO_2 and phosphate concentration on the growth, elemental composition, photosynthesis, and toxin production of the marine HAB diatom *Pseudo-nitzschia multiseries*. The aim of this study was to gather basic information on DA production and growth rates of *Pseudo-nitzschia* under potential future conditions of elevated CO_2 and P availability, in order to contribute toward understanding how damaging blooms of this toxic diatom species may respond to likely future conditions in the changing coastal ocean.

Methods

Cultures and growth conditions—Stock cultures of the marine diatom *Pseudo-nitzschia multiseries* (Hasle; the Provasoli-Guillard National Center for Culture of Marine Phytoplankton 2708, originally isolated from Eastern Canada) were maintained at 17°C in 0.2-μm-filtered, microwave-sterilized natural seawater, enriched with levels of phosphate, nitrate, silicate, vitamins, and trace nutrients as in Price et al. (1988). Light was provided on a 12 h dark : 12 h light cycle using cool white fluorescent bulbs at 120 μmol photons m^{-2} s^{-1}.

Experimental design and determination of growth rates—Semicontinuous culturing methods were used in order to measure the effects of P availability or pCO_2 levels during acclimated, steady-state growth (Fu et al. 2008*a*,*b*; Hutchins et al. 2007). Cultures were diluted daily with medium that was previously adjusted to the appropriate temperature and pCO_2. Each bottle was diluted back to the same cell density present in that bottle directly after the previous day's dilution. In this way, each replicate was allowed to independently reach its own steady-state growth rate under a given experimental treatment. Thus, unlike a chemostat culture in which growth rates are determined by the dilution rate, in this semicontinuous method dilution rates are instead adjusted to the growth rate. In this way, growth rate is a dependent variable that is a function of the independent variables chosen for the experiment, in this case pCO_2 and P availability. Many previous studies of the effects of pCO_2 and other variables on algal growth and physiology have used this same technique with other algae (Fu et al. 2007; Hutchins et al. 2007; Feng et al. 2008). Cultures were harvested following $\sim$ 4–6 weeks of semicontinuous incubation when they were fully acclimated to the experimental conditions, after statistically invariant growth rates were recorded for $\geq$ 4–6 consecutive dilutions.

Samples from each culture bottle were always taken at the same time in the diel cycle (Fu et al. 2008*a*), between 09:00 h and 10:00 h in the morning, to measure cell density and, thus, determine changes in growth rate. Dilutions were done in real time using biomass estimates made by in vivo fluorescence, and were subsequently validated using preserved cell-count samples. Chlorophyll *a* (Chl *a*) per cell in *Pseudo-nitzschia multiseries* was not affected by the availability of CO_2 (data not shown), suggesting that the use of in vivo fluorescence was a valid and practical way to monitor the biomass of *Pseudo-nitzschia multiseries*. Unchanged cellular Chl *a* in response to changing availability of dissolved inorganic carbon (DIC) or pCO_2 has been reported in both cyanobacteria and eukaryotic algae (Fu et al. 2008*a*,*b*). Growth rates were calculated based on the equation: $\mu = \ln N_b - \ln N_a / t_b - t_a$, where N_a and N_b are the average cell density at times t_a (directly after a dilution) and t_b (directly before the next day's dilution). For cell counts, whole-culture samples were fixed with glutaraldehyde (2.5% v to v final concentration) and counted in triplicate. About 1000 cells per replicate were enumerated in a 1-mL Sedgewick–Rafter counting chamber, using an Olympus BX51 epifluorescence microscope at 100-fold magnification.

Triplicate bottles at two conditions of phosphate availability were equilibrated at three different CO_2 concentrations by gentle bubbling with commercially prepared

Table 1. Measured pH, dissolved inorganic carbon (DIC), concentrations (μmol L^{-1}), and calculated CO_2 concentration (μmol L^{-1}), and pCO_2 (Pa) on the final sampling day of the incubation. Numbers in parentheses are the standard deviations of triplicate samples.

Treatment	Measured pH	Measured DIC concentration (μmol L^{-1})	Calculated [CO_2] (μmol L^{-1})	Calculated pCO_2 (Pa)
P-limited, 22 Pa	8.38(0.05)	1917(38)	7.4(0.6)	23(2)
P-limited, 41 Pa	8.15(0.02)	2029(8)	13.9(0.7)	42(2)
P-limited, 74 Pa	7.94(0.01)	2145(9)	24.5(0.6)	75(2)
P-replete, 22 Pa	8.40(0.03)	1970(4)	7.1(0.5)	22(2)
P-replete, 41 Pa	8.19(0.02)	2066(11)	12.8(0.8)	40(3)
P-replete, 74 Pa	7.96(0.01)	2177(6)	23.9(0.4)	73(1)

certified standard air and CO_2 gas mixtures (Praxair Gas). CO_2 concentrations examined included preindustrial atmospheric levels ($\sim$ 22 Pa), near present-day concentrations ($\sim$ 41 Pa), and values predicted to occur before the end of this century ($\sim$ 74 Pa; IPCC 2007). In-line high-efficiency particulate air filters were used to avoid contamination from particles in the gas tanks or lines. Phosphate levels used were 20 μmol L^{-1} (P replete) and 0.5 μmol L^{-1} (P limited). Six different phosphate and CO_2 conditions were used in this study: 20 μmol L^{-1} P and $\sim$ 22 Pa CO_2; 20 μmol L^{-1} P and $\sim$ 41 Pa CO_2; 20 μmol L^{-1} P and $\sim$ 74 Pa CO_2; 0.5 μmol L^{-1} P and $\sim$ 22 Pa CO_2; 0.5 μmol L^{-1} P and $\sim$ 41 Pa CO_2; and 0.5 μmol L^{-1} P and $\sim$ 74 Pa CO_2.

Carbonate buffer system measurements and pCO_2 *treatments*—The pH in each bottle was monitored daily using a high-sensitivity microprocessor pH-meter (Orion EA 940), calibrated with pH 4, 7, and 10 buffer solutions. The relative precision of this instrument is $\sim$ 0.01 and accuracy is $\sim$ 0.03 pH units. For the analysis of total DIC, DIC samples were stored in 2-mL capped borosilicate vials free of air bubbles and were preserved with 20-μL saturated $HgCl_2$ L^{-1}, and stored at 4°C until analyzed. Total DIC was measured by acidifying 2-mL 10% of H_3PO_4 and quantifying the CO_2 trapped in an acid sparging column (model CM 5230) with a model CM 140 UIC carbon coulometer (Beman et al. 2010; King et al. 2011). Certified reference materials obtained from Andrew Dickson (University of California, San Diego; <http://andrew.ucsd.edu/co2qc/index.html>) were measured periodically during the run and used for calibration. pH values remained invariant before and after the dilution, suggesting that bubbling rates were sufficient to maintain the target CO_2 equilibration levels in the medium, regardless of diel changes in photosynthesis and respiration. Based on the daily measurements of pH and DIC, pCO_2 stabilized during the early part of the semicontinuous growth period and then remained steady throughout the latter part of the incubation period. Calculated pCO_2 values for the three CO_2 treatments in both P treatments ranged from 22 Pa to 23 Pa, 40 Pa to 42 Pa, and 73 Pa to 75 Pa (Table 1), very close to the certified standard gas mixture values. For convenience, these values were averaged and rounded to 22 Pa, 41 Pa, and 74 Pa when referring to the three pCO_2 treatments throughout the paper.

Determination of P–E curves and primary production—Photosynthesis vs. irradiance (P–E) curves were performed by measuring ^{14}C fixation rates at a range of light intensities using a photosynthetron (Composite High Pressure Technologies), following the method described in Fu et al. (2008*a*,*b*). Five mL of scintillation cocktail was added and the filters were stored in the dark overnight, and then counted using a Wallac System 1400 liquid scintillation counter.

All ^{14}C uptake rates were corrected for dark uptake and carbon assimilation values were subsequently normalized to Chl *a*. The initial slope of the P vs. E curve (i.e., the photosynthetic efficiency α [mg C (mg Chl *a*)$^{-1}$ h^{-1} (μmol quanta m^{-2} s^{-1})$^{-1}$]) and the maximum chlorophyll-specific carbon fixation rate P^B_{max} [mg C (mg Chl *a*)$^{-1}$ h^{-1}] were calculated from least-squares nonlinear regression using the exponential function of Platt et al. (1980). E_k (μmol quanta m^{-2} s^{-1}), the light-saturation point and index of light adaptation, was calculated as $P^B_{max} : \alpha$.

Primary production was measured in triplicate using 24-h incubations with $H^{14}CO_3$ under the appropriate experimental growth conditions of light and temperature for each treatment (Fu et al. 2008*b*). A final concentration of 0.037 MBq ^{14}C sodium bicarbonate was added to 20-mL culture samples prior to incubation. All carbon fixation rates for both primary productivity and P–E curves were calculated using measured initial experimental DIC and Chl *a* concentrations for each treatment.

Analysis of POC, PON, POP, and BSi—Samples for the analysis of particulate organic carbon (POC) and particulate organic nitrogen (PON) were collected on precombusted GF/F glass-fiber filters (450°C for 5 h) under low vacuum and dried at 55°C. The samples were then analyzed on an Elemental Analyzer (Costech Instruments, model 4010) as described in Fu et al. (2007). Particulate organic phosphorus (POP) was measured followed by the protocol in Fu et al. (2005). Cellular biogenic silica (BSi) was analyzed according to the spectrophotometric method of Brzezinski and Nelson (1995).

Analysis of DA concentrations—Particulate and dissolved DA was measured using ASP-enzyme–linked immunosorbent assay (ELISA) kits available from Biosense Laboratories. This method has been validated with chemical methods (i.e., liquid chromatography–mass spectrometry), and the results indicate that the ASP ELISA kit can be applied to the routine determination and monitoring of DA (Kleivdal et al. 2007). Particulate DA samples were collected on uncombusted Whatman GF/F filters and

frozen at −20°C until analyzed. The filtrate from each sample was also collected, frozen, and later analyzed for dissolved DA. Sample preparation and ELISA tests were carried out following the protocol of Biosense Laboratories (2005 version). The limit of detection for the ELISA method for particulate DA is 6.8 ng L^{-1}. Total DA produced per cell (including the sum of both particulate and dissolved DA) was calculated by dividing the DA content of the whole-culture sample by the cell density.

Statistical analysis—Growth rates, photosynthetic parameters, and cell quotas and elemental ratios in different experiments were assessed using a two-way ANOVA. Differences between treatment groups were tested by Tukey honest significant difference multiple comparisons. Differences are termed significant when $p < 0.05$. All data analyses were conducted using the Software package Statistica.

Results

DA production—DA production occurred to varying degrees in all treatments (Fig. 1). The toxin cell quotas were much higher in P-limited cultures relative to P-replete ones, consistent with previous work (Pan et al. 1998; Fehling et al. 2004). P-limited cultures at corresponding pCO_2 levels had cell-normalized DA contents that were 30–50-fold higher (Fig 1A; P-limited vs. P replete: 22 Pa: $F_{1,12} = 53.4$, $p < 0.001$; 41 Pa, $F_{1,12} = 90.6$, $p < 0.001$; 74 Pa, $F_{1,12} = 1436$, $p < 0.001$) than those of the P-replete cultures. The dissolved DA normalized to cell abundances (Fig. 1B; P-limited vs. P replete, 22 Pa, $F_{1,12} = 426.2$, $p < 0.001$; 41 Pa, $F_{1,12} = 146.8$, $p < 0.001$; 74 Pa, $F_{1,12} = 281.6$, $p < 0.001$) and total cell-normalized DA contents (dissolved + particulate, Fig. 1C; 22 Pa: $F_{1,12} = 66.4$, $p < 0.001$; 41 Pa: $F_{1,12} = 100.8$, $p < 0.001$; 74 Pa: $F_{1,12} = 120.8$, $p < 0.001$) showed similar trends to the cell-normalized particulate DA contents.

Cellular DA toxin content (Fig. 1A) also increased with increasing pCO_2 regardless of P availability. This trend was especially evident at 74 Pa CO_2, where values were significantly higher than those at 22 Pa and 41 Pa (P-limited: 22 Pa vs. 74 Pa: $F_{1,12} = 957$, $p < 0.0001$; 41 Pa vs. 74 Pa: $F_{1,12} = 819$, $p < 0.001$; P-replete: 22 Pa vs. 74 Pa: $F_{1,12} = 27.6$, $p < 0.001$; 41 Pa vs. 74 Pa: $F_{1,12} = 13.4$, $p = 0.003$). This effect was, however, much more pronounced under P-limited conditions, where cellular DA content in P-limited cultures (Fig. 1B) increased by ~ four-fold (74 Pa) relative to the cultures grown at 22 Pa CO_2 levels. Although DA production was greatly reduced in P-replete cultures, toxin levels were still a function of pCO_2. Cellular DA content at 22 Pa CO_2 was only 64% and 45% as high as cellular content in the P-replete 41-Pa and 74-Pa treatments, respectively.

Dissolved DA levels in the cultures were nearly an order of magnitude lower than particulate DA concentrations, but showed similar trends across the pCO_2 treatments (Fig. 1B). Regardless of P conditions, dissolved DA concentrations normalized to cell number were significantly higher in the 74-Pa pCO_2 treatment than the two lower

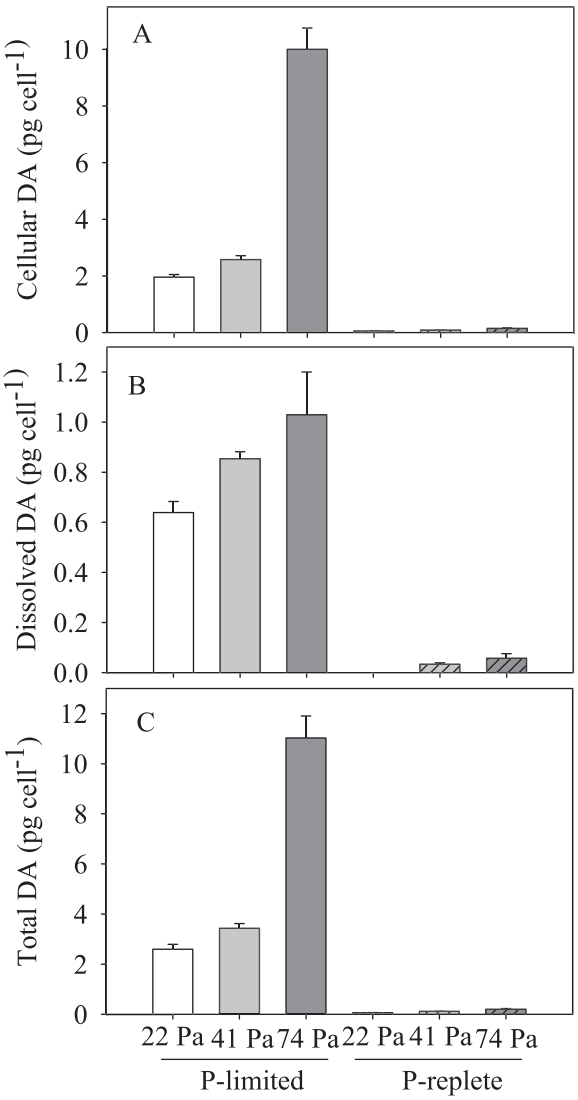

Fig. 1. Domoic acid production (DA) by *Pseudo-nitzschia multiseries* CCMP 2708 (Hasle) in the six P and CO_2 treatments. (A) Particulate DA, pg $cell^{-1}$; (B) dissolved DA, pg $cell^{-1}$; and (C) total DA (particulate + dissolved), pg $cell^{-1}$. Open bars are the P-limited cultures, and cross-hatched bars are the P-replete treatments; increasing grey shades represent increasing pCO_2 levels in the three treatments. Values are the means and error bars are the standard deviations of triplicate samples.

pCO_2 treatments (P-limited: 22 Pa vs. 41 Pa: $F_{1,12} = 6.1$, $p = 0.03$; 22 Pa vs.74 Pa: $F_{1,12} = 13.7$, $p = 0.003$; 41 Pa vs. 74 Pa: $F_{1,12} = 5.3$, $p = 0.04$; P-replete: 22 Pa vs. 74 Pa: $F_{1,12} = 369.5$, $p < 0.001$; 41 Pa vs. 74 Pa: $F_{1,12} = 82.1$, $p < 0.001$). For the 22-Pa pCO_2 P-replete cultures, the dissolved DA level was less than the limit of detection of the ELISA assay (~ 0.2 pg L^{-1}). A similar trend was observed with the total DA content normalized to cell abundance, in that higher values were always found under higher pCO_2 levels. For P-limited cultures, the values were significantly higher under 74 Pa CO_2 compared to those at 22 Pa CO_2, and significantly higher values were found under 74 Pa and 41 Pa CO_2 compared to 22 Pa (Fig. 1C; P-limited: 22 Pa vs. 41 Pa: $F_{1,12} = 6.1$, $p = 0.03$; 41 Pa vs. 74 Pa: $F_{1,12} = 3.8$, p

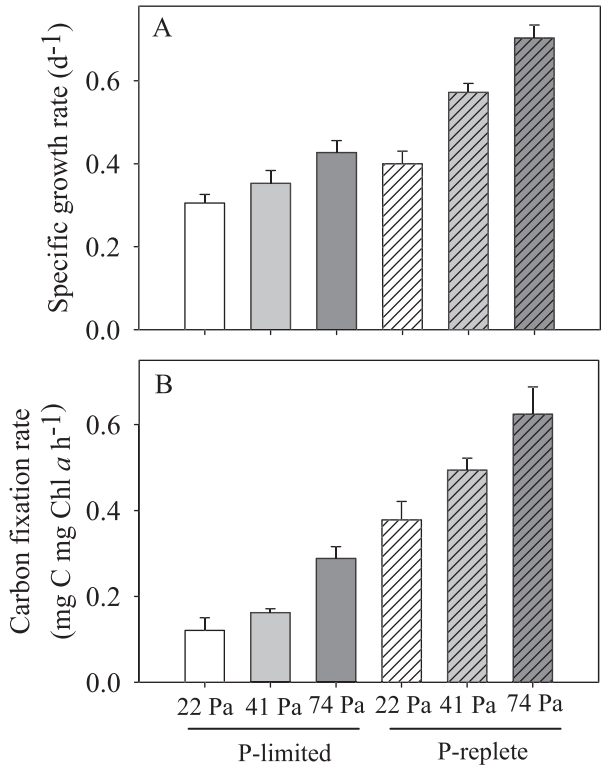

Fig 2. (A) Specific growth rates and (B) carbon fixation rates of *Pseudo-nitzschia multiseries* CCMP 2708 (Hasle) in the six P and pCO_2 treatments (symbols and error bars as in Fig. 1).

= 0.08; 22 Pa vs. 74 Pa: $F_{1,12} = 14.4$, $p = 0.003$; P-replete: 22 Pa vs. 41 Pa: $F_{1,12} = 61.6$, $p < 0.001$; 41 Pa vs. 74 Pa: $F_{1,12} = 6.2$, $p = 0.03$; 22 Pa vs. 74 Pa: $F_{1,12} = 17.9$, $p = 0.001$).

In addition, there was a significant two-way interactive effect between pCO_2 and phosphate availability on toxin production. For all forms (particulate, dissolved, and total) of cellular DA, by far the greatest enhancement of total DA levels always occurred in the treatments combining higher pCO_2 and decreased P availability (two-way ANOVA, interactions: cellular DA: $F_{2,12} = 9.3$, $p = 0.004$; cell-normalized dissolved DA: $F_{2,12} = 22.0$, $p < 0.001$; cell-normalized total DA: $F_{2,12} = 8.4$, $p = 0.005$).

Growth rate and primary production—Growth rates of *Pseudo-nitzschia multiseries* scaled with pCO_2 in both P-replete and P-limited cultures (Fig. 2A). As expected, the growth rates of *Pseudo-nitzschia multiseries* were higher in P-replete cultures than under P-limitation, increasing by 34% (22 Pa), 58% (41 Pa), and 65% (74 Pa) under equivalent CO_2 conditions (Fig. 2A, 22 Pa: $F_{1,12} = 16.9$, $p = 0.001$; 41 Pa: $F_{1,12} = 43.5$, $p < 0.001$; 74 Pa, $F_{1,12} = 73.6$, $p < 0.001$). For P-limited cultures, growth rates in the 74-Pa pCO_2 treatment were significantly higher than in the two lower pCO_2 treatments ($F_{1,12} = 28.4$, $p < 0.001$; $F_{1,12} = 10.3$, $p = 0.007$), but the latter two treatments were not significantly different from each other ($F_{1,12} = 4.3$, $p = 0.06$). The effect of changing pCO_2 on growth rates of P-replete cultures was more dramatic. Growth rates in the P-replete medium increased significantly with increasing pCO_2 across all three pCO_2 treatments (22 Pa vs. 41 Pa: $F_{1,12} = 57.3$, $p < 0.001$; 41 Pa vs. 74 Pa: $F_{1,12} = 33.1$, $p < 0.001$; 22 Pa vs. 74 Pa: $F_{1,12} = 177.6$, $p < 0.001$).

The influence of pCO_2 on daily primary production by *P. multiseries* cultures was in general similar to that observed for growth rates (Fig. 2B). Increases in CO_2 and phosphate together stimulated the 24-h primary productivity of *P. multiseries* under the experimental conditions (Fig. 2B). For the P-replete cultures grown under elevated CO_2, primary production increased substantially. Carbon fixation rates in the 41-Pa and 74-Pa pCO_2 treatments were 1.3–1.7-fold higher than in the P-replete 22-Pa treatment (22 Pa vs. 41 Pa: $F_{1,12} = 15.4$, $p = 0.002$; 22 Pa vs. 74 Pa: $F_{1,12} = 34.5$, $p < 0.001$). However, in the P-limited cultures pCO_2 only affected primary production significantly at 74 Pa (22 Pa vs. 74 Pa: $F_{1,12} = 82.2$, $p < 0.001$; 41 Pa vs. 74 Pa: $F_{1,12} = 256.5$, $p < 0.001$), and no significant difference was observed between the two lower pCO_2 treatments ($F_{1,12} = 3.4$, $p = 0.09$).

Photosynthesis vs. irradiance—Photosynthetic carbon-fixation rates vs. irradiance (P–E curves) in both P treatments reached a plateau with no sign of photoinhibition at any light or CO_2 levels (Fig. 3). Maximum light-saturated carbon fixation rates (P^B_{max}) were invariably lower in P-limited cultures than in P-replete ones at the same levels of pCO_2, with P^B_{max} values in P-replete cultures being 2.2-, 2.0-, and 1.8-fold higher at 22 Pa, 41 Pa, and 74 Pa pCO_2, respectively (Table 2).

CO_2 enrichment effects on P^B_{max} were dependent on P availability. P^B_{max} in P-replete cultures at 74-Pa pCO_2 cultures increased by 21% compared to 22-Pa CO_2 levels ($F_{1,12} = 5.5$, $p = 0.037$; Fig. 3; Table 2). The values of P^B_{max} in the 74-Pa pCO_2 treatment were not significantly different from those in the 41-Pa pCO_2 treatment ($F_{1,12} = 4.3$, $p = 0.06$). The P^B_{max} values in P-limited cultures at 22-Pa pCO_2 were significantly lower than in the other two elevated pCO_2 levels ($F_{1,12} = 11.1$, $p = 0.006$; $F_{1,12} = 97.4$, $p < 0.001$; compared with 41 Pa and 74 Pa CO_2 respectively). The values of P^B_{max} in the 74-Pa cultures were also significantly higher than the ones in the 41-Pa treatment ($F_{1,12} = 117.4$, $p < 0.001$).

The slope of the light-limited portion of the photosynthetic curve (α) in P-replete cultures was significantly greater than in P-limited ones under 41-Pa and 74-Pa pCO_2 conditions (Table 2; 41 Pa: $F_{1,12} = 9.3$, $p = 0.01$; 74 Pa: $F_{1,12} = 11.1$, $p = 0.006$). Under both P-limited and P-replete conditions, α values in the three pCO_2 treatments were statistically invariant except for significantly increased α values at 41 Pa relative to those at 22-Pa CO_2 for P-limited cultures ($F_{1,12} = 7.2$, $p = 0.02$).

E_k, the ratio of $P^B_{max}:\alpha$, represents the light saturation point at which light absorption is in balance with the capacity of the photosynthetic system to process this incoming energy. Regardless of P conditions, there was no significant difference among the three pCO_2 enrichments (P-limited: 22 Pa vs. 41 Pa: $F_{1,12} = 3.7$, $p = 0.08$; 41 Pa vs. 74 Pa: $F_{1,12} = 4.3$, $p = 0.06$; 22 Pa vs. 74 Pa, $F_{1,12} = 2.6$, $p = 0.14$; P-replete: 22 Pa vs. 41 Pa: $F_{1,12} = 0.64$, $p = 0.44$;

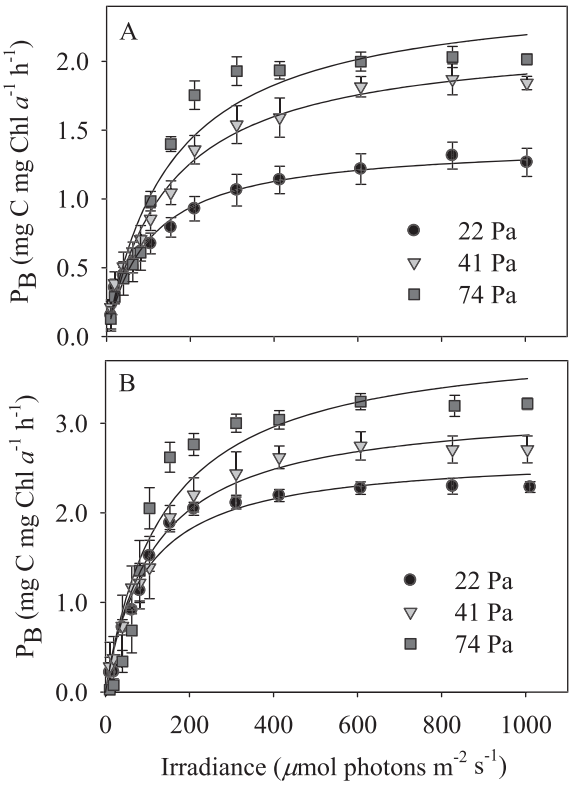

Fig. 3. P–E curves (photosynthesis vs. irradiance curves) of *Pseudo-nitzschia multiseries* CCMP 2708 (Hasle) in the six P and CO_2 treatments. (A) P-limited treatments and (B) P-replete treatments. Symbols for the three pCO_2 treatments are: circles, 22 Pa pCO_2; triangles, 41 Pa pCO_2; squares, 74 Pa pCO_2. Values are the means and error bars are the standard deviations of triplicate samples.

41 Pa vs. 74 Pa: $F_{1,12} = 0.7$, $p = 0.43$; 22 Pa vs. 74 Pa: $F_{1,12} = 0.8$, $p = 0.4$). Also, significant differences between the two P conditions were only found at 41 Pa CO_2 ($F_{1,12} = 9.0$, $p = 0.01$). The ratios of E_k to the culture irradiance (120 μmol photons $m^{-2}s^{-1}$) in P-limited cultures ranged from 0.9 to 1.4 regardless of different pCO_2 levels or P conditions, while the E_k values under P-replete conditions were $\sim$ 1.2 (data not shown).

Elemental composition—The cellular quotas of silicon (Q_{Si}, pmol Si $cell^{-1}$) for the experimental *P. multiseries* cultures were affected by P or pCO_2 conditions, or by both

Table 2. Photosynthetic parameters from P–E curve fitting, including P^B_{max} (mg C mg Chl a^{-1} h^{-1}), alpha (mg C h^{-1} [mg Chl a]$^{-1}$ [μmol m^{-2} s^{-1}]$^{-1}$), and E_k (μmol $m^{-2}s^{-1}$). Values are the means of triplicate measurements ($\pm$SD).

Treatment	P^B_{max}	α	E_k
P-limited, 22 Pa	1.22(0.09)	0.010(0.001)	124.5(6.5)
P-limited, 41 Pa	1.53(0.01)	0.014(0.001)	107.3(11.0)
P-limited, 74 Pa	1.84(0.05)	0.011(0.002)	167.4(39.2)
P-replete, 22 Pa	2.73(0.10)	0.021(0.002)	130.5(13.7)
P-replete, 41 Pa	3.13(0.08)	0.020(0.002)	156.9(15.6)
P-replete, 74 Pa	3.30(0.09)	0.024(0.003)	142.1(24.0)

(Fig. 4A). Growth at the lowest experimental pCO_2 resulted in a significant increase in Q_{Si} of the cells. Q_{Si} values at 22 Pa pCO_2 were 66% and 52% higher in P-limited cultures compared to cells in the 41-Pa and 74-Pa CO_2 treatments, respectively (22 Pa vs. 41 Pa: $F_{1,12} = 15.4$, $p = 0.002$; 22 Pa vs. 74 Pa: $F_{1,12} = 8.7$, $p = 0.01$) and 25% and 46% higher in P-replete cultures (22 Pa vs. 41 Pa: $F_{1,12} = 7.2$, $p = 0.02$; 22 Pa vs. 74 Pa: $F_{1,12} = 11.8$, $p = 0.005$) (Fig. 4A). However, Q_{Si} was not significantly different between the two elevated pCO_2 treatments regardless of P conditions (P-limited: $F_{1,12} = 0.6$, $p = 0.45$; P-replete: $F_{1,12} = 3.4$, $p = 0.09$). Cellular Si quotas in P-limited cultures were always much higher than in P-replete cultures, within the same pCO_2 levels (22 Pa: $F_{1,12} = 105.3$, $p < 0.001$; 41 Pa: $F_{1,12} = 10.6$, $p = 0.008$; 74 Pa: $F_{1,12} = 9.3$, $p = 0.01$).

The cellular Si : C ratios showed similar trends to the Q_{Si} values (Fig. 4B). At 22 Pa pCO_2 under P-limited conditions, Si : C ratios were significantly higher compared to the other two elevated pCO_2 levels (Fig. 4B; 22 Pa vs. 41 Pa: $F_{1,12} = 5.5$, $p = 0.04$; 22 Pa vs. 74 Pa: $F_{1,12} = 9.3$, $p = 0.01$). However, there was no significant difference in the ratios of Si : C between these two higher CO_2 levels ($F_{1,12} = 0.8$, $p = 0.4$). Under P-replete conditions, cultures grown under the two lower pCO_2 levels showed significantly higher ratios of Si : C (by 69% and 38% for the 22-Pa and 41-Pa levels, respectively) than cells grown at 74 Pa pCO_2 ($F_{1,12} = 7.84$, $p = 0.02$; $F_{1,12} = 8.3$, $p = 0.01$). Ratios of Si : C in P-replete cultures were generally higher than P-limited cultures (due largely to their lower cellular C content), but this trend was only significant for the 41 Pa treatment (22 Pa: $F_{1,12} = 0.6$, $p = 0.45$; 41 Pa: $F_{1,12} = 6.1$, $p = 0.03$; 74 Pa: $F_{1,12} = 4.3$, $p = 0.06$).

The molar ratios of C : P in P-limited cultures were $\sim$ 400, significantly higher than those of P-replete cultures at the same pCO_2 levels (Fig. 4C; 22 Pa: $F_{1,12} = 15.4$, $p = 0.002$; 41 Pa: $F_{1,12} = 57.8$, $p < 0.001$; 74 Pa: $F_{1,12} = 11.8$, $p = 0.005$). This was due to the much lower cellular P content under P-limited growth conditions; such elevated ratios are a diagnostic feature of P-limited cells (Fu et al. 2005). A significant interactive effect between phosphate availability and pCO_2 on the ratios of C : P was not observed ($F_{2,12} = 0.56$, $p = 0.61$). Under P-replete conditions, stepwise increases in pCO_2 marginally increased the ratios of C : P from 84 to 99, with a significantly higher ratio at 74 Pa compared to the two lower pCO_2 levels (22 Pa vs. 74 Pa: $F_{1,12} = 5.3$, $p = 0.04$; 41 Pa vs. 74 Pa: $F_{1,12} = 5.3$, $p = 0.04$). However, the molar C : P ratios were not significantly different among these three pCO_2 levels under P-limited conditions (22 Pa vs. 41Pa: $F_{1,12} = 0.9$, $p = 0.37$; 41 Pa vs. 74 Pa: $F_{1,12} = 0.9$, $p = 0.37$; 22 Pa vs. 74 Pa: $F_{1,12} = 0.01$, $p = 0.91$).

Molar C : N ratios of *P. multiseries* cells ranged from 7.7 to 9.5, and there was no systematic influence of the three pCO_2 treatments regardless of phosphate conditions (Fig. 4D; $F_{2,12} = 1.72$, $p = 0.22$). There was also no difference in C : N between P-limited and P-replete conditions ($F_{1,12} = 0.13$, $p = 0.73$).

Discussion

DA production and growth rates relative to pCO_2 *and P availability*—Increases in CO_2 levels significantly induced

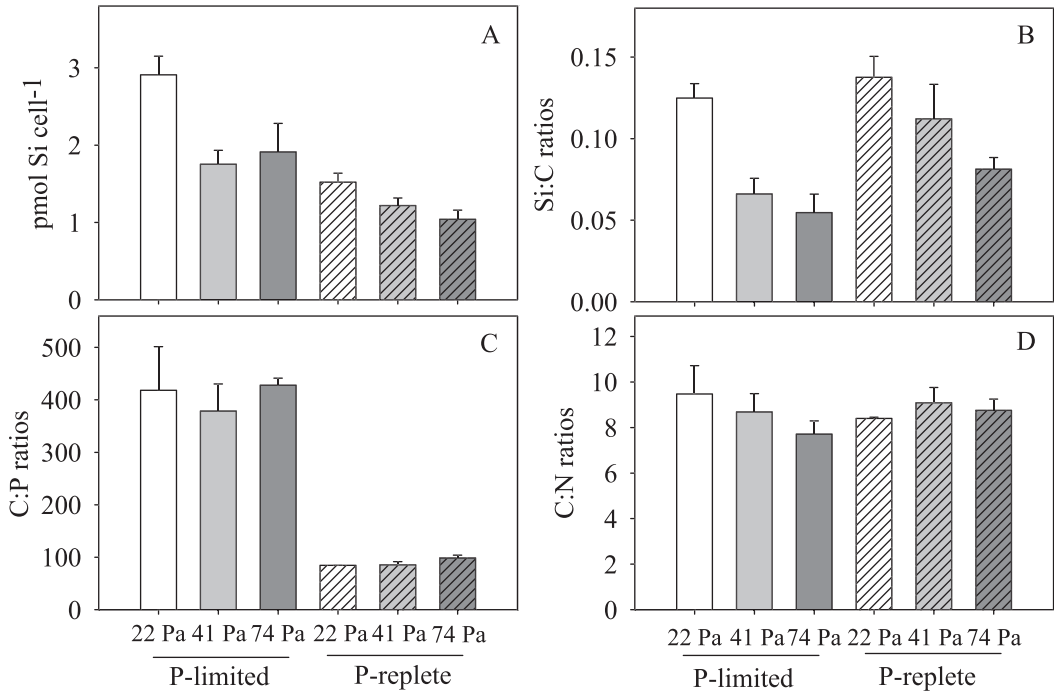

Fig. 4. Elemental compositions in *Pseudo-nitzschia multiseries* CCMP 2708 (Hasle) in the six P and CO_2 treatments (A) Cellular quotas of silicon, Q_{Si} (pmol cell^{-1}), (B) cellular Si : C (mol : mol), (C) cellular C : P (mol : mol), and (D) cellular C : N (mol : mol). Symbols and error bars as in Fig. 1.

DA production by *Pseudo-nitzschia multiseries.* Toxin concentrations were also strongly enhanced by P-limitation. Elevated CO_2 concentrations have also been reported to greatly increase the toxicity of P-limited cultures of the harmful bloom dinoflagellate *Karlodinium veneficum,* through changes in the relative production rates of two differentially toxic karlotoxin congeners (Fu et al. 2010).

P-limitation has previously been shown to stimulate DA production (Pan et al. 1998; Fehling et al. 2004). P-limited cells may be arrested in the G1 phase of the cell cycle, which has been suggested to result in accumulation of toxins (John and Flynn 2000). Our study supports the notion that P availability plays an important role in controlling DA production.

Two previous studies of *P. multiseries* showed an inverse relationship between cell-specific toxin levels and pH values (Lundholm et al. 2004; Trimborn et al. 2008), implying greater toxin production at lower CO_2 concentrations. We observed the opposite trend with higher pCO_2 (lower pH) significantly promoting the cellular DA content of *P. multiseries*. These contradictory results could potentially be due to differences in the species and strains employed, to differing cell physiology due to growth phase of the cultures, or to different experimental protocols. Lundholm et al. (2004) and Trimborn et al. (2008) both used different culture isolates from the one employed here. DA production in these prior studies was measured during late exponential or stationary growth phase using batch cultures, in which cell growth rates and physiology were changing throughout the course of the experiment. In contrast, we employed a semicontinuous incubation technique to maintain the cells in early exponential growth phase throughout the experiment.

The actual biochemical mechanism by which CO_2 availability or pH affects toxin production remains to be determined. Enzymatic processes, carbon limitation, changes in metal speciation, and bacterial community composition shifts have all been suggested to affect the production of DA (Lundholm et al. 2004). This prior study also suggested that there could be particular optimum pH values for DA production.

CO_2-mediated variations in toxin production could also potentially be an indirect effect, resulting from alleviation of C limitation of carbon fixation. Increased photosynthesis at elevated CO_2 could potentially reduce competition for energy between carbon fixation and toxin production pathways. A similar argument has been used to explain enhanced nitrogen fixation at high CO_2 by the marine cyanobacterium *Trichodesmium* (Hutchins et al. 2007). Hence, carbon availability could potentially be a factor limiting the production of DA toxin. However, we saw the greatest stimulation of DA production at high pCO_2 in P-limited cells, in which carbon fixation rates were lower than in P-replete treatments. This implies that fixed carbon limitation is likely not the cause of the observed effect.

External pH changes may affect the speciation of trace metals such as iron and, hence, could reduce their bioavailability to phytoplankton (Shi et al. 2010). DA production in *Pseudo-nitzschia* has been linked to the availability of Fe and Cu (Maldonado et al. 2002). However our cultures were grown under trace-metal–replete conditions (0.45 μmol L^{-1} iron) and were not

maintained using rigorous trace-metal clean-culture methods, so iron limitation due to pH effects seems unlikely.

Accelerated growth and carbon fixation of *P. multiseries* with rising pCO_2 levels is in agreement with both field and lab studies of a variety of marine diatoms (Riebesell et al. 1993; Burkhardt and Riebesell 1997; Kim et al. 2006), dinoflagellates (Dason et al. 2004; Fu et al. 2008*a*, 2010), raphidophytes (Fu et al. 2008*a*), and cyanobacteria (Hutchins et al. 2007; Fu et al. 2008*b*). Stimulation of growth by elevated CO_2 in these studies ranged from a few percent to 50%. Although some field incubation experiments have shown that growth of natural diatom assemblages was unaffected by the availability of pCO_2 (Hare et al. 2007), Tortell et al. (2002) found that the relative abundance of diatoms in the equatorial Pacific was 50% lower in a low-pCO_2 treatment compared to high pCO_2.

Most marine phytoplankton operate a CCM to actively elevate pCO_2 around the key carbon-fixing enzyme ribulose-1,5-bisphosphate carboxylase oxygenase (RubisCO; Raven and Johnston 1991). Several bloom-forming diatom species employ an efficient and well-regulated CCM that can maintain fast growth of the cells even under low CO_2 concentrations (Burkhardt et al. 2001; Rost et al. 2003). One recent study investigated the specific DIC acquisition strategies of *Pseudo-nitzschia multiseries* (Trimborn et al. 2008). They found that this species has a low $K_{1/2}(CO_2)$ for photosynthesis, indicating that it possesses a highly efficient and regulated CCM and suggesting that its growth may not be easily limited by DIC.

In contrast, our results instead showed that rising CO_2 significantly stimulated growth rates. As noted above though, our culture strain and experimental protocols were quite different from this previous study. Another possible explanation for our findings of CO_2-stimulated growth is that they are due to biochemical changes not directly related to photosynthesis. For instance, there are substantial energetic costs associated with maintaining a stable intracellular pH despite changes in external pH, such as those needed for cross-membrane transport of protons and other ions (Smith and Raven 1979; Pucéat 1999).

Interactive effects of CO_2 and P on domoic acid, growth rate, and photosynthesis—In general, many harmful algal species often show increased toxin production when their growth is limited by nutrients (Sunda et al. 2006). As noted above, our results comparing P-replete and P-limited cultures are consistent with this suggestion, and with previous DA studies (Bates et al. 1991; Pan et al. 1998). Exceptions to this general trend have been reported for some HAB species as well. For instance, the toxicity of the dinoflagellate *Cochlodinium polykrikoides* is highest in early exponential growth phase (Tang and Gobler 2009), similar to the physiological state of our semicontinuous cultures.

Despite the strong overall negative correlation between DA levels and P availability in our experiments, there is still a significant positive relationship of DA concentration with growth rates within each of the two P treatments (Fig. 5). In the P-replete cultures DA production increased (although absolute concentrations were quite low) stepwise with both pCO_2 and growth rate (Fig. 5B, $r^2 = 0.70$, $p = 0.005$), and these pCO_2-mediated increases were much more dramatic at the higher concentrations produced in the P-limited cultures (Fig. 5A; $r^2 = 0.66$, $p = 0.007$).

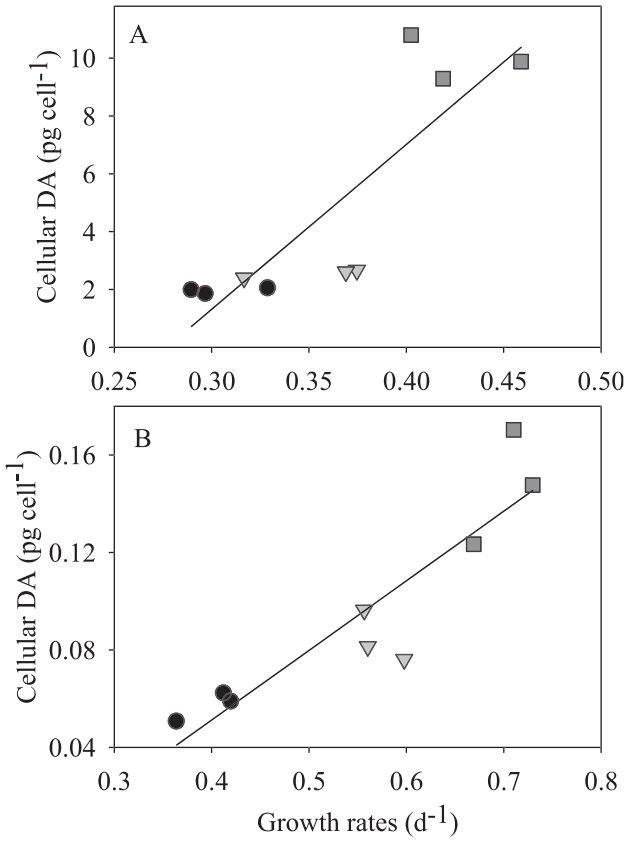

Fig. 5. Cellular domoic acid (DA; pg cell^{-1}) plotted vs. growth rates (d^{-1}) for *Pseudo-nitzschia multiseries* CCMP 2708 (Hasle) in both P treatments, across all three pCO_2 conditions. Each data point represents a single replicate bottle (three replicates for each of the three pCO_2 treatments). (A) P-limited cultures and (B) P-replete cultures. Note the scale differences between the two panels for DA concentration (y-axes) and growth rates (x-axes). (Symbols as in Fig. 3).

Growth rates were stimulated by increased CO_2 even in P-limited cultures. A similar co-limitation effect between CO_2 and P was documented for the cyanobacterium *Trichodesmium* by Hutchins et al. (2007). Our results suggest that pCO_2 also may have a complex co-limiting relationship with P-limitation in the diatom *Pseudo-nitzschia*, in terms of its effects on both growth rate and toxin production. The physiological causes of this net pCO_2 and P-limitation effect remain to be determined, but these results do suggest that toxin and growth interactions with pCO_2 may be fundamentally different than the effects of limitation by single nutrients such as P, Si, and Fe.

This relationship between pCO_2, P availability, and growth rate could potentially increase the cell density of toxic *P. multiseries* blooms under future high-CO_2 seawater conditions. In general, diatom blooms can be driven by eutrophication because they require higher nutrient concentrations for growth relative to other smaller taxa (Bates and Trainer 2006). Because elevated pCO_2 increased

growth rates of *P. multiseries* even during severely P-limited growth, though, the growth response of this species to future increasing atmospheric pCO_2 is likely to be positive regardless of the availability of nutrients, other factors being equal.

In our semicontinuous experimental design growth rates were allowed to vary as a function of the experimental pCO_2 and P conditions, in concert with toxin production and photosynthetic parameters. It would also be an instructive exercise to design continuous culture (chemostat) experiments in which growth rates are instead set by the dilution rate according to the supply rate of a limiting nutrient. Such experiments have potential to directly examine the physiological connections between growth rates, other variables, and toxin production in HAB species. However, our method is arguably more relevant ecologically, because growth rates of natural algal populations obviously do fluctuate as a function of nutrients and pCO_2.

An additive effect of simultaneously elevated P and CO_2 on photosynthesis was also observed in the present study. The rate of photosynthetic carbon fixation is an enzymatically controlled process under saturating light (Falkowski and Raven 1997). A reduced capacity to synthesize phosphorus-containing nucleotides such as adenosine triphosphate, adenosine diphosphate, and nicotinamide adenine dinucleotide phosphate under P-limited conditions may decrease photosynthetic rates. Under P-limitation, cellular Rubisco content of some algae can be much reduced, and such changes in the activity of Rubisco have been linked to reduced light-saturated photosynthetic rates (Geider et al. 1998). A decreased P^B_{max} under P-limiting condition has been commonly reported in other studies as well (Litchman et al. 2003). The photosynthetic efficiency value α (carbon fixed per unit light absorbed) under P-limited conditions was also lower, so that photosynthetic efficiency and total DA production were affected in opposite ways by P-limitation. This could suggest a tradeoff between the energy demands for photosynthesis and DA production under P-limited conditions.

Implications of elemental composition changes—Cell-normalized Si quotas (Q_{Si}) and Si : C ratios were much higher in the cells grown under the lowest pCO_2 conditions, irrespective of P conditions. This result coincides with the previous work of Milligan et al. (2004), who found an increase in Si quotas and Si : C ratios at low pCO_2 in the diatom *Thalassiosira weissflogii*. They suggested that an elevated Si quota in cells grown in low-pCO_2 treatments is caused by reduction in cellular losses of Si through efflux and dissolution.

It is also noteworthy that cellular Si quotas in P-limited cultures were higher than in P-replete cultures, within the same pCO_2 levels. Claquin et al. (2002) showed that diatom cellular Si quotas increase with decreasing growth rates, along with an increase in cell size and elongation of the gap phase G and mitosis. In our study, an increase in Si quota also coincides with a reduced growth rate in low pCO_2 and P-limited cultures.

pCO_2-induced changes in diatom silicification could potentially affect silicon biogeochemistry. High pCO_2 appears to favor lower Si : C ratios, potentially increasing diatom C flux relative to Si consumed. A recent review of the literature suggests that the effects of rising pCO_2 on phytoplankton elemental ratios are not consistent across taxa and regimes, and so it is still questionable how phytoplankton elemental ratios in general are likely to respond (Hutchins et al. 2009).

Because both cellular carbon and phosphorus quotas changed in parallel across pCO_2 treatments in cells grown under P-limited conditions (data not shown), there was no significant difference between C : P ratios due to pCO_2 (Fig. 4C). In contrast, we observed a modest pCO_2-induced increase in C : P ratios in P replete cultures ($\sim$ 20% across all three CO_2 concentrations; Fig. 4C). Some recent studies have revealed variations in algal stoichiometry as a consequence of CO_2 enrichments (Fu et al. 2007; Feng et al. 2008; Hutchins et al. 2009). These studies often observed an increase in C : P or N : P ratios due to higher CO_2 availability. However, compared to the effects of nutrient availability, these stoichiometry changes due to changing CO_2 availability may be relatively small.

Ecological and environmental implications—Our observation of increased DA production by *P. multiseries* at higher pCO_2 suggests the possibility of increasingly toxic blooms of these species with projected future trends of atmospheric CO_2 enrichment. The range of pCO_2 values we examined is also relevant today, because it spans the variability commonly observed between contemporary freshly upwelled water (which can reach 101 Pa or higher; Feely et al. 2008), and older, CO_2-depleted surface water under postbloom conditions (which can be $<$ 20 Pa). Extrapolating from our results based on pCO_2 alone, we would predict that *Pseudo-nitzschia* cells growing in newly upwelled water might exhibit higher levels of cell-specific toxicity. The tendency toward lower toxicity at higher nutrient levels (P, in the case examined here), however, could act to counteract the effects of high pCO_2 on DA production. The complex interactions we documented between CO_2 and nutrient availability may need to be considered to make truly accurate environmental predictions.

Pseudo-nitzschia blooms and elevated DA production are usually found in coastal areas, bays, and upwelling regions (Trainer et al. 2000; Schnetzer et al. 2007). In these areas, nutrients are supplied plentifully either by natural replenishment processes or anthropogenic eutrophication. However, P depletion can likely occur during the late stages of *Pseudo-nitzschia* blooms (Trainer et al. 2009), and the trend of increasing DA production at high pCO_2 we observed was more severe under P-limitation.

Blooms of *Pseudo-nitzschia* are rarely dominated by a single species (Trainer et al. 2009). Because only one species was tested in this study, we cannot draw firm conclusions about how future increasing CO_2 levels may affect the toxicity of these mixed-species blooms. However, it is possible to use our results to calculate potential increases in cellular toxicity for this species. For instance, in one *P. multiseries*–dominated bloom in the California coastal upwelling zone, DA levels ranged from 0.1 pg cell^{-1} to

0.7 pg cell^{-1} (Trainer et al. 2000). If our experimental results can be directly extrapolated to *P. multiseries* growing in these coastal waters, by the year 2100 cell-specific toxin content could increase to between 0.17 pg and 1.2 pg DA (74% increase under P-replete conditions) and 0.4–2.7 pg DA (288% increase under P-limited condition) because of anticipated CO_2 increases alone. This calculation assumes that the relative influences of other limiting factors such as Fe, N, Si, and light availability do not change. Because there are $>$ 10 species of *Pseudo-nitzschia* distributed along the west coast of the U.S. alone that can form mixed blooms (Stehr et al. 2002), the responses of more species need to be determined in order to accurately predict future DA production by *Pseudo-nitzschia* spp. in general under changing CO_2 conditions.

In summary, our results have shown that both phosphorus availability and pCO_2 can affect the growth and DA production of *Pseudo-nitzschia multiseries.* There is also a strong interactive effect of these two factors on toxin production. We would predict that CO_2 stimulation of DA production will be most significant when *P. multiseries* with a high intrinsic capacity for toxin production grow in P-limited environments, whereas the effect of pCO_2 on toxicity will probably be smaller under P-replete growth conditions.

A number of previous studies have demonstrated that DA production can be influenced by a large number of factors, including pH (Lundholm et al. 2004; Trimborn et al. 2008), P, Si (Bates et al. 1991; Pan et al. 1998), Fe, Cu (Maldonado et al. 2002) and N speciation (Howard et al. 2007). Our study strongly suggests that CO_2 availability also needs to be considered. An understanding of the precise nature of the interactions between all of these many DA-active variables would go a long way toward the formulation of a universal hypothesis regarding regulation of DA synthesis. However, complex multivariate experiments incorporating pCO_2 changes along with shifts in Si, N, Fe, and Cu availability in all possible combinations will undoubtedly be needed to unravel the complexities of this relationship. A complete picture of how and why DA levels are affected by all of these environmental factors, alone and in combination, may also have to await discovery of the cellular biochemical toxin synthesis pathways and their genetic regulation.

Further work will be needed to determine whether DA production by other toxic *Pseudo-nitzschia* strains and species could also be regulated by changing CO_2. Equally important is determining whether their responses to rising pCO_2 may be modulated by other interactive environmental factors such as changes in nutrient availability, irradiance, and temperature. This is information that will be critically needed in order to establish the ecological, environmental, and economic consequences of toxin production by *Pseudo-nitzschia* blooms in a future changing ocean.

Acknowledgments

We thank Cynthia Breene for assisting with the analysis of domoic acid. Grant support was provided by United States Environmental Protection Agency Ecology and Oceanography of Harmful Algal Blooms R83-3221 and National Science Foundation Biological Oceanography Ocean Sciences 0722337 to D. A. Hutchins; Ocean Sciences 0850730 and a University of Southern California Women in Sciences and Engineering grant to F.-X. Fu; National Oceanic and Atmospheric Administration NA05-NOS4781228 to D. A. Caron; Knowledge Innovation Project of The Chinese Academy of Sciences KZCX2-YW-QN205, National Basic Research Program of China 2009CB421202, and National Natural Science Foundation of China 40776093, 40676089 to J. Sun. The authors also wish to thank two anonymous reviewers for helpful comments.

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Associate editor: John Albert Raven

Received: 04 September 2010
Accepted: 10 January 2011
Amended: 21 January 2011