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High CO_2 and Silicate Limitation Synergistically Increase the Toxicity of *Pseudo-nitzschia fraudulenta*

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Abstract

Anthropogenic CO_2 is progressively acidifying the ocean, but the responses of harmful algal bloom species that produce toxins that can bioaccumulate remain virtually unknown. The neurotoxin domoic acid is produced by the globally-distributed diatom genus *Pseudo-nitzschia*. This toxin is responsible for amnesic shellfish poisoning, which can result in illness or death in humans and regularly causes mass mortalities of marine mammals and birds. Domoic acid production by *Pseudo-nitzschia* cells is known to be regulated by nutrient availability, but potential interactions with increasing seawater CO_2 concentrations are poorly understood. Here we present experiments measuring domoic acid production by acclimatized cultures of *Pseudo-nitzschia fraudulenta* that demonstrate a strong synergism between projected future CO_2 levels (765 ppm) and silicate-limited growth, which greatly increases cellular toxicity relative to growth under modern atmospheric (360 ppm) or pre-industrial (200 ppm) CO_2 conditions. Cellular Si:C ratios decrease with increasing CO_2, in a trend opposite to that seen for domoic acid production. The coastal California upwelling system where this species was isolated currently exhibits rapidly increasing levels of anthropogenic acidification, as well as widespread episodic silicate limitation of diatom growth. Our results suggest that the current ecosystem and human health impacts of toxic *Pseudo-nitzschia* blooms could be greatly exacerbated by future ocean acidification and 'carbon fertilization' of the coastal ocean.

Introduction

The relentless consumption of fossil fuels is forcing carbon dioxide (CO_2) into the oceans at the rate of ~20–25 million tons day^{-1}, driving a steady decrease in seawater pH often termed ocean acidification [1,2]. Marine phytoplankton can be sensitive to the rising partial pressure of CO_2 (pCO_2) and lowered pH, as well as other concurrent environmental changes such as decreases in nutrient supplies to surface waters due to enhanced stratification [3]. Only of late, however, have oceanographers begun to examine how these ocean global change trends will affect the phytoplankton species that cause ecosystem-damaging toxic algal blooms [4]. Recent documented global increases in the frequency and severity of harmful algal blooms are hypothesized to be linked to changing anthropogenic atmospheric emissions and nutrient supplies, but direct causal evidence is lacking [5]. In particular, the possible synergistic effects of multiple climate change variables remain largely unexplored [3].

Among the most widely distributed and environmentally destructive harmful bloom species are diatoms of the genus *Pseudo-nitzschia* [6]. Many *Pseudo-nitzschia* species produce the potent toxin domoic acid, which binds with high affinity to vertebrate kainite-type ionotropic glutamate neuroreceptors [7]. Domoic acid is the agent of Amnesic Shellfish Poisoning, which causes illness or mortality in humans, marine mammals, and seabirds that ingest fish or shellfish that have been contaminated through bioaccumulation in coastal food webs [8,9]. Previous studies have highlighted multiple triggers for increased domoic

acid production, including nutrient limitation, extreme pH increases, trace metal availability and nitrogen source changes [10–16]. Recently Sun *et al.*, (2011) notably demonstrated that culture toxicity increased substantially when phosphorous-limitation is combined with higher pCO_2 in a *Pseudo-nitzschia multiseries* clone from Nova Scotia, Canada [17].

Extremely elevated levels of domoic acid have recently been reported from *Pseudo-nitzschia* blooms in coastal waters of Southern California [18]. One of the common species within these mixed blooms is *P. fraudulenta*. Our study examined how the availability of the required diatom nutrient silicate ($Si(OH)_4$) interacts with changing pH and pCO_2 (8.4 and 200 ppm pre-industrial; 8.2 and 360 ppm modern day; and 7.9 and 765 ppm, projected year 2100) to influence toxicity, growth, and silicon utilization in a *P. fraudulenta* clone isolated from Southern California waters.

Results and Discussion

We found that the concentration of domoic acid in *P. fraudulenta* cells increases significantly under $Si(OH)_4$ limitation, as has been reported in prior studies for other *Pseudo-nitzschia* species [10,11,19,20]. Our results demonstrate, however, that this previously recognized $Si(OH)_4$ effect on cellular toxin production rates is dramatically magnified (>250%) during growth at projected end-of-century atmospheric pCO_2 levels (765 ppm). Thus, these $Si(OH)_4$-limited diatom cells produce more than twice as much toxin when grown in acidified seawater. This nutrient/acidification synergism is environmentally relevant, as the

California coast where this species was isolated is among the first ocean regimes where anthropogenically-acidified surface seawater has been documented during upwelling events [21]. Coincidentally, Si(OH)$_4$ limitation of diatoms is also a common biogeochemical feature in parts of this regime [22].

This strong toxin production synergism between Si(OH)$_4$ limitation of *Pseudo-nitzschia fraudulenta* and ocean acidification is shown in Fig. 1a. Relative to nutrient-replete cultures grown at the same pH, the cellular domoic acid production rates (pg cell^{-1} day^{-1}) of Si(OH)$_4$-limited diatom cells were elevated ~4–7 fold (Fig. 1a, p<0.0001). Within these Si(OH)$_4$-limited treatments, cellular domoic acid production rates were highest in cultures acclimated at projected year 2100 seawater pH (~7.9). Toxin production rates within this acidified treatment were increased ~4.2-fold compared to cells maintained at pre-industrial pH (~8.4, p = 0.001), and 2.5-fold relative to those maintained at modern pH levels of ~8.2 (Fig. 1a, p = 0.004). There was a highly significant negative linear correlation between domoic acid production rates and pH in these Si(OH)$_4$-limited cultures (r^2 = 0.94). Toxin production rates of nutrient-replete cells were substantially reduced, but also increased significantly (p<0.01) in a linear manner (r^2 = 1.0) with successive decreases in pH (Fig. 1a, inset).

Similar to cellular production rates, cellular domoic acid quotas (total cellular toxin content, pg cell^{-1}) were 7–18-fold higher in Si(OH)$_4$-limited cultures relative to nutrient-replete cultures (Fig. 1b, p<0.0001). Domoic acid quotas in these Si(OH)$_4$-limited treatments were 3-fold higher at pH 7.9 than at pH 8.4 (p = 0.0003), and increased 1.4-fold at pH 7.9 relative to pH 8.2 (p = 0.002). The inverse relationship between toxin quotas and pH under Si(OH)$_4$-limitation was again highly linear (r^2 = 0.94) across the three pH levels. Due to the inherent chemical relationships of the seawater carbonate buffer system, in the Si(OH)$_4$-limited treatments cellular domoic acid quotas (and production rates, not shown) were also strongly positively correlated with pCO$_2$ (r^2 = 0.99, Fig. 1c). The much lower domoic acid content of nutrient-replete cells also increased at lower pH and higher pCO$_2$, but these toxin quota changes were not significant (panel insets, Fig. 1b,c, p>0.05).

As intended in our experimental design, specific growth rates of Si(OH)$_4$-limited *P. fraudulenta* cultures were significantly reduced compared to nutrient-replete cells in all three pCO$_2$ treatments (p<0.01, Fig. 2a). In the nutrient-replete cultures, there were significant progressive increases in growth rates across all three pCO$_2$ levels, with increases of 48% and 66% relative to the 200 ppm treatment at 360 and 765 ppm, respectively (p<0.0009). Si(OH)$_4$-limited culture growth rates also increased significantly with pCO$_2$, but to a lesser degree (p<0.03, Fig. 2a, inset). These trends were confirmed in the semi-continuous cultures by steady-state concentrations of particulate organic carbon and nitrogen, proxies for total diatom biomass in the treatments (Fig. 3a,b).
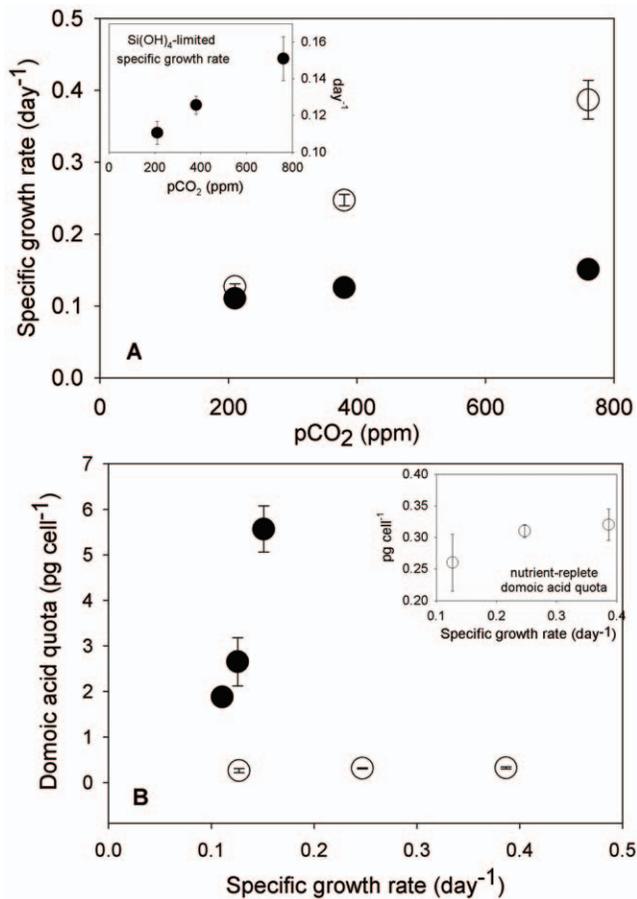
The relationship between growth rate and cellular domoic acid quota was bimodal. Elevated domoic acid levels were only produced by cultures below a specific growth rate threshold of ~0.15 d^{-1}; at all higher growth rates in the nutrient-replete cultures, cellular toxin quotas were low, and there was no evident relationship between growth rate and cellular toxin content across the data set as a whole (Fig. 2b). However, due to the stimulatory effect of CO$_2$ on both growth and toxicity, cellular domoic acid quotas were positively linearly correlated with growth rates within each nutrient treatment (Si(OH)$_4$-limited, r^2 = 0.99; nutrient-replete, r^2 = 0.96, Fig. 2b and inset).

The trends we observed in toxin levels could be due in part to changes in individual cell mass or biovolume under our
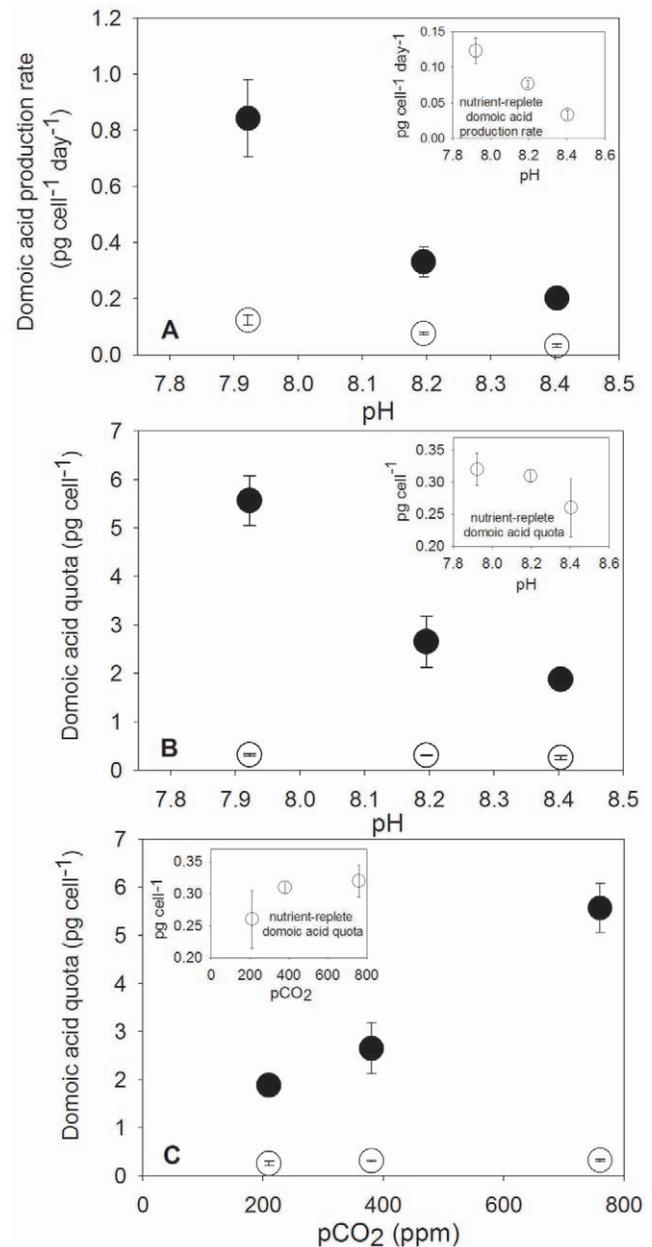
Figure 1. Interactive effects of pH and pCO$_2$ with nutrient limitation control *Pseudo-nitzschia fraudulenta* toxicity. Cellular domoic acid production rates (pg cell^{-1} day^{-1}) versus pH (**a**) and domoic acid quotas (pg cell^{-1}) versus pH (**b**) and pCO$_2$ (**c**) in *Pseudo-nitzschia fraudulenta* cultures grown under Si(OH)$_4$-limited (●) and nutrient-replete (○) conditions at seawater CO$_2$ concentrations of 200 ppm (preindustrial atmospheric levels), 360 ppm (modern levels), and 765 ppm (projected year 2100 levels). Panel insets present the nutrient-replete data with an expanded Y-axis scale for clarity. Error bars represent standard deviations of triplicates for each treatment.
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experimental treatments. Measurements of particulate organic carbon and nitrogen cell quotas (mol cell^{-1}) suggested a 1.5 to 2-fold increase in cell size in all Si(OH)$_4$-limited cultures relative to nutrient-replete treatments grown at the same pCO$_2$ levels (Fig. 3c,d, p<0.0001), a not-uncommonly observed phenomenon in slowly growing, Si(OH)$_4$-limited diatoms. Cell mass also increased slightly but not significantly with pCO$_2$ (Fig. 3c,d,

Figure 2. Relationships between nutrient-limited growth rates, pCO$_2$, and toxicity in *Pseudo-nitzschia fraudulenta*. Specific growth rates (day^{-1}) versus pCO$_2$ (**a**) and cellular domoic acid quota (pg cell^{-1}) versus specific growth rates (**b**) under Si(OH)$_4$-limited (●) and nutrient-replete (○) conditions at three seawater CO$_2$ concentrations (200, 360 and 765 ppm). Panel insets present the Si(OH)$_4$-limited (**a**) or nutrient-replete (**b**) data with an expanded Y-axis scale for clarity. Error bars represent standard deviations of triplicates for each treatment.
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p>0.05). Thus the higher cell-normalized toxin levels in Si(OH)$_4$-limited, high CO$_2$-grown cells could be partially related to increases in the size and volume of individual *P. fraudulenta* cells. However, the observed increases in cellular toxin quotas in our experimental Si(OH)$_4$-limited treatments (7–18-fold) were up to an order of magnitude greater than the corresponding increases in cell mass suggested by the data in Fig. 3c,d, so both nutrient limitation and ocean acidification appear to have physiological consequences resulting in large increases in toxin production per unit of diatom biomass.

Cellular biogenic silica to particulate organic carbon (Si:C) ratios were significantly lower at 765 than at 200 ppm ppm CO$_2$ in both Si(OH)$_4$ treatments (p<0.05), although neither was significantly different from Si:C ratios at 360 ppm (p>0.05, Fig. 4a). These data are corroborated by the findings of Sun *et al.*, (2011) in a *P. multiseries* clone grown at a similar range of pCO$_2$ [17]. Milligan *et al.*, (2004) observed lower Si cell quotas (mol Si cell^{-1}) under more acidic conditions in cultures of the centric diatom *Thalassiosira weissflogii* due to higher cellular Si efflux:influx ratios [23]. However, unlike the results of Milligan et al., cellular Si quotas in our *P. fraudulenta* isolate did not differ significantly between CO$_2$ treatments (data not shown), and the Si:C trends we

observed were instead due entirely to increasing cellular particulate organic carbon quotas at higher CO$_2$ concentrations (Fig. 3c). It is notable that in our pennate diatom, Si:C increases at low CO$_2$ occurred even in Si(OH)$_4$-limited cultures (Fig. 4a).

Cellular Si:C ratios were negatively linearly related to culture growth rates in both Si(OH)$_4$-limited (r^2 = 0.99) and Si(OH)$_4$-replete (r^2 = 0.77) cultures (Fig. 4b). This observation that Si:C ratios are highest in diatoms under CO$_2$-limited growth conditions (Fig. 4b) is similar to the Si:C increases typically seen in diatoms growing under iron limitation [22,24]. The inverse trends in cellular Si:C ratios relative to CO$_2$ levels and growth rates are however the opposite of the positive linear correlations we observed for toxin production rates and quotas versus these same two variables (Figs. 1c, 2b).

The synergism between elevated pCO$_2$ and nutrient limitation in a widespread *Pseudo-nitzschia* species suggests that ocean acidification or 'carbon fertilization' could dramatically amplify the already considerable worldwide ecosystem impacts of toxic blooms, especially under commonly occurring nutrient-limited conditions. The west coast of North America where we isolated this clone is the first region where upwelling of anthropogenically-acidified seawater has been unambiguously demonstrated [21]. This same coastline is also plagued by large, frequently recurring toxic *Pseudo-nitzschia* blooms [18,25,26]. When fossil fuel-derived ocean acidification combines with naturally elevated pCO$_2$ from upwelled deeper waters, surface water pH values here can be as low as 7.6 and pCO$_2$ levels can reach 1000–1200 ppm [21]. By comparison, pH values of ~7.9 and pCO$_2$ of 765 ppm more than doubled diatom toxin production rates in our cultures, relative to modern atmospheric pCO$_2$ levels (Fig. 1a).

In our experiments, CO$_2$-driven increases in toxin production were much more evident in cultures limited by Si(OH)$_4$ than in nutrient-replete treatments. Regulation of domoic acid production has been previously linked to Si(OH)$_4$ limitation in several other *Pseudo-nitzschia* species [10,11,19,20]. Inverse correlations between Si(OH)$_4$ levels and domoic acid concentrations have been demonstrated during *Pseudo-nitzschia* blooms in southern California waters, and modeling approaches have suggested a role for Si(OH)$_4$ limitation in determining domoic acid toxicity throughout much of central and southern California [18,27,28]. Chronic iron limitation of diatoms in some California upwelling centers also leads to ecosystem-level Si(OH)$_4$-limitation, since iron-stressed cells produce heavier silica frustules and so deplete Si(OH)$_4$ from surface waters long before other nutrients [22,24].

The combination of high pCO$_2$, acidified water with frequent and widespread Si(OH)$_4$ limitation of toxic *Pseudo-nitzschia* assemblages could have major consequences for marine environmental health in the California region, and in other areas where similar conditions exist. Indeed, an environmental survey data set from the northern Gulf of Mexico shows correlations between low Si(OH)$_4$ availability, elevated dissolved inorganic carbon concentrations, and increased toxin levels in *Pseudo-nitzschia* cells [29]. Our study is also validated by the findings of Sun *et al.*, (2011), who found elevated domoic acid levels in another species (*P. multiseries*) cultured under phosphorous-limited, low pH (high pCO$_2$) conditions [17]. However, our results may have considerably broader environmental implications since previous studies suggest that Si(OH)$_4$-limitation of *Pseudo-nitzschia* growth is likely to be much more common and widespread than phosphorous-limitation [18,27,28].

In addition to increased toxicity, increasing pCO$_2$ resulted in significantly higher specific growth rates for both nutrient-replete and Si(OH)$_4$-limited cells (Fig. 2a), as well as increased carbon fixation rates (data not shown). Thus, ocean acidification could not
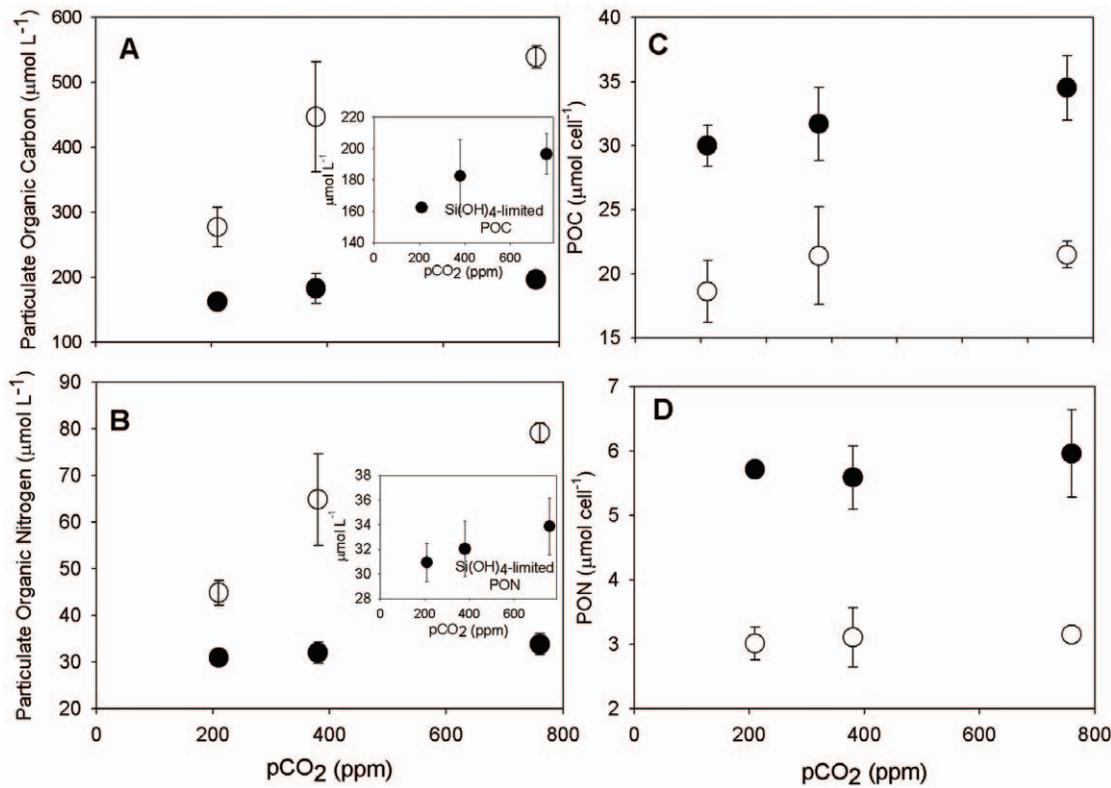
Figure 3. Steady-state particulate organic carbon (POC) (a) and nitrogen (PON) (b) concentrations (μmol L^{-1}), particulate organic carbon (POC) (c) and nitrogen (PON) (d) cell quotas (mol cell^{-1}) in semi-continuous *Pseudo-nitzschia fraudulenta* cultures grown under Si(OH)$_4$-limited (black circles, ●) and nutrient-replete (open circles, ○) conditions at three seawater CO$_2$ concentrations (200, 360 and 765 ppm). Panel insets present the Si(OH)$_4$-limited data with an expanded Y-axis scale for clarity. Error bars represent standard deviations of triplicates for each treatment.
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only enhance the toxicity of *Pseudo-nitzschia* blooms, but may also increase growth rates and biomass, even under Si(OH)$_4$-limited conditions when cellular domoic acid production is elevated.

The physiological and genetic mechanisms underlying this CO$_2$-nutrient limitation toxicity synergism are undetermined at present, but may have to await elucidation of the full biosynthetic pathway of domoic acid. Complete synthesis results from condensation of an alpha-ketoglutarate derivative stemming from the tricarboxylic acid cycle with a C-10 isoprenoid unit likely originating from an unknown compartmentalized pathway within the cell. As this latter component of the synthetic pathway is poorly understood [30], our ability to obtain a truly mechanistic picture of how domoic acid production is regulated by environmental variables is limited. Application of modern transcriptomic and proteomic methods to toxic *Pseudo-nitzschia* cultures of varying potencies is likely to be productive in this regard; the ability to quantitatively and sensitively up- and down-regulate cellular domoic acid levels by manipulating the nutrient conditions and pCO$_2$ at which cultures are grown may prove invaluable for such mechanistic molecular studies. Future work also needs to examine how the interactions between rising pCO$_2$ and other global change trends such as warming temperatures and increased irradiance in shallower ocean mixed layers [3,4] will affect *Pseudo-nitzschia* growth and toxicity. In particular, increasing pCO$_2$ combined with iron limitation, which was also demonstrated to regulate domoic acid production [14,15,31], should be considered in future studies, as well as co-limitation by multiple factors including iron, light, silicate, and nitrate [32].

The implications of our results are not limited to the distant future. In fact, the range of pCO$_2$ levels found in anthropogenically-acidified waters of the California upwelling regime today already encompasses those used in our study, including projected average year 2100 atmospheric concentrations [21]. Thus, *Pseudo-nitzschia* bloom toxicity may already be affected by changing pCO$_2$ levels here, and this area may be considered a natural laboratory in which to evaluate the effects of future global ocean acidification on harmful algal blooms. Strong synergistic effects of rising pCO$_2$ with other variables such as nutrient limitation emphasize the need for heightened future vigilance by marine environmental regulatory and protection agencies, as well as a possible need to add new parameters such as seawater carbonate system measurements to existing harmful algal bloom detection and monitoring efforts. Such unprecedented measures may well be necessary to help mitigate escalating environmental and economic damage from increasingly toxic *Pseudo-nitzschia* blooms in a rapidly acidifying ocean.

Materials and Methods

Culture, Media, and Sampling

Pseudo-nitzschia fraudulenta was micropipette isolated from public nearshore water collected at 34.08 N, 119.05 W in Ventura County, California in March, 2010. This isolate, designated USC WWA7, was maintained at 16°C on a 12-h light: 12-h dark cycle in modified f/2 enriched seawater growth media [33] under 90 photons m^{-2} s^{-1} of cool white fluorescent illumination. Silicate
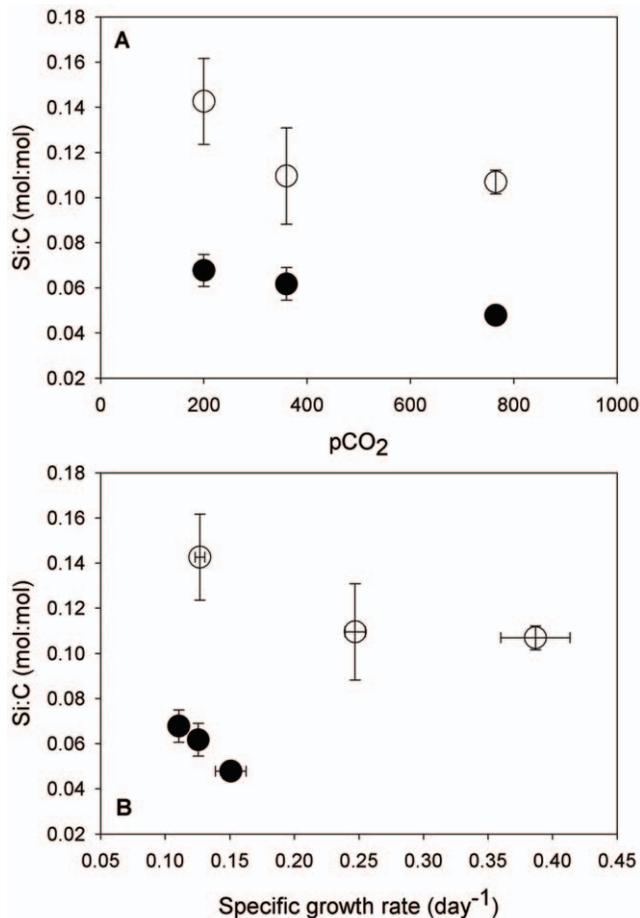
Figure 4. Relationships between cellular Si:C ratios, pCO₂, and growth rates in _Pseudo-nitzschia fraudulenta_. Cellular silica to particulate organic carbon (Si:C, mol:mol) ratios versus pCO₂ (**a**) and cellular Si:C versus specific growth rates (**b**) under Si(OH)₄-limited (●) and nutrient-replete (○) conditions at three seawater CO₂ concentrations (200, 360 and 765 ppm). Error bars represent standard deviations of triplicates for each treatment.
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(Si(OH)₄) final concentrations in the medium were 10.6 μM for the Si(OH)₄-limited treatment and 106.1 μM for the nutrient-replete treatment; these concentrations were chosen based on previous experience with cultures of _Chaetoceros_ and _Thalassiosira_ spp. Dissolved CO₂ concentrations were controlled by gentle bubbling with commercially prepared air/CO₂ mixtures (Praxair Gas) at approximately 120 bubbles·min⁻¹ with in-line HEPA filters to avoid particulate contamination.

Cell Counts and Growth Rates

Steady state semi-continuous culture methodology was employed to maintain cultures in exponential state and specific growth rates were calculated as described in Sun _et al._, (2011) [17]. Growth rate as determined by bi-weekly microscopic cell counts and _in vivo_ fluorescence determined the dilution rate of each bottle. Cells of _P. fraudulenta_ preserved in acidified Lugol's solution were vortexed and enumerated by direct counts using an Accu-Scope 3032 inverted microscope according to the Utermöhl method [34]. A minimum of 300 cells were counted to guarantee a 95% confidence interval with +/−11.5% accuracy [35]. Cultures were acclimated for a period of three months to the respective

experimental conditions prior to splitting into triplicates, which were then further acclimated for one month (5–17 cell divisions) prior to final sampling.

Domoic acid by high performance liquid chromatography

High performance liquid chromatography with ultraviolet detection (HPLC-UV) of domoic acid was performed using a SCL-10ADVP controlled system (Shimadzu). The UV detector was programmed for sample and reference wavelengths of 242 and 280 nm respectively and the system was operated by EZ START software version 7.4 SP1 (Shimadzu).

Cellular concentrations of domoic acid were determined according to Mafra et al., (2009) with slight modifications [36]. Culture subsamples of 10–20 ml were carefully measured and cells were collected by gentle filtration on 25 mm GF/F filters (Whatman). The filters were stored in the dark at −20°C. Filters were subsequently subject to sonication in 10% aqueous methanol for 2 min. at 40W in a water bath. The cell extracts were then clarified by centrifugation at 3000× _g_ for 10 min at 4°C. The pellet was discarded and the clarified extracts were transferred to 300 μl polyspring inserts (National Scientific) placed inside clean 2.0 ml Target DP™ vials (National Scientific). Prior to analysis, all samples were treated with 0.15% trifluoroacetic acid (TFA).

Briefly, the chromatographic separation was carried out on a reversed phase Luna C18 (2) column (3 μm, 2×100 mm, Phenomenex) at 25° C with a mobile phase system consisting of water with 0.1% TFA (A), and acetonitrile (MeCN) with 0.1% TFA (B). The elution gradient began with a 10–35% B transition over 10 min, then was held at 35% B until 15 min, followed by a subsequent decrease to 10% B at 16 min, and held at 10% B. The flow rate was 0.2 ml min⁻¹ and the injection volume 5–10 μl. Quantification of domoic acid was determined using certified reference material CRM-domoic acid-e obtained from the National Resource Council, Canada at a range of concentrations.

Calibration curves of CRM-domoic acid-e were determined by linear regressions (r^2 values≥0.99) for each sample treatment. Interpolation from the standard curves was used to calculate the amount of compound injected from the peak areas of each sample under the same experimental conditions. Domoic acid per cell concentrations were determined by the ratio of reconstituted volume (300 μl) to the volume injected (5–10 μl) and dividing by the total cell count in the original sample. Reported values represent means of the results (n = 3). Domoic acid production rates were calculated by multiplying the growth rate by toxin per cell.

Carbonate Buffer System analysis

Dissolved inorganic carbon analysis was performed using a CM5230 CO₂ coulometer (UIC) [17]. Samples were transferred carefully without agitation into 25 ml liquid scintillation vials fitted with Teflon (PFTE) coated caps, poisoned with 200 μl of 5% HgCl₂ l⁻¹, and stored at 4°C until analysis. pH was determined on freshly collected samples using a calibrated Orion 5-star plus pH meter using an NBS buffer system with three-point calibration. The pCO₂ in the experimental media was calculated from these two parameters using CO₂SYS software [37]. Measured NBS scale pH values averaged across all the replicates in the three treatments were 8.43, 8.23, and 7.95; measured total dissolved inorganic carbon concentrations were 1965, 2107, and 2249 μmol kg⁻¹; and the corresponding calculated pCO₂ values were 198, 357, and 764 ppm. For clarity, pCO₂ treatments in the cultures are referred to in the text using rounded-off values of

200 ppm, pre-industrial atmospheric levels; 360 ppm, modern atmospheric levels: and 765 ppm, projected year 2100 levels [38].

Chlorophyll *a*

For chlorophyll *a* (Chl *a*) measurements, samples were filtered in duplicate onto 25 mm GF/F filters. Five ml of 90% acetone was later added and each vial was allowed to extract overnight in the dark at -20°C. After twenty-four hours, Chl *a* was determined using a Turner Designs 10-AU fluorometer [39].

Particulate organic carbon and nitrogen

For particulate organic carbon (POC) and nitrogen (PON), sample volumes of 20 ml were collected onto pre-combusted (450°C for 5 h) GF/F glass fiber filters, stored at -20°C, and dried at 55°C before analysis. Molar POC and PON content was analyzed using a 4010 Costech Elemental Combustion System calibrated with methionine and atropine as reference materials according to the methods in Hutchins *et al.*, (1998) and Fu *et al.*, (2007) [40,41].

Biogenic Silica

Sample volumes of 20 ml were collected onto 0.6 μM polycarbonate filters, dried and stored at ambient temperature

until analysis. Cellular biogenic silica (BSi) was measured according to Brzezinski and Nelson (1995) [42].

Statistics

Differences between treatments for all parameters were tested using two-way ANOVA and the individual treatments were compared using Tukey's Multiple Comparison Test or Student's t-test. All analyses were performed using SigmaPlot 10 software.

Author Contributions

Conceived and designed the experiments: AOT DAH FF. Performed the experiments: AOT FF. Analyzed the data: AOT DAH FF. Contributed reagents/materials/analysis tools: AOT DAH FF. Wrote the paper: AOT DAH.

References

1. Doney SC, Fabry VJ, Feely RA, Kleypas JA (2009) Ocean acidification: The other CO$_2$ problem. Annual Rev Mar Sci 1: 169–192.
2. Dybas CL (2006) On a collision course: oceans plankton and climate change. BioScience 56(8): 642–646.
3. Boyd PW, Strzepzk R, Fu F, Hutchins DA (2010) Environmental control of open-ocean phytoplankton groups: Now and in the future. Limnol Oceanogr 55(3): 1353–1380.
4. Hutchins DA, Mulholland MR, Fu F (2009) Nutrient cycles and marine microbes in a CO$_2$-enriched ocean. Oceanography 22: 128–145.
5. Hallegraeff G (2010) Ocean climate change, phytoplankton community responses and harmful algal blooms: A formidable predictive challenge. J Phycol 46(2): 220–225.
6. Hasle GR (2002) Are most of the domoic acid-producing species of the diatom genus *Pseudo-nitzschia* cosmopolites? Harmful Algae 1(2): 137–146.
7. Peng YG, Ramsdell JS (1996) Brain fos induction is a sensitive biomarker for the lowest observed neuroexcitatory effects of domoic acid. Fund Appl Toxicol 31: 162–168.
8. Scholin CA, Gulland F, Doucette GJ, Benson S, Busman M, et al. (2000) Mortality of sea lions along the central California coast linked to a toxic diatom bloom. Nature 403: 80–84.
9. Bates SS, Trainer VL (2006) Diatoms. In: Graneli E, Turner JT, eds. Ecology of Harmful Algae. Heidelberg: Springer-Verlag. pp 81–93.
10. Pan Y, Bates SS, Cembella AD (1998) Environmental stress and domoic acid production by *Pseudo-nitzschia*: a physiological perspective. Nat Toxins 6: 127–135.
11. Bates SS, Garrison DL, Horner RA (1998) Bloom dynamics and physiology of domoic acid-producing *Pseudo-nitzschia* species. In: Anderson DM, Cembella AD, Hallegraeff GM, eds. Physiological Ecology of Harmful Algal Blooms. Heidelberg: Springer-Verlag. pp 267–292.
12. Lundholm N, Hansen PJ, Kotaki Y (2004) Effect of pH on growth and domoic acid production by potentially toxic diatoms of the genera *Pseudo*-nitzschia and Nitzschia. Mar Ecol Prog Ser 273: 1–15.
13. Trimborn S, Lundholm N, Thoms S, Richter KU, Krock B, et al. (2008) Inorganic carbon acquisition in potentially toxic and non-toxic diatoms: the effect of pH-induced changes in seawater carbonate chemistry. Physiol Plant 133: 92–105.
14. Maldonado MT, Hughes MP, Rue EL, Wells ML (2002) The effect of Fe and Cu on growth and domoic acid production by *Pseudo-nitzschia multiseries* and *Pseudo-nitzschia australis*. Limnol Oceanogr 47: 515–526.
15. Wells ML, Trick CG, Cochlan WP, Hughes MP, Trainer VL (2005) Domoic acid: The synergy of iron, copper, and the toxicity of diatoms. Limnol Oceanogr 50(6): 1908–1917.
16. Armstrong-Howard MD, Cochlan WP, Ladizinsky N, Kudela RM (2007) Nitrogenous preference of toxigenic *Pseudo-nitzschia australis* (Bacillariophyceae) from field and laboratory experiments. Harmful Algae 6(2): 206–217.
17. Sun J, Hutchins DA, Feng Y, Seubert EL, Caron DA, et al. (2011) Effects of changing pCO$_2$ and phosphate availability on domoic acid production and physiology of the marine harmful bloom diatom *Pseudo-nitzschia multiseries*. Limnol Oceanogr 56(3): 829–840.
18. Schnetzer A, Miller P, Schaffner RA, Stauffer B, Jones B, et al. (2007) Blooms of *Pseudo-nitzschia* and domoic acid in the San Pedro Channel and Los Angeles harbor areas of the Southern California Bight, 2003-2004. Harmful Algae 6: 372–387.
19. Pan Y, Subba Rao DV, Mann KH, Brown RG, Pocklington R (1996b) Effects of silicate limitation on production of domoic acid, a neurotoxin, by the diatom *Pseudo-nitzschia multiseries*. I. Batch culture studies. Mar Ecol Prog Ser 131: 225–33.
20. Pan Y, Subba Rao DV, Mann KH, Li WKW, Harrison WG (1996c) Effects of silicate limitation on production of domoic acid, a neurotoxin, by the diatom *Pseudo-nitzschia multiseries* II. Continuous culture studies. Mar Ecol Prog Ser 131: 235–243.
21. Feely RA, Sabine CL, Hernandez-Ayon JM, Ianson D, Hales B (2008) Evidence for upwelling of corrosive "acidified" water onto the continental shelf. Science 320(5882): 1490–1492.
22. Hutchins DA, Bruland KW (1998) Iron-limited diatom growth and Si:N uptake ratios in a coastal upwelling regime. Nature 393: 561–564.
23. Milligan AJ, Varela D, Brzezinski MA, Morel FMM (2004) Dynamics of silicon metabolism and silicon isotopic discrimination in a marine diatom as a function of pCO$_2$. Limnol Oceanogr 49(2): 322–329.
24. Firme GF, Rue EL, Weeks DA, Bruland KW, Hutchins DA (2003) Spatial and temporal variability in phytoplankton iron limitation along the California coast and consequences for Si, N, and C biogeochemistry. Global Biogeochem Cy 17(1): 1016.
25. Wood ES, Benitez-Nelson C, Morton S, Anderson C, Burrell C, et al. (2011) *Pseudo-nitzschia* and domoic acid fluxes in Santa Barbara Basin (CA) from 1993 to 2008. Harmful Algae 10(6): 567–575.
26. Anderson CR, Brzezinski MA, Washburn L, Kudela RM (2006) Circulation and environmental conditions during a toxigenic *Pseudo-nitzschia australis* bloom in the Santa Barbara Channel, California. Mar Ecol Prog Ser 327: 119–133.
27. Lane JQ, Raimondi PT, Kudela RM (2009) Development of a logistic regression model for the prediction of toxigenic *Pseudo-nitzschia* blooms in Monterey Bay, California. Mar Ecol Prog Ser 383: 37–51.
28. Anderson CR, Siegel DA, Kudela RM, Brzezinski MA (2009) Empirical models of toxigenic *Pseudo-nitzschia* blooms: potential use as a remote detection tool in the Santa Barbara Channel. Harmful Algae 8(3): 478–492.
29. Macintyre HL, Stutes AL, Smith WL, Dorsey CP, Abraham A, et al. (2011) Environmental correlates of community composition and toxicity during a bloom of *Pseudo-nitzschia* spp. in the northern Gulf of Mexico. J Plank Res 33(2): 273–295.
30. Ramsey UP, Douglas DJ, Walter JA, Wright JLC (1998) Biosynthesis of domoic acid by the diatom *Pseudo-nitzschia multiseries*. Nat Toxins 6(3–4): 137–146.
31. Trick CG, Bill BD, Cochlan WP, Wells ML, Trainer VL, et al. (2010) Iron enrichment stimulates toxic diatom production in high nitrate, low-chlorophyll areas. Proc Natl Acad Sci USA 107: 5887–5892.
32. Saito MA, Goepfert TJ, Ritt JT (2008) Some thoughts on the concept of co-limitation: Three definitions and the importance of bioavailability. Limnol Oceanogr 53: 276–290.
33. Guillard RRL (1975) Culture of phytoplankton for feeding marine invertebrates. In Smith WL, Chanley MH, eds. Culture of Marine Invertebrate Animals. New York: Plenum Press. pp 26–60.

34. Utermöhl H (1931) Neue Wege in der quantitativen Erfassung des Planktons. (Mit besonderer Berücksichtigung des Ultraplanktons). Verh Int Verein Limnol 5: 567–596.

35. Guillard RRL (1973) Division Rates. In: Stein J, ed. Culture Methods and Growth Measurements. New York: Cambridge University Press. pp 289–312.

36. Mafra LL, Jr., Leger C, Bates SS, Quilliam MA (2009) Analysis of trace levels of domoic acid in seawater and plankton by liquid chromatography without derivatization, using UV or mass spectrometry detection. J Chromatogr A 1216(32): 6003–11.

37. Lewis E, Wallace DWR Program developed for CO₂ system calculations, Carbon Dioxide Information Analysis Center, Report ORNL/CDIAC-105, Oak Ridge National Laboratory, Oak Ridge, Tennessee, USA.

38. Alley RB, Berntsen T, Bindoff, NL, Chen Z, Chidthiasong A, et al. (2007) Summary for policymakers. In: Solomon S, Qin D, Manning M, Chen Z, Marquis M, et al. (2007) Climate change 2007: The physical science basis. Contribution of Working Group I to the fourth assessment report of the Intergovernmental Panel on Climate Change. New York: Cambridge University Press.

39. Welschmeyer NA (1994) Fluorometric analysis of chlorophyll a in the presence of chlorophyll b and phaeopigments. Limnol Oceanogr 39: 1985–1992.

40. Hutchins DA, DiTullio GR, Zhang Y, Bruland KW (1998) An iron limitation mosaic in the California coastal upwelling regime. Limnol Oceanogr 43: 1037–54.

41. Fu F, Warner ME, Zhang Y, Feng Y, Hutchins DA (2007) Effects of increased temperature and CO₂ on photosynthesis, growth and elemental ratios of marine *Synechococcus* and *Prochlorococcus* (Cyanobacteria). J Phycol 43: 485–496.

42. Brzezinski MA, Nelson DM (1995) The annual silica cycle in the Sargasso Sea near Bermuda. Deep Sea Res 42: 1215–1237.