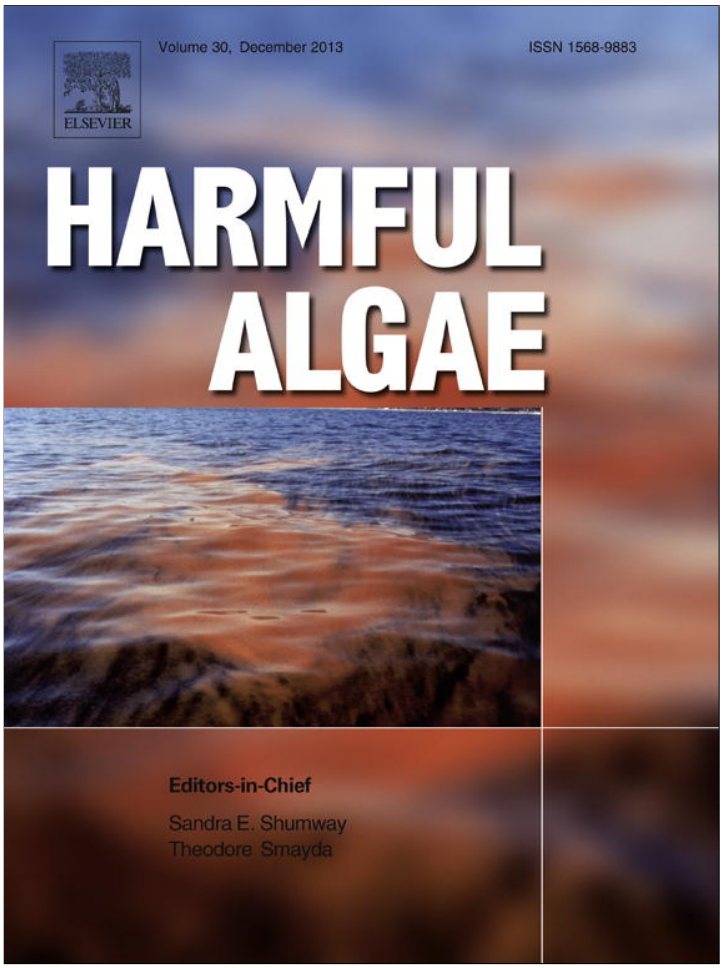
Provided for non-commercial research and education use.
Not for reproduction, distribution or commercial use.



This article appeared in a journal published by Elsevier. The attached copy is furnished to the author for internal non-commercial research and education use, including for instruction at the authors institution and sharing with colleagues.

Other uses, including reproduction and distribution, or selling or licensing copies, or posting to personal, institutional or third party websites are prohibited.

In most cases authors are permitted to post their version of the article (e.g. in Word or Tex form) to their personal website or institutional repository. Authors requiring further information regarding Elsevier's archiving and manuscript policies are encouraged to visit:

http://www.elsevier.com/authorsrights

Author's personal copy

Harmful Algae 30 (2013) 37–43

Contents lists available at ScienceDirect

Harmful Algae

journal homepage: www.elsevier.com/locate/hal

High CO_2 promotes the production of paralytic shellfish poisoning toxins by *Alexandrium catenella* from Southern California waters

Avery O. Tatters [a], Leanne J. Flewelling [b], Feixue Fu [a], April A. Granholm [b], David A. Hutchins [a,*]

[a] Department of Biological Sciences, University of Southern California, 3616 Trousdale Parkway, Los Angeles, CA 90089, United States
[b] Florida Fish and Wildlife Conservation Commission, Fish and Wildlife Research Institute, 100 8th Avenue SE, St. Petersburg, FL 33701, United States

ARTICLE INFO

Article history:
Received 6 July 2013
Received in revised form 27 August 2013
Accepted 28 August 2013

Keywords:
Alexandrium catenella
Dinoflagellate
Global warming
Harmful algae
Ocean acidification
Paralytic shellfish poisoning toxins
Saxitoxin

ABSTRACT

In many dinoflagellates, cellular toxin levels have been demonstrated to increase when growth is limited by essential nutrients such as phosphorus. Despite the recognized importance of nutrient limitation to dinoflagellate toxicity, interactions with current and future global environmental change variables have been relatively unexplored. This is a critical question, as dissolution of anthropogenic CO_2 emissions into seawater is leading to progressively lower pH values, or ocean acidification. Sea surface temperatures are concurrently increasing, a trend that is also projected to continue in the future. We conditioned a clonal culture of paralytic shellfish poisoning toxin producing *Alexandrium catenella* (A-11c) isolated from coastal Southern California to factorial combinations of two temperatures, two pCO_2 levels, and two phosphate concentrations for a period of eight months. Interactions between these variables influenced growth and carbon fixation rates and although these treatments only elicited minor differences in toxin profile, total cellular toxicity was dramatically affected. Cells conditioned to high pCO_2 (levels projected for year 2075) and low phosphate at low temperature (15 °C) were the most toxic, while lower pCO_2, higher phosphate levels, and warmer temperature (19 °C) alleviated this toxicity to varying degrees. Overall increased pCO_2 generally led to enhanced potency. Our results suggest that future increased ocean acidification may exacerbate the toxic threat posed by this toxic dinoflagellate, especially when combined with nutrient limitation, but that future warmer temperatures could also offset some of this enhanced toxicity.

© 2013 Elsevier B.V. All rights reserved.

1. Introduction

Dinoflagellates of the class Dinophyceae are prominent members of microalgal communities, particularly in estuaries and coastal oceans. Recent observations suggest an increasing frequency and intensification of noxious proliferations or harmful blooms caused by some members of this group (Hallegraeff, 1993, 2010). This phenomenon could be related to increased sea surface temperatures, ocean acidification, and their associated interactive effects with other environmental drivers like nutrient availability (Fu et al., 2012). The propensity of some harmful dinoflagellates to opportunistically form dense, sometimes visible blooms coupled with an ability to synthesize a variety of biologically active and often toxic chemicals underscores their importance to human and ecosystem health.

The genus *Alexandrium* is comprised of over thirty species, of which more than half are confirmed producers of the neurotoxin saxitoxin (STX) and its derivatives, collectively known as paralytic shellfish poisoning toxins (PSPs) (Kodama, 2010; Anderson et al., 2012). Paralytic shellfish poisoning (PSP) is a potentially life-threatening syndrome that is primarily caused by the consumption of contaminated shellfish. PSPs are heat stable tetrahydropurines that act as potent, selective muscular and neuronal sodium channel blockers (Catterall, 1980; Anderson et al., 1990). These chemicals ($n > 20$) are divided into four subgroups (carbamate, N-sulfo-carbamoyl, decarbamoyl and deoxydecarbamoyl) that are indicative of their potencies, which span orders of magnitude (Boyer et al., 1987; Hall et al., 1990; Hallegraeff et al., 1995; Quilliam et al., 2001) (Table 1).

The production of PSPs is influenced by numerous environmental conditions including salinity, light and nutrient concentrations (White, 1978; Hall et al., 1982; Ogata et al., 1987; Boyer et al., 1987; Parkhill and Cembella, 1999). Although there have been considerable efforts to investigate these environmental influences on *Alexandrium* toxicity, surprisingly few genus-wide correlations were observed. These findings emphasize the well-known interspecific variability and less explored intraspecific variability reported from this genus, as well as from other

* Corresponding author. Tel.: +1 213 740 5616.
E-mail address: dahutch@usc.edu (D.A. Hutchins).

1568-9883/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.hal.2013.08.007

Author's personal copy

Table 1
PSP congeners detected in *Alexandrium catenella* A-11c. Specific toxicity of each compound expressed as mouse units (MU) per μmole of toxin in which 1 MU is a dose sufficient to kill a ~20 g male mouse (strain ddy) in 15 min (Oshima, 1995).

Compound	Potency	Compound	Specific toxicity
STX	2483	C2	239
GTX-1	2468	GTX-5	160
neoSTX	2295	C1	15
GTX-4	1803		

dinoflagellates (Anderson et al., 1990; Gribble et al., 2005; Burkholder and Glibert, 2009; Masseret et al., 2009; Adolf et al., 2009; Bachvaroff et al., 2009; Tillmann et al., 2009; Kremp et al., 2012).

The buildup of anthropogenic carbon dioxide in the surface ocean is progressively contributing to a phenomenon known as ocean acidification (Sabine et al., 2004; Feely et al., 2008). Simultaneously, the 'greenhouse' effects of atmospheric CO_2 increases are leading to the elevation of sea surface temperatures in many regions (Cane et al., 1997; Xie et al., 2010). The responses of a variety of marine phytoplankton to these two global change variables are being increasingly examined (Beardall and Raven, 2004; Hutchins et al., 2009; Hallegraeff, 2010; Fu et al., 2012). There are, however, only a few reports of how ocean acidification may affect the toxicity and physiology of harmful algal species (Fu et al., 2008, 2010; Sun et al., 2011; Tatters et al., 2012a, 2013; Kremp et al., 2012; Flores-Moya et al., 2012; reviewed by Fu et al., 2012). Even fewer studies have examined the combined effects of ocean acidification and temperature on species within the genus *Alexandrium* (Kremp et al., 2012; Flores-Moya et al., 2012). The interactive effects of ocean acidification, temperature and nutrient limitation on toxin-producing *Alexandrium* have not been documented, even though it is evident that future blooms of PSP-producing species will often be affected by all three of these factors concurrently. To investigate this, we measured growth and PSP toxin production in an *Alexandrium catenella* (Whedon and Kofoid) Balech clone isolated from Southern California coastal waters following eight months of conditioning to an experimental matrix of two pCO_2 concentrations, two temperatures and two conditions of phosphate-availability.

2. Materials and methods

2.1. Sample collection, cell isolation and culture maintenance

The strain of *Alexandrium catenella* used in this study, designated clone A-11c, was micropipette isolated from a water sample collected at Jalama Beach, Santa Barbara County, California, USA in March of 2011. This isolate was maintained in autoclaved L1/20 enriched-seawater growth media minus silicate (Guillard and Hargraves, 1993) at a salinity of 35 on a 12-h light:12-h dark cycle under a near-saturation photon fluence rate of 90 μmol m^{-2} s^{-1} of cool white fluorescent illumination at 15 and 19 °C. A subculture was transferred into L1/20 experimental media (replete: 32 μm NO_3^- and 2 μm PO_4^{3-} and phosphate-limited: 32 μm NO_3^- and 0.4 μm PO_4^{3-}) within sterile tissue culture flasks approximately one week after isolation. After one month, aliquots of these cultures were inoculated into experimental media and subject to gentle bubbling in 100 mL volumes using commercially prepared air/CO_2 mixtures (Praxair) at two concentrations. A preliminary investigation revealed no significant differences in growth rates for unbubbled cultures compared to gently aerated cultures ($p > 0.05$). For phosphate-replete treatments at 15 °C the unbubbled cultures grew at 0.17 ± 0.01 day^{-1} and bubbled at 0.16 ± 0.02 day^{-1}. For phosphate-limited treatments at 19 °C, growth rates were 0.07 ± 0.01 day^{-1} and 0.09 ± 0.01 day^{-1} for unbubbled and bubbled cultures, respectively. This methodology has been employed for other ocean acidification experiments focusing on dinoflagellates (Fu et al., 2008, 2010; Tatters et al., 2013).

Table 2
Experimental conditions representing combinations of pCO_2, temperature and phosphate concentrations employed in this study.

Experimental treatments	
Control replete	380 μatm pCO_2; 15 °C; 2 μm PO_4^{3-}
High temperature-replete	380 μatm pCO_2; 19 °C; 2 μm PO_4^{3-}
High pCO_2-replete	750 μatm pCO_2; 15 °C; 2 μm PO_4^{3-}
Greenhouse-replete	750 μatm pCO_2; 19 °C; 2 μm PO_4^{3-}
Control limited	380 μatm pCO_2; 15 °C; 0.4 μm PO_4^{3-}
High temperature-limited	380 μatm pCO_2; 19 °C; 0.4 μm PO_4^{3-}
High pCO_2-limited	750 μatm pCO_2; 15 °C; 0.4 μm PO_4^{3-}
Greenhouse-limited	750 μatm pCO_2; 19 °C; 0.4 μm PO_4^{3-}

2.2. Experimental conditioning and treatments

The three variable factorial matrix design of the experiment used phosphate-replete (2 μm) and phosphate-limited (0.4 μm) cultures, each grown in triplicate under four pCO_2/temperature conditions: 'control' (15 °C, low pCO_2), 'high pCO_2' (15 °C, high pCO_2), 'high temperature' (19 °C, low pCO_2), and 'greenhouse' (19 °C, high pCO_2). This eight-treatment experimental matrix is shown in Table 2. To insure that the experimental cultures were fully acclimated to the temperature, pCO_2 and nutrient treatments, they were maintained under these conditions for a period of eight months prior to the experiments using semi-continuous culture methodology (Tatters et al., 2012a). Each flask was diluted to a predetermined in vivo chlorophyll *a* fluorescence values using autoclave sterilized L1/20 experimental media on a weekly basis over the conditioning period. The approximate number of generations for this relatively slow-growing species during this eight-month time period encompassing all treatments were: control, 14–48; high pCO_2, 20–65; high temperature, 25–55; and greenhouse 25–63.

2.3. Cell counts, growth rates and primary production (carbon fixation)

For cell counts, measured culture volumes were fixed with acidified Lugol's iodine solution, settled in 5 mL settling chambers and enumerated according to the Utermöhl method (Utermöhl, 1931) using an Accu-Scope 3032 inverted microscope. Specific growth rates for all treatments were determined in individual culture flasks over the eight month conditioning period (data not shown), as well as from triplicate replicates during the final two dilutions prior to sampling (Fig. 1). Growth rates were calculated from samples obtained seven days apart (post-dilution and prior to subsequent dilution) using the equation $\mu = \ln(N_t/N_o)/t_1 - t_0$ (where N is the number of cells at time t_1 and t_0 (in days)). Primary production was measured from triplicate replicates after incubation with $H^{14}CO_3^-$ under identical environmental conditions as described in Fu et al. (2008) and analyzed with a Wallac System 1400 Liquid Scintillation Counter. A total radioactivity of 3.7×10^{-4} MBq was added to 30 mL of culture.

2.4. Analytical methods

2.4.1. PSP toxin detection

Seven days after the final dilution, cells from culture volumes of up to 200 mL from each replicate were collected by gentle vacuum

Author's personal copy

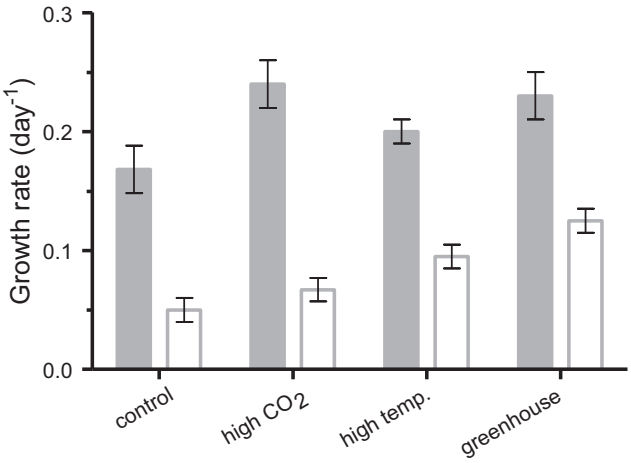


Fig. 1. Specific growth rates assessed after eight months of conditioning in phosphate-replete (shaded gray bars) and phosphate-limited (white open bars) *Alexandrium catenella* conditioned to an experimental matrix of four temperature and pCO_2 treatments. Values are averages of triplicates with standard deviation depicted in error bars.

filtration onto 25 mm GF/F filters (Whatman). The filters were extracted by homogenization into 1% acetic acid using a Tenbroeck tissue grinder and the resulting extracts were filtered (0.45 μm) prior to analysis. PSPs were detected using high performance liquid chromatography with fluorescence detection (HPLC-FL) with pre-column oxidation according to Lawrence et al. (2005). The suite of PSPs constituting the toxin profile of A-11c (Table 1) was identified and quantified by comparison to certified reference standards from the National Research Council of Canada, including: saxitoxin (STX), neosaxitoxin (neoSTX), gonyautoxins (GTX-1 and 4; GTX-2 and 3; GTX-5), C1 and 2, and the decarbamoyl analogs (dcSTX, dcNEO, dcGTX-2 and 3). Peaks that were believed to be C3 and C4 were observed in all replicates of all treatments. These peaks could not be confirmed or quantified due to the lack of standards and represented only a minor fraction of the toxins present. Toxicity in saxitoxin equivalents (STX eq) was calculated using conversion factors based on the specific toxicity of each derivative reported by Oshima (1995).

2.4.2. Carbonate buffer system characterization

Samples for carbonate chemistry analysis were taken monthly (data not shown) and at the conclusion of the experiment (Table 3). Spectrophotometric pH was determined using a Shimadzu 1800UV dual-beam spectrophotometer according to Clayton and Byrne (1993). Dissolved inorganic carbon was analyzed using a CM5230 CO_2 coulometer (UIC) according to King et al. (2011). Experimental pCO_2 (in μatm) was calculated using CO_2SYS software (Lewis and Wallace, 1998) using these two measured parameters (Table 3). pCO_2 concentrations between 260 and 319 ppm are referred to as 'low' and values between 571 and 602 ppm as 'high' pCO_2.

Table 3
Seawater carbonate chemistry system values: spectrophotometric pH and total dissolved inorganic carbon (DIC). Calculated pCO_2 values (in μatm) were obtained from the two measured parameters after eight months of conditioning from all treatments. pCO_2 concentrations between 260 and 319 μatm are referred to as 'low' and values between 571 and 602 μatm as 'high' pCO_2.

Treatment	pH	Total DIC	Calculated pCO_2
Phosphate-replete			
Control replete	8.169	1957	285
High temperature-replete	8.135	1986	319
High CO_2-replete	7.925	2103	571
Greenhouse-replete	7.941	2123	555
Phosphate-limited			
Control-limited	8.205	1949	261
High temperature-limited	8.199	1976	264
High CO_2-limited	7.912	2145	603
Greenhouse-replete	7.925	2126	579

2.5. Statistics

Differences between specific growth rates, carbon fixation rates, cellular STX equivalents, as well as individual PSP derivatives and ratios after eight months of conditioning for the temperature, pCO_2 and phosphate combinations were tested by one-way ANOVA using GraphPad Prism®.

3. Results

3.1. Semi-continuous growth rates

3.1.1. Phosphate-replete cultures

Semi-continuous growth rates for phosphate-replete cultures of *Alexandrium catenella* at 15 °C were significantly lower at low pCO_2 (0.16 ± 0.02 day^{-1}) than at high pCO_2 (0.24 ± 0.02 day^{-1}, Fig. 1, $p < 0.001$). Growth rates were also significantly higher in the greenhouse cultures (19 °C and high pCO_2) relative to the high temperature treatment (low pCO_2) of 19 °C (0.23 ± 0.02 day^{-1} versus 0.20 ± 0.01 day^{-1}, respectively, $p < 0.001$; Fig. 1). The effects of temperature in the phosphate-replete treatments at low pCO_2 concentration were significant ($p < 0.001$), but not at high pCO_2 ($p > 0.05$).

3.1.2. Phosphate-limited cultures

Specific growth rates of phosphate-limited control cultures at 15 °C were 0.05 ± 0.01 day^{-1} at low pCO_2 and 0.07 ± 0.01 day^{-1} at high pCO_2 (Fig. 1). At the high temperature, phosphate-limited cultures grew at an average of 0.09 ± 0.010 div day^{-1} and under greenhouse conditions 0.10 ± 0.013 div day^{-1} (Fig. 1). For each temperature, the differences between pCO_2 treatments were not significant ($p > 0.05$), but all pairwise temperature variances consistently were ($p < 0.01$). However, the growth rates of the cultures grown under greenhouse conditions were approximately 2-fold higher compared to control conditions. As expected, compared to phosphate-replete cultures, growth rates of phosphate-limited treatments were significantly lower at each temperature and pCO_2 combination ($p < 0.001$). Compared to pCO_2 or temperature, phosphate had the most obvious effect on growth rates.

3.2. Carbon fixation/primary production

The relationship between primary production and pCO_2 concentration for both phosphate-replete and phosphate-limited cultures strongly resembled growth (Fig. 2). In general, regardless of the effect of pCO_2 and temperature, the carbon fixation rates were significantly higher under phosphate-replete than phosphate-limited treatments ($p < 0.05$, 0.01, 0.001). Regardless of the temperature, the enrichment of pCO_2 did not stimulate the carbon fixation rates of the phosphate-limited cultures. However, under phosphate-replete conditions, the carbon fixation rates of the cultures at high pCO_2 and greenhouse treatments significantly increased relative to the control and high temperature treatments, respectively ($p < 0.05$). Under phosphate-replete conditions, there was no significant effect of temperature on the carbon fixation rates, but under phosphate-limitation, an increase of 4 °C resulted in stimulation. Except for the control compared to the greenhouse treatment ($p < 0.01$), phosphate-limited carbon fixation rates were not significantly different ($p > 0.05$).

Author's personal copy

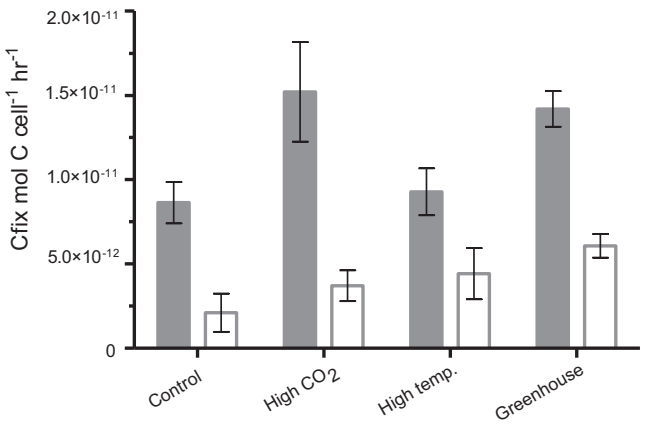


Fig. 2. Carbon fixation rates assessed after eight months of conditioning in phosphate-replete (shaded gray bars) and phosphate-limited (white open bars) *Alexandrium catenella* conditioned to an experimental matrix of four temperature and pCO_2 treatments. Values are averages of triplicates with standard deviation depicted in error bars.

3.3. Cellular saxitoxin equivalents

3.3.1. Phosphate-replete cultures

Phosphate-replete cultures grown at 15 °C had total cellular toxicity quotas that were more than twice as high at high pCO_2 (4.64 ± 0.38 fmol STX equivalents cell^{-1}) than in the control treatment (1.99 ± 0.06 fmol cell^{-1}, $p < 0.001$; Fig. 3A). Greenhouse conditioned cultures also had toxicity quotas that were significantly higher (~1.5×) compared to the high temperature at low pCO_2 (3.01 ± 0.46 fmol cell^{-1} versus 2.06 ± 0.14 fmol cell^{-1}, $p < 0.05$, Fig. 3A). For the phosphate-replete cultures there was no significant difference in STX equivalents per cell for control compared to high temperature, but toxicity quotas were significantly higher in the high pCO_2 relative to the greenhouse treatment ($p < 0.001$; Fig. 3A).

3.3.2. Phosphate-limited cultures

Phosphate-limited cells had total toxin contents that were roughly an order of magnitude higher than those in phosphate-replete cells in each experimental treatment (Fig. 3A and B). However, toxicity quotas at 15 °C were again significantly elevated under high pCO_2 (44.46 ± 2.13 fmol cell^{-1}) compared to the control treatment at low pCO_2 (35.28 ± 3.95 fmol cell^{-1}, $p < 0.01$, Fig. 3B). Likewise, greenhouse conditioned cultures again contained significantly more toxin equivalents per cell (17.66 ± 1.19 fmol cell^{-1}) than those at the high temperature grown under low pCO_2 (12.66 ± 0.89 fmol cell^{-1}, $p < 0.05$, Fig. 3B). At both pCO_2 levels, total cellular toxicity decreased dramatically at the higher temperature ($p < 0.001$; Fig. 3B).

3.4. Toxin profiles for phosphate-replete treatments

3.4.1. Relative toxin profile of PSP derivatives per replete treatment

For phosphate-replete cultures the relative toxin profile is depicted for control, high CO_2, high temperature and greenhouse conditioned treatments (Fig. 4A and B). The relative contribution of GTX-5 was 67.7% (control treatment), 56.4% (high CO_2 treatment), 56.3% in the high temperature treatment and 64.7% in the greenhouse treatment. For STX, the relative composition per treatment was 4.3% (control), 3.3% (high CO_2), 4.5% (high temperature) and 3.2% (greenhouse). For the C-toxins (C1/2), composition per treatment was 26.4% (control), 38.4% (high CO_2), 38.4% (high temperature) and 29.7% (greenhouse). Regarding neoSTX, the composition of the control treatment was 1.15%, 0.66% at high CO_2, 0.99% at high temperature and 0.91% for greenhouse.

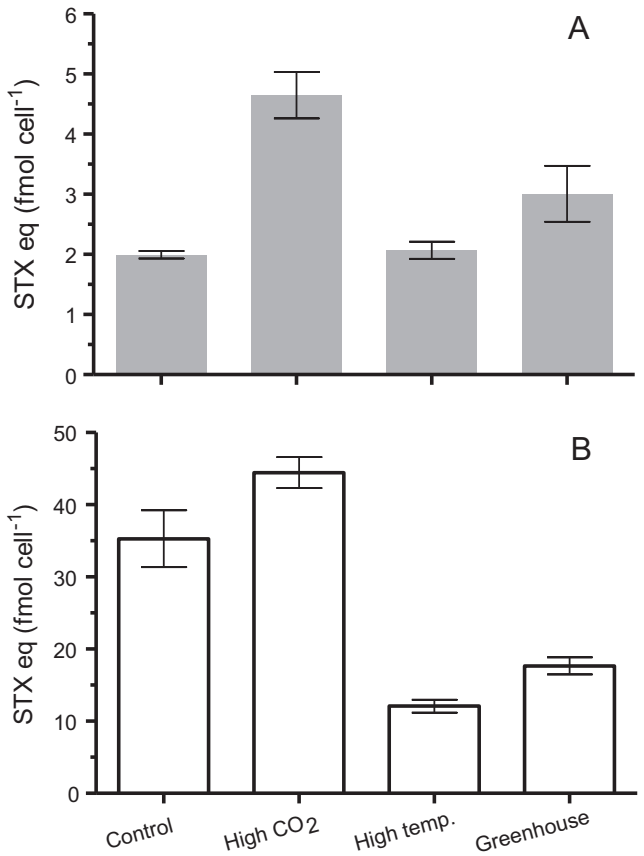


Fig. 3. Cellular saxitoxin equivalents (fmol cell^{-1}) in cultures of *Alexandrium catenella* grown in an experimental matrix of four temperature and pCO_2 treatments under (A) phosphate-replete (shaded gray bars) and (B) phosphate-limited (open white bars) conditions. Note *Y*-axis scale differences. All values are the average of triplicates with standard deviations depicted in error bars.

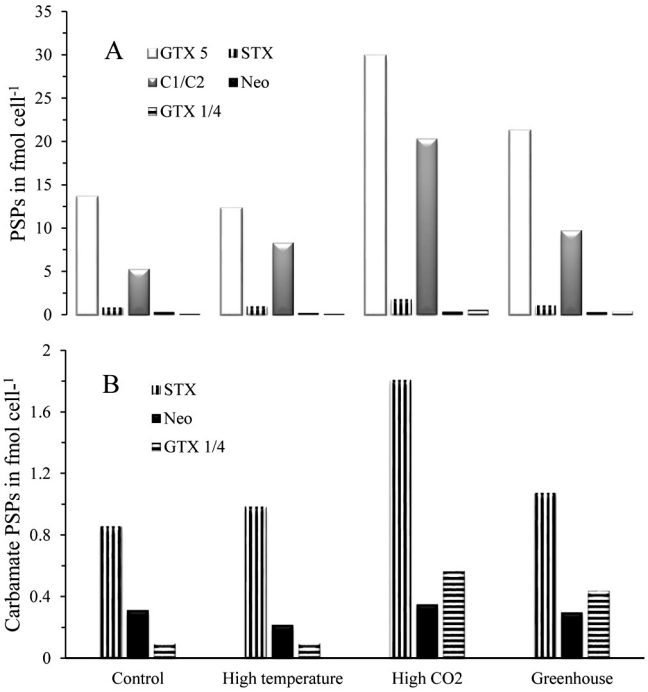


Fig. 4. Paralytic shellfish poisoning toxin profile of *Alexandrium catenella* cultures conditioned to an experimental matrix of eight temperature, pCO_2 and phosphate combinations. Total congener abundance representations in fmol cell^{-1} (Panel A) and carbamate compounds (Panel B).

Author's personal copy

For GTX1-4, the percent composition for both control and high temperature was below the limit of detection, but for high CO_2 and greenhouse treatments was 1.1% and 1.3%, respectively.

Pairwise comparisons of individual PSPs revealed a significant difference between control versus high temperature ($p < 0.05$), but not for control versus high CO_2 or greenhouse conditions ($p > 0.05$). For the C-toxins (C1/2), STX and neoSTX there was no significant difference in experimental treatments compared to the control ($p > 0.05$). Regarding the minor constituents GTX-1 and 4, there were differences between the control compared to high CO_2 and greenhouse, but the significance level cannot be determined due to the fact that the values obtained from control and high temperature cultures were below the limit of detection. Despite the large differences in total STX equivalents (Fig. 3A and B), the toxin profiles (both congeners and ratios) in each treatment of the phosphate-replete cultures (Fig. 4A and B) were virtually identical to corresponding treatments in phosphate-limited cultures (data not shown).

3.4.2. Concentrations of various PSP derivatives per replete treatment

For control cells, the toxin profile (all values reported in fmol $cell^{-1}$) was: 13.61 (GTX-5), 0.86 (STX), 5.30 (C1/2) and 0.31 (neoSTX). GTX-1 and 4 were either not present or below the limit of detection (Fig. 4A and B). On average at high CO_2, cells contained 29.89 fmol of GTX-5, 1.81 of STX, 20.33 of C1/2, 0.35 of neoSTX and 0.56 of GTX 1 and 4. At high temperature, the profile consisted of 12.28 fmol (GTX-5), 0.98 (STX), 9.77 (C1/2) and 0.21 (neoSTX) with no detectable GTX 1 and 4. For the greenhouse treatment, the concentrations were 21.25 fmol (GTX-5), 1.17 (STX), 10.76 (C1/2), 0.30 (neoSTX) and 0.43 (GTX 1 and 4) (Fig. 4A and B).

Pairwise comparisons of individual toxin derivatives revealed that for concentrations of GTX-5, the control treatment was significantly different compared to both high CO_2 and high temperature treatments, but not compared to the greenhouse treatment ($p < 0.01$ and $p > 0.05$). For STX, the control treatment was significantly different from the high CO_2 treatment ($p < 0.001$), but the control was not different from the high temperature and greenhouse treatments ($p > 0.05$). There was no significant difference between treatments regarding concentrations of the minor component neoSTX ($p > 0.05$). For the C toxins (C1/C2) there was a significant difference between the control compared to both high CO_2 and greenhouse, but not compared to the high temperature treatment ($p < 0.001$, $p < 0.01$, and $p > 0.05$).

4. Discussion

Despite the environmental and economic importance of harmful bloom-forming marine dinoflagellates, few investigations have focused on their responses to ocean acidification and climate change (Rost et al., 2006; Fu et al., 2010; Tatters et al., 2013; Kremp et al., 2012; Flores-Moya et al., 2012; reviewed by Fu et al., 2008, 2012). These gaps in our holistic autecological understanding of these organisms are a barrier to accurately predicting their impacts on marine food webs in the future. In our isolate of *Alexandrium catenella*, high CO_2 led to increased cellular toxin levels regardless of temperature or phosphate status examined. High CO_2, phosphate-limited cells conditioned to low temperature were the most toxic both in terms of collective STX equivalents (Fig. 3A), absolute concentration of STX per cell (Fig. 4A and B) as well as total concentrations of PSP toxins per cell.

We hypothesize that this effect is due, at least in part, to the shunting of 'excess' fixed carbon through secondary metabolic pathways under conditions of high CO_2 availability (Fu et al., 2012). It is likely that enhanced net photosynthetic rates, simply due to inorganic carbon 'fertilization', is a plausible explanation for these findings. The pronounced effect under phosphate-limitation and reduced toxicity in faster growing cells at higher temperature lends further support to this hypothesis, as does our previous work with the domoic acid-producing diatom genus *Pseudo-nitzschia* (Sun et al., 2011; Tatters et al., 2012a).

PSPs are carbon and nitrogen rich compounds. Both carboxylation by Rubisco as well as nitrate reduction via nitrate reductase take place within the plastid, and in microalgae, these events are transcriptionally regulated and controlled by light. Given that both processes utilize energy created by the light reactions of photosynthesis, assimilation of NO_3^- and CO_2 may compete for reductant produced during photosynthetic electron transport. There is evidence that enhancement of carbon assimilation and growth under elevated CO_2 is highly dependent on the adequate supply (Gordillo et al., 2001, 2003) and speciation (Clark and Flynn, 2000) of inorganic nitrogen in some marine algae. These results suggest the possibility of a positive feedback scenario between elevated CO_2 and nitrate enrichment on algal physiology and toxicity, which should be investigated in future studies in addition to the phosphorus availability interactions examined here.

Effects from CO_2 led to general trends regardless of nutrient status or temperature, suggesting ocean acidification conditions (co-varying increased CO_2 and decreased pH) significantly impacted the biochemistry of *Alexandrium catenella*. In the nutrient replete treatments, growth rates were significantly increased at both temperatures by higher CO_2. This observation is suggestive of alleviation from carbon limitation when other resources are sufficient. Growth rates were also slightly higher at elevated CO_2 in phosphate-limited cells, but these differences were not significant. Carbon fixation rates generally mirrored the trends of growth and toxicity (Figs. 2 and 3A,B). As carbon is required to biosynthesize these chemicals, additional fixed carbon not utilized in growth is likely available to be allocated to secondary metabolite production.

Warmer temperatures stimulated growth at low CO_2 (but not high CO_2) in phosphate-replete cells. In phosphate-limited cultures, growth was significantly influenced by temperature at both CO_2 levels. It has been repeatedly demonstrated that the toxicity of *Alexandrium* spp. is inversely correlated with temperature (Hall et al., 1982; Anderson et al., 1990; Cembella, 1998; Navarro et al., 2006). In our experiments effects of temperature on toxicity were generally as anticipated, except for phosphate-replete treatments under low CO_2 where the difference between the two temperatures was not significant. In general cultures at the higher temperature, although growing faster, contained less STX equivalents per cell (Fig. 3A and B). This could be reflective of either toxin production rates decreasing with increasing temperature or 'growth dilution', where faster growth promotes partitioning of toxin among daughter cells.

In bloom-forming phytoplankton species, rapid proliferation of biomass can often lead to nutrient limitation during later stages of the bloom. In addition, periodic altered nutrient ratios due to anthropogenic loading are common in today's estuaries and coastal oceans. Thus, future simulations that incorporate nutrient limitation in conjunction with global change variables are environmentally relevant. Phosphate-limitation was previously demonstrated to increase toxicity in several toxin-producing *Alexandrium* species (Boyer et al., 1987; Jauzein et al., 2010; Tatters et al., 2012b), as well as in some other toxic dinoflagellates (Adolf et al., 2009; Fu et al., 2010). In our experiments the dramatically enhanced cellular toxicity we observed under phosphate-limitation was expected, but was further exacerbated by ocean acidification conditions. Increased temperature however suppressed toxicity in high-CO_2 grown cells, especially in phosphate limited cultures which were on average at least 50% less toxic at the warmer temperature (Fig. 3B). This suggests that under future "greenhouse" conditions, increases in the toxicity of this strain

Author's personal copy

driven by high CO_2 and/or phosphate-limitation may be partially (but not entirely) offset by warmer temperatures.

Two other studies have examined the mechanism of cell specific enhancement of toxicity by CO_2 in dinoflagellates. In a Chesapeake Bay isolate of the dinoflagellate *Karlodinium veneficum* a dramatic shift was observed in karlotoxin (KmTx) congeners, not in the amount of toxin produced, as a function of CO_2 (Fu et al., 2010). They found that high CO_2 promoted the production of the highly potent KmTx-1, relative to the much less toxic KmTx-2 that predominated in cells grown at present-day CO_2. Some clones of *K. veneficum* are also known to exclusively produce KmTx-1, 2, or other derivatives, but the effects of CO_2 on the toxin profiles of these strains are unknown.

In a study by Kremp et al. (2012), only two of eight *Alexandrium ostenfeldii* clones were stimulated by high CO_2. There were few reported differences in total PSPs, but the combined CO_2 and temperature treatments led to different proportions of PSPs in most clones (Kremp et al., 2012). In our study, there were few changes in the profiles of PSPs produced by *Alexandrium catenella* on a percent basis. One difference was the enhancement of GTX 1 and 4 concentrations at high CO_2. The fact that low CO_2 led to values below the limit of detection but high CO_2 levels were consistently twice this level suggests promotion of these compounds with increasing CO_2. Although GTX 1 and 4 were very minor constituents of the toxin profile, PSP toxin profiles of other dinoflagellates are dominated by these derivatives (Franco et al., 1994; Pitcher et al., 2007). As GTX 1 and 4 are very toxic derivatives, further investigation is warranted. Although there was little change in composition, the various treatments certainly modulated total cellular toxicity (Fig. 3A and B). There are clearly differences in the ways cells arrived the higher potency levels in these studies. The issue of quantity versus quality of toxin biosynthesis is an important facet of examining how these organisms respond to any variable and the interactive outcomes of multiple variables, including increasing CO_2 and temperature.

Another recent study grew two clones of *Alexandrium minutum* from the Rio de Vigo, NW Spain at two pH levels (7.5 and 8.0, adjusted with HCl) and two temperatures (20 and 25 °C) for a duration of 720 days (Flores-Moya et al., 2012). The authors attributed observed deviations in growth and toxicity to mutation, and suggested that future blooms of *A. minutum* could increase in frequency, but that effects on cellular toxicity were unpredictable (Flores-Moya et al., 2012). The findings of this study relative to growth were in agreement with a study conducted by Hwang and Lu (2000), who examined another isolate of *A. minutum* and observed more robust growth at pH 7.5 versus 5.5 or 8.5. Siu et al. (1997) however reported that optimal pH was around 8.5 for a clone of *Alexandrium catenella* from Hong Kong. More recently, Kremp et al. (2012) examined eight strains of *Alexandrium ostenfeldii* from the North Sea grown at 20 and 24 °C in batch culture under CO_2 concentrations of 385 and 750 ppm. The authors reported a variety of responses from the different clones, but overall there was a trend of increasing STX concentrations with increasing pCO_2 and increasing temperature individually, as well as with these variables in concert (Kremp et al., 2012). However, these various studies are difficult to directly compare with ours not only because different species were used, but also because they employed different experimental techniques (acid additions versus CO_2 bubbling, batch versus semi-continuous culturing and toxin detection methods, etc.). Increased cellular toxicity appears to be a general effect of ocean acidification on our *A. catenella* A-11c isolate, but the effects on other strains may differ.

Aside from these methodological uncertainties, it is no surprise to find non-uniform responses to environmental stressors given the sheer number of species within this wide-ranging genus, coupled with the existence of biogeographically isolated populations of *Alexandrium* in various marine environments worldwide. Temperature imposes biogeographic boundaries and exerts direct control on cellular metabolic processes (Boyd et al., 2010). The effects of warming on the physiology and biochemistry of harmful dinoflagellates including *Alexandrium* spp. have long been investigated, yet the potential synergistic effects of accompanying global change variables like ocean acidification have been inadequately explored.

Currently, there are wide-reaching impacts from seasonal accumulations of PSPs throughout marine food webs in numerous regions around North America, including the Gulf of Maine (Shumway et al., 1988; White et al., 1993), Alaska (Gessner and Schloss, 1996), Washington state (Horner et al., 1997; Moore et al., 2010), and numerous parts of coastal California (Meyer et al., 1928; Sommer and Meyer, 1937; Hall et al., 1990; Jester et al., 2009). The objective of this study was to examine the effects of simulated greenhouse ocean conditions on the growth and toxin production of a California clone of the wide-ranging species *Alexandrium catenella*. This study provides the first insight into how this important species may respond to impending climate change impacts. If the responses of our isolate are broadly representative, then it is possible that rising dissolved inorganic carbon concentrations may lead to increased cellular toxicity in this species. Concurrent temperature increases however may offset a portion of this enhancement.

Our findings demonstrate that *Alexandrium catenella* responds to CO_2, but this response depends on other co-occurring variables, emphasizing the need to avoid making general conclusions about ocean acidification effects until potentially interacting factors have been fully considered (Boyd and Hutchins, 2012). In particular, nutrient limitation has seldom been included in experimental designs of previous ocean acidification/warming experiments, despite its obvious relevance to phytoplankton growth in the present day and future coastal ocean. Experiments testing the interactive effects of other variables such as trace metals, vitamins and irradiance should also be considered. In addition, there is a need to examine the responses of toxic dinoflagellates to medium or long-term environmental changes by acclimation versus adaptation (Flores-Moya et al., 2012; Tatters et al., 2013). Ongoing global change processes such as acidification and warming that are occurring over timescales of years to decades make it critical to address questions about evolutionary processes, and their consequences for harmful toxic blooms in the future ocean.

Acknowledgements

This study was funded by USC Sea Grant, NSF OCE-0962309 and the Florida Fish and Wildlife Conservation Commission.[SS]

References

Adolf, J.E., Bachvaroff, T.R., Place, A.R., 2009. Environmental modulation of karlotoxin levels in strains of the cosmopolitan dinoflagellate *Karlodinium veneficum* (Dinophyceae). J. Phycol. 45, 176–192.

Anderson, D.M., Kulis, D.M., Sullivan, J.J., Hall, S., Lee, C., 1990. Dynamics of physiology of saxitoxin production by the dinoflagellates *Alexandrium* spp. Mar. Biol. 104, 511–524.

Anderson, D.M., Alpermann, T.J., Cembella, A.D., Collos, Y., Masseret, E., Montresor, M., 2012. The globally distributed genus *Alexandrium*: multifaceted roles in marine ecosystems and impacts on human health. Harmful Algae 14, 10–35.

Bachvaroff, T.R., Adolf, J.E., Place, A.R., 2009. Strain Variation in *Karlodinium veneficum* (Dinophyceae): toxin profiles, pigments and growth characteristics for strains grown under common garden conditions. J. Phycol. 45, 137–153.

Beardall, J., Raven, J.A., 2004. The potential effects of global climate change on microalgal photosynthesis, growth and ecology. Phycologia 43 (1) 26–40.

Boyd, P.W., Strzepek, R., Fu, F.-X., Hutchins, D.A., 2010. Environmental control of open ocean phytoplankton groups: now and in the future. Limnol. Oceanogr. 55, 1353–1376.

Author's personal copy

Boyd, P.W., Hutchins, D.A., 2012. Understanding the responses of ocean biota to a complex matrix of cumulative anthropogenic change. Mar. Ecol. Prog. Ser. 470, 125–135, http://dx.doi.org/10.3354/meps10121.

Boyer, G.L., Sullivan, J.J., Anderson, R.J., Harrison, P.J., Taylor, F.J.R., 1987. Effects of nutrient limitation on toxic production and composition in the marine dinoflagellate *Protogonyaulax tamarensis*. Mar. Biol. 96, 123–128.

Burkholder, J.M., Glibert, P.M., 2009. The importance of intraspecific variability in harmful algae. Preface to a collection of topical papers. Harmful Algae 8 (5) 744–745.

Cane, M.A., Clement, A.C., Kaplan, A., Kushnir, Y., Pozdnyakov, D., Seager, R., Zebiak, S.E., Murtugudde, R., 1997. Twentieth-century sea surface temperature trends. Science 275 (5302) 957–960, http://dx.doi.org/10.1126/science.275.5302.957.

Catterall, W.A., 1980. Neurotoxins that act on voltage-sensitive sodium channels in excitable membranes. Annu. Rev. Pharmacol. Toxicol. 20, 15–43.

Cembella, A.D., 1998. Ecophysiology and metabolism of paralytic shellfish toxins in marine microalgae. In: Anderson, D.M., Cembella, A.D., Hallegraeff, G.M. (Eds.), Physiological Ecology of Harmful Algal Blooms. Springer, Berlin, pp. 381–403.

Clayton, T.D., Byrne, R.H., 1993. Spectrophotometric seawater pH measurements: total hydrogen ion concentration scale calibration of m-cresol purple and at-sea results. Deep-Sea Res. Pt. I 40, 2115–2129, http://dx.doi.org/10.1016/0967-0637(93)90048-8.

Clark, D.R., Flynn, K.J., 2000. The relationship between the dissolved inorganic carbon concentration and growth rate in marine phytoplankton. Proc. R. Soc. B: Biol. Sci. 267, 953–959.

Feely, R.A., Sabine, C.L., Hernandez-Ayon, J.M., Ianson, D., Hales, B., 2008. Evidence for upwelling of corrosive "acidified" water onto the continental shelf. Science 320, 1490–1492.

Flores-Moya, A., Rouco, M., Garcia-Sanchez, M.J., Garcia-Balboa, C., Gonzalez, R., Costas, E., Lopez-Rodas, V., 2012. Effects of adaptation, chance, and history on the evolution of the toxic dinoflagellate *Alexandrium minutum* under selection of increased temperature and acidification. Ecol. Evol. 2 (6) 1251–1259, http://dx.doi.org/10.1002/ece3.198.

Franco, J.M., Fernández, P., Reguera, B., 1994. Toxin profiles of natural populations and cultures of *Alexandrium minutum* Halim from Galician (Spain) coastal waters. J. Appl. Phycol. 6, 275–279.

Fu, F.-X., Zhang, Y., Warner, M.E., Feng, Y., Hutchins, D.A., 2008. A comparison of future increased CO_2 and temperature effects on sympatric *Heterosigma akashiwo* and *Prorocentrum minimum*. Harmful Algae 7, 76–90.

Fu, F.-X., Place, A.R., Garcia, N.S., Hutchins, D.A., 2010. CO_2 and phosphate availability control the toxicity of harmful bloom dinoflagellate *Karlodinium veneficum*. Aquat. Microb. Ecol. 59, 55–65, http://dx.doi.org/10.3354/ame01396.

Fu, F.-X., Tatters, A.O., Hutchins, D.A., 2012. Global change and the future of harmful algal blooms in the ocean. Mar. Ecol. Prog. Ser. 470, 207–233, http://dx.doi.org/10.3354/meps10047.

Gessner, B.D., Schloss, M.S., 1996. A population-based study of paralytic shellfish poisoning in Alaska. Alaska Med. 38 (2) 54–58.

Gordillo, F.J.L., Niell, F.X., Figueroa, F.L., 2001. Non-photosynthetic enhancement of growth by high CO_2 level in the nitrophilic seaweed *Ulva rigida* C. Agardh (Chlorophyta). Planta 213, 64–70.

Gordillo, F.J.L., Jimenez, C., Figueroa, F.L., Niell, F.X., 2003. Influence of elevated CO_2 and nitrogen supply on the carbon assimilation performance and cell composition of the unicellular alga *Dunaliella viridis*. Physiol. Plant. 119, 513–518.

Gribble, K.E., Keafer, B.A., Quilliam, M.A., Cembella, A.D., Kulis, D.M., Manahan, A., Anderson, D.M., 2005. Distribution and toxicity of *Alexandrium ostenfeldii* (Dinophyceae) in Gulf of Maine, USA. Deep-Sea Res. Pt. II 52, 2745–2763.

Guillard, R., Hargraves, P.E., 1993. *Stichochrysis immobilis* is a diatom, not a chrysophyte. Phycologia 32, 234–236.

Hall, S., Reichardt, P.B., Neve, R.A., Boyer, G.L., Wichman, C.F., Schroes, H.K., 1982. Studies on the origin and nature of toxicity in Alaskan bivalves: toxins from *Protogonyaulax* of the Northeast Pacific. J. Shellfish Res. 2 (1) 119.

Hall, S., Strichartz, G., Moczydlowski, E., Ravindran, A., Reichart, P.B., 1990. The saxitoxins: sources, chemistry and pharmacology. In: Hall, S., Strichartz, G. (Eds.), Marine Toxins: Origin, Structure and Molecular Pharmacology, vol. 418. Am. Chem. Soc. Symp. Ser, pp. 29–65.

Hallegraeff, G.M., 1993. A review of harmful algal blooms and their apparent global increase. Phycologia 32, 79–99.

Hallegraeff, G.M., McCausland, M.A., Brown, R.K., 1995. Early warning of toxic dinoflagellate blooms of *Gymnodinium catenatum* in southern Tasmanian waters. J. Plankt. Res. 17, 1163–1176.

Hallegraeff, G.M., 2010. Ocean climate change, phytoplankton community responses, and harmful algal blooms: a formidable predictive challenge. J. Phycol. 46 (2) 220–235.

Horner, R.A., Garrison, D.L., Plumley, F.G., 1997. Harmful algal blooms and red tide problems on the U.S. West Coast. Limnol. Oceanogr. 42 (5 (Pt 2)) 1076–1088, The ecology and oceanography of harmful algal blooms.

Hutchins, D.A., Mulholland, M.R., Fu, F.-X., 2009. Nutrient cycles and marine microbes in a CO_2-enriched ocean. Oceanography 22, 128–145.

Hwang, D.F., Lu, Y.H., 2000. Influence of environmental and nutritional factors on growth, toxicity, and toxin profile of dinoflagellate *Alexandrium minutum*. Toxicon 38, 1491–1503.

Jauzein, C., Labry, C., Youenou, A., Quéré, J., Delmas, D., Collos, Y., 2010. Growth and phosphorus uptake by the toxic dinoflagellate *Alexandrium catenella* (Dinophyceae) in response to phosphate limitation. J. Phycol. 46 (5) 926–936.

Jester, R., Baugh, K.A., Lefebvre, K.A., 2009. Presence of *Alexandrium catenella* and paralytic shellfish toxins in finish, shellfish and rock crabs in Monterey Bay, California, USA. Mar. Biol. 156 (3) 493–504, http://dx.doi.org/10.1007/s00227-008-1103-z.

King, A.L., Sañudo-Wilhelmy, S.A., Leblanc, K., Hutchins, D.A., Fu, F.-X., 2011. CO_2 and vitamin B_{12} interactions determine bioactive trace metal requirements of a subarctic Pacific diatom. Intl. Soc. Microb. Ecol. J. 5 (8) 1388–1396.

Kodama, M., 2010. Paralytic shellfish toxins: biochemistry and origin. Aqua BioSci. Monogr. 3 (1) 1–38.

Kremp, A., Godhe, A., Egardt, J., Dupont, S., Suikkanen, S., Casabianca, S., Penna, A., 2012. Intraspecific variability in the response of bloom-forming marine microalgae to changed climate conditions. Ecol. Evol. 2 (6) 1195–1207, http://dx.doi.org/10.1002/ece3.245.

Lewis, E., Wallace, D.W.R., 1998. Program developed for CO_2 system calculations, Carbon Dioxide Information Analysis Center. Report ORNL/CDIAC-105. Oak Ridge National Laboratory, Oak Ridge, Tennessee, USA.

Lawrence, J.F., Niedzwiadek, B., Menard, C., 2005. Quantitative determination of paralytic shellfish poisoning toxins in shellfish using prechromatographic oxidation and liquid chromatography with fluorescence detection: collaborative study. J. AOAC Int. 88 (6) 1714–1732.

Masseret, E., Grzebyk, D., Nagai, S., Genovesi, B., Lasserre, B., et al., 2009. Unexpected genetic diversity among and within populations of the toxic dinoflagellate *Alexandrium catenella* as revealed by nuclear microsatellite markers. Appl. Environ. Microb. 75, 2037–2045.

Meyer, K.F., Sommer, H., Schoenholz, P., 1928. Mussel poisoning. J. Prevent. Med. 2, 365–394.

Moore, S.K., Mantua, N.J., Hickey, B.M., Trainer, V.L., 2010. The relative influences of El Niño-Southern Oscillation and Pacific Decadal Oscillation on paralytic shellfish toxin accumulation in northwest Pacific shellfish. Limnol. Oceanogr. 55 (6) 2262–2274, http://dx.doi.org/10.4319/lo.2010.55.6.2262.

Navarro, J.M., Munoz, M.G., Contreras, A.M., 2006. Temperature as a factor regulating growth and toxin content in the dinoflagellate *Alexandrium catenella*. Harmful Algae 5, 762–769.

Ogata, T., Ishimaru, T., Kodama, M., 1987. Effect of water temperature and light intensity on growth rate and toxicity change in *Protogonyaulax tamarensis*. Mar. Biol. 95, 217–220.

Oshima, Y., 1995. Post-column derivation liquid chromatographic method for paralytic shellfish toxins. J. AOAC Int. 78 (2) 528–532.

Parkhill, J.P., Cembella, A.D., 1999. Effect of salinity, light and temperature and inorganic nitrogen in growth and toxicgenicity of the marine dinoflagellate *Alexandrium tamarense* from northeastern Canada. J. Plank. Res. 21 (5) 939–955.

Pitcher, G.C., Cembella, A.D., Joyce, L.B., Larsen, J., Probyn, T.A., Ruiz Sebastián, C., 2007. The dinoflagellate *Alexandrium minutum* in Cape Town harbour (South Africa): bloom characteristics, phylogenetic analysis and toxin composition. Harmful Algae 6 (6) 823–836.

Quilliam, M.A., Dell'Aversano, C., Hess, P., 2001. Analysis of PSP toxins by liquid chromatography–mass spectrometry. In: Book of Abstract. Second International Conference on Harmful Algae Management and Mitigation, 83, 12–16 November, Qingdao, China.

Rost, B., Richter, K., Riebesell, U., Hansen, P.J., 2006. Inorganic carbon acquisition in red tide dinoflagellates. Plant Cell Environ. 29, 810–822.

Sabine, C.L., Feely, R.A., Gruber, N., Key, R.M., Lee, K., Bullister, J.L., et al., 2004. The oceanic sink for anthropogenic CO_2. Science 305, 367–371.

Siu, G.K.Y., Young, M.L.C., Chan, D.K.O., 1997. Environmental and nutritional factors which regulate population dynamics and toxin production in the dinoflagellate *Alexandrium catenella*. Hydrobiologia 352, 117–140.

Sommer, H., Meyer, K.F., 1937. Paralytic shellfish poisoning. Arch. Pathol. 24, 560–598.

Sun, J., Hutchins, D.A., Feng, Y., Seubert, E.L., Caron, D.A., Fu, F.-X., 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.

Tatters, A.O., Fu, F.-X., Hutchins, D.A., 2012a. High CO_2 and silicate limitation synergistically increase the toxicity of *Pseudo-nitzschia fraudulenta*. PLoS ONE 7 (2) e32116, http://dx.doi.org/10.1371/journal.pone.0032116.

Tatters, A.O., Van Wagoner, R.M., Wright, J.L.C., Tomas, C.R., 2012b. Regulation of spiroimine neurotoxins and hemolytic activity in laboratory cultures of the dinoflagellate *Alexandrium peruvianum* (Balech & Mendiola) Balech & Tangen. Harmful Algae 19, 160–168, http://dx.doi.org/10.1016/j.hal.2012.07.002.

Tatters, A.O., Schnetzer, A., Fu, F.-X., Lie, A.A.Y., Caron, D.A., Hutchins, D.A., 2013. Short-versus long-term responses to changing CO_2 in a coastal dinoflagellate bloom: implications for interspecific competitive interactions and community structure. Evolution 67 (7) 1879–1891, http://dx.doi.org/10.1111/evo.12029.

Tillmann, U., Alpermann, T.L., da Purificacao, R.C., Krock, B., Cembella, A., 2009. Intra-population clonal variability in allelochemical potency of the toxigenic dinoflagellate *Alexandrium tamarense*. Harmful Algae 8, 759–769.

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

White, A.W., 1978. Salinity effects on growth and toxin content of *Gonyaulax excavata*, a marine dinoflagellate causing paralytic shellfish poisoning. J. Phycol. 14, 475–479.

Xie, S.-P., Deser, C., Vecchi, G.A., Ma, J., Teng, H., Wittenberg, A.T., 2010. Global warming pattern formation: sea surface temperature and rainfall. J. Climate 23, 966–986, http://dx.doi.org/10.1175/2009JCLI3329.1.