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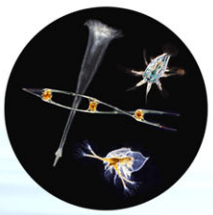

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Interactive effects of temperature, CO_2 and nitrogen source on a coastal California diatom assemblage

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Diatoms are often considered to be a single functional group, yet there is a great deal of morphological, genetic and ecological diversity within the class. How these differences will translate into species-specific responses to rapid changes in the ocean environment resulting from climate change and eutrophication is currently poorly understood. We investigated the response of a natural diatom-dominated assemblage in coastal California waters to interactions between the variables nitrogen source (nitrate and urea), temperature (19 and 23°C) and CO_2 (380 and 800 ppm) in a factorial experimental matrix using continuous culture (ecostat) methods. The community included diatoms of the cosmopolitan genera *Pseudo-nitzschia* and *Chaetoceros*, as well as *Leptocylindrus* and *Cylindrotheca*. Our results

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demonstrate strong interactive effects of these variables on community composition; notably, nitrogen source alone and nitrogen and CO_2 together had a much greater influence on diatom community structure at 23°C compared with 19°C. In addition, warming and acidification interactions significantly increased cellular quotas of the neurotoxin domoic acid produced by *Pseudo-nitzschia multiseries*. In general, the effects observed for the factors tested differed significantly between the various diatom genera in this assemblage, suggesting potentially divergent responses of some of these ecologically and biogeochemically important phytoplankton taxa to interactions between global-scale and local-scale anthropogenic stressors in a changing ocean.

KEYWORDS: global change; diatom community structure; interactive effects

INTRODUCTION

The accelerated input of carbon dioxide (CO_2) into the atmosphere is increasing sea surface temperatures and decreasing seawater pH (Cane *et al.*, 1997; Sabine *et al.*, 2004; Feely *et al.*, 2008). Warming and acidification are occurring on a global scale, but their combined effects on ocean biology are poorly understood and only beginning to be examined. Another important anthropogenic impact on the ocean is excessive nitrogen inputs into near-shore waters, stemming from coastal development and agriculture. Recent studies have also implicated eutrophication as a factor that enhances ocean acidification, by promoting respiration of the resulting excess production of organic carbon in coastal waters (Cai *et al.*, 2011; Sunda and Cai, 2012; Wallace *et al.*, 2014; Gobler and Baumann, 2016).

Diatoms are a globally distributed phytoplankton group that has tremendous biogeochemical significance in the ocean (Mann, 1999; Boyd *et al.*, 2012). These organisms represent a substantial portion of the microalgal community throughout the year in many regions, and they are especially prominent in upwelling regimes including the one along the California coastline. Despite the ecological and environmental significance of this diverse group, only a few studies have examined the interactive effects of multiple global change factors on natural diatom-dominated plankton assemblages (Kim *et al.*, 2006; Hare *et al.*, 2007a,b; Feng *et al.*, 2008; Feng *et al.*, 2010; Tatters *et al.*, 2013b).

The physiology and the biochemistry of the cosmopolitan pennate diatom genus *Pseudo-nitzschia* are influenced by a variety of environmental parameters (Bates *et al.*, 1998). Some species can biosynthesize the neurotoxin domoic acid, which threatens human and environmental health (Bates and Trainer, 2006). Although blooms of some potentially toxic *Pseudo-nitzschia* spp. are common in the majority of the world's coastal ocean, little is known about how these diatoms will respond to multiple global environmental change factors in field populations. Several culture-based studies have highlighted experimental conditions that influence *Pseudo-nitzschia* dominance and cellular domoic acid quotas, including selective nutrient limitation, trace metal availability, allelopathy, nitrogen source, CO_2, pH and temperature (Pan *et al.*, 1998; Maldonado *et al.*, 2002; Lundholm *et al.*, 2004; Trimborn *et al.*, 2008; Sun *et al.*, 2011; Tatters *et al.*, 2012; Xu *et al.*, 2015). Most notably, the responses to temperature and CO_2/pH manipulations are the least explored (Lelong *et al.*, 2012a, b). Information obtained from these culture studies is useful, but lacks the complexity and stochasticity inherent in natural assemblages.

The principal objective of this study was to examine how a mixed marine diatom assemblage that included *Pseudo-nitzschia* spp. responds to a suite of environmental change factors. To investigate this, we used an "ecostat" continuous culture system (Hutchins *et al.*, 2003; Hare *et al.*, 2007; Feng *et al.*, 2009) to incubate a natural California coastal diatom community in an experimental matrix of temperature (19 and 23°C), CO_2 (present-day and predicted year 2100, 380 and 800 ppm, respectively) and major nitrogen source (nitrate (NO_3^-) and urea). Here we report on the interactive effects of these variables on the final diatom community composition and cellular quotas of domoic acid in *Pseudo-nitzschia*. Our results suggest that global-scale stressors such as increasing sea surface temperature, ocean acidification and local-scale stressors such as eutrophication-driven changes in nitrogen sources may interactively influence total diatom community composition. These changes may also affect the toxicity of particular component species such as members of the genus *Pseudo-nitzschia*.

METHOD

Experimental design

We investigated the response of a mixed natural diatom-dominated assemblage to three-way interactions between warming, acidification, and inorganic or organic nitrogen sources. Seawater collections for the experiment occurred on 10 May 2012 in Fish Harbor, Terminal Island, Long

Beach, CA, USA (33°44′59″N; 118°12′54″W). Seawater within the Long Beach/Los Angeles harbor system exchanges tidally with Southern California Bight coastal water on a semi-diurnal basis, and so phytoplankton communities here are representative of local coastal assemblages, including the common presence of the toxic diatom *Pseudo-nitzschia* spp. (Schnetzer *et al.*, 2013). Near-surface seawater at an ambient temperature of 19°C containing the intact phytoplankton community was pumped into acid-washed 20 L plastic cubitainers using an acid-cleaned plastic hand pump, and immediately transported back to the laboratory to fill the experimental bottles.

The experimental design used a factorial matrix of temperature (19 and 23°C), CO_2 (present-day, 380 ppm; and predicted for year 2100, 800 ppm), and major nitrogen source (nitrate and urea). The three variable factorial matrix design of the experiment is shown in Table I. The treatments were designated as the following: 19°C, 380 ppm CO_2 (Control), 23°C, 380 ppm CO_2 (+Temp) 19°C, 800 ppm CO_2 (+CO_2) and 23°C, 800 ppm CO_2 (Combined); each of these was tested with either nitrate or urea addition. Total final dissolved nitrogen concentrations were 22.8 μM (nitrate-amended seawater) and 23.1 μM (urea-amended seawater); these values describe the total N available to the phytoplankton, whereby urea contains two N per mol and nitrate one. Since we used natural coastal seawater, it originally contained ambient background levels of both NO_3^- and urea. The NO_3^- diluent was dominated by this nitrogen species, with an average NO_3^-:urea N ratio of 12.5 to 1 (referred to as the nitrate or NO_3^- treatment). For the urea-dominated diluent, the urea N: NO_3^- N ratio was 2.6 to 1 (referred to as the urea treatment) (Spackeen *et al.*, 2017). Silicate (SiO_2, 42 μM) and phosphate (PO_4^{3-}, 2 μM) were provided to both treatments in the ecostat diluent (see below) to mimic typical freshly upwelled water nutrient conditions in the California Upwelling (Hutchins *et al.*, 1998; Firme *et al.*, 2003). Stable carbonate buffer system conditions in both CO_2 treatments were maintained by continuous bubbling of all experimental bottles with commercial CO_2/air mixtures (Praxair). Thermostatically controlled, recirculating heater/chiller systems (Hare *et al.*, 2007; Feng *et al.*, 2009, 2010) were used to maintain constant temperatures of 19 and 23°C ± 1°C in two incubators located on the roof of the Alan Hancock Foundation building at the University of Southern California.

Table I: Factorial experimental design combining different temperatures, CO_2 levels and major nitrogen sources into eight distinct treatments

Temperature	CO_2	N-source	Total *N* (μM)
23°C	380 ppm	Nitrate	22.8
	–	Urea	23.1
	800 ppm	Nitrate	22.8
	–	Urea	23.1
19°C	380 ppm	Nitrate	22.8
	–	Urea	23.1
	800 ppm	Nitrate	22.8
	–	Urea	23.1

To incubate the diatom assemblage under this eight-treatment triplicated experimental matrix of temperature, CO_2 and nitrogen source, we employed two 12-bottle natural community continuous culture systems or "ecostats" (Hutchins *et al.*, 2003; Hare *et al.*, 2005). The ecostats function in a manner that is similar to laboratory continuous culture systems, but are designed for use in temperature-controlled outdoor incubators with natural phytoplankton communities under natural sunlight. Seawater diluent medium consisting of nutrient-amended 0.2 μm cartridge-filtered local coastal seawater was continuously supplied to the twenty-four 2.7 L polycarbonate experimental bottles in the incubators (experimental volume ~2.5 L) through Teflon tubing from two 50 L reservoirs (one containing nitrate-amended seawater and one urea-amended seawater) located inside the lab using peristaltic pumps. The uniform dilution rate for all treatments was 0.3 d^{-1}, a typical whole phytoplankton community growth rate in local California coastal waters (Hutchins unpublished data). The natural community was kept continually gently suspended using a compressed air-driven rotating rack system that inverted the bottles at 5 min intervals; outflow tubes were located at the shoulders of the experimental bottles to provide constant quantitative removal of biomass, thereby allowing community loss rates to come into balance with growth rates. The advantage of the ecostat system is that the constant inflow of seawater diluent and the removal of cells through the outflows allow the community to be maintained for long periods without entering stationary phase as occurs rapidly in a batch or "growout" experiment, so community biomass and structure stabilizes (i.e. does not change) after a few days of equilibration under any given set of experimental conditions. Ecostat systems have been successfully used to conduct long-term natural community incubations examining various combinations of variables including temperature, CO_2, iron, irradiance and nutrients in environments ranging from polar to tropical seas (Hutchins *et al.*, 2003; Hare *et al.*, 2005, 2007a, b; Feng *et al.*, 2009, 2010; Rose *et al.*, 2009).

The ecostats were run for a 10-day incubation period; biogeochemical parameters such as nitrogen utilization rates are presented in Spackeen *et al.* (2017). Here, we present results detailing the interactive effects of warming, acidification and nitrogen source on diatom community structure and toxin concentrations, using samples obtained initially at the time of collection, and at the end of the 10-day

continuous culture period after the ecostat systems had equilibrated biologically to the experimental treatments.

Carbonate buffer system characterization

Spectrophotometric pH of the seawater incubations at final sampling was determined using a Shimadzu 1800 UV dual-beam spectrophotometer according to Clayon and Byrne. The concentration of dissolved inorganic carbon in $HgCl_2$ preserved samples was determined using a CM5230 CO_2 coulometer (UIC) according to King *et al.* (2011). Experimental pCO_2 (in µatm) was calculated using CO2SYS software (Lewis and Wallace, 1998) using these two measured parameters for quality control. pCO_2 values calculated from the pH and DIC measurements varied slightly day to day but were always within 10–15% of the target values of 380 and 800 ppm.

Cell counts

Diatom cells were preserved in acidified Lugol's solution and enumerated using inverted compound light-microscopy with an Accu-Scope 3032 according to the Utermöhl method (Utermöhl, 1931). Diatoms were identified according to Tomas (1997) under 20–40× agnification.

Domoic acid analysis

Two hundred mL from each bottle was gently filtered onto 25 mm GF/F filters and promptly stored at −20°C until extraction. The filters were extracted with 1 mL of 10% aqueous MeOH, clarified by syringe filtration split into three vials. Domoic acid was detected using HPLC-UV according to Mafra *et al.* (2009) with slight modification using an Agilent 1260 Infinity UHPLC 1260. Separation was performed with a reverse-phase C-18(2) Luna column (Phenomenex) as in Tatters *et al.* (2012) using one-fourth strength trifluoroacetic acid additions prior to injection. Quantification of domoic acid was enabled with certified reference material obtained from NRC Canada.

Statistics

Multivariate analyses were conducted using the PRIMER v6 statistics package (Clarke and Warwick, 2001) with the multivariate analysis of variance (PERMANOVA) add-on. Final cell abundances were square-root transformed and Bray–Curtis similarities computed. To compare the individual effects of each temperature, the two CO_2 concentrations and the two types of nitrogen source on individual genera/species and on overall community structure we conducted ANOSIM permutation analyses. These tests resulted in global R values that indicated no difference among treatments ($R = 0$ or low) or maximal group separation ($R = 1$ or high) (Clarke and Warwick, 2001). PERMANOVA analyses were used to test for significant differences among and within predefined groups in response to combined factors (temperature, pCO_2 level and nitrogen source) where Pseudo-F significance levels of 1 implied a large overlap among sample groups or treatments and Pseudo-$F > 1$ indicated little or no overlap between them (Anderson *et al.*, 2008). Also tested were the interactive effects of two of the factors or all three in forcing overall community structure (PERMANOVA).

RESULTS

Initial natural community composition

The natural coastal California phytoplankton community collected for the experiment was composed of various diatom taxa, with two dominant species (Fig. 1a and b). Taxa other than diatoms made up <1% of the initial community. Within the 5–80 µm size fraction, the two most abundant species were *Pseudo-nitzschia multiseries* and *Leptocylindrus danicus* (Fig. 1a) whose relative abundances were 48% and 39%, respectively (Fig. 1b). Other minor components of the diatom assemblage were *Chaetoceros* spp. (5%), *Pseudo-nitzschia hasleana* (4%), *Cylindrotheca fusiformis* (3%) and five other rare diatoms (1%) (Fig. 1a and b).

Final abundances of individual species

After 10 days of continuous incubation, the experimental variables CO_2, warming, and nitrogen source produced distinct diatom-dominated communities in the various treatments. Final overall relative abundances are shown at 19°C in Fig. 2a, and at 23°C in Fig 2b. Trends in relative abundances of individual species were significantly related to individual variables, and to varying degrees also driven by interactive effects of multiple variables.

Leptocylindrus danicus *L. danicus* was a strong competitor and was the dominant diatom in all final communities, despite comprising less than half of the original community. Final abundances ranged from 53.3% in the 23°C 800 NO_3^- treatment (Fig. 2b), to 94.8% at 19°C 800 NO_3^- (Fig. 2a). The major form of nitrogen present was the strongest and only significant individual forcing factor for *L. danicus* ($P = 0.001$) (Table SI), with nitrate being more influential compared with urea (ANOSIM) (Table SII). PERMANOVA indicated a moderate synergism between temperature and CO_2, and a similar interactive effect between all three variables combined ($P = 0.003$, $P = 0.004$, respectively) (Table SI).

Pseudo-nitzschia multiseries *P. multiseries* was the most abundant diatom species in the natural sample

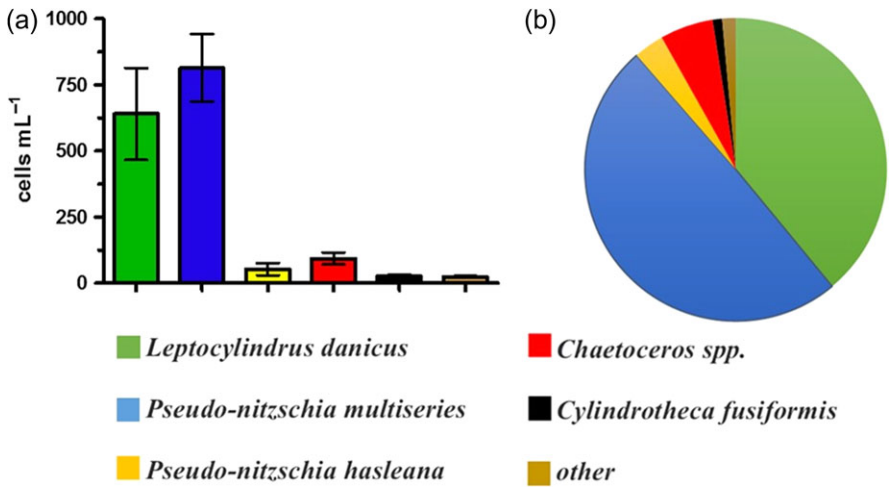

Fig. 1. (**a**) Absolute abundances (cells mL^{-1}) of the five most common diatom species and a combined category of five other rare species in the natural community collected at the beginning of the incubation experiment. Values are the means and error bars are the standard deviations of triplicate samples. (**b**) Initial relative abundances (% of total cells counted) of the same six diatom groups.

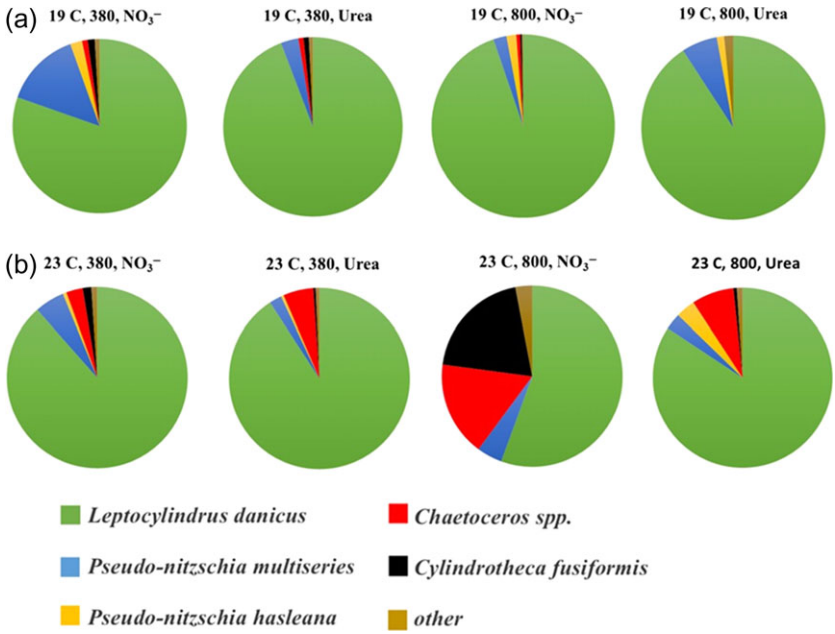

Fig. 2. Final relative abundances of the six diatom groups within each CO_2 and nitrogen treatment at (**a**) 19°C and (**b**) 23°C. Values are the means of triplicate bottles.

(Fig. 1), but failed to maintain this dominance in any of the experimental treatments. The final relative abundance of this potentially toxic species ranged from 2.3% in the 19°C 800 NO_3^-, treatment, to 14.2% in the 19°C 380 NO_3^- treatment (Fig. 2a). Temperature was the only significant variable in terms of the three factors individually ($P = 0.002$) (Table SI). There were pronounced synergistic interactive effects between CO_2 and major nitrogen source ($P < 0.001$) (Table SI). In addition, nitrate was more influential with increasing CO_2, and urea had more effect in combination with both increasing CO_2 and temperature (ANOSIM) (Table SII).

Pseudo-nitzschia hasleana *P. hasleana* was a minor species in the community throughout the study. The final relative abundance of *P. hasleana* ranged from 0% in the 23°C 800 NO_3^- treatment to 3.5% in the 23°C 800 urea treatment (Fig. 2b). CO_2 was the only significant variable in isolation ($P = 0.001$) (Table SI). All two-way interactions were significant ($P = 0.001$) (Table SI), but the interactions between all three variables were not significant. The major

nitrogen source (urea) was most and equally important at high CO_2 and high temperature (ANOSIM) (Table SII).

***Chaetoceros* spp.** The final relative abundance of *Chaetoceros* spp. ranged from 0% in the 19°C 800 urea treatment (Fig. 2a) to 18% in the 23°C 800 NO_3^- treatment (Fig. 2b). Temperature was the strongest driver among the individual variables ($P = 0.000$) (Table SI), but all were significant ($P = 0.001$). The relative abundance of *Chaetoceros* spp. at 19°C was always less than 1.0% (Fig. 2a). There were minor interactive effects from temperature and CO_2 ($P = 0.007$) (Table SI), temperature and major nitrogen source ($P = 0.005$) (Table SI), CO_2 and major nitrogen source ($P = 0.003$) (Table SI), and all three in concert ($P = 0.001$) (Table SI). ANOSIM analyses showed that high CO_2 and major nitrogen source were the most important influences on the abundance of this species (Table SII).

Cylindrotheca fusiformis Relative abundance of *Cylindrotheca* sp. in the final communities varied from 0% in the 19°C 800 urea treatment (Fig. 2a) to 21.3% in the 23°C 800 NO_3^- treatment (Fig. 2b). The effects of the individual variables were all significant, including temperature ($P = 0.001$) (Table SI), CO_2 ($P = 0.001$) (Table SI) and major nitrogen source ($P = 0.001$) (Table SI). Two-way interactions were all significant with temperature and major nitrogen source being the strongest ($P = 0.0005$) (Table SI) compared with temperature and CO_2 ($P = 0.001$) (Table SI) or CO_2 and major nitrogen source ($P = 0.003$) (Table SI). CO_2 was most influential at 23°C (ANOSIM) and nitrate was a stronger driver than urea (ANOSIM) (Table SII).

Overall community structure responses

Although overall forcing from the individual variables was relatively weak, each of the factors did significantly structure the communities. Non-parametric multi-dimensional scaling plots for the final nitrate- or urea-amended communities grouped by CO_2 (Fig. 3a and b) and temperature (Fig. 3c and d) indicated a degree of dissimilarity among assemblages in different treatments, and consistency among replicates from the same treatment. Global R values were 0.47 for temperature ($P = 0.001$, ANOSIM; Table SIII), 0.20 for pCO_2 ($P = 0.001$, Table SIII) and 0.06 for nutrients ($P = 0.025$, Table SIII).

Treatments grouped by temperature

At 19°C, CO_2 and nutrients both significantly influenced community structure ($P = 0.006$, $P = 0.002$, PERMANOVA) (Table SIV). PERMANOVA also indicated interactive effects of CO_2 and N-source on community structure ($P = 0.002$) (Table SIV). CO_2 was revealed to be a stronger driver than major nitrogen source at 19°C (Global R $pCO_2 = 0.51$, $P = 0.004$, Global R N source $= 0.01$, $P = 0.405$; ANOSIM) (Table SIII).

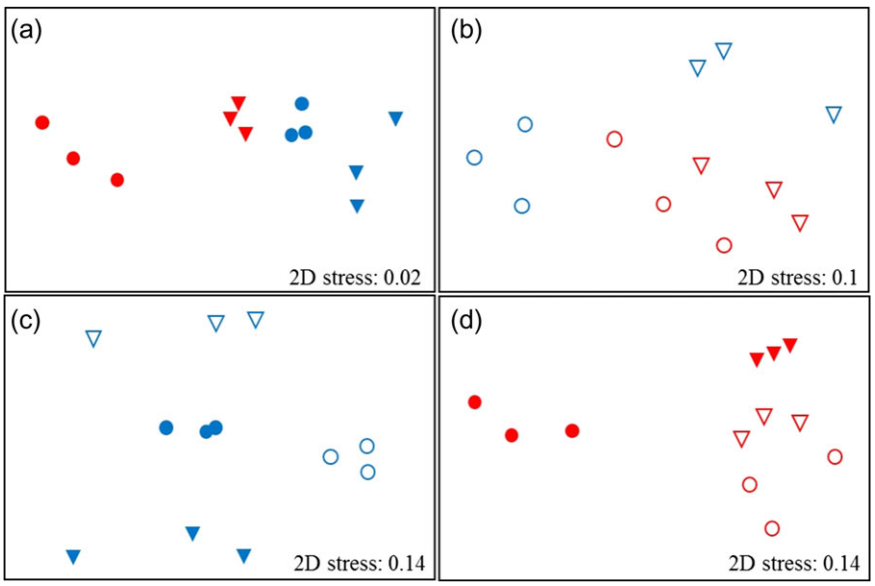

Fig. 3. MDS plots based on Bray–Curtis similarities among final phytoplankton communities. (**a**) 800 ppm CO_2 conditions (closed symbols) at 23°C (red symbols) and 19°C (blue symbols) receiving either urea (triangles) or nitrate (circles) as a major nitrogen source. (**b**) 380 ppm CO_2 conditions (open circles) at 23°C (red symbols) and 19°C (blue symbols) receiving either urea (triangles) or nitrate (circles) as a major nitrogen source. Similarities among the same communities at (**c**) 19°C and (**d**) 23°C.

At 23°C, the effects of CO_2 and major nitrogen source both significantly influenced diatom community structure ($P = 0.002$, $P = 0.001$) (Table SIV). PERMANOVA also indicated interactive effects from the two variables in concert ($P = 0.002$) (Table SIV) and ANOSIM revealed an enhanced effect at 23°C compared with the lower temperature of 19°C ($P = 0.002$). Notably, when compared with the low temperature, the major nitrogen source effect was enhanced by warming (Global $R = 0.42$ at 23°C; ANOSIM) (Table SIII). CO_2, however, was a much weaker driver of community structure at higher temperature (Global $R = 0.26$ $P = 0.0035$; ANOSIM) versus (19°C) (Table SIII).

Domoic acid

In the original natural sample, domoic acid concentrations were 1.11 pg cell^{-1}. Cell-normalized domoic acid concentrations varied among treatments after 10 days, based on abundances of *P. multiseries* (the only recognized domoic acid producing species in the community) (Fig. 4). One-way ANOVA was used to determine significant differences among treatments. Overall, the highest domoic acid quotas of 2.65 pg cell^{-1} were found in the 23°C 800 NO_3^- communities (Fig. 4), although *P. multiseries* was only a minor contributor to the overall community in this treatment (Fig. 2). The lowest cellular concentrations of domoic acid (0.88 pg cell^{-1}) were observed in the 19°C 380 NO_3^- treatments (Fig. 4), the treatment that also had the highest abundances of *P. multiseries* (Fig. 2).

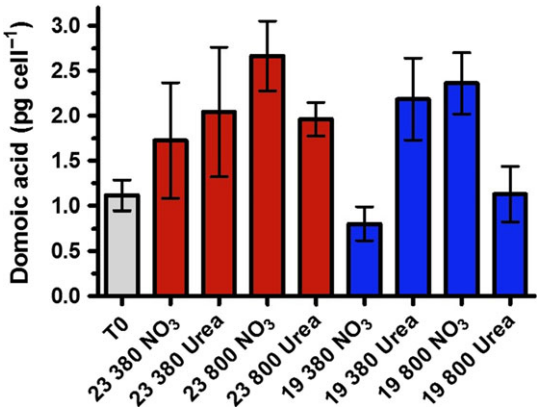

Fig. 4. Domoic acid concentrations normalized to *Pseudo-nitzschia multiseries* cell numbers at the time of collection (gray bars) and in final samples after 10 days incubation within each temperature, CO_2 and major nitrogen source treatment. Values are reported in pg cell^{-1} with cellular concentrations in the 19°C communities depicted in blue bars and 23°C shown in red. Values are the means and error bars are the standard deviations of triplicates.

Table II: Pairwise comparisons between treatments for domoic acid

Treatment	Versus treatment	*P*-value
19°C 380 ppm CO_2 nitrate	23°C 380 ppm CO_2 urea*	$P = 0.0446$
19°C 380 ppm CO_2 nitrate	23°C 800 ppm CO_2 nitrate*	$P = 0.0017$
19°C 380 ppm CO_2 nitrate	23°C 800 ppm CO_2 urea*	$P = 0.0015$
19°C 800 ppm CO_2, urea	23°C 800 ppm CO_2 nitrate*	$P = 0.0058$
19°C 380 ppm CO_2 nitrate	19°C 800 ppm CO_2 nitrate*	$P = 0.0022$
19°C 380 ppm CO_2 urea*	19°C 800 ppm CO_2 urea	$P = 0.0288$
19°C 380 ppm CO_2 nitrate*	19°C 380 ppm CO_2 urea	$P = 0.0081$
19°C 800 ppm CO_2 nitrate*	19°C 800 ppm CO_2 urea	$P = 0.0095$
23°C 800 ppm CO_2 nitrate*	23°C 800 ppm CO_2 urea	$P = 0.0480$

Higher value is depicted with an asterisk*.

Temperature comparisons

In general, warmer temperatures resulted in higher cellular domoic quotas in most cases. Cellular domoic acid levels in the 19°C 380 NO_3^- treatment were significantly lower than those in the 23°C 800 treatments with either nitrate ($P = 0.002$) or urea ($P = 0.002$) (Table II), as well as lower than those in the 23°C 380 ppm CO_2 treatments with urea ($P = 0.04$) (Table II). The 19°C 800 urea treatment also had lower domoic acid concentrations than both the 23°C 800 urea ($P = 0.03$) and nitrate ($P = 0.006$) (Table II) treatments (Fig. 4).

CO_2 comparisons

In the 19°C nitrate treatments, levels of domoic acid in the 380 ppm CO_2 bottles were significantly lower than those in the 800 ppm CO_2 bottles ($P = 0.002$) (Table II). However, in the 19°C urea treatments, the opposite trend was observed relative to CO_2 levels, with higher toxin concentrations in the 380 ppm than in the 800 ppm treatments ($P = 0.03$) (Table II) (Fig. 4). At 23°C, there were no significant differences in cellular domoic acid quotas between CO_2 levels with the same major nitrogen sources; levels were considerably higher in the 23°C 800 NO_3^- bottles than in the 23°C 380 NO_3^- bottles, but due to large variability between replicates at the lower CO_2 level this difference was not significant at the $P < 0.05$ level (Fig. 4).

Nitrogen source comparisons

There were significantly higher levels of cell-normalized domoic acid in the 19°C 380 CO_2 treatments with urea as a major nitrogen source, compared with nitrate ($P = 0.008$) (Table II). However, toxin quotas were significantly higher with nitrate than with urea under elevated CO_2 at both 19°C ($P = 0.009$) and 23°C ($P = 0.048$) (Table II). At 23°C, there were no significant differences in cellular domoic acid levels as a function of major nitrogen source at 380 ppm CO_2 ($P > 0.05$, Fig. 4).

DISCUSSION

Global change experimental methods and their implications

A variety of global change incubation experiments have been performed with marine diatom-dominated assemblages (Kim *et al.*, 2006; Hare *et al.*, 2007; Feng *et al.*, 2008, 2010; Tortell *et al.*, 2008; Tatters *et al.*, 2013b). All of these previous studies noted significant responses such as community composition shifts and altered productivity. For instance, responses attributed to elevated CO_2 included changes in the growth of centric versus pennate species (Feng *et al.*, 2008; Tatters *et al.*, 2013b), increasing growth of larger and/or chain-forming diatoms (Tortell *et al.*, 2008; Feng *et al.*, 2010) and increases in primary productivity (Tortell *et al.*, 2008). A few diatom community global change studies have also included an assessment of warming effects. Tatters *et al.* (2013b) found that increases in temperature were a stronger driver of community shifts than CO_2 changes, mostly resulting in decreases in community species diversity. Feng *et al.* (2010) reported shifts away from diatoms and towards nanoeukaryote groups in warming treatments during the North Atlantic spring bloom. These studies imply that global change-driven community shifts both within the diatom community, and between diatoms and other groups, could have potentially large-scale direct and indirect consequences for marine ecosystems.

The ecostat continuous culture system employed in this study allowed us to incubate a California coastal diatom-dominated community for 10 days supplied with environmentally relevant nutrient concentrations. As in all of the previous natural community studies cited above, the major caveat on our study relative to global change processes is that the duration of our experiment was necessarily much shorter than the timescale of ongoing anthropogenic changes in temperature and acidification. Thus, any extrapolation of our results to decadal or longer responses of natural assemblages needs to consider appropriate qualifications. In addition, although we focus here on the abiotic variables we tested, biotic interactions such as allelopathy could have also significantly influenced the composition of these assemblages (Tatters *et al.*, 2013a).

Despite the need to consider these qualifiers when interpreting our results, it is clear that the timescales of our experimental shifts in temperature and pCO_2 are certainly relevant today with respect to California coastal upwelling events. In fact, during active upwelling similar biogeochemical changes often occur over timescales of days or weeks (Hutchins *et al.*, 1998; Firme *et al.*, 2003). Our results can, therefore, offer insights into present-day biological responses to these three variables in this region. While our results should not be interpreted as providing unambiguous, detailed predictions of the exact responses of future coastal ocean phytoplankton communities, we suggest that with appropriate caution they provide a benchmark to formulate hypotheses to be tested in future long-term observational studies of responses to global change variables, and to possible shifts in major nitrogen sources due to eutrophication with expanding coastal development and population.

To our knowledge, no incubation experiments with natural diatom communities have attempted to manipulate nutrient sources simultaneously with temperature and CO_2. A number of recent studies examining cultured cyanobacteria, diatoms or coccolithophores have addressed two-way interactions between CO_2 and temperature (Hutchins *et al.*, 2007; De Bodt *et al.*, 2010; Schlüter *et al.*, 2014; Pancic *et al.*, 2015; Taucher *et al.*, 2015), CO_2 and light (Rokitta and Rost, 2012), or CO_2 and nutrients (Lefebvre *et al.*, 2012; Rouco *et al.*, 2013). Other work with mixed natural communities has tested CO_2 interactions with temperature (Feng *et al.*, 2009; Tatters *et al.*, 2013b; Sommer *et al.*, 2015). Only relatively few studies have attempted to manipulate three or more climate change variables simultaneously (Feng *et al.*, 2008, 2010; Shi *et al.*, 2015; Boyd *et al.*, 2016). Like these latter experiments, our study was undertaken in an effort to move beyond examining single and two-variable effects in climate change scenarios, by considering the combined effects of three variables simultaneously.

Nitrogen source interactions

Our study combined effects of nutrient sources that might result from local eutrophication impacts, with those of the global change variables temperature and CO_2. Each of the eight combined multivariate treatments yielded distinct communities. Although all the final assemblages were dominated by the diatom species *Leptocylindrus danicus*, they were nevertheless significantly structured to varying degrees by the three variables and their mutual interactions. Notably, although multiple forms of nitrogen were present naturally in the collected water, community structure was clearly a significant function of the major nitrogen species present in the highest concentration (the added nitrate or urea), either alone or interactively with temperature and CO_2. One of our most striking results was that changing the major nitrogen source had a far stronger influence on diatom community structure at the elevated temperature, whereas CO_2 interacted with warming to a lesser degree. The interactive effects of nitrogen source and acidification were also significantly enhanced in the warming treatment. This may indicate an increasingly important role for eutrophication as a

controlling factor on diatom assemblages in a future warmer coastal ocean. For an examination of uptake rates of various inorganic and organic nitrogen sources under our eight experimental treatments, we refer the reader to Spackeen *et al.* (2017).

Temperature interactions

Temperature is a key climate change variable, and has been repeatedly shown to be fundamental in determining phytoplankton community structure (Hare *et al.*, 2007; Feng *et al.*, 2010; Tatters *et al.*, 2013b; Hutchins and Boyd, 2016). Diatom community changes associated with temperature changes have been documented in both a contemporary and historical context (Thomas *et al.*, 2012; Irwin *et al.*, 2015). Some experimental studies have suggested that warming has neutral (Sommer *et al.*, 2015) or positive (Yvon-Durocher *et al.*, 2015) effects on phytoplankton community diversity. Conversely, our work shows that warming and diversity may also be inversely correlated, at least on short timescales (Tatters *et al.*, 2013b, this study); either way, such altered diversity may have implications for food web structure and function. Less is known about the interplay of temperature with other factors, or the potential for non-linear responses; such interactive effects often tend to be unpredictable at this time and more effectively characterized by empirical studies. Although observing the net outcome of these interactive relationships is useful, we still have much to learn about the mechanistic basis of these complex multivariate responses.

Global change and phytoplankton diversity

Due to natural variability in the coastal ocean, contemporary phytoplankton are exposed to a range of environmental variables on a diurnal and seasonal basis (Duarte *et al.*, 2013). When the extremes of future global change are realized as well, coping populations may fundamentally change. Whether these differences generally manifest in terms of genotype, phenotype or both remains to be seen. Our experiments and previous studies demonstrate that exposure to multivariable scenarios almost certainly results from differences in competitive ability among co-occurring diatoms. (Hoffmann *et al.*, 2008; Tatters *et al.*, 2013b). These could be due to trait-based tradeoffs (Litchman *et al.*, 2012). In addition, we demonstrate a "layering" effect of additional variables that can enhance, counteract or even reverse the trajectory of the observed response to a single variable.

Using diverse natural populations within a relatively large volume in the course of our ecostat experiment could have resulted in species or strain sorting of existing variation in the populations of diatoms. In our experiment, the continuous culture dilution may have facilitated sorting, especially if particular phenotypes were performing optimally while slower growing "losers" were being washed out (Hutchins *et al.*, 2003). Sorting allows for the selection of standing variation that may occur because of exposure to different environmental conditions (Ackerly, 2003; Litchman *et al.*, 2012; Panicic *et al.*, 2015), and so may influence competitive outcomes. The ability of diatoms to proliferate over a broad range of environmental conditions is likely influenced by a spectrum of genetic composition linked to physiological capability and plasticity within populations. In fact, the variability in the growth rates among isolates of the diatom *Ditylum brightwellii* suggests that in natural populations, genotypic frequencies could change dramatically on a timescale of weeks (Rynearson and Armbrust, 2004). Distinct seasonal populations of this diatom have been documented (Gallagher, 1980; Rynearson *et al.*, 2006). Likewise, it has been noted that the composition of both artificial and natural communities of a number of phytoplankton groups can shift dramatically in response to temperature and CO_2 increases (Collins, 2014).

Diatoms and global change

Diatoms are often considered to be a biogeochemically coherent functional group, yet there is tremendous morphological and genetic diversity within the class. Relatively small differences in traits such as uptake rates of different nutrient sources and other physiological parameters among species may contribute to differences in competitive ability, and so alter community composition. It is likely that these observed community shifts in the present study were a result of physiological and competitive differences that were magnified by the non-linear nature of multivariable interactions. Interestingly, the observed synergistic and antagonistic interactive effects were not consistent among any of the diatom genera examined, suggesting that taxon-specific responses to multiple stressors in a changing ocean are likely (Boyd and Hutchins, 2012), as has been previously noted in other studies (Hoffmann *et al.*, 2008; Low-Décarie *et al.*, 2013; Tatters *et al.*, 2013b).

It was striking that *L. danicus* dominated all of the final communities to varying degrees. Previous studies have highlighted the response of *Leptocylindrus spp.* to a variety of variables, both in culture and in field examinations. These include the determination that optimal temperature for growth was between 15 and 20°C (Verity, 1982) and reported domination of enclosed communities (Davis *et al.*, 1980; Egge and Aksnes, 1992; Reul *et al.*, 2014), including at decreased pH (Pedersen and Hansen, 2003). In our mixed natural community continuous culture system experiments, dominance of this

species may have also been facilitated by the characteristic sorting action that iteratively increases the relative abundance of rapidly growing species relative to slower growing ones, as discussed above. Thus, the overall proliferation of *L. danicus* across all treatments was not unexpected.

In our experiments, harmful algal bloom-forming (HAB) species of *Pseudo-nitzschia* co-occurred with phytoplankton species that are considered innocuous. Due to human and environmental health significance, a wealth of HAB experiments have been conducted investigating the effects of single variables such as nutrients or light. Our work adds to the relatively few multivariate studies focused on HAB species, especially in a global change context (Fu *et al.*, 2010; Sun *et al.*, 2011; Kremp *et al.*, 2012; Tatters *et al.*, 2012, 2013a, b). In addition, many single variable culture investigations have used one or few clonal isolates, while obviously understanding relevant impacts also requires studies using natural communities containing mixtures of populations. Moving beyond the examination of cultured organisms allows for a more realistic interpretation and better understanding of natural responses to interactive variables both currently and potentially in the future ocean (Hutchins and Fu, 2017).

Pseudo-nitzschia toxicity

Pseudo-nitzschia cellular quotas of domoic acid in our experiments were influenced by temperature, CO_2 and major nitrogen source. As domoic acid quotas were different among all treatments, it is tempting to suggest that interactive effects of these variables were responsible. Although particulate concentrations of domoic acid were also affected in the final communities, the differences between treatments were not as pronounced as for community composition. Due to limited replication and sample number, multivariate analyses for cellular domoic acid were not performed. Instead, simple statistics were applied and yielded numerous significant pairwise comparisons between the eight treatments. Any aforementioned strain sorting that occurred during the experiment could also help explain variability among treatments, but it is unclear to what degree this comes into play. Despite the fact that there was no clear trajectory to the domoic acid response, there were general trends to the combined treatments. Individually, temperature seemed to have the strongest influence, followed by CO_2 and major nitrogen source providing similar results (regardless of which N source was tested).

Nitrogen and domoic acid

Of the three separate variables examined in this study, nitrogen speciation is the most thoroughly researched in terms of *Pseudo-nitzschia*-related physiology. We found inconclusive trends in cellular domoic acid in the final communities supplied with either nitrate or urea as the major nitrogen source, depending on the other combined variables. This is in line with previous studies that demonstrate nitrogen speciation produces contrasting results in both laboratory and field experiments. Experiments examining N-sources have demonstrated the importance of nitrogen speciation in domoic acid production (Armstrong-Howard *et al.*, 2007; Calu *et al.*, 2009). Another study examined isolated cells from the same water sample and found that isolates had different growth rates and domoic acid content (Thessen *et al.*, 2009), thus highlighting the intra- and interspecific diversity of *Pseudo-nitzschia* and/or associated microflora (Stewart, 2008). The latter authors inferred that this was due to changing nitrogen source alone, but interactive effects with temperature and CO_2 were not studied.

Temperature and domoic acid

Temperature has been documented to modulate domoic acid concentrations in *Pseudo-nitzschia*, with both positive and negative correlations reported in the literature. Domoic acid levels increased from 4 to 15°C in *P. seriata*, and from 5 to 25°C in *P. multiseries* (Lundholm *et al.*, 2004). Lewis *et al.* (1993) also reported increased toxin with increasing temperature in *P. multiseries*, but not for other species in the genus. In contrast, cellular domoic acid concentrations in *P. multiseries* were found to be lower at 27°C versus 18°C (Amato *et al.*, 2010). A general increase in domoic acid production with warming has been reported for *P. australis* (Zhu *et al.*, 2017), as well as when this species was grown in a matrix of light and temperature (Thorel *et al.*, 2014). Our community study, where the major toxic species was *P. multiseries*, supports these latter results as cellular toxicity was generally increased by warming. Ours and early studies clearly indicate that temperature modulates domoic acid production, but the mechanisms, which may involve enzymatic activity and associated bacterial flora, are poorly understood.

CO_2 and domoic acid

The co-varying nature of CO_2 and pH in seawater has also been shown to influence domoic acid concentrations. Two culture studies with two different *Pseudo-nitzschia* species have demonstrated increases in cellular domoic acid upon exposure to year 2100 (~800 ppm) CO_2 levels compared to current concentrations, especially when combined with phosphorus limitation (Sun *et al.*, 2011) or silicon limitation (Tatters *et al.*, 2012). In our mixed natural assemblage study, cellular toxicity was also generally increased by CO_2, despite the continuous supply of both

of these nutrients to the cells. However, other studies have conversely demonstrated increases in cellular domoic acid with increasing pH, corresponding to lower pCO_2 (Lundholm *et al.*, 2004; Trimborn *et al.*, 2008). These apparently opposing results are suggestive of contrasting potential implications for climate change and/or current bloom scenarios, but it is important to note that these results were obtained using quite different methodologies. More research in this area is needed using consistent techniques, particularly with respect to the interactions between acidification and changing nutrient availability.

The highest toxin levels on a per cell basis were detected in our high temperature, high CO_2, nitrate major treatment, conditions that are likely to occur episodically in the present-day upwelling region, and perhaps generally in the future California coastal ocean. Enhanced *Pseudo-nitzschia* toxicity could result in additional domoic acid input and circulation through already affected marine food webs. The fact that *P. multiseries* comprised less than 10% of the population, however, under all 23°C treatments suggests potential limitation of growth by temperature. This phenomenon may occur in a regionally specific manner where some *Pseudo-nitzschia* populations in generally cooler areas may grow more rapidly and may be inhibited by temperature increases, while cell-specific toxin production reacts in an opposite fashion. Indeed, exceptional levels of domoic acid accumulation in marine food webs were observed along the U.S. West Coast in 2015 due to interactions between anomalously warm conditions and normal coastal upwelling processes, resulting in major economic losses to coastal fishing industries (Bond *et al.*, 2015; McCabe *et al.*, 2016). Warming has been shown to enhance the toxicity of a *P. australis* culture isolated near the beginning of this regional warming occurrence (Zhu *et al.*, 2017). Future warming events of similar magnitude are likely to continue, and toxic bloom occurrences are anticipated to become increasingly common.

CONCLUSIONS

Our results suggest that marine diatom species could respond differentially to environmental changes resulting from both local (nitrogen source) and global (warming and acidification) anthropogenic impacts. The effects of eutrophication-driven changes in nitrogen sources could be magnified under warmer sea surface temperatures. Our experiments also suggest that future observational and process studies should be alert to the potential for longer-term changes in community structure, as well as to possible environmentally and ecologically significant physiological responses such as modulated domoic acid production. Coupled with natural upwelling and anthropogenic acidification and changes in nitrogen sources in an increasingly human-impacted coastal zone, these processes may cumulatively affect the productive upwelling-based marine food webs of coastal California.

SUPPLEMENTARY DATA

Supplementary data can be found online at *Journal of Plankton Research* online..

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DATA ARCHIVE

To be archived at BCO-DMO.

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