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Simulated upwelling and marine heatwave events promote similar growth rates but differential domoic acid toxicity in *Pseudo-nitzschia australis*

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A R T I C L E I N F O

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A B S T R A C T

Along the west coast of the United States, highly toxic *Pseudo-nitzschia* blooms have been associated with two contrasting regional phenomena: seasonal upwelling and marine heatwaves. While upwelling delivers cool water rich in pCO_2 and an abundance of macronutrients to the upper water column, marine heatwaves instead lead to warmer surface waters, low pCO_2, and reduced nutrient availability. Understanding *Pseudo-nitzschia* dynamics under these two conditions is important for bloom forecasting and coastal management, yet the mechanisms driving toxic bloom formation during contrasting upwelling vs. heatwave conditions remain poorly understood. To gain a better understanding of what drives *Pseudo-nitzschia australis* growth and toxicity during these events, multiple-driver scenario or 'cluster' experiments were conducted using temperature, pCO_2, and nutrient levels reflecting conditions during upwelling (13 °C, 900 ppm pCO_2, replete nutrients) and two intensities of marine heatwaves (19 °C or 20.5 °C, 250 ppm pCO_2, reduced macronutrients). While *P. australis* grew equally well under both heatwave and upwelling conditions, similar to what has been observed in the natural environment, cells were only toxic in the upwelling treatment. We also conducted single-driver experiments to gain a mechanistic understanding of which drivers most impact *P. australis* growth and toxicity. These experiments indicated that nitrogen concentration and N:P ratio were likely the drivers that most influenced domoic acid production, while the impacts of temperature or pCO_2 concentration were less pronounced. Together, these experiments may help to provide both mechanistic and holistic perspectives on toxic *P. australis* blooms in the dynamic and changing coastal ocean, where cells interact simultaneously with multiple altered environmental variables.

1. Introduction

The biologically-rich California Current System is increasingly impacted by two contrasting extreme phenomena: stronger upwelling events and marine heatwaves. These events differentially impact marine ecosystem function through distinct alteration of the bottom-up controls that influence phytoplankton physiology and community structure. Seasonal upwelling-favorable wind conditions vertically transport cold, carbon dioxide- (CO_2) and nutrient-rich deeper water to the surface, fueling high rates of primary productivity (Chavez and Messié 2009; Gruber et al., 2012; Capone and Hutchins 2013). On the other hand, surface ocean warming during marine heatwaves can enhance stratification, consequentially reducing the availability of inorganic nutrients and carbon available for primary producers (Cheung and Frölicher 2020; Sen Gupta et al., 2020).

Climate change is predicted to amplify the intensity of both of these contrasting coastal processes in the California Current System, which may fundamentally alter the marine environment and conditions for phytoplankton (Du and Peterson 2013; Zhu et al., 2017; Smith et al., 2018; Barth et al., 2020; Trainer et al., 2020). Stronger and more

Abbreviations: NUEs, nitrogen use efficiencies; CCMs, carbon concentrating mechanisms; DA, domoic acid; ENSO, El Niño-Southern Oscillation; pCO_2, partial pressure of carbon dioxide; PDO, Pacific Decadal Oscillation; POC, particulate organic carbon.

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frequent upwelling favorable winds could increase the influx of cold water and nutrients, counteracting the effects of warming and enhanced stratification that accompany rising global temperature (Bakun et al., 2015). These conditions may favor dense and diverse diatom-dominated phytoplankton communities, as this group thrives with high inputs of nutrients (Lassiter et al., 2006; Du and Peterson 2018). Additionally, ocean acidification is likely to intensify with the increased vertical transport of CO_2-rich bottom waters (Gruber et al., 2012; Hauri et al., 2012; Capone and Hutchins 2013). For some phytoplankton groups, this combined exposure to low-pH upwelled waters and global ocean acidification could exceed physiological tolerances and cause cellular stress (Hurd et al., 2009; Hutchins and Fu 2017). On the other hand, upwelling could supplement the amount of inorganic carbon available for photosynthesis, decreasing cellular energy expenditure on carbon concentrating mechanisms (CCMs), especially in phytoplankton groups with low efficiency CCMs and weak Ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) affinities for CO_2 (Giordano et al., 2005). Therefore, altered temperature, nutrient concentrations, and inorganic carbon availability with a projected increase in upwelling may impact marine phytoplankton community structure and function (Lassiter et al., 2006; Du and Peterson 2018).

Extreme heat events represent the other face of climate change in this coastal upwelling system. Climate models and meta-analyses have attributed the increased frequency, duration, and intensity (e.g., maximum temperature) of marine heatwaves to anthropogenic climate change (Di Lorenzo and Mantua 2016; Oliver et al., 2018; Laufkötter et al., 2020). This increased heatwave severity (extreme heatwaves) also plays a role in changing conditions that drive phytoplankton community structure and system productivity (Soulié et al., 2022). For instance, during the 2013–2015 "blob" heatwave event in the North Pacific Ocean, surface warming and enhanced stratification caused weaker upwelling that reduced nutrient input and therefore overall primary productivity of the system (Cavole et al., 2016; Yang et al., 2018; Peña et al., 2019). In some regions of the North Pacific, this combination of warming and upwelling relaxation led to a shift in phytoplankton community composition away from diatoms, and towards a high abundance and diversity of dinoflagellates (Du and Peterson 2018). Lab experiments mimicking the temperature effects of heatwaves on natural communities have also demonstrated shifts to low diversity, dinoflagellate-dominated assemblages (Remy et al., 2017). Furthermore, warm water phytoplankton species can invade normally colder temperate regimes during extended surface ocean warming (Ajani et al. 2020). Additionally, during marine heatwaves CO_2 concentrations may be lower due to thermally-reduced gas solubility or following drawdown by phytoplankton (Murata et al., 2002; Chavez and Messié 2009). Decreased inorganic carbon availability could limit rates of carbon fixation by primary producers, or favor species with strong CCMs (Giordano et al., 2005).

Although upwelling and marine heatwaves differentially alter the environmental conditions in the California Current System that control phytoplankton community structure and function, blooms of the toxic diatom *Pseudo-nitzschia australis* have been associated with both of these climatic extremes. This diatom often produces domoic acid, a potent mammalian and avian neurotoxin, which can be harmful to human health, marine ecosystems, and commercial fisheries (Bates et al., 2018). High abundances of *Pseudo-nitzschia* spp. and domoic acid have frequently been observed during springtime upwelling in the California Current System (Lange et al., 1994; Trainer et al., 2000; Brzezinski and Washburn 2011; Schnetzer et al. 2013; Smith et al., 2018). The correlation of toxic blooms and upwelling has led HAB researchers to categorize *Pseudo-nitzschia* as a cold-water genus that blooms following large, pulsed inputs of nutrients.

In contrast, a marine heatwave in 2015 induced a persistent, highly toxic *Pseudo-nitzschia* bloom of unprecedented size. In the northern portion of the California Current System, several species, including *P. australis, P. fraudulenta*, and *P. pungens* thrived in these warmer, low-nutrient, low-CO_2 conditions, resulting in record concentrations of domoic acid in shellfish and marine mammals (McCabe et al., 2016). Over longer time periods, increased concentrations of domoic acid in the Northern California Current System have been correlated with the warm phases of the El Niño-Southern Oscillation (ENSO) and the Pacific Decadal Oscillation (PDO; McKibben et al., 2017, Sandoval-Belmar et al., 2023). This indicates that in some regions heatwaves (and extreme heatwaves) are another trigger of toxic *Pseudo-nitzschia* bloom formation, in addition to upwelling. The cellular strategies that support the remarkable ability of *P. australis* to bloom under these vastly different environmental conditions remain unknown. However, it should be noted that other studies have found little association between heatwaves and *Pseudo-nitzschia* blooms in the Southern California Current system, thus considerations of regional differences are important (Barron et al., 2013; Ryan et al. 2017).

The physiological responses of *P. australis* to single environmental drivers have been well documented (reviewed in Lelong et al., 2012; Trainer et al., 2012; Bates et al., 2018). For instance, warming can increase the growth rate and toxicity of several species of *Pseudo-nitzschia* (Lewis et al., 1993; Zhu et al., 2017). However, one study measured more domoic acid per cell in *P. seriata* at 4°C, compared to cells grown at 15°C (Lundholm et al., 1994). Furthermore, in the absence of other triggering factors such as nutrient limitation, decreases in pH associated with increases in pCO_2 did not themselves greatly alter the amount of total domoic acid produced by *P. multiseries* and *P. fraudulenta* (Sun et al., 2011; Tatters et al., 2012). However, more domoic acid per *Pseudo-nitzschia* spp. cell was measured in a natural phytoplankton community exposed to 800 ppm pCO_2, relative to 380 ppm (Tatters et al., 2018). Furthermore, growth phase plays an important role, as more domoic acid per *P. australis* cell was measured at higher pCO_2 concentrations in early stationary phase, but not during exponential growth (Wingert and Cochlan, 2021). Lastly, phosphate and silicate limitation have been implicated in increasing both dissolved and cellular domoic acid (Pan et al., 1998; Sun et al., 2011; Tatters et al., 2012). However, sufficient quantities of nitrogen are necessary for domoic acid biosynthesis, as the amino acid glutamate is a building block in its biosynthesis (Bates et al., 1991).

Despite this extensive body of knowledge about the individual impacts of temperature, pCO_2, and nutrients on *Pseudo-nitzschia* spp., little is known about the synergistic effects between multiple variables that may contribute to toxic bloom formation during upwelling and marine heatwaves. Previous multiple-driver studies with *Pseudo-nitzschia* spp. have demonstrated synergistic effects between multiple variables that can exacerbate growth and/or toxicity (Sun et al., 2011; Tatters et al., 2012; Kelly et al., 2021). This suggests that single stressor experiments cannot fully elucidate bloom dynamics in the natural environment where multiple drivers are at play; however, these single stressor experiments are critical to understanding the mechanisms driving observed responses to multiple drivers. To our knowledge, multiple driver experiments have not been used to directly compare the impacts of upwelling and marine heatwaves of *P. australis* toxic bloom formation.

The objective of our experiment was to examine the physiological changes that allow *P. australis* to thrive under these disparate conditions. We studied the impacts of simulated upwelling, marine heatwaves, and extreme marine heatwaves on *P. australis* physiology and toxicity to gain a better understanding of how blooms may be impacted by these events. Our in-depth approach to this question involved (a) examining relevant variables (temperature, pCO_2, and nutrients) clustered together into a scenario approach for a holistic understanding, and (b) single-driver experiments for a mechanistic understanding of how individual variables may be impacting the responses we see. We hypothesized that similar to what has been observed in the field, *P. australis* would be able to grow and produce toxins under both heatwave and upwelling conditions.

2. Materials and methods

2.1. Culture maintenance

Pseudo-nitzschia australis (strain NWFSC 731) was isolated from Long Beach, Washington State, USA on November 3, 2020. The temperature and salinity were 14°C and 27 ppt, respectively at the time of collection. In the lab, cultures were maintained in modified *f*/2 medium under 120 μmol photons m^{-2} s^{-1} LED light on a 12:12 light:dark cycle. The salinity of waters off the Washington coast can fluctuate between 19.7 and 33 ppt dependent on riverine input (Aguilar-Islas and Bruland 2006). The salinity of the natural seawater used to make maintenance media was 33 ppt and cultures were maintained at this salinity for all experiments. To make modified *f*/2 medium, natural seawater was collected from the San Pedro Ocean Time Series site (33° 33′ N, 118° 24′ W). The natural seawater was then filter-sterilized (0.2 μm Whatman Polycap 150 TC cartridge filter) and microwaved. A Panasonic Genius Sensor microwave was used to sterilize 2 L bottles of seawater for approximately 16 min (until boiling began) at 1250 W, mixing halfway through to ensure homogeneity in temperature. The salinity of the natural seawater collected from the California Bight was reduced from 36 to 33 ppt by diluting with microwave sterilized Milli-Q water. Lastly, the sterilized seawater was amended with nutrients (100 μM nitrate, 100 μM silicate, 6 μM phosphate; standard trace metals and vitamins; Guillard and Ryther, 1962; Guillard, 1975). The seawater used in this study was collected at the surface in the summer. The background nutriment concentrations of phosphate and silicate were 0.3 μM and 1.62 μM respectively. Our values were very close to the concentrations (0.2–0.4 μM phosphate; 2–3 μM silicate) at the surface in the study of Caron et al. (2017), who found that the nitrate concentrations in the surface seawater at this site were almost undetectable (<1 μM). Although the nitrate concentration in the seawater was not measured, it can be assumed to be almost zero since our other two nutrient measurements were close to the values in the study of Caron et al. (2017).

2.2. Experimental conditions

In each experiment, cultures were acclimated to the respective conditions (described below and in Tables 1 & 2) for at least eight generations before sampling. All cultures were maintained semi-continuously in 1 L polycarbonate bottles with experimental base medium (sterilized natural seawater + standard *f*/2 trace metals and vitamins; Guillard and Ryther, 1962; Guillard, 1975). Culture volume was also maintained at 1 L. Nitrate, silicate, and phosphate were added directly into culture flasks after dilution for final concentrations specific to each treatment (Tables 1 & 2). These semi-continuous culturing methods allowed *P. australis* to remain in the exponential phase of growth by diluting with fresh medium every other day. This repetitive reduction in the number of cells not only prevents cultures from ever reaching the stationary phase of growth, which would lead to self-shading and depletion of nutrients and CO_2, but also allows cultures to determine their own growth rates, based on the conditions to which they are exposed (Kelly et al., 2021; Chen and Gao, 2021). The percentage of the total volume replaced was dependent on the biomass increase over the dilution cycle; cultures were diluted down to approximately the same initial biomass with each dilution (estimated with in vivo fluorescence).

Table 1
Levels of treatment for cluster experiments, with altered temperature, pCO_2, and nutrients[1].

Treatment	Upwelling	LTCN	Heatwave	Extreme heatwave
Temperature	13 °C	13 °C	19 °C	20.5 °C
pCO_2	900 μatm	230 μatm	255 μatm	240 μatm
Nutrients	3 μM PO_4	0.5 μM PO_4	0.5 μM PO_4	0.5 μM PO_4
	40 μM $Si(OH)_4$	10 μM $Si(OH)_4$	10 μM $Si(OH)_4$	10 μM $Si(OH)_4$
	30 μM NO_3	5 μM NO_3	5 μM NO_3	5 μM NO_3

[1] LTCN = low temperature, carbon dioxide, and nutrients.

Table 2
Experimental treatments in the nutrient-temperature matrix experiment. Each treatment below was grown under two temperature conditions: 13 and 19 °C.

N:P ratio	Total nutrient concentration	Nitrate (μM)	Phosphate (μM)	Silicate (μM)
5	Low	5	1	10
	High	30	6	40
10	Low	5	0.5	10
	High	30	3	40
50	Low	5	0.1	10
	High	30	0.6	40

In the cluster experiments, cultures were simultaneously exposed to a combination of temperature, pCO_2, and nutrient concentrations representative of upwelling, heatwave, and extreme heatwave conditions (Table 1). Values were chosen according to nutrient concentrations measured during the blob heatwave (Du et al., 2016; Gentemann et al., 2017; Gómez-Ocampo et al., 2018; Bif et al., 2019; Jiménez-Quiroz et al., 2019) and upwelling events (Feely et al., 2008; Schnetzer et al. 2013; Siedlecki et al., 2016; Messié et al. 2017; Larkin et al., 2020) off the West Coast of the United States. An additional treatment with low temperature, pCO_2, and nutrient concentrations (LTCN), although less environmentally relevant, was included to tease apart the potential interactive effects due to temperature. In order to change the carbonate chemistry of these cluster experiments, commercial pre-mixed gas was gently bubbled directly into cultures. pH measurements were used during the acclimation phase to characterize the carbonate system (*see Carbonate buffer system measurements* and Table 3).

For the thermal gradient response curve, a temperature gradient was established using a thermal block connected to a VWR 1180-S recirculating chiller and a VWR 1130–2S recirculating heater (Qu et al. 2022). Cultures were grown in modified *f*/2 replete medium (100 μM total nitrate, 100 μM silicate, 6 μM phosphate; standard trace metals and vitamins; Guillard and Ryther, 1962; Guillard, 1975) in triplicate 100 mL polystyrene vials at the following temperatures: 8.4, 11.0, 12.5, 14.1, 15.2, 16.6, 17.7, 19.3, 20.2, 22.7°C. For simplification, each temperature was rounded to the nearest whole number when referenced in the text: 8, 11, 12, 14, 15, 17, 18, 19, 20, 23 °C.

In the pCO_2 single-factor experiments, all cultures were grown at 19°C with modified *f*/2 replete medium (described above). Triplicate bottles for each treatment were gently bubbled with commercially premixed gas at concentrations of 200 ppm (pre-industrial), 600 ppm (ocean acidification), and 1040 ppm (extreme ocean acidification). The extreme ocean acidification treatment only had two replicates due to an accidental culture loss the day of sampling. pH was measured daily to characterize the carbonate system (*see Carbonate buffer system measurements* and Table 3). The extreme ocean acidification treatment only had two replicates for physiological measurements as the third replicate was lost during final sampling. A replicate was also removed from the pre-industrial pCO_2 DA quota and production measurements due to a sample processing error.

Two-factor nutrient-temperature experiments were conducted to examine interactive effects between temperature and nutrients, as well as the impacts of N:P ratios on growth and domoic acid biosynthesis. Cultures were grown across a matrix of three nitrogen to phosphorus ratios (N:*P* = 5, 10, 50), two temperatures (13 vs. 19°C), and low vs. high total nutrient concentrations (Table 2). Cultures were diluted semi-continuously with experimental base medium (described above). Post-dilution, cultures were directly spiked with nitrate, phosphate, and silicate for final concentrations specific to each treatment (Table 2).

Table 3
Calculated carbonate buffer system based on pH and DIC measurements. Values in parentheses represent the standard deviation of the mean[2].

		Measured pH	Measured DIC (μmol/kg)	Calculated bulk alkalinity (μmol/kg)	Calculated pCO_2 (μatm)
Cluster experiments	**Upwelling**	7.7 (0.01)	2098.0 (17.8)	32.9 (0.7)	889.1 (29.0)
	LTCN	8.2 (0.01)	1868 (16.9)	90.1 (0.8)	229.3 (4.6)
	Heatwave	8.24 (0.02)	1785 (4.6)	93.5 (2.5)	254.3 (9.4)
	Extreme heatwave	8.24 (0.04)	1787.1 (24.8)	95.2 (3.9)	240.2 (23.2)
Single-factor experiments	**Pre-industrial**	8.29 (0.08)	1884 (23.2)	117.7 (14.6)	201.8 (36.5)
	Ocean acidification	7.89 (0.01)	2058 (13.1)	53.5 (1.1)	592.2 (14.3)
	Extreme ocean acidification	7.68 (0.01)	2166 (26.2)	34.4 (0.5)	1038.9 (4.7)

[2] LTCN = low temperature, carbon dioxide, and nutrients.

2.3. Sample collection and analysis

2.3.1. Growth rates

Cultures were sampled once fully acclimated to the experimental conditions (as determined by steady-state growth ±10% for at least 8 generations). Fresh medium in 1 L polycarbonate bottles was inoculated with approximately 650 cells/mL and incubated under respective experimental conditions for 48 h.

For the single factor temperature and pCO_2 experiments, specific growth rates were calculated using in vivo chlorophyll a fluorescence measurements. Fluorescence at $T_{initial}$ and T_{final} was measured on a Turner Designs 10-AU fluorometer. For all other experiments, growth rates were calculated using extracted chlorophyll a measurements collected at $T_{initial}$ and T_{final} (see *Pigment analysis*). Specific growth rates were calculated using the following equation:

$$\mu = \frac{\ln\left(\frac{N_{Tfinal}}{N_{Tinitial}}\right)}{T_{final} - T_{initial}}$$

where μ is the specific growth rate (per day) and N is the in vivo fluorescence or chlorophyll a concentration (for temperature-only; or cluster, nutrient, and pCO_2 experiments, respectively) at $T_{initial}$ and T_{final} (Kling et al., 2021)

2.3.2. Elemental analysis

For particulate organic carbon (POC) and nitrogen measurements, cells were filtered onto pre-combusted glass microfiber (GF/F) filters (500°C for 3 h) and placed in the drying oven at 60 °C for at least 48 h. Filters were pelleted in foil capsules for analysis on a Costech 4010 Elemental Analyzer (Fu et al., 2007).

2.3.3. Domoic acid analysis

Samples for particulate domoic acid were filtered onto Supor 0.2 μm 47 mm PES filters and stored in 15 mL centrifuge tubes at −20°C for 1–4 months (Smith et al. 2017, Harðardóttir et al., 2018; Jennings et al., 2020). The filtrate was also collected and frozen in 100 mL amber HDPE bottles for dissolved domoic acid analysis.

For particulate domoic acid analyses, filters were extracted for four hours in 90% methanol and 10% water, vigorously vortexing each hour following the methods described in Wang et al. (2012) where DA recoveries exceeded 90% in cultured phytoplankton samples. Extracts were passed through a 0.2 μm syringe filter directly into a 1.5 mL LC-MS vial for LC-MS/MS analysis on a Prominence UFLC system (Shimadzu, Kyoto, Japan) coupled to a SCIEX 4500 QTRAP mass spectrometer (AB Sciex, Framingham, MA, USA). The mass spectrometry methods followed those of Sterling et al. (2022) with some minor modifications (the eluent was sent to waste during the first 5 min of the run rather than 2 min and the CRM for DA was obtained from the National Research Council of Canada rather than Sigma-Aldrich). The peak of DA eluted at 11.00 min. LC-MS/MS with multiple reaction monitoring (MRM) was employed for detection and quantification. Analysis was carried out in positive mode, and three transitions from the protonated DA molecule were used: m/z 312 → 266 (quantitation transition), m/z 312 → 248, and m/z 312 → 193. Plankton-associated domoic acid was quantified to ng particulate domoic acid L^{-1} of filtered seawater using an external calibration curve created from pure domoic acid standards of increasing concentrations (CRM, National Research Council of Canada), included in each analysis.

One DA sample was removed from analysis in the pre-industrial treatment in the CO_2 single factor experiment, and from the NP=50, high nutrient, 19°C treatment in the N:P ratio experiment, due to a sampling and/or analytical error.

For environmental relevance and to make inferences about trophic transfer of toxins, domoic acid was normalized to cells per L. Domoic acid was normalized also to moles of POC per liter; because *Pseudo-nitzschia* spp. cell size and volume can change with nutrient limitation, normalizing only to cell counts may result in skewed results (Tatters et al., 2012). In the text, discussion of "DA quotas" refers to this POC normalized value, unless otherwise stated.

Dissolved DA was measured from acidified filtered seawater (50 mL, 0.2 μm) passed over a C18 solid phase extraction column and eluted with methanol based on the methods of Wang et al., 2012 with some modification. The eluent was placed directly into a 1.5 mL LC-MS vial and analyzed using the LC-MS method detailed above.

Domoic acid production rates were calculated by multiplying specific growth rates by DA quotas. This value provides an estimate of how toxic a bloom might be, based on the ability of *Pseudo-nitzschia* to increase cell abundances and produce high DA quotas (per mol POC). For instance, if *Pseudo-nitzschia* is present in high abundances but not toxic, the bloom might not have negative ecosystem implications (Kelly et al., 2021).

2.3.4. Chlorophyll a analysis

Samples for chlorophyll a were filtered on GF/F filters and stored in scintillation vials at −20°C for ~24–48 h. Samples were extracted in 6 mL of 90% acetone at −20 °C for 24 h, then analyzed using a Turner 10AU field fluorometer (Welschmeyer 1994; Fu et al., 2007).

2.3.5. Net carbon fixation and elemental use efficiencies

Primary production was determined by measuring the uptake of radiolabeled bicarbonate (Fu et al., 2008). ^{14}C-bicarbonate was added to 45 mL sub-cultures at $T_{24\ h}$ and incubated for 24 h (approximating net carbon fixation) under the respective experimental conditions. After the incubation period, cells were collected on GF/F filters and placed in a scintillation vial containing scintillation cocktail. Samples were stored for 24 h before being read on a Wallac System 1400 liquid scintillation counter. Carbon fixation rates were calculated by converting raw counts of disintegrations per minute to μmol of carbon (based on total activity of the radiolabeled bicarbonate), then normalizing to the incubation time. Dissolved inorganic carbon (DIC) values used in the calculation were adjusted for each treatment based on measured values of DIC (see below). These rate measurements were then normalized to POC. In the cluster experiment, one sample from the extreme heatwave treatment was removed from primary production analyses due to an error made during the assay.

Nitrogen use efficiencies (NUEs) were calculated by normalizing net carbon fixation rates to particulate organic nitrate (mols C fixed hour^{-1} mol N^{-1}; Yang et al., 2022).

2.3.6. Carbonate buffer system measurements

pH measurements were made on a Mettler Toledo SevenCompact pH meter using a three-point calibration curve and total pH scale (Cooley and Yager 2006). Samples for total DIC analysis were collected at T_{final}. Seawater from undisturbed culture bottles was removed with a sterile syringe, ejected into pre-evacuated borosilicate Exetainers, and poisoned with 5% $MgCl_2$. Samples were stored at 4°C until analysis. Total DIC was measured using a Picarro cavity ring-down spectrophotometer according to Subhas et al. (2015). Experimental seawater pCO_2 and total alkalinity were calculated from measured DIC and pH using CO2SYS version 2.1 software (Table 3; Lewis and Wallace, 1998).

2.3.7. Cell counts

For cell count samples, 1 mL of the final experimental culture was preserved with 40 ul glutaraldehyde and stored at 4°C in the dark. Cells were counted on a Olympus BX51 microscope using a Sedgewick Rafter Chamber.

2.3.8. Statistical methods

Multivariate analyses were conducted in R version 1.3.1093 (http://www.r-project.org) using statistical tools in Rallfun-v38 (https://dornsife.usc.edu/labs/rwilcox/software/). For global analyses, percentile bootstrap methods for comparing 20% trimmed means were used to detect significant differences between treatments. t1way was used in the single-factor and cluster experiments, while t3way was used in the nutrient-temperature experiments. To test for significant differences between any two treatments, pairwise analyses using percentile bootstrapping of 20% trimmed means (trimpb2) were conducted. These statistical tools are analogous to ANOVAs and post-hoc and Welch's *t*-test pairwise analyses but are more robust as they do not assume normality or homoscedasticity (Wilcox, 2003).

3. Results

3.1. Multiple driver cluster experiments

Specific growth rates were greatest in the heatwave treatment, but only 17% lower in the upwelling treatment (p-value < 0.01), averaging 0.44 and 0.37 day^{-1} (respectively; Fig. 1A). In contrast, *P. australis* growth rates in the extreme heatwave treatment were 61% lower than in the heatwave treatment, although these experimental conditions only differed by 1.5°C in temperature. Furthermore, when *P. australis* was grown with lower heatwave concentrations of nutrients and pCO_2, but also at a reduced temperature (i.e., low temperature, CO_2, and nutrients; LTCN), growth rates were not significantly different from those measured in the extreme heatwave treatments.

Dissolved DA (dDA) concentrations were a negligible fraction of the total DA in all treatments (data not shown), likely due to the constant exponential phase growth and regular culture medium dilutions used in our semi-continuous incubations. POC normalized pDA quotas were highest in the upwelling treatment, averaging 0.9 ng DA μmol C^{-1} (Fig. 1B). In contrast, toxins were not detected in the heatwave treatment, and pDA concentrations were 93% lower than upwelling values under extreme heatwave conditions. Furthermore, pDA concentrations for the LTCN treatment were 41% lower than upwelling concentrations. Trends in DA quotas were the same when normalized to cell abundance and to volume (mL), with the greatest quotas measured in the upwelling treatment Table 4). DA production rates demonstrated a similar trend, with the highest production rates measured under upwelling conditions (0.33 ng DA μmolC^{-1} day^{-1}) and zero or near zero for the heatwave and extreme heatwave treatments (Fig. 1C). DA production rates in the LTCN treatment were 92% higher than in the extreme heatwave treatment ($p < 0.01$).

Net C-specific primary productivity trends were similar to those observed for growth rates (Fig. 2A). The highest rates were measured in upwelling and heatwave treatments (0.012 and 0.013 day^{-1},

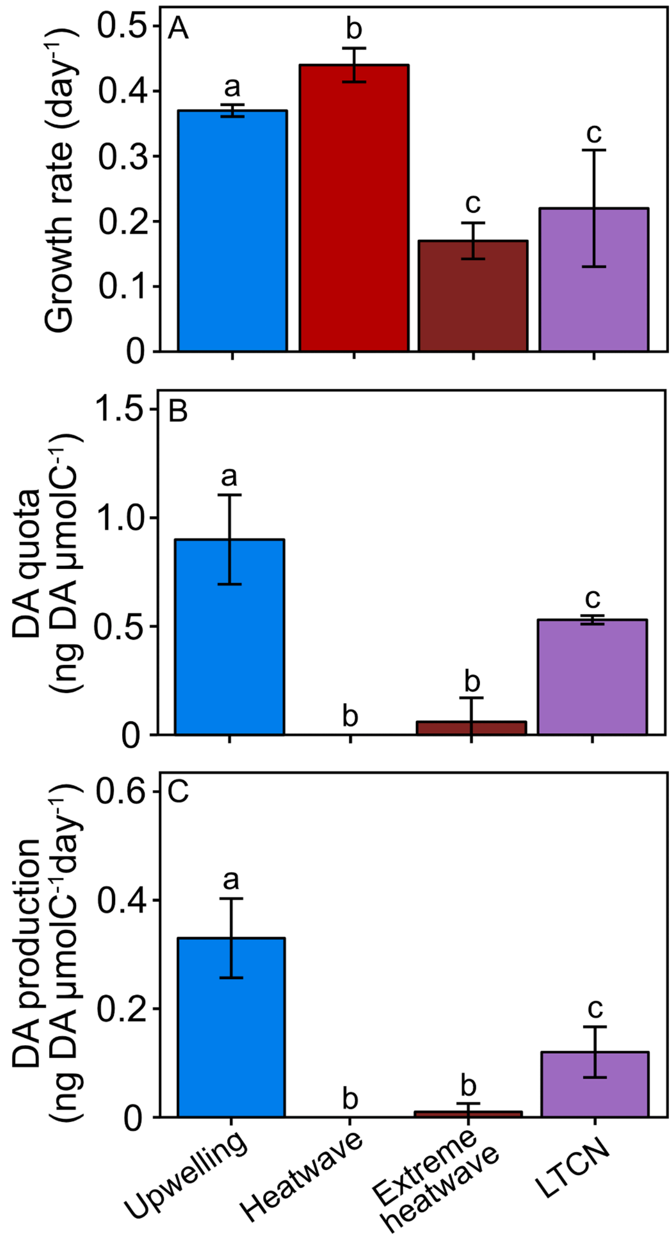

Fig. 1. Average growth rates (a), particulate domoic acid quotas (b), and domoic acid production rates for scenario experiments with combined CO_2, temperature, and nutrients representative of upwelling, heatwaves, extreme heatwave, and low temperature, CO_2, and nutrients (LTCN) conditions. Different letters indicate that differences between treatments are statistically significant, while the same letters indicate that differences between treatments are not statistically significant. Error bars represent standard deviation of the mean.

Table 4
Average domoic acid (DA) quotas for the cluster experiment, normalized to cell abundance.

Treatment	Average ng DA/cell (standard deviation)	Average ng DA/mL (standard deviation)
Upwelling	7×10^{-5} (4.3×10^{-5})	0.07 (0.01)
Heatwave	0 (0)	0 (0)
Extreme heatwave	6.9×10^{-6} (1.2×10^{-5})	0.01 (0.01)
LTCN	2.61×10^{-5} (8.65×10^{-6})	0.04 (0.04)

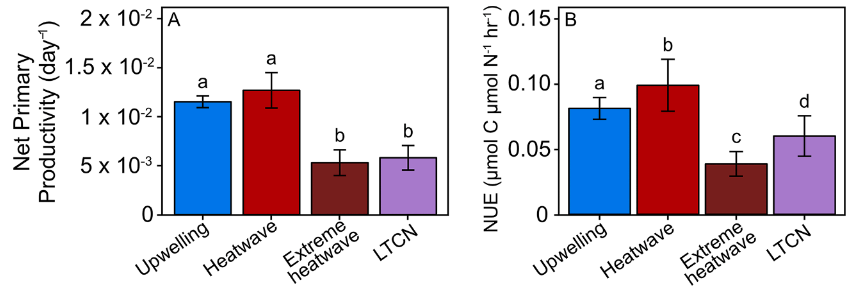

Fig. 2. Net primary productivity (A), nitrogen use efficiency (NUE; B) of *P. australis* under upwelling, LTCN, heatwave, and extreme heatwave conditions. Different letters indicate that differences between treatments are statistically significant, while the same letters indicate that differences between treatments are not statistically significant. Error bars represent standard deviation of the mean.

respectively), which were not significantly different from one another ($p = 0.221$). Rates for the LTCN and extreme heatwave conditions were 54% lower that the heatwave treatment ($p < 0.01$).

Nitrogen use efficiencies (NUEs) measure how efficiently the cells use their cellular nitrogen quotas to fix carbon under different experimental conditions. NUEs were highest under heatwave conditions and decreased by 17% in the upwelling treatment ($p = 0.028$ Fig. 2B). For the LTCN treatment, NUEs were slightly lower than those for the upwelling treatment ($p < 0.01$). Finally, NUEs for the extreme heatwave treatment declined 61% relative to the heatwave treatments ($p < 0.01$).

3.2. Single factor experiments

When temperature alone was considered, the fastest specific growth rates were measured between 14 and 19°C, reaching a maximum of 0.35 day^{-1} at 18°C (t1way p-value <0.01; Fig. 3A). At temperatures above 19°C or below 13°C, growth rates declined rapidly. Furthermore, *P. australis* grew extremely slowly (0.02 day^{-1}) and was unable to persist stably at temperatures ≤9°C and could not survive when temperatures exceeded 23°C.

Particulate domoic acid cellular quotas (pDA, ng DA µmol C^{-1}) had an inverse relationship with temperature, whereby pDA decreased as temperature increased, except at 8°C where cold-stressed cultures did not produce any detectable toxin (Fig. 3B). The R^2 value was 0.88 for a linear regression that excluded the 8°C values, indicating a strong linear relationship between temperature and pDA. The highest concentrations of pDA were measured at 11°C, averaging 3.52 ng DA µmol C^{-1}. Between 13 and 18°C, pDA quotas were slightly lower than the maximum, though similar to each other. Relative to the maximum, pDA declined by 72% with warming to 19°C and 20°C, and no pDA was measured at 23°C. DA production rates (ng DA µmol C^{-1} d^{-1}) were highest and not significantly different from one another between 11 and 18°C, averaging 0.68 ng DA µmolC^{-1} d^{-1} and decreased by 62% to 19°C. At 8 and 23°C, DA production rates were at or near zero (Fig. 3C).

In the single factor CO_2 experiment, the highest growth rates were measured in the 600 µatm treatment (Fig. 4A). Growth rates were 23% and 11% lower in the 200 and 1040 µatm treatments, although this difference was only significant for the 200 µatm treatment (pairwise p-value < 0.01). On the other hand, cellular DA content was greatest at the extreme ends of the CO_2 concentration gradient (Fig. 4B). In the 600 µatm treatment, DA quotas were 66% and 56% lower relative to 200 µatm and 1040 µatm (respectively, p-values <0.01 and 0.021). Lastly, DA production rates followed a trend similar to DA quotas; the lowest rates were observed at 600 µatm, with a 54% and 50% increase compared to 200 and 1040 µatm (although differences were only statistically significant between 200 and 600 µatm; $p < 0.01$).

3.3. N:P ratio experiments

Within each combination of temperature and total nutrient concentration (i.e., low vs. high nutrients), growth rates were greatest for the

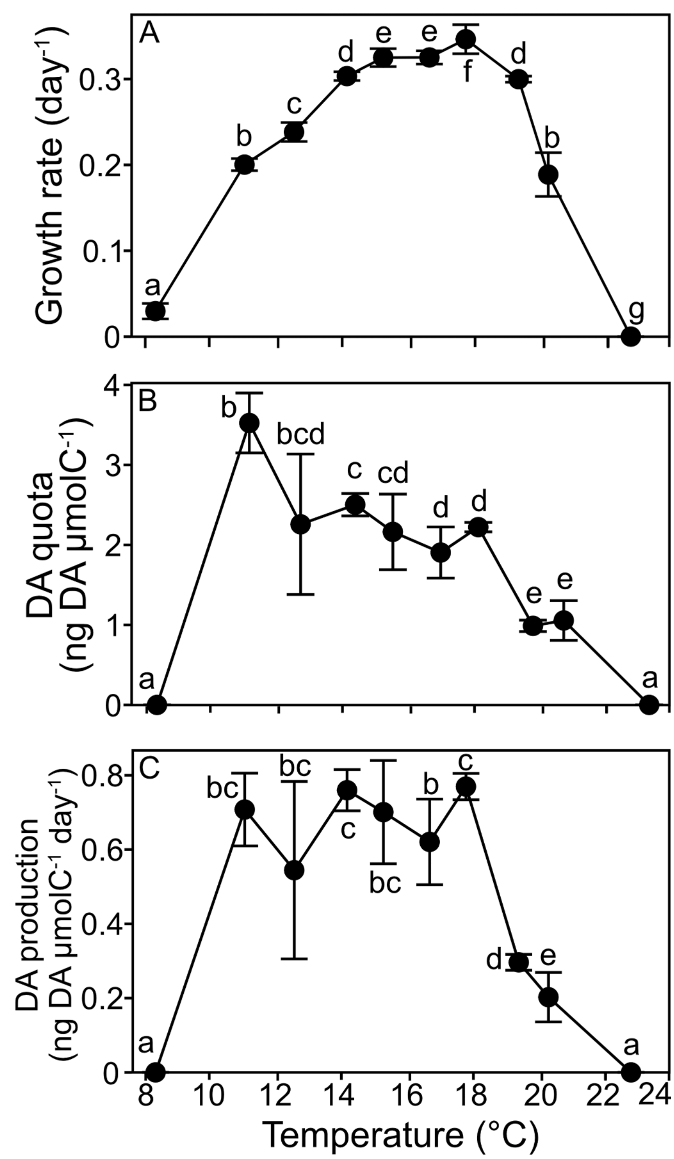

Fig. 3. N Average *P. australis* growth rates (A), particulate domoic acid (pDA) cellular quotas (ng DA µmol C^{-1}) (B), and domoic acid production rates (ng DA µmol C^{-1} d^{-1}) (C) across a range of temperatures. Different letters indicate different statistically significant differences between temperatures, and error bars represent standard deviations of the mean ($n = 3$).

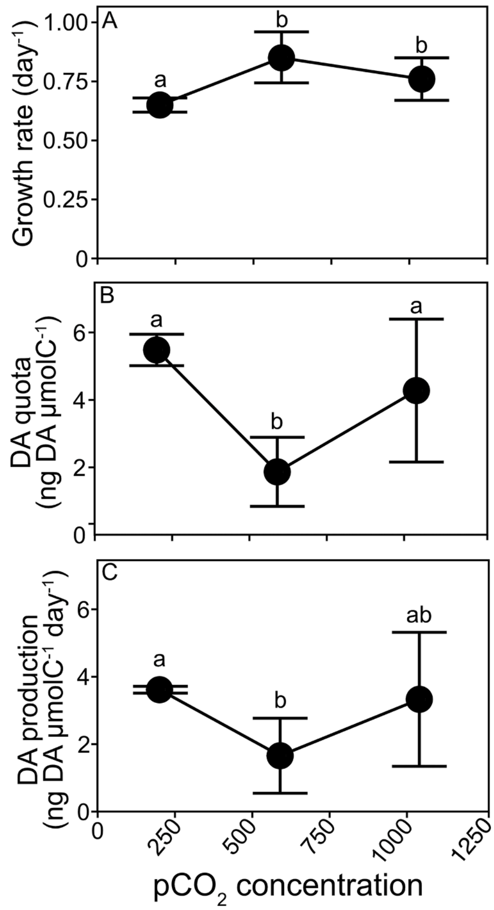

Fig. 4. Growth rates (A), particulate domoic acid concentrations (B), and domoic acid production rates (C) measured in the single factor pCO_2 experiments. Different letters indicate statistically significant differences between temperatures, and error bars represent standard deviations of the mean.

treatments with an N:P ratio of 50 (Fig. 5A). Within each temperature for the N:*P* = 5 and N:*P* = 10 treatments, differences in growth rates between high and low total nutrient treatments were very minor, yet statistically significant (t3way N:P ratio affects p-value = 0.0001).

There were no other significant 2- or 3-way interactive effects between variables for growth rates in this experiment. Furthermore, growth rates increased with both temperature and higher total nutrient concentrations (high nutrient treatments), except for the 19 °C high nutrient N:*P* = 50 treatment.

Particulate domoic acid was not detected in the N:*P* = 5 treatments. The most pDA was produced in the N:*P* = 50 treatments, which were 71 to 90% higher than the N:*P* = 10 treatments (t3way single variable N:P ratio effects p-value = 0.0001; Fig. 5B). Furthermore, within the N:*P* = 50 treatments lower concentrations of pDA were measured in the treatments with lower total nutrient concentrations (t3way interactive effects between N:P ratio and total nutrient concentration p-value = 0.014). Differences between N:*P* = 10 treatments were minor at 13°C, yet at 19°C the high total nutrient concentration treatment was 96% higher than the treatment with a low total nutrient concentration (t3way interactive effects between all variables p-value = 0.046).

4. Discussion

4.1. Single factor experiments and potential interactions reveal the influence of each environmental variable the cluster experiment

The single and dual factor experiments provided a mechanistic understanding of how the drivers examined contributed to the responses in the cluster experiment. We found that the trends observed in the single-factor treatments could not fully explain the results of the multiple-driver experiments. This provides evidence that non-linear, interactive effects may be influencing trends observed in the multivariate scenario experiments.

4.1.1. Growth rates

Other studies have shown that optimal growth temperatures are highly species and strain dependent (Lelong et al., 2012; Bates et al., 2018). The heatwave temperature was within the broad optimal temperature range for growth for this *P. australis* strain, while the upwelling temperature was suboptimal. This aligns with results from the cluster experiment, where growth rates for the heatwave treatment were slightly higher than those in the upwelling cluster, indicating that temperature did influence growth rates in the cluster experiment. However, temperature was only partially responsible for the decreased growth rates in the extreme heatwave treatment, as growth rates declined more than expected from temperature alone.

On the other hand, pCO_2 alone had only a slight impact on *P. australis* growth rates. However, inorganic carbon concentration did not influence growth rates in the cluster experiments as expected based on the single factor experiment; growth increased slightly with pCO_2 concentration alone, yet growth was higher in the heatwave treatment with less pCO_2. Similar to the trends observed in the present single-factor study with *P. australis*, increases in growth rates have been observed from 200 to 765 ppm for *P. multiseries* and from 220 to 730 ppm for *P. fraudulenta*. However, when *P. multiseries* and *P. fraudulenta* were both exposed to pCO_2 and limited for either phosphate or silicate, growth rates further

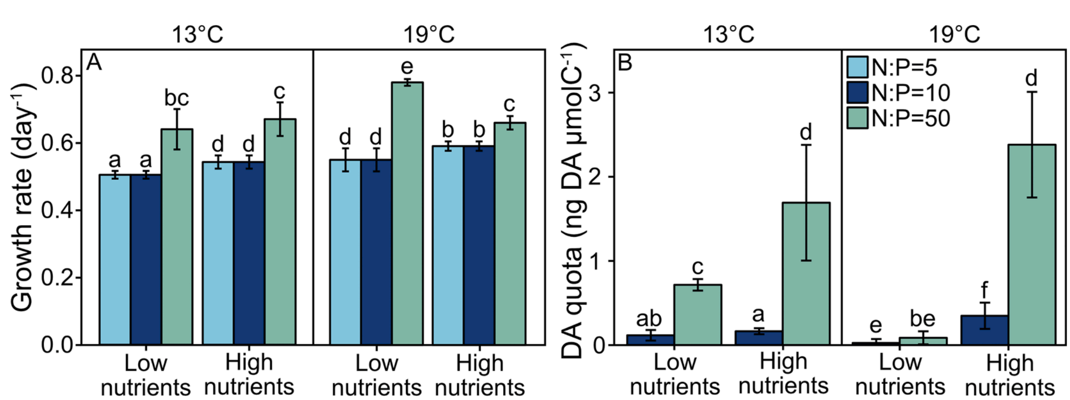

Fig. 5. Specific growth rates for all N:P ratio experiments (A) and particulate domoic acid quotas for the N:*P* = 10 and N:*P* = 50 experiments (B). Domoic acid was not detected in the N:*P* = 5 experiments. Different letters indicate different statistically significant differences between temperatures, and error bars represent standard deviations of the mean.

declined, suggesting that there were interactive effects between nutrient limitation and pCO_2 concentration (Sun et al., 2011; Tatters et al., 2012). Zhu et al. (2017) also demonstrated that there were interactive effects of pCO_2 and temperature on *P. subcurvata*; growth rates under suboptimal temperature and high pCO_2 conditions were lower than growth rates at optimal temperatures combined with low pCO_2.

Single-factor nutrient experiments were not conducted in the present study, as individual nutrient effects on *Pseudo-nitzschia* growth have been studied extensively. As with many other diatom species, nutrient limitation typically reduces *Pseudo-nitzschia* growth (Fehling et al., 2004; Hagström et al., 2011; Sun et al., 2011; Tatters et al., 2012; Auro and Cochlan 2013). In the nutrient-temperature matrix experiment, growth rates were higher for treatments with higher total nutrient concentrations. However, in the cluster experiments *P. australis* growth rates were slightly higher in the heatwave treatment, despite having lower nutrient concentrations relative to the upwelling treatment. Furthermore, regardless of equal nutrient concentrations in both heatwave treatments, growth rates in the extreme heatwave treatment were lower. This indicates that there was a temperature-nutrient interaction in the cluster experiments (see below).

4.1.2. Domoic acid quotas

Temperature alone had a significant impact on DA quotas in the present study. In contrast to our observation that DA quotas decreased with warming, most published *Pseudo-nitzschia* studies on temperature have observed increases in DA with warming (Lelong et al., 2012; Thorel et al., 2014; Zhu et al., 2017; Kelly et al., 2021); however, one found that *P. seriata* produced more DA at relatively colder temperatures, similar to observations in the present study (Lundholm et al., 1994). Furthermore, *P. australis* has been associated with relatively cold temperatures in Narraganset Bay, RI (Roche et al., 2022). In March 2017, a highly toxic *P. australis* bloom occurred at temperatures ranging from 1 to 5°C in Narraganset Bay, RI (Sterling et al., 2022). The diversity of thermal tolerances documented in the literature demonstrate that different species and strains of *Pseudo-nitzschia* have distinct thermal triggers for toxin production, though the data in the present study indicates that this strain of *P. australis* specializes in being more toxic at colder temperatures.

Because temperature alone (under nutrient replete conditions) had a strong inverse relationship with DA concentrations in the single factor experiments, it likely contributed to the differences in DA between cluster treatments where more DA was measured in the cold, upwelling treatment compared to the warm, heatwave treatments. However, in the heatwave and extreme heatwave treatments the decrease in DA quotas were greater than expected based on the temperature single factor experiments. Furthermore, it should be noted that in the upwelling treatment and temperature experiment, nitrogen availability was replete and ample to support DA biosynthesis, which may not have been the case in the heatwave and extreme heatwave cluster treatments (the interactive effects are discussed more below).

Similar to our observation that more DA was measured at the extreme ends of the pCO_2 concentration gradient, another strain of *P. australis* was highly toxic at low and extremely high pCO_2 concentrations (371 and 1849 μatm), and only moderately toxic at an intermediate pCO_2 concentration (785 μatm; Ayache et al., 2021). In contrast, other strains of *P. australis* have demonstrated decreases in DA along the same pCO_2 gradient described above, or no change in DA across pCO_2 gradients during the exponential phase of growth (Sun et al., 2011; Tatters et al., 2012; Ayache et al., 2021; Wingert and Cochlan 2021). While there are species and strain differences with regard to pCO_2 concentration, toxicity of the *P. australis* strain used in the present study is more sensitive to changes in CO_2 alone.

Inorganic carbon may have influenced the upwelling cluster DA quotas, as the extreme ocean acidification pCO_2 concentration in the single factor experiment was similar to the concentration used in the upwelling experiment. However, it is likely that pCO_2 alone did not trigger DA biosynthesis in the heatwave and extreme heatwave cluster treatments. Although the pre-industrial pCO_2 level, similar to levels used in the heatwave and extreme heatwave treatments, did trigger DA biosynthesis, very little to no DA was observed in these clusters.

Previous investigations have robustly demonstrated that phosphate and/or silicate limitation trigger DA biosynthesis in toxic strains of *Pseudo-nitzschia* sp. (Pan et al., 1996; Fehling et al., 2004; Sun et al., 2011; Tatters et al., 2012). This suggests that silicate and phosphate limited conditions should have triggered DA biosynthesis in the heatwave and extreme heatwave treatments, yet little to no DA was measured. However, these treatments were also nitrate limited, and sufficient nitrogen is required for synthesis of DA, a nitrogen-containing molecule (Lelong et al., 2012). Therefore, the total amount of nitrogen in these treatments may have strongly negatively impacted DA quotas (see more below).

4.2. Mechanistic studies and potential interactive effects inform dynamics in the cluster experiment

The three drivers interacted in the cluster experiment to alter growth rates more than expected from individual variables or additive effects. We hypothesize that there was a tradeoff between temperature, nutrients, and carbon dioxide: at cooler temperatures, high nutrients and inorganic carbon offset the growth limitation of low temperature. On the other hand, warming supported higher growth rates, even if limited by nutrients and inorganic carbon. This indicates that there was a synergistic interactive effect between these three variables.

In terms of bloom toxicity, temperature and inorganic carbon may have partially contributed to the increased DA measured in the upwelling cluster. However, it is likely that the main driver of differences in toxicity between treatments was nitrogen. Both the heatwave treatments were supplied with less nitrogen, while the upwelling treatment had ample nitrate, which is necessary for the biosynthesis of DA molecules (Lelong et al., 2012). Therefore, it is possible that the lack of nitrogen in the heatwave and extreme heatwave treatments had an antagonistic effect, inhibiting DA biosynthesis regardless of the CO_2 conditions that may trigger toxin production. Therefore, we surmise that nitrate concentration was a key factor influencing DA production in the cluster experiments.

DA is a specialized metabolite and biosynthesis is an energetically expensive process. Therefore, DA is typically produced when there is sufficient energy for both processes, or when silicate or phosphate availability limit or reduce cell division (Pan et al., 1998). However, this tradeoff does not always seem to be the case in the present study. In the temperature experiment, slower growth rates and high DA quotas at 11°C were associated with high DA production rates, while higher growth rates at slightly warmer temperatures were associated with lower DA quotas yet near equal DA production rates. For the pCO_2 experiments, differences in growth rates were marginal and DA production rates closely followed DA quota trends. In this case, growth rates were decoupled from bloom toxicity. Together, these experiments demonstrate that high biomass does not necessarily indicate high toxicity, and low biomass does not indicate a low threat of DA poisoning. Similarly, observations of high *Pseudo-nitzschia* sp. biomass yet low DA concentrations have been detected in the natural environment (Roche et al., 2022).

DA concentrations in the cluster treatments can also be examined through the lens of cellular energetics and the tradeoff between growth and toxicity. In addition to the need for sufficient cellular energy, nitrogen is also required for both growth and DA synthesis. This tradeoff between growth and toxicity is not evident in the cluster experiments: upwelling and heatwave treatments grew equally well yet differed in terms of toxicity. Instead, this may be explained by the total amount of nitrogen available in each treatment.

Considering growth rate results, DA measurements, and previous studies with nitrogen, we hypothesize that the upwelling treatment had more than enough nitrogen for growth, and the excess was shunted to DA biosynthesis. *P. australis* cells in the heatwave and extreme heatwave treatments had less total nitrogen available. In the heatwave treatment, nitrogen did not limit growth, carbon fixation, or NUEs, but there might not have been enough for concurrent DA synthesis. This indicates that the absolute amount of nitrogen plays a role in the tradeoff between growth and toxicity. Additionally, in the extreme heatwave treatment, both growth rates and DA concentrations were reduced. Previous studies have observed declines in growth rates due to warming, concurrent with increases in toxicity, indicating that the energy and nitrogen not used for growth was instead shunted to DA biosynthesis (Zhu et al., 2017). However, for this strain of *P. australis* the low DA quotas in the extreme heatwave treatment may be due to a lack of sufficient nitrogen and a warming-induced reduction in DA biosynthesis.

The LTCN cluster treatment, though not environmentally realistic, further aids in teasing apart the nutrient-temperature interactive effects on DA quotas. These LTCN cells were more toxic than the heatwave and extreme heatwave treatments, though not as toxic as the upwelling treatment. Low temperature and pCO_2 are triggers of DA for this strain of *P. australis*, which could partially explain the increased toxicity relative to the heatwave treatments. However, growth rates for the LTCN treatment were lower. Therefore, if all of the available nitrogen was not used for growth in this treatment, there may have been enough left over for DA synthesis. Furthermore, both the upwelling and LTCN treatments efficiently used the available nitrate to support carbon fixation, as NUEs were similar. This indicates that temperature does indeed affect the ability of *P. australis* to use nutrients and synthesize DA.

Results from the N:P ratio experiments also demonstrate this temperature-nutrient interaction. While growth rates changed minimally across the $N{:}P = 50$ treatments, there were significant differences in DA quotas within each temperature. This indicates a decoupling between growth rates and DA biosynthesis. Furthermore, when total nutrient concentrations were low, warming constrained DA quotas, yet under high total nutrient concentrations differences in DA quotas were not considerable. This suggests that temperature is more influential on DA quotas when total nutrient concentrations are high. Consequently, under these high total nutrient conditions with excess nitrogen (relative to phosphorus), cells can both double their population size approximately once per day and produce substantial DA, generating a large toxic bloom.

4.3. Cluster experiments: a holistic view and ecosystem implications

Examining the data empirically using cluster experiments can provide a holistic view of how *P. australis* bloom formation and toxicity may be impacted by complex events like upwelling and marine heatwaves. In the present study's simulated upwelling and heatwave conditions, similarly high growth rates for both treatments indicate that both conditions could be triggers for bloom formation. These results are consistent with what has been observed in the natural environment: blooms occurring during both upwelling and marine heatwave events (Schnetzer et al. 2013; McCabe et al., 2016; McKibben et al., 2017; Smith et al., 2018). With more frequent heatwave and upwelling events expected as climate change progresses, rapid bloom-forming growth may be triggered more frequently (Bakun et al., 2015). However, if extreme heatwaves exceed the broad thermal optimum for *P. australis* growth, climate change may impede bloom formation. Therefore, the severity of future heatwaves will determine whether *P. australis* will be able to rapidly grow.

Despite similar growth rates between upwelling and heatwave treatments, only the upwelling conditions led to substantial DA biosynthesis. This suggests that blooms triggered by upwelling may be more toxic than blooms occurring during a heatwave or extreme heatwave event. Furthermore, DA production rates were only substantial in the upwelling treatment. This parameter combines growth rates and toxin quotas to estimate the impact of the bloom (Kelly et al., 2021). A high DA production rate suggests that upwelling events may promote both rapid increases in population size and high concentrations of toxins per cell. Therefore, *P. australis* may be able to form a large, toxic bloom rapidly under these conditions, supporting predictions that blooms will worsen with climate change (Fu et al., 2012; Smith et al., 2018; Gobler 2020; Trainer et al., 2020). Consequentially, blooms triggered by upwelling may be especially harmful to the marine ecosystem, with severe implications for the coastal California system. More frequent, highly toxic blooms could have devastating consequences for ecosystem health and fisheries (McCabe et al., 2016). However, in the present experiment pDA quotas normalized to volume were very low compared to maximum concentrations of pDA observed during the Blob bloom (McCabe et al., 2016). These values are also well below the regulatory limit for DA (20 ppm). Additionally, DA quotas normalized to cell abundance were a few orders of magnitude lower than a highly toxic *P. australis* bloom triggered by upwelling in 2006 (Schnetzer et al., 2013). Therefore, the risk of trophic transfer of domoic acid may not be high for this strain of *P. australis* under upwelling conditions.

In contrast, although cells in the heatwave treatment grew faster, DA was not detected and thus DA production rates were inconsequential. *P. australis* in the extreme heatwave treatment had neither high growth rates nor high DA quotas. This indicates that despite the ability of *P. australis* to multiply rapidly and form a bloom, toxin production may not be triggered by heatwave conditions. The rapid growth of *Pseudo-nitzschia* spp. under heatwave conditions was also observed during the Blob bloom, with cells persisting in the warm waters, reducing nutrient concentrations in the surface layer of these stratified waters (McCabe et al., 2016). Similar to our experiment, the large population of cells in the northern region of the California Current System did not produce toxins without the availability of nitrogen. However, upwelling occurred in the spring of 2015, fueling the eastern edge of the Blob with nutrients, including nitrogen, which likely allowed the *Pseudo-nitzschia* spp. present to produce DA (McCabe et al., 2016). These seed populations of highly abundant and persistent *Pseudo-nitzschia* sp. populations, triggered by warming, pose a looming threat ecosystem health, as their toxicity may be triggered by episodic nitrogen inputs. Though our study did not mimic both the heatwave and upwelling aspects of the Blob bloom, future studies should consider heatwave conditions along with a pulse of nutrients.

In contrast, Ryan et al. (2017) found that in Monterey Bay, the high *Pseudo-nitzschia* cell abundance and toxicity was not due the warming anomaly (as temperatures were near normal), but instead correlated with the cold water, upwelling phases. Similarly, Barron et al. (2013) found no correlation between *P. australis* presence in the sediment record and warm anomalies in the Santa Barbara Channel. These studies in the Southern Portion of the California Current System contrast studies that occurred in the Northern portion of the California Current System (McCabe et al., 2016; McKibben et al., 2017). Furthermore, Sandoval-Belmar et al. (2023) found that toxic *Pseudo-nitzschia* blooms in Northern California have been associated with the warm phases of ENSO and PDO, while blooms in Southern California were associated with the cool phases. The strain of *P. australis* used in the present study was isolated from the Northern California Current System (Washington State) and thrived under heatwave conditions. This indicates that regional differences exist regarding the ability of heatwaves to trigger bloom formation.

On the other hand, although heatwaves may trigger a lot of growth, those cells are not expected to be notably toxic without nitrogen input from simultaneous upwelling or an anthropogenic source. While high nutrient concentrations occur in the natural environment during upwelling, dissolved N:P ratios of this water are typically slightly below the Redfield Ratio ($N{:}P = \sim 12$; Feely et al., 2008; Schnetzer et al. 2013; Siedlecki et al., 2016; Larkin et al., 2020). However, increased flow of anthropogenic nitrogen from land to sea (fertilizers and wastewater treatment facility discharge) may increase the N:P ratio of coastal surface waters (Howard et al., 2014). Increased growth and DA quotas in the N:$P = 50$ treatments of the temperature-nutrient matrix experiment suggest that upwelling combined with anthropogenic nutrient inputs might trigger especially toxic *P. australis* blooms, more than upwelling alone. If anthropogenic nutrient inputs occurred during a heatwave, a highly toxic bloom could be triggered due to the excess nitrogen, relative to phosphorus. Furthermore, considerable species and strain specificity exists with regard to the ability of *Pseudo-nitzschia* to utilize different nitrogen sources for growth and toxin production (Howard et al. 2007; Kudela et al. 2008; Thessen et al. 2009). Therefore, it is possible that DA biosynthesis for this strain of *P. australis* is not optimized for nitrate, and other sources of nitrogen (e.g., urea and ammonia) should be tested with this strain in the future to further explore the implications of anthropogenic nutrient inputs and their potential interactions with bloom formation and toxicity.

5. Conclusions

This study is among the few to examine unialgal *Pseudo-nitzschia* cultures in a multiple driver context, and the first to examine both upwelling and heatwave scenarios in holistic laboratory cluster experiments. These experiments are important for improving our understanding of *P. australis* specific responses to these conditions in order to make better predictions about future bloom dynamics in the California Current System. Furthermore, the single factor experiments here provided a unique mechanistic understanding of specific triggers of toxicity and bloom formation for this strain of *P. australis*. This has the potential to improve our ability to predict the occurrence of toxic blooms in the natural environment.

This experiment only examined one strain of *Pseudo-nitzschia australis*, and these responses may be strain specific. Similar experiments should be conducted with different species and strains isolated from different areas in the California Current System. Furthermore, while we chose to focus on only temperature, nutrients, and pCO_2, the clusters are not truly complete without other factors that have been shown to impact domoic acid synthesis, including biological factors like grazing, competition with other phytoplankton, and the presence of associated bacteria and fungi. Other physical and chemical factors like light intensity and quality, iron concentration, and alternative nitrogen sources are also clearly important (Howard et al. 2007; Thessen et al. 2009; Lelong et al., 2012; Bates et al., 2018; Radan and Cochlan 2018). Considering these factors as well could make these experiments more realistic. Nevertheless, these cluster experiments provide a valuable start towards obtaining a more holistic picture of *P. australis* dynamics during these contrasting events in the future rapidly changing coastal ocean.

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CRediT authorship contribution statement

F-XF, KJK, and DAH conceptualiazed and designed the experimental methodology with help from BDJ and MJB. F-XF, KJK, AM, and CL carried out the experimental investigation. Subsequent sample processing was done by AM, KJK, and F-XF. MJB, AMK, and LAM performed domoic acid extraction and quantification. Formal data analyses, curation, and visualization were performed by KJK, with help from F-XF and DAH. KJK wrote the manuscript with contributions from DAH, F-XF, BDJ, and MJB. All authors reviewed, edited, and gave their approval for the final manuscript. Funding acquisition, project administration, and supervision were done by F-XF, DAH, BDJ, and MJB.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The data presented in this study can be found in the following online repository: https://www.bco-dmo.org/project/855,438.

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