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Interactions Between Ultraviolet B Radiation, Warming, and Changing Nitrogen Source May Reduce the Accumulation of Toxic *Pseudo-nitzschia multiseri*s Biomass in Future Coastal Oceans

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Understanding the environmental conditions that trigger *Pseudo-nitzschia* bloom formation and domoic acid (DA) production is critical as the frequency and severity of these toxic blooms increases in the face of anthropogenic change. However, predicting the formation of these harmful blooms in a future ocean remains a challenge. Previous studies have examined the effects of single environmental drivers on *Pseudo-nitzschia* spp. growth and toxin production, but few have considered the interactions between them. In this multiple driver study with *Pseudo-nitzschia multiseri*s, we used a full factorial matrix experimental design to examine the simultaneous effects of temperature (20 and 25°C), nitrogen source (nitrate and urea), and irradiance (photosynthetically active radiation with and without ultraviolet B radiation; UVB). This strain of *P. multiseri*s was unable to withstand prolonged exposures (>0.5 h) to 0.06 mw·cm⁻² UVB light, with implications for near-surface bloom formation if future shallower mixed layers increase UVB exposure. Growth rates were inhibited by UVB, but photosynthesis and carbon fixation continued at a reduced capacity. Additionally, DA synthesis continued despite UVB-induced growth inhibition. Warming by 5°C enhanced cellular DA quotas three-fold. Within these warmer treatments, urea-grown cultures exposed to UVB had the highest amount of DA per cell, suggesting that interactive effects between UVB exposure, warming, and urea can synergistically enhance toxin production. However, overall production of toxic biomass was low, as growth-integrated DA production rates were near zero. This indicates that although *Pseudo-nitzschia multiseri*s cell-specific toxicity could worsen in an anthropogenically-altered future ocean, bloom formation may

be inhibited by increased exposure to UVB. This multi-variable experimental approach revealed previously unknown interactions that could not have been predicted based on combined effects of single-variable experiments. Although *P. multiseriata* DA production may be enhanced in a future ocean, inherent sensitivity to prolonged UVB exposure may moderate trophic transfer of toxin to coastal food webs.

Keywords: multiple drivers, *Pseudo-nitzschia multiseriata*, domoic acid, climate change, harmful algal blooms, ultraviolet radiation

INTRODUCTION

Harmful algal blooms (HABs) threaten human health and are responsible for millions of dollars lost to fisheries and tourism every year (Anderson et al., 2000). In coastal areas around the globe, annual blooms of the diatom *Pseudo-nitzschia* spp. pose a serious threat to human and ecosystem health. Members of this genus produce domoic acid (DA), an amino acid-like molecule that is a potent neurotoxin that affects humans, marine mammals, and sea birds (Figure 1; Lelong et al., 2012; Trainer et al., 2012; McCabe et al., 2016). DA bioaccumulates in the food web, most notably in commercially harvested species such as shellfish, crustaceans, and finfish (Hallegraeff, 1993), which can lead to strandings and mass mortality of cetaceans, otters, and pinnipeds (McCabe et al., 2016). Human consumption of DA-contaminated seafood causes amnesic shellfish poisoning, a neurological disorder that in severe cases can cause death (Perl et al., 1990). Thus, commercial fisheries must close during toxic bloom events, leading to massive economic losses (Ritzman et al., 2018). The increasing frequency and severity of *Pseudo-nitzschia* spp. blooms may be linked to climate and anthropogenic change (Fu et al., 2012; Smith et al., 2018; Gobler, 2020; Trainer et al., 2020), but the direct causes of bloom formation and toxin production remain poorly understood.

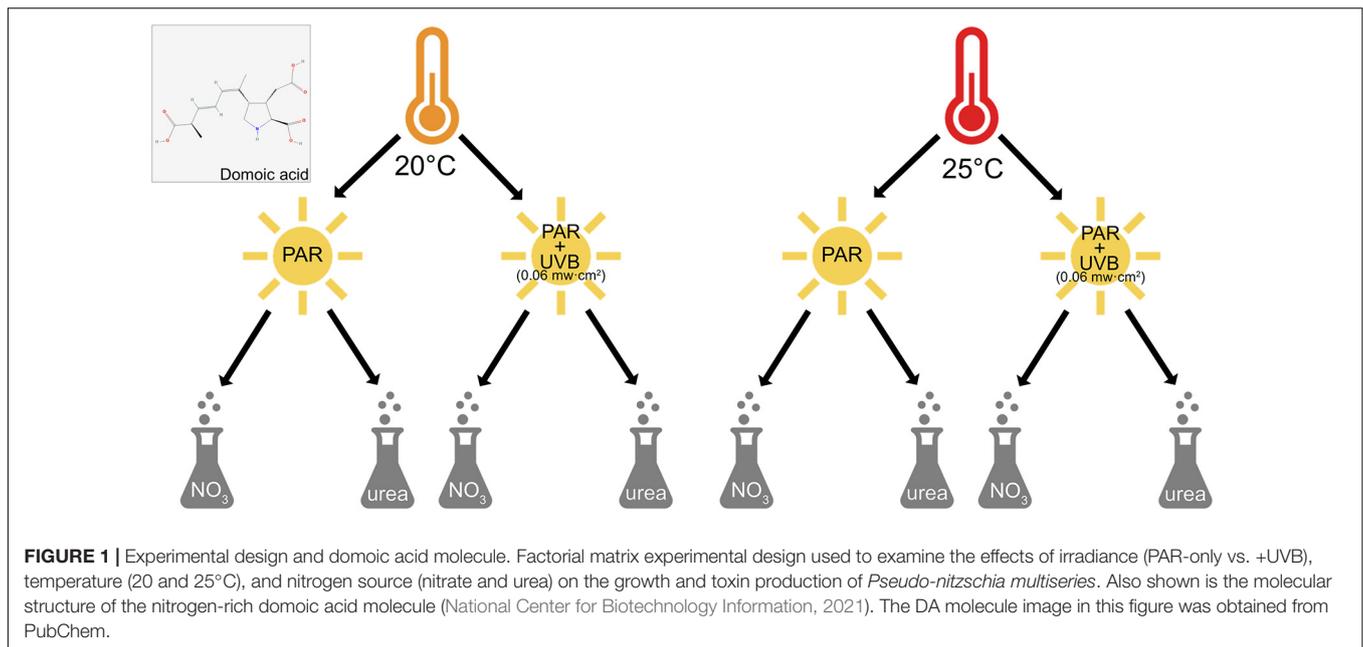
Climate change and other anthropogenic influences are increasingly altering many aspects of coastal environments, and consequentially, the provenance and occurrence of HABs. Laboratory experiments have demonstrated relationships between DA production and individual environmental drivers. For instance, because warming is an unequivocal consequence of climate change, temperature effects on *Pseudo-nitzschia* spp. growth and toxicity have been well studied (Lelong et al., 2012; Trainer et al., 2012; Bates et al., 2018). A consistent trend among various *Pseudo-nitzschia* species is an enhancement of toxin production as temperatures warm above optimal growth temperatures (Bates et al., 1998; Zhu et al., 2017).

However, water temperature is not the only way in which the coastal ocean will be altered in the future. Warming itself modifies other key environmental variables, and in fact induces a cascade of numerous changes. Warming-enhanced stratification of the water column may cause the mixed layer to shoal, and decrease the delivery of nutrients like nitrate to phytoplankton in the surface ocean (Hutchins and Fu, 2017). At the same time, anthropogenic nitrogen sources, such as urea from agricultural runoff and wastewater effluent, are expected to become increasingly important to coastal phytoplankton. It has been suggested that the magnitude of these anthropogenic

inputs could rival the natural inputs of nitrate via upwelling or mixing in some places. Additionally, because natural and anthropogenic nitrogen inputs are often separated temporally (i.e., inputs from upwelling and rainwater runoff are seasonal while wastewater effluent is more or less constant), situations may arise whereby urea is the dominant nitrogen source available to phytoplankton (Kudela et al., 2008; Auro and Cochlan, 2013; Howard et al., 2014).

Because nitrogen is required for both growth and DA biosynthesis, the effects of excess nitrogen loading (eutrophication) and chemical speciation on *Pseudo-nitzschia* spp. have been well documented (Glibert et al., 2005). In fact, both natural and anthropogenic inputs of nitrogen can enhance *Pseudo-nitzschia* spp. growth and toxicity (Heisler et al., 2008), and blooms have been associated with both upwelling and eutrophication (Lange et al., 1994; Schnetzer et al., 2013). In 2014–2015, a massive, highly toxic *Pseudo-nitzschia* spp. bloom occurred along the West Coast of the United States that was thought to be the result of high nutrient influxes from upwelling combined with warming (McCabe et al., 2016; McKibben et al., 2017). An accident washing >2,750 tons of urea-dominant fertilizer into Danish waters induced a highly toxic multi-species *Pseudo-nitzschia* bloom in 2016 (Olesen et al., 2020). Furthermore, *Pseudo-nitzschia* abundances have been positively correlated with high rainfall, as this runoff can deliver large amounts agricultural nitrogen to the ocean (Rees et al., 2009; Downes-Tettmar et al., 2013). However, both inter- and intra-specific differences in nitrogen preference constrain predictions of how changing nitrogen sources may influence *Pseudo-nitzschia* bloom dynamics. Examining growth and DA production in the context of nitrogen speciation in a strain-dependent manner thus becomes increasingly important with ongoing anthropogenic change.

Another indirect consequence of warming is that phytoplankton cells will be exposed to more intense light regimes in a shallower mixed layer, including both photosynthetically active radiation (PAR; 400–700 nm) and shallow-penetrating ultraviolet B radiation (UVB; 280–315 nm; Beardall et al., 2009). UVB radiation can cause oxidative stress and damage to nucleic acids in diatoms (Mengelt and Prézelin, 2005). Field studies have demonstrated that UV radiation can significantly impact phytoplankton mortality, leading some to suggest that UVB partially inhibits net community production in some areas of the ocean (Llabrés and Agustí, 2006; Agustí and Llabrés, 2007; Llabrés et al., 2010; Godoy et al., 2012). Studies have demonstrated mixed responses of *Pseudo-nitzschia* spp. to ultraviolet radiation (UVR; 280–400 nm) exposure. One experiment found that UVB



caused a loss of fixed carbon in a *Pseudo-nitzschia*-dominated natural community (Mengelt and Prézélin, 2005); however, DA production was not measured. Another study found that UVR exposure enhanced toxin production in stationary phase batch cultures of *P. pungens* var. *multiseriis*; growth under these conditions declined for *P. fraudulenta* and *P. pungens* var. *pungens*, yet had no effect on *P. pungens* var. *multiseriis*, suggesting species-specific responses (Hargraves et al., 1993). Clearly, much remains unresolved regarding how more intense UVR exposures will affect *Pseudo-nitzschia* spp. growth and toxicity in future oceans.

Despite the multitude of studies identifying particular environmental variables that affect *Pseudo-nitzschia* growth and toxin production in the lab, the oceanic conditions that fuel blooms in the natural environment remain poorly understood. The majority of these experiments have focused on studying responses to a single factor, yet these conditions do not accurately reflect the natural coastal environment where multiple drivers interactively impact bloom dynamics. Indeed, the few studies that have examined multiple factors have often shown interactive effects between variables (Pan et al., 1998; Sun et al., 2011; Tatters et al., 2012, 2018).

The objective of the present study was to elucidate the potential interactive effects of multiple environmental change drivers on the physiology and toxicity of *Pseudo-nitzschia multiseriis*. We used a full factorial matrix experimental design to examine the combined effects of three environmentally relevant anthropogenic and climate change variables, including temperature (20 vs. 25°C), nitrogen source (nitrate vs. urea), and light (PAR-only vs. UVB-exposed). We hypothesized that experiments examining the complex interactions between these key drivers would better reflect the dynamics occurring in natural coastal ecosystems. Our results suggest that while warming is the main individual driver of increased DA production, there are

interactive effects between UVB, warming, and nitrogen source that exacerbate toxicity to a greater extent than could have been predicted by examining each variable in isolation.

MATERIALS AND METHODS

Culture Conditions

Pseudo-nitzschia multiseriis was isolated from Sungo Bay, Yellow Sea, China (37° 02' N, 122° 33' E). The ambient sea surface temperature was 19.8°C at the time of isolation. In the laboratory, cells were maintained semi-continuously on modified F/2 medium at 20°C on a 12-h light:12-h dark cycle under 130 $\mu\text{mol m}^{-2} \text{s}^{-1}$ cool white fluorescent light. Modified F/2 medium (100 μM total nitrogen, 100 μM silicate, 6 μM phosphate; Guillard and Ryther, 1962; Guillard, 1975) was made using natural seawater filtered through an ultrafiltration water purifier (MUF1580-2T, Midea, China), autoclaved, and bubbled with air filtered through a 0.22 μm PVD syringe filter (Membrane Solutions, Nantong) to equilibrate with ambient atmospheric gas concentrations. Quartz culture bottles were used to allow UVB wavelengths to pass through (Zhu et al., 2020).

UVB Intensity and Dose Experiments

Preliminary experiments using *in vivo* fluorescence (measured with a Trilogy Laboratory Fluorometer, Turner Designs, United States) to calculate growth rates demonstrated that *P. multiseriis* was unable to survive relatively high UVB intensities of 0.18 $\text{mw}\cdot\text{cm}^{-2}$ for 4 h each day over a 2-day period (Supplementary Table 2). This led us to lower the UVB intensity and analyze the dose duration-dependent responses of *P. multiseriis* photosystem (PS) health.

To determine the duration of ultraviolet B radiation (UVB; 280–315 nm) exposure to use in our full factorial matrix experiments, effective quantum yield of PSII (Fv/Fm) was measured as a proxy for photosynthetic efficiency for *P. multiseriis* exposed to UVB (+UVB; Philips FS20T12-UVB Broadband bulb), concurrent with PAR irradiance (>400 nm; irradiance conditions described in section “Culture Conditions”), for varying durations on a daily basis. Cultures maintained semi-continuously in the exponential growth phase were incubated at 20°C in modified F/2 medium with nitrate (see section “Culture Conditions”) under 130 $\mu\text{mol m}^{-2} \text{s}^{-1}$ cool white fluorescent light and allowed to acclimate for 2 weeks (~10 generations) until steady state growth was achieved. New medium was then inoculated at a concentration of ~20,000 cells per liter, and triplicate quartz bottles were incubated under four different durations of 0.06 $\text{mw}\cdot\text{cm}^{-2}$ +UVB: 0, 0.5, 1, and 2 h each day, over a 2-day period. This regimen was chosen to simulate transitory mixing of cells to near-surface, high-UVB waters in the natural environment (Denman and Gargett, 1983; Helbling et al., 2003; Tedetti and Sempéré, 2006; Llabrés et al., 2013). Fv/Fm was measured just after inoculation (T_0), before and after each UVB exposure (T_3 , T_{24} , and T_{27}), and at the time of final sampling (T_{48}). *P. multiseriis* was only able to tolerate short term exposures (<0.5 h) to low intensity UVB (0.06 $\text{mw}\cdot\text{cm}^{-2}$). We found that longer exposure periods caused Fv/Fm to decline to zero by T_{48} , indicating complete inhibition of *P. multiseriis* PSII activity (see section “Determining Sensitivity of Photosynthesis to UVB With Dosage Experiments”). Thus, this dosage was chosen for our full factorial matrix experiments.

Multiple Driver Experimental Design

A three-by-two full factorial matrix experimental design was used to examine all three-way combinations of temperature, nitrogen source, and irradiance, each at two treatment levels (Figure 1). The treatments for each variable were selected based on conditions reflecting contemporary oceans, and those predicted for future oceans altered by climate and anthropogenic change (Duarte, 2014; Hutchins and Fu, 2017). Nitrate treatments used concentrations of 100 μM , whereas concentrations of urea were 50 μM . Total nitrogen concentrations were thus equivalent (100 μM) across nitrogen treatments, as the urea molecule contains two nitrogen atoms.

Cultures were not acclimated to UVB in order to simulate a short-term mixing event, whereby cells residing deeper in the euphotic zone are advected closer to the surface ocean and suddenly exposed to shallow penetrating UVB light (Helbling et al., 2003). Therefore, physiological responses to UVB can be considered consequences of short-term stress rather than acclimated responses.

Cultures were acclimated and grown in their respective temperature-nitrogen source combinations using semi-continuous culturing methods, which allowed the cells to remain in the exponential growth phase. By reducing culture cell density every other day to a concentration in the early exponential growth phase, cells were allowed to grow at rates determined by acclimation to temperature and nitrogen, rather than having a growth rate imposed on them (as in continuous

cultures) or growing into nutrient-starved stationary phase (as in batch cultures). Therefore, in our *P. multiseriis* experiments near steady-state exponential growth rates, physiology, and toxicity were measured as they were influenced by temperature and nitrogen source.

Once cultures had achieved acclimated steady-state growth under experimental conditions, fresh medium was inoculated with ~20,000 cells per liter. All treatments were incubated in 500 mL quartz experimental tubes under their respective conditions continuously for 48-h. Under PAR irradiance, +UVB treatments were exposed to 0.06 $\text{mw}\cdot\text{cm}^{-2}$ UVB from the UVB bulb for 0.5 h per day at midday (11:45-12:15), twice over the 2-day experimental period. This exposure regimen represents transitory vertical mixing. Incident UVB radiation was measured with a digital ultraviolet radiometer (Solarmeter). PAR-only treatments were grown under the same conditions, but on a different shelf in the same incubator. These PAR-only quartz tubes were wrapped in a UVB-absorbing Ultraphan film 395 (UV Opak, Digefra), which only allows the penetration of PAR wavelengths (400–700 nm; Cai et al., 2017; Zhu et al., 2020).

Analytical Methods

Cell Counts and Growth Rates

Cell count samples were obtained at the beginning of the experiment just after medium inoculation (T_0), and again during final sampling (T_{48}). Samples were preserved in a 10% Lugol's solution, stored at room temperature until counted on a Nikon Eclipse Ts2-FL light microscope (NIKON, Tokyo, Japan) using a Sedgewick Rafter Chamber. Specific growth rates were determined using cell counts (only counting frustules that contained chloroplasts) and the following equation:

$$\mu = \frac{\ln\left(\frac{N_{T48}}{N_{T0}}\right)}{T_{48} - T_0}$$

where μ is the specific growth rate (per day) and N is the number of cells at T_0 and T_{48} .

Elemental Analysis

To measure particulate organic carbon and nitrogen, 20-100 mL of culture was filtered on glass microfiber (GF/F) filters (pre-combusted at 500°C for 2 h), then dried for at least 48 h at 60°C in a drying oven. Dried filters were pelleted for analysis on a Costech 4010 Elemental Analyzer (Fu et al., 2007). For biogenic silica samples, 30–50 mL of culture was filtered on 0.5 μm polycarbonate filters and analyzed using an alkaline digestion and colorimetric assay (Nelson et al., 1995). To measure particulate organic phosphorus, 20–60 mL of culture was filtered on pre-combusted GF/F filters and rinsed with 0.17 M Na_2SO_4 , placed in borosilicate scintillation vials with 2 mL of 0.017 M MgSO_4 and dried at 60°C for at least 48 h (Fu et al., 2007). These were then combusted for 4 h at 500°C in order to convert the organic phosphorus to inorganic orthophosphate. Samples were then acidified in 0.2 M hydrochloric acid and analyzed colorimetrically on a spectrophotometer.

Domoic Acid

Samples for cellular toxin quotas (particulate DA) were collected at the end of the experiment (T_{48}) by filtering 50 mL of culture onto 0.5 μm polycarbonate filters and stored at -20°C until subsequent analysis. Sample extraction and cleanup were carried out according to Wang et al. (2012) with minor modifications to the protocol. For each sample, 8 mL of 10% methanol/water (methanol:water, 1:9, v/v) was added to a 10 mL plastic conical centrifuge tube containing the filter, then homogenized by vortexing at room temperature for 10 min. Cells were disrupted by sonication using a 650 W sonic disrupter at 30% amplitude of power setting for 2 min on an ice slurry to release intracellular DA into solution. The extraction was centrifuged at 10,000 g for 5 min and then filtered with a 0.22 μm MCM syringe filter into an LC vial for liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) analysis.

Liquid chromatography coupled with tandem mass spectrometry analysis was performed using an U3000 HPLC system (Thermo Scientific, United States) linked to a Thermo TSQ Endura mass spectrometer equipped with an ESI source operated in positive ionization mode. The chromatographic system equipped with a binary pump and an autosampler with a 100 μL sample loop. To obtain maximum abundance of molecular ions and generate higher sensitivity for analyte, optimization of MS/MS parameters was necessary. Using flow injection analysis at 10 $\mu\text{L min}^{-1}$, the mass spectrometer was tuned using the CRM-DA-f DA standard solution (1 $\mu\text{g mL}^{-1}$; National Resource Council, Canada) and the final ion source conditions were as follows: spray voltage (kV) of +3.50, sheath gas flow 25 (arb), auxiliary gas flow 15 (arb), sweep gas flow 0 (arb), ion transfer tube temperature of 300°C , and vaporizer temperature of 250°C .

Full scan MS in the positive ion mode gave the predominant peak at $[\text{M}+\text{H}]^+ m/z$ 312.0, which was selected as precursor ion. The SRM transitions from the protonated DA ion were m/z 312.0 > 266.0 ($[\text{M}-\text{HCOOH}+\text{H}]^+$) at collision energy (CE) = 15 V used for quantitative analysis, and two confirmatory transitions of m/z 312.0 > 248.0 ($[\text{M}-\text{HCOOH}-\text{H}_2\text{O}+\text{H}]^+$) at CE = 16 V and m/z 312.0 > 161.0 ($[\text{M}-\text{HCOOH}-\text{C}_2\text{H}_3\text{O}_2\text{N}-\text{H}_2\text{O}-\text{CH}_2+\text{H}]^+$) at CE = 22 V, all with a RF lens of 142 V, a dwell time of 250 ms, and a collision induced dissociation gas setting of 1.5 mTorr. Resolution of both quadrupoles Q1 and Q3 were set at 0.7 FWHM. Chromeleon and Xcalibur software were used for the entire MS tune, instrument control, data acquisition, and data analysis. Quantitative analysis was attained by an external standard method with DA calibration solutions at a concentration of 1,000 ng mL^{-1} . DA had a retention time of around 9.49 min. The flow was diverted to waste by a six-way valve for the 0–7.5 min and 11.5–25.0 min of each run to keep the ion source clean.

Photophysiology

The maximum quantum yield of PSII (F_v/F_m), effective quantum yield of PSII (F_v'/F_m'), non-photochemical quenching (NPQ),

relative electron transport rate (rETR), and light saturation point (I_k) of live cultures was assessed using a Multi-Color-PAM Chlorophyll Fluorometer (MC-PAM; Heinz Walz GmbH, Germany; Schreiber et al., 2011) at the time of final sampling.

F_v/F_m was measured after 15 min of dark adaptation of the cultures and calculated as

$$F_v/F_m = \frac{F_m - F_o}{F_m}$$

F_v'/F_m' was measured at an actinic light value close to the experimental PAR irradiance (230 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) and calculated using the following the equation:

$$F_v'/F_m' = \frac{F_m' - F_t}{F_m'}$$

Non-photochemical quenching was calculated as:

$$\text{NPQ} = \frac{F_m - F_m'}{F_m'}$$

Relative electron transport rate was calculated using the following equation:

$$r\text{ETR} = Y(II) \times \text{PAR} \times 0.5 \times 0.84$$

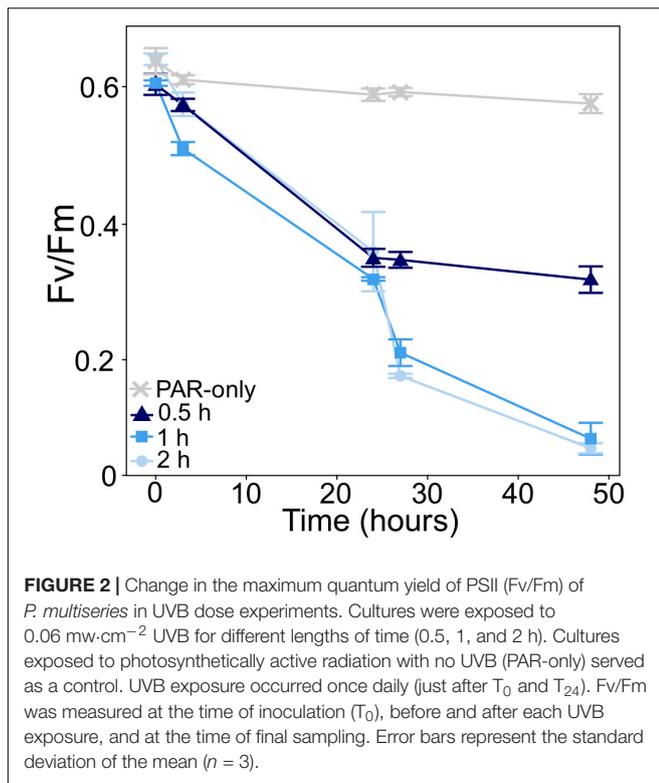
where 0.5 is the equal distribution of photons between PSII and PSI, and 0.84, the homogeneous absorption factor, is a widely accepted absorptivity value (Bjrkman and Demmig, 1987; Ralph et al., 2005). I_k was calculated according to Jassby and Platt (1976).

Statistical Methods

Multivariate analyses were conducted in R version 4.0.2¹ using statistical tools in Rallfun-v38². A percentile bootstrap method for multiple comparisons of 20% trimmed means (t3way) was used to detect significant one-, two-, and three-way interactions between variables (temperature, nitrogen, and irradiance) in the three-by-two factorial matrix experiment. A percentile bootstrap method for comparing 20% trimmed means (t1way) was used to detect significant differences in final (T_{48}) F_v/F_m values in UVB dosage experiments. Pairwise analyses using percentile bootstrapping of 20% trimmed means (trimpb2) were also conducted to test for significant differences between any two matrix treatments for growth rates, DA quotas and production rates, and photophysiology measurements. All tests were performed at the $\alpha = 0.05$ level. These robust methods were chosen over classical one- and three-way ANOVA tests as they do not assume normality or homoscedasticity (Wilcox, 2003).

¹<http://www.r-project.org>

²<https://dornsife.usc.edu/labs/rwilcox/software/>



RESULTS

Determining Sensitivity of Photosynthesis to UVB With Dosage Experiments

In initial short-term UVB dosage experiments, measurements of the maximum quantum yield of PSII (Fv/Fm) showed that *P. multiseriis* PSII activity was unable to tolerate exposure to 0.06 mw·cm⁻² UVB for >2 h per day, or any dose of UVB greater than 0.06 mw·cm⁻² (Supplementary Tables 1, 2). The average Fv/Fm of all treatments at T₀ was 0.623 (Figure 2). From T₀ to T₂₄ (following one dose of UVB), Fv/Fm declines were similar across all treatments: 44, 47.3, and 42% for 2, 1, and 0.5 h of exposure, respectively (Figure 2). Fv/Fm was significantly different between treatments at T₄₈ (t1way, $p = 0.000004$), after two UVB doses. In cultures exposed for 2 or 1 h per day, Fv/Fm further decreased to 0.072 and 0.086 (respectively) after a second UVB exposure, declining by a total of 88.7 and 85.8% from T₀ to T₄₈. However, Fv/Fm of cultures exposed to UVB for 0.5 h per day only declined to 0.319, representing a 47% decline from T₀ to T₄₈ (Figure 2).

Growth Rates

Spectral quality of irradiance had the greatest impact on *P. multiseriis* growth (t3way, $p = 0.0001$). Cell-specific growth rates were <0.05 day⁻¹ for all UVB-exposed (+UVB) treatments (Figure 3A and Supplementary Table 3). In contrast, a temperature-nitrogen source interaction impacting growth rates

was observed for the PAR-only treatments (t3way three-way interaction, $p = 0.046$; Figure 3B, Supplementary Table 4). Growth rates for treatments with nitrate differed between temperatures (20 vs. 25°C), while those of cells grown with urea did not. For nitrate-supported cultures, growth rates declined from 0.58 to 0.39 day⁻¹ (46.3% decrease) with warming from 20 to 25°C (pairwise comparison between two treatments, $p < 0.001$, and Supplementary Table 5). Conversely, growth rates for urea-supported cells were not significantly different at 0.51 and 0.50 day⁻¹ for 20 and 25°C, respectively.

Domoic Acid

Cellular DA quotas at 25°C were on average three orders of magnitude greater than DA quotas in the 20°C incubations, regardless of nitrogen source or irradiance (Figure 4A and Supplementary Table 3). Similarly, DA production rates for PAR-only treatments were on average two orders of magnitude greater at 25°C, relative to 20°C (Figure 4B and Supplementary Table 3). Additionally, DA production was greatly reduced by UVB exposure (t3way, $p = 0.0001$). On average, DA production rates for all +UVB treatments were 8.1×10^{-3} pg cell⁻¹ day⁻¹, while maximum DA production rates, measured for 25°C PAR-only with nitrate or with urea treatments, were two orders of magnitude greater (0.22 and 0.18 pg cell⁻¹ day⁻¹, respectively).

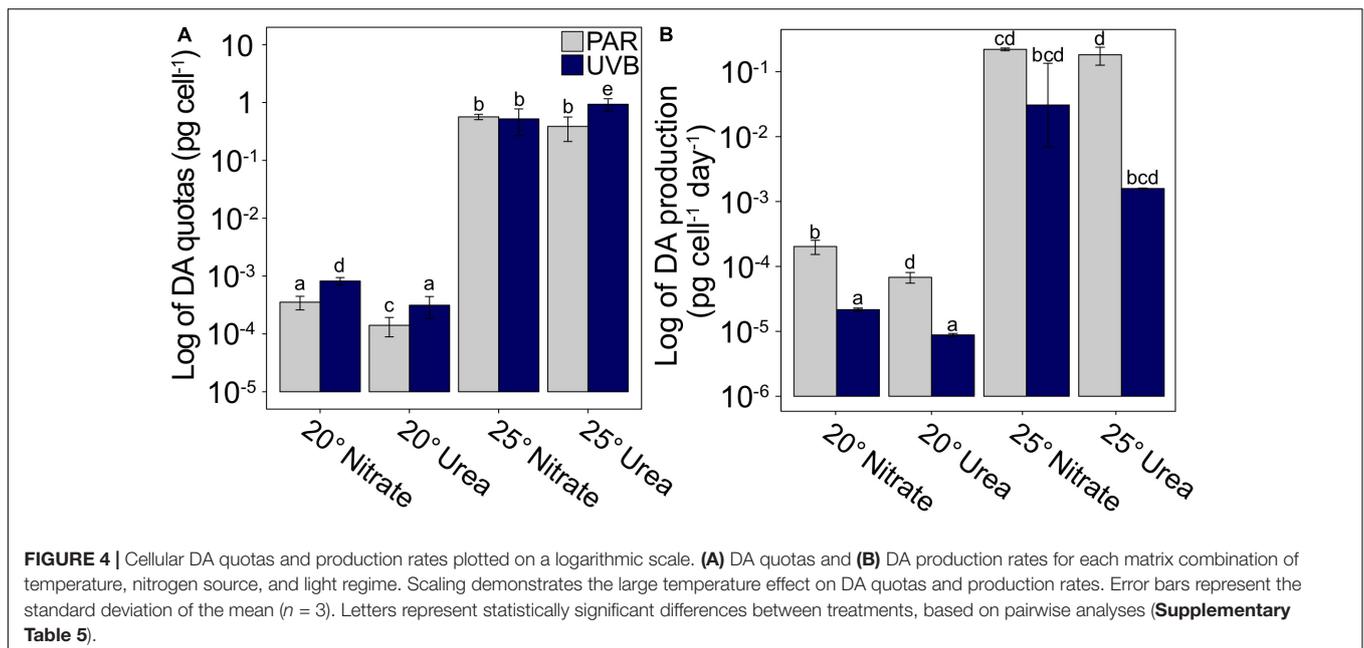
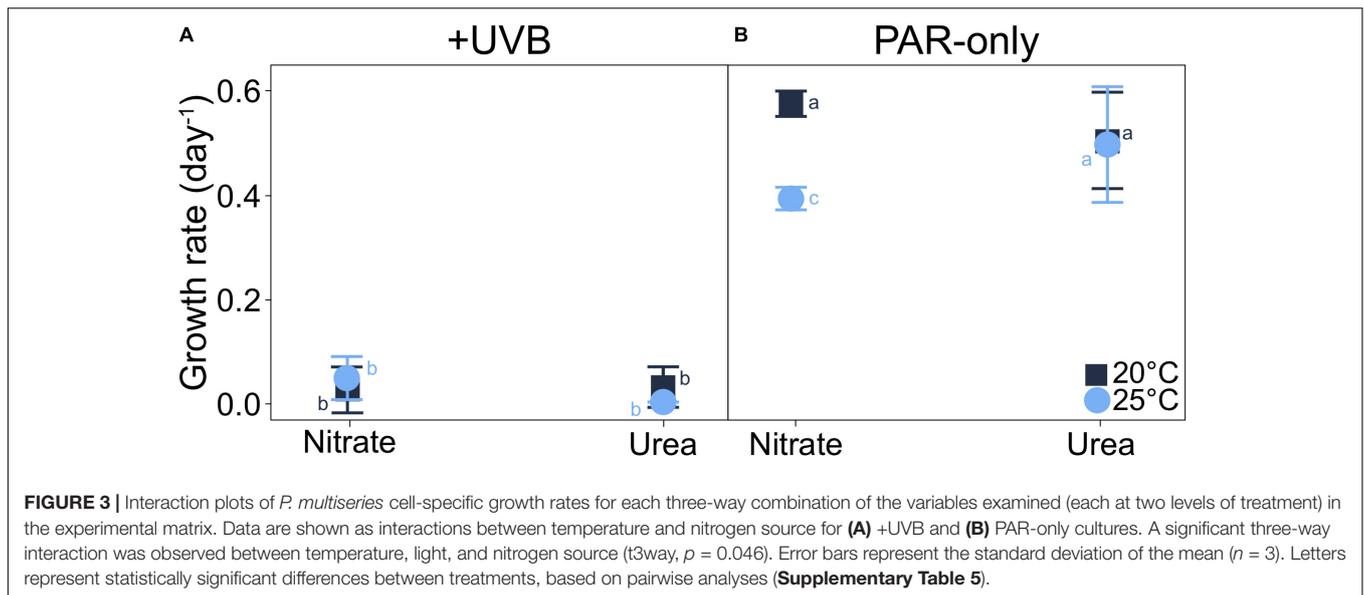
The three-way interaction between nitrogen source, temperature, and irradiance was deemed significant for DA quotas (t3way, $p = 0.032$; Figures 5A,B and Supplementary Table 4). Interactions between light and nitrogen source (t3way, $p = 0.032$) were observed at each respective temperature treatment (i.e., within 20°C treatments, and within 25°C treatments); therefore, results are presented separately for each temperature treatment in the following sections. DA quotas have been plotted as interaction plots to aid in the comparison of trends both across and within treatments, and to highlight the primary drivers of differences between treatments.

Domoic Acid Quotas at 20°C

Within each light treatment, DA quotas were significantly greater with nitrate than with urea (pairwise comparison, PAR $p < 0.001$; UVB $p = 0.001$; Figure 5A and Supplementary Tables 3, 5). This difference was greater for +UVB treatments: cultures with nitrate contained 8.21×10^{-4} pg DA cell⁻¹, while cultures with urea contained 3.13×10^{-4} pg DA cell⁻¹. DA quotas also differed between light treatments, especially for nitrate-grown cells; DA quotas significantly increased from 3.54×10^{-4} pg DA cell⁻¹ when grown under PAR-only, to 8.21×10^{-4} pg DA cell⁻¹ with UVB exposure (pairwise comparison, $p < 0.001$). DA quotas also differed between PAR-only with urea and +UVB with urea treatments (1.41×10^{-4} and 3.13×10^{-4} pg cell⁻¹, respectively; pairwise comparison, $p = 0.001$), but the magnitude of this difference was smaller compared to nitrate-grown cells (Figure 5A).

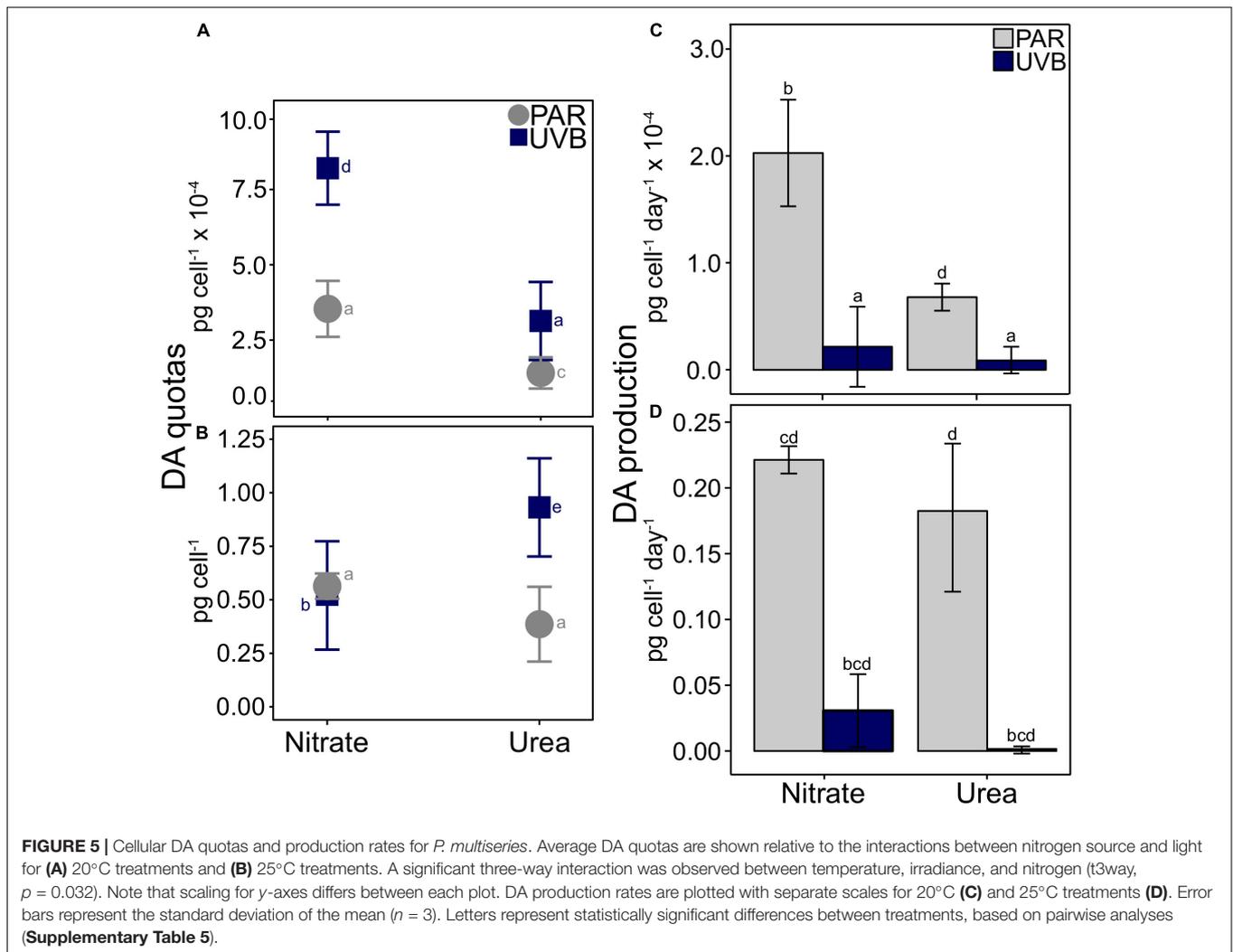
Domoic Acid Quotas at 25°C

The greatest DA quotas were observed at 25°C, relative to 20°C treatments (Figures 5A,B and Supplementary Table 3). For the two 25°C treatments with nitrate, cellular DA quotas did not



significantly differ between light regimes; PAR-only with nitrate DA quotas were on average $0.56 \text{ pg cell}^{-1}$, similar to those of +UVB with nitrate ($0.52 \text{ pg cell}^{-1}$; Figure 5B). Conversely, a large difference in cellular DA quotas was observed between PAR-only with urea and +UVB with urea treatments (pairwise comparison, $p < 0.001$, and Supplementary Table 5). When 25°C urea treatments were exposed to UVB, cellular DA quotas increased from 0.39 to $0.93 \text{ pg cell}^{-1}$ (Figure 5B). Despite higher cellular DA quotas, DA production rates were much lower in UVB treatments than in PAR treatments at both 20°C (Figure 5C) and 25°C (Figure 5D) for both nitrogen sources, due to the greatly reduced growth rates in all treatments after UVB exposure (Figure 3).

Examining the same four treatments in the context of nitrogen source-effects within light treatments (i.e., 25°C PAR with nitrate vs. with urea, and 25°C +UVB with nitrate vs. with urea) allows for interpretation of the data from another perspective, revealing additional key differences between treatments (Figure 5B). DA quotas differed between nitrogen sources within each light treatment, both in terms of magnitudes and direction of change. Under a PAR-only light regime, DA production was 1.46 times higher with nitrate ($0.56 \text{ pg cell}^{-1}$) than urea ($0.39 \text{ pg cell}^{-1}$), but the difference was marginally not significant at $p = 0.058$ (pairwise comparison). In contrast, +UVB treatments produced significantly more DA with urea ($0.93 \text{ pg cell}^{-1}$) compared to treatments with nitrate ($0.52 \text{ pg cell}^{-1}$; $p < 0.001$).



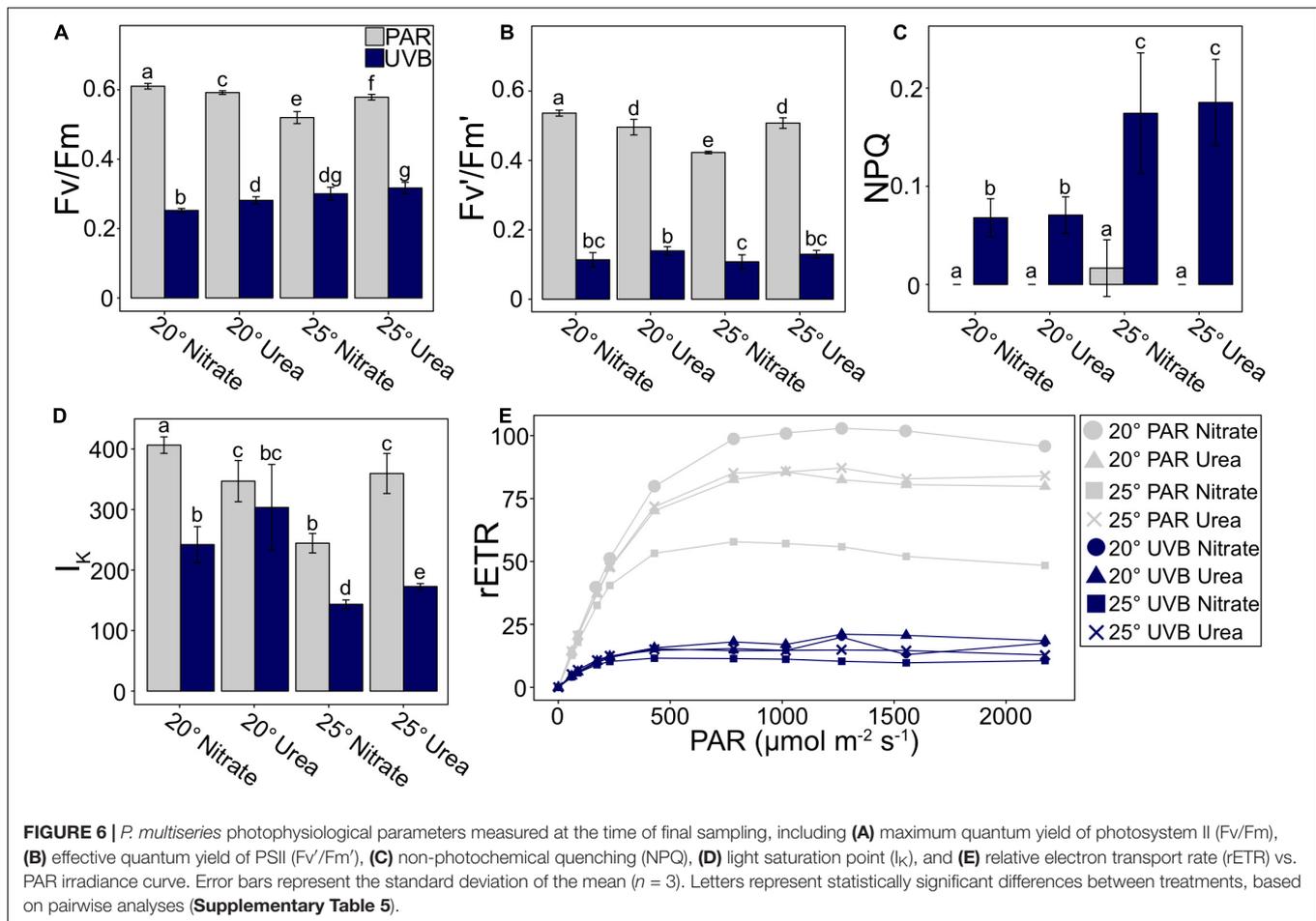
Photophysiology

Large differences were observed between PAR-only and +UVB treatments for F_v/F_m and effective quantum yield (F_v'/F_m' ; Figures 6A,B and Supplementary Table 6). At 20°C, F_v/F_m decreased with UVB exposure by 58.7% for nitrate and 52.5% for urea. For 25°C treatments with nitrate and with urea, F_v/F_m declined in +UVB treatments by 42.2 and 45.2%, respectively (Figure 6A). The magnitude of decline was greater for F_v'/F_m' : 78.8 and 71.9% for 20°C treatments with nitrate and with urea, and 74.5 and 74.5% for 25°C treatments with nitrate and with urea (Figure 6B). Furthermore, a significant temperature-nitrogen interaction was observed for both F_v/F_m and F_v'/F_m' (t3way, $p = 0.007$ and $p = 0.001$, Supplementary Table 4). For PAR-only treatments, F_v/F_m did not significantly differ between 20 and 25°C treatments, with urea averaging 0.592 and 0.579, respectively (Figure 6A). A similar trend was observed for F_v'/F_m' values, which were 0.496 and 0.508, respectively (Figure 6B). However, F_v/F_m and F_v'/F_m' for PAR-only with nitrate treatments did differ between temperatures; with an increase in temperature, F_v/F_m decreased by 14.8% from

0.520 (Figure 6A), while F_v'/F_m' decreased by 21.1% from 0.536 to 0.432 (Figure 6B). These temperature-dependent nitrogen source effects were not observed for +UVB treatments.

Non-photochemical quenching was 0 for all PAR-only treatments, except for a very low value of 0.017 at 25°C with nitrate (Figure 6C and Supplementary Table 6). The temperature-light interaction was deemed significant at the $p = 0.003$ level (t3way). For +UVB treatments, NPQ values were higher at 25°C with both nitrate and with urea (0.174 and 0.185, respectively) relative to 20°C treatments (0.068 and 0.071; Figure 6C). Differences between nitrogen treatments were not significant within each temperature.

Measured differences in the irradiance at the light saturation point (I_k) were mainly driven by light (t3way, $p = 0.0001$; Figure 6D, Supplementary Tables 4, 6). Decreases were observed from PAR-only to +UVB treatments at 20°C with nitrate (40.5%), 25°C with nitrate (41.4%), and 25°C with urea (51.9%), but 20°C with urea only decreased by 12.5%. Furthermore, trends within PAR-only treatments were under different controls than UVB-exposed treatments. For PAR-only, urea treatments



did not vary between temperatures, while the I_k for nitrate treatments decreased with an increase in temperature. In contrast, within +UVB treatments, measured I_k values were greater at 20°C relative to 25°C within each nitrogen source treatment. Additionally, at each respective temperature, I_k was greater for treatments with urea compared to treatments with nitrate (Figure 6D).

Furthermore, maximum relative electron transport rates (rETR_{max}) for +UVB treatments were on average 5.37x lower than PAR-only treatments (Figure 6E and Supplementary Table 6). Differences between UVB treatments were not large in magnitude. In contrast, rETR_{max} for PAR-only treatments were dependent on interactions between nitrogen source and temperature (t3way, $p = 0.001$, Supplementary Table 4); urea treatments were similar between temperatures, yet there was an inverse relationship between temperature and rETR_{max} for nitrate treatments (Figure 6E).

Elemental Ratios and Quotas

The amount of carbon per cell (cellular carbon quota) significantly increased with UVB exposure (t3way, $p = 0.0003$; Table 1 and Supplementary Tables 4, 7). This increase was observed to a greater extent for 25°C treatments; relative to PAR-only, +UVB increased carbon quotas by 30 and 50% at 25°C

with nitrate and 25°C with urea treatments, respectively. This +UVB effect was not observed for 20°C treatments. Similar light effects were observed for cellular nitrogen quotas (Table 1 and Supplementary Table 7). Smaller cellular nitrogen pools were measured at 20°C and were little influenced by UVB-exposure, yet at 25°C UVB-exposure increased nitrogen quotas relative to PAR-only treatments by 27.5 and 46.7% for both nitrate and urea treatments, respectively (Table 1 and Supplementary Table 7). However, while temperature alone did not significantly influence cellular carbon pools, it did influence nitrogen pools, demonstrated by increased nitrogen per cell for 25°C treatments (t3way, $p = 0.012$).

Cellular phosphorus quotas were most influenced by irradiance (t3way, $p = 0.002$, and Supplementary Table 4), as each paired treatment showed a percent increase of at least 30% with UVB-exposure (Table 1 and Supplementary Table 7). Warming also influenced differences in measured cellular phosphorus quotas (t3way, $p = 0.007$). The smallest cellular phosphorus pools were measured for 20°C PAR-only with nitrate ($5.17 \times 10^{-8} \mu\text{mol P cell}^{-1}$), while the largest pools were an order of magnitude greater for 25°C +UVB with urea treatments ($1.18 \times 10^{-7} \mu\text{mol P cell}^{-1}$). Change in nitrogen source did not result in significant differences in phosphorus quotas between treatments (Table 1).

TABLE 1 | *P. multiseriis* cellular elemental quotas and ratios.

Treatment	Cell quotas ($\mu\text{mol}\cdot\text{cell}^{-1}$)				Elemental ratios			
	Carbon	Nitrogen	Phosphorus	Biogenic silica	C:N	C:P	N:P	BSi:C
20°C Nitrate	3.53×10^{-6}	5.17×10^{-7}	5.17×10^{-8}	8.76×10^{-7}	7.250*	137.525*	14.098*	0.124*
PAR	(7.16×10^{-7})	(3.02×10^{-7})	(4.87×10^{-9})	(6.75×10^{-8})	(1.7)	(14.77)	(1.05)	(0.01)
20°C Nitrate	4.60×10^{-6}	9.38×10^{-7}	8.33×10^{-8}	9.02×10^{-7}	6.890*	142.951*	14.758	0.082*
UVB	(9.19×10^{-7})	(2.86×10^{-7})	(1.00×10^{-8})	(1.43×10^{-7})	(1.30)	(42.62)	(2.54)	(0.03)
20°C Urea	4.65×10^{-6}	7.08×10^{-7}	5.39×10^{-8}	8.06×10^{-7}	7.697	133.590*	15.030	0.112*
PAR	(4.29×10^{-7})	(1.21×10^{-7})	(2.47×10^{-9})	(7.48×10^{-8})	(0.32)	(8.74)	(0.58)	(0.01)
20°C Urea	4.77×10^{-6}	8.83×10^{-7}	7.41×10^{-8}	8.25×10^{-7}	6.827*	113.250*	14.336*	0.099*
UVB	(9.96×10^{-7})	(2.54×10^{-7})	(5.11×10^{-9})	(4.63×10^{-8})	(0.27)	(7.16)	(1.79)	(0.01)
25°C Nitrate	7.73×10^{-6}	9.45×10^{-7}	6.17×10^{-8}	8.07×10^{-7}	8.179*	120.871*	15.214	0.109*
PAR	(3.62×10^{-7})	(1.09×10^{-7})	(4.01×10^{-9})	(1.97×10^{-7})	(0.18)	(11.56)	(1.56)	(0.03)
25°C Nitrate	1.11×10^{-5}	1.30×10^{-6}	9.69×10^{-8}	1.17×10^{-6}	8.037*	95.919	12.951*	0.127*
UVB	(1.22×10^{-6})	(2.13×10^{-7})	(1.67×10^{-8})	(1.58×10^{-7})	(0.86)	(2.49)	(2.85)	(0.01)
25°C Urea	6.28×10^{-6}	8.52×10^{-7}	8.30×10^{-8}	1.01×10^{-6}	7.474	91.147*	11.157	0.142*
PAR	(5.10×10^{-7})	(7.90×10^{-8})	(2.79×10^{-8})	(1.19×10^{-7})	(0.35)	(16.68)	(2.34)	(0.01)
25°C Urea	1.28×10^{-5}	1.60×10^{-6}	1.18×10^{-7}	1.12×10^{-6}	7.613	92.567*	12.829*	0.103*
UVB	(2.67×10^{-7})	(3.32×10^{-7})	(1.43×10^{-8})	(8.85×10^{-8})	(0.34)	(4.84)	(1.89)	(0.004)

For elemental ratios, * indicates values that are significantly different from Redfield Ratio. Numbers in parentheses represent standard deviations of the mean ($n = 3$).

Temperature significantly increased biogenic silica (BSi) quotas (t3way, $p = 0.014$, **Supplementary Table 4**) for all treatments except 25°C PAR-only with nitrate (**Table 1** and **Supplementary Table 7**). However, irradiance effects were marginally insignificant at the $p = 0.054$ level, and interactive effects between nitrogen and temperature were not significant. Cellular BSi quotas at 20°C were not significantly impacted by nitrogen source (**Table 1**).

C:N and C:P ratios were significantly impacted by temperature (t3way, $p = 0.016$ and $p = 0.012$, **Supplementary Table 4**). The largest C:N ratio was measured in the 25°C PAR with nitrate treatment, while the smallest was observed for 20°C UVB with urea (**Table 1** and **Supplementary Table 7**). All C:P ratios measured for 20°C treatments were significantly above the Redfield Ratio, while 25°C treatments were below (with the exception 25°C +UVB with nitrate). Nitrogen, irradiance, and interactive effects on C:P were not deemed statistically significant. Temperature effects on BSi:C ratios were marginally insignificant at the $p = 0.056$ level, with irradiance being a more significant driver of differences between treatments (t3way, $p = 0.026$). PAR-only treatments had larger BSi:C ratios than their paired UVB-exposed treatments, except for 25°C with nitrate. No significant differences in N:P ratios were detected between treatments (**Table 1** and **Supplementary Table 7**).

DISCUSSION

Dependency of Growth Under PAR-Only Conditions on Temperature-Nitrogen Interactions

Our multiple-driver experimental design revealed previously unknown interactions between temperature, nitrogen, and UVB in this HAB species. *P. multiseriis* growth rates under

PAR-only conditions were moderately affected by nitrogen source in a temperature-dependent manner. Growth rates for nitrate treatments declined with warming, while growth rates for cultures with urea did not differ between temperatures. Previous studies examining the effects of nitrogen source on *Pseudo-nitzschia* spp. growth rates have demonstrated a great degree of variability between both species and strains. Growth rates measured for two strains of *P. multiseriis* were slower for urea-supported cultures relative to nitrate, while growth rates for another strain did not differ between nitrogen treatments. For some strains of *P. calliantha* and *P. fraudulenta*, growth rates decreased in urea-supported cultures compared to nitrate and ammonium. In contrast, no difference between nitrogen substrates was observed for *P. australis* (Thessen et al., 2009; Martin-Jézéquel et al., 2015). Based on a meta-analysis of nitrogen source-effects on growth rates for several species of *Pseudo-nitzschia*, Radan and Cochlan (2018) hypothesized that differences in culture conditions (PAR irradiance, temperature, and nitrogen concentration) partially contributed to differences in growth rates. This demonstrates that interactive effects between variables are important to consider. Nevertheless, there is a great deal of both intra- and inter-specific variability in terms of *Pseudo-nitzschia* nitrogen source preference (Thessen et al., 2009; Auro and Cochlan, 2013). The mechanisms driving *P. multiseriis* nitrogen source preference and utilization remain unclear, yet it is evident that this interactive effect on nitrogen utilization has implications for bloom formation and photosynthesis in future oceans with altered nitrogen source availability.

Climate and anthropogenic change may modify the inorganic nitrogen species available to phytoplankton in future oceans. In contemporary coastal oceans, primary productivity is primarily supported by naturally-supplied nitrate delivered to the surface ocean via mixing and upwelling (Dugdale and Goering, 1967). However, future warming-induced stratification of the water

column may reduce the intensity of upwelling and mixing, thereby decreasing the delivery of nitrate to the surface ocean (Hutchins and Fu, 2017). Additionally, the supply of nitrogen from anthropogenic sources (e.g., urea from agricultural runoff and wastewater effluent) is expected to increase as global fertilizer use and human populations in coastal areas expand (Glibert et al., 2006). Anthropogenic inputs of alternative nitrogen species may eventually rival or exceed natural inputs of nitrate, becoming the dominant species of nitrogen available to phytoplankton in coastal areas (Howard et al., 2014). Because of the importance of nitrogen to growth, photosynthesis, and toxin biosynthesis, and the previously documented impacts of nitrogen species on *Pseudo-nitzschia* toxin biosynthesis (Howard et al., 2007; Thessen et al., 2009; Auro and Cochlan, 2013; Martin-Jézéquel et al., 2015; Radan and Cochlan, 2018), the effects of this predicted eutrophication and changing nitrogen speciation on *Pseudo-nitzschia* spp. may have important implications for HAB modeling and forecasting (Glibert et al., 2005).

Despite potential changes to nitrogen speciation and availability in coastal zones, our data suggest that *P. multiseriis* grows at the same rate on both urea and nitrate at temperatures representative of a future, warmer ocean (25°C). Temperature and nitrogen also interacted to impact photosynthetic parameters like Fv/Fm, Fv'/Fm', I_k, and rETR_{max} in a similar manner; measurements for nitrate-supported treatments decreased with warming, while temperature had negligible effects on the photophysiology of urea-supported cells. Therefore, at warmer temperatures (under PAR-only conditions), *P. multiseriis* blooms and primary productivity may not be strongly influenced by shifting nitrogen sources. This aligns with previous evidence demonstrating that diatom nitrogen metabolism is highly flexible, enabling cells to alter uptake and assimilation mechanisms quickly and efficiently when other nitrogen sources become available (Smith et al., 2019). However, in the present study nitrate-supported growth rates at 25°C were slightly lower than maximum observed rates at 20°C for PAR-only treatments. This suggests that future warming may incrementally reduce *P. multiseriis* growth, with implications for bloom development and primary productivity relative to contemporary nitrate-dominated systems. However, the trends just described are only applicable to PAR-only treatments. Therefore, these results are most relevant to populations of cells residing deeper in the water column where UVB is largely attenuated (*see below*).

***P. multiseriis* Growth Under UVB Exposure**

Despite the differential effects alternative nitrogen sources and warming may have on *P. multiseriis* growth rates, increased UVB exposure in a future surface ocean may have an overpowering influence on bloom formation. Inhibition of cellular division across all treatments exposed to 0.06 mw·cm⁻² UVB demonstrates that the growth of this strain of *P. multiseriis* is strikingly sensitive to UVB. This dose of UVB is similar to near-surface measurements in the natural environment. For example, summertime UVB radiation measured just below the surface in the coastal Red Sea was 0.084 mw·cm⁻² (Al-Aidarous

et al., 2014). Other studies examining the impact of UVR on diatoms have also used UVB doses similar to or lower than those measured at the air-sea interface, reasoning that these lower irradiances are more reflective of the light regimes present at the depths to which phytoplankton are mixed (Nilawati et al., 1997; Liang et al., 2006; Helbling et al., 2011).

Many studies have demonstrated the sensitivity of phytoplankton to UVB, causing declines in both growth and photosynthesis (Cullen and Lesser, 1991; Helbling et al., 1992; Smith et al., 1992; Neale et al., 1994; Nilawati et al., 1997; Gao et al., 2007a,b; Guan and Gao, 2010; Li Y. et al., 2012; Cai et al., 2017; Zhang et al., 2019). However, only a handful of studies have specifically examined *Pseudo-nitzschia* sp. in this regard. For instance, UVB exposure of polar *P. seriata* caused growth rates to decline by 50% and Fv/Fm to decline by 27% (Nilawati et al., 1997). UVR contributed to inhibition of both growth and Fv'/Fm' in *P. pungens*, with warming playing a role in alleviating the severity of inhibition (Chen et al., 2018). Hargraves et al. (1993) reported declines in *P. fraudulenta* and *P. pungens* var. *pungens* growth rates with exposure to either artificial or natural UV light, respectively, yet growth was not fully inhibited. On the other hand, *P. pungens* var. *multiseriis* growth was unaffected by either type of UVR exposure. Furthermore, field studies found that in a mixed diatom community dominated by *P. australis*, loss of fixed carbon was greater in treatments exposed to UVB (Mengelt and Prézélin, 2005). Therefore, it is clear that UVB alone can impose controls on phytoplankton growth and photosynthesis, with implications for *Pseudo-nitzschia* blooms in the natural environment.

We propose that the co-occurrence of UVB inhibition of growth and increased toxicity for some treatments is a situation similar to growth limitation by low silicate or phosphate availability. Growth rates are typically inversely correlated with DA production, since as cellular division decreases due to nutrient limitation or other growth-limiting stressors, DA production increases (Bates et al., 1991; Zhu et al., 2017). Because DA is a secondary metabolite, only produced when there is enough energy, this inverse correlation is hypothesized to be a function of re-allocation of energy resources within the cell (Pan et al., 1998). This has previously been studied in the context of growth limitation by silicate and phosphate. These studies have shown that growth inhibition by macronutrient limitation can enhance cellular toxin quotas (Bates et al., 1991, 1996; Pan et al., 1996; Fehling et al., 2004; Sun et al., 2011; Tatters et al., 2012). In a chemostat culture experiment using silicate limitation to control *P. multiseriis* growth rates, DA quotas were inversely correlated with growth rates (Bates et al., 1996). The authors concluded that DA was “produced during conditions of stress” as part of secondary metabolism occurring when cellular division is not the main energy sink, or when there is sufficient energy for growth. Therefore, when cellular division is slowed or inhibited by macronutrient limitation, unused energy is reallocated toward toxin production, increasing cellular DA quotas. This is similar to our growth-inhibited UVB exposed cultures. UVB-exposure stressed cellular growth rates and, in some treatments, enhanced cell-specific toxicity. We hypothesize that these observed increases in toxicity could therefore be a stress

response to UVB-limited growth, whereby DA production is a sink for energy not being used for growth.

Other research suggests that ultraviolet A radiation (UVA; 320–400 nm) wavelengths can help to mitigate cellular UVB damage (Quesada et al., 1995) by stimulating photo-repair mechanisms (DNA repair and antioxidant mechanisms; Mitchell and Karentz, 1993), the synthesis of photoprotective pigments (mycosporine-like amino acids; Lesser, 1996; Xu and Gao, 2010), and increased photosynthetic carbon fixation (Mengelt and Prézelin, 2005; Gao et al., 2007a; Li et al., 2011). Our study did not incorporate UVA wavelengths, but we hypothesize that the inclusion of this portion of the light spectrum in our experimental design may have decreased the sensitivity of *P. multiseriis* to damaging UVB rays. Therefore, our measurements may represent a maximum estimate of damaging UVB-related effects.

Furthermore, in the natural environment, self-shading can occur in dense phytoplankton blooms, reducing effective light exposure and decreasing oxidative stress within the population (Barros et al., 2003). Self-shading thus acts as a photoprotection mechanism for *in situ* blooms. However, our use of semi-continuous culture techniques maintained cultures at low cell abundances, such that photoprotection from harmful UVB irradiance as a result of self-shading was minimal. Accordingly, our estimates of UVB-impacts on *P. multiseriis* physiology and toxicity represent an upper estimate. Our results nevertheless suggest that UVB could be an important control on *P. multiseriis* growth in the natural environment. Future studies using full spectrum natural sunlight at a range of intensities are needed to validate our findings and examine the potential synergistic or antagonistic effects of both UVA and UVB.

The extent to which UVB impacts *P. multiseriis* in the natural environment will depend on changing physical oceanography. Projected ocean warming will intensify stratification of surface waters and cause the mixed layer to shoal, increasing the residence time of cells in the near-surface ocean. Consequentially, light exposure integrated over depth will increase, exposing cells to more intense PAR and especially to additional shallow penetrating UVB rays (Beardall et al., 2009; Gonçalves et al., 2010; Steinacher et al., 2010; Gao et al., 2019). Therefore, the inhibition of growth by UVB exposure observed in the present study suggests that the ability of *P. multiseriis* to form near-surface blooms in a future ocean may be inhibited by increases in UVB exposure and intensity.

Cells Continued to Function Despite Inhibition of Growth and Damage to Photosystems

Pseudo-nitzschia multiseriis photosystem health was also impaired by UVB but was less sensitive compared to cellular division. Fv/Fm, Fv'/Fm', I_k, and rETR_{max} decreased with UVB exposure relative to PAR-only treatments, indicating stress, damage to photosynthetic proteins, and a diminished capacity of photosystems to absorb and process light energy. In another study, the marine cyanobacterium *Trichodesmium* exhibited sharp declines in Fv'/Fm' just 10 min after initial exposure to UVB, concurrent with measured reductions in

carbon fixation rates (Cai et al., 2017). Similarly, sharp declines in Fv/Fm soon after UVB exposure were observed in the UVB-dosage experiments of the present study (section “Determining Sensitivity of Photosynthesis to UVB With Dosage Experiments”) in addition to the decreases in Fv/Fm and Fv'/Fm' for all UVB treatments relative to PAR-only in the matrix experiments. This suggests that UVB damage to *P. multiseriis* photosystems may have also reduced, but not fully inhibited, carbon fixation rates. Furthermore, NPQ increased with UVB exposure, suggesting diversion of a greater proportion of energy away from damaged reaction centers that are unable to handle the amount of incoming light energy. NPQ increased to a greater degree for 25°C treatments, indicating that cells increased photosynthetic stress-responses as a result of warming. Although +UVB impaired photosynthetic processes, photosystem function was not fully inhibited. Therefore, despite the suppression of cellular division, cells were able to survive and continue photosynthesis under UVB stress. This allows *P. multiseriis* to obtain energy for cellular functions such as carbon fixation, nutrient uptake, and DA synthesis, as well as stress mitigation and repair.

Increases in cellular elemental quotas with UVB exposure and warming provide additional evidence for continued carbon fixation and nutrient uptake in stressed cells. Carbon per cell increased with UVB exposure for 25°C treatments. This effect was not observed at 20°C, indicating an interactive effect whereby UVB exposure magnifies the response of cells previously stressed by warming. Similarly, nitrogen and silica cell quotas were higher for 25°C UVB-exposed treatments, relative to the respective PAR-only treatments, though silica differences were marginal for the 25°C urea-supported treatments. Phosphorus quotas increased with UVB exposure at both temperatures, but the increase at 25°C was greater. These increased carbon, nitrogen, silica, and phosphorus pools suggest that both photosynthesis and nutrient uptake mechanisms continued to function in stressed cells. Because cellular division was inhibited by UVB, the fixed carbon and nutrients taken up by cells could not be used for growth related to cellular division. In other diatoms, cellular nutrient storage pools have increased when growth was inhibited by other stressors such as phosphate, silicate, nitrogen, and iron limitation (Kilham et al., 1977; Hutchins and Bruland, 1998; Tantanararit et al., 2013; Liefer et al., 2019). This nutrient storage capacity has implications for growth resumption and potential bloom formation upon relief and recovery from stress.

The continued survival and functioning of cells under stress raises questions about post-stress recovery. Depending on the severity and duration of exposure to UVB, it is possible that *P. multiseriis* may be able to recover and resume normal functioning. Studies have shown that cells can recover from UVB-caused photosynthetic inhibition once the stressor has been removed (Villafañe et al., 2007; Roncarati et al., 2008; Halac et al., 2009; Helbling et al., 2011; Cai et al., 2017). Chen et al. (2018) demonstrated that recovery rates of *P. pungens* exposed to UVR were enhanced with warming. We hypothesize that the kinetics of recovery may play an important role in bloom formation for *P. multiseriis* residing in environments with dynamic light regimes. Damage from short-term vertical

mixing of *P. multiseriata* to the surface ocean may be mitigated by subsequent mixing back down to depth, which provides stressed cells respite from shallow penetrating UVB rays and time to repair damage and recover. Reduced PAR-irradiance is also beneficial to recovery from photoinhibition (Helbling et al., 2011). Previous research suggests that shorter mixing events are not as inhibitory to phytoplankton community carbon fixation, as these short periods of exposure are not terribly damaging to photosystems (Helbling et al., 2003). However, future shoaling of the mixed layer may prevent mixing to depths at which UVB wavelengths are completely attenuated, reducing the ability of cells to recover. This cellular recovery aspect was not investigated in the present study, and thus further research with long-term tracking of post-stress growth and photophysiology is needed to draw further conclusions.

Multiple Stressor Effects on *P. multiseriata* Toxicity

Temperature was the main driver of differences in cellular DA toxin quotas between treatments, regardless of nitrogen source or light spectrum. Although there were significant differences between 20°C nutrient and irradiance treatments, they were small. Therefore, any interactive effects occurring at 20°C are unlikely to have a great influence on the severity of a toxic bloom. On average, DA quotas were three orders of magnitude greater for 25°C treatments compared to 20°C incubations. This suggests that during current heatwave events or in a future consistently warmer ocean, *P. multiseriata* toxicity per cell will increase. Previous laboratory studies have also demonstrated that warming above optimal growth temperatures can enhance toxin production in several *Pseudo-nitzschia* species (Bates et al., 1998; Zhu et al., 2017). Field-based observational studies have also positively correlated temperature and DA (Downes-Tettmar et al., 2013; McKibben et al., 2015). In contrast, there has been one documented occurrence of a negative correlation between DA quotas and temperature, whereby greater DA quotas were measured in *P. seriata* incubated at 4°C compared to 15°C (Lundholm et al., 1994). This demonstrates heterogeneity in warming responses of the *Pseudo-nitzschia* genus, indicating that more species should be studied with regard to temperature effects. This becomes problematic as global ocean mean temperatures rise, and the frequency of marine heat waves increases (Oliver et al., 2018). The effects of warm anomalies are already being felt in coastal ecosystems; the extensive and highly toxic 2014–2015 “Blob” bloom occurred when an anomalously warm water mass developed off the West Coast of North America (McCabe et al., 2016; McKibben et al., 2017). More frequent events such as these could have devastating impacts for coastal ecosystems. Adding another layer of complexity, within the 25°C treatments, DA quotas appear to be related to nitrogen source and irradiance. This implies that although temperature was the main driver of increased toxicity, it likely cannot be the sole parameter used to draw accurate conclusions about the threat of toxic bloom events in warmer coastal oceans.

Nitrogen source and UVB interacted with warming to influence DA quotas. The most DA was measured in the 25°C

+UVB treatment with urea. In addition to warming stress, UVB also caused an increase in DA quotas for most treatments, relative to respective PAR-only treatments. However, the magnitude of this increase between PAR-only and +UVB treatments was temperature- and nitrogen source-dependent. DA biosynthesis and cellular division, both energetically demanding processes, tend to be decoupled: as cells become thermally-stressed, growth rates decline and DA production increases (Pan et al., 1998). This is hypothesized to be caused by a reallocation of cellular energy and resources within the cell away from growth and toward DA biosynthesis (Lelong et al., 2012; Trainer et al., 2012; Bates et al., 2018). Previous studies have attributed this increased DA to growth suppression by nutrient limitation (Si, P, and Fe) or warming stress (Maldonado et al., 2002; Sun et al., 2011; Tatters et al., 2012; Zhu et al., 2017). Our study suggests that UVB, which also suppresses *P. multiseriata* growth, triggers increased DA production. Few studies have examined the impacts of UVB on *Pseudo-nitzschia* spp., and to our knowledge only one has also measured DA. Hargraves et al. (1993) found that *P. pungens* var. *multiseriata* growth was unaffected by UV exposure using natural sunlight (representative of a 1.5 m depth in a coastal area), but DA quotas were greater in treatments without UVB exposure measured in stationary phase batch cultures. Differences between results from Hargraves et al. and our own study may lie in the severity of UVB exposure and effects on growth. While UVB did not affect growth rates in the other study, we hypothesize that the marked UVB inhibition of growth contributed to greater DA yields in our study.

Light spectral quality strongly influenced calculated DA production rates. The calculation for DA production integrates growth rates and DA quotas, assuming DA production is proportional to growth. This provides us with an estimate of how much DA could be produced by a bloom, based on both the toxicity per cell and the rate at which this toxin-containing cellular biomass accumulates. *P. multiseriata* was not able to grow with UVB exposure, so under this assumption the calculated DA production was also zero. Although this was true for two of the temperature-nitrogen pairings (20°C with urea and 25°C with nitrate), as DA quotas were equal between PAR and +UVB light treatments, DA quotas were greater with UVB relative to PAR-only for the other two temperature-nitrogen combinations (20°C with nitrate and 25°C with urea). This contradicts the assumption of the DA production rate calculation, whereby cells must be growing in order to produce DA. Therefore, calculated production rates can be somewhat deceptive because of this growth rate effect. In the present study, *P. multiseriata* cells were able to continue photosynthesis and the uptake of nutrients. This excess energy and nitrogen not being used for growth may instead be used in other secondary metabolic processes, such as DA production. Some studies have found an inverse relationship between *Pseudo-nitzschia* DA production and growth rates. As cells become stressed for growth, DA production increases (Lelong et al., 2012; Trainer et al., 2012; Bates et al., 2018).

Our results suggest that although UVB treatments had near-zero growth rates, it is likely that the surviving cells were producing DA. In spite of this increased cell-specific toxicity, the amount of DA produced over time is what determines the

ultimate accumulation of toxic biomass in a bloom. Although cells in UVB-exposed 20°C treatments with nitrate and 25°C treatments with urea were more toxic relative to their PAR counterparts, the inhibition of growth due to UVB prevents rapid biomass accumulation. Therefore, a toxic bloom is not likely to manifest under these conditions.

Because we did not measure dissolved DA (i.e., the fraction released into the medium), it is important to consider that our estimates of DA production may be low. Godinho et al. (2018) found that in *P. multiseriis* batch cultures, 63–98% of total measured DA was released into culture medium during exponential growth, and 99% was released during stationary phase. They hypothesized that cell death and lysis beginning in late exponential phase, and increasing during stationary phase, was the cause of increased dissolved DA. In the present semi-continuous experiment, final sampling occurred during steady state exponential growth phase for PAR-only cultures; dissolved DA can account for as little as 10% of the total under these growth conditions (Sun et al., 2011). However, growth was arrested in UVB-exposed cultures, and it is likely that many cells died due to exposure to this stressor, as indicated by empty (yet intact) frustules observed under the microscope. It is therefore possible that our estimates including only particulate DA may represent a minimum estimate of total DA produced, especially in UVB treatments. Most published studies have focused on particulate DA, because this is the form usually assumed to be involved in trophic transfer and biomagnification of the toxin. Nonetheless, future studies should measure both fractions, as extracellular DA could be important to consider in UVB-induced cell mortality.

Interactions Between Irradiance and Nitrogen Source May Impact Cellular Energetics

Our study also showed that UVB reduces photosystem function, which may limit light harvesting capabilities. Light energy plays an important role in nitrogen uptake; when PAR irradiance is limiting, the uptake of urea, a reduced form of nitrogen, is less energetically expensive relative to nitrate (Legrand et al., 1998; Li J. et al., 2012). This interaction between nitrogen source and light, and its impacts on cellular energetics, has been demonstrated in *P. cuspidata* using PAR irradiance. In this study, nitrogen preference was a function of light availability; at saturating irradiances, growth rates were nearly identical between nitrate or ammonium-supported cultures, and slower on urea. In contrast, at a low photon flux density, urea-supported cultures grew fastest (Auro and Cochlan, 2013). A potential mechanism for these differences in nitrogen use efficiencies could be the reallocation of energy from nitrogen uptake to growth when supported by urea. From this, we surmise that light-dependent uptake of nitrate may have been reduced when photosystems are damaged by UVB, yet the uptake of urea may not have been as impacted. Because cellular energetic allocation is important for DA production, this may partially explain observed increases in DA quotas between nitrogen sources for some UVB-exposed treatments. However, uptake kinetic experiments with nitrogen

use efficiency calculations will need to be performed to validate these suggestions.

Previous studies examining the effects of nitrogen source on *P. multiseriis* DA production have demonstrated a large degree of variability between strains. For instance, one strain of *P. multiseriis* produced more DA per cell in urea-supported cultures relative to those supported by nitrate, while two separate strains both produced more DA per cell on nitrate compared to urea (Thessen et al., 2009; Martin-Jézéquel et al., 2015). This variability has also been described across the whole *Pseudo-nitzschia* genus, and several studies with other species and strains have observed increases in toxicity in urea-supported cultures of *Pseudo-nitzschia* (Howard et al., 2007; Thessen et al., 2009; Radan and Cochlan, 2018). Because of this variability within the diatom genus, elucidating the precise mechanisms for increased DA quotas with regard to nitrogen source is challenging and speculative.

Prior studies interrogating the question of nitrogen source preference used measurements of nitrogen uptake kinetics as a proxy for preference. These revealed that *P. fryxelliana* preferred ammonium over nitrate, with urea being the least preferred nitrogen substrate. This low preference for urea was also observed in *P. australis* and *P. delicatissima* (Cochlan et al., 2008; Loureiro et al., 2009; Auro and Cochlan, 2013). However, uptake preference does not indicate how the nitrogen is used within the cell. Future studies should not only include a broader representation of the genus, but also mechanistically investigate the role of different nitrogen sources in toxin biosynthesis, possibly using stable isotopic methods.

Multiplicative Interactions Between Multiple Drivers Provide New Insights Into *P. multiseriis* Toxicity and Bloom Dynamics

The highest observed yield of DA per cell in this experiment may be a consequence of the interactive effects between urea, UVB, and warming that synergistically enhanced cellular DA quotas. This interaction was multiplicative, meaning the resulting DA quotas from the combination of these three variables could not have been predicted by adding together the effects of each individual driver (Boyd et al., 2018). First, trends within 20°C treatments differed from those observed within the 25°C treatments. Therefore, interactive effects observed at 25°C could not have been predicted based on trends observed at 20°C. At 25°C, UVB did not alter DA quotas between nitrate with PAR-only versus nitrate +UVB cultures, as the two treatments had similar cellular DA quotas. PAR treatments also had DA quotas that were nearly 1.5x greater for cells grown on nitrate than those supported by urea. Based on these results, we would expect DA quotas for the 25°C +UVB urea-supported treatment to be similar to the 25°C PAR-only urea-supported treatment, as +UVB does not increase DA quotas for nitrate-supported cells at 25°C. However, measured DA quotas did not follow these predictions in the present experiment, as UVB exposure of 25°C urea treatments enhanced DA production more than any of the other treatments. This result was unexpected and shows

that the impact of UVB enhances DA production more than would have been expected due to interactions between light and nitrogen source.

This three-way interaction between warming, urea, and +UVB indicates that a future warmer, more stratified ocean with a greater influence of anthropogenic nutrients could enhance the toxicity of *P. multiseriis*. However, these results also point to a potential decrease in *P. multiseriis* bloom events, as increased UVB exposure may inhibit cellular division and growth that could offset enhanced toxicity. Despite increased toxicity per cell, harmful effects will not be so impactful if cellular abundances are low. Therefore, projected increases in cellular toxicity may be mitigated by UVB inhibition of bloom formation in a future ocean.

Alternatively, *P. multiseriis* may find refuge from harmful UVB wavelengths in the deep chlorophyll maximum (DCM). Field surveys have detected high abundances of *Pseudo-nitzschia* spp. in the DCM (McManus et al., 2008; Velo-Suárez et al., 2008; Durham and Stocker, 2012). It has been suggested that *Pseudo-nitzschia* spp. blooms may be induced by upwelling or mixing of these seed populations from the dim DCM to the well-lit surface ocean (Seegers et al., 2015). Our multiple stressor experimental results both support and build upon these ideas by demonstrating that *P. multiseriis* growth can be extremely sensitive to UVB, and thus may be strongly inhibited by extended exposures in the surface ocean. The thin layers of *Pseudo-nitzschia* spp. that reside in the DCM are protected from UVB rays, which only penetrate the first 10–12 m of the water column in clear coastal areas (Denman and Gargett, 1983; Tedetti and Sempéré, 2006). In more turbid coastal regions, these wavelengths are attenuated even more rapidly, with only ~10% of incident UVB radiation reaching a depth of 0.5 m (Llabrés et al., 2013). In these systems, the DCM may serve as a refugium for *P. multiseriis* in between bloom events. Mengelt and Prézelin (2005) suggested that *Pseudo-nitzschia* does best at mid-depths in the water column, where UVB is attenuated but UVA remains. If high concentrations of these cells are advected to the surface, exposure to UVB may be a stressor that inhibits growth and consequentially promotes DA production. The persistence of these cells and their subsequent impact on coastal ecosystems will then depend on surface conditions, including prolonged exposure to intense UVB rays, which may inhibit further growth and reduce the impact of the toxic bloom.

These results demonstrate that multiple driver experimental approaches are necessary for revealing the complex interactive effects between climate-relevant environmental variables, with consequences for toxic *Pseudo-nitzschia* blooms. *In situ* observational studies and long-term monitoring datasets are also a convenient system for studying multiple stressors, as correlations can be made between numerous environmental parameters, *Pseudo-nitzschia* abundance, and particulate DA (Fehling et al., 2006; Downes-Tettmar et al., 2013; Schnetzer et al., 2013; McKibben et al., 2017; Anjani et al., 2020). Laboratory-based multi-factorial experiments are advantageous in their ability to mechanistically elucidate potential interactive effects between multiple drivers, which

cannot be teased apart using observational *in situ* studies. Furthermore, while these experiments are limited in their capacity to incorporate additional variables and levels of treatment, they portray a more comprehensive view of ocean dynamics compared to single-driver experiments. The incorporation of additional drivers would yield even more information about *P. multiseriis* bloom dynamics, yet experiments using more than three drivers become exponentially larger in scale and are thus infeasible (Boyd et al., 2018). Therefore, based on previous single-driver literature and future projected ocean conditions, we chose three drivers that could be key controls on *P. multiseriis* growth and toxicity, but have not been examined together before. For instance, single-driver studies have demonstrated that trace metals (iron and copper), pCO₂/pH, and silicon or phosphorus limitation impact *Pseudo-nitzschia* toxicology, and thus should be incorporated into these multi-driver experimental designs (Bates et al., 1998; Maldonado et al., 2002; Sun et al., 2011). Future studies should combine these two approaches, using *in situ* observational studies to identify key drivers, and laboratory experiments to gain a mechanistic understanding of how these environmental parameters impact toxic blooms.

CONCLUSION

Our study is among the few to use more than two environmental drivers to test hypotheses regarding drivers of *Pseudo-nitzschia* toxicity and bloom formation (Tatters et al., 2018). These experiments suggest that certain combinations of environmental parameters can create conditions that synergistically or antagonistically enhance or reduce DA production and growth. The inclusion of multiple variables in experimental designs more accurately reflect the natural coastal environment where *Pseudo-nitzschia* spp. interacts with multiple environmental parameters simultaneously. Multiple stressor experiments therefore help us to gain a more holistic view of bloom dynamics *in situ*.

Because these three-way interactions cannot be predicted based on one variable alone, it is important to consider what might be missed by only including single variable experiments in models and forecasts. For instance, phosphorus and silicon limitation also seem to be a key driver of toxicity, yet we do not know how they interact in the context of multiple drivers (other than with ocean acidification; Sun et al., 2011; Tatters et al., 2012). These blooms threaten human health, the marine ecosystem, and economically important fisheries, thus accurately forecasting their occurrence is important to our ability to prepare rapid and effective responses to bloom events. However, our current HAB forecasting models are incomplete in their predictive powers, reducing our ability to anticipate toxic blooms. Although some predictive models include multiple variables, they typically do not include the interactive effects between them. Improvement of existing models may require more accurate representation of interactive effects between multiple environmental drivers on toxicity and growth of a diverse selection of toxic

Pseudo-nitzschia species, in order to better understand HAB dynamics in a rapidly changing ocean.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the corresponding authors upon request, without undue reservation.

AUTHOR CONTRIBUTIONS

F-XF and DH conceived of and designed the experiment with help from KK and KG. KK carried out the experiment, with help from XJ and HL for PAM photophysiological measurements. KK, XJ, and HL conducted final sampling. Subsequent sample processing was done by KK, JK, and NYang. The data analyses were performed by KK, with help from MD. DX and NYe provided cultures and domoic acid sample processing. KK wrote the manuscript with contributions from DH, F-XF, KG, XJ, DX, NYe, and NYang. All authors reviewed and gave their approval for the final manuscript.

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SUPPLEMENTARY MATERIAL

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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