

ARTICLE



Plastic responses lead to increased neurotoxin production in the diatom *Pseudo-nitzschia* under ocean warming and acidification

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Ocean warming (OW) and acidification (OA) are recognized as two major climatic conditions influencing phytoplankton growth and nutritional or toxin content. However, there is limited knowledge on the responses of harmful algal bloom species that produce toxins. Here, the study provides quantitative and mechanistic understanding of the acclimation and adaptation responses of the domoic acid (DA) producing diatom *Pseudo-nitzschia multiseries* to rising temperature and $p\text{CO}_2$ using both a one-year in situ bulk culture experiment, and an 800-day laboratory acclimation experiment. Ocean warming showed larger selective effects on growth and DA metabolism than ocean acidification. In a bulk culture experiment, increasing temperature $+4^\circ\text{C}$ above ambient seawater temperature significantly increased DA concentration by up to 11-fold. In laboratory when the long-term warming acclimated samples were assayed under low temperatures, changes in growth rates and DA concentrations indicated that *P. multiseries* did not adapt to elevated temperature, but could instead rapidly and reversibly acclimate to temperature shifts. However, the warming-acclimated lines showed evidence of adaptation to elevated temperatures in the transcriptome data. Here the core gene expression was not reversed when warming-acclimated lines were moved back to the low temperature environment, which suggested that *P. multiseries* cells might adapt to rising temperature over longer timescales. The distinct strategies of phenotypic plasticity to rising temperature and $p\text{CO}_2$ demonstrate a strong acclimation capacity for this bloom-forming toxic diatom in the future ocean.

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INTRODUCTION

One of the great public health challenges for marine ecosystems is determining how oceanic primary producers and their nutritional or toxin content will change in response to global change [1, 2]. Some species of the diatom genus *Pseudo-nitzschia* are well known to produce the neurotoxin domoic acid (DA). This toxin can harm wild and cultured marine animals, and human consumers through the food chain [3]. To date, 58 *Pseudo-nitzschia* species have been described, 26 of which are known to be toxigenic and global distribution patterns have been recorded, covering both coastal and open ocean regions [4–6]. However, no general uniform response of harmful algal bloom (HAB) events to environmental changes has yet been found at a global scale [7]. Evidence suggests that bloom dynamics and toxin concentration of *Pseudo-nitzschia* will be altered under future climate change conditions at regional and local scales [8–10].

While many investigations aim to study the effect of environmental factors on the eco-physiology of *Pseudo-nitzschia*, DA regulation has primarily been investigated using field observations

of discrete bloom events and in laboratory-based short-term culture experiments [5, 11–18]. For example, the well documented toxic *Pseudo-nitzschia* bloom which affected nearly the entire West Coast of North America during 2015–2016, was reported to be driven by the “warm blob” of ocean water and anomalous nutrient concentrations present throughout this period [18, 19]. Culture experiments further found that warming increases toxicity and abundance of the harmful bloom diatoms *P. australis* and *P. delicatissima* in California coastal waters [13]. In addition to ocean warming, Wingert and Cochlan reported that the increasingly acidic seawater caused by elevated $p\text{CO}_2$ influences cellular growth, photosynthetic performance, and domoic acid production of a strain of *P. australis* isolated from the California Current System [15]. Sun et al. found that the DA content of a toxic *P. multiseries* isolate from eastern Canada was regulated by both $p\text{CO}_2$ and phosphate availability. P-limited cultures had 30–50 times higher DA concentrations compared to P-replete cultures at ambient $p\text{CO}_2$, and increasing $p\text{CO}_2$ further stimulated DA concentration, by up to 4-fold, under both nutrient conditions [12].

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Tatters et al. confirmed that high $p\text{CO}_2$ and silicate limitation synergistically increased the toxicity of *P. fraudulenta* isolated from southern California [20]. However, others have also reported that elevated pH resulted in a 70-fold increase in cellular DA concentrations in Japanese and eastern Canadian strains of *P. multiseriis* [21, 22]. While cultures typically acclimate to experimental conditions after 3–5 consecutive transfers or 4–6 weeks of semicontinuous incubation, little is known about the fate of *Pseudo-nitzschia* diatoms under complex climate change scenarios, and especially about their potential for evolutionary adaptation to global change conditions.

The molecular basis of DA biosynthesis in *P. multiseriis* has been delineated by Brunson et al. [23]. Four candidate genes have been implicated in the DA biosynthetic pathway, annotated as DabA (terpene cyclase), DabB (hypothetical protein), DabC (α -ketoglutarate (α KG)-dependent dioxygenase), and DabD (CYP450) involved in terpenoid and redox biochemistry [23]. Additionally, the SLC6 amino acid transporter was previously shown to be involved in the depuration of DA [24]. This genetic knowledge offers the opportunity for a deeper understanding of the basis and diversity of *Pseudo-nitzschia* toxicity, the physiological functions of DA, and the environmental conditions that promote harmful algal blooms (HABs). However, the mechanisms that underpin acclimation or evolution of DA metabolism and genetic responses involved in the DA biosynthetic pathway under multiple climate-relevant conditions remain unclear. To address this issue, both short-term (more than 20 generations) and long-term (approximately 800 generations) responses of *P. multiseriis* to projected ocean warming were studied in a factorial design with ocean acidification, using both semi-natural bulk culture experiments and laboratory acclimation experiments. Changes in their reproductive fitness were evaluated using growth rate measurements and quantification of changes in DA concentrations, as well as other physiological traits including cell size, cellular quotas and ratios of particulate organic carbon (POC) and nitrogen (PON), cellular biogenic silica (BSi), and Si/C. Since the magnitude of growth and DA metabolic responses under ocean warming was much larger than the responses under ocean acidification, a reciprocal transfer or shift experiment was conducted to test for evolutionary adaptation in warming-selected cell lines by transferring them to the higher or lower temperature ranges, and comparing them with ambient temperature selected ones. To understand the molecular evolutionary mechanisms involved, transcriptome and western blot analyses were applied to identify genetic divergence and expression variations between the ancestral lineage and selected populations under different warming and acidification regimes. This study aimed to address the following questions: 1) What is the strategy that *P. multiseriis* employs to respond to rising temperature and increasing CO_2 emissions, individually and in combination? and 2) What are the molecular mechanisms that underpin these phenotypic changes?

MATERIALS AND METHODS

Algal isolation and culture

An experimental culture of *Pseudo-nitzschia multiseriis* was isolated from the coastal waters of Sungo Bay in the Yellow Sea, China (37°02'N, 122°33'E) (Fig. S1), at an ambient surface seawater temperature of 19.8 °C and ambient seawater pH of 8.23. Cells were isolated using a glass micro-pipette under an inverted Eclipse Ti-U microscope (Nikon, Tokyo, Japan). In the laboratory, monoclonal cultures were established by serial dilution and streaking the isolate onto agar plates infused with f/2 medium. Colonies were then picked under a microscope and transferred back into liquid axenic culture with modified f/2 medium containing 100 $\mu\text{mol L}^{-1}\text{N}$, 6 $\mu\text{mol L}^{-1}\text{P}$, and 100 $\mu\text{mol L}^{-1}\text{Si}$, made with 0.2 μm -filtered seawater collected from the same location that the cultures were sampled from. Cultures were kept at 20 ± 1 °C and 120 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ with a 12 h/12 h light–dark cycle (light on at 08:00 and off at 20:00) using cool white fluorescent bulbs. To study the effects of ocean warming and acidification on physiological performance and DA concentration of *P. multiseriis*, three sets of experiments were conducted: 1) bulk culture experiments in the field, 2) long-term acclimation experiments

and 3) shift experiments in the laboratory (see the details of laboratory experiments in Fig. S2).

Bulk culture experiments to study acclimation responses to ocean warming and acidification under semi-natural conditions

The mesocosm facility for Climate Change Impacts Study of Yellow Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences consists of 20 independently operated culture units made out of acrylic, each holding 100 L (Fig. S3). The site is located in the southeastern Sungo Bay in the Yellow Sea, China (37°02'N, 122°33'E), where *P. multiseriis* was isolated. Six treatments were replicated in triplicate to simulate ocean warming and acidification in accordance with the Intergovernmental Panel on Climate Change (IPCC) representative concentration pathway (RCP) 8.5 “business-as-usual” emissions scenario: 1) +4 °C at 400 $\mu\text{atm } p\text{CO}_2$, 2) +4 °C at 1000 $\mu\text{atm } p\text{CO}_2$, 3) +4 °C at 2000 $\mu\text{atm } p\text{CO}_2$, 4) ambient temperature at 400 $\mu\text{atm } p\text{CO}_2$, 5) ambient temperature at 1000 $\mu\text{atm } p\text{CO}_2$ and 6) ambient temperature at 2000 $\mu\text{atm } p\text{CO}_2$ (Eighteen culture units in total; Fig. S3B). Warming was achieved by an electronic heating element connected to a thermocouple (MC-6; Pengyu, Qingdao, China), which monitored and recorded the temperature variations (a constant 4 degrees above ambient temperature was maintained). The $p\text{CO}_2$ levels of 400 μatm , 1000 μatm , and 2000 μatm were established by bubbling the medium with air or air/ CO_2 premixed gas using a CO_2 Enrichlor (CE-100B; Wuhan Ruihua Instrument & 25 Equipment Ltd).

To compare the responses between different seasons, the effects of ocean warming and acidification on growth rates and domoic acid (DA) concentrations of *P. multiseriis* was investigated at four time points from 2018 to 2019. Experiment dates include, March 15 to April 15 (spring, ambient seawater surface temperature (ASST) 6.93 ± 1.13 °C and warming temperature (WT) 10.86 ± 1.28 °C); June 16 to July 16 (summer, ASST 19.22 ± 0.60 °C and WT 23.64 ± 0.78 °C); August 25 to September 25 (autumn, ASST 23.33 ± 0.40 °C and WT 27.79 ± 0.71 °C); January 29 to February 29 (winter, ASST 2.99 ± 0.72 °C and WT 7.21 ± 0.32 °C). The ancestral *P. multiseriis* lineage was inoculated into 18 independent 100 L culture units with CO_2 -modified natural seawater at a starting density of 2×10^4 cells mL^{-1} . The seawater was vacuum-filtered through GF/F filters (47 mm, Whatman), but without sterilization. After acclimation under ambient and elevated $p\text{CO}_2$ for 10 days (two batch cycles), three replicates per $p\text{CO}_2$ level were warmed gradually by 4 °C ($+1$ °C day^{-1}), while the other three were kept at the ambient natural temperature. The experiments lasted for 30 days, and algal cells were diluted into fresh medium every five days (six batch cycles) at 2×10^4 cells mL^{-1} to maintain stable carbonate chemistry. The seawater was supplemented with modified f/2 medium (100 $\mu\text{mol L}^{-1}\text{N}$, 6 $\mu\text{mol L}^{-1}\text{P}$, and 100 $\mu\text{mol L}^{-1}\text{Si}$) to prevent nutrient limitation during each transfer. The pH in each culture medium treatment was measured every day using a pH meter (Orion ROSS, Fisher Scientific Instruments) calibrated with National Bureau of Standards (NBS) buffers (pH variations $< \pm 0.05$). Temperature, salinity, and total alkalinity (TA) were also measured simultaneously throughout the experiment. Other carbonate system parameters were calculated with CO2SYS using pH and TA (Fig. S4) [25]. In the bulk culture experiments, temperature and $p\text{CO}_2$ were the two principal abiotic factors; other variables such as nutrient availability, light, and day length were consistent between different temperature and $p\text{CO}_2$ combinations.

Cell density, particulate organic carbon (POC), particulate DA (pDA) and dissolved DA (dDA) were measured on the 4th day of each batch cycle during exponential growth phase. One 2 mL sample was collected at the same time (11:00 AM to 12:00 PM) across different seasons. Samples were preserved in Lugol's solution to estimate growth using a 1 mL Sedgewick-Rafter counting chamber using an optical microscope (Nikon, Tokyo, Japan). At the beginning of each batch cycle, the initial concentration was measured using a particle counter (Z2, Beckman Instruments). Exponential growth rate (day^{-1}) was calculated as follows:

$$\text{Growth rate} = (\ln N_1 - \ln N_0) / (t_1 - t_0) \quad (1)$$

where N_1 and N_0 represent cell density at times t_1 and t_0 , respectively. At the same sampling time, 100 mL cultures filtered onto pre-combusted (500 °C for 6 h) Whatman GF/F filters and used for particulate carbon content (POC) determination. A further 100 mL was filtered onto un-combusted Whatman GF/F filters for pDA analysis, and 50 mL filtrate samples were collected for dDA analysis. The DA concentrations presented in the results are the sum of the pDA and dDA, which represents approximately 90% and 10% of total DA, respectively. The averages for

growth rate, particulate organic carbon (POC), and total DA concentration, over six batch cycles, were calculated and presented as acclimation responses to ocean warming and acidification.

Laboratory acclimation experiment to study long-term responses to ocean warming and acidification

Long-term acclimation experiments. To study long-term responses of *P. multiseriis* to ocean warming and acidification, algal cultures were maintained under semi-continuous culture conditions, under three temperatures of 20 °C, 25 °C, and 30 °C, combined with three $p\text{CO}_2$ levels (400, 1,000, and 2,000 μatm) at each temperature (Fig. S2). Triplicate populations for each regime ($9 \times 3 = 27$ cultures) were maintained in 1000 mL Schott Duran flasks containing 800 mL sterile seawater enriched with modified f/2 medium (100 $\mu\text{mol L}^{-1}$ N, 6 $\mu\text{mol L}^{-1}$ P, and 100 $\mu\text{mol L}^{-1}$ Si). The control culture conditions were maintained at 20 °C at 400 μatm $p\text{CO}_2$, consistent with the in situ conditions at the time of sampling. Clonal ancestral lineages in exponential growth phase were split into three independent cultures and semi-continuously cultured under different CO_2 conditions (400, 1000, and 2000 μatm) at 20 °C, for 30 days (more than 20 generations). For each $p\text{CO}_2$ condition, cultures were then split into three independent replicates under one of three different temperature conditions (20 °C, 25 °C, and 30 °C), resulting in nine experimental units in total. Cultures maintained at 25 °C and 30 °C were gradually elevated by 1 °C per day. The acclimation experiment lasted for 800 days (160 batch cycles) corresponding to 251 ± 12.1 to 750 ± 3.5 generations, depending on different seasonal growth rates under different experimental conditions (Table S1).

During the acclimation phase, 2×10^4 cells were transferred into fresh medium every five days to initiate the next batch cycle. Culture medium was bubbled either with air ($\sim 400 \mu\text{atm}$) or air/ CO_2 mixed gas in a programmed CO_2 chamber (HP1000G-D, China) to achieve the desired $p\text{CO}_2$ levels and temperature treatments. Samples were shaken gently three times daily to avoid settling and biofilm formation. The seawater carbonate system parameters were maintained at stable levels by adjusting the aeration rate, and by maintaining cell concentrations within the range of $2.0\text{--}30 \times 10^4 \text{ cells mL}^{-1}$ (pH variations $< \pm 0.05$). The pH in each treatment was measured before and after each transfer using a pH meter (Orion ROSS, Fisher Scientific Instruments) that was calibrated with National Bureau of Standards (NBS) buffers. Temperature, salinity, and total alkalinity (TA) were also measured periodically (but not at every transfer due to logistical limitations) throughout the experiment. Other carbonate system parameters were calculated with CO2SYS using the average pH and TA (Table S2).

On the 4th day of each batch cycle, 2 mL samples were collected to monitor cell concentrations using a 1 mL Sedgewick-Rafter counting chamber under an optical microscope (Nikon, Tokyo, Japan). At the same sampling time, 50 mL samples were filtered onto Whatman GF/F filters to collect algal cells. The filters and filtrates were stored at $-20 \text{ }^\circ\text{C}$ and used for particulate DA (pDA) and dissolved DA (dDA) analysis. 20 mL cultures were filtered onto pre-combusted (450 °C, 5 h) GF/F glass filters for POC and PON determination in the same time interval. The average values of cell concentrations and total DA concentrations across 20 days (4 batch cycles) are presented as the physiological responses to different acclimation treatments. To examine the potential bacterial effects on DA content, bacterial abundance was monitored regularly via staining with SYBR gold and enumerating with flow cytometry (FACS Calibur, BD, USA) throughout the acclimation period. The proportion of bacteria remained stable at low quantities and contributed $< 1\%$ of total biomass throughout.

To study and compare other phenotypic changes between the short-term acclimation (30 days) and long-term acclimation (800 days) experiments, particulate organic carbon (POC) and nitrogen (PON) content, cellular biogenic silica (BSi), and cell size were measured. For POC and PON, 20 mL samples were filtered onto pre-combusted (500 °C, 6 h) GF/F glass filters and placed onto pre-combusted aluminum foil and analysed using a Vario EL III automatic elemental analyzer (Elementar Analysensysteme). For BSi, samples of 20 mL were collected onto 0.6 μm polycarbonate filters and analyzed according to the spectrophotometric method of Brzezinski and Nelson [26]. Algal cell length was monitored using a Eclipse 80i microscope (Nikon, Tokyo, Japan) with NISelements BR software (Nikon Co., Japan).

Shift experiments after long-term acclimation. Changes in physiological responses following long-term acclimation to temperature were measured across a gradient of seven temperatures (5–35 °C), including growth rates

and DA concentrations. The low and high temperatures of 5 °C and 35 °C were chosen to coincide with the lower and upper thermal tolerance limits for *P. multiseriis*. Pilot experiments revealed that the culture almost stopped growing above 30 °C (Table S3), while the chosen experimental temperature was well within the environmental range for this widely distributed species. As in Schlüter et al. [27], due to logistical restrictions and a prohibitively large number of replicates temperature switch experiments were only performed for samples selected under low (20 °C), and high (30 °C) temperature at respective $p\text{CO}_2$ values of ambient (400 μatm), moderate (1000 μatm), and high (2000 μatm) levels (Fig. S2). After having been grown under ambient and elevated temperature and $p\text{CO}_2$ regimes for 800 days, these lineages were transferred into 800 mL fresh media across a temperature gradient of 5 °C, 10 °C, 15 °C, 20 °C, 25 °C, 30 °C, and 35 °C at their respective selection $p\text{CO}_2$. Ancestral populations were also challenged under the same conditions to distinguish plastic and evolved responses. After acclimation for 35 days (seven batch cycles), growth rates and DA concentrations were measured in the last (7th) batch cycle when no significant changes were found with longer culture periods. The pH, temperature, salinity, and total alkalinity (TA) were also measured periodically (but not at every transfer due to logistical restrictions) throughout the experiment, and carbonate system parameters were calculated with CO2SYS (Table S4).

Thermal tolerance curves for growth rate were fitted using the four-parameter Sharpe–Schoolfield model [28], which assumes that there is a single rate-limiting enzymatic reaction that is reversibly inhibited by temperature:

$$\ln(b(T)) = E_a \left(\frac{1}{kT_c} - \frac{1}{kT} \right) + \ln(b(T_c)) - \ln \left(1 + e^{\frac{E_h}{T_h} \left(\frac{1}{T} - \frac{1}{T_h} \right)} \right) \quad (2)$$

Where $b(T)$ represents the per capita metabolic rate, E_a represents an activation energy (in eV) during the metabolic process, k is Boltzmann's constant ($8.62 \times 10^{-5} \text{ eV K}^{-1}$), T represents Kelvin temperature (K), T_h is the temperature when half of the enzyme is inactivated, and E_h indicates the temperature above T_h -induced inactivation of enzyme kinetics. $b(T_c)$ represents the rate of metabolism normalized to an arbitrary reference temperature, and T_{opt} is the maximum growth rate at an optimum temperature, which was calculated from the following equation.

$$T_{\text{opt}} = \frac{E_h T_h}{E_h + k T_h \ln \left(\frac{E_h}{E_a} - 1 \right)} \quad (3)$$

The parameters $b(T_c)$, E_a , E_h , T_h , and T_{opt} represent traits that together characterize the metabolic thermal response. Parameters of this model were considered to be traits that were expected to vary between different acclimation regimes [28].

Molecular responses involved in DA metabolic pathways of *P. multiseriis*

To explore the potential for genetic adaptation using transcriptomes from the different warming and acidification regimes and between the acclimated and ancestral lineages, transcriptome resequencing was performed at the beginning (t_0) and after 800 days (t_{800}) of acclimation in the following five groups in triplicate: 1) ancestor (algal isolate without any acclimation at t_0), 2) low temperature 20 °C and low $p\text{CO}_2$ 400 μatm at t_{800} (20/400), 3) low temperature 20 °C and high $p\text{CO}_2$ 1000 μatm at t_{800} (20/1000), 4) high temperature 30 °C and low $p\text{CO}_2$ 400 μatm at t_{800} (30/400), and 5) high temperature 30 °C and high $p\text{CO}_2$ 1000 μatm at t_{800} (30/1000). During exponential growth phase, 250 mL of algal culture was sampled from the ancestral and long-term acclimation lineages, 30 h after inoculation during a batch cycle (at 14:00 pm on the second day). A pilot study showed that *P. multiseriis* produced a peak in gene expression at this time point. Algal cells were centrifuged at 10,000 g , 4 °C for 5 min and immediately frozen in liquid nitrogen and stored at $-80 \text{ }^\circ\text{C}$ for RNA extraction, transcriptional sequencing, and gene RT-qPCR. The transcriptome data have been deposited in the Sequence Read Archive (SRA) at the National Center for Biotechnology Information (NCBI) and are accessible through accession number PRJNA686847.

Gene sequences involved in the DA metabolic pathways including DabA (terpene cyclase), DabB (hypothetical protein), DabC (α -ketoglutarate (αKG)-dependent dioxygenase), DabD (CYP450), and SLC6 amino acid transporter were identified in the transcriptome data. The housekeeping gene Histone H3 was selected as a reference gene and used for RT-qPCR of *P. multiseriis* [24]. The primers designed for reference and target genes are listed in Table S5. To determine protein expression involved in DA

metabolic pathway, total protein from ancestral *P. multiseriis* cells and the long-term acclimation lineages (same samples as used in transcriptome analysis) was extracted using a Protein Extraction Kit (KeyGEN, Nanjing, China). Proteins were separated by SDS-PAGE on a 10% gel and electrotransferred to a polyvinylidene fluoride membrane for western blot analysis. Actin served as an internal control. To further test for any adaptation due to long-term warming acclimation, the relative expression of the core genes *Daba*, *DabB*, *DabC*, *DabD*, and *SLC6* was measured in the shift experiment at their respective $p\text{CO}_2$, and differences in expression were compared between treatments: of 20 °C/400 $p\text{CO}_2$, 20 °C/1000 $p\text{CO}_2$, 30 °C/400 $p\text{CO}_2$, 30 °C/1000 $p\text{CO}_2$, 20 °C/400 $p\text{CO}_2$ to 30 °C/400 $p\text{CO}_2$, 20 °C/1000 $p\text{CO}_2$ to 30 °C/1000 $p\text{CO}_2$, 30 °C/400 $p\text{CO}_2$ to 20 °C/400 $p\text{CO}_2$, and 30 °C/1000 $p\text{CO}_2$ to 20 °C/1000 $p\text{CO}_2$.

DA determination

Whatman GF/F glass fiber filters (0.45 μm , 25 mm diameter, Whatman, Germany) were used to collect cells and filtrate from the cultures to determine particulate DA (pDA) and dissolved DA (dDA). Particulate DA (pDA) was extracted using 10% methanol/water (8 mL methanol:water, 1:9, v/v) [29]. Each filter was placed in a 10 mL plastic conical centrifuge tubes and stored at $-20\text{ }^\circ\text{C}$ until analysis. 8 mL of 10% methanol/water was added to the 10 mL plastic conical centrifuge tubes. The filtrates were homogenized by vortexing at room temperature for 10 min. Cells were disrupted to release intracellular DA into solution by sonication using a 650 W sonic disrupter at 30% amplitude, for 2 min on an ice slurry. The extraction was centrifuged at 4500 r min^{-1} for 5 min and then filtered with a 0.22 μm MCM syringe filter into an LC vial for liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. Dissolved DA (dDA) was measured in the filtrate without cells. 50 mL filtered cultures were placed into 50 mL plastic conical centrifuge tubes and acidified with 20% aqueous formic acid to yield 0.2% formic acid in the filtered cultures. After vortex mixing, the filtered cultures were transferred into the pre-activated solid-phase extraction (Bond Elut C18 LRC10 mL column, 200 mg, 40 μm ; Agilent) for desalination and extraction. Next, the 50 mL plastic conical centrifuge tubes were washed with 10 mL 0.2% aqueous formic acid, and this was added to the solid-phase extraction and eluted with 1.5 mL 50% methanol/water into a 2 mL microcentrifuge tube. The eluate was filtered through a 0.22 μm MCM syringe filter into an LC vial for LC-MS/MS analysis.

LC-MS/MS analysis was performed using an U3000 HPLC system (Thermo Scientific, USA) linked to a Thermo TSQ Endura mass spectrometer equipped with an ESI source operated in positive ionization mode. The chromatographic system was 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 $\mu\text{g mL}^{-1}$) 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 (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 $[M + H]^+ m/z$ 312.0, which was selected as precursor ion. The SRM transitions from the protonated DA ion were m/z 312.0 > 266.0 ($[M - \text{HCOOH} + H]^+$) at collision energy (CE) = 15 V used for quantitative analysis, and two confirmatory transitions of m/z 312.0 > 248.0 ($[M - \text{HCOOH} - \text{H}_2\text{O} + H]^+$) at CE = 16 V and m/z 312.0 > 161.0 ($[M - \text{HCOOH} - \text{C}_2\text{H}_3\text{O}_2\text{N} - \text{H}_2\text{O} - \text{CH}_2 + H]^+$) at CE = 22 V, all with a RF lens of 142 V, a dwell time of 250 msec 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. The sample of 10 μL was injected into a LC Kinetex C18 column (2.1 \times 100 mm, 2.6 μm , Phenomenex, USA). The system was operated under the following gradient elution program: solution A (2 mmol L^{-1} ammonium formate in H_2O) and solution B (100% MeOH) at a flow rate of 0.3 mL min^{-1} as follows: 0–1 min, 20% B, 1–3 min, 20–90% B, 3.0–3.1 min, 90–20% B, 3.1–8.0 min, 20% B. The column and the autosampler were maintained at 35 °C and 15 °C, respectively. The retention time of DA was around 2.86 min. The flow was diverted to waste by a six-way valve for the 0–2.2 min and 3.5–8.0 min of each run to prevent early eluting matrix material from blocking the heated capillary region of the MS system and contaminating the ion source. DA quantitation was obtained using a calibration curve of DA standard reference material. Because of the variation of cell size during the culture period, the total DA concentration was normalized to algal particulate organic carbon (POC) and expressed as mg DA gC^{-1} .

Statistical analysis

All statistics were conducted in R (version 3.5.1; The R Foundation for Statistical Computing, Vienna, Austria) and model selection was based on Akaike Information Criterion (AIC) using the “MuMIn” package. The effects of temperature, $p\text{CO}_2$, and the combined effects of increasing temperature and $p\text{CO}_2$ on growth rate and DA concentration for the bulk culture experiment were analyzed with a mixed effects model using the lmer function within the package lme4 and lmerTest. When modelling the individual effect of temperature or $p\text{CO}_2$ alone, temperature or $p\text{CO}_2$ was used as a fixed factor, and when modelling the combined effects of temperature and $p\text{CO}_2$, both temperature and $p\text{CO}_2$ were used as fixed factors. Using the lm function within R, the interactions between temperature, $p\text{CO}_2$, and season were included in a linear model (the additive effects between temperature, $p\text{CO}_2$, and season were dropped from the model based on AIC). Growth rate or DA concentration were used as random factors in both models.

In the laboratory acclimation experiment, the lm function was used to test the differences in growth rates or DA contents over acclimation times of 160 batch cycles for *P. multiseriis* acclimated under different temperatures and $p\text{CO}_2$. The effect of acclimation time, temperature, $p\text{CO}_2$, and the interaction between time, temperature, $p\text{CO}_2$ were included in the model and used as fixed factors. The effect of measurement type, either growth rate or DA content or other phenotypic parameters, were used as random factors. Additionally, the individual effects of acclimation time, temperature, or $p\text{CO}_2$, or the combined effects between two of them were analyzed using a mixed effects model with the lmer function.

RESULTS

Acclimation responses of toxic *P. multiseriis* to ocean warming and acidification under semi-natural conditions

Controlled laboratory studies have investigated the effects of warming, elevated CO_2 , or nutrient limitation on growth and toxin production of *Pseudo-nitzschia* species [12, 20, 30]. However, there is no consensus regarding whether any one factor or combination of factors leads to predictable DA concentration in a realistic ecological context [4, 31]. These results reveal that changes in specific growth rate (SGR) and DA concentration are driven by temperature and $p\text{CO}_2$ across all four seasons (Fig. 1, Table S6). *P. multiseriis* showed maximal SGR in the summer when ambient seawater temperature was $19.2 \pm 0.1\text{ }^\circ\text{C}$. Warming treatments (OW) decreased SGR by 61% and 54% when ambient seawater temperatures were higher than $19.2\text{ }^\circ\text{C}$ in summer and autumn, respectively (Fig. 1A; $F_{1, 104} = 1341.9$, $p < 0.001$ for summer, and $F_{1, 104} = 271.9$, $p < 0.001$ for autumn). However, warming increased SGR in spring and winter when ambient seawater temperature was less than $6.9\text{ }^\circ\text{C}$ (Fig. S5; $F_{1, 106} = 75.6$, $p < 0.001$ for spring and $F_{1, 106} = 409.1$, $p < 0.001$ for winter). Although elevated $p\text{CO}_2$ showed no significant effects on SGR in spring, autumn and winter, it increased SGR by 17% in summer when ambient seawater was around $19\text{ }^\circ\text{C}$. The DA concentration increased more than four orders of magnitude in the high temperature seasons of summer and autumn (Fig. 1B) compared to the low temperature seasons of spring and winter (Fig. S5B). OW consistently increased DA concentrations by 30.5%, 1045.9%, 2339.2%, and 12.5% in spring, summer, autumn, and winter, respectively. While OA showed no significant effect on DA concentration in spring and winter, DA levels increased by 52.6% and 46.6% respectively in summer and autumn. Moreover, OW and OA synergistically increased DA concentration in the high temperature seasons of summer and autumn at the maximal level occurred in autumn at $26.8\text{ }^\circ\text{C}$ and 2000 $\mu\text{atm } p\text{CO}_2$ (Fig. 1B; $F_{15, 416} = 1140.1$, $p < 0.001$).

Long term responses of toxic *P. multiseriis* to ocean warming and acidification in laboratory acclimation experiments

Consistent with the bulk culture experiment, warming treatments (MT and HT) significantly reduced SGR at all three $p\text{CO}_2$ levels across the acclimation period of 800 days (Fig. 2A; Table S7, $F_{1, 356} = 1204.3$, $p < 0.001$). Acidification treatments (HC and MC) generally increased SGR at low and medium temperature conditions (LT and MT, Fig. 2A; Table S7, $F_{1, 356} = 21.1$,

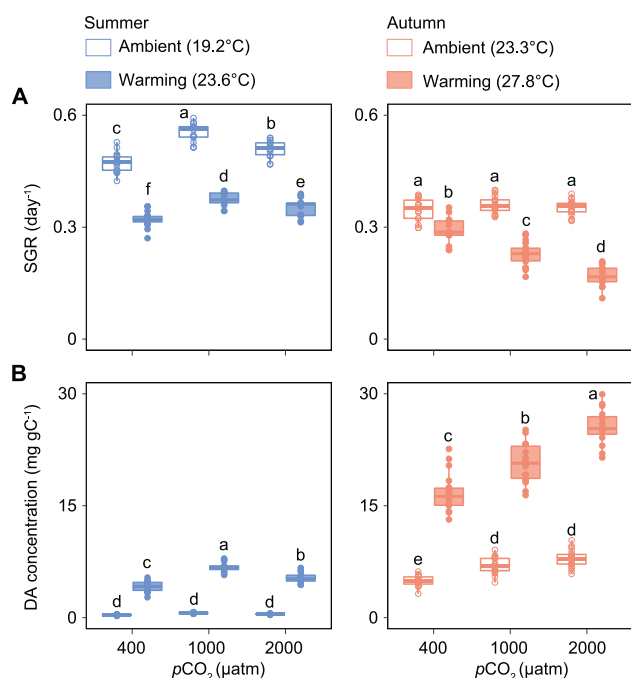


Fig. 1 Growth rates and DA concentrations of *P. multiseriis* under six different temperature and $p\text{CO}_2$ combinations in bulk culture experiments during summer and autumn. Ambient seawater temperatures were 19.2 °C and 23.3 °C, respectively. In each season, treatments were a matrix of three CO_2 levels (400, 1000 and 2000 μatm) and two temperatures, ambient and warming (ambient +4 °C). Boxplot symbols indicate ambient (open symbols) and warmer (filled symbols). **A** Specific growth rates (SGR, day^{-1}). **B** DA concentrations expressed as mg DA gC^{-1} . Boxplots show the bold line indicating the median, and the tops and bottoms of the boxes corresponding to the 25th and 75th percentiles, and whiskers extending to highest and lowest values that are within 1 interquartile range (IQR) of lower/upper quartile. $n = 3$ for each combination of $p\text{CO}_2$ and temperature. Boxplots marked with different letters are significantly different ($p < 0.05$).

$p < 0.001$), but showed no significant effect on SGR at high temperature (HT) at the end of acclimation. In contrast to SGR, warming treatments resulted in increased DA concentration in all three CO_2 levels (Fig. 2B; Table S7, $F_{1, 356} = 1171.8$, $p < 0.001$); elevated $p\text{CO}_2$ interacted with warming synergistically and up-regulated DA concentration significantly, so the maximal value of DA occurred in the HTHC combination condition (Table S7, $F_{15, 416} = 325.2$, $p < 0.001$).

Next, changes in cell size and cell quotas under warming selection in combination with ocean acidification were analysed and compared response differences between short-term (30 days) and long-term (800 days) acclimation treatments (Fig. 3). Warming selection resulted in significant reduction of cell length after short-term acclimation (30 days) for all cells selected under ambient or moderate or high $p\text{CO}_2$ (Fig. 3A; $F_{1, 23} = 17.0$, $p < 0.001$). Compared with the short-term acclimation, *P. multiseriis* underwent progressive cell size reduction by 11.4% in length after long-term acclimation (Fig. 3A; $F_{1, 48} = 103.5$, $p < 0.001$). Although temperature and $p\text{CO}_2$ showed significant effects on cell length after short-term acclimation ($F_{3, 23} = 103.5$, $p = 0.003$), there were no significant differences among treatments after long-term acclimation ($F_{3, 23} = 0.6$, $p = 0.66$).

As an immediate physiological response to ocean acidification, POC increased linearly with $p\text{CO}_2$ under all temperatures (Fig. 3B; $F_{1, 23} = 61.6$, $p < 0.001$). However, short-term warming acclimation at all $p\text{CO}_2$ levels produced a pronounced decline in POC content (Fig. 3B; $F_{1, 23} = 77.9$, $p < 0.001$). This response was smaller after long-term acclimation. Similar to POC, PON content increased by

7.91% and 11.96% under MC and HC at low temperature ($p < 0.05$), but elevated $p\text{CO}_2$ did not change PON significantly at MT and LT ($p > 0.05$). After long-term acclimation, there were no significant differences in PON among treatments (Fig. 3C; $F_{3, 23} = 2.7$, $p = 0.07$). As a result of the simultaneous variation of POC and PON, the ratio of POC/PON did not change significantly with elevated $p\text{CO}_2$ at low temperature ($p > 0.05$), but increased linearly with $p\text{CO}_2$ under MT and LT ($p < 0.05$) (Fig. 3D). Under long-term acclimation, the ratio showed no significant variation among treatments, except for that between MC and HC at moderate temperature (25 °C).

Like other diatoms, *P. multiseriis* requires silicic acid to produce its silica cell wall. In contrast to POC, cellular biogenic silica (BSi) decreased linearly with elevated $p\text{CO}_2$ under both short-term (Fig. 3E; $F_{1, 25} = 26.7$, $p < 0.001$) and long-term acclimation (Fig. 3E; $F_{1, 23} = 33.2$, $p < 0.001$). Warming treatments did not influence BSi contents regardless of $p\text{CO}_2$ ($p > 0.05$). Due to increasing POC quotas and decreasing BSi quotas, the ratio of Si/C decreased linearly with the increasing $p\text{CO}_2$ under both short-term (Fig. 3F; $F_{1, 23} = 63.6$, $p < 0.001$) and long-term acclimation (Fig. 3F; $F_{1, 25} = 58.0$, $p < 0.001$).

Rapid reversible changes in neurotoxin concentrations of long-term acclimated *P. multiseriis* in shift experiments

To test whether *P. multiseriis* had the capacity to adapt to elevated temperatures after 800 generations, growth rates and DA concentrations were measured across a gradient of seven temperatures (5–35 °C) and responses were compared between different acclimation regimes. Due to a prohibitively large number of replicates, temperature transition experiments were performed for samples selected under low (20 °C), and high (30 °C) temperature at respective $p\text{CO}_2$ values of ambient (400 μatm), moderate (1000 μatm), and high (2000 μatm) levels (Fig. 4). A typical “increase-peak-decline” pattern of specific growth rate (SGR) was found in thermal reaction norms of all selected cell lines (Fig. 4A). Under ambient $p\text{CO}_2$ (400 μatm) conditions and high temperature ranges (25 °C, 30 °C, and 35 °C), growth rates were higher for the high temperature selected lines (30 °C) than the ancestral and low temperature selected lines (20 °C). However, high temperature selected lines had similar growth rates to ancestral or low temperature selected lines when assayed at low temperature ranges (5 °C, 10 °C, 15 °C, and 20 °C). While warming acclimation (30 °C) resulted in significantly higher values of T_h (the upper temperature at which growth rates are 50% inactivated) and T_{opt} (the thermal optimum) under ambient $p\text{CO}_2$ (400 μatm), T_h of the long-term warming acclimation lines varied under high $p\text{CO}_2$, and the ancestral lines showed the highest T_h level (Table S8). This indicates that although ocean warming had a larger effect on algal fitness than ocean acidification, the selective effect of warming was changed by elevated $p\text{CO}_2$ [32]. This is supported by the significant interaction between selective ocean warming and acidification (mixed-effects model, interaction $p\text{CO}_2 \times \text{Temperature}$, $p < 0.001$).

After thermal acclimation for 800 days, long-term 30 °C-selected *P. multiseriis* cells showed higher concentrations of DA than 20 °C-selected cells (Fig. 4B). During the shift experiments, the long-term warming acclimated *P. multiseriis* cells demonstrated rapid acclimation rather than adaptation to temperature from 5 °C to 30 °C (Fig. 4B). When the warming selected lines (30 °C) were transferred to the low temperature range from 5 to 25 °C, their DA concentration decreased linearly with significantly lower levels than at a selection temperature of 30 °C. Although the long-term 30 °C-selected cultures showed rapid acclimation, they still showed higher concentrations of DA than low temperature selected (20 °C) and ancestral lines when assayed under the temperature range from 10 to 25 °C. Therefore, it is unknown whether adaptation might have occurred later with longer acclimation time. In contrast, long-term moderate and high $p\text{CO}_2$ acclimation did not change the DA trends across the assay

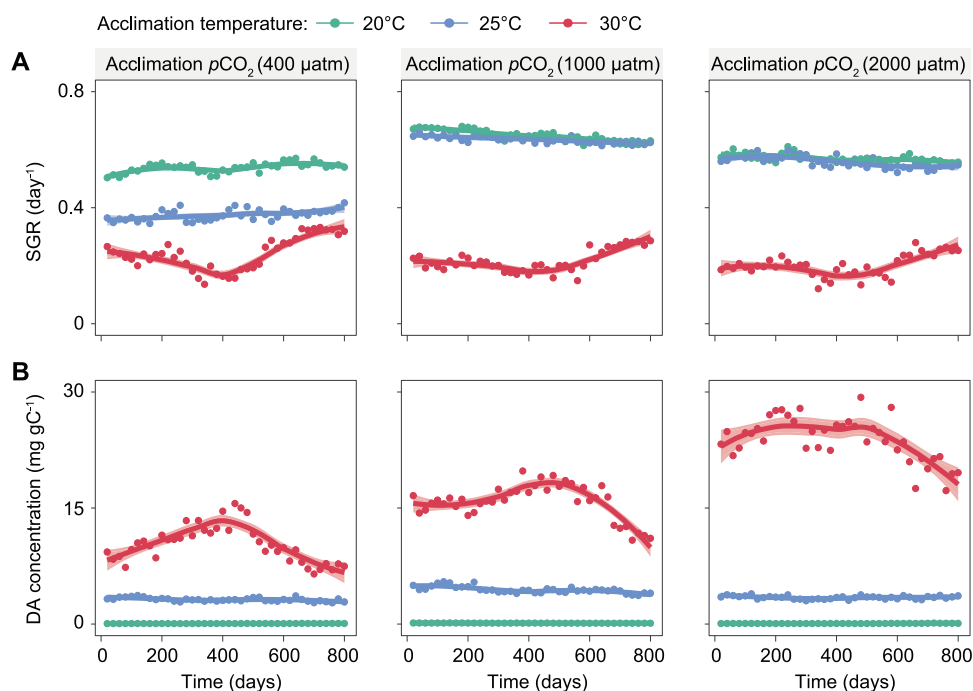


Fig. 2 Growth rates and DA concentrations of *P. multiseriis* laboratory cultures over 800 days of acclimation to nine different temperature and $p\text{CO}_2$ combinations. Treatments were a matrix of three three CO_2 levels (400, 1000 and 2000 μatm) and three temperatures (20 °C, 25 °C, 30 °C) **A** Specific growth rates (SGR, day^{-1}). **B** DA concentrations expressed as mg DA gC^{-1} . Fitted lines are based on an autoregressive moving average model that incorporates significant autocorrelation terms, and shaded areas depict 95% prediction intervals.

temperature ranges, although they significantly increased DA levels compared with ambient $p\text{CO}_2$ selected cultures ($F_{1, 153} = 47.4$, $p < 0.001$). Note that the DA concentration was log-normalized DA concentration (LogDA) because it showed relatively low values and was close to the detection limit in the low temperature range from 5 to 15 °C.

Molecular mechanisms underpinning increasing DA concentration of *P. multiseriis* under climate change

Significant differences were found in the transcriptomes under different acclimation regimes, yielding a total of 8,102 differentially expressed genes (DEGs) (Fig. S6A) including eight main clusters (Fig. S6B). By comparing ancestral or 20 °C/400 $p\text{CO}_2$ with 30 °C/400 $p\text{CO}_2$ or 30 °C /1000 $p\text{CO}_2$, it was found that most genes involved in carbon metabolism such as the CO_2 concentration mechanism (CCM), calvin cycle, glycolysis and tricarboxylic acid (TCA) cycle were significantly downregulated (Fig. S7). Genes involved in nutrient metabolism such as NiT, NR, NiR, GS, PHT, and SiT were also significantly downregulated. In contrast, isocitrate dehydrogenase (ICDHm) and glutamine amidotransferase (GOGAT) were up-regulated under 30/400, likely to produce Glu as one of the substrates for DA biosynthesis. Based on the gene expression profile (Fig. 5A) and western blot analysis (Fig. 5B), all of the five core genes *DabA*, *DabB*, *DabC*, *DabD*, and *SLC6* in the DA metabolic pathway were significantly up-regulated under high temperature and elevated $p\text{CO}_2$ (Fig. 5C).

To test for any adaptive changes arising at the transcriptome level due to long-term warming acclimation, the long-term selected cultures were transferred from high to low or low to high temperature at their respective $p\text{CO}_2$, and the relative expression levels of core genes involved in the DA metabolic pathway were determined. As expected, all core genes were significantly up-regulated when cultures acclimated in low temperature treatments were transferred to high temperature at respective $p\text{CO}_2$ (20 °C/400 $p\text{CO}_2$ to 30 °C/400 $p\text{CO}_2$ and 20 °C/1000 $p\text{CO}_2$ to 30 °C/1000 $p\text{CO}_2$). Transfers between low temperature lines showed higher levels than warming-selected and high temperature grown lines (30 °C/400

$p\text{CO}_2$ and 30 °C/1000 $p\text{CO}_2$) and warming-selected transferred to low temperature switch ones (30 °C/400 $p\text{CO}_2$ to 20 °C/400 $p\text{CO}_2$ and 30 °C/1000 $p\text{CO}_2$ to 20 °C/1000 $p\text{CO}_2$) ($p < 0.05$) (Fig. 6). However, when the warming-selected lines were transferred to low temperature conditions at respective $p\text{CO}_2$ (30 °C/400 $p\text{CO}_2$ to 20 °C/400 $p\text{CO}_2$ and 30 °C/1000 $p\text{CO}_2$ to 20 °C/1000 $p\text{CO}_2$), the relative expression of *DabA*, *DabB*, *DabC*, *DabD*, and *SLC6* were 34.8, 12.3, 75.2, 10.9, and 8.2 times higher, respectively, than those of low temperature-selected and low temperature grown ones (20 °C/400 $p\text{CO}_2$ and 20 °C/1000 $p\text{CO}_2$), but showed similar expression levels to the warming-selected and high temperature grown ones (30 °C/400 $p\text{CO}_2$ and 30 °C/1000 $p\text{CO}_2$). Thus, the warming-selected lines were unable to down-regulate the core gene expression when moved back to the low temperature level, which was suggestive of adaptation at the transcriptome level.

DISCUSSION

Biomass and DA productivity are among the most important traits indicating the ecological roles of toxigenic microorganisms, yet it remains difficult to predict how these traits may change under future global climate change [5, 7, 23]. The current understanding is based on simplified, short-term laboratory experiments [12, 33, 34], that may underestimate or overestimate the evolutionary potential of marine phytoplankton to adapt to global change [28, 35–37]. This work builds on this knowledge based on one-year semi-natural field experiments and 800-day long-term acclimation laboratory experiments using the model toxin producing diatom *P. multiseriis*. Long-term acclimation experiments showed that *P. multiseriis* acclimate rapidly to elevated temperatures and $p\text{CO}_2$ resulting in changes in algal growth and increased DA concentration. Warming by 4 °C or 5 °C had greater selective effects on physiological responses than acidification under 1000 μatm $p\text{CO}_2$, regardless of field or laboratory experiments. While long-term warming improved algal growth rate and DA concentration under high temperature conditions, this trend was reversed and both growth rates and DA declined when

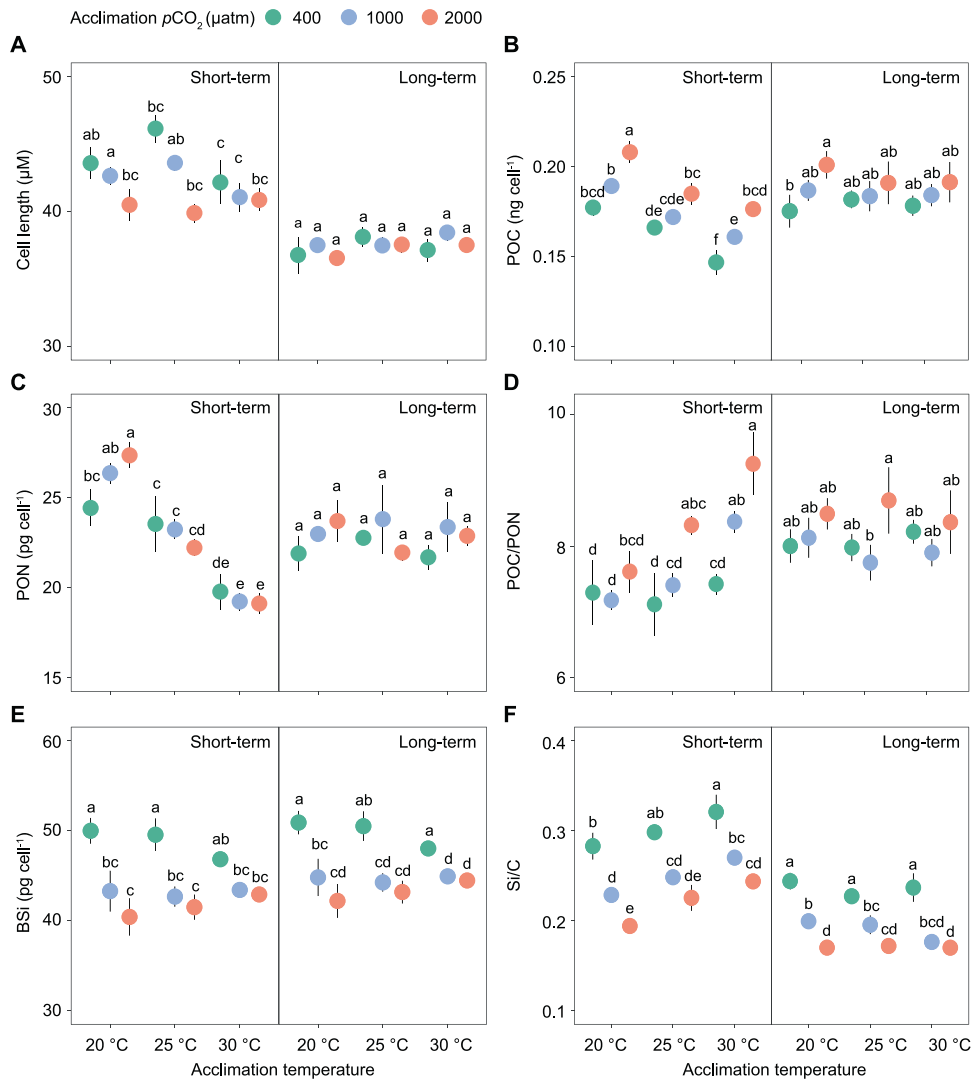


Fig. 3 Combined effects of ocean warming and ocean acidification on traits of *P. multiseriis* after short-term or long-term acclimation. Results are from the same experiment shown in Fig. 2, sampled at acclimation periods of 30 days and 800 days. **A** Cell lengths (μM). **B** Particulate organic carbon per cell (POC, ng cell^{-1}). **C** Particulate organic nitrogen per cell (PON, pg cell^{-1}). **D** Ratio of POC/PON. **E** Cellular biogenic silica (BSi, pg cell^{-1}). **F** Ratio of Si/C. Values are displayed as means and ± 1 SEM of triplicate cultures and symbols marked with different letters are significantly different. ($p < 0.05$).

cultures were switched back to low temperature regimes in a shift experiments. In contrast to physiological acclimation, the warming-selected lines showed adaptation at the transcriptome level, where the core gene expression was unable to decline when moved back to the low temperature level. Thus, results suggest that *P. multiseriis* cells might adapt to rising temperature with longer acclimation.

The combined effects of ocean warming and acidification on *P. multiseriis*

Bulk culture experiments highlight that ocean warming and acidification could stimulate *P. multiseriis* growth or even promote blooms at a suitable temperature range, but inhibit growth when warming and acidification becomes too high (27.8 °C and 2000 μatm $p\text{CO}_2$). Ocean warming and acidification are two major anthropogenic perturbations that influence phytoplankton physiology and productivity [32, 34, 38]. Complex interactions between CO_2 concentration and temperature have been reported to cause antagonistic, synergistic, or additive effects on different phytoplankton species [27, 39]. Schlüter et al. reported that temperature adaptation of the

coccolithophore *Emiliania huxleyi* occurred independently of ocean acidification levels [27]. In two other diatom species *Phaeodactylum tricornutum* and *Thalassiosira weissflogii*, Xu et al. found that the effect of ocean acidification on phytoplankton growth depends on growth temperature [39]. Here, our results using semi-natural bulk culture experiments found that ocean acidification had different effects on the growth rate of *P. multiseriis*, depending on the ocean temperature and under different seasonal and warming scenarios. In the low temperature seasons of spring and winter when ambient seawater temperature was less than 6.9 °C, there were no significant effects of elevated $p\text{CO}_2$ on growth rate under either ambient seawater temperature or warming (+4 °C) scenarios, whereas warming stimulated growth at all $p\text{CO}_2$ levels. Similar to the findings of Xu et al., high $p\text{CO}_2$ enhanced growth rates in summer when seawater temperatures ranged from 19.2 °C to 23.6 °C, suggesting ocean acidification and warming had antagonistic effects [39]. However, when temperatures reached 27.8 °C in autumn, high $p\text{CO}_2$ interacted synergistically with warming to reduce growth rate by 22.0% and 42.0% at 1000 μatm and 2000 μatm , respectively.

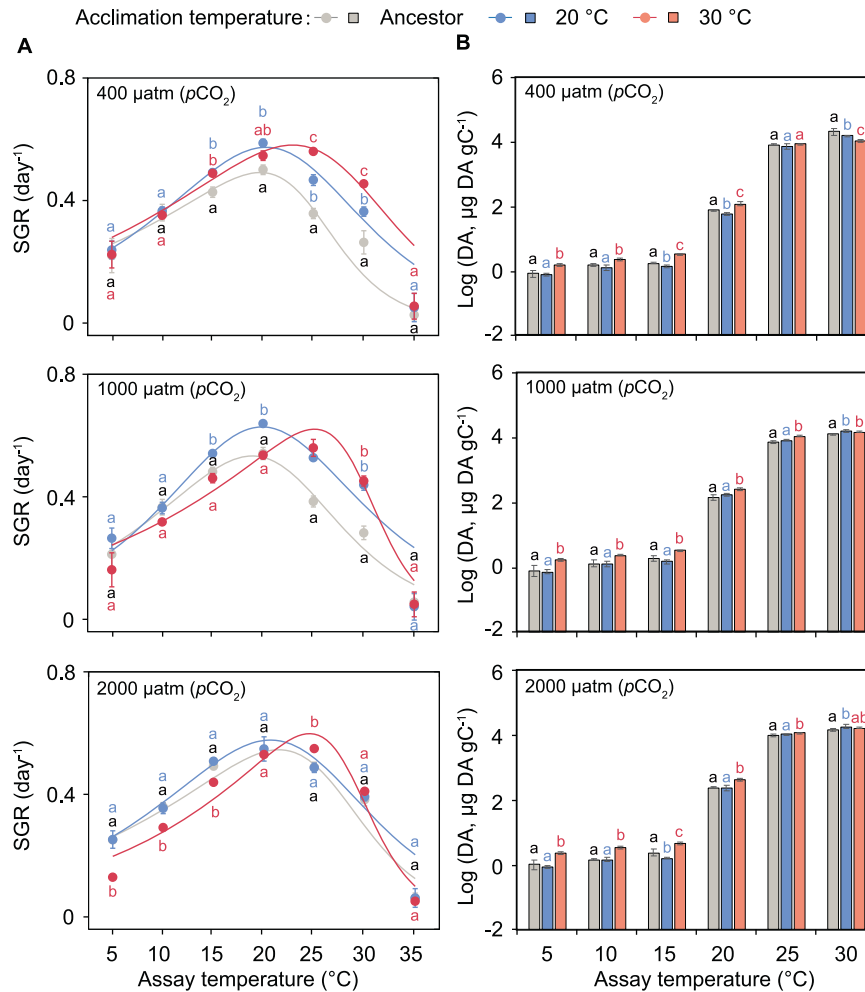


Fig. 4 Growth rates and DA concentrations of *P. multiseriis* in a shift experiment after long-term acclimation. Results are from short-term transfers to assay conditions (5 °C–30 °C, 400, 1000 or 2000 μatm) for cell lines acclimated long term (800 d) to a matrix of temperatures (20 °C, 30 °C) and $p\text{CO}_2$ levels (400, 1000 and 2000 μatm). Comparisons to the ancestral cell line are also included. **A** Fitted lines of specific growth rates (SGR) at each acclimation condition across replicates ($n = 3$) derived from non-linear least squares regression using the modified Sharpe-Schoolfield model. **B** Carbon-normalized log DA concentrations ($\text{Log}(\text{DA}, \mu\text{g DA gC}^{-1})$) of *P. multiseriis* when assayed under different temperature and $p\text{CO}_2$ regimes. Values are displayed as means and ± 1 SEM of triplicate cultures, and symbols marked with different letters among different acclimation regimes at each assay temperature are significantly different. ($p < 0.05$).

Acclimation and adaptation potential of *P. multiseriis* to ocean warming and acidification

Long-term acclimation experiments demonstrated that warming temperatures showed larger selective effects on growth and DA metabolism than elevated $p\text{CO}_2$, and temperature and CO_2 interact synergistically. It is important to study the acclimation and adaptation of HAB species to fully understand their responses to future climate change [40]. Acclimation usually involves phenotypic changes without genetic variation, while adaptation is generally associated with irreversible evolution [41]. Many studies have found different responses between short-term acclimation and long-term adaptation of phytoplankton to CO_2 and temperature perturbation [42–44]. This is the first study to test the adaptive response of *Pseudo-nitzschia* to ocean warming and acidification, individually and in combination, however, this finding is in agreement with other studies. Schlüter et al. found that the adaptation of *E. huxleyi* to a elevated temperatures at the upper thermal tolerance was up to six times faster than to elevated CO_2 [27]. Increasing temperature has been highlighted as a key factor in determining phytoplankton distribution, production and metabolic function [45–47]. Since most enzymes are temperature dependent, there is possibly a larger mutational

target for thermal adaptation than CO_2 adaptation, accelerating the speed of thermal adaptation [27, 47].

Reciprocal transplant experiments (also called shift experiments) can be used to compare trait optima between ancestral and evolved populations after long-term selection [48]. Our shift experiments further suggested that long-term warming acclimation resulted in the entire thermal reaction norm shifting to higher temperatures. However, the lower fitness and DA concentration of the high-temperature acclimation lines when moved back to low temperature regimes indicate that *P. multiseriis* cells use rapid plastic acclimation to respond to temperature changes, even after an 800-day acclimation period. Similar expression levels of core genes involved in the DA metabolic pathway suggest adaptation at the transcriptome level. Plastic acclimation or evolutionary adaptation have been extensively debated in different phytoplankton taxa in response to environmental changes, and both are viable ways to maintain fitness under long-term climatic acclimation [49]. Previous studies have found that plasticity may facilitate evolution by maintaining a large population size, with great genetic and mutational variance [50–52]. Phenotypic acclimation of DA production and adaptation of transcriptomic

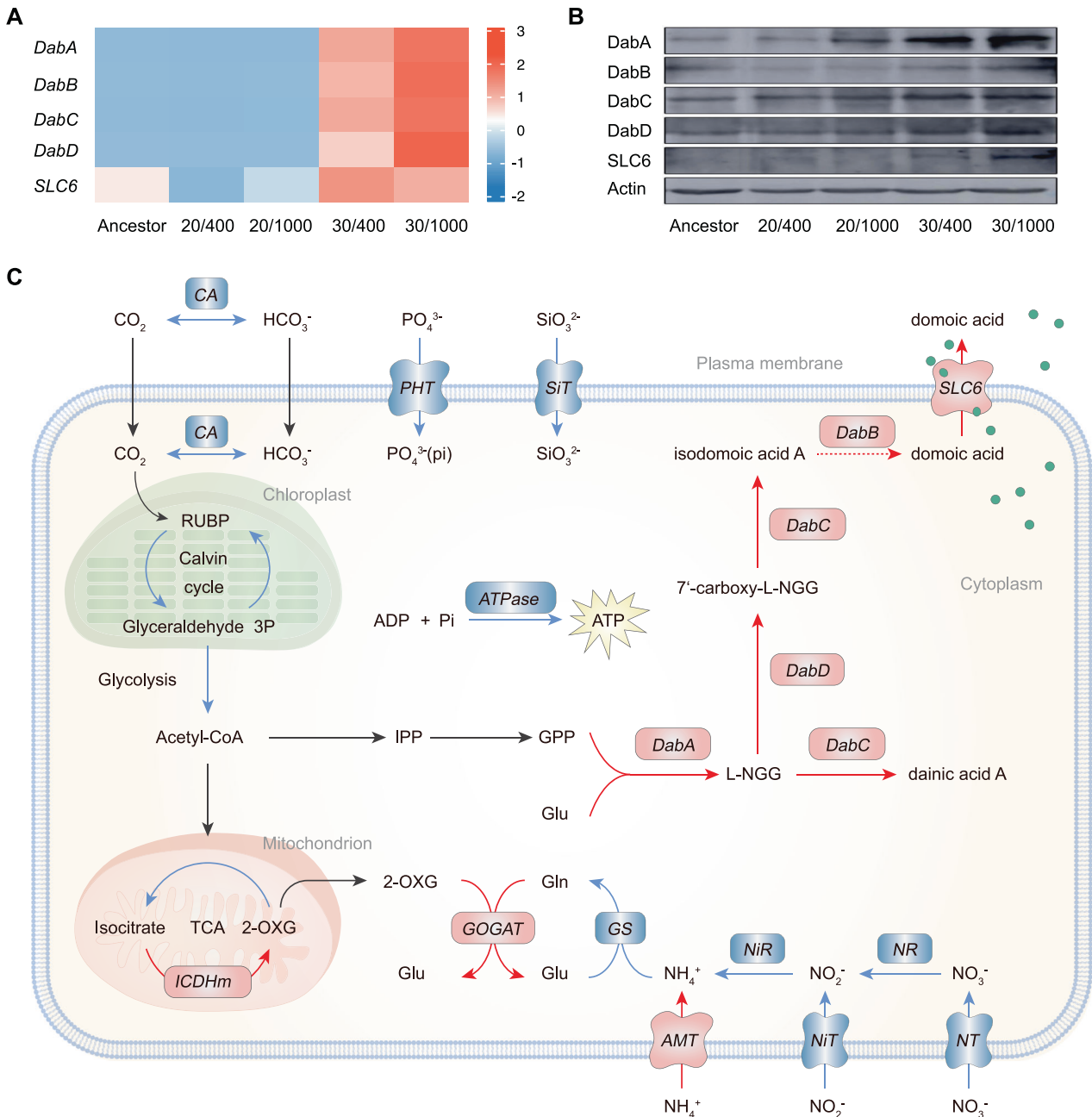


Fig. 5 Molecular understanding of altered DA biosynthetic potential of *P. multiseriis* under different warming and $p\text{CO}_2$ combinations. **A** Variation of core gene expression involved in the DA biosynthetic pathway under different warming and $p\text{CO}_2$ combinations. The color of heatmap represents the z-score of the FPKM value. **B** Western blot analysis of core protein expression involved in the DA metabolic pathway using Actin as an internal control. **C** Diagram representing altered DA biosynthetic potential of *P. multiseriis* by comparison between the ambient condition (low temperature and low $p\text{CO}_2$) and HTHC (high temperature and high $p\text{CO}_2$). The red and blue symbols represent up and downregulated genes or processes, respectively. CA carbonic anhydrase involved in CCM, ICDHm isocitrate dehydrogenase, NT nitrate transporter, NiT nitrite transporter; NR nitrate reductase, NiR nitrite reductase, AMT ammonia transporter; GS glutamine synthetase, GOGAT glutamine amidotransferase, PHT phosphate transporter, SiT silicate transporter, DabA terpene cyclase, DabB hypothetical protein, DabC α -ketoglutarate (α KG)-dependent dioxygenase, DabD CYP450, SLC6 sodium and chloride-dependent amino acid transporter.

expression under long-term warming indicates that *P. multiseriis* had a capacity to adapt to rising temperatures. This suggests that future ocean warming may increase DA production of *P. multiseriis*, and could enhance trophic transfer of toxins to food webs in the marine ecosystem. However, caution is needed when linking *P. multiseriis* growth and DA production potential with global change since not all strains produce the toxin, and some strains produce more than others [4].

Physiological differences between short-term and long-term acclimation

Studies have shown that different timescales of acclimation to a perturbation can result in different physiological responses, and acclimation to multiple drivers or extreme changes may be slower [53, 54]. During the 800-day long-term acclimation experiment, physiological traits including cell size and cell quotas of POC, PON, BSi were measured, and compared the differences between

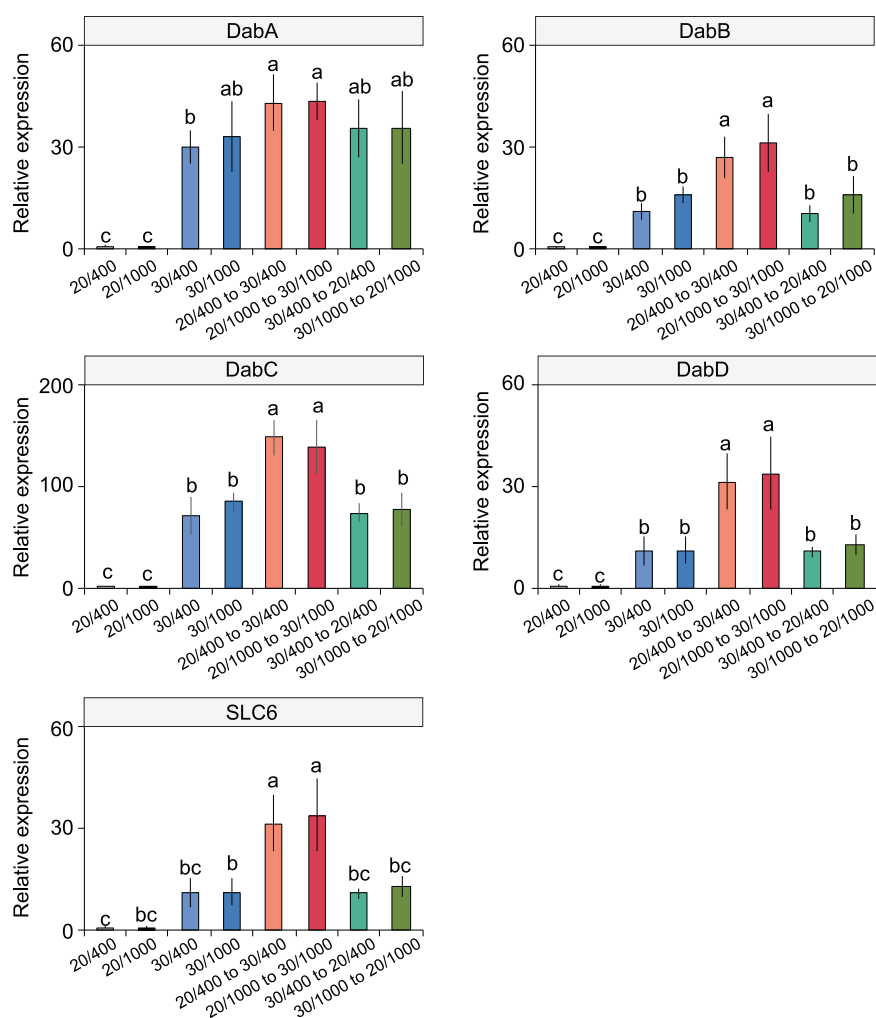


Fig. 6 Relative expression of the core genes involved in the DA metabolic pathway of long-term acclimated *P. multiseriis* in shift experiments. Transcriptome results are from the same temperature and CO₂ acclimation/shift experiments shown in Fig. 4. DabA: terpene cyclase; DabB: hypothetical protein; DabC: α -ketoglutarate (α KG)-dependent dioxygenase; DabD: CYP450; SLC6: sodium and chloride-dependent amino acid transporter. Values are displayed as means and ± 1 SEM of triplicate cultures and symbols marked with different letters among different acclimation regimes at each assay temperature are significantly different. ($p < 0.05$).

short-term and long-term acclimation. Cell size is an important master trait affecting nutrient acquisition, sinking velocity and the trophic role of plankton [55, 56]. Cell length in *P. multiseriis* decreased significantly under high temperatures for all cells after short-term acclimation under all p CO₂ levels, but showed no significant differences among treatments after long-term acclimation. Therefore, high DA concentrations under long-term acclimation of high temperature and/or elevated p CO₂ cannot be attributed to physiological changes in cell size, but rather to plastic acclimation. These findings are similar to a previous study by Bates et al., who reported that *P. multiseriis* underwent progressive cell reduction by 11.4% in length after long-term culture [4]. It should be noted that along with reductions in cell size, cellular DA concentration decreased gradually after 800-day acclimation (data not shown) regardless of whether they were grown at low or high temperature or p CO₂. Therefore, we terminated the long-term acclimation experiments at 800 days and conducted short-term shift experiments after 30 days acclimation. Possible reasons for this may be that laboratory constant conditions are very different from natural dynamic environments [4, 57, 58]. Zooplankton grazers, UVB exposure, and nutrient limitation can also induce and increase DA production in *Pseudo-nitzschia*, and thus long-term, semi-continuous

maintenance of the cultures in the laboratory without these factors could explain gradual loss or decline of DA metabolic capacity [4, 58].

Particulate organic carbon (POC) and (PON) content as well as POC/PON ratios are important predictors of nutritional quality to higher trophic levels [59]. Similar to elevated POC quotas under high p CO₂ [12, 20], POC content of *P. multiseriis* increased linearly under medium and high p CO₂ at all acclimation temperature gradients. However, after long-term warming acclimation (25 °C and 30 °C) there were no significant differences among different treatments. In contrast to the effects of increasing p CO₂, short-term warming treatments resulted in linear decreases of POC and PON at all three p CO₂ levels, but there were no significant differences in PON content or POC/PON among treatments after long-term acclimation. Stable POC/PON suggests the maintenance of one aspect of nutritional quality owing to adaptive evolution under high temperature or p CO₂ individually or in combination [46]. Our results also support previous studies showing that ocean acidification significantly reduced BSi levels in both short-term and long-term acclimation experiments [12, 60]. Decreasing BSi quotas and increasing or stable POC quotas resulted in lower Si/C ratios under medium and high p CO₂ at all selective temperatures. As with other diatoms, the reduced BSi quotas and Si/C of *P.*

multiseries under ocean acidification may have significant impacts on global carbon and silicon cycles by altering the vertical flux of these elements to the deep ocean [60].

Ecological implications

With increasing discovery and reporting of new species of *Pseudo-nitzschia*, the known distribution of this genus has expanded globally [4–6]. Although apparent trends are hard to distinguish from increased observational efforts, the extended distributions and increased number of toxigenic species worldwide has important consequences for ecosystem health and human seafood safety [1, 2, 4, 7]. Our work suggests that global warming associated with increasing CO₂ emissions could be one of the most important environmental drivers influencing growth rate and DA production of *P. multiseries*. However, experiments considering a more complex matrix of changing environmental variables under more realistic conditions may be necessary to determine the full range of evolutionary responses of these toxin producing organisms to an altered future ocean. Kelly et al. indicated that UVB exposure, warming, and urea availability can synergistically enhance cell-specific toxin production, while at the same time bloom formation may be inhibited by increased exposure to UVB [58]. Despite these necessary qualifications, our results provide new insights into the DA exposure potential for ocean ecosystems and harvested marine resources under continuing climate change conditions.

DATA AVAILABILITY

Transcriptome sequencing data are available at the National Center for Biotechnology Information (NCBI) with the accession number PRJNA686847.

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AUTHOR CONTRIBUTIONS

NY designed the project with contributions from DX, NY, DX, GCZ, ZW, TJ, KS, XF, CB, DAH, and ZJT analyzed the data. DX, NY, GB, ZW, and DAH wrote the manuscript; all authors contributed to editing the manuscript.

COMPETING INTERESTS

The authors declare no competing interests.

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