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1 Differential toxin response of *Pseudo-nitzschia multiseries* as a function of nitrogen speciation in
2 batch and continuous cultures, and during a natural assemblage experiment.
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14 *Formerly known as the Romberg Tiburon Center for Environmental Studies
15 Running Title: N & toxicity in *P. multiseries*
16
17 Abstract
18
19 The toxigenic diatom *Pseudo-nitzschia multiseries* Hasle, isolated from the U.S. Pacific
20 Northwest, was examined in unialgal laboratory cultures and in natural assemblages during
21 shipboard experiments, to examine cellular growth and domoic acid (DA) production as a
22 function of nitrogen (N) substrate and availability expected during bloom development and
23 decline. Laboratory experiments utilizing batch cultures conducted at saturating (120 µmol

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1 photons·m^{-2}·s^{-1}) photosynthetic photon flux density (PPFD), demonstrated that *P. multiseries*
2 (strain NWFSC-245) grows equally well on the three N substrates tested (nitrate [NO_3^-],
3 ammonium [NH_4^+] and urea), and achieved an average specific growth rate of 0.83 d^{-1}. Despite
4 equivalent growth rates, cellular toxicity (particulate DA concentration normalized to cell
5 abundance) varied as a function of N substrate, with urea-grown cells demonstrating 1.3- and
6 3.4-fold more toxicity than both NH_4^+- and NO_3^--grown cells. Cellular toxicity of the N-limited
7 chemostat cultures, grown at a dilution rate of 0.48 d^{-1}, were less than the cellular toxicity
8 measured for the N-replete batch cultures for all three N substrates, but again cellular toxicity
9 varied as a function of N substrate and the urea-supported cells were 3.5- and 4.3-fold more toxic
10 than the respective NH_4^+- and NO_3^--supported cells. Starved cultures of *P. multiseries* showed no
11 decline in cellular toxicity or change in the order of toxicity as a function of N substrate, and cells
12 previously supported by urea were 13- and 5-fold more toxic than NH_4^+- and NO_3^--supported
13 cells. At all three levels of N-sufficiency, the urea-grown cells consistently produced the highest
14 concentration of particulate DA per cell compared to cells grown on either NO_3^- or NH_4^+.
15 Shipboard N enrichment experiments using natural phytoplankton assemblages were
16 conducted off the west coast of Washington in an area characterized by elevated concentrations
17 of macronutrients and iron. All N (NO_3^-, NH_4^+ and urea) treatments showed significant increases
18 in biomass (as measured by total and size-fractionated chlorophyll *a*) and the abundance of
19 *Pseudo-nitzschia* species over the 6-d experiment. As with the unialgal laboratory experiments,
20 cellular toxicity varied as a function of the N source supporting growth, and the planktonic
21 assemblages enriched with either NH_4^+ or urea demonstrated greater cellular toxicity than the
22 assemblages supported solely by NO_3^-. These laboratory and field results demonstrate that N
23 substrate can regulate the toxicity of *Pseudo-nitzschia* species, and that N source should be

1 considered when evaluating the potential effects of cultural eutrophication on the growth of
2 toxigenic diatoms.
3
4 Key Index Words: ammonium, chemostat, continuous-culture, domoic acid, nitrate, nitrogen
5 uptake, *Pseudo-nitzschia multiseries*, urea
6
7 Abbreviations: N, nitrogen; NH_4^+, ammonium; NO_3^-, nitrate; DCMU, 3-[3,4-dichlorophenyl]-
8 1,1-dimethylurea; DA, domoic acid; pDA particulate domoic acid; cELISA, indirect cellular
9 enzyme-linked immunosorbent assay; photosynthetic photon flux density (PPFD).
10

11 1. Introduction

12 Diatoms of the *Pseudo-nitzschia* genus Pergallo (Heterokonta, Bacillariophyceae) have
13 been the subject of numerous field and culture studies since the discovery of domoic acid (DA)
14 production by *Pseudo-nitzschia multiseries* (then termed *Nitzschia pungens* f. *multiseries* Hasle
15 1995) resulting in the first report of an amnesic shellfish poisoning (ASP) in Atlantic Canada
16 (Subba Rao et al., 1988; Bates et al., 1989). Since then, the number of species of *Pseudo-*
17 *nitzschia* thought to synthesize DA has increased to 26 (Lundholm, 2017) worldwide, and at least
18 ten of these species are reported in the coastal waters of the West Coast of North America
19 (Trainer et al., 2012). The environmental factors that regulate the growth of these toxigenic cells
20 and/or their production of this potent neurotoxin by *Pseudo-nitzschia* have been reviewed in
21 detail (Lelong et al., 2012; Trainer et al., 2012), and it is clear that many of the factors needed to
22 promote the growth of these toxigenic cells, such as adequate light and macronutrients, are not
23 necessarily those responsible for enhancement of DA synthesis or its cellular accumulation; in

1 fact the reverse can be true.

2 The relationship between the specific growth rate of *Pseudo-nitzschia* and DA
3 production, in particular, is not fully understood and may be more complex than originally
4 envisioned. It has been widely accepted, based on studies conducted primarily with Atlantic
5 strains of *P. multiseries* and often extrapolated to other species, that DA production, specifically
6 the amount of DA per cell (DA cellular quota) is generally minimal or non-detectable during
7 nutrient-replete exponential growth, and increases during nutrient-depleted stationary growth,
8 provided the limiting nutrient responsible for the induction of the stationary growth phase is
9 either phosphorus or silicon. The same relationship is not found for nitrogen (N) due to the N
10 requirement for the synthesis of DA – a secondary amino acid (cf., reviews by Bates, 1998;
11 Bates and Trainer, 2006). As a consequence, for the few *Pseudo- nitzschia* species tested, N-
12 depleted cells found in the stationary growth phase are generally less toxic than cells found
13 growing exponentially under N-replete conditions (e.g., Auro and Cochlan, 2013).

14 The association of increased toxicity with slower-growing cells has been demonstrated in
15 continuous culture experiments where growth rate is controlled by the supply rate of the limiting
16 nutrient for growth. Studies have shown that DA production is inversely related to the cellular
17 growth rate in both P-limited (Pan et al., 1996a; Hagström et al., 2011) and Si-limited (Bates et
18 al., 1996; Pan et al., 1996b; Kudela et al., 2004) continuous cultures of *P. multiseries and P.*
19 *australis*. But beyond the knowledge that N is required for DA production (cf. review by Bates,
20 1998), the relationship between N and toxicity is not well understood. Based on cultures studies
21 it is still unclear if the specific growth rate achieved by exponentially growing cells influences
22 the resultant toxicity of either nutrient-sufficient or -limited *Pseudo-nitzschia* cells, or if toxicity
23 is simply a function of the N source used for growth.

1 Until recently the majority of N studies of *Pseudo-nitzschia* species have been conducted
2 using batch cultures supplied with N in excess of the cellular requirements to support maximal
3 growth rates. These N-replete batch studies have demonstrated that all three of the N substrates
4 commonly found in marine and estuarine waters: nitrate [NO_3^-], ammonium [NH_4^+] and urea,
5 can adequately support the growth of *Pseudo-nitzschia* species tested to date, with N preference
6 varying widely among species and even between strains of the same species (e.g., Thessen et al.,
7 2009 and references therein). In one such study, using a *P. australis* strain isolated from
8 Monterey Bay, CA, the slower growing cells supported by urea were 3- to 5-fold more toxic than
9 the faster growing NO_3^--, and NH_4^+-supported cells (Cochlan et al., 2006; Howard et al., 2007).
10 Similar results of urea-enhanced DA production have been reported for nutrient-amended deck
11 'grow-out' experiments using natural assemblages dominated by *P. australis* off San Francisco
12 Bay (Howard et al., 2007) and by *P.* cf. *seriata* in Bizerte Lagoon in the SW Mediterranean Sea
13 (Garali et al., 2016). Apparently the toxicity of *Pseudo-nitzschia* species may be influenced by a
14 number of factors, including N source, cellular phase of growth, or the growth rate achieved by
15 the cells, but it is still unknown whether cellular toxicity varies as a direct result of the N
16 substrate utilized for growth, or indirectly due to the growth rate achieved on one substrate
17 versus another. The challenge then is to determine the potential effects of growth rate and N
18 substrate on DA production independent of each other.
19 Continuous cultures are highly controlled systems that provide a dynamic equilibrium
20 between nutrient input and growth, and can be used to determine DA production as a function of
21 N source independent of the potential growth rate effects. Unlike batch cultures that have a
22 continuously changing environment, continuous cultures provide a constant growth environment
23 (Rhee, 1980) where cells are maintained in exponential growth phase independent of time, and

1 the effects of one environmental parameter can be assessed while holding all the others constant
2 (Herbert et al., 1956; Rhee, 1980). In the present study, both batch cultures and continuous
3 cultures were employed to assess the effects of NO_3^-, NH_4^+ and urea on the cellular toxicity of
4 *Pseudo-nitzschia multiseries* Hasle at three degrees of N sufficiency: N-replete, N-limited and N-
5 depleted. In the continuous culture systems used, the growth rates achieved by three different N
6 sources were equal and controlled by the dilution rate set by the experimenter. When specific
7 growth rate and dilution rate are balanced, phytoplankton biomass remains constant over time,
8 and the system is considered to be in steady-state. Such continuous cultures, termed chemostats
9 (e.g., MacIntyre and Cullen, 2005), were used to examine differential toxicity as a function of N
10 substrate independent of the growth achieved on the different N substrates. These N-limited
11 chemostats were then allowed to starve without N amendment to examine toxicity in N-depleted
12 cells; a situation normally expected following bloom decline in natural marine systems.
13 To assess the impact of N sources on natural *Pseudo-nitzschia* species, field studies were
14 conducted with phytoplankton assemblages collected off Washington in macronutrient-and
15 micronutrient-replete coastal waters. A multi-day, deck-board incubation experiment was
16 conducted to determine the differential growth and DA toxin response of natural assemblages of
17 *Pseudo-nitzschia* after N amendment by NO_3^-, NH_4^+ or urea. These field studies were conducted
18 as part of the ECOHAB-PNW (Ecology and Oceanography of Harmful Algal Blooms-Pacific
19 Northwest) project investigating the physiology, toxicology, ecology and oceanography of toxic
20 *Pseudo-nitzschia* species off the Pacific coast of Washington and British Columbia.
21
22 ## 2. Materials and Methods
23

1 *2.1 Cell Culturing. Pseudo-nitzschia multiseries* culture NWFSC-245, isolated from Sequim
2 Bay, WA by B. Bill in June 2006, was used for the batch and continuous culture experiments of
3 this study. Cultures were maintained on sterile-filtered (0.2-µm, Whatman® PolyCap™ 150 TC
4 Filter; Whatman, Florham Park, NJ, USA) artificial seawater (ESAW; Harrison et al. 1980);
5 following Berges et al. (2001 and subsequent Corrigendum 2004), with the following
6 modifications: Metals Stock I: $FeCl_3 \cdot 6H_2O$, 1.77 g L^{-1}, Na_2-EDTA, 2.44 g L^{-1}; Metals Stock II:
7 Na_2-EDTA, 3.09 g L^{-1}, $MnSO_4 \cdot H_2O$, 0.1512 g L^{-1}, $Na_2MoO_4 \cdot 2H_2O$, 0.0148 g L^{-1}, $NiCl_2 \cdot 6H_2O$,
8 0.0149 g L^{-1}. Copper, as $CuSO_4 \cdot 5H_2O$ and Selenium, Na_2SeO_3 were prepared as separate stocks
9 and added to the medium at double the concentrations of Berges et al. (2001) to achieve final
10 concentrations of 1×10^{-15} M and 6.36×10^{-9} M, respectively. All other enrichments were
11 unchanged except silicic acid, which was doubled to ensure a minimum Si:N ratio of 2:1, and
12 phosphate, which was reduced from 22 to 11 µM to facilitate, automated nutrient analysis.
13 Nitrate, the sole nitrogen source, was reduced from 550 to ≤ 80 µM, and the cultures were
14 maintained on NO_3^- for a minimum of 3 months before experiments to determine growth and
15 toxin response as a function of N substrate were initiated.

16 Cultures were maintained at 13.1°C (± 0.2 °C) in a temperature-controlled environmental
17 chamber, stirred at 60 rpm, bubbled with sterile air, and continuously illuminated from one side
18 using four Vita-Lite® Plus Tower Twist fluorescent tubes (Color Rendering Index-91; Duro-
19 Test® Corporation). Average PPFD, measured using a 4π collector (QSL-100 Quantum scalar
20 irradiance meter; Biospherical Instruments Inc.) when immersed in medium-filled culture vessels
21 was 120 µmol photons $m^{-2} s^{-1}$. This irradiance was experimentally determined to be saturating for
22 the growth of this strain at 13°C (see Results: Figure 1). Continuous (24 h) illumination was
23 employed during all maintenance and experimental culturing to avoid the potential effects of

1 synchronous cell division from diel periodicity (e.g., MacIntyre and Cullen, 2005, and references
2 therein).
3 All glassware and polycarbonate containers used for culturing and media storage were
4 first washed in dilute detergent (Contrad 70®; Decon Laboratories Inc.), soaked in weak
5 hydrochloric acid (5% v/v) for at least 24 hours, rinsed thoroughly with ultra-pure water (18.2
6 MΩ·cm; Milli-Q®; EMD Millipore Corp.), and autoclaved prior to use. Culture transfers and pre-
7 experimental handling were conducted in a positive pressure hood equipped with an MAC 10®
8 HEPA filtration unit (Enviroco Corp.™), and aseptic technique was employed to prevent fungal
9 growth and minimize bacterial contamination throughout all experimental and sample
10 manipulations. Despite these precautions, cultures were unialgal but not axenic.
11
12 *2.2 Analytical Methods*
13 *2.2.1 Cell growth.* Cell growth was monitored by either measuring cell abundance or *in vivo*
14 fluorescence as a function of time during the laboratory experiments Samples (1 ml) for cell
15 enumeration were fixed with 100 µL of Lugol's iodine solution (10% final concentration), and
16 stored at 10°C until counted using a nanoplankton counting chamber (PhycoTech® Inc.,) and
17 phase contrast microscopy (Eclipse E4000, Nikon® Instruments Inc.) at 100X magnification.
18 Samples for *in vivo* fluorescence were measured using a Turner Designs 10-AU fluorometer
19 (Turner Designs). During the initial experiments to determine growth as a function of PPFD, 50-
20 mL borosilicate (Pyrex®) culturing tubes were inserted directly into the fluorometer as described
21 by Brand and Guillard (1981), whereas in all other experiments 8-mL subsamples were removed
22 from culture vessels and fluorescence measured with the fluorometer.
23

1 *2.2.2 Chlorophyll and DCMU.* Phytoplankton biomass (as chlorophyll *a*) was determined by
2 using the acidification *in vitro* fluorometric technique (Parsons et al., 1984) after filtration onto
3 Whatman GF/F filters (0.7 µm nominal pore size). Samples were stored frozen before extraction
4 in 90% acetone for approximately 24 h at -20°C, and the fluorescence subsequently measured
5 with a Turner Designs 10-AU fluorometer calibrated with pure Chl *a* obtained from Turner
6 Designs. *In vivo* cellular fluorescence capacity was measured in 8-mL subsamples, and
7 determined as the ratio of variable (F_V) to maximum (F_m) fluorescence measured after dark
8 acclimation (10 min) using the 3-[3,4-dichlorophenyl]-1,1-dimethylurea (DCMU) technique
9 (Parkhill et al., 2001).
10
11 *2.2.3 Nutrients.* Samples for inorganic nutrient analysis were filtered (combusted Whatman®
12 GF/F filters) and filtrates stored frozen in pre-cleaned 15-mL polypropylene, conical tubes
13 (Falcon®; Becton Dickinson Labware) prior to analysis. Samples were analyzed for nitrate plus
14 nitrite (NO_3^- + NO_2^-; hereafter referred to as nitrate, NO_3^-), orthophosphate (PO_4^{3-}), and silicic
15 acid ($Si[OH]_4$) with a Lachat Instruments Flow Injection Analysis system (8000 series; Hach
16 Co.) using according to the Quick-Chem® colorimetric techniques (Smith and Bogren, 2001;
17 Knepel and Bogren, 2002; Wolters, 2002, respectively). Samples collected for urea were stored
18 frozen at -20˚C in 50-mL polypropylene, centrifuge tubes (Corning®), until spectrophotometric
19 analysis using an UV-visible spectrophotometer (model 8453E; Agilent Technologies) equipped
20 with a 10-cm cell to increase the sensitivity and limit of detection. The diacetyl monoxime
21 thiosemicarbizide technique (Price and Harrison, 1987), modified slightly to account for a longer
22 (72 h) and lower digestion temperature (22°C), was employed for manual analysis of urea
23 samples. Ammonium was analyzed manually following the fluorometric method described by

1 Holmes et al. (1999) using a Turner Designs 10-AU fluorometer equipped with the ammonium
2 optical kit (part number 10-303); high concentration samples were diluted with Milli-Q® water
3 when necessary.
4
5 *2.2.4 Domoic acid.* Samples for particulate domoic acid (pDA) concentrations were analyzed
6 using an indirect cellular enzyme-linked immunosorbent assay (cELISA; Garthwaite et al., 1998)
7 employing flat-bottomed, 96-well polystyrene high binding stripwell plates (Costar, Corning,
8 USA) pre-coated with DA-carboxy-linked BSA conjugate (Beacon Analytical Systems Inc,).
9 Samples for pDA were filtered onto 0.45µm HAWP025 Millipore MF™ Membrane filters,
10 transferred into polypropylene conical tubes containing 4-mL Milli-Q® water, macerated to
11 release cells from the filter, sonicated to lyse the cells and then frozen (-20 °C) for later analysis
12 at the Northwest Fisheries Science Center (Seattle, WA). Domoic acid concentrations in all
13 samples were calculated based on the interpolation from a standard curve with known DA
14 concentrations (DACS-1 standard, NRC, Canada) ranging from 0.64 to 50 ng mL^{-1} using the non-
15 linear four-parameter logistic curve fit model. Particulate DA concentrations normalized to cell
16 abundance are termed cellular DA concentrations (or DA cell quota), and are the mean of
17 triplicate samples collected from each laboratory culture or natural phytoplankton assemblage
18
19 *2.2.5. Statistics* Data processing and statistical analyses were conducted using Excel®
20 (Microsoft® Corp.) and KaleidaGraph® (Synergy Software) software programs, respectively.
21 Results are considered significantly different at the 5% level for all statistical tests utilized
22 including, analysis of variance (ANOVA), least significant difference (LSD) post-hoc
23 comparison test, and the Student-Newman-Keuls multiple comparison test.

1

2 *2.3 Experimental Procedures*

3 *2.3.1. Cultures at varying degrees of N sufficiency.* Cells of *P. multiseries* were studied at three
4 levels of N sufficiency in the laboratory: replete, limited and depleted. Batch cultures were used
5 to provide N-replete conditions for growth, continuous cultures were used for N-limited
6 conditions, and the continuous cultures - after deprived of external N sources for 2-3 generations
7 - were used to emulate the N-depleted (or starved) condition. The N-replete batch cultures were
8 initiated by transferring 15.6 x 10^3 cells from their early stationary phase of growth to duplicate,
9 6-L flat-bottomed glass flasks (Pyrex®) containing 5 L of ESAW with either: 45.6, 51.2 or 42.3
10 µM-N of NO_3^-, NH_4^+, or urea, respectively. Note that the N concentrations reported in this study
11 are in terms of nitrogen (i.e., 2 µM-N of urea is equivalent to 2.0 µg-at N L^{-1} which equals 1 µM
12 urea).

13 Sub-samples were taken daily for monitoring of growth using cell abundance and *in vivo*
14 fluorescence. Specific growth rates were calculated from a least-squares linear regression
15 analysis of the exponential growth phase, determined from semi-log plots of cell density over
16 time, and using the exponential (K_e) growth equation (Guillard, 1973). The mean growth rates
17 reported were determined over a minimum of four days of exponential growth prior to depletion
18 of N in the media. Samples for pDA, cell abundance, and *in vivo* cellular fluorescence capacity
19 were collected during the mid-exponential phase of growth. The batch cultures were monitored
20 daily for nutrients; once depleted of external nitrogen they were then used to initiate the
21 continuous cultures; NO_3^--grown batch cultures were used to initiate NO_3^--limited continuous
22 cultures, NH_4^+-grown batch cultures were used to initiate NH_4^+-limited cultures, and urea-grown
23 batch cultures used for urea-limited cultures.

2.3.2. Continuous cultures. *P multiseries* was grown in duplicate cultures under N limitation for each of the three N (NO_3^-, NH_4^+, urea) treatments using cells grown in the previous batch cultures recently depleted of their external N reserves. Six, 2-L flat-bottomed glass boiling flask (Pyrex®) containing 1.5 L of culture served as culture vessels, and cultures were gently stirred to maintain algal cells in suspension, and to ensure homogeneity as new medium was added drop-wise to the surface with peristaltic pumps (see below). The cultures were aerated with sterile, ammonia-free, filtered air (0.2-µm polycarbonate syringe filters; Corning®) by pumping air through activated charcoal, and bubbling through weak sulfuric acid (5% v/v) and two vessels of Milli Q® water before the air entered the culture vessels. Digital console peristaltic pumps (Masterflex, Cole-Palmer Instruments) were used to pump the medium from 20-L polycarbonate reservoir carboys through silicone (Nalgene® 50) tubing into the culture vessels, and a common reservoir supplied medium to the duplicate cultures of each N treatment. The concentrations of NO_3^-, NH_4^+ and urea in the three reservoir carboys were 40.2, 39.4, and 44.6 µM-N, respectively, ensuring the biomass achieved for each N treatment was the same once steady-state conditions were achieved. The Masterflex pumps were manually calibrated to provide a constant flow rate of 30.0 mL h^{-1} (720 mL $\cdot d^{-1}$) to each culture vessel, and maintained a constant dilution rate of 0.48 d^{-1} across all three N treatments. Daily monitoring of cell abundance, *in vivo* fluorescence, and dissolved nutrients (from both the center of the flasks and effluent tubes) were conducted to determine when steady-state conditions were achieved. Cultures was assumed to be at steady state conditions when the N substrate supporting growth was below its limit of detection (0.01 - 0.03 µM-N), and cell abundance and *in vivo* fluorescence did not vary by more than ± 10% for a minimum of three consecutive days. At this time, additional samples were sampled for DA toxin analyses and *in*

1 *vivo* cellular fluorescence capacity.

2 The remaining cells in the culture vessels were then allowed to grow in 'batch' mode
3 without additional N amendment for 2 days (equivalent to ca. 2.4 generations based on the
4 average growth rate achieved during their N-replete condition) and were then considered N-
5 depleted or starved (e.g., Dortch et al., 1982; Cochlan and Harrison, 1991). Although neither
6 macro- nor micro-nutrients were added during this time, only N was fully depleted, and all other
7 nutrients were at growth-saturating concentrations. After this 2-d period, samples were collected
8 for cellular toxin, cell abundance and *in vivo* cellular fluorescence capacity from the duplicate
9 cultures of each of the three N (NO_3^-, NH_4^+, urea) treatments.

10

11 *2.3.2. Field experiment* Nutrient enrichment experiments were conducted aboard the R/V
12 *Thomas G. Thompson* during September 2006 as part of ECOHAB-PNW VI cruise using water
13 collected at 5-m depth (*ca.* 40-50% light penetration depth) from 20 km off the coast of
14 Washington (latitude: 47° 45.95' N; longitude: 124° 48.20' W). The water was collected while
15 underway at *ca.* 2 knots using a trace metal (TM) clean, all-Teflon® sampling system comprised
16 of a weighted, plastic tow-fish suspended *ca.* 7-8 m outboard from the vessel using the ship's
17 crane to avoid contamination associated with the vessel. Water was pumped onboard at ~3-4 L
18 min^{-1} using an all-Teflon double diaphragm pump which is gentle on phytoplankton and
19 microzooplankton (Wells et al., 2009), through Kevlar-encased Teflon® perfluoroalkoxyalkane
20 (PFA) tubing into a fabricated, positive pressure clean room, where all water dispensing and
21 sample manipulations were conducted under a class 100 HEPA airflow unit (MAC 10®, Enviroco
22 Corp.). The seawater was course-sieved through pre-rinsed 200-µm nylon mesh (Nitex®) to
23 exclude macrozooplankton grazers, directly into two TM-clean, thoroughly acid-cleaned and

1 rinsed, 50-L polypropylene carboys. The use of these large carboys ensured uniformity of the
2 seawater when subdividing the collected water into eight, acid-cleaned, 10-L polycarbonate
3 carboys that were rinsed three times with the filtered seawater prior to being filled.

4 The ambient nutrient concentration of the seawater collected for the experiments (prior
5 to nutrient enrichment) was 12.5, 0.20, and 0.15 µM-N of NO_3^-, NH_4^+ and urea, respectively, and
6 29.8 µM $Si(OH)_4$, and 1.14 µM PO_4^{3-}. All of the 10-L polycarbonate carboys were enriched
7 with 20 µM $Si(OH)_4$, and 1.85 µM PO_4^{3-}, including the controls, to ensure that these
8 macronutrients were present in excess throughout the duration of the experiment. The
9 experimental N treatments (conducted in duplicate) were enriched with either ca. 20 µM-N of
10 NO_3^- or NH_4^+, or ca. 30 µM-N urea, whereas N was not added to the two carboys that served as
11 controls. Carboys were thoroughly mixed and sampled for initial concentrations of
12 macronutrients and biomass prior to placement in clear, deckboard Plexiglas® incubators.
13 Temperature was maintained at sea-surface temperature with flowing surface seawater (ca. 12 °C
14 at the initiation of the experiment) and the incident PPFD was reduced by *ca.* 40% using a
15 combination of neutral density screening and blue Plexiglas®. All carboys remained sealed
16 throughout the 6-d experiment, but were sub-sampled using compressed filtered air overpressure
17 (Coale et al., 1991) every second day (days 1, 3 and 5) between 10:00 and 11:00 h for
18 chlorophyll *a,* pDA, and phytoplankton, whereas the control natural assemblages were sampled
19 on days 2, 4 and 6.

20 Size-fractionated (> 5 µm) and total phytoplankton chlorophyll *a* concentrations were
21 collected using parallel filtrations with polycarbonate (Poretics®, pore size = 5 µm) and glass-
22 fiber (Whatman® GF/F, nominal pore size = 0.7 µm) filters, and analyzed by *in vitro*
23 fluorometry. For both size fractions, the values reported for each carboy are the means of

1 duplicate filtrations and analyses. Subsamples (20 mL) for phytoplankton species identification
2 and enumeration were collected in high-density, polyethylene (HDPE) plastic scintillation vials
3 (Wheaton), preserved with 4 drops of acidic Lugol's Iodine solution, and stored in the dark at
4 room temperature for later analysis ashore. Taxonomic identification and enumeration were
5 performed using the Utermöhl technique, using a Zeiss Axiostar phase contrast light microscope
6 (100 X total magnification). Inorganic and organic nutrient concentrations were collected and
7 analyzed onboard as previously described.
8
9 3. Results
10 *3.1. Laboratory Experiments*
11 *3.1.1. Growth rates* The optimal PPFD for non-axenic cultures of *Pseudo-nitzschia multiseries*
12 (strain NWFSC-245) was 120 µmol photons$\cdot$m$^{-2}\cdot$s^{-1} when grown at 13.1 ± 0.2 °C (Figure 1).
13 This PPFD was employed for all of the culture experiments reported here. Maximum specific
14 growth rates (µ) of *P. multiseries*, determined from cell density measurements over time, and
15 using either NO_3^-, NH_4^+or urea as the sole N source of N treatments, were evaluated using an
16 one-way ANOVA approach. Specific growth rates from two different experiments, (denoted a
17 cultures 1 and 2 in Figure 2), and calculated over a minimum of four days of exponential growth
18 prior to N depletion, were 0.85 ± 0.02 d^{-1}, 0.83 ± 0.02 d^{-1}, and 0.81 ± 0.05 d^{-1} for NO_3^--, NH_4^+-
19 and urea-grown cells, respectively (mean ± range of duplicates), and are statistically
20 indistinguishable (p = 0.718, $F_{df=5}$ = 0.37). It should be noted that exponential growth rates
21 estimated above using cell density measurements over time did not differ significantly from rates
22 determined using *in vivo* fluorescence measurements over time (R.L. Radan, unpubl. results).
23

1 *3.1.2 Cellular fluorescence capacity* The *in vivo* cellular fluorescence capacity (CFC; determined
2 using DCMU) indicates that these cultures were photosynthetically "healthy" for all three N
3 substrates at all three levels of N sufficiency. For the batch cultures, the average F_v/F_m ratios
4 measured during mid-exponential growth phase, were 0.71 ± 0.02, 0.68 ± 0.01, and 0.65±0.01
5 (mean ± range of duplicate cultures) for the respective NO_3^-, NH_4^+, and urea treatments, and
6 were statistically indistinguishable ($p = 0.106$, $F_{df=5} = 5.19$). The F_v/F_m ratios did not decline in
7 the chemostat cultures, and F_v/F_m ratios during steady-state conditions averaged 0.68 ± 0.01,
8 0.65 ± 0.00, and 0.66 ± 0.04 for the NO_3^-, NH_4^+, and urea treatments, respectively, and also were
9 statistically indistinguishable ($p = 0.610$, $F_{df=5} = 5.85$). Nitrogen-starved cells, initially grown in
10 the continuous culture system and then allowed to continue without any additional N supply, did
11 not demonstrate a substantial decrease in their respective F_v/F_m values. The average F_v/F_m
12 ratios for these cells during this phase of growth were 0.62 ± 0.00, 0.63 ± 0.01, and 0.62 ± 0.05
13 for NO_3^-, NH_4^+, and urea, respectively, and were also statistically indistinguishable across N
14 treatments ($p = 0.976$, $F_{df=5} = 0.025$).
15
16 *3.1.3 Domoic Acid*. Cultures of *P. multiseries* were analyzed for particulate DA (pDA) using the
17 cELISA method to determine if DA production was influenced by the N substrate used for
18 growth and the degree of N sufficiency. DA values are presented as volumetric concentrations,
19 and also as cellular DA quotas (pDA concentration normalized to cell density) to account for
20 differing *Pseudo-nitzschia* abundances in each culture (Table 1; Fig. 3). Because of the varying
21 cell densities, it is difficult to compare volumetric pDA concentrations as either a function of N
22 sufficiency or N source; however, suffice it to say that pDA concentrations were consistently
23 greatest in the urea-grown cultures, followed by the NH_4^+ and the NO_3^--grown cultures, except in

1 the NO_3^--depleted cultures where pDA levels were slightly greater than the NH_4^+ -depleted
2 cultures (Fig. 3A-C). Nitrogen depletion only resulted in substantially increased concentrations
3 of pDA in the previously urea-grown cultures, whereas pDA increased < 2 fold for the NO_3^--
4 grown cultures, and declined by 52-76% for the NH_4^+-grown cultures relative to the pDA
5 concentrations measured during N-limited, steady-state conditions.
6 Cellular DA concentrations varied both as a function of N source and N sufficiency. In
7 the N-replete batch cultures, although the three N sources supported statistically identical specific
8 growth rates for these cultures, the cells grown on urea produced 2.57 ± 0.19 fg pDA cell^{-1} &
9 6.03 ± 0.42 fg pDA cell^{-1} (mean values ± 1 SD are based on triplicate analytical DA
10 determinations per culture), and cellular DA quotas were similar for the NH_4^+-grown cells (2.69
11 ± 0.03 & 3.77 ± 0.02 fg DA cell^{-1}), but lower for the NO_3^--grown cells (1.70 ± 0.06 & 0.83 ±
12 0.05 fg pDA cell^{-1}) (Fig. 3D). On average, the urea-grown cells of *P. multiseries* produced 1.3
13 and 3.4-fold more pDA than cells grown on NH_4^+ or NO_3^- with cellular toxicity following the
14 order: urea > NH_4^+ > NO_3^-. Although when examined using a One-Way ANOVA, the differences
15 in cellular toxicity of these N-replete, exponentially growing cells are not considered
16 significantly different as function of N source supporting growth ($p = 0.275$, $F_{df=5} = 2.05$). This
17 is, presumably, in part due to the small sample size and the sizeable variation between replicates
18 of each N treatment.
19 Nitrogen-limited chemostat cultures were harvested after achieving steady-state
20 conditions, and analyzed similarly for DA production (Fig. 3E). Duplicate cultures grown on
21 urea produced 2.66 ± 0.21 and 1.67 ± 0.10 fg pDA cell^{-1}, whereas lower cellular DA quotas were
22 measured for both NH_4^+-grown (0.74 ± 0.10 and 0.49 ± 0.03 fg pDA cell^{-1}) and NO_3^--grown
23 cells (0.40 ± 0.07 and 0.61 ± 0.09 fg pDA cell^{-1}). Cells grown on urea produced on average 3.5

1 and 4.3 times more particulate DA per cell than those growing on NH_4^+ or NO_3^- which were
2 indistinguishable from each other. Comparison of the mean cellular DA concentration as a
3 function of N source using a One-Way ANOVA indicates a significant difference among N
4 treatments ($p = 0.0489$, $F_{df=5} = 9.72$). Due to the substantial difference between the duplicate
5 urea-limited cells, a least significant difference (LSD) post-hoc comparison test was used to
6 compare between the N source groupings, instead of the typical Student-Newman-Keuls multiple
7 comparison test. The LSD analysis indicates significant differences in DA production between
8 urea and both the NH_4^+-and the NO_3^--limited cultures ($p = 0.035$ and 0.029 respectively);
9 however, there is no significant difference in DA production between the NO_3^-- and NH_4^+-
10 limited cultures ($p = 0.814$).
11 Immediately following collection of samples from the N-limited cultures, the dissolved N
12 inputs to the cultures were terminated, and the cells allowed to grow for an additional 2 days
13 before N-starved cells were collected for DA analyses (Fig. 3F). Again urea-grown cells
14 produced more particulate DA per cell (3.69 ± 0.35 and 2.18 ± 0.19 fg pDA $cell^{-1}$) than the *P.*
15 *multiseries* cells grown previously on NH_4^+ (0.33 ± 0.02 and 0.11 ± 0.01 fg pDA $cell^{-1}$) or NO_3^-
16 (0.43 ± 0.06 and 0.71 ± 0.09 fg pDA $cell^{-1}$). Cells previously grown on urea produced an average
17 of 13- and 5-fold more particulate DA per cell than those grown previously on NH_4^+ or NO_3^-. A
18 One-Way ANOVA indicates a significant difference in cellular DA for cells previously grown
19 on urea, compared to those maintained with NH_4^+ or NO_3^- ($p = 0.04264$, $F_{df=5} = 10.79$). When
20 using a LSD post-hoc comparison test, there is a significant difference in cellular DA
21 concentrations between urea- and NH_4^+-grown cells ($p = 0.0236$), and between urea and NO_3^--
22 grown cells ($p = 0.0338$), but as before no significant difference between the NH_4^+- and NO_3^--
23 maintained cells ($p = 0.6215$).

1 Cellular DA concentrations in all of the N-limited chemostat cultures were on an average
2 40, 19, and 50% of the cellular DA quotas of the respective N-replete batch cultures grown with
3 NO_3^-, NH_4^+ or urea. The LSD post-hoc comparison tests indicate that the declines in cellular
4 pDA production in the urea- and NO_3^--grown cultures were not statistically significant (p =
5 0.349 and 0.121, respectively), whereas the decrease in the NH_4^+-grown cultures was statistically
6 significant ($p = 0.011$). In the N-starved cultures, the NO_3^-- and urea-grown cells slightly
7 increased cellular pDA production by an average of 13 and 36%, respectively, compared to the
8 N-limited cultures. The cellular toxicity of NH_4^+-grown cells on the other hand, continued to
9 decrease in the N-starved cultures by an average of 64%. LSD post-hoc comparison tests indicate
10 that the 2-d starvation period did not result in statistically significant changes in DA toxicity in
11 any of the N treatments from those achieved during N-limited exponential growth ($p > 0.05$).
12
13 *3.2 Field Experiment*
14 *3.2.1 Environmental conditions*
15 The six-day deckboard experiment began on September 23, 2006 on a clear, sunny day;
16 weather that continued for the next three days of the experiment. Beginning on the fourth day,
17 conditions turned chilly, windy, and foggy with light rains and only occasional sun, so
18 unfortunately relatively constant light conditions were not experienced during the complete 6 d
19 experiment. The temperature of the surface waters used to cool the incubators ranged from 10.3
20 to 14.0°C throughout the experiment. Initial macronutrient concentrations at the 5 m sampling
21 depth of collection were 29.8 µM $Si(OH)_4$, 1.14 µM PO_4^{3-}, 0.20 µM-N of NH_4^+, 0.15 µM-N of
22 urea, and 12.5 µM of NO_3^- + NO_2^- (hereafter referred to as NO_3^-). The initial chl *a* concentration
23 was 3.87 µg chl $a \cdot L^{-1}$ for the total planktonic assemblage collected on GF/F filters (nominal pore

1 size = 0.7 μm), and was composed primarily of larger phytoplankton (3.18 μg chl *a*·L^{-1} in the >5
2 μm size-fraction), and *Pseudo-nitzschia* only represented ca. 1.5 % of the total cell density of the
3 natural assemblage when first collected.
4
5 *3.2.2.Nutrients*

6 The deck-board cultures of the natural phytoplankton assemblages were all initially N
7 replete, and total dissolved N concentrations were ca. 30-40 μM-N in the N-treated assemblages,
8 and ca.13 μM-N in the un-enriched control assemblages at the start of the field experiment (Fig.
9 4A-D). Nitrate declined steadily in all of the natural assemblages except for those enriched with
10 NH_4^+, where ambient concentrations of NO_3^- only decreased by <1 μM during the first 3 days of
11 the experiment (Fig. 4C) compared to drawdowns of 10-12 μM- N in the NO_3^-- and urea-
12 enriched assemblages (Fig. 4 B and D). Nitrate was still present on day 5 in the NO_3^-- and NH_4^+-
13 enriched assemblages, but below the Method Detection Level (MDL) of 0.01 μM in the control
14 and urea-enriched assemblages. Ammonium was initially present at very low concentrations
15 (0.20 μM) in the ambient water collected for the experiment, and remained at concentrations
16 below the MDL of 0.02 μM in all of the non NH_4^+-enriched treatments except for slight
17 increases up to concentrations of 0.05 μM in the NO_3^-- and urea-enriched assemblages on the
18 final sampling day. During the first three days, NH_4^+ declined at the same rate in the NH_4^+-
19 enriched assemblages as the NO_3^- drawdown rate in the NO_3^--enriched assemblages (ca. 6.2-6.4
20 μM d^{-1}), but slowed over the next 2 days until NH_4^+ was fully depleted on day 5 although 2 μM
21 NO_3^- was still present (Fig. 4C). Urea, initially present at 0.15 μM-N in the non-urea enriched
22 treatments, increased over time to an average of 0.74 μM-N (range 0.58–1.05 μM-N) on the final
23 sampling day. Urea did not decline in the urea-enriched assemblages until after day 3, and the

1 natural phytoplankton assemblages instead utilized the high ambient NO_3^- concentrations during
2 the first 3 days of incubation; urea only declined by < 4 µM-N over the course of the experiment,
3 and was still present in abundance (26.1 µM-N) on the final sampling day (Fig. 4D).
4 Concentrations of $Si(OH)_4$, and PO_4^{3-} were present at non-limiting concentrations throughout the
5 experiment (results not shown); on the final day of sampling (day 5) $Si(OH)_4$ averaged 14.7, 9.1
6 and 18.4 µM in NO_3^--, NH_4^+- and urea-enriched assemblages, whereas PO_4^{3-} averaged 1.5, 1.3
7 and 1.6 µM in the respective assemblages. Concentrations in the control assemblages were even
8 greater on their final sampling day (day 6), and averaged 23.6 µM $Si(OH)_4$, and 1.9 µM PO_4^{3-} in
9 the control assemblages where phytoplankton were clearly limited by N availability by day 4.
10
11 *3.2.3. Chlorophyll and Growth rates*
12 For all three N treatments, total chl *a* concentration increased over the duration of the
13 experiment by > 2 fold (Fig. 4 E-H). The average chl *a* concentration (mean ± range of
14 duplicates) for the whole phytoplankton community was slightly greater in the NO_3^- (9.37 ± 0.18
15 µg chl $a{\cdot}L^{-1}$) treatment on day three than either the NH_4^+ (8.22 ± 0.54 µg chl $a{\cdot}L^{-1}$) or the urea
16 (8.31 ± 0.65 µg chl $a{\cdot}L^{-1}$) enriched assemblages (Fig. 4F-H). Although by day five, the biomass
17 achieved by the NO_3^- (10.53 ± 0.11µg chl $a{\cdot}L^{-1}$) and NH_4^+ (10.44 ± 1.75 µg chl $a{\cdot}L^{-1}$) treatments
18 were indistinguishable, whereas the urea-enriched assemblages (9.23 ± 0.32 µg chl $a{\cdot}L^{-1}$)
19 produced 12% less chl *a* L^{-1} than the others. For the larger size (>5-µm) fraction, the biomass
20 achieved on day 3 for the NO_3^- (3.47 ± 0.01 µg chl $a{\cdot}L^{-1}$) and NH_4^+ (3.53 ± 0.24 µg chl $a{\cdot}L^{-1}$)
21 treatments were equal, and only 12-14% slightly greater than the average biomass achieved (3.05
22 ± 0.37 µg chl $a{\cdot}L^{-1}$) in the urea-enriched assemblages (Fig. 4F-H). By day five, chl *a*
23 concentrations in the larger fraction were similar for the three N treatments: 4.60 ± 0.30, 4.96 ±

1 0.46, and 4.99 ± 0.43 µg chl $a \cdot L^{-1}$ for NO_3^-, NH_4^+ and urea assemblages, respectively; the
2 greatest biomass achieved was only 6% greater than the lowest. At this time the ambient N
3 conditions in the NH_4^+-enriched assemblages were low enough (0.15 µM of NH_4^+ and < 2 µM of
4 NO_3^-) to limit phytoplankton growth, whereas the NO_3^-- and urea-enriched assemblages still
5 contained an average of 3.3 µM NO_3^- and 26.1 µM-N urea, respectively.
6 Whole community growth rates, estimated by the increases in total chl *a* over the first 2-d
7 period (days 1 to 3) were: 0.27 ± 0.01, 0.24 ± 0.04 and 0.28 ± 0.007 d^{-1} (mean ± range of
8 duplicate natural cultures) for the NO_3^-, NH_4^+ and urea treatments, respectively. From day 3 to 5,
9 the growth rates in the NO_3^-, NH_4^+, and urea-amended treatments decreased to 0.06 ± 0.004, 0.11
10 ± 0.05, and 0.05 ± 0.02 d^{-1} presumably due to the reduced PAR resulting from the overcast
11 weather conditions during the last days of the experiment and possibly the reduction in N
12 reserves in the NO_3^- and NH_4^+ treatments. Net growth rates for the > 5-µm fraction during the
13 first 2-d period (days 1 to 3) were 0.36 ± 0.01, 0.31 ± 0.10, and 0.22 ± 0.03 d^{-1} for the NO_3^-,
14 NH_4^+ and urea treatments, and although the growth rates for NO_3^--, NH_4^+-enriched communities
15 decreased during the next 2 days to 0.14 ± 0.03 and 0.17 ± 0.01 d^{-1} as external N reserves
16 declined to potentially sub-saturating concentrations, the growth rate for the urea-replete
17 assemblages increased to 0.25 ± 0.10 d^{-1}. Neither the total community growth rates nor the rates
18 for the > 5-µm fraction differed significantly as a function of N treatment during days 1-3 of the
19 experiment (total: $p = 0.57758$, $F_{df=5} = 0.663$; > 5-µm fraction: $p = 0.37731$, $F_{df=5} = 1.3727$) or
20 during the final days 3 - 5 (total: $p = 0.47605$, $F_{df=5} = 0.9603$; > 5-µm fraction: $p = 0.55177$, $F_{df=5}$
21 $= 0.72972$).
22 The estimated net growth rates of *Pseudo-nitzschia* spp. (based on cell abundance over
23 time; Fig. 5) for all three N treatments were consistently greater than both the total and size-

1 fractionated chl-*a* based community growth rates. During the N-replete conditions from day 1 to
2 3, *Pseudo-nitzschia* growth rates averaged 0.77 ± 0.17, 0.65 ± 0.03 and 0.66 ± 0.16 d^{-1} (mean ±
3 range of duplicate natural cultures) for the respective NO_3^-, NH_4^+ and urea treatments, but did not
4 differ significantly as a function of N treatment ($p = 0.78771$, $F_{df=5} = 0.2587$). In the NO_3^-- and
5 NH_4^+ -treated assemblages, *Pseudo-nitzschia* growth rates then decreased over the next two days
6 to 0.51 ± 0.03 and 0.41 ± 0.01 d^{-1} as nutrient concentrations became potentially limiting (2-3
7 µM), but increased to 0.85 ± 0.15 d^{-1} for the N-replete, urea-enriched assemblages. The increased
8 growth rates of the urea assemblages were only significantly greater than the growth rates
9 estimated for NH_4^+- enriched assemblages ($p = 0.039$), and only during these last 2 days were
10 phytoplankton, including *Pseudo-nitzschia* cells supported entirely by growth-saturating
11 concentrations of urea having previously exhausted all of the residual NO_3^- from the source
12 water. In contrast, ambient N concentrations in both the NO_3^- and NH_4^+ assemblages were at
13 levels considered limiting to growth during this latter period.
14

15 *3.2.4. Community Composition*

16 The dominant phytoplankton species in all of the N treatments was the centric diatom
17 *Chaetoceros debilis,* followed by *Pseudo-nitzschia* spp. (Table 2). *Pseudo-nitzschia* spp. never
18 exceeded 8% of the total phytoplankton cellular abundance in these experiments. Other diatoms
19 present in lesser abundances were species of the genus *Skeletonema, Thalassiosira,*
20 *Thalasionema, Navicula, Cylindrotheca, Stephanopyxix,* and other *Chaetoceros* species. *Pseudo-*
21 *nitzschia* abundance increased across all N treatments over time and increased from a minimum
22 of 1.33 ± 0.39 x 10^5 to a maximal density of 2.70 ± 0.41 x 10^6 cells L^{-1} from day 1 to 5 (Fig. 5;

1 Table 2). On day 3, maximal concentrations of *Pseudo-nitzschia* spp. were present in the NH_4^+-
2 enriched assemblages ($6.33 \pm 0.23 \times 10^5$ cells L^{-1}), but by day 5 the greatest densities ($2.70 \pm$
3 0.41×10^6 cells L^{-1}) were observed in the urea-replete assemblages, with lower densities in the
4 NO_3^- and NH_4^+ assemblages due to potentially growth-limiting N concentrations. *Pseudo-*
5 *nitzschia* abundance did not increase beyond day 4 in the controls, apparently due to NO_3^-
6 depletion, and the cell density on the final sampling day was 2-4 fold less than the N-amended
7 assemblages. The species of *Pseudo-nitzschia* identified in this study were divided into three
8 categories: (1) *P. pseudodelicatissima/delicatissima* group (pd/d), which are small and narrow,
9 (2) *P. australis/fraudulenta/heimii* group (a/f/h), which are long and broad, and (3) *P.*
10 *pungens/multiseries* group (p/m) which are long and narrow (Trainer and Suddleson, 2005; Table
11 3). Overall, the smaller *Pseudo-nitzschia* spp. were present in greater abundance than the
12 medium or larger species, and represent > 80% of the pennate diatoms present. Nitrogen
13 treatment did not have any obvious impact on the resultant size distribution of *Pseudo-nitzschia*
14 species present. Note that the 95% confidence limits presented here are relatively large, and are
15 due to the very low densities of *Pseudo-nitzschia* present in the natural assemblages.
16 Nevertheless, despite their relatively low densities, pDA levels were still measurable.
17
18 *3.2.5. Domoic acid*
19 Particulate domoic acid (pDA) concentrations during the N-enrichment experiment were
20 analyzed fresh at sea using the cELISA method, and are presented both volumetrically (Fig. 5),
21 and normalized to total *Pseudo-nitzschia* spp. abundance to estimate cellular DA quotas (pDA
22 $cell^{-1}$; Fig. 6) and are reported below as the mean ± range of duplicates. From day 1 to day 3,
23 ambient concentrations of pDA increased in all of the N enriched assemblages. Among N

1 treatments, the greatest increase (2.8 fold) and highest ambient pDA concentrations (18.8 ± 4.2
2 pg DA mL^{-1}) were measured in the NH_4^+-amended assemblages, followed by the NO_3^- (8.7 ±
3 0.29 pg DA mL^{-1}) and urea-amended assemblages (7.8 ± 0.18 pg DA mL^{-1}) with respective
4 increases of 2.2 and 1.4 fold. NH_4^+-amended assemblages produced significantly more DA than
5 urea-amended assemblages from day 1 to day 3 ($p = 0.0495$), but not more than the NO_3^--
6 amended assemblages ($p = 0.0608$). Even greater increases (4.5 fold) and greater absolute pDA
7 concentrations (37.7 ± 0.27 pg DA mL^{-1}) were measured in the controls on day 4 and 6 following
8 depletion of all available NO_3^-. During the next two days of incubation (from day 3 to 5),
9 average pDA concentrations decreased modestly by ca. 30% in all of the N treatments, except for
10 the urea- enriched assemblages where pDA continued to increase by 2.6-fold under N-replete
11 conditions to a maximal mean value of 19.3 ± 9.0 pg DA mL^{-1} compared to lower pDA
12 concentrations in the NO_3^--(6.0 ± 3.4 pg DA mL^{-1}) and NH_4^+-amended assemblages (13.3 ± 5.93
13 pg DA mL^{-1}). These maximal DA concentrations, however, were not significantly different from
14 DA concentrations measured in NH_4^+- and NO_3^--amended assemblages ($p > 0.05$).
15 Cellular DA concentrations declined in all N-treated assemblages from day 1 to 5: mean
16 decreases of 90, 77 and 84% were observed in the NO_3^-, $NH_4^+$and urea treatments, respectively
17 (Fig. 6). In contrast the mean cellular pDA quotas for the control assemblages only varied
18 slightly throughout the experiment from 44.2 ± 0.13 fg pDA $cell^{-1}$ to 38.7 ± 21.4 fg pDA $cell^{-1}$ as
19 *Pseudo-nitzschia* cell density stopped increasing following N depletion on day 4. Although
20 considerably variable, the concentrations of cellular DA (mean ± range of duplicates) were
21 approximately equal in all of the N treatments by day 1: 35.5 ± 16.9 fg pDA $cell^{-1}$ in the NO_3^--
22 amended assemblages, 39.4 ± 9.3 fg pDA $cell^{-1}$ in the NH_4^+-amended assemblages and 41.4 ±
23 5.0 fg pDA $cell^{-1}$ in the urea-amended assemblages ($p = 0.93467$); concentrations very similar to

1 those found in the controls on day 2 (44.2 ± 0.13 fg pDA $cell^{-1}$). Following another two days of
2 incubation, the concentrations of cellular DA were greatest in the NH_4^+-amended assemblages
3 (29.5 ± 5.5 fg pDA $cell^{-1}$) followed by the urea- (16.2 ± 2.7 fg pDA $cell^{-1}$) and NO_3^- (14.7 ± 0.10
4 fg pDA $cell^{-1}$) assemblages on day 3, but these differences were not statistically significant (p =
5 0.10621). By day 5, the NH_4^+-grown assemblages showed the most cellular DA (9.1± 3.6 fg
6 pDA $cell^{-1}$), followed by urea (6.8 ± 2.3 fg pDA $cell^{-1}$) and NO_3^- (3.6 ± 2.0 fg pDA $cell^{-1}$), and
7 none of the N-amended assemblages were fully depleted in N, except the controls. Again no
8 significant differences were found among the three N treatments (p =0.45685). The highest, but
9 highly variable cellular DA concentrations, were measured in the control (N-depleted)
10 assemblages on the final day of sampling (38.7± 21.4 fg pDA $cell^{-1}$), and were greater than those
11 measured for any of the N-amended assemblages. Among the N treatments, the lowest mean
12 values of cellular DA were observed in the NO_3^--amended assemblages, and the highest in the
13 NH_4^+-amended assemblages during the final two sampling days.
14
15 4. Discussion

16 Nitrogen is the macronutrient responsible for growth limitation of natural phytoplankton
17 assemblages in most coastal systems (e.g., Ryther and Dunstan, 1971; Downing, 1997), and is
18 required for the production of DA - a secondary amino acid (e.g., Bates et al., 1991). Yet
19 previous studies have shown that neither the relationship between N source and the specific
20 growth rate achieved (e.g., Thessen et al., 2009; Auro and Cochlan, 2013), nor the relationship
21 between N source and level of cellular toxicity (e.g., Thessen et al., 2009; Martin-Jézéquel et al.,
22 2015) are uniform among the various species or strains of *Pseudo-nitzschia.* Although the
23 relationship between growth rate and the N substrate supporting cellular growth is relatively

1 straightforward and simple to determine, it can vary as a function of light availability employed
2 during experimentation (Hillebrand and Sommer, 1996; Cho et al., 2001; Auro and Cochlan,
3 2013), and by potential inhibitory or toxic effects of high NH_4^+concentrations (e.g., Hillebrand
4 and Sommer, 1996), the later of which appears to be strain dependent for at least one species of
5 *Pseudo-nitzschia* – *P. multiseries* (c.f. Martin-Jézéquel et al., 2015). Nevertheless, it is still
6 unclear whether cellular toxicity of *Pseudo-nitzschia* species varies as a direct result of the N
7 substrate utilized for growth, or indirectly due to the growth rate achieved on one N substrate
8 versus another. The challenge then, in term of understanding cellular toxicity, is to determine the
9 potential effects of growth rate and N substrate on DA production independent of each other.
10 The present study examined the effects of the three most common N substrates (NO_3^-,
11 NH_4^+ and urea) found in coastal systems in terms of achievable growth rates and cellular toxicity
12 by laboratory cultures of *P. multiseries,* and by a natural assemblage of mixed species of *Pseudo-*
13 *nitzschia* from the U.S. Pacific Northwest. These cultures were examined under both resource-
14 saturated and -limited conditions, and using a variety of culturing systems – N-replete batch
15 cultures, N-limited continuous cultures and N-starved cultures as well as N-amended deck
16 cultures of natural assemblages collected from upwelled waters off the Washington coast in an
17 attempt to unravel the relationship between N source, growth rate achieved and cellular toxicity.
18
19 *4.1 Laboratory experiment*
20 *4.1.1. Laboratory Growth Rates.*
21 The irradiance (120 µmol photons $m^{-2}\cdot s^{-1}$), temperature (13 °C) and N concentrations
22 (40-50 µM-N) employed during the laboratory experiments reported in the present study
23 approximate the conditions expected *in situ* in the Pacific Northwest during the time of collection

1 (e.g., Trainer et al. 2009a, b), and thus can be considered ecologically relevant. Although with
2 the exception of irradiance, exponential growth as a function of temperature or N concentration
3 was not tested here. It should also be noted that although ambient concentrations of NH_4^+ and
4 urea would normally not be found at such at elevated ambient concentrations in natural systems
5 of the PNW, they are provided at the equivalent concentrations expected for NO_3^- in these
6 upwelled waters to assess N preference. Preference for a particular N substrate can be assessed
7 by comparing maximum uptake rates (V_{max}) or maximum growth rates (μ_{max}) for one N substrate
8 in the absence of the other substrates (e.g., Dortch 1990). The N preference experiments for *P.*
9 *multiseries* using N-replete batch cultures demonstrate that NO_3^-, NH_4^+ and urea support
10 statistically identical specific growth rates which averaged 0.83 d^{-1}.

11 These results support the early results of Bates et al. (1991) who found equivalent growth
12 of *P. multiseries* on NO_3^- and NH_4^+ when supplied at relatively low (55 and 110 µM-N)
13 concentrations. Other studies, directly reporting specific growth rates as a function of various N
14 substrates, have shown that both the large-celled *Pseudo-nitzschia* species including *P.*
15 *multiseries* (Hillebrand and Sommer, 1996; Bates et al., 1993; Cho et al., 2001; Calu et al., 2009;
16 Thessen et al., 2009; Martin-Jézéquel et al., 2015), *P. pungens* (Cho et al., 2001), *P. australis*
17 (Cochlan et al., 2006; Howard et al., 2007; Martin-Jézéquel et al., 2015), *P. fraudelenta* (Thessen
18 et al., 2009) as well as the smaller-celled species - *P. calliantha* (Thessen et al., 2009; Garali et
19 al., 2016) and *P. cuspidata* (Auro and Cochlan, 2013) can grow on both inorganic and organic N
20 substrates (Table 4). Generally, it appears that most species can grow equally well on either NO_3^-
21 or NH_4^+, but that urea often supports slower growth, unlike that reported here where urea
22 supported statistically indistinguishable growth rates from those achieved on NO_3^- or NH_4^+.
23 Determining a generalized synopsis of N preference of these published studies is nonetheless

1 difficult given the considerable interspecies and interstrain variability demonstrated by *Pseudo-*
2 *nitzschia*, even for strains isolated from the same water sample (Thessen et al., 2009).
3 The cellular fluorescence capacity of the *P. multiseries* cultures in the present study
4 remained relatively constant in the N-replete batch cultures regardless of the N substrate
5 supporting growth, and did not substantially decline when limited by N availability in the
6 chemostat cultures or when starved of N for 2 days. F_v/F_m determined with using a conventional
7 fluorometer and DCMU averaged 0.65 ± 0.04 (mean ± SD; n= 18) for all of the cultures and
8 indicates that the physiological status, as measured by this method, was relatively unaffected by
9 N sufficiency or N substrate supporting growth. It is not novel that F_v/F_m remained constant
10 during the balanced growth conditions experienced by *P. multiseries* in the N-limited chemostat
11 cultures, and this result agrees with those of Parkhill et al. (2001) for NO_3^--limited chemostat
12 cultures of the neritic diatom *Thalassiosira pseudonana.* They found this measurement to be an
13 inadequate measure of nutrient stress under balanced growth (steady-state) conditions such as
14 ours, whereas under unbalanced growth conditions such as during N starvation, they reported
15 F_v/F_m to decline by ~50% after 2 days of N starvation. We observed only a minor (~5%) decline
16 in the values of F_v/F_m in the N-starved cultures of *P. multiseries* compared to the N-replete batch
17 cultures. Similarly, no decreases were observed for batch cultures of *P. australis* from the mid-
18 exponential growth phase to the late exponential growth phase when N stressed and unbalanced
19 growth conditions could be expected (Howard et al., 2007). These results may indicate that these
20 *Pseudo-nitzschia* species have the ability to utilize N from internal reserves when starved of
21 external nutrients, although internal pools were not measured in either study.
22
23 *4.1.2. Laboratory Domoic Acid.*

1 The maximum concentration of 6.03 fg DA $cell^{-1}$ for *P. multiseries* was measured during
2 urea-replete, exponential growth, and is below the reported range of maximal cellular DA
3 concentrations for *P. multiseries* reported in other studies from around the World (0.02 – 67 pg
4 DA $cell^{-1}$; Trainer et al., 2012). This is likely due to relatively old age of the culture (>2 years
5 since isolation and the initiation of experiments) as DA production usually decreases with time in
6 *Pseudo-nitzschia* cultures (e.g., Kudela et al., 2004; Howard et al., 2007), or perhaps due to the
7 25% diminution in cell size since isolation of our clonal culture of *P. multiseries* (110 to 83 µm
8 in length; B. Bill, pers. comm.). Clonal cultures of pennate diatoms such as *Pseudo-nitzschia* do
9 not have an opposite mating type, and therefore can only reproduce asexually and will become
10 smaller over time (e.g., Davidovich and Bates, 1998). We speculate that the lack of sexual
11 reproductive capability in our cultures and their inability to limit diminution in cell size may
12 have contributed to the decreased concentrations of cellular DA measured in our study. Our
13 reasoning is based on the significant positive relationship found between new large cells
14 resulting from auxosporulation and DA concentrations measured in razor clams on Kalaloch
15 Beach during *Pseudo-nitzschia* blooms in coastal Washington waters (Holtermann et al., 2010).
16 Despite the low cellular quotas in our experiments, DA production varied measurably as a
17 function of N source and of N sufficiency in our study.

18 At all levels of N sufficiency, urea-supported cells of *P. multiseries* produced more
19 particulate DA per cell than those grown on either NH_4^+ or NO_3^-, which did not statistically differ
20 from one another. Although this enhanced toxicity was only statistically greater in the N-limited
21 and N-starved cultures, cellular toxicity was indeed a function of N source, and not a function of
22 the growth rate supported by an individual N source. Nitrate, NH_4^+ and urea all supported
23 indistinguishable specific growth rates during N-replete exponential growth in the batch cultures,

1 during N-limited balanced growth experimentally controlled in the chemostat cultures, or
2 following N-depletion during unbalanced stationary growth in the starved cultures. These results
3 initially appear to support the early findings of Cochlan et al. (2006) and Howard et al. (2007)
4 who first examined toxicity as a function of N source in cultures and natural assemblages of *P.*
5 *australis*. These earlier studies reported that NO_3^- and NH_4^+ supported 2-3 times less DA
6 production (both particulate DA and dissolved DA concentrations) than urea-supported cells
7 during exponential growth, although it should be noted that the urea-grown *P. australis* cells
8 grew almost half as fast as those supported by NO_3^- and NH_4^+, whereas the N-replete growth
9 rates achieved here for *P. multiseries* did not differ by more than 5% regardless of N source. The
10 present results also differ from those obtained for N-replete batch cultures of *P. multiseries* and
11 *P. australis*, where NH_4^+ not urea, produced much greater mean concentrations of pDA per cell
12 during the exponential phase, with lower and indistinguishable cellular pDA quotas for NO_3^--
13 and urea-supported cells (Martin-Jézéquel et al., 2015). Only during the Si-induced stationary
14 phase did urea-grown cells produce substantially greater pDA per cell than the other N sources
15 including arginine, glutamine and glutamate. Unlike our study, they found obvious differences in
16 N preference with NH_4^+ supporting the fastest growth and producing the most toxic cells under
17 N-replete conditions.

18 Domoic acid production as a function of N source has also been measured for other
19 *Pseudo-nitzschia* species, but these studies measured DA either in N-limited continuous cultures
20 (Calu et al., 2009) or in batch cultures after the cells had reached Si-limited, stationary growth
21 (Thessen et al., 2009), and thus are difficult to compare with the present study. In NH_4^+- and
22 NO_3^--grown batch cultures, Bates et al. (1993) found for two clones of *P. multiseries*, NH_4^+-
23 grown cells had either equivalent or 2-3-fold greater DA per cell than NO_3^--grown cultures

1 depending on whether high (220 or 440 µM) or lower (55 and 110 µM) concentrations were
2 employed. In another study of *P. multiseries*, intracellular DA was greater during the early
3 stationary phase for batch cultures grown on urea compared to NO_3^-, but no significant
4 differences were observed for middle and late stationary growth, although the replacement of
5 NO_3^- by urea in continuous cultures of *P. pungens* resulted in slightly increased intracellular DA
6 (Calu et al., 2009). In contrast, an intensive study that examined two strains of *P. multiseries*,
7 five strains of *P. fraudulenta* and two strains of *P. calliantha*, toxicity (measured as total DA,
8 particulate DA and dissolved DA) showed no consistent pattern with N source, and toxicity
9 demonstrated considerable strain and species variability as a function of N source (Thessen et al.,
10 2009).

11 Studies using batch cultures have shown that most species of *Pseudo-nitzschia* are more
12 toxic during the stationary phase of growth, where cell division is slow or absent, compared to
13 the exponential phase of growth where maximal division rates are expected, and DA production
14 rates are low resulting in cellular DA quotas (pDA per cell) that are either low or undetectable
15 (cf., reviews by Lelong et al., 2012; Trainer et al., 2012). In the present study, cellular
16 concentrations of pDA were consistently greater during the exponential growth phase than
17 during N-limited growth achieved in the chemostat cultures or the very slow growth (mean μ =
18 0.21 ± 0.097 d^{-1}) following N-starvation in the stationary phase. This is not surprising since N is
19 required for the production of this secondary amino acid, and unlike other studies where growth
20 was limited by either Si or P, here both the balanced growth achieved in the chemostats or the
21 unbalanced growth in the N-starved cultures was induced by N stress, as the growth medium was
22 specifically designed such that cultures would exhaust the dissolved N (either NO_3^-, NH_4^+ or
23 urea), but remain P and Si replete in order to better emulate the natural macronutrient conditions

1 expected in the episodic upwelling systems off North America (cf. review by Wilkerson and
2 Dugdale, 2008).

3 When comparing the results of our unialgal laboratory experiments to the response(s) of
4 natural phytoplankton in the World's oceans, the continuous and batch cultures employed here
5 suffer in their ecological interpretation as losses from grazing pressure and the
6 sedimentation/sinking of cells are not simulated well, the competitive impacts of other species
7 are not included, and N substrates are only tested independently and not in combination. Still
8 batch cultures can effectively simulate the nutrient-replete condition of cells expected after
9 upwelling events and the unbalanced growth condition as nutrients are exhausted, while
10 continuous cultures offer the unique capability to determine the production of a secondary
11 metabolite such as DA as function of N source independent of cellular growth rate. Using these
12 culturing systems our study clearly demonstrates that the toxicity of *P. multiseries* is regulated by
13 the N source governing growth, where urea supports the greatest cellular toxicity (as measured
14 by pDA per cell) compared to NO_3^- and NH_4^+, and that decreased growth rate resulting from N
15 stress neither enhances cellular toxicity nor alters the change in relative toxicity as a function of
16 N source. Whereas saturating concentrations of urea and NH_4^+ are unlikely to be realized in
17 natural systems resulting in *Pseudo-nitzschia* blooms, except perhaps those regions highly
18 impacted by anthropogenic activities (e.g., Trainer et al., 2007; Howard et al., 2014), low
19 ambient concentrations of these more reduced N substrates may be supplied within the mixed
20 layer by *in situ* heterotrophic regenerative activities or by diffusive flux from deeper waters
21 below (*sensu* Dugdale and Goering, 1967) following the exhaustion of upwelled-supplied NO_3^-
22 reserves, and therefore may contribute to increased DA production and more toxic cells.
23

1 *4.2 Field Experiment*

2 In natural systems, *Pseudo-nitzschia* cells experience a variety of nutrient conditions,
3 ranging from nutrient replete to nutrient deplete for varying periods of time depending on the
4 geographic location and season – degrees of nutrient sufficiency that contribute to the wide
5 variability in cellular growth and toxicity in a given region. The Pacific Northwest (PNW) region
6 off the coast of Washington State and Vancouver Island, British Columbia, includes the Juan de
7 Fuca Eddy and regions of coastal upwelling off Washington State, and is well known for the
8 widespread presence and variable toxicity of many species of toxigenic diatoms, including *P.*
9 *multiseries* (e.g., Trainer et al., 2002; Marchetti et al., 2004). In particular, the cold core Juan de
10 Fuca eddy, a nutrient-rich, physically retentive feature, supports the initiation and growth of
11 toxigenic diatom blooms, (e.g., Trainer et al., 2009a) and can serve as an advective source of
12 toxic *Pseudo-nitzschia* blooms to the Washington State coast (Adams et al., 2006; MacFadyen et
13 al., 2005; 2008). Normally, *Pseudo-nitzschia* species only comprise a minor portion of the total
14 phytoplankton assemblage in the PNW, even during toxic bloom events, although there have
15 been exceptions including massive, dense blooms of *P. cuspidata* (Trainer et al., 2009b) and *P.*
16 *australis* (McCabe et al., 2015).

17 The NO_3^--rich (>10 µM) conditions experienced during our field experiment were typical
18 of Fall upwelling conditions in the PNW, and as expected during incubation experiments,
19 phytoplankton biomass (chl. *a*) increased in all the N treatments, including the un-enriched
20 control assemblages presumably due to increased light availability and reduced grazing pressure
21 (i.e., exclusion of macrozooplankton). Although the natural phytoplankton assemblages were
22 exposed to elevated levels of dissolved N in all of the N treatments, the absolute concentrations
23 of N enrichment ranged from ca. 20 µM-N for NO_3^- or NH_4^+ to 30 µM-N for urea. Despite these

1 differences, the various N substrates were each available at growth-saturating concentrations
2 based on N kinetic parameters derived from short-term N uptake experiments of cultured
3 *Pseudo-nitzschia* species and field assemblages dominated by *Pseudo-nitzschia* (see review by
4 Auro and Cochlan, 2013), thereby permitting a relative comparison of toxicity and growth of
5 *Pseudo-nitzschia* as a function of N source in these field assemblages.
6
7 *4.2.1 Field Growth Rates*
8 The mean growth rates, based on the chlorophyll data of the field study, did not differ as
9 function of N source during the initially N-replete conditions of the deck-board experiment (days
10 1-3). Whereas all N sources supported identical growth rates for the whole phytoplankton
11 assemblage, urea appeared to support slower rates for the larger cells (> 5 µm) during this time.
12 It should be noted that during these first three days both the urea-enriched and the NH_4^+-
13 enriched assemblages contained an abundance of NO_3^- as well, and similar growth rates to those
14 maintained by NO_3^- alone were observed for 5-µm cells later in the experiment when fueled
15 predominately by urea.
16 Howard et al. (2007) also reported a lack of N preference in similarly N-amended natural
17 assemblages conducted off San Francisco, CA, using either the concentrations of chlorophyll or
18 counts of whole cell probes of the dominant toxic diatom – *P. australis* over time, whereas urea
19 supported faster growth rates for Mediterranean Sea assemblages dominated by *P*. cf. *seriata*
20 (Garali et al., 2016). The specific growth rates of the collective species of the *Pseudo-nitzschia*
21 assemblage in the present study were consistently greater than the net growth rates estimated for
22 the total phytoplankton community or the >5 µm fraction; a result also observed in the same
23 region by Olson et al. (2008) during dilution experiments with natural assemblages. As seen for

1 the growth rates calculated from increases in chlorophyll, the mean specific growth rates based
2 on increases in the cellular density of *Pseudo-nitzschia* in the present study did not differ as a
3 function of N source, and all three N substrates supported statistically indistinguishable growth
4 rates over the course of the 5-day incubation experiment, although fastest rates were found for
5 the urea-enriched assemblages.

6 Cell densities increased from 1.33 x 10^5 to 2.70 x 10^6 cells L^{-1} (Table 2), and such
7 concentrations of *Pseudo-nitzschia* spp. have been reported previously along the Washington
8 (e.g., Bill et al., 2006; Trainer et al., 2009a), and further south along the U.S. West coast during
9 toxic events (e.g., Schnetzer et al., 2007; 2013; Du et al., 2016). Despite exceeding
10 concentrations of *ca.* $\geq 10^5$ cells L^{-1} - generally considered a threshold density for major *Pseudo-*
11 *nitzschia* blooms in the PNW (e.g., Trainer et al., 2007), and southern California (e.g., Seubert et
12 al., 2013), the phytoplankton community consisted primarily of the centric diatom *Chaetoceros*
13 *debilis* which averaged 89 % of the total cell abundance within 2 days of incubation, followed by
14 *Pseudo-nitzschia* spp. (8 %) and various flagellates (1.6 %); the relative species composition
15 changed very little over the remainder of the experiment. As reported previously in this region
16 *Pseudo-nitzschia* spp. are not the dominant diatom in these waters in terms of biomass (Trainer
17 et al., 2002, 2008, 2009a; Olson et al., 2008), and averaged only 8% of the abundance of
18 phytoplankton cells in the natural assemblage of the field experiment.

19

20 *4.2.2. Field Domoic acid*

21 Cellular DA levels decreased in all of the N-enrichments over time in the deck-board
22 incubation study. These results are in contrast to those of Howard et al. (2007), where pDA
23 normalized to chl *a* increased over time across all N treatments including the control, where no N

was added. Cellular DA concentrations in our coastal Washington incubation experiment were low, and ranged from values of 2 to 60 fg DA $cell^{-1}$ compared to much greater values reported for Washington waters including maximal cellular concentrations of 20-63 pg DA $cell^{-1}$ for *P. cuspidata* blooms, 0.5 pg DA $cell^{-1}$ during coastal blooms of *P.* cf. *delicatissima* (Adams et al., 2000), 35 pg DA $cell^{-1}$ for inland waters of WA (Bill et al., 2006), and 17-76 pg DA $cell^{-1}$ for *P. pseudodelicatissima* blooms in Sequim Bay following anthropogenic inputs of NH_4^+ (Trainer et al., 2007).

The toxin response in the present experiment to N enrichment cannot be attributed solely to a specific N source due to the availability of multiple N substrate at any given time; however following day 1, the NH_4^+-amended assemblages produced the highest cellular DA quotas throughout the remainder of experiment followed by the urea-amended assemblages and lastly those growing solely on NO_3^- (Figure 6). Similarly, urea- and NH_4^+-amended phytoplankton resulted in the greatest chlorophyll-normalized pDA quotas during the San Francisco experiment dominated by *P. australis*, (Howard et al., 2007) during N-replete and optimal light conditions. In addition here we found that N stress, at least for NO_3^- and NH_4^+, did not enhance cellular toxicity, presumably because N is needed for DA production whereas cell division can continue, albeit at a slower rate, provided other macronutrients are still found in abundance. These results differ from the San Francisco experiment in that we measured the greatest cellular DA quotas at the beginning rather than the end of these multi-day experiments when *Pseudo-nitzschia* cells were accustomed to growth on high ambient concentrations of NO_3^-. Still, in both of the these natural assemblage experiments, the overall toxin response to N enrichment under optimal light conditions was greater cellular toxicity in diatoms grown on reduced N forms (NH_4^+ and urea) rather than those grown exclusively on NO_3^-.

1

2 *4.2.3. Utilization of N sources*

3 Ammonium inhibition of NO_3^- uptake, and NO_3^- inhibition of urea uptake were clearly
4 demonstrated during the first three days of the deckboard experiment. During this period < 10%
5 of the NO_3^- was utilized in the NH_4^+-enriched assemblages compared to ca. 13 µM of NH_4^+, and
6 ca. 12 µM of NO_3^- in the solely NO_3^--enriched assemblages. Inhibition of NO_3^- uptake by NH_4^+
7 has been well studied in both natural assemblages and cultured populations of phytoplankton (cf.
8 reviews by Dortch, 1990; Cochlan and Bronk, 2003), and in its most extreme form is thought to
9 result in the complete cessation of NO_3^- utilization above a certain and highly variable threshold
10 concentration. Here, simultaneous utilization of NO_3^- and NH_4^+ was not observed until the
11 ambient NH_4^+ concentrations declined to ca. 5 µM. Similarly, urea uptake was completely
12 inhibited by NO_3^- in the urea-enriched assemblages until an even lower threshold of ca. 2 µM
13 NO_3^- was reached followed by simultaneous utilization of both N sources. Interactions between
14 urea and other N forms have been studied less, but it is generally thought from unialgal culture
15 studies that urea suppresses NO_3^- uptake, but to a lesser degree than NH_4^+, not the other way
16 around (e.g., Molloy and Syrett, 1998; Cochlan and Harrison, 1991b). NO_3^- inhibition of urea
17 uptake has been reported previously in field culture studies using coastal phytoplankton
18 assemblages off southern California (McCarthy and Eppley, 1972), and the inland waters of
19 British Columbia (frontal waters in the Strait of Georgia; Price et al., 1985) as well as Bizerte
20 Lagoon (SW Mediterranean Sea) when dominated by *P.* cf. *seriata* (Garali et al., 2016) during
21 similar N- enrichment experiments. Such interactions among the N substrates used to fuel the
22 actual growth of these mixed *Pseudo-nitzschia* assemblages cannot be determined from our

1 study, and inhibitory effects of one substrate on its utilization by the *Pseudo-nitzschia*
2 assemblages are unknown.
3

4 ## 5. Conclusions

5 The toxic threat of DA to coastal ecosystems and the health of marine mammals, birds
6 and humans, is directly linked to the concentration of particulate DA (pDA) - the form of DA
7 that is directly transferable to the food chain. Environmental conditions that permit rapid growth
8 of *Pseudo-nitzschia* cells, or conditions that trigger greater biosynthesis rates of DA will increase
9 this toxic threat by producing elevated concentrations of pDA, since the volumetric
10 concentration of pDA is defined by the number of *Pseudo-nitzschia* cells per volume multiplied
11 by the DA concentration per cell (or DA cellular quota). Here, using laboratory culture
12 experiments of a PNW isolate of *P. multiseries*, and a field experiment using the natural
13 phytoplankton assemblage from the coastal waters of the PNW, it is clearly demonstrated that all
14 three commonly found N substrates support statistically indistinguishable specific growth rates,
15 and that urea does not support slower growth under optimal resource conditions. Although pDA
16 production demonstrates variability as a function of N source, cellular DA levels were
17 consistently greater in urea-grown *P. multiseries* cultures than either NH_4^+- or NO_3^--grown
18 cultures at all levels of N sufficiency – N-replete, limited and starved.

19 In the field experiment, *Pseudo-nitzschia* species, dominated by the short and narrow *P.*
20 *pseudodelicatissima/ delicatissima* group (pd/d), were only a minor portion of the natural
21 community experiments, so it is difficult to ascribe their impact on the N utilization and
22 dynamics observed in the deck incubation study. Despite low cell densities of *Pseudo-nitzschia*,
23 cellular toxicity still differed as a function of N enrichment; although not statistically significant,

1 pDA $cell^{-1}$ was greatest in the NH_4^+-enriched natural assemblages followed by the urea- and
2 NO_3^--enriched assemblages. These results highlight the potential importance of these reduced N
3 substrates in understanding the threat of DA toxicity in natural systems. Although their ambient
4 concentrations are not sufficiently elevated to support bloom concentrations of *Pseudo-nitzschia*
5 in the PNW, their impact on DA production and cellular growth cannot be ignored, especially
6 during periods of N limitation where these substrates likely maintain seed populations of these
7 toxic diatoms. Furthermore, there are coastal upwelling regions further south of the PNW that are
8 decidedly impacted by anthropogenic inputs of reduced N sources, including wastewater effluent
9 (composed of primarily NH_4^+) from sewage treatment facilities in highly urbanized regions of
10 southern California (Howard et al., 2014), and riverine runoff from agricultural activities in
11 northern California (e.g., Kudela et al., 2007).

12 Nitrification rates are usually sufficiently high in southern California to rapidly oxidize
13 ammonium to nitrate in close proximity to effluent outfalls (McLaughlin et al., 2017), thus
14 limiting the availability of NH_4^+ at elevated concentrations to coastal phytoplankton. However
15 nitrification, an obligately aerobic process, is highly sensitive to hypoxia and acidification, both
16 of which are expected to intensify in the California upwelling system as a result of climate
17 change (c.f. review by Capone and Hutchins, 2013) potentially resulting in decreased
18 nitrification rates and increased availability of NH_4^+ to support growth and domoic acid
19 production by *Pseudo-nitzschia* species. Future impacts on N cycling by other co-varying
20 stressors associated with climate change are likely to impact N speciation in these coastal waters
21 with relatively unknown, but probably highly variable effects on diatom toxicity in the coastal
22 upwelling waters along the west coast of the United States, and in the other coastal upwelling
23 systems associated with eastern boundary currents of the Pacific and Atlantic Oceans.

1

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1 Table 1. Concentrations of *Pseudo-nitzschia multiseries* and chlorophyll *a* for the replicate

2 laboratory cultures of each nitrogen treatment at the time of domoic acid sampling.

3 (-- indicates no data available)

4

Treatment	Chlorophyll *a* ($\mu g\ L^{-1}$)	Cells (x 10^3 mL^{-1})
N-replete (batch)		
Nitrate-1	--	46.9
Nitrate-2*	--	50.9
Ammonium-1	--	35.5
Ammonium-2*	--	30.9
Urea-1	--	42.8
Urea-2*	--	24.1
N-limited (continuous)		
Nitrate-1	62.8	93.1
Nitrate-2	65.3	67.3
Ammonium-1	53.8	103.9
Ammonium-2	40.6	101.5
Urea-1	60.0	55.5
Urea-2	61.1	75.7
N-depleted (batch)		
Nitrate-1	--	13.7
Nitrate-2	--	10.1
Ammonium-1	--	14.5
Ammonium-2	--	14.4
Urea-1	--	12.6
Urea-2	--	9.18

5

6 * batch culture used to initiate duplicate continuous cultures of the same N treatment

Table 2. Cell abundance (cells L^{-1}) of dominant phytoplankton over the 5-d incubation period of the Washington coast N-enrichment experiment for the three N treatments (NO_3^-, NH_4^+ and urea). Other diatoms present include species of *Skeletonema, Thalassiosira, Thalasionema, Navicula, Cylindrotheca, Stephanopyxix,* and *Chaetoceros* other than *C. debilis*. Flagellates include species of *Dinophysis, Protoperidinium, Dictyocha, Gymnodinium,* and various tintinids. Species were identified using phase-contrast light microscopy, and estimates are the average of the duplicate (n = 2) deck-board cultures of natural assemblages.

Sampling Day	N Treatment	*Pseudo-nitzschia* spp.	*Chaetoceros debilis*	Flagellates	Other diatoms
0	-------------	2.25×10^4	9.76×10^5	4.55×10^5	7.25×10^4
1	Nitrate	1.33×10^5	1.72×10^6	2.34×10^4	7.81×10^3
	Ammonium	1.72×10^5	2.68×10^6	4.69×10^4	0
	Urea	1.33×10^5	1.74×10^6	3.91×10^4	4.69×10^4
3	Nitrate	5.94×10^5	1.11×10^7	2.66×10^5	7.03×10^4
	Ammonium	6.33×10^5	1.07×10^7	1.33×10^5	1.80×10^5
	Urea	4.92×10^5	7.63×10^6	7.81×10^4	2.19×10^5
5	Nitrate	1.64×10^6	1.84×10^7	1.02×10^5	2.19×10^5
	Ammonium	1.43×10^6	2.75×10^7	1.09×10^5	6.25×10^5
	Urea	2.70×10^6	2.39×10^7	1.88×10^5	1.64×10^5

Table 3. Cellular abundance of *Pseudo-nitzschia* species identified and counted by phase-contrast light microscopy for the four sampling days of the Washington coast N-enrichment experiment for the three N treatments (NO_3^-, NH_4^+ and urea) and their upper and lower 95% confidence limits (Ricker, 1937 as used in Lund et al., 1958). Classifications are: **pd/d**: *Pseudo-nitzschia pseudodelicatissima/delicatissima* group (short and narrow), **a/f/h:** *Pseudo-nitzschia australis/fraudulenta/heimii* group (long and broad), and **p/m**: *Pseudo-nitzschia pungens/multiseries* group (long and narrow). Cell densities and 95% confidence limits are estimated from the means of duplicate (n =2), deck-board cultures of natural assemblages.

Sampling Day	N Treatment	pd/d cell Abundance	Lower Limit (%)	Upper Limit (%)	a/f/h cell Abundance	Lower Limit (%)	Upper Limit (%)	p/m cell Abundance	Lower Limit %	Upper Limit %
0	----------	2.67×10^4	43	42	0	0	100	2.00×10^4	37	84
1	Nitrate	1.17×10^5	53	53	0	0	100	1.56×10^4	34	88
	Ammonium	1.72×10^5	46	46	0	0	100	0	0	100
	Urea	1.17×10^5	53	53	1.56×10^4	35	88	0	0	100
3	Nitrate	5.86×10^5	28	28	7.81×10^3	29	92	0	0	100
	Ammonium	6.02×10^5	14	28	3.13×10^4	69	75	0	0	100
	Urea	4.30×10^5	33	32	6.25×10^4	63	64	0	0	100
5	Nitrate	1.46×10^6	19	19	1.41×10^5	50	50	3.91×10^4	32	80
	Ammonium	1.31×10^6	10	20	9.38×10^4	28	56	2.34×10^4	91	80
	Urea	2.48×10^6	15	15	1.25×10^5	52	52	9.38×10^4	61	58

Table. 4. Exponential growth rate (μ; d^{-1}) of *Pseudo-nitzschia* species as a function of N source. Growth rates were determined in N-replete batch cultures, and reported as the mean ± one standard deviation (SD) where reported or calculated. -- indicates not determined or reported.

Species/ Strain No.	N conc. (μM-N)	PPFD[1] / L:D cycle	NO_3^-	NH_4^+	Urea	Origin of Isolate	Reference
P. multiseries							
CLN47	88	150-200 /14:10	0.65 ± 0.018	0.61 ± 0.054	0.68 ± 0.070	Eastern Canada	Thessen et al. (2009)
Pn-1	88	150-200 /14:10	0.76 ± 0.104	0.76 ± 0.175	0.30 ± 0.100	Choptank R., MD, USA	Thessen et al. (2009
NWFSC-245	42-51	120 / 24:0	0.85 ± 0.027	0.83 ± 0.030	0.81 ± 0.074	Sequim Bay, WA, USA	Present Study
Inje Univ.	100	100 / 16:8	0.32	0.33	0.33	Chinhae Bay, Korea	Cho et al. (2001)[2]
	200	100 / 16:8	0.31	0.29	0.22		
KP 84	200-300[3]	230 / 14:10	0.49 ± 0.063	no growth	--	Prince Edward Island, CAN	Hillebrand & Sommer (1996)[4]
	200	25 / 14:10	0.54 ± 0.170	0.35 ± 0.021	0.56 ± 0.057		
CCL70	440/441	--- / 12:12	0.56 ± 0.06	--	0.67 ± 0.07	Thames Estuary, UK	Calu et al. (2009)
CCL70	440	110 / 12:12	0.80 ± 0.45	1.12 ± 0.17	0.60 ± 0.01	Thames Estuary, UK	Martin-Jézéquel et al. (2015)
P. pungens							
Inje Univ.	100	100 / 16:8	0.29	0.29	0.33	Chinhae Bay, Korea	Cho et al. (2001)[2]
	200		0.27	0.28	0.18		
P. australis							
AU221-a	50	100 / 12:12	0.89 ± 0.077	0.93 ± 0.001	0.52 ± 0.099[5]	Monterey Bay, CA, USA	Howard et al. (2007)
DOMA-1	54	22 / 12:12	0.80			Monterey Bay, CA, USA	Garrison et al. (1992)
DOMA-2	54	32 / 12: 12	0.82				
PNC1	440	110 / 12:12	0.48 ± 0.004	0.56 ± 0.004	0.44 ± 0.014	Bay of Crozon, Atlantic coast, France	Martin-Jézéquel et al. (2015)
cultures 1-4	760	12 / 16:8	0.78 ± 0.092	--	--	South coast of Ireland	Cusack et al. (2002)
cultures 5-6	760	115 / 12:12	0.84 ± 0.148	--	--		
P. calliantha							
Pn-3	88	150-200 /14:10	0.55 ± 0.047	0.87 ± 0.154	0.42 ± 0.056	Choptank R., MD, USA	Thessen et al. (2009)
Pn-13	88	150-200 /14:10	0.72 ± 0.071	0.71 ± 0.064	0.44 ± 0.028	Choptank R., MD, USA	

--	10-40[6]	100 / 12:12	0.84[7]	0.95[7]	1.03[7]	Bizerte Lagoon, Tunisia SW Mediterranean Sea	Garali et al. (2016)
P. fraudulenta							
Pn-9	88	150-200 /14:10	0.75 ± 0.051	0.62 ± 0.038	1.02 ± 0.137	Assateague I., MD, USA	Thessen et al. (2009)
Pn-10	88	150-200 /14:10	0.84 ± 0.035	0.85 ± 0.058	0.58 ± 0.067	Assateague I., MD, USA	
Pn-11	88	150-200 /14:10	0.73 ± 0.036	1.22 ± 0.177	0.55 ± 0.103	Assateague I., MD, USA	
Pn-12	88	150-200 /14:10	0.75 ± 0.039	0.88 ± 0.048	0.58 ± 0.181	Assateague I., MD, USA	
Pn-15	88	150-200 /14:10	1.05 ± 0.033	1.16 ± 0.020	1.05 ± 0.030	Asilomar, CA, USA	
P. cuspidata	40	120 / 24:0	0.88 ± 0.024	0.89 ± 0.036	0.83 ± 0.030	Offshore Washington State,	Auro and Cochlan
NWFSC-221	40	40 / 24:0	0.55 ± 0.028	0.51 ± 0.048	0.64 ± 0.006	USA	(2013)
P.* cf. *seriata field assembl.	10-40[6]	~1,850	0.34 ± 0.015	0.22 ± 0.022	0.37 ± 0.039	Bizerte Lagoon, Tunisia SW Mediterranean Sea	Garali et al. (2016)
P. fryxelliana NWFSC-241	40	120 / 24:0	1.30 ± 0.134	--	--	Coastal Washington USA	Auro and Cochlan (2013)

[1]Photosynthetic photon flux density (PPFD) measured in μmol photons $m^{-1}\cdot s^{-1}$

[2] Growth rates are estimated from rates (divisions$\cdot d^{-1}$) reported in figures of Cho et al. (2001)

[3] NO_3^- conc. = 200 μM; NH_4^+ conc. = 300 μM

[4] Growth on glutamine = 0.74 ± 0.127 d^{-1}

[5]As reported/corrected in Auro & Cochlan (2013); incorrect SD provided in Howard et al. (2007)

[6]NO_3^- conc. = 40 μM; NH_4^+ conc. = 10 μM; Urea conc. = 40 μM-N as provided in Garali et al. (2016)

[7]Growth rates in text/table differ from those reported in the abstract of Garali et al. (2016): NO_3^- = 0.90 d^{-1}; NH_4^+ = 0.80 d^{-1}; urea = 1.50 d^{-1}

1 Figure Legends

2

3 Figure 1. The specific growth rate (μ; d^{-1}) of *P. multiseries* (NWFSC-245) as a function of
4 photosynthetic photon flux density (PPFD) for cells grown on enriched, sterile-filtered
5 seawater and 40 μM of NO_3^- as the sole N source. Values are the means of duplicate
6 cultures (n= 2); error bars represent the range of duplicates.

7

8 Figure 2. Cell density of *P. multiseries* as a function of time for duplicate cultures grown on:
9 NO_3^- (A), NH_4^+ (B) and urea (C). Insert graphs are semi-log plots of cell abundance versus
10 time during the exponential phase of growth. Dashed lines denote the least-squares, linear
11 regressions for culture one (○) and culture two (●), and their corresponding coefficients of
12 determination (r^2); slopes of these lines were used to calculate specific growth rates (μ; d^{-1}) as
13 a function of N source. Line equations are: nitrate 1 & 2: $y = 3.688 + 0.8690x$, $y = 4.046 +$
14 $0.8421x$; ammonium 1 & 2: $y = 1.946 + 0.8564x$, $y = 4.407 + 0.8087x$; urea 1 & 2: $y = 3.888 +$
15 $0.861x$, $y = 3.733 + 0.7563x$. Note the change in scale of ordinal axes.

16

17 Figure 3. Particulate domoic acid (pDA) concentrations for laboratory cultures of *P.*
18 *multiseries* as a function of N source (NO_3^-, NH_4^+ and urea). Concentrations are reported
19 volumetrically (left plots; pg pDA·mL^{-1}) and normalized to cellular density (right plots;
20 fg pDA·$cell^{-1}$) in N-replete, batch cultures during mid-exponential growth (A, D), N-limited
21 chemostat cultures during steady-state conditions (B, E), and N-depleted chemostat cultures
22 'starved' of nitrogen for 2 days (C, F). Values are the means of duplicate (n=2) cultures;
23 error bars represent the range of analytical triplicates from each culture. Note the change in

1 scale of the ordinal axes.

2

3 Figure 4. Time-course measurements of coastal Washington natural phytoplankton

4 assemblages incubated under simulated *in situ* conditions over six days. The draw-down of

5 nitrate (●), ammonium (○) and urea (△) in deck-board cultures with no addition of nitrogen

6 (A; 'control'), nitrate enriched (B), ammonium enriched (C), and urea enriched (D). Size-

7 fractionated (< 5 µm; > 5 µm) and total chlorophyll *a* are reported in addition to total

8 phytoplankton cell density ( )for cultures grown with no additional nitrogen added (E), or the

9 addition of nitrate (F), ammonium (G), or urea (H). Reported values are the averages of

10 biological replicate (n=2) cultures. Error bars represent the range of duplicate deck-board

11 cultures; no error bars indicate errors are smaller than the symbol size.

12

13 Figure 5. Concentrations of particulate domoic acid (pDA) and *Pseudo-nitzschia* (*PN*) cell

14 density (•) in deck-board cultures of coastal Washington natural phytoplankton assemblages

15 over time. Biological duplicate (n=2) cultures with no addition of nitrogen (A; 'control'), or

16 enriched with nitrate (B), ammonium (C) or urea (D). Values for pDA are reported as the

17 average of analytical (n=3) measurements of pDA from each of the biological replicate

18 natural assemblage cultures; except for days 5 and 6 when pDA was averaged using duplicate

19 analytical measurements; error bars for pDA are the range of the analytical measurements. *P.*

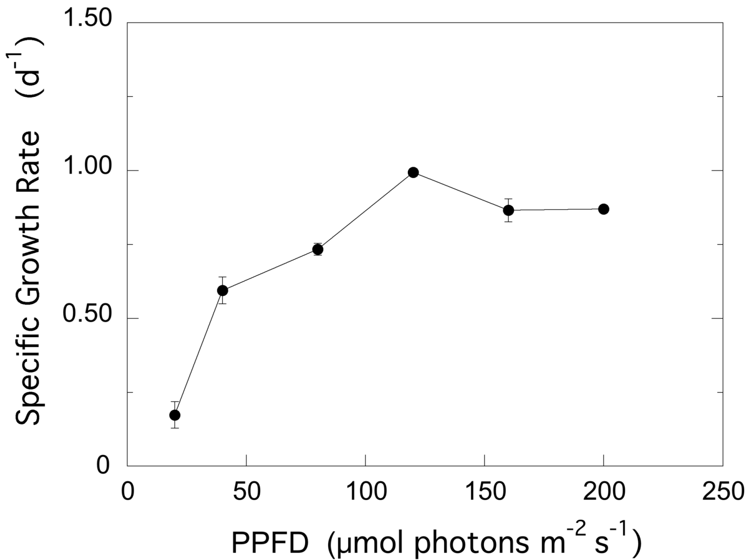
20 *nitzschia c*ell density is reported as the average of the duplicate, deck-board natural

21 assemblage cultures; errors bars are the range of replicate cultures. No error bars for all

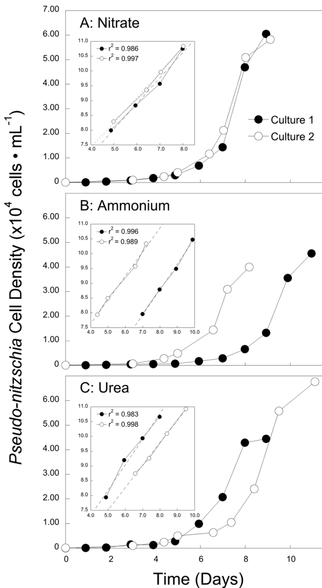
22 measurements indicate that errors are smaller than the symbol size.

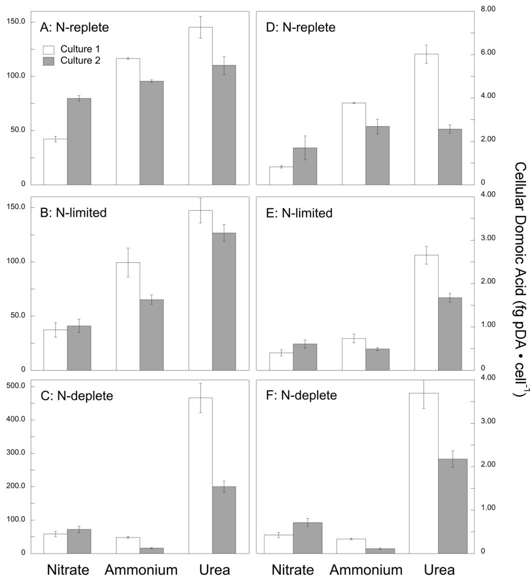
23

1 Figure 6. Cellular domoic acid (pg DA cell $^{-1}$) of deck-board cultures of coastal Washington
2 natural assemblages over time. Biological duplicate (n=2) cultures with no addition of nitrogen
3 (A; ‘control’), or enriched with nitrate (B), ammonium (C) or urea (D). Values are the mean of
4 analytical (n=3) measurements of pDA divided by the cell density from each biological
5 replicate culture; except for days 5 and 6 when only duplicate analytical measurements were
6 used. Error bars are the range of analytical replicates divided by the cell density. No error bars
7 indicate that errors that are smaller than the symbol size.
8



1.50
1.00
0.50
0
0
50
100
150
200
250
Specific Growth Rate (d-1)
PPFD (μmol photons m-2 s-1)


A: Nitrate
B: Ammonium
C: Urea
Culture 1
Culture 2
r² = 0.986
r² = 0.997
r² = 0.996
r² = 0.989
r² = 0.983
r² = 0.998
Pseudo-nitzschia Cell Density (x10⁴ cells • mL⁻¹)
Time (Days)



A: N-replete
Culture 1
Culture 2
B: N-limited
C: N-deplete
D: N-replete
E: N-limited
F: N-deplete
Particulate Domoic Acid Concentration (pg pDA • mL-1)
Cellular Domoic Acid (fg pDA • cell-1)
150.0
100.0
50.0
0
500.0
400.0
300.0
200.0
8.00
6.00
4.00
2.00
3.00
1.00
Nitrate
Ammonium
Urea

A
Nitrate
Ammonium
Urea

B

C

D

Nitrate (µM)

Ammonium (µM)

Urea (µM-N)

Time (Days)

E
Chl *a* < 5 µm
Chl *a* > 5 µm
Total Cell Density

F

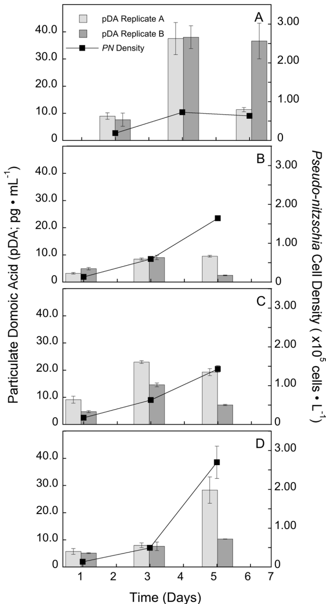
G

H

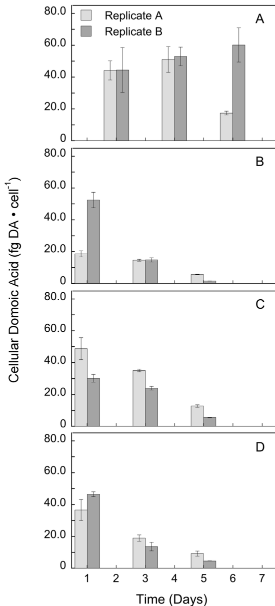
Chlorophyll *a* (µg • L^{-1})

Total Phytoplankton Cell Density (x10^{7} cells • L^{-1})

Time (Days)



pDA Replicate A
pDA Replicate B
PN Density
A
B
C
D
40.0
30.0
20.0
10.0
0
3.00
2.00
1.00
0
Particulate Domoic Acid (pDA; pg • mL-1)
Pseudo-nitzschia Cell Density (x105 cells • L-1)
1
2
3
4
5
6
7
Time (Days)



Replicate A
Replicate B
A
B
C
D
80.0
60.0
40.0
20.0
0
Cellular Domoic Acid (fg DA • cell^{-1})
1
2
3
4
5
6
7
Time (Days)