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Differential toxin response of *Pseudo-nitzschia multiseries* as a function of nitrogen speciation in batch and continuous cultures, and during a natural assemblage experiment

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ABSTRACT

The toxigenic diatom *Pseudo-nitzschia multiseries* Hasle, isolated from the U.S. Pacific Northwest, was examined in unialgal laboratory cultures and in natural assemblages during shipboard experiments, to examine cellular growth and domoic acid (DA) production as a function of nitrogen (N) substrate and availability expected during bloom development and decline. Laboratory experiments utilizing batch cultures conducted at saturating (120 μmol photons m^{-2} s^{-1}) photosynthetic photon flux density (PPFD), demonstrated that *P. multiseries* (strain NWFSC-245) grows equally well on the three N substrates tested (nitrate [NO_3^-], ammonium [NH_4^+] and urea), and achieved an average specific growth rate of 0.83 d^{-1}. Despite equivalent growth rates, cellular toxicity (particulate DA concentration normalized to cell abundance) varied as a function of N substrate, with urea-grown cells demonstrating 1.3- and 3.4-fold more toxicity than both NH_4^+- and NO_3^--grown cells. Cellular toxicity of the N-limited chemostat cultures, grown at a dilution rate of 0.48 d^{-1}, were less than the cellular toxicity measured for the N-replete batch cultures for all three N substrates, but again cellular toxicity varied as a function of N substrate and the urea-supported cells were 3.5- and 4.3-fold more toxic than the respective NH_4^+- and NO_3^--supported cells. Starved cultures of *P. multiseries* showed no decline in cellular toxicity or change in the order of toxicity as a function of N substrate, and cells previously supported by urea were 13- and 5-fold more toxic than NH_4^+- and NO_3^--supported cells. At all three levels of N-sufficiency, the urea-grown cells consistently produced the highest concentration of particulate DA per cell compared to cells grown on either NO_3^- or NH_4^+.

Shipboard N enrichment experiments using natural phytoplankton assemblages were conducted off the west coast of Washington in an area characterized by elevated concentrations of macronutrients and iron. All N (NO_3^-, NH_4^+ and urea) treatments showed significant increases in biomass (as measured by total and size-fractionated chlorophyll *a*) and the abundance of *Pseudo-nitzschia* species over the 6-d experiment. As with the unialgal laboratory experiments, cellular toxicity varied as a function of the N source supporting growth, and the planktonic assemblages enriched with either NH_4^+ or urea demonstrated greater cellular toxicity than the assemblages supported solely by NO_3^-. These laboratory and field results demonstrate that N substrate can regulate the toxicity of *Pseudo-nitzschia* species, and that N source should be considered when evaluating the potential effects of cultural eutrophication on the growth of toxigenic diatoms.

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1. Introduction

Diatoms of the *Pseudo-nitzschia* genus Pergallo (Heterokonta, Bacillariophyceae) have been the subject of numerous field and culture studies since the 1987 discovery of domoic acid (DA) production by *Pseudo-nitzschia multiseries* (then termed *Nitzschia pungens* f. *multiseries* Hasle, 1995) resulting in the first report of amnesic shellfish poisoning (ASP) in Atlantic Canada (Subba Rao et al., 1988; Bates et al., 1989). Since then, the number of species of

Abbreviations: N, nitrogen; NH_4^+, ammonium; NO_3^-, nitrate; DCMU, 3-[3,4-dichlorophenyl]- 1,1-dimethylurea; DA, domoic acid; pDA, particulate domoic acid; cELISA, indirect cellular enzyme-linked immunosorbent assay; PPFD, photosynthetic photon flux density; chlorophyll *a*, chl *a*.

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Pseudo-nitzschia thought to synthesize DA has increased to 26 (Lundholm, 2017) worldwide, and at least ten of these species are reported in the coastal waters of the West Coast of North America (Trainer et al., 2012). The environmental factors that regulate the growth of these toxigenic cells and/or their production of this potent neurotoxin by *Pseudo-nitzschia* have been reviewed in detail (Pan et al., 1998; Lelong et al., 2012; Trainer et al., 2012), and it is clear that many of the factors needed to promote the growth of these toxigenic cells, such as adequate light and macronutrients, are not necessarily those responsible for enhancement of DA synthesis or its cellular accumulation; in fact the reverse can be true.

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

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

Until recently the majority of N studies of *Pseudo-nitzschia* species have been conducted using batch cultures supplied with N in excess of the cellular requirements to support maximal growth rates. These N-replete batch studies have demonstrated that all three of the N substrates commonly found in marine and estuarine waters: nitrate [NO_3^-], ammonium [NH_4^+] and urea, can adequately support the growth of *Pseudo-nitzschia* species tested to date, with N preference varying widely among species and even between strains of the same species (e.g., Thessen et al., 2009 and references therein). In one such study, using a *P. australis* strain isolated from Monterey Bay, CA, the slower growing cells supported by urea were 3- to 5-fold more toxic than the faster growing NO_3^--, and NH_4^+-supported cells (Cochlan et al., 2006; Howard et al., 2007). Similar results of urea-enhanced DA production have been reported for nutrient-amended deck 'grow-out' experiments using natural assemblages dominated by *P. australis* off San Francisco Bay (Howard et al., 2007) and by *P.* cf., *seriata* in Bizerte Lagoon in the SW Mediterranean Sea (Garali et al., 2016). Apparently the toxicity of *Pseudo-nitzschia* species may be influenced by a number of factors, including N source, cellular phase of growth, or the growth rate achieved by the cells, but it is still unknown whether cellular toxicity varies as a direct result of the N substrate utilized for growth, or indirectly due to the growth rate achieved on one substrate versus another. The challenge then is to determine the potential effects of growth rate and N substrate on DA production independent of each other.

Continuous cultures are highly controlled systems that provide a dynamic equilibrium between nutrient input and growth, and can be used to determine DA production as a function of N source independent of the potential growth rate effects. Unlike batch cultures that have a continuously changing environment, continuous cultures provide a constant growth environment (Rhee, 1980) where cells are maintained in exponential growth phase independent of time, and the effects of one environmental parameter can be assessed while holding all the others constant (Herbert et al., 1956; Rhee, 1980). In the present study, both batch cultures and continuous cultures were employed to assess the effects of NO_3^-, NH_4^+ and urea on the cellular toxicity of *Pseudo-nitzschia multiseries* Hasle at three degrees of N sufficiency: N-replete, N-limited and N- depleted. In the continuous culture systems used, the growth rates achieved by three different N sources were equal and controlled by the dilution rate set by the experimenter. When specific growth rate and dilution rate are balanced, phytoplankton biomass remains constant over time, and the system is considered to be in steady-state. Such continuous cultures, termed chemostats (e.g., MacIntyre and Cullen, 2005), were used to examine differential toxicity as a function of N substrate independent of the growth achieved on the different N substrates. These N-limited chemostats were then allowed to starve without N amendment to examine toxicity in N-depleted cells; a situation normally expected following bloom decline in natural marine systems.

To assess the impact of N sources on natural *Pseudo-nitzschia* species, field studies were conducted with phytoplankton assemblages collected off Washington in macronutrient- and micronutrient-replete coastal waters. A multi-day, deck-board incubation experiment was conducted to determine the differential growth and DA toxin response of natural assemblages of *Pseudo-nitzschia* after N amendment by NO_3^-, NH_4^+ or urea. These field studies were conducted as part of the ECOHAB-PNW (Ecology and Oceanography of Harmful Algal Blooms in the Pacific Northwest) project investigating the physiology, toxicology, ecology and oceanography of toxic *Pseudo-nitzschia* species off the Pacific coast of Washington and British Columbia.

2. Materials and methods

2.1. Cell culturing

Pseudo-nitzschia multiseries culture NWFSC-245, isolated from Sequim Bay, WA by B. Bill in June 2006, was used for the batch and continuous culture experiments of this study. Cultures were maintained on sterile-filtered (0.2-μm, Whatman® PolyCap™ 150 TC Filter) artificial seawater (ESAW; Harrison et al., 1980); following Berges et al. (2001 and subsequent Corrigendum 2004), with the following 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: 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}, $NiCl_2 \cdot 6H_2O$, 0.0149 g L^{-1}. Copper, as $CuSO_4 \cdot 5H_2O$, and Selenium, as Na_2SeO_3, were prepared as separate stocks and added to the medium at double the concentrations of Berges et al. (2001) to achieve final concentrations of 1×10^{-15} M and 6.36×10^{-9} M, respectively. All other enrichments were unchanged except silicic acid, which was doubled to ensure a minimum Si:N ratio of 2:1, and phosphate, which was reduced from 22 to 11 μM to facilitate, automated nutrient analysis. Nitrate, the sole nitrogen source, was reduced from 550 to ≤ 80 μM, and the cultures were maintained on NO_3^- for a minimum of three months before experiments to

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