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Control of domoic acid toxin expression in *Pseudo-nitzschia multiseries* by copper and silica: Relevance to mussel aquaculture in New England (USA)

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ABSTRACT

The production of the toxin Domoic Acid (DA) by the diatoms *Pseudo-nitzschia* spp. is affected by several environmental factors, among them copper and silica. The effects of these nutrients upon DA production have been studied individually, but not in combination. There is evidence, however, that in diatoms copper can enter the cell *via* the silicic-acid transport site. The goal of this study was to analyze the effect of the interaction between copper and silicic-acid supply upon DA production in *Pseudo-nitzschia multiseries*. The study was motivated by concerns about the risk of toxigenic *Pseudo-nitzschia* spp. impacting mussel aquaculture in New England (USA). The results of the present study do not indicate that copper uses the silicic acid transport site to enter the cell; nevertheless, there is an interaction between these two nutrients that produces a synergistic affect upon toxin production. A small increase in copper, without a simultaneous increase in silicate, as well as an increase in both copper and silicate, leads to DA up-regulation. Furthermore, the field component of this study reports the presence of species of *Pseudo-nitzschia* on the New England coast that are capable of producing DA. Together these findings indicate that risk of DA impacting mussel aquaculture along the coast of New England would be increased by an unusual enrichment of copper in the vicinity of mussel farms.

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1. Introduction

Mussel aquaculture is a growing industry, with world production dominated by Europe, China, Canada and the United States (FAO, 2012). Three species of mussel are cultured in North America, *Mytilus trossulus* and *Mytilus galloprovincialis* on the West Coast, and *Mytilus edulis* on the East Coast (Hilbish et al., 2000). East-Coast production of this species has increased enormously during the last two decades. The main production center of eastern North America is Prince Edward Island (PEI), Canada, which produces nearly 20 thousand tonnes annually, according to the Fisheries and Oceans Canada (2010). As most of the eastern US mussel market is supplied by imports from Canada, the US government, through H.R. 2010, the National Offshore Aquaculture Act of 2007, provided support for the commercial fishing industry to stimulate a sustainable, offshore aquaculture industry in New England and nationwide. Consequently, mussel aquaculture is a developing industry in New England.

Shellfish harvests, including those from mussel farming, are affected every year by Harmful Algal Blooms (HABs), which can cause long-term harvest closures resulting in great economic losses. In the northeastern United States, HAB episodes are a serious and recurrent problem, mainly associated with Paralytic Shellfish Poisoning (PSP) (Hoagland et al., 2002). Several monitoring networks have been implemented to manage the impact of PSP blooms. One of the most notorious human poisoning events attributable to HABs occurred in 1987 when more than 100 people became ill and three persons died after consuming mussels harvested off PEI (Bates et al., 1998). These mussels were found to be contaminated by domoic acid (DA) produced by the diatom *Pseudo-nitzschia multiseries*. Recent evidence suggests that *Pseudo-nitzschia*, a genus that blooms regularly on the US west coast, is expanding in range and has also been detected more frequently off the East Coast of United States. Species of *Pseudo-nitzschia* have been reported in Massachusetts Bay (Villareal et al., 1994), Narragansett Bay (Hargraves et al., 1993), and recently in Florida, Chesapeake Bay, and Maine (Phlips et al., 2004; Marshall et al., 2005; Van Dolah and Leighfield, unpublished data). In the February, 2007 issue of Plankton News (http://www.chbr.noaa.gov/pmn/_docs/PlanktonNews/PlanktonNews_February2007.pdf.), a bloom of *Pseudo-nitzschia* also was reported in North Carolina. Furthermore, Shuler et al. (2012) reported the presence of *Pseudo-nitzschia* spp. from North Carolina through northern Florida, with six bloom

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events (three of them with detectable levels of DA) throughout nine years of sampling. Given that this genus apparently is expanding throughout the North American East Coast, it is crucial to assess risks to protect the public from Amnesic Shellfish Poisoning (ASP) that can result from the ingestion of DA-contaminated shellfish.

Domoic acid is a tricarboxylated amino acid – an analog of the neurotransmitter L-glutamic acid – which binds to the glutamate receptor, causing neuronal depolarization in the brain resulting in permanent memory loss in mammals in severe cases. Blooms of DA-producing *Pseudo-nitzschia* spp. have been associated with mortalities of both wildlife and humans (reviewed by Lelong et al., 2012a; Trainer et al., 2012). Presence of *Pseudo-nitzschia* spp. in the water, though, does not necessarily indicate that DA is being produced that could contaminate shellfish and make them unfit for human consumption. Production of DA by *Pseudo-nitzschia* spp. is extremely variable, depending upon species (Lelong et al., 2012a; Trainer et al., 2012), strain, and physiological status of the population. Furthermore, the interaction between algae and bacteria has been shown to enhance DA production (Bates et al., 1995). This toxin variability makes monitoring and management of shellfish-harvesting activities very challenging. Research has started to address the question of how specific environmental conditions and resulting physiological changes may influence DA production in these diatoms. Suggested environmental stimuli leading to DA production in *Pseudo-nitzschia* spp. include macronutrients such as silica. Low concentrations of silica have been reported to increase DA production (Pan et al., 1996; Amato et al., 2010), which may involve diversion of energy from cell division to production of toxin. In addition, micronutrients also have been reported to influence DA production (Rue and Bruland, 2001; Wells et al., 2005). Experiments have shown that iron levels are important in the promotion of *Pseudo-nitzschia* spp. blooms (Hutchins et al., 1998; Tsuda et al., 2003; Silver et al., 2010; Trick et al., 2010), and it has been hypothesized that DA could be involved indirectly in the acquisition of iron by facilitating copper uptake (Wells et al., 2005). Usually, in eukaryotic organisms, copper is taken up by membrane-associated copper-importers (Balamurugan and Schaffner, 2006); however, Rueter et al. (1981) suggested that copper might also enter diatom cells using the silicic-acid transport site, as copper inhibits silicic acid uptake. If copper uses the silicic-acid transport system, then high copper could induce silica limitation, thereby increasing DA production. It is, however, not clear yet how the interaction of these two nutrients affects production of DA in *Pseudo-nitzschia* spp.

In aquaculture settings, copper is used as an algicide in gear paint (Schiff et al., 2003), as a feed supplement (Clearwater et al., 2002), and to treat and prevent fungal and bacterial diseases (Fernandes et al., 2009). Various human practices, from shipping to aquaculture itself, may change water chemistry locally in ways that could potentially induce DA production by endemic *Pseudo-nitzschia* spp. populations. It is necessary, therefore, to understand environmental triggers for up-regulation of DA production to assess the risk of ASP affecting this industry as it develops. Thus far, the role of DA in copper uptake, or otherwise how the copper-uptake system works, is not known. Furthermore, there have been no studies evaluating the effects of copper and silica interaction upon DA toxicity in *Pseudo-nitzschia*, which motivated the present research.

In this study we investigated how the interaction of silica and copper affects toxin production by a culture of *Pseudo-nitzschia multiseries* so that the risk of DA toxicity impacting the developing mussel-aquaculture industry can be managed effectively. We chose to use *P. multiseries* in this study not only because this is one of the most studied species of *Pseudo-nitzschia*, but also because the entire genome sequence for this species will soon be available (http://genome.jgi-psf.org/Psemu1/Psemu1.home.html). It is, however, important to consider species variability in toxin production, as well as species-specific sensitivity to nutrient stress (Lelong et al., 2012a), before generalizing the results from this study. We used a cultured isolate of *P. multiseries* to: 1) determine possible effects of copper concentrations on DA production; and 2) characterize the copper-uptake system by analyzing copper and silicate uptake under different concentrations of external copper and silicate. In addition, we sampled proposed mussel farming areas on the New England coast for the presence of potentially toxigenic *Pseudo-nitzschia* species.

2. Materials and methods

2.1. Cultures and culture experiments

Pseudo-nitzschia multiseries strain (CCL69) was used as a model organism. This axenic strain was obtained from the Culture Collection of Algae and Protozoa (CCAP, Scotland, United Kingdom). Batch cultures were maintained in artificial seawater enriched with nutrients at f/2 (Guillard, 1975) concentrations at 16 ± 1 °C on a 12 h:12 h light:dark cycle with light intensity of 110 μmol photons m^{-2} s^{-1}. A factorial experiment was designed to analyze possible interactions between copper and silica upon the production of DA in this species. Several nutrient concentrations (20–300 μM silica and 0–3.92×10^{-7} M copper) were tested to determine an appropriate range based upon the growth rate of *P. multiseries*. The factorial grid consisted of three concentrations of copper: Low (no copper enrichment); Sufficient (pCu 14.7 = 1.96×10^{-8} Cu^{2+} M); and High (pCu 13.4 = 3.92×10^{-7} Cu^{2+} M) and three concentrations of sodium silicate: low (75 μM), sufficient (150 μM), and high (300 μM), with $N = 4$ in each treatment block. Cupric ion activities were determined using the chemical equilibrium program MINEQL (Environmental Research Software).

Pseudo-nitzschia multiseries cells were collected from an exponentially-growing culture and, to remove any copper or silicate left in the media, the cells were centrifuged at 1000 × *g* for 10 min at 16 °C. The overlying water was decanted, and the cells were washed twice with artificial seawater purified in a Chelex 100 ion-exchange resin (Bio-Rad). Five-hundred-ml polystyrene culture flasks containing 250 ml of Aquil medium (Price et al., 1988/89) were inoculated with a starting cell count of approximately 5,000 cells ml^{-1}. The cultures were maintained at the temperature, light intensity, and light-cycle conditions indicated above. The growth of each culture was determined three times a week for two weeks from flow-cytometer counts (FACScan, BD BioSciences). Samples of 300 μl were taken from each culture and vortexed before being measured in the flow cytometer so that individual cells rather than groups of cells were counted. Separation of cells from chains was verified in a light microscope (Zeiss Axioscop 2 MOT Plus). Using a flow rate of 60 μl min^{-1}, each sample was counted for 30 s. Cells were distinguished based upon particle size (Forward Scatter) and chlorophyll fluorescence (FL3 detector, >650 nm). Nutrient concentrations were quantified with a TRAACS 800 autoanalyzer (Bran and Luebbe), following the protocol described by Hansen and Koroleff (1999). The determination of silica is based upon formation of a blue silicomolybdic complex (detection limit 0.10 μM). Silica uptake was calculated by subtracting final dissolved silicate concentrations from the initial amount. Copper concentrations were quantified by Inductively Coupled Plasma Mass Spectrometry (ICP-MS) (detection limit, Cu < $8.5e^{-09}$ M), which was performed by an external contract laboratory (Applied Speciation and Consulting LLC). DA concentrations were quantified 2 days after the beginning of the stationary phase using a cELISA kit (detection limit 300 pg DA ml^{-1}) (Biosense