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Impact of copper exposure on *Pseudo-nitzschia* spp. physiology and domoic acid production

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ABSTRACT

Microalgae have differing sensitivities to copper toxicity. Some species within the genus *Pseudo-nitzschia* produce domoic acid (DA), a phycotoxin that has been hypothesised to chelate Cu and ameliorate Cu toxicity to the cells. To better characterise the effect of Cu on *Pseudo-nitzschia*, a toxic strain of *P. multiseries* and a non-toxic strain of *P. delicatissima* were exposed to Cu(II) for 96 h (50 $\mu g\,l^{-1}$ for *P. delicatissima* and 50, 100 and 150 $\mu g\,l^{-1}$ for *P. multiseries*). Physiological measurements were performed daily on *Pseudo-nitzschia* cells using fluorescent probes and flow cytometry to determine the cell density, lipid concentration, chlorophyll autofluorescence, esterase activity, percentage of dead algal cells, and number of living and dead bacteria. Photosynthetic efficiency and O_2 consumption and production of cells were also measured using pulse amplitude modulated fluorometry and SDR Oxygen Sensor dish. The DA content was measured using ELISA kits. After 48 h of Cu exposure, *P. delicatissima* mortality increased dramatically whereas *P. multiseries* survival was unchanged (in comparison to control cells). Cellular esterase activity, chlorophyll autofluorescence, and lipid content significantly increased upon Cu exposure in comparison to control cells (24 h for *P. delicatissima*, up to 96 h for *P. multiseries*). Bacterial concentrations in *P. multiseries* decreased significantly when exposed to Cu, whereas bacterial concentrations were similar between control and exposed populations of *P. delicatissima*. DA concentrations in *P. multiseries* were not modified by Cu exposure. Addition of DA to non-toxic *P. delicatissima* did not enhance cells survival; hence, extracellular DA does not protect *Pseudo-nitzschia* spp. against copper toxicity. Results suggested that cells of *P. delicatissima* are much more sensitive to Cu than *P. multiseries*. This difference is probably not related to the ability of *P. multiseries* to produce DA but could be explained by species differences in copper sensitivity, or a difference of bacterial community between the algal species.

1. Introduction

In coastal waters, copper concentrations fluctuate due to both natural and anthropogenic activities, such as volcanoes, industrial discharges (Paul and Pillai, 1983), mining (Castilla, 1996; Stauber et al., 2000), algaecides (McKnight et al., 1983) and antifouling paints (Hall and Anderson, 1999). Copper is an essential trace metal for microalgae, as a component of key proteins such as Cu-superoxide dismutase, cytochrome c oxidase and plastocyanin (Raven et al., 1999; Balamurugan and Schaffner, 2006; Peers and Price, 2006). However, at higher concentrations, copper becomes toxic inducing a range of metabolic changes in phytoplanktonic cultures, such as inhibition of cell division (Lage et al., 2001; Perales-Vela et al., 2007), photosynthesis (Perales-Vela et al., 2007), respiration (Xia and Tian, 2009), pigments synthesis (Perales-Vela et al., 2007), cell mobility inhibition (Lage et al., 2001) and changes in membrane potential (Cid et al., 1996). Copper toxicity is likely to occur through mechanisms such as inhibition of enzymes and oxidative damage within the cell (Knauert and Knauer, 2008). Copper sensitivity in microalgae varies vastly between species, with dissolved copper inhibiting population growth by 50% (IC-50) at concentrations < 1 $\mu g\,Cu\,l^{-1}$ in *Minutocellus polymorphus* (Levy et al., 2007), and 630 $\mu g\,Cu\,l^{-1}$ in *Parachlorella kessleri* (Nugroho and Frank, 2011).

The effects of copper toxicity in microalgae have been widely studied, mainly to identify potential bioindicators of contamination (e.g. Ahmed and Hader, 2010). These studies typically focus measurements on population growth and cell death, sometimes observing physiological changes such as photosynthetic rates or ATP production (Cid et al., 1995). Only a few studies have investigated the effects of metal exposure on *Pseudo-nitzschia* species, most of which were centred on domoic acid (DA) production

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(Ladizinsky, 2003; Maldonado et al., 2002). DA is an amnesic toxin, which chelates both copper and iron (Rue and Bruland, 2001), and is produced by some species of *Pseudo-nitzschia* (see a review in Lelong et al., 2012). Maldonado et al. (2002) hypothesised that DA is produced in response to copper exposure to chelate copper, reducing its toxicity to cells by decreasing its bioavailability. *Pseudo-nitzschia multiseries* and *P. australis* have been shown to release approximately 20-fold more DA when exposed to toxic concentrations of copper (Ladizinsky, 2003; Maldonado et al., 2002). The effects of copper exposure on toxic and non-toxic *Pseudo-nitzschia* species have not been reported in the literature. A comprehensive understanding of factors governing DA production in *Pseudo-nitzschia* spp. is of global interest to commercial fisheries, as bivalves, molluscs and fishes contaminated by DA pose a threat to human health (Bejarano et al., 2008). So far, the factors triggering DA production in algae remains unclear, but as Cu concentrations can become elevated in coastal waters, particularly following pulse wastewater discharges from industry (Paul and Pillai, 1983), it is important to know if DA is produced by cells in response to copper exposure.

This study investigated the effects of copper toxicity on the physiology of two species of *Pseudo-nitzschia*: a non-toxic (non-DA producing) *P. delicatissima* and a toxic (DA producing) *P. multiseries*. The first step was to examine the impacts of copper on different physiological parameters simultaneously, to allow comparison between the two species. All physiological measurements were performed during acute (24 h) and chronic (24–96 h) copper stress. Flow cytometry was used to measure the concentration and percentage of dead *Pseudo-nitzschia* and associated free-living bacteria, and the lipid content and enzymatic activity of algal cells. A pulse-amplitude modulated (PAM) fluorometer and an oxygen sensor dish (SDR) were used to measure photosynthesis efficiency and quantify O_2 consumption and release during cell respiration and photosynthesis. For each experiment, DA was also quantified to estimate its potential as a protective agent against copper. To fully evaluate the protective role of DA in copper toxicity, DA was added to *P. delicatissima* cultures to allow a comparison with the DA producing *P. multiseries* cultures.

2. Material and methods

2.1. Culture conditions

The two species of *Pseudo-nitzschia* used in this study were *P. multiseries* (strain CLNN-16, isolated from the Bay of Fundy, Canada) and *P. delicatissima* (strain Pd08RB, isolated from the Rade de Brest, France). Both strains were 2.5 years old at the time of the experiment. They were grown in sterilised f/2 medium (Guillard and Hargraves, 1993) at 17.2 °C (±0.5 °C) and 155 ± 10 μmol photons m^{-2} s^{-1} (with a light:dark photoperiod of 12:12 h). Cultures were xenic and grown without antibiotics.

2.2. Bioassays

Experiments were always performed on cultures in exponential growth phase (3–5 days old). Before each experiment, cultures were homogenised by gentle manual stirring. Aliquots of the cultures were centrifuged 3 times (5 min, 16 °C, 780 g) and the remaining pellet was rinsed with sterile seawater (>0.22 μm filtered, Sartorius bottle-top filter). After the last rinse, the pellet was resuspended in sterile seawater. Almost all the cells in the cultures were present as single cells, with <5% forming two cell chains. Therefore, for flow cytometry analyses, they were all considered as single cells.

Cells were inoculated into flasks (TPP cell culture flasks 75 cm^2, Dutscher, Brumath, France) at a cell concentration of ~5000 cells ml^{-1}. Nutrient enriched sterile seawater (containing 26 μM $NaNO_3$, 13 μM NaH_2PO_4 and 10.6 μM Na_2SiO_3) was added to each inoculate. A stock solution of 50 mg Cu l^{-1} was prepared from $CuSO_4 \cdot 5H_2O$ in 0.1% of HCl. Copper exposure concentrations were selected based on Maldonado et al. (2002). Treatments were prepared in triplicate at 0 (controls), 50, 100 or 150 μg l^{-1} for both *P. multiseries* and *P. delicatissima*, however *P. delicatissima* proved to be more sensitive to copper and only 50 μg l^{-1} exposures are reported here. The flasks were incubated for 4 days (96 h) under the above conditions. Flasks were rotated within the cabinet and shaken twice daily by hand to ensure sufficient gas exchange. The pH remained at 8.0 ± 0.2 throughout the test (recorded initially and at test completion).

2.3. Copper analyses

Copper measurements were conducted using an octopol collision cell-inductively coupled plasma-mass spectrometer (OCR-ICP-MS, Agilent 7500cs) utilising both standard and collision/reaction gas modes where applicable. Calibration standards were prepared in 0.32 M HNO_3 (Choice Analytical, Suprapur, 69%) using a multi-element standard (IV-ICP-MS-71D, Inorganic Ventures, USA). Blanks, duplicates and spiked recoveries were performed on at least 10% of all samples. Method blanks were below the 0.89 μg l^{-1} limits of reporting, duplicates were within 12% of each other and spike-recoveries were 92–107%. On day 4, samples were filtered (0.45 μm, Minisart syringe filter, Sartorius), acidified to 0.32 M HNO_3 and copper was measured.

2.4. Specific growth rate, morphological and physiological measurements

Specific growth rate (μ, d^{-1}) was determined by linear regression of the natural log of cell concentration (cell ml^{-1}) over time (days). A flow cytometer FACScalibur (BD Biosciences, San Jose, CA, USA) with an argon blue laser (488 nm) was used. Flow cytometer flow-rates were calculated daily by analysing samples for 45 s, as per Marie et al. (1999). Morphological information of cell complexity was determined using forward scatter (FSC) and side scatter (SSC) as *Pseudo-nitzschia* is a pennate diatom, not a spherical cell. FL3 fluorescence (red fluorescence at 670 nm) was also determined as an indicator of cell chlorophyll content, as Galbraith et al. (1988) showed that autofluorescence of cells is linearly linked to chlorophyll content.

Cell density, morphological and physiological measurements of *Pseudo-nitzschia* spp., quantification of free-living bacteria associated to *Pseudo-nitzschia* populations and percentage of dead bacteria in the treatments were assessed using fluorescent stains and flow cytometry according to Lelong et al. (2011). A range of commercial stains from Invitrogen (Molecular Probes, Invitrogen, Eugene, Oregon, USA) were used to assess physiological changes associated with copper exposure in *P. delicatissima* and *P. multiseries*. Physiological changes included mortality (30 min incubation in 0.1 μM of SYTOX Green), intracellular lipid content (30 min incubation in 10 μM of BODIPY 493/503), and esterase activity (6 min incubation in 3 μM of fluorescein di-acetate (FDA)). A 300 μM working solution of FDA was freshly prepared before each experiment. Cells were considered dead when they were permeable to SYTOX Green (Fig. 1A and B). Esterase activity is a measure of metabolic activity (Jochem, 1999) and cells with no FDA staining were considered as inactive cells (as opposed to stained and active cells, Fig. 1C and D). The concentration of free-living bacteria (both living and dead) were measured after 15 min incubation with SYBR Green I (a 1/10,000 dilution of the commercial solution), and