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Influence of dissolved organic nitrogen on Ni bioavailability in *Prorocentrum donghaiense* and *Skeletonema costatum*

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ABSTRACT

Dissolved organic nitrogen (DON) is an important nutrient in the aquatic environment. This study examined the influence of DON addition on the adsorption, absorption, and distribution in macromolecular forms of environmentally deleterious trace metal (Ni) in *Prorocentrum donghaiense* and *Skeletonema costatum* over eight days. Ni adsorption and absorption of two species increased with the addition of urea, while Ni adsorption and absorption of two species in the presence of humic substances (HS) decreased. Meanwhile, Ni adsorption and absorption of *P. donghaiense* were higher than that of *S. costatum*. Furthermore, Ni contents in the protein fraction of the cells, both in *P. donghaiense* and *S. costatum*, were increased with both urea and HS addition. Thus, urea and HS input could impact Ni biogeochemistry and bioavailability, and then affect the biodynamics thereafter.

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1. Introduction

In many estuarine and coastal waters, eutrophication has become a serious environmental problem (De Jong, 2006). Nutrient enrichment, accompanied by a variation in nutrient ratio due to disproportionate inputs of nutrients, has been shown to affect phytoplankton species composition and production (Smith et al., 1999). It might also affect the structure and functioning of the entire marine ecosystem (Bu-Olayan et al., 2001; Li et al., 2013). Nutrient enrichment mainly refers to nitrogen and phosphorus. Nitrogen include dissolved inorganic nitrogen (i.e., NO_2^-, NO_3^-, and NH_4^+) and DON (i.e., urea, amino acid, humic substances (HS), and so on) in the ocean (Peers et al., 2000). While, HS are often the predominant components of DON in estuarine and coastal waters because of riverine input (Dyhrman and Anderson, 2003; Liu et al., 2011).

Phytoplankton are not only able to use inorganic nitrogen (NH_4^+ and NO_3^-), but also can take up and assimilate a variety of DON compounds (Berg et al., 1997; Cawley et al., 2013). For instance, dinoflagellates have been associated with low nitrate and high DON concentrations, which suggests that these phytoplankton may selectively use organic nitrogen sources (Dyhrman and Anderson, 2003). The bioavailability of DON (including particularly urea and HS) showed almost the same significance as that of inorganic N for phytoplankton (Bronk et al., 2007).

Usually, urea contribute to the DON load in the East China Sea, where its concentration ranges from 5.78 to 25.26 μmol L^{-1} (Li et al., 2010). Urease activity has been demonstrated in phytoplankton urea uptake with urea addition (Berg et al., 1997; Berg et al., 2002; Dyhrman and Anderson, 2003), although not for many phytoplankton. Either the enzyme urease or adenosine triphosphate (ATP) urea amidolyase in marine phytoplankton used urea through its hydrolysis to ammonia (Antia et al., 1991). Thus far, marine phytoplankton appear to use nickel-dependent urease enzyme, except chlorophytes (Worms et al., 2007).

HS could be adsorbed on the cell surface of algae, which could influence trace metal uptake (including that of Ni (Koukal et al., 2003)). The availability of trace metals is determined by the amount of the free trace metal ion, instead of by its total concentration which also includes non-bioavailable trace metal complexes, i.e. cheated metals by HS (Koukal et al., 2003). Thus, the bioavailability of trace metals, especially for Ni, is logically influenced by the input of DON, such as urea and HS, in natural waters.

However, the influence of DON on trace metal uptake, especially for Ni, is little understood. So it is essential to examine the interaction of trace metal and DON in phytoplankton, and thus

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the functioning and fate of trace metals in the context of marine biogeochemical cycles. Nitrate status can increase Ni uptake in phytoplankton and facilitates the transfer from phytoplankton to copepods (Hong et al., 2009; Wang et al., 2007). Furthermore, Ni is an essential trace metal for aquatic organisms and it is toxic at higher concentrations (Hong et al., 2009). Thus, Ni uptake influenced by urea and HS should be examined.

It is important to distinguish Ni absorption (intracellular assimilation), adsorption (cellular surface uptake) and Ni distribution in macromolecular (Li et al., 2007). The bioavailability or toxicity of Ni that is absorbed or adsorbed by marine phytoplankton is quite different. Does the DON affect Ni availability to, or toxicity in, coastal phytoplankton over long periods of exposure? Are the influences of DON on Ni absorption, adsorption, and distribution in macromolecular forms similar, or not? What relationships are there between Ni absorption, adsorption, and distribution in macromolecular forms? All of these questions are pertinent to a deeper understanding of phytoplankton-Ni dynamics.

Prorocentrum donghaiense and *Skeletonema costatum* are key algal bloom species in the coastal waters of China (Ou et al., 2008). In the past few years, they have formed extensive blooms in the East China Sea which coexisted with an abundance of DON. So, DON might significantly affect the bioavailability of Ni in this area, which may have influenced the growth of phytoplankton. However, no work has been done to investigate this possibility. In this study, we investigated the influence of DON (urea and HS) on the biological adsorption, absorption, and distribution in macromolecular forms of Ni by *P. donghaiense* and *S. costatum*, so as to reveal the impacts and mechanisms of DON on Ni bioavailability or risk assessment in the phytoplankton.

2. Materials and methods

2.1. Seawater and marine phytoplankton cultures

The seawater used in all experiments was collected from Zhangzhou Bay, Fujian Province, China. Seawater was filtered through a 0.2-μm membrane filter, and then stored at 20 °C for about six months before testing. The background nutrient concentrations in the seawater were measured by a flow injection analyser (FIA), and were 2.44 (as nitrate), 0.04 (as nitrite), 0.12 (as NH_4^+), and 0.28 (as reactive P) μmol L^{-1}, respectively (Li et al., 2014). The background concentrations of total dissolve nitrogen (TDN) were measured using a Shimadzu TOC–TN analyser (Shimadzu Corp., Kyoto, Japan), and the urea concentration was measured using the diacetylmonoxime reagent method for seawater at room temperature (Goeyens et al., 1998). The seawater was added to a closed vessel with mixed acid (HNO_3:H_2O_2, v:v = 2:1), microwave digested for 10 min at 10 atm, and then used for determining the background concentration of Ni using inductively coupled plasma mass spectrometry (ICP-MS). The amount of Ni in the seawater was 1.23 μmol L^{-1}, and the relative standard deviation was 1.3%. Consequently, this coastal seawater, with both Ni and nutrient enrichment, could be used for Ni bioavailability and risk assessment by phytoplankton experiments.

P. donghaiense and *S. costatum* were chosen for this study because: they are common species which have caused harmful blooms in the East China Sea (Ou et al., 2008). They were collected from Yangtze River estuary in 2003, and cultured in the State Key Laboratory for Marine Environmental Science, Xiamen University. Under light illumination at 140 μmol photons m^{-2} s^{-1} over a light:dark cycle of alternately 14 h:10 h, they were maintained in seawater (with f/10 levels of phosphate, Si (only for *S. costatum*) and vitamins added, but without trace metals) at different N (added as $NaNO_3$, urea, and HS) concentrations at 20 °C. The cells were transferred to a new medium every three to six days, to ensure that the cells were acclimated to these nutrient conditions.

2.2. Isolation of HS and analysis

HS were isolated according to the procedure recommended by International Humic Substances Society (IHSS) (http://www.humicsubstances.org/aquatichafa.html). This protocol has a long tradition within the scientific community and reference materials are available. This allows a direct comparison to existing data on HS from various environments. Based on this method, quantities of low-ash (<1.0 wt%) aquatic HS can be obtained by adsorption chromatography on Amberlite XAD-8 resin, ion exchange and freeze-drying (Thurman and Malcolm, 1981). Isolation procedure started with the acidification of the water sample (pH = 2.0) which made dissolved HS, including humic acid (HA) and fulvic acid (HA), sufficiently hydrophobic to be adsorbed on the XAD-8 resin. After alkaline elution, the eluate was acidified to separate HA from FA since HA precipitates at pH = 1 and FA remains in solution. After cation-exchange using Varion KSM resin, HA and FA were obtained. The HS of Jiulong River were obtained from IHSS as a reference material. The isolated HS were investigated by elemental analysis, FTIR spectroscopy, and ESI-FT-ICR/MS, respectively. Elemental analysis (N, C, H, and S) was performed on a NA 1500 NCS analyser (Fisons Instruments) at 1010 °C and O content was calculated as a reference (Kovács et al., 2012). The total HS content was 6.4 mg L^{-1}, and the elemental composition of N, C, H, S, and O were 59.4, 4.23, 2.12, 1.23 and 33.01%, respectively. Furthermore, the concentrations of Ni in the HS were measured using inductively coupled plasma mass spectrometry (ICP-MS) after microwave digestion for 10 min at 10 atm in a closed vessel with mixed acid (HNO_3:H_2O_2, v:v = 2:1), and was 0.31 μmol L^{-1} in the HS solution.

2.3. Ni adsorption and absorption under different nutrient regimes

Filtered seawater (0.22 μm) was added into 1000 mL acid-cleaned polycarbonate bottles, and then sterilised in an autoclave at 120 °C for 30 min. Algal cells in their exponential growth phase were used in these experiments. The cells were added to sterile seawater at a cell concentration of 2×10^6 cells L^{-1} (*P. donghaiense*) and 4×10^6 cells L^{-1} (*S. costatum*). The experimental N addition included 20, 80, and 160 μmol N L^{-1} with different species of N (nitrate, HS, and urea), and a fixed concentration (f/10 levels) of phosphate, Si (only for *S. costatum*) and vitamins added, but without trace metals. NaOH was added to adjust the pH value to 8.0 in the medium. Three replicates were established for each treatment. The experiments lasted for eight days at 20 °C under light illumination at 140 μmol photons m^{-2} s^{-1} over a light:dark cycle of 14 h:10 h.

The cells contained in 1000 mL of the medium at the eight day were filtered through a 0.45 μm membrane filter, and twice rinsed with 20 mL artificial seawater. Then the cells were re-suspended in 30 mL of trace metal clean reagent (Li et al., 2007; Tovar-Sanchez et al.,2003), stirred (at 200 rpm and 20 °C) for 1 h to elute surface-bound Ni (adsorbed Ni) from marine phytoplankton, and filtered through a pre-weighed 0.45 μm membrane filter. The filtrate, with any eluted Ni adsorbed on the cells, was added to a closed vessel with mixed acid (HNO_3:H_2O_2, v:v = 2:1), microwave digested for 10 min at 10 atm, and then used for determining the concentration of Ni adsorbed by *P. donghaiense* and *S. costatum* cells using inductively coupled plasma mass spectrometry (ICP-MS). Ni adsorption was calculated as relative surface sorption metal amount to cellular amount. After removing surface sorption metals, the cell dry mass was measured by filtering the sample onto a pre-weighed GF/F filter, rinsed with ammonium formate, and dried at 105 °C for 1 day. Half of the dry cells were weighed,

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