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Acquisition of organically complexed copper by marine phytoplankton and bacteria in the northeast subarctic Pacific Ocean

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ABSTRACT

Copper (Cu) is an essential micronutrient for marine phytoplankton, but can cause toxicity at elevated intracellular concentrations. The majority of Cu (>99.9%) in oceanic surface waters is bound to strong organic ligands, presumably produced by prokaryotes to detoxify Cu. Although laboratory studies have demonstrated that organically complexed Cu may be bioavailable to marine eukaryotic phytoplankton, the bioavailability of Cu organic complexes to indigenous marine phytoplankton has not been examined in detail. Using the carrier free radioisotope ^{67}Cu at an iron limited station in the northeast subarctic Pacific Ocean, we performed size fractionated short-term Cu uptake assays with three Cu(II)-chelates, and ^{67}Cu bound to the strong in situ ligands, with or without additions of weak Cu(I) ligands. Estimates of the maximum supply of inorganic Cu (Cu′) to the cell surface of eukaryotic phytoplankton were unable to account for the observed Cu uptake rates from the in situ ligands and two of the three added Cu(II)-chelates. Addition of 10 nM weak organic Cu(I) ligands enhanced uptake of Cu bound to the in situ ligands. Thus, Cu within the in situ and strong artificial Cu(II) organic ligands was accessible to the phytoplankton community via various possible Cu uptake strategies, including: cell surface enzymatically mediated reduction of Cu(II) to Cu(I), the substrate of the high-affinity Cu transport system in eukaryotes; or ligand exchange between weak Cu-binding ligands and the cellular Cu transporters. During a 14-hour uptake assay, particulate Cu concentrations reached a plateau in most treatments. Losses were observed in some treatments, especially in the small size fractions (<5 μm), corresponding with faster initial Cu uptake rates. This may indicate that Cu cycling is rapid between particulate and dissolved phases due to cellular efflux or remineralization by micrograzers. The acquisition of Cu from the strong in situ ligands puts into question the historic role attributed to Cu binding ligands in decreasing Cu bioavailability.

1. Introduction

Copper (Cu) is a metabolically essential micronutrient for marine phytoplankton, and is involved in a number of important electron transfer reactions, including iron (Fe) uptake via the high-affinity Fe transport system (Peers et al., 2005; Wells et al., 2005; Maldonado et al., 2006), electron transfer between photosystem II and photosystem I via plastocyanin (Peers and Price, 2006), extracellular amine oxidation (Palenik and Morel, 1991), quenching of reactive oxygen species via superoxide dismutase (Raven et al., 1999), and respiration via cytochrome *c* oxidase. Indeed, intracellular Cu requirements are on par with those of other essential micronutrients like Fe and zinc (Zn; Sunda and Huntsman, 1992; Maldonado and Price, 1996), and limitation of growth rates by Cu has been observed in 4 of 18 laboratory phytoplankton strains examined thus far (Annett et al., 2008; Guo et al., 2012). However, Cu is also toxic to marine phytoplankton at nanomolar concentrations (Brand et al., 1986), and growth rates decrease as intracellular Cu increases (Sunda and Guillard, 1976). Some phytoplankton groups are more susceptible to Cu toxicity (e.g., cyanobacteria; Brand et al., 1986) and Cu limitation (e.g., oceanic diatoms; Peers et al., 2005), and so elucidating the bioavailability of in situ Cu to marine phytoplankton communities would provide a first order approximation of the Cu nutritional status of natural phytoplankton populations.

Total dissolved Cu concentrations ($[Cu]_d$) in open ocean surface waters vary between 0.5 to 3 nM (Coale and Bruland, 1988; Moffett and Dupont, 2007; Bundy et al., 2013; Jacquot et al., 2013), and the speciation of Cu is dominated by strong organic complexes that comprise >99% of total dissolved Cu (van den Berg, 1984). A strong ligand class, with conditional stability constants ($\log K^{cond}_{CuL,Cu^{2+}}$) ranging $10^{11.5}$ to 10^{16}, is present in concentrations ranging from 1 to 10 nM (van den Berg, 1984; Coale and Bruland, 1988; Moffett and Dupont, 2007; Buck et al., 2010; Bundy et al., 2013; Jacquot et al., 2013). The resulting calculated "free" cupric ion concentrations are between $10^{-13.5}$ and $10^{-16.3}$ M in surface waters, and are within the range that can cause Cu-limitation in diatoms and prymnesiophytes (10^{-15} M; Peers et al.,

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2005; Annett et al., 2008; Guo et al., 2012). There is evidence that the strongest ligands found in seawater are produced by cyanobacteria and heterotrophic bacteria to alleviate Cu toxicity by complexing Cu extracellularly (Moffett and Brand, 1996; Gordon et al., 2000), while eukaryotes produce weaker ligands (Croot et al., 2000). Indeed, in the absence of strong organic chelators, Cu concentrations in surface waters (0.5 to 3 nM) would cause inhibition of growth in many marine phytoplankton groups (Brand et al., 1986). Although the structures of the strong in situ Cu binding ligands remain unknown, the ligands may contain thiol and amine functional groups (Ross et al., 2003), and could be phytochelatins, phytochelatin precursors (e.g., glutathione and cysteine), humic and fulvic acids, or other low molecular weight compounds (Leal and van den Berg, 1998; Laglera and van den Berg, 2003; Tang et al., 2004; Dupont et al., 2006; Dryden et al., 2004; Yang and van den Berg, 2009).

To date, the substrate for Cu transport by in situ marine phytoplankton communities has yet to be fully elucidated. Early laboratory studies suggested that inorganic Cu (Cu′) was the sole substrate for Cu transport in isolated marine phytoplankton strains (Sunda and Guillard, 1976). Various studies support the hypothesis that prokaryotes produce strong ligands to detoxify Cu via extracellular complexation, thereby lowering the [Cu′] in the growth media and decreasing the potential for Cu toxicity (Moffett and Brand, 1996; Gordon et al., 2000). However, subsequent work has demonstrated that organically complexed Cu appears to be bioavailable to many marine phytoplankton phyla. Copper uptake rates by phytoplankton can exceed the diffusive supply of inorganic Cu to the cell surface by 2 to 1000-fold, when Cu is complexed by the artificial chelators ethylenediamine tetraacetic acid (EDTA) (Hudson, 1998; Quigg et al., 2006; Annett et al., 2008; Guo et al., 2010, 2012) or nitrilotriacetic acid (NTA) (Croot et al., 2003). A preliminary study in the northeast subarctic Pacific Ocean demonstrated that Cu was acquired ~5 times faster from strong in situ ligands than from a strong artificial Cu(II) ligand of comparable strength (Semeniuk et al., 2009). Thus, the bioavailability of in situ Cu does not appear to be mediated solely by Cu′ availability.

Although the mechanism allowing eukaryotic marine phytoplankton to acquire Cu from strong ligand complexes has not been described, the mechanisms of Cu transport have been elucidated in other eukaryotes. In *Saccharomyces cerevisiae*, *Chlamydomonas reinhardtii*, and most eukaryotes, Cu(II) is reduced to Cu(I) by a FRE-encoded surface reductase, and the Cu(I) produced is subsequently internalized by the CTR-encoded high-affinity Cu transport system (reviewed by Pope et al., 2012). The FRE reductases in diatoms are capable of reducing strongly complexed Fe(III) (Maldonado and Price, 2001; Shaked et al., 2005; Kustka et al., 2007), and so strongly complexed Cu(II) may also be reduced by these reductases prior to internalization (Guo et al., 2015).

Weak ligands may also play an important role in metal acquisition. A ligand shuttle mechanism has recently been described for Zn uptake in marine diatoms (Aristilde et al., 2012). Complexation of Zn′ by weak ligands draws down the concentration of Zn′, and pushes the equilibrium of strongly bound Zn towards dissociation. Thus, the total concentration of Zn′ remains relatively constant while weakly complexed Zn species increase in concentration. Both inorganic and weak organic complexes of Zn and Fe appear to be bioavailable (Maldonado et al., 2002; Hassler et al., 2011; Aristilde et al., 2012), so weak Cu binding ligands may similarly facilitate transport in marine phytoplankton.

Given the uncertain role of organic complexation in determining the bioavailability of Cu to marine phytoplankton, and the potential for Cu limitation and toxicity in surface waters, the aim of the present study was to expand on our preliminary work examining the substrates for Cu transport in phytoplankton and bacteria at an Fe-limited station in the northeast subarctic Pacific Ocean (Semeniuk et al., 2009). We monitored Cu uptake from three Cu(II)–ligand complexes and Cu bound to the in situ strong ligands using the carrier free gamma emitting radionuclide ^{67}Cu. We also investigated how additions of weaker ligands influenced in situ Cu bioavailability.

2. Materials and methods

2.1. Sampling and incubation

Seawater was sampled from Station P26 (50°N 145°W) during the September 2008 Line P Cruise (2008–26) aboard the C.C.G.S. J.P. Tully. Station P26 is a perennially Fe-limited station along the Line P transect. Approximately 3 h before sunrise on June 10, 2008, seawater was pumped from the mixed layer (10 m depth) into trace metal clean 2 L polycarbonate bottles (Nalgene) using a trace metal clean pumping system and class 100 laminar flow hood (Johnson et al., 2005; Semeniuk et al, 2009). To remove large grazers, the water was filtered through a 250 μm trace metal cleaned nylon mesh. The average PAR was calculated for the mixed layer (56 m), and this corresponded to 14% of the surface irradiance (I_o). Thus, the bottles were immediately placed into an on-deck Plexiglas shipboard incubator, and the PAR (10% I_o) and temperature were maintained using neutral density screening and continuously pumped seawater from 5 m depth.

2.2. Determination of initial chemical and biological parameters

Size-fractionated chlorophyll *a* concentrations ([chl *a*]) were sampled by filtering 500 mL onto stacked 20, 5, 1 and 0.22 μm polycarbonate filters (AMD) separated by nylon drain disks (Millipore) (Semeniuk et al., 2009). Filters were archived at −20 °C until analysis in the lab. The chl *a* was extracted in 90% acetone at 4 °C overnight, and [chl *a*] was determined using a Turner Designs Model 10 fluorometer (Parsons et al., 1984). Nutrients (nitrate, phosphate, and silicic acid) were analyzed on board using freshly collected samples (Barwell-Clarke and Whitney, 1996). A sample for total dissolved Cu (0.22 μm Opticap® cartridge filter) was collected in trace metal clean low-density polyethylene bottles using a Teflon pump and laminar flow hood as previously described (Johnson et al., 2005). The sample bottles were rinsed three times before being filled and acidified to pH 1.7 using ultraclean HCl (Seastar) in a Class 100 laminar flow hood. Total dissolved Cu was measured after UV-oxidation by adsorptive cathodic stripping voltammetry (ACSV) with salicylaldoxime (SA) (Buck and Bruland, 2005).

2.3. Determination of conditional stability constants for Cyclam and Cyclen

Ten milliliter subsamples of open ocean surface seawater were UV-irradiated for 8 h and chelexed prior to being aliquoted into acid cleaned Teflon cups. Cyclam or Cyclen (10 nM) was then added to each cup, followed by boric acid buffer (pH 8.2), and $CuSO_4$ additions ranging from 0 to 100 nM. Subsamples were allowed to equilibrate for 2 h before adding 25 μM SA. After equilibrating for 15 min with SA, samples were then analyzed by competitive ligand-exchange adsorptive cathodic stripping voltammetry (CLE-ACSV) according to Bundy et al. (2013).

2.4. Preparation of the copper–ligand complex additions for copper uptake assay

Using the carrier free radioisotope ^{67}Cu (courtesy of TRIUMF), we measured the time-course accumulation of Cu by microorganisms at Station P26. Three strong Cu(II)–ligands and three weak Cu(I)–ligands were chosen with differing Cu-binding functional groups and conditional stability constants (Table 1). Ligand solutions were prepared as described elsewhere (Wiramanaden, 2007; Semeniuk et al., 2009). Briefly, Cyclam and Cyclen powders were dissolved in a few drops of HPLC grade methanol, and subsequently diluted to 12.5 mM in ultrapure water (Millipore). Ethylenediamine tetraacetic acid disodium salt (EDTA; Sigma), reduced glutathione (GSH; Sigma), cysteine (Sigma), and bathocuproinedisulfonic acid disodium salt hydrate (BCDS; Sigma) were dissolved in water immediately prior to complexation to Cu to ensure oxidation of the sulfhydryl groups by O_2 did not occur.