

Nickel limitation and zinc toxicity in a urea-grown diatom

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Abstract

When growing on urea as a nitrogen source, diatoms must accumulate nickel (Ni), a cofactor in the urease enzyme, which hydrolyzes urea. The uptake of Ni at low ambient concentrations is particularly challenging in view of the slow rate of reaction of the Ni²⁺ ion with uptake ligands. As expected, cultures of the model diatoms *Thalassiosira weissflogii* and *Thalassiosira pseudonana* become limited at very low Ni concentrations when growing on urea but not on nitrate or ammonium as a nitrogen source. At high Ni concentrations, urea-grown cultures of *T. weissflogii* exhibit similar accumulation of various other metals to nitrate-grown cultures and the same sensitivity to zinc and copper (Zn and Cu) concentrations. But at low Ni concentrations, *T. weissflogii* cells growing on urea accumulate excess Zn and exhibit extreme sensitivity to Zn toxicity. It appears that Zn accumulates in cells growing at low Ni concentrations by uptake through the up-regulated Ni transport system. The resulting sensitivity of Ni-limited cells to Zn (or to other metals that may be taken up via the Ni transport system) may limit the use of urea as a source of nitrogen in the oceans by some phytoplankton species.

Ocean productivity depends on the ability of phytoplankton to acquire both major nutrients, C, N, and P (+ Si for diatoms), and a host of micronutrients, including the metals Mn, Fe, Co, Ni, Cu, Zn, and Cd (Morel and Price 2003). The requirements for major and trace nutrients are not independent of one another, for a number of trace elements are involved in the acquisition and assimilation of C, N, and P. For example, Zn requirements are dependent on CO₂ availability (Morel et al. 1994), and Fe requirements in some marine diatoms change depending on the primary source of nitrogen (Maldonado and Price 1996).

Urea is an important source of N in the world's oceans, after NO₃⁻ and NH₄⁺, accounting for up to 50% of N utilization in some areas (McCarthy 1972). In order for urea to be utilized as a source of N, it must first be hydrolyzed. There are two pathways of urea hydrolysis: the urease pathway and the adenosine triphosphate (ATP):urea amidolyase pathway (Syrett 1981). The ATP:urea amidolyase pathway has been demonstrated only in *Chlorophyceae*, while other classes of phytoplankton apparently use urease, as shown in two diatom species, *Phaeodactylum tricorutum* and *Thalassiosira weissflogii* (Leftley and Syrett 1973; Price and Morel 1991; Fan et al. 2003). Urease is the predominant pathway of urea hydrolysis in the ocean (Oliveira and Antia 1986). Urease

is a Ni metalloenzyme (Rees and Bekheet 1982) such that growth on urea requires the presence of Ni in the medium (Syrett and Peplinska 1988). Ni also serves as a cofactor in several other enzymes, including hydrogenase and superoxide dismutase in cyanobacteria (Mulrooney and Hausinger 2003), but has no other known use than as a cofactor in urease in eukaryotic phytoplankton, including diatoms. In such organisms, the use of urea as a source of nitrogen should thus impose a unique Ni requirement, although this has been quantified only once, in the diatom *T. weissflogii* (Price and Morel 1991). This Ni requirement is particularly interesting because Ni reacts slower than the other metals used by phytoplankton: the water loss constant for Ni²⁺ is $3 \times 10^4 \text{ s}^{-1}$ compared to water loss constants between $1 \times 10^6 \text{ s}^{-1}$ and $5 \times 10^9 \text{ s}^{-1}$ for the other essential metal cations (Margerum et al. 1978). At the very low free metal concentrations of interest in the surface ocean and in phytoplankton cultures (nmol L⁻¹ to pmol L⁻¹), this rate of water loss for Ni²⁺ results in typical reaction times of minutes to days for binding to a ligand in excess. As a result, the uptake of Ni presumably requires a large number of uptake ligands (Hudson and Morel 1993). Since uptake systems for divalent metals are rarely very selective (Sunda and Huntsman 1998b), the need to acquire Ni may lead to the accumulation of other metals and result in complex cellular interactions.

Here we examine the effect of urea as a nitrogen source on metal requirements and metal accumulation in the model diatoms *T. weissflogii* and *Thalassiosira pseudonana*. Our results confirm and extend the results of Price and Morel (1991) showing limitation of growth of both species at low unchelated Ni concentrations. We also show that cultures of *T. weissflogii* grown on urea accumulate high concentrations of Zn at low nickel concentrations, resulting in an extremely high sensitivity to zinc toxicity.

Acknowledgments

We thank Jason Wilcox for assistance in collecting data on Cu toxicity, J. P. Bellenger and Degui Tang for assistance with the ICP-MS, and Adam Kustka for help with short-term Ni uptake experiments. We express gratitude to two anonymous reviewers for their insightful comments that resulted in a stronger and more complete final manuscript. This work was supported in part by the Center for Environmental BioInorganic Chemistry, NSF grant CHE-0221978.

Methods

Experiments were conducted with cultures obtained from the Provasoli-Guillard National Center for Culture of Phytoplankton (CCMP): *T. weissflogii* (CCMP 1336) originally isolated from Gardner's Island, Long Island, New York, and *T. pseudonana* (CCMP 1335) originally isolated from Mariches Bay, Long Island, New York. Both stocks were maintained under sterile conditions in the chemically defined Aquil medium (Morel et al. 1979; Price et al. 1988–1989) prepared with Gulf Stream seawater, which has a lower background metal concentration than artificial seawater (Sunda et al. 2005). Chelation of the trace metals by 100 $\mu\text{mol L}^{-1}$ ethylenediaminetetraacetic acid (EDTA) overwhelms any natural ligand that may be present and allows a precise control of unchelated metal concentrations (e.g., $\text{Zn}' = 15 \text{ pmol L}^{-1}$). For Ni and Zn limitation experiments, Aquil was modified by replacing nitrate (NO_3^-) with 50 $\mu\text{mol L}^{-1}$ urea ($\text{CO}[\text{NH}_2]_2$) and manipulating Ni and Zn concentrations, taking into account measured background concentrations (in particular for Ni whose background was at times as high as 4 nmol L^{-1}). Cells were grown at 20°C at a pH of 8.2 ± 0.5 in acid-cleaned 250-mL polycarbonate bottles containing 100–150 mL of culture medium under continuous fluorescent light ($\sim 300 \mu\text{mol quanta m}^{-2} \text{ s}^{-1}$) and exposed to atmospheric concentrations of CO_2 ($\sim 380 \text{ ppmv}$).

Prior to experiments, cells were transferred from standard stock Aquil medium to the experimental medium, acclimated for at least 20 generations, and transferred to new medium at least four times to dilute the nitrate and higher metal concentrations of the stock medium. The diatoms were then grown for 9–10 generations to the end of the exponential growth phase, and cell concentrations and average cell size were monitored with a Z2 Beckman-Coulter multichannel electronic particle counter. Specific growth rates of cultures were computed from linear regressions of the natural log of cell density (cells mL^{-1}) vs. time of the exponential phase of growth. It was not uncommon during growth on urea and low Ni' to observe artificially high growth rate due to NH_4^+ contamination. We traced the NH_4^+ to bacterial contamination and obtained new axenic culture stocks whenever contamination was observed.

Cellular concentrations of trace metals were measured by inductively coupled plasma emission mass spectroscopy (ICP-MS), ThermoFinnegan Element 2. One hundred milliliters of exponentially growing cultures containing approximately 10,000–40,000 cells mL^{-1} were filtered through acid-cleaned 5- μm polycarbonate filters in a trace metal-clean room. The cells were washed with 5 mL of oxalic acid wash for 5 min and then rinsed three times with 5 mL of sterile chelexed NaCl (0.5 mol L^{-1}) (Tovar-Sanchez et al. 2003). The filters were then placed in 10-mL Teflon tubes, digested in 0.8 mL of 50% HNO_3 at 100°C for 4 h, and allowed to sit overnight. After dilution with 7.2 mL of Milli-Q water (Millipore Corporation), the samples were centrifuged to remove any remaining silicon frustules and analyzed by ICP-MS (Tang and Morel 2006).

Short-term metal uptake by *T. weissflogii* grown on urea or NO_3^- was measured by following the method of Price and Morel (1991). The quantity of ^{63}Ni incorporated over the course of 2 h was counted on a Beckman-Coulter LS 65000 multipurpose scintillation counter, and uptake rates were calculated by taking into account the ^{63}Ni -specific activity. Cells were harvested in midexponential phase (10,000–50,000 cells mL^{-1}) and filtered onto acid-cleaned 3- μm polycarbonate filters. They were then resuspended in metal-free filter-sterilized Aquil medium and kept in light at 20°C. Experiments were started by adding 10 nmol L^{-1} unchelated Ni. Zn was simultaneously added to some Ni uptake experiments in order to determine the effect of Zn on Ni uptake. At various time points, 5-mL subsamples were filtered onto 3- μm polycarbonate filters. Cells were rinsed for 2 min with 5 mL of 1 mol L^{-1} 8-hydroxyquinoline-5-sulfonate (sulfoxine) dissolved in Aquil medium to remove surface labile Ni, followed by 5 mL of sterile chelexed NaCl. After rinsing, the subsamples were placed in 10-mL cuvettes, and activity was measured in the scintillation counter. Some subsamples were rinsed only with NaCl and not with sulfoxine in order to measure the amount of exchangeable Ni bound to the cell surface.

Results

Growth rates—Cultures of *T. weissflogii* and *T. pseudonana* were grown on urea, ammonium, and nitrate as nitrogen sources over a range of unchelated nickel concentrations, Ni', from 0.0016 pmol L^{-1} to 1 nmol L^{-1} (Fig. 1). As expected, both diatoms maintained maximum growth rates, independent of Ni', when grown on NH_4^+ or NO_3^- , showing that their Ni requirements under these conditions are nil or very small. When urea was the nitrogen source, *T. weissflogii* grew at near its maximum rate for Ni' > 1 pmol L^{-1} and much slower for Ni' \leq 1 pmol L^{-1} , with a clear cutoff in between. *T. pseudonana* maintained near maximum growth rates down to much lower Ni' when grown on urea, with a clear decrease in growth rate observed only for Ni' \leq 0.016 pmol L^{-1} . At the maximum concentration tested, Ni' = 1 nmol L^{-1} , nickel appeared to be toxic to *T. pseudonana*. These results confirm that Ni is required for the utilization of urea by diatoms as previously reported (Syrett and Peplinska 1988; Price and Morel 1991).

Intracellular metal concentrations—In view of the large number of transport ligands presumably necessary to take up the slow-reacting Ni^{2+} ion and the relatively poor selectivity of divalent metal transporters, Ni limitation may affect trace metal homeostasis in diatoms. We compared the cellular concentrations of Ni, Zn, Co, Cu, and Mn in cultures of *T. weissflogii* grown on urea at Ni' = 1 pmol L^{-1} and Ni' = 10 pmol L^{-1} and *T. pseudonana* grown on urea at Ni' = 0.004 pmol L^{-1} and Ni' = 0.3 pmol L^{-1} on either side of the cutoff concentrations where Ni becomes limiting (Fig. 2). In both diatoms, the metal content was similar at both Ni concentrations, with one major exception: *T. weissflogii* cultures grown at 1 pmol L^{-1} Ni' had nearly 20 times higher cellular Zn

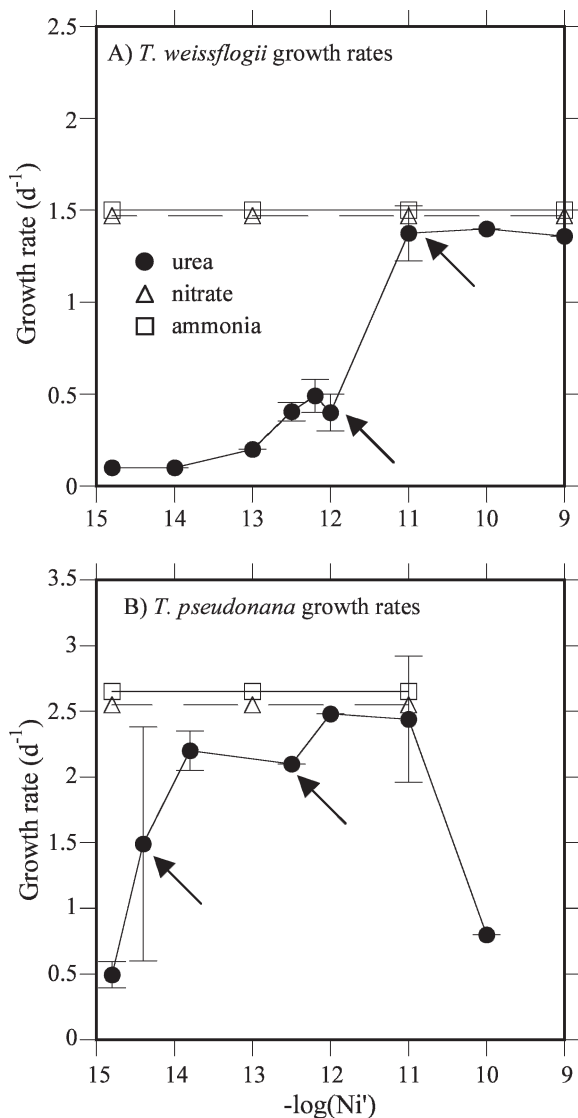


Fig. 1. (A) Growth rates of *T. weissflogii* and (B) *T. pseudonana* as a function of unchelated Ni concentration (Ni') in cultures with different sources of N. Growth on nitrate and ammonia is independent of Ni', while growth on urea varies with Ni'. Arrows indicate experiments for which cellular metal concentrations are reported in Fig. 2. Error bars represent 1 standard deviation ($n > 3$).

concentrations than those grown at 10 pmol L⁻¹ Ni' despite the fact that both cultures were grown at the same external zinc concentration (Zn' = 15 pmol L⁻¹). *T. weissflogii* cultures grown on urea and 100 pmol L⁻¹ Ni' had cellular Ni concentration in the range 25–70 amol cell⁻¹ (Table 1), somewhat larger than the previously reported cellular concentration of 19 amol cell⁻¹ for cells growing at 1 nmol L⁻¹ Ni' (Price and Morel 1991).

Interactions between Ni and Zn—To better understand the relationship between the elevated cellular Zn concentrations and Ni' in *T. weissflogii*, we grew cultures of this organism over a range of external Ni and Zn concentrations covering metal limitation and sufficiency: Ni' from

0.3 pmol L⁻¹ to 10 pmol L⁻¹ and Zn' from 3 pmol L⁻¹ to 15 pmol L⁻¹. The resulting growth rates showed a remarkable pattern (Fig. 3). In Ni-replete cultures with 10 pmol L⁻¹ Ni', decreasing Zn' to 10 pmol L⁻¹ and 3 pmol L⁻¹ induced Zn limitation and decreased growth rates as expected. But in Ni-limited cultures, at Ni' ≤ 1 pmol L⁻¹, decreasing Zn' actually increased the growth rate, with the highest growth rate observed in the cultures with the smallest Zn' of 3 pmol L⁻¹. At this Zn', growth rates declined as Ni' fell below 1 pmol L⁻¹ but remained well above growth rates in cultures with higher Zn concentrations. There appears to be a threshold nickel concentration between 1 pmol L⁻¹ and 10 pmol L⁻¹ Ni', below which 10 pmol L⁻¹ Zn' is toxic and above which it is limiting.

A comparison of the cellular Zn concentrations (Q_{Zn}) in *T. weissflogii* cultures grown on urea and 1 pmol L⁻¹ or 10 pmol L⁻¹ Ni' as a function of Zn' shows the effect of Ni limitation on Zn homeostasis (Fig. 4). In the cultures grown at 10 pmol L⁻¹ Ni', Q_{Zn} remained approximately constant at 20–30 amol cell⁻¹ for all Zn concentrations. In other words, increasing Zn' from limiting to sufficient allowed the cells to grow faster while maintaining a constant cellular Zn concentration. In contrast, in cultures grown at 1 pmol L⁻¹ Ni', Q_{Zn} increased from 38 amol cell⁻¹ at 3 pmol L⁻¹ Zn' to 185 amol cell⁻¹ at 15 pmol L⁻¹ Zn', indicating an inability to maintain Zn homeostasis under these conditions.

Short-term uptake—The simplest explanation for the high cellular Zn concentrations observed under conditions of limiting Ni concentrations is that Zn is taken up by the Ni transport system. If so, it might be possible to demonstrate a competition between Zn and Ni for uptake. In short-term uptake experiments, we observed a regulation of Ni uptake kinetics as a function of the Ni concentration in the growth medium. The short term Ni uptake rate (measured at 10 nmol L⁻¹ Ni') increased by a factor of two when the concentration of Ni in the preconditioning growth medium was decreased from Ni' = 10 pmol L⁻¹ to Ni' = 0.3 pmol L⁻¹ (Fig. 5A). At the same time, the concentration of sulfoxine-exchangeable cellular Ni increased by 70%, presumably reflecting an increase in the concentration of Ni uptake sites (Price and Morel 1991). This up-regulation of the Ni uptake system at low Ni' concomitant with a decrease in growth rate explains why the cellular Ni concentrations of cultures grown at 1 pmol L⁻¹ Ni' are actually higher than those grown at 10 pmol L⁻¹ Ni' (Fig. 2). When high concentrations of Zn (Zn' = 20 nmol L⁻¹ and 100 nmol L⁻¹) were added to short-term Ni uptake experiments performed at high Ni concentration (Ni' = 10 nmol L⁻¹) with previously Ni-limited cells, we observed a systematic decrease in Ni uptake rates and a simultaneous decrease in sulfoxine-exchangeable cellular Ni. The relationship between Ni':Zn' ratios and inhibition of Ni uptake indicates binding of both Ni and Zn by the Ni uptake ligands, with an 8–10-fold greater affinity for Ni. These results are consistent with a competitive uptake of Zn via the Ni transport system.

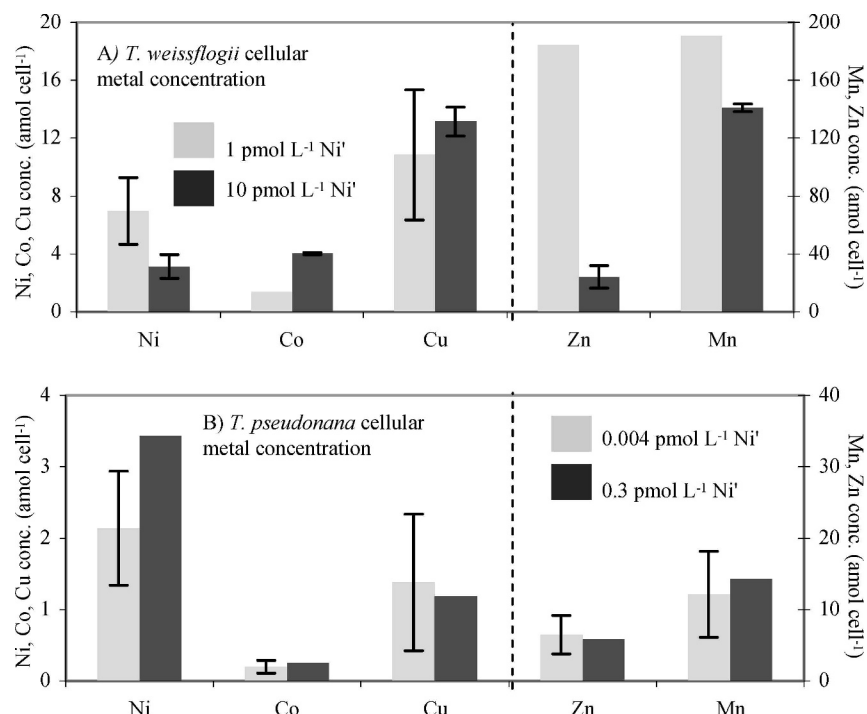


Fig. 2. Cellular metal concentrations in (A) *T. weissflogii* and (B) *T. pseudonana* measured at Ni' both just below and above values at which Ni becomes limiting when grown on urea (as indicated by arrows in Fig. 1).

Table 1. Cellular metal concentrations* (amol cell⁻¹) and growth rates (d⁻¹) in *T. weissflogii* cultures grown in varying Ni', Zn', and Cu'.

A	Urea + 1 pmol L ⁻¹ Ni'			B	Urea + 10 pmol L ⁻¹ Ni'		
	3 pmol L ⁻¹ Zn'	10 pmol L ⁻¹ Zn'	15 pmol L ⁻¹ Zn'		3 pmol L ⁻¹ Zn'	10 pmol L ⁻¹ Zn'	15 pmol L ⁻¹ Zn'
Ni	5.36 (±3.08)	6.51 (±0.99)	6.97 (±2.30)	5.05 (±1.34)	4.84 (±2.63)	3.11 (±0.82)	
Zn	48.36 (±25.7)	128.57 (±41.3)	184.52	28.11 (±11.9)	22.77 (±5.40)	24.19 (±7.79)	
Mn	144.46 (±12.1)	142.47 (±2.44)	190.94	130.71	101.12 (±33.7)	141.48 (±2.82)	
Co	3.86 (±2.46)	1.05 (±0.00)	1.36	5.08	4.68 (±0.98)	4.02 (±0.08)	
Cu	20.32 (±2.30)	8.78 (±1.60)	10.85 (±4.50)	12.74 (±0.60)	10.78 (±1.10)	13.16 (±1.00)	
μ	0.92 (±0.01)	0.67 (±0.01)	0.40 (±0.10)	0.93 (±0.02)	1.21 (±0.03)	1.38 (±0.15)	
C	Urea + 100 pmol L ⁻¹ Ni'			Urea + 100 pmol L ⁻¹ Ni'			
	12 pmol L ⁻¹ Zn'	100 pmol L ⁻¹ Zn'	1 nmol L ⁻¹ Zn'	10 nmol L ⁻¹ Zn'	32 nmol L ⁻¹ Zn'	50 nmol L ⁻¹ Zn'†	
Ni	39.86 (±11.0)	69.74 (±75.3)	40.33 (±27.7)	20.89 (±8.36)	26.11 (±1.46)	626.50	
Zn	102.44 (±42.2)	137.55 (±0.65)	191.59 (±51.0)	1609.53 (±1096)	1963.18 (±336)	13,600.61	
Mn	237.58 (±31.0)	222.13 (±3.54)	180.17 (±45.1)	80.38 (±5.79)	58.55 (±1.54)	227.59	
Co	11.35 (±2.74)	3.41 (±0.07)	2.46 (±0.48)	2.00 (±0.03)	2.56 (±0.11)	15.05	
Cu	9.89 (±3.24)	7.45 (±1.07)	8.05 (±0.48)	7.34 (±1.41)	21.31 (±8.32)	64.19	
μ	1.39	1.47	1.47	1.54	0.77	0.31	
D	Nitrate			Nitrate			
	12 pmol L ⁻¹ Zn'	100 pmol L ⁻¹ Zn'	1 nmol L ⁻¹ Zn'	10 nmol L ⁻¹ Zn'	32 nmol L ⁻¹ Zn'		
Ni	10.14	8.32 (±1.91)	13.16 (±4.26)	10.62 (±0.23)	8.04 (±1.11)		
Zn	47.14	136.50 (±32.6)	600.75 (±22.6)	1878.96 (±391)	2400.92 (±682)		
Mn	243.23	198.01 (±25.7)	213.16 (±28.7)	95.47 (±2.90)	72.42 (±4.45)		
Co	15.11	2.48 (±0.36)	2.68 (±0.13)	3.05 (±0.52)	2.78 (±0.13)		
Cu	11.69	8.21 (±1.96)	11.62 (±0.30)	8.40 (±0.51)	12.06 (±0.03)		
μ	1.47	1.47	1.50	1.54	1.14		
E	Copper toxicity			Copper toxicity			
		0.016 pmol L ⁻¹ Cu'	0.16 pmol L ⁻¹ Cu'	1.6 pmol L ⁻¹ Cu'	16 pmol L ⁻¹ Cu'	160 pmol L ⁻¹ Cu'	
μ	1 pmol L ⁻¹ Ni'	0.43	0.5	0.47	0.46	0.38	
μ	10 pmol L ⁻¹ Ni'	1.55	1.57	1.54	1.54	0.82	

* Average cellular P for *T. weissflogii*: 0.13 pmol cell⁻¹ (this study); average cellular C for *T. weissflogii*: 7.7 pmol cell⁻¹ (Sunda and Huntsman 1992).

† Quotas for extremely slow growing cultures may not be accurate.

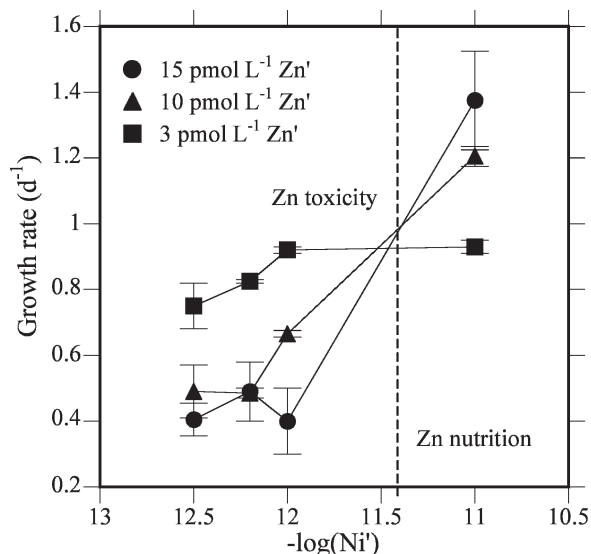


Fig. 3. Growth rates for *T. weissflogii* cultures grown on urea as a function of unchelated Ni concentration (Ni') at three levels of Zn' : 15 pmol L^{-1} , 10 pmol L^{-1} , and 3 pmol L^{-1} . Note the increase in growth rate with increasing Zn' at nonlimiting Ni' (10 pmol L^{-1}) and the decreasing growth rate with increasing Zn' at limiting Ni' (1 pmol L^{-1}). Error bars represent 1 standard deviation ($n \geq 3$).

Zn toxicity—The results of Fig. 3 showing an increase in growth rate with decreasing concentrations of Zn (and decreasing cellular Zn; Fig. 4) at low Ni' imply that Zn is somehow toxic to Ni-limited *T. weissflogii*. To better understand this phenomenon, we investigated the effect of Zn concentration (Zn' from 12 pmol L^{-1} to 50 nmol L^{-1}) on *T. weissflogii* cultures grown on NO_3^- and on urea at high Ni ($\text{Ni}' = 100 \text{ pmol L}^{-1}$). Under both conditions, these cultures maintained maximum growth rates for $\text{Zn}' \leq 10 \text{ nmol L}^{-1}$ and exhibited a decrease in growth rate at $\text{Zn}' > 30 \text{ nmol L}^{-1}$ (Table 1, C and D). These results are very similar to other published studies of Zn toxicity in *T. weissflogii* cultures grown on NO_3^- (Sunda and Huntsman 1992; Reinfeldt et al. 2000; Miao et al. 2005). At $\text{Ni}' = 10 \text{ pmol L}^{-1}$, growth increases with Zn' and the data are quantitatively consistent with the data on Zn limitation of *T. weissflogii* grown on NO_3^- (Sunda and Huntsman 1992). But the data from cultures grown on urea at Ni' of 1 pmol L^{-1} and 0.3 pmol L^{-1} do not follow the general pattern, exhibiting decreasing growth rates with increasing Zn' in a range where the other cultures are limited by Zn (Table 1A).

Cultures grown on NO_3^- or on urea and high Ni' have decreasing growth rates for Q_{Zn} above $2000 \text{ amol cell}^{-1}$ (or below $50 \text{ amol cell}^{-1}$), while cultures grown on urea and low Ni' have decreasing growth rates for Q_{Zn} above $100 \text{ amol cell}^{-1}$. Thus, the increase in the Q_{Zn} of low Ni cultures does not by itself explain their decreasing growth rate (Table 1). Somehow, Ni-limited cells are much more sensitive to Zn toxicity—by a factor of about 20—than *T. weissflogii* cells that are not limited by Ni.

Other metals—Many divalent metal ion transporters have low selectivity and transport other metals such as Cu

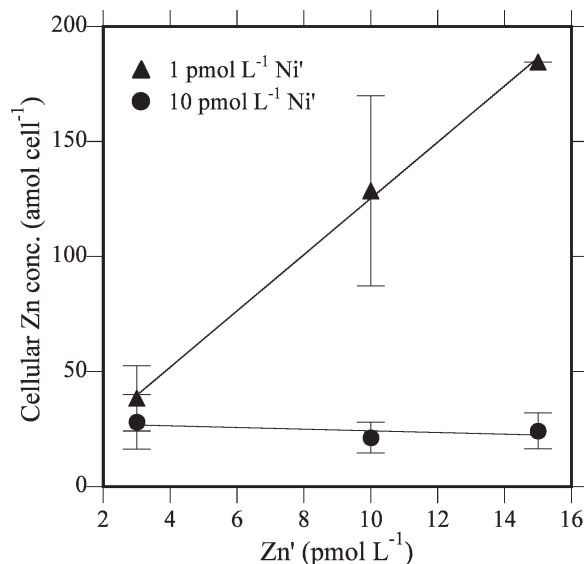


Fig. 4. Cellular Zn concentrations in *T. weissflogii* cultures grown on urea as a function of Zn' at limiting (1 pmol L^{-1}) and nonlimiting (10 pmol L^{-1}) unchelated Ni concentrations (Ni').

and Mn along with Ni or Zn (Sunda and Huntsman 1998a; Watt and Ludden 1999). Since Cu is particularly toxic to diatoms, we examined the growth rate and cellular metal concentrations in *T. weissflogii* cultures grown on urea over a range of Ni and Cu concentrations (Ni' from 0.3 pmol L^{-1} to 10 pmol L^{-1} and Cu' from $0.016 \text{ pmol L}^{-1}$ to 160 pmol L^{-1}). The growth rates of the cultures remained constant up to $\text{Cu}' = 16 \text{ pmol L}^{-1}$ and declined at $\text{Cu}' = 160 \text{ pmol L}^{-1}$ (Table 1E). This level of sensitivity to Cu' is the same as that reported for *T. weissflogii* grown in *f/2* medium with NO_3^- as an N source (Miao et al. 2005). There was no variation in response between cultures grown at different Ni' and no increase in cellular Cu concentrations at low Ni' . Thus, Ni-limited cultures, which accumulate Zn and are very sensitive to Zn toxicity, do not accumulate Cu and are not affected by it. This must correspond either to an ability of the Ni transporter to discriminate more effectively against Cu than against Zn or, perhaps more likely, to the existence of an effective efflux system for Cu but not for Zn (Ahner and Morel 1995; Lee et al. 1996).

In the diatom *T. pseudonana*, high Zn concentrations inhibit Mn uptake, leading to a decrease in cellular Mn concentration and growth rate (Sunda and Huntsman 1983, 1996). In our experiments with *T. weissflogii*, as Zn' increased from 12 pmol L^{-1} to 50 nmol L^{-1} , steady-state Mn uptake rates derived from cellular metal concentrations and growth rates fell from $360 \text{ amol cell}^{-1} \text{ d}^{-1}$ down to $45 \text{ amol cell}^{-1} \text{ d}^{-1}$, while Mn' remained constant. The decrease in growth rate for *T. weissflogii* occurred between $\text{Zn}' = 10 \text{ nmol L}^{-1}$ and 32 nmol L^{-1} when the cellular Mn concentration decreased from 80 to $95 \text{ amol cell}^{-1}$ to 59 to $72 \text{ amol cell}^{-1}$ (Table 1C). This corresponds to a decrease in the Mn:C mol ratio from about 7 to $8 \text{ } \mu\text{mol mol}^{-1}$ down to 5 to $6 \text{ } \mu\text{mol mol}^{-1}$, respectively, in the same range observed to cause a decrease in the growth rate in *T.*

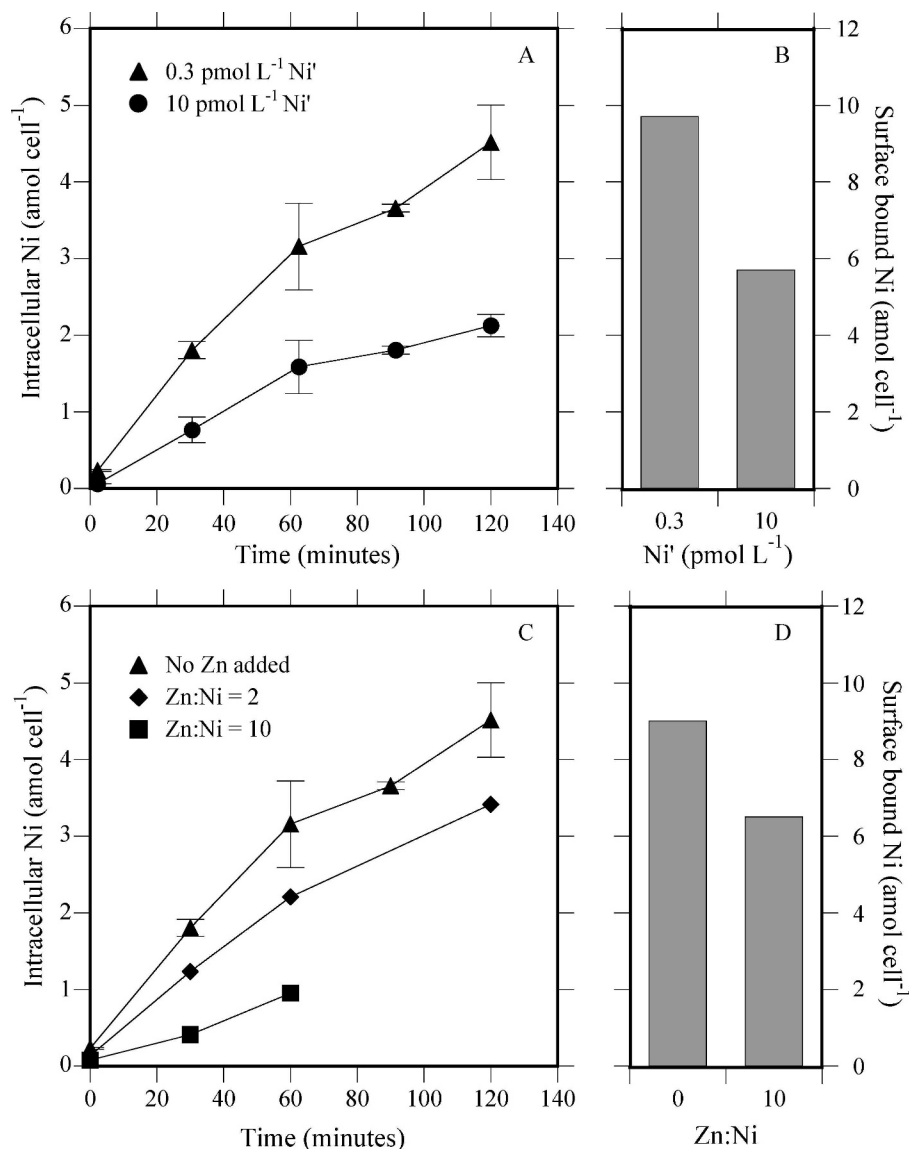


Fig. 5. Short-term Ni uptake and sulfoxine exchangeable Ni concentration experiments in *T. weissflogii* grown on urea. Experiments are in assay medium with 10 nmol L⁻¹ Ni'. (A) Effect of Ni' in preconditioning medium of 10 pmol L⁻¹ (51 amol cell⁻¹ d⁻¹) and 0.3 pmol L⁻¹ (25 amol cell⁻¹ d⁻¹) on Ni uptake rates. Panel presents time-course measurements of the amount of Ni internalized by each cell (nonsulfoxine-exchangeable) over a 2-h period from which uptake rates can be calculated. (B) Sulfoxine-exchangeable surface bound Ni as a function of preconditioning Ni', an estimate of the number of Ni binding sites on the cell surface. (C) The effect of Zn concentration in the assay medium on Ni uptake rates in cells preconditioned in 0.3 pmol L⁻¹ Ni': no Zn added (51 amol cell⁻¹ d⁻¹), 20 nmol L⁻¹ unchelated Zn added (40 amol cell⁻¹ d⁻¹), and 100 nmol L⁻¹ unchelated Zn added (33 amol cell⁻¹ d⁻¹). (D) Sulfoxine-exchangeable surface-bound Ni as a function of Zn : Ni in cells preconditioned in 0.3 pmol L⁻¹.

pseudonana that occurred at a threshold Mn:C ratio of about 5–10 $\mu\text{mol mol}^{-1}$ (Sunda and Huntsman 1996).

Co is known to alleviate Zn limitation in diatoms, including *T. weissflogii*, presumably through replacement in the active site of some Zn enzymes such as carbonic anhydrase (Yee and Morel 1996). Co uptake is regulated by external Zn' in several species of *Thalassiosira*, with the two metals likely sharing an uptake system (Sunda and Huntsman 1995). In our experiments, with the exception of those grown at 1 pmol L⁻¹ Ni', all cells growing at Zn'

≤ 12 pmol L⁻¹ had much higher cellular Co concentration than cells grown at higher Zn'. At a given Zn', cellular Co concentrations also increased noticeably with increasing Ni' (Table 1). These results might be consistent with Co uptake occurring primarily via the Zn uptake system, the negative effect of Zn being caused by competitive inhibition, and the positive effect of Ni possibly reflecting a down-regulation of the Zn-Co uptake system in response to elevated cellular Zn concentrations in low Ni' cultures.

Discussion

Where they can be compared, our data are generally in qualitative and quantitative agreement with the results of earlier studies. Cultures grown on urea at high Ni' behaved similarly to cultures grown on NO_3^- and were affected by the concentrations of other metals in the same way. Cultures grown on urea at low Ni' became limited by N, although there are significant quantitative differences between the two species we studied, and *T. weissflogii* cultures also demonstrated an interesting effect of Zn toxicity.

High Ni' cultures—Our growth experiments with *T. weissflogii* and *T. pseudonana* showed no significant differences between cultures grown on urea at high Ni' and cultures grown on NO_3^- . In both cases, we observed Zn limitation and Zn toxicity over the same ranges of Zn' as previous authors who used NO_3^- as a nitrogen source (Sunda and Huntsman 1992; Reinfelder et al. 2000). We also observed Cu toxicity in *T. weissflogii* over the same range of Cu' as previous authors (Miao et al. 2005).

A previous study with *T. pseudonana* grown on NO_3^- (Sunda and Huntsman 1996) demonstrated a decrease in Mn uptake rates at high Zn concentrations. *T. weissflogii* cultures grown on NO_3^- or on urea at high Ni' showed a similar decrease in Mn uptake. The threshold cellular Mn concentration below which growth rate decreased in our experiments and in the previous study are essentially identical: Mn:C ratio between 5 and $10 \mu\text{mol mol}^{-1}$. This quantitative agreement makes it likely that Mn limitation, caused by an interference of Zn with Mn uptake, is the reason for the low growth rate of those cultures, as demonstrated in the previous study (Sunda and Huntsman 1996).

Growth of low Ni' cultures—The response to low nickel concentrations we observed is qualitatively identical to that previously reported for urea-grown cultures (Price and Morel 1991), although the cutoff Ni' below which *T. weissflogii* growth decreased was a factor of 10 higher in the earlier study. The reason for this difference is unknown. It may have been caused by the different chelating agents used as metal buffers in the two studies: EDTA in our study and the faster-reacting diethylenetriaminepentaacetate (DTPA) in the other. It is also possible that the strains of *T. weissflogii* used in the two studies 16 yr apart are actually not identical and respond differently to Ni limitation. Whatever the cause for the difference, it is consistent with the greater accumulation of Ni by the cells in our experiments compared to the previous one. In any case, it is clear that *T. weissflogii* and *T. pseudonana* have a negligible Ni requirement when growing on NO_3^- or NH_4^+ and an absolute Ni requirement when growing on urea as an N source. This result is consistent with previous data showing that diatoms depend on urease for urea utilization (Syrett 1981).

Zn uptake by *T. weissflogii* at low Ni'—Our concentration and uptake data for Ni and Zn in *T. weissflogii* provide

evidence that Zn is taken up via the Ni transport system of this organism under conditions of Ni limitation. This is seen in the high cellular Zn concentrations (Fig. 2) and the increased Zn uptake rate (Fig. 5) in Ni-limited cells. The resulting prediction that high Zn concentrations should interfere with Ni uptake is actually seen in the short-term uptake data (Fig. 5C). Such lack of specificity for metal uptake in phytoplankton has been well documented before. For example, the metal pairs Mn and Cu, Mn and Zn, Mn and Cd (Sunda and Huntsman 1998a), Zn and Cd (Lee et al. 1995), and Zn and Co (Sunda and Huntsman 1995) have all been shown to compete for uptake with one another in various species. One reason for this lack of specificity may be the very low ambient concentration of essential metals in surface seawater that makes it necessary for the cells to internalize all metal ions that become bound to uptake ligands on their surface. The specificity afforded by the differential kinetics of the dissociation reaction is then lost, and the uptake rate becomes limited by the rate of metal binding to the uptake ligands (Hudson and Morel 1993). Ni^{2+} being the slowest reacting of the essential metal cations, the corresponding number of uptake ligands must be very large at low Ni', creating an opportunity for significant uptake of other metals through the same transport system.

At this point, nothing is known of the molecular aspects of Ni uptake in phytoplankton. In bacteria, Ni is taken up by two types of high-affinity transport systems, Ni-specific permeases, and ATP-binding cassette (ABC)-type transporters (Mulrooney and Hausinger 2003). Homologues of both types of transport systems are expressed in eukaryotic organisms, including *T. pseudonana* (Armbrust et al. 2004). Both types are used to transport several divalent metal ions beside Ni^{2+} , and neither is usually very specific. For example, a bacterial permease known to transport both Co and Ni in *Rhodococcus rhodochrous* has a slight preference for Co (Degen et al. 1999). Another permease which is used for Ni transport in *Rhodospirillum rubrum* is inhibited by Co, Cd, and Cu (Watt and Ludden 1999). The Ni-binding protein NikA, a member of the ABC family of transporters in *Escherichia coli*, has only a 25-fold higher affinity for Ni than for Co (Hedde et al. 2003). The binding characteristics of NikA have been debated, but the protein seems able to bind both hydrated and chelated forms of Ni (Cherrier et al. 2005; Addy et al. 2007). The binding of these forms of Ni overcomes the slow kinetics of water loss from Ni^{2+} but at the cost of lower affinity and limited specificity. This is consistent with the need for a very high affinity uptake system for trace metals in marine organisms.

Possible mechanism of Zn toxicity at low Ni'—While Zn toxicity in *T. weissflogii* cultures grown on NO_3^- or urea with high Ni' appears to result from Mn limitation (see previous discussion), this is clearly not the case in cultures grown on urea at low Ni', which have high cellular Mn concentrations (corresponding to Mn:C ratios above $10 \mu\text{mol mol}^{-1}$). So it seems that the high sensitivity of these cultures to intracellular Zn is somehow linked to Ni limitation. A possibility is that Zn interferes with intracel-

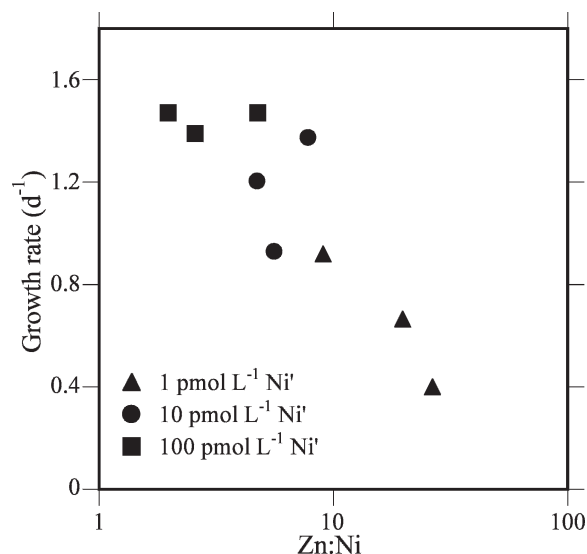


Fig. 6. Growth rates of *T. weissflogii* grown on urea as a function of the intracellular Zn:Ni ratio (data from cultures at $Zn' < 1 \text{ nmol L}^{-1}$, below the value where Mn becomes limiting).

lular Ni binding to urease. Urease requires two Ni^{2+} ions at its active site, and the metal must be delivered to the apoprotein by the metallochaperone UreE to produce active urease (Lee et al. 1990). Zn binding has been documented to inactivate urease (King and Zerner 1997), and UreE has been shown to have an affinity for Zn^{2+} 13- to 20-fold lower than for Ni^{2+} (Grossoehme et al. 2007). If the toxic effect of Zn on cells grown on urea at low Ni' is due to the competitive binding of Zn replacing Ni in urease or its accessory proteins, we might expect that the growth rate of the organism may be controlled by the Zn:Ni ratio in the cells. We thus plotted the growth rate of *T. weissflogii* cultures grown on urea over a range of Ni and Zn concentrations as a function of the cellular Zn:Ni ratio—excluding the cultures at very high Zn concentration ($Zn' \geq 10 \text{ nmol L}^{-1}$) in which Zn toxicity is caused by Mn limitation. The results (Fig. 6) exhibit a reasonable correlation, with a decrease in growth rate occurring for a cellular Zn:Ni ratio between 5 and 30 mol mol⁻¹, roughly in accord with the relative Zn and Ni affinities for the UreE metallochaperone. So Zn toxicity in Ni-limited cultures may indeed result from an inability of the cells to bind Ni at the active site of urease. Although this explanation is speculative, it is worth emphasizing that the extracellular Zn' and the cellular Zn concentrations that caused a decrease in growth rate in Ni-limited cells in our experiments are far lower than ever reported for Zn toxicity. It is possible that such extreme sensitivity to Zn toxicity at low Ni' is the reason for the relatively poor growth observed previously in urea-grown cultures with no added Ni of *T. weissflogii* (Fan et al. 2003) and of the cyanobacterium *Agmenellum quadruplicatum* (Syrett 1981).

Differences between species and environmental significance—Although the responses of urea-grown cultures of *T. weissflogii* and *T. pseudonana* to low Ni are qualitatively the same, they are very different quantitatively, as seen in

Fig. 1. In *T. weissflogii*, Ni becomes limiting at unchelated concentrations on the order of pmol L^{-1} , similar to the limiting concentrations of other metals, such as Zn (Sunda and Huntsman 1992). At such a concentration, the supply of metal to the cell (which needs to take it up at a rate of a few $\text{amol cell}^{-1} \text{ d}^{-1}$ for maximum growth) becomes limited by molecular diffusion to the cell surface (Hudson and Morel 1993). Ni limitation of *T. weissflogii* thus occurs over the expected range of Ni' . But *T. pseudonana* is somehow able to maintain a sufficient Ni uptake rate (which is also on the order of a few $\text{amol cell}^{-1} \text{ d}^{-1}$) down to $Ni' = 0.02 \text{ pmol L}^{-1}$. Such a very high rate of uptake of a metal at very low concentration, in excess of the supply of unchelated metal by diffusion, has been observed before in phytoplankton (Hudson 1998; Xu et al. 2007). A possible though seemingly improbable explanation is that the cells are somehow able to obtain the metal from the EDTA chelate. Alternatively, the bioavailable metal concentration may be increased by formation of a weak complex—one that can exchange the metal with surface ligands more rapidly than the diffusion time—with some exudate in solution. In this second alternative, the complexation of Ni by the weak ligand exudate, which leads to an increase in Ni' , would provide another opportunity for specificity of the overall uptake system and might explain why, unlike *T. weissflogii*, *T. pseudonana* is not affected by Zn toxicity at low Ni' .

The extrapolation of our laboratory results to the ocean is not straightforward. First, the unchelated nickel concentrations, Ni' , over which we observed a limitation of growth is much lower than the total Ni concentration in the surface ocean, ca. 2 nmol L^{-1} (Bruland 1980). Ni limitation would occur only if chelation lowered Ni' about three orders of magnitude below total Ni. Unfortunately, the question of whether Ni in oceanic surface waters is actually chelated is unresolved at this point (Bruland et al. 1991; Mackey et al. 2002; Saito et al. 2004). Second, for Zn to be toxic to some species of phytoplankton at low Ni' requires a ratio $Zn':Ni'$ in excess of 10. This is not impossible in principle since the affinity of many complexing agents is higher for Ni than for Zn, and this higher affinity may compensate for the usually higher concentrations of Zn than Ni in surface seawater (Morel and Hering 1993). But, again, testing this possibility requires new information on potential Ni chelating agents in seawater.

Aside from the specifics of the Ni-Zn interactions, the result of our study showing high toxicity of a trace metal resulting from the limitation by another may be generalizable. It seems that the superposition of the very low concentrations of essential metals in the surface ocean, the relatively poor selectivity of the uptake systems that ensues, and the inactivation of metalloproteins by metals others than those necessary for activity may result in a situation where several metals may be simultaneously limiting and toxic to the oceanic biota. Similar interactions have been previously shown in the laboratory for various metals; for example, Zn can become simultaneously limiting and toxic at high Cu and low Mn concentrations (Sunda 1998a). Some data indicate that Cu (Mann et al. 2002) and possibly Cd (Saito et al. 2003) may be toxic or nearly toxic to some

phytoplankton species in the ocean. It is possible that the relatively high unchelated Ni concentration in surface seawater is necessary not just because of slow uptake kinetics by the plankton but also to avoid toxicity by other metals that may be transported along with Ni. This, in turn, may limit the use of urea as a source of nitrogen for primary production or favor species that do not suffer from Zn toxicity in regions with sufficient Zn concentrations.

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Received: 12 November 2007

Accepted: 20 June 2008

Amended: 21 June 2008