

## Iron and zinc enrichments in the northeastern subarctic Pacific: Ligand production and zinc availability in response to phytoplankton growth

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### Abstract

Iron- and zinc-enrichment experiments were carried out at Ocean Station Papa in the subarctic North Pacific. In iron-enriched treatments, phytoplankton chlorophyll *a* (Chl *a*) increased 20-fold ( $9.7 \mu\text{g L}^{-1}$ ) above the concentration on day zero. No stimulation of Chl *a* production or nitrate drawdown was observed on addition of zinc alone compared to the control. In the iron-enriched treatment, bioavailable zinc concentration decreased to  $0.2 \text{ pmol L}^{-1}$  lower than that which is known in culture experiments to limit some phytoplankton growth. Theoretical analyses suggest that this zinc concentration would cause diffusion-limited growth of large diatom cells present at the end of the incubation. Direct measurements of zinc-binding ligands indicate that the natural microbial planktonic assemblages have the ability to respond rapidly to conditions of high dissolved zinc concentrations. Rapid ligand production may be a mechanism by which certain phytoplankton reduce zinc toxicity or for maintaining zinc concentrations in the upper water column. Zinc-binding ligands were observed to be both produced and removed on the timescale of 1 d. We suggest that these zinc-binding ligands are produced to assist assimilation, particularly under iron-enriched conditions when concentrations of bioavailable zinc were extremely low, thereby alleviating the effects of zinc limitation.

Over the past two decades, there has been mounting evidence to suggest that in some oceanic environments, very low total dissolved zinc concentrations ( $2\text{--}10 \text{ pmol L}^{-1}$ ) may limit phytoplankton growth (Anderson and Morel 1978; Morel et al. 1991; Sunda and Huntsman 1992). At these low zinc concentrations, microalgae are unable to make enough functional carbonic anhydrase, which may limit carbon acquisition and consequently the rate of algal growth (Morel et al. 1994; Tortell et al. 2000). Zinc limitation is particularly important for diatoms, which are responsible for fixing the bulk of carbon exported from the surface to the deep ocean (Tortell et al. 1997, 2000). In contrast, Fukuda et al. (2000) suggest that the suppression of synthesis of bacterial leucine aminopeptase (a zinc-containing enzyme) can accelerate the carbon and nitrogen pump because of increased degradation of proteins in the euphotic zone. Recent attention has focused on another zinc-containing enzyme, alkaline phosphatase, which enables phytoplankton to utilize dissolved organic phosphorus when inorganic phosphate is limiting (Riegman et al. 2000; Dyhrman and Palenik 2003). In oligotrophic gyres where both zinc and inorganic phosphate concentrations are low, the calcite coccoliths of *Emiliania huxleyi* have been demonstrated to enhance the extracellular

activity of alkaline phosphatase and hence use organic phosphate (Shaked pers. comm.). Thus, if zinc concentrations in oligotrophic gyres are sufficiently low, this may limit the ability of coccolithophores to utilize organic phosphorus. In oceanic regions where zinc concentrations are limiting, the corresponding decrease in coccolithophorid growth is not accompanied by a decrease in the production of calcium carbonate, resulting in highly calcified cells and hence altering carbon sequestration in the ocean (Schulz et al. 2004).

There is conflicting evidence as to whether zinc is limiting phytoplankton growth in the open ocean. In high nutrient–low chlorophyll (HNLC) regions such as the subarctic North Pacific, phytoplankton growth is known to be limited by iron (Martin and Gordon 1988; Boyd et al. 1996; Crawford et al. 2003). In the subarctic North Pacific, phytoplankton growth may also be limited by the extremely low total dissolved zinc concentrations ( $40 \text{ pmol L}^{-1}$ ; Lohan et al. 2002). In the North Atlantic, Ellwood and van den Berg (2000) showed from zinc speciation experiments that the phytoplankton in these waters are not zinc limited. Yet in the Southern Ocean, another HNLC region, complexation by organic ligands reduced the bioavailable zinc concentration to  $0.2 \text{ pmol L}^{-1}$  and could potentially limit phytoplankton growth (Ellwood 2004).

Phytoplankton excrete organic substances that may act as metal ligands in surface oceanic waters to help keep dissolved zinc in solution as opposed to being adsorbed onto particle surfaces (Bruland 1989). Degradation products from zooplankton grazing have also been hypothesized as a source of these organic ligands (Bruland et al. 1991). Depending on their physiochemical properties, these ligands can influ-

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ence metal availability to the algal species that produce them and also to their competitors (Whitfield 2001) by lowering the concentration of the free metal ion. At any point during the cycle of ligand production and degradation, microorganisms will seek to optimize their use of essential metals according to their cellular quotas (Morel et al. 1991), uptake rates (Hudson and Morel 1990), and concentration mechanisms (Hudson and Morel 1993; Sunda and Huntsman 1995), leading to changes in community structure with time (Muller et al. 2003).

Previous studies at Ocean Station Papa (50°N, 145°W), northeastern Pacific Ocean, have examined the impact of additions of trace metals on phytoplankton growth (Martin et al. 1989; Boyd et al. 1996; Crawford et al. 2003). While these studies have revealed that iron strongly limits phytoplankton growth, they have not assessed the production of complexing ligands by phytoplankton and the impact these ligands may have on the speciation and availability of iron and zinc to phytoplankton. Coale (1991) did investigate the effect of added copper (Cu) on the production of copper-complexing ligands and free  $\text{Cu}^{2+}$  ion concentration in waters from the subarctic Pacific incubated over 7 d. Most laboratory phytoplankton culture studies (e.g., Brand et al. 1983; Sunda and Huntsman 1992) have been carried out using natural seawater supplemented with trace metal buffer systems (e.g., ethylenediamine tetra-acetic acid [EDTA] and nitrilotriacetic acid [NTA]), where the natural organic ligands (including those released during growth) have been ignored.

To date, the few field studies in which incubations of surface HNLC waters have been supplemented with zinc have suggested minimal effects on phytoplankton growth and have primarily concentrated on the biological response, rather than the impact of the natural microbial community on the chemical speciation of the metal added (Scharek et al. 1997; Franck et al. 2000; Crawford et al. 2003). Although the concentration of zinc at Ocean Station Papa is extremely low in near-surface waters (Lohan et al. 2002), results from our earlier study suggested that zinc has only a minor effect in stimulating growth and production of phytoplankton in these HNLC waters (Crawford et al. 2003). The addition of iron and zinc in combination, compared to iron alone, however, resulted in a significant shift in physiology and size distribution of chlorophyll within the population (Crawford et al. 2003).

In this study, we investigated the effect of zinc and iron enrichments on zinc speciation, and in particular the zinc-binding ligand and free zinc concentrations in order to examine their role in the bioavailability of zinc to phytoplankton.

## Materials and methods

*Shipboard incubations*—An experimental strategy was designed to incubate on deck a series of aliquots of surface water from Ocean Station Papa supplemented with iron (+Fe), zinc plus iron (+Zn and +Fe), zinc (+Zn), and a control (no additions). The 4-liter low-density polyethylene (LDPE) flexible cubic containers (“cubitainers”; VWR Canlab) were acid cleaned according to the protocol in Crawford

et al. (2003). The cubitainers were rinsed in open ocean water and left to soak in oceanic water for 2 d before arriving at Ocean Station Papa, in order to permit equilibration and thus minimize adsorption of trace metals onto the container walls. Once arriving on station, the cubitainers were rinsed three times with Ocean Station Papa surface (15-m depth) seawater prior to filling.

Samples for the incubation experiment were collected from Ocean Station Papa during the hours of darkness between 17 and 18 June 2001 onboard the CCGS *John P. Tully*. Seawater was collected from 15-m depth with a Teflon pump (PFD-1 Asti) and Teflon tubing. The mixed-layer depth at the time was 75 m. Samples were pumped directly into the 32 acid-cleaned cubitainers in an on-deck PVC ultralow-penetration air clean hood. The water samples were not prefiltered to exclude grazing organisms prior to incubation. Rinsing and filling the cubitainers took most of the night and therefore the species composition could have changed during that time. In order to minimize any bias, the filled cubitainers were randomly assigned to one of the four treatments.

Once filled, additions of zinc (1,000 ppm Zn stock solution [Fisher Scientific]) as  $\text{ZnCl}_2$  equivalent to  $10 \text{ nmol L}^{-1}$  were made to eight cubitainers; another eight cubitainers were spiked to  $10 \text{ nmol L}^{-1}$  iron (1,000 ppm Fe stock solution [Fisher Scientific]) as  $\text{FeCl}_3$ ; and  $10 \text{ nmol L}^{-1}$  iron plus  $10 \text{ nmol L}^{-1}$  zinc was added to a further eight cubitainers. The remaining eight cubitainers served as a control. In many previous incubation experiments, iron was added in a 1:1.5 molar ratio with EDTA solution to ensure initial solubility of iron (Coale 1991; Boyd et al. 1996). Following the addition of EDTA, it is argued that the supply of iron to the cell is automatically related to the concentration of free iron, as the EDTA-bound iron is not directly available (Boye and van den Berg 2000). However, as the natural speciation of zinc was to be measured in all treatments, the addition of EDTA was not possible, as it would affect the speciation measurements. All cubitainers were double-bagged in clear heavy-duty polyethylene to minimize contamination from the incubator coolant water and then before dawn placed in on-deck incubators supplied with surface seawater to maintain mixed-layer temperatures ( $\sim 7^\circ\text{C}$ ). Neutral density screening provided spectrally unmodified shading to 30% of the surface ambient light level, estimated to correspond to the irradiance level at the depth of collection. Cubitainers were randomly placed in the incubators to minimize any shading bias due to position on-deck.

The cubitainers were not repetitively sampled, thereby avoiding contamination during the subsampling procedure. Filtered samples were taken from the pumped supply at the time of collection ( $t_0$ ) for total dissolved Zn and Fe analysis using a  $0.22\text{-}\mu\text{m}$  acid-cleaned polycarbonate membrane filter (Nuclepore). Samples were also taken from the pumped supply at  $t_0$  for chlorophyll *a* (Chl *a*), particulate organic carbon (POC), and inorganic nutrient analysis.

During the course of the experiment, one cubitainer from each of the four treatments was randomly removed each evening at 18:00 h toward the end of the light period and was sampled for Chl *a*, POC, nutrients, total dissolved Zn and Fe, and zinc speciation. Because of the limited water

volume available (4 liters), no replication of any samples was possible. In a previous incubation experiment carried out in 1999, each treatment was triplicated with very little difference measured between replicates (Crawford et al. 2003). Daily measurements (without replication) over 8 d allowed a more detailed study of trace metal interaction with the biota rather than having fewer time point samples with replication.

### Analytical methods

**Trace metals**—Seawater samples for Zn and Fe analysis were immediately vacuum filtered through acid-cleaned 0.22- $\mu\text{m}$  polycarbonate membrane filters (Nuclepore) into two replicate 500-ml LDPE bottles. Sample collection, filtration, and analysis were carried out using trace metal clean techniques. One 500-ml aliquot of each sample was acidified to pH 2 with 0.5 ml ultrapure HCl (SeaStar) for total dissolved Zn and Fe analysis. The other 500-ml sample was used for Zn speciation studies, which were analyzed within 3 h of collection. Samples for total dissolved zinc analysis were analyzed 6 months later by adsorptive cathodic stripping voltammetry (AdCSV). Measurements of total dissolved Zn (not reported here) and Fe were also carried out with a chelation solvent extraction method (Statham 1985).

**Total dissolved zinc and zinc speciation analysis**—The voltammetric system consisted of a Metrohm (model 663 VA Stand) static mercury drop interfaced with a  $\mu\text{Autolab}$  voltammeter (Eco Chemie), which was controlled by a Vigen 486 computer. The reference electrode was a double-junction, Ag/AgCl, KCl- (3 mol L<sup>-1</sup>) saturated AgCl electrode, and the counter electrode was a glassy carbon rod. A 0.01 mol L<sup>-1</sup> stock solution of ammonium pyrrolidinedithiocarbamate (APDC) (Sigma) was prepared every 4 d in a 1% (v/v) isothermally distilled high-purity ammonium solution (Q-NH<sub>3</sub>) and was cleaned by extraction with three 3-ml aliquots of trace metal clean chloroform. A 1.5 mol L<sup>-1</sup> stock borate solution was prepared following the method described by Ellwood and van den Berg (2000). Zinc standards were prepared in 0.05% Q-HCl by serial dilution of a 1,000-ppm standard (BDH).

Total dissolved zinc was determined by AdCSV with APDC as the competing ligand, as described in detail in Lohan et al. (2002). The concentration of free zinc and zinc-binding ligands was analyzed using competitive ligand equilibrium (CLE)-AdCSV. This involves the establishment of a competitive equilibrium between zinc-complexing ligands naturally present in the sample and a competing organic ligand, APDC, added to the sample (van den Berg 1985; Donat and Bruland 1990; Ellwood and van den Berg 2000). The theory of determining zinc speciation in seawater has been previously described in detail (van den Berg 1985; Donat and Bruland 1990; Ellwood and van den Berg 2000). Zinc-speciation measurements were performed onboard ship, in order to minimize possible changes to chemical equilibria in samples caused by their storage and transport and to prevent loss of analyte due to changes in redox speciation or adsorption on the container walls.

The method involves a titration procedure to determine

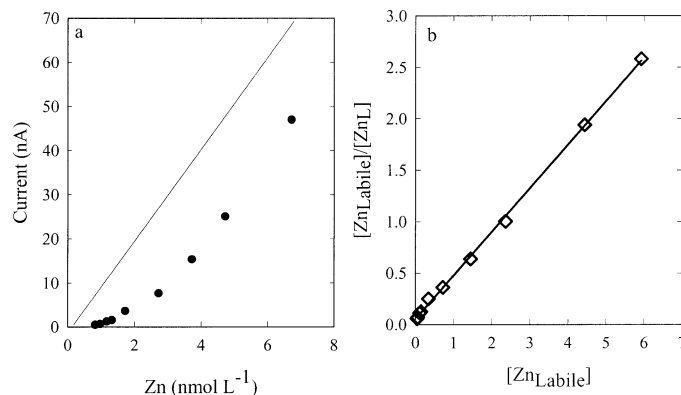


Fig. 1. Example of Zn titration data for day 5, control. (a) Peak current versus added Zn concentration. The line indicates the response if no ligand were present. (b) Linearization of data from which a ligand concentration of 0.26 nmol L<sup>-1</sup> and  $K'_{\text{ZnL}}$  of 10.8 was calculated.

the concentration of natural metal complexing ligands (L) and their conditional stability constants ( $K'_{\text{ZnL}}$ ). Before use, Teflon vials were acid cleaned and then conditioned (3 $\times$  with seawater and zinc additions) to minimize the effects of Zn-PDC (pyrrolidinedithiocarbamate) adsorption onto the vial walls. To each of the 10 15-ml Teflon vials (Cole-Palmer), 10 ml of filtered seawater plus 60  $\mu\text{l}$  of borate buffer (1.5 mol L<sup>-1</sup>) was added. Each vial was then spiked with increasing zinc concentrations and allowed to equilibrate for 3–4 h. As two of the incubations included additions of dissolved zinc (10 nmol L<sup>-1</sup>), the final spiked addition of zinc was 30 nmol L<sup>-1</sup> to ensure that the ligand was completely titrated with added zinc. After equilibration with added zinc concentrations, 20  $\mu\text{l}$  (0.01 mol L<sup>-1</sup>) APDC was added to each vial and allowed to equilibrate at room temperature for a further 10–12 h. The first two vials were not spiked with zinc and were used as replicates for the starting point of the titration. Voltammetric parameters were deoxygenation time 4 min, adsorption potential  $-0.3$  V, adsorption time 60 s to 300 s, reoxidation potential  $-0.8$  V for 10 s, and a potential scan from  $-0.8$  V to 1.3 V using square-wave modulation (50 Hz) with a step potential of 2.5 mV. Typically the zinc peak was observed at  $-1.1$  V.

Figure 1 shows a typical example of a Zn-complexing ligand titration for the control. In both treatments without added zinc and in +Zn treatments, the AdCSV response of the titrations with zinc showed suppression at low zinc concentrations indicating the ligand competition by natural zinc complexing ligands present in the seawater. As the total zinc concentration was increased, the electrode response became linear, indicating that effectively all of the Zn-complexing ligands had been titrated with the additional zinc. Total zinc-binding ligand (ZnL) and the conditional stability constant ( $K'_{\text{ZnL}}$ ) were calculated using Langmuir linearization of the titration data (Fig. 1b). As these plots were linear, a simple one-ligand one-metal model could be applied (Ruzic 1982; van den Berg 1985; Ellwood and van den Berg 2000). In the +Zn treatments, the concentration of zinc added was in excess of the concentration of the zinc-binding ligands, which may lead to some errors in using this linearization

technique. However, clear curvature was observed at the lower additions of these titrations and the Langmuir linearization plots were linear, indicating the presence of a zinc-binding ligand and that a simple one-ligand one-metal model could be used.

**Total dissolved zinc and iron**—Zinc and iron were also analyzed in acidified seawater samples with chelation and organic extraction using ammonium 1-pyrrolidinedithiocarbamate/diethylammonium diethyldithiocarbamate (APDC/DDDC) into chloroform. This technique is similar to that of Bruland and Franks (1979) but modified with a higher complexant concentration to allow the simultaneous additional extraction of manganese (Statham 1985). All sample extracts were analyzed for their metal content on a Perkin-Elmer 1000B equipped with an HGA-700 atomic adsorption graphite furnace within 1 week of completing the solvent extraction procedure. A comparison between measurements of dissolved Zn using AdCSV and solvent extraction method revealed a close 1:1 relationship over a wide range of concentrations from 0.03 nmol L<sup>-1</sup> to 11.7 nmol L<sup>-1</sup> (Lohan unpubl. data).

**Quantifying adsorption of zinc and iron during shipboard experiments**—Previous experiments have reported a problem of adsorption of metals onto the container walls during incubation experiment (Martin et al. 1989; Coale 1991). Therefore, to examine whether removal of trace metals during the incubation experiment was an effect of adsorption onto particles or phytoplankton or to uptake by the phytoplankton, and not an effect of adsorption onto the container walls, the following experiment was conducted. Seawater was collected in two additional acid-cleaned cubitainers at the same time as the samples used in the shipboard incubation experiments. The seawater was not filtered and was stored at 4°C in the dark for 6 months prior to analysis. Therefore, no live phytoplankton or zooplankton would be expected to be present in the water. Additions of 10 nmol L<sup>-1</sup> Fe (FeCl<sub>3</sub>) and 10 nmol L<sup>-1</sup> Zn (ZnCl<sub>2</sub>) were made to one cubitainer, and no additions were made to the second control cubitainer. Each day for 8 d, 250 ml of sample was removed and analyzed for total dissolved Zn and Fe by chelation and solvent extraction. At the end of 8 d, the remaining 2 liters of sample in the cubitainer was removed, and the inside of the cubitainer was rinsed with subboiled distilled water and then 10% HCl. These solutions were then analyzed to assess if any material had adsorbed to the container walls.

**Chlorophyll and inorganic nutrients**—Nitrate, phosphate, and silicic acid were analyzed onboard ship on a Technicon autoanalyzer using a modification of standard Technicon procedures as outlined in Barwell-Clarke and Whitney (1996). Chl *a* was measured in acetone extractions using a Turner Design fluorometer (Model 10) calibrated with Chl *a* standard (Sigma) on a spectrophotometer, and the concentration of Chl *a* was calculated with standard formulas (e.g., Strickland and Parsons [1972]).

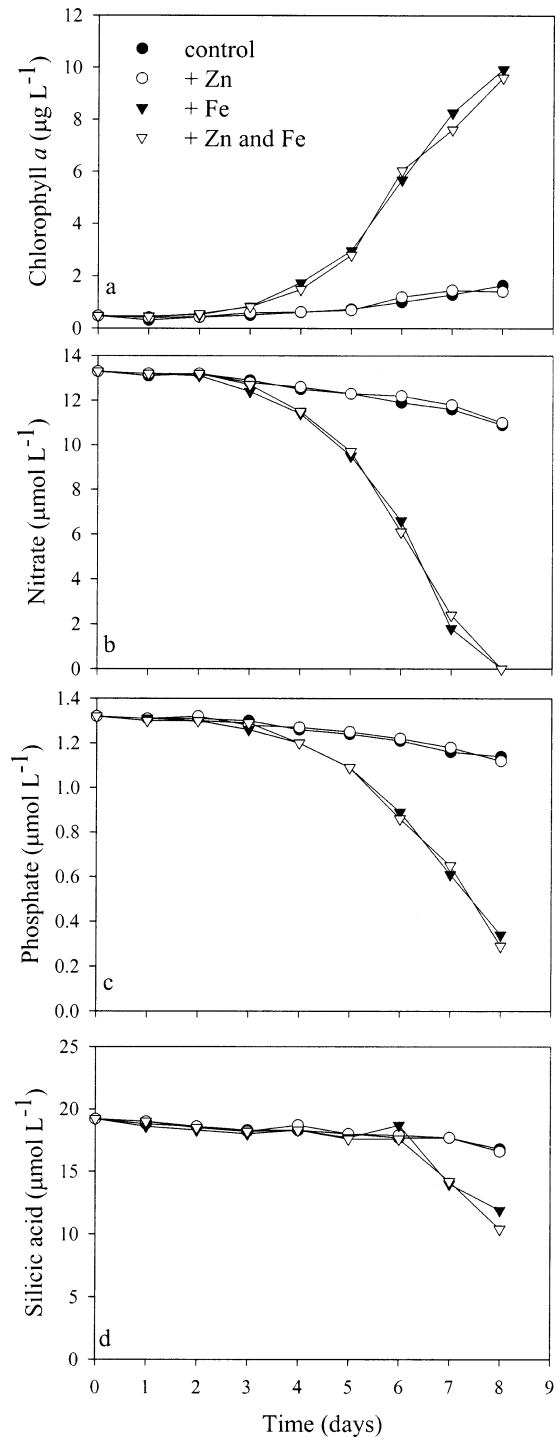


Fig. 2. Changes in (a) chlorophyll *a*, (b) nitrate, (c) phosphate, and (d) silicic acid in all four treatments.

## Results

**Chlorophyll and nutrients**—Chl *a* did not change significantly in all treatments (0.47–0.44 µg L<sup>-1</sup>) during the first 3 d of the incubation, but concentrations diverged dramatically between days 3 and 4 (Fig. 2). The +Fe treatments showed a large stimulation of phytoplankton growth with a

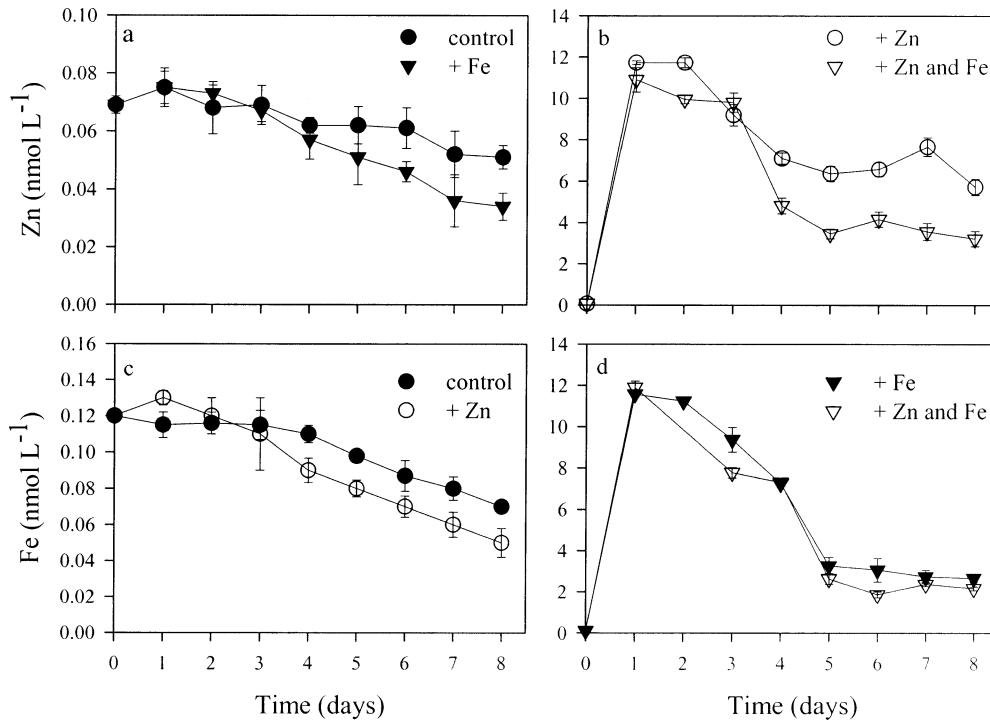


Fig. 3. Total dissolved Zn concentrations in (a) the control and +Fe treatment, and (b) the +Zn treatments. Total dissolved Fe concentrations in (c) the control and +Zn treatment and (d) in the +Fe treatments. Error bars represent the standard deviation on three replicate measurements.

20-fold increase (9.9 μg L<sup>-1</sup>) in comparison to the t<sub>0</sub> concentration (0.47 μg L<sup>-1</sup>). The control showed a 3-fold increase after 8 d (1.6 μg L<sup>-1</sup>) in comparison to the initial concentration. There was little difference in Chl *a* concentrations between the control and the +Zn treatment.

Changes in the nutrient concentrations reflected the above changes in Chl *a* (Fig. 2). Iron addition caused removal of all measurable nitrate after 8 d (from 13.3 μmol L<sup>-1</sup> to concentration below limits of detection) and phosphate concentrations decreased from 1.32 μmol L<sup>-1</sup> to 0.29 μmol L<sup>-1</sup>. The phytoplankton community was able to remove only 18%

(a decrease of 2.4 μmol L<sup>-1</sup> over 8 d) of the available nitrate and 15% (a decrease of 0.2 μmol L<sup>-1</sup>) of the phosphate in the control and the +Zn treatment. Silicic acid showed a strong removal between day 6 and the end of the experiment in the +Fe treatments, resulting in 38% (a decrease of 7.3 μmol L<sup>-1</sup>) uptake of available silicic acid (Fig. 2). The control and +Zn treatments showed only a slight removal of silicic acid and only between days 6 and 8.

**Trace metal concentrations**—There is no indication of contamination of the seawater during sampling and filling of the cubitainers. A small increase in Zn (<0.01 nmol L<sup>-1</sup>) was observed between days 0 and 1 in the control and +Fe treatment (Fig. 3a). The Fe concentration showed a small increase (<0.02 nmol L<sup>-1</sup>) in the +Zn treatment and a minor decrease in the control between days 0 and 1 (Fig. 3c). Although problems with adsorption and desorption have been suggested in previous incubation experiments, the results from the adsorption experiment in this study showed no evidence for significant adsorption and/or desorption to/from the walls of the cubitainers (Fig. 4). The amount of added Zn and Fe (10 nmol L<sup>-1</sup> each) adsorbed onto the walls of the cubitainers after 8 d was only 0.34 nmol L<sup>-1</sup> (3.4%) Zn and 0.26 nmol L<sup>-1</sup> (2.6%) Fe, much less than the 9.3% adsorption of iron that was reported by Martin et al. (1989). Coale (1991) reported that 39% of Zn added was adsorbed onto the container walls by assuming that all the Zn removed from the dissolved phase throughout the 7-d experiment was adsorbed onto the container walls and no uptake by organisms occurred. In the present experiment, there was no detectable removal of either Zn or Fe in the control with con-

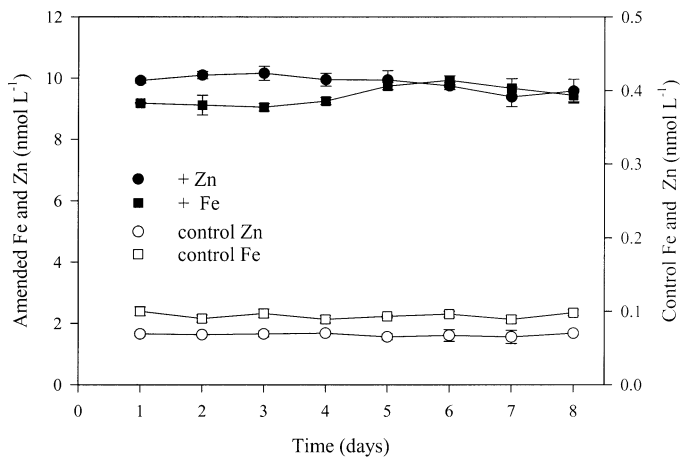


Fig. 4. Experiment to investigate possible loss of total dissolved Zn and Fe to cubitainer walls where Zn- and Fe-amended treatments are on the left y axis and the control on the right.

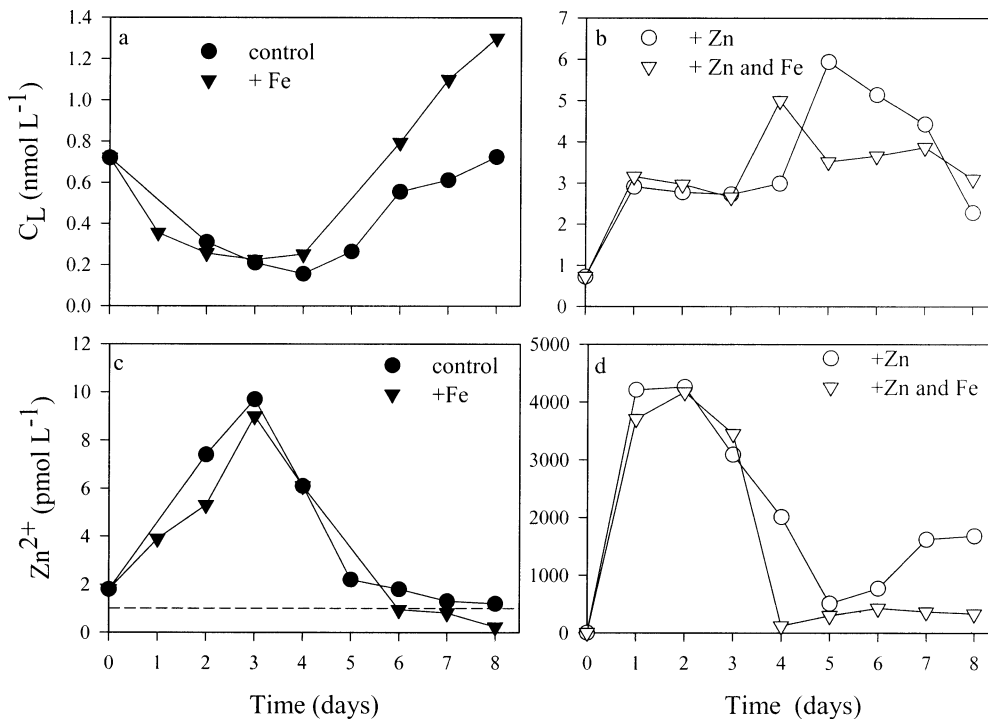


Fig. 5. Zinc-binding ligand concentrations in (a) the control and +Fe treatment; and in (b) the +Zn treatments. Free Zn<sup>2+</sup> concentrations in (c) the control, and the +Fe treatment; and (d) the +Zn treatments. The dashed line indicates the concentration (~1 pmol L<sup>-1</sup>) at which [Zn<sup>2+</sup>] becomes limiting to some phytoplankton growth (Sunda and Huntsman 1992).

concentrations remaining relatively constant at 0.08 nmol L<sup>-1</sup> Zn and 0.1 nmol L<sup>-1</sup> Fe (Fig. 4), similar to results reported by Takeda and Obata (1995) in the equatorial Pacific. Results from this experiment suggest phytoplankton were absent from the stored seawater, as only small changes in Zn and Fe concentrations were detected when compared to the incubation experiments. Two hundred fifty milliliters was removed for analysis each day for the 8 d and therefore the surface area available for adsorption decreased to half (2 liters) during the incubation period. Vasconelos et al. (2002) measured metal concentrations in algae (extracellular adsorption plus intracellular uptake) and found that the metal concentration in the algae balanced the metal lost from seawater, thus indicating negligible adsorption onto container walls. Therefore, given the minimal removal of trace metals to the walls of containers, the loss of metal from the dissolved phase in the four treatments can be assumed to be predominately through extracellular adsorption onto and uptake into the cells.

Both total dissolved Zn and Fe concentrations showed a general trend of decreasing concentrations as the experiment progressed in all four treatments (Fig. 3). In the +Fe treatments, a rapid removal of iron was observed until day 5, reducing the concentration of iron from 11.3 nmol L<sup>-1</sup> to 3 nmol L<sup>-1</sup>, after which less iron removal was observed (Fig. 4d) despite the increase in phytoplankton biomass and continued nutrient uptake (Fig. 2). The +Zn treatments significantly showed an increased removal of iron in comparison to the control (Fig. 3c). The control showed relatively con-

stant iron concentrations until day 4, after which the Fe concentration showed a steady decrease to day 8.

A similar trend was observed in the total dissolved zinc concentrations, where additions of Fe increased the uptake of Zn compared to the control (Fig. 3a). However, unlike the rate of iron removal, the zinc uptake during the start of the growth phase (days 1 to 3) was relatively small (11.9 nmol L<sup>-1</sup> to 10.3 nmol L<sup>-1</sup>) in the +Zn treatment. Between days 3 and 6, Zn concentrations decreased in all treatments, although the control showed a reduced rate of removal compared to the +Fe treatment (Fig. 3a,b).

*Zinc speciation data*—Figure 5 and Table 1 show the changes observed in the concentration of the natural zinc-binding ligand concentration in the four treatments. Both the zinc-binding ligand concentration on day 0 and the calculated stability constants ( $K'_{ZnL}$ ) in the control and +Fe treatment, are in good agreement with those of previous studies (Bruland 1989; Donat and Bruland 1990; Ellwood and van den Berg 2000; Ellwood 2004). In the +Zn treatments, the conditional stability constants were slightly lower over the first 4 d (8.9 to 10.1) than those previously reported (Bruland 1989; Donat and Bruland 1990; Ellwood and van den Berg 2000; Ellwood 2004). This may be an experimental artifact, rather than a thermodynamic result, as the concentration of zinc-binding ligand is lower than the total dissolved zinc concentration present. Concentrations and conditional stability constants for zinc and estimates for Zn<sup>2+</sup> are presented in Figs. 3 and 5 and Table 1. Addition of 10 nmol L<sup>-1</sup> of

Table 1. Zinc speciation data for all four treatments throughout the 8-d trace metal enrichment experiment.  $[Zn_T]$  and  $[Fe_T]$ , total dissolved Zn and Fe concentrations, respectively;  $[C_L]$ , concentration of the Zn-binding ligand;  $[Zn^{2+}]$ , free Zn ion concentration;  $[Zn']$ , labile inorganic species;  $\log K'_{ZnL}$ , stability constant.

Time (d)	$[Zn_T]$ (nmol L <sup>-1</sup> )	$[Fe_T]$ (nmol L <sup>-1</sup> )	$[C_L]$ (pmol L <sup>-1</sup> )	$[Zn^{2+}]$ (pmol L <sup>-1</sup> )	$[Zn']$ (pmol L <sup>-1</sup> )	$\log K'_{ZnL}$
Control						
0	0.069	0.12	720	1.8	3.8	10.5
1	0.075	0.12				
2	0.068	0.11	310	7.4	15.54	10.4
3	0.069	0.11	210	9.7	20.37	10.5
4	0.062	0.11	156	6.1	12.81	10.9
5	0.062	0.098	264	2.2	4.62	11.1
6	0.061	0.08	556	1.8	3.78	10.8
7	0.052	0.08	612	1.3	2.73	10.8
8	0.051	0.07	725	1.2	2.52	10.7
+Fe						
1	0.075	11.57	355	3.9	8.19	10.8
2	0.073	11.23	256	5.3	11.13	10.8
3	0.067	9.37	224	9	18.90	10.5
4	0.057	7.25	251	6.1	12.81	10.5
5	0.051	3.27				
6	0.046	3.07	795	0.94	1.97	10.8
7	0.036	2.75	1.1	0.8	1.68	10.6
8	0.034	2.68	1.3	0.2	0.42	10.8
+Zn						
1	11.73	0.13	2,910	4,210	8,841	8.9
2	11.73	0.12	2,780	4,260	8,946	9.41
3	9.19	0.11	2,730	3,090	6,489	10.1
4	7.1	0.09	2,990	2,010	4,221	10.1
5	6.36	0.08	5,940	510	1,071	9.71
6	6.57	0.07	5,140	771	1,619	9.92
7	7.65	0.06	4,430	1,620	3,402	10.1
8	5.71	0.05	2,280	1,680	3,528	10.1
+Zn and +Fe						
1	10.9	11.9	3,160	3,710	7,791	9.1
2	9.95		2,970	4,170	8,757	9.31
3	9.8	7.78	2,660	3,450	7,245	9.64
4	4.81	7.3	5,000	120	252	10.1
5	3.46	2.64	3,520	300	630	9.85
6	4.15	1.87	3,660	428	899	10.3
7	3.56	2.38	3,870	365	767	10.1
8	3.21	2.17	3,100	330	693	10.1

zinc increased the ligand concentration from 0.7 nmol L<sup>-1</sup> to 3 nmol L<sup>-1</sup> on day 1 (Fig. 5b). The ligand concentration in the +Zn treatments was much lower than the concentration of total dissolved zinc in the sample, but there is no evidence of a second weaker ligand. Extracellular production of Zn-binding ligands was observed to increase on days 4 and 5 in +Zn treatments, which coincided with a rapid removal of total dissolved zinc and a concomitant decrease in the bioavailable Zn<sup>2+</sup> ion. These ligands were then partially removed or broken down and by day 8 were slightly lower than observed on day 1 (Fig. 5b).

In the +Fe treatment and the control, zinc-binding ligands decreased over the first 3–4 d, followed by a rapid increase between days 4 and 6 and continued to increase until the

end of the experiment (Fig. 5a). The higher chlorophyll biomass observed in the +Fe treatment may account for the increased level of Zn-binding ligand in the +Fe treatment than in the control.

The free Zn<sup>2+</sup> ion concentration  $[Zn^{2+}]$  shown in Fig. 5 was calculated using

$$[Zn^{2+}]^2 \alpha_{Zn} K'_{ZnL} + [Zn^{2+}](K'_{ZnL} C_L - K'_{ZnL} C_{Zn} + \alpha_{Zn}) - C_{Zn} = 0,$$

where  $\alpha_{Zn}$  is the inorganic side-chain reaction coefficient for Zn<sup>2+</sup> (Ringbom and Still 1972), which was calculated to be 2.1 (Turner et al. 1981),  $K'_{ZnL}$  and  $C_L$  were determined from Langmuir linearization of titration data, and  $C_{Zn}$  is the concentration of total dissolved zinc in solution.

Between days 3 and 5, there was a large reduction in  $[Zn^{2+}]$  available to phytoplankton in all four treatments (Fig. 5c,d). After day 5, the  $[Zn^{2+}]$  in the +Zn treatment increased, reflecting the decrease in ligand concentration. In both the control and the added iron treatment (Fig. 5a), there was an initial increase in the  $[Zn^{2+}]$  concomitant with the decrease in ligand production. By day 6, the  $[Zn^{2+}]$  in both the control (1.8 pmol L<sup>-1</sup>) and +Fe treatment (0.94 pmol L<sup>-1</sup>) were lower than those previously observed in field studies in the Pacific (Donat and Bruland 1990) and the Atlantic (Ellwood and van den Berg 2000) but similar to that reported in the subantarctic region (Ellwood 2004).

## Discussion

Both the biological and chemical parameters measured from the incubations show clear trends throughout the 8 d, and the consistency of the data gives confidence in the experimental design employed. These experiments were performed in a closed on-deck incubator while in transit, and it was therefore not possible to compare changes in the control cubitainers with those changes occurring in situ. Several factors other than the metal additions have the potential to influence the experimental results. The absence of some grazers, particularly migrating zooplankton, from incubation experiments has been hypothesized as a cause of increased biomass in the control containers (Martin et al. 1989; Coale 1991). Small variations between in situ and on deck irradiance levels may also have been an influence (Crawford et al. 2003). However, such factors are not expected to significantly influence the interactions between phytoplankton, bacteria, and metals present in the cubitainers we used. All samples were collected at a similar time and under similar conditions, and therefore relative changes observed can be assumed to be caused by the trace metals added.

In a previous experiment carried out in September 1999 at Ocean Station Papa (Crawford et al. 2003), very little growth was observed in the control, which was attributed to the low concentrations of Zn and Fe. The total dissolved Zn and Fe were measured daily in the experiment described here, and the concentrations of both trace metals decreased throughout the 8 d (Fig. 3), whereas in the previous experiment the Zn and Fe concentrations in the control were slightly higher after 8 d (Crawford et al. 2003). The growth in the control in the present study is similar to that observed

by Martin et al. (1989) and Coale (1991) in their incubations and also to reported growth rates of the natural phytoplankton assemblage at Ocean Station Papa in spring (Boyd and Harrison 1999).

**Macronutrients and biomass response**—As observed in all previous incubation experiments, nitrate was depleted from  $13.3 \mu\text{mol L}^{-1}$  to below limits of detection ( $\leq 0.2 \mu\text{mol L}^{-1}$ ) in the +Fe treatments over the 8 d, which is comparable to phytoplankton nitrate uptake rates observed in productive upwelling regions (Dugdale and Wilkerson 1991). Similar enhancement of nitrate uptake upon the addition of iron has been observed at Ocean Station Papa (Boyd et al. 1996).

Addition of iron caused a dramatic increase in Chl *a* biomass (Fig. 2a) with the community dominated by large diatoms. The lack of Fe in the control and +Zn treatment was reflected in the smaller numbers of diatoms present after 8 d. The small increase in Chl *a* biomass in the +Zn treatment and the control (Fig. 2a), compared to the large increase in Chl *a* observed upon the addition of Fe, indicates that Zn is not significantly limiting the phytoplankton biomass production at Ocean Station Papa.

**Changes in total dissolved trace metals**—As adsorption onto the container walls was shown to be minimal, the loss of trace metals from solution can be assumed to be predominately through extracellular adsorption onto and uptake into cells. In all four treatments, trace metal removal from solution was observed throughout the 8 d (Fig. 4). The removal of total dissolved Zn from the control is similar to that reported by Coale (1991). While there have been a few incubation experiments that have studied the effect of added zinc and zinc plus iron (Franck et al. 2000; Cochlan et al. 2002), no measurements of dissolved zinc concentrations were determined at the end of these experiments and therefore no comparison of zinc uptake can be made. Coale et al. (2003) observed a  $2\text{-nmol L}^{-1}$  removal of zinc throughout the course of a season in the Ross Sea, Southern Ocean, as part of the Assessing the Effects of Submesoscale Ocean Parameterizations (AESOP's) study, suggesting that the zinc requirement for algal growth controls zinc concentrations in that region. A large uptake of added zinc and iron was reported in an earlier incubation experiment (Crawford et al. 2003).

The rapid uptake of iron and zinc in the first few days is consistent with observations from culture studies, which indicate that initial rapid uptake rates of Fe- or Zn-deficient cells is followed by a decrease in trace metal uptake similar to those observed for cells growing under replete iron and zinc concentrations (Sunda and Huntsman 1992). Both iron- and zinc-deficient phytoplankton have been reported to accumulate surplus metals in response to episodic metal inputs such as dust events or upwelling (Sunda and Huntsman 1992; Takeda and Obata 1995). The hypothesis of "luxury uptake" is supported by our data, where the removal of total dissolved Zn and Fe concentrations is observed following their addition to the natural phytoplankton assemblage. Metal to carbon ratios were calculated on each day of the incubation using POC measurements and the decrease in total dissolved trace metal concentration.

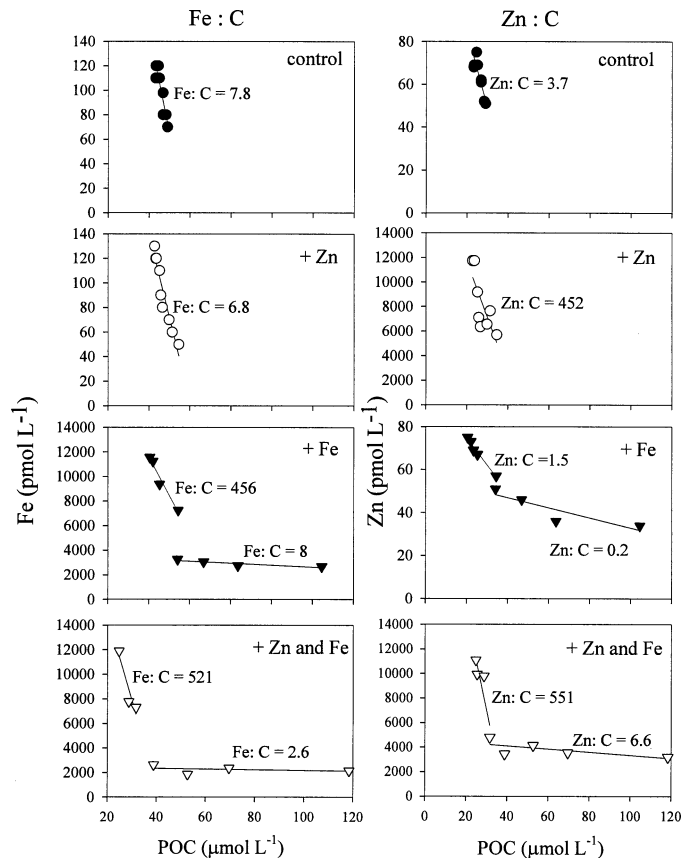


Fig. 6. Iron:carbon and zinc:carbon ratios in all treatments. Fe:C and Zn:C units are  $\mu\text{mol}:\text{mol}$ .

Microplankton communities in the +Fe incubation had higher Fe:C ratios ( $465 \mu\text{mol}:\text{mol}$  and  $521 \mu\text{mol}:\text{mol}$ ; Fig. 6) over the first 4 d when total dissolved iron concentrations were high. Iron:carbon ratios then decreased to  $8 \mu\text{mol}:\text{mol}$ , which is similar to that observed in the control ( $7.8 \mu\text{mol}:\text{mol}$ ). In the case of zinc, Zn:C ratios were also higher in the +Zn and +Fe treatment ( $551 \mu\text{mol}:\text{mol}$ ; Fig. 6) over the first 4 d and then decreased to  $6.6 \mu\text{mol}:\text{mol}$ , which is slightly higher than the  $3.7 \mu\text{mol}:\text{mol}$  observed in the control. In the control both Zn:C and the Fe:C ratios are within the range reported from culture studies for phytoplankton cells grown under low iron (Fe:C  $6\text{--}9 \mu\text{mol}:\text{mol}$ ) and zinc (Zn:C  $0.9\text{--}4.2 \mu\text{mol}:\text{mol}$ ) concentrations (Sunda 1991; Sunda and Huntsman 1992). The higher Zn:C and Fe:C ratios suggest that phytoplankton cells in the metal-enriched incubations have elevated zinc and iron quotas during the incubation when the respective metal is present in higher concentrations. The lag phase in phytoplankton growth observed in trace metal incubations could perhaps be due to the initial assimilation of trace metals. During an upwelling event, for example, phytoplankton require time to accumulate both macro- and micronutrients. The Zn:C ratio in the control was  $3.7 \mu\text{mol}:\text{mol}$ , while in the +Fe treatment the ratio was lower at  $1.5 \mu\text{mol}:\text{mol}$  and decreased to  $0.2 \mu\text{mol}:\text{mol}$  at the end of the incubation. A similar result was observed in incubation experiments conducted in subantarctic waters (Ellwood 2004). Cullen et al. (2003) have also

shown that phytoplankton contain elevated zinc levels when iron is limiting. The enhanced uptake of Zn by iron-stressed algae would explain the low zinc concentrations observed at Ocean Station Papa.

**Zn-binding ligands**—The addition of dissolved Zn to the cubitainers caused a remarkably rapid production of Zn-binding ligands with a 4-fold increase in ligand concentrations in <1 d (Fig. 5; Table 1). No artificial chelators were utilized in our experiment and therefore the rapid production of these ligands indicates a specific response from the natural microbial community to the added zinc. Two different hypotheses can be put forward to explain the production of these ligands: detoxification and/or a mechanism to enhance uptake and storage for future growth via Zn–ligand interaction.

As has been observed for copper, complexation of trace metals with organic ligands often ameliorates trace metal toxicity (Leal and van den Berg 1999). In culture experiments with high concentrations of copper, the Cu-binding ligand increased for the duration of the experiment and only when free copper  $\text{Cu}^{2+}$  ion concentrations were no longer toxic was an increase in cell numbers observed (Moffett and Brand 1996). Martin et al. (1993) have shown that the addition of  $10 \text{ nmol L}^{-1}$  of zinc can reduce primary production rates in the equatorial Pacific by 25%. If the  $10 \text{ nmol L}^{-1}$  of dissolved zinc added was all in the bioavailable form, the  $[\text{Zn}^{2+}]$  would only just reach the order  $10^{-8} \text{ mol L}^{-1}$  required for zinc toxicity to begin to be observed (Sunda and Huntsman 1992). However, the immediate complexation of dissolved zinc by these zinc-binding ligands reduces the  $[\text{Zn}^{2+}]$  to  $\sim 4 \text{ nmol L}^{-1}$  (Table 1), a level which is not toxic and at which the growth rate of *E. huxleyi* or *Thalassiosira oceanica* is increased (Sunda and Huntsman 1992). Because these shipboard incubations were conducted with the natural microbial community and not with monospecific cultures, the production of zinc-binding ligands may be a toxicity response from some phytoplankton species or bacteria.

Previous laboratory and field studies have demonstrated that phytoplankton are capable of producing Fe-binding ligands or siderophores that keep Fe in solution and are utilized for iron uptake (Barbeau et al. 2001; Rue and Bruland 1997). However, as there are no immediate solubility constraints on the zinc concentrations typical of ocean waters and the metal does not undergo the redox cycling of iron, these benefits conferred by complexation on Fe availability do not apply to Zn (Whitfield 2001). In the +Zn and +Fe treatment, the large increase in ligand production on day 4 coincides with a large decrease in total dissolved zinc concentration (from  $9.8 \text{ nmol L}^{-1}$  to  $4.8 \text{ nmol L}^{-1}$ ; Fig. 3b) and an increase in Chl *a* biomass (Fig. 2). No significant adsorption onto the container walls occurred and therefore the decrease in dissolved zinc concentration was due either to uptake into phytoplankton cells or on the cell surface. On day 5 in the +Zn and +Fe treatment, a decrease in both the total dissolved zinc concentration and the Zn-binding ligand ( $1.35 \text{ nmol L}^{-1}$  and  $1.48 \text{ nmol L}^{-1}$ , respectively) was observed concomitant with an increase in Chl *a* biomass. The decrease in both the concentration of the zinc-binding ligand and the total dissolved zinc were similar. This implies that

the zinc-binding ligand complex was readily available, rather than being nonavailable like a copper–ligand complex (Moffett and Brand 1996). Although photochemical and/or bacterial breakdown of these ligands was not accounted for during this study, the uptake of total dissolved zinc ( $1.35 \text{ nmol L}^{-1}$ ) between day 4 and 5 was much larger than the bioavailable Zn  $[\text{Zn}']$  present ( $0.25\text{--}0.63 \text{ nmol L}^{-1}$ ; Table 1). This provides strong evidence for the biological uptake of ligand-bound zinc.

From our study, when Zn is either in excess supply or when concentrations are extremely low ( $0.07 \text{ nmol L}^{-1}$ ), Zn-binding ligands are produced by the planktonic microbial community (Fig. 5a,b). Because of the sampling strategy employed, any fluctuations in the ligand concentrations over the course of the day cannot be resolved. In treatments without Zn additions, the concentration of Zn-binding ligands was observed to increase over the 8 d (Fig. 5a). The addition of iron increased both the phytoplankton biomass and the production of zinc-binding ligands compared to the control. These ligands were positively correlated with Chl *a* ( $r^2 = 0.934$ ), which provides the first evidence of a link between zinc-binding ligands and biota as previous studies in the open ocean revealed no relationship with Chl *a* (Ellwood and van den Berg 2000). However, in the +Fe and +Zn treatment no correlation between ligand production and Chl *a* was observed. The large increase in Zn-binding ligands observed within 1 d after the addition of zinc (Fig. 5) masked any relationship with Chl *a*.

This is the first field study to show zinc-binding ligands being produced and removed by the microbial community. These results also provide evidence that suggests that zinc-binding ligands may be a type of zinc siderophore compound whereby certain microorganisms may gain an advantage through selective uptake of this potentially biolimiting element.

**Dissolved inorganic zinc concentrations**—The  $[\text{Zn}^{2+}]$  in the +Fe treatment by day 8 was  $0.2 \text{ pmol L}^{-1}$ , which is lower than the concentration established in culture experiments to limit phytoplankton growth (Sunda and Huntsman 1992). There is increasing evidence that, along with the free metal ions and lipid-soluble fraction of the metal complexes (Croft et al. 2000), the labile fraction of metal complexes  $[\text{Zn}']$  in the diffusion layer of the organisms is also bioavailable and may alleviate the low concentrations of  $[\text{Zn}^{2+}]$ . In the +Fe treatment the lowest  $[\text{Zn}^{2+}]$  of  $0.2 \text{ pmol L}^{-1}$  was measured while the  $[\text{Zn}']$  was slightly higher at  $0.42 \text{ pmol L}^{-1}$ . Low levels of  $\text{Zn}'$  have also been reported in subantarctic waters (Ellwood 2004). Despite the very low bioavailable zinc present in this natural phytoplankton population, it is clear that in the +Fe treatment high growth rates occurred, suggesting there must be an adequate supply of zinc to the phytoplankton.

A theoretical approach (Hudson and Morel 1990) can be used to estimate whether the diffusional supply of  $[\text{Zn}']$  is sufficient to account for the growth rates observed. Assuming a conservative Zn:C ratio of  $1 \mu\text{mol}:\text{mol}$  compared to Zn:C ratios presented here, and a growth rate of 0.65 divisions per day (taken from Fig. 2; +Fe treatment), the theoretical growth demand for spherical cells of  $10\text{-}\mu\text{m}$  and  $20\text{-}\mu\text{m}$

$\mu\text{m}$  diameter is estimated at  $6 \times 10^{-18}$  mol Zn cell<sup>-1</sup> d<sup>-1</sup> and  $45 \times 10^{-18}$  mol Zn cell<sup>-1</sup> d<sup>-1</sup>, respectively. The maximum rate for diffusion ( $\rho$ ) of labile inorganic Zn' species to the cell was computed using the relationship  $\rho = 4\pi rD[\text{Zn}']$  by assuming that the cells are spherical in shape with a radius of  $r$ .  $D$  is the diffusion rate constant for Zn' at 10°C ( $5.6 \times 10^{-6}$  cm<sup>2</sup> s<sup>-1</sup>), and  $[\text{Zn}']$  is the inorganic Zn' species calculated from the speciation measurements made each day throughout the experiment. At a  $[\text{Zn}']$  of 4 pmol L<sup>-1</sup>, the maximum diffusion flux would be  $12 \times 10^{-18}$  mol Zn cell<sup>-1</sup> d<sup>-1</sup> and  $24 \times 10^{-18}$  mol Zn cell<sup>-1</sup> d<sup>-1</sup> into 10- $\mu\text{m}$  and 20- $\mu\text{m}$  diameter cells, respectively, sufficient to satisfy growth demand of 10- $\mu\text{m}$  but not 20- $\mu\text{m}$  cells. At a  $[\text{Zn}']$  of 1 pmol L<sup>-1</sup>, the maximum diffusion flux would have fallen to  $3 \times 10^{-18}$  mol Zn cell<sup>-1</sup> d<sup>-1</sup> and  $6 \times 10^{-18}$  mol Zn cell<sup>-1</sup> d<sup>-1</sup> into 10- $\mu\text{m}$  and 20- $\mu\text{m}$  diameter cells, respectively, and this would be insufficient to satisfy growth demand of either cell size. This diffusion limitation would be further exacerbated with increasing cell size, suggesting that the growth of very large pennate diatoms in the +Fe treatment (Crawford et al. pers. comm.) could have been supported by the uptake of zinc via zinc–ligand complexes.

Results from this study support previous work in demonstrating that the availability of dissolved iron strongly limits phytoplankton growth in the northeastern subarctic Pacific. The addition of zinc to the incubations showed no major Chl *a* biomass change in comparison to the control. Therefore it appears that Zn availability is not a major limiting factor for phytoplankton growth at Ocean Station Papa. Our results additionally show that the production of zinc-binding ligands is influenced by the natural microbial community in these northeastern Pacific HNLC waters. The biological response to Zn and Fe additions, such as those from aeolian and upwelling sources, is very rapid. Complexation of dissolved zinc in surface waters would be beneficial to the microbial community by removing any effect of toxicity, allowing total dissolved zinc to be longer retained in the surface water column and allowing luxury uptake of this complex. The large increase in phytoplankton growth observed at bioavailable Zn concentrations previously thought to be limiting may provide indirect evidence for ligand-mediated uptake of Zn. Such a process can help explain why only small changes in community structure have been observed upon the addition of zinc despite the low concentrations (Crawford et al. 2003). The planktonic microbial community has a major role in controlling the speciation of Zn in surface oceanic waters, and it can interact with the environment to optimize access to the essential micronutrient Zn when present at both high and low concentrations.

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