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Zinc isotope fractionation during high-affinity and low-affinity zinc transport by the marine diatom *Thalassiosira oceanica*

Abstract—We have measured the isotopic fractionation of zinc (Zn) during uptake by the marine diatom *Thalassiosira oceanica* cultured at a range of free Zn²⁺ concentrations representative of the natural range from coastal and oligotrophic regions of the ocean. Harvested cells were rinsed with either plain seawater or a wash designed to remove adsorbed extracellular metals. Unwashed cells had much higher levels of Zn and were isotopically heavier than the media, indicating a positive isotope effect for extracellular Zn adsorption. Internalized Zn, measured in washed cells, was isotopically lighter than the media. The magnitude of Zn isotope fractionation changed with free Zn²⁺ concentration, corresponding to a switch on the part of *T. oceanica* between the predominance of high- and low-affinity Zn transport pathways. The total isotope effect for uptake ($\Delta^{66}\text{Zn}$) was -0.2% for high-affinity uptake at low Zn concentrations and -0.8% at the highest Zn concentrations, where low-affinity uptake is dominant. To our knowledge, this is the first study to describe a physiological basis for biological metal isotope fractionation during transport across the cell membrane. Similar high- and low-affinity Zn transport pathways are common among marine phytoplankton, suggesting that the processes described here are an important factor in natural marine Zn isotope variations.

Zinc (Zn) is an essential biological nutrient in the oceans where the concentration distribution of Zn is controlled largely by phytoplankton Zn uptake and remineralization. When micronutrients such as Zn are transported across a cell membrane, some isotopes may be taken up more quickly than others. This biological fractionation may be recorded as variations in the ratio of ⁶⁶Zn : ⁶⁴Zn found in environmental samples. Biological processes have been invoked to explain Zn isotope variations in seawater (Bermin et al. 2006), marine sediments (Maréchal et al. 2000; Pichat et al. 2003), manganese nodules, and marine particles (Maréchal et al. 2000). Measuring the magnitude of biological Zn isotope fractionation is crucial to interpreting these natural signals. Zn isotope effects of up to 0.5‰ in $\delta^{66}\text{Zn}$ have been observed in land plants (Weiss et al. 2005) and phytoplankton (Gélabert et al. 2006) and are substantial compared to a total reported range for natural $\delta^{66}\text{Zn}$ values of about 1.5‰ (Wilkinson et al. 2005), where

$$\delta^{66}\text{Zn} = \left(\frac{(^{66}\text{Zn}/^{64}\text{Zn})_{\text{sample}}}{(^{66}\text{Zn}/^{64}\text{Zn})_{\text{standard}}} - 1 \right) \times 1,000 \quad (1)$$

By accurately measuring the magnitude of biological Zn isotope fractionation, changes in the isotopic composition of Zn in seawater or ancient sediments can be directly related to the extent of biological Zn uptake in surface waters.

High- and low-affinity transport systems for Zn have been identified in many organisms, including several species of fish, yeast (Zhao and Eide 1996), plants such as wheat (Hacisalihoglu et al. 2001), and several species of marine phytoplankton (Sunda and Huntsman 1992, 1998). ZIP-family proteins, many of which are specific Zn transporters, have been found in bacteria, archaea, and many eukaryotes, and putative ZIP homologs have recently been identified in the genome of the diatom *Thalassiosira pseudonana*. For all eukaryotic phytoplankton studied, including three species of diatoms, the coccolithophore *Emiliania huxleyi*, and the alga *Chlamydomonas*, the switch between high- and low-affinity transport systems occurs under similar conditions, at free Zn²⁺ concentrations of around 10⁻¹⁰ mol L⁻¹ (Sunda and Huntsman 1992, 1998). This is in the middle of the natural range of the marine environment, from 10⁻¹² mol L⁻¹ in the nutrient-poor central ocean gyres (Bruland 1989) up to 10⁻⁸ mol L⁻¹ in anthropogenically affected coastal waters, such as Narragansett Bay (Kozelka and Bruland 1998), and suggests that the Zn isotope fractionations observed in *T. oceanica* have broad applicability to understanding the marine biological Zn isotope cycle (Fig. 1).

Methods—The diatom *T. oceanica* (CCMP 1005) was maintained in acid-cleaned polycarbonate bottles at 20°C under 85 $\mu\text{mol m}^{-2} \text{s}^{-1}$ constant light. Media were prepared from filtered Sargasso surface seawater collected with a towed trace element clean Fish modified with a rigid rubber tube in place of the polyvinyl chloride pipe used for support (Vink et al. 2000) and amended with major and trace element nutrients and vitamins (Sunda and Huntsman 1995) with 10 $\mu\text{mol L}^{-1}$ Fe, 25 nmol L⁻¹ Co, and varying amounts of Zn. Added Zn was a laboratory standard prepared with Zn from the CEZ (Canadian Electrolytic Zinc) Corporation. This Zn has an isotopic composition similar to natural Zn (John unpubl. data); thus, small amounts of Zn contamination would not have a large effect on $\delta^{66}\text{Zn}$. Seawater was microwave sterilized, and all nutrients, vitamins, and trace elements were sterilized by syringe filtration with an acid-cleaned rubber/silicone-free syringe (NormJect, HSW) through an acid-cleaned 0.4- μm polycarbonate filter (Nuclepore) and added to the seawater after it had cooled. One percent inocula by volume for

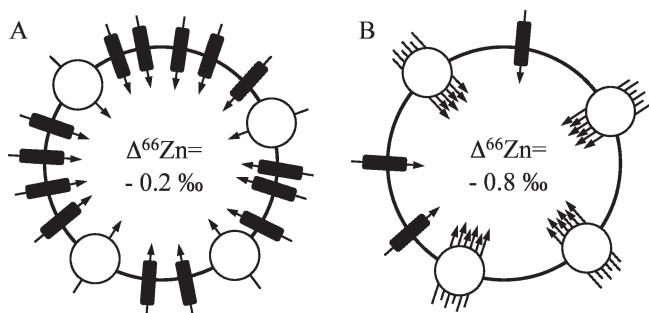


Fig. 1. A switch in the predominance of Zn transport through high- versus low-affinity transport pathways is accompanied by a change in the isotope effect for Zn uptake. Zn uptakes (arrows) by high-affinity (rectangles) and low-affinity (circles) pathways are shown under (A) low-Zn and (B) high-Zn conditions. The high-affinity pathway is effective at transporting Zn even at low Zn concentrations, but it is either saturated or down-regulated at higher Zn concentrations. The low-affinity pathway is less efficient at low Zn concentrations but does not become saturated when Zn concentrations are high. These two “pathways” are distinguished by the rate at which they take up Zn and their apparent saturation constant, so that each “pathway” may, in fact, amalgamate the effects of many different Zn transport proteins. When Zn concentrations are low (e.g., central oceans), high-affinity transport accounts for most Zn uptake, with a suggested isotope effect of $\Delta^{66}\text{Zn} = -0.2\text{‰}$. Under high-Zn concentrations (e.g., upwelling regions, coastal waters), Zn uptake will occur primarily by low-affinity transport with an isotope effect of $\Delta^{66}\text{Zn} = -0.8\text{‰}$.

experiments were taken from cultures in log-phase growth that were maintained in media with Zn concentrations within 1 log unit of experimental conditions. Growth in 1-liter polycarbonate bottles at maintenance conditions was monitored by measuring *in vivo* chlorophyll *a* fluorescence with a Synergy HT Microplate reader (BioTek). Specific Zn uptake rate was calculated by multiplying the log-phase growth rate by the Zn:P ratio of the final cultures.

Cultures were harvested near the end of log-phase growth and filtered onto a 1- μm polycarbonate filter (Whatman Nuclepore). Each culture was split into two equal parts, one of which was rinsed once with metal-free seawater and the other with an oxalate–ethylenediamine-tetraacetic acid (EDTA) reagent to remove adsorbed extracellular metals (Tovar-Sanchez et al. 2003) modified to pH = 7 for two 5-min rinses to ensure complete removal of extracellular ferric oxides (Tang and Morel 2006). This wash is also effective for removing extracellular Zn (Tang and Morel 2006). Cells and filters were transferred to $\sim 5\text{-mL}$ quartz beakers, and organics were partially oxidized by reacting for several hours with 2 mL of warm HNO_3 and 200 μL of H_2O_2 . When the liquid had evaporated, the remaining organics were oxidized by combusting samples for 8 h at 450°C. Combusted material was dissolved in warm 6 mol L^{-1} HCl for column purification of Fe and Zn, and small subsamples were diluted for measurement of Zn:P ratios.

Zn concentrations were measured by isotope dilution ICP-MS (inductively coupled plasma–mass spectrometry) with a ^{68}Zn spike. P concentrations were performed colorimetrically according to standard procedures for measurements of P in natural seawater. Zn and Fe were

purified by anion exchange chromatography (Maréchal et al. 1999) with small column volumes to reduce metal contamination from acids (Archer and Vance 2004), and the Zn fraction was eluted with 0.1% HCl to avoid coelution of other elements (Chapman et al. 2006). Samples were evaporated and then reacted overnight in 5-mL PFA (perfluoroalkoxy) capsules (Savillex) at high temperature with 200 μL of HF and 100 μL of H_2O_2 to drive off silicates remaining from the diatom frustules and organics that may have leached off the column. Samples were evaporated to dryness, evaporated again with 100 μL of 16 mol L^{-1} HNO_3 to drive off remaining F^- , and dissolved in 2% HNO_3 for isotopic analysis. Procedural blanks for our method were between 6 and 16 ng of Zn, comprising as much as 5% of the total Zn in the sample for cultures grown at the lowest Zn concentrations. Zn blanks for column purification are typically between 1 and 4 ng, indicating that the blank seen here was largely due to Zn contained in the polycarbonate filters.

Samples were analyzed for Zn isotopes on an IsoProbe multicollector ICP-MS (Thermo Electron Corporation, formerly MicroMass) equipped with an Apex Q inlet system without the optional desolvator. Samples were prepared with 50 ppb of Cu and 25, 50, or 100 ppb of Zn. Signal was monitored on masses 60, 63, 64, 65, 66, 67, and 68. $^{64}\text{Ni}^+$ was subtracted from $^{64}\text{Zn}^+$ by monitoring $^{60}\text{Ni}^+$ and applying a Cu mass bias correction, although these corrections were insignificant. Corrections for instrumental mass bias were made by the empirical external normalization (EEN) method by monitoring the $^{65}\text{Cu} : ^{63}\text{Cu}$ ratio of the Cu spike in samples and standards to establishing a linear mass bias relationship between the natural log of $^{65}\text{Cu} : ^{63}\text{Cu}$ and the natural log of $^{66}\text{Zn} : ^{64}\text{Zn}$ (Maréchal et al. 1999), combined with sample-standard bracketing of the EEN-corrected values. $\delta^{68}\text{Zn}$ values showed twice the fractionation compared to $\delta^{66}\text{Zn}$ values, ensuring the absence of polyatomic interferences. Extensive error analysis based on hundreds of samples was performed in conjunction with a study of Zn isotopes in hydrothermal systems (John unpubl. data). External reproducibility is 0.069‰ (2σ SD) independent of the Zn concentration in the analysis. Internal error for triplicate analysis of individual samples was generally smaller than external error.

Results and discussion—The internalized specific Zn uptake rate measured in our experiments shows a sigmoidal relationship to Zn concentration, consistent with the shift between low- and high-affinity Zn transporters previously observed in marine phytoplankton (Sunda and Huntsman 1992) (Fig. 2). This sigmoidal uptake curve has been described as the cumulative uptake of high- and low-affinity transporters governed by the following equation:

$$V = \frac{V_{\max}[\text{Zn}^{2+}]}{[\text{Zn}^{2+}] + K_M} + A[\text{Zn}^{2+}] \quad (2)$$

The uptake rate (V) is the cumulative effect of two terms. High-affinity Zn uptake predominates at low $[\text{Zn}^{2+}]$ and is governed by the first term, following classical Michaelis–Menten kinetics dependent on the maximal uptake rate (V_{\max})

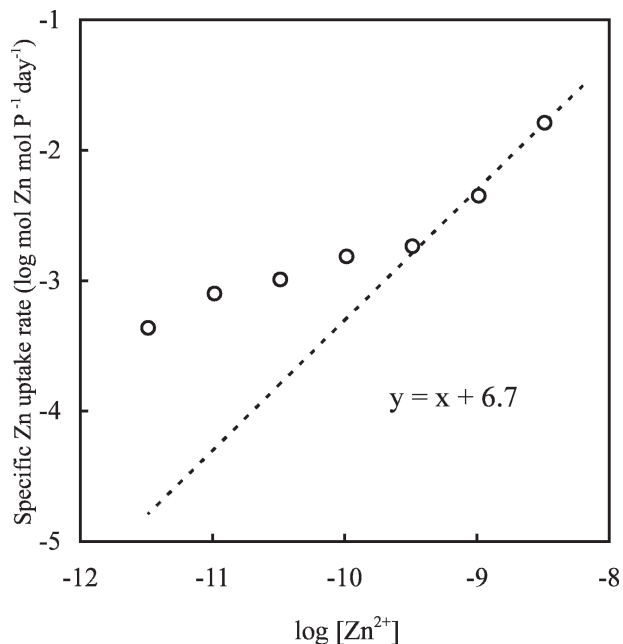


Fig. 2. Specific Zn uptake rates for washed *T. oceanica* cells (circles) have a sigmoidal relationship to Zn^{2+} concentration in the media, indicating a switch in the predominance of high- versus low-affinity uptake pathways. The predicted specific Zn uptake rate by the low-affinity transport system (dashed line) varies linearly with Zn^{2+} concentration. Any Zn uptake in excess of this quantity is assumed to occur via the high-affinity uptake pathway.

and the Michaelis–Menten constant (K_M). At high Zn concentrations, low-affinity transport dominates with an uptake rate related to $[\text{Zn}^{2+}]$ concentration by a constant (A). The decreasing contribution of high-affinity uptake at a higher $[\text{Zn}^{2+}]$ can also result from down-regulation of the high-affinity transporters. In seawater, only 66% of the inorganic (nonorganically bound) Zn is aqueous Zn^{2+} , though all inorganic species are in rapid equilibrium and are considered available for uptake (Sunda and Huntsman 1992).

We have modeled our specific Zn uptake rate data as the sum of high- and low-affinity uptake, assuming that nearly all the uptake at $10^{-8.5}$ mol L^{-1} Zn^{2+} occurs through low-affinity transport (Fig. 2). According to Eq. 2, Zn uptake through the low-affinity transport system decreases linearly with Zn concentration. Any uptake in excess of the predicted low-affinity Zn uptake is assumed to have occurred through the high-affinity Zn transport pathway. Accordingly, nearly 100% of Zn uptake occurs through the low-affinity transport system at 10^{-9} mol L^{-1} Zn^{2+} and above, there is a transition between the predominance of these two uptake pathways between $10^{-10.5}$ and $10^{-9.5}$ mol L^{-1} Zn^{2+} , and low-affinity transport accounts for less than 10% of total Zn uptake when the concentration of Zn^{2+} is 10^{-11} mol L^{-1} and below (Fig. 3).

The Zn isotope ratio in our diatoms is correlated to the switch in Zn transport systems (Fig. 3). The values of $\Delta^{66}\text{Zn}$ ($\delta^{66}\text{Zn}_{\text{diatoms}} - \delta^{66}\text{Zn}_{\text{media}}$) change rapidly over the same range of Zn concentrations where the Zn uptake switches from predominantly high-affinity uptake to predominantly low-affinity uptake when Zn^{2+} is between

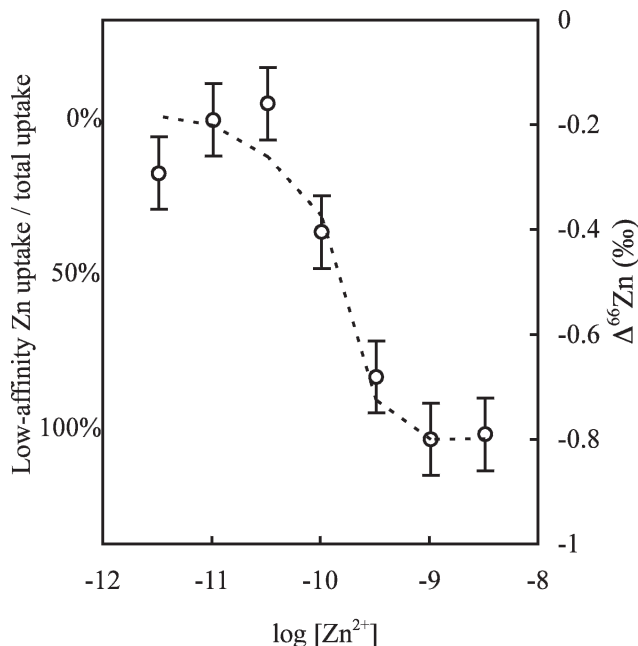


Fig. 3. The transition between high- and low-affinity transport is coincident with a change in the biological isotope effect. Predicted specific Zn uptake through the low-affinity transport system as a percentage of the total specific Zn uptake rate (dashed line) is shown along with the isotopic offset between $\delta^{66}\text{Zn}$ of *T. oceanica* cells and the $\delta^{66}\text{Zn}$ of the seawater media they were grown in ($\Delta^{66}\text{Zn}$) (circles) at different concentrations of Zn^{2+} . The shift in $\Delta^{66}\text{Zn}$ occurs within a similar range of Zn concentrations as the shift between the predominance of high- and low-affinity uptake, indicating that each transport pathway has a unique isotope effect. Error bars represent external reproducibility (2σ SD).

$10^{-10.5}$ and $10^{-9.5}$ mol L^{-1} . $\Delta^{66}\text{Zn}$ is around -0.2‰ at the lowest Zn concentrations and around -0.8‰ at the highest Zn concentrations, with intermediate values when there is significant uptake through both the high- and low-affinity transport systems. We have averaged values of $\Delta^{66}\text{Zn}$ at the three lowest Zn concentrations and the two highest Zn concentrations, resulting in the first estimates of the isotopic fractionation factors for Zn transport, with $\Delta^{66}\text{Zn} = -0.2\text{‰}$ for high-affinity transport and $\Delta^{66}\text{Zn} = -0.8\text{‰}$ for low-affinity transport.

We hypothesize that the fractionation observed during high-affinity transport probably does not occur at the transporter itself but occurs because the $\delta^{66}\text{Zn}$ of free Zn arriving at the transporter is lighter than the Zn in the bulk media. At equilibrium, free Zn is -0.16‰ lighter than EDTA-bound Zn in aqueous solution (Ban et al. 2002). As in EDTA-buffered cultures, Zn in the open ocean is predominantly ($<98\%$) bound to strong organic ligands (Bruland 1989). The binding strength of natural ligands ($K'_{\text{cond}} = 10^{11.0}$) is not so different from EDTA ($K'_{\text{cond}} = 10^{7.8}$) that we expect a large difference in the isotope effect. An additional isotope effect will be conferred by the difference in diffusivity between the different Zn isotopes. $^{64}\text{Zn}^{2+}$ will diffuse toward the cell more quickly than $^{66}\text{Zn}^{2+}$, and the difference in aqueous diffusivity of free Zn is equivalent to a preference for light Zn uptake by $\Delta^{66}\text{Zn}$

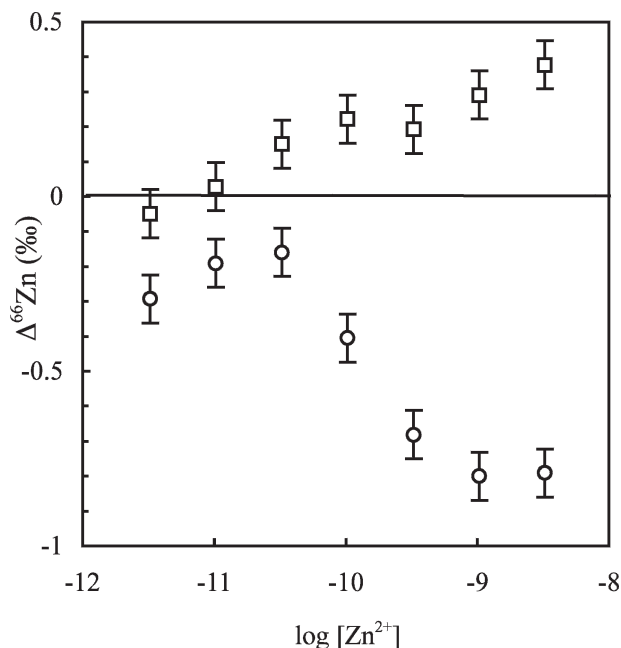


Fig. 4. Comparison of cells washed to remove extracellular precipitates (circles) with unwashed cells (squares) shows that extracellular precipitates are isotopically heavy and can greatly influence the total cell Zn isotope ratio if not removed.

= -0.06‰ (Rodushkin et al. 2004). Together, the equilibrium isotope effect between free Zn and Zn-EDTA plus the difference in isotope diffusivity can account for the entire -0.2‰ fractionation observed under high-affinity transport. Zn concentrations in the open ocean are nearly low enough to limit phytoplankton growth (Ellwood and Van Den Berg 2000; Lohan et al. 2002); thus, it follows that high-affinity transporters efficiently acquire all Zn diffusing to the transporters, regardless of isotope. By contrast, the isotope effect associated with the low-affinity uptake pathway is much larger. This isotope effect could result from the preferential retention of the heavier Zn isotopes to a low-affinity transport binding site or a more rapid translocation of light Zn isotopes across the cell membrane, such as would result from faster diffusion through an ion channel.

When cells are not washed to remove extracellular metals, external Zn can have a major effect on the total cellular $\Delta^{66}\text{Zn}$ (Fig. 4; Table 1). In this experiment, the $\Delta^{66}\text{Zn}$ of unwashed cells ranged from -0.05‰ at the lowest Zn concentration to $+0.38\text{‰}$ at the highest Zn concentration. The contribution of internal Zn isotopes can be subtracted to calculate values of $\Delta^{66}\text{Zn}$ for the external Zn pool alone. We have found that external $\Delta^{66}\text{Zn}$ increases almost linearly from $+0.09\text{‰}$ at $10^{-11.5} \text{Zn}^{2+}$ to $+0.52\text{‰}$ at $10^{-8.5} \mu\text{mol L}^{-1} \text{Zn}^{2+}$. These results are consistent with other studies showing that heavy Zn isotopes are preferentially adsorbed or precipitated onto diatom exteriors (Gélabert et al. 2006). In previous experiments (unpubl. data), we have found the amount of externally bound Zn and the consequent values of cell total $\Delta^{66}\text{Zn}$ are highly variable between different experiments. Cell-surface precipitation may be a major contribution to total Zn when

Table 1. $\delta^{66}\text{Zn}$ of washed and unwashed diatoms grown at a range of Zn concentrations. Error is the internal reproducibility for triplicate analysis of each sample.

-log [Zn] _{total}	-log [Zn ²⁺]	Unwashed cells		Washed cells	
		$\delta^{66}\text{Zn}$ (‰)	SD (2 σ)	$\delta^{66}\text{Zn}$ (‰)	SD (2 σ)
4.5	8.5	0.38	0.02	-0.79	0.03
5	9	0.29	0.08	-0.80	0.01
5.5	9.5	0.19	0.06	-0.68	0.02
6	10	0.22	0.03	-0.41	0.09
6.5	10.5	0.15	0.06	-0.16	0.10
7	11	0.03	0.12	-0.19	0.02
7.5	11.5	-0.05	0.08	-0.29	0.05

cells are grown in EDTA-buffered seawater media because of high concentrations of total Zn and high rates of iron oxide precipitation (Tang and Morel 2006). In fact, simply reducing the concentration of free Fe in the media can reduce the levels of extracellular Zn to a fraction of the intracellular concentration (Tang and Morel 2006). Laboratory studies are potentially confounded by significant extracellular precipitation, a phenomenon that is likely to be relevant only in coastal and/or polluted marine environments.

Our experiments distinguish between several distinct processes that contribute to the total phytoplankton “biological fractionation” in the oceans. A switch between the predominance of either high- or low-affinity uptake, depending on the ambient concentrations of free Zn, can lead to large differences in the isotopic composition of internal cellular Zn. Additionally, because Zn in seawater is generally complexed to organic ligands, the isotopic offset between free Zn and organic ligand-bound Zn may contribute to the magnitude of biological fractionation. Cell-surface adsorption will constitute a small fraction of total cellular Zn for growing cells in most of the open ocean, but when it occurs, we expect a preference for heavy Zn to adsorb onto cell exteriors.

Because of the similarity in Zn uptake kinetics and Zn transport pathways between many species of marine phytoplankton (e.g., Sunda and Huntsman 1996, 1998), the isotope effects measured in this study may be applied to interpret natural $\delta^{66}\text{Zn}$ signals. For example, dissolved $\delta^{66}\text{Zn}$ variations of 0.2‰ have been measured between surface and deeper northeast Pacific seawaters (Bermin et al. 2006), attributed to a biological drawdown of Zn. The magnitude of this isotopic signal is consistent with phytoplankton Zn uptake via a high-affinity transport pathway, suggesting that Zn concentrations in this region are low enough that cells had to up-regulate specific Zn transport proteins to maintain a sufficient supply of cellular Zn. The observation that surface waters have a lighter $\delta^{66}\text{Zn}$ signature than deeper waters suggests a possible role for surface adsorption in carrying Zn out of the surface ocean. With a knowledge of the physiological and chemical mechanisms responsible for isotopic fractionation, we may soon be able to use Zn isotopes to trace Zn supply through upwelling or dust deposition, to quantify Zn utilization by

natural plankton, and to understand how biological Zn is regenerated in the deep ocean.

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