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Bioavailability and Electroreactivity of Zinc Complexed to Strong and Weak Organic Ligands

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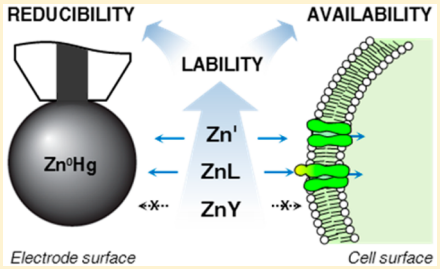
Supporting Information

ABSTRACT: Laboratory experiments have established the importance of complexation by organic ligands in determining the bioavailability of trace metals to marine phytoplankton, while electrochemical measurements with field samples have demonstrated that a large fraction of bioactive trace metals are complexed to strong organic ligands in seawater. Using the model organic ligands, EDTA and histidine, we show a quantitative correspondence between the bioavailability of Zn to the diatom *Thalassiosira weissflogii*, and its reduction at −1.2 V (vs Ag/AgCl) on a hanging mercury drop electrode. Equilibrium calculations and polarographic data indicate that Zn bound in inorganic complexes and the 1:1 Zn-histidine complex, but not in the 1:2 Zn-histidine complex or the Zn−EDTA complexes, is taken up by the organism and reduced at the electrode surface, confirming a previous report of the bioavailability of weak Zn complexes. Electrochemical measurements of Zn speciation in seawater do not generally reveal the presence of weak (and potentially bioavailable) complexes; but such measurements (particularly by Anodic Stripping Voltammetry) should nonetheless often provide good estimates of the bioavailable Zn concentrations. These results can likely be generalized to other bioactive divalent trace metals.



INTRODUCTION

As demonstrated by many laboratory experiments, the availability of essential trace metals to microorganisms, including marine phytoplankton, depends on their chemical speciation. For example, in media containing a strong chelating agent such as EDTA, the fraction of the total concentration of metals such as Fe, Zn, Cu, Cd, or Ni that is chelated is not available for biological uptake. In this situation, the bioavailability of a metal, as measured, for example, by its uptake rate, has been shown experimentally to be controlled by the "free" or "inorganic" metal concentration: Fe′, Zn′, Cu′, Cd′, or Ni′.[1−4] This inorganic concentration is defined as the sum of the concentrations of the solvated metal ion (M^{n+}) and complexes with inorganic ligands (principally HCO_3^-, CO_3^{2-}, Cl^-, SO_4^{2-}, in addition to OH^- and H_2O), which can be calculated on the basis of thermodynamic data and the composition of the medium. In the case of Fe, the question of bioavailability is complicated by the formation of Fe(III)−siderophore complexes which promotes Fe acquisition by organisms that possess the corresponding specialized uptake machinery and by the ability of some organisms to access Fe bound to strong Fe(III) chelators via extracellular reduction to Fe(II).[5,6]

The chemical speciation of several trace metals in natural waters has been measured by a variety of analytical methods, including, in particular, electrochemical techniques. In open ocean surface seawater samples, the two techniques of choice have been anodic stripping voltammetry (ASV) and cathodic stripping voltammetry (CSV). The first quantifies the inorganic or labile concentration of a metal on the basis of its reducibility at an electrode surface, while the second quantifies it by its reactivity with a surface-active complexing agent. In this way it has been shown that a large fraction of the dissolved concentration of biologically important trace metals, including Fe, Zn, and Cu, in surface seawater is bound to strong chelating agents of largely unknown identity and origin.[7−10]

On the basis of the laboratory studies with model chelating agents, it is often assumed that, with the exception of Fe, the labile concentration of a bioactive metal measured electrochemically corresponds to its bioavailable concentration, or at least gives a measure of its bioavailability. Surprisingly, this correspondence has never been tested directly by simultaneous measurements of metal uptake rates and electrochemical reactivity in laboratory cultures.

The question of the correspondence between the chemically measured labile concentration of a metal and its bioavailability is complicated by the demonstration that complexes of some trace metals with "weak" complexing agents are actually available for uptake. This is the case for example for the ZnCys complex which makes Zn available to diatoms with the net result that addition of cysteine to an EDTA buffered medium increases the rate of Zn uptake by these organisms.[11,12] In this situation, the electrochemically measured labile concentration of Zn, $Zn_{labile,}$ corresponds to the bioavailable concentration Zn″ only if it includes the concentration of the

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weak complex: Zn″ = Zn′ + ZnCys, or more generally M″ = M′ + ML, where ML represents the concentration of the bioavailable complexes of M with organic ligands. As discussed by Aristilde et al. (2012), the bioavailability of a Zn complex depends on its ability to exchange the metal with a cellular uptake ligand via formation of a ternary surface complex. Thus, the bioavailability of the metal depends on the denticity of the complex and the rate of ligand exchange, which is related to the thermodynamic stability of the metal−ligand complex. The same coordination and thermodynamic properties govern the lability of an organic metal complex, which determines whether the bound metal is measured by ASV or CSV.

To compare quantitatively the bioavailability of a trace metal to a microorganism with its chemical speciation requires that (1) a convenient radioactive tracer be available to measure uptake rates in the presence of picomolar bioavailable concentrations; and (2) the speciation of the metal be measurable at very low concentrations by a direct electrochemical technique such as anodic stripping voltammetry (ASV), so that complexes of varying labilities can be measured conveniently by adjusting the reducing potential. The only essential trace metal that fulfills both requirements is Zn(II), the uptake of which is conveniently measured by tracer additions of ^{65}Zn and which amalgamates readily on Hg electrodes upon reduction to Zn(0) at an accessible negative potential.[8,13−15] We note that Cd, which is able to replace a fraction of the Zn required for growth in a number of marine phytoplankton species is also amenable to convenient uptake studies with the ^{109}Cd tracer and to measurements by ASV.[9,13−16]

The choice of weak and strong metal complexing agents is also important for a successful quantitative comparison of metal bioavailability and electrochemical reactivity. EDTA is the most obvious choice for a strong chelating agent as there is extensive experimental data showing that EDTA metal complexes are not available to phytoplankton. The hexadentate nature of EDTA complexes makes them kinetically inert, and hence electrochemically unreactive, while the effective thermodynamic affinity of EDTA for trace metals in seawater is relatively weak as a result of its binding to Ca^{2+}, allowing for an efficient buffering of the free metal ion over a wide range of concentrations.[17] Two obvious choices of "weak" complexing agents for Zn^{2+} are cysteine (Cys) and histidine (His), the amino-acids responsible for metal coordination in most Zn proteins, which thus represent possible Zn-coordinating moieties in natural seawater. Additionally, the 1:1 Zn complexes with cysteine and histidine, ZnCys and ZnHis, have been shown to make Zn available for uptake by diatoms.[12] The use of cysteine may be problematic in electrochemical experiments, however, as a result of the possible redox reactions of cysteine itself (the reversible formation of cystine) or those of the reducing agent TCEP, tris(2-carboxyethyl)phosphine, which has been used in biological experiments to avoid the oxidation of cysteine to cystine.

In this study, we compare the rate of uptake of Zn by the model diatom *Thalassiosira weissflogii* to its chemical speciation in the presence of EDTA and His (and in some cases Cys). Uptake rates are quantified from both short-term experiments with ^{65}Zn and in cultures where steady state uptake rates are obtained as the product of the specific growth rate of the organism and the cellular Zn concentration. The concentrations of the labile chemical species of Zn in the culture media are measured via a combination of pseudopolarography and anodic stripping voltammetry with a hanging mercury drop electrode (HMDE) and compared to the speciation predicted from thermodynamic calculations. The results show a quantitative correspondence between the bioavailability of Zn and its electrochemical reactivity and confirm the role of weak complexes in enhancing metal bioavailability in the presence of strong complexing agents.

MATERIALS AND METHODS

Zn Speciation Calculation and Electrochemistry. The Zn speciation in each experiment was computed using the thermodynamic equilibrium modeling program, MINEQL+,[18] and stability constants taken from the MINEQL database and from Martell and Smith (1974)[19] and Morel and Hering (1993)[20] after correction to an ionic strength of 0.5. Voltammetric measurements were made by using a static mercury drop electrode (663VA Stand and PGSTAT 128N potentiostat, Metrohm-Autolab), an Ag/AgCl reference electrode with a 3 M KCl salt bridge, and a glassy carbon counter electrode. Pseudopolarograms were obtained in the presence of a 45 mM borate buffer adjusted to pH 8.0 with samples initially purged with high-purity N_2 (5.0, Airgas) for 200s and before each measurement for 20s. They were obtained from the stripping peak currents in ASV runs at increasingly negative deposition potentials (−0.9 to −1.5 V, ΔE = 0.01 V). After an equilibration time of 10 s, the potential was ramped from −1.2 to 0 V using a square wave voltammetry pulse modulation (scan rate 0.12 V s^{-1}; amplitude 0.02 V; frequency 60 Hz). In the Sargasso Sea water used for all our experiments, but without addition of ligands, we observed the half wave potential of inorganic Zn at ∼ −1.05 V and the pseudopolarograms were not significantly affected by UV irradiation (Figure S1). Anodic stripping voltammetry titrations were performed in 60 mL Teflon bottles spiked with varying Zn concentrations (0 nM−150 μM). The samples were allowed to equilibrate overnight in seawater in the presence of a 50 mM borate buffer adjusted to pH 7.9 (±< 0.1 pH units throughout the titrations). Only Cys and TCEP were spiked with Zn 2−3 h in advance of the titration to avoid oxidation of Cys. The deposition time was varied from 90s (EDTA, EDTA+His at high concentrations, and EDTA+Cys) to 3600s (EDTA and EDTA+His at low concentrations) at −1.2 V and the data were normalized by using the average ratio of the sensitivities measured with both deposition times in the intermediate concentration range (Zn′ = 30−500 nM; Zn_T = 50−90 μM with EDTA = 100 μM). Stripping was performed by square wave voltammetry analogous to the pseudopolarographic analyses. Conditional stability constants with respect to inorganic Zn ($K'_{ZnL,Zn'}$) and complexing ligand concentrations (L′) were determined by nonlinear regression using a 1 or 2 ligand model.[21]

Culture and Growth. *T. weissflogii* CCMP1336 was obtained from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton in Maine. Acid-cleaned polycarbonate bottles were used for culturing and uptake experiments. Culture media were prepared using 0.2 μm filtered and microwave-sterilized Gulf Stream seawater enriched with chelexed and filter-sterilized macronutrients (100 μM NO_3^-, 10 μM PO_4^{3-}, 100 μM $Si(OH)_4$) filter-sterilized f/2 vitamin and trace metals (20 nM Cu, 120 nM Mn, 10 nM Se, 1 μM Fe) buffered with 100 μM EDTA, and various concentrations of Zn added as the ZnEDTA complex (1:1.1).[17] Cells for cultures and short-term uptake were grown at 20 °C under continuous light (80−100 μmol quanta m^{-2} s^{-1}). Specific growth rates were

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determined from linear regressions of the natural log of number of cells versus time. Cell density and volume were determined with a Coulter Counter (Beckman).

Cellular Zn Quota. For long-term experiments, cellular Zn concentrations were determined by inductively coupled plasma mass spectrometry (ICP-MS, Element 2, Thermo Scientific).[22] Cells were harvested at a concentration ~120 000 cells mL^{-1}. About 50 mL of cell culture was filtered onto 3 μm acid-cleaned polycarbonate membranes under low vacuum. Cells were washed twice with an oxalate-EDTA solution for 5 min, and then rinsed with chelexed NaCl. Washed cells on filter were then digested in Teflon tubes with 50% HNO_3 (Optima grade, Fisher) at 100 °C for 4 h. After digestion, Milli-Q water was added to give a final concentration of 5% HNO_3 and the samples were centrifuged.

Short-Term Uptake of Zn. The uptake media were prepared using 0.2 μm filtered Gulf Stream seawater.[23] The seawater was equilibrated overnight with 100 μM EDTA before adding Zn and the other ligand (L-histidine or cysteine). ^{65}Zn (Oak Ridge National Laboratory) was used as tracer with a specific activity adjusted in each experiment. L-histidine (His) and cysteine (Cys) + TCEP were equilibrated first with Zn in a small volume of water at about pH 8.0 for 2−4 h. Zn or Zn-weak ligand complex was added into the EDTA-buffered seawater to equilibrate for an additional 2−4 h before adding cells. The uptake media contained Zn_T = 50 nM, and in some experiments His = 40 μM or Cys = 2 μM + TCEP = 20 μM. Cells were preacclimated in the EDTA-buffered seawater under the condition of 29 nM Zn_T. Zn-limited exponentially growing cells were filtered onto acid-cleaned polycarbonate membrane filters, rinsed five times with 0.2 μm filtered Gulf Stream seawater, resuspended in seawater, and dispensed into the uptake bottles. At intervals of 0.5−1 h for a total incubation period of 2−4 h, 20 mL aliquots from each bottle were removed and filtered onto 3 μm polycarbonate membrane filters. Cells were then washed twice with an oxalate-EDTA solution for 5 min,[22] and ^{65}Zn retained on the membrane was measured via liquid scintillation counting. The pH was measured at the beginning and the end of the uptake experiment by thymol blue. To quantify the cell-normalized uptake rate, the cell concentration in each bottle was measured at the end.

Steady-State Uptake of Zn. The steady-state rate of cellular Zn uptake (mol Zn $cell^{-1}$ d^{-1}) during exponential growth was obtained by multiplying the intracellular Zn quota by the specific growth rate.

RESULTS

Calculated Chemical Speciation of Zn in the Presence of EDTA and Histidine or Cysteine. According to equilibrium calculations, the complexation of Zn by the strong chelating agent EDTA in excess results in very low free Zn concentration in seawater (e.g., Zn′ = 10 pM for $EDTA_T$ = 100 μM, Zn_T = 50 nM and pH 7.9; Figure 1). The addition of the weak ligand histidine has little effect on this free concentration as the bulk of the Zn in solution remains bound to the stronger ligand EDTA so long as the His concentration remains below 1 mM ($His_T \leq 100$ μM in our experiments). Nonetheless the concentration of Zn bound in His complexes (ZnHis and $Zn(His)_2$) becomes larger than the free Zn, Zn′, when His exceeds 6 μM (Figure 1). Similar calculations for cysteine show that the concentration of Zn bound to Cys (ZnCys and $Zn(Cys)_2$ complexes) becomes larger than Zn′ upon addition of 0.9 μM Cys to the same EDTA-buffered medium.

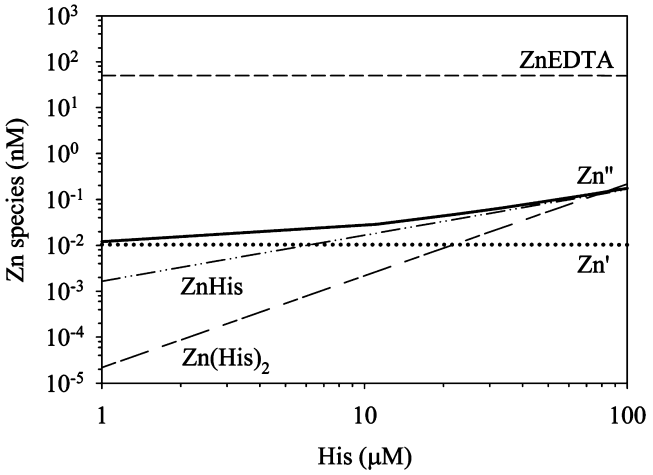

Figure 1. Calculated chemical speciation of the total free Zn (Zn′), ZnHis, $Zn(His)_2$, the sum of total free Zn and ZnHis (Zn″ = Zn′ + ZnHis), and ZnEDTA complexes as a function of His concentration. The total metal and ligand concentration are 50 nM Zn and 100 μM EDTA (pH 7.9). The conditional equilibrium constants (log K) were taken from Morel and Hering 1993 and the MINEQL database (ZnEDTA 18.3, ZnHis 7.3 and $Zn(His)_2$ 13.0, at I = 0 M). The constants were corrected for an ionic strength (I) of 0.5.

According to previous work, the free Zn and the ZnCys complex, but not the $Zn(Cys)_2$ or the ZnEDTA complexes, are available for uptake by marine phytoplankton.[12] This is explained by the difficulty in forming a quaternary Zn complex involving two cysteines and an uptake ligand on the cell surface to promote ligand exchange and uptake. The same situation should obtain with histidine and we expect the ZnHis but not the $Zn(His)_2$ complex to be available for uptake. The addition of 40 μM His is thus expected to increase the bioavailable Zn concentration, Zn″ (=Zn′ + [ZnHis]), by a factor of nearly 8 in our example (Zn″ = 77 pM vs Zn′ = 10 pM; Table S1), while the addition of 2 μM Cys should increase Zn″ (=Zn′ + [ZnCys]) by a factor of about 2 (Zn″ = 23 pM vs Zn′ = 10 pM) as observed by Aristilde et al. (2012).[12]

Reducible Zn Concentrations As a Function of Redox Potential in the Presence of Histidine or Cysteine (Pseudopolarograms). The relative concentration of reducible Zn as a function of redox potential in the presence of excess His or Cys in seawater was measured by ASV. This was achieved by conducting the electrodeposition step at potentials varying from −0.9 V to −1.5 V (vs Ag/AgCl) and measuring the stripping current. The resulting pseudopolarograms shown in Figure 2 (Zn_T = 50 nM and His = 40 μM or Cys = 2 μM in seawater at pH 7.9) exhibit three distinct inflection points, with the middle one being somewhat blurred in the case of His. The reducing potentials corresponding to three inflection points seen in each pseudopolarogram are interpreted as corresponding to the reduction of Zn′, ZnHis, and $Zn(His)_2$ respectively, or Zn′, ZnCys, and $Zn(Cys)_2$. Based on these data, a reduction potential of −1.2 V was selected for the following titration experiments, as it appears to reduce the inorganic Zn and the ZnHis and ZnCys complexes but not the $Zn(His)_2$ or $Zn(Cys)_2$ (or the ZnEDTA) complexes.

Labile Zn Concentrations in Media Containing EDTA only, EDTA+Histidine, EDTA+Cysteine (ASV titrations). We obtained titration curves of our media with model ligands

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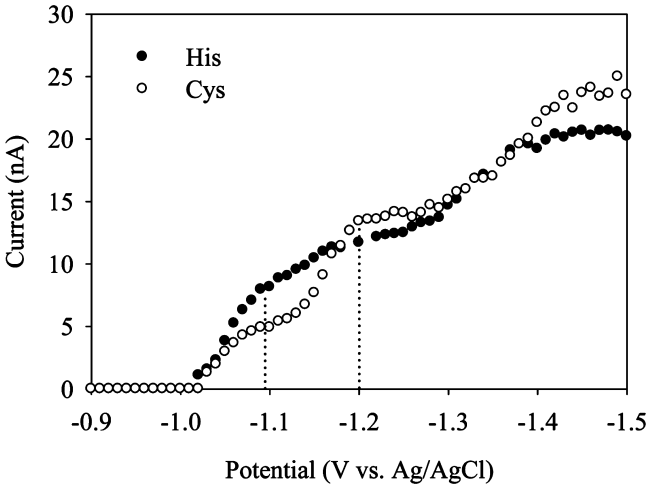

Figure 2. Pseudopolarograms for the reduction of Zn in seawater medium in the presence of weak organic binding ligands, His (filled circles) and Cys (open circles). The curvatures represent Zn′, Zn−histidine complexes (ZnHis and $Zn(His)_2$), and Zn−cysteine complexes (ZnCys and $Zn(Cys)_2$) in the presence of 50 nM Zn_T at pH 7.9 (90s deposition time). The concentration of His and Cys are 40 μM and 2 μM, respectively.

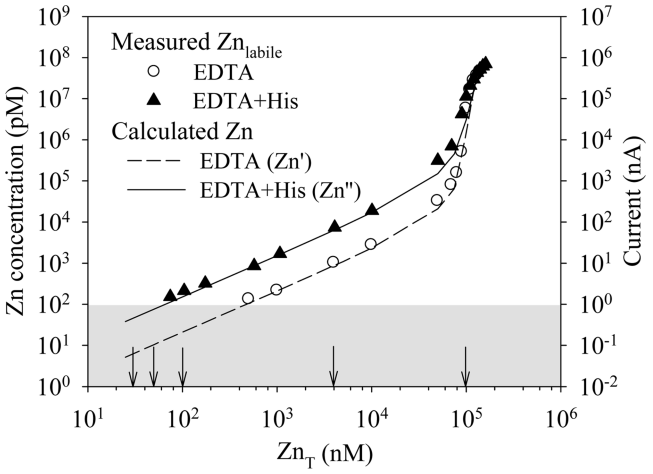

Figure 3. ASV titration results showing the response of reducible Zn concentration at −1.2 V deposition potential (Zn_{labile}) with increasing Zn additions (pH 7.9). Each titration curve was obtained in the presence of 100 μM EDTA (open circles), and in the presence of 100 μM EDTA and 40 μM His (filled triangles). The deposition time was varied from 90s (>100 μM Zn_T for EDTA, and >80 μM Zn_T for EDTA + His) to 3600s and all the data points were normalized to 3600s. The shading represents the lower limit of detection using a plating time of 3600 s. The dashed line and the solid line represent the calculated Zn′ concentration in EDTA only control and Zn″ concentration in EDTA+His treatment, respectively. The conditional equilibrium constants taken for the calculation were the same as the constants used in Figure 1. The arrows point to the total Zn concentrations at which the Zn uptake of phytoplankton was tested in this study.

by ASV using a plating potential of −1.2 V and the long deposition time of 1 h at low concentrations (see Materials and Methods), which provided dependable data down to Zn_{labile} ~ 0.1 nM (limit of quantitation, corresponding to a stripping current ~0.9 nA). Varying the total Zn concentrations from 70 nM to 150 μM in culture media containing 100 μM EDTA only or 100 μM EDTA + 40 μM His yielded titrations curves Zn_{labile} vs Zn_T that are nearly identical to those calculated for Zn″ (=Zn′ + [ZnHis]) vs Zn_T on the basis of thermodynamic data (Figure 3). In particular, at low Zn concentrations, the addition of 40 μM His to the EDTA buffered medium increased the measured Zn_{labile} by a factor of 7.8 on average, close to the calculated ratio of 7.3 for Zn″/Zn′ and significantly smaller than the ratio of (Zn″ + $Zn(His)_2$)/Zn′ = 10.6 (see Table S1). This confirms that the plating potential of −1.2 V (vs Ag/AgCl) reduces the ZnHis but not the $Zn(His)_2$ complex as intended. The labile Zn concentration, Zn_{labile}, is thus expected to be a measure of the bioavailable Zn.

Despite the long plating time, it was not possible to obtain experimental data at the lowest Zn concentrations used in the uptake experiments (indicated by arrows on the *x* axis of Figure 3). But the linear portions of the titration curves (with slopes of unity on the log−log graphs of Figure 3) at low Zn concentrations are well-defined by the data and can be extrapolated with confidence to lower concentrations.

The titrations curves Zn_{labile} vs Zn_T in the presence of 100 μM EDTA and 2 μM Cys exhibited a systematic offset compared to the equilibrium calculations, with the measured Zn_{labile} concentration consistently larger than the calculated Zn″ (=Zn′ + [ZnCys]) (Figure S2). The reasons for this offset are not precisely known but they seem to be linked to the presence of TCEP in the medium as a control experiment showed a significant increase in current at −1.2 V upon addition of TCEP (Figure S3). This experimental problem justifies a posteriori the choice of His rather than Cys as a model ligand for comparing electrochemical and biological data.

Short-Term Uptake Rates of Zn in the Presence of EDTA-Only and EDTA+Histidine or Cysteine. The bioavailability of Zn in the presence of EDTA only and of EDTA+His was quantified by measuring the uptake rate of Zn by Zn-limited *T. weissflogii* in short-term experiments. As expected, the uptake rate of Zn in the presence of EDTA + His (6.2 amol Zn $cell^{-1}$ h^{-1}) was significantly higher than the rate observed in the presence of EDTA only (0.75 amol Zn $cell^{-1}$ h^{-1}) at the same total Zn concentration (Figure 4B). The quantitative agreement between the increase in uptake rate upon addition of His (× 8.3, Figure 4B) and the measured increase in Zn_{labile} (× 8.0, Figure 4A), near the average (×7.8 in Figure 3) under the same conditions, is remarkable.

Addition of 2 μM Cys to the same EDTA-buffered medium resulted in an increase in Zn uptake rate by a factor of 2 (Figure 4D). This is very similar to the result obtained by Xu et al. (2012),[11] which showed an increase in Zn uptake rate by a factor of 2.2 by the same organism under the same conditions. This result is in good agreement with the calculated increase in Zn″ under those conditions (× 2.2; Figure 4C). As expected from the results of the titration experiments, however, this increase is significantly smaller than the measured increase in Zn_{labile} (Figure S2 and Figure 4C).

Growth and Steady State Zn uptake rates as a function of Zn in the presence of EDTA-only and EDTA+Histidine. The effect of His addition on growth and steady state Zn uptake rates in EDTA-buffered media was examined in growth experiments over a range of Zn concentrations. As observed in previous studies, in medium containing EDTA only, the growth rate, μ, was limited for Zn′ (= Zn″) ≤ 0.8 nM and reached the maximum value of μ = 2.1 d^{-1} at a concentration Zn′ > 20 pM (Figure 5A). At the highest concentrations (Zn′ = 19 nM), there seem to be a slight

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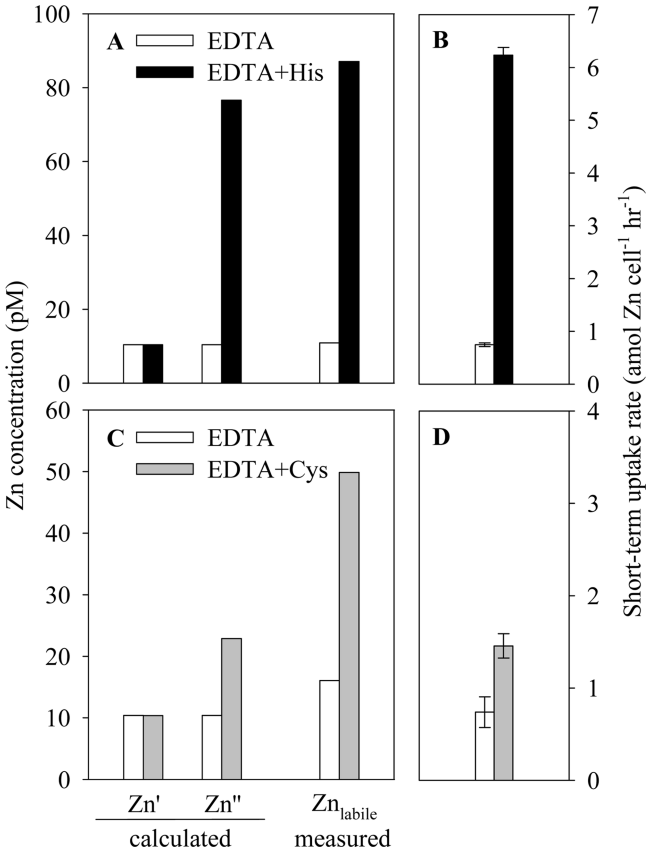

Figure 4. Zn speciation and the short-term Zn uptake rate by Zn-limited *T. weissflogii*. (A, C) The concentration of calculated Zn′, Zn″, and measured reducible Zn at −1.2 V (Zn_{labile}, obtained by extrapolation of the calibration curve of Figure 3), and (B, D) the Zn uptake rate. The total Zn concentration is 50 nM. The ligand concentrations in EDTA control (white bars): 100 μM EDTA; EDTA + His (black bars): 100 μM EDTA and 40 μM His; EDTA+Cys (gray bars): 100 μM EDTA, 2 μM Cys and 20 μM TCEP (pH 7.9). The conditional equilibrium constants (log K) taken for the Zn speciation calculation in the presence of His were the same as the constants used in Figure 1. The constants for Cys were taken from Morel and Hering 1993 (ZnCys 10.1 and $Zn(Cys)_2$ 19.1, at I = 0 M). The constants were corrected for an ionic strength (I) of 0.5.

decrease in growth rate, implying a degree of Zn toxicity. This is consistent with previous studies showing an inhibition of the growth of *T. weissflogii* above Zn′ = 10 nM.[3] Under Zn-limited conditions, μ increased in response to His addition, demonstrating that the enhancement of Zn bioavailability is permanent rather than transient (Figure 5A). In contrast there was no effect of His on μ at high Zn concentration when cells were Zn-replete. The positive effect of His addition on the growth rate of Zn-limited cells is accounted for by the increased in Zn bioavailability as all growth data follow a single graph when μ is plotted as a function of Zn″ (Figure 5B).

In contrast to its effect on growth, the effect of His addition on cellular Zn concentration (the cellular Zn quota Q) was not significant when Zn was limiting growth but became large at high Zn concentrations (Figure 5C). A positive effect of increased Zn bioavailability on growth at limiting Zn concentrations while cells maintain a minimum cellular Zn quota is consistent with theory and previous results.[24,25] The increase in Zn quota in the presence of His at high Zn concentrations corresponds to an increase in the steady state Zn uptake rate, which is obtained by multiplying the quota by the growth rate ($\mu \times Q$) (Figure 5E). As is the case for the growth rate, the positive effect of His addition on the steady state Zn uptake rate of Zn-limited cells is accounted for by the increased in Zn bioavailability as all the data follow a single graph when $\mu \times Q$ is plotted as a function of Zn″ (Figure 5F).

DISCUSSION

Our results confirm the bioavailability of weak Zn complexes and, in the case of histidine, demonstrate a tight correspondence between the reducibility of Zn at an electrode surface and its bioavailability to phytoplankton (Figure 4). As previously observed, the addition of histidine or cysteine to an EDTA-buffered medium increases the Zn uptake rate and the growth rate of Zn-limited *T. weissflogii*. This increase in Zn bioavailability is quantitatively accounted for by the calculated increase in Zn″ = Zn′+[ZnHis] or Zn′ + [ZnCys] as demonstrated in the consistency of the short-term uptake data obtained in this study and previous studies by Aristilde et al. (2012) and Xu et al. (2012) (Figure 6).

In the case of histidine, our data also show a tight correspondence between Zn uptake rates by phytoplankton and the concentration of reducible Zn at a potential of −1.2 V (vs Ag/AgCl). The ability of histidine, a bidentate ligand, to deliver Zn for cellular uptake as the ZnHis complex depends on metal exchange with uptake ligands at the surface of the cells.[12] The ZnHis complex is also sufficiently labile to allow reduction of the Zn(II) at a potential only slightly more negative (by ∼100 mV; Figure 2) than the Zn^{2+} ion or its inorganic complexes. This is not the case for the $Zn(His)_2$ complex which is not able to form a quaternary complex with uptake ligands and is more inert, requiring a significantly more negative potential for Zn(II) reduction. The ability of a ligand, L, to exchange a metal, M with an uptake ligand and the reducibility of M in the ML complex both depend on the lability of the M complex such that there should be a good correlation, albeit not always a perfect one, between the two. In the case of Zn, it appears that the reducible concentration at a potential of −1.2 V (vs Ag/AgCl) should generally provide a good approximation of the bioavailable metal concentration.

Our results obtained with histidine differ in an interesting way from those obtained earlier by Aristilde et al. (2012) with cysteine.[12] In our steady-state experiments, the addition of histidine increased the Zn uptake rate at the highest Zn concentrations when the cells were clearly Zn-replete and may have even suffered from Zn toxicity. The implication is that the ZnHis complex is available to Zn-replete *T. weissflogii*. This is in contrast with the results obtained for Cys, which showed no significant effect of Cys addition on Zn uptake in Zn-replete cells.[12] This difference between the results obtained for His and Cys may indicate that the divalent metal transporters expressed by the cells under Zn-replete conditions have an affinity for Zn that is between that of His and Cys; that is, ZnHis but not ZnCys can exchange the metal with low affinity Zn transporters. In contrast the high affinity transporters expressed under Zn-limiting conditions are able to take Zn from both the ZnHis and the ZnCys complexes as shown by the similar effects of His and Cys on growth and Zn uptake rates when normalized to the increase in Zn″.

Implications for Electrochemical Measurements in the Field. The speciation of Zn in ocean surface waters has been measured by both ASV and CSV.[8,9,26−30] Although some experiments indicate the possible presence of weak ligands,[31,32]

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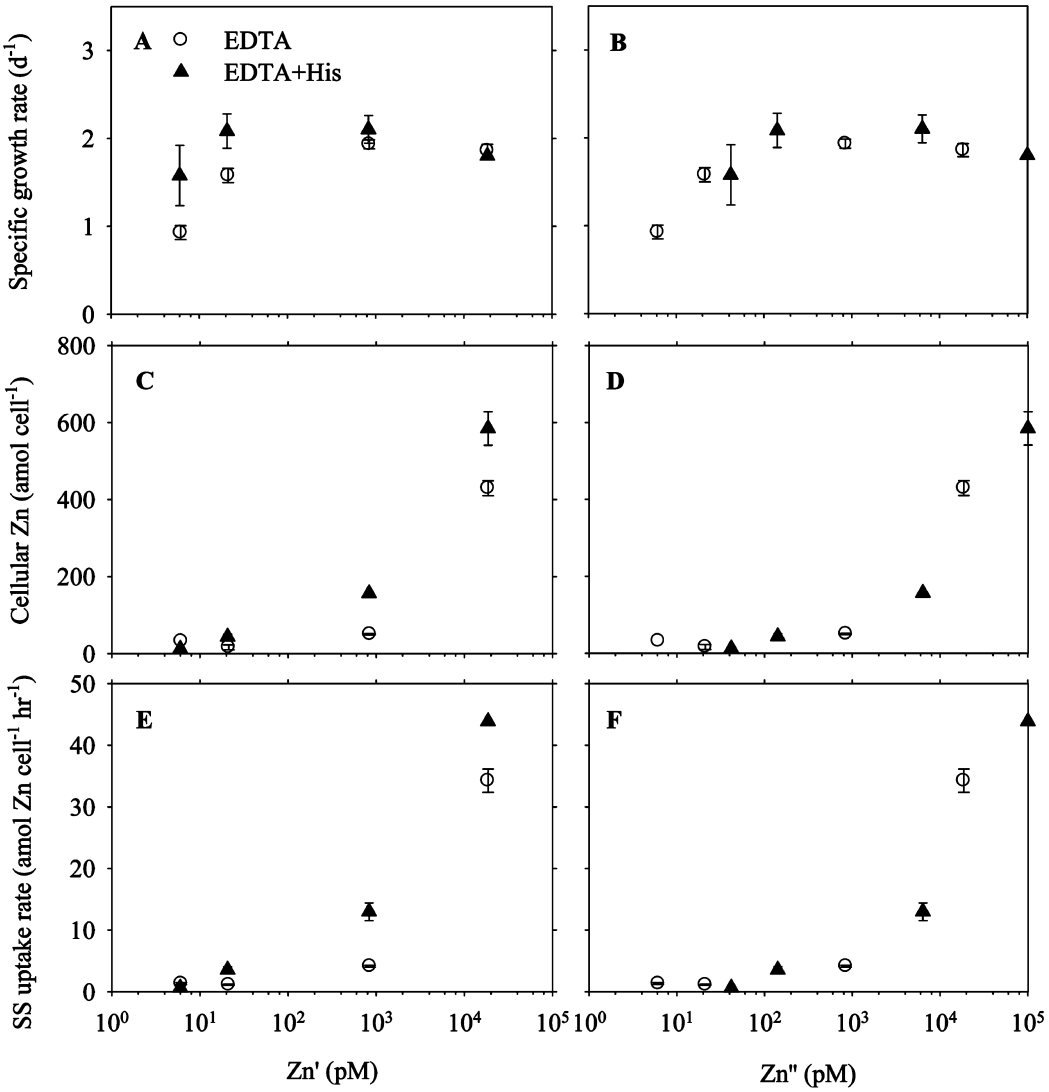

Figure 5. (A, B) Growth rate, (C, D) cellular Zn concentration, and (E, F) steady-state (SS) Zn uptake rate ($=\mu \times Q$) of *T. weissflogii* at 100 μM EDTA (open circles); 100 μM EDTA and 40 μM His (filled triangles). All parameters are plotted against the calculated concentrations of the Zn species.

many data sets can be interpreted by assuming a single strong ligand, X, with log K'_X = 9.1−11.3 and X_T = 0.2−2.5 nM). In view of our results, it is pertinent to consider whether such experiments would reveal the presence of a weak ligand, and if the ambient unchelated Zn concentration that is estimated from such data provides a good approximation of the bioavailable Zn.

To examine these questions concretely, we generated ideal data for a sample containing an initial Zn concentration of 0.2 nM, a strong chelator Y, with $Y_T = 10^{-9}$ M and log K'_Y = 10.5, and a weaker ligand L, with $L_T = 10^{-6.5}$ M and log K'_L = 6.5. The values Y_T and log K'_Y are in the range observed experimentally;[31] K'_L is chosen to correspond to a Zn complex that can be reduced at −1.2 V (vs Ag/AgCl); and L_T is chosen so that $K'_L \times L_T = 1$, the minimum value of practical interest (otherwise the weak complex ZnL would be less abundant than the inorganic Zn, Zn′; note that these conditional equilibrium constants are defined with respect to Zn′, not $[Zn^{2+}]$). The calculated values of inorganic and bioavailable Zn are Zn′ = 7.2 pM and Zn″ = Zn′ + ZnL = 14.4 pM, respectively. We note that the high concentration L_T, which is necessary to increase significantly the bioavailability of Zn, is far in excess of the Zn concentrations added in typical ASV or CSV titrations of natural samples, which are generally stopped at Zn_{added} = 10−15 nM. The net results are that the bulk of the weak ligand remains free throughout the titration ($[ZnL] \ll L_T \approx L$) and the titration data become linear when $Zn_{added} > Y_T$.

ASV titrations of such a sample conducted at the typical potential of −1.2 V, would reduce both ZnL and Zn′ and the stripping current at various Zn additions would yield experimental data as shown in Figure 7, using the line obtained at high Zn concentrations for calibration. These data are perfectly fitted by considering a single ligand X with the same total concentration as Y: $X_T = Y_T$ = 1 nM and a stability constant equal to half that of Y: log K'_X = log (K'_Y/2) = 10.2. As a result, the extrapolated value for the initial labile Zn concentration is equal to the calculated Zn″ = 14.4 pM. This is a generalizable result: if the weak complex is reduced and L is in excess of the added Zn, the ASV titration is well fitted with a single strong ligand X, with $X_T = Y_T$ and $K'_X = K'_Y/(1 + K'_L \times L_T)$ (Text S1). In all such cases, if the initial Zn concentration is smaller than that of the strong ligand, fitting of the ASV data

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Environ. Sci. Technol. 2015, 49, 10894−10902

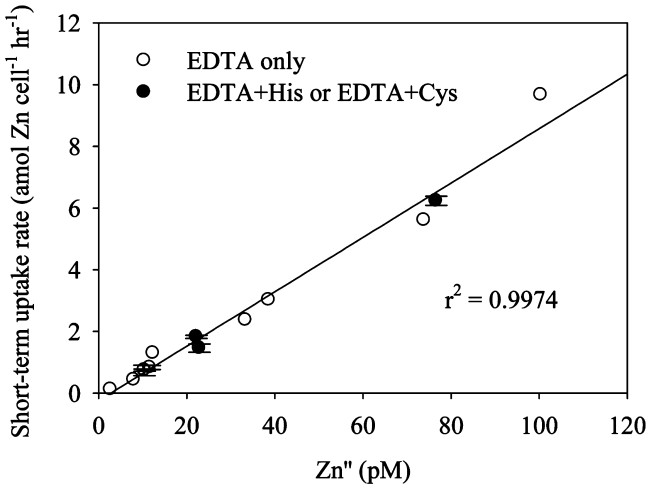

Figure 6. Short-term Zn uptake rate by Zn-limited *T. weissflogii* in the presence of EDTA only and EDTA + weak ligand (His or Cys). Data were taken from this study (Figure 4B, D) and previous studies (Figure 7B from Xu et al. 2012 and corrected from Figure 4E in Aristilde et al. 2012; Figure S4) and plotted versus calculated Zn″. Open circles: the uptake rate of Zn bound to EDTA only; filled circles: the uptake rate of Zn in the EDTA + His or EDTA + Cys. Detailed experimental conditions are described in Table S2.

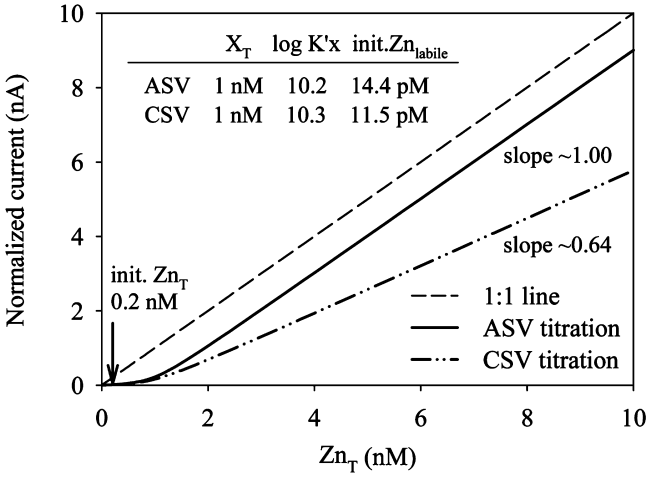

Figure 7. Simulated ideal titrations for ASV and CSV in a two ligand case relevant for surface ocean Zn speciation ($Y_T = 10^{-9}$ M, log $K'_{Zn'Y}$ = 10.5 and $L_T = 10^{-6.5}$ M, log $K'_{Zn'L}$ = 6.5; α_{ZnPDC} for CSV with respect to free Zn = 1.68). At an initial Zn_T = 0.2 nM, the calculated inorganic and weak complex concentrations are Zn′ = ZnL = 7.2 pM, such that the bioavailable concentration is Zn″ = 14.4 pM. Both, the ASV and the CSV data, can be fitted by a one ligand model with the log K'_X and X_T values shown in the inset.

yields the bioavailable concentration Zn″, even though the data do not reveal the presence of the weak ligand L.

In CSV titrations, the stripping current measures the concentration of the Zn complex with the adsorbing ligand added for the analysis, often PDC (1-pyrrolidinedithiocarbamate), and Zn_{labile} is calculated by dividing the ZnPDC concentration (obtained from the stripping current and the calibration slope) by the side reaction coefficient $\alpha_{ZnPDC} = K_{ZnPDC} \times [PDC]$. If α_{ZnPDC} is larger than the side reaction coefficient of the weak ligand $\alpha_{ZnPDC} \gg \alpha_L = K_L \times [L_T]$, L is effectively outcompeted by PDC and invisible during the titration (Text S1). Fitting of the data then simply yields a strong ligand X identical to Y; in our case: $X_T = Y_T = 1$ nM and log K'_X = log K'_Y = 10.5. The extrapolated labile Zn concentration is equal to the inorganic Zn concentration, Zn′ = 7.2 pM, half of the bioavailable concentration in our example.

In the opposite case where the side reaction coefficient of the plating ligand is much smaller than the side reaction coefficient of the weak ligand, $\alpha_{ZnPDC} \ll \alpha_L = K_L \times [L_T]$, the linear response observed after titration of the strong ligand Y is generally taken as the calibration slope. But this slope is in fact too low because a large fraction of the Zn remains bound to the weak ligand L (Figure 7). The net result is an overestimation of the labile Zn concentrations if the slope is taken to be unity. This overestimation is exactly what is necessary to measure Zn″ instead of Zn′, and the mathematics are in fact identical to the ASV case discussed above (Text S1), such that fitting of the data can be done with a single strong ligand X with $X_T = Y_T$ and $K'_X = K'_Y/(1 + K'_L \times L_T)$. In our numerical example, the side reaction coefficient of the plating ligand (α_{ZnPDC} = 1.68) has been chosen near the top of the range reported in the literature (corresponding to $K_{ZnPDC} = 10^{4.4}$ and [PDC] = 67 μM). The corresponding conditional side reaction coefficient for Zn′ is a $\alpha_{ZnPDC}' = 0.764$ ($=\alpha_{ZnPDC}/\alpha_{Zn}$). This coefficient is slightly smaller than $K_L \times [L_T] = 1.0$, such that the CSV measurements do not overestimate the labile Zn concentration sufficiently to yield Zn″. The strong ligand that fits the data (X_T = 1 nM and log K'_X = 10.3) is slightly weaker than necessary to yield the correct bioavailable Zn concentration: Zn_{labile} = 11.5 pM < Zn″. This approximation of the bioavailable Zn concentration is obtained without any evidence of the presence of weak ligands.

As a matter of caution, it must be noted that voltammetric data for low concentrations of metals in seawater are subject to measurement errors, which add substantial uncertainty to the estimations of bioavailable concentrations, regardless of whether ASV or CSV is used. But the well-known problem of obtaining the correct sensitivity (nM/nA) has very different consequences for the determination of bioavailable concentrations by ASV and CSV. In ASV, as long as the plating potential selectively reduces the bioavailable metal, free or bound to a weak complex, a sufficiently sensitive and precise titration should, in principle, yield the sought for bioavailable concentration. In CSV, if the correct sensitivity (nM/nA) is obtained (e.g., the slope of the line in Figure 7 is known to be 0.64 rather than 1.0), then the fortuitous misinterpretation of the data discussed above (overestimate of the sensitivity yields correct bioavailable concentration) no longer obtains. A correct estimation of the bioavailable metal concentration then depends on the ability to correctly evaluate the concentration and thermodynamic stability of the metal complexes as discussed for example in Pižeta et al.[33] In this respect, substantial differences between ASV and CSV data may provide an important clue regarding the presence of weak and potentially bioavailable metal complexes.

Our parallel biological and electrochemical experiments with the model ligands EDTA and histidine demonstrate an excellent correspondence between the bioavailability of Zn, as measured by its uptake rate by the diatom *T. weissflogii*, and its electrochemical lability, measured by its reduction at −1.2 V (vs Ag/AgCl) at an electrode surface. This highly satisfying result reflects the fact that both bioavailability and electrochemical lability are governed by the same coordination and thermodynamic properties. As demonstrated by the good correspondence with equilibrium calculations, Zn bound in inorganic

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complexes or in a 1:1 complex with histidine can be reduced at the electrode surface and taken up by the organism, confirming previous reports on the bioavailability of Zn bound in weak organic complexes. In light of our findings, previous electrochemical measurements of unchelated Zn in seawater (particularly those obtained by ASV) should often provide good estimates of the bioavailable concentrations of the metal, even though they do not generally reveal the presence of weak bioavailable complexes that may be present in the samples. Because our results depend on the fundamental properties of zinc and its complexes, there is every reason to believe that they can be extended, mutatis mutandis, to other divalent metals.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.5b02098.

The SI contains the speciation of Zn under experimental conditions (Table S1), the short-term uptake data and the experimental conditions of present and previous studies (Table S2 and Figure S4), pseudopolarograms of Sargasso seawater (Figure S1), ASV titration results obtained in the presence of EDTA and EDTA+Cys (Figure S2), pseudopolarograms for the reduction of Zn in the presence of Cys with and without TCEP (Figure S3), and a description of the estimation of labile Zn concentrations from ASV and CSV titrations (Text S1) (PDF)

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Notes

The authors declare no competing financial interest.

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