Zinc association with surface-bound iron-hydroxides on cultured marine diatoms: A zinc stable isotope perspective

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Köbberich, Michael; Vance, Derek

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1 **Zinc association with surface-bound iron-**
2 **hydroxides on cultured marine diatoms: A zinc**
3 **stable isotope perspective**

4 **Michael Köbberich* and Derek Vance**

5 *To whom correspondence should be addressed

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11 **Affiliation**

12 Institute of Geochemistry and Petrology

13 Department of Earth Sciences, ETH Zurich

14 Clausiusstrasse 25 , NW C 81.1

15 CH-8092 Zurich, Switzerland

16 michael.koebberich@erdw.ethz.ch

18 **Running head**

19 Diatom surface-bound Zn isotopes

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22

23 **Taxonomy**

24 Trace Metal, Isotope Geochemistry, Phytoplankton, Biogeochemistry, Oceanography,
25 Adsorption.

26

27 ***Abstract***

28 Uptake of trace metals by marine phytoplankton for metabolic use exerts a fundamental control
29 on their marine geochemical distributions. Moreover, such trace metals limit primary
30 productivity over large areas of the surface ocean. As such, an understanding of the
31 mechanisms and extent of phytoplankton uptake are essential components of oceanic trace
32 metal chemistry. Efforts to quantify intra-cellular quotas of phytoplankton are complicated by
33 the presence of metals adsorbed to external surfaces, including surface-bound Fe-hydroxides,
34 both in nature and in culturing experiments. In the relatively new discipline focused on oceanic
35 metal isotopes, these surface-bound metal hydroxides may be of particular importance in that
36 they could result in isotope signatures that complicate studies that seek to understand intra-
37 cellular signatures related to metabolic uptake.

38 In this contribution, we assess the extent to which heavy Zn isotopes are preferentially adsorbed
39 to surface-bound Fe-hydroxides on marine diatoms. For this purpose, the marine diatom
40 *Thalassiosira oceanica* has been cultured at low versus high inorganic Fe concentrations in the
41 medium, while two further diatoms strains have been compared at elevated Fe levels. The
42 formation of surface bound Fe-hydroxides at elevated Fe was further stimulated by reducing the
43 trace metal buffering capacity of the experimental medium, lowering the concentration of the
44 used organic chelator. We also investigate an alternative procedure for quantifying intra-
45 cellular metal quotas, the analysis of the contents of deliberately lysed cells.

46 In good agreement with previous work, we find that biomass associated Fe/P ratios represent a
47 good proxy for the absolute quantity of Fe-precipitates on diatom surfaces. Zn sorption to these
48 surface-bound Fe-hydroxides can drive bulk biomass $\delta^{66}\text{Zn}$ compositions up. On the other
49 hand, the loss of heavy Zn from the experimental medium causes the biomass $\Delta^{66}\text{Zn}$, if referred
50 to the starting medium, to be biased in the other direction, towards more negative values. To
51 avoid any such complications, likely to occur at high Fe or low buffer capacities, we conclude
52 that diatoms cultured at low Fe are most likely to record the $\Delta^{66}\text{Zn}$ signatures of Zn uptake into
53 the phytoplankton cell.

54

55 **1. Introduction**

56 Extreme scarcity of Zn and Fe in the euphotic zone, coupled to deep enrichments (Bruland et
57 al., 2014), is consistent with biological uptake at the surface and regeneration at depth. It is
58 well-established that transition metals such as zinc (Zn) and iron (Fe) are required by marine
59 phytoplankton as essential micronutrients (for a review, see Morel et al., 2014). Both elements
60 fulfill important structural functions, often as cofactors in enzymes required for growth, for the
61 fixation of atmospheric CO_2 (Zn) into biomass (Morel et al., 1994), and for electron transport
62 (Fe) within the cell (Greene et al., 1991). The physiology of diatoms, which accounts for as
63 much as 20% of carbon fixation on Earth (Armbrust, 2009), is particularly closely linked to the
64 bioavailability of these metals (Anderson et al., 1978; Harrison and Morel, 1986; Sunda and
65 Huntsman, 1992; Sunda and Huntsman, 1997). A recent modeling study has suggested that
66 diatom uptake, particularly in the Southern Ocean, dominates the oceanic cycle of Zn (Vance et
67 al., 2017).

68 Recently, efforts have begun to harness the power of stable isotopes (e.g. for Zn, Conway and
69 John, 2014; Zhao et al., 2014) to unravel the oceanic biogeochemical cycling of transition
70 metals, including the impact of biological uptake and regeneration. The first studies have
71 revealed a remarkable homogeneity in Zn isotopes beneath the permanent thermocline, with

72 variable signatures confined to the euphotic zone (Conway and John, 2014; Zhao et al., 2014).
73 In addition to the measurement of isotopic signatures in samples of the real ocean, the
74 exploitation of stable isotopes demands an understanding of the isotope fractionations that
75 accompany key oceanic processes, including those of biological origin. Thus far, only two
76 culturing studies have been performed to study isotope fractionation upon uptake of Zn into
77 marine diatoms (John et al., 2007; Köbberich and Vance, 2017). Further culturing evidence,
78 elucidating key controlling mechanisms that might potentially cause the observed variability in
79 the upper ocean, is required. However, culturing approaches, that aim to study the distribution
80 of metal isotopes between marine diatoms and seawater, might be complicated by experimental
81 difficulties, some of which are addressed in this contribution.

82 Of prime interest is the isotopic signature associated with cellular uptake in the photic zone, a
83 signature that is ultimately transferred to the deep ocean, and regenerated there, by export
84 productivity. In order to isolate and characterize this signal, culturing studies must maintain the
85 integrity of the isotopic signature internal to the cell right up to the point of analysis.
86 Complications can arise from the potentially strong tendency of metals to sorb to external cell
87 surfaces (Gélabert et al., 2006), resulting in the requirement to remove this metal pool (Hudson
88 and Morel, 1989) while preserving the intra-cellular pool for analysis. Transition metals in
89 artificial culturing media are commonly well-buffered by strong organic chelators, such as
90 EDTA (Anderson and Morel, 1982; Sunda et al., 2005). This is done to keep the inorganically
91 bound, bioavailable, metal concentration at a low, and constant, level over the duration of the
92 culturing experiment. However, the fast oxidation kinetics of ferrous iron in ventilated waters
93 can lead to the formation of colloidal or solid Fe-hydroxides phases (Stumm and Sulzberger,
94 1992) that tend to nucleate on surfaces, including those provided by marine phytoplankton
95 (Gélabert et al., 2006). Zn has a high affinity for the surface sites of solid Fe-oxyhydroxides
96 (Dzombak and Morel, 1990), a process that should be accompanied by the preferential
97 adsorption of heavy Zn isotopes (Gélabert et al., 2006; Juillot et al., 2008). Pioneering work by
98 John et al. (2007), the first to explore the fractionation of Zn isotopes associated with uptake

99 into a marine diatom, dealt with this difficulty by applying a cleaning procedure designed to
100 remove surface bound Fe-hydroxides (Tovar-Sanchez et al., 2003), including Zn adsorbed to
101 them, in order to isolate uptake related isotope effects (John et al., 2007).

102 The currently most accepted protocol for the removal of cell surface-bound Fe-oxyhydroxides
103 and other elements associated with them (Hudson and Morel, 1989) relies on reductive
104 dissolution. However, the study of stable metal isotopes introduces special challenges in that all
105 cleaning reagents are required to have low metal blanks. Thus, the John et al. (2007) study used
106 a novel cleaning protocol suggested by Tovar-Sanchez et al. (2003), involving oxalate as a
107 reducing agent. The advantage of this approach is that a liquid-liquid extraction can be used to
108 reduce metal blanks to sufficiently low levels (Tovar-Sanchez et al., 2003). Tang and Morel
109 (2006), however, rigorously compared results for such a strong reductive cleaning reagent
110 versus rinsing with a metal clean sodium chloride solution. The results are striking in that, for a
111 range of metals, including those that are currently subject to isotope studies such as Zn, Cd, Cu,
112 the two methods produce almost identical metal to phosphorus (P) ratios in the final cleaned
113 cells at low Fe concentrations, suggesting that the two cleaning procedures isolate the same
114 intra-cellular metal pool. These findings raise the important question of whether reductive
115 cleaning, in culturing studies that aim to study metal isotopes, is necessary at all.

116 In this contribution, we seek to elucidate the impact of surface-bound Fe-hydroxides on the
117 distribution of Zn isotopes in cultured marine diatoms. Specifically, we have three main
118 objectives. Firstly, we aim to compare Zn isotope fractionation between three distinct diatom
119 strains and their culturing medium, all cultured at elevated Fe and low chelator concentrations
120 to induce large quantities of surface-bound Fe-hydroxides. Secondly, we aim to investigate
121 whether variable total Fe levels in the culturing medium influence Fe-hydroxide formation on
122 diatom surfaces, including the degree to which these might affect the Zn isotope compositions
123 of the bulk biomass. Thirdly, we evaluate a novel method, which deliberately lyses the cells for
124 analysis of the products, with the aim of quantifying intra-cellular isotope ratios in a potentially
125 more robust manner than established procedures. The comparison of diatoms treated with this

126 newly developed approach with cells rinsed only with metal-free seawater aims to address the
127 maximum impact that elevated quantities of surface associated Fe-hydroxides might have on
128 the distribution of Zn isotopes associated with cultured marine diatoms.

129

130 **2. Materials & Methods**

131 All reagents and solutions used in this study were either of trace metal purity or were cleaned
132 using a chelating resin (Chelex[®] 100, Bio-Rad, USA). Reagent grade acids were twice purified
133 by sub-boiling distillation (DST-1000, Savillex, USA). 18.2 MΩ·cm ultrapure water came from
134 a Milli-Q[®] integral water purification system (Merck, Millipore, Germany). Preparation of
135 artificial seawater, manipulation of phytoplankton cultures, and handling of all samples was
136 carried out under ‘Class 100’ clean laboratory conditions at constant humidity of around 10%, a
137 temperature of 21.2 ± 0.2 °C, maintaining sterile techniques. All stock solutions required for
138 culturing were stored exclusively in fluorinated ethylene propylene (FEP) bottles, preventing
139 the sorption of metals to the bottle. Furthermore, this material allows rigorous cleaning which
140 helps to prevent contamination.

141 **2.1 Diatom culturing**

142 Three different axenic diatom strains were obtained from the National Center for Marine Algae
143 and Microbiota (NCMA), formerly known as Provasoli-Guillard Center for Culture of Marine
144 Phytoplankton (CCMP), Bigelow Laboratories, USA. The *Thalassiosira* strains, *T. oceanica*
145 (CCMP 1005), *T. pseudonana* (CCMP 1015), and *T. weissflogii* (CCMP 1336) were cultured at
146 a temperature of $24 \text{ °C} \pm 1 \text{ °C}$. Light was supplied to phytoplankton cultures in 15- to 9-hour
147 light to dark cycles with an hour long gradual dusk and dawn. 24 W Master TL5 HO
148 fluorescent light bulbs (Philips, Germany) of known light spectrum (near natural, low UV)
149 were used for all culturing experiments. Photosynthetically active radiation (PAR) was mapped
150 with a spherical quantum sensor LI-193 (LI-COR[®], Nebraska, USA), achieving an angular
151 response of 82% as estimated by deviation from the sensor’s azimuth. Photon flux densities of

152 50 $\mu\text{mol m}^{-2} \text{ s}^{-1}$ have been achieved by varying the distance to the light source. All
153 phytoplankton strains were illuminated and cultured at the media compositions used later for
154 experimentation. This was done for at least 10 transfer cycles prior to the experiment, and
155 aimed at complete physiological acclimation of the cell to the prevailing experimental
156 conditions.

157 The artificial culturing medium developed for this study aims to vary Fe over a wide range of
158 concentrations at elevated, but comparable, Zn levels. This was done to study the impact of
159 diatom surface-bound Fe precipitates on the distribution of Zn isotopes between the culturing
160 medium and biomass-associated Zn.

161 The seawater base and vitamin supplements of the artificial medium used here are identical to
162 those described by Berges et al. (2001), a medium sometimes referred to as ESAW. Whereas
163 the major nutrients nitrate and phosphate were also as given in Berges et al. (2001), silicate
164 concentrations were kept at a slightly lower level (Provasoli, 1968). Including all additions
165 described below, total dissolved solids are in the range 30.6 – 31.2 g l⁻¹. The transition metal
166 content of the final culturing medium has been modified with respect to Fe, Ni, and Zn. In
167 contrast to Berges et al. (2001), manganese (Mn) and cobalt (Co) were added as hydrated
168 chloride salts, and molybdenum (Mo) and selenium (Se) as dehydrated and hydrated disodium
169 salts, respectively. Whereas, the final Co concentration follows Harrison et al. (1980), those of
170 Mn, Mo, and Se were kept at values as given by Berges et al. (2001). Total Ni and Zn
171 concentrations in the initial medium were kept in the range 0.78 – 0.83 and 1.16 – 1.17 $\mu\text{mol l}^{-1}$,
172 respectively. For the experiments performed as part of this study, the total Fe concentrations
173 were adjusted to be 1.71 $\mu\text{mol l}^{-1}$ (here referred to as ‘intermediate’). The results are compared
174 to selected previously reported experiments on *T. oceanica* cells (Köbberich and Vance, 2017),
175 performed at total Fe concentrations around 2.98 $\mu\text{mol l}^{-1}$ (here referred to as ‘high’) and at
176 0.07 – 0.14 $\mu\text{mol l}^{-1}$ (here referred to as ‘low’). These latter ‘low’ cultures were neither Fe
177 limited, nor high enough in Fe for Fe precipitates to occur. All together, the three Fe
178 concentrations from ‘low’, through ‘intermediate’, to ‘high’, cover a range previously reported

179 to yield very different biomass Fe/P ratios (Tang and Morel, 2006), up to values that far exceed
180 those that could possibly be explained by uptake into the cell alone. EDTA was used as a
181 transition metal buffer to maintain relatively constant inorganically bound metal concentrations
182 over the duration of the culturing experiment.

183 Total EDTA concentrations were set to be in the range between 8.2 and 15 $\mu\text{mol l}^{-1}$, similar to
184 Berges et al. (2001). In this context, it is furthermore important to note that the ESAW like
185 medium used for the intermediate and high Fe concentrations presented here is a generally low
186 EDTA medium, prone to form Fe-hydroxides both on diatom surfaces and, at elevated Fe
187 concentrations, free in solution. The ESAW like medium was deliberately used here to
188 stimulate Fe precipitation to cell surfaces. This medium differs from that previously used by
189 Köbberich and Vance (2017), where a total EDTA concentrations of 97 $\mu\text{mol l}^{-1}$ was used,
190 similar to Sunda and Huntsman (1992), or close to the 100 $\mu\text{mol l}^{-1}$, as recommended *e.g.* by
191 Sunda et al. (2005). The aqueous speciation of the transition metals has been calculated
192 according to (Sunda et al., 2005) and references therein. The free divalent (Me^{2+})
193 concentrations of Zn and Ni have been kept in the range 0.06 – 0.24 nmol l^{-1} and at around 1 –
194 3 pmol l^{-1} , respectively, for the intermediate and high Fe concentration media presented here.
195 We consider comparison to data obtained at lower Fe possible as previously reported Zn^{2+}
196 levels of around 0.105 nmol l^{-1} (Köbberich and Vance, 2017) are not very different from those
197 used here.

198 All culture media were sterilized by using 0.2 μm polyethersulfone (PES) membrane filtration
199 instead of microwave sterilization, which has previously been reported to promote the
200 precipitation of Fe-hydroxides (Andersen et al., 2005). The final medium was stored in acid
201 pre-cleaned polycarbonate (PC) bottles, easily allowing visual inspection for precipitates after
202 storage and immediately before use. Preliminary experiments on ventilated and illuminated
203 media showed no precipitation over a time period of at least 2 months. However, we note that
204 colloidal Fe phases are likely to occur at intermediate and high Fe concentrations, and represent

205 important precursors to Fe precipitates, potentially growing with time (Crosby et al., 1983; Wu
206 et al., 2001).

207 Cultures were harvested at mid exponential growth by filtration onto 0.2 μm polycarbonate
208 (PC) filters embedded in polystyrene (PS) filter housings, both acid pre-cleaned before use. We
209 cannot definitively rule out the possibility that this procedure also collected solid Fe oxides, not
210 associated with cells, on the filter. We do not know of any *a priori* basis for selecting a pore
211 size that permits the passing of solid Fe oxides to the filtrate while retaining cells on the filter,
212 given that the size range of solid Fe oxides in the medium is poorly constrained. However, such
213 a problem, obviously, must result in retention of non-cell-associated Fe-oxide particles greater
214 than 0.2 μm on the filter, and microscopic inspection here did not reveal their presence.
215 Residual media remnants were removed by washing the collected cells with pre-cleaned NaCl
216 solutions, adjusted to seawater ionic strength, or by washing with UV-treated equatorial
217 Atlantic seawater. The latter has very low metal concentrations, notably extremely low Zn in
218 the range of 0.01 – 0.05 nmol kg^{-1} (Zhao, 2011).

219 **2.2 Inverse wash**

220 An important aim of this contribution is to investigate whether the analysis of the contents of
221 deliberately lysed cells represents an alternative method for quantifying intra-cellular isotope
222 ratios. We refer to this new procedure as an ‘Inverse wash’. Most previous washing procedures
223 use strong chemical reagents to remove surface bound components. Our procedure is inverse in
224 its character in that the biomass fraction is leached through the filtration membrane, leaving
225 behind a residue containing glassy diatom frustules, surface-bound Fe-hydroxides, and
226 transition metals potentially associated with either of these phases (Figure 1).

227 Dense 50 ml *T. weissflogii* cultures, designed to provoke elevated biomass Fe/P ratios at a total
228 medium Fe concentration of around 1 $\mu\text{mol l}^{-1}$, and elevated EDTA levels, were split into two
229 20 ml aliquots, both passed through 0.2 μm polycarbonate (PC) filters embedded in polystyrene
230 (PS) filter housings, both acid pre-cleaned as usual. The biomass collected from both splits was
231 washed with a UV treated low-trace-metal equatorial Atlantic seawater. The ‘control

experiment' was not treated any further, so that it serves as a reference that represents intact, but potentially insufficiently cleaned, cells. Rinsing with metal-free seawater is non-destructive, avoids cell-lysis, and removes media remnants trapped between diatom cells, but it is considered to insufficiently remove surface bound transition metals such as Zn (Tovar-Sanchez et al., 2003). On the filter membrane collected cells of the other aliquot were brought into contact with a pre-cleaned 70% (v/v) ethanol-water mixture (EtOH, Scharlau absolute ethanol, Scharlab, Switzerland). Contact times of about a minute burst open all diatom cells, intentionally causing cell lysis. The initially transparent reagent quickly becomes intensely green in color, indicating chlorophyll leakage from the interior of the cell to the surrounding solution. The formerly golden-brown biomass on the filter paper loses its color if the 70 % EtOH mixture is pushed through the membrane, leaving behind almost transparent glassy diatom frustules.

The cells retained on the filter, and the filtrate solutions of both, control and experimental, splits were taken onward for trace metal analysis as described in the next section.

2.3 Elemental and stable isotope analysis

All biomass samples were digested at 120 °C for 16 hours in double distilled 65% HNO₃, with a 0.1‰ (v/v) addition of H₂O₂ for the quantitative oxidation of organic matter. Initial and residual media were acidified to a pH in the range 1.8 – 2.0 for storage. All experimental solutions were dried-down and re-dissolved in 2% HNO₃ for elemental and isotopic analysis.

Elemental analyses were performed on a ThermoScientific Element XRTM inductively-coupled plasma mass spectrometer (ICP-MS) at ETH Zurich, Switzerland. Two secondary standards, a natural river water (SLRS-5, National Research Council (NRC), Canada) and a carbonate-rich shale (Green River Shale, SGR-1, United States Geological Survey (USGS), USA) were used to monitor accuracy and precision. Our key elements (Fe, Ni, Zn, and P) reproduced to 5.2, 8.2, 8.2 and 8.8%, (2 relative standard deviations, 2 RSD) respectively.

Isotope analyses were performed on a ThermoScientific Neptune Plus™ multiple-collector inductively-coupled plasma mass spectrometer (MC-ICPMS) at ETH Zurich, Switzerland, using methods previously described by Bermin et al. (2006) and Zhao et al. (2014). Sample purification was done by anion exchange chromatography, after Maréchal et al. (1999) and as modified by Archer and Vance (2004) and Bermin et al. (2006). Correction of mass fractionation, occurring on the ion exchange column or associated with the analytical instrumentation itself, was done using the double spike approach as described by Bermin et al. (2006). Samples were introduced into the mass spectrometer in 2% (v/v) nitric acid via an Aridus desolvating system (Cetac, USA) using a low flow rate (50 µl min⁻¹) nebulizer, argon (Ar) as sweep gas, but with no nitrogen (N₂) to avoid argon-nitride (ArN₂) interferences on ⁶⁸Zn. Double spike raw data reduction followed Siebert et al. (2001). The double spike was calibrated relative to the reference standard Lyon JMC 3-0749 (Maréchal et al., 1999). Standard delta notation, in per mil, is used to report data relative to this standard as follows: $\delta^{66}\text{Zn} (\text{‰}) = [({}^{66}\text{Zn}/{}^{64}\text{Zn})_{\text{sample}} / ({}^{66}\text{Zn}/{}^{64}\text{Zn})_{\text{JMC-Lyon}}] - 1$. Accuracy and precision was monitored through double spike analysis of a secondary standard, IRMM-3702, previously determined to yield a value of +0.32‰ (Cloquet et al., 2008; Ponzevera et al., 2006), or +0.30‰ (Moynier et al., 2017) for $\delta^{66}\text{Zn}$ relative to JMC-Lyon. We obtain $\delta^{66}\text{Zn} = 0.30 \pm 0.06\text{‰}$ (2SD, n = 163 over 380 days) for IRMM-3702 relative to JMC-Lyon.

Zn isotope fractionation observed in culturing experiments is reported as $\Delta^{66}\text{Zn}$, defined as the difference between the culturing medium and the separated biomass: $\Delta^{66}\text{Zn} (\text{‰}) = \delta^{66}\text{Zn}_{\text{biomass}} - \delta^{66}\text{Zn}_{\text{medium}}$. External reproducibility was estimated to be $\pm 0.06\text{‰}$ based on the replicate analyses of IRMM-3702.

3. Results

Table 1 summarizes the results of this study, and compares them with previous work (John et al., 2007; Köbberich and Vance, 2017) to arrive at a dataset for *Thalassiosira* cultures grown at

total medium Fe concentrations ranging over two orders of magnitude. The *Thalassiosira* biomass from these cultures were found to yield variable biomass-associated Fe/P ratios (Figure 2), consistent with previous results presented by Tang and Morel (2006). These previous observations establish the framework within which we further discuss our results. We distinguish between three different classes of Fe/P ratios ranging from low, through intermediate, to high (see Section 2.1). In good agreement with previous culture studies done at intermediate and high Fe concentrations (Tang and Morel, 2006), all diatoms were found to grow very fast, with specific growth rates of $>1 \text{ d}^{-1}$.

3.1 Diatom culturing

Two different representatives of the genus *Thalassiosira*, grown at intermediate total Fe concentrations, were found to yield high Fe/P ratios in the range $122 - 232 \text{ mmol mol}^{-1}$, in good agreement with Tang and Morel (2006), and Zn/P ratios in the range $10.3 - 26.7 \text{ mmol mol}^{-1}$ (Table 1). Biomass associated Ni/P ratios, in contrast to Fe/P and Zn/P, were found to be much lower, typically below $0.08 \text{ mmol mol}^{-1}$, because of much lower bioavailable Ni levels in the medium. The currently most reliable estimates of intra-cellular Fe/P ratios in natural diatom cells derive from synchrotron based X-ray fluorescence imaging techniques (Twining and Baines, 2013; Twining et al., 2004), and are $4.6 - 5.2 \text{ mmol mol}^{-1}$. The Fe/P ratios reported here (Table 1) are in most cases significantly higher than this, and indicate the presence of surface-bound Fe-hydroxides (Tang and Morel, 2006). This finding provides the basis for the evaluation of two different phenomena discussed in this paper. First, the species-dependent potential to accumulate surface-bound Fe-hydroxides. Second, whether moderate quantities of surface bound Fe-hydroxides impact the distribution of Zn isotopes in the Fe concentration range we refer to as ‘intermediate’ (Figure 2).

Consistent with the precipitation of Fe-hydroxides, all *Thalassiosira* associated Fe/P ratios increase with increasing Fe' (Figure 3A). Six *T. oceanica* cultures grown at high Fe', above the previously reported threshold, show extremely high Fe/P ratios, in the range $315 - 429 \text{ mmol mol}^{-1}$, but very low Zn/P ratios, below $4.2 \text{ mmol mol}^{-1}$ ($3.4 - 4.2 \text{ mmol mol}^{-1}$). The highest Ni/P

ratio of 0.10 mmol mol⁻¹ is only marginally higher than at intermediate Fe/P, with similar variability. Whereas Ni/P is always low in all experiments, Fe/P and Zn/P show an anti-correlation, with high Zn/P ratios associated with low Fe/P ratios in cultures grown in media at low Fe' (Figure 3A and B).

In contrast to Tang and Morel (2006) we report biomass metal to P ratios as a function of the free inorganic Fe concentration (Sunda et al., 2005) at the harvesting time (Figure 3 and 4), here denoted as Fe'_{RM} (RM stands for residual medium), rather than as a function of total Fe (c.f. Figure 2). This is done because Fe' is not only an important parameter for Fe acquisition by eukaryotic marine algae (Shaked et al., 2005), but also represents the pool of aqueous precursor species required for the nucleation of solid Fe-hydroxides (Cornell and Schwertmann, 2003; Morgan and Lahav, 2007). Their progressive formation over the duration of the culturing experiment rapidly reduces Fe' levels, by many orders of magnitude. This very large reduction is mainly driven by the photochemical reduction of Fe(III) to Fe(II) off the EDTA complex (Sunda and Huntsman, 2003), followed by fast re-oxidation (Morgan and Lahav, 2007). Fe'_{RM} is thus a measure for the final state of the system, indicating whether Fe is still likely to be precipitating at the harvesting time. Early work by Sunda and Huntsman (1995b) and Sunda and Huntsman (1997) describe an Fe' threshold concentration of about 0.7 nmol l⁻¹, above which Fe precipitates are likely to occur, not only on diatom surfaces but also freely in the culturing medium. Fe'_{RM} of all *Thalassiosira* cells cultured here to have intermediate Fe/P ratios were found to be below this threshold concentration after the experiment. This indicates that precipitation is likely to have ended before harvesting. In contrast, six replicates of *T. oceanica*, cultured to yield high Fe/P ratios show final Fe concentrations in the medium above 0.7 nmol l⁻¹. These higher Fe'_{RM} values indicate that the precipitation of Fe-hydroxides may still be ongoing in cultures starting from higher initial total Fe concentrations.

3.2 Surface bound Fe dependent $\Delta^{66}\text{Zn}$

A key feature of all *Thalassiosira* strains cultured to have intermediate and high Fe/P ratios is that the calculated bulk isotopic composition of measured products at the end of the

experiment, the residual medium plus the biomass, is isotopically lighter than the initial medium (Table 1). Thus, heavy Zn isotopes must be lost through the seawater wash, and $\Delta^{66}\text{Zn}$ calculated with respect to the initial medium ($\Delta^{66}\text{Zn}_{\text{biomass} - \text{initial medium}}$, Figure 4A) is not identical to that with respect to the residual medium ($\Delta^{66}\text{Zn}_{\text{biomass} - \text{residual medium}}$, Figure 4B). In other words, the derived fractionation is shifted by about +0.14‰ on average if referenced to the medium at the harvesting time, instead of its initial Zn isotope composition.

In this context, it is important to note that surface bound Fe-hydroxides are likely to precipitate over the duration of the culturing experiment (see Section 3.1). Thus, the initial medium represents a poor reference, as its composition will be variable with time. All culturing results presented here are in marked contrast to those previously presented for *T. oceanica* cells cultured at low total Fe and elevated EDTA concentrations (Köbberich and Vance, 2017). For these low Fe' cultures, no heavy Zn isotopes were lost in seawater washes, as indicated by the fact that $\Delta^{66}\text{Zn}_{\text{biomass} - \text{residual medium}}$ and $\Delta^{66}\text{Zn}_{\text{biomass} - \text{initial medium}}$ were found to be within analytical error. In other words, the Zn isotope composition of the previous used initial culturing medium has barely changed over the course of the experiment.

The overall variability in $\Delta^{66}\text{Zn}$ of the **two** diatom species at intermediate Fe/P (Figure 5, left) is compared with **that** found for *Thalassiosira* cells investigated at variable Fe/P ratios (Figure 5, right). The heaviest biomass associated Zn isotope compositions in the datasets of all replicates increase from low, through intermediate, to high Fe/P ratios. $\Delta^{66}\text{Zn}$ signatures significantly lighter than the minimum values observed at intermediate or high Fe/P ratios, are associated with low Fe/P ratios.

3.3 Inverse wash

We also investigated the potential for quantification of intra-cellular metal quotas using a newly developed method, which analyzes the contents of lysed cells rather than trying to remove surface-bound metals. This approach is 'inverse' in its character in that the biomass fraction is leached through the filtration membrane, leaving behind a residue containing the diatom

363 frustules, surface-bound Fe-hydroxides, and transition metals potentially associated with either
364 or both of these phases.

365 One half of a split culture bottle was filtered and the collected biomass was washed with low-
366 trace-metal equatorial Atlantic seawater and not treated any further. This treatment serves as a
367 'control' experiment (Figure 1, left). Different volumes of seawater used for washing did not
368 cause any differences in the distribution of Fe, Zn, and P between the residue fraction and the
369 filtrate (Figure 6A and B). For the inverse wash (see Figure 1, right), in contrast to the control
370 experiment, the volume of the 70% EtOH solution brought into contact with the diatom cells
371 does affect the proportion of Zn and P that is mobilized and transported through the membrane
372 in the filtrate solution (Figure 6C and D). Importantly, the majority of Fe in the filter residue
373 stays associated with the diatom frustules, whereas Zn and P are co-mobilized with each other.

374 The mobility of Zn and P and immobility of Fe, as observed for the inverse wash, causes
375 changes in Fe/P and Zn/P ratios that are also dependent on the volume of the 70% EtOH
376 solution used for washing. Frustule associated Fe/P ratios, in the residue fraction, increase from
377 below 90 to almost 280 mmol mol⁻¹ (Figure 6E). The Fe/P ratios of the corresponding filtrate
378 solution decreases from about 14.3 to less than 4.5 mmol mol⁻¹ as increasing quantities of P are
379 brought through the filtration membrane (Figure 6F). Zn/P ratios were consistently found to be
380 around 2 mmol mol⁻¹ (Figure 6E and F).

381 Six fully reproduced culture experiments, conducted either with 5 ml rinses of seawater or
382 equivalent volumes of 70% EtOH, have been compared for their Zn isotope fractionation with
383 respect to the culturing medium (Figure 7). The seawater rinse of the control experiment was
384 found to be characterized by heavy signatures, with a mean $\Delta^{66}\text{Zn}$ of $0.09 \pm 0.06\text{‰}$. The
385 biomass of the control experiment (residue) and the inverse wash (filtrate) were found to yield a
386 mean of $-0.07 \pm 0.06\text{‰}$ and $-0.09 \pm 0.07\text{‰}$, respectively. Five replicates of the inverse residue,
387 containing diatom frustules, potentially adsorbing metals, and the fraction of biomass not
388 carried in the filtrate, gave the lightest mean of $-0.15 \pm 0.06\text{‰}$.

389

390 4. Discussion

391 4.1 Species dependent differences: metal/P ratios

392 Our diatom culturing experiments at intermediate Fe concentrations allow comparison of two
393 different *Thalassiosira* strains (Table 1), all characterized by intermediate Fe/P ratios (Figure
394 2). We find Fe/P ratios conform to those previously reported by Tang and Morel (2006) for *T.*
395 *weissflogii*, but lower values for *T. pseudonana*. Figure 3 compares these results to *T. oceanica*
396 cells grown in high Fe' media. For all six replicates the initial Fe content was high enough to
397 obtain Fe'_{RM} values (c.f. Section 3.1) above 0.7 nmol l⁻¹, levels at which Fe-hydroxides were
398 previously described to occur on diatom surfaces and possibly free in the culturing medium
399 (Sunda and Huntsman, 1995b; Sunda and Huntsman, 1997). Indeed, all diatoms plotted in
400 Figure 3 are characterized by Fe/P ratios that are at least 20 times higher than those obtained
401 from synchrotron based X-ray imaging techniques (Twining and Baines, 2013). On these
402 grounds, we interpret most Fe to be associated with the diatom surface rather than with the
403 intra-cellular pool. This view is further supported by the fact that the initially set Fe'
404 concentration is reduced by many orders of magnitude between the beginning of the experiment
405 and harvesting (c.f. Section 3.1). This is unlikely to be explained by cellular uptake alone.

406 The data in Figure 3B provide no evidence that large amounts of metals other than Fe are
407 associated with larger quantities of Fe precipitates. The distribution of Ni is clearly not to the
408 same extent dependent on the concentration of Fe, or on the quantity of Fe-hydroxides formed
409 on cell surfaces. In direct comparison to Zn, a larger proportion of Ni is associated with the
410 organic chelator, in EDTA buffered solutions. The free, and non-chelated, inorganic Ni pool is
411 thus much smaller in comparison to that of Zn. These lower Ni levels might explain both
412 reduced uptake and reduced association with surface-bound Fe-hydroxides, despite the fact that
413 Ni also generally has a high propensity to sorb to solid Fe phases. In contrast, the data suggest
414 decreasing Zn/P ratios with increasing Fe' and biomass-associated Fe/P ratios. The lowest Zn/P
415 (3.4 – 4.2 mmol mol⁻¹) comes with the highest levels of Fe-hydroxides. Previous culturing work
416 done with the same *Thalassiosira* strain suggests cellular Zn quotas equivalent to Zn/P ratios of

417 1 – 2 mmol mol⁻¹ (Sunda and Huntsman, 1992; Sunda and Huntsman, 1995a; reviewed in
418 Twining and Baines, 2013), at the bioavailable Zn level used here. X-ray images of intra-
419 cellular metal quotas illustrate a larger range, extending to more than 5 mmol mol⁻¹ (Twining et
420 al., 2011), encapsulating the full spectrum of observations in the experiments documented here.

421 For our purposes here, the lack of any positive correlation between Fe/P and Ni/P or Zn/P is the
422 most important – and first order – observation. The apparent inverse trend seen for Zn/P in
423 Figure 3B is intriguing, but at this stage, its interpretation is rather speculative. One possibility,
424 however, is that differences in Fe-hydroxide precipitation, either between the **two** different
425 *Thalassiosira* strains with intermediate Fe/P or between the cells with intermediate versus high
426 Fe/P, on diatom cell surfaces could inhibit Zn uptake. This inhibition could be a direct
427 consequence of Fe on the diatom surface, or an indirect physiological effect triggered by the
428 suppression of growth towards increasingly lower Fe'. It is also possible that at high Fe,
429 continued precipitation of Fe-hydroxides (*c.f.* Section 3.1) will inhibit Zn adsorption, whereas
430 such sorption might be favored if Fe precipitation stops part way through the experiment, as
431 may occur in the intermediate Fe experiments.

432 **4.2 Species dependent differences: Zn isotope compositions**

433 An important implication of the data presented here, in the context of the small number of
434 previous culturing experiments and for the future, is the observation that heavy Zn isotopes
435 must have been lost from the bulk experimental system through washing of cells. Our study
436 represents a rather extreme case, given that it constitutes a deliberate attempt to promote Fe
437 oxide precipitation in order to investigate its impact on metal isotopes in culture experiments,
438 so that cells were cultured at elevated total Fe but low EDTA concentrations.

439 However, this finding does have implications for past and future culturing experiments, where
440 Fe oxide precipitation is occurring and where isotope fractionation associated with uptake into
441 cells is a target for study. In previous experiments the emphasis has been placed on the removal
442 of surface-bound Fe oxides because they affect the measured biomass, and thus the target
443 isotopic signal for uptake (John et al., 2007; Köbberich and Vance, 2017). But, the progressive

444 precipitation of surface bound Fe-hydroxides over the course of the culturing experiment, and
445 the fact that this process removes heavy Zn isotopes from the bio-available pool in the medium,
446 implies that the medium Zn isotope composition must be evolving to lighter isotope
447 compositions. While the usual practice is to calculate the isotope fractionation associated with
448 uptake via referencing the final biomass to the initial medium, [here](#), the true fractionation must
449 be somewhere between that calculated relative to the initial medium and that calculated relative
450 to the residual medium. From the Zn recoveries calculated as the sum of the residual medium
451 and the biomass (Table 1), it is apparent that the fraction of Zn that is lost through washing is
452 small. The isotopic imbalance, then, implies that such Zn must be [quite](#) heavy.

453 In terms of elemental ratios, all our findings are consistent with those of Tang and Morel
454 (2006). These authors furthermore observe intra-cellular Zn/P ratios identical to those of the
455 total biomass at the lowest Fe/P ratios. With increasing Fe/P ratios, interpreted as increasing
456 quantities of surface-bound Fe-hydroxides, bulk biomass Zn/P ratios are consistently found to
457 be slightly higher than the intra-cellular proportion alone (*c.f.* Figure 3.5 in Tang and Morel,
458 2006). The absolute difference is small, indicating that only a tiny fraction of the bulk Zn is
459 associated with the diatom surface at increased Fe/P ratios. This is where Zn isotopes might
460 offer additional insights, in that all here presented cultures with intermediate and high Fe/P are
461 generally heavier than the currently lightest numbers previously reported (John et al., 2007) for
462 similar bioavailable Zn^{2+} concentrations (*c.f.* Table 1).

463 [In this context, it is important to note that the low trace metal buffering capacities of the](#)
464 [medium, used here to facilitate the precipitation of Fe-hydroxides, also causes \$\text{Zn}^{2+}\$ to drop](#)
465 [beyond what would be expected from the reduction of total Zn from the initial to the residual](#)
466 [medium. The bioavailable – non-EDTA-bound – level of many divalent cations will generally](#)
467 [be high under in low buffering capacity media, leading to uptake of various cations into](#)
468 [diatoms. Assuming that the total EDTA concentration remains constant, lower total cation](#)
469 [concentrations provide more EDTA available to complex Zn, which in turn lowers the](#)
470 [bioavailable \$\text{Zn}^{2+}\$ concentrations. As Fe-hydroxides were deliberately precipitated from](#)

471 solution, particularly large quantities of Fe-EDTA complexes would dissociate and the Fe
472 incorporated into newly formed solids, thereby explaining some excess EDTA that can then
473 bind Zn. Acknowledging some unavoidable variability in Zn^{2+} in weakly buffered and
474 precipitating cultures, we argue that Zn^{2+} at the time of harvesting is more likely to reflect the
475 concentration relevant for comparison.

476 **4.3 Assessment of the inverse wash: elemental metal/P ratios**

477 Here, we propose that the analysis of the contents of deliberately lysed cells may be a useful
478 alternative approach for assessing intra-cellular metal quotas and their isotope compositions.
479 Below we discuss these experiments in the context of Figure 6, which compares elemental
480 abundances for a control experiment involving analysis of diatom cells washed only with low-
481 trace-metal seawater (A, B) with the results for the inverse wash (C, D). Element ratios
482 obtained for the inverse wash are shown on the right (E, F).

483 The first key feature of Figure 6 is that as P is increasingly transported through the membrane
484 using increasing amounts of 70% EtOH to induce cell lysis, Fe is left behind. The latter results
485 in changing Fe/P ratios in the residual fraction left on top of the membrane, and in the filtrates
486 (Figure 6E and F). Moreover, filtrate Fe/P ratios converge towards a value of around 4.4 ± 3.6
487 mmol mol^{-1} . This Fe/P ratio is completely consistent with the range obtained for intra-cellular
488 quotas from synchrotron X-ray fluorescence work on natural diatom cells (4.6 - 5.2 mmol mol^{-1}
489 ¹, Twining and Baines, 2013). In contrast, Fe/P ratios in the residue rise to values near 280
490 mmol mol^{-1} as cell lysis proceeds, documenting the retention of extra-cellular iron on the
491 membrane.

492 It is known from synchrotron based X-ray fluorescence imaging techniques that Zn and P are
493 co-located within cultured *T. weissflogii* cells (Twining et al., 2003). Thus, a second key feature
494 of our experiments is that the EtOH solution co-mobilizes Zn and P. Thus, as the volume of
495 reagent increases, an increasing proportion of the biomass Zn and P is mobilized in the filtrate,
496 leaving progressively less material behind as a residue on the membrane. Zn/P ratios are almost
497 identical in both fractions, residue and filtrate, and remain the same throughout the experiment.

498 As suggested by previous research, based on culturing (Sunda and Huntsman, 1992; Sunda and
499 Huntsman, 1995a) and imaging techniques (Twining and Baines, 2013; Twining et al., 2003),
500 there exists only one significant source of Zn in the biomass, one that is associated with P and
501 not with Fe.

502 Critical evaluation of the dataset (Figure 6) obtained with this approach allows us to identify
503 the following two potential drawbacks. Firstly, P (and Zn) transfer from the initial bulk biomass
504 to the filtrate fraction is non-quantitative. If P is interpreted as a mainly intra-cellular associated
505 element, then ~20 to 30% of this pool remains on top of the filter membrane and is not carried
506 in the filtrate solution even at the largest volume of EtOH (Figure 6C and D). This, of course, is
507 not a concern if intra-cellular metal to P ratios, or the distribution of Zn isotopes (see below),
508 are the focus of interest. The exact reasons as to why some P is left behind are currently
509 unclear. Elevated Fe concentrations, however, could initiate the co-precipitation of the
510 remaining P as increasing amounts of mobilized biomass are transported across the filtration
511 membrane. Moreover, the experimental approach that has been chosen to provoke partial cell-
512 lysis is also highly dependent on the contact time of the diatom cells with the 70% EtOH
513 mixture, and on sample agitation. Our experiments kept the contact time constant and varied
514 the volume of the EtOH solution that is used to re-suspend cells instead. This was done to
515 achieve different degrees of cell-lysis, but it requires contact times to be comparatively short
516 and of identical length, in order to interrupt the cell lysis process. It seems possible that further
517 experiments with variable contact time at constant volumes, possibly also agitating the sample,
518 would yield more easily reproducible results and mobilize more biomass.

519 **4.4 Assessment of the inverse wash: Zn isotope compositions**

520 In Section 4.3 we proposed that the analysis of cell lysates represents an alternative approach
521 for quantifying intra-cellular metal quotas. In this section, we further discuss the distribution of
522 Zn isotopes between partially extracted biomass separated from other cellular remnants
523 remaining on a filtration membrane after an EtOH treatment, as measured on six full culturing
524 replicates. Figure 7 compares the cellular soft-tissue mobilized with 5 ml of the 70% EtOH

525 solution with those of a control experiment (*c.f.* with Section 2.2 and 3.3) in which the filtered
526 biomass is only washed with seawater. As a reminder, isotope fractionation in this diagram is
527 defined as $\Delta^{66}\text{Zn}$, expressed as the $\delta^{66}\text{Zn}$ composition of the fraction of interest relative to the
528 starting culture medium.

529 Our expectation, given the small impact on Zn isotopes from surface-bound Fe-hydroxides
530 suggested in previous sections of this discussion, is that $\Delta^{66}\text{Zn}$ for seawater-washed diatoms
531 ('control' residue) and intra-cellular biomass ('inverse' filtrate) should be identical. The data in
532 Figure 7 are consistent with this expectation. The 'control' residue on Figure 7 represents the
533 bulk biomass (plus adsorbed Zn), after washing only with seawater, and is characterized by
534 intermediate Fe/P ratios of $123 \pm 44 \text{ mmol mol}^{-1}$ ($n = 6$). This fraction is essentially identical in
535 Zn isotopes to the soft-tissue extracted with the inverse EtOH leach. The population means and
536 variances of the two fractions are not significantly different at a confidence level of 95%.
537 Whereas the mean difference is as small as 0.03‰, the 25th percentile of the 'inverse' filtrate
538 and the 75th percentile of the 'control' residue differ from each other by 0.11‰.

539 The residual media of all inverse experiments are, within our external reproducibility of
540 $\pm 0.06\text{‰}$, indistinguishable from the initial medium and the 'system' as a whole, since only a
541 small amount of the medium Zn is taken up or adsorbed from solution. This is an important
542 difference with respect to the *T. weissflogii* cultures described earlier, for intermediate Fe/P
543 ratios (Section 4.1 and 4.2). The discrepancy might simply be explained by lower total Fe
544 concentrations used for inversely washed culture experiments – close to half that used for
545 species inter-comparison. Despite biomass associated Fe/P ratios that are more than 20 times
546 higher than our currently best estimates of average intracellular ratios (Twining and Baines,
547 2013), only slightly heavy isotopes are lost through the wash, while the residual medium is
548 analytically indistinguishable from the initial.

549 Figures 6C and D suggest that the quantitative transfer of all biomass from the residue to the
550 filtrate fraction has not been achieved with the approach chosen here (*c.f.* Section 4.3). At the
551 chosen EtOH volume of 5 ml, only ~30 – 50% of the soft-tissue Zn and P was extracted from

the diatoms and leached into the filtrate fraction, leaving behind ~50 – 70% of the biomass, the diatom frustules, surface-bound Fe-hydroxides, and the Zn adsorbed with either of the last two phases. Given that the latter should drive the bulk signature heavy, it is somewhat surprising that the mean of the residue fraction (‘inverse wash’) appears to be isotopically lighter than the filtrate fraction (Figure 7). These differences, however, are small and statistically neither population means, nor variances, are significantly different at a confidence level of 95%.

5. Conclusion

In this study, we investigated the Zn isotope fractionation between seawater washed diatoms and their culturing medium. Two different *Thalassiosira* strains were cultured at intermediate Fe' concentrations, and compared to *T. oceanica* cells grown at low versus high Fe' levels. Consistent with previous elemental work (Tang and Morel, 2006), we found bulk biomass Fe/P ratios to represent a good predictor of the impact from surface-bound Fe-hydroxides on Zn isotopes. Our culturing results suggest that low Fe/P ratios, near those expected inside the cell (Twining and Baines, 2013), do not show any or only a small impact on Zn isotopes arising through adsorption of heavy Zn isotopes to surface-bound Fe-hydroxides. Cultures grown at low Fe' concentrations are thus most likely to record the $\Delta^{66}\text{Zn}$ fractionation of uptake, as little surplus Fe is available to form the Fe-hydroxide associated surface complexation sites to bind heavy Zn in the first place.

This does not mean that the same result could not be achieved by the removal of surface-bound Fe-hydroxides. While Fe-rich media certainly help to avoid cultures slipping into Fe-limitation, this approach could require potentially large corrections of the measured $\Delta^{66}\text{Zn}$. The narrow compositional interval between Fe-limitation and Fe concentrations high enough to trigger significant amounts of precipitates might be the ideal range to avoid any complication caused by surface-bound Fe-hydroxides. However, exactly when the phytoplankton of interest starts to become Fe-limited is often not available or difficult to obtain, and this bears the potential to

578 complicate the design of future culture experiments. Fe' levels below, but not too far away,
579 from the previously established threshold of 0.7 nmol l⁻¹ seem to be our currently best estimate
580 of an empirical Fe concentration that works for several different types of phytoplankton
581 (Köbberich and Vance, 2017; Sunda and Huntsman, 1995b; Sunda and Huntsman, 1997).

582 A newly developed 'inverse' approach suggests that the analysis of the contents of deliberately
583 lysed cells could represent an alternative method to assess intra-cellular metal/P ratios and
584 metal isotope compositions (Hudson and Morel, 1989; Twining et al., 2003). Although this
585 newly developed method is not able to quantitatively separate biological tissue from surface-
586 bound Fe-phases, biomass leachates could help to get around the need to quantitatively remove
587 all surface-bound Fe-hydroxides before intra-cellular ratios are experimentally accessible.
588 Rather simple improvements, such as longer contact times or agitation might help to obtain
589 quantitative recovery of the biomass in future experiments.

590 An important feature of the entire dataset reported here, similar to previous findings by John et
591 al. (2007), is that Zn sorption to surface-bound Fe-hydroxides can drive biomass $\delta^{66}\text{Zn}$
592 signatures up. The degree, to which this happens, however, depends on the absolute quantity of
593 Fe precipitated on the diatom surface. On the other hand, the loss of heavy Zn from the overall
594 experiment, as indicated by the isotopic mass balance in our experiments, and occurring via the
595 precipitation of Fe oxides followed by washing off material collected on filters, drives the
596 medium from which the cultured organisms are taking up Zn to isotopically light values. This
597 latter effect, if isotope fractionation during uptake is calculated relative to initial medium $\delta^{66}\text{Zn}$,
598 will cause biomass $\Delta^{66}\text{Zn}$ to be biased in the other direction, to negative values.

599

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768

769 ***Figure captions***

770 **Table 1.** Elemental and isotopic data for cultured marine *Thalassiosira* strains (this study),
 771 compared with literature data (John et al., 2007; Köbberich and Vance, 2017).

772 **Figure 1.** Schematic illustration of a newly developed method that aims to investigate whether
 773 the analysis of the products of lysed cells represents a viable alternative approach to the
 774 quantification of intra-cellular metal quotas. Whereas most previous procedures use strong
 775 reductive cleaning reagents for the removal of surface-bound metals (left), our novel approach
 776 is ‘inverse’ in its nature in that the soft-tissue of the diatom is extracted with a 70% EtOH
 777 solution. This leachate is then brought through the filtration membrane (right, green), leaving

778 behind a residue containing glassy diatom frustules, surface-bound Fe-hydroxides, and
779 transition metals potentially associated with either of these phases (right, red).

780 **Figure 2.** Bulk biomass-associated Fe/P ratios shown as a function of the total medium Fe
781 concentration. Previous observations presented by Tang and Morel (2006), establish the
782 framework we use to discuss our results. We distinguish between three different classes of Fe/P
783 ratios ranging from low, through intermediate, to high.

784 **Figure 3.** Comparison of metal (Me) to P ratios as obtained for three different *Thalassiosira*
785 strains cultured at intermediate and high total Fe concentrations. The results are reported as a
786 function of the free inorganic Fe concentration at the time of harvesting (Fe'_{RM}). This quantity
787 is not only important for Fe acquisition by marine diatoms, but also includes the aqueous
788 precursor species required for the nucleation of solid Fe-hydroxides. Above the Fe' threshold of
789 about 0.7 nmol l^{-1} , Fe precipitates are likely to occur on diatom surfaces and free in the
790 culturing medium. Color coding and symbol shape are the same as in Figure 2.

791 **Figure 4.** Zn isotope fractionation between biomass and the culturing medium ($\Delta^{66}\text{Zn}$), with
792 respect to the initial (A) and residual medium (B) at the time of harvesting, as observed for
793 three different *Thalassiosira* strains grown at intermediate (colored diamonds) and high Fe'
794 (black squares). Referenced to the residual medium the derived $\Delta^{66}\text{Zn}$ is shifted by about
795 $+0.14\text{‰}$ versus that referenced to the Zn isotope composition of the initial medium. Thus, the
796 $\Delta^{66}\text{Zn}$ values likely result in an over-estimate of biological fractionation at elevated Fe and low
797 EDTA levels, if the reference point is the starting medium. Above the Fe' threshold of about 0.7
798 nmol l^{-1} , Fe precipitates are likely to occur not only on diatom surfaces, but also free in the
799 culturing medium (*c.f.* Figure 3). Color coding and symbol shape are in the same as in Figure 2
800 and 3.

801 **Figure 5.** Whisker plot comparing the Zn isotope fractionation between the biomass and
802 culturing media ($\Delta^{66}\text{Zn}$) as found for *T. pseudonana* and *T. weissflogii* (left panel). Also shown
803 is the dependency of Zn isotope fractionation of *Thalassiosira* strains with variable Fe/P ratios
804 (right panel). On each box, the median is represented by the central mark (red), the 25th and 75th

percentiles are marked by the bottom and top edges of the colored box (blue or grey), and the most extreme data points are given as extended lines (black). $\Delta^{66}\text{Zn}$ is either defined as the difference between the biomass and the initial culture medium (grey whiskers at the bottom), or with respect to the residual medium (blue whiskers at the top).

Figure 6. Elemental results of intra-cellular metal quotas obtained by analyzing the products of lysed phytoplankton cells. Diatoms rinsed only with trace-metal-free equatorial Atlantic seawater serve as ‘control’ experiment, representing unwashed cells (A and B). The latter results are compared with the new approach, intentionally causing cell-lysis with a 70% EtOH solution (C to F). Note that for A – D the amount of each element found in the filtrate versus the residue are shown as proportions of the total in these two fractions. All residual media fractions, whether they belong to the control or inverse experiment, were found to be essentially identical in their chemical and isotope composition to the initial medium. The metal distribution and metal (Me) to P ratios as obtained for the filter residue (top row) and all filtrate solutions (bottom row) are shown as a function of the volume of 70% EtOH that was brought into contact with the cells. The error estimates were obtained with 6 full culturing replicates at a volume 5 ml, and were assumed also to be representative of samples measured at different volumes.

Figure 7. Whisker plot comparing the Zn isotope fractionation between biomass and the culturing medium ($\Delta^{66}\text{Zn}$) of the residue and filtrate fractions of EtOH extracted and seawater washed *T. weissflogii* cells (*c.f.* Figures 1 and 6). Residual media solutions have identical isotopic compositions to the initial medium and the system as a whole (see horizontal lines) because comparatively little Zn has been removed from the large initial medium pool. While the biomass is leached through the filter paper in the ‘inverse wash’, it remains as a residue on top of the filtration membrane in the case of the ‘control’ experiment. On each box, the median is represented by the central mark (red), the 25th and 75th percentiles are marked by the bottom and top edges of the box (blue), and the most extreme data points are given as extended lines (black).

Table 1

Fe/P	Species Strain	Initial medium (IM)					Residual medium (RM)					Biomass (B)					Reco- very ²	Δ ⁶⁶ Zn		Reference								
		[Fe] _{total} μM	Fe' _{IM} nM	EDTA μM	Zn ²⁺ nM	δ ⁶⁶ Zn ‰	2 σ ‰	[Fe] _{total} μM	Fe' _{RM} nM	Zn ²⁺ nM	f _{IM} ⁻¹ ‰	δ ⁶⁶ Zn ‰	2 σ ‰	Fe/P	Ni/P	Zn/P		f _{IM} ⁻¹ ‰	δ ⁶⁶ Zn ‰		2 σ ‰	%	%	B-IM ³	B-RM ⁴	%	%	
low	artificial seawater																											
		<0.15	0.056	97	0.10	0.08	0.04			0.10	0.95	0.11	0.05	washed with clean seawater											97	-0.09	-0.12	Köbberich & Vance (2017)
									0.10	0.95	0.13	0.04	3.5	0.03	2.10	0.02	-0.06	0.04	98	-0.14	-0.19							
	<i>T. oceanica</i>		0.084						0.10	0.96	0.12	0.05	5.5	0.02	2.64	0.04	-0.07	0.02	100	-0.16	-0.18							
	CCMP 1005		0.112						0.10	0.96	0.12	0.05																
intermediate	artificial seawater																											
		1.71	>0.7	8.2	0.24	-0.03	0.03	0.014	0.057	0.07	0.78	-0.21	0.04	122	0.05	16.2	0.16	-0.13	0.03	94	-0.11	0.07	This study					
								0.012	0.044	0.06	0.74	-0.21	0.04	135	0.04	21.9	0.22	-0.13	0.03	96	-0.10	0.08						
								0.009	0.032	0.05	0.69	-0.20	0.03	132	0.04	26.2	0.26	-0.21	0.03	95	-0.18	-0.01						
								0.009	0.035	0.06	0.75	-0.14	0.03	167	0.05	22.1	0.21	-0.15	0.03	96	-0.12	-0.01						
<i>T. pseudonana</i>							0.007	0.027	0.06	0.71	-0.21	0.04	149	0.04	24.4	0.27	-0.19	0.04	98	-0.16	0.02							
high	artificial seawater																											
		1.71	>0.7	8.2	0.24	-0.03	0.03	0.011	0.038	0.06	0.69	-0.18	0.04	156	0.04	26.7	0.26	-0.18	0.03	95	-0.15	0.00	This study					
								0.043	0.195	0.09	0.89	-0.16	0.04	207	0.05	10.3	0.07	-0.25	0.03	96	-0.22	-0.09						
								0.029	0.134	0.09	0.90	-0.18	0.04	163	0.08	10.6	0.08	-0.20	0.04	98	-0.17	-0.01						
								0.028	0.127	0.09	0.85	-0.15	0.04	180	0.05	11.3	0.08	-0.22	0.04	94	-0.19	-0.07						
<i>T. weissflogii</i>							0.043	0.202	0.09	0.89	-0.17	0.04	205	0.08	11.9	0.08	-0.21	0.03	96	-0.19	-0.04							
not reported	artificial seawater																											
		2.98	>0.7	15	0.06	-0.02	0.03	0.030	0.140	0.09	0.88	-0.13	0.04	232	0.06	13.9	0.12	-0.17	0.03	100	-0.14	-0.03	Köbberich & Vance (2017)					
								0.028	0.130	0.09	0.86	-0.18	0.04	201	0.07	12.7	0.09	-0.16	0.03	95	-0.13	0.02						
								washed with clean seawater																				
								0.868	1.82	0.04	0.97	-0.19	0.03	429	0.07	3.45	0.01	-0.17	0.06	98	-0.16	0.02						
<i>T. oceanica</i>							0.836	1.76	0.04	0.97	-0.18	0.03	382	0.08	3.41	0.01	-0.05	0.04	98	-0.03	0.14							
not reported	enriched natural seawater																											
		10						0.737	1.55	0.04	0.92	-0.20	0.02	377	0.08	4.17	0.01	0.06	0.06	93	0.08	0.26	John et al. (2007)					
								1.014	2.13	0.04	0.97	-0.16	0.03	379	0.09	3.50	0.01	-0.01	0.05	98	0.01	0.15						
								0.816	1.72	0.04	0.97	-0.18	0.03	376	0.10	3.58	0.01	-0.27	0.04	98	-0.25	-0.09						
								0.817	1.72	0.05	0.98	-0.20	0.04	315	0.08	3.70	0.01	-0.06	0.04	99	-0.04	0.14						
<i>T. oceanica</i>							reductively cleaned cells ⁵																	-0.41	-0.16			
not reported	unwashed biomass																											

¹Fraction of the initially added Zn as measured after the experiment, ²Zn recovery as obtained by the sum of the residual medium and the biomass, ³Δ⁶⁶Zn of the biomass minus the initial medium (B-IM), ⁴Δ⁶⁶Zn of the biomass minus the residual medium (B-RM), ⁵The reductive removal of surface bound Fe-hydroxides was performed after a protocol originally suggested by Tovar- Sanchez et al. (2003).

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Fe/P	<i>Species</i> Strain	Initial medium (IM)						Residual medium (RM)						Biomass (B)						$\Delta^{66}\text{Zn}$		Reference
		[Fe] _{total}	Fe' _{IM}	EDTA	Zn ²⁺	$\delta^{66}\text{Zn}$	2 σ	[Fe] _{total}	Fe' _{RM}	Zn ²⁺	f_{IM}^1	$\delta^{66}\text{Zn}$	2 σ	Fe/P	Ni/P	Zn/P	f_{IM}^1	$\delta^{66}\text{Zn}$	2 σ	Recovery ²	B-IM ³	B-RM ⁴
		μM	nM	μM	nM	‰	‰	μM	nM	nM		‰	‰	mmol	mol ⁻¹	...		‰	‰	%	‰	‰
low		artificial seawater												washed with clean seawater								Köbberich & Vance (2017)
		<0.15	0.056	97	0.10	0.08	0.04			0.10	0.95	0.11	0.05	2.0	0.02	1.89	0.02	-0.01	0.06	97	-0.09	-0.12
	<i>T. oceanica</i>		0.084			0.08	0.04			0.10	0.95	0.13	0.04	3.5	0.03	2.10	0.02	-0.06	0.04	98	-0.14	-0.19
	CCMP 1005		0.112			0.09	0.05			0.10	0.96	0.12	0.05	5.5	0.02	2.64	0.04	-0.07	0.02	100	-0.16	-0.18
intermediate		artificial seawater												washed with clean seawater								This study
		1.71	>0.7	8.2	0.24	-0.03	0.03	0.014	0.057	0.07	0.78	-0.21	0.04	122	0.05	16.2	0.16	-0.13	0.03	94	-0.11	0.07
								0.012	0.044	0.06	0.74	-0.21	0.04	135	0.04	21.9	0.22	-0.13	0.03	96	-0.10	0.08
								0.009	0.032	0.05	0.69	-0.20	0.03	132	0.04	26.2	0.26	-0.21	0.03	95	-0.18	-0.01
								0.009	0.035	0.06	0.75	-0.14	0.03	167	0.05	22.1	0.21	-0.15	0.03	96	-0.12	-0.01
	<i>T. pseudonana</i>							0.007	0.027	0.06	0.71	-0.21	0.04	149	0.04	24.4	0.27	-0.19	0.04	98	-0.16	0.02
	CCMP 1015							0.011	0.038	0.06	0.69	-0.18	0.04	156	0.04	26.7	0.26	-0.18	0.03	95	-0.15	0.00
		artificial seawater												washed with clean seawater								This study
		1.71	>0.7	8.2	0.24	-0.03	0.03	0.043	0.195	0.09	0.89	-0.16	0.04	207	0.05	10.3	0.07	-0.25	0.03	96	-0.22	-0.09
								0.029	0.134	0.09	0.90	-0.18	0.04	163	0.08	10.6	0.08	-0.20	0.04	98	-0.17	-0.01
								0.028	0.127	0.09	0.85	-0.15	0.04	180	0.05	11.3	0.08	-0.22	0.04	94	-0.19	-0.07
								0.043	0.202	0.09	0.89	-0.17	0.04	205	0.08	11.9	0.08	-0.21	0.03	96	-0.19	-0.04
	<i>T. weissflogii</i>							0.030	0.140	0.09	0.88	-0.13	0.04	232	0.06	13.9	0.12	-0.17	0.03	100	-0.14	-0.03
	CCMP 1336							0.028	0.130	0.09	0.86	-0.18	0.04	201	0.07	12.7	0.09	-0.16	0.03	95	-0.13	0.02
high		artificial seawater												washed with clean seawater								Köbberich & Vance (2017)
		2.98	>0.7	15	0.06	-0.02	0.03	0.868	1.82	0.04	0.97	-0.19	0.03	429	0.07	3.45	0.01	-0.17	0.06	98	-0.16	0.02
								0.836	1.76	0.04	0.97	-0.18	0.03	382	0.08	3.41	0.01	-0.05	0.04	98	-0.03	0.14
								0.737	1.55	0.04	0.92	-0.20	0.02	377	0.08	4.17	0.01	0.06	0.06	93	0.08	0.26
								1.014	2.13	0.04	0.97	-0.16	0.03	379	0.09	3.50	0.01	-0.01	0.05	98	0.01	0.15
	<i>T. oceanica</i>							0.816	1.72	0.04	0.97	-0.18	0.03	376	0.10	3.58	0.01	-0.27	0.04	98	-0.25	-0.09
	CCMP 1005							0.817	1.72	0.05	0.98	-0.20	0.04	315	0.08	3.70	0.01	-0.06	0.04	99	-0.04	0.14
not reported		enriched natural seawater																				John et al. (2007)
		10			0.10									reductively cleaned cells ⁵							-0.41	
					0.03																-0.16	
	<i>T. oceanica</i>				0.10									unwashed biomass							0.22	
	CCMP 1005				0.03																0.15	

¹Fraction of the initially added Zn as measured after the experiment, ²Zn recovery as obtained by the sum of the residual medium and the biomass, ³ $\Delta^{66}\text{Zn}$ of the biomass minus the initial medium (B-IM), ⁴ $\Delta^{66}\text{Zn}$ of the biomass minus the residual medium (B-RM), ⁵The reductive removal of surface bound Fe-hydroxides was performed after a protocol originally suggested by Tovar- Sanchez et al. (2003).

Control

20 ml

Inverse wash

20 ml

Fractions

Biomass

Frustule + adsorbed

2. Residual medium

3. Rinse

4. Filtrate

Surface wash

Biomass

70 % ethanol solution

CCMP strain

1015
n = 6

1336
n = 6

1005
n = 3

1015/1336
n = 12

1005
n = 6