



# Distinguishing between cellular and Fe-oxide-associated trace elements in phytoplankton

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## Abstract

In cultures and in nature, ferric (oxyhydro-)oxides ( $\text{FeO}_x$ ) precipitate and become associated with phytoplankton surfaces. Other trace elements adsorb on  $\text{FeO}_x$  and it is thus difficult to differentiate between cellular- and oxide-associated concentrations of both iron and these elements. Existing techniques to selectively dissolve the  $\text{FeO}_x$  associated with phytoplankton surfaces often contaminate the sample or necessitate elaborate pre-cleaning procedures and/or proceed by unknown and thus uncontrolled mechanisms.

Here we examine the efficacy of various washing techniques, the mechanisms effecting  $\text{FeO}_x$  dissolution, and the methods for controlling contamination from the wash solutions. Solutions containing a single chelating agent are ineffective at dissolving  $\text{FeO}_x$ . A wash solution containing two types of chelating agents, oxalate and EDTA, is effective for dissolving fresh precipitates via a ligand-promoted process, in which oxalate and EDTA function synergistically. This is in contrast with a Ti-citrate-EDTA wash technique, which effectively dissolves fresh or aged  $\text{FeO}_x$  by a reductive mechanism. Contaminating trace elements in the wash solutions that are complexed by excess EDTA can be effectively eliminated from filters by appropriate rinsing with a clean NaCl solution. For those elements that are not bound by excess EDTA, it is necessary to add specific chelating agents to the wash solution and, in some cases, to pre-clean the reagents.

We show that under defined culture conditions, Ba and V in the diatom *Thalassiosira weissflogii* are mostly adsorbed on the extracellular  $\text{FeO}_x$  so that their apparent cellular concentrations increase with the concentration of Fe in the culture medium. In contrast, the cellular concentrations of Cu, Zn, Co, Cd, and Mn, which are dominated by their intracellular pools, are independent of the Fe concentration in the medium and can be measured directly on rinsed filters without dissolving the  $\text{FeO}_x$ .  
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## 1. Introduction

There is currently great interest in the iron and other trace metal nutrition of marine phytoplankton

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which is being studied in laboratory cultures and in the field. But in many culture media, ferric (oxyhydro-)oxide ( $\text{FeO}_x$ ) is supersaturated and precipitates on cell surfaces. In the surface waters of the oceans, a large fraction of Fe is in colloidal form (Wu et al., 2001) and an unknown fraction is thus collected along with phytoplankton samples. Other solutes potentially adsorb on the extracellular  $\text{FeO}_x$  (Dzombak and Morel, 1990) and this adsorption may account for a large fraction of the “cellular” concentrations that are measured, as recently demonstrated for barium in cultures of the diatom *Thalassiosira weissflogii* (Sternberg et al., 2005). An understanding of the trace requirements in phytoplankton, of the uptake mechanisms of trace elements, or of their geochemistries requires that we be able to distinguish between the cellular and Fe-oxide-associated concentrations of bioactive trace elements in the laboratory and in the field.

Several washing techniques have been used to selectively dissolve the  $\text{FeO}_x$  attached to phytoplankton cell surfaces. These typically involve chelating agents such as EDTA (ethylenediaminetetraacetic acid) or DTPA (diethylenetriaminepentaacetic acid) (Knauer et al., 1997; Hutchins et al., 1999; Chang and Reinfelder, 2000), or reductants, such as Ti(III) or ascorbic acid (Anderson and Morel, 1982; Hudson and Morel, 1990). Although widely used, wash solutions containing a single chelator have been found ineffective at dissolving  $\text{FeO}_x$  and associated trace metals (Hutchins et al., 1999). In contrast, a wash solution containing a citrate–Ti(III)–EDTA tertiary

## 2.2. Culture medium

Cells were cultured in Gulf Stream water (GSW) enriched with filter-sterilized nutrients, vitamins and trace metals based on the Aquil recipe (Price et al., 1988/1989). All labware were soaked in 5% detergent overnight, followed by 10% HCl overnight and finally rinsed with Milli-Q water. The GSW was filtered through a 0.2  $\mu\text{m}$  filter cartridge and microwave sterilized. The final concentrations of nitrate, phosphate and silicate were 150, 10, and 100  $\mu\text{M}$ , respectively. In the GSW medium, Fe levels were varied and other trace metals were added at total concentrations of:  $\text{Mn}_T=120$  nM,  $\text{Cu}_T=20$  nM,  $\text{Zn}_T=80$  nM,  $\text{Co}_T=50$  nM,  $\text{Mo}_T=100$  nM,  $\text{Se}_T=10$  nM. All cultures contain 100  $\mu\text{M}$  EDTA to buffer trace metals at the following unchelated concentrations:  $\text{Mn}'=10$  nM,  $\text{Cu}'=0.2$  pM,  $\text{Zn}'=12$  pM, and  $\text{Co}'=17$  pM (Sunda et al., 2005). In this culture medium, Fe precipitation may occur at a total concentration as low as 80 nM, corresponding to unchelated  $\text{Fe}' \approx 0.16$  nM, depending on the temperature, pH and light regime of the culture (Hudson and Morel, 1990; Ho et al., 2003; Sunda and Huntsman, 2003).

## 2.3. Culture methods

Laboratory studies were conducted with the marine diatom *T. weissflogii*, CCMP 1336 (CCMP, Bigelow, Maine, USA). *T. weissflogii* cells were maintained in the enriched Gulf Stream water with 304 nM Fe. Prior to experiments, cells were transferred to the growth medium with different Fe levels and grown under constant light (150  $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ ) at 20 °C. The growth rates were monitored daily with a Coulter Multisizer. Generally, 120 ml of diatom culture at mid-exponential phase (usually 5 days after inoculation) were filtered through an acid-cleaned polycarbonate filter (25 mm in diameter, 5  $\mu\text{m}$  pore size) placed in acid-cleaned polypropylene filter holder (Fisher), and washed according to the experimental design. Filters with cells were then placed into 10 ml Teflon tubes and digested as described in Section 2.4.

Algal cell membrane integrity upon washes was tested using methylamine (Hudson and Morel, 1989). In this method, *T. weissflogii* cells, grown at

$\text{Fe}_T=304$  nM, were exposed to  $^{14}\text{C}$ -labeled methylamine for two h. The cells were then filtered, re-suspended in GSW, and half the re-suspended culture was treated with 0.3% glutaraldehyde. After one h incubation, the cells were filtered in triplicate and washed with either the NaCl solution, the oxalate–EDTA solution (pH 7.07), or the Ti–citrate–EDTA reagent. The  $^{14}\text{C}$  activity was counted with a scintillation counter (Beckman Coulter LSC6500), and activity readings were normalized to that from the live cells after the NaCl rinse. Concomitantly, elemental quotas from a parallel experiment were also measured with an additional treatment of heat-killing (70 °C, 10 min).

To test the extent of Fe(III) reduction upon applying the wash solutions, ferrozine (FZ: 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-*p,p'*-disulfonic acid) was added directly in the wash solutions to extract any Fe(II) produced. In one experiment with a culture grown at  $\text{Fe}_T=840$  nM spiked with radioactive  $^{59}\text{Fe}$ , the production of radioactive Fe(II) was detected according to the method reported by Shaked et al. (2004), using 50 mM of FZ. In this method, the Fe(II)–FZ<sub>3</sub> complexes and the labile Fe(III) were extracted by Sep-Pak C18 cartridges and subsequently a mild acid wash was applied to separate them. The  $^{59}\text{Fe}$  radioactive activities for both Fe(III) and Fe(II) were measured using  $\gamma$ -counting. In a separate experiment, we measured the Fe(II) production from abiotic precipitation of  $\text{FeO}_x$  in the absence of cells. The precipitates were formed by the addition of 2  $\mu\text{M}$   $\text{FeCl}_3$  in GSW and aging for 1 day. After filtration through a 0.2  $\mu\text{m}$  polycarbonate filter, filters with the collected  $\text{FeO}_x$  precipitates were placed in test tubes and washing solutions were added in the presence of 50 mM FZ. The Fe(II) produced from the reduction of  $\text{FeO}_x$  in the wash solutions was measured by spectrophotometry at a wavelength of 566 nm for the Fe(II)–FZ<sub>3</sub> complex and corrected for the formation of the Fe(II)–EDTA complex. For the Ti–citrate–EDTA wash, the reaction solution was bubbled with air to oxidize the Ti(III) in the solution (which becomes clear) before spectrophotometric measurement of the Fe(II)–FZ<sub>3</sub> complex.

## 2.4. Analysis

The elemental concentrations of trace elements were determined using a magnetic sector inductively

coupled plasma mass spectrometer (Element2, Thermo Finnigan, Bremen, Germany). Sample digestion and instrumental settings were similar to those reported by Cullen et al. (2001). For digestion, filters with algal cells were placed in 10-ml Teflon tubes and 800  $\mu\text{l}$  of 50%  $\text{HNO}_3$  (Fisher Optima Grade) were added. The tightly capped tubes were heated below the boiling point for 4 h. The tubes were then brought up to a final volume of 8 ml using Milli-Q water and centrifuged (3000 rpm for 10 min at 20  $^\circ\text{C}$ ) to separate the silica frustules from the acid-soluble fraction. The supernatant was spiked with Sc and In as internal standards (2  $\mu\text{g l}^{-1}$  each) and introduced with an autosampler (Cetac ASX-100) placed in a class-100 clean bench. A Teflon PFA free-aspirating nebulizer ( $\mu\text{Flow-100}$ , Elemental Scientific) was used with a Scott double-bypass spray chamber coupled with a PFA end cap for sample introduction. The measurement was conducted in the medium resolution mode for all the elements but Se, for which the high-resolution mode was used. Procedural blanks for filters were obtained from filtering Gulf Steam water medium as described above for cultures. The instrument was calibrated with diluted, certified standards (High Purity Standards) in 5%  $\text{HNO}_3$  (Fisher Optima Grade). Direct measurement of standard river water (SLRS-4, National Research Council Canada) ensured detection accuracy after its acid content was raised to 5% with  $\text{HNO}_3$ .

### 3. Results and discussion

#### 3.1. Choice of wash solutions

In addition to the commonly used technique of washing cells with a solution of a single strong complexing agent like EDTA (Hassler et al., 2004), there have been two main techniques used to dissolve the  $\text{FeO}_x$  associated with phytoplankton and to measure the true “cellular” concentrations of various elements: i) the Ti–citrate–EDTA wash solution, which uses a ternary complex of Ti(III) with citrate and EDTA (Hudson and Morel, 1989) and ii) the oxalate–EDTA–citrate wash, which has a similar composition with replacement of Ti(III) by oxalate (Tovar-Sanchez et al., 2003). The oxalate–EDTA–citrate solution is amenable to thorough pre-cleaning by a solvent extraction procedure.

Using cultures of the diatom *T. weissflogii*, grown at various total Fe concentrations, we first tested the efficacy of wash solutions containing oxalate only, EDTA only, and oxalate and EDTA together and compared the results with those obtained using the Ti–citrate–EDTA wash and a simple NaCl rinse (Fig. 1). The apparent cellular Fe concentrations measured after either the oxalate-only (100 mM) or EDTA-only (50 mM) washes were similar and both were lower than the concentrations obtained after the NaCl rinse. The oxalate–EDTA and Ti–citrate–EDTA washes yielded similar cellular Fe concentrations that were

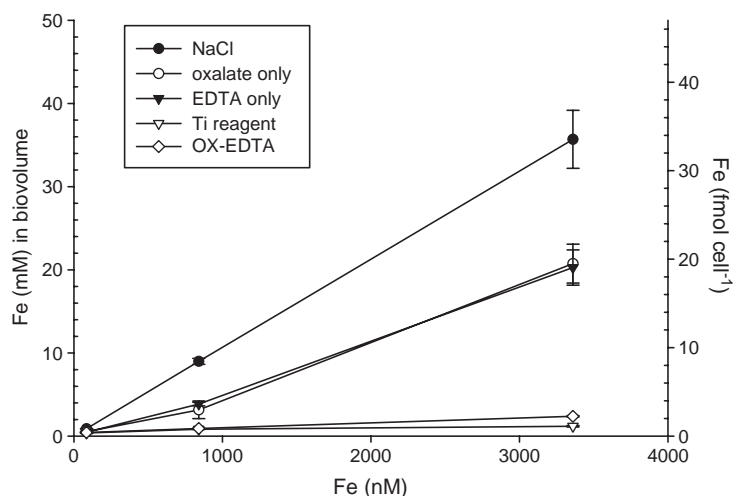


Fig. 1. Measured Fe quotas in *T. weissflogii* after different washes as a function of the total Fe concentration in the growth medium.

significantly lower than those from the oxalate-only or EDTA-only washes. Thus, while oxalate or EDTA alone is not effective at dissolving  $\text{FeO}_x$  in these samples, the combination of the two works quite well. Only at the highest Fe concentration used were the values for the Ti-citrate-EDTA wash lower than those for the oxalate-EDTA wash (Fig. 1).

The reagent proposed by Tovar-Sanchez et al. (2003) substitutes oxalate for Ti(III) in the original Ti-citrate-EDTA solution proposed by Hudson and Morel (1989). EDTA and citrate which are no longer necessary to stabilize Ti(III) are retained because they might increase the removal efficiency of the reagent for metals. We found, however, that the presence of citrate had no significant effect on the effectiveness of the oxalate-EDTA-citrate wash (Table 1), thus citrate was omitted from the oxalate-EDTA-citrate solution in our study. The elimination of citrate also decreases the concentrations of the contaminants from the wash solution by about a factor of two (data not shown).

It appears that the oxalate-EDTA wash is not quite as effective as the Ti-citrate-EDTA wash in dissolving extracellular  $\text{FeO}_x$  as shown by the results obtained with cells grown at the highest Fe concentration (3.36  $\mu\text{M}$ ; Fig. 1). This was confirmed by comparing the abilities of these two wash solutions to dissolve an  $\text{FeO}_x$  sample precipitated in the absence of cells (1  $\mu\text{M}$   $\text{FeCl}_3$  addition in GSW), heated at 70 °C for 20 min and stored for 14 h before filtration (Fig. 2). Ten percent of the iron was retained on the filter after the oxalate-EDTA wash, compared to only 0.4% for the Ti-citrate-EDTA wash (and 65% after the NaCl rinse, showing that not all the  $\text{FeO}_x$  is retained by the filter).

### 3.2. Mechanisms of $\text{FeO}_x$ dissolution

The Ti-citrate-EDTA wash was designed as a mildly reducing solution that would be effective in

Table 1

Comparison of Fe:P ratios and P quotas measured in *T. weissflogii* (grown at  $\text{Fe}_T=840$  nM) after washing with oxalate-EDTA with and without citrate at pH 8.1

	Fe:P ( $\text{mmol mol}^{-1}$ )		P (mM)	
	-Citrate	+Citrate	-Citrate	+Citrate
Light	38.7 ( $\pm 1.6$ )	31.1 ( $\pm 6.5$ )	30.2 ( $\pm 2.2$ )	37.6 ( $\pm 2.0$ )
Dark	29.7 ( $\pm 1.1$ )	26.7 ( $\pm 2.7$ )	40.6 ( $\pm 2.5$ )	45.6 ( $\pm 2.5$ )

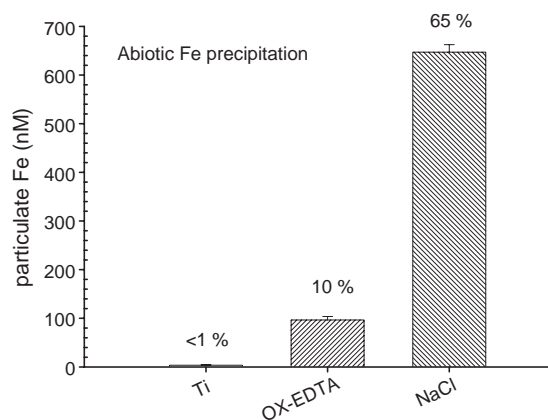


Fig. 2. Concentrations of particulate  $\text{FeO}_x$  remaining on 0.2  $\mu\text{m}$  filters after various washes (the percent value on top of each bar represents the calculated retention of Fe on the filter compared with the total Fe added).

dissolving  $\text{FeO}_x$  by reduction of Fe(III) to Fe(II) (Hudson and Morel, 1989). Tovar-Sanchez et al. (2003) implied that their oxalate wash also dissolves the  $\text{FeO}_x$  associated with cell surfaces by a reductive mechanism, an implication fully accepted recently by Sanudo-Wilhelmy et al. (2004). Fe(III)-citrate and Fe(III)-oxalate are photoreactive and both ligands can reduce iron oxide in the light but not in the dark (Waite and Morel, 1984; Sulzberger et al., 1989) and only at low pH does the photo-reductive dissolution of Fe-oxides outcompete the re-oxidation of Fe(II) (Sulzberger and Laubscher, 1995). It is thus unlikely that the dissolution of iron oxide would result from photo-reduction at the high pH and low light intensity that prevail in the filtering apparatus during the wash. Indeed, the measured Fe quotas of *T. weissflogii* were similar to each other when the washes were conducted in the dark or under room light (Table 1). In view of its efficacy with pure  $\text{FeO}_x$  suspensions (Fig. 2), we also know that the oxalate-EDTA wash is effective in the absence of reductant (as might be provided by cellular exudates). A reductive process is thus very unlikely. This was confirmed by measuring the extent of Fe(II) production during the washing process, by using the ferrozine trapping technique on  $\text{FeO}_x$  precipitated in the absence of cells (2  $\mu\text{M}$   $\text{FeCl}_3$  in GSW). Less than 1% of the Fe was measured as Fe(II) as a result of the oxalate-EDTA wash (at pH 7) or the oxalate-EDTA + citrate wash (at pH 8) while the Ti-citrate-EDTA reagent (at pH 8) reduced at

least 75% of the Fe(III) in the system (Fig. 3). Using the trapping and column extraction method of Shaked et al. (2004), we obtained a similar result in the presence of cells: a negligible fraction of the Fe dissolved by the oxalate–EDTA wash from cells grown at  $Fe_T=840$  nM (and spiked with  $^{59}Fe$ ) was measured as Fe(II). Thus, as expected, the Ti–citrate–EDTA wash promotes  $FeO_x$  dissolution by Fe(III) reduction, but the oxalate–EDTA (or oxalate–EDTA–citrate) wash does not.

The only possible mechanism for  $FeO_x$  dissolution by the oxalate–EDTA wash must be a ligand-promoted process. Cheah et al. (2003) have documented a synergistic effect of oxalate and desferrioxamine B (DFB) in the dissolution of goethite, where the presence of the siderophore increased the rate of dissolution by oxalate by an order of magnitude. The proposed mechanism involves the formation of an Fe(III)–oxalate complex at the surface, followed by a transfer of Fe(III) from oxalate to DFB either through formation of a surface ternary complex or by ligand exchange in solution after detachment of the Fe(III)–oxalate complex. The key to such a synergistic effect between two ligands is that one ligand (oxalate) adsorbs effectively on the Fe(III) oxide at the pH of interest and that the other (DFB) is an effective scavenger of Fe(III). We postulate that a similar mechanism explains the strong synergistic effects of

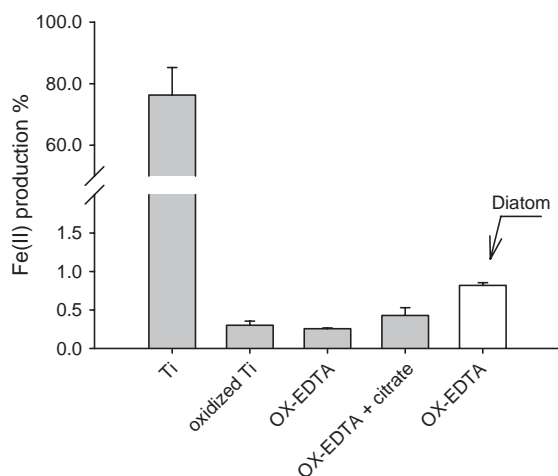


Fig. 3. Fe(II) production from various washing techniques of  $FeO_x$  precipitated in absence of cells (gray bars; 2  $\mu$ M addition of  $FeCl_3$  in Gulf Stream water) and in a *T. weissflogii* culture (white bar; cells grown at  $Fe_T=840$  nM spiked with  $^{59}Fe$ ).

oxalate and EDTA in the dissolution of  $FeO_x$  from phytoplankton surfaces (Fig. 1), with EDTA acting as a strong scavenger of Fe(III).

### 3.3. Optimization

The kinetics of  $FeO_x$  dissolution by the Ti–citrate–EDTA wash technique have been well explored by Hudson and Morel (1989) and a wash time of 2 min has been found effective. In contrast, the oxalate–EDTA wash is not always sufficiently effective at dissolving  $FeO_x$  (Figs. 1 and 2). Therefore, we performed experiments to see if the effectiveness of the oxalate–EDTA wash could be increased and to quantify how much time was necessary to dissolve  $FeO_x$ .

According to the two-ligand mechanism, the rate of  $FeO_x$  dissolution by the oxalate–EDTA wash should increase with the adsorbed oxalate concentration and be relatively independent of the EDTA concentration, as long as it is high enough to scavenge all the dissolved Fe(III) in the system (Cheah et al., 2003). Published data on oxalate adsorption on goethite show that the extent of adsorption decreases sharply as pH increases (Eick et al., 1999). Thus, we maintained high reagent concentrations in our wash solution and performed a series of experiments with filtered *T. weissflogii* cells from cultures grown at various Fe concentrations, varying the pH and the duration of the washing step (Fig. 4). As seen in Fig. 4A, the measured cellular Fe concentration increased with the total Fe in the medium. At high Fe concentrations, the effectiveness of the oxalate–EDTA wash in dissolving  $FeO_x$  decreased sharply above pH=7.5. At pH=7, the maximum Fe dissolution (minimum apparent Fe quota) was obtained in 10 min or less at all but the highest Fe concentration tested (Fig. 4B). Two consecutive 5-min washes should thus be generally sufficient to obtain reasonable results at pH 7 with the oxalate–EDTA wash. However, under conditions where the Fe concentration is high or the  $FeO_x$  precipitate is less labile, longer wash times or lower pHs are needed.

### 3.4. Cell membrane integrity

To determine whether the wash techniques cause membrane damage and intracellular material leakage, we followed the method used by Hudson and Morel

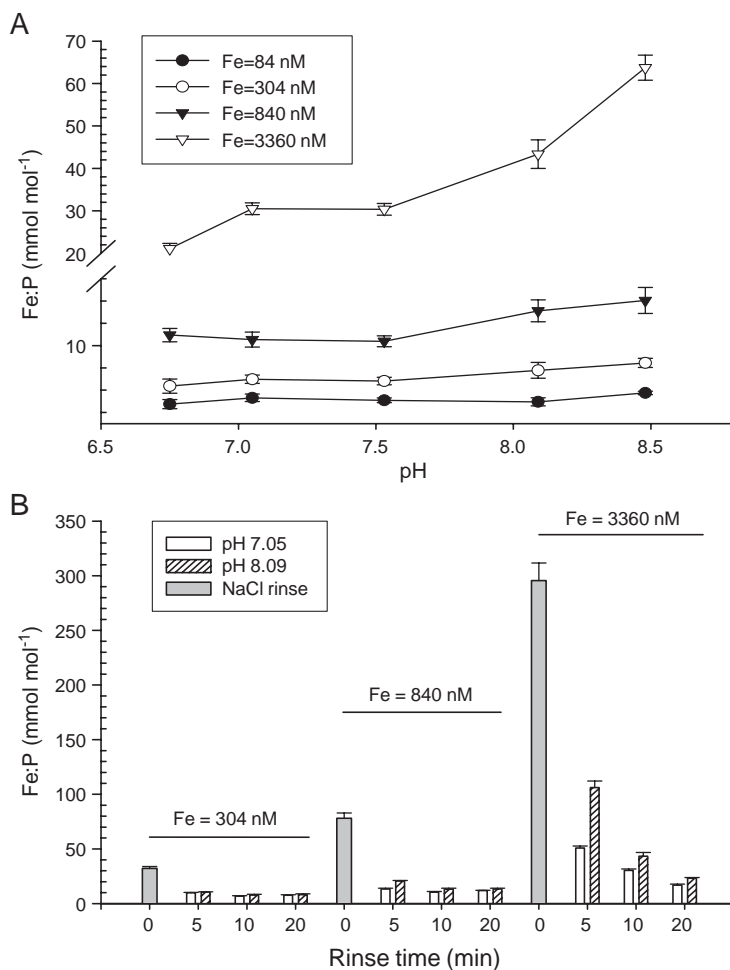


Fig. 4. Effect of pH and washing time on the measured cellular Fe:P quotas after oxalate–EDTA wash of *T. weissflogii* cells grown at various Fe concentrations: (A) effect of pH for a 10-min wash and (B) effect of wash time at two different pHs.

(1989) to measure the cellular retention of  $^{14}\text{C}$ -labeled methylamine, a substrate that is taken up but not assimilated by diatoms (Wheeler, 1979). Both the Ti–citrate–EDTA and the oxalate–EDTA washes were tested in parallel with *T. weissflogii* cells (grown with 304 nM Fe) that were either untreated (live cells) or fixed with glutaraldehyde (0.3%). We found little loss of methylamine from live cells in all treatments, indicating no membrane damage resulted from these washes (Fig 5). However, a 90% loss of methylamine was observed as a result of fixation with glutaraldehyde. It is notable that the cell leakage caused by glutaraldehyde also resulted in about a 50% loss of cellular P but negligible loss of Fe,

consistent with the fact that a large fraction of P is contained in the soluble cellular pool but that the filtered Fe concentration is dominated by the  $\text{FeO}_x$  on the surface of the cells.

### 3.5. Contamination from wash solutions

High contaminant concentrations of many elements of interest are found in the oxalate–EDTA and, even more so, in the Ti–citrate–EDTA wash solutions (but not in the NaCl rinse solution; Table 2). This is a major reason why the Ti solution has been mainly used in radioactive tracer studies and why Tovar-Sanchez et al. (2003) looked for an alternative

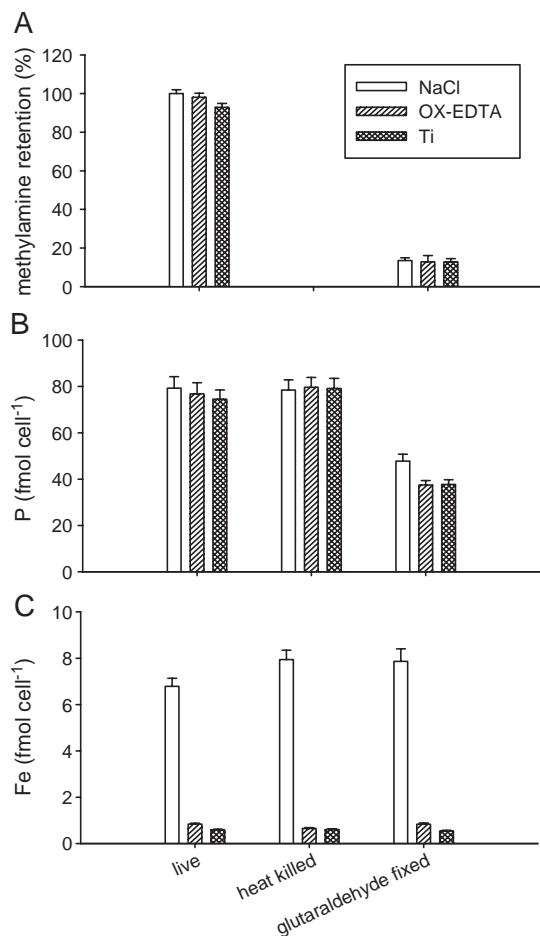


Fig. 5. Retention of  $^{14}\text{C}$ -methylamine and cellular P and Fe quotas in live and dead *T. weissflogii* cells after different washing treatments.

and developed stringent pre-cleaning procedures for their oxalate–EDTA–citrate solution. In the presence of excess chelator, however, the metal contaminants introduced by the wash solution should remain bound as hydrophilic species and be removable by sufficient rinsing with a clean salt solution.

Indeed, extensive rinsing of filters after passing 2.8 ml of the oxalate–EDTA wash solution ( $5 \times 1.5$  ml NaCl solution rinse) gave blanks that were similar to those from NaCl rinsing only (Table 3) and comparable with those reported using ultra-clean techniques (Cullen and Sherrell, 1999). The filter blanks measured after the 1.4 ml Ti–citrate–EDTA wash and extensive rinsing ( $10 \times 1.5$  ml NaCl) were also low but not quite as good for Co, Cu, and Zn. Good blanks

were obtained using a Ti–citrate–EDTA+ wash solution containing extra EDTA (75 mM total, see Section 2.1) after a simple cleaning step and with addition of BCDS (see below). All these filter blanks are much lower than the values from a sample of  $5 \times 10^6$  cells (i.e., 120 ml of a mid exponential phase culture; approximately 1.2 mg dry weight) grown at low Fe concentration (84 nM) and rinsed with NaCl only.

To see if extensive rinsing is effective to remove contaminants from cells as well as filters, we measured the cellular quotas using both wash techniques and the same extensive rinses that had been found effective for filter blanks. The results are exemplified for Zn, the worst contaminant in the wash reagents. Similar cellular Zn quotas were obtained by both the Ti–citrate–EDTA ( $\text{Zn:P} = 0.38 \pm 0.04 \text{ mmol mol}^{-1}$ ) and the oxalate–EDTA ( $\text{Zn:P} = 0.40 \pm 0.07 \text{ mmol mol}^{-1}$ ) techniques for cells grown at high Fe concentration (840 nM). These quotas are similar to that measured for cells grown at low Fe concentration and rinsed with NaCl only ( $\text{Zn:P} = 245/477\,000 = 0.51 \text{ mmol mol}^{-1}$ ; Table 3). As shown later, the Fe concentration in the medium has little or no influence on the Zn quota and these values are thus accurate. Note that the value obtained by NaCl rinse of a low Fe culture is a bit higher than those obtained by Ti–citrate–EDTA and oxalate–EDTA washes; we attribute the difference to a small but significant concentration of metal attached to the surface of the cells (but not to the  $\text{FeO}_x$ ). In this view, the values obtained from the Ti–citrate–EDTA or oxalate–EDTA wash are intracellular concentrations.

Table 2  
Typical contamination levels in wash solutions<sup>a</sup>

Element (nM)	Wash solution		
	NaCl	Oxalate–EDTA	Ti–citrate–EDTA <sup>+</sup>
Cd	0.09 (0.10)	0.18 (0.31)	2.29 (0.22)
Co	0.2 (0.3)	11.5 (1.6)	50.5 (1.0)
Cu	3.5 (1.3)	49.5 (14.8)	1230 (48)
Fe	2.3 (1.6)	338.8 (45.3)	6840 (90)
Mn	3.3 (1.8)	25.8 (2.1)	665.5 (2.0)
P	80 (37)	327 (110)	7160 (115)
Sr	210 (1)	242 (10)	319 (4)
Zn	5.4 (2.4)	39.1 (8.2)	65\,700 (1450)

Values are average from triplicates with standard deviation in parenthesis.

<sup>a</sup> See text for the definition of oxalate–EDTA and Ti–citrate–EDTA+–plus solutions.

Table 3

Filter blanks from NaCl, oxalate–EDTA, and Ti–citrate–EDTA washes<sup>a</sup> and comparison with published filter blanks and a typical culture of *T. weissflogii* (unit: pmol filter<sup>-1</sup>)

	Osmonics Poretics (batch #1) blank		Osmonics Poretics (batch #2) blank			Osmonics Poretics <sup>b</sup> blank (Cullen and Sherrell, 1999)	Total biomass <sup>c</sup> ( $5.2 \times 10^6$ cells, ca 1.2 mg d.w.)
	NaCl rinse <sup>d</sup>	oxalate–EDTA <sup>e</sup>	NaCl rinse <sup>d</sup>	Ti–citrate–EDTA wash <sup>f</sup>	Ti–citrate–EDTA+ wash <sup>g</sup>		
Al	53 (1)	67 (1)	148 (3)	134 (2)	142 (1)	141 (64)	3880 (118)
Ba	0.35 (0.01)	0.43 (0.01)	0.23 (0.01)	0.29 (0.01)	0.12 (0.01)		0.41 (0.03)
Cd	0.075 (0.013)	0.073 (0.008)	0.086 (0.022)	0.072 (0.016)	0.072 (0.010)	0.112 (0.022)	3.3 (0.3)
Co	0.33 (0.01)	0.33 (0.01)	0.45 (0.02)	0.74 (0.02)	0.36 (0.02)	0.19 (0.06)	30.8 (1.2)
Cu	3.46 (0.06)	3.81 (0.08)	13.43 (0.93)	22.87 (0.86)	16.41 (0.01)	4.10 (2.21)	62.6 (2.7)
Fe	63 (1)	82 (1)	102 (2)	78 (2)	81.4 (0.4)	68 (33)	3730 (75)
Mn	1.53 (0.03)	1.74 (0.03)	3.40 (0.15)	2.36 (0.13)	1.09 (0.01)	1.83 (0.77)	1790 (18)
Mo	0.93 (0.04)	0.60 (0.02)	0.95 (0.11)	1.07 (0.14)	0.47 (0.01)		6.30 (0.49)
P	118 (1)	107 (2)	310 (16)	316 (12)	220 (3)	124 (27)	477 000 (8060)
Se	1.1 (0.4)	0.9 (0.3)	2.0 (1.1)	1.5 (1.0)	1.5 (0.9)		88.9 (14.2)
Sr	16.4 (0.1)	14.4 (0.2)	33.0 (0.7)	12.4 (0.4)	3.22 (0.01)		130.6 (5.5)
V	0.22 (0.02)	0.17 (0.01)	0.35 (0.07)	0.19 (0.11)	0.20 (0.01)		11.6 (1.0)
Zn	7.56 (0.18)	6.82 (0.16)	8.27 (1.12)	16.67 (1.98)	8.90 (0.03)	6.99 (2.53)	245 (8)

<sup>a</sup> Average values from individual measurements of 6 polycarbonate filters (25 mm in diameter, 5  $\mu$ m pore size; each measured 12 times) with standard deviation in parenthesis.

<sup>b</sup> Calculated from the 47 mm polycarbonate filter (Osmonics Poretics), reported in Table 1 in Cullen and Sherrell (1999). The larger filter size probably explains the larger blank values compared to those obtained for the 25 mm filters.

<sup>c</sup> The collected total biomass of *T. weissflogii* on the Osmonics Poretics filter after NaCl rinse (120 ml of a culture grown at  $Fe_T=84$  nM was filtered at a cell concentration of  $43 \times 10^6$  cells  $l^{-1}$ ). Average values determined from measurements of two separate samples (each measured 12 times) with standard deviation in parenthesis.

<sup>d</sup> NaCl rinse ( $4 \times 2$  ml) of chelex-cleaned NaCl solution (0.56 M) with 2.38 mM  $HCO_3^-$  added (pH 8.2).

<sup>e</sup> Oxalate–EDTA wash ( $2 \times 1.4$  ml, 5 min each, at pH 7), followed by NaCl rinse.

<sup>f</sup> Ti–citrate–EDTA wash (1.4 ml, 2 min each, at pH 8), followed by NaCl rinse.

<sup>g</sup> Ti–citrate–EDTA+ wash from the reversed-phase cleaned Ti-plus solution with addition of 5 mM BCDS (1.4 ml, 2 min each, at pH 8), followed by NaCl rinse.

It thus appears that both the oxalate–EDTA and the Ti–citrate–EDTA washes can be applied directly to measure intracellular concentrations of many elements in phytoplankton, provided that an appropriate follow-up rinsing of the filters with a clean solution is carried out. This technique will fail, however, for elements that are not effectively chelated in the wash solution, such as Ba, and Cu in the case of the Ti–citrate–EDTA wash. As demonstrated by Sternberg et al. (2005), intracellular Ba concentrations are exceedingly small and most of the Ba measured as “cellular” in phytoplankton cultures is actually adsorbed on  $FeO_x$ . Although the filter blanks for Ba are reasonably low (Table 3), the measured Ba quotas after the oxalate–EDTA wash are higher than after NaCl rinse only (see Section 3.4). Ba contamination was eliminated by adding the Ba complexing agent, Kryptofix-222, at a concentration of 5 mM to the wash solution.

The Cu contamination from the Ti–citrate–EDTA wash remained high, even after extensive rinsing with clean NaCl solution (Table 3). This is expected since Ti(III) should reduce Cu(II) to Cu(I) which is weakly complexed by EDTA and cannot be washed away easily. Without any treatment, the direct use of both the Ti–citrate–EDTA or Ti–citrate–EDTA+ solution resulted in much higher Cu quotas (Fig. 6B). We tried a number of techniques to remove the Cu contamination: adding extra EDTA and the Cu(I) complexing agent bathocuproine disulfonate (BCDS; 5 mM) and pre-washing the Ti–citrate–EDTA solution by reversed-phase extraction. (The effectiveness of Cu removal from the wash solution by the reversed-phase cartridges is shown in Fig. 6A: Cu decreased from 1.24 to 0.12  $\mu$ M after the second cartridge; while Fe decreased only slightly.) All these modifications together yielded good filter blanks (Table 3) and a Cu quota for cells grown at high Fe concentration

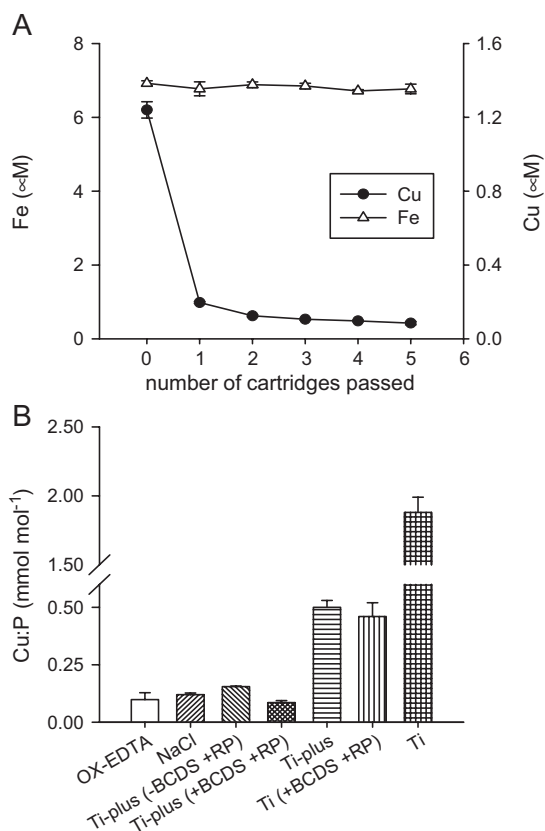


Fig. 6. (A) The decrease in Cu concentration in the Ti-plus solution after passing through reversed-phase cartridges; (B) Cu:P ratios measured in *T. weissflogii* (grown at  $Fe_T=840$  nM) after various washing treatments (RP: reversed-phase extraction; BCDS: bathocuproine disulfonate).

(840 nM) that was similar to that obtained with the oxalate–EDTA wash (Fig. 6B). Ironically, a simple NaCl rinse gave a value that was only slightly higher. This is because the concentration of Cu adsorbed on the  $FeO_x$  associated with the cells is in fact negligible compared to the intracellular Cu concentration (see Section 3.4). (As discussed above for Zn, the slightly higher value obtained with the NaCl rinse likely corresponds to Cu bound to the surface of the cells).

### 3.6. Extracellular and intracellular Fe and associated trace metals

To assess the relative importance of cellular uptake and adsorption for various trace elements, we compared the apparent cellular concentrations measured

after rinsing with NaCl only and washing with the oxalate–EDTA (+Kryptofix-222) solution (followed by extensive rinses of NaCl) for cultures grown over a range of Fe concentrations (Table 4 and Fig. 7). Except at the lowest concentration, the filtered particulate Fe accounted for a major fraction of the total Fe in the medium such that the “total cellular” Fe increased linearly with the total Fe concentration. The intracellular Fe quotas increased by a factor of ten over the range of concentrations tested, with an Fe:P ratio varying from 4.4 to 43.6  $mmol\ mol^{-1}$ . The cellular P concentration measured by the oxalate–EDTA wash technique remained constant at about 80  $fmol\ cell^{-1}$  and was roughly 82–88% of the cellular P obtained after rinse with NaCl, independent of Fe levels.

Despite significant extracellular precipitation of  $FeO_x$  and its potential to adsorb various elements, only a small fraction of the cellular concentration of the other trace nutrients, Cu, Zn, Co, Cd and Mn, was dissolved by the oxalate–EDTA wash along with the  $FeO_x$ . Further the total cellular concentrations of these elements were practically unaffected by the total Fe concentration in the medium. Only at the lowest Fe concentration ( $Fe_T=84$  nM), which slightly limited growth and may have affected the requirements for other micronutrients, did the cellular concentrations of some elements slightly increase (Cu and Mn) or decrease (Co and Cd). It thus appears that the intracellular pools of Cu, Zn, Co, Cd and Mn dominated their respective cellular concentrations and that the concentrations of these elements adsorbed on extracellular  $FeO_x$  were negligible.

In contrast with those of the essential trace metals, the total cellular concentrations of both V and Ba increased with the total Fe concentration in the medium, while their intracellular concentrations remained constant. We have previously reported that a major fraction of the Ba is typically associated with extracellular  $FeO_x$  (Sternberg et al., 2005); clearly, the same is true of  $VO_4^{3-}$ . (Note that without addition of Kryptofix-222, the oxalate–EDTA wash yielded higher intracellular Ba quotas; see the cross symbols on the Ba graph of Fig. 7.) It may seem paradoxical that  $Ba^{2+}$ , a cation that does not adsorb particularly strongly on  $FeO_x$ , and  $VO_4^{3-}$ , an anion which adsorbs poorly at high pH, should be largely adsorbed on  $FeO_x$ , while strongly adsorbing metals like  $Cu^{2+}$ ,  $Zn^{2+}$ ,  $Co^{2+}$  or  $Cd^{2+}$  are not. This results from the facts that the free

Table 4  
Elemental quotas for *T. weissflogii* grown at different Fe concentrations<sup>a</sup>

Growth conditions										
Fe level (nM)	84		304		840		1680		3360	
Growth rate (d <sup>-1</sup> )	1.14		1.29		1.27		1.23		1.27	
pH	8.95		8.98		8.93		9.08		8.96	
Elemental quotas										
	NaCl rinse					OX-EDTA wash				
Fe level (nM)	84	304	840	1680	3360	84	304	840	1680	3360
P (fmol cell <sup>-1</sup> )	92.1	91.4	99.7	102.4	95.3	78	77	88	88	78
	(1.2)	(3.4)	(7.1)	(4.9)	(3.6)	(1.4)	(3.1)	(7.3)	(4.8)	(2.6)
P (mmol l <sup>-1</sup> ) <sup>b</sup>	113	113	111	106	107	96	95	98	91	87
	(2)	(4)	(8)	(5)	(4)	(2)	(4)	(8)	(5)	(3)
Fe:P (mmol mol <sup>-1</sup> )	7.9	31.2	106.9	213.7	424.3	4.4	8.6	21.0	35.4	43.6
	(0.2)	(1.9)	(9.7)	(14.7)	(28.2)	(0.2)	(0.5)	(2.0)	(2.1)	(3.4)
Mn:P (mmol mol <sup>-1</sup> )	3.8	3.0	3.1	3.5	3.3	4.1	3.4	3.1	3.5	3.3
	(0.07)	(0.22)	(0.36)	(0.21)	(0.17)	(0.17)	(0.25)	(0.31)	(0.29)	(0.21)
Zn:P (mmol mol <sup>-1</sup> )	0.51	0.51	0.46	0.55	0.44	0.53	0.47	0.39	0.52	0.37
	(0.020)	(0.034)	(0.042)	(0.030)	(0.031)	(0.032)	(0.035)	(0.040)	(0.045)	(0.024)
Cu:P (mmol mol <sup>-1</sup> )	0.13	0.11	0.09	0.12	0.10	0.092	0.089	0.077	0.092	0.082
	(0.006)	(0.005)	(0.008)	(0.006)	(0.006)	(0.006)	(0.008)	(0.007)	(0.012)	(0.008)
Co:P (mmol mol <sup>-1</sup> )	0.064	0.068	0.060	0.060	0.058	0.070	0.076	0.066	0.068	0.066
	(0.003)	(0.003)	(0.005)	(0.003)	(0.003)	(0.004)	(0.005)	(0.006)	(0.004)	(0.003)
Cd:P (μ mol mol <sup>-1</sup> )	7.0	8.4	7.4	6.7	6.9	6.6	8.0	7.9	8.3	7.5
	(0.74)	(0.81)	(0.64)	(0.64)	(0.76)	(0.77)	(0.77)	(0.75)	(1.0)	(1.0)
Ba:P (μ mol mol <sup>-1</sup> )	0.85	0.57	0.62	1.01	3.60	0.43	0.67	0.43	0.47	0.29
	(0.078)	(0.083)	(0.11)	(0.061)	(0.20)	(0.069)	(0.069)	(0.077)	(0.051)	(0.040)
V:P (μ mol mol <sup>-1</sup> )	24.2	37.8	56.5	63.7	130.3	17.8	18.9	18.4	20.0	19.0
	(2.1)	(4.5)	(7.1)	(5.0)	(7.1)	(2.2)	(1.8)	(2.5)	(2.2)	(2.5)

<sup>a</sup> Average quotas from duplicate samples with errors in parenthesis.

<sup>b</sup> Concentrations in bio-volume calculated using the cell volume measured with Coulter Multisizer for each sample.

concentrations of Ba<sup>2+</sup> and VO<sub>4</sub><sup>3-</sup> (ca 40 and 25 nM, respectively) are much higher in the culture medium than those of Cu<sup>2+</sup>, Zn<sup>2+</sup>, Co<sup>2+</sup> or Cd<sup>2+</sup> (0.2, 12, 17 and ~1 pM, respectively) and that Ba and V are nonetheless comparatively less accumulated by the cells. Barium has no known biological function and vanadium, though it is used in some nitrogenases and bromoperoxidases, is not known to be useful to eukaryotic phytoplankton. Calculations based on published data for the adsorption of cations and anions on FeO<sub>x</sub> (Dzombak and Morel, 1990) show that indeed, even at the highest Fe concentration (4 μM), the amount of Zn, Cu, Co or Cd adsorbed on FeO<sub>x</sub> should account for only about 10% of the corresponding total cellular concentrations. According to these calculations, the extracellular adsorbed fraction of these elements should only become significant when the FeO<sub>x</sub> concentration reaches 10 μM and above.

#### 4. Conclusions

The results shown on Fig. 7 are quite encouraging: under most circumstances, one should obtain accurate cellular concentrations of many elements of interest in phytoplankton by measuring total particulate concentrations on filters rinsed with a NaCl solution. Even in the presence of a significant concentration of iron oxide precipitate, the fractions of elements such as Cu, Zn, Co, Cd and Mn that are adsorbed on the precipitate account for a negligible portion of the corresponding particulate concentrations. (This result can probably be extended to manganese or aluminum oxide precipitates, which have typically lower affinities than FeO<sub>x</sub> for most solutes.) This result should apply to laboratory cultures in which the trace metals are suitably buffered by a strong chelating agent and to field samples

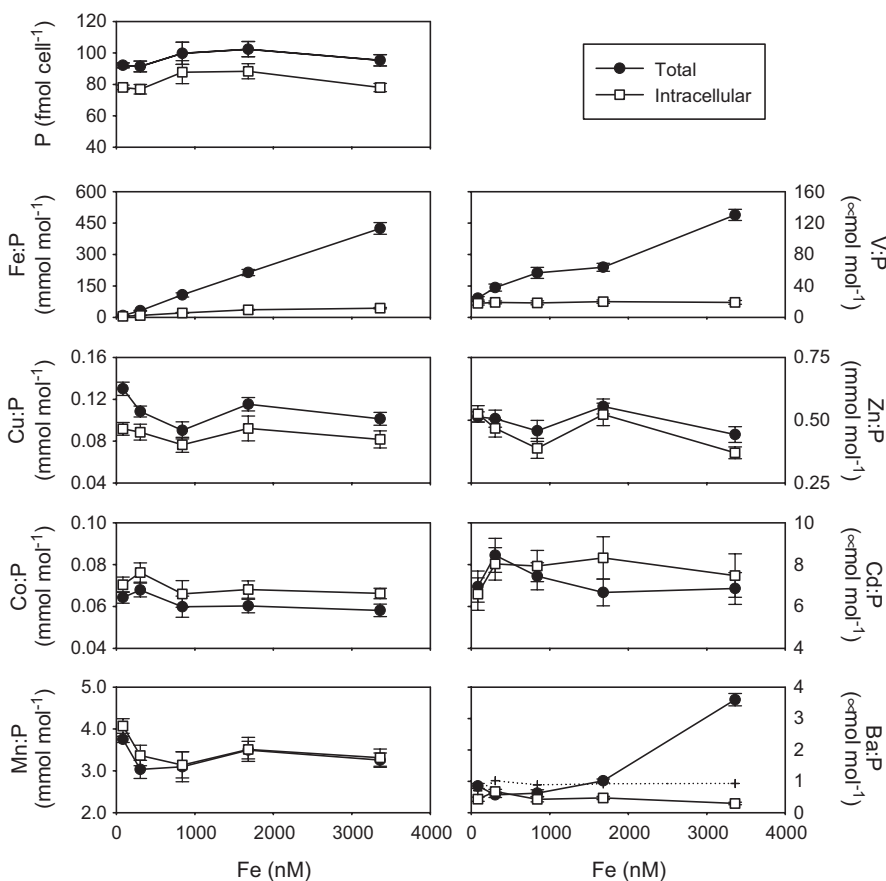


Fig. 7. The cellular P concentration and metal:P quotas in *T. weissflogii* as function of Fe concentrations in the growth medium. Open squares: intracellular fraction from oxalate-EDTA wash and filled circles: total cellular quota from NaCl rinse. (For Ba, extra crosses represent data from oxalate-EDTA wash without Kryptofix-222 addition from a different experiment.)

when the lithogenic fraction of the suspended particles is not too large. Thus, for example, an accurate elemental composition should have been obtained by Ho et al. (2003) for various phytoplankton species in culture and by a number of previous authors (Martin and Knauer, 1973; Martin et al., 1976; Collier and Edmond, 1984; Kuss and Kremling, 1999) for the composition for the ambient biomass in field samples.

For some elements, chiefly Fe but also elements such as Ba or V (or even Mn which may precipitate on its own), an accurate measure of the true cellular concentration requires a washing step to dissolve the  $\text{FeO}_x$  (or the  $\text{MnO}_x$ ) associated with the cells. Two types of wash solutions are effective for this purpose. Solutions of mild reductants (such as a Ti(III) com-

plex) and mixtures of two ligands that act synergistically (such as oxalate+EDTA) can both dissolve  $\text{FeO}_x$ , without damaging the integrity of the cells. One approach or the other may be preferable depending on the particulars of the situation. For elements that are effectively bound to chelator(s) in the wash solution, it is generally possible to eliminate much of the contamination introduced by this solution through extensive rinsing of the filters. For elements that are not chelated in the wash solution, as exemplified here for Ba and Cu(I), it may be necessary to add specific chelators and/or to pre-clean the wash solution. When the concentration of  $\text{FeO}_x$  is high or the precipitate is relatively inert, a reducing solution appears generally more effective than a two-ligand solution.

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