Quantification of Si, P, S, Mn, Fe, and Zn in Cultured Phytoplankton and Southern Ocean Protists

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Introduction

Heterotrophic and autotrophic aquatic protists have an obligate requirement for iron [1, 2]. Despite the abundance of Fe in the earth's crust, however, its low solubility in oxygenated seawater results in low dissolved Fe concentrations in most parts of the ocean [3]. In particular, dissolved Fe is so low (<100 pM) in the three high-nutrient, low-chlorophyll (HNLC) oceanic regions that it limits the growth of resident plankton [4], reducing the amount of carbon dioxide that can be converted to organic material and potentially sequestered in the ocean via the "biological pump" [5]. Large-scale Fe fertilization has been proposed as a means of increasing oceanic C sequestration in order to offset increased anthropogenic C emissions, allowing the subsequent sale of tradeable C credits in a global C marketplace [6]. This idea has spurred an active debate among oceanographers regarding the feasibility and ecological consequences of such a plan [7, 8].

In order to assess the role of Fe as a limiting nutrient in the Southern Ocean, the Southern Ocean Iron Experiment (SOFeX) was conducted in early 2002. This project involved the fertilization of two patches of water (each initially 15 km × 15 km) with Fe. Plankton samples were collected before and after sequential fertilizations for subsequent analysis of the Fe content of the cells with a synchrotron-based x-ray fluorescence (SXRF) microprobe at the APS. The Si, P, S, Mn, and Zn content of each individual cell was also measured with SXRF, allowing us to observe shifts in cell composition and stoichiometry in response to increased Fe availability.

Given the lack of appropriate biological standard reference materials for this type of microprobe analysis [9], we also performed comparative analyses of cultured phytoplankton cells with SXRF and graphite furnace atomic absorption spectrometry (GFAAS). In this manner, we were able to prove the quantitative merits of SXRF and demonstrate that our SXRF sample preparation protocols do not introduce artifacts.

Methods and Materials

Southern Ocean Samples

Two water patches were fertilized with Fe during SOFeX. The first patch was located north of the Antarctic Polar Front Zone (APFZ) in water characterized by high concentrations of nitrate and phosphate but low silicate. A 15 × 15-km square patch was fertilized with iron sulfate to a concentration of ~1 nM. Samples were collected at a station immediately prior to the first fertilization and also at stations within the patch following each fertilization. A station outside the patch was also sampled to test for temporal changes in the unfertilized water. A second patch of water south of the APFZ and characterized by high nitrate, phosphate, and silicate was fertilized four times. Again, samples were collected prior to fertilization, after each of the first two Fe additions, and at a station outside the patch.

Samples were collected for SXRF analysis from the mixed layer at each station following stringent trace-metal cleaning techniques [10]. Water was transferred directly from Teflon®-lined Niskin sampling bottles into acid-washed 2.5-L polycarbonate bottles that were then enclosed in two ziplock bags and stored in the dark at ambient temperature until processing (<1 hour). All subsequent manipulations were performed in a Class 100 laminar-flow hood. Water samples were gently mixed, and aliquots were decanted into acid-washed polyethylene centrifuge tubes containing gold London Finder electron microscopy grids (Electron Microscopy Sciences) coated with a carbon/Formvar® film and mounted on acid-washed Araldite™ bases. These samples were preserved with 0.25% (final concentration) ice-cold electron-microscopy-grade glutaraldehyde (buffered to pH 8 with SupraPur NaOH and stripped of trace metal contaminants with cation exchange resin) immediately prior to centrifugation. The grids were removed with Teflon-coated forceps, rinsed with several drops of Milli-Q deionized water, and allowed to dry in a laminar-flow hood that was darkened to minimize Chl *a* degradation. Once dry, the grids were examined with light and epifluorescence microscopy at 400×. Individual cells were located and identified, and both light and epifluorescence (blue excitation) micrographs were taken of each target cell. Finally, the grids were transferred to acid-washed plastic grid boxes and stored in a dessicator until analysis.

The elemental composition of each target cell was analyzed with SXRF following the protocols outlined elsewhere [10]. All analyses were performed with the

side-branch x-ray fluorescence microprobe at APS beamline station 2-JD-E. The sample chamber was filled with He to reduce fluorescence from atmospheric Ar. Pixel step size was generally 0.5 μm except for the larger diatom cells, which were scanned in 1-μm steps. Dwell time was adjusted to ensure <10% counting error for fluorescent counts and ranged from 1 to 8 seconds per pixel. For each target, the fluorescence spectra from the pixels corresponding to the cell were averaged (summed for all working detectors); an averaged spectrum was also generated for a neighboring background region for each target. Both cell and background spectra were fit with a summed exponentially modified Gaussian peak model, generating peak areas for each element. The averaged cell peak areas were corrected for background signal (generally <15% of cell signal) and then normalized to upstream x-ray flux and dwell time and converted to element areal concentrations (μg cm^{-2}) by comparison to National Institute of Standards and Technology (NIST) thin-film standards. Areal concentrations were multiplied by the area of the cellular region of interest to produce whole-cell element concentrations.

Comparative Analyses of Cultured Cells

The freshwater diatom *Stephanodiscus hantzschii* (clone UTCC 267) was cultured in WCL-1 media [11] and analyzed with both SXRF and GFAAS to provide an independent measure of the SXRF values. Cells were fixed with glutaraldehyde at the end of log-phase growth and centrifuged onto grids. Additionally, an aliquot of *S. hantzschii* was mounted without glutaraldehyde fixation for comparison to the fixed treatment. Aliquots of the culture were also collected on acid-cleaned membrane filters for subsequent bulk analysis of Si, Mn, Fe, and Zn.

Results and Discussion

The cellular contents (mol $cell^{-1}$) of Si, Mn, Fe, and Zn in *S. hantzschii* as measured by spectrophotometry, GFAAS, and SXRF are shown as box plots in Fig. 1. For all four elements, the cellular content as determined with GFAAS on filtered samples (aggregating millions of cells) was similar to that from using single-cell SXRF. The only significantly different (*t*-test, P<0.05) treatment was found with Fe. SXRF measurements of Fe in DI-rinsed cells were slightly lower than measurements of both glutaraldehyde-fixed cells analyzed with SXRF and DI-rinsed cells analyzed with GFAAS. A comparison of the element contents of the glutaraldehyde-preserved cells with those left unfixed reveals no notable differences. Glutaraldehyde penetrates cellular membranes and cross-links proteins, and two possible artifacts may be introduced during chemical fixation: internal elements may leach out of the cell when membranes are compromised, and

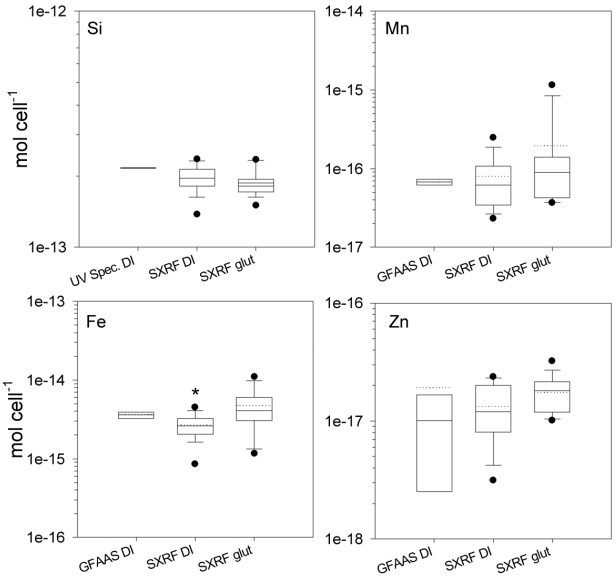

*FIG. 1. Si, Mn, Fe, and Zn contents of **S. hantzschii** cells as measured by ultraviolet (uv) spectrophotometry, GFAAS, and SXRF. The cells analyzed with spectrophotometry (UV Spec. DI, n = 2 filters) and GFAAS (GFAAS DI, n = 8 filters) were collected on filters and rinsed with deionized water. Cells analyzed with SXRF were treated the same way (SXRF DI, n = 16 cells), but an additional treatment was fixed with glutaraldehyde prior to centrifugation (SXRF glut, n = 15 cells). In these plots, the solid line represents the median, the dotted line is the arithmetic mean, the shaded box delineates the 25th and 75th percentile confidence intervals, and the error bars encompass the 10th and 90th percentile confidence intervals. Data falling outside of these ranges are plotted individually. Treatments found to be significantly different (**t**-test, P <0.05) are noted with an asterisk (*).*

contaminant elements may be introduced to the cell with the glutaraldehyde. The glutaraldehyde was stripped of metal contaminants with cation exchange resin prior to use, reducing the likelihood of the latter artifact. The good agreement between the element contents of fixed and unfixed cells suggests that fixation did not allow elements to leach from the cells prior to drying. In fact, by stabilizing cell structure, the chemical fixative may enhance retention of elements that might otherwise have leached during the rinsing and drying process. These results are describing more fully elsewhere [10].

The mean element-to-C ratios for the protist cells collected from the Southern Ocean are presented in Table 1. The three cell types were found to have significantly different elemental compositions. Diatoms contained the least amount of P, relative to C, followed by autotrophic then heterotrophic flagellated cells. The

TABLE 1. Elemental composition (cell contents normalized to cell C) of protists collected from the Southern Ocean during SOFeX. Shown are geometric mean stoichiometries for each cell type (diatoms, autotrophic flagellated cells = A flag, heterotrophic flagellated cells = H flag) collected from either unfertilized or Fe fertilized waters.

	Unfertilized			Fertilized		
Element	Diatom	A flag	H flag	Diatom	A flag	H flag
P[a]	9.0	16.4	22.0	10.4	18.7	23.7
S[a]	6.8	11.3	11.9	8.3	11.7	11.0
Mn[b]	3.4	2.7	3.0	4.5	4.4	4.4
Fe[b]	6.0	8.7	14.1	22.8	36.1	21.9
Zn[b]	67.8	22.2	46.9	70.7	34.0	60.0

[a] mmol mol C^{-1}.
[b] μmol mol C^{-1}.

flagellated cells also contained more S than the diatoms, although there was not a notable difference between the two types of flagellates. Manganese concentrations were similar in the three cell types but were somewhat higher following the addition of Fe to the Southern Ocean waters. Not surprisingly, Fe, which also varied between cell types, increased dramatically following the fertilization. Finally, Zn:C ratios were found to be highest in diatoms, followed by heterotrophic and then autotrophic flagellates.

These data provide the first demonstration of variations in the elemental composition of like-sized marine protists collected from a natural ecosystem. The data indicate that different types of cells may have different nutrient requirements and possibly different abilities to obtain these nutrients, particularly the limiting trace metal Fe. This unique type of information can be used to understand the partitioning of Fe within a pelagic ecosystem [12] and also to constrain the predicted response of Fe-limited communities to fertilization events [13].

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