Quantification of Trace Elements in Cultured Phytoplankton Cells by Using an X-ray Fluorescence Microprobe

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Introduction

The accumulation of trace metals by planktonic protists influences the growth of primary producers, metal biogeochemical cycling, and metal bioaccumulation in aquatic food chains. Fe is of particular interest because it limits phytoplankton production in large oceanic regions [1]. Cellular accumulation of this metal thus influences the contribution of these regions to oceanic C sequestration [2]. Most inferences about oceanic trace metal cycling by protists come from laboratory studies on metal bioaccumulation in monospecific cultures or from metal concentration profiles in oceanic water columns. There are only a few reliable field measurements of the trace metal contents of phytoplankton [3], and there are none of other protists, because of our inability to isolate components of the plankton from complex natural particle assemblages and analyze their metal contents accurately. Bulk size-fractionation techniques cannot distinguish between biotic and abiotic particles or between different species of plankton of similar size. Although these techniques are valuable tools for geochemists, the general nature of the data makes it less useful for studying the cycling of metals within pelagic plankton communities.

We are developing sample preparation and data analysis protocols that allow us to accurately and confidently quantify the trace elements Si, Fe, Cu, and Zn in individual nanoplankton cells (2-20 µm) collected from natural marine waters with a hard x-ray fluorescence (XRF) microprobe at the APS. Here we describe work done to test the effect of chemical fixation with glutaraldehyde on trace element concentrations in cultured diatom cells. We also demonstrate the importance of using spectral mapping instead of standard region-of-interest (ROI) energy windows for obtaining accurate element quantitation.

Materials and Methods

The diatom *Thalassiosira weissflogii* was grown in Aquil media [4] in 20-L batch cultures containing either 450 nM ("high-Fe") or 45 nM ("low-Fe") total Fe. Cells were harvested at the beginning of the stationary phase by centrifugation onto carbon/Formvar-coated Au electron microscopy (EM) grids. An aliquot from each culture was mounted without fixation or rinsing. A second aliquot of the high-Fe culture was fixed with 0.25% (final concentration) EM-grade buffered glutaraldehyde (pH adjusted to 8 with NaOH) before centrifugation and subsequent rinsing with Milli-Q water for comparison to the unfixed treatment. All grids were allowed to dry in a laminar-flow hood.

Samples were analyzed at the 2-ID-E hard x-ray microprobe at the APS. X-ray photons with an energy of 10 keV were focused to a spot of approximately 0.5×0.3 µm full width at half maximum (FWHM). Elemental maps were acquired by scanning the sample through the focused beam and recording the full x-ray fluorescence spectra at each pixel with an energy-dispersive, three-element germanium detector optimized for low-Z detection (Canberra ultra-LEGe detector). Pixel step sizes and dwell times were varied to match the size of the cell and were typically 0.5-1 µm and 1-2 s, respectively. The spectra were averaged over the area of the cell by using Cl as a proxy for the cell biomass, which we found produces a strong fluorescence signal for a range of marine protists. A spectrum of the background region near the cell was then subtracted.

The ability to quantify trace constituents in target cells with complex elemental matrices has required improvements in spectral data acquisition and analysis. We have developed a protocol for modeling the fluorescence spectra of the pixels representing the cell. By using a Marquardt-Levenberg iterative search algorithm in Matlab (version 5.3, MathWorks Corp), background-corrected spectra were fit to a summed exponentially modified Gaussian (EMG) peak model with a sigmoidal baseline (tolerance = 0.00001). In its raw form, this model contains 150-200 parameters, depending on the incident energy of the excitation beam. Such complex formulations of peaked distributions are notoriously difficult to fit to data. To reduce the problem to a tractable size, several constraints and simplifications were employed. First, a universal standard deviation was fit for all peaks. The ratio of the amplitudes of the elemental K_α and K_β emission lines were also tightly constrained to conform to well-established theoretical expectations. Finally, we presumed that the relative position of the peak midpoints matched the established emission lines for x-ray fluorescence and that the differences in absolute position could be explained as a linear transformation of the vector of expected midpoints. These assumptions

allowed us to center all 20 peaks of interest by using only two parameters, an offset factor and a multiplier. The sigmoidal baseline was employed to empirically account for low energy noise in the detector. In all, 29 parameters were allowed to float unconstrained during the fitting process. The models and data were square-root transformed before fitting to stabilize variance and reduce the influence of large peaks on the solution. Finally, peak heights were converted to element concentrations (µg/area) by using peak height:concentration ratios determined from National Institute of Standards and Technology (NIST) standards.

Element contents of *T. weissflogii* were normalized to estimates of cellular C, which were calculated by applying published C:vol ratios to cell sizes estimated from the x-ray scans. Diatoms were assumed to be cylindrical, and the conversion factor 0.14 pg C μm^{-3} measured by Sunda and Huntsman [5] was used to convert the volume to C content.

Results and Discussion

Figure 1 shows the modeled spectra for a *T. weissflogii* cell. The EMG model accounts and corrects for the overlap of neighboring peaks (e.g., S K_α and Cl K_α). It also enables the identification of detector irregularities, such as the "pile-up" of Cl counts at two times the Cl K_α peak (masking the Cr K_α). The sigmoidal baseline allows us to account for low energy noise in the detector, which can lead to spurious results for the lower Z elements such as Si at the bottom end of the spectrum. Figure 2 shows the concentrations of Si, Fe, Cu, and Zn in the diatoms of the various treatments as calculated by using both simple ROI energy windows for the elements and spectral modeling. Although the concentrations calculated by using these two data analysis approaches are fairly close for Fe, Cu, and Zn, the ROI approach overestimates the Si contents in the unrinsed samples, on which dried salt results in large Cl K_α peaks. The resulting increase in the electronic noise at the low end of the spectrum leads to erroneously high estimates of Si. Spectral analysis allows us to account for this, and the spectral estimate of the Si content in the rinsed cells is within the range (indicated by the pink lines) of Si:C ratios measured with other techniques for this diatom clone. This demonstrates the importance of rinsing marine samples before analysis and of using the spectral modeling approach.

The concentrations of Si, Fe, Cu, and Zn in the diatoms are also shown in Fig. 2. The cellular Fe concentrations measured with XRF for cultured *T. weissflogii* cells were 29 ±4 and 64 ±13 µmol Fe per mol C in the low-Fe and high-Fe unfixed cells (n = 3 for both treatments). These fall within the range of concentrations measured with radioisotopes for the same diatom strain cultured in the same media at similar Fe concentrations [5, 6]. Unlike these previous studies, we did not remove extracellularly bound Fe before analysis, since the Ti(III)-citrate/EDTA rinse developed by Hudson and Morel [7] is appropriate only for radioisotope studies. This may explain slight differences between our measured Fe concentrations. In addition, the concentrations of Fe, Cu, and Zn in the unfixed and fixed cells were not statistically different. This indicates that glutaraldehyde is not a significant source of contamination and that the process of fixation is not allowing trace elements to leak out of the cells during rinsing and drying.

Advances in the sampling and analysis of trace metals in marine environments have allowed oceanographers to develop an understanding of the distribution of metals between dissolved, colloidal, and various particulate phases. However, since bulk techniques cannot differentiate the trace metal compositions of diverse constituents of the same size class, plankton-trace metal studies have been limited to whole particle assemblages, with the complication that abiotic and living particles of comparable size are combined. By using the sample preparation and data analysis protocols described here, we believe the hard x-ray microprobe can reveal trace metal interactions among different members of the same planktonic assemblage that will allow us to examine metal cycling within the microbial loop for the first time.

Acknowledgments

Use of the APS was supported by the U.S. Department of Energy, Office of Science, Office of Basic Energy Sciences, under Contract No. W-31-109-ENG-38.

References

[1] J. H. Martin, R. M. Gordon, and S. E. Fitzwater, Limnol. Oceanogr. **36**(8), 1793 (1991); K. H. Coale, K. S. Johnson, and S. E. Fitzwater et al., Nature **383** (6600), 495 (1996).

[2] J. H. Martin, R. M. Gordon, and S. Fitzwater et al., Deep-Sea Res. **36**(5), 649 (1989).

[3] J. H. Martin and G. A. Knauer, Geochim. Cosmochim. Acta **37**, 1639 (1973); R. Collier and J. Edmond, Prog. Oceanogr. **13**(2), 113 (1984).

[4] N. M. Price, G. I. Harrison, and J. G. Hering et al., Biol. Oceanogr. **6**, 443 (1988/1989).

[5] W. G. Sunda and S. A. Huntsman, Mar. Chem. **50**(1-4), 189 (1995).

[6] R. F. Strzepek and N. M. Price, Mar. Ecol. Prog. Ser. **206**, 107 (2000).

[7] R. J. M. Hudson and F. M. M. Morel, Limnol. Oceanogr. **34**(6), 1113 (1989).

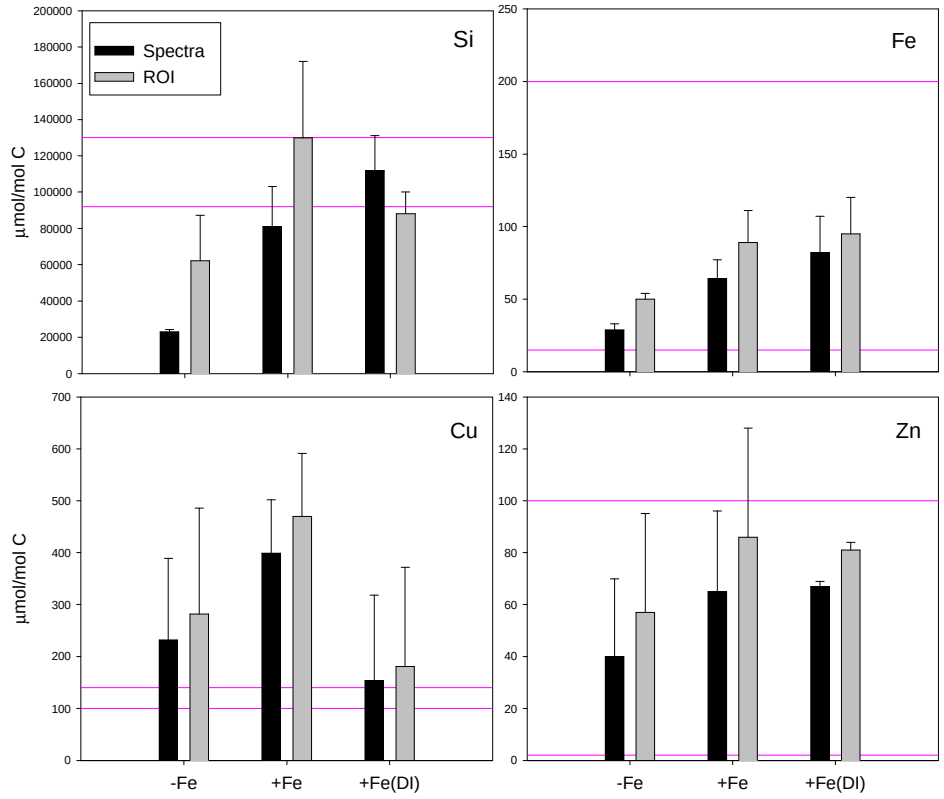

FIG. 1. Fluorescence spectra for the marine diatom T. weissflogii. The fitted sigmoidal baseline is shown in black, while the fitted element peaks are displayed in other colors. The K_a peaks of certain elements are indicated above the peaks, along with a spurious peak identified by the EMG model.

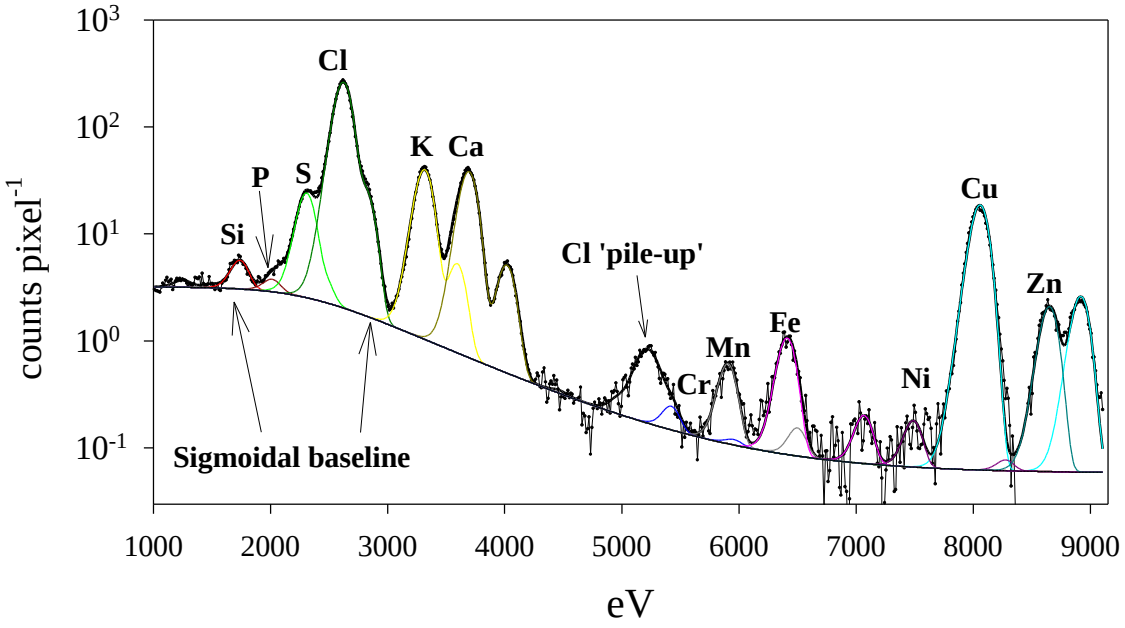

FIG. 2. Concentrations (normalized to cellular C) of the trace elements Si, Fe, Cu, and Zn in T. weissflogii. The concentrations calculated by using ROI energy windows are compared with those calculated by using spectral modeling. The horizontal pink lines indicate the upper and lower range of the element concentrations reported for this diatom clone as reported in the literature, as measured with radioisotopes and atomic absorption spectrophotometry (AAS).