ABSTRACT. The biogeochemical cycles of many elements in the ocean are linked by their simultaneous incorporation into protists. In order to understand these elemental interactions and their implications for global biogeochemical cycles, accurate measures of cellular element stoichiometries are needed. Bulk analysis of size-fractionated particulate material obscures the unique biogeochemical roles of different functional groups such as diatoms, calcifying protists, and diatzooptroths. Elemental analysis of individual protist cells can be performed using electron, proton, and synchrotron X-ray microprobes. Here we review the capabilities and limitations of each approach and the application of these advanced techniques to cells collected from natural communities. Particular attention is paid to recent studies of plankton biogeochemistry in low-iron waters of the Southern Ocean. Single-cell analyses have revealed significant inter-taxon differences in phosphorus, iron, and nickel quotas. Differences in the response of autotrophs and heterotrophs to iron fertilization were also observed. Two-dimensional sub-cellular mapping indicates the importance of iron to photosynthetic machinery and of zinc to nuclear organelles. Observed changes in diatom silicification and cytoplasm content following iron fertilization modify our understanding of the relationship between iron availability and silification. These examples demonstrate the advantages of studying ocean biogeochemistry at the level of individual cells.

Key Words. Iron fertilization, phytoplankton, plankton, Southern Ocean, synchrotron X-ray fluorescence.

THE BIOGEOCHEMISTRY OF PLANKTONIC PROTISTS

The field of biogeochemistry concerns, in part, the impact of biological processes on the chemical composition of the environment. Many critical transformations of biologically active elements in natural ecosystems are mediated by protists. Autotrophic protists are often responsible for entry of elements into food webs. As consumers, mixotrophic and heterotrophic protists play a key role in returning elements to the abiotic world. Our knowledge of the mechanisms driving these transformations is poised to rapidly expand in the near future as a molecular level understanding of cellular physiology, biochemical composition, and gene expression develops. Such an understanding will be needed if we are to anticipate the consequences of anthropogenic alterations to global geochemical cycles. Many of the changes already underway will in turn impact protist biology and ecology in the future in ways that may depend on their elemental requirements.

The importance of protists to global biogeochemistry is perhaps most obvious in the sea. Covering 70% of the Earth’s surface, the oceans play numerous critical roles in global biogeochemistry and climate. The oceans buffer temperature fluctuations, provide an important food supply for maritime countries, and mediate changes in the global C cycle. The oceans have absorbed approximately 120 petagrams (1 Pg = 10^{15} g) of anthropogenic carbon dioxide in the past two centuries, about half of the total amount released by human activities during this time (Sabine et al. 2004). Carbon in surface waters is “pumped” to deep waters and the sea floor via the sinking of planktonic organisms. Through this “biological pump”, phytoplankton play a major role in the global C cycle (Longhurst 1991; Sarmiento and Gruber 2006). Calcifying protists such as coccolithophores and foraminifera further impact the ocean C cycle through the formation and export of external shells composed of CaCO\textsubscript{3} (Langer, this issue). Ocean sediments in some regions are described as calcareous “oozes” because of the abundance of calcareous shells of protists. The growth and metabolism of marine phytoplankton may also impact global climate through the production of dimethylsulphide (DMS). Certain phytoplankton species produce DMS and its precursor dimethylsulphoniopropionate (DMSP) for osmonic regulation and antioxidant protection in the cell (Sunda et al. 2002). Phytoplankton DMS can diffuse into the overlying atmosphere and may serve as a significant source of non-sea salt sulfate aerosols and cloud condensation nuclei (Charlson et al. 1987). This process may impact global climate through the enhancement of cloud albedo (Bates, Charlson, and Gammon 1987).

Marine protists also control the biogeochemical cycling of many elements in the ocean besides C. The nutrient elements N, P, Si, Fe, Mn, Ni, Cu, and Zn are accumulated by phytoplankton in surface waters and exported to depth as sinking biogenic particles. This process results in the surface-depleted “nutrient profile” characteristic of many bioactive elements (Donat and Bruland 1995). Protistan metabolic activities can also alter the chemical form of many elements. Eukaryotic phytoplankton reduce nitrate to ammonia upon uptake, convert silicic acid to opaline shells, and modify the chemical speciation and reactivity of dissolved metals (e.g. Coale and Bruland 1988; Rue and Bruland 1995). Grazing processes can further change the physico-chemical speciation of metals and non-metals (Hutchins and Bruland 1995; Sato, Takeda, and Furuya 2007).

Numerous approaches have been taken to study the biogeochemical interactions between ocean protists and their marine environment. Incubations with isotopically labeled compounds have revealed differences in the relative availability of different nutrient substrates to ambient phytoplankton populations (Dugdale and Goering 1967; Hutchins et al. 1999; McCarthy 1972), enzyme activities have been measured as markers of nutrient utilization and limitation (Gilbert et al. 2004; Webb, Moffett, and Waterbury 2001), and gene expression has been used to determine nutrient cycling by specific phenotypes in field assemblages (Church et al. 2005). Comparisons of nutrient stoichiometries have also been critical to our understanding of ocean biogeochemistry. Alfred

1Invited presentation delivered during the joint annual meeting of the Phycological Society of America and the International Society of Protistologists, Providence, Rhode Island, August 5–9, 2007.

EXPLORING OCEAN BIOGEOCHEMISTRY BY SINGLE-CELL MICROPROBE ANALYSIS OF PROTIST ELEMENTAL COMPOSITION

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1Invited presentation delivered during the joint annual meeting of the Phycological Society of America and the International Society of Protistologists, Providence, Rhode Island, August 5–9, 2007.
Redfield famously noted similarities in the stoichiometries of $N$ and $P$ in bulk surface plankton and dissolved nutrients at depth (Redfield 1934; Redfield 1958), and this approach has since been applied to a number of other bioactive elements, including trace metals (e.g. Löschler 1999; Sunda 1997).

THE NEED FOR A SINGLE-CELL APPROACH

The biogeochemical cycles of bioactive elements are linked by their simultaneous incorporation into protists. Therefore, in order to understand these elemental interactions, accurate measures of cellular element stoichiometries are needed. These are typically obtained for field communities using bulk size-fractionation (Collier and Edmond 1984; Copin-Montegut and Copin-Montegut 1983; Martin and Knauer 1973). However, this macroscopic approach obscures significant internal processes and limits our ability to link ecology and biogeochemistry. Protists play unique ecological roles in pelagic plankton communities. A simplified model of such a system is shown in Fig. 1. Photoautotrophs form the base of the food web and provide organic substrates to heterotrophic bacteria, protists, and mesozooplankton, as well as mixotrophic protists (Caron 2000). Photoautotrophs occur in each of the traditional plankton size classes (0.2–2, 2–20, 20–200 $\mu$m). Heterotrophic nanoflagellates, dinoflagellates, and ciliates consume autotrophs and bacteria, moving carbon through the food web and also contributing dissolved organic compounds for use by bacteria and dissolved inorganic and organic nutrients for use by autotrophs. These trophic relationships are organized by size in Fig. 1, although it is unlikely that predation and grazing occur only between these clear size delineations. Each size class contains both autotrophs and heterotrophs (and likely mixotrophs), and bulk elemental analyses performed on all cells collected on 0.2-, 2-, and 20-$\mu$m membranes would obscure differences between ecological functional groups in the same size range. Because of the presence of abiotic particulate material and the sorption of dissolved and colloidal metals to filters, bulk chemical analyses often cannot be used to estimate the average cellular elemental stoichiometries for the entire protistan community. Even in open-ocean high-nutrient low chlorophyll (HNLC) areas, biogenic iron may only account for a small fraction of total particulate iron (Strzepek et al. 2005). Further complicating matters, delicate cells may burst upon contact with the filter membrane under vacuum.

Diverse pelagic protists from the same size class can perform unique biogeochemical roles. Eukaryotic autotrophs such as coccolithophores and diatoms produce mineral shells composed of calcite and silicate, respectively, that provide relatively dense “ballast” to the cells. It is thought that these taxa are more likely to sink from surface waters and contribute to carbon export than non-ballasted autotrophs (Armstrong et al. 2002; Klaas and Archer 2002). Coccolithophores and diatoms will also impact carbon and silicon geochemical cycles differently than non-mineralized taxa by removing silicon and additional carbon from surface waters and reducing alkalinity. Given their different biogeochemical roles, it is desirable to separate ballasted and non-ballasted cells for elemental analysis. Diazotrophic (N-fixing) organisms such as *Trichodesmium* and Crocosphaera are also present in the same size classes (and in the case of the endosymbiont Richelia, in the same organism) as non-diazotrophs, but they clearly play a unique and very important role in nitrogen biogeochemistry. Given the high iron requirements for nitrogenase (Kustka et al. 2003), diazotrophs are also likely to impact iron biogeochemistry differently than other cells types (Berman-Frank et al. 2001; Falkowski 1997). Specific taxa can also impact sulfur geochemistry, because prymnesiophytes such as *Emiliania huxleyi* and *Phaeocystis* sp. produce far more intracellular DMS/DMSP than other algal species (Matrai and Keller 1994; Stefels and Vanboeckel 1993). To advance our understanding of biogeochemistry, we must collect elemental information at the level of ecological and biogeochemical functional groups. That is, since taxonomy influences biogeochemistry, biogeochemical measurements should reflect taxonomic differences.

![Fig. 1. Description of common particulate material in pelagic waters, including a hypothetical food web demonstrating the diversity of ecological and biogeochemical functional groups contained within common size classes. Picoplankton (<2 $\mu$m) are typically composed of heterotrophic and autotrophic prokaryotes, including *Synechococcus*, *Prochlorococcus*, and some pico-eukaryotes. Nanoplankton (2–20 $\mu$m) generally comprise the bulk of the protist biomass. This size class includes heterotrophs, mixotrophs, and “naked” and “ballasted” autotrophs. Microplankton (>20 $\mu$m) can include large diatoms, dinoflagellates, foraminifera, radiolarians, and zooplankton nauplii.](image-url)
Recent modeling efforts have begun to recognize these varied biogeochemical roles and have incorporated multiple plankton functional groups. Moore, Doney, and Lindsay (2004) and Moore et al. (2002) include three phytoplankton groups—small phytoplankton, diatoms, and diazotrophs—and assign unique parameters to each, including different nutrient uptake kinetics, grazing mortality, and minimum cell quotas. Salihoglu and Hofmann (2007) further separate the picoautotrophs into low light-adapted Prochlorococcus, high light-adapted Prochlorococcus, and Synechococcus, in addition to autotrophic eukaryotes and diatoms. Others have chosen to separate the behavior of herbivorous microzooplankton grazers and bactovorous heterotrophic nanoflagellates (Lancelot et al. 2000). In order to accurately parameterize these models and ground truth the output, it is necessary to make elemental measurements of each relevant group. Given the broadly overlapping sizes of the autotrophic and heterotrophic functional groups, group quotas cannot be resolved with standard bulk chemical analysis techniques.

There is substantial evidence from laboratory culture work that element quotas are dynamic and can vary significantly in protists as a function of taxonomy, trophic function, and nutrient substrate. Much of the work has focused on the micronutrient iron. Brand (1991) found the iron requirements of eukaryotes to be significantly lower than those of prokaryotes. Minimum iron quotas (often normalized to cellular carbon or phosphorus) required for growth have been shown to be 2- to 4-fold lower in oceanic diatoms compared to coastal diatoms (Maldonado and Price 1996; Sunda, Swift, and Huntsman 1991). It also appears that mixotrophic and heterotrophic phagotrophs require 2- to 3-fold more iron than autotrophic protists (Chase and Price 1997; Maraner, Bird, and Price 1998; Sunda et al. 1991). The cell quotas of other bioactive metals also vary between taxa. Diatoms contain less iron, cobalt, copper, and molybdenum (normalized to P) than dinoflagellates grown in the same media (Ho et al. 2003), and selenium concentrations in phytoplankton can vary by more than four orders of magnitude among species at ambient levels of selenite (Baines and Fisher 2001). Furthermore, phytoplankton metal quotas are sensitive to the ambient nutrient environment. The additional iron required for nitrate reductase raises iron quotas of diatoms grown on nitrate compared to those grown on ammonium (Maldonado and Price 1996). Trichodesmium iron requirements increase 5-fold during nitrogen fixation as a result of nitrogenase synthesis (Kustka et al. 2003). Other trace nutrients such as manganese, copper, and nickel may also vary in cells in response to iron status and nutrient utilization patterns (Peers and Price 2004; Peers, Quesnel, and Price 2005; Price and Morel 1991). Furthermore, phytoplankton have the ability to accumulate and store considerable amounts of trace metals in response to environmental concentrations (Sunda and Huntsman 1998a).

Further complicating the extrapolation of culture studies to the field is the uncertainty surrounding bioavailability of Fe and other trace elements in the field. In the sea, the dissolved fraction of Fe and many other trace elements exists predominantly as complexes with dissolved organic ligands (Coale and Bruland 1988; Ellwood and Van den Berg 2000; Rue and Bruland 1995; Saito, Rocap, and Moffett 2005). In the free-ion model, the ligand-bound metal is assumed to be unavailable to cells (Sunda and Huntsman 1998a). This assumption allows modelers to calculate Fe quotas using simple Michaelis– Menten uptake kinetics, measured Fe concentrations, and presumed concentrations of Fe-binding ligands (e.g., Salihoglu and Hofmann 2007). However, at least some Fe bound to organic ligands in nature may be directly accessible to protists (Maldonado and Price 1999; Maldonado et al. 2005). Alternatively, phagotrophic protists may sidestep the dissolved pool by directly ingesting colloidal Fe or bacteria (Chase and Price 1997; Maranger et al. 1998). Iron is not the only element for which bioavailability of dissolved fractions is open to question. Both nitrogen and selenium are accumulated not only as inorganic ion, but also in the dissolved organic form (Baines et al. 2001; Bronk et al. 2007).

Given the plasticity of protist metal quotas and the uncertainty regarding bioavailability of dissolved trace elements, it is problematic to assume that field quotas match those measured in laboratory cultures. Perhaps more importantly, valuable information regarding phytoplankton physiology, biogeochemical cycling, and ambient nutrient bioavailability can be gained from measurements of in situ protist cell quotas. The elemental content of individual protists cells can be assayed through one of several microscope-based technologies (Table 1). Light microscopy can be used in conjunction with fluorescent dyes to detect various metal ions within cells (Kikuchi, Komatsu, and Nagano 2004; Thomas et al. 1999; Yang et al. 2005). Such dyes are commercially available, utilize relatively common light and confocal microscopes, and have become widely used. However, only labile forms of the metals that are available to bind with the probes are detected, so absolute quantification of metal quotas is difficult. Fluorescent probes are also prone to interferences from competing metal ions, further complicating quantitation. More promising for analytical work are microscopy techniques which base detection on the characteristic radiation absorption and fluorescence properties of individual elements.

A REVIEW OF APPLICABLE MICROPROBE APPROACHES

Fluorescence X-rays are emitted when atomic electrons transition from outer shells to lower energy inner shell vacancies created by incident ionizing radiation. The fluorescence energies associated with these transitions correspond to the difference between inner and outer shell electron binding energies, and are thus unique to each element, making it possible to deduce the elemental composition from the X-ray fluorescence spectrum. For example, for iron the predominant X-ray fluorescence emission has an energy of 6.404 keV, corresponding to the transition of an L-shell electron into a K-shell vacancy. The number of detected X-ray photons for each element generally scales directly as a function of atomic abundance, thus elemental content can be quantified in a straightforward manner. The yield of fluorescence photons is lowest for the lighter elements since the excess energy of the relaxed electron for such elements is more likely to be carried away through ejection of a secondary emitted electron (Auger electron) rather than via a fluorescent X-ray photon.

Characteristic X-rays can be generated by one of several forms of higher energy ionizing radiation, and each technique has advantages and disadvantages (Table 1). Electron microprobes are commercially available and fairly common, and electron beams can be easily focused, although the high flux requirements to detect trace elements in biological samples limit the achievable spatial resolution in analytical mode to 20 nm typically. Electrons are rapidly absorbed by light elements (Z < 10) in biological samples, typically limiting the depth of penetration to several micrometers at best. More importantly, multiple inelastic scattering will cause the electron beam to broaden rapidly, thus increasing the excited volume in the sample significantly and lowering achievable spatial resolution (Ingram et al. 1999). To circumvent this problem thin sections (<100 nm) are typically used, and larger protist targets may need to be embedded and sectioned prior to analysis. Lower ionization cross-sections for high mass (Z > 20) transition metals and significant Bremsstrahlung background combine to limit the sensitivity of electron microprobe X-ray microanalysis (XRMA) for the heavier bioactive metals (e.g. Mn, Fe, Cu, Zn).

Cellular elemental quantification and sub-cellular mapping can also be formed by measuring energy loss of electrons as they pass...
Table I. Comparison of some available techniques for trace element mapping in protists.

<table>
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<th>Typ. spatial resolution (nm)</th>
<th>Typ. sample thickness (μm)</th>
<th>Resolution limitation</th>
<th>Sample preparation</th>
<th>Advantages (+)/disadvantages (−)</th>
</tr>
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<tbody>
<tr>
<td>Light microscope</td>
<td>200</td>
<td>30</td>
<td>Wavelength</td>
<td>Requires use of fluorescent dyes</td>
<td>+ Changes in living cells can be monitored /− Can only detect &quot;labile&quot; ions in solution and not total element content</td>
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<td></td>
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<td>Cells generally need to be hydrated</td>
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<td>− Absorption quantification is difficult</td>
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<td>+ Simultaneously detect &gt; 10 elements</td>
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<td>− Analyses are slow</td>
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<td></td>
<td>− Significant radiation damage</td>
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<tr>
<td>Analytical electron microprobe (XRMA, EPXMA)</td>
<td>20</td>
<td>0.1</td>
<td>Sample thickness</td>
<td>Thick samples need to be sectioned</td>
<td>− Only some elements are readily accessible (e.g., P, Fe)</td>
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<td></td>
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<td></td>
<td>Samples typically need to be dried, or imaged frozen-hydrated</td>
<td>− Co-localization can be difficult (EFTEM) or slow (EELS)</td>
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<td>+ Simultaneously detect &gt; 20 elements</td>
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<td>+ High sensitivity</td>
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<td>− Analyses are slow</td>
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<td>+ Very high sensitivity, low background, selective excitation of analytes</td>
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<td>+ Simultaneously detect &gt; 10 elements</td>
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<td>+μ-XANES for chemical state mapping</td>
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<td>− Analyses are slow</td>
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<tr>
<td>EELS/EFTEM</td>
<td>2</td>
<td>0.005–0.05</td>
<td>Radiation damage</td>
<td>Requires ultrathin sections</td>
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<tr>
<td>Proton microprobe</td>
<td>1,000</td>
<td>50</td>
<td>Radiation damage; Flux</td>
<td>Samples typically need to be dried</td>
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<td>− Significant radiation damage</td>
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<td>Synchrotron X-ray microscope</td>
<td>30–200</td>
<td>10</td>
<td>Optics (currently)</td>
<td>Samples typically need to be dried, or imaged frozen-hydrated</td>
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<td>+ Very high sensitivity, low background, selective excitation of analytes</td>
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XRMA, X-ray microanalysis; EPXMA, electron probe X-ray microanalysis; EELS, electron energy-loss spectroscopy; EFTEm, energy-filtered transmission electron microscopy; PIXE, proton-induced X-ray emission; SXRF, synchrotron X-ray fluorescence; μ-XANES, micro X-ray absorption near-edge structure.

Through thin biological samples, electron energy loss spectroscopy (EELS) measures the energy loss of electrons at a single focused spot while the energy of the incident electron beam is systematically shifted across the absorption edge for the element of interest (Table 1). A closely related technique, energy-filtered transmission electron microscopy (EFTEM), involves the collection of a series of images of the whole specimen at specific energies spanning the absorption edge. The “stack” of hyperspectral images can then be processed to produce element maps of extremely high spatial resolution (Leapman 2004). These techniques are also capable of remarkable elemental sensitivity; for example EELS has been used to detect single atoms of Ca and Fe in biomolecules mounted directly onto TEM grids (Leapman 2003). These techniques require samples <100 nm in thickness to avoid multiple scattering interactions between the electrons and the sample matrix, so their applicability to natural protist samples is limited.

Protons may also be used to generate characteristic X-ray fluorescence in protist cells, a technique termed proton-induced X-ray emission (PIXE). Although less common than analytical electron microprobes, nuclear (proton) microprobes are more readily available and less expensive than synchrotron facilities. A result of their higher momentum, protons generate less Bremsstrahlung background than electrons and have higher sensitivity for transition metals (Garman 1999). Lower absorption coefficients also eliminate the need for sample sectioning for most protist samples. Protons can be focused to only ~1 μm at the required fluxes, and larger beam sizes are commonly used to analyze marine samples (Iwata et al. 2005; Pallon et al. 1999). Proton-induced X-ray emission is therefore generally inappropriate for studies of subcellular element distributions. Radiation damage can also be significant. Proton-induced X-ray emission analyses can be supplemented with direct carbon and nitrogen measurements via proton backscattering, and sample thickness can be quantified by measurement of proton absorption by the sample (scanning transmission ion microscopy) (Pallon et al. 1999).

Synchrotron-based X-ray fluorescence (SXRF) microprobes are rapidly becoming a powerful tool for single-cell element analysis. With low Bremsstrahlung background and high ionization cross-sections for heavier elements, SXRF has the highest sensitivity for transition metals (Sparks 1980). Improvements in Fresnel zone-plate optics are allowing spatial resolution to approach that of XRMA (Wang, Yun, and Jacobsen 2003; Yun et al. 1999). The development of high brightness third-generation synchrotron X-ray sources has provided the needed sensitivity to measure transition metal stoichiometries in cells collected from the most pristine regions of the ocean. Radiation damage is significantly reduced in SXRF. Synchrotron-based X-ray fluorescence analyses do not require high vacuum conditions and can be performed on frozen-hydrated samples to reduce radiation damage and preserve cell structure optimally. However, it is often most practical to prepare dried protist samples in order to immobilize mobile species and preserve elemental composition until analysis. Synchrotron-based X-ray fluorescence measurements can also be supplemented with microscale chemical speciation measurements that provide redox and coordination environment information (e.g., Bacquart et al. 2007).

A representative SXRF spectrum for a marine protist is shown in Fig. 2. The spectrum shown is the sum of 156 individual spectra collected during a raster scan of a small pennate diatom collected from the equatorial Pacific Ocean. The Kα fluorescence peaks for Si, P, S, Cl, Ar, K, Ca, Mn, Fe, Co, Ni, Cu, and Zn are clearly visible, as well as Kα peaks (for Ca and Cu especially). Using custom designed software (Vogt 2003), each peak in the spectrum is iteratively fit to an exponentially modified Gaussian curve to find the peak areas. At the same time background is estimated from the data using a peak stripping algorithm (SNIP; Ryan et al. 1988). Fitting of a complex series of peaks to data can be time consuming and prone to artifacts due to the number of free parameters. To make the fitting procedure tractable and efficient, ratios of the Kα and Kα peak areas for an element are tightly constrained according to the ratios observed in the thin-film X-ray fluorescence standards from NIST. Also, relative...
positions of the peaks are fixed in accordance with the known X-ray emission lines for each element. Quantitation is achieved by comparing the areas under the curves with corresponding peaks from the NIST standard.

Synchrotron-based X-ray fluorescence also provides two-dimensional maps of element distribution in target cells. Given a typical focused beam spot size of 200–300 nm for 10 keV hard X-ray microprobes, informative maps of sub-cellular element localization can be generated for larger protists. An example is shown in Fig. 3. This silicoflagellate was collected from the Southern Ocean during the Southern Ocean Iron Experiment (SOFeX) project. The edge of a neighboring pennate diatom can be seen to the right of the cell. The chloroplasts are clustered in the middle of the cell. Silicon maps onto the silicate test, while S is broadly indicative of the internal cytoplasmic components. In this cell Fe and Zn are most highly localized in the chloroplasts, although Zn distributions are often found to correlate with P in other cells. In addition to providing information about the biological functions of the elements in target cells, these maps are extremely useful for identifying extra-cellular abiotic particles that can easily confound bulk analyses (Twining et al. 2003b).

SINGLE-CELL ANALYSIS OF PLANKTON BY ELECTRON MICROPROBE

Electron microprobe X-ray fluorescence analysis, often termed XRMA, energy-dispersive X-ray microanalysis or electron probe X-ray microanalysis has been used to study the elemental composition of eukaryotic algae and cyanobacteria collected from freshwater systems. Sigee, Levdov, and Dodwell (1999) analyzed the diatom Biddulphia nematode collected from a stratified lake and consistently detected Mg, Si, P, and S in single cells. Iron concentrations in epilithic diatoms were comparable to those measured in cultured Prochlorococcus and Synechococcus. The range of C:N:P stoichiometries spanned the Redfield ratio, but inter-population variability was observed. Carbon:phosphorous ratios ranged from 156 ± 6 to 215 ± 9 and 73 ± 8 to 350 ± 30 for Prochlorococcus and Synechococcus, respectively. Sodium, Mg, Cl, K, and Ca were also detected in the Prochlorococcus cells, but the bioactive transition metals were not detectable via XRMA. Reliable XRMA measurements of trace metals such as Mn and Fe appear limited to metal-sequestering bacteria which contain 1,000- to 10,000-fold more metal in extracellular appendages than typical microorganisms (Heldal et al. 1996).

PLANKTON ELEMENTAL ANALYSIS WITH PIXE

Within the marine sciences, PIXE has primarily been used to study the elemental composition of inorganic particulate material collected on aerosol filters (e.g. Reis et al. 2006), but several groups have applied PIXE for element analysis of algae. Zhang et al. (1997) investigated the binding of Ag, Ba, Cd, Cu, Hg, and Pb to the cell wall of Chlorella vulgaris under controlled laboratory conditions. The metals were easily detected in the samples, which were composed not of individual cells but of a layer of freeze-dried cells collected on a membrane filter. A similar approach was used by Iwata and colleagues to measure metal bioaccumulation by actively growing phytoplankton cultures (Iwata 2001; Iwata et al. 2005). These researchers used a 2-mm proton beam to assay mats of cells collected on filters. More recently, Gisselson, Graneli, and Pallon (2001) mapped C, N, P, S, Cl, K, and Ca concentrations in individual cells of Dinophysis norvegica collected from the Baltic Sea. Carbon and N were quantified by proton backscattering, and the heavier elements were assayed with PIXE. Absolute cellular quotas (mol/cell) of C, N, and P were lower when measured with the nuclear microprobe than with
traditional bulk techniques, but C:P and N:P ratios were similar. More intra-population variability was observed for N quotas (6.4-fold variation) than C or P quotas (2.2- and 3.4-fold, respectively), which the authors suggested might indicate mixotrophic or heterotrophic ingestion of prey by some of the *D. norvegica* cells to obtain N.

SINGLE-CELL SXRF ANALYSIS OF MARINE PROTISTS

Synchrotron X-ray fluorescence was first used to analyze planktonic protists in culture and collected from the coastal Atlantic (Twining et al. 2003a). Following method development (Twining et al. 2003b), the first large-scale field sampling program came during SOFeX (Cosse et al. 2004). Single autotrophic and heterotrophic protist cells were collected within and outside the fertilized patch and analyzed with SXRF. Here we will summarize and expand upon the results of this study, which demonstrates the power of the single-cell approach to studying ocean biogeochemistry.

The importance of Fe to the functioning of HNLC regions was not apparent until the adoption of stringent trace metal “clean” techniques to oceanographic sampling and analysis protocols.
(Martin and Fitzwater 1988; Martin, Gordon, and Fitzwater 1990; Martin, Gordon, and Fitzwater 1991). The resulting observations of low dissolved Fe led Martin (1990) to hypothesize that variations in delivery of airborne Fe-rich dust to the Southern Ocean over time may have caused past glacial periods by generating large blooms of phytoplankton. This hypothesis has stimulated a series of large-scale Fe addition experiments, modeling exercises, and even proposals to geoengineer atmospheric CO$_2$ by adding Fe to stimulate phytoplankton growth and reduce atmospheric CO$_2$ (Kintisch 2007). Cellular Fe:C ratios in the plankton are of particular interest because they affect the relationship between C sequestration and Fe addition, and therefore the economic viability of attempts to bioengineer atmospheric carbon through Fe fertilization. Quotas of other nutrient elements such as P, Mn, Ni, and Zn are also important for their potential to limit cellular growth. Cellular quotas of these elements were measured with SXRF on single diatom, autotrophic flagellate and heterotrophic flagellate cells.

Iron quotas were detectable even under HNLC low Fe conditions, and community wide averages of Fe:C agreed well with radioisotopic measurements of C and Fe uptake (Twining et al. 2004b). However several interesting differences between cell types emerged. First, diatoms collected from unfertilized waters had lower cellular Fe:C (with carbon estimated from biovolume) than did flagellates (Fig. 4). While cell volume-normalized elemental contents might be expected to be lowest for diatoms with large vacuoles, the differences in Fe:C ratios may reflect more interesting ecological or biological distinctions. For example, diatoms are constrained to obtain Fe from the dissolved fraction and may thus be more susceptible to limitation than flagellates, which in the open ocean are widely believed to feed upon other cells which have already done the work of concentrating Fe from the environment (Maranger et al. 1998). Indeed, the Fe:C ratios of heterotrophic flagellates more closely resembled the uptake ratio of Fe and C into the bacterial size fraction as determined by radioisotopes. Alternatively, the higher Fe content in heterotrophs may reflect a greater requirement. Respiratory enzymes that make up the electron transport system have a higher Fe content than does the photosynthetic apparatus that makes up much of autotrophic cell mass (Chase and Price 1997; Raven 1988).

Cell types differed in a number of other important respects as well. For example, estimated P:C and P:S ratios were lower for diatoms than for flagellated cells (Fig. 4), a finding in opposition to culture studies indicating that diatoms as a group have high P contents (Ho et al. 2003). Diatoms also exhibited much higher Ni and Zn quotas. Elemental fluorescence maps indicate that most, but not all, of the Ni in diatoms was associated with the frustule, and in particular with the densely silicified girdle region (Twining et al. 2003b). The only known Ni enzyme in eukaryotes is urease, which can be used to assimilate reduced urea nitrogen from the environment. Although nitrate was present at non-limiting concentrations (Coale et al. 2004), urease expression in cultured phytoplankton is not generally suppressed in the presence of nitrate (Lomas 2004; Peers, Milligan, and Harrison 2000). Furthermore, diatoms in the Southern Ocean may favor the use of reduced N when cellular energy is limited by light and Fe availability (Price, Ahner, and Morel 1994). In contrast with Ni, Zn seemed to be associated mostly with the nucleus (Twining, Baines, and Fisher 2004a), perhaps reflecting the importance of Zn-containing finger proteins in the overall Zn budget of the cell. This observation is interesting because most discussion of Zn-containing finger proteins is largely unknown. Furthermore, higher Fe quotas might result from physiological adjustments to low-light conditions in the Southern Ocean (Sunda and Huntsman 1997). As might be predicted for a region with excess dissolved phosphate, cellular P was also unusually high in Southern Ocean cells, being >2-fold more abundant in flagellates than predicted by Redfield. Diatom Zn:P ratios were elevated relative to bulk plankton from the Pacific Ocean but similar to bulk plankton from Southern Ocean (Twining et al. 2004a). This suggests there may be unique ecological or biogeochemical aspects of Southern Ocean taxa that are reflected in the elemental composition. Measured diatom Zn quotas were more than an order of magnitude higher than those reported for cultured diatoms by Ho et al. (2003). This may indicate greater bioavailability of Zn bound to natural organic ligands than typically assumed. Diatom Zn quotas in culture may also have been lowered by relatively high Mn concentrations in the media, as Mn can inhibit Zn uptake in marine phytoplankton (Sunda and Huntsman 1998b). These comparisons highlight the uncertainties introduced when culture data is applied to natural systems and the benefit of field measurements.

The cellular concentrations of several elements changed markedly in response to Fe addition. Iron cellular concentrations changed most, increasing in all cell types by a factor of 3–6 over the course of 6 days and two additions. While the Fe content of diatoms and autotrophic flagellates increased after both Fe additions, the Fe:C ratio in heterotrophic flagellates exhibited a delayed response which probably reflected a particulate food supply that was continuously changing in Fe content (Fig. 5). Such metal cycling within plankton communities is extremely difficult to study without single-cell analytical tools, because the dominant autotrophs and heterotrophs have similar sizes. Interestingly, P and S content of diatoms increased markedly after Fe addition, perhaps reflecting an increase in cytoplasm within the frustule. It
may be that reduction of the cytoplasmic mass (or cell volume) is a general response to Fe limitation. In addition to these changes, cellular concentrations of the bioactive metals Mn, Ni, and Zn also increased 1.5- to 4-fold after Fe addition. In general, these results suggest that relaxation of Fe limitation caused a physiological cascade in protist cells leading to a high growth, high metabolic rate condition.

Some of the most interesting findings regarding cellular silica in diatoms. Diatoms in the Southern Ocean were heavily silified prior to Fe fertilization. The average diatoms had Si:C ratios of 0.5 mol mol$^{-1}$, which is nearly 4 times the typical value for diatoms in culture (Brzezinski 1985). These measurements imply that SiO$_2$ constitutes $\sim 20\%$ of wet mass and $\sim 50\%$ of the dry mass of the diatoms in the Southern Ocean. The high degree of silicification may explain why the Southern Ocean plays such an important role in the global C cycle since dense silica ballast enhances sinking rates of aggregates and fecal material produced in these regions. It is widely believed that high silicification is typical of Fe-limited regions because silica incorporation is inexpensive in energy terms and tends to continue even as cellular growth rates slow in response to limitation by other nutrients (Franck et al. 2000). However, Fe addition in the Southern Ocean produced only a transient 40% decline in Si per cell, after which cellular Si contents rebounded to match those of cells collected before fertilization and from a control patch after the experiment. There was a 2-fold decline in Si:P and Si:S ratios, but this reflected the increase in cellular P and S rather than a decrease in Si. Clearly, the maintenance of high cellular silicification in the Southern Ocean requires an explanation other than Fe limitation.

The SXRF data collected during the SOFeX project demonstrate the power of single-cell element measurements for studying the role of protists in the biogeochemistry of natural systems. This project has been recently followed up by SXRF studies in the Eastern Equatorial Pacific, which is also believed to be Fe-limited, and in the Sargasso Sea, which is nutrient poor but generally considered Fe-replete. These analyses are ongoing and as yet unpublished, but they generally confirm many trends noted for diatoms in the Southern Ocean, most notably higher than expected cellular Fe, lack of an effect of Fe availability on cellular silicification, an increase in cellular S and P content when Fe limitation is relieved, and high cellular Ni and Zn. Large differences among cell types were also noted, although not all patterns conformed to those observed in the Southern Ocean. In addition, recent improvements in analytical sensitivity of the SXRF microprobe have allowed trace element contents to be measured in prokaryotes for the first time, and these have proven to be distinct from those of eukaryotic cells.

**FUTURE METHODOLOGICAL DEVELOPMENTS**

Synchrotron X-ray microprobes offer arguably the best combination of analytical sensitivity, analytical breadth and spatial resolution while imparting the least radiation damage to protist samples. There are several limitations to their usefulness for environmental and other microbiologists, however. First, SXRF typically cannot measure the major components of organic matter (C, N, and O), which complicates interpretation of cellular concentrations and two-dimensional element maps. Second, localization of elements within the cell is currently only possible in two dimensions, which makes it difficult to determine how much of an element is associated with, for example, the cell surface or cellular organelles embedded in protoplasm. Third, routine spatial resolutions are currently too low to resolve cell structures smaller than the nucleus or chloroplast.

Estimation of cellular mass simultaneous with SXRF analyses is possible in one of two ways. Just like visible light passing through water or air, X-rays passing through biological samples are scattered in proportion to the X-ray density of the substance. These scattered X-rays are registered by the detector and the area under the Compton scattering peak can be used as a proxy for cell mass at a particular pixel. Without a vacuum chamber, the method is fairly insensitive, because background scattering peaks due to He atmosphere are large, resulting in a high ratio of noise to signal and high error. Also, the XRF detector is typically positioned to minimize detection of scattered radiation (and thus improve sensitivity), though use has been made of a second detector at a position optimized for detection of increased scatter (Golosio et al. 2003). A much more sensitive method that can be used for thin biological samples without a vacuum is X-ray phase contrast microscopy (Hornberger, Feser, and Jacobsen 2007). X-rays are subject to phase shift as they pass through biological samples to a degree dependent on the thickness and composition of the sample. Using a specially designed and fabricated segmented detector positioned downstream of the sample source, differences in phase can be recorded to produce a differential phase contrast image (Feser et al. 2006). Given assumptions about the C, O, and N composition of the sample, and direct measurements of other elements available from the fluorescence spectra, the phase shift can be reconstructed (de Jonge et al. submitted; Hornberger et al. 2007) and used to estimate cellular mass at each pixel. Because the method uses the same X-ray beam to construct a map of fluorescence and phase contrast (Hornberger et al. 2006), the two maps coincide perfectly (Fig. 6). This method has been calibrated on latex beads, and a comparison with standard chemical determinations of elemental composition of algal cells is underway (Hornberger et al. 2007).

X-ray fluorescence computed tomography (XFCT) offers the possibility of visualizing both elemental distributions and phase contrast maps in three dimensions (Boisseau and Grodzins 1987; Golosio et al. 2003; La Riviere and Vargas 2006; La Riviere et al. 2006). In XFCT the target is repeatedly imaged in two dimensions under different angular positions. Once the desired angular range is covered, the three-dimensional elemental or mass distribution that best reconciles all of the images is determined. This process can be complicated for larger cells due to the absorption of lower

![Graph](image-url)
energy fluorescent X-ray photons by cellular organic matter (Golosio et al. 2003). However, recent developments in statistical fitting techniques that also estimate this self absorption using penalized-maximum likelihood have been able to correctly reconstruct the three dimensional test structures for which self absorption is significant, making quantitative tomography possible (La Riviere et al. 2007). This approach effectively increases the size of targets that can be imaged. Anticipated improvements in terms of X-ray source brightness should improve the speed of such techniques in the future.

The focused spot sizes (i.e. spatial resolution) of hard X-ray microprobes continue to improve with advances in X-ray optics (Hignette et al. 2005; Kang et al. 2006). Current instrumentation at third-generation synchrotron facilities is capable of <200 nm spatial resolution with 10 keV X-rays, with ongoing efforts to reduce this to 30 nm. Beamlines utilizing intermediate energy X-rays (2–3 keV), such as 2-ID-B at the Advanced Photon Source, have already achieved <100 nm resolution (Fig. 6D) (McNulty et al. 2003). This level of focus will allow elemental measurements in viruses and the smallest prokaryotes, which can be important food resources for protists. It will also enable more precise sub-cellular element mapping in eukaryotes, expanding our ability to study the biological roles of metals in protists.

CONCLUSIONS

No microprobe technique is perfectly suited to all potential environmental applications. Electron microprobes are widely available and are useful when studying major elements in small prokaryotes. Proton microprobes can detect a wide range of elements but at limited spatial resolution. Synchrotron-based X-ray fluorescence combines unmatched sensitivity for transition metals and spatial resolution that approaches XRMA, at the expense of light element quantification and instrument availability. Each of these microprobe techniques can be used to help open the ‘‘black box’’ of plankton elemental composition with single-cell measurements. Only by making geochemical (elemental) measurements of individual functional groups will we advance our understanding of the relationships between protist biology, ecology, and biogeochemistry.

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LITERATURE CITED


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