

Consideration of the bioavailability of iron in the North American Great Lakes: Development of novel approaches toward understanding iron biogeochemistry

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There is increasing recognition that iron distribution and availability is significant in terms of global oceanic production. Low availability of iron and other nutritive trace metals may also constrain productivity in the North American Great Lakes. Despite its importance, the biogeochemistry of iron in the water column of lacustrine systems remains poorly characterized. In addressing the current state of iron biogeochemistry, a workshop organized a decade ago at the Bermuda Biological Station for Research brought together a cross-disciplinary team of chemists and biologists who sought to synthesize current knowledge and identify research priorities in this field. Key among goals identified during the workshop, and one that remains today for the most part unfulfilled, was to 'develop techniques to quantify those fractions of Fe that are accessible to phytoplankton.' Here we review recent progress toward meeting this objective, drawing on specific examples from Lake Superior where these approaches have been applied.

Keywords: bioreporter, ferredoxin, flavodoxin, fluorescence, Lake Superior, phytoplankton, trace metal speciation

Introduction

The limitation of primary production by phosphorus availability is a central tenet of modern day Great Lakes limnology. Indeed, the importance of P-availability in constraining phytoplankton growth has long directed lake management decisions, perhaps most notably via the Great Lakes Water Quality Agreement which has resulted in reduced P-loading and contributed to en-

hanced water quality in the Laurentian Great Lakes system over the past two decades (Neilson et al., 1995). In light of the seemingly overwhelming evidence in support of P-limitation, it is tempting to overlook the often-important role played by other elements in regulating lake productivity. Specifically, the potential for trace metal or 'micronutrient' limitation in the Great Lakes has received limited attention. Compared to macronutrients (C, N and P), micronutrients

(Fe, Zn, Mn, Cu, Ni, Co) are required in very small amounts to support cellular metabolic processes. However, recent findings in marine systems identify a need to reexamine trace metal-phytoplankton interactions. Indeed, current opinion favors Fe availability as the factor constraining productivity in ‘high nutrient, low chlorophyll’ oceanic provinces such as the equatorial Pacific (Martin et al., 1994; Coale et al., 1996), the subarctic Pacific (Tsuda et al., 2003) and regions of the Southern Ocean (Boyd et al., 2000).

Although present consideration is limited to the bioavailability of Fe in the Great Lakes, with particular focus on Lake Superior, many of the same principles and approaches can be extended to other bioactive trace metals and to other systems. In this review, we provide a discussion on the biogeochemistry of Fe in freshwater systems and the methods available to assess both speciation of dissolved Fe and bioavailability of Fe to phytoplankton. Recent work from the authors’ laboratories will describe biochemical and bioreporter-based methods aimed at determining the fraction of dissolved Fe that is readily bioavailable to biota.

Relevance of Fe deficiency to lacustrine systems

Consideration of Fe availability as a factor constraining phytoplankton growth is not limited to the open ocean. Supporting Fe limitation in ‘non-traditional’ marine environments, Hutchins and colleagues described Fe limitation in terms of a ‘mosaic’ throughout the California coastal upwelling region (Hutchins and Bruland, 1998; Hutchins et al., 1998) and recently demonstrated Fe limitation associated with the Peruvian upwelling (Hutchins et al., 2002). Coastal Fe limitation has also been described for regions of the northern Japan Sea (Suzuki et al., 1995), the southern Ross Sea (Sedwick et al., 2000; Coale et al., 2003) and in a coral reef environment (Entsch et al., 1983).

Furthermore, Fe limitation is not restricted to the marine environment. It has long been recognized that some lakes are inherently subject to Fe limitation as a result of lake chemistry (e.g., hard-water calcareous lakes: Schelske, 1962; Schelske et al., 1962; Wetzel, 1966; prairie saline lakes of high alkalinity: Evans and Prepas, 1997). Consideration of Fe limitation for many lakes, however, has received little attention. Much of this is driven by the importance placed on phosphate as a limiting element of lacustrine systems. Taken together with the extent to which the Great Lakes are sub-

ject to anthropogenic influence (e.g., Dolan et al., 1993; Nriagu et al., 1996), trace metal deficiency is rarely afforded consideration when identifying factors limiting lake productivity. The view of metals as ‘toxic’ rather than ‘tonic’ was further reinforced by results of available field surveys that documented relatively high levels of dissolved trace metals in the Great Lakes system. The adoption of ultraclean metal sampling protocols by geochemists working on the Great Lakes, however, has resulted in a change in perception and reveals that dissolved levels of many metals are quite low and several are within the range commonly reported in the open ocean (Flegal et al., 1989; Nriagu et al., 1993, 1996). In general, dissolved Fe is reported to be present in low nanomolar concentrations (Rossmann and Barres, 1988; Nriagu et al., 1996; Twiss et al., 2000; Porta et al., 2003).

Renewed confidence in measurements of trace metal levels in the Great Lakes sheds light on the interpretation of enrichment bioassay results reported over two decades ago by Schelske and colleagues for various locations throughout the upper Great Lakes (Schelske et al., 1972, 1978; Stoermer et al., 1978; Lin and Schelske, 1981; Schelske and Sicko-Goad, 1990). These studies demonstrate that chelating agents and trace metals could enhance the phytoplankton growth response over that obtained by addition of phosphate alone in bioassays of natural lake water. However, because of prevailing attitudes regarding metal abundance, the positive response elicited by trace metals and chelating agents on phytoplankton growth was difficult to reconcile at the time their observations were made and it was unclear whether growth was promoted by decreasing metal toxicity or, alternatively, by increasing metal availability. Our current perspective on absolute trace metal levels lends support to the latter interpretation.

Chemical speciation of Fe in aquatic systems

Although total concentrations of dissolved trace metals provide a first-order indication of potential limitations to productivity, they do not directly relate to biological availability. The availability of a given trace metal depends ultimately on the chemical speciation of the element (Sunda, 1988/89; Campbell, 1995). Iron exists in solution in two oxidation states, Fe(II) and Fe(III), the latter of which is recognized as the thermodynamically stable form under oxic conditions. Whereas inorganic speciation of Fe(III) in seawater is dominated by hydrolysis products ($\text{Fe}(\text{OH})_3^{(3-i)}$)

(Byrne and Kester, 1976; Millero et al., 1995; Kuma et al., 1996; Byrne et al., 2000), the free hydrated ferric ion (Fe^{3+}) is the form that is conventionally recognized as relevant for direct uptake by phytoplankton. Further highlighting the difficulty in relating Fe chemistry to phytoplankton nutrient dynamics, there is no clear consensus on i) the extent of inorganic speciation of Fe(III) in situ, and ii) the solubility of Fe(III), estimates of which range from 0.1 nM to 10 nM in seawater (Wells et al., 1995; Kuma et al., 1998; Millero, 1998) depending on the concentration of dissolved organic Fe ligands present in the specific system.

Contributing to this lack of consensus is the wide range of detection methods that are currently employed. Atomic absorption spectroscopy or alternatively, the use of a mass spectrometer can quantify low nanomolar amounts of Fe but provide no information on redox speciation or ligand complexation. During the past decade, analytical voltammetry, in particular competitive ligand exchange—adsorptive cathodic stripping voltammetry (CLE-ACSV), has been used to quantify low levels of iron and also provide insight into Fe speciation in seawater (Gledhill and Van den Berg, 1994; Rue and Bruland, 1995, 1997; Wu and Luther, 1995; Witter et al., 2000). Arguably, the most significant outcome of this approach is the finding that the speciation of Fe and other trace metals in natural waters is dominated, not by inorganic forms, but by natural organic ligands. Results from these electrochemical approaches indicate that upwards of 99.9% of Fe(III) in ocean surface waters is complexed to organic ligands belonging to two major functional classes (the stronger 'L1' and the weaker 'L2' ligand classes) distinguished by their stability constants (Rue and Bruland, 1995, 1997; Lewis et al., 1995; Witter et al., 2000). A similar dominance of chemical speciation by strong organic ligands for other trace metals has been reported in both seawater (Zhang et al., 1990; Donat and Bruland, 1990; Bruland et al., 1991; Moffett, 1995; Saito and Moffett, 2001; Twiss and Moffett, 2002) and freshwaters (Xue et al., 1995; Xue and Sunda, 1997; Qian et al., 1998; M.R. Twiss, unpubl. data). Although the Fe-complexing ligands are of unknown type, they possess Fe-specific conditional stability constants within the range reported for siderophores and for the Fe-porphyrin moiety of cytochrome (Rue and Bruland, 1995, 1997; Witter et al., 2000). They may serve as a 'biological reservoir' of Fe when released from cells during grazing or viral lysis (Hutchins and Bruland, 1994; Hutchins, 1995; Hutchins et al., 1999; Wilhelm and Suttle, 1999).

That Fe speciation in seawater is dominated by organic complexation has substantially impacted our

understanding of phytoplankton—trace metal interactions. Upon accounting for organic complexation, the calculated equilibrium concentration of Fe' (the sum of dissolved inorganic species) is estimated to be in the low picomolar range, a value well below the concentration required to support diffusion-mediated delivery of Fe to the cell surface of nanoplankton (Hudson and Morel, 1993; Sunda and Huntsman, 1995) and perhaps even picoplankton. This comparison suggests that at least some fraction of the organically-complexed Fe must be accessible via direct or indirect means (Hutchins et al., 1999).

Fe biogeochemistry in lacustrine systems warrants additional consideration. First, the contribution of Fe(II) to the dissolved Fe pool is generally considered important only in association with lakes of low pH (McKnight et al., 1988; Auclair, 1995). Fe(II) is considered to be a biologically available form and its formation via reduction of Fe(III) is a common phenomenon in aquatic systems (Francko and Heath, 1982; Jones et al., 1987; Wells et al., 1995; Croot et al., 2001; Weger et al., 2002). In seawater, high concentrations of Cl^- and SO_4^- complex Fe(II) and retard its oxidation (Millero et al., 1987). As a result, transient accumulation of Fe(II) must be considered in marine systems. In contrast, rapid re-oxidation of Fe(II) is expected in most lakes since $[\text{Cl}^-]$ and $[\text{SO}_4^-]$ are much lower and hydroxide contributes to the oxidation of Fe(II) at the rate proportional to $[\text{OH}^-]^2$. An apparent exception to this trend is the recent observation from Lake Kinneret, Israel that phytoplankton assemblages both mediate the reduction of Fe(III) and the stabilization of Fe(II) resulting in the accumulation of nanomolar levels of reduced Fe in surface waters of this lake (Shaked et al., 2002).

Organic complexation of Fe and other trace metals in lakes also warrants special attention. As mentioned, the dominant Fe-complexing ligands of the open ocean are generally described in the context of biomolecules (e.g., siderophores, porphyrins) rather than allochthonous or autochthonous humic substances. Consistent with this, marine humic substances are of rather low abundance, rarely exceeding concentrations of 0.25 mg C l^{-1} (Harvey and Boran, 1985). In contrast, dissolved humic substances (fulvic acid, and to a lesser extent, humic acid) in freshwaters are generally more abundant ($>1 \text{ mg C l}^{-1}$) and comprise up to 80% of total dissolved organic C (Kemp and Johnston, 1979; Steinberg and Muenster, 1985). Although the Fe complexing ability of freshwater humic substances has been recognized for some time (Francko and Heath, 1982; Wetzel, 1983; Aiken et al., 1985; Francko, 1990), the bioavailability of

humic-complexed Fe to phytoplankton is unclear. Whereas stimulation of algal production has been reported coincident with the presence of high levels of aquatic humic substances (Prakash et al., 1973), other studies are consistent with an inhibitory role for humics (Jackson and Hecky, 1980; Guildford et al., 1987; Kawaguchi et al., 1997). The role played by humic substances is that affecting both the bulk water chemistry and also the chemistry of the cell surface (Campbell et al., 1997). Recently, Imai et al. (1999) provided a quantitative assessment of Fe(III) complexation by humic substances isolated from Lake Kasumigaura, Japan and demonstrated that Fe complexed to fulvic acid was not readily available to the toxic cyanobacterium *Microcystis aeruginosa*, concluding that organic complexation of Fe likely plays a role in regulating the formation of blooms of this species.

Dissolved Fe in the Great Lakes

For the Great Lakes, however, there is as yet little direct information about the composition or binding strength of potential soluble organic Fe ligands. A chemical speciation model (WHAM) has been used to estimate that >99% of the dissolved Fe species in Lake Erie are complexed to organic ligands (Twiss et al., 2000). This is consistent with voltammetric measurements indicating that >99% of dissolved Cu and Co are complexed to strong organic ligands in this lake (M. Saito, Woods Hole Oceanographic Inst., Woods Hole, MA, and M.R. Twiss, unpubl. data). Moreover, the results of recent Fe enrichment assays performed using trace metal-clean techniques at various locations throughout Lake Erie report variable stimulation of phytoplankton growth and increased photosynthetic efficiency following additions of Fe to lake water (Twiss et al., 2000).

For the most part, evidence for biological cycling of Fe and possible Fe deficiency comes from measurements of dissolved Fe concentrations and from bioassay experiments. The study of Nriagu et al. (1996) brought measurements of trace metals in the Great Lakes into the modern analytical era, producing accurate data for several biologically important elements for the first time. Concentrations of dissolved Fe were found to be 5 to 10 nM for pelagic waters of the central and eastern basins of Lake Erie (Nriagu et al., 1996; Twiss et al., 2000), and as low as 1 to 2 nM for the epilimnia of some isolated stations in Lakes Ontario and Superior. Very recently, a study employing higher resolution spatial and temporal sampling has re-investigated dissolved trace metal distributions

in western Lake Superior (Field and Sherrell, 2003; R.M. Sherrell, unpubl. data). The relatively large spatial gradients suggested by the earlier data of Nriagu et al. (1996) were confirmed, with the added dimension of seasonal variability in Lake Superior being investigated. Whereas dissolved Fe in the epilimnion was generally <6 nM, nearshore regions generally had elevated Fe, and it was possible to find quite low (<2 nM) dissolved Fe in waters just tens of km from nearshore sites where dissolved Fe in excess of 100 nM was measured. Seasonal variability was substantial as well. A temporal drawdown of dissolved Fe amounting to 2.0 to 2.5 nM at open lake sites between early June and late July, 2000 was about twice the drawdown of dissolved Zn over the same time period, the 2:1 ratio roughly consistent with estimates of metal quotas in marine plankton (Sunda and Huntsman, 1995) and with preliminary measurements of Fe:Zn ratios in several species of Lake Superior phytoplankton (R.M. Sherrell, unpubl. data) grown in media intended to mimic Lake Superior water. These findings, coupled with the observation of dissolved Fe minima at the depth of the sub-surface chlorophyll *a* maximum, suggest that distributions of both Fe and Zn may be driven by biological activity. Later in the same year, however, dissolved Fe increased at mid-lake sites, while Zn continued to decrease, indicating that Fe input events, either from the atmosphere or from lake-edge sources, can dominate biological effects on short time scales. Based on these chemical data, it seems likely that conditions for Fe deficiency would be found only at mid-lake sites under a particular set of conditions. However, it appears that the residence time of dissolved Fe is sufficiently short that such conditions could be met within a summer growing season.

While the lowest measured dissolved Fe concentrations in Lake Superior are higher than the subnanomolar concentrations measured in ocean regions characterized as Fe-limited, we cannot yet demonstrate whether the bioavailable Fe fractions are similarly scaled. The likely differences in the composition of organic Fe complexing molecules in fresh versus marine water, as discussed above, suggest as well that the bioavailable fraction could be smaller in the Great Lakes. Demonstration of biological Fe deficiency at these levels of dissolved Fe relies at present on bioassay approaches, which are well-developed, or biochemical approaches, which are still in their infancy.

Bioassays

As indicated by reference to previous work by Schelske and colleagues, the enrichment bioassay

approach to identifying limiting nutrients is not new. Recently, it has been used to assess both macro- and micronutrient limitation of phytoplankton and bacteria in western Lake Superior (Sterner et al., 2004). We demonstrated that even nominal increases in phytoplankton growth resulting from amendment with phosphorus induced Fe limitation. This suggests that phytoplankton in Lake Superior may be on the cusp of co-limitation by P and Fe. These experiments were conducted using trace metal clean techniques, and the results, along with those of others (Twiss et al., 2000), confirm earlier studies that indicated metal deficiency of freshwater phytoplankton in the Great Lakes.

Bioassays can be used not only to assess nutrient limitation, but the results of these experiments may also be analyzed in such a way as to assess the strength of the limitation (Sterner et al., 2004; Downing et al., 1999). Because organisms respond to nutrient limitation on different time scales, the length of time during which these experiments are conducted is an important consideration. In general, these experiments assess the responses of plankton to chronic nutrient limitation, as opposed to limitation occurring transiently.

Although experiments of this design have provided investigators the means with which to manipulate nutrient composition in a controlled manner, results must be interpreted with care (Schelske, 1984). Critics of this approach argue that ‘bottle assays’ do not adequately mimic the natural environment; grazing is disrupted, physical mixing is negated and phytoplankters are isolated at a fixed optical depth (Banse, 1991; Cullen, 1991; Carpenter, 1996). That biomass periodically increases, albeit to reduced levels, in unamended control bottles during enrichment experiments lends support to this view. Furthermore, despite the implementation of metal-clean protocols, concerns over contamination are always valid when applying this experimental approach to study phytoplankton-trace metal interactions.

In response to these concerns, effort has been directed in recent years toward developing chemical and biological approaches with which to directly or indirectly assess Fe bioavailability *in situ*. The remainder of this review focuses on some of the recent developments toward meeting this objective.

Chemical assessment of ‘labile’ Fe: A proxy for bioavailable Fe?

Several methods are employed by chemists to quantify ‘labile’ Fe. One approach is to measure labile Fe by incubating natural water samples with the chelat-

ing agent 8-hydroxyquinoline followed by retention of oxine-Fe complexes by an ion-exchange resin (Wells et al., 1991). Other procedures involve voltammetric assessment of labile species of Fe using CLE-ACSV. The basis of the CLE-ACSV technique is the addition of a known amount of an Fe-binding ligand that has a known thermodynamic stability constant for the Fe-ligand complex. The added ligand establishes equilibrium in solution between labile forms of Fe and Fe bound with other natural Fe-binding ligands. The amount of Fe bound to the added ligand can be determined voltammetrically by depositing the Fe-added ligand complex on a hanging mercury drop electrode (cathode). By scanning the voltage, the change in amperage at the reduction potential of Fe(III) can be measured and this is proportional to the concentration of the Fe-added ligand complex in solution. By titrating the sample containing the added ligand with Fe, the concentration of Fe³⁺ in addition to the effective natural Fe-binding ligand concentration and conditional stability constant can be determined. This method has been used effectively for several model ligands including 1-nitroso-2-naphthol (Van den Berg, 1995; Wu and Luther, 1995; Gledhill et al., 1998) and salicylaldoxime (Rue and Bruland, 1995). There have been no reports of CLE-ACSV measurements made of Fe speciation in fresh water.

The Diffusion Gradient in Thin-film (DGT) gel technique incorporates spatially separate ion-exchange resin and hydrogel matrix components in a diffusion-controlled system to assess labile trace metal species (Davison and Zhang, 1994). The technique has been used with modest success to predict labile Cu levels in impacted marine harbors (DGT response compared to CLE-ACSV; Twiss and Moffett, 2002) and was recently used to assess Mn levels in Lake Superior (Twiss et al., *in press*). However, in those environments where the Cu speciation is dominated by organic complexation, as seen in pristine environments, the DGT technique consistently overestimated labile [Cu] measured voltammetrically (Twiss and Moffett, 2002). These results imply that some metal-organic complexes may enter the probe and exchange the metal with that on the complexing resin surface. In addition, it has been reported that non-ideal diffusion of trace metal cations may occur within the gel exposed to a fresh water environment (A. Tessier, Centre Eau, Terre et Environnement, Université du Québec, pers. comm.) due to the counter-flux of ions from the resin. Despite the current problems with gel matrix selectivity and constraining diffusivity, the DGT technique may provide a readily useable technique for monitoring labile metal

concentrations in natural waters if metal flux through the hydrogels can be adequately described.

Despite advances in analytical chemistry, the key question remains: ‘is the biological availability of Fe directly related to its chemical lability?’ An attractive alternative is to look toward the organism to assess the bioavailability of a given element. By comparing chemical estimates of labile metal with the specific nutrient status of the endemic phytoplankters, a measure of bioavailability is afforded.

Assessing photosynthetic efficiency: Radiocarbon and fluorescence-based approaches

Measures of photoconversion efficiency as determined by ^{14}C photosynthesis-irradiance (P-I) curves have long been used to monitor the effects of environmental stress (including nutrient limitation) on phytoplankton physiology in both laboratory and field settings (reviewed by Cullen et al., 1992). Although providing a general measure of stress, this approach lacks diagnostic specificity and potentially diagnostic parameters of the P-I curve (P_{\max} , α) are not always informative (Cullen et al., 1992; Falkowski et al., 1992). Furthermore, the technique is labor intensive, susceptible to ‘bottle effects’ and generates radioactive waste.

An alternative approach, and one capable of providing a rapid, non-invasive assessment of photoconversion efficiency, is analysis of *in vivo* chlorophyll fluorescence. This approach exploits the inherent relationship between chlorophyll fluorescence and photosystem II (PSII) photochemistry for which a significant correlation has been reported both in the laboratory (Falkowski et al., 1986) and the field (Falkowski et al., 1991; Kolber and Falkowski, 1993; Boyd et al., 1997). Application of fluorescence technology in the field has been traditionally synonymous with the Turner field fluorometer (Turner Designs, Sunnyvale, CA) and data obtained from DCMU-enhanced *in vivo* chlorophyll fluorescence. This approach has been particularly useful for the determination of *in situ* photoinhibition (e.g., Putt et al., 1987), although it is also useful for assessing phytoplankton nutrient deficiency (Geider et al., 1993). Increasingly, additional properties of chlorophyll fluorescence have been characterized and exploited as diagnostic parameters of phytoplankton physiology, including nutrient status (Falkowski et al., 1992). Reflecting the Fe-rich biochemical composition of the photosynthetic electron transport chain (Raven, 1990), Fe

limitation imparts a characteristic signature upon the *in vivo* chlorophyll *a* fluorescence profile of phytoplankton. Recognizing this, Paul Falkowski and Zbigniew Kolber developed pump-and-probe (Falkowski et al., 1992; Falkowski and Kolber, 1995), and later, fast-repetition-rate fluorometry (FRRF; Falkowski and Kolber, 1995). This technology permits quantitative evaluation of the quantum efficiency of photochemistry (Φ_{PSII}), the effective absorption cross-section of PSII (σ_{PSII}) and rates of photosynthetic electron transport, each of which is influenced by Fe nutritional status. With consideration given to the light history of the cells and in choosing an appropriate blank (Cullen and Davis, 2003), FRRF can serve as a rapid diagnostic tool to detect Fe deficiency. Notably, this approach has been used with success to assess the phytoplankton response to Fe amendment in conjunction with several mesoscale ocean fertilization efforts (Behrenfeld et al., 1996; Boyd et al., 2000).

An inability to resolve taxon-specific response has been cited as a concern over use of the fluorescence approach. In response to this, German manufacturer Walz (Heinz Walz GmbH, Effeltrich, Germany) has developed the PHYTO-PAMTM, a pulse amplitude-modulated fluorometer capable of simultaneously measuring chlorophyll fluorescence and providing limited taxonomic resolution, differentiating between green algae, diatoms and cyanobacteria (Schreiber, 1998). They have also developed the MICROSCOPY-PAMTM, a combination epifluorescence microscope and fluorometer providing fluorescence kinetics analysis at the level of single cell (Snel and Dassen, 2000; Villareal and Morton, 2002) or even single chloroplast (Schreiber, 1998). These exciting developments should prove to be valuable diagnostic aids to oceanographers and limnologists alike.

Biochemical approaches

During periods of Fe deficiency, cellular changes influencing the net Fe quota of phytoplankton occur that can be used as diagnostics of the *in situ* Fe status of the population. This includes the photosynthetic electron transport components where flavodoxin (Flvd) replaces the Fe-containing electron transfer catalyst ferredoxin (Fd) as a means of relieving the cellular Fe burden in cyanobacteria (Straus, 1994) and numerous algae (Geider and La Roche, 1994). As such, Flvd is identified as a candidate biochemical marker of Fe deficiency in phytoplankton; its detection providing an *in situ* assessment of cellular Fe nutrition thereby avoiding the

complicating factors associated with manipulation experiments. Among criteria satisfied to serve in the role of diagnostic indicator, induction of Flvd synthesis is specific in response to Fe deficit (La Roche et al., 1993; Erdner et al., 1999). Further, accumulation of Flvd can be related to parameters of physiological limitation, especially when expressed in terms of a Fd Index, a parameter defined as relative cellular Fd/(Fd + Flvd) (Doucette et al., 1996; Erdner et al., 1999). Accumulation of Flvd and Fd in samples collected from the field is measured using high performance liquid chromatography (HPLC) (Doucette et al., 1996; Erdner and Anderson, 1999; Erdner et al., 1999) or alternatively, assessed by immunological means, either by Western blot (La Roche et al., 1993, 1995, 1996; McKay et al., 1997, 1999, 2000; Davey and Geider, 2001; Maldonado et al., 2001; Xia et al., 2003) or single-cell immunofluorescence (La Roche et al., 1996; Boyd et al., 2000). Whereas HPLC detection carries no bias with respect to phylogeny and measures total community response to endemic levels of Fe, immunoassay is generally more selective, providing a measure of taxonomic resolution. This is particularly true if the immunofluorescence approach is adopted where immunoassay is combined with microscopic analysis of intact cells. The immunological approach is also generally more sensitive; depending on antibody affinity, detection limits can be 3 orders of magnitude lower than that achieved with HPLC (femtomolar vs. picomolar). This limits application of the HPLC approach when dealing with oligotrophic waters where difficulties in sampling sufficient amounts of biomass are likely to be encountered (Erdner and Anderson, 1999).

Extending the immunological approach to preliminary studies of Fe bioavailability in the Great Lakes has been facilitated by the availability of appropriate antisera. Despite initial concerns over the efficacy of a ‘marine’ probe to study Fe deficiency in freshwater systems, antisera directed against flavodoxin (La Roche et al., 1995) isolated from a marine diatom cross-react with antigens of appropriate size in freshwater diatoms (Figure 1). Antisera directed against diatom ferredoxin (McKay et al., 1999) are similarly cross-reactive with antigen from freshwater forms. These antisera have been used to demonstrate the presence of a Fe-replete diatom assemblage during the period of vernal mixing in Lake Superior (R.M. McKay, unpubl. data) and have been similarly used to demonstrate a nearshore—offshore gradient in Fe deficiency along a transect located perpendicular to the Keweenaw Peninsula during mid-summer, due to the increased levels of flavodoxin detected in extracts of diatoms retrieved at

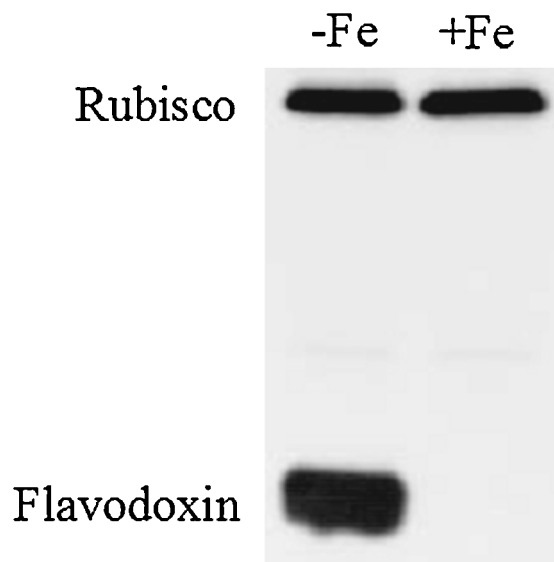


Figure 1. Western immunoblot showing the Fe-responsive accumulation of flavodoxin in a freshwater diatom, *Cyclotella* sp. UTCC 432. Flavodoxin accumulates in cells from Fe-deficient culture (–Fe) but not in cells from Fe-replete culture (+Fe). A constitutively expressed protein (large subunit of Rubisco) is detected in cells from each culture and serves as a procedural control.

offshore stations (Figure 2, left two lanes). This pattern of staining is interpreted as meaning that diatoms sampled from offshore waters were exposed to Fe deficiency to a greater degree than those sampled closer to shore and may reflect a higher terrigenous particle base constrained close to shore by the strong Keweenaw current that sweeps up the peninsula.

Similarly, antibodies directed against flavodoxin and ferredoxin purified from both freshwater cyanobacteria (Yakunin et al., 1993a,b) and chlorophytes (Inda and Peleato, 2002; Moseley et al., 2002) should prove useful in a diagnostic capacity. Considered together, these markers provide a measure of taxonomic resolution in specifically recognizing diatoms, cyanophytes and green algae. Additional antisera may similarly prove useful in assessing phytoplankton Fe status or the nutritional status of other trace metals. For example, immunological detection of the *idiA* gene product was recently used to demonstrate Fe deficiency among marine cyanobacteria (Webb et al., 2001). Likewise, antisera recognizing the Fe and Mn isoforms of superoxide dismutase (SOD) (Matta et al., 1992) may be informative as an additional probe of phytoplankton Fe status, and perhaps simultaneously of Mn status. This serum preparation is broadly reactive, directed against antigen purified from *Escherichia coli*, yet

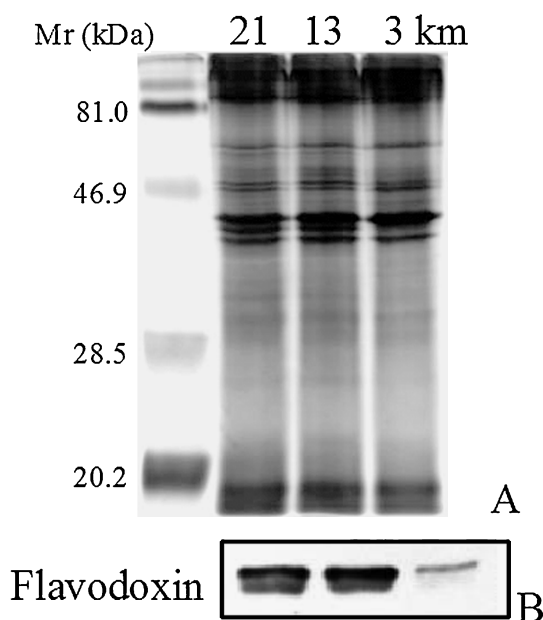


Figure 2. A nearshore—offshore profile of flavodoxin accumulation in diatoms collected along a transect extending from the mouth of the Keweenaw Waterway which bisects the Keweenaw Peninsula. Samples collected during a July, 1998 research cruise using a plankton net (75 μm mesh) at hydrographic stations located 3 km, 13 km and 21 km from shore were processed and analyzed as described in McKay et al. (1999). Flavodoxin accumulated to higher levels in diatoms collected from offshore sites compared to the nearshore (3 km) site suggesting the existence of a nearshore—offshore gradient in Fe deficiency. (A) Profile of total soluble proteins resolved by SDS-PAGE (15% acrylamide) and silver stained. Location of pre-stained M_r standards (Bio-Rad, low range) is shown. (B) Corresponding immunoblot stained with anti-flavodoxin and detected by chemiluminescence. Flavodoxin has an apparent $M_r \sim 24$ kDa.

recognizing SOD isoforms from diverse phytoplankton (Matta et al., 1992; R.M. McKay, unpubl. data).

A more conventional biochemical approach is assay of ectoenzyme activity, in this case ferric chelate reductase. It was suggested recently that assay of ferric chelate reductase (FCR) activity can serve in a diagnostic capacity to determine Fe deficiency among phytoplankton that use Strategy I Fe acquisition (Weger, 1999; Weger et al., 2002). This strategy has been documented for a variety of green algae (as reviewed in Weger et al., 2002), diatoms (Maldonado and Price, 2000) and for the brown tide pelagophyte *Aureococcus anophagefferens* (Nichols et al., 2001). The strategy involves enzymatic reduction of ferric chelate complexes at the cell surface with resulting transport of Fe(II) across the cell membrane (Schmidt, 1999). The assay involves spectrophotometric determination of ferrous-

chelate complexes (bathophenanthroline disulphonate or ferrozine) following enzymatic reduction of the ferric moiety (Weger, 1999). Alternatively, extracellular reduction of Fe(III) to Fe(II) can be measured directly by chemiluminescence detection (Maldonado and Price, 2000). Iron deficiency results in the induction of FCR activity, although curiously, for several taxa assessed, enzyme activity was maximal under moderate Fe deficiency with activity decreasing under more severe Fe deficit (Weger, 1999; Weger et al., 2002). This pattern of activity may limit the broad application of this approach since in several instances, it was reported that cells under severe Fe deficiency showed levels of FCR activity only marginally higher than in Fe replete cells (Weger, 1999; Weger et al., 2002).

A luminescent bioreporter of Fe?

Arguably the most direct approach to assessing Fe bioavailability is through construction of cell-based reporter systems. In general, such bioreporters yield a detectable and quantifiable signal (usually bioluminescence or fluorescence) when an environmental stress is applied to the cell. In the case of Fe, a cell would be engineered so that it emits light when it experiences Fe deficiency. This approach has been used successfully for the specific detection of toxic metals and xenobiotics in the environment (as reviewed by van der Lelie et al., 1994; Daunert et al., 2000; Kohler et al., 2000) and more recently has been adopted to detect nutrient deficiency in aquatic systems (Gillor et al., 2002, 2003; Bachmann, 2003; Wilhelm et al., 2003). Likewise, several groups have adopted this approach to develop a microbial bioreporter of Fe for use in the rhizosphere (Loper and Lindow, 1994, 1996, 1997; Khang et al., 1997; Yong-Ho et al., 1997). The availability of Fe in soil, much like that in water, is affected by a multitude of factors and is not readily evaluated using conventional analytical methods. By applying contemporary principles of genetic engineering, however, evaluation of biologically available Fe in this microenvironment is made possible.

Toward developing bioreporters of Fe for use in the aquatic environment, a number of factors must be considered in choosing an organism and reporter system appropriate for the task. Foremost, Fe-responsive genes and their regulatory elements must be identified and the organism must be amenable to genetic manipulation. Satisfying both criteria are freshwater cyanophytes, particularly of the genus *Synechococcus*. The Fe stress response of *Synechococcus* has been well-characterized (Riethman et al., 1988), and

Fe-regulated genes and their regulatory elements have been identified (Straus, 1994; Ghasseman and Straus, 1996). Notably, many of the promoter elements of these genes bear a sequence resembling the *fur* (ferric uptake regulator) consensus sequence of *E. coli*, a feature ensuring transcriptional control of these genes by intracellular Fe(II) levels (Straus, 1994). Other Fe-regulated genes (*dpsA*, *idiA*, *irpA*) appear to be under the control of other transcription factors (Sen et al., 2000; Michel et al., 2001; Durham et al., 2003) and afford additional opportunities for bioreporter construction. In addition, *Synechococcus* PCC 7942 is amenable to genetic manipulation by high-frequency genetic transformation. A further consideration should be the ecological relevance of the organism to the study area. Picoplanktonic cyanophytes, such as *Synechococcus* are an important component of lacustrine systems, both in terms of their contribution to biomass and overall production (Stockner and Antia, 1986; Fahnenstiel and Carrick, 1992; Carrick and Schelske, 1997). This is particularly applicable to oligotrophic systems such as Lake Superior.

In choosing a reporter system for the Fe bioreporter, two important factors must be considered: i) the assay is designed for use in the field and ii) it must accommodate detection of gene expression from low amounts of biomass. The first criterion is readily satisfied by any number of systems. Satisfying the latter criterion, however, is realized using a high sensitivity luminescent reporter such as bacterial luciferase (*luxAB*; Chatterjee and Meighen, 1995), aequorin (Falciatore et al., 2000) or alternatively, green fluorescent protein (GFP) (Chalfie, 1995). A natural product of marine cnidarians, GFP is now exploited as a trans-kingdom marker of gene expression as well as a tag for localizing fusion proteins (Cubitt et al., 1995). In either case, bioreporter response to metal availability can be ascertained in situ using a luminometer or a fluorometer. In addition, luminescence or fluorescence can be calibrated in the laboratory where Fe chemistry can be controlled. Ancillary measures of dissolved Fe and speciation in the field will be helpful in interpreting bioreporter response.

In this direction, we recently developed a series of luminescent cyanobacterial bioreporters designed to detect bioavailable forms of Fe in freshwater environments (Durham et al., 2002, 2003; Porta et al., 2003). In each case, we introduced into *Synechococcus* sp. PCC 7942 an iron-regulated promoter fused to the *Vibrio harveyi luxAB* luciferase genes. Whereas each of the promoter fusions that we constructed (*dpsA*, *isiAB* and *irpA*) was shown to be Fe responsive, the *isiAB* promoter fusion provided the most robust lumi-

nescent signal in response to low Fe availability. This is consistent with reported high, steady-state levels of *isiAB* transcription in cultures of the cyanobacterium *Synechocystis* PCC 6803 rendered Fe deficient (Kunert et al., 2000). Moreover, it was recently reported that this gene is widely distributed among strains in taxonomic Sections I–V of the cyanobacteria and that it might serve as an appropriate marker for Fe deficiency in the natural environment (Giess et al., 2001). For these reasons, we chose the *psiAB* fusion (strain KAS101) for field-testing (Durham et al., 2002; Porta et al., 2003). Detailed physiological characterization of this construct yielded the experimental conditions under which a rapid and reproducible luminescent signal could be obtained under field and laboratory conditions (Porta et al., 2003). To date, we have tested the Fe bioreporter with water sampled from Lake Erie (Durham et al., 2002), Lake Huron (Porta et al., 2003) and Lake Superior (Table 1). We observed differences in Fe availability between nearshore and offshore locations in Lake Superior. Illustrating this, the nearshore-offshore transect running perpendicular to the Keweenaw Peninsula highlighted by data presented in Figure 2, showed higher Fe availability nearshore (1 km) compared to a station located 21 km offshore (Table 1). This was manifested through a luminescent response one order of magnitude higher at the offshore station compared to the nearshore site (Table 1). Consistent with these results were measures of dissolved Fe from these stations with epilimnetic water sampled from the nearshore station containing more than double the concentration of Fe compared

Table 1. A nearshore—offshore comparison of bioreporter response along a transect extending from the mouth of the Keweenaw Waterway which bisects the Keweenaw Peninsula. Samples collected during a July, 2001 research cruise using a metal clean pump system (Field and Sherrell, 2003) at hydrographic stations located 1 km and 21 km from shore were filtered in-line through a 0.45 μ m pore size polypropylene cartridge filter and frozen in acid-rinsed polycarbonate bottles. Dissolved Fe was determined on sub-samples by HR-ICP-MS (Field and Sherrell, 2003) and bioreporter luminescent response was assayed essentially as described by Porta et al. (2003).

Station	Depth	RLU ¹	[Fe] (nM)
HN010 ² (1 km)	5 m ³	3.76 \pm 0.6 ⁴	23.7
HN210 ² (21 km)	5 m ³	34.88 \pm 14	10.9

¹RLU: Relative luminescent units per second normalized to in vivo chlorophyll fluorescence of the bioreporter.

²HN: Hancock-North transect.

³Epilimnion.

⁴Mean \pm SD.

to the offshore site. Taken together with the results obtained by the immunoblotting approach (Figure 2), a clear gradient in Fe availability along this nearshore-offshore transect was apparent for both eukaryotic (Figure 2) and prokaryotic organisms (Table 1).

Whereas our initial efforts in developing a Fe bioreporter have been focused on using the *isiAB* promoter, we plan to conduct parallel work using *idiA*, which has been identified as a candidate marker for Fe deficiency in cyanophytes (Webb et al., 2001) as well as *irpA* (Durham et al., 2003), *dpsA* (Sen et al., 2000) and *mapA* (Webb et al., 1994). As demonstrated through the dose-response characterization (Porta et al., 2003), it is anticipated that each promoter construct will have a limited dynamic range over which its respective bioreporter will serve as a quantitative tool. Thus the use of several bioreporters, each developed using a different promoter fusion, can be viewed as a complementary approach that will serve to increase the overall dynamic range of bioreporter response.

Along the same lines, parallel studies have resulted in the construction of a similar bioluminescent reporter system in heterotrophic bacteria (Mioni et al., 2003). This base construct, contained in a mobile transpososome, involves the regulation of the *luxCDABE* cassette by the promoter of a siderophore (Enterobactin) biosynthesis gene regulated by the FUR protein of the host cell. Construction on the transpososome has the added advantage of making the reporter cassette highly mobile: it can be mated into different strains of heterotrophic prokaryotes and as such provide a reporter for a variety of different microbes. Although no tests have been as-of-yet carried out in Lake Superior, studies in Lake Erie have demonstrated the utility of this tool in distinguishing between growth limitation by dissolved organic material (DOM) and Fe. One interesting finding has been the importance of particulate ($>0.2 \mu\text{m}$) material in the supply of bioavailable Fe: results from studies during thermal stratification have demonstrated that a significant portion of the bioavailable Fe in the lake is associated with the $>0.2 \mu\text{m}$ particulate size class (Mioni et al., 2003).

We believe the bioreporter approach provides advantages over alternative approaches. Whereas the chemical approaches assess chemical 'lability' of Fe, it is not clear that this is equivalent to 'bioavailability.' A major advantage of a bioreporter over the biochemical and immunoassay techniques is that concerning problems of accumulating biomass sufficient for the latter. Whereas larger plankton can be concentrated using

nets, collecting sufficient picoplankton for biochemical analysis requires large volume filtration (Erdner and Anderson, 1999) or concentration using vortex (Paul et al., 1999) or tangential flow filtration units (R.M. McKay, unpubl. data). Even with filtration of upwards of 600 L of seawater, Erdner and Anderson (1999) were unable to detect ferredoxin or flavodoxin signals from a picoplankton-dominated open ocean environment.

We also believe the bioreporter approach provides an advantage over that using a model organism whose growth and physiological characteristics following inoculation into natural water are monitored (e.g., Timmermans et al., 2001a,b). Specifically, monitoring the growth and physiological response of a model organism may yield a situation in which the physiological response of the inoculated organism will alter the chemical properties of the water over time. Over an extended period (as little as 1–2 d) the model organism may affect the pH (which will affect Fe availability) and draw down Fe and other nutrients as its biomass increases. Potential ligand production by the model organism is also expected to alter Fe availability. In contrast, the much shorter response time (within hours) of a suitably characterized bioreporter should ensure that the 'biology reflects the chemistry' of the system rather than the biology acting to 'modify' the chemistry.

The development of a bioreporter for bioavailable Fe represents a compromise taking into consideration several factors. Foremost, one is confronted with present limitations on the genetic engineering of photoautotrophs, particularly eukaryotic forms (Stevens and Purton, 1997). An exception to this observation is the green chlorophyte *Chlamydomonas reinhardtii* where the development of nuclear and organellar transformation systems is highly advanced. The ecological relevance of this alga to natural aquatic systems, however, is questionable. Recent success at stable nuclear transformation of marine diatoms (as reviewed by Falcatore et al., 1999; Roessler, 2000; Zaslavskaja et al., 2001) is an exciting development and offers the potential of a complementary bioreporter system representative of the diversity of Fe acquisition strategies exhibited by phytoplankton (Hutchins et al., 1999). Falcatore et al. (2000) describe a transgenic diatom that detects and responds to broad physiochemical stimuli, including variations in external Fe content, using sensing systems based on changes in cytosolic $[\text{Ca}^{2+}]$. Notably, transient changes in cytosolic $[\text{Ca}^{2+}]$ were not observed in response to fluctuations in the external levels of major nutrients (N, P and Si), only to variable $[\text{Fe}]$.

Concluding remarks

There are many areas in which future efforts should be directed. For example, the accurate assessment of labile Fe in freshwater by CLE-ACSV will help in the identification of the bioavailable Fe species present. Secondly, whereas the Fe bioreporter system described here has been very useful, it exhibits a restricted dynamic range for detection of bioavailable Fe. Construction of second generation bioreporters, employing different Fe-responsive promoters, will provide a clearer picture with respect to the degree of Fe deficiency experienced by phytoplankton. Lastly, experiments in which bioreporter cells are tested against different size fractions in water will determine to what degree regeneration contributes to the pool of bioavailable Fe in the Great Lakes.

Acknowledgments

This material is based upon work supported by the National Science Foundation under Grant numbers OCE-9911592 and DBI-0070334 (R.M.L.M. and G.S.B.), MCB-9634049 (G.S.B.), OCE-000298 (S.W.W.) and OCE-9902658 (R.M.L.M., R.M.S., E.T.B. and R.W.S.). Additional support for preliminary data behind many of the ideas presented in this contribution comes from Ohio Sea Grant under contract number R/ER-49-PD (R.M.L.M.), the Natural Sciences and Engineering Research Council of Canada (M.R.T.), Environment Canada and the University of Tennessee Office of Research (S.W.W.). R.M.L.M. would like also to thank Sarah Green (Michigan Technological University) for the opportunity to collect field samples as part of the NSF-CoOP KITES project. The authors thank the captains and crew of the *R/V Laurentian* and the *R/V Blue Heron* for assistance with field collections.

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