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Manipulating iron availability in nearshore waters

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Abstract

The consideration of iron effects on marine ecosystems has focused mainly on high-nitrate low-chlorophyll regions, but iron likely has an equally important regulatory role in coastal waters. Iron requirements of neritic phytoplankton not only are comparatively high but also differ substantially among species, so that iron fluctuations within metal-replete systems should strongly influence the composition and distribution of phytoplankton assemblages. But unlike the simplicity of testing iron effects in iron-depleted waters, ascertaining iron effects in apparently replete waters has been forestalled by a lack of experimental tools that can regulate iron availability independent of other bioactive metals in seawater. I present here the results of size-fractionated shipboard culture experiments using the fungal siderophore desferriferrioxime B (DFB) to regulate iron availability in coastal upwelling waters. Addition of excess DFB essentially eliminated ^{59}Fe uptake by both phytoplankton and heterotrophic bacteria over a 6-h period and allowed only marginal iron uptake over 5 d of incubation. Carbon uptake by small (0.2–5.0- μm) phytoplankton was immediately curtailed upon addition of DFB, signifying a rapid onset of iron stress. In contrast, short-term (0–6 h) carbon uptake by larger (>5.0- μm) phytoplankton was not affected, indicating that larger cells contained significant iron reserves. Nonetheless, carbon assimilation was substantially lower in DFB treatments relative to the controls after 5 d of incubation. Uptake of ^{54}Mn , ^{65}Zn , and ^{57}Co was not immediately affected by DFB but then slowed after 4 h and was significantly lower after 5 d, presumably because iron limitation lowered the cellular requirements for these bioactive metals. These findings demonstrate that DFB can be used to manipulate biologically accessible iron to determine how iron affects algal community structure and carbon cycling in iron-replete waters.

It is now well demonstrated that low concentrations of iron play a pivotal role in controlling primary production in open ocean high-nitrate low-chlorophyll (HNLC) regions (Martin and Fitzwater 1988; Martin et al. 1990; Coale et al. 1996) and in upwelling HNLC regions along the California coast (Hutchins and Bruland 1998). Heterotrophic bacterial production in HNLC waters also can be directly limited by iron (Pakulski et al. 1996), demonstrating that iron can profoundly influence both the production and remineralization segments of the marine carbon cycle (Tortell et al. 1996). But even in regions where iron additions have little or no impact on community-level production, iron still should affect the composition of algal assemblages and the distribution of phytoplankton species because iron requirements differ dramatically among phytoplankton (and presumably bacteria) species (Brand et al. 1983; Brand 1991; Sunda and Huntsman 1995). As a result, temporal and spatial variations in the concentration and chemical speciation of iron should likely influence the character and expression of phytoplankton species succession (Wells et al. 1995).

Ascertaining whether iron limits phytoplankton production in surface waters is experimentally straightforward; iron is added to on-deck incubations of the seawater, and the resultant phytoplankton response is monitored. The challenge in these experiments is the consistent elimination of iron contamination so that the controls indeed reflect ambient seawater conditions. However, it has been considerably more difficult to probe iron effects on algal composition and

trophic relationships in regimes where iron additions have little effect on community-level production. What is needed to facilitate testing of iron-related hypotheses in apparently iron-replete systems is the ability to regulate iron availability independent of other bioactive metals.

One novel approach to achieve this level of discrimination is to use a metal-specific chelator that renders iron unavailable to the microbial community. If the conditional stability constant of the iron complex were well defined, one could vary the chelator additions to induce accurate and reproducibly different intensities of iron stress in natural population cultures. The phytoplankton species and community-level responses to these manipulations would then provide keen insight to the effects that natural iron fluctuations exert on both autotrophic and heterotrophic microbial communities.

Wells et al. (1994) utilized such an iron-removal approach to investigate whether picoplankton in equatorial Pacific waters was limited by iron. They found that addition of excess concentrations of the fungal siderophore desferriferrioxime B (DFB) essentially eliminated iron uptake in this picoplankton-dominated community. DFB (sold commercially under the tradename Desferal) is a small trihydroxamate molecule that specifically complexes inorganic Fe(III) with an extremely high conditional stability constant in seawater ($K_{\text{FeL,Fe}}^{\text{cond}} = 10^{16.5} \text{ M}^{-1}$; Rue and Bruland 1995). However, the efficacy of using this terrestrially derived siderophore to regulate iron availability in seawaters has been brought into serious question by recent work suggesting that a marine diatom clone (Soria-Dengg and Horstmann 1995) and phytoplankton communities in the subarctic Pacific (Maldonado and Price 1999) can utilize iron bound to DFB.

I present here the results of natural population incubation experiments from coastal upwelling waters off the central California coast where DFB was added to curtail iron assim-

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ilation. Short-term (h) and long-term (d) iron uptake was measured with and without DFB additions in two size fractions broadly representative of: (1) heterotrophic bacteria, picoplankton, and nanoplankton (the ultraplankton); and (2) microplankton communities. In addition, the indirect influence of DFB-induced iron stress on phytoplankton assimilation rates of Mn, Zn, Co, and Cd also was measured. These findings are used to explore new manipulative strategies for investigating the effects of iron stress in coastal waters or other iron-replete regimes.

Materials and methods

Water collection—Surface seawater was collected for the incubation experiments approximately 1 km off the California and Oregon coasts at 38°41'N (long-term experiment) and 42°33'N (short-term experiment) in the early mornings of 26 June and 2 July 1997, respectively. Seawater was pumped from ~11 m depth through 9.6-mm inner diameter Teflon tubing using an all-Teflon double diaphragm pump at a flow rate of ~4 liters min⁻¹. The tubing intake was attached to a nylon line suspended from the forward boom extended 6 m away from the ship to avoid contamination from the hull. The nylon line and tubing were kept taut with a plastic encased weight attached 2 m below the plastic vane and tubing intake. The vane ensured that the sample intake was directed ahead of the nylon rigging line as the ship moved forward.

After flushing the sample tubing with seawater for several minutes, sample water was pumped into a 50-liter polyethylene carboy and homogenized. The 500-ml polycarbonate incubation bottles were rinsed and filled from this homogenized sample. Two opaque polyethylene bottles also were rinsed and filled for the dark controls.

All sample tubing and containers were thoroughly acid washed before use by sequentially soaking in trace-metal grade 6 N HCl and 7 N HNO₃ for 1–2 weeks. All container rinsing, sample transfer, and isotope additions (below) were done under a clean air bench (Class 100 conditions) using standard trace-metal clean handling precautions.

Metal isotope mix—A mixed metal isotope stock containing ⁵⁹Fe, ⁵⁴Mn, ⁶⁵Zn, ⁵⁷Co, and ¹⁰⁹Cd (New England Nuclear) at 1 μCi ml⁻¹ for each was prepared in acidified (pH 2) deionized (Milli-Q) water. For the light treatments, 0.5 ml of this isotope stock was added to each 500-ml polycarbonate bottle along with 10 μCi of ¹⁴C bicarbonate solution (New England Nuclear). The total metal additions with this isotope solution, calculated from the specific activities of the primary stocks, were 200 pM Fe, 52 pM Mn, 119 pM Zn, and 0.6 pM Co, values ~10–100× lower than typical concentrations found in coastal waters. Half the bottles received additions of 100 nM DFB (Sigma). Equilibrium calculations indicate that even with dissolved iron concentrations up to 10 nM inorganic Fe(III) species would be limited to ~10⁻¹⁶ M, or several orders of magnitude below the levels needed to support phytoplankton growth. Dark controls for measuring the dark assimilation of ¹⁴C bicarbonate were prepared with and without DFB addition (but no metal isotope additions).

The amended controls and treatments were vacuum sealed in polyethylene bags and transferred to an on-deck flowing seawater incubator. The incubator, designed for use with 20-liter carboys, is fabricated of 12.8-mm-thick plexiglass and lined with 3.2-mm-thick blue plexiglass to simulate the light intensity and spectral composition at the 50% incident light depth in the water column.

For the short-term bioassay, replicate DFB treatment and control bottles were withdrawn at 2, 4, and 6 h after isotope addition. Subsamples of each replicate were gently vacuum filtered (≤5 mm Hg) onto 47-mm-diameter 0.2-μm and 5.0-μm pore-sized polycarbonate (Poretics) filters and allowed to stand for 3 min in Ti(III) ethylenediaminetetraacetate (EDTA)–citrate reducing solution to desorb metals from cell and particle surfaces (Hudson and Morel 1989). Filters were then rinsed three times with filtered seawater, transferred to 47-mm-diameter sealing petri dishes, and frozen until analysis. The dark controls were withdrawn after 6 h, filtered (0.2 μm and 5.0 μm), washed with the Ti(III) EDTA–citrate solution, and transferred to 6-ml plastic scintillation vials.

A replicate short-term bioassay experiment was aborted after the first sampling time because the increasing sea state made filtration of the isotope-spiked solutions unwise. However, one set of these light treatments was left in the incubator and harvested 5 d later (as described above). The net uptake of carbon and metals in this incubation illustrates the long-term effect of DFB; however, uptake values are not quantitatively comparable to results from the short-term experiment because phytoplankton abundances differed between these coastal sites.

The intracellular ⁵⁹Fe, ⁵⁴Mn, ⁶⁵Zn, ⁵⁷Co, and ¹⁰⁹Cd gamma activities were measured simultaneously by placing the petri dishes containing the flat filters directly onto an intrinsic germanium detector. The measured values were corrected for losses due to decay and were converted to isotope uptake (pmol ml⁻¹). The filters were then folded, transferred into 6-ml scintillation vials containing fluor, and the ¹⁴C uptake measured by liquid scintillation. Values were corrected for quench and also for the contribution of beta and electron capture emission of the metal isotopes. For the latter correction, a series of separate standards was prepared for each isotope in scintillation vials and counted by both liquid scintillation and gamma detection to determine the linear relationships between metal isotope activity (gamma counts) and apparent ¹⁴C activity (liquid scintillation counts). The sample filters in the 6-ml scintillation vials then were recounted by gamma detection, the different proximity of the folded filters to the detector yielding lower counts than initial measurements made with flat filters in the petri dishes. These gamma measurements were then converted to liquid scintillation counts using the calibration curves determined above. The contributions from the individual metal isotopes were then summed for each sample filter and subtracted from the total apparent ¹⁴C counts to give the actual ¹⁴C activity for that sample filter. Precision of the ¹⁴C counts (±15%) estimated from the worst case propagation of errors is therefore less than for standard ¹⁴C analyses.

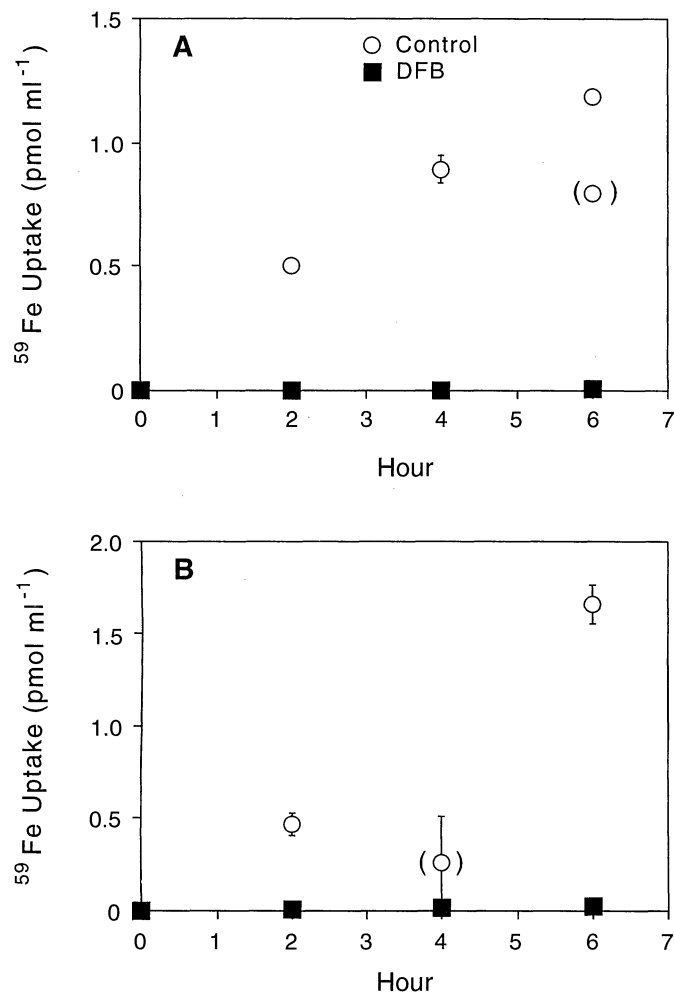


Fig. 1. Short-term ^{59}Fe uptake in controls and treatments containing an excess (100 nM) of DFB: (A) $>5.0\ \mu\text{m}$; (B) $0.2\text{--}5.0\ \mu\text{m}$. Error bars give the range of replicate measurements where visible.

Results

Size-fractionated iron uptake—Total ^{59}Fe uptake in the controls was split roughly equally between the large ($>5.0\ \mu\text{m}$) and small ($0.2\text{--}5.0\ \mu\text{m}$) size fractions, with uptake rates being linear over the first 6 h (Fig. 1). Based on a measured detector efficiency of 2%, the combined ^{59}Fe assimilation rate of both size fractions was $\sim 0.5\ \text{pmol h}^{-1}$. In contrast, there was no detectable assimilation of ^{59}Fe in the DFB treatments in either size fractions (Fig. 1). The effect of DFB on Fe uptake persisted over at least 5 d, with ^{59}Fe assimilation in the DFB treatments of the second bioassay being $\sim 4\%$ of that measured in the controls (Fig. 2). Absolute ^{59}Fe uptake of controls in the second, long-term bioassay was significantly lower than in the short-term bioassay, but uptake again was split equally between large ($>5.0\text{--}\mu\text{m}$) and small ($0.2\text{--}5.0\text{--}\mu\text{m}$) organisms.

Effect on C assimilation—Carbon assimilation increased exponentially ($R^2 > 0.97$) in all controls and treatments over the 6-h incubation, demonstrating that the population was

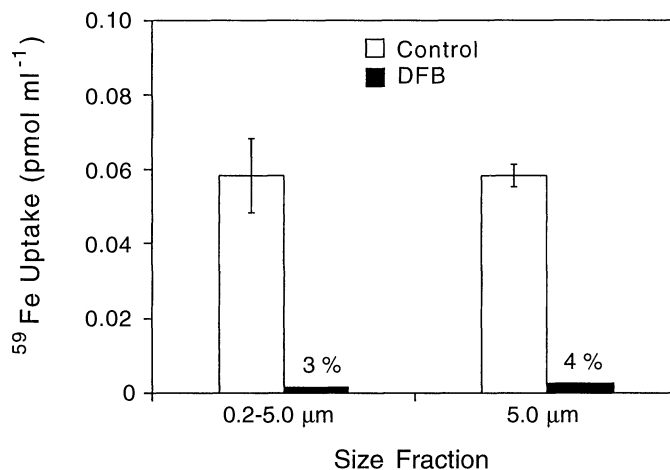


Fig. 2. The percent decrease of net ^{59}Fe uptake in DFB treatments after 5 d of incubation: control and DFB treatment. Error bars give the range of replicate measurements where visible.

actively growing from the start (Fig. 3a,b). Net carbon uptake in the $0.2\text{--}5.0\text{--}\mu\text{m}$ controls was roughly double that in the $>5.0\text{--}\mu\text{m}$ fraction by the end of 6 h. Addition of DFB had no discernable impact on short-term carbon uptake in the large size class but decreased uptake in the small phytoplankton class by a factor of 5 after 6 h.

There was less difference in net carbon assimilated by the two size classes in controls of the long-term bioassay, with uptake in the $>5.0\text{--}\mu\text{m}$ fraction being 25% lower than in the $0.2\text{--}5.0\text{--}\mu\text{m}$ fraction (Fig. 4). Net carbon assimilation by the two size classes in the DFB treatments was nearly identical and was $<20\%$ of that in their respective controls.

Effect of DFB addition on the uptake of other bioactive metals—In contrast to the linear increase for ^{59}Fe , uptake of ^{54}Mn , ^{65}Zn , and ^{57}Co in the controls was exponential ($R^2 > 0.96$), with the absolute uptake for each tracer increasing dramatically between 4 and 6 h (Fig. 5a-f). This increase occurred in both large and small size classes but was most intense for smaller cells. The short-term uptake of ^{109}Cd was below our detection for the sample volumes filtered.

Addition of DFB had little discernable impact on the uptake of ^{54}Mn , ^{65}Zn , or ^{57}Co over the first 4 h of incubation. Between 4 and 6 h, metal uptake in the DFB treatments was lower relative to the controls, with the exception of ^{57}Co uptake in the $>5.0\text{--}\mu\text{m}$ fraction (Fig. 5a-f). Uptake of these tracers also was exponential in most cases but at lower rates than in the controls. Of the bioactive tracers measured, DFB had the largest impact on ^{54}Mn , decreasing its uptake by 80% ($0.2\text{--}5.0\ \mu\text{m}$) and 65% ($>5.0\ \mu\text{m}$) compared to the controls. DFB also reduced ^{65}Zn and ^{57}Co uptake by $\sim 50\%$ in the $0.2\text{--}5.0\text{--}\mu\text{m}$ DFB fraction but had a much smaller effect on the $>5.0\text{--}\mu\text{m}$ cells. It is important to note that because the specific activity of these isotopes in the starting seawater is not known, the measured uptake of these tracers does not accurately reflect differences in absolute metal assimilation; that is, the reason ^{54}Mn uptake is lower than ^{65}Zn is because ambient dissolved Mn concentrations are much higher than

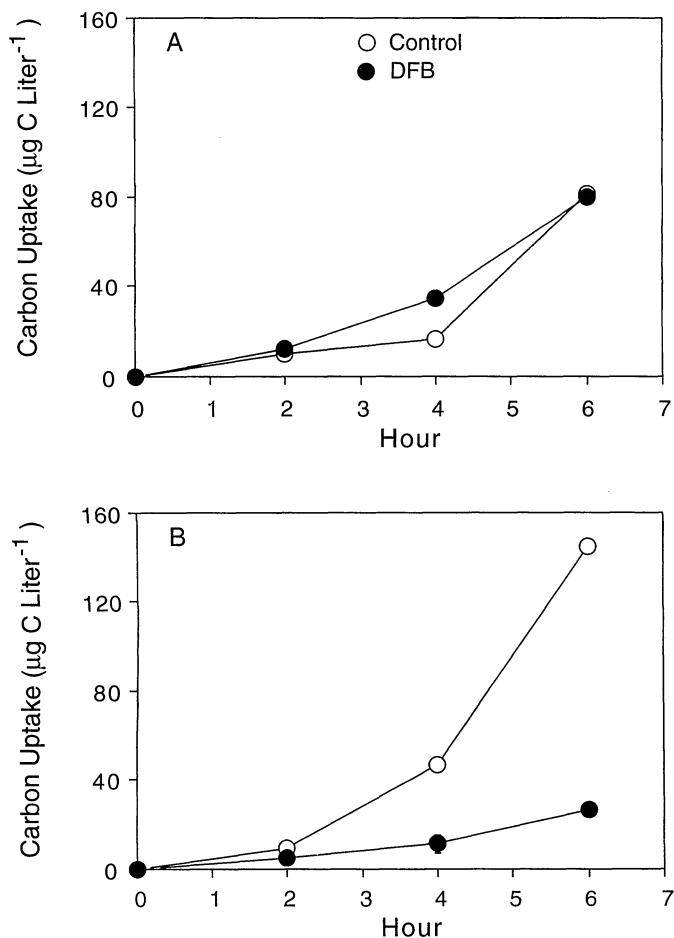


Fig. 3. Short-term ¹⁴C uptake in the controls and DFB treatments of two size fractions: (A) >5.0 μm; (B) 0.2–5.0 μm. Uptake rates were exponential and described as follows: control >5.0-μm fraction, $y = 3.11 \times e^{0.514x}$, $R^2 = 0.97$; DFB >5.0-μm fraction, $y = 5.15 \times e^{0.46x}$, $R^2 = 1.00$; control 0.2–5.0-μm fraction, $y = 2.59 \times e^{0.683x}$, $R^2 = 0.99$; DFB 0.2–5.0-μm fraction, $y = 1.98 \times e^{0.435x}$, $R^2 = 1.00$. The range of replicate measurements were smaller than the symbol size.

ambient Zn concentrations in these coastal waters (Wells unpubl. data).

The disparity in net uptake of ⁵⁴Mn, ⁶⁵Zn, and ⁵⁷Co between DFB treatments and controls was much greater in both size classes after 5 d of incubation (Fig. 6a–c). Again, the inhibition of long-term tracer uptake was greatest for ⁵⁴Mn (~88%) followed by ⁵⁷Co (~84%) and ⁶⁵Zn (~77%).

Discussion

Addition of DFB to the cultures sharply curtailed iron uptake by both phytoplankton and heterotrophic bacteria over these short-term (0–6-h) experiments. This result supports the earlier findings in the equatorial Pacific (Wells et al. 1994) and suggests that DFB is equally effective at reducing iron uptake in both offshore (picoplankton-dominated) and nearshore (nanoplankton-dominated) communities. Moreover, DFB inhibited iron uptake over at least 5 d in

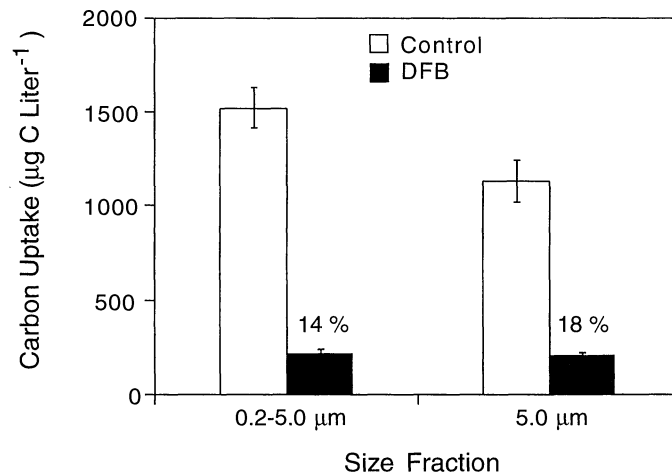


Fig. 4. The percent decrease in net ¹⁴C uptake in DFB treatments after 5 d of incubation: control and DFB treatment. Error bars give the range of replicate measurements where visible.

coastal waters and 8 d in the equatorial Pacific (Wells et al. 1994), indicating that the DFB–Fe complex is stable with respect to photochemical degradation. These results support an earlier finding that the DFB–Fe complex is not significantly photolabile (Finden et al. 1984). The DFB–Fe complex also may be stable against competition with strong, specific iron chelators found in seawater (Gledhill and van den Berg 1994; Rue and Bruland 1995), although this resistance cannot be confirmed here because of uncertainty over whether iron bound to natural chelators is directly accessed by phytoplankton. Regardless, the stability of DFB–Fe complexes in natural population cultures makes DFB an ideal tool for studying the interactions among iron, phytoplankton, and heterotrophic bacteria.

These findings complement those of Hutchins et al. (1999) who conducted a parallel experiment in the same general iron-replete upwelling region. They report that addition of DFB-generated nutrient and biological conditions resembling those found in HNLC regions; a mirror image of past iron addition experiments. They showed that diatoms and other phytoplankton bloomed in control incubations but not in DFB treatments. My results here suggest that this iron limitation initiated some time after the first several hours of incubation when internal iron reserves of the diatoms became depleted (see below). Hutchins et al. (1999) also show that bacterial numbers and zooplankton grazing activity were diminished in the DFB treatments, demonstrating a cascading impact of the DFB-induced iron limitation shown in our related experiments.

As found by Hutchins et al. (1999), ⁵⁹Fe uptake in these recently upwelled coastal waters was divided roughly equally among large (>5.0-μm) and small (0.2–5.0-μm) cells. While uptake in the >5.0-μm fraction can be attributed exclusively to phytoplankton, uptake in the small size fraction would reflect that of both heterotrophic bacteria and phytoplankton. Heterotrophic bacteria account for more than half of the total iron uptake in offshore waters (Tortell et al. 1996; Wells and Cochlan unpubl. data) and likely were a large component of the uptake measured here in nearshore waters.

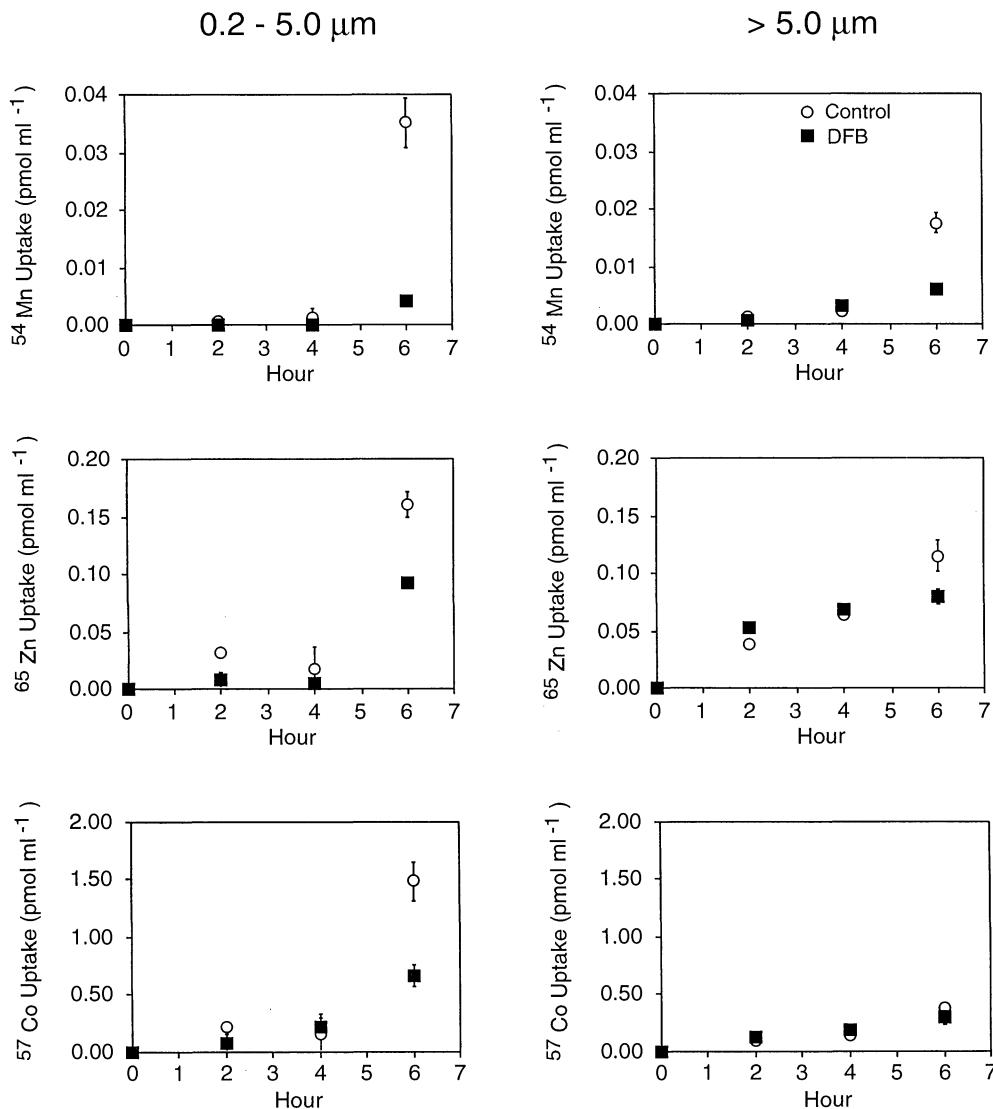


Fig. 5. Short-term uptake of ^{54}Mn , ^{65}Zn , and ^{57}Co in the large ($>5.0\text{-}\mu\text{m}$) and small ($0.2\text{-}5.0\text{-}\mu\text{m}$) size fractions. Error bars give the range of replicate measurements where visible.

A major distinction among the bioactive metals was that ^{59}Fe uptake in the controls was linear and rapid from time zero while the uptake of ^{54}Mn , ^{65}Zn , and ^{57}Co lagged by several hours before increasing. The absence of any lag in iron uptake implies that the microbial community was more highly tuned for iron assimilation than for other essential metals at the time of collection. Linear uptake of ^{59}Fe also is reported for equatorial Pacific surface waters (Wells et al. 1994) where iron is limiting phytoplankton production (Coale and al. 1996). This similarity between nearshore and offshore waters may imply that the coastal organisms were iron stressed at the time of collection, despite the presence of comparatively high concentrations of dissolved iron (typically $\geq 1\text{ nM Fe}$ in these upwelling waters). Alternatively, the linear uptake may reflect a general strategy of marine phytoplankton to maintain high iron uptake at all times, whereby cells accumulate and store iron regardless of cellular metabolic requirements for use when the iron supply

ultimately becomes less optimal (e.g., during later stages of bloom development). Luxury uptake of iron has been measured in laboratory cultures (Sunda and Huntsman 1995), and there is recent evidence that coastal upwelling regions can become iron limited (Hutchins and Bruland 1998), suggesting that a combination of both interpretations is likely closer to the truth.

The findings here and in Hutchins et al. (1999) contrast with recent reports that iron bound by DFB is available for uptake by diatoms in laboratory cultures (Soria-Dengg and Horstmann 1995) and natural plankton communities in the subarctic Pacific (Maldonado and Price 1999). It is possible that this disagreement results because marine algal species from disparate ocean regions utilize different mechanisms for iron assimilation. Soria-Dengg and Horstmann (1995) and Maldonado and Price (1999) hypothesize that the DFB-Fe complex can be reductively dissociated at the cell surface, allowing the released iron to be assimilated. If true, this re-

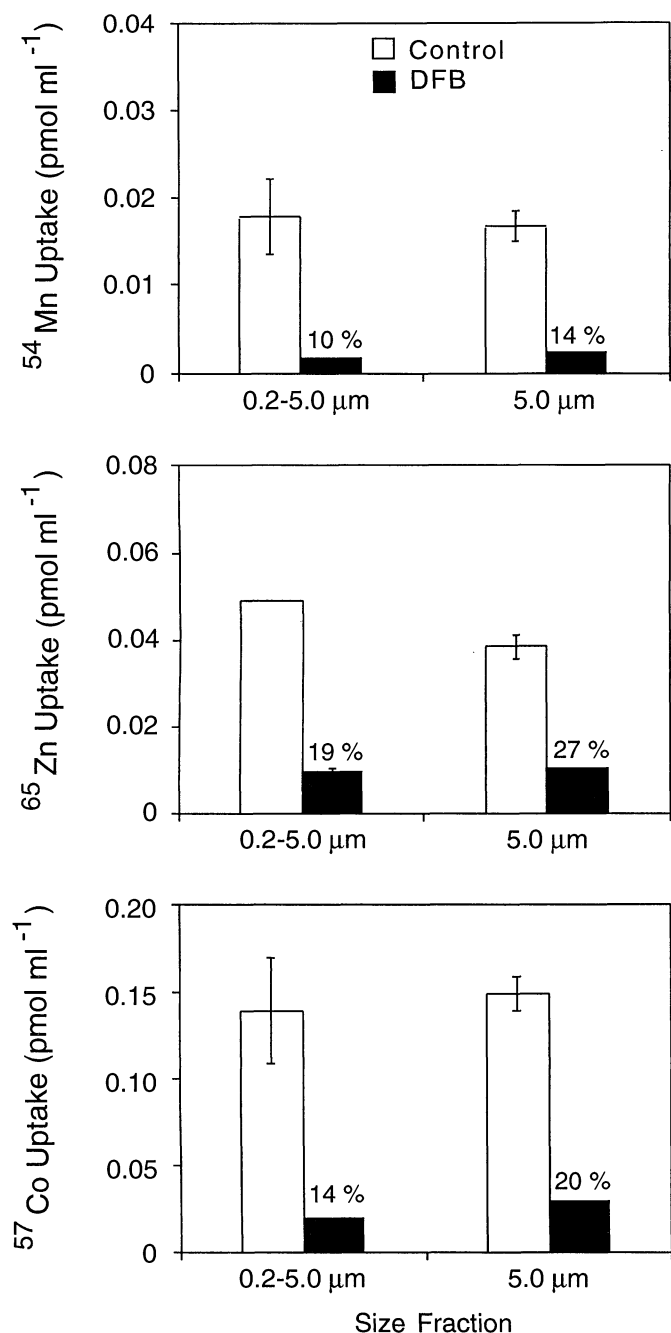


Fig. 6. The percent decrease in net ⁵⁴Mn, ⁶⁵Zn, and ⁵⁷Co uptake after 5 d of incubation: control and DFB treatment. Error bars give the range of replicate measurements where visible.

ductive capability may not be ubiquitous among marine algae. But it is also possible that the disagreement instead results from the different methods employed to quantify iron uptake in those experiments. Hutchins et al. (1999) argue that discrepancies between the results of Soria-Dengg and Horstmann (1995) and Maldonado and Price (1999) make definitive interpretations and comparisons difficult and suggest that a complete understanding of reductive iron uptake by phytoplankton awaits the development of more sensitive methods.

The results here demonstrate that it is possible to apply DFB to determine how abrupt curtailing of iron acquisition affects short-term carbon uptake by phytoplankton in coastal waters. This manipulation is of interest because it affords a window to the intracellular balance of iron reserves and metabolic demand. The addition of DFB had no measurable impact on the short-term carbon uptake of large (>5.0-μm) phytoplankton (Fig. 3a), but net carbon assimilation was significantly decreased after 5 d (Fig. 4). This finding suggests that larger cells had sufficient iron to sustain growth over several hours before depleting the intracellular iron reserves. In contrast, addition of DFB immediately decreased the carbon uptake of small (0.2–5.0-μm) autotrophs (Fig. 3b). It is very unlikely that this inhibition resulted from any direct toxicity of DFB to the cells because there was no toxicity in the large fraction, and other bioactive metals were assimilated in the 0.2–5.0-μm DFB treatments (Fig. 5). There also have been no observations of any toxic effects of DFB in other natural population and laboratory culture studies (Soria-Dengg and Horstmann 1995; Maldonado and Price 1999). Instead the immediate decrease in carbon uptake implies that the intracellular iron reserves of the smaller phytoplankton were not large enough to meet the metabolic demand when the extracellular source of iron was suddenly restricted. This explanation seems at odds with the expected competitive advantage that smaller size would provide for iron uptake; small cells should be able to acquire excess iron reserves faster than large cells. One possible explanation is that because small phytoplankton are grazed at higher rates than large phytoplankton they have less time to accumulate iron reserves than their larger, longer-lived counterparts. Regardless, these results illustrate how short-term manipulations of iron availability by DFB additions can provide unique insights to the cellular iron metabolism of plankton.

These results have important implications for designing experiments to study the role of iron in coastal waters, where pulsed inputs can generate large spatial and temporal variability in iron concentrations. The growing use of rapid analytical methods (flow injection analysis) and experimental methods (short-term P versus I experiments, pump/probe fluorescence) has led oceanographers toward employing very short incubation times to minimize potential bottle effects. However, unlike the chronic iron limiting conditions of HNLC regions, where iron additions cause increased photosynthetic capacities on the time scale of hours (Behrenfeld et al. 1996), iron manipulations in coastal waters may have very little short-term impact. Instead, the timing and magnitude of the response to iron manipulation will depend more on the recent chemical history of the seawater than the chemistry measured at the start of the experiment. This situation makes the study of iron effects on microbial processes in coastal waters much more complicated than in comparatively stable open ocean systems.

Given the high specificity of DFB for iron, it was not expected that Desferal would affect the instantaneous uptake of other bioactive metals. Indeed, DFB had no discernable effect on ⁵⁴Mn, ⁶⁵Zn, and ⁵⁷Co uptake over the first 4 h of incubation. However, between 4 and 6 h there was a slightly to moderately lower uptake of these metals in the DFB treatments that was also seen, and in some cases magnified, after

5 d of incubation. This diminished uptake likely reflects the lower biological demand for these metals due to iron stress.

The findings presented here and in the companion paper (Hutchins et al. 1999) confirm that addition of high (100 nM) concentrations of the fungal siderophore DFB specifically decreases iron availability in natural population cultures. The strong specificity and high conditional stability constant of DFB for iron, combined with its very rapid complexation kinetics (Hudson et al. 1992), make it possible to regulate iron availability in natural population cultures independent of the availability of other bioactive metals. DFB therefore will be a highly useful tool for probing the role iron exerts in natural waters. For example, by titrating iron in neritic waters with increasing additions of DFB it should be possible to determine precisely how changing iron availability impacts the algal community structure, primary production, and carbon remineralization by heterotrophic bacteria. It also opens the door to developing more sophisticated ligand competition experiments to probe how accessible the dissolved iron complexed by natural organic ligands is to marine phytoplankton. These experiments will bring us closer to understanding the true nature of biologically available iron in seawater.

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