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The FeL model of iron acquisition: Nondissociative reduction of ferric complexes in the marine environment

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Abstract

Recently there has been recognition of the importance of reductive processes in the acquisition of iron by microorganisms in marine environments with Fe(III) reduction induced by either membrane-bound reductases or by superoxide, a powerful Fe(III) reducing agent generated either by photochemical or biological means. We have measured the relative rates of iron uptake achieved by the cyanobacterium *L. majuscula* in the presence of a variety of model- and naturally-derived organic ligands exhibiting a broad range of conditional ferric and ferrous stability constants. Additionally, we have investigated the effect upon iron uptake rate of varying the concentration of both iron and the iron-binding ligands. We have reconciled this data with previous work in which we measured rates of reduction by exogenous superoxide of Fe(III) bound to these same complexes. We show that the rate of formation of ferrous iron and the rate of uptake of iron by *Lyngbya majuscula* are each independent of the concentration of Fe′ and demonstrate that this is consistent with our previous finding that this organism acquires iron via nondissociative reduction of ferric complexes. We also show that the rate of reoxidation of organically complexed Fe(II) is a critical determinant of the subsequent bioavailability of iron, a feature not previously addressed in the literature. In view of the central importance of the complexation and redox behavior of the iron–organic complexes to iron uptake rate, we propose a new kinetic model of iron acquisition, termed the FeL model, that is consistent with presented and previously published data and which describes processes both in natural and artificial conditions.

Iron is an essential micronutrient for all known forms of marine life (Crichton 2001; Sunda 2002) yet is quite insoluble under pH and redox conditions typical of marine systems, and the resultant particulate form is generally considered relatively nonbioavailable. Consequently, a variety of iron acquisition strategies have been developed by marine organisms, in some cases requiring great expenditure of other metabolic resources.

Many common metabolic activities require iron as a cofactor, particularly those involving redox exchanges such as oxidative respiration. Photosynthesis and nitrogen fixation are two complex aspects of metabolism with a high requirement for iron (Rueter and Petersenc 1987; Raven 1988; Rueter 1988; Geider and La Roche 1994). Each is prevalent among marine microorganisms, which make up the great majority of marine biota (Whitman et al. 1998); thus, in combination, they determine a high proportion of the total biological iron requirement in both oceanic and coastal waters (Capone et al. 1997).

In natural waters, iron exists predominantly in either the reduced ferrous (Fe[II]) or the oxidized ferric (Fe[III]) redox states, each of which can react with various oxygen species present in the aquatic system (Gledhill and van den Berg 1995; Millero et al. 1995; Waite et al. 1995). These oxygen species include the fully oxidized form, dioxygen (O_2), and partially reduced forms known as reactive oxygen species. Given the redox potentials of the iron and oxygen systems, ferric iron is the thermodynamically favored form of iron in oxygenated marine waters. Ferric iron is highly insoluble at circumneutral pH and is rapidly hydrolyzed to form amorphous, particulate oxyhydroxides that are biologically unavailable. Importantly however, terrestrially

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derived dissolved natural organic matter (NOM) is often found at relatively high concentrations in estuarine and coastal waters and has been shown to be capable of maintaining iron in solution for periods of hours to days (Rose and Waite 2003*a*). Additionally, much of the iron in the open ocean appears to be maintained in dissolved form through complexation by strong ligands (Rue and Bruland 1995, 1997).

Various general models of iron acquisition in marine systems advanced as technological developments have provided increasingly sophisticated and accurate measurements of iron species in such environments (Johnson et al. 1997; Rue and Bruland 1995, 1997). Predominant is the Fe′ model (Hudson and Morel 1990, 1993; Sunda and Huntsman 1995), which proposes that iron is acquired by the binding of unchelated iron (Fe′), typically of the ferric form, at the surface of the organism with subsequent internalization of the bound iron across the plasma membrane. The Fe′ concentration in the model is governed by equilibrium in the aquatic milieu surrounding the organism. This model has been used in studies of iron physiology of phytoplankton and other algae over a long period and a broad and varied array of experimental evidence has been produced that is consistent with this model (Hudson and Morel 1990, 1993; Sunda and Huntsman 1995; Shaked et al. 2005).

Despite the weight of evidence consistent with this model, its robustness in marine environments is now under doubt. As mentioned previously, it has been established that the majority of dissolved iron in seawater is bound by strong organic ligands, decreasing the concentration of Fe′ to levels below that required to support growth of phytoplankton (Gledhill and van den Berg 1994; Rue and Bruland 1995; Sunda and Huntsman 1997).

The importance of extracellular reduction in acquisition of iron has long been recognized in dicots and nongraminaceous monocots (strategy I–type plants) such as pea, tomato, and soybean (Guerinot 1994; Guerinot and Yi 1994) and in yeast (*Saccharomyces cerevisiae*; Dancis et al. 1992; De Freitas et al. 2003). Recently, a report of iron acquisition that uses reductive processes in the marine diatom *Thalassiosira oceanica* was published (Maldonado and Price 2000) with a subsequent analysis of the influence of iron and ligand concentration on the system (Maldonado and Price 2001). Data were presented showing a stronger correlation of iron uptake with total concentration of the Fe–ligand complex than the concentration of unchelated iron (Fe′). This has recently been followed by a more complete analysis of reduction-mediated iron acquisition of two other *Thalassiosira* species (Shaked et al. 2005) that concluded that the concentration of Fe′ moderated ferric reduction and subsequent uptake, except in the case of very strong ligands or at high ligand concentrations. The data of Maldonado and Price (2001) was thus reconciled with previous data by extension of the Fe′ model of iron acquisition to the so-called Fe(II)s model (Shaked et al. 2005).

In the work presented by Shaked et al. (2005), complexation with ferrozine (FZ), a strong Fe(II) chelator, was used to measure formation of Fe(II)′ during reduction by the *Thalassiosira* species. A critical and untested assumption of the Fe(II)s model is that dissociation to form free ligand and inorganic Fe(II) immediately follows the reduction of ferric complexes. This assumption allowed an equation of $Fe(FZ)_3$ formation with total generation of Fe(II); thus, the authors assert, enabling measurement of the rate of iron reduction. This assumption, however, is inconsistent with a recent demonstration that reduction of ferric complexes typically leads to immediate formation of corresponding ferrous complexes without intermediate dissociation (Rose and Waite 2005). Indeed, such a mechanism was assumed in the first studies of iron reduction by a species of *Thalassiosira* (Maldonado and Price 2001).

Furthermore, the analysis presented by Shaked et al. (2005) is incompatible with a previous report that the rate of reduction of Fe(III) by superoxide is independent of ethylenediaminetetraacetate (EDTA) concentration (Rose et al. 2005). Importantly, in that study, Fe(II) was detected with the FeLume chemiluminesence technique, which is capable of measuring complexed Fe(II) in addition to unchelated Fe(II).

We have previously established that reduction of ferric iron by endogenously produced superoxide is a prerequisite to the majority of iron uptake by the benthic, diazotrophic, cyanobacterium *Lyngbya majuscula* (Rose et al. 2005). It is possible that this reductive process is of significance to many organisms because a wide variety of marine algae, particularly ichthyotoxic raphidophytes and gymnodinioid dinoflagellates, are now recognized to be active superoxide producers (Twiner and Trick 2000; Marshall et al. 2005). The possibility that superoxide-mediated iron reduction is important to iron acquisition by the diatom *Thalassiosira weissflogii* was recently investigated and, in contrast to our results with *L. majuscula* (Rose et al. 2005), was shown to play a minimal role under the conditions used in their investigations despite measurable production of superoxide (Kustka et al. 2005).

The reduction of organically complexed ferric iron by superoxide in natural waters has been investigated in recent work (Rose and Waite 2005). A variety of organic ligands were studied, including synthetic chelators and representative NOM from the region of interest, and the reducibility of their respective ferric complexes was determined. In this work, we investigate the effect of these same organic complexes on uptake of iron by *L. majuscula*. We determine the relative rates of iron uptake and correlate these measurements with the previously determined rates of superoxide-mediated reduction and conditional stability constants of these same complexes. Finally, we present a model for reductive iron uptake that is consistent with the iron uptake mechanism employed by *L. majuscula*, as well as those reported for other marine microorganisms, and discuss its potential wider relevance to iron acquisition in the marine environment.

Materials and methods

Collection, treatment, and culturing of L. majuscula—Fresh *L. majuscula* biomass was harvested from Moreton Bay and maintained in clean, filtered seawater with

Table 1. Natural organic matter extracts used in this study.

Sample*	Origin (vegetation type)	pH	[Fe] (μmol L^{-1})	TOC (mg L^{-1})
CLC	Sugar cane	7.60	27.0	191
MLC	Melaleuca	7.78	132.3	307

* CLC, sugar cane NOM extract; MLC, melaleuca NOM extract.

Table 2. Physicochemical parameters of the organic ligands used in this study.

Ligand	log $K_{Fe^{3+}}$	log $K_{Fe^{2+}}$	log R*	log k_{RED}†
DFB‡	31.82	20.32	−9.4	4.24
EDTA§	28.03	18.35	2.6	5.10
DTPA‖	27.74	—	—	—
Salicylate‡	17.02	7.04	7.9	5.29
5–Sulfosalicylate‡	15.86	6.81¶	6.8	5.28
Citrate#	10.36	8.81	14.3	4.96
CLC**	11.4††	7.4††	−4.0	4.48
MLC**	11.4††	9.8††	−1.6	4.71
SRFA**	10.4††	7.5††	−2.9	5.17

* Value shown is for the most stable 1 : 1 Fe : ligand complex.
† Rose and Waite (2005).
‡ Morel and Hering (1993).
§ Delgado et al. (1997).
‖ Martell and Smith (1997).
¶ Reported for 20°C.
# Königsberger et al. (2000).
** Rose and Waite (2003*b*). CLC, sugar cane NOM extract; MLC, melaleuca NOM extract.
†† Conditional stability constants at pH 8.1 in seawater.

continuous aeration under natural room lighting at room temperature. No changes in metabolic activity (photosynthetic rate and nitrogen fixation rate) were detected in the organism during the period of 1 week over which experiments were performed (Rose et al. 2005). Individual experimental incubations were performed under the same environmental conditions but without aeration.

Preparation of reagents—Unless otherwise stated, all solutions were made up in 18 MΩ-cm resistivity Milli-Q water. All pH measurements were performed with a calibrated Hanna HI9025 pH meter, and pH adjustments were made with the use of high-purity 30% (w/v) HCl and 32% (w/v) NaOH (Fluka puris p.a plus). Clean seawater was collected from the Sydney Offshore Reference Station, filtered through 0.22-μm Millipore membranes and stored in the dark at 4°C when not in use.

Solutions of high-purity 100 mmol L^{-1} HCl and 100 mmol L^{-1} NaOH were each made by respective dilution of 30% (w/v) HCl and 32% (w/v) NaOH (Fluka puris p.a plus). A stock solution of 3 mmol L^{-1} $FeCl_3 \cdot 6H_2O$ (Ajax Chemicals) was prepared in 100 μmol L^{-1} HCl with an appropriate volume of ^{55}Fe (New England Nuclear) such that 1–10% of the iron was ^{55}Fe. The same stock solution of ^{55}Fe-labeled iron was used for all experiments, and the experiments were performed over short duration compared with the half-life of ^{55}Fe, such that the relationship between activity and total iron was the same in all experiments. Stock solutions of defined ligands 5-sulfosalicylate (Sigma), salicylate (Sigma), citrate (Sigma), EDTA (Ajax Chemicals), desferrioxamine B (DFB, Sigma), and diethylenetriaminetetraacetate (DTPA, Fluka) were freshly prepared before use.

In addition, samples of several types of dissolved NOM were provided by the Queensland Department of Natural Resources and Mines. These samples were used in this study because the kinetic and thermodynamic properties of their ferrous and ferric complexes and the rates of reduction of their ferric complexes had been determined previously (Rose and Waite 2003*a*), thus providing an opportunity to relate these properties to their behavior in mediating the rates of uptake of iron by *L. majuscula*. Briefly, the solutions were produced by leaching with rainwater the coarse (>2 mm) and fine (<2 mm) sieved fractions of leaf litter from six representative vegetation types in the vicinity of Moreton Bay, Queensland, followed by filtration through 0.22-μm membrane filters (Millipore). The total iron and total organic carbon (TOC) content of each sample was determined with a Perkin-Elmer ICP-OES and Shimadzu TOC analyzer, respectively. Sample details are shown in Table 1. All solutions were stored in the dark at 4°C when not in use.

For uptake experiments from iron–ligand complexes, intermediate stocks of each of the defined ligands complexed with iron were made to final concentrations of 1 mmol L^{-1} and 100 μmol L^{-1} in filtered seawater. Similarly, a Fe(III)–Suwannee River fulvic acid (SRFA, International Humic Substances Society) was prepared by appropriate dilution of the 3 mmol L^{-1} ^{55}Fe stock and a 2 g L^{-1} SRFA stock in filtered seawater to concentrations of 100 μmol L^{-1} and 500 mg L^{-1}, respectively. The other NOM samples were prepared as in Rose and Waite (2005) to the concentrations shown in Table 1 and with additions of the 3 mmol L^{-1} ^{55}Fe to a final concentration of 100 μmol L^{-1}. For incubations with amorphous ferric oxyhydroxide (AFO), suspensions of hydrolyzed ferric iron were freshly prepared by spiking appropriate volumes of the 3 mmol L^{-1} ^{55}Fe stock directly into filtered seawater. The added iron was assumed to immediately undergo spontaneous hydrolysis and precipitation. All solutions were stored in the dark at 4°C when not in use.

For examination of the effect of Fe(III) concentration, intermediate stocks of iron/EDTA were made as described above to iron concentrations of 10, 50, 100, and 500 μmol L^{-1} with a constant EDTA concentration of 1 mmol L^{-1}. For titration of EDTA, DFB, and citrate, intermediate stocks were set up, each with 100 μmol L^{-1} radiolabeled Fe(III) and a series of ligand concentrations (10, 100, and 500 μmol L^{-1} and 1 mmol L^{-1}). The known relevant physicochemical parameters for each ligand used in this study are shown in Table 2. The stability constants for the defined ligands have been obtained from the indicated literature sources, whereas those for the naturally occurring organic ligands were obtained by Rose and Waite (2003*b*). The rate constants for the superoxide-mediated reduction of Fe(III) complexed by the various defined and

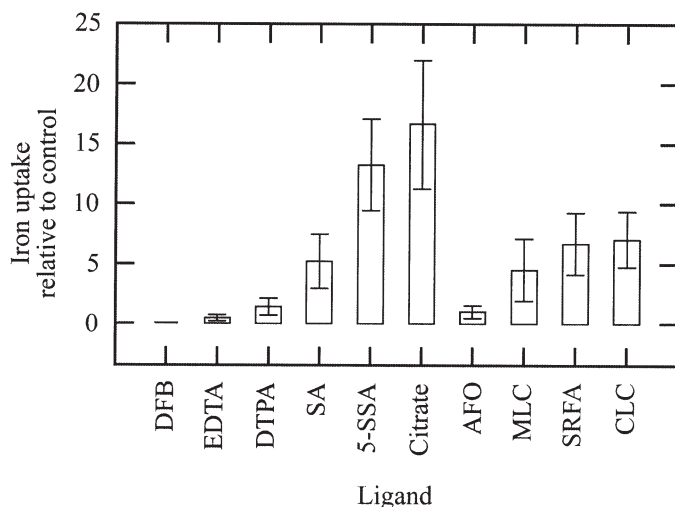


Fig. 1. Relative rates of iron uptake by *L. majuscula* in the presence of various organic ligands. In each case, 1 μmol L^{-1} of ferric iron was supplied bound to 10 μmol L^{-1} of ligand (0.5 mg L^{-1} in the case of SRFA and without ligand in the case of AFO). For each condition, error bars represent a single standard deviation of the measured uptake. DFB, desferrioxamine B; EDTA, ethylenediaminetetraacetate; DTPA, diethylenetriaminetetraacetate; SA, salicylate; SSA, sulfosalicylate; MLC, melaleuca NOM extract; CLC, sugar cane NOM extract.

natural organic ligands were obtained previously (Rose and Waite 2005).

For rinsing of adsorbed ^{55}Fe from the surface of the biomass in assays, a titanium (Ti)–citrate–EDTA solution was prepared in a manner identical to that described by Hudson and Morel (1989). This solution, which contains 47 mmol L^{-1} Ti(IIII), 47 mmol L^{-1} citrate, 47 mmol EDTA, 0.61 mol L^{-1} Na^+, 0.35 mol L^{-1} Cl^-, and 10 mmol L^{-1} K^+ at pH 8.0, has been shown to be effective in dissolving extracellular iron without causing cell breakage or toxicity (Hudson and Morel 1989).

^{55}Iron uptake experiments—Incubations were performed in 150 mL of filtered seawater. Aliquots (1.5 mL) of the stock solutions were added to the respective incubations and premixed thoroughly before addition of approximately 500 mg (wet weight) *L. majuscula* at time zero.

Incubations were each performed for 4 h under moderate ambient (indoor) illumination at room temperature without aeration. Incubations were halted by removal of the assayed biomass from the incubator in quadruplicate samples and immediate immersion of these samples in separate 20-mL aliquots of the Ti–citrate–EDTA complex (Hudson and Morel 1989) for 2 min with agitation to remove any particulate iron oxyhydroxide adsorbed to the exterior of the organism so that only iron uptake was measured. (Note that although two of the ligands used in the wash solution are identical to those used in the uptake studies, the wash process should not interfere in any way with iron uptake behavior.) The *L. majuscula* was then separated from the culture medium containing the Ti–citrate–EDTA rinse and any removed iron by rapidly filtering onto coarse GFC (Millipore) filters that were then rinsed with a further 10 mL of the Ti–citrate–EDTA complex. The samples were arranged in 24-well polycarbonate titer plates (Invitrogen) and air dried overnight at 110°C. From each dried sample, a subsample of ~20 mg was taken, weighed, and placed in a glass scintillation vial to which 5 mL of Beckman ReadyScint scintillation fluid was added; ^{55}Fe activity, which was proportional to total iron uptake, was determined with a Packard TriCarb liquid scintillation counter.

Note that extensive studies of iron uptake (and superoxide generation) by *L. majuscula* against a variety of controls (e.g., organism dead/alive, superoxide production on/off, etc.) have been reported previously (Rose et al. 2005) and are not reiterated here.

Results

Effect of ligand on iron uptake—The relative effects of various ligands on iron uptake by *L. majuscula* are shown in Fig. 1. AFO is used as a standard measure because it is found in all natural or simulated natural systems and does not involve an organic ligand. Compared with this standard, significant increases (tested with a single-tailed Student's *t*-test assuming different variances, $p < 0.05$) are observed in iron uptake in treatments with salicylate, 5-sulfosalicylate, citrate, and each NOM extract ($p < 0.05$ in all cases) over that observed with AFO. A significant decrease (again, relative to the uptake observed in the presence of AFO) is observed with DFB as the complexing agent ($p < 0.02$), whereas the observed changes in uptake in the presence of EDTA and DTPA were not found to be statistically significant ($p > 0.05$) from those with AFO.

Figure 2a–f shows correlations between the relative iron uptake and various known physicochemical parameters of the ligands present. DTPA is only included in Fig. 2a (uptake vs. ferric stability) because the data for the other comparisons are not available. A clear correlation is observable (Fig. 2a) between uptake and the ferric stability constant $K_{Fe^{3+}}$ of the respective ligand. Generally, as the complex stability increases, the rate of uptake by *L. majuscula* decreases. The correlation between these values for defined ligands is well supported ($R^2 = 0.89$, $n = 6$) but less clearly supported for all ligands ($R^2 = 0.80$, $n = 9$).

A similarly strong inverse correlation ($R^2 = 0.90$, $n = 8$) for all ligands is seen (Fig. 2b) for the relationship between iron uptake and stability of the ferrous complex of the respective ligand. A positive correlation ($R^2 = 0.82$, $n = 8$) can be seen, as is common with iron-binding ligands, between the stabilities of the ferric and ferrous complexes (Fig. 2c).

Although there does appear to be a slight correlation between the rate of reduction k_{RED} and iron uptake (Fig. 2d) when considering only the five defined ligands ($R^2 = 0.57$, $n = 5$), that relationship is not supported with the inclusion of the remaining ligands ($R^2 = 0.29$, $n = 8$). In comparison, there appears to be no relationship between the strength of the ferric complex and the rate of reduction of that complex by superoxide.

A good correlation ($R^2 = 0.82$, $n = 8$) exists between iron uptake and the rate of formation of the ferrous/

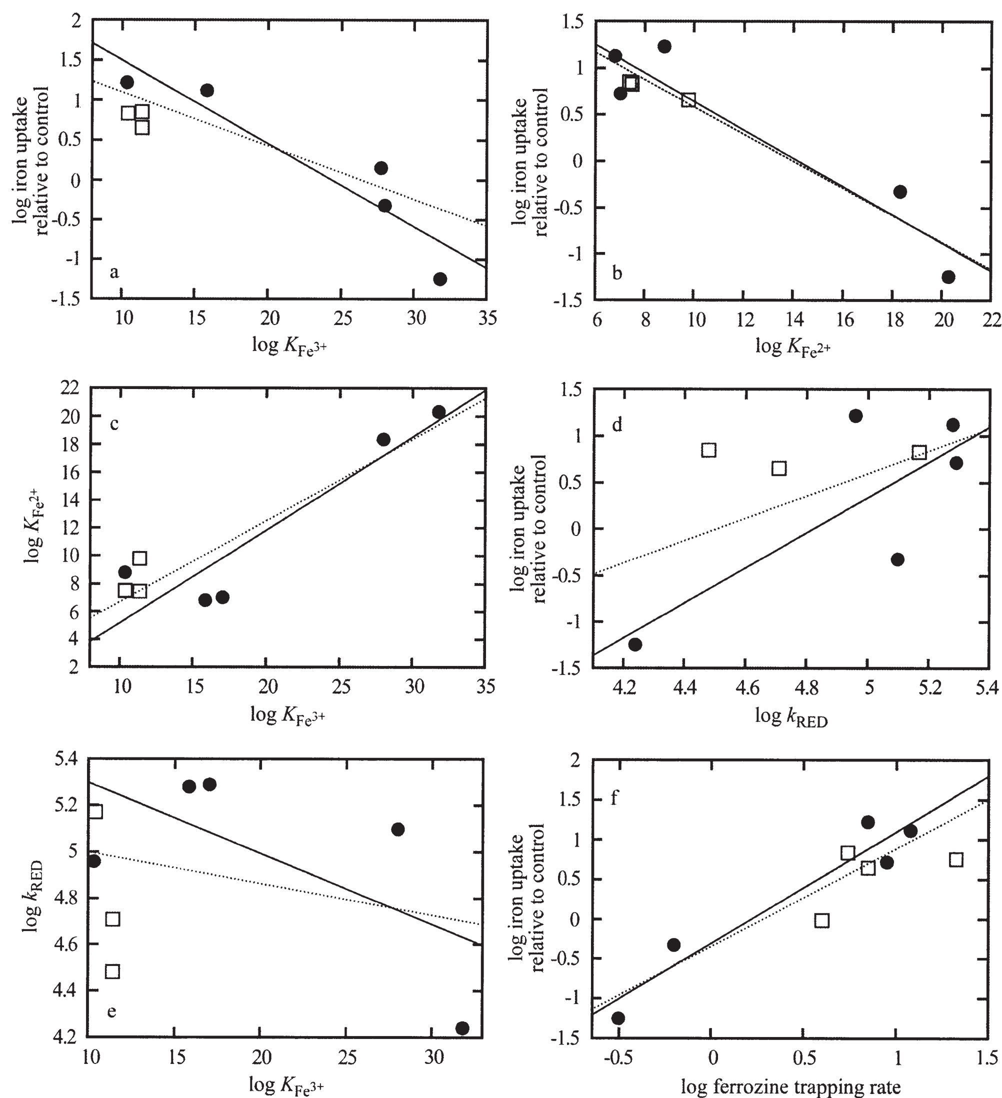


Fig. 2. Correlation of physicochemical characteristics of iron complexes and iron uptake by *L. majuscula*. Plots represent the (dual logarithmic) relationships between (a) ferric complex stability $(K_{Fe^{3+}})$ and iron uptake relative to that achieved from iron when present as amorphous ferric oxide, (b) ferrous complex stability $(K_{Fe^{2+}})$ and relative iron uptake, (c) ferric and ferrous complex stability, (d) ferric complex reduction rate (k_{RED}) and relative iron uptake, (e) ferric complex stability and reduction rate, and (f) ferrozine trapping rate and relative iron uptake. In each of the figures, the dark circles and solid line indicate the data and fit for defined ligands only, whereas the open squares represent the data for the natural ligands and the dashed line represents the fit for all ligands.

ferrozine complex in the presence of superoxide-mediated reduction of the ferric–ligand complexes (Fig. 2f). This relationship becomes more definite when considering only the defined ligands ($R^2 = 0.93$, $n = 5$).

Effect of iron and ligand concentration on iron uptake—The effect of variation of initial total dissolved iron concentration at fixed EDTA concentration (10 μmol L^{-1}) is illustrated in Fig. 3a. Figure 3b,c shows, respectively, the effect of variation of the concentration of strong iron–ligand complexes (EDTA and DFB) and a weak iron–ligand complex (citrate) in the presence of fixed initial dissolved iron concentration (of 1 μmol L^{-1}) on iron uptake by *L. majuscula*. Note that large differences in relative iron uptake are observed as a result of the marked differences in initial dissolved iron concentration (up to 5 μmol L^{-1} in Fig. 3a and only 1 μmol L^{-1} in Fig. 3b,c). The solution to the Michaelis–Menten relationship plotted in Fig. 3a yields a value for the half-saturation constant, K_m, of 3.63 μmol L^{-1}. It can be seen that a general decrease in uptake occurs in the presence of increasing concentrations of each of EDTA and DFB (Fig. 3b), whereas increased concentration of citrate correlates with increased uptake (Fig. 3c) of iron by *L. majuscula*.

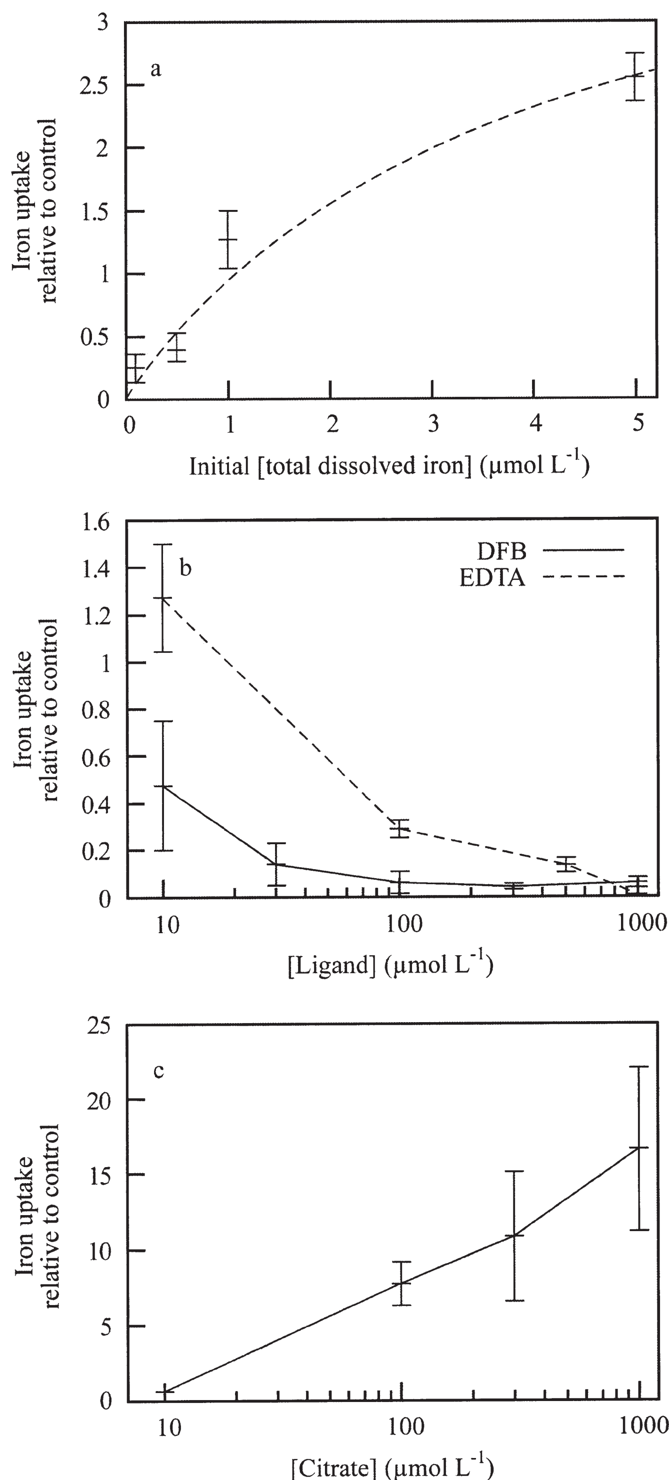


Fig. 3. Effect of varying concentration of (a) initial total dissolved ferric iron at a fixed EDTA concentration of 10 μmol L^{-1}, (b) strongly binding iron ligands (DFB and EDTA) at a fixed initial total dissolved ferric iron concentration of 1 μmol L^{-1}, and (c) a weakly binding iron ligand (citrate) at a fixed initial total dissolved ferric iron concentration of 1 μmol L^{-1}. The dashed line in panel a represents a fit of the discrete data to a Michaelis–Menten relationship between substrate concentration and uptake.

Discussion

Relationship between iron–ligand complex characteristics and iron uptake behavior—Correlation of iron uptake rate with many of the physicochemical characteristics of the ligands appears to yield a number of distinct relationships (Fig. 2), particularly when considering only the defined ligands. Relationships are not so clear when also considering the NOM extracts and amorphous ferric oxyhydroxide. It can be seen in Fig. 3b,c that ligand concentration is of critical importance to uptake. Because we were unable to standardize the molar concentrations of the nondefined samples in the various analyses, it is not surprising that they did not conform in all cases to the relationships established by the defined compounds.

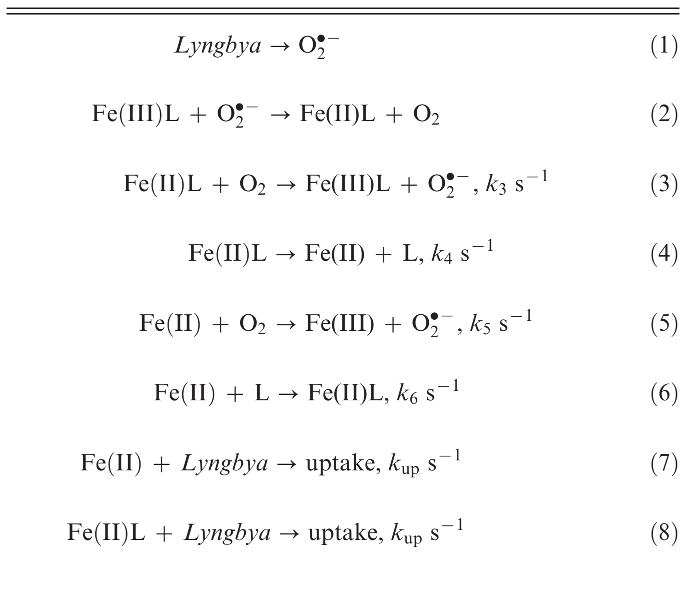
For the range of ligands studied, there is a clear correlation between the rate of iron uptake and the logarithm of each of the ferric and ferrous stability constants (Fig. 2a,b). Importantly, there is also a strong correlation between the logarithms of the ferric and ferrous complex stabilities, as seen in Fig. 2c. It is arguable then that at least one of the correlations between complex stability and iron uptake is at least partially misleading and merely a function of the innate relationship between the stabilities of the respective ferrous and ferric complexes.

In isolation, the clear inverse correlation of ferric–ligand complex stability and iron uptake in Fig. 2a immediately suggests a mechanistic link between the two quantities. We have previously demonstrated (Rose et al. 2005) that reduction of ferric iron is an essential precursor to iron importation by *L. majuscula*. It could be postulated then that stability of the ferric complex determines the reducibility of that complex. This is contradicted, however, by comparison of the stability of the ferric–ligand complex with the rate of reduction, k_{RED} (Fig. 2e). This comparison reveals no clear correlation between these parameters; the reducibility of the ferric complex is independent of its stability. This is consistent with a similar finding by Maldonado and Price (2001); for several complexes in which stability constants varied by >15 orders of magnitude, their reduction rates varied by only a factor of two.

It is most interesting then that a comparison of rates of iron uptake in the presence of the various complexes and reduction of those same complexes yields no direct correlation. It is also illuminating that rates of reduction vary between the different complexes by an order of 10, whereas those of uptake vary by close to 1,000. The implication that ferric reduction is not the rate-limiting step in the iron uptake process is consistent with a prior observation that the rates of reduction exceed the rates of subsequent uptake (Rose et al. 2005).

Importance of ferrous iron and its rate of oxidation—It follows that it is the acquisition of the pooled ferrous iron that is the rate-limiting step in iron acquisition and that the mechanism is such that the rate of this acquisition is determined at least in part by the strength of the ferrous complex. In the case that reduction of iron occurs remotely from the organism (i.e., not localized at any part of its continuous structure), *L. majuscula* is dependent on

Table 3. Reaction scheme underlying the FeL model of iron uptake by *L. majuscula*. The numerical labels correspond to the numbered processes shown in Figure 4.



Reaction	No.
Lyngbya $\rightarrow O_2^{\bullet -}$	(1)
$Fe(III)L + O_2^{\bullet -} \rightarrow Fe(II)L + O_2$	(2)
$Fe(II)L + O_2 \rightarrow Fe(III)L + O_2^{\bullet -}$, k_3 s^{-1}	(3)
$Fe(II)L \rightarrow Fe(II) + L$, k_4 s^{-1}	(4)
$Fe(II) + O_2 \rightarrow Fe(III) + O_2^{\bullet -}$, k_5 s^{-1}	(5)
$Fe(II) + L \rightarrow Fe(II)L$, k_6 s^{-1}	(6)
$Fe(II)$ + *Lyngbya* $\rightarrow$ uptake, k_{up} s^{-1}	(7)
$Fe(II)L$ + *Lyngbya* $\rightarrow$ uptake, k_{up} s^{-1}	(8)

delivery of ferrous ion by diffusion to its importation mechanism. Under these conditions, *L. majuscula* must compete both with other ferrous-scavenging entities and with the rapid reoxidation of ferrous iron that occurs in oxic systems.

Most importantly, it has been established in previous work (Rose et al. 2005) that *L. majuscula* is able to acquire both inorganic and organically complexed ferrous iron. Additionally, preliminary genetic analysis of *L. majuscula* (Salmon unpubl. data) has revealed an 827–base pair putative fragment of a gene that might encode a transmembrane ferrous iron transport protein. This sequence has been shown by BLASTX analysis (Altschul et al. 1997) to translate to a continuous polypeptide that is 79% identical to a continuous fragment of a ferrous transporter (FeoB) identified in another cyanobacterium, *Trichodesmium erythreum* IMS101 (GenBank 48892101). FeoB-mediated ferrous uptake is thought to involve hydrolysis of either guanine triphosphate or adenosine triphosphate (Kammler et al. 1993). The discovery of this genetic element is consistent with both the data presented here and our previous conclusion that the iron is imported in the reduced ferrous state (Rose et al. 2005). Of immediate interest, then, are the influences of the nature and concentration of the organic ligand in the ferrous complex on (1) the concentration of ferrous iron in solution and (2) the ability of *L. majuscula* to acquire iron from that pool.

Direct reduction of the ferric complex results in immediate formation of a corresponding ferrous complex, the nature of which determines the reoxidation rate of the ferrous iron (Rose and Waite 2003*a*). Additionally, unchelated ferrous iron produced from reduction of unchelated ferric iron could become complexed before reoxidation. Considering Fig. 3c, it is apparent that increasing the concentration of citrate, a relatively weak iron-binding ligand, increases iron uptake by *L. majuscula*, whereas increasing the concentration of stronger iron-binding ligands (EDTA and DFB; Fig. 3b) decreases iron uptake. Intuitively this seems reasonable; we might expect that increasing the concentration of a weak ligand would promote iron solubility while maintaining the iron in a highly labile form that might be accessible to *L. majuscula*. To examine this phenomenon more quantitatively, we used our knowledge of the mechanism for iron uptake by *L. majuscula* to devise a simple reaction scheme to describe the process as shown in Table 3.

Under the assumed condition that $[Fe(III)L] >> [O_2^{\bullet -}]$, such that superoxide reacts exclusively with Fe(III) complexes, Eqs. 1 and 2 can be considered in combination to establish a zero-order superoxide production rate, P (mol L^{-1} s^{-1}). Because the only sink of superoxide in this simple model is reaction with ferric iron, this leads to a zero-order generation rate of Fe(II)L of P also. Similarly, a rate of uptake, k_{up}, is determined from the combination of Eqs. 7 and 8, if *L. majuscula* is considered to take up iron in the ferrous form (either inorganic or organically complexed; Rose et al. 2005). The remaining rate constants, k_3 to k_6, can be treated as first order, as long as dissolved oxygen and the organic ligand are in considerable excess of iron. This model has an analytical solution given by Eq. 9.

$$\text{iron uptake rate} = Pk_{up}\left[k_{up} + k_3 + k_4\left(\frac{k_5 - k_3}{k_4 + k_5 + k_6 + k_{up}}\right)\right]^{-1} \quad (9)$$

For any particular ligand, increasing the concentration of ligand will only affect (increase) the rate constant k_6, whereas decreasing the ligand concentration will decrease k_6. It is thus evident from Eq. 9 that increasing the ligand concentration will decrease the absolute value of the term inside the inner parentheses.

If the rate of oxidation of the complexed ferrous iron is greater than that of unchelated ferrous iron, then $k_3 > k_5$ and the described inner term will be negative. An increase in ligand concentration, then, will decrease the absolute value of the inner term and result in an overall decrease in uptake. If, however, the nature of the ligand is such that the complexed iron is oxidized less rapidly than the unchelated form, then $k_3 < k_5$ and the inner term will become positive. Increasing the ligand concentration will thus have the opposite effect, with a resultant increase in the rate of iron uptake. This result, as previously suggested, is intuitively consistent with the high concentrations of such organic ligands resulting in greater concentrations of soluble ferrous iron in the form of labile complexes.

This is fully consistent with the observation that citrate, which inhibits Fe(II) oxidation at pH 8.1 (Rose and Waite 2003*a*), increases the iron uptake with increasing ligand concentration, whereas EDTA (Santana-Casiano et al. 2000) and DFB (Cowart 2002) each enhance Fe(II) oxidation and restrict uptake. It is also noteworthy that if the organism is only able to access unchelated ferrous iron,

then increasing the ligand concentration will decrease the iron uptake rate, regardless of the chemical properties of the ligand.

The critical factor determining the influence of organic complexation on iron availability to *L. majuscula* therefore appears to be the rate at which the complexed Fe(II) oxidizes. We would similarly expect that natural organic ligands that inhibited iron oxidation would increase the availability of iron to the organism.

Relationship between rate of ferrous iron oxidation and complex stability—According to the theory of Marcus (1976), the logarithm of the rate constant for oxidation of a ferrous complex will be proportional to the logarithm of the ratio of the stability constants of the ferrous and ferric forms of the complex (Rose and Waite 2003*a*),

$$\log k_3' \propto \log\left(\frac{K_{Fe^{3+}}}{K_{Fe^{2+}}}\right) \Rightarrow \log\left(\frac{K_{Fe^{3+}}}{K_{Fe^{2+}}}\right) = \mathrm{A} \log k_3' \quad (10)$$

where k_3' is the second-order rate constant for the reaction in Eq. 3, and A is a constant of proportionality. As the stabilities of the ferrous and ferric constants are also logarithmically proportional, at least in the case of the ligands used in this work (Fig. 2c), it is also true that

$$\log K_{Fe^{3+}} = \mathrm{B} \log K_{Fe^{2+}} \quad (11)$$

where B is the constant of proportionality. It follows that $K_{Fe^{3+}} = (K_{Fe^{2+}})^{\mathrm{B}}$ and thence from Eq. 10 that

$$\log k_3' = [(\mathrm{B} - 1)/\mathrm{A}] \log K_{Fe^{2+}}$$
$$(\text{i.e., that } \log k_3' \propto \log K_{Fe^{2+}})$$

The correlation between the iron uptake rate and the rate of ferrozine trapping of ferrous iron produced by superoxide-mediated reduction of the complexes (Fig. 2f) suggests that the ferrozine trap technique might be a useful means of assessing the tendency for reductive iron uptake by *L. majuscula*. However, the reasons for this strong relationship are not immediately apparent because a critical difference between ferrozine and the uptake machinery of *L. majuscula* exists. Namely, ferrozine is able to complex only inorganic ferrous iron (i.e., that which has dissociated from the organic ligand), whereas *L. majuscula* appears to be able to access both inorganic and organically complexed iron (Rose et al. 2005). Careful consideration of the chemical processes involved offers a suggestion as to why this relationship functions well.

Because $K_{Fe^{2+}} = k_f/k_d$ (where k_f and k_d are the rate constants for complex formation and dissociation, respectively) and k_f is typically relatively constant for metals such as iron (Morel and Hering 1993), the rate at which inorganic ferrous iron is released from an organic complex depends on the stability of that complex. Thus, the ability of ferrozine to access organically complexed ferrous iron depends on $K_{Fe^{2+}}$. Given that the availability of iron to *L. majuscula* is governed primarily by the rate of oxidation of organically complexed ferrous iron and that this depends on $K_{Fe^{2+}}$, as demonstrated above, it follows that the trapping of ferrous iron by ferrozine should be related to the rate of iron uptake by *L. majuscula* despite an important difference in the exact chemical process involved.

Definition of the FeL model and comparison with other models—Consideration of the implications of the nature of the iron–ligand complex on iron uptake is a significant advance on the approach used in many of the previous ligand addition studies (Hudson and Morel 1990; Hutchins et al. 1999; Wells and Trick 2004), in which only the steady-state concentrations and bioavailabilities of complexed and free iron were considered. To reconcile this analysis with our experimental results with *L. majuscula* and with results from previous studies, we present a model of iron acquisition by reductive processes, which we have named the FeL model, as an alternative to both the Fe′ model and the Fe(II)s model. It is consistent with the data that has been used to support each; however, it differs from them in that it assumes that (1) organically complexed iron is reducible without dissociation of the complex and (2) the rate of reoxidation of complexed ferrous iron is critical in determining the availability of the reduced pool. These processes are shown with the other elements of the FeL model in Fig. 4. Because of their irrelevance to the FeL model, the process of dissociation of the ferric complex and the fate of unchelated ferric iron are neglected. The FeL model provides a sound explanation for the sophisticated influence of ligand concentration and the nature of the ferrous complex on iron availability to *L. majuscula*. Without these integral components, the Fe′ and Fe(II)s models are each unable to explain the data presented here.

The assumption that only Fe′ is available for uptake (with or without preceding reduction) is inconsistent with the data showing the increase in availability as citrate concentration increases. In the presence of increased citrate concentration, as with other ligands, the concentration of Fe′ decreases. If Fe′ were the only available iron irrespective of the subsequent processes, this would result in lower uptake rates. It therefore follows that factors other than the concentration of Fe′ control iron uptake rate in the system examined here.

The FeL model comprises three major components: biologically mediated reduction of Fe(III)L, abiotic transformations of iron in the surrounding milieu, and uptake of transformed iron by the organism. The first and third components, namely reduction and uptake, are presented as general processes for which the precise mechanism can vary between different organisms. For example, it is acknowledged that the precise mechanism of ferric iron reduction for iron uptake by two members of the genus *Thalassiosira*, as reported by Shaked et al. (2005), and on which the Fe(II)s model is primarily based, does not appear to involve superoxide (Kustka et al. 2005), in contrast with the mechanism for iron reduction by *L. majuscula* (Rose et al. 2005). However, it is reasonable to expect a similar mechanism for iron reduction by an outer sphere pathway to apply to a range of reductants. In the case of the uptake

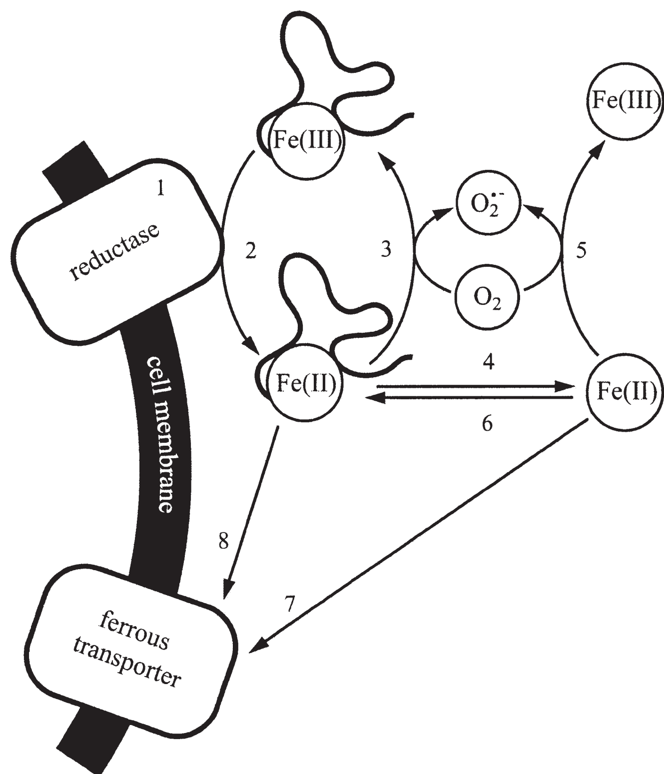


Fig. 4. The FeL model of reductive iron acquisition by *L. majuscula*. The proposed model involves reduction of organically complexed Fe(III) either directly by a membrane-located reductase (1) or by superoxide generated by this reductase. The reduction of organically complexed ferric iron to its corresponding ferrous complex (2) is followed by reoxidation of organically complexed ferrous iron to its corresponding ferric complex (3). The Fe(II) complexes are typically relatively weak with (potentially) significant dissociation to the inorganic Fe(II) form (4). This inorganic Fe(II) can be oxidized by oxygen to inorganic Fe(III) (5) or can be recomplexed by the organic ligand (6). *L. majuscula* appears capable of uptake of both inorganic ferrous iron (7), and possibly of organically complexed ferrous iron (8), by a membrane-spanning ferrous transporter. The fate of the inorganic dissolved Fe(III) species is ignored in our model because it is not considered important for *L. majuscula*.

component of the FeL model, an important difference between our work with *L. majuscula* and that on two members of the genus *Thalassiosira* studied by Shaked et al. (2005) is illustrated by the effect of the addition of high concentrations of ferrozine, which has a high affinity for unchelated ferrous iron but which will acquire already-complexed ferrous iron more slowly. The elimination of uptake by the *Thalassiosira* species on addition of ferrozine implies uptake only of unchelated Fe(II), whereas the lack of effect on uptake by *L. majuscula* implies that organically complexed Fe(II) is also available to this organism. Although the result with *Thalassiosira* is consistent with the Fe(II)s model, which proposes the production of unchelated ferrous iron as an intermediate step before uptake, it is not inconsistent with the FeL model because it does not rule out formation of unchelated ferrous iron during subsequent processes.

In any case, it is possible that the results of Shaked et al. (2005) could be explained not by the concentration of Fe' but by the effect of EDTA on the concentration of Fe(II)' (via complexation and complex oxidation) predicted by the FeL model. The importance of reoxidation is an issue that is unresolved in the studies described by Shaked et al. (2005), with reoxidation of the reduced ferrous iron considered to be merely a contributor to the inefficiency of the reductive process. This is consistent with their Fe(II)s model because it considers reduction of either Fe′ or FeL to yield unchelated ferrous iron, of which the rate of reoxidation is independent of ligand concentration. On the other hand, the demonstration by Maldonado and Price (2001) that uptake is eliminated in *T. oceanica* by the addition of ascorbate, a reductant, suggests that reoxidation might be a critical postreductive step before uptake for this organism.

The influence of ferrous iron reoxidation on iron acquisition by members of the *Thalassiosira* genus can be determined by measurement of iron uptake (1) in the presence of ascorbate and (2) in a number of different concentrations of a weak iron ligand such as citrate. The former test will demonstrate the possible requirement of reoxidation for uptake, and the latter will provide insight into the acquisition of bound iron and the influence of reoxidation of the ferrous complex.

It thus appears that at least two mechanisms of iron acquisition might exist involving reductive processes that are implemented by marine organisms. With the limited number of species that have been examined and the unresolved issues that exist within analysis of the genus *Thalassiosira*, it is too early to make generalizations as to the distribution of these and, probably more, alternate mechanisms of iron acquisition. It remains to be seen whether they occur along phylogenetic lines, for example, with a division between eukaryotes and prokaryotes. Alternatively, a general alignment is possible with ecological occupation, for example, between coastal and open-ocean organisms or between benthic and planktonic species.

Typically, the relatively eutrophic waters of the coastal environment are dominated by dissolved organic material with reasonably strong iron binding properties. High concentrations of these organic ligands will assist in preventing ferric iron precipitation, and advantage will be conferred on organisms that can efficiently acquire iron from these complexes. It is to be expected that acquisition of iron from these complexes by organisms employing the pathway proposed in the Fe(II)s model would lose efficiency because effort expended in reduction would be wasted by the formation of unavailable ferrous complexes either by reduction of ferric complexes or rapid complexation of ferrous iron by strong ligands. It could then be that organisms capable of reducing ferric complexes to the equivalent ferrous complex and then accessing the whole complex (whether subsequent acquisition proceeds by dissociative or nondissociative processes) might have an advantage in these organic-rich coastal environments over organisms that cannot directly reduce complexed ferric iron.

Although a key attraction of the FeL model lies in its generality and relative simplicity, aspects of the process of the acquisition of iron by *L. majuscula* (and other organisms) are still not addressed by this model. Specifically, we do not yet know the means of acquisition of the complexed ferrous iron. We suggest that uptake is tightly coupled to dissociation of the complex such that it limits the accessibility of unchelated ferrous iron to competing processes, such as complexation by ferrozine. Assisting this process could be a low-oxygen environment at the organism's surface, produced by the rapid reduction of oxygen to superoxide. This relatively reduced environment would favor the stabilization of ferrous iron by preventing reoxidation to the ferric form. Further work investigating aspects of the microenvironment, such as pH and oxygen concentration at the surface of the organism and within its sheath, might yield important insights into the process leading to internalization.

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