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Plasma membrane ferric reductase activity of iron-limited algal cells is inhibited by ferric chelators

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Abstract Iron-limited cells of the green alga *Chlorella kesslerii* use a reductive mechanism to acquire Fe(III) from the extracellular environment, in which a plasma membrane ferric reductase reduces Fe(III)-chelates to Fe(II), which is subsequently taken up by the cell. Previous work has demonstrated that synthetic chelators both support ferric reductase activity (when supplied as Fe(III)-chelates) and inhibit ferric reductase. In the present set of experiments we extend these observations to naturally-occurring chelators and their analogues (desferrioxamine B mesylate, schizokinen, two forms of dihydroxybenzoic acid) and also two formulations of the commonly-used herbicide *N*-(phoshonomethyl)glycine (glyphosate). The ferric forms of the larger siderophores (desferrioxamine B mesylate, schizokinen) and Fe(III)-*N*-(phoshonomethyl)glycine (as the isopropylamine salt) all supported rapid rates of ferric reductase activity, while the iron-free forms inhibited reductase activity. The smaller siderophores/siderophore precursors, 2,3- and 3,4-dihydroxybenzoic acids, did not support high rates of reductase in the ferric form but did inhibit reductase activity in the iron-free form. Bioassays indicated that Fe(III)-chelates that supported high rates of ferric reductase activity also supported a large stimulation in the growth of iron-limited cells, and that an excess of iron-free chelator decreased the growth rate. With respect to *N*-(phosphonomethyl)glycine, there were differences between the pure compound (free acid form) and the most common commercial formulation (which also contains isopropylamine) in terms of supporting and inhibiting ferric reductase activity and growth. Overall, these results suggest that photosynthetic organisms that use a reductive strategy for iron acquisition both require, and are potentially simultaneously inhibited by, ferric chelators. Furthermore, these results also may provide an explanation for the frequently contradictory results of *N*-(phosphonomethyl)glycine application to crops: we suggest that low concentrations of this molecule likely solubilize Fe(III), making it available for plant growth, but that higher (but sub-lethal) concentrations decrease iron acquisition by inhibiting ferric reductase activity.

Keywords Algae · Chelators · *Chlorella* · Desferrioxamine B · Ferric reductase · Glyphosate · Iron limitation · Schizokinen · Siderophores

Abbreviations

BICINE	*N,N*-bis(2-hydroxyethyl)glycine
BPDS	Bathophenanthroline disulfonic acid
DFB	Desferrioxamine B
2,3-DHBA	2,3-Dihydroxybenzoic acid
3,4-DHBA	3,4-Dihydroxybenzoic acid

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HBED	*N,N′*-bis(2-hydroxybenzyl)-ethylenediamine-*N,N′*-diacetic acid
HEDTA	Hydroxyethylethylenediaminetriacetic acid
HEPES	*N*-(2-hydroxyethyl)piperazine-*N′*-(2-ethanesulfonic acid)
IPA	Isopropylamine
PMG	*N*-(phosphonomethyl)glycine

Introduction

Among plants and algae there are two well-documented iron acquisition mechanisms. The vast majority of vascular plants (all non-grasses) use a reductive system, sometimes called "Strategy I", which is also exhibited by many eukaryotic algal species. In this mechanism iron limitation leads to increased plasma membrane ferric reductase activity and Fe(II) transport capacity. The ferric reductase uses intracellular reducing power to reduce extracellular Fe(III)-chelates, and the resulting Fe(II) is transported across the plasma membrane by a separate Fe(II) transport system (Schmidt 2003). This system is necessitated by the fact that in aerobic situations iron tends to exist in the poorly soluble Fe(III) state, which occurs in soil and aquatic environments as Fe(III)-oxyhydroxides, Fe(III)-organic complexes, and/or as a mixture of inorganic and organic Fe(III) complexes (Dolfing et al. 1999; Gunnars et al. 2002; Gustafsson et al. 2007). Therefore, Fe(III) is not directly biologically available, and the ferric reductase serves to generate the much more soluble Fe(II) which can be transported across the plasma membrane.

In vascular plants, the increase in ferric reductase capacity typically is localized to the plasma membrane of root cells; for example in iron-limited pea plants the ferric reductase *FRO1* mRNA is highest in the root epidermal cells, which are in contact with the soil (Waters et al. 2002). However, leaf mesophyll cells also exhibit plasma membrane ferric reductase activity (Brüggemann et al. 1993; de la Guardia and Alcántara 1996). Although less investigated than the vascular plants, several species of green algae, including *Chlorella kessleri*¸ the focus of this work, have been demonstrated to exhibit a reductive mechanism of iron acquisition (e.g. Allnutt and Bonner 1984; Keshtacher-Liebson et al. 1999; Eckhardt and Buckhout 2000; Weger et al. 2002).

Among the vascular plants, members of the grass family (Poaceae/Graminae) are unique in not using a reductive mechanism of iron acquisition. The grass iron acquisition mechanism, often called "Strategy II", is based on the iron limitation-induced release, by roots, of phytosiderophores (nicotianamine-based Fe(III) chelating molecules) that serve to scavenge iron from the environment (Mori 1999). The intact Fe(III)-phytosiderophore complex is then transported across the plasma membrane via a specific transport system (Schaaf et al. 2004). A very similar iron acquisition mechanism is exhibited by many cyanobacteria, bacteria and fungi, which produce siderophores in response to iron limitation. However, these latter siderophores have distinctly different chemical structures from the phytosiderophores, and are not based on the nicotianamine molecule (Barry and Challis 2009).

With respect to the reductive iron acquisition mechanism, Weger et al. (2009) recently presented a model for the interactions between ferric chelators and ferric reductase activity by iron-limited cells of the green alga *Chlorella kessleri*. This model suggests that ferric chelators interact with ferric reductase in several ways: (1) Fe(III)-chelates in which the chelator has a higher affinity for Fe(III) will support lower ferric reduction rates, (2) higher affinity chelators will more effectively compete with the Fe(II) transport system for Fe(II) produced by ferric reductase activity, thus decreasing the Fe(II) transport rate, and (3) iron-free higher affinity chelators will directly inhibit ferric reductase activity.

The model was based primarily on results of experiments using the synthetic chelators HEDTA and HBED, both of which are structurally related to the well-known Fe(III) chelator EDTA. In this paper, we focus on the third aspect of that model, the inhibition of ferric reductase activity by iron-free chelators. We examine the effects of common chelators that potentially are found in the natural environment, including the herbicide *N*-(phosphonomethyl)glycine (PMG, "glyphosate"), on plasma membrane ferric reductase activity in iron-limited *Chlorella* cells.

The chelators examined include two isomers of dihydroxybenzoic acid (DHBA): 2,3-DHBA and 3,4-

DHBA. Both 2,3-DHBA (López-Goñi et al. 1992) and 3,4-DHBA (Calugay et al. 2006) are bacterial siderophores in their own right, and are also components of simple DHBA-amino acid conjugates/siderophores that are produced by certain bacteria under iron-limited conditions (e.g. Ito and Neilands 1958; Hantke 1990; Temirov et al. 2003). 2,3-dihydroxybenzoylysine (2,3-DHBA with a conjugated lysine) will solubilize precipitated ferric hydroxide (Duhme et al. 1996). As well, 2,3-DHBA is the common source of catechol Fe(III) chelating moieties in large multidentate catecholate siderophores (e.g. enterobactin, bacillibactin, and numerous other bacterial siderophores; Drechsel and Jung 1998). 3,4-DHBA is less commonly found in catecholate siderophores than is 2,3-DHBA, but is a component of petrobactin (Bergeron et al. 2003) and petrobactin sulfonate (Hickford et al. 2004). Both 2,3- and 3,4-DHBA have also been used as analogues for the phenolic components of humic substances in studies of metal complexation (e.g. Gerard et al. 1987; Borges et al. 2005).

We have also investigated the trihydroxamate molecule desferrioxamine B (DFB) mesylate, which is the commercially available form (has medical utility in chelation therapy) of the bacterial siderophore DFB (Imbert et al. 1995). DFB and/or DFB mesylate have been used to impose artificial iron limitation in aquatic systems (e.g. Timmermans et al. 2001; Eldridge et al. 2004; Wells and Trick 2004). Addition of this compound induces iron limitation in a wide variety of marine algal groups, including cyanobacteria and different size classes of eukaryotic cells (Eldridge et al. 2004). Interestingly, Fe(III)-DFB is a viable iron source for growth of cells of the diatom *Thalassiosira oceanica* (Maldonado and Price 2001) but not for the diatom *Chaetoceros sociale* (Iwade et al. 2006). And Weger et al. (2006), in a comparison of reductive iron acquisition by two species of green algae, demonstrated that iron-limited cells of *Chlorella kessleri* rapidly reduce, and assimilate iron from, Fe(III)-DFB, while iron-limited cells of *Chlamydomonas reinhardtii* reduce Fe(III)-DFB much more slowly and also grow much more slowly on that iron source.

The work with iron-limited Strategy I plant species examining Fe(III)-DFB as an iron source suggests that Fe(III)-DFB is either not a substrate for the ferric reductase of roots from iron-limited plants (e.g. Manthey et al. 1996; Johnson et al. 2002) or is a poor substrate (Bar-Ness et al. 1992). However, it is clear that soils contain hydroxamate siderophores of microbial origin (e.g. Powell et al. 1980), and it is generally conceded that siderophores produced by soil bacteria may positively influence plant iron acquisition (Rroço et al. 2003).

In terms of naturally occurring siderophores, we also examined the dihydroxamate molecule schizokinen. This molecule is produced by some bacteria (Budzikiewicz et al. 1997) and by members of the freshwater cyanobacterial genus *Anabaena* (Simpson and Neilands 1976; Nicolaisen et al. 2008). The presence of schizokinen inhibits the growth of iron-limited cells of the green alga *Chlamydomonas reinhardtii* (Matz et al. 2004). Subsequent further work with iron-limited green algal cells demonstrated that, similar to the situation with DFB, there are species differences in the abilities of iron-limited cells to acquire iron from Fe(III)-schizokinen, with *Chlorella kessleri* being much more efficient at utilizing that iron source than *Chlamydomonas* (Weger et al. 2006). And classic work by Murphy et al. (1976) provided evidence that hydroxamate siderophores produced by iron-limited cyanobacteria inhibit the growth of green algae, presumably by sequestering iron in a form that is biologically unavailable to green algae.

Lastly, we examined the potential of the herbicide PMG to inhibit ferric reductase activity by iron-limited cells. PMG is one of the world's most commonly used herbicides, and a number of recent studies have pointed towards a negative interaction between PMG application and metal micronutrient (including iron) metabolism (Franzen et al. 2003; Jolley et al. 2004; Eker et al. 2006). While the well-known propensity of PMG to form chelates with metal cations (Motekaitis and Martell 1985) has led to suggestions that the inhibitory effects of sub-lethal PMG applications may be caused by formation of poorly soluble metal-PMG complexes within plant tissues and/or the rhizosphere (e.g. Jolley et al. 2004; Eker et al. 2006), more recent work has supplied evidence that PMG has a negative effect on root plasma membrane ferric reductase activity (Ozturk et al. 2008; Bellaloui et al. 2009).

In this paper, we further examine the model of reductive iron acquisition put forward by Weger et al. (2009). Specifically, we examine the potential of

potentially common soil and aquatic molecules (siderophores/humic acid analogues, and the herbicide PMG) to both support and inhibit iron acquisition by an organism that uses a reductive (Strategy I) iron acquisition mechanism.

Materials and methods

Chlorella kessleri Fott et Nováková UTEX 263 was obtained from the University of Texas Culture Collection. Cells were grown in iron-limited chemostat cultures (Weger 1999) at a dilution rate of 0.15 d^{-1} in water-jacketed glass vessels at a temperature of 25°C. Chemostat cultures are a type of continuous culture in which the culture growth rate is set by the rate of supply of the limiting nutrient (in this case, iron). The use of chemostat cultures allows for the reproducible production of physiologically uniform nutrient-limited cells.

Light for algal cultures was supplied by a bank of high output, cool white fluorescent lamps (Sylvania F48T12/CW/HO); photosynthetic photon flux density was approximately 80 μmol m^{-2} s^{-1}. Iron concentration in the medium was 100 nM (from an acidified stock of $Fe(NO_3)_3 \cdot 9H_2O$), and was chelated with 16 μM hydroxyethylethylenediaminetriacetic acid (HEDTA). The medium was a modification of that of Hughes et al. (1958); Si and carbonate were omitted, and N was supplied as 8 mM $NaNO_3$. Minor elements were supplied as in Allen (1968), supplemented with Se and Ni (0.05 and 0.15 μM final concentrations, respectively). The medium was buffered at pH 8 with 5 mM *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid) (HEPES), and the cultures were aerated with 0.5% CO_2 in air (continuously monitored via an IRGA [Model S153; Qubit Systems, Kingston, ON, Canada]); steady-state culture pH was approximately 7.6. All chemostat components were washed with 5% HNO_3 prior to sterilization.

The siderophore schizokinen was isolated from iron-limited cultures of the cyanobacterium *Anabaena flos-aquae* (Lyng.) Brèb. (UTEX 1444, which was obtained from the University of Texas Culture Collection). *Anabaena* cells were grown in iron-limited chemostat cultures under conditions similar to those described above for *Chlorella*. The medium was buffered at pH 8 with 10 mM *N,N*-bis (2-hydroxyethyl)glycine (BICINE), iron was supplied at 0.25 μM.

Ferric reductase activity

Ferric reductase activity was quantified via two different methods: (1) colorimetrically using bathophenanthroline disulfonic acid (BPDS), and (2) using an oxygen electrode (both methods are described in Weger et al. 2007). For both methods, iron-limited *Chlorella* cells were harvested by centrifugation (1 min at 2000 g) and re-suspended in assay buffer (5 mM HEPES, pH 8.0, 245 μM $CaCl_2$) at varying cell densities (ranging from 1/10th the chemostat culture cell density up to 10× the chemostat density). Chemostat culture cell density was approximately 20×10^6 cells ml^{-1}.

For the colorimetric assay, when using Fe(III)-DFB as the substrate for ferric reductase, the molar absorptivity of the Fe(II)-$BPDS_3$ complex (22140 M^{-1} cm^{-1} at 535 nm; Blair and Diehl 1961) was adjusted to take into account the absorption of the Fe(III)-DFB complex at 535 nm (determined to be 626 M^{-1} cm^{-1}). A similar adjustment was necessary when using Fe(III)-HBED as the iron source (molar absorptivity at 535 nm 2560 M^{-1} cm^{-1}; Weger et al. 2009). This adjustment was not necessary when using Fe(III)-HEDTA, as the absorption at 535 nm was very low (11.5 M^{-1} cm^{-1}; Weger et al. 2009).

For the oxygen electrode-based ferric reductase measurements, the stimulation of O_2 consumption upon addition of Fe(III)-chelate was used to estimate ferric reductase activity (see Weger et al. 2007 for details). Oxygen consumption was quantified using an oxygen electrode (Hansatech, Norfolk, UK).

Bioassays/growth experiments

To determine the ability of various ferric-chelates to support growth, 30 ml aliquots (subcultures) were aseptically removed from the chemostats, pelleted by centrifugation in sterile centrifuge tubes, re-suspended in sterile, iron-free medium, and then placed into sterile, acid-washed Erlenmeyer flasks. These subcultures were constantly stirred, and aerated with 0.5% CO_2 in air, and received the same illumination

as the chemostat cultures. Iron was added at a final concentration of 1000 nM chelated with one of HEDTA, DFB, schizokinen, PMG-isopropylamine (PMG-IPA), PMG, isopropylamine (IPA), 2,3 DHBA or 3,4 DHBA. The above experiments were carried out in the presence and absence of BPDS (when present, final concentration was 500 μM). 500 μM BPDS completely inhibits the growth of *Chlorella* in the presence of Fe(III)-HEDTA by binding the reduced ferrous iron, preventing uptake by the cells (Weger et al. 2009). Bioassays were run over a 116 h time course.

Schizokinen isolation

For isolation of schizokinen, cells were removed from the medium by centrifugation. One litre of supernatant was passed over an Amberlite XAD-4 (Supelco, Bellefonte, PA, USA) column (9 cm × 6.35 cm). The column was washed with distilled water, and then eluted with 100% methanol. Schizokinen-containing fractions were detected using the CAS assay (Schwyn and Neilands 1987) and combined. Ammonium sulphate was added to precipitate proteins and prevent foaming during rotary evaporation. The combined fractions were rotary evaporated to dryness, and a small amount of methanol was added to dissolve the schizokinen and maintain the extracellular polymeric substances (EPS) and ammonium sulphate in the precipitate. The EPS strongly bound the dye alcian blue 8GX, indicating that it was likely composed of largely of negatively charged polysaccharides (Alldredge et al. 1993). After centrifugation, the methanolic solution was applied to a column of Sephadex LH-20 (GE Healthcare, Uppsala, Sweden; column dimensions 12.5 cm × 6.35 cm). The column was eluted with 100% methanol, the schizokinen-containing fractions were again combined and the volume reduced to approximately 2 ml by rotary evaporation. The eluent was then applied to a second LH-20 column (20 cm × 3.8 cm), and 90% water/10% methanol was used as the mobile phase to elute the schizokinen. Schizokinen-containing fractions were combined and dried under a stream of N_2. Purity of the schizokinen preparation was checked by thin-layer chromatography (silica gel 60 F254, from Selecto Scientific, Suwannee, GA, USA; 100% methanol as the mobile phase). Schizokinen was visualized on the TLC plates using a saturated methanolic solution of ferric nitrate; purified schizokinen yielded a single burgundy-coloured spot with an R_f of approximately 0.65. The *Anabaena* UTEX 1444 siderophore co-migrates on TLC with schizokinen from *Anabaena* ATCC 19213 (Gress and Weger unpubl. results). Schizokinen preparations were stored in a freezer over silica gel until needed.

Other methods

Chlorophyll was quantified spectrophotometrically after extraction in 100% methanol (Porra et al. 1989). Cells were enumerated using a hemacytometer after preservation in Lugol's solution. Iron for cell cultures and for all experiments was a commercially-prepared atomic absorption standard of 1000 mg iron ($Fe(NO_3)_3{\cdot}9H_2O$) per l in 2% HNO_3 (Spex Certi-Prep, Metuchen, NJ, USA).

We evaluated the potential toxicity of the maximum concentrations of all tested chelators by evaluating the effects on photosynthesis. Chlorophyll *a* fluorescence was used to determine the maximum quantum yield of Photosystem II of dark-adapted cells (F_v/F_m, 30 min in darkness). Fluorescence was quantified using an OS5-FL pulse-modulated fluorometer (Opti-Sciences, Tyngsboro, MA, USA). Saturation pulse duration was 0.8 s (see Schreiber et al. 1994 for details about the theory underlying chlorophyll *a* fluorescence measurements). Algal cells were harvested by centrifugation and re-suspended in assay buffer at twice the chemostat culture cell density. The chelators were added to the algal suspension prior to dark adaptation.

DFB, HEDTA and schizokinen were dissolved in distilled water. PMG was used in two forms: a 40% aqueous solution of the PMG-IPA salt, and the PMG free acid (powder). The aqueous solution was diluted with distilled water as needed, and the free acid was dissolved in water. Fe(III)-DFB, (III)-HEDTA, Fe(III)-schizokinen and Fe(III)-PMG stocks were prepared in a ratio of 1:2 Fe(III):chelator. Both 2,3- and 3,4-DHBA were dissolved in 0.8 M KOH, and Fe(III):chelator was ratio was 1:6.

BPDS was from Alfa Aesar (Ward Hill, MA, USA) and IPA was from Anachemia (Winnipeg, MB, Canada). All other chemicals were from Sigma-Aldrich (Oakville, ON, Canada).

Results

With respect to the trihydroxamate siderophore DFB, Fe(III)-DFB supported high rates of ferric reductase activity; these rates decreased with increasing DFB concentrations (Fig. 1) in a manner consistent with the decreases associated with increasing HEDTA or HBED concentrations described by Weger et al. (2009). Increasing DFB levels also decreased the iron-limited culture growth rates, with 2500 μM supporting poor culture growth (Fig. 2). Similar effects on ferric reductase activity were obtained with the dihydroxamate siderophore schizokinen, with the exception that overall ferric reduction rates were lower (Fig. 1). As well, similar to DFB, high schizokinen concentrations (2500 μM) lead to zero culture growth (Fig. 2). Thus, both of the hydroxamate siderophores supported ferric reductase activity at low external hydroxamate concentrations, but higher hydroxamate levels lead to greatly diminished activity.

Neither Fe(III)-DFB nor Fe(III)-schizokinen would support growth of iron-limited cells in the presence of the Fe(II) chelator BPDS (Fig. 2). This indicates that extracellular reduction of Fe(III) to Fe(II) was an obligate part of the iron acquisition mechanism.

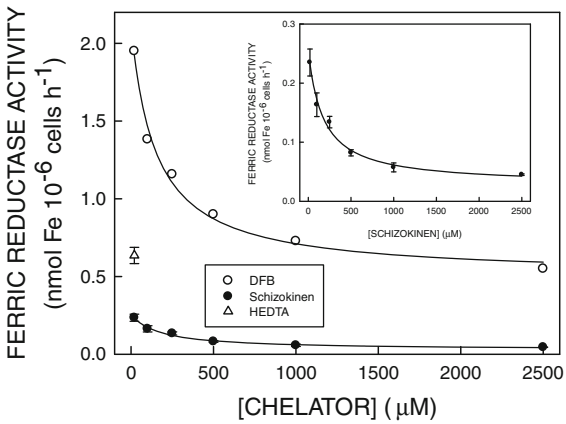

Fig. 1 Effects of increasing siderophore (DFB mesylate, schizokinen) concentrations on ferric reductase activity (measured spectrophotometrically) by iron-limited *Chlorella* cells. Fe(III) was provided at 10 μM, with various concentrations of siderophore. The ferric reductase rate with 10 μM Fe(III) and 20 μM HEDTA is shown for comparison. Inset: data for ferric reductase activity with Fe(III)-schizokinen. Data are the means of three determinations (bars represent SE)

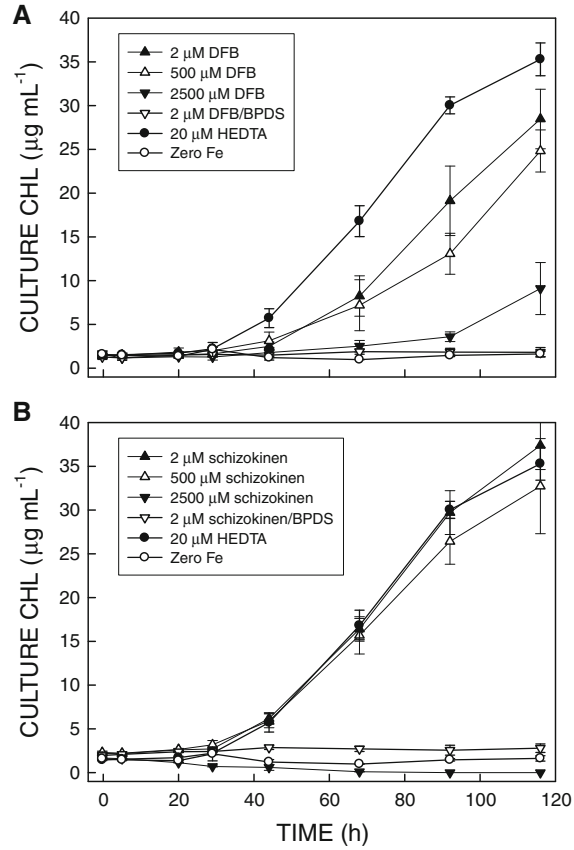

Fig. 2 Bioassays examining the effects varying concentration of siderophores on the growth of iron-limited *Chlorella* cultures. Iron was added at 1 μM, which is 10× greater than the chemostat culture iron concentration. **a** DFB mesylate, **b** schizokinen. Bars represent SE ($n = 3$–4)

In terms of the catecholate molecules, rates of reduction of Fe(III)-3,4-DHBA by iron-limited *Chlorella* cells were very low; virtually indistinguishable from the cell-free ferric reduction rate and much lower than the cell-mediated rate of Fe(III)-HEDTA reduction (Fig. 3). This suggests that low rates of non-biological 3,4-DHBA mediated Fe(III) reduction explained the majority of the reduction observed under these conditions. Reduction of Fe(III)-2,3-DHBA was substantially higher than 3,4-DHBA (and higher than the cellular rate of Fe(III)-HEDTA reduction), but the reduction appeared to be mainly non-cellular as well (Fig. 3). These results suggest that dihydroxybenzoic acids are poor substrates for the *Chlorella* ferric reductase. Nonetheless, Fe(III) chelates of both 2,3- and 3,4-DHBA supported the growth of iron-limited *Chlorella* cells, and this could

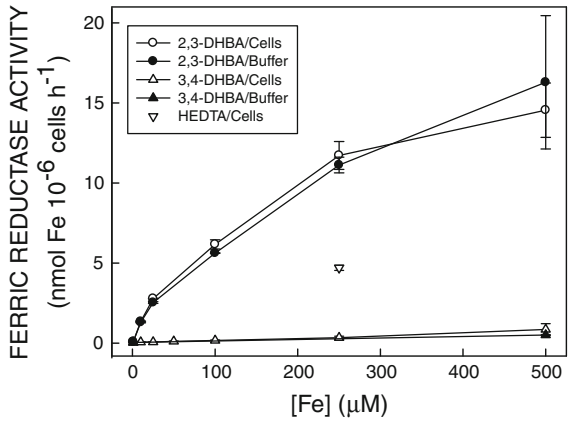

Fig. 3 Effects of Fe(III) concentration on apparent ferric reductase activity by iron-limited *Chlorella* cells and in assay buffer, quantified spectrophotometrically. The chelated Fe(III) sources were Fe(III)-2,3-DHBA and Fe(III)-3,4-DHBA. DHBA was present in 6-fold excess over Fe(III) at all Fe(III) concentrations. For both forms of DHBA there was no detectable effect of the presence of cells, and we conclude that most of the apparent ferric reductase activity was non-cellular. Cellular ferric reduction using Fe(III)-HEDTA (1:2 ratio) is shown for comparison. Data are the means of three determinations (bars represent SE)

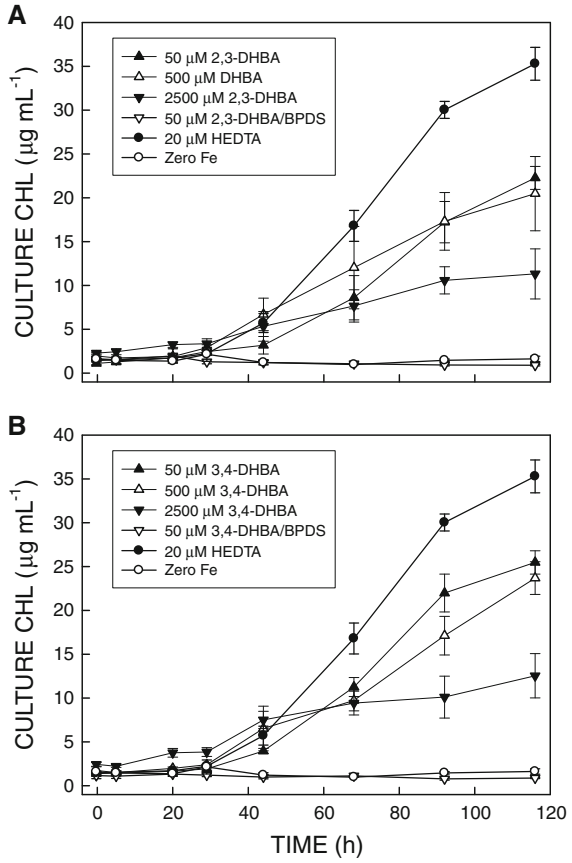

Fig. 4 Bioassays examining the effects varying concentrations of 2,3- and 3,4-DHBA on the growth of iron-limited *Chlorella* cultures. Iron was added at 1 μM. **a** 2,3-DHBA, **b** 3,4-DHBA. Bars represent SE ($n = 3$–4)

be inhibited by the addition of the Fe(II) chelator BPDS, indicating that a reductive mechanism was occurring (Fig. 4). We speculate that the non-cellular Fe(III) reduction rate was sufficient to supply (Fe(II) and support growth in the absence of BPDS.

However, despite the fact that the Fe(III)-DHBAs were poor substrates for *Chlorella* ferric reductase, they could act as inhibitors of the reaction when Fe(III)-HBED was the substrate (Fig. 5). For experiments examining the inhibition of ferric reductase by DHBAs, we used Fe(III)-HBED as the iron source, rather than Fe(III)-HEDTA. Preliminary spectrophotometric investigations indicated that at high levels of DHBA, Fe(III)-HEDTA lost Fe(III) to DHBA. In contrast, the much stronger Fe(III) chelator HBED was able to retain Fe(III) in the presence of 2.5 mM DHBA (not shown). Both DHBAs inhibited ferric reductase by iron-limited cells in a concentration-dependent manner (Fig. 5).

With respect to phosphonomethylglycine (PMG), we tested one of the common commercial formulations, a 1:1 mixture of PMG and isopropylamine (IPA), and also the PMG free acid (no IPA), and IPA in the absence of PMG. Both PMG and IPA are Fe(III) chelators, and both PMG formulations were effective inhibitors of ferric reductase activity (Fig. 6), with PMG-IPA having a slightly larger effect. As well, IPA, in the absence of PMG, was also inhibitory of ferric reductase (Fig. 6).

However, bioassays with PMG-IPA did not survive past 1 day when PMG-IPA was used at 500 μM (Fig. 7). This was consistent with the negative effects of PMG-IPA on photosynthesis (Table 1). In fact, of all of the tested chelators, only PMG-IPA inhibited photosynthesis (Table 1), implying that this formulation exerted a non-specific toxic effect on the cells. On the other hand, both Fe(III)-PMG and Fe(III)-IPA could support the growth of iron-limited cells at low chelator concentrations, but exhibited growth inhibition at higher concentrations (Fig. 7).

In order to confirm that the apparent inhibition of ferric reductase activity by the various Fe(III) chelators was not simply due to an artefact associated

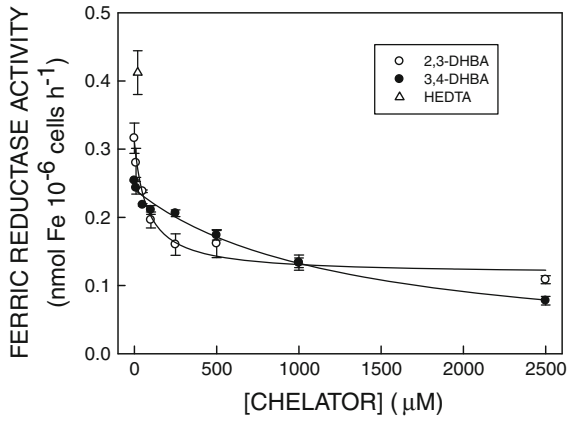

Fig. 5 Effects of increasing DHBA concentrations on ferric reductase activity (measured spectrophotometrically) by iron-limited *Chlorella* cells. Fe(III) was provided at 10 μM, chelated with 20 μM HBED, and the effects of increasing DHBA concentrations on reduction of Fe(III)-HBED was examined. The ferric reductase rate with 10 μM Fe(III) and 20 μM HEDTA is shown for comparison. Data are the means of three determinations (bars represent SE)

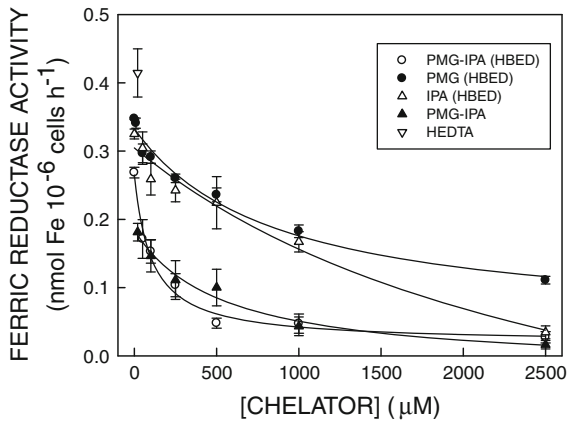

Fig. 6 Effects of increasing PMG, PMG-IPA and IPA concentrations on ferric reductase activity (measured spectrophotometrically) by iron-limited *Chlorella* cells. In the figure legend, "(HBED)" indicates that Fe(III) was provided at 10 μM, chelated with 20 μM HBED. For PMG-IPA, Fe(III) was supplied as 10 μM Fe(III) chelated with 20 μM PMG-IPA. The ferric reductase rate with 10 μM Fe(III) and 20 μM HEDTA is shown for comparison. Data are the means of three determinations (bars represent SE)

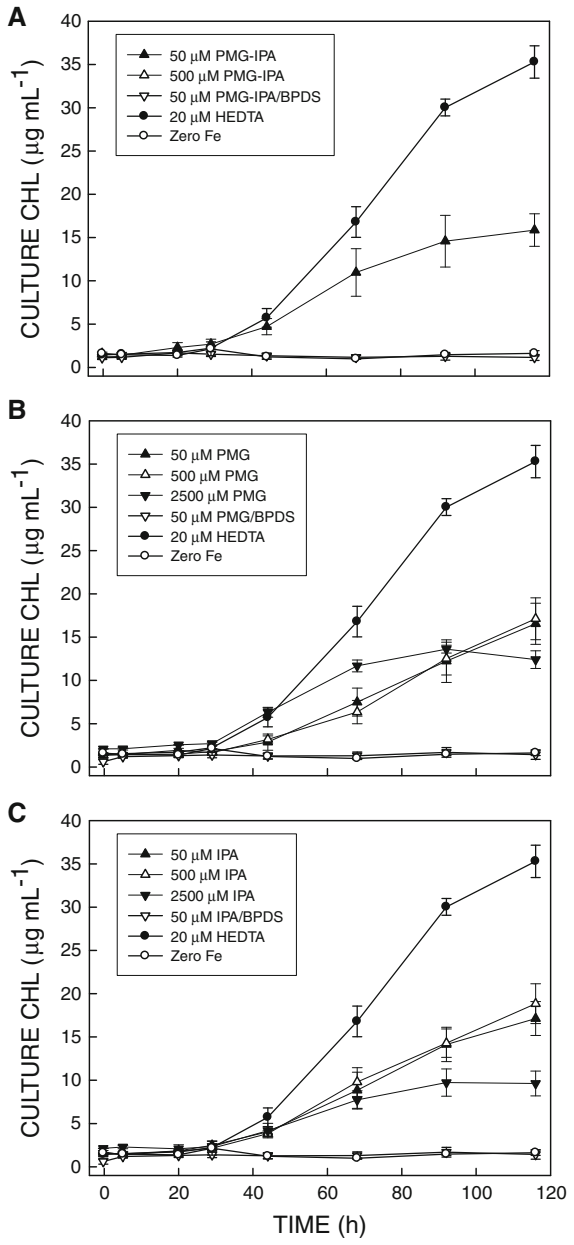

Fig. 7 Bioassays examining the effects varying concentrations of **a** PMG-IPA, **b** PMG (free acid), and **c** IPA, on the growth of iron-limited *Chlorella* cultures. Iron was added at 1 μM. Bars represent SE ($n = 3$). For **a**, addition of 2500 μM PMG-IPA resulted in rapid culture death, and no results are shown

with the spectrophotometric (colorimetric) enzyme assay (i.e. due to competition between BPDS and chelators for Fe(II) formed as a result of ferric reductase activity), we performed two additional checks. First, for the highest concentrations for every tested chelator, we re-ran the colorimetric assays at three times the standard BPDS concentration; in all cases there was no detectable difference in ferric reductase rates (not shown). Second, we used an oxygen electrode to measure ferric reductase activity in a BPDS-independent manner. While this method is

Table 1 Maximum quantum yield (F_v/F_m) as affected by various ferric chelators

Treatment	F_v/F_m
Control	0.587 ± 0.008
2,3-DHBA	0.584 ± 0.005
3,4-DHBA	0.585 ± 0.011
DFB	0.555 ± 0.026
Schizokinen	0.576 ± 0.006
PMG-IPA	0.230 ± 0.024
PMG	0.576 ± 0.009
IPA	0.598 ± 0.021

Chelators (2500 μM) were added to the cells immediately prior to the 30 min dark adaptation period. Numbers are the means of 5 determinations (±SE)

not as sensitive as the BPDS-dependent spectrophotometric method, it provides an independent estimate of the effects of chelators on ferric reductase activity. The oxygen electrode data showed the same patterns as the spectrophotometric data: increasing inhibition of ferric reductase activity with increasing iron-free chelator concentration (Fig. 8).

Effects of biomass were also tested for the inhibition of ferric reductase activity by DFB (Fig. 9). The inhibitory effect of DFB increased as biomass decreased, suggesting that the effect of DFB was not simply due to general toxicity, but that DFB interacted with a specific molecule on the algal plasma membrane.

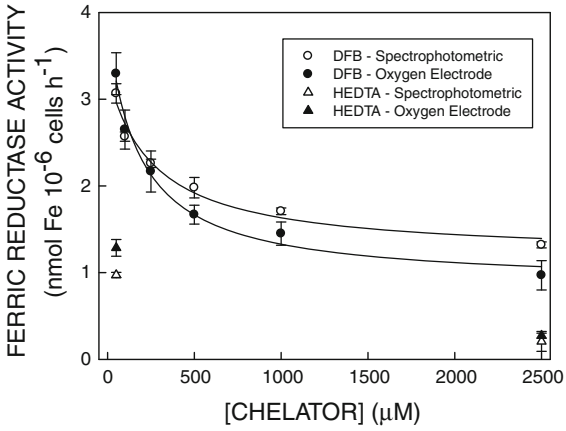

Fig. 8 Comparison of spectrophotometric and oxygen electrode-based measurements of the inhibition of ferric reductase activity by chelators. Iron-limited *Chlorella* cells were re-suspended in assay buffer at two times the original cell density, and the same batch of cells was assayed simultaneously using the two techniques. Data are the means of five determinations (bars represent SE)

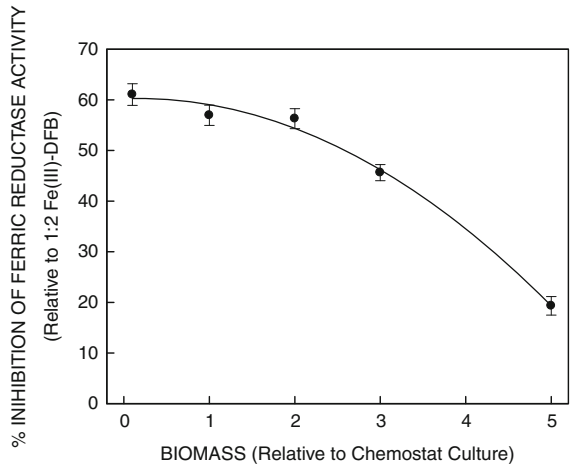

Fig. 9 Effects of relative biomass on the inhibition of ferric reductase activity (measured spectrophotometrically) by 500 μM DFB. Iron-limited *Chlorella* cells were harvested by centrifugation and re-suspended in assay buffer at various cell densities. Fe(III) was added at 10 μM, and rates are expressed relative to the ferric reductase activity with 10 μM and 20 μM DFB. Data are the means of three determinations (bars represent SE)

Discussion

Weger et al. (2009) proposed a model for the effects of ferric chelators on iron acquisition by organisms that use a reductive iron acquisition mechanism. According to this model, iron-free Fe(III) chelators decrease iron acquisition by two separate mechanisms: 1) competition between the Fe(II) transport system and the chelators for the Fe(II) produced via ferric reductase activity (Fe(III) chelators will also bind Fe(II)), and 2) direct inhibition of ferric reductase activity by chelators. Increasing concentrations of the synthetic chelators HEDTA or HBED resulted in progressively decreasing ferric reductase activity (Weger et al. 2009). In the present work, we show that naturally-occurring chelators/siderophores, and also a commonly-used herbicide, inhibit ferric reductase activity and also inhibit the growth of iron-limited cells when applied at high concentrations, but stimulate growth when applied at lower concentrations. These observations have potentially important implications for agricultural and aquatic systems.

Siderophores and siderophore precursors/humic acid analogues

Both of the tested siderophores (DFB, schizokinen) inhibited ferric reductase activity in a concentration-dependent manner. However, the Fe(III)-siderophore complexes were also a viable iron source for growth as long the iron-free siderophore concentration was not high. The fact that BPDS inhibited growth of iron-limited cells in the presence of Fe(III)-siderophore indicated that a reductive step was required of the iron uptake process under these conditions.

DFB has been used to induce iron limitation in marine systems. The hypothesis for the effect of DFB has been that DFB sequesters Fe(III), thereby making it unavailable for uptake by marine algal species (Wells and Trick 2004). Similarly, Murphy et al. (1976) showed that iron-limited cells of the cyanobacterium *Anabaena* inhibited the growth of a green alga, and suggested that siderophore production by the *Anabaena* sequestered the aquatic Fe(III). Later work by Matz et al. (2004) demonstrated that only iron-limited *Anabaena* cells inhibited the growth of iron-limited cells of the green alga *Chlamydomonas reinhardtii* (which, like *Chlorella*, uses a reductive mechanism of iron acquisition; Eckhardt and Buckhout 2000); these iron-limited *Anabaena* cells produced the siderophore schizokinen. The basis of the inhibition was suggested to be the poor bioavailability of Fe(III)-schizokinen to *Chlamydomonas* (Weger et al. 2006).

Interestingly, iron-limited cells of *Chlorella* are able to acquire iron from Fe(III)-schizokinen, and also from Fe(III)-DFB, much more effectively than *Chlamydomonas* (Weger et al. 2006). In other words Fe(III) complexes of hydroxamate siderophores exhibit differential bioavailability between green algal species. However, the present work showed that even for *Chlorella*, high levels of DFB or schizokinen proved to be inhibitory. We suggest that the inhibitory effect was due to hydroxamate siderophore inhibition of ferric reductase activity.

The presence of BPDS prevented culture growth on Fe(III)-DFB (and also Fe(III)-schizokinen), indicating that an extracellular reduction step was essential for acquiring iron from Fe(III)-hydroxamate complexes. This stands in contrast to work with some plants that suggest that a moderate amount of iron uptake from Fe(III)-DFB or from Fe(III)-pyoverdine (a siderophore produced by fluorescent pseudomonads) may occur through a mechanism that does involve plasma membrane ferric reduction (Wang et al. 1993; Manthey et al. 1996; Johnson et al. 2002; Vansuyt et al. 2007).

In contrast to the hydroxamates, the simple dihydroxybenzoic acids (2,3-DHBA, 3,4-DHBA) did not support substantial rates of ferric reductase activity when supplied as Fe(III)-DHBA. Nonetheless, both Fe(III)-2,3-DHBA and Fe(III)-3,4-DHBA supported low rates of growth of iron-limited *Chlorella*, which could be inhibited by the presence of BPDS. In this case, we suggest that the DHBA-catalyzed non-cellular reduction of Fe(III) produced sufficient Fe(II) to support some growth of iron-limited cells. While Fe(III)-DHBA did not support high rates of cellular ferric reduction or growth, iron-free DHBA inhibited ferric reduction and growth.

N-(Phosphonomethyl)glycine (PMG)

There is a growing body of work that suggests that PMG (glyphosate), at sub-lethal concentrations, and/or when applied to glyphosate-resistant crop cultivars, interferes with plant iron metabolism. While some reports suggest that the interference may be due to PMG-mediated sequestration of metal micronutrients (see "Introduction"), others have provided evidence that PMG has a more direct effect on reductive iron acquisition. For example, PMG inhibits root ferric reductase activity in iron-deficient sunflower plants (Ozturk et al. 2008), and simulated PMG spray drift inhibits root ferric reductase activity and decreases leaf iron content in both PMG-sensitive and—resistant soybean cultivars (Bellaloui et al. 2009). However, the situation is made more complex by the fact that much of the PMG-associated research is performed with commercial formulations, which include not only PMG and a cation (e.g. IPA), but sometimes also one or more adjuvants. Ozturk et al. (2008) used a PMG-IPA formulation in their experiments. As both PMG and IPA are iron chelators, it may be difficult to separate the effects of PMG and IPA. Bellaloui et al. (2009) used RoundUp WeatherMAX® in their work; this formulation contains the potassium salt of PMG (49% by mass) and 51% of trade secret "other ingredients".

In this work, it was apparent that both PMG and the common cation IPA were independently capable

of inhibiting ferric reductase activity, although only the combination of PMG and IPA together, led to cell death. However, both can also support iron-limited growth by acting as sources of chelated Fe(III), but also inhibited ferric reductase activity and reductive iron acquisition at higher concentrations.

Mechanism of ferric reductase inhibition

The increasing inhibitory nature of DFB on ferric reductase activity as biomass decreases suggests that there is a specific site of action of the chelators: as biomass decreases, the ratio of chelator to target would increase. The mechanism of chelator-induced inhibition may be chelation of iron molecules in the ferric reductase enzyme. While the ferric reductase from *Chlorella* has not yet been characterized at the biochemical or molecular level, all ferric reductases characterized to date are di-iron (as heme) enzymes, including the yeast (*Saccharomyces cerevisiae*) ferric reductase FRE1 (Finegold et al. 1996) and the mammalian duodenal Dcytb (McKie 2008). Among vascular plants, the *Arabidopsis* root ferric reductase FRO2 exhibits a very similar structure to yeast FRE1 and is also postulated to be a di-heme enzyme with four histidine residues that coordinate the heme groups (Schagerlöf et al. 2006), as is the *Chlamydomonas reinhardtii* FRE1 ferric reductase that is induced under iron limitation (Allen et al. 2007).

Inhibition of metalloenzyme activity by metal chelators, while not previously demonstrated for a ferric reductase, has been documented for several other enzymes. For example, inhibitors of mammalian matrix metalloproteinases (which contain two Zn(II) molecules in the active site) contain functional groups (e.g. hydroxamate or carboxylate) capable of chelating the active site Zn(II) (Whittaker et al. 1999). Newsome et al. (2007) showed that apolactoferrin, but not Zn(II)-lactoferrin inhibits matrix metalloproteinase activity, presumably by chelating the active site Zn(II). As well, small iron chelators (including DFB) inhibit the activities of the non-heme iron-containing enzymes deoxyhypusine hydroxylase and 4-prolyl hydroxylase (Clement et al. 2002). Small chelators also inhibit activity of the iron-containing enzyme lipoxygenase (Liu et al. 2002).

Agricultural and aquatic implications

Application of Fe(III)-chelates as a foliar spray has been extensively investigated as a potential mechanism to overcome iron-deficiency chlorosis in agricultural settings (Fernández et al. 2009). Typically, millimolar concentrations of synthetic chelators or siderophores are applied in the spray (e.g. Fernández et al. 2005; Fernández et al. 2008; Rodríguez-Lucena et al. 2010). Based on the work presented here and in Weger et al. (2009), these concentrators of chelators (synthetic, or siderophores) may potentially inhibit ferric reductase activity, both the well-characterized iron limitation-induced root form (Jeong and Connolly 2009) and also including the leaf cell plasma membrane ferric reductase that is postulated to be involved in leaf mesophyll uptake of Fe(III) delivered by the plant vascular system (Brüggeman et al. 1993; de la Guardia and Alcántara 1996; Jeong and Connolly 2009). That is, the molecules used to keep Fe(III) in soluble form may also decrease the iron acquisition rate by plant species that use a reductive mechanism.

In terms of aquatic systems, it has been suggested during cyanobacterial blooms the cyanobacteria may suppress the growth of eukaryotic algae due to siderophore release into the water (Murphy et al. 1976). The original formulation of this hypothesis suggested that the siderophores acted to sequester Fe(III), making it unavailable for algal uptake. However, the current results suggest that the situation may be slightly more complicated. Fe(III)-siderophore complexes were reduced by iron-limited *Chlorella* cells, and could support the growth of iron-limited cultures, as long as the iron-free siderophore concentration was not too high. However at elevated siderophore levels, cultures of iron-limited *Chlorella* cells did not grow and the ferric reductase activity was greatly decreased.

Conclusions

Chelators (synthetic or naturally-occurring) are clearly important in maintaining Fe(III) in bioavailable form for plants that use a reductive iron acquisition strategy. The obligate substrate for both plant (e.g. Holden et al. 1995) and green algal (e.g. Weger et al. 2007) ferric reductase is Fe(III)-chelate. Thus chelators play

an important role in providing substrate (both as a direct substrate for the enzyme, and indirectly by potentially solubilizing iron from Fe(III)-oxyhydroxides and/or from Fe(III)-organic complexes) for that key enzyme in iron acquisition. Conversely, high concentrations of chelators also potentially inhibit ferric reductase activity.

This has implications for both plants and algae. The rhizosphere is expected to contain elevated levels of siderophores, for example as produced as plant growth-promoting rhizobacteria, and in agricultural situations there may be substantial levels of synthetic chelators present. With respect to aquatic systems containing green algae, the presence of bacterial and cyanobacterial siderophores may both promote iron acquisition (by chelating Fe(III)) and potentially inhibit ferric reductase activity.

Similarly, in both terrestrial and aquatic systems, the presence of humic substances, which possess substantial Fe(III)-chelating capacities, may thus potentially promote or inhibit iron acquisition by photosynthetic organisms that use a reductive iron acquisition mechanism. Similar arguments can be made with respect to the herbicide PMG. Our experiments indicate that both PMG and the cation IPA independently inhibited ferric reductase activity. However, both also supported iron-limited growth by acting as sources of chelated Fe(III).

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References

Alldredge AL, Passow U, Logan BE (1993) The abundance and significance of a class of large, transparent organic particles in the ocean. Deep-Sea Res I 40:1131–1140

Allen MM (1968) Simple conditions for growth of unicellular blue-green algae on plates. J Phycol 4:1–4

Allen MD, Del Campo JA, Kropat J, Merchant SS (2007) FEA1, FEA2, and FRE1, encoding two homologous secreted proteins and a candidate ferrireductase, are expressed coordinately with FOX1 and FTR1 in iron-deficient *Chlamydomonas reinhardtii*. Eukaryot Cell 6: 1841–1852

Allnutt FCT, Bonner WD Jr (1984) Characteristics of iron uptake from hydroxamate siderophores by *Chlorella vulgaris* and the correlation between uptake and reduction. J Plant Nutr 7:427–435

Bar-Ness E, Hadar Y, Chen Y, Shanzer A, Libman J (1992) Iron uptake by plants from microbial siderophores—a study with 7-nitrobenz-2 oxa-1,3-diazole-desferrioxamine as fluorescent ferrioxamine B-analog. Plant Physiol 99:1325–1329

Barry SM, Challis GL (2009) Recent advances in siderophore biosynthesis. Curr Opin Chem Biol 13:205–215

Bellaloui N, Reddy KN, Zablotowicz RM, Abbas HK, Abel CA (2009) Effects of glyphosate application on seed iron and root ferric (III) reductase in soybean cultivars. J Agr Food Chem 57:9569–9574

Bergeron RJ, Huang GF, Smith RE, Bharti N, McManis JS, Butler A (2003) Total synthesis and structure revision of petrobactin. Tetrahedron 59:2007–2014

Blair D, Diehl H (1961) Bathophenanthroline disulphonic acid and bathocuproïne disulphonic acid, water soluble reagents for iron and copper. Talanta 7:163–174

Borges F, Guimarães C, Lima JL, Pinto I, Reis S (2005) Potentiometric studies on the complexation of copper(II) by phenolic acids as discrete ligand models of humic substances. Talanta 66:670–673

Brüggemann W, Maaskantel K, Moog PR (1993) Iron uptake by leaf mesophyll-cells—the role of the plasma membrane-bound ferric-chelate reductase. Planta 190:151–155

Budzikiewicz H, Munzinger M, Taraz K, Meyer JM (1997) Bacterial constituents. 69. Schizokinen, the siderophore of the plant deleterious bacterium Ralstonia (Pseudomonas) solanacearum ATCC 11696. Z Naturforsch C 52:496–503

Calugay RJ, Takeyama H, Mukoyama D, Fukuda Y, Suzuki T, Kanoh K, Matsunaga T (2006) Catechol siderophore excretion by magnetotactic bacterium *Magnetospirillum magneticum* AMB-1. J Biosci Bioeng 101:445–447

Clement PMJ, Hanauske-Abel HM, Wolff EC, Kleinman HK, Park MH (2002) The antifungal drug ciclopirox inhibits deoxyhypusine and proline hydroxylation, endothelial cell growth and angiogenesis in vitro. Int J Cancer 100: 491–498

de la Guardia MD, Alcántara E (1996) Ferric chelate reduction by sunflower (*Helianthus annuus* L.) leaves: Influence of light, oxygen, iron-deficiency and leaf age. J Exp Bot 47:669–675

Dolfing J, Chardon WJ, Japenga J (1999) Association between colloidal iron, aluminum, phosphorus, and humic acids. Soil Sci 164:171–179

Drechsel H, Jung G (1998) Peptide siderophores. J Pept Sci 4:147–181

Duhme AK, Hider RC, Khodr H (1996) Spectrophotometric competition study between molybdate and Fe(III) hydroxide on *N,N′*-bis(2,3-dihydroxybenzoyl)-L-lysine, a naturally occurring siderophore synthesized by Azotobacter vinelandii. Biometals 9:245–248

Eckhardt U, Buckhout TJ (2000) Analysis of the mechanism of iron assimilation in *Chlamydomonas reinhardtii*: a model system for strategy I plants. J Plant Nutr 23:1797–1807

Eker S, Ozturk L, Yazici A, Erenoglu B, Römheld V, Cakmak I (2006) Foliar-applied glyphosate substantially reduced uptake and transport of iron and manganese in sunflower (*Helianthus annuus* L.) plants. J Agric Food Chem 54: 10019–10025

Eldridge ML, Trick CG, Alm AB, DiTullio GR, Rue EL, Bruland KW, Hutchins DA, Wilhelm SW (2004) Phytoplankton community response to a manipulation of bioavailable iron in HNLC waters of the subtropical Pacific Ocean. Aquat Microb Ecol 35:79–91

Fernández V, Ebert G, Winkelmann G (2005) The use of microbial siderophores for foliar iron application studies. Plant Soil 272:245–252

Fernández V, Del Río V, Pumariño L, Igartua E, Abadía J, Abadía A (2008) Foliar fertilization of peach (*Prunus persica* (L.) Batsch) with different iron formulations: Effects on re-greening, iron concentration and mineral composition in treated and untreated leaf surfaces. Sci Hort 117:241–248

Fernández V, Orera I, Abadía J, Abadía A (2009) Foliar iron-fertilisation of fruit trees: present knowledge and future perspectives—a review. J Hortic Sci Biotechnol 84:1–6

Finegold AA, Shatwell KP, Segal AW, Klausner RD, Dancis A (1996) Intramembrane bis-heme motif for transmembrane electron transport conserved in a yeast iron reductase and the human NADPH oxidase. J Biol Chem 271: 31021–31024

Franzen DW, O'Barr JH, Zollinger RK (2003) Interaction of a foliar application of iron HEDTA and three postemergence broadleaf herbicides with soybeans stressed from chlorosis. J Plant Nutr 26:2365–2374

Gerard C, Njomgang R, Pierrard J-C, Rimbault B, Hugel RP (1987) Modelling the interactions of metal cations with soil organic matter. Part 2. Thermodynamic stability of iron(III) and manganese(II) complexes with three dihydroxybenzoic acids. J Chem Res S 12:294–295

Gunnars A, Blomqvist S, Johansson P, Andersson C (2002) Formation of Fe(III) oxyhydroxide colloids in freshwater and brackish seawater, with incorporation of phosphate and calcium. Geochim Cosmochim Acta 66:745–758

Gustafsson JP, Persson I, Kleja DB, Van Schaik JWJ (2007) Binding of iron(III) to organic soils: EXAFS spectroscopy and chemical equilibrium modeling. Environ Sci Technol 41:1232–1237

Hantke K (1990) Dihydroxybenzolyserine—a siderophore for *E. coli*. FEMS Microbiol Lett 67:5–8

Hickford SJH, Kupper FC, Zhang GP, Carrano CJ, Blunt JW, Butler A (2004) Petrobactin sulfonate, a new siderophore produced by the marine bacterium *Marinobacter hydrocarbonoclasticus*. J Nat Prod 67:1197–1199

Holden MJ, Crimmins TJ, Chaney RL (1995) Cu^{2+} reduction by tomato root plasma membrane vesicles. Plant Physiol 108:1093–1098

Hughes EO, Gorham PR, Zehnder A (1958) Toxicity of a unialgal culture of *Microcystis aeruginosa*. Can J Microbiol 4:225–236

Imbert M, Béchet M, Blondeau R (1995) Comparison of the main siderophores produced by some species of *Streptomyces*. Curr Microbiol 31:129–133

Ito T, Neilands JB (1958) Products of "low-iron fermentation" with Bacillus subtilis: isolation, characterization and synthesis of 2,3-dihydroxybenzoylglycine. J Am Chem Soc 80:4645–4647

Iwade S, Kuma K, Isoda Y, Yoshida M, Kudo I, Nishioka J, Suzuki K (2006) Effect of high iron concentrations on iron uptake and growth of a coastal diatom *Chaetoceros sociale*. Aquat Microb Ecol 43:177–191

Jeong J, Connolly EL (2009) Iron uptake mechanisms in plants: Functions of the FRO family of ferric reductases. Plant Sci 176:709–714

Johnson GV, Lopez A, La Valle Foster N (2002) Reduction and transport of Fe from siderophores—reduction of siderophores and chelates and uptake and transport of iron by cucumber seedlings. Plant Soil 241:27–33

Jolley VD, Hansen NC, Shiffler AK (2004) Nutritional and management related interactions with iron-deficiency stress response mechanisms. Soil Sci Plant Nutr 50:973–981

Keshtacher-Liebson E, Hadar Y, Chen Y (1999) Fe nutrition demand and utilization by the green alga *Dunaliella bardawil*. Plant Soil 215:175–182

Liu ZD, Kayyali R, Hider RC, Porter JB, Theobald AE (2002) Design, synthesis, and evaluation of novel 2-substituted 3-hydroxypyridin-4-ones: structure-activity investigation of metalloenzyme inhibition by iron chelators. J Med Chem 45:631–639

López-Goñi I, Moriyón I, Neilands JB (1992) Identification of 2,3-dihydroxybenzoic acid as a Brucella abortus siderophore. Infect Immun 60:4496–4503

Maldonado MT, Price NM (2001) Reduction and transport of organically bound iron by *Thalassiosira oceanica* (Bacillariophyceae). J Phycol 37:298–309

Manthey JA, Tisserat B, Crowley DE (1996) Root responses of sterile-grown onion plants to iron deficiency. J Plant Nutr 19:146–161

Matz CJ, Christensen MR, Bone AD, Gress CD, Widenmaier SB, Weger HG (2004) Only iron-limited cells of the cyanobacterium *Anabaena flos-aquae* inhibit growth of the green alga *Chlamydomonas reinhardtii*. Can J Bot 82:436–442

McKie AT (2008) The role of Dcytb in iron metabolism: an update. Biochem Soc Trans 36:1239–1241

Mori S (1999) Iron acquisition by plants. Curr Opin Plant Biol 2:250–253

Motekaitis RJ, Martell AE (1985) Metal chelate formation by *N*-phosphonomethylglycine and related ligands. J Coord Chem 14:139–149

Murphy TP, Lean DRS, Nalewajko C (1976) Blue-green algae: their excretion of iron-selective chelators enables them to dominate other algae. Science 192:900–902

Newsome AL, Johnson JP, Seipelt RL, Thompson MW (2007) Apolactoferrin inhibits the catalytic domain of matrix metalloproteinase-2 by zinc chelation. Biochem Cell Biol 85:563–572

Nicolaisen K, Moslavac S, Samborski A, Valdebenito M, Hantke K, Maldener I, Muro-Pastor AM, Flores E, Schleiff E (2008) Alr0397 is an outer membrane transporter for the siderophore schizokinen in Anabaena sp strain PCC 7120. J Bact 190:7500–7507

Ozturk L, Yazici A, Eker S, Gokmen O, Römheld V, Cakmak I (2008) Glyphosate inhibition of ferric reductase activity in iron deficient sunflower roots. New Phytol 177:899–906

Porra RJ, Thompson WA, Kriedemann PE (1989) Determination of accurate extinction coefficients and simultaneous-equations for assaying chlorophyll-*a* and chlorophyll-*b* extracted with 4 different solvents—verification of the concentration of chlorophyll standards by atomic-absorption spectroscopy. Biochim Biophys Acta 975:384–394

Powell PE, Cline GR, Reid CPP, Szaniszlo PJ (1980) Occurrence of hydroxamate siderophore iron chelators in soils. Nature 287:833–834

Rodríguez-Lucena P, Hernández-Apaolaza L, Lucena JJ (2010) Comparison of iron chelates and complexes supplied as foliar sprays and in nutrient solution to correct iron chlorosis of soybean. J Plant Nutr Soil Sci 173:120–126

Rroço E, Kosegarten H, Harizaj F, Imani J, Mengel K (2003) The importance of soil microbial activity for the supply of iron to sorghum and rape. Eur J Agron 19:487–493

Schaaf G, Ludewig U, Erenoglu BE, Mori S, Kitahara T, von Wiren N (2004) ZmYS1 functions as a proton-coupled symporter for phytosiderophore- and nicotianamine-chelated metals. J Biol Chem 279:9091–9096

Schagerlöf U, Wilson G, Hebert H, Al-Karadaghi S, Hägerhäll C (2006) Transmembrane topology of FRO2, a ferric chelate reductase from Arabidopsis thaliana. Plant Mol Biol 62:215–221

Schmidt W (2003) Iron solutions: acquisition strategies and signaling pathways in plants. Trends Plant Sci 8:188–193

Schreiber U, Bilger W, Neubauer C (1994) Chlorophyll fluorescence as a nonintrusive indicator for rapid assessment of in vivo photosynthesis. In: Schulze E-D, Caldwell MM (eds) Ecophysiology of photosynthesis. Springer, Berlin, pp 40–70

Schwyn B, Neilands JB (1987) Universal chemical assay for the detection and determination of siderophores. Anal Biochem 160:47–56

Simpson FB, Neilands JB (1976) Siderochromes in cyanophyceae—isolation and characterization of schizokinen from *Anabaena*-Sp. J Phycol 12:44–48

Temirov YV, Esikova TZ, Kashparov IA, Balashova TA, Vinokurov LM, Alakhov YB (2003) A catecholic siderophore produced by the thermoresistant *Bacillus licheniformis* VK21 strain. Russ J Bioorg Chem 29:542–549

Timmermans KR, Davey MS, van der Wagt B, Snoek J, Geider RJ, Veldhuis MJW, Gerringa LJA, de Baar HJ (2001) Co-limitation by iron and light of *Chaetoceros brevis, C.* dichaeta and C. calcitrans (Bacillariophyceae). Mar Ecol Prog Ser 217:287–297

Vansuyt G, Robin A, Briat JF, Curie C, Lemanceau P (2007) Iron acquisition from Fe-pyoverdine by *Arabidopsis thaliana*. Mol Plant Microbe Interact 20:441–447

Wang Y, Brown HN, Crowley DE, Szaniszlo PJ (1993) 0 Evidence for direct utilization of a siderophore, ferrioxamine B, in axenically grown cucumber. Plant Cell Environ 16:579–585

Waters BM, Blevins DG, Eide DJ (2002) Characterization of *FRO1*, a pea ferric-chelate reductase involved in root iron acquisition. Plant Physiol 129:85–94

Weger HG (1999) Ferric and cupric reductase activities in the green alga *Chlamydomonas reinhardtii*: experiments using iron-limited chemostats. Planta 207:377–384

Weger HG, Middlemiss JK, Petterson CD (2002) Ferric chelate reductase activity as affected by the iron-limited growth rate in four species of unicellular green algae (Chlorophyta). J Phycol 38:513–519

Weger HG, Matz CJ, Magnus RS, Walker CN, Fink MB, Treble RG (2006) Differences between two green algae in biological availability of iron bound to strong chelators. Can J Bot 84:400–411

Weger HG, Walker CN, Fink MB (2007) Ferric and cupric reductase activities by iron-limited cells of the green alga *Chlorella kessleri*: quantification via oxygen electrode. Physiol Plant 131:322–331

Weger HG, Lam J, Wirtz NL, Walker CN, Treble RG (2009) High stability ferric chelates result in decreased iron uptake by the green alga *Chlorella kessleri* owing to decreased ferric reductase activity and chelation of ferrous iron. Botany 87:922–931

Wells ML, Trick CG (2004) Controlling iron availability to phytoplankton in iron-replete coastal waters. Mar Chem 86:1–13

Whittaker M, Floyd CD, Brown P, Gearing AJH (1999) Design and therapeutic application of matrix metalloproteinase inhibitors. Chem Rev 99:2735–2776