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Geochemical control of microbial Fe(III) reduction potential in wetlands: comparison of the rhizosphere to non-rhizosphere soil

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Abstract

We compared the reactivity and microbial reduction potential of Fe(III) minerals in the rhizosphere and non-rhizosphere soil to test the hypothesis that rapid Fe(III) reduction rates in wetland soils are explained by rhizosphere processes. The rhizosphere was defined as the area immediately adjacent to a root encrusted with Fe(III)-oxides or *Fe plaque*, and non-rhizosphere soil was >0.5 cm from the root surface. The rhizosphere had a significantly higher percentage of poorly crystalline Fe ($66 \pm 7\%$) than non-rhizosphere soil ($23 \pm 7\%$); conversely, non-rhizosphere soil had a significantly higher proportion of crystalline Fe ($50 \pm 7\%$) than the rhizosphere ($18 \pm 7\%$, $P < 0.05$ in all cases). The percentage of poorly crystalline Fe(III) was significantly correlated with the percentage of FeRB ($r = 0.76$), reflecting the fact that poorly crystalline Fe(III) minerals are labile with respect to microbial reduction. Abiotic reductive dissolution consumed about 75% of the rhizosphere Fe(III)-oxide pool in 4 h compared to 23% of the soil Fe(III)-oxide pool. Similarly, microbial reduction consumed 75–80% of the rhizosphere pool in 10 days compared to 30–40% of the non-rhizosphere soil pool. Differences between the two pools persisted when samples were amended with an electron-shuttling compound (AQDS), an Fe(III)-reducing bacterium (*Geobacter metallireducens*), and organic carbon. Thus, Fe(III)-oxide mineralogy contributed strongly to differences in the Fe(III) reduction potential of the two pools. Higher amounts of poorly crystalline Fe(III) and possibly humic substances, and a higher Fe(III) reduction potential in the rhizosphere compared to the non-rhizosphere soil, suggested the rhizosphere is a site of unusually active microbial Fe cycling. The results were consistent with previous speculation that rapid Fe cycling in wetlands is due to the activity of wetland plant roots.

Keywords: Rhizosphere; Fe(III) reduction; Fe-reducing bacteria; Fe plaque; Mineralogy; Freshwater wetland

1. Introduction

Dissimilatory Fe(III)-oxide reduction is an important carbon-consuming process in freshwater and marine environments [1]. The contribution of Fe(III) reduction to anaerobic carbon metabolism in a given environment depends on many factors, but most importantly on the availability of labile organic matter and reducible Fe(III) [2]. Because both substrates are abundant in mineral wetland soils, many wetland ecosystems are considered "hotspots" of Fe(III) reduction [3]. Several previous studies have linked enhanced substrate availability and rapid rates of Fe cycling to the presence of vegetation, particularly in salt marshes [4,5]. Kostka and Luther [6] reported that vegetated salt marsh sediments (i.e. soils) contained more reactive Fe(III) than marine sediments, and proposed that O_2 leaking from living *Spartina* roots is an important mechanism for regenerating reactive Fe-oxides. Similar observations were made in a freshwater *Juncus* marsh by comparing vegetated and unvegetated patches of soil [7], and in a rice paddy by comparing soils within and below the root zone [8]. The presence of vegetation may also enhance

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Fe cycling through the production of labile carbon and organic ligands [4,9,10]. In each of these studies, the influence of plants was inferred by comparing the properties of bulk soils (i.e. samples that were mixtures of both rhizosphere and non-rhizosphere soil) across sites that differed in root abundance.

An important implication of linking plant activity to rapid Fe cycling is that substrate availability and microbial Fe(III) reduction should be higher immediately adjacent to wetland plant roots (i.e. the rhizosphere) than in soil located several millimeters or more outside the rhizosphere (i.e. non-rhizosphere soil). Weiss et al. [11] found higher densities of Fe(III)-reducing bacteria (FeRB) in the rhizosphere than in non-rhizosphere soil, suggesting that there is substantial small-scale spatial variability in Fe(III) mineralogy, carbon availability and microbial community composition that can be attributed to plant roots. However, to our knowledge, no previous study has directly compared the reactivity of Fe(III) minerals in the rhizosphere to non-rhizosphere soil, including studies of root-associated Fe(III) mineral deposits known as *Fe plaque*.

Fe plaque is an Fe-dominated crust that forms on the surface of wetland plant roots. It is composed primarily of Fe(III)-oxides deposited on the root surface by abiotic and biotic Fe(II) oxidation, the later attributable in part to microaerophilic Fe(II)-oxidizing bacteria (FeOB) [11–13]. Fe plaque is a large reservoir of Fe(III) is some environments and has been studied because of its role as a sink for heavy metals and phosphorus (see [14] for a review). The oxides in Fe plaque are commonly dominated by poorly crystalline forms such as ferrihydrite [15–17], but it can contain more crystalline Fe forms such as lepidocrocite (γ-FeOOH) and/or goethite (β-FeOOH) [18]. It is uncertain how Fe plaque mineralogy compares to nearby non-rhizosphere soil. In the present study, we defined *rhizosphere* as the area surrounding a root that is encrusted with Fe plaque. At an operational level, the rhizosphere Fe pool was largely limited to the Fe plaque itself with any attached soil removed in the washing process. The terms 'rhizosphere' and 'Fe plaque' are used interchangeably in this article. Non-rhizosphere soil was defined as soil located >0.5 cm from the root surface.

The goal of this study was to compare the relative mineralogy and reduction potential of rhizosphere and non-rhizosphere Fe(III) pools. Based on previous observations of the distribution of FeOB and FeRB [11], we hypothesized that Fe(III) minerals in the rhizosphere would be more reactive than those in the bulk soil and support higher rates of enzymatic Fe(III) reduction. We report increased amounts of amorphous Fe(III) and increased Fe(III) reduction potentials in the rhizosphere compared to the non-rhizosphere soil, indicating that the roots are sites of relatively rapid Fe cycling.

2. Materials and methods

2.1. Sampling sites and collection of samples

On several occasions, we sampled six wetlands located in Virginia and Maryland, including three freshwater natural marshes (Linton Hall, Poplar Tree, and Huntley Meadows), a freshwater constructed marsh (Braun), a tidal freshwater marsh (Great Marsh), and a brackish marsh (SERC). The soil organic matter content at Great Marsh was 38% and that at SERC was 69%, making these histosols. The non-tidal soils had soil organic contents ranging from 2% to 16%, making them mineral soils. The total Fe content of the soils generally varied inversely to the soil C content, and all sites were circumneutral in pH. Full descriptions of the sites can be found in [11]. *Typha latifolia* (common cattail) was collected at all sites, except for the Smithsonian Environmental Research Center (SERC) site where *Typha angustifolia* occurred due to brackish conditions. *Typha* is an emergent macrophyte with high rates of radial oxygen loss [19].

In order to prevent changes in the rhizosphere Fe chemistry, intact soil blocks (approximately $15 \times 15 \times 25$ cm deep) were removed containing individuals of *Typha* and the vast majority of its root system. The intact blocks were transported to the laboratory, stored at 4 °C, and dissected under anoxic conditions either in an anaerobic glove box or under a continuous stream of N_2 gas. Dissection was usually done within 2–3 hrs of collection, but always within 24 h. Anoxic conditions within the intact blocks of mineral soils was confirmed by the persistence of reduced soil in the interior of the soil block, in contrast to the outside of the blocks which changed color. Soil samples were collected from the reduced layer (at least 0.5 cm and generally 1–3 cm from the root surface), then homogenized under anaerobic conditions by passing through a 2 mm sieve. Roots varying in color from white to dark orange or red were collected from many positions along the root system in order to sample a full range of Fe plaque concentrations. The root samples were cut into small (<1 cm) pieces and stored anaerobically at 4 °C for up to 48 h before analysis.

2.2. Geochemical characterization

Three approaches were used to geochemically characterize Fe forms in Fe plaque and non-rhizosphere soil: quantification of Fe(II) and Fe(III) [20], sequential extraction based primarily on crystallinity [21], and reducibility of Fe(III)-oxides as determined by the reactive continuum method [22]. Fe(II) and Fe(III) concentrations were quantified with the dual-chelation technique of Wang and Peverly [23] because it does not require drying or other extensive processing prior to analysis.

Approximately 1 g (wet wt) of roots or soil was extracted at least five times with 20 ml of 1 mM ethylenediamine tetraacetate (EDTA)/1 mM bathophenanthroline disulfonate (BPDS) to chelate the Fe(III) and Fe(II), respectively [20]. Fe(II) bound to BPDS resulted in a red color that was quantified on a spectrophotometer. During the extractions, samples were shaken in the dark at 125 rpm for 48 h, centrifuged at 3500 rpm for 20 min, and an aliquot of the supernatant read at 535 nm to determine [Fe(II)]. Absorption was measured again after exposing this aliquot to fluorescent light for 1 week, which resulted in the photoreduction of all Fe(III) to Fe(II). Fe(III) was calculated as the difference between total Fe and Fe(II). Fe(II) and Fe(III) standards were made with ferrous ammonium sulfate and ferric chloride.

To determine the crystallinity and potential bioavailability of the Fe(III) in root plaque and non-rhizosphere soil, a sequential extraction procedure modified from Wieder and Lang [21] and Faulkner [24] was used. This extraction method was chosen largely due to its wide use in freshwater wetlands and its ability to easily distinguish between poorly crystalline and crystalline Fe-oxides. Four of the six sites (Linton Hall, Poplar Tree, Great Marsh, and Huntley Meadows) were sampled for this analysis. Because no significant differences were found between aerobic and anaerobic sequential extractions (unpublished results), all extraction steps were performed aerobically on ~1 g wet weight of root or soil, with samples shaken for varying lengths of time at 125 rpm, then centrifuged at 3400 rpm for 30 min. An initial 1-h extraction was performed with 15 ml of 1.0 M $MgCl_2$ (pH 5) to remove exchangeable Fe, representing the adsorbed fraction [25]. This was followed by a 16-h extraction with 40 ml of 0.1 M sodium pyrophosphate to obtain the organically bound fraction. The term ''organically bound'' is operational because sodium pyrophosphate can also extract small amounts of some Fe(III)-oxides [26]. After low-speed centrifugation, the supernatant was amended with 0.5 ml of 1 $g\,L^{-1}$ superfloc (Cytec Industries, West Paterson, NJ), a flocculent used to clarify the supernatant by removing finely suspended materials, then centrifuged at 10,000 rpm for 30 min. Poorly crystalline Fe (a.k.a. ferrihydrite or amorphous Fe) was extracted in the dark for 4 h with 40 ml of 0.2 M acid ammonium oxalate (pH 3). Next, the root or soil samples were extracted for 16 h with 40 ml of a DCB solution to remove the strongly crystalline forms of Fe [27]. Finally, residual Fe was extracted by shaking for 2 h in 20 ml of concentrated nitric acid. All extractants were analyzed for Fe on an atomic absorption spectrometer (Perkin–Elmer model 5100).

To further characterize the mixed mineralogy of Fe in the root plaque and non-rhizosphere soil at one site, Linton Hall Marsh, we used the reactive continuum method [22], which yields the following general rate equation to describe the dissolution of the reactive Fe pool:

$$J/m_0 = v/a(m/m_0)^{1+1/v}, \quad (1)$$

where J is the overall rate of dissolution ($mol\,s^{-1}$), m_0 is the initial mass of reactive Fe ($mol\,g\,dw^{-1}$), m is the mass of undissolved Fe ($mol\,g\,dw^{-1}$), and v and a are unitless parameters calculated by fitting curves to m/m_0 vs. time. The expression v/a is a rate constant for the mixture, and $1 + 1/v$ is a statistical term describing heterogeneity in Fe(III)-oxide reactivity. The parameter m_0 was determined by an initial dithionate extraction and m was monitored by measuring Fe(II) production due to Fe(III) reduction by 10 mM ascorbic acid. Ascorbic acid reduction experiments were performed in 100-ml serum bottles that were capped and purged with N_2 to ensure anaerobic conditions. The bottles were inoculated with 50 ml of anoxic 10 mM ascorbic acid and ~2 g wet weight of either soil or *T. latifolia* roots collected from Linton Hall marsh in the fall of 2001. Samples were shaken at 125 rpm for 5 h and subsampled for Fe(II) production every 5 min during the first hour, with sampling intensity decreasing thereafter. Subsamples were filtered (0.45 μm) and measured with ferrozine at 562 nm [28]. Total DCB-extractable Fe was determined on a subset of samples to estimate the percentage of total Fe reduced at each time interval.

2.3. Issues regarding biotic vs. abiotic Fe-reduction

Autoclave sterilization is often used to assess the importance of biotic vs. abiotic Fe(III) reduction [7,29,30]. We investigated the possibility that autoclaving might change the initial Fe(II) and Fe(III) pools and found that it increased Fe(II) concentrations 6-fold in both rhizosphere and non-rhizosphere samples ($P < 0.05$, Fig. 1). Autoclaving decreased the Fe(III) content of the poorly crystalline pool, which is most susceptible to reduction. We tested the possibility that the absence of Fe(III) reduction in autoclaved samples (see Section 3) was due to a decrease in the Fe(III) content. A subset of autoclaved samples were exposed to sterile ambient air to regenerate Fe(III), then incubated anaerobically as described above. The amount of reduced Fe(III) in reoxidized, sterilized soils was 7–10 $\mu mol\,g\,dw^{-1}$ in contrast to 120 $\mu mol\,g\,dw^{-1}$ in the unsterilized samples (data not shown), demonstrating that the differences between autoclaved controls and untreated samples were due to the presence or absence of FeRB. Pasteurization at 70 °C for 30 min did not change the total amount and Fe(III) content of 0.5 M-extractable Fe pool (Fig. 1) or the abundance of strongly crystalline Fe-oxides (data not shown), but it also did not entirely eliminate FeRB (see results). Thus, pasteurization was used in experiments where the goal was to reduce the size of the indigenous FeRB populations before spiking them with *G. metallireducens*, and autoclaving was

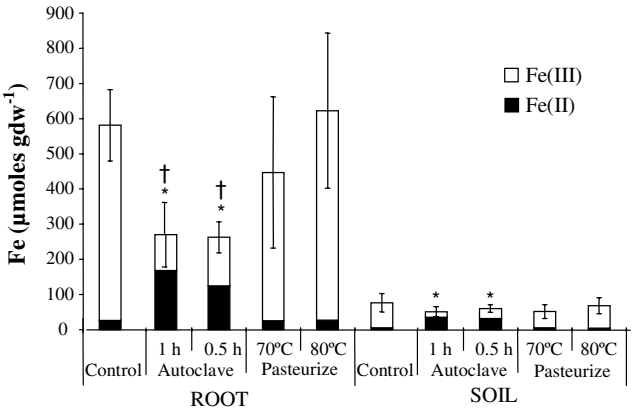

Fig. 1. A comparison of two sterilization techniques on Fe(II) and Fe(III) concentrations in the non-rhizosphere soil and in root plaque (rhizosphere) of *Typha latifolia*. Values represented by the entire columns are the average ± 1 sd ($n = 3$) of the total Fe concentration determined with 0.5 M HCl/0.5 M hydroxylamine. Fe(II) was determined by a 1-hr extraction with 0.5 M HCl. For clarity, no error bars are presented on the individual Fe(II) and Fe(III) amounts. Both treatments of pasteurization (70 and 80 °C) were for 30 min. A "*" indicates a significant difference ($P < 0.05$) in [Fe(II)] between the treatment and the control; a "†" indicates a significant difference in total Fe between the treatment and control.

used in experiments where the goal was to establish that Fe(III) reduction was dominated by enzymatic reduction.

2.4. Fe(III) reduction potential

To assess how differences in Fe(III)-oxide mineralogy influenced microbial Fe(III) reduction potential, we performed anaerobic incubations of *T. latifolia* roots and non-rhizosphere soils collected from Linton Hall marsh. Initial Fe(III) reduction assays were performed without microbial amendments to evaluate the reduction potential of the indigenous Fe(III)-reducing bacteria. Root or soil samples were placed into Hungate tubes containing 10 ml of Fe-free, sodium bicarbonate-buffered media (pH 7) [31] and 20 mM sodium acetate to minimize differences in labile carbon availability. Tubes were inoculated in the anaerobic hood then flushed with 80:20 N_2:CO_2 to eliminate residual H_2. One-half of the inoculated tubes were autoclaved at 121 °C for 1 h to test for abiotic Fe(III) reduction. The tubes were incubated at 30 °C in the dark. At each sampling interval, Fe(II) concentrations were measured in five tubes of live and autoclaved controls by adding 10 ml of 1.0 M HCl (final concentration = 0.5 M HCl). After 1 h, Fe(II) was immediately measured with ferrozine on a spectrophotometer at 562 nm. Total reactive Fe, constituting primarily the poorly crystalline Fe(III) fraction, was determined on a sub-set of the samples by adding 0.5 ml of the HCl extract to 4.5 ml of 0.25 M hydroxylamine/0.25 M HCl solution, then measuring Fe(II) with ferrozine; Fe(III) was calculated as the difference between total Fe(II) and initial Fe(II). The remaining non-reducible Fe(III) was extracted with DCB (see above). Total Fe was calculated as the sum of the 0.5 M HCl-extractable Fe and the DCB-extractable Fe fractions.

2.5. Fe(III) reduction potential experiments with Geobacter metallireducens

Geobacter metallireducens was grown in a bicarbonate-buffered Fe(III)-citrate media containing 20 mM acetate as a carbon/energy source [31]. The inoculum was prepared from cultures growing at log-phase in 100 ml of medium. The cells were centrifuged at 12,000 rpm for 10 min at 4 °C, then washed and resuspended in 80 ml of Fe(III)-reducing media lacking Fe(III)-citrate. All cell manipulations were performed under a stream of 80:20 N_2:CO_2. Hungate tubes containing pasteurized roots or soil were inoculated with 1 ml of the *G. metallireducens* (final concentration between 10^8 and 10^9 cells ml^{-1}), and incubated at 30 °C. Controls were not inoculated with *G. metallireducens*. At intervals ranging from 4 to 48 h over 7 days, 3 to 5 replicate tubes were harvested in an anaerobic glove box. Fe(II), Fe(III), and total Fe were measured as described above. An initial sequential extraction was performed on a subset of samples in order to relate the Fe pool composition to Fe(III) reduction rates, and to determine if pasteurization, like autoclaving, influenced Fe(III)-oxide crystallinity.

2.6. Effect of humic substances on Fe(III) reduction potential

It has been shown that humic acids can shuttle electrons between *G. metallireducens* and insoluble Fe(III)-oxides [32]. To investigate the influence of humic acids on Fe(III) reduction in situ, we spiked one-half of the samples prepared for the Fe(III) reduction experiment (Section 2.5 above) with the humic acid analog 2, 6-anthraquinone disulphonate (AQDS) (final concentration = 100 μM AQDS) after 48 h of incubation. Replicate tubes ($n = 3$ to 5) were harvested at each time point as described above. A subset of the tubes lacking AQDS (described in Section 2.5) was harvested at the end of the incubations to quantify the 'intrinsic non-Fe(III) electron-accepting capacity' of the roots and soil [33]. An aliquot (1 ml) of the supernatant was filtered using a 0.2 μm filter to remove the cells, mixed with 10 mM Fe(III)-citrate (final concentration = 1 mM) for 15 min at room temperature, then assayed with ferrozine. After adjusting for soluble Fe(II) in the initial solution, the excess Fe(II) produced during the 15 min incubation was assumed to be derived from the abiotic reduction of Fe(III)-citrate by reduced substances such as humic

substances, nitrite, H_2S or Mn(II). The total electron-accepting capacity of the samples was calculated as the sum of this non-Fe(III) electron-capacity and the amount of 0.5 M HCl-extractable Fe(III).

The procedure for estimating the intrinsic non-Fe(III) electron-accepting capacity [33] does not distinguish between possible reductants. However, concentrations of H_2S and nitrite are expected to be negligible in anaerobic freshwater sediments such as those of Linton Hall marsh, and soluble Mn is limited by low abundance in most crustal rocks. We speculate that most of the Fe(III)-citrate consumed in the assay was reduced by humic substances, but we did not measure the concentrations of reductants. The assay also assumes that Fe(III)-citrate is the primary oxidant in the filtrate. This is reasonable because sulfate is not reduced abiotically under these conditions and nitrate concentrations would have been negligible because of initially low levels in this anaerobic soil.

2.7. Statistical analyses

All statistics were performed in Microsoft Excel Version 2002 using the add-in statistical package XLSTAT version 5 (Kovach Computing Services, Wales, UK). Fe extraction methods were compared using a one-way ANOVA followed by a Tukey–Kramer test to determine which of the relationships were statistically different. The ANOVAs were applied separately to each substrate (root or soil) within each site. Paired *t*-tests were used to compare the contribution of each Fe fraction in root vs. soil samples; roots and soils were paired by replicate soil blocks. Non-paired *t*-tests were used to determine the effects of sterilization techniques on 0.5 M HCl-extractable Fe and [Fe(II)], and to evaluate the effect of AQDS addition on Fe(III) reduction rates. In cases where a complete data set existed, Pearson correlation analyses were performed on the percentage of Fe(II) in the root and the soil, or between the percentage of FeRB and poorly crystalline Fe. All differences were considered significant at $\alpha = 0.05$. Eq. (1) describing the chemical dissolution rates Fe minerals on roots and soil was fit to empirical data using Sigma Plot Version 7.0.

3. Results

3.1. Fe(II):Fe(III) ratio and sequential Fe fractionation

Using the dual-chelation technique, Fe(II) averaged 21% of the Fe plaque. This value ranged from 17% to 39% in mineral-dominated soils, but increased to >65% at the SERC site which had a soil organic matter content of 69% (Fig. 2, [11]). Fe(II) averaged 26% of the soil Fe pool and was significantly correlated to the proportion of Fe(II) on roots from the same site ($r = 0.91$, $n = 18$, $P < 0.001$).

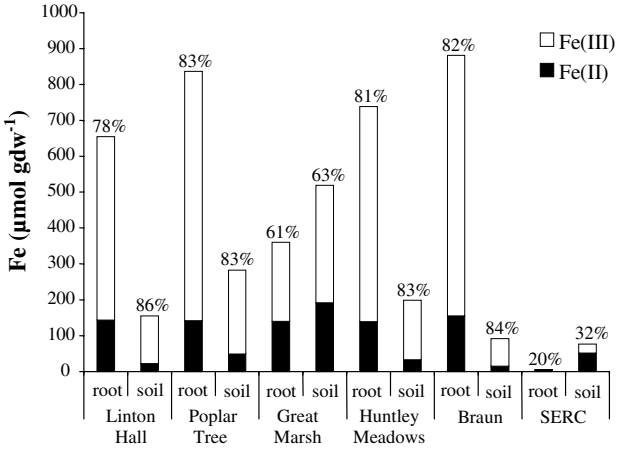

Fig. 2. The relative contributions of Fe(II) and Fe(III) to Fe pools in the root plaque (rhizosphere) of *Typha* spp. and in the non-rhizosphere soil. Each bar is the average of three replicates. The percentage of total Fe as Fe(III) is given at the top of each bar.

Using a sequential extraction method, the root plaque was found to contain significantly more poorly crystalline Fe than the non-rhizosphere soil (606 μmol Fe $g\,dw^{-1}$ root vs. 136 μmol $g\,dw^{-1}$ soil) whereas the soil had higher amounts of crystalline Fe (231 μmol $g\,dw^{-1}$ soil vs. 122 μmol Fe $g\,dw^{-1}$ root). When calculated as a percentage of the total Fe in each environment, the roots had a significantly higher percentage of poorly crystalline Fe ($66 \pm 7\%$) than the non-rhizosphere soil ($23 \pm 7\%$); conversely, the soil had significantly more crystalline Fe ($50 \pm 7\%$) than the roots ($18 \pm 7\%$) (Fig. 3). There was a significant correlation between percent poorly crystalline Fe and percent FeRB reported by Weiss et al. [11] ($r = 0.76$, $n = 5$ sites, $P < 0.05$). The exchangeable and residual fractions were higher in the soil than the root plaque, though only the differences in the residual fraction were statistically different ($P = 0.01$, Fig. 3). The proportion of sodium pyrophosphate-extractable (organically bound) Fe was statistically similar, ~11.0%, in the root plaque and non-rhizosphere soil.

3.2. Relative Fe reactivity

The initial rate of abiotic Fe(III) reductive dissolution was more rapid in the root plaque Fe pool than soil Fe pool at Linton Hall marsh (Fig. 4). The dissolution rate decreased dramatically after about half of the plaque Fe had been dissolved. About 75% of the Fe pool had been removed from the roots after 4 h compared to 23% for the soil Fe pool. These values are similar to the amount of Fe extracted in the same time period with oxalate (i.e. poorly crystalline Fe, Fig. 3).

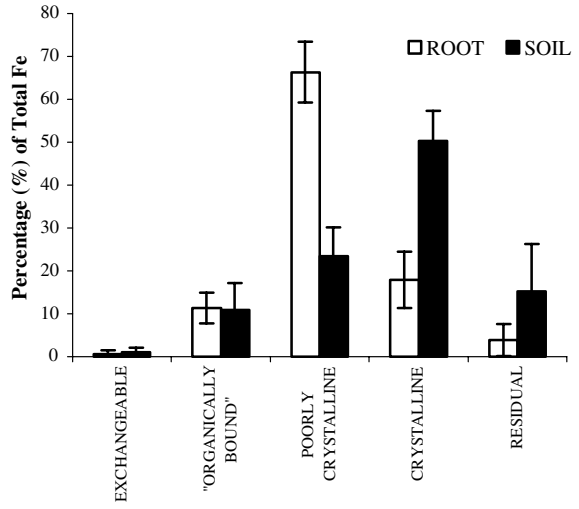

Fig. 3. A comparison of Fe fractions in root plaque (rhizosphere) vs. non-rhizosphere soil. All columns represent the average ($n = 5$) of four different wetland sites, one of which was sampled on two occasions. All comparisons of the root plaque and soil within an Fe fraction were significantly different ($P < 0.05$), with the exception of the organically bound fraction. Error bars represent ±1 sd.

Fitting these data to Eq. (1) produced the following relationships ($r^2 \geqslant 0.98$):

$$J/m_0(\text{root}) = 1.1 \times 10^{-2}(m/m_0)^{1.78}, \quad (2)$$

$$J/m_0(\text{soil}) = 4.0 \times 10^{-3}(m/m_0)^{5.14}. \quad (3)$$

The exponent $1 + 1/v$ for the root plaque (1.78), was greater than the value of 1.1 reported for the reduction of synthetic ferrihydrite [22], indicating that the root Fe plaque pool was heterogeneous with respect to abiotic reduction. The relatively large exponent for the soil Fe curve indicates a much slower abiotic dissolution rate than the Fe plaque and a more diverse assemblage of Fe(III)-oxides (Fig. 4). Soil Fe dissolution rates varied by 5 orders of magnitude between the initial reduction of the Fe(III)-oxide pool and the most recalcitrant Fe pool, compared to a range of <2 orders of magnitude for the root plaque Fe pool. As discussed by Roden [34], differences between the two pools in the rate of enzymatic Fe(III) reduction are probably less dramatic than this abiotic assay suggested. Thus, we also measured enzymatic Fe(III) reduction rates.

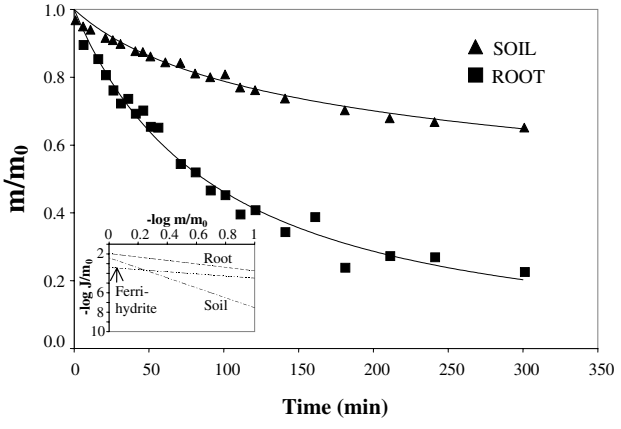

Fig. 4. Reduction of Fe(III)-oxides on the roots (rhizosphere) of *Typha latifolia* and in non-rhizosphere soil by 10 mM ascorbic acid (pH 3.0). Each data point is an average of five samples. The ratio m/m_0 is the fraction of undissolved Fe oxide. The inset is a comparison of Fe(III)-oxide reduction rates, normalized to the initial mass (J/m_0), vs. the fraction (m/m_0) remaining in the solid phase. The relationship for ferrihydrite is from Postma [22].

3.3. Fe(III) reduction rates without microbial amendments

As expected, Fe plaque in the samples used for the Fe(III) reduction experiments was dominated by poorly crystalline Fe (~80%) while the soil was dominated by the crystalline fraction (~50%). We observed overall Fe(III) reduction rates of 18 μmol Fe(III) $g\,dw^{-1}$ d^{-1} and 8 μmol Fe(III) $g\,dw^{-1}$ d^{-1} for the plaque and soil Fe(III) pool, respectively. The amount of Fe(III) reduced from unsterilized, unamended roots after 350 h was 75 ± 14% compared to 29 ± 2% in the soils (Fig. 5). After a lag period, both root and soil Fe(III) reduction rates increased and peaked between 24 and 78 h, then decreased and became asymptotic after 200 h. No net reduction was observed in the autoclaved samples, indicating that the process was microbially mediated. There was no evidence of magnetite formation in any of the tubes monitored for Fe(III) reduction, including those inoculated with *G. metallireducens* (see below).

It is important to note that the use of 0.5 M HCl provided a better estimate of the microbial-reducible Fe(III) in the rhizosphere than it did in the non-rhizosphere soil. Over 70% of the Fe in the non-rhizosphere soil was 0.5 M HCl-extractable but only

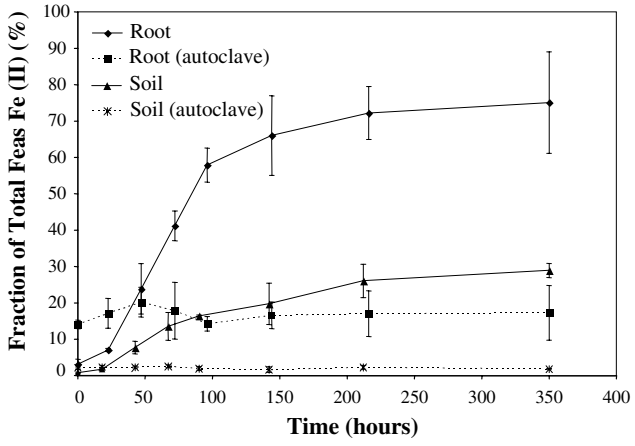

Fig. 5. Fe(III) reduction during anaerobic incubation of roots (rhizosphere) from *Typha latifolia* and bulk soil. The control was sterilized by autoclaving at 121 °C for 1 h. Data points represent the average of three replicates ±1 standard error.

~60% of this amount (42% of total non-rhizosphere soil Fe) was microbially reduced. A similar observation was reported from a slightly acidic freshwater marsh [2]. In contrast, >90% of the HCl-extractable Fe in the rhizosphere samples was microbially reduced. This difference probably reflects the relatively high quantities of *moderately* crystalline Fe(III) in soils, which are HCl-extractable, but not readily reducible by FeRB [2].

3.4. Effect of G. metallireducens on Fe(III) reduction potential

Roots and soil from Linton Hall marsh were amended with *G. metallireducens* to determine if the observed differences in Fe(III) reduction rates were due to the availability of reducible Fe(III) and not in situ differences in FeRB microbial population densities [11]. In both the rhizosphere and non-rhizosphere samples, an initial period of Fe(III) reduction occurred in the first 6 to 12 h (Fig. 6). A lag period followed, potentially due to the cells adjusting from growth on Fe(III) in a soluble form (ferric citrate) to a solid form [35]. Fe(III) reduction rates increased dramatically after 48 h and leveled off at 137 h. The total Fe reduced was 77% and 30% of the initial Fe pool in the root plaque and soil, respectively. Pasteurized samples had a much longer lag phase than untreated samples, but eventually had Fe(III) reduction rates comparable to unpasteurized samples (Fig. 6).

3.5. Role of humic substances in Fe(III) reduction

The addition of 100 μM AQDS after 48 h of incubation significantly stimulated Fe(III) reduction in the root samples by >20% ($P = 0.04$, Fig. 6). The addition of AQDS to the soil samples increased Fe(III) reduction by 8%, but this difference was significant only at 92 h. It is possible that high levels of phosphate in the medium (4 mM) reduced the stimulatory effect of AQDS on crystalline Fe(III) reduction (especially in the soil), as has been observed for hematite reduction by *S. putrefaciens* strain CN32 [36]. However, it is unlikely that adding AQDS in the absence of P would have eliminated the large difference in Fe(III) reduction rates between the rhizosphere and the soil.

In addition to the experiments where a humic acid analog was added, we compared roots and soils for their potential to produce non-Fe(III) electron acceptors such as humic substances (i.e. *intrinsic non-Fe(III) electron-accepting capacity*). At the end of the Fe(III) reduction experiments, we filtered the supernatant from AQDS-free root and soil samples and incubated it with 10 mM Fe(III)-citrate. The total electron-accepting capacity of the samples was assumed to be the sum of the Fe(III)-citrate reduced by humic substances (and perhaps reduced inorganic compounds) and the 0.5 M HCl-extractable Fe(III) (Table 1). The results indicate that 11% and 2% more Fe(III) could have been reduced in the roots and soil, respectively, if additional poorly

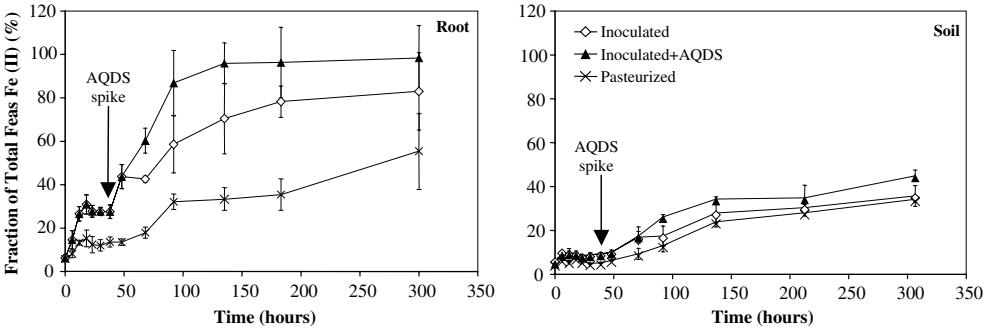

Fig. 6. Fe(II) accumulation in incubations of *Typha latifolia* roots (rhizosphere) and non-rhizosphere soil inoculated with *Geobacter metallireducens*. Fe(II) is expressed as a percentage of the total Fe present in each sample. At 48 h, 100 μM AQDS was added to half of the samples. Data points are the average of 3–5 replicates and error bars are 1 standard error.

Table 1
The electron accepting capacity of substances other than Fe(III) (e.g. humic substances) measured during laboratory incubations of *Typha latifolia* roots and non-rhizosphere soil

	Non-Fe(III) electron accepting capacity (μmol g dw^{-1})[a]	Microbially reducible Fe(III) (μmol g dw^{-1})[b]	Total electron-accepting capacity due to Fe(III) (%)[c]
Rhizosphere	36.75 (16.18)	282.5 (18.9)	88.5
Non-rhizosphere soil	6.14 (1.83)	259.3 (32.1)	97.7

Values in brackets represent the standard deviation of six samples.
[a] As determined by incubation of filtrate with 10 mM ferric citrate.
[b] As determined with 0.5 M HCl.
[c] Calculated as {microbially reducible-Fe(III)/[(non-Fe(III) electron accepting capacity) + (microbially reducible Fe(III))]} × 100 (see [33]).

crystalline Fe(III) had been available. These values are low in comparison to values obtained from other sources of humic substances [33] and may reflect the relatively high amounts of poorly crystalline Fe(III) in wetland soils vs. unvegetated sediments. The data suggest that humic substances (in the form of AQDS) have a larger stimulatory effect on Fe(III) reduction rates in the rhizosphere than in the surrounding soil, but roots may also be a slightly larger source of humic substances than the soil on a mass basis.

4. Discussion

Previous studies have shown that wetland soils are 'hotspots' of Fe cycling due to the presence of plants, and proposed that this is caused by enhanced availability of reactive Fe(III) minerals and organic carbon in the rhizosphere [2,8,37]. We tested this hypothesis by comparing Fe(III) minerals and potential Fe(III) reduction rates in the rhizosphere vs. non-rhizosphere soils. The Fe-plaque on the roots of wetland plants collected at several different sites had a much higher percentage of reactive Fe(III) minerals than the surrounding non-rhizosphere soil (66% vs. 23%), even though the two were separated by distances of 0.5 to 3 cm. Furthermore, over 75% of the total Fe in the root plaque was reduced in anaerobic incubations, compared to 29% in the soil, demonstrating that differences in Fe(III)-oxide mineralogy translated into different rates of microbial metabolism. Thus, the properties of rhizosphere Fe(III) minerals were consistent with the notion that plant roots promote rapid Fe(III) reduction in wetland soils.

A significant correlation between the relative abundances of amorphous Fe and FeRB suggests that FeRB were Fe-limited, a relationship also identified by Lowe et al. [38]. Factors other than the surface area and thermodynamic properties of Fe minerals [34] that could have favored relatively rapid Fe(III) reduction rates in the root plaque include higher initial densities of native FeRB [11,34], a larger pool of labile organic carbon, or a larger pool of electron-shuttling humic substances. However, amendments of *G. metallireducens*, acetate, and AQDS did not eliminate the dramatic difference in Fe(III) reduction rates between the root plaque and the non-rhizosphere soil. In fact, because acetate was added to all Fe(III) reduction trials, the reduction potential of the soil Fe pool may have been overestimated because soils are C-poor compared to the rhizosphere [39]. The differences between rhizosphere and non-rhizosphere Fe(III) reduction potential are likely to be more significant in situ than this study suggests.

Although the percentage of amorphous Fe(III) in non-rhizosphere soils was substantially less than in the rhizosphere, it was relatively high compared to unvegetated sediments. This was the case even at continuously saturated sites such as Great Marsh, a tidal freshwater wetland. Converting the fraction of amorphous Fe(III) to a mass basis based on the data reported here (Figs. 2, 3) and previously published bulk density data [11], the amorphous Fe(III) content of non-rhizosphere soils averaged 35 $\mu mol\,cm^{-3}$. This value is higher than 36 of 38 sediment samples collected from 6 anaerobic marine sites [1]. Previous studies have noted higher reactive Fe(III) in bulk soils (i.e. rhizosphere + non-rhizosphere soil) than sediments that lacked roots altogether [6–8]. We propose that wetland vegetation not only promoted the accumulation of poorly crystalline Fe in the active rhizosphere, but that much of the poorly crystalline pool in the non-rhizosphere soil was also derived from senesced roots. Furthermore, organic carbon from decomposing roots and root exudates may help stabilize this rhizosphere-derived Fe(III) after it transitions to the non-rhizosphere soil Fe pool. Thus, cycles of new root growth, poorly crystalline Fe(III) deposition, root death, and Fe plaque dissolution may cumulatively influence the Fe mineralogy of the entire root zone over a period of years.

4.1. Geochemical characterization of Fe plaque

Results from both the reactive continuum method and a sequential extraction indicated that the plaque was dominated by poorly crystalline or amorphous Fe(III) (>65%). Previous studies employing chemical extractants [15,40], electron microscopy [16,41], and X-ray absorption near-edge structure (XANES) spectroscopy [17] also reported that root plaque was dominated by amorphous Fe, primarily in the form of ferrihydrite. These estimates of amorphous Fe ranged from 63% to 84% of the total Fe pool. A few studies using X-ray diffraction analysis have reported the presence of crystalline Fe forms such as lepidocrocite (γ-FeOOH) and/or goethite (β-FeOOH), but not ferrihydrite [18,42,43]. However, X-ray diffraction does not easily detect highly amorphous Fe [44,45]. In contrast to crystalline Fe-oxide reduction, which rarely exceeds 20% of the total Fe(III) present [36,46–48], amorphous Fe(III)-oxides are subject to near or complete dissolution [1,49]. This difference explains the high Fe(III) reduction rates of the root plaque Fe, comprised predominately of poorly crystalline Fe, compared to the much lower rates of the more strongly crystalline soil Fe pool. Based on the small percentages of crystalline and residual Fe in the root plaque, ~80% of the Fe in the plaque is actively involved in Fe cycling in the rhizosphere.

Iron coatings on the roots of aquatic plants have been assumed to consist primarily of Fe(III)-oxides [14,50–52]. However, Fe(II) can account for 17-70% of the total Fe plaque depending on the age and morphology of the

root, and the wetland environment [15,20]. In the current study, Fe(II) averaged 21% of the plaque Fe pool. Because $MgCl_2$-exchangeable Fe was <1% of the total Fe pool, Fe(II) must have existed primarily as a precipitate. High [Fe(II)] on older portions of the root system [20], where oxidation capacity is negligible [53], suggest that this Fe(II) was derived from reduction of the plaque itself. The Fe(II) content of Fe plaque is an important topic because adsorption and/or surface precipitation of Fe(II) on Fe(III)-oxide and FeRB surfaces can significantly retard enzymatic Fe(III) reduction [54,55].

4.2. Influence of organic carbon on Fe(III) reduction potential

Fe(III) reduction potential is regulated in part by the availability of labile organic carbon [1], which is more abundant in the rhizosphere than the soil [39,56]. Roots are likely sources of acetate and other organic acids [9,57,58]. They may also influence Fe(III) reduction by producing organic chelators, that maintain Fe(III) in a soluble form [4] and allow it to diffuse to anaerobic microzones [59], or humic substances, that serve as an alternative electron acceptor [30]. The latter possibility was investigated at one of the sites, Linton Hall marsh, by estimating the Fe(III) reduction capacity of humic substances derived from roots vs. non-rhizosphere soil organic matter. This assay suggested that 11% of the potential Fe(III) reduction capacity of root samples was due to agents other than Fe(III), which we assume were humic substances in this freshwater system; the figure for non-rhizosphere soils was 2%. Thus, the roots appear to be a relatively abundant source of compounds that can be used either as electron donors or electron shuttles in the enzymatic reduction of Fe(III). In contrast to these experiments, which were amended with organic carbon and the humic acid analog AQDS, it is likely that even larger differences in Fe(III) reduction potential exist between the rhizosphere and the non-rhizosphere soil in situ where carbon often limits microbial activity in the soil [2,60].

4.3. Implications for in situ Fe(III) reduction and cycling

Although the rhizosphere is typically characterized as a zone of Fe(III) deposition [14], we show that it is also a zone of potentially rapid Fe(III) reduction under anaerobic conditions. The peak rate of Fe(III) reduction was ~60 μmol Fe(III) $g\,dw^{-1}\,d^{-1}$ on roots compared to a rate of 25 μmol Fe(III) $g\,dw^{-1}\,d^{-1}$ in the soil. Root plaque Fe(III) reduction rates between 134 and 531 μmol Fe(III) $g\,dw^{-1}\,d^{-1}$ were reported previously for three freshwater species, including *T. latifolia* [30]. However, these rates were measured after addition of exogenous Fe(III)-oxides; Fe(III) reduction rates in unamended samples of *T. latifolia* were 5–15 times lower (calculated based on Fig. 2B in [30]) and are similar to those observed in the current study. Of course, all of these Fe(III)-reduction rates were determined under favorable laboratory conditions whereas rates under in situ conditions will be influenced by fluctuations in temperature, carbon inputs during the growing season, and varying amounts of radial O_2 loss.

The rhizosphere appears to be an ideal oxic-anoxic interface for the coupling of bacterial Fe(III) reduction and Fe(II) oxidation. Freshly precipitated rhizosphere Fe(III)-oxides stimulates the activity of FeRB (present study). A significant correlation between percent poorly crystalline Fe and percent FeRB ($r = 0.76$, $n = 5$ sites, $P < 0.05$, FeRB data from [11]) can be explained by the combination of poorly crystalline Fe(III) and labile organic C [2], which are concentrated in the rhizosphere. Fe(III) reduction rates have been reported to increase with FeRB densities in culture studies [34]. The result of these processes over time appear to enhance rates of Fe(III) reduction throughout the root zone (i.e. rhizosphere and non-rhizosphere soil).

A key reason for the large amounts of poorly crystalline Fe in the rhizosphere is that O_2 release by plant roots creates microaerophilic zones suitable to the activity of Fe(II)-oxidizing bacteria (FeOB). We previously showed that lithotrophic FeOB are abundant in the rhizosphere and they are likely to account for a large fraction of overall Fe(III) oxidation activity in such environments [13]. Anaerobic Fe(III) oxidation coupled to nitrate reduction [61–64] may also support Fe cycling in the rhizosphere. Lithotrophic FeOB factored significantly in a proposed model for continuous microscale Fe cycling by forming amorphous Fe-oxides and forcing the development of steep O_2 gradients [65].

Aerobic Fe(II) oxidation and anaerobic Fe(III) reduction are expected to be coupled by diurnal and seasonal variations in plant development and photosynthetic activity that cause the rate of O_2 release by roots to vary hourly, daily and seasonally [66]. Thus, regions of the root system may support net deposition of poorly crystalline Fe(III)-oxides when O_2 is being released, then switch to net Fe(III)-reduction when O_2 is absent. This model for rhizosphere Fe cycling is consistent with the observation that substantial numbers of FeRB and FeOB occur on the same 1-cm lengths of plant roots [11], indicating a tolerance of varying concentrations of O_2. There are also non-biological phenomena such as variations in water table depth that influence Fe cycling. The actual contribution of these physiological, ecological and physical processes to Fe cycling remains to be determined. However, the presence of Fe plaque on wetland plants under many different environmental conditions indicates that Fe(III)-oxide reduction lags behind the

Fe(II)-oxidation in situ [67]. Since we have shown that the potential for Fe(III) reduction of the plaque is high, more studies are needed to determine the factors governing the balance between Fe(II) oxidation and Fe(III) reduction, both spatially and on a seasonal basis.

5. Summary and conclusions

Previous speculation that rapid Fe cycling in wetlands is due to the activity of wetland plant roots was supported by our comparisons of Fe(III) mineralogy and Fe(III) reduction potential of root Fe plaque (defined here as the rhizosphere) vs. non-rhizosphere soil. It was previously reported that FeRB represent up to 32% of all bacterial cells in the rhizosphere vs. <1% in non-rhizosphere soil. Further differences between the two pools reported here include a relatively high proportion of poorly crystalline Fe(III)-oxides and higher rates of Fe(III) reduction in the rhizosphere than the non-rhizosphere soil. In laboratory assays, in which initial differences in labile organic carbon availability and FeRB populations were controlled, Fe(III)-oxide reduction rates were about 2-times higher in the rhizosphere than in soil >0.5 cm from a root surface. Thus, Fe(III)-oxide mineralogy contributed strongly to differences between the two pools in Fe(III) reduction potential. It is likely that in situ differences between the rhizosphere and non-rhizosphere soil with respect to Fe(III) reduction are further widened by higher concentrations of labile organic carbon and humic substances in the rhizosphere compared to the soil. The high reduction potential of the rhizosphere Fe(III) pool has important biogeochemical implications such as suppressing methane production [7,60,68], regulating P availability to plants [69], and enhancing the mobility of trace metals [17]. The high Fe(III) reduction potential of the Fe plaque is one factor promoting a microbially mediated Fe cycle around the roots of wetland plants.

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