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Microbial iron metabolism in natural environments

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Abstract

The aim of this project was to assess the diversity of iron metabolizing bacteria in several ecological niches by culture-dependent and culture-independent methods. In particular, we wanted to determine whether non-phototrophic, anaerobic nitrate-dependent Fe(II)-oxidizers coexist with aerobic Fe(II)-oxidizers and facultatively anaerobic Fe(III)-reducers in natural habitats. To this end, we sampled iron-rich sites in freshwater, estuarine and marine environments and enriched for non-phototrophic anaerobic and aerobic Fe(II)-oxidizers. The identification of the two environmentally most important facultatively anaerobic Fe(III)-reducers and one aerobic heterotrophic Fe(II)-oxidizer in the sediment samples was carried out by means of fluorescent *in situ* hybridization (FISH). We were able to culture aerobic Fe(II)-oxidizers in gradient tubes from each sample site. Nitrate-reducing Fe(II)-oxidizers were only found in sediments from a Cranberry Bog. *Geobacter, Shewanella* and *Leptothrix* coexist in Salt Pond at the oxic/anoxic interface.

Introduction

The significance of bacteria in the biogeochemical cycling of iron has been broadly recognized over the past two decades. In Fig. 1 the role of dissimilatory anaerobic Fe(III)-reducers as well as acidophilic and neutrophilic aerobic Fe(II)-oxidizers, anaerobic phototrophic Fe(II)-oxidizers and facultatively anaerobic nitrate-reducing Fe(II)-oxidizers in the microbial cycling of iron is shown.

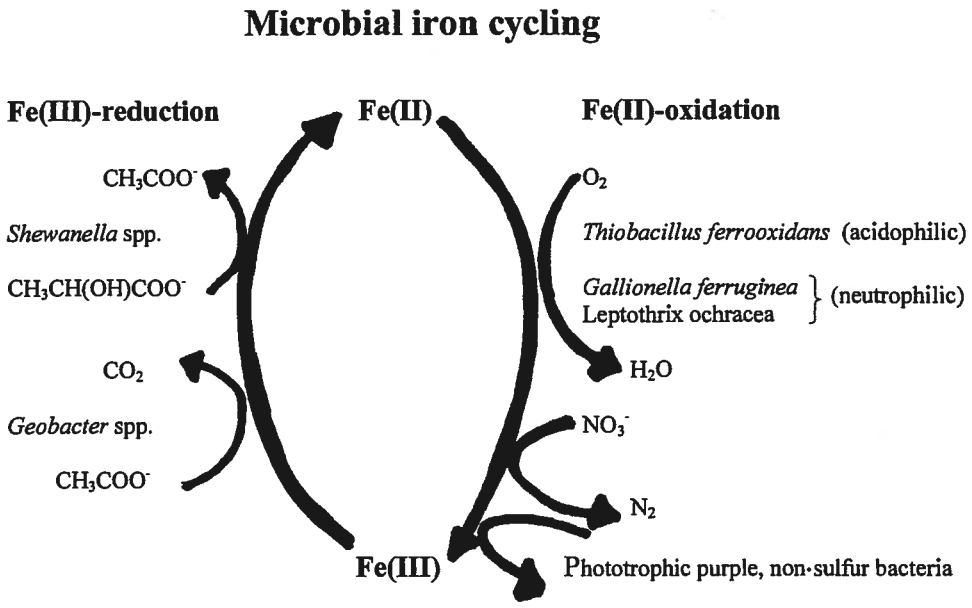

Fig. 1. The microbial iron cycle.

In the following sections, the iron reducing and oxidizing microorganisms responsible for the biological cycling of iron and their habitats are introduced.

Fe(III) reduction

Nonenzymatic (chemical) processes were previously considered to account for much of the Fe(III) reduction in subsurface environments. Dissimilatory Fe(III)-reducing bacteria that gain energy by coupling the oxidation of hydrogen or organic compounds to the reduction of ferric iron oxides have been known for many years but their biogeochemical importance was recognized only a decade ago (reviewed by Lovley, 1997). Manganese and iron reduction are, with a few exceptions, carried out by the same organisms (iron is usually 5-10 times more abundant than manganese). Fe(III) reduction accounts for a major fraction of the carbon oxidation in many different environments and in the presence of large amounts of reactive Fe(III), microbial Fe reduction may even inhibit sulfate reduction and methanogenesis (King, 1990).

A wide diversity of Fe(III)-reducing microorganisms which fall in a number of different phylogenetic groups are known today. Both organisms that grow by respiration and by fermentation have been isolated and identified. H_2, short- and long-fatty acids, amino acids,

sugars and aromatic compounds may serve as electron donors for Fe(III) reduction. The enzyme responsible for dissimilatory Fe(III) reduction is a membrane associated ferric reductase. Fe(III)-reducers may utilize alternative electron acceptors such as O_2, nitrate, S^0, sulfate, humic substances, contaminant metals and metalloids and chlorinated solvents.

The first organism shown to couple respiratory growth to dissimilatory iron reduction was *Pseudomonas ferrireductans* now known as *Shewanella oneidensis* but previously classified as *Alteromonas putrefaciens* and *Shewanella putrefaciens* (Balashova et al., 1980). Various dissimilatory Fe(III)-reducers, including the renowned *Geobacter sp.* and *Shewanella sp.*, have been isolated from marine and freshwater sediments. The obligate anaerobic *Geobacter* sp. belong to the δ-proteobacteria whereas the facultatively anaerobic *Shewanella* sp. belong to the γ-proteobacteria. Since *Shewanella* and *Geobacter* are the dominant species *in situ* we will deal with these Fe(III)-reducers exclusively in this project.

The redox potentials of oxidized and reduced iron couples, and thus the energy available from ferric iron reduction and ferrous iron oxidation, depend strongly on the specific iron phases involved. In soil and aquatic environments, iron oxides mainly occur in association with other sediment particles as aggregates or coatings. Amorphous and poorly crystalline iron oxides are the main products of abiotic and biotic iron oxidation in sediments and they constitute the most important phases for microbial iron reduction. Amorphous/ poorly crystalline iron oxides usually make up 20% or less of the iron content in a sediment.

The maximum energy that *Shewanella oneidensis* can gain from reducing amorphous ferrihydrite and oxidizing lactate at pH 7.0 is

$$4(am)Fe(OH)_3 + CH_3CH(OH)COO^- + 7H^+ = 4Fe^{2+} + CH_3COO^- + HCO_3^- + 10H_2O$$

$\Delta_r G = -46.4$ kJ/mol $\Rightarrow$ -11.6 kJ/mol Fe

The maximum energy that *Geobacter metallireducens* can gain from reducing amorphous ferrihydrite to magnetite and oxidizing acetate at pH 7.0 is

$$24(am)Fe(OH)_3 + CH_3COO- = 8Fe_3O_4 + 2HCO_3^- + H^+ + 36H_2O$$

$\Delta_r G = -683.5$ kJ/mol $\Rightarrow$ -28.5 kJ/mol Fe

Fe(II) oxidation

Since ancient times iron has been mined from bog deposits where anaerobic groundwater containing ferrous iron enters aerobic environments and is oxidized and precipitated both spontaneously and by the action of bacteria such as *Leptothrix* and *Gallionella*. The iron in subsurface deposits is mobilized by reduction of ferric deposits catalyzed by bacteria that oxidize dissolved organic carbon. The carbon is derived from decomposition of plant material in natural ecosystems. More recently, contamination by landfill leachates and other contaminant plumes from chemical and fuel spills have produced conditions of low redox potential and low pH that are appropriate for mobilization of the iron. When water containing dissolved organic material moves through the subsurface, bacteria can oxidize the organic material and reduce

inorganic electron acceptors such as ferric iron. The process lowers the redox potential and often lowers the pH so that ferrous iron minerals become soluble and are transported through the matrix. When the water emerges to the atmosphere in seeps or springs the ferrous iron is oxidized rapidly and precipitates as ferric hydroxides. Such deposits accumulate over time and were a substantial source of iron in preindustrial times. Some of the iron oxidation is spontaneous, but much of it is catalyzed by bacteria that gain energy from the process. Aerobic oxidation of ferrous iron minerals by lithotrophic acidophilic and neutrophilic bacteria has been known for many years. Such iron-oxidizing bacteria have been isolated from acidic ecosystems (*Thiobacillus ferrooxidans*), organic-rich systems (*Sphaerotilus, Leptothrix*) and oligotrophic systems (*Gallionella,* SPB OUT-1-- closest relative *Stenotrophomonas*, and *Leptothrix)*. The degree of overlap and the interactions among the above bacteria are not clear.

At neutral pH, ferrous iron is unstable in the presence of oxygen and is rapidly oxidized to the insoluble ferric iron. Hence, the only pH neutral environments where soluble ferrous iron is present are interfaces between oxic and anoxic conditions. The neutrophilic Fe(II)-oxidizers, *Leptothrix* sp., *Gallionella* sp. and *Sphaerotilus* sp., live at such interfaces and are usually associated with the characteristic (orange flocculent) ferric deposits that they form. *Leptothrix* produces long, polysaccharide sheaths with associated clumps of flocculent iron. Often the cells are not obvious in unstained preparations.

True autotrophy has been found only in *Gallionella ferruginea,* a vibrioid beta-proteobacteria that forms a twisted stalk (http:\link.springer.de/link/service/books/10125/papers/2003009/10030425.htm). The aerobic oxidation of ferrous iron to ferric iron is an energy yielding reaction for only a few bacteria. The maximum energy that *Gallionella ferruginea* can gain from oxidizing ferrous iron to amorphous ferrihydrite at pH 7.0 is

$$4Fe^{2+} + 10H_2O + O_2 = 4(am)Fe(OH)_3 + 8H^+$$

$\Delta_r G = -431.9$ kJ/mol $\Rightarrow$ -108.0 kJ/mol Fe

It is not clear whether *Leptothrix* gains energy from the oxidation of iron, but it is clear that it contains enzymes that catalyze the process (http:\link.springer.de/link/service/books/10125/papers/2003009/10030425.htm). The type species, *Leptothrix orchracea*, has not been studied in pure culture so the physiological implications of iron oxidation have not been determined rigorously. It is not clear how much of the iron oxidation that takes place in the seeps is catalyzed by bacteria and how much is abiotic.

Over the past several years there has been a growing recognition that other, less readily identifiable, types of bacteria are involved in iron oxidation in circumneutral ecosystems. They seem to produce large amounts of amorphous iron oxides that were previously attributed to abiotic deposition. For example, obligate chemolithotrophs that use ferrous iron as the terminal electron acceptor have been isolated from hydrothermal vents (Emerson et al., 2002). Neutrophilic Fe-oxidizing bacteria are abundant at the Loihi seamount hydrothermal vents and play a major role in Fe oxide deposition. Similarly, unidentified, obligate lithotrophs have also been isolated from the roots of wetlands plants where they are closely associated with amorphous deposits of iron (Emerson et al., 1999).

In the past it has been thought that crystalline iron deposits associated with sheaths were produced biologically and that amorphous deposits not associated with cells were produced abiotically. Recently the deposition of amorphous ferric iron in gradient tubes has been attributed to the action of iron-oxidizing bacteria (Sobolev et al., 2001). The authors attribute 90% of the oxidation to biological processes and indicate that the organisms seem to produce a mobile form of Fe(III) that diffuses away from the cells before it precipitates so that encrustation of the cells is avoided. They suggest that such soluble ferric iron complexes might be substrates for closely associated iron reducing bacteria. Such an arrangement might allow close coupling between Fe(II) oxidation and Fe(III) reduction within millimeters of the oxic-anoxic interface.

Anaerobic ferrous iron oxidation by phototrophic purple, non-sulfur bacteria utilizing ferrous iron as an electron donor in the light was recognized only a decade ago (Widdel et al., 1993). Subsequently, it was demonstrated that the biological oxidation of ferrous iron in the absence of oxygen is possible by light-independent chemotrophic microbial activity using nitrate as the electron acceptor (Straub et al., 1996). In addition, studies conducted in gradient cultures have revealed that nitrate-reducing strains can oxidize ferrous iron with molecular oxygen (Benz et al., 1998). Both lithoheterotrophic (depending on organic cosubstrates such as acetate) and strictly lithoautotrophic nitrate-reducing iron oxidizers have been found in various marine and freshwater sediments. However, most isolates depend on organic cosubstrates for cell biosynthesis (Benz et al., 1998). MPN estimations showed that nitrate-reducing Fe(II)-oxidizing bacteria accounted for 0.0006-0.8% of the acetate-oxidizing denitrifying microbial population. Lithotrophic Fe(II)-oxidizing bacteria accounted for less than 0.0001% of the total bacterial community and attempts to isolate CO_2-fixing nitrate-dependent Fe(II)-oxidizers from lithotrophic cultures failed (Straub et al., 1998). Mixotrophic Fe(II)-oxidizers accounted for 0.004-0.04% of the total bacterial community. Since it is not restricted to sunlight exposed habitats, it is believed that microbial nitrate-dependent Fe(II)-oxidation is more important on a global scale than anaerobic Fe(II)-oxidation by phototrophic bacteria.

No ferrous iron-oxidizing enrichment or culture has produced ammonium from nitrate. The microbial oxidation of ferrous iron was coupled to stoichiometric reduction of nitrate to N_2, in only one strain traces of N_2O formed as a side product (Straub et al., 1996; Benz et al., 1998). The ferric iron mineral formed was identified as amorphous 2-line ferrihydrite. The maximum energy that nitrate-reducing Fe(II)-oxidizers may gain from oxidizing siderite to amorphous ferrihydrite and reducing nitrate at pH 7.0 is

$$5FeCO_3 + NO_3^- + 12\ H_2O \quad = \quad 5(am)Fe(OH)_3 + \tfrac{1}{2}N_2 + 5HCO_3^- + 4H^+$$

$\Delta_r G^\circ = -302.9$ kJ/mol $\Rightarrow$ -60.6 kJ/mol Fe

The chemical reduction of nitrate by ferrous iron requires a catalyst, e.g. at least 10 µM Cu^{2+}, in order to take place and will not be significant under the conditions applied here (Moraghan et al., 1976). The chemical oxidation of ferrous iron with nitrous oxide has not been observed. However, nitrite can oxidize ferrous iron chemically (Moraghan et al., 1977; Straub et al., 1996) but this process is considered insignificant under the conditions studied here.

The aim of this project was to assess the diversity of iron metabolizing bacteria in several ecological niches by culture-dependent and culture-independent methods. In addition, we wanted to determine whether non-phototrophic, anaerobic nitrate-dependent Fe(II)-oxidizers coexist with aerobic Fe(II)-oxidizers and facultatively anaerobic Fe(III)-reducers in natural habitats. To this end, we sampled iron-rich sites in freshwater, estuarine and marine environments and enriched for non-phototrophic anaerobic and aerobic Fe(II)-oxidizers. The identification of the two environmentally most important facultatively anaerobic Fe(III)-reducers and one aerobic heterotrophic Fe(II)-oxidizer in the sediment samples was carried out by means of fluorescent *in situ* hybridization (FISH).

Experimental work

Sampling sites

Three iron-rich ecosystems were sampled for this project. The first was a cranberry bog on the Coonamessett River near John Parker Rd. in East Falmouth (Fig. 2; Appendix). The water emerging from a 10" black plastic pipe produced a copious red precipitate as it entered the main stream. The pH of the emerging water was 5.2. There were two pipes that emerge into the section of the bog, one at the north end adjacent to a herring ladder and one at the south end adjacent to a bridge in the road across the river.

The second sampling site was along the shore of Red Brook Harbor just north of Cataumet in the town of Bourne (Fig. 3; Appendix). There are several seeps at the shoreline between Scotch House Cove Rd. and Harborside Lane. At low tide the seeps are above the waterline. There is some evidence that a contaminant plume from the nearby Massachussetts Military Reservation feeds the seeps, but the name of the bay suggests that iron has been seeping at the site for a long time. There is also anecdotal evidence that bog iron was mined near the site in colonial times. The pH of the water emerging from the seeps was 4.75.

The third sampling site was Salt Pond in the south part of Falmouth (Fig. 4). The site was in the middle of the eutrophic pond and has been studied extensively by Katrina Edwards, WHOI. There is a stratification of the water in the pond with respect to oxygen, salinity, and iron speciation (Fig. 5 & 6) and samples were collected near the oxic/anoxic interface.

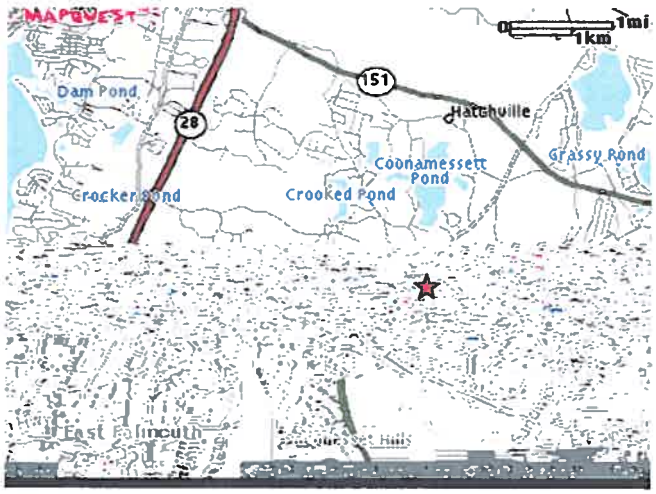

Fig. 2. Cranberry bog in Falmouth, Cape Cod, MA, USA.

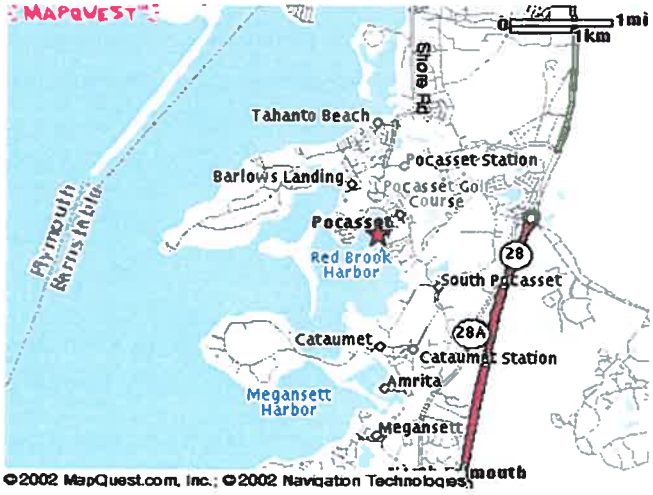

Fig. 3. Red Brook Bay, Cape Cod, MA, USA.

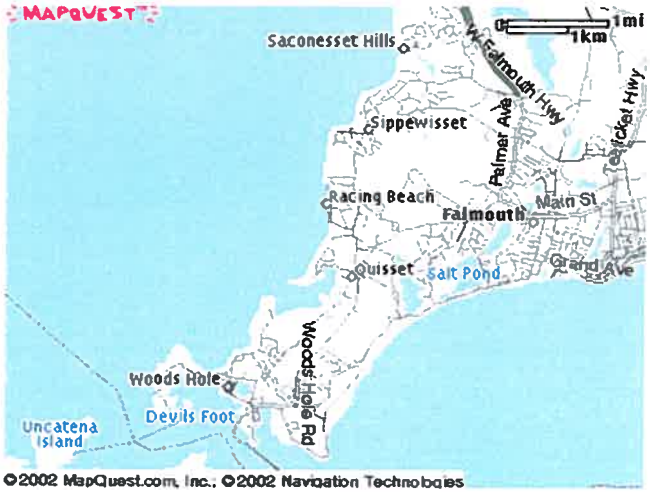

Fig. 4. Salt Pond, Falmouth, Cape Cod, MA, USA.

Salinity, pH, temperature, oxygen concentration and iron speciation were measured every foot from the surface down till 12.5 ft (Fig. 5 & 6).

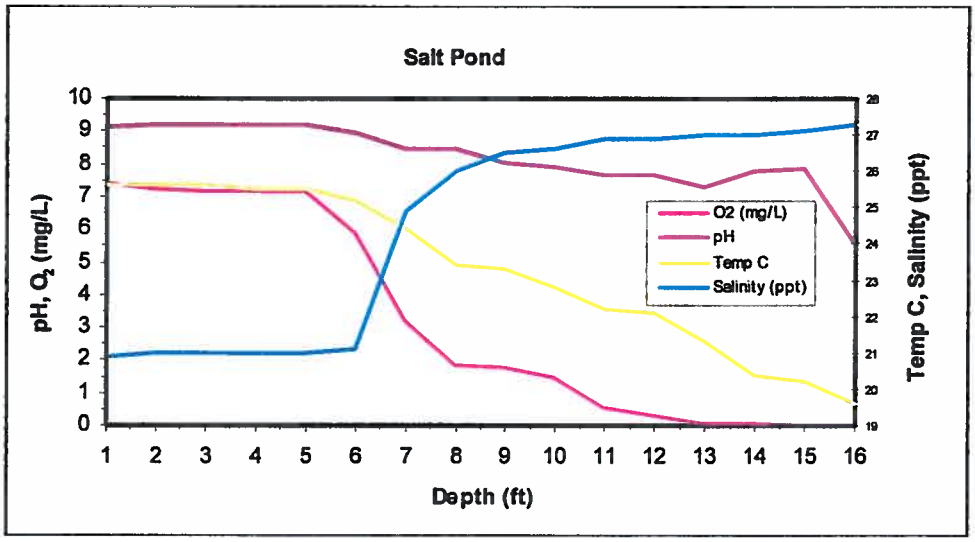

Fig. 5. Salinity, pH, temperature and oxygen concentration in Salt Pond.

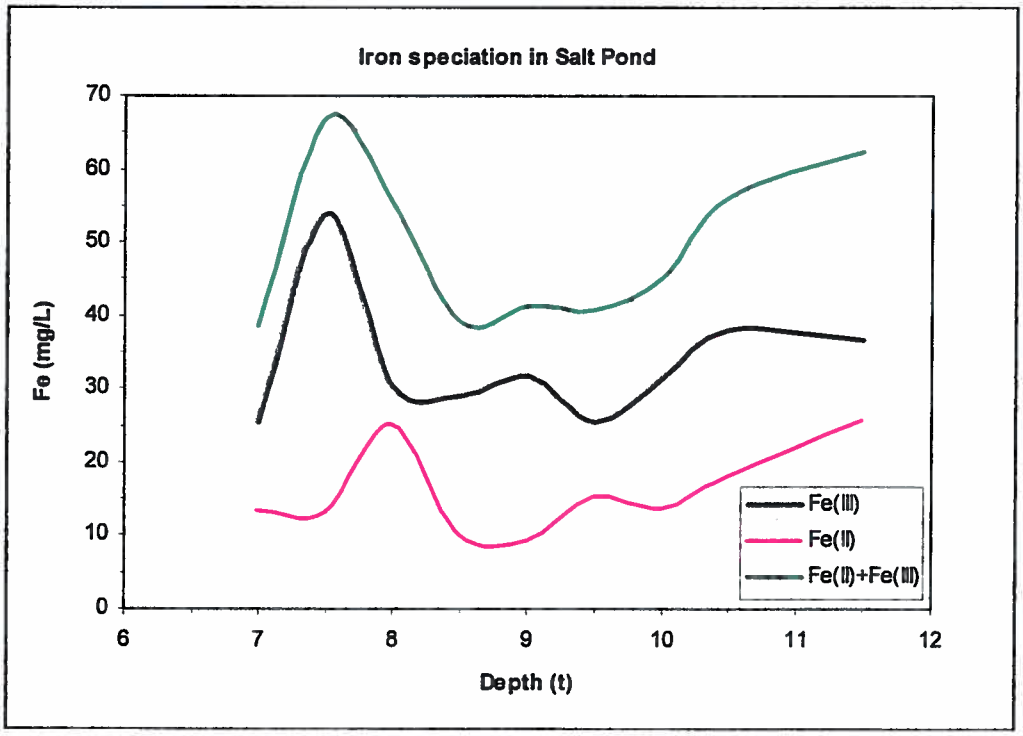

Fig. 6. Iron speciation in Salt Pond.

Fluorescent *in situ* hybridization (FISH)

Fluorescently labeled rRNA-targeted nucleic acid probes allow an *in situ* phylogenetic identification of individual microbial cells in their natural habitats without prior cultivation. *Geobacter sp.* and *Shewanella sp.* were identified and quantified using group-specific oligonucleotide probes SRB-687 and SPN3, respectively. Probe SRB687 (5'-TACGGATTTCACTCCT-3') was originally designed to target the sulfate-reducing bacteria genera, *Desulfovibrio* (Devereux et al., 1992), but the probe also detects some Fe(III)-reducers that belong to the *Geobacter metallireducens* subgroup (284 sequences matching the probe). Probe SPN3 (5'-ECCGGTCCTTCTTCTGTAGGTAACGTCACAG-3', target position 477-506 on the 16SrDNA genes in the E. coli numbering system) is complementary to a 30-nucleotide region that distinguishes *S. oneidensis* and *S. algae* from other members of the genus *Shewanella* and all other eubacteria in the rRNA database (77 sequences matching the probe; DiChristina et al., 1993). *Leptothrix sp.* was identified using the oligonucleotide probe PS-1 (5'-GATTGCTCCTCTACCGT-3') specific for five different *Leptothrix* strains and one *Spaerotilus natans* strain (6 sequences matching the probe; Siering et al., 1997). All probes were labeled with the indocarbocyanine fluorescent dye, Cy3, linked to the 5'-end of the oligonucleotide (100 nmole unit size; purchased from IDT, Inc.). Sediment samples taken from iron-rich anoxic/suboxic zones in marine and freshwater habitats and water samples from a brackish water habitat (see above) were fixed with formaldehyde, hybridized and stained for epifluorescence microscopic analysis according to the method described in Pernthaler et al., 2001. Samples incubated with the SRB-687 (0% formamide in hybridization buffer) and the PS-1 (20% formamide in hybridization buffer) probe were hybridized for 2 h at 46°C. Unhybridized and nonspecifically bound probes were removed by washing in buffer for 15 min at 48°C. Samples incubated with the SPN-3 probe were washed successively with 50%, 75% and 90% ethanol (3 min steps) prior to hybridization (0% formamide in hybridization buffer), incubated 16 h at 46°C and subsequently washed in buffer for 10 min at 65°C (DiChristina et al., 1993).

Enrichment for aerobic neutrophilic Fe(II)-oxidizers in gradient tubes

Gradient tubes were prepared as described by Hanert with the exception that $FeCl_2$ was substituted for FeS (Hanert, 1981). Briefly, a plug of agar (1.5%) containing minimal salts and ferrous iron was poured in the bottom of 16 x 150 mm screw capped culture tubes and allowed to solidify in an anaerobic glove box under an atmosphere of N_2-H_2. A soft agar upper layer containing 0.15 % low melting agarose, 5 mM bicarbonate, trace metals, and vitamins in minimal medium was then added and sparged with carbon dioxide to achieve a pH of 5.5 for freshwater media and 7.0 for brackish medium (15 ppt NaCl). The tubes were inoculated with material from natural habitats (Red Brook Bay (marine), Salt Pond (estuarine), Cranberry Bog (freshwater)) and incubated in the dark at room temperature. Air was allowed to enter the headspace of the tubes during inoculation.

Enrichment for non-phototrophic nitrate-dependent Fe(II)-oxidizers from Cranberry Bog

Sediment samples were collected from the Cranberry bog in Falmouth, Cape Cod, MA, USA where rusty iron occurrence was particularly recognizable (fig. 2). The bog water had pH 5.2. Enrichment cultures were grown in anoxic and bicarbonate buffered (30 mM, pH 7.0, 90% N2-

10% CO_2) freshwater mineral media containing 10 mM $FeCl_2$(aq) and 4 mM $NaNO_3$ in the presence or absence of acetate (1 mM) as an auxiliary substrate.

In an anoxic glove-box, small amounts of sediment samples were imbedded in 30 mL anoxic 0.5% agar in 100 mL serum vials followed by addition of 70 mL anoxic fresh water medium after the agar bed had stiffened. This set-up prevented initial mud-colouring of the overlying medium and hence enabled monitoring the activity of Fe(II)-oxidizing bacteria through the color change (from white (siderite) to red-brown (amorphous ferrihydrite)) in the medium over time. The rubber-stoppered vials were taken out of the glove-box and a 90% N_2/10% CO_2 headspace was reestablished. Each vial was amended with Fe(II), nitrate and acetate according to the list below and incubated at 30°C in dim incandescent light.

Vial	
1,2	Media amended 10 mM Fe(II) + 4 mM nitrate
3	Media amended with 10 mM Fe(II)
6	Media amended with 10 mM Fe(II) + 1 mM acetate
7,**9**	Media amended with 10 mM Fe(II) + 4 mM nitrate
11,**14**	Media amended with 10 mM Fe(II) + 4 mM nitrate + 1 mM acetate

Vials 1 & 2 were controls (not inoculated). Vials marked in bold did not contain agar plugs. Vials 3,6,7,9 & 11 were inoculated with Cranberry Bog sediment samples.

Enrichment for non-phototrophic nitrate-dependent Fe(II)-oxidizers from Salt Pond

Water samples were collected from Salt Pond (fig. 4), at a depth within the upper anoxic zone (10 ft; pH 7.3). Enrichment cultures were grown in anoxic and bicarbonate buffered (30 mM, pH 7.0, 90% N_2-10% CO_2) estuarine mineral media containing 10 mM $FeCl_2$(aq) and 4 mM $NaNO_3$ in the presence or absence of acetate (1 mM) as an auxiliary substrate.

Due to low cell density in the water samples, cells were harvested and concentrated by centrifugation before inoculation. Inocula were transferred into 25 mL rubber-stoppered and crimp-capped culture tubes containing 20 mL anoxic estuarine media amended with Fe(II), nitrate and acetate according to the list below and incubated at 30°C.

Culture tube	
1	Media amended 10 mM Fe(II) + 4 mM nitrate
3	Media amended with 10 mM Fe(II)
5,6	Media amended with 10 mM Fe(II) + 1 mM acetate
7,8	Media amended with 10 mM Fe(II) + 4 mM nitrate
9,10	Media amended with 10 mM Fe(II) + 4 mM nitrate + 1 mM acetate

Tube 1 was a control (not inoculated).

Preparation of media

Either fresh water or estuarine mineral media was autoclaved in a Widdel flask equipped with a filter inlet for flushing headspace with sterile anoxic gas, screw-cap inlets for addition of thermally unstable trace element solutions and a 7-vitamin solution after autoclaving, a tubing

connection for dispensing media and a magnetic stirring bar. After autoclaving, the flask was cooled to room temperature while flushing the headspace with 90% N_2-10% CO_2 at low pressure. Upon reaching room temperature, the media was amended with 30 mL of buffer (1 M $NaHCO_3$) as well as 1 mL of trace elements solutions and vitamins. Basal mineral media, trace element solutions and 7-vitamin solution as described in the course hand-outs were used.

Other reagents required

A 1 M $NaHCO_3$ buffer solution was autoclaved in a tightly sealed screw-cap bottle with ~ 60% headspace (90% N_2-10% CO_2).

Anoxic 0.4 M $NaNO_3$ stock solution
Anoxic 1 M CH_3COONa stock solution
Both stock solutions were autoclaved and stored cold.

A 0.5 M $FeCl_2$ stock solution was made by heat-catalyzed oxidation of metallic iron. 100 mL 1 M HCl was transferred to a 100 mL serum vial equipped with a N_2 gas inlet and a thin hypodermic needle for gas outlet and capped with a rupper stopper. The solution was flushed with N_2 for 30 min under magnetic stirring. After stopping the agitation and pulling the N_2 inlet above the solution surface, 3.63 g iron powder was quickly added and the vial was resealed. The vial was heated and slowly stirred until the hydrogen production had nearly stopped (1.5-2 h). The gas outlet needle was removed and an overpressure was alowed to form in the vial before removing gas inlet.

Results

Phase contrast microscopy

Direct examination of samples from the cranberry bog and Red Brook Bay revealed extensive mats of sheaths typical of *Leptothrix* surrounded by precipitated iron (Fig. 7). The south part of the cranberry bog and the samples from Salt Pond were dominated by rod-shaped bacteria of various sizes mixed in with the iron precipitates.

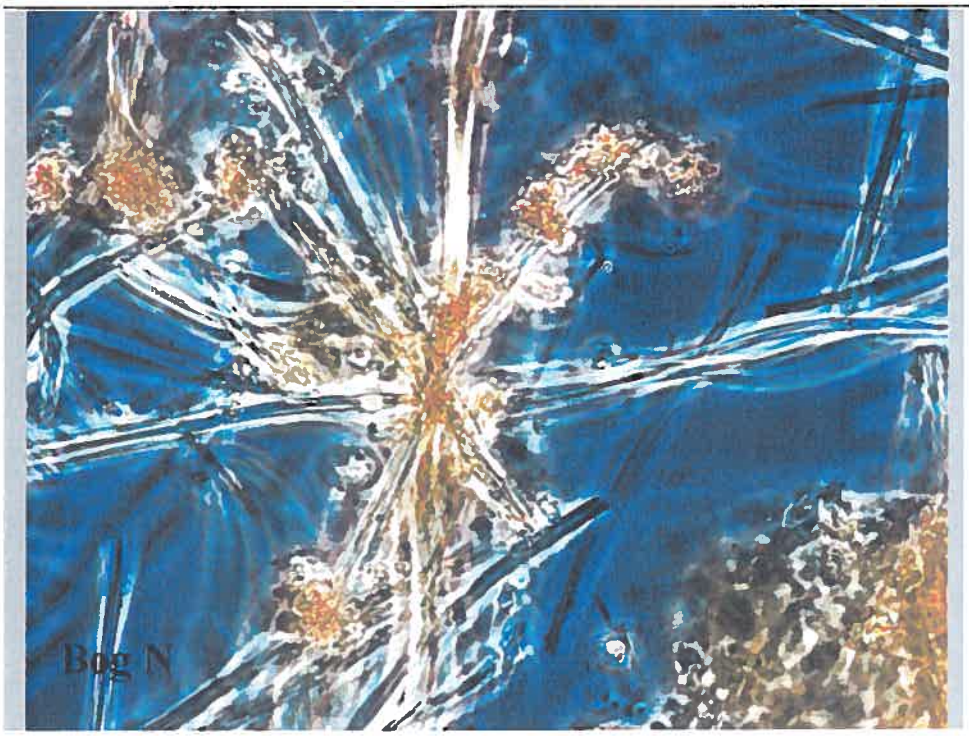

Fig. 7. Phase-contrast micrograph of a sample from the cranberry bog.

FISH Identification of *Shewanella* sp., *Geobacter* sp. and *Leptothrix* sp.

FISH analyses were conducted with samples from each of the field sites. Filters were prepared and stained according to the methods described in the course handouts. Each set of samples contained a diverse community as indicated by DAPI staining. The specific probes for *Geobacter, Shewanella, and Leptothrix* stained substantially smaller subpopulations (Table 1). The probes for γ-proteobacteria were included because there is a growing awareness that unidentified γ-proteobacteria play a major role in iron cycling in a variety of habitats (Sobolev et al., 2001; Emerson et al., 2002). The β-proteobacteria probe was included because both *Leptothrix* and *Gallionella* are beta-proteobacteria.

Sample site	pH	Geobacter	Shewanella	Leptothrix	γ	β
Salt Pond	7.5	+	+	+	+	++
Red Brook Bay	4.7	-		+	++	++
Cranberry Bog	4.8	-		+	+++	+++

Table 1. Identification of *Shewanella* sp., *Geobacter* sp. and *Leptothrix* sp. by means of FISH. + denotes 5-10%, ++ denotes 10-30% and +++ denotes 30-50% of total microbial population. No sign denoted means that no data were collected.

Leptothrix sp. were found at all three sampling sites. *Geobacter, Shewanella* and *Leptothr* coexist in Salt Pond at the oxic/anoxic interface. It is clear that other β and γ proteobacteri constituted a major part of the microbial community at each site. Their identities and roles remain to be determined.

Enrichment for aerobic neutrophilic Fe(II)-oxidizers in gradient tubes

Aerobic Fe(II)-oxidizers were cultured in gradient tubes from each sample site. Fig. 8 shows a typical gradient tube 5 days after inoculation with material from the Cranberry Bog. The orange precipitate was similar in inoculated and uninoculated tubes because there is substantial abiotic oxidation of iron. The iron deposition is, therefore, limited by oxygen and iron mass transfer rather than biological activity. Microscopic examination of the material from the orange layers revealed massive microbial communities in the inoculated tubes and none in the uninoculated controls. No sheaths or stalked bacteria were detected in the upper layers of the gradient tubes. The tubes from the cranberry bog formed a second, lighter band below the main band which indicated the presence of microbes that occupy a slightly different niche. The black precipitate at the interface between the agar plug and the overlying mediumcould indicate either Fe(III)-reducers or Fe(II)-oxidizers producing black magnetite (Fe_3O_4) or sulfate-reducers from the inoculum producing black ferrous sulfide (FeS). Phase contrast microscopy revealed extensive formation of filamentous growth in the deposits (Fig. 9). This black deposits were only found in gradient tubes inoculated from Cranberry Bog.

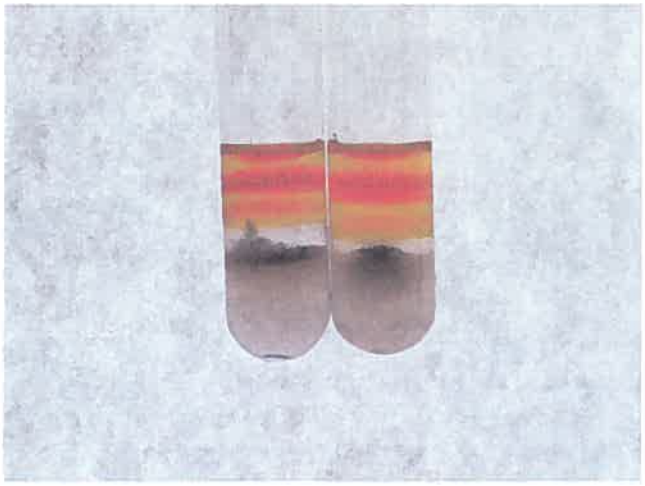

Fig. 8. A gradient tube 5 days after inoculation from Cranberry Bog.

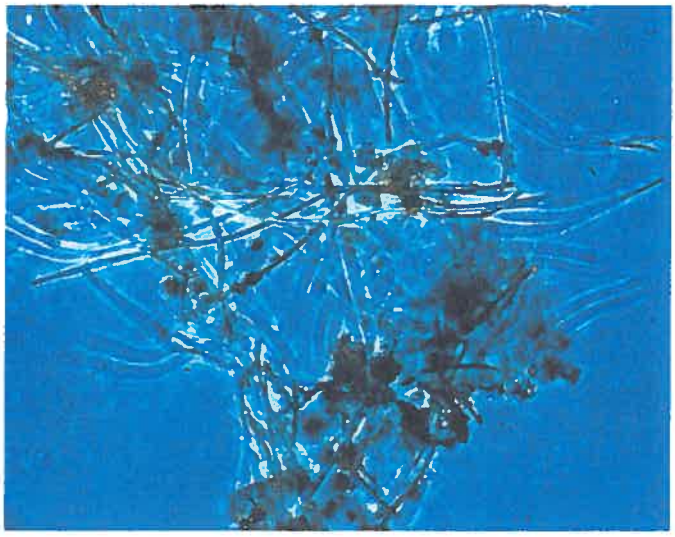

Fig. 9. Phase-contrast micrograph of black deposits in gradient tubes associated with sheathed bacteria.

Enrichment for non-phototrophic nitrate-dependent Fe(II)-oxidizers from Cranberry Bog

After 7 days vials 7 & 11 but in particular vial 7 exhibited signs of Fe(II) oxidation (orange brownish bands at the glass wall) in the upper layer of the agar plug as well as green colouring of the siderite flocs (left vial Fig. 10). In vials 3,6 & 8 the agar plug and the overlaying siderite flocs turned completely black within the first week indicating activity of sulfate reducing bacteria producing sulfide which subsequently precipitates as black ferrous sulfide (right vial Fig. 10). Due to this color, any Fe(II) oxidation would be undetectable in these vials.

Fig. 10. Visual indication of activity of denitrifying Fe(II)-oxidizers in left vial.

From vial 7 inocula were withdrawn from the agar plug upper layer and transferred into culture tubes containing liquid freshwater mineral media amended with Fe(II), nitrate and acetate according to the list below and incubated at 30°C in the dark or in dim incandescent light.

Culture tube	
1,2	Media amended 10 mM Fe(II) + 4 mM nitrate
3,4	Media amended with 4 mM nitrate
5,6	Media amended with 10 mM Fe(II) + 1 mM acetate
7,8	Media amended with 1 mM acetate
9,10	Media amended with 10 mM Fe(II) + 4 mM nitrate + 1 mM acetate

The color changed over time according to the contents of the tubes (Fig. 11) Culture tubes 9 and 10 amended with both ferrous iron, nitrate and acetate appeared more brownish red than the other tubes which might indicate activity of lithoautotrophic or mixotrophic nitrate-reducing Fe(II)-oxidizers. Measurement of nitrate consumption over time would indicate whether the Fe(II)-oxidizing bacteria are nitrate-dependent but the available resources did not allow nitrate measurements. Culture tubes 8 and 9 both contained a black precipitate which could be indicative of either Fe(III)-reducers producing black magnetite (Fe_3O_4) from Fe(III) in the inoculum or sulfate-reducers from the inoculum producing black ferrous sulfide (FeS) while growing on acetate. There were no apparent color differences between tubes incubated in the light or in the dark.

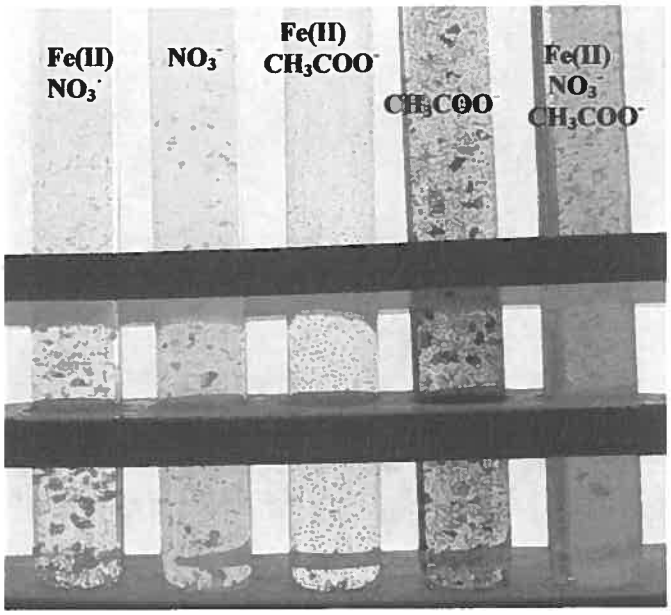

Fig. 11. Color development after 7 days in fresh water media amended with Fe(II), nitrate and acetate. Inoculum from left serum vial in Fig. 10.

Enrichment of microorganisms provide information about their presence but not about their abundance and activity in the original habitat.

Enrichment for non-phototrophic nitrate-dependent Fe(II)-oxidizers from Salt Pond

There was no visual sign of denitrifying Fe(II)-oxidizers in any of the tubes.

Discussion

It is clear that there are thriving communities of iron oxidizing bacteria in each of the ecosystems examined. The most obvious populations are the sheathed bacteria, but there is growing evidence that the rods and vibrioid γ-proteobacteria can play a major role in iron cycling. The recent suggestion that such bacteria release ferric iron in a diffusible form that precipitates at some distance from the cells seems to be borne out by our results in gradient tubes, where the iron precipitates did not seem to be attached to the often highly motile cells. Such diffusible ferric iron would also support the existence of iron reducing bacteria in close juxtaposition to the iron oxidizers and allow the cycling to take place over a very narrow interface.

The relative amount of abiotic and biological oxidation of iron in the circumneutral ecosystems is not known. Many of the bacteria have not been cultured and the standing biomass has not been determined across a variety of sites. It would be very useful to clone and sequence the 16S ribosomal genes from the various sites to determine the diversity of the communities. It would be even more interesting to find a way to measure the biotic vs abiotic contribution to the flux in the systems.

Conclusions

Aerobic Fe(II)-oxidizers were cultured in gradient tubes from each sample site. Nitrate-reducing Fe(II)-oxidizers were only found in sediments from the Cranberry Bog. *Geobacter, Shewanella* and *Leptothrix* coexist in Salt Pond at the oxic/anoxic interface. The roles of the various bacteria in the global iron and carbon cycles remain to be determined.

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Cranberry Bog

Red Brook Bay