Ecology of neutrophilic iron-oxidizing bacteria in wetland soils

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Ecology of neutrophilic iron-oxidizing bacteria in wetland soils

De ecologie van neutrofiele ijzeroxiderende bacteriën in wetlandbodems

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General introduction

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1.1 Iron geochemistry

Iron (Fe) is the fourth most abundant element in the Earth's crust and the most prevalent redox active metal (Hauck et al., 2001). Iron is absolutely required for life of all forms and it participates in many major biological processes, such as photosynthesis, N_2 fixation, methanogenesis, H_2 production and consumption, respiration, TCA cycle, oxygen transport, gene regulation and DNA biosynthesis (Andrews et al., 2003). Iron plays an important role in the metabolism of a variety of bacteria as trace element, as well as an electron donor and acceptor (Kumaraswamy et al., 2006). Hence, iron has been the focus of many studies because of its importance in biogeochemical redox reactions in the environment (Straub et al., 2001; Emerson and Weiss, 2004; Roden, 2004; Borch et al., 2010).

There are two main valence states of iron in the environment, *i.e.* ferrous iron Fe(II) and ferric iron Fe(III). The standard redox potential (E_0) of the Fe(III)(aq)/Fe(II)(aq) transition is +0.77V. Under strongly acidic conditions, the transition from Fe(II) to Fe(III) occurs at Eh = + 0.77 V, however, at pH 7.0 the transition involves iron minerals, such as $FeCO_3$ and $Fe(OH)_3$, and occurs at lower Eh, typically +0.1 to +0.2 V (Widdel et al., 1993).

Ferric and ferrous irons have very different solubility properties. Under sub-oxic conditions, ferric iron is readily reduced either chemically or by microbial processes (Figure 1). It is a potentially important electron acceptor for organic matter degradation (Lovley and Phillips, 1988). Aqueous ferrous iron is stable only under acidic conditions. In the presence of molecular oxygen, the oxidation rate of Fe(II) can be described by (Armstrong, 1967; Sung and Morgan, 1980):

$$-d[Fe(II)]/dt = k\,[OH^-]^2\,pO_2\,[Fe(II)] \quad (1)$$

where k is the rate constant, [Fe(II)] denotes that concentration of ferrous iron, while pO_2 is the partial pressure of oxygen.

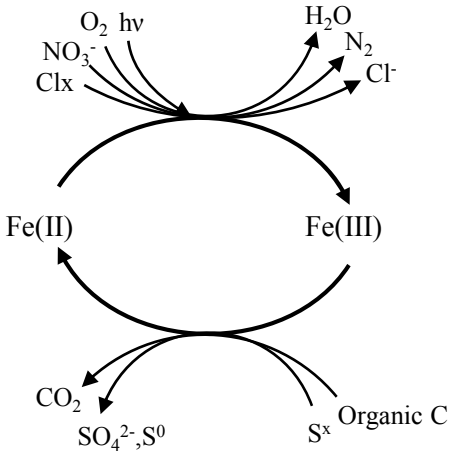

Figure 1. Iron redox cycle. The reduction of iron always occurs under anoxic conditions, while iron oxidation can occur under both oxic and anoxic conditions. The reactions are always associated with other elemental cycles.

1.2 Microbial iron interactions

The cycling of iron is driven by abiotic and biotic reactions. Bacteria can act both directly and indirectly to oxidize or reduce iron (Fortin and Langley, 2005). Direct interactions involve the enzymatic conversion of iron to both oxidized and reduced forms in order to gain energy (Ghiorse, 1984; Widdel et al., 1993). Indirect interactions involve localized alteration of pH and *Eh* conditions through their metabolic activities, which then promotes either mineral precipitation or solubilisation with subsequent metal mobilization (Fortin et al., 1998). Also, bacteria can accumulate iron by passive physicochemical sorption of Fe(III) and Fe(II) ions onto the negatively charged microbial and extracellular polymeric substance (EPS) surfaces (McLean et al., 1996; Jiao et al., 2010), which may accelerate the rate of iron precipitation.

Iron oxides formed in close association with bacteria (either as internal or external precipitates) are referred to as biogenic minerals. The formation, occurrence, and potential use of biogenic iron-rich minerals have been thoroughly reviewed (Fortin and Langley, 2005).

1.2.1 Microbial iron oxidation

As for the abiotic reaction, biotic iron oxidation depends on the pH and the O_2 concentration. Microbial oxidation of Fe(II) to Fe(III) can be accelerated by a wide range of iron-oxidizing bacteria under both acidic and neutral pH and oxic

and anoxic conditions. Microorganisms that are able to oxidize Fe(II) are diverse in their phylogeny and overall physiology. Those that oxidize iron and/or accumulate iron oxides are, in general, placed into one of two groups: the acidophilic and the neutrophilic prokaryotes. Representative examples of bacteria and archaea capable of coupling Fe(II) oxidation to growth include psychro-, hyperthermo- and mesophiles that couple Fe(II) oxidation to the reduction of nitrate or oxygen, and the anaerobic Fe(II)-oxidizing phototrophs.

Acidophilic iron oxidation

In the presence of oxygen and under acidic conditions, ferrous iron is relatively stable. A wide number of studies have been carried out on iron oxidation under acidic conditions and some microbes have been observed of being capable of aerobic respiration on both Fe(II) and reduced forms of sulfur (H_2S, S_0, $S_2O_3^{2-}$) (Leduc and Ferroni, 1994; Baker and Banfield, 2003). *Acidothiobacillus ferrooxidans* is widely known to catalyze the oxidation of Fe(II) in natural systems at low pH, and thus accelerates the rate of Fe (II) oxidation (Baker and Banfield, 2003b). It is an industrially and environmentally important microorganism (Suzuki, 2001) and has been the focus of extensive genetic, genomic, and physiological studies. Other important species that have been extensively studied include *Leptospirillum ferooxidans* that can grow only by aerobically oxidizing Fe(II) ions (Rohwerder et al., 2003).

The A. *ferrooxidans* respiratory chain follows the pathway: Fe(II)→Cyc2 →rusticyanin →cytochrome c4→cytochrome oxidase aa3→O_2 (Appia-Ayme et al., 1999). Recently investigations revealed more detailed mechanisms of acidophilic iron oxidation (Carlos et al., 2008; Osorio et al., 2008) and novel enzymes involved in iron respiratory chain were identified (Castelle et al., 2008). Progress has also been made in characterizing their biology and ecology by using comparative genomics (Coram et al., 2005) and by looking at the gene expression patterns in relation to environmental parameters in natural ecosystems (Parro and Moreno-Paz, 2003; Parro et al., 2007).

16S rRNA gene-based approaches have been used effectively to elucidate microbial community structure in extremely acidic, metal-leaching environments (Baker and Banfield, 2003; Tan et al., 2009). Community genome sequencing (Tyson et al., 2004; Dick et al., 2009) and proteomics (Ram et al., 2005; Bouchal et al., 2006) has also been used to evaluate the diversity of acidophilic iron-oxidizing community and their gene expression, identify key activities, and examine partitioning of metabolic functions in a natural acid mine drainage (AMD) microbial biofilm communities.

Neutrophilic iron oxidation

Most physiological studies on ferrous iron oxidation have been carried out with aerobic acidophilic bacteria (Straub et al., 2001). Iron (II) oxidation at near-neutral pH has long been thought to occur abiotically (Kasama and Murakami, 2001). Indeed, at circumneutral pH, Fe(II) is subject to rapid chemical oxidation by dissolved O_2, and the Fe(III) produced quickly hydrolyzes and precipitates as Fe(III) oxyhydroxides. Due to the rate of this spontaneous reaction, the feasibility of circumneutral bacterial Fe(II) oxidation with O_2 as an electron acceptor, though energetically more favorable than Fe(II) oxidation to Fe(III) at low pH, has generally been considered doubtful (Sobolev and Roden, 2002).

Another study (Neubauer et al., 2002) shows that Fe-oxidizing bacteria (FeOB), which gain energy for growth from the oxidation of Fe(II) at circumneutral pH, face several challenges. First, the energy available from Fe(II) oxidation is low. Also the half-life time of Fe(II) in natural circumneutral freshwaters is approximately 2 to 10 min under air-saturated conditions, whereas the shortest reported doubling time for a lithotrophic Fe(II) oxidizer is about 8 h (Emerson and Moyer, 1997). These kinetic parameters limit growth of Fe(II) oxidizers to microaerophilic zones at the interface between oxic and anoxic environments.

Lately, the presence of Fe(III) precipitates and substantial numbers of FeOB obtained from a variety of environments suggest that microbial Fe(II) oxidation may be significant in circumneutral environments (Neubauer et al., 2002; Weiss et al., 2004, 2005; Duckworth et al., 2009). The study of Neubauer et al. (2002) indicates that the bacteria can successfully compete with abiotic oxidation for Fe(II), and account for 50 to 60% of the total Fe(II) oxidation in laboratory cultures despite Fe(II) limitation in their bioreactor eperiments. Several iron oxidizers are isolated from circumneutral environments, including *Gallionella* and *Leptothrix* species.

Environments where microbes are likely to play a significant role in circumneutral Fe(II) oxidation are redox interfaces, where diffusion-limited O_2 transport leads to low dissolved O_2 partial pressure within the zone of Fe(II)-O_2 overlap, *i.e.* environments characterized by opposing diffusion gradients of O_2 and Fe(II) (Emerson and Moyer, 1997; Sobolev and Roden, 2002).

An interesting and potentially very widespread environment where bacterial Fe(II) oxidation may occur is the rhizosphere of aquatic plants, where release of O_2 from respiring plants roots provides an input of O_2 into otherwise anoxic sediment, which might contain significant quantities of Fe(II). Recent studies

(Emerson et al., 1999; Sobolev and Roden, 2001) suggest that bacterial Fe(II) oxidation is likely to generate reactive amorphous Fe(III) compounds, which are readily available for Fe(III)-reducing bacteria at oxic-anoxic interfaces. This dynamic network of oxic-anoxic interfaces is typically found in the rhizosphere of wetland plants where rapid Fe-cycling is known to occur (Weiss et al., 2004).

However, our knowledge concerning the physiology, phylogenetic diversity, and environmental abundance and distribution of neutrophilic Fe-oxidizing microorganisms has remained poorly elucidated to date. The reasons remain largely in the difficulties in growing and studying organisms in pure culture in the laboratory (Emerson and Moyer, 1997; Edwards et al., 2003b) and the lack of sufficient molecular tools to detect them in the environment.

Anaerobic iron oxidation

Studies have shown that microbial Fe(II) oxidation is not limited to oxygenated environments, but also occurs under fully anoxic conditions (Straub et al., 2001; Sobolev and Roden, 2004; Straub et al., 2004; Weber et al., 2006). Anaerobic Fe(II)-oxidizing bacteria are found in a variety of oxygen-poor freshwater and marine settings with elevated Fe(II) concentrations (Posth et al., 2010). In anoxic habitats, Fe(II), ferrous iron can form minerals such as siderite, vivianite or iron sulfide under weakly acidic to neutral conditions (Straub et al., 2001), but can also be oxidized by a number of bacteria, which use Fe(II) as electron donor.

Anaerobically, ferrous iron can be oxidized either by phototrophic bacteria (Widdel et al., 1993; Ehrenreich and Widdel, 1994; Kappler et al., 2005b; Poulain and Newman, 2009) or by chemotrophic, denitrifying bacteria and archaea (Hafenbradl et al., 1996; Benz et al., 1998; Blothe and Roden, 2009b; Muehe et al., 2009; Weber et al., 2009). In both cases, iron serves as an electron donor for redox processes in anoxic habitats (Widdel et al., 1993), schematically:

$$HCO_3^- + 4Fe^{2+} + 10H_2O = (CH_2O) + 4Fe(OH_3) + 7H^+ \quad (2)$$

Photoautotrophic bacteria including green sulfur bacteria (Heising et al., 1999), purple non-sulfur and purple sulfur bacteria (Ehrenreich and Widdel, 1994; Kappler and Newman, 2004; Poulain and Newman, 2009) gain energy from the oxidation of Fe(II) and use light energy for CO_2 fixation. Ferrous iron can also be oxidized anaerobically in the dark. Typical examples are nitrate-reducing bacteria that gain energy for growth by oxidizing ferrous iron

anaerobically (Straub et al., 1996; Straub and Buchholz-Cleven, 1998; Lack et al., 2002; Kappler et al., 2005a; Weber et al., 2009). Anaerobic Fe (II) oxidation coupled to nitrate reduction may also support Fe cycling in the rhizosphere (Straub et al., 2004).

1.2.2 Ferric iron reduction

Where limited oxygen diffusion and vigorous heterotrophic microbial activity create anoxic conditions, as found in waterlogged soils, and aquatic sediments, ferric iron may act as an electron sink and be reduced to ferrous iron.

Reduction of Fe (III) may involve both chemical reduction and microbial dissimilatory reduction. Chemically sulfide oxidation may be coupled to iron reduction, which is responsible for the majority of reduced iron (Jacobson, 1994). Many bacteria, in both oxic and anoxic environments, are able to reduce iron. Microbial reduction of ferric iron plays an important role in the iron geochemistry and organic matter mineralization of aquatic sediments (Lovley et al., 1987). They reduce iron by coupling its reduction to the oxidation of hydrogen or organic carbon (Brown et al., 1999). A wide diversity of Fe(III)-reducing microorganisms have been described, which can oxidize fatty acids, aromatic compounds, and some amino acids to carbon dioxides with the concomitant reduction of Fe(III) (Lovley, 1997).

Microbiologists have extensively studied dissimilatory iron-reducing bacteria (FeRB), which use ferric iron as electron acceptor in anoxic environments (Lovley et al., 2004). Ferric iron-reducing bacteria have been isolated from a great variety of anoxic environments, including sediments, soils, deep terrestrial sub-surfaces, and hot springs (Hauck et al., 2001; Straub et al., 2004; Shelobolina et al., 2007). Significant progress has been made toward the understanding of their biochemistry and physiology (Magnuson et al., 2001).

2 Iron oxidizing bacteria (FeOB)

The remarkable feature of some Fe-oxidizing bacteria are the unique morphological structures they produce, such as sheaths or stalks, that act as organic matrices upon which the deposition of hydrous ferric oxides can occur (Emerson and Moyer, 2002). They are capable of accumulating metals by binding them as cations to the cell surface in a passive process (Gadd and White, 1993), as well gaining energy for growth from the oxidation of ferrous iron with O_2 as terminal electron acceptor.

A variety of iron-oxidizing bacteria have been isolated from acidic mine drainage or places where an ore body is naturally exposed to water and the

atmosphere. The iron-oxidizing bacteria found in these 'natural', ambient temperature conditions are ubiquitous and most of them have been identified as *Acidithiobacillus ferrooxidans* and *Leptospirillum ferroxidans*. The former has been well studied for ferrous iron oxidation compared to the latter (Widdel et al., 1993). It is by far the most studied group of iron-oxidizing bacteria and has been used as a model microorganisms capable of gaining energy from the oxidation of Fe(II) to Fe(III) at low pH (Baker and Banfield, 2003; Wakai et al., 2007; Carlos et al., 2008; Castelle et al., 2008).

Knowledge concerning neutrophilic FeOB is far scarcer than for their acidophilic counterparts. The stalked bacterium *Gallionella ferruginea* and sheathed bacteria belonging to the genus *Leptothrix* are the classical examples of bacteria catalyzing the aerobic oxidation of ferrous iron at neutral pH. They are found growing at the interface between the oxidizing and the reducing zones (Lutters-Czekalla, 1990) in neutral pH environment and are classified as gradient bacteria according to the oxygen concentrations where they occur.

Gallionella ferruginea was first described in 1836 by Ehrenberg (c.f.(Hallbeck et al., 1993)) and many years later successfully enriched in the laboratory (Hallbeck and Pedersen, 1991). The small, bean-shaped cells of this organism produce long cell-associated stalks, presumably consisting of excretion products from the cell. It was suggested that the stalks are protective mechanism against the precipitated iron or oxygen toxicity (Hallbeck and Pedersen, 1995). They belong to the Betaproteobacteria and able to utilize Fe(II) and the reduced sulfur compounds sulfide and thiosulfate as electron donor and energy source and CO_2 as a carbon source (Ghiorse, 1984; Lutters-Czekalla, 1990). First thought to be obligate autotrophs, they have also been found to be able to grow mixotrophically on CO_2 and small organic compounds (Hallbeck and Pedersen, 1991).

Leptothrix ochracea is perhaps the most commonly recognized Fe(II) oxidizer due to its iron-encrusted sheaths and cell filaments. It is a heterotrophic microorganism that obtains its energy from the oxidation of organic matter (Katsoyiannis and Zouboulis, 2004). So far, it has been found to be the predominant bacterial group associated with iron precipitations (Emerson and Revsbech, 1994; James and Ferris, 2004). While other species of *Leptothrix* have been successfully grown in the laboratory, the physiology of *L. ochracea* itself is largely unknown, because it has not been isolated in pure culture (Spring, 2006).

Research on neutrophilic FeOB has identfied a number of morphologically distinct species from different study sites, including water iron seeps, hydrothermal vents, groundwater and rhizosphere of wetland plants (Table 1).

In addition to *Gallionella sp*. and *L. ochracea*, novel strains, *i.e.* ES-1, ES-2 (Emerson and Moyer, 1997) and PV-1, JV-1 (Emerson and Moyer, 2002) have been isolated from a Michigan groundwater site and Loihi Seamount vents, respectively, using gradient tubes (Figure 2). Phylogenetically these four stains belong to the alpha- and gamma-proteobacteria (Table 1). They don't produce morphologically distinct sheaths or stalks, however.

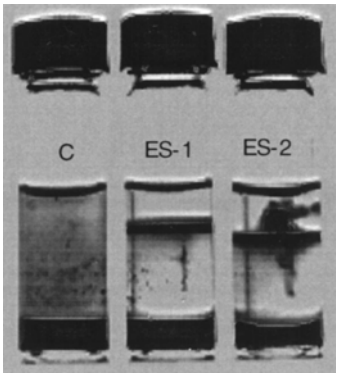

Figure 2. Gradient growth tubes. The black layer on the bottom of the tubes is the FeS agarose plug. The tube on the left (C) is a control tube that was not inoculated with cells (Emerson and Moyer, 1997).

Weiss et al. (2007) have isolated a new obligate lithotrophic Fe(II)-oxidizing bacterium, strain BrT, from the rhizosphere of a wetland plant. Analysis of the 16S small subunit rRNA gene indicates that BrT is part of a novel lineage of the Gamma-proteobacteria and that it is closely related to another FeOB strain CCJ (Emerson et al., 1999). Another recently isolated strain, TW2 (Sobolev, 2002), falls within the Beta-proteobacteria with 91% 16S rRNA gene sequence similarity with *G. ferruginea*. The phylogenetic identification of some other neutrophilic FeOB recently isolated from seafloor (Edwards et al., 2003b) is still in progress.

A special characteristic of FeOB is that they favor oxic-anoxic boundaries, where low oxygen concentrations allow the bacteria to compete for ferrous iron with chemical oxidation processes (Rentz et al., 2009). Gradient tubes that mimic these boundary conditions have been used successfully to isolate these organisms (Kucera and Wolfe, 1957; Emerson and Moyer, 1997) .

Table 1 Neutrophilic Fe-oxidizing bacteria described recently

Strains	Phylogeny	Morphology	Isolation sites	References
FO1,2,3	Alphaproteobacteria	N.A	sea floor	Edwards et al., 2003b
ES-1	Betaproteobacteria	curved/helical rod	ground water	Emerson and Moyer, 1997
ES-2	Betaproteobacteria	curved rod	ground water	Emerson and Moyer, 1997
TW2	Betaproteobacteria	curved rod	wetlands	Sobolev and Roden, 2004
PV-1	Gammaproteobacteria	curved rod	hydrothermal vents	Emerson et al., 2007
JV-1	Gammaproteobacteria	curved rod	hydrothermal vents	Emerson et al., 2007
BrT	Gammaproteobacteria	curved rod	root of *Typha latifolia*	Weiss et al., 2007
CCJ	Gammaproteobacteria	straight rod	root of *Juncus effusus*	Weiss et al., 2007
FO 4,5,6,8,9,15	Gammaproteobacteria	N.A	sea floor	Edwards et al., 2003b

FeOB play an important role in iron oxidation throughout a wide variety of environments. They have been shown to increase the rate of iron oxidation by up to four orders of magnitude over the rate of strictly chemical oxidation (Kasama and Murakami, 2001; Sogaard et al., 2001). Emerson et al. (2002) estimated that up to 60% of the iron oxides deposition at a number of the Loihi vents is directly attributable to microbial activity. This value falls in the range of 50-80% of the iron-oxidizing activity accounted for by microbes in a freshwater microcosm experiment (Emerson and Revsbech, 1994). Using bioreactor batch cultures, Neubauer (Neubauer et al., 2002) showed that one iron-oxidizing strain could mediate 18 to 53% of the total Fe(II) oxidation under circumneutral conditions. Sobolev and Roden (2002) also reported that a pure culture of a Fe-oxidizing bacterium accounted for up to 90% of the iron oxidation in the environment.

The recent isolation of novel iron-oxidizing strains of neutrophilic bacteria, distantly related to the classical iron oxidizers and morphologically different (Emerson and Moyer, 1997), indicating that there might be a wide variety of microorganisms capable of iron oxidation (Hauck et al., 2001) in pH neutral environments.

3 Mechanisms of iron oxidation

It is believed that iron-oxidizing bacteria couple the energy derived from the oxidation of ferrous iron to the production of reducing power (NADPH) and the synthesis of ATP for construction of cellular materials (Leduc and Ferroni, 1994). Most of the knowledge on the biochemistry of iron oxidation comes from studies of the acidophile *Acidithiobacillus ferrooxidans* (Newman, 2010).

Fe(II) is oxidized at the outer membrane where rusticyanin receives one electron, and transfers it to a periplasmic c-type cytochrome, which subsequently reduces cytochrome a. The later interacts with oxygen to from H_2O via cytochrome oxidase.

The mechanism of bacterial iron oxidation in neutral pH conditions, however, is still poorly understood. By using isotope fractionations (Kappler et al., 2010) identified three different locations where $Fe(OH)_3$ precipitation occurs after oxidation of Fe(II)(aq) by *Acidovorax* sp. strain BoFeN1, ***i.e.*** in the periplasm, at the cell surface or away from the cells (***e.g.***, at EPS). The periplasm was revealed as a key location for iron oxidation, while EPS may play a role in the precipitation of Fe(III) mineral phases (Miot et al., 2009b).

In order to get sufficient energy for growth, FeOB need to oxidize tremendous amount of ferrous iron, rendering this a poor lifestyle. In addition, the products may be harmful, which will hamper bacterial growth and sometimes can even lead to cell death. One of the daunting phenomena is how the bacteria deal with iron oxides that precipitate rapidly under neutral pH conditions and avoid encrustation. Previous work showed a microenvironment with lowered pH values in close vicinity of the cells (Kappler and Newman, 2004; Roden et al., 2004), and some reasonable hypotheses have been proposed (Miot et al., 2009a; Weber et al., 2009). One general explanation is that bacteria prevent cell entombment and death by mineral metabolic by-products (Fortin, 2004). Chan et al., (2004) proposed that the oxidation of ferrous iron by iron-oxidizing bacteria increases the pH gradient across the cell membrane, which generates a proton motive force and thus increases the energy generating profit from encrusting. A clear understanding and demonstration of the mechanisms will help to better understand bacterial-iron interaction in environments.

One other enigma is how the bacteria defend themselves against oxygen toxicity. Their primary substrate, Fe(II), can be a potential toxin in the presence of oxygen, generating reactive oxygen species. Ghiorse (1984) suggested that H_2O_2, when produced in the periplasm space of these bacteria during oxidative metabolism, might diffuse outward and be eliminated extracellularly by either enzymatic oxidation or non-enzymatic reduction. If such a mechanism would be operational under oligotrophic and microaerophilic conditions, low levels of H_2O_2 would be produced and used in peroxidase-oxidation and subsequent deposition of metal oxides (Ghiorse, 1984).

In the case of neutrophilic iron-oxidizers such as *Leptothrix* and *Gallionella*, the production of layers of exopolysaccharides (*i.e.* sheath) or twisted stalks around the cells protects the cell itself from encrustation (Hallberg and Ferris, 2004). Also, the oxidation of ferrous iron on the stalks of *G. ferruginea* produces highly reactive (and toxic) oxygen species. Localization of iron oxidation to the stalk may serve to protect the cell from the damaging effects of the oxygen radicals (Hallbeck and Pedersen, 1995).

4 Iron oxidation in wetlands

4.1 Characteristics of wetlands and wetland plants

Wetland ecosystems are sites of rapid biogeochemical cycling due to the interactions between the oxic soil surface an deeper anoxic soils (Weiss et al., 2003). In wetlands, long-lasting water-logging results in reduced soil conditions. Since the diffusion of gases in water is about 10,000 times slower than in air (Colmer, 2003), oxygen supply is greatly impeded and oxygen is depleted rapidly by roots, microorganisms, and soil reductants. The processes that follow the oxygen depletion include denitrification, reduction of manganese, iron and sulfate, as well as changes in soil pH and Eh (Pezeshki, 2001). The accumulation of reduced ions such as ammonium, Fe(II), Mn(II) and sulfide is known to be toxic to the plants.

Wetland plants are adapted to survive anoxic conditions in oxygen-limited soils or sediments by the formation of aerenchymatous tissue (Visser et al., 2000) in their roots, stems and leaves (Armstrong, 1964). By means of the aerenchyma wetland plants provide an important conduit for gas exchange between the atmosphere and water-saturated, anoxic soils (Colmer, 2003). Oxygen from the atmosphere or oxygen originating from photosynthesis can be transported to the belowground organs, while CO_2, ethylene, and methane move from the soil to the shoots and the atmosphere.

Perhaps the most important influence plants have on gaseous fluxes into anoxic soils is oxygen transport from stems to the roots and the subsequent release of O_2 into the rhizosphere. Wetland plants are known to release oxygen from their roots, a phenomenon called radial oxygen loss (ROL) (Armstrong, 1964). The oxic-anoxic interface in wetlands is therefore extended by plant roots that leak oxygen via ROL. As a result the soil redox potential locally increases and various molecules are re-oxidized at this interface.

4.2 Iron cycling in wetlands

In wetlands pore water containing dissolved Fe(II), radial oxygen loss promotes the formation of iron precipitates along the roots. Wetland ecologists have long recognized that Fe oxidation occurs in the rhizosphere of many wetland plants based on the presence of orange-brown crusts of iron oxides precipitates that often coat root surfaces (Mendelssohn et al., 1995). Moreover, other activities such as bioturbation or mixing of sediments by waves or storm events (Koretsky et al., 2005) provide conditions for microbial iron oxidation in otherwise anoxic circumstances.

These Fe(II) deposits are commonly referred to as Fe-plaques (Armstrong, 1967). Fe-plaque is composed primarily of Fe(III)-oxides deposited on the root surface by abiotic and biotic Fe(II) oxidation. It may be amorphous or crystalline, in the form of ferric hydroxides, goethite (γ-FeOOH) and lepidocrocite (β-FeOOH) (Liu and Zhu, 2005). Iron oxides are widely observed at the oxic-anoxic boundary (Figure 3) and on the roots of wetland plants and submersed aquatic macrophyte species where it can account for a large reservoir of Fe(III) and influence the mobility of trace metals and phosphorus (Mendelssohn et al., 1995; Weiss et al., 2003) because of its role as a sink for these elements.

Figure 3. Iron oxidation observed at Waarde, a brackish tidal marsh site

Both acidophilic as well as neutrophilic FeOB are associated with the Fe-plaque on the roots of wetland plants (Emerson et al., 1999). Lithotrophic Fe(II) oxidizers have been enriched from the roots of wetland plants growing at circumneutral pH (Emerson et al., 1999; Neubauer et al., 2002; Sobolev and Roden, 2002). Microaerobic, chemolithotrophic FeOB are abundant in the rhizosphere of wetland plants and are likely to account for a large fraction of overall ferrous Fe oxidation in such environments. In an extensive survey, Weiss et al. (2003) observed the presence of FeOB in the root zone of 92% of plants representing 25 species.

Weiss et al.'s (2003) observation of high abundances of FeOB and FeRB on the same 1-cm subsection of a root suggests that oxidation and reduction are occurring simultaneously and both processes are mediated, at least in part, by bacteria. In this cycle, Fe(II) is oxidized by both autocatalytic and biotic mechanisms using O_2 from roots as a terminal electron acceptor. Under anoxic conditions, FeRB use root-derived poorly crystalline Fe(III) and labile carbon for Fe(III) reduction. Relatively high proportions of FeOB, FeRB, poorly-crystalline Fe(III), O_2, and labile C suggest that the rhizosphere is a 'hotspot' of microbially-mediated iron cycling in wetlands.

Chemolithotrophic FeOB factor prominently in a model for continuous microscale Fe-cycling by forming amorphous Fe-oxides and forcing the development of a steep O_2 gradient (Sobolev and Roden, 2002). Rapid rates of Fe cycling in the presence of vegetation both in salt and in freshwater marshes have been related to enhanced availability of reactive Fe(III) minerals and labile organic carbon in the rhizosphere (Kostka and Luther, 1994; Roden and Wetzel, 1996).

There are also non-biological phenomena such as variations in water table depth that influence Fe-cycling. The actual contribution of physiological, ecological and physical processes to Fe-cycling remains to be determined. The presence of Fe-plaques on wetland plants under many different environmental conditions indicates that ferric Fe reduction lags behind the ferrous Fe oxidation in situ. Since the potential for ferric Fe 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 (Weiss et al., 2004).

4.3 The ecological implication of iron oxidation in wetlands

Wetland ecosystems are important as sites of rapid biogeochemical cycling. A mechanistic understanding of carbon and nutrient cycling in wetlands is thus important for global scale climate modeling efforts, as well as for regional scale restoration and protection of wetland systems (Gutknecht et al., 2006). The oxidation Fe(II) produces relatively small amounts of energy and that means a large amount of Fe(II) has to be oxidized in order to produce a relatively large quantity of microbial biomass (Katsoyiannis and Zouboulis, 2006a,b).

The precipitation of iron oxyhydroxides in the rhizosphere leads to a gradient of decreasing concentration of dissolved iron towards the plant roots and accumulation of a few elements (Doyle and Otte, 1997). This gradient of

oxygen and elements will thus affect the growth of plants and other activities associated with plant roots.

Because of their high specific surface area, iron oxides, especially biogenic iron oxides (Fortin and Langley, 2005), act as important sorbents for dissolved compounds, particularly heavy metals, phosphate, and arsenate (Rozan et al., 2002; Rentz et al., 2009). Sorption on and co-precipitation with iron oxides may immobilize the metals and anions (Jacob and Otte, 2003). By doing this, the plaque may simply act as a physical barrier which thus lowers the concentration of heavy metals in the surrounding (Batty et al., 2000). However, the mechanisms behind metal and phosphate sequestration remain incompletely understood.

For a number of years, wetlands, both natural and constructed, have been used to remove potentially toxic metals from contaminated drainage (Hallberg and Johnson, 2005). Iron plaques have been observed on a number of wetland plant species, including those growing in contaminated areas, which shows that iron plaques can act as deposits of heavy metals (Batty et al., 2000). Iron-oxidizing bacteria have also been exploited in artificial wetlands constructed for waste water treatment (Hallberg and Johnson, 2005; Nicomrat et al., 2006; Nicorarat et al., 2008; Faulwetter et al., 2009).

As stated earlier, Fe(II) oxidation and Fe(III) reduction are closely connected at the oxic-anoxic interface in wetlands including the rhizosphere of wetland plants. By the formation of amorphous iron oxides, FeOB appear to have the potential to induce rapid microscale coupling of Fe oxidation and reduction at oxic-anoxic interfaces (Roden et al., 2004). Studies have revealed that both FeOB and FeRB are closely associated in wetland soils and sediments (Weiss et al., 2003). Indirectly this also influences other elemental cycles. For example, it is well known that Fe(III)-reducing bacteria are able to outcompete methanogenesis for electron donors in anoxic sediments (Sobolev and Roden, 2004) and thus suppress CH_4 production in iron-rich freshwater wetlands. Fe(III)-oxide reduction accounts for 65% of total carbon metabolism in rhizosphere sediment incubations compared to 22% for methanogenesis (Roden and Wetzel, 1996).

Therefore, it is crucial to understand the limits of Fe(III) formation in wetland environments. Since iron-oxidizing bacteria take an important role in this process, special attention should be paid to the effect bacteria may have on Fe(III) (hydr)oxide formation as well as to the chemical and microbial controls of bacterial oxidation of Fe(II).

5 FeOB and environmental constraints

Being dependent on ferrous Fe and O_2 for growth, FeOB have to compete with other chemical and microbial processes for these compounds. As described above, Fe(II) will be oxidized spontaneously to Fe(III) in environments of circumneutral pH and elevated levels of oxygen. Hence, at the oxic-anoxic interface around plants roots in waterlogged soils or sediments, FeOB have to compete with chemical processes for available oxygen and Fe(II), the latter abundantly available from the surrounding anoxic environment.

It has been suggested that some iron oxidizers produce extracellular organic matrix which helps prevent the cells from being entrapped in the insoluble Fe(III) oxides and temporarily bind Fe(II) thus making it less available for chemical oxidation (Neubauer et al., 2002). This may be an additional evolutionary strategy developed by these organisms in order to compete with the rapid rates of abiotic Fe(II) oxidation in circumneutral environments. However, this matrix has not been characterized yet.

Moreover, some plant species exert a definite effect on iron oxidation. For example, organic compounds released by plants roots require an oxidizing compound to be oxidized. It has been suggested (Emerson and Revsbech, 1994) that *L. ochracea* can only grow in the presence of ferrous iron when no other major electron donors are present.

With respect to the oxidation of organic compounds, the use of electron acceptors is governed by thermodynamics in such a way that they are used in the order oxygen >nitrate >ferric iron > sulfate >carbon dioxide (Laanbroek, 1990; Rivett et al., 2008) (Table 2). In this way the reduction of ferric Fe can suppress the reduction of carbon dioxide to methane when sufficient Fe is available (Van Der Nat et al., 1998). However, hardly anything is known about preferential oxygen-consuming processes under limiting availability of O_2.

The microbial iron cycle interacts with many other wetland processes (Gutknecht et al., 2006). There are quite a few biotic processes that will compete for oxygen with FeOB such as the oxidation of ammonium, methane and sulfide, which are the products of anaerobic microbial processes.

The calculation of Gibbs free energy of the following oxidation processes based on Thauer et al. (1977) shows an order of ferrous iron > methane > sulfide > ammonium (Table 3). Though theoretically Fe oxidizers could act as successful competitors under limiting amounts of oxygen, the interactions

among these biotic oxidation processes are far more complicated in the environment.

Table 2 Standard Gibbs free energy for the oxidation of molecular hydrogen by various oxidants

Substrates	Products	$-\Delta G^{o'*}$	
		kcal/ H_2	kJ/ H_2
$O_2 + H_2$	$2H_2O$	28.3	118.4
$2NO_3^- + 5H_2 + 2H^+$	$N_2 + 6H_2O$	26.7	112.1
$2Fe(OH)_3 + H_2 + 4H^+$	$2Fe^{2+} + 6H_2O$	12.2	51.2
$SO_4^{2-} + 4H_2 + H^+$	$HS^- + 4H_2O$	4.5	18.8
$CO_2 + 4H_2$	$CH_4 + 2H_2O$	3.9	16.4

*$\Delta G^{o'}$ for the different reaction was calculated from $\Delta Gf^{o'}$ values (Thauer et al., 1977)

Table 3 Standard Gibbs free energies of oxidative processes

Substrates	Products	$-\Delta G^{o'}$	
		kcal/ H_2	kJ/ H_2
$Fe^{2+} + 0.25O_2 + 2.5H_2O$	$Fe(OH)_3 + 2H^+$	26.1	109
$CH_4 + O_2$	$CO_2 + 2H_2O$	24.2	101.3
$HS^- + 2O_2$	$SO_4^{2-} + H^+$	23.8	99.6
$NH_4^+ + 3/2O_2$	$H_2O + NO_2^-$	9.3	38.9

Roy, Knowles et al. (1996) found that methane-oxidizing bacteria are dominant over ammonia-oxidizing bacteria with respect to their competitive abilities under oxygen-limited conditions. In a soil incubation study (van Bodegom et al., 2001), it has been shown that microbial oxygen consumption is dominated by heterotrophic and methanotrophic respiration. Ammonium-oxidizing activities are found to be suppressed by heterotrophs (Verhagen et al., 1992) and by plant uptake both at limiting amounts of ammonium (Bodelier et al., 1996). The complex interaction between iron-oxidizing bacteria and methanotrophs, ammonium oxidizers, sulfide oxidizers and heterotrophs may also be critical for the iron precipitation in the rhizosphere of wetland plants. However, almost nothing is known about a preference for electron donors used in the oxidation reactions when oxygen is only supplied in limiting amounts (Laanbroek, 1990).

The competitive abilities of a population with respect to the use of limiting amounts of substrates is dependent on the specific substrate-scavenging abilities of the individual cells as determined by the apparent affinity for the substrate (K_m) as well as on the value of the substrate-consuming potential (V_{max}), which depends on the size of the population (Healey, 1980). Hence, a limited substrate-scavenging ability of a certain population might be

compensated by the number of cells as was argued by Bodelier and Laanbroek (Bodelier and Laanbroek, 1997) for the competitive abilities of *Nitrosomonas europaea*. In addition to discriminating between different oxidation processes at limiting amounts of O_2, oxygen availability may also differentially affect members of the same functional group of microorganisms as was observed during the enrichment of chemolithotrophic ammonia-oxidizing bacteria from the Scheldt estuary (Bollmann and Laanbroek, 2002).

6 Summary

Iron-oxidizing bacteria under acidic conditions have been widely studied in the past decades, while far fewer studies have been devoted to neutrophilic FeOB, especially those associated with Fe-plaque in the rhizosphere of wetland plants. The reason for this is partly due to the long-held view that ferrous iron oxidizes abiotically under neutral oxic conditions, and partly because of difficulties in isolating and cultivating the responsible bacteria.

Wetlands play an important role on earth as a host for all kinds of organisms, as well as a recovery site for pollutants. The formation of aerenchyma of wetland plants creates an oxic-anoxic interface in the rhizosphere where active physical, chemical and microbial processes occur simultaneously, among which iron oxidation is prominent because it has a strong influence on soil chemistry as well on the metabolism of plants and microorganisms.

Despite the importance of iron oxidation in wetlands, our knowledge of the physiology and biochemistry of the responsible microorganisms is still very limited. In contrast to the oxidation of manganese(II), sulfide and ammonium, for which the quantitative importance of enzymatic catalysis by chemolithotrophic bacteria is well recognized (Sobolev and Roden, 2002), the role of neutrophilic FeOB in the oxidation of iron in the rhizosphere of wetlands is still largely unknown.

As reviewed, more work needs to be done to assess the activity of iron-oxidizing microbes in wetland environments and to establish their interaction with other elemental cycles. To date, few studies have been done demonstrating the spatial and temporal distribution of neutrophilic FeOB, however, and information on environmental constraints (*e.g.* chemical iron oxidation and oxygen availability, temperature, influence of plants or other microorganisms) is hardly available. It is important to scale up from individual cells to complete microbial communities and ecosystems. Besides, most of the

relevant studies have been done only on a few Fe-oxidizing strains retrieved from a rather limited geographical area.

Moreover, many former experiments failed to get satisfactory results because of difficulties in obtaining appropriate cultures. The gradient tube culture method (Emerson 1997) has been applied successfully, but only a small number of cultures have been obtained so far. The efficiency of gradient plates (Emerson and Floyd, 2005) and bioreactor cultures (Neubauer et al., 2002) need to be further developed, and new methods of isolation and culturing FeOB have to be developed.

7 Objectives of this study

In this thesis the focus will be on diversity and distribution of iron-oxidizing bacteria from freshwater and brackish wetlands of circumneutral pH that differ with respect to the prevailing ecohydrological and geochemical conditions. The main goals of the research include:

1) To study the distribution of iron-oxidizing populations present in different wetlands by developing novel culture independent techniques;

2) To reveal the possible environmental factors controlling the FeOB community structure and the interaction between FeOB and other elemental cycles;

3) To explore the spatial distribution of abundance and community structure of FeOB and of potentially steering environmental factors, among which the distribution of other chemolitho-autotrophic bacteria, such as the methane-oxidizing bacteria.

4) To isolate and characterize novel neutrophilic iron-oxidizing bacteria.

Realizing these aims involved the development of specific 16S rRNA primers (Chapter 2), the study on the temporal and spatial changes in their diversity and abundance in relation to environmental parameters (Chapter 3), the exploration and prediction of the distribution of iron-oxidizing bacteria and methane oxidizers at different spatial and elevation scales (Chapter 4) and the enrichment and isolation of novel iron-oxidizing bacteria (Chapter 5).

Soil samples were taken from four different wetland environments. Two of them are situated along the Scheldt estuary comprising one tidal freshwater marsh site (Appels, Belgium) and one tidal brackish marsh site (Waarde, the Netherlands), the third site is an irregular flooded grassland (Huis den Doorn, the Netherlands) and the last locaton is a riparian wetland (Ewijk, the Netherlands).

Chapter 2 describes the results of enrichment cultures with soil from one of the locations and the distribution of FeOB in the environment investigated by the application of molecular tools based on the genetic information from the enrichment cultures. A clone library of enriched cultures was constructed and 16S rRNA probes and primers were designed based on the sequence information. Specific primers were applied on environmental samples using a nested PCR-DGGE method to detect the special group of iron-oxidizing bacteria in the environments.

In Chapter 3, a study is described based on soil samples taken from a tidal freshwater march near Appels, at 5 different locations in April, July and October 2007. Samples were obtained at different depths and newly designed specific 16S rRNA primers were applied to compare the community structure of the iron-oxidizing bacteria in the samples. The results were compared with chemical properties of the soil samples in order to constrain the major controlling factors on distribution of iron-oxidizing bacteria.

Chapter 4 is devoted to the development of a real-time PCR (qPCR) assay that is used in combination with the previously applied nested PCR-DGGE methods to detect the abundance and diversity of iron-oxidizing bacteria in a riparian zone at different sampling scales along an elevation gradient. Geostatistics was used to predict the distribution pattern of FeOB and groups of methane oxidizers, in order to find out the interaction between these two gradient bacteria.

In Chapter 5, the gradient culture system described by Emerson et al. (1999) was applied to mimic the environmental conditions at an oxic-anoxic interface for the enrichment and isolation of FeOB. Bacterial iron oxidation distinguished itself from chemical iron oxidation in that the bands are formed sharply at the oxic-anoxic boundary. Novel FeOB obtained from serial dilution were identified and described.

In Chapter 6, the results of the various analyses are discussed in the broader perspective of the ecology of neutrophilic FeOB in wetlands.

Chapter 2

Diversity of iron oxidizers in wetland soils revealed by novel 16S rRNA primers targeting Gallionella-related bacteria

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ABSTRACT

Neutrophilic iron-oxidizing bacteria (FeOB) are important catalysts of iron cycling in wetland environments. However, little is known about their diversity and distribution in various environments. The aim of this study was to develop a PCR-DGGE assay enabling the detection of neutrophilic iron oxidizers in wetland habitats. Gradient tubes were used to enrich FeOB. From these enrichments, a clone library was established on the basis of the almost complete 16S rRNA gene using the universal bacterial primers 27F and 1492R. This clone library consisted of mainly alpha- and beta-proteobacteria, among which two major clusters were closely related to *Gallionella* spp. Specific probes and primers were developed on the basis of this 16S rRNA gene clone library. The newly designed *Gallionella*-specific 16S rRNA gene primer set 122F/998R was applied to community DNA obtained from three contrasting wetland environments, followed by Denaturing Gradient Gel Electrophoresis (DGGE) analysis. A second 16S rRNA gene clone library was constructed using the PCR products from one of our sampling sites amplified with the newly developed primer set 122F/998R. The cloned 16S rRNA gene sequences all represented novel culturable iron oxidizers most closely related to *Gallionella* spp. On the basis of their nucleotide sequences, four groups could be identified that were comparable to the DGGE banding pattern obtained before with the same PCR products as used for the second clone library. Using these *Gallionella*-specific 16S rRNA gene based primers, in combination with DGGE, first insights into the diversity and distribution of these bacteria in wetland soils were obtained.

INTRODUCTION

Iron is the fourth most abundant element in the Earth's crust and the most prevalent redox active metal (Sudek et al., 2009). It participates in many key biological processes, such as photosynthesis, N_2 fixation, methanogenesis, H_2 production and consumption, respiration, the trichloroacetic acid cycle, oxygen transport, gene regulation and DNA biosynthesis (Andrews et al., 2003). Iron also acts as an electron donor and acceptor for microbial energy generation. It has been the focus of many studies because of its importance in biogeochemical redox reactions in the nvironments (Straub et al., 2001; Emerson and Weiss, 2004; Roden et al., 2004).

Microorganisms can accelerate iron oxidation through a variety of mechanisms, such as changing local pH (Fortin et al., 1998), passive physicochemical sorption (Hallbeck and Pedersen, 1990; McLean et al., 1996; Ferris et al., 1999; Hanert, 2002), and enzymatic conversion of iron to oxidized forms (Ghiorse, 1984; Widdel et al., 1993). Iron-oxidizing bacteria (FeOB) have been detected in a wide variety of environments and have been shown to increase the rate of iron oxidation by up to four orders of magnitude compared with the rate of strictly abiotic oxidation(Søgaard et al., 2001).

Compared with acidophilic iron oxidizers that have been studied extensively (Leduc and Ferroni, 1994; Auernik et al., 2008) much less is known about the eco-physiology of FeOB inhabiting environments of neutral pH. This is largely due to the fact that their competitive ability in relation to abiotic oxidation still remains controversial (Sobolev and Roden, 2002), and to the difficulties associated with obtaining and maintaining them in pure culture (Edwards et al., 2003a). In the last decade, a number of studies (Hallbeck and Pedersen, 1995; Emerson and Moyer, 2002; Neubauer et al., 2002; Sobolev and Roden, 2002) showed that neutrophilic FeOB could successfully compete with abiotic oxidation for Fe(II); in some batch culture experiments, they accounted for 50-60% of the total Fe (II) oxidation.

The typical characteristic of these neutrophilic FeOB is their lithoautotrophic metabolism using iron as the only energy source. They are found growing at oxic-anoxic interfaces (Lutters-Czekalla, 1990) in neutral pH environments and are classified as micro-aerophilic bacteria. Among them, *Gallionella ferruginea* is known as the characteristic iron-oxidizing bacterium (Engel and Hanert, 1967; Hanert, 1968; Hallbeck and Pedersen, 1991). The rhizosphere of wetland plants has been shown to be one of the niches for these organisms because of the fact that plant roots release excessive oxygen into the soil (Armstrong, 1964) creating suboxic conditions required by the iron oxidizers. Research on neutrophilic FeOB has recently moved forward with the isolation of a number of morphologically

distinct species from different environments (Emerson and Moyer, 1997; Hanert, 2002; Edwards et al., 2003b; Edwards et al., 2004; Roden et al., 2004; Weiss et al., 2007). Most of them were classified as beta- or gamma-proteobacteria.

Despite the progress in isolation and the biogeochemical evidence supporting their potential role in Fe-cycling, knowledge concerning their diversity and environmental distribution is still lacking. In this study, FeOB were enriched and identified. A 16S rRNA gene clone library of the enrichments was constructed and specific probes and primers were designed subsequently. The distribution of FeOB in wetland environments was further investigated by the application of specific primers targeting the 16S rRNA gene of *Gallionella*-related bacteria, in combination with PCR-DGGE, cloning and phylogenetic analysis.

MATERIALS AND METHODS

Soil sampling. Three sampling sites were chosen for this study. The first location, Appels (A; Belgium, 51°2'0" N, 4°4'0" E), is situated along the Scheldt estuary and has been characterized extensively (Lin et al., 2007). It is an intertidal freshwater marsh area typically colonized by *Typha latifolia, Bolboschoenus maritimus* and *Phragmites australis*. Waarde (W; The Netherlands, 51°25'2" N, 4°4'8" E) is a salt marsh where iron oxidation is very common and *Spartina anglica* is the dominating plant species. The sediments at Appels and Waarde contain significant amounts of ascorbate extractable Fe at near neutral pH. The pore water Fe (II) concentration reached values as high as 400 mM (Hyacinthe and Van Cappellen, 2004; Lin et al., 2007). The third location, Huis Den Doorn (H; The Netherlands, 52°32'60" N, 6°7'60" E), is an irregularly flooded inland wetland site in the center of the Netherlands, which has a remarkably high concentration of Fe (total Fe 702 mmol g^{-1} dry soil and oxalate-extracted Fe 459 mmol g^{-1} dry soil) (Loeb et al., 2008). *Glyceria maxima* is the dominating plant species.

Approximately 300 g of sieved soil (mesh size 1mm) from each sampling site was put in a glass beaker (350 ml) and flooded with distilled water. One seedling (15 days old, two-leaf stage) of *Glyceria maxima* (for Appels and Huis den Doorn soil) or *Spartina anglica* (for Waarde soil) was transplanted into the glass beakers when the soil was completely saturated with water. Eight replicates for each soil type were prepared. The flood water level was kept constant at 2 cm above the soil surface by replenishing with sterile distilled water. After six weeks' growth at 20°C (16 h/light and 8 h/dark) and 70% humidity, the plants were harvested. Soil from 1 cm below the soil surface (T), rhizosphere (R) and bulk (B) sections were sampled separately. A portion of each sample was used for enrichment of FeOB. Other aliquots were freeze-dried and stored for further molecular analysis.

Enrichment and isolation. Fresh soil samples were mixed at a ratio of 1:1 with Milli-Q water and used as inocula for enrichment of bacteria. Plant roots containing visible iron plaques were rinsed in sterile deionized water to remove adhering soil particles, cut into sections and used directly for inoculation.

Gradient tubes with opposing gradients (Emerson and Floyd, 2005) of oxygen and ferrous iron sulfide were used for the initial enrichment, isolation and subsequent maintenance of the culture. The system consisted of two layers of agarose in 16mm screwcap glass tubes. A bottom layer contained 1.25 ml FeS and modified wolfe mineral medium (MWMM) (Emerson and Floyd, 2005) at a ratio of 1:1, amended with 1% (wt/vol) agarose. The top layer consisted of 6.75 ml mineral medium supplemented with vitamins and minerals (http://www.lgcstandards-atcc.org, MDVS, MD-TMS) and stabilized with 0.15% (wt/vol) agarose. Before autoclaving, the solutions for the upper layer were added together with $NaHCO_3$ to a concentration of 0.5mM $NaHCO_3$ and flushed with CO_2 for 1 min to reach a final pH of 6.5.

The gradient tubes were always inoculated 24 h after preparation to facilitate the diffusion of Fe(II) into the top layer where bacterial growth occurs. The tubes were inoculated by pulling a micropipette vertically through the medium while the inoculum was expelled. The inoculum was serially diluted in sterile Modified Wolfe Mineral Medium. Abiotic controls were prepared without bacteria. Positive cultures were identified by observation of a discrete band of Fe (III) oxide in contrast to the diffuse deposition of oxides in abiotic controls (Emerson and Moyer, 1997). The presence of bacteria was confirmed by both microscopy and molecular analysis.

DNA extraction and PCR. DNA was extracted using a modified DNA isolation procedure (Zhou et al., 1996). The purification of DNA was carried out with DNA Clean and concentrator ^{TM} kit (Zymo Research, Orange, CA, USA). The quantity and quality of the extracted DNA was analyzed by spectrophotometry using NanoDrop ND-1000^{TM} (Nano-Drop Technologies, Wilmington, DE, USA) and by agarose gel electrophoresis. The genomic DNA was stored at-20°C for later use. 16S rRNA genes were amplified by PCR using different primer sets (see Table 1 for details).

Denaturing Gradient Gel Electrophoresis (DGGE). DGGE was performed with the Protean II system as described earlier (Muyzer et al., 1993). An 8% polyacrylamide gel with a vertical gradient of 30-60% denaturants (100% denaturants constitute a mixture of 7M urea and 40% (vol/vol) formamide) was used to analyze the 550 bp PCR products. The running conditions were 100V at a constant temperature of 60°C in 23L of 0.5×TAE buffer (20mM Tris acetate,

0.5mM EDTA, pH 8.0) for 18 h. The DGGE gels were visualized using a UV transilluminator after ethidium bromide staining. DNA fragments were excised from the gels, re-amplified, purified using the Gel DNA Recovery Kit (Zymoclean, Orange, CA, USA) and subjected to sequencing.

Clone library of 16S rRNA genes. Two clone libraries were constructed in this experiment using the TA cloning kit (Invitrogen, Carlsbad,CA, USA, version V). For analyzing the gradient tube enrichments, cloning of nearly complete 16S rRNA gene fragments was performed. For the application of *Gallionella*-specific primers on environmental soil samples, another clone library was constructed with the PCR products obtained using the specific primers. Ninety-six positive clones from each clone library were randomly picked for sequence analysis. The clones were inoculated into liquid LB medium amended with 20mg ml^{-1} ampicillin and incubated on shakers at 37°C overnight. The cells were harvested and plasmid DNA was isolated using the Zyppy plasmid miniprep kit (Zymo Research). The plasmid DNA was sequenced in both directions using M13F/M13R primers (Table 1) by Macrogen (http://www.macrogen.co.kr).

Comparative sequence analysis. Sequences of both excised DGGE bands and clones were manually checked and modified with Sequencher software (Sequencher 4.1.4) and then compared with existing sequences using the online database of BLAST (http://www.ncbi.nlm.nih.gov/). Sequences were aligned and Phylogenetic trees were reconstructed in ARB (Ludwig et al., 2004; Pruesse et al., 2007) using the neighbor joining algorithm (http://www.arb-home.de/). Probes for sequences that are closely related phylogenetically to defined iron-oxidizing species were designed using the probe design tool from the ARB package. The newly designed probes were aligned against sequences from the online sequence database (http://rdp.cme.msu.edu/probematch/search.jsp), and seven with less than 2 mismatches were chosen as specific probes.

On the basis of these probes, three primer sets were designed, which were 122F/998R, 122F/997R and 645F/996R. Primers 122F and 998R, specific to *Gallionella*-related FeOB, were selected for further analysis. The PCR conditions were optimized by running gradient PCRs at different annealing temperatures (50.3, 51.9, 53, 54.3, 55 and 58°C) and 55°C was found to yield products with the best quality and was used for this newly designed primer set. To test the specificity of the primers, plasmid DNA of clones of almost complete 16S rRNA gene sequences from non-related species (that is, MWE_C17, MWE_C40, MWE_N10, MWE_N19, MWE_C10 in Figure 4) were used as templates.

RESULTS

Enrichment of FeOB. Enrichment experiments started in winter time when no plant growth occurs in the field. Hence, plants were grown in growth chambers to enable differentiation between surface layers, rhizosphere and bulk soils. After six weeks of growth under controlled conditions, the soils in the beakers were fully rooted. Deposits of oxidized iron could be seen both around the roots and close to the soil surface (Figure 1a). Three days after inoculation of the soil samples into the gradient tubes, all the inoculated tubes showed growth. In each tube, one brownish iron oxide band mixed with bacterial biomass was formed approximately 1 cm below the surface. It was defined as growth because the shape and position of iron oxides were distinct from those in non-inoculated controls (as shown in Figure 1b). In the tubes inoculated with bulk or rhizosphere soil, similar iron oxide band patterns had been formed, whereas in tubes inoculated with fresh roots, the oxide band developed close to the headspace. Microscopic test and DNA isolation further confirmed bacterial growth associated with the iron oxide bands.

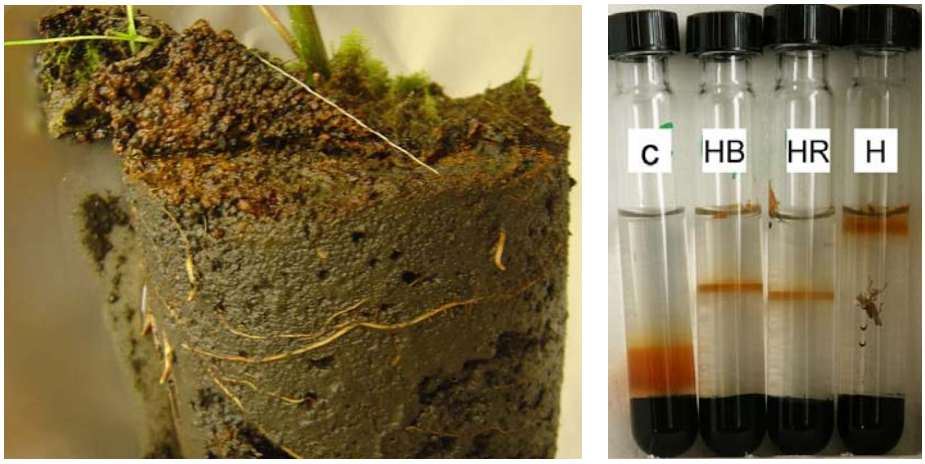

Figure 1. (a) Iron oxidation at the soil surface and around the roots of wetland plants. (b) Gradient tubes showing bacterial (HB, HR, H) and chemical iron oxidation (C). HB: Huis den Doorn bulk soil, HR: Huis den Doorn rhizosphere soil, H: Huis den Doorn root inoculum.

It was expected that pure cultures would be obtained after a few transfers, as described by Emerson and Moyer (1997). However, in our case, a mixture of several species remained together till the later stage of isolation. Among them, one was closely related to *G. ferruginea* on the basis of the 16S rRNA gene information (96% sequence identity). With the increase in transfer times, the brown-colored iron bands developed much slower. It took approximately 24 h to see a bright orange iron band at the beginning of the enrichment, whereas after a few transfers,

it took several days to form a thick white band. A brown color was observed only a week later. Typically, these bands were thinner and tended to move deeper toward the bottom of the tubes compared with the early gradient cultures.

DGGE analysis of enrichment cultures. The cultures obtained in the gradient tubes during the first transfers were analyzed using PCR-DGGE on the basis of general bacterial primers. On average, 15 different DGGE bands were detected in the enrichments (Figure 2a), which were excised and sequenced. The sequences were quite diverse, including different species of alpha- and beta-Proteobacteria, and Bacteroidetes as well as Firmicutes (Figure 3). After another four transfers, the total number of DGGE bands diminished in each of the samples (for example, Figure 2b).

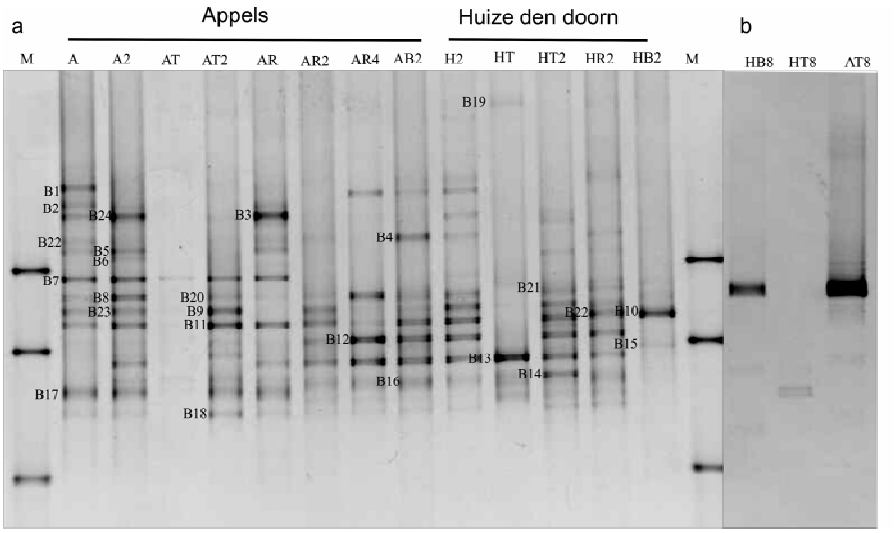

Figure 2. Denaturing gradient gel electrophoresis (DGGE) patterns showing diverse bacterial communities after the first (a) and later (b) transfers in gradient tube enrichments. A and H refer to the sampling sites Appels and Huis Den Doorn, respectively; B, Tand M indicate bulk soil, surface soil and markers, respectively; numbers refer to numbers of transferring times. B1–B24 are cut and sequenced bands, and the sequence information can be found in Figure 3.

Clone library. The enrichments from the gradient tubes (5-6 transfers of AT, HB and HT) were pooled and used for DNA isolation and cloning. In total, 96 clones were screened. Figure 4 shows the Phylogenetic affiliation of the different clone types (coded MWE) found in the enrichments. In the clone library, over 20% of the clones were closely related to *G. ferruginea* (Hallbeck et al., 1993), which is atypical neutrophilic iron oxidizer (Hallberg and Ferris, 2004). They clustered either with *Siderooxydans lithotrophicus* LD-1 and *Siderooxydans lithoauto-trophicus* ES-1, which are two recently identified iron oxidizers (Weiss et al.,

2007), or with uncultured *Gallionellaceae* (Winderl et al., 2008). Apart from the iron oxidizer group, in the clone library, we also found sequences that are related to other genera such as *Azospira*, *Rhodocylclaceae*, *Janthinobacterium*, *Thiobacillus* (Figure 4).

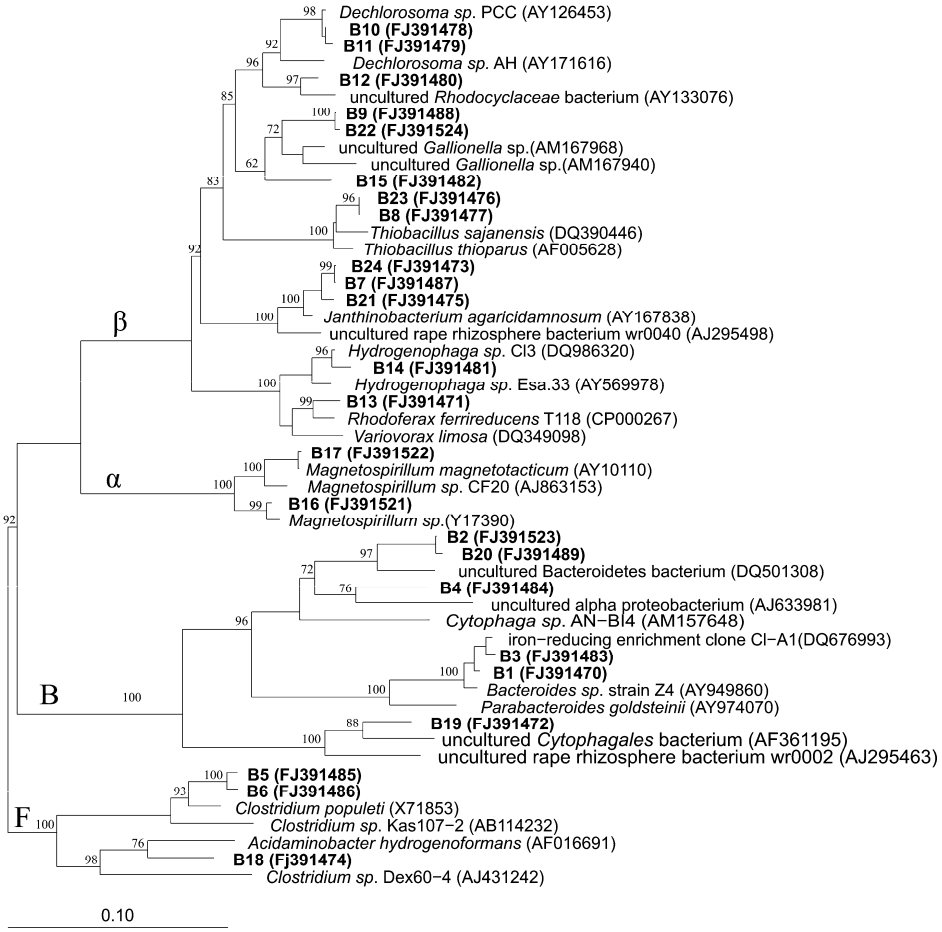

Figure 3. Neighbor-joining tree of sequences obtained from gradient tube enrichments shown in Figure 2a. Sequences determined in this study are printed in bold type. Bootstrap values are shown near the nodes (based on 1000 replicate trees). The bar indicates 10% sequence difference. *Thermotoga maritima* was used as an outgroup, but was pruned from the tree. α= Alphaproteobacteria, β = Betaproteobacteria, γ = Bacteroidetes, F = Firmicutes.

Design of specific primers and probes. Sequences from three clusters of interest (see arrows in Figure 4), including six representative clones on the bases of their assumed iron-oxidizing capacity, were chosen for specific probe or primer development. MWE_C19, MWE_N34, MWE_N10 and MWE_C13 fall into the

Gallionella group. MWE_C19 and MWE_N34 share 95% similarity to *G. ferruginea* and 98%, similar to *Gallionella capsiferriformans* (Weiss et al., 2007), whereas MWE_N10 and MWE_C13 are closely related to *Siderooxydans* species.

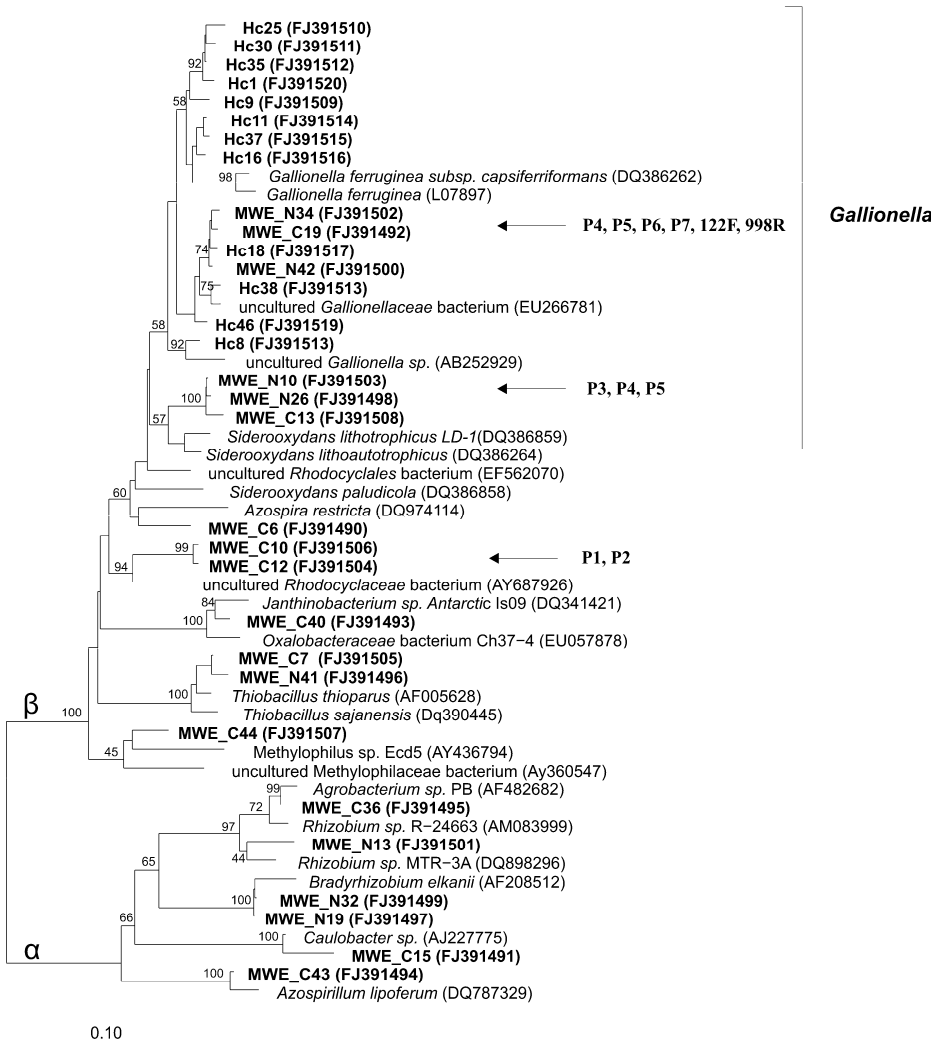

Figure 4. Phylogenetic tree of 16S rRNA-based clones derived from gradient tube enrichments using the 27F/1492R primer set (coded MWE), as well as from iron-rich Huis den Doorn soil samples using primer 122F/998R developed in this study (coded Hc). The sequences in bold were selected clones. Bootstrap values are shown near the knots (based on 1000 replicate trees). The bar indicates 10% sequence difference. *Thermotoga maritima* was used as an outgroup, but was pruned from the tree. α= Alphaproteobacteria, β = Betaproteobacteria, γ = Bacteroidetes, F = Firmicutes.

MWE_C10, MWE_C12 are related to some uncultured beta-Proteobacterium found in iron-rich areas (Juretschko et al., 2002). In total, seven probes (that is, P1-P7) were developed and their specificity was checked using a probe-matching program (Table 2). Finally, three primer sets were designed on the basis of the probes, that is, 122F/998R, 122F/997R and 645F/997R. Primer set 122F/998R is specific to two almost identical sequences that share 98% similarity to a recently described iron-oxidizing bacterium *Gallionella capsiferriformans* on the basis of the 16S rRNA gene. Clones MWE_N10 and MWE_C13, clustering with *Siderooxydans* spp., share the same forward primer 122F with clones MWE_C19 and MWE_N34, but have their own specific reverse primer 997R. The third primer set 645F/997R was designed for clones MWE_C10 and MWE_C12, which have uncultured *Rhodocyclaceae* spp. as their closest relative.

Table1 Primers employed in this study

Primer	Sequence (5´ to 3 ´)	Application	Reference
341F-GC	CCTACGGGAGGCAGCAG	DGGE analysis	Muyzer et al., 1993
907R	CCGTCAATTCMTTTGAGTTT		Muyzer et al., 1998
27F	GTGCTGCAGAGAGTTTGATCCTGGCTCAG	Clone library of gradient tube enrichments	Lane, 1991
1492R	CACGGATCCTACGGGTACCTTGTTACGACTT		Lane, 1991
M13F	GTAAAACGACGGCCAG	Sequencing DNA from clones	Huey and Hall, 1989
M13R	CAGGAAACAGCTATGAC		Huey and Hall, 1989
122F	ATATCGGAACATGTCCGG	Detecting *Gallionella*-like organisms in soil samples	This study
998R	CTCTGGAAACTTCCTGAC		This study

Table 2 Specific 16S rRNA-targeted oligonucleotide probes designed according to the clone library from the mixed enrichment cultures of the gradient tubes.

Probe Name	Probe Sequence (5' – 3')	Position	Clone specificity
P1_996	CAG CAG GAT TCC AGG CAT	996-1013	MWE_C10, MWE_C12
P2_645	GCC ACA CTC GAG TCT TGC	645-662	MWE_C10, MWE_C12
P3_997	TCT TCT GGA TTC TCG GCA	997-1014	MWE_N10, MWE_C13
P4_460	CAC TCC ACG TAT TAG GTG	460 - 477	MWE_N10, MWE_C13
P5_122	CCG GAC ATG TTC CGA TAT	122 - 140	MWE_C19, MWE_N34
			MWE_N10, MWE_C13
P6_645	GCT ACA CTC TAG CCT TCC	645-662	MWE_C19, MWE_N34
P7_998	CTC TGG AAA CTT CCT GAC	998-1015	MWE_C19, MWE_N34

Detection of *Gallionella*-like bacteria in soil samples. Confirmed to be specific for one novel cluster of culturable iron oxidizers, the primer set 122F/998R designed for *Gallionella*-like organisms was then used to detect these organisms in environmental samples. After a nested-PCR using the universal Eubacterial primer set 341F-GC/907R, the PCR products were checked on DGGE gel. The result showed that these organisms were present in most of the soil samples tested (Figure 5). The bands migrated to or around the position of the marker band that belongs to the clone on which the primers were designed (Marker c in Figure 5). Differences in band patterns can be seen among the samples. As to the samples from Huis den Doorn, no bands were detected from the bulk soil (HB) and rhizosphere (HR), whereas the most intensive band was obtained in the surface soil (HT). Appels soil sample (AB, AR and AT) appeared to be more diverse with respect to the presence of *Gallionella*-like bacteria than the other two soils.

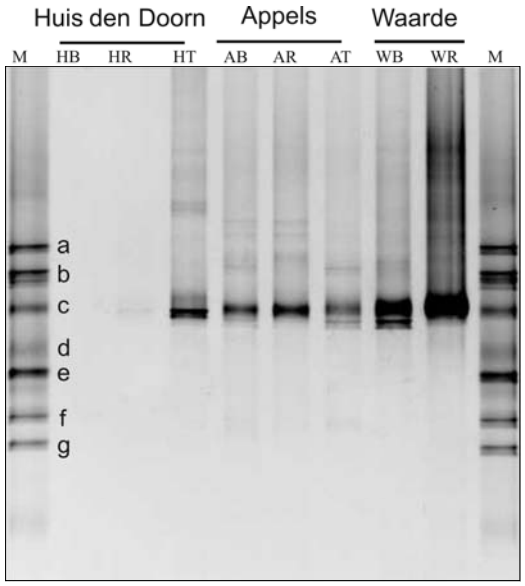

Figure 5. Denaturing gradient gel electrophoresis (DGGE) patterns showing DNA of Gallionella-like iron oxidizers from different wetland environments being amplified by using a nested approach (*Gallionella*-specific primers and universal DGGE primers 341GC/907R, respectively). A and H refer to the sampling sites Appels and Huis Den Doorn, respectively; B, T and M indicate bulk soil, surface soil and markers, respectively. Marker sequences are clones from the library of the gradient tube enrichments; from a to g: MWE_C40, MWE_N41, MWE_N34, MWE_N13, MWE_N19, MWE_C13 and MWE_N26 (see also Figure 4). Marker c indicates the clone from which the *Gallionella*-specific primers are developed.

To validate the PCR-DGGE method, a clone library was constructed using the PCR products of the *Gallionella*-specific primers (122F and 998R). Soil DNA was

taken from one of the sampling sites, Huis den Doorn. Within 96 sequenced clones retrieved from these soil samples, four clusters could be identified for their differences in nucleotide sequences. Twelve sequences were chosen as representatives to be included in the phylogenetic tree on the basis of the clones from the gradient tube enrichments (Figure 4). Compared with the other clones, Hc8 is less closely related to *G. ferruginea* and other clones. Clones Hc37, Hc11 and Hc16 are identical to some uncultured *Gallionella* spp., whereas clones Hc9, Hc25, Hc30 and Hc1 share more similar sequences. Clones Hc18, Hc46 and Hc38 shared 97% similarity with *G. ferruginea*. By comparing the obtained sequences, the most discriminative variable region could be identified, which corresponds to *Escherichia coli* 16S rRNA range 455-479. These 12 representative sequences were checked again on DGGE (Figure 6). According to their migrating positions in the gradient gel, different groups could be distinguished. This was compared with the result obtained with the nested PCR-DGGE method (Lane HT in Figure 5). As expected, more bands were obtained after cloning, but most of them ended up at the same positions.

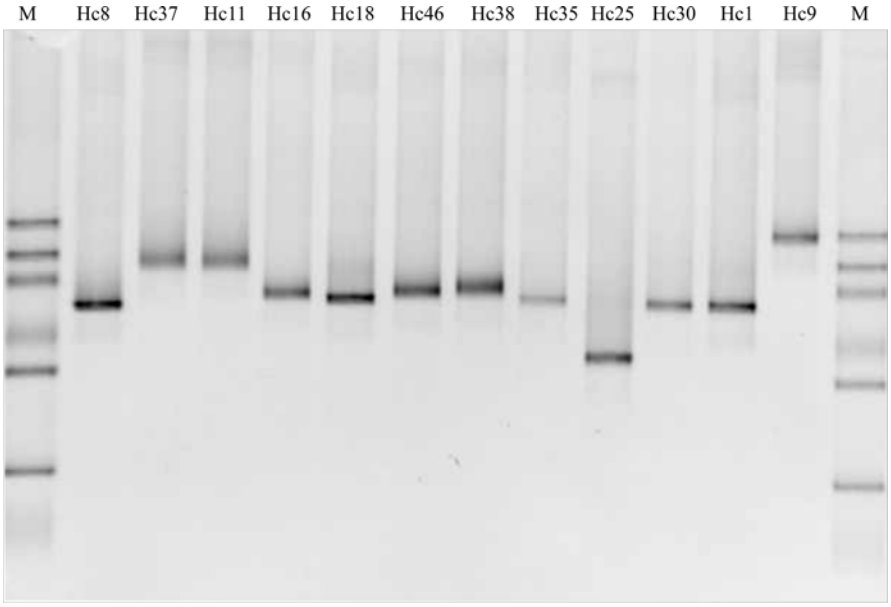

Figure 6. Denaturing gradient gel electrophoresis (DGGE) patterns sho/?wing cloned sequences amplified with the *Gallionella*-specific primers 122F and 998R. Corresponding clones can be found in Figure 4. Hc refer to the codes of the clones, and M is the marker (same as Figure 5).

DISCUSSION

Microbial iron oxidation under neutral conditions has been reported earlier (Hallbeck and Pedersen, 1990; Straub et al., 2001; Sobolev and Roden, 2002), but progress in this research area is hampered by the difficulty of obtaining the responsible organisms in pure culture. The development of the gradient tube technique (Emerson and Floyd, 2005) made it possible to obtain these bacteria in pure cultures. In this study, it was observed that when fresh roots were used as inoculation, they formed iron oxide bands close to the headspace where the oxygen concentration is relatively high. This is probably due to the depletion of oxygen by root respiration, which results in the upward movement of microaerophilic bacteria.

The success of isolation depends in large part on experience. In our study, the repeated dilution and inoculation of gradient tubes reduced the bacterial diversity significantly (as can be seen comparing Figures 2a and b). With the increase of the time between transfers, the iron oxide bands in the gradient tubes developed much slower and were thinner, which might be the consequence of a decrease in the number of many non-dominant FeOB, as well as of those microorganisms that might benefit the growth of the remaining iron-oxidizing species. A pure iron-oxidizing isolate was obtained in this study, which shared the same 16S rRNA sequences as the clone used for designing the *Gallionella*-specific primers.

Despite its advantages of accuracy and efficiency, molecular tools are hardly applied in the study of FeOB in environments of approximately neutral pH, mainly because of the lack of sequence information to design specific primers or probes for conservative regions of the 16S RNA genes (Konstantinidis and Tiedje, 2007) as well as for functional genes (Parro et al., 2007). We developed 16S rRNA gene-based probes and primers, which can be used directly on environmental samples to study the occurrence of these microbes and their temporal and spatial distribution. In combination with laboratory cultivation, this opens up the ecological studies on this environmentally relevant group of bacteria.

By using a nested PCR-DGGE approach, we were able to detect *Gallionella*-like organisms in different compartments of freshwater as well as brackish wetland environments. As shown in Figure 5, the bands ended up at slightly different positions in DGGE gels, showing that the diversity of *Gallionella* related iron oxidizers is higher than recently known. The slightly higher diversity revealed by cloning compared with a nested PCR-DGGE approach showed that cloning has decreased the detection limit as compared with DGGE. In fact, in this study, the species detected by cloning, but missed by DGGE, that is, Hc8, Hc9 and Hc25, count for only 5% of the total clones obtained. Hc8 is the only one out of the 96

clones that are distantly related to the other sequences. The low density of this species might increase the difficulty in detecting it by DGGE.

It was reported that in wetland environments iron oxidation mainly occurs close to the plant roots where sufficient oxygen is available owing to its release (Neubauer et al., 2007). We have indeed detected *Gallionella*-related organisms not only in the rhizosphere but also in the bulk soil. Detection based on the 16S rRNA gene indicates the presence but not the activity of microbial cells. Hence, cells detected in the rhizosphere may still be more active than those found in the bulk soil.

Iron oxidizers are phylogenetically so diverse that they fall in evolutionarily distinct groups (Roden et al., 2004). Beside the facts that not many culturable FeOB are available, the known sequences are so diverse that it is almost not possible to design group-specific probes or primers for them. With our specific primers, four different groups were selectively picked up from environmental wetland samples. The comparison of sequences showed that they vary in a specific region. Therefore, primers can be developed to detect *Gallionella*- related sequences separately from each other using real time PCR.

To our knowledge, this is the first study using specific primers for detecting neutrophilic FeOB in environmental samples. Although bacterial iron oxidation has been claimed to be important especially in wetland environments (Neubauer et al., 2007), studies on this subject are still limited. The lack of efficient molecular tools to study these microbes seriously hampers progress in this field. This fact will make the detection of their distributions in the environments a challenge. Isolation of pure cultures and sequencing of their genomes will help to develop and define primers on the basis of functional genes, which will also facilitate mRNA studies on these microbes.

ACKNOWLEDGEMENTS

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Chapter 3

Distribution and diversity of Gallionella-like neutrophilic iron oxidizers in a tidal freshwater marsh

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ABSTRACT

Microbial iron oxidation is an integral part of the iron redox cycle in wetlands, where it is intimately coupled to the cycling of carbon and nutrient elements. Nonetheless, relatively little is known about the composition and ecology of iron oxidizing communities in soils and sediments of wetlands. In this study, sediment cores were collected across a freshwater tidal marsh to characterize the iron oxidizing bacteria (FeOB) and link their distributions to geochemical properties of the sediments. We applied recently designed 16S rRNA primers targeting *Gallionella*-related FeOB using a nested PCR-DGGE approach, combined with a novel quantitative PCR (qPCR) assay. The presence of *Gallionella*-related FeOB was detected in most of the samples. The diversity and abundance of the FeOB were generally higher in the upper 5-12 cm of sediment compared to deeper sediment, and higher in samples collected in April compared to July and October. Oxygen supply by plant roots and macrofauna appears to be a major forcing controlling the spatial and temporal variations in FeOB communities. The higher abundance of *Gallionella*-related FeOB in April coincided with elevated concentrations of extractable Fe(III) in the sediments. However, overall, the distributions of FeOB did not exhibit a simple relationship to the redox zonation inferred from the geochemical depth profiles.

INTRODUCTION

Wetlands are among the most productive ecosystems on Earth providing crucial ecosystem services (Costanza et al., 1997). Located at the transition between the terrestrial and aquatic realms, wetlands function as major nutrient-transforming ecosystems (Mitsch and Gosselink, 2000). Many of the key biogeochemical transformations affecting the mobility and bioavailability of nutrients in wetland environments take place within soils and sediments (Laverman et al., 2007). A characteristic of wetland soils and sediments is the close juxtaposition of oxic and anoxic conditions that enable intense cycling of carbon, nutrients and metals (Roden and Wetzel, 1996). The elemental redox cycles are driven by O_2 entering the anoxic zone, not only at the sediment surface, but also at greater depths due to O_2 input via aerenchymatous roots of wetland plants and macrofaunal burrows (Armstrong, 1964; Doyle and Otte, 1997; Koretsky et al., 2005). Redox conditions in wetland soils and sediments are highly dynamic, because of variations in primary productivity, tidal forcing, temperature, sediment deposition and groundwater inputs, among others. These environmental characteristics promote the establishment of metabolically versatile microbial communities that are able to rapidly respond to changes in their chemical environment (Koretsky et al., 2005; Thullner et al., 2005).

As the most abundant transition metal at the Earth's surface, iron (Fe) plays a particularly important role in environmental biogeochemistry (Weber et al., 2006; Borch et al., 2010). Oxidized Fe(III) as well as reduced Fe(II) minerals are effective sorbents for trace metals and organic contaminants in soils and sediments (Ferris et al., 2000; Fortin and Langley, 2005). In addition, the surfaces of Fe(II) and Fe(III) minerals catalyze numerous important redox transformations, while aqueous Fe(II) is a powerful environmental reductant (Liger et al., 1999; Jeon et al., 2003; Kappler and Straub, 2005). In many wetlands, the availability of phosphate as a nutrient is closely coupled to the redox cycling of iron (Frossard E., 1995; Hyacinthe and Van Cappellen, 2004).

Microorganisms can both oxidize and reduce iron. Microbial iron reduction has received abundant attention from both microbiologists and biogeochemists (Lovley et al., 1991; Bonneville et al., 2009). Most work on microbial iron oxidation has focused on acid environments where competing abiotic oxidation of Fe(II) tends to be negligible (Baker and Banfield, 2003; Tan et al., 2009). Mounting evidence, however, indicates that specialized bacteria are able to oxidize iron under circum-neutral pH conditions at oxic to anoxic boundaries,

where low O_2 levels slow down the chemical oxidation of Fe(II) (Emerson and Moyer, 1997; Sobolev and Roden, 2001). *Gallionella ferruginea* was among the first iron-oxidizing bacteria isolated from this type of environment (Vatter and Wolfe, 1956; Hallbeck and Pedersen, 1990). More recently, Fe-oxidizing bacteria (FeOB) have been detected in various wetland environments (Emerson and Moyer, 1997; Weiss et al., 2004; Weiss et al., 2007). A number of isolates have been obtained directly from the rhizosphere of wetland plants (Sobolev and Roden, 2004; Weiss et al., 2007).

It is becoming increasingly evident that FeOB are ubiquitous in wetland soils and sediments where they play a major role in the oxidative part of the iron cycle. Nonetheless, our knowledge concerning the distribution and environmental role of neutrophilic iron oxidizers remains rather poor, in part due to the lack of efficient molecular tools to detect FeOB. In a previous study we designed and applied specific primers targeting the 16S rRNA gene of *Gallionella*-like iron-oxidizing bacteria and revealed a much higher diversity in wetlands soils than previously known (Wang et al., 2009). The aim of the present study was to delineate the environmental factors, including the presence of plants as well as pore water and solid phase geochemistry, that influence the distribution and diversity of FeOB in a tidal freshwater marsh.

MATERIALS AND METHODS

Site description and sampling. Sediments were sampled in a tidal freshwater marsh located in the vicinity of the village of Appels, Belgium (51°2'0" N, 4°4′0" E), 127 km upstream of the mouth of the Scheldt estuary. The upper marsh is flooded only during exceptionally high tides and is vegetated by willow trees (*Salix alba*), while the lower mudflat is vegetated by bulrush (*Scirpus lacustris*) and common reed (*Phragmites australis*), and is flooded twice a day. In between upper and lower marsh vegetation consists mainly of cattail (*Typha latifolia*). Sediment cores were collected in April (A), July (J) and October (O) 2007 from 3-5 locations within the marsh. The sampling locations were characterized by the absence of vegetation (No), or by the presence of *S. lacustris* (Sc), *P. australis* (Ph), *T. latifolia* (Ty) or *S. alba* (Sa), respectively. Previous work has shown intense redox cycling of iron in sediments collected in the marsh (Hyacinthe et al., 2006; Lin et al., 2007).

In April all five locations were sampled. Cores for iron extractions were sectioned in the field, those for pore water measurement were processed in the laboratory under argon atmosphere. In July and October, three locations were

sampled, including a non-vegetated (No), as well as the *Scirpus* (Sc), and *Phragmites* (Ph) sites. The cores were processed in a glove box within two days after sampling. Each core was cut in intervals of 1 cm from 0 to 10 cm and in steps of 2 cm until the bottom of the core. Samples for iron extraction were stored under an argon atmosphere and samples for molecular analysis were freeze-dried.

Pore water and sediment analyses. Pore water was obtained by centrifugation. The supernatant was filtered (0.2 µm nylon filter) and the pH was measured. An aliquot of pore water was used to measure alkalinity spectrophotometrically with bromophenol blue (Sarazin et al., 1999). The remaining pore water was acidified with concentrated HCl (10 µl ml^{-1}) for subsequent chemical analyses by inductively coupled plasma optical emission spectroscopy (ICP-OES) and ion chromatography.

A modified method of Lovley and Phillips (Lovley and Phillips, 1987) was used to determine Fe(II) and Fe(III) in amorphous or poorly crystalline iron phases. Aliquots of 0.1-0.2 g wet sediment were added in a glove box to 0.5 mol L^{-1} HCl to extract Fe(II) and to 0.5 mol L^{-1} HCl plus 0.25 mol L^{-1} hydroxyl ammonium chloride to extract Fe(II) and Fe(III). Suspensions were shaken for an hour and the extract was subsequently separated from the sediment by centrifugation. Iron concentrations were determined spectrophotometrically with Ferrozine at 562 nm using standards of $Fe(NH_4)_2(SO_4)_2 \bullet 6H_2O$ in 0.5 mol L^{-1} HCl. Extractable Fe(III) was calculated as the difference of the HCl (*i.e.* Fe(II)) and HCl plus hydroxyl ammonium (*i.e.* Fe(II) plus Fe(III)) extracts. Extractions were done in triplicate or duplicate.

Water content (weight loss at 105^{o}C) and organic matter content (weight loss between 105^{o}C and 550^{o}C) were determined by thermogravimetric analysis (TGA). Elemental concentrations of the solid fraction were measured by X-ray fluorescence (XRF). Extractable Fe(II) and Fe(III) were expressed as percentages of the total XRF iron concentrations.

DNA extraction and PCR-DGGE. DNA was extracted with a modification of Zhou's DNA isolation procedure (Zhou et al., 1996). Purification of DNA was done using the DNA Clean & Concentrator™ kit (Zymo Research). The quantity and quality of the extracted DNA was analyzed by spectrophotometry using a NanoDrop ND-1000 TM (Nano-Drop Technologies, Wilmington, DE, USA) and by agarose gel electrophoresis. The genomic DNA was stored at -20°C for future use.

16S rRNA genes were amplified by PCR using the newly designed *Gallionella*-specific primer set 122F/998R (Wang et al., 2009). One µl of a 50 ng μl^{-1} soil DNA template was used for a 50 µl PCR reaction volume for each sample, followed by a nested PCR using the primer set 357F-GC/907R specific for bacteria in general, and the PCR products were separated by DGGE analyses (Muyzer et al., 1993). Representative bands were excised and sequenced.

Quantitative PCR. qPCR primers targeting the 16s rRNA gene of iron-oxidizing bacteria were designed based on known specific primers and probes developed for *Gallionella*-related bacteria. The primer set includes a degenerate forward primer 628F (GBMAGGCTAGAGTGTAGC) and a reverse primer 998R, which has previously been used in conventional PCR (Wang et al., 2009). Primers were then compared via a BLAST search, and against the RDP II and ARB databases using the Probe Match function (Ludwig et al., 2004; Cole et al., 2005) to ascertain primer specificity. The primer pairs were also analyzed for dimer formation using Primer Premier (http://www.premierbiosoft.com).

A conventional gradient PCR (55-67°C) was performed to test the specificity and to optimize the annealing temperature. The PCR conditions were: 1 cycle at 95°C for 4 min and 40 cycles of 94°C for 45s, 55-67°C for 20s, 72°C for 45s, and 1 cycle at 72°C for 5 min. On the basis of the results, a temperature of 56°C was chosen as annealing temperature.

The real-time detection was performed in a 25 µl reaction volume containing 2.5 µl DNA and 22.5 µl SYBR® Green PCR Master Mix (Invitrogen); 5 pmol μl^{-1} primers and 10 ng μl^{-1} purified environmental DNA were used. PCR was run in 45 cycles, with 1 cycle consisting of denaturation at 95°C for 20 s, annealing at 56°C for 20 s and extension at 72°C for 45s. Data acquisition was done at 82°C for 10 s, to avoid signals from primer dimer formation. Samples were only regarded as being above the detection limit when a PCR product of the correct size was obtained on agarose gel after completing a qPCR run. Each sample was run in duplicate.

Clone MWE_N34, which was used to design the primers, served as a positive control to generate standard curves. The dilution series were made to construct a standard regression line by plotting the cycle threshold (Ct) values versus the logarithm of the starting DNA concentration. Negative controls consisted of clones selected from a clone library described previously (Wang et al., 2009) (MWE_N10, MWE_C10, MWE_C7, MWE_C36, MWE_N19,

MWE_C15, MWE_N10) that are distantly related to the target sequences. Plasmid DNA of the clones was amplified using M13 primers (Huey and Hall, 1989), purified and diluted to serial concentrations in duplicates. The DNA copy number of iron-oxidizing bacteria in each sample was estimated by comparing the Ct value of each sample to the Ct values of the standard regression line. qPCR amplification products were analyzed by electrophoresis in an agarose gel in 0.5×TBE buffer to check for the specificity of the amplification. Sequences were aligned and phylogenetic trees were reconstructed with ARB (Ludwig et al., 2004; Pruesse et al., 2007), using the neighbor joining algorithm (http://www.arb-home.de/).

Data analysis. DGGE gels were analyzed using the Phoretix gel analysis software (Phoretix International, Newcastle upon Tyne, UK). The number of bands of each lane was defined and a matrix of band intensity was created. Lanes were created manually, with a fixed width of 5% of the standard lane width. Each lane represents one sample. Background noise was subtracted by using the Rolling Ball algorithm with a radius of 50 pixels. Bands were detected automatically with a minimum slope of 100 and a noise reduction of 4. The bands were then assessed and corrected visually, matched to the reference lane (Markers), and quantified. The relative abundance of each band was defined as the intensity ratio of each band to the total intensity of individual lanes of each sample.

A similarity matrix (Bray-Curtis coefficient) was created to assess the similarity in patterns among sites and sampling times. The data were ordinated by a Non-metric Multidimensional Scaling (NMDS, 10 restarts) and Cluster analyses with the PRIMER software (version 5.2.6, PRIMER-E Ltd, Plymouth, UK). With the one-way ANOSIM method (analysis of similarities, 999 permutations) the differences among the samples were evaluated. Note that the stability index R describes the extent of similarity between each pair in the ANOSIM, with values close to unity indicating that the two groups are entirely separate and a zero value indicating that there is no difference between the groups.

To correlate environmental variables to community composition of iron-oxidizing bacteria, the BVSTEP (Clarke and Warwick, 1998) procedure was used (Bray-Curtis similarity coefficient, Spearman rank correlation method, Rho>0.95, delta Rho<0.001). A similarity matrix was first generated for both biological and environmental data, upon which a pair wise rank correlation was executed. Moreover, a correlation matrix of the biological and environmental

dataset was created (Statistica 9, StatSoft, Inc., Tulsa, USA) to check for possible correlations between different variables.

RESULTS

Sediment and pore water characteristics. The sediments of the tidal marsh were organic-rich, with organic matter concentrations up to 10 (w/w). Sediment porosity generally decreased with depth (Figure 1). The porosity gradients, however, were irregular with local minima and maxima, reflecting variations in sediment texture. The local porosity minima correlated with higher solid-phase silicon concentrations (Figure 1), indicating the presence of coarser, sandy sediment layers. Pore water pH was around 7.5 and did not vary systematically with depth, location or sampling time. In April, when roots were not yet well developed, orange-brown coatings were visually observed around burrow tubes down to a depth of 12 cm below the sediment surface.

Pore water profiles indicated ongoing Fe(III) and SO_4^{2-} reduction in all the sediments (Figure 1). Build-up of dissolved Fe(II) was typically already detected in the topmost sediment layers. Pore water sulfate gradients implied sulfate reduction in the upper 5 to 20 cm of the sediments. High pore water alkalinities (5.7-15.7 meq L^{-1}) were consistent with a dominance of anaerobic respiration processes. The presence and type of vegetation had a marked influence on the pore water profiles. The steepest pore water gradients were observed at the non-vegetated location. At the location with *Scirpus* vegetation, the NH_4^+ concentrations were 15-20 times lower than at the non-vegetated location. The July and October sulfate profiles exhibited a subsurface maximum at the two vegetated locations. Such a subsurface maximum was not detected at the non-vegetated location.

Solid-phase iron was mainly present as Fe(II) (Figure 2). Extractable Fe(III) was only detected in April. Note that measurable Fe(III) concentrations were observed down to the bottom of the cores collected in April. The highest extractable Fe(III) concentrations were found at the non-vegetated location. Comparison of the concentrations of extracted iron and total iron as measured by XRF implied different iron reactivities at the three locations. At the *Phragmites* location, 80-100% of total iron was extractable below 11 cm depth. At the non-vegetated and *Scirpus* locations only around 30% total iron was extractable (data not shown).

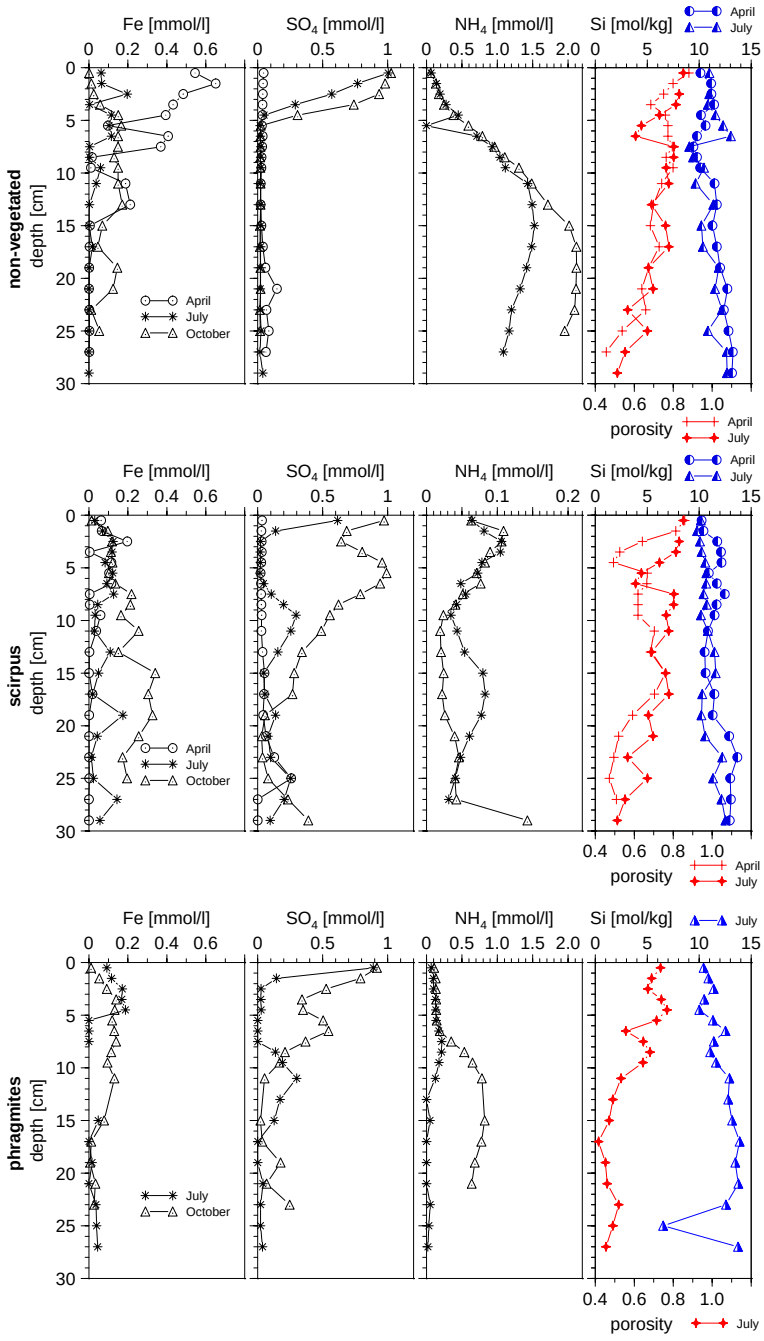


Figure 1. Examples of vertical profiles of pore water dissolved iron, sulfate and ammonium, porosity and solid-phase silicon collected in April, July and October. The upper, middle and lower panels correspond to the non-vegetated, *Scirpus lacustris* and *Phragmites australis* sites, respectively.

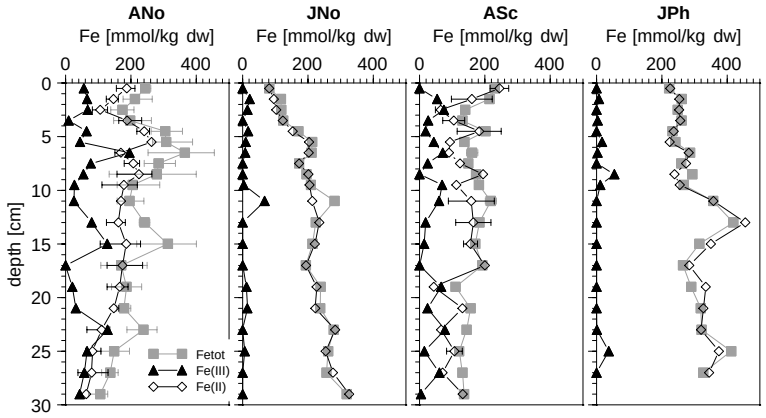


Figure 2. Depth distributions of total, ferric and ferrous extractable iron at the different locations sampled in April and July (ANo: April, non-vegetated; JNo: July, non-vegetated; ASc: April; *Scirpus lacustris*; JPh: July; *Phragmites australis*). Fe_{tot} is the total concentration of extractable iron: Fe_{tot} = Fe(II) + Fe(III). Error bars correspond to the standard deviations of measured Fe_{tot} and Fe(II) concentrations in replicate extractions. For the sake of clarity, error bars for the calculated Fe(III) concentrations are not shown.

FeOB abundance and community composition: qPCR measurements. In all sediment samples analyzed qPCR revealed 16S rRNA gene copies of *Gallionella*-related iron-oxidizing species. Averages of the duplicate measurements obtained in April are shown in Figure 3. In about half the samples from April the total numbers of 16S rRNA gene copies from iron-oxidizing bacteria were above the detection limit of 30 copies per gram sediment. The copy numbers ranged from 3.2×10^{1} to 7.87×10^{5} per gram sediment. The highest copy number was found in the surface layer (0-1 cm) of sediment from the *Phragmites* site in April, though it was only one of the two samples that gave products for this site. Most gene copies were detected in samples from the non-vegetated and the *Scirpus* locations in April with a general tendency toward decreasing copy numbers with increasing depth. For the other sampling times, the 16S rRNA gene copies of FeOB fell below the detection limit (data not shown).

FeOB abundance and community composition: PCR-DGGE. DGGE analyses implied differences in FeOB community composition between the different sites, as well as with depth (Figure 4). This was especially pronounced in the sediment samples collected in April (Figure 4a, b). Generally speaking, band patterns for the *Phragmites* and *Typha* sites were similar in the April samples and exhibited the highest numbers and relative abundances of bands. The non-vegetated and *Scirpus* sites showed also similar patterns. At each site

the FeOB communities varied with depth. For example, at the non-vegetated, *Scirpus* and *Phragmites* sites, band 1 disappeared in the two deepest sediment samples, while a different band (band 2B) became present. This latter band was dominant at the *Phragmites* and *Typha* sites (Figure 4a, b). Only a couple of bands were retrieved from the core collected at the *Salix* location, which is seldom exposed to flooding (data not shown).

Fewer bands were detected in July as compared to April (Figure 4a-c). The relative intensity of the bands also varied between sampling times. For example, band 3 was the most dominant band in July at the non-vegetated and the *Scirpus* sites, while in April band 1 was dominant at these sites. In October (Figure 4d), even fewer bands were detected compared with the other two sampling times. Three weak bands were detected at different depths in the non-vegetated zone, while there was only one band expressed in the top layers of sediment at the *Scirpus* and *Phragmites* sites.

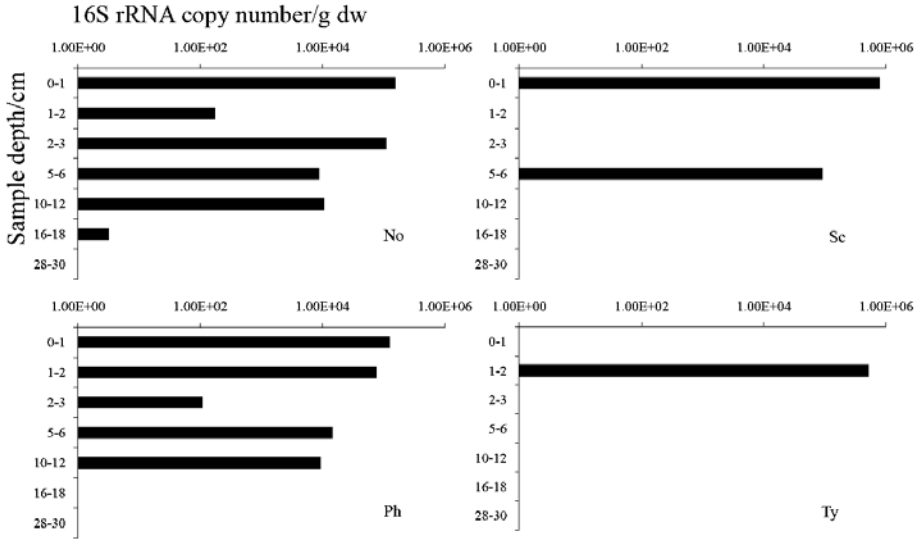
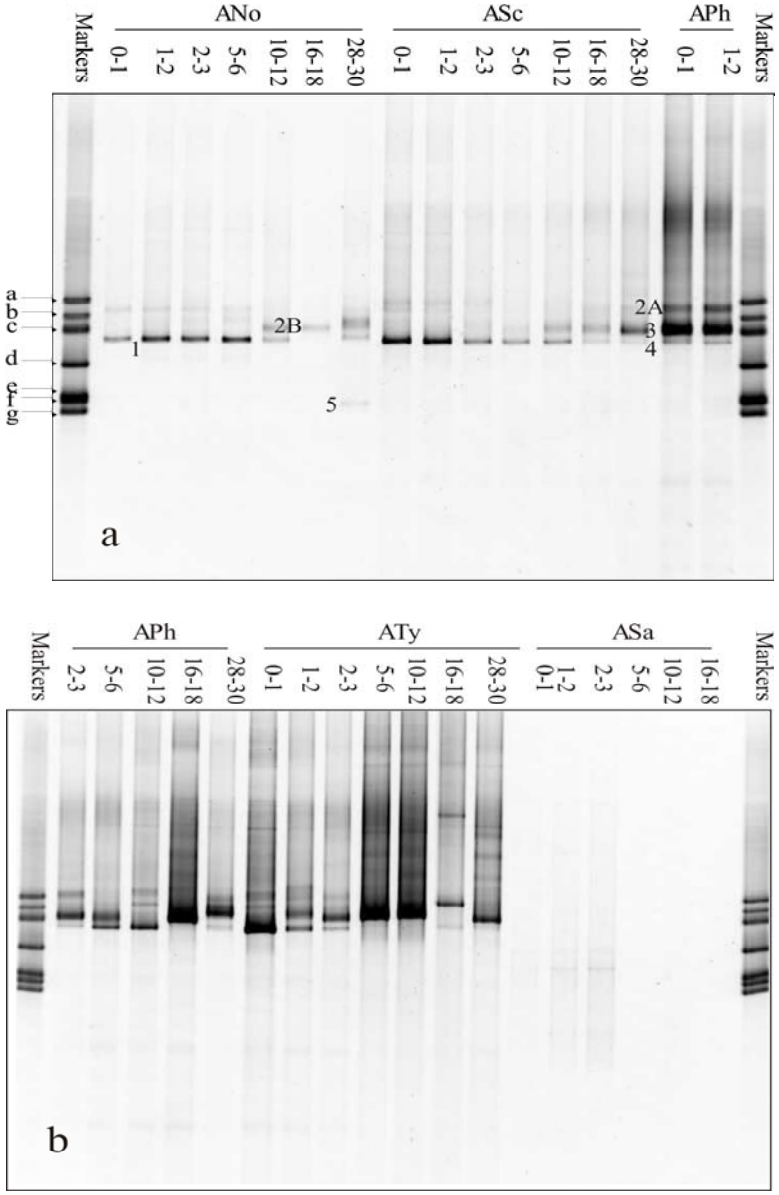


Figure 3. Abundance of 16S rRNA copy numbers from *Gallionella*-related FeOB in samples from different locations of the tidal freshwater marsh collected in April as assessed using a newly developed qPCR assay. ANo: non-vegetated; Sc: *Scirpus lacustris*; Ph: *Phragmites australis;* Ty*: Typha latifolia.*

In total, four out of the six bands were successfully sequenced. Among these, bands 2B and 3 were closely related to *Gallionella ferruginea* (Figure 5), while bands 1 and 4 were related to sequences of uncultured bacteria, possibly representing unknown iron oxidizers.

Similarities between community compositions of FeOB are analyzed in the NMDS plot (Figure 6). ANOSIM analyses revealed no significant differences

among sites and depths, when combining the results from all sampling times and depths, or from all sites, respectively. However, significant differences were observed between the sampling times. Iron-oxidizing bacterial communities were significantly dissimilar between April and July (R=0.604, p=0.001), and between July and October (R=0.577, p=0.001). Differences were not significant when comparing data from April and October.



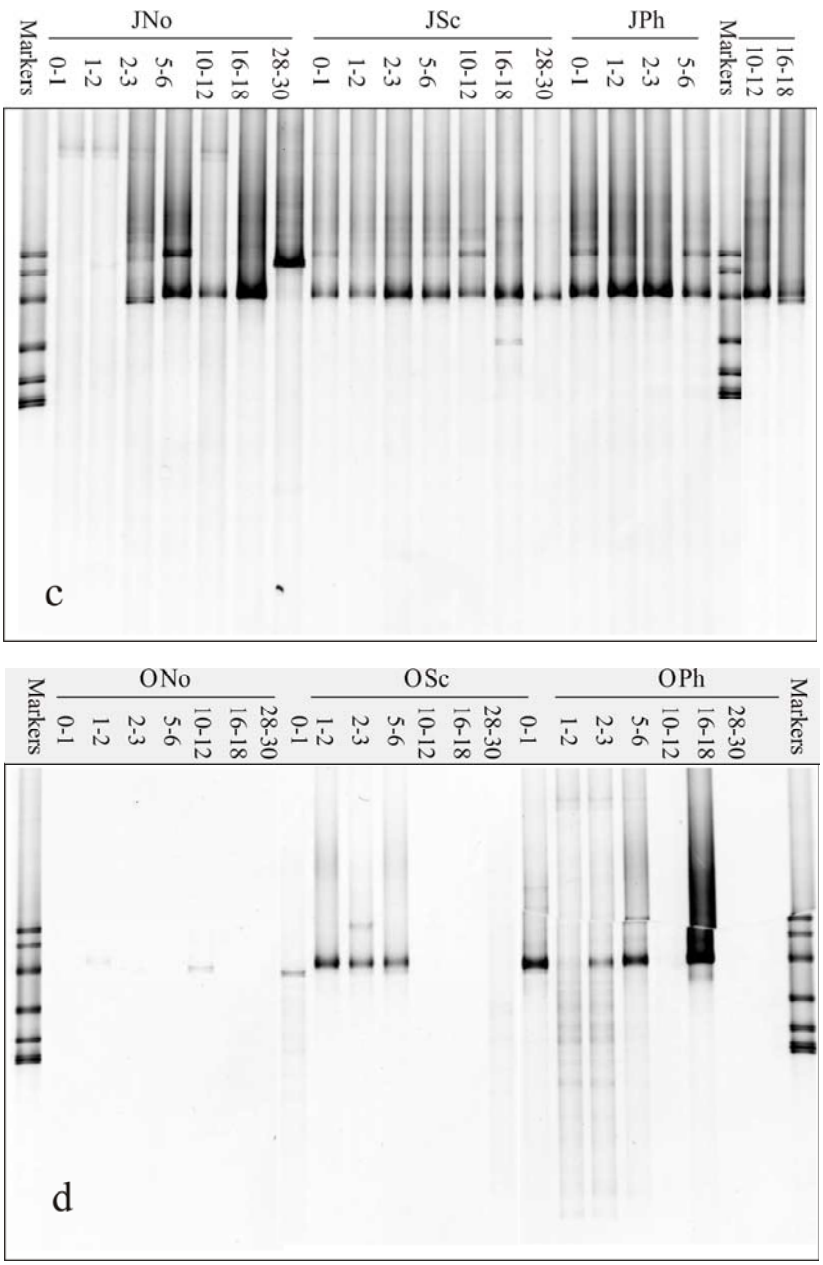


Figure 4. DGGE patterns showing 16S rRNA genes of *Gallionella*-like iron oxidizers from different sampling locations within the tidal marsh. The genes were amplified using a nested PCR approach. Panels a and b correspond to samples collected in April, panels c and d to samples collected in July and October, respectively. Sample identifiers include three parts: Time, Site, and Depth. For example, ANo0-1 means that the sample was taken in April at the non-vegetated site from 0-1 cm depth.

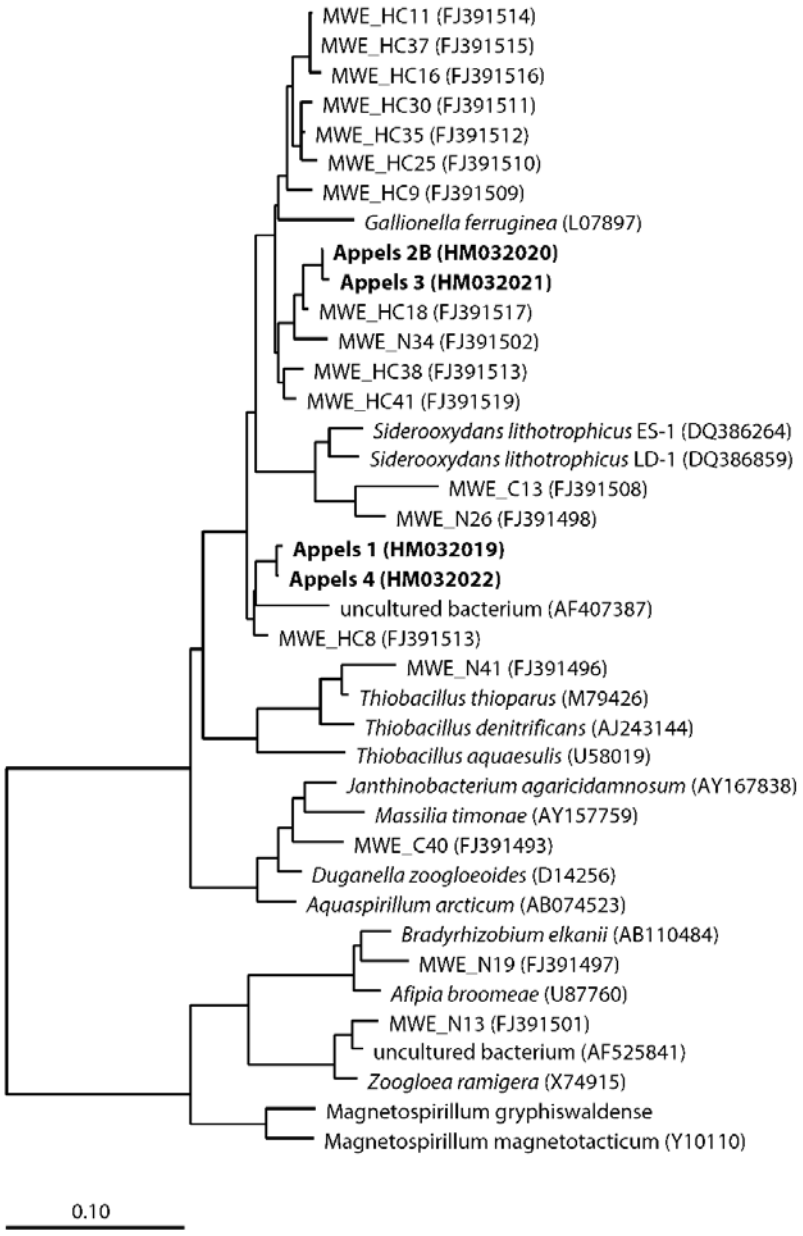


Figure 5. Phylogenetic tree of *Gallionella*-like partial 16S rRNA gene sequences of DGGE bands (bold) as well as of cloned sequences (MWE codes) obtained from enrichments in gradient tubes (Wang et al., 2009). Bootstrap values based on 1000 replicate trees are shown near the nodes. The bar indicates 10% sequence difference.

Correlation of 16S rRNA data with environmental variables. The BVSTEP analysis revealed that, among all the environmental variables tested, the

concentration of extractable Fe(III) was the most influential factor (ρ=0.314). Additionally, the non-pair-wised correlation results showed that the relative abundance of band 1 was significantly correlated to solid-phase extractable Fe(III) (r^2=0.5054). Interestingly, the total copy number of 16S rRNA genes was also positively correlated to the relative abundance of band 1 (r^2 = 0.41), indicating that band 1 was the numerically most abundant FeOB.

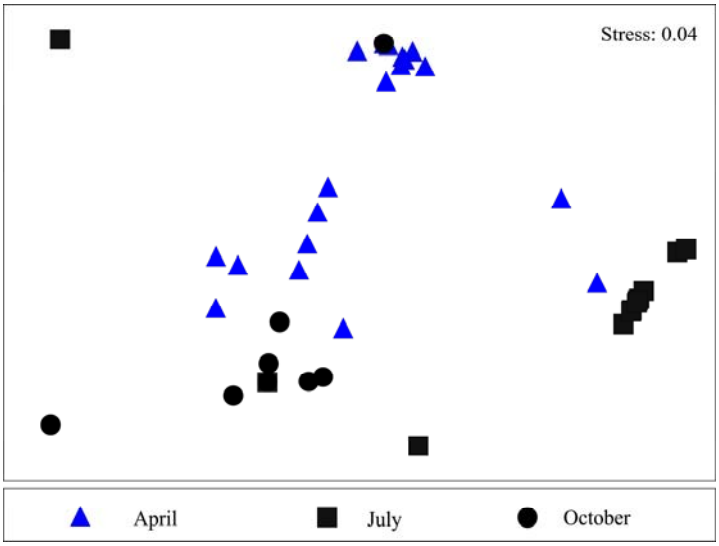


Figure 6. Ordination by non-metric multidimensional scaling of log(*x*+1) transformed relative species abundance of the samples taken from non-vegetated, *Scirpus lacustris* and *Phragmites australis* sites in April (triangles), July (squares) and October (circles).

DISCUSSION

Our results are consistent with previous studies, suggesting that neutrophilic FeOB are widely distributed in wetland soils and sediment. The DGGE analyses demonstrate the presence of several *Gallionella*-like species in the freshwater marsh sediments (Emerson and Revsbech, 1994; Hanert, 2002; Weiss et al., 2003; James and Ferris, 2004). The ANOSIM analysis further implies significant variations in abundance and composition of the *Gallionella*-related community from one sampling time to another. This is not entirely unexpected as the freshwater estuarine environment from where the sediment cores were collected exhibits large seasonal changes in freshwater discharge, tidal forcing, temperature, biological productivity and supply of allochtonous organic matter (Wollast, 1988; Baeyens et al., 1998).

The highest FeOB diversity as determined by PCR-DGGE, and FeOB abundance as determined by qPCR were observed in the cores collected in April. The *Gallionella*-related community is dominated by one species corresponding to band 1 in the DGGE pattern. The relative intensity of this band roughly correlates with the extractable Fe(III) concentrations ($y=8.5823+48.0966x$; $r^2=0.5054$), and with the visual presence of Fe(III)-enriched coatings along burrows. Although the number of sampling times in the present study is limited, it would appear that iron-oxidizing bacteria flourish during spring, leading to localized accumulations of Fe(III) mineral phases in the sediments. The highest number of 16S rRNA gene copies measured is around $8x10^5$ cells per gram of soil, that is, an FeOB density comparable to direct cell counts of 10^5-10^6 per gram soil reported for wetland soils and bacterial mats (Emerson and Moyer, 1997).

Enhanced iron-oxidizing activity in anoxic salt marsh sediments during spring has been reported by Sundby (Sundby et al., 2003), who ascribed it to enhanced oxygen supply by growing roots. Oxygen availability in the rhizosphere is known to vary both temporally (e.g., diurnal and seasonal fluctuations) and spatially along root systems (Weiss et al., 2003). Root activity clearly influences the observed geochemical pore water profiles in the marsh sediments (Figure 1). It should be noted, however, that no aboveground parts of *Scirpus* plants were observed during the sampling in April, in contrast to the other two sampling times. Hence, an oxygen-releasing rhizosphere does not appear to explain the observed higher abundance of *Gallionella*-like species at the *Scirpus* site in April. In addition, similar trends in the depth distribution of 16s rRNA gene copy numbers in the non-vegetated and *Scirpus* sites also point to a limited role of root activity by *Scirpus* plants in April. The high abundance of FeOB at the *Scirpus* sampling location could be stimulated by the growth of macro-benthos during spring months, which enhances macrofaunal introduction of oxygen into the otherwise anoxic zones of the sediment (Beukema, 1974).

An abundant macrofaunal community, mainly oligochaete worms, has been reported for the non-vegetated mudflat sediments at the Appels site (Seys et al., 1999). Active flushing of macrofaunal burrows introduces oxygenated water well below the depth to which molecular diffusion can resupply oxygen from the overlying water (Koretsky et al., 2005). The presence down to depths of about 10 cm of active ammonia-oxidizing bacteria in non-vegetated intertidal sediments from the same Appels site has been linked to pore water irrigation by

worms (Coci et al., 2005). By analogy, we hypothesize that faunal activity exerts a major forcing on the FeOB populations in sediments from the non-vegetated sampling location and, most likely, the location vegetated by *Scirpus*. An additional factor that may help explain the high abundance of FeOB in April is temperature. Heinzel (Heinzel et al., 2009) reported high microbial iron oxidation rates at relatively low temperatures. Thus, enhanced oxygen supply through the aerenchyma systems of plants or via irrigation of macrofaunal burrows combined with lower temperatures may create more favorable conditions for FeOB during spring compared to the summer situation.

The highest numbers of *Gallionella*-related bacteria are found in the upper 5-12 cm of the sediments, that is, the zone where root and macrofaunal activities most strongly impact local redox conditions. At the non-vegetated and the *Scirpus* locations, a noticeable change in the composition of the community of *Gallionella*-related bacteria is also observed between the upper and lower sections of the sediment cores (Figure 4). Although the results point to more diverse and abundant FeOB populations in the upper portions of the sediments, they nevertheless imply that FeOB are present at depths where the geochemical profiles indicate globally anoxic conditions (Figure 1). Koretsky et al. (2005) similarly reported the persistence of viable aerobic bacteria at depths well within the sulfidic zone of salt marsh sediments.

Overall, the distributions of FeOB within the Appels marsh sediments exhibit high spatial and temporal heterogeneity. Yu and colleagues also found large changes in the phylogenetic diversity of iron-oxidizing bacteria across short vertical distances in a contaminated aquifer site (Yu et al., 2010). When integrated over all sampling times and depths, however, no statistically significant relationship emerges between vegetation type and the distribution of the *Gallionella*-related communities, with the exception of the near-absence of detectable FeOB in sediments from the *Salix*-vegetated marsh. Similarly, the community composition of chemolithotrophic ammonia-oxidizing beta-proteobacteria in the same tidal freshwater marsh does not appear to be related to the presence or type of plants, but rather by the elevation within the marsh (Laanbroek and Speksnijder, 2008). We speculate that differences in the flooding regime between the upper and lower portions of the marsh could be a primary forcing of iron redox cycling in the sediments and, consequently, the presence and structure of FeOB communities.

In conclusion, *Gallionella*-related FeOB inhabit vegetated and non-vegetated sediments of the tidal marsh at Appels in the upper freshwater part of

the Scheldt estuary. Cell densities range from below detection to up to 10^6 per gram sediment. Together with previous studies our results thus support a widespread distribution of neutrophilic FeOB in wetland soils and sediments. Although several FeOB species are present in the sediments of the Appels marsh, one dominant species appears to be closely associated with the abundance of reactive Fe(III) phases. The highest diversity and abundance of the FeOB are found in the upper 5-12 cm of the sediments retrieved in April, probably due to enhanced root and macrofaunal activity during the spring season. However, the composition and abundance of the *Gallionella*-related FeOB do not exhibit otherwise straightforward relationships with the geochemical conditions in the sediments. The lack of simple correlations between geochemistry and microbial communities is probably common in marsh sediments (Koretsky et al., 2005).

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Chapter 4

Spatial aspects of iron-oxidizing bacterial communities and their interactions in an irregularly flooded, riparian wetland

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ABSTRACT

Information on the ecology of microbes oxidizing ferrous iron at circum-neutral pH is very scarce. The objective of this study was to assess the spatial distribution of iron-oxidizing bacterial (FeOB) communities across an elevation gradient in an irregularly flooded riparian wetland. In addition, the co-occurrence of methane-oxidizing bacteria (MOB) was investigated, as they represent a biological component that may potentially compete with FeOB under conditions of oxygen limitation. For this purpose, soil samples were collected at different spatial scales within a flooding gradient in the riparian zone of the river Waal in the Netherlands. The diversity of FeOB was assessed using PCR-DGGE and the abundance of both FeOB and MOB by qPCR. Geostatistical methods were applied to evaluate the spatial distribution of FeOB. Results showed higher abundances of FeOB and MOB in samples taken at the large (10m×10m) and medium scales (1m×1m) compared to the small scale (20cm×20cm), probably due to the underlying flooding gradient. The abundance of iron oxidizers also increased with elevation. The copy numbers of 16S rRNA genes belonging to FeOB ranged from 1×10^3 to 3.9×10^8, whereas numbers of MOB, assessed by *pmoA* gene copy numbers, ranged from 5.7×10^5 to 4.0×10^8 per gram dry soil. The abundance of FeOB correlated positively to that of MOB. Cell numbers of FeOB, as well as type Ia, Ib and II of the MOB, were negatively correlated to soil moisture content.

INTRODUCTION

Wetland ecosystems are sites of intense biogeochemical cycling due to the interactions between the oxic soil surface an deeper anoxic soils (Weiss et al., 2003). In wetlands, long-lasting water-logging results in reduced soil conditions. Wetland plants are known to release oxygen from their roots, a phenomenon called radial oxygen loss (ROL) (Armstrong, 1964). The oxic-anoxic interface in wetlands is therefore extended by plant roots that leak oxygen via ROL. Oxygen is also introduced into anoxic soils by other physical or biological processes (Doyle and Otte, 1997; Coci et al., 2005). As a result, various chemical compounds can be oxidized and reduced along the sharp redox gradients characterizing wetland soils and sediments.

Iron oxides are widely distributed in wetland environments. These iron oxides will be reduced rapidly under anoxic conditions by anaerobic iron(III)-reducing bacteria, that couple the oxidation of organic carbon compounds to the reduction of iron(III) (Lovley, 1997). Plant roots can serve as sources of labile organic carbon, and therefore can catalyze both iron reduction and subsequent iron oxide formation (Weiss et al., 2004). As one of the most important elements in biogeochemical redox reactions (Fortin and Langley, 2005), iron interacts with other elemental cycles, which in turn affect the growth and activities of plants and microorganisms.

In addition to chemical oxidation, microbial activity accounts for a large part of the production of iron oxides under circum-neutral pH conditions. Studies have been carried out to detect iron-oxidizing bacteria (FeOB) associated with iron oxides in samples from circumneutral environments (Emerson and Moyer, 1997; Weiss et al., 2003; Chan et al., 2009). Actual bacterial mediation of iron oxidation was also confirmed, further pointing to the important role of FeOB in the formation of iron oxides (Neubauer et al., 2002). To date a number of FeOB have been isolated and our understanding of the role of these microorganisms in iron oxidation has significantly progressed (Emerson, 2010).

Nevertheless, knowledge about the environmental factors affecting the diversity and distribution of FeOB is still rudimentary. Sundby and coworkers have proposed that seasonal changes of O_2 concentration and pore water Fe^{2+} availability resulting from the growth and death of roots exert a major control on the precipitation of iron oxides (Sundby et al., 2003). Neubauer et al. (2007) reported significant temporal changes in the iron-oxidizing community in

microcosm studies with plants, which suggests that plant biomass and activity play a key role in rhizospheric Fe(II) oxidation. Changes in the sediment FeOB community composition have been observed to depend on sampling time, flooding intensity and elevation in a tidal freshwater wetland (Wang et al., submitted).

Being dependent on O_2 for growth, FeOB have to compete with chemical as well as other microbial processes for oxygen. There are quite a few biotic processes that consume oxygen, such as the oxidation of ammonium, methane and sulfide, which are all products of anaerobic microbial processes. Thermodynamically, Fe(II) oxidation yields more energy than other chemolitrotrophic reactions under oxygen-limiting conditions (Thauer et al., 1977). However, no information is available on the interactions between iron-oxidizing bacteria and other oxygen-consuming organisms, such as methane oxidizers. The outcome of these interactions will depend on the oxygen-scavenging abilities of the different organisms, but also by their spatial distribution, and hence, by the environmental heterogeneity.

The soil is a highly heterogeneous environment and soil components and properties may exhibit high spatial variations (Franklin and Mills, 2003; Baker et al., 2009; Ferreira et al., 2010). In particular, the microbial community structure may depend on the spatial scale of observation, in the range of centimeters to a few hundred meters (Ettema and Wardle, 2002). Unlike other organisms, the spatial patterns of FeOB have not been studied yet (Philippot et al., 2009).

The aim of this paper is to assess the distribution of the FeOB community in an irregularly flooded riparian wetland, representing a gradient in flooding intensity and, hence, in redox conditions. Special attention is paid to the influence of sampling scale and elevation within the flooding gradient on the distribution of FeOB. It is hypothesized that the community structure of iron-oxidizing bacteria changes with sampling scale and elevation, and that it is affected the presence of methane-oxidizing bacteria. By using previously designed nested PCR-DGGE and qPCR assays, the absolute and relative abundances of iron-oxidizing bacteria are measured and linked to environmental variables.

MATERIALS AND METHODS

Site and sampling. Soil samples were collected from the Ewijkse Waard (51°88´ N, 5°73´ E), a riparian wetland, in November 2006. The soil properties

have been described previously in detail (Kemnitz et al., 2004; Steenbergh et al., 2010). To assess spatial dependence and heterogeneity a nested design was used as described by Franklin and Mills (Franklin and Mills, 2003). In total 73 soil cores (1.8cm×5cm) were collected in a 10m×10m plot (Fig. 1a). The large-scale spatial level of sampling comprised 24 cores arranged evenly distributed along the sides and diagonals of the plot as depicted in Figure 1a. Nested around the intersection of the diagonals of the large plot, again 24 samples were taken using the same distribution but now on a 1m×1m grid (medium scale; Fig.1b). Nested around the diagonal of the medium scale plot again 25 samples were obtained in a 20cm×20cm grid (small scale; Fig. 1c). The 10m×10m plot was situated on a slope of the riparian area resulting in an elevation difference of 1.1m between the lowest and highest point of the plot. The samples taken at the 3 spatial scales were classified according to their elevation in the following elevation level classes, low (0-0.33m), intermediate (0.34-0.66m) and high (0.67-1.1m). Soil water content (dry weight), soil pH, soil density, total organic matter, water filled pore space were analyzed using standard methods.

DNA extraction. DNA was extracted using a modification of the method described by Yeates and Gillings (Yeates and Gillings, 1998), based on the FastDNA spin kit for soil (MP Biomedicals, LLC, Solon, OH, USA), as described by Yao et al. (Yao Pan, 2010). Soil (0.3 g) and 780 µl lysis buffer [200 mM $NaPO_4$ pH 7.0; 1% (w/v) CTAB; 1.5 M NaCl; 2% (w/v) Polyvinylpyrrolidone K30; 5 mg ml^{-1} lysozyme (added directly before use)] was added into a multimix FastPrep tube and incubated at 37°C for 30 min. MT buffer (122 µl), provided with the kit, was added and tubes were shaken in the FastPrep instrument (MP, Biomedicals, LLC, Solon, OH, USA) for 30 s at 5.5 m s^{-1}. Subsequently, samples were centrifuged for 15 min at 10000 rpm and 700 µl supernatant was collected. The pellet was re-extracted by adding lysis buffer (500 µl) and 50 µl MT buffer to the FastPrep tubes, shaken in the FastPrep instrument for 30 seconds at 5.5 m s^{-1} again followed by the transfer of the second 700 µl of supernatant into separate Eppendorf tubes. At this step, 2×700 µl supernatant was obtained from each sample. 5 µl of 10 mg ml^{-1} freshly made proteinase K was added to each tube. Tubes were incubated at 65°C for 30 min. Samples were extracted with phenol-chloroform-isoamyl alcohol (25:24:1), followed by a chloroform-isoamyl alcohol (24:1) extraction. 125 µl of 7.5 M potassium acetate was added, samples were incubated on ice for 5 min and then centrifuged at 10000 rpm for 10 min. Supernatants (2×700

µl per soil sample) were transferred to new tubes, 700 µl Binding Matrix was added and tubes were mixed for 5 min on a rotator. Binding Matrix, with bound DNA, was pelleted by 1 min centrifugation at 10000 rpm. The supernatant was discarded and pellet was resuspended in 500 µl wash buffer. The resulting suspension was added into a Spin filter, and centrifuged for 1 min at 10000 rpm. The eluate was discarded and the pellet was washed again in 500 µl wash buffer. After discarding the second eluate, the Spinfilter was centrifuged for another 10 s to dry the pellet. The filter was taken into a new tube and 50 µl of TE pH 8.0 was added. The filter was incubated at room temperature for 1 min and centrifuged for 1 min. The filter was re-eluted in the same way with 50 µl of TE pH 8.0. The eluate collected in the catch tube contained the purified DNA.

PCR-DGGE. A nested PCR-DGGE was performed using a previously developed assay (Wang et al., 2009). Briefly, 1µl of 50ng μl^{-1} purified environmental DNA was used for the first step PCR with primer set 122F/998R. The PCR was run in a 50 µl reaction volume containing 5 µM of each primer, 2.5mM of dNTPs, 1.5mM $MgCl_2$, 2 mM BSA, and 1.25 units Taq DNA polymerase. The PCR conditions include 1 cycle of 5 min at 94°C, followed by 30 cycles of 1 min at 94°C, 1 min at 55°C, and 1.5 min at 72°C, the final extension cycle was 10 min at 72°C. The PCR products were diluted 10 times and 2 µl of the dilution was used for a second-step PCR using universal primers set 357F-GC and 907R following a touch-down PCR cycle (Schafer et al., 2001). The previously cloned sequences, representing different phylogenetic taxa, were re-amplified using primers G357F-GC and 907R (Muyzer et al., 1993), and used as markers.

DGGE was performed with the Bio-Rad Protean II system as described previously (Muyzer et al., 1993). An 8% polycarylamide gel with a vertical gradient of 30-60% of the denaturant was used to analyze the 550 bp PCR products. The running conditions was 100V at a constant temperature of 60 °C in 23 L of 0.5×TAE buffer (20mM Tris acetate, 0.5 mM EDTA, pH 8.0) for 18 hours. The DGGE gels were visualized using an UV transilluminator after ethidium bromide-staining.

The representative bands were excised, purified and sequenced. The sequences were checked and manually modified using Sequencher (4.1.4) software, then compared with existing sequences using the online database of BLAST (http://www.ncbi.nlm.nih.gov/).

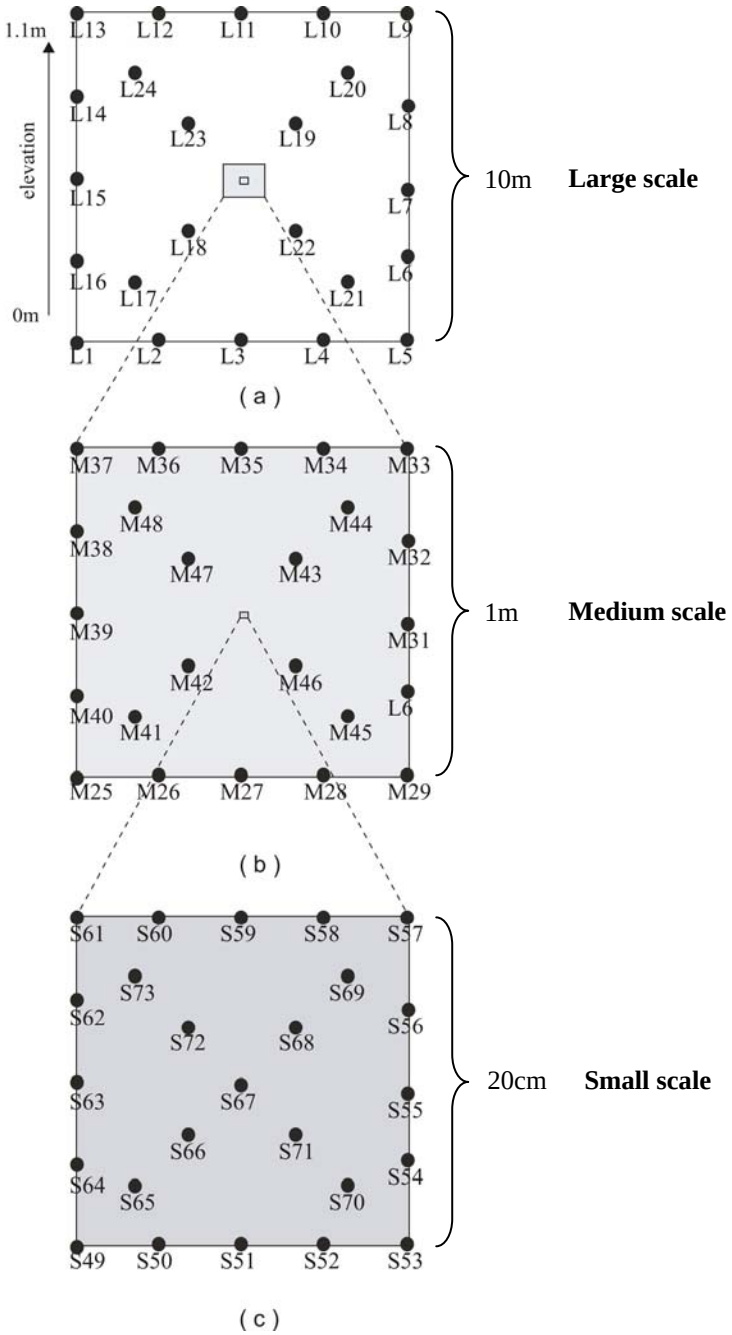

Figure 1. Sampling scheme: (a) is the primary plot (10m×10m) with an elevation from 0 to 1.1m. Samples were taken at 2.5m intervals along the sides and 2.35m intervals along the diagonals; (b) is the medium plot (1m×1m) with sampling interval of 25cm along the sides and 23.5cm along the diagonals; (c) is the small plot (20cm×20cm) with samples intervals of 5.0cm along the sides and 4.7cm along the diagonals.

Real-time PCR. A recently developed real-time PCR assay (Wang et al. submitted) was used to quantify the iron-oxidizing bacteria in the samples collected. The primer set includes a degenerate forward primer 628F (GBMAGGCTAGAG TGTAGC) and a reverse primer 998R (CTCTGGAAAC TTCCTGAC). DNA from soil samples was purified and diluted accurately to a concentration of 10 ng μl^{-1}. The real-time PCR was performed in a 25 µl reaction volume containing 2.5 µl purified DNA and 22.5 µl SYBR® Green PCR Master Mix (Invitrogen). The concentration of each primer was 5 pmol μl^{-1}. The PCR involved 45 cycles, with each cycle consisting of denaturation at 95°C for 20 s, annealing at 56°C for 20 s and extension at 72°C for 45 s. Data acquisition was done at 82°C for 10 s, to avoid signal from primer dimer formation. The DNA copy number of iron-oxidizing bacteria in each sample was estimated by comparing the C_t value of each sample to those of the standard regression line, which was made by using the reference cloned sequences (Wang et al., 2009).

Three methanotrophic sub-groups were quantified by *pmoA*-based quantitative PCR based on the assays described by Kolb and coworkers (Kolb et al., 2003). The type Ia and II assays were carried out as previously described (Bodelier, 2009). For the type Ib assay, DNA standards were prepared by dilution of a known amount of PCR product amplified from a reference clone by using the 189-Mc468 primer set (Kolb et al., 2003). 25 µl reaction containing 12.5 µl 2×SYBR green mix (AB gene, Epsom, UK), 2.5 µl of diluted DNA template and 0.8 mM each of primers. The samples were diluted accurately to 1 ng μl^{-1}. The thermal cycle started with an initial denaturation at 95°C for 15 min, followed by 45 cycles of denaturation at 95°C for 20 sec, annealing at 64°C for 20 sec, and extension at 72°C for 45 sec. Fluorescence was recorded at 84°C and DNA melting curve analysis was performed at temperatures ranging from 70°C to 99°C. All of three assays were performed with a Rotor Gene 6000 thermal cycling system (Corbett Research, Eight Mile Plains, Qld, Australia), where samples were added to aliquots of the master mixture using a CAS-1200 (Corbett Robotics Eight Mile Plains, Qld, Australia) liquid handling system. Every sample was performed in duplicate. Quantification analysis was performed by the RotorGene software.

Data analysis. DGGE gels were analyzed using Phoretix gel analysis software (Phoretix International, Newcastle upon Tyne, UK). The number of bands of each lane was defined and a matrix of band intensity was created. Lanes were created manually, with a fixed width of 5% of the standard lane width. Each

lane represents one sample. Background noise was subtracted by using the Rolling Ball algorithm with a radius of 50 pixels. Bands were detected automatically with a minimum slope of 100 and a noise reduction of 4. Then bands were assessed and corrected by eye, matched to a reference lane (Markers), and quantified. The relative abundance of each band was defined as the intensity ratio of each band to the total intensity of individual lanes.

Similarity between DGGE community profiles was analyzed using multivariate analyses in PRIMER 5 software (Plymouth Marine Laboratory, Plymouth, UK) The relative abundance or presence/absence data derived from the DGGE gels were used in non-metric Multidimensional Scaling (MDS) analyses. The input of MDS analyses were Bray-Curtis similarity matrices generated using Log(x+1) transformed relative abundances or presence/absence data. The MDS analyses results in a 2-dimensional plot were the distance between samples indicates the similarity of these samples relative to other samples in the plot. The accuracy of the 2-dimensional representation is indicated by the "Stress" value (Kruskall's stress formula). Stress values <0.1 indicate a good ordination with no prospect of misleading interpretation. Stress values <0.2 still give a good 2-dimensional representation where not too much reliance should be put on the detail. Theoretical aspects of the MDS analyses used are described by Clarke and Warwick (Clarke and Warwick, 2001). One-way ANOSIM in PRIMER software test was used to compare samples between different scales. This was followed by BIOENV procedure to evaluate the effect of environmental parameters on the distribution of microbial community structures (Clarke and Warwick, 1998).

In addition to the samples shown on the gel, a total of 18 out of 73 samples (L1-5, M25, M31, M 46, M 49, S50, S52, S53, S56, S60, S63, S65, S67, and S72) did not give products during PCR procedures and were excluded from analysis.

Data of the relative abundance of each band was log-transformed and analyzed using Statistica software (Statistica 9, StatSoft, Inc., Tulsa, USA), together with the qPCR results, and certain environmental parameters. The distribution of bacterial abundance at different elevation levels and sampling scales was compared using ANOVA. Post hoc Turkey analysis was used to further compare the difference among samples. The abundance between iron-oxidizing bacteria and methane-oxidizing bacteria was done using non-parametric pair wise correlation in Statistica.

Geostatistics. To model spatial structures for both iron oxidizing bacteria and environmental parameters geostatistics were applied. Geostatistics originate from mineral and soil science and is a common tool to identify and model spatial patterns (Legendre and Legendre, 1998). It is based on the assumption that spatial variability is autocorrelated, i.e. locations close to each other are more similar than those further apart (Ettema and Wardle, 2002). In order to model the spatial structure first a variogram analysis has to be carried out. In Brief, semi-variances between samples are calculated and plotted against their spatial separation; the slope indicates whether a spatial structure is present (Ettema and Wardle, 2002). In a second analysis theoretical variogram models are used to fit to the values of the empirical variograms. The Hawkins and Cressie's modulus estimator were calculated to create empirical variograms and exponential spatial model were fitted to the semi-variance values for biological data and residual maximum likelihood (REML) for environmental data (Cressie, 1993). The goodness of fit of the variogram model has been checked by cross validation. Ordinary kriging using global neighborhood was used to model the spatial structures of the total abundance of iron oxidizing bacteria (logarithm of the total 16S rRNA gene copy number) and the moisture content (percentage of moist in a sample) at the study site.

All geostatistical analyses were performed using the geostatistical data analysis software geoR as implemented in the statistical software R (Ribeiro JR., 2001; R Development Core Team, 2008).

RESULTS

Community structure of FeOB. Between one and four bands in each sample were retrieved from the DGGE gels (Figure 2). On average samples taken at the medium-scale had more bands than the large-scale samples, but no significant difference in number of bands was detected. The banding patterns of samples collected at the small scale (20×20cm) were rather different from those of large and medium-scales (figures not shown) and the average number of bands from small scale samples was less than that of the large and medium scale samples.

Similarities between community compositions of FeOB were represented in the MDS plot (Figure 3). In terms of the relative abundance of the bands retrieved from DGGE gels (Figure 3a), samples taken from large and medium scale differed from the samples collected at small scale (R=0.42, large & small, R=0.43 medium & small). There was no clear separation in the presence/

absence of the bands from the samples taken at different scales (Figure 3b), as could be seen from the overlaps of the samples in the figure.

A total of 5 bands were retrieved from the DGGE gels and sequenced. They were distinctly related to known cultures, with uncultured clones as most close relatives. The most dominant band (G35) from the medium-scale and large-scale samples was closely related to uncultured bacterial sequences (99%), while the most closely related cultured organism is *Gallionella* strain ES-2 (Emerson 2010), which shares 95% percent similarity in the 16S rRNA gene sequences. The most closely isolated relative to Band G37 is *Sideroxydans lithotrophicus* strain LD-1 (Weiss et al., 2007), a microaerophilic iron-oxidizing bacterium, which shares 97% identify in the 16S rRNA sequence. Band G33 was most closely related to uncultured *Gallionella* clones, among which Hc9 with 100% identity on the level of the 16S rRNA gene (Wang et al., 2009). G34 and G 36 are almost identical (99%) to uncultured clones Hc16 and Hc18 retrieved previously from wetlands (Wang et al., 2009), representing uncultured species from two separate clusters.

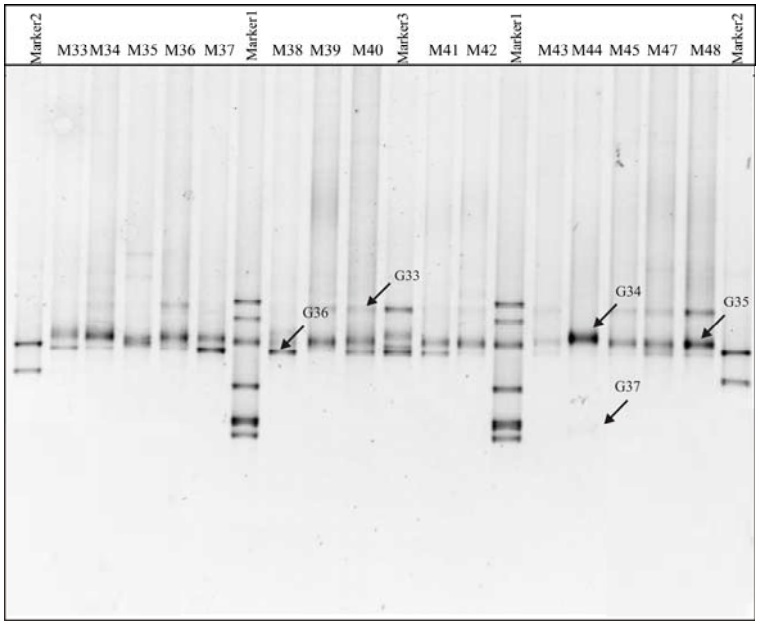

Figure 2. DGGE patterns showing the distribution of iron-oxidizing bacteria at different sampling sites. G33-37 were sequenced and shown in Fig 4. Sequenced clones obtained previously were used as markers. Marker1: MWE_C40(FJ391490), MWE_N41(FJ391496), MWE_N34(FJ391502), MWE_N13(FJ391501), MWE_N19(FJ391497), and MWE_N26(FJ391498), Marker2: Hc1(FJ391520), and Hc25(FJ391510), Marker3: Hc9(FJ391509), Hc37(FJ391515), Hc16 (FJ391516)and Hc8(FJ391513)(Wang et al., 2009).

Effects on the abundance and diversity of FeOB and MOB: Spatial scale

The abundance of FeOB as determined by total copy numbers of 16S rRNA genes, ranged from 1×10^3 to 3.9×10^8 per gram dry soil. The highest copy number was measured for sample L20, taken in the high elevation part. The average copy number of 16S rRNA from samples taken at large-scale, *i.e.* 4.6×10^7 copies per g dry weight, was significantly higher than that of the small-scale samples (5.4×10^6 copies per g dry weight) (Figure 4a). Four out of 24 medium-scale samples didn't yield product in the qPCR measurement, indicating that the value of their 16S rRNA copy numbers were below the detection limit, and thus were excluded from the analyses.

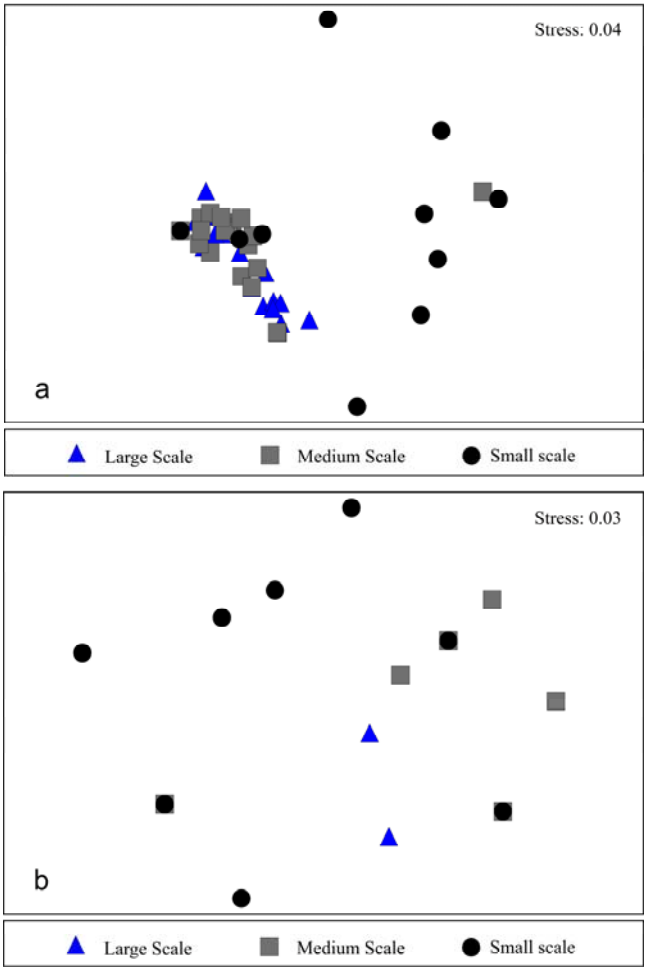

Figure 3. Ordination by non-metric multidimensional scaling of log(x+1) transformed relative abundance (a) and presence/absence of the DGGE bands of the samples taken from at different spatial scales (b).

One-way ANOVA test indicated significant variation of iron-oxidizing bacteria as well as methane oxidizers across spatial scales (Table 1). Post hoc Turkey test indicated that the abundance of both FeOB and MOB in samples taken at small-scale was significantly lower than those at large- and medium-scale plots ($p<0.05$). No difference was observed between large- and medium-scale samples.

Table 1 Effects of spatial and elevation factors on microbial community abundance

Factor	SS	DF	MS	F	P
Spatial					
FeOB	2.20	2	1.10	6.820	0.001982*
Type II	20.18	2	10.88	30.482	0.000000*
Type1a	2.62	2	1.31	4.941	0.009959*
Type Ib	15.05	2	7.52	7.550	0.001410*
Elevation					
FeOB	16.80	2	8.40	9.749	0.000576*
Type II	0.62	2	0.31	1.670	0.195695
Type1a	5.98	2	2.99	5.152	0.008773*
Type Ib	1.36	2	0.68	2.400	0.098485

* indicates significant difference

At large- and medium-sampling scales, the total gene copy numbers of the different bacterial groups followed the sequence, FeOB > MOB type II > type Ib > type Ia. Significant differences were detected between all the groups except for type II and type Ib MOB. In the small-scale sampling plot, the abundance of MOB type Ia was significantly lower than the rest of all samples (Figure 4a).

A diverse pattern of relative abundance of the bands retrieved from the DGGE gels was obtained. Among all the bands detected, Band G35 and Band G36 showed a similar trend to the average abundance detected using qPCR (Figure 5a). The relative abundance of Band G35 dropped significantly at small-sampling scale as compared to large- and medium-scale samples. The rest of the bands did not show a clear trend given spatial level as a factor.

Effects on the abundance and diversity of FeOB and MOB: Elevation scale

With the increase of elevation, the abundance of FeOB increased linearly (Figure 4b). Again in this case, band G35 was found to contribute the most to the increase. Moreover, the other dominant band G36 also showed a positive trend with elevation (Figure 5b).

MOB showed a similar trend except for type Ia, which displayed maximum abundance in the intermediate elevation level. There was a significant difference between the abundance of type Ia in samples taken from medium (33-46 cm) and low level (0-33 cm). No difference was observed among the abundance of other types of methane oxidizers along the elevation gradient.

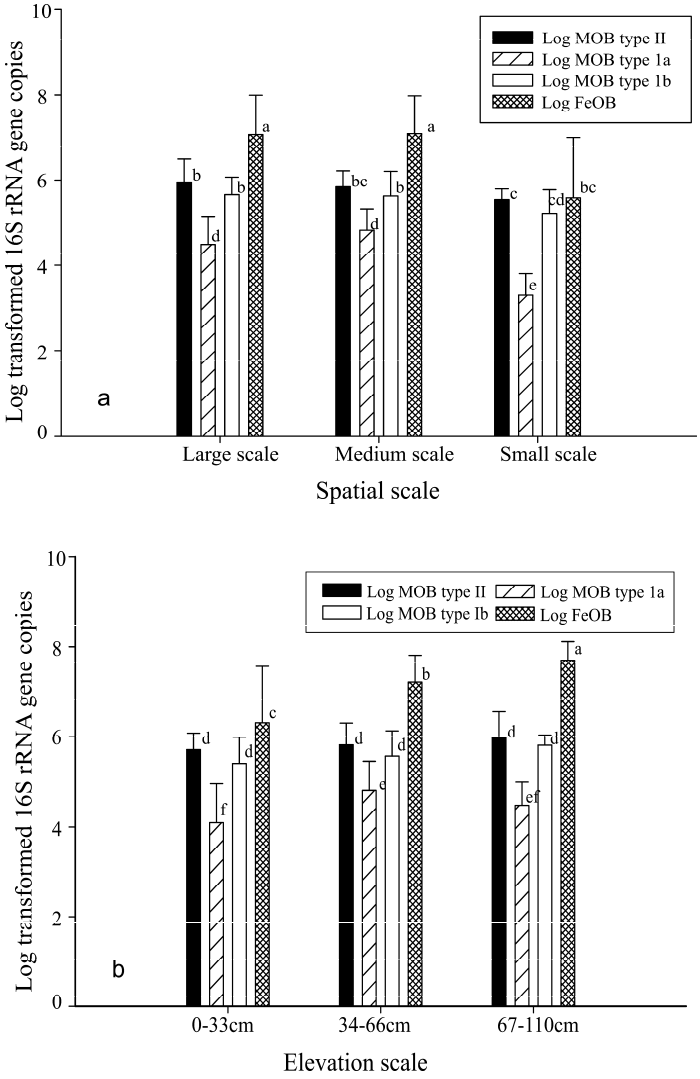

Figure 4. The distribution of 16s rRNA copy numbers of FeOB and MOB at different spatial scales (a) and elevation (b) levels. Significant differences ($P < 0.01$) between potential activities are indicated by different letters.

The total 16S rRNA gene copy numbers detected for FeOB was much higher (10 to 100 fold) than the *pmoA* gene numbers of the different types of

methane oxidizers at all elevation levels. Although the total copies of 16S rRNA genes in FeOB cells are uncertain, the conclusion can still be made that there are more cells of FeOB than total numbers of methane oxidizers. A positive correlation was detected between the abundance of FeOB and the methane oxidizers. Among the three types, type Ia showed higher positive correlation to FeOB ($r=0.65$, $p<0.05$) than type II ($r=0.46$, $p<0.05$) and type Ib ($r=0.48$, $p<0.05$).

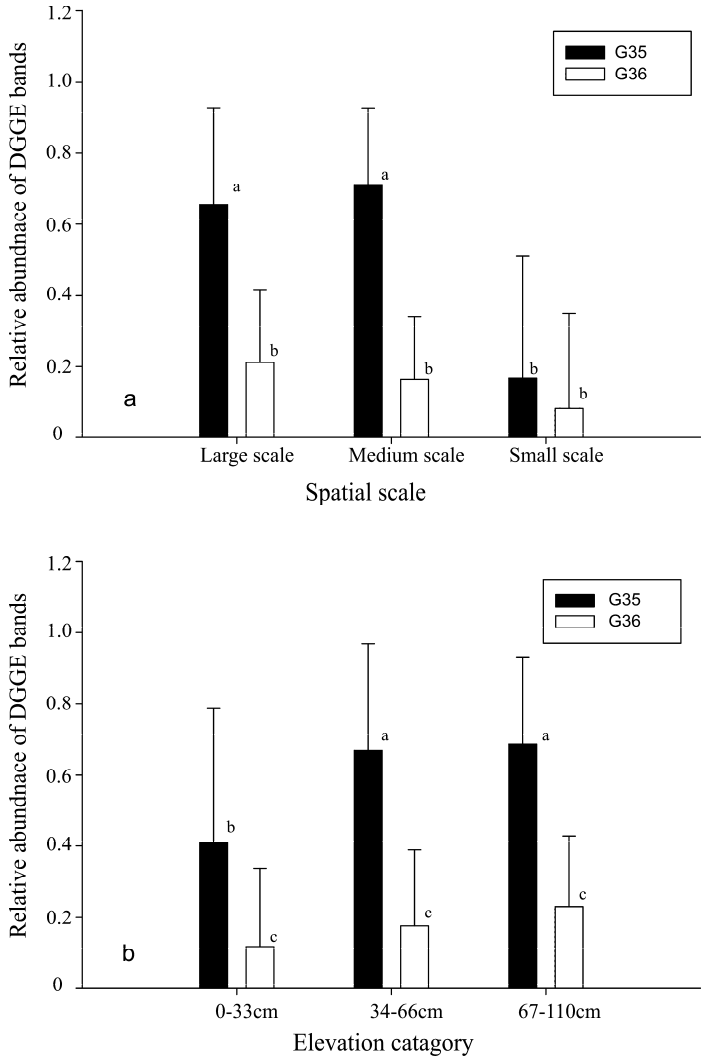

Figure 5. The distribution of relative abundance of the dominant DGGE bands of FeOB at different sampling (a) and elevation levels (b). Significant differences ($P < 0.01$) between potential activities are indicated by different letters.

Effects of moisture contents. Using ordinary kriging, the FeOB abundance and distribution of moisture was interpolated for the whole plot using the measured points as reference (Figure 6). A clear gradient was shown in the abundance of FeOB within the flooding gradient, in the elevation with increasing distance to the river. The higher parts of the plot contained the highest numbers of FeOB. The distribution pattern of moisture content was opposite to that of FeOB abundance (Figure 6). Interestingly the lowest moisture content was observed not at the top of sampling plot, but to the side of the plot with the highest plant biomass (*i.e.* 0.56 to 1.12 m higher than the lowest point of the plot). The moisture content was negatively correlated to the abundance of FeOB (slope=-0.14, r=0.36, p=0.01).

Similarly, MOB abundance also correlated negatively with the soil moisture content. Abundance of type Ia (slope=-0.10, r=0.35, p=0.01) and type II (slope=-0.05, r=0.3, p=0.01) decreased significantly with the increase of moisture content. The decrease of abundance of MOB type Ib (slope=-0.05, r=0.22, p=0.06) with the increase of moisture content was not significant.

DISCUSSION

The diversity and abundance of iron-oxidizing bacterial communities varies with the spatial scale of observation. There is no significant difference detected between samples taken at large- and medium-scales (Figure 4), but the diversity and abundance decreases markedly at the small-sampling scale. This could be caused by soil heterogeneity, including the heterogeneous distribution of iron geochemistry, as described by Ferreira and colleagues (Ferreira et al., 2010). These authors demonstrated that the position of soils within an estuary affects the distribution of a number of important soil components and attributes.

The increase in numbers of iron-oxidizing bacteria with elevation is almost linear. Soil moisture content could be one of the important environmental variables that control the distribution of the bacterial community. With elevation, soil moisture content decreases, likely increasing the availability and penetration depth of oxygen into the soil, which in itself should favor bacterial iron oxidation. However, when soil oxygenation increases, more ferrous iron may be consumed by chemical iron oxidation. A decrease in soil moisture content may also lead to less iron reduction, which consequently slows down the redox turnover of iron (Blothe and Roden, 2009a). Together, this would lead to low ferrous iron supply, a key factor for iron-oxidizing activity.

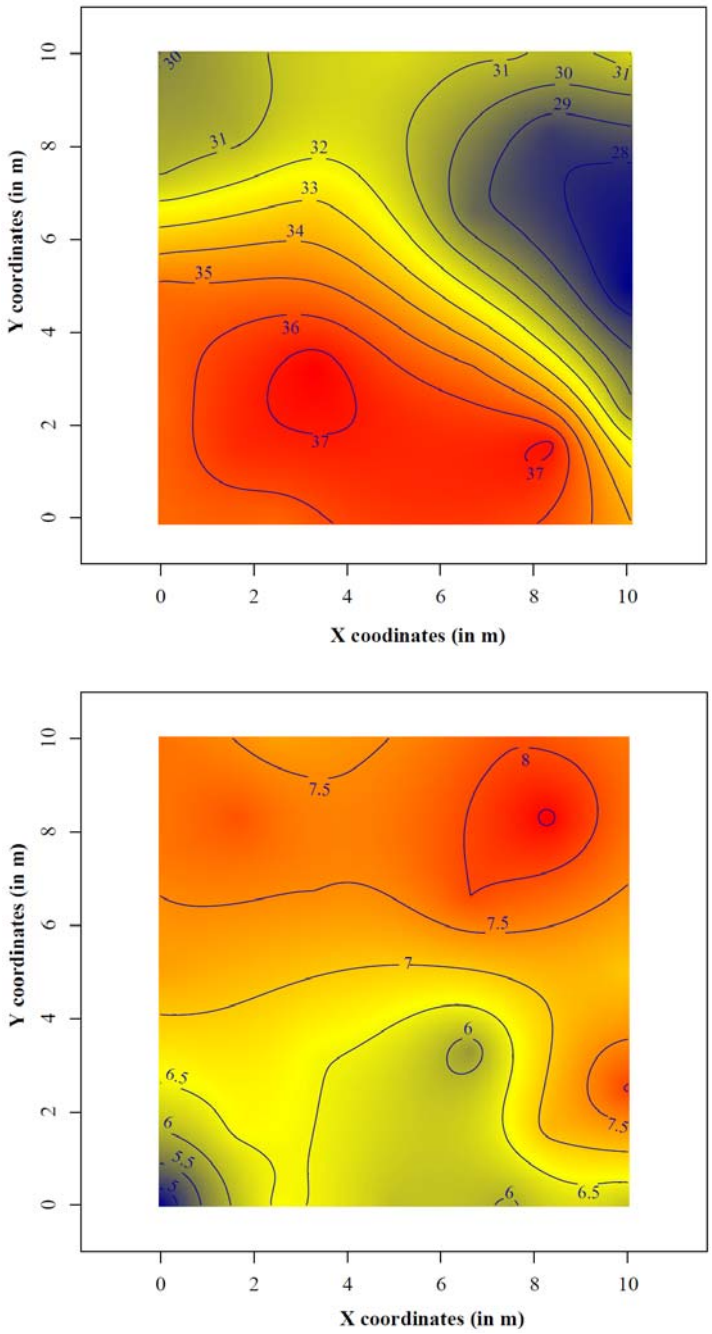

Figure 6. Interpolated maps of the spatial distribution of iron-oxidizing bacteria (left) and soil moisture content (right). The color scalesindicate the extrapolated total abundance values of iron-oxidizing bacteria (logarithm of the total 16S rRNA gene copy number) or moisture content (in percent).

In an earlier study, FeOB could not be detected at a dry site in a freshwater marsh, which is seldom exposed to flooding (Wang et al., submitted). In the present study, the increase in abundance of iron-oxidizing bacterial with elevation levels off at the highest elevations of the sampling plot, indicating that the linear correlation between FeOB abundance and elevation is restricted to a certain range.

Except for type Ia the abundance of methane-oxidizing bacteria does not change with elevation. Type Ia is known to display an ecological strategy different from the other types as it reproduces rapidly when conditions are favorable, including the presence of oxic/anoxic interfaces such as rhizospheres and surface layers of lake sediments (Bodelier et al., 2009a; Steenbergh et al., 2010). In this case, probably the conditions halfway the elevation gradient are optimal for their growth.

The results show that the abundances of FeOB and MOB are positively correlated with each other, though the abundance of FeOB is much higher than that of MOB at the different sampling and elevation scales. These two groups of microorganisms are very much influenced by the soil redox conditions (van Bodegom et al., 2001). In this present study, both groups decreased in abundance with increasing soil moisture content. However, concluding from the observed differences in the effect of soil moisture content on gene abundances, with linear slopes amounting to -0.14, -0.10, -0.05 and -0.05 for FeOB, type Ia MOB, type Ib MOB and type II MOB, respectively, iron-oxidizing bacteria seems to be more sensitive to moisture stress than the methane-oxidizing bacteria.

It seems likely that moisture stress is coupled to stress due to oxygen limitation. According to thermodynamics (Thauer et al., 1977; Sobolev and Roden, 2002), the oxidation processes should follow the order of ferrous iron > methane > sulfide > ammonium oxidation under conditions of oxygen limitation. It has been suggested by Neubauer (Neubauer et al., 2002) that neutrophilic Fe(II)-oxidizing bacteria may compete for limited O_2 in the rhizosphere and therefore influence other wetland biogeochemical cycles. Van Bodegom and co-workers (van Bodegom et al., 2001) also reported that iron oxidation was the most important oxidative process in a rice paddy rhizosphere and accounted initially for 97% of the consumed oxygen. Hence, other factors besides oxygen limitation may be responsible for the observed relationships between the numbers of FeOB and MOB and moisture content.

The interpolated map (Figure 6) also reveals a gradient in the distribution of 16S rRNA genes of iron-oxidizing bacteria along the elevation gradient. Nevertheless, the driest spot is not at the highest elevation but somewhere between the middle and top part of the large-scale sampling plot. The abundance of iron-oxidizing bacteria is not correlated with soil moisture content in this particular region. Therefore, there are more environmental factors than soil moisture content that contribute to the distribution of FeOB. The statistical BIOENV tool using soil properties such as elevation, soil organic matter content and soil density as independent variables, did not explain the variability in FeOB distribution in better way, however.

When comparing the FeOB community of the irregularly flooded riparian soil with the one in a tidal freshwater marsh (Wang et al., submitted), it is striking to see that the species compositions are comparable. The dominating iron-oxidizing species are slightly different at these two sites, as shown by the major bands on the DGGE gels. In terms of absolute abundance, however, the irregularly flooded riparian site at Ewijk shows higher 16S rRNA copy numbers than the tidal freshwater marsh at Appels. Both sites contain high amounts of iron and are influenced by human activities. The site at Appels is flooded twice a day by tide and is likely more dynamic with respect to redox conditions and processes than the irregularly flooded site at Ewijk. This difference in redox dynamics will probably affect the iron-oxidizing community as well. Dedicated microcosms studies with defined cultures of FeOB isolated from both sites may elucidate the effect of flooding on the behavior and niche differentiation of this group of microorganisms.

In conclusion, the results of this study show that the iron-oxidizing bacterial abundance in the soil increases with elevation, and that the scale of sampling influences the observed community structure of both FeOB and MOB. The abundances of both groups are negatively correlated with soil moisture content, with FeOB more sensitive to changes in moisture content.

Chapter 5

Ferrocurvibacter nieuwersluisensis gen. nov., sp. nov., a neutrophilic iron-oxidizing bacterium from an irregularly flooded grassland

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ABSTRACT

A novel neutrophilic iron-oxidizing bacterium, strain HDD^T, was isolated from an iron-rich grassland. Cells are extremely motile, curved rods, around 1.2 µm long and 0.5 µm wide. The optimum temperature for growth is 28 °C and the optimum pH range is from 6.0 to 7.0. The cells were able to grow chemolitho-autotrophically in the presence of ferrous iron and oxygen. Heterotrophic growth was not observed on any of the following substrates: pyruvate, acetate, lactate, formate, ethanol, glucose, succinate, glycine, and fumarate. Phylogenetic analysis showed that strain HDD^T is a member of the *Betaproteobacteria* and its closest relative is *Gallionella ferruginea* (94% identity of the 16S rRNA gene). The major cellular fatty acids in the polar lipids (PLFA) are 16:0 (72%), 16:1ω7c (22%) and 18:2ω6c (15%). The presence of large amounts of the latter PLFA distinguishes it from the previously isolated neutrophilic iron oxidizers strains ES1 and ES2. On the basis of phenotypic properties and phylogenetic analysis, the isolate is proposed to represent a novel genus and species *Ferrocurvibacter nieuwersluisensis* gen. nov., sp. nov. (type stain HDD^T=JCM16104^T =NCC B10032^T).

INTRODUCTION

Iron oxidation under neutral pH conditions has generally been ascribed to abiotic reactions until bacteria associated with iron oxides were convincingly demonstrated to be involved (Emerson and Moyer, 1997). The first record of an iron-oxidizing bacterium (FeOB), *Gallionella ferruginea,* dates from the early 1800s (c.f. (Hallbeck et al., 1993). Wolfe and colleague (Kucera and Wolfe, 1957) introduced an FeS-containing medium and showed that *G. ferruginea* was a redox boundary-loving organism that required opposing gradients of oxygen and iron(II) to grow. Hallbeck et al. (Hallbeck et al., 1993) isolated a new strain of *G. ferruginea* and demonstrated the mixotrophic growth of these organisms. The Wolfe cultivation technique has been modified by Emerson to enrich and isolate FeOB using Fe^{2+}-O_2 gradient tubes with semi-solid agarose (Emerson and Moyer, 1997).

Microbial iron oxidation has been observed in various environments of circumneutral pH (Sobolev and Roden, 2002; Weiss et al., 2003; Blothe and Roden, 2009a). A number of isolates have been obtained, including the isolates ES-1and ES-2 obtained from a groundwater-fed iron seep (Emerson and Moyer, 1997). Phylogenetically these isolates belong to the genera '*Siderooxydans'* and *Gallionella*.

Wetlands are environments characterized by rapid redox cycling. Wetland plants transport molecular oxygen into otherwise anoxic soils where ferrous iron is readily available. This results in the formation of iron plaques, which are widely observed in wetland soils and sediments (Du Laing et al., 2009). Studies have shown that a diverse microbial community is associated with the Fe-coated root surfaces (Weiss et al., 2003; Wang et al., 2009). TW2, an oxygen-dependent lithotrophic FeOB belonging to the *Rhodocyclales* group was isolated from an iron-rich freshwater wetland, (Sobolev and Roden, 2004). Several iron-oxidizing strains have been isolated from wetlands, representing new genera of *Sideroxydans* and *Ferritrophicum*, respectively (Weiss et al., 2007).

Neutrophilic FeOB are typically found at the oxic-anoxic boundary where chemical oxidation is relatively slow. This may represent a survival strategy allowing the FeOB to outcompete the otherwise fast abiotic Fe(II) oxidation under near-neutral pH conditions (Kucera and Wolfe, 1957; Hallbeck and Pedersen, 1990; Emerson and Moyer, 1997). Neutrophilic FeOB are not only found in terrestrial habits, but also in deep-sea hydrothermal habitats, which are

both well oxygenated and with approximately neutral pH (Kamimura et al., 2001; Bazylinski et al., 2004; Edwards et al., 2004; Emerson et al., 2007; Schubbe et al., 2009).

In this study, we have isolated a new strain HDD^T from an irregularly flooded grassland using a modified gradient tube approach. The strain is an obligate chemolitho-autotroph that utilizes ferrous iron as the sole energy source and CO_2 as carbon source. Phylogenetically it belongs to the Betaproteobacteria, sharing 94% sequence similarity to the 16S rRNA gene sequence of its most closely relative *Gallionella ferruginea*.

MATERIAL AND METHODS

Sample collection. Strain HDD^T (=JCM 16104^T, =NCCB10032^T) was isolated from an irregularly flooded inland wetland site in the center of the Netherlands (52°32'60" N, 6°7'60" E), which has a remarkably high concentration of Fe (total Fe 702 µmol/g dry soil and oxalate-extracted Fe 459 µmol/g dry soil) (Loeb et al., 2008). The soil pH is 6.0 and *Glyceria maxima* is the dominating plant species.

Enrichment and isolation. The soil was planted with *G. maxima* and incubated under flooded conditions in the lab for six weeks (Wang et al., 2009). Fresh soil samples were mixed at a ratio of 1:1 with Milli-Q water and used as inocula for the enrichment of bacteria.

A modified method of Emerson and co-workers (Emerson et al., 1999) was used to prepare Fe(II)-O_2 gradient tubes for the initial enrichment, isolation and subsequent maintenance of the culture. The system consisted of two layers of ultra-pure agarose (Invitrogen) in 16 mm screw cap glass tubes. The bottom layer contained 1.25 ml FeS and modified wolfe mineral medium, (MWMM) (Emerson and Floyd, 2005) at a ratio of 1:1, amended with 1% (wt/vol) agarose. The top layer consisted of 6.75 ml mineral medium supplemented with vitamins and minerals (http://www.lgcstandards-atcc.org, MDVS, MD-TMS) and stabilized with 0.05% (wt/vol) agarose.

Preparation of the FeS stock solution, which is critical for the successful isolation of FeOB, has been described in detail (Emerson and Floyd, 2005). The quality of FeS varied from batch to batch, which affected the bacterial growth significantly. In our study, 500ml of dH_2O was heated to 50°C and divided into two beakers. 46.2 g ferrous sulfate ($FeSO_4 \cdot 4H_2O$) and 39.6 g of sodium sulfide ($Na_2S \cdot 9H_2O$) were weighed and dissolved in each beaker

separately. The ferrous sulfate solution was added to the sodium sulfide solution, while stirring with a glass rod. The mixture was stirred continuously for a couple of minutes, followed by the procedure described by Emerson and Floyd. Every step of the preparation of FeS was performed in a fume hood.

The solutions for making the upper layer of the tubes were prepared in sealed serum bottles with $NaHCO_3$ at a concentration of 10 mM and flushed with CO_2 for 1 min. After autoclaving at 121°C for 30 min, the final pH of media was stable and around 6.5.

After autoclaving, 1.25 ml of the bottom layer medium was pipetted into the tubes while still hot. The top layer was allowed to cool down to around 40°C and 6.75 ml of the top layer medium supplemented with trace element and vitamin solutions was pipetted onto the solidified bottom layer. After a few hours, the tubes were inoculated with series dilutions of soil samples. The tubes were capped with butyl rubber stoppers leaving a headspace filled with air. A positive sample, defined by the appearance of a sharp orange band at the lowest dilution was taken, diluted into MWMM (Emerson and Floyd, 2005) for further inoculation.

At the later isolation stage, serial dilutions were made in liquid medium (Emerson and Floyd, 2005). A concentration of 10mM of $FeCl_2$ was used as iron source and the headspace was supplied with N_2 and O_2 at a ratio of 80:20. The pH was adjusted by adding $NaHCO_3$ and bubbling with CO_2. Pure cultures were obtained from the 10^7 dilution. Subsequently, these cultures were transferred back to gradient tubes and maintained. The purity was checked by observing universal morphology under the microscope, growth on culture medium rich in organic compounds (LB and R_2A), as well as DGGE analysis of the PCR-amplified 16S rRNA gene fragment.

Growth media. The gradient tube method was used for routine cultivation as previously described. The following substrates were tested in tubes. Pyruvate, acetate, lactate, formate, ethanol, glucose, succinate, glycine, and fumarate at 10 mM were added to MWMM, amended with vitamins and trace elements. The pH was adjusted as done for the gradient tubes. Final concentrations of 5 mM of $FeCl_2$, $FeCO_3$, $MnCl_2$, Na_2SO_3, Na_2S were used to replace FeS as electron donor either in gradient tubes or in liquid cultures.

Similarly, 2.5 mM NO_2^- or NO_3^- was added to 1×MWMM to replace oxygen as electron acceptor. The medium was prepared in sealed serum bottles, flushed vigorously with N_2 to replace oxygen. The medium was buffered with 0.5 mM $NaHCO_3$ and adjusted to a final pH of 6.5 by flushing CO_2 for one

minute, and then autoclaved. Filter sterilized $FeCl_2$ was amended to a final concentration of 10 mM. The bottles were inoculated with 1 ml fresh cultures using sterile syringes.

Salt resistance of bacteria was tested at concentrations of 1, 3, 5, 7, 9, 10, 12, 20, 30, 40, and 50 g L^{-1} NaCl. The isolate was tested for growth over a range of different pH values (pH 3 to 9 at 0.5 unit intervals). The pH range for growth was determined by buffering the top layer of the gradient tubes with $NaHPO_4$ and citric acid for the pH range 3 to 6, $NaHCO_3$ and CO_2 for the pH range 6 to 7.5, and $NaHCO_3$ and $NaCO_3$ for pH between 7.5 and 9. The optimal growth temperature range was tested from 4°C to 40°C, at 5 degree intervals.

Polar lipid fatty acid (PLFA) analyses. Bands of bacteria mixed with iron oxides were collected from the gradient tubes, freeze-dried and used for fatty acid analysis. The phospholipids were extracted and their PFLA composition analyzed as described previously (Bodelier et al., 2009b). Briefly, lipids were extracted using a modified Bligh and Dyer extraction procedure (Boschker et al., 1998). The lipid extract was fractionated on silicic acid columns into different polarity classes by sequential elution with chloroform, acetone and methanol. The methanol fraction containing the PLFA was subjected to mild-alkaline methanolysis to yield fatty acid methyl esters (FAME). FAME concentrations were determined using a gas chromatograph (Thermo Finnagan TRACE GC) equipped with a flame ionization detector (GC-FID) and a polar capillary column (SGE, BPX-70, 50m_0.32mm_0.25 mm). To determine the double-bond position of mono-unsaturated PLFAs that could not be identified by the retention index and GC-MS data, dimethyl disulphide (DMDS) derivatization was performed as described by Nichols et al. (Nichols et al., 1985) .

Phylogenetic analysis. Bacterial cells were collected from the gradient tubes and the genomic DNA was extracted using a modified protocol after Zhou (Zhou et al., 1996). Primers 27 F and 1492 R (Lane, 1991) were used to amplify the almost complete 16S rRNA gene. The PCR product was purified with Gel Extraction kit (Qiagen) and sequenced by the commercial company Macrogen (http://www.macrogen.co.kr). The primers used for sequencing include 27F, 357F, 907R and 1492 R. The sequences were assembled in Sequencer and compared to database (http://www.arb-silva.de) using ARB (Ludwig et al., 2004; Pruesse et al., 2007). The sequence of this isolate was

aligned with previously described FeOB. A phylogenetic tree was constructed using the Neighbor-joining method.

RESULTS

Morphology. Cells of strain HDD^T are curved rods (Fig 1), with an average length of 1.25 µm and a width of 0.5 µm. They were mostly attached to iron oxides and were extremely motile when released into the liquid phase. When reaching the exponential growth stage, the cells encrusted themselves inside iron oxides and it was hardly possible to see them using phase contrast microscopy. Only the new cells that were not attached to iron oxides could be seen. With SYTO 13 stain the cells were easily observed by fluorescence microscopy.

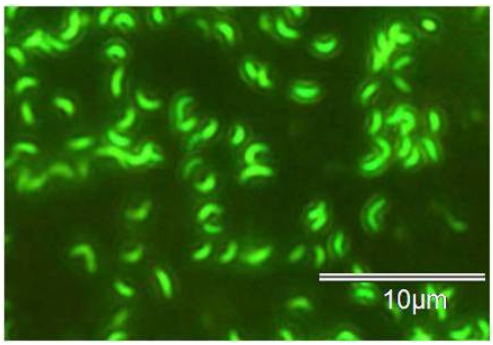

Figure 1. Photomicrograph of fluorescent cells of strain HDD^T, obtained from gradient tubes.

Growth Substrates. For gradient tube cultures, positive growth was defined both by the formation in the agar of a band containing bacteria, and by the observation of live cells by microscopy. Growth was only observed when ferrous iron compounds were used as energy source, i.e. FeS, $FeCl_2$, and $FeCO_3$, but not with sulfur species or Mn^{2+}. The growth on Fe(II) was dependent on oxygen and no anaerobic growth associated with NO_3^- or $NO_2^-$was observed. Strain HDD^T is likely a microaerophile, since the growth was maximal 1 to 2 cm down from the surface of the tubes, but the culture was not inhibited by oxygen. The cells revived well after a few days exposure to air. Heterotrophic growth was not observed in the presence of the various substrates and strain HDD^T was described as an obligate chemolithtroph.

Salt, pH and temperature optima. The novel isolate grew best without salt and was inhibited at NaCl concentrations exceeding 10 g L^{-1}. No clear difference was observed between the growth without and with lower concentrations of salt. The pH range of growth was between 6 and 7.5. When

the pH was higher than 7 the formation of a bacterial band in the agar was a little slower, than that with pH at around 6.5. This might have been caused by a faster chemical oxidation under higher pH conditions. This is comparable to iron-oxidizing strains ES-1 and ES-2, which have an optimum pH range for growth between pH 6.0 and 6.5 (Emerson and Floyd, 2005). The temperature range for growth of strain HDD^T was between 15°C and 35°C with an optimum of 28°C.

PLFA and phylogenetic analysis. The major fatty acids of strain HDD^T are 16:0 (40.72%), followed by 16:1ω7c (22.2%) and 18:2ω6c,9c (15.23%) (Table 1). A phylogenetic tree was constructed based on the almost complete 16S rRNA gene sequence of strain HDD^T (Fig 2). It shared 94% sequence identity to *Gallionella ferruginea* (Hallbeck et al., 1993) and 95% to strain ES-2 (*Gallionella ferruginea sub. sp. capsiferrifomas*), which was classified as a *Gallionella* strain based on the 98.5% sequence identity to *G. ferruginea*. According to the result of a BLAST analysis, the closest relatives of strain HDD^T were uncultured organisms from an iron-oxidizing enrichment culture (Blothe and Roden, 2009a). Within the phylogenetic tree, there were three major clusters formed by iron-oxidizers and closely related clone sequences. Two of them belonged to the genera *Gallionella* and '*Siderooxydans'* respectively, while stain HDD^T was the only cultured representative in the third cluster (Fig 2).

Table 1 Phospholipid Fatty acid composition of strain HDD^T

Fatty acid	% of total
C10:0	0.43
C11:0	0.19
C12:0	0.57
C13:0	0.16
C14:0	1.19
C15:0	3.89
C16:0	40.72
C16:1ω7c	22.20
C17:0	1.39
C18:0	4.60
C18:1ω?*9c	3.78
C18:1ω7c	1.10
C18:2ω6c,9c	15.23
C18:3ω?*	4.55

* Can be cis or trans configuration.

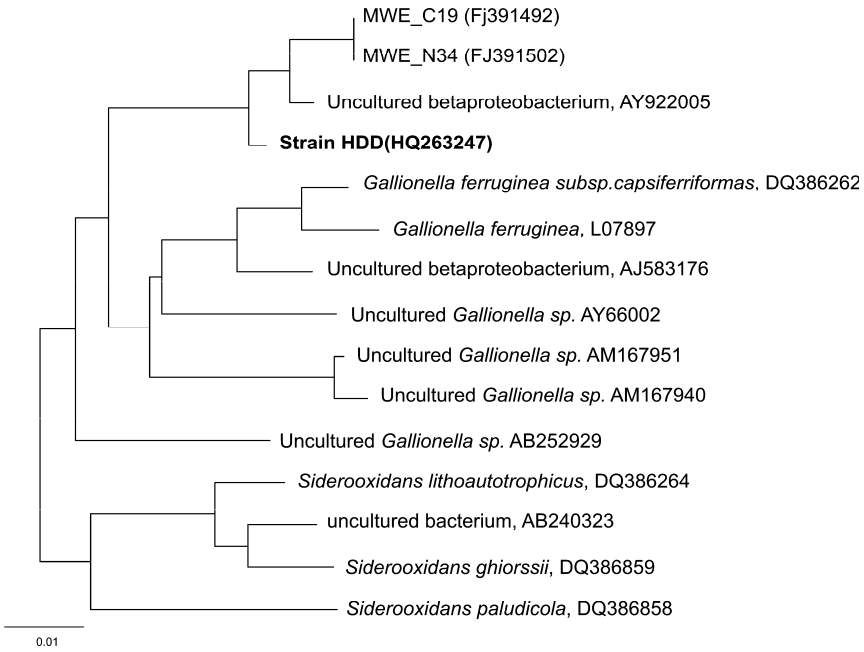

Figure 2. Phylogenetic tree based on nearly complete 16S rRNA gene sequences. The sequence of the new strain is printed in bold. The bar indicates 1% sequence difference. *Nitrosomonas europaea* was used as an out-group, but was pruned from the tree.

DISCUSSION

It is now well recognized that bacteria play an important role in iron oxidation at neutral pH, and advances in gradient cultivation techniques has allowed several phylogenetically different isolates to be obtained. One of the common characteristics of the isolates is that they are from environments with an oxic-anoxic interface, where ferrous iron is readily accessible and low oxygen conditions prevail. Strains ES-1 and ES-2 are from a ground water-fed seep, while strains BrT, Br-1 and CCJ were isolated from the rhizosphere of wetland plants. *Gallionella ferruginea* was originally isolated from a deep drinking-water well, i.e. an oligotrophic environment (Hallbeck and Pedersen, 1990). Strain HDD^T was obtained from an irregularly flooded grassland soil with a dynamic redox interface.

The isolation and cultivation of neutrophilic FeOB is not a straightforward task. First of all, ferrous iron oxidises quickly abiotically according to the rate law ($-d[Fe(II)]/dt = k\ [OH^-]^2\ pO_2\ [Fe(II)]$ (Sung and Morgan, 1980)). With increasing pH, the chemical oxidation process accelerates, which increasingly limits the remaining amount of ferrous iron for bacterial growth. Gradient tubes

were specifically designed to create a low partial oxygen pressure environment which slows down the rate of chemical oxidation. However, at a certain stage, shortage of oxygen in the tube will inevitably limit bacterial growth, hence requiring frequent transfer of the cultures to fresh medium for continued growth.

The novel FeOB isolate described here is comparable with other lithotrophic FeOB in that they have simlilar morphology, either bean-shaped or curved rods. In general, cells of strain HDD^T are smaller compared to the isolates obtained within the past decades. None of the latter produced unique structures, like the stalks or sheaths formed by *G. ferruginea* and *Leptothrix ochracea,* respectively. Instead, amorphous iron oxide was produced, as was also observed for HDD^T.

The growth condtions of strain HDD^T are slightly different from *G. ferruginea*, which grows optimally at a slightly lower pH (5.0-6.0) (Hallbeck et al., 1993) and is also found in moderately acidic environments (Heinzel et al., 2009). In contrast, HDD^T did not grow well when the pH dropped below 6. Exept for some marine isolates that were defined as psychrophilic with temperature optima ranging from 3 to 10°C (Edwards et al., 2003b), previously described FeOB and strain HDD^T are mesophiles. The preference of the strains for different temperatures could be related to the environments from which they were isolated.

Previous studies have shown that the classic iron oxidizer *G. ferruginea* is also able to grow heterophically on glucose when no iron is available. However, other isolates have been shown to grow solely on ferrous iron as an energy source, except for strain TW2, whose growth was accelerated by adding acetate to the medium (Sobolev and Roden, 2004). With respect to oxygen, it was noticed that strain HDD^T survived after exposure to full oxygen for a few days. Similarly, strains ES-1 and ES-2 preferred to grow at the oxic-anoxic boundary, but can tolerate environments saturated with O_2 at least temporarily.

Phylogenetic analysis shows that HDD^T doesn't fall into any clade of known FeOB (Fig 2). Based on the 16S rRNA it shares 94% identify with *G. ferruginea*. However, since the culture of *G. ferruginea* was not available as a reference, direct physiological comparison was not possible. There are clone sequences that are related to HDD^T from environments characterized by ongoing iron cycling. It is not known, however, whether the organisms to which those sequences belong to are actually taking part in the oxidation of iron in these environments.

Another interesting feature of the novel FeOB isolate is its PLFA composition. Unfortunately, PLFA data are not available for the classic iron oxidizer *G. ferruginea*. The profiles of the two other novel iron-oxidizing strains ES-1 and ES-2 are slightly different from each other in their fatty acid compositions (Emerson, personal communication). As in these 2 strains, the most dominant fatty acid of HDD^T is 16:0. However, we detected also relatively high percentages of C16:1ω7c- and C18:2ω6c,9c-type fatty acids that were not found for strain ES-1 and ES-2. The PLFA C18:2ω6c,9c has not been described in iron oxidizers to date.

Based on both phylogeny and physiology, we propose the new strain HDD^T as a novel genus and species. This finding further extend our knowledge concerning the diversity of FeOB. The energetically unfavourable process of circumneutral iron oxidation apparently can be performed by a larger diversity of microbes than thought before. Unlike acidophilic FeOB that have been thoroughly investigated (Baker and Banfield, 2003), we know little about the mechanism of bacterial iron oxidation at neutral pH. Therefore, further efforts need to be taken to get more FeOB isolates available for more detailed characterizations of the physiology, biochemistry and genomics of these organisms.

Description of *Ferrocurvibacter* gen. nov

Ferrocurvibacter (fer.ro.cur.vi.bac'ter. L. n. *ferrum*, iron; L.adj. *curvus*, bent, curved; N.L. masc. n. bacter, rod; N.L. masc. n. *Ferrocurvibacter*, a curved rod using ferrous iron as energy source)

Cells are curved motile rods. Neutrophilic, mesophilic and microaerophilic. Utilize ferrous iron as a sole energy source. Belong to Betaproteobacteria. The type species is *Ferrocurvibacter nieuwersluisensis*.

Description of *Ferrocurvibacter nieuwersluisensis* sp. nov.

Ferrocurvibacter nieuwersluisensis (ni.eu.wers.lui.sen'sis. N.L. masc. adj. *nieuwersluisensis*, of or belonging to Nieuwersluis)

Ferrocurvibacter. The cells are curved rods with a length and width of approximately 1.25 and 0.5 μm and are extremely motile. The growth temperature ranges between15 and 35°C, with an optimum of 28-30°C. The pH range for growth is between 6.0 and 7.5. Non-halophilic, tolerates NaCl concentrations of 0-10 g L^{-1}. Obligate chemolitho-autotroph utilizing ferrous iron as the sole electron donor and oxygen as the only electron acceptor. The major fatty acids in the cellular polar lipids are 16:0, 16:1ω7c and 18:2ω6c,9c,

of which 16:0 constitutes 40.72% of the total. Isolated from an irregularly flooded iron-rich grassland using gradient tubes. The type strain is HDD^T (=JCM16104^T =NCCB10032^T). The 16S rRNA gene sequence GeneBank accession number is HQ263247.

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Chapter 6

General discussion and perspectives

Wetlands are essential ecosystems which play vital roles by harboring high biodiversity, purifying water, preventing soil erosion and flooding, and providing sites for recreation activities. They are also sites of rapid biogeochemical cycling due to the presence of extensive and steep redox gradients. The redox cycles of elements are directly related to the biological and geochemical functions of wetlands and, as such, redox processes are gaining wide attention (Borch et al., 2010). A key redox process is iron oxidation, which results in the formation of ferric iron (hydr)oxides that are abundantly found in the rhizosphere of wetland plants, as well as at other oxic-anoxic interfaces. In addition to acting as barriers of nutrient for plants, iron oxides are also known to sequester numerous metals or metalloids that may be toxic in soluble form, and to act as catalysts in contaminant degradation.

The recognition of iron-oxidizing bacteria (FeOB) dates back to the mid-1800s (c.f. (Hallbeck et al., 1993)). Though some early attempts were made to isolate these organisms (Kucera and Wolfe, 1957; Hanert, 1968), significant progress on microbial iron oxidation in circum-neutral pH environments has only been made since the 1990s (Emerson and Revsbech, 1994). The reasons for this are that ferrous iron oxidizes spontaneously and rapidly at neutral pH under oxic conditions, but also because of difficulties in isolating and cultivating FeOB. In the past decades, a number of studies have been carried out to identify and isolate iron-oxidizing bacteria from various environments (see **Introduction)**. By using the gradient tube method several phylogenetically different isolates were obtained from ground water (Emerson and Moyer, 1997), wetlands (Sobolev and Roden, 2004; Weiss et al., 2007) and the sea floor (Edwards et al., 2003b; Emerson et al., 2007). Batch and microcosm experiments suggest that iron-oxidizing bacteria can account for a large portion of total iron oxidation (Emerson and Revsbech, 1994; Neubauer et al., 2002; Sobolev and Roden, 2002).

Despite the importance of iron oxidation in wetland soils and sediments, we know little about the iron-oxidizing microbial communities in these environments. As formulated in the introductory chapter of this thesis, knowledge remains poor as to their abundance and diversity, their interactions with other microorganisms, and steering environmental processes. Therefore, the main objectives of this study were as follows.

1) To gain insight into the distribution of iron-oxidizing populations present in different wetland soils and sediments by developing novel culture-independent techniques.

2) To reveal the possible environmental factors controlling the community structure of iron-oxidizing bacteria (FeOB) and the interaction between iron oxidation and other redox processes.

3) To explore the spatial distribution of abundance and community structure of FeOB and of potentially steering environmental factors, among which the distribution of other chemolitho-autotrophic bacteria, in particular methane-oxidizing bacteria (MOB).

4) To isolate and characterize novel neutrophilic iron-oxidizing bacteria.

Construction of molecular tools and environmental applications

In this study, bacteria were enriched from various iron-rich soils and sediments, and clone libraries were constructed on the basis of these enrichments. The 16S rRNA sequences of the clone libraries showed that they belong to a diverse group of bacteria (**Chapter 2**). Despite their advantages of accuracy and efficiency, molecular techniques are hardly applied in the study of FeOB in environments of circum-neutral pH, mainly due to the lack of sequence information to design specific primers or probes for conservative regions of the 16S RNA genes (Konstantinidis and Tiedje, 2007) or for functional genes (Parro et al., 2007). Therefore, I started with enriching FeOB and developed 16S rRNA gene-based probes and primers, based on the clusters of interest (*i.e.* the clusters most closely related to known iron-oxidizing bacteria).

The primers and probes developed as part of my research can in principle be applied directly on environmental samples to study the distributions of FeOB. With one pair of these specific primers, I was indeed able to detect closely related sequences in the environment. This confirmed that the newly developed fingerprinting assay using nested PCR-DGGE was reliable and could be used to detect iron-oxidizing bacteria as described in the subsequent chapters. The results, which are based on the first use of molecular methods for FeOB, revealed that iron-oxidizing bacteria are quite abundant in the wetland

ecosystems investigated. The species compositions found at the Appels and Ewijk sites are comparable. In terms of absolute abundance, however, the irregularly flooded riparian soils at Ewijk exhibit higher 16S rRNA copy numbers than the tidal freshwater marsh sediments at Appels. Both sites have high concentrations of soil iron and are influenced by human activities. The Appels marsh is tidally flooded twice a day, which likely induce dynamic changes in redox condition, which in turn influence the structure of the iron-oxidizing bacterial community.

FeOB were detected in samples from both rhizosphere and bulk soils. It has been proposed that the rhizosphere is the ideal environment for microbial iron oxidation (Neubauer et al., 2002). Nonetheless, the comparative analyses of Weiss and coworkers have shown higher absolute abundances of aerobic, chemolithotrophic FeOB in bulk soil than in the rhizosphere (Weiss et al., 2003). In the latter study no clear difference in the composition of the iron-oxidizing bacteria community was further observed between bulk soil and rhizosphere. Neither the abundance nor the diversity alone could explain the differences in microbial iron oxidation rates between rhizosphere and bulk soils as the activity of bacteria remains unknown. Besides, there could be controlling factors on the community structures of iron-oxidizing bacteria other than the presence and activity of plant roots.

Environmental factors: wetland plants and tidal flooding

Previous work has shown that bacterial iron oxidation may be influenced by a number of soil variables, including chemical and physical factors (Neubauer et al., 2007). Also, the structural arrangement of iron hydroxide deposits varies among plant species (Hansel et al., 2001), which may subsequently cause variations in their role in metal sequestration. The differences in iron oxide formation may be indirectly influenced by plants though the activities of iron-oxidizing bacteria, yet no direct evidence of a link between plant activity and iron-oxidizing bacterial communities has so far been presented. Therefore, in **Chapter 3** I explore the distribution of iron-oxidizing bacteria at a number of sites colonized by different plant species in a tidal freshwater marsh near the village of Appels (Belgium). In addition, the diversity and distribution at three different sampling times was also investigated.

A significant temporal variation in the composition of the iron-oxidizing community was observed in the marsh sediments. Iron-oxidizing bacteria were most abundant during the spring sampling (April). Simultaneously, a high Fe-oxidizing activity was reflected by the accumulation of extractable iron(III)

oxides in the sediments. Sundby and co-workers (2003) attributed the higher iron-oxidizing activity in anoxic salt marsh sediments during spring to the penetration of growing and oxygen-releasing roots in the reduced soil. However, our sampling campaign in April occurred before the start of the growing season of the plants; fresh above-ground plant biomass still had to fully develop and, consequently, activity of the roots must have been minor. Hence, differences in the flooding regime between the upper and lower portions of the marsh and pore water irrigation by macrofauna are proposed to be the primary forcing on iron redox cycling in spring and, consequently, on the abundance and structure of the FeOB communities. Plant root activity, however, clearly influences the depth distributions of redox-active pore water and sediment chemical species.

A supplementary factor explaining the abundance of iron-oxidizing bacteria and the dynamics in the community structure could be the temperature variations during the year. High microbial iron oxidation rates at relatively low temperatures have been reported (Heinzel et al., 2009), implying that iron oxidizers might be competitively favored in relation to chemical oxidation at the relatively low sediment temperatures in April. This is in accordance with the observations of higher extractable soil Fe(III) and increased abundance of FeOB in samples taken in April compared to those in July and October.

Differences in diversity and relative abundance were observed with depth in the sediments and soils. Certain bands on the DGGE gels disappeared below 10 cm depth, while other bands appeared at deeper sediment layers, for which pore water geochemistry indicates ongoing iron and sulfate reduction. Transport of oxygen to the deeper parts of the sediments by biological or physical processes could offer an explanation for this phenomenon. An earlier study found that the distribution of ammonium-oxidizing bacteria in the non-vegetated intertidal sediment of the marsh at Appels was significantly impacted by burrow flushing by worms, which kept the sediment supplied with molecular oxygen, at least locally, till 10 cm depth (Coci et al., 2005). In this study the field data indeed showed worm tubes surrounded with iron oxides in the deeper sediment layers.

Under neutral pH and micro-aerobic conditions, FeOB are apparently able to compete for oxygen with the chemical process of iron oxidation that usually predominates at higher oxygen concentrations (Sobolev and Roden, 2001). However, this probably leads to another dilemma, i.e., the competition of FeOB for limiting amounts of oxygen with other chemical or biological oxidation processes.

Environmental factors: spatial distribution, irregular flooding and interacting bacteria.

In **Chapter 4,** the distribution of FeOB was studied in a riparian wetland that experiences irregular flooding. In order to account for the heterogeneity of the riparian soils, samples were collected at different distances from each other (spatial scales). As expected, differences in diversity and abundance of the FeOB communities were observed at different spatial scales. A clear trend in increasing abundance of iron-oxidizing bacteria was observed with elevation and hence, decreasing flooding intensity. The spatial sampling design enabled the application of geostatistical methods to interpolate FeOB abundance in the sampled plots. The distribution pattern of FeOB, as simulated for the whole sampling area clearly indicated a negative correlation with soil moisture content. Therefore, high numbers of FeOB prevailed in the "dry" parts of the wetland and low numbers in the "wet" parts. As also indicated by the results from the tidal flooded riverbank (**Chapter 3**), it seems that oxygen availability is an important factor, influencing the distribution of the FeOB in wetland soils.

I also compared the abundances of iron- and methane-oxidizing bacteria (**Chapter 4**). Hardly anything is known about the interaction between FeOB and other oxygen-consuming organisms. One of the consequences of the gradient in flooding regime in the riparian area sampled is a decreasing availability of oxygen with decreasing elevation and increasing flooding frequency and duration. On thermodynamic grounds it can be hypothesized that iron oxidation would out-compete methane oxidation under oxygen-limited conditions (see **Introduction**). The results presented in Chapter 4 show that both groups decrease in abundance with increasing soil moisture content. However, the FeOB seem to be more sensitive to moisture stress than the MOB. Hence, it was concluded that flooding-related factors other than oxygen availability affect the ecological interactions between iron- and methane-oxidizing bacteria.

Methane could have been a limiting factor for the methane oxidizers, which precludes any conclusions about a possible competition for oxygen. It has been suggested that CH_4 emission remains low if water-table fluctuations allow re-oxidation of ferrous iron, sustaining iron reduction as the most important process in terminal carbon mineralization (Jerman et al., 2009). The high abundance of iron-oxidizing bacteria at both medium and high elevations may maintain a high supply of oxidized iron for iron reducers (Blothe and Roden, 2009a), which could then out-compete methanogens thereby lowering the CH_4

supply, leading to the low abundances of methane oxidizers. The high clay content and compaction of the soils should favor the presence of anoxic microsites or "patches", where fast iron reduction may occur. This latter anaerobic process could happen even in the drier parts of the floodplain (Paul et al., 2006), especially after rainfalls. Therefore, O_2 may not have been the ultimate controlling factor in the distributions of iron and methane oxidizers. A dedicated study using defined cultures of iron- and methane-oxidizing bacteria in model systems such as microcosms or chemostats would provide information into the kinetic controls on microbial iron and methane oxidation that could help interpret in a more definitive way the interaction between both groups of chemolithotrophs.

A new iron-oxidizing organism

I have modified the commonly used gradient tube techniques and used it to isolate a new FeOB strain from an irregularly flooded riparian zone along the River Overijsselse Vecht, i.e., Huis den Doorn, the Netherlands (**Chapter 5**). Based on the phylogeny and physiology of the organism it is proposed to be a representative of a new genus of iron-oxidizing bacteria, i.e. *Ferrocurvibacter*. The strain shares a similar growth pattern with previously described isolates, but differs in its 16S rRNA sequence and the composition of phospholipid fatty acids.

Interestingly, the sequences detected with the designed primer set (**Chapter 2**) fall into the same cluster of the Betaproteobacteria as the isolate. It is likely that the isolate represents the most dominant iron-oxidizing bacterium at Huis den Doorn site, from which it was isolated. The differences in the dominant bands retrieved by PCR-DGGE from the sampling sites imply the presence of diverse uncultured iron-oxidizing bacteria and a variable community structure in the different wetland environments.

Iron oxidizers are phylogenetically quite diverse based on their 16S rRNA sequences (Weber et al., 2006; Emerson, 2010), making it almost impossible to design group-specific probes or primers. However, it is likely that FeOB share some common strategies in iron oxidation. To date the genomic analysis of *Acidothiobaccilus ferrooxidans* has revealed a full picture of iron oxidation pathways (Belnap et al., 2010; Cardenas et al., 2010). With a model strain it is possible to explore the genes or proteins involved in iron oxidation, which may then be applied to identify iron-oxidizing bacteria in environments. Working with isolates allows one to better understand the organisms' physiology and phylogeny (Emerson, 2010).

In conclusion, this thesis contributes to the knowledge about the ecology of FeOB by exploring their distribution in various wetlands and by enriching and isolating the organisms. The distributions of the FeOB correlate to soil properties and other environmental factors, including elevation and vegetation. This work also opens up opportunities for using molecular tools to study iron oxidation in circum-neutral pH environments, and to better assess the importance of this biogeochemical process in freshwater ecosystems, including wetlands. Based on the findings, new hypotheses and research questions are proposed as detailed in the following sections.

Perspectives

In the past decades there have been an increasing number of studies done on iron-oxidizing bacteria. The analysis of the 16S rRNA gene has shown that iron-oxidizing bacteria are phylogenetically diverse. They are distributed among the alpha-, beta-, gamma- and even zeta-subclasses of the *Proteobacteria.* Compared to other important microorganisms mediating elemental cycles, however, they are still rather poorly understood (Emerson, 2010). Lack of information on functional genes makes it difficult to study the ecology and functioning of these organisms and probably leads to an underestimation of the environmental importance of the group. The role of bacteria in the iron cycle needs to be further elucidated.

Remarkable progress has been made in detecting the key iron-oxidizing proteins in acidophilic iron-oxidizing bacteria. To boost the research in the field of iron oxidation at circum-neutral pH, similar work should be done on neutrophilic iron-oxidizing bacteria, assuming that the core metabolism is comparable for these two groups of iron oxidizers. After metagenomics, the development of proteomics and single cell genomics will help to learn more about the structures and functions of the microbial communities, as well as the physiology of iron-oxidizing bacteria.

Iron oxides can efficiently sorb heavy metals and metalloids, some of which are toxic in soluble forms. Arsenic in drinking water, for example, represents a worldwide health problem. Previous work has shown that some iron-oxidizing bacteria can effectively immobilize arsenic (Hohmann et al., 2010). However, the mechanisms involved in this process, as well as the interaction between bacteria, iron oxides, heavy metals and other nutrients, remains to be unraveled. Moreover, the abundance and diversity of the micoorganisms involved is largely unknown.

Improvement of cultivation methods is important. Since only a few isolates are available, it is not possible yet to select representative cultures. Only after successful isolation of many strains can we compare them and select those that are common in different environments or different regions for further study. Experiments with defined cultures of FeOB may elucidate the environmental effects on the behavior and niche differentiation of this group of microorganisms and help to better understand the interaction between iron-oxidizing bacteria and other bacteria. The success of isolation depends largely on the media preparation, and it is necessary to modify the known cultivation methods (Emerson and Floyd, 2005) and to seek out new techniques. Moreover, it is important to study the extent to which iron-oxidizing bacteria relate to the activity of other microorganisms, such as bacteria oxidizing CH_4, S^{2-}, and NH_4^+, and iron-reducing organisms.

References

Andrews, S.C., Robinson, A.K., and Rodriguez-Quinones, F. (2003) Bacterial iron homeostasis. *FEMS Microbiology Reviews* **27**: 215-237.

Appia-Ayme, C., Guiliani, N., Ratouchniak, J., and Bonnefoy, V. (1999) Characterization of an operon encoding two c-type cytochromes, an aa(3)-type cytochrome oxidase, and rusticyanin in *Thiobacillus ferrooxidans* ATCC 33020. *Applied and Environmental Microbiology* **65**: 4781-4787.

Armstrong (1967) Oxidising activity of roots in waterlogged soils. *Physiologia Plantarum* **20**: 920-926.

Armstrong, W. (1964) Oxygen diffusion from roots of some British bog plants. *Nature* **204**: 801-802.

Auernik, K.S., Maezato, Y., Blum, P.H., and Kelly, R.M. (2008) The genome sequence of the metal-mobilizing, extremely thermoacidophilic archaeon *Metallosphaera sedula* provides insights into bioleaching-associated metabolism. *Applied and Environmental Microbiology* **74**: 682-692.

Baeyens, W., van Eck, B., Lambert, C., Wollast, R., and Goeyens, L. (1998) General description of the Scheldt estuary. *Hydrobiologia* **366**: 1-14.

Baker, B.J., and Banfield, J.F. (2003) Microbial communities in acid mine drainage. *FEMS Microbiology Ecology* **44**: 139-152.

Baker, K.L., Langenheder, S., Nicol, G.W., Ricketts, D., Killham, K., Campbell, C.D., and Prosser, J.I. (2009) Environmental and spatial characterisation of bacterial community composition in soil to inform sampling strategies. *Soil Biology and Biochemistry* **41**: 2292-2298.

Batty, L.C., Baker, A.J.M., Wheeler, B.D., and Curtis, C.D. (2000) The effect of pH and plaque on the uptake of Cu and Mn in *Phragmites australis* (Cav.) Trin ex. Steudel. *Annals of Botany* **86**: 647-653.

Bazylinski, D.A., Dean, A.J., Williams, T.J., Long, L.K., Middleton, S.L., and Dubbels, B.L. (2004) Chemolithoautotrophy in the marine, magnetotactic bacterial strains MV-1 and MV-2. *Archives of Microbiology* **182**: 373-387.

Belnap, C.P., Pan, C., VerBerkmoes, N.C., Power, M.E., Samatova, N.F., Carver, R.L. et al. (2010) Cultivation and quantitative proteomic analyses of acidophilic microbial communities. *ISME Journal* **4**: 520-530.

Benz, M., Brune, A., and Schink, B. (1998) Anaerobic and aerobic oxidation of ferrous iron at neutral pH by chemoheterotrophic nitrate-reducing bacteria. *Archives of Microbiology* **169**: 159-165.

Beukema, J.J. (1974) Seasonal changes in the biomass of the macro-benthos of a tidal flat area in the Dutch Wadden Sea. *Netherlands Journal of Sea Research* **8**: 94-107.

Blothe, M., and Roden, E.E. (2009a) Microbial iron redox cycling in a circumneutral-pH groundwater seep. *Applied Environmental Microbiology* **75**: 468-473.

Blothe, M., and Roden, E.E. (2009b) Composition and activity of an autotrophic Fe(II)-oxidizing, nitrate-reducing enrichment culture. *Applied and Environmental Microbiology* **75**: 6937-6940.

Bodelier, P.L.E., and Laanbroek, H.J. (1997) Oxygen uptake kinetics of *Pseudomonas chlororaphis* grown in glucose- or glutamate-limited continuous cultures. *Archives of Microbiology* **167**: 392-395.

Bodelier, P.L.E., Libochant, J.A., Blom, C., and Laanbroek, H.J. (1996) Dynamics of nitrification and denitrification in root-oxygenated sediments and adaptation of ammonia-oxidizing bacteria to low-oxygen or anoxic habitats. *Applied Environmental Microbiology* **62**: 4100-4107.

Bodelier, P.L.E., Kamst, M., Meima-Franke, M., Stralis-Pavese, N., and Bodrossy, L. (2009a) Whole-community genome amplification (WCGA) leads to compositional bias in methane-oxidizing communities as assessed by pmoA-based microarray analyses and qPCR. *Environmental Microbiology Reports* **1**: 434-441.

Bodelier, P.L.E., Gillisen, M.J.B., Hordijk, K., Damste, J.S.S., Rijpstra, W.I.C., Geenevasen, J.A.J., and Dunfield, P.F. (2009b) A reanalysis of phospholipid fatty acids as ecological biomarkers for methanotrophic bacteria. *ISME Journal* **3**: 606-617.

Bodelier, P.L.E., Kamst, M., Meima-Franke, M., Stralis-Pavese, N., Bodrossy, L. (2009) Whole-community genome amplification (WCGA) leads to compositional bias in methane-oxidizing communities as assessed by *pmoA*-based microarray and qPCR. *Environmental Microbiology Reports* **1**: 434-441.

Bollmann, A., and Laanbroek, H.J. (2002) Influence of oxygen partial pressure and salinity on the community composition of ammonia-oxidizing bacteria in the Schelde estuary. *Aquatic Microbial Ecology* **28**: 239-247.

Bonneville, S., Behrends, T., and Van Cappellen, P. (2009) Solubility and dissimilatory reduction kinetics of iron(III) oxyhydroxides: A linear free energy relationship. *Geochimica et Cosmochimica Acta* **73**: 5273-5282.

Borch, T., Kretzschmar, R., Kappler, A., Van Cappellen, P., Ginder-Vogel, M., Voegelin, A., and Campbell, K. (2010) Biogeochemical redox processes and their impact on contaminant dynamics. *Environmental Science and Technology* **44**: 15-23.

Boschker, H.T.S., Nold, S.C., Wellsbury, P., Bos, D., de Graaf, W., Pel, R. et al. (1998) Direct linking of microbial populations to specific biogeochemical processes by C-13-labelling of biomarkers. *Nature* **392**: 801-805.

Bouchal, P., Zdrahal, Z., Helanova, S., Janiczek, O., Hallberg, K.B., and Mandl, M. (2006) Proteomic and bioinformatic analysis of iron- and sulfur-oxidizing *Acidithiobacillus ferrooxidans* using immobilized pH gradients and mass spectrometry. *Proteomics* **6**: 4278-4285.

Brown, D.A., Sherriff, B.L., Sawicki, J.A., and Sparling, R. (1999) Precipitation of iron minerals by a natural microbial consortium. *Geochimica et Cosmochimica Acta* **63**: 2163-2169.

Cardenas, J.P., Valdes, J., Quatrini, R., Duarte, F., and Holmes, D.S. (2010) Lessons from the genomes of extremely acidophilic bacteria and archaea with special emphasis on bioleaching microorganisms. *Applied Microbiology and Biotechnology* **88**: 605-620.

Carlos, C., Reis, F.C., Vicentini, R., Madureira, D.J., and Ottoboni, L.M.M. (2008) The rus operon genes are differentially regulated when *Acidithiobacillus ferrooxidans* LR is kept in contact with metal sulfides. *Current Microbiology* **57**: 375-380.

Castelle, C., Guiral, M., Malarte, G., Ledgham, F., Leroy, G., Brugna, M., and Giudici-Orticoni, M.T. (2008) A new iron-oxidizing/O_2-reducing super complex spanning both inner and outer membranes, isolated from the extreme acidophile *Acidithiobacillus ferrooxidans*. *Journal of Biological Chemistry* **283**: 25803-25811.

Chan, C.S., Fakra, S.C., Edwards, D.C., Emerson, D., and Banfield, J.F. (2009) Iron oxyhydroxide mineralization on microbial extracellular polysaccharides. *Geochimica et Cosmochimica Acta* **73**: 3807-3818.

Clarke, K.R., and Warwick, R.M. (1998) Quantifying structural redundancy in ecological communities. *Oecologia* **113**: 278-289.

Clarke, K.R., and Warwick, R.M. (2001) A further biodiversity index applicable to species lists: variation in taxonomic distinctness. *Marine Ecology Progress Series* **216**: 265-278.

Coci, M., Riechmann, D., Bodelier, P.L.E., Stefani, S., Zwart, G., and Laanbroek, H.J. (2005) Effect of salinity on temporal and spatial dynamics of ammonia-oxidising bacteria from intertidal freshwater sediment. *FEMS Microbiology Ecology* **53**: 359-368.

Cole, J.R., Chai, B., Farris, R.J., Wang, Q., Kulam, S.A., McGarrell, D.M. et al. (2005) The Ribosomal Database Project (RDP-II): sequences and tools for high-throughput rRNA analysis. *Nucleic Acids Research* **33**: D294-D296.

Colmer, T.D. (2003) Long-distance transport of gases in plants: A perspective on internal aeration and radial oxygen loss from roots. *Plant, Cell and Environment* **26**: 17-36.

Coram, N.J., van Zyl, L.J., and Rawlings, D.E. (2005) Isolation, sequence analysis, and comparison of two plasmids (28 and 29 kilobases) from the biomining bacterium *Leptospirillum ferrooxidans* ATCC 49879. *Applied And Environmental Microbiology* **71**: 7515-7522.

Costanza, R., dArge, R., deGroot, R., Farber, S., Grasso, M., Hannon, B. et al. (1997) The value of the world's ecosystem services and natural capital. *Nature* **387**: 253-260.

Cressie, N.A.C. (1993) Statistics for spatial data *Wiley-Interscience; Rev Sub edition*

Dick, G.J., Andersson, A.F., Baker, B.J., Simmons, S.L., Yelton, A.P., and Banfield, J.F. (2009) Community-wide analysis of microbial genome sequence signatures. *Genome Biology* **10**.

Doyle, M.O., and Otte, M.L. (1997) Organism-induced accumulation of iron, zinc and arsenic in wetland soils. *Environmental Pollution* **96**: 1-11.

Du Laing, G., Rinklebe, J., Vandecasteele, B., Meers, E., and Tack, F.M.G. (2009) Trace metal behaviour in estuarine and riverine floodplain soils and sediments: A review. *Science of the Total Environment* **407**: 3972-3985.

Duckworth, O.W., Holmstrom, S.J.M., Pena, J., and Sposito, G. (2009) Biogeochemistry of iron oxidation in a circumneutral freshwater habitat. *Chemical Geology* **260**: 149-158.

Edwards, K.J., Bach, W., and Rogers, D.R. (2003a) Geomicrobiology of the ocean crust: A role for chemoautotrophic Fe-bacteria. *Biological Bulletin* **204**: 180-185.

Edwards, K.J., Rogers, D.R., Wirsen, C.O., and McCollom, T.M. (2003b) Isolation and characterization of novel psychrophilic, neutrophilic, Fe-oxidizing, chemolithoautotrophic alpha- and gamma-Proteobacteria from the deep sea. *Applied and Environmental Microbiology* **69**: 2906-2913.

Edwards, K.J., Bach, W., McCollom, T.M., and Rogers, D.R. (2004) Neutrophilic iron-oxidizing bacteria in the ocean: Their habitats, diversity, and roles in mineral deposition, rock alteration, and biomass production in the deep-sea. *Geomicrobiology Journal* **21**: 393-404.

Ehrenreich, A., and Widdel, F. (1994) Anaerobic oxidation of ferrous iron by purple bacteria, a new type of phototrophic metabolism. *Applied and Environmental Microbiology* **60**: 4517-4526.

Emerson, D., and Revsbech, N.P. (1994) Investigation of an iron-oxidizing microbial mat community located near Aarhus, Denmark: Field studies. *Applied and Environmental Microbiology* **60**: 4022-4031

Emerson, D., and Moyer, C. (1997) Isolation and characterization of novel iron-oxidizing bacteria that grow at circumneutral pH. *Applied And Environmental Microbiology* **63**: 4784-4792.

Emerson, D., and Moyer, C.L. (2002) Neutrophilic Fe-Oxidizing bacteria are abundant at the Loihi Seamount hydrothermal vents and play a major role in Fe oxide deposition. *Applied and Environmental Microbiology* **68**: 3085-3093.

Emerson, D., and Weiss, J.V. (2004) Bacterial iron oxidation in circumneutral freshwater habitats: Findings from the field and the laboratory. *Geomicrobiology Journal* **21**: 405-414.

Emerson, D., and Floyd, M.M. (2005) Enrichment and isolation of iron-oxidizing bacteria at neutral pH. *Methods in Enzymology* **397**: 112-123

Emerson, D., Weiss, J.V., and Megonigal, J.P. (1999) Iron-oxidizing bacteria are associated with ferric hydroxide precipitates (Fe-plaque) on the roots of wetland plants. *Applied And Environmental Microbiology* **65**: 2758-2761.

Emerson, D., Rentz, J.A., Lilburn, T.G., Davis, R.E., Aldrich, H., Chan, C., and Moyer, C.L. (2007) A novel lineage of proteobacteria involved in formation of marine Fe-oxidizing microbial mat communities. *PLOS One* **2**: e667.

Emerson, E.J.F., and J. M. McBeth (2010) Iron-oxidizing bacteria: an environmental and genomic perspective. *Annual Review of Microbiology* **64**: 561-583.

Engel, H., and Hanert, H. (1967) Isolierung von *Gallionella ferruginea* ehrenberg. *Naturwissenschaften* **54**: 147-&.

Ettema, C.H., and Wardle, D.A. (2002) Spatial soil ecology. *Trends in Ecology & Evolution* **17**: 177-183.

Faulwetter, J.L., Gagnon, V., Sundberg, C., Chazarenc, F., Burr, M.D., Brisson, J. et al. (2009) Microbial processes influencing performance of treatment wetlands: A review. *Ecological Engineering* **35**: 987-1004.

Ferreira, T.O., Otero, X.L., de Souza, V.S., Vidal-Torrado, P., Macias, F., and Firme, L.P. (2010) Spatial patterns of soil attributes and components in a mangrove system in Southeast Brazil (So Paulo). *Journal of Soils and Sediments* **10**: 995-1006.

Ferris, F.G., Konhauser, K.O., Lyven, B., and Pedersen, K. (1999) Accumulation of metals by bacteriogenic iron oxides in a subterranean environment. *Geomicrobiology Journal* **16**: 181-192.

Ferris, F.G., Hallberg, R.O., Lyven, B., and Pedersen, K. (2000) Retention of strontium, cesium, lead and uranium by bacterial iron oxides from a subterranean environment. *Applied Geochemistry* **15**: 1035-1042.

Fortin, D. (2004) What biogenic minerals tell us. *Science* **303**: 1618-1619.

Fortin, D., and Langley, S. (2005) Formation and occurrence of biogenic iron-rich minerals. *Earth-Science Reviews* **72**: 1-19.

Fortin, D., Ferris, F.G., and Scott, S.D. (1998) Formation of Fe-silicates and Fe-oxides on bacterial surfaces in samples collected near hydrothermal vents on the Southern Explorer Ridge in the northeast Pacific Ocean. *American Mineralogist* **83**: 1399-1408.

Franklin, R.B., and Mills, A.L. (2003) Multi-scale variation in spatial heterogeneity for microbial community structure in an eastern Virginia agricultural field. *FEMS Microbiology Ecology* **44**: 335-346.

Frossard E., B.M., Hedley M. J. and Metherell A. (1995) Reactions controlling the cycling of P in soils. In *Phosphorus in the Global Environment, ed HTiessen John Wiley and Sons, New York.*

Gadd, G.M., and White, C. (1993) Microbial treatment of metal pollution - a working biotechnology. *Trends In Biotechnology* **11**: 353-359.

Ghiorse, W.C. (1984) Biology of iron- and manganese-depositing bacteria. *Annual review of microbiology* **38**: 515-550.

Gutknecht, J.L.M., Goodman, R.M., and Balser, T.C. (2006) Linking soil process and microbial ecology in freshwater wetland ecosystems. *Plant And Soil* **289**: 17-34.

Hafenbradl, D., Keller, M., Dirmeier, R., Rachel, R., Roßnagel, P., Burggraf, S. et al. (1996) *Ferroglobus placidus* gen. nov., sp. nov., a novel hyperthermophilic archaeum that oxidizes Fe^{2+} at neutral pH under anoxic conditions. *Archives of Microbiology* **166**: 308-314.

Hallbeck, L., and Pedersen, K. (1990) Culture parameters regulating stalk formation and growth-rate of *Gallionella ferruginea. Journal of General Microbiology* **136**: 1675-1680.

Hallbeck, L., and Pedersen, K. (1991) Autotrophic and mixotrophic growth of *Gallionella ferruginea*. *Journal of General Microbiology* **137**: 2657-2661.

Hallbeck, L., and Pedersen, K. (1995) Benefits associated with the stalk of *Gallionella-ferruginea*, evaluated by comparison of stalk-forming and a non-stalk forming strain and biofilm studies in-situ. *Microbial Ecology* **30**: 257-268.

Hallbeck, L., Stahl, F., and Pedersen, K. (1993) Phylogeny and phenotypic characterization of the stalk-forming and iron-oxidizing bacterium *Gallionella ferruginea*. *Journal of General Microbiology* **139**: 1531-1535.

Hallberg, K.B., and Johnson, D.B. (2005) Microbiology of a wetland ecosystem constructed to remediate mine drainage from a heavy metal mine. *Science of the Total Environment* **338**: 53-66.

Hallberg, R., and Ferris, F.G. (2004) Biomineralization by *Gallionella*. *Geomicrobiology Journal* **21**: 325-330.

Hanert, H. (1968) Investigations on isolation physiology and morphology of *Gallionella Ferruginea* ehernberg. *Archiv Fur Mikrobiologie* **60**: 348-&.

Hanert, H.H. (2002) Bacterial and chemical iron oxide deposition in a shallow bay on Palaea Kameni, Santorini, Greece: Microscopy, electron probe microanalysis, and photometry of in situ experiments. *Geomicrobiology Journal* **19**: 317-342.

Hansel, C.M., Fendorf, S., Sutton, S., and Newville, M. (2001) Characterization of Fe plaque and associated metals on the roots of mine-waste impacted aquatic plants. *Environmental Science and Technology* **35**: 3863-3868.

Hauck, S., Benz, M., Brune, A., and Schink, B. (2001) Ferrous iron oxidation by denitrifying bacteria in profundal sediments of a deep lake (Lake Constance). *FEMS Microbiology Ecology* **37**: 127-134.

Heinzel, E., Janneck, E., Glombitza, F., Schlomann, M., and Seifert, J. (2009) Population dynamics of iron-oxidizing communities in pilot plants for the treatment of acid mine waters. *Environmental Science and Technology* **43**: 6138-6144.

Heising, S., Richter, L., Ludwig, W., and Schink, B. (1999) *Chlorobium ferrooxidans* sp nov., a phototrophic green sulfur bacterium that oxidizes ferrous iron in coculture with a "*Geospirillum*" sp strain. *Archives of Microbiology* **172**: 116-124.

Hohmann, C., Winkler, E., Morin, G., and Kappler, A. (2010) Anaerobic Fe(II)-oxidizing bacteria show as resistance and immobilize as during Fe(III) mineral precipitation. *Environmental Science and Technology* **44**: 94-101.

Huey, B., and Hall, J. (1989) Hypervariable DNA fingerprinting in *Escherichia-Coli*-minisatellite probe from bacteriophage-M13. *Journal of Bacteriology* **171**: 2528-2532.

Hyacinthe, C., and Van Cappellen, P. (2004) An authigenic iron phosphate phase in estuarine sediments: composition, formation and chemical reactivity. *Marine Chemistry* **91**: 227-251.

Hyacinthe, C., Bonneville, S., and Van Cappellen, P. (2006) Reactive iron(III) in sediments: chemical versus microbial extractions. *Geochimica et Cosmochimica Acta* **70**: 4166-4180.

Jacob, D.L., and Otte, M.L. (2003) Conflicting processes in the wetland plant rhizosphere: Metal retention or mobilization? *Water, Air, and Soil Pollution: Focus* **3**: 91-104.

Jacobson, M.E. (1994) Chemical and biological mobilization of Fe(III) in marsh sediments. *Biogeochemistry* **25**: 41-60.

James, R.E., and Ferris, F.G. (2004) Evidence for microbial-mediated iron oxidation at a neutrophilic groundwater spring. *Chemical Geology* **212**: 301-311.

Jeon, B.H., Dempsey, B.A., and Burgos, W.D. (2003) Kinetics and mechanisms for reactions of Fe(II) with iron(III) oxides. *Environmental Science and Technology* **37**: 3309-3315.

Jerman, V., Metje, M., Mandic-Mulec, I., and Frenzel, P. (2009) Wetland restoration and methanogenesis: the activity of microbial populations and competition for substrates at different temperatures. *Biogeosciences* **6**: 1127-1138.

Jiao, Y.Q., Cody, G.D., Harding, A.K., Wilmes, P., Schrenk, M., Wheeler, K.E. et al. (2010) Characterization of extracellular polymeric substances from acidophilic microbial biofilms. *Applied and Environmental Microbiology* **76**: 2916-2922.

Juretschko, S., Loy, A., Lehner, A., and Wagner, M. (2002) The Microbial community composition of a nitrifying-denitrifying activated sludge from an industrial sewage treatment plant analyzed by the full-cycle rRNA approach. *Systematic and Applied Microbiology* **25**: 84-99.

Kamimura, K., Kunomura, K., Wakai, S., Murakami, K., and Sugio, T. (2001) Some properties of a novel obligately autotrophic iron-oxidizing bacterium isolated from seawater. *Hydrometallurgy* **59**: 373-381.

Kappler, A., and Newman, D.K. (2004) Formation of Fe(III)-minerals by Fe(II)-oxidizing photoautotrophic bacteria. *Geochimica et Cosmochimica Acta* **68**: 1217-1226.

Kappler, A., and Straub, K.L. (2005) Geomicrobiological cycling of iron. In *Molecular Geomicrobiology*, pp. 85-108.

Kappler, A., Schink, B., and Newman, D.K. (2005a) Fe(III) mineral formation and cell encrustation by the nitrate-dependent Fe(II)-oxidizer strain BoFeN1. *Geobiology* **3**: 235-245.

Kappler, A., Pasquero, C., Konhauser, K.O., and Newman, D.K. (2005b) Deposition of banded iron formations by anoxygenic phototrophic Fe(II)-oxidizing bacteria. *Geology* **33**: 865-868.

Kappler, A., Johnson, C.M., Crosby, H.A., Beard, B.L., and Newman, D.K. (2010) Evidence for equilibrium iron isotope fractionation by nitrate-reducing iron(II)-oxidizing bacteria. *Geochimica et Cosmochimica Acta* **74**: 2826-2842.

Kasama, T., and Murakami, T. (2001) The effect of microorganisms on Fe precipitation rates at neutral pH. *Chemical Geology* **180**: 117-128.

Katsoyiannis, I.A., and Zouboulis, A.I. (2004) Biological treatment of Mn(II) and Fe(II) containing groundwater: kinetic considerations and product characterization. *Water Research* **38**: 1922-1932.

Kemnitz, D., Chin, K.J., Bodelier, P., and Conrad, R. (2004) Community analysis of methanogenic archaea within a riparian flooding gradient. *Environmental Microbiology* **6**: 449-461.

Kolb, S., Knief, C., Stubner, S., and Conrad, R. (2003) Quantitative detection of methanotrophs in soil by novel pmoA- targeted real-time PCR assays. *Applied and Environmental Microbiology* **69**: 2423-2429.

Konstantinidis, K.T., and Tiedje, J.M. (2007) Prokaryotic taxonomy and phylogeny in the genomic era: advancements and challenges ahead. *Current Opinion in Microbiology* **10**: 504-509.

Koretsky, C.M., Van Cappellen, P., DiChristina, T.J., Kostka, J.E., Lowe, K.L., Moore, C.M. et al. (2005) Salt marsh pore water geochemistry does not correlate with microbial community structure. *Estuarine Coastal and Shelf Science* **62**: 233-251.

Kostka, J.E., and Luther, G.W. (1994) Partitioning and speciation of solid-phase iron in salt-marsh sediments. *Geochimica et Cosmochimica Acta* **58**: 1701-1710.

Kucera, S., and Wolfe, R.S. (1957) A selective enrichment method for *Gallionella ferruginea*. *Journal of Bacteriology* **74**: 344-349.

Kumaraswamy, R., Sjollema, K., Kuenen, G., van Loosdrecht, M., and Muyzer, G. (2006) Nitrate-dependent [Fe(II)EDTA]$^{2-}$ oxidation by P*aracoccus ferrooxidans* sp nov., isolated from a denitrifying bioreactor. *Systematic and Applied Microbiology* **29**: 276-286.

Laanbroek, H.J. (1990) Bacterial cycling of minerals that affect plant growth in waterlogged soils: a review. *Aquatic Botany* **38**: 109-125.

Laanbroek, H.J., and Speksnijder, A. (2008) Niche separation of ammonia-oxidizing bacteria across a tidal freshwater marsh. *Environmental Microbiology* **10**: 3017-3025.

Lack, J.G., Chaudhuri, S.K., Chakraborty, R., Achenbach, L.A., and Coates, J.D. (2002) Anaerobic biooxidation of Fe(II) by *Dechlorosoma suillum*. *Microbial Ecology* **43**: 424-431.

Lane, D.J. (1991) 16S/23S rRNA sequencing. Nucleic Acid Techniques in Bacterial Systematics, E. Stackebrandt and M. Goodfellow, eds. In *Wiley, New York*, pp. 115-175.

Laverman, A.M., Meile, C., Van Cappellen, P., and Wieringa, E.B.A. (2007) Vertical distribution of denitrification in an estuarine sediment: Integrating sediment flowthrough reactor experiments and microprofiling via reactive transport modeling. *Applied and Environmental Microbiology* **73**: 40-47.

Leduc, L.G., and Ferroni, G.D. (1994) The chemolithotrophic bacterium *Thiobacillus ferrooxidans*. *FEMS Microbiology Reviews* **14**: 103-119.

Legendre, P., and Legendre, L. (1998) Numerical ecology. *Elsevier Science BV*, Amsterdam.

Liger, E., Charlet, L., and Van Cappellen, P. (1999) Surface catalysis of uranium(VI) reduction by iron(II). *Geochimica et Cosmochimica Acta* **63**: 2939-2955.

Lin, B., Hyacinthe, C., Bonneville, S., Braster, M., Van Cappellen, P., and Roling, W.F.M. (2007) Phylogenetic and physiological diversity of dissimilatory ferric iron

reducers in sediments of the polluted Scheldt estuary, Northwest Europe. *Environmental Microbiology* **9**: 1956-1968.
Liu, W.J., and Zhu, Y.G. (2005) Iron and Mn plaques on the surface of roots of wetland plants. *Acta Ecologica Sinica* **25**: 358.
Loeb, R., Lamers, L.P.M., and Roelofs, J.G.M. (2008) Prediction of phosphorus mobilisation in inundated floodplain soils. *Environmental Pollution* **156**: 325-331.
Lovley, D.R. (1997) Microbial Fe(III) reduction in subsurface environments. *FEMS Microbiology Reviews* **20**: 305-313.
Lovley, D.R., and Phillips, E.J.P. (1987) Rrapid assay for microbially reducible ferric iron in aquatic sediments. *Applied and Environmental Microbiology* **53**: 1536-1540.
Lovley, D.R., and Phillips, E.J.P. (1988) Novel mode of microbial energy-metabolism - organic-carbon oxidation coupled to dissimilatory reduction of iron or manganese. *Applied and Environmental Microbiology* **54**: 1472-1480.
Lovley, D.R., Phillips, E.J.P., and Lonergan, D.J. (1991) Enzymatic versus nonenzymatic mechanisms for fe(iii) reduction in aquatic sediments. *Environmental Science and Technology* **25**: 1062-1067.
Lovley, D.R., Holmes, D.E., and Nevin, K.P. (2004) Dissimilatory Fe(III) and Mn(IV) reduction. In *Advances in Microbial Physiology,* **49**, 219-286.
Lovley, D.R., Stolz, J.F., Nord, G.L., and Phillips, E.J.P. (1987) Anaerobic production of magnetite by a dissimilatory iron-reducing microorganism. *Nature* **330**: 252-254.
Ludwig, W., Strunk, O., Westram, R., Richter, L., Meier, H., Yadhukumar et al. (2004) ARB: a software environment for sequence data. *Nucleic Acids Research* **32**: 1363-1371.
Lutters-Czekalla, S. (1990) Lithoautotrophic growth of the iron bacterium *Gallionella ferruginea* with thiosulfate or sulfide as energy source. *Arch Microbiol* **154**: 417-421.
Magnuson, T.S., Isoyama, N., Hodges-Myerson, A.L., Davidson, G., Maroney, M.J., Geesey, G.G., and Lovley, D.R. (2001) Isolation, characterization and gene sequence analysis of a membrane-associated 89 kDa Fe(III) reducing cytochrome c from *Geobacter sulfurreducens*. *Biochemical Journal* **359**: 147-152.
McLean, R.J.C., Fortin, D., and Brown, D.A. (1996) Microbial metal-binding mechanisms and their relation to nuclear waste disposal. *Canadian Journal of Microbiology* **42**: 392-400.
Mendelssohn, I.A., Kleiss, B.A., and Wakeley, J.S. (1995) Factors controlling the formation of oxidized root channels - a review. *Wetlands* **15**: 37-46.
Miot, J., Benzerara, K., Obst, M., Kappler, A., Hegler, F., Schadler, S. et al. (2009a) extracellular iron biomineralization by photoautotrophic iron-oxidizing bacteria. *Applied and Environmental Microbiology* **75**: 5586-5591.
Miot, J., Benzerara, K., Morin, G., Kappler, A., Bernard, S., Obst, M. et al. (2009b) Iron biomineralization by anaerobic neutrophilic iron-oxidizing bacteria. *Geochimica et Cosmochimica Acta* **73**: 696-711.
Mitsch, W.J., and Gosselink, J.G. (2000) The value of wetlands: importance of scale and landscape setting. *Ecological Economics* **35**: 25-33.
Muehe, E.M., Gerhardt, S., Schink, B., and Kappler, A. (2009) Ecophysiology and the energetic benefit of mixotrophic Fe(II) oxidation by various strains of nitrate-reducing bacteria. *FEMS Microbiology Ecology* **70**: 335-343.
Muyzer, G., Dewaal, E.C., and Uitterlinden, A.G. (1993) Profiling of complex microbial-populations by denaturing gradient gel-electrophoresis analysis of

polymerase chain reaction-amplified genes-coding for 16s ribosomal-RNA. *Applied and Environmental Microbiology* **59**: 695-700.

Neubauer, S.C., Emerson, D., and Megonigal, J.P. (2002) Life at the energetic edge: Kinetics of circumneutral iron oxidation by lithotrophic iron-oxidizing bacteria isolated from the wetland-plant rhizosphere. *Applied and Environmental Microbiology* **68**: 3988-3995.

Neubauer, S.C., Toledo-Duran, G.E., Emerson, D., and Megonigal, J.P. (2007) Returning to their roots: Iron-oxidizing bacteria enhance short-term plaque formation in the wetland-plant rhizosphere. *Geomicrobiology Journal* **24**: 65-73.

Newman, D.K. (2010) Feasting on Minerals. *Science* **327**: 793-794.

Nichols, P.D., Smith, G.A., Antworth, C.P., Hanson, R.S., and White, D.C. (1985) Phospholipid and lipopolysaccharide normal and hydroxy fatty-acids as potential signatures for methane-oxidizing bacteria. *FEMS Microbiology Ecology* **31**: 327-335.

Nicomrat, D., Dick, W.A., and Tuovinen, O.H. (2006) Microbial populations identified by fluorescence in situ hybridization in a constructed wetland treating acid coal mine drainage. *Journal of Environmental Quality* **35**: 1329-1337.

Nicorarat, D., Dick, W.A., Dopson, M., and Tuovinen, O.H. (2008) Bacterial phylogenetic diversity in a constructed wetland system treating acid coal mine drainage. *Soil Biology and Biochemistry* **40**: 312-321.

Osorio, H., Martínez, V., Veloso, F.A., Pedroso, I., Valdés, J., Jedlicki, E. et al. (2008) Iron homeostasis strategies in acidophilic iron oxidizers: Studies in *Acidithiobacillus* and *Leptospirillum*. *Hydrometallurgy* **94**: 175-179.

Parro, V., and Moreno-Paz, M. (2003) Gene function analysis in environmental isolates: The *nif* regulon of the strict iron oxidizing bacterium *Leptospirillum ferrooxidans*. *Proceedings of The National Academy of Sciences of The United States of America* **100**: 7883-7888.

Parro, V., Moreno-Paz, M., and Gonzalez-Toril, E. (2007) Analysis of environmental transcriptomes by DNA microarrays. *Environmental Microbiology* **9**: 453-464.

Paul, S., Kusel, K., and Alewell, C. (2006) Reduction processes in forest wetlands: Tracking down heterogeneity of source/sink functions with a combination of methods. *Soil Biology and Biochemistry* **38**: 1028-1039.

Pezeshki, S.R. (2001) Wetland plant responses to soil flooding. *Environmental and Experimental Botany* **46**: 299-312.

Philippot, L., Cuhel, J., Saby, N.P.A., Cheneby, D., Chronakova, A., Bru, D. et al. (2009) Mapping field-scale spatial patterns of size and activity of the denitrifier community. *Environmental Microbiology* **11**: 1518-1526.

Posth, N.R., Huelin, S., Konhauser, K.O., and Kappler, A. (2010) Size, density and composition of cell-mineral aggregates formed during anoxygenic phototrophic Fe(II) oxidation: Impact on modern and ancient environments. *Geochimica et Cosmochimica Acta* **74**: 3476-3493.

Poulain, A.J., and Newman, D.K. (2009) *Rhodobacter capsulatus* catalyzes light-dependent Fe(II) oxidation under anaerobic conditions as a potential detoxification mechanism. *Applied and Environmental Microbiology* **75**: 6639-6646.

Pruesse, E., Quast, C., Knittel, K., Fuchs, B.M., Ludwig, W.G., Peplies, J., and Glockner, F.O. (2007) SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. *Nucleic Acids Research* **35**: 7188-7196.

Ram, R.J., VerBerkmoes, N.C., Thelen, M.P., Tyson, G.W., Baker, B.J., Blake, R.C. et al. (2005) Community proteomics of a natural microbial biofilm. *Science* **308**: 1915-1920.

Rentz, J.A., Turner, I.P., and Ullman, J.L. (2009) Removal of phosphorus from solution using biogenic iron oxides. *Water Research* **43**: 2029-2035.

Ribeiro JR., P.J.a.D., P.J. (2001) geoR: A package for geostatistical analysis. *NEWS Vol 1, No 2 ISSN 1609-3631*.

Rivett, M.O., Buss, S.R., Morgan, P., Smith, J.W.N., and Bemment, C.D. (2008) Nitrate attenuation in groundwater: A review of biogeochemical controlling processes. *Water Research* **42**: 4215-4232.

Roden, E.E. (2004) Analysis of long-term bacterial vs. chemical Fe(III) oxide reduction kinetics. *Geochimica Et Cosmochimica Acta* **68**: 3205-3216.

Roden, E.E., and Wetzel, R.G. (1996) Organic carbon oxidation and suppression of methane production by microbial Fe(III) oxide reduction in vegetated and unvegetated freshwater wetland sediments. *Limnology and Oceanography* **41**: 1733-1748.

Roden, E.E., Sobolev, D., Glazer, B., and Luther, G.W. (2004) Potential for microscale bacterial Fe redox cycling at the aerobic-anaerobic interface. *Geomicrobiology Journal* **21**: 379-391.

Rohwerder, T., Gehrke, T., Kinzler, K., and Sand, W. (2003) Bioleaching review part A: Progress in bioleaching: fundamentals and mechanisms of bacterial metal sulfide oxidation. *Applied Microbiology and Biotechnology* **63**: 239-248.

Rozan, T.F., Taillefert, M., Trouwborst, R.E., Glazer, B.T., Ma, S., Herszage, J. et al. (2002) Iron-sulfur-phosphorus cycling in the sediments of a shallow coastal bay: Implications for sediment nutrient release and benthic macroalgal blooms. *Limnology and Oceanography* **47**: 1346-1354.

Sarazin, G., Michard, G., and Prevot, F. (1999) A rapid and accurate spectroscopic method for alkalinity measurements in sea water samples. *Water Research* **33**: 290-294.

Schafer, H., Bernard, L., Courties, C., Lebaron, P., Servais, P., Pukall, R. et al. (2001) Microbial community dynamics in Mediterranean nutrient-enriched seawater mesocosms: changes in the genetic diversity of bacterial populations. *FEMS Microbiology Ecology* **34**: 243-253.

Schubbe, S., Williams, T.J., Xie, G., Kiss, H.E., Brettin, T.S., Martinez, D. et al. (2009) Complete genome sequence of the chemolithoautotrophic marine *Magnetotactic Coccus* Strain MC-1. *Applied and Environmental Microbiology* **75**: 4835-4852.

Seys, J., Vincx, M., and Meire, P. (1999) Spatial distribution of oligochaetes (*Clitellata*) in the tidal freshwater and brackish parts of the Schelde estuary (Belgium). *Hydrobiologia* **406**: 119-132.

Shelobolina, E.S., Nevin, K.P., Blakeney-Hayward, J.D., Johnsen, C.V., Plaia, T.W., Krader, P. et al. (2007) *Geobacter pickeringii* sp nov., *Geobacter argillaceus* sp nov and *Pelosinus fermentans* gen. nov., sp nov., isolated from subsurface kaolin lenses. *International Journal of Systematic and Evolutionary Microbiology* **57**: 126-135.

Sobolev, D., and Roden, E.E. (2001) Suboxic deposition of ferric iron by bacteria in opposing gradients of Fe(II) and oxygen at circumneutral pH. *Applied and Environmental Microbiology* **67**: 1328-1334.

Sobolev, D., and Roden, E.E. (2002) Evidence for rapid microscale bacterial redox cycling of iron in circumneutral environments. *Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology* **81**: 587-597.

Sobolev, D., and Roden, E.E. (2004) Characterization of a neutrophilic, chemolithoautotrophic Fe(II)-oxidizing beta-proteobacterium from freshwater wetland sediments. *Geomicrobiology Journal* **21**: 1-10.

Sogaard, E.G., Aruna, R., Abraham-Peskir, J., and Bender Koch, C. (2001) Conditions for biological precipitation of iron by *Gallionella ferruginea* in a slightly polluted ground water. *Applied Geochemistry* **16**: 1129.

Spring, S. (2006) The Genera *Leptothrix* and *Sphaerotilus*. *The Prokaryotes* **5**: 758-777.

Steenbergh, A.K., Meima, M.M., Kamst, M., and Bodelier, P.L.E. (2010) Biphasic kinetics of a methanotrophic community is a combination of growth and increased activity per cell. *FEMS Microbiology Ecology* **71**: 12-22.

Straub, K.L., and Buchholz-Cleven, B.E.E. (1998) Enumeration and detection of anaerobic ferrous iron-oxidizing, nitrate- reducing bacteria from diverse European sediments. *Applied and Environmental Microbiology* **64**: 4846-4856.

Straub, K.L., Benz, M., and Schink, B. (2001) Iron metabolism in anoxic environments at near neutral pH. *FEMS Microbiology Ecology* **34**: 181-186.

Straub, K.L., Benz, M., Schink, B., and Widdel, F. (1996) Anaerobic, nitrate-dependent microbial oxidation of ferrous iron. *Applied and Environmental Microbiology* **62**: 1458-1460.

Straub, K.L., Schönhuber, W.A., Buchholz-Cleven, B.E.E., and Schink, B. (2004) Diversity of ferrous iron-oxidizing, nitrate-reducing bacteria and their involvement in oxygen-independent iron cycling. *Geomicrobiology Journal* **21**: 371.

Sudek, L.A., Templeton, A.S., Tebo, B.M., and Staudigel, H. (2009) Microbial ecology of Fe (hydr)oxide mats and basaltic rock from vailulu'u seamount, American Samoa. *Geomicrobiology Journal* **26**: 581-596.

Sundby, B., Vale, C., Caetano, M., and Luther, G.W. (2003) Redox chemistry in the root zone of a salt marsh sediment in the Tagus Estuary, Portugal. *Aquatic Geochemistry* **9**: 257-271.

Sung, W., and Morgan, J.J. (1980) Kinetics and product of ferrous iron oxygenation in aqueous systems. *Environmental Science and Technology* **14**: 561-568.

Suzuki, I. (2001) Microbial leaching of metals from sulfide minerals. *Biotechnology Advances* **19**: 119-132.

Tan, G.L., Shu, W.S., Zhou, W.H., Li, X.L., Lan, C.Y., and Huang, L.N. (2009) Seasonal and spatial variations in microbial community structure and diversity in the acid stream draining across an ongoing surface mining site. *FEMS Microbiology Ecology* **70**: 277-285.

R Development Core Team (2008) R: a language and environment for statistical computing [WWW document]. URL http://wwwR-projectorg.

Thauer, R.K., Jungermann, K., and Decker, K. (1977) Energy conservation in chemotrophic anaerobic bacteria. *Bacteriological Reviews* **41**: 100-180.

Thullner, M., Van Cappellen, P., and Regnier, P. (2005) Modeling the impact of microbial activity on redox dynamics in porous media. *Geochimica et Cosmochimica Acta* **69**: 5005-5019.

Tyson, G.W., Chapman, J., Hugenholtz, P., Allen, E.E., Ram, R.J., Richardson, P.M. et al. (2004) Community structure and metabolism through reconstruction of microbial genomes from the environment. *Nature* **428**: 37-43.

van Bodegom, P., Stams, F., Mollema, L., Boeke, S., and Leffelaar, P. (2001) Methane oxidation and the competition for oxygen in the rice rhizosphere. *Applied Environmental Microbiology* **67**: 3586-3597.

Van Der Nat, F.J.W.A., Middelburg, J.J., Van Meteren, D., and Wielemakers, A. (1998) Diel methane emission patterns from *Scirpus lacustris* and *Phragmites australis*. *Biogeochemistry* **41**: 1-22.

Vatter, A.E., and Wolfe, R.S. (1956) Electron microscopy of *Gallionella-ferruginea.* *Journal of Bacteriology* **72**: 248-252.

Verhagen, F.J.M., Duyts, H., and Laanbroek, H.J. (1992) Competition for Ammonium between nitrifying and heterotrophic bacteria in continuously percolated soil columns. *Applied Environmental Microbiology* **58**: 3303-3311.

Visser, E.J.W., Bogemann, G.M., Van de Steeg, H.M., Pierik, R., and Blom, C.W.P.M. (2000) Flooding tolerance of Carex species in relation to field distribution and aerenchyma formation. *New Phytologist* **148**: 93-103.

Wakai, S., Tsujita, M., Kikumoto, M., Manchur, M.A., Kanao, T., and Kamimura, K. (2007) Purification and characterization of sulfide : quinone oxidoreductase from an acidophilic iron-oxidizing bacterium, *Acidithiobacillus ferrooxidans*. *Bioscience Biotechnology and Biochemistry* **71**: 2735-2742.

Wang, J.J., Muyzer, G., Bodelier, P.L.E., and Laanbroek, H.J. (2009) Diversity of iron oxidizers in wetland soils revealed by novel 16S rRNA primers targeting *Gallionella*-related bacteria. *ISME Journal* **3**: 715-725.

Weber, K.A., Achenbach, L.A., and Coates, J.D. (2006) Microorganisms pumping iron: anaerobic microbial iron oxidation and reduction. *Nature Reviews Microbiology* **4**: 752-764.

Weber, K.A., Hedrick, D.B., Peacock, A.D., Thrash, J.C., White, D.C., Achenbach, L.A., and Coates, J.D. (2009) Physiological and taxonomic description of the novel autotrophic, metal oxidizing bacterium, *Pseudogulbenkiania* sp strain 2002. *Applied Microbiology and Biotechnology* **83**: 555-565.

Weiss, J.V., Emerson, D., and Megonigal, J.P. (2004) Geochemical control of microbial Fe(III) reduction potential in wetlands: comparison of the rhizosphere to non-rhizosphere soil. *FEMS Microbiology Ecology* **48**: 89-100.

Weiss, J.V., Emerson, D., and Megonigal, J.P. (2005) Rhizosphere iron(III) deposition and reduction in a *Juncus effusus* L.-dominated wetland. *Soil Science Society of America Journal* **69**: 1861-1870.

Weiss, J.V., Emerson, D., Backer, S.M., and Megonigal, J.P. (2003) Enumeration of Fe(II)-oxidizing and Fe(III)-reducing bacteria in the root zone of wetland plants: Implications for a rhizosphere iron cycle. *Biogeochemistry* **64**: 77-96.

Weiss, J.V., Rentz, J.A., Plaia, T., Neubauer, S.C., Merrill-Floyd, M., Lilburn, T. et al. (2007) Characterization of neutrophilic Fe(II)-oxidizing bacteria isolated from the rhizosphere of wetland plants and description of *Ferritrophicum radicicola* gen. nov sp nov., and *Sideroxydans paludicola* sp nov. *Geomicrobiology Journal* **24**: 559-570.

Widdel, F., Schnell, S., Heising, S., Ehrenreich, A., Assmus, B., and Schink, B. (1993) Ferrous iron oxidation by anoxygenic phototrophic bacteria. *Nature* **362**: 834-836.

Winderl, C., Anneser, B., Griebler, C., Meckenstock, R.U., and Lueders, T. (2008) Depth-resolved quantification of anaerobic toluene degraders and aquifer microbial community patterns in distinct redox zones of a tar oil contaminant plume. *Applied and Environmental Microbiology* **74**: 792-801.

Wollast, R. (1988) The Scheldt estuary. In Pollution of the North Sea: An Assessment. W., Blayne, B., Duursma, E., and Forstmer, U. (eds). *Berlin, Germany: Springer-Verlag,*: 183-193.

Yao Pan, L.B., Peter Frenzel, Anne-Grethe Hestnes, Sascha Krause,Claudia Lu¨ke, Marion Meima-Franke, Henri Siljanen,Mette M. Svenning, and Paul L. E. Bodelier (2010) Impacts of inter- and intralaboratory variations on the reproducibility of microbial community analyses. *Applied And Environmental Microbiology* **In press**.

Yeates, C., and Gillings, M.R. (1998) Rapid purification of DNA from soil for molecular biodiversity analysis. *Letters in Applied Microbiology* **27**: 49-53.

Yu, R., Gan, P., MacKay, A.A., Zhang, S.L., and Smets, B.F. (2010) Presence, distribution, and diversity of iron-oxidizing bacteria at a landfill leachate-impacted groundwater surface water interface. *FEMS Microbiology Ecology* **71**: 260-271.

Zhou, J.Z., Bruns, M.A., and Tiedje, J.M. (1996) DNA recovery from soils of diverse composition. *Applied and Environmental Microbiology* **62**: 316-322.

Summary

Wetland ecosystems are important as sites of rapid biogeochemical cycling of bioactive elements, among which iron features prominently. The redox cycling of iron exerts a strong influence on soil chemistry and the metabolism of plants and microorganisms. Studies have shown that bacteria play an important role in the process of iron oxidation in wetlands, although little is known about the ecology of these organisms, mainly due to the lack of molecular techniques to study them in their natural habitat.

This study explores the diversity and distribution of iron-oxidizing bacteria (FeOB) in soils and sediments of freshwater and brackish wetlands of circum-neutral pH, and analyzes the potential environmental factors influencing them. Gradient tubes were used to enrich FeOB from soil and sediment samples. From these enrichments, a clone library was established on the basis of the almost complete 16S rRNA gene. Specific probes and primers were developed using *Gallionella*-related sequences from this library. The newly designed *Gallionella*-specific 16S rRNA gene primer set was applied to community DNA obtained from three contrasting wetland environments, followed by Denaturing Gradient Gel Electrophoresis (DGGE) analysis and cloning. The retrieved 16S rRNA gene sequences yielded novel iron oxidizers most closely related to *Gallionella* spp.

A novel quantitative PCR (qPCR) assay was developed. In combination with the nested PCR-DGGE approach, it was used to delineate the spatial and temporal distributions of FeOB at a number of locations characterized by different plant species in a tidal freshwater marsh (Appels, Belgium). The presence of *Gallionella*-related FeOB was confirmed in vegetated and non-vegetated sediments of the tidal marsh. A high temporal variability of the composition of the iron-oxidizing community was observed. Iron-oxidizing bacteria flourished especially in early spring. The simultaneous accumulation of iron (hydr)oxides further indicated increased iron oxidizing activity. Overall, the results implied a highly dynamic FeOB community structure.

The spatial distribution of FeOB communities was also analyzed along a flooding gradient in an irregularly flooded riparian wetland (Ewijk, Netherlands). In addition, the co-occurrence of methane-oxidizing bacteria (MOB) was investigated, as MOB represent a biological component that may affect the distribution of FeOB under conditions of oxygen limitation. A clear trend of increasing abundance of FeOB was observed with increasing elevation and, hence, decreasing flooding intensity and soil moisture content. The abundance of FeOB exceeded that of MOB.

With the modified gradient tube method, we successfully isolated a novel FeOB strain, representing a new genus. This neutrophilic iron-oxidizing bacterium, *Ferrocurvibacter nieuwersluisensis* gen. nov., sp. nov., was isolated from an iron-rich grassland. This isolate belongs to the same group as detected by the specific primers.

In conclusion, this thesis describes novel findings about the occurrence of neutrophilic iron-oxidizing bacteria in a number of wetland ecosystems in the delta area of the Netherlands and Belgium, by characterizing the distribution of FeOB in various wetlands and by enriching and isolating these organisms. This work opens up new opportunities for using molecular tools to study microbial iron oxidation at circum-neutral pH and contributes to the knowledge of iron cycling in redox-stratified environments. Future work should focus on the spatial and temporal distributions of FeOB in additional environment settings, the mechanism of bacterial iron oxidation, and its interactions with other key biotic and abiotic processes, in particular microbial iron reduction.

Samenvatting

Wetlands worden gekenmerkt door snelle biogeochemische omzettingen, waaronder de oxidatie van ijzer. De oxidatie van ijzer is belangrijk vanwege haar sterke invloed op de chemie van de bodem en op het metabolisme van planten en micro-organismen. Studies hebben aangetoond dat bacteriën een belangrijke rol spelen in het proces van ijzeroxidatie. Over de ecologie van deze organismen bij neutrale pH is echter weinig bekend, hetgeen in belangrijke mate wordt veroorzaakt door een gebrek aan moleculaire technieken, die het mogelijk maken hen in hun natuurlijk milieu te bestuderen.

Het doel van deze studie was onderzoek te doen naar de diversiteit en de verspreiding van ijzeroxiderende bacteriën in zoetwater en brakke moerasgebieden van neutrale pH en naar de milieufactoren, die hierop van invloed zouden kunnen zijn. Voor het ophopen van ijzeroxiderende bacteriën werden z.g. gradiëntbuizen gebruikt. Uit de verschillende ophopingsculturen werd op basis van bijna het hele 16S rRNA gen een kloonbibliotheek gemaakt. Deze bibliotheek werd op haar beurt weer gebruikt voor de ontwikkelingen van probes en primers op basis van DNA-kenmerken, die gerelateerd zijn aan het bacteriegeslacht Gallionella. Natuurlijk DNA, dat afkomstig was uit drie contrasterende wetlandtypen, werd met behulp van de ontwikkelde primer set vermenigvuldigd en vervolgens geanalyseerd met de z.g. Denaturing Gradient Gel Electrophoresis (DGGE) techniek en met kloneren. De gevonden 16S rRNA genen behoorden allen tot nieuwe soorten ijzeroxiderende bacteriën, die verwant zijn aan Gallionella.

Op basis van dezelfde kloonbibliotheek werd ook een kwantitatieve PCR methode ontwikkeld. In combinatie met de nieuwe DGGE methode werd de verspreiding van ijzeroxiderende bacteriën bestudeerd in verschillende vegetatietypen in een getijde zoetwatermoeras langs de Schelde bij Appels in België. Behalve naar de ruimtelijke verspreiding werd ook gekeken naar veranderingen in de diversiteit en de aantallen in de tijd. De aanwezigheid van ijzeroxiderende bacteriën, die gerelateerd zijn aan Gallionella, werden zowel in de begroeide als in de niet-begroeide delen van het moeras gevonden. Ook bleek er een grote temporele variatie in de samenstelling van de ijzeroxiderende gemeenschap. IJzeroxiderende bacteriën kwamen vooral in het vroege voorjaar naar voren en tegelijkertijd was er een hoge ijzeroxiderende activiteit waarneembaar in de vorm van ophoping van geoxideerde ijzerdeeltjes in het sediment. Dit alles liet zien dat de samenstelling van en de activiteit van de ijzeroxiderend gemeenschap heel dynamisch was.

De ruimtelijk verspreiding van ijzeroxiderende, bacteriële gemeenschappen werd ook onderzocht in een wetland dat gelegen was in een onregelmatig

overstroomde uiterwaard van de Waal bij Ewijk in Nederland. Het verspreidingspatroon van deze bacteriegemeenschappen werd vergeleken met die van methaanoxiderende, microbiële gemeenschappen. Van beiden wordt aangenomen dat ze bij overstroming met elkaar concurreren om zuurstof. Van de ijzeroxiderende bacteriën werden hogere aantallen aangetroffen dan van de methaanoxiderende bacteriën. De aantallen ijzeroxiderende bacteriën namen bovendien dramatisch toe met de hoogte in het wetland en correleerden negatief met het vochtgehalte van de bodem.

Met de gradiëntbuizen werd tenslotte uit een ijzerrijk en vochtig grasland een nieuwe ijzeroxiderende bacteriestam geïsoleerd, die een nieuw geslacht vertegenwoordigt. Deze ijzeroxiderende bacterie, Ferrocurvibacter nieuwersluisensis gen. nov., sp. nov., behoort tot dezelfde groep waartoe ook de soorten behoren, waarop de kloonbibliotheek van ijzeroxiderende bacteriën is gebaseerd.

Concluderend kan worden gezegd dat dit proefschrift de eerste studie is die het voorkomen van ijzeroxiderende bacteriën in wetlands met een neutrale pH in het deltagebied van Nederland en België beschrijft. De resultaten van het onderzoek zijn gebaseerd op het ophopen en isoleren van deze bacteriën. Met de daaruit, nieuw ontwikkelde, moleculaire technieken, die gebaseerd zijn op kloonbibliotheken van opgehoopte en geïsoleerde ijzeroxiderende bacteriën, worden nieuwe wegen geopend voor onderzoek naar de mechanismen van ijzeroxidatie bij neutrale pH. Het draagt zodoende bij aan vergroting van de kennis over de rol van microorganismen in de kringloop van ijzer. Toekomstig werk zou zich moeten richten op de verspreiding van deze organismen in verschillende milieus, het mechanisme van bacteriële ijzeroxidatie en de interactie tussen deze organismen en andere natuurlijke biotische en niet-biotische processen.

摘　要

湿地不仅具有丰富的生物多样性，而且可以净化污水、防止土壤冲蚀和洪水，是地球上重要的生态系统。其淹水的特殊状况决定了各种化学元素的迅速转变。铁循环是湿地重要的地球化学过程之一。铁的氧化还原深刻影响着土壤化学、植物及微生物代谢过程。研究表明，在自然界铁化过程中细菌的作用至关重要。然而，由于研究手段如分子生物学技术应用的欠缺，对该类微生物生态学的研究目前尚处于初级阶段。

本论文研究了中性铁氧化细菌（FeOB）在土壤及淡、咸水湿地沉积物中的分布，包括数量与多样性，并分析了影响其群落分布的环境因素。应用铁氧梯度试管方法，富集与分离了铁氧化细菌。基于此建立了16S rRNA基因文库，并设计了针对*Gallionella*属铁氧化细菌的基因探针与引物。结合变性梯度凝胶电泳（DGGE）和基因克隆技术，该引物被成功用于探测此类细菌在三种不同湿地（不定期淹水湿地，受潮汐影响淡水湿地，受潮汐影响咸水湿地）中铁氧化细菌的群落分布情况。

此外，本论文还建立了定量PCR测定方法。与之前的PCR-DGGE方法相结合，研究了一受潮汐影响淡水湿地（Appels，比利时）的铁氧化细菌的空间与时间分布变化。在有植被与无植被覆盖条件下均检测到了铁氧化细菌。这类细菌最大数量值出现在四月初的采样。该结果与大量的铁氧化产物积累也有一定相关。结果表明，铁氧化细菌群落的时空变异性较大。

在另一个无定期淹水河岸湿地（Ewijk，荷兰），同时也研究了FeOB群落的空间分布特征，并将其数量与甲烷氧化细菌（MOB）相比较。甲烷氧化细菌与中性铁氧化细菌的生长环境相似，均喜好微氧环境。可以假定，这两种微生物在某种程度上存大一定的消长关系。结果表明，铁氧化细菌的数量随河岸离水面高度增加而增加，与淹水强度及土壤湿度呈负相关。在各种条件下，铁氧化细菌数量均高于甲烷氧化细菌，两者随河岸高度变化趋势一致。

本研究采用改进的铁氧梯度试管装置，成功地分离一种新型的中性铁氧化细菌菌株。该菌从含铁量高的淹水草地土壤中分离，被命名为*Ferrocurvibacter nieuwersluisensis* gen. nov., sp. nov.。之前用引物从各湿地环境检测到的新序列，与此菌株接近，为同一分类单元。

综上所述，本论文通过对湿地铁氧化细菌的富集分离培养和对其在环境中分布的研究，描述了荷兰与比利时三角洲一些湿地生态系统的中性铁细菌的新发现。该工作率先使用分子生物学技术研究中性条件下微生物氧化铁过程，对淹水环境铁循环过程相关科学知识有一定的贡献。未来的相关研究应着重于在其它环境中铁氧化细菌的时空分布特征、微生物铁氧化机制及其与其它重要生物及化学过程，尤其是其与铁还原微生物过程的相互关系。

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Curriculum Vitae

Juanjuan Wang was born on July 24th, 1979 in Sihong, China. She did her Bachelor study in soil and agro-chemistry at Yangzhou University, China. In 2000 she started a Master study in Botany at the same University. The subject of this Master study was the effect of different forms of nitrogen fertilizers and water supply on the growth characteristics of rice and the environments. During the study she also taught Bachelor courses for two semesters.

After graduation, she followed a second Master program in Environmental Biology at Leiden University, the Netherlands. Two projects, 'Dutch farmland biodiversity' and 'Taxonomy of bio-control bacteria active against *Fusarium oxysporum*' were involved in this Master degree.

Following that she started as PhD student at the Netherlands Institute of Ecology (NIOO-KNAW). The project aimed at understanding the diversity and distribution of iron-oxidizing bacteria in neutrophilic wetlands. During her PhD program she attended several graduate courses and taught in the Aquatic Ecology BSc courses. She paid visits and presented her work to (inter)national labs and various conferences in China, Europe and the USA.

Since November 1st 2010 she has been working at University of Tübingen, Germany.

Publications

Wang J., Muyzer G., Bodelier P. L. E., and Laanbroek H. J. (2009): Diversity of iron oxidizers in wetland soils revealed by novel primers targeting *Gallionella*-related bacteria. The ISME Journal, **3**, 715-725.

Wang J., Vollrath S., Behrends T., Bodelier P. L. E., Muyzer G., Meima-Franke M., den Oudsten F., Van Cappellen P., Laanbroek H. J. Spatial and temporal aspects of diversity and abundance of *Gallionella*-like neutrophilic iron oxidizers in a tidal freshwater marsh. Applied and Environmental Microbiology, under review.

Wang J., Muyzer G., Bodelier P. L. E., and Laanbroek H. J. *Ferricurvibacter nieuwersluisensis* gen. nov., sp. nov., a neutrophilic iron-oxidizing bacterium from an irregularly flooded grassland. International Journal of systematic and evolutionary Microbiology, under review

Wang J., Meima-Franke M., Muyzer G., den Oudsten F., Laanbroek H. J., Bodelier P. L. E. Spatial patterns of iron-oxidizing bacterial community in an irregularly flooded wetland, in preparation

Validov S., Kamilova F., Qi S., Stephan D., **Wang J**., Makarova N. and Lugtenberg B. (2007): Selection of bacteria able to control *Fusarium oxysporum f. sp. radicis- lycopersici* in stonewool substrate. Journal of Applied Microbiology **102**, 461-471.

Qian X., Shen Q., Xu G., **Wang J**. (2004): Nitrogen form effects on yield and nitrogen uptake of rice crop grown in aerobic soil. Journal of Plant Nutrition **27**, 1061-1076.