7

Living on Iron

Stefanie Becker, Allison M. L. Enright, and Andreas Kappler

Geomicrobiology, Center for Applied Geoscience, University of Tübingen,
Schnarrenbergstrasse 94–96, D-72076 Tübingen, Germany
<andreas.kappler@uni-tuebingen.de>

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Abstract: Reduced and oxidized iron is present in virtually all of Earth's environments. Iron is essential to all living organisms because it is a critical component of many biomolecules. It can also be used as an electron donor or terminal electron acceptor by microorganisms for metabolic redox reactions which generate energy and drive growth. In this chapter we introduce the environmental distribution and redox activity of iron and discuss how different types of Fe(II)-oxidizing (aerobic, microaerophilic, anoxygenic phototrophic, and anaerobic nitrate-reducing) and Fe(III)-reducing (ammonium-oxidizing, organic matter-oxidizing, methanotrophic, sulfur-oxidizing) microorganisms use the oxidation and reduction of Fe(II) and Fe(III), respectively, to generate energy and to produce biomass. In addition, we present some of the many biotechnological and environmental applications of iron-cycling microorganisms.

Keywords: biogeochemical iron cycling · geomicrobiology · heavy metal sequestration · microbial Fe(II) oxidation · microbial Fe(III) reduction

1. INTRODUCTION

1.1. Iron Speciation

Iron is present in the environment in the form of poorly crystalline and crystalline iron minerals, dissolved iron-organic-matter complexes, colloids, as well as dissolved ions [1]. The two main redox species relevant for environmental processes and biogeochemical iron cycling are Fe(III) and Fe(II) species. At pH 5.0–8.0 (circumneutral pH), Fe(III) is poorly soluble, with solubility products for Fe(III) minerals in the range of 10^{-38}–10^{-42}. Because of this, in the absence of Fe(III)-complexing ligands, concentrations of dissolved Fe^{3+} at circumneutral pH are in the low nM range. In contrast, at circumneutral pH, Fe(II) is relatively

soluble. Environmental concentrations of dissolved Fe^{2+} can be on the order of high μM or even low mM. This solubility is impacted by the rapid abiotic oxidation of Fe(II) in the presence of O_2; in fully aerated water, the half-life of Fe(II) is ~15 minutes [2]. By contrast, at pH < 4.0, both Fe^{3+} and Fe^{2+} are soluble, which makes iron redox transformations even more energetically favorable in low pH environments.

1.2. Iron Mineralogy

Fe(II) and Fe(III) mineral species are important to environmental microbiology, because of their global ubiquity, redox-activity, and their impact on biogeochemistry. Iron-bearing minerals present in soils and sediments include Fe(III) (oxyhydr)oxides, such as ferrihydrite ($Fe_{10}O_{14}(OH)_2$) [3], goethite (α-FeOOH), Fe(III) oxides (e.g., hematite, α-Fe_2O_3), and mixed-valent Fe(II)-Fe(III) oxides including magnetite (Fe_3O_4) and various green-rusts [4]. Common non-oxide Fe(II) minerals include siderite ($FeCO_3$), vivianite ($Fe_3(PO_4)_2$), pyrite (FeS_2), and mackinawite (FeS), as well as detrital minerals such as biotite, hornblende, and iron-containing clay minerals (e.g., illite, smectite, chlorite) [5]. Many iron-bearing minerals form as secondary phases during rock weathering, i.e., by a combination of physical, chemical, and biological processes.

The crystallinity and oxidation state of these minerals also have a dramatic impact on the bioavailability, especially in the case of Fe(III) [6], where being limited to solid-state electron acceptors means that the electron transfer processes must take place outside the cell, as the solid substrate cannot be assimilated into cells [6, 7]. Semi-conductive minerals, such as hematite and magnetite, can function as conductors to transfer electrons between different microbial species [7, 8].

1.3. The Effect of Iron Redox Potentials on Energy-Yielding Processes

Fe(II) can be oxidized to provide an electron for either: (i) chemolithotrophic microbial processes coupled to the reduction of O_2 or NO_3^-, and CO_2 fixation; or, (ii) photosynthesis and CO_2 fixation. By contrast, Fe(III) can act as a terminal electron acceptor for anaerobic respiration coupled to oxidation of organic and inorganic compounds (e.g., H_2, OM, NH_4^+, CH_4).

The potential of the Fe(II)/Fe(III) redox couple, and therefore the energy available for microbial processes, depends on the iron speciation and pH. It ranges from less than −400 mV (strongly reducing) to more than +700 mV (strongly oxidizing). This means that the iron species of both the oxidant and reductant effect the energetics of their corresponding chemotrophic process.

In the case of Fe(II) oxidation, the energy released is determined in part by whether the reductant is dissolved Fe^{2+}, a Fe(II)-organic matter (OM) complex, or a Fe(II)-containing mineral. Similarly, in case of Fe(III)-reduction, where

Fe(III) acts as the terminal electron acceptor, the energy released is determined in part by whether Fe^{3+} is dissolved (i.e., pH < 4.0), or present in either poorly crystalline or crystalline Fe(III) minerals (i.e., ferrihydrite or goethite; pH > 4.0).

Generally, more crystalline minerals such as hematite or goethite have lower (less positive/more negative) redox potentials and thus provide less energy than less crystalline minerals such as ferrihydrite or even dissolved Fe(III)-OM complexes.

In the case of photoautotrophic Fe(II)-oxidizing processes, the energy driving the redox processes is derived from light. As such, this process is not thermodynamically dependent on the oxidation of Fe(II). Instead, the amount of CO_2 fixed (and biomass produced) is stoichiometrically linked to the amount of the electron donor oxidized (see Section 2.2.1; Equation 6).

1.4. Significance of Iron to Global-Scale Biogeochemical Processes

Until the oxidation of Earth's oceans ca. 2.4 Ga, dissolved Fe(II) was highly concentrated in the oceans. This early abundance is thought to make Fe(II) oxidation one of the most ancient metabolic pathways exploited by microbes. This longevity, as well as the perpetual abundance of iron in modern terrestrial environments, has the consequence of linking the microbial iron cycle to global biogeochemical cycling of nitrogen, carbon, phosphate, oxygen, and sulfur [9–11]. The relatively large amount of energy available in the iron redox state transformations is likely the reason that microbes adopted Fe(II) as electron donor in phototrophic or chemosynthetic processes or Fe(III) as electron acceptor in anaerobic respiration [11]. It is important to note that high concentrations of iron are not always necessary to indicate the importance of biogeochemical iron cycling in a certain habitat. It has been shown that iron, and in particular, Fe-OM complexes, can be efficiently cycled by microbial and abiotic Fe(II)-oxidizing and Fe(III)-reducing processes [12–14]. An illustration of several mechanisms of microbial iron cycling is presented in Figure 1.

2. MICROBIAL GROWTH BY OXIDIZING IRON(II)

2.1. Chemolithotrophic Iron(II) Oxidation

Chemolithotrophic Fe(II)-oxidizing microorganisms gain energy from a redox reaction where Fe(II) is oxidized to Fe(III) and donates electrons to an oxidant with a more positive redox potential. The two-half reactions are spatially separated and coupled by an electron transport chain through the cytoplasmic membrane. During the process of electron transport, energy is released by redox reactions and coupled to the dislocation of H^+ over the cytoplasmic membrane, building up or maintaining the H^+ concentration gradient. This results in an

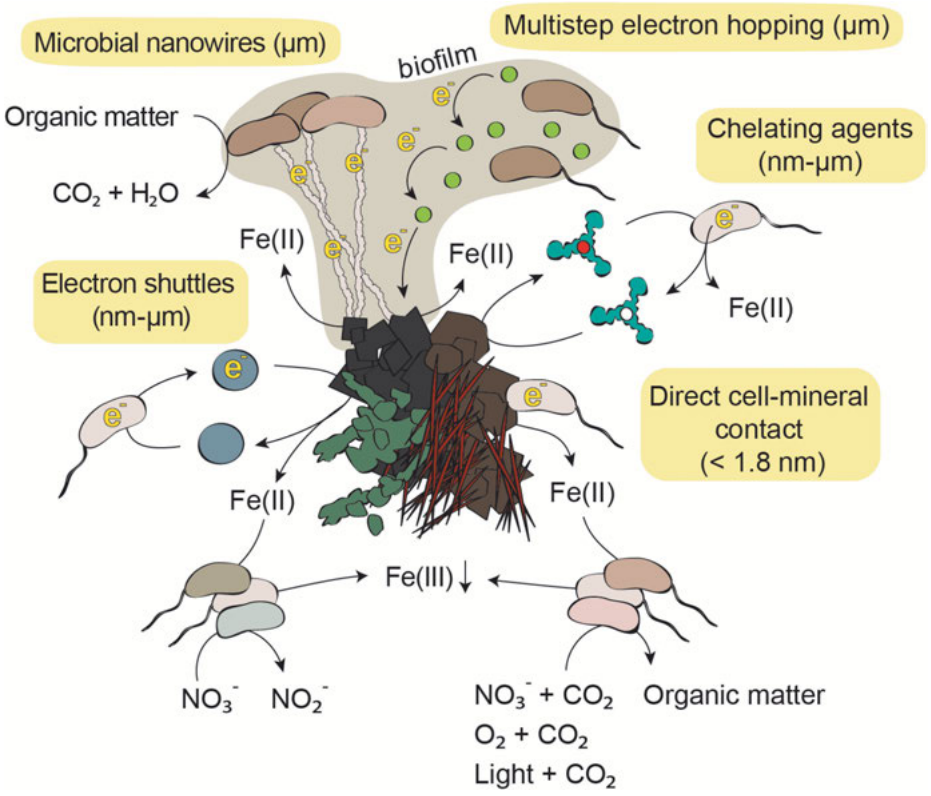

Figure 1. Illustration of processes involved in electron exchange between microbes, iron minerals, and dissolved iron. Mechanisms of electron transfer utilized for microbial Fe(III) reduction are highlighted in yellow. **Yellow top left**: Cells use microbial nanowires to directly attach to solid Fe(III)-bearing substrates, these nanowires are conductive. **Yellow top right**: Cells transfer electrons to redox-active intermediates, the electrons then can hop along a chain of small molecules which are quickly reoxidized and can cycle more electrons. **Yellow bottom left**: Cells donate electrons to redox-active electron shuttles, which in turn transfer electrons to the Fe(III)-bearing mineral substrate. **Yellow middle right**: Cells produce chelating ligands that complex with Fe(III), making it available for microbial reduction. **Yellow bottom right**: Cells in direct contact with their substrates of interest can transfer electrons directly to mineral surfaces. **Bottom left**: Fe(II) solely used as energy source by nitrate-dependent iron-oxidizing bacteria. **Bottom right**: Fe(II) used as electron donor for CO_2 fixation in organic matter by nitrate-dependent iron-oxidizing bacteria, microaerophilic and acidophilic bacteria, and phototrophs.

electrochemical proton gradient over the membrane and consequently to a proton motive force (PMF). The PMF powers an ATP synthase regenerating ATP by the phosphorylation of ADP. This mechanism of energy conservation is called electron transport-linked phosphorylation.

Considering redox processes like an energetic tower, electrons will move down a gradient of electrochemical potential to their oxidant in the course of electron

transport-linked phosphorylation. This is called a down-hill electron pathway. Anabolic processes (e.g., carbon assimilation) are essential for cell growth and proliferation, and require reducing agents, in particular, NADPH. Since these molecules are regenerated by reduction, a cell needs a consistent supply of electrons. However, the reducing potential of Fe(II) is insufficient to reduce $NAD(P)^+$ (NAD^+ or $NADP^+$). As such, the PMF is used to both: (i) to regenerate ATP, and, (ii) to power the up-hill pathway of $NAD(P)^+$ reduction. This mechanism is called reverse electron transport. The process of both down-hill (exergonic) and up-hill (endergonic) pathway is a bifurcated electron-transport chain.

2.1.1. Acidophilic Iron(II) Oxidation

2.1.1.1. Iron(II)-Driven Energy Conservation

For acidophilic Fe(II)-oxidizers, O_2 functions as a terminal electron acceptor in the energy conservation pathway (Equation 1). The half-reaction of Fe(II) oxidation takes place at the outer membrane (~pH 2) while the half-reaction of O_2 reduction takes place in the pH neutral cytoplasm (~pH 6.5) (Equation 1).

$$4\,Fe^{2+} + O_2 + 4\,H^+ \rightarrow 4\,Fe^{3+} + 2\,H_2O \quad (1)$$

Considering the pH dependence of redox potentials, the overall redox reaction seems thermodynamically unfavorable *(*$E_{m\ pH2}$ Fe(II)/Fe(III): ~ +0.77 V; $E_{m\ pH6.5}$ O_2/H_2O: ~ +0.82 V). However, as the reduction of O_2 to H_2O is localized in an environment with an overall pH of 2, the redox potential of the O_2/H_2O couple increases by 0.3 V ($E_{m\ pH2}$ O_2/H_2O: ~ +1.12 V). Because of this, the redox gradient of Fe(II) oxidation to Fe(III) and O_2 reduction to H_2O is positive and an overall exergonic reaction [15–17]. Additionally, the formation of H_2O from the reaction of reduced O_2 with protons, neutralizes protons that have entered the cell via the ATPase complex coming from a pH 2 environment. For this reason, the oxidative phosphorylation does theoretically not require additional H^+ transport against the pH gradient, although it is possible that the final oxidase translocates H^+ over the cytoplasmic membrane [16].

Maintaining a circumneutral cytoplasmic pH is a challenge for all acidophiles [18], and pH homeostasis in acidophiles was reviewed by Baker-Austin and Dopson in 2007 [19]. A neutral cytoplasmic pH is accomplished by maintaining a positive intracellular electrical potential; known as an inverted transmembrane electrical potential. Consequently, the electrical potential ($\Delta\Psi$) is unfavorable for the potential energy of the PMF, however, the net result facilitates a chemiosmotic mechanism of energy conservation. This means the PMF is entirely due to the pH gradient. In contrast, in neutrophiles, both the chemical potential (ΔpH) and the electrical potential ($\Delta\Psi$) contribute to the PMF. Down-hill electron transport supporting ATP synthesis and up-hill electron transport supporting reconstitution of reducing equivalents (e.g., NAD(P)H), are both connected to proton flux. Therefore, it is thought that these pathways are homeostatically regulated [20].

The genomics of Fe(II) oxidation and iron uptake strategies of acidophiles were reviewed by Bonnefoy and Holmes [20, 21]. Here, we discuss *Acidithiobacillus ferrooxidans,* the best-studied representative of the Fe(II)-oxidizing acidophiles. Multi-omic studies on *Leptospirillum ferriphilum* have been published by Christel et al. [22].

2.1.1.2. Environmental Distribution

Because abiotic Fe(II) oxidation (Fe(II) autoxidation by O_2) occurs rapidly at alkaline and neutral pH, it has been difficult to quantify the contribution of microbial Fe(II) oxidation, i.e., enzymatically catalyzed Fe(II) oxidation, at neutral pH. As such, aerobic microbial Fe(II) oxidation has been a subject of controversy for decades [23]. In contrast, this has not been an issue for the research on acidophilic Fe(II)-oxidizers [24, 25]. Acidophilic Fe(II)-oxidizers include members of the domain Bacteria and Archaea and have been identified in mesophilic environments such as mine drainage water [26] and thermophilic environments, such as solfatara fields and marine hydrothermal systems [27]. Examples for microorganisms isolated from these environments are listed in Table 1.

Table 1. Examples of mesophilic and thermophilic acidophilic aerobic Fe(II)-oxidizing microorganisms, including Bacteria and Archaea.

Bacteria		**Reference**
Mesophiles	*Acidithiobacillus ferrooxidans*	[23]
	Acidithiobacillus ferridurans	[288]
	Acidithiobacillus prosperus	[289]
	Leptospirillum ferrooxidans	[290]
	Metallogenium	[291]
	Ferromicrobium acidophilum	[292]
	Ferrovum myxofaciens	[293]
Thermophiles	*Sulfobacillus thermosulfidooxidans*	[294]
	Sulfobacillus acidophilus	[295, 296]
	Acidimicrobium ferrooxidans	[297, 298]
	Acidiferrobacter thiooxydans	[299]
Archaea		
Mesophiles	*Ferroplasma acidiphilum*	[300]
	Ferroplasma acidarmanus	[301]
	Ferroplasma thermophilum	[302]
Thermophiles	*Acidianus brierleyi*	[303]
	Sulfolobus acidocaldarius	[304]

2.1.1.3. *Acidithiobacillus ferrooxidans*

(a) Environmental impact and biotechnological application

At. ferrooxidans (basionym: *Thiobacillus ferrooxidans*) is a Gram-negative bacterium that oxidizes Fe(II) (di)sulfide minerals (i.e., pyrite or marcasite), thereby contributing to microbial sulfur cycling in the environment [23, 28]. Because bacterial pyrite oxidation results in the formation of ferric iron and sulfuric acid

which perpetuates dissolution of sulfide minerals, causing acid mine drainage and solubilizing metals from minerals [29–31]. *At. ferrooxidans'* ability to oxidize metals makes it interesting for industrial applications such as bioleaching of metals from ores or from electronic waste [32–38]. $FeSO_4$ oxidation catalyzed by *At. ferrooxidans* can be used to improve schwertmannite production [20]. Schwertmannite is a mineral that promotes the natural passivation of heavy metals and can be used to decrease environmental pollution [39–42].

(b) Metabolic pathway of Fe(II) oxidation

At. ferrooxidans became a model organism for acidophilic chemotrophic life and aerobic iron oxidation [16, 20, 43, 44]. Therefore, its iron respiration chain is one of the best-studied Fe(II) oxidation mechanisms. A model for up-hill and down-hill electron transport during Fe(II) oxidation by *At. ferrooxidans* is shown in Figure 2.

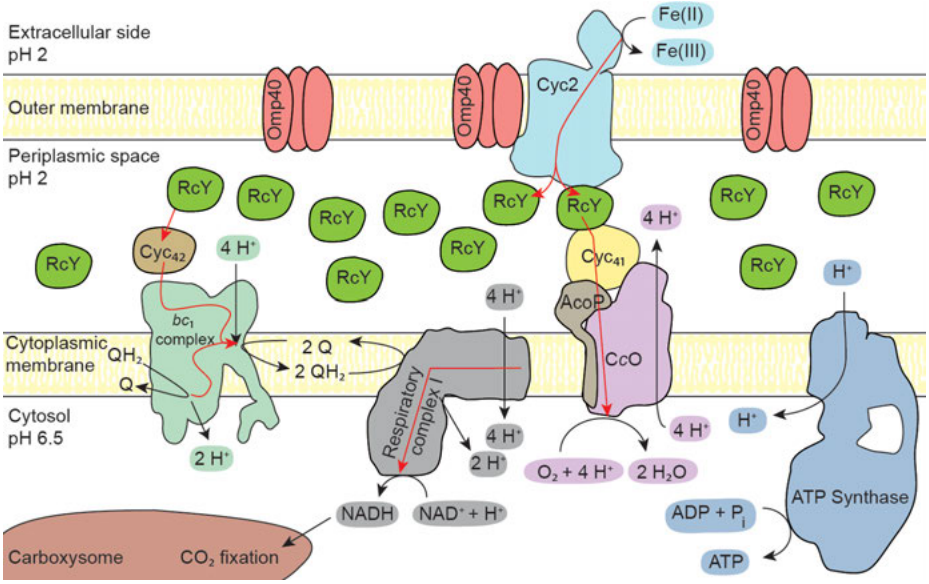

Figure 2. Model for electron transport from Fe(II) to the electron transport chain via free rusticyanin (RcY), and the respirasome in the cytoplasmic membrane. Fe(II) becomes oxidized by a high-molecular-weight cytochrome c_4 (Cyc2) located in the outer membrane. Cyc2 has been proposed to bind to a 40-kD major outer membrane protein (Omp40) [308]. In the course of the down-hill electron chain, the electrons are transported by the respirasome, compromising Cyc2, RcY, a dihemic cytochrome c_4 (Cyc_{41}), a green copper protein (AcoP) and an aa_3-type cytochrome *c* oxidase (C*c*O). C*c*O builds up the proton motive force which is used by either an ATP synthase to produce ATP from ADP and inorganic phosphate (P_i) or by the respiratory complex I to regenerate NADH. In the up-hill electron chain, it has been proposed that the electrons travel via RcY, Cyc_{42} and bc_1 complex which reduces quinone by using the proton motive force. The electron of reduced quinone can be then used to regenerate NADH by the respiratory complex I. The figure contains data from Li et al. [50], Castelle et al. [56], and Wang et al. [54].

The proposed iron respiratory chain (down-hill electron transport) is composed of five electron transfer proteins which are encoded by the *rus* operon [45, 46]. The Fe(II)-oxidation itself is hypothesized to take place extracellularly by an outer membrane high-molecular-weight cytochrome c_4 (Cyc2, encoded by *cyc2* [47, 48]). The harvested electrons are passed to rusticyanin (RcY), a type I blue copper protein which was found to form a complex with Cyc2 [49]. The abundance of RcY is relatively high compared to other cellular proteins [49]. Because of the cells' RcY concentration, it has been concluded that there is also free RcY present in the periplasm [49, 50]. At the next step, the electron is transferred via a dihemic cytochrome c_4 (Cyc_{41}, encoded by *cyc1*) [51] located in the periplasm, to an aa_3-type cytochrome *c* oxidase (C*c*O) that belongs to the subgroup of heme-copper O_2 reductases and spans the cytoplasmic membrane, where it reduces molecular oxygen to one molecule of water [52, 53]. The *rus* operon also encodes a green copper protein (AcoP: "acidophile C*c*O partner") of unknown function which interacts with C*c*O and Cyc_{41}. Two different functions have been proposed: (i) AcoP accepts electrons from Cyc_{41} as a linker to C*c*O [54, 55], (ii) as a chaperone-like protein to protect C*c*O against acidic damage [55].

The proteins encoded by the *rus* operon have been proposed to form a super-protein complex [45], which could be reconstituted [56], and has been termed respirasome. Looking at the relative ratios of the proteins involved in the respirasome and the periplasmic concentration of RcY, one can conclude that most of the RcY is either free or undergoes other protein-protein interactions. Free RcY has a redox midpoint potential of +490 mV (pH 4.8) which is increased upon protein complex formation with cytochrome c_4 to +590 mV (pH 4.8) [49]. Li et al. [50] observed that the entirety of all available respiratory electron transfer proteins is organized as a network that is reducing and oxidizing concomitantly at a common functional reduction potential. As stated by the authors, this observation stands in contrast to the proposed well-defined linear series of electron transfer accomplished by a respirasome. Considering that the experiments by Li et al. [50] were performed *in situ* and that the formation of a protein complex is highly specific, both findings are biologically relevant. Consequently, this leads to the hypothesis that the efficient electron transfer is based on a protein network influenced by both, specific interactions due to super-complex formation (respirasome) and non-specific interactions that can be traced back to the macromolecular crowding of electron transfer proteins in the periplasmic space [57]. The latter mechanism would explain how the organism can change between different electron donors without the observation of a lag phase. Considering the bifurcated chain, accomplishing both ATP formation and $NAD(P)^+$ reduction, the implementation of both models might be crucial for the understanding of the regulation of up- and down-hill pathways. The branching point of these pathways has been proposed to be at the level of RcY [58]. The electrons for $NAD(P)^+$ reduction are hypothesized to be transferred from RcY through a dihemic cytochrome c_4 (Cyc_{42}) [59], a bc_1 complex [60], the quinone pool, and a NAD(P)H dehydrogenase [48, 52, 61]. Cyc_{42} and the bc_1 complex are encoded by the *petI* operon.

2.1.2. Microaerophilic Iron(II) Oxidation

2.1.2.1. Iron(II)-Driven Energy Conservation

Microaerophilic Fe(II)-oxidizers are a group of bacteria which are proposed to be among the first to have taken advantage of rising levels of oxygen in the atmosphere [62]. This group of organisms grows in circumneutral environments and uses oxygen as electron acceptor to oxidize Fe(II) (Equation 1). Unlike at acidic pH; Fe^{3+} will subsequently react with water and precipitate Fe(III) (oxyhydr)oxide mineral phases (Equation 2).

$$10\,Fe^{3+} + 16\,OH^- \rightarrow Fe_{10}O_{14}(OH)_2\downarrow + 14\,H^+ \quad (2)$$

The Gibbs free energy ($\Delta G^{0'}$) yield from neutrophilic oxygen-dependent Fe(II) oxidation (oxidation of Fe^{2+} to Fe^{3+}) is relative low ($-29\ kJ\,mol^{-1}$) compared with all other potential lithotrophic energy sources. However, considering that Fe^{3+} precipitates as a Fe(III) (oxyhydr)oxide at pH 7, the energetic yield doubles, which also shows that depending on the identity of the Fe(III) mineral product, the free energy can vary.

2.1.2.2. Environmental Distribution and Biomineral Formation

Fe(II) oxidation at circumneutral pH must overcome two challenges: (i) Fe(III) (oxyhydr)oxide precipitation during microbial Fe(II) oxidation causes cell encrustation, which can lead to cell death [63]; and (ii) autoxidation of Fe(II) by O_2. This process (autoxidation) is pH-dependent and in circumneutral-pH oxic waters, Fe(II) has a half-life of less than 15 minutes [2, 63].

To minimize Fe(II) autoxidation, neutrophilic Fe(II)-oxidizers typically grow in microoxic habitats, increasing the half-life of Fe(II) 300-fold [64]. Furthermore, OM-complexed Fe(II) slows autoxidation [65]. Microaerophilic Fe(II)-oxidizers are found in freshwater and marine iron-rich flocs and sediments, the rhizosphere of wetland plants and paddy soils [66, 67], interfaces of ground and surface water [68], slow moving streams [69, 70], creeks, ditches, and marine hydrothermal vents [71].

To prevent Fe(III) mineral encrustation of cells, microaerophilic Fe(II)-oxidizers produce extracellular biofilaments forming either sheaths, stalks, Y-shaped tubular filaments, or induce the formation of particulate Fe(III) (oxyhydr)oxides [70, 72–74] (Figure 3). Sheaths and stalks can, in turn be colonized (i.e., *Siderocapsa* [71, 75]). These colonies appear as spherical structures that are fibrillary and resemble rounded nests [71]. Model organisms for each type extracellular biofilament structure can be found in Table 2.

The characteristic extracellular twisted stalks can be easily spotted under a microscope and are evidence for the presence of *Gallionellaceae*, and thus are often used as indicator for microaerophilic Fe(II) oxidation activity [71, 72, 76]. Such stalks have been suggested to be found in ancient rock formations. If true, their presence would allow the reconstruction of geochemical conditions and the evolution of early microbial life, and provide evidence for the availability of reduced iron and oxygen on early Earth [62, 77]. However, this evidence is

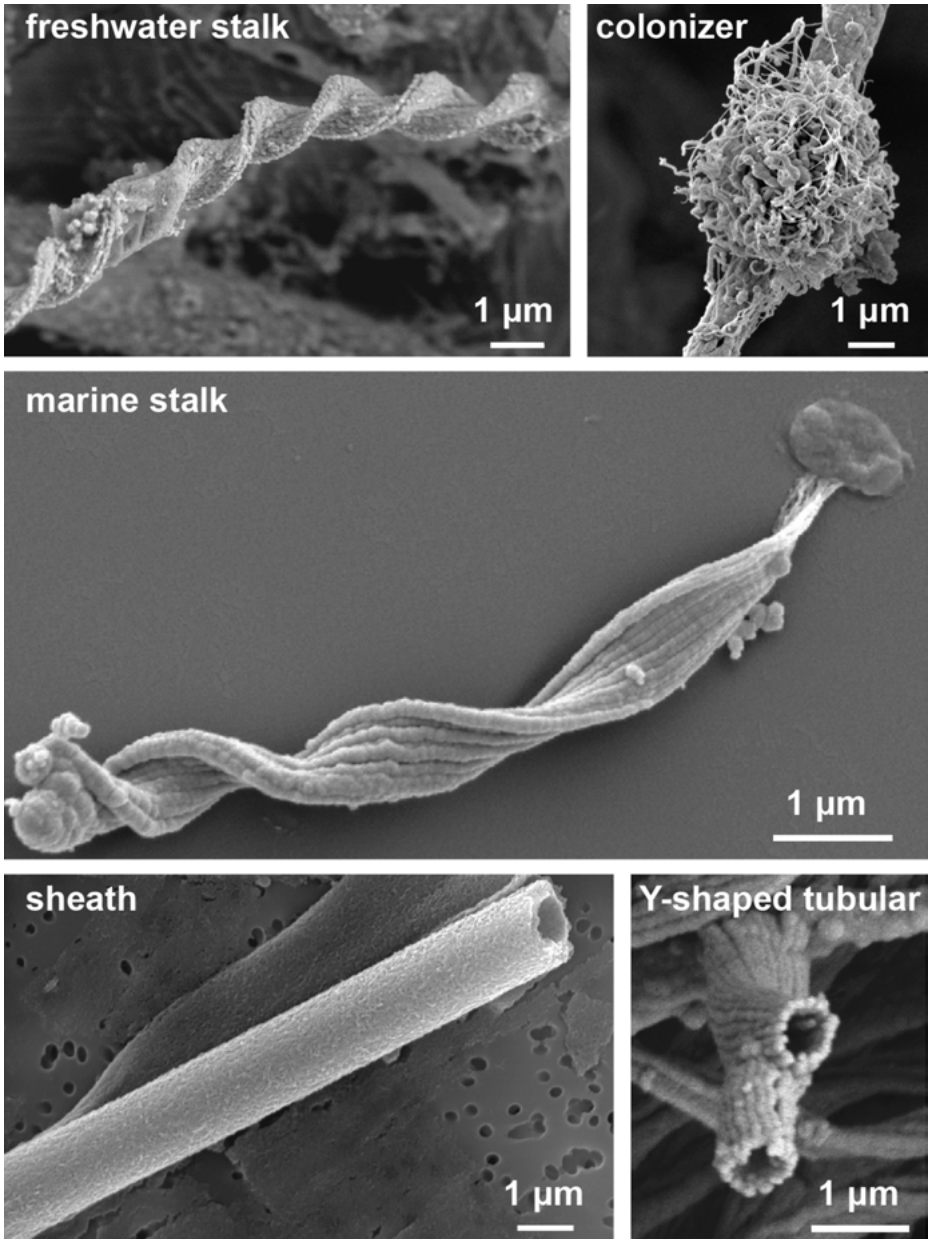

Figure 3. Morphologies of Fe(III) (oxyhydr)oxide biominerals known or suspected to be formed by microaerophilic Fe(II)-oxidizers. Micrographs were kindly provided by Clara Chan (University of Delaware, USA). The figure is modified from Chan et al. [71] and McAllister et al. [73].

Table 2. Examples of freshwater and marine microaerophilic Fe(II)-oxidizers sorted by Fe(III) (oxyhydr)oxide formation [79].

Freshwater microaerophilic Fe(II)-oxidizers (Betaproteobacteria)		
Fe(III) (oxyhydr)oxide-stalk-formers	**particulate Fe(III) (oxyhydr)oxides of no specific shape**	**Fe(III) (oxyhydr)oxide-sheath/-dreads-formers**
Ferriphaselus amnicola OYT1 [79] *Ferriphaselus* sp. R-1 [79] *Gallionella ferruginea* [71]	*Sideroxydans lithotrophicus* ES-1 [82] *Gallionella capsiferriformans* ES-2 [82]	*Leptothrix ochracea* [71] *Ferriphaselus amnicola* OYT1 [79] *Ferriphaselus* sp. R-1 [79]
Marine microaerophilic Fe(II)-oxidizers (Zetaproteobacteria)		
Fe(III) (oxyhydr)oxide-stalk-formers	**Fe(III) (oxyhydr)oxide-sheath/-dreads-formers**	**Y-shaped tubular filaments and colonizers**
Mariprofundus ferrooxydans PV-1 [305] *Mariprofundus* sp. M34 [306] *Mariprofundus* sp. EKF-M39 [306]	*Mariprofundus aestuarium* CP-5 [81] *Mariprofundus ferrinatatus* CP-8 [81]	Uncultured Zetaproteobacteria [71]

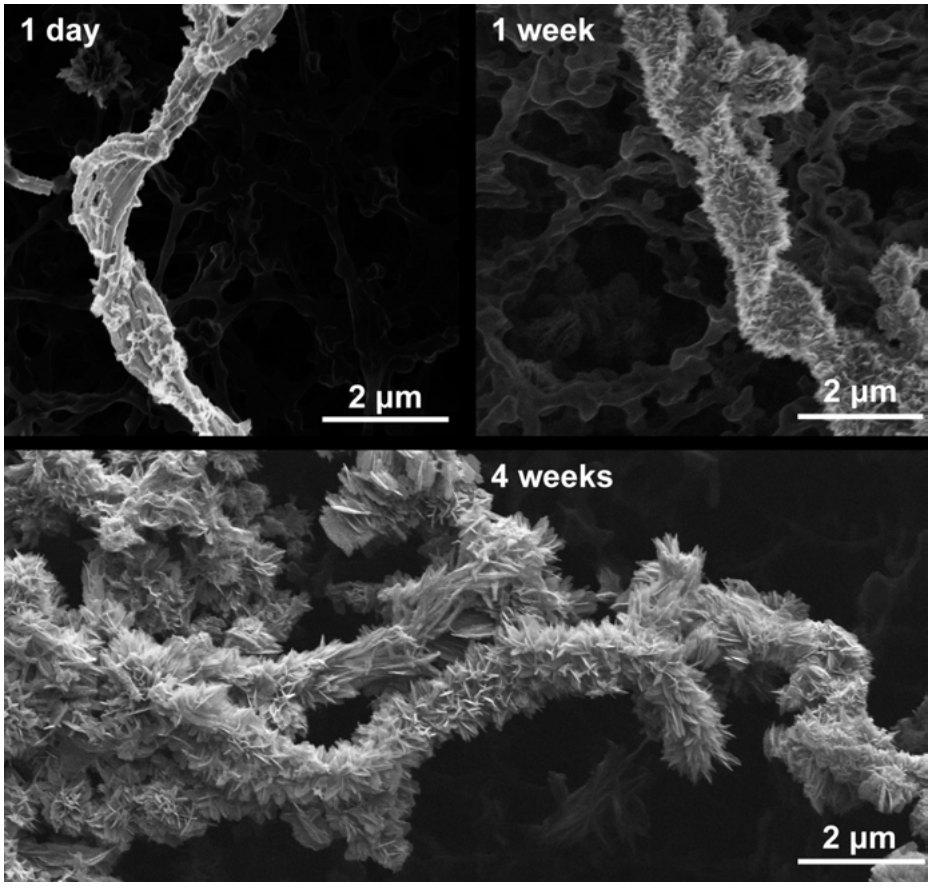

Figure 4. Aging twisted stalk produced by microaerophilic Fe(II)-oxidizing bacteria. Micrographs were collected by using a helium ion microscope and kindly provided by James Byrne (University of Bristol, UK). The figure is modified from Byrne et al. [72].

limited and still controversial. Stalk formation is proposed to be induced by acidic polysaccharide-containing fibrils excreted by the cell [78]. These fibrils then act as a template for Fe(III) (oxyhydr)oxide precipitation (Figure 4).

It is thought that the formation of stalks has the dual purpose of preventing cell encrustation and supporting cell mobility [79, 80]. The stalk anchors the cell to surfaces, and while the stalk grows, the cell moves forward, for example, towards an optimum O_2 concentration. Stalk formation has been observed for *Gallionella ferruginea* and most Zetaproteobacteria isolates. As stated previously, some microaerophilic Fe(II)-oxidizers produce other biomineral morphologies to avoid encrustation. For example, *Leptothrix ochracea*, *Mariprofundus ferrinatatus* CP-8, and *Mariprofundus aestuarium* CP-5 form shorter filaments that look like tubular sheaths [65, 73, 81]. Cultures of *Ferriphaselus amnicola* OYT1 and *Ferriphaselus* strain R-1 showed both stalks and tubular sheaths [79]. The freshwa-

ter Betaproteobacteria *Sideroxydans lithotrophicus* ES-1 and *Gallionella capsiferriformans* ES-2 do not form a specific shape of particulate Fe(III) (oxyhydr)oxides [82].

2.1.2.3. Biotechnological Application

Fe(III) (oxyhydr)oxides produced by neutrophilic Fe(II)-oxidizers have been investigated for their possible application in wastewater treatment as a metal remediation strategy [83] and in nanotechnology for their magneto-electronic applications as biosensors [84].

2.1.2.4. Metabolic Pathway of Iron(II) Oxidation

The neutrophilic Fe(II) oxidation metabolism has not been as extensively studied as acidophilic Fe(II) oxidation metabolism. Models for the metabolic pathway were proposed based on comparative genome studies; insights into freshwater and marine neutrophilic Fe(II)-oxidizers were published by Kato et al. in 2015 [79] and He et al. in 2017 [85]. A genomic study of marine neutrophilic Fe(II)-oxidizers, in particular Zetaproteobacteria, was published by McAllister et al. [73], and a comparative genomic study of freshwater neutrophilic Fe(II)-oxidizers was published by Emerson et al. [82].

Based on genome analysis, these researchers hypothesized, that either a homologous gene to *cyc2* or *mtoA/B* encodes the Fe(II) oxidase. Nevertheless, the authors also state that there might be an alternative Fe(II) oxidase, too. Since Cyc2 in *Acidithiobacillus ferrooxidans* was shown to oxidize Fe(II), its homologs are likely candidates for Fe(II) oxidation [56]. MtoA/B and MtrA/B of *Shewanella* spp. (Fe(III)-reducing bacteria, see Section 3.4.1.) are homologous proteins [86, 87]. MtrA/B is a porin-cytochrome complex located in the outer membrane and binds MtrC on the outside of the cell [88–91]. These proteins are involved in extracellular electron transfer linked to metal reduction (Mtr) [88–90]. If Fe(II)-oxidizers can oxidize Fe(II) on the outside of the cell instead of inside the cell, they could avoid intracellular mineral precipitation. Because of this, MtrA/B homologs such as MtoA/B, have also been proposed to be involved in metal oxidation (Mto). In addition to MtoA/B, Fe(II)-oxidizing homologs to MtrA/B include PioA/B in *Rhodopseudomonas palustris* strain TIE-1, a phototrophic Fe(II)-oxidizer (see Section 2.2) [92, 93].

Furthermore, MtoA has been demonstrated to be a decaheme cytochrome with Fe(II) oxidation activity *in vitro* by Liu et al. [93]. Based on the genomic analyses, microaerophilic Fe(II)-oxidizers have either a homologous protein to MtoA or Cyc2 functioning as Fe(II) oxidase with differing electron transport chains. In Figure 5 we present an overview of proposed Fe(II) oxidation models, corresponding to freshwater isolates and marine Zetaproteobacteria. He et al. presented a comparative genome analysis of neutrophilic Fe(II)-oxidizer where they suggest alternative Fe(II) oxidases [85]. Their findings indicate two novel porin-cytochrome *c* complexes, a transmembrane multicopper oxidase and a porin-multicopper complex as putative Fe(II) oxidases in addition to the commonly suggested transmembrane cytochrome *c* oxidase (similar to Cyc2) and the porin-cytochrome *c* complex (similar to MtoA/B and PioA/B).

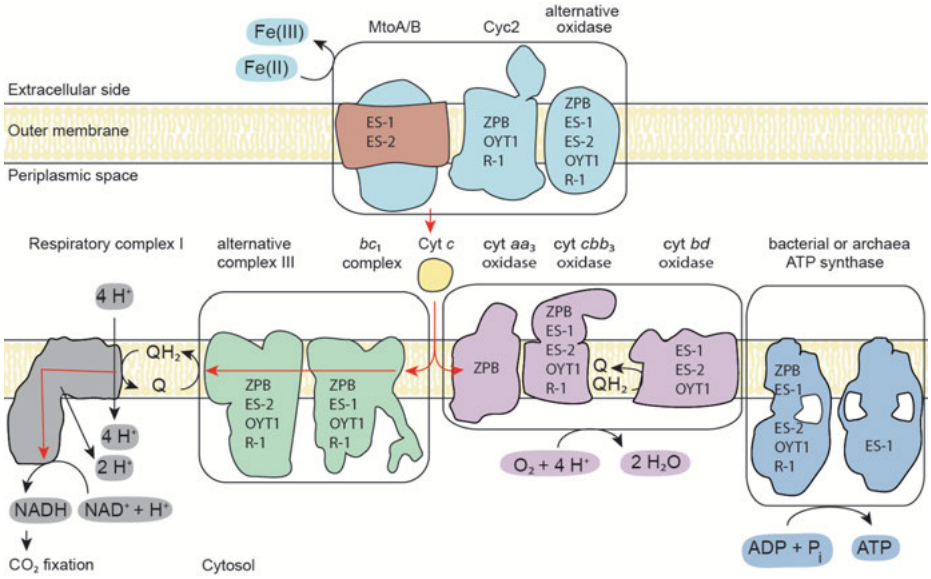

Figure 5. Model for electron transport from Fe(II) to the electron transport chain in the cytoplasmic membrane for the freshwater isolates *Sideroxydans lithotrophicus* ES-1 (ES-1) and *Gallionella capsiferriformans* ES-2 (ES-2) [82], *Ferriphaselus amnicola* OYT1 (OYT1) and *Ferriphaselus* sp. R-1 (R-1) [79], and for marine microaerophilic Fe(II)-oxidizing bacteria, in particular Zetaproteobacteria (ZPB) [73]. Proteins that have been suggested for one of these organisms are marked with the corresponding organisms short name in the site of the protein envelope. After oxidation of Fe(II) by an iron oxidase (light blue), the electron is passed to a *c*-type cytochrome (yellow) and from there it enters either the up-hill or down-hill electron chain. The alternative complex III or bc_1 complex (green) is proposed to pass the electrons to oxidized quinone by using the proton motive force. The NADH regeneration is catalyzed by the respiratory complex I (grey) by oxidizing reduced quinone and use of the proton motive force. Via the down-hill electron chain, the electrons are passed to their terminal electron acceptor oxygen, by an oxygen reductase (violet; respiratory oxidases). It should be noted that the *bd*-type cytochrome oxidase receives the electrons from reduced quinone but not directly from a *c*-type cytochrome as it is the case for the other shown oxidases. The ATP synthase phosphorylates ADP by the use of the proton motive force, generated by the oxygen reductase.

2.1.3. Nitrate-Reducing Iron(II) Oxidation

2.1.3.1. Iron(II)-Driven Energy Conservation

Nitrate-dependent Fe(II)-oxidizers couple enzymatic Fe(II) oxidation to the reduction of NO_3^- to NO_2^-, N_2, or NH_4^+ as a source of energy and electrons. The chemolithotrophic process of nitrate reduction to NO_2^- coupled to Fe(II) oxidation, has a proposed change in Gibbs free energy of -96.23 kJ mol^{-1} for standard conditions at pH 7 [94]. Equation (3) describes the nitrate-dependent Fe(II) oxidation with nitrate reduction to molecular nitrogen:

$$10\,Fe^{2+} + 2\,NO_3^- + 12\,H^+ \rightarrow 10\,Fe^{3+} + 2\,N_2 + 6\,H_2O \quad (3)$$

At circumneutral environments, Fe^{3+} will subsequently react with water and precipitate Fe(III) (oxyhydr)oxide mineral phases (Equation 2).

Fe(II) oxidation is not only observed due to direct enzymatic nitrate-reducing Fe(II) oxidation activity, but also due to heterotrophic denitrifying bacteria, reducing nitrate to nitrite. This process is called chemodenitrification and nitrite oxidizes Fe(II) abiotically.

The equations of nitrite production by denitrifying bacteria (Equation 4) and of abiotic Fe(II)-oxidation by nitrite to nitrous oxide (Equation 5) are as follows:

$$NO_3^- + 2\,e^- + 2\,H^+ \rightarrow NO_2^- + H_2O \quad (4)$$

$$20\,Fe^{2+} + 10\,NO_2^- + 17\,H_2O \rightarrow 2\,Fe_{10}O_{14}(OH)_2\downarrow + 5\,N_2O + 30\,H^+ \quad (5)$$

Heterotrophic denitrifying bacteria do not belong to the group of nitrate-reducing Fe(II)-oxidizers (NRFeOx). By contrast, in this case, Fe(II)-oxidation is an abiotic process, caused by nitrite released from the cells, and is not an enzymatic process [63]. The uncontrolled Fe(III) (oxyhydr)oxide precipitation at the cell

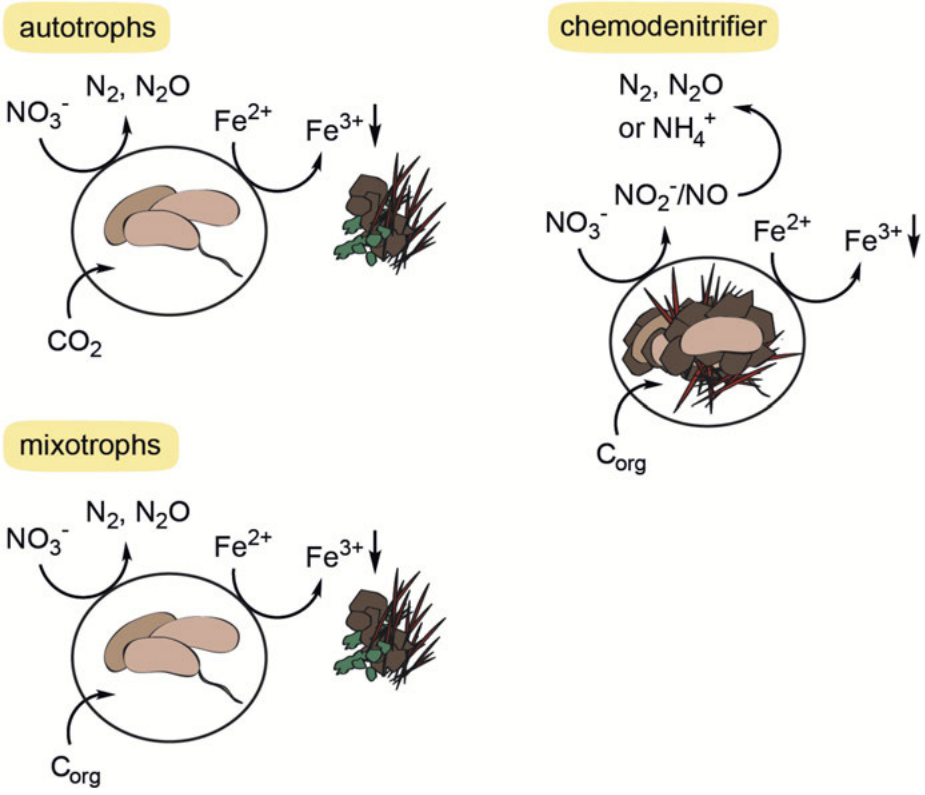

Figure 6. Schematic of autotrophic and mixotrophic NRFeOX, and heterotrophic denitrifiers.

surface, in their periplasm and cytoplasm [63, 95] can cause cell encrustation even leading to cell death (Figure 6).

In addition to these heterotrophic denitrifying bacteria indirectly oxidizing Fe(II), nitrate-reducing Fe(II) oxidation has been proposed for autotrophic and mixotrophic enrichment cultures and isolates. However, this remains controversial. A true autotrophic NRFeOx maintains its ability to conserve energy by nitrate-dependent Fe(II)-oxidation over several transfers into fresh autotrophic media and does not require any additional carbon source beyond CO_2 to building biomass. So far, this was only demonstrated for the enrichment culture KS by culture maintenance on autotrophic media over several years (in two different laboratories) and by incorporation of labelled CO_2 into biomass [96–99]. Most NRFeOx cultures are proposed to require addition of organic substrates for continuous Fe(II)-oxidation. These NRFeOx were termed 'mixotrophs'. By definition, this means that the cells use both organic and inorganic compounds as sources for carbon fixation and/or energy conservation. Depending on the context, mixotrophy can mean different combinations of obligate or facultative metabolic processes. However, so far, such a dependence on both inorganic and organic compounds for NRFeOx has not been demonstrated.

In the context of NRFeOx, mixotrophy has not been further defined beyond the dependence on organic substrates in combination with Fe(II)-oxidation. If some of these strains are true mixotrophs, the role of organic substrates (used during Fe(II)-oxidation) could be to regenerate reducing equivalents due to the lack of a reverse electron transport pathway linked to Fe(II)-oxidation. Further, it could be an essential substrate for CO_2 assimilation similar to acetate assimilation via the ethylmalonyl-CoA pathway. The metabolic mode of mixotrophic and autotrophic NRFeOx has been recently reviewed by Bryce et al. [100]. It must be noted that for all NRFeOx that have been called "mixotrophs", it seems more likely these strains are heterotrophic denitrifiers producing nitrite that is abiotically oxidizing Fe(II). There is currently no example of a true mixotrophic NRFeOx.

2.1.3.2. Environmental Distribution

Nitrate-dependent Fe(II)-oxidation has been observed for several pure and enrichment cultures derived from anoxic sediments and soils at circumneutral pH [11, 101]. Habitats where NRFeOx have been found include freshwater lake sediments [102], ponds, paddy soils [103], ditches, a brackish water lagoon [97], and groundwater aquifers [104, 105].

2.1.3.3. Biotechnological Application

Nitrate-reducing Fe(II)-oxidizing bacteria can be harnessed as a whole cell biocatalyst for the removal of nitrate pollution in groundwater [106]. High levels of nitrate in groundwater are caused by agricultural practices, in particular the application of inorganic fertilizer and animal waste [107, 108]. Nitrate pollution is causing environmental problems such as eutrophication of surface waters due to excess nutrients [109]. In subsurface environments or groundwater aquifers containing pyrite, NRFeOx could potentially couple pyrite oxidation to denitrification for nitrate removal [110, 111]. However, nitrate-reducing Fe(II)-oxidizing

bacteria may cause environmental problems due to the accumulation of NO_2^- or the release of N_2O gas. While NO_2^- in drinking water directly affects human health [112], N_2O is a greenhouse gas. Additionally, depending on the Fe(II) source (e.g., aqueous OM complexes) the identity of the reduced nitrogen species varies [113].

2.1.3.4. Metabolic Pathway of Iron(II) Oxidation

(a) Diversity of nitrate-reducing pathways

Microbial nitrate reduction is part of various metabolic pathways. Nitrate can be reduced to nitrite in a two-electron transfer step ($NO_3^- \rightarrow NO_2^-$) [114], to N_2 by full denitrification ($NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$), or to ammonium by dissimilatory nitrate reduction to ammonium (DNRA; $NO_3^- \rightarrow NO_2^- \rightarrow NH_4^+$) [115]. Nitrite and ammonium, obtained by either of these processes can be further metabolized to N_2 by anaerobic ammonium oxidation (Anammox) ($NH_4^+ + NO_2^- \rightarrow N_2$) [116, 117]. Thus it is possible that NRFeOx have a common Fe(II) oxidation mechanism via homologs of MtoA/B or Cyc2 but differ in the metabolic pathway for nitrate reduction. The best model for the nitrate-reducing Fe(II)-oxidation mechanism is derived from metagenome analyses of the enrichment culture KS and genome analysis of *Gallionellaceae* sp., main Fe(II)-oxidizer of culture KS [85, 118].

(b) Culture KS and the proposed *Gallionellaceae* sp. Fe(II)-oxidation metabolic pathway

The enrichment culture KS was originally isolated from sediment in a freshwater pond in Bremen, Germany [97], it is a chemolithoautotrophic nitrate-reducing Fe(II)-oxidizing culture and has been used as a model system to study nitrate-reducing Fe(II)-oxidation. However, the putative Fe(II)-oxidizer, a species from the family *Gallionellaceae*, has not yet been isolated from this co-culture [119].

This *Gallionellaceae* sp. is closely related to *Sideroxydans lithotrophicus* ES-1, a microaerophilic Fe(II)-oxidizer. Culture KS also contains relatives of the heterotrophic nitrate-reducing bacteria *Comamonas badia*, *Parvibaculum lavamentivorans* and *Rhodanobacter thiooxidans* [120]. He et al. [118] analyzed the culture community composition and in particular the flanking community of two KS cultures maintained in different laboratories and demonstrated that the communities of these two subcultures of culture KS can indeed vary substantially but both subcultures still perform autotrophic nitrate-reducing Fe(II) oxidation [118]. The cultures were maintained on autotrophic media in both laboratories, at the University of Tübingen (Germany) and at the University of Wisconsin (Madison, USA), over several years. In KS-Madison, *Gallionellaceae* accounted for 42 % of the total community whereas *Gallionellaceae* of KS-Tübingen accounted for 96 % of the total community [85]. Since both cultures are able to grow autotrophically, the composition of the flanking community seems to have a minor impact on the predominant occurring *Gallionellaceae* sp. which is the putative Fe(II)-oxidizer. However, since the *Gallionellaceae* sp. has not been isolated, it is hypothesized that the flanking community has an essential role for

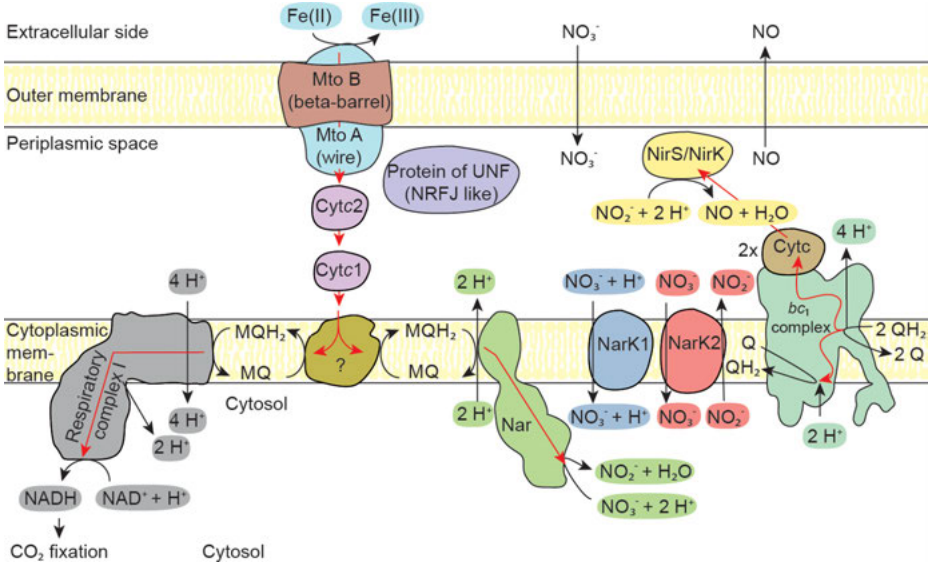

Figure 7. Schematic of nitrate-reducing Fe(II) oxidation pathway in culture KS. The figure is modified from Bryce et al. [100]. Fe(II) is oxidized by multiheme *c*-type cytochrome (MtoA) associated with a porin (MtoB). *c*-type cytochromes 1 and 2 (Cytc1 and Cytc2) are located in the same genome cluster as MtoAB and are therefore suggested being the next proteins in the electron chain. However, there is also a protein of unknown function (Protein of UNF) located in the same gene cluster. The electrons are proposed to reduce quinones via the bc_1 complex. This would require a proton motive force that might not be regenerated by the reduction of nitrate alone. Therefore, the figure shows a question mark for the reduction of the quinones. The quinones can be used by the respiratory complex I to regenerate NADH by using the proton motive force. The proton motive force is supposed to be built by the dissimilatory nitrate reductase complex (Nar) catalyzing the reaction of nitrate to nitrite. NarK1 and NarK2 are nitrate:nitrite antiporter. NirS and NirK are cytochrome cd_1- and copper-type nitrite reductase, respectively and catalyze the reduction of nitrite to nitric oxide.

the physiology of the main Fe(II)-oxidizer in the co-culture, e.g., these community members could degrade a toxic product derived from the Fe(II)-oxidation metabolism in *Gallionellaceae* sp.

The metabolic pathway model resulting from comparative genome analysis suggests that nitric oxide cannot be degraded by *Gallionellaceae* sp. as it lacks a nitric oxide reductase. However, the flanking community members have the genomic capability to reduce nitric oxide to N_2. The schematic of the proposed Fe(II)-oxidation metabolic pathway in culture KS is presented in Figure 7. The pathway is similar to the proposed pathways in *Sideroxydans* ES-1 and *Gallionella* ES-2 (Figure 5), suggesting a similar Fe(II)-oxidizing pathway in neutrophilic freshwater Fe(II)-oxidizers. The putative Fe(II)-oxidase is MtoA, which is thought to form a porin-cytochrome complex with MtoB. The *mtoB* gene is located downstream next to *mtoA*. The *mto* operon encodes two more cytochromes that are potentially involved in electron transfer as well.

After passing the electrons to the quinone pool they can enter either an up-hill electron transfer pathway via complex I resulting in NAD(P)H generation or a down-hill pathway towards nitrate and further nitrite reduction. It should be acknowledged that the neutrophilic Fe(II)-oxidation pathways remain speculative and are mainly based on *in silico* analysis. He et al. [85] published a genome study where several alternative putative Fe(II)-oxidases of neutrophilic Fe(II)-oxidizers are discussed [85]. Further information about putative Fe(II)-oxidases in nitrate-dependent Fe(II)-oxidizers can be found in the supplementary information of He et al. [118].

2.2. Phototrophic Iron(II)-Oxidation

2.2.1. Iron(II)-Driven Energy Conservation

Phototrophic Fe(II)-oxidizing bacteria, so-called photoferrotrophs, conserve light energy and reduce CO_2 to biomass by the use of electrons stemming from Fe(II). It is proposed that light does not support the oxidation of Fe(II), however, builds up a PMF that in turn powers the up-hill transportation of the electrons that reduce $NAD(P)^+$ to NAD(P)H. This process is anoxygenic, which means oxygen is not a product of phototrophic Fe(II)-oxidation, thus this metabolism is an example of anoxygenic photosynthesis. It may have evolved earlier than oxygenic photosynthesis [121, 122] and might be an ancestor in its evolution [123–125].

Fe(II)-oxidation by photoferrotrophy follows the stoichiometry described by Equation (6):

$$20\,Fe^{2+} + 5\,HCO_3^- + 22\,H_2O + C_n(H_2O)_m \xrightarrow{h\nu} Fe_{10}O_{14}(OH)_2\downarrow + C_{n+5}(H_2O)_{m+5} + 35\,H^+ \quad (6)$$

2.2.2. Environmental Distribution

Phototrophic Fe(II)-oxidizing bacteria can be isolated from freshwater sediments of ditches [126, 127], stratified lakes and marshes [128], and from marine sediments at the North Sea coast [129] and coastal marine sediments [130], among other locations. Examples are listed in Table 3 and an extended list was recently published by Bryce et al. [100].

2.2.3. Biological Availability of the Iron(II) Source

Rhodobacter ferrooxidans strain SW2, *Chlorobium ferrooxidans* strain KoFox and *Thiodictyon* sp. strain F4 were shown to metabolize dissolved Fe(II) and highly soluble minerals such as FeS and $FeCO_3$, but not poorly soluble Fe(II)-bearing minerals (e.g., Fe_3O_4 and FeS_2 [131]). There is evidence that another photoferrotroph, *Rhodopseudomonas palustris* strain TIE-1, can oxidize magnet-

Table 3. Cultures of phototrophic Fe(II)-oxidizers.

Freshwater strains	**Reference**
Rhodobacter ferrooxidans strain SW2	[126]
Rhodopseudomonas palustris strain TIE-1	[307]
Chlorobium ferrooxidans strain KoFox	[127]
Thiodictyon sp. strain F4	[128]
Rhodomicrobium vannielii strain BS-1	[154]
Marine strains	
Rhodovulum iodosum	[129]
Rhodovulum robiginosum	[129]
Chlorobium sp. strain N1	[130]

ite, however, the oxidation was shown to depend on the magnetite particle size [132]. Fe-OM complexes are suggested to play a significant role in biogeochemical iron cycling in the photic zones of aquatic environments [133]. Here, Fe(III)-OM reduction can be induced by abiotic photoreduction and Fe(II)-OM oxidation is promoted by photoferrotrophic bacteria. This suggests a light-driven cryptic iron cycle that may play a role in the photic zone of aquatic habitats [134]. A cryptic iron cycle is so-called because it describes such a rapid turnover of Fe(II) and Fe(III) that a change in their concentration cannot be measured using traditional sampling or analytical approaches. Despite rapid Fe(II) oxidation and Fe(III) re-reduction, the Fe(II) concentration would remain low and steady.

Cultivation studies with *Rhodopseudomonas palustris* strain TIE-1 demonstrated that: (i) Fe(II)-organic matter complexation promotes Fe(II)-oxidation in comparison to free Fe^{2+}, and, (ii) the choice of organic ligand influenced the bioavailability of Fe(II) [133, 134]. This might be due to different redox kinetics between Fe(II) and *c*-type cytochromes based on the organic ligand or free Fe^{2+}, respectively. Further steric effects of the complexing ligand could be an additional factor [135].

2.2.4. Environmental Impact

Microbial iron oxidation and reduction processes are tightly coupled and allow efficient iron cycling [136]. It is thought that one iron atom can undergo up to 300 redox cycles until it is converted to a bio-unavailable form [137]. In anoxic environments phototrophic and nitrate-reducing Fe(II) oxidation activity can play an especially important role in iron cycling [97, 138]. During phototrophic Fe(II) oxidation a variety of Fe(II) minerals can be oxidized and Fe(III) minerals can be formed, e.g., ferrihydrite, goethite, lepidocrocite, and magnetite [131]. If phototrophic Fe(II)-oxidizing bacteria colonized the early Earth, they may have contributed to the deposition of banded iron formations (BIFs), some of the most economically significant iron deposits worldwide [139–141].

2.2.5. *Biotechnological Application*

Photoferrotrophs are promising candidates for whole-cell catalysts in combination with electrodes as electron donors. It was demonstrated for *Rhodopseudomonas palustris* strain TIE-1 that electrons derived from solid-phase conductive matrices (e.g., electrodes) can be linked to carbon dioxide fixation by extracellular electron uptake [142]. Such a photoelectron autotrophic system was applied for producing bioplastic (polyhydroxybutyrates) [143].

2.2.6. *Metabolic Pathway of Iron(II) Oxidation*

The best studied photoferroautotrophic strains are the freshwater isolates *Rhodobacter ferrooxidans* strain SW2 and *Rhodopseudomonas palustris* strain TIE-1. Notably, they have different metabolic pathways for Fe(II)-oxidation and electron transport. In strain SW2, the *fox* operon; and, in strain TIE-1 the *pio* operon, have been shown to be essential for Fe(II)-oxidation.

The first gene in the *pio* operon encodes PioA, a decaheme *c*-type cytochrome. It is proposed to function as the Fe(II)-oxidase similar to Cyc2 in acidophilic Fe(II)-oxidizers [144]. Sequence analysis of PioA indicates that it is located in the periplasm because it lacks typical features of an outer membrane protein [144]. PioA has a putative secretory signal peptide (amino acids 1–40) [145] and can be further divided in a C-terminal domain that shows a multiheme cytochrome *c* family profile (amino acids 271–529) similar to MtrA and MtoA [92] and an N-terminal domain of unknown function (amino acids 41–270) which includes a glycine-rich region (amino acids 90–133) [146].

PioB and MtrB of *Shewanella* spp. (Fe(III)-reducing bacteria, see Section 3.4.1.) are homologous proteins. MtrB is an outer membrane porin with 26 transmembrane β-strands [91]. According to the high number of β-strands MtrB, and consequently its homologs (i.e., PioB and MtoB), are relatively large outer membrane porins. MtrA and B of the metal-reducing bacterium *Shewanella oneidensis* strain MR-1 are assembled in a porin-cytochrome complex. These findings are based on MtrA, MtrA/B, and MtrA/B/C models derived from small-angle x-ray scattering (SAXS) data [88–90]. The X-ray crystal structure of the whole Mtr complex from *Shewanella baltica* OS185 has been published recently by Edwards et al. [91]. Because of this, it has been suggested that PioB and PioA are assembled in a similar complex [85, 147], however, the localization of the N-terminal domain remains unclear.

The third gene in the *pio* operon encodes PioC, a putative high potential iron-sulfur protein (HiPIP). Its function is proposed to be an electron carrier from PioA to the photosynthetic reaction center (RC). However, HiPIPs could also substitute for cytochrome c_2 and therefore function as an electron carrier that shuttles electrons between the cytochrome bc_1 complex and the RC in the periplasm. In the case of strain TIE-1, the extracellular electrons derived from Fe(II)-oxidation are transferred to reducing equivalents and can finally be used for the reduction of CO_2 to build up biomass via the Calvin-Benson-Bassham cycle [142].

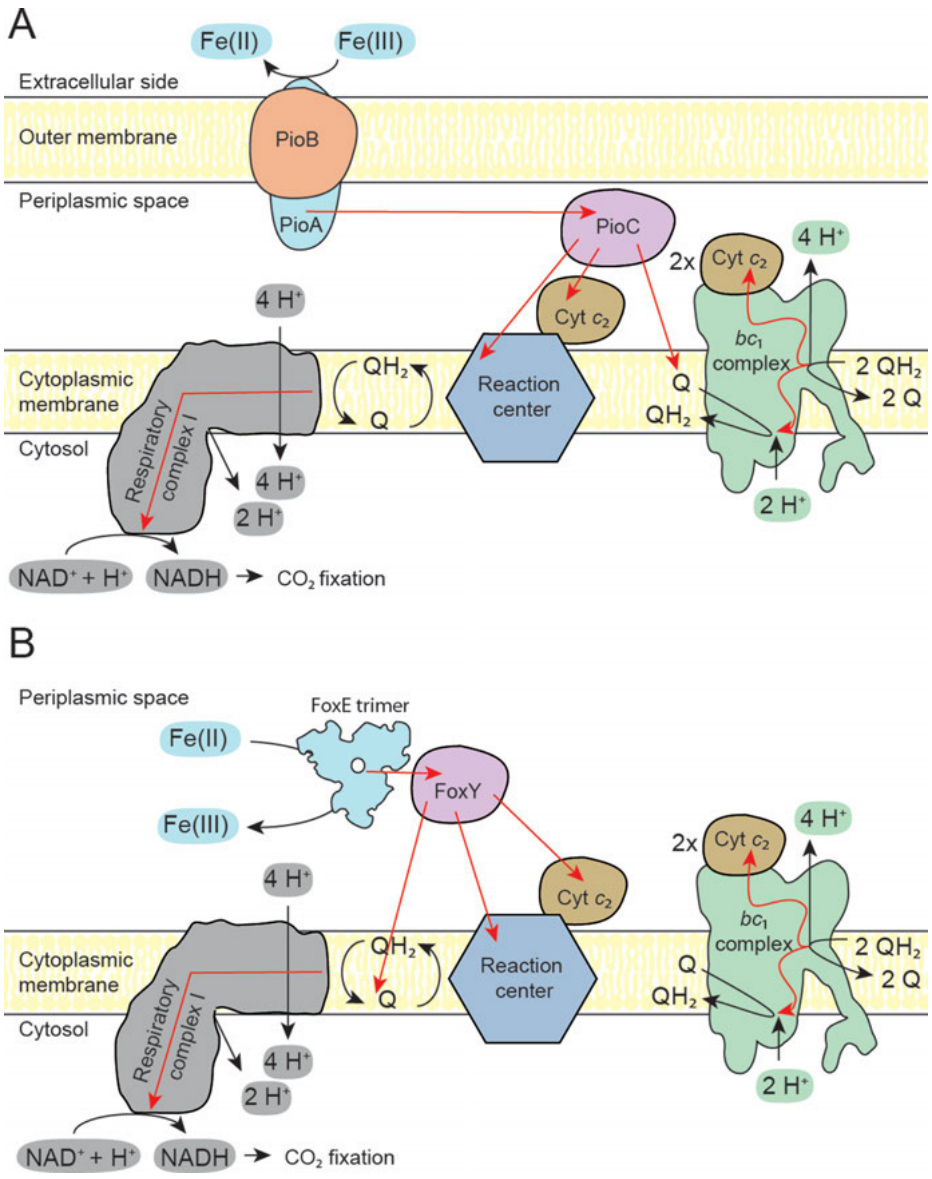

Figure 8. Schematic for electron transport from Fe(II) to the electron transport chain in the cytoplasmic membrane for the freshwater isolates *Rhodopseudomonas palustris* strain TIE-1 (A) and *Rhodobacter ferrooxidans* strain SW2 (B) [309]. FoxE is shown as a trimer [153].

The *fox* operon of strain SW2 encodes three essential proteins that are different to those encoded by the *pio* operon [148, 149] (Figure 8). The first gene in the *fox* operon encodes a *c*-type cytochrome, FoxE, and is the proposed Fe(II)-

oxidase. It has been shown that the protein is thermodynamically and kinetically able to perform Fe(II)-oxidation [149] and its location is hypothesized for the periplasm based on the lack of β-sheets and a lipoprotein profile, as is the case for Cyc2, OmcA or OmcB [47, 86, 148]. The suggested redox partner of FoxE is FoxY encoded by the second gene of the *fox* operon. The protein is similar to PioC and has a binding site for pyrroloquinoline quinone. According to its predicted isoelectric point (5.34), it is negatively charged at neutral pH, and therefore it could possibly interact with the convex side of the FoxE trimer. The last gene in the *fox* operon encodes a putative transport protein, FoxZ.

Strain SW2 seems to lack an extracellular electron transport system which stands in agreement with the observation, that it cannot oxidize solid Fe(II) phases [148].

All the proposed photoferrotrophic Fe(II)-oxidation pathways do not answer the question, why Fe(III) production does not lead to cell encrustation. Strain SW2 seems to oxidize Fe(II) in the periplasm, where it theoretically precipitates as Fe(III) (oxyhydr)oxide at pH 7. This would lead to mineral deposition inside the cell. However, experiments indicate that photoferrotrophs must have strategies to avoid mineral formation inside or on the cell [74, 126, 131, 150].

For strain TIE-1, the expression of genes involved in active efflux mechanisms upon Fe(II) addition and long period cultivations with Fe(II) was demonstrated by Bryce et al. [100]. It is possible that efflux proteins contribute to the mineralization away from the cell. Further, it is hypothesized that the cells produce Fe(III) chelators that transport Fe(III) to the outside of the cell [149]. This is consistent with the observation that: (i) strain SW2 grows better with nitrilotriacetic acid (NTA, a complexing agent) [148], and, (ii) the supernatant of Fe(II)-grown culture increases the solubility of Fe(III) [149, 151]. The chelators do not seem to be siderophores, as they have not been found in high concentrations in Fe(II)-grown cultures. Additionally, their synthesis would be energetically expensive [149]. It has been demonstrated that FoxE is active at low pH, thus a low-pH microenvironment around the cell could be an alternative strategy to avoid cell encrustation [152].

Pereira et al. [153] showed that the distances of hemes within the FoxE trimer structure are relatively large (16 Å, within the monomer; 22 Å, the closest heme between neighbors). Therefore, the authors propose a slow, intermolecular electron transfer that prevents product accumulation and further spontaneous Fe(III) precipitation in the cytosol. In general, it has been proposed that (i) not all photoferrotophs share the same strategy to avoid Fe(III) precipitation inside or at the surface of the cell, and, (ii) their strategies have different efficiencies [150, 154].

3. MICROBIAL GROWTH BY REDUCTION OF IRON(III)

Fe(III)-reducing microbes couple the reduction of Fe(III), including Fe(III) minerals and dissolved Fe(III)-OM complexes, to the oxidation of a variety of elec-

tron sources, including organic matter (OM), sulfur (S^0), ammonium (NH_4^+), methane (CH_4) or dihydrogen (H_2). Fe(III)-reducing microbes are phylogenetically diverse, and are present in virtually every environmental habitat on Earth, including soils, sediments, and subsurface environments. Combined with the abundance of iron in the Earth's crust, and the phylogenetic and spatial breadth of this process, these interactions, which underpin biogeochemical cycling for so many elements, make the Fe(III)-reduction metabolic process globally and geologically significant.

3.1. Dissimilatory Iron(III) Reduction

Dissimilatory Fe(III) reduction is a metabolic process where microbes obtain energy for growth and cellular maintenance by oxidizing organic (e.g., acetate, lactate, benzene) or inorganic (i.e., H_2) electron donors, and transferring electrons to minerals that contain Fe(III), thereby reducing the Fe(III). Myers and Nealson [155] first identified metal reduction as a cellular metabolic process linked to growth, in the year 1988.

Many types of microbes can perform Fe(III) reduction; the substrates used by microorganisms to reduce Fe(III) are varied and readily available in most terrestrial environments. Observed electron donors include organic compounds such as: glucose [156, 157], acetate [158, 159], and CH_4 [160]; as well as H_2 [161], NH_4^+ [162], S^0 [163, 164], and electrically conductive carbon materials; e.g., activated carbon [165, 166] and carbon cloth [167]. Fe(III)-bearing electron acceptors include minerals such as ferrihydrite [160, 168, 169] and magnetite [156, 157].

In addition, dissimilatory Fe(III)-reducing bacteria have been shown to be capable of degrading complex polysaccharides [170, 171]; thus, the potential exists for Fe(III)-reducing bacteria to participate directly in the metabolism of humic substances, in addition to oxidizing the products of hydrolytic and fermentative metabolism [172]. Coupling humic substance degradation to dissimilatory iron reduction dramatically increases the pathways for soil organic carbon degradation, since microbial Fe(III)-reduction can lead to the release of dissolved organic carbon associated with Fe(III)-oxide surfaces [173, 174].

3.2. Metabolic Pathways and Substrates

3.2.1. Fe-Ammox

Initially suggested in 2005 [175], Fe-ammox is a recently discovered metabolic pathway that links nitrogen and iron cycling by coupling the reduction of Fe(III) to ammonium oxidation [175–177]. This process has been observed in a forested riparian wetland in New Jersey [162, 175, 176, 178, 179], tropical upland soils [177], wetland soils in South Carolina [179], and various forested and wetland locations in Southern China, including paddy soils [179]. The Fe-ammox reaction appears to be more common in acidic, iron-rich wetland environments [162, 177–

180]. The stoichiometry for the Fe-ammox process with ferrihydrite as the iron source is described by Equation (7).

$$6\,Fe_{10}O_{14}(OH)_2 + 98\,H^+ + 11\,NH_4^+ \rightarrow 60\,Fe^{2+} + 80\,H_2O + 11\,NO_2^- \quad (7)$$

The first Fe-ammox bacterium, *Acidimicrobiaceae* sp. A6, was isolated and characterized by Huang and Jaffe in 2018 [162]. Fe-ammox may be an autotrophic Fe(III) reduction process [162], although at present, data does not support this.

3.2.2. Anaerobic Methane Oxidation and Methanotroph Symbiosis

Beal et al. [160] first observed the coupling of the reduction of Fe(III)-bearing minerals by Archaea to the anaerobic oxidation of methane (AOM). AOM is most often coupled to sulfate reduction. However, in low-sulfate environments where abundant reactive Fe(III)-(oxyhydr)oxide phases and CH_4 co-occur, such as in freshwater and brackish environments [181], the coupling of Fe(III)-reduction to AOM can act as a mechanism to remove CH_4, which is a potent greenhouse gas.

Summers et al. [182] demonstrated the direct involvement of microbial nanowires and multiheme *c*-type cytochromes in interspecies electron transfer, and identified the process of direct interspecies electron transfer. In the context of microbial Fe(III)-reduction coupled to methanogenesis, a syntrophic interaction requires a Fe(III)-reducing bacterium, which oxidizes OM to interspecies-transferable molecules, and a methanotrophic partner to scavenge the transferable molecules. The reaction proceeds as follows for the Fe(III) minerals, bernite (Equation 8) and goethite (Equation 9) [4].

$$CH_4 + 8\,Fe(OH)_3 + 15\,H^+ \rightarrow HCO_3^- + 8\,Fe^{2+} + 21\,H_2O \quad (8)$$

$$CH_4 + 8\,FeOOH + 15\,H^+ \rightarrow HCO_3^- + 8\,Fe^{2+} + 13\,H_2O \quad (9)$$

Several studies have subsequently shown evidence for CH_4-oxidation coupled to Fe(III)-reduction [181, 183, 184]. Most recently, Cai et al. demonstrated that the anaerobic methanotrophic Archaea, *Candidatus* "Methanoperedens ferrireducens" could directly couple AOM to Fe(III) reduction [169].

3.2.3. S^0-Oxidation Coupled to Iron(III)-Reduction

At pH < 4.0, Fe(III) is soluble, removing the barrier of a solid-phase electron acceptor for acidophilic bacteria [185]. Dissimilatory Fe(III)-reduction is widespread among moderately acidophilic and extremely acidophilic bacteria [186]; the best known bacterium which utilizes this metabolic pathway is *Acidothiobacillus ferroxidans*. This strain is important in bioleaching applications, and can contribute to the creation of acid mine drainage [164]. Brock and Gustafson

[187] reported that *A. ferrooxidans* anaerobically reduced Fe(III) using elemental sulfur as the electron donor, proposing bacterial growth (Equation 10) [164]:

$$S^0 + 6\,Fe^{3+} + 4\,H_2O \rightarrow HSO_4^- + 6\,Fe^{2+} + 7\,H^+ \quad (10)$$

3.3. Mechanisms of Iron(III)-Reduction

Two physical constraints act to direct the mechanisms of electron transfer adopted by Fe(III)-reducing microorganisms at circumneutral pH: (1) the poor solubility of Fe(III) (oxyhydr)oxide minerals, and (2) the maximum hopping distance of 1.8–2.0 nm [188] for an electron moving between redox-active molecules. Despite this, both *Shewanella* and *Geobacter* species can transfer electrons to Fe(III) minerals located at considerable (mm- to cm-scale) distances from the cell [189–192] via non-local electron transfer strategies [193]. Two mechanisms have been proposed for the extracellular transfer of electrons from the microorganism to solid surfaces. These are: (i) electron transfer via direct contact of outer-membrane enzymes (cytochromes) or with outer cell membrane structures, such as pili and nanowires; and (ii) the use of soluble electron shuttles, such as humic substances and quinones. *Geobacter* spp. can also use Fe(III) (oxyhydr)oxides as conductors for interspecies electron transfer [8].

3.3.1. Electron Shuttling by Humic Substances

The process of electron shuttling between the bacterial cell and Fe(III) minerals via redox-active organic matter happens in a two-step process. First, the microbe donates electrons to the electron shuttle, reducing it. Second, the electrons are abiotically donated from the reduced shuttle to the Fe(III) mineral [11, 172, 194–199]. Many of the redox-active organic compounds which are common in soils and sediments can be used as electron shuttles for dissimilatory iron reduction. These include humic substances [194], plant exudates [200], biochar [201], and antibiotics [172, 192].

The ability to reduce humic substances is not constrained to metal-respiring organisms: many bacterial groups, including fermenting bacteria, methanogens, sulfate reducers, halorespirers, and hyperthermophilic Archaea [202–205] in diverse environments, such as lake and marine sediments and pristine and contaminated wetland sediments, were shown to be able to transfer electrons to humic substances [194, 198, 206, 207]. Because of the environmental and phylogenetic ubiquity of humic substance reduction, the abiotic reduction of Fe(III) by electron shuttles implicates biogeochemical iron-redox transformations even for microorganisms lacking the enzymatic machinery to directly reduce Fe(III) [208].

3.3.2. Pili and Nanowires to Iron Minerals

Conductive, redox-active pili, often called nanowires, have been implicated in extracellular electron transfer in both *Shewanella* spp. and *Geobacter* spp. [209,

210]. These nanowires play a critical role in long-range (~10 μm) extracellular electron transfer for respiration (i.e., cell-to-mineral) [209, 211] and interspecies (i.e., cell-to-cell) electron exchange [182, 212]. Wang et al. [135] show that conductive *G. sulfurreducens* pili are chains solely composed of OmcS, a six-heme *c*-type cytochrome. The hemes are closely stacked along the micrometer length of the filament, establishing the molecular basis for electronic conductivity in these nanowires.

3.3.3. Enzymatics

Electron transport pathways for Fe(III) reduction in different microorganisms often contain functionally similar components, but are different in their biochemistry [11, 213]. Genome sequence information from *Shewanella* spp. [214] and *Geobacter* spp. [215] helped to identify the genes involved in Fe(III) reduction pathways. Porin-cytochrome homologs have been identified in all sequenced *Geobacter* species and in bacteria from six different phyla, including *Anaeromyxobacter dehalogenans* 2CP-1, '*Candidatus* Kuenenia stuttgartiensis', *Denitrovibrio acetiphilus* DSM12809, *Desulfurispirillum indicum* S5, *Ignavibacterium album* JCM16511, and *Thermovibrio ammonificans* HB-1 [7, 11].

Electrons that originate from intracellular catabolism are transferred to cell surface-localized *c*-type cytochromes, which catalyze the extracellular electron transfer for the reduction of Fe(III) (oxyhydr)oxides [216]. The outer membrane cytochromes are connected to respiratory electrons of the intracellular quinone pool by outer membrane porin-cytochrome complexes, such as MtrA, MtrB, and CymA [217, 218]. This is suggestive of a general design principle for transferring electrons during the extracellular reduction of Fe(III)-bearing minerals [219, 220].

3.4. Most Important Representatives of Iron(III)-Reducing Microorganisms

The most notable examples of Fe(III)-reducing microorganisms include *Geobacter* spp. [159, 221, 222], *Shewanella* spp. [155, 161, 223–226], *Albidiferax ferrireducens* (formerly known as *Rhodoferax*) [227], *Geothrix fermentans* [228], and various hyperthermophilic Archaea [205, 229, 230]. Among the isolated microorganisms, Fe(III)-reducing extremophiles including hyperthermophilic, thermophilic, psychrophilic, acidophilic and alkaliphilic Archaea and bacteria have been described in pure culture [9, 203, 231–235]. One such isolate (strain 121, member of Archaea, most closely related to *Pyrodictium occultum* and *Pyrobaculum aerophilum*) surviving in hydrothermal vents has pushed the upper temperature limit for life to 121 °C [203].

3.4.1. Shewanella spp.

Shewanella oneidensis MR-1 was among the first identified microorganisms capable of using minerals that contain Fe(III) as terminal electron acceptors [224,

225]. The genus *Shewanella* is widespread in sedimentary environments and is characterized by a wide metabolic diversity [224, 226, 236–239]. *Shewanella* spp. reduce ferrihydrite to Fe(II) with lactate or formate (Equation 11):

$$5\,CH_3CH(OH)COO^- + 2\,Fe_{10}O_{14}(OH)_2 + 35\,H^+ \rightarrow 20\,Fe^{2+} + 5\,CH_3COO^- + 5\,HCO_3^- + 22\,H_2O \quad (11)$$

S. oneidensis MR-1 produces and secretes flavins that mediate extracellular electron transfer and facilitate the interaction between bacteria and their solid ferric substrate [240–243]. These chemically reduced flavins are proposed to function as diffusive electron shuttles [240, 241, 243], redox-active compounds that can be reduced and then transfer electrons directly to minerals that contain Fe(III) [244, 245].

Genetic studies of *Shewanella* spp. revealed the direct involvement of six multiheme *c*-type cytochromes – CymA, Fcc3 (also known as FccA), MtrA, MtrC, OmcA, and a small tetraheme cytochrome (STC) – and the porin-like outer membrane protein MtrB in the extracellular reduction of minerals that contain Fe(III) [92, 242, 246–249]. CymA, Fcc3, MtrA, MtrB, MtrC, OmcA, and STC form a pathway that oxidizes quinol in the cytoplasmic membrane and transfers the released electrons across the entire width of the cell envelope to the surface of minerals [7]. MtrA, B, and C from a complex and their protein structure has been recently solved by Edwards et.al. using X-ray crystallography techniques [91].

3.4.2. Geobacter spp.

Microorganisms from the family *Geobacteraceae* play a significant role in environmental Fe(III) reduction and the oxidation of organic matter in circumneutral surface and subsurface environments. The metabolic activity of *Geobacter* spp. is flexible and varied. These taxa are capable of utilizing a broad variety of carbon sources for growth, including monoaromatic compounds [250, 251], alcohols and fatty acids [252], acetate, lactate, pyruvate, and formate. In addition, *Geobacter* spp. can completely mineralize organic carbon to CO_2 [222]. Of special interest for engineering applications, *Geobacter* spp. are capable of generating electricity by transferring electrons directly to electrodes.

Two examples of the stoichiometric reduction of Fe(III) coupled to the oxidation of organic matter (acetate; Equation 12) and the reductive formation of magnetite from ferrihydrite (Equation 13) are as follows:

$$CH_3COO^- + 8\,Fe^{3+} + 4\,H_2O \rightarrow 2\,HCO_3^- + 8\,Fe^{2+} + 9\,H^+ \quad (12)$$

$$5\,CH_3COO^- + 12\,Fe_{10}O_{14}(OH)_2 \rightarrow 40\,Fe_3O_4 + 5\,HCO_3^- + 5\,CO_2 + 17\,H_2O \quad (13)$$

G. sulfurreducens does not synthesize electron shuttle molecules but requires direct contact with an electron acceptor via conductive filaments for long-range

extracellular electron transfer [209]. However, *Geobacter* spp. are known to make use of electron shuttles if present (see e.g., *Geobacter metallireducens* [194]). *Geobacter* spp. secrete extracellular cytochromes, such as the hexaheme OmcS in *G. sulfurreducens.* These cytochromes have been associated with conductive pili nanowires [211, 253], which mediate the conduction of current along the length of the wire or function as a contact point for mineral Fe(III) reduction [254].

In addition to cytochromes, *G. sulfurreducens* requires the outer membrane porin OmpJ for Fe(III) reduction [255]. In *G. sulfurreducens* DL-1 and *G. sulfurreducens* PCA, the key players in electron transfer across the cell envelope include the putative quinol oxidases ImcH and CbcL in the cytoplasmic membrane [256, 257], PpcA and PpcD in the periplasm [258, 259], and OmaB, OmaC, OmcB and OmcC in the outer membrane. The latter form porin–cytochrome trans-outer membrane protein complexes with the porin-like outer membrane proteins OmbB and OmbC [219, 254, 260]. In addition to cytochromes, *G. sulfurreducens* requires the outer membrane porin OmpJ for Fe(III) reduction [255].

4. APPLICATIONS AND CONSEQUENCES

4.1. Link to Other Biogeochemical Cycles

As explained in the previous sections, iron cycling microorganisms have been shown to occur in various aquatic and terrestrial environments, using different species of Fe(II) and Fe(III) for energy generation and growth. However, these processes are not only relevant for biogeochemical iron cycling. Iron metabolism is closely linked to most other important biogeochemical cycles. The oxidation of organic molecules (including methane) during microbial Fe(III) reduction (respiration with Fe(III)) as well as CO_2 fixation during microbial Fe(II) oxidation (with nitrate or O_2 as electron acceptor) link the iron to the carbon cycle. Additionally, the sequestration of organic molecules by sorption and co-precipitation to Fe(III) minerals and the release of the organic carbon during reductive dissolution of these carbon-loaded Fe(III) minerals shows how Fe(III) minerals have the potential for controlling the cycling of carbon [261].

Furthermore, a series of abiotic and biotic reactions such as microbially-catalyzed nitrate-dependent Fe(II) oxidation (see Section 2.1.3.), enzymatic ammonium oxidation coupled to Fe(III) mineral reduction, or abiotic oxidation of Fe(II) by reactive nitrogen species (nitric oxide or nitrite) couple the iron to the nitrogen cycle. Similarly, the oxidation of several sulfur species (sulfide, elemental sulfur, etc.) can be linked abiotically and biotically to Fe(III) mineral reduction (see Section 3.2.3.), thus linking the iron cycle to the sulfur cycle.

4.2. Consequences for Pollutants

In addition to these connections of the biogeochemical iron cycle to all other major element cycles, iron-metabolizing microorganisms can also influence the

fate and environmental behavior of pollutants, in particular toxic metals. On the one hand, Fe(III)-reducing bacteria can directly interact with toxic metals such as chromium and uranium, and can be used for remediation purposes by reductive immobilization of Cr(VI) as Cr(III) oxide (Cr_2O_3) and U(VI) as U(IV) oxide (UO_2) [262]. On the other, Fe(III) mineral-reducing bacteria were shown to be responsible for reductive dissolution of arsenic-bearing Fe(III) minerals and thus mobilization of arsenic into ground water and drinking water [263, 264]. On the oxidative side, Fe(II)-oxidizing bacteria and the resulting biogenic Fe(III) (oxyhydr)oxides have been suggested to be useful for immobilizing toxic metals such as arsenic, for example in drinking water filters [265–267].

4.2.1. Bioremediation and Biotechnology

Fe(III)-reducing bacteria contribute to bioremediation and bioattenuation of many contaminants through a variety of processes. Both, *Shewanella* spp. and *Geobacter* spp. have been reported to directly respire a number of metals other than Fe(III) and Mn(IV), including U(VI) [268, 269], sequestering radioactive uranium in the solid phase as the oxidized form U(IV).

4.2.2. Oxidation of Hydrocarbons

Fe(III)-reducing microorganisms such as *Geobacter metallireducens* GS-15 and *Geobacter* strain Ben couple the oxidative degradation of aromatic hydrocarbon contaminants, such as benzoate, toluene, phenol and *p*-cresol, to the reduction of Fe(III) [250, 250, 270–275]. Acetate oxidation by *Geobacter sulfurreducens* PCA is also electrically coupled to the reductive degradation of the contaminant trichloroethene by *Desulfitobacterium* spp. and *Dehalococcoides* spp. through conductive minerals [276]. In hydrocarbon-contaminated groundwater, Amos et al. [277] were able to link Fe(III) reduction to the oxidation of CH_4 under anoxic conditions.

4.2.3. Sequestration of Heavy Metals

In addition to the direct respiration of metals, the microbially-mediated reduction of Fe(III) regulates the solubility and sequestration of heavy metals by causing the reduction, and subsequent precipitation of heavy metals in Fe(III) (oxyhydr)oxide minerals [278–280]. This is because Fe(II) is a strong reductant to many heavy metals. Minerals, such as Fe(III) (oxyhydr)oxides (e.g., goethite), hematite, or maghemite that contain microbially-formed Fe(II) also reduce, and thereby sequester Cr(VI), Se(IV), Se(VI), and Tc(VII). Further, some Cr(III) can be incorporated into the mineral structure [102, 168, 278, 281–285], and the precipitates provide a reactive surface for the adsorption of PO_4^{3-}, Zn(II), As(V), and Co(II) [286].

5. OUTLOOK AND FUTURE DIRECTIONS

Although a lot is known about microbial iron metabolisms, there are several fascinating new areas of research that emerged in the last years, which require study by the next generations of iron biogeochemists and iron geomicrobiologists. These areas include (i) the isolation, cultivation, and characterization of microbial representatives of new iron-related metabolisms, (ii) the mechanisms of electron transfer being utilized by iron-cycling bacteria, and (iii) the role of iron-metabolizing microbes in the environment.

One obvious research need is that microbial representatives of new iron-related metabolisms need to be isolated, cultured, and characterized. This includes autotrophic nitrate-reducing Fe(II)-oxidizing microorganisms where so far only one promising example exists (culture KS, see Section 2.1.3.4. (b) [98]). However, this culture is currently still a mixed culture, and most other cultures that have been suggested to be autotrophic are either questionable or have been shown to need an organic co-substrate for sustainable growth and cultivation [100]. The isolation of new autotrophic nitrate-reducing Fe(II)-oxidizers (including isolation of the Fe(II)-oxidizer from culture KS) will then also allow to study the Fe(II) oxidation mechanisms and enzymes in these systems. Additionally, isolating more novel strains capable of coupling methane and ammonium oxidation to Fe(III) reduction [160, 177, 287] will provide the opportunity to investigate these metabolisms in more detail and to evaluate their potential environmental relevance.

The mechanisms of electron transfer being exploited by iron-cycling bacteria are complex and varied, and recent discoveries about the underlying structures have accelerated in the last few years. This is a promising and important area of active research, as these complex microbial interactions are teased apart in detail. Finally, while links to nearly every known biogeochemical cycle have been established, current knowledge only scratches the surface of understanding the intricacies of the role of iron minerals and iron-cycling microbes in the environment.

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ABBREVIATIONS AND DEFINITIONS

ADP	adenosine 5′-diphosphate
Anammox	anaerobic ammonium oxidation
AOM	anaerobic oxidation of methane
ATP	adenosine 5′-triphosphate
C*c*O	cytochrome *c* oxidase
Cyc	cytochrome *c*
DNRA	dissimilatory nitrate reduction to ammonium
EDTA	ethylenediaminetetraacetic acid
Fe-ammox	iron-reducing anaerobic oxidation of ammonium
HiPIPs	high potential iron-sulfur proteins
NADH	reduced nicotinamide adenine dinucleotide
NAD^+	nicotinamide adenine dinucleotide
$NADP^+$	nicotinamide adenine dinucleotide phosphate
NADPH	reduced nicotinamide adenine dinucleotide phosphate
NRFeOx	nitrate reducing Fe(II)-oxidizer
NTA	nitrilotriacetic acid
OM	organic matter
PMF	proton motive force
P_i	inorganic phosphate
RcY	rusticyanin
RC	photosynthetic reaction center
STC	small tetraheme cytochrome

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