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Minireview

Microbial anaerobic Fe(II) oxidation – Ecology, mechanisms and environmental implications

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Summary

Iron is the most abundant redox-active metal in the Earth's crust. The one electron transfer between the two most common redox states, Fe(II) and Fe(III), plays a role in a huge range of environmental processes from mineral formation and dissolution to contaminant remediation and global biogeochemical cycling. It has been appreciated for more than a century that microorganisms can harness the energy of this Fe redox transformation for their metabolic benefit. However, this is most widely understood for anaerobic Fe(III)-reducing or aerobic and microaerophilic Fe(II)-oxidizing bacteria. Only in the past few decades have we come to appreciate that bacteria also play a role in the anaerobic oxidation of ferrous iron, Fe(II), and thus can act to form Fe(III) minerals in anoxic settings. Since this discovery, our understanding of the ecology of these organisms, their mechanisms of Fe(II) oxidation and their role in environmental processes has been increasing rapidly. In this article, we bring these new discoveries together to review the current knowledge on these environmentally important bacteria, and reveal knowledge gaps for future research.

Biogeochemistry of iron

A substantial amount of iron (Fe) is present in soils, as well as freshwater, marine and subsurface sediments. Under oxidizing conditions, iron is found mainly in Fe(III) (oxyhydr) oxide minerals such as ferrihydrite ($Fe(OH)_3$), goethite (α-FeOOH) and haematite (α-Fe_2O_3), as well as, to a lesser extent, in dissolved Fe(III)-natural-organic-matter complexes (Fe(III)-NOM complexes) (Carlson and Schwertmann, 1981; Schwertmann and Murad, 1988; Kostka and Luther, 1994). Under reducing conditions iron is mostly found in mixed-valent Fe minerals such as magnetite and green rust, or in Fe(II) minerals such as vivianite ($Fe_3(PO_4)_2 \cdot 8H_2O$) and siderite ($FeCO_3$), or even as dissolved Fe^{2+} ions (Thompson *et al*., 2011; Daugherty *et al*., 2017; Ginn *et al*., 2017; Herndon *et al*., 2017). Under both oxidizing and reducing conditions, iron can be found as structural components of silicate minerals such as clays (Pentráková *et al*., 2013) and in iron sulfide minerals. It has also been suggested that dissolved Fe(III)- and Fe(II)-complexes and colloids are important for iron biogeochemical processes (Luther *et al*., 1992; Taillefert *et al*., 2000). The fate of iron in the environment is controlled by a series of abiotic and microbially catalysed redox reactions that lead to Fe mineral formation, transformation and dissolution (Melton *et al*., 2014a). Abiotic reactions include the oxidation of Fe(II) by manganese oxides, by oxygen and nitrogen species, or by reactive radicals from natural organic matter (NOM). Additionally, Fe(II) can be formed by photochemical reduction of Fe(III), particularly in water columns, as well as by reduction of Fe(III) by sulfide and reduced NOM (Melton *et al*., 2014a).

Microbial Fe(III) reduction can reduce Fe(III) minerals, and in turn provide Fe(II) as either a dissolved or solid phase electron donor for Fe(II)-oxidizing bacteria. Under acidic and pH-neutral conditions, aerobic and microaerophilic Fe(II)-oxidizing bacteria can use O_2 as an electron acceptor for oxidation of Fe(II) to Fe(III). Almost 200 years ago, Ehrenberg (1836) described microbially-produced stalk-like iron oxide structures reminiscent of those now known to be produced by microaerophilic Fe(II)-oxidizing bacteria. However, in the last two decades, two other metabolic types of Fe(II)-oxidizers were discovered which require anoxic conditions and oxidize Fe(II) coupled to either nitrate reduction (NRFeOx, Eq. 1) or to photosynthesis (pFeOx, Eq. 2) (Widdel *et al*., 1993; Straub *et al*., 1996).

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$$10Fe^{2+} + 2NO_3^- + 24H_2O \rightarrow 10Fe(OH)_3 + N_2 + 18H^+ \quad (1)$$

$$HCO_3^- + 4Fe^{2+} + 10H_2O \rightarrow (CH_2O) + 4Fe(OH)_3 + 7H^+ \quad (2)$$

Oxygen is not abundant in all habitats on Earth: for example, vast regions of oceans and lakes are either seasonally or permanently anoxic. Oxygen can only be measured down to a few millimetres depth in soils and sediments, particularly if they are waterlogged and organic-rich. Similarly, the deep sea and the deep continental crust are oxygen-poor. Thus, the discovery of these anaerobic Fe(II)-oxidizers revealed that microorganisms could contribute to Fe(II) oxidation in environments which had previously been overlooked, rapidly broadening our appreciation of the importance of Fe(II)-oxidizing bacteria in the environment. Several strains of NRFeOx and pFeOx have been isolated and more and more data from genomic, metagenomic and metatranscriptomic studies are available (He *et al*., 2016; Jewell *et al*., 2016), yet our understanding of the ecological contribution, the physiology and of the enzymatic mechanism(s) of anaerobic Fe(II) oxidation is still very limited compared with our understanding of microbial Fe(III) reduction or microaerophilic Fe(II) oxidation. The physiology of these organisms is very different from those known from oxic environments and, since oxygen is one of the primary factors controlling biogeochemical cycling, their interaction with the surrounding environment is markedly different (Melton *et al*., 2014a). Thus, specific focus on this anoxic part of the iron cycle is required to fully appreciate the role of these bacteria in the earth system. In this review article, we compile the current knowledge on these anaerobic Fe(II)-oxidizing bacteria, highlight existing open research questions and discuss the potential environmental importance and biotechnological applications of these microorganisms.

Nitrate-reducing Fe(II) oxidation

Links between nitrate-reducers and Fe(II)

The reduction of nitrate coupled to Fe(II) oxidation was first observed more than 20 years ago (Brons *et al*., 1997; Hafenbradl *et al*., 1996; Straub *et al*., 1996). Since then we have come to appreciate that nitrate-reducing bacteria can interact with Fe(II) in three distinct ways (Fig. 1). Firstly, autotrophic nitrate-reducing Fe(II)-oxidizing bacteria (NRFeOx) use Fe(II) as an electron donor for energy generation and fixation of CO_2, from which they build biomass. These organisms do not need organic carbon in addition to the Fe(II). In contrast to autotrophic NRFeOx, most other nitrate-reducing bacteria which have been shown to oxidize Fe(II) require addition of an organic substrate such as acetate to continually oxidize Fe(II) (Straub *et al*., 2004; Kappler *et al*., 2005b). Such organisms are often referred to in the literature as 'mixotrophs'. In order to be a true mixotroph, these organisms must utilize Fe(II) as an electron donor (by an enzymatic oxidation of the Fe(II)) in addition to the organic C source. Alternatively, some of the organisms described in the literature to catalyse Fe(II) oxidation do so as a result of abiotic reactions with reactive nitrogen-containing by-products of heterotrophic denitrification, such as nitrite and nitric oxide. For our purposes, we will refer to all organisms described to simultaneously oxidize Fe(II) and reduce nitrate, but for which no enzymatic component has been proven, as 'chemodenitrifiers', but will not consider them true nitrate-reducing Fe(II)-oxidizers. Given that these reactions, as discussed in subsequent sections, seem to be common to typical denitrifiers such as *Escherichia coli* (Brons *et al*., 1997), we expect that such a classification would apply to any heterotrophic denitrifier grown with high concentrations of Fe(II).

Deciphering which mechanism is applicable to which strains is a subject of significant debate in the literature. In the following sections we will highlight this debate, which centres around two key issues. Firstly, there have been many strains proposed to be autotrophic NRFeOx yet evidence for continued Fe(II) oxidation in the absence of additional carbon sources is often lacking. Secondly, for a number of strains which do require additional organic carbon, there is much debate regarding whether or not there is an enzymatic component of Fe(II) oxidation, that is, whether or not these strains are 'mixotrophs' or simply 'chemodenitrifiers'.

Autotrophic NRFeOx

While Fe(II) oxidation coupled to nitrate reduction is thermodynamically favourable ($\Delta G^{\circ\prime}$ = –96.23 kJ mol^{-1} Fe), large quantities of Fe(II) would be required by autotrophic NRFeOx bacteria to grow (ca., 26 mol Fe(II) to fix 1 mol carbon) (Laufer *et al*., 2016a). Despite this, several studies claim to have identified autotrophic NRFeOx organisms. In Table 1 we have listed all the isolates which, to our knowledge, have been proposed to be autotrophic NRFeOx. We suggest that a true autotrophic culture would (i) require no organic carbon source, (ii) show growth of cells with only Fe(II), nitrate and CO_2 provided, (iii) maintain Fe(II) oxidation over several transfers without organic carbon addition and (iv) demonstrate CO_2 uptake during Fe(II) oxidation by incorporation of labelled CO_2 into biomass. As can be seen in Table 1, only one enrichment culture, the so-called 'KS Culture', fulfils all of these criteria while the other strains either do not fulfil these criteria, or the necessary supporting information is not described.

The chemolithoautotrophic co-culture known as Culture KS, enriched in the mid-1990s (Straub *et al*., 1996; Blöthe and Roden, 2009; Nordhoff *et al*., 2017), is therefore the most robust example of a purely autotrophic NRFeOx

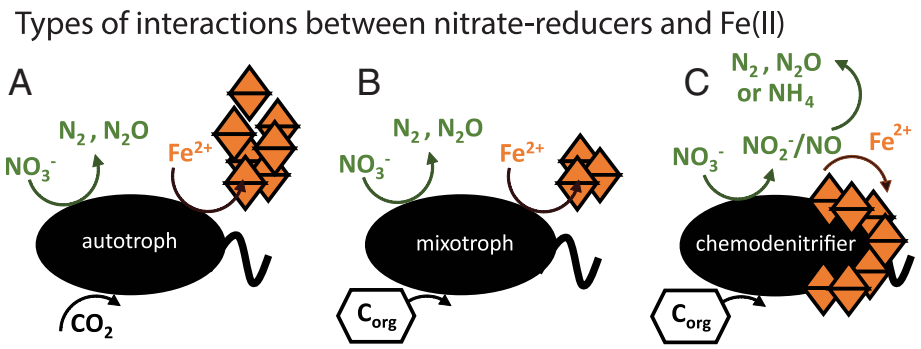

Fig. 1. Overview of the three different types of interaction between nitrate-reducing bacteria and Fe(II). (A) Autotrophic NRFeOx obtain carbon from CO_2 and oxidize Fe(II) enzymatically. (B) Mixotrophic NRFeOx require additional organic carbon as a carbon source, and Fe(II) oxidation has some enzymatic component (although there may also be some abiotic component). (C) Chemodenitrifiers require organic carbon and have no enzymatic component of Fe(II) oxidation. The position of the minerals (orange) relative to cells (black) indicates whether or not encrustation is expected.

system to date. It has been cultivated continuously for over 20 years with Fe(II) as the sole electron donor, nitrate as the sole electron acceptor, and CO_2 as the only carbon source in at least two different laboratories (Blöthe and Roden, 2009; He *et al*., 2016; Nordhoff *et al*., 2017; Tominski *et al*., 2018a). In this culture, complete oxidation of aqueous Fe(II) is observed and is coupled to denitrification to produce N_2. The dominant strain in 'Culture KS' belongs to the family *Gallionellaceae*, with a flanking community including species of *Rhizobium/Agrobacterium, Bradyrhizobium, Comamonadaceae, Nocardioides, Rhodanobacter, Polaromonas* and *Thiobacillus* which are chemodenitrifiers (Tominski *et al*., 2018a). The *Gallionellaceae* is designated as the autotrophic Fe(II)-oxidizer. The relative abundance and composition of the flanking community and the Fe(II)-oxidizer is dependent on the laboratory in which the culture is cultivated. It is hypothesized that the autotroph fixes CO_2 for the heterotrophic community members while the heterotrophic organisms detoxify nitric oxide which the autotroph does not have the genetic machinery to reduce (He *et al*., 2016; Tominski *et al*., 2018a, 2018b). The contribution of the *Gallionellaceae* strain varies between 42% and 96%, apparently as a result of different inoculum concentrations in different laboratories with lower inoculum concentrations (1%) favouring higher abundance of the Fe(II)-oxidizer (He *et al*., 2016; Nordhoff *et al*., 2017). The effect of the different community composition on the rate of Fe(II) oxidation has not been directly compared.

Mixotrophic NRFeOx and chemodenitrification

Many studies have also been conducted with bacteria which reduce nitrate and oxidize Fe(II) only in the presence of an additional carbon source, yet there is much debate over whether these organisms are mixotrophs or chemodenitrifiers (defined in Fig. 1). Some studies have shown an energetic benefit and increase in cell number when such bacteria are grown on acetate and nitrate with Fe(II) compared with setups without Fe(II) which could be seen as evidence of an enzymatic component (Muehe *et al*., 2009; Chakraborty *et al*., 2011). However, it was later suggested that this effect is probably a response to the nutritional benefit of iron addition, and not due to enzymatic Fe(II) oxidation (Klueglein and Kappler, 2013). Determination of an enzymatic component in potential mixotrophs is technically challenging to decipher and requires intricate analysis of all reactive intermediates (see most recently Jamieson *et al*., 2018). These authors claimed to have shown a requirement for an enzymatic component of oxidation in *Acidovorax* sp. strains BoFeN1 and 2AN, as well as *A. ebreus* strain TPSY, *Paracoccus denitrificans* Pd 1222 and *Pseudogulbenkiania* sp. strain 2002 by compilation and model-based interpretation of previously published experimental data. However, while this study is extensive and makes an impressive attempt at accounting for all potential abiotic processes, all previous studies on which these models were based on only account for extracellular substrate concentrations, that is, those substrates which were exported out of the cell. In the case of all of these strains, significant reactive nitrogen species production and Fe(II) oxidation occurs in the periplasm. We would argue that until these intracellular processes can be fully considered, we do not have concrete evidence that an enzymatic component is required to explain observed Fe(II) oxidation in any of the strains commonly studied as NRFeOx. As such, we will include all bacteria which have been shown to require an additional organic substrate together under 'chemodenitrifiers', of which some of the most well studied are listed in Table S1 (although this is not intended to be an exhaustive list).

Ecology of nitrate-reducing Fe(II)-oxidizers

A map displaying all isolated strains of anaerobic Fe(II)-oxidizing bacteria is shown in Fig. 2. Chemodenitrifiers have been isolated from a range of habitats including freshwater sediments (Straub *et al*., 1996), soils (Shelobolina *et al*., 2012a), hypersaline sediments (Emmerich *et al*., 2012), marine sediments (Laufer *et al*., 2016b) and hydrothermal vents (Hafenbradl *et al*., 1996; Edwards *et al*.,

Table 1. Summary of isolates/enrichment cultures of NRFeOx proposed in previous studies to be capable of autotrophic growth on Fe(II) and an indication of whether or not they meet the criteria for autotrophy proposed here. Number represents position in the map in Figure 2. This shows that all except one of the strains/cultures published as an 'autotroph' do not meet this criteria. Strain marked '*' required H_2 for Fe(II) oxidation.

No.	Culture/strain name	Class Order Family	Origin of sample	Identity of iron(III) minerals	Met criteria for autotrophy: *growth*	*Fe(II) oxidation over several generations*	*Absence of organic C source*	*Uptake of labelled CO_2 during Fe(II) oxidation*	Reference
Stable enrichment cultures									
1	Enrichment culture KS	Fe(II)-oxidizer: Betaproteobacteria Gallionellales Gallionellaceae	Freshwater sediment, Bremen, Germany	Goethite, magnetite	+	+	+	+	(Straub *et al*., 1996; He *et al*., 2016; Nordhoff *et al*., 2017; Tominski *et al*., 2018a)
Isolated strains									
2	Azoarcus Strain ToN1	Betaproteobacteria; Rhodocyclales; Zoogloeaceae	Weser river sediment, Bremen, Germany	n.a.	n.a.	n.a.	+	n.a.	(Rabus and Widdel, 1995; Straub *et al*., 1996)
3	*Pseudomonas stutzeri*	Gammaproteobacteria, Pseudomonadales; Pseudomonadaceae	Marine sample off the Californian Coast	n.a.	n.a.	n.a.	+	n.a.	(ZoBell and Upham, 1944; Straub *et al*., 1996; Peña *et al*., 2012)
4	*Ferroglobus placidus* (hyperthermophilic archaea)	Archaeoglobi; Archaeoglobales; Archaeoglobaceae	Shallow submarine hydrothermal system, Vulcano, Italy	Rusty ferric precipitates	+	–	–	n.a.	(Hafenbradl *et al*., 1996)
5	Aquabacterium Strain BrG2	Betaproteobacteria, Burkholderiales, Comamonadaceae	Freshwater sediment, Bremen, Germany	Rusty ferric precipitates	n.a.	n.a.	+	n.a.	(Straub *et al*., 1996; Buchholz-Cleven *et al*., 1991)
6	*Thiobacillus denitrificans* ATCC 25259*	Betaproteobacteria; Nitrosomonadales; Thiobacillaceae;	Sewage sludge, The United Kingdom	n.a.	–	+	+	n.a	(Straub *et al*., 1996; Beller *et al*., 2006)
7	*Geobacter metallireducens* strain GS-15	Deltaproteobacteria; Desulfuromonadales; Geobacteraceae	U-contaminated aquifer sediments, San Juan River, Shiprock	n.a.	n.a.	n.a.	+	n.a.	(Lovley, 1997; Finneran *et al*., 2002; Weber *et al*., 2006)
8	*Marinobacter* related species	Gammaproteobacteria; Alteromonadales; Alteromonadaceae	Deep sea hydrothermal system, Juan de Fuca ridge	n.a.	–	n.a.	+	n.a.	(Edwards *et al*., 2003)
9	*Hyphomonas* related species	Alphaproteobacteria; Rhodobacterales; Hyphomonadaceae;	Deep sea hydrothermal system, Juan de Fuca ridge	n.a.	–	n.a.	+	n.a.	(Edwards *et al*., 2003)
10	*Paracoccus ferrooxidans* strain BDN-1	Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae	Sewage sludge from bioreactor system	Ferric iron precipitates	+	–	–	n.a.	(Kumaraswamy *et al*., 2006)
11	*Pseudogulbenkiania* sp. strain 2002	Betaproteobacteria; Neisseriales; Chromobacteriaceae	Freshwater lake sediment, Illinois University	n.a.	+	–	+	+	(Weber *et al*., 2006)
12	*Azospira* bacterium TR1	Betaproteobacteria; Rhodocyclales; Rhodocyclaceae	Bioremediation site, BC, Canada	Very fine ferric iron oxides	–	–	–	n.a.	(Mattes *et al*., 2013)

(Continues)

Table 1. Continued

No.	Culture/strain name	Class Order Family	Origin of sample	Identity of iron(III) minerals	Met criteria for autotrophy				Reference
					growth	*Fe(II) oxidation over several generations*	*Absence of organic C source*	*Uptake of labelled CO_2 during Fe(II) oxidation*	
13	*Citrobacter freundii* PXL1	Gammaproteobacteria; Enterobacterales; Enterobacteriaceae	Sewage sludge, China	Amorphous to poorly crystalline ferric iron oxides	n.a.	n.a.	–	n.a.	(Li *et al*., 2014)
14	*Pseudomonas* sp. SZF15	Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae	Freshwater sediment, Tang Yu reservoir, China	n.a.	+	–	+	n.a.	(Su *et al*., 2015)
15	*Microbacterium* sp. strain W3	Actinobacteria; Micrococcales; Microbacteriaceae	Deep freshwater sediment, Lake Wuhan, China	Yellow-brown to orange rusty precipiates (assumed to be Fe(III) oxyhydroxide)	+	–	+	n.a.	(Zhang *et al*., 2015)
16	Strain W5	Actinobacteria; Micrococcales; Microbacteriaceae	Deep freshwater sediment, Lake Wuhan, China	n.a.	–	–	+	n.a.	(Zhou *et al*., 2016)

2003). Simultaneous Fe(II) oxidation and nitrate reduction has been demonstrated in enrichment cultures and natural sediment samples from freshwater (Melton *et al*., 2012), marine (Laufer *et al*., 2016c), estuarine (Robertson *et al*., 2016), fluvial (Coby *et al*., 2011) and subsurface (Benzine *et al*., 2013) environments as well as sewage sludge (Oshiki *et al*., 2013) and aquifers (Lovley *et al*., 1987). As shown in Table 1, isolation of NRFeOx organisms which fulfil all the criteria for autotrophy has proven to be more difficult. However, cultivation-independent techniques have been used to identify potentially active autotrophic NRFeOx microbes in the environment. A recent study by Laufer *et al*. (2016c) using coastal marine sediment in ^{14}C-labelled incubations provided, for the first-time, unequivocal evidence for the existence of autotrophic NRFeOx organisms in the environment. Using metagenomics and metatranscriptomics, Jewell *et al*. (2016) showed an increase in members belonging to the Fe(II)-oxidizing *Gallionellaceae* (accounting for up to 80% of the transcriptome) when nitrate was pumped into a groundwater aquifer. Another study showed that chemolithoautotrophy was dominant in an organic poor aquifer and that high fractions of the denitrifying communities were represented by OTUs closely related to the Fe(II)-oxidizer *Sideroxydans lithotrophicus* ES-1 (Herrmann *et al*., 2017). Recent studies have investigated the abundance and distribution of NRFeOx bacteria in freshwater lake sediments (Melton *et al*., 2012, 2014c) and in coastal marine sediments (Laufer *et al*., 2016b). In the freshwater lake sediments, the results indicate that NRFeOx bacteria are likely to be in competition for Fe(II) with phototrophic Fe(II)-oxidizers in the presence of light (Melton *et al*., 2012). Furthermore, while the distribution of these microbes is expected to adhere to the thermodynamically controlled geochemical stratification of substrates, this is not always the case. In marine sediments, autotrophic NRFeOx and denitrifiers which abiotically catalyse Fe(II) oxidation (as well as photoferrotrophs) may exist together in the sediment layers (Laufer *et al*., 2016b).

Mechanisms of oxidation

Mechanisms used by anaerobic NRFeOx bacteria to oxidize Fe(II) are still unclear, but appear to be different depending on whether the bacteria are autotrophs, mixotrophs or chemodenitrifiers (see Fig. 1). For autotrophic NRFeOx, three mechanisms have been proposed for Fe(II) oxidation whereby: (i) there is a dedicated Fe(II) oxidoreductase, (ii) there is an unspecific activity of the nitrate reductase or (iii) the bc1 complex accepts electrons from Fe(II) and reduces the quinone pool (Ilbert and Bonnefoy, 2012).

In recent years much research has focused on the first scenario and attempted to identify a possible dedicated outer membrane Fe(II) oxidoreductase present in autotrophic NRFeOx microorganisms (Beller *et al*.,

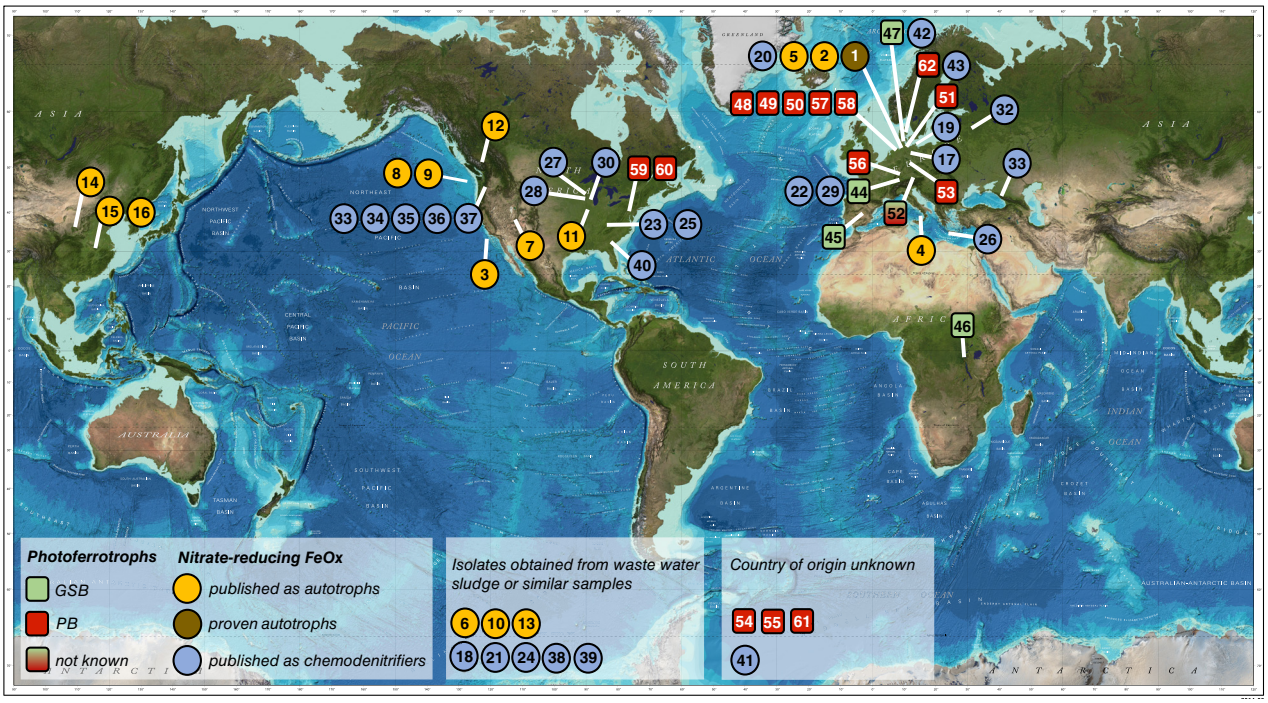

Fig. 2. Map displaying isolated strains (or stable enrichment cultures) of photoferrotrophs or nitrate-reducing bacteria implicated in Fe(II)-oxidation, as well as their source of origin. Further detail on each strain can be found in Tables 1 and 2; Supporting Information Table S1. 'Proven' autotrophs were designated based on their ability to meet the four criteria for autotrophy proposed in Table 1. Red squares include both purple sulfur bacteria and purple non-sulfur bacteria (PB). GSB represents Green Sulfur Bacteria. Map obtained from the GEBCO world map 2014 (www.gebco.net).

2013; He *et al*., 2017). A metagenomics analysis of the NRFeOx Culture KS identified homologues of the cytochrome c putative Fe(II) oxidase Cyc2 (found in other known Fe(II)-oxidizers) in the draft genomes of the *Gallionellaceae* sp. and in the *Rhodanobacter* sp. present within Culture KS (He *et al*., 2016). Homologues of the porin cytochrome c porin complex MtoAB were also found in the *Gallionellaceae* sp. in Culture KS and as well as in *D. aromatica* RCB (which was proposed to be autotrophic but has not been confirmed) (He *et al*., 2017). Figure 3A displays a potential mechanism by which the proposed autotrophic Fe(II)-oxidizer in the KS culture could oxidize Fe(II) in which an electron is obtained via Fe(II) oxidation and passed along the electron transport chain where nitrate is reduced stepwise to NO. Outside the cell, there is potential for NO to be either consumed by the flanking community or react with aqueous Fe(II).

As already discussed, there is continuing controversy as to whether some NRFeOx bacteria make use of an enzymatic machinery to oxidize Fe(II), making them true mixotrophs. Many hypothesize that Fe(II) oxidation is driven by an abiotic chemical side reaction of denitrification (Brons *et al*., 1997; Klueglein and Kappler, 2013; Klueglein *et al*., 2014). It has been proposed that nitrate-dependent Fe(II) oxidation can be promoted by all heterotrophic denitrifiers (Carlson *et al*., 2013). This is evidenced by the fact that many nitrate-reducers can oxidize Fe(II) when an organic compound is provided in combination with Fe(II), even *E. coli* (Brons *et al*., 1997). The abiotic reduction of nitrate by dissolved Fe(II) is slow (Buresh and Moraghan, 1978; Colman *et al*., 2008), but nitrite reduction by Fe(II) to N_2O is kinetically favourable under environmental conditions and likely to occur if reactive chemical substrates are present as catalysts (Zhu-Barker *et al*., 2015). For example, the reduction of nitrogen species by Fe(II) can occur via heterogeneous surface catalysis where viable surfaces include crystalline Fe(III) oxyhydroxides, green rust, pyrite (FeS_2) and cell surfaces (Moraghan and Buresh, 1977; Ottley *et al*., 1997; Kampschreur *et al*., 2011; Bosch *et al*., 2012; Dhakal *et al*., 2013; Jones *et al*., 2015; Buchwald *et al*., 2016; Grabb *et al*., 2017). Microbially driven NRFeOx, Fe-ammox (Fe(III)-coupled ammonium oxidation) and heterotrophic nitrate-reduction (denitrification) can lead to the formation of reactive nitrogen species as a metabolic intermediate (nitrite, NO_2^- or nitric oxide, NO) (Weber *et al*., 2001; Picardal, 2012; Klueglein and Kappler, 2013; Oshiki *et al*., 2013), providing ample supply of compounds which could react quickly with Fe(II).

A summary of the proposed mechanism for Fe(II) oxidation by chemodenitrification is shown in Fig. 3B. This represents an end-member mechanism for NRFeOx where there is no enzymatic component to

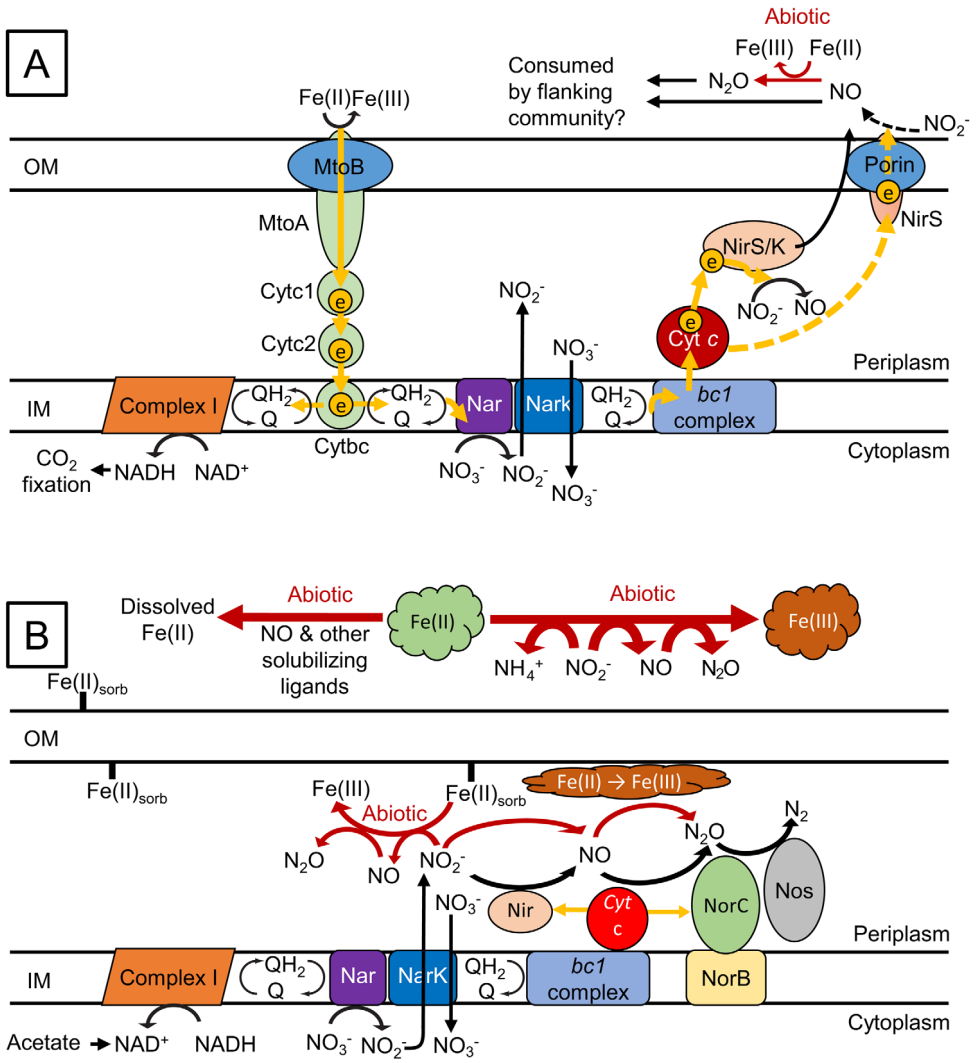

Fig. 3. Schematics of the current hypotheses on the mechanism of Fe(II) oxidation in (A) the Gallionellaceae species proposed to be the autotrophic nitrate-reducing Fe(II)-oxidizer in the KS culture (modified from He *et al*., 2016) and (B) the proposed mechanism of Fe(II) oxidation by chemodenitrification (modified from Carlson *et al*., 2013). In (B), nitrate reduction may also be catalysed by Nap instead of Nar, and nitric oxide reduction may be facilitated by NorZ instead of NorC, which accepts electrons from quinols rather than cytochrome c.

Fe(II) oxidation. In this case electrons are obtained from organic carbon oxidation, leading to stepwise reduction of nitrate to nitrogen. Intermediates may leak out at any of the steps and react with Fe(II) either inside or outside of the cell. While some Fe(II)-oxidizing bacteria such as photoferrotrophs and the autotrophic Culture KS have developed strategies for avoiding cell encrustation by Fe(III) minerals (Hegler *et al*., 2010), no such strategies have been demonstrated by chemodenitrifying bacteria (Schaedler *et al*., 2009; Klueglein *et al*., 2014). Cell encrustation inhibits respiratory complexes and other periplasmic sites which leads to decreased nitrate-dependent Fe(II) oxidation (Carlson *et al*., 2013) and eventually results in cell death (though not always for all community members, e.g., Miot *et al*., 2015).

Fe(II) sources and Fe(III) minerology

Different sources of Fe(II) species including dissolved Fe^{2+}, complexed Fe(II) [e.g., Fe(II)-EDTA] and mineral bound Fe(II) can undergo oxidation as a result of NRFeOx. Fe(II)-OM complexes such as Fe(II)-EDTA and Fe(II)-NTA were shown to be oxidized by several different species including *Dechloromonas* sp. strain UWNR4, *Paracoccus ferrooxidans* sp. nov., BDN-1, *Pseudogulbenkiania* sp. 2002 (Kumaraswamy *et al*., 2006) and *Desulfitobacterium frappieri* (Shelobolina *et al*., 2003). *Acidovorax* sp. BoFeN1 is able to promote oxidation of Fe(II)-citrate, Fe(II)-EDTA, Fe(II)-humic-acid, and Fe(II)-fulvic-acid, but only in the presence of aqueous Fe(II) (Peng *et al*., 2018). Potential toxicity of ligands may also be a controlling factor in these processes.

In addition to dissolved Fe(II), different microbial species are also able to oxidize Fe(II) mineral phases via NRFeOx. The autotrophic enrichment Culture KS, can oxidize Fe(II) in the form of microbially reduced goethite, biogenic magnetite (Fe_3O_4) and chemically precipitated siderite ($FeCO_3$) but only a small amount of biogenic siderite (Weber *et al*., 2001). Oxidation of biogenically reduced goethite, chemically precipitated siderite, biogenic magnetite and magnetite nanoparticles by a chemodenitrifier has also been observed (Chakraborty *et al*., 2011; Byrne *et al*., 2015). Sulfide minerals, such as ferrous sulfide (FeS), was observed to be oxidized by an enriched nitrate-reducing culture from marine sediment although pyrite (FeS_2) was not bioavailable for oxidation by the same culture (Schippers and Jorgensen, 2002). Vivianite can be oxidized by *Acidovorax* sp. BoFeN1 in the presence of dissolved free Fe^{2+} (Miot *et al*., 2009a), or also in cell suspension experiments with *Acidovorax ebreus* (Carlson *et al*., 2013); however, vivianite cannot be oxidized by *Acidovorax* sp. Strain BoFeN1 in the absence of dissolved Fe^{2+} (Kappler *et al*., 2005b; Miot *et al*., 2009b). Fe(II)-containing phyllosilicates, such as biotite, can be oxidized by the autotrophic enrichment Culture KS (Shelobolina *et al*., 2012b). Clay minerals such as illite, smectite and nontronite, are also available for Fe(II) oxidation by *Desulfitobacterium frappieri* (Shelobolina *et al*., 2003) and by *Pseudogulbenkiania* sp. strain 2002 (Zhao *et al*., 2017). Culturing of microbes on solid mineral substrates has, however, proved difficult. Poised electrodes could provide an alternative strategy for enrichment of mineral transforming bacteria as demonstrated by Rowe *et al*. (2015) in sediment microcosms.

The oxidation of Fe(II) species coupled to microbial nitrate reduction can lead to the precipitation of a variety of Fe(III) minerals and/or mixed-valent Fe(II)–Fe(III) mineral phases. These phases can precipitate either on the surface of the bacteria or in close association between bacteria and minerals as cell mineral aggregates. For many chemodenitrifiers, precipitation occurs in the periplasm, as shells which completely enclose the cell, or in extracellular globules or as Fe filaments on extracellular polymeric substances (EPS) (Kappler *et al*., 2005b; Miot *et al*., 2009b; Schaedler *et al*., 2009; Schmid *et al*., 2014). The extent of encrustation increasingly limits the metabolic capabilities of these bacteria until encrustation is so severe that they are killed (Miot *et al*., 2015). The extent to which encrustation is an artefact caused by high Fe(II) concentrations in lab conditions is unclear, but such structures have been observed in the environment (Miot *et al*., 2016). In addition to goethite, mineral encrustation of the bacterial periplasm may also be comprised of Fe(III) phosphate minerals, which accumulate on proteins within the periplasm of cells (Miot *et al*., 2009b). *Acidovorax* sp. strain BoFeN1 can also form extracellular magnetite when green rust is provided as an Fe(II) source (Miot *et al*., 2014).

The mineralogy of biogenic Fe(III) minerals is dependent on a myriad of variables ranging from the microbial species, growth medium (i.e., phosphate or buffer concentrations), substrate availability, as well as geochemical and physical conditions such as pH and temperature (Posth *et al*., 2014; Miot and Etique, 2016). Because solution chemistry is such a strong driver in biogenic mineral precipitation, the same strain of bacteria, *Acidovorax* sp. Strain BoFeN1 for example, is capable of forming multiple Fe(III) mineral phases (Kappler and Newman, 2004; Kappler *et al*., 2005a; Hohmann *et al*., 2009; Miot *et al*., 2009b; Posth *et al*., 2010). For instance, lepidocrocite (γ-FeOOH) can be formed directly via NRFeOx by *Acidovorax* sp. strain BoFeN1 (Larese-Casanova *et al*., 2010). However, in the presence of strong complexing ligands such as carbonate, Fe(III) mineral precipitation is directed toward goethite (α-FeOOH) (Carlson and Schwertmann, 1981; Larese-Casanova *et al*., 2010). Similarly, when phosphate concentration is high, the precipitation of both poorly crystalline or crystalline Fe(III)-phosphate minerals is favoured. In addition to Fe(III) minerals, mixed-valent minerals such as green rust (as an intermediate phase) or magnetite can also result from microbial Fe(II) oxidation coupled to nitrate reduction, by species such as *Dechlorosoma sullium* strain PS (this species is capable of using perchlorate or nitrate as an electron acceptor) (Achenbach *et al*., 2001; Chaudhuri *et al*., 2001) or by *Acidovorax* sp. strain BoFeN1 (Pantke *et al*., 2012; Klueglein and Kappler, 2013; Miot *et al*., 2014). Magnetite formation by NRFeOx has been shown in the presence of magnetite nucleation sites which function as seeds for further magnetite formation (Dippon *et al*., 2012). Green rust has also been shown to form as an intermediate phase during the oxidation of Fe^{2+} to Fe(III) minerals by *Acidovorax* sp. BoFeN1 (Pantke *et al*., 2012), *Klebsiella mobilis* (Etique *et al*., 2014) and Culture KS (Nordhoff *et al*., 2017). The formation of this metastable mineral gives an indication of the mineralization pathway, that is, via precipitation of green rust by NRFeOx followed by solid-state transformation into Fe(III) or Fe(II)–Fe(III) mineral phases.

Environmental implications

The proposed stoichiometric reaction for NRFeOx organisms is presented in Eq. 1 whereby the stepwise reduction of nitrate via denitrification coupled to Fe(II) oxidation results in the formation of N_2. While some studies have shown nitrate reduction to N_2 without the accumulation of intermediates (Chaudhuri *et al*., 2001; Straub *et al*., 2004;

Blöthe and Roden, 2009), a smaller number of studies using sediment enrichments have shown that ammonium accumulation can also occur during NRFeOx (Weber *et al*., 2006; Coby *et al*., 2011). More recent studies using sediment incubations have identified the concentration of dissolved Fe(II) to be the controlling factor on the relative contribution of denitrification and dissimilatory nitrate reduction to ammonium (DNRA) in nitrate removal (Roberts *et al*., 2014; Robertson *et al*., 2016; Robertson and Thamdrup, 2017). In addition to contributing to the accumulation of ammonium, anaerobic ammonium oxidizing (annamox) bacteria can also mediate nitrate-reducing Fe(II) oxidation (Oshiki *et al*., 2013). The role of NRFeOx in the transformation of ammonium can have important implications for wastewater treatment processes.

The abiotic reduction of nitrite by Fe(II) can produce N_2O (Eq. 3) which is not quickly reduced by Fe(II).

$$4\,Fe^{2+} + 2\,NO_2^- + 5\,H_2O \rightarrow 4\,FeOOH + N_2O + 6\,H^+ \qquad (3)$$

Thus, the abiotic reduction of nitrite by Fe(II) (Eq. 3) has the potential to be a crucial source of nitrous oxide (N_2O) (Burgin and Hamilton, 2007; Picardal, 2012; Zhu-Barker *et al*., 2015). N_2O belongs to the group of greenhouse gases (Wuebbles, 2009), has 300 times the global warming potential of CO_2 on a time scale of 100 years (IPCC, 2013) and contributes to stratospheric ozone depletion (Crutzen, 1974; Ravishankara *et al*., 2009). It is known that abiotic nitrite reduction by redox-active Fe(II) has the potential to be an important source of N_2O in Fe- and carbon-rich habitats (Burgin and Hamilton, 2007; Picardal, 2012; Zhu-Barker *et al*., 2015). Chemodenitrification occurs in forest, grassland and cropland soils (van Cleemput and Samater, 1995; Zhu *et al*., 2013; Heil *et al*., 2015), paddy soils (Liu *et al*., 2012; Wang *et al*., 2016b), activated sludge (Wang et al., 2016a), hypersaline ponds and brines in Antarctica (Samarkin *et al*., 2010; Peters *et al*., 2014; Zhu-Barker *et al*., 2015; Ostrom *et al*., 2016), and marine coastal sediments (Wankel *et al*., 2017). However, the contribution of this process to the global N_2O budget is currently unknown.

The identity and morphology of iron minerals formed by NRFeOx can be influenced by the presence of metal species, such as arsenic (As) or silicon (Si) (Kleinert *et al*., 2011; Picard *et al*., 2016). For example, during initial formation, the presence of As can direct mineral formation toward the poorly crystalline Fe(III) mineral ferrihydrite as opposed to more crystalline Fe(III) mineral goethite (Kleinert *et al*., 2011). Arsenic co-precipitated with ferrihydrite could potentially be mobilized upon microbial Fe(III) reduction whereas As-goethite co-precipitates would likely be more stable (Hohmann *et al*., 2009). This transformation process may be inhibited if minerals are co-precipitated with Si, which has been shown to stabilize phases such as ferrihydrite and goethite (Picard *et al*., 2016). Repeated redox cycling between oxidizing and reducing conditions can influence the products which form as a result of nitrate-reducing Fe(II) oxidation (Mejia *et al*., 2016) which may influence the interactions between the minerals and other environmental factors. Immobilization of iron by oxidation is itself an important environmental process. For example, it has been shown that NRFeOx processes can act to limit iron transport from oceanic oxygen minimum zones (Scholz *et al*., 2016). Since iron is a major limiting nutrient in the world's oceans (Moore *et al*., 2013), the limitation of iron transport from zones where dissolved iron is higher than average seawater can thus have an influence on global oceanic productivity.

It should be noted that the concentrations of nitrate in an environment do not need to be high to lead to Fe(II) oxidation, because nitrate production and nitrate consumption can proceed simultaneously. Therefore the net concentrations which are measured (e.g., in porewater) do not necessarily reflect the amount of nitrate that is available to interact with NRFeOx bacteria. Additionally, the abiotic oxidation of Fe(II) is primarily driven by reaction with reactive nitrogen intermediates (NO, NO_2^-) which are unstable and rapidly react, such that they would not be detected in bulk measurements of porewater substrate concentrations.

Anoxygenic phototrophic Fe(II)-oxidizers

Physiology, existing isolates/cultures and ecology

The second group of anoxygenic bacteria which can oxidize Fe(II) are anoxygenic phototrophs. These bacteria harvest energy from light and oxidize Fe(II) in order to produce reducing equivalents for CO_2 fixation. The existence of phototrophic organisms which could oxidize Fe(II) was first proposed as an explanation for the formation of vast, economically important iron-rich rock formations by Hartman (1984) who disagreed with the geologist Preston Cloud that these were formed as a result of cyanobacterial oxygenic photosynthesis (Cloud, 1973). Hartman suggested that anoxygenic photosynthetic microorganisms thrived in Earth's ancient, oxygen-poor and iron-rich oceans, and utilized the abundant Fe(II) as an electron donor. Nevertheless, it took almost a decade for the first anoxygenic phototrophic Fe(II)-oxidizer, described as a purple non-sulfur bacteria, to be isolated (Widdel *et al*., 1993).

Anoxygenic phototrophic Fe(II)-oxidizers have since been isolated from both the purple sulfur (Gammaproteobacteria) and non-sulfur bacteria (Alphaproteobacteria), and from the green sulfur bacteria (see Table 2). Isolated purple non-sulfur bacteria include *Rhodobacter ferrooxidans* strain

Table 2. Summary of isolated phototrophic Fe(II)-oxidizers (pFeOx) which all oxidize Fe(II) for CO_2 fixation and gain energy from light with the exception of *R. capsulatus* (*). Number represents position in the map in Figure 2.

No.	Strain	Class, Order, Family	Origin of sample	Identity of iron(III) minerals	Reference
Green sulfur bacteria (Chlorobiaceae)					
44	*Chlorobium ferrooxidans* KoFox [co-culture with KoFum (*Geospirillum arsenophilum*)]	Chlorobia Chlorobiales Chlorobiaceae	Freshwater sediment, Lake Constance, Germany	bulbous, spiky and needle like amorphous Fe(III) hydroxides, ferrihydrite; encrustation mainly on KoFum; no encrustation of KoFox	(Heising *et al*., 1999; Gauger *et al*., 2016)
45	*Chlorobium* sp. [co-culture: *Chlorobium* sp. (80%) and *Acidobacteria* sp. (20%)]	Chlorobia Chlorobiales Chlorobiaceae	Water column of Lake La Cruz, Spain	Fe(III) oxides	(Walter *et al*., 2014)
46	*Chlorobium phaeoferrooxidans*	Chlorobia Chlorobiales Chlorobiaceae	Water column of Lake Kivu, Rep. Congo	Fe(III) oxides	(Llirós *et al*., 2015; Crowe *et al*., 2017)
47	*Chlorobium* sp. strain N1	Chlorobia Chlorobiales Chlorobiaceae	Marine sediment, Aarhus, Denmark	Fe(III) oxyhydroxides, amorphous to poorly crystalline Fe(III) phase, ferrihydrite, akageneite and/ or lepidocrocite; no encrustation of cells	(Laufer *et al*., 2017)
Purple sulfur and non-sulfur bacteria					
48	*Rhodomicrobium* isolate	Alphaproteobacteria Rhizobiales Hyphomicrobiaceae	Marine sediment Jadebusen, North Sea, Germany	Fe(III) oxides, cells covered with Fe(III) precipitates	(Widdel *et al*., 1993)
49	*Rhodopseudomonas palustris*	Alphaproteobacteria Rhizobiales Bradyrhizobiaceae	Marine sediment Jadebusen, North Sea, Germany	Fe(III) oxides	(Widdel *et al*., 1993)
50	*Thiodyction* sp.	Gammaproteobacteria	Marine sediment Jadebusen, North Sea, Germany	Fe(III) oxides	(Widdel *et al*., 1993)
51	Strain L7 (closest relative *Chromatium* sp.)	NA	Freshwater sediment, Lübeck, Germany	Ochre to light brown, rusty ferric precipitates; cells are only loosely associated with precipitates	(Ehrenreich and Widdel, 1994)
52	Strain SF4	NA	Forest soil, Munich, Germany	Orange-brown precipitates with fluffy appearance; cells mainly free of encrustation	(Ehrenreich and Widdel, 1994)
53	*Rhodobacter ferrooxidans* SW2	Alphaproteobacteria Rhodobacterales Rhodobacteraceae	Freshwater sediment, Hanover, Germany	Ferrihydrite, lepidocrocite, goethite, poorly crystalline Fe-carbonates; encrustation away from cell on EPS	(Ehrenreich and Widdel, 1994; Kappler and Newman, 2004; Miot *et al*., 2009)
54	*Rhodobacter capsulatus* DSM 1710 *(photoheterotrophic)*	Alphaproteobacteria Rhodobacterales Rhodobacteraceae	n.a.	Green to dark green Fe(II)/Fe(III) minerals	(Ehrenreich and Widdel, 1994)
55	*Rhodopseudomonas palustris* DSM 123	Alphaproteobacteria Rhizobiales Bradyrhizobiaceae	Surface water or mud, country of origin unknown	Green to dark green Fe(II)/Fe(III) minerals	(Ehrenreich and Widdel, 1994)
56	*Rhodomicrobium vannielii* BS-1	Alphaproteobacteria Rhizobiales Hyphomicrobiaceae	Freshwater sediment, Tübingen, Germany	Cell encrustation by ferric minerals	(Heising and Schink, 1998)
57	*Rhodovulum iodosum*	Alphaproteobacteria Rhodobacterales Rhodobacteraceae	Marine sediment Jadebusen, North Sea, Germany	No cell encrustation, ferric mineral precipitation on EPS	(Straub *et al*., 1999)

(Continues)

Table 2. Continued

No.	Strain	Class, Order, Family	Origin of sample	Identity of iron(III) minerals	Reference
58	*Rhodovulum robiginosum*	Alphaproteobacteria Rhodobacterales Rhodobacteraceae	Marine sediment Jadebusen, North Sea, Germany	Green to dark green Fe(II)/Fe(III) minerals	(Straub *et al*., 1999)
59	*Thiodictyon* sp. strain F4	Gammaproteobacteria	Marsh sediment, Woods Hole, US	Rusty Fe(III) precipitates, two-line ferrihydrite, potential trace amounts of goethite, vivianite and siderite	(Croal *et al*., 2004; Hegler *et al*., 2008, 2010)
60	*Rhodopseudomonas palustris* TIE-1	Alphaproteobacteria Rhizobiales Bradyrhizobiaceae	Marsh sediment, Woods Hole, US	Poorly crystalline iron(oxy)hydroxides, goethite, magnetite	(Jiao *et al*., 2005)
61	*Rhodobacter capsulatus* SB 1003*	Alphaproteobacteria Rhodobacterales Rhodobacteraceae	n.a.	n.a.	(Poulain and Newman, 2009; Kopf and Newman, 2012)
62	*Rhodobacter* sp.	Alphaproteobacteria Rhodobacterales Rhodobacteraceae	Marine sediment, Kalo Vig, Denmark	Cells closely associated with rusty-orange Fe(III) minerals	(Laufer *et al*., 2016b)

**Rhodobacter capsulatus* SB1003 can catalyse Fe(II) oxidation but does not appear to be able to grow from this metabolism.

SW2 (Ehrenreich and Widdel, 1994), *Rhodopseudomonas palustris* strain TIE-1 (Jiao *et al*., 2005), *Rhodovulum iodosum* and *Rhodovulum robiginosum* (Straub *et al*., 1999; Wu *et al*., 2014). The only purple sulfur bacteria isolate available is *Thiodycton* sp. strain F4 (Croal *et al*., 2004). All isolated photoferrotrophs from the green sulfur bacteria belong to the genus Chlorobium. These include *Chlorobium ferrooxidans* strain KoFox (Heising *et al*., 1999) and two recently isolated species: *Chlorobium phaeoferrooxidans* (Crowe *et al*., 2017; Thompson *et al*., 2017) and *Chlorobium* sp. strain N1 which is closely related to *Chlorobium luteolum* (Laufer *et al*., 2017). These organisms typically oxidize aqueous Fe(II) completely under normal culture conditions, similarly to Culture KS and in contrast to most other NRFeOx bacteria. *Rhodobacter capsulatus* can also oxidize Fe(II) but does not appear to use this process to support growth (Poulain and Newman, 2009). This organism instead utilizes Fe(II) oxidation as a detoxification mechanism. Fe(II) can also be oxidized by purple non-sulfur bacteria *Rhodomicrobium vannielli* strain BS-1, however, the physiological role of Fe(II) oxidation by this organism seems to be somewhat of an enigma. Heising and Schink (1998) showed that while this strain could oxidize Fe(II), this process is significantly aided by the presence of an organic substrate and can only be maintained for two to three generations. This led them to suggest that Fe(II) oxidation was of minor physiological importance and was merely a side-reaction of normal metabolism. However, *Rhodomicrobium vannielli* strain BS-1 does have the same set of genes for Fe(II) oxidation as *Rhodopseudomonas palustris* strain TIE-1 (He *et al*., 2017) and, thus, it is unclear why this strain does not use Fe(II) as the sole electron donor whereas *Rhodopseudomonas palustris* TIE-1 does.

Purple non-sulfur bacteria and green sulfur bacteria that oxidize Fe(II) live in both high and low salinity environments. Marine strains include *Chlorobium* sp. strain N1, *Rhodovulum iodosum* and *Rhodovulum robiginosum* while all other isolates stem from freshwater habitats. Phototrophic Fe(II)-oxidizers from the green sulfur bacteria can utilize lower light intensities than the purple sulfur bacteria due to differences in their pigments such as bacteriochlorphylls and carotenoids (Kappler *et al*., 2005a). Some green sulfur bacteria utilize extremely low light intensities (down to > 0.005% of surface irradiance; Manske *et al*., 2005) and have a light saturation much lower than that of purple bacteria (< 50 lux for *Chlorobium ferrooxidans* strain KoFox compared with 400 lux for *Rhodobacter ferrooxidans* strain SW2 or 800 lux for *Thiodycton* sp. strain F4) (Hegler *et al*., 2008). Green sulfur bacteria such as *Chlorobium* sp. strain N1, however, utilize higher light intensities (saturation at 400 lux) thus they are not limited to very low light environments (Laufer *et al*., 2017). All isolated anoxygenic phototrophic Fe(II)-oxidizers are metabolically flexible and have the ability to

utilize multiple electron donors for CO_2 fixation instead of Fe(II), such as H_2 and H_2S (Croal *et al*., 2009). Alternatively, they are able to use organic carbon compounds instead of CO_2 (Melton *et al*., 2014b).

Possibly as a result of their metabolic flexibility, anoxygenic phototrophs are widespread in both aquatic and terrestrial habitats. Most of the isolated species were obtained from either freshwater or marine sediments with the exception of *Chlorobium phaeoferrooxidans* which was isolated from the water column of ferruginous Lake Kivu, East Africa (Crowe *et al*., 2017). However, despite the lack of pelagic isolates, these bacteria have been shown (using microcosms and in situ observations) to substantially contribute to Fe(II) oxidation in numerous stratified lakes. For example, in ferruginous Lake La Cruz, Spain, an anoxygenic phototrophic Fe(II)-oxidizer closely related to *Chlorobium ferrooxidans* strain KoFox was enriched from the water column and shown to contribute to Fe(III) mineral formation in situ (Walter *et al*., 2014). These bacteria even thrive in low iron environments like mermomictic Lake Cadagno, where they are thought to account for up to 10% of total carbon fixation (Berg *et al*., 2016). Given the metabolic flexibility of anoxygenic phototrophic Fe(II)-oxidizers, it is challenging to determine whether or not they contribute to Fe(II) oxidation in their environment of origin simply from the analysis of in situ DNA or RNA.

Mechanisms of Fe(II) oxidation by photoferrotrophs

The mechanisms involved in Fe(II) oxidation by anoxygenic phototrophic Fe(II)-oxidizing bacteria are still not fully understood. Questions remain about how these bacteria can oxidize different forms of Fe(II) at circumneutral pH including dissolved Fe^{2+}aq, ligand bound Fe(II) and solid phase minerals (Byrne *et al*., 2015, 2016) as well as poised electrodes (Bose *et al*., 2014). Furthermore, it is not clear exactly how these bacteria are able to deal with the solid phase mineral precipitates (e.g., ferrihydrite) that are formed as a result.

The mechanism of phototrophic microbial Fe(II) oxidation has been most widely studied in *Rhodopseudomonas palustris* TIE-1. In this organism, electron transfer by Fe(II) oxidation is thought to require the *pioABC* operon, where *pio* stands for 'photosynthetic Fe(II)-oxidation' (Jiao and Newman, 2007). This is a 3 gene operon containing genes encoding for the proteins PioA (a periplasmic decaheme c-type cytochrome), PioB (an outer membrane beta-barrel protein) and PioC (a periplasmic high potential iron–sulfur cluster protein) (Jiao and Newman, 2007). PioA and PioB are homologous with MtrA and MtrB, respectively, that are for instance expressed by the Fe(III)-reducer *Shewanella oneidensis* MR-1 (Jiao and Newman, 2007). PioC is similar to the putative Fe(II) oxidoreductase Iro in *Acidothiobacillus ferrooxidans*. *Rhodopseudomonas palustris* TIE-1 most likely transfers electrons from PioA to PioC, which then donates electrons to the bc1 complex. Some authors have also suggested that the electrons could be passed to the inner membrane phototrophic reaction centre (Bird *et al*., 2014) (Fig. 4A). A *pioABC* operon was also found in *Rhodomicrobium vannielii* that probably functions similarly to that of *Rhodopseudomonas palustris* TIE-1 (He *et al*., 2017).

Deletion of PioA in *Rhodopseudomonas palustris* TIE-1 results in almost complete loss of Fe(II)-oxidizing ability whereas PioB and PioC deletions only result in partial loss compared with the wild type (Bose and Newman, 2011). The *pio* genes show highest expression with Fe(II) as an electron donor, but are transcribed and translated under all anoxic growth conditions (Bose and Newman, 2011). Expression of the *pio* operon is regulated by the global regulator FixK (Bose and Newman, 2011). Transcriptome-level insights have generated additional information on how Fe(II) shapes cellular processes in *Rhodopseudomonas palustris* TIE-1. These show that high levels of Fe(II) induce stress responses even under anoxic conditions where classical Fe(II) toxicity via oxidative stress induced by the Fenton reaction should not be an issue. The cellular response is primarily characterized by the induction of numerous metal efflux mechanisms (Bird *et al*., 2013).

To date, evidence relating to the location of PioA in the cell is somewhat contradictory. On one hand, based on sequence information, PioA is predicted to be a periplasmic protein (Jiao and Newman, 2007). Recent findings, however, show that *Rhodopseudomonas palustris* TIE-1 can oxidize the solid phase mixed-valent Fe(II)–Fe(III) mineral magnetite Fe_3O_4 (Byrne *et al*., 2015), and utilized electrons directly from poised electrodes (Bose *et al*., 2014). This suggests that PioA, with its electron transport mechanism and thus the ability to oxidize Fe(II), must be present on the outer membrane of the cell. Furthermore, it was shown that *Rhodopseudomonas palustris* TIE-1 is only able to access surface bound Fe(II) in the magnetite which further suggests a direct surface-mineral contact mechanism might be required (Byrne *et al*., 2016).

The other widely studied mechanism of anoxygenic phototrophic Fe(II) oxidation is that of *Rhodobacter ferrooxidans* SW2. This organism oxidizes Fe(II) via the *foxEYZ* operon (Croal *et al*., 2007). *foxE* encodes a c-type cytochrome with no significant similarity to other known Fe(II)-oxidizing or Fe(III)-reducing proteins. *foxE* and *foxY* are co-transcribed in the presence of Fe(II) and/or hydrogen, whereas *foxZ* is only transcribed in the presence of Fe(II) (Croal *et al*., 2007). It is thought that FoxE is positioned in the periplasm (Saraiva *et al*., 2012). It has further been proposed that electrons from Fe(II) are transferred to FoxE, then to FoxY and from there to the bc1 complex or,

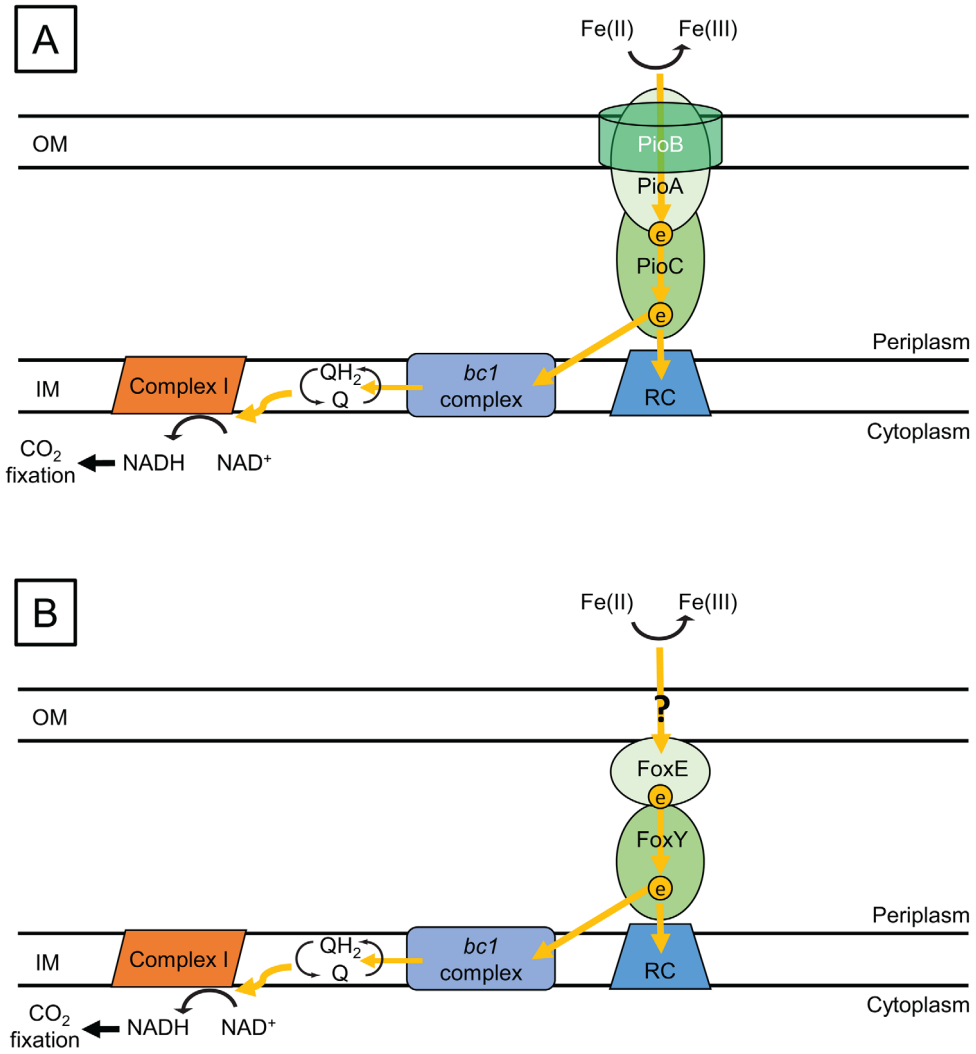

Fig. 4. Schematics of the current hypotheses on the mechanism of Fe(II) oxidation: (**A**) proposed Fe(II) oxidation mechanism in *Rhodopseudomonas palustris* TIE-1 and (**B**) proposed Fe(II) oxidation mechanism for *Rhodobacter ferrooxidans* SW2 (modified from Bird *et al*., 2011).

possibly, to the reaction centre (Bird *et al*., 2011) (Fig. 4A). To date, there is no evidence which shows the ability for *Rhodobacter ferrooxidans* SW2 to oxidize solid phase Fe(II) mineral phases.

The recently sequenced genome of *Chlorobium phaeoferrooxidans* suggests that yet another mechanism exists in these green sulfur bacteria. This genome encodes for an outer membrane cytochrome ($cyc2_{PV\text{-}1}$), which is known to be responsible for Fe(II) oxidation in the microaerophilic Fe(II)-oxidizer *Mariprofundus ferrooxidans* PV-1 (Crowe *et al*., 2008). $Cyc2_{PV\text{-}1}$ is a distant homologue of Cyc2, which is widely present in many known obligate lithotrophic Fe(II)-oxidizing bacteria (He *et al*., 2017). Cyc2 is also encoded in the genome of *Chlorobium ferrooxidans* DSM13031 (He *et al*., 2017).

The electron transfer mechanisms and proteins responsible for Fe(II) oxidation are probably diverse and one single mechanism is neither universally present among all physiological types of Fe(II)-oxidizers nor within one group of Fe(II)-oxidizers such as the phototrophs.

Mineral formation by photoferrotrophs

Phototrophic Fe(II)-oxidizing bacteria are able to oxidize a variety of Fe(II) species (see previous paragraph) resulting in the formation of poorly soluble Fe(III) (oxyhydr)oxides (Ehrenreich and Widdel, 1994; Straub *et al*., 1999; Kappler and Newman, 2004; Jiao *et al*., 2005; Gauger *et al*., 2015). Several studies observed the transformation of these amorphous to low crystalline initial precipitates to higher crystalline and thermodynamically more stable Fe mineral phases, such as goethite, lepidocrocite and magnetite over time (Straub *et al*., 1999; Kappler and Newman, 2004; Jiao *et al*., 2005; Miot *et al*., 2009c; Schaedler *et al*., 2009; Wu *et al*., 2014). Partial

dissolution and re-precipitation processes in combination with mineral transformation by sorption of residual Fe(II) represent potential transformation pathways (Posth *et al*., 2014). Laufer *et al*. (2016c) suggested that a close association between minerals and organics might constrain mineral growth and subsequent transformation, thus preserving the poorly crystalline ferrihydrite. The final mineral product of the Fe(II) oxidation therefore depends on various factors such as the geochemical conditions and/or the microbial community in the surrounding environment, and the Fe(II) concentration (e.g., Schaedler *et al*., 2009; Posth *et al*., 2010, 2013). In the case of strain *R. palustris* TIE-1 the pH seemed to be of particular importance, since at low pH conditions, poorly crystalline Fe(III) oxyhydroxides and goethite were formed while at pH conditions above 7.2, the formation of magnetite was observed (Jiao *et al*., 2005).

It has been suggested that Fe(II) oxidation occurs at different, strain specific localities, such as the cell surface or the periplasm. In close proximity to bacteria, freshly formed Fe(III) minerals, are expected to strongly adsorb to the negatively charged cell surface (Kappler and Newman, 2004; Schaedler *et al*., 2009). However, in contrast to nitrate-reducing Fe(II)-oxidizing bacteria, a lack of encrustation seems characteristic for photoferrotrophs (Schaedler *et al*., 2009; Wu *et al*., 2014). Yet, the extent to which cells and minerals are associated seems to be strain-specific. Furthermore, varying cell-mineral aggregate morphologies, ranging from irregular and bulbous to more symmetric (e.g., flower- and star-like shapes) have been reported and seem to depend on the mineralogy and age of culture (Ehrenreich and Widdel, 1994; Kappler and Newman, 2004; Jiao *et al*., 2005; Posth *et al*., 2010; Wu *et al*., 2014; Gauger *et al*., 2015; Laufer *et al*., 2017).

Much research has focused on the question as to why mineral precipitates and cells are closely associated while the cell surface itself remains (mostly) free of mineral precipitates and no encrustation occurs in the case of most photoferrotroph strains. Miot *et al*. (2009c) demonstrated an occurrence of goethite only outside of the cell and along organic fibres produced by *Rhodobacter ferrooxidans* sp. SW2. Likewise, Wu *et al*. (2014) found that in the case of *Rhodovulum iodosum*, the final precipitation product was mainly localized at the exopolysaccharides (EPS) and suggested the excretion of EPS as a strategy to prevent mineral encrustation on the cell surface. Another potential mechanism was presented by Hegler *et al*. (2010), who suggested that a low-pH environment around the cell would prevent Fe(III) mineral precipitation on the cell surface, probably in combination with the presence of EPS structures that direct the precipitation away from the cell surface. Avoidance of encrustation may also be a result of the location of the Fe(II) oxidase in the outer membrane and not in the periplasm as is likely to be the case in *Rhodopseudomonas palustris* TIE-1. This process may also be aided by the location of the Fe(II) oxidase within an outer membrane porin (Fig. 4A), which could facilitate the extrusion of Fe(III) from the cell before it has time to precipitate (Bird *et al*., 2011). The precipitation of Fe(III) outside of the cell on organic fibres in *Rhodobacter ferrooxidans* SW2 (Miot *et al*., 2009c) is consistent with either extracellular oxidation or rapid removal of Fe(III) from the cell. Alternatively, the structure of the Fe(II) oxidation protein itself may aid in the avoidance of encrustation as proposed by Pereira et al. (2017) who suggested that the structure of FoxE in *Rhodobacter ferrooxidans* SW2 discourages precipitation of Fe(III) within the periplasm following Fe(II) oxidation. A comparison between the iron oxidation mechanisms in these two strains is shown in Fig. 4.

A clear exception among the phototrophic Fe(II)-oxidizing bacteria is the strain *R. vannielii* BSI, which has been shown to produce Fe(III) oxyhydroxides that precipitated directly on the cell surface, forming crusts, which hindered further metabolic activity and hence, impeded Fe(II) oxidation after prolonged cultivation (Heising and Schink, 1998). The authors suggest that, other than during a laboratory cultivation, in a natural environment, this strain is likely able to re-dissolve the crusts of Fe(III) minerals and hence, maintain microbial activity and cell growth. In co-cultures of the green sulfur bacteria *Chlorobium ferrooxidans* KoFox and *Geospirillum* sp. KoFum, mineral encrustations are also observed but only prominently on KoFum cells (Schaedler *et al*., 2009). This suggests photoferrotrophs have active mechanisms to avoid encrustation.

Photoferrotrophs and their interactions with metals

Similar to biogenic Fe minerals produced by NRFeOx bacteria, biogenic Fe minerals produced by phototrophs react with and can even remove heavy metals from solution through sorption and/or co-precipitation processes. This association is mediated by forming Fe-metal bonds, rather than metal bonds with organic carbon. For example, when nickel (Ni) is reacted with biogenic phases produced by phototrophic bacteria, this heavy metal demonstrates a clear preference for associations with Fe on EPS, rather than with carbon (Eickhoff *et al*., 2014). Similarly, when the association of Ni with Si in biogenic Fe minerals produced by either *Rhodobacter ferrooxidans* SW2 or *Rhodovulum iodosum* is examined using scanning transmission X-ray microscopy (STXM), it is clear that Ni preferentially is associated with Fe rather than with Si (Eickhoff *et al*., 2014).

The preferential association of trace metals with Fe in biogenic Fe(III) minerals produced via phototrophic bacteria does not necessarily ensure decreased mobility of the

trace metal. For example, biogenic ferrihydrite produced by *Rhodobacter ferrooxidans* SW2 removes 99% of both As(V) and As(III) added (Hohmann *et al*., 2009). While this poorly crystalline phase adsorbs and incorporates As to a greater extent compared with more crystalline phases, ferrihydrite is also more susceptible to microbial Fe(III) reduction. Microbial Fe(III) reduction may induce dissolution of the solid and potentially release of As back into solution. Phosphate, which behaves similarly to arsenic in the environment, will also bind to Fe(III) minerals thus dissolution of Fe(III) minerals can release P and lead to eutrophication (Orihel *et al*., 2015). This effect may be enhanced in biogenic Fe minerals compared with abiogenic minerals due to the presence of organic carbon on the mineral surface and within the mineral structure itself due to co-precipitation with organics. In addition to organic carbon content, abiogenic and biogenic minerals produced by phototrophic bacteria differ in the extent of metal sorption and/or incorporation into the mineral structure. For example, a two to three-fold lower Ni/Fe ratio is observed for biogenic phases than for abiogenic phases. This decrease is likely due to surface site blockage by organic carbon (Eickhoff *et al*., 2014).

Co-existence of anaerobic Fe(II)-oxidizers in sediments

One of the key recent discoveries in the field of sedimentary Fe(II) oxidation is that anaerobic Fe-transforming metabolisms (NRFeOx, pFeOx, Fe(III)-reduction) are found to co-exist in both freshwater and marine sediments (Melton *et al*., 2012; Laufer *et al*., 2016b; Otte *et al*., 2018). Based on the thermodynamically-controlled stratification of redox-active compounds in sediments, it was previously suggested that Fe(II)-oxidizing microorganisms would be spatially separated over very short distances based on where the optimum geochemical conditions are found (Schmidt *et al*., 2010). The distribution of these metabolisms in shallow water sediments from Aarhus Bay (marine) shows that none of the physiological groups of Fe(II)-oxidizing bacteria in these sediments showed strong correlations with geochemical gradients (Laufer *et al*., 2016b; Otte *et al*., 2018), however some correlation with the abundance of cable bacteria was observed (Otte *et al*., 2018). It should be noted however, that these groups did show some correlation with geochemical gradients in deeper, profundal sediments in Lake Constance (Melton *et al*., 2012). Of the three physiological types, MPN studies showed microaerophilic Fe(II)-oxidizers were the most dominant, followed by nitrate-reducing Fe(II) oxidizers, with anoxygenic phototrophic iron oxidizers being the least abundant.

Possible applications in biotechnology

These anaerobic Fe(II)-oxidizing bacteria are not only environmentally important, but may have some useful biotechnological applications. Firstly, the ability of Fe(II)-oxidizers to remove nitrate may be harnessed for the improvement of drinking water, waste water and sludge in sewage treatment plants (Davidson *et al*., 2003; Zhang *et al*., 2015; Wang *et al*., 2016a; Kiskira *et al*., 2017). This may already occur naturally in aquifers where Fe(II)-oxidizing bacteria could couple nitrate reduction to oxidation of Fe(II)-rich clays or Fe(II)-containing minerals such as pyrite (Haaijer *et al*., 2007; Vaclavkova *et al*., 2015; Jessen *et al*., 2017). There is also potential to use minerals produced by these anaerobic Fe(II)-oxidizers, particularly reactive Fe(II)–Fe(III) phases like green rust or magnetite in remediation of metals and contaminants (reviewed in Usman *et al*., 2018). The importance of (biogenic) iron minerals in controlling As mobility has already been well documented (Hohmann *et al*., 2010). In anoxic Fe- and As-rich systems such as rice paddy soil or As-contaminated aquifers, the formation of iron minerals can act to bind arsenic and limit its dispersal in the environment (Seyfferth *et al*., 2010; Yamaguchi *et al*., 2014; Smith *et al*., 2017; Vega *et al*., 2017), although nothing is yet known about the importance of biogenic Fe(III) minerals in As-contaminated aquifers. The As-binding properties of iron are also harnessed in drinking water filters in arsenic-rich areas to provide low cost filtration solutions for contaminated water in developing counties (Nitzsche *et al*., 2015). On the one hand, Fe(II)-oxidizing bacteria could help remove As by co-precipitation (Hohmann *et al*., 2010). On the other hand, it has been shown that less As binds to biogenic Fe(III) minerals in such filter systems than to abiogenic Fe(II) minerals, probably due to surface sorption competition with the organic material stemming from the bacteria (Kleinert *et al*., 2011).

Conclusions

The extensive research conducted on these two distinct groups of anaerobic Fe(II)-oxidizing bacteria has provided a fundamental understanding of their physiology, Fe(II) oxidation mechanisms and role in the environment. However, there is much work still to be done. Regarding their physiology, there is still an important need to further test the proposed models for Fe(II) oxidation mechanisms for both types of anaerobic Fe(II) oxidation and establish how universal or variable these mechanisms are. Many of the proposed autotrophic NRFeOx bacteria also need to be re-tested to establish whether they are indeed autotrophic. We suggest that the four criteria used in Table 1 all need to be demonstrated in order to make this claim. There also remains some doubt as to whether organisms which require additional organic C for NRFeOx have

some enzymatic component of Fe(II) oxidation or whether it is all abiotic. We suggest that the role of intracellular reactions needs to be considered before this can be proven. Further understanding of how some Fe(II)-oxidizers avoid encrustation by minerals whereas some do not, and why some anoxygenic phototrophs can grow using Fe(II) as an electron donor and others cannot, is also needed. Another key knowledge gap is in the role of solid substrates as an electron donor for such bacteria which can often prove challenging. A promising avenue of research in this regard is the use of poised electrodes to simulate soil mineral substrates.

In terms of the environmental implications of these metabolisms, while bacteria have been isolated from many different environments (Fig. 2), we are still lacking a complete overview of the abundance and environmental distribution of these metabolisms. For example, almost all isolates of anaerobic Fe(II)-oxidizing bacteria have come from freshwater or marine sedimentary environments in Europe and North America (Table 1 and Supporting Information Table S1). However, their demonstrated ubiquity in these environments, as well as in freshwater stratified lakes, suggests that these bacteria are widespread. NRFeOx processes in particular are likely to play a key role in agricultural soils and aquifers where nitrate contamination is high and iron is generally plentiful. There is also a distinct European and North American bias in our knowledge of the environmental role of these organisms. Future work should focus on determining the role of these organisms in more tropical settings, or in more extreme climatic conditions such as arid or cold regions.

We also still need to better understand how our chosen growth conditions influence the minerals which are formed by Fe(II)-oxidizers, and why only some isolates can oxidize solid substrates. The knowledge of what minerals form under environmentally relevant conditions, for example, under low iron concentrations or in the presence of competing (organic or inorganic) substrates, has been barely studied yet is critical for transferring our knowledge of these processes out of the lab. A focus on this may also aid in our understanding of the distribution of these organisms within redox gradients in the environment by determining if their even distribution can be explained by metabolic flexibility.

It is an exciting time to work on the anoxic side of the microbial iron cycle. Our appreciation of the role of these bacteria in many different environments is increasing rapidly, and we are beginning to establish the fundamental mechanisms underpinning these metabolisms, and the effects they have on mineral formation and element cycling under environmental conditions. Over the coming years, we hope that more and more researchers will begin to look for these types of organisms in their own anoxic systems and consider their potential biotechnological usage when faced with an environmental problem.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Appendix S1. SUPPORTING INFORMATION

Table S1. Selection of the more well-studied bacteria which are thought to be chemodenitrifiers (i.e., an enzymatic contribution to Fe(II) oxidation is not confirmed), displaying their genetic diversity, point of origin and minerals which can be formed.