Microbial anaerobic oxidation of Fe(II)-organic matter complexes

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Content

1. Summary

1.1 Abstract

The oxidation of Fe(II) to Fe(III) influences both the behavior of environmental contaminants and many other biogeochemical cycles. In anoxic environments, Fe(II) can be microbially oxidized by two different types of anaerobic Fe(II)-oxidizing bacteria. Phototrophic Fe(II)-oxidizing bacteria utilize light as an energy source and oxidize Fe(II) coupled with carbon fixation. Nitrate-reducing Fe(II)-oxidizing bacteria (NRFeOx) oxidize Fe(II) while reducing nitrate (NO_3^-). These two types of bacteria have been identified in a variety of habitats and are thought to play an important role in determining Fe speciation in the environment.

Research on anaerobic microbial Fe(II) oxidation has been conducted for a few decades, however, most of these previous studies focused on the oxidation of either non-organically-bound, dissolved free Fe(II) or Fe(II) minerals. In natural environments, free Fe(II) and Fe(II)-containing minerals are not the only Fe(II) sources available. Fe(II) can also be complexed by organic-matter (Fe(II)-OM) in solution. However, there is still a knowledge gap when it comes to the effect of Fe(II)-OM complexation on microbial Fe(II) oxidation. To fill this knowledge gap, this thesis combined geochemical modeling and microbial incubation experiments to study microbial Fe(II) oxidation when Fe(II) was fully complexed by OM and when both free Fe(II) and Fe(II)-OM complexes were present in the medium.

Using these approaches, this thesis determined the rates and extent of oxidation of Fe(II)-OM complexes by the nitrate-reducing Fe(II)-oxidizing bacteria *Acidovorax* sp. BoFeN1 (Chapter 3), and the phototrophic Fe(II)-oxidizing bacteria *Rhodopseudomonas palustris* TIE-1 and *Rhodobacter ferrooxidans* SW2 (Chapters 4 and 5). Moreover, this thesis has also investigated the roles of free Fe(II) and nitrite in the oxidation of Fe(II)-OM complexes (Chapter 3), has characterized the products of

microbial phototrophic oxidation of Fe(II)-OM complexes (Chapter 4). Moreover, results presented in this thesis have demonstrated a new type of light-driven cryptic Fe cycle involving abiotic photochemical reduction of Fe(III)-OM complexes and microbial phototrophic Fe(II) oxidation (Chapter 5).

For nitrate-reducing Fe(II)-oxidizing bacteria, Fe(II)-OM complexation inhibited the oxidation of Fe(II) by *Acidovorax* sp. BoFeN1. The colloidal and negatively charged Fe(II)-OM complexes showed much lower oxidation rates than free Fe(II). In addition, accumulation of nitrite and fast oxidation of Fe(II)-OM complexes only happened in the presence of free Fe(II) which probably interacted with denitrifying enzymes in the cell periplasm causing nitrite accumulation in the cell periplasm and in the solution outside the cells. Compared to free Fe(II), Fe(II)-OM complexes can probably not enter into the periplasm and cause these changes due to their differences in charge, molecular size and solubility. These results suggest that Fe(II) oxidation by mixotrophic nitrate-reducers in the environment depends on Fe(II) speciation and free Fe(II) plays a critical role in regulating microbial denitrification processes.

For phototrophic Fe(II)-oxidizing bacteria, Fe(II)-OM complexation significantly accelerated the rates of Fe(II) oxidation by *Rhodopseudomonas palustris* TIE-1, compared to the oxidation of free Fe(II). Different types of Fe(II)-OM complexes showed different Fe(II) oxidation rates, although a fraction of the Fe(II) present as colloidal Fe(II)-OM complexes seemed to resist to microbial oxidation. In addition to *Rhodopseudomonas palustris* TIE-1, Fe(II)-OM complexes also accelerated Fe(II) oxidation by another phototrophic Fe(II)-oxidizing bacteria, *Rhodobacter ferrooxidans* SW2, but this stimulating effect was weaker and did not apply to all of the Fe(II)-OM complexes. Moreover, our results showed that *Rhodobacter ferrooxidans* SW2 was capable of re-oxidizing Fe(II)-citrate produced by photochemical reduction of Fe(III)-citrate, which kept the dissolved Fe(II)-citrate concentration at low and stable concentrations (<10 μM) with a concomitant increase in cell numbers. This result demonstrated the potential for active cryptic Fe-cycling in the photic zone of anoxic

aquatic environments, despite low measurable Fe(II) concentrations. These results indicate that Fe-cycling in photic anoxic environment could be much more active than previously thought.

Taken together, the result presented in this thesis revealed that Fe(II)-OM complexation can play an important role for the microbial oxidation of Fe(II), and microbial Fe(II) oxidation in OM rich environments may strongly depend on the metabolic type of Fe(II)-oxidizing bacteria and the speciation of Fe(II), e.g. the identity of Fe(II)-OM complexes and the existence of free Fe(II). These new findings improve our understanding of microbial Fe-cycling and highlight the importance of Fe(II)-OM complexation in many environmental processes.

1.2 Zusammenfassung

Die Oxidation von Fe(II) zu Fe(III) beeinflusst sowohl das Verhalten von Umweltschadstoffen als auch viele andere biogeochemische Zyklen. In anoxischen Milieus kann Fe(II) mikrobiell durch zwei verschiedene Klassen anaerober Fe(II)-oxidierender Bakterien oxidiert werden. Phototrophe Fe(II)-oxidierende Bakterien nutzen Licht als Energiequelle und oxidieren Fe(II) um Kohlenstoff zu fixieren. Nitrat-reduzierende, Fe(II)-oxidierende Bakterien (NRFeOx) oxidieren Fe(II) während sie Nitrat (NO_3^-) reduzieren. Diese zwei Klassen von Bakterien wurden in einer Vielzahl an Habitaten nachgewiesen und es wird angenommen, dass diese eine wichtige Rolle für die Eisen-Speziierung in der Umwelt spielen.

Seit einigen Jahrzehnten bereits beschäftigt sich die Forschung mit anaerober mikrobieller Fe(II) Oxidation, wobei sich die meisten Studien nur auf die Oxidation von unorganisch-gebundenem Fe(II), gelöstem freien Fe(II) oder Fe(II) Mineralen konzentrierten. In der Natur, jedoch, sind freies Fe(II) und Fe(II) haltige Minerale nicht die einzigen verfügbaren Fe(II)-Quellen. Fe(II) kann dabei auch komplexiert mit organischen Substanzen (Fe(II)-OM) in Lösung vorkommen. Welchen Effekt die Fe(II)-OM Komplexierung auf die mikrobielle Fe(II) Oxidation hat, stellt heute noch eine große Wissenslücke dar. Um diese Wissenslücke zu schließen, kombiniert die vorliegende Thesis geochemische Modellierung mit mikrobiellen Inkubationsversuchen um mikrobielle Fe(II) Oxidation von freiem gelösten Fe(II) und Fe(II)-OM Komplexen in flüssigen Medien zu untersuchen.

Mit Hilfe dieser Ansätze wurde in der vorliegenden Arbeit die Rate und das Ausmaß der Oxidation von Fe(II)-OM Komplexen durch Nitrat-reduzierende Fe(II)-oxidierende Bakterien der Gattung *Acidovorax* sp. BoFeN1 (Kapitel 3), und durch phototrophe Fe(II)-oxidierende Bakterien *Rhodopseudomonas palustris* TIE-1 und *Rhodobacter ferrooxidans* SW2 (Kapitel 4 und 5) bestimmt. Darüber hinaus untersuchte die vorliegende Studie die Bedeutung von freiem Fe(II) und Nitrit bei der

Oxidation von Fe(II)-OM Komplexen (Kapitel 3), charakterisierte die Produkte der mikrobiellen phototrophen Oxidation v+on Fe(II)-OM Komplexen (Kapitel 4) und wies einen neuen lichtabhängigen, kryptischen Fe-Zyklus zwischen abiotischer photochemischer Reduktion von Fe(III)-OM Komplexen und mikrobieller phototropher Fe(II) Oxidation nach (Kapitel 5).

Die Fe(II)-OM Komplexierung hemmte die Fe(II) Oxidation durch Nitrat-reduzierende Bakterien der Gattung *Acidovorax* sp. BoFeN1. Die kolloidalen und negativ-geladenen Fe(II)-OM Komplexe zeigten eine sehr viel langsamere Oxidationsrate als freies gelöstes Fe(II). Eine Akkumulation von Nitrit und schnelle Oxidation von Fe(II)-OM Komplexen trat nur auf, wenn freies gelöstes Fe(II) vorhanden war. Dieses interagierte wahrscheinlich mit denitrifizierenden Enzymen im Zell-Periplasma und führte zu einer Anreicherung von Nitrit im Periplasma und außerhalb der Zellen. Fe(II)-OM Komplexe können aufgrund ihrer anderen Ladung, der Größe und veränderten Löslichkeiten wahrscheinlich nicht in das Periplasma gelangen, um ähnliche Effekte zu verursachen. Diese Ergebnisse legen nahe, dass die Fe(II) Oxidation durch mixotrophe Nitrat-Reduzierer in der Umwelt von der Fe(II)-Speziierung abhängt und freies Fe(II) eine wichtige Rolle bei der Regulation mikrobieller Denitrifikations-Prozesse spielt.

Die Fe(II)-OM Komplexierung führte zu einer deutlichen Beschleunigung der Fe(II) Oxidationsraten durch das phototrophe Fe(II)-oxidierende Bakterium *Rhodopseudomonas palutris* TIE-1 gegenüber der Oxidationsrate von freiem Fe(II). Verschiedene Arten an Fe(II)-OM Komplexen zeigten unterschiedliche Fe(II) Oxidationsraten, wobei ein Teil des verfügbaren Fe(II), das als kolloidale Fe(II)-OM Komplexe vorlag, sogar nicht mikrobiell oxidierbar zu sein schien. Neben *Rhodopseudomonas palustris* TIE-1 beschleunigten Fe(II)-OM Komplexe auch die Fe(II) Oxidation durch einen weiteren phototrophen Mikroorganismus, *Rhodobacter ferrooxidans* SW2. Hierbei war der beschleunigende Effekt jedoch schwächer ausgeprägt und konnte nicht bei allen Fe(II)-OM Komplexen beobachtet werden.

Zusätzlich zeigten unsere Ergebnisse, dass *Rhodobacter ferrooxidans* SW2 in der Lage war Fe(II)-Citrat, das sich durch photochemische Reduktion aus Fe(III)-Citrat gebildet hatte, zu reduzieren. Dabei konnte gelöstes Fe(II)-Citrat nur in konstant niedrigen Konzentration nachgewiesen werden, während aber gleichzeitig die Zelldichte stetig zunahm. Dieses Ergebnis zeigt, dass obwohl nur geringe Fe(II) Konzentrationen gemessen werden können, ein aktiver aber kryptischer Eisen-Kreislaufs in der lichtdurchfluteten Zone anoxischer aquatischer Milieus existieren kann. Das legt nahe, dass der Eisen-Kreislauf in lichtdurchfluteten sauerstofffreien Umwelten deutlich aktiver sein kann als bisher angenommen.

Zusammenfassend zeigen die Ergebnisse der vorliegenden Thesis, dass die Fe(II)-OM Komplexierung eine wichtige Rolle für die mikrobielle Fe(II) Oxidation spielen kann. Zudem kann die Rate und das Ausmaß der mikrobiellen Fe(II) Oxidation in einer Organik-reichen Umwelt stark von der jeweiligen Gattung,dem Metabolismus Fe(II)-oxidierender Bakterien und der Speziierung von Fe(II) abhängen. Insbesondere jedoch beeinflussen die Identität an Fe(II)-OM Komplexen und die Verfügbarkeit von freiem Fe(II) die mikrobielle Fe(II) Oxidation. Diese neuen Erkenntnisse verbessern unser Verständnis mikrobieller Eisen-Kreisläufe und verdeutlichen die Rolle der Fe(II)-OM Komplexierung in vielen Umweltprozessen.

2. Introduction

2.1 Iron in the environment

General introduction of Fe

Iron(Fe) is a highly abundant element on the earth and present in almost all aquatic and terrestrial environments. It is an essential element for nearly all living organisms (Ilbert & Bonnefoy, 2013) and it is involved in many geological and environmental processes (Borch *et al.*, 2010, Melton *et al.*, 2014). Most of the Fe compounds on the earth's surface have either +2 (Fe(II)), or +3 (Fe(III)) oxidation states as a consequence of the Fe electron configuration (Greenwood & Earnshaw, 1984). More specifically, one of the 3d and two of the 4s orbital electrons of Fe are relatively easy to loose. Although there is only a difference of one electron, Fe(II) and Fe(III), the two oxidation states of Fe, differ significantly from each other in many important physical and chemical parameters. These parameters include water solubility, redox activity, and adsorption or binding of other elements and compounds. In addition to the difference between Fe(II) and Fe(III), as the redox potentials of Fe(III)/Fe(II) couple lies between the redox potentials of many other elements and compounds (Borch *et al.*, 2010), the redox reactions between Fe(II) and Fe(III) could also drive redox reactions of other elements. Therefore, it is crucial to have a better understanding of the redox reactions between Fe(II) and Fe(III).

Importance of Fe(II) oxidation

The oxidation of Fe(II) to Fe(III) at neutral pH leads to changes in many physical and chemical parameters in the environment. Therefore oxidation of Fe(II) could significantly influence many biological and geological processes on earth.

The most intuitive change of Fe(II) oxidation is probably the formation of Fe(III) minerals, as the Fe(III)-containing minerals are poorly soluble at neutral pH, whereas Fe(II) is far more soluble (Schwertmann, 1991, Millero, 1998). The formation of Fe minerals as a result of Fe(II) oxidation could greatly influence bioavailability of Fe (Boyd *et al.*, 2017), which is critical for the metabolisms of many living organisms such as plankton and cyanobacteria (Geider & La Roche, 1994). Therefore, via these organisms, Fe(II) oxidation could contribute significantly to

global primary production, nitrogen fixation, and water quality on a regional scale (Lis *et al.*, 2015). In addition to life, the mineral formation also directly affects the fate of other elements and compounds. This is because Fe(III) minerals, the products of Fe(II) oxidation, are usually characterized by high surface areas and can bind or adsorb a lot of metal(loid)s and therefore lower their mobility and toxicity (Borch *et al.*, 2010). An example of this is the Fe(III) minerals and organic matter association in ocean sediment, which is thought to protect OM from microbial degradation (Lalonde *et al.*, 2012), and the mobility of toxic metals such as arsenic (Hohmann *et al.*, 2010, Zhu *et al.*, 2017) and cadmium (Muehe *et al.*, 2013) which could adsorb or co-precipitate with Fe(III).

Besides the formation of minerals, Fe(II) can also undergo redox reaction with many other elements and compounds. These elements and compounds are not limited to those directly reacting with Fe(II) like sulfur (Hansel *et al.*, 2015) and oxygen, but also the elements and compounds which indirectly react with Fe(II) via intermediates during Fe(II) oxidation. For example, antibiotics in the water could be transformed by the highly reactive ROS produced during abiotic Fe(II) oxidation by molecular oxygen (Wang *et al.*, 2016).

In addition to the fate of elements and compounds, Fe(II) oxidation can lead to the corrosion of iron-based industrial facilities (Maeda *et al.*, 1999, Starosvetsky *et al.*, 2001, Starosvetsky *et al.*, 2008), and can also contribute to the global warming and stratospheric ozone depletion, because the greenhouse gas N_2O was biologically and chemically produced during microbial heterotrophic nitrate-reducing Fe(II) oxidation (Ravishankara *et al.*, 2009, Wuebbles, 2009).

2.2 Fe(II) oxidation

Fe(II) is redox-active. At neutral pH, Fe(II) can be rapidly oxidized to Fe(III) abiotically by molecular oxygen, by reactive nitrogen species and by manganese, and biotically by Fe(II)-oxidizing microorganisms which are able to use either O_2, light, or nitrate to oxidize Fe(II) (Melton *et al.*, 2014). In the following sections I will discuss these different Fe(II)-oxidizing processes in detail.

Abiotic Fe(II) oxidation

Abiotic Fe(II) oxidation by molecular oxygen

In oxygenated aquatic environments, Fe(II) is oxidized by oxygen and reactive oxygen species (ROS), including $O_2^{\cdot-}$, H_2O_2, and $OH^{\cdot}$. These abiotic Fe(II) oxidation reactions take place via so called Fenton reactions with 4 steps (Weiss, 1935):

$$Fe^{2+}+O_2\,(aq) \rightarrow Fe^{3+}+O_2^{\cdot-}\,(aq) \quad (1)$$

$$Fe^{2+}+ O_2^{\cdot-}\,(aq)+2H^{+}\rightarrow Fe^{3+}+H_2O_2\,(aq) \quad (2)$$

$$Fe^{2+}+ H_2O_2\,(aq)+H^{+}\rightarrow Fe^{3+}+OH^{\cdot}(aq) \quad (3)$$

$$Fe^{2+}+ OH^{\cdot}\,(aq)+H^{+}\rightarrow Fe^{3+}+H_2O \quad (4)$$

The oxidation product, Fe(III), is poorly soluble in water (Schwertmann, 1991, Millero, 1998). Thus, Fe(III) can precipitate and function as a surface catalyst for further chemical Fe(II) oxidation (Emerson, 2012). The kinetics of this reaction is written as:

$$-d[Fe(II)]/dt=k*[Fe(II)]*pO_2*[OH^-]^2 \quad (5)$$

Where the rates of Fe(II) oxidation are higher at higher pH (Stumm & Lee, 1961), due to of the formation of Fe(II)-hydroxo complexes. Therefore, the acidification of samples to pH lower than 2 has been widely applied for the preservation of Fe(II) in oxic solution.

Abiotic Fe(II) oxidation by reactive nitrogen

In anoxic environments, where there is no oxygen present, Fe(II) could be abiotically oxidized by other redox reactive compounds which have higher redox potentials than the Fe(II)/Fe(III) couple. One of the most common abiotic Fe(II) oxidants in anoxic environments are the so called reactive nitrogen species (RNS), including nitrite (NO_2^-), nitrogen monoxide (NO), and nitrogen dioxide (NO_2). The abiotic oxidation of Fe(II) with RNS takes place via several elementary reaction steps (Nelson & Bremner, 1970, Bonner & Pearsall, 1982, Park & Lee, 1988):

$$NO_2^-+H^+ \leftrightarrows HNO_2 \quad (6)$$

$$2HNO_2\rightarrow NO_2+NO+H_2O \quad (7)$$

$$2Fe^{2+}+NO_2+2H^+ \rightarrow 2Fe^{3+}+NO+H_2O \quad (8)$$

$$Fe^{2+}+NO+H^+ \rightarrow Fe^{3+}+HNO \quad (9)$$

$$2HNO \rightarrow N_2O+H_2O \quad (10)$$

And the overall net reaction of Fe(II) oxidation by nitrite is expressed as:

$$4Fe^{2+}+2NO_2^-+6H^+ \rightarrow 4Fe^{3+}+N_2O+3H_2O \quad (11)$$

The produced Fe(III) minerals could also function as a catalyst and lead to high abiotic Fe(II) oxidation rates (Klueglein & Kappler, 2013). However, the oxidation of Fe(II) by nitrite is faster at lower pH, in contrast to the inhibition of Fe(II) oxidation by molecular oxygen at low pH. Lower pH is thus expected to lead to a rapid production of NO_2 and NO according to reaction 6 and 7 and result in a rapid abiotic Fe(II) oxidation by reactive NO_2 and NO (Klueglein & Kappler, 2013). Therefore, dissolving samples with sulfamic acid (H_3NSO_3), which rapidly reacts with nitrite (Granger & Sigman, 2009), has been widely applied to prevent abiotic Fe(II) oxidation by nitrite at acidic pH during sample preparation (Klueglein & Kappler, 2013).

Abiotic Fe(II) oxidation by manganese

Another important group of anoxic-abiotic Fe(II) oxidants in the environment is manganese oxides (MnO_2), which usually co-exist with Fe in sediments (Thamdrup, 2000). During Fe(II) oxidation, solid MnO_2 is reduced to dissolved Mn(II) when Fe(II) is oxidized to Fe(III) according to reaction 12 (Postma, 1985):

$$MnO_2+2Fe^{2+}+2H_2O \rightarrow Mn^{2+}+2FeOOH+2H^+ \quad (12)$$

Microbial Fe(II) oxidation

In many ancient and modern environments, solely abiotic oxidation of Fe(II) is not sufficient to explain the observed spatial distribution of Fe(II) and Fe(III). Microbial Fe(II) oxidation also needs to be taken into account in many environments (Rentz *et al.*, 2007, Carlson *et al.*, 2012, Klueglein & Kappler, 2013).

Although the first microbial Fe(II) oxidation had been described for almost two centuries, microbial Fe(II) oxidation did not receive much attention until a few decades ago, after the discovery of several neutrophilic Fe(II)-oxidizing bacteria. Based on the mechanism by which the Fe(II) was oxidized, neutrophilic Fe(II)-oxidizing bacteria have been classified into three

different types: microaerophilic Fe(II)-oxidizing bacteria, nitrate-reducing Fe(II)-oxidizing bacteria and phototrophic Fe(II)-oxidizing bacteria, which oxidize Fe(II) while utilizing O_2, nitrate and light, respectively (Melton *et al.*, 2014).

Microaerophilic Fe(II) oxidation

Microaerophilic Fe(II)-oxidizing bacteria oxidize Fe(II) in environments containing low levels of oxygen (Emerson *et al.*, 2010), for example 20-40 µM (Lueder *et al.*, 2018). These type of Fe(II)-oxidizing bacteria have been isolated from a variety of environments (Emerson *et al.*, 2010, Melton *et al.*, 2014) and belong to a few genera in the phylum Proteobacteria, including freshwater genera *Leptothrix*, *Gallionella* and *Sideroxydans* and the marine genus *Mariprofundus*. Except for a few recent isolates, such as *Sideroxydans* ES-1, most of the microaerophilic Fe(II)-oxidizers are obligate Fe(II)-oxidizing lithotrophic bacteria which only grow by oxidizing Fe(II) with O_2 while fixing CO_2 (Emerson *et al.*, 2013). For microaerophilic Fe(II)-oxidizing bacteria, Fe(II) is thought to be oxidized by Fe(II)-oxidizing proteins embedded in their outer membrane (Shi *et al.*, 2016). DNA sequence analysis reveal that the Fe(II)-oxidizing proteins of microaerophilic Fe(II)-oxidizing bacteria are c-type cytochromes and fused with an outer membrane protein. This structure allows the bacteria to oxidize Fe(II) outside the cell membrane to prevent cell encrustation by precipitated Fe(III) minerals. The electrons from Fe(II) oxidation are transferred via several other proteins, and finally to the reaction center in the inner membrane of the cells.

In contrast to abiotic oxidation of Fe(II) by oxygen (reaction 1-4), Fe(II) is oxidized enzymatically by the microaerophilic Fe(II)-oxidizing bacteria rather than ROS. Overall the oxidation of Fe(II) by microaerophilic Fe(II)-oxidizing bacteria is written as:

$$4Fe^{2+} + 10H_2O + O_2 \rightarrow 4Fe(OH)_3 + 8H^+ \qquad (13)$$

Fe(II) is used in this case as both an electron donor in energy metabolism and a reductant for CO_2 fixation ($CO_2 \rightarrow CH_2O$, chemoautotrophy).

Nitrate-reducing Fe(II) oxidation

Nitrate-reducing Fe(II)-oxidizing bacteria (NRFeOx) can oxidize Fe(II) anaerobically while reducing nitrate. These types of Fe(II)-oxidizing bacteria include both autotrophic and heterotrophic consortia and pure cultures (Straub *et al.*, 1996, Kappler *et al.*, 2005, Kiskira *et al.*, 2017).

The heterotrophic nitrate-reducing Fe(II)-oxidizing bacteria oxidize Fe(II) only in the presence of reduced carbon compounds, e.g., acetate, as an additional electron donor (Kappler *et al.*, 2005, Klueglein *et al.*, 2014), and they have been isolated from a variety of habitats (Hafenbradl *et al.*, 1996, Straub *et al.*, 1996, Emmerich *et al.*, 2012, Melton *et al.*, 2012, Shelobolina *et al.*, 2012, Rowe *et al.*, 2014). No specific enzymatic machinery for the heterotrophic nitrate-reducing Fe(II) oxidation has been identified (Carlson *et al.*, 2013, Bryce *et al.*, 2018), and a large portion of Fe(II) is supposed to be abiotically oxidized by the reactive nitrogen species (Carlson *et al.*, 2013, Klueglein & Kappler, 2013, Klueglein *et al.*, 2014), such as NO_2^- and NO (reactions 6-10). Although Fe(II) is oxidized abiotically by RNS, however, as the nitrate reducing step can only be done by bacteria (reaction 14) and the abiotic reaction between Fe(III) and nitrate (NO_3^-) is extremely slow without a catalyst (Ottley *et al.*, 1997), the role of the NRFeOx bacteria in Fe(II) oxidation is also important. Since those RNS are continuously produced during the step by step microbial denitrification process inside the cells (Madigan *et al.*, 2010) (reaction 14), Fe(II) oxidation is thought to be an innate capability of all nitrate-reducing bacteria that encompass many different phyla (Carlson *et al.*, 2013). This coupled biotic-abiotic Fe(II) oxidation mechanism may also explain the Fe(II) oxidation observed for many other heterotrophic nitrate-reducing bacteria (Figure 2.1) (Klueglein *et al.*, 2014, Chen *et al.*, 2018, Li *et al.*, 2018).

$$NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2 \qquad (14)$$

The oxidation of Fe(II) occurs both in the periplasm and at the surface of the cells (Kappler *et al.*, 2005, Schmid *et al.*, 2014, Chen *et al.*, 2018, Li *et al.*, 2018), as both the cell surface and Fe(III) minerals could function as a catalyst and lead to high abiotic Fe(II) oxidation rates with RNS (Klueglein & Kappler, 2013). Fe(III) minerals have been observed both outside the cells and in the periplasm (Figure 2.2) (Kappler *et al.*, 2005, Miot *et al.*, 2009). The precipitation of Fe(III) minerals in the periplasm would lead to the encrustation of the cells and hamper the metabolism of the organisms (Hanert, 1981, Hallberg & Ferris, 2004).

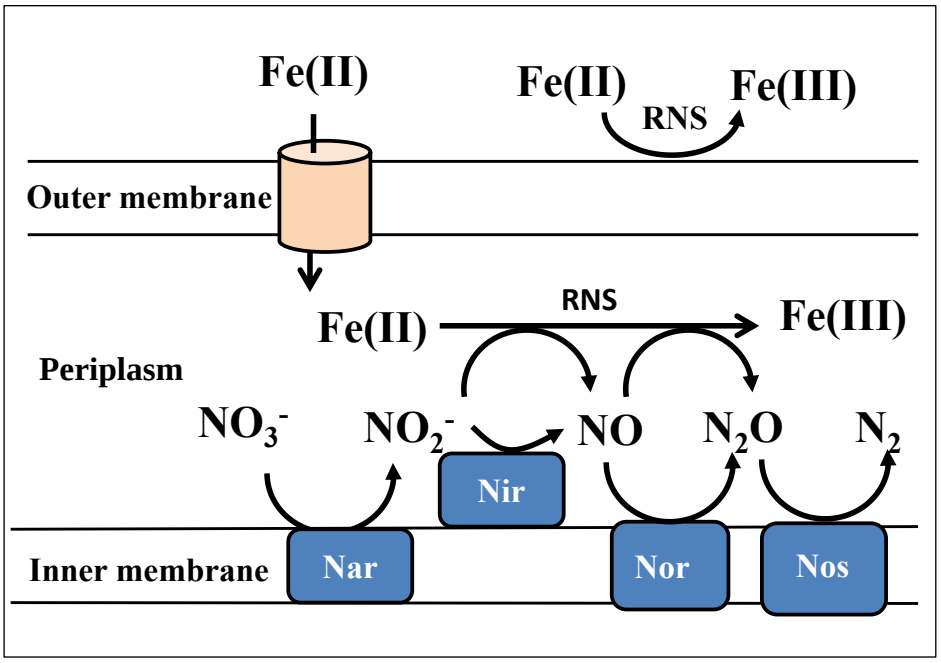

Figure 2.1 Schematics of the current hypotheses on the mechanism of Fe(II) oxidation by NRFeOx bacteria e.g. *Acidovorax* sp. BoFeN1 (modified from Bryce et al. 2018).

Although many cultures have been suggested to perform autotrophic Fe(II) oxidation, the ultimate proof for their autotrophic lifestyle is, in many cases, still missing (Weber *et al.*, 2006, Li *et al.*, 2015, Zhang *et al.*, 2015). Only one nitrate-reducing Fe(II)-oxidizing consortia (culture KS) has been demonstrated unequivocally to maintain autotrophic growth with Fe(II) over more than two decades for many generations and transfers (Straub *et al.*, 1996, Kiskira *et al.*, 2017). This KS culture is a mixture of a supposed autotrophic Fe(II)-oxidizer in the family *Gallionellaceae* and several heterotrophic strains (He *et al.*, 2016) which including *Rhizobium, Agrobacterium*, *Bradyrhizobium, Comamonadaceae*, *Nocardioides, Rhodanobacter, Polaromonas* and *Thiobacillus*. Metagenomic studies of this autotrophic Fe(II)-oxidizing culture suggested that both the *Gallionellaceae* sp. and the heterotrophic strains contribute to the Fe(II) oxidation (He *et al.*, 2016). The oxidation of Fe(II) by *Gallionellaceae* sp. is supposed to be enzymatic via a similar c-type cytochrome system to that identified in neutrophilic microaerobic Fe(II)-oxidizing bacteria.

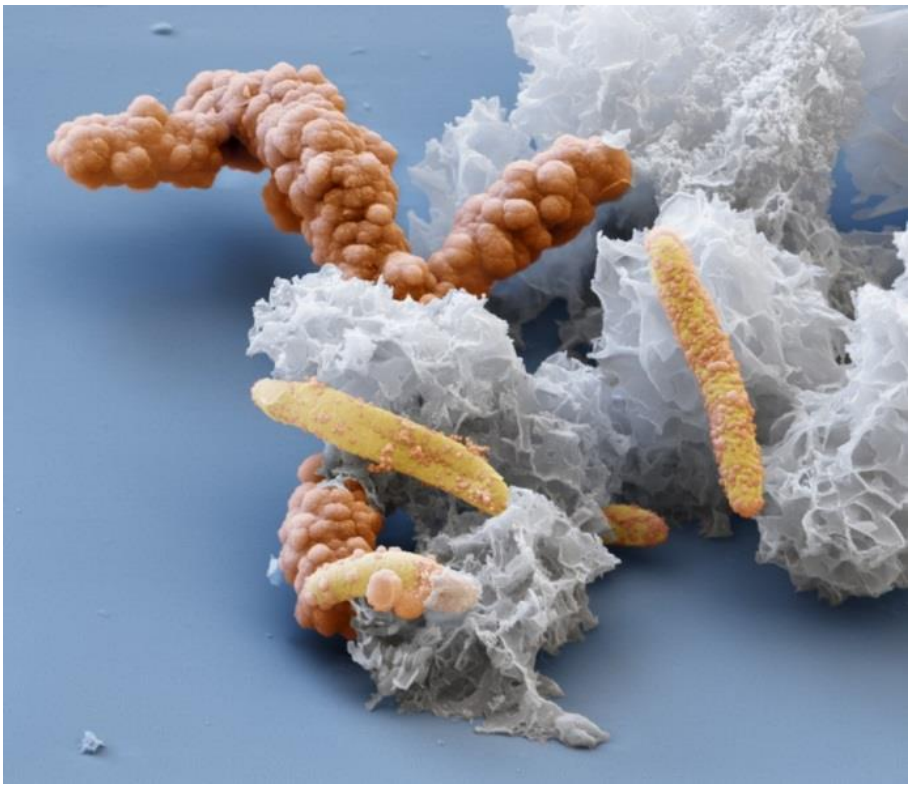

Figure 2.2 Scanning electron micrographs of nitrate-reducing Fe(II)-oxidizing bacteria *Acidovorax* sp. BoFeN1 grown in the presence of Fe(II). Fe(III) minerals and the cells are colored in brown and light yellow respectively. Image: Eye-of-Science/Andreas Kappler.

Phototrophic Fe(II) oxidation

Phototrophic Fe(II)-oxidizing bacteria utilize light as energy source and anaerobically oxidize Fe(II) for carbon fixation. These microorganisms belong to the purple sulfur bacteria (Gammaproteobacteria), purple non-sulfur bacteria (Alphaproteobacteria) and green sulfur bacteria (Chlorobi). Probably as a result of the metabolic flexibility of phototrophic Fe(II)-oxidizing bacteria, they have been isolated in a variety of different environments (Bryce *et al.*, 2018). Most of the phototrophic Fe(II)-oxidizing bacteria are able to autotrophically and enzymatically oxidize Fe(II) without organic compounds. The electrons from Fe(II) oxidation are thought to be transferred via several other proteins and finally reach the reaction center in the inner membrane of the cells. However, there are some exceptions, for example, Fe(II) oxidation by *Rhodomicrobium vannielii* strain BS-1 could only be maintained for up to 3 generations and Fe(II) oxidation by *Rhodobacter capsulatus* strain SB1003 could occur only in the presence of organic ligands (Kopf & Newman, 2012). The two most well

studied strains of phototrophic Fe(II)-oxidizing bacteria are *Rhodobacter ferrooxidans* SW2 and *Rhodopseudomonas palustris* TIE-1. The mechanisms of Fe(II) oxidation by these two strains are slightly different. For *Rhodobacter ferrooxidans* SW2, it is thought that Fe(II) is oxidized by its c-type cytochrome, FoxE, located in the periplasm (Figure 2.3) (Saraiva *et al.*, 2012). The oxidation products, Fe(III) minerals, were associated with organic carbon (Miot *et al.*, 2009) and the cells were not encrusted like the heterotrophic nitrate-reducing Fe(II)-oxidizing bacteria. However the mechanism by which strain SW2 excretes Fe(III) minerals out of the cells is still unknown. For *Rhodopseudomonas palustris* TIE-1, although Fe(II) is also thought to be oxidized by a periplasmic c-type cytrochrome, this c-type cytrochrome, named PioA, it is thought to be embedded inside an outer membrane porin, PioB (Figure 2.3). Interestingly PioA and PioB are respectively found to be homologous with the Fe(III) reductase and outer membrane porin MtrA and MtrB expressed by the Fe(III)-reducer *Shewanella oneidensis* MR-1 (Jiao & Newman, 2007). This unique structure of PioA and PioB is thought to allow the *Rhodopseudomonas palustris* TIE-1 to oxidize Fe(II) on the surface of the cell's outer membrane, obtain electrons from solid Fe(II)-containing minerals e.g. magnetite (Byrne *et al.*, 2015) and harvest electrons directly from poised electrodes (Bose *et al.*, 2014). In summary, though different enzymes are involved in the oxidation of Fe(II), the reaction of Fe(II) by phototrophic Fe(II)-oxidizing bacteria can be written in the following reaction:

$$HCO_3^- + 4Fe^{2+} + 10H_2O \rightarrow (CH_2O) + 4Fe(OH)_3 + 7H^+ \qquad (15)$$

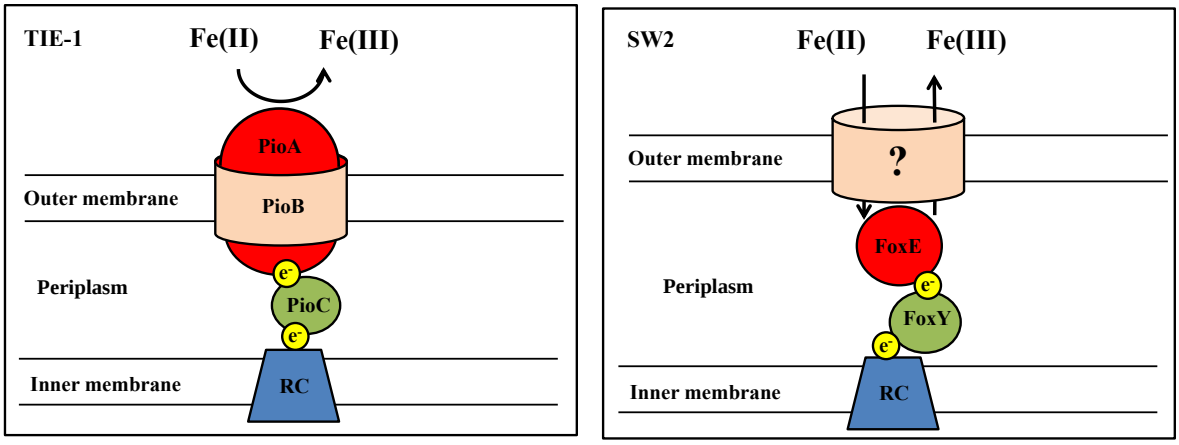

Figure 2.3 Schematics of the current hypotheses on the mechanism of Fe(II) oxidation of two phototrophic Fe(II)-oxidizing bacteria. Left: proposed Fe(II) oxidation mechanism in

Rhodopseudomonas palustris TIE-1. Right: proposed Fe(II) oxidation mechanism for *Rhodobacter ferrooxidans* SW2 (modified from Bryce et al. 2018).

Distribution of different types of Fe(II) oxidation

As different Fe(II) oxidants have different redox potentials, at thermodynamic conditions, we would expect that specific Fe(II) oxidation reactions could dominate under different redox regimes (Figure 2.4) (Melton *et al.*, 2014). For example, Fe(II) would mainly be abiotically oxidized by molecular oxygen in oxygen-rich environments like oxic surface waters. in environments containing low levels of oxygen (Emerson *et al.*, 2010), for example 20-40 µM (Lueder *et al.*, 2018), for example in the upper layers of sediments and in stratified lakes, microbial microaerophilic Fe(II) oxidation would be the dominate type of Fe(II) oxidation. In anoxic environments, like sediments, soils, and stratified lakes, Fe(II) could be oxidized abiotically by RNS or microbially by phototrophic Fe(II)-oxidizing bacteria and NRFeOx. It should be noted that, those RNS in the environment are usually the products of microbial nitrogen metabolites, for example the microbial nitrate reducing process, therefore the oxidation of Fe(II) by RNS and NRFeOx are cross-linked. In environments where light cannot penetrate and in the absence of nitrate, e.g. the deeper sediment, abiotic Fe(II) oxidation by MnO_2 would be the dominate Fe(II) oxidation pathway.

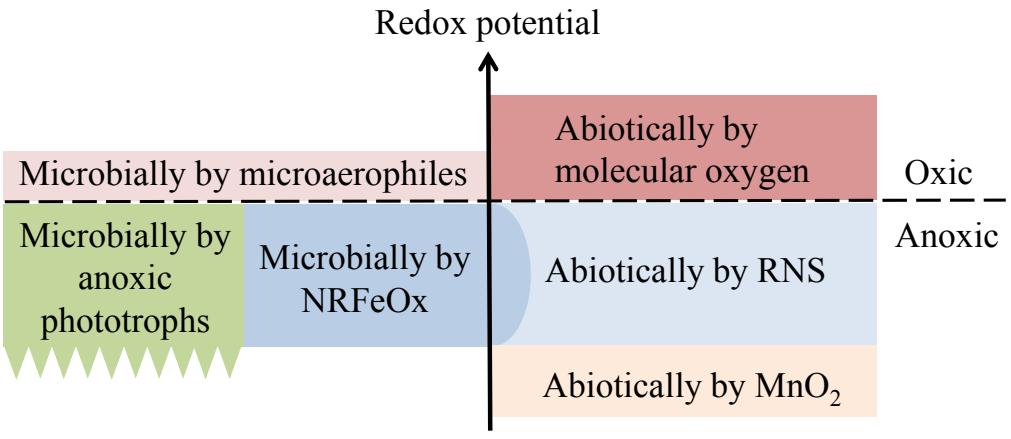

Figure 2.4 Distribution of different types of Fe(II) oxidation.

2.3 Fe-organic-matter complexes

Fe(II) present not only in the form of pure free Fe(II) ions or minerals, but also in the form of Fe(II)-organic-matter complexes (Fe(II)-OM) in natural environments such as soils, rivers, wetlands, ocean sediments and hydrothermal plumes (Luther *et al.*, 1996, Kleja *et al.*, 2012, Sundman *et al.*, 2013, Sundman *et al.*, 2014, von der Heyden *et al.*, 2014, Hopwood *et al.*, 2015, Yu *et al.*, 2015, Bhattacharyya *et al.*, 2018). Previous studies have shown that the complexation of Fe-OM significantly changed the chemical behaviors of both Fe(II) and Fe(III), and remarkedly influenced the concentration, distribution and redox state of Fe.

Abiotic oxidation of Fe(II)-OM

Fe(II)-OM complexation has been suggested to influence the abiotic oxidation rates of Fe(II) by O_2 (Rose & Waite, 2003, Daugherty *et al.*, 2017, Lee *et al.*, 2017) and by nitrite (Kopf *et al.*, 2013). Depending on the type of OM, the effect of Fe(II)-OM complexation on rates of abiotic Fe(II) oxidation are different (Rose & Waite, 2003, Kopf *et al.*, 2013, Daugherty *et al.*, 2017, Lee *et al.*, 2017). In the case of Fe(II)-natural-organic-matter complexes, such as Fe(II)-humic-acid complexes, Fe(II)-OM complexation was suggested to retard (Daugherty *et al.*, 2017) and accelerate (Rose & Waite, 2003, Lee *et al.*, 2017) the oxidation of Fe(II) by molecular oxygen, and slightly alter the oxidation rate of Fe(II) by NO_2^- (Kopf *et al.*, 2013). In contrst, the oxidation rates of simpler Fe(II)-OM complexes, such as Fe(II)-citrate and Fe(II)-EDTA (ethylenediaminetetraacetic acid) by molecular oxygen and nitrite (NO_2^-), are both found to be accelerated by the complexation compared to non-complexed Fe(II) (Kopf *et al.*, 2013, Jones *et al.*, 2015).

Several mechanisms have been proposed to explain these effects of Fe(II)-OM complexation on abiotic Fe(II) oxidation. The complexation of Fe(II) by OM may cause the changes in the rate constants *k* of Fe(II) oxidation, affect the reaction mechanisms (Rose & Waite, 2003, Kopf *et al.*, 2013) or cause a thermodynamics change (Straub *et al.*, 2001). OM itself could also directly influence Fe(II) oxidation, due to the reduced functional groups of OM which are able to react with oxygen (Daugherty *et al.*, 2017) and nitrite (Stevenson & Swaby, 1964, Stevenson *et al.*, 1970, Zhu-Barker *et al.*, 2015). Besides, the formation of Fe(II)-OM-NO complexes (Jin *et al.*, 2005), and the adsorption of OM onto the surface of Fe(III) minerals, i.e. goethite, (Klueglein & Kappler, 2013) could thereby inhibit the

autocatalysis of Fe(II) oxidation.

Potential influence of OM complexation on microbial oxidation of Fe(II)

The influence of Fe-OM complexation has not been extensively studied. However, there are a few studies indicated that Fe-OM complexation could potentially influence the microbial Fe(II) oxidation process. Different reaction kinetics between different Fe(II)-OM and bacterial c-type cytochromes have been observed. For example MtoA, the putative Fe(II) oxidase of the microaerophilic Fe(II)-oxidizing bacterium *Sideroxydans lithotrophicus* ES-1 (Liu *et al.*, 2012), and MtrC and OmcA of Fe(III)-reducing bacterium *Shewanella oneidensis* MR-1 (Wang *et al.*, 2008). It is currently not clear why these c-type cytochromes show different reaction kinetics with different forms of Fe(II). Moreover, Fe(II)-OM complexation enabled *Rhodobacter capsulatus* SB1003, a bacterium which can not oxidize dissolved non-OM-complexed Fe(II) (Croal *et al.*, 2007), to oxidize and grow on Fe(II) (Caiazza *et al.*, 2007). And Fe(II)-OM complexation also promoted the growth of the Fe(II)-oxidizer *Thiobacillus denitrificans* (Kanaparthi & Conrad, 2015). Although a previous publication has suggested Fe(II)-OM complexes promote oxidation of Fe(II) by nitrite, which was produced by the nitrate-reducer *Paracoccus denitrificans* (Kopf *et al.*, 2013). However, in this study, the Fe(II)-OM complexes were added to the cultures containing NO_2^- which may lead to abiotic Fe(II) oxidation. Therefore, it still remains inconclusive whether the NRFeOx bacteria could directly oxidize Fe(II)-OM complexes.

Formation of Fe(III)-OM complexes

Fe(III), the product of Fe(II) oxidation, Fe(III) also forms complexes with OM. The properties of OM-complexed Fe(III) are different from its non-OM-complexed Fe(III) counterpart. One of the most obvious difference is the high solubility of Fe(III)-OM, whereas the non-OM-complexed Fe(III) is barely soluble at neutral pH. Previous studies suggest that the complexation of Fe(III)-OM is critical in regulating solubility and bioavailability of Fe in oxic environments and more than 99% of the dissolved Fe(III) is present as organic complexes in the open ocean (Rose & Waite, 2003). Secondly, Fe(III)-OM complexes can be chemically photolysised, resulting in the formation of Fe(II) and breakdown of OM in photic zones. This photochemical process plays an important role in the cycle of Fe in oxic environments. The photochemical reduction of Fe(III)-OM was thought to be one of the explanations for the

higher than expected abundance of Fe(II) in many oxic natural aquatic environments (Kuma *et al.*, 1992, Borer *et al.*, 2005, Barbeau, 2006) and could also lead to an abiotic Fe cycling when Fe(II) is abiotically re-oxidized by molecular oxygen (Voelker *et al.*, 1997, Barbeau *et al.*, 2001, Lukas *et al.*, 2001, Sunda & Huntsman, 2003, Boyd & Ellwood, 2010). The complexation of Fe(III)-OM have also been shown to influence the microbial reduction of Fe(III) as the OM can serve as electron shuttle for microbial Fe(III)-reducing bacteria (Lovley *et al.*, 1996). And the complexation of Fe(II)-OM was shown to influence rates and extent of microbial reduction of Fe(III)-OM by *Shewanella putrefaciens* (Shimizu *et al.*, 2013) and *Shewanella alga* (Urrutia *et al.*, 1999).

2.4 Objectives of this study

Fe(II) oxidation and the complexation of Fe(II)-OM have significant impacts on many biological and geological processes in natural environment. Although the oxidaiton of non-organically-bound Fe(II) (free Fe(II)) and the abiotic oxidation of Fe(II)-OM have been extensively studied. Most of the previous studies on microbial Fe(II) oxidation did not consider the complexation of Fe(II) by OM, the effect of Fe(II)-OM complexation on anaerobic microbial Fe(II) oxidation is so far unknown. Therefore the objectives of this thesis are:

1. Determine whether and how fast anaerobic Fe(II)-oxidizing bacteria can oxidize Fe(II)-OM complexes (Chapters 3, 4 and 5).

2. Investigate the mechanism by which Fe(II)-OM complexation influences the rates and extent of microbial Fe(II) oxidation (Chapters 3 and 4).

3. Investigate the roles of Fe(II)-OM in the geochemical cycles of nitrogen (Chapter 3) and carbon (Chapter 5)

4. Characterise the products of microbial phototrophic oxidation of Fe(II)-OM complexes, in terms of the size distribution of Fe(II) and Fe(III) compounds (Chapter 4).

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3. Oxidation of Fe(II)-organic-matter complexes in the presence of the mixotrophic nitrate-reducing Fe(II)-oxidizing bacterium *Acidovorax* sp. BoFeN1

3.1 Abstract

Fe(II)-organic-matter (Fe(II)-OM) complexes are abundant in the environment and may play a key role for the behavior of Fe and pollutants. Mixotrophic nitrate-reducing Fe(II)-oxidizing bacteria (NRFeOx) reduce nitrate coupled to the oxidation of organic compounds and Fe(II). Fe(II) oxidation may occur enzymatically or abiotically by reaction with nitrite that forms during denitrification. However, it is unknown whether Fe(II)-OM complexes can be oxidized by NRFeOx. We used cell-suspension experiments with *Acidovorax* sp. strain BoFeN1 to reveal the role of non-organically-bound Fe(II) (free Fe(II)) and nitrite in the oxidation rates and extent of Fe(II)-OM-complexes (Fe(II)-citrate, Fe(II)-EDTA, Fe(II)-humic-acid, and Fe(II)-fulvic-acid). We found that Fe(II)-OM complexation inhibited microbial nitrate-reducing Fe(II) oxidation; colloidal and negatively charged complexes showed lower oxidation rates than free Fe(II). Accumulation of nitrite and fast oxidation of Fe(II)-OM complexes only happened in the presence of free Fe(II) that probably interacted with (nitrite-reducing) enzymes in the periplasm causing nitrite accumulation in the periplasm and outside of the cells, whereas Fe(II)-OM complexes probably could not enter the periplasm and cause these changes. These results suggest that Fe(II) oxidation by mixotrophic nitrate-reducers in the environment depends on Fe(II) speciation, and that free Fe(II) potentially plays a critical role in regulating microbial denitrification processes.

3.2 Introduction

Iron (Fe) is present in almost all aquatic and terrestrial environments (Jickells *et al.*, 2005, Boyd & Ellwood, 2010). It is an essential element for nearly all organisms and influences both the behavior of environmental contaminants (Borch *et al.*, 2010) and many other biogeochemical cycles (Melton *et al.*, 2014). The oxidation of Fe(II) to Fe(III) influences Fe bioavailability, as the Fe(III)-based oxidation product is poorly soluble at neutral pH whereas Fe(II) is far more soluble. Additionally, Fe(II) oxidation also influences the mobility and toxicity of many toxic metal(loid)s such as arsenic (Hohmann *et al.*, 2010, Zhu *et al.*, 2017) and cadmium (Muehe *et al.*, 2013) by sorption to the resulting Fe(III) minerals.

At neutral pH, Fe(II) can be rapidly oxidized by molecular oxygen to Fe(III) (Fenton reactions) or by reactive N-species and biotically by Fe(II)-oxidizing microorganisms which are able to use either O_2, light, or nitrate to oxidize Fe(II) (Melton *et al.*, 2014). Nitrate-reducing Fe(II)-oxidizing bacteria (NRFeOx) have been isolated from a variety of habitats (Hafenbradl *et al.*, 1996, Straub *et al.*, 1996, Emmerich *et al.*, 2012, Melton *et al.*, 2012, Shelobolina *et al.*, 2012, Rowe *et al.*, 2014). NRFeOx microorganisms include both autotrophic and heterotrophic consortia and pure cultures (Straub *et al.*, 1996, Kappler *et al.*, 2005, Kiskira *et al.*, 2017). *Acidovorax* sp. strain BoFeN1 has been isolated from Lake Constance freshwater sediments (Kappler *et al.*, 2005). It is a mixotrophic nitrate-reducing Fe(II)-oxidizing bacterium that can oxidize Fe(II) in the presence of reduced carbon compounds, e.g., acetate, as an additional electron donor (Kappler *et al.*, 2005, Klueglein *et al.*, 2014). Several *Acidovorax* sp. relatives have been found in arsenic-contaminated aquifers, town ditches, ground water, and freshwater sediments (Sutton *et al.*, 2009, Kiskira *et al.*, 2017). Until now, a specific enzymatic machinery for nitrate-reducing Fe(II) oxidation has not been identified (Carlson *et al.*, 2013). So far, only one nitrate-reducing Fe(II)-oxidizing mixed culture has been demonstrated unequivocally to maintain autotrophic growth with Fe(II) over more than two decades for many generations and transfers (Straub *et al.*, 1996, Kiskira *et al.*, 2017). More cultures have been suggested to also perform autotrophic Fe(II) oxidation although ultimate proof for their autotrophic lifestyle is, in many cases, still missing (Weber *et al.*, 2006, Li *et al.*, 2015, Zhang *et al.*, 2015). In contrast, for most nitrate-reducing Fe(II)-oxidizers reactive nitrogen species such as NO_2^- and NO (intermediates of microbial heterotrophic denitrification) (reaction 1) have been suggested to be the oxidants for Fe(II) (Carlson *et al.*,

2013, Klueglein & Kappler, 2013, Klueglein *et al.*, 2014). Although the enzymatic steps of microbial denitrification take place inside the cell (Madigan *et al.*, 2010), precipitation of Fe(III) minerals, the products of Fe(II) oxidation, have been observed both in the periplasm and at the surface of the cells (Kappler *et al.*, 2005, Schmid *et al.*, 2014, Chen *et al.*, 2018, Li *et al.*, 2018). Consequently, these initially formed Fe(III) minerals could function as catalyst and lead to high abiotic Fe(II) oxidation rates(Klueglein & Kappler, 2013). This coupled biotic-abiotic Fe(II) oxidation mechanism may also explain the Fe(II) oxidation observed for many other heterotrophic nitrate-reducing bacteria(Klueglein *et al.*, 2014, Chen *et al.*, 2018, Li *et al.*, 2018).

$$NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2 \quad (1)$$

It has been shown that NRFeOx can oxidize dissolved Fe(II), Fe(II)-containing minerals such as siderite and magnetite (Chakraborty *et al.*, 2011), clay minerals (Shelobolina *et al.*, 2003, Zhao *et al.*, 2017), and simple organic Fe(II) complexes such as Fe(II)-EDTA and Fe(II)-NTA (Shelobolina *et al.*, 2003, Kumaraswamy *et al.*, 2006, Chakraborty & Picardal, 2013). However, in nature Fe(II)-organic-matter (Fe(II)-OM) complexes are present (Luther *et al.*, 1996, Kleja *et al.*, 2012, Sundman *et al.*, 2013, Sundman *et al.*, 2014, von der Heyden *et al.*, 2014, Hopwood *et al.*, 2015, Yu *et al.*, 2015, Bhattacharyya *et al.*, 2018). Previous studies have shown that Fe-OM complexes, that in many cases are present as colloids, can significantly influence the concentration, distribution and redox state of Fe and (in)organic contaminants, for example arsenic, via the formation of ternary OM-Fe-As complexes (Mikutta & Kretzschmar, 2011, Sharma *et al.*, 2011, Catrouillet *et al.*, 2016). OM complexation also influences the abiotic oxidation rates of Fe(II) by O_2 (Rose & Waite, 2003, Daugherty *et al.*, 2017, Lee *et al.*, 2017, Chen & Thompson, 2018) and by nitrite (Kopf *et al.*, 2013). It was also suggested that complexation and stabilization with organic ligands is the reason for the higher than expected abundance of Fe(II) and Fe(III) in many oxic natural aquatic environments(Barbeau, 2006, Neubauer *et al.*, 2013, Sundman *et al.*, 2014, Bhattacharyya *et al.*, 2018).

Although there is a lot of evidence that OM complexation, including Fe(II) colloid formation, affects abiotic Fe(II)/Fe(III) redox reactions, the effect of OM on microbial Fe redox reactions, particularly on microbial Fe(II) oxidation, is poorly understood. Previous studies have suggested that the content of OM in marine sediments can influence the ratio

of nitrate reduction to Fe(II) oxidation (Laufer *et al.*, 2016). The oxidation of simple Fe(II)-EDTA and Fe(II)-NTA complexes has been demonstrated with several nitrate-reducing Fe(II)-oxidizing strains (Shelobolina *et al.*, 2003, Kumaraswamy *et al.*, 2006, Chakraborty & Picardal, 2013). In contrast, large Fe(II)-OM complexes such as Fe(II)-NTA-agarose complexes were not oxidized by NRFeOx bacteria, probably because their size is too large to enter the cells (Carlson *et al.*, 2013). A previous study has analyzed the oxidation rates for several Fe(II)-OM complexes compared to free Fe(II) by nitrite produced by the nitrate-reducer *Paracoccus denitrificans* (Kopf *et al.*, 2013). However, in these experiments the Fe(II)-OM complexes were added to the cultures after they had accumulated 5 mM NO_2^- (leading to abiotic Fe(II) oxidation by nitrite) and therefore these setups are not suited for investigation of direct microbial oxidation of Fe(II)-OM. Interestingly, mixotrophic nitrate-reducing Fe(II)-oxidizing microorganisms, such as *Acidovorax* sp. strain BoFeN1, did not accumulate nitrite, when there is only acetate/nitrate but no dissolved Fe(II) present (Figure S3.1). In the absence of Fe(II), they perform complete denitrification and the nitrite gets reduced stepwise via NO, and N_2O to N_2 (Madigan *et al.*, 2010). As previous studies on mixotrophic NRFeOx have reported an encrustation of both the cell surface and the periplasm (Kappler *et al.*, 2005, Klueglein *et al.*, 2014, Schmid *et al.*, 2014, Chen *et al.*, 2018, Li *et al.*, 2018), this suggests that at least a part of the Fe(II) entered the periplasm, became oxidized, and precipitated there. This allows us to hypothesize that on the one hand, since the outer membrane is not permeable to proteins or other large molecules (Madigan *et al.*, 2010), it also presents a barrier for large Fe(II)-OM complexes (colloids) preventing that they enter the periplasm. On the other hand, we can hypothesize that unique chemical conditions in the periplasm, e.g. lower pH and higher concentrations of nitrite, make the periplasm a potential hotspot of abiotic Fe(II) oxidation. In summary, we do not know the effect of Fe(II)-OM complexation and Fe(II)-colloid formation on the formation of nitrite and the kinetics and extent of oxidation of Fe(II)-OM complexes by such strains.

Therefore, the objectives of this study are to determine the rates and extent of oxidation of Fe(II)-OM complexes compared to free Fe(II) by the mixotrophic nitrate-reducing Fe(II)-oxidizer *Acidovorax* sp. BoFeN1, and to investigate the roles of free Fe(II) and nitrite in the oxidation of Fe(II)-OM species.

3.3 Materials and Methods

Synthesis of Fe(II)-OM complexes

All Fe(II)-OM complexes were synthesized anoxically in a 20 mM PIPES buffer amended with 20 mM NaCl. Stock solutions of Fe(II)-citrate and Fe(II)-EDTA complexes were synthesized by mixing $FeCl_2$:citrate in a 1:2 and $FeCl_2$:EDTA in a 1:1.2 molar ratio, respectively, pH was adjusted to 7 with HCl. Fe(II)-citrate and Fe(II)-EDTA complexes were synthesized as stock solutions (100 mM), filter-sterilized (0.2 µm, polyethersulfone, PES) and stored in sterile anoxic glass serum bottles with butyl rubber stoppers in the dark at room temperature. Fe(II)-PPHA (Pahokee peat humic acid) and Fe(II)-SRFA (Suwannee river fulvic acid) complexes were synthesized by dissolving either PPHA and SRFA (purchased from the International Humic Substances Society) with media containing 20 mM PIPES and 20 mM NaCl at a final concentration of 2.5 mg/ml (118 and 109 mM carbon/L, respectively, for PPHA and SRFA), the pH was adjusted to approximately 10 then neutralized to 7 with anoxic NaOH and HCl solution. $FeCl_2$ was added to the solutions to a final concentration of 3 mM Fe(II). At this pH and concentration of Fe(II), there is no $Fe(OH)_2$ precipitate forming. The solubility of Fe(II) is about 80 mM at pH 7 (Gayer & Woontner, 1956), which is much higher than 3 mM, and we have confirmed the absence of $Fe(OH)_2$ precipitate by PHREEQC modeling (Table S3.1). To minimize abiotic redox reactions between Fe(II) and PPHA and SRFA, $FeCl_2$ was added approx. 2 h before the Fe(II) oxidation cell suspension experiments (see below). All experiments were performed in an anoxic glovebox (100% N_2), based on the oxygen concentration in the glovebox (<10 ppm), less than 1.5 nM oxygen was in solution.

Bacterial strain and pre-cultivation

Acidovorax sp. BoFeN1 was isolated from Lake Constance sediments and kept in the authors' laboratory since then (Kappler *et al.*, 2005). The culture was continuously transferred in freshwater medium with 10 mM $FeCl_2$, 10 mM $NaNO_3$ and 5 mM Na-acetate (for medium composition see Table S3.2).

To prepare the cell suspension experiments, *Acidovorax* sp. BoFeN1 was transferred twice in a basal medium with nitrate and acetate without Fe(II) to get rid of remaining Fe(III) minerals stemming from the inoculum; each time the bacteria were cultured under anoxic

conditions until the late exponential phase (after about 40 hours of incubation). Cells were harvested by centrifugation (7000 g, 20 min, 25°C), washed twice with 20 mM PIPES buffer containing 20 mM NaCl, and re-suspended in anoxic PIPES buffer with 20 mM NaCl. This cell suspension was used for the Fe(II) oxidation experiments. An aliquot of the cell suspension was fixed using 2% paraformaldehyde and stored at 4°C for quantification of cell numbers by a flow cytometer (Attune Nxt Acoustic focusing cytometer, Thermo Fisher Scientific). All plastics used in the glovebox were pre-degassed in the vacuum chamber and stored in the glovebox for at least 24 h before use. Chemicals used were of analytical reagent grade. Volumetric flasks and vessels were cleaned by oxalic acid and soaked in 1 M HCl, rinsed several times with deionized water, and sterilized at 180°C for 40 min.

Setup of Fe(II) oxidation experiments

Basal non-growth medium was prepared containing anoxic 20 mM PIPES buffer and 20 mM NaCl. This simple medium was chosen as typical medium components such as mM concentrations of calcium, phosphate or bicarbonate can lead to dissociation of Fe(II)-OM complexes (based on our thermodynamic calculations using PHREEQC with the minteq.v4 database; data not shown). Our experiments have shown that there was no or only a minor toxic effect of OM on BoFeN1 nitrate reduction (see suppoting information). For experiments containing free Fe(II), Fe(II)-citrate or Fe(II)-EDTA complexes (or a mixture of these), stock solutions of these components were added to the basal medium as required. For Fe(II)-PPHA/Fe(II)-SRFA complexes, PPHA or SRFA was dissolved in the basal medium (pH 7) to a concentration of 2.5 mg/ml before addition of 3 mM $FeCl_2$ (experimental procedures described above). In the experiments with a mixture of Fe(II)-PPHA or Fe(II)-SRFA and free Fe(II), 3 mM $FeCl_2$ was added following the dissolution of PPHA and SRFA in the basal medium to a concentration of 0.5 mg/ml (ca. 24 and 22 mM carbon/L, respectively, for PPHA and SRFA).

10 mM $NaNO_3$ and 5 mM sodium acetate were added as electron acceptor and donor for *Acidovorax* sp. BoFeN1. The washed cells were added into the non-growth medium at a concentration of between $4.00*10^8$ and $6.82*10^8$ cells/ml (Table S3.3) as determined by flow cytometry. The cell suspensions were then incubated in Hungate tubes closed with air-tight butyl-stoppers in the dark at 28°C. In experiments with different types of OM, the Fe(II)

oxidation rate in setups with free Fe(II) only was determined in each case (for each Fe(II)-OM experiment) individually for comparison. Abiotic Fe(II) oxidation experiments (with nitrite as oxidant) were carried out in the same way as the cell suspension experiments, however, without cells but instead with the addition of 2 mM of nitrite. All experiments were carried out in an anoxic glovebox (100% N_2) in duplicate.

Sample analysis

Samples were taken hourly in the anoxic glovebox. Fe(II) concentrations were determined anoxically using a slightly modified ferrozine assay (Stookey, 1970). For the quantification of Fe(II) in samples without PPHA and SRFA ($FeCl_2$, Fe(II)-citrate and Fe(II)-EDTA samples), samples were first diluted with 40 mM anoxic sulfamic acid to prevent Fe(II) oxidation by nitrite at low pH (Klueglein & Kappler, 2013). The Fe(II) concentration was determined by first mixing samples with 1M anoxic HCl, followed by addition of anoxic ferrozine solution (0.1% w/v) dissolved in ammonium acetate ($C_2H_7NO_2$, 50% w/v). The samples were then incubated in the dark for about 5 minutes, and were finally (when the Fe(II) was stabilized for analysis) taken out of the glovebox for absorbance measurements. The purple ferrozine-Fe(II) complex formed was quantified at 562 nm with a microplate reader (Thermo Scientific). The previous reported interference of EDTA with the ferrozine assay (Kopf *et al.*, 2013) was not observed when the assay was performed anoxically. For quantification of Fe(II) in samples with PPHA and SRFA, samples were first diluted with anoxic Milli-Q H_2O, immediately after sampling Fe(II) concentration was determined by adding ferrozine solutions (described above) directly into the H_2O-diluted samples without using 1M HCl. Light absorption by PPHA and SRFA at 562 nm was determined and subtracted from the absorbance of the samples. All ferrozine measurements were done in at least triplicate and the results reported as an average. Linear fits of Fe(II) concentrations during Fe(II) oxidation phases were used for the calculation of Fe(II) oxidation rates. As there was an obvious lag phase in the system without OM, in this system, the reactive period was distinguished from the lag phase by the time points after which the Fe(II) oxidation rates determined with two adjacent time points were similar as in the rates determined from the linear regressions.

Samples for nitrite and nitrate quantification were taken in the glovebox and centrifuged (14,000 g, 10 mins) to remove cells and minerals. The supernatant was diluted

with anoxic Milli-Q H_2O and stored at 4°C. Nitrite and nitrate concentrations were quantified by a continuous-flow analysis containing a dialysis membrane for the removal of Fe and organic ligands to prevent side reactions during analysis (Seal Analytical, Norderstedt). In this automated system, nitrate is reduced to nitrite with hydrazine sulfate and quantified colorimetrically with N-1-naphtylethylendiamin at 550 nm.

3.4 Results

Oxidation of free Fe(II) and OM-complexed Fe(II)

In order to determine the effect of Fe(II) complexation by OM on oxidation of Fe(II) by the mixotrophic nitrate-reducing Fe(II)-oxidizer *Acidovorax* sp. strain BoFeN1, we incubated free Fe(II), different Fe(II)-OM complexes or a mixture of free Fe(II) and Fe(II)-OM complexes with nitrate, acetate and strain BoFeN1 (Figure 3.1). We found that in all experiments, free Fe(II) was oxidized with rates of up to 0.19-0.46 mM/h (Table S3.4). From one experiment to another, the oxidation rates for free Fe(II) varied slightly but generally all Fe(II) was oxidized to near completion within 9-12 hours. However, oxidation of the Fe(II)-OM complexes was found to be significantly slower than free Fe(II). Among the four different complexes, the Fe(II)-citrate complex showed the fastest oxidation rates (0.11 mM/h; Table S3.4) followed by Fe(II)-EDTA (0.06-0.08 mM/h). The Fe(II)-SRFA (0.02 mM/h) and Fe(II)-PPHA complexes (<0.01 mM/h) had the slowest oxidation rates (Table S3.4). The average oxidation rates for the Fe(II)-citrate, Fe(II)-EDTA, Fe(II)-SRFA complexes were approximately 2-, 4.3-, and 23-fold slower than the average oxidation rates of free Fe(II) (Table S3.4). For the Fe(II)-PPHA complexes hardly any oxidation was observed during 24 hours of incubation, while the Fe(II)-citrate, Fe(II)-EDTA, and the Fe(II)-SRFA complexes showed ca. 67%, 46-50% and 37-45% oxidation of the total Fe(II) present within 24 h, respectively.

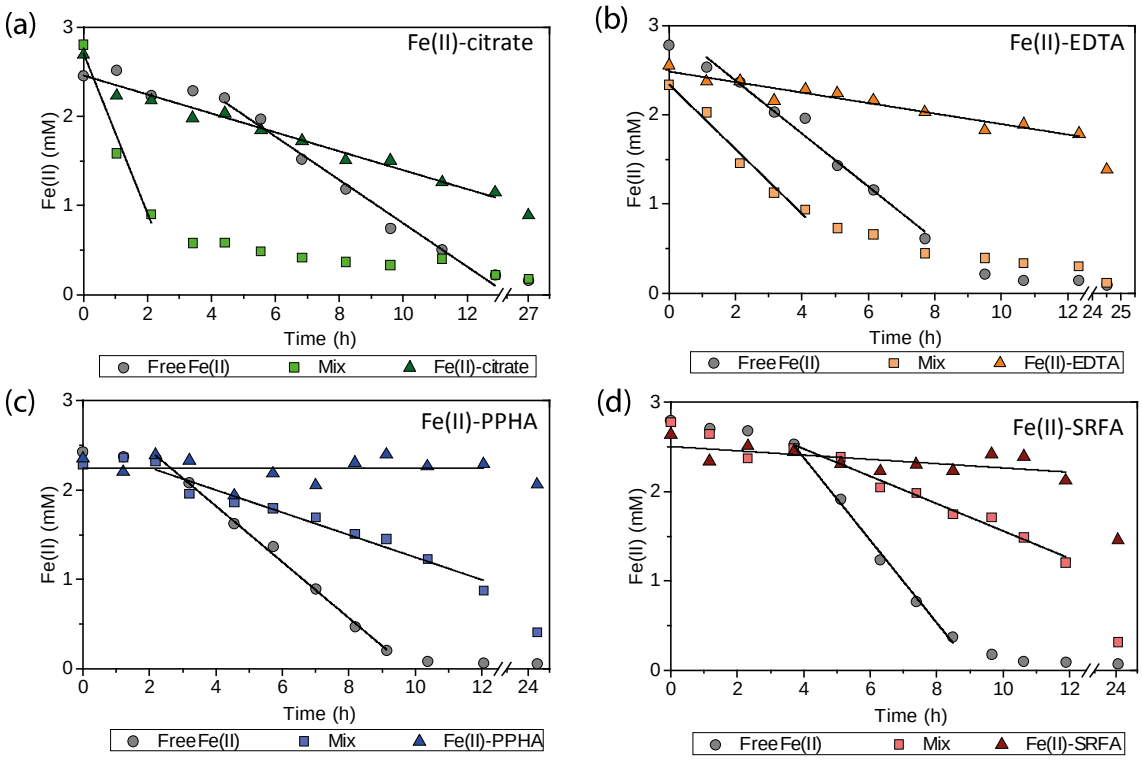

Figure 3.1 Oxidation of Fe(II) in the presence of 10 mM nitrate, 5 mM acetate and *Acidovovax* sp. strain BoFeN1. The Fe(II) was present in the form of either free (non-OM-complexed) Fe(II) (circles), different fully-complexed Fe(II)-OM complexes (triangles), or a mixture of free Fe(II) and Fe(II)-OM complexes (squares). Data is shown for Fe(II)-citrate (a), Fe(II)-EDTA (b), Fe(II)-PPHA complexes (c) and Fe(II)-SRFA complexes (d). The solid black lines indicate the linear fits of Fe(II) concentrations for the calculation of Fe(II) oxidation rates shown in table S3.4. Since duplicate microbiological setups showed very similar results, representative experiments out of these duplicate setups are shown (the second one is shown in the supporting information, Figure S3.5), and the data are shown as averages of triplicate spectrophotometric measurements.

However, these trends for Fe(II) oxidation changed when the setups contained a mixture of both free Fe(II) and Fe(II)-OM complexes in the presence of strain BoFeN1. In these mixtures, all Fe(II)-OM complexes were oxidized, and the rates of Fe(II) oxidation were much faster than in setups with Fe(II)-OM complexes only. Fe(II) was oxidized at 0.68-0.90 mM/h, at ca. 0.37, ca. 0.13, and at 0.15 mM/h in the mixtures of free Fe(II) and Fe(II)-citrate, Fe(II)-EDTA, Fe(II)-PPHA, and Fe(II)-SRFA complexes, respectively (Table S3.4). In the mixed setups containing both free Fe(II) and Fe(II)-citrate or both free Fe(II) and Fe(II)-EDTA

complexes, Fe(II) was oxidized 3.7-fold (free Fe(II) and Fe(II)-citrate mixture), and 1.2-fold (free Fe(II) and Fe(II)-EDTA mixture) faster than in the setups with free Fe(II) only. Even in the mixed setups with both free Fe(II) and Fe(II)-PPHA or free Fe(II) and Fe(II)-SRFA complexes, the average Fe(II) oxidation rates were only about 33-43% slower than the rates in setups with only free Fe(II), whereas in the absence of free Fe(II) in the setup with fully-complexed Fe(II)-PPHA and Fe(II)-SRFA complex, there was almost no or only a small amount of Fe(II) oxidized during the first 12 hours. After one day of incubation, a large fraction of Fe(II) in the setups with fully-complexed Fe(II)-OM still remained, whilst the Fe(II) in the mixed setups with both free Fe(II) and Fe(II)-OM was nearly completely oxidized. The concentrations of Fe(II) remaining in the free Fe(II) and Fe(II)-OM mixed setups of Fe(II)-citrate, Fe(II)-EDTA, Fe(II)-PPHA and of Fe(II)-SRFA were close to the setups with only free Fe(II).

In addition to the Fe(II) oxidation rates, the time after which Fe(II) started to oxidize was always earlier in the mixture of free Fe(II) and Fe(II)-citrate or free Fe(II) and Fe(II)-EDTA complexes than in the setups with free Fe(II) alone, and this effect was even more obvious in setups with lower cell numbers (Figure S3.2). During the first few hours, depending on the cell numbers, only a small amount of Fe(II) was oxidized in the setups with only free Fe(II) (a lag-phase of Fe(II) oxidation) and fast Fe(II) oxidation started after a few hours of incubation. In contrast, in the presence of citrate or EDTA, Fe(II) oxidation started almost immediately, independently of whether there was free Fe(II) present or not. The earlier oxidation of Fe(II) in the setups with mixtures of free Fe(II) and the Fe(II)-complexes is also evidenced by the color changes of the medium as Fe(III) complexes and Fe(III) minerals usually have a darker brownish color (Figure S3.2). However, due to the dark color of the HA and FA, this effect cannot be seen in the setups with Fe(II)-PPHA and Fe(II)-SRFA complexes.

Nitrite accumulation in BoFeN1 cultures

Nitrite is an intermediate product of microbial denitrification and considered as one of the main chemical oxidants of Fe(II) during mixotrophic NRFeOx (Klueglein *et al.*, 2014). In order to further investigate the role of free Fe(II) in nitrite accumulation and thus in the (abiotic) oxidation of Fe(II)-OM species, we determined nitrite concentrations during the incubation of either free Fe(II), Fe(II)-OM complexes or mixtures of both free Fe(II) and Fe(II)-OM complexes in the presence of nitrate, acetate and strain BoFeN1 (Figure 3.2). We

found that nitrite only accumulated in BoFeN1 cultures that contained free Fe(II). Specifically, nitrite was observed in BoFeN1 cultures with only free Fe(II) and in cultures that contained a mixture of both free Fe(II) and either Fe(II)-citrate or Fe(II)-EDTA complexes. The highest nitrite concentration determined was 2.8 mM in the presence of mixtures of free Fe(II) and Fe(II)-citrate after 12 h of incubation. In BoFeN1 cultures with only free Fe(II) and with mixtures of free Fe(II) and Fe(II)-EDTA, nitrite accumulated as well and reached concentrations of around 200-300 μ*M.* In some cases, as soon as Fe(II) was almost, but not completely consumed, nitrite concentrations started to decrease again. As an example, in cultures with lower cell numbers (ca. 50% of the typical cell abundance, i.e. $2.35*10^8$ cells/ml), 1.3 mM and 1 mM nitrite accumulated in the setups with only free Fe(II) and with a mixture of free Fe(II) and Fe(II)-EDTA, respectively (Figure S3.2), compared to 0.18 mM and 2 mM nitrite when $4.51*10^8$ and $4.00*10^8$ cells/ml were present, respectively, in these two setups (Figure 3.2). However, in cultures with only fully complexed Fe(II)-OM complexes, there was no nitrite accumulation in both cases with either low or high cell numbers (Figure 3.2 and Figure S3.2). The presence of nitrite paralleled the observed trends in Fe(II) oxidation: nitrite accumulated only in the BoFeN1 cultures where there was relatively fast Fe(II) oxidation, and did not accumulate in the cultures where Fe(II) oxidation rates were relatively slow.

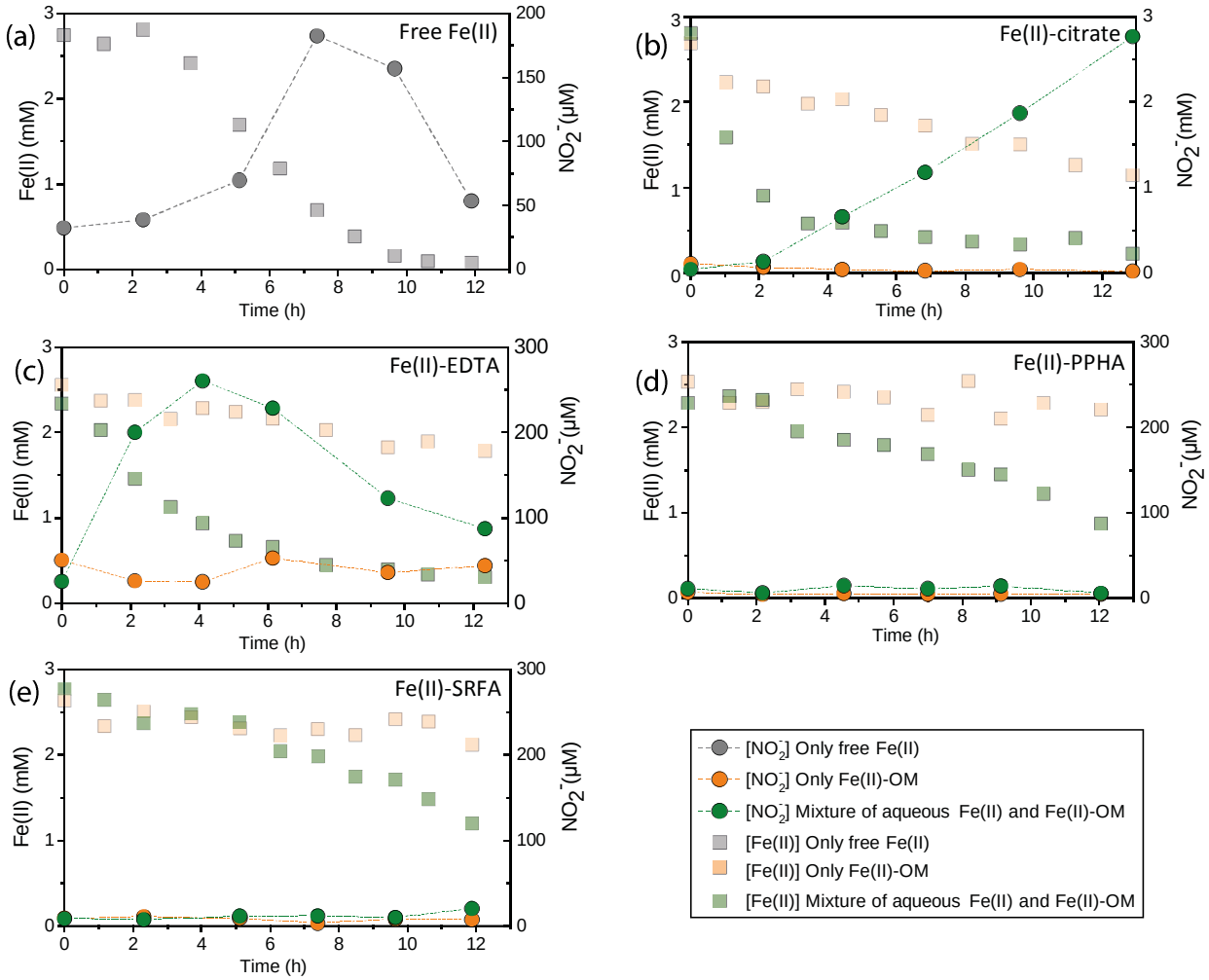

Figure 3.2 Concentrations of nitrite (NO_2^-, circles, dashed lines) and total Fe(II) (squares) over time in the presence of *Acidovorax* sp. strain BoFeN1 with free Fe(II) (a) and fully complexed Fe(II)-OM complexes and mixtures of free Fe(II) and different Fe(II)-OM complexes (b-e) for Fe(II)-citrate (b), Fe(II)-EDTA (c), Fe(II)-PPHA (d), and Fe(II)-SRFA (e). Since duplicate microbiological setups showed very similar results, representative experiments out of these duplicate setups are shown (the second one is shown in the supporting information, Figure S3.6).

Abiotic Fe(II) oxidation with nitrite

To evaluate the influence of OM on the chemical oxidation of Fe(II) by nitrite, we followed abiotic oxidation of Fe(II) in the presence of 2 mM nitrite with either Fe(II)-OM complexes, free Fe(II) or Fe(II)-OM/free-Fe(II) mixtures (Figure 3.3). We found that setups with simple Fe(II)-OM complexes, such as Fe(II)-citrate and Fe(II)-EDTA, independent of

whether there was free Fe(II) or not, showed much faster abiotic Fe(II) oxidation rates than setups with only free Fe(II) (Figure 3.3). Almost no free Fe(II) was oxidized by nitrite within 3 days of incubation. This result contradicts the result of the microbial Fe(II) oxidation experiment with *Acidovorax* sp. BoFeN1, acetate and nitrate, where free Fe(II) not only could be oxidized but also had even faster oxidation rates than fully complexed Fe(II)-citrate and Fe(II)-EDTA complexes (Figure 3.1). In setups with either Fe(II)-PPHA or Fe(II)-SRFA, both in the presence or absence of free Fe(II), almost no Fe(II) was oxidized abiotically by nitrite. Unlike Fe(II) oxidation in the presence of *Acidovorax* sp. BoFeN1, which started earlier and was faster when setups contained both free Fe(II) and Fe(II)-OM complexes compared to Fe(II)-OM complexes only (Figure 3.1), we did not observe the same stimulating effect for the chemical oxidation of Fe(II)-OM complex by nitrite. No matter whether there was free Fe(II) or not, samples with the same type of OM were both oxidized abiotically at similar rates. Although the setup with a mixture of free Fe(II) and Fe(II)-citrate initially showed a faster Fe(II) oxidation rate (ca. 0.19 mM/h) than the setup with the Fe(II)-citrate complex only (ca. 0.06 mM/h) (Figure 3.3), the Fe(II) oxidation rate decreased after the first day to a value of ca. 0.08 mM/h. Approximately 2.3 and 2.0 mM Fe(II) were oxidized within of 1 day of incubation with nitrite in the setups with Fe(II)-citrate only and with the mixture of free Fe(II) and Fe(II)-citrate while 0.9 and 0.7 mM Fe(II) were oxidized within of 1 day of incubation with nitrite in the setups with Fe(II)-EDTA only and with the mixture of free Fe(II) and Fe(II)-EDTA. The remaining Fe(II) concentration after 2 ½ days of incubation was similar in the setups with the same type of OM.

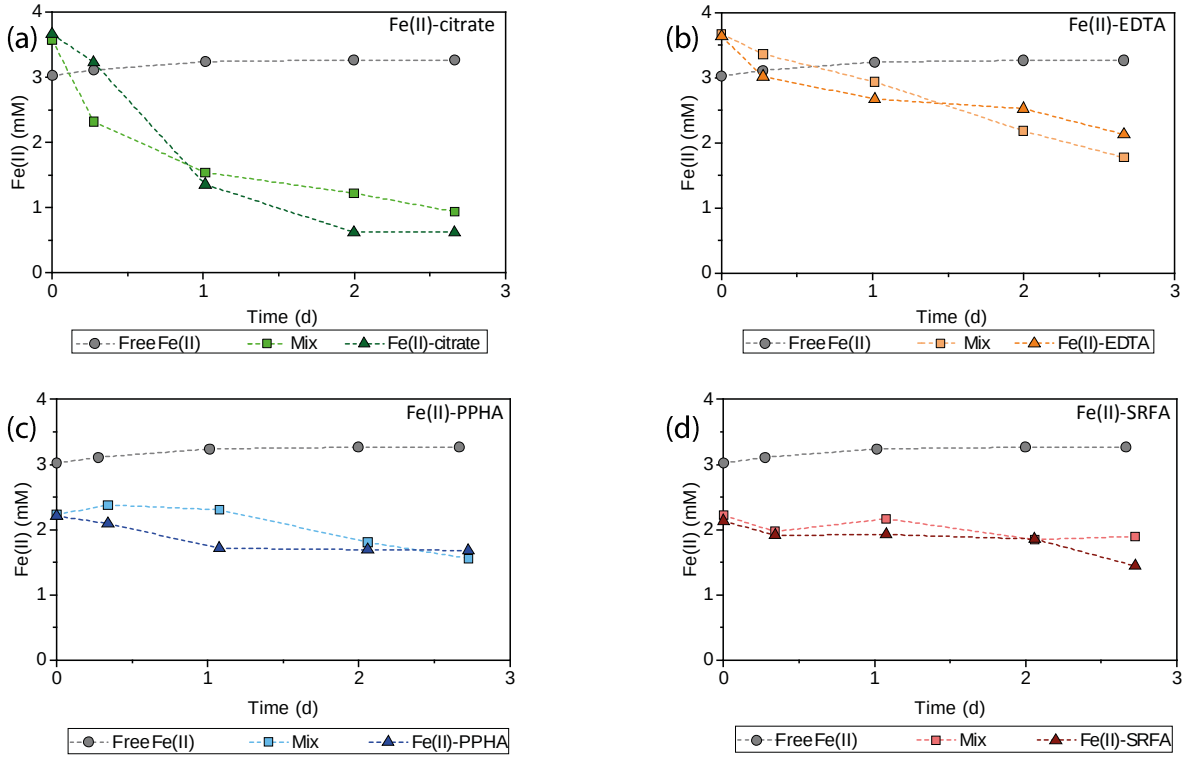

Figure 3.3 Abiotic Oxidation of Fe(II) by 2 mM nitrite. The Fe(II) was present in the form of either free (non-OM-complexed) Fe(II) (grey circles), different fully-complexed Fe(II)-OM complexes (triangles), or a mixture of free Fe(II) and Fe(II)-OM complexes (squares). Data is shown for Fe(II)-citrate (a), Fe(II)-EDTA (b), Fe(II)-PPHA complexes (c) and Fe(II)-SRFA complexes (d). Since duplicate microbiological setups showed very similar results, representative experiments out of these duplicate setups are shown (the second one is shown in the supporting information, Figure S3.7), and the data are shown as averages of triplicate spectrophotometric measurements.

3.5 Discussion

Abiotic and biotic oxidation of Fe(II) in the presence of organic matter

Fe(II) complexation by OM has been suggested to influence the rate of abiotic Fe(II) oxidation (Rose & Waite, 2003, Kopf *et al.*, 2013, Daugherty *et al.*, 2017, Lee *et al.*, 2017). Indeed, our experimental results showed that Fe(II) complexation by citrate and EDTA promoted chemical Fe(II) oxidation by nitrate (Figure 3.3), while Fe(II) complexation by PPHA and SRFA dramatically slowed down abiotic Fe(II) oxidation. This agrees with a previous study

that observed an increase in abiotic Fe(II) oxidation rates by nitrite following OM complexation by citrate (Kopf *et al.*, 2013). Different rates and extent of abiotic Fe(II) oxidation in the presence of citrate/EDTA, could be either a result of a change in the rate constants *k* of Fe(II) oxidation for different reaction mechanisms (Kopf *et al.*, 2013) or a change in the thermodynamics, as Fe(III)-citrate/-EDTA complexes are water-soluble and more stable than Fe(II)-citrate/-EDTA complexes at pH 7 (Martell & Smith, 1974). In contrast, the formation of Fe(II)-EDTA-NO complexes via the reaction of Fe(II)-EDTA and nitrite may slow down the oxidation of Fe(II)-EDTA complexes (Jin *et al.*, 2005). Both PPHA and SRFA did not promote the abiotic oxidation of Fe(II) by nitrite, this could be a result of i) adsorption of PPHA and SRFA onto the surface of Fe(III) minerals such as goethite (Klueglein & Kappler, 2013) which inhibits the autocatalysis of Fe(II) oxidation or ii) the ability of HA and FA to reduce and thus consume nitrite (Stevenson & Swaby, 1964, Stevenson *et al.*, 1970, Zhu-Barker *et al.*, 2015).

In contrast to the abiotic Fe(II) oxidation experiments, all Fe(II)-OM complexes studied here showed a slower Fe(II) oxidation compared to free Fe(II) in the presence of strain BoFeN1, acetate and nitrate (Figure 3.1) – conditions under which nitrite is known to be formed by BoFeN1 that then abiotically reacts with the free Fe(II) (Klueglein & Kappler, 2013). This suggests that Fe(II)-OM complexation inhibits Fe(II) oxidation by the mixotrophic nitrate-reducer *Acidovorax* sp. strain BoFeN1. In the following sections we will evaluate possible mechanisms that lead to the observed effects of OM on Fe(II) oxidation by strain BoFeN1.

Effect of Fe(II) complexation by OM on Fe(II) oxidation

Since the OM did not significantly change nitrate reduction by BoFeN1 (Figure S3.3), i.e. the OM is not toxic to the cells (see supporting information), there must be other mechanisms by which the presence of OM influences Fe(II) oxidation by BoFeN1. OM-complexation of Fe(II) changes the thermodynamics (i.e. the redox potentials) as well as the kinetics of abiotic Fe(II)/Fe(III) redox reactions (Straub *et al.*, 2001, Weber *et al.*, 2006, Kopf *et al.*, 2013). However, solely abiotic Fe(II)/Fe(III) redox reactions cannot fully explain why, in the absence of free Fe(II), the rates of abiotic oxidation of Fe(II) by nitrite were in disagreement with the oxidation of Fe(II) in the presence of the nitrite-producing *Acidovorax*

sp. strain BoFeN1. In particular it was unclear why nitrite oxidized Fe(II)-EDTA and Fe(II)-citrate complexes abiotically faster than free Fe(II), while in the presence of strain BoFeN1 these complexes were oxidized slower than free Fe(II) (Figure 3.1, Figure 3.3). Although with *Acidovorax* sp. BoFeN1, a large extent, if not all, of oxidation of free Fe(II) was shown to be abiotically caused by nitrite formed during nitrate reduction (Carlson *et al.*, 2013, Klueglein *et al.*, 2014), Fe(II)-OM complexation has been recognizedS to have promoted Fe(II) oxidation with other NRFeOx bacteria before (Miot *et al.*, 2009, Chakraborty *et al.*, 2011, Kopf *et al.*, 2013). The Fe(III)-stabilizing ligands can obviously not only promote the rates of abiotic Fe(II) oxidation by nitrite by changing the species-specific rate constants (Kopf *et al.*, 2013), but could also help to maintain the activity of microorganisms by preventing the formation of cell encrustation via the formation of Fe(III)-OM complexes (Miot *et al.*, 2009, Chakraborty *et al.*, 2011, Liao *et al.*, 2017).

An additional, and probably the most important, point that has to be considered in the case of BoFeN1 cells is the location of nitrite production and the location of Fe(II) oxidation. In previous BoFeN1 studies, it was shown that Fe(II) was initially oxidized in the periplasm (Miot *et al.*, 2009, Schmid *et al.*, 2014). Therefore it has been suggested that Fe(II) has to cross the outer membrane for oxidation to occur (Carlson *et al.*, 2013). Specifically, Fe(II) must pass through negatively charged cell pores, i.e. the porins (French *et al.*, 2013). However, Fe(II)-OM complexation is expected to change the charge of the Fe(II) ions from positive towards neutral and negative (Grinberg, 2013), therefore, complexation could hamper Fe(II) from entering the periplasm (Figure 3.4b). As a result, the interaction between Fe(II) and periplasmic components, such as nitrite reductase enzymes, could be inhibited (Madigan *et al.*, 2010). In addition to the changes in the charge of the Fe(II)-species, Fe(II)-OM complexation also changes the size of Fe(II). Without OM, the hydrated free Fe(II) has a radius of ca. 0.21 nm (2.1 Å) (Marcus, 1988) which is much smaller than the size of Fe(II)-OM complexes. The tridentate Fe(II)-citrate and polydentate Fe(II)-EDTA complexes are approximately 2-3 times larger than the free Fe(II), the Fe(II)-PPHA and Fe(II)-SRFA complexes would be even larger, as they are expected to be in the colloidal size fraction (1-200 nm) due to the coagulation of HA particles (Liao *et al.*, 2017). This coagulation of HA could further enhance electrostatic and/or steric repulsion (Liao *et al.*, 2017), and hamper the diffusion of negatively charged Fe(II)-OM complexes through the negatively charged porin channels. In

our experiment with a C:Fe molar ratio of 35, more than 98% of the Fe(II)-OM complexes were larger (>3 kDa, i.e. larger than approx. 10 Å) than free Fe(II), as determined by ultracentrifugation (Amicon Ultra, Millipore, data not shown). Additionally, previous studies showed that the content of Fe(II) in the large size fraction increased with C:Fe ratios (Liao *et al.*, 2017). Even with a lower C:Fe ratio (ca. 23) than we used (ca. 35), more than 85% of the Fe(II)-OM complexes had sizes in the 3-200 nm range (Liao *et al.*, 2017). Pore-forming proteins (porins) provide channels only about 1-2 nm in size (Brunen *et al.*, 1991). Therefore, the cell's outer-membrane could be a potential barrier for the transportation of Fe(II)-PPHA and Fe(II)-SRFA colloids into the BoFeN1 periplasm, where nitrite forms and is essential to initiate extensive NRFeOx (Madigan *et al.*, 2010, Carlson *et al.*, 2013). Taken together, the change of charge and size of the Fe(II) ion induced by complexation, in particular the formation of Fe(II)-colloids, could potentially inhibit the passage of the Fe(II)-OM complexes into the periplasm. With no free Fe(II) in the periplasm, nitrite does not accumulate, and fast Fe(II) oxidation does not occur (Figure 3.2; Figure 3.4b). However, when free Fe(II) is provided alongside the Fe(II)-OM complexes, free Fe(II) can enter the periplasm, where it promotes nitrite accumulation and causes the oxidation of Fe(II)-OM complexes outside of the cells (Figure 3.4c). The oxidation of Fe(II) also leads to changes of Fe(II) speciation over the Fe(II) oxidation process. The Fe(III)-containing oxidation products also form complexes with OM and thus can release free Fe(II) from Fe(II)-OM complexes; the released free Fe(II) could therefore lead to an even higher extent of Fe(II) oxidation.

Another interesting observation was the fact that the presence of OM ligands in addition to free Fe(II) also resulted in a shortened lag-phase for Fe(II) oxidation by BoFeN1. We believe that this is due to the faster abiotic nitrite reduction by Fe(II) (leading to Fe(II) oxidation) stimulated by the organic matter (citrate and EDTA) (Figure 3.3), while in the absence of OM, nitrite has to accumulate to a higher concentration to oxidize free Fe(II).

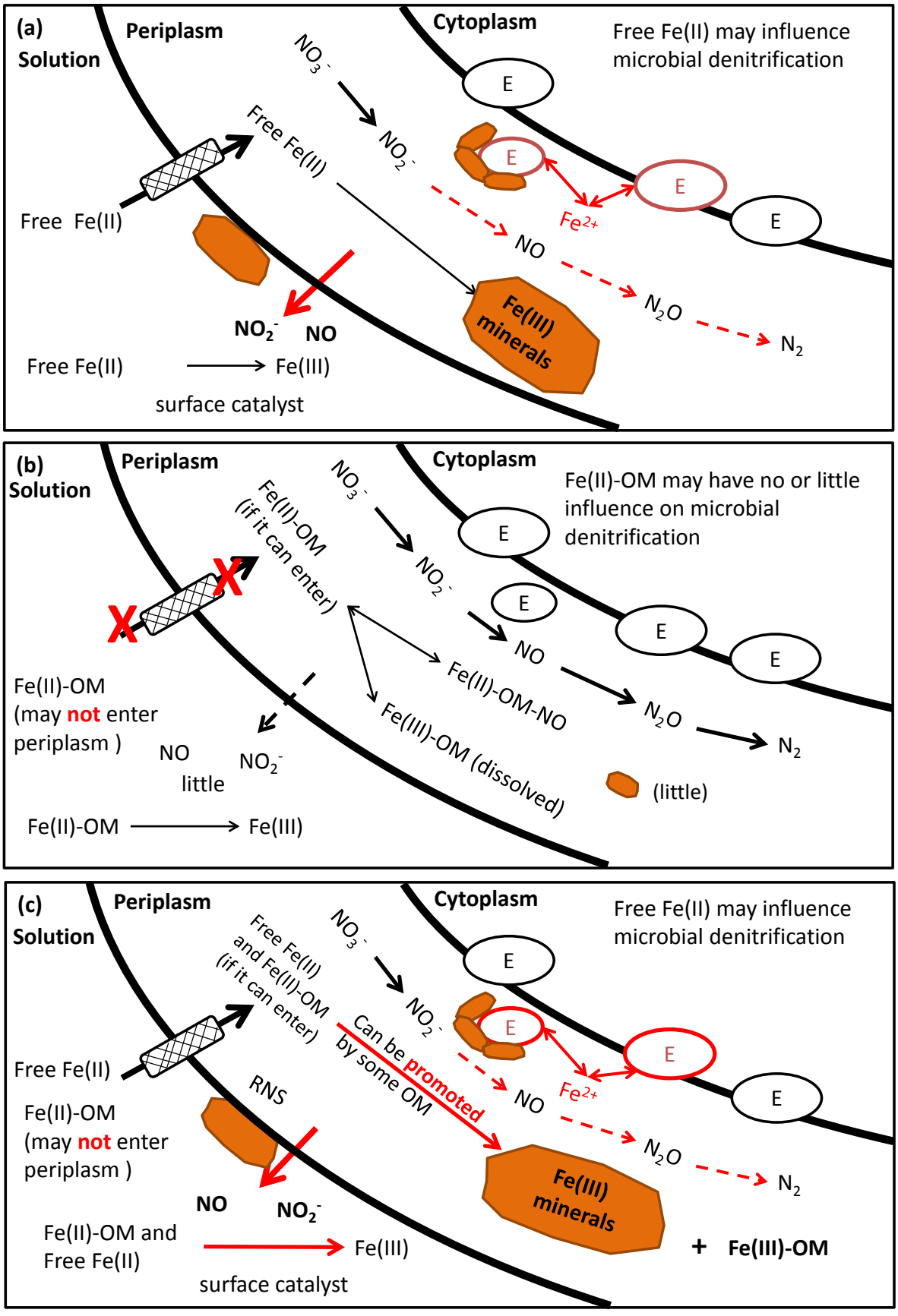

Figure 3.4 Proposed mechanism of Fe(II) oxidation by *Acidovorax* sp. strain BoFeN1 in samples with only free Fe(II) (a), only Fe(II)-OM complexes (b), and a mixture of free Fe(II)

and Fe(II)-OM complexes (c). For simplicity, only the few relevant enzymes are shown. (a) Free Fe(II) is transported into the periplasm and potentially interacts with microbial denitrification enzymes stimulating the accumulation of reactive nitrogen species (RNS) such as NO_2^- and NO resulting in Fe(II) oxidation in the periplasm and outside of the cells. (b) In the presence of only Fe(II)-OM complexes, Fe(II)-OM complexes may either not be transported into the periplasm (because of their large size and their negative charge, etc.), or they have little influence on microbial denitrification enzymes leading to no nitrite accumulation. Therefore, *Acidovorax* sp. strain BoFeN1 further reduces NO_2^- to N_2 preventing Fe(II)-OM oxidation. (c) When both free Fe(II) and Fe(II)-OM complexes are present, the accumulated RNS produced by the interaction of free Fe(II) with the microbial denitrification enzymes stimulate Fe(II) oxidation as nitrite could oxidize both Fe(II)-OM and free Fe(II).

The importance of free Fe(II) for nitrite accumulation

During denitrification, nitrite accumulates due to a slower rate of nitrite reduction compared to nitrate reduction (Betlach & Tiedje, 1981). Interestingly, even though abiotic Fe(II) oxidation by nitrite consumes nitrite and could in theory lead to a lower nitrite concentration, nitrite accumulated in our experiments only when there was fast Fe(II) oxidation (Figure 3.2, Figure S3.2, Figure S3.6). The occurrence of simultaneous Fe(II) oxidation and nitrite accumulation only when free Fe(II) was present, could be explained by the interaction of free Fe(II) with enzymatic components in the periplasm (Carlson *et al.*, 2013). This interaction could lead to slower microbial nitrite reduction rates. Mechanisms that may explain how Fe(II) can influence the enzymatic machinery in the periplasm include, but are not limited to i) disruption of protein stability, ii) replacement of active-site metal cofactors (Gurd & Wilcox, 1956), and iii) precipitation of poorly soluble Fe(III) minerals on cellular components such as NO_2^- and NO reductase enzymes. The extent of these effects may also be different with different types and percentages of Fe(II)-OM, as a result of different location and amount of Fe(III) mineral precipitation. As different Fe(III)-OM complexes have different stability constants (Martell & Smith, 1974), this could lead to different amounts of Fe(III) precipitates depending on whether the present OM is sufficient to form dissolved Fe(III)-OM complexes or not. In particular, mineral precipitation at the

periplasmic nitrite reductase (Madigan *et al.*, 2010), would decrease nitrite reductase enzyme activity and could thus lead to an accumulation of nitrite (Miot *et al.*, 2009, Schädler *et al.*, 2009, Carlson *et al.*, 2013, Klueglein *et al.*, 2014). As the nitrate reductase is an inner membrane protein (Madigan *et al.*, 2010), nitrate reduction probably is influenced to a lesser extent by mineral precipitation in the periplasm than the nitrite reductase and thus nitrite reduction. The accumulation of nitrite would then favor the abiotic oxidation of Fe(II) by nitrite and cause precipitation of more Fe(III) minerals (Figure 3.4a, 3.4c) result in an even stronger inhibition of microbial nitrite reduction explaining the accumulation of nitrite in the presence of free Fe(II) (Figure 3.2, Figure. S3.2).

Although our abiotic Fe(II) oxidation experiments showed that oxidation of free Fe(II) by 2 mM of nitrite was very slow at pH 7 (Figure 3.3), this reaction could be much faster in the periplasm because in the periplasm, the pH and the concentration of nitrite could be very different from what we measured outside the cells (Wilks & Slonczewski, 2007). During microbial denitrification, protons are translocated from the cytoplasm into the periplasm by the NADH dehydrogenase (complex I), bc1 complex (complex III), and the cytoplasmic nitrate reductase (Chen & Strous, 2013), potentially creating a low-pH hotspot in the periplasm favoring oxidation of free Fe(II) by nitrite.

In contrast to the experiments where free Fe(II) was present, all experiments with fully complexed Fe(II)-OM (no free Fe(II)) showed no nitrite accumulation. This could be due to the lack of free Fe(II) in the periplasm to initiate Fe(II) oxidation. The absence of Fe(III) mineral precipitation would result in no encrustation and no inhibition of periplasmic enzymatic components. As a consequence, the microorganisms themselves could have reduced the nitrite further, preventing nitrite accumulation (Figure 3.4b). This suggests that although some Fe(II)-OM complexes react abiotically faster with nitrite than free Fe(II), free Fe(II) rather than the Fe(II)-OM complexes plays the key role in causing Fe(II) oxidation coupled to denitrification. This is because the free Fe(II) causes nitrite accumulation in the periplasm. However, when there are both free Fe(II) and Fe(II)-OM complexes present, the nitrite accumulation caused by free Fe(II) can then stimulate oxidation of Fe(II)-OM in the presence of *Acidovorax* sp. BoFeN1 (Figure 3.2, Figure 3.4c). For instance, rapid Fe(II) oxidation was observed for the Fe(II)-citrate and Fe(II)-EDTA complexes, as they can react with nitrite abiotically already at low nitrite concentration and have fast rates of abiotic Fe(II)

oxidation by nitrite (Figure 3.3).

Implications for Fe(II) oxidation the environment

This study suggests that Fe(II) oxidation by mixotrophic nitrate-reducers in the environment strongly depends on Fe(II) speciation, specifically on the content of free Fe(II) and Fe(II)-NOM complexes. Such complexes have been identified in soils, rivers and sediments (Luther *et al.*, 1996, Kleja *et al.*, 2012, Sundman *et al.*, 2013, Sundman *et al.*, 2014, von der Heyden *et al.*, 2014, Hopwood *et al.*, 2015, Bhattacharyya *et al.*, 2018). In our study, the highest molar ratio of dissolved organic carbon to Fe(II) (DOC:Fe(II)) was 35 which is higher than the ratio which allows full Fe(II) complexation by OM according to the Fe(II)-OM binding model (Tipping *et al.*, 2011, Catrouillet *et al.*, 2014). Although the Fe(II) speciation depends also on other parameters such as the absolut Fe(II) and DOC concentrations, Fe(II)-HA colloids have been shown to form even at very low C:Fe molar ratios (e.g. 0.2) and low OM concentrations (e.g. 1 mg/L, ca. 80-90 µM carbon) (Liao *et al.*, 2017). In real environments such as the pore water of the freshwater sediment from which *Acidovorax* sp. strain BoFeN1 was isolated (Melton *et al.*, 2012), the DOC:Fe(II) ratio can be as high as 40, and the ratios can be even higher in soils (Vogel *et al.*, 2014). The higher DOC:Fe(II) ratios in the environment suggest that most Fe(II) is present as OM-complexes/colloids, thus inhibiting direct oxidation by mixotrophic NRFeOx microorganisms. In contrast, in environments with lower DOC:Fe(II) ratios, such as DOC:Fe(II) ratios of 5-16 as recently studied in marine coastal sediments (Laufer *et al.*, 2016), not all Fe(II) is expected to be complexed and nitrate-dependent oxidation of free Fe(II) may take place and contribute to Fe(II) oxidation. Interestingly, these authors also reported that the ratio of $nitrate_{reduced}$ to $Fe(II)_{oxidized}$ changed with the OM content of the sediment (Laufer *et al.*, 2016). Potential reasons for this could be the effect of Fe(II)-OM complexation on the rates of Fe(II) oxidation by NRFeOx (as shown in our study) or on the products of abiotic vs. biotic nitrite reduction (the abiotic reaction of Fe(II) with nitrite is expected to lead to N_2O while biological reduction of nitrite is expected to lead to N_2).

Because mixotrophic nitrate-reducing Fe(II)-oxidizers have been found in various environments (Gallus & Schink, 1994, Straub *et al.*, 1996, Buchholz-Cleven *et al.*, 1997, Achenbach *et al.*, 2001, Lack *et al.*, 2002, Kappler *et al.*, 2005, Senko *et al.*, 2005, Strous *et al.*,

2006, Handley *et al.*, 2009, Byrne-Bailey *et al.*, 2010, Chakraborty *et al.*, 2011, Coby *et al.*, 2011, Shelobolina *et al.*, 2012, Sorokina *et al.*, 2012, Benzine *et al.*, 2013, Oshiki *et al.*, 2013, Park *et al.*, 2014, Hu *et al.*, 2017), the effect of Fe(II)-OM complexation on mixotrophic nitrate-reducing Fe(II)-oxidizing bacteria can potentially also influence the global iron biogeochemical cycle. Additionally, our results also suggest a link between the microbial iron and the nitrogen cycles, as the accumulation of nitrite, a toxic reactive nitrogen species (Fan & Steinberg, 1996), depends on whether Fe(II) is available in its free Fe(II) form or is complexed by OM (Figure 3.2, Figure S3.2). As a consequence, the accumulation of nitrite could further cause the formation of the important greenhouse gas N_2O by the reaction of Fe(II) with nitrite (Jones *et al.*, 2014, Buchwald *et al.*, 2016).

3.6 Supporting information

Minor toxic effect of OM on BoFeN1 nitrate reduction

It is necessary to evaluate a potential harmful influence of the added OM-species (the OM-ligands) on the BoFeN1 metabolism. In our cell suspension experiments, the added nitrate can only be consumed by *Acidovorax* sp. BoFeN1 cells, as Fe(II) does not react abiotically with nitrate without a catalyst (Hansen *et al.*, 1996, Ottley *et al.*, 1997, Hansen *et al.*, 2001). We found that although the Fe(II) oxidation rates were different with and without free Fe(II) and OM (Figure 3.1, Table S3.4), the nitrate (NO_3^-) consumption (reduction) in all samples containing acetate and either free Fe(II), Fe(II)-OM complexes or both, were not as significantly different as the Fe(II) oxidation rates (Figure 3.1, Figure S3.3). In all the cases, although there were slight variations, there were about 4-5 mM $NaNO_3$ consumed during 12 hour incubation. This clearly suggests that the presence of OM did not impair or stimulate the reduction of nitrate using electrons from NADH that stems from acetate oxidation. Additionally, even if the OM would be toxic to a small extent, the OM alone did not influence the Fe(II) oxidation by strain BoFeN1. In setups with the same amount of OM ligands (1.5 mM EDTA), samples containing a mixture of both free Fe(II) and Fe(II)-EDTA had much faster Fe(II) oxidation rates than samples with Fe(II)-EDTA only (i.e. without free Fe(II)) (Figure S3.4), similar to the result in Figure 3.1 with a similar amount of Fe(II) and different amounts of OM. These data suggest that OM itself has no (or, at most, only a minor) toxic effect on strain BoFeN1.

Table S3.1 Speciation of Fe(II) in experiments with different Fe(II) complexes and free Fe(II) in the basal non-growth medium used in this study. The medium contains different Fe(II) sources, PIPES buffer, NaCl, $NaNO_3$ and sodium acetate. The speciation was determined by PHREEQC with the minteq.v4 database and a previously published model for Fe(II) binding to humic and fulvic acids (Catrouillet *et al.*, 2014).

Table S3.1a: presence of only Free Fe(II) in the medium.

Fe(II) species	Percentage %
Fe^{2+}	99.82
$FeOH^{+}$	0.18
$Fe(OH)_2$	<0.01
$Fe(OH)_3^{-}$	<0.01

Table S3.1b: presence of Fe(II)-citrate in the medium.

Fe(II) species	Percentage %
$Fe(II)citrate^{-}$	99.47
FeH(citrate)	0.10
Fe^{2+}	0.42
$Fe(OH)^{+}$	<0.01
$Fe(OH)_2$	<0.01
$Fe(OH)_3^{-}$	<0.01

Table S3.1c: presence of Fe(II)-EDTA in the medium.

Fe(II) species	Percentage %
$Fe(EDTA)^{2-}$	98.90
$FeOH(EDTA)^{3-}$	1.08
$FeH(EDTA)^{-}$	0.01
$Fe(OH)_2(EDTA)^{4-}$	0.00
Fe^{2+}	<0.01
$FeOH^{+}$	<0.01
$Fe(OH)_2$	<0.01
$Fe(OH)_3^{-}$	<0.01

Table S3.1d: presence of Fe(II)-PPHA in the medium.

Fe(II) species	Percentage %
Fe(II)-PPHA	99.59
Fe^{2+}	0.41
$FeOH^{+}$	<0.01
$Fe(OH)_2$	<0.01
$Fe(OH)_3^{-}$	<0.01

Table S3.1e: presence of Fe(II)-SRFA in the medium.

Fe(II) species	Percentage %
Fe(II)-SRFA	99.77
Fe^{2+}	0.23
$FeOH^{+}$	<0.01
$Fe(OH)_2$	<0.01
$Fe(OH)_3^{-}$	<0.01

Table S3.1f: presence of a mixture of free Fe(II) and Fe(II)-citrate in the medium.

Fe(II) species	Percentage %
$Fe(II)citrate^{-}$	65.79
FeH(citrate)	0.06
Fe^{2+}	34.01
$FeOH^{+}$	0.06
$Fe(OH)_2$	<0.01
$Fe(OH)_3^{-}$	<0.01

Table S3.1g: presence of a mixture of free Fe(II) and Fe(II)-EDTA in the medium.

Fe(II) species	Percentage %
$Fe(EDTA)^{2-}$	39.56
$FeOH(EDTA)^{3-}$	0.44
$FeH(EDTA)^{-}$	<0.01
$Fe(OH)2(EDTA)^{4-}$	<0.01
Fe^{2+}	59.91
$FeOH^{+}$	0.10
$Fe(OH)_2$	<0.01
$Fe(OH)_3^{-}$	<0.01

Table S3.1h: presence of a mixture of free Fe(II) and Fe(II)-PPHA in the medium.

Fe(II) species	Percentage %
Fe(II)-PPHA	37.10
Fe^{2+}	62.90
$FeOH^{+}$	0.11
$Fe(OH)_2$	<0.01
$Fe(OH)_3^{-}$	<0.01

Table S3.1i: presence of a mixture of free Fe(II) and Fe(II)-SRFA in the medium.

Fe(II) species	Percentage %
Fe(II)-SRFA	48.07
Fe^{2+}	51.93
$FeOH^{+}$	0.09
$Fe(OH)_2$	<0.01
$Fe(OH)_3^{-}$	<0.01

Table S3.2. Medium composition (Emerson & Merrill Floyd, 2005) for cultivation of *Acidovorax* sp. BoFeN1.

NH_4Cl	5.61 mM
KH_2PO_4	4.41 mM
$CaCl_2 \cdot 2H_2O$	0.68 mM
$MgSO_4 \cdot 7H_2O$	2.0 mM
$NaHCO_3$	22 mM
Trace elements:	
$FeSO_4$ * 7 H_2O	4.0 nM
$CoCl_2$ * 6 H_2O	0.80 nM
$ZnCl_2$	0.31 nM
$NiCl_2$ * 6 H_2O	0.10 nM
Na_2MoO_4 * 2 H_2O	0.074 nM
H_3BO_4	4.8 nM
$CuCl_2$ * 2 H_20	0.01 nM
MnCl2* 4 H20	0.25 nM
Vitamins:	
4 Aminobenzoic acid	0.29 µM
D (+) biotin	41 nM
Nicotinic acid	0.81 nM
Ca-(+) pantothenate	0.21 nM
Pyridoxamine dihydrochloride	0.41 nM
Thiaminium dichloride	0.29 nM
Riboflavin	1.32 nM

Table S3.3. Abundance of cells in the non-growth medium at the beginning of the cell suspension experiments, results are averaged from duplicate analyses.

Fe(II)-source	cells/ml
Fe(II)-citrate	5.48E+08
Fe(II)-EDTA	4.00E+08
Fe(II)-PPHA	6.82E+08
Fe(II)-SRFA	4.51E+08
Fe(II)-EDTA- in Figure S1	2.35E+08

Table S3.4. Maximum Fe(II) oxidation rates (mM/h) for free Fe(II) and for the four different Fe(II)-OM complexes (Tables S3.3a and S3.3b) as well as the ratios of Fe(II) oxidation rates for free Fe(II) and Fe(II)-OM complexes or the ratios of Fe(II) oxidation rates for free Fe(II) and mixtures of free Fe(II) and Fe(II)-OM complexes (Table S3.4c). Results of the two biological replicates are given in Tables S3.4a and S3.4b separately. Because of slightly different lag phases and reactive phases (periods where Fe(II) oxidation takes place), only a few data points were used in the calculation of the rates of Fe(II) oxidation. The reactive periods were distinguished from the lag phase from the time points after which Fe(II) was oxidized at rates similar to the rate obtained in the linear regressions, for details please refer to Table S3.4. The rate is the slope of the linear regression of the of Fe(II) concentrations calculated with the origin software. The errors given are the standard error of the regression slopes.

Table S3.4a

Citrate -exp.	Rate (mM/h)	EDTA-exp.	Rate (mM/h)	PPHA -exp.	Rate (mM/h)	SRFA -exp.	Rate (mM/h)
Free Fe(II)	0.24 ±0.02	Free Fe(II)	0.30 ±0.02	Free Fe(II)	0.31 ±0.007	Free Fe(II)	0.46 ±0.02
Fe(II)-citrate	0.11 ±0.01	Fe(II)-EDTA	0.06 ±0.06	Fe(II)-PPHA	<0.01 ±0.01	Fe(II)-SRFA	0.02 ±0.01
Mix (citrate)	0.90 ±0.16	Mix (EDTA)	0.36 ±0.03	Mix (PPHA)	0.13 ±0.01	Mix (SRFA)	0.15 ±0.01

Table S3.4b

Citrate-exp.	Rate (mM/h)	EDTA-exp.	Rate (mM/h)	PPHA-exp.	Rate (mM/h)	SRFA-exp.	Rate (mM/h)
Free Fe(II)	0.19 ±0.02	Free Fe(II)	0.30 ±0.03	Free Fe(II)	0.30 ±0.01	Free Fe(II)	0.46 ±0.02
Fe(II)-citrate	0.11 ±0.01	Fe(II-EDTA	0.08 ±0.01	Fe(II)-PPHA	<0.01 ±0.01	Fe(II)-SRFA	0.02 ±0.01
Mix (citrate)	0.68 ±0.11	Mix (EDTA)	0.37 ±0.04	Mix (PPHA)	0.12 ±0.01	Mix (SRFA)	0.15 ±0.02

Table S3.4c

Average Fe(II) oxidation rates in different experiments							
Citrate -exp.	Rate (mM/h)	EDTA-exp.	Rate (mM/h)	PPHA-exp.	Rate (mM/h)	SRFA-exp.	Rate (mM/h)
Free Fe(II)	0.21±0.02	Free Fe(II)	0.30±0	Free Fe(II)	0.30±0.01	Free Fe(II)	0.46±0
Mix(citrate)	0.11±0	Mix (EDTA)	0.07±0.01	Mix (PPHA)	NC	Mix (SRFA)	0.02±0
Fe(II)-citrate	0.79±0.11	Fe(II)-EDTA	0.36±0.05	Fe(II)-PPHA	0.12±0.01	Fe(II)-SRFA	0.15±0

Table S3.4d

Difference of average Fe(II) oxidation rates							
Free Fe(II): Fe(II)-citrate	2.0	Free Fe(II): Fe(II)-EDTA	4.3	Free Fe(II): Fe(II)-PPHA	NC	Free Fe(II): Fe(II)-SRFA	23
Mix(citrate): Free Fe(II)	3.7	Mix (EDTA): Free Fe(II)	1.2	Free Fe(II): Mix (PPHA)	2.4	Free Fe(II): Mix (SRFA)	3.0

±: standard error (table S3.4c), and standard error of slopes (table S3.4a, 3.4b)

NC: not calculated (one value <0.01 mM/h)

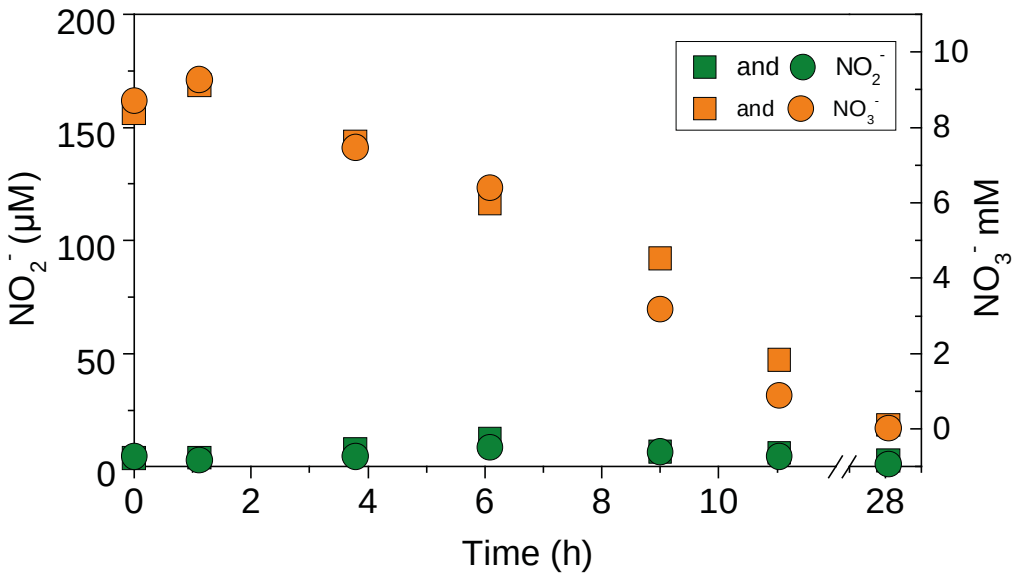

Figure S3.1 Concentrations of nitrite (NO_2^-, green squares and circles) and nitrate (NO_3^-, yellow squares and circles) in an experiment with *Acidovorax* sp. strain BoFeN1 incubated with acetate and nitrate (no Fe(II)). Symbols with the same color and different shapes (squares and circles) represent the two biological/incubation replicates.

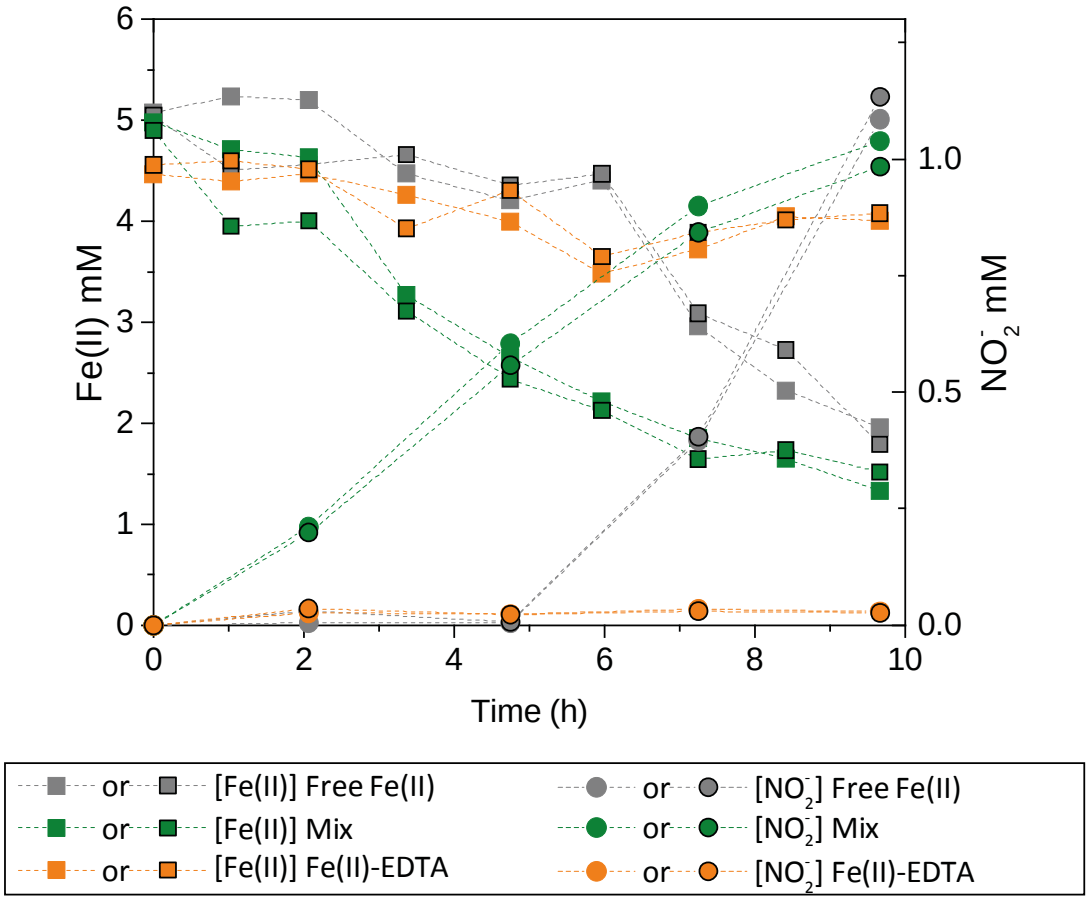

Figure S3.2 Concentrations of nitrite (NO_2^-, circles, dashed lines) and total Fe(II) (squares) over time in experiment with lower cell abundance (1.75E+8 cells/ml) of *Acidovorax* sp. strain BoFeN1. The different colors represent samples with different percentages of free Fe(II): grey: samples with only free Fe(II) without Fe(II)-EDTA, orange: samples with only Fe(II)-EDTA complex without free Fe(II), green: samples containing 60% free Fe(II) and 40% Fe(II)-EDTA complex. Symbols with and without black borderlines represent the two biological/incubation replicates. The two photos underneath show the color of the medium after 2 and 6 hours incubation. The color of the samples were initially the same (white-transparent), the color of the mixture sample turned brown earlier than the sample with free Fe(II) only. However, the sample with Fe(II)-EDTA did not show any orange color,

implying no oxidation of Fe(II). Samples with a mixture of free Fe(II) and Fe(II)-EDTA accumulated nitrite to a higher concentration than the samples with free Fe(II), however, samples with Fe(II)-EDTA showed no accumulation of nitrite.

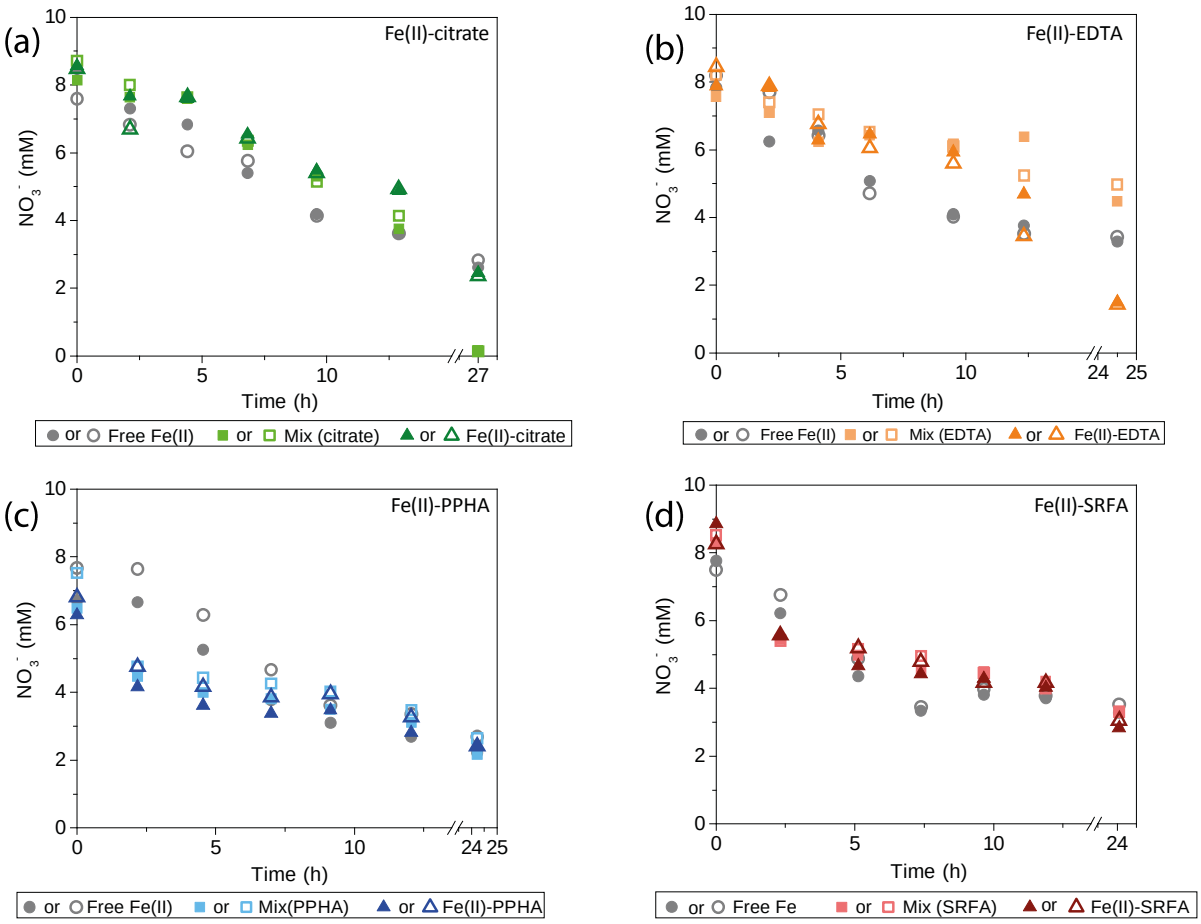

Figure S3.3 Concentrations of nitrite (NO_3^-) over time in the presence of *Acidovorax* sp. strain BoFeN1 with either free Fe(II) (circles), different fully-complexed Fe(II)-OM complexes (triangles), or a mixture of free Fe(II) and Fe(II)-OM complexes (squares). Open and closed circles represent two biological/incubation replicates. Fe(II)-OM complexes used were (a) Fe(II)-citrate, (b) Fe(II)-EDTA, (c) Fe(II)-PPHA, and (d) Fe(II)-SRFA complexes. Although there were variations in the nitrite concentration after the first 2 hours (c, d), and 1 day incubation (a, b), the consumption (reduction) rates of nitrate (NO_3^-) in all samples containing either free Fe(II), Fe(II)-OM complexes or both, were not significantly different. The details regarding the percentages of free Fe(II) and Fe(II)-OM complexes values can be found in the materials and methods.

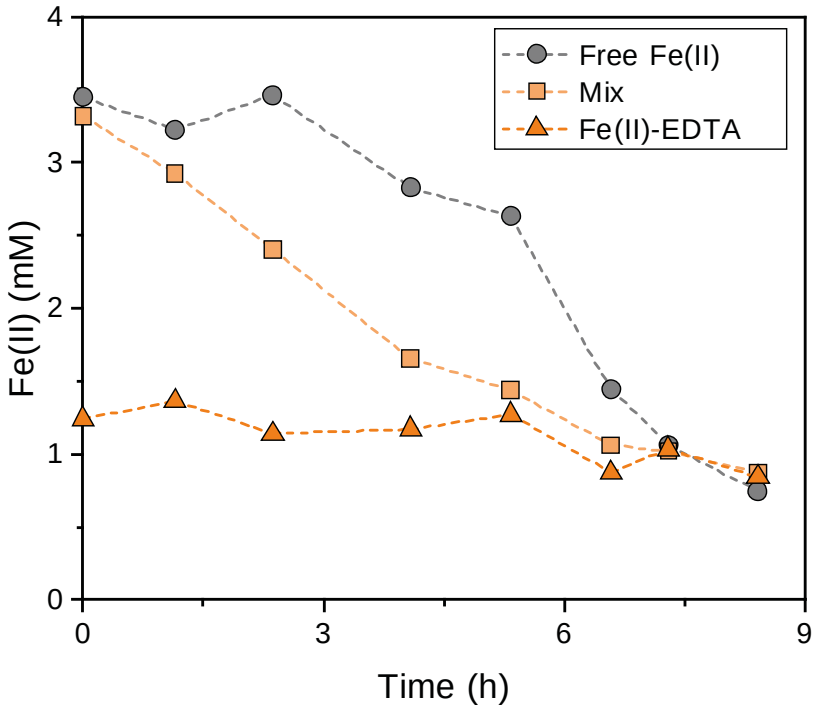

Figure S3.4 Oxidation of Fe(II) with free Fe(II) only (no EDTA, grey cycle), with Fe(II)-EDTA only including 1.5 mM EDTA (orange square), or a mixture of Fe(II)-EDTA and free Fe(II) including the same amount of 1.5 mM EDTA (yellow triangles).

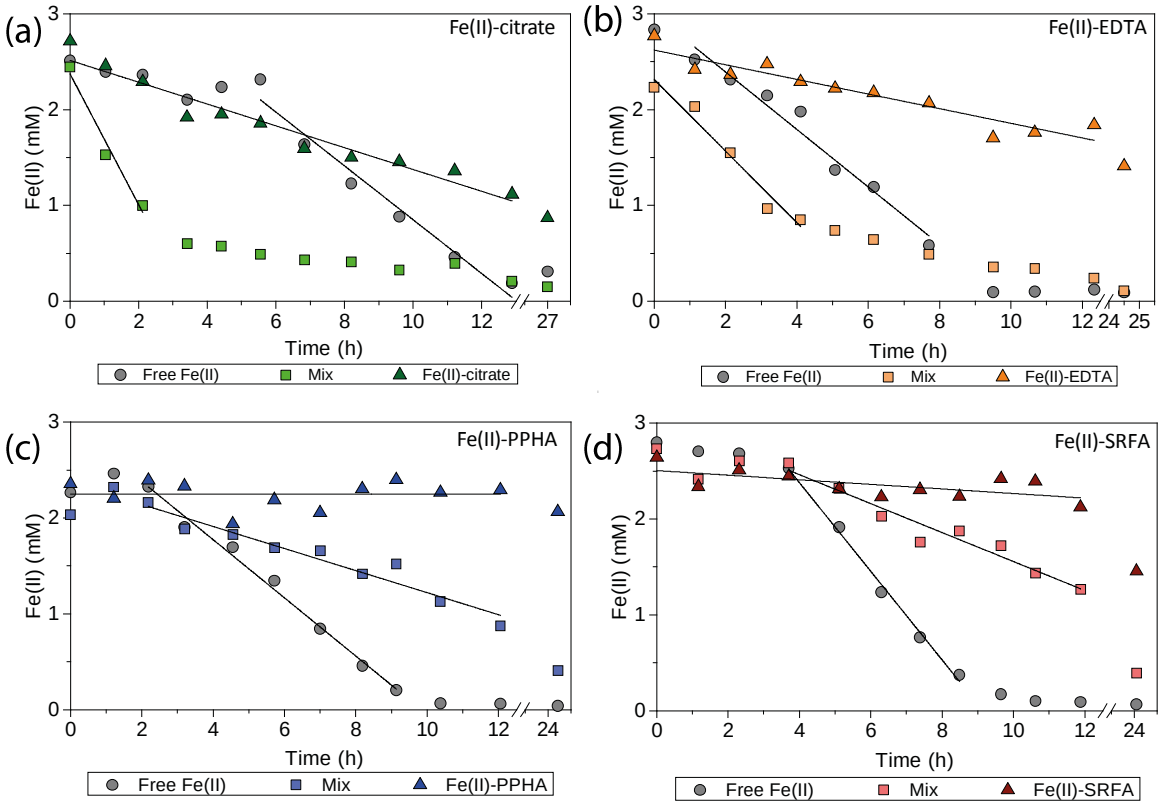

Figure S3.5 Oxidation of Fe(II) in the incubation/biological replicates in the presence of 10 mM nitrate, 5 mM acetate and *Acidovorax* sp. strain BoFeN1. The Fe(II) was present in the form of either free Fe(II) (circles), different fully-complexed Fe(II)-OM complexes (triangles), or a mixture of free Fe(II) and Fe(II)-OM complexes (squares). Data is shown for Fe(II)-citrate (a), Fe(II)-EDTA (b), Fe(II)-PPHA complexes (c) and Fe(II)-SRFA complexes (d). The solid black lines indicate the linear fits of Fe(II) concentrations for the calculation of Fe(II) oxidation rates shown in table S5.1b. The data are shown as averages of triplicate spectrophotometric measurements.

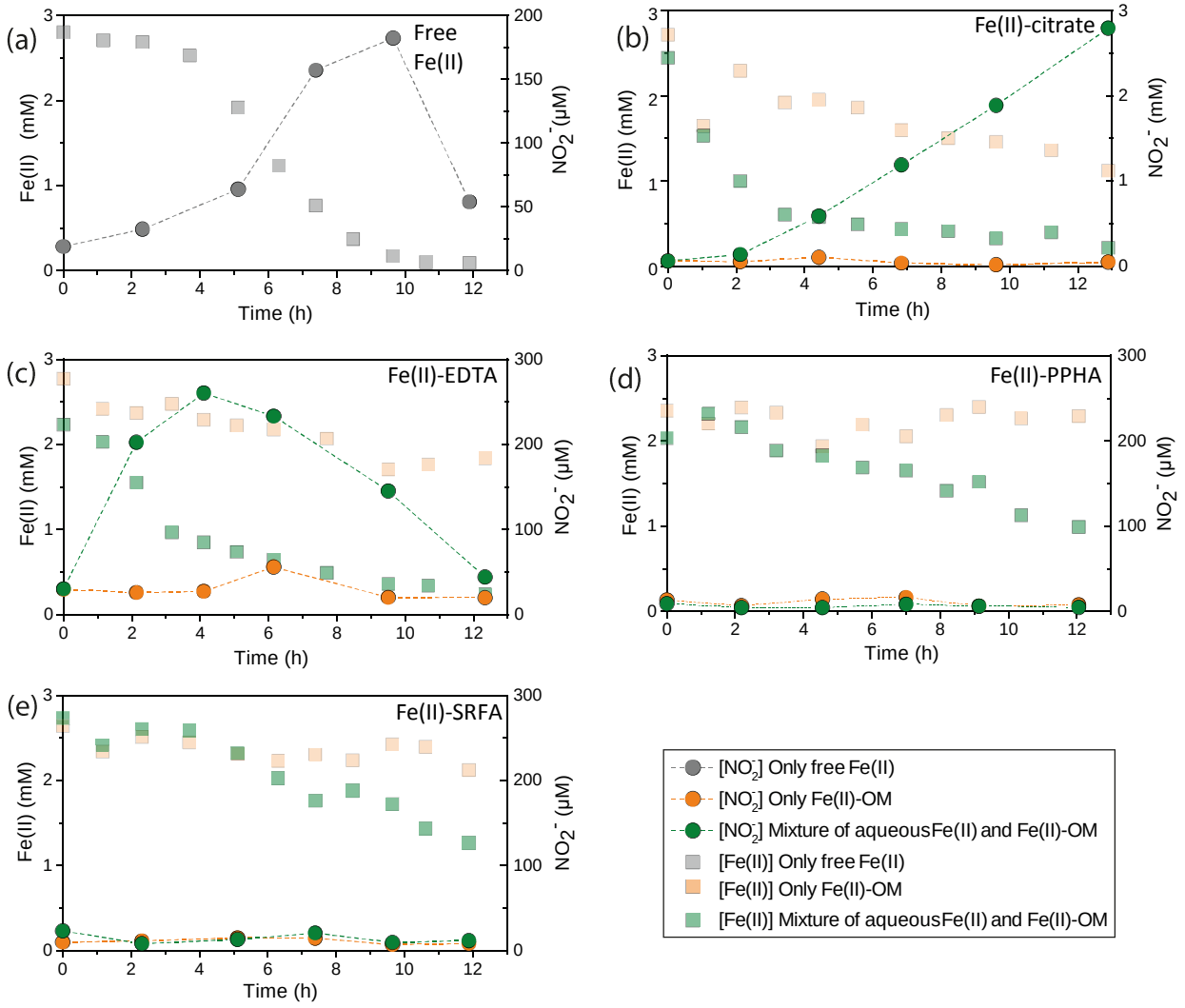

Figure S3.6 Concentrations of nitrite (NO_2^-, circles, dashed lines) and total Fe(II) (squares) in the incubation/biological replicates over time in the presence of *Acidovorax* sp. strain BoFeN1 with (a) only free Fe(II), (b) with Fe(II)-citrate, (c) Fe(II)-EDTA, (d) Fe(II)-PPHA, and (e) Fe(II)-SRFA (e) complexes, and mixtures of free Fe(II) and different Fe(II)-OM complexes. The details regarding these percentage values can be found in the materials and methods.

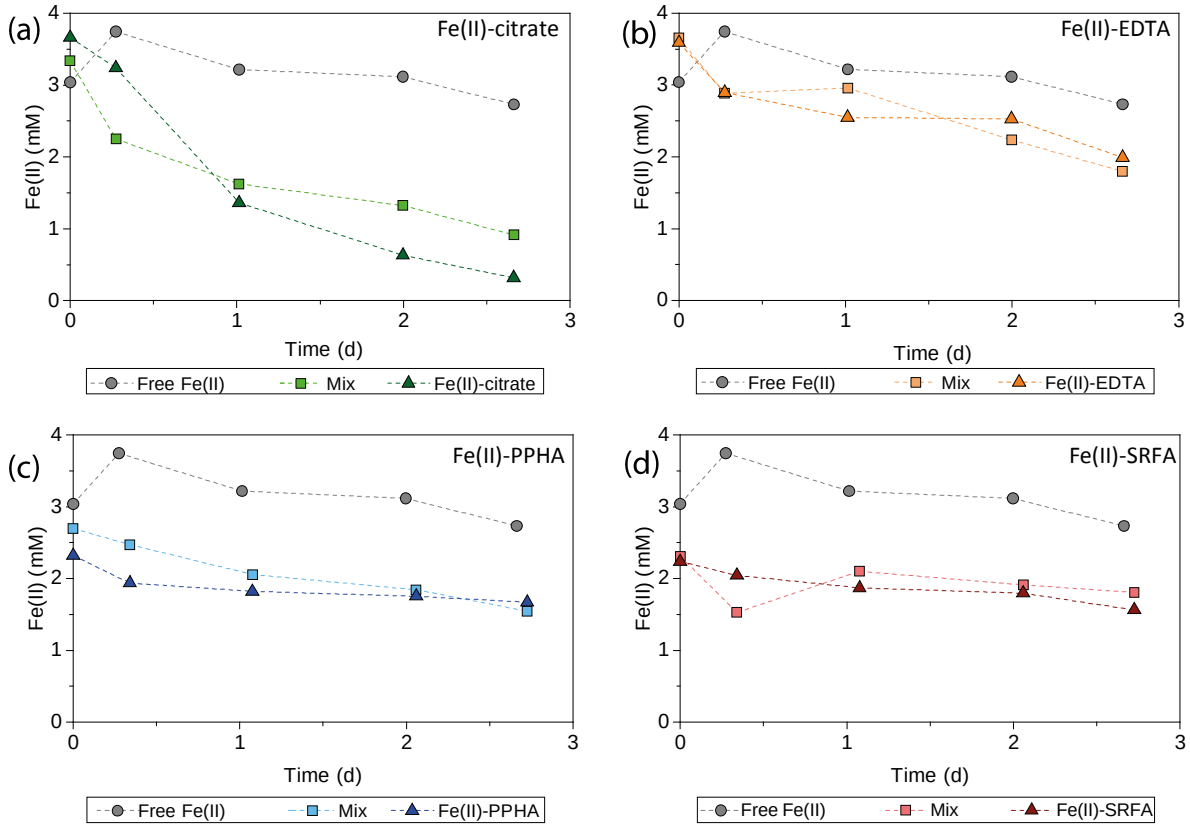

Figure S3.7 Abiotic Oxidation of Fe(II) by 2 mM nitrite in the incubation replicates. The Fe(II) was present in the form of either free Fe(II) (grey circles), different fully-complexed Fe(II)-OM complexes (triangles), or a mixture of free Fe(II) and Fe(II)-OM complexes (squares). Data is shown for Fe(II)-citrate (a), Fe(II)-EDTA (b), Fe(II)-PPHA complexes (c) and Fe(II)-SRFA complexes (d). The data are shown as averages of triplicate spectrophotometric measurements.

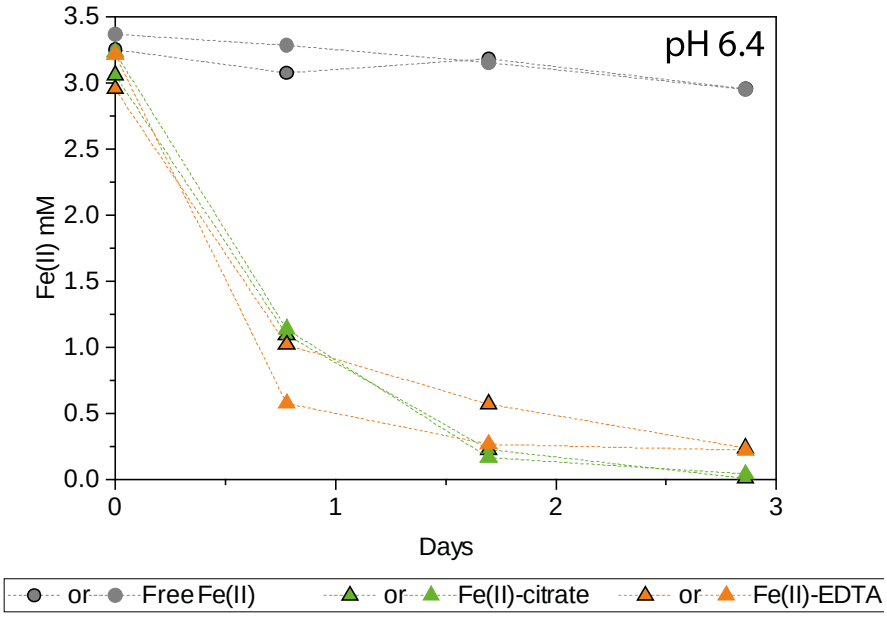

Figure S3.8 Abiotic Oxidation of Fe(II) by 2 mM nitrite at pH 6.4. The Fe(II) was present in the form of either free Fe(II) (grey circles), fully-complexed Fe(II)-citrate complexes (green triangles) and Fe(II)-EDTA complexes (orange triangles). The results showed that Fe(II) oxidation (especially the oxidation of Fe(II)-OM complexes) was promoted at pH 6.4 compared to pH 7 (Figure 3.3 and Figure S3.7). The data are shown as averages of triplicate spectrophotometric measurements.

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4. Organic matter complexation promotes Fe(II) oxidation by the phototrophic Fe(II)-oxidizer *Rhodopseudomonas palustris* TIE-1

4.1 Abstract

Fe(II)-organic matter (Fe(II)-OM) complexes are present in the photic zone of aquatic environments and due to their reactivity may play an important role in biogeochemical cycles. Complexation of Fe has been shown to influence the rates and extent of many chemical and microbial redox reactions. However, it is currently unknown whether, how fast, and to which extent Fe(II)-OM complexes can be oxidized by anoxygenic phototrophic Fe(II)-oxidizing bacteria which are widespread in photic habitats and use electrons from Fe(II) to fix CO_2. Here we used the phototrophic Fe(II)-oxidizer *Rhodopseudomonas palustris* TIE-1 to demonstrate that Fe(II) complexation by OM significantly accelerated the rates of Fe(II) oxidation by strain TIE-1 compared to the oxidation of non-organically bound, free Fe(II), although a fraction of the Fe(II) present as Fe(II)-OM complexes seemed to resist microbial oxidation. Analysis of Fe-OM aggregate sizes showed that the remaining, non-oxidized Fe(II) and almost all of the Fe(III) in the Fe(II)-humic and Fe(II)-fulvic acid oxidation products were in the form of colloids (3-200 nm). In summary, this study shows that Fe(II)-OM complexes can be oxidized microbially in the photic zone, and the complexation of Fe(II) by OM controls the rates and extent of Fe(II) oxidation.

4.2 Introduction

The oxidation of Fe(II) at neutral pH forms poorly soluble Fe(III) which typically leads to precipitation of Fe(III) minerals with reactive surfaces and large binding capacities. This immobilization of Fe(III) in minerals influences iron bioavailability in the environment, and can also influence the fate and turnover of other elements and compounds, from organic carbon (Lalonde *et al.*, 2012) to toxic heavy metal(loid)s such as As and Cd (Hohmann *et al.*, 2010, Muehe *et al.*, 2013, Zhu *et al.*, 2017) and other environmental contaminants (Wang *et al.*, 2016). At neutral pH, Fe(II) can be rapidly oxidized abiotically by molecular oxygen to Fe(III). Alternatively, Fe(II)-oxidizing microorganisms can also catalyze this reaction coupled to reduction of O_2 or nitrate, or photosynthetically during CO_2 fixation (Melton *et al.*, 2014). Phototrophic Fe(II)-oxidizing bacteria are widely distributed in freshwater and marine habitats including photic zones of water columns and sediments (Bryce *et al.*, 2018). These bacteria gain energy from anoxygenic photosynthesis, oxidizing Fe(II) and utilizing the electrons for CO_2 fixation (Madigan *et al.*, 2010, Melton *et al.*, 2014). The phototrophic Fe(II)-oxidizer, *Rhodopseudomonas palustris* TIE-1 (Jiao *et al.*, 2005) is thought to be able to enzymatically oxidize Fe(II) via a c-type cytochrome (Jiao & Newman, 2007) at the surface of the cell membrane (Shi *et al.*, 2016). Strain TIE-1 has been shown to be capable of oxidizing Fe(II) in the mineral magnetite (Byrne *et al.*, 2015) and utilizing electrons directly from poised electrodes (Bose *et al.*, 2014).

In the natural environment, Fe(II) is not only found as free Fe(II) or in Fe(II)-containing minerals, it is often complexed with organic matter which has been shown to strongly influence the environmental behavior of Fe. Such Fe(II)-organic-matter (Fe(II)-OM) complexes are abundant in nature (Luther *et al.*, 1996, Kleja *et al.*, 2012, Sundman *et al.*, 2013, Sundman *et al.*, 2014, von der Heyden *et al.*, 2014, Hopwood *et al.*, 2015, Yu *et al.*, 2015, Bhattacharyya *et al.*, 2018). For example, more than 99% of the Fe in the ocean is found to be complexed by OM (Barbeau, 2006). OM-complexation influences the rates of abiotic oxidation of Fe(II) by O_2 (Rose & Waite, 2003, Daugherty *et al.*, 2017, Lee *et al.*, 2017, Chen & Thompson, 2018) and by nitrite (Kopf *et al.*, 2013). Indeed, the complexation and stabilization of Fe(II) by organic ligands was suggested to be the main reason for

higher than expected abundances of Fe(II) in many oxic aquatic environments (Barbeau, 2006, Neubauer *et al.*, 2013, Sundman *et al.*, 2014, Bhattacharyya *et al.*, 2018). Recent work has also shown that Fe(II)-OM complexation strongly inhibits microbial nitrate-reducing Fe(II) oxidation (Peng *et al.*, 2018). However, it is currently unknown whether and how Fe(II)-OM complexation influences microbial phototrophic Fe(II) oxidation.

In this study, we aim to determine how Fe(II)-OM complexation influences the oxidation of Fe(II) by the phototrophic Fe(II)-oxidizing strain *Rhodopseudomonas palustris* TIE-1. The rates, extent of oxidation of Fe(II)-OM complexes, and the size fractions of Fe in their oxidation products are compared to non-OM-complexed free Fe(II).

4.3 Materials and Methods

In order to determine how Fe(II)-OM complexation influences microbial phototrophic Fe(II) oxidation, we performed separate cell suspension experiments with the phototrophic Fe(II)-oxidizer *Rhodopseudomonas palustris* TIE-1 with either Fe(II)-citrate, Fe(II)-EDTA, Fe(II)-PPHA (Pahokee peat humic acid) or Fe(II)-SRFA (Suwannee river fulvic acid). In parallel, we also performed one cell suspension experiment with only non-OM-bound, free Fe(II). Oxidation of OM-complexed and free Fe(II) was compared by following concentrations of Fe(II) over time.

Bacterial strain and pre-cultivation

The phototrophic Fe(II)-oxidizing bacterium *Rhodopseudomonas palustris* TIE-1 isolated from a marsh sediment (Jiao *et al.*, 2005) is routinely cultivated in the authors' lab with 10 mM $FeCl_2$ in a basal medium (Melton *et al.*, 2012). To avoid transfer of Fe(III) minerals from the pre-culture, strain TIE-1 was grown and transferred three times in the basal medium without Fe(II) with H_2/CO_2 (80:20) as electron donor in the headspace before starting the incubation experiments with the different Fe(II) compounds.

Medium and chemicals

The effect of four different ligands on Fe(II) oxidation rates were determined, and compared to the rate and extent of oxidation of free non-OM-complexed Fe(II). The ligands tested were citrate, EDTA, PPHA and SRFA. Experiments were conducted in PIPES-buffered media (pH 7) (prepared as in Peng et al., 2018) to which 0.1 mM $FeCl_2$ and one of the chosen ligands were added. The concentration of ligand added varied between experiments to ensure complete complexation of the Fe(II). These concentrations were 0.2 mM, 0.12 mM, 0.2 mg/ml and 0.2 mg/ml for citrate, EDTA, PPHA, and SRFA, respectively. Under these conditions, more than 99% of the Fe(II) was present as Fe(II)-OM complexes in each of the four individual Fe(II)-OM treatments according to geochemical modeling using PHREEQC coupled with a previously published humic Ion-binding model (Model VI) (Catrouillet *et al.*, 2014). This model takes the change of complexation capacity with concentration of OM into consideration and assumes that the complexation of Fe(II) by OM occurs through eight discrete sites. 5 mL of anoxic medium were dispensed into 15 ml glass Hungate tubes with air-tight butyl-stoppers, and amended with 1 mM $NaHCO_3$ and 0.5% CO_2/99.5% N_2 in the headspace.

Setup of experiments

Rates and extent of microbial oxidation of Fe(II)-OM complexes were determined in cell suspension experiments. In such experiments no vitamins or trace metals are included in the medium so the cells are not expected to grow. For these experiments, the bacteria were cultured to the late exponential phase, harvested by centrifugation (7000 g, 20 min, 25°C), washed twice and re-suspended in 20 mM anoxic PIPES buffer containing 20 mM NaCl in an anoxic glovebox (100% N_2). D/L staining (Thermo Fisher Scientific) showed that almost all the cells were alive after the washing steps (data not shown). An aliquot of the cell suspension (final cell number ca. $3.6x10^7$ cells/ml) was added to the medium containing either Fe(II)-citrate, Fe(II)-EDTA, Fe(II)-PPHA or Fe(II)-SRFA complexes (four individual setups, no mixtures of complexes) in an anoxic glovebox. The tubes with the cell suspensions were incubated at 20°C horizontally under a 40 watt incandescent light bulb with a light intensity of ca. 550 lux, the

thickness of the water layer in the tubes is ca. 5 mm.

Sample analysis

Samples were taken using syringes in an anoxic glovebox (100% N_2) every 1-2 hours. Fe(II) concentrations were determined anoxically using the ferrozine assay (Stookey, 1970) modified as in Peng et al. (2018). In brief, samples with non-OM-bound, free Fe(II), Fe(II)-citrate and Fe(II)-EDTA complexes were first diluted with anoxic 1M HCl to accelerate the formation of the Fe(II)-ferrozine complex, the last step of the ferrozine assay. Samples with Fe(II)-PPHA and Fe(II)-SRFA complexes were immediately mixed with ferrozine solution without 1M HCl to avoid precipitation of HA and to avoid potential redox reactions of Fe with PPHA and SRFA during acidification. Total Fe concentrations were quantified by reduction of Fe(III) to Fe(II) by hydroxylamine hydrochloride 10% (w/v) in 1 M HCl.

The microbial oxidation products of free Fe(II) and Fe(II)-OM complexes were size-fractionated into dissolved (3 kDa MWCO; ca. 3 nm), colloidal (3 kDa to 0.2 µm) and particulate (>0.2 µm) fractions, and the concentrations of Fe(II) and Fe(III) within each fraction were determined. The fractionation was done anoxically in the glovebox using Amicon Ultra-0.5 3 kDa ultrafiltration membranes (Millipore) and 0.2 µm PES filters (VMS). Cells were stained using BacLight Green (Thermo Fisher Scientific), and cell numbers were quantified by an Attune Nxt flow cytometer (Thermo Fisher Scientific) equipped with a 488 nm laser as an excitation source. Cells were distinguished from noise or debris based on their properties in the side scatter and BL1 channel (with emission filter 530/30 nm).

Maximum rates of Fe(II) oxidation for the individual setups were calculated from the steepest slope between two subsequent data points of Fe(II) concentrations. All incubation and measurements were conducted in at least duplicates.

4.4 Results and Discussion

Kinetics of phototrophic Fe(II) oxidation

To determine how Fe(II)-OM complexation influences the rates and extent of phototrophic Fe(II) oxidation, we performed four separate cell suspension experiments with the phototrophic Fe(II)-oxidizer *Rhodopseudomonas palustris* TIE-1 with either Fe(II)-citrate, Fe(II)-EDTA, Fe(II)-PPHA, or Fe(II)-SRFA complexes in parallel to a control setup with only non-OM-bound, free Fe(II), and followed concentrations of Fe(II) over time. We observed that the oxidation rates of Fe(II) by strain TIE-1 were higher when Fe(II) was present as Fe(II)-OM complexes compared to non-OM-bound, free Fe(II), although the extent of this stimulating effect varied for the different Fe(II)-OM complexes (Figure 4.1, Table 4.1). Fe(II)-EDTA complexes showed the fastest Fe(II) oxidation rates (126.7±4.4 µM/h) followed by Fe(II)-citrate (100.7±0.02 µM/h), Fe(II)-SRFA (45.9±1.5 µM/h) and Fe(II)-PPHA (20.7±1.3 µM/h), while the oxidation rate of free Fe(II) was only 6.1±1.3 µM/h (the ± values indicate the range of rates calculated from duplicate experiments). The oxidation rates of Fe(II)-EDTA, Fe(II)-citrate, Fe(II)-PPHA and Fe(II)-SRFA complexes were calculated to be 20.6, 16.3, 3.4, and 7.5 fold faster, respectively, than for free Fe(II). No Fe(II) was oxidized in abiotic controls without cells (Figure S4.1).

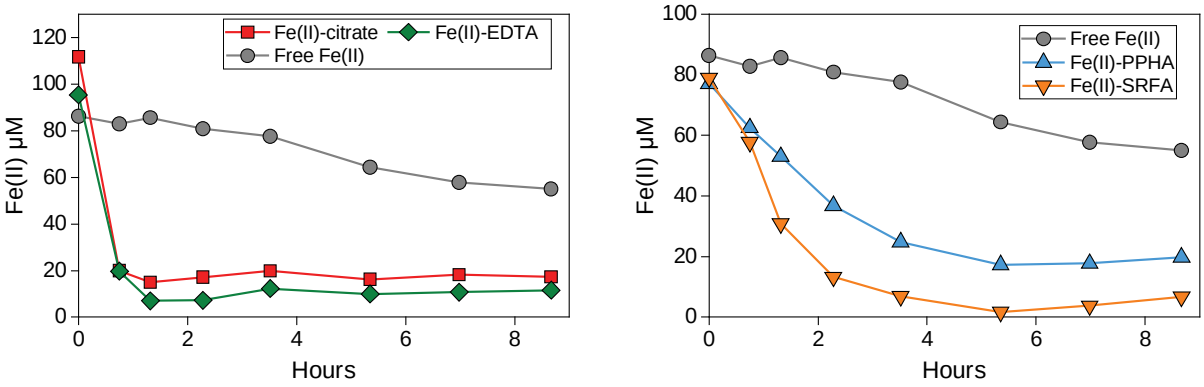

Figure 4.1 Oxidation of different Fe(II)-OM complexes by *Rhodopseudomonas palustris* TIE-1. Fe(II) was present in the form of either non-OM-bound, free Fe(II) (grey circles), as Fe(II)-citrate (red squares) or Fe(II)-EDTA (green diamonds) (left panel) or as Fe(II)-PPHA complexes (blue triangles) or Fe(II)-SRFA complexes (yellow

triangle) (right panel). The results are reported as an average of triplicate measurements. Since duplicate microbiological setups showed very similar results, representative results out of these duplicate setups are shown here and the second one is shown in the supporting information Figure S4.2

At pH 7 and 25°C, the calculated redox potentials of Fe(II)-citrate and Fe(II)-EDTA complexes are more negative than the redox potential of aqueous Fe(II) (Buerge & Hug, 1999, Naka *et al.*, 2006). Hence, the oxidation of Fe(II)-OM is thermodynamically more favorable than the oxidation of free Fe(II). This promotion of Fe(II) oxidation rates by OM may also explain what has been observed in some biotechnological studies, where systems containing Fe-OM complexes, e.g. Fe-citrate (Doud & Angenent, 2014), and Fe-hexacyanoferrate (Rengasamy *et al.*, 2018), showed a higher electron uptake by the phototrophic Fe(II)-oxidizer strain TIE-1 than from the electrodes.

Table 1. The maximum oxidation rates of free Fe(II) compared to the four individual Fe(II)-OM complexes by the phototrophic Fe(II)-oxidizing bacterium strain TIE-1.

Form of Fe(II)	Maximum oxidation rate	
	Total (μM/h)	Per cell pM/h/cell
Fe(II)-EDTA	126.7±4.4	3.5
Fe(II)-citrate	100.7±0.02	2.8
Fe(II)-SRFA	45.9±1.5	1.3
Fe(II)-PPHA	20.7±1.3	0.58
Free Fe(II)	6.1±1.3	0.17

In strain TIE-1, a decaheme c-type cytochrome, PioA, is thought to be the Fe(II)-oxidase, however, so far the reaction rates for different Fe(II)-OM complexes with PioA have not been determined. In other cases with comparable microbial Fe redox systems as in this study, it has been shown that different Fe(II)-OM complexes have different reaction kinetics with bacterial c-type cytochromes, for example with MtoA of the microaerophilic Fe(II)-oxidizing bacterium *Sideroxydans lithotrophicus*

ES-1 (Liu *et al.*, 2012), MtrC and OmcA of Fe(III)-reducing bacterium *Shewanella oneidensis* MR-1 (Wang *et al.*, 2008), and an equine c-type cytochrome (Wang *et al.*, 2017). It is currently not fully understood why these c-type cytochromes show different reaction kinetics with different forms of Fe(II). One plausible explanation could be a lower chemical activation energy for the oxidation of Fe(II)-OM complexes, which is related to both the reorganization energy and Gibbs free energy when Fe(II) is complexed by OM (Wang *et al.*, 2008). Another possible explanation for the higher Fe(II) oxidation rates could be a better accessibility of the cytochrome to the Fe(II)-OM than to the free Fe(II). The surface of the cytochrome is known to be unevenly charged (Leys *et al.*, 2002, Pereira *et al.*, 2017), and Fe(II)-OM complexation is expected to change the overall charge of the Fe(II) from positive to neutral or even negative (Grinberg, 2013) potentially allowing a better interaction of the cytochrome with the Fe(II)-species. Preferred oxidation of Fe(II)-OM compared to free Fe(II) may also provide a physiological benefit as the product of Fe(II)-OM oxidation could be water-soluble or colloidal Fe(III)-OM complexes, while the oxidation of non-organically bound Fe(II) would produce solid Fe(III) mineral particles that could be harmful to the cells due to encrustation. Preferential oxidation of Fe(II)-OM could thus negate the need to synthesize Fe(III)-binding ligands or adopt other energy-consuming mechanisms to avoid encrustation (such as establishment of low pH microenvironments) (Chan *et al.*, 2004, Miot *et al.*, 2009, Hegler *et al.*, 2010).

Extent of Fe(II) oxidation

Although we found that the Fe(II) oxidation rate was promoted by Fe(II)-OM complexation, there was still a significant portion of Fe(II) left at the end of the incubation (Figure 4.1). In order to better understand how OM complexation influences the products of phototrophic Fe(II) oxidation, we analyzed the particle/aggregate size of the products of oxidation of free Fe(II) and of Fe(II)-citrate, Fe(II)-PPHA and Fe(II)-SRFA complexes (Figure 4.2). We chose to determine the size of the present Fe(II) and Fe(III) species after 8.6 hours when Fe(II) concentrations were stable, in order to minimize the potential effect of abiotic self-reorganization and recrystallization of Fe after the microbial oxidation of Fe(II)-OM complexes. For Fe-EDTA we only determined the size for Fe(II)-EDTA complexes, as the strong binding

between Fe(III) and EDTA (stability constant logK=16) (Martell *et al.*, 1993) seemed to interfere with the reduction of Fe(III) by hydroxylamine hydrochloride during the quantification of the total Fe (data not shown).

We found that Fe-OM complexation significantly changed the size fraction of the Fe oxidation products. Without OM complexation, after 8.6 h the oxidation of free Fe(II) was not complete due to the relatively slow Fe(II) oxidation rate, and all Fe(III) produced (ca. 30% of the total Fe) was in the particulate fraction (>200 nm). Interestingly, there was also approximately 15% Fe(II) in this particulate fraction, resulting in a ratio of Fe(II):Fe(III) of 1:2, probably in the form of mixed-valent Fe(II)-Fe(III)-phases such as magnetite (Fe_3O_4), green rust or simply Fe(III) oxyhydroxides with associated Fe(II). This agrees with previous reports of Fe(II) being oxidized by strain TIE-1 resulting in the formation of Fe(III) oxyhydroxides and potentially magnetite (Jiao *et al.*, 2005). However, in the presence of OM, the size of the Fe(III) species produced was much smaller. Most of the Fe(III) was dissolved (ca. < 3 nm) in the oxidation products of Fe(II)-citrate or was present as colloids (between ca. 3-200 nm) in the oxidation products of Fe(II)-PPHA and Fe(II)-SRFA. The Fe(II) remaining at the end of the incubation with Fe(II)-citrate was in the particulate and dissolved fractions (ca. 5.6% and 11.6%, respectively) (Figure 4.2). In the experiments with Fe(II)-EDTA, the remaining Fe(II) was only in the dissolved fraction (data not shown), whereas the Fe(II) remaining at the end of the incubation with Fe(II)-PPHA and Fe(II)-SRFA was nearly all in the colloid fraction, which accounted for ca. 23.5% and 10.7% of the total Fe, respectively.

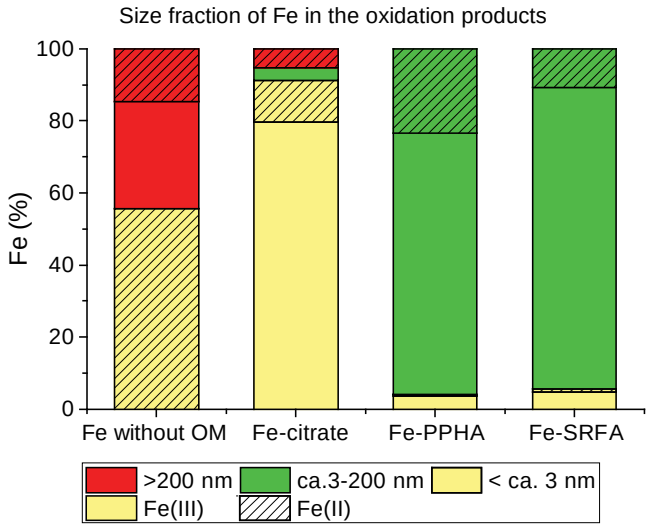

Figure 4.2 Size fractions of Fe in the oxidation products of free Fe(II) (Fe without OM), Fe(II)-citrate, Fe(II)-PPHA and Fe(II)-SRFA at steady-state conditions after ca. 8.6 hours of incubation (the last data point in Figure 4.1 when oxidation of free Fe(II) was not complete yet). The y-axis shows the percentages of Fe(II) and Fe(III) in a certain size fraction relative to the total concentration of Fe. Different size fractions are labeled with different colors, Fe(II) and Fe(III) are labeled with and without hatched patterns, respectively. The results are reported as an average calculated from two independent parallels and triplicate measurements.

The size fractionation indicated that Fe(II)-OM complexation influences microbial phototrophic Fe(II) oxidation in several ways. Firstly, the presence of colloidal Fe(II) remaining in the samples with PPHA and SRFA suggested that aggregation of Fe-PPHA and Fe-SRFA complexes as colloids may protect Fe(II) from microbial Fe(II) oxidation. This is probably due to a lack of accessibility of the c-type cytochromes to Fe(II) that is present in the inner part (core) of the Fe-OM colloids (the 3-200 nm fraction), as the maximum distance for electron transfer is very short such as 2 nm (Gray & Winkler, 2009). Secondly, other possible factors, such as the simultaneously occurring photoreduction of Fe(III)-citrate to Fe(II) (Frahn, 1958) and the change of redox potentials of Fe(II)/Fe(III) compared to free Fe(II) systems (Straub *et al.*, 2001) may also influence the remaining Fe(II) content. When the Fe(II)-OM and Fe(III)-OM complexes are not in the colloidal form but are completely dissolved, as it is the case for Fe(II)-/Fe(III)-citrate (Figure 4.2), these effects could be even more important than

the size of the Fe(II)-species present.

Environmental Implications

This study showed that Fe(II) complexation by OM significantly promoted microbial phototrophic Fe(II) oxidation. The higher oxidation rates observed for Fe(II)-OM complexes and the high abundance of such complexes in photic environments suggest that microbial Fe(II) oxidation in photic anoxic environments could be much more important than previously thought, since most previous studies only focused on oxidation of non-organically bound free Fe(II) (Ehrenreich & Widdel, 1994, Jiao *et al.*, 2005, Hegler *et al.*, 2008, Laufer *et al.*, 2016). Since photoautotrophic Fe(II) oxidation provides electrons during photosynthesis for CO_2 fixation (Widdel *et al.*, 1993, Jiao & Newman, 2007, Bird *et al.*, 2011), the Fe(II) oxidation stimulated by OM complexation may also promote the fixation of CO_2 and thus decrease CO_2 emission in Fe- and OM-rich environmental habits including surface layers of rice paddy soils and peatlands. On the other hand, our results have also revealed that part of the colloidal Fe(II)-OM was resistant to microbial phototrophic Fe(II) oxidation (Figure 4.2). This fraction of Fe(II) could be a sink for Fe(II) in the environment, as shown to be the case in the presence of a high C:Fe ratio (Liao *et al.*, 2017), where protection against abiotic oxidation by oxygen (Daugherty *et al.*, 2017), a decrease in the bioavailability of Fe (Oleinikova *et al.*, 2017), and less oxidation by nitrate-reducing Fe(II)-oxidizing bacteria was observed (Peng *et al.*, 2018). Overall, this study indicates a close relationship between OM and Fe in the environment, and shows that OM does not only influence iron cycling by providing e-donors for microbial Fe(III) reduction (Jiang & Kappler, 2008, Roden *et al.*, 2010, Amstaetter *et al.*, 2012, Shimizu *et al.*, 2013), but also by Fe-complexation which can strongly influence microbial phototrophic Fe(II) oxidation.

4.5 Supporting information

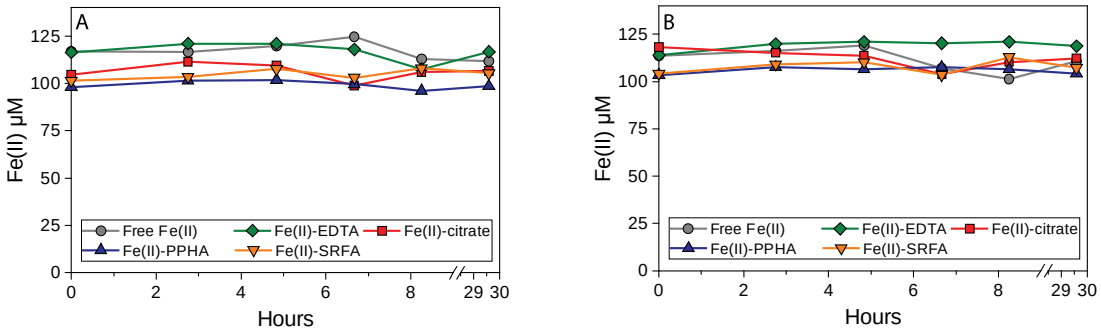

Figure S4.1 Abiotic controls (without *Rhodopseudomonas palustris* TIE-1 but incubated in the light) of experiments with different Fe(II)-OM complexes. Results of two independent parallel incubations for each type of Fe(II)-complex are shown on the left (A) and right (B), respectively. Fe(II) was present in the form of either non-OM-bound free Fe(II) (grey circles), as Fe(II)-citrate (red squares), Fe(II)-EDTA (green diamonds), Fe(II)-PPHA complexes (blue triangles) or Fe(II)-SRFA complexes (yellow triangle). The result showed that there was no Fe(II) oxidation in the abiotic controls.

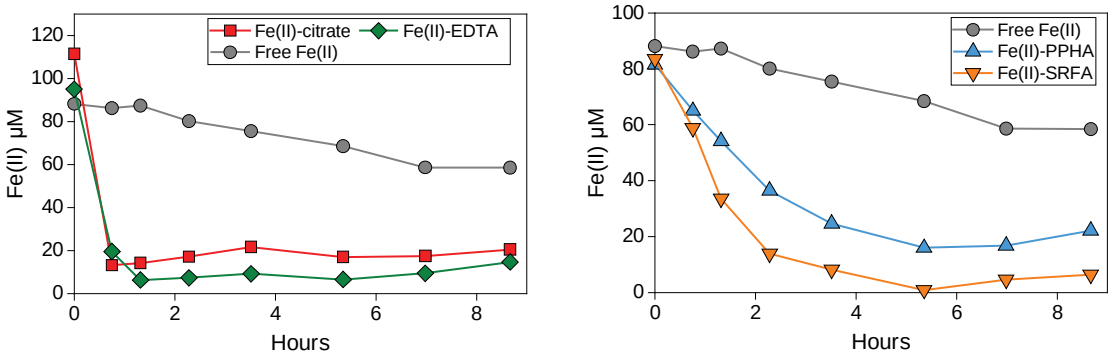

Figure S4.2 Oxidation of different Fe(II)-OM complexes by *Rhodopseudomonas palustris* TIE-1 in the second biological replicate (the first one is shown in the main paper as Figure 4.1). Fe(II) was present in the form of either non-OM-bound, free Fe(II) (grey circles), as Fe(II)-citrate (red squares), Fe(II)-EDTA (green diamonds), Fe(II)-PPHA complexes (blue triangles) or Fe(II)-SRFA complexes (yellow triangle). The data are shown as averages of triplicate spectrophotometric measurements.

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5. Cryptic cycling of Fe(III)-organic matter complexes by phototrophic Fe(II)-oxidizing bacteria

5.1 Abstract

Fe-organic matter (Fe-OM) complexes are abundant in the environment and, due to their mobility, reactivity and bioavailability, play a significant role in the biogeochemical Fe cycle. In photic zones of aquatic environments, Fe-OM complexes can potentially be reduced and oxidized, and thus cycled, by light-dependent processes including abiotic photoreduction of Fe(III)-OM complexes and microbial oxidation of Fe(II)-OM complexes by anoxygenic phototrophic bacteria. This could lead to a cryptic iron cycle in which continuous oxidation and re-reduction of Fe could result in a low and steady-state Fe(II) concentration despite rapid Fe turnover. However, the coupling of these processes has never been demonstrated experimentally. In this study we grew a model anoxygenic phototrophic Fe(II)-oxidizer, *Rhodobacter ferrooxidans* SW2, with either citrate, Fe(II)-citrate or Fe(III)-citrate. We found that strain SW2 was capable of re-oxidizing Fe(II)-citrate produced by photochemical reduction of Fe(III)-citrate, which kept the dissolved Fe(II)-citrate concentration at low (<10 μM) and stable concentrations with a concomitant increase in cell numbers. Cell suspension incubations with strain SW2 showed that it can also oxidize Fe(II)-EDTA, Fe(II)-humic-acid and Fe(II)-fulvic-acid complexes. This work demonstrates the potential for active cryptic Fe-cycling in the photic zone of anoxic aquatic environments, despite low measurable Fe(II) concentrations which are controlled by the rate of microbial Fe(II) oxidation and the identity of the Fe-OM complexes.

5.2 Importance

Iron cycling, including reduction of Fe(III) and oxidation of Fe(II), involves the formation, transformation and dissolution of minerals and dissolved iron-organic-matter compounds. It has been shown previously that Fe can be cycled so rapidly that no measurable changes in Fe(II) and Fe(III) concentrations occur, leading to a so-called cryptic cycle. Cryptic Fe cycles have been shown to be driven either abiotically by a combination of photochemical reduction of Fe(III)-OM complexes and re-oxidation of Fe(II) by O_2, or microbially by a combination of Fe(III)-reducing and Fe(II)-oxidizing bacteria. Our study demonstrates a new type of light-driven cryptic Fe cycle that is relevant for the photic zone of aquatic habitats involving abiotic photochemical reduction of Fe(III)-OM complexes and microbial phototrophic Fe(II) oxidation. This new type of cryptic Fe cycle has important implications for biogeochemical cycling of iron, carbon, nutrients and heavy metals, and can also influence the composition and activity of microbial communities.

5.3 Introduction

The biogeochemical cycling of elements plays an important role in shaping the modern environment and has contributed to the evolution of the Earth system throughout its history (Borch *et al.*, 2010, Druschel & Kappler, 2015). However, many important microbial element cycles are said to be 'cryptic' due to the fact that rapid production and consumption rates of the relevant compounds maintains extremely low and stable concentrations despite rapid turnover (Kappler & Bryce, 2017). In the last decade, several studies have identified cryptic cycles in different environments illustrating that cryptic elemental cycles are widely distributed and can influence, or even drive, the cycling of other elements (Canfield *et al.*, 2010, Holmkvist *et al.*, 2011, Durham *et al.*, 2015, Hansel *et al.*, 2015, Berg *et al.*, 2016). So far, the majority of

studies on cryptic elemental cycles have focused on sulfur and carbon (Hansel *et al.*, 2015). Cryptic cycling of Fe has only been observed in a few aqueous environments and can involve combinations of abiotic or biotic reactions. For example, it was suggested that abiotic cryptic Fe cycling in the oxic surface ocean was driven by the simultaneous photochemical reduction of Fe(III)-OM complexes followed by the abiotic re-oxidation of Fe(II) by oxygen/reactive oxygen species (Voelker *et al.*, 1997, Barbeau *et al.*, 2001, Lukas *et al.*, 2001, Sunda & Huntsman, 2003, Boyd & Ellwood, 2010).

Fe-OM complexes, including both Fe(II)-OM and Fe(III)-OM complexes, are widespread in the environment (Luther *et al.*, 1996, Boyd & Ellwood, 2010, Neubauer *et al.*, 2013, Sundman *et al.*, 2014, Hopwood *et al.*, 2015, Yu *et al.*, 2015, Bhattacharyya *et al.*, 2018). For example, almost all of the dissolved Fe (>99%) in seawater was shown to be associated with organics according to modeling and speciation analysis (Rue & Bruland, 1995, Barbeau *et al.*, 2001, Barbeau, 2006). Additionally, organic matter and the formation of OM-metal complexes were shown to determine the speciation, concentration and distribution of Fe in soils and seawater (Kenshi *et al.*, 1996, Liu & Millero, 2002, Kraemer, 2004, Kraemer *et al.*, 2005, Boyd & Ellwood, 2010). In photic zones, Fe(III)-OM complexes can be photochemically reduced forming Fe(II), a process which was suggested to be one of the reasons for the higher than expected abundance of Fe(II) in many oxic natural aquatic environments (Kuma *et al.*, 1992, Borer *et al.*, 2005, Barbeau, 2006). Depending on the conditions, e.g. the wavelength of the light and type of organic ligands (Barbeau *et al.*, 2003), photochemical Fe(III) reduction can happen either directly or indirectly (Barbeau, 2006). In the direct photochemical Fe(III) reduction, an electron is directly transferred from the photo-excited organic ligand to Fe(III) (Lever, 1974), whereas in the indirect photochemical Fe(III) reduction, Fe(III) is reduced by reactive radical species that are produced photochemically by ligands, e.g. the superoxide radical (O_2^-) (Voelker & Sedlak, 1995). Independent of whether

photochemical Fe(III) reduction occurs directly or indirectly, both processes are accompanied with the transformation or loss of functional groups of the organic matter (Barbeau, 2006). Therefore, photochemical Fe(III) reduction was also suggested to play an important role in the degradation of organic matter (Lockhart & Blakeley, 1975, Barbeau *et al.*, 2001, Fisher *et al.*, 2006, Martin *et al.*, 2006).

Fe can also be oxidized or reduced by bacteria, but biotically catalyzed cryptic Fe cycling is much less well characterized. In photic environments, phototrophic Fe(II)-oxidizing bacteria can anaerobically oxidize Fe(II) and use the electrons for carbon fixation. Together with Fe(III)-reducing bacteria, which reduce Fe(III) using organic or inorganic compounds as electron donors, phototrophic Fe(II)-oxidizing bacteria were suggested to be one of the key players in cryptic Fe cycling in a few redox-stratified lakes by re-oxidation of the Fe(II) produced from microbial Fe(III) reduction (Llirós *et al.*, 2015, Berg *et al.*, 2016). However, it is currently unknown whether cryptic Fe-cycling could also occur from the combined action of phototrophic Fe(II)-oxidizing bacteria and photochemical reduction of Fe(III)-OM complexes.

In this study we therefore used a model anoxygenic phototrophic Fe(II)-oxidizer (*Rhodobacter ferrooxidans* SW2) to determine whether these bacteria can oxidize different Fe(II)-OM complexes, and whether they can re-oxidize the Fe(II)-species produced from abiotic photochemical reduction of Fe(III)-OM, thus closing a cryptic iron cycle under photic conditions.

5.4 Materials and methods

Bacterial strain and pre-cultivation

The phototrophic Fe(II)-oxidizing bacterium *Rhodobacter ferrooxidans* SW2 was isolated from a freshwater pond (Ehrenreich & Widdel, 1994) and routinely cultivated in the author's lab with 10 mM $FeCl_2$ in a basal medium which was prepared anoxically as described previously (Hegler *et al.*, 2008). In order to remove remaining Fe(III) minerals from the inoculum prior to the experiment, strain SW2 was transferred 3-4 times in the basal medium with H_2/CO_2 (90:10) in the headspace as an electron donor instead of Fe(II).

Medium and chemicals

Different media were made for different experiments. In total three different types of medium were prepared. Firstly, for the non-growth cell suspension experiment, a anoxic PIPES buffered medium (pH 7) with Fe(II)-OM complexes were prepared following a slightly modified method published previously (Peng *et al.*, 2018). To guarantee complexation of most of the Fe(II) present by the different sources of OM tested, the final concentrations of Fe(II), citrate, EDTA, PPHA and SRFA were 0.1 mM, 0.2 mM and 0.12 mM, 0.2 mg/ml, and 0.2 mg/ml, respectively. Speciation calculation using an Fe(II)-OM geochemical model (Catrouillet *et al.*, 2014) showed that more than 99% of the Fe(II) was present as Fe(II)-OM complexes with these chosen concentrations. Medium was dispensed into 15 ml Hungate tubes with a final volume of 5 ml, and amended with 1 mM $NaHCO_3$ and 0.5% CO_2/99.5% N_2 in the headspace. Secondly, for photochemical Fe(III) reduction experiments, 0.1 mM $FeCl_3$, instead of $FeCl_2$, was added into the anoxic PIPES medium with the same concentrations of OM, $NaHCO_3$ and CO_2. Thirdly, for the growth experiment, 25 ml anoxic basal medium (Hegler *et al.*, 2008) was dispensed into 50 ml serum bottles and was amended with 4 mM $HOC(COONa)(CH_2COONa)_2 \cdot 2H_2O$ (sodium citrate), either without Fe or with 2

mM $FeCl_2$ or $FeCl_3$.

Experimental design

To evaluate the possibility of cryptic Fe-cycling with Fe-OM complexes, we first performed independent SW2 cell suspension experiments with different Fe(II)-OM complexes and photochemical Fe(III)-OM reduction experiments. For the cell suspension experiment, the bacteria were first cultured to the late exponential phase. Cells were harvested by centrifugation (7000 g, 20 min, 25°C), washed twice, and re-suspended in 20 mM PIPES buffer containing 20 mM NaCl. An aliquot of the cell suspension was added to the non-growth medium in Hungate tubes containing different Fe(II)-OM complexes, including Fe(II)-citrate, Fe(II)-EDTA, Fe(II)-PPHA and Fe(II)-SRFA. The final cell number was ca. $1.5x10^8$ cells/ml. The Hungate tubes with the cell suspension were placed horizontally under a 40W incandescent light bulb, and incubated at 20°C and a light intensity of ca. 550 lux. Abiotic photochemical Fe(III) reduction experiments were carried out in the same way as the cell suspension experiments, however, without cells and with Fe(II) replaced by Fe(III) (added as $FeCl_3$). Additionally, to evaluate the effect of the dark color of humic acids on Fe(II) oxidation (humic acids may absorb part of the light and decrease light intensity for the phototrophic bacteria) (Figure S5.4), we have performed a cell suspension experiment to determine the oxidation of Fe(II)-PPHA complexes by *Rhodobacter ferrooxidans* SW2 at different light intensities of ca. 1800 lux, 500 lux, and 200 lux (Figure S5.5).

To determine if the anoxygenic phototrophic strain SW2 can grow by cryptic Fe-OM cycling, we performed a growth experiment. Strain SW2 was inoculated in the anoxic basal medium containing either citrate only, or citrate with Fe(II) or citrate with Fe(III), and an initial cell number of ca. $3x10^7$ cells/ml. The initial concentrations of Fe were about 2 mM for Fe(II) and Fe(III), and the initial concentration of citrate was 4 mM. According to the stoichiometry of Fe(III) reduction to Fe(II) by citrate

(Frahn, 1958, Buchanan, 1970), 1 mole of citrate can reduce 2 moles of Fe(III), meaning that Fe(III) can be cycled a maximum of 4 times in our experiment with initial concentrations of 2 mM Fe and 4 mM citrate. This also implies that after each Fe cycle the remaining citrate could still form a complex with Fe(III). The cells in the serum bottles were incubated at 25°C with a light intensity of ca. 1000 lux generated by a 40W light bulb.

Sample analysis

Samples were taken in an anoxic glovebox (UNIIlab Plus, MBraun, Germany) (100% N_2) every 3-4 days for the growth experiment or every 1-2 hours for cell suspension and abiotic photochemical Fe(III) reduction experiments. Fe(II) concentrations were determined anoxically using the ferrozine assay (Stookey, 1970) modified as in Peng et al. (2018) (Ehrenreich & Widdel, 1994). The quantification of Fe(II) in samples without PPHA and SRFA was performed using 1M anoxic HCl and anoxic ferrozine solution (0.1% w/v) dissolved in ammonium acetate ($C_2H_7NO_2$, 50% w/v). Samples with PPHA and SRFA were first diluted with anoxic Milli-Q H_2O and immediately mixed with ferrozine solutions without 1M HCl to avoid potential redox reactions of Fe with PPHA and SRFA during acidification. All ferrozine measurements were conducted in triplicate and the results reported as an average. Maximum rates of Fe(II) oxidation for the individual experiments were calculated from the slopes of the linear fits of Fe(II) concentrations at the steepest part of the experiments; at least three data points were used in the calculation of the Fe(II) oxidation rates. Cell numbers were quantified by an Attune Nxt flow cytometer (Thermo Fisher Scientific) equipped with a blue laser beam as an excitation source (488 nm). Prior to flow cytometry, an aliquot of the cells was stained using BacLight Green stain (Thermo Fisher Scientific). Cells were distinguished from debris by their properties in the side scatter and fluorescence parameters. All measurements were conducted in duplicate and the results reported as an average.

5.4 Results

Oxidation of Fe(II)-OM complexes *by Rhodobacter ferrooxidans strain* SW2

In order to better understand the role of microorganisms in the cryptic Fe-cycle, and to evaluate whether cryptic light-dependent Fe cycling could occur with different Fe(II)-OM complexes, we performed cell suspension experiments with the phototrophic Fe(II)-oxidizer *Rhodobacter ferrooxidans* strain SW2. We incubated $1.5x10^8$ cells/ml of strain SW2 with either Fe(II)-citrate, Fe(II)-EDTA (ethylenediaminetetraacetic acid), Fe(II)-PPHA (Pahokee peat humic acid), or Fe(II)-SRFA (Suwannee river fulvic acid) complexes. This non-growth medium contains only those Fe(II)-OM complexes plus 1 mM $NaHCO_3$, 20 mM PIPES and NaCl, i.e. no other salts, nutrients or vitamins. This guarantees that cells are actively metabolizing but no cell growth is possible and that therefore changing cell numbers do not influence the quantification of metabolic rates.

In these cell suspension experiments we observed that all Fe(II)-OM complexes tested were oxidized by strain SW2, while the Fe(II) oxidation rates varied for the different Fe(II)-OM complexes (Figure 5.1). The Fe(II)-EDTA complex showed the fastest oxidation rates (ca. 48 µM/h, or 32.1 fM/h per cell) followed by Fe(II)-citrate (ca. 31 µM/h, or 20.7 fM/h per cell), Fe(II)-SRFA (ca. 28 µM/h, or 18.6 fM/h per cell) and Fe(II)-PPHA (ca. 14 µM/h, or 9.6 fM/h per cell). With the exception of Fe(II)-PPHA, the Fe(II) oxidation rates for all Fe(II)-OM complexes were higher than the rate for non-OM-bound free Fe(II) (ca.24 µM/h, or 15.8 fM/h per cell). Increased light intensities also increased the oxidation rates of Fe(II)-PPHA (Figure 5.2).

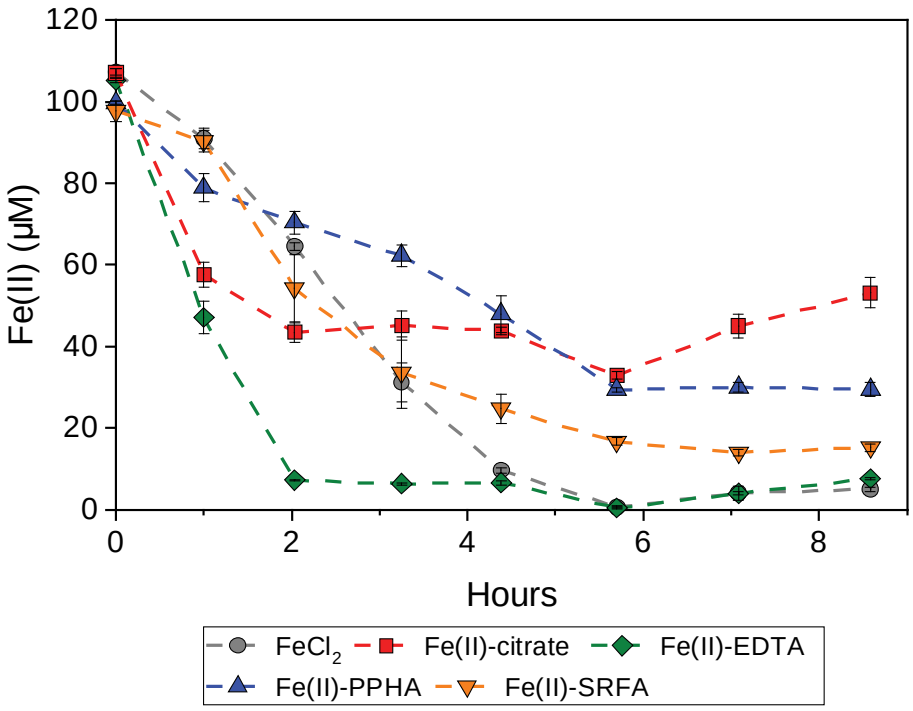

Figure 5.1 Oxidation of Fe(II) by *Rhodobacter ferrooxidans* SW2. Fe(II) was present in the form of either non-OM-bound free Fe(II), as Fe(II)-citrate, Fe(II)-EDTA, Fe(II)-PPHA complexes or Fe(II)-SRFA complexes (yellow triangle). The results are reported as an average and error bars indicate standard errors calculated from two independent parallels.

Not only the rates but also the extent of Fe(II) oxidation varied in experiments with these different Fe(II)-OM complexes (Figure 5.1). Although initially the oxidation of Fe(II)-citrate was faster than the non-OM-bound free Fe(II), the decrease in Fe(II) concentration virtually halted after 2 hours with a remaining 40% of Fe(II) after more than 8 hours of incubation. In addition to Fe(II)-citrate, Fe(II)-PPHA and Fe(II)-SRFA showed a lower extent of Fe(II) oxidation than the non-OM-bound free Fe(II). About 30% and 15% of Fe(II) still remained in the treatments with Fe(II)-PPHA and Fe(II)-SRFA, respectively. Only Fe(II)-EDTA showed a high extent of Fe(II) oxidation as the non-OM-bound Fe(II) (with <5% remaining Fe(II)).

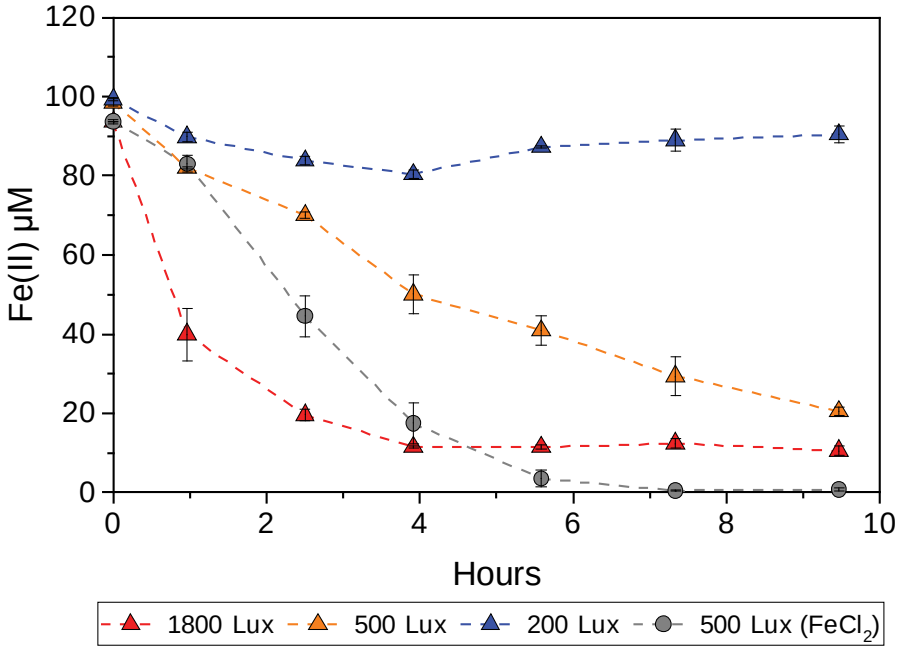

Figure 5.2 Oxidation of Fe(II)-PPHA complexes by *Rhodobacter ferrooxidans* SW2 at different light intensities of ca. 1800 lux, 500 lux, and 200 lux. For comparison, at 500 lux, duplicate treatments were amended with $FeCl_2$ without PPHA. Error bars indicate standard errors calculated from two independent parallels.

Abiotic photoreduction of Fe(III)-OM complexes

Photochemical Fe(II) reduction represents the second half of the cryptic photic Fe cycle (i.e. the Fe(II) formation). To quantify photochemical Fe(III) reduction in the presence of different OM, we incubated non-OM-bound Fe(III), Fe(III)-citrate, Fe(III)-EDTA, Fe(III)-PPHA and Fe(III)-SRFA under the same conditions as present in the cell suspension experiment (pH 7, light intensity of 550 lux) but in the absence of microbes. We found that the rates and extents of photochemical Fe(III) reduction by OM vary for the four different types of OM (Figure 5.3). Fe(III)-citrate had the fastest photochemical Fe(III) reduction rates among all the Fe(III)-OM complexes. About 20% of the Fe(III) (ca. 20 µM) was reduced to Fe(II) within 8 hours of incubation (ca. 2.5 µM/h), and Fe(III) photoreduction continued until all the Fe(III) was reduced after 2

days. Fe(III)-EDTA had the second fastest Fe(III) reduction rates with about 5.6% and 30% of the Fe(III) being reduced after 8 hours and 2 days respectively (ca. 0.7 µM/h). From Fe(III)-PPHA and Fe(III)-SRFA complexes, ca. 12 and 14 µM of Fe(II) (12 and 14% of the initial Fe(III)) were produced within 2 days (ca. 0.25 and 0.3 µM/h), respectively. This was less Fe(II) than formed from Fe(III)-citrate and Fe(III)-EDTA, but still more than in the OM-free controls that showed only 7 µM free Fe(II) within 2 days (0.15 µM/h).

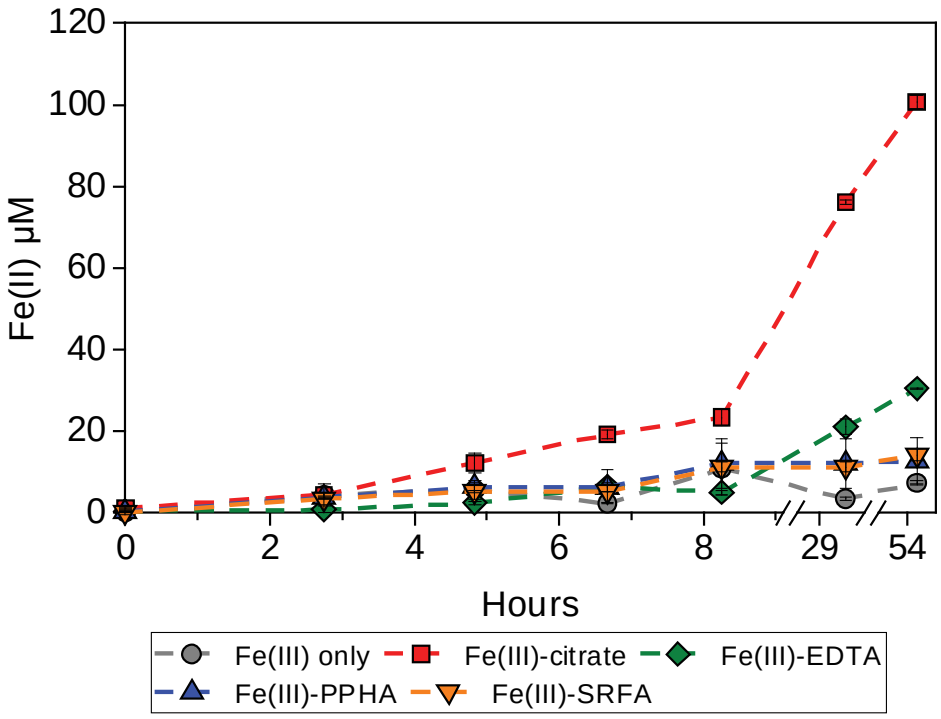

Figure 5.3 Abiotic photochemical reduction of Fe(III). Fe(III) was photochemically reduced to Fe(II) in the absence of organic chelators or in the presence of citrate, EDTA, Fe(II)-PPHA complexes or Fe(II)-SRFA complexes. Error bars indicate standard errors calculated from two independent parallels.

Cryptic cycling of Fe-citrate complexes by *Rhodobacter ferrooxidans* strain SW2

In order to determine if Fe(II) and Fe(III) can be re-cycled by microbial phototrophic oxidation of Fe(II)-OM complexes and abiotic photoreduction of Fe(III)-OM complexes, we incubated the phototrophic Fe(II)-oxidizing bacterium *Rhodobacter ferrooxidans* SW2 with either Fe(II)-citrate, Fe(III)-citrate, or citrate only, and quantified the concentrations of Fe(II) and cell numbers over time. We chose the Fe citrate system because Fe(II)-citrate showed one of the two fastest microbial Fe(II) oxidation rates (Figure 5.1), Fe(III)-citrate showed the fastest photochemical reduction rate (Figure 5.3), and citrate is an important environmental ligand which is known to be produced and released in many biotic systems, particularly by bacteria (Sandy & Butler, 2009).

The presence of bacteria had a significant impacton the Fe(II) concentration over time in the treatments with Fe(III)-citrate (Figure 5.4). We found that in the absence of phototrophic Fe(II)-oxidizing bacteria, i.e. in the abiotic controls, almost all of the Fe(III) present as an Fe(III)-OM complex was photochemically reduced to Fe(II) within 3 days of incubation. After these three days the concentration of Fe(II) remained constant at ca. 1.8 mM (Figure 5.4A). In contrast, when Fe(II)-oxidizing bacteria were present initially, there was no significant accumulation of Fe(II) during the incubation and Fe(II) stayed constant at a very low concentration (10-30 µM). When strain SW2 was incubated with Fe(II)-citrate, Fe(II) was oxidized to Fe(III) within three days of incubation, then Fe(II) remained constant at a low-µM concentration, similar to the treatments with Fe(III)-citrate and strain SW2.

Although the steady-state concentration of Fe(II) was low, we observed significant growth of strain SW2 cells independent of whether the growth medium was initially amended with Fe(III)-citrate or with Fe(II)-citrate (Figure 5.4B). In both cases, the cell abundance increased 24 and 23-fold within 14 days of incubation and

reached their maximum numbers of $6x10^8$ and $9x10^8$ cells/ml in the treatments with Fe(III)-citrate and with Fe(II)-citrate, respectively. In addition to the slightly different maximum cell numbers, the growth rates and the length of the lag phases were also different depending on whether Fe(II)-citrate or Fe(III)-citrate was provided. Growth of strain SW2 in the treatment with Fe(III)-citrate started much earlier (within 3 days of incubation) than in the treatments with Fe(II)-citrate where an obvious increase of cell numbers was observed only after 3 days (Figure 5.4B). However, despite the longer lag phase of cell growth in the treatment with Fe(II)-citrate, during the exponential phase strain SW2 grew significantly faster than in the treatment with Fe(III)-citrate. The doubling time of strain SW2 in the treatment with Fe(II)-citrate was about 2.9 days in the exponential phase, which is more than 2-fold faster than the doubling time of SW2 in the treatment with Fe(III)-citrate (ca. 6.5 days).

In the absence of Fe(II) or Fe(III) (citrate only), strain SW2 did not show any growth demonstrating that growth in the Fe(II)-/Fe(III)-citrate amended treatments was due to enzymatic Fe(II) oxidation and not based on utilization of the citrate (Figure 5.4B).

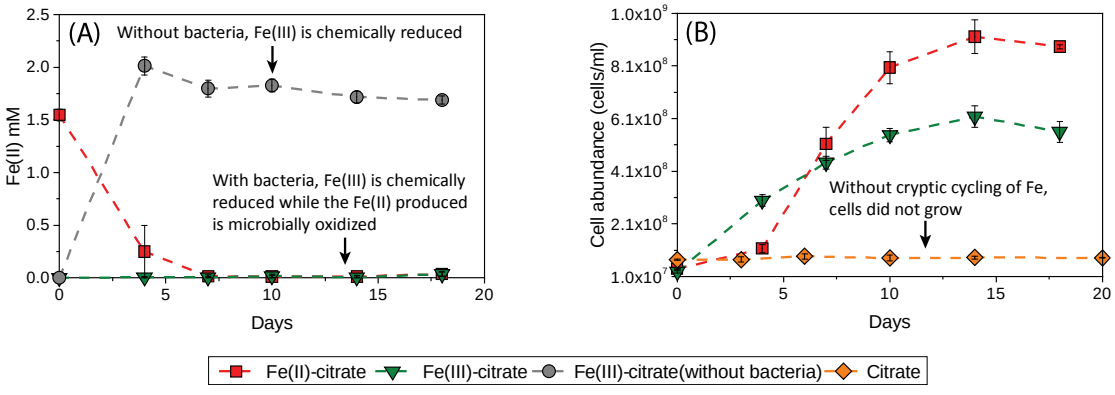

Figure 5.4 Fe(II) concentrations and cell abundances in *Rhodobacter ferrooxidans* SW2 growth experiments (20°C, light incubation). (A) Concentration of Fe(II) over time in the presence of either Fe(III)-citrate or Fe(II)-citrate. The grey symbols represent abiotic controls with Fe(III)-citrate without *Rhodobacter ferrooxidans* SW2.

(B) Cell abundance of *Rhodobacter ferrooxidans* SW2 in the growth experiment with Fe(III)-citrate, Fe(II)-citrate, and only citrate without Fe(II). The data are shown as averages of duplicate flow cytometry measurements. Error bars indicate standard errors calculated from two independent parallels.

5.5 Discussion

Cryptic Fe-cycling composed of photochemical reduction of Fe(III)-OM complexes and phototrophic Fe(II)-oxidizing bacteria

Redox cycling of Fe plays an important role for many other biogeochemical cycles, and can strongly influence the fate of pollutants and nutrients (Borch et al., 2010, Melton et al., 2014). However, Fe cycling may be masked by balanced oxidation/reduction rates which lead to low and stable iron concentrations which can be difficult to measure. Two types of cryptic Fe cycles have been identified before. Firstly, a purely abiotic Fe-cycle consisting of photochemical reduction of Fe(III)-OM and chemical Fe(II) oxidation by O_2 has been proposed (Barbeau et al., 2001). Secondly, a biotic cryptic Fe-cycle combining Fe(III)-reducing and Fe(II)-oxidizing bacteria has been observed in a stratified lake (Berg et al., 2016). Based on the results of the present study, we propose a new type of cryptic Fe cycle where Fe is cycled in photic zones by photochemical reduction of Fe(III)-OM complexes and microbial phototrophic Fe(II) oxidation (Figure 5.5). The potential for this cryptic Fe cycle is demonstrated by the fact that multiple Fe(III)-OM complexes can be photochemically reduced to Fe(II) (Figure 5.3), and the corresponding Fe(II)-OM complexes can be oxidized by phototrophic Fe(II)-oxidizing bacteria (Figure 5.1). In our experiments, the Fe(II)-/Fe(III)-citrate system showed the highest Fe turnover rates, in which the phototrophic Fe(II)-oxidizer *Rhodobacter ferrooxidans* SW2 showed significant cell growth while the steady-state concentration of Fe(II)

remained stable in the low µM range (Figure 5.4). Additionally, we were also able to confirm growth by cryptic Fe cycling when both Fe(II)-citrate and Fe(III)-citrate complexes were initially present together in the medium (Figure S5.1).

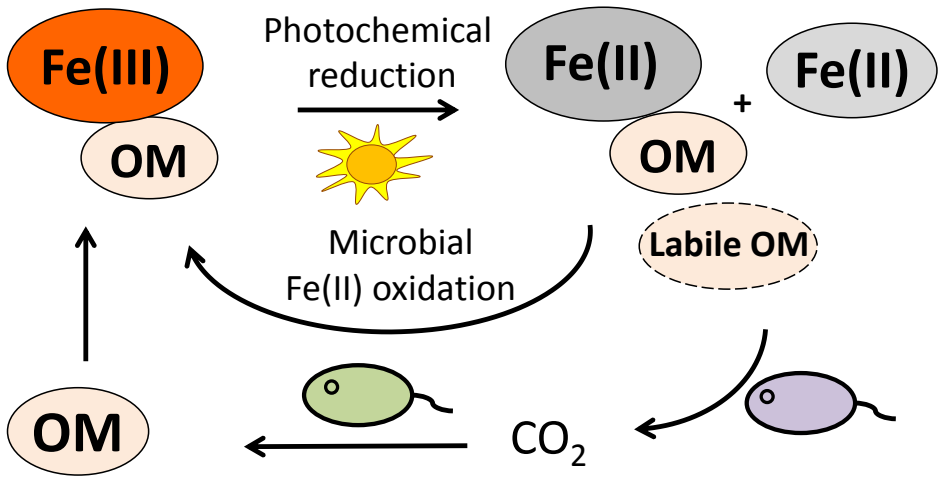

Figure 5.5 Proposed mechanism of cryptic Fe cycling by *Rhodobacter ferrooxidans* SW2. Fe(III) is photochemically reduced to Fe(II) in the presence of OM producing Fe(II)-OM complexes and labile OM. The Fe(II) produced, including free Fe(II) and Fe(II)-OM complexes, can be re-oxidized to Fe(III) by microbial phototrophic Fe(II) oxidation. The electrons from microbial Fe(II) oxidation are used to fix CO_2, therefore potentially further increasing the content of OM and thus stimulating further photochemical Fe(III) reduction. During Fe(III) photochemical reduction of Fe(III)-OM complexes, part of the photolysed OM may become labile and can serve as carbon and energy source for the growth of bacteria.

Consequences of photochemical reduction of Fe(III)-OM

Several abiotic and biotic reactions are involved in the photic cryptic Fe cycle with OM and bacteria. First of all, abiotic photochemical Fe(III) reduction drives the reductive side of the cryptic Fe cycle. In our study, different Fe(III)-OM complexes

showed different rates of photochemical Fe(III) reduction (Figure 5.3), supporting previous findings that different Fe(III)-OM complexes have different half-life times in light (Svenson *et al.*, 1989, Faust & Zepp, 1993, Voelker *et al.*, 1997). These different photochemical reduction rates for different Fe(III)-OM complexes could be due to several reasons such as the type and abundance of different functional groups, as well as the structure of the Fe-OM complexes (Barbeau, 2006). For example, the abundance of highly reactive alpha-hydroxyl carboxylate groups (Barbeau *et al.*, 2003, Miller *et al.*, 2013) present in citrate could explain why Fe(III)-citrate had higher photochemical reduction rates than the other Fe(III)-OM complexes in our experiments.

Regardless of how fast the reduction of different Fe(III)-OM was, Fe(III) reduction reactions are accompanied with the transformation or loss of functional groups of the organic matter (Barbeau, 2006). For example, during the reduction of Fe(III)-citrate to Fe(II), citrate is oxidized forming acetone and carbon dioxide via several intermediates, e.g. *α*-ketoglutarate (Frahn, 1958, Buchanan, 1970, Abrahamson *et al.*, 1994). The general reaction could be represented as:

$$C(OH)(COOH)(CH_2COOH)_2+2Fe^{3+}+h\nu \rightarrow CH_3COCH_3+2Fe^{2+}+3CO_2+2H^+$$

These organic compounds formed as the products of OM-photolysis could also serve as a carbon and electron source for bacteria, as was previously demonstrated in *Rhodobacter capsulatus* SB1003, a bacterium which could not oxidize dissolved non-OM-complexed Fe(II) (Croal *et al.*, 2007) but could grow on Fe(III)-citrate utilizing the products of Fe(III)-citrate photochemical reactions (Caiazza *et al.*, 2007). However, control experiments showed that *Rhodobacter ferrooxidans* SW2 did not grow on 1 mM acetone (Figure S5.2), the product of photochemical citrate degradation. Therefore, it is not possible that the cells grow by oxidizing only the OM without microbial oxidation of Fe(II). if the microbes did not re-oxidize Fe(II) to Fe(III), there would be Fe(II) accumulation as shown in our abiotic controls with Fe(III)-citrate

without cells (Figure 5.4). This clearly showed that the Fe(II) is microbially re-oxidized thus closing the cryptic iron cycle. Overall, those organic compounds formed by photochemical Fe(III) reduction may also have stimulated the growth of some other heterotrophic bacteria in the environment, but it did not prevent the phototrophic Fe(II) oxidation microbes from oxidizing Fe(II) (Figure 5.4A).

Microbial oxidation of Fe(II) in the presence of OM

So far it was unknown whether phototrophic Fe(II)-oxidizing bacteria, e.g. those that can oxidize and grow on non-OM-complexed Fe(II), are also capable of oxidizing Fe(II)-OM complexes. The results of this study (Figure 5.1 and 5.4) demonstrated that Fe(II)-OM complexes can be oxidized by the phototrophic Fe(II)-oxidizing bacterium *Rhodobacter* ferrooxidans strain SW2. This is interesting because strain SW2 was proposed to oxidize Fe(II) intracellularly with a putative iron oxidase FoxE embedded in the periplasm of the cell (Croal et al., 2007, Pereira et al., 2017). The observed oxidation of large Fe(II)-OM complexes, such as the Fe(II)-PPHA and Fe(II)-SRFA complexes which were determined to be in the colloidal size range (Liao et al., 2017), suggests that strain SW2 may also be able to oxidize Fe(II) extracellularly, similar to many other bacteria with enzymatic Fe(II) oxidation pathways (Shi et al., 2016), since the large Fe(II)-PPHA and Fe(II)-SRFA complexes may not be easily transported into the periplasm.

The slightly slower oxidation of Fe(II)-PPHA is probably due to the dark color of the Fe(II)-PPHA complex which absorbs light thus lowering the light intensity that is available for the phototrophic microorganisms (Figure 5.2). The different rates of Fe(II) oxidation for the different Fe(II)-complexes (compared to free Fe(II)) may also have several other explanations. For example, the reactivity of Fe(II) and Fe(II)-OM complexes with the Fe(II)-oxidizing proteins may be different (Liu et al., 2012), or complexation of Fe(II) by OM may be beneficial for other microbial processes involved in Fe(II) oxidation as well. One possible benefit of the presence of organic

ligands for the bacteria could be that complexation of the product of Fe(II) oxidation, i.e. the Fe(III), by the OM keeps the Fe(III) in solution and thus may negate the need to protect the cell from Fe(III) mineral encrustation. The cells thus may not need to synthesize organic polymer fibers which keep the Fe(III) minerals away from the membrane (Miot et al., 2009) as the Fe(III)-OM complexes are water soluble.

In addition to the rates, also the extents of microbial Fe(II) oxidation were different for the different Fe(II)-OM complexes (Figure 5.1). This can have several reasons, since many parameters change when Fe(II) is complexed by different types of OM and at different concentrations of Fe(II)-OM and Fe(III)-OM complexes. These parameters include potential differences in Fe-speciation and/or in the structure and size of the Fe(II)-OM complexes (Liao et al., 2017), varying redox potentials, different availability of Fe(II)- and Fe(III)-binding functional groups of the humic and fulvic acids (Tipping et al., 2011), and also different Fe(III)-OM photoreduction rates (Kuma et al., 1992). All these differences are expected to influence not only the rates but also the extent of microbial Fe(II) oxidation, as also shown in an experiment with different concentrations of Fe(II)-OM complexes (Figure S5.3).

Environmental/Geochemical implications

It is likely that the photic cryptic Fe-cycle we describe here also exists and is widespread in many natural habitats. Firstly, microbial Fe(II) oxidation is widespread, and phototrophic Fe(II)-oxidizing bacteria were commonly found in many environments including lakes, soils, and freshwater and marine sediments (Ehrenreich & Widdel, 1994, Melton et al., 2014, Laufer et al., 2016, Bryce et al., 2018, Otte et al., 2018). Secondly, Fe-OM complexes are abundant in the environment and many biologically produced Fe-chelating siderophores contain alpha-hydroxy carboxylate groups which can reduce Fe(III) to Fe(II) in the light (Haygood et al., 1993, Reid et al., 1993, Martinez et al., 2000, Barbeau et al., 2001, Barbeau et al., 2002, Barbeau et al., 2003). While in our batch growth experiment Fe

cycling is limited by the amount of initially present citrate (that allows photoreduction and thus the regeneration of Fe(II) as substrate for the Fe(II)-oxidizing microorganisms), in the environment the organic ligands (e.g. citrate) are constantly produced and therefore would allow continuous Fe cycling (Mimmo et al., 2014, Lehmann & Kleber, 2015). Future studies determining the concentration of organic ligands needed for maintaining photochemical Fe(III) reduction are required to evaluate the relevance of cryptic cycling of Fe(III)-OM in different environments.

In the environment, cryptic cycling of Fe could also greatly influence other biogeochemical cycles (Raiswell & Canfield, 2012), such as cycling of Mn-species and As-species which co-occur with Fe in many anoxic environments (Thamdrup, 2000, Zhu et al., 2017). The cryptic cycling of Fe may also influence the transport of toxic metals in the form of Fe-OM-Metal complexes, as the Fe(III)-/Fe(II)-OM complexes may have different binding capacities and binding mechanisms to heavy metals (Sharma et al., 2011, Catrouillet et al., 2016). The re-oxidation of Fe(II)-OM by phototrophic Fe(II)-oxidizing bacteria could not only increase the extent of Fe(III) photochemical reduction, but also result in a larger extent of photolysis of OM and generation of labile OM for the growth of heterotrophic bacteria (Figure 5.5). This could potentially influence microbial community composition as a whole, since the cryptic cycling of Fe could not only support the growth and carbon fixation of Fe(II)-oxidizing bacteria in habitats with low Fe(II) concentration, but also continuously promote release of labile carbon for a heterotrophic bacterial community. Overall, the results of this study suggest that, despite low steady-state concentrations of Fe, Fe cycling in photic zones of aqueous and terrestrial habitats could be more prominent and have a larger influence on carbon cycling than expected based on the low Fe concentrations alone.

5.6 Supporting information

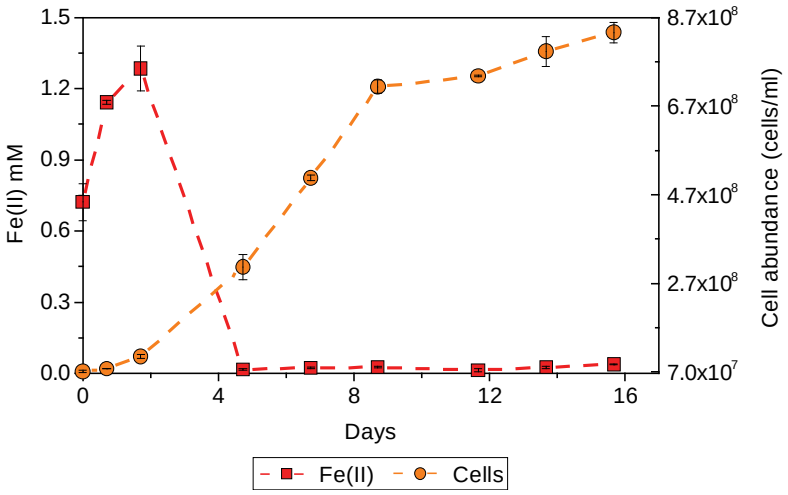

Figure S5.1 Fe(II) concentration and abundance of *Rhodobacter ferrooxidans* SW2 cells in growth experiments (20°C, light incubation) using medium that initially contained both Fe(II) and Fe(III) (ca. 0.8 mM each) and 4 mM citrate.

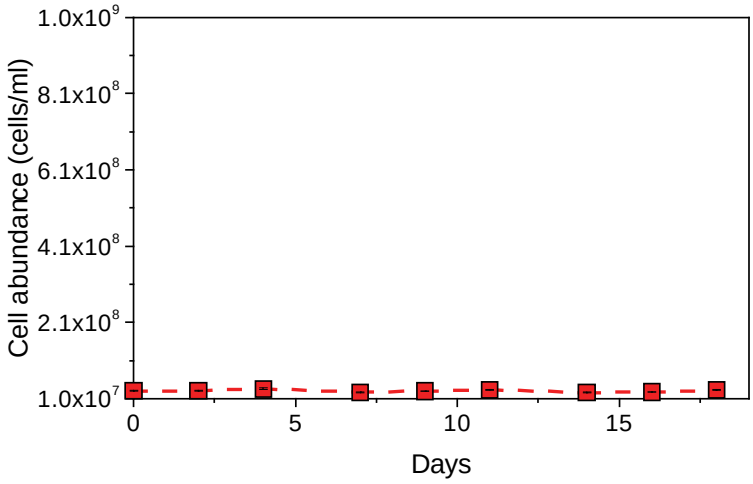

Figure S5.2 Cell abundance of *Rhodobacter ferroxidans* SW2 during incubation with 1 mM acetone showing that there was no significant growth of *Rhodobacter ferroxidans* SW2 on acetone.

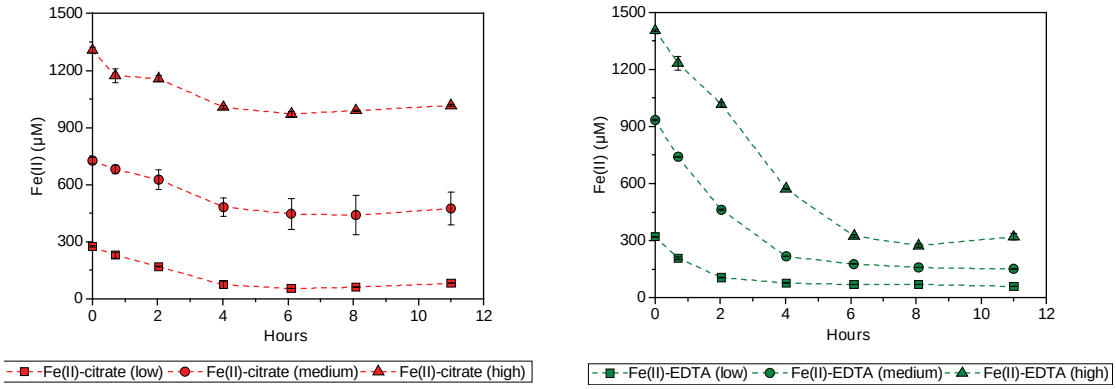

Figure S5.3 Oxidation of Fe(II)-citrate (left) and Fe(II)-EDTA (right) at three different Fe(II)-complex concentrations by *Rhodobacter ferrooxidans* SW2 (1.4x10^8 cells/ml). The initial Fe(II) concentrations varied from ca. 300 µM to 1300 µM. The results are reported as an average and error bars indicate standard errors calculated from two independent parallels.

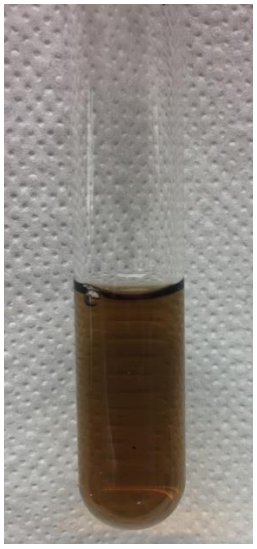

Figure S5.4. Fe(II)-PPHA complexes (0.1 mM Fe(II) and 0.2 mg/ml PPHA) in Hungate tubes. The PPHA has a dark color and absorbs light thus lowering the light intensity available for the phototrophic microorganisms.

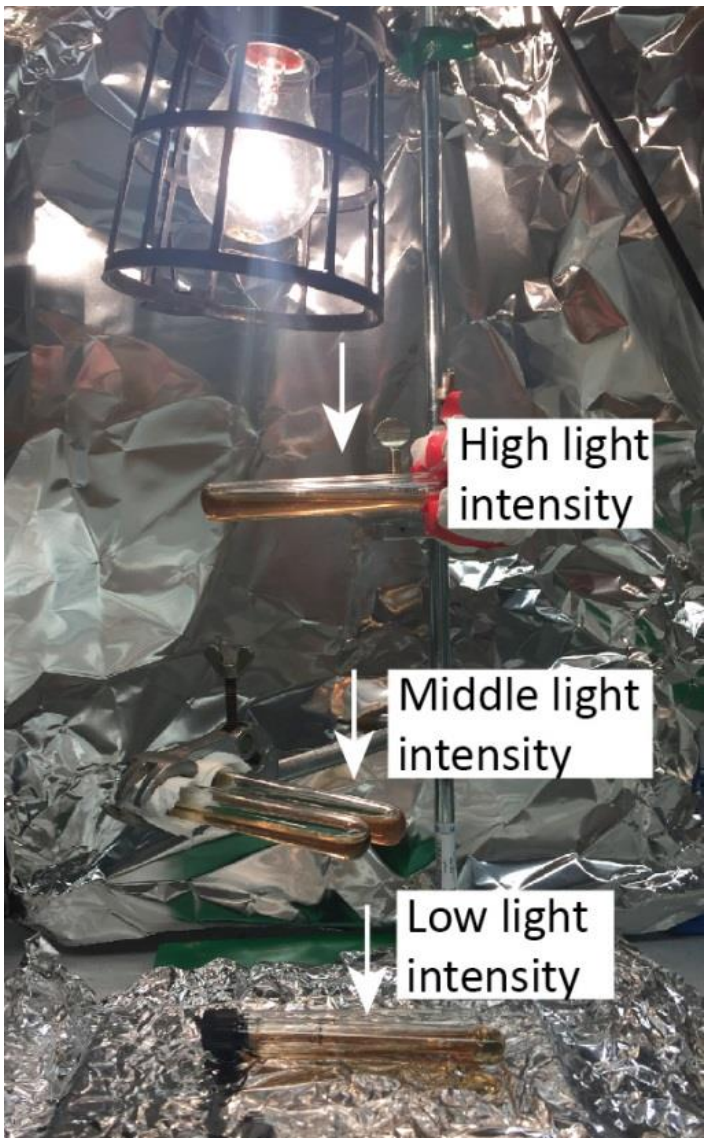

Figure S5.5. Photograph of an experiment to determine how light intensity influences the oxidation of Fe(II)-PPHA complexes by *Rhodobacter ferrooxidans* SW2.

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6. General conclusion and discussion

6.1 Effect of Fe(II)-OM complexation on bacterial Fe(II) oxidation

The work presented in this thesis showed that the influence of Fe(II)-OM complexes on bacterial Fe(II) oxidation strongly depends on the type of Fe(II)-oxidizing bacteria (Figure 6.1). Chapter 3 showed that Fe(II)-OM complexation inhibited the oxidation of Fe(II) by nitrate-reducing Fe(II)-oxidizing bacteria *Acidovorax* sp. BoFeN1. The oxidation of Fe(II)-OM complexes by *Acidovorax* sp. BoFeN1 was much slower compared to the oxidation of free Fe(II). Chapter 4 showed that in contrast to nitrate-reducing Fe(II)-oxidizing bacteria, the oxidation of Fe(II) by phototrophic Fe(II)-oxidizing bacteria *Rhodopseudomonas palustris* TIE-1 was significantly accelerated by all the Fe(II)-OM complexes tested, including Fe(II)-citrate, Fe(II)-EDTA and Fe(II)-PPHA and Fe(II)-SRFA. In addition, different phototrophic Fe(II)-oxidizing bacterial species with slightly different enzymatic Fe(II) oxidation mechanisms (Chapter 3, 4, 5) may respond differently to Fe(II)-OM complexation. Compared to *Rhodopseudomonas palustris* TIE-1, *Rhodobacter ferrooxidans* SW2 oxidized Fe(II)-OM complexes only slightly faster than free Fe(II). Therefore it is important to consider the type of Fe(II)-oxidizing bacteria and their oxidation mechanisms when it comes to the effect of Fe(II)-OM complexation on microbial Fe(II) oxidation.

Moreover, the work presented in this thesis thesis showed that the types and speciations of Fe(II)-OM also play a critical role in determining the rate and extent of microbial Fe(II) oxidation. Chapter 3 showed that fully complexed Fe(II) inhibits Fe(II) oxidation by NRFeOx, however, the extension of this inhibition effect is different among different OMs. The inhibition effect of Fe(II)-PPHA complexes on Fe(II) oxidation was stronger than that of Fe(II)-SRFA complexes and the other simpler

Fe(II)-OM, such as Fe(II)-EDTA and Fe(II)-citrate complexes. However, although Fe(II)-EDTA and Fe(II)-citrate have a weaker inhibition effect on Fe(II) oxidation by NRFeOx compared to Fe(II)-PPHA and Fe(II)-SRFA complexes, they had a much stronger promotion effect on the Fe(II) oxidaiton by phototrophic Fe(II)-oxidizing bacteria *Rhodopseudomonas palustris* TIE-1 (Chapter 4). The oxidation of Fe(II)-citrate and Fe(II)-EDTA complexes was significantly faster than the oxidation of Fe(II)-PPHA and Fe(II)-SRFA. These different Fe(II)-OM complexes all have similar mechanisms of binding with Fe(II), mainly via carbonyl functional groups (Daugherty *et al.*, 2017), however they differ in many chemical properties such as redox potentials, stabilities, charges and sizes. Those chemical properties were thought to be one of the main reasons for the different rates and extents of microbial oxidation of Fe(II) (Chapter 3, 4). However, though the existence of Fe(II)-OM complexes in the environment have been proved, little is known about the chemical properties of those Fe(II)-OM complexes. To further understand the effect of Fe(II)-OM complexation on microbial Fe(II) oxidation in environment, future studies determining the speciation of Fe(II)-OM complexes and their chemical properties are needed.

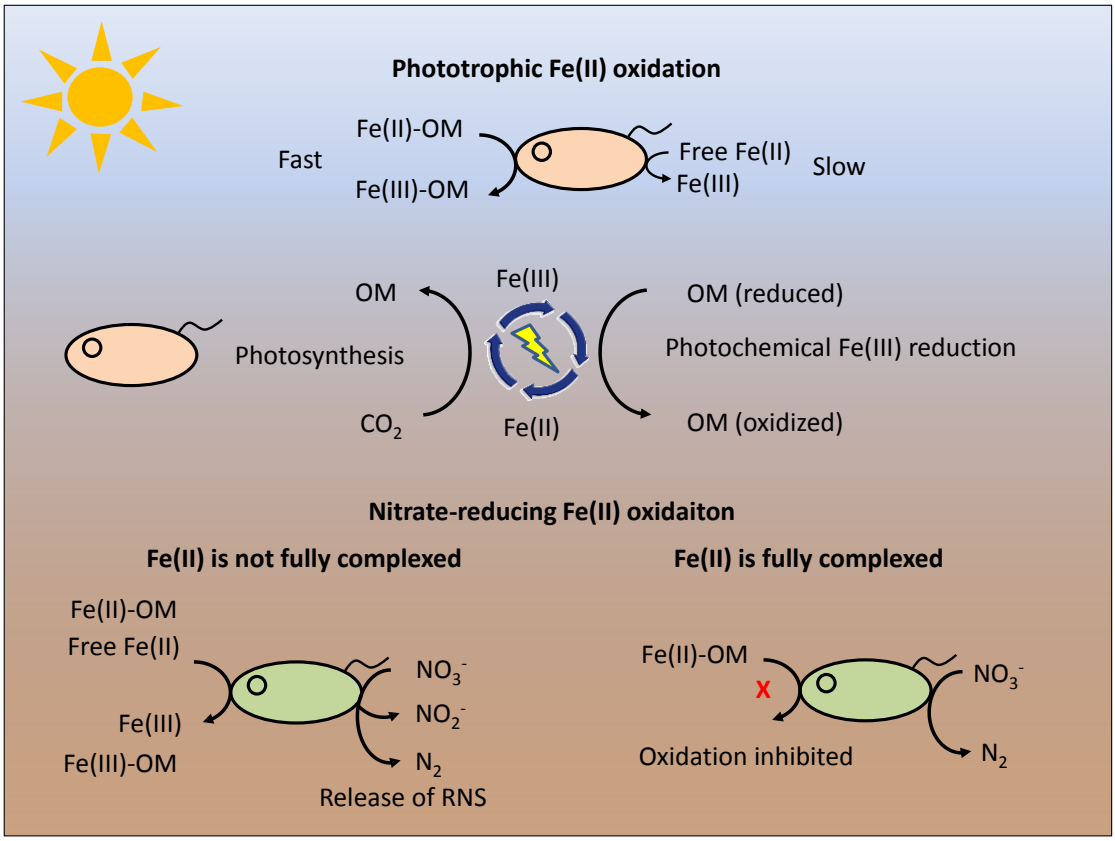

Figure 6.1 Summary of the influence Fe(II)-OM complexation on the different Fe(II)-oxidizing bacteria. The influences of Fe(II)-OM complexation on microbial oxidation of Fe(II) strongly depend on the type of Fe(II)-oxidizing bacteria and the environmental conditions, such as the chemical properties of Fe(II)-OM complexes and the speciation of Fe(II), e.g. whether Fe(II) is fully complexed by OM.

6.2 The crosslink between Fe(II) oxidation and denitrification

Results in this thesis revealed that the percentages of free Fe(II) and Fe(II)-OM complexes played an important role in regulating the Fe(II) oxidation rates and the accumulation of nitrite. The previously proposed Fe(II) oxidation mechanisms by NRFeOx could not explain the result in presented this thesis (Chapter 3). For example, the previous proposed mechanisms could not explain why Fe(II)-EDTA promotes chemical oxidation of Fe(II) by nitrite, but the microbial oxidation of fully complexed

Fe(II)-EDTA with NRFeOx is inhibited, as even for NRFeOx a large portion of Fe(II) is supposed to be abiotically oxidized by the reactive nitrogen species. Based on the experimental result, this thesis proposed a revised mechanism of Fe(II) oxidation by NRFeOx and hypothesized as to how Fe(II)-OM complexation influences the oxidation mechanism (Chapter 3). This proposed mechanism of Fe(II) oxidation by the NRFeOx indicates that microbial Fe(II) oxidation and denitrification are much more linked with each other than previously understood. As shown in Chapter 3, the oxidation of Fe(II) was found to influence the microbial denitrification systems ($NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$) and lead to the accumulation of nitrite.

Compared to the previous model of Fe(II) oxidation by NRFeOx, there are two new factors that need to be taken into consideration:

1) At least a part of the Fe(II) in the solution has to be transported into the periplasm to initiate the oxidation of Fe(II) by NRFeOx.

2) In the periplasm, non-organically-bounded, free Fe(II) and its oxidation can influence the microbial denitrification system in a way which leads to the accumulation of RNS such as nitrite (NO_2^-).

These two factors emphasize the importance of free Fe(II) in both the Fe(II) oxidation and denitrification systems. Several explanations were listed in chapter 3. The key argument is that free Fe(II) may more easily enter the periplasm and thus can disrupt the stability of microbial denitrifying system. Once Fe(II) is oxidized in the periplasm, poorly soluble Fe(III) minerals will precipitate on critical periplasmic proteins. This precipitation of Fe(III) minerals on the nitrite (NO_2^-) reductase would probably lead to an imbalance in nitrate reduction and nitrite reduction rates, which then lead to the abiotic oxidaiton of Fe(II) by nitrite. Whereas the nitrate (NO_3^-) reductase is embedded in the cytoplasmic membrane thus may be less influenced by the mineral precipitates on proteins in the periplasm. These effects are expected to be much weaker when OM ligands are present. Because, on one hand, Fe(II)-OM

complexation may inhibit the transport of Fe(II) into the periplasm, as when Fe(II) is in the form of Fe(II)-OM, the overall charge of Fe(II)-OM molecule is expected be more neutral or negative than free Fe(II), and the size of the Fe(II)-OM complexes is expect to be larger than free Fe(II). One the other hand, as the OM could also complex Fe(III) and keep it in the dissolved form, there would be less precipitation on these critical proteins in the periplasm. Therefore, as a result, fully complexed Fe(II)-OM complexes ultimately lead to the inhibition of nitrate-reducing Fe(II) oxidation.

6.3 Locations of biological and chemical reactions in NRFeOx

Another important concept in this thesis is the importance of the locations of Fe(II) oxidation reactions, especially for the NRFeOx where both biological and chemical reactions occur simultaneously. This is because that all the detitrifying proterins are located inside the cell (Madigan *et al.*, 2010). There is a cell membrane outside the denitrifying proteins and it works like a door allowing some compounds pass but keeps some out. However, chemicals including Fe(II), nitrate and RNS, which we added into the experimental system or we measured during the experiment were all in the solution outside the cell membrane. It is debatable if the chemical conditions in the periplasm may be different from the conditions we determine in the solution (Figure 6.2). Because inside the cells, pH, concentrations of different chemical compounds and the type of catalyst could be very different from what we measured in the solution outside the cells (Wilks & Slonczewski, 2007). Therefore characterizing the chemical conditions within the periplasm is needed in future studies.

This concept could also explain the contrasting results of the purely abiotical Fe(II) oxidation by nitrite and the Fe(II) oxidaiton by NRFeOx of which large extend of

Fe(II) is supposed to be oxidized abiotically (Carlson *et al.*, 2013, Klueglein *et al.*, 2014). For example, several studies have shown that chemical Fe(II) oxidation rates, even in the presence of dead cells and minerals as catalysts, were still several orders lower than Fe(II) oxidation rates with live nitrate-reducing bacteria and nitrate (Klueglein *et al.*, 2014, Chen *et al.*, 2017). In addition, as the results in this thesis have shown, the accumulated nitrite in the medium is not necessary to start the abiotic oxidation of Fe(II) by RNS (Chapter 3). Nitrite produced during denitrification can be consumed not only chemically by Fe(II) oxidation but also biotically in the periplasm of the denitrifying bacteria before it gets released into the solution (Figure 6.2).

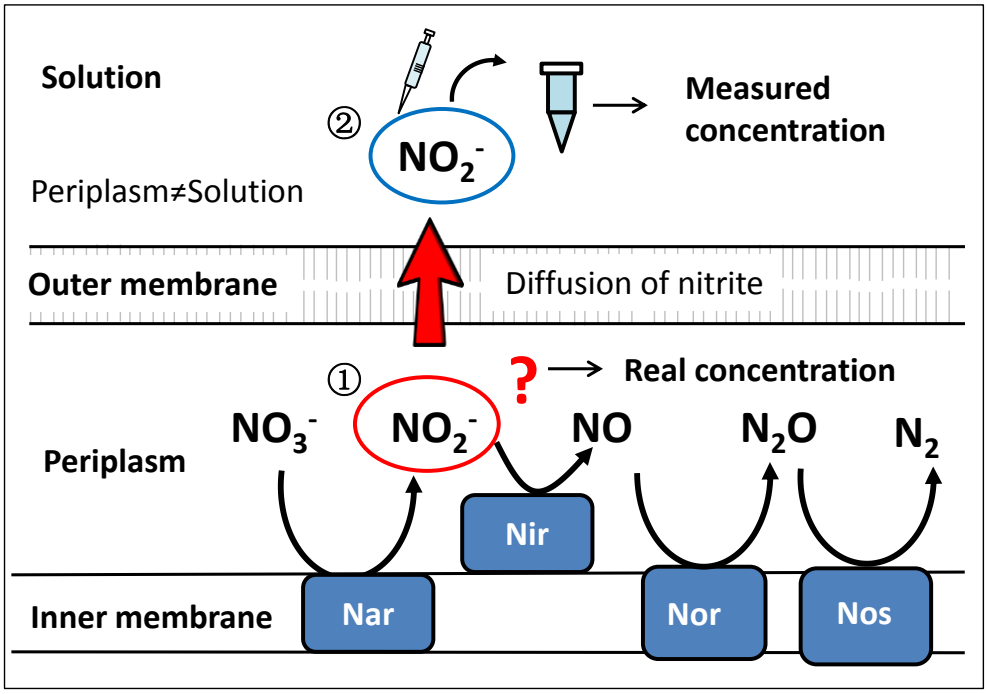

Figure 6.2 A simplified model of bacterial denitrification process, including the limitation of the sampling process. Part of the nitrite (①, with a red circle in the graph) produced in the denitrification respiratory chain could oxidize part of the Fe(II) in the periplasm. While part of the nitrite may be released to the solution via the bacterial outer membrane. This released nitrite (②, with a blue circle in the graph) is what has been measured with current sampling method, e.g. directly determing the concentration of nitrite in the solution. The concentrations of nitrite in the periplasm

and in the solution are not necessarly the same. The graph shows the limitation of nitrite measurement with current sampling technique.

6.4 Phototrophic Fe(II) oxidation

It is interesting that Fe-OM complexation has a distinctly different effect on phototrophic Fe(II)-oxidizing bacteria than NRFeOx bacteria, because these two types of Fe(II)-oxidizing bacteria have different mechanisms of Fe(II) oxidation (Chapter 2). Fe(II)-OM complexation could promote the oxidation of Fe(II) in two ways. This effect could be both directly increasing the oxidation rates (Chapter 4) and indirectly increasing the total amount of Fe(II) oxidized by phototrophic Fe(II)-oxidizing bacteria via reducing Fe(III) back (Chapter 5). Independent of how Fe(II)-OM complexation promoted the oxidation of Fe(II), the fast oxidation of Fe(II) by phototrophic Fe(II)-oxidizing bacteria in the presence of OM ligands indicates that the contribution of phototrophic Fe(II)-oxidizing bacteria to Fe biogeochemical cycling could be much more than previously expected. It is therefore necessary to reevaluate the contribution of phototrophic Fe(II)-oxidizing bacteria to the cycling of Fe in the environment.

Although no further investigation is conducted in this thesis to explain how phototrophic Fe(II) oxidation is promoted by Fe(II)-OM complexation, Chapter 4 has listed several possible reasons explaining this effect. Among these possible explanations, the most likely reason may lie in the different reaction kinetics of Fe(II)-OM with bacterial c-type cytochromes and/or the accessibility of the cytochrome to the Fe(II)-OM. In addition, when Fe(II) is in the form of Fe(II)-OM complexes, the oxidation of Fe(II) is probably not limited to the previously identified putative Fe(II) oxidoreductases like PioA (Jiao & Newman, 2007) and FoxE (Croal *et al.*, 2007), because Fe(II)-OM complexation changed Fe(II)/Fe(III) redox potential and

accessibility. Although the exact mechanism by which OM complexation promoted the oxidation rates of Fe(II) is still unknown, it is reasonable that bacteria have this preferential oxidation of Fe(II)-OM complexes, as Fe(II)-OM complexation may bring a physiological benefit. For example, OM complexation with Fe(III) could keep Fe(III) in the dissolved phase to avoid encrustation of the cell.

Beside the promotion of photrophic Fe(II) oxidation by Fe(II)-OM complexation, another interesting finding in this thesis is the oxidation of Fe(II)-OM complexes by *Rhodobacter ferrooxidans* SW2, expecially the large Fe(II)-PPHA complexes. This result contradicts the current understanding of the Fe(II) oxidation mechanism of *Rhodobacter ferrooxidans* SW2 (Chapter 2). In the current model of Fe(II) oxidation by *Rhodobacter ferrooxidans* SW2, Fe(II) is thought to be oxidized intracellularly with a putative iron oxidase FoxE in the periplasm of the cell (Croal *et al.*, 2007). However, Fe(II)-OM complexes, especially the colloidal Fe(II)-PPHA complexes, had sizes between 3-200 nm (Aiken *et al.*, 2011, Liao *et al.*, 2017) thus it is likely that these colloid Fe(II)-OM complexes may not be easily transported between the solution and periplasm (Figure 6.3). One possibility is the bacteria may also be able to oxidize Fe(II) extracellularly with another set of Fe(II) oxidation enzymes. Future studies to determine the Fe(II)-oxidizing mechanism of those phototrophic bacteria are necessary.

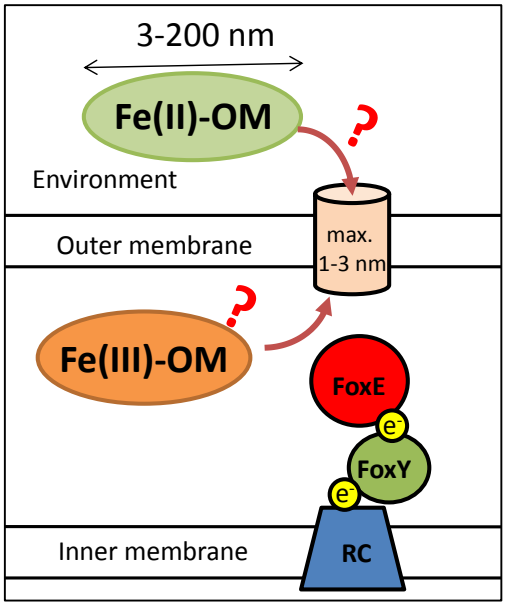

Figure 6.3 The questionable mechanism of Fe(II) oxidation by *Rhodobacter ferrooxidans* SW2. Previous studies suggested that *Rhodobacter ferrooxidans* SW2 oxidize Fe(II) intracellularly with a putative Fe(II) oxidase FoxE in the periplasm of the cell. However, this contradicts the results of this thesis that large Fe molecules like colloidal Fe(II) were still able to be oxidized.

6.5 The design of experiments

This thesis is an another example showing the importance of experimental design in studying microbial Fe(II) oxidation, especially with of Fe(II)-OM complexes. The primary and original goal of this thesis is to determine whether Fe(II)-oxidizing bacteria are able to oxidize Fe(II)-OM complexes which are present in many environments. To reach this objective, we synthesized Fe(II)-OM complexes and designed a set of experiments which are suitable to study the microbial oxidation of Fe(II)-OM complexes. The method development is necessary as Fe(II)-OM complexes were not stable in nearly all the medium previously designed to grow anaerobic bacteria (Emerson & Merrill Floyd, 2005) according to theromodynamic calculation using PHREEQC with humic ion-binding model VI (Catrouillet *et al.*, 2014). In many

previously designed media for anaerobic bacteria, several chemicals such as phosphate (PO_4^{3-}) and bicarbonate (HCO_3^-), have relatively high concentrations in the mM level in order to stimulate and support the fast growth of bacteria. However, those nutrients usually are not present in such a high concentrations in natural environments and they could interfere with the binding between Fe(II) and OM, as they could form minerals with Fe(II), like vivianite ($Fe_3(PO_4)_2 \cdot 8H_2O$), and siderite ($FeCO_3$). Therefore, we designed experiments to study microbial oxidation of Fe(II)-OM complexes, only basic compounds that are necessary for the microbial Fe(II) oxidation are included in the solution. Specifically, in addition to Fe(II), a pH buffer (PIPES) and NaCl, for NRFeOx, the solution only contains nitrate and acetate (Chapter 3), and for phototrophic Fe(II)-oxidizing bacteria, the solution contains only a small amount (1 mM) of bicarbonate and 0.5 % CO_2 in the headspace of the container (Chapter 4 and 5). We have examined that 1 mM inorganic carbon is enough for the microbial phototrophic Fe(II)-oxidation while Fe(II) is still in the form of Fe(II)-OM complexes. However, as bacteria could not grow in non-growth medium in the absence these compounds, e.g. no phosphate (PO_4^{3-}) and calcium (Ca^{2+}), the cells were cultivated to the late exponential phase in the general growth medium without adding Fe(II) then harvested and transferred into the solution we designed.

The experimental approaches used in the thesis allowed us to study the microbial oxidation of Fe(II) not only when Fe(II) was fully complexed by OM, but also when both free Fe(II) and Fe(II)-OM complexes were present in the medium. It is important to consider the second situation that free Fe(II) and Fe(II)-OM complexes are both present in the solution, because these two species could always co-exist in natural environments. This is supported by the results in Chapter 3 of this thesis which showed that the oxidation of Fe(II) and the release of nitrite by NRFeOx were dramatically different when there was only Fe(II)-OM complexes and when there were both Fe(II)-OM and free Fe(II) in the solution. In summary, due to the relatively low stability of Fe(II)-OM complexes in the solution and the

Fe(II)-speciation-dependent microbial oxidation of Fe(II), more attention should be paid to the experimental design when studying the microbial oxidation of Fe(II)-OM complexes in future.

6.6 General environmental implications

Results presented in this thesis revealed that OM complexation of Fe(II) could play a significant role in anaerobic microbial Fe(II) oxidation, and that different Fe(II)-OM complexes can have different effects on different types of microbial Fe(II) oxidation. Because Fe(II)-OM complexes have been identified in many different environments including soils, rivers and sediments (Luther *et al.*, 1996, Kleja *et al.*, 2012, Sundman *et al.*, 2013, Sundman *et al.*, 2014, von der Heyden *et al.*, 2014, Hopwood *et al.*, 2015, Bhattacharyya *et al.*, 2018), and microbially mediated Fe(II) oxidation is a dominant pathway in Fe cycling under various environmental conditions (Melton *et al.*, 2014), Fe(II)-OM complexation is expected to have a profound influence on the fate of Fe in natural environments. For example, the colloidal Fe(II)-OM complexes could be a sink for Fe(II) in the soil and sediment. Because this fraction of Fe was found to be protected against abiotic oxidation by oxygen (Daugherty *et al.*, 2017), decrease in the bioavailability of Fe (Oleinikova *et al.*, 2017), and inhibit Fe(II) oxidation by nitrate-reducing Fe(II)-oxidizing bacteria (Chapter 3), and be resistant to fast microbial phototrophic Fe(II) oxidation (Chapter 4).

Moreover, the influence of Fe(II)-OM complexation on microbial Fe(II) oxidation are not only limited to the oxidation states of Fe, Fe(II)-OM complexation may also influence many other environmental processes:

Firstly, results presented in the thesis suggested that Fe(II)-OM complexation could further contribute to the transport of heavy metals in the environment via

influencing anaerobic microbial Fe(II) oxidation. The results showed that the complexation of Fe(II)-OM strongly influenced the microbial Fe(II) oxidation reactions, these influences in turn would affect the binding between the heavy metals and Fe(III)-OM complexes or Fe(III) minerals (Chapter 2), and thereby affect the mobility and transportation of these toxic metals in Fe- and OM-rich anoxic environments.

Secondly, results in this thesis indicate that Fe plays an even more important role in the turnover of carbon in the environment than previously expected. Because photoautotrophic Fe(II) oxidation provides electrons during photosynthesis for CO_2 fixation (Widdel *et al.*, 1993, Jiao & Newman, 2007, Bird *et al.*, 2011), the stimulated Fe(II) oxidation by OM complexation may also promote the fixation of CO_2 in OM-rich environmental habits, such as surface layers of rice paddy soils and peatlands. In addition to CO_2 fixation, the re-oxidation of Fe(II)-OM by phototrophic Fe(II)-oxidizing bacteria could also result in a larger extent of photolysis of OM (Chapter 5). These two processes together form not only a cryptic Fe cycle but also a continuous transformation of the OM in the environment.

Last but not least, results in this thesis suggest a stronger crosslink between the microbial iron and the nitrogen cycles in the environment. For example, the results showed that the accumulation of nitrite, a toxic reactive nitrogen species (Fan & Steinberg, 1996), depends on whether Fe(II) is available in its free Fe(II) form or is complexed by OM. This result may explain the different ratios of microbial nitrate reducing rates to Fe(II) oxidation rates in the sediments with different the OM concentrations (Laufer *et al.*, 2016), as there may be different amount of Fe(II)-OM complexes and free Fe(II) in these sediments. In addition, Fe(II)-OM complexation may influence the global warming. Because nitrite only accumulated during the oxidaiton of free Fe(II), and the reactions of Fe(II) and nitrite produce an important greenhouse gas N_2O which is 300 times more potent than CO_2 for global warming (Jones *et al.*, 2014, Buchwald *et al.*, 2016).

Taken all together, these new findings in this thesis showed that Fe(II)-OM complexes has a profound and diverse influences on anarobic microbial Fe(II) oxidation. These new findings improve our understanding of microbial Fe-cycling and highlight the importance of Fe(II)-OM complexation in many environmental processes.

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Statement of personal contribution

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Experiments were conceptualized by myself or together with Prof. Andreas Kappler and/or Dr. Casey Bryce and/or Dr. Anneli Sundman and were carried out by me. The discussion and analysis of the obtained result as well as writing all manuscripts were completed in cooperation with Prof. Andreas Kappler, Dr. Casey Bryce and Dr. Anneli Sundman for chapter 3, 4, 5 and with Prof. Thomas Borch for chapter 3, 5.

Other contributions of other people in the laboratory were: Dr. Anneli Sundman taught me the experimental technique to synthesis Fe(II)-OM complexes. Dr. Charlotte Catrouillet taught me PHREEQC modeling software. Nitrate and nitrite concentrations in Chapter 3 were measured by Ellen Roehm. Dr. Casey Bryce and Lars Grimm helped me doing cell counts using flow cytometer in Chapter 3, 4, and 5.

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Academic Education

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