

# Oxidation of Fe(II)–Organic Matter Complexes in the Presence of the Mixotrophic Nitrate-Reducing Fe(II)-Oxidizing Bacterium *Acidovorax* sp. BoFeN1

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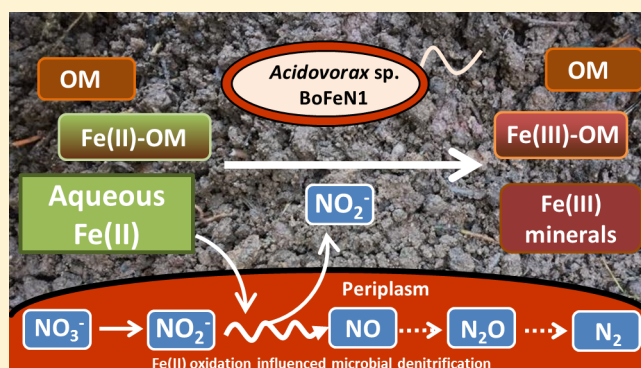
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## Supporting Information

**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 heterotrophic denitrification. However, it is unknown whether Fe(II)–OM complexes can be oxidized by NRFeOx. We used cell-suspension experiments with the mixotrophic nitrate-reducing Fe(II)-oxidizing bacterium *Acidovorax* sp. strain BoFeN1 to reveal the role of nonorganically bound Fe(II) (aqueous Fe(II)) and nitrite for the rates and extent of oxidation 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; large colloidal and negatively charged complexes showed lower oxidation rates than aqueous Fe(II). Accumulation of nitrite and fast abiotic oxidation of Fe(II)–OM complexes only happened in the presence of aqueous 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 nitrite accumulation. These results suggest that Fe(II) oxidation by mixotrophic nitrate reducers in the environment depends on Fe(II) speciation, and that aqueous Fe(II) potentially plays a critical role in regulating microbial denitrification processes.



## INTRODUCTION

Iron (Fe) is present in almost all aquatic and terrestrial environments.<sup>1,2</sup> It is an essential element for nearly all organisms and influences both the behavior of environmental contaminants<sup>3</sup> and many other biogeochemical cycles.<sup>4</sup> 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<sup>5,6</sup> and cadmium<sup>7</sup> 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<sub>2</sub>, light, or nitrate to oxidize Fe(II).<sup>4</sup> Nitrate-reducing Fe(II)-oxidizing bacteria (NRFeOx) have been isolated from a variety of habitats.<sup>8–13</sup> NRFeOx micro-

organisms include both autotrophic and heterotrophic consortia and pure cultures.<sup>13–15</sup> *Acidovorax* sp. strain BoFeN1 has been isolated from Lake Constance freshwater sediments.<sup>15</sup> 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.<sup>15,16</sup> Several *Acidovorax* sp. relatives have been found in arsenic-contaminated aquifers, town ditches, groundwater, and freshwater sediments.<sup>14,17</sup> Until now, a specific enzymatic machinery for nitrate-reducing Fe(II) oxidation has not been identified.<sup>18</sup> So far, only one nitrate-reducing Fe(II)-oxidizing mixed culture has been demonstrated unequivocally

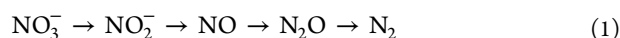
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to maintain autotrophic growth with Fe(II) over more than two decades for many generations and transfers.<sup>13,14</sup> More cultures have been suggested to also perform autotrophic Fe(II) oxidation although ultimate proof for their autotrophic lifestyle is, in many cases, still missing.<sup>19–21</sup> In contrast, for most nitrate-reducing Fe(II) oxidizers reactive nitrogen species such as NO<sub>2</sub><sup>-</sup> and NO (intermediates of microbial heterotrophic denitrification) (reaction 1) have been suggested to be the oxidants for Fe(II).<sup>16,18,22</sup> Although the enzymatic steps of microbial denitrification take place inside the cell,<sup>23</sup> 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.<sup>15,24–26</sup> Consequently, these initially formed Fe(III) minerals could function as catalyst and lead to high abiotic Fe(II) oxidation rates.<sup>22</sup> This coupled biotic–abiotic Fe(II) oxidation mechanism may also explain the Fe(II) oxidation observed for many other heterotrophic nitrate-reducing bacteria.<sup>16,24,25</sup>



It has been shown that NRFeOx can oxidize dissolved Fe(II), Fe(II)-containing minerals such as siderite and magnetite,<sup>27</sup> clay minerals,<sup>28,29</sup> and simple organic Fe(II) complexes such as Fe(II)–EDTA and Fe(II)–NTA.<sup>28,30,31</sup> However, in nature, Fe(II)–organic matter (Fe(II)–OM) complexes are present.<sup>32–39</sup> 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.<sup>40–42</sup> OM complexation also influences the abiotic oxidation rates of Fe(II) by O<sub>2</sub><sup>43–46</sup> and by nitrite.<sup>47</sup> 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.<sup>36,37,48,49</sup>

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.<sup>50</sup> The oxidation of simple Fe(II)–EDTA and Fe(II)–NTA complexes has been demonstrated with several nitrate-reducing Fe(II)-oxidizing strains.<sup>28,30,31</sup> 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.<sup>18</sup> A previous study has analyzed the oxidation rates for several Fe(II)–OM complexes compared to aqueous Fe(II) by nitrite produced by the nitrate-reducer *Paracoccus denitrificans*.<sup>47</sup> However, in these experiments the Fe(II)–OM complexes were added to the cultures after they had accumulated 5 mM NO<sub>2</sub><sup>-</sup> (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 (Supporting Information (SI), Figure S1). In the absence of Fe(II), they perform complete denitrification and the nitrite gets reduced stepwise via NO and N<sub>2</sub>O to N<sub>2</sub>.<sup>23</sup> As previous studies on mixotrophic NRFeOx have reported an encrustation of both the cell surface and the

periplasm,<sup>15,16,24–26</sup> this suggests that at least a part of the Fe(II) that entered the periplasm, became oxidized, and precipitated there. This allows us to hypothesize that on the one hand, because the outer membrane is not permeable to proteins or other large molecules,<sup>23</sup> it also presents a barrier for large Fe(II)–OM complexes (colloids) preventing their entrance into 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 aqueous Fe(II) by the mixotrophic nitrate-reducing Fe(II) oxidizer *Acidovorax* sp. BoFeN1, and to investigate the roles of aqueous Fe(II) and nitrite in the oxidation of Fe(II)–OM species.

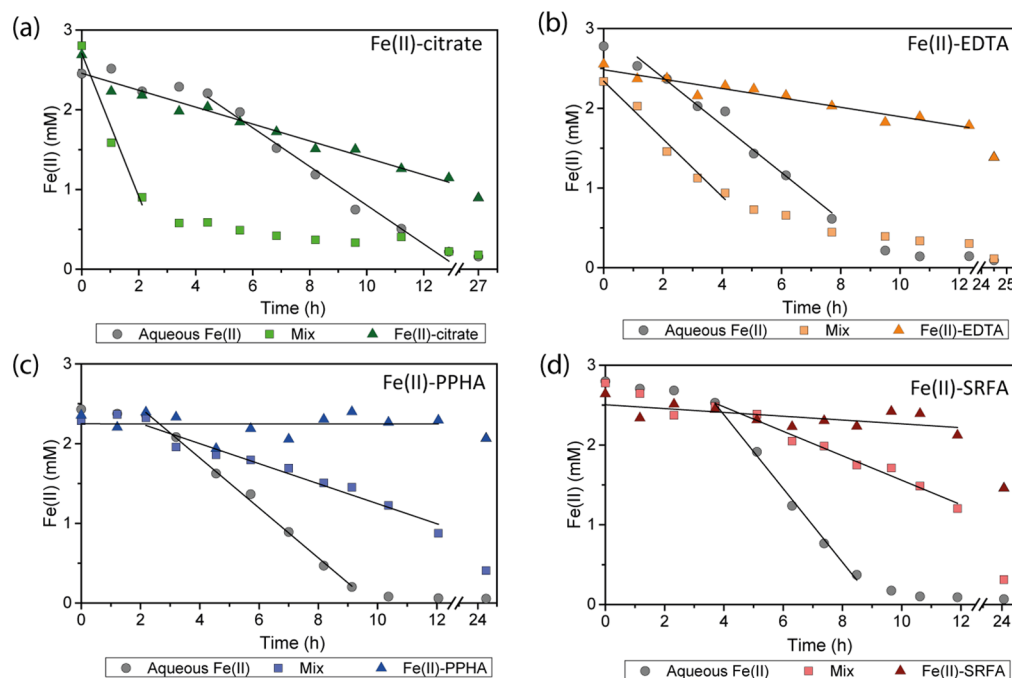
## ■ MATERIALS AND METHODS

**Synthesis of Fe(II)–NOM Complexes.** All Fe(II)–OM complexes were synthesized anoxically in a 20 mM PIPES buffer amended with 20 mM NaCl. Fe(II)–citrate and Fe(II)–EDTA complexes were synthesized by mixing FeCl<sub>2</sub>:citrate in a 1:2 and FeCl<sub>2</sub>:EDTA in a 1:1.2 molar ratio, respectively, followed by adjustment to pH 7 and filter sterilization (0.2 μm). Fe(II)–PPHA (Pahokee peat humic acid) and Fe(II)–SRFA (Suwannee river fulvic acid) complexes were synthesized by dissolving either PPHA and SRFA at a final concentration of 2.5 mg/mL (118 and 109 mM carbon, respectively), followed by adjustment to pH 7 and mixed with 3 mM FeCl<sub>2</sub> (for detailed experimental procedures, see section 1.1 in the SI). The speciation of Fe(II) was determined by thermodynamic calculations using PHREEQC with the “minteq.v4” database and a previously published model for Fe(II) binding to humic and fulvic acids.<sup>51</sup> This calculation showed that more than 99% of the Fe(II) was present as Fe(II)–OM complexes (SI, Table S1). All experiments were performed in an anoxic glovebox (100% N<sub>2</sub>), the oxygen concentration in the solution was below 1.5 nM (calculated based on the O<sub>2</sub> in the glovebox <10 ppm).

**Bacterial Strain and Precultivation.** *Acidovorax* sp. BoFeN1 was isolated from Lake Constance sediments and kept in the authors’ laboratory since then.<sup>15</sup> The culture was continuously transferred in freshwater medium with 10 mM FeCl<sub>2</sub>, 10 mM NaNO<sub>3</sub>, and 5 mM sodium acetate (for medium composition see SI, Table S2).

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. The bacteria were cultured under anoxic conditions to the late exponential phase. Cells were harvested by centrifugation (7000g, 20 min, 25 °C), washed twice, and resuspended in 20 mM PIPES buffer containing 20 mM NaCl. An aliquot of the cell suspension was fixed using 2% paraformaldehyde and stored at 4 °C for quantification of cell numbers by a Attune NxT flow cytometer (Thermo Fisher Scientific).

**Setup of Fe(II) Oxidation Experiments.** Basal nongrowth medium containing only PIPES buffer, NaCl, and electrolyte (10 mM NaNO<sub>3</sub> and 5 mM sodium acetate) was prepared for the determination of Fe(II) oxidation rates. For experiments



**Figure 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 aqueous (non-OM-complexed) Fe(II) (circles), different fully complexed Fe(II)–OM complexes (triangles), or a mixture of aqueous 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 SI, Table S4. Because duplicate microbiological setups showed very similar results, representative experiments out of these duplicate setups are shown (the second one is shown in the SI, Figure S5), and the data are shown as averages of triplicate spectrophotometric measurements.

containing aqueous Fe(II), Fe(II)–citrate, or Fe(II)–EDTA complexes (or a mixture of these), stock solutions of these components were added to the anoxic basal medium containing 20 mM PIPES buffer and NaCl as required. For the experiments with a mixture of Fe(II)–PPHA or Fe(II)–SRFA and aqueous Fe(II), 3 mM FeCl<sub>2</sub> was added following the dissolution of PPHA and SRFA in the basal medium to a concentration of 0.5 mg/mL (24 and 22 mM carbon, respectively, for PPHA and SRFA; for detailed experimental procedures, see section 1.3 in the SI). According to our thermodynamic calculations, the percentages of aqueous Fe(II) were approximately 66, 60, 63, and 52% in the mixtures of aqueous Fe(II) and Fe(II)–citrate, Fe(II)–EDTA, Fe(II)–PPHA, and Fe(II)–SRFA, respectively (SI, Table S1); the rest of the Fe(II) was determined to be present as Fe(II)–OM complexes.

The washed cells were added into the nongrowth medium at a concentration of between  $4.00 \times 10^8$  and  $6.82 \times 10^8$  cells/mL (SI, Table S3). The cell suspensions were then incubated in Hungate tubes closed with airtight butyl stoppers in the dark at 28 °C. In experiments with different types of OM, the Fe(II) oxidation rate in setups with aqueous Fe(II) only was determined 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<sub>2</sub>) in duplicate.

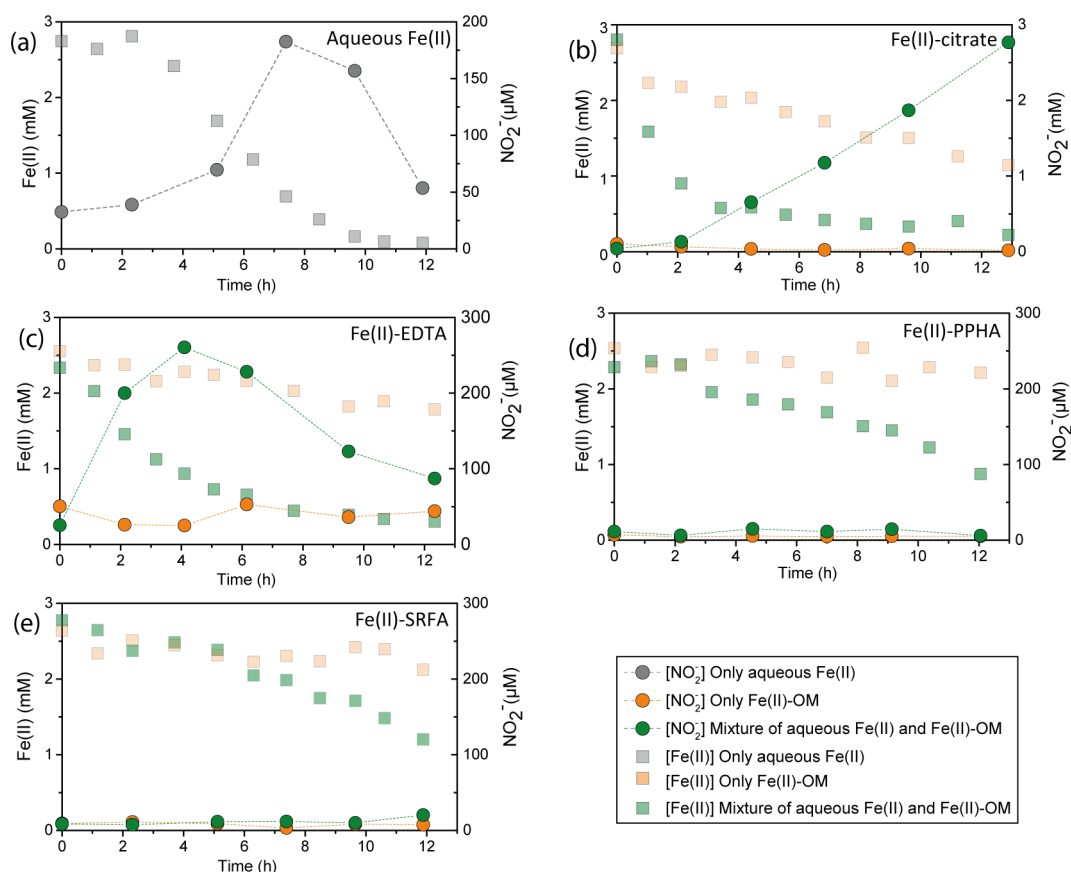
**Sample Analysis.** Samples were taken hourly in the glovebox. Fe(II) concentrations were determined anoxically using a slightly modified ferrozine assay.<sup>52</sup> For the quantification of Fe(II) in samples without PPHA and SRFA (FeCl<sub>2</sub>, Fe(II)–citrate, and Fe(II)–EDTA samples), samples were first

diluted with 40 mM sulfamic acid to prevent Fe(II) oxidation by nitrite at low pH.<sup>22</sup> The Fe(II) concentration was determined using 1 M anoxic HCl and anoxic ferrozine solution (0.1% w/v) dissolved in ammonium acetate (C<sub>2</sub>H<sub>7</sub>NO<sub>2</sub>, 50% w/v), and the purple ferrozine–Fe(II) complex formed was quantified at 562 nm with a microplate reader (Thermo Scientific). For quantification of Fe(II) in samples with PPHA and SRFA, samples were first diluted with anoxic Milli-Q H<sub>2</sub>O. Immediately after sampling the Fe(II) concentration was determined by adding ferrozine solutions directly into the H<sub>2</sub>O diluted samples without using 1 M 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 (for detailed experimental procedures, see section 1.4 in the SI).

Samples for nitrite and nitrate quantification were taken in the glovebox and centrifuged (14000g, 10 min). The supernatant was diluted with anoxic Milli-Q H<sub>2</sub>O 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-naphthylethylenediamine at 550 nm.

## RESULTS

**Oxidation of Aqueous Fe(II) and OM-Complexed Fe(II).** To determine the effect of Fe(II) complexation by



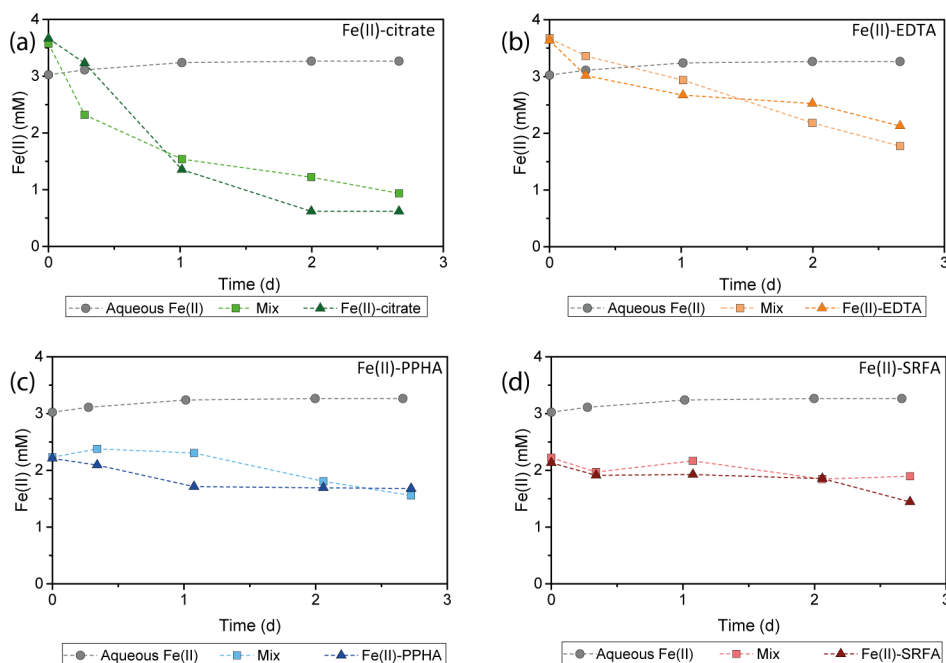
**Figure 2.** Concentrations of nitrite ( $\text{NO}_2^-$ , circles, dashed lines) and total Fe(II) (squares) over time in the presence of *Acidovorax* sp. strain BoFeN1 with aqueous Fe(II) (a) and fully complexed Fe(II)-OM complexes and mixtures of aqueous Fe(II) and different Fe(II)-OM complexes (b-e) for Fe(II)-citrate (b), Fe(II)-EDTA (c), Fe(II)-PPHA (d), and Fe(II)-SRFA (e). Because duplicate microbiological setups showed very similar results, representative experiments out of these duplicate setups are shown (the second one is shown in the SI, Figure S6).

OM on oxidation of Fe(II) by the mixotrophic nitrate-reducing Fe(II) oxidizer *Acidovorax* sp. strain BoFeN1, we incubated aqueous Fe(II), different Fe(II)-OM complexes or a mixture of aqueous Fe(II) and Fe(II)-OM complexes with nitrate, acetate, and strain BoFeN1 (Figure 1). We found that in all experiments, aqueous Fe(II) was oxidized with rates of up to 0.19–0.46 mM/h (SI, Table S4). From one experiment to another, the oxidation rates for aqueous Fe(II) varied slightly, but generally all Fe(II) was oxidized to near completion within 9–12 h. However, oxidation of the Fe(II)-OM complexes was found to be significantly slower than aqueous Fe(II). Among the four different complexes, the Fe(II)-citrate complex showed the fastest oxidation rates (0.11 mM/h; SI, Table S4) 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 (SI, Table S4). The average oxidation rates for the Fe(II)-citrate, Fe(II)-EDTA, and Fe(II)-SRFA complexes were approximately 2-, 4.3-, and 23-fold slower than the average oxidation rates of aqueous Fe(II) (SI, Table S4), respectively. For the Fe(II)-PPHA complexes, hardly any oxidation was observed during 24 h 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.

However, these trends for Fe(II) oxidation changed when the setups contained a mixture of both aqueous 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 aqueous Fe(II) and Fe(II)-citrate, Fe(II)-EDTA, Fe(II)-PPHA, and Fe(II)-SRFA complexes, respectively (SI, Table S4). In the mixed setups containing both aqueous Fe(II) and Fe(II)-citrate or both aqueous Fe(II) and Fe(II)-EDTA complexes, Fe(II) was oxidized 3.7-fold (aqueous Fe(II) and Fe(II)-citrate mixture) and 1.2-fold (aqueous Fe(II) and Fe(II)-EDTA mixture) faster than in the setups with aqueous Fe(II) only. Even in the mixed setups with both aqueous Fe(II) and Fe(II)-PPHA or aqueous 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 aqueous Fe(II), whereas in the absence of aqueous 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 h. After 1 day of incubation, a large fraction of Fe(II) in the setups with fully complexed Fe(II)-OM still remained, while the Fe(II) in the mixed setups with both aqueous Fe(II) and Fe(II)-OM was nearly completely oxidized. The concentrations of Fe(II) remaining in the aqueous 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 aqueous 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



**Figure 3.** Abiotic oxidation of Fe(II) by 2 mM nitrite. The Fe(II) was present in the form of either aqueous (non-OM-complexed) Fe(II) (gray circles), different fully complexed Fe(II)–OM complexes (triangles), or a mixture of aqueous 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). Because duplicate microbiological setups showed very similar results, representative experiments out of these duplicate setups are shown (the second one is shown in the SI, Figure S7), and the data are shown as averages of triplicate spectrophotometric measurements.

aqueous Fe(II) and Fe(II)–citrate or aqueous Fe(II) and Fe(II)–EDTA complexes than in the setups with aqueous Fe(II) alone, and this effect was even more obvious in setups with lower cell numbers (SI, Figure S2). During the first few hours, depending on the cell numbers, only a small amount of Fe(II) was oxidized in the setups with only aqueous 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 aqueous Fe(II) present or not. The earlier oxidation of Fe(II) in the setups with mixtures of aqueous 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 (SI, Figure S2). 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.<sup>16</sup> To further investigate the role of aqueous 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 aqueous Fe(II), Fe(II)–OM complexes, or mixtures of both aqueous Fe(II) and Fe(II)–OM complexes in the presence of nitrate, acetate, and strain BoFeN1 (Figure 2). We found that nitrite only accumulated in BoFeN1 cultures that contained aqueous Fe(II). Specifically, nitrite was observed in BoFeN1 cultures with only aqueous Fe(II) and in cultures that contained a mixture of both aqueous 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 aqueous Fe(II) and Fe(II)–citrate after 12 h of incubation. In BoFeN1

cultures with only aqueous Fe(II) and with mixtures of aqueous Fe(II) and Fe(II)–EDTA, nitrite accumulated as well and reached concentrations of around 200–300  $\mu\text{M}$ . 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.,  $2.35 \times 10^8$  cells/mL), 1.3 mM and 1 mM nitrite accumulated in the setups with only aqueous Fe(II) and with a mixture of aqueous Fe(II) and Fe(II)–EDTA, respectively (SI, Figure S2), compared to 0.18 and 0.26 mM nitrite when  $4.51 \times 10^8$  and  $4.00 \times 10^8$  cells/mL were present, respectively, in these two setups (Figure 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 2 and SI, Figure S2). 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.

**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, aqueous Fe(II), or Fe(II)–OM/aqueous Fe(II) mixtures (Figure 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 aqueous Fe(II) or not, showed much faster abiotic Fe(II) oxidation rates than setups with only aqueous Fe(II) (Figure 3). Almost no aqueous 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 aqueous 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 1). In setups with either Fe(II)–PPHA or Fe(II)–SRFA, both in the presence or absence of aqueous 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 aqueous Fe(II) and Fe(II)–OM complexes compared to Fe(II)–OM complexes only (Figure 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 aqueous 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 aqueous 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), 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 aqueous 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 aqueous Fe(II) and Fe(II)–EDTA. The remaining Fe(II) concentration after 2 and 1/2 days of incubation was similar in the setups with the same type of OM.

## DISCUSSION

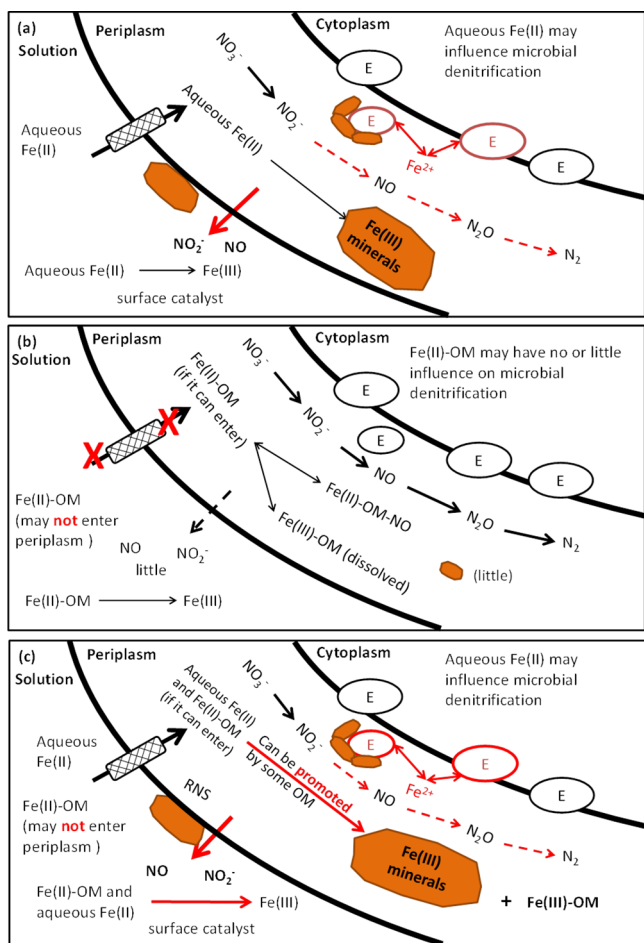
**Abiotic and Biotic Oxidation of Fe(II) in the Presence of Organic Matter.** Fe(II) complexation by OM has been suggested to influence the rate of abiotic Fe(II) oxidation.<sup>43–45,47</sup> Indeed, our experimental results showed that Fe(II) complexation by citrate and EDTA promoted chemical Fe(II) oxidation by nitrate (Figure 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.<sup>47</sup> 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<sup>47</sup> 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.<sup>53</sup> 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.<sup>54</sup> 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,<sup>22</sup> which inhibits the autocatalysis of Fe(II) oxidation, or (ii) the ability of HA and FA to reduce and thus consume nitrite.<sup>55–57</sup>

In contrast to the abiotic Fe(II) oxidation experiments, all Fe(II)–OM complexes studied here showed a slower Fe(II) oxidation compared to aqueous Fe(II) in the presence of strain BoFeN1, acetate, and nitrate (Figure 1), conditions under which nitrite is known to be formed by BoFeN1 that then abiotically reacts with the aqueous Fe(II).<sup>22</sup> 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.** Because the OM did not significantly change

nitrate reduction by BoFeN1 (SI, Figure S3), i.e., the OM is not toxic to the cells (see section 2 in the SI), 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.<sup>47,58,59</sup> However, solely abiotic Fe(II)/Fe(III) redox reactions cannot fully explain why, in the absence of aqueous 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 aqueous Fe(II), while in the presence of strain BoFeN1 these complexes were oxidized slower than aqueous Fe(II) (Figure 1, Figure 3). Although with *Acidovorax* sp. BoFeN1, a large extent, if not all, of oxidation of aqueous Fe(II) was shown to be abiotically caused by nitrite formed during nitrate reduction,<sup>16,18</sup> Fe(II)–OM complexation has been recognized to have promoted Fe(II) oxidation with other NRFeOx bacteria before.<sup>27,47,60</sup> 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,<sup>47</sup> 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.<sup>27,60,61</sup>

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.<sup>26,60</sup> Therefore, it has been suggested that Fe(II) has to cross the outer membrane for oxidation to occur.<sup>18</sup> Specifically, Fe(II) must pass through negatively charged cell pores, i.e., the porins.<sup>62</sup> However, Fe(II)–OM complexation is expected to change the charge of the Fe(II) ions from positive toward neutral and negative,<sup>63</sup> therefore, complexation could hamper Fe(II) from entering the periplasm (Figure 4b). As a result, the interaction between Fe(II) and periplasmic components, such as nitrite reductase enzymes, could be inhibited.<sup>23</sup> 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 aqueous Fe(II) has a radius of ca. 0.21 nm (2.1 Å),<sup>64</sup> 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 aqueous 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.<sup>61</sup> This coagulation of HA could further enhance electrostatic and/or steric repulsion,<sup>61</sup> 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 approximately 10 Å) than aqueous 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.<sup>61</sup> 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.<sup>61</sup> Pore-forming proteins (porins) provide channels only about 1–2 nm in size.<sup>65</sup> Therefore, the cell's outer membrane could be a potential barrier for the transportation of Fe(II)–PPHA and Fe(II)–SRFA colloids



**Figure 4.** Proposed mechanism of Fe(II) oxidation by *Acidovorax* sp. strain BoFeN1 in samples with only aqueous Fe(II) (a), only Fe(II)–OM complexes (b), and a mixture of aqueous Fe(II) and Fe(II)–OM complexes (c). For simplicity, only the few relevant enzymes are shown. (a) Aqueous Fe(II) is transported into the periplasm and potentially interacts with microbial denitrification enzymes stimulating the accumulation of reactive nitrogen species (RNS) such as  $\text{NO}_2^-$  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  $\text{NO}_2^-$  to  $\text{N}_2$ , preventing Fe(II)–OM oxidation. (c) When both aqueous Fe(II) and Fe(II)–OM complexes are present, the accumulated RNS produced by the interaction of aqueous Fe(II) with the microbial denitrification enzymes stimulate Fe(II) oxidation as nitrite could oxidize both Fe(II)–OM and aqueous Fe(II).

into the BoFeN1 periplasm, where nitrite forms and is essential to initiate extensive NRFeOx.<sup>18,23</sup> 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 aqueous Fe(II) in the periplasm, nitrite does not accumulate and fast Fe(II) oxidation does not occur (Figure 2, Figure 4b). However, when aqueous Fe(II) is provided alongside the Fe(II)–OM complexes, aqueous 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 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 aqueous Fe(II) from Fe(II)–OM complexes; the released aqueous 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 aqueous 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), while in the absence of OM, nitrite has to accumulate to a higher concentration to oxidize aqueous Fe(II).

**The Importance of Aqueous Fe(II) for Nitrite Accumulation.** During denitrification, nitrite accumulates due to a slower rate of nitrite reduction compared to nitrate reduction.<sup>66</sup> 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 2; SI, Figures S2, S5). The occurrence of simultaneous Fe(II) oxidation and nitrite accumulation only when aqueous Fe(II) was present, could be explained by the interaction of aqueous Fe(II) with enzymatic components in the periplasm.<sup>18</sup> 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,<sup>67</sup> and (iii) precipitation of poorly soluble Fe(III) minerals on cellular components such as  $\text{NO}_2^-$  and NO reductase enzymes. The extent of these effects may also be different with different types and percentages of Fe(II)–OM complexes as a result of different location and amount of Fe(III) mineral precipitation. As different Fe(III)–OM complexes have different stability constants,<sup>53</sup> 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<sup>23</sup> would decrease nitrite reductase enzyme activity and could thus lead to an accumulation of nitrite.<sup>16,18,60,68</sup> As the nitrate reductase is an inner membrane protein,<sup>23</sup> 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 4a,c) result in an even stronger inhibition of microbial nitrite reduction explaining the accumulation of nitrite in the presence of aqueous Fe(II) (Figure 2; SI, Figure S2).

Although our abiotic Fe(II) oxidation experiments showed that oxidation of aqueous Fe(II) by 2 mM of nitrite was very slow at pH 7 (Figure 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.<sup>69</sup> 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,<sup>70</sup> potentially creating a low-pH hotspot in the periplasm favoring oxidation of aqueous Fe(II) by nitrite.

In contrast to the experiments where aqueous Fe(II) was present, all experiments with fully complexed Fe(II)–OM (no aqueous Fe(II)) showed no nitrite accumulation. This could be

due to the lack of aqueous 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 4b). This suggests that although some Fe(II)–OM complexes react abiotically faster with nitrite than aqueous Fe(II), aqueous 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 aqueous Fe(II) causes nitrite accumulation in the periplasm. However, when there are both aqueous Fe(II) and Fe(II)–OM complexes present, the nitrite accumulation caused by aqueous Fe(II) can then stimulate oxidation of Fe(II)–OM in the presence of *Acidovorax* sp. BoFeN1 (Figure 2, Figure 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).

#### 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 aqueous Fe(II) and Fe(II)–NOM complexes. Such complexes have been identified in soils, rivers, and sediments.<sup>32–38</sup> 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.<sup>51,71</sup> Although the Fe(II) speciation depends also on other parameters such as the absolute 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  $\mu$ M carbon).<sup>61</sup> In real environments such as the pore water of the freshwater sediment from which *Acidovorax* sp. strain BoFeN1 was isolated,<sup>10</sup> the DOC:Fe(II) ratio can be as high as 40 and the ratios can be even higher in soils.<sup>72</sup> 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,<sup>50</sup> not all Fe(II) is expected to be complexed and nitrate-dependent oxidation of aqueous Fe(II) may take place and contribute to Fe(II) oxidation. Interestingly, these authors also reported that the ratio of nitrate<sub>reduced</sub> to Fe(II)<sub>oxidized</sub> changed with the OM content of the sediment.<sup>50</sup> 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<sub>2</sub>O while biological reduction of nitrite is expected to lead to N<sub>2</sub>).

Because mixotrophic nitrate-reducing Fe(II) oxidizers have been found in various environments,<sup>12,13,15,27,73–86</sup> 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,<sup>87</sup> depends on whether Fe(II) is available in its aqueous Fe(II) form or is complexed by OM (Figure 2; SI, Figure S2). As a consequence, the accumulation of nitrite could further

cause the formation of the important greenhouse gas N<sub>2</sub>O by the reaction of Fe(II) with nitrite.<sup>88,89</sup>

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.8b00953.

Detailed experimental procedures, information on toxic effect of OM, figures, and tables (PDF)

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### Notes

The authors declare no competing financial interest.

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