

# Oxidation of Fe(II)-EDTA by nitrite and by two nitrate-reducing Fe(II)-oxidizing *Acidovorax* strains

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## ABSTRACT

The enzymatic oxidation of Fe(II) by nitrate-reducing bacteria was first suggested about two decades ago. It has since been found that most strains are mixotrophic and need an additional organic co-substrate for complete and prolonged Fe(II) oxidation. Research during the last few years has tried to determine to what extent the observed Fe(II) oxidation is driven enzymatically, or abiotically by nitrite produced during heterotrophic denitrification. A recent study reported that nitrite was not able to oxidize Fe(II)-EDTA abiotically, but the addition of the mixotrophic nitrate-reducing Fe(II)-oxidizer, *Acidovorax* sp. strain 2AN, led to Fe(II) oxidation (Chakraborty & Picardal, 2013). This, along with other results of that study, was used to argue that Fe(II) oxidation in strain 2AN was enzymatically catalyzed. However, the absence of abiotic Fe(II)-EDTA oxidation by nitrite reported in that study contrasts with previously published data. We have repeated the abiotic and biotic experiments and observed rapid abiotic oxidation of Fe(II)-EDTA by nitrite, resulting in the formation of Fe(III)-EDTA and the green Fe(II)-EDTA-NO complex. Additionally, we found that cultivating the *Acidovorax* strains BoFeN1 and 2AN with 10 mM nitrate, 5 mM acetate, and approximately 10 mM Fe(II)-EDTA resulted only in incomplete Fe(II)-EDTA oxidation of 47–71%. Cultures of strain BoFeN1 turned green (due to the presence of Fe(II)-EDTA-NO) and the green color persisted over the course of the experiments, whereas strain 2AN was able to further oxidize the Fe(II)-EDTA-NO complex. Our work shows that the two used *Acidovorax* strains behave very differently in their ability to deal with toxic effects of Fe-EDTA species and the further reduction of the Fe(II)-EDTA-NO nitrosyl complex. Although the enzymatic oxidation of Fe(II) cannot be ruled out, this study underlines the importance of nitrite in nitrate-reducing Fe(II)- and Fe(II)-EDTA-oxidizing cultures and demonstrates that Fe(II)-EDTA cannot be used to demonstrate unequivocally the enzymatic oxidation of Fe(II) by mixotrophic Fe(II)-oxidizers.

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## INTRODUCTION

In the absence of high concentrations of sulfide, phosphate, and carbonate, divalent iron, that is, Fe(II), is highly soluble under anoxic conditions at circumneutral pH. It is therefore bioavailable and can function as an electron source for microaerophilic, phototrophic, and nitrate-reducing iron(II)-oxidizing bacteria (Kappler & Straub, 2005; Weber *et al.*, 2006; Konhauser *et al.*, 2011; Melton *et al.*, 2014). These organisms have been the focus of intensive research over more than a decade because of their

apparent ability to affect Fe(II) oxidation, their potential involvement in many geochemical cycles and processes (Kendall *et al.*, 2012), their effects on Fe(III) mineral formation, and their influence on sorption and degradation of contaminants (Borch *et al.*, 2010; Posth *et al.*, 2014). In anoxic environments, nitrate reduction was suggested to be coupled to Fe(II) oxidation and many strains have been isolated that are able to perform this metabolism (Straub *et al.*, 1996; Benz *et al.*, 1998; Kappler *et al.*, 2005; Weber *et al.*, 2009). Typical heterotrophic denitrifiers have also been shown to be able to cause Fe(II) oxidation under

specific conditions in batch reactors (Kumaraswamy *et al.*, 2006; Klueglein *et al.*, 2014). In those nitrate-reducing, Fe(II)-oxidizing cultures, continued growth has not been demonstrated in the absence of an organic electron donor additional to ferrous iron. However, in these mixotrophic cultures, reactive nitrogen compounds, for example, nitrite and NO, can be formed during denitrification using the organic co-substrate. These reactive compounds can oxidize iron(II) abiotically, not only at acidic pH during sample analysis (acidic extraction), but also at neutral pH during culture incubation in the presence of a catalyst, for example, minerals, cell surfaces, chelators, or metal ions (Sorensen & Thorling, 1991; Tai & Dempsey, 2009). These possible abiotic side reactions raise the question whether and to what extent the observed Fe(II) oxidation in denitrifying cultures is enzymatically driven or occurring due to chemical reactions between nitrite/NO and Fe(II) (Buresh & Moraghan, 1976; Van Cleemput & Baert, 1983; Coby & Picardal, 2005; Picardal, 2012; Klueglein & Kappler, 2013; Kopf *et al.*, 2013).

To obtain further insights into the processes involved in nitrate-dependent Fe(II) oxidation, Chakraborty and Picardal (Chakraborty & Picardal, 2013) cultured *Acidovorax* strain 2AN with EDTA-complexed Fe(II), that is, Fe(II)-EDTA, rather than aqueous Fe<sup>2+</sup>. Their rationale was that chelated Fe(II) would minimize formation of poorly soluble Fe(III) minerals, thereby preventing cell encrustations that eventually lead to reduced metabolic activity or cell death (Miot *et al.*, 2009, 2011). As part of their studies, they presented evidence that Fe(II)-EDTA is not oxidized abiotically by nitrite and therefore concluded that all oxidized Fe(II) must originate from direct enzymatic activity. However, the absence of Fe(II)-EDTA oxidation by nitrite in their work differed from the results of other studies that have shown chemical oxidation of Fe(II)-EDTA by nitrite and NO (Zang *et al.*, 1988; Van Der Maas *et al.*, 2004; Kumaraswamy *et al.*, 2006). It should be noted that some of these other studies measured Fe(II) concentrations with ferrozine as the colorimetric agent (Stokey, 1970) to follow Fe(II)-EDTA oxidation, while others used phenanthroline as the colorimetric complexant (Tamura *et al.*, 1974). It is therefore unclear whether the differences observed are due to the different reagents used.

In addition to the absence of abiotic Fe(II)-EDTA oxidation, Chakraborty & Picardal (2013) found that the growth yield of *Acidovorax* strain 2AN increased by 25% in comparison with similar cultures lacking Fe(II)-EDTA. Fe(II)-oxidation-dependent enhanced growth of another nitrate-reducing Fe(II)-oxidizer, *Acidovorax* strain BoFeN1, in the presence of aqueous Fe<sup>2+</sup> was previously reported (Muehe *et al.*, 2009), but such growth enhancement could also be achieved by providing Fe(III) oxyhydroxide (Klueglein & Kappler, 2013), suggesting a

nutritional effect. In contrast, Chakraborty & Picardal (2013) did not observe higher cell numbers using Fe(III)-EDTA, questioning the possibility of a nutrient effect of iron (Andrews *et al.*, 2003). As *Acidovorax* strains 2AN and BoFeN1 are highly similar (98.3% 16S rRNA gene similarity) and were cultured at a similar pH and with similar concentrations of electron donors and acceptors, the observed differences in Fe(II) oxidation and growth response to the presence of various forms and redox species of iron might be a result of the different iron sources, but the exact reasons are not yet clear.

The overall goals of the current studies were to clarify some of these conflicting results and to (i) specifically determine whether Fe(II)-EDTA is oxidized abiotically by nitrite, (ii) evaluate the relative contribution of abiotic and enzymatic Fe(II) oxidation in *Acidovorax* strains BoFeN1 and 2AN cultures, and (iii) compare the similarities and differences between the two closely related *Acidovorax* strains under identical culture conditions. Our results help clarify discrepancies in the existing literature and further elucidate the process of Fe(II) oxidation in nitrate-reducing cultures.

## MATERIAL AND METHODS

### Micro-organisms and growth media

*Acidovorax* strain BoFeN1 is a chemoorganotrophic, nitrate-reducing Fe(II)-oxidizer isolated from Lake Constance sediments and was kept in the authors' laboratory since its original isolation (Kappler *et al.*, 2005). *Acidovorax* strain 2AN, also a chemoorganotrophic nitrate-reducing Fe(II)-oxidizer, was isolated using an iron-rich river sediment from Wisconsin, USA (Chakraborty *et al.*, 2011). For routine cultivation of strain BoFeN1 and 2AN, 10 mM Na-nitrate and 5 mM Na-acetate were added to 22 mM bicarbonate-buffered low-phosphate mineral medium (pH 7.1), which was prepared anoxically as described in detail by Hegler *et al.* (2008).

### Preparation of Fe-EDTA and nitrite solutions

Fe(II)- and Fe(III)-EDTA stock solutions were prepared by following the protocol of Chakraborty & Picardal (2013). Anoxic Na<sub>2</sub>-EDTA solution was mixed with an equimolar concentration of anoxic Fe(II)Cl<sub>2</sub> or Fe(III)Cl<sub>3</sub> in anoxic, deionized water (final concentration approximately 100 mM). All steps were carried out under anoxic conditions, and the solutions were prepared freshly for every experiment. Nitrite solutions were prepared by dissolving Na-nitrite in anoxic deionized water followed by sterile filtration. The solution was stored under anoxic conditions at room temperature in the dark. The nitrite concentration of the stock solution was quantified before each

experiment to confirm that no abiotic degradation of nitrite has been taken place.

### Experimental protocols

For abiotic oxidation experiments, culture tubes (15 mL) were filled with 9 mL of anoxic medium containing 22 mM bicarbonate buffer (headspace N<sub>2</sub>/CO<sub>2</sub> (90/10, v/v)) or 5 mM PIPES buffer (headspace 100% N<sub>2</sub>) and sealed with butyl rubber stoppers. All tubes were amended with 1 mL of anoxic Fe(II)-EDTA solution (final concentration approximately 11 mM) and anoxic sodium nitrite stock solution to achieve final concentrations of 0, 2, and 4 mM nitrite. For biotic oxidation experiments, culture tubes (15 mL) were filled with 9 mL of anoxic medium (headspace N<sub>2</sub>/CO<sub>2</sub> (90/10, v/v)) and sealed with butyl rubber stoppers. Experimental tubes were amended with 1 mL of anoxic Fe-EDTA solution (final concentration 7–10 mM), Na-nitrate (10 mM), and Na-acetate (5 mM). The Fe(III)-EDTA stock solution (approximately 100 mM) contained 10% Fe(II) as contamination (10 mM), and therefore, 1 mM was subtracted from all Fe(II) values measured in tubes containing Fe(III)-EDTA. Control tubes contained 10 mL medium without Fe-EDTA addition. BoFeN1 or 2AN cultures grown on acetate/nitrate for 72 h were added as inoculum (5% inoculum), and tubes were incubated at 28 °C statically in the dark.

### Analytical methods

For quantification of Fe(II) and Fe(III), the samples were diluted in 40 mM sulfamic acid to avoid abiotic Fe(II) oxidation by nitrite during acidification (Klueglein & Kappler, 2013). Samples of cultures (100 µL) were withdrawn anoxically from tubes with a syringe and mixed with 900 µL sulfamic acid for 1 h at room temperature. For Fe(II) and Fe(III) quantification, we compared two spectrophotometric assays with two different Fe(II)-complexing dyes, that is, ferrozine and phenanthroline. The goal of this comparison was to determine whether both assays are generally suitable for quantifying Fe(II) in the presence of the strong complexing agent EDTA that potentially interferes with the Fe(II)-complexing dyes. The solutions for these assays were prepared as described in Amstatter *et al.* (2012) and Verschoor & Molot (2013). The absorbance of the ferrozine-Fe(II) complex was quantified at 562 nm (Stookey, 1970) and the phenanthroline-Fe(II) complex at 510 nm (Tamura *et al.*, 1974) using a microtiter plate reader (FlashScan 550; Analytik Jena, Jena, Germany). EDTA interfered with both assays, and a calibration curve was therefore prepared with standards containing similar concentrations of EDTA (10 mM). Maximum rates of microbial iron oxidation for the cultures were calculated from the steepest slope between two subsequent data

points of Fe(II) concentrations. Acetate was quantified by HPLC (Class vp with RID 10 A & DAD SPM 10 A vp detectors; Shimadzu, Kyoto, Japan; pre-column: Micro guard cation H cartridge; main column: Aminex HPX-87H Ion exclusion column 300 mm × 7.8 mm; Biorad, Vienna, Austria; eluent: 5 mM H<sub>2</sub>SO<sub>4</sub> in MQ water). For quantification of the Fe(II)-EDTA-NO complex, the absorbance was measured at 635 and 430 nm, and Fe(III)-EDTA was quantified at 340 and 258 nm (Zang *et al.*, 1988). As Fe(II)- and Fe(III)-EDTA interfered with our standard photometrical nitrate/nitrite assays, the presence of nitrite was demonstrated semiquantitatively with diagnostic test sticks (Merck, Darmstadt, Germany).

### Microscopy

For scanning electron microscopy (SEM), cells from Fe(II)-EDTA-containing cultures were fixed with 2.5% glutaraldehyde on ice overnight (Schaedler *et al.*, 2009). The samples were washed three times with PBS and successively dehydrated with stepwise increasing ethanol concentrations. After critical point drying, the samples were mounted on a carbon grid, coated with platinum, and examined with a LEO Model 1450 VP SEM at 5 kV.

## RESULTS

### Abiotic Fe(II)-EDTA oxidation by nitrite quantified with ferrozine and phenanthroline

Abiotic Fe(II)-EDTA oxidation by nitrite was quantified in tubes containing approximately 11 mM Fe(II)-EDTA and various concentrations of nitrite (0, 2, and 4 mM) by monitoring Fe(II) concentrations over time using spectrophotometric assays with two different Fe(II)-complexing dyes, that is, ferrozine and phenanthroline (Fig. 1). The two different assays have been used in various previous studies of nitrate-dependent Fe(II) oxidation and iron biogeochemistry in general (Urrutia *et al.*, 1999; Coby *et al.*, 2011; Chakraborty & Picardal, 2013; Muehe *et al.*, 2013; Verschoor & Molot, 2013; Kappler *et al.*, 2014), and our goal was to determine whether both assays are generally suitable for quantifying Fe(II) in the presence of the strong complexing agent EDTA. We observed that addition of nitrite to Fe(II)-EDTA led to a relatively rapid abiotic oxidation of Fe(II) (Fig. 1). Analyzing the same samples from these experiments with either ferrozine or phenanthroline showed only small differences in the total amount of Fe(II) (Fig. 1). Adding 2 mM nitrite to Fe(II)-EDTA resulted in an abiotic loss of 37 ± 4% Fe(II) (ferrozine) and 43 ± 1% Fe(II) (phenanthroline) within 7 days of incubation. Addition of 4 mM nitrite resulted in the loss of 49 ± 6% Fe(II) (ferrozine) and 54 ± 5% Fe(II) (phenanthroline), respectively. Without nitrite, no significant Fe(II) oxidation was

observed (<5% with ferrozine and <3% with phenanthroline). There was no measurable difference in Fe(II) oxidation when PIPES buffer was used instead of bicarbonate buffer (data not shown). The pH was stable around 7 and did not change significantly over time (data not shown). As Zang *et al.* (1988) showed that reaction rates increased at lower pH, similar experiments were carried out at pH 6.5 and 7.5. In experiments with ca. 2.0 mM nitrite and 5 mM Fe(II)-EDTA, approximately 2.3 mM Fe(II) was oxidized after 6 days at both pH 6.5 and 7.5, showing that pH was not an important factor over this pH range (data not shown).

Immediately after addition of nitrite to abiotic systems containing Fe(II)-EDTA, a green color developed (Fig. 2), indicating the formation of the Fe(II)-EDTA-NO nitrosyl complex. The intensity of the green color increased with increasing nitrite concentrations. The presence of the nitrosyl complex and Fe(III)-EDTA was confirmed by absorption measurements at indicative wavelengths (nitrosyl complex: 635, 430 nm; Fe(III)-EDTA: 340, 258 nm) (Figs S1 and S2). The green color was stable in both bicarbonate and PIPES buffer, even during long-term incubations (2 months).

#### Fe speciation changes in microbial systems containing Fe(II)-EDTA or Fe(III)-EDTA

Two *Acidovorax* strains, BoFeN1 and 2AN, were cultivated individually in the presence of 10 mM nitrate and 5 mM acetate amended with either 10 mM Fe(II)-EDTA, 7 mM Fe(III)-EDTA, or without iron as a control. After a lag phase of 6 days, strain BoFeN1 oxidized  $4.7 \pm 0.6$  mM Fe(II)-EDTA at a maximum rate of  $2.5$  mM day<sup>-1</sup> (Fig. 3A). Strain 2AN had a shorter lag phase of 4 days and was able to oxidize  $7.5 \pm 1.5$  mM Fe(II)-EDTA at a maximum rate of  $2.6$  mM day<sup>-1</sup> (Fig. 3A). After 25 days of incubation,  $2.4 \pm 0.3$  mM Fe(III) was quantified in BoFeN1 cultures and  $4.7 \pm 1.5$  mM Fe(III) in the cultures of 2AN (Fig. 3B) using the ferrozine assay. When both strains were incubated individually in the presence of 7 mM Fe(III)-EDTA, only  $5.3 \pm 0.2$  mM total Fe(III) was measurable at the end of the experiment for BoFeN1 and

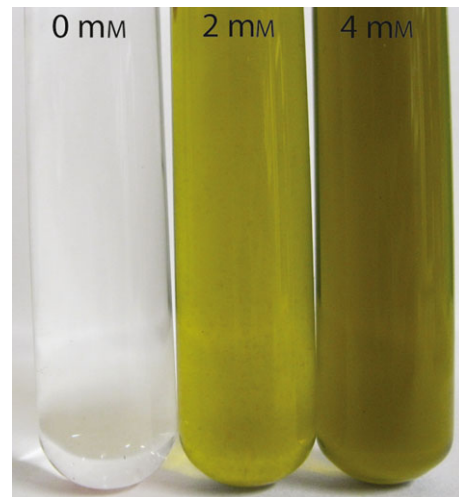


Fig. 2 Photograph of culture tubes containing bicarbonate-buffered freshwater medium with approximately 11 mM Fe(II)-EDTA and different concentrations of nitrite (0, 2, and 4 mM) after 1 day of incubation.

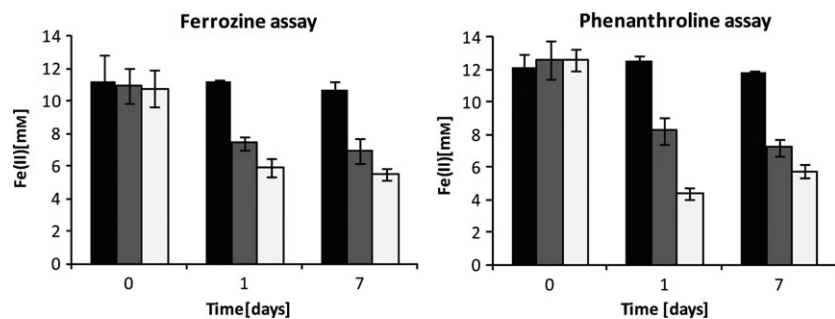
$5.5 \pm 0.1$  mM for 2AN (Fig. 3B), and  $1.0 \pm 0.1$  mM Fe(II) was formed in BoFeN1 and  $0.7 \pm 0.0$  mM Fe(II) in 2AN cultures over the same period (Fig. 3A).

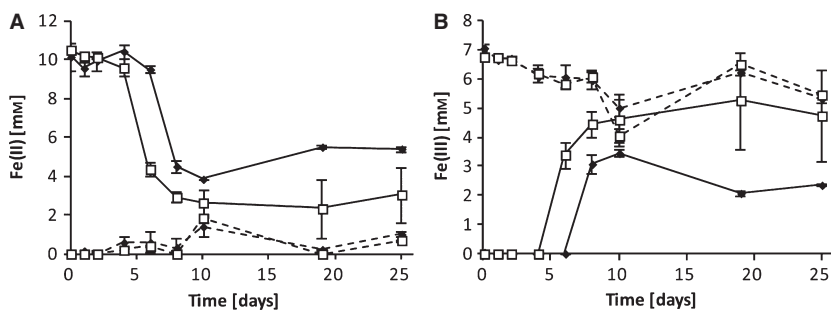
In *Acidovorax* 2AN cultures amended with 10 mM Fe(II)-EDTA, a green color developed in the tubes after 5 days, which turned and remained brownish-orange after two more days (Fig. 4). In contrast, in the cultures of BoFeN1 at day 5, only a slight green color was visible. This intensified over the next several days, and the cultures of BoFeN1 still showed the intense green color after 25 days. Using the absorbance maxima of Fe(II)-EDTA-NO (430 nm) and Fe(III)-EDTA (340 nm), we were able to confirm the formation of these both compounds (Fig. S3). The formation of biogenic nitrite was confirmed in all Fe(II)-EDTA-containing cultures by qualitative analysis using test sticks.

#### Effects of Fe on microbial acetate consumption

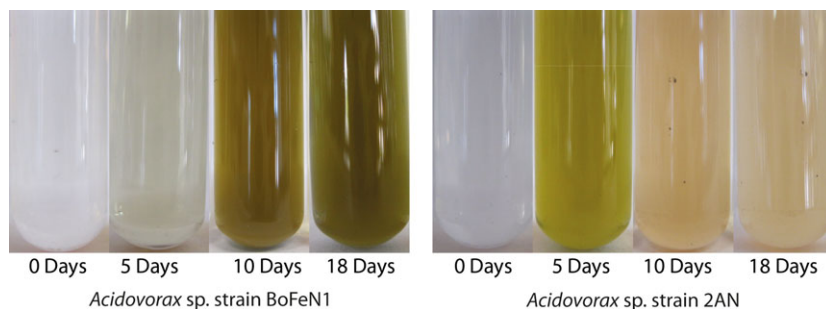
The long lag phase of Fe(II) oxidation in both *Acidovorax* cultures raises the question of whether Fe(II)-EDTA is toxic to these strains. In cultures of both strains, acetate

Fig. 1 Oxidation of approximately 11 mM Fe(II)-EDTA by 2 mM nitrite (gray bars) or 4 mM nitrite (white bars) quantified over time with the ferrozine assay (left panel) or the phenanthroline assay (right panel) in freshwater medium containing 22 mM bicarbonate buffer. The controls contained no nitrite (black bars). Error bars indicate standard deviation calculated from three replicates.





**Fig. 3** Fe(II) (A) and Fe(III) (B) concentrations over time for *Acidovorax* strain BoFeN1 (◆) and *Acidovorax* strain 2AN (□) in freshwater medium containing 10 mM nitrate, 5 mM acetate, and either 10 mM Fe(II)-EDTA (solid lines) or 7 mM Fe(III)-EDTA (dashed lines). Fe(II) and Fe(III) were analyzed using the ferrozine assay. Sterile controls show no change in iron concentrations (not shown). Error bars indicate the range of duplicates.



**Fig. 4** Photographs of culture tubes of *Acidovorax* strain BoFeN1 (left) and *Acidovorax* strain 2AN (right) during the growth on 10 mM nitrate, 5 mM acetate, and 10 mM Fe(II)-EDTA.

consumption (Fig. 5) started concurrently with Fe(II) oxidation, that is, with the same lag phase of several days (Fig. 3A). In BoFeN1 cultures with 10 mM Fe(II)-EDTA, acetate consumption started at day 6 with a maximum rate of  $1.4 \pm 0.0 \text{ mM day}^{-1}$ . In cultures of 2AN, acetate consumption started at day 4 with a maximum rate of  $2.0 \pm 0.0 \text{ mM day}^{-1}$ . In cultures amended with 7 mM Fe(III)-EDTA, we also observed a slower acetate consumption rate (BoFeN1 =  $1.7 \pm 0.1 \text{ mM day}^{-1}$ ; 2AN =  $3.2 \pm 0.0 \text{ mM day}^{-1}$ ) and a short lag phase compared to the rates of acetate consumption in the iron-free cultures (BoFeN1 =  $2.5 \pm 0.0 \text{ mM day}^{-1}$ ; 2AN =  $4.0 \pm 0.0 \text{ mM day}^{-1}$ ).

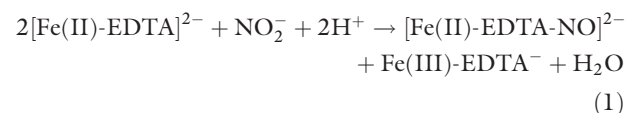
## DISCUSSION

There is ongoing discussion about whether nitrate-reducing Fe(II)-oxidizing bacteria have the enzymatic machinery to oxidize Fe(II) or whether, and to what extent, Fe(II) oxidation is caused by a chemical reaction between Fe(II) and biologically produced nitrite. Previous studies in batch cultures were mostly conducted with aqueous  $\text{Fe}^{2+}$ , but some authors have used chelated Fe(II)-EDTA to minimize Fe(III) oxyhydroxide mineral formation that ultimately limits cell activity or otherwise produces experimental complications. Chakraborty & Picardal (2013) did not see any chemical oxidation of Fe(II)-EDTA by nitrite, but observed Fe(II) oxidation after inoculation with the nitrate-reducing *Acidovorax* strain 2AN. This result was interpreted as a direct evidence for enzymatic Fe(II)

oxidation. However, several other reports in the literature have shown abiotic Fe(II)-EDTA oxidation by nitrite. We wanted to evaluate these conflicting results, and our data clearly showed that Fe(II)-EDTA is indeed abiotically oxidized by nitrite under conditions relevant for these microbial experiments. The observed Fe(II)-EDTA oxidation in cultures of *Acidovorax* sp. 2AN and BoFeN1 is, at least partially, therefore due to abiotic side reactions.

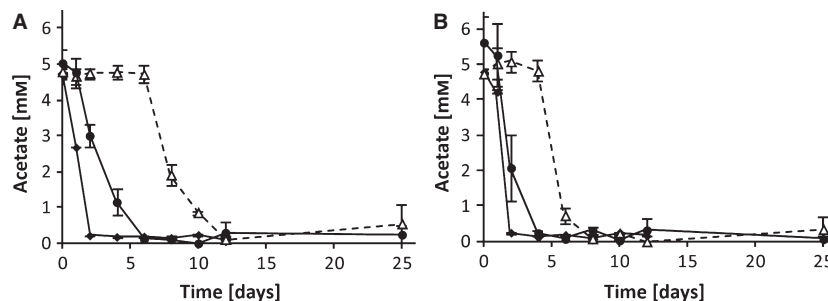
### Abiotic Fe(II)-EDTA oxidation by nitrite

Addition of nitrite to a buffered Fe(II)-EDTA solution led to rapid oxidation of Fe(II)-EDTA within a few days (Fig. 1). Zang *et al.* (1988) showed that the reaction of Fe(II)-EDTA under anoxic conditions with an excess of nitrite produces Fe(III)-EDTA and Fe(II)-EDTA-NO in a 1:1 ratio (equation 1).



In our abiotic experiment (Fig. 1), we observed the loss of approximately 4 mM Fe(II) after addition of 2 mM nitrite. Considering the proposed stoichiometric equation for this reaction (equation 1), 2 mM of Fe(III)-EDTA and 2 mM of Fe(II)-EDTA-NO are expected to have formed. The presence of both compounds was confirmed by measuring absorbance at specific wavelengths (Zang *et al.*, 1988) (Fig. S1). However, the loss in Fe(II)-EDTA could

**Fig. 5** Acetate consumption of *Acidovorax* strain BoFeN1 (A) and *Acidovorax* strain 2AN (B). Both strains were cultivated in the presence of 10 mM nitrate, 5 mM acetate, and either 10 mM Fe(II)-EDTA ( $\Delta$ , dashed line) or 7 mM Fe(III)-EDTA ( $\bullet$ ) or without iron ( $\blacklozenge$ ). Error bars indicate the range of duplicates.



not be balanced with the measured increase in Fe(III). One reason for this might be the fact that we did not use an excess of nitrite as in the experiments of Zang *et al.*, which might have resulted in a different stoichiometry. Another reason might be that we were likely not able to measure the Fe(II) present in the Fe(II)-EDTA-NO complex with our spectrophotometric quantification methods. We diluted our samples in sulfamic acid to stabilize Fe(II) against chemical oxidation because we performed both the ferrozine and the phenanthroline assay under oxic conditions. Maybe this acidification leads to an unknown reaction which caused neither ferrozine nor phenanthroline to complex Fe(II) (to outcompete the Fe(II)-EDTA-NO or any other formed complex), which led to an absence in a detectable color change. After addition of 4 mM nitrite, only 5–7 mM Fe(II) was lost instead of the 8 mM predicted by equation 1.

Chakraborty & Picardal (2013) did not measure any abiotic oxidation of 5 mM Fe(II)-EDTA by 2.5 mM nitrite, in contrast to previous studies (Van Der Maas *et al.*, 2004; Kumaraswamy *et al.*, 2006) that reported such a reaction. Direct evidence for the reaction between nitrite and Fe(II)-EDTA is the development of the strong green color caused by the formation of the Fe(II)-EDTA-NO nitrosyl complex (equation 1). We also observed this green color (Fig. 2), although such color development was not previously observed by Chakraborty and Picardal. Both the ferrozine and the phenanthroline spectrophotometric iron quantification assays have been used by many research groups for Fe(II) and Fe(III) quantification, and the application of these two methods may have resulted in different Fe(II)-EDTA quantification efficiencies and therefore varying results. We therefore compared the very commonly used spectrophotometric assays with ferrozine or phenanthroline as colorimetric agents. Both assays showed the same results, and we found that both are equally suitable for quantifying Fe(II) in cultures containing Fe(II)-EDTA. Although ferrozine is generally not effective for use in measuring EDTA-complexed Fe(II), equilibrium speciation modeling predicts that our acidification step releases Fe<sup>2+</sup> from the Fe(II)-EDTA complex (data not shown) which would allow successful quantification with the ferrozine protocol. Even when replacing bicarbonate buffer by PIPES buffer, we observed the same extent of Fe(II)-EDTA oxida-

tion. In summary, major differences due to the use of different iron quantification methods can be ruled out; therefore, it is currently unclear why Chakraborty & Picardal (2013) saw no abiotic Fe(II)-EDTA oxidation by nitrite. Most probably, the nitrite stability observed by Chakraborty & Picardal (2013) was an artifact of the analytical method used to quantify nitrite, and the lack of strong green color formation in their experiments may have been a result of their use of lower Fe(II)-EDTA concentrations. As the results presented here are in agreement with the results of Van Der Maas *et al.* (2004), Kumaraswamy *et al.* (2006), and several others (Sada *et al.*, 1984; Zang *et al.*, 1988; Littlejohn & Chang, 1990), we are confident that there is indeed significant abiotic Fe(II)-EDTA oxidation by nitrite under the conditions of our studies.

#### Fe(II)-EDTA oxidation by *Acidovorax* strains BoFeN1 and 2AN

Chakraborty & Picardal (2013) showed that *Acidovorax* strain 2AN is able to oxidize Fe(II)-EDTA and forms Fe(III)-EDTA. Considering the results of our abiotic experiments, we wanted to compare *Acidovorax* strain BoFeN1 and strain 2AN in their ability to oxidize EDTA-chelated Fe(II). Both strains are nitrate-reducing bacteria and have both been described to oxidize aqueous Fe(II) (Kappler *et al.*, 2005; Chakraborty *et al.*, 2011). Cultivating both strains with 10 mM nitrate, 5 mM acetate, and 10 mM Fe(II)-EDTA, we observed that after 25 days 71% (2AN) or 47% (BoFeN1) of the initial Fe(II) was lost (Fig. 3). At the same time, Fe(III) increased (although total Fe decreased, data not shown) but never fully balanced the loss of Fe(II). This supports our conclusions drawn from the results of the abiotic experiments that our spectrophotometric assay probably did not measure the Fe(II)-EDTA-NO complex. In cultures of strain BoFeN1, 2.4 mM Fe(III) was formed within 25 days, and when comparing this concentration to the total loss of 4.7 mM Fe(II), we can conclude that 2.3 mM of Fe was undetected. Considering the possible formation of Fe(III)-EDTA/Fe(II)-EDTA-NO in a 1:1 ratio (see equation 1), the missing 2.3 mM Fe may have been present as the green nitrosyl complex (Fig. 4). The culture tubes of BoFeN1 kept the

strong green color of the nitrosyl complex even after long-term incubations (2 months) (data not shown), suggesting that this complex is very stable and could not be further oxidized by strain BoFeN1. Alternatively, oxidation of Fe(II) in the nitrosyl complex may first require reduction of the complexed NO, leading to release of Fe(II)-EDTA (equation 2, below). In that case, strain BoFeN1 may instead be incapable of reduction of the complexed NO.

If all Fe(II)-EDTA disappearance in BoFeN1 cultures resulted from an abiotic reaction with nitrite, approximately 2.4 mM nitrite would have been required in BoFeN1 cultures to explain the observed loss of 4.8 mM Fe(II). Although we were unfortunately not able to measure nitrite concentrations due to interferences of Fe(II)-EDTA with our standard nitrite quantification methods, such nitrite concentrations have been previously observed in cultures of strain BoFeN1 (Klueglein & Kappler, 2013).

In contrast, *Acidovorax* strain 2AN cultures initially showed formation of the green complex, but this color subsequently disappeared to form the brownish-to-orange color stemming from the Fe(III)-EDTA. In the cultures of 2AN, approximately 7.5 mM Fe(II) was ultimately oxidized or bound as the nitrosyl complex, and the loss rate arguably exceeded that observed in abiotic experiments. At the same time, 4.7 mM Fe(III) was formed; therefore, 2.8 mM of the Fe(II)-EDTA-NO complex should be present, which would implicate the formation of 2.8 mM nitrite. This shows that in contrast to the expected 1:1 ratio of Fe(III)-EDTA and Fe(II)-EDTA-NO, there is a higher Fe(III) concentration present in the cultures compared to the Fe(II)-EDTA-NO complex. This also can be observed in the color change from green to brown at day 6 (Fig. 4). This suggests that the Fe(II) in the Fe(II)-EDTA-NO complex is further biologically oxidized in the 2AN culture, either directly or after reduction of complexed NO (see equation 2 below).

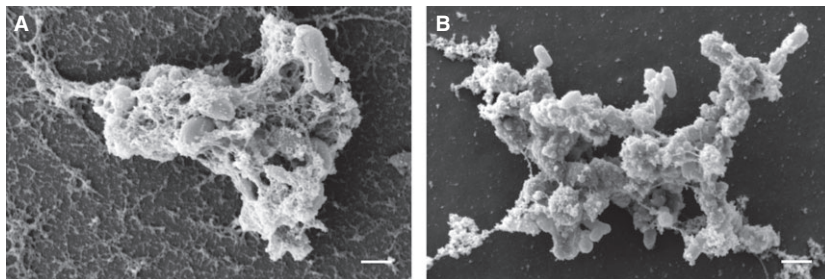
#### Fe-EDTA toxicity and its influence on acetate consumption

EDTA is a very strong chelating agent, and although biodegradation by bacteria has been described (Nörtemann, 1999), unchelated EDTA is toxic to most bacteria due to disruption of membranes by removal of the divalent

cations, calcium, and magnesium (Oviedo & Rodríguez, 2003). We also observed no growth of *Acidovorax* strains BoFeN1 and 2AN when unchelated EDTA (10 mM) was present in the medium (data not shown). Chelation of a metal (e.g., Fe(II)) by EDTA reduces the toxicity of EDTA because complexed EDTA cannot bind other metals in the cell membrane. However, in our experiments with Fe(II)- and Fe(III)-EDTA, we still saw delayed and slower acetate consumption (Fig. 5). This effect was especially pronounced in strain BoFeN1 when cultivated with Fe(II)-EDTA, where we measured a lag phase of 6 days prior to acetate consumption. Strain BoFeN1 therefore showed a high sensitivity to iron-bound EDTA, whereas acetate consumption by strain 2AN displayed a short lag phase when cultivated with Fe(II)-EDTA and only a minor response to Fe(III)-EDTA. Inhibition might have resulted from low concentrations of the more toxic, unchelated EDTA still in solution, although previous equilibrium speciation calculations (Chakraborty & Picardal, 2013) showed that only submicromolar concentrations of free EDTA should be present. Although the inhibitory mechanism remains speculative, it may be due to a combined toxicity effect of Fe(II) and EDTA. Aqueous Fe<sup>2+</sup> was described to be toxic to cells under anoxic conditions in the mM range (Poulain & Newman, 2009; Bird *et al.*, 2013) and, as a possible toxicity response, led to exopolysaccharide (EPS) formation at high concentrations (Klueglein *et al.*, 2014). SEM images of *Acidovorax* strains BoFeN1 and 2AN cultivated in the presence of Fe(II)-EDTA also showed significant amounts of EPS surrounding and agglutinating the cells (Fig. 6). This observation suggests that Fe(II)-EDTA may be more toxic than aqueous Fe<sup>2+</sup>, at least to BoFeN1, and that metabolic activity may require an adaptation time of several days.

#### Implications for nitrate-dependent Fe(II) oxidation

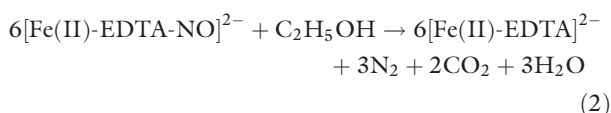
Based on our experiments, it is still uncertain whether both tested *Acidovorax* strains are able to oxidize Fe(II) enzymatically. The fact that the BoFeN1 culture was not able to oxidize all available Fe(II)-EDTA, despite an excess of provided nitrate, and that the green color of the Fe(II)-EDTA-NO complex persisted in BoFeN1 cultures for at least 2 months shows that the abiotic Fe(II) oxidation by



**Fig. 6** Scanning electron microscopy images of *Acidovorax* strain BoFeN1 (A) and *Acidovorax* strain 2AN (B) cultivated with 10 mM nitrate, 5 mM acetate, and 10 mM Fe(II)-EDTA. Samples were taken after 25 days of incubation. Scale bars are 1  $\mu$ m.

nitrite rather than an enzymatic oxidation of Fe(II) played the major role in our experiments with this strain. Another possibility is a potential direct inhibition of enzymatic Fe(II) oxidation in strain BoFeN1 due to a higher sensitivity to the toxic effects of Fe(II)-EDTA compared to the cultures of *Acidovorax* strain 2AN. In strain 2AN, we observed further oxidation of either Fe(II) or the reduction of NO present in the Fe(II)-EDTA-NO complex, which must be biologically controlled as the Fe(II)-EDTA-NO does not become oxidized abiotically by nitrite as shown by our abiotic controls. The fact that a brownish-orange color was produced in 2AN cultures upon disappearance of the green nitrosyl complex, however, suggests that Fe(II)-EDTA was ultimately oxidized to Fe(III)-EDTA.

There is, however, extensive literature available dealing with Fe(II)-EDTA-NO in the so-called BioDeNOx technique, in which industrial NOx gases are bound to Fe(II)-EDTA (Jin *et al.*, 2005). In this context, many authors observed biological reduction of NO chelated to Fe(II)-EDTA (Van Der Maas *et al.*, 2006; Zhang *et al.*, 2007) (equation 2).



In addition, several strains have been isolated that are able to reduce the NO in Fe(II)-EDTA-NO (Kumaraswamy *et al.*, 2006; Zhang *et al.*, 2007; Dong *et al.*, 2013). It may be that *Acidovorax* strain 2AN is also able to further reduce the chelated NO. This would lead to free Fe(II)-EDTA, which again could be oxidized by nitrite. Further experiments and genetic studies with both strains are necessary to clarify the utility of using Fe(II)-EDTA-NO as electron acceptor.

In contrast to the previous work of Chakraborty & Picardal (2013), our study shows that abiotic oxidation of Fe(II)-EDTA by nitrite is an important factor in Fe(II)-EDTA oxidation by nitrate-reducing bacteria. Interestingly, although both strains are members of the genus *Acidovorax*, they apparently behave differently in their ability to deal with the toxic effects of iron-EDTA and the further reduction of the Fe(II)-EDTA-NO nitrosyl complex. Therefore, results from one bacterial strain cannot be easily transferred to other strains, and further experiments with a variety of described nitrate-reducing Fe(II)-oxidizers are necessary to answer the question whether Fe(II) oxidation is truly enzymatic.

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## SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

**Fig. S1** (a) Absorbance spectra of 10 mM Fe(II)-EDTA reacted with 4 mM nitrite forming the Fe(II)-EDTA-NO nitrosyl-complex. Absorption maxima are at 635 and 430 nm. The theoretical maximum at 340 is completely covered by an intense absorption of Fe(III)-EDTA. (b) Absorbance spectra of 10 mM Fe(II)-EDTA and 10 mM Fe(III)-EDTA.

**Fig. S2** Absorbance of test tubes containing approximately 10 mM Fe(II)-EDTA and 2 mM nitrite in (A) 22 mM bicarbonate buffered medium or (B) 5 mM PIPES buffered medium over time. Diamonds = 0 h; squares = 4 h; triangles = 24 h; circles = 48 h. Absorbance at 630, 430, 340 and 258 nm is indicative for the Fe(II)-EDTA-NO nitrosyl-complex. Error bars indicate standard deviation of three replicates. The absence of error bars indicates that the error was smaller than the symbol size.

**Fig. S3** Absorbance at 430 nm (A) or 340 nm (B) of cultures of *Acidovorax* strain BoFeN1 (square) or *Acidovorax* strain 2AN (triangle) cultivated with 10 mM nitrate, 5 mM acetate and approximately 10 mM Fe(II)-EDTA (filled symbols, solid line) or approximately 7 mM Fe(III)-EDTA (open symbols) or without iron (filled symbols, dashed lines). OD 3 = maximum.