Environmental Science & Technology

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Ligand-Enhanced Abiotic Iron Oxidation and the Effects of Chemical versus Biological Iron Cycling in Anoxic Environments

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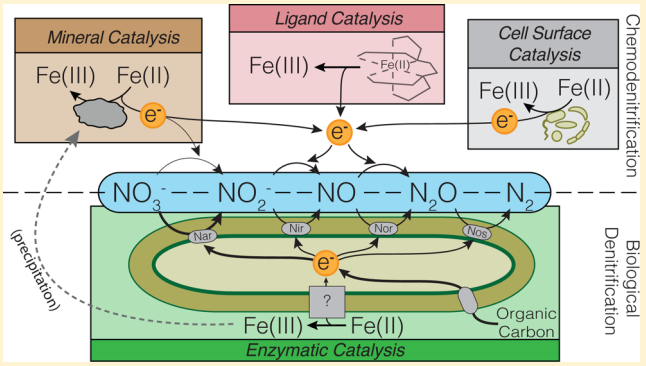
Supporting Information

ABSTRACT: This study introduces a newly isolated, genetically tractable bacterium (*Pseudogulbenkiania* sp. strain MAI-1) and explores the extent to which its nitrate-dependent iron-oxidation activity is directly biologically catalyzed. Specifically, we focused on the role of iron chelating ligands in promoting chemical oxidation of Fe(II) by nitrite under anoxic conditions. Strong organic ligands such as nitrilotriacetate and citrate can substantially enhance chemical oxidation of Fe(II) by nitrite at circumneutral pH. We show that strain MAI-1 exhibits unambiguous biological Fe(II) oxidation despite a significant contribution (~30−35%) from ligand-enhanced chemical oxidation. Our work with the model denitrifying strain *Paracoccus denitrificans* further shows that ligand-enhanced chemical oxidation of Fe(II) by microbially produced nitrite can be an important general side effect of biological denitrification. Our assessment of reaction rates derived from literature reports of anaerobic Fe(II) oxidation, both chemical and biological, highlights the potential competition and likely co-occurrence of chemical Fe(II) oxidation (mediated by microbial production of nitrite) and truly biological Fe(II) oxidation.



INTRODUCTION

Fe(II)/Fe(III) is an important redox couple in natural environments.[1] In anoxic systems, iron oxidation can be mediated by several biological agents, such as anoxygenic phototrophs[2,3] and nitrate-dependent chemotrophs.[4,5] While the enzymatic machinery for Fe(II) oxidation has been identified and characterized for two anoxygenic phototrophs,[3,6,7] comparable catalysts have not yet been identified for nitrate-dependent chemotrophs. Toward this end, we isolated a fast growing Fe(II) oxidizing, nitrate-dependent chemotroph from the iron-rich tropical Lake Matano,[8] with the intention of developing it into a model genetic system. However, work with the isolate highlighted a second, often overlooked aspect of Fe(II) oxidation in anoxic environments: direct chemical interaction with nitrite (a form of chemodenitrification[9]). Being able to distinguish the mechanisms and turnover rates of direct biological versus abiotic components of anaerobic Fe(II) oxidation is necessary to gain a complete understanding of the biogeochemical coupling of the N and Fe redox cycles. Here, we expand our understanding of chemodenitrification by experimental elucidation of how organic ligands promote abiotic Fe(II) oxidation by nitrite, and discuss its relevance to assessing the potential co-occurrence of chemical and biological Fe(II) oxidation.

The isolation and characterization of an increasing number of microorganisms capable of nitrate-dependent anaerobic Fe(II) oxidation in recent years[4,5,10−16] has revealed the potential for chemotrophic recycling of Fe(II) in anoxic systems. However, deconvolving the chemical and biological aspects of this process remains challenging in many environmental settings[17,18] and even laboratory studies.[19,20] The complication arises whenever denitrifying organisms reduce nitrate in iron-rich anoxic systems, where the metabolic intermediate nitrite can oxidize Fe(II).[21−26] This was recently highlighted in a review by Picardal,[27] which underscored that while biologically induced (through the production of nitrite during biological denitrification), Fe(II) oxidation can be abiotically catalyzed and proceed by chemodenitrification. Because Fe(II) oxidation may also be directly catalyzed by (potentially the same) denitrifying organisms, two competing pathways exist whose precise mechanisms and relative importance in nature are poorly understood. While the physiology of nitrate-dependent Fe(II)-oxidizing bacteria has been the subject of a growing number of studies,[16,24,28−30] the chemical aspect of anaerobic Fe(II)

Received: March 22, 2012
Revised: February 6, 2013
Accepted: February 12, 2013

dx.doi.org/10.1021/es3049459 | Environ. Sci. Technol. XXXX, XXX, XXX−XXX

oxidation by nitrite has received less attention,[27,31] despite its relevance to constraining the extent of its microbial counterpart.

Rapid oxidation of Fe(II) by nitrite in strongly acidic conditions was described as early as 1936,[32] with high reaction rates linked to the generation and subsequent degradation of nitrous acid (pK_a = 3.4). At circumneutral pH, nitrite is stable and anaerobic Fe(II) oxidation requires a catalyst or suitable Fe(II)-containing mineral to proceed at appreciable rates. Acceleration of this process has been reported with a number of specific Fe(II) mineral phases and catalysts, such as Cu^{2+},[33] iron oxides and hydroxides,[31,34−37] green rust,[25,38] as well as siderite[39] and vivianite,[23] and even microbial surfaces,[22] providing possible reaction mechanisms for Fe(II)-oxidizing chemodenitrification. The same is true for nitrate, which is generally less reactive toward Fe(II) than nitrite at circumneutral pH,[40] but can similarly benefit from metal and mineral catalysis.[41,42] However, metals and surfaces are not the only agents for chemical catalysis. While the kinetic effects of ligands (including EDTA, NTA, and citrate) on iron redox processes in oxic environments have been explored before[43−46] and often lead to acceleration of Fe(II) oxidation, much less is known about their effects in the absence of molecular oxygen. Several studies have investigated the effect of ligands on iron redox processes in acidic conditions and solvents,[47,48] but with the notable exception of studies on microbial Fe(II) oxidation in the presence of EDTA,[13,49] little is known about the impact of ligands at circumneutral pH.

Here, we investigate the effect of several Fe(II)-chelating ligands on iron-oxidizing chemodenitrification to (1) assess true biological Fe(II) oxidation in the newly isolated β-proteobacterium *Pseudogulbenkiania* sp. strain MAI-1 and (2) elucidate the role ligands could play more generally in abiotic Fe(II) oxidation in laboratory and environmental settings. We use *Paracoccus denitrificans* as a model strain to show how Fe(II) oxidation can appear to be directly biologically catalyzed when, in fact, much of this activity may only be indirectly biologically mediated. We describe the kinetics and potential reaction mechanism of the chemical oxidation of Fe(II) by nitrite observed in these experiments and discuss their relevance for the interpretation of laboratory and environmental studies. We place our findings in the context of chemical and biological oxidation rates reported in the literature to evaluate their relative importance in anaerobic Fe(II) oxidation.

MATERIALS AND METHODS

Media. All reagent solutions were autoclaved or filter-sterilized prior to use. The basal medium for all experiments was a freshwater medium containing 500 mg/L $MgSO_4 \cdot 7H_2O$, 300 mg/L NH_4Cl, 100 mg/L $CaCl_2 \cdot 2H_2O$, and 5.4 mg/L $KH_2PO_4 \cdot H_2O$[2]. For microbial cultures, the medium was amended with a 1000× vitamin mix (final concentrations in the medium: 40 μg/L 4-aminobenzoic acid, 10 μg/L D-biotin, 100 μg/L nicotinic acid, 50 μg/L Ca pantothenate, 100 μg/L pyridoxamine·2HCl, 100 μg/L thiamine·2Cl) and a 1000× trace element solution (final concentrations in the medium: 1.1 mg/L $FeSO_4 \cdot 7H_2O$, 42 μg/L $ZnCl_2$, 50 μg/L $MnCl_2 \cdot 4H_2O$, 190 μg/L $CoCl_2 \cdot 6H_2O$, 2 μg/L $CuCl_2 \cdot 2H_2O$, 24 μg/L $NiCl_2 \cdot 6H_2O$, 18 μg/L $Na_2MoO_4 \cdot 2H_2O$, 300 μg/L H_3BO_3).[50] For aerobic cultures, the medium was buffered to pH 7.2 with 20 mM phosphate. For anoxic experiments, the medium was pH buffered with 22 mM $NaHCO_3$ and adjusted to pH 7 with 1 M HCl under an oxygen free atmosphere containing 15% CO_2. Phosphate addition was minimal (but not microbially growth inhibiting) to avoid precipitation of vivianite ($Fe_3(PO_4)_2 \cdot 8H_2O$) at high Fe(II) concentrations. The final ionic strength was ∼0.04 M. Anoxic solutions were prepared using O_2-free deionized water and stored anoxically for at least three days prior to use. Reactant solutions containing nitrite were always prepared fresh from an anoxic stock solution kept at pH 11 to avoid degradation through self-decomposition. All glassware and plastics were autoclaved and stored anoxically for at least three days prior to use.

Bacterial Strains. *Paracoccus denitrificans* strain ATCC 19367 was obtained from the United States Department of Agriculture culture collection and was grown routinely in anoxic freshwater medium under denitrifying conditions with succinate as the growth substrate. *Pseudogulbenkiania* sp. strain MAI-1 is a newly isolated β-proteobacterium that was routinely grown in anoxic freshwater medium under denitrifying conditions with acetate as the growth substrate.

Isolation. Cultures of anaerobic Fe(II) oxidizing chemotrophs were enriched by inoculating freshwater medium supplemented with 10 mM $FeCl_2$, 10 mM Na_3NTA, 2 mM Na acetate, and 5 mM $NaNO_3$ with samples from a microbial mat in the litterol zone of iron-rich tropical Lake Matano, Sulawesi Island, Indonesia.[8] Enrichments were incubated at 30 °C in the dark. After a few days, some enrichments developed the characteristic dark green color of Fe(III)-NTA, indicating Fe(II) oxidation. Cultures exhibiting fast Fe(II) oxidation were transferred successively to fresh Fe(II)-containing medium. After four transfers, serial dilutions of enrichments were plated on YP agar pates (0.3% yeast extract, 0.3% Difco Bacto Peptone, 1.2% agarose) and incubated aerobically at 30 °C in the dark to identify strains potentially suitable for genetic manipulation. Colonies were picked and subcultured in the Fe(II) enrichment medium. Fast Fe(II) oxidizers were plated again, and the purity was assessed by phase-contrast microscopy. The 1497-bp 16S rRNA gene sequence of strain MAI-1 was deposited in the GenBank database under the accession number HQ714499. The pure strain was deposited with the American Type Culture Collection under the ATCC number BAA-2177.

Analytical Techniques. The concentration of Fe(II) was determined colorimetrically at 562 nm using the ferrozine [3-(2-pyridyl)-5,6 bis(4-phenylsulfonic acid)-1,2,4-triazine, monosodium salt] assay[51] without prior acidification of analyte. Sample acidification in the presence of nitrite led to underestimation of Fe(II) concentrations[31] and was therefore avoided (see Supporting Information Figure S4). The assay was calibrated using ferrous ammonium sulfate hexahydrate of known concentration. Nitrite was determined colorimetrically at 520 nm using sulfanilamide and N-1-napthylethylenediamine dihydrochloride.[52] The chelator EDTA is incompatible with this assay,[53] but none of the ligands used in this study interfere with nitrite determination (Supporting Information Figure S5). The assay was calibrated using a commercial nitrite standard (Fluka Analytical TraceCERT). Samples for Fe(II) and nitrite determination in microbial cultures were obtained with a sterile disposable syringe flushed for 30 s with 20%CO_2/80%N_2. The evolution of N_2O in abiotic reactions was assessed qualitatively by gas-chromatography using a Hewlett-Packard 5890 Series II Plus Gas Chromatograph equipped with a Thermal Conductivity Detector. Samples were injected onto a HP-MOLSIV column (30m, 0.32 mm inner diameter (ID), 12 μm film) and eluted with helium at a flow rate of 10 mL/min using a

dx.doi.org/10.1021/es3049459 | Environ. Sci. Technol. XXXX, XXX, XXX−XXX

temperature gradient from 35 to 240 °C (4 min at 35 °C, 35 °C/min up to 140 °C, 25 °C/min up to 240 °C). Formation of the nitrosyliron-NTA complex (Fe(II)-NTA-NO) was assessed qualitatively by monitoring its characteristic absorption peaks (440 nm and 600 nm)[54,55] spectroscopically. Growth of microbial cultures was followed by optical density at 600 nm (OD_{600}) in cultures without iron and at 700 nm (OD_{700}) in cultures with iron. This wavelength was used to decrease distortion by Fe(III)-NTA, which absorbs strongly at 600 nm. OD_{700} underestimates optical density as compared to OD_{600}.

Experimental Procedure. Kinetic Fe(II) oxidation experiments were conducted inside an anaerobic chamber (Coy Laboratory Products, Inc.) equipped with palladium catalysts for O_2 removal. The chamber contained ~3%H_2/15% CO_2/82% N_2 and experiments were performed at 25 °C using a digital heat block. Samples were taken at varying time points and analyzed immediately for Fe(II) and nitrite concentrations using a BioTek Synergy 4 Microplate Reader housed inside the chamber. Oxidation experiments were conducted in sterile basal freshwater medium containing 2 mM Fe(II) and 2 mM NO_2^- and were amended alternatively with 2 mM nitrilotriacetate (NTA), 300 mg/L Pahokee Peat Humic Acid (PPHA, International Humic Substances Society), 0.1, 0.5, or 2 mM citrate, 300 mg/L PPHA + 2 mM citrate. PPHA was selected as the humic acid of choice due to its high solubility and low capacity for storing redox equivalents that could rereduce Fe(III) and interfere with the experiment.[56] Control experiments included incubations of Fe(II) with or without NTA in the absence of nitrite or in the presence of 2 mM nitrate. pH was measured at the beginning and conclusion of each experiment.

Pseudogulbenkiania sp. strain MAI was grown in triplicate at 30 °C in the dark in freshwater medium amended with 0.5 mM acetate, 4 mM Fe(II), and 8 mM NTA, and a headspace of ~3%H_2/15% CO_2/82% N_2. Cultures were sampled regularly for nitrite accumulation and Fe(II) oxidation.

Paracoccus denitrificans was grown in triplicate at 30 °C in the dark in freshwater medium amended with 10 mM succinate and 20 mM nitrate and sampled regularly for nitrite accumulation. Upon reaching a nitrite concentration of ~5 mM, 5 mL of each culture was withdrawn and processed anaerobically as follows: each withdrawn sample was divided in four. Two aliquots were left unchanged while the other two were filter sterilized using a 0.2 μm syringe filter. All aliquots were spiked with ~5 mM Fe(II) and one of each set (one unfiltered *P. denitrificans* and one filter-sterilized aliquot) was further amended with 10 mM citrate (all from 1 M stock solutions to avoid sample dilution). No citrate was present in cultures prior to spiking. Aliquots were incubated at 25 °C for 4 h and sampled at regular intervals as described in the kinetic Fe(II) oxidation experiments. The remaining cultures were reincubated at 30 °C for continued monitoring of growth and nitrite accumulation.

Computation. Nonlinear least-squares model fits and parameter estimates for kinetic data were computed using the statistical model analysis functionality provided by Wolfram *Mathematica* (v. 8.0). Fe(II) speciation in solution was estimated using the Visual MINTEQ equilibrium speciation model (v. 3.0) with stability constants provided by King[57] (Fe(II)-carbonate complexes) and the MINTEQ database[58] (all other Fe(II) species) and precomputed humic substance properties based on the NICA-Donnan model.[59] Chemical oxidation of Fe(II) with nitrite produced by MAI-1 was modeled using Euler's method to calculate stepwise solutions of eq 5. Nitrite concentrations at each time step were calculated by linear interpolation between closest measurement time points. Chemical oxidation with concomitant biological NO consumption was modeled by assuming complete NO removal and subsequent lack of Fe(II)-NTA-NO complex formation.

RESULTS

The enrichment of fast growing anaerobic Fe(II) oxidizing chemotrophs lead to the successful isolation of *Pseudogulbenkiania* sp. strain MAI-1, a novel β-proteobacterium closely related to the lithoautotrophic Fe(II) oxidizer *Pseudogulbenkiania* sp. 2002[16,28] (96.9% 16S rRNA gene sequence similarity, 97.3% to the type strain *Pseudogulbenkiania subflava* BP-5[60]). MAI-1 has several key characteristics necessary for routine genetic manipulation: the strain forms colonies on plates (aerobically within 24 h), grows rapidly both aerobically and anaerobically (overnight at 30 °C), is sensitive to antibiotics, and cryopreserves well. Most importantly, it displays the desired phenotype: rapid nitrate dependent Fe(II) oxidation (10 mM in less than 24 h, Supporting Information Figure S1) in the presence of a chelator, nitrilotriacetate (NTA), that prevents the formation of mineral precipitates (which could obscure cells in automated assays) but does not serve as a growth substrate for the organism (Supporting Information Figure S2). When first isolated, MAI-1 appeared to be an ideal candidate for elucidating the genes required for nitrate dependent Fe(II) oxidation. However, although Fe(II)-NTA is highly stable in abiotic controls in the presence of nitrate (Figure 1; Supporting Information Figure S1), adding Fe(II)-NTA to filter-sterilized spent MAI-1 growth medium that had

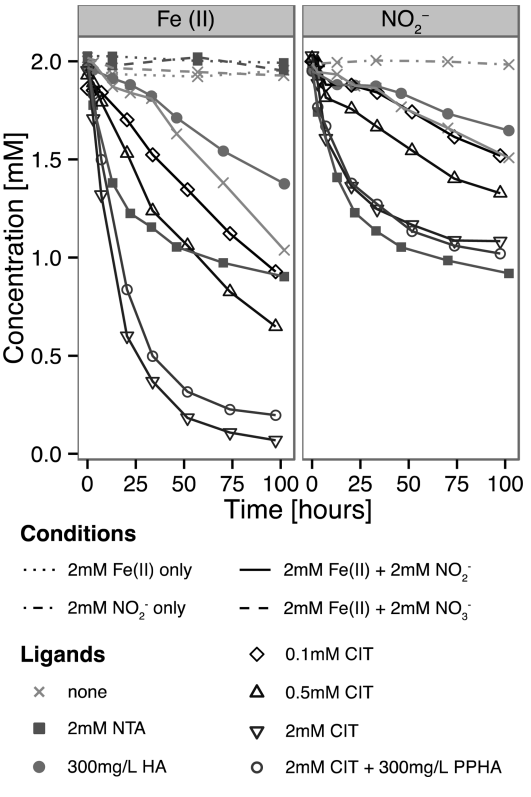


Figure 1. Ligands affect the abiotic oxidation of Fe(II) by NO_2^-. Error bars omitted for clarity (relative standard deviation of Fe(II) and NO_2^- quantitation from all seven experiments estimated at 3% and 2%, respectively).

dx.doi.org/10.1021/es3049459 | Environ. Sci. Technol. XXXX, XXX, XXX–XXX

Table 1. Summary of Kinetic Fe(II) Oxidation Experiments by Nitrite[a]

	reactant changes within ~100 h			Fe(II) oxidation		NO_2^- reduction	
	ΔFe(II) [μM] (%[b])	ΔNO_2^- [μM] (%[b])	$\Delta Fe(II)/\Delta NO_2^-$ (±1σ)[c]	model R^2	k_{app} (LCI;UCI[d]) [10^{-3} M^{-1} s^{-1}]	model R^2	k_{app} (LCI;UCI[d]) [10^{-3} M^{-1} s^{-1}]
Controls							
2 mM NO_2^- only		−3 (0%)					
2 mM Fe(II) only	−3 (0%)						
+2 mM NTA	−35 (2%)						
+2 mM NO_3^-	−91 (5%)						
+2 mM NTA + 2 mM NO_3^-	−64 (3%)						
Kinetically Unresolved							
2 mM Fe(II) + 2 mM NO_2^-	−963 (48%)	−478 (24%)	2.0 ± 0.3				
+0.1 mM citrate	−933 (50%)	−480 (24%)	1.9 ± 0.2				
+300 mg/L PPHA	−592 (30%)	−303 (16%)	2.0 ± 0.4				
Second-Order Kinetics							
+0.5 mM citrate	−1281 (66%)	−686 (34%)	1.9 ± 0.2	0.9995	0.98 (0.92;1.04)	0.9995	1.04 (0.88;1.19)
+2 mM citrate	−1883 (96%)	−945 (47%)	2.0 ± 0.1	0.9979	4.67 (4.18;5.17)	0.9992	4.31 (3.57;5.06)
+2 mM citrate +300 mg/L PPHA	−1773 (90%)	−931 (48%)	1.9 ± 0.1	0.9963	3.31 (2.85;3.78)	0.9997	3.59 (3.24;3.93)
+2 mM NTA	−1119 (55%)	−1065 (54%)	1.1 ± 0.1	0.9987	6.66 (5.19;8.13)	0.9993	6.11 (5.15;7.07)

[a]The rate constant k_{app} is reported for reactions that are described well by second-order kinetics. The experiments were conducted at 25°C, pH 6.9 to 7.1. The p-values for the model parameter k_{app} are <0.001 for all conditions. R^2 is the adjusted regression coefficient for the least-squares fit. [b]Percentage change of [Fe(II)] and [NO_2^-] relative to starting concentrations. [c]Derived by error propagation from measurement errors (relative standard deviation of Fe(II) and NO_2^- quantitation during experiments estimated at 3% and 2% respectively). [d]Lower (LCI) and upper (UCI) 95% confidence interval of parameter derived from model fit.

accumulated substantial amounts of nitrite lead to rapid Fe(II) oxidation with concomitant nitrite reduction (Supporting Information Figure S3). The strain's ability to use a wide range of chelators as a carbon substrate (e.g., citrate, humic acids, DTPA) and its inability to grow and oxidize free Fe^{2+} (Supporting Information Figure S1) precluded avoiding NTA. Additionally, MAI-1 cannot use alternate electron acceptors (e.g., DMSO, TMAO, fumarate), requiring the use of nitrate (and consequentially risking the production of nitrite) for anaerobic culturing.

To quantitatively assess the effect of Fe(II) chelation on chemical oxidation by nitrite at circumneutral pH, we conducted kinetic experiments with NTA as well as two environmentally relevant Fe(II)-chelating ligands (citrate, CIT, and Pahokee Peat Humic Acid, PPHA). Attempts to investigate the effect of Fe(II) chelation with the siderophore desferroxamine (DFO) and the organic pollutant ethylenediaminetetraacetate (EDTA) proved unsuccessful because of interference with the ferrozine assay and the nitrite assay, respectively (Supporting Information Figure S5). They were not pursued further. Figure 1 shows the oxidation of Fe(II) and concomitant reduction of NO_2^- over the course of ~100 h (4.2 days) for each condition. Nitrite-free controls without any oxidant or amended with nitrate show little Fe(II) oxidation (a maximum of 2% without oxidant, 5% with nitrate; see Table 1) over the course of the experiment. This provided confidence that O_2 contamination is not a significant source of error in our experimental setup and suggested that nitrate is relatively unreactive toward Fe(II) even in the presence of ligands (see abiotic control in Supporting Information Figure S1). Nitrite in the absence of iron shows high stability, confirming the expected absence of nitrite self-decomposition that occurs at acidic pH.[61] In the absence of any chelating moieties, less than 9% of Fe(II) is oxidized by nitrite within the first 22 h. Similar control experiments in previous reports have yielded Fe(II) oxidation rates at ~8% Fe(II) within 10 h,[36] ~9% within 20 h,[39] and ~1% within 24 h.[37] Complexation by both citrate and NTA, however, leads to rapid depletion of Fe(II) and nitrite, indicating that these organic ligands can accelerate Fe(II) oxidation by nitrite (Figure 1).

Equipped with an estimate for the extent of chemical Fe(II) oxidation by nitrite in the presence of NTA, we grew MAI-1 in the presence of Fe(II)−NTA while closely monitoring the accumulation of nitrite (Figure 2) to model the maximal abiotic Fe(II) oxidation resulting from an abiotic reaction with nitrite. Given the strong effect of citrate on the chemical oxidation of Fe(II) by nitrite, we also tested the hypothesis that abiotic Fe(II) oxidation could be mediated by the biological production of nitrite during denitrification in general. For this purpose, *P. denitrificans*, a model denitrifying microorganism, was grown anaerobically on succinate and nitrate, such that substantial quantities of nitrite accumulated during early exponential growth (Supporting Information Figure S6). After accumulation of ~5 mM nitrite, filter sterilized culture medium as well as active cultures of *P. denitrificans* were amended with ~5 mM Fe(II) with or without 10 mM citrate. Figure 3 illustrates the resulting oxidation of Fe(II) over the course of 4 h. Moderate oxidation occurred in the absence of chelation both with *P. denitrificans* cultures as well as in spent medium (up to 21% and 12%, respectively). Higher oxidation rates for cultures are likely a consequence of continued denitrification by *P. denitrificans*, increasing the measured pool of nitrite by up to 13%. However, the most striking feature is the rapid depletion of Fe(II) and nitrite (up to 76% Fe(II), 38% NO_2^-) observed with the addition of 10 mM citrate, regardless of the presence of *P. denitrificans* (Table 2, Figure 3).

DISCUSSION

Reaction Mechanism and Kinetics. Understanding the kinetics of Fe(II) oxidation in the presence of ligands provides the tools for predicting the potential effects of ligand-enhanced Fe(II) oxidation in microbial systems. The total consumption of Fe(II) and nitrite (Table 1) suggests that Fe(II) oxidation by nitrite proceeds with 2:1 Fe(II)/NO_2^- stoichiometry regardless

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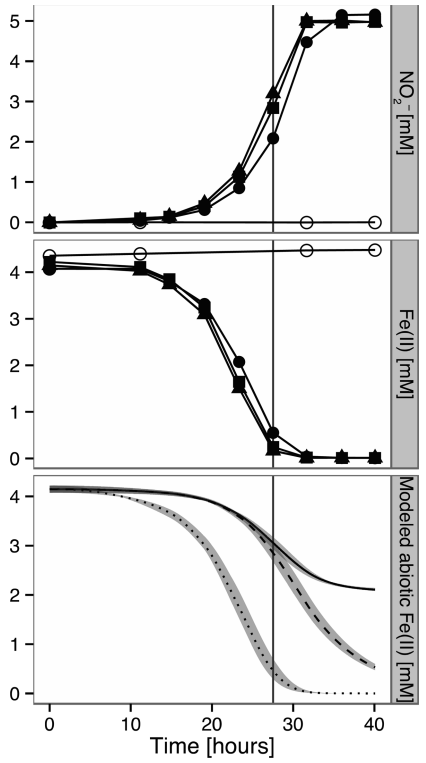

Figure 2. Fe(II) oxidation by *Pseudogulbenkiania* sp. strain MAI-1 during anaerobic growth with nitrate. Nitrite accumulation during growth depicted in top panel, concomitant Fe(II) oxidation in middle panel, modeled abiotic Fe(II) oxidation in bottom panel (see Materials and Methods for details on computation). Solid and dashed lines indicate Fe(II) oxidation without/with biological NO consumption, respectively. Dotted line indicates Fe(II) oxidation with 6× higher rate constant and NO consumption. Model range for three biological replicates shaded in gray. Vertical line indicates time point addressed in text. Experiment conducted in biological triplicates (solid markers) and with abiotic control (empty circles, ○). All data are shown.

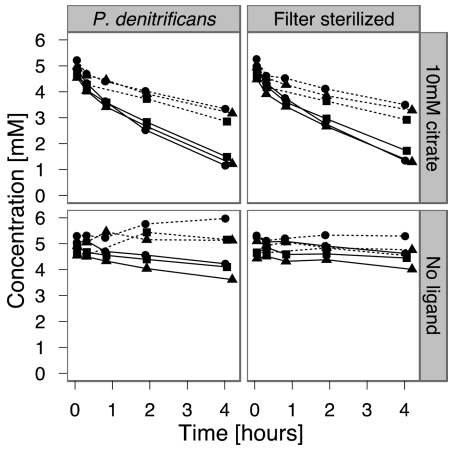

Figure 3. Fe(II) oxidation in *P. denitrificans* cultures and filter-sterilized spent medium. Fe(II) concentrations shown as solid lines, NO_2^- concentrations as dashed lines. Samples are drawn from triplicate cultures (Supporting Information Figure S6) after accumulation of ~5 mM NO_2^- and spiked with Fe(II) ± citrate at 0 h. All data are shown.

of complexation (no ligand, PPHA, citrate), with the notable exception of NTA, which appears to deplete Fe(II) and NO_2^- in a 1:1 ratio. The 2:1 stoichiometry is in agreement with

Table 2. Summary of Kinetic Fe(II) Oxidation Experiments by Nitrite in *P. denitrificans* Cultures and Spent Medium[a]

	reactant changes within ~4 h		Fe(II) oxidation		NO_2^- reduction	
	$\Delta Fe(II)$ [mM] (%[b])	ΔNO_2^- [mM] (%[b])	model R^2	k_{app} (LCI;UCI[c]) [10^3 M^{-1} s^{-1}]	model R^2	k_{app} (LCI;UCI[c]) [10^3 M^{-1} s^{-1}]
			P. denitrificans			
#1	−3.7 (76%)	−1.9 (36%)	0.9991	12 (11;14)	0.9991	11 (8;15)
#2	−3.3 (73%)	−1.8 (36%)	0.9984	11 (9;13)	0.9996	10 (8;12)
#3	−3.2 (69%)	−1.8 (38%)	0.9977	10 (7;13)	0.9985	11 (6;17)
			Filter Sterilized			
#1	−3.6 (73%)	−1.8 (33%)	0.9990	11 (9;12)	0.9981	10 (6;15)
#2	−3.2 (71%)	−1.7 (34%)	0.9985	11 (9;13)	0.9995	10 (8;12)
#3	−3.2 (65%)	−1.7 (37%)	0.9983	9 (7;11)	0.9988	12 (7;17)

[a]The experiment was conducted at 25 °C. P-values for the model parameter k_2 are <0.01. R^2 is the adjusted regression coefficient for the least-squares fit. [b]Percentage change of [Fe(II)] and [NO_2^-] relative to starting concentrations. [c]Lower (LCI) and upper (UCI) 95% confidence interval of parameter derived from model fit.

literature reports that the predominant product of nitrite reduction at pH regimes between 6 and 8 is N_2O,[22,33,36,37,62] according to the following representative net reaction:

$$k_1 \quad 4Fe^{2+} + 2NO_2^- + 6H^+ \rightarrow 4Fe^{3+} + N_2O + 3H_2O \tag{1}$$

where Fe^{2+} can be unbound Fe^{2+} or a ligand-bound Fe(II)-L species, and Fe^{3+} can be ligand-bound Fe(III)-L or contained within an (oxy)hydroxide mineral (e.g., FeOOH). This net reaction likely comprises a number of elementary reaction steps; we consider the following three to contextualize our observations:

$$k_2(\text{slow}) \quad Fe^{2+} + NO_2^- + 2H^+ \rightarrow Fe^{3+} + NO_{aq} + H_2O \tag{2}$$

$$k_3(\text{fast}) \quad Fe^{2+} + NO_{aq} \rightarrow (Fe(II) - NO)^{2+} \tag{3}$$

$$k_4(\text{fast}) \quad (Fe(II) - NO)^{2+} + H^+ \rightarrow Fe^{3+} + \frac{1}{2}N_2O + \frac{1}{2}H_2O \tag{4}$$

Equations 3[63] and 4[64] proceed rapidly at circumneutral pH, with eq 2 being the rate limiting step ($k_1 \approx k_2$). Accordingly, the reaction consumes 2 Fe(II) for every NO_2^-, except in the case of NTA. Both citrate and NTA complexes with ferrous iron can bind nitric oxide such that the following reactions can occur in competition with eq 3:

$$k_5 \quad (Fe(II) - CIT)^- + NO_{aq} \rightarrow (Fe(II) - CIT - NO)^- \tag{5}$$

$$k_6 \quad (Fe(II) - NTA)^- + NO_{aq} \rightarrow (Fe(II) - NTA - NO)^- \tag{[6]}$$

dx.doi.org/10.1021/es3049459 | Environ. Sci. Technol. XXXX, XXX, XXX−XXX

However, Fe(II)-NTA forms a considerably stronger complex with NO ($k_6 \approx 2.1 \times 10^7$ M^{-1} s^{-1}, $K_{eq} = 10^{6.26}$)[54,65,66] than Fe(II)-citrate ($k_5 \approx 4.4 \times 10^5$ M^{-1} s^{-1}, $K_{eq} = 10^{2.83}$)[66] or Fe^{2+} alone ($k_3 \approx 6.2 \times 10^5$ M^{-1} s^{-1}, $K_{eq} = 10^{2.65}$),[63] potentially preventing eq 4 from proceeding. For example, if 100 μM Fe(II) reacted with 100 μM NO_2^- to form NO in the presence of 2 mM NTA, more than 99.98% of the produced NO would form the highly stable Fe(II)-NTA-NO complex. The 1:1 stoichiometry of Fe(II) oxidation by nitrite observed in the presence of NTA is likely a consequence of this stable Fe(II)−NTA−NO complex formation. As expected, we confirmed evolution of N_2O during Fe(II) oxidation by nitrite by gas chromatography in the presence of citrate, but no N_2O formed in the presence of NTA (Supporting Information Figure S8); the formation of the Fe(II)−NTA−NO complex could be observed instead (Supporting Information Figure S9).

Based on the rate-limiting, Fe(II) and NO_2^- dependent first reaction step (eq 2), a plausible scheme for the overall reaction kinetics is a second-order rate expression with overall rate constant k_{app} in analogy with oxidation of Fe(II) and Mn(II) by O_2[57,67]

$$\mathrm{dFe(II)}/\mathrm{d}t = -2k_{app}[\mathrm{Fe(II)}][\mathrm{NO_2^-}] \quad (7)$$

$$\mathrm{dNO_2^-}/\mathrm{d}t = -k_{app}[\mathrm{Fe(II)}][\mathrm{NO_2^-}] \quad (8)$$

where Fe(II) comprises the total pool of ferrous iron (free Fe^{2+} as well as all complexed Fe(II)). Given the equimolarity of initial total Fe(II) and NO_2^- in our experimental setup, we integrate eqs 7 and 8 to yield the following decay equations (see the Supporting Information for details):

$$\mathrm{Fe(II)}(t) = \frac{\mathrm{Fe(II)}_0}{-1 + 2\,e^{\mathrm{Fe(II)}_0 k_{app} t}} \quad (9)$$

$$\mathrm{NO_2^-}(t) = \frac{\mathrm{NO_2^-}_0\, e^{\mathrm{NO_2^-}_0 k_{app} t}}{-1 + 2\,e^{\mathrm{NO_2^-}_0 k_{app} t}} \quad (10)$$

Least-squares fits of eqs 9 and 10 to our experimental results for Fe(II) and NO_2^- depletion provide two separate estimates of the overall rate constant k_{app} for each condition (Tables 1 and 2). Reactions without a ligand and with low citrate or PPHA are better described by a linear least-squares fit (apparent zero-order kinetics) and are therefore considered kinetically unresolved (no k_{app} determined). Elementary reaction steps and kinetic constraints for these conditions cannot be deduced from our observations, and it remains unclear why the reactions appear to be zero-order. Oxidation in these conditions likely proceeds as a consequence of ferric (oxy)hydroxide precipitation (observed visually) and subsequent heterogeneous autocatalysis as reported by Tai and Dempsey (2009).[37] Apparent zero-order kinetics could reflect the complex balance between the generation of catalytic mineral surfaces and depletion of dissolved Fe(II) and nitrite.

At higher concentrations of citrate and NTA, the reactions remained homogeneous and are in agreement with a second-order kinetic interpretation of our data (Tables 1 and 2 and Supporting Information Figure S7). Rate constants derived from Fe(II) oxidation and nitrite reduction agree well within their 95% confidence intervals, lending further credence to the model. The pH remained close to 7.0 in all conditions, with an average change of 0.1 by the end of the experiment (Supporting Information Table S1), suggesting that the presence of the ligands, rather than fluctuations in pH are responsible for the observed differences in reaction kinetics. The reaction progression observed in the presence of PPHA suggests that chelation of Fe(II) by the humic acid moieties (10% of the initial Fe(II) pool is organically complexed) has little to no effect on the kinetics of iron oxidation (see Figure 1, PPHA and CIT + PPHA). Rather than accelerating Fe(II) oxidation, PPHA appears to have a slight retarding effect. In contrast to experiments without a ligand, PPHA is likely to impede iron oxide formation and autocatalysis as a result of its high affinity for Fe(III). In combination with citrate, PPHA leads to diminished formation of the Fe(II)-citrate complex (Supporting Information Table S2), which appears to reduce the overall reaction rate (Table 1).

Additional information for predicting the contribution of chemical Fe(II) oxidation, especially in well-defined laboratory systems, can be gained from identifying the reactive species. In analogy to Fe(II) and Mn(II) oxidation by O_2, the overall rate constant k_{app} observed in our experiments can likely be explained in terms of the weighted sum of the oxidation rates of individual Fe(II) species[57,67] $k_{app} = \sum k_i \alpha_i$ where α_i is the fraction of each Fe(II) species in solution and k_i the species-specific second-order rate constant for oxidation by nitrite. A comparison of k_{app} with the extent of Fe(II) complexation for each experimental condition (Figure 4; Supporting Information Table S2) suggests that the Fe(II)-L complex is involved in accelerating Fe(II) oxidation, although the effect is ligand-specific (no effect for PPHA, variable magnitude for citrate and NTA). The observed reaction rates at low species fractions of Fe(II)-L (<20%) suggest the existence of other Fe(II) species with appreciable nitrite-dependent oxidation rates. We speculate that the carbonate species Fe(II)−CO_3−OH^- and Fe(II)−$(CO_3)_2^{2-}$ (Supporting Information Table S2) could provide such reactive species in analogy to their role in Fe(II) oxidation by molecular oxygen.[57] However, the precise mechanism and species-specific reaction rates k_i for the

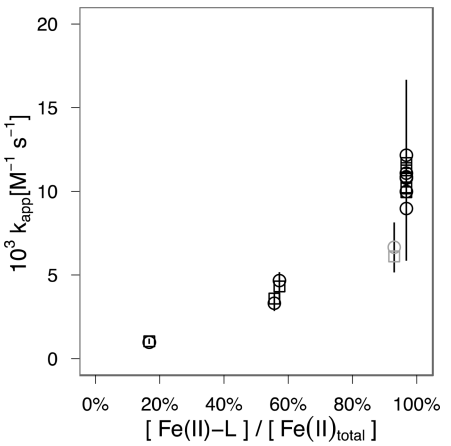

Figure 4. Rate constants increase with increasing degree of Fe(II) complexation. Second-order rate constants for oxidation experiments in the presence of citrate (black symbols) and NTA (gray symbols) are plotted against the degree of Fe(II) complexation by citrate/NTA. Rate constants derived from [Fe(II)] depicted as circles (○), constants derived from [NO_2^-] as squares (□). Error bars indicate 95% confidence intervals (Tables 1 and 2). Details on speciation can be found in Supporting Information Table S1. Larger confidence intervals for data reported in Table 2 are a consequence of reduced temporal resolution and greater deviation from the assumption that initial Fe(II) and NO_2^- concentrations are equimolar.

dx.doi.org/10.1021/es3049459 | Environ. Sci. Technol. XXXX, XXX, XXX−XXX

Table 3. Maximal Rates of Fe(II) Oxidation Reported for Various Anaerobic Processes at Circumneutral pH (25−30 °C, Except Where Otherwise Indicated)

	experimental conditions					max. rates	
	pH	buffer	Fe(II)	nitrite	nitrate	ΔFe(II) [μM/h]	reference
Chemical (Abiotic)							
+30 mg/L lepidocrocite (γ-FeOOH)	7.5	autotitration	0.2 mM	0.2 mM		−7	36, Figure 5
+30 mg/L lepidocrocite (γ-FeOOH)	8.5	autotitration	0.2 mM	0.2 mM		−40	36, Figure 5
Fe(II) as siderite (10 g/L ~ 80 mM)	6	MES/PIPES/ HEPES	10 g/L	4.6 mM		−265	39, Figure 5
Fe(II) as siderite (10 g/L ~ 80 mM)	6.5	MES/PIPES/ HEPES	10 g/L	4.6 mM		−169	39, Figure 5
Fe(II) as siderite (10 g/L ~ 80 mM)	7.9	MES/PIPES/ HEPES	10 g/L	4.6 mM		−140	39, Figure 5
+2.5 mM Fe(II) as HFO, 64 μM average solid-bound Fe(II)	6.8	PIPES	0.38 mM	0.38 mM		−158	37, Table 1, #6
+17.5 mM Fe(III) as HFO, 188 μM average solid-bound Fe(II)	6.8	PIPES	0.34 mM	0.32 mM		−301	37, Table 1, #11
F(II) as green rust	8.25	autotitration	10.81 mM		14.2 mM	−139	42, Table 1
+2 mM NTA	7	bicarbonate	2 mM	2 mM		−192	this study, Table 1
+2 mM CIT	7	bicarbonate	2 mM	2 mM		−134	this study, Table 1
+10 mM CIT, *P. denitrificans* spent medium	7	bicarbonate	5 mM	5 mM		−1695	this study, Table 2
+10 mM CIT, *P. denitrificans* culture	7	bicarbonate	5 mM	5 mM		−1910	this study, Table 2
Mixed (Chemical + Biological)							
D. frappieri strain G, Fe(II) complexed by 10 mM NTA	~7	bicarbonate	4.8 mM	1.4 mM	2.5 mM	−294	20, Figure 5
D. frappieri strain G, Fe(II) as smectite	~7	bicarbonate	3 mM	1.4 mM	5 mM	−175	20, Figure 6
Pseudogulbenkiania sp. MAI-1, Fe(II)-NTA	7	bicarbonate	4 mM	5 mM	10 mM	−360	this study, Figure 2
Chemotrophic							
enrichment culture, +1 mM acetate	7	bicarbonate	10 mM	?	3 mM	−106	4, Figure 1
enrichment culture containing *Sideroxydans* species	6.8	bicarbonate	10 mM	?	4 mM	−156	29, Figure 1a
Pseudogulbenkiania strain 2002	6.8	bicarbonate	10 mM	?	2.2 mM	−74	16, Figure 4
strain HidR2, +1 mM acetate	6.7	bicarbonate	6 mM	<30 μM	5 mM	−66	14, Figure 2
Ferroglobus placidus, 85C	7	bicarbonate	2 mM	up to 550 μM	0.64 mM	−173	5, Figure 4
cell suspension of *D. suillum*, grown on acetate + nitrate	6.8	bicarbonate	10 mM	?	10 mM	−4700	12, Figure 3a
Paracoccus ferrooxidans, +25 mM EDTA, +1 mM ethanol	7	bicarbonate	25 mM	?	5 mM	−1600	13, Figure 3a
Acidovorax sp. strain BoFeN1, +2 mM acetate	6.8	bicarbonate	2.5 mM	<1 mM	5 mM	−48	15, Figure 2
Acidovorax sp. strain BoFeN1, +5 mM acetate	7	bicarbonate	10 mM	0 mM	10 mM	−240	30, Figure 1a
Acidovorax sp. strain 2AN, +1.6 mM acetate	6.85	bicarbonate	8.3 mM	up to 1 mM	5 mM	−158	24, Figure 2a
Acidovorax sp. strain 2AN, + 4 mM EDTA, +1.2 mM ethanol	7	PIPES	4 mM	?	5 mM	−970	49, Figure 3c
Dechloromonas sp. UWNR4, + 4 mM EDTA, +1.2 mM ethanol	7	PIPES	4 mM	?	5 mM	−950	49, Figure 3d
lake sediment slurry	~7	bicarbonate	1.4 mM	0.01 mM	1 mM	−6	69, Figure 3
Phototrophic							
Rhodopseudomonas palustris strain TIE-1, + 0.2 mM citrate	7	bicarbonate	4.5 mM			−21	3, Figure 2
Rhodobacter capsulatus strain SB1003, +0.2 mM citrate	7	bicarbonate	0.1 mM			−34	3, Figure 4
Rhodobacter capsulatus strain SB1003, +1 mg/L HA	7	bicarbonate	0.1 mM			−50	70, Figure 4
Rhodobacter capsulatus strain SB1003, +0.2 mM NTA	7	bicarbonate	0.1 mM			−112	70, Figure 4

observed oxidation of Fe(II) by nitrite are beyond the scope of this report and await further study. Due to the uncertainty surrounding the reactive species involved, we recommend caution in applying the rate constants derived in Tables 1 and 2 to aqueous environments with widely differing Fe(II) complexation, pH, or ionic strength.

Biological Fe(II) Oxidation by *Pseudobulkeniania* sp. Strain MAI-1. Using the kinetic rate constants derived for the oxidation of Fe(II) by nitrite in the presence of NTA with the nitrite accumulation measured in culture of MAI-1 (Figure 2), we modeled the purely abiotic Fe(II) oxidation that would result from the interaction of Fe(II) with the accumulated nitrite (Figure 2, bottom), assuming the presence of cell

dx.doi.org/10.1021/es3049459 | Environ. Sci. Technol. XXXX, XXX, XXX−XXX

surfaces[22] to have negligible effects on purely chemical oxidation. Even if we conservatively assume the upper 95% confidence interval for the rate constant (8.13 M^{-1} s^{-1}; see Table 1) and that produced NO is biologically consumed (thus leaving more Fe(II) free to react by preventing formation of the highly stable Fe(II)-NTA-NO complex), abiotic oxidation would maximally account for ~30%/35% (solid vs dashed curve) of the observed Fe(II) oxidation after 28 h (time point indicated by vertical line in Figure 2). In fact, a 6× higher rate constant (combined with biological consumption of any produced NO) would be required to attribute observed Fe(II) oxidation to purely chemical processes (Figure 2, dotted model). Based on the kinetic quantification of chemical oxidation of Fe(II), it thus becomes evident that *Pseudogulbenkiania* sp. MAI-1 can directly oxidize Fe(II), establishing the organism as a novel neutrophilic nitrate-dependent chemotroph with unambiguous biological Fe(II)-oxidizing activity. The potential to easily genetically manipulate this strain makes it a good candidate for elucidating the machinery involved in biological Fe(II) oxidation. Whether the biological component of Fe(II) oxidation in MAI-1 occurs via a dedicated enzyme system or via nonspecific reactions with redox active components of the cell, such as periplasmic thiols or components of the electron transport chain,[25,26] is a question that could be addressed in the future.

Chemical vs Biological Fe(II) Oxidation in Laboratory and Environmental Studies. Given the aforementioned difficulty in discriminating between chemical and biological contributions to anaerobic Fe(II) oxidation in many systems, it can be informative to compare Fe(II) oxidation rates observed in a variety of environmental and laboratory settings. Table 3 provides an overview of the maximal Fe(II) oxidation rates reported in a number of publications on chemical and biological Fe(II) oxidation in nitrite/nitrate rich anoxic environments at circumneutral pH. Several observations are particularly noteworthy:

(i) The majority of observed maximal rates of chemical and biological Fe(II) oxidation fall within a similar range of values (~10−100 μM/h), highlighting the likely competition and co-occurrence of chemical and biological processes involved in the coupled biogeochemical cycling of iron and nitrogen. Moreover, because nitrite is produced and often accumulates during the microbial denitrification process, they are intrinsically coupled. This biologically induced chemical oxidation of iron (via the microbial production of nitrite) in organic rich environments such as soils and wetlands is likely to contribute significantly to the cycling of iron and immobilization of metal contaminants and organic pollutants on iron (oxy)hydroxides. High oxidation rates reported for environmental samples with mixed contributions from biological and chemical catalysis[20] illustrate the interplay of these processes and call for caution in interpreting an observed effect to stem from solely one or the other mechanism.

(ii) In the case of mineral accelerated Fe(II) oxidation, the presence of amorphous hydrous ferric oxide (HFO/ferrihydrite)[9,31,37] and green rust[42] appears to cause the most significant acceleration of Fe(II) oxidation (see Table S3 for additional detail on rate constants derived for mineral catalysis). This effect is likely to be highly relevant in natural settings where poorly crystalline iron oxides are ubiquitous. However, it is also important to consider this effect in laboratory studies where iron oxides precipitate over the course of an experiment and can provide catalytic surfaces for chemodenitrification as suggested previously.[23−25]

(iii) In the case of ligand-enhanced Fe(II) oxidation by nitrite, the absence of a major effect of the humic acid representative PPHA and low environmental abundance of the anthropogenic ligand NTA (maximal levels of 10−100 nM in aqueous systems),[1] suggests that citrate (detected in soil solutions in appreciable quantities, ~100 μM range)[68] is likely to be the only ligand investigated in this study that could be relevant in natural systems. In laboratory studies of iron oxidizing microorganisms in the presence of citrate or NTA, the ligands' effect on oxidation kinetics is a crucial aspect of Fe(II) depletion that cannot be disregarded. This is particularly clear from the experiment reported in Figure 3 that confirms ligand-enhanced chemical oxidation of Fe(II) by nitrite can be an important side effect of microbial denitrification. Here, chemical Fe(II) oxidation could be mistaken for direct biological catalysis by *P. denitrificans*; while direct catalysis may indeed be at play, it would simply be challenging to unambiguously identify without appropriate controls. In conclusion, this study serves as a reminder of the complex interplay between direct and indirect biological effects involving metal transformations. In the case of denitrifying microorganisms, the extent to which these different processes catalyze Fe(II) oxidation likely depends on the precise culturing conditions and must be evaluated on a case-by-case basis.

■ ASSOCIATED CONTENT

Supporting Information

Derivation of reaction equations and additional tables and figures as described in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Jim Morgan for many insightful conversations and inspiring S.H.K. to pursue this project, Sean Crowe, CarriAyne Jones, Arne Sturm, Sulung Nomosatryo, David Fowle, and Don Canfield for sample acquisition and fieldwork in Indonesia, Nathan Dalleska and the Caltech Environmental Analysis Center for instrumentation that benefited this project, Andreas Kappler, Nicole Klüglein, and Jay Labinger for helpful discussions, members of the Newman Lab and the anonymous reviewers for constructive criticism that improved the manuscript. This work was supported by grants to D.K.N from the Dreyfus Foundation and the Howard Hughes Medical Institute (HHMI). D.K.N. is an HHMI Investigator. S.H.K. is an HHMI International Student Research Fellow.

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Ligand-enhanced abiotic iron oxidation and the effects of chemical vs. biological iron cycling in anoxic environments

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AVAILABLE SUPPORTING INFORMATION

- Derivation of reaction equations
- Table S1: pH of reactant solutions at the beginning and end of kinetic Fe(II) oxidation experiments.
- Table S2: Theoretical Fe(II) inorganic and organic speciation in bicarbonate-buffered freshwater medium at pH 7.
- Table S3: Overview of rate constants reported for chemical oxidation of Fe(II) by NO_2^-.
- Figure S1. Anaerobic growth of *Pseudogulbenkiania* sp. strain MAI-1.
- Figure S2. MAI-1 growth on various Fe(II)-chelating ligands.
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- Figure S4. Oxidation test of Fe(II) in the presence of nitrite during sample dilution for the ferrozine assay.

- Figure S5. Reduction test of nitrite in the presence of Fe(II) (+ ligands) during diazotization for the nitrite assay.
- Figure S6. Anaerobic growth and nitrite production of *Paracoccus denitrificans*.
- Figure S7: Model fits for abiotic Fe(II) oxidation by nitrite.
- Figure S8. Evolution of N_2O during reaction of Fe(II)-citrate with nitrite.
- Figure S9. Absorption spectrum of Fe(II)-NTA solution reacted with nitrite.

Derivation of Fe(II) and NO_2^- reaction equations:

$d[Fe(II)]/dt = -2\, k_{app}\, [Fe(II)][NO_2^-]$
$[Fe(II)] = [Fe(II)]_0 + \Delta[Fe(II)]$; $[NO_2^-] = [Fe(II)]_0 + ½\, \Delta[Fe(II)]$
➔ $d([Fe(II)]_0 + \Delta[Fe(II)]) / dt = -2\, k_{app}\, ([Fe(II)]_0 + \Delta[Fe(II)])\, ([Fe(II)]_0 + ½\, \Delta[Fe(II)])$
➔ $d\, \Delta[Fe(II)] / dt = -2\, k_{app}\, ([Fe(II)]_0 + \Delta[Fe(II)])\, ([Fe(II)]_0 + ½\, \Delta[Fe(II)])$ [eq. S1]

$d[NO_2^-]/dt = -k_{app}\, [Fe(II)][NO_2^-]$
$[NO_2^-] = [NO_2^-]_0 + 2\, \Delta[NO_2^-]$; $[NO_2^-] = [NO_2^-]_0 + \Delta[NO_2^-]$
➔ $d([NO_2^-]_0 + \Delta[NO_2^-]) / dt = -k_{app}\, ([NO_2^-]_0 + 2\, \Delta[NO_2^-])\, ([NO_2^-]_0 + \Delta[NO_2^-])$
➔ $d\, \Delta[NO_2^-] / dt = -k_{app}\, ([NO_2^-]_0 + 2\, \Delta[NO_2^-])\, ([NO_2^-]_0 + \Delta[NO_2^-])$ [eq. S2]

For 2:1 stoichiometry, $\Delta[Fe(II)] = -[Fe(II)]_{ox} = -([Fe(II)]_0 - [Fe(II)]_{obs})$ and $\Delta[NO_2^-] = -[NO_2^-]_{red} = -([NO_2^-]_0 - [NO_2^-]_{obs})$, and [S1] and [S2] integrate to yield:

$$Fe(II)_{obs}(t) = \frac{Fe(II)_0}{-1 + 2\, e^{Fe(II)_0\, k_{app}\, t}} \qquad NO_2^-(t) = \frac{NO_{2\,0}^-\, e^{NO_{2\,0}^-\, k_{app}\, t}}{-1 + 2\, e^{NO_{2\,0}^-\, k_{app}\, t}}$$

For 1:1 stoichiometry observed in the presence of NTA: The Fe-NTA-NO complex does not appear to be reactive towards NO_2^- such that [S1] describes Fe(II) oxidation even in the presence of NTA, with the caveat that measured concentrations of Fe(II) (which include the Fe(II)-NTA-NO^+ complex) require a correction for Fe-NTA-NO. Assuming all NO that is generated complexes with Fe(II)-NTA such that it no longer participates in a redox reaction with nitrite, but is still measured as Fe(II) by the ferrozine assay and assuming the reactions are coupled such that $[Fe(II)]_{ox} = [Fe(II)\text{-NTA-NO}]$, then $\Delta[Fe(II)] = -([Fe(II)]_{ox} + [Fe(II)\text{-NTA-NO}]) = -2\, [Fe(II)]_{ox} = -2\, ([Fe(II)]_0 - [Fe(II)]_{obs})$ and $\Delta[NO_2^-] = -[NO_2^-]_{red} = -([NO_2^-]_0 - [NO_2^-]_{obs})$. This leads [S1] to integrate to:

$$Fe(II)_{obs}(t) = \frac{Fe(II)_0\, e^{Fe(II)_0\, k_{app}\, t}}{-1 + 2\, e^{Fe(II)_0\, k_{app}\, t}}$$

SUPPORTING TABLES

Table S1

pH of reactant solutions at the beginning and end of kinetic Fe(II) oxidation experiments.

Condition	Start	End	Change
2mM Fe(II) + 2mM NO_2^-	7.03	6.88	-0.15
+ 2mM NTA	7.00	7.12	0.12
+ 300mg/L PPHA	6.99	7.03	0.04
+ 100µM Citrate	6.95	7.02	0.07
+ 500µM Citrate	6.97	7.07	0.10
+ 2mM Citrate	6.96	7.06	0.10
+ 2mM Citrate + 300mg/L PPHA	6.94	7.13	0.19

Table S2

Theoretical Fe(II) inorganic and organic speciation in bicarbonate-buffered freshwater medium at pH 7. Species with relative abundance < 0.01% for all experimental conditions are not shown. Species suggested to be relevant for Fe(II) oxidation by nitrite are highlighted in gray.

		2mM Fe(II)							5mM Fe(II)
	Ligand	none	PPHA (300mg/L)	Citrate (0.1mM)	Citrate (0.5mM)	Citrate (2mM)	Citrate + PPHA (2mM+300mg/L)	NTA (2mM)	Citrate (10mM)
$[Fe(II)_{species}] / [Fe(II)_{total}]$	Fe^{2+}	26.66%	23.80%	25.75%	22.26%	11.49%	9.64%	1.89%	0.91%
	$Fe\text{-}OH^{+}$	0.06%	0.05%	0.06%	0.05%	0.03%	0.02%	< 0.01%	< 0.01%
	$Fe\text{-}HCO_3^{+}$	4.37%	3.91%	4.22%	3.64%	1.86%	1.57%	0.31%	0.13%
	$Fe\text{-}CO_{3\ (aq)}$	65.68%	58.82%	63.39%	54.60%	27.86%	23.44%	4.57%	1.93%
	$Fe\text{-}CO_3\text{-}OH^{-}$	0.15%	0.14%	0.15%	0.13%	0.07%	0.06%	0.01%	< 0.01%
	$Fe\text{-}(CO_3)_2^{2-}$	0.09%	0.08%	0.08%	0.07%	0.04%	0.03%	0.01%	< 0.01%
	$Fe\text{-}Cl^{+}$	0.09%	0.08%	0.08%	0.07%	0.04%	0.03%	0.01%	< 0.01%
	$Fe\text{-}NH_3^{2+}$	0.02%	0.02%	0.02%	0.01%	0.01%	0.01%	< 0.01%	< 0.01%
	$Fe\text{-}HPO_{4\ (aq)}$	0.32%	0.30%	0.32%	0.28%	0.16%	0.14%	0.03%	0.01%
	$Fe\text{-}H_2PO_4^{+}$	0.08%	0.07%	0.08%	0.07%	0.04%	0.03%	0.01%	< 0.01%
	$Fe\text{-}SO_{4\ (aq)}$	2.48%	2.24%	2.39%	2.06%	1.06%	0.90%	0.17%	0.07%
	$^{\#}Fe\text{-}L^{-}$			3.46%	16.73%	57.26%	55.67%	93.00%	96.79%
	$^{\#}Fe\text{-}HL$			0.01%	0.03%	0.09%	0.09%	< 0.01%	0.15%
	Fe-HA (complexed)		8.26%				7.04%		
	Fe::HA (weakly bound)		2.23%				1.33%		

#: $Fe\text{-}L^{-}$ = $Fe\text{-}NTA^{-}$ or $Fe\text{-}Citrate^{-}$, Fe-HL = Fe-HNTA or Fe-HCitrate

Table S3

Overview of rate constants reported for chemical oxidation of Fe(II) by NO_2^-.

	Experimental conditions			Kinetic parameters				Source
	pH	**Temp**	**buffer**	**Order**	**Rate constant (k)**		**d[Fe(II)]/dt =**	**Reference**
Oxidation by nitrite								
Fe(II) as siderite (10g/L ~ 80mM)	6	25C	MES/PIPES/HEPES	2nd	1.00E-04	$M^{-1} s^{-1}$	- 2 k $[FeCO_{3(s)}]$ $[NO_2^-]$	Rakshit et al. (2008)(*1*), Fig. 5
Fe(II) as siderite (10g/L ~ 80mM)	6.5	25C	MES/PIPES/HEPES	2nd	6.39E-05	$M^{-1} s^{-1}$	- 2 k $[FeCO_{3(s)}]$ $[NO_2^-]$	Rakshit et al. (2008)(*1*), Fig. 5
Fe(II) as siderite (10g/L ~ 80mM)	7.9	25C	MES/PIPES/HEPES	2nd	5.28E-05	$M^{-1} s^{-1}$	- 2 k $[FeCO_{3(s)}]$ $[NO_2^-]$	Rakshit et al. (2008)(*1*), Fig. 5
Fe(II) as goethite	6.8	30C	carbonate	1st	3.18E-06	s^{-1}	- k [Fe(II)]	Weber et al. (2001)(*2*), Table 3
Fe(II) as biogenic magnetite	6.8	30C	carbonate	1st	3.38E-05	s^{-1}	- k [Fe(II)]	Weber et al. (2001)(*2*), Table 3
Fe(II) as HFO	6.8	26-28	PIPES	3rd	3.83E+03	$M^{-2} s^{-1}$	- k $[Fe(II)_{diss}]$ $[Fe(II)_{bound}]$ $[NO_2^-]$	Tai et al. (2009)(*3*)
+2mM NTA	7	25C	carbonate	2nd	6.67E-03	$M^{-1} s^{-1}$	- 2 k [Fe(II)] $[NO_2^-]$	This study, Table 1
+2mM CIT	7	25C	carbonate	2nd	4.67E-03	$M^{-1} s^{-1}$	- 2 k [Fe(II)] $[NO_2^-]$	This study, Table 1
+10mM CIT, *P. denitrificans* spent medium	7	25C	carbonate	2nd	9.42E-03	$M^{-1} s^{-1}$	- 2 k [Fe(II)] $[NO_2^-]$	This study, Table 2
+10mM CIT, *P. denitrificans* culture	7	25C	carbonate	2nd	1.06E-02	$M^{-1} s^{-1}$	- 2 k [Fe(II)] $[NO_2^-]$	This study, Table 2
Oxidation by nitrate								
Fe(II) as green rust	8.25	25C	auto-titration	2nd	4.93E-05	$M^{-1} s^{-1}$	- 8 k $[Fe(II)_{GR}]$ $[NO_3^-]$	Hansen et al. (1996)(*4*), Table 1

SUPPORTING FIGURES

Figure S1

Anaerobic growth and concomitant Fe(II) oxidation of *Pseudogulbenkiania* sp. strain MAI-1 in freshwater medium amended with 10mM nitrate and different concentrations of Fe(II), NTA and acetate, and a headspace containing ~3% hydrogen. In the presence of NTA, up to 10mM Fe(II) is oxidized within 24hours (in yellow), however, in the absence of NTA, neither growth nor Fe(II) oxidation is observed (in green). Replicate culture (duplicates or triplicates) indicated with solid, dashed and dotted lines, respectively.

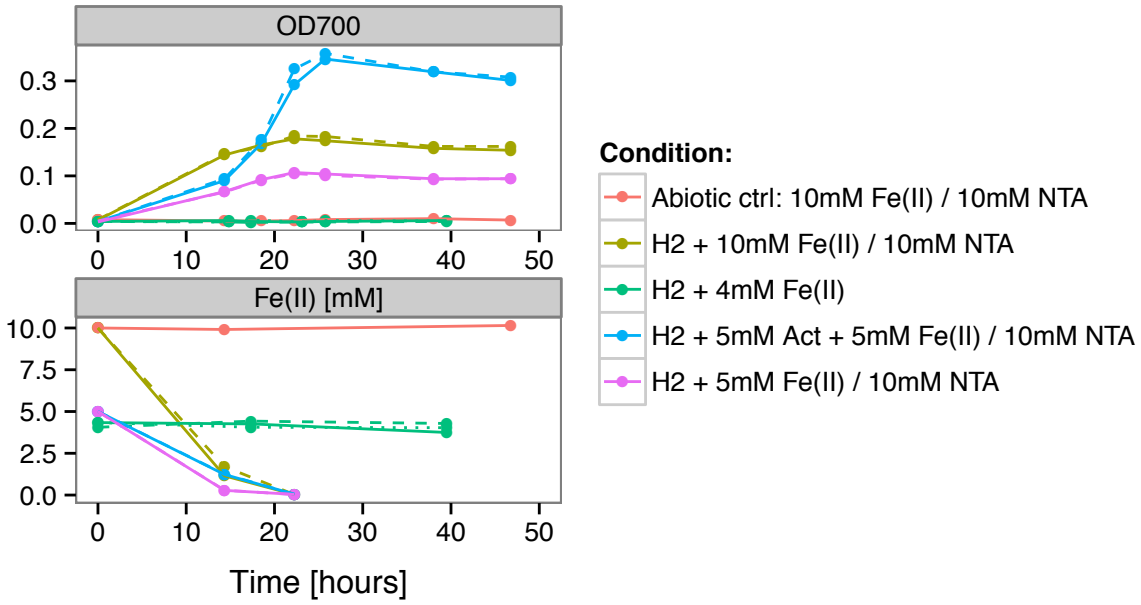


Figure S2

Growth of MAI-1 on various Fe(II) chelating ligands. The organism is grown aerobically in freshwater medium in a 96 well plate (OD_{600} is measured every 5 minutes) with different ligands as the sole carbon source. Citrate (Cit), humic acids (HA), acetate (Act) and diethylene triamine pentaacetic acid (DTPA) can all serve as growth substrates for MAI-1. The strain's ability to use siderophore desferioxamine (DFO) as a carbon source is ambiguous. No growth could be observed in the presence of nitrilotriacetate (NTA) as sole carbo source. This makes NTA a suitable choice for anaerobic growth experiments with MAI-1 as a chelator for Fe(II) that does not supply extra carbon. Replicate cultures indicated with dashed and solid lines, respectively.

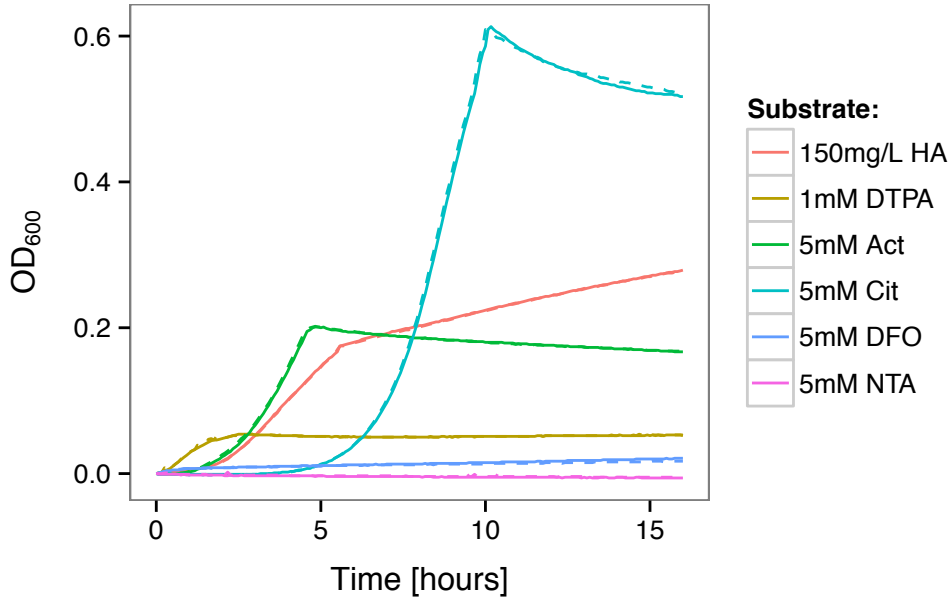


Figure S3

Oxidation of Fe(II)-NTA in spent MAI-1 growth medium. Triplicate cultures of *Pseudogulbenkiania* sp. strain MAI-1 (solid, dashed and dotted line) were grown in freshwater medium amended with 10mM nitrate and 1.25mM acetate, with ~3% H_2 present in the headspace. During growth of MAI-1 (upper left panel), significant amounts of nitrite accumulated in the medium (lower left panel). Accumulated nitrite was stable at the end of growth but upon addition of ~3mM Fe(II)-NTA to filer sterilized spent medium, Fe(II) oxidation and concomitant nitrite reduction could be observed (right panels).

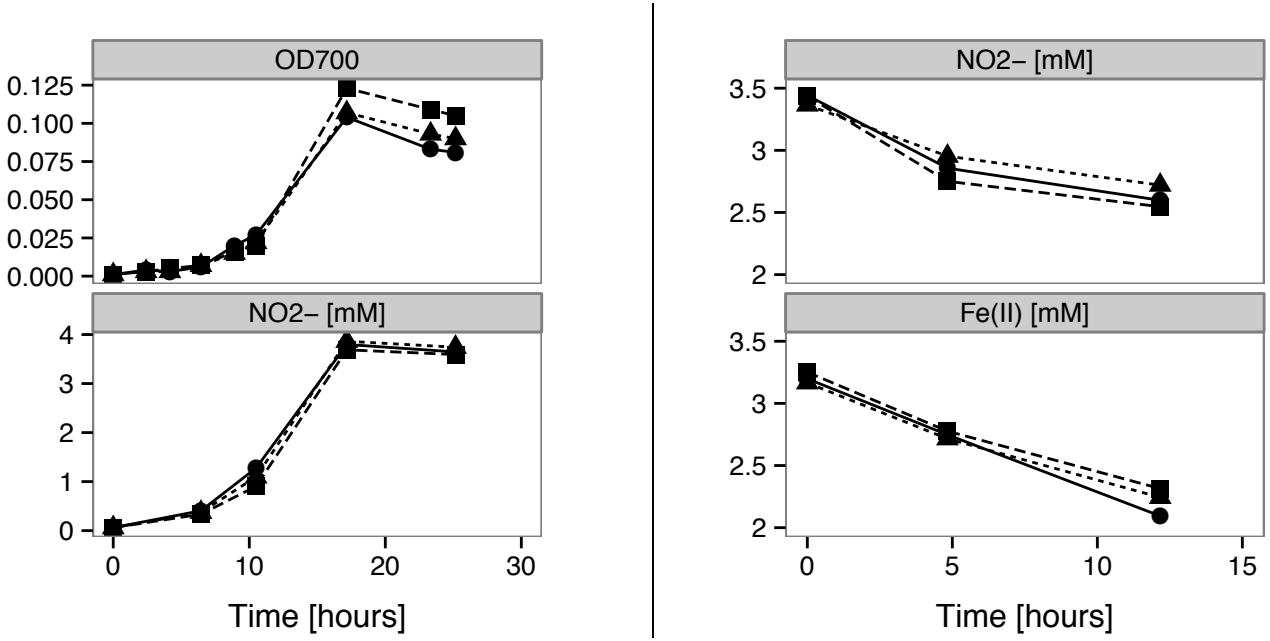


Figure S4

Oxidation test of Fe(II) in the presence of nitrite during sample dilution for the ferrozine(*5*) assay. The ferrozine assay often includes an acid dilution step prior to spectrophotometric determination of Fe(II) with the ferrozine reagent. Acidification aids in the desorption of strongly coordinated Fe(II) from mineral surfaces and other strong sorption sites and is an important preparative step for environmental samples. However, at acidic pH, nitrite is protonated (pKa=3.4) to nitrous acid, which can self- decompose to form reactive N-oxides(*6*) as well as oxidize Fe(II) directly(*7*, *8*). To assess the effect of acidification in the presence of nitrite for our experimental setup, an anoxic freshwater solution containing ~650µM Fe(II) and ~1mM NO_2^- was diluted 1:10 with 1M HCl, and Fe(II) concentrations were measured after varying time intervals using the ferrozine assay (depicted in grey). Within 10 seconds of acidification, >20% of Fe(II) was oxidized and could no longer be detected by the ferrozine assay. After 1 minute, >60% of Fe(II) was lost. Without the acidification step (e.g. by direct dilution of the sample with the ferrozine reagent), Fe(II) concentrations did not significantly decrease within several minutes (black line). Since our experimental conditions included relatively high concentrations of nitrite, but little to no risk of sorptive loss of Fe(II), all ferrozine measurements were conducted without acidification.

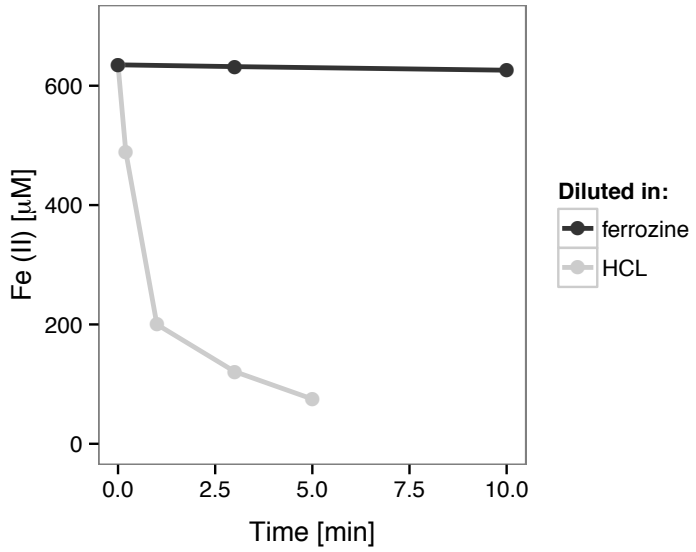


Figure S5

Reduction test of nitrite in the presence of Fe(II) during incubation with sulfanilamide in phosphoric acid for the nitrite assay used in this study. To assess the effect of free and chelated Fe(II) on the assay, an anoxic freshwater solution containing ~1.7mM nitrite was amended with 2mM Fe(II) and no ligand / 2mM citrate / 2mM EDTA / 2mM NTA / 300mg/L PPHA, and immediately diluted 1:10 with 1% sulfanilamide in 5% phosphoric acid for diazodization. Nitrite concentrations were determined colorimetrically after varying time intervals by addition of 0.1% N-1-napthylethylenediamine. The true concentration of nitrite measured in the absence of Fe(II) is indicated as a grey band with 95% confidence intervals. As previously observed(*9*), the presence of Fe(II)-EDTA leads to rapid disappearance of nitrite and significant underestimation of nitrite concentrations by this assay. The addition of Fe(II) without a ligand, as well as with the ligands used in this study did not significantly affect the determination of nitrite by this assay (all measurements were conducted within 3 minutes of sulfanilamide addition to prevent nitrite loss).

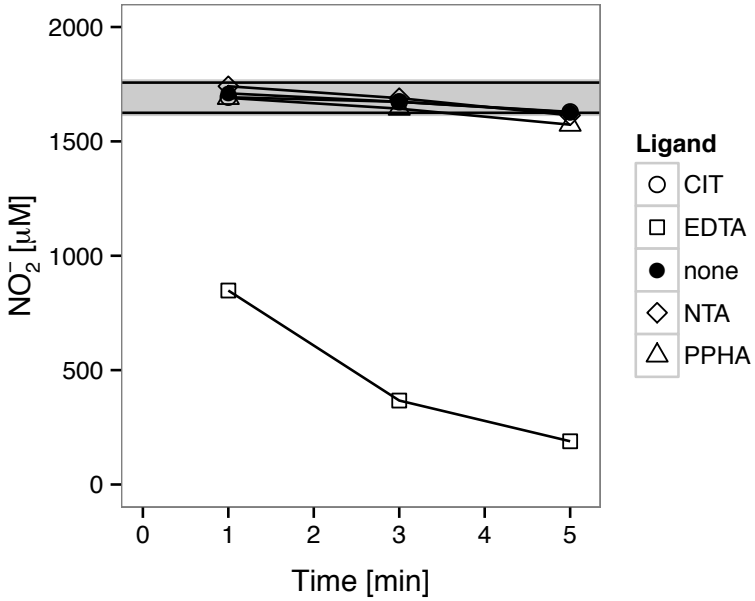


Figure S6

NO_2^- production by *P. denitrificans* (B) during anaerobic growth on succinate (A). Samples for Fe(II) oxidation assays (Figure 3) were taken after accumulation of ~5mM NO_2^- for each biological replicate, respectively (grey shaded area indicated by arrow in panel B). Experiment conducted in biological triplicates. All data are shown.

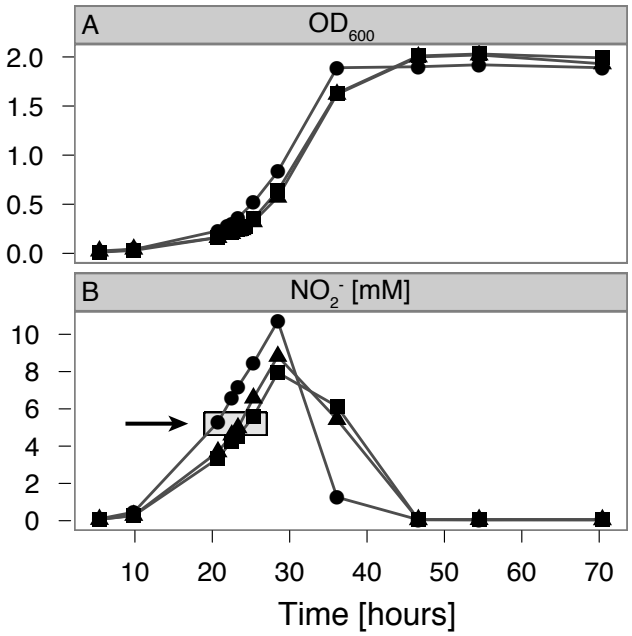


Figure S7

Model fits for abiotic Fe(II) oxidation by nitrite. Low citrate, no ligand, PPHA are best described by a zero-order (i.e. linear) reaction model (linear least squares fit illustrated for these conditions instead of 2nd order decay).

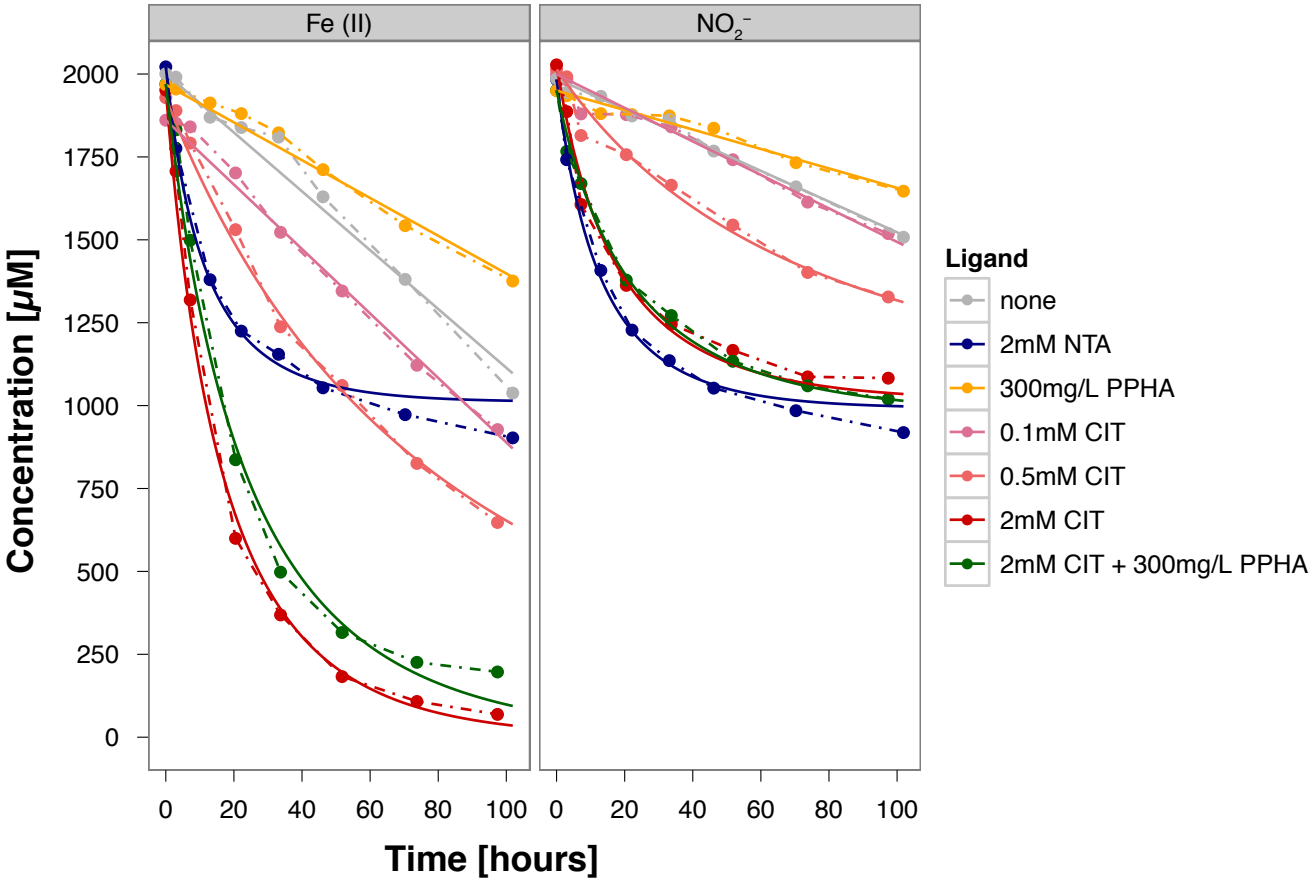


Figure S8

Evolution of N_2O in the headspace of sealed septum bottles during the reaction of 5mM nitrite with ~3mM Fe(II) complexed by citrate vs. NTA (peaks normalized to Ar). Retention times of the gases in the headspace were 2.2min (Ar), 3.0min (N2), 10.8min (N_2O) and 12-13min (CO_2, poorly resolved). The accumulation of N_2O (gray band) as a reaction product could only be observed in the presence of citrate, but not in the presence of NTA. Varying trace amounts of N_2 were present in the Ar/CO_2 headspace of the reaction vessels at the start of the experiment but did not change significantly with reaction progress.

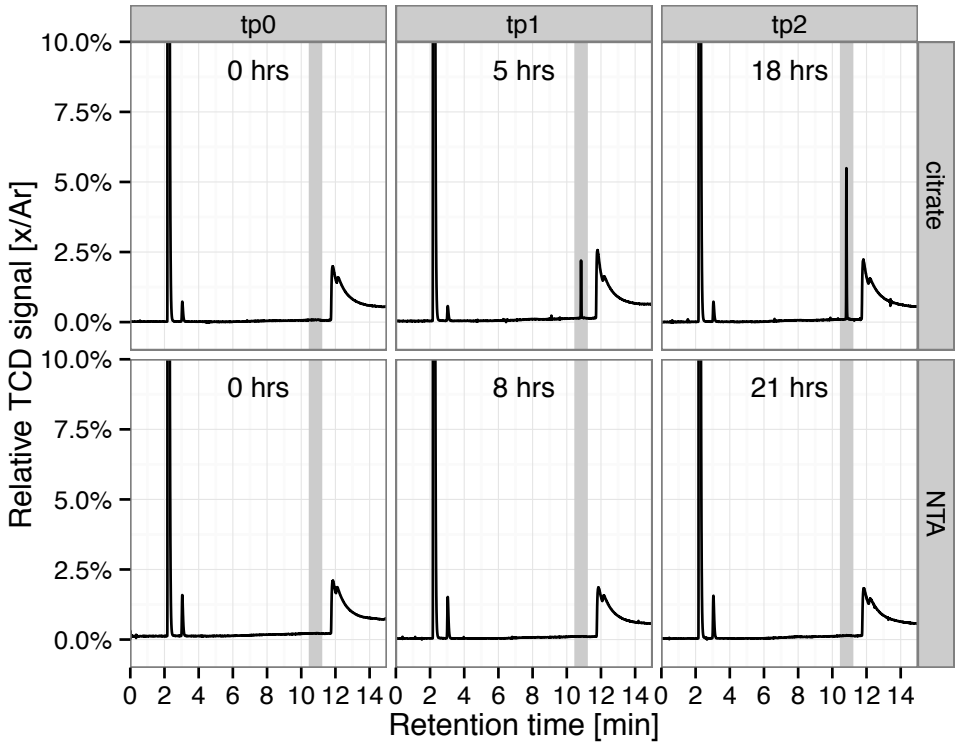
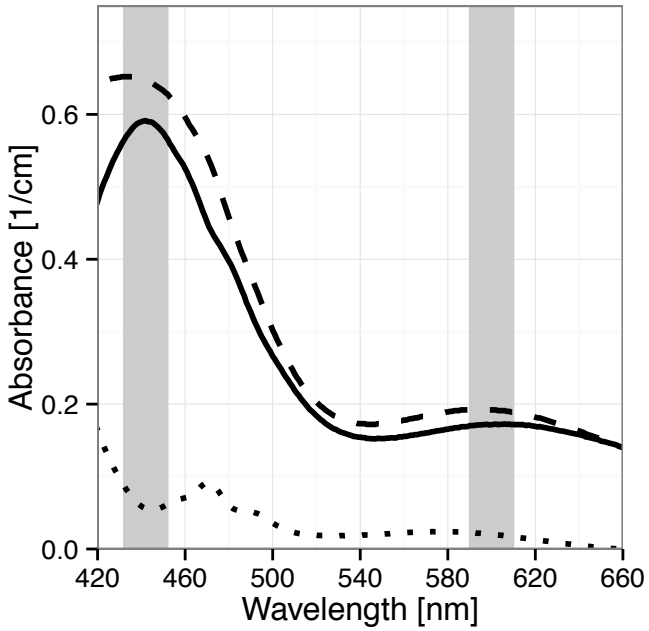


Figure S9

Absorption spectrum of a ~3mM Fe(II)-NTA solution (dashed line) after 950µM NO_2^- was lost by abiotic oxidation of 1086µM Fe(II) (21hrs data point in S3). Fe(II)-NTA by itself does not absorb in this wavelength range. The oxidized Fe forms a complex with NTA that absorbs light weakly with a characteristic peak at 470nm(dotted line). Residual light absorption (solid line) after accounting for the effect of Fe(III)-NTA in solution is indicative of Fe(II)-NTA-NO^- complex formation. Characteristic absorption peaks of the Fe(II)-NTA-NO^- complex (440nm and 600nm)(*10*) are indicated in gray.



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