

Interference of Nitrite with Pyrite under Acidic Conditions: Implications for Studies of Chemolithotrophic Denitrification

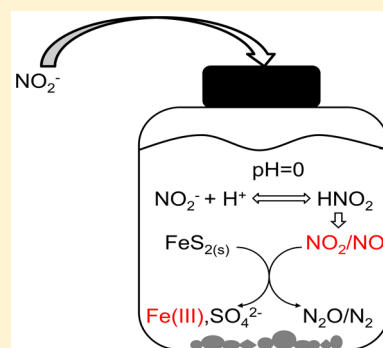
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Supporting Information

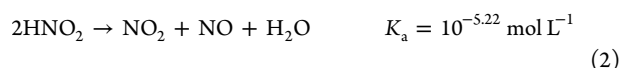
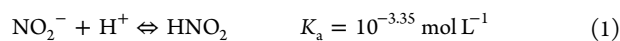
ABSTRACT: Chemolithotrophic denitrification coupled to pyrite oxidation is regarded a key process in the removal of nitrate in aquifers. A common product is nitrite, which is a strong oxidant under acidic conditions. Nitrite may thus interfere with Fe(II) during acidic extraction, a procedure typically used to quantify microbial pyrite oxidation, in overestimating Fe(III) production. We studied the reaction between pyrite (5–125 mM) and nitrite (40–2000 μM) at pH 0, 5.5, and 6.8 in the absence and presence of oxygen. Significant oxidation of pyrite was measured at pH 0 with a yield of 100 μM Fe(III) after 5 mM pyrite was incubated with 2000 μM nitrite for 24 h. Dissolved oxygen increased the rate at pH 0. No oxidation of pyrite was observed at pH 5.5 and 6.8. Our data imply a cyclic model for pyrite oxidation by Fe(III) on the basis of the oxidation of residual Fe(II) by NO and NO₂. Interference by nitrite could be avoided if nitrite was removed from the pyrite suspensions through a washing procedure prior to acidic extraction. We conclude that such interferences should be considered in studies on microbially mediated pyrite oxidation with nitrate.



INTRODUCTION

The disappearance of nitrate coupled to sulfate generation as observed in several pyrite-bearing aquifers has been attributed to microbial chemolithotrophic denitrification linked to pyrite oxidation^{1–6} and has fueled a series of laboratory studies to resolve the mechanisms underlying this reaction.^{7–11} A common product of denitrification is nitrite.^{8–11} Significant formation of nitrite has been determined in laboratory column experiments with sediments from groundwater aquifers.^{9,12} Nitrite was generated as a prominent intermediate compound in anaerobic denitrification with pyrite as electron donor in the presence of the nitrate-reducing bacterium *Thiobacillus denitrificans*.^{9–11}

Pyrite oxidation is typically quantified by acidic extraction of Fe(III) that is assumed to have formed upon oxidation of pyrite in experiments under circumneutral conditions.^{10,13} Such techniques may, however, bear the risk of producing artifacts if nitrite is present because under acidic conditions, nitrite decomposes into highly reactive compounds after protonation to nitrous acid (HNO₂) (eq 1). Nitrous acid is unstable at pH < 5 and spontaneously decomposes to nitrogen dioxide (NO₂) and nitric oxide (NO) (eq 2).^{14–16}



NO₂ and NO are known as strong oxidants toward Fe(II).^{16–18} It was demonstrated that NO₂ and NO lead to a significant overestimation of the Fe(II) oxidation rate in cultures of the nitrate-reducing Fe(II) oxidizer *Acidovorax sp.* strain *BoFeN1*.¹⁹

On the basis of this observation, the authors questioned the occurrence of enzymatic Fe(II) oxidation coupled to nitrate reduction that was postulated in previous studies.¹⁹

In this study, we therefore aim to test whether similar processes may also trigger the abiotic oxidation of pyrite under acidic conditions and thus generate a risk of producing artifacts and data misinterpretations.²⁰ To these ends, we have performed batch experiments that cover the range of pyrite concentrations used in previous studies (5–125 mM) as well as the concentration range of nitrite determined in these studies (40–2000 μM).^{7–11}

MATERIALS AND METHODS

Preparation and Characterization of Pyrite. Crystalline pyrite (3–6 mm in diameter) from Peru (Georg Maisch Import; Freising, Germany) was ground by milling it in a ball mill with an agate mortar under atmospheric conditions. After sieving, a size fraction between 63 and 200 μm was added to a 1 L glass bottle filled with deaerated ultrapure water (Millipore). The headspace of the bottle was flushed with nitrogen, and the bottle was sealed with a butyl stopper. This bottle was placed in an ultrasonic bath for 1 h to remove fine particles attached to the pyrite surface. To remove ferric iron that may have formed from oxidation of pyrite surfaces during crushing as well as residual acid-extractable sulfur species, we shook the material in 1 M HCl for 1 h, washed it with

Received: November 4, 2014

Revised: September 2, 2015

Accepted: September 3, 2015

Published: September 3, 2015

ultrapure water, and ultrasonically cleaned it for an additional hour. This procedure was repeated nine times. After the last extraction step, the extraction solution was free of Fe(III). However, substantial amounts of Fe(II) were still extractable but not quantifiable due to significant mass loss of material during the washing process. The material was freeze-dried and then washed three times with deaerated cyclohexane to remove elemental sulfur. The residual fraction of elemental sulfur in pyrite was 0.001 mass % (cf. below for a description of the analytical protocol). Residual cyclohexane was evaporated by continuous nitrogen purging. It appears that even though the material was purged by nitrogen for ca. 1 h to remove the residual cyclohexane, there is still some solvent adsorbed, which was detected as carbon in the elemental analysis by EDX spectra (see Figure S1 and Tables S1–S2). The material was stored anoxically in a 250 mL brown Schott bottle sealed with a gas-tight butyl stopper. The headspace of the Schott bottle was flushed for 2 min with nitrogen. The washed pyrite was characterized by scanning electron microscopy (Zeiss Leo 1530 FE-SEM) and by X-ray diffractometry (XRD) (D5000; Siemens, Germany) using Co $K\alpha$ radiation (40 kV, 40 mA) from 10° – 85° 2θ .

The BET surface area (Gemini V series, Micromeritics; Aachen, Germany) of the ground pyrite was $0.17 \text{ m}^2 \text{ g}^{-1}$. Although the ground pyrite was washed several times with HCl, nanometer-sized structures were still detectable by SEM on the surface of pyrite (Figure 1). EDX spectra (Figure S1 and Tables

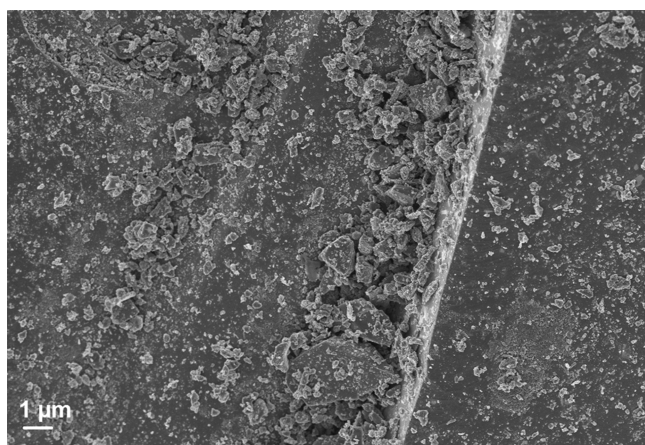


Figure 1. Scanning electron micrograph of the ground pyrite after preparation.

S1–S2) taken from these nanometer-sized structures sample displayed a Fe/S ratio of approximately 1:2. XRD revealed that the ground material was pure pyrite (Figure S2).²¹

Experimental Setup. Batch experiments in the absence of oxygen were performed in an anoxic glovebox (Innovative Technology; Massachusetts, USA; 100% N_2) at room temperature ($20 \pm 2^\circ \text{C}$). All solutions were purged with N_2 for dissolved-oxygen removal before being transferred into the glovebox. To test the influence of initial nitrite concentration on anoxic pyrite oxidation at pH 0, we placed 0.03 g of pyrite (5 mM) and 50 mL of HCl (1 M) in 120 mL glass serum bottles. The bottles were sealed with butyl stoppers and crimped. At the beginning of each experiment, small aliquots of a deaerated NaNO_2 stock solution (10 or 100 mM) were added to the bottles to obtain NO_2^- concentrations between 40 and 2000 μM . A control experiment with 5 mM pyrite was performed without the addition of nitrite.

The influence of the initial pyrite concentration on the reaction rate at pH 0 was tested with various concentrations of pyrite (5, 25, and 125 mM) at a nitrite concentration of 1000 μM in HCl (1 M). Control experiments with the same amounts of pyrite were performed in the absence of nitrite. To test the importance of oxygen in this process, we also carried out experiments with 5 mM pyrite and 1000 μM nitrite under oxic conditions. These batch experiments under oxic conditions were prepared as described above. Here, the contact between the headspace of the bottles and the atmosphere was maintained by needles that were inserted into the butyl stoppers.

To evaluate the effect of pH, we performed additional batch experiments under circumneutral pH conditions (pH = 5.5 and 6.8). Sodium acetate and NaHCO_3 were used as buffers in these experiments at a pyrite concentration of 50 mM and a constant nitrite concentration of 400 μM to achieve two constant pH values of 5.5 (0.05 M acetate) and 6.8 (0.05 M NaHCO_3), respectively. These pH values were adjusted with 1 M HCl. Control experiments with 400 μM nitrite in the absence of pyrite or with 50 mM pyrite in the absence of nitrite were conducted at pH 5.5 and pH 6.8, respectively. All suspensions were removed from the glovebox and shaken overnight before the addition of nitrite. Experiments and controls were performed in three independent replicates.

Analytical Methods. In experiments performed in 1 M HCl under anoxic conditions, Fe(II) and $\text{Fe}(\text{HCl})_{\text{tot}}$ (total HCl-extractable Fe) were quantified by the ferrozine assay.²² Fe(III) was calculated as the difference between $\text{Fe}(\text{HCl})_{\text{tot}}$ and Fe(II). Individual samples were withdrawn and filtered through a 0.45 μm pore size filter (nylon) to remove residual pyrite particles and thereby to stop the reaction. For the determination of $\text{Fe}(\text{HCl})_{\text{tot}}$, hydroxylamine hydrochloride was added into filtered samples and followed by a 30 min incubation to reduce Fe(III) to Fe(II). All samples of Fe(II) and $\text{Fe}(\text{HCl})_{\text{tot}}$ were removed from the glovebox after the addition of the ferrozine reagent and exposed to air for only approximately 5 min during the measurement. The absorbance of samples was measured at 570 nm using a microplate reader (Infinite F200 PRO; Tecan, Switzerland). Each sample was analyzed in triplicate.

For the quantification of sulfate, it was necessary to use two different analytical methods. In acidic samples (pH 0), sulfate was measured turbidimetrically following a modification of the turbidimetric BaSO_4 method²³ because dissolved ferric iron will tend to precipitate during ion chromatography measurements; barium–gelatin reagent was prepared using the standard procedure. A 2.5 mL sample and 125 mL of barium–gelatin reagent were placed into a cuvette. The absorbance was measured after a reaction time of 24 h at 420 nm. All filtered acidic samples were collected and removed from the glovebox after the end of the experiments. The reaction time had to be extended compared to the original instruction to account for the slow formation rate of the BaSO_4 precipitate under acidic conditions (data not shown). Hence, the partial oxidation of intermediate sulfur compounds during this period and an overestimation of sulfate concentrations cannot be ruled out.

Total concentrations of S and Fe were determined in samples from experiments performed at different nitrite concentrations using inductively coupled plasma optical emission spectrometry (ICP-OES PerkinElmer Optima 3200 XL). The samples were withdrawn from the serum bottles after 24 h and filtered via 0.45 μm filters inside the glovebox. They were diluted 1:1 with 1 M HCl shortly before the measurement.

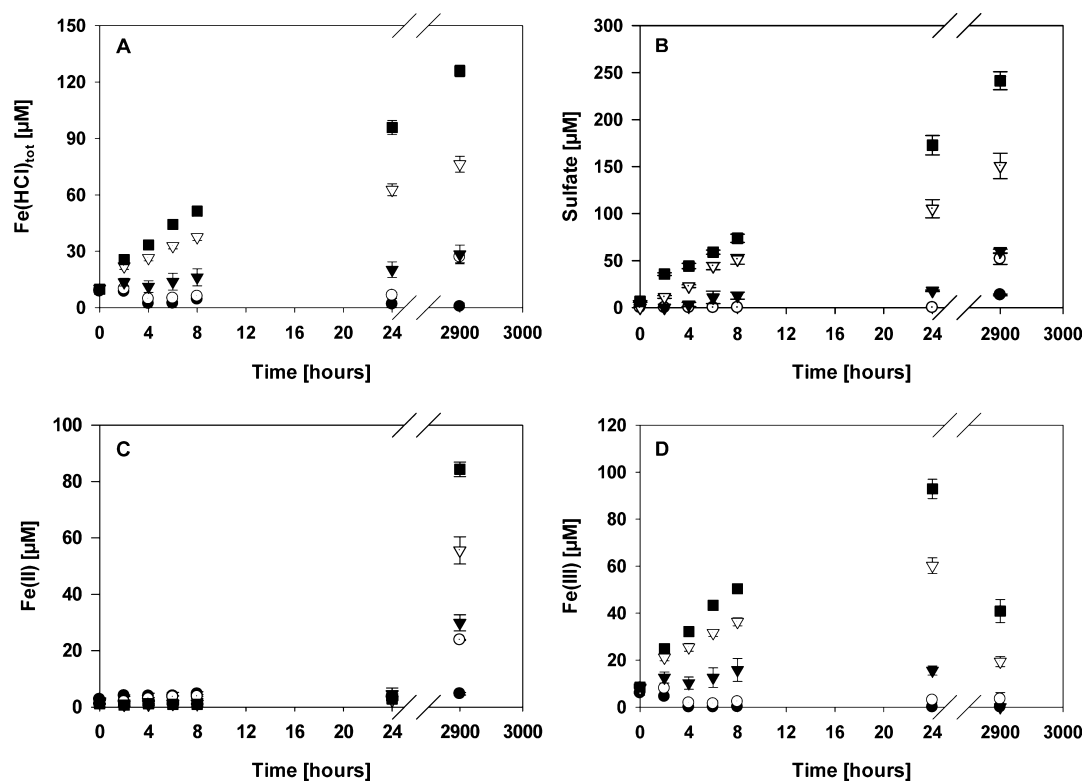


Figure 2. Concentration of (A) $\text{Fe(HCl)}_{\text{tot}}$, (B) sulfate, (C) Fe(II) , and (D) Fe(III) of the reaction between pyrite (5 mM) with different concentrations of nitrite (0 (●), 40 (○), 200 (▼), 1000 (▽), and 2000 μM) in 1 M HCl. Error bars represent standard deviations calculated from three independent replicates.

In experiments performed at circumneutral pH (pH 5.5 and 6.8), sulfate was determined by ion chromatography (IC) to prevent the precipitation of BaCO_3 from the reaction between the barium–gelatin reagent and NaHCO_3 used as a buffer in the experiments, which would lead to an overestimation of the concentration of measured sulfate. A sample of approximately 1.5 mL was filtered through a 0.22 μm pore size filter (Nylon), diluted to a ratio of 1:5 with ultrapure water, and then analyzed by ion chromatography with chemical suppression and conductivity detector using a Supp 4 anion column (Metrohm) to determine the concentrations of sulfate. The eluent was a mixture of 4 mM NaHCO_3 and 1 mM Na_2CO_3 with a flow rate of 1 mL min^{-1} . In addition to sulfate, IC also allowed for the determination of nitrite at neutral pH values. Nitrite, as well as other nitrogen species, could not be quantified under acidic conditions, in which these species are not stable. In experiments performed in 1 M HCl under oxic conditions, the sampling and analysis of Fe(II) , $\text{Fe(HCl)}_{\text{tot}}$ and sulfate were performed in the presence of air.

For the quantification of elemental sulfur associated with pyrite, 0.5 g of ground pyrite was added to a 120 mL glass serum bottle. The bottles were sealed and crimped. The headspace of the bottles was filled with nitrogen. Variable volumes of deaerated methanol (5, 10, and 20 mL) were added to the serum bottles with a glass syringe. Experiments for each volume were performed in two independent replicates. The headspace of the bottles was flushed with nitrogen for 1 min. Suspensions were shaken for 24 h to extract elemental sulfur. Thereafter, ca. 1.5 mL samples were taken and filtered through a 0.22 μm pore size filter (Nylon) and then analyzed by HPLC (UV–vis detector, 265 nm).

RESULTS AND DISCUSSION

Kinetics of Pyrite Oxidation by Nitrite in Anoxic 1 M HCl. Oxidation of pyrite was fast in the presence of nitrite in 1 M HCl under anoxic conditions. At high initial nitrite concentrations, distinct increases of extractable $\text{Fe(HCl)}_{\text{tot}}$, sulfate and Fe(III) were observed (Figure 2), and their formation rates clearly depended on the initial nitrite concentrations ($0.4 \pm 0.16 \mu\text{M h}^{-1}$ $\text{Fe(HCl)}_{\text{tot}}$ and $0.8 \pm 0.03 \mu\text{M h}^{-1}$ sulfate at 200 μM nitrite, $2.2 \pm 0.09 \mu\text{M h}^{-1}$ $\text{Fe(HCl)}_{\text{tot}}$ and $4.4 \pm 0.41 \mu\text{M h}^{-1}$ sulfate at 1000 μM nitrite, and $3.6 \pm 0.18 \mu\text{M h}^{-1}$ $\text{Fe(HCl)}_{\text{tot}}$ and $6.9 \pm 0.46 \mu\text{M h}^{-1}$ sulfate at 2000 μM nitrite). Formation rates were calculated as the mean ($n = 3$) linear concentration increase within 24 h.

Initial pyrite oxidation rates appear to follow a first-order reaction rate with respect to nitrite concentration, as indicated by the slopes of the logarithmic plots of the formation rates of sulfate and $\text{Fe(HCl)}_{\text{tot}}$ against the logarithm of the corresponding initial nitrite concentrations ($n_{\text{Fe(HCl)}_{\text{tot}}} = 0.95$ and $n_{\text{sulfate}} = 0.99$) (Figure S3).

Kinetics were different in experiments under anoxic conditions where the nitrite concentration was kept constant ($c = 1000 \mu\text{M}$) but in which the pyrite concentration was varied. Sulfate and $\text{Fe(HCl)}_{\text{tot}}$ accumulated within 24 h at 5 mM pyrite. At higher pyrite concentrations of 25 mM and 125 mM, there was a fast initial increase of $\text{Fe(HCl)}_{\text{tot}}$ and sulfate, which remained nearly unchanged after 8 h in the presence of 25 mM pyrite and after 2 h in the presence of 125 mM pyrite (Figure 3). Contrary to the first-order rate dependency on initial nitrite concentrations, a fractional order ($n_{\text{Fe(HCl)}_{\text{tot}}} = 0.51$ and $n_{\text{sulfate}} = 0.64$) was determined with respect to initial pyrite concentrations (Figure S4).

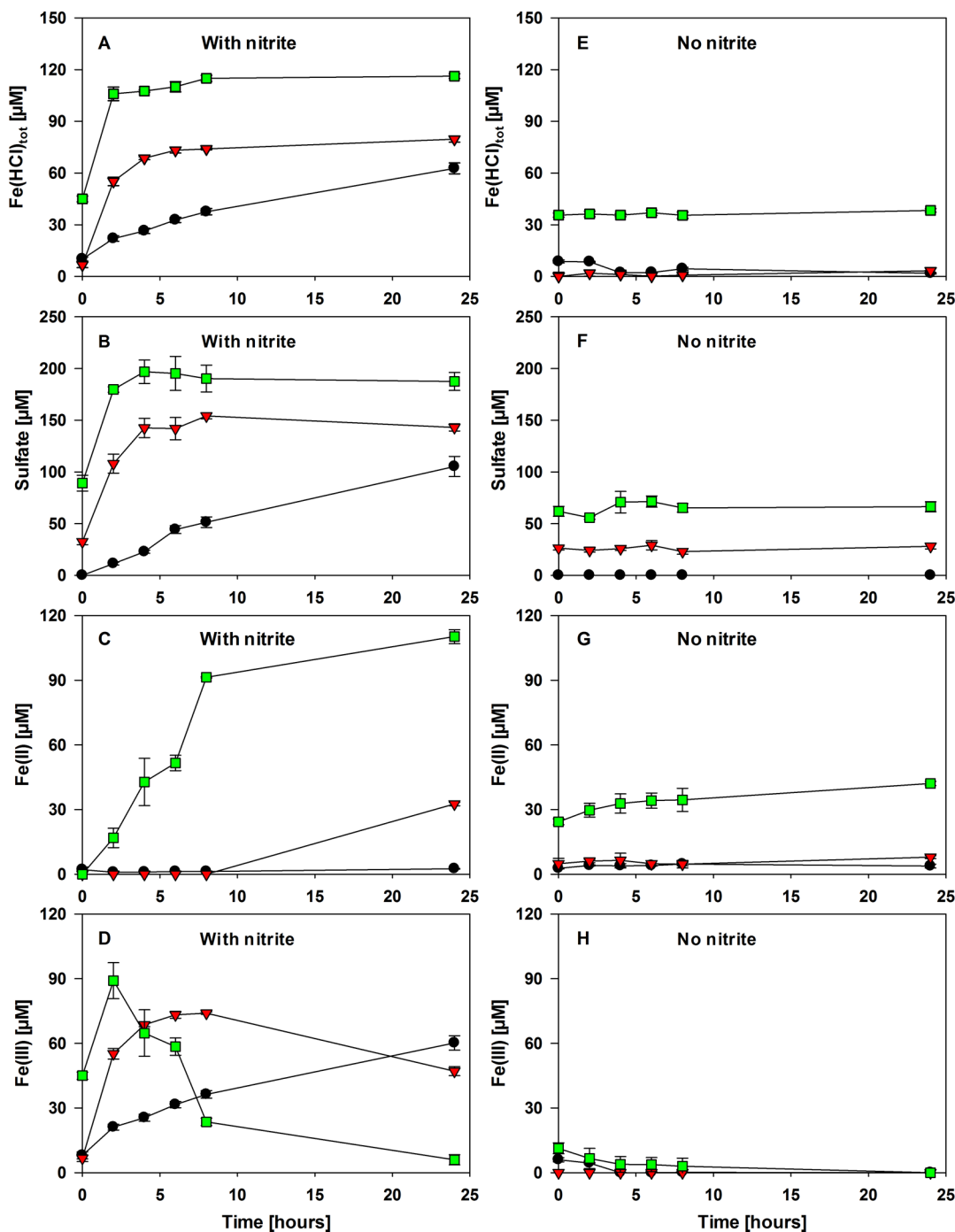


Figure 3. Product concentration after reaction with pyrite in the presence of 1000 μM nitrite (A–D) and in the absence of nitrite (E–H) with different concentrations of pyrite (5 mM (●), 25 mM (▼), and 125 mM (■)) in 1 M HCl under anoxic conditions. Error bars represent standard deviation calculated from three independent replicates.

Initial rates of pyrite oxidation were estimated as the mean value ($n = 3$) of the product formation rates ($\text{Fe}(\text{HCl})_{\text{tot}}$ and sulfate) within the first 2 h. Initial rates increased with increasing pyrite concentrations ($6.0 \pm 0.31 \mu\text{M Fe}(\text{HCl})_{\text{tot}}$ and $5.7 \pm 0.71 \mu\text{M}$ sulfate at 5 mM pyrite, $24.2 \pm 0.83 \mu\text{M Fe}(\text{HCl})_{\text{tot}}$ and $37.8 \pm 3.37 \mu\text{M}$ sulfate at 25 mM pyrite, and $30.5 \pm 2.45 \mu\text{M Fe}(\text{HCl})_{\text{tot}}$ and $45.3 \pm 4.19 \mu\text{M}$ sulfate at 125 mM pyrite).

The reaction order determined in this study is consistent with values determined in earlier studies on pyrite oxidation by dissolved oxygen under acidic conditions (Table 1)^{24–26} indicating that pyrite oxidation by NO and NO₂ is a surface-

Table 1. Comparison of Reaction Order for Pyrite Oxidation by Nitrite at Different Pyrite Concentrations in Our Study and by Dissolved Oxygen under Acidic Conditions

pH range	T range	reaction order	reference
0	20 °C	0.51, 0.64 (NO ₂ ⁻)	this study
2–4	20–40 °C	0.49 (O ₂)	Mckibben and Barnes ²⁴
-0.1–1.2	30–70 °C	0.81 (O ₂)	Mathews and Robins ²⁵
2	20–35 °C	0.70 (O ₂)	Smith and Shumate ²⁶

controlled reaction.²⁷ In the presence of oxygen, the oxidation of pyritic Fe(II) is generally regarded to be preceded by the

oxidation of the disulfide (S_2^{2-}) surface group by Fe^{3+} , which binds to the disulfide group and forms a surface complex, allowing for electron transfer.²⁸ In analogy to this model, we propose that NO and NO_2 (rather than oxygen) play a role in the oxidation of Fe(II) to Fe(III), with Fe(III) being the oxidant for the disulfide (S_2^{2-}) surface group.

Identity of Products from Anoxic Pyrite Oxidation in 1 M HCl. Product formation differed between the different kinetic pyrite oxidation experiments. As expected, the concentration of extractable Fe(II) was below the detection limit ($<5 \mu M$) within the first 24 h in all experiments where nitrite concentration was varied at a concentration of 5 mM pyrite (Figure 2) because crystalline pyrite is not dissolvable in HCl. Hence, the concentration of extractable $Fe(HCl)_{tot}$ was equal to the concentration of Fe(III) within this time period.

Only slight increases in $Fe(HCl)_{tot}$ and sulfate concentrations occurred within the following time until 2900 h. However, a significant fraction of the $Fe(HCl)_{tot}$ (88% with $40 \mu M$ nitrite, 105% with $200 \mu M$ nitrite, 73% with $1000 \mu M$ nitrite, and 67% with $2000 \mu M$ nitrite) was Fe(II) after this time in all experiments, with Fe(III) concentrations decreasing correspondingly.

The concentration ratios between SO_4^{2-} and $Fe(HCl)_{tot}$ were lower than 2:1 in the first 24 h and increased with time, approaching the expected stoichiometric ratio of 2:1 (1.9–2.1) after 2900 h (Table 2). In contrast, concentrations ratios between

Table 2. Change of Ratios between Sulfate and $Fe(HCl)_{tot}$ Concentrations with Time at pH = 0 (1 M HCl) and 5 mM Concentration of Pyrite at Different Initial Nitrite Concentrations from 40 to 2000 μM at Room Temperature under Anoxic Conditions^a

time (h)	SO_4^{2-}/Fe ratios			
	40 μM NO_2^-	200 μM NO_2^-	1000 μM NO_2^-	2000 μM NO_2^-
0	0.0	0.0	0.0	0.7
2	0.0	0.0	0.5	1.4
4	0.0	0.3	0.9	1.3
6	0.0	0.8	1.3	1.3
8	0.0	0.8	1.4	1.4
24	0.0	0.9	1.7	1.8
2900	1.9	2.1	2.0	1.9
24 (ICP)	2.1	1.9	1.9	1.8

^aThe bottom row displays ratios between total S and Fe concentrations as measured by ICP after 24 h.

total S and total Fe determined by ICP after 24 h were close to 2:1 (1.8–2.1) (Table 2), indicating the presence of intermediate sulfur compounds in the first 24 h. This observation suggests that pyritic disulfide is initially oxidized to intermediate sulfur species. The oxidation of disulfide (S_2^{2-}) to the final product of sulfate (SO_4^{2-}) requires the transfer of seven electrons per sulfur atom. However, more than two electrons are typically not transferred in a single reaction step.²⁹ Therefore, the overall process must consist of several steps, and several sulfur species of intermediate oxidation state, such as sulfite (SO_3^{2-}), thiosulfate ($S_2O_3^{2-}$), and polythionates ($S_nO_6^{2-}$, $n = 4, 5,$ and 6) are expected to form. The rate of conversion to sulfate depended on the initial nitrite concentration, which indicates the involvement of reactive N species in the oxidation process. At low nitrite concentrations, the ratios were clearly lower (0 for $40 \mu M$ nitrite and 0.9 for $200 \mu M$ nitrite) after 24 h, while ratios were much closer to the

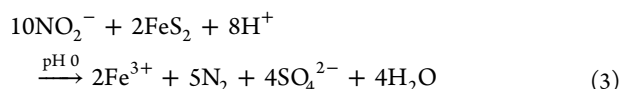
stoichiometric ratio for $1000 \mu M$ (1.7) and $2000 \mu M$ (1.8) nitrite.

Product formation was different in experiments with varying pyrite concentrations. Iron was ferric until a plateau of constant $Fe(HCl)_{tot}$ concentration was achieved after 8 h in the presence of 25 mM pyrite and after 2 h in the presence of 125 mM pyrite (Figure 3). Thereafter, the Fe(III) concentration decreased with a concomitant increase of the Fe(II) concentration. In control experiments in the absence of nitrite with different pyrite concentrations (5, 25, and 125 mM), the average concentrations of Fe(III) were less than $5 \mu M$ in all control experiments. No sulfate and only very little Fe(II) ($<5 \mu M$) was detectable with 5 mM pyrite. With increasing pyrite concentrations, Fe(II) and sulfate were already detectable right after the beginning. In the control experiment with 25 mM pyrite, the concentration of Fe(II) was measurable but quite low ($\sim 6 \mu M$). Sulfate concentration was detectable but below the determination level. Both Fe(II) ($33 \mu M$) and sulfate ($65 \mu M$) were well-quantifiable in the control experiment with 125 mM pyrite. The concentrations of Fe(II), $Fe(HCl)_{tot}$, and sulfate remained stable at 25 and 125 mM pyrite within the time frame of the control experiments (Figure 3E–G). This observation indicates the occurrence of Fe(II) already in the initial suspensions if the pyrite concentration is high. Acidic extraction of the ground material (cf. the Materials and Methods section) revealed that there is, in addition to acid-insoluble pyritic Fe(II), an additional acid-soluble source for Fe(II) in the initial suspension. We propose that the occurrence of acid-soluble Fe(II) is due to the reaction of water with defect or nonstoichiometric sites on pyrite,³⁰ leading to the dissolution of nanometer-size pyrite particles as identified with SEM (Figure 1) and the formation of dissolved Fe(II) and sulfate. In the experiment with 5 mM pyrite, the S/Fe ratio increased with time, whereas there was no significant change at reactions with 25 and 125 mM pyrite (Table 3).

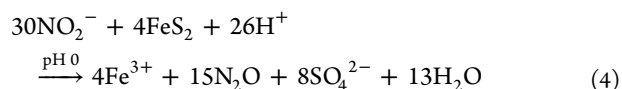
Table 3. Change of Ratios between Sulfate and $Fe(HCl)_{tot}$ Concentrations with Time at pH = 0 (1 M HCl) and NO_2^- Concentration of 1000 μM at Different Initial Pyrite Concentrations from 5 to 125 mM at Room Temperature under Anoxic Conditions

time (h)	$SO_4^{2-}/Fe(HCl)_{tot}$ ratios		
	5 mM FeS_2	25 mM FeS_2	125 mM FeS_2
2	0.5	2.0	1.7
4	0.9	2.1	1.8
6	1.3	1.9	1.8
8	1.4	2.1	1.7
24	1.7	1.8	1.6

Mechanism of Anoxic Pyrite Oxidation by Nitrite in 1 M HCl. Our results provide clear evidence that pyrite can be oxidized in the presence of nitrite at pH 0, and that the rate and extent of pyrite oxidation depends on the initial nitrite concentrations. The question arises whether NO_2^- is also the oxidant. The experimentally determined yields of $Fe(HCl)_{tot}$ and sulfate were distinctly lower in these experiments than those stoichiometrically predicted for the complete turnover of nitrite to either N_2 or N_2O . For example, we observed that $126 \mu M$ $Fe(HCl)_{tot}$ and $241 \mu M$ sulfate were formed in the presence of $2000 \mu M$ nitrite. The complete reduction of this nitrite concentration to N_2 would, however, stoichiometrically generate $400 \mu M$ and $800 \mu M$ of $Fe(HCl)_{tot}$ and sulfate, respectively (eq 3),



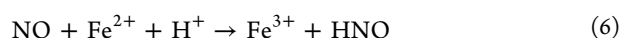
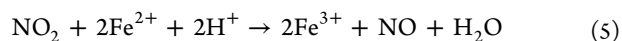
or with the final product being N_2O , 267 μM and 533 μM , respectively (eq 4):



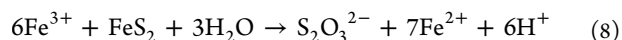
Lower yields of $\text{Fe}(\text{HCl})_{\text{tot}}$ and sulfate than those expected were also observed in experiments in which concentrations of pyrite were varied and indicate that pyrite oxidation in the presence of nitrite was nonstoichiometric under our experimental conditions.

It is well-known that $\text{Fe}(\text{III})$ is a major oxidant for pyrite under acidic conditions, with the role of the dissolved oxygen being reoxidizing $\text{Fe}(\text{II})$ to $\text{Fe}(\text{III})$ forming a cycle of iron.³¹ We therefore propose a pathway for pyrite oxidation that is based on the cyclic oxidation of $\text{Fe}(\text{II})$ by reactive NO_2 and NO to $\text{Fe}(\text{III})$ and the regeneration of $\text{Fe}(\text{II})$ upon the reaction of $\text{Fe}(\text{III})$ with pyrite.

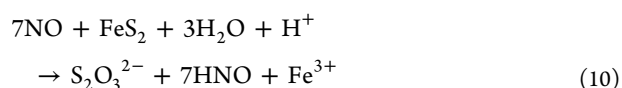
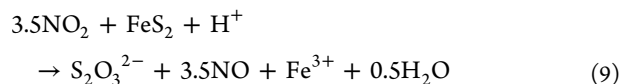
Significant amounts of dissolved $\text{Fe}(\text{II})$ and sulfate with an average concentration of 33 μM and 65 μM , respectively, reflecting a S/Fe ratio of 2:1 were observed in the initial suspension of the control experiment with 125 mM pyrite in the absence of nitrite. Acid-extractable $\text{Fe}(\text{II})$ completely disappeared from the initial suspension if nitrite was present (Figure 3C), indicating that acid-soluble $\text{Fe}(\text{II})$ becomes rapidly oxidized by reactive NO and NO_2 . Similar observations were made in previous studies that investigated the abiotic oxidation of $\text{Fe}(\text{II})$ to $\text{Fe}(\text{III})$ with nitrite under acidic or weak acidic conditions^{16,17,19,32,33} and which proposed the following reactions (eqs 5–7):¹⁸



The Fe^{3+} ion is a potential oxidant for pyrite under acidic conditions, thereby typically forming thiosulfate (eq 8):²⁸



Provided that the oxidation rate of $\text{Fe}(\text{II})$ by NO and NO_2 is faster than the oxidation of pyrite with $\text{Fe}(\text{III})$, the buildup of $\text{Fe}(\text{II})$ occurs only if the reactive NO and NO_2 become exhausted. Combining eqs 5 and 6 with eq 8 yields overall stoichiometries that predict the accumulation of Fe^{3+} (eqs 9 and 10):



Once NO and NO_2 are depleted, the residual $\text{Fe}(\text{III})$ is steadily consumed to build up the $\text{Fe}(\text{II})$ pool observed after 2900 h (Figure 2) upon reaction with pyrite. The proposed cyclic model also explains the pyrite-concentration-dependent turnover rate of $\text{Fe}(\text{III})$ to $\text{Fe}(\text{II})$ observed in Figure 3. The higher the

concentration of pyrite, the faster $\text{Fe}(\text{III})$ will be converted into $\text{Fe}(\text{II})$.

The current data do not exclude the direct oxidation of pyrite by NO_2 and NO . A possible mechanism is that under acidic conditions, reactive NO_2 and NO forming from the self-decomposition of HNO_2 formed from NO_2^- (eqs 1 and 2)^{14–16} directly react with pyrite. Due to a dynamic equilibrium, only a certain fraction of HNO_2 decomposes to NO_2 and NO being available for reaction with pyrite during the reaction in 1 M HCl . Additionally, the degassing of gaseous NO_2 and NO may contribute to the observed nonstoichiometric pyrite oxidation. However, this model does not explain the increase in $\text{Fe}(\text{II})$ concentration observed after 2900 h (Figure 2), and the observed dynamic behavior of $\text{Fe}(\text{II})$ in Figure 2 and 3 strongly supports the cyclic model.

Effect of Oxygen and pH on Pyrite Oxidation by Nitrite.

The presence of oxygen clearly affected the oxidation of pyrite by nitrite (Figure 4). Pyrite (5 mM) was incubated with 1000 μM

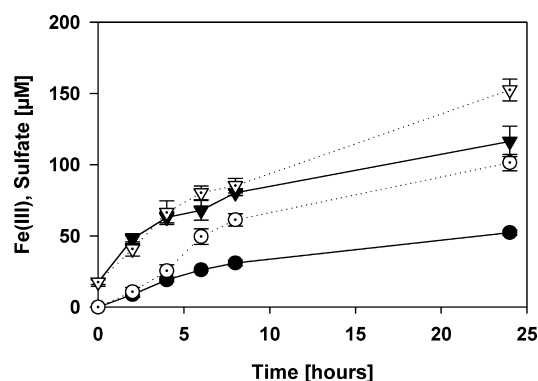


Figure 4. Concentration of $\text{Fe}(\text{III})$ (●) and sulfate (▼) in experiments of reactions of nitrite (1000 μM) with pyrite (5 mM) in 1 M HCl under anoxic (closed symbols and solid line) or oxic (open symbols and dotted line) conditions. Error bars represent standard deviations calculated from three independent replicates.

nitrite in 1 M HCl under anoxic and oxic conditions. The concentrations of acid-extractable $\text{Fe}(\text{II})$ were below the detection limit ($<5 \mu\text{M}$) in both anoxic and oxic experiments within 24 h. The initial concentration increase of $\text{Fe}(\text{III})$ and sulfate was almost the same under oxic and anoxic conditions within the first 4 h. After this initial time period, the concentrations of both $\text{Fe}(\text{III})$ and sulfate increased much more rapidly in the presence of oxygen. After 24 h, $116 \pm 10.7 \mu\text{M}$ sulfate and $52.4 \pm 1.9 \mu\text{M}$ $\text{Fe}(\text{III})$ were observed under anoxic conditions, corresponding to a ratio of 2.2, whereas concentrations reached values of $152 \pm 7.6 \mu\text{M}$ and $102 \pm 5.8 \mu\text{M}$, respectively, under oxic conditions, with a ratio of 1.5.

Oxygen clearly enhanced the extent of pyrite oxidation by NO and NO_2 under acidic conditions. Oxygen itself is a weak oxidant for pyrite even under acidic conditions,^{28,31,34,35} but it may interfere with the intermediate reactive nitrogen species forming during the reaction. Previous studies suggest that NO can be oxidized by oxygen to NO_2 (eq 11),^{36,37} being a stronger oxidant for the oxidation of $\text{Fe}(\text{II})$ under acidic conditions than NO :¹⁹



We therefore propose that this very reactive NO_2 is able to oxidize pyrite more efficiently than NO . Additionally, oxygen may accelerate the oxidation of Fe more strongly relative to that of S because the increase in $\text{Fe}(\text{III})$ yield after 24 h (50 $\mu\text{mol/L}$)

Table 4. Concentrations of Fe(II) and Fe(HCl)_{tot} Measured in Suspension before the Addition of Nitrite and after 24 h in Unwashed and in Washed Samples from Batch Experiments^a

anoxic extraction with 1 M HCl after 24 h								
setup	pyrite [mM]	nitrite [mM]	samples before the addition of nitrite		unwashed samples		washed samples	
			Fe(HCl) _{tot} (μM)	Fe(II) (μM)	Fe(HCl) _{tot} (μM)	Fe(II) (μM)	Fe(HCl) _{tot} (μM)	Fe(II) (μM)
control	5	0	15 ± 3.25	7 ± 1.27	12 ± 2.73	5 ± 2.81	13 ± 2.26	8 ± 0.16
addition of nitrite	5	1	14 ± 2.88	6 ± 0.62	87 ± 9.01	2 ± 0.87	11 ± 1.29	8 ± 0.21

^aExperiments were conducted with 50 mM pyrite and 10 mM nitrite at pH 6.4 after acidic extraction diluted 1:10 with 1 M HCl under anoxic conditions. The pyrite and nitrite concentrations were 5 mM and 1 mM during acidic extraction, respectively.

in the presence of oxygen was greater than that of sulfate (36 μmol/L, Figure 4)

Anoxic oxidation of pyrite in the presence of nitrite appeared to be not effective at pH 5.5 and 6.8 (Figure S5). Concentrations of nitrite and sulfate remained at the same level as those in the control experiments; the pH also did not vary by more than 0.05 pH units. Contrary to the oxidation of ferrous iron by nitrite^{32,33} and the oxidation of pyrite in the presence of other oxidants such as Fe(III) or dissolved oxygen that are observable at circum-neutral pH,³⁸ abiotic pyrite oxidation by nitrite seems to not occur under these conditions. Hence, the contribution of nitrite to abiotic oxidation of pyrite in anoxic circumneutral groundwater aquifers seems to be not an important pathway. The reason probably is that HNO₂ as the precursor of reactive (i.e., pyrite-oxidizing) NO₂ and NO occurs at relevant concentrations only at pH < 5 (pK_a = 3.35), while the ionic species nitrite, i.e., NO₂⁻, is not reactive toward pyrite.

Implications for Studies on Microbial Nitrate-Dependent Pyrite Oxidation. This study has demonstrated that ground pyrite material contained a small but quantifiable pool of acid-extractable Fe(II) even after intensive washing with HCl. We assume that this fraction of extractable Fe(II) is due to the tiny surface-bound particles identified with SEM (Figure 1) that are either of pyritic (FeS₂) origin or Fe(HSO₄)₂ as the product of the pyrite dissolution. This assumption is supported by the EDX spectra (Figure S1 and Tables S1–S2) taken from these structures that displays an S/Fe ratio of 2:1. The same ratio was determined in the control experiment with 125 mM pyrite in the absence of nitrite, with initial concentrations of 65 μM and 33 μM for sulfate and extractable Fe(II), respectively. Extractable Fe(II) in this experiment made up approximately 0.26 mol % of the initial pyrite content (Figure 3G). Similar nanostructures were observed on ground pyrite crystals not pretreated with HCl¹⁰ that were interpreted as nanopyrite. Thus, two fractions of Fe(II) have to be considered when performing oxidation studies with pyrite, and great care has to be taken when attributing experimental results to one of these fractions.

Both of these fractions appeared to react with NO₂⁻ derived reactive N species under acidic conditions. Studies about the microbial nitrate-dependent oxidation of pyrite should consider these interferences. An assessment of possible interferences from nitrite in previous studies is difficult because their experimental approaches cannot be directly compared to our study (see Table S3, in which we compiled previous laboratory studies). Several studies did not perform acid extractions of pyrite-containing samples to determine the formation of Fe(III).^{7–9,11} Fe(II) extracted with 1 M HCl from the pyrite suspension was completely (0.13 mM) oxidized to Fe(III) after 24 h of extraction in 1 M HCl in the presence of nitrite (100–800 μM).¹⁰ Given the results obtained in the present study, one cannot exclude

interference of nitrite in the oxidation process described by these authors.

Revised Protocol for Acidic Fe Extraction in Nitrite-Containing Pyrite Suspensions. To avoid the interferences described above, we are proposing to remove nitrite by washing the pyrite suspensions with nitrite-free water prior to the acidic extraction. This protocol was tested in batch experiments by comparing unwashed pyrite suspensions (100 mL, c_{pyrite} = 50 mM, pH 6.4) in the presence of nitrite (c_{nitrite} = 10 mM) with washed pyrite suspensions in the absence of nitrite and in the absence of nitrate-reducing cells. The pyrite was pretreated as described in the Material and Methods section. Experimental details are provided in the Supporting Information.

Table 4 demonstrates that there is a clear increase in Fe(III) by a factor of >6 in the unwashed samples in the presence of nitrite compared to that in the washed samples, in which initial concentrations remained constant after 24 h of acidic extraction. We therefore recommend the consideration of this protocol in any acid extraction procedure with suspensions containing nitrite and pyrite or other Fe(II)-containing solid phases that may be subject to interference with nitrite.

■ ASSOCIATED CONTENT

📄 Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.5b02981.

EDX data and X-ray diffractogram of ground pyrite after preparation, kinetic data from experiments with different concentrations of nitrite and with different concentrations of pyrite, experimental data of pyrite oxidation experiments at pH 5.5 and pH 6.8, overview of previous batch studies on chemolithotrophic denitrification coupled to pyrite oxidation, experimental details for the testing of the revised protocol for nitrite-free acidic Fe extraction in nitrite containing pyrite suspension. (PDF)

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was funded by the research group FOR 580 of the German Research Foundation (DFG) “Electron Transfer Processes in Anoxic Aquifers”. We thank the staff of our laboratory for their technical and analytical support, Regina Lohmayer (Department of Environmental Geochemistry of the University of Bayreuth) for assistance in iron measurement, and

Nicole Klueglein from the University of Tuebingen and Julian Bosch from the Helmholtz Centre Munich for helpful discussions. We are grateful to the reviewers of this manuscript whose comments helped to substantially improve this manuscript.

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