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ABSTRACT

We compared the response at neutral pH of some denitrifiers to different electron donors such as reduced sulfur (pyrite, S(0), and marcasite) and reduced Fe. Chemolithoautotrophic oxidation of pyrite with nitrate as electron acceptor was not possible when the pyrite was in a pure crystalline form, whereas oxidation of synthesized FeS_2 of low crystallinity and of S(0) with nitrate as electron acceptor was possible. Neither nitrite nor sulfate was formed when Fe(II)-oxidizing strain *Acidovorax* sp. BoFeN1 was tested. Microbial reduction of nitrate appears to be induced via S oxidation but not via Fe oxidation.

Introduction

Denitrification is an important anaerobic nitrate attenuation process, which has been observed in many groundwater systems (Hiscock et al. 1991). Heterotrophic denitrification is driven by organic electron donors, whereas autotrophic denitrification is driven by inorganic electron donors (Korom 1992). For decades, it has been postulated that denitrification may be coupled to the oxidation of pyrite, mediated by chemoautolithotrophs such as *Thiobacillus denitrificans* (Kölle et al. 1983). In pyrite-bearing aquifers, the net consumption of nitrate with concomitant generation of sulfate and dissolved Fe(II) is generally regarded indicative of this process (Pauwels et al. 2000; Postma et al. 1991; Tesoriero et al. 2000; Zhang et al. 2009). The release of pyrite-associated trace metals such as As, Ni, Co, Zn (Broers 1998; Evangelou and Zhang 1995; Van Beek et al. 1989; Zhang et al. 2009), and aqueous uranium (van Berk and Fu 2017) concomitant with nitrate removal was regarded as further evidence of pyrite oxidation. In addition, natural-gradient, anoxic tracer injections with nitrate into nitrate-free, Fe(II)-containing groundwater indicated that nitrate-dependent Fe(II) oxidation could occur rapidly and that the process can impact the mobility of other chemical species (e.g., phosphate and arsenic) (Smith et al. 2017).

A series of laboratory studies were undertaken to resolve the mechanisms underlying pyrite-dependent nitrate reduction (Bosch et al. 2012; Haaijer et al. 2007; Jørgensen et al. 2009; Torrentó et al. 2010). However, results of these studies were contradictory. Incubation of natural sediment to which ground pyrite was added did not provide any evidence of denitrification coupled to pyrite oxidation (Haaijer et al. 2007; Schippers and Jorgensen 2002). In contrast, accelerated nitrate reduction and sulfate generation has been observed on incubation of natural pyrite-containing sediment from a sandy aquifer and in accompanying batch experiments to which ground pyrite had been added (Jørgensen et al. 2009). Nitrate reduction rates in the presence of the autotrophic denitrifying bacterium *T. denitrificans* increased with decreasing pyrite grain size and were dependent on initial nitrate concentration and nitrate-loading rate in anaerobic batch and flow-through experiments to which ground pyrite was added (Torrentó et al. 2010). Both studies, therefore, revealed indirect evidence for the presence of microbially mediated denitrification with pyrite as the electron donor. Bosch et al. (2012) have described oxidation of pyrite nanoparticles by the nitrate-reducing bacterium *T. denitrificans*. Their conclusion was based on an electron balance involving the formation of ferric iron and sulfate at the expense of nitrate reduction to nitrite. However, using acid extraction to determine the amount of ferric hydroxide that may be formed in pyrite oxidation may lead to an overestimation of ferric iron when nitrite is present. This is because nitrite is able to oxidize pyrite under acidic conditions

(Yan et al. 2015). Hence, detection of ferric iron as a product of pyrite oxidation may be misleading unless great care is taken to prevent such artifacts. To shed light on these contradictory observations, we set up a systematic series of experiments to compare the ability of different sources of reduced S (pyrite, elemental sulfur, and marcasite) and of reduced Fe [pyritic Fe(II), dissolved Fe(II)] to serve as electron donors in bacterial denitrification. To do so, we used two types of pyritic materials: (1) a synthesized pyritic mixture of small-grain-size, which consisted of pyrite, marcasite and elemental sulfur; and (2) a pure crystalline pyrite, which was carefully treated prior to the experiments to remove impurities.

Materials and methods

Preparation and characterization of iron disulfides

Two kinds of iron sulfides were used in the batch experiments. Ground pyrite (from Peru, Georg Maisch Import, Freising, Germany) was prepared and purified as described by Yan et al. (2015). Additionally, iron disulfide (FeS_2) was synthesized following a procedure described by Peiffer and Stubert (1999) and Berner (1970). Contrary to the earlier work, we synthesized FeS_2 in an anoxic glovebox (Innovative Technology, Newburyport, MA, USA) in an atmosphere of 100% N_2 at room temperature ($20 \pm 2\,^{\circ}C$). A solution of 0.1 M Na_2S was prepared from 15.6 g Na_2S and 2 L ultrapure water (Millipore) in a glass bottle and acidified with 32% HCl to a pH of 8. To this solution, 39.75 g $FeCl_2 \bullet 4H_2O$ and 12.8 g S(0) were added to reach final concentrations of 0.1 and 0.2 M, respectively. The bottle was closed with a plastic cap and sealed gas-tight with silicon gel, removed from the glovebox, stored in an oven at $60\,^{\circ}C$, and shaken by hand twice a day. After two weeks, the supernatant was decanted and the solid residue was sieved (mesh-size of sieves: 0.63 mm and 1.4 mm) to remove unreacted elemental sulfur particles. The fraction between 0.63 mm and 1.4 mm was collected and washed three times with 1–2 L oxygen-free ultrapure water and then boiled in 1 M HCl under N_2 for 1 h in order to remove acid-volatile sulfide and then washed twice with oxygen-free ultrapure water. The solid residue was washed three times with acetone to remove water and elemental sulfur and then washed nine times with petroleum ether to remove remaining elemental sulfur. In spite of these treatments, the residual elemental sulfur content was 4.6 mass% (as detected by HPLC, cf. below). After the washing procedure, the solid residue was dried under continuous nitrogen flow to remove the residual solvent, sieved with a 20-μm and a 100-μm sieve, and stored in an anoxic glovebox. The fraction between 20 and 100 μm was used for the experiments. The two iron disulfide specimen were characterized using scanning electron microscopy (Zeiss Leo 1530 FE-SEM, Germany), X-ray diffractometry (D5000, SIEMENS, Germany) using Co Kα radiation (40 kV, 40 mA) and the BET-method (Gemini V Series, Micromeritics, Germany).

Cultivation of microorganisms

Thiobacillus denitrificans DSM 12475 was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ), Braunschweig, Germany. The strain was grown at pH 6.8 in medium 113 (DSMZ 2010). The medium consisted of 14.7 mM KH_2PO_4, 19.8 mM KNO_3, 18.7 mM NH_4Cl, 3.25 mM $MgSO_4 \bullet 7H_2O$, 20.1 mM $Na_2S_2O_3 \bullet 5H_2O$, 30.0 mM $NaHCO_3$, 0.007 mM $FeSO_4 \bullet 7H_2O$, and trace element solution SL-4.

Acidovorax sp. BoFeN1 isolated from Lake Constance sediments is a mixotrophic bacterium that is able to grow with acetate plus Fe(II) as electron donors and nitrate as electron acceptor (Kappler et al. 2005). *Acidovorax* sp. BoFeN1 was grown in anoxic 22 mM bicarbonate-buffered low-phosphate mineral medium (pH = 7.0), which contained 10 mM nitrate as electron acceptor and 5 mM acetate as sole carbon source and was prepared as described by Hegler et al. (2008) and Hohmann et al. (2010).

Thiobacillus denitrificans and *Acidovorax* sp. BoFeN1 were grown at $30\,^{\circ}C$ in an atmosphere of 80% N_2 and 20% CO_2 in the dark and unshaken. Growth of the cultures was determined by following the optical density (OD) of the culture media at a wavelength of 600 nm (OD_{600}) in a spectrophotometer (DR3800, Hach Lange). Total cell number was determined by direct counting with a light microscope with a counting grid. After growth to the late exponential phase, both cultures were harvested by centrifugation, washed, and resuspended in modified medium (see later) before the start of the experiments.

Experimental set-up

Two types of batch experiments were conducted. Synthesized FeS_2 (characterized as a mixture of pyrite, marcasite, and elemental sulfur) was inoculated with the nitrate-reducing sulfide-oxidizing bacterium *T. denitrificans* or a mixotrophic culture of nitrate-reducing Fe(II)-oxidizing bacterium *Acidovorax* sp. BoFeN1. Control experiments were performed with *T. denitrificans* in the presence of (1) dissolved Fe(II) to test whether Fe(II) may be oxidized with nitrate as electron acceptor; and (2) dissolved Fe(III) to test whether abiotic FeS_2 oxidation is stimulated by dissolved Fe(III) (Peiffer and Stubert 1999). Ground, pure pyrite, free of elemental sulfur, which generally exists in pyrite as an impurity in natural and synthesized samples, was inoculated with *T. denitrificans* to study nitrate-dependent pyrite oxidation in the absence of elemental sulfur. The details of the batch experiments including controls are described in Table 1.

To avoid interference by sulfur in the medium when determining rates of sulfate formation from pyrite, the medium for the pyrite oxidation experiments with *T. denitrificans* did not contain thiosulfate or iron, unlike the medium for preparing *T. denitrificans* by cultivation. The modified reaction medium (pH 6.8) contained 15 mM KH_2PO_4, 19 mM NH_4Cl, 3.2 mM $MgCl_2 \bullet 6H_2O$, 30 mM $NaHCO_3$, and the same concentration of trace element solution SL-4 as described above. The medium used for the

Table 1. Overview of the setups of the batch experiments and their controls.

Experiments	Material	Initial nitrate concentration [mM]	Bacterial strain	Cell concentration [cells ml^{-1}]	Initial material concentration [mM]	Dissolved Fe(II) concentration [μM]	Dissolved Fe(III) concentration [μM]
Inoculated setup with synthesized FeS$_2$ (pyrite and marcasite with residual elemental sulfur)	synthesized FeS$_2$	~10	Thiobacillus denitrificans	9.3×10^6 or 9.3×10^7	8.3		
	synthesized FeS$_2$	~10	Acidovorax sp.BoFeN1	1.2×10^7 or 1.2×10^8	8.3		
	synthesized FeS$_2$	~10	Thiobacillus denitrificans	9.3×10^6 or 9.3×10^7	8.3	100	
	synthesized FeS$_2$	~10	Thiobacillus denitrificans	9.3×10^6 or 9.3×10^7	8.3		100
Control experiment	synthesized FeS$_2$	~10	Thiobacillus denitrificans	1.8×10^8	8.3		
Inoculated setup with ground pyrite	ground pyrite	~10	Thiobacillus denitrificans	2×10^4 or 2×10^5	50		
Control experiments	ground pyrite	~10	Thiobacillus denitrificans	2×10^4 or 2×10^5	50		
	ground pyrite		Thiobacillus denitrificans	2×10^4 or 2×10^5	50		
	elemental sulfur	~10	Thiobacillus denitrificans	2×10^4 or 2×10^5	50		

batch experiment with *Acidovorax* sp. BoFeN1 was the same as the nutrient medium for cultivation.

For batch experiments with synthesized FeS$_2$, 100 mL of medium and 0.1 g of the synthesized FeS$_2$ (final concentration 8.3 mM) were added into each autoclaved glass serum bottle inside an anoxic, hydrogen-free, UV-sterilized stainless-steel glovebox (Mecaplex, Grenchen, Switzerland) containing a 100% N$_2$ atmosphere. Bottles were sealed with butyl stoppers, crimped, and then removed from the glovebox. The headspace of each serum bottle was flushed with a mixture of 80% N$_2$ and 20% CO$_2$. At the beginning of each batch experiment with synthesized FeS$_2$, 1 mL of anoxic KNO$_3$ (1 M) stock solution was injected into each serum bottle through the butyl stopper (final concentration of approximately 10 mM) using a syringe that had been flushed several times with N$_2$. A volume of 0.1 mL or 1 mL of the pure culture of *T. denitrificans* was added to each serum bottle resulting in a cell concentration of 9.3×10^6 or 9.3×10^7 cells mL^{-1}, respectively. Serum bottles for experiments with *Acidovorax* sp. BoFeN1 were prepared in a similar way. Prior to inoculation with bacteria (cell concentration 1.2×10^7 or 1.2×10^8 cells mL^{-1}), solutions of oxygen-free NaNO$_3$ (1 M), and Na acetate (1 M) were added to yield approximately 10 mM and 5 mM concentrations, respectively.

Parallel batch experiments were performed in order to test a potential stimulating effect of redox-active substances. A volume of 100 μL of 100 mM sterile FeCl$_2$•4H$_2$O or 100 μL of 100 mM FeCl$_3$•6H$_2$O was added to the serum bottles containing synthesized FeS$_2$, nitrate, and *T. denitrificans* as described above, to obtain a final Fe(II) concentration of 100 μM and a final Fe(III) concentration of 100 μM. All cultures were incubated at 30 °C in the dark. Batch experiments and controls were conducted in two independent replicates.

For batch experiments with ground pyrite, the procedure was essentially the same as for the experiments with synthesized FeS$_2$. Medium was added to the serum bottles from a Widdel flask outside the glovebox. Thus, to each autoclaved glass serum bottle, 100 mL of medium was added under an atmosphere of 80% N$_2$ and 20% CO$_2$ using two sterilized needles. The bottles were then sealed with butyl stoppers and crimped. All bottles were placed in the glovebox containing an atmosphere of 100% N$_2$ (Innovative Technology, Massachusetts, USA, 100% N$_2$) and then opened. Ground pyrite (0.6 g: final concentration 50 mM) was added to each serum bottle. The bottles were sealed with butyl stoppers, crimped, and then removed from the glovebox. The head space of each serum bottle was flushed with gas of a composition of 80% N$_2$ and 20% CO$_2$.

At the beginning of each batch experiment with ground pyrite, 1 mL of oxygen-free, sterile 1 M KNO$_3$ stock solution was added to a final nitrate concentration of approximately 10 mM. Since substantial denitrification occurred in the presence of high cell concentrations of *T. denitrificans* which accumulated sulfur intracellularly (cf. Results and Discussion sections), we tried to keep the cell concentration as low as possible. Therefore, we added 0.1 or 1 mL of a pure culture of *T. denitrificans* to each serum bottle, resulting in cell

concentrations of 2×10^4 and 2×10^5 cells mL^{-1}, respectively. After the additions of nitrate and cells, the headspace of the bottles was flushed again with N$_2$/CO$_2$ (80/20) for 10 min. These serum bottles were incubated at 30 °C in the dark.

For control experiments, some serum bottles contained only pyrite and nitrate but no *T. denitrificans*, others contained only pyrite and *T. denitrificans* but no nitrate, and still, others contained only nitrate and *T. denitrificans* but no pyrite. These controls allowed monitoring of background reactions.

To demonstrate that the cells in a cell suspension were viable, a control experiment was run with 50 mM elemental sulfur and 10 mM nitrate and inoculated with *T. denitrificans*. Each of the different experimental setups were run in triplicate, whereas the control setups were run in duplicate.

Chemical analyses

Aliquots of approximately 2 mL were anoxically withdrawn from serum bottles. Concentrations of nitrate, nitrite and sulfate were quantified using ion chromatography with chemical suppression and conductivity detector using an A-supp 4 anion column (Metrohm, Herisau, Switzerland) after filtration of the sample through a Nylon filter having a 0.22 μm pore size to stop the microbial reaction and remove the residual particles. Filtered samples were diluted with ultrapure water prior to analysis. Samples from experiments with *T. denitrificans* in the presence of synthesized FeS$_2$ and nitrate were diluted 50-fold. Samples from experiments with *Acidovorax* sp. BoFeN1 in the presence of synthesized FeS$_2$ and nitrate were diluted 10-fold. Samples from experiments with *T. denitrificans* in the presence of ground pyrite and nitrate were also diluted 10-fold.

To quantify the elemental sulfur in the solid phase, 0.5 g of ground pyrite and 0.5 g of synthesized FeS$_2$ were added to separate 120-mL glass serum bottles. The bottles were sealed and crimped, and the headspace of the bottles was flushed with nitrogen. To each serum bottle, 20 mL of oxygen-free methanol were subsequently added with a glass syringe. The methanol was the reagent that extracted the elemental sulfur. Experiments were performed in two independent replicates. The headspace of the bottles was again flushed with nitrogen for 1 min. Suspensions were shaken for 24 h to extract elemental sulfur. Thereafter, an aliquot of ca. 1.5 mL of each sample was removed and filtered through a 0.22-μm pore size filter (Nylon). The concentration of the elemental sulfur in the methanol filtrate was analyzed using HPLC (PerkinElmer 2000 pump and autosampler, Fa. linear-UV – VIS detector and software peak-sample 409, 265 nm).

Attempts to determine ferric iron of samples from cultures of the nitrate-reducing, Fe(II) oxidizer *Acidovorax* sp. BoFeN1 failed because of interference by nitrite (Klueglein and Kappler 2013), of which we were unaware at the time at which the measurements were attempted. In these attempts, samples to determine the amount of FeOOH formed during the oxidation of pyritic iron were acidified with HCl (pH 1) in order to dissolve the ferric iron for further quantification. However, pyritic Fe(II) oxidizes rapidly in the presence of nitrite under these conditions (Klueglein and Kappler 2013).

Measurements performed revealed the absence of Fe(II) but the occurrence of Fe(III), the origin of which remaining uncertain. We, therefore, did not consider the results from these determinations in the Discussion.

Results

Characterization of pyrite

X-ray diffractogram (XRD) patterns revealed that the ground material was pure pyrite (Figure 1), whereas the synthesized FeS$_2$ was a mixture of pyrite and marcasite (Figure 2). Diffraction intensities indicated a mixture of pyrite and marcasite in a ratio of approximately 1:2 (data not shown). The ground pyrite had a crystalline structure (Figure 3) and contained only very small amounts of residual elemental sulfur (0.001 mass % as detected using HPLC, cf. below). Although the ground pyrite was washed several times with HCl, nanosized structures were still visible on the surface of the pyrite in the SEM images (Figure 3). EDX spectra (see Supporting Information (SI), Figure S1, Tables S1 and S2) derived from these nanosized structures displayed a Fe:S ratio of approximately 1:2 suggesting that the nanosized particles also consisted of pyrite. Consistent with the classification by Ainsworth and Blanchar (1984), the synthesized FeS$_2$ consisted of conglomerates with irregular surfaces composed of cemented particles (Figure 4). It still contained a large amount of elemental sulfur (4.6 mass%). The BET surface area of the ground pyrite and the synthesized FeS$_2$ was 0.17 and 0.41 m^2 g^{-1}, respectively.

Oxidation of synthesized FeS$_2$ in the presence of nitrate

Throughout an entire 43-day experiment, S-oxidizing, nitrite-generating *T. denitrificans* at a cell concentration of 9.3×10^6 or 9.3×10^7 cells mL^{-1} generated sulfate in a medium containing 8.3 mM synthesized FeS$_2$ and 9.2 mM nitrate. Nitrate reduction resulted in the formation of nitrite (Figure 5(A)). Nitrate reduction and sulfate generation proceeded without a lag from the beginning of the experiment. The rate of reaction with 9.3×10^7 cells mL^{-1} was faster (0.16 mM nitrate consumption day^{-1} and 0.15 mM sulfate formation day^{-1}) than that at lower cell concentration of 9.3×10^6 cells mL^{-1} (0.12 mM nitrate consumption day^{-1} and 0.13 mM sulfate formation day^{-1}) from the ninth day to the 43rd day. By contrast, neither nitrite nor sulfate formation was observed in the experiment with 8.3 mM synthesized FeS$_2$ in the presence of Fe(II)-oxidizing strain *Acidovorax* sp. BoFeN1 at a cell concentration of 1.2×10^7 or 1.2×10^8 cells mL^{-1} (Figure 5(B)). Abiotic control experiments with synthesized FeS$_2$ and nitrate but without either *T. denitrificans* or strain *Acidovorax* sp. BoFeN1 showed no reaction (Figure 5(C)). However, a control experiment containing only nitrate, and a cell suspension of *T. denitrificans* without synthesized FeS$_2$ led to consumption of nitrate accompanied by the formation of sulfate and nitrite (Figure 5(D)), which we attribute to oxidation of sulfur being introduced into the reaction vessel with the cell suspension
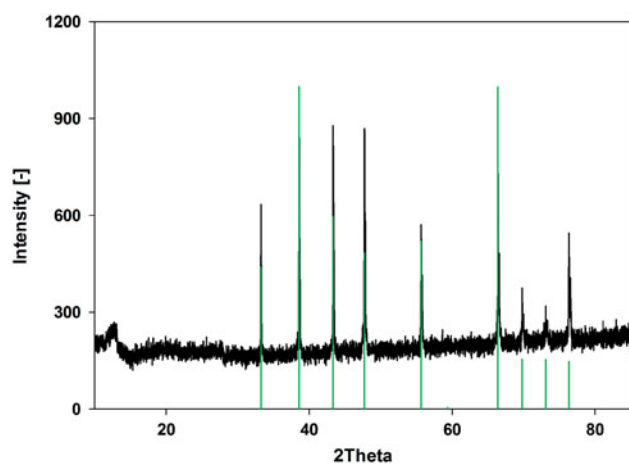
Figure 1. X-ray diffractogram of ground pyrite. Green lines represent the predicted diffractogram of pyrite (Brostigen and Kjekshus 1969).
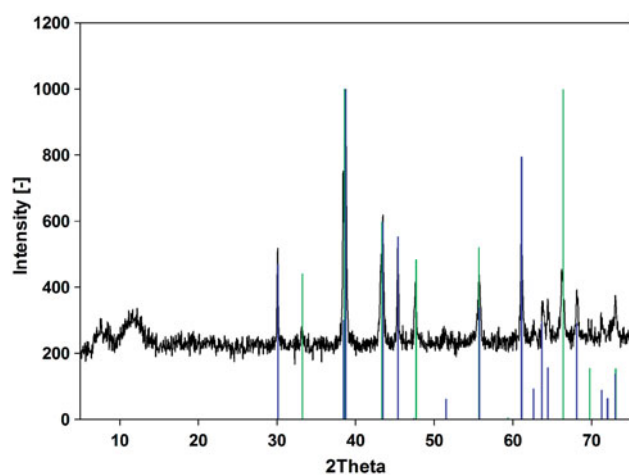

Figure 2. X-ray diffractogram of synthesized FeS$_2$. Green lines reflect the predicted diffractogram of pyrite (Brostigen and Kjekshus 1969) and blue lines represent the predicted diffractogram of marcasite (Rieder et al. 2007).
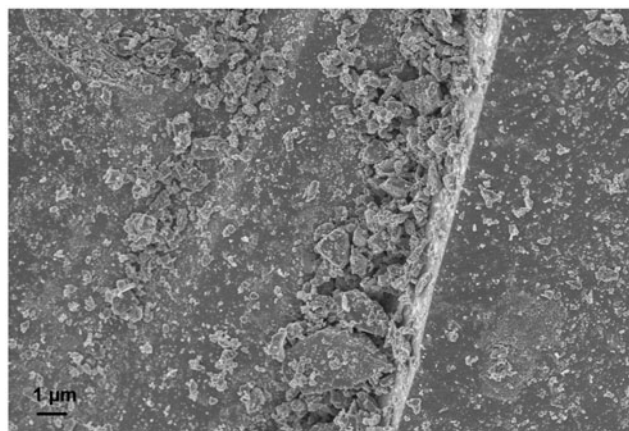

Figure 3. Scanning electron micrograph of the ground pyrite after preparation.

(cf. Discussion). But the consumption of nitrate in the control experiment ($\Delta NO_3^- = 4.6$ mol L^{-1}) was distinctly lower than in the presence of the synthesized FeS$_2$ ($\Delta NO_3^- = 7.5$ mol L^{-1}) suggesting the occurrence of chemolithoautotrophic reduction of nitrate by the synthesized FeS$_2$ added in the *T. denitrificans* in the reaction mixture.
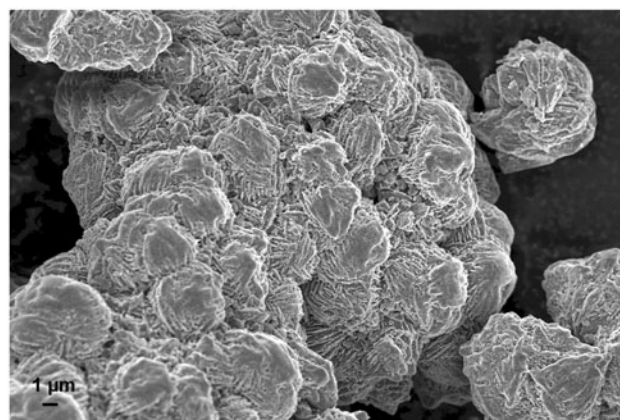

Figure 4. Scanning electron micrograph of the synthesized FeS$_2$ after preparation.

In control experiments, in which 100 μM Fe(II) or 100 μM Fe(III) were added to the reaction mixture, no increase in nitrate consumption and sulfate formation was observed compared to experiments in which no Fe(II) or Fe(III) were added (Figure 6(A,B)). Instead, the rates of nitrate consumption and sulfate formation were even slower. Addition of Fe(III) decelerated the formation of nitrite (Figure 6(C)). In summary, the addition of Fe(II) or Fe(III)) did not stimulate the oxidation of synthesized FeS$_2$ by nitrate under our experimental conditions. Similar results were obtained at the lower cell concentration of 9.3×10^6 cells mL^{-1} (data not shown).

Potential of pure ground crystalline pyrite as an electron donor for nitrate reduction

To prevent denitrification by sulfur associated with the inoculum of *T. denitrificans*, as observed in the control experiments at high cell concentration, experiments using ground crystalline pyrite were run at a low cell concentration.

In an 87-day experiment using 50 mM ground crystalline pyrite, 10 mM nitrate, and cell concentrations of 2×10^4 cells mL^{-1} and 2×10^5 cells mL^{-1}, the concentration of nitrate remained stable (Figure 7). The concentration of nitrite was below the detection limit, and the concentration of sulfate was approximately constant between 0.02 and 0.04 mM, indicating that no pyrite oxidation occurred with pure pyrite (no other associated sulfur species) within the timeframe of our experiments and at the cell concentration of this experiment (Figure 7).

No reaction was observed in control experiments with (1) ground crystalline pyrite and nitrate in the absence of *T. denitrificans*, with (2) ground pyrite and *T. denitrificans* but no added nitrate, or with (3) nitrate and *T. denitrificans* but no added ground crystalline pyrite. The cell concentration under experimental conditions (2) and (3) was 2×10^5 cells mL^{-1} (Figure 8(A–C)). In a control experiment with elemental sulfur as electron donor and nitrate as electron acceptor in the presence of 2×10^5 *T. denitrificans* cells mL^{-1}, the nitrate was reduced to nitrite accompanied by sulfate production (Figure 8(D)), demonstrating that the cells
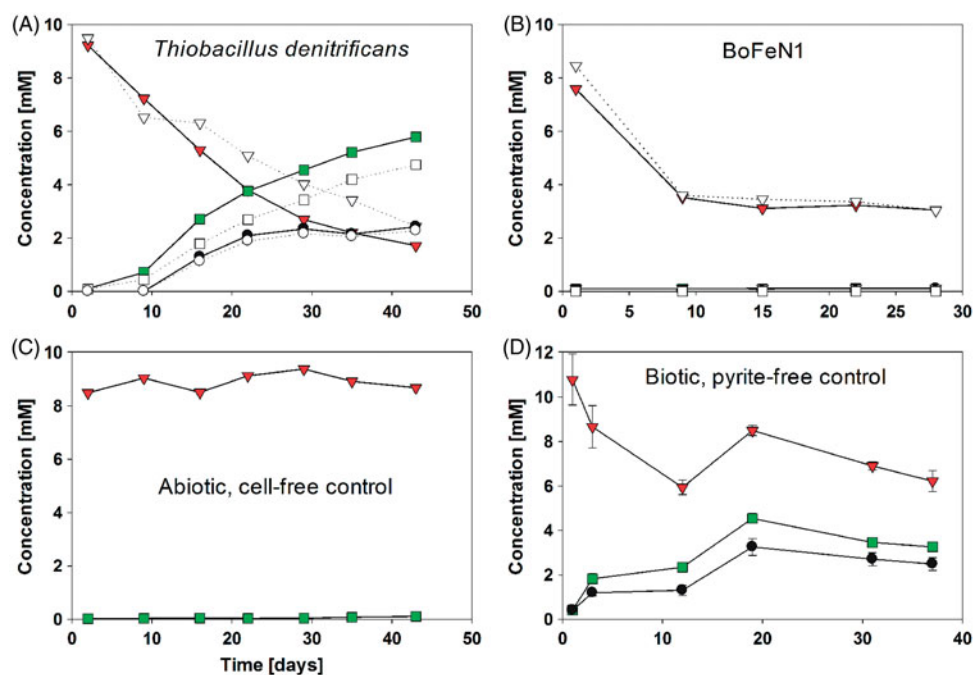
Figure 5. Product concentrations (nitrite (●), nitrate (▼), sulfate (■)) from the reaction between synthesized FeS$_2$ (8.3 mM) and nitrate (approximately 10 mM) in the presence of (A) *Thiobacillus denitrificans* at a cell concentration of 9.3×10^6 (open symbols and dotted line) or 9.3×10^7 cells ml-1 (closed symbols and solid line), (B) BoFeN1 at a cell concentration of 1.2×10^7 (open symbols and dotted line) or 1.2×10^8 cells mL^{-1} (closed symbols and solid line) and (C) in the absence of cells under anoxic, pH-neutral conditions. (D) Pyrite-free control experiment with nitrate (approximately 10 mM), in the presence of *T. denitrificans* at a cell concentration of 1.8×10^8 cells mL^{-1}. Concentrations of (A)–(C) were calculated from the mean values of two independent replicates. Concentrations of (D) were calculated from the mean values of three independent replicates. Error bars are standard deviations calculated from three independent replicates.

were active under these conditions. The oxidation of elemental sulfur was detected only after 29 days of incubation and continued until the end of the experiment (87 days).

At a *T. denitrificans* concentration of 2×10^4 cells mL^{-1}, no reaction was observed in the experiment with pyrite and nitrate nor in any of the controls including the control with elemental sulfur (see Supporting Information (SI)). The results in Figure S2 and S3 suggest that the number of active cells was too low for observing elemental sulfur oxidation within the time frame of the experiments.

Discussion

In this study of nitrate-dependent FeS$_2$ oxidation, pure ground crystalline pyrite was not oxidized in the presence of *T. denitrificans* during 87 days at a cell concentration of 2×10^5 cells mL^{-1} (Figure 7). In contrast, synthesized FeS$_2$ served as electron donor in chemolithoautotrophic reduction of nitrate at *T. denitrificans* cell concentrations of 9.3×10^6 or 9.3×10^7 cells mL^{-1} (Figure 5(A)). However, a significant portion of the observed nitrate reduction may be attributed to denitrification by sulfur introduced with the inoculum of *T. denitrificans*. This interpretation is supported by the results from a control experiment in the absence of synthesized FeS$_2$ with a cell suspension of *T. denitrificans* containing nitrate. In this experiment, consumption of nitrate was observed accompanied by the formation of sulfate and nitrite (Figure 5(D)). As shown in Figure 8D, *T. denitrificans* cells were able to oxidize elemental sulfur with nitrate, generating sulfate and nitrite, albeit with a distinct lag of 27 days before the reaction became detectable. This

observation implies that part of the denitrification observed in experiments with synthesized FeS$_2$ may have been due to chemolithoautotrophic oxidation of the residual elemental sulfur associated with synthesized FeS$_2$. Similar observation in a previous study showed that denitrification and accumulation of nitrite occurred in a chemolithotrophic denitrifying mixed culture utilizing elemental sulfur as electron donor (Cardoso et al. 2006).

To distinguish between denitrification from oxidation of sulfur introduced with the inoculum of *T. denitrificans* and denitrification from oxidation of residual sulfur associated with the synthesized FeS$_2$, we established a mass balance based on the experimental data shown in Figure 5(A). This mass balance will be discussed in the following section.

The reactive species in chemolithoautotrophic denitrification

The reaction between synthesized FeS$_2$ and nitrate in the presence of *T. denitrificans* consumed 7.5 mM nitrate and generated 2.5 mM nitrite and 5.7 mM sulfate, respectively (Figure 5(A), Table 2, row 1). In a control experiment with nitrate and comparable cell numbers of *T. denitrificans* but without synthesized FeS$_2$, 4.6 mM nitrate was consumed, whereas 2.0 mM nitrite and 2.9 mM sulfate were generated (Figure 5(D), row 2 in Table 2). The fraction of nitrate reduced by oxidation of elemental sulfur can be estimated based on a stoichiometry derived from the mass balance in a corresponding control experiment (Figure 8(D)). Although this experiment was performed at a much lower cell concentration than in the experiment shown in Figure 5
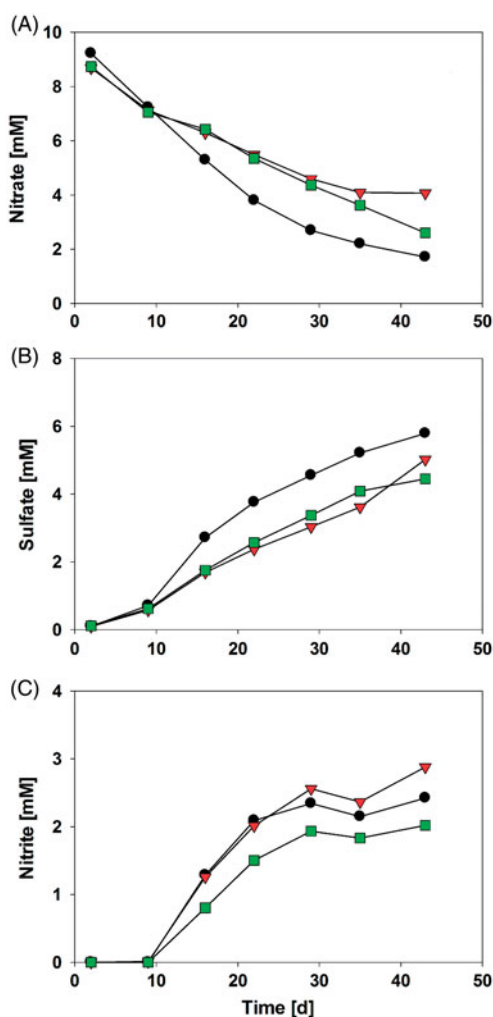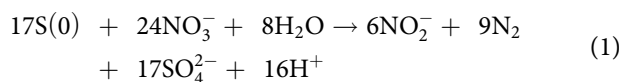
Figure 6. Product concentrations (nitrate (A), sulfate (B), nitrite (C)) from the reaction between synthesized FeS₂ (8.3 mM) and nitrate (approximately 10 mM) in the presence of *Thiobacillus denitrificans* at a cell concentration of 9.3 × 10⁷ cells mL⁻¹ in the presence and absence of either Fe(II) or Fe(III) (in the absence of Fe(II) and Fe(III) (●)) under anoxic, pH-neutral conditions. The data were the same as in Figure 5(a) at a cell concentration of 9.3 × 10⁷ cells mL⁻¹, with Fe(II) (▼), with Fe(III) (■)). Concentrations were calculated from the mean values of two independent replicates.

Figure 7. Product concentrations (nitrite (●), nitrate (▼), sulfate (■)) from the reaction between ground pyrite (50 mM) and nitrate (approximately 10 mM) in the presence of *T. denitrificans* at a cell concentration of 2 × 10⁵ cells mL⁻¹ under anoxic, pH-neutral conditions. Error bars are standard deviations calculated from three independent replicates. The nitrite symbols are hidden behind the sulfate ones.

(cf. Table 1), it provides a reliable estimate of the stoichiometry because the reaction was close to a steady state. The consumption of 8 mM nitrate generated 2 mM nitrite and 5 mM sulfate (Figure 8(D)) is close to the stoichiometry displayed in Equation (1):

$$17S(0) + 24NO_3^- + 8H_2O \rightarrow 6NO_2^- + 9N_2 + 17SO_4^{2-} + 16H^+ \tag{1}$$

The concentration of nitrite presumably produced from denitrification by reaction with intracellularly accumulated stored sulfur (2.0 mM) (Figure 5(D), Table 2, row 2) can be subtracted from the total concentration of nitrite produced in the experiment with synthesized FeS₂ (2.5 mM) (Figure 5(A), Table 2, row 1), leaving 0.5 mM nitrite maximally produced by reaction with elemental sulfur associated with synthesized FeS₂. Considering Equation (1), this concentration of nitrite corresponds stoichiometrically to an oxidation of 1.4 mM elemental sulfur, matching the measured concentration of 1.4 mM elemental sulfur associated with synthesized FeS₂
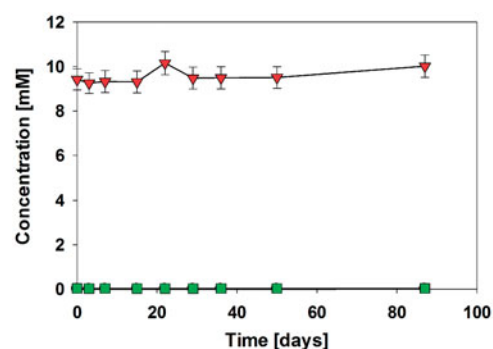
(calculated based on 4.6 mass% of the residual elemental sulfur content), and a consumption of 2.0 mM nitrate. Subtraction of this quantity of nitrate and the 4.6 mM nitrate consumed during denitrification owing to reaction of nitrate with stored sulfur from the total nitrate consumption of 7.5 mM in the experiment shown in Figure 5(A), results in a residual amount of 0.9 mM nitrate potentially consumed by chemolithoautotrophic oxidation of synthesized FeS₂-sulfur. This reaction can be summarized using the stoichiometry for denitrification coupled to pyrite oxidation in anoxic groundwater environments usually described by Equation (2) (Jørgensen et al. 2009; Kölle et al. 1983; Korom 1992; Postma et al. 1991; Tesoriero et al. 2000):

$$5FeS_2 + 14NO_3^- + 4H^+ \rightarrow 5Fe^{2+} + 7N_2 + 10SO_4^{2-} + 2H_2O \tag{2}$$
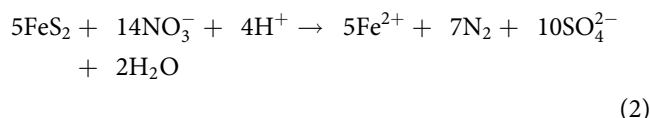
It has been reported that *T. denitrificans* is able to oxidize Fe(II) with nitrate under autotrophic conditions (Straub et al. 1996). However, attempts to demonstrate the occurrence of reaction (2) by measuring changes in Fe(II) concentrations failed because of the interference of nitrite in the determination (cf. Materials and methods) (Klueglein and Kappler 2013). The control experiments demonstrated that addition of Fe(II) did not stimulate denitrification by *T. denitrificans* (Figure 6), leading to the conclusion that *T. denitrificans* cannot oxidize Fe(II) with nitrate under the experimental conditions and that liberated Fe(II) does not need be considered in Equation (2) when establishing an electron balance for denitrification. This may be explained by *T. denitrificans* not being able to oxidize Fe(II) with nitrate when sulfur compounds are present in the reaction system. *Thiobacillus denitrificans* is a well-known chemolithoautotrophic bacterium that is able to couple denitrification to the oxidation of inorganic sulfur compounds (Beller et al. 2006, 2013).

Despite the uncertainties inherent in the assumptions about the nature of the N products, our data provide evidence that the synthesized FeS₂ (Table 2, row 4), residual elemental sulfur associated with synthesized FeS₂ (Table 2, row 3), and intracellular stored sulfur (Table 2, row 2) were
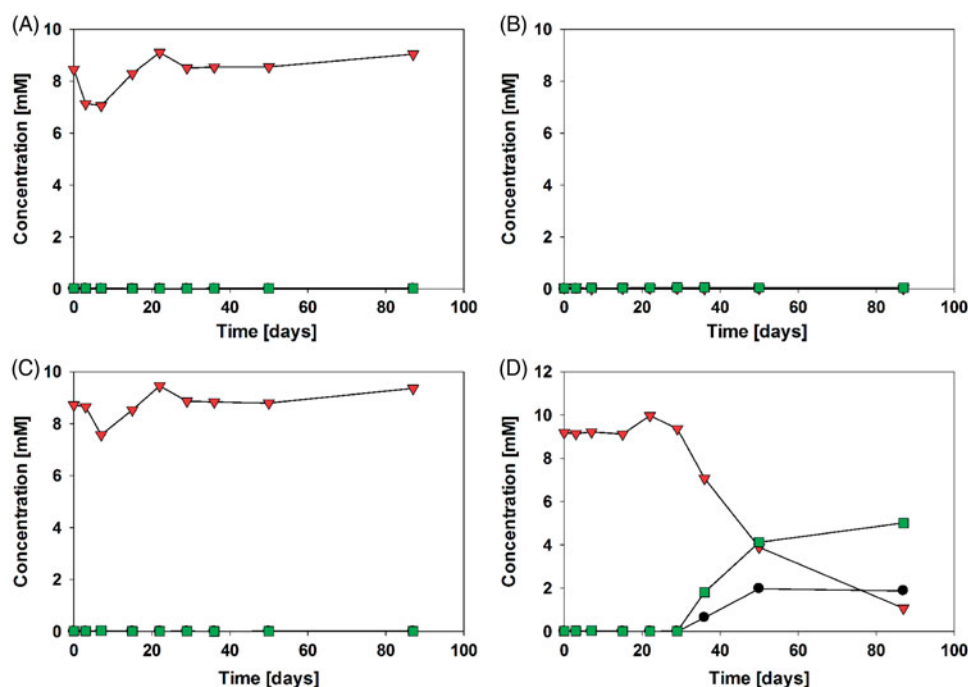
Figure 8. Product concentrations (nitrite (●), nitrate (▼), sulfate (■)) from the control experiment of (A) ground pyrite (50 mM) and nitrate (approximately 10 mM) in the absence of *Thiobacillus denitrificans*, (B) ground pyrite (50 mM) and *T. denitrificans* at a cell concentration of 2×10^5 cells mL^{-1} in the absence of nitrate, (C) nitrate (approximately 10 mM) and *T. denitrificans* at a cell concentration of 2×10^5 cells mL^{-1} in the absence of pyrite, (D) elemental sulfur (50 mM) and nitrate (approximately 10 mM) in the presence of *T. denitrificans* at a cell concentration of 2×10^5 cells mL^{-1} under anoxic, pH-neutral conditions. Concentrations were calculated from the mean values of two independent replicates. The nitrite symbols are hidden behind the sulfate ones (A–C).

Table 2. Mass balance of substrates and products of the reaction between synthesized FeS₂ (initial concentration 8.3 mM containing 1.5 mM elemental sulfur and approximately 10 mM nitrate in the presence of *Thiobacillus denitrificans* at high cell concentration of 9.3×10^7 cells $m1^{-1}$ under anoxic, pH-neutral conditions.

	NO_{3-} **depleted** [mM]	NO_2^- **produced** [mM]	S(0) **depleted** [mM]	SO_4^{2-} **produced** [mM]
Experiment with synthesized FeS₂ (Figure 5A)	7.5[a]	2.5[a]	1.4[a]	5.7[a]
Control experiment with sulfur stored in bacterial cells (Figure 5D)	4.6[a]	2.0[a]		2.9[a]
Mass balance Equation (1)	2.0[c]	0.5[b]	1.4[c]	1.4[c]
Mass balance Equation (2)	0.9[b]			0.6[d]
Calculated mass balance				4.9

[a]Measured concentrations changes (calculated as the difference between the initial and final concentration of the experiment).
[b]Calculated from experimental mass balance.
[c]Calculated using Equation (1).
[d]Calculated using Equation (2).

used as electron donors for chemolithoautotrophic denitrification (Table 2). This interpretation is supported by the mass balance for sulfate that can explain the 86% of the measured sulfate concentration [(calculated from mass balance/measured concentrations) × 100%], suggesting that our assumptions and estimates are reasonable.

Field and laboratory studies of nitrate-dependent anaerobic FeS₂ oxidation

This study highlights the importance of the speciation of reduced sulfur in mediating chemolithoautotrophic denitrification. It is interesting to note from Table 3 that speciation was not considered in previous laboratory studies on nitrate-dependent pyrite oxidation. Generation of nitrite and sulfate upon consumption of nitrate was observed and attributed to the oxidation of pyrite (Bosch et al. 2012; Jørgensen et al. 2009; Torrentó et al. 2010; Vaclavkova et al.

2015) in experiments in which no attempts were made to remove elemental sulfur during preparation of pyrite and to employ a cell concentration that would cause minimal interference from intracellular stored sulfur and compounds in the medium in denitrification. The absence of XRD-reflection characteristics for S(0) is not an essential criterion to exclude its occurrence because it merely indicates that the elemental sulfur content was lower than 3–5 mass% or the crystallinity of the S(0) too low or the overall S(0) crystal size too small. Unless the content of elemental sulfur is quantified, it remains unclear whether the reduction of nitrate is coupled to pyrite oxidation or simply related to the oxidation of elemental sulfur associated with pyrite. In previous studies, when elemental sulfur was removed from the reaction mixture in the preparation of pyrite (Haaijer et al. 2007; Schippers and Jørgensen 2001; this study), no pyrite oxidation was observed (Table 3).

Additional complications could arise from interference of reactants with pyrite oxidation. It has been demonstrated

Table 3. Overview of previous studies on chemolithotrophic denitrification coupled to pyrite oxidation in the presence of nitrate-reducing strains or in environmental samples.

Bacterial strain	Reference	Type of pyrite (particle size of pyrite)	Remove of elemental sulfur from pyrite material	Evidence for nitrate reduction and sulfate generation	Fe speciation data as proof of pyrite oxidation
Thiobacillus denitrificans	Jørgensen et al. (2009) supporting information	Ground natural crystalline pyrite (45–200 μm)	None	Yes	None
Thiobacillus denitrificans	Torrento et al. (2010)	Ground natural crystalline pyrite (25–50 μm and 50–100 μm)	None	Yes	None
Thiobacillus denitrificans	Bosch et al. (2012)	Ground natural crystalline pyrite (<200 μm)	None	Yes	Yes
Thiobacillus cultures	Vaclavkova et al. (2014)	Ground natural crystalline pyrite (<200 μm)	None	Yes	None
Thiobacillus denitrificans	Present study with synthesized Pyrite	Synthesized less crystalline pyrite (630–1400 μm)	None	Yes	None
Soil samples from fresh water lake	Haaijer et al. (2007)	Ground natural crystalline pyrite (500–3000 μm)	Washed once with acetone	None	None
Marine sediment	Schippers and Jorgensen (2001)	Coarse Pyrite originated from an ore processing flotation plant (50–100 μm)	Washed three times with acetone	None	None
Thiobacillus denitrificans	Present study with ground pyrite	Ground natural crystalline pyrite (63–200 μm)	Washed three times with cyclohexane	None	None

that nitrite is able to oxidize pyrite abiotically in 1 M HCl leading to the formation of ferric iron (Yan et al. 2015). The occurrence of ferric iron may, therefore, be misinterpreted as proof of pyrite oxidation (Bosch et al. 2012). To overcome such interferences, a revised protocol was recommended for acid extraction of suspensions containing nitrite and pyrite or other Fe(II)-containing solid phases that may be subject to interference by nitrite (Yan et al. 2015).

Thus, nitrate-dependent microbial pyrite oxidation in the presence of *T. denitrificans* postulated in previous studies cannot be ruled out. However, its contribution to the observed production of sulfate and the consumption of nitrate is probably much less than assumed. The findings of the present study suggest that laboratory studies on microbially mediated pyrite oxidation may be subject to several misinterpretations. The experimental design of the present study may provide explanations for contradictory observations, i.e., consideration was given to removal of elemental sulfur during pyrite preparation, to the effect of nitrite formation from nitrate, and to cell concentration in the reaction mixture.

There is a clear indication from field studies that nitrate consumption and pyrite oxidation are interrelated (Broers 1998; Evangelou and Zhang 1995; Pauwels et al. 2000; Postma et al. 1991; Tesoriero et al. 2000; Van Beek et al. 1989; Zhang et al. 2009). The consumption of nitrate with concomitant generation of sulfate and dissolved Fe(II) is generally regarded as indirect evidence for denitrification coupled to pyrite oxidation, which calls for a closer inspection of the chemical nature of the reacting sulfur species.

Our present study shows that under our experimental conditions, chemolithoautotrophic oxidation of FeS_2 by *T. denitrificans* with nitrate as electron acceptor was not possible with pure crystalline pyrite that did not contain elemental sulfur as an impurity. In contrast, it is possible to determine a mass balance for chemolithoautotrophic oxidation of synthesized FeS_2 having low crystallinity, i.e., pyrite or marcasite, even in the presence of side reactions. Such side reactions may be represented by denitrification involving stored sulfur in *T. denitrificans* and by microbial reduction of nitrate by residual elemental sulfur associated with synthesized FeS_2 (Table 2). The oxidation of synthesized FeS_2 appeared to be induced via S oxidation but not via Fe oxidation because the Fe(II)-oxidizing nitrate-reducing strain *Acidovorax* sp. BoFeN1 did not stimulate FeS_2-dependent nitrate reduction.

Addition of Fe(II) and Fe(III) to the reaction mixture of synthesized FeS_2, nitrate, and *T. denitrificans* resulted even in a slight decrease in the rates of nitrate reduction and sulfate generation, supporting the hypothesis that Fe is not involved in the oxidation. The larger peak widths in the XRD of the synthesized FeS_2, as well as the SEM images, suggest that this material had a smaller mean particle size and less crystallinity compared to the ground crystalline pyrite, which may explain its higher reactivity. Also, the BET surface area (0.41 m^2 g^{-1}) of the synthesized FeS_2 was greater than that of the ground pyrite (0.17 m^2 g^{-1}) although this difference was not very large.

The synthesized FeS_2 consisted of pyrite, marcasite, and elemental sulfur. It remains unclear which S source (pyrite, marcasite, or elemental sulfur) plays the predominant role in the reaction. Our study suggests that field observations on denitrification being linked to oxidation of reduced sulfur (Kölle et al. 1983; Pauwels et al. 2000; Postma et al. 1991; Tesoriero et al. 2000; Zhang et al. 2009) are indicative of biologically active zones where an active sulfur cycle may take place rather than zones of geological ripening. We, therefore, suggest that quantitative differentiations among the sulfur sources, pyrite, marcasite, and elemental sulfur, as well as their mineralogical characterization, are key factors in pyrite oxidation studies, both in the field and in the laboratory. Contradictory findings obtained so far from potential chemolithoautotrophic oxidation of FeS_2 with nitrate as electron acceptor may have resulted from reaction with reduced sulfur species present in natural or synthetic pyrite phases or in sediments.

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