



Review

Towards a standardized protocol for studying chemolithoautotrophic denitrification with pyrite at circumneutral pH

Ruiwen Yan^{a,b,e}, Andreas Kappler^c, Marcus A. Horn^d, Stefan Peiffer^{e,*}^a State Key Laboratory of Coal Resources and Safe Mining, China University of Mining and Technology (Beijing), Beijing, 100083, China^b College of Geoscience and Surveying Engineering, China University of Mining and Technology (Beijing), Beijing, 100083, China^c Geomicrobiology Group, Eberhard-Karls-University Tuebingen, 72076, Tuebingen, Germany^d Institute of Microbiology, Leibniz University Hanover, 30419, Hanover, Germany^e Department of Hydrology, BayCEER, University of Bayreuth, 95440, Bayreuth, Germany

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ABSTRACT

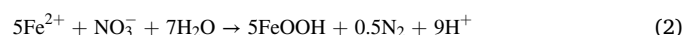
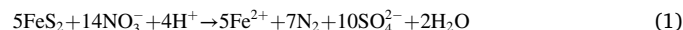
Laboratory studies on chemolithoautotrophic microbial denitrification coupled to pyrite oxidation at circumneutral pH yielded conflicting results. Some studies indicated that microbial oxidation of pyrite does occur, but several reports have shown that no microbial pyrite oxidation took place in experiments with pyrite as electron donor and nitrate as electron acceptor. We propose that inconsistent experimental and analytical protocols may cause substantial uncertainty in the interpretation of results from such laboratory studies, and may even produce artifacts. In this study, a comprehensive overview of possible pitfalls and artifacts in relation to geochemical and microbiological interferences is provided. Key interferences are impurities of reduced sulfur species associated with pyrite, interferences of nitrite and dissolved Fe(III) with quantitative spectrophotometric determination of Fe(II) and Fe(III) during acidic extractions, interference of oxygen, occurrence of residual iron and sulfur compounds in the reaction medium, and interference of cell-associated and stored sulfur. Therefore, we propose a series of experimental standard protocols to overcome these interferences in future studies on chemolithoautotrophic denitrification with pyrite.

1. Introduction

Nitrate is a common inorganic pollutant in shallow groundwater aquifers due to agricultural fertilizer or manure application (Yeshno et al., 2019; Kolbe et al., 2019). In these catchments, the concentrations of nitrate usually exceed the World Health Organization (WHO) guideline of 50 mg/L and therefore threaten the supply of drinking water (Ward et al., 2018). Effective removal of nitrate from groundwater occurs primarily through denitrification, the microbially mediated reduction of nitrate using organic or inorganic electron donors (Knoll et al., 2020). Field observations in many natural systems clearly indicated denitrification to occur in the absence of organic carbon which is commonly attributed to chemoautotrophic denitrification by iron sulfides (Schippers and Jørgensen 2002; Knöller et al., 2005; Haaijer et al., 2007; Vaclavkova et al., 2014).

Pyrite (FeS₂) is the most abundant iron- and sulfur-bearing mineral in the earth's crust (Gregory and Kohn 2020). It plays an important role in the global biogeochemical cycles of iron and sulfur. The process of

pyrite oxidation coupled to denitrification received increasing attention in the recent two decades (Schippers and Jørgensen 2002; Zhang et al., 2009; Haaijer et al., 2007; Jørgensen et al., 2009; Torrentó et al., 2010; Bosch et al., 2012; Yan et al., 2015, 2019; Vaclavkova et al., 2015; Pang and Wang 2020). It has great impact on the removal of nitrate from the aquifers and sediments. However, it may cause pollution of soils and waters by trace heavy metals released from the pyrite at the same time. Therefore, the redox process is of great significance for environmental protection. The pathway is generally expressed as summarized in equations (1) and (2):



Anaerobic pyrite oxidation with nitrate as electron acceptor is thermodynamically possible at circumneutral pH albeit denitrification coupled to pyrite oxidation is controversially discussed in the literature. Field data provide clear evidence that there is denitrification linked to

* Corresponding author.

E-mail address: s.peiffer@uni-bayreuth.de (S. Peiffer).<https://doi.org/10.1016/j.apgeochem.2021.104995>

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the oxidation of reduced sulfur in pyrite-containing aquifers (Tesoriero et al., 2000; Zhang et al., 2009). However, laboratory experiments to link these observations to chemolithoautotrophic pyrite oxidation are contradictory. Incubation of natural sediment to which ground pyrite was added did not provide any evidence for denitrification coupled to pyrite oxidation (Schippers and Jørgensen 2002; Haaijer et al., 2007). In contrast, accelerated nitrate reduction and sulfate generation has been observed in incubation experiments with naturally pyrite-containing sediment from a sandy aquifer and accompanying batch experiments to which ground pyrite was added (Jørgensen et al., 2009). Nitrate reduction rates in the presence of the chemolithoautotrophic denitrifying bacterium *Thiobacillus 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. Moreover, Bosch et al. (2012) described the oxidation of pyrite nanoparticles coupled to reduction of nitrate to nitrite by a pure culture of *Thiobacillus denitrificans*. Recently, Pang and Wang (2020) demonstrated pyrite-based denitrification by an indigenous mixed culture with nitrous oxide (N₂O) as an intermediate product. Based on these studies, pyrite coupled to denitrification has been considered as a promising bioprocess for removal of the nitrate from groundwater or wastewater. Pyrite has been utilized as a low-cost electron donor in autotrophic denitrification for nitrate-contaminated groundwater remediation and nitrified domestic wastewater (Pu et al., 2014; Tong 2015).

However, our previous study revealed that acidic extraction of pyrite suspensions to quantify ferric hydroxide as the product of pyrite oxidation may lead to significant overestimation of ferric iron if nitrite is present, because these reactive N-species formed from nitrate reduction are able to oxidize pyrite under acidic conditions (Yan et al., 2015), leading to overestimation of denitrification-dependent pyrite oxidation. Moreover, it is important to note that nitrite and sulfate generated upon consumption of nitrate were observed and attributed to the oxidation of pyrite (Jørgensen et al., 2009; Torrentó et al., 2010; Bosch et al., 2012; Vaclavkova et al., 2015; Pang and Wang 2020) in previous laboratory studies in which no attempts were made to remove elemental sulfur during the preparation of pyrite. In contrast, no pyrite oxidation was observed in previous studies in which elemental sulfur was removed in the preparation of pyrite (Schippers and Jørgensen 2001; Haaijer et al., 2007; Yan et al., 2019). We therefore propose that the existing, contradictory observations may be related to inconsistent experimental protocols that allow for the presence or absence of reactive species, such as reduced sulfur species, iron species, or reactive N-species. These species may form from impurities present in natural or synthetic samples of pyrite or be generated as an intermediate during denitrification, eventually leading to several possibilities for interference. The objective of this review is to discuss potential geochemical and microbiological interferences that may occur in laboratory studies focusing on chemolithoautotrophic denitrification with pyrite, aiming to propose experimental and analytical standard protocols to overcome these interferences in future experiments and studies.

2. Geochemical challenges

2.1. Interference of alternative reduced sulfur species associated with pyrite

Natural pyrite is often found to be associated with other reduced sulfur species. Reduced sulfur compounds are well known as electron donors for denitrification by the chemolithoautotrophic denitrifying microorganism *Thiobacillus denitrificans* (Beller et al., 2006; Kelly and Wood 2000). Bacteria of the genus *Thiobacillus* are able to derive energy from the oxidation of reduced sulfur compounds (sulfide, elemental sulfur, thiosulfate) to sulfate. The presence of reduced inorganic sulfur

compounds such as elemental sulfur in natural pyrite or sediments may lead to consumption of nitrate accompanied by the generation of nitrite and sulfate which complicates the conclusions whether oxidation of pyrite or rather an oxidation of other reduced sulfur species takes place.

A previous study demonstrated that elemental sulfur could be utilized as an electron donor for denitrification by a chemolithoautotrophic denitrifying enrichment culture (Cardoso et al., 2006). As a result, nitrite accumulated and elemental sulfur was converted to sulfate. More recently, we illustrated that the S-oxidizing nitrate-reducing bacterium *Thiobacillus denitrificans* is able to oxidize elemental sulfur with nitrate to generate sulfate and nitrite under anoxic, pH-neutral conditions (Yan et al., 2019). Elemental sulfur associated with a typical impurity of the synthesized pyrite mineral (4.6 mass % of elemental sulfur) also served as an electron donor for reduction of nitrate. In contrast, pure ground crystalline pyrite (0.001 mass % of elemental sulfur), which was prepared with great care to remove elemental sulfur, could not be microbially oxidized with nitrate as electron acceptor in the presence of *Thiobacillus denitrificans* (Yan et al., 2019). Our observations implied that part of the denitrification observed in experiments with synthesized pyrite may have been due to microbial oxidation of the residual elemental sulfur. Microbial oxidation of pyrite with nitrate as electron acceptor was not possible if the pyrite source was pure crystalline pyrite that did not contain elemental sulfur contaminations.

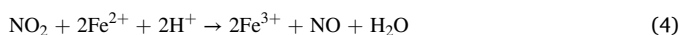
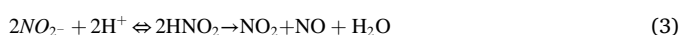
In order to rule out interference of reduced sulfur species with pyrite oxidation, it is clearly imperative that the reduced sulfur species associated with pyrite materials are removed from the material during the preparation of pyrite, e.g., using approaches which have been used in previous studies (Yan et al., 2015, 2019). Specifically, pyrite grains are washed with 1 M HCl to remove ferric iron (Fe(III)) which may have formed from oxidation of pyrite surfaces during crushing and residual acid-extractable sulfur species. The oxidation of pyrite by dissolved Fe (III) may take place in this process. However, the products such as dissolved sulfate and Fe(II) from pyrite oxidation will be washed by HCl. Thereafter, the material is washed with deaerated acetone or petrolether to remove elemental sulfur (Peiffer and Stubert 1999; Yan et al., 2019; Schippers and Jørgensen 2001; Haaijer et al., 2007). Nevertheless, a complete removal of elemental sulfur from synthetic or natural pyrite is difficult, necessitating the quantification of elemental sulfur in pyrite materials. Thus, in a previous study, we provide an analytical protocol for determining elemental sulfur content (Yan et al., 2015).

In summary, we suggest that quantitative differentiation between the sulfur components and their mineralogical characterization of initial pyrite mineral are key requirements in pyrite oxidation studies, both in field samples and in more pure systems in the laboratory. Moreover, pyrite or pyrite-containing material used in microbial experiments should be prepared carefully to exclude the interference of residual sulfur species. This can be done by removal of and quantification of such sulfur species.

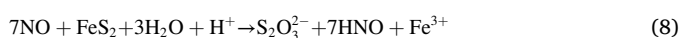
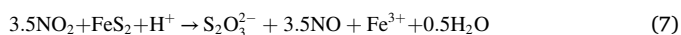
2.2. Interference of nitrite and dissolved Fe(III) with quantitative spectrophotometric determination of Fe(II) and Fe(III)

The first reaction product of microbial denitrification, stemming from the reduction of nitrate, is nitrite (Albina et al., 2019; Bi et al., 2020). In cultures of chemolithotrophic denitrifying bacteria with inorganic sulfur compounds coupled to nitrate reduction, nitrite appeared to be formed as an important intermediate nitrogen compound (Cardoso et al., 2006; Haaijer et al., 2007). Nitrite was also found to be present as intermediate formed during microbial nitrate reduction coupled to Fe(II) oxidation (Picardal 2012; Klueglein and Kappler 2013). Recently, laboratory studies presented evidence on the accumulation of nitrite during chemolithoautotrophic denitrification coupled to pyrite oxidation in the presence of *Thiobacillus denitrificans* (Torrentó et al., 2010, 2011; Bosch et al., 2012), which is the most prominent obligate chemolithoautotrophic model organism for conserving energy from the oxidation of inorganic sulfur compounds coupled to denitrification

(Beller et al., 2006; Kelly and Wood 2000). Pyrite oxidation is typically quantified by acidic extraction and quantification of Fe(II) and Fe(HCl)_{tot} (total HCl-extractable Fe), which is assumed to have formed upon pyrite oxidation under circumneutral conditions (Bosch et al., 2012). Using the standard ferrozine/phenanthroline assay (Stookey 1970; Tamura et al., 1974), nitrite-containing pyrite samples from microbial experiments are often acidified with 1 M HCl at room temperature for stabilization of Fe(II) and extraction of Fe(HCl)_{tot} before measurement (Porsch and Kappler 2011). However, previous studies have determined the abiotic oxidation of Fe(II) to Fe(III) with nitrite at acidic or circumneutral pH (Klueglein and Kappler 2013; Klueglein et al., 2014; Jamieson et al., 2018). Nitrite is protonated to nitrous acid (HNO₂) which spontaneously decomposes to nitrogen dioxide (NO₂) and nitric oxide (NO). Both reactive N-species are able to abiotically oxidize Fe(II) according to equations (3)–(6) (Nelson and Bremner 1970; Van Cleemput and Samater 1995; Klueglein and Kappler 2013).

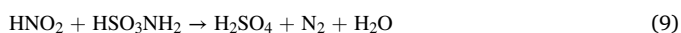


In a previous paper, we provided clear evidence that pyrite is abiotically oxidized by reactive NO and NO₂ formed from decomposition of HNO₂ at pH 0 under anoxic conditions (equations (7) and (8)) (Yan et al., 2015). The presence of nitrite in pyrite samples can lead to an overestimation of Fe(III) production during acidic extraction and thus generate the risk of producing artifacts and data misinterpretations.



In order to quantify Fe(II)/Fe(III) values accurately in nitrite-containing pyrite samples from experiments investigating chemolithoautotrophic denitrification coupled to pyrite oxidation, there are two methods to remove or stabilize the nitrite in nitrite-containing pyrite samples. The first method is to remove nitrite by washing the nitrite-containing pyrite samples with nitrite-free water prior to the acidic extraction during a revised protocol. This washing process should be performed several times until no nitrite could be detected by nitrite indicator strips with a range of 0.05–25 mg/L (Yan et al., 2015). The samples from experiments of nitrate-dependent chemolithotrophic pyrite oxidation for Fe measurement should first be filtered or centrifuged to remove the nitrite from the solid phase before an acidic extraction. The residue on the filter paper or the pellet after centrifugation should be washed several times with ultrapure water to remove dissolved/-bound nitrite and then be extracted with 1 M HCl to dissolve Fe(III) (oxyhydr)oxides and quantify Fe(II) and Fe(HCl)_{tot}. For the calculation of Fe(III) concentrations, the concentration of Fe(II) is subtracted from Fe(HCl)_{tot} concentration.

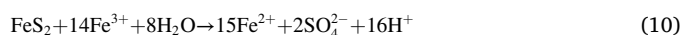
Alternatively, sulfamic acid (HSO₃NH₂) is a moderately strong acid (pK_a = 1.3) which is able to react rapidly with nitrite to form N₂ and sulfuric acid (equation (9)) (Marouf-Khelifa et al., 2006; Granger and Sigman 2009):



Application of sulfamic acid (pH approximately 1.7) instead of HCl as extracting agent has been proven to be an effective method to remove nitrite without oxidizing dissolved Fe(II) in nitrite-containing samples (Klueglein and Kappler 2013). However, the nitrite concentrations and pH of the samples are two important factors for the removal of nitrite with sulfamic acid. Sulfamic acid should be added in relative excess to nitrite and the pH of the reaction should be kept at or below the pK_a of sulfamic acid (pK_a = 1.3). A pH higher than 3 should be avoided to

prevent the formation of reactive NO and NO₂ (equation (3)) (Granger and Sigman 2009). Low pH conditions are also necessary for efficient Fe extraction. For these reasons, the protocol whereby sulfamic acid is used to remove nitrite has been developed. A further study provided a revised Fe extraction protocol to use a combination of 40 mM sulfamic acid with 1 M HCl in carbonate-enriched samples which allows to maintain low pH conditions for an efficient Fe extraction and preserve the capability of sulfamic acid to remove nitrite from the sample (Schaedler et al., 2018). Therefore, it is assumed that, for studies of chemolithoautotrophic denitrification with pyrite, nitrite-containing pyrite samples should be extracted using a combination of sulfamic acid with 1 M HCl as another approach to remove nitrite but avoid its abiotic oxidation of pyrite during acidic extraction.

Fe(III) (oxyhydr)oxides is generated in the reactions between pyrite and nitrate in the presence of bacteria. Moreover, dissolved Fe(III) is an efficient oxidant for pyrite during acidic extraction (equation (10)) (Peiffer and Stubert 1999; Chen et al., 2014).



The reaction takes place very rapidly and continues until ferric iron is depleted when pyrite is in excess. Therefore, we suggest that the generated Fe(III) from pyrite oxidation by microbial denitrification could be calculated by measured concentration of dissolved Fe(II) via Eq. (10).

2.3. Interference of oxygen

When oxygen and water are available, pyrite can be oxidized by microorganisms (Simate and Ndlovu 2014; Kefeni et al., 2017), which leads to the formation of acid mine drainage. The results of experiments studying chemolithoautotrophic denitrification coupled to pyrite oxidation can be significantly affected when oxygen is present. Two studies reported that the measured ratio of S/N was higher than the theoretical ratio (Pu et al., 2014; Pang and Wang 2020). This can be explained by the chemical oxidation of pyrite by small amounts of oxygen introduced during preparation of solutions, sampling or measuring procedures. Moreover, the presence of oxygen clearly accelerated chemical oxidation of pyrite by nitrite during acidic extraction of nitrite-containing pyrite samples, leading to overestimation of Fe(III) production (Yan et al., 2015). Therefore, it is necessary to avoid the interference of oxygen during the batch experiment, sampling, and measuring procedures.

3. Microbiological challenges

3.1. Interference of remaining iron and sulfur compounds in the reaction medium

Varying concentrations of thiosulfate, sulfate, and Fe(II) can be present in the cultivation medium for the pre-growth of chemolithoautotrophic denitrifying bacterial strains, which are probably not completely consumed when the culture is used following batch experiments. The problem is that the residual thiosulfate, sulfate and iron (some of which are potentially even stored within the cells; see following paragraph) could interfere with nitrate reduction and sulfate production in the following batch experiment and provide false positive results.

In previous studies, *Thiobacillus denitrificans* was cultured with thiosulfate in an anoxic (pH 6.8) nutrient medium as recommended by the German Collection of Microorganisms and Cell Culture (DSMZ) (Yan et al., 2019; Vaclavkova et al., 2015; Torrentó et al., 2010). The medium consisted of 14.7 mM KH₂PO₄, 19.8 mM KNO₃, 18.7 mM NH₄Cl, 3.25 mM MgSO₄•7H₂O, 20.1 mM Na₂S₂O₃•5H₂O, 30.0 mM NaHCO₃, 0.007 mM FeSO₄•7H₂O, and trace element solution SL-4. If the medium is directly used in batch experiments, it will cause an interference of sulfur and iron sources and it cannot be easily determined whether pyrite is

responsible for chemolithoautotrophic denitrification or other sulfur compounds.

In order to exclude interferences of sulfur and iron from the previous incubation medium in the experiments, cells of the pre-culture (the inoculum for the following experiment) after growth to the late exponential phase should be centrifugated, washed and resuspended in modified medium without sulfur and iron species several times before the start of the experiments to avoid interference of sulfur and iron from the medium in the determination of formation rates of sulfate from pyrite. When the modified medium is used to wash the pre-culture cells and in the batch experiments, it should be adjusted without thiosulfate, and iron, using chloride salts instead of sulfate salts (Torrentó et al., 2010; Vaclavkova et al., 2015; Yan et al., 2019). As an example, in our previous microbial experiments, a modified reaction medium (pH 6.8) in the absence of thiosulfate contained 15 mM KH_2PO_4 , 19 mM NH_4Cl , 3.2 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ instead of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 30 mM NaHCO_3 and the same concentration of trace element solution SL-4 (Yan et al., 2019).

3.2. Interference of cell associated and stored sulfur

A previous study demonstrated that *Thiobacillus denitrificans* could be grown in a medium which contains thiosulfate as the electron donor under anoxic conditions (Schedel and Trüper 1980). When thiosulfate was present, elemental sulfur accumulated transiently within the cells. However, when thiosulfate was completely consumed, intracellular elemental sulfur appeared to be rapidly oxidized to sulfate (Schedel and Trüper 1980). Therefore, during the pre-growth phase of chemolithoautotrophic denitrifying bacterial strains, sulfur is probably stored within the cells or attached to the cells and can act as electron donor. Thus, the stored sulfur could interfere with the nitrate reduction and sulfate production in the batch experiment and provide false positive results.

In previous microbial experiments with pyrite and nitrate, the measured concentration of sulfate was stoichiometrically more than expected, corresponding to the observed nitrate reduction (Yan et al., 2019; Jørgensen et al., 2009; Pang and Wang, 2020; Bosch et al., 2012; Pu et al., 2014). Furthermore, a control experiment containing only nitrate and a cell suspension of *Thiobacillus denitrificans* without pyrite led to consumption of nitrate accompanied by the formation of sulfate and nitrite (Yan et al., 2019). This data confirmed the observation of the previous study (Schedel and Trüper 1980) that cells of the pre-cultures used for inoculation grown in the thiosulfate containing medium lead to accumulation of sulfur attached to cells that was chemolithoautotrophically oxidized with nitrate during the experiments.

One solution would be to pre-incubate the cells with an electron acceptor, without addition of an electron donor, to deplete the cells from the stored electron donor. Alternatively, batch and control experiments for studying chemolithoautotrophic denitrification with pyrite should be set up with an appropriate cell density. When the cell density is too high, the generation of sulfate and reduction of nitrate due to stored sulfur may be dominant so that the reaction products upon pyrite oxidation may be neglected. When the cell density is too low, it may not be able to provide enough active cells to trigger the reaction. A control experiment with nitrate in the absence of pyrite and the same cell density of bacteria as in the batch experiment is required. The contribution of reaction products due to denitrification fueled by stored sulfur should be subtracted from the total contribution in batch experiments. A positive control experiment with a well-known sulfur compound as the electron donor (e.g., elemental sulfur, thiosulfate) should be set up to test the viability of the cell cultures at this given cell density.

4. A standardized protocol for studying chemolithoautotrophic denitrification with pyrite

The large numbers of possible artifacts might provide some explanations why previous observations are contradictory. We highlighted

possible pitfalls and are presenting a revised protocol avoiding or at least minimizing the impact of geochemical and microbiological pitfalls in studying chemolithoautotrophic denitrification with pyrite (Table 1). Laboratory studies on denitrification coupled to pyrite oxidation generally consists of four procedures: pyrite preparation and characterization, cultivation of microorganisms, pyrite oxidation experiment, and chemical analysis (Fig. 1).

4.1. Pyrite preparation and characterization

Of particular importance is that the material should be pure pyrite, which is carefully prepared prior to the experiments to remove impurities. To exclude ferric iron, FeS, ferrous sulfate minerals, or marcasite (a polymorph of FeS_2) that may have formed from oxidation of pyrite surfaces during milling as well as residual acid-extractable iron and

Table 1

Overview of potential interferences and appropriate protocols for studies on chemolithotrophic denitrification coupled to pyrite oxidation by denitrifying strains.

	Problem	Potential interference	Appropriate protocol
Geochemical interference	Reduced sulfur species associated with pyrite	Overestimation of sulfate production and nitrate reduction	(1) Quantitative differentiation between the sulfur components (2) Removal all potential reduced sulfur species besides pyrite
	NO_2^- oxidizing pyrite during acidic extraction	Overestimation of Fe(III) production	(1) Nitrite-containing pyrite samples should be filtered or centrifuged and then washed with nitrite-free water before the acidic extraction to remove nitrite (2) Acidic extraction of nitrite-containing pyrite samples in a combination of sulfamic acid with 1 M HCl
	Dissolved Fe (III) oxidizing pyrite during acidic extraction	Underestimation of Fe(III) production	Quantification of Fe (III) via stoichiometrical calculation with measured concentration of dissolved Fe(II)
	Oxygen	Overestimation of Fe(III) and sulfate production	Preparation of solutions, sampling and chemical analysis of Fe measurement under anoxic conditions
Microbiological interference	Iron and sulfur compounds in the reaction medium Stored sulfur within the cells or on the outside of its cells but attached to the cells	Overestimation of sulfate production and nitrate reduction Overestimation of sulfate production and nitrate reduction	Modified reaction medium without thiosulfate, sulfate and iron (1) Depletion of cells (preincubation) or set up of an appropriate cell density (2) Quantification of Fe(II) and sulfate in the control experiment

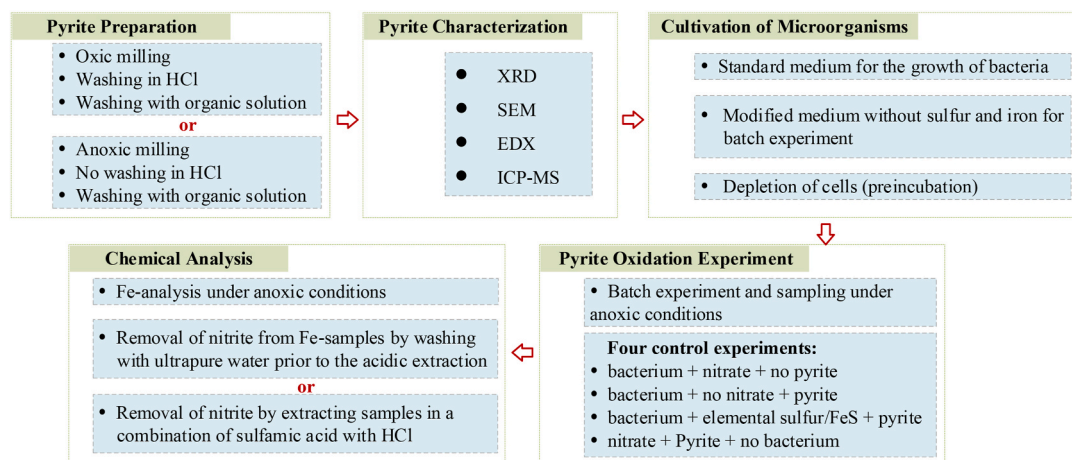


Fig. 1. A standardized protocol for studying chemolithoautotrophic denitrification with pyrite. XRD = X-ray diffractometry, SEM=Scanning Electron Microscopy, EDX = Energy Dispersive X-Ray Spectroscopy, ICP-MS = inductively coupled plasma atomic emission spectrometry.

sulfur species, we suggest that the pyrite grains should be washed in HCl. In a particular case, washing in HCl can be omitted to preserve the nanoparticulate fraction on the pyrite surface (Bosch et al., 2012). However, an anoxic milling procedure to avoid the oxidation of pyrite is required. To remove elemental sulfur, the pyrite grains need to be washed with a deaerated organic solution (e.g., cyclohexane) until the residual fraction of elemental sulfur in pyrite is neglectable. After the preparation, the pyrite is characterized by X-ray diffractometry (XRD) for structural analysis of chemical compounds, and by Scanning Electron Microscopy (SEM) to obtain information about the surface topography. The elemental composition of the sample can also be analyzed by Energy Dispersive X-Ray Spectroscopy (EDX). In addition, an inductively coupled plasma atomic emission spectrometry (ICP-MS) can be used to search for contaminating trace elements. All these methods are used to identify whether the material is pure pyrite.

4.2. Cultivation of microorganisms

Standard media should be used to ensure the growth of bacteria. In order to exclude interference of sulfur and iron in the main pyrite oxidation experiment, the culture need to be washed by modified medium prior to the main experiment. The modified medium that is used to wash the pre-culture and in batch experiments should be prepared without sulfur and iron.

4.3. Pyrite oxidation experiment

Batch experiments should be performed under anoxic conditions to avoid the interference of pyrite with oxygen: The modified medium and pyrite are added into each autoclaved glass serum bottle inside an anoxic glovebox (100% N₂). Bottles are sealed with butyl stoppers, crimped, and then removed from the glovebox. The headspace of each serum bottle is flushed with CO₂/N₂ (20/80%). At the beginning of each batch experiment with pyrite, anoxic nitrate stock solution is injected into each serum bottle through the butyl stopper using a syringe that have been flushed several times with N₂.

It should be noted that four control experiments are necessary. The first abiotic control experiment should be setting up with nitrate and pyrite but no bacteria. The background concentrations of reaction products from the control experiment should be subtracted from the concentrations in the microbial experiment with pyrite. Furthermore, the medium for the cultivation of bacterium may contain sulfur (e.g., thiosulfate). During the incubation, sulfur accumulates intracellularly and cannot be washed out. The second control experiment needs to be set up with bacterium and nitrate in the absence of pyrite. The

background concentration of nitrite or N₂O produced from denitrification by reaction with intracellularly accumulated stored sulfur should be subtracted from the total concentration of nitrite or N₂O produced in the experiment with pyrite. The third control experiment should be set up with bacteria and pyrite in the absence of nitrite to monitor the background values of Fe(II) and sulfate due to the dissolution of nanometer-sized pyrite particles. In addition, a series of cell densities are suggested to be investigated in the pyrite experiment to figure out an appropriate cell density, which is high enough so that the reaction is triggered, but also not so high that the generated products dominate due to stored sulfur, causing the reaction products upon pyrite oxidation to be neglected. The fourth control experiment need to be done with a verified electron donor like elemental sulfur, thiosulfate or FeS, to prove the viability of the cell suspension at the given cell density in modified medium.

4.4. Chemical analysis

For analysis under anoxic conditions, samples for Fe measurement are withdrawn, incubated, and reacted with the ferrozine reagent inside of an anoxic glovebox (100% N₂) and are exposed to air for only approximately 5 min during absorbance measurement.

Due to the interference of nitrite with quantitative spectrophotometric determination of Fe(II) and Fe(III), the samples for Fe measurement should first be filtered or centrifuged from the solid phase before an acidic extraction. The residue on the filter paper or the pellet after centrifugation should be washed several times with ultrapure water to remove dissolved/bound nitrite and then be extracted with HCl to dissolve Fe(III) (oxyhydr)oxides and quantify Fe(II)/Fe(III). Alternatively, nitrite-containing pyrite samples for studies of pyrite-based denitrification can be extracted in a combination of sulfamic acid with 1 M HCl.

4.5. Implications for studies of chemolithotrophic denitrification with pyrite

In order to verify whether the pyrite-based denitrification occurs, most of the previous studies have measured the concentration changes of reaction products during the reaction process. However, the results of these studies may be affected by the reduced sulfur replacing pyrite as electron donor for microbial nitrate reduction. Future studies should pay special attention to reduced sulfur species that can be impurities associated with pyrite or from other sources such as cell-associated and stored sulfur.

Chemolithoautotrophic denitrification with pyrite is a complex

redox process, which includes not only sulfur oxidation, but also iron oxidation. The quantification of Fe(III) (oxyhydr)oxides which is formed upon oxidation of pyrite is therefore assumed to be a strong evidence for the microbial pyrite oxidation. However, the previous studies did not consider the interference of nitrite and dissolved Fe(III) with quantitative spectrophotometric determination of Fe(II) and Fe(III), which could lead to an overestimation of Fe(III) production during acidic extraction. In order to quantify Fe(II)/Fe(III) values accurately in nitrite-containing pyrite samples, removing or stabilizing the nitrite is necessary. Moreover, the abiotic pyrite oxidation by dissolved Fe(III) during acidic extraction can neither be ignored.

The present review presents potential geochemical and microbiological interferences that may occur in experimental studies on chemolithoautotrophic denitrification with pyrite. Only when these interferences are ruled out can man verify whether denitrification coupled to pyrite oxidation exists.

5. Conclusions

The potential geochemical and microbiological interferences for microbial oxidation of pyrite presented from this study including (1) impurities of reduced sulfur species associated with pyrite, (2) nitrite and dissolved Fe(III) with quantitative spectrophotometric determination of Fe(II) and Fe(III) during acidic extractions, (3) oxygen, (4) residual iron and sulfur compounds in the reaction medium, and (5) cell-associated and stored sulfur.

Therefore, we propose the following experimental standard protocols:

1. The pyrite minerals should be pure and needs to be carefully prepared prior to the experiments to remove impurities such as ferric iron, FeS, ferrous sulfate minerals, marcasite and elemental sulfur. A comprehensive mineralogical characterization of initial pyrite minerals by XRD, SEM, EDX and ICP-MS are recommended.
2. The samples for Fe measurement should first be centrifuged and the upper solution needs to be removed. The pellet after centrifugation should be washed with ultrapure water to remove nitrite and then be extracted with HCl to dissolve Fe(III) (oxyhydr)oxides and quantify Fe(II)/Fe(HCl)_{tot}. Based on Fe stoichiometry, the generated Fe(III) from microbial pyrite oxidation by nitrate could be calculated by measured concentration of dissolved Fe(II).
3. The cultivation of microorganisms, the pyrite oxidation experiment, the sampling and chemical analysis should be performed anoxically to avoid the interference of oxygen.
4. The modified medium without sulfur and iron species are recommended to wash the pre-culture and to use in batch experiments, in order to obtain accurate formation rates of products from pyrite oxidation and nitrate reduction.
5. Sulfur accumulated intracellularly during the incubation cannot be washed out by modified medium. One solution would be pre-incubating the cells with an electron acceptor, without addition of an electron donor, to deplete the cells from the stored electron donor. Alternatively, A control experiment should be set up with bacterium and nitrate in the absence of pyrite."

Author contributions

RY and SP contributed to all aspects of this work. RY wrote the main manuscript text. SP, AK and MH gave some useful comments and suggestions to this work. All authors reviewed the manuscript.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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