Effects of reduced sulfur speciation and nitrite on the chemolithoautotrophic pyrite oxidation with nitrate

-implications for studies of chemolithoautotrophic denitrification

Dissertation

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List of Abbreviations

Nitrate	NO_3^-
Nitrite	NO_2^-
Oxygen	O_2
Manganese oxide	MnO_2
Pyrite	FeS_2
Ferric iron	Fe(III) or Fe^{3+}
Ferrous iron	Fe(II) or Fe^{2+}
Total HCl-extractable Fe	$Fe(HCl)_{tot}$
Total iron	Fe(tot)
Arsenic	As
Nickel	Ni
Cobalt	Co
Zinc	Zn
Sulfate	SO_4^{2-}
Elemental sulfur	S(0)
Sulfur	S
Thiosulfate	$S_2O_3^{2-}$
Heavy oxygen isotope	^{15}N
Heavy oxygen isotope	^{18}O
Nitrous acid	HNO_2
Nitrogen dioxide	NO_2
Nitric oxide	NO
Dinitrogen	N_2
Carbon dioxide	CO_2
Sulfur	S
High performance liquid chromatography	HPLC
Optical density	OD
Ion chromatography	IC
X-ray diffractometry	XRD
Inductively coupled plasma optical emission spectrometry	ICP-OES
Scanning electron microscopy	SEM
Energy-dispersive X-ray spectroscopy	EDX
Hour	h
Day	d

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Summary

Pyrite (FeS_2) is a major iron- and sulfur-containing mineral phase in earth's crust. It plays an important role in the global biogeochemical cycles of iron and sulfur. Nitrate (NO_3^-) is a common inorganic pollutant in shallow groundwater aquifers, drinking water wells and streams, and is strongly linked to agricultural fertilizers or manure. The interaction between pyrite and nitrate under anaerobic conditions is of great importance in many pyrite-bearing anoxic aquifers. Even though the natural occurrence of this process has been proved based on geochemical and stable isotope field data, the results of laboratory studies are partly contradictory. Some of these studies indicated that a microbial oxidation of pyrite occurred, whereas the results of other studies with pyrite as the electron donor and nitrate as the electron acceptor indicated the contrary. Hence, the mechanism of this process is still unclear. The objectives of this dissertation are (1) to further analyze the mechanism of denitrification coupled to pyrite oxidation, and (2) to identify potential geochemical and microbiological interferences related to species which may form from impurities in natural or synthetic pyrite, or be generated as an intermediate of the denitrification process.

The first study investigates the interference with Fe measurement of nitrite-containing pyrite suspensions during acidic extraction by nitrite as an intermediate of nitrate reduction. The results demonstrate a significant oxidation of pyrite by nitrite in 1 M HCl under anoxic conditions, and imply a cyclic model for pyrite oxidation by Fe(III) based on oxidation of Fe(II) by reactive N-species NO and/or NO_2. The interference by nitrite should be considered in future studies on microbially mediated pyrite oxidation with nitrate. In this sense, a revised protocol on the removal of nitrite from the pyrite suspensions through a washing procedure prior to acidic extraction is provided. The results also demonstrate that the abiotic oxidation of pyrite by nitrite under acidic conditions is strongly affected by dissolved oxygen. An explanation is that NO can be oxidized to NO_2 by dissolved oxygen, NO_2 being a stronger oxidant than NO for the oxidation of Fe(II) under acidic conditions. No oxidation of pyrite

was detected at pH 5.5 and 6.8. Hence, abiotic oxidation of pyrite by nitrite seems not to be a possible pathway in anoxic circumneutral groundwater aquifers.

The second study investigates the anaerobic, nitrate-dependent oxidation of two pyrites by *Thiobacillus denitrificans*: ground crystalline pyrite (high purity, high crystallinity, low BET surface area) and synthesized pyrite (a mixture of pyrite, marcasite and elemental sulfur, low crystallinity, high BET surface area). Pure ground crystalline pyrite could not be oxidized microbially with nitrate as an electron acceptor. In contrast, the results of mass balance calculation suggest that chemoautotrophic oxidation of synthesized pyrite species of low crystallinity is possible. This study also deals with the effects of the nitrate-dependent, sulfur-oxidizing, and iron-oxidizing bacterium *Thiobacillus denitrificans*, and the nitrate-dependent, iron-oxidizing bacterium *Acidovorax sp.* BoFeN1 as a catalyst for the microbial reaction between pyrite and nitrate. Consumption of nitrate accompanied by the formation of sulfate and nitrite was observed in the presence of sulfur-oxidizing strain *Thiobacillus denitrificans*, whereas no reaction was detected in the experiments with iron-oxidizing bacterium *Acidovorax sp.* BoFeN1. Iron-oxidizing nitrate-reducing strain *Acidovorax sp.* BoFeN1 did not stimulate pyrite-dependent nitrate reduction, and the addition of Fe(II) and Fe(III) to the reaction even slightly decreased the rates of nitrate reduction and sulfate generation.

The third study exposes the possible geochemical and microbiological interferences in previous studies on the chemolithoaoutotrophic pyrite with nitrate. Key interferences include i) impurities of reduced sulfur species associated with pyrite, ii) formation of nitrite and its interference during acidic extractions, and iii) occurrence of residual iron and sulfur compounds in the reaction medium. Experimental standard protocols are provided to overcome these interferences in future studies on chemolithoautotrophic denitrification with pyrite.

In summary, three key findings of this dissertation are:

1) Nitrite can abiotically oxidize pyrite under acidic conditions. The interference by nitrite, which formed as an major intermediate of nitrate reduction, may lead to overestimation of pyrite oxidation by denitrifying bacteria.

2) Reduced sulfur species play an important role in chemolithoautotrophic pyrite oxidation with nitrate. The microbial interaction between pyrite and nitrate appears to be stimulated via S oxidation but not via Fe oxidation. These results call for the characterization of different sulfur components and the investigation whether only pyrite is microbially oxidized or some other sulfur minerals such as elemental sulfur or marcasite.

3) Geochemical and microbiological interferences may cause biased results of anaerobic nitrate-dependent pyrite oxidation.

Zusammenfassung

Pyrit (FeS_2) ist ein in der Erdkruste sehr häufig auftretendes, eisen- und schwefelhaltiges Mineral, welches eine wichtige Rolle in den globalen biogeochemischen Zyklen von Eisen und Schwefel spielt. Hauptsächlich beeinflusst von landwirtschaftlichen Düngemittel ist zunehmend Nitrat als anorganischer Schadstoff in oberflächlichen Grundwasserleitern, Trinkwasserbrunnen und Fließgewässern enthalten. Die Wechselwirkung zwischen Pyrit und Nitrat unter anaeroben Bedingungen ist in vielen pyrithaltigen anoxischen Grundwasserleitern von großer Bedeutung. Obwohl das Auftreten dieses Wechselwirkungsprozesses durch geochemische und stabile isotope Felddaten nachgewiesen ist, sind die Ergebnisse aus Laboruntersuchungen in einer Reihe von Studien teilweise widersprüchlich. In einigen der Studien konnte gezeigt werden, dass eine mikrobielle Oxidation von Pyrit auftrat. Wohingegen in andere Studien keine mikrobielle Pyritoxidation in Versuchen mit Pyrit als Elektronendonor und Nitrat als Elektronenakzeptor beobachtet werden konnte. Der genaue Mechanismus bei der Wechselwirkung von Pyrit und Nitrat ist somit noch unklar und bedarf weiterer Forschung.

Ziel dieser Arbeit ist es, den Mechanismus der Denitrifikation verbunden mit der Pyrit-Oxidation näher zu untersuchen sowie potenzielle geochemische und mikrobiologische Interferenzen im Zusammenhang mit Spezies, die sich aus Verunreinigungen von natürlichem oder synthetischem Pyrit ergeben oder als Zwischenprodukte des Denitrifikationsprozesses erzeugt werden, zu identifizieren.

In der ersten Studie wurde die Interferenz von Nitrit als Zwischenprodukt der Nitratreduktion auf die Fe-Messung von nitrithaltigen Pyrit-Suspensionen bei der sauren Extraktion betrachtet. Die Ergebnisse zeigten eine signifikante Oxidation von Pyrit durch Nitrit in 1 M HCl unter anoxischen Bedingungen und deuten auf ein zyklisches Modell der Pyritoxidation durch Fe(III) basierend auf der Oxidation von Fe(II) durch reaktive N-Spezies NO und/oder NO_2 hin. Die Interferenz von Nitrit sollte in zukünftigen Studien zur mikrobiellen Pyritoxidation mit

Nitrat in Betracht gezogen werden. Aus diesem Grund wurde ein modifiziertes Protokoll zur Entfernung von Nitrit aus Pyrit-Suspensionen durch einen vorherigen Waschvorgang vor der eigentlichen Säure-Extraktion erstellt. Die Ergebnisse zeigten, dass die abiotische Oxidation von Pyrit durch Nitrit unter sauren Bedingungen stark vom gelösten Sauerstoff beeinflusst wird. Eine Erklärung hierfür ergibt sich aus der Oxidation von NO durch gelösten Sauerstoff zu NO_2, welches als ein stärkeres Oxidationsmittel für die Oxidation von Fe(II) unter sauren Bedingungen dient. Bei pH-Werten von 5,5 und 6,8 konnte keine Oxidation von Pyrit beobachtet werden. Deshalb ist es nicht möglich, Pyrit durch Nitrit in anoxischen Grundwasserleitern mit nahezu neutralen pH Werten abiotisch zu oxidieren.

In der zweiten Studie wurde die anaerobe, nitratabhängige Oxidation von zwei Pyriten durch *Thiobacillus denitrificans* untersucht. Bei den Pyriten handelt es sich um gemahlenen kristallinen Pyrit (hohe Reinheit, hohe Kristallinität, niedrige BET-Oberfläche) und synthetischen Pyrit (eine Mischung aus Pyrit, Markasit und elementarem Schwefel; niedrige Kristallinität, hohe BET-Oberfläche). Rein gemahlener kristalliner Pyrit konnte nicht durch Nitrat als ein Elektronenakzeptor mikrobiell oxidiert werden. Im Gegensatz dazu deutet die Massenbilanz für den synthetischen Pyrit darauf hin, dass eine chemoautotrophe Oxidation von synthetischen Pyrit mit geringerer Kristallinität durch Nitrat möglich ist. Außerdem befasste sich diese Studie mit den Wirkungen des nitratabhängigen S-oxidierenden und Fe-oxidierenden Bakteriums *Thiobacillus denitrificans* sowie des nitratabhängigen Fe-oxidierenden Bakteriums *Acidovorax sp.* BoFeN1 als Katalysator für die mikrobielle Reaktion zwischen Pyrit und Nitrat. Die mit der Reduktion von Nitrat einhergehende Bildung von Sulfat und Nitrit konnte bei Experimenten mit dem Bakterium *Thiobacillus denitrificans* beobachtet werden, während bei Experimenten mit dem Bakterium *Acidovorax sp.* BoFeN1 keine Reaktion festgestellt werden konnte. Das Fe-oxidierende nitratreduzierende Bakterium *Acidovorax sp.* BoFeN1 stimulierte nicht die Pyrit-abhängige Nitratreduktion. Die Zugabe

von Fe(II) sowie Fe(III) zur Reaktion verringerte sogar die Rate der Nitratreduktion und der Sulfatbildung geringfügig.

Die dritte Studie befasste sich mit potenziellen geochemischen und mikrobiologischen Interferenzen in früheren Studien anderer Autoren von chemolithoautotrophen Pyrit mit Nitrat zusammengefasst. Wichtige Interferenzen sind i) Verunreinigungen von reduzierten Schwefelspezies, die mit Pyrit assoziiert sind, ii) die Bildung von Nitrit und deren Interferenz bei sauren Extraktionen sowie iii) die Anwesenheit von noch verbleibenden Eisen- und Schwefel-Bestandteilen im Reaktionsmedium. Experimentelle Standardprotokolle wurden zur Verfügung gestellt, um diese Interferenzen in zukünftigen Studien der chemolithoautotrophen Denitrifikation mit Pyrit zu vermeiden.

Drei wichtige Befunde lassen sich in dieser Dissertation zusammenfassen:

1) Pyrit kann durch Nitrit unter sauren Bedingungen abiotisch oxidiert werden. Die Interferenz durch Nitrit, die sich als Hauptzwischenprodukt der Reduktion von Nitrat bildete, kann zu einer Überbewertung der Pyritoxidation durch denitrifizierende Bakterien führen.

2) Reduzierte Schwefelspezies spielen bei der chemolithoautotrophen Pyritoxidation durch Nitrat eine wichtige Rolle. Die mikrobielle Wechselwirkung zwischen Pyrit und Nitrat scheint durch S-Oxidation, aber nicht durch Fe-Oxidation stimuliert zu werden. Diese Ergebnisse zeigten, dass die Charakterisierung von verschiedenen Schwefelbestandteilen sowie die Untersuchung noch zu ergänzen ist, ob nur Pyrit mikrobiell oxidiert wird oder andere Schwefelmineralien wie elementarer Schwefel und Markasit auch oxidiert werden.

3) Geochemische und mikrobiologische Interferenzen könnten abweichende Ergebnisse einer anaeroben Nitrat-abhängigen Oxidation von Pyrit verursachen.

1. General introduction

1.1 Introduction

1.1.1 Denitrification coupled to pyrite oxidation in natural systems

Denitrification

Nitrate is a common inorganic pollutant in shallow groundwater aquifers due to agricultural fertilizers or manure (Postma et al. 1991; Korom 1992; Devlin et al. 2000; Darbi et al. 2003; Strebel et al. 1989). In these catchments, the concentration of nitrate usually exceed the EU drinking water guideline of 50 mg/l and therefore threaten the supply of drinking water (Hiscock et al. 1991). Effective removal of nitrate from groundwater occurs primarily through denitrification which is microbially mediated reduction of nitrate to the gaseous products N_2O or N_2 (Korom 1992).

Various organic or inorganic electron donors (e.g iron sulfides) drive heterotrophic or autotrophic denitrification in the environment, respectively (Korom 1992). In comparison, heterotrophic denitrification which use organic carbon as electron donor is thermodynamically preferred to autotrophic denitrification which use inorganic compounds as electron donors (Korom 1992). Numerous laboratory and field studies have been focused on the occurrence of heterotrophic denitrification with organic electron donors (Bragan et al. 1997; Bradley et al. 1992; Laverman et al. 2007; Korom 1992; Trudell et al. 1986; Cey et al. 1999; Mengis et al. 1999; Hill et al. 2000; Vidon and Hill 2005). The reaction theoretically follows the equation below:

$$5CH_2O + 4NO_3^- \Leftrightarrow 2N_2 + 4HCO_3^- + CO_2 + 3H_2O \quad (1)$$

However, even denitrification by autotrophic bacteria using inorganic compounds as electron donors is less well-known. Clear indication for denitrification was detected in several groundwater systems in the absence of organic carbon. The denitrification observed can only be attributed to autotrophic denitrification coupled to the oxidation of mineral containing

inorganic electron donors (Pauwels et al. 2000; Böttcher et al. 1990; Beller et al. 2004; Knöller et al. 2005). Furthermore, even with the presence of organic carbon, in several field studies, autotrophic denitrification (inorganic electron donors) has been found to be dominant (Postma et al. 1991; Broers 1998; Pauwels et al. 1998; Tesoriero et al. 2000; Prommer and Stuyfzand 2005). Therefore, minerals containing reduced sulfur species e.g pyrite have been suggested as electron donors for denitrification in natural aquifers.

Pyrite oxidation

Pyrite (FeS_2) is a major iron- and sulfur-bearing mineral in earth's crust. It plays an important role in the global biogeochemical cycles of iron and sulfur (Howarth 1979; Berner and Petsch 1998). By comparison to other iron monosulfide such as mackinawite, because of its highly crystalline structure, naturally formed pyrite as an iron disulfide is extremely stable against acidic dissolution.

The oxidation of pyrite is generally considered to be a complicated process. Several potential electron acceptors such as oxygen (O_2), ferric iron (Fe(III)), manganese oxide (MnO_2) and nitrate (NO_3^-) have been investigated in previous studies (Schippers and Jørgensen 2001; Lowson 1982; Moses et al. 1987; Kölle et al. 1983). In recent decades, pyrite oxidation by molecular oxygen has been extensively discussed (equation 2) (Moses et al. 1987; Rimstidt and Vaughan 2003). It could lead to the formation of acid mine drainage and the release of pyrite associated heavy metals like arsenic and uranium, which could seriously threaten water quality.

$$FeS_2 + 3.5O_2 + H_2O \Leftrightarrow 2SO_4^{2-} + Fe^{2+} + 2H^+ \quad (2)$$

Fe(III) is a potential oxidant for pyrite under acidic conditions (equation 3). At low pH values, the role of Fe(III) is suggested to be much more efficient than oxygen (Singer and Stumm 1970; Nordstrom 1982; Mckibben and Barnes 1986).

$$FeS_2 + 14Fe^{3+} + 8H_2O \Leftrightarrow 2SO_4^{2-} + 15Fe^{2+} + 16H^+ \quad (3)$$

Schippers and Jørgensen (2001) described the oxidation of pyrite by manganese oxide in marine sediment according to the following reaction (equation 4):

$$FeS_2 + 7.5MnO_2 + 11H^+ \Leftrightarrow 2SO_4^{2-} + Fe(OH)_3 + 7.5Mn^{2+} + 4H_2O \quad (4)$$

Dissolved Fe(III) can only be available in a significant quantity when pH values are below pH 3 (Stumm and Morgan 1996), which is below the typical pH values of groundwater. The oxidation of pyrite by manganese oxide occurs in marine sediment but not in groundwater sediment. Therefore, it is generally assumed that the oxidation of pyrite is coupled to in situ denitrification processes in anoxic groundwater environments (Postma et al. 1991; Korom 1992; Kölle et al. 1983; Kölle et al. 1985; Böttcher et al. 1985; Robertson et al. 1996; Tesoriero et al. 2000; Garcia-Gil and Golterman 1993; Korom et al. 2005).

Chemolithoautotrophic microorganisms

Since pure chemical interaction between pyrite and nitrate cannot occur kinetically in nature at significant rates (Stumm and Morgan 1996), this redox process has been assumed to be catalyzed by microorganisms (Kölle et al. 1983; Kölle et al. 1985; Jørgensen et al. 2009). *Thiobacillus denitrificans* is the only well-known obligate chemolithoautotrophic bacterium which is able to couple denitrification to the oxidation of inorganic sulfur compounds such as thiosulfate, polythionate, elemental sulfur and sulfide and to catalyze anaerobic nitrate-dependent oxidation of Fe(II) (Beller et al. 2006; Straub et al. 1996; Beller et al. 2013). Environmentally relevant capabilities of *Thiobacillus denitrificans* have been reported to catalyze the removal of nitrate, which is a widespread pollutant of shallow groundwater, by anaerobic nitrate-dependent oxidation of minerals such as pyrite (Kölle et al. 1983). Beside nitrate-dependent sulfur-oxidizing bacterium *Thiobacillus denitrificans*, the denitrification coupled to pyrite oxidation is assumed to be catalyzed by nitrate-dependent iron-oxidizing

bacterium e.g *Acidovorax sp.* BoFeN1. The latter is characterized as a chemoorganotrophic, anaerobic nitrate-dependent Fe(II)-oxidizing bacterium and was isolated from a freshwater sediment (Kappler et al. 2005).

Field evidence for denitrification coupled to pyrite oxidation

Since the pyrite minerals are often unevenly distributed in natural groundwater sediments, the quantitative analysis of pyrite is difficult (Jacobsen et al. 1990). 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 (Postma et al. 1991; Tesoriero et al. 2000; Zhang et al. 2009; Pauwels et al. 2000). Theoretically, the pathway of nitrate-dependent pyrite oxidation follows the equations (5) and (6):

$$5FeS_2 + 14NO_3^- + 4H^+ \rightarrow 5Fe^{2+} + 7N_2 + 10SO_4^{2-} + 2H_2O \quad (5)$$

$$5Fe^{2+} + NO_3^- + 7H_2O \rightarrow 5FeOOH + 0.5N_2 + 9H^+ \quad (6)$$

Sedimentary pyrite often includes significant amounts of trace metals (Morse 1994). The mobilization of pyrite-associated trace metals such as As, Ni, Co, Zn (Figure 1.1) (Zhang et al. 2009; Van Beek et al. 1989; Evangelou and Zhang 1995; Broers 1998) and aqueous uranium (van Berk and Fu 2017) concomitant to nitrate removal was regarded as further evidence of pyrite oxidation.

Beside geochemical data, numerous field studies provided evidence of natural denitrification coupled to pyrite oxidation by utilizing stable isotopes of heavy oxygen isotope (^{15}N) and heavy oxygen isotope (^{18}O) in groundwater. Microorganisms prefer to use the lighter isotopes ^{14}N and ^{16}O during denitrification. This leads to the remaining nitrate being increasingly enriched in ^{15}N and ^{18}O. There is no other process than anaerobic pyrite oxidation which seemed plausible for the enrichment of ^{15}N and ^{18}O in nitrate in concert with ^{34}S enrichment in sulfate (Pauwels et al. 2000; Beller et al. 2004; Schwientek et al. 2008; Böttcher et al. 1990).

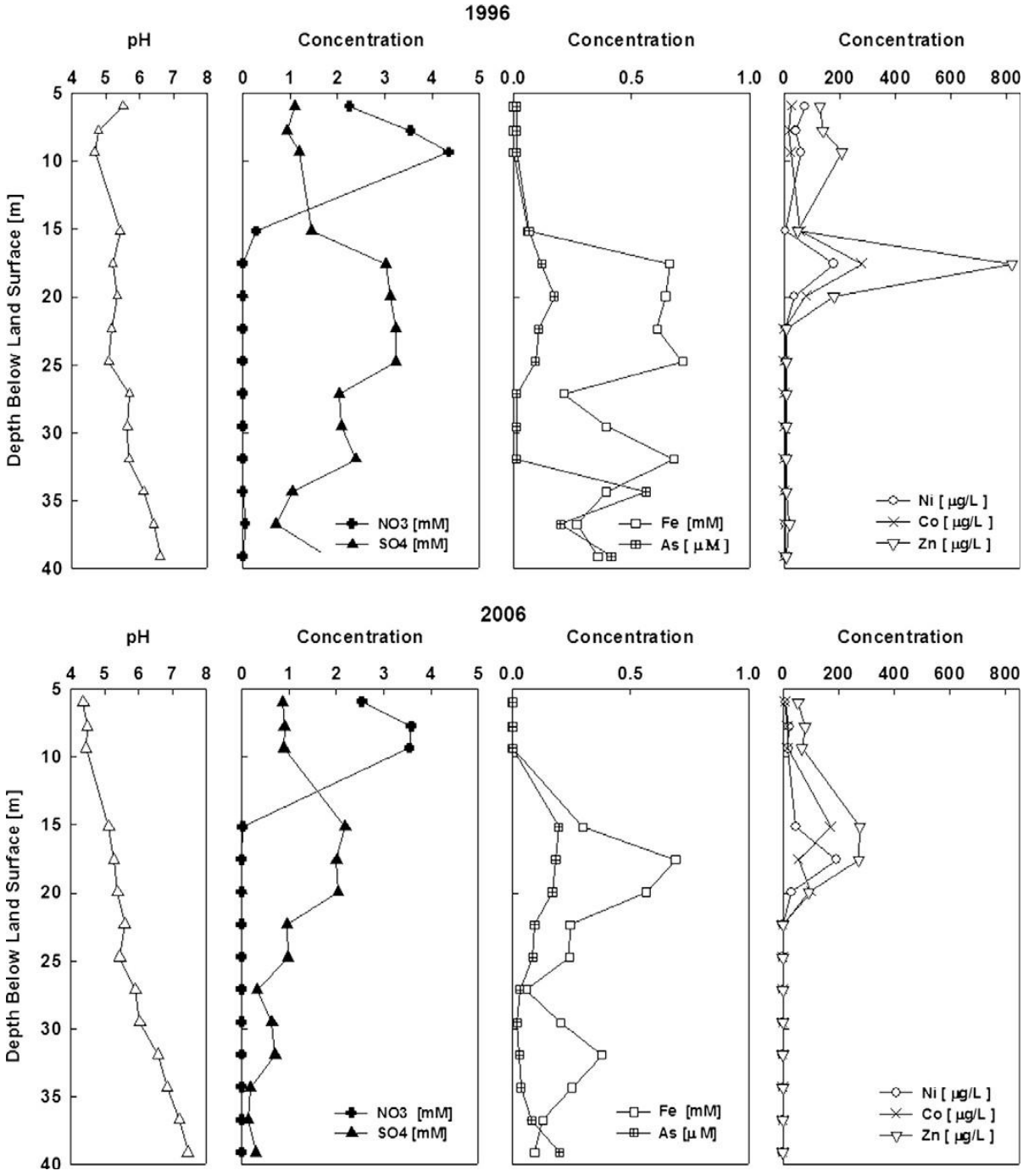

Figure 1.1 Depth distributions of pH, dissolved NO_3^-, SO_4^{2-} and Fe^{2+} (in mM) and selected trace metals (As in µM, Ni, Co and Zn, in µg/l) in groundwater of well 40 (farmland) in a sandy aquifer in 1996 and 2006. The groundwater table in this area is located approximately 4 m below the land surface (Figure taken from Zhang et al., 2009).

1.1.2 Laboratory studies on denitrification coupled to pyrite oxidation

Even though field studies provide geochemical and isotopic evidence for denitrification coupled to pyrite oxidation, the mechanism of this process are still not clear. A series of laboratory studies was therefore initiated to resolve the mechanisms underlying pyrite-dependent nitrate reduction (Haaijer et al. 2007; Jørgensen et al. 2009; Torrentó et al. 2010; Bosch et al. 2012). However, results of these studies are contradictory. The first study has performed well-defined batch experiments with material from marine sediment. Still, bacterial growth could not be observed in enrichment experiments with pyrite as electron donor and nitrate as electron acceptor (Schippers and Jorgensen 2002). In an anoxic slurry experiment with $^{55}FeS_2$ and marine sediments, there appeared no dissolution of $^{55}FeS_2$ (Schippers and Jorgensen 2002). Both observations showed negative results on microbial pyrite oxidation by nitrate. Neither did another study of incubation in a soil-containing reactor in which ground pyrite was added, provide any evidence of denitrification coupled to pyrite oxidation (Haaijer et al. 2007). In contrast, two laboratory studies with natural sediment confirmed the positive field observations on anaerobic nitrate-dependent pyrite oxidation. In incubation experiments with naturally pyrite-containing sediment from a sandy aquifer and accompanying batch experiments in which ground pyrite was added, accelerated nitrate reduction and sulfate generation have been observed (Figure 1.2) (Jørgensen et al. 2009). In anaerobic batch and flow-through experiments where ground pyrite was added, the rates of nitrate reduction in the presence of the autotrophic denitrifying bacterium *Thiobacillus denitrificans* increased as pyrite grain size decreased and were dependent on initial nitrate concentration and nitrate-loading rate (Torrentó et al. 2010). Both studies therefore revealed indirect evidence for the presence of a microbially mediated denitrification with pyrite as the electron donor. A recent study described oxidation of pyrite nanoparticles by the nitrate-reducing bacterium *Thiobacillus denitrificans*. Its conclusion was based on an

established electron balance with regard to the formation of ferric iron and sulfate along with the reduction of nitrate to nitrite (Bosch et al. 2012).

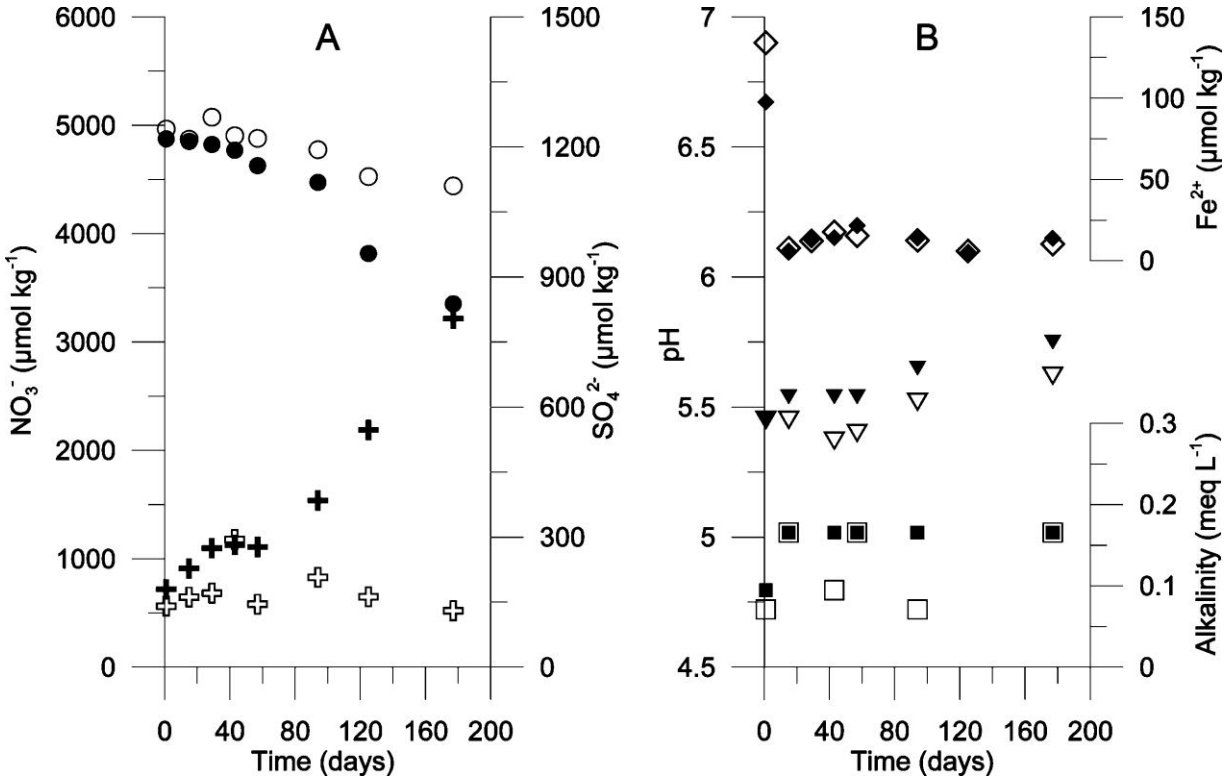

Figure 1.2 Chemical development of NO_3^- (circles), SO_4^{2-} (crosses), Fe^{2+} (diamonds), pH (triangles) and alkalinity (squares) of a single natural and pyrite amended reactor incubated at 21.5 °C over 177 days. Open symbols represent concentrations in the natural reactor and filled symbols represent concentrations in the pyrite-amended reactor (Figure taken from Jørgensen et al. 2009).

1.1.3 Interference of nitrite with Fe measurement during acidic extraction

A common product of denitrification is nitrite. Due to difficulties of in-situ measurement or the limited amount of it, nitrite is generally either not measured or not detected in the field. The role of nitrite in the process of pyrite oxidation by nitrate is often neglected. However, significant formation of nitrite has been determined in laboratory column experiments with sediments from groundwater aquifers (Torrentó et al. 2010; Leson and Wisotzky 2012).

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* (Torrentó et al. 2010; Bosch et al. 2012).

However, nitrite is a major interfering compound for Fe(II)/Fe(III) measurement (Klueglein and Kappler 2013). Under acidic conditions, nitrite decomposes into highly reactive compounds after protonation to nitrous acid (HNO_2) (equation 7). Nitrous acid is unstable at pH<5 and spontaneously decomposes to nitrogen dioxide (NO_2) and nitric oxide (NO) (equation 8) (Nelson and Bremner 1970a; Park and Lee 1988; Ibrahim et al. 2001).

$$NO_2^- + H^+ \Leftrightarrow HNO_2 \qquad K_a = 10^{-3.35} \text{ mol } L^{-1} \qquad (7)$$

$$2HNO_2 \rightarrow NO_2 + NO + H_2O \qquad K_a = 10^{-5.22} \text{ mol } L^{-1} \qquad (8)$$

NO_2 and/or NO are strong oxidants which are able to oxidize Fe(II) abiotically according to the equations 9-12 (Bonner and Pearsall 1982; Van Cleemput and Samater 1995; Nelson and Bremner 1970b).

$$2NO_2^- + 2H^+ \Leftrightarrow 2HNO_2 \rightarrow NO_2 + NO + H_2O \qquad (9)$$

$$NO_2 + 2Fe^{2+} + 2H^+ \rightarrow 2Fe^{3+} + NO + H_2O \qquad (10)$$

$$NO + Fe^{2+} + H^+ \rightarrow Fe^{3+} + HNO \qquad (11)$$

$$2HNO \rightarrow N_2O + H_2O \qquad (12)$$

A recent study demonstrated that NO_2 and/or NO lead to a significant overestimation of the Fe(II) oxidation rate in cultures of the nitrate-reducing Fe(II) oxidizer *Acidovorax sp.* BoFeN1 (Klueglein and Kappler 2013). However, numerous previous studies did not consider the abiotic Fe(II) oxidation by nitrite during acidic Fe extraction (Table 1.1).

Table 1.1 Overview of isolated nitrate-reducing, Fe(II)-oxidizing strains or environmental samples (including sediments) with nitrate-dependent Fe(II) oxidation capacity that has been published in the last years. In some cases, approaches to prevent abiotic Fe(II) oxidation by nitrite during sampling/analysis are described (Table taken from Klueglein and Kappler, 2013).

Name bacterial strain	**Author (year of publication)**	**Nitrite accumulation**	**Approach to prevent abiotic Fe(II) oxidation by nitrite**	**Samples for Fe(II) and Fe(III) analysis diluted in**
Isolate HidR2	Benz *et al.*, (1998)	No	Anoxic centrifugation	500 mm phosphate buffer/pellet in 1 m HCl
Thiobacillus denitrificans DSMZ 739	Bosch *et al.*, (2012)	Yes	No	1 m HCl
Acidovorax strain 2AN	Chakraborty *et al.*, (2011)	Yes	Anoxic centrifugation	Pellet in 0.5 m HCl
Lake sediment	Hauck *et al.*, (2001)	Not measured	No	1 m HCL
Acidovorax strain BoFeN1	Kappler *et al.*, (2005)	Yes	No	HCl
Isolate FW33AN	Senko *et al.*, (2005)	Yes	No	0.5 m HCl
Sediment & water samples	Straub *et al.*, (1996)	Not shown but stated in text	First dilution in Na_2CO_3 & Anoxic centrifugation	Pellet in 1 m HCl
Isolates BrG1, 2, 3	Straub *et al.*, (2004)	Not stated	No	0.7 m Na-acetate buffer pH 5
Enrichment culture	Weber *et al.*, (2001)	Yes	Anoxic centrifugation	Pellet in 0.5 m HCl
*Pseudogulbenkiania*strain 2002	Weber *et al.*, (2006a; 2006b)	Yes	No	0.5 m HCl or directly in ferrozine

1.2 Research hypotheses and objectives

The goal of this dissertation is further understanding of the mechanism of denitrification coupled to pyrite oxidation and to identify potential geochemical and microbiological interferences which may produce artifacts to influence experimental results. To this purpose, the following hypotheses were generated and studied in this thesis.

(1) The type of pyrite may be a key factor which affects the microbial pyrite oxidation by nitrate.

(2) The microbial pyrite oxidation by nitrate may be catalyzed not only by nitrate-dependent sulfur-oxidizing iron-oxidizing bacterium *Thiobacillus denitrificans* but also by nitrate-dependent iron-oxidizing bacterium *Acidovorax sp.* BoFeN1.

(3) Formation of nitrite due to nitrate reduction may lead to overestimation of Fe(III) formation during acidic extraction in nitrite-containing pyrite samples.

(4) Reduced sulfur species may strongly affect the Fe(III) and sulfate production in the batch experiment and provide false positive results.

Based on these hypotheses, the following objectives were investigated in this thesis:

(1) To identify whether nitrite can oxidize pyrite abiotically in nitrite-containing pyrite samples.

(2) To investigate two types of pyrite (different crystallinities, different BET surface areas) in the anaerobic nitrate-dependence oxidation of pyrite by *Thiobacillus denitrificans*.

(3) To evaluate the effects of nitrate-dependent sulfur-oxidizing iron-oxidizing bacterium *Thiobacillus denitrificans* and nitrate-dependent iron-oxidizing bacterium *Acidovorax sp.* BoFeN1 as a catalyst for the microbial reaction between pyrite and nitrate.

(4) To investigate the effects of reduced sulfur species on microbial oxidation of pyrite by nitrate.

With these hypotheses and objectives, the following three studies were performed in this dissertation.

Study 1: Interference of nitrite with pyrite under acidic conditions – implications for studies of chemolithotrophic denitrification

In order to quantitatively understand the interaction between pyrite and nitrite, and evaluate the interference of nitrite with the determining Fe(II)/$Fe(HCl)_{tot}$ in nitrite-containing pyrite samples during acidic extraction, batch experiments are designed to cover the range of pyrite concentrations (5–125 mM) used in previous studies as well as the concentration range of nitrite determined in these studies (40–2000 µM) (Haaijer et al. 2007; Jørgensen et al. 2009; Torrentó et al. 2010; Bosch et al. 2012; Vaclavkova et al. 2014). The effects of oxygen and pH on pyrite oxidation by nitrite are also investigated in study 1.

Study 2: The effect of reduced sulfur speciation on the chemolithoautotrophic pyrite oxidation with nitrate

In order to further understand the mechanism of chemolithoautotrophic pyrite oxidation by nitrate, study 2 performed a systematic series of experiments to compare different sources of reduced S (pyrite, elemental sulfur and marcasite) and reduced Fe (pyritic Fe(II), dissolved Fe(II)) with regard to their ability to act as electron donor. The nitrate-dependent, sulfur-oxidizing, and iron-oxidizing bacterium *Thiobacillus denitrificans*, and the nitrate-dependent, iron-oxidizing bacterium *Acidovorax sp.* BoFeN1 are applied to function as catalysts for interaction between nitrate and pyrite.

Study 3: Towards a standardized protocol for studying chemolithoautotrophic denitrification with pyrite

The existing, contradictory results of previous laboratory studies on the chemolithoautrophic denitrification coupled to pyrite oxidation, which have been published in the last years, are

considered to be related to inconsistent experimental protocols. In study 3, possible geochemical and microbiological interferences in these previous studies are illustrated and revised protocols are recommended.

1.3 Materials and methods

1.3.1 Crystalline pyrite and synthesized pyrite

Previous studies reveal that grain size of pyrite could play an important role in the microbial pyrite oxidation (Torrentó et al. 2010; Bosch et al. 2012). In this dissertation, two kinds of pyrite were applied for different research purposes. In order to rule out any interference by other sulfur compounds or ferric iron (study 1 and 2), crystalline pyrite from Peru, Georg Maisch Import (Freising, Germany) was carefully prepared to achieve very high purity. These procedures to remove impurities include the following steps: With an aim of removing ferric iron which may have formed from oxidation of pyrite surfaces during crushing, and residual acid-extractable sulfur species, the material was washed with 1 M HCl and ultrapure water. In addition, the material was freeze-dried and then washed 3 times with deaerated cyclohexane to remove elemental sulfur.

A synthesized pyrite was also used in microbial batch experiments (study 2). It has been characterized as a mixture of pyrite, marcasite and elemental sulfur. Reduced sulfur compounds such as marcasite and elemental sulfur could typically arise from impurities present in natural or synthetic samples of pyrite or groundwater sediments. This iron disulfide was synthesized following procedure described by Peiffer and Stubert (1999) and Berner (1970). Contrary to the previous work, the synthesis was conducted in an anoxic glovebox against the oxidation of oxygen. Compared to the ground crystalline pyrite, the synthesized material has a smaller mean particle size and lower crystallinity, which is assumed to have a higher reactivity. Also the BET surface area (0.41 $m^2 g^{-1}$) of the synthesized pyrite is higher than that of the ground pyrite (0.17 $m^2 g^{-1}$), though this difference is not very large.

1.3.2 Culture cultivation

Nitrate-dependent sulfur-oxidizing iron-oxidizing bacterium *Thiobacillus denitrificans* or nitrate-dependent iron-oxidizing bacterium *Acidovorax sp.* BoFeN1 was used as a catalyst for the reaction between pyrite and nitrate. *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). *Acidovorax sp.* BoFeN1 isolated from Lake Constance sediments is a mixotrophic bacterium that grows with acetate plus Fe(II) and nitrate as electron acceptor (Kappler et al. 2005). *Acidovorax sp.* BoFeN1 was grown in an 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 substrate and was prepared as described by Hegler et al. (2008) and Hohmann et al. (2009). *Thiobacillus denitrificans* and *Acidovorax sp.*BoFeN1 were grown at 30 °C under an atmosphere of 80% N_2 and 20% CO_2 in the dark and unshaken. Growth of the cultures was measured by following the optical density (OD) of the culture media at a wavelength of 600 nm (OD_{600}) in a spectrophotometer. Total cell number was measured 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 without thiosulfate and iron before the start of the experiments.

1.3.3 Experimental set up

Chemical reaction between pyrite and nitrite

Previous study revealed that during acidic extraction of Fe(II) nitrite samples from *Acidovorax sp.* BoFeN1 cultures, the nitrite present forms nitrous acid by protonation that showed spontaneous self-decomposition into NO_2 and/or NO, which lead to a significant overestimation of the enzymatic Fe(II) oxidation (Klueglein and Kappler 2013). In study 1 we therefore aim to test whether similar processes may also cause abiotic oxidation of pyrite by

nitrite under acidic conditions. The experimental set-up modified depending on the experimental purposes. There are 4 variables in the batch experiments of chemical reaction between pyrite and nitrate: the concentration of nitrite, the concentration of pyrite, pH value, with/or without the presence of oxygen (study 1). In order to test the influence of initial nitrite concentration on anoxic pyrite oxidation at pH 0, batch experiments were conducted at a constant pyrite concentration (5 mM) and a constant pH value (1 M) in an anoxic glovebox. The concentration of nitrite varied from 40 to 2000 µM. The influence of the initial pyrite concentration on the reaction rate at pH 0 was tested with various concentrations of pyrite (5, 25, 125 mM) at a nitrite concentration of 1000 µM in HCl (1 M) under anoxic conditions. To evaluate the effect of pH, batch experiments with the same concentrations of pyrite and nitrite in the absence of oxygen were performed at pH 0, 5.5 and 6.8, respectively. Furthermore, in order to test the effect of oxygen in this process, 5 mM pyrite were incubated with 1000 µM nitrite in 1 M HCl under anoxic and oxic conditions.

Experiments for the testing of the revised protocol for nitrite-free acidic Fe extraction in nitrite containing pyrite suspensions.

In order to avoid abiotic oxidation of pyrite by nitrite during acidic extraction, our suggestion is to remove nitrite from pyrite nitrite samples by washing the pyrite suspensions with nitrite-free water prior to the acidic extraction. We performed therefore an additional test by comparing unwashed pyrite suspensions in the presence of nitrite with washed pyrite suspensions in the absence of nitrite. Aliquots of 0.1 mL were withdrawn from serum bottles (100 mL, c_{pyrite}=50 mM, $c_{nitrite}$=10 mM, pH 6.4) as unwashed and immediately diluted in 1 M HCl. Similarly, for the washed samples, aliquots of 0.1 mL were taken from the serum bottles (100 mL, c_{pyrite}=50 mM, pH 6.4) in the absence of nitrite then washed three times with ultrapure water until no nitrite could be detected by nitrite indicator strips. Unwashed samples

in the presence of nitrite and washed samples in the absence of nitrite were placed in a gas-tight container, removed from the glovebox and shaken for 24 h.

Microbial reaction between pyrite and nitrate

Four types of microbial batch experiments between pyrite and nitrate were conducted under anoxic, pH-neutral conditions:

(1) Batch experiments of synthesized pyrite (8.3 mM) and nitrate (approximately 10 mM) in the presence of nitrate-reducing sulfide-oxidizing bacterium *Thiobacillus denitrificans* with a cell density of 9.3×10^6 or 9.3×10^7 cells ml^{-1} were carried out within a period of 43 days.

(2) Batch experiments of synthesized pyrite (8.3 mM) and nitrate (approximately 10 mM) were performed with *Thiobacillus denitrificans* at a cell density of 9.3×10^7 cells ml^{-1} within a period of 43 days in the presence of i) dissolved Fe(II) (100 µM). This was in order to test whether Fe(II) will be oxidized with nitrate as electron acceptor and ii) dissolved Fe(III) (100 µM) to test whether abiotic oxidation of pyrite by Fe(III) (Peiffer and Stubert 1999) may stimulate pyrite oxidation.

(3) Batch experiments of synthesized pyrite (8.3 mM) and nitrate (approximately 10 mM) in the presence of Fe(II)-oxidizing nitrate-reducing strain *Acidovorax sp.* BoFeN1 with a cell density of 1.2×10^7 or 1.2×10^8 cells ml^{-1} were conducted during a period of 28 days.

(4) Batch experiments of pure ground crystalline pyrite (proven free of elemental sulfur, 50 mM) and nitrate (10 mM) in the presence of nitrate-reducing sulfide-oxidizing bacterium *Thiobacillus denitrificans* with cell densities of 2×10^4 and 2×10^5 cells ml^{-1} were performed within a period of 87 days. This was in order to test the occurrence of nitrate-dependent pyrite oxidation in the absence of sulfur as potential impurity in natural and synthesized samples.

The medium for the batch experiments with *Thiobacillus denitrificans* was prepared without thiosulfate and iron (as being used in the growth medium) to avoid interference of sulfur from the medium in the determination of formation rates of sulfate from pyrite. The medium used

for the batch experiment with *Acidovorax sp.* BoFeN1 was the same as the nutrient medium for cultivation. The headspace of each serum bottle was flushed with a mixture of 80% N_2 and 20% CO_2. All batch experimental serum bottles were incubated at 30°C in the dark.

1.3.4 Chemical analytic methods

Sulfur species determination: Elemental sulfur was determined by HPLC (PerkinElmer 2000 HPLC-system, C18-column, 0.4 ml min^{-1} flow rate, UV-VIS detection at 265 nm for elemental sulfur) (study 1 and 2). For the quantification of sulfate it was necessary to use two different analytical methods in our studies. In acidic samples (pH 0) (study 1), sulfate was measured turbidimetrically following a modification of the turbidimetric $BaSO_4$ method (Tabatabai 1974) since dissolved Fe(III) will tend to precipitate during ion chromatography measurements: In pH-circumneutral samples taken from experiments at pH 5.5, 6.8 (study 1) and pH 7 (study 2), 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. Total concentrations of S was determined in samples from experiments performed at different nitrite concentrations using inductively coupled plasma optical emission spectrometry (ICP-OES Perkin Elmer Optima 3200 XL) (study 1).

Iron species determination: Fe(II) and $Fe(HCl)_{tot}$ (total HCl-extractable Fe) were quantified by the ferrozine assay (Stookey 1970), since ferrozine reacts with Fe(II) to form stable colored complexes. Fe(III) was calculated as the difference between $Fe(HCl)_{tot}$ and Fe(II). To determine of $Fe(HCl)_{tot}$, hydroxylamine hydrochloride was added to samples followed by a 30 min incubation in order to reduce Fe(III) to Fe(II). In order to avoid the oxidation of samples by oxygen, all samples of Fe(II) and $Fe(HCl)_{tot}$ were removed from the glovebox after addition of the ferrozine reagent and exposed to air only approximately 5 min during the

measurement. Absorbance of samples was measured at 570 nm using a microplate reader (Infinite F200 PRO, Tecan, Switzerland). Total concentrations of Fe was determined in samples from experiments performed at different nitrite concentrations using inductively coupled plasma optical emission spectrometry (ICP-OES Perkin Elmer Optima 3200 XL) (study 1).

Nitrogen species determination: concentrations of nitrate and nitrite at circumneutral and neutral pH values were quantified by ion chromatography (IC) with chemical suppression and conductivity detector using an A-supp 4 anion column (Metrohm, Herisau, Switzerland) (study 1 and 2). Neither nitrite nor other nitrogen species could be quantified under acidic conditions, at which these species are not stable.

1.4 Summary of results and discussion

1.4.1 Kinetics and mechanisms of pyrite oxidation by nitrite under acidic conditions

Our results in study 1 provide clear evidence that pyrite can be significantly oxidized in the presence of nitrite in 1 M HCl under anoxic conditions and that the rate and extent of pyrite oxidation depends on the initial nitrite and pyrite concentrations. In experiments performed with a constant pyrite concentration (c=5 mM) and various nitrite concentrations (c=40-2000 µM), initial pyrite oxidation rates appear to follow a first order reaction rate with respect to nitrite concentration. Kinetics were different in experiments where the nitrite concentration was kept constant (c=1000 µM) but in which the pyrite concentration was varied (c=5-125 mM), a fractional order was determined with respect to initial pyrite concentrations. Our experimental data imply a cyclic model to explain the mechanisms of pyrite oxidation by nitrite under acidic conditions. This process is suggested to be a cyclic oxidation of Fe(II) by reactive NO_2 and/or NO to Fe(III) and regeneration of Fe(II) upon reaction of Fe(III) with pyrite, since Fe(III) is a major oxidant for pyrite under acidic conditions, with the role of the

dissolved oxygen being to re-oxidize Fe(II) to Fe(III) forming a cycle of iron (Singer and Stumm 1970).

The presence or absence of oxygen and pH value clearly affected the oxidation of pyrite by nitrite. Oxygen clearly enhanced the extent of pyrite oxidation by NO and/or NO_2 under acidic conditions. The reason is probably that oxygen can oxidize NO to NO_2 (Prather and Miyamoto 1974; Van Cleemput and Samater 1995) which is suggested a stronger oxidant for the oxidation of Fe(II) under acidic conditions than NO (Klueglein and Kappler 2013). In addition, no anoxic oxidation of pyrite in the presence of nitrite was observed at pH 5.5 and 6.8. This observation can be explained as follows: HNO_2 is the precursor of reactive NO_2 and/or NO, and occurs at relevant concentrations only at pH<5 (pK_a=3.35), while the ionic species nitrite, i.e. NO_2^-, is not reactive towards pyrite.

The revised protocol on the removal of nitrite from the nitrite-containing pyrite samples is recommended, since the concentration of Fe(III) remained stable after 24 h of acidic extraction in the washed samples, compared to the unwashed samples, in which a significant increase of Fe(III) was observed.

1.4.2 Potential of *Thiobacillus denitrificans* and *Acidovorax sp.* BoFeN1 as catalysts for chemolithoautotrophic nitrate reduction coupled to pyrite oxidation

Throughout the entire batch experiment with synthesized pyrite and nitrate in the presence of *Thiobacillus denitrificans*, sulfate was generated from synthesized pyrite in the presence of the S-oxidizing nitrate-reducing bacterium *Thiobacillus denitrificans*. Nitrate reduction was accompanied by significant formation of nitrite. In contrast, neither formation of nitrite nor sulfate was observed in the presence of the Fe(II)-oxidizing nitrate-reducing strain *Acidovorax sp.* BoFeN1. The reaction appears to be induced via S oxidation but not via Fe oxidation.

1.4.3 Potential of dissolved Fe(II) and Fe(III) as chemical catalysts for chemolitho-autotrophic nitrate reduction coupled to pyrite oxidation

In additional batch experiments with synthesized pyrite and nitrate in the presence of *Thiobacillus denitrificans* where Fe(II) or Fe(III) was added, no increase in the consumption of nitrate and the formation of sulfate could be observed relative to experiments in the absence of these species. Therefore, the addition of Fe(II) or Fe(III)) did not stimulate the oxidation of pyrite by nitrate under our experimental conditions.

1.4.4 Potential of synthesized pyrite and pure ground crystalline pyrite as an electron donor for chemolithoautotrophic nitrate reduction

The ground material was characterized as a pure crystalline pyrite while the synthesized pyrite was a mixture of pyrite, marcasite and elemental sulfur. Under our well-defined experimental conditions, chemolithoautotrophic oxidation of pyrite with nitrate as electron acceptor was not observed when the pyrite source is pure crystalline pyrite, while oxidation of synthesized pyrite species of low crystallinity in the presence of elemental sulfur is possible.

1.5 Conclusions and perspective

Based on the previous laboratory studies of chemolithoautotrophic denitrification coupled to pyrite oxidation, this dissertation is able to further elucidate the mechanism of this process by comparing different pyrite sources and different denitrifying bacteria strains in batch experiments under anoxic, circumneutral conditions. Also, by means of careful material preparation and optimization of chemical analytical methods of determining reaction products, geochemical and microbiological interferences which affect experimental results are avoided. Three key findings from this thesis can be highlighted as providing helpful implications for future studies on chemolithoautotrophic pyrite oxidation with nitrate.

(1) Study 1 determines that nitrite is able to oxidize pyrite abiotically during acidic extraction which leads to the formation of ferric iron. The occurrence of ferric iron may therefore be misinterpreted as proof of pyrite oxidation (Bosch et al. 2012). A revised protocol was recommended in the case of any acid extraction procedure with suspensions containing nitrite and pyrite or other Fe(II)-containing solid phases that may be subject to interference by nitrite. Interference by nitrite could be avoided if nitrite was removed from the pyrite suspensions through a washing procedure prior to acidic extraction. The finding of Study 1 therefore calls for an investigation of anaerobic, nitrate-dependent oxidation of pyrite by denitrifying bacteria without the interference by nitrite during acidic extraction.

(2) Study 2 highlights the importance of the speciation of reduced sulfur in mediating chemolithoautotrophic denitrification. Results demonstrated that chemolithoautotrophic oxidation of pyrite with nitrate as electron acceptor was not possible if the pyrite source is pure crystalline pyrite that does not contain elemental sulfur contaminations. By contrast, our study suggested that chemolithoautotrophic oxidation of synthesized less crystalline pyrite with nitrate may be possible. The synthesized pyrite consisted of pyrite, marcasite, and elemental sulfur, allowing the question of which kind of S source (pyrite, marcasite or elemental sulfur) plays the predominant role in the reaction. The findings of the present study

imply that contradictory results from previous studies (Haaijer et al. 2007; Jørgensen et al. 2009; Torrentó et al. 2010; Bosch et al. 2012; Vaclavkova et al. 2014; Schippers and Jorgensen 2002) on the potential chemolithoautotrophic oxidation of pyrite with nitrate obtained so far may arise from impurities of reduced sulfur species present in natural or synthetic pyrite phases or sediments. A quantitative differentiation between the sulfur components as well as their mineralogical characterization is required in future field and laboratory studies of pyrite oxidation.

(3) Study 2 reveals that the microbial reaction between pyrite and nitrate appears to be induced via S oxidation but not via Fe oxidation, since the Fe(II)-oxidizing nitrate-reducing strain *Acidovorax sp.* BoFeN1 did not stimulate pyrite-dependent nitrate reduction. Also, the addition of Fe(II) and Fe(III) to the reaction even slightly decreased the rates of nitrate reduction and sulfate generation. The finding of Study 2 calls for an investigation of the role of reduced sulfur components e.g thiosulfate, elemental sulfur as chemical catalysts in nitrate-dependent pyrite oxidation.

(4) Study 3 summarizes possible geochemical and microbiological interferences in previous laboratory studies that may arise from impurities of reduced sulfur species associated with pyrite, formation of nitrite and its interference during acidic extractions, and occurrence of residual iron and sulfur compounds in the reaction medium. To further improve the understanding of chemolithoautotrophic denitrification with pyrite, a well-defined systematic study with good consideration of all possible interferences is required to provide direct evidence of this process.

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2. Study 1: Interference of nitrite with pyrite under acidic conditions – implications for studies of chemolithotrophic denitrification

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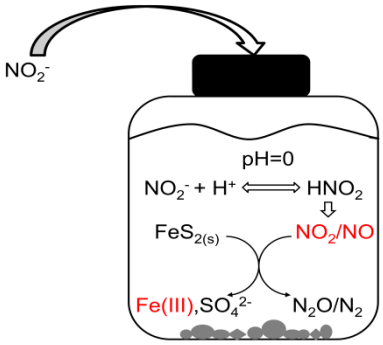

2.1 Abstract

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 24 h of incubation of 5 mM pyrite with 2000 µM nitrite. 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) based on oxidation of residual Fe(II) by NO and/or NO_2. 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.

Key words: Denitrification, pyrite oxidation, nitrite, interference, acidic extraction

2.2 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 (Kölle et al. 1983; Pauwels et al. 2000; Postma et al. 1991; Schwientek et al. 2008; Zhang et al. 2009; Van Beek et al. 1989) and fueled a series of laboratory studies to resolve the mechanisms underlying this reaction (Haaijer et al. 2007; Jørgensen et al. 2009; Torrentó et al. 2010; Bosch et al. 2012; Vaclavkova et al. 2014). A common product of denitrification is nitrite (Jørgensen et al. 2009; Torrentó et al. 2010; Bosch et al. 2012; Vaclavkova et al. 2014). Significant formation of nitrite has been determined in laboratory column experiments with sediments from groundwater aquifers (Torrentó et al. 2010; Leson and Wisotzky 2012). 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* (Torrentó et al. 2010; Bosch et al. 2012; Vaclavkova et al. 2014).

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 (Moses and Herman 1991; Bosch et al. 2012). 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 (HNO_2) (equation 1). Nitrous acid is unstable at pH<5 and spontaneously decomposes to nitrogen dioxide (NO_2) and nitric oxide (NO) (equation 2) (Nelson and Bremner 1970a; Park and Lee 1988; Ibrahim et al. 2001).

$$NO_2^- + H^+ \Leftrightarrow HNO_2 \qquad K_a = 10^{-3.35} \text{ mol } L^{-1} \qquad (1)$$

$$2HNO_2 \rightarrow NO_2 + NO + H_2O \qquad K_a = 10^{-5.22} \text{ mol } L^{-1} \qquad (2)$$

NO_2 and/or NO are known as strong oxidants towards Fe(II) (Wullstein and Gilmour 1966; Bonner and Pearsall 1982; Ibrahim et al. 2001). It was demonstrated that NO_2 and/or NO lead

to a significant overestimation of the Fe(II) oxidation rate in cultures of the nitrate-reducing Fe(II) oxidizer *Acidovorax sp.* BoFeN1 (Klueglein and Kappler 2013). Based on this observation the authors questioned the occurrence of enzymatic Fe(II) oxidation coupled to nitrate reduction that was postulated in previous studies (Klueglein and Kappler 2013).

In this study we therefore aim to test whether similar processes may also trigger abiotic oxidation of pyrite under acidic conditions and thus generate the risk of producing artifacts and data misinterpretations (Melton et al. 2014). To these ends we have performed batch experiments which 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) (Haaijer et al. 2007; Jørgensen et al. 2009; Torrentó et al. 2010; Bosch et al. 2012; Vaclavkova et al. 2014).

2.3 Materials and methods

2.3.1 Preparation and characterization of pyrite

Crystalline pyrite (3-6 mm in diameter) from Peru, Georg Maisch Import (Freising, Germany) was ground by milling in a ball mill with an agate mortar under atmospheric conditions. After sieving, a size fraction between 63-200 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. In order to remove ferric iron which may have formed from oxidation of pyrite surfaces during crushing, and residual acid-extractable sulfur species, the material was shaken in 1 M HCl for 1 h, washed with ultrapure water and ultrasonically cleaned for an additional hour. This procedure was repeated 9 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 3 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 the Supporting Information (SI), Figure S2.1, Tables S2.1-S2.2). 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 minutes with nitrogen. The washed pyrite was characterized by scanning electron microscopy (Zeiss Leo 1530 FE-SEM, Germany) and by X-ray diffractometry (D5000, SIEMENS, Germany) using Co Kα 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 m^2 g^{-1}. Although the ground pyrite was washed several times with HCl, nm-sized structures were still detectable by SEM on the surface of pyrite (Figure 2.1). EDX spectra (SI, Figure S2.1, Tables S2.1-S2.2) taken from the these nm-sized structures sample displayed a Fe:S ratio of approximately 1:2. XRD revealed that the ground material was pure pyrite (SI, Figure S2.2) (Brostigen and Kjekshus 1969).

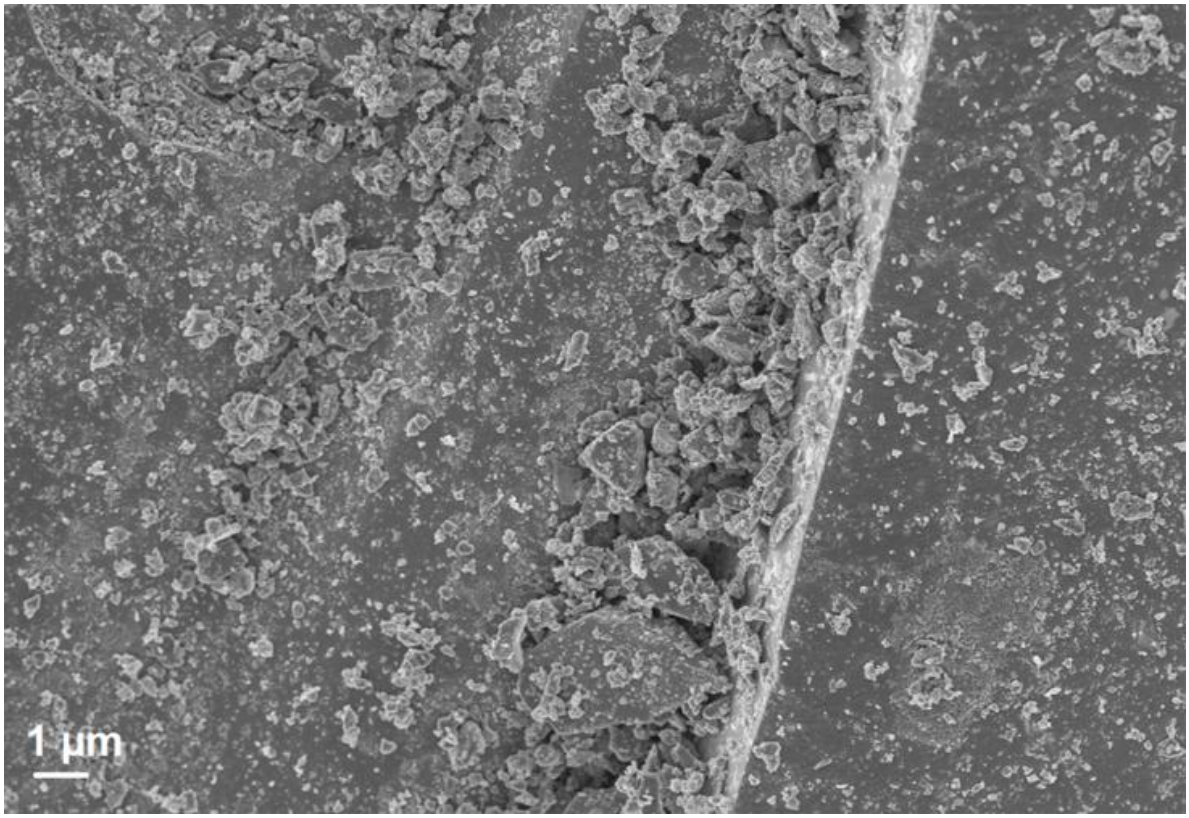

Figure 2.1 Scanning electron micrograph of the ground pyrite after preparation.

2.3.2 Experimental set up

Batch experiments in the absence of oxygen were performed in an anoxic glovebox (Innovative Technology, Massachusetts, USA, 100% N_2) at room temperature (20±2°C). All solutions were purged with N_2 for dissolved oxygen removal before being transferred into the glovebox. In order to test the influence of initial nitrite concentration on anoxic pyrite oxidation at pH 0, 0.03 g pyrite (5 mM) and 50 mL HCl (1 M) were placed 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 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, 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. In order to test the importance of oxygen in this process, experiments with 5 mM pyrite and 1000 µM nitrite were carried out also 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, additional batch experiments were performed 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 over night before the addition of nitrite. Experiments and controls were performed in three independent replicates.

2.3.3 Analytical methods

In experiments performed in 1 M HCl under anoxic conditions, Fe(II) and $Fe(HCl)_{tot}$ (total HCl-extractable Fe) were quantified by the ferrozine assay (Stookey 1970). Fe(III) was calculated as the difference between $Fe(HCl)_{tot}$ 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 $Fe(HCl)_{tot}$, hydroxylamine hydrochloride was added into filtered samples followed by a 30 min incubation in order to reduce Fe(III) to Fe(II). All samples of Fe(II) and $Fe(HCl)_{tot}$ were removed from the glovebox after addition of the ferrozine reagent and exposed to air only approximately 5 min during the measurement. Absorbance of samples was measured at 570 nm using a microplate reader (Infinite F200 PRO, Tecan, Switzerland). Each sample was analyzed in triplicates.

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 $BaSO_4$ method (Tabatabai 1974), since dissolved ferric iron will tend to precipitate during ion chromatography measurements: Barium-gelatin reagent was prepared using the standard procedure. 2.5 mL sample and 125 mL 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, 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 Perkin Elmer Optima 3200 XL). The samples were withdrawn from the serum bottles after 24 h and filtered via 0.45 um filters inside the glovebox. They were diluted 1:1 with 1 M HCl shortly before the measurement.

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. Approximately 1.5 mL of a sample were filtered through 0.22 µm pore size filter (Nylon), 1:5 diluted with ultrapure water and then analyzed by ion chromatography with chemical suppression and conductivity detector using a supp 4 anion column (Metrohm) to determine 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, at which these species are not stable. In experiments performed in 1 M HCl under oxic conditions, sampling and analysis of Fe(II), $Fe(HCl)_{tot}$ and sulfate were performed in the presence of air.

For quantification of elemental sulfur associated with pyrite, 0.5 g of grinded 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, 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).

2.4 Results and discussion

2.4.1 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 $Fe(HCl)_{tot}$, sulfate and Fe(III) were observed (Figure 2.2) and their formation rates clearly depended on initial nitrite concentrations (0.4±0.16 µM h^{-1} $Fe(HCl)_{tot}$ and 0.8±0.03 µM h^{-1} sulfate at 200 µM nitrite, 2.2±0.09 µM h^{-1} $Fe(HCl)_{tot}$ and 4.4±0.41 µM h^{-1} sulfate at 1000 µM nitrite, 3.6±0.18 µM h^{-1} $Fe(HCl)_{tot}$ and 6.9±0.46 µM h^{-1} sulfate at 2000 µM nitrite). Formation rates were calculated as the mean (n=3) linear concentration increase within 24 h.

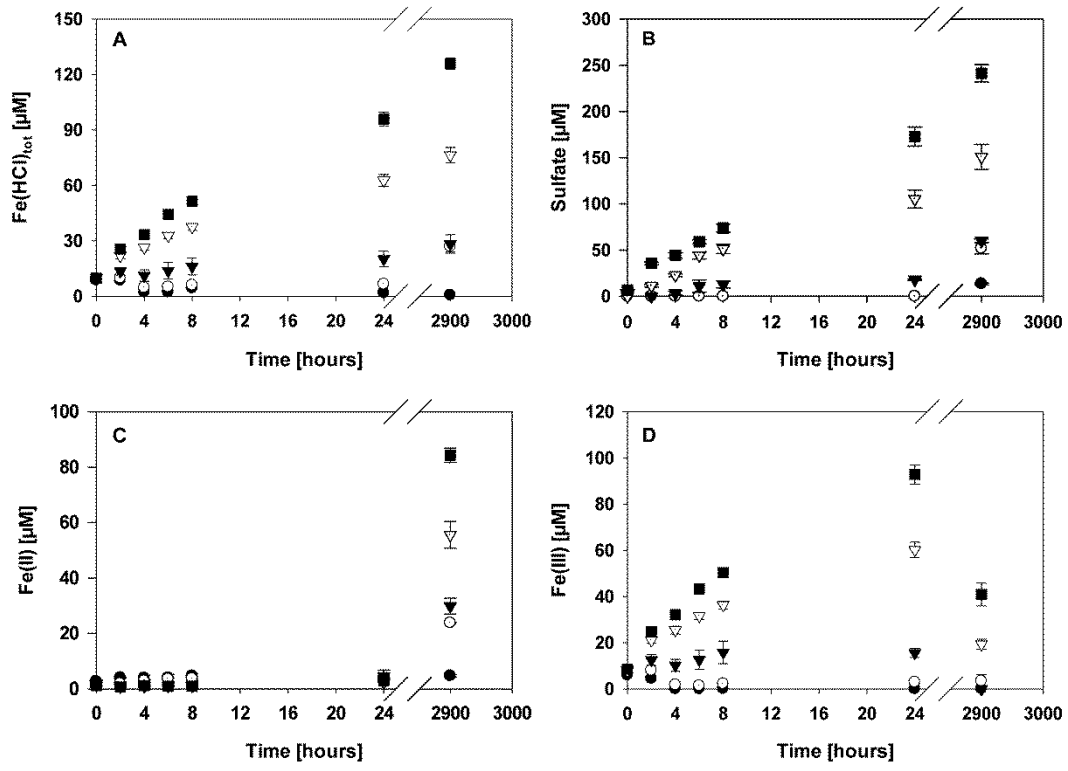

Figure 2.2 Concentration of ((A) $Fe(HCl)_{tot,}$ (B) sulfate, (C) Fe(II), (D) Fe(III)) of the reaction between pyrite (5 mM) with different concentrations of nitrite (0 µM (●), 40 µM (○), 200 µM (▼), 1000 µM (▽), 2000 µM (■)) in 1 M HCl. Error bars shown represent standard deviations calculated from three independent replicates.

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 $Fe(HCl)_{tot}$ against the logarithm of the corresponding initial nitrite concentrations ($n_{Fe(HCl)tot}$=0.95, $n_{Sulfate}$=0.99) (SI, Figure S2.3).

Kinetics were different in experiments under anoxic conditions where the nitrite concentration was kept constant (c=1000 µM) but in which the pyrite concentration was varied. Sulfate and $Fe(HCl)_{tot}$ 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 $Fe(HCl)_{tot}$ 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 2.3). Contrary to the first order rate dependency on initial nitrite concentrations, a fractional order ($n_{Fe(HCl)tot}$=0.51, $n_{Sulfate}$=0.64) was determined with respect to initial pyrite concentrations (SI, Figure S2.4).

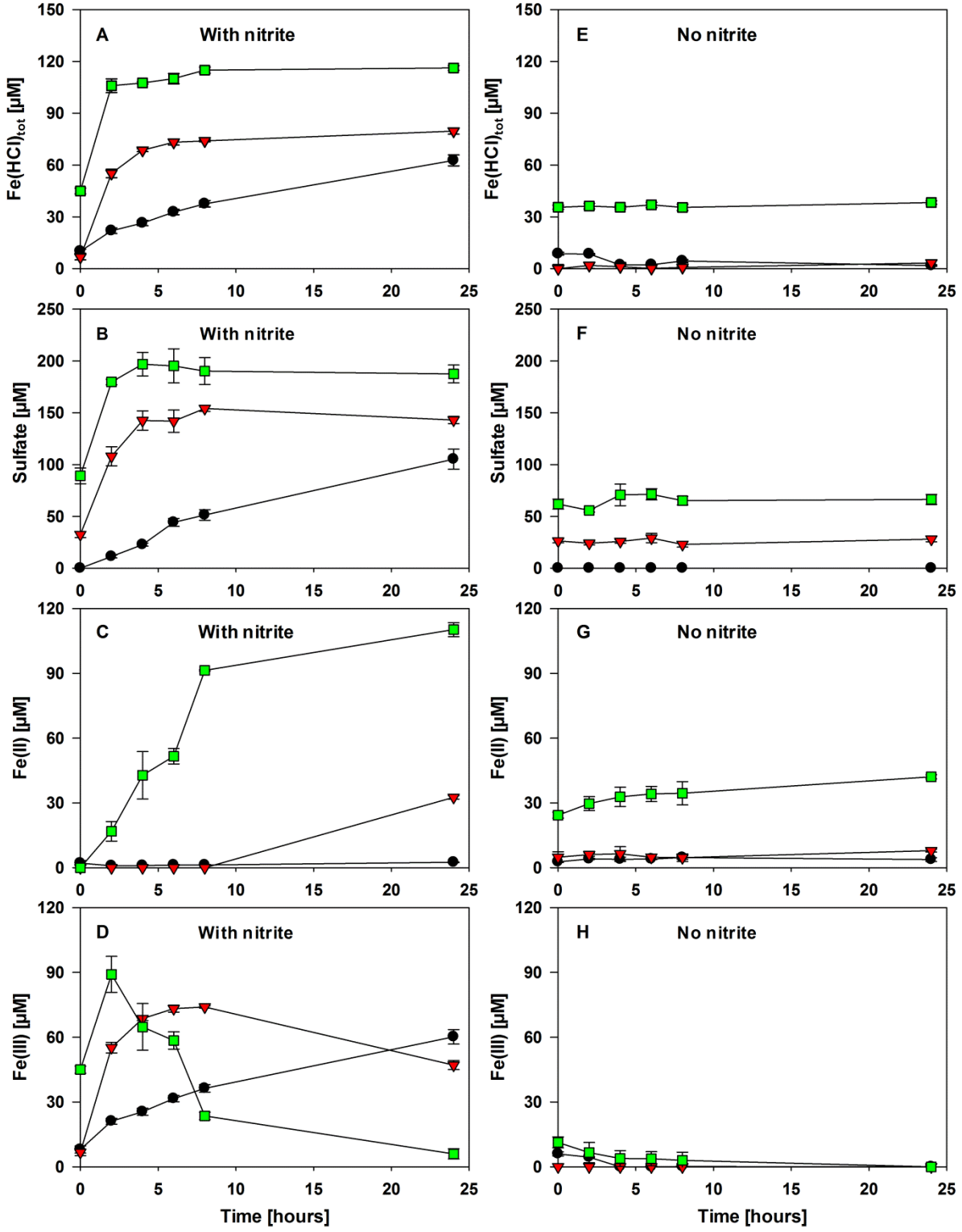

Figure 2.3 Product concentration after reaction with pyrite in the presence of 1000 µM nitrite (A-D) and in the absence of nitrite (E-H) with different concentrations of pyrite (5 mM (●), 25 mM (▼), 125 mM (■)) in 1 M HCl under anoxic conditions. Error bars were standard deviation calculated from three independent replicates.

Initial rates of pyrite oxidation were estimated as the mean value (n=3) of the product formation rates ($Fe(HCl)_{tot}$ and sulfate) within the first 2 h. Initial rates increased with increasing pyrite concentrations (6.0±0.31 µM $Fe(HCl)_{tot}$ and 5.7±0.71 µM sulfate at 5 mM pyrite, 24.2±0.83 µM $Fe(HCl)_{tot}$ and 37.8±3.37 µM sulfate at 25 mM pyrite, 30.5±2.45 µM $Fe(HCl)_{tot}$ and 45.3±4.19 µM 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 2.1) (Mckibben and Barnes 1986; Mathews and Robins 1974; Smith and Shumate 1970) indicating that pyrite oxidation by NO and/or NO_2 is a surface controlled reaction (Lowson 1982). 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(III) which binds to the disulfide group and forms a surface complex which allows for electron transfer (Luther III 1987). In analogy to this model, we propose NO and/or NO_2 instead of 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.

Table 2.1 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	
0	20 ^{o}C	0.51, 0.64 (NO_2^-)	This study
2 - 4	20 - 40 ^{o}C	0.49 (O_2)	Mckibben and Barnes (1986)
-0.1 - 1.2	30 - 70 ^{o}C	0.81 (O_2)	Mathews and Robins (1974)
2	20 - 35 ^{o}C	0.70 (O_2)	Smith and Shumate (1970)

2.4.2 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 µM) within the first 24 h in all experiments where nitrite concentration was varied at a concentration of 5 mM pyrite (Figure 2.2) since 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 µM nitrite, 105% with 200 µM nitrite, 73% with 1000 µM nitrite, 67% with 2000 µM 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 and approached the expected stoichiometric ratio of 2:1 (1.9~2.1) after 2900 h (Table 2.2). In contrast, concentrations ratios between total S and total Fe determined by ICP after 24 h were close to 2:1 (1.8~2.1) (Table 2.2) 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 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 (Basolo and Pearson 1967). 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 conversion rate to sulfate depended on the initial nitrite concentration indicating the involvement of reactive N-species in the oxidation process. At low nitrite concentrations the ratios were clearly lower (0 at 40 µM nitrite and 0.9 at 200 µM nitrite) after 24 h, while ratios were much closer to the stoichiometric ratio at 1000 µM nitrite (1.7) and 2000 µM (1.8).

Table 2.2 Change of ratios between sulfate and $Fe(HCl)_{tot}$ concentrations with time at pH = 0 (1 M HCl), c(pyrite) = 5 mM at different initial nitrite concentrations from 40 to 2000 µM at room temperature under anoxic conditions. The bottom row displays ratios between total S and Fe concentrations as measured by ICP after 24 h.

Time	**SO_4^{2-}/Fe ratios**			
(Hours)	**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

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 2.3). 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, 125 mM), average concentrations of Fe(III) were less than 5 µM in all control experiments. No sulfate and only very little Fe(II) (<5 µM) was detectable at 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 measureable but quite low (~6 µM). Sulfate concentration was detectable but below the determination level. Both Fe(II) (33 µM) and sulfate (65 µM) 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 control experiments (Figure 2.3E-G). This observation indicates the occurrence of Fe(II) already in the initial suspensions if the pyrite concentration is high. Acidic extraction of the grinded material (cf. 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 non-stoichiometric sites on pyrite (Guevremont et al. 1998), leading to the dissolution of nm-size pyrite particles as identified with SEM (Figure 2.1) 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 2.3).

Table 2.3 Change of ratios between sulfate and $Fe(HCl)_{tot}$ concentrations with time at pH = 0 (1 M HCl), $c(NO_2^-)$ = 1000 µM at different initial pyrite concentrations from 5 to 125 mM at room temperature under anoxic conditions.

Time	**$SO_4^{2-}/Fe(HCl)_{tot}$ ratios**		
[Hours]	**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

2.4.3 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 stoichiometrically predicted for complete turnover of nitrite to either N_2 or N_2O. For example, we observed that 126 µM $Fe(HCl)_{tot}$ and 241 µM sulfate were formed in the presence of 2000 µM nitrite. Complete reduction of this nitrite concentration to N_2 would, however, stoichiometrically generate 400 µM and 800 µM of $Fe(HCl)_{tot}$ and sulphate, respectively (equation 3),

$$10NO_2^- + 2FeS_2 + 8H^+ \xrightarrow{pH\,0} 2Fe^{3+} + 5N_2 + 4SO_4^{2-} + 4H_2O \quad (3)$$

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

$$30NO_2^- + 4FeS_2 + 26H^+ \xrightarrow{pH\,0} 4Fe^{3+} + 15N_2O + 8SO_4^{2-} + 13H_2O \quad (4)$$

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

It is well known that Fe(III) is a major oxidant for pyrite under acidic conditions, with the role of the dissolved oxygen being re-oxidizing Fe(II) to Fe(III) forming a cycle of iron (Singer and Stumm 1970). We therefore propose a pathway for pyrite oxidation that is based on cyclic oxidation of Fe(II) by reactive NO_2 and/or NO to Fe(III) and regeneration of Fe(II) upon reaction of Fe(III) with pyrite.

Significant amounts of dissolved Fe(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 Fe(II) completely disappeared from the initial suspension if nitrite was present (Figure 2.3C) indicating that acid-soluble Fe(II) becomes rapidly oxidized by reactive NO and/or NO_2. Similar observations were made in previous studies that investigated the abiotic oxidation of

Fe(II) to Fe(III) with nitrite under acidic or weak acidic conditions (Wullstein and Gilmour 1966; Buresh and Moraghan 1976; Ibrahim et al. 2001; Klueglein and Kappler 2013; Van Cleemput and Baert 1983) and who proposed the following reactions (equations 5-7) (Bonner and Pearsall 1982).

$$NO_2 + 2Fe^{2+} + 2H^+ \rightarrow 2Fe^{3+} + NO + H_2O \quad (5)$$

$$NO + Fe^{2+} + H^+ \rightarrow Fe^{3+} + HNO \quad (6)$$

$$2HNO \rightarrow N_2O + H_2O \quad (7)$$

The Fe^{3+} ion is a potential oxidant for pyrite under acidic conditions thereby typically forming thiosulfate (equation 8) (Luther III 1987):

$$6Fe^{3+} + FeS_2 + 3H_2O \rightarrow S_2O_3^{2-} + 7Fe^{2+} + 6H^+ \quad (8)$$

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

$$3.5NO_2 + FeS_2 + H^+ \rightarrow S_2O_3^{2-} + 3.5NO + Fe^{3+} + 0.5H_2O \quad (9)$$

$$7NO + FeS_2 + 3H_2O + H^+ \rightarrow S_2O_3^{2-} + 7HNO + Fe^{3+} \quad (10)$$

Once NO and/or NO_2 are depleted, the residual Fe(III) is steadily consumed to build up the Fe(II) pool observed after 2900 h (Figure 2.2) upon reaction with pyrite. The proposed cyclic model also explains the pyrite-concentration dependent turnover rate of Fe(III) to Fe(II) observed in Figure 2.3. The higher the concentration of pyrite the faster Fe(III) being converted into Fe(II).

The current data do not exclude direct oxidation of pyrite by NO_2 and/or NO. A possible mechanism is that under acidic conditions reactive NO_2 and/or NO that form from self-decomposition of HNO_2/NO_2^- (equations 1 and 2) (Nelson and Bremner 1970a; Park and Lee 1988; Ibrahim et al. 2001) directly react with pyrite. Due to a dynamic equilibrium only a certain fraction of HNO_2 decomposes to NO_2 and/or NO being available for reaction with

pyrite during the reaction in 1 M HCl. Additionally, degassing of gaseous NO_2 and/or NO may contribute to the observed non-stoichiometric pyrite oxidation. However, this model does not explain the increase in Fe(II) concentration observed after 2900 h (Figure 2.2) and the observed dynamic behaviour of Fe(II) in Figure 2.2 and 2.3 strongly supports the cyclic model.

2.4.4 Effect of oxygen and pH on pyrite oxidation by nitrite

The presence of oxygen clearly affected the oxidation of pyrite by nitrite (Figure 2.4). 5 mM pyrite were incubated with 1000 µM nitrite in 1 M HCl under anoxic and oxic conditions. The concentration of acid-extractable Fe(II) were below the detection limit (<5 µM) in both anoxic and oxic experiments within 24 h. The initial concentration increase of Fe(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 Fe(III) and sulfate increased much more rapidly in the presence of oxygen. After 24 h, 116±10.7 µM sulfate and 52.4±1.9 µM Fe(III) were observed under anoxic conditions corresponding to a ratio of 2.2, whereas concentrations reached values of 152±7.6 µM and 102±5.8 µM, respectively, under oxic conditions with a ratio of 1.5.

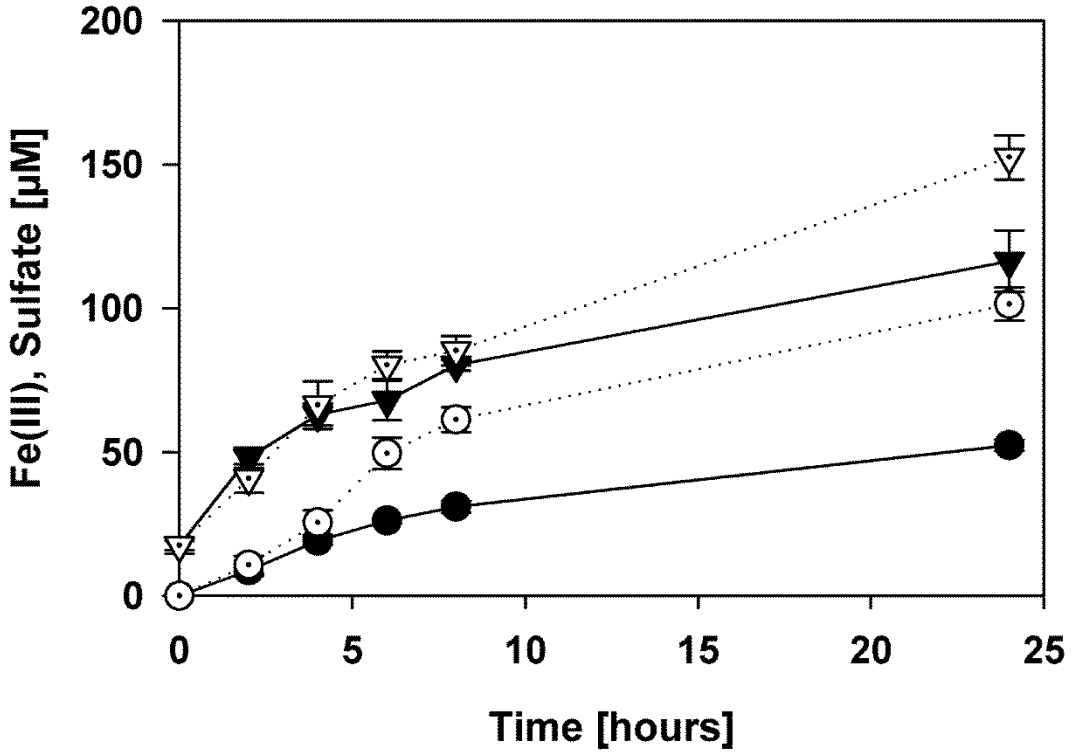

Figure 2.4 Concentration of Fe(III) (●) and sulfate (▼) in experiments of reactions of nitrite (1000 µM) with pyrite (5 mM) in 1 M HCl under anoxic (closed symbols and solid line) or oxic (open symbols and dotted line) conditions. Error bars shown represent standard deviations calculated from three independent replicates.

Oxygen clearly enhanced the extent of pyrite oxidation by NO and/or NO_2 under acidic conditions. Oxygen itself is a weak oxidant for pyrite even under acidic conditions (Singer and Stumm 1970; Nordstrom 1982; Luther III 1987; Moses et al. 1987), 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 (equation 11) (Prather and Miyamoto 1974; Van Cleemput and Samater 1995) being a stronger oxidant for the oxidation of Fe(II) under acidic conditions than NO (Klueglein and Kappler 2013).

$$NO + O_2 \rightarrow 2NO_2 \qquad (11)$$

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 since the increase in Fe(III) yield after 24 h (50 µmol/L) in the presence of oxygen was greater than of sulfate (36 µmol/L, Figure 2.4)

Anoxic oxidation of pyrite in the presence of nitrite appeared not to be effective at pH 5.5 and 6.8 (SI, Figure S2.5). Concentrations of nitrite and sulfate remained at the same level as those in the control experiments and also the pH did not vary by more than 0.05 pH units. Contrary to the oxidation of ferrous iron by nitrite (Buresh and Moraghan 1976; Van Cleemput and Baert 1983) and the oxidation of pyrite in the presence of other oxidants like Fe(III) or dissolved oxygen which are observable at circumneutral pH (Peiffer and Stubert 1999), abiotic pyrite oxidation by nitrite seems not to occur under these conditions. Hence, contribution of nitrite to abiotic oxidation of pyrite in anoxic circumneutral groundwater aquifers seems not to be an important pathway. The reason probably is that HNO_2 as the precursor of reactive (i.e. pyrite-oxidizing) NO_2 and/or NO occurs at relevant concentrations only at pH<5 (pK_a=3.35), while the ionic species nitrite, i.e. NO_2^-, is not reactive towards pyrite.

2.5 Implications for studies on microbial nitrate-dependent pyrite oxidation

This study has demonstrated that grinded 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 2.1) which is either of pyritic (FeS_2) origin or $Fe(HSO_4)_2$ as the product of the pyrite dissolution. This assumption is supported by EDX spectra (SI, Figure S2.1, Tables S2.1-S2.2) taken from these structures displaying 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 approx. 0.26 mol ‰ of the initial pyrite content (Figure 2.3G). Similar nanostructures were observed on grinded pyrite crystals not pretreated with HCl (Bosch et al. 2012), which 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 NO_2^--derived reactive N-species under acidic conditions. Studies about microbial nitrate-dependent oxidation of pyrite should consider these interferences. An assessment of possible interferences from nitrite in previous studies is difficult since their experimental approaches cannot be directly compared to our study (SI, Table S2.3 in the Supporting Information where we compiled previous laboratory studies). Several studies did not perform acid extractions of pyrite containing samples to determine the formation of Fe(III) (Jørgensen et al. 2009; Torrentó et al. 2010; Vaclavkova et al. 2014; Haaijer et al. 2007). Fe(II) extracted with 1 M HCl from the pyrite suspension was completely (0.13 mM) oxidized to Fe(III) after 24 h extraction in 1 M HCl in the presence of nitrite (100-800 µM) (Bosch et al. 2012). Given the results obtained in the present study one cannot exclude interference of nitrite in the oxidation process described by these authors.

2.6 A revised protocol for acidic Fe extraction in nitrite containing pyrite suspensions

In order 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 2.4 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 the washed samples, in which initial concentrations remained constant after 24 h of acidic extraction. We therefore recommend to consider 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.

Table 2.4 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 with 50 mM pyrite and 10 mM nitrite at pH 6.4 after acidic extraction 1:10 diluted in 1 M HCl under anoxic conditions. The pyrite and nitrite concentrations were 5 mM and 1 mM during acidic extraction, respectively.

Anoxic extraction with 1 M HCl after 24 h								
Set up	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

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2.7 References

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2.8 Supporting information for Study 1

Figure S2.1 Scanning electron microscopy image of ground pyrite.

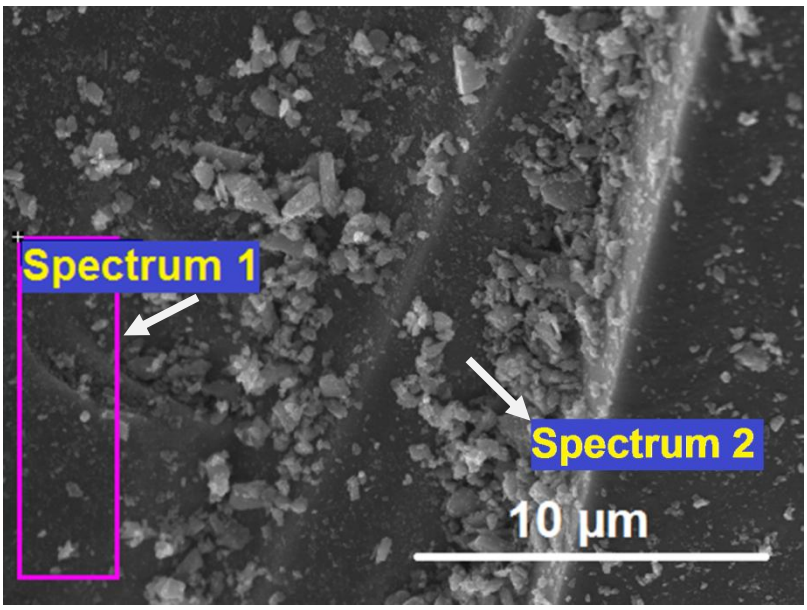

Table S2.1 Elemental analysis by EDX of a fraction of the pyrite surface (spectrum 1) with only few nano-particles.

Element	Mass %	Atom %
C	4	11
S	51	58
Fe	46	30
total	100	

Table S2.2 Elemental analysis by EDX of a fraction of the pyrite surface (spectrum 2) covered with a high density of nano-particles.

Element	Mass %	Atom %
C	3	11
S	50	59
Fe	46	31
total	100	

Figure S2.2 X-ray diffractogram of ground pyrite. Green lines reflect the expected diffractogram of pyrite (Brostigen and Kjekshus 1969).

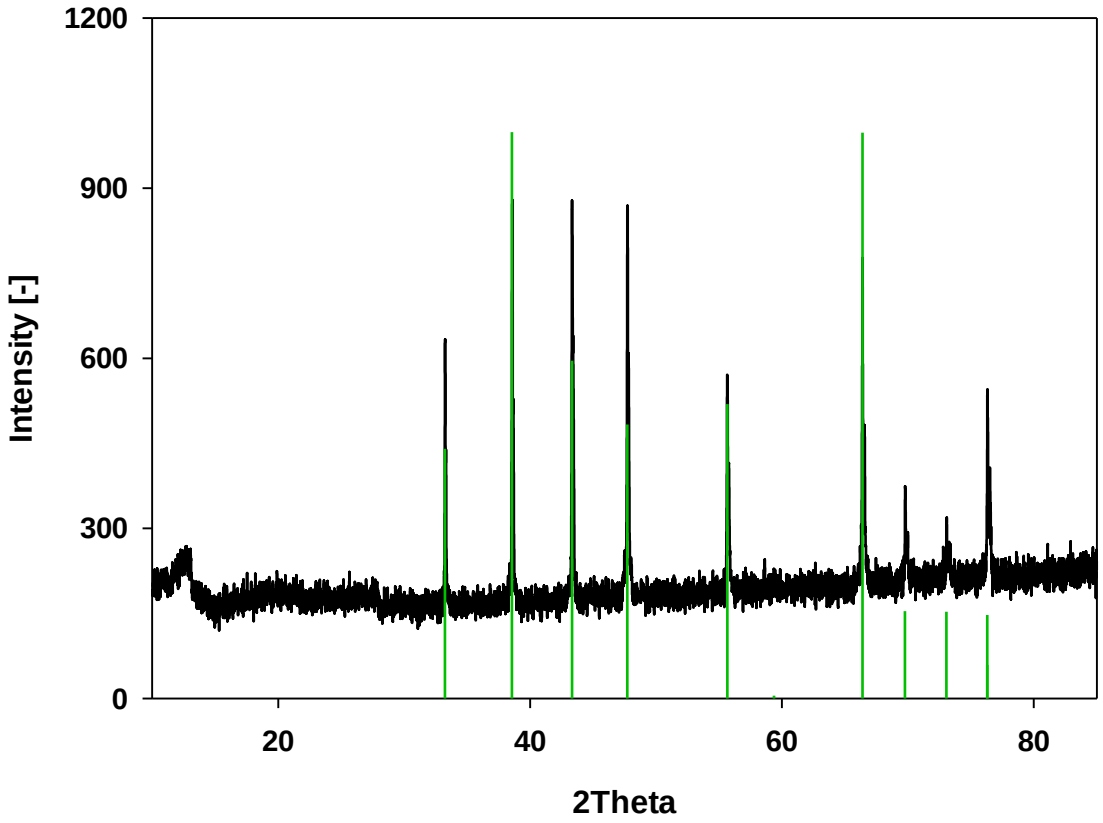

Figure S2.3 Kinetic data from experiments with different concentrations of nitrite. Plot of the logarithm of the initial formation rates (24 h) of $Fe(HCl)_{tot}$ and sulfate against the logarithm of the corresponding initial nitrite concentrations (40 µM to 2000 µM)

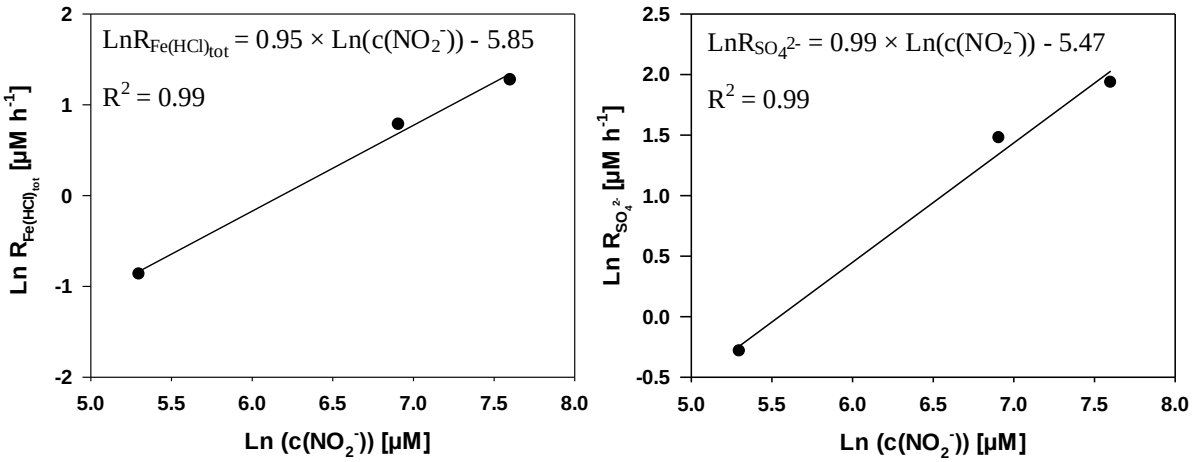

Figure S2.4 Kinetic data from experiments with different concentrations of pyrite. Plot of the logarithm of the turnover (2 h) of $Fe(HCl)_{tot}$ and sulfate against the logarithms of the corresponding initial pyrite concentration (5 mM to 125 mM)

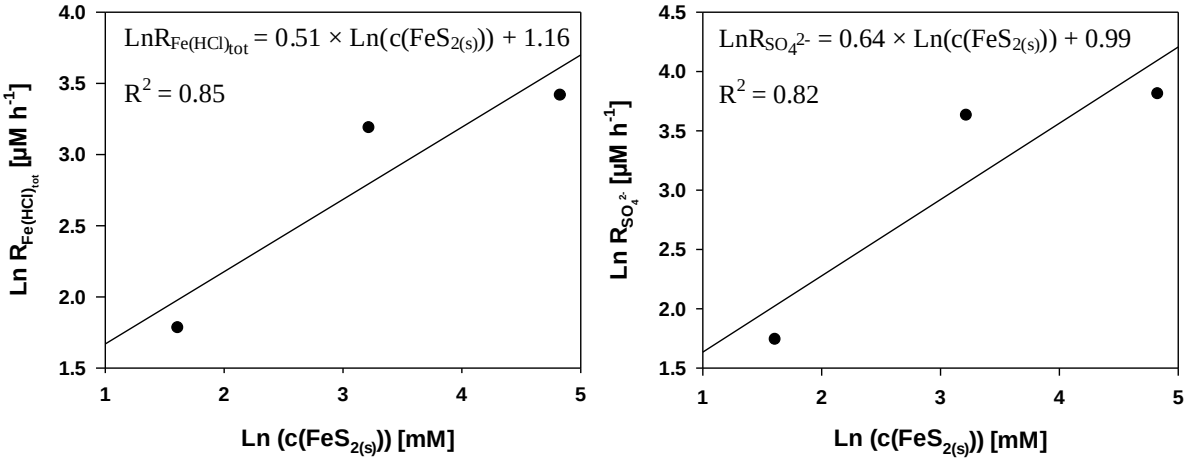

Figure S2.5 Reaction of nitrite (400 µM) with 50 mM pyrite at pH 5.5 (left), pH = 6.8 (right): concentrations of NO_2^- in the presence (●), and absence of pyrite (○), SO_4^{2-} concentration in the presence (▼), and absence of nitrite (∇). Error bars were standard deviation calculated from three independent replicates.

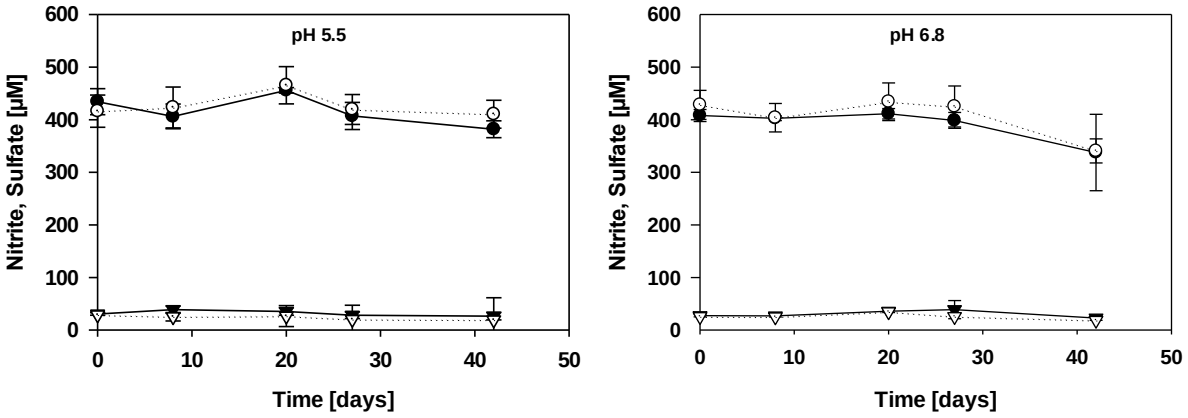

Table S2.3 Overview of previous batch studies on chemolithotrophic denitrification coupled to pyrite oxidation.

Reference	Haaijer et al., (2007)	Jørgensen et al., (2009)	Torrentó et al., (2010)	Bosch et al., (2012)	Vaclavkova et al., (2014)
Experimental approach	Batch incubation	Batch incubation	Batch incubation	Batch incubation	Batch incubation
Pyrite species	Ground crystals of 0.5–3 mm	Ground crystals of 45–200 μm	Ground crystals of 25-50 and 50–100 μm	Ground pyrite (nanoparticles of 0.1-1.2 μm)	Various ground pyrites (no information on particle size)
Concentrations of pyrite added [mM]	33.3	18.5	1667	3.96	87
Concentrations of nitrite [μM]	840, 1700, 1400	0-200	0-1000	100-800	0-300
Filtration of samples	No	No	Yes	No	No
Centrifugation of samples	Yes	No	No	No	No
Acidification of samples/pH	No	No	Yes (diluted in nitric acid)/the nitric acid concentration and dilution factor was not shown	Yes (diluted in 1 M HCl)/pH ~ 0	No
Extraction time	No	No	Data not shown	24 h	No
Method of Fe quantification	Photometric	No	ICP-AES	Photometric	No
Fe data	Water-insoluble and dissolved Fe(III) was measured but data not shown	Not measured	Total dissolved Fe, data not shown.	Complete oxidation of initially extractable Fe(II) (0.13 mM)	Not measured
Fe data as a proof for pyrite oxidation	No	No	No	Yes	No
Evidence for pyrite oxidation/based on	No/nitrate, nitrite and sulfate remained the same as that of the medium-feed.	Yes/decreasing nitrate with production of sulfate and nitrite	Yes/decreasing nitrate with production of sulfate and nitrite	Yes/formation of Fe(III)	Yes / decreasing nitrate with production of sulfate and nitrite

Experimental details for the testing of the revised protocol for nitrite-free acidic Fe extraction in nitrite containing pyrite suspensions.

Experiments and controls were performed in three independent replicates. 0.06 g NaCl as electrolyte and 0.6 g pyrite were weighed into 120 mL glass serum bottle in the glovebox. 100 mL anoxic ultrapure water was added to get a final concentration of 10 mM and 50 mM, respectively. These bottles were sealed with butyl stoppers, crimped and shaken. The pH value was adjusted with NaOH in each serum bottle to pH 6.4. Samples were taken before the addition of nitrite to determine background values of Fe(II) and $Fe(HCl)_{tot}$. Thereafter, 1 mL of a 100 mM deaerated $NaNO_2$ stock solution was added to 3 bottles to obtain a NO_2^- concentrations of 10 mM. Control experiments were performed without the addition of nitrite. For the unwashed samples, aliquots of 0.1 mL were withdrawn from each serum bottle and immediately diluted 1:10 in 1 M HCl. For the washed samples, aliquots of 0.1 mL were taken from the serum bottles and filled in Eppendorf tubes and then washed with 1 mL ultrapure water. After centrifugation, 1 mL supernatant was removed with a pipette. This washing process was performed three times when no nitrite could be detected any more by nitrite indicator strips with a range of 0.05-25 mg/L. The residual suspension (0.1 mL) was diluted 1:10 in 1 M HCl. Samples before the addition of nitrite, unwashed samples and washed samples in Eppendorf tubes were placed in a gas-tight container, removed from the glovebox and shaken for 24 h. Fe(II) and Fe(tot) were then measured with the ferrozine assay.

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3. Study 2: The effect of reduced sulfur speciation on the chemolithoautotrophic pyrite oxidation with nitrate

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3.1 Abstract

We compared different sources of reduced S (pyrite, S(0) and marcasite) and reduced Fe to act as electron donor for denitrifying bacteria strains at neutral pH. Chemolithoautotrophic oxidation of pyrite with nitrate as electron acceptor was not possible if the pyrite source is pure crystalline pyrite, while oxidation of synthesized pyrite species of low crystallinity as well as S(0) is possible. Neither formation of nitrite nor sulfate was observed in the presence of Fe(II)-oxidizing strain *Acidovorax sp.* BoFeN1. The reaction appears to be induced via S oxidation but not via Fe oxidation.

Keywords: denitrification, iron-oxidizng bacteria, pyrite oxidation, sulfur-oxidizing, bacteria, reduced sulfur.

3.2 Introduction

Denitrification is an important anaerobic attenuation process for nitrate which has been observed in many groundwater systems (Hiscock et al. 1991).Various organic or inorganic electron donors (e.g iron sulfides) drive heterotrophic or autotrophic denitrifers in the environment, respectively (Korom 1992). It has been postulated since decades that denitrification can be coupled to the oxidation of pyrite mediated by chemolithoautotrophic strains such as *Thiobacillus denitrificans* (Kölle et al. 1983). The net consumption of nitrate with concomitant generation of sulfate and dissolved Fe(II) is generally regarded to be indicative for this process in pyrite bearing aquifers (Postma et al. 1991; Tesoriero et al. 2000; Zhang et al. 2009; Pauwels et al. 2000). The release of pyrite-associated trace metals such as As, Ni, Co, Zn (Zhang et al. 2009; Van Beek et al. 1989; Evangelou and Zhang 1995; Broers 1998) and aqueous uranium (van Berk and Fu 2017) concomitant to nitrate removal was regarded as further evidence for 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 (Haaijer et al. 2007; Jørgensen et al. 2009; Torrentó et al. 2010; Bosch et al. 2012). However, results of these studies are contradicting. Incubation of natural sediment to which ground pyrite was added did not provide any evidence for denitrification coupled to pyrite oxidation (Schippers and Jorgensen 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 autotrophic 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 a microbially mediated denitrification with pyrite as the electron donor. Bosch et al. (2012) have described oxidation of pyrite nanoparticles by the nitrate-reducing bacterium *Thiobacillus denitrificans*. Their conclusion was based on an electron balance established based on the formation of ferric iron and sulfate along with the reduction of nitrate to nitrite. However, acidic extraction of pyrite suspensions to determine ferric hydroxide as the reactant of pyrite oxidation may lead to significant overestimation of ferric iron if nitrite is present, because this N-species is able to oxidize pyrite under acidic conditions (Yan et al. 2015). Hence, detection of ferric iron as the product of pyrite oxidation may be misleading unless great care is taken to prevent such or other artifacts. To shed light on these contradictory observations, we have, therefore, set up a systematic series of experiments to compare different sources of reduced S (pyrite, elemental sulfur and marcasite) and reduced Fe (pyritic Fe(II), dissolved Fe(II)) with regard to their ability to act as electron donor for denitrifying bacterial strains. To do so, we used two types of pyrite materials: 1) a low-grain-size synthesized pyrite, which was a mixture of pyrite, marcasite and sulfur; and 2) a pure crystalline pyrite, which was carefully treated prior to the experiments to remove impurities.

3.3 Materials and methods

3.3.1 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 was synthesized following a procedure described by Peiffer and Stubert (1999) and Berner (1970). Contrary to the previous work, the synthesis was conducted in an anoxic glovebox (Innovative Technology, Massachusetts, USA, 100% N_2) at room temperature (20±2°C). A solution of 0.1 mol L^{-1} 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 a final concentration of 0.1 mol L^{-1} and 0.2 mol L^{-1}, respectively. The bottle was closed with a plastic cap and gas-tight sealed with silicon gel, removed from the glovebox, stored in an oven at 60°C and shaken by hand twice a day. After two weeks, the supernatant was decanted and the sediment was sieved to remove unreacted sulfur particles. The fraction between 0.63 mm and 1.4 mm was collected and washed 3 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 material was washed 3 times with acetone to remove water and elemental sulfur and then washed 9 times with petroleum ether to remove the remaining elemental sulfur. In spite of these treatments the residual sulfur content was 4.6 mass% (as detected by HPLC, cf. below). After the washing procedure, the material 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 by 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).

3.3.2 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 $MgSO_4 \cdot 7H_2O$, 20.1 mM $Na_2S_2O_3 \cdot 5H_2O$, 30.0 mM $NaHCO_3$, 0.007 mM $FeSO_4 \cdot 7H_2O$, and trace element solution SL-4.

*Acidovorax sp.*BoFeN1 isolated from Lake Constance sediments is a mixotrophic bacterium that grows with acetate plus Fe(II) 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 containing 10 mM nitrate as electron acceptor and 5 mM acetate as sole carbon substrate and was prepared as described by Hegler et al. (2008) and Hohmann et al. (2009).

Thiobacillus denitrificans and *Acidovorax sp.* BoFeN1 were grown at 30 °C under an atmosphere of 80% N_2 and 20% CO_2 in the dark and unshaken. Growth of the cultures was measured by following the optical density (OD) of the culture media at a wavelength of 600 nm (OD_{600}) in a spectrophotometer. Total cell number was measured 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.

3.3.3 Experimental set up

Two types of batch experiments were conducted: (1) Inoculation of synthesized pyrite (characterized as a mixture of pyrite, marcasite and elemental sulfur) with the nitrate-reducing sulfide-oxidizing bacterium *Thiobacillus denitrificans* or the nitrate-reducing Fe(II)-oxidizing bacteria *Acidovorax sp.* BoFeN1. Control experiments were performed with *Thiobacillus denitrificans* in the presence of i) dissolved Fe(II) to test whether Fe(II) will be oxidized with

nitrate as electron acceptor and ii) dissolved Fe(III) to test whether abiotic oxidation of pyrite by Fe(III) (Peiffer and Stubert 1999) may stimulate pyrite oxidation; (2) Inoculation of ground pure pyrite, proven free of elemental sulfur with *Thiobacillus denitrificans* to test the occurrence of nitrate-dependent pyrite oxidation in the absence of sulfur as potential impurity in natural and synthesized samples.

In order to avoid interference of sulfur from the medium in the determination of formation rates of sulfate from pyrite, the medium for the batch experiments with *Thiobacillus denitrificans* was prepared without thiosulfate and iron (as being used in the growth medium). The modified reaction medium (pH 6.8) contained 15 mM KH_2PO_4, 19 mM NH_4Cl, 3.2 mM $MgCl_2 \cdot 6H_2O$, 30 mM $NaHCO_3$ and the same concentration of trace element solution SL-4 as described above. The medium used for the batch experiment with *Acidovorax sp.*BoFeN1 was the same as the nutrient medium for cultivation.

For batch experiments with synthesized pyrite, 100 mL of medium and 0.1 g of the material (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) under 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 pyrite, 1 mL of anoxic KNO_3 (1M) stock solution was injected to the serum bottles through the butyl stopper (final concentration of approximately 9 mM) using a syringe that was several times flushed with N_2. 0.1 mL or 1 mL of the pure cultures of *Thiobacillus denitrificans* were added into each serum bottle to obtain cell density of 9.3×10^6 or 9.3×10^7 cells ml^{-1}. Serum bottles used for experiments with *Acidovorax sp.* BoFeN1 were prepared in a similar way. Prior to inoculation with bacteria (cell density 1.15×10^7 or 1.15×10^8 cells ml^{-1}), aliquots of oxygen-free $NaNO_3$ (1 M) and Na acetate (1 M) solutions were added to obtain final concentration of 9 mM and 5 mM, respectively. Parallel batch experiments were

performed in order to test a potentially stimulating effect of redox-active substances. 100 µl of 100 mM sterile $FeCl_2 \cdot 4H_2O$ or 100 µl of 100 mM $FeCl_3 \cdot 6H_2O$ were added to the serum bottles containing synthesized pyrite, nitrate and *Thiobacillus 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 with synthesized pyrite that the medium was added to the serum bottles outside the glovebox using a Widdel flask. 100 mL medium were filled into each autoclaved glass serum bottle under an atmosphere of 80% N_2 and 20% CO_2 using two needles. The bottles were then sealed with butyl stoppers and crimped. All bottles were brought into the anoxic 100% N_2-filled glovebox (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 aliquots of a oxygen-free KNO_3 stock solution (1 M) were added to the medium to obtain a final nitrate concentration of 10 mM. Since we observed substantial denitrification due to stored sulfur by *Thiobacillus denitrificans* in control experiments with high cell numbers of *Thiobacillus denitrificans* (cf. results and discussion sections), we tried to keep cell densities as low as possible. Therefore, 0.1 mL or 1 mL of the pure cultures of *Thiobacillus denitrificans* were added into each serum bottle to obtain cell densities 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. Control experiments contained only pyrite and nitrate without *Thiobacillus denitrificans*, only pyrite and *Thiobacillus denitrificans* without nitrate, as well as only nitrate and *Thiobacillus denitrificans* without pyrite to monitor

the background reaction. To proof the viability of the cell suspension, a control experiment was set up with 50 mM elemental sulfur and 10 mM nitrate in the presence of *Thiobacillus denitrificans*. Batch experiments were conducted in three independent replicates and controls in two independent replicates.

3.3.4 Chemical analyses

Aliquots of approximately 2 mL were anoxically withdrawn from serum bottles. Concentrations of nitrate, nitrite and sulfate were quantified by ion chromatography with chemical suppression and conductivity detector using an A-supp 4 anion column (Metrohm, Herisau, Switzerland) after filtration of a sample through 0.22 µm pore size filter (Nylon) to stop the microbial reaction and remove the residual particles. Filtered samples were diluted with ultrapure water prior to analysis.

For the quantification of the elemental sulfur content of the solid phases, 0.5 g of ground pyrite and synthesized pyrite were added to a 120 mL glass serum bottle. The bottles were sealed and crimped and the headspace of the bottles was flushed with nitrogen. 20 mL oxygen-free methanol were subsequently added to the serum bottles with a glass syringe. 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 hours to extract elemental sulfur. Thereafter, an aliquot of ca. 1.5 mL of each sample was extracted and filtered through a 0.22 µm pore size filter (Nylon) and then analyzed by HPLC (PerkinElmer 2000 pump and autosampler, Fa. linear-UV−VIS detector and software peaksample 409, 265 nm).

Attempts to determine ferric iron failed due to interference with nitrite (Klueglein and Kappler 2013) and which we were not aware about by the time the measurements were done. Samples to determine the amount of FeOOH formed from oxidation of pyritic Fe were acidified with HCl (pH=1) in order to dissolve ferric iron for further quantification. However,

it turned out that pyritic Fe(II) becomes rapidly oxidized under these conditions in the presence of nitrite (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 further consider results from these measurements in the discussion.

3.4 Results

3.4.1 Characterization of pyrite

XRD patterns revealed that the ground material was pure pyrite (Figure 3.1) while the synthesized pyrite was a mixture of pyrite and marcasite (Figure 3.2). Diffraction intensities indicated a mixture of pyrite and marcasite at a ratio of approximately 1:2 (data not shown). In the following we will use pyrite as a synonym for both minerals. The ground pyrite was of crystalline structure (Figure 3.3) and contained only very small amounts of residual elemental sulfur (0.001 mass % as detected by HPLC, cf. below). Although the ground pyrite was washed several times with HCl, nm-sized structures were still visible in the SEM images on the surface of pyrite (Figure 3.3). EDX spectra (see Supporting Information (SI), Figure S3.1, Tables S3.1-S3.2) taken from these nm-sized structure samples displayed a Fe:S ratio of approximately 1:2 indicating that the nm-sized particles also consisted of pyrite. According to the classification by Ainsworth and Blanchar (1984), the synthesized pyrite consisted of conglomerates with irregular surfaces composed of cemented particles (Figure 3.4). It still contained a large amount of elemental sulfur (4.6 mass%). The BET surface area of the ground pyrite and the synthesized pyrite was 0.17 and 0.41 $m^2 g^{-1}$, respectively.

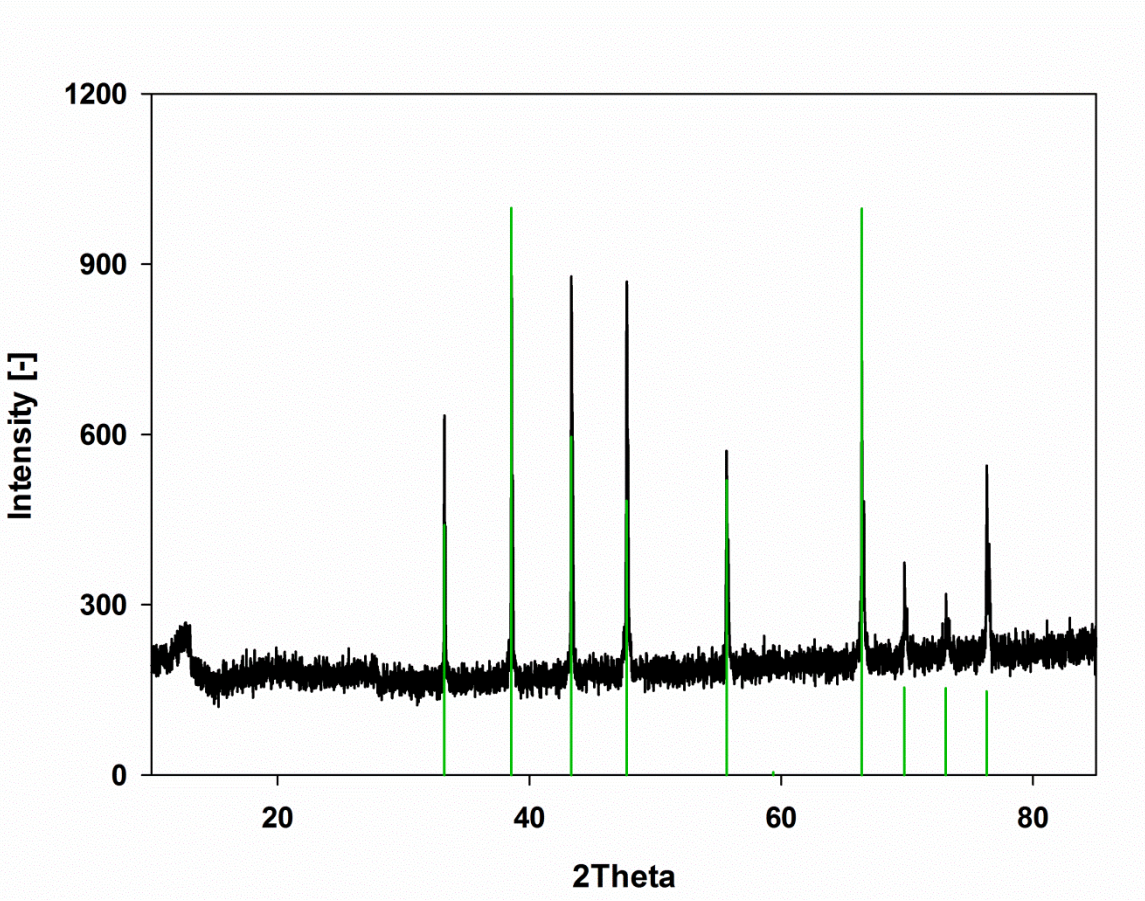

Figure 3.1 X-ray diffractogram of ground pyrite. Green lines reflect the expected diffractogram of pyrite (Brostigen and Kjekshus 1969).

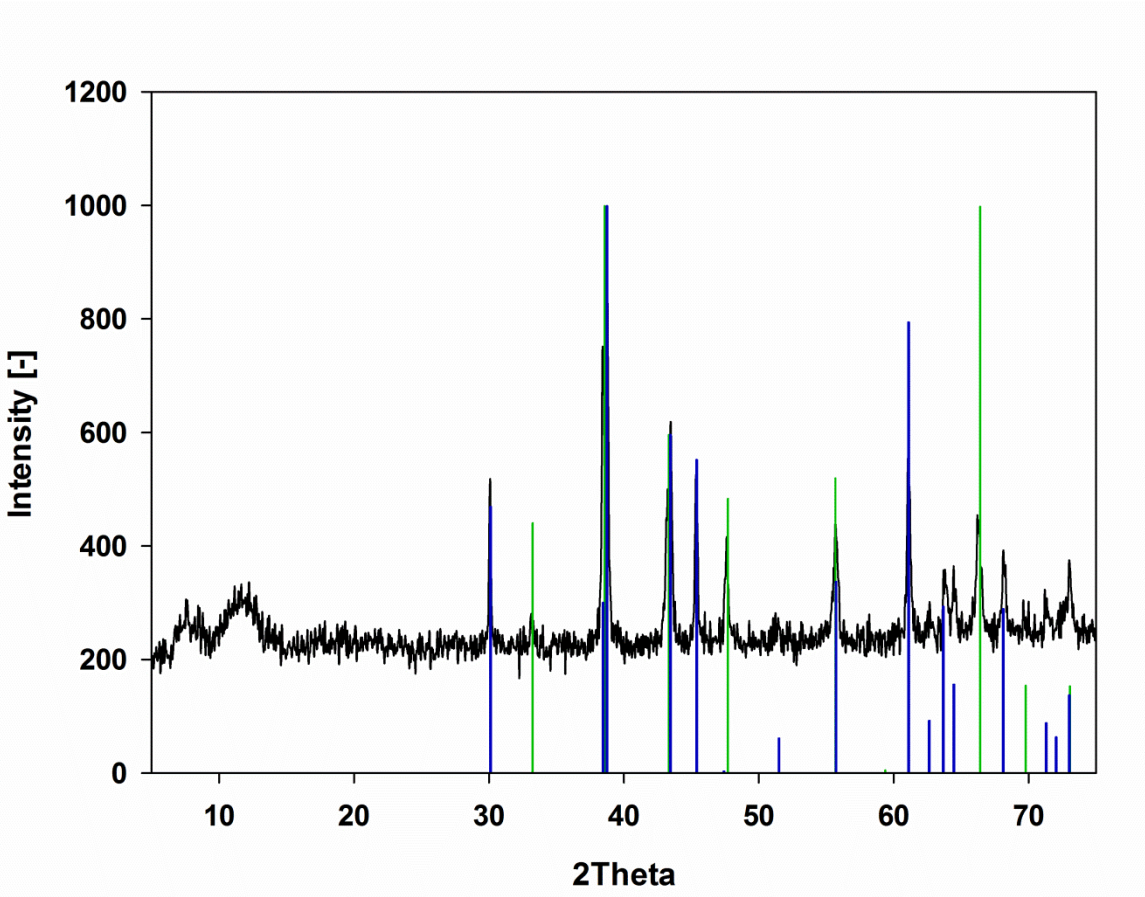

Figure 3.2 X-ray diffractogram of synthesized pyrite. Green lines reflect the expected diffractogram of pyrite (Brostigen and Kjekshus 1969) and blue lines reflect the expected diffractogram of marcasite (Rieder et al. 2007).

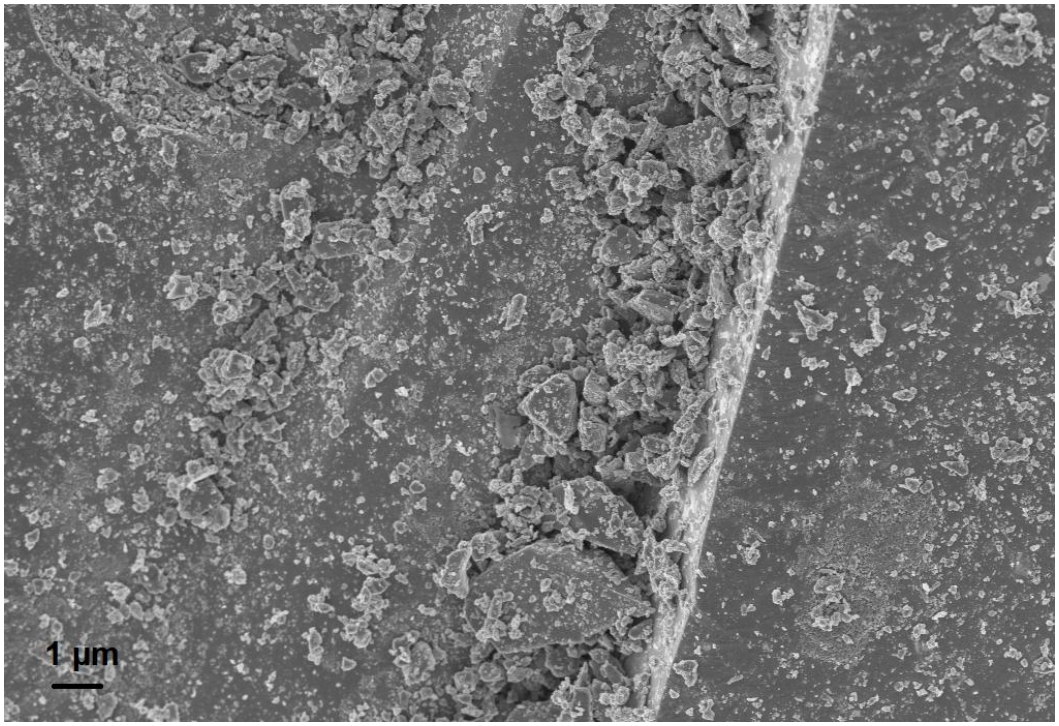

Figure 3.3 Scanning electron micrograph of the ground pyrite after preparation.

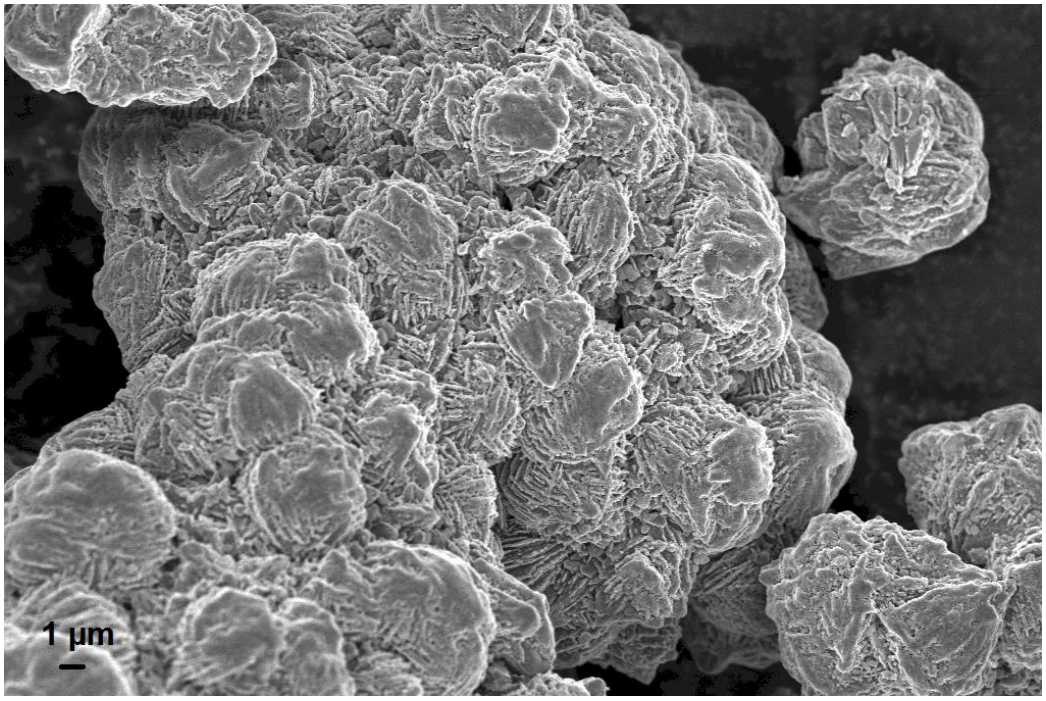

Figure 3.4 Scanning electron micrograph of the synthesized pyrite after preparation.

3.4.2 Oxidation of synthesized pyrite in the presence of nitrate

Throughout the entire experiment (43 d) with 8.3 mM synthesized pyrite and 9.2 mM nitrate in the presence of *Thiobacillus denitrificans* with a cell density of 9.3×10^6 or 9.3×10^7 cells ml^{-1}, sulfate was generated from synthesized pyrite in the presence of the S-oxidizing nitrate-reducing bacterium *Thiobacillus denitrificans* and nitrate reduction was accompanied by significant formation of nitrite (Figure 3.5A). Nitrate reduction and sulfate generation started directly from the beginning of the experiment and the rates of the reactions at higher cell density (9.3×10^7 cells ml^{-1}) were higher (0.16 mM nitrate consumption day^{-1} and 0.15 mM sulfate formation day^{-1}) compared to those at lower cell density (0.12 mM nitrate consumption day^{-1} and 0.13 mM sulfate formation day^{-1}) from the 9th day to 43rd day. In contrast, no formation of either nitrite or sulfate could be observed concomitant to nitrate consumption in the experiment with 8.3 mM synthesized pyrite in the presence of Fe(II)-oxidizing strain *Acidovorax sp.* BoFeN1 with a cell density of 1.2×10^7 or 1.2×10^8 cells ml^{-1} (Figure 3.5B). Abiotic control experiments containing pyrite and nitrate but no cell suspension of either *Thiobacillus denitrificans* or *Acidovorax sp.* BoFeN1 showed no reaction (Figure 3.5C). However, a control experiment containing only nitrate, and a cell suspension of *Thiobacillus denitrificans* without pyrite lead to consumption of nitrate accompanied by the formation of sulfate and nitrite (Figure 3.5D). However, the consumption of nitrate in the control experiment (ΔNO_3^- = 4.6 mol L^{-1}) was distinctly lower than in the presence of the pyrite species (ΔNO_3^- = 7.5 mol L^{-1}) suggesting the occurrence of chemolithoautotrophic reduction of nitrate by the reduced sulfur species added.

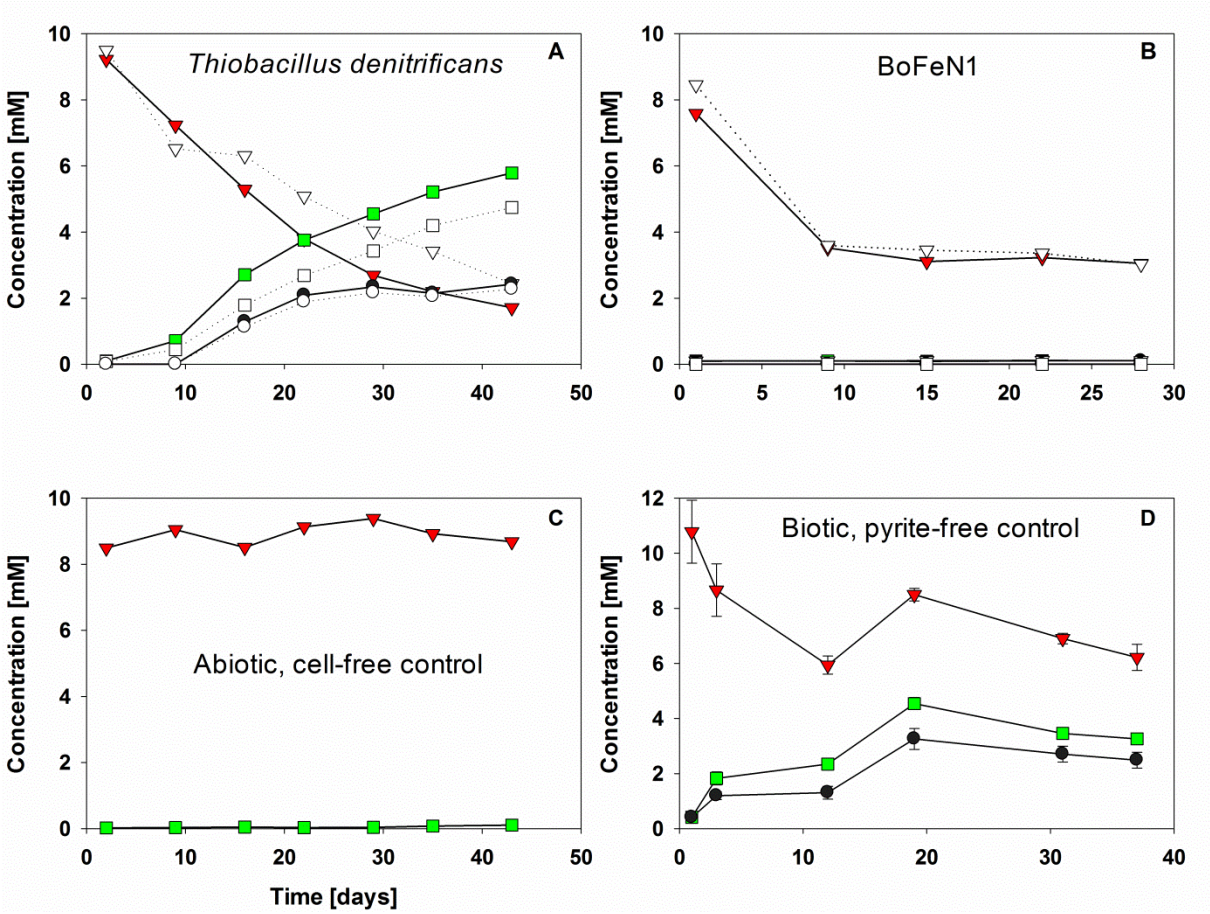

Figure 3.5 Product concentration (nitrite (●), nitrate (▼), sulfate (■)) of the reaction between synthesized pyrite (8.3 mM) and nitrate (approximately 10 mM) in the presence of (A) *Thiobacillus denitrificans* with a cell density of 9.3×10^{6} (open symbols and dotted line) or 9.3×10^{7} cells ml^{-1} (closed symbols and solid line), (B) *Acidovorax sp.* BoFeN1 with a cell density of 1.2×10^{7} (open symbols and dotted line) or 1.2×10^{8} cells ml^{-1} (closed symbols and solid line) and and (C) abiotic, cell-free control under anoxic, circumneutral conditions. (D) biotic, pyrite-free control experiment with nitrate (approximately 10 mM) in the presence of *Thiobacillus denitrificans* with a cell density of 1.8×10^{8} cells ml^{-1}. Concentrations were calculated as the mean values of two independent replicates. Concentrations were calculated as the mean values of three independent replicates and error bars are standard deviations calculated from three independent replicates.

In control experiments to which Fe(II) or Fe(III) were added, no increase in the consumption of nitrate and the formation of sulfate could be observed relative to experiments in the absence of these species (Figure 3.6A, 3.6B). Rather, nitrate consumption and sulfate production rates appeared to be even smaller. Addition of Fe(III) decelerated the formation of nitrite (Figure 3.6C). In summary, the addition of Fe(II) or Fe(III)) did not stimulate the oxidation of pyrite by nitrate under our experimental conditions. Similar results were obtained at the lower cell density of 9.3×10^{6} cells ml^{-1} (data not shown).

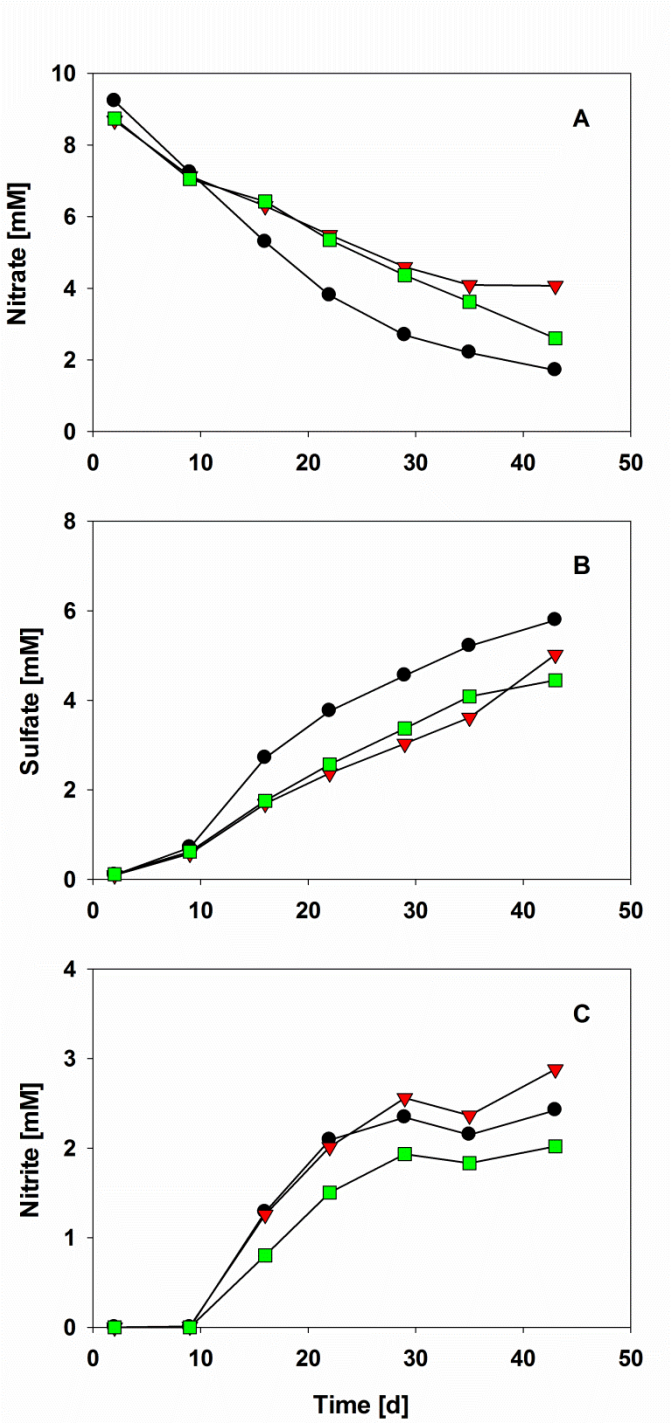

Figure 3.6 Product concentration (nitrate (A), sulfate (B), nitrite (C)) of the reaction between synthesized pyrite (8.3 mM) and nitrate (10 mM) in the presence of *Thiobacillus denitrificans* (9.3×10^7 cells ml^{-1}) in the presence and absence of either Fe(II) or Fe(III) (without Fe(II) and Fe(III) (●) the data were the same as in Figure 3.5A at a cell density of 9.3×10^7 cells ml^{-1}, with Fe(II) (▼), with Fe(III) (■)). Concentrations were calculated as the mean values of two independent replicates.

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

In order to minimize the interference of denitrification due to stored sulfur by *Thiobacillus denitrificans* observed in the control experiments with high cell densities, the experiments with ground crystalline pyrite were set up with a low cell density.

Throughout the entire experiment (87 d) with 50 mM ground crystalline pyrite and 10 mM nitrate in the presence of *Thiobacillus denitrificans* with a cell density of 2×10^4 cells ml^{-1} and 2×10^5 cells ml^{-1}, the concentration of nitrate remained stable (Figure 3.7). 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 density of this experiment (Figure 3.7).

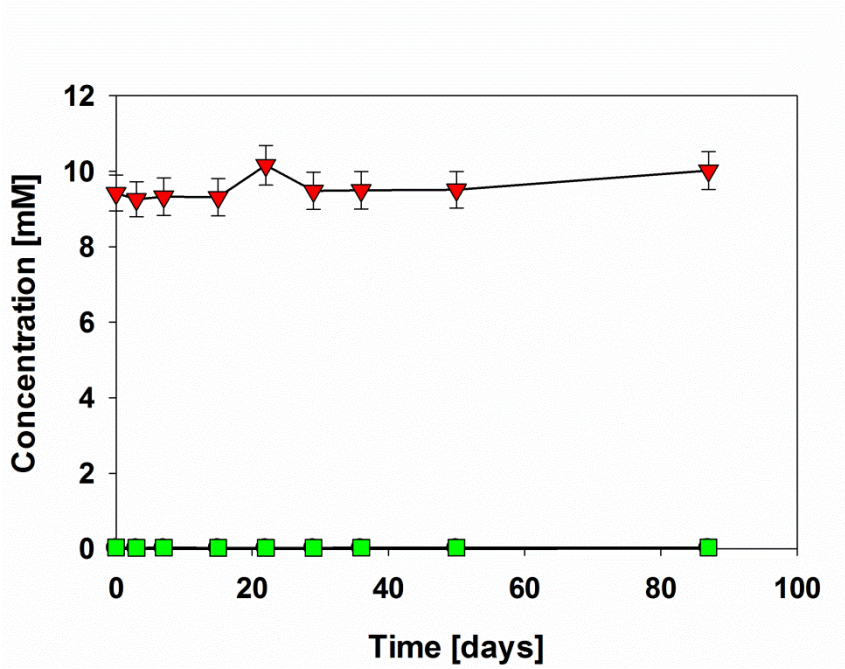

Figure 3.7 Product concentration (nitrite (●), nitrate (▼), sulfate (■)) of the reaction between ground pyrite (50 mM) and nitrate (10 mM) in the presence of *Thiobacillus denitrificans* with a cell density of 2×10^5 cells ml^{-1} under anoxic, pH-neutral conditions. Error bars are standard deviations calculated from three independent replicates. The nitrite symbols are hidden behind the sulfate ones.

Control experiments with 1) ground crystalline pyrite and nitrate without *Thiobacillus denitrificans*, 2) ground pyrite and *Thiobacillus denitrificans* without nitrate and 3) nitrate and *Thiobacillus denitrificans* without ground crystalline pyrite in the presence of 2×10^5 cells ml^{-1} showed no reaction (Figure 3.8A-C). In the control experiment with elemental sulfur as the electron donor in the presence of *Thiobacillus denitrificans* (2×10^5 cells ml^{-1}), nitrate was reduced to nitrite accompanied by the formation of sulfate (Figure 3.8D), demonstrating that the cells were alive and active under the experimental conditions. The reaction started only after 29 days and appeared to continue until the end of the experiment (87 days).

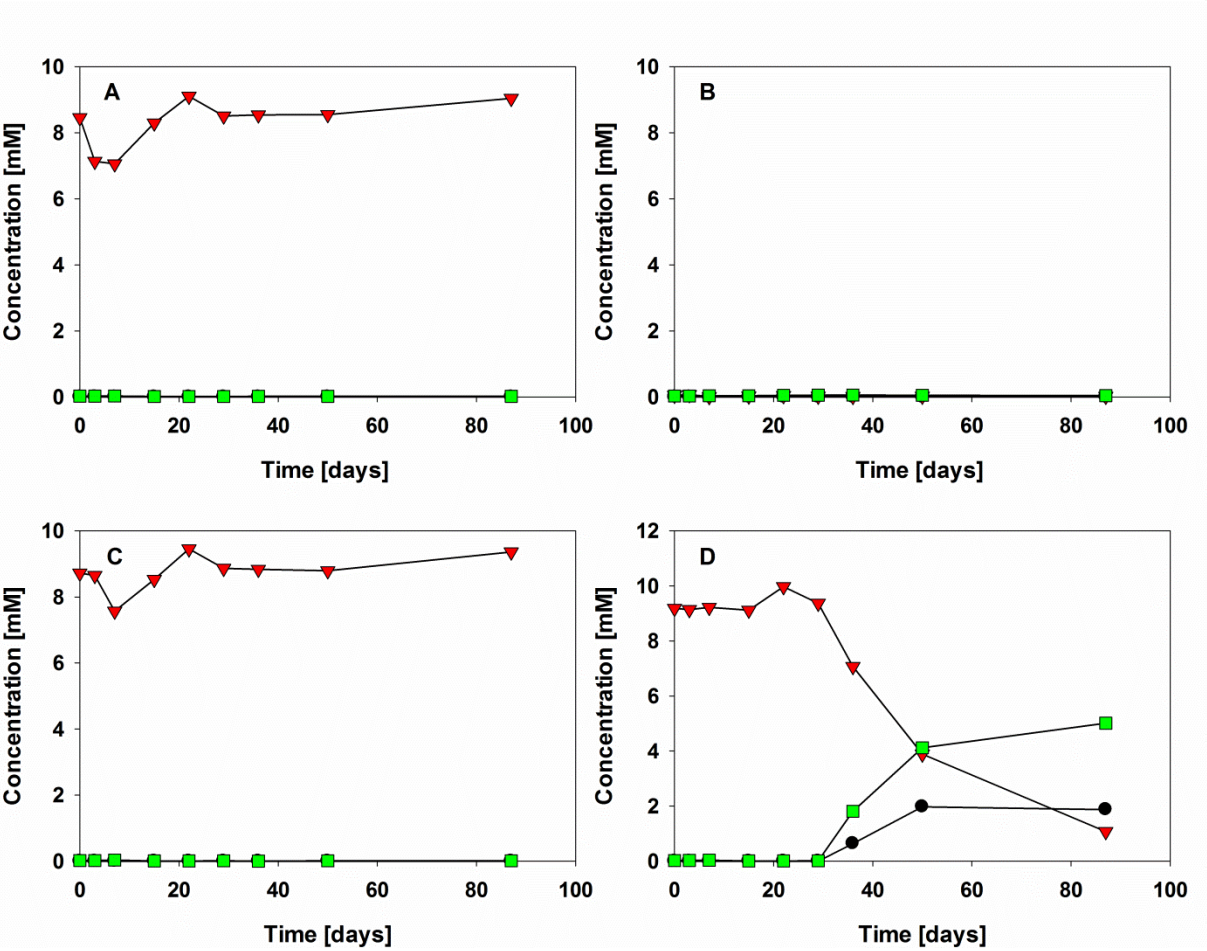

Figure 3.8 Product concentration (nitrite (●), nitrate (▼), sulfate (■)) of the control experiment of (A) 50 mM ground pyrite and 10 mM nitrate in the absence of *Thiobacillus denitrificans*, (B) 50 mM ground pyrite and *Thiobacillus denitrificans* (2×10^5 cells ml^{-1}) in the absence of nitrate, (C) 10 mM nitrate and *Thiobacillus denitrificans* (2×10^5 cells ml^{-1}) in the absence of pyrite, (D) 50 mM elemental sulfur and 10 mM nitrate in the presence of *Thiobacillus denitrificans* (2×10^5 cells ml^{-1}) under anoxic, pH-neutral conditions. Concentrations were calculated as the mean values of two independent replicates. The nitrite symbols are hidden behind the sulfate ones (A-C).

At the lower cell density of *Thiobacillus denitrificans* (2×10^4 cells ml^{-1}), no reactions were observed in the experiment with pyrite and nitrate and all controls concluding the positive control with elemental sulfur (see Supporting Information (SI), Figure S3.2-S3.3) suggesting that the number of active cells was too low to cause sulfur oxidation within the time frame of our experiment.

3.5 Discussion

Our study on nitrate-dependent pyrite oxidation revealed that pure ground crystalline pyrite could not be oxidized microbially with nitrate as electron acceptor within a period of 87 days at the cell density of 2×10^5 cells ml^{-1} used in the experiments. In contrast, reduced sulfur associated as typical impurities with the synthesized pyrite mineral served as an electron donor for chemolithoautotrophic reduction of nitrate in the experiments with a cell density of 9.3×10^6 or 9.3×10^7 cells ml^{-1}. This reaction was, however, accompanied by a significant contribution from denitrification due to stored sulfur by *Thiobacillus denitrificans*. Even at low cell numbers, *Thiobacillus denitrificans* was able to oxidize elemental sulfur with nitrate to generate sulfate and nitrite (Figure 3.8D), albeit with a prominent delay of 27 days delay in the onset of the reaction. Accumulation of nitrite upon chemolithoautotrophic oxidation of S(0) was also observed in batch experiments using inocula from an anaerobic sludge bed reactor (Cardoso et al. 2006). Hence, our observation implies that part of the denitrification observed in experiments with synthesized may also be due to chemolithoautotrophic oxidation of the residual elemental sulfur. In order to separate the contribution of this reaction and of denitrification due to stored sulfur by *Thiobacillus denitrificans* from chemolithoautotrophic denitrification with pyrite, we have established a mass balance based on the experimental data shown in Figure 3.5A. This mass balance will be discussed in the following section.

3.5.1 The reactive species in chemolithoautotrophic denitrification

The reaction between synthesized pyrite and nitrate in the presence of *Thiobacillus denitrificans* consumed 7.5 mM nitrate and generated 2.5 mM nitrite and 5.7 mM sulfate, respectively (Figure 3.5A). In the control experiment with nitrate and *Thiobacillus denitrificans* but without pyrite, 4.6 mM nitrate were reduced while 2.0 mM nitrite and 2.9 mM sulfate were generated (Figure 3.5D). The cause for these observations is unclear. The increase in sulfate remains speculative. An explanation would be that pre-growth of the cells used for inoculation in the thiosulfate containing growth medium lead to accumulation of sulfur attached to cells that was chemolithoautotrophically oxidized with nitrate during the experiments (Schedel and Trüper 1980).

From the oxidation of elemental sulfur with nitrate, a stoichiometry can be derived based on the measured NO_3^-/NO_2^- ratio. The consumption of 8 mM nitrate generated 2 mM nitrite and 5 mM sulfate (Figure 3.8D) resulting in the following stoichiometry:

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

The amount of nitrite produced by denitrification due to stored sulfur (2.0 mM) can be subtracted from the total amount of nitrite produced (2.5 mM) to constrain the fraction of nitrite generated from elemental sulfur to be 0.5 mM at maximum. This concentration stoichiometrically (Equation 1) corresponds to an oxidation of 1.4 mM of elemental sulfur, matching the measured concentration of 1.4 mM elemental sulfur, and a consumption of 2.0 mM nitrate. Subtracting this value and the 4.6 mM nitrate consumed during denitrification due to stored sulfur (in total=6.6 mM NO_3^-), one obtains a residual amount of 0.9 mM nitrate potentially consumed by chemolithoautotrophic oxidation of synthesized pyrite-sulfur according to the following reaction which is in situ denitrification by pyrite oxidation in anoxic groundwater environments usually expressed (Equation 2) (Jørgensen et al. 2009; Postma et al. 1991; Korom 1992; Kölle et al. 1983; Tesoriero et al. 2000):

$$5FeS_2 + 14NO_3^- + 4H^+ \rightarrow 5Fe^{2+} + 7N_2 + 10SO_4^{2-} + 2H_2O \quad (2)$$

Fe(II) was found to be able to act as an electron donor for *Thiobacillus denitrificans* during nitrate-dependent Fe(II) oxidation (Beller et al. 2013). It could be argued that also Fe(II) may be oxidized serving as an electron source for reduction of nitrate. However, measurements of the concentration of Fe(II) to proof the occurrence of reaction (2) failed due to the interference of nitrite (cf. Materials and Methods) (Klueglein and Kappler 2013). Yet, the control experiments demonstrated that addition of Fe(II) did not stimulate denitrification by *Thiobacillus denitrificans* (Figure 3.6). We therefore conclude that *Thiobacillus denitrificans* is not able to oxidize Fe(II) and that we do not have to account for the liberated Fe(II) in equation (2) when establishing an electron balance for denitrification. An explanation would be that *Thiobacillus denitrificans* preferred the sulfur compounds as better electron donors (stored and pyrite associated) and thus did not perform the activity of Fe(II) oxidation under the experimental conditions.

In spite of the uncertainties inherent to the assumptions about N products, our data provide evidence that the synthesized pyrite with associated sulfur compounds was used as an electron donor for chemolithoautotrophic denitrification (Table 3.1). This assumption is supported by the mass balance for sulfate that could explain after all 86% (calculated mass balance/measured concentrations ×100%) of the measured sulfate concentration suggesting that our assumptions and estimates are reasonable.

Table 3.1 Mass balance of substrates and products of the reaction between synthesized pyrite (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 density under anoxic, pH-neutral conditions.

Synthesized pyrite	NO_3^- depleted	NO_2^- produced	S(0) depleted	SO_4^{2-} produced
	[mM]	[mM]	[mM]	[mM]
measured concentrations	7.5[a]	2.5[a]	1.4[a]	5.7[a]
denitrification due to stored sulfur by *Thiobacillus denitrificans*	4.6[a]	2.0[a]		2.9[a]
mass balance eq.(1)	2.0[c]	0.5[b]	1.4[c]	1.4[c]
mass balance eq.(2)	0.9[b]			0.6[d]
calculated mass balance				4.9

[a] measured values

[b] calculated from experimental mass balance

[c] calculated using equation (1)

[d] calculated using equation (2)

3.5.2 Field and laboratory studies on nitrate-dependent anaerobic pyrite oxidation

This study highlights the importance of the speciation of reduced sulfur in mediating chemolithoautotrophic denitrification. It is interesting to note in Table 3.2 that speciation has not been considered in previous laboratory studies on nitrate-dependent pyrite oxidation. Generation of nitrite and sulfate accompanied by consumption of nitrate was observed and attributed to the oxidation of pyrite (Jørgensen et al. 2009; Torrentó et al. 2010; Bosch et al. 2012; Vaclavkova et al. 2014) in experiments in which no attempts had been made to remove elemental sulfur during the preparation of pyrite and minimize cell density to reduce the interference of denitrification due to stored sulfur between nitrate and medium compounds.

The absence of XRD-reflection characteristics for S(0) is not an essential criterion to exclude its occurrence because it simply states that the sulfur content could be 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. If, however, elemental sulfur was removed from the material during the preparation of pyrite (Haaijer et al.(2007), Schippers and Jørgensen (2001), this study), no pyrite oxidation could be observed (Table 3.2).

Table 3.2 Overview of previous studies on chemolithotrophic denitrification coupled to pyrite oxidation in the presence of nitrate-reducing strains or in environmental samples.

Name bacterial strain	Reference	Type of pyrite (particle size of pyrite)	Approach to remove elemental sulfur from pyrite material	Evidence for nitrate reduction and sulfate generation	Fe speciation data as a proof for pyrite oxidation
Thiobacillus denitrificans	Jørgensen et al. (2009) supporting information	Ground natural crystalline pyrite (45 -200 µm)	No	Yes	No
Thiobacillus denitrificans	Torrento et al. (2010)	Ground natural crystalline pyrite (25-50 µm and 50-100 µm)	No	Yes	No
Thiobacillus denitrificans	Bosch et al. (2012)	Ground natural crystalline pyrite (<200 µm)	No	Yes	Yes
Thiobacillus cultures	Vaclavkova et al. (2014)	Ground natural crystalline pyrite (<200 µm)	No	Yes	No
Thiobacillus denitrificans	Present study with synthesized Pyrite	Synthesized less crystalline pyrite (630-1400 µm)	No	Yes	No
Soil samples from fresh water lake	Haaijer et al. (2007)	Ground natural crystalline pyrite (500-3000 µm)	Washed once with acetone	No	No
Marine sediment	Schippers and Jorgensen (2001)	Coarse Pyrite originated from an ore processing flotation plant (50-100 µm)	Washed three times with acetone	No	No
Thiobacillus denitrificans	Present study with ground pyrite	Ground natural crystalline pyrite (63-200 µm)	Washed three times with cyclohexane	No	No

Additional complexity may arise from the interference of reactants with pyrite. It has been demonstrated that nitrite is able to oxidize pyrite abiotically in 1 M HCl which leads to the formation of ferric iron (Yan et al. 2015). The occurrence of ferric iron may therefore be misinterpreted as a proof for pyrite oxidation (Bosch et al. 2012). A revised protocol in our present study was recommended 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 (Yan et al. 2015). In conclusion, the nitrate-dependent microbial pyrite oxidation in the presence of *Thiobacillus denitrificans* postulated in previous studies cannot be ruled out, but its contribution to the observed production of sulfate and consumption of nitrate is probably much lower than assumed. The findings of the present study imply that laboratory studies on microbially mediated pyrite oxidation may be subject to several misinterpretations and our systematic study design may also provide explanations for the contradictory observations (cf. introduction). Nevertheless, there is a clear indication from field studies that nitrate consumption and pyrite oxidation are interrelated (Postma et al. 1991; Tesoriero et al. 2000; Zhang et al. 2009; Pauwels et al. 2000; Van Beek et al. 1989; Evangelou and Zhang 1995; Broers 1998), which calls for a closer inspection of the chemical nature of the reacting sulfur species.

Our study demonstrates that chemolithoautotrophic oxidation of pyrite with nitrate as electron acceptor was not possible if the pyrite source is pure crystalline pyrite that does not contain elemental sulfur contaminations. In contrast, the mass balance suggests that chemolithoautotrophic oxidation of synthesized less crystalline pyrite with nitrate, it being pyrite or marcasite, may be possible, even if one accounts for side reactions such as denitrification due to stored sulfur and the reduction of nitrate by elemental sulfur (Table 3.1). The reaction appears to be induced via S oxidation but not via Fe oxidation, since the Fe(II)-oxidizing nitrate-reducing strain *Acidovorax sp.* BoFeN1 did not stimulate pyrite-dependent nitrate reduction. Moreover, addition of Fe(II) and Fe(III) to the reaction

even slightly decreased the rates of nitrate reduction and sulfate generation. The larger peak widths in the X-ray diffractogram of the synthesized pyrite as well as the SEM images suggest that this material has a smaller mean particle size and lower 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 pyrite is higher than that of the ground pyrite (0.17 m^2 g^{-1}) though this difference is not very large. The synthesized pyrite consisted of pyrite, marcasite, and elemental sulfur and it remained unclear, which kind of S source (pyrite, marcasite or elemental sulfur) plays the predominant role in the reaction. Our study therefore 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 even an active sulfur cycle may take place rather than zones of geological ripening. Thus, we propose that quantitative differentiation between the sulfur components pyrite, marcasite, and elemental sulfur as well as their mineralogical characterization is a key requirement in pyrite oxidation studies, both in the field and in the laboratory. Contradictory results on the potential chemolithoautotrophic oxidation of pyrite with nitrate obtained so far may arise from impurities of reduced sulfur species present in natural or synthetic pyrite phases or sediments.

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3.7 Supporting information for Study 2

EDX data of ground pyrite after preparation, product concentration of the reaction between ground pyrite and nitrate in the presence of *Thiobacillus denitrificans* with a cell density of 2× 10^4 cells ml^{-1} under anoxic, pH-neutral conditions, product concentration of the control experiment in the presence of *Thiobacillus denitrificans* with a cell density of 2× 10^4 cells ml^{-1} under anoxic, pH-neutral conditions are shown in the supporting information.

Figure S3.1 Scanning electron microscopy image of ground pyrite.

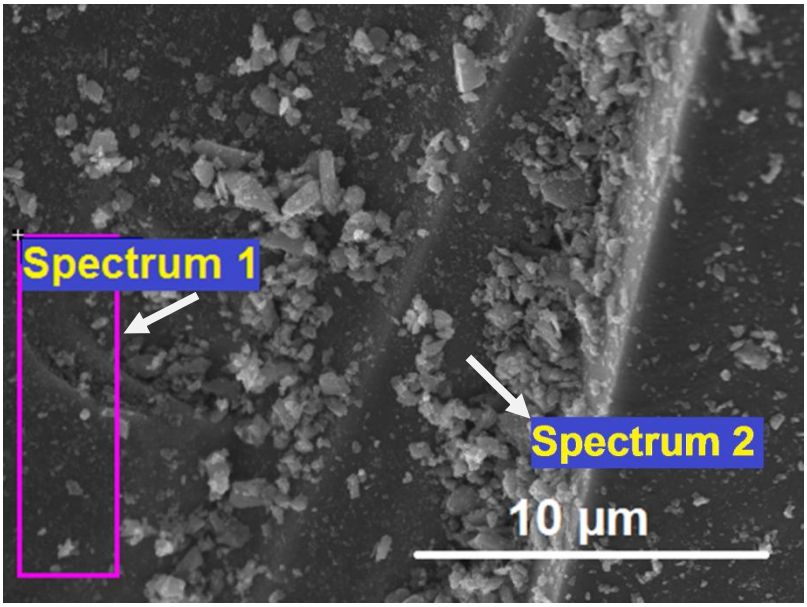


Table S3.1 Elemental analysis by EDX of a fraction of the pyrite surface (spectrum 1) with only few nano-particles.

Element	Mass %	Atom %
C	4	11
S	51	58
Fe	46	30
total	100	

Table S3.2 Elemental analysis by EDX of a fraction of the pyrite surface (spectrum 2) covered with a high density of nano-particles.

Element	Mass %	Atom %
C	3	11
S	50	59
Fe	46	31
total	100	

Figure S3.2 Product concentration (nitrite (●), nitrate (▼), sulfate (■)) of the reaction between ground pyrite (50 mM) and nitrate (10 mM) in the presence of *Thiobacillus denitrificans* with a cell density of 2×10^4 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.

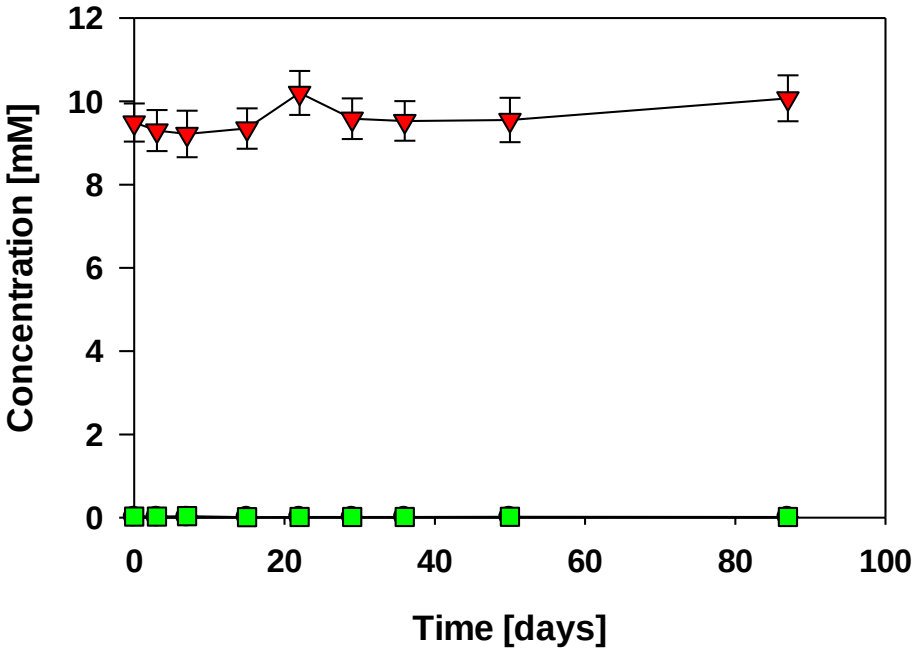
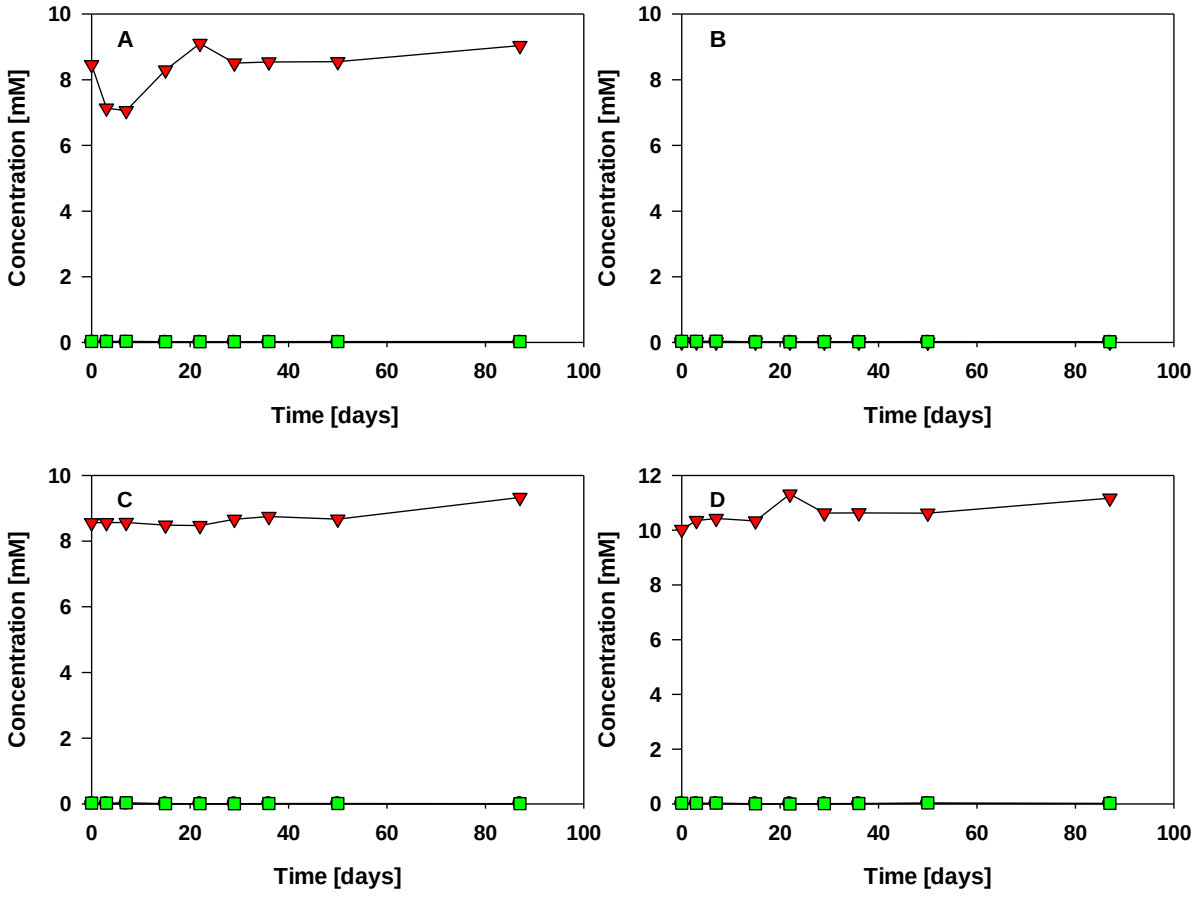


Figure S3.3 Product concentration (nitrite (●), nitrate (▼), sulfate (■)) of the control experiment of (A) 50 mM ground pyrite and 10 mM nitrate in the absence of *Thiobacillus denitrificans*, (B) 50 mM ground pyrite and *Thiobacillus denitrificans* (2×10^4 cells ml^{-1}) in the absence of nitrate, (C) 10 mM nitrate and *Thiobacillus denitrificans* (2×10^4 cells ml^{-1}) in the absence of pyrite, (D) 50 mM elemental sulfur and 10 mM nitrate in the presence of *Thiobacillus denitrificans* (2×10^4 cells ml^{-1}) under anoxic, pH-neutral conditions. Concentrations were calculated as the mean values of two independent replicates. The nitrite symbols are hidden behind the sulfate ones (A-D).



4. Study 3: Towards a standardized protocol for studying chemolithoautotrophic denitrification with pyrite

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4.1 Abstract

Laboratory studies results on the chemolithoautrophic denitrification coupled to pyrite oxidation are partly contradicting. Some of these studies indicated that a microbial oxidation of pyrite occurred, whereas the results of other studies with pyrite as the electron donor and nitrate as the electron acceptor indicated the contrary. Recent findings revealed that inconsistent experimental protocols may cause substantial uncertainty in the interpretation of results from laboratory studies or may even produce artifacts. In this paper, we are providing a comprehensive overview of possible interferences that may arise from both geochemical and microbiological interferences. Key interferences are i) impurities of reduced sulfur species associated with pyrite, ii) formation of nitrite and its interference during acidic extractions, and iii) occurrence of residual iron and sulfur compounds in the reaction medium. We present experimental standard protocols to overcome these interferences in future studies on chemolithoautotrophic denitrification with pyrite.

Key words: artifact, protocol, denitrification, pyrite oxidation, reduced sulfur species, nitrite, interference

4.2 Introduction

Denitrification coupled to pyrite oxidation is controversially discussed in the literature. The pathway is generally expressed as equation (1) and (2):

$$5FeS_2 + 14NO_3^- + 4H^+ \rightarrow 5Fe^{2+} + 7N_2 + 10SO_4^{2-} + 2H_2O \quad (1)$$

$$5Fe^{2+} + NO_3^- + 7H_2O \rightarrow 5FeOOH + 0.5N_2 + 9H^+ \quad (2)$$

Field data provide clear evidence that there is denitrification linked to oxidation of reduced sulfur in pyrite containing aquifers (Kölle et al. 1983; Pauwels et al. 2000; Postma et al. 1991; Tesoriero et al. 2000; Zhang et al. 2009). However, laboratory experiments to link these observations to chemolithoautotrophic pyrite oxidation are contradicting. Incubation of natural sediment to which ground pyrite was added did not provide any evidence for denitrification coupled to pyrite oxidation (Schippers and Jorgensen 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 a microbially mediated denitrification with pyrite as the electron donor. Bosch et al. (2012) have described oxidation of pyrite nanoparticles by the nitrate-reducing bacterium *Thiobacillus denitrificans*. Their conclusion was based on an electron balance established based on the formation of ferric iron and sulfate along with the reduction of nitrate to nitrite. However, acidic extraction of pyrite suspensions to determine ferric hydroxide as the reactant of pyrite oxidation may lead to significant overestimation of ferric iron if nitrite is present, because this N-species is able to oxidize pyrite under acidic conditions (Yan et al. 2015). 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 generate as an intermediate of denitrification process may lead to several possibilities for interference. In the present review, we will discuss potential geochemical and microbiological interferences as well as suggestions as to how such interferences could be avoided by applying experimental standard protocols.

4.3 Geochemical interferences

4.3.1 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 donor for denitrification by the chemolithoautotrophic denitrifying microorganism *Thiobacillus denitrificans* (Schedel and Trüper 1980; 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 electron donor for chemolithotrophic denitrification by a denitrifying enrichment culture. As a result, nitrite accumulated and elemental sulfur was converted to sulfate (Cardoso et al. 2006). 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. 2017). Moreover, elemental sulfur associated as a typical impurity with the synthesized pyrite mineral (4.6 mass % of elemental sulfur) served as an electron donor for chemolithoautotrophic 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. 2017). Our observations implied that part of the denitrification observed in experiments with synthesized pyrite may have been due to chemoautotrophic oxidation of the residual elemental sulfur. Chemolithoautotrophic oxidation of pyrite with nitrate as electron acceptor was not possible if

the pyrite source is pure crystalline pyrite that does not contain elemental sulfur contaminations.

In order to rule out interference of reduced sulfur species with pyrite oxidation, it is obvious that it is absolutely necessary that the reduced sulfur species associated with pyrite materials are removed from the material during the preparation of pyrite, e.g. using approaches which were used in previous studies (Yan et al. 2015; Yan et al. 2017). Specifically, in order to remove residual acid-extractable sulfur species, the material was washed several times with 1 M HCl. Thereafter, the material was washed several times with cyclohexane or petrolether to remove elemental sulfur. Nevertheless, a complete removal of elemental sulfur from synthetic or natural pyrite is difficult, therefore, the elemental sulfur content of material in pyrite materials should be quantified. For this reason, an analytical protocol for determining elemental sulfur content was provided in our previous study (Yan et al. 2015): 0.5 g of pyrite were added to a 120 mL glass serum bottle. The bottles were sealed and crimped and the headspace of the bottles was flushed with N_2. 20 mL oxygen-free methanol were subsequently added to the serum bottles with a glass syringe. Experiments were performed in two independent replicates. The headspace of the bottles was again flushed with N_2 for 1 min. Suspensions were shaken for 24 hours to extract elemental sulfur. Thereafter, an aliquot of ca. 1.5 mL of each sample was extracted and filtered through a 0.22 µm pore size filter (Nylon) and then analyzed by HPLC (PerkinElmer 2000 pump and autosampler, Fa. linear-UV−VIS detector and software peaksample 409, 265 nm).

In summary we suggest that quantitative differentiation between the sulfur components as well as their mineralogical characterization of initial pyrite mineral is a key requirement 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 either by its removal or by quantifying its content.

4.3.2 Quantitative spectrophotometric determination of Fe(II) and Fe(III) in nitrite-containing pyrite samples.

The first reaction product of microbial denitrification, stemming from the reduction of nitrate, is nitrite (Glass and Silverstein 1998; Betlach and Tiedje 1981). 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). Recently, laboratory studies presented evidence on accumulation of nitrite during chemolithoautotrophic denitrification coupled to pyrite oxidation in the presence of *Thiobacillus denitrificans* (Bosch et al. 2012; Torrentó et al. 2010; Torrentó et al. 2011) which is known as the most famous obligate chemolithoautotrophic species to conserve energy from the oxidation of inorganic sulfur compounds to denitrification (Kelly and Wood 2000; Schedel and Trüper 1980; Timer-ten Hoor 1981). Pyrite oxidation is typically quantified by acidic extraction and quantification of Fe(II) and $Fe(HCl)_{tot}$ (total HCl-extractable Fe) that is assumed to have formed upon pyrite oxidation under circumneutral conditions (Moses and Herman 1991; Bosch et al. 2012). Using the standard ferrozine/phenantroline assay (Stookey 1970; Tamura et al. 1974), nitrite-containing pyrite samples from microbial experiments are often acidified with 1 M HCl for stabilization of Fe(II) and extraction of $Fe(HCl)_{tot}$ (total HCl-extractable Fe) before measurement. However, previous studies have determined the abiotic oxidation of Fe(II) to Fe(III) with nitrite under acidic (Wullstein and Gilmour 1966; Buresh and Moraghan 1976; Ibrahim et al. 2001; Klueglein and Kappler 2013) or weak acidic conditions (Van Cleemput and Baert 1983). Nitrite is protonated to nitrous acid (HNO_2) which spontaneously decomposes to nitrogen dioxide (NO_2) and nitric oxide (NO). The both reactive N species are able to abiotically oxidize Fe(II) according to the equations 3-6 (Bonner and Pearsall 1982; Van Cleemput and Samater 1995; Nelson and Bremner 1970b).

$$2NO_2^- + 2H^+ \Leftrightarrow 2HNO_2 \rightarrow NO_2 + NO + H_2O \quad (3)$$

$$NO_2 + 2Fe^{2+} + 2H^+ \rightarrow 2Fe^{3+} + NO + H_2O \quad (4)$$

$$NO + Fe^{2+} + H^+ \rightarrow Fe^{3+} + HNO \quad (5)$$

$$2HNO \rightarrow N_2O + H_2O \quad (6)$$

In a recent paper, we provided clear evidence that pyrite is abiotically oxidized in the presence of nitrite 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.

$$3.5NO_2 + FeS_2 + H^+ \rightarrow S_2O_3^{2-} + 3.5NO + Fe^{3+} + 0.5H_2O \quad (7)$$

$$7NO + FeS_2 + 3H_2O + H^+ \rightarrow S_2O_3^{2-} + 7HNO + Fe^{3+} \quad (8)$$

In order to quantify Fe(II)/Fe(III) values accurately in nitrite-containing pyrite samples from experiments investigating chemolithoautotrophic denitrification coupled to pyrite oxidation, it is essential to remove or stabilize the nitrite in nitrite-containing pyrite samples. We proposed to remove nitrite by washing the nitrite-containing pyrite samples with nitrite-free water prior to the acidic extraction during a revised protocol (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 before an acidic extraction is applied. 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)/Fe(III).

Alternatively, sulfamic acid (HSO_3NH_2) is a moderately strong acid (pK_a = 1.3) which is able to react rapidly with nitrite to form N_2 and sulfuric acid (equation 9) (Granger and Sigman 2009; Marouf-Khelifa et al. 2006):

$$HNO_2 + HSO_3NH_2 \rightarrow H_2SO_4 + N_2 + H_2O \quad (9)$$

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 nitrate and pH of the reaction should be kept at or below the pK_a of sulfamic acid (pK_a = 1.3), a higher pH than 3 should be avoided to prevent the formation of reactive NO and NO_2 (equation 3) (Granger and Sigman 2009). Low pH conditions is also for an efficient Fe extraction necessary. For these reasons, the protocol by using sulfamic acid to remove nitrite has been further developed. A recent study provided a revised Fe extraction protocol to use a combination of 40 mM sulfamic acid with 1 M HCl 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. 2017). Therefore, it is assumed that nitrite-containing pyrite samples for studies of chemolithoautotrophic denitrification with pyrite should be extracted in sulfamic acid instead of HCl or in a combination of sulfamic with 1 M HCl as another approach to remove nitrite without abiotic oxidizing pyrite by nitrite during acidic extraction.

4.3.3 Interference of pyrite nanoparticles

Studies on pyrite-containing sedimentary material or synthesized pyritic material might provide indirect evidence for chemolithoautotrophic denitrification with pyrite (Jørgensen et al. 2009; Yan et al. 2017). However, in order to obtain direct evidence for this process, it is suggested to use a pure pyrite without any contaminations of other reduced iron- and sulfur compounds. Preparation of pyrite with a great care is therefore advised to be applied in the microbial experiments of chemolithoautotrophic denitrification coupled to pyrite oxidation. The first step of pyrite preparation is the milling of crystalline pyrite to achieve an appropriate particle size for microbial batch experiments (Bosch et al. 2012; Vaclavkova et al. 2014; Torrentó et al. 2010; Yan et al. 2015; Jørgensen et al. 2009). A previous study investigating denitrification with pyrite in the presence of *Thiobacillus denitrificans* demonstrated that the nitrate reduction rates are dependent on pyrite grain size (Torrentó et al. 2010). The procedure

to prepare pyrite e.g. the milling of pyrite is therefore considered to be related to the rate of pyrite oxidation. Depending on experimental purposes, the milling of pyrite in previous studies was carried out with two different procedures. Studies investigating the role of pyrite nanoparticles in microbial nitrate reduction performed the milling of crystalline pyrite under anoxic conditions to avoid the oxidation of pyrite by oxygen and the pyrite material was not washed with HCl preserve the nanoparticulate fraction (Bosch et al. 2012; Vaclavkova et al. 2014). Nanoparticles are broad, heterogeneous size distributed on the surface of larger pyrite crystals. The problem of this pyrite preparation is that two kinds of pyrite crystals (nanoparticles and larger pyrite crystals) with different particle sizes existed in the reaction system allowing the question of which kind of pyrite particle was actually microbial oxidized. Without washing with HCl, the presence of possible iron and sulfur impurities on the pyrite surface cannot be completely ruled out.

In order to exclude the interference of nanoparticles and other possible iron and sulfur impurities, the milling of pyrite in our study (Yan et al. 2015) was carried out under oxic condition following an intensive washing with HCl in order to remove pyrite nanoparticles, sulfur impurities and iron oxides which may have formed from oxidation of pyrite surfaces during crushing. Our study demonstrated that the ground pyrite material contained a small but quantifiable pool of acid-extractable Fe(II) even after intensive washing with HCl (Yan et al. 2015). We assume that this fraction of extractable Fe(II) is due to the tiny surface bound particles identified with SEM (Yan et al. 2015) which is either of pyritic (FeS_2) origin or $Fe(HSO_4)_2$ as the product of the pyrite dissolution. An explanation of the pyrite dissolution is due to the reaction of water with defect or non-stoichiometric sites on pyrite (Guevremont et al. 1998), leading to the dissolution of nm-size pyrite particles and the formation of dissolved Fe(II) and sulfate, which could interfere with the Fe(III) and sulfate production in the batch experiment and provide false positive results. Therefore, we suggest that a quantification of

Fe(II) and sulfate in the initial solution is necessary and it cannot be related to microbial pyrite oxidation.

4.4 Microbiological interference

4.4.1 Interference of remaining iron and sulfur compounds in the reaction medium

Thiosulfate, sulfate and Fe(II) are generally 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 for a following batch experiments. The problem is that the residual thiosulfate, sulfate and iron (potentially even stored within the cells) could interfere with the nitrate reduction and sulfate production in the following batch experiment and provide false positive results.

In our previous study (Yan et al. 2017), *Thiobacillus denitrificans* was cultured with thiosulfate in an anaerobic (pH 6.8) nutrient medium 113 specially designed for *Thiobacillus denitrificans*. The medium consisted of 14.7 mM KH_2PO_4, 19.8 mM KNO_3, 18.7 mM NH_4Cl, 3.25 mM $MgSO_4•7H_2O$, 20.1 mM $Na_2S_2O_3•5H_2O$, 30.0 mM $NaHCO_3$, 0.007 mM $FeSO_4•7H_2O$, and trace element solution SL-4. If the culture 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 interference of sulfur and iron from the previous medium incubation in the experiments, the grown culture (the inoculum for the following experiment) should be washed and resuspended in modified medium several times before the start of the experiments to avoid interference of sulfur from the medium in the determination of formation rates of sulfate from pyrite. The modified medium to wash pre-culture and also using in the batch experiments should be adjusted without thiosulfate, and iron, using chloride salts instead of sulfate salts. 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 $MgCl_2•6H_2O$ instead of $MgSO_4•7H_2O$, 30 mM $NaHCO_3$ and the same concentration of trace element solution SL-4 (Yan et al. 2017).

4.4.2 Interference of denitrification due to stored sulfur and verification of the viability of the cell cultures

A previous study demonstrated that *Thiobacillus denitrificans* could be grown in a medium which contained thiosulfate as electron donor under anoxic conditions (Schedel and Trüper 1980). When thiosulfate was present, elemental sulfur accumulated transiently within the cells. However, when thiosulfate had been 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 on the outside of its cells but attached to the cells can act a electron donors. The problem is that the stored sulfur could interfere with the nitrate reduction and sulfate production in the batch experiment and provide false positive results.

In our previous microbial experiment with synthesized pyrite and nitrate in the presence of *Thiobacillus denitrificans*, the measured concentration of sulfate was stoichiometrically more than expected corresponding to the observed nitrate reduction (Yan et al. 2017). 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. 2017). This data confirmed the observation of the previous study (Schedel and Trüper 1980) that pre-growth of the cells used for inoculation in the thiosulfate containing growth medium lead to accumulation of sulfur attached to cells that was chemolithoautotrophically oxidized with nitrate during the experiments.

For this reason, 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 due to stored sulfur should be subtracted from the total contribution in batch experiments. A positive control experiment with well-known sulfur compound as electron donor (e.g. elemental sulfur, thiosulfate) should be set up to test the viability of the cell cultures at this cell density.

4.5 Conclusions

The large numbers of artifacts make it not surprising that previous observations are contradicting. We are presenting a revised protocol on geochemical as well as microbiological side for studying chemolithoautotrophic denitrification with pyrite (Table 4.1). Further works should pay special attention to a comprehensive mineralogical characterization of initial pyrite mineral and quantitative differentiation between the sulfur components. An interference of denitrification due to stored sulfur should be considered and the viability of the cell cultures should be proved. A reaction medium for batch experiment should be setup with a great care to exclude the interference of iron and sulfur compounds. In summary, the geochemical and microbiological interference of reduced sulfur species with the pyrite oxidation is difficult to be completely excluded. The quantification of Fe(III) (oxyhydr)oxides which formed upon oxidation of pyrite is therefore assumed to be strong evidence for the microbial pyrite oxidation. Fe(II) and Fe(III) in nitrite-containing samples should be quantitative determined with a revised protocol using an approach to remove or stabilize the nitrite to protect Fe(II) against abiotic oxidation.

Table 4.1. 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^- oxdizing 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 sulfamic acid instead of HCl or in a combination of sulfamic with 1 M HCl
	Pyrite nanoparticles	Confusion whether the oxidation of pyrite nanoparticles or the oxidation of possible iron and sulfur impurities on the pyrite surface	Washing material with HCl to remove pyrite nanoparticles, sulfur impurities and iron oxides, quantification of Fe(II) and sulfate in the initial solution
Microbiological interference	Iron and sulfur compounds in the reaction medium	Overestimation of sulfate production and nitrate reduction	Modified reaction medium without thiosulfate, sulfate and iron
	Stored sulfur within the cells or on the outside of its cells but attached to the cells	Overestimation of sulfate production and nitrate reduction	An appropriate cell density

4.6 References

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Contributions to different studies

Study 1 (Chapter 2): Interference of nitrite with pyrite under acidic conditions – implications for studies of chemolithotrophic denitrification

Authors: Ruiwen Yan, Andreas Kappler, Stefan Peiffer

Own and author contributions statement:

Own contribution: concept and study design 60%, data acquisition 100%, analyses of samples 100%, data analyses and figures 100%, discussion of results 70%, manuscript writing 70%

Ruiwen Yan, Stefan Peiffer designed the research.

Ruiwen Yan performed the experiments and analyzed the data.

Samples were analyzed in the **Hydrology department at University of Bayreuth,** Germany

Ruiwen Yan, Andreas Kappler, and Stefan Peiffer interpreted and discussed results.

Figures and tables were created by **Ruiwen Yan**.

Ruiwen Yan wrote the first draft of the manuscript.

The manuscript was revised and finished by **Ruiwen Yan, Andreas Kappler, and Stefan Peiffer**

Study 2 (Chapter 3): The effect of reduced sulfur speciation on the chemolithoautotrophic pyrite oxidation with nitrate

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Ruiwen Yan, E. Marie Muehe, Alexander Poser, and Regina Lohmayer performed the experiments and analyzed the data.

Samples were analyzed in the **Hydrology department at University of Bayreuth,** Germany and in the **Geomicrobiology Group at Eberhard-Karls-University of Tuebingen**, Germany

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Study 3 (Chapter 4): Towards a standardized protocol for studying chemolithoautotrophic denitrification with pyrite

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