Rates and potential mechanism of anaerobic nitrate-dependent microbial pyrite oxidation

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Abstract
Pyrite (FeS$_2$) is a major iron- and sulfur-containing mineral phase in the environment. Oxidation of pyrite by aerobic micro-organisms has been well investigated. However, the reactivity of pyrite under anoxic conditions is still an open question. In the present paper, we summarize field and laboratory data on this chemolithotrophic respiration process with nitrate as terminal electron acceptor. Geochemical and stable isotope field data indicate that this process is occurring. Laboratory studies are more ambiguous, but recent positive results provide evidence that anaerobic microbial pyrite oxidation can, in fact, occur with nitrate as electron acceptor.

Pyrite in the global sulfur cycle
Pyrite is by mass as well as by reactivity a significant mineral in ore and coal deposits, or in marine and freshwater sediments [1]. Pyrite is also a major sink within the global biogeochemical sulfur and iron cycles. Sedimentary pyrite burial occurs mainly via bacterial sulfate reduction, subsequent reaction to FeS and aging with elemental sulfur to FeS$_2$. This flow of elements has widespread implications for the related global biogeochemical cycles of redox equivalents, carbon and oxygen, as does the reverse process of pyrite weathering [2]. In general, pyrite weathering under oxic conditions proceeds both abiotically and via biotic catalysis [3].

Under anoxic conditions, when positive electron acceptors such as nitrate are present, the pathway corresponds to redox eqns (1) and (2).

$$\text{FeS}_2(s) + 3\text{NO}_3^-(aq) + 2\text{H}_2\text{O} \rightarrow \text{Fe(OH)}_3(s) + 2\text{SO}_4^{2-}(aq) + 1.5\text{N}_2(g) + \text{H}^+$$

(1)

$$\text{FeS}_2(s) + 7.5\text{NO}_3^-(aq) + 3.5\text{H}_2\text{O} \rightarrow 2\text{Fe(OH)}_3(s) + 2\text{SO}_4^{2-}(aq) + 4\text{H}^+$$

(2)

However, this process of microbially mediated anaerobic pyrite oxidation has eluded scientific investigation to some extent. Nevertheless, it should be expected in a multitude of anoxic nitrate-contaminated aquifers, where organic electron donors are lacking or not reactive, such as recalcitrant humic acids. Coupled to nitrate instead of oxygen reduction, pyrite oxidation (as lithotrophic denitrification) still yields up to 2439 kJ mol$^{-1}$, depending on the oxidation state of the possible end products (N$_2$ or NO$_2^-$). Nitrate is well known to serve as electron acceptor in the microbial oxidation of ferrous monosulfide [4].

In comparison with monosulfide ferrous minerals (FeS), however, the disulfide pyrite is highly crystalline. Its valence bands are entirely derived from its metal orbitals, rendering the crystal inert against proton/acid-promoted dissolution and therefore also against direct microbial dissolution [3]. Nevertheless, pyrite can be rapidly oxidized by ferric iron under acidic conditions [5]. Widespread microbial oxidation of non-pyritic ferrous iron can therefore lead to an indirect pyrite oxidation mechanism, in which the oxidized ferric iron promotes the initial attack and starts an electron-shuttling system. Yet the initial oxidation and cyclic regeneration of dissolved ferrous iron would still require oxygen as terminal electron acceptor. Consequently, strictly anaerobic microbial pyrite oxidation was assumed to be non-existing. However, the question remained whether this dissolution process can be coupled to microbial denitrification.

Field evidence for anaerobic pyrite oxidation
Denitrification is a widespread anaerobic respiration process that occurs heterotrophically and lithotrophically in the environment [6]. Yet many field studies could rule out heterotrophic denitrification or the impact of non-pyritic minerals to some extent. In unconfined sandy aquifers, anoxic, pyrite-containing and organic carbon-poor zones were identified, in which high Fe$^{2+}$ and sulfate concentrations appeared simultaneously with pronounced denitrification [7,8]. A similar geochemical pattern was observed for a schist river catchment aquifer [9] and a bedrock aquifer [10]. The schist aquifer study already postulated the risk of ‘pyrite exhaustion’ under sustained nitrate influx from agricultural activities. Another study observed denitrification indicators in concurrence with an increase in dissolved pyrite-associated metals such as arsenic [11].
Furthermore, several field studies confirmed lithotrophic denitrification by isotope measurements of $^{15}$N and $^{18}$O in geochemical settings, where no other process than anaerobic pyrite oxidation seemed plausible for the enrichment of heavy nitrogen and oxygen isotopes, or where even $^{34}$S enrichment in dissolved sulfate emphasized the role of pyrite as electron donor [9, 10, 12, 13].

Even though there is some evidence from field studies, several factors biased the definite geochemical discovery of anaerobic nitrate-dependent pyrite oxidation: a complete assessment of all mineral phases in an aquifer is usually not performed. A conclusive mineralogical analysis of the overall conditions is therefore difficult, allowing the question of whether only pyrite was anaerobically oxidized or some other mineral phase such as marcasite or other metal-bearing phases was oxidized. Furthermore, the impact of already oxidized ferric iron on the reaction is hard to assess, as is subsequently the share of biotic catalysis in the overall geochemical reaction of anaerobic pyrite oxidation [14].

**Laboratory studies on anaerobic pyrite oxidation**

The *in situ* geochemical and isotopic evidence for anaerobic pyrite oxidation stimulated subsequent laboratory studies to clearly prove the process. In well-defined batch experiments with marine sediment slurries and ferric iron amendment, however, only denitrification and sulfate production, but no clear nitrate-dependent pyrite oxidation, could be observed [15]. This was explained by the precipitation of non-complexed ferric iron, as these authors argue that only complexed dissolved ferric iron could act as the initial crystal structure-attacking agent [15]. In another study, no anaerobic pyrite oxidation could be detected in a soil-containing bioreactor, whereas FeS was clearly oxidized in a parallel reactor [16].

In contrast, two sediment column experiments provided evidence for pyrite oxidation [17, 18]. The first study showed a simultaneous decrease in nitrate and increase in sulfate in a pyrite-amended anoxic sediment. Pyrite-free columns showed no reaction [17]. The second study applied flowthrough and batch systems. Clearly, denitrification could be demonstrated, and even though sulfate production did not entirely match theoretical expectations, $^{15}$N and $^{18}$O isotope fractionation in nitrate supported the occurrence of anaerobic nitrate-dependent pyrite oxidation [18]. However, to our judgement, the presence of initial ferric iron, ferrous iron and already oxidized pyrite surfaces in these two sediment column studies cannot be fully ruled out. As mentioned above, this leads to a certain degree of uncertainty whether the process is fully microbially induced and definitely related to anaerobic pyrite oxidation.

A recent study examined whether pyrite nanoparticles might be more susceptible to anaerobic oxidation [19]. In this study, pyrite nanoparticles were produced by anoxic milling, and the mineral structure was carefully examined by X-ray diffraction, energy-dispersive X-ray spectroscopy, atomic emission spectroscopy and transmission electron microscopy to rule out any mineral composition other than pure pyrite. This nanoparticle was used as electron donor in sediment-free and iron-free batch incubation with nitrate as sole electron acceptor at neutral pH. It was demonstrated that the reaction occurred rapidly at a rate of 38 $\mu$M·h$^{-1}$ NO$_3^-$ with a pure strain of *Thiobacillus denitrificans* [20, 21]. Furthermore, it could be shown for the first time that the measured reaction product concentrations came close to the expected theoretical stoichiometry of eqn (2) [19].

**Rates of anaerobic pyrite oxidation**

The available set of successful experiments has been produced under a wide range of conditions. So far, there have been batch incubations with variations in the initial nitrate concentrations, nitrate loading rates and cell densities [18], all of which had an impact on the nitrate-removal rates. Crystal size seemed to have a strong impact, as a higher surface area increased the reactivity by an order of magnitude [19] (Table 1). This fitted into the general picture of the enhanced reactivity of environmental nanominerals [22]. In sediment-containing flow columns, a higher flow rate and a higher pyrite loading naturally increased the removal efficiency [17, 18].

These experiments showed a wide range of denitrification rates, but the individual maxima of all rates mentioned of 0.05–38.56 $\mu$M·h$^{-1}$ NO$_3^-$ fell into a strikingly narrow range of only two orders of magnitude (Table 1). The conditions of the experiments ranged from pure nanoparticles in batch incubations with no limiting factors except pyrite reactivity to the simulated *in situ* conditions in flowthrough reactors. The narrow range therefore tentatively indicated that the underlying microbial process is more or less the same in all observations. Furthermore, rough calculations of pyrite-attributed denitrification rates in the field fall into the very same range [11], supporting the confidence in the available data (Table 1).

**Mechanisms of anaerobic pyrite oxidation**

Despite the initial negative results on pyrite reactivity in lithotrophic denitrification studies [15, 16], there is now some good indication from geochemical field data, isotope fractionation and laboratory studies of different scale and complexity that pyrite can be oxidized with nitrate as electron acceptor. However, the mechanism of microbial pyrite oxidation remains unclear. Considering the stability of metal disulfides in comparison with monosulfides, the underlying mechanism must be different from proton-promoted dissolution, as suggested for microbial FeS oxidation.

The general pyrite oxidation reaction, in disregard of the terminal electron acceptor, involves seven elementary steps, which occur at different mineral sites due to the semiconducting nature of pyrite [5]. The difficulties in
Pyrite oxidation rates taken from different studies

<table>
<thead>
<tr>
<th>Reference</th>
<th>Data source</th>
<th>Pyrite species</th>
<th>Pyrite oxidation rate ($\mu$M·h$^{-1}$ NO$_3^-$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Torrentó et al. (2010) [18]</td>
<td>Batch incubation</td>
<td>Crystals of 25-100 μm</td>
<td>0.07</td>
</tr>
<tr>
<td>Bosch et al. (2012) [19]</td>
<td>Batch incubation</td>
<td>Nanoparticles of ~1 μm</td>
<td>2.04</td>
</tr>
<tr>
<td>Torrentó et al. (2010) [18]</td>
<td>Columns</td>
<td>Crystals of 25-100 μm amended in sediment</td>
<td>38.56</td>
</tr>
<tr>
<td>Zhang et al. (2009) [11]</td>
<td>Field study</td>
<td>Sedimentary pyrite deposit</td>
<td>0.05</td>
</tr>
</tbody>
</table>

Consequences of pyrite depletion in anoxic sediments

Sedimentary pyrite deposits, especially when located over shallow aquifers and under a high anthropogenic nitrate influx by, e.g., agricultural fertilizers, serve as a buffer against nitrate transport into groundwater resources. Nitrate is a known contaminant of aquifers [6]. The depletion of this buffer will result in increasing nitrate contamination. As it is being depleted, pyrite oxidation can result in the release of pyrite-associated groundwater contaminants such as arsenic species or heavy metals [31–33]. Free sulfides from pyrite oxidation might lead to the dissolution of iron–phosphate minerals and in turn cause eutrophication [16]. Yet, in comparison with aerobic pyrite oxidation, anaerobic pyrite oxidation occurs at modest rates. One reason for this might be the relatively low environmental concentrations of dissolved nitrate in comparison with atmospheric O$_2$ concentrations.

With a ferric–ferrous electron-shuttling system putatively being relevant for anoxic nitrate-dependent pyrite oxidation, colloidal ferric nanoparticles could be involved in the reaction. Environmental iron oxide colloids have been shown to be highly reactive in microbial iron reduction [34].

Conclusions

Evidence for anaerobic nitrate-dependent pyrite oxidation is growing, and the first experimental demonstrations have been achieved. Yet the detailed molecular mechanisms are far from being understood, e.g. in comparison with the electron-transfer processes in microbial iron reduction. Whereas in microbial metal reduction, a solid electron acceptor is used, anaerobic pyrite oxidation can serve as an example for microbial solid electron donor utilization such as putatively the use of biotite [35]. Further research is needed to clarify the analogy to aerobic pyrite oxidation reactions. If an iron-mediated indirect mechanism is prevalent, a strong impact of pH and the applied putative electron-shuttling compounds [36] should be observed. When direct attachment of bacteria is of relevance, the cellular compounds involved should be identified. Further studies are needed on the...
responsible microbial communities and pH-dependency in the field, and the reaction kinetics in relation to particle size. Special attention should be paid to a comprehensive initial pyrite mineral and surface characterization [37] and a proper end-product analysis towards sulfur and iron.

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**References**


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