Different isotope and chemical patterns of pyrite oxidation related to lag and exponential growth phases of Acidithiobacillus ferrooxidans reveal a microbial growth strategy

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A B S T R A C T

The solution chemistry during the initial (slow increase of dissolved iron and sulfate) and main stage (rapid increase of dissolved iron and sulfate) of pyrite leaching by Acidithiobacillus ferrooxidans (Af) at a starting pH of 2.05 shows significant differences. During the initial stage, ferrous iron (Fe2+) is the dominant iron species in solution and the molar ratio of produced sulfate (SO4 2−) and total iron (FeTot) is 1.1, thus does not reflect the stoichiometry of pyrite (FeS2). During the main stage, ferric iron (Fe3+) is the dominant iron species in solution and the SO4 2−:FeTot ratio is 1.9, close to the stoichiometry of FeS2. Another difference between initial and main stage is an initial trend to slightly higher pH values followed by a drop during the main stage to pH 1.84. These observations raise the question if there are different modes of bioleaching of pyrite, and if there are, what those modes imply in terms of leaching mechanisms.

Different oxygen and sulfur isotope trends of sulfate during the initial and main stages of pyrite oxidation confirm that there are two pyrite bioleaching modes. The biochemical reactions during initial stage are best explained by the net reaction FeS2 + 3O2 ⇒ Fe3+ + SO4 2− + SO2(g). The degassing of sulfur dioxide (SO2) acts as sink for sulfur depleted in 34S compared to pyrite, and is the cause of the SO4 2−:FeTot ratio of 1.1 and the near constant pH. During the exponential phase, pyrite sulfur is almost quantitatively converted to sulfate, according to the net reaction FeS2 + 15/4O2 + 1/2H2O ⇒ Fe3+ + 2SO4 2− + H+. We hypothesize that the transition between the modes of bioleaching of pyrite is due to the impact of the accumulation of ferrous iron, which induces changes in the metabolic activity of Af and may act as an inhibitor for the oxidation of sulfur species. This transition defines a fundamental change in the growth strategy of Af. A mode, where bacteria gain energy by oxidation of elemental sulfur to sulfate but show little growth is switched into a mode, where bacteria gain a smaller amount of energy by the oxidation of ferrous iron, but induce much faster pyrite leaching rates due to the production of ferric iron.

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1. Introduction

Bacteria are important mediators of geochemical reactions. Due to its contribution to acid mine drainage problems, the bacterial oxidation of pyrite by Acidithiobacillus ferrooxidans (Af) is one of the best-studied examples of such a process. Pyrite oxidation rates are dramatically increased by the role of Af in the oxidation of ferrous to ferric iron. While bacterial oxidation of ferrous iron has been studied intensely and is well understood, the initiation of pyrite leaching by Af has only been investigated in a few studies (e.g. Mustin et al.; 1992; Yu et al.; 2001; Mielke et al.; 2003) and characterization of the chemical reactions resulting in sulfur and oxygen isotope fractionations is lacking so far. A deeper understanding of the bacterial mechanisms in the initial stage of pyrite leaching is essential in terms of ecology and evolution of Af (survival strategies) and for the evaluation of isotope patterns related to oxidation of pyrite that could indicate presence or absence of life (biomarkers).

During the initial stage of pyrite oxidation in the presence of Af, iron is mostly accumulated in its ferrous form, Fe2+, and the accumulation rates of iron and sulfate do not correspond to the 2:1 stoichiometry for sulfur and iron in pyrite (e.g. Mustin et al.; 1992; Yu et al.; 2001). In the main stage of pyrite leaching by Af, the leaching products are sulfate and ferric iron (Fe3+), and their ratio is close to the 2:1 sulfur–ferric stoichiometry of pyrite (FeS2) (Yu et al.; 2001). The
curious discrepancy in the solution chemistry between different stages of pyrite leaching by Af leads to the hypothesis that the pyrite oxidation mechanism in the presence of Af in the initial stage of pyrite oxidation is different from the one in the main stage.

1.1. The Yu et al. (2001) experiments

Yu et al. (2001) carried out batch pyrite leaching experiments with Af with open access to air in acidic conditions at a pH of 2.04 and they monitored the extent of evaporation of the media. They observed two stages of pyrite leaching, an initial stage where Fe\(^{2+}\) and sulfate were the main products, and after approximately 400 h, a second stage with Fe\(^{3+}\) and sulfate as main products (Fig. 1). In the initial stage, the production of dissolved iron and sulfate was generally low (2.5 mmol SO\(_4^{2-}\)/L, 1.7 mmol Fe\(^{2+}\)/L in 381 h), and decreased with time. However, compared to nonbiological (uninoculated) leaching of pyrite under the same conditions, Af considerably increases the leaching rate during the initial stage of pyrite leaching (Fig. 1). The second stage of pyrite leaching is marked by strongly increased production rates of iron and sulfate, and therefore, is called the main stage of pyrite leaching. During the initial phase of pyrite leaching, the number of bacteria stayed low and most of the bacteria were attached to the pyrite surface. At the end of the initial leaching stage there is a significant reduction in cell count. During the main stage of pyrite oxidation, the number of bacteria grew dramatically and most bacteria were detached from the pyrite surface (Fig. 1; Table 1).

The change between the modes of pyrite leaching is marked by a change in the ratio of produced sulfate to iron (Fig. 2). During the initial stage, the SO\(_4^{2-}\)/Fetot ratio was 1.1±0.1, a behavior that has been called nonstoichiometric, referring to ratios that are not equal to the 2:1 stoichiometry of pyrite (FeS\(_2\)). During the main stage, the SO\(_4^{2-}\)/Fetot ratio was 1.9±0.0, thus almost stoichiometric. This change is accompanied by a change in the pH of the solution: the pH of the solution stayed constant or slightly increased (2.08±0.04) during the initial stage and dropped to pH 1.84±0.04 in the main stage of pyrite leaching (Fig. 2).

The nonstoichiometric behavior of the SO\(_4^{2-}\)/Fetot ratio in the solution implies that not as much sulfur as iron is leached from pyrite as one would expect from strict dissolution of FeS\(_2\), or that other sulfur species than sulfate (e.g. elemental sulfur S\(_0\), sulfite SO\(_3^{2-}\), thiosulfate S\(_2\)O\(_3^{2-}\)) are accumulated. Yu et al. (2001) neither detected any elemental sulfur by Soxhlet extraction (detection limit, 10 µmol), nor dissolved sulfur species besides sulfate by ICP-AES (detection limit, 1 ppm). We, therefore, refer to this undetected sulfur species as “missing sulfur”. The SO\(_4^{2-}\)/Fetot ratio (Fig. 2) clearly shows that the “missing sulfur” was not converted to sulfate at the beginning of the main stage of pyrite leaching, but remained missing. As indicated by the SO\(_4^{2-}\)/Fetot ratio of 1.9 during the main stage, “sulfur loss” still continues.

1.2. Nonstoichiometric leaching of pyrite in abiotic experiments

Interestingly, nonstoichiometric leaching of pyrite has also been observed in abiotic experiments: Descostes et al. (2004) report SO\(_4^{2-}\)/Fetot ratios of 1.5 to 1.6 at pH of 1.2 to 2 in abiotic experiments with perchloric and hydrochloric acid and present a data compilation that indicates that the observed ratio depends on the pH of the solution. Descostes et al. (2004) suggest that the “missing sulfate” may have degassed as sulfur dioxide (SO\(_2\)).

1.3. Speciation of sulfate, and potential degassing of sulfur dioxide (SO\(_2\))

Sulfite is an important intermediate in biologic oxidation of sulfur compounds (e.g. Vestal and Lundgren, 1971; Eccleston and Kelly, 1978; Pronk et al., 1990; Hirose et al., 1991; Suzuki et al., 1992; Wodara et al., 1997; Masau, 1999; Friedrich et al., 2001; Rohwerder and Sand, 2003; Waki et al., 2004; Rawlings, 2005; Sugio et al., 2006) and likely to be an important intermediate in the pyrite leaching mechanism. In the absence of an oxidant (e.g. Fe\(^{3+}\)), it may accumulate in solution. At a pH of 2, speciation programs (such as visual Minteq®), calculate that 37% of sulfite is present as H\(_2\)SO\(_3\) (sulfurous acid molecule). However, there is no evidence for the existence of H\(_2\)SO\(_3\) (Betts and Voss, 1970; Horner and Connick, 2003 and references therein), the most acidic species is sulfur dioxide (SO\(_2\)). Thus, 37% of the sulfite species in an acid solution must exist as volatile sulfur dioxide.

1.4. Observations from sulfur and oxygen isotope studies

The sulfur in sulfate of pyrite leaching solutions is ultimately derived from pyrite. If pyrite is quantitatively oxidized to sulfate, no sulfur isotope fractionation can occur and the sulfur isotope composition of sulfate reflects that of pyrite. However, if sulfur pools other than sulfate are formed, sulfur isotope fractionation can occur and the actual oxidation mechanism of pyrite sulfur becomes important. Sulfur isotope fractionation may occur during the stepwise oxidation of pyrite sulfur, when existing sulfur species are consumed (e.g. breaking of S–S bond when thiosulfate is transformed into elemental sulfur and sulfite) and new sulfur species are formed (e.g. oxidation of elemental sulfur to sulfite), or also when sulfur isotopes are exchanged (e.g. sulfur isotope exchange between sulfonate and sulfane sulfur of the thiosulfate molecule, Uyama et al., 1985; Chu et al., 2004). Thus, the sulfur isotope composition of sulfate from leaching of pyrite preserves information about the isotope composition of the leached pyrite, as well as information about potential diversion of sulfur species in pools other than sulfate. Commonly, sulfur isotope fractionation related to abiotic and biotic oxidation of sulfur species is relatively small (Taylor et al., 1984; Toran and Harris, 1989; Balci et al., 2007; Pisapia et al., 2007 and references therein).

Under neutral conditions (pH 7) and at ambient temperatures (25 °C), oxygen isotope exchange between sulfate and water is
extremely slow (Lloyd, 1968; Zak et al., 1980). Even at pH 2, oxygen isotope exchange is slow (half time ~10 yr, extrapolated from Lloyd, 1968; half time ~10,000 yr, extrapolated from Chiba and Sakai, 1985). Thus, sulfate is likely to preserve its oxygen isotope composition once it is formed. Therefore, sulfate derived from leaching of pyrite preserves information about 1) the source of oxygen (in most cases oxygen is either derived from water or from dissolved oxygen), 2) the oxygen isotope fractionation related to biochemical reactions that oxidize pyrite sulfur and sulfur intermediates, and 3) the oxygen isotope exchange processes between intermediate sulfur species (i.e. $SO_3^{2-}$) and water. So far, most studies have focused on the first two mechanisms, assuming that oxygen isotope exchange between intermediate sulfur compounds and water is of minor importance (Toran and Harris, 1989). Taylor et al. (1984) estimate an isotope fractionation of ~11.4% for the Af-mediated oxidation of pyrite with dissolved oxygen under submersed conditions and an isotope fractionation of +8.9‰ to +10.9‰ for the incorporation of oxygen derived from water under the same conditions. For the same process, Balci et al. (2007) measured fractionations of ~9.8‰ to ~10.9‰ and +3.5‰, respectively; and Pisapia et al. (2007) measured fractionations of ~25‰ and +16‰, respectively. The oxygen and sulfur isotope effects related to the degassing of SO$_3$ from the solution of the remaining sulfite pool has yet not been investigated, however, due to the kinetic nature of such an escape, one would expect that the sulfur dioxide remaining in solution would be enriched in $^{34}$S and $^{18}$O (Biegeleisen, 1949).

1.5. Summary

- Nonstoichiometric leaching of pyrite has been observed both in abiotic and biological experiments with Af.
- The solution chemistry in the initial and main stage leaching of pyrite by Af indicates that there are different biochemical mechanisms between the different leaching modes.
- Sulfur seems to be missing or is present in a yet undetected form.
- Because it is likely that sulfite is one of the sulfur intermediates formed both in abiotic and biological experiments, degassing of sulfur dioxide may account for the missing sulfur, however, this has not been observed so far.
- The sulfur and oxygen isotope composition of formed sulfate could give crucial information for understanding the processes involved, i.e. about the existence of a “missing sulfur pool”.

In this paper, we report the results of experimental work that investigates the potential degassing of sulfur dioxide during pyrite oxidation and its effect on the sulfur and oxygen isotope data of the remaining sulfur species, and we report and interpret sulfur and oxygen isotope analysis of water that was produced in the course of the experiments by Yu et al. (2001).

2. Methods

Stable isotopic compositions of water and dissolved sulfate were measured for the samples collected by Yu et al. (2001), who performed a batch experiment of pyrite oxidation with Af. The details of the experimental procedures and the chemical compositional information on the collected samples are described in Yu et al. (2001). The analyzed isotopic compositions include the oxygen isotope composition of water, the oxygen and sulfur isotope composition of sulfate and the sulfur isotope composition of pyrite. The isotopic composition is reported with respect to the standards, Vienna Standard Mean Oceanic
Water (VSMOW) for oxygen and Vienna Canyon Diablo Troilite (VCDT) for sulfur. The oxygen isotope composition of water was measured using CO₂ equilibration method (Epstein and Mayeda, 1953). Measurements were performed with a stable isotope ratio mass spectrometer (SIR-MS) model VG SIRA 10 at the Postgraduate Research Institute for Sedimentology (PRIS), Reading University, United Kingdom. The reference material used for the calibration of the analytical data was PRIS laboratory standard DW-1 #35 (δ²⁷⁸O=−6.7‰) for oxygen. Dissolved sulfate in the experimental solutions was extracted by precipitation in the form of BaSO₄ (Kolthoff et al., 1969). For sulfur isotope analysis 0.6 mg of the precipitated BaSO₄ and 1.2 mg V₂O₅ was transferred to a silver capsule and thermochemically reduced at 1450 °C in the presence of graphite and glassy carbon in the Finnigan Thermal Conversion/Elemental Analyzer (TC/EA) to produce CO. The evolved SO₂ and CO were converted to sulfate. During the initial stage of pyrite leaching the pH is higher than the initial pH, but no clear trend can be observed. During the main stage of pyrite leaching, the pH drops continuously. Regression lines are calculated for 95% confidence level. Symbols: crosses: number of cells; filled squares: produced sulfate (uninoculated); hollow circles: pH (Af); hollow circles: pH (uninoculated).

Initial experiments were carried out to test the possibility of degassing of SO₂ in abiotic pyrite leaching (Table 2a, b, c). One set of experiments investigated if abiotic leaching of pyrite with hydrochloric acid produces SO₂ in the headspace of serum bottles flushed with argon gas (Table 2a), a second set tested the same for leaching of pyrite with sulfuric acid (Table 2b). Gas from the headspace from the experiments with hydrochloric acid was flushed by an Ar gas stream into a Ba(OH)₂ solution, where SO₂ precipitates as barium sulfate. The formed precipitates were checked by XRD to examine for the possible presence of BaSO₄. The presence of SO₂ in the experiments with sulfuric acid was tested by an alternate method: A 10 μl gas sample was drawn from the headspace of the serum bottles with a gas-tight syringe and transferred to a 1 L Tedlar sample bag containing pure (99.999%) helium. The sample bag was connected to a carboxen trap, with gas being drawn from the bag for 2 min to adsorb SO₂ onto the carboxen in the process. Following the sampling process, the trap was isolated and purged with helium prior to performing temperature programmed desorption of the carboxen. The desorbed SO₂ was then injected into a prototype gas chromatograph mass spectrometer designed at JPL, Caltech (Shortt et al., 2005). The detection limit of this mass spectrometer for sulfur dioxide gas is below 10 ppt. The abiotic leaching experiments were performed to investigate if degassing of SO₂ is possible, but do not allow to make conclusive estimates of the quantity of degassed SO₂. In order to do so, a series of experiments that would focus on how experimental parameters affects the degassing would need to be performed. Parameters, such as duration of experiment, flushing of headspace of serum bottles with Ar gas, amount of pyrite and periodic shaking of bottles were chosen arbitrarily. Argon was used to flush the headspace of the serum bottles because we assumed that produced SO₂ would be less prone to be oxidized to sulfate under inert conditions. However, low pO₂ in the headspace may also have reduced the amount of pyrite that was oxidized by the attack of dissolved oxygen, thus could also have led to a lower accumulation of SO₂.

Since very small amounts of SO₂ were produced in the acid leaching experiments (Table 2b) no isotope analysis could be carried out on the produced gas. Therefore, a third experiment investigated the sulfur isotope effect related to the degassing of SO₂ from sulfuric acid (Table 2c). Twenty-five ml of 0.1 M H₂SO₄-K₂SO₄ solution (pH 1.5) was injected through a septum into an Ar-flushed serum bottle (125 ml) that contained 0.1 g Na₂SO₃. After 5 min, headspace samples were transferred with a gas-tight syringe into the He-flow of the elemental analyzer used for sulfur isotope analysis. The sulfur isotope measurements were calibrated with the reference materials RM 8557 (NBS 127; δ³⁴S=+20.32‰), RM 8553 (Soufre de Lacq; δ³⁴S=+16.00‰) and RM 8556 (NBS 123; δ³⁴S=−0.3‰) of National Institute of Standards and Technology. The oxygen isotope measurements were calibrated with NBS 127 (δ¹⁸O=8.6‰, Boscetti and lacumin, 2005) and JPL laboratory BaSO₄ standard (δ¹⁸O=11.6‰). For the measurement of the sulfur isotope composition of pyrite, 0.07 mg of the starting pyrite was mixed with 1.2 mg V₂O₅ and analyzed by following the procedures for sulfur isotope analysis of BaSO₄. The standard errors (σ₁) of the measurements were less than 0.1‰ for δ¹⁸Oₛₒ₄, 0.2‰ for δ¹⁴Nₛₒ₃, and 0.3‰ for δ¹⁸Oₛₒ₃. The sulfur isotope composition of pyrite was found to be −3.7±0.3‰ (p=0.05, n=6).

### Table 2a

<table>
<thead>
<tr>
<th>Pyrite powder RM 8455 NIST (g)</th>
<th>HCl-KCl 0.1 M (ml)</th>
<th>pH</th>
<th>Ar²</th>
<th>Duration (weeks)</th>
<th>Shaking</th>
<th>Treatment</th>
<th>XRD analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.017</td>
<td>30</td>
<td>1.9</td>
<td>30</td>
<td>3</td>
<td>12 times</td>
<td>Transfer of headspace into Ba(OH)₂</td>
<td>BaCO₃&gt;90%, no BaSO₄</td>
</tr>
<tr>
<td>0.05</td>
<td>30</td>
<td>1.9</td>
<td>30</td>
<td>3</td>
<td>12 times</td>
<td>trap by Ar stream</td>
<td>BaCO₃&gt;90%, no BaSO₄</td>
</tr>
<tr>
<td>0.105</td>
<td>30</td>
<td>1.9</td>
<td>30</td>
<td>3</td>
<td>12 times</td>
<td>BaCO₃&gt;90%, BaSO₄&gt;53</td>
<td></td>
</tr>
</tbody>
</table>

* The headspace was flushed with Argon gas. This treatment does not ensure that dissolved oxygen is removed from the acid solution.
composition of evolved SO$_2$ was directly compared to the SO$_2$ produced by the combustion of Na$_2$SO$_3$ (0.3 mg).

### 3. Results

The results of the isotope measurements are summarized in Table 1. The sulfur isotope composition of samples drops from 3.5‰ at the beginning of the initial stage to 2.4‰ at the end of the initial stage of pyrite leaching (380 h). It further drops from 1.3‰ at the beginning of the main stage of pyrite leaching to −1.7‰ at the end of the main stage of pyrite leaching (770 h). The sulfur isotope composition of the sulfur from the uninoculated experiments is invariant over the course of the experiment.

The average oxygen isotope composition of sulfate in the initial stage is 7.5‰, ranging from 6.5‰ to 8.6‰. The data scatter and there is no trend. During the main stage of pyrite leaching, the oxygen isotopic value of accumulated sulfate drops from 6.2‰ to 3.2‰ at the end of the experiment. The oxygen isotope composition of water rises from −5.6‰ at the beginning to −2.4‰ at the end of the initial stage, as a result of the evaporation of the media also measured by the increase in Mg. During the main stage of pyrite leaching, the oxygen isotope composition of water is between 0.3‰ and 0.6‰, with oneoutlying data point at +4.6‰.

The initial experiments to explore the possibility of degassing of SO$_2$ from acid solutions containing pyrite yielded the following results: The flushing of the headspace from the pyrite leaching experiments into a Ba(OH)$_2$ solution caused the formation of precipitates in all 3 experiments. The precipitates were identified by XRD to be dominantly BaCO$_3$ (≥90%), most likely derived from CO$_2$ by contamination with air during the transfer of the headspace into the barium hydroxide trap. BaSO$_3$ (<5%) was identified by XRD in the experiment with most pyrite (0.01 g), whereas no sulfate was detected in the other two experiments (Table 2a). The analysis of the gas in the headspace, with the prototype mass spectrometer, clearly showed a presence of SO$_2$ (Fig. 3) for the sample containing the most pyrite (0.105 g). In the case of the blanks and the samples with less pyrite (0.017 g, 0.05 g), no SO$_2$ was detected (Table 2b). The SO$_2$ level in the experiments with 0.105 g pyrite was very close to the detection limit of the mass spectrometer, thus below 10 ppt. Taking the dilution factor from the sampling procedure into account (10 μl headspace gas injected into a 1 L Tedlar sample bag containing pure helium) the concentration of SO$_2$ in the headspace of the serum bottle is calculated to be below 1 ppm. Repeated analysis of the sample gas from the Tedlar bag showed a decrease in the amount of detected SO$_2$ (Fig. 3). This implies that SO$_2$ is lost over time, most likely due to adsorption on the sample bag. Thus, we may underestimate the actual amount of SO$_2$ in the serum bottles. The sulfur dioxide that evolved from the experiment, where sodium sulfate was dissolved in acid, was depleted in $^{34}$S by −12±2‰, with respect to the Na$_2$SO$_3$ used (Table 2c).

### 4. Discussion

The collected sulfate samples consist of initial sulfate in the experiment and sulfate accumulated in solution during the progressive leaching of pyrite. Thus, the sulfur and oxygen isotope composition measured does not reflect the isotope composition of sulfate produced at a certain time, but an average of the isotope composition of initial sulfate and the isotope composition of sulfate produced between the start of the experiment and the time the sample was taken. In order to derive the isotope composition of produced sulfate from our data, we plot the isotope data against the relative amount of produced sulfate to total sulfate (Fig. 4 and 6) according to:

$$X = \frac{\text{produced sulfate}}{\text{(initial sulfate + produced sulfate)}}$$

At the start of the experiment, where all sulfate is initial sulfate, X equals 0, towards the end of the experiment, where the amount of produced sulfate is much larger than the initial amount of sulfate, X approaches 1. In such plots, linear trends in the isotope composition of sulfate indicate the production of sulfate with a constant isotope composition, and this composition can be deduced by extrapolation of the regression line to X=1.

Fig. 4 shows linear isotope trends with respect to the relative amount of produced sulfate. During the main stage of pyrite leaching, there is a strong linear correlation between the sulfur isotope composition of sulfate in solution and X ($R^2=0.99$). Extrapolation of the regression line for the main stage to X=1 indicates the production of sulfate with a $^{34}$S of −3.9±1.8‰ for the main stage which is close to the $^{34}$S of pyrite of −3.7±0.3‰. No sulfur isotope fractionation between pyrite and produced sulfate in aerobic leaching by AF was observed in the experiments by Balci et al. (2007) for the main stage of pyrite leaching as well.

During the initial stage of pyrite leaching, the sulfur isotope trend is not as steep as during the main stage of pyrite leaching, and there is only a weak linear correlation between the sulfur isotope data and X ($R^2=0.60$), indicating that processes other than the addition of sulfur from pyrite leaching contribute to the sulfur isotope composition of produced sulfate, i.e. that sulfur isotope fractionation occurs. This is confirmed by extrapolating the regression line for the main stage of pyrite leaching to X=0: If the sulfur isotope composition of produced sulfate would have been the same over the whole course of the experiment, the initial composition would be 5.1±1.0, which is significantly different from the actual measured initial composition of 3.5±0.3‰. Thus, sulfate produced during the initial stage of pyrite

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**Table 2b**

<table>
<thead>
<tr>
<th>Pyrite powder RM 8455 NIST (mg)</th>
<th>H$_2$SO$_4$–K$_2$SO$_4$ 0.1 M (ml)</th>
<th>pH</th>
<th>Ar* (s)</th>
<th>Duration (weeks)</th>
<th>Shaking</th>
<th>Treatment</th>
<th>Ion Trap analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 g (blank) 30</td>
<td>0.15</td>
<td>3</td>
<td>3</td>
<td>12 times</td>
<td>Injection of 10 μl headspace gas into 1 L Tedlar bag for subsequent analysis by ION Trap</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>0.05 g</td>
<td>30</td>
<td>1.57</td>
<td>30</td>
<td>3</td>
<td>No</td>
<td>No</td>
<td>Yes, &lt;10 ppt</td>
</tr>
<tr>
<td>0.105 g</td>
<td>30</td>
<td>1.57</td>
<td>30</td>
<td>3</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
</tbody>
</table>

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* The headspace was flushed with Argon gas. This treatment does not ensure that dissolved oxygen is removed from the acid solution.

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**Table 2c**

Sulfur isotope effect by degassing of SO$_2$ from a Na$_2$SO$_3$ solution by acidification with H$_2$SO$_4$ in a sealed serum bottle (125 ml)

<table>
<thead>
<tr>
<th>Na$_2$SO$_3$ (g)</th>
<th>Ar* (s)</th>
<th>H$_2$SO$_4$–K$_2$SO$_4$ 0.1 M (ml)</th>
<th>pH</th>
<th>Duration (weeks)</th>
<th>Treatment</th>
<th>Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>30</td>
<td>25</td>
<td>1.5</td>
<td>5 min</td>
<td>Injection of headspace into Elemental Analyzer IRMS</td>
<td>$^{34}$S (SO$_2$)–$^{34}$S (Na$_2$SO$_3$)</td>
</tr>
</tbody>
</table>

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<table>
<thead>
<tr>
<th>Na$_2$SO$_3$ (g)</th>
<th>Ar* (s)</th>
<th>H$_2$SO$_4$–K$_2$SO$_4$ 0.1 M (ml)</th>
<th>pH</th>
<th>Duration (weeks)</th>
<th>Treatment</th>
<th>Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>30</td>
<td>25</td>
<td>1.5</td>
<td>5 min</td>
<td>Injection of headspace into Elemental Analyzer IRMS</td>
<td>$^{34}$S (SO$_2$)–$^{34}$S (Na$_2$SO$_3$)</td>
</tr>
</tbody>
</table>

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* The serum bottle was flushed with Argon gas after addition of Na$_2$SO$_3$. The bottle was capped and the H$_2$SO$_4$ solution injected with a syringe.

* Direct comparison of 10 replicates (1σ) to SO$_2$ produced by combustion of 0.3 mg Na$_2$SO$_3$. 
Af
d sulfate produced by nonstoichiometrical leaching of pyrite by circles mean value of the background count rate. The sulfur dioxide signal is averaged hollow circles: δ of copper absorption/reaction of SO2 with the walls of the sample bag. We observe an increase in Cu2+. This could indicate a preferential leaching stage of pyrite leaching. Together with the increase in sulfate, they found only a minor enrichment in 34S (Pisapia et al. 2007) is strongly different from the one by Yu et al. (2001); Pisapia et al. (2007) is from +4.1 to –6‰ (Taylor et al., 1984) and references therein. For the main stage of pyrite leaching by Af, Balci et al. (2007) found an enrichment in 18O of +3.5‰ in sulfate compared to water. The discrepancy between the results of Balci et al. (2007) and ours may be partly due to the use of different values for the standard NBS 127. Balci et al. (2007) use the internationally accepted value of +9.3‰ while we use a value of +8.6‰ which has been suggested by Boschetti and Iacumin (2005). We prefer the latter value for NBS 127 since it has been determined by direct comparison to water standards (Böhlke et al., 2003). This is important because most oxygen in sulfate from pyrite oxidation is derived from water. Using the value +9.3‰ for NBS 127, the oxygen isotope fractionation in the main stage of our experiment would be calculated as 2.0±2.6‰, which, compared to the result of Balci et al. (2007) is within error.

During the main stage of pyrite leaching, the oxygen isotope composition of sulfate shows a clear trend and correlates well with X (R2=0.91). By extrapolation of the regression line to X=1 a δ18O of 1.3±2.6‰ for produced sulfate results, which is close to the oxygen isotope composition of water for the latter part of the experiment (0‰ to 1‰). Dependent on the pyrite oxidation pathway, different oxygen isotope fractionations can be determined, the range for the isotope fractionation related to the incorporation of oxygen derived from H2O is from +4.1 to –6‰. The nonlinear trend in the oxygen isotope composition of water is due to the fact that sulfate accumulation is not linear with time. Thus, small sulfate accumulation towards the end of the initial stage of pyrite leaching causes the oxygen isotope curve for water to appear steeper, while high sulfate production during the main stage of pyrite causes the oxygen isotope curve for water to appear more flat.

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The oxygen isotope data for sulfate from the initial stage of pyrite leaching scatter strongly, there is no linear correlation between the oxygen isotope composition of sulfate and $X$ ($R^2=0.04$) despite a progressive 6‰ increase of water $\delta^{18}O$. This indicates that there may be a difference in the processes that control the oxygen isotope composition of produced sulfate for the initial stage of pyrite leaching compared to the mechanism in the main stage, where the oxygen isotope composition of produced sulfate is similar to the oxygen isotope composition of water. We explore this by extrapolating the regression line for the main stage of pyrite leaching to $X=0$: If the oxygen isotope composition of produced sulfate would be derived from water with an isotope composition of 1.3‰, the oxygen isotope composition of the initial sulfate would be 10.18±1.3, which is significantly different from the actual measured initial composition of 7.4±0.3‰. Taking into account that the oxygen isotope composition of water during the initial stage was depleted in $\delta^{18}O$ compared to the water of the main stage, the back calculated initial value would be even higher. For example, using an isotope composition of $-3$‰ for sulfate produced during the phase $X=0$ to $X=0.22$, followed by sulfate with a composition of 1.3‰, an oxygen isotope of initial sulfate of more than 11‰ would result (Fig. 6). This is clear evidence that, sulfate produced during the initial stage of pyrite leaching is enriched in $\delta^{18}O$ compared to water. Since there is no clear trend in the oxygen isotope composition of sulfate produced in the initial stage its average oxygen isotope composition must be roughly equal to the isotope composition of initial sulfate (7.4±0.3‰), however, the scatter in the data shows that the actual oxygen isotope composition of produced sulfate varies strongly.

The sulfur isotope composition of produced sulfate from the initial stage can be estimated by extrapolation of the regression lines for the data to $X=1$. However, this estimate must be taken with caution, since there is scatter in the data the linear correlation between $X$ and sulfur isotopes is weak ($R^2=0.60$). Extrapolation of the regression lines to $X=1$ indicates the formation of sulfate with a $\delta^{34}S$ of 0.3 ± 1.4‰ for the initial stage. This indicates the formation of two sulfur pools, sulfate enriched in $\delta^{34}S$ by roughly 4.0‰ compared to pyrite and another “missing sulfur” pool, which is depleted in $\delta^{34}S$ with respect to pyrite. Using the nonstoichiometric relationship between sulfate and total iron of 1.1 the isotope composition of the “missing sulfur” pool can be estimated to be around $-9.0$‰. Thus, the “missing sulfur” is depleted in $\delta^{34}S$ by roughly $-5.3$‰ relative to pyrite and by $-9.3$‰ relative to sulfate.

5. Interpretation

5.1. Leaching mode of pyrite in the initial stage and degassing of SO$_2$

Our data show that the initial stage of pyrite leaching by $Af$ produces sulfate that is different in its oxygen and sulfur isotope composition from the one produced in the main stage of pyrite leaching. This is evidence for differences in the mechanism of the leaching process (i.e., kinetics and/or reaction pathways) that need to be explained in the context of the observation of the changes in the solution chemistry (Fe species, pH and sulfate–iron ratio), microbial growth and abiotic pyrite oxidation mechanism.

Actual degassing of sulfur dioxide related to abiotic or biological leaching of pyrite has not been recorded so far. A correlation between the population size of $Af$ in acid sulfate soils and the evolution of SO$_2$ has been observed by Dürs et al. (2004). Emission of sulfur dioxide from sulfuric soils is related to the oxidation of pyrite; the mechanism causing these emissions is currently not resolved (Macdonald et al. 2004; Kinsela et al., 2007). Descostes et al. (2004) report $SO_2^2$/Fe$_{tot}$ ratios of 1.5 to 1.6 at pH of 1.2 to 2 in abiotic experiments with perchloric and hydrochloric acid and present a data compilation that indicates that the observed ratio depends on the pH of the solution. Since the degassing rate of SO$_2$ is higher at lower pH, Descostes et al. (2004) suggest that the “missing sulfate” may have degassed as sulfur dioxide (SO$_2$). Our initial experiments with acid leaching of pyrite by hydrochloric and sulfuric acid show that small quantities of SO$_2$ can be produced. This provides evidence for the formation of sulfate species during abiotic leaching of pyrite but no proof that degassing of SO$_2$ accounts for the nonstoichiometric sulfate:iron ratios in acid pyrite leaching experiments. Druschel and Borda (2006) showed that nonstoichiometric sulfate:iron ratios can be caused by other processes: At pH below 3, Fe-SSO$_3$ surface structures on the pyrite surface are the first product of pyrite leaching (Fig. 6), allowing subsequent reactions to break the S–S bond (Rimstidt and Vaughan, 2003), releasing sulfate or sulfite species to the solution (Borda et al.)

![Fig. 6. Thiosulfate pathway (modified from Druschel and Borda, 2006): Formation of a Fe-SSO$_3$ surface structure, which can detach as thiosulfate (right side, occurs at pH<3 Luther, 1987; Williamson and Rimstidt, 1993) or as sulfate/sulfite under the production of a residual sulfur pool (left side, likely to occur at pH below 3). Left side: Formation of sulfate or sulfite under the production of monosulfide, which may be oxidized to elemental sulfur or sulfonate ions (Druschel and Borda, 2006). Right side: Release of thiosulfate (S$_2$O$_3^{2-}$) with subsequent competition between two reactions: Decomposition into elemental sulfur and sulfite (SO$_2^{2-}$) under acidic conditions or formation of tetrathionate (S$_4$O$_6^{2-}$) in the presence of ferric iron (Fe$^{3+}$). However, unless the supply of ferric iron is limited, the latter reaction dominates (Williamson and Rimstidt, 1993). Dark boxes: Sulfite is an important intermediate in most of the depicted pathways. Under acidic conditions, it rapidly equilibrates oxygen isotopes with water and allows for degassing of sulfur dioxide (SO$_2$), which is driven by the pH and venting conditions. Loss of SO$_2$ (dashed arrows, $\Delta^{34}S$) or as sulfur is weak (Luther, 1987; Williamson and Rimstidt, 1993) or as sul}
The abiotic oxidation of pyrite to sulfate is composed by two half-reactions (Rimstidt and Vaughan, 2003):

- Cathode site of pyrite: \(3/2\text{O}_2 + 6\text{H}^+ \rightarrow 3\text{H}_2\text{O}\) (1.1)
- Anode site of pyrite: \(\text{FeS}_2 + 3\text{H}_2\text{O} \rightarrow \text{Fe}^{3+} + \text{S}^0 + \text{SO}_4^{2-} + 6\text{H}^+\). (1.2)

And, reactions to sulfate, respectively are:

- Cathode site of pyrite: \(2\text{O}_2 + 8\text{H}^+ \rightarrow 4\text{H}_2\text{O}\) (2.1)
- Anode site of pyrite: \(\text{FeS}_2 + 4\text{H}_2\text{O} \rightarrow \text{Fe}^{3+} + \text{S}^0 + \text{SO}_4^{2-} + 8\text{H}^+\). (2.2)

Either sulfite or sulfate could be the reaction product and both reactions are pH neutral. This scenario includes the formation of monosulfide, which may be oxidized to elemental sulfur and sulfur oxonium species (Druschel and Borda, 2006). If not all monosulfide is subsequently oxidized to sulfite, this chemical pathway can cause nonstoichiometric \(\text{SO}_4^{2-}/\text{Fe}_{\text{tot}}\) ratios as low as 1. However, detectable amounts of those sulfur species should remain in the solution or on the pyrite surface. Descostes et al. (2004) did not detect any dissolved sulfur species besides sulfate by ion chromatography; the presence of elemental sulfur was not investigated in their experiments. In the here investigated pyrite leaching experiments with \(Af\) by Yu et al. (2001) neither elemental sulfur nor dissolved sulfur species besides sulfate were detectable, and the “missing sulfur” was not converted to sulfate at the beginning of the main stage of pyrite leaching (Fig. 2), but remained missing. This leads us to the conclusion that accumulation of sulfur species and water is very rapid (Betts and Voss, 1970; Horner and Connick, 2003), however, the exact oxygen isotope fractionation between sulfate and water is currently not known. The equilibrium oxygen isotope fractionation between sulfur dioxide and water vapor is +24.3‰ (Holt et al., 1983). At pH 7 sulfite is enriched in \(^{18}\text{O}\) at least by 11‰ compared to water (Brunner et al., 2006). This implies, that if sulfate species are present, and exist long enough to allow for oxygen isotope exchange, sulfite would be enriched in \(^{18}\text{O}\) compared to water. The possibility of oxygen isotope fractionation between sulfate and water has been mentioned by Lloyd (1968) and it has been speculated that this process may be responsible for the oxygen isotope effects observed in sulfate related to dissimilatory sulfate reduction and disproportionation of sulfur (e.g. Fritz et al., 1989; Böttcher et al., 2005; Brunner et al., 2005; Turcyn and Schrag, 2006). Thus, both degassing of \(\text{SO}_3\) and oxygen isotope exchange between water and sulfate should lead to sulfite enriched in \(^{18}\text{O}\) compared to water. Correspondingly, sulfate derived from the oxidation of this sulfite should be enriched in \(^{18}\text{O}\) as well. This accords well with the sulfite produced during the initial stage of pyrite leaching which is enriched in \(^{18}\text{O}\) compared to water by roughly 10‰. The strong scatter in the data may be due to the competition between processes deriving oxygen from water and processes that derive oxygen from \(\text{O}_2\) for the oxidation of the sulfur species to sulfate.

### 5.2. Leaching mode of pyrite in the initial stage and degassing of \(\text{SO}_2\)

During the main stage, pyrite sulfate is almost quantitatively converted to sulfate, according to the net reaction

\[
\text{FeS}_2 + 15/4\text{O}_2 + 1/2\text{H}_2\text{O} \rightarrow \text{Fe}^{3+} + 2\text{SO}_4^{2-} + \text{H}^+.
\] (4.1)

This reaction is composed of the abiotic attack of ferric iron on pyrite, where the oxygen of sulfate is derived from water

\[
\text{FeS}_2 + 14\text{Fe}^{3+} + 8\text{H}_2\text{O} \rightarrow 15\text{Fe}^{2+} + 2\text{SO}_4^{2-} + 16\text{H}^+.
\] (4.2)

and by the oxidation of ferrous iron with dissolved oxygen to ferric iron, a reaction which is catalyzed by \(Af\) \(\Delta G^\circ = -33 \text{kJ/mol Fe}^{2+}; -131.7 \text{kJ/mol O}_2\)

\[
15\text{Fe}^{2+} + 15/4\text{O}_2 + 15\text{H}^+ \rightarrow 15\text{Fe}^{3+} + 15/2\text{H}_2\text{O}.
\] (4.3)

Due to the quantitative conversion of sulfur from pyrite to sulfate, no sulfur isotope fractionation is observed, and the oxygen isotope composition is close to that of water.

### 5.3. Offset between lag phase of growth of \(Af\) and change in leaching stage of pyrite

The cell counts of bacteria in solution indicate that the transition between the lag phase of growth (no increase in cell counts) and the exponential phase of growth (linear increase in counts on a logarithmic scale) occurs at a time around 70 h of incubation (Fig. 1). The major change in the solution chemistry and in the isotope composition of produced sulfate, however, takes place at around 400 h of incubation. This raises the question if the chemical changes are directly related to...
changes in the growth phases of the bacteria. During the initial stage of pyrite leaching, the major increase in sulfate and iron in solution occurs in the first 100 h, while in the time between 100 and 400 h, the sulfate and iron concentration remains almost constant (Fig. 1). This indicates that the leaching mode of Af during the lag phase is replaced by a leaching mode that belongs to the initial part of the exponential growth phase. Since ferrous iron is readily available in the solution, the cells in solution are likely to gain their energy from its oxidation to ferric iron Eq. (4.3). In fact, the cell count just before 400 h shows a decrease (Table 1), which we interpret as adaptation to the new metabolic pathway. This reduction is similar to the reduction at the beginning of the experiment when an inoculum taken from an exponential growth culture adapts to pyrite oxidation in the absence of ferrous iron. At the start of the exponential growth, the number of bacteria in solution is very low and the rate of production of ferric iron is smaller than the rate of ferrous iron reduction in the abiotic attack on pyrite Eq. (4.2). Therefore, ferrous iron remains the dominant iron species in solution. In the absence of ferric iron that would oxidize sulfite, sulfur dioxide degassing and thus nonstoichiometric leaching of pyrite may still occur, however, the very small amount of change in the concentration of sulfate and ferrous iron during the time between 100 and 400 h makes it hard to explore this in more detail. Thus, there is an offset between the lag phase of growth of Af and the change in the solution chemistry of pyrite; however, the chemical changes are linked to the change in the growth phases.

During the lag phase, the bacteria are attached to the pyrite surface, where elemental sulfur is formed, and the oxidation of this elemental sulfur seems to be the energy source. Preferred oxidation of elemental sulfur over ferrous iron, as is the case in the initial stage of pyrite leaching by Af, has been observed before at pH values below 1.3 (Sand, 1989). Interestingly, the production rate of ferrous iron and sulfate declines almost to zero after the end of the lag phase in growth of Af (70–100 h). This indicates that the nonstoichiometric pyrite leaching mechanism was limited, either by substrate availability (i.e. reactive sites on the pyrite surface) or by accumulated products, i.e. ferrous iron and sulfite, triggering changes in the metabolic activity of Af. Inhibition of enzymatic activities of Af has been observed for sulfite (Sugio et al., 1994; Takeuchi and Suzuki, 1994; Rohwerder and Sand, 2003) for ferrous iron (Sugio et al., 1990; Sugio et al., 1992; Das et al., 1993) and for pH below 1.3 (Sand, 1989). In the initial stage of pyrite leaching of the Yu et al. (2001) experiments, the sulfite concentrations were below the detection limits (< 1 ppm), the concentration of ferrous iron reached 2 mM and the pH stayed above 2. Thus, accumulation of ferrous iron is the most likely candidate that may have caused inhibition of certain metabolic processes of Af. Sugio et al. (1990) found that hydrogen sulfide:ferric iron oxidoreductase and sulfite:ferric iron oxidoreductase were completely inhibited by 20 mM and 1 mM Fe2+, respectively. The use of elemental sulfur as energy source by Af was completely inhibited above 108 mM of Fe2+. Thus, the accumulation of ferrous iron may have a negative influence on the capability of Af to oxidize sulfite compounds. Margalith et al. (1966) observed that the oxidation rate of Fe2+ by Af decreased in the presence of elemental sulfur, indicating that there might be a competition between microbial oxidation of sulfite compounds and oxidation of ferrous iron. Recent investigations into the protein expression during growth of Af on ferrous iron and sulfur compounds (Brasseur et al., 2004; Ramirez et al., 2004; Yarzabal et al., 2004) confirm the strong influence of the concentration of Fe2+ on the metabolic activity of Af. Thus, high concentrations of ferrous iron may trigger the transition from a sulfur oxidation mechanism to a mechanism where oxidation of ferrous iron dominates. The transition between initial and main stage of pyrite leaching by Af sheds light on an interesting physiological aspect: A mode, where bacteria gain energy by oxidation of elemental sulfur (ΔG° = −272.2 kJ/mol O2) but show little growth in population due to a slow abiotic pyrite leaching mechanism is switched into a mode, where bacteria gain a smaller amount of energy by the oxidation of ferrous iron (ΔG° = −131.7 kJ/mol O2), but induce much faster pyrite leaching rates due to the production of ferric iron. Compared to the main stage, where bacteria mainly depend on the availability of ferrous iron from the solution, the initial phase of pyrite oxidation seems to be a much more secure way to establish a sustainable population. Thus, the intriguing change in the leaching mechanism of pyrite by Af may reflect a survival and growth strategy.

6. Outlook

This work puts emphasis on four aspects related to oxidation of pyrite by Af. The importance of the initial stage of pyrite leaching, the degassing of sulfur dioxide during biological and abiotic acid pyrite leaching, the importance of sulfite as an intermediate in the oxidation of pyrite and the influence of ferrous iron on the metabolic activity of Af. None of these aspects is new in the literature, however, yet, they have not been explored thoroughly. We hope that our work stimulates further research in this direction.

7. Conclusions

Sulfur and oxygen isotope analysis of sulfate that is produced during pyrite leaching by Af confirms that the leaching mechanism during the initial stage of pyrite leaching is different from the mechanism in the main stage. Enrichment in the 34S of produced sulfates in the initial stage indicates the formation of sulfite as an intermediate that is not immediately converted to sulfate, therefore allowing for oxygen isotope exchange between sulfite species and water and allowing for degassing of sulfur dioxide under acidic conditions. Loss of 34S-depleted SO2 causes enrichment of 34S in formed sulfate and explains nonstoichiometric sulfur–iron ratios during the initial stage of pyrite leaching. This is consistent with observed degassing of SO2 in abiotic acid pyrite leaching experiments.

The transition from initial stage of pyrite stage may be triggered by the accumulation of ferrous iron. It defines a fundamental change in the growth strategy of Af. A mode, where bacteria gain energy by oxidation of elemental sulfur (ΔG° = −272.2 kJ/mol O2) but show little growth in population due to a slow abiotic pyrite leaching mechanism is switched into a mode, where bacteria gain a smaller amount of energy by the oxidation of ferrous iron (ΔG° = −131.7 kJ/mol O2), but induce much faster pyrite leaching rates due to the production of ferric iron.

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