

# Interactions of the extremely acidophilic archaeon *Ferroplasma acidiphilum* with acidophilic bacteria during pyrite bioleaching

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**Abstract:** Bioleaching has been applied as a successful technique for metal recovery from various mineral sources like low-grade ores, waste materials and tailings. Mixed cultures of bioleaching microorganisms have a high performance in mineral dissolution. Thus far, microbial interactions in bioleaching communities are poorly understood. In this paper, the acidophilic archaeon *Ferroplasma acidiphilum* and the bacteria *Leptospirillum ferriphilum* and *Sulfobacillus thermosulfidooxidans* were chosen to study their interactions during pyrite leaching. The initial attachment to pyrite and pyrite leaching efficiency of pure and mixed populations were investigated. The data indicate: (i) attachment and bioleaching efficiency of *L. ferriphilum* was reduced in the presence of *F. acidiphilum*. However, the combination of *F. acidiphilum* and *S. thermosulfidooxidans* showed increased leaching, although the initial attachment rate was reduced, when compared to pure cultures. Thus, synergistic or antagonistic interactions may exist between *F. acidiphilum* and *S. thermosulfidooxidans* or *F. acidiphilum* and *L. ferriphilum*, respectively; (ii) pre-established biofilms of *L. ferriphilum* inhibited initial attachment to pyrite by cells of *F. acidiphilum* and did not promote pyrite leaching by *F. acidiphilum*. In contrast, inactivated biofilm cells of *S. thermosulfidooxidans* enhanced pyrite bioleaching by *F. acidiphilum*; (iii) adhesion forces of cells to an AFM tip ( $\text{Si}_3\text{N}_4$ ) seemed to be not correlated to attachment and bioleaching capacity; and (iv) lectins were applied to show and distinguish single species in mixed biofilm populations. Physical contact between cells of *S. thermosulfidooxidans* and *F. acidiphilum* was visible.

**Keywords:** pyrite, biofilm, bioleaching, *Ferroplasma*, interspecies interactions

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**Received:** April 23, 2016; **Accepted:** July 4, 2016; **Published Online:** September 23, 2016

**Citation:** Maulani N, Li Q, Sand W, *et al.* 2016, Interactions of the extremely acidophilic archaeon *Ferroplasma acidiphilum* with acidophilic bacteria during pyrite bioleaching. *Applied Environmental Biotechnology*, vol.1(2): 43–55.  
<http://dx.doi.org/10.26789/AEB.2016.02.006>.

## 1. Introduction

Bioleaching can be used as a biomining technology to achieve metal extraction from metal sulfides (MS) or waste materials by microbes<sup>[1,2]</sup>. Compared to traditional MS processing technologies like ore smelting/toasting, biomining is more attractive because of reduced energy costs and also lesser impact on the environment. It is estimated worldwide that 15% of copper, 5% of gold and smaller amounts of other metals (such as nickel and zinc) are currently produced by this technology. If bioleaching occurs under natural conditions without control it may cause serious environmental problems like acid mine/rock drainage (AMD/ARD)<sup>[3,4]</sup>.

Several species of acidophilic microorganisms from the genera *Acidithiobacillus*, *Leptospirillum*, *Acidiferrobacter*, *Acidiphilium*, *Ferroplasma*, *Acidimicrobium*, *Ferrimicrobium*, *Sulfobacillus*, *Metallosphaera* and *Acidianus*, among others, are commonly found at AMD sites as well as in biomining operations in all temperature ranges from minus up to more than 100°C<sup>[5,6]</sup>. Attachment of microorganisms to MS surfaces and a subsequent biofilm formation are of great importance for the MS dissolution process<sup>[7,8]</sup>. Microbial surface colonization may occur via diffusion, convection, electrostatic interactions and/or chemotaxis. Quorum Sensing (QS), cell to cell communication, plays a role in microbial attachment and biofilm formation among bioleaching microorganisms<sup>[9–11]</sup>.

*Ferroplasma* spp., extremely acidophilic archaea, are often found in different biomining operations as well as in natural environments such as the Iron Mountain and the Rio Tinto<sup>[12–14]</sup>. Strains of *Ferroplasma* can grow autotrophically using iron(II) ions or MS. Moreover, they can use organic compounds to sustain heterotrophic growth<sup>[15]</sup>. *Ferroplasma* contributes to the generation of AMD and is involved in the global sulfur and iron cycling<sup>[13,16]</sup>. *Ferroplasma* cells usually promote the growth and metabolic activity of other leaching bacteria by detoxifying leach liquors and thus, maintains robust bioleaching microbial communities<sup>[16–18]</sup>. Although pyrite leaching by *L. ferriphilum* was suppressed, when combined with *F. acidiphilum* (strain MT17), the inclusion of *Acidithiobacillus caldus* to the mixed culture resulted in a 30% increase of leaching efficiency over a pure culture of *L. ferriphilum*<sup>[19]</sup>. Recently, a study confirmed that the addition of *Ferroplasma thermophilum* improved the copper concentrate dissolution by *At. caldus* and *Lep*

*tospirillum ferriphilum*. Furthermore, a significant difference in the planktonic and attached population dynamics was observed<sup>[18]</sup>.

Recent evidence suggests the presence of microbial interactions regarding pyrite leaching. In binary species biofilms, the presence of active biofilms of iron-oxidizers influences the subsequent cell attachment by other species. *Acidithiobacillus thiooxidans* cells attached 40% more to pyrite pre-colonized with biofilms of *Acidithiobacillus ferrooxidans* or *L. ferrooxidans* than to non-pre-colonized pyrite. Interestingly, cell attachment of *F. acidiphilum* was faster to pyrite, if pre-colonized with *L. ferrooxidans*, than if pre-colonized with *At. ferrooxidans*<sup>[11]</sup>. Since *L. ferrooxidans* leaches pyrite more efficiently than *At. ferrooxidans*, the increased attachment observed for *At. thiooxidans* has been suggested to be probably related to a chemotactic response towards RISCs like polythionates, which are known to be released during pyrite leaching. In contrast, cell attachment of *At. ferrooxidans* to pyrite grains pre-colonized with *L. ferrooxidans* was strongly dependent on its pre-cultivation. Thiosulfate-grown cells were positively influenced by the presence of *L. ferrooxidans*, while iron(II)-grown cells were not<sup>[11]</sup>. Thus, the presence of iron oxidizers, which have been described as primary colonizers in natural AMD biofilms<sup>[20]</sup>, may be a relevant factor for sulfur-oxidizers to efficiently attach to MS.

Investigations in biomining systems have shown that microbial consortia are more robust than pure cultures of mineral-oxidizing acidophiles, and also tend to be more effective for bioleaching of ores and concentrates. However, the behavior of metal-oxidizing bacterial species in mixed cultures is largely unknown. We have previously shown that pre-established inactivated biofilms of *L. ferriphilum* decreased the initial attachment of *S. thermosulfidooxidans* to pyrite<sup>[21]</sup>. In this study, we focused on cell attachment and pyrite leaching by *F. acidiphilum* DSM 28986 in the presence of *S. thermosulfidooxidans* DSM 9293<sup>T</sup> (sulfur and iron oxidizer) and *L. ferriphilum* DSM 14647<sup>T</sup> (iron oxidizer). Attachment of microbes from suspensions containing *F. acidiphilum* either with *S. thermosulfidooxidans* or *L. ferriphilum* was comparatively studied. Additionally, the attachment of *F. acidiphilum* to pyrite pre-colonized either by *S. thermosulfidooxidans* or *L. ferriphilum* was compared to the attachment of *F. acidiphilum* to virgin pyrite. Moreover, adhesion forces between cells of *F. acidiphilum* and a silicon nitrate probe were compared by using atomic

force microscopy (AFM). In addition, advanced microscopic techniques in combination with fluorescent lectins were applied to visualize binary-species biofilms. Results are helpful to improve our understanding of the interactions between the acidophilic archaeon *F. acidiphilum* with acidophilic bacteria during pyrite bioleaching.

## 2. Materials and Methods

### 2.1 Strains and Growth Conditions

Cells of *F. acidiphilum* DSM 28986, *S. thermosulfidooxidans*<sup>T</sup> and *L. ferriphilum*<sup>T</sup> were grown in Mac medium<sup>[22]</sup>. *F. acidiphilum* DSM 28986 and *L. ferriphilum*<sup>T</sup> were grown at 37°C with 4 g/L iron(II) ions at an initial pH of 1.7. *S. thermosulfidooxidans*<sup>T</sup> was cultivated at 45°C with 10 g/L S<sup>0</sup> and an initial pH of 2.5. Yeast extract (0.2 g/L) was added to the Mac medium for cells of *F. acidiphilum* DSM 28986 and *S. thermosulfidooxidans*<sup>T</sup>. All strains were grown on a rotary shaker at 130 rpm.

### 2.2 Pyrite Slice

Pyrite slices with an approximate size of 1 cm × 1 cm × 2 mm were cut from cubes (origin: Navajun, Spain). Ground pyrite with a size of 50–200 µm was used for attachment and bioleaching tests. Both slices and grains, were incubated with boiling 6 M HCl for 30 min and rinsed with deionized water until neutral pH was reached. Subsequently, pyrite samples were treated three times with acetone. After cleaning, pyrite was dried at 80°C for 12 h and sterilized for 24 h at 120°C under a nitrogen atmosphere.

### 2.3 Pre-colonization and Attachment Tests

The pyrite grains pre-colonized with live and inactivated cells of *L. ferriphilum*<sup>T</sup> or *S. thermosulfidooxidans*<sup>T</sup> were prepared as previously described<sup>[11]</sup>. Cell attachment of single species of the three tested cultures to clean pyrite as well as *F. acidiphilum* DSM 28986 to pre-colonized pyrite with live or inactivated biofilms of *L. ferriphilum*<sup>T</sup> or *S. thermosulfidooxidans*<sup>T</sup> was monitored. Briefly, 1×10<sup>8</sup> cells/mL of each species were inoculated with clean pyrite or pyrite with live or inactivated biofilms and cultivated at 37°C with constant shaking at 130 rpm. Experiments were done in duplicates. The number of planktonic cells was determined by direct counting with a Thoma chamber within 6 h. The amount of attached cells was calculated by subtracting the number of planktonic

cells from the initial cell number. Theoretical attachment rate of mixed cultures was estimated by averaging the sum of the attachment rate from each member in the pure culture. The calculation was done according to the following formula:

$$A_{F,a+L,f} = (A_{F,a} + A_{L,f})/2 \text{ or } A_{F,a+S,t} = (A_{F,a} + A_{S,t})/2$$

$A$  = Attachment rate,  $F, a = F. acidiphilum$  DSM 28986,  $L, f = L. ferriphilum$ <sup>T</sup>,  $S, t = S. thermosulfidooxidans<sup>T</sup>$

### 2.4 Bioleaching of Pyrite

Erlenmeyer flasks (100 mL) containing 10 g of pyrite grains, 50 mL of Mac medium (pH 1.7) and 0.2 g/L yeast extract were inoculated with pure or mixed cultures of the three strains with an initial total cell number of 2×10<sup>8</sup> cells/mL. The following cultures were used for inoculation: (i) pure cultures of *F. acidiphilum* DSM 28986, *S. thermosulfidooxidans*<sup>T</sup> or *L. ferriphilum*<sup>T</sup>; (ii) mixed cultures of *F. acidiphilum* DSM 28986 and *S. thermosulfidooxidans*<sup>T</sup> or *F. acidiphilum* DSM 28986 and *L. ferriphilum*<sup>T</sup>. Abiotic controls were also prepared. In order to test whether pre-established biofilms can influence pyrite bioleaching by *F. acidiphilum* DSM 28986, additional assays were included by using pyrite either pre-colonized by *S. thermosulfidooxidans*<sup>T</sup> or by *L. ferriphilum*<sup>T</sup> as described<sup>[11,21]</sup>. All the leaching experiments were done in duplicates. Cell numbers were determined by direct microscopic counts and pH of the leachates was measured using a digital pH meter (Model pH 537, WTW). Iron ions were quantified using the phenanthroline method (according to DIN 38406-1).

### 2.5 Visualization of Biofilms on Pyrite by Confocal Laser Scanning Microscopy

For visualization of biofilms formed by the three species in pure and mixed populations on pyrite, biofilm cells were stained by nucleic acid specific stains 4',6-diamidino-2-phenylindole (DAPI) or SYTO 9. The matrix of extracellular polymeric substances (EPS) was stained by fluorescent-labeled lectins as described<sup>[23]</sup>. Briefly, pyrite colonized with cells was washed with sterile Mac medium and then neutralized by using filtered tap water. Staining dyes were applied directly to cover pyrite surfaces. Direct light exposure was avoided during the staining process.

Examination of stained biofilms was performed via confocal laser scanning microscopy (CLSM) using a TCS SP5X (Leica, Heidelberg, Germany), controlled

by the LASAF 2.4.1 build 6384. The system was equipped with an upright microscope and a super continuum light source (470–670 nm) as well as a 405 nm laser diode. Images were collected with a  $63\times$  water immersion lens with a numerical aperture (NA) of 1.2 and a  $63\times$  water immersible lens with an NA of 0.9. CLSM data sets were recorded in sequential mode in order to avoid cross talk of the fluorochromes between two different channels. Surface topography and texture of the pyrite surface were recorded by using the CLSM in reflection mode.

## 2.6 Force Measurements using Atomic Force Microscopy

Microbial cells were attached to polyethyleneimine (PEI: Mr 1200, Alfa Aesar) coated glass slides as described<sup>[24]</sup>. AFM measurements were performed at room temperature (20°C) in Mac medium at pH 1.7 using a Nano Wizard-II atomic force microscope (JPK Instruments, Germany). A silicon cantilever CSC38 AL (Mikromasch, Estonia) with the following features was used: typical length, 350  $\mu\text{m}$ ; width, 32.5  $\mu\text{m}$ ; thickness, 1  $\mu\text{m}$ ; resonance frequency, 10 kHz; and nominal force/spring constant, 0.02–0.09 N/m. Each AFM image consisted of  $512\times 512$  pixels. Each force measurement generates two force-distance curves: the approach force curve and the retraction force curve. The retraction force features the adhesion force between the AFM probe surface and the cell surface. Adhesion force refers to the force needed to prevent two surfaces from separation and is defined as the equivalent force at the maximum cantilever deflection in the retraction force curve. Force curve normalization and alignment was done according to a previous report<sup>[25]</sup>.

## 2.7 Digital Image Analysis

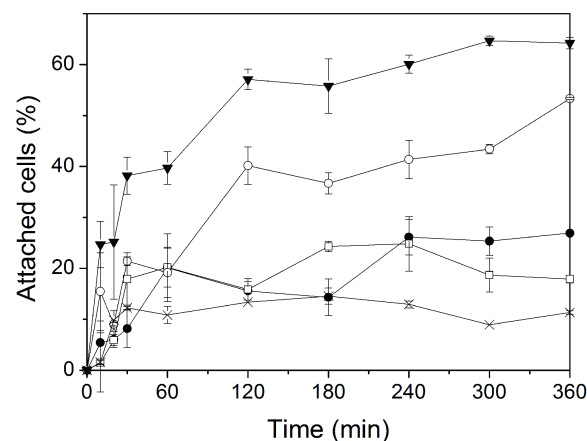
Fluorescence images were analyzed using an extended version of the ImageJ software. Maximum intensity (MIP) and XYZ projections of 3-dimensional data sets were produced with the software IMARIS version 7.3.1 (Bitplane AG, Zurich, Switzerland).

## 3. Results and Discussion

### 3.1 Initial Attachment to Pyrite by Pure and Mixed Cultures

As shown in Figure 1, the initial attachment to pyrite of the three strains amounted to 64% for *L. ferriphilum*<sup>T</sup>, 53% for *F. acidiphilum* DSM 28986, and 11% for *S. thermosulfidooxidans*<sup>T</sup> after 6 h of cultivation, respectively. This is in agreement with previous stu-

dies, in which *L. ferriphilum*<sup>T</sup> showed a high affinity to pyrite, while *S. thermosulfidooxidans*<sup>T</sup> did not attach well<sup>[21,26]</sup>. An attachment of leaching microorganisms to the pyrite surface is an essential step for gaining energy. It is known that cell attachment to pyrite potentially accelerates the bioleaching process. Cells in the bulk solution (planktonic cells), which attach to pyrite, become sessile cells and degrade pyrite<sup>[23,27]</sup>. The cell attachment is influenced by cell surface properties like cellular appendages and EPS production, and mineral surface characteristics<sup>[28]</sup>. This has been demonstrated in several studies, which all agreed that flagella play a pivotal role in the initial attachment to a solid surface<sup>[29–31]</sup>. *L. ferriphilum*<sup>T</sup> has been shown to rapidly attach to pyrite surfaces. A possible explanation is the presence of flagella that mediate the contact between cells and pyrite surfaces. It has been shown that both *Leptospirillum* group II and III possess a chemotaxis system and a flagellar operon<sup>[32]</sup>. *L. ferrooxidans* can sense substrates via its chemotaxis system<sup>[33]</sup>. In addition, EPS production by cells of *L. ferrooxidans* is generally higher than by cells of *At. ferrooxidans* or *At. thiooxidans*<sup>[28,34]</sup>.



**Figure 1.** Binary species cultures containing *F. acidiphilum* showed reduced attachment compared to single species ones. *F. acidiphilum* (○), *L. ferriphilum* (▼), *S. thermosulfidooxidans* (×), mixed culture of *F. acidiphilum* with *L. ferriphilum* (●), mixed culture of *F. acidiphilum* with *S. thermosulfidooxidans* (□).

Unlike attachment behavior in assays with pure cultures, *F. acidiphilum* and *L. ferriphilum* seem to have reduced attachment ability when cultivated in mixed culture (Figure 1). Cells of *F. acidiphilum* DSM 28986 mixed with cells of *L. ferriphilum*<sup>T</sup> or with *S. thermosulfidooxidans*<sup>T</sup> had attachment rates of 27%



and 18% after 6 h, respectively. If these data are compared with theoretical attachment values for *F. acidiphilum* co-cultured with *L. ferriphilum* ( $59\% = (64\% + 53\%)/2$ ) and *S. thermosulfidooxidans* ( $32\% = (11\% + 53\%)/2$ ), around a two-fold decrease in the attachment was noticeable.

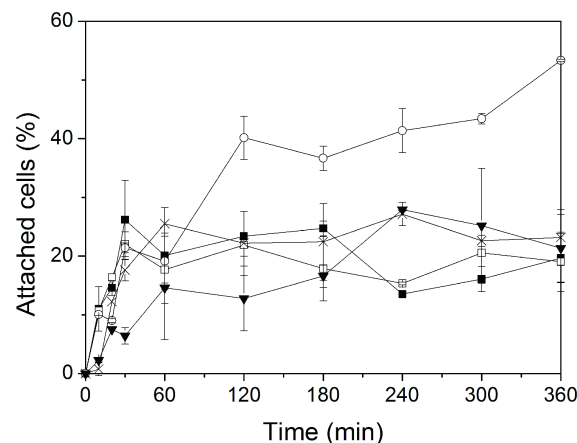
Our previous work has shown that mixed cultures possess substantially different attachment behavior from pure ones. In mixed cultures of *At. ferrooxidans* ATCC 23270<sup>T</sup> with *Acidiphilum cryptum* JF-5, both EPS production and attachment to pyrite were enhanced, as compared to the pure cultures. Strain *A. caldus* S2 does not attach to pyrite in pure cultures, but in the presence of cells of *L. ferriphilum* its EPS production and biofilm formation are stimulated (Sand et al., unpublished). Work on the competitive adsorption of binary mixtures of *L. ferriphilum* LF-104 with *A. caldus* MTH-04 onto pyrite surfaces indicates that *L. ferriphilum* and *A. caldus* compete in their adsorption to pyrite, while *L. ferriphilum* dominated in the competitive adsorption process<sup>[35]</sup>. We assume that there are antagonistic interactions between *F. acidiphilum* and *S. thermosulfidooxidans* or *L. ferriphilum* in the attachment process.

### 3.2 Initial Attachment of *F. acidiphilum* to Pyrite Pre-colonized by cells of *L. ferriphilum* or of *S. thermosulfidooxidans*

As shown in Figure 2, initial attachment of *F. acidiphilum* showed mostly the same trend within the first 1 h, namely 20%–25% of the cell population attached to pyrite pre-colonized with cells of *L. ferriphilum* or of *S. thermosulfidooxidans*. Cell attachment to pyrite pre-colonized with inactivated cells of *S. thermosulfidooxidans* showed the lowest value (<10%). A two-fold reduction in the initial attachment of *F. acidiphilum* to pyrite pre-colonized either by *L. ferriphilum* or by *S. thermosulfidooxidans* was measured as compared to cell attachment to non-colonized pyrite (after 6 h). Obviously, the presence of either *L. ferriphilum* or *S. thermosulfidooxidans* in the form of living or inactivated cells reduced the initial attachment of *F. acidiphilum*.

### 3.3 Bioleaching Efficiency of Pure or Mixed Cultures

Pyrite leaching activities can be estimated by measuring total dissolved iron ions as well as by the ratio of iron(III)/iron(II) ions<sup>[36]</sup>. In general, a high ratio of iron(III)/iron(II) is favorable for metal dissolution<sup>[37]</sup>. As shown in Figure 3, pyrite dissolution was found



**Figure 2.** Initial attachment of cells of *F. acidiphilum* to non-colonized pyrite (○) or pyrite grains pre-colonized with living cells of *L. ferriphilum* (□), or inactivated cells of *L. ferriphilum* (■), living cells of *S. thermosulfidooxidans* (×), inactivated cells of *S. thermosulfidooxidans* (▼), respectively.

to be slightly higher in the assay with *L. ferriphilum* (iron(III)/iron(II) = 16, iron total = 6.9 g/L) than in the assays with mixed cultures containing *F. acidiphilum* (iron(III)/iron(II) = 15, iron total = 6.5 g/L). Of all cultures, *F. acidiphilum* showed the lowest pyrite dissolution efficiency (iron total = 1.5 g/L). Mixed cultures of *F. acidiphilum* with *S. thermosulfidooxidans* showed relatively higher leaching efficiency (iron(III)/iron(II) = 11, iron total = 5 g/L) compared to pure cultures of *F. acidiphilum* or *S. thermosulfidooxidans* (iron(III)/iron(II) = 0.3 and 0.6, iron total = 1.5 and 2.4 g/L, respectively). This indicates that pyrite leaching was enhanced when these two species were present together.

However, leaching efficiency by mixed cultures of *F. acidiphilum* with *L. ferriphilum* was relatively higher than by mixed cultures of *F. acidiphilum* with *S. thermosulfidooxidans*. This is possibly due to the fact that *L. ferriphilum* has the strongest leaching ability among the three tested species.

Numbers of planktonic cells in the mixed cultures of *F. acidiphilum* with *L. ferriphilum*, in *F. acidiphilum* with *S. thermosulfidooxidans*, and in the pure culture of *L. ferriphilum* tended to increase gradually from an initial cell density of about  $2 \times 10^8$  cells/mL to  $9 \times 10^8$ ,  $7 \times 10^8$ , and  $9 \times 10^8$  cells/mL, respectively, at the end of the incubation. In comparison, cell numbers of *F. acidiphilum* doubled after one week, and then decreased slowly to a  $2 \times 10^8$  cells/mL until the end of the incubation. Meanwhile, cell numbers of *S. thermosulfidooxidans* had a ten-fold decrease over the period of

incubation. Due to pyrite oxidation, the pH in the test cultures either as pure cultures or in mixed cultures declined moderately (Figure 3). Cultures with a decrease of pH from the highest to the lowest were *F. acidiphilum* and *L. ferriphilum* (pH 0.56), *F. acidiphilum* and *S. thermosulfidooxidans* (pH 0.54), *S. thermosulfidooxidans* (pH 0.48), *L. ferriphilum* (pH 0.45), and *F. acidiphilum* (pH 0.40).

Specifically, in both mixed cultures the iron(III) ion concentration increased until the end of the experiment. In our study, this also occurred in the case of the pure cultures of *L. ferriphilum*. Even though mixed cultures showed a low ability of attachment to pyrite surfaces (Figure 2), their performance in leaching was at a high level. *F. acidiphilum* and *S. thermosulfidooxidans* showed a mutually beneficial interaction regarding pyrite dissolution. The ability of a culture for pyrite leaching tends to be dissimilar for the pure and for mixed cultures<sup>[37]</sup>. As reported by Noël<sup>[38]</sup>, the leaching process is more effectively performed by mixed cultures than by pure cultures. In the aforementioned case, both strains are able to oxidize iron(II) ions to iron(III) ions. The attack of iron(III) ions on pyrite releases RISCs that are oxidized into sulfuric acid by *S. thermosulfidooxidans*<sup>[38]</sup>. The elimination of RISCs on mineral surfaces generally increases pyrite leaching rate<sup>[39]</sup>. The presence of a heterotroph or a mixotroph in mixed cultures favors mineral leaching by degrading dissolved organic molecules produced by the microbial communities. Therefore, an accumulation of organic carbon in the bulk solution can be detoxified<sup>[39]</sup>. However, it has been shown that the heterotroph *Acidiphilum* sp. only enhances metal solubilization by cells of *L. ferrooxidans*, but not the

one by cells of *A. ferrooxidans*<sup>[40]</sup>.

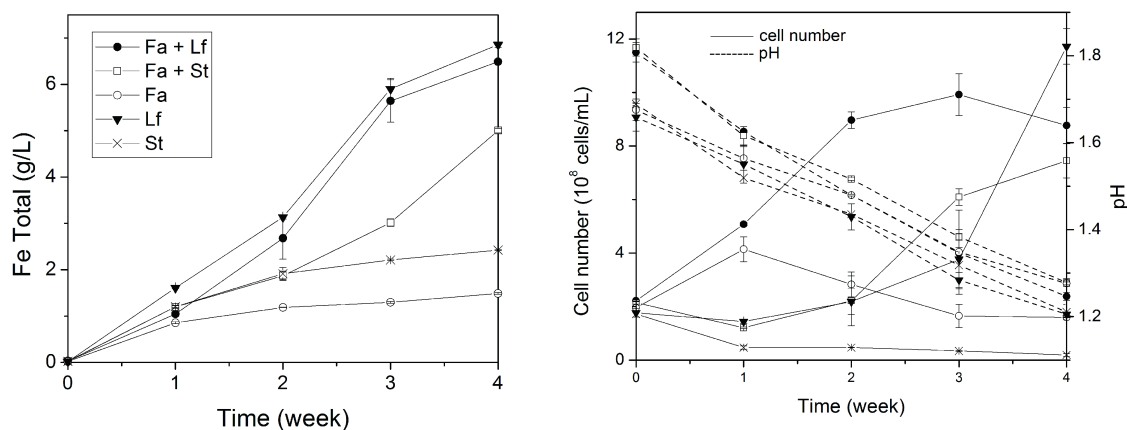
In the case of mixed cultures of *F. acidiphilum* with *L. ferriphilum*, a slight decrease in leaching efficiency was noticed compared to the values reached by a pure culture of *L. ferriphilum*. Furthermore, mixed cultures of *Acidiferrobacter* sp. SPIII/3 and *L. ferrooxidans* showed a reduced pyrite leaching efficiency (95%) compared to a pure culture of *L. ferrooxidans*<sup>[11]</sup>.

Among pure cultures tested, *L. ferriphilum* presented the highest initial attachment and the most favorable leaching activity. Attachment of cells of *Acidithiobacillus*, *Leptospirillum* and *Sulfobacillus* were shown to be proportional to their mineral dissolution rates<sup>[41,42]</sup>. In the present study, such phenomenon only occurred in case of a pure culture of *L. ferriphilum* (Figures 1 and 3).

The most likely explanation for the negative interaction is that in mixed cultures, *F. acidiphilum* and *L. ferriphilum* compete for the energy source, iron(II) ions. This result is consistent with the previous finding: a reduced pyrite oxidation rate resulted, when *L. ferriphilum* as an autotrophic iron-oxidizer grew in co-culture with *F. acidiphilum* as a heterotrophic iron oxidizer<sup>[19]</sup>. However, these reports seem to be contradictory to a recent metabolic model constructed for a mixed culture of *F. acidiphilum* and *L. ferriphilum*. It was expected that the main interaction of both species involved metabolic exchange such as the EPS secreted by *L. ferriphilum* are used by *F. acidiphilum* as an energy source<sup>[43]</sup>.

### 3.4 Effects of a Pre-established Biofilm on Pyrite Leaching by *F. acidiphilum*

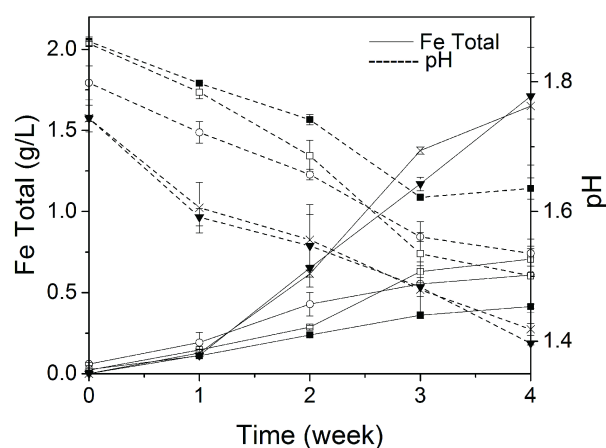
The leaching of pyrite colonized with living *L.*



**Figure 3.** Bioleaching of pyrite by pure cultures of *F. acidiphilum* (○), *L. ferriphilum* (▼), *S. thermosulfidooxidans* (×), and mixed cultures of *F. acidiphilum* with *L. ferriphilum* (●), and *F. acidiphilum* with *S. thermosulfidooxidans* (□).

*ferriphilum* cells by *F. acidiphilum* showed an upward trend with respect to detected dissolved iron ions, which reached around 0.7 g/L at the end of incubation (Figure 4). The leaching efficiency of *F. acidiphilum* with inactivated *L. ferriphilum* or without *L. ferriphilum* cells was low (iron total = 0.4 or 0.6 g/L, respectively). Thus, it seems that biofilm cells of *L. ferriphilum* did not significantly contribute to pyrite leaching by *F. acidiphilum*. Even so, the presence of inactivated cells of *L. ferriphilum* showed an inhibitive effect on pyrite leaching by *F. acidiphilum* (Figure 4). This phenomenon has been observed in the case of biofilm cells of *L. ferriphilum*<sup>[21]</sup>. Due to the heterotrophic growth ability of *F. acidiphilum*, we assume that cells of *F. acidiphilum* might use biomass of inactivated *L. ferriphilum* and thus pyrite leaching efficiency was reduced. However, this needs further biochemical evidence.

Leaching by *F. acidiphilum* with pre-colonized cells of *S. thermosulfidooxidans* was slightly higher than in a pure culture. The dissolved total iron ion concentration was around 1.7 g/L, when cells of *S. thermosulfidooxidans* were present (inactivated or alive). These values are considerably higher than the ones of a pure culture of *F. acidiphilum* (0.6 g/L). In case of bioleaching of pyrite pre-colonized by living cells of *S. thermosulfidooxidans*, *S. thermosulfidooxidans* cells were present, but accounted only for a minor fraction of the total planktonic cells in the bulk solution. In the other culture with pyrite pre-colonized by inactivated cells of *S. thermosulfidooxidans*, a few



**Figure 4.** Bioleaching by *F. acidiphilum* with clean pyrite (○) and pyrite grains precolonized by living cells of *L. ferriphilum* (□), inactivated cells of *L. ferriphilum* (■), living cells of *S. thermosulfidooxidans* (×), inactivated cells of *S. thermosulfidooxidans* (▼), respectively.

planktonic cells of *S. thermosulfidooxidans* were observed, but only at the end of the incubation. It indicates that *S. thermosulfidooxidans* spores previously produced due to the heat treatment for inactivation of the vegetative cells had germinated. However, pyrite leaching was not observed by inactivated biofilm cells alone in the control experiments (results not shown). In contrast, living biofilm cells of *S. thermosulfidooxidans* alone showed some leaching ability (data not shown). This leads to an assumption that the leaching performed by *F. acidiphilum* with pyrite pre-colonized by living biofilms of *S. thermosulfidooxidans* was partially caused by attached cells of *S. thermosulfidooxidans*.

### 3.5 Adhesion Forces Between Cells of *F. acidiphilum*, *L. ferriphilum* or *S. thermosulfidooxidans* and an AFM Silicon Nitride Cantilever

Probing nanoscale interactions between an atomic force microscope cantilever and a microbial cell may provide useful information on cellular adhesion. In this study, the cohesive and adhesive strengths of bioleaching microorganisms were investigated by measuring interactions between a bare silicon nitride AFM tip and PEI-immobilized cells attached to glass slides. As shown in Table 1, the adhesion force between a bare AFM probe and iron-grown cells of *L. ferriphilum* was with 77–83 pN higher than those of iron-grown cells of *F. acidiphilum* with 60–84 pN. However, the highest values were measured for iron-grown cells of *S. thermosulfidooxidans* with 246 pN. Sulfur-grown cells of *S. thermosulfidooxidans* had the lowest adhesion force to a bare silicon nitride with 58 pN. These values are in the range of known interaction forces between bacteria and a silicon nitride probe with 26–5000 pN<sup>[44]</sup>. However, these values were in general lower than the adhesion forces measured for the leaching bacteria *L. ferrooxidans*, *A. ferrooxidans* and *A. thiooxidans* to pyrite and chalcopyrite, which amounted to 500–1000 pN<sup>[45,46]</sup>.

It has been shown that *At. ferrooxidans* cells grown

**Table 1.** Adhesion force values (pN) for three acidophilic microorganisms grown with different substrates to silicon nitride probes

Substrate	<i>F. acidiphilum</i>	<i>L. ferriphilum</i>	<i>S. thermosulfidooxidans</i>
Iron(II) ions	Range	60–84	77–83
	Average	69.14 ± 12.98	80.38 ± 3.09
S <sup>0</sup>	Range	NA	NA
	Average	NA	57.90 ± 15.23

NA: not applicable. ND: not determined



on chalcopyrite had the strongest interaction force with the same substrate. The forces for cells grown with iron(II)-ions or  $S^0$  were considerably reduced. In addition, cell attachment and chalcopyrite leaching rate of *A. ferrooxidans*, *A. thiooxidans* and *L. ferrooxidans* shall be positively related to the bacterial adhesion force<sup>[45,47]</sup>. However, no such correlation could be derived from our experimental data.

### 3.6 Visualization of Mixed-species Biofilms on Pyrite

#### *Mixed Biofilms on Pyrite Visualized by Non-specific Nucleic Acid Staining*

In order to investigate cell distribution and interactions in mixed biofilms, CLSM combined with several fluorescent stains were applied to visualize dual-species biofilms. As shown in Figure 5, in mixed cultures of *F. acidiphilum* with *S. thermosulfidooxidans*, the two species were distinguishable by cell morphology. Cells of *F. acidiphilum* show a tiny round appearance, but *S. thermosulfidooxidans* cells are rod-shaped. Also, cells of *S. thermosulfidooxidans* show brighter signals than the ones of *F. acidiphilum* (Figure 5A, red arrows). In general, mixed samples containing *F. acidiphilum* with *S. thermosulfidooxidans* cells exhibited a tendency to form colonies/communities of up to 50  $\mu\text{m}$  in diameter. Cell contact between both species was often observed. In case of the mixed cultures of *F. acidiphilum* with *L. ferriphilum*, biofilm cells mainly remained separated from each other. Due to the similar cell sizes of these two

species, the recognition of single species within the mixed populations is not possible.

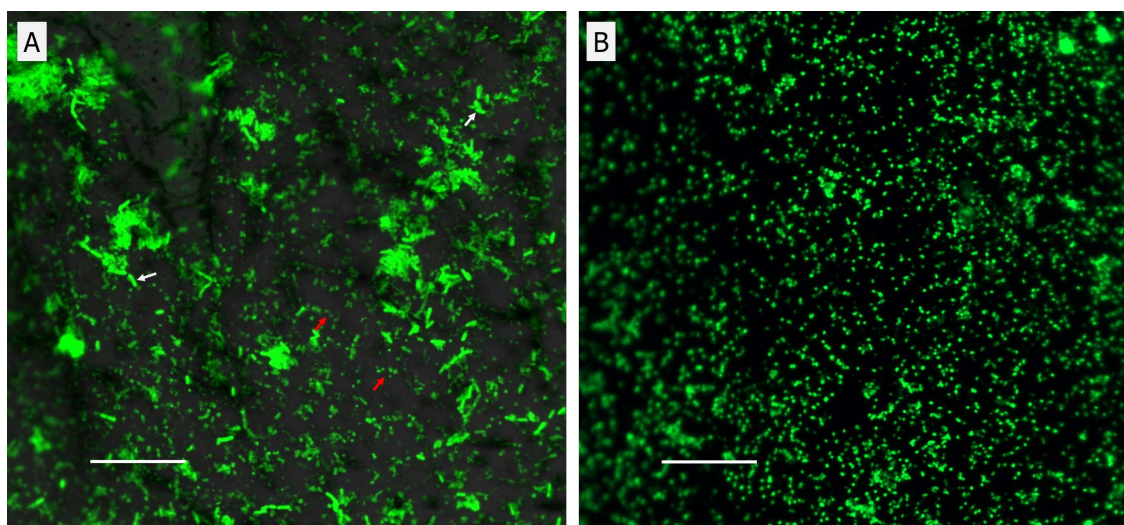
Physical contact between cells of *S. thermosulfidooxidans* and cells of *L. ferriphilum* on pyrite has been observed<sup>[21]</sup>. In mixed cultures with *Leptospirillum ferriphilum* P3A and *At. caldus* S2, the former plays the central role in biofilm formation on pyrite<sup>[48]</sup>. The hyperthermophilic archaea *Pyrococcus furiosus* and *Methanopyrus kandleri* formed bi-species biofilms on glass surfaces. Interactions between *P. furiosus* and *M. kandleri* were mediated by flagella and direct cell contact<sup>[49]</sup>.

Also *L. ferrooxidans* forms microcolonies or flocks in the presence of *Acidiphilum* sp. cells<sup>[40]</sup>. These observations indicate that the forming of aggregates or biofilm clusters in mixed cultures may be a specific interaction among bioleaching microorganisms mediated by until now unknown mechanisms.

#### *Mixed Biofilms on Pyrite Visualized by Specific Lectin Staining*

Lectins are able to interact with many polysaccharides in a specific way<sup>[50]</sup>. Thus, they may be used to mark a target strain in mixed biofilm populations. The recently developed fluorescence lectin-binding analysis has been used to visualize and analyze biofilms of bioleaching archaea<sup>[51,52]</sup>; e.g., the lectin PSA (*Pisum sativum* agglutinin), has been used to distinguish cells of *Sulfolobus metallicus* from those of *Acidianus* sp. DSM 29099 or *Acidianus* sp. 29038 on pyrite<sup>[52,53]</sup>.

The lectin AAL (*Aleuria aurantia* agglutinin) has



**Figure 5.** Mixed culture colonization of pyrite by cells of *F. acidiphilum* with *S. thermosulfidooxidans* (A) or cells of *F. acidiphilum* with *L. ferriphilum* (B). Biofilms were stained by SYTO 9 and visualized by EFM. Red and white arrows in A indicate cells of *F. acidiphilum* and *S. thermosulfidooxidans*, respectively. Bars represent 20  $\mu\text{m}$ .



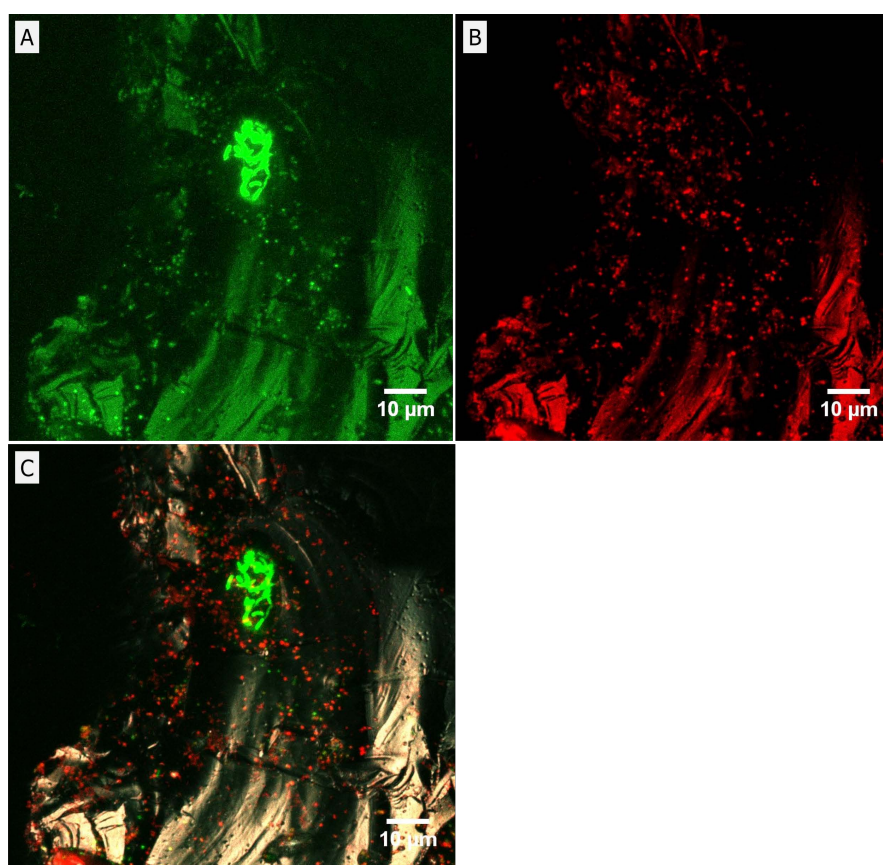
been shown to stain cells of *F. acidiphilum*<sup>[23]</sup>, but not cells of *S. thermosulfidooxidans* or *L. ferriphilum* (R. Y. Zhang and W. Sand, unpublished). Thus, this lectin was selected to distinguish cells of *F. acidiphilum* from those of *S. thermosulfidooxidans* or *L. ferriphilum* in biofilms on pyrite. As shown in Figure 6, cells of *F. acidiphilum* exhibited a lectin signal, whereas those of *S. thermosulfidooxidans* remained unstained. Also, a direct contact between cells of the two species was observed.

Correspondingly, in mixed cultures of *F. acidiphilum* and *L. ferriphilum*, cells of *F. acidiphilum* were recognized by the lectin AAL (Figure 7). The dominating species was stained by SYBR Green, but remained without a lectin signal. These were the cells of *L. ferriphilum*. As shown in Figure 5B, major parts of the mixed populations were attached as single cells on the pyrite surface. No direct contact between cells of *F. acidiphilum* and those of *L. ferriphilum* was observed. During CLSM recording, the pyrite surface reflection

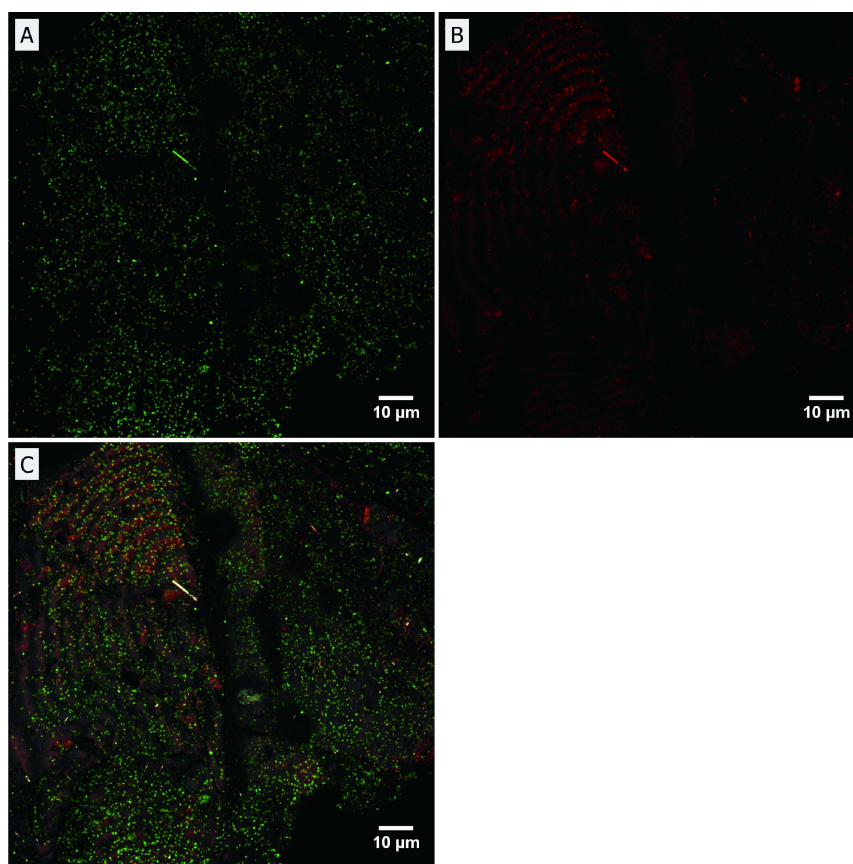
seemed to disturb cell and EPS signals in the green and red channels, respectively (Figures 6 and 7). This may be attributed to the fact that the pyrite grains were freshly crushed. More tests are still necessary to separate cell and EPS signals from the surface reflection.

#### 4. Conclusion

In summary, the present study provides new insight into microbial interactions between cells of *F. acidiphilum* and of *S. thermosulfidooxidans* or of *L. ferriphilum* during initial attachment to pyrite and the leaching process. Mixed cultures of *F. acidiphilum* and *L. ferriphilum* showed reduced initial attachment to pyrite. No improved leaching was measured by the mixed cultures compared to pure cultures. By comparison, mixed cultures of *F. acidiphilum* and *S. thermosulfidooxidans* showed an increased leaching, although the initial attachment was reduced. Pre-established biofilms of *S. thermosulfidooxidans* or *L. ferriphilum* did not promote pyrite leaching by *F. acidiphilum*.



**Figure 6.** Mixed cultures of *F. acidiphilum* with *S. thermosulfidooxidans* on pyrite. Biofilm cells were stained by AAL-Alexa568 (red channel) and SYBR Green (green channel) and visualized by an upright mode CLSM. SYBR Green stains nucleic acid of cells of both species (A). The signals from AAL-Alexa568 represent cells of *F. acidiphilum* (B). The merged image from both channels (C) indicates that cells of *F. acidiphilum* seem to be predominant. The pyrite surface was recorded in reflection mode (grey).



**Figure 7.** Mixed cultures of *F. acidiphilum* with *L. ferriphilum* on pyrite. Biofilm cells were stained by AAL-Alexa568 (red channel) and SYBR Green (green channel) and visualized by an upright mode CLSM. SYBR Green stains the nucleic acids of both species (A). The signals from AAL-Alexa568 represent cells of *F. acidiphilum* (B). The merged images from both channels (C) indicate that *L. ferriphilum* seems to be the predominant species. The pyrite surface was recorded in reflection mode (grey)

Initial attachment and pyrite leaching were found to be correlated only for *L. ferriphilum* but not for *F. acidiphilum* or *S. thermosulfidooxidans*. Adhesion forces between the three species and an AFM probe ( $\text{Si}_3\text{N}_4$ ) did not show correlation with initial attachment and bioleaching capability.

### Conflict of Interest and Funding

There is no conflict of interest claimed by the authors.

### Acknowledgements

The authors greatly acknowledge Dr. Thomas R. Neu and Ute Kuhlicke from the Department of River Ecology, Helmholtz Centre for Environmental Research–UFZ, for their assistance in CLSM preparation and image processing. N.M. thanks the German Academic Exchange Service (DAAD) and the Federal Ministry of Education and Research (BMBF) for financial support. Q.L. acknowledges China Scholarship Council (CSC) for the PhD program funding. R.Z.

thanks Fraunhofer Institute for Environmental, Safety, and Energy Technology UMSICHT (Oberhausen, Germany) and Dr. Brill + Partner GmbH (Hamburg, Germany) for financial support.

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