CONTROL OF BACTERIAL METAL SULFIDE LEACHING PROCESSES

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Abstract

Biofilm formation is considered to be very important in the bioleaching of metal sulfides by microorganisms. Attachment of microorganisms as the first step in biofilm formation is the critical one. The role played by various bacterial species, which are involved in bioleaching, with respect to initial attachment processes and biofilm formation is still largely unknown. To develop methods to enhance or reduce bioleaching, our investigations are focused on the initial processes of attachment and biofilm formation.

In mixed cultures, with *Leptospirillum spp., Acidithiobacillus ferrooxidans, Acidithiobacillus thiooxidans, Ferromicrobium acidophilum* and *Acidithiobacillus caldus* initial attachment and biofilm formation was mostly dominated by *Leptospirillum spp* in mesophilic as well as in moderately thermophilic temperature ranges. Another finding is that large areas of the surface of minerals remain uncolonized whereas at some places bacteria attach in clusters. In general, interaction of different species resulted in increased production of extracellular polymeric substances (EPS) increased attachment and leaching rates. Furthermore they stimulate bacteria with fewer attachment tendencies, to be part of a mixed biofilm.

Based on these findings we conclude that attachment and thereby bacterial leaching can be increased by the combination of several strains especially in presence of *Leptospirillum spp.*

Introduction

Bioleaching is a process whereby bacteria dissolve metal sulfides by oxidation processes. This process is used for metal winning in tank or heap leaching. Metals such as copper, gold or nickel are produced by this process from low grade ores (Sand 1992). Bioleaching is occurring as well as a natural process e.g. during coal mining and has a high environmental impact. Acidification of water bodies concomitant with heavy metal pollution often is the consequence (González-Toril 2006).

Various bacterial and archaeal species are involved in the process of sulfide dissolution by "contact" and "non-contact" mechanisms. While the non-contact mechanism assumes that microorganisms oxidize dissolved iron(II) ions to iron(III) ions which attack the metal sulfides and are chemically reduced to iron(II) ions, the contact mechanism requires microorganisms attached to the mineral surface (Rohwerder 2003). It is commonly accepted that EPS are needed for the attachment of cells to the metal-sulfide surface, biofilm formation and the general leaching process (Gehrke 1995). Consequently, the attachment of microorganisms as the first step in biofilm formation is the critical one.

Therefore, we quantified and visualized initial colonization and biofilm formation on pyrite for mesophilic and moderately thermophilic species of the genera *Acidithiobacillus, Ferrimicrobium* and *Leptospirillum* as well as a novel γ-proteobacterium by using DAPI, fluorescently labeled lectin or fluorescence *in situ* hybridization (FISH) in combination with atomic force microscopy.

It is necessary to understand the attachment and leaching of mesophilic and moderately thermophilic microorganisms to optimize the composition of leaching bacteria to advance different process-sections depending on temperature ranges. Furthermore it is important to understand the way of bacterial attachment and biofilm formation on mineral surfaces on the

molecular level. With this knowledge commercial leaching processes may be optimized or unwanted natural leaching processes maybe come controllable.

Materials and Methods

Microorganisms and conditions of cultivation. Iron-oxidizing bacteria *Acidithiobacillus ferrooxidans* ATCC23270, *Leptospirillum ferrooxidans* DSM2391, L*eptospirillum ferriphilum* DSM14647 and a γ- proteobacterium (undescribed, designated SPIII) were grown in a Mackintosh medium (Mackintosh, 1978) with 4g iron(II)-ions/L and an addition of 0,02% yeast extract for *F. acidiphilum.* Sulfur-oxidizing bacteria *Acidithiobacillus thiooxidans* DSM 622 and *Acidithiobacillus caldus* DSM 8584 were grown in Mackintosh medium (Mackintosh, 1978) with 5g/L elemental sulfur.

Attachment and leaching experiments with pyrite grains. Attachment tests were performed in 50 mL Mackintosh salt solution (Mackintosh, 1978) with 5-10 g pyrite (50-100µm particle size) and $5*10^8$ cells per mL. Aliquots of 1 mL were taken at times 0, 0.5, 5, 10, 20, 30, 60, 90,120, 240, 300, 360 and 420 min. Leaching experiments were done in 150 mL Mackintosh salt solution with 5 g pyrite (50-100µm particle size) and $1x10^8$ cells per mL. Samples were taken 3 times a week. To calculate leaching rates, iron(II)- and iron(III)- ions were measured by the phenanthroline method (DEV 1984). Attachment and leaching experiments were performed with pure or defined cultures in different compositions. Control was performed in the same experimental set up without cells.

Attachment tests with pyrite coupons. From museum grade pyrite crystals (size approx. 3,5 cm^3) slices were cut off in a thickness of about 1-2 mm by a diamond saw. These coupons were incubated for 1, 5 and 8 h in cell suspension of pure cultures of *A. ferrooxidans*,

L. ferrooxidans and *A. thiooxidans* with $1x10^8$ cells/mL. After sampling coupons were washed in phosphate buffer, deionised water and stained with DAPI.

Visualization of cells. Cells and EPS were visualized by epifluorescence microscopy (EFM) (Noël 2008) or a confocal laser scanning microscope (CLSM) (Leon Morales 2008). 4',6-diamidino-2-phenylindole (DAPI) and Syto 9 were used to visualize whole cells. A lectin (ConA- TRITC) was used to stain parts of biofilms. The FISH probe EUB338 was used to visualize bacteria in a culture from a lignite mine in eastern Germany (Mangold et al, 2008a, González-Toril et al, 2006, Strathmann, 2003). Additionally, atomic force microscopy (AFM, BioMaterial™Workstation, JPK Instruments) was used for the investigation of cell morphology and distribution of cells on surfaces of pyrite coupons (Mangold et al, 2008a, Mangold et al, 2008b).

Results

Attachment tests with pyrite grains

Attachment to pyrite of bacteria in pure and mixed cultures was tested for two temperatures. The moderately thermophilic species *L. ferriphilum* and *At. caldus* were tested at 45°C. All other mesophilic strains were tested at 28°C. The numbers of planktonic cells were determined by direct counting with a Thoma chamber. The amount of attached cells was calculated by subtracting the planktonic from the initial cell number.

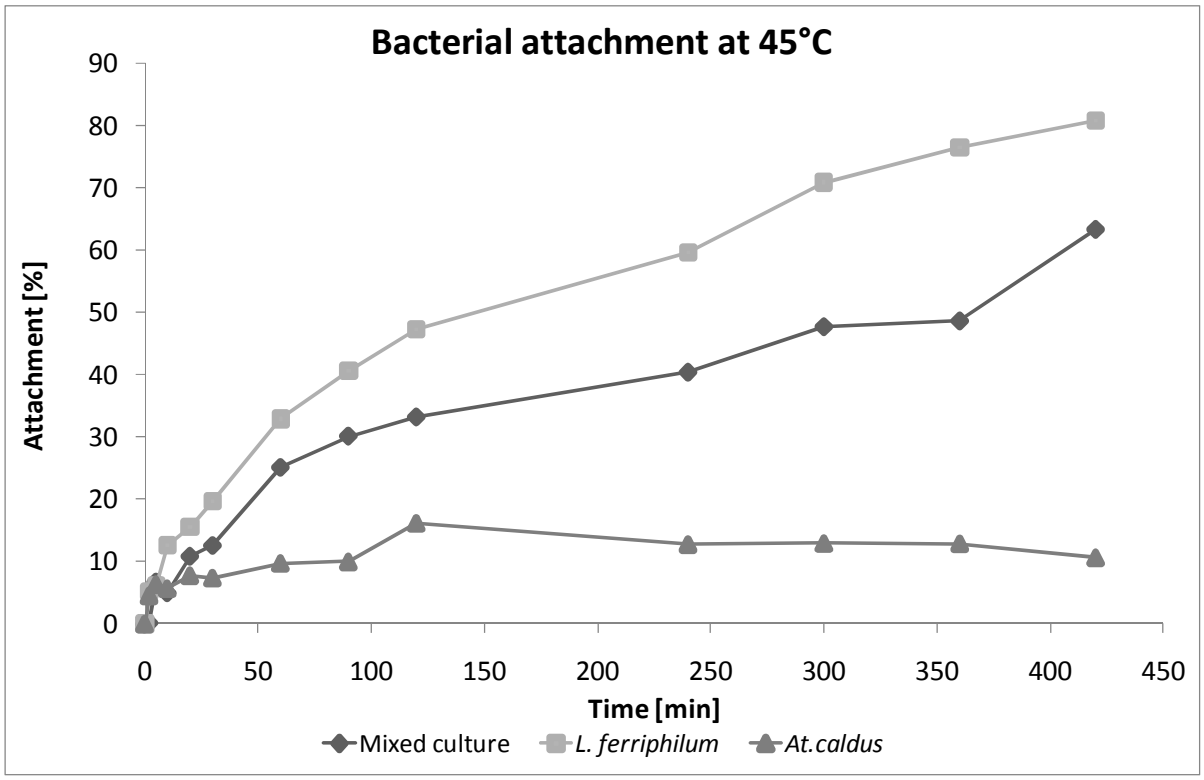

Fig. 1.: Attachment of *L. ferriphilum* and *At. caldus* in pure and mixed culture to pyrite. Initial cell number of $5x10^8$ cells/L in pure cultures or $2.5x10^8$ of *L.f.* and $2.5x10^8$ of *At.c.* in mixed culture, 50 mL salt solution, 45°C, rotary shaker (120 rpm), pyrite grains 50-100µm, n= 3, standard deviation equal or less than 20% for mixed culture, 10% for *L. ferriphilum*, 10% for *At. caldus*.

At. caldus showed little attachment during the whole experimental time (Fig.1) with only up to 10%. In contrast, 80% of the cells of *L. ferriphilum* attached to pyrite. In mixed cultures the attachment amounted to 60% adverted to the total cell number.

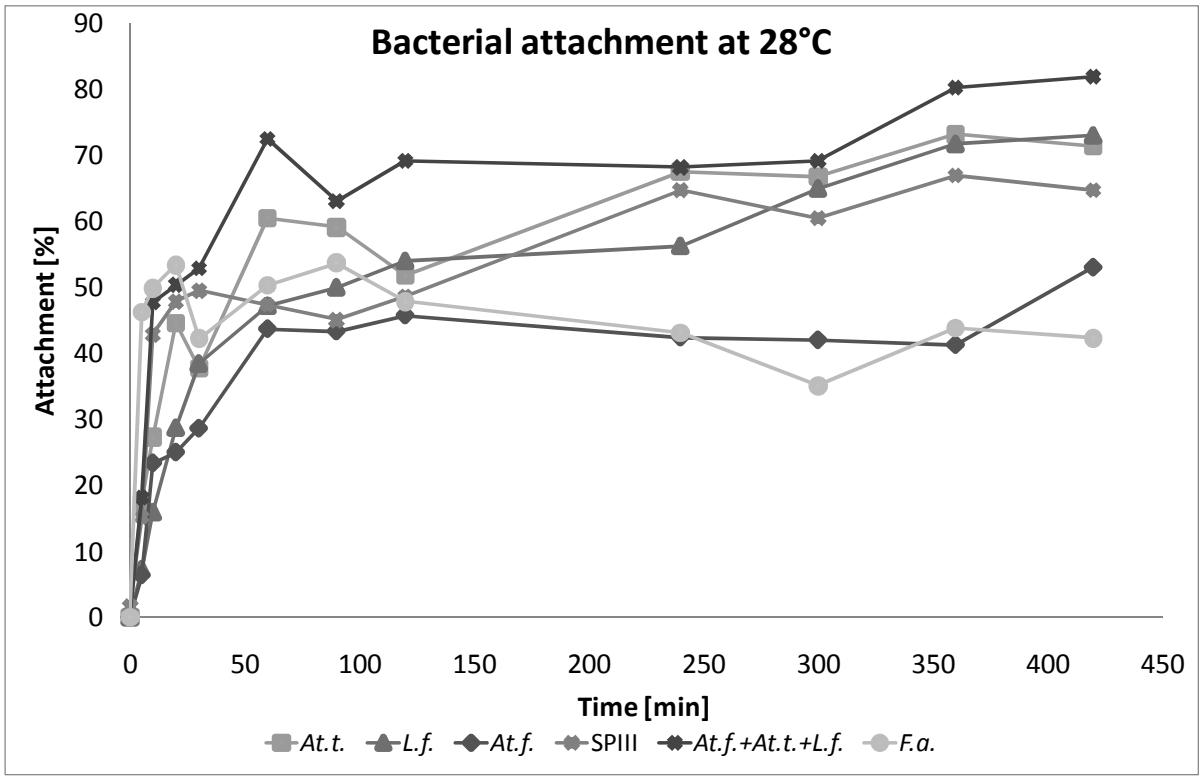

Fig. 2.: Attachment of *F. acidiphilum* (*F.a.*), *At. ferrooxidans* (*At.f.*), SPIII, *At. thiooxidans* (*At.t.*) and *L. ferrooxidans (L.f.)* pure and mixed cultures to pyrite grains (1/3 *L.f.*, 1/3 *At.t.*, 1/3 *At.f.*). Initial cell number of $1x10^8$ cells/L, 50 mL salt solution, 28°C, rotary shaker (120 rpm), pyrite grains 50-100µm, n= 3, standard deviation equal or less than 20% for *At.f. At.t.,L.f.*, 20% for *L. f.*, 30% for *At.f.*, 20% for *At. t.*, 20% for *F.a.*, 10% for SPIII.

SD.: ±1-20%.

In Fig.2 progress of bacterial attachment of mesophilic organisms is shown. Cells of *F. acidiphilum* and *At. ferrooxidans* exhibit with 40%-50% the lowest attachment to pyrite grains. SPIII cells attached to 60%, whereas cells of *L. ferrooxidans* and *At. thiooxidans* attached with 70% to the mineral surface. The mixed culture consisting of *At.f., At.t.* and *L.f.* produced the highest attachment of 80%.

Leaching test with pyrite grains

Leaching rates of the previously described pure and mixed cultures at 28°C and 45°C were calculated by the amount of total iron ions in the solution.

In Fig.3 the leaching rates of the two moderately thermopiles *L. ferriphilum* and *At. caldus* in pure and mixed culture are shown.

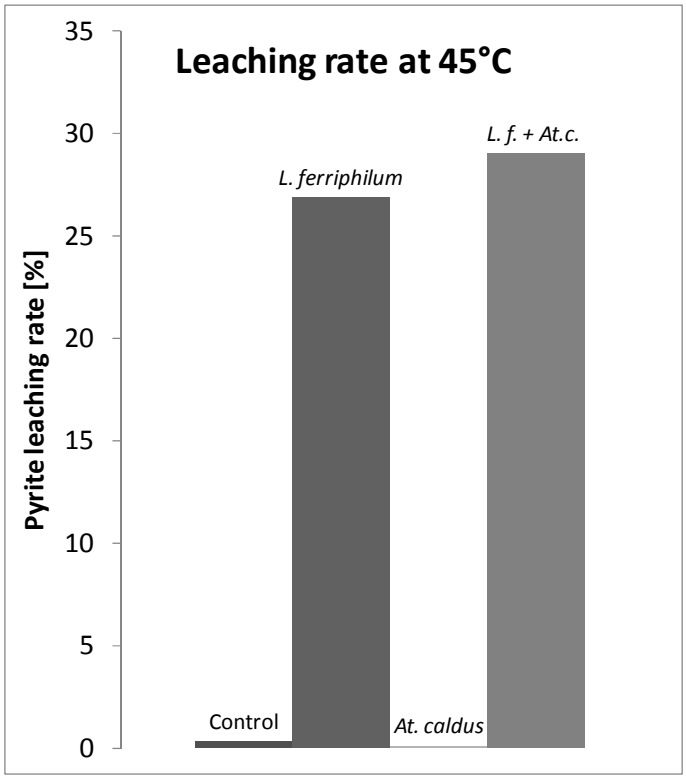

Fig. 3.: Leaching rates for pyrite of *L. ferriphilum* and *At. caldus* in pure and mixed culture. Initial cell number of $1x10^8$ cells/L, 150 mL Machintosh salt solution, 45°C, 21d, rotary shaker (120 rpm), pyrite grains 50-100µm, n= 3, standard deviation equal or less than 9% for *L. ferriphilum*, 32% for *Ac. caldus*, 2% for mixed culture.

Significant differences in leaching rates are obvious: Cells of *L. ferriphilum* exhibited rates 27% pyrite, whereas cells of *At. caldus* where without leaching activity. Leaching rates for the mixed cultures showed a slight increase of 29% over pure cultures of *L. ferriphilum*.

Leaching rates for the mesophilic bacteria *F. acidiphilum*, *At. ferrooxidans*, SPIII, *At. thiooxidans* and *L. ferrooxidans* in pure and mixed cultures are shown in Fig.4.

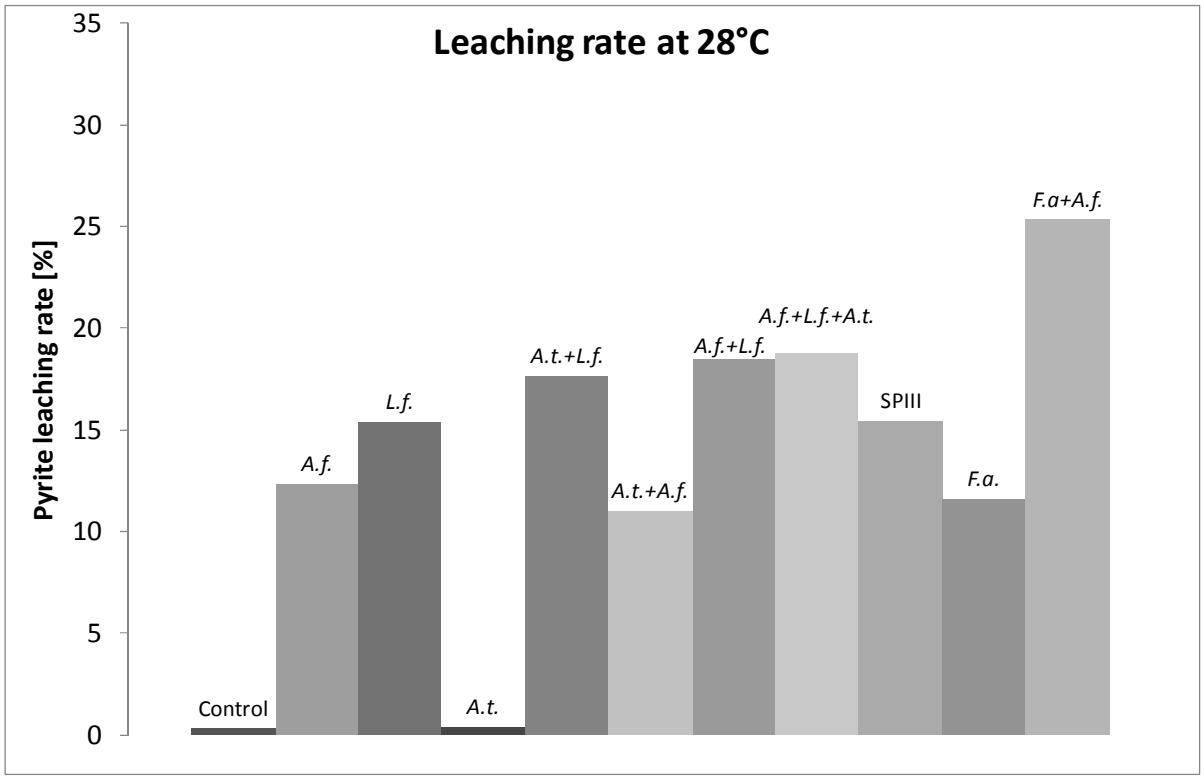

Fig. 4.: Leaching rates of cells of *F. acidiphilum* (*F.a.*), *At. ferrooxidans* (*At.f.*), SPIII, *At. thiooxidans (At.t.)* and *L. ferrooxidans (L.f.)* pure and mixed cultures with pyrite. Mixed cultures of two strains were composed of 50% of each strain, mixed cultures of three strains were composed of 1/3 of each strain. Initial cell number of $1x10^8$ cells/L, 150 mL Machintosh salt solution, 28°C, 21d, rotary shaker (120 rpm), pyrite grains 50-100µm, n= 3, standard deviation equal or less than 5% for *A.f.*, 10% for *L.f.*, 30% for *At.t.*, 5% for *A.t.+L.f.*, 5% for *At.t.+At.f.*, 5% for *At.f.+L.f.*, 5% for *At.f.+L.f.+At.t.*, 10% for *F.a.*, 5% for *At.f.+F.a.*, 10% for SPIII.

In general, leaching rates were lower for pure cultures than for mixed cultures except for mixed culture of *At. thiooxidans* and *At. ferrooxidans*. With pure cultures, cells of *L. ferrooxidans* and SPIII reached the highest leaching rate of 15 %, whereas the leaching rate of *At. thiooxidans* remained negligible. Cells of *At. ferrooxidans* and *F. acidiphilum* exhibited similar leaching activities of 12%. The mixed culture with cells of *At. ferrooxidans* and *F. acidiphilum* demonstrated the highest leaching rate of 25%. All other combinations produced reduced leaching rates around 18-19%.

Attachment tests with pyrite coupons

Attachment to pyrite coupons of pure cultures of *At. ferrooxidans, L. ferrooxidans* and *At. thiooxidans* was visualized by EFM. A statistical evaluation of the cell distribution on the coupon surface was done (data in Tab.1). Furthermore, the topographical distribution of bacterial on the mineral surface was analyzed (Fig.5).

Tab. 1: Time dependent counts of DAPI stained cells of pure cultures of *At. ferrooxidans, L. ferrooxidans* and *At. thiooxidans* on surfaces of pyrite-coupons. Incubation of coupons (fixed on a glass slide) in cultures of iron- or sulfur- grown cells, $1x10^8$ cells/mL, 28°C, vertical submerged, aerated, n=8, standard deviation equal or less than 40%.

	A. ferrooxidans [cells/mm²]	*A. thiooxidans* [cells/mm²]	*L. ferrooxidans* [cells/mm²]
1h	4,6	0,96	112
5h	7,04	6,4	>500
8h	>500	36,09	>500

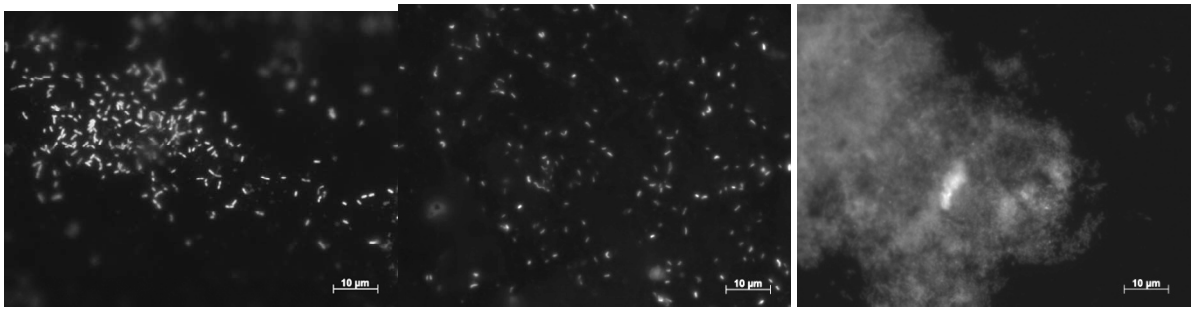

Fig.5a: EFM image of DAPI stained cells from a pure culture of *A. ferrooxidans* on the surface of a pyrite- coupon after 5h incubation.

Fig. 5b: EFM image of DAPI stained cells from a pure culture of *A. thiooxidans* on a surface of a pyrite-coupon after 5h incubation.

Fig. 5c: EFM image of DAPI stained cells from a pure culture of *L. ferrooxidans* on a surface of a pyrite-coupon after 5h incubation.

Cells of *At. ferrooxidans*, *L. ferrooxidans* and *At. thiooxidans* (pure culture) show differing attachment behavior to pyrite coupons. Whereas cells of *At. thiooxidans* are homogeneously distributed over the whole pyrite surface in low cell numbers, cells of *At. ferrooxidans* attach non homogenously, but with increased cell numbers. Cells of *L. ferrooxidans* tend to attach in clusters to the mineral surface in high cell numbers after 5h, tendering large areas free.

Visualization of sessile cells on pyrite by a combination of AFM and EFM

A combination of atomic force microscopy and epifluorescence microscopy was used to investigate differences in cell morphology, aggregation behavior and biofilm formation. EPS-compounds and cells were visualized by DAPI- and lectin- staining and FISH. In Fig. 6 cells of a mixed culture from a lignite mining area in eastern Germany on a pyrite surface are shown. The cells were labeled with fluorescence probes (FISH-probe EUB338) and DAPI stained.

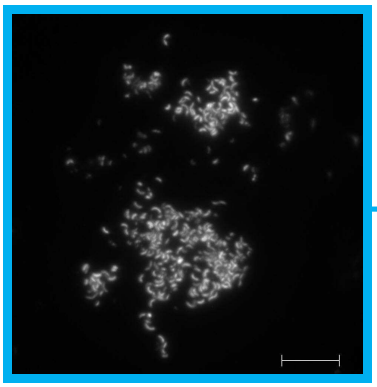

Fig. 6a: EFM image of DAPI stained cells of a mixed leaching culture on a pyrite surface.

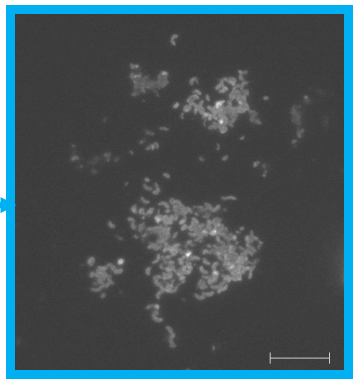

Fig. 6b: EFM image of cells of a mixed leaching culture on a pyrite surface, visualized by FISH probe EUB338.

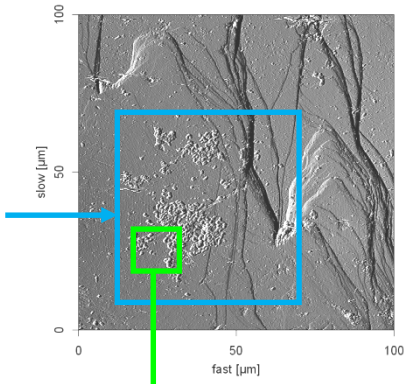

Fig. 6c: AFM image of cells of a mixed leaching culture on a pyrite surface after DAPI staining and FISH.

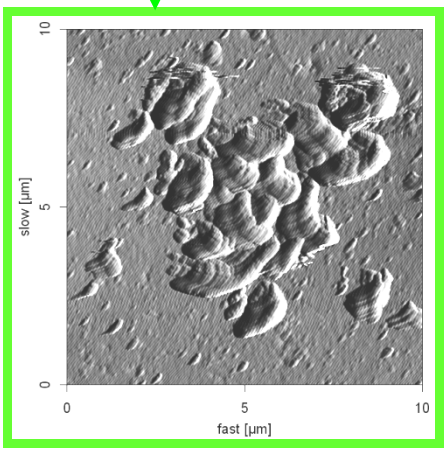

Fig. 6d: Detail of Fig. 6c.

Using this combination the cell distribution according to the mineral topography can be studied. Fig.6a-d indicate that cells are attached to the mineral in aggregates, whereas large parts of the pyrite surface remain uncolonized. Due to the positive signal of DAPI stain and FISH it was proven that the bright objects are bacteria.

Visualization of biofilms

Biofilm formation of leaching bacteria was investigated by the use of fluorescence marked lectin (ConA). Lectins were used to stain parts of EPS. Syto 9 and DAPI were used to visualize the cells and to determine the total cell number. ConA binds to certain structures found in various sugars like glycoproteins, glycolipids and mainly internal and non reducing terminal alpha-mannosylgroups and thus can be used to stain EPS containing this sugar.

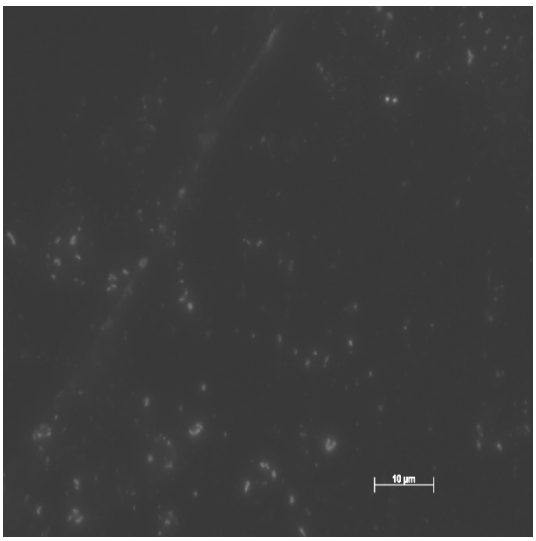

Fig. 7a: EFM image of lectin (ConA-TRITC) and DAPI stained cells from a pure culture of *L. ferrooxidans* on pyrite ($5*10^8$ cells/mL) after 2 h incubation, 28°C, vertical submerged, aerated .

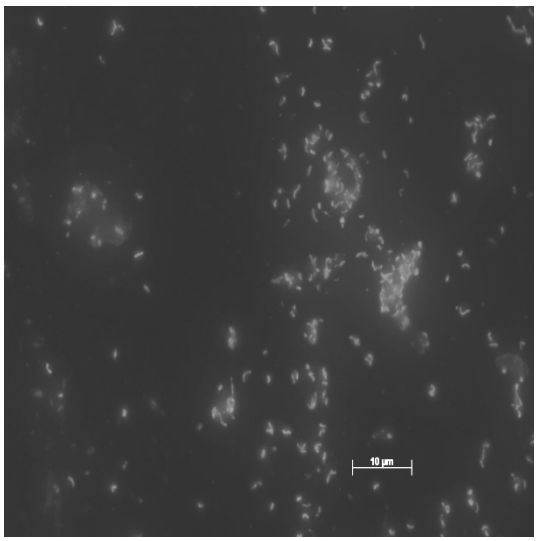

Fig. 7b: EFM image of lectin (ConA-TRITC) and DAPI stained cells of a mixed culture of *L. ferrooxidans, At. ferrooxidans* and *At. thiooxidans* on pyrite ($5*10^8$/mL) after 2 h incubation28°C, vertical submerged, aerated .

After 2 hours incubation of pyrite coupons in cultures of *Leptospirillum ferrooxidans* cells and parts of EPS became detectable (Fig.7a). In mixed cultures of *L. ferrooxidans* with

At. ferrooxidans and *At. thiooxidans* it becomes obvious that not only the amount of attached cells is increased, but also that EPS production is increased (Fig.7b).

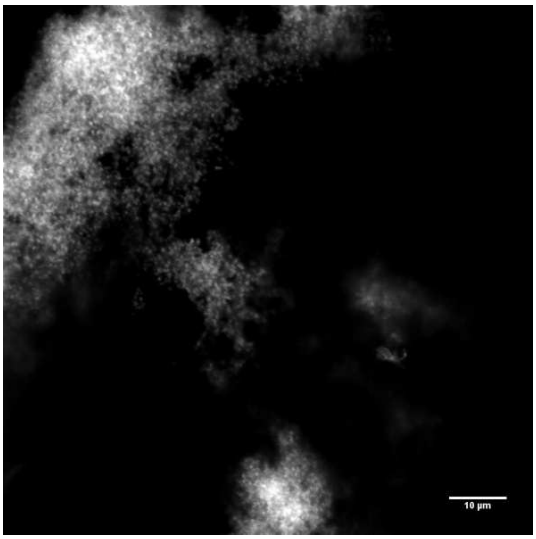

Fig. 8a: CLSM image of cells of a pure culture of iron(II) grown SPIII on a glass slide as stained by Syto^{TM} 9.

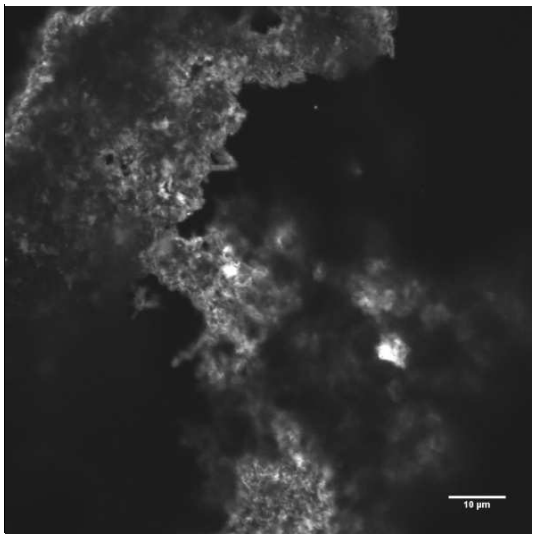

Fig.8b: CLSM image of cells of a pure culture of iron(II) grown SPIII on a glass slide as stained by lectin (ConA- TRITC).

Cells of SPIII tend to form flocks at the end of logarithmic growth in liquid media. Fig. 8 shows such a stained flock attached to a glass slide. The cells were enclosed by a strong lectin signal.

Discussion

To improve industrial heavy metal recovery processes and to reduce unwanted naturally occurring leaching processes e.g. acid rock drainage (ARD) it is necessary to understand bacterial attachment and biofilm formation.

Iron- and/or sulfur- oxidizers grow/live in at different temperature ranges. Mesophiles are important for natural leaching processes and are common in industrial applications. Especially at the beginning of leaching processes mesophiles are very fast and effective. Oxidation of pyrite is an exothermic process. Consequently, if rapid mineral oxidation is achieved

temperatures increase. Constructed ore heaps or commercial scale bioreactors often operate at temperatures between 40-50°C. Processes for heavy metal recovery become more efficient at these temperatures. Consequently, we studied adhesion and leaching rates at mesophilic and moderately thermophilic temperatures.

In general, cells of mixed cultures exhibit increased attachment and leaching rates than those from pure cultures. Attachment and leaching experiments with moderately thermophiles demonstrated that *L. ferriphilum* is the important organism. Amount of cell attachment and leaching rates with mixed cultures of *At. caldus* and *L. ferriphilum* show an slight increase over pure cultures of *L. ferriphilum*. Obviously, *At. caldus* cannot attach alone, but together with *L. ferriphilum* the cells complement each other and form mixed species biofilms on pyrite (Noël 2008; Florian et. al, 2010; Noël et. al, 2010). *L. ferriphilum* is able to oxidize iron(II) ions to iron(III) ions, the attacking agents for pyrite. The sulfur oxidizer *At. caldus* is not able to dissolve metal sulfides but in mixed cultures they complement each other.

By oxidizing the sulfur compounds they keep the acidity constant and prevent the formation of a passivation layer on mineral surfaces. The same effect is caused by cells of mesophilic *At. thiooxidans*.

Little is known about novel yet unclassified microorganism SPIII or *F. acidiphilum*. In leaching experiments cells of *F. acidiphilum* and *At. ferrooxidans* complement each other (highest leaching rate). One of the reasons may be that *F.a.* uses for growth organic compounds, which are inhibitory for *At. ferrooxidans*.

Attachment of the novel organism SPIII demonstrated comparable results to those of cells of *Leptospirillum spp.* (Fig.2). Also the EPS formation of these cells is important for attachment (Gehrke et. al.1995). The strong lectin signal indicates a high amount of polysaccharides within the EPS. The result is in agreement with data for *At.f.* and *L.f.*

Cells from pure cultures of *Leptospirillum spp.* exhibit attachment in high cell numbers and high amounts of EPS on pyrite, as compared to other leaching bacteria. In combination with other strains, cells of *Leptospirillum spp.* further the attachment of other bacteria, resulting in a mixed species biofilm on pyrite surfaces (Fig.5c, Fig.7). EPS production (chemical composition and amount) is species-specific; some strains produce much EPS, others almost none. *Leptospirillum spp.* (Fig.7) or SPIII (Fig. 8) are among copious amounts of EPS producing bacteria.

Summarizing, it is clear that the composition of leaching communities need to be optimized with regard to the industrial application. These findings, creates the necessity to combine chemolithoautotrophic organisms with chemolithoheterotrophic organisms like *F. acidophilum* in mesophilic temperatures. In consequence of increasing temperatures during leaching processes, *L. ferriphilum* in combination with a sulfur oxidizer is a combination of choice and environmental conditions should be optimized for this organism.

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