Attachment of acidophilic bacteria to solid substrata

Dissertation

zur Erlangung des akademischen Grades eines
Doktors der Naturwissenschaften
- Dr. rer. Nat. -

vorgelegt von

Nanni Noël

geboren in Oberhausen

Fakultät für Chemie
der
Universität Duisburg-Essen

2013

Die vorliegende Arbeit wurde im Zeitraum von Oktober 2008 bis September 2013 im Arbeitskreis von Prof. Dr. W. Sand Fakultät für Chemie, Biofilm Center der Universität Duisburg-Essen durchgeführt.

Tag der Disputation: 18.12.2013

Gutachter:	Prof. Dr. W. Sand
	Prof. Dr. A. Schippers
Vorsitzender:	Prof. Dr. S. Barcikowski

… to my mum, Ben & in memory of my dad …

-Science is the poetry of reality-
(Richard Dawkins)

Acknowledgments

At first, I would like to thank my Ph.D. supervisor **Prof. Dr. Wolfgang Sand** not only for the guidance and support during this study, but also for the understanding of private problems in hard times of my life. Thanks for the warm words and the free space which I needed to find my own way to deal with the situation.

I' am grateful to **Prof. Dr. Axel Schippers** for accepting the task for being my co-adviser.

Special thanks go to **Dr. Mario Vera** for his great support during this thesis and the helpful discussions about the communication pathways of bacteria. Thanks for proofreading and discussions of this work. I 'am grateful that I was allowed to travel to Chile and to be received in a very friendly way in a foreign country.

I' am grateful to **Fraunhofer-Umsicht**, Oberhausen for financial support. I would like to thank **Dr. Holger Wack**, **Damian Hintemann** and **Frank Pape**. It was a pleasure to work with them in different biocorrosion projects during the last years.

Thanks to **Prof. Dr. N. Guiliani** for a nice cooperation via DAAD and helpful discussions about communication pathways of bacteria.

Great thanks to **Prof. Dr. Wolfgang Streit**, Universität Hamburg, **Claudia Hornung** and **Frederike Haak** for the help with the biosensor bacterium HH01.

Thanks to **Prof. Dr. Thorsten Schmidt**, **Jens Laaks** and **Xochitli Osorio** for the help with the GC-MS and the interpretation of MS- spectra.

I would like to thank **all the persons** from Prof. Sand's working group who helped and supported me during the last years whether they are still members of this group (Petra, Qian, RuiYong, Sören, Til) or already find their ways in their working career (Bianca, Claudia, David, Igor, Jürgen, Mariël & Felipe). Special thanks go to:

Bianca, who was every time interested in my work, pushed me forward and for her talent to be a "headshrinker". Thanks for this huge friendship and also for proofreading of this thesis.

Bea, "my little thunderstorm" for her big heart, helpfulness and friendship.

Claudia, for discussions about "the great *At. caldus"*, molecular biology or the unpredictability's of life.

Andrzej and Christian, for technical advice and handling with the AFM-EFM, IC or HPLC. Also thanks for funny discussions about diverse passions, music/movies and drinks. That made long days during sample preparations/processing much easier.

Igor, Jens, Mark and Robert for funny good times especially during sampling sessions or after work to keep the "bad twins" alive.

Conny & Jürgen and **Mariël & Felipe**, for a great friendship and moral support during the last years.

Mia, "my travel companion", for the right interruptions of work, for travelling around the world to enjoy the most beautiful things of life.

All my friends, above all "J", Farina, Karin, Sandi, for their never ending support during the last years.

The biggest thanks go to **my family,** first of all:

my lovely mum, my brother Ben and his wife **Maike, my granny** and **my dad**
-it was not granted to him to undergo this day-

Table of Contents

List of Figures

List of Tables

Glossary

Acyl-ACP	acyl-carrier-protein
AI	autoinducer
AFM	atomic force microscopy
AHL	N-acyl-homoserine-lactone
AMD	acid mine drainage
ARD	acid rock drainage
BRGM	*Bureau de recherches géologiques et minières*
CLSM	Confocal laser scanning microscope
ConA	concanavalin A (Lectin of Canavalia ensiformis)
DAPI	4’,6-diamino-2-phenylindole
DMSO	dimethylsulfoxide
DPD	dihydroxypentane-2,3-dione
DSMZ	Deutsche Stammsammlung von Mikroorganismen und Zellkulturen
EFM	epifluorescence microscopy
EPS	extracellular polymeric substances
FISH	fluorescence in situ hybridization
GC	gas chromatography
HPLC	High-performance liquid chromatography
H_2S^+	sulfide cation radical
H_2S_n	polysulfide
IC	Ion-exchange chromatography
M	molarity
M^{2+}	metal cation
MAC	Mackintosh
MS	metal sulfide
n	numbers of repetition
NMR	nuclear magnetic resonance
OD	optical density
PBS	phosphate buffer solution

QS	quorum sensing
RISC	reduced inorganic sulfur compound
rRNA	ribosomal ribonucleic acid
rpm	resolutions per minute
TLC	thin layer chromatography
TCC	total cell count

Abstract

Bioleaching is the dissolution of metal sulfides such as pyrite (FeS_2) by bacterial and archaeal oxidation-driven processes. Leaching microorganisms attach to mineral surfaces, thus enhancing metal sulfide dissolution. Bioleaching has two contrary aspects: The negative aspect of acid mine/rock drainage (AMD/ARD), which results in water pollution, and the positive aspect, the environmentally friendly application in industry. Consequently, there are two targets: the prevention of AMD/ARD and the optimization of bioleaching as industrial technology.

In industrial applications such as in reactors, bioleaching is controlled and conditions are optimized for leaching microorganisms to achieve high oxidation rates. One important factor for the optimization is a high temperature. Moderately thermophilic leaching organisms are beneficial for such applications. The attachment and biofilm formation and the physiology of pure and mixed cultures of moderately thermophilic bacteria were investigated in this thesis. Leaching activities of the metal sulfide and interactions in between the consortium were of interest.

Attachment and leaching experiments under moderately thermophilic conditions indicated that *Leptospirillum ferriphilum* is the first colonizer of a pyrite surface. Furthermore, it was shown that mixed cultures are more effective than pure cultures with attachment and leaching. Precolonization tests exhibited that *Acidithiobacillus caldus* needs precolonization of active *L. ferriphilum* cells to establish itself in a biofilm on metal sulfide surfaces. Quorum sensing or chemotaxis related effects were determined because dead *L. ferriphilum* cells and their EPS residues did not increase attachment of At. caldus cells to the metal sulfide. However, active *L. ferriphilum* cells had an increasing effect on attachment of *At. caldus* cells to pyrite.
Further experiments indicated that attachment and biofilm formation of *L. ferriphilum* and *At. caldus* are influenced by addition of certain N-acetyl-homoserine-lactones (AHLs). Especially, pyrite leaching with cells of *L. ferriphilum* was strongly inhibited by addition of 3-hydroxy-C14-AHL. Consequently, pyrite leaching by *L. ferriphilum* cells becomes manipulable. Biofilm formation of *At. caldus* on pyrite coupons was increased by the use of the C8-AHL family. *L. ferriphilum* and *At. caldus* do not produce AHLs on their own but they were able to respond to an external addition of AHLs.

The addition of (5Z)-4-bromo-5-(bromomethylene)2(5H)-furanone inhibited growth, biofilm formation and leaching of *L. ferriphilum*, *At. caldus* and *Acidimicrobium ferrooxidans*. The inhibition is a reversible process.

Extracts from supernatants of pyrite-grown *L. ferriphilum* cells were tested in growth experiments with other species. *L. ferriphilum* supernatants had a strongly inhibiting effect on iron-oxidation in several microorganisms such *Leptospirillum* spp., *At. ferrooxidans, Acidimicrobium ferrooxidans, Acidithiobacillus ferrivorans* and *Sulfobacillus thermosulfidooxidans*. Sulfur oxidation was not affected by the addition of the "*L. ferriphilum*- extract". The inhibitory effect on iron-oxidation was also caused by an addition of extracts from three other *Leptospirillum* strains.

A novel QS- related autoinducer molecule was detected in cultures of *L. ferriphilum.* The utilization of a novel *Janthinobacterium* based biosensor test indicated its presence. It is highly likely that this autoinducer is a derivative of a α-hydroxyketones. Bioinformatic studies indicated that genomes of *Leptospirillium* spp. include a homologous gene for the enzyme, 8-amino-7-oxononanoate synthase, of the *jqsA* gene of the *Janthinobacterium*. Whether the inhibiting effect on iron oxidation can be attributed to the unknown autoinducer(s), still needs to be demonstrated. Nevertheless, the data indicated that a cross-communication via AHLs and by the unknown autoinducer(s) occurs in moderately thermophilic bioleaching bacteria.

1. Introduction

1.1. Bioleaching

Bioleaching is the conversion of metal sulfides (MS) to water soluble ions by bacterial and archaeal oxidation processes (Brandl, 2001; Rohwerder et al., 2002). Microorganisms oxidize different sulfidic ores such as pyrite (FeS_2), chalcopyrite ($CuFeS_2$) or sphalerite (ZnS) resulting in a type of water pollution called acid rock drainage (ARD) or acid mine drainage (AMD) (Silverman, 1967). AMD is also an environmental problem in regions where coal mining or lowering of ground water takes place, as coal deposits regularly contain metal sulfides such as pyrite or marcarsite. ARD is present in several natural MS rich environments such as the RioTinto region in Spain (Davis et al., 1999). Acidification and heavy metal pollution are the main consequences of ARD/AMD.

However, bioleaching is also established in biotechnology. As an industrial process it is used for heavy metal recovery such as gold, copper, zinc or nickel from low grade ores. Microbial recovery of heavy metals is an environmental friendly process and has a significant economic impact. Commercial applications such as heap or tank leaching are wide-spread for copper, gold or uranium extraction. Bioleaching has also a potential for detoxification of industrial products such as sewage sludge or heavy metal contaminated soil (Bosecker, 1997; Schippers, 1998; Rohwerder et al, 2007). However, basic research on the fundamental mechanisms and the physiology of the bioleaching consortia need to be performed to understand the whole process. With this knowledge industrial aspects could be improved and ARD/AMD could be prevented.

1.2. Bioleaching Mechanisms

Two mechanisms have been proposed to describe bacterial and archaeal leaching processes, the direct and the indirect leaching mechanism. However, only the indirect mechanism is generally accepted and described as an oxidative attack of ferric iron or protons on the mineral sulfide (Sand et al., 1995; Rohwerder et al., 2003). The two MS oxidation mechanisms have been named after the first sulfur intermediates in the process of MS oxidation, the polysulfide- or the thiosulfate- mechanism (Schippers, 1998), see figure 1.

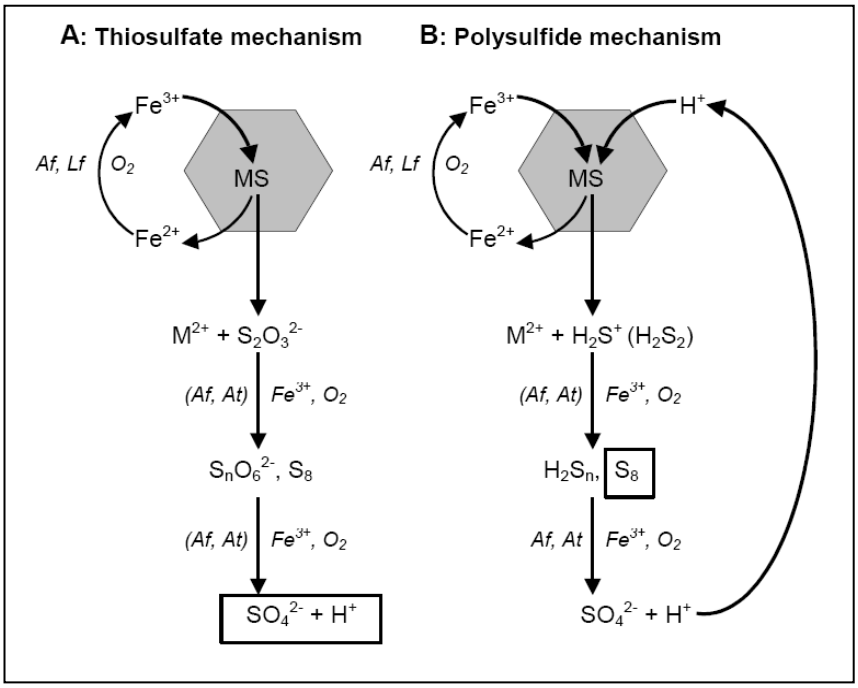

Figure 1: Scheme of bioleaching mechanisms. MS= metal sulfide, M^{2+}= metal cation, Af= *At. ferrooxidans*, Lf= *L. ferrooxidans*, At= *At.thiooxidans* (taken from Rohwerder et al., 2013)

Both mechanisms depend on the acid solubility of ores. Via the thiosulfate pathway, acid-insoluble metal sulfides e.g. pyrite (FeS_2), molybdenite (MoS_2) and tungstenite (WS_2) are oxidized. This oxidation process occurs via electron extraction by iron (III) ions. Six one-electron oxidation steps are necessary to break the chemical bonds between sulfur- and metal-atom. The S_2 group is oxidized to thiosulfate (equation 1) which is further oxidized via tetrathionate or other polythionates to sulfate (equation 2). Iron (III) ions serve as oxidation agent and are reduced to iron (II) ions. The regeneration of the oxidation agent (equation 3) is achieved by bacterial oxidation (Schippers & Sand, 1998; Rohwerder et al., 2003). Sulfur oxidizers such as *At. thiooxidans* or *At. caldus* convert reduced forms of sulfur compounds (RISCs) to sulfate and protons (equation 4) (Schippers, 2007).

Thiosulfate mechanism:

$$FeS_2 + 6\ Fe^{3+} + 3\ H_2O \rightarrow S_2O_3^{2-} + 7\ Fe^{2+} + 6\ H^+ \quad \text{(Equation 1)}$$

$$S_2O_3^{2-} + 8\ Fe^{3+} + 5\ H_2O \rightarrow 2\ SO_4^{2-} + 8\ Fe^{2+} + 10\ H^+ \quad \text{(Equation 2)}$$

$$2\ Fe^{2+} + 0.5\ O_2 + 2\ H^+ \rightarrow 2\ Fe^{3+} + H_2O \quad \text{(Equation 3)}$$

$$0.125\ S_8 + 1.5\ O_2 + H_2O \rightarrow SO_4^{2-} + 2H^+ \quad \text{(Equation 4)}$$

Acid soluble metal sulfides such as sphalerite (ZnS), galena (PbS), arsenopyrite (FeAsS), chalcopyrite ($CuFeS_2$) or hauerite (MnS_2) are dissolved by a combined action of protons and iron (III) ions. The protons attack the chemical bonds between metal and sulfur moiety. Theoretically, after binding of two protons, hydrogen sulfide (H_2S) could be produced (Rohwerder et al., 2003). However, in the presence of iron (III) ions, the first free intermediate is a sulfide cation radical (H_2S^+), which ends after some reactions in a polysulfide (H_2S_n) (equation 5). Via different oxidation processes these intermediate compounds break down to H_2SO_4 and elemental sulfur, providing protons for this pathway (eq.6-7) (Rohwerder et al., 2002).

Polysulfide mechanism:

$$MS + Fe^{3+} + H^+ \rightarrow M^{2+} + 0.5\ H_2S_n + Fe^{2+}\ (n \geq 2) \quad \text{(Equation 5)}$$

$$0.5\ H_2S_n + Fe^{3+} \rightarrow 0.125\ S_8 + Fe^{2+} + H^+ \quad \text{(Equation 6)}$$

$$0.125\ S_8 + 1.5\ O_2 + H_2O \rightarrow SO_4^{2-} + 2\ H^+ \quad \text{(Equation 7)}$$

During dissolution of MS, bacteria and archaea have the pivotal role to regenerate iron (III) ions and to produce H_2SO_4. Two mechanisms are proposed for the biological dissolution of MS: the “contact” and “non-contact” mechanism as shown in Figure 2.

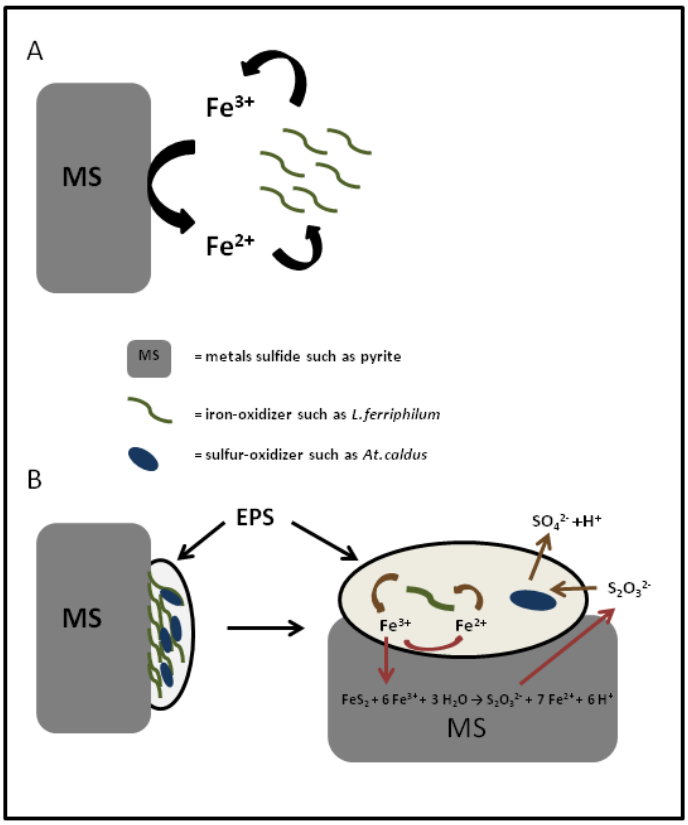

Figure 2: "Contact" und "non-contact" mechanism as reviewed by Rohwerder et al., 2003, Crundwell, 2003; A= "non-contact mechanism, where microbial oxidation of iron (II) to iron (III) ions occurs in the bulk solution followed by a chemical attack of iron (III) ions on the MS. B= "contact" mechanism, cells are embedded in EPS and form a monolayer biofilm on the MS. In between this reaction space iron (II) ions are oxidized to iron (III) ions via iron-oxidizers (green spiral). Iron (III) ions attack the mineral and are chemically reduced to iron (II) ions. Thiosulfate is released and oxidized by sulfur-oxidizers (blue oval) to sulfate and protons leading to a decrease of pH in the environment. Brown arrows indicate microbial reactions, whereas red arrows describe chemical reactions.

The contact mechanism postulates an attachment of microorganisms to the MS-surface. A reaction space between the MS-surface and the microorganism is established where electrochemical processes take place and lead to the dissolution of the mineral. This space is filled with extracellular polymeric substances (EPS). Iron (III) ions are the oxidizing agent, produced by microorganisms and chemically reduced back to iron (II) ions (Schippers & Sand, 1998; Rohwerder et al, 2003). However, the non-contact mechanism assumes that microorganisms oxidize iron (II) ions to iron (III) ions in the bulk solution, which can also attack the MS (Rohwerder et al, 2003).

1.3 Attachment and biofilm formation on pyrite

Attachment of cells to the MS-surface is a prerequisite for dissolution of the mineral and is mainly influenced by EPS. Electrostatic interactions effect attachment of the positively charged cells to the negatively charged pyrite (Gehrke et al., 1998; Rohwerder et al., 2003). Iron (III) ions are complexed in the EPS and further the dissolution of pyrite. Bacteria adapt their EPS composition according to the substrate and growth conditions (Rohwerder et al., 2007). In previous studies it was demonstrated that cells which grew only in the planktonic phase e.g. with iron (II) sulfate, did not produce large amounts of EPS. Whereas, cells grown attached on pyrite had a higher amount of EPS. This reaction space is composed of different sugars e.g. glucose, mannose, fructose, fatty acids, glucuronic acids and iron (III) ions (Gehrke et al., 1998; Kinzler et al., 2003). However, cells grown on elemental sulfur produce a high amount of EPS, but they do not attach to pyrite. This is explained by the lack of iron (III) ions or other positive charged groups in the EPS of sulfur-grown cells. Here, hydrophobic interactions are the relevant ones which allow attachment to elemental sulfur (Gehrke et al., 1998; Rohwerder et al., 2007).

Since it is commonly accepted that EPS are needed for microbial attachment to MS, biofilm formation and especially the attachment of microorganisms need to be investigated (Rohwerder et al., 2010; Florian et al., 2011). Biofilm formation implies three different parts: transport of the cell to the solid surface, primary or initial attachment and colonization or development of a continuous biofilm including detachment of cells (van Loosdrecht et al., 1990).

Transport of cells to the solid surface occurs in different ways: diffusion, convection or active movement. Diffusion is the slowest process caused by Brownian movement. In comparison, convective movement is fast and a result of liquid flow. However, active movement requires motility of microorganisms using flagella or cilia (van Loosdrecht et al., 1990; Watnick & Kolter, 2000).

The next step is the primary or initial attachment of cells to the solid surface. That process is reversible or irreversible. Reversible attachment is defined as adhesion, where bacteria can easily be removed by mild shear forces or active movement. In contrast, irreversible attachment needs stronger shear forces for cell detachment (van Lossdrecht et al., 1990).

The last step of biofilm formation is defined as surface colonization. EPS is produced and enhances the microbial adhesion followed by increased attachment and surface colonization (Yee et al., 2000). On the one hand, already attached cells start to grow and multiply. In

addition to that, new planktonic cells can also attach, resulting in the formation of microcolonies (Watnick & Kolter, 2000).

Development of microcolonies and monolayered biofilms on pyrite surfaces was observed in studies on *At. ferrooxidans* (Harneit et al., 2006). Atomic force microscopy (AFM) was used to visualize cells covered by EPS attached on a metal sulfide surface. *At. ferrooxidans* has also been observed as a monolayered biofilm on elemental sulfur particles (Jerez, 2009). Furthermore, cells of another leaching bacterium *L. ferrooxidans* were detected. A part of a monolayered biofilm, developed on a pyrite surface, was released to the surrounding environment (Rojas-Chapana & Tributsch, 2004).

It is often described that leaching organisms attach to crystal defect sites including visible scratches or dislocation sites of the MS (Andrews, 1988; Gehrke et al., 1998; Jerez, 2009). Cross-sectional shapes of the mineral are critical and effect bacterial adhesion (Edwards & Rutenberg, 2001).

But how do leaching organisms detect or sense attractive attachment sites on the mineral? Chemotaxis is described as a way to sense the environment. It consists of the activity of bacteria to direct their movement according to signal molecules in their surrounding environment. Motile bacteria sense the environment and detect changes of chemicals which can be an attractant or a repellent. Therefore, bacteria utilize chemosensory and chemotactic transduction systems for regulation of their flagellar movement (Baker et al., 2005; Delgado et al., 2008; Jerez, 2009). Chemotaxis allows leaching organisms to detect gradients of useable substrates released from MS resulting in an increased attachment to favorable sites (Jerez, 2009). By this way, bioleaching organisms could find specific sites on the MS surface to adhere and to start oxidation to obtain their energy. Motility by means of flagella has been described for *At. ferrooxidans, At. thiooxidans* and *L. ferrooxidans* (DiSpirito et al., 1982; Ohmura et al., 1996). Furthermore, it was demonstrated that *L. ferrooxidans* possesses a chemotatic response to aspartate and Ni^{2+}, whereas, aspartate seems to be a repellent and Ni^{2+} an attractant (Delgado et al., 1998; Jerez, 2001). In addition, it was reported that *L. ferrooxidans* was attracted by Fe^{2+} and Cu^{2+} (Acuña et al., 1986, 1992). The chemotactic response of *At. ferrooxidans* to thiosulfate has also been described (Chakrabarty et al., 1992) and also Fe^{2+} and tetrathionate had an attracting effect on this leaching organism (Meyer et al., 2002). For *L. ferrooxidans* a gene encoding a putative chemotatic receptor (Lcrl) has been found (Jerez, 2001) and in *L. ferriphilum* strain ML-04 different chemotactic sensory transducers are encoded in the genome (Mi et al., 2011).

1.4 Quorum sensing (QS)

Biofilm formation is a complex process and is partially regulated by Quorum Sensing (QS) mechanisms (Ng & Bassler, 2009). Furthermore, QS is known as one of the main regulatory ways for EPS production and biofilm formation in bacteria (Ruiz et al., 2008). QS allows a bacterial population to control their number and to modulate gene expression in a growth dependent manner. Therefore, diffusible autoinducers (AIs) are used to regulate cellular processes (Camilli & Bassler, 2006, Boyer & Wisniewski-Dye, 2009).

Constitutive expression of AI- biosynthetic genes at low cell densities causes a basic production of AIs, which are released into the surrounding environment. High-population densities result in an accumulation of AIs in and around the cells. AIs bind to specific transcriptional regulator proteins which are activated and further bind to specific DNA sequences. The transcription of QS- regulated genes is enhanced and diverse phenotypes are expressed. These include regulation of biofilm formation, EPS production, virulence factor production and motility (Gonzalez and Keshavan, 2006).

Gram-negative and Gram-positive bacteria utilize QS as communication system. The molecular bases of these are different and classified in three main groups (Miller & Bassler, 2001; Persson et al., 2005; Waters & Bassler, 2005).Gram- negative bacteria use N-acyl homoserine lactones (AHLs) as autoinducers and the QS-system (generically known as AI 1), whereas Gram-positive bacteria utilize mainly small peptides as signaling molecules. The third group is known as AI-2-system, which includes 4, 5-dihydroxypentane-2,3-dione (DPD) derived molecules. This family of molecules is considered to be an interspecies communication system (Camilli & Bassler, 2006; Valenzuela et al., 2010).

1.4.1 N-acyl homoserine lactones (AHLs) and the LuxI/R- QS-system

Cell-cell communication in Gram-negative bacteria is mostly mediated by AHLs. The QS-system is based on the LuxI/R-system, which was first described for *Vibrio fischeri,* and consists of two proteins: LuxI and LuxR (NG & Bassler, 2009) as illustrated in figure 3. LuxI is the AHL synthase protein, whereas LuxR is the transcriptional regulator (Valenzuela et al. 2010). LuxI synthesizes AHLs catalyzed by two substrates, S-adenosyl methionine (SAM) and an acylated acyl carrier protein (acyl-ACP). In general they consist of a lactone ring carrying acyl chains in a length of C_4 to C_{18} and different functionalities (alcohol, hydroxy or ketone) at the C_3 atom (Ng & Bassler, 2009; Valenzuela et al., 2010).

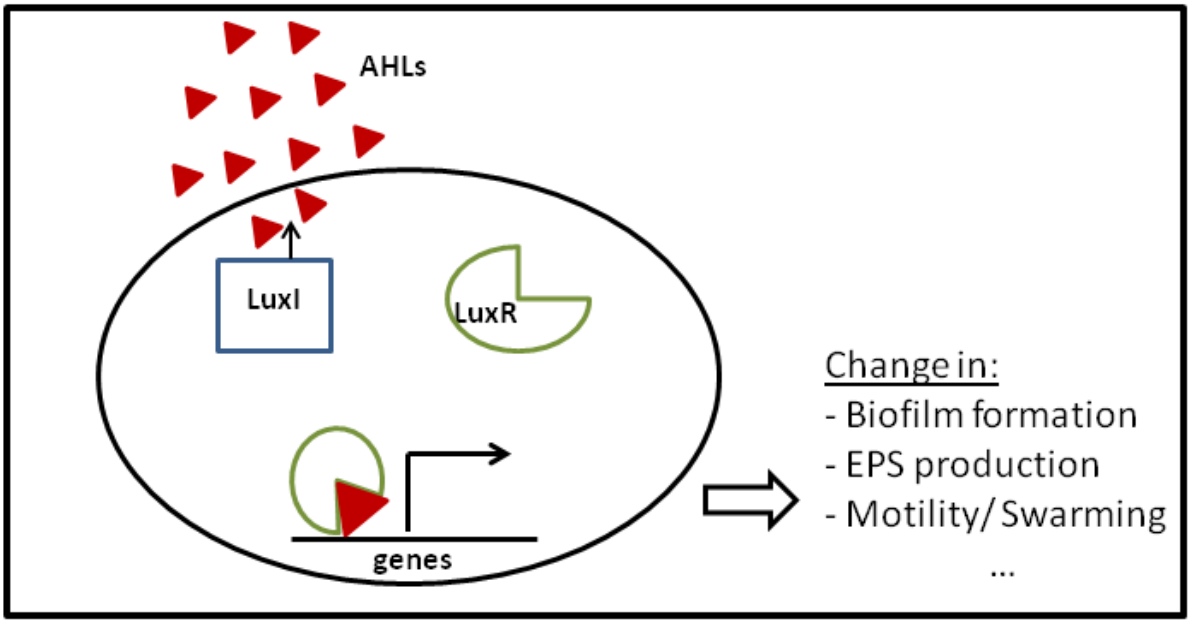

Figure 3: LuxI/LuxR quorum sensing system in Gram- negative bacteria (adapted and modified based on Henke & Bassler, 2004; Ng & Bassler, 2009; Valenzuela et al, 2010). Red triangles= AHLs, autoinducing molecules; LuxI= AHL synthase protein and responsible for formation of specific AHLs, which diffuse out of the cell and accumulate at high cell densities; LuxR= transcriptional regulator protein, which interacts with AHLs and dimerizes. This complex binds to target gene promoters and starts their transcription resulting in regulation of physiological functions.

1.4.2 A novel QS system regulated by α-hydroxyketone signaling

Recently, a novel class of signaling molecules, the α-hydroxyketones (AHKs), was discovered in *Legionella pneumophila* and *Vibrio cholera* (Tiaden et al., 2010). The AHK signaling circuit is linked to the stationary growth phase of *L. pneumophila*. It comprises the autoinducer synthase LqsA, which produces the autoinducer LAI-1 (3-hydroxypentadecan-4-one). The system also includes the putative sensor kinase LqsS and the response regulator LqsR which is controlled by the alternative σ factor RpoS and the two-component system LetA/LetS (Spirig et al., 2008; Tiaden et al., 2010).

V. cholerae includes a similar system with an autoinducer synthase CqsA, which produces the autoinducer CAI-1 (3-hydroxytridecan-4-one). The CqsS is the corresponding sensor kinase. In *Vibrio cholerae* the AHK signaling is coupled with a convergent density-dependant regulatory circuit. At low cell densities, LuxO (response regulator) which is bound by LuxU plus the sigma factor RpoN, induces the expression of small non coding RNAs. These RNAs and the nucleoid protein FIS bind and thus destabilize the HapR which prevent the production of QS master regulator protein, HapR (Tiaden et al., 2010). HapR can act in two ways: once as transcriptional repressor (biofilm formation) or as activator such as Hap proteases. At high

cell densities, LuxO is inactive and the production of HapR is not prevented. (Tiaden et al., 2010).

Recently, a homologue of the *cqsA* and *lqsA* autoinducer synthases encoding gene was found in *Janthinobacterium* and was designated *jqsA* which is linked to a cognate sensor kinase jqsS and this is flanked by the response regulator *jqsR* (Hornung et al., 2013). The *lqs/cqs* gene cluster and homologues were found in several environmental bacteria (Tiaden et al., 2010; Hornung et al., 2013). Thus, it was suggested that AHKs are commonly used as signaling molecules.

1.4.3 Quorum sensing and bioleaching

Earlier studies reported that *At. ferrooxidans* possesses a functional type AI-1 QS system that involves acyl-homoserine lactones (AHLs) as autoinducing molecules including 3-hydroxy-C8-AHL, 3-hydroxy-C10-AHL, C12-AHL, 3-oxo-C12-AHL, 3-hydroxy-C12-AHL, C14-AHL, 3-oxo-C14-AHL, 3-hydroxy-C14-AHL, and 3-hydroxy-C16-AHL (Farah et al. 2005, Rivas et al., 2005). Recently, it has been shown that an external addition of synthetic AHL pools enhanced attachment of *At. ferrooxidans* to pyrite and sulfur surfaces. This enhancement was mediated by a concomitant increase of capsular polysaccharides in the EPS (Gonzalez et al., 2012). In addition, the fact that some other strains of *At. ferrooxidans* and *At. thiooxidans* produce AHLs suggests them as potential interspecies communication molecules in bioleaching communities (Valenzuela et al., 2010). In this context, with the aim to influence microbial leaching processes, QS should be investigated as a way to manipulate cell-cell communication for inhibition or improvement of bioleaching. Furthermore, chemically synthesized agonist and antagonist molecules and their effect on EPS- and biofilm formation on minerals could be tested.

1.5 Bioleaching community

Generals

Biofilms on MS provide an environment for leaching communities where intercellular interactions are favored. Not only QS is useful, also the different types of metabolism are favorable to create an environment, where a diversity of organisms is able to survive. One part of the biofilm consortium will generate compounds which are useful for other members of the community. Others will benefit from metabolic by-products (Jerez, 2009).

Leaching communities are composed of organisms with different metabolic properties. Iron-oxidizers oxidize iron (II) to iron (III) ions, hence producing the MS- oxidizing agent. In addition, RISCs are released by the attack of iron (III) ions. In many studies both types of organisms were present in mixed leaching communities and furthered the dissolution of the mineral (Dopson & Lindström, 2003; Okibe & Johnson, 2004; Noel, 2008; Florian 2012). Efficiency and the capability of resistance against stress situations were much higher in mixed cultures corresponding to pure ones (Johnson, 1998). But not only the benefit for each other via metabolic products further the dissolution of MS, also EPS production was stimulated. Little attaching organisms started to show increased attachment and establish themselves in the mixed biofilm on the pyrite surface. *At. caldus* did not attach to pyrite alone, but it benefits from the presence of *L. ferriphilum* in mixed cultures. Increased attachment and an increase of EPS production and, consequently, biofilm formation are the result of this mixed consortium (Noel, 2008; Florian, 2012).

Many interactions, which occur in the bioleaching environments, have been described and include competition, predation, mutualism and synergy. Leaching organisms compete for substrates or environmental parameters such as temperature or pH (Johnson, 1998). *L. ferrooxidans* is described to be more effective for pyrite leaching as compared with *At. ferrooxidans*, because it is more tolerant against ferric ions and low pH. *At. ferrooxidans* grows faster at a pH around 2 and high ferrous iron concentrations than *L. ferrooxidans* (Norris et al., 1988; Rawlings et al., 1999). Predation was specified in the case of an *Eutreptia*-like flagellate grazing *At. ferrooxidans* in preference to *L. ferrooxidans* in a mixed consortium of both (Johnson, 1998). Mutualism was detected in cultures of *At. ferrooxidans* or *L. ferrooxidans* when *Acidiphilium* spp. or *Ferromicrobium acidophilium* were present. MS dissolution was increased in mixed cultures, because organic acids inhibited the chemolithotrophic organisms. The presence of the heterotrophs avoided its accumulation by metabolizing the organic material (Hallmann et al., 1992; Johnson & Roberto, 1997). Synergism is described in situations where organisms complement each other and are more efficient together as on their own. Chalcopyrite dissolution was enhanced by mixed consortia of *L. ferrooxidans* and *At. thiooxidans* or *At. caldus* (Norris et al., 1990). Examples for an increased dissolution of pyrite is given by *At. caldus* and *L. ferriphilum* (Noel, 2008) or *At. ferrooxidans* and *At. thiooxidans* (Florian et al., 2011; Florian, 2012).

Diversity of leaching organisms

In general, bioleaching organisms can be divided into three groups according to their optimal growth temperature. These are mesophiles growing up to 40°C, moderately thermophiles in the range of 40-55°C and thermophiles in the range of 55-80°C (Schippers, 2007). Often industrial heap and tank bioleaching processes run below 40°C. However, pyrite oxidation is an exothermic process and fast reaction rates are developed which lead to high temperatures (Okibe & Johnson, 2004). Therefore, moderately thermophilic microorganisms are important for industry and will be discussed in this study.

Moderately thermophilic leaching organisms

Bioleaching organisms of the moderately thermophilic temperature range have an optimal growth temperature between 40-55° (Johnson & Hallberg, 2009). The diversity of microorganisms in this temperature range is large and includes autotrophic, mixotrophic and heterotrophic bacteria and archaea which utilize different electron- donors and acceptors (Okibe & Johnson, 2004). An overview of physiological characteristics of four different species of moderate thermophiles is given in Table 1. This study was focused on these four organisms due to their different physiological traits.

Table 1: Phylogeny and physiological traits of four main bioleaching organisms at moderately thermophilic temperature conditions

SPECIES	PHYLUM	PHYSIOLOGICAL TRAITS	REFERENCE
Leptospirillum ferriphilum	Nitrospira	Autotrophic, Fe^{2+}-oxidizer	Okibe et al., 2003
Acidithiobacillus caldus	Proteobacteria	Autotrophic, S°-oxidizer	Hallberg & Lindström, 1994
Acidimicrobium ferrooxidans	Actinobacteria	Mixotrophic, Fe^{2+}-oxidizer, Fe^{3+}-reducer	Clark & Norris, 1996
Ferroplasma acidiphilum	Euryarchaeota	Heterotrophic, Fe^{2+}-oxidizer, Fe^{3+}-reducer	Johnson et al., 2001

L. ferriphilum was first described by Coram and Rawlings (2002) as a chemolithotrophic Fe^{2+}-oxidizer. It grows up to 45°C with an optimal growth temperature of 37°C. This bacterium uses iron (II) as electron donor and oxygen as electron acceptor. The optimum growth is between pH 1.3-2.0. Cells are 0.3 to 0.6 µm wide and 0.9 to 3.5 µm long and spiral

shaped (Coram & Rawlings, 2002). Furthermore, the strains of this species form a distinct phylogenetic cluster separated from *L. ferrooxidans.* This Gram-negative bacterium fixes carbon dioxide via the reductive citric acid (rTCA) cycle (Levican et al., 2008; Mi et al., 2011). *L. ferriphilum* is described as the most dominant iron-oxidizer in industrial biooxidation tanks (Okibe & Johnson, 2004).

At. caldus was described by Hallberg & Lindström (1994). It is an aerobic, autotrophic, Gram-negative sulfur-oxidizer, able to use diverse RISCs such as elemental sulfur, sulfide, sulfite, thiosulfate or tetrathionate (Hallberg et al., 1996). *At. caldus* is not able to grow on pyrite or iron (II) ions (Hallberg and Lindstrom, 1994). Its cell sizes are 0.4 -0.6 by 1 - 2µm and it grows optimally at pH 2.5 and 45°C (Zhou et al., 2007). Semenza et al. (2002) reported that under certain conditions *At. caldus* can dominate the sulfur oxidizing population in bioleaching and biooxidation plants and that this organism has two roles during bioleaching processes. On the one hand the continuous acidification of the medium is achieved which allows the solubilization of metal compounds. On the other hand the dissolution of the sulfur "passivation" layers is effected, which may be deposited on the sulfide surface during bioleaching processes.

Am. ferrooxidans was described by Clark & Norris (1996). This Gram-positive bacterium is rod-shaped with cell sizes of 0.4 by 1-1.5µm. The optimal growth temperature and pH are between 45-50°C and pH 2 respectively (Clum et al., 2009). Autotrophic growth on Fe (II) ions and heterotrophic growth with yeast extract were reported. Cells are motile during heterotrophic growth (Clum et al., 2009; Clark & Norris, 1996). In mixed consortia, *L. ferriphilum* and *Am. ferrooxidans* compete for the same energy source (Fe (II) ions) (Okibe & Johnson, 2004).

F. acidiphilum was isolated by Golyshina et al. (2000). This archaeon grows at temperatures between 37-50°C with an optimum at 37°C. Oxidation of Fe (II) ions is possible, if an organic carbon source is available. The pH optimum is around 1.7. It has a pleomorphic shape with a size of 0.3-3µm. *Ferroplasma spp.* are most abundant in leaching areas with very low pH and high ionic strength (Golyshina & Timmis, 2005).

2. Aims of this study

Bioleaching has two contrary aspects: The negative aspect of AMD/ARD, which results in water pollution, and the positive aspect, the environmentally friendly application in industry. Consequently, there are two targets: the prevention of AMD/ARD and the optimization of bioleaching as industrial technology.

Industrial applications of bioleaching have a significant economic impact, especially for the recovery of gold, copper and zinc. In reactors, the process is controlled and conditions are optimized for leaching organisms to achieve high oxidation rates. One important factor for the optimization is a high temperature. However, economic efficiency should be kept in mind (e.g. reduction of cooling cost). Moderately thermophilic leaching organisms are beneficial for such applications, thus the attachment and biofilm formation should be investigated and the physiology of pure and mixed cultures has to be understood. These findings should be correlated with leaching activities of the MS and both, ecology and interactions in between the consortium need to be investigated.

This study is based on the dissolution of pyrite by bacteria or archaea. Pyrite is one of the most frequent MS on earth and is often present in the field of bioleaching. Four leaching microorganisms, *L. ferriphilum*, *At. caldus*, *Am. ferrooxidans* and *F. acidiphilum* were chosen to evaluate the leaching process under moderately thermophilic conditions.

There are still several open questions to understand the process of bioleaching in this temperature range. The following questions were addressed in this study:

- Which member of a defined mixed consortium is the first colonizer of a pyrite surface?
- Which member needs a precolonization by other bacteria to be able to attach or to establish itself in a biofilm?
- Is there an effect of QS signal molecules on cultures of moderately thermophiles and do they influence bioleaching?

3. Materials & Methods

3.1. Microorganisms and growth

Bacterial and archaeal strains

The strains of *L. ferriphilum*, *At. caldus*, *Am. ferrooxidans* and *F. acidiphilum* used in this study are listed in table 2. The strains were maintained in the culture collection of the Biofilm Centre, Aquatic Biotechnology group, University Duisburg-Essen, and grown from these stocks at the conditions specified.

Table 2: Microorganisms used in this study

SPECIES	STRAIN	ORIGIN	REFERENCES
L. ferriphilum	DSM14647	Enrichment culture, Peru, 1984	Coram and Rawlings (2002)
At. caldus	DSM8584	Enrichment culture of P. Norris, Univ. of Warwick	Hallberg & Lindström (1995); Kelly & Wood (2000)
Am. ferrooxidans	DSM10331	Enrichment culture, hot spring run-off, Iceland	Clark & Norris (1996)
F. acidiphilum	BRGM4	Enrichment culture	Golyshina et al. (2000)

Growth media

L. ferriphilum

L. ferriphilum was grown in MAC basal salt solution according to Mackintosh (1978) at 37°C. 4 g/L iron (II) ions were added as substrate. A stock solution of 200 g/L $FeSO_4 \times 7H_2O$ was prepared. To prevent iron (II) oxidation and precipitations the $FeSO_4$ solution was acidified with sulfuric acid to a pH of 1.3.

At. caldus

At. caldus was cultivated in MAC basal salt solution (Mackintosh 1978). Elemental sulfur with a concentration of 5 g/L was used as a substrate. The initial pH of the medium was adjusted to 2.8. Cells were incubated at 45°C.

Am. ferrooxidans and F.acidiphilum

Cells were grown in MAC basal salt solution (Mackintosh, 1978) at 37°C. 4 g/L iron (II) ions were added as substrate. Additionally 0.2 g/L yeast extract was added after autoclaving.

Stock cultures

Cultures were grown in a 100 mL narrow- neck Erlenmeyer flask containing 50 ml growth medium and were incubated at 37°C or 45°C. Cultures were shaken at 120 rpm on a rotary shaker in darkness. Cells were harvested after reaching the stationary phase of growth checked by total cell count determination (using a Thoma counting chamber see 3.3.5).

Pre- and mass-cultures

Pre- or mass- cultures of the respective microorganism were inoculated by 10% (v/v) and grown in 1 L Erlenmeyer flasks or 5 L glass- bottles. 5 L glass-bottles got a supply of filtered pressurized air and were stirred (instead of shaking).

Purity control

Cell morphology was checked by using a light microscope (1000 x magnified, Leica DMLS, Wetzler GmbH). All strains were assayed on Harrison media (Harrison, 1981) for the detection of contaminating acidophilic chemoorganotrophic bacteria. *L. ferriphilum* and *At. caldus* were also checked by extracting DNA and the use of PCR (Janosch, 2013).

Cell harvest

All cultures were harvested via centrifugation (Thermo Scientific, Sorvall RC 6+ centrifuge, rotor F109-6x500Y) for 10 min at 20°C at 8000 rpm (11270 G). Cells were washed to remove iron- or sulfur residuals and resuspended in mineral salt solution.

3.2 Substrate Pyrite

Pyrite (FeS_2) was used as ground particles (50- 100 µm, as shown in Fig. 4) for the attachment and leaching experiments or as coupons (10 x10 mm, shown in Fig. 5) for visualization experiments. Pyrite crystals were roughly crushed by a jaw-crusher (BB 1/A, Retsch) and then ground by a disc-swing-mill (HSM 100M, Herzog, 1988) in the Geology department of the University Duisburg-Essen. Crushed pyrite was wet sieved (Test sieves, Retsch, Germany) and sterilized by boiling in 6 N HCl for 30 min, then washed with deionized water until the

pH was neutral. Afterwards, it was rinsed three times in acetone for the removal of iron (III) ions and sulfur compounds (Schippers et al., 1999; Moses et al., 1987). Finally, it was dried at 60°C and sterilized for 24 h at 120°C under nitrogen atmosphere.

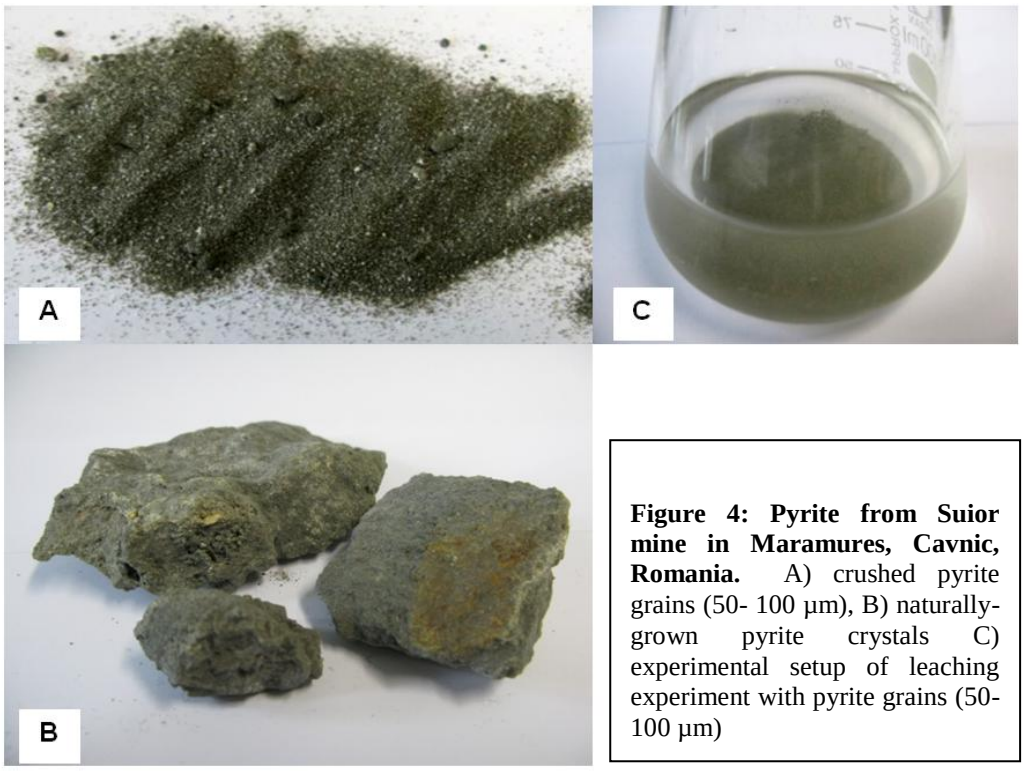

Figure 4: Pyrite from Suior mine in Maramures, Cavnic, Romania. A) crushed pyrite grains (50- 100 µm), B) naturally-grown pyrite crystals C) experimental setup of leaching experiment with pyrite grains (50-100 µm)

Pyrite grains were obtained from the Suior mine in Maramures, Cavnic, Romania, and were analyzed by X-ray fluorescence spectroscopy at the Geology department, Faculty for Biology and Geography, Universität Duisburg – Essen. The composition of pyrite is shown in table 3.

Table 3 Composition of pyrite grains from Suior mine in Maramures, Cavnic, Romania

SYMBOL	CONCENTRATION		ERROR	
Na_2O	< 0,27	%	(0,0)	%
MgO	0,716	%	0,067	%
Al_2O_3	< 0,026	%	(0,0)	%
SiO_2	0,432	%	0,013	%
P_2O_5	< 0,013	%	(0,0)	%
S	17,47	%	0,02	%
K_2O	< 0,012	%	(0,0099)	%
CaO	< 0,014	%	(0,0052)	%
TiO_2	0,03121	%	0,00045	%
V	< 15	µg/g	(6,2)	µg/g
Cr	< 5,0	µg/g	(0,0)	µg/g
MnO	< 0,00039	%	(0,0)	%
Fe_2O_3	35,32	%	0,04	%
Co	< 18	µg/g	(0,0)	µg/g
Ni	30,2	µg/g	2,6	µg/g
Cu	380,2	µg/g	5,1	µg/g
Zn	52,5	µg/g	1,7	µg/g
Ga	< 1,1	µg/g	(0,0)	µg/g
Ge	< 0,9	µg/g	(0,0)	µg/g
As	1083	µg/g	5	µg/g
Se	1,6	µg/g	0,2	µg/g
Rb	< 0,8	µg/g	(0,0)	µg/g
Sr	2,4	µg/g	0,4	µg/g
Y	2,1	µg/g	0,5	µg/g
Zr	4,5	µg/g	0,5	µg/g
Nb	1,9	µg/g	0,5	µg/g
Mo	< 0,7	µg/g	(0,0)	µg/g
Ag	12,8	µg/g	1,1	µg/g
Cd	4,6	µg/g	0,7	µg/g
In	< 0,2	µg/g	(0,0)	µg/g
Sn	3,7	µg/g	0,4	µg/g
Sb	22,5	µg/g	0,6	µg/g
Te	< 1,3	µg/g	(0,0)	µg/g
Cs	< 1,3	µg/g	(0,0)	µg/g
Ba	< 1,8	µg/g	(0,0)	µg/g
La	< 2,4	µg/g	(0,9)	µg/g
Hf	< 0,1	µg/g	(0,0)	µg/g
Ta	< 21	µg/g	(0,0)	µg/g
Hg	< 1,8	µg/g	(0,0)	µg/g
Tl	4,6	µg/g	1,4	µg/g
Pb	97,5	µg/g	2,9	µg/g
Bi	< 3,1	µg/g	(0,0)	µg/g
Th	< 3,0	µg/g	(1,1)	µg/g
U	< 1,9	µg/g	(0,0)	µg/g

Pyrite coupons, which were used for the visualization of attached cells, were sterilized in the same manner as the pyrite grains. Coupons were cut from museum grade pyrite crystals originating from dump Victoria, Navajun/district of Rioja in Spain. A Logitech Model 15 saw

with a diamond cut-off wheel (B 127, ø 127, thickness 0.48, ATM GMBH) was used. Obtained slices were broken to small pieces with a total area of about 0.5 – 1 cm^2.

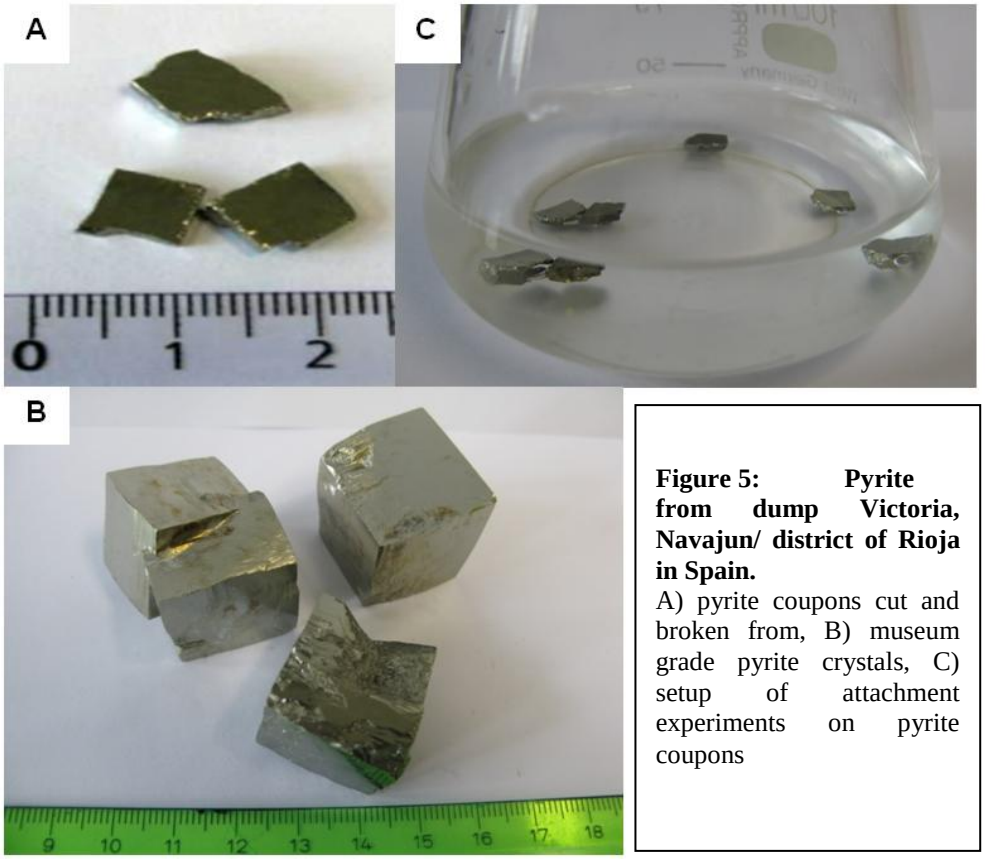

Figure 5: Pyrite from dump Victoria, Navajun/ district of Rioja in Spain.
A) pyrite coupons cut and broken from, B) museum grade pyrite crystals, C) setup of attachment experiments on pyrite coupons

3.3 Part 1: Attachment and Leaching experiments

3.3.1 Attachment experiments

Attachment to pyrite grains

Attachment experiments were done in sterile 100 ml wide-neck Erlenmeyer flasks, each containing 50 mL MAC basal salt solution. An inoculum of $5x10^8$cells/mL for pure cultures or $2.5x10^8$ cells/mL of each culture in mixed consortia was used. First, 1 mL sample was taken (time: 0 min), prior to the addition of 10 g pyrite grains. Afterwards, the second sample was taken (time: 1.5 min). Further sampling was carried out in order of: 10, 20, 30, 60, 90, 120, 240, 300, 360, 420 and 480 min. During the experiment the flasks were shaken (120 rpm) at 37°C. Unspecific attachment to the glass wall was tested in equal experiments without the addition of pyrite. All samples were centrifuged (Biofuge A, Heraeus Sepatech) at 1000 rpm for 5 min to remove mineral residues that might have been taken with the sample. The supernatant was used for planktonic cell counting. Numbers of attached cells were quantified by subtracting the number of planktonic cells from the number of cells inoculated.

Attachment to pyrite coupons

Pyrite coupons were incubated in 100 mL Erlenmeyer flasks containing 50 mL MAC basal salt solution and an inoculum of $1x10^8$ cells/mL. The coupons were removed after 1, 3 and 5 or 7 days. Cells and/or biofilm formation were visualized by Epifluorescence microscopy (EFM) by the use of cell stainings such as FISH (Fluorescent-in-Situ-Hybridization), DAPI (4‘,6-diamidino-2-phenylindol) or lectin (Con A) (see 3.4).

3.3.2 Leaching experiments

Inocula in a range of 1.7×10^8 to 5×10^8 cells/mL of pure and/or mixed cultures were tested in different combinations, (Table 4).

Table 4: Overview and mixing ratios of pure and mixed cultures for leaching experiments on pyrite grains (50- 100 µM)

CULTURE	RATIO	[CELLS/ML]			
		L. f	***At. c***	***Am. f***	***F. a***
Leptospirillum ferriphilum (L. f)	-	$5x10^8$	-	-	-
Acidithiobacillus caldus (A. c)	-	-	$5x10^8$	-	-
Acidimicrobium ferrooxidans (Am.f)	-	-	-	$5x10^8$	-
Ferroplasma acidiphilum (F. a)	-	-	-	-	$5x10^8$
L. f + At. c	1:1	$2.5x10^8$	$2.5x10^8$	-	-
L. f + Am. f	1:1	$2.5x10^8$	-	$2.5x10^8$	-
L. f + F. a	1:1	$2.5x10^8$	-	-	$2.5x10^8$
At. c + Am. f	1:1	-	$2.5x10^8$	$2.5x10^8$	-
At. c + F. a	1:1	-	$2.5x10^8$	-	$2.5x10^8$
Am. f + F. a	1:1	-	-	$2.5x10^8$	$2.5x10^8$
L. f + At. c +Am. f	1:1:1	$1.7x10^8$	$1.7x10^8$	$1.7x10^8$	-
L. f + At. c +F. a	1:1:1	$1.7x10^8$	$1.7x10^8$	-	$1.7x10^8$

Cells were incubated in 300 mL wide-neck Erlenmeyer flasks containing 150 mL MAC basal salt solution and 3 g Pyrite at 37°C-45°C in the dark with shaking (120 rpm) over a period of 3 weeks. Every second day 2 samples of 1 mL were taken. After pH was determined, samples were stored in the freezer at -20°C for the determination of sulfur- and iron- species.

3.3.3 Precolonization experiments

Pyrite coupons

Precolonization experiments were done as described in “attachment of pyrite coupons” except that coupons were removed after 4 h and directly added in an Erlenmeyer flask containing another leaching species. This allows to test, which bacterium needs precolonization of the pyrite surface by another species for its own attachment (Fig. 6).

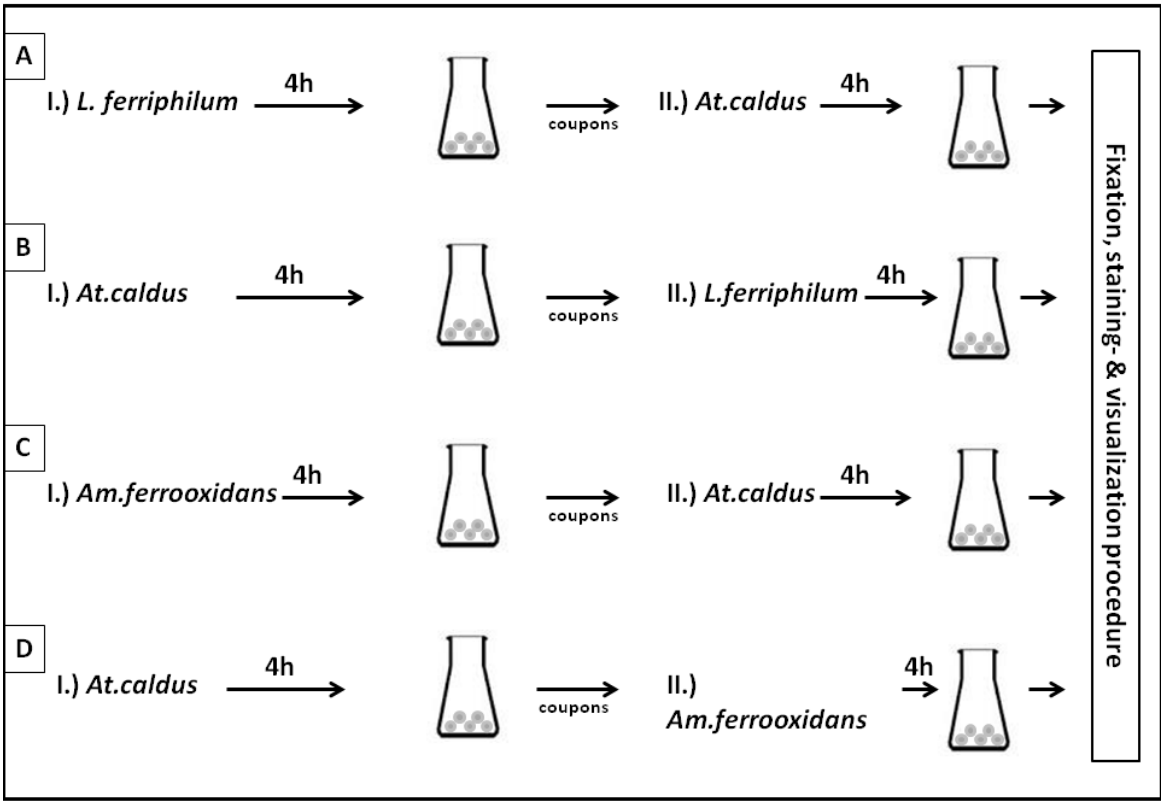

Figure 6: Experimental setup of precolonization experiments with pyrite coupons. Pyrite coupons were first incubated for 4 h at 37°C in culture I. and afterwards for 4h in cultures II. The concentration of bacteria in I & II was $1x10^8$ cells/mL; after 8 h cells were fixed on coupons, hybridized by FISH, stained with DAPI (3.3.2) and visualized by EFM (3.4.6).

Different incubation times for both microorganisms were tested (2, 4, 8, 10, 12, 24 and 48 h) to find the most suitable one. For every run also an 8h control of pure cultures was done for determination of total cells attached to the mineral.

Pyrite grains

Precolonized pyrite grains were used for attachment experiments of *At. caldus* cells to clarify if attachment is depending on the presence of live *L. ferriphilum* or *Am. ferrooxidans* cells, or if the presence of their EPS was sufficient to influence the attachment of *At. caldus*.

Therefore, attachment experiments were done in sterile 100 ml wide-neck Erlenmeyer flasks containing 50 ml MAC basal salt solution. Cells of *L. ferriphilum* or *Am. ferrooxidans* ($1x10^8$ cells/mL) were inoculated with 1g pyrite grains (50- 100 µm) for 2 days at 37°C. Afterwards, a) attached cells were inactivated by heat-drying at 80°C for 2 h and washed twice with MAC basal salt solution or b) cells were directly washed twice with MAC basal salt solution to remove planktonic cells and were used for further experiments (figure 7). Later, cells of *At.*

caldus ($1x10^8$ cells/mL) were added to the precolonized pyrite grains with dead or living precolonized cells in 50 mL mineral salt solution and were incubated for 3 days at 37°C. Daily, 1mL samples were taken and used for total cell count determination (see chapter 3.3.4). Furthermore, a control with pyrite-grains and addition of *At. caldus* cells, 1 mM $Na_2S_2O_3$ and iron (III) ions was carried out under the same experimental conditions.

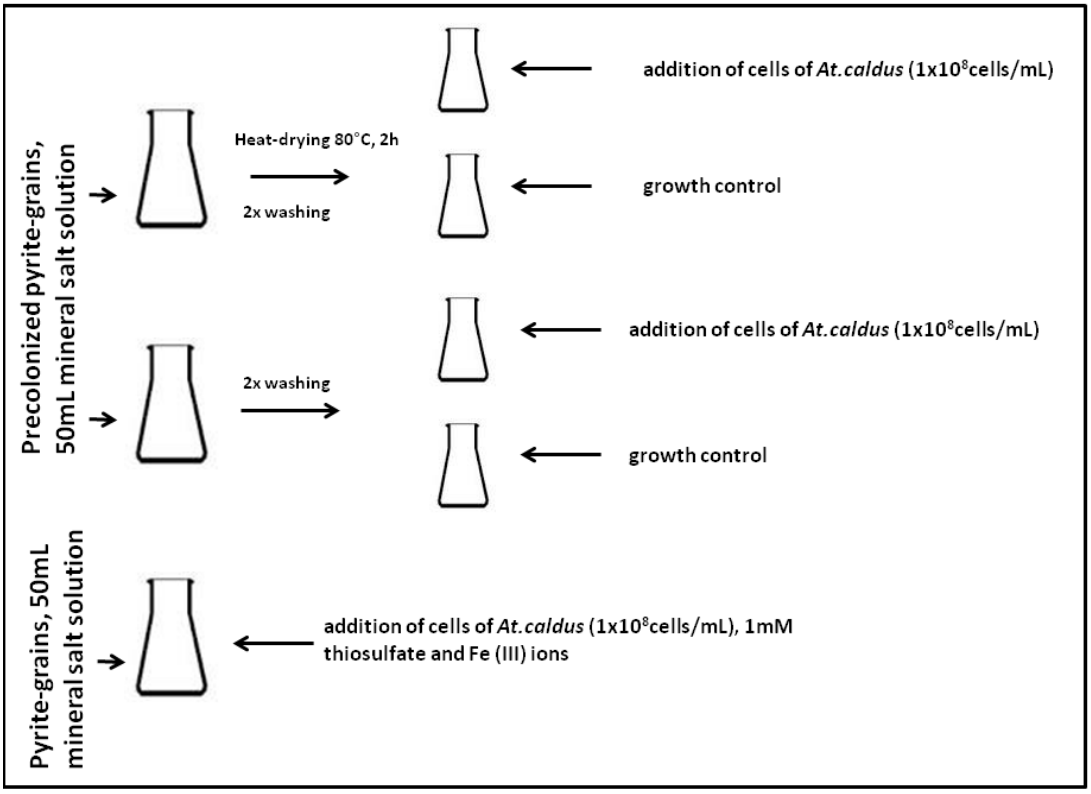

Figure 7: Experimental setup for precolonization experiments with pyrite grains (50- 100 µm). Precolonized pyrite grains (with *L. ferriphilum* or *Am. ferrooxidans*) were washed or killed by heat-drying (80°C, 2 h) for further attachment experiments of *At. caldus* ($1x10^8$ cells/mL), incubation at 37°C for 3days.

Growth controls were done to test whether cells were inactivated after heat-drying. Therefore, precolonized pyrite grains were inoculated in fresh basal salt solution with or without addition of Fe (II) ions. Cell growth was checked by TTC determination.

3.3.4 Analytical procedures

Total cell count determination (TTC)

The planktonic cell number per mL was determined using a Thoma counting chamber (depth = 0.1 mm, area per small square = 0.0025 m^2, Assistant, Germany) and a light microscope (Leica DMLS, Wetzlar GmbH) in phase contrast mode with 40 0x magnification.
Samples were diluted, if cell numbers were higher than 100 cells per counting-field with MAC mineral salt solution. The number of counted cells was multiplied by the dilution factor.

pH determination

The pH was measured with a digital pH meter (Model pH 537, WTW, in Lab® 422 Combination Semi-micro pH Electrode, Mettler Toledo).

Iron ions determination

Iron ion determination was performed according to the German standard methods for the examination of water, waste water and sludge; cations; determination of iron (DIN 38406-1) with the "Phenanthrolin test". Iron (II) ions and 1,10-Phenanthrolin build a red color complex, which can be determined spectrophotometrically at 492 nm. Upon addition of hydroxylamine iron (III) ions are reduced to iron (II) ions, whereby the total iron ion concentration was determined. As calibration a standard iron (II)-sulfate- heptahydrate solution in a range between 0.8 – 4 mg/L was used. Every sample was measured in triplicate using a UV-VIS spectrophotometer (Cary 50, Varian INC.) equipped with software ADL Shell (modified by F. Leon-Morales).

Sulfate and thiosulfate determination

The sulfate and thiosulfate concentrations were determined by the use of ion exchange chromatography (IC). For this purpose, the Dionex DX500 system was used in combination with an eluent generator (EG 50), a conductivity detector (CD 20) and an autosampler (AS 3500). The whole system was controlled using the chromatographic software Chromeleon Version 6.7. As stationary phase an analytical column with anion exchange resin IonPac AS17 (2 x 250 mm, Dionex USA) and a guard column IonPac AG17 (50 mm, Dionex, USA)) were used.

A flow rate of 0.5 mL/min and a current for the suppressor (SRS current) of 50 mA were applied for the measurements. The injection volume of the autosampler was 10 µL and potassium hydroxide was used as eluent as previously described (Thyssen, 2008). The frozen samples were defrosted and centrifuged at 10000 rpm (Heraeus Biofuge A) for 10 min to remove cells and particles. The supernatant was diluted 1:100 with ultrapure water and phosphate buffer (5 mM, pH= 7) to dilute iron ions. Samples were measured in duplicate.

3.4 Visualization techniques

Different microscopic visualization procedures were used to visualize cell attachment and biofilm formation. Fluorescently labelled bacteria were visualized by Epi-fluorescence microscopy (EFM). Confocal-Laser-Scanning-Micoscopy (CLSM) was also used for the three-dimensional visualization of biofilms with the combination of Syto^{9}- and lectin- staining (ConA). Fluorescence *in situ* hybridization (FISH) was performed for the identification of different bacterial strains and Live/Dead®- Kit for testing bacterial viability.

3.4.1 4', 6-diamino-2-phenylindole (DAPI)- staining

DAPI is a fluorescence dye and stains all DNA- and RNA- containing microorganisms, including algae, protozoa and nucleic-acid containing cell structures (Porter and Feig, 1980). Therefore, it can be used for total cell counts and as counterstaining for FISH (Amann et al. 1995, Amann et al. 2001). In this study DAPI (Sigma Aldrich) was prepared at 0.01% (w/v) and dissolved in 2% formaldehyde. Pyrite coupon-attached cells were washed with phosphate buffer (PBS: NaCl 4 g; KCl 0.1 g; Na_2HPO_4 x $2H_2O$ 0.72 g; KH_2PO_4 0.1 g filled up to 1000 mL with distilled water) to get pH 7. Afterwards, coupons were washed with particle-free water to eliminate particles. Coupons were covered completely with DAPI solution for 2 min and washed again with particle-free water for the removal of crystals and precipitates. Samples were dehydrated with ethanol and air-dried.

3.4.2 Fluorescent-in-Situ-Hybridization FISH

FISH is a powerful method for specific identification and quantification of cells in their natural environment (Amann et al., 2001). A fluorescent specific ribosomal-RNA (rRNA) targeted oligonucleotide probe is used for the identification and quantification of the microorganism (Gonzalez-Toril et al., 2006). The oligonucleotide is complementary to a

certain region within the RNA of the target organism. Most frequently r-RNA is used as target molecule.

FISH probes used in this study were purchased from Operon, Biotechnologies, Germany (Table 5). Experiments were carried out as described by Gonzalez-Toril et al. (2006) and Florian (2012). The probe specificity was checked by hybridization with a reference system containing the target sequence (positive control) and with microorganisms from collections with sequences having some mismatch with the probe (negative control).

Table 5: Details about fluorescent-labeled oligonucleotide probes (25 nM)

NAME	SPECIFICITY	SEQUENCE
LEP 634	*Leptospirillum*	[TAMRA]AGTCTCCCAGTCTCCTTG
Caldus_liu	*Acidithiobacillus caldus*	[Cy3]TTGGCGCCTTAGGTGCTGA
ACD638	*Acidimicrobium*	[FI]CTCAAGACAACACGTCTC
FER656	*Ferroplasma*	[Cy3]CGTTTAACCTCACCGATC
EUB 338	Eubacteria	[Cy3]GCTGCCTCCCGTAGGAGT
Arch915	Archeae	[Cy3]GTGCTCCCCCGCCAATTCCT
NON 338	*Negative control*	*[Cy3]ACTCCTACGGGAGGCAGC*

Fixation

Cells were fixed directly on pyrite coupons as described (Gonzalez-Toril et al., 2006) using a final concentration of 4% (v/v) formaldehyde in acidified water (pH= 2) for 30 min at 4°C. Afterwards, they were washed twice in mineral salt solution and twice in PBS (1x) to neutralize the sample. Then drying steps in ethanol (50% → 75% → 100%) were used, each for 1 min. Samples were stored at -20°C until visualization.

Hybridization

Hybridization buffer (NaCl 0.9 M, $NaC1_2H_{25}SO_4$ 0.01%, Tris-HCl 20 mM, formamide 35% was prepared freshly. Samples were covered with 20 µL hybridization buffer and mixed with 4 µL of probe.

Samples were placed in a 50 mL falcon tube. A slip of paper was put in the tube soaked with 1 mL of hybridization buffer to get a humidity saturated atmosphere. Samples were hybridized in horizontal position for 1.5 h at 45.5°C in a hybridization oven in the dark. 50mL washing buffer (NaCl 0.9 M, $NaC1_2H_{25}SO_4$ 0.01%, Tris-HCl 20 mM) were prepared and preheated at 45.5°C.

After hybridization, samples were covered with washing buffer for 15 min at 37°C in darkness. Then they were rinsed with PBS (1x) and air dried. Afterwards, samples were counterstained with DAPI (0.01% (w/v) in formaldehyde) for 1 min and rinsed with PBS. After air drying, samples were visualized by EFM (AxioImager A1m, Zeiss, Germany).

3.4.3 Lectin staining

Lectins are sugar-binding proteins and occur e.g. in bacteria, viruses and plants. They are highly specific for their sugar moieties (Doyle 1994). As previously described, carbohydrates are a main part of EPS. Therefore, fluorescently-labeled lectins were used to stain parts of the EPS. Lectin staining has already been tested for moderately thermophilic leaching bacteria attached to pyrite (Holuscha, 2010). Especially Concanavalin A (ConA) has been used in biofilm studies of *At. ferrooxidans* (Bellenberg et al., 2012). Con A originates from *Canavalin ensiformis* and has an affinity for α-D-mannosyl and α-D-glucosyl residues branched α-mannosidic structures (Marchalonis, 1987).

ConA conjugated with tetramethylrhodamine-5-isothiocyanate (TRITC) (Invitrogen) was used. Stock solutions were prepared to a final concentration of 1 mg/mL in 10 mM PBS (pH 7.5). Aliquots were stored at -20°C in the dark until use. Particle-free deionized water was used to prepare a working solution of 50 µg/mL and stored in darkness at 4°C less than two weeks. Coupons were pretreated as described in section 3.4.1. Instead of DAPI samples were covered by ConA working solution and incubated for 20 min in darkness. Afterwards, coupons were rinsed carefully with particle-free water to remove unbound lectin. Syto^{9} staining followed (Gonzalez et al. 2012).

3.4.4 Biofilm formation on “Floating-filters”

Sterile polycarbonate filters (GTTB, Ø 2.5 cm, 0.22 µm pore size, Millipore®) were inoculated by filtration of 10^{6}-10^{7} cells/mL thiosulfate- or iron-grown cells as described (de Bruyn et al. 1990). Inoculated filters were immediately transferred to Erlenmeyer flasks containing 50 mL basal salt solution and 2 g/L iron (II) ions or 5 g/L thiosulfate and incubated by floating at 37-45°C without agitation (Gonzalez et al. 2012). Liquid samples for iron- or sulfate/thiosulfate determination were taken on the first and last day of the experiment to follow the substrate metabolism (see 3.3.4).

3.4.5 Epifluorescence microscopy (EFM)

Visualization of fluorescently stained cells on pyrite coupons was carried out using an epifluorescence microscope (Zeiss® AxioImager™ A1m) equipped with a HBO 100 mercury vapor lamp using Zeiss® filterset 49, air-objectives (Zeiss® EC plan NEOFLUAR 440480/9903 and EC epiplan NEOFLUAR 422393/9900). The software AxioVision 4 and a digital microscope camera (Zeiss® AxioCam™MRm) were used.

3.4.6 Confocal-Laser-Scanning-Microscopy (CLSM)

A laser scanning module (LSM 510 Carl Zeiss® Jena) coupled to an inverted Axiovert 100 MBP microscope (Zeiss®) was used for imaging of biofilms. All images were done with the plan-apochromatic 100 x 0.79 oil DIC objective. The microscope was operated with LSM 510 Release 3.2 software (Zeiss®). Basic visualization and image manipulation was performed using the program ImageJ (http://rsbweb.nih.gov./ij/).

3.5 PartII: Effect of N-acetyl-homoserine-lactones (AHLs) and other signal molecules

AHLs used in this study were purchased from The University of Nottingham Quorum Sensing Research Group (http://www.nottingham.ac.uk/quorum/index.htm) and from Sigma. Stock solutions of 50 mM of different AHLs were dissolved in DMSO and stored at -20°C.
The furanone (5Z)-4-bromo-5-(bromomethylene)2(5H)-furanone was purchased from Sigma. A stock solution of 50 mM was prepared with ethanol and stored at -20°C until use.

3.5.1 Effect of AHLs

Effects of the external addition of AHLs (C6-, C8-,3-oxo-C8-, 3-hydroxy-C8, C10-, 3-oxo-C10-, 3-hydroxy-C10-, C12-, 3-oxo-C12-, 3-hydroxy-C12-, C14-, 3-oxo-C14-, 3-hydroxy-C14-, 3-oxo-C16, C18-AHL) were tested with cells of *L. ferriphilum* or *At. caldus* and in mixed cultures of both. Pyrite- leaching experiments as well as attachment to pyrite coupons were tested. Also biofilm formation experiments on floating filters were done, when previous experiments showed an inhibiting or enhancing effect.

Leaching experiments

Leaching experiments were carried out as described in 3.3.2. MAC basal salt solution (100 mL) was used with 3 g pyrite grains (50- 100 µm particle size) and $1x10^8$ cells/mL. AHLs were added in a concentration of 5 µM. Samples were taken periodically. For the determination of leaching efficiency, iron (II)- and iron (III)- ions were measured by the phenanthroline method (described in 3.3.4).

Attachment experiments

Pyrite coupons were pretreated and experiments were done as described (Gonzalez et al. 2012). $1x10^8$ cells/mL were incubated at 37°C and shaken at 120 rpm. Attachment and biofilm formation was visualized by EFM with a combined DAPI- and lectin- staining.

Floating filter experiments

Floating filter experiments were carried out as previously described (see 3.4.5.) 10^6- 10^7 cells/mL were filtered and incubated at 37°C or 45°C depending on the microrganism. AHLs were added in concentrations between 0.2 and 5 µM. Biofilm formation was visualized by CLSM with a combined Syto^9- and lectin- staining. Additionally, samples for iron determination were taken to follow the bacterial oxidation of their substrate.

3.5.2. Extraction of AHLs

AHLs were extracted as previously described (Farah et al., 2005). Briefly, cells of *L. ferriphilum* and *At. caldus* were harvested by centrifugation and the supernatants were extracted three-times with 1 volume of dichloromethane. Afterwards, three drying steps by addition of anhydrous $MgSO_4$, rota-evaporation and final drying by flushing with N_2-gas were done. Extracts were stored at -20°C. Extracts were dissolved in dimethylsulfoxide (DMSO) before use.

3.5.3 Identification of AHLs

Thin-layer-liquid-chromatography (TLC)

An AHL biosensor assay was used to detect the presence of AHLs in extracts of leaching bacteria (Shaw et al. 1997; Farah et al. 2005). *C.violaceum* and *A. tumefaciens* were used as reporter strains (Table 6) and extracts were tested as described with analytical TLC (Shaw et al. 1997). Biosensor strains were spread over the TLC plate. A positive signal was recorded via the ß-galactosidase reaction or violacein production, when AHLs were present. The 3-oxo-AHLs give tear-shaped spots, whereas the alkanoyl- and hydroxyl-AHLs produce circles (Steindler & Venturi 2007). This method leads to a rapid and visible result of the presence or absence of AHLs in the extracts of leaching bacteria. However, to characterize AHLs clearly, more accurate methods such as mass spectroscopy or magnetic resonance spectroscopy (NMR) should be used.

Table 6: Information of biosensors used in this study

ORGANISM	TYPE	TYPE OF REACTION	REFERENCE
A. tumefaciens	NTL4 (pCF218)(pCF372)	ß-galactosidase reaction	Fuqua und Winans, 1996
C. violaceum	Wild type, DSM 30191	Violacein production	Kimmel and Maier, 1969

Gas chromatography coupled with mass-spectrometry (GC-MS)

For determination of possible AHLs in extracts of leaching bacteria and their exact chemical structure gas chromatography was carried out using a Trace GC (Thermo Quest) with a RTX-440 column (Restek; 30 m length, 0.25 mm inner diameter and 0.25 µm film thickness). Extracts were dissolved in methanol or ethyl-acetate and 1µL was injected without split (injector temperature = 200°C, flow 2 mL/min; oven temperature = 150°C for 1 min followed by 15°C/ min steps till 330°C were reached, 5 min holding time). The mass spectrometer PolarisQ (Finnigan) was used with an interface at 250°C and samples were scanned in an area from m/z = 57-300.

3.5.4 Effect of furanone

The effect of a furanone addition was tested on cells of *L. ferriphilum, At. caldus,* and *Am. ferrooxidans*. At first growth experiments followed by leaching experiments (grains) were performed as described in 3.3.1 and 3.3.2. Afterwards, attachment to pyrite coupons was also tested. Floating filter experiments were carried out to determine the effect of these compounds on biofilm formation. Experiments were carried out as described for AHLs (see 3.5.2.) with concentrations between 0.2-5 µM of furanone.

3.6 Part III: Preliminary studies on the identification of an unknown cell-cell signaling molecule produced by cells of *L. ferriphilum*

3.6.1 Extraction procedures

Cell free supernatants from iron- and pyrite- grown cultures of *L. ferriphilum* between 1 -12 weeks were used. Extraction was done with dichloromethane as described in 3.5.3. Dry extracts were stored at -20°C.

3.6.2 Inhibition experiments

Solubility tests

Different types of solvents (acetonitrile, chloroform, DMSO, hexane, isooctane and methanol) were tested to solve the "*L. ferriphilum*-extracts" in order to test their effect in growth experiments of *L. ferriphilum*. Therefore, the dried powder-extract was solved in 100 µl of each solvent and added to 50 mL MAC basal salt solution including 4 g/L ferrous iron and a fresh inoculum of *L. ferriphilum*. Daily, a 1 mL sample was taken and the iron species were determined.

The extracts were also tested with cultures of other acidophilic leaching bacteria and cultures of *Escherichia coli* or *Bacillus subtilis* (table 7). The latter two bacteria were used for reference purposes.

Table 7: Overview of experimental conditions and microorganisms tested with the unknown QS-substance from *L. ferriphilum*

ORGANISM	STRAIN	INCUBATION TEMPERATURE	GROWTH MEDIUM	DETECTION OF EFFECT ON MICROBIAL GROWTH OR ACTIVITY
At.caldus	DSM 8584	45°C	MAC basal salt sol., 5g/L elemental sulfur	Sulfate see 3.3.4
Am. ferrooxidans	DSM 10331	37°C	MAC basal salt sol., 4g/L ferrous iron	Iron ions see 3.3.4
S. thermosulfidooxidans	DSM 9293	45°C	MAC basal salt sol., 4g/L ferrous iron	Iron ions see 3.3.4
S. thermosulfidooxidans	DSM 9293	45°C	MAC basal salt sol., 5g/L elemental sulfur	Sulfate see 3.3.4
At. ferrooxidans	ATTC 23270	28°C	MAC basal salt sol., 4g/L ferrous iron	Iron ions see 3.3.4
At. ferrooxidans	ATTC 23270	28°C	MAC basal salt sol., 5g/L elemental sulfur	Sulfate see 3.3.4
At. ferrivorans	CF 27	28°C	MAC basal salt sol., 4g/L ferrous iron	Iron ions see 3.3.4
L. ferrooxidans	L3, R31	28°C	MAC basal salt sol., 4g/L ferrous iron	Iron ions see 3.3.4
L. ferrooxidans	L4, R32	28°C	MAC basal salt sol., 4g/L ferrous iron	Iron ions see 3.3.4
L. ferrooxidans	L7, S53	28°C	MAC basal salt sol., 4g/L ferrous iron	Iron ions see 3.3.4
E. coli	K12, DH5α	37°C	standard I solution (Merck)	OD- measurement
B. subtilis	DSM23778	37°C	standard I solution (Merck)	OD- measurement

Substrate conversion was followed during the experiments. Depending on the type of microorganism iron ions- or sulfur- determination or OD- measurements using a cell density meter (model 40 Fisher Scientific) were done.

Floating filter experiments

Floating filter experiments were done as previously described (see 3.4.5.) Cells of *L.ferriphilum* (10^7 cells/mL) were filtered and incubated at 37°C without agitation. The dried powder extract from *L. ferrooxidans* supernatant was solved in DMSO and 10- 100 µL were added to the floating filter assay. Biofilm formation was visualized by CLSM and a combined Syto^9- and lectin- staining. Additionally, samples for iron determination were taken to follow the substrate conversion of bacteria.

3.6.3 Biosensor tests

Among the biosensor tests, which were described for the detection of AHLs (3.5.4), a biosensor test based on *Janthinobacterium* sp. HH01 was used to see if the addition of the "*L.ferriphilum*-extract" had any effect on its violacein production. The genome of HH01 apparently lacks the AHL-dependent signaling system. Instead it encodes a homologue of the *Legionella*- and *Vibrio*-like autoinducer (*lqsA/cqsA*) synthase gene (Hornung et al. 2013).
5mL of R2A medium supplemented with ampicillin were inoculated with 1% of the HH01 biosensor. The dried powder from "*L. ferriphilum*–extracts" was dissolved in 400 µl DMSO and added in different dilutions (1:10, 1:1000) to the biosensor culture. Incubation was done at 22°C for 48 h. The violacein production was measured as described (Hornung et al. 2013).

4. Results

Part I:

The first part of this study deals with attachment of pure and mixed cultures to pyrite. Furthermore, leaching activities and biofilm formation on pyrite were investigated. *L. ferriphilum*, *At. caldus*, *Am. ferrooxidans* and *F. acidiphilum* were chosen for attachment and leaching experiments and also their interactions in mixed cultures were investigated.

4.1 Attachment experiments to pyrite grains

A standardized attachment test (Kock, 2003; Stein, 2004, Noël, 2008; Florian, 2012) was used to quantify attachment of planktonic cells to pyrite grains. The percental attachment after 8h in pure cultures (Figure 8) indicated that *L. ferriphilum* reached the highest attachment with around 80% followed by *Am. ferrooxidans* with 60%. *F. acidiphilum* exhibited an attachment of 50%, whereas *At. caldus* showed the lowest attachment of 10%.

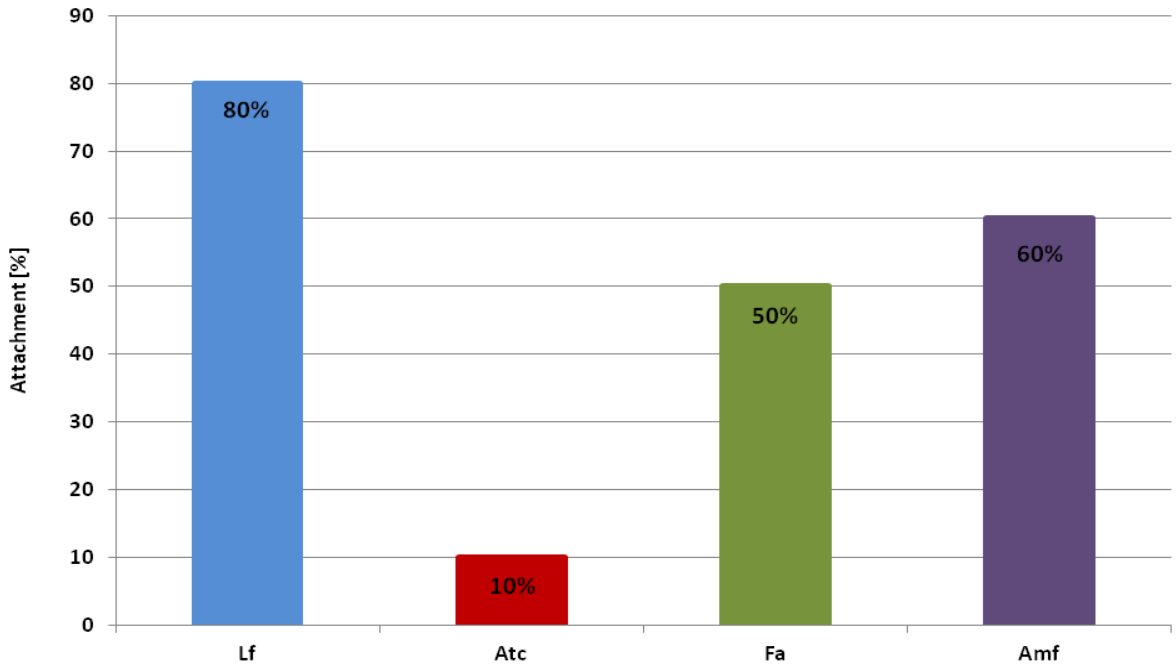

Figure 8: Attachment of pure cultures of moderately thermophilic leaching organisms to pyrite grains after 8h incubation.
Percental attachment of *L. ferriphilum* (L.f= 80%), *At. caldus* (At.c= 10%), *F. acidiphilum* (F.a= 50%) and *Am. ferrooxidans* (Am.f= 60%) in pure cultures ($1x10^8$ cells/mL) to pyrite grains (10 g; 50- 100 µm) at 37°C under shaking (120 rpm) in 50 mL MAC basal salt solution. Standard error (n= 5): *L.f*= 10%; *At.c*= 5%; *Am.f*= 10%; *F.a*= 11%.

The results of the attachment experiments in mixed cultures are shown in Figure 9. Attachment of individual strains in mixed cultures was different from the attachment of each strain in pure cultures. For the mixture of *At. caldus* and *F. acidiphilum* the calculated theoretical attachment was equal compared with the experimental one (30%). The highest attachment with 60% was achieved by the mixture of *L. ferriphilum* and *At. caldus.* Given the values for the pure cultures an attachment of 45% should have resulted (80% *L.ferriphilum* plus 10% *At.caldus* divided by 2).

Mixtures of *At. caldus* and *Am. ferrooxidans* exhibited also a slight increase of attached cells (40%) compared to the calculated value (35%), however this may not be significant due to the deviations. All other assays resulted in decreased attachment compared to the calculated values.

Controls without the addition of pyrite indicated unspecific attachment to glass at around 5 % for all pure and mixed cultures (data not shown). Cell division experiments showed that the bacteria had a generation time of more than 8h. Thus, cell division in between attachment experiments can be neglected.

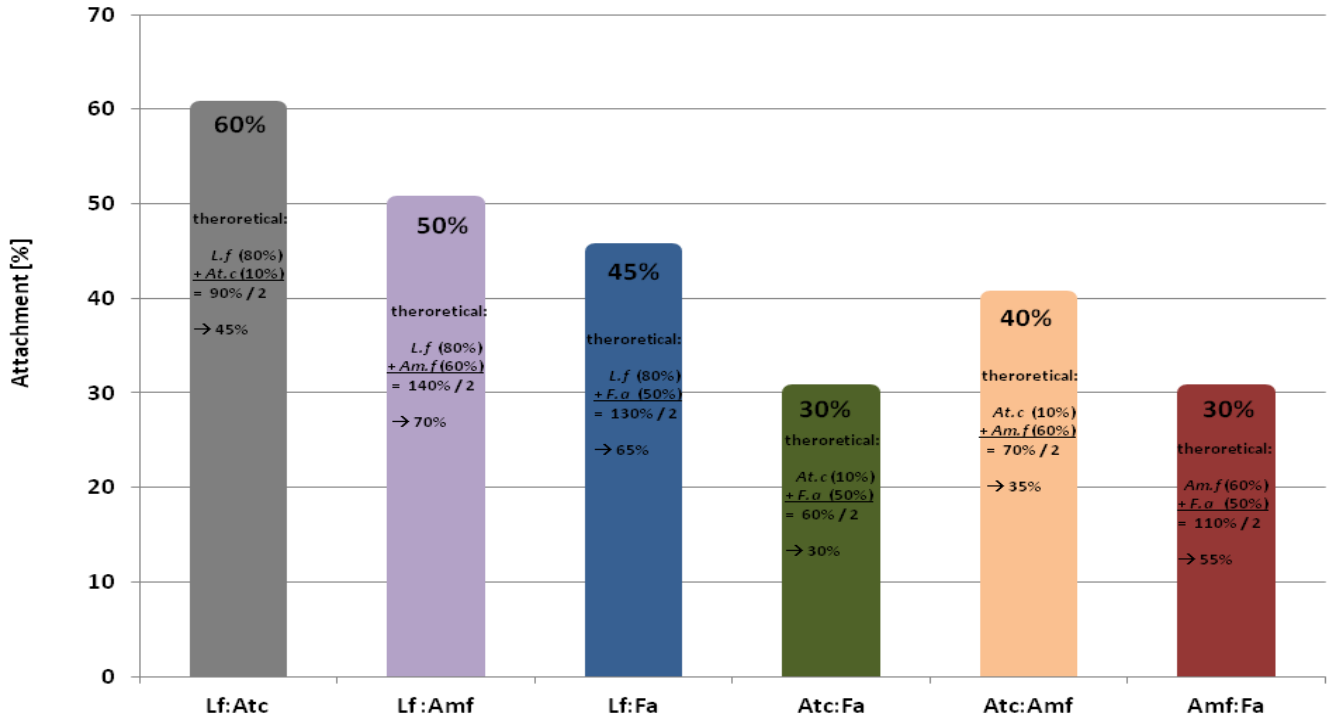

Figure 9: Attachment of mixed cultures of moderately thermophilic leaching organisms to pyrite grains after 8h incubation.
Percental attachment of mixed cultures of *L. ferriphilum*, *At. caldus* , *F. acidiphilum* and *Am. ferrooxidans*. The total initial cell number was $5x10^8$ cells/mL (each strain inoculated at 50%, $2.5x10^8$ cells/mL, respectively); pyrite grains (10 g; 50- 100 µm); incubation at 37°C under shaking (120 rpm) in 50 mL MAC basal salt solution. Bars represent percential attachment of cells in mixed cultures. Theoretical values for attachment in mixed cultures were calculated by the addition of percential attachment of each member in pure cultures divided by the number of strains.
Standard error (n= 5): *L.f*+*At.c*= 5%; *L.f*+*Am.f*= 12%; *At.c*+*Am.f*= 7%; *L.f*+*F.a*= 10%; *At.c*+*F.a*= 8%.

4.2 Leaching experiments with pyrite grains

Besides the attachment tests, standardized leaching experiments were carried out to quantify the oxidation activities of *L. ferriphilum*, *At. caldus*, *Am. ferrooxidans* and *F. acidiphilum* for pyrite. Different combinations of pure and mixed cultures were tested. Cells were incubated for 3 weeks at 37°C. The dissolution of pyrite was monitored by an increase of iron (III) ions which accumulate in the bulk solution as a result of the oxidative attack on pyrite.

Results for the leaching of pyrite grains with pure cultures are shown in Figure 10. Cultures of *L. ferriphilum* reached with 3800 mg/L the highest iron (III) ion concentration after 3 weeks followed by cultures of *Am. ferrooxidans* with 2300 mg/L. Reduced amounts of iron (III) ions were detected for *F. acidiphilum* with 30 mg/L and *At. caldus* with 5 mg/L. Furthermore,

abiotic controls were carried out yielding an iron (III) ion concentration below 5 mg/L (data not shown).

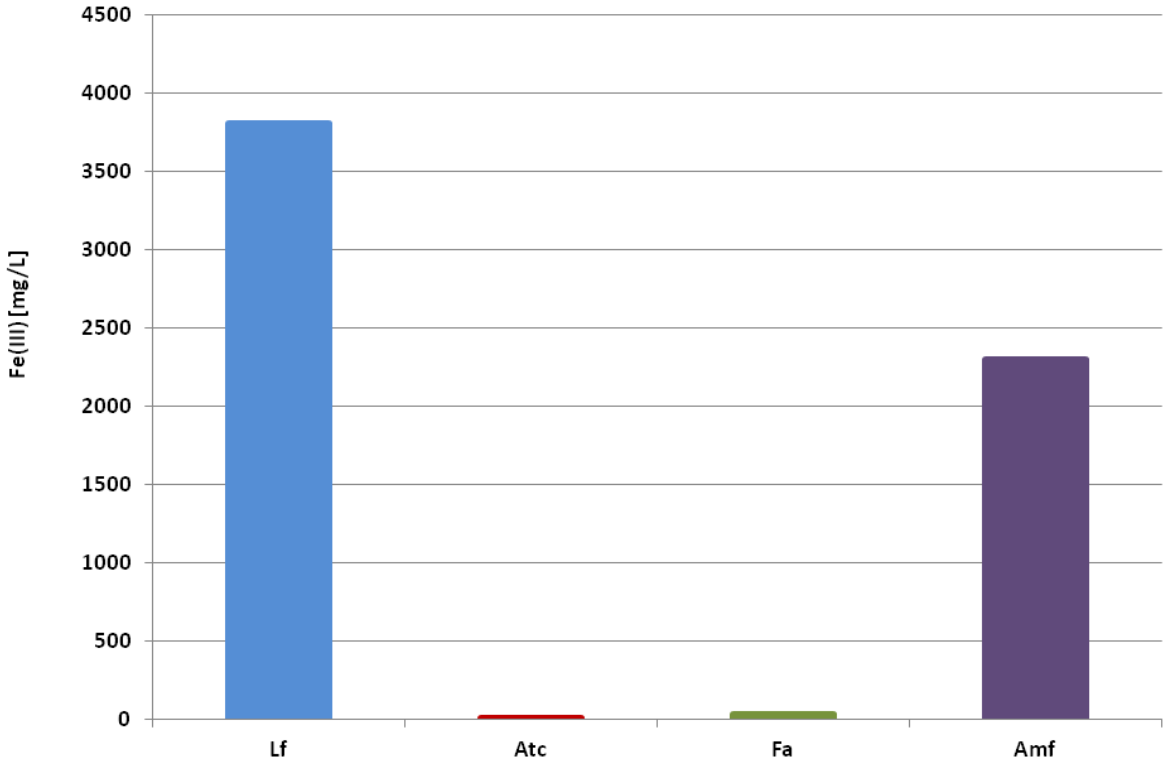

Figure 10: Leaching of pyrite grains by pure cultures of moderately thermophilic microorganisms.
Leaching of pyrite grains characterized by iron (III) ion concentration in [mg/L]. Pure cultures ($5x10^8$ cells/mL) were incubated with pyrite grains (4.5 g; 50- 100 µm) at 37°C under shaking (120 rpm) in 150 mL MAC basal salt solution. Standard error (n= 3) less than 8%.

Results for pyrite leaching in mixed cultures as shown in Figure 11 indicated that the oxidation activity was increased in every combination except for the mixtures of *L. ferriphilum* with *Am. ferrooxidans* and for *At. caldus* with *F. acidiphilum*. The best leaching results were determined for mixtures of *L. ferriphilum* with *At. caldus* (4050 mg/L iron (III) ions) and for the combination of *L. ferriphilum*, *At. caldus* and *F. acidiphilum* (3950 mg/L iron (III) ions). Mixtures of *L. ferriphilum* and *Am. ferrooxidans* dissolved less pyrite than the pure culture of *L. ferriphilum* alone, but were similar to the *Am. ferrooxidans* result. *At. caldus* and *F. acidiphilum* produced values, which are in agreement with their pure culture data.

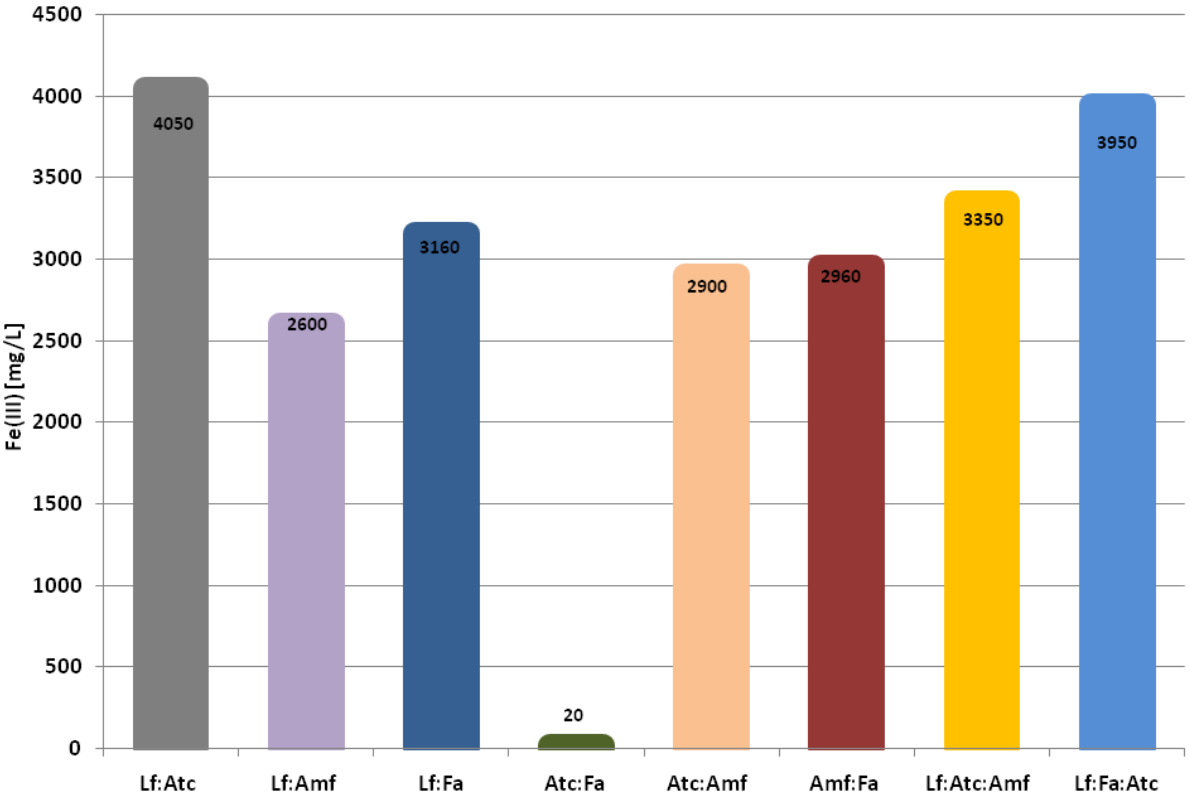

Figure 11: Leaching of pyrite grains by mixed cultures of moderately thermophilic leaching microorganisms.
Leaching of pyrite grains characterized by iron (III) ion concentration in [mg/L]. Initial cell number $5x10^8$ cells/mL (two strains= each strain incubated with 50%, ($2.5x10^8$ cells/mL), respectively; three strains= each strain incubated with 33%, ($1.7x10^8$ cells/mL), respectively); pyrite grains (4.5 g; 50- 100 µm); incubation at 37°C under shaking (120 rpm) in 150 mL MAC basal salt solution. Standard error (n= 3) less than 10%.

4.3 Attachment to and biofilm formation on pyrite coupons

Attachment to and biofilm formation on pyrite coupons was investigated in pure and mixed cultures of *L. ferriphilum*, *At. caldus* and *Am. ferrooxidans*. Pyrite coupons were incubated for 3 and 7 days at 37°C with shaking. Afterwards, attached cells were visualized by DAPI (TTC) and parts of the EPS were stained by the lectin ConA (Figure 12). For each organism three runs were done and sampling was carried out in duplicate.

The highest attachment to pyrite coupons was determined for cultures of *L. ferriphilum* (B1+B3) followed by *Am. ferrooxidans* (C1+C3). *At. caldus* showed nearly no attachment after 3 days of incubation (A1), but a few attached cells could be detected after 7 days (A3). The strongest lectin signal was detected with cultures of *L. ferriphilum* (B2+B4). Signals of DAPI stained cells and the lectin signal were difficult to detect on coupons colonized by

L. ferriphilum after 3 days of incubation (B3+B4). A high amount of cells and a strong lectin signal complicated the imaging.

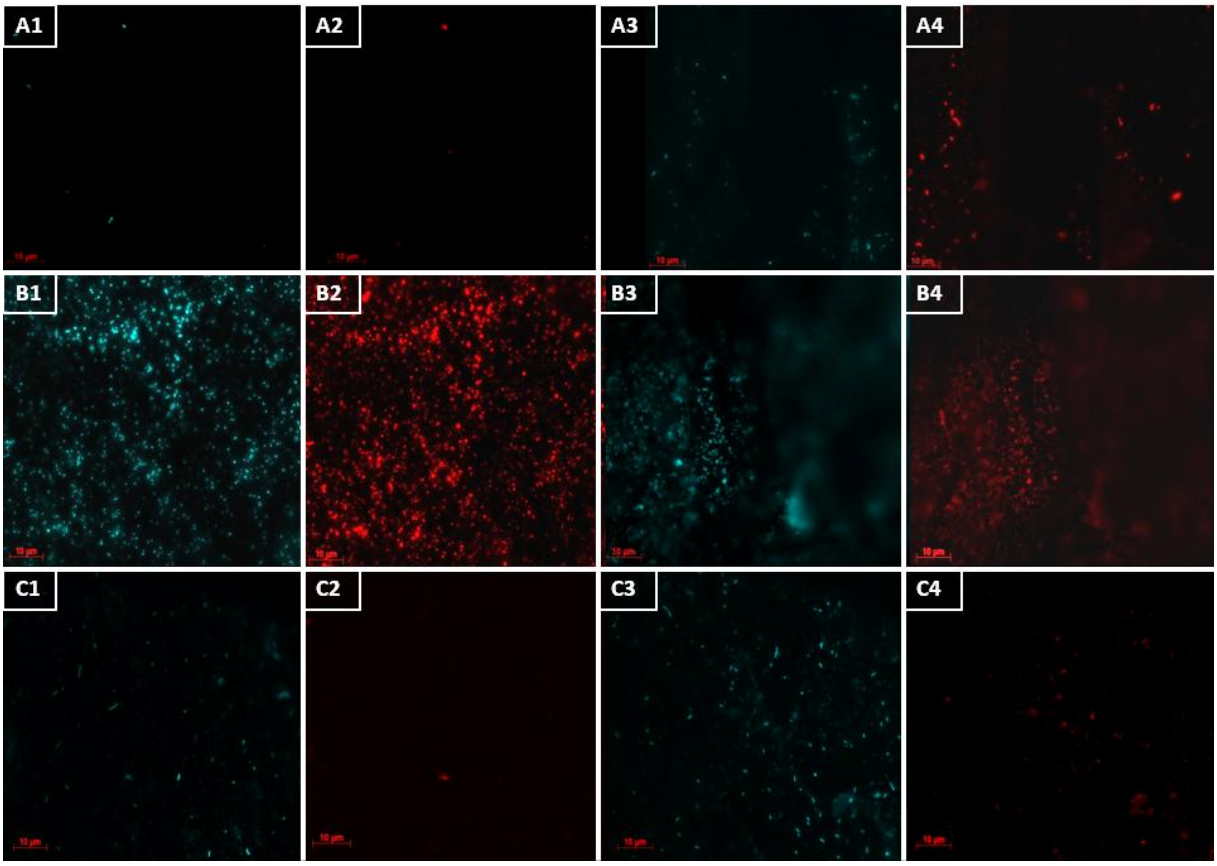

Figure 12: Attachment to and biofilm development of moderately thermophilic microorganisms on pyrite coupons.
Attachment and biofilm development of *At. caldus* (A1-A4), *L. ferriphilum* (B1-B4) and *Am. ferrooxidans* (C1-C4) on pyrite coupons stained by DAPI (blue= total cells) and lectin ConA (red= biofilm development). Initial cell number was $1x10^8$ cells/mL in 50 mL MAC basal salt solution incubated at 37°C under shaking (120 rpm); A1+A2= *At. caldus* after 3 days of incubation, A3+A4= *At. caldus* after 7 days of incubation; B1+B2= *L.f* after 3 days of incubation, B3+B4= *L.f* after 7 days of incubation; C1+C2= *Am. ferrooxidans* after 3 days of incubation, C3+C4= *Am. ferrooxidans* after 7 days of incaldusubation.

Leaching and attachment experiments indicated that mixed cultures of *L. ferriphilum* and *At. caldus* yielded the best results. Thus, attachment to and biofilm formation on pyrite coupons of this mixed culture was also investigated (Figure 13). Compared to pure cultures, biofilm and aggregate formation increased after 7 days of incubation.

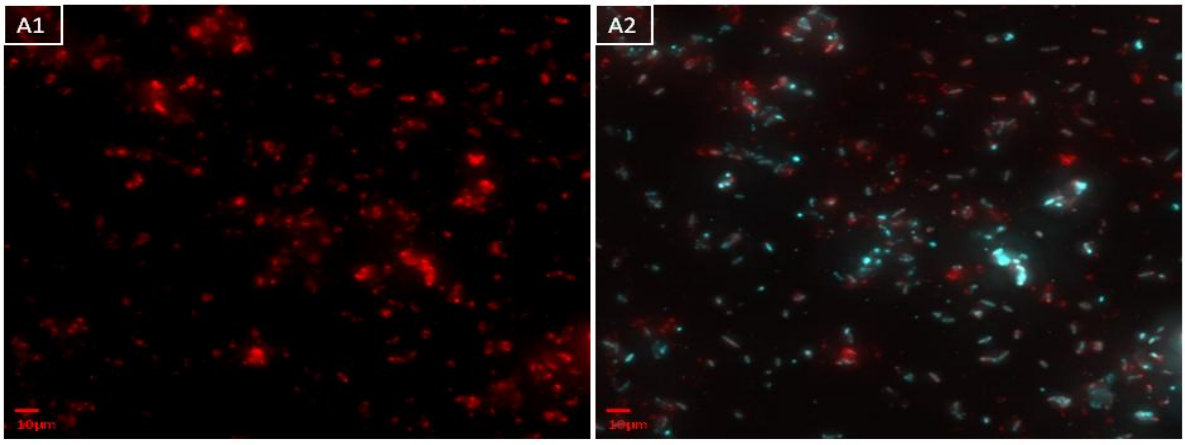

Figure 13: Attachment to and biofilm formation on a pyrite coupon of a mixed culture of *L. ferriphilum* and *At. caldus*
Visualization of a mixed culture of *L. ferriphilum* and *At. caldus* on pyrite coupons after 7 days of incubation; A1= lectin signal (ConA), A2= combined lectin and DAPI signal; initial cell number was $1x10^8$ cells/mL in 50 mL MAC basal salt solution incubated at 37°C under shaking (120 rpm).

4.4 Precolonization experiments

Pyrite coupons

Precolonization experiments on pyrite coupons were carried out to investigate, which organism is the primary colonizer and/or needs precolonization of the mineral by another bacterium. First, different incubation times for the precolonization were tested (2, 4, 8, 10, 12, 24 and 48 h) and a total incubation time of 8 h (4 h incubation in each culture) was used for further experiments. Prolonged incubation periods caused problems with the imaging of the pyrite-attached cells, especially with cultures of *L. ferriphilum.* The problem was mainly due to the formation of cell aggregates.

For every test also an 8 h control with pure cultures was done for comparison of total cells attached to the mineral (Figure 14, A0= *L.ferriphilum* und B0= *At.caldus*). DAPI images of *L. ferriphilum* (A0) and the mixed culture of *L. ferriphilum* and *At. caldus* (A1) showed a similar amount of cells after 8h of incubation, independent of a precolonization for *At.caldus*. However, aggregate formation was increased, if both organisms were present. In contrast,

At. caldus alone attached only in low numbers to pyrite coupons. But in the presence of *L. ferriphilum* cell attachment of *At. caldus* was slightly increased.

Mixed cultures of *At. caldus* and *Am. ferrooxidans* attached to pyrite coupons showed no differences after 8 h as compared to pure cultures (data not shown).

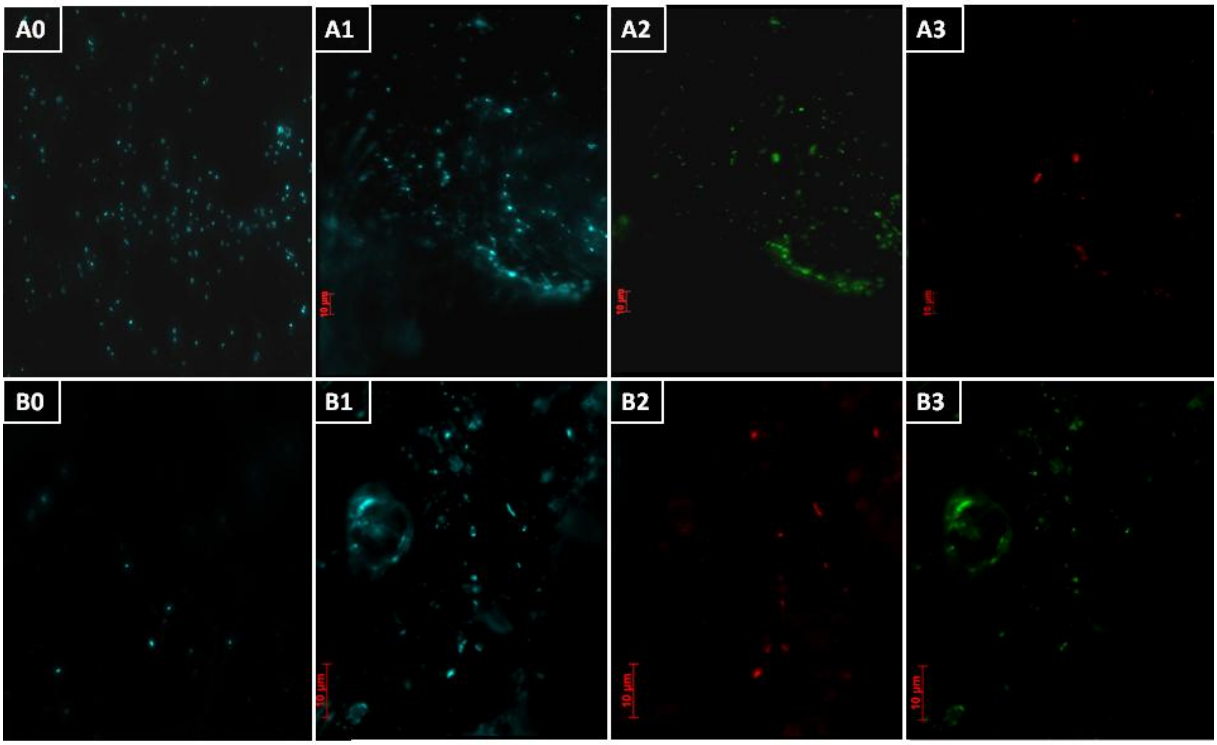

Figure 14: Effect of precolonization of pyrite coupons with *L. ferriphilum* and *At. caldus* in moderately thermophilic leaching conditions.
Visualization of pure and mixed cultures of *L. ferriphilum* and *At. caldus* on pyrite coupons; A0= total cells of *L. ferriphilum* after 8 h incubation, A1= total cells of *At. caldus* and *L. ferriphilum* (blue) stained with DAPI, A2= cells of *L. ferriphilum* (green= specific FISH probe LEP634) after 8 h incubation (4 h incubation in each culture), A3= cells of *At. caldus* (red= specific FISH probe Caldus_liu) after 8 h of incubation (4 h incubation in each culture) on precolonized pyrite coupons with *L. ferriphilum* cells; B0= total cells of *At. caldus* after 8 h of incubation, B1= total cells of *At. caldus* and *L. ferriphilum* (blue) stained with DAPI, B2= cells of *At. caldus* (red= specific FISH probe Caldus_liu) after 8 h incubation (4h in each culture), B3= cells of *L. ferriphilum* (green= specific FISH probe LEP634) after 8 h of incubation (4h in each culture) on precolonized pyrite coupons with *At. caldus* cells; initial cell number was $1x10^8$ cells/mL in 50 mL MAC basal salt solution incubated at 37°C under shaking (120 rpm).

Pyrite grains

Precolonization experiments on pyrite grains were done, too. It was investigated whether *At. caldus* needs a precolonization of the mineral by cells of *L. ferriphilum* of the mineral and the corresponding EPS production or if the presence of active *L. ferriphilum* cells is necessary (considering the possible discharge of QS molecules). Therefore, pyrite grains were precolonized with *L. ferriphilum* cells. One set was inactivated by heat-drying at 80°C for 2h; the other one was washed and directly used for attachment experiments with *At. caldus*. Furthermore, a control with uncolonized pyrite and one with *At. caldus* cells were investigated. Attachment was determined by counting of planktonic cells and calculated as percentage as previously described. The results of the attachment experiments are shown in Figure 15.

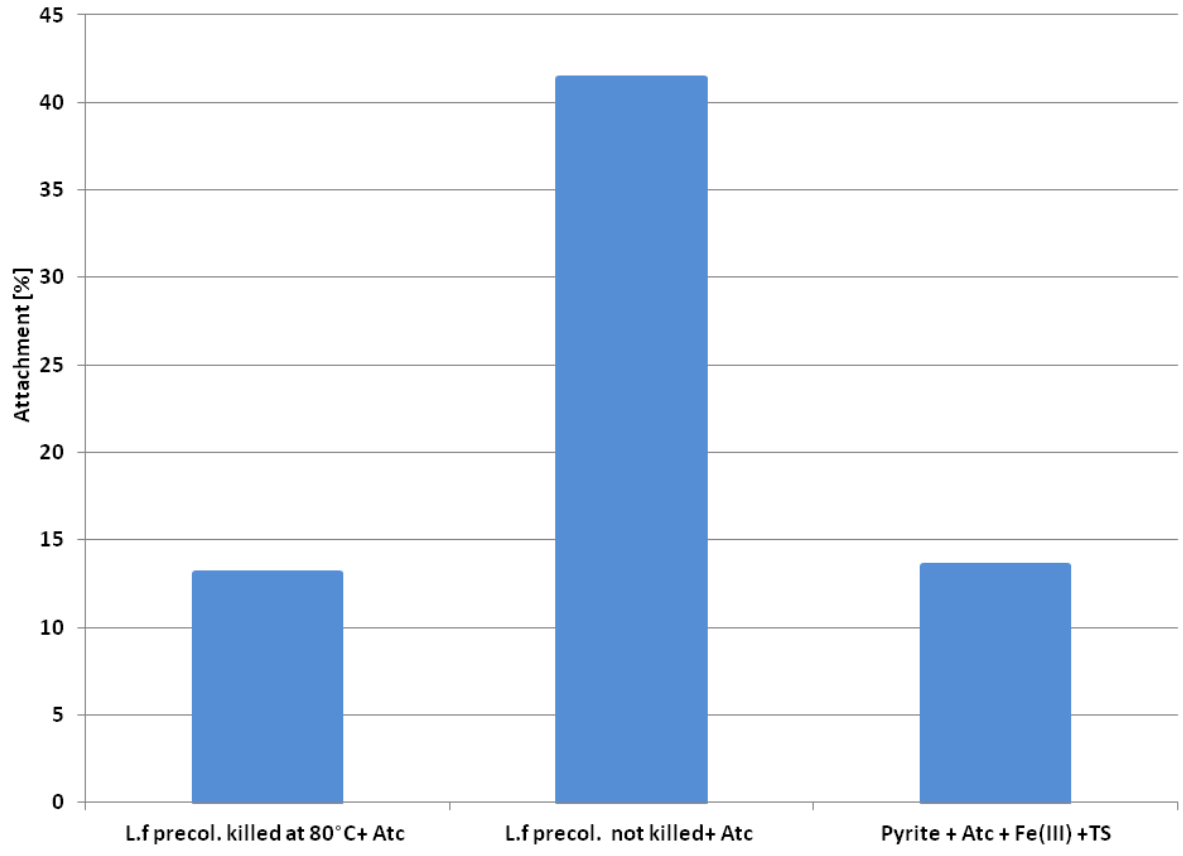

Figure 15: Effect of precolonization of pyrite coupons with *L. ferriphilum* on attachment of *At. caldus* in moderately thermophilic leaching conditions.
Attachment tests of *At. caldus* to precolonized pyrite grains with inactivated (80°C, 2 h) or active *L. ferriphilum* cells are shown; a control with attachment of *At. caldus* to pyrite grains with addition of iron (III) and thiosulfate (TS) was also done; initial cell number of *At. caldus* was $1x10^8$ cells/mL in 50 mL MAC basal salt solution incubated for 3 days at 37°C under shaking (120 rpm). Standard error (n=3) less than 7%.

Attachment of *At. caldus* to precolonized pyrite grains with heat-inactivated *L. ferriphilum* cells exhibited the same results as to the uncolonized grains. Cells attached to less than 15%, which is also comparable with the results reached in standardized attachment experiments with cells of *At. caldus* (see 4.1). However, precolonized pyrite grains with *L. ferriphilum* cells, which were not heat-inactivated, furthered the attachment of *At. caldus* by around 25% to 40% in total.

PartII:

Some of the detected results in attachment and leaching experiments could maybe explained by the effect of quorum sensing molecules. Thus, the second part of the study deals with their possible effects on pure and mixed cultures of moderately thermophilic leaching microorganisms. Different QS molecules were chosen and their effect was tested on attachment, leaching and biofilm formation.

4.3 Effect of AHLs and other signal molecules

Previous studies (Farah et al., 2005; Rivas et al., 2007; Ruiz et al., 2007) have shown that the mesophilic leaching bacterium *At. ferrooxidans* produces AHLs and that these molecules have an effect on the attachment of *At. ferrooxidans* to pyrite. Furthermore, furanones seem to influence attachment too (Ruiz et al., 2007). Thus, different synthetic AHLs and (5Z)-4-bromo-5-(bromomethylene)2(5H)-furanone were tested whether they influence the attachment and the leaching of moderately thermophilic leaching organisms. Only Gram-negative organisms were tested, because AHLs are the autoinducer molecules in the LUX I/R QS system, which only occurs in Gram-negative bacteria (see 1.4).

Effect of AHLs on attachment to pyrite coupons

Attachment to pyrite coupons was tested with cultures of *L. ferriphilum* and *At. caldus*. Pyrite coupons were incubated for 3 and 5 days. Afterwards, coupons were removed, stained with DAPI and imaged by EFM. The effect of AHLs was tested in different concentrations (0.5-5 µM). Finally, 5 µM was selected as the most appropriate concentration. The effect of AHLs at this concentration was clearly visible and an imaging of cells attached to pyrite coupons still remained possible. Attachment of *L. ferriphilum* to pyrite coupons was influenced by the use

of C14-, 3-oxo-C14- and 3-hydroxy-C14-AHL (Figure 16). 3-hydroxy-C14-AHL had the strongest effect and inhibited the attachment of cells. However, the addition of C14-, 3-oxo-C14-AHL had a reduced inhibiting effect.

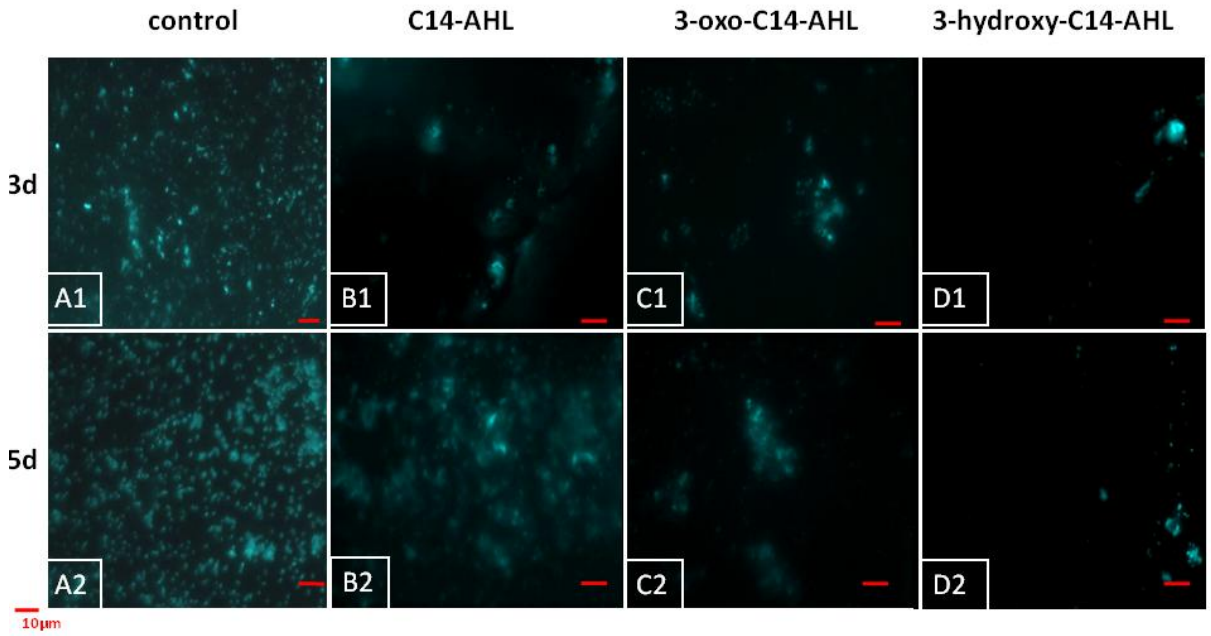

Figure 16: Effect of AHLs in attachment assays of cells of *L. ferriphilum* to pyrite coupons.
Cells of *L. ferriphilum* attached to pyrite coupons with and without addition of specific AHLs (5µM); initial cell number was $1x10^8$ cells/mL in 50 mL MAC basal salt solution incubated for 3 and 5 days at 37°C under shaking (120 rpm). Cells were stained with DAPI (blue); A1+A2= control, *L.f* incubated without addition of AHL; B1+B2= *L. ferriphilum* cells incubated with addition of C14-AHL; C1+C2= *L. ferriphilum* cells incubated with addition of 3-oxo-C14-AHL; D1+D2= *L. ferriphilum* cells incubated with addition of 3-hydroxy-C14-AHL.

Attachment of cells of *At. caldus* to pyrite coupon surfaces was influenced by an addition of C8-, 3-oxo-C8- and 3-hydroxy-C8-AHL (Figure 17). Attachment increased especially by the addition of C8- and 3-hdroxy-C8-AHLs after 5 days of incubation. The remaining ones (see 3.5.2) had no detectable influence on attachment of *L. ferriphilum* or *At. caldus* to pyrite coupons.

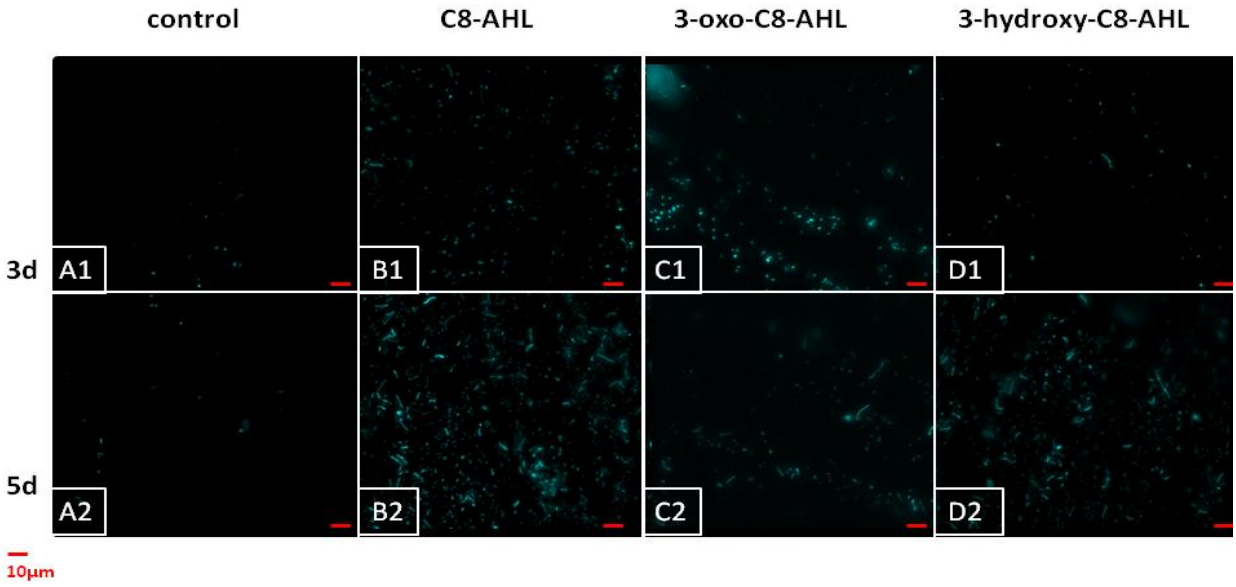

Figure 17: Effect of AHLs in attachment assays of cells of *At. caldus* to pyrite coupons.
Cells of *At. caldus* attached to pyrite coupons with and without addition of specific AHLs (5 µM); initial cell number was $1x10^8$ cells/mL in 50 mL MAC basal salt solution incubated for 3 and 5 days at 37°C under shaking (120 rpm). Cells were stained with DAPI (blue); A1+A2= control, *At. caldus* incubated without addition of AHL; B1+B2= *At. caldus* cells incubated with addition of C8-AHL; C1+C2= *At. caldus* cells incubated with addition of 3-oxo-C8-AHL; D1+D2= *At.c* cells incubated with addition of 3-hydroxy-C8-AHL.

Effect of AHLs on leaching of pure and mixed cultures of *L. ferriphilum* and *At. caldus* to pyrite grains

The effect of AHLs on pyrite leaching was also tested in standardized leaching experiments with pure and mixed cultures. Three AHLs (C8-, 3-hydroxy-C8- and 3-hydroxy-C14-AHL) were chosen as a result of the attachment experiments as described in chapter 4.3. Figure 18 presents the results of the iron (III) ions produced during pyrite leaching experiments with pure and mixed cultures of *L. ferriphilum* and *At. caldus*.

The addition of C8-AHL caused only a slight increase of the leaching effect with a mixed culture of *L. ferriphilum* and *At. caldus*. No effect was detectable with pure cultures of both organisms. The use of 3-hydroxy-C8-AHL produced an increase of 16% for leaching with pure cultures of *L. ferriphilum* and for mixed cultures of both organisms. 3-hydroxy-C14-AHL had the strongest effect and inhibited pyrite leaching by pure cultures of *L. ferriphilum* and also by mixed cultures of both species. Leaching by *At. caldus* cultures was always

negligible.

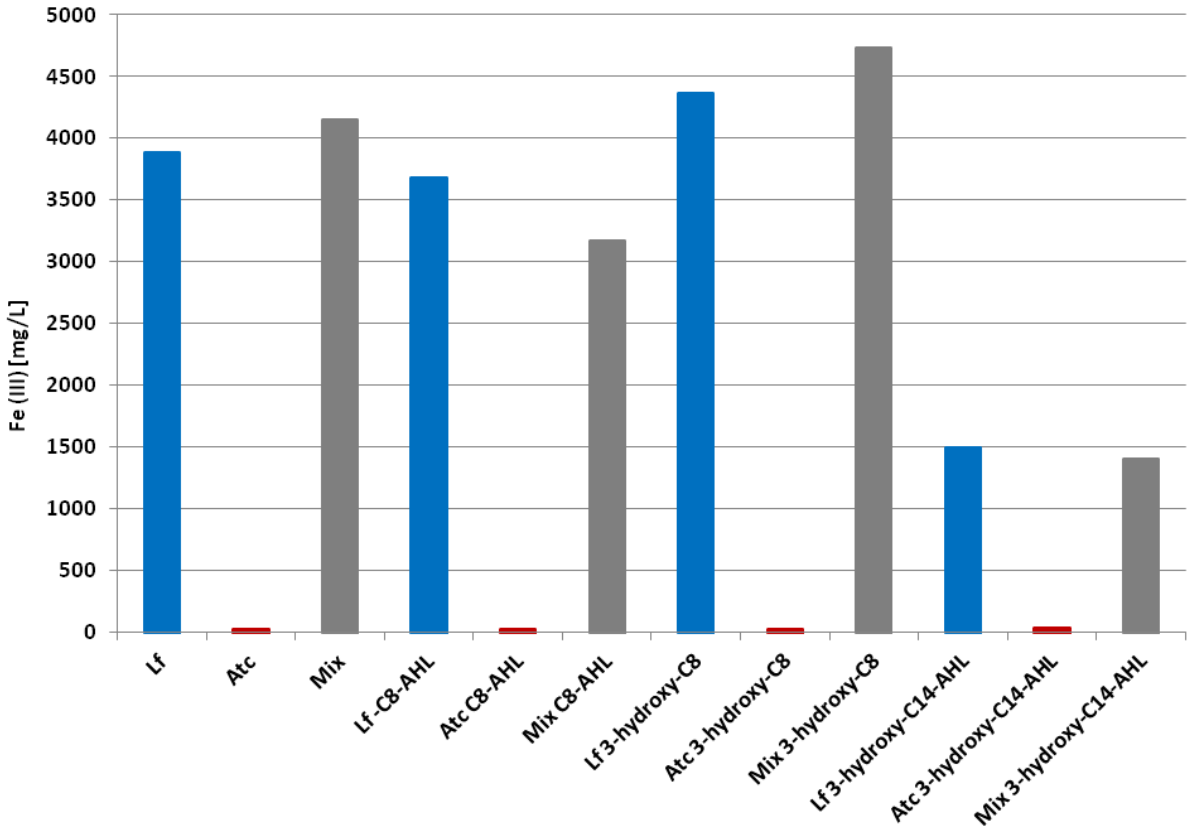

Figure 18: Effect of AHLs in pyrite- leaching assays with pure and mixed cultures of *L. ferriphilum* and *At. caldus*.
Leaching of pyrite grains characterized by iron (III) ion concentration in [mg/L] by addition of C8-, 3-hydroxy-C8- and 3-hydroxy-C14-AHL (5 µM) and controls without addition of AHLs. Initial cell number $1x10^8$cells/mL (two strains= 50%, $5x10^7$ cells/mL of each strain respectively); pyrite grains (3 g; 50- 100 µm); incubation at 37°C under shaking (120 rpm) in 100 mL MAC basal salt solution for 21 d. Standard error (n= 3) less than 10%.

Effect of external addition of AHLs on biofilm formation in floating filter experiments

The inhibition of attachment and leaching in cultures of *L. ferriphilum* by addition of C14-, 3-oxo-C14- and 3-hydroxy-C14-AHLs was also investigated with floating filter experiments. *L. ferriphilum* cells had been filtered on polycarbonate filters, which then had been added to MAC basal salt solution containing 4 g/L iron (II) ions. Filters were sampled after 5 days of incubation and stained by the lectin ConA and by Syto[9] for further imaging by CLSM. Additionally, samples for iron determination were taken.

The iron ion values indicate that the addition of 3-hydroxy-C14-AHL inhibited the oxidation of iron (II) ions in cultures of *L. ferriphilum* (Figure 19, A).

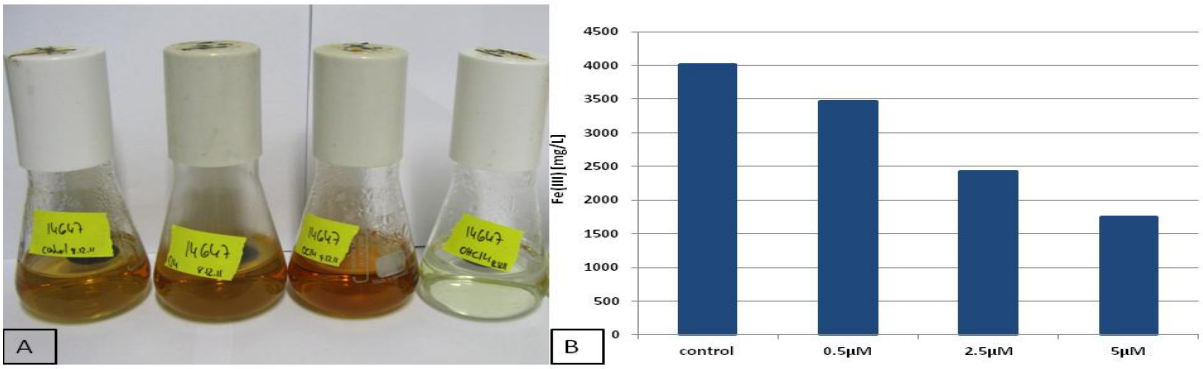

Figure 19: Visual and graphical presentation of the results from floating filter experiments with cultures of *L. ferriphilum* and addition AHLs.

Cells of *L. ferriphilum* grown on polycarbonate filter without and with addition of AHLs. A: From left to right: a control experiment without addition of AHLs, with addition (5 µM) of C14-, 3-oxo-C14-, or 3-hydroxy-C14-AHL are shown; The initial cell number was $1x10^7$ cells/mL. Filters were incubated in 50 mL MAC medium (with 4 g/L iron (II) ions) for 5 days at 37°C without shaking; B: Iron (III) ion concentration of floating filter experiments with addition of 3-hydroxy-C14-AHL in different concentrations (0.5 -5 µM). Standard error (n= 3) < 7%.

The data in Figure 19 indicate that the addition of C14- and 3-oxo-C14-AHL had no effect on iron oxidation, if *L. ferriphilum* cells were grown on the filter surface. But an addition of 5 µM 3-hydroxy-C14-AHL showed a strong iron-oxidation inhibition just visible by eye. The iron ion values exhibited that an addition of 0.5 µM 3-hydroxy-C14-AHL already inhibited iron oxidation in cells of *L. ferriphilum*. The strongest inhibition was detectable for 5 µM of 3-hydroxy-C14-AHL. However, imaging of cells by microscopy exhibited that cells were present and formed colonies and a monolayer biofilm on the polycarbonate filter surface (Figure 20, A1-D1). Lectin staining indicated that a strong signal was produced for/by cultures even though of C14- and 3-hydroxy-C14-AHL (B2+D2) addition to the cultures. Weaker ConA-signals were observed for control cultures of *L. ferriphilum* (A2) than for cultures with addition of C14- and 3-hydroxy-C14-AHL.

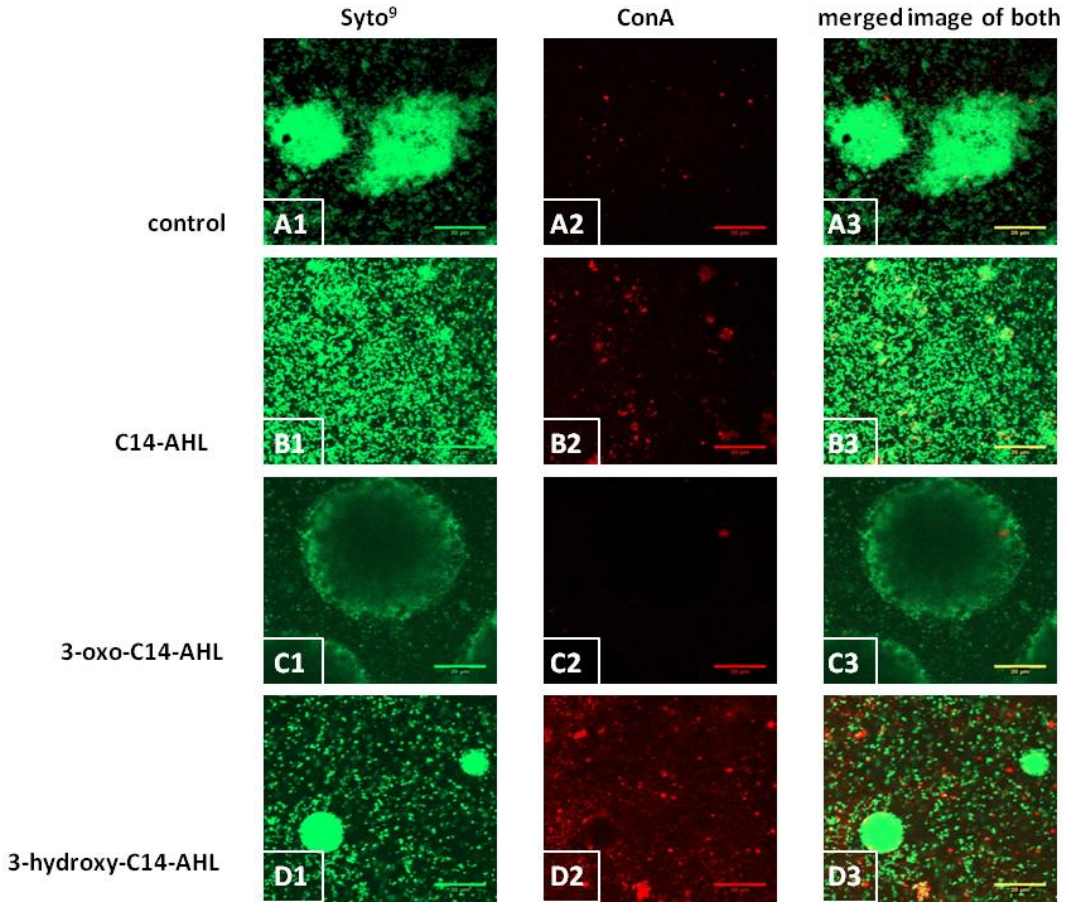

Figure 20: Effect of three different C14-AHLs on biofilm formation of *L. ferriphilum* on floating filters. Cells of *L. ferriphilum* were grown on floating polycarbonate filters without (A) and with addition of 5 µM C14-AHL (B), 3-oxo-C14-AHL (C), 3-hydroxy-C14-AHL (D). The initial cell number filtered was $1x10^7$ cells/mL. Flasks were incubated in 50 mL MAC medium (with 4 g/L iron (II) ions) for 5 days at 37°C without shaking. Filters were stained with Syto^9(green) and lectin ConA (red). All image rows show Syto^9 signal (left), lectin signal (middle) and the merged images of both channels (right).

AHL extraction from cultures of moderately thermophiles

Both organisms, *L. ferriphilum* and *At. caldus*, responded to the external addition of AHLs in attachment and leaching experiments. *L. ferriphilum* was inhibited by an addition of 3-hydroxy-C14 and *At. caldus* showed increased biofilm formation by the use of C8-AHLs. Thus, AHL biosensor assays were used to detect a possible presence of AHLs in supernatants of these leaching bacteria. *C. violaceum* and *A. tumefaciens* pNTL-04 were used as reporter strains. A dichlormethane- extraction was done and extracts from the supernatants of grown cultures of *L. ferriphilum* and *At. caldus* were tested as described for analytical TLC (3.5.4). Extracts resulting from growth with different substrates were tested. Neither *C. violaceum* nor *A. tumefaciens* gave positive signals for these extracts. Controls with synthetic AHLs as standards showed positive results. Furthermore, GC-MS measurements were carried out with

extracts of both organisms, but positive signals were obtained only for controls with AHL-standards.

Bioinformatic search for *afeI/afeR* genes in the genomes of moderately thermophilic microorganisms

Since both organisms, *L. ferriphilum* and *At. caldus*, react on an external addition of AHLs a bioinformatic search was carried out to clarify, whether genes involved in QS mechanisms are present in the genomes of *At. caldus* or *Leptospirillum* spp.. BlastP search was done to compare *afeR/I* genes from *At. ferrooxidans* with the genomes of the moderately thermophiles table 8). Results indicated that homologous genes encoding the QS-regulatory protein of *At. ferrooxidans*, AfeR (Farah et al., 2005) seem to be also present in the genomes of *L. ferriphilum* and other *Leptospirillum* spp..

Table 8: BlastP search for the gene AfeR using the genome of *At. ferrooxidans* against several *Leptospirillum* species genomes

ORGANISM	ANNOTATED PROTEIN FUNCTION	LOCUS_TAQ	MAX. IDENTITY BY BLASTP	CORRESPONDING GENE
L. ferriphilum ML-04	LuxR family two component transcriptional regulator	LFML04_0547	36%	*afeR*
L. ferrooxidans C2-3	transcriptional activator	LFE_1606	29%	*afeR*
L. ferrooxidans C2-3	two component transcriptional regulator, LuxR family	LFE_1172	34%	*afeR*
L. rubarum	putative two component transcriptional regulator, LuxR family	UBAL2_79310380	36%	*afeR*
L. ferroodiazotrophum	two component transcriptional regulator, LuxR family	UBAL3_95450025	38%	*afeR*

The search for AfeR in the genome of *L. ferriphilum* indicated 36% identity via BlastP search. BlastP search also exhibited that genomes of *Leptospirillum* spp. from all subgroups had encoded proteins with identities between 30-40% to AfeR. The search for the gene *afeI* was negative for all tested *Leptospirillum* genomes. In *At. caldus* both genes were not detectable.

Bioinformatic research in the genome of *L. ferriphilum* was also done for genes homologous to those of *At. ferrooxidans* putatively involved in EPS and biofilm formation. AHLs operate as mediator for CPS/EPS production and subsequently the colonization of a MS surface (Gonzalez et al., 2012). An increase of biofilm formation of *At. ferrooxidans* on pyrite surfaces is attributed amongst other factors to two outer membrane polysaccharide export proteins, AFE_1339 and AFE_2975 (Bellenberg et al., 2012). Expression levels of AFE_2975 were enhanced in biofilm and planktonic cells of *At. ferrooxidans* subpopulated on pyrite (Bellenberg et al., 2011; Vera et al., 2011; Vera et al, 2013).

Results of the BlastP search for the genes AFE_1339, AFE_2975 and their neighboring genes in the genome of *L. ferriphilum* are shown in Table 9. The hits for those genes in the genome of *L. ferriphilum* showed between 22- 51% maximal identity by BlastP compared to the genome of *At. ferrooxidans*.

Table 9: BlastP search for some genes involved in EPS and biofilm formation of *At. ferrooxidans* against the genome of *L. ferriphilum* ML-04

ORGANISM	ANNOTATED PROTEIN FUNCTION	LOCUS_TAQ	MAX. IDENTITY BY BLASTP	CORRESPON-DING GENE	ANNOTATED PROTEIN FUNCTION
L. ferriphilum	mannose-1-phosphate guanylyltransferase	LFML04_1022	39%	AFE_2960	mannose-1-phosphate guanylyltransferase/mannose-6-phosphate-isomerase
L. ferriphilum	polysaccharide/polyol phosphate ABC transporter ATPase	LFML04_1800	36%	AFE_2963	capsule polysaccharide exporter, ATP-binding protein
L. ferriphilum	glycosyltransferase	LFML04_1797	29%	AFE_2967	group 1 glycosyl transferase
L. ferriphilum	translation elongation factor Tu	LFML04_1582	27%	AFE_2971	sulfate adenylyltransferase
L. ferriphilum	ISAfe1, transposase	LFML04_0017	25%	AFE_2972	ISAfe3, transposase
L. ferriphilum	inositol monophosphatase	LFML04_2475	25%	AFE_2973	3'(2'),5'-bisphosphate nucleotidase
L. ferriphilum	polysaccharide export protein	LFML04_0791	22%	AFE_2975	capsule polysaccharide export protein, BexD/CtrA/VexA family
L. ferriphilum	dTDP-D-glucose 4,6-dehydratase(rfbB)	LFML04_0724	25%	AFE_2977	GDP-mannose 4,6-dehydratase
L. ferriphilum	UDP-glucose 4-epimerase(galE)	LFML04_0896	46%	AFE_1342	UDP-glucose 4-epimerase
L. ferriphilum	UDP-glucose 6-dehydrogenase	LFML04_1455	51%	AFE_1343	UDP-glucose 6-dehydrogenase
L. ferriphilum	SAM-dependent methyltransferase	LFML04_2292	32%	AFE_1345	hypothetical protein
L. ferriphilum	group 1 glycosyl transferase	LFML04_0785	36%	AFE_1351	glycoside hydrolase
L. ferriphilum	glycosyltransferase	LFML04_0786	37%	AFE_1354	glycoside hydrolase

Effect of furanone addition to growth of moderately thermophilic microorganisms

Changes in QS mediated bacterial properties such as swarming, bioluminescence or different phenotypes have been reported after addition of furanones to bacterial cultures (Givskov et al., 1996; Manefield et al., 1999). Furthermore, furanones are described to display antimicrobial and antifouling properties (Givskov et al., 1996). Therefore, the effect of (5Z)-4-bromo-5-(bromomethylene)2(5H)-furanone was tested in growth experiments with cells of *L. ferriphilum, At. caldus* and *Am. ferrooxidans*.

Different concentrations of the furanone (between 0.2- 2 µM) were used. At first, typical growth experiments were done with furanone addition to MAC medium containing 4 g/L iron (II) ions (*L. ferriphilum* and *Am. ferrooxidans*) or 5 g/L elemental sulfur (*At.caldus*). Substrate conversion of the organisms was used for evaluation of the effect of the addition. An addition of 0.2 µM furanone already caused a strong inhibition of iron- (Figure 21) or sulfur- (Figure 22) oxidation.

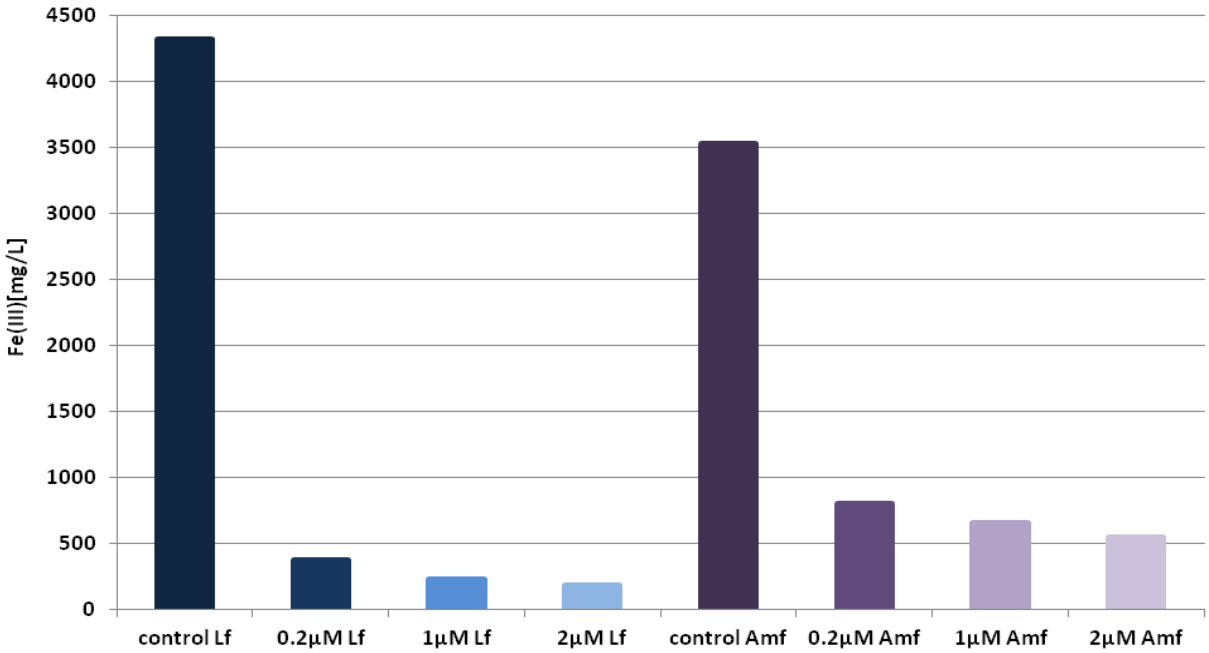

Figure 21: Effect of furanone on substrate oxidation by *L. ferriphilum* and *Am. ferrooxidans*.
Substrate conversion was characterized by iron (III) ion concentration in [mg/L] with and without addition of (5Z)-4-bromo-5-(bromomethylene)2(5H)-furanone (0.2- 2µM). Initial cell number was $1x10^7$ cells/mL; incubation at 37°C under shaking (120 rpm) in MAC medium (incl. 4 g/L iron (II) ions). Standard error (n= 3) < 5%.

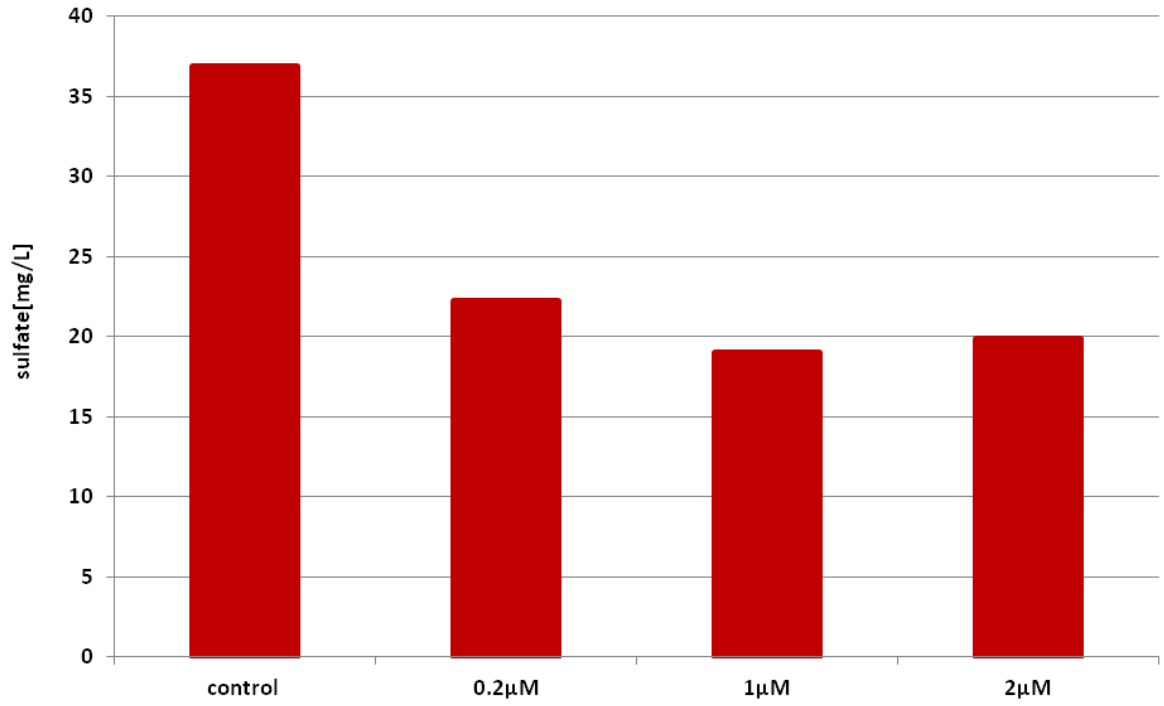

Figure 22: Effect of a furanone on substrate oxidation by *At. caldus*.
Substrate conversion was characterized by sulfate concentration in [mg/L] without and with addition of (5Z)-4-bromo-5-(bromomethylene)2(5H)-furanone (0.2- 2 µM). Initial cell number was $1x10^7$ cells/mL; incubation at 37°C under shaking (120 rpm) in MAC medium (including 5 g/L elemental sulfur). Standard error (n= 3) < 5%.

Effect of furanone addition on pyrite leaching

In growth experiments with an addition of (5Z)-4-bromo-5-(bromomethylene)2(5H)-furanone a concentration of 2 µM produced the strongest effect and seriously inhibited the growth of *L. ferriphilum*, *Am. ferrooxidans* and *At. caldus*. Consequently, this concentration was also chosen for leaching experiments with *L. ferriphilum* and *Am. ferrooxidans*. The dissolution of pyrite was monitored by the concentration of iron (III) ions in the bulk solution.

Results of iron determination indicate that a concentration of 2 µM furanone inhibited pyrite dissolution strongly (Figure 23). In assays with *L. ferriphilum* only 20% of the iron (III) ions of the control assays were detectable. For *Am. ferrooxidans* only 17% were measureable (against control).

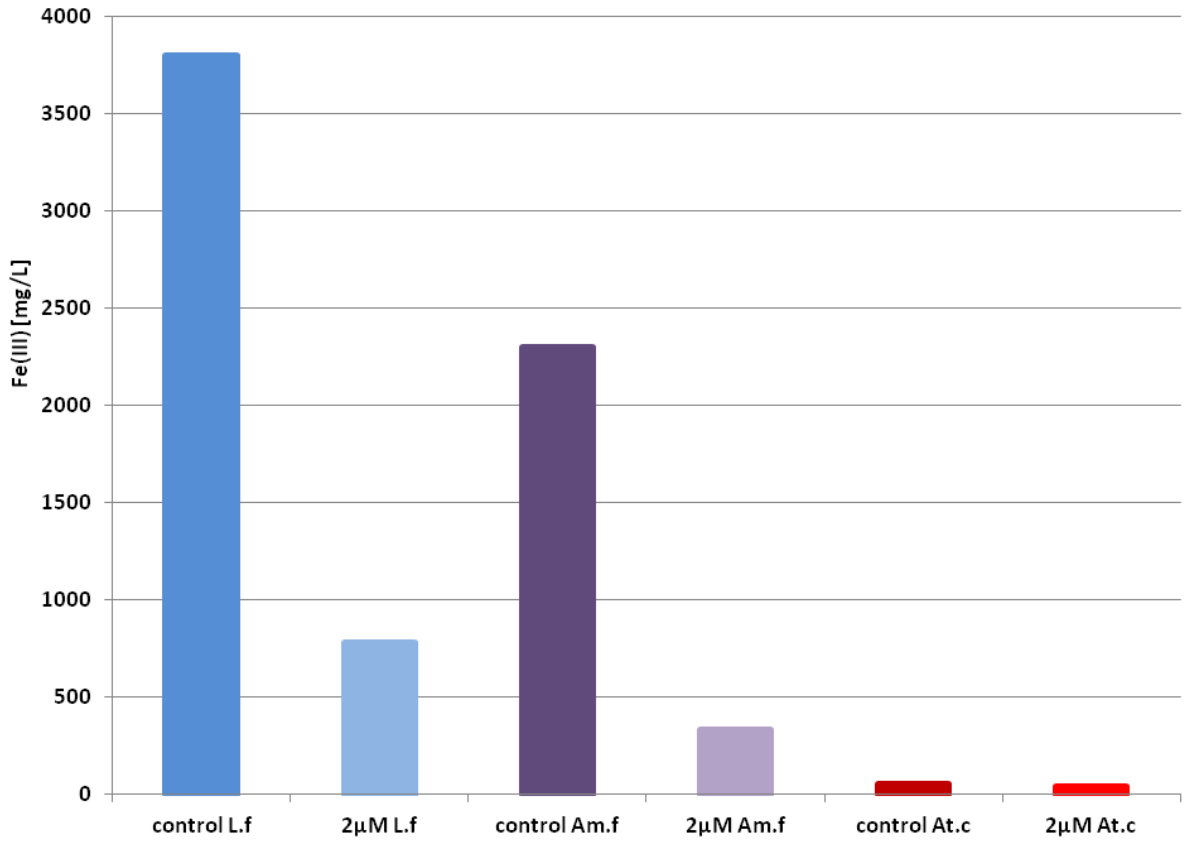

Figure 23: Effect of furanone to pyrite- leaching pure cultures of *L. ferriphilum*, *Am. ferrooxidans* and *At. caldus*.
Leaching of pyrite grains was characterized by iron (III) ion concentration in [mg/L] after addition of (5Z)-4-bromo-5-(bromomethylene)2(5H)-furanone (2 µM). Initial cell number was $1x10^8$ cells/mL; pyrite grains (3 g; 50- 100 µm); incubation at 37°C under shaking (120 rpm) in 100 mL MAC basal salt solution. Standard error (n= 3) < 10%.

Effect of a furanone addition on biofilm formation in floating filter experiments

The effect of (5Z)-4-bromo-5-(bromomethylene)2(5H)-furanone on growth and biofilm formation by cells of *L. ferriphilum* on floating filters was investigated using a concentration of 2 µM. This furanone concentration reduced the growth and the biofilm formation of *L. ferriphilum* (Figure 24). After 6 days only a few microcolonies had developed, whereas the control showed dense cell growth.

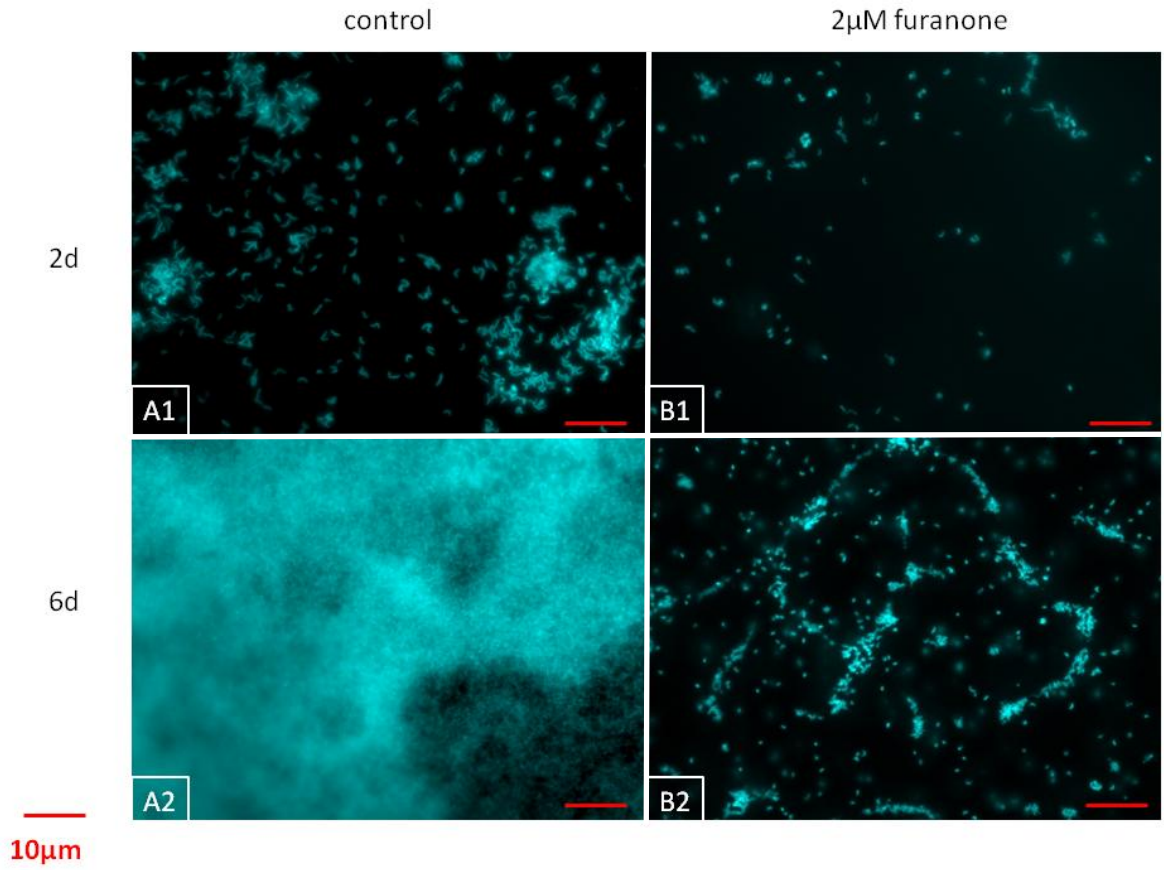

Figure 24: Furanone addition and biofilm formation of cells of *L. ferriphilum* on floating filters.
Cells of *L. ferriphilum* grown on floating polycarbonate filter with or without addition of (5Z)-4-bromo-5-(bromomethylene)2(5H)-furanone (2 µM); initial cell number ($1x10^7$ cells/mL) was filtered on polycarbonate filter; filter were incubated in 50 mL MAC basal salt solution for 2 and 6 days at 37°C without shaking. Cells were stained with DAPI; A1and A2 are controls without addition of furanone after 2 and 6 days of incubation, respectively; B1and B2 are with addition of furanone after 2 and 6 days of incubation, respectively.

The filters were removed from the assays after 6 days to test, whether the furanone addition had caused cell death/ inactivation. The cells were washed and subcultured. The results indicated normal growth and corresponding substrate conversion (data not shown).

Part III:

The third part of this study deals with the search for a possible novel QS molecule produced by cells of *L. ferriphilum*. During the search for possible AHLs in extracts of grown cultures of *At. caldus* and *L. ferriphilum*, concentrated extracts were also tested for an effect on bacterial growth. Therefore, the dried extract of *At. caldus* and *L. ferriphilum* were dissolved in DMSO and added at 0.2% (v/v) to growing cultures of *At. caldus* and *L. ferriphilum*, respectively. For cultures of *At. caldus* no effect was detected, whereas iron oxidation in cultures of *L. ferriphilum* became inhibited upon addition of the "*L. ferriphilum*-extract". Thus, further experiments were carried out to investigate the origin of this inhibiting effect of the "*L. ferriphilum*-extract".

4.4 Identification of cell-cell signaling molecule(s) produced by cells of *L. ferriphilum*

Solubility and inhibition tests

The "*L. ferriphilum*-extract" originated from iron- or pyrite- grown cultures of *L. ferriphilum*. Cultures were grown between 1 and 12 weeks and then used for extraction of the unknown inhibiting compound(s). Furthermore, the culture time should be determined, at which the highest concentration of these compounds became available. Thus, a dichlormethane extraction was done as described for extraction of AHLs (Farah et al., 2005). The possible presence and effect of this/these unknown molecule/s was tested at first by growth experiments with *L. ferriphilum* cultures. For this purpose extracts from iron- as well as from pyrite-grown cultures, were used. The data indicated that both extracts, originating from iron- or pyrite-grown cultures, showed an inhibitory effect on cells of *L. ferriphilum* (data not shown). If the extracts originated from cultures with a long incubation time on pyrite, the effect on cells of *L. ferriphilum* was higher than with extracts from short term cultures. In addition, extracts from ferrous iron grown cultures had a decreased effect.

Solubility tests were done to determine the best solvent for the compounds in the dried "*L. ferriphilum*-extracts". The extracts were well soluble in hydrophobic solvents such as hexane and octane. The solvents were tested for their effect on growth of cells of *L. ferriphilum* to clarify any solvent-related effect. Results are shown in Figure 25.

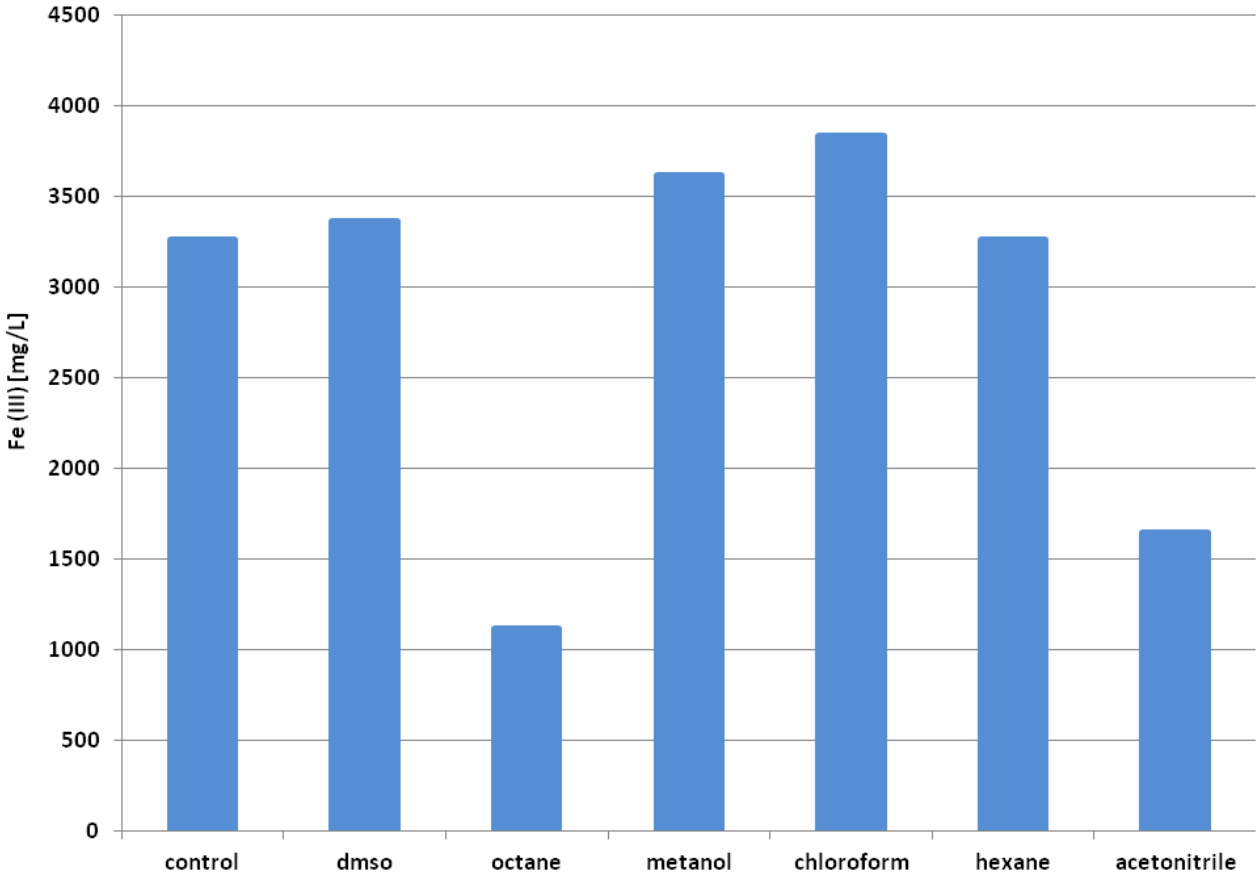

Figure 25: Effect of solvent addition on iron (II) ion oxidation by cultures of *L. ferriphilum*.
Substrate conversion characterized by iron (III) ion concentration in [mg/L] with addition of 0.2% (v/v) DMSO, octane, methanol, chloroform, hexane or acetonitrile. Flasks were incubated at 37°C under shaking (120 rpm) in MAC medium (including 4 g/L iron (II) ions) and 5% inoculum of *L. ferriphilum*. Standard error (n= 3) < 5%.

Octane or acetonitrile in a concentration of 0.2% (v/v) to the assays exhibited a clear inhibiting effect on iron (II) ion oxidation by cultures of *L. ferriphilum*. DMSO, methanol, chloroform or hexane had no detectable effect. Thus, these four solvents were chosen for further experiments. The hexane-dissolved "*L. ferriphilum*-extract" caused the strongest inhibition of iron (II) ion oxidation for cultures of *L. ferriphilum* (data not shown). The tests with various amounts of this hexane extract indicated that a concentration of 0.2% (v/v) produced the strongest effect on iron (II) ion oxidizing cultures of *L. ferriphilum* (Figure 26). Consequently, this concentration was used further on.

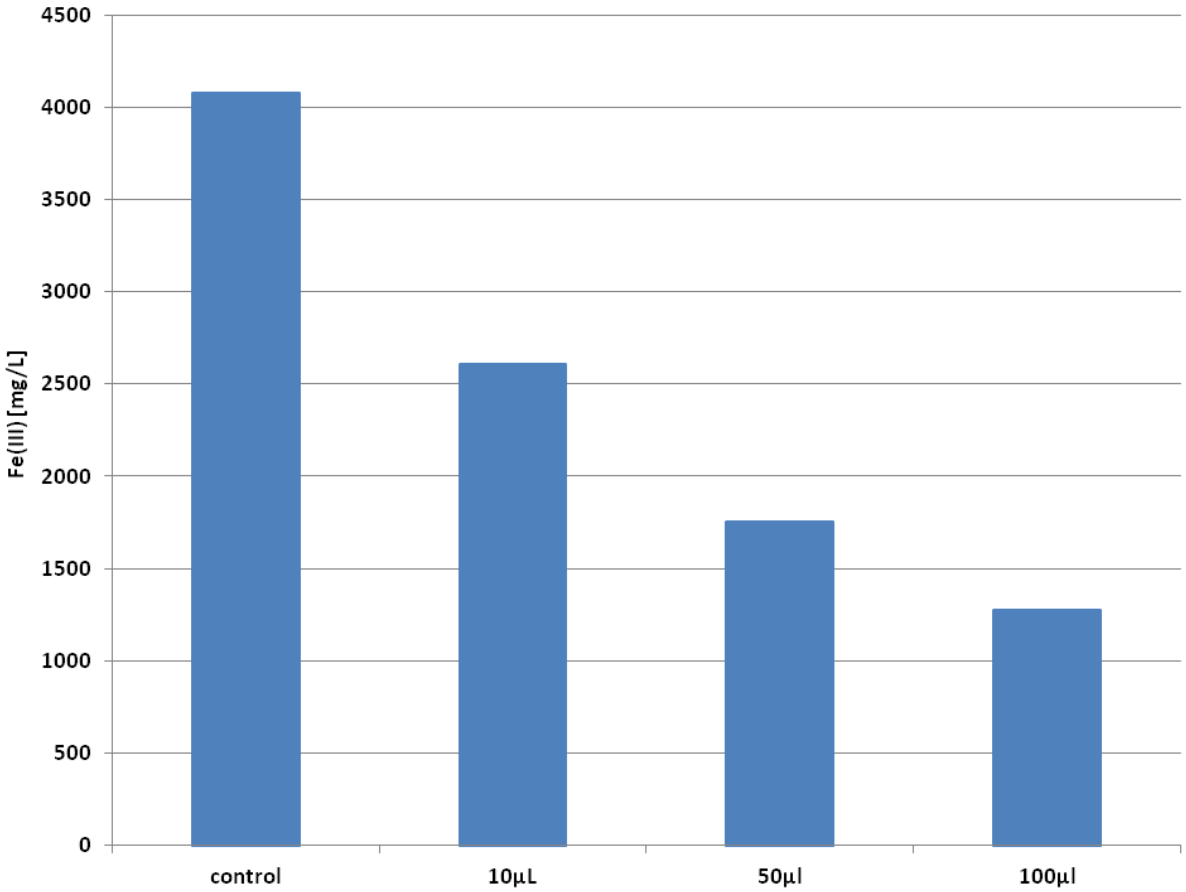

Figure 26: Inhibition of iron (II) ion oxidation of *L.ferriphilum* cells by an addition of the "*L. ferriphilum*-extract".
Substrate conversion characterized by iron (III) ion concentration in [mg/L] with or without addition of 0.02, 0.1 or 0.2% (v/v) of "*L. ferriphilum*-extract" dissolved in hexane. Incubation at 37°C under shaking (120 rpm) in MAC medium (including 4 g/L iron (II) ions) and 5% inoculum of *L.f.* Standard error (n= 3) < 5%.

Furthermore, the effect of the extract was also tested with cultures of other bacteria. For these tests, different iron-oxidizers such as *At. ferrooxidans*, *Am. ferrooxidans*, *At. ferrivorans* and *Sulfobacillus thermosulfidooxidans* were chosen. All of them were not influenced by pure hexane addition, but iron-oxidation was clearly inhibited by an addition of 0.2% (v/v) of the "*L. ferriphilum*-extract" (Figure 27). Interestingly, the addition of the "*L. ferriphilum*-extract" to a sulfur oxidizing culture of *At. caldus* as well as to sulfur oxidizing cultures of *At. ferrooxidans* and *S. thermosulfidooxidans* had no detectable effect. Additionally, similar experiments using *Bacillus subtilis* and *Escherichia coli* were done. No influence on growth, as measured by OD-determination, was observed after addition of the "*L. ferriphilum*-extract" (data not shown).

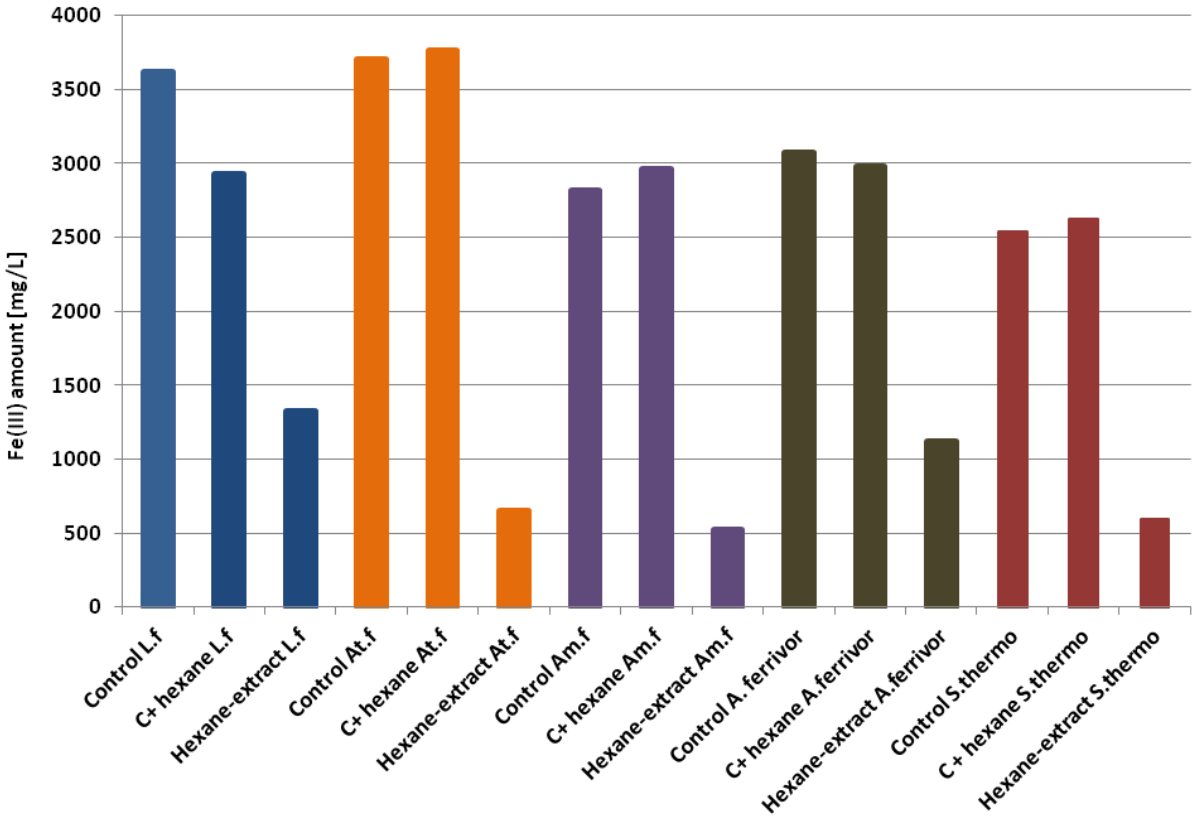

Figure 27: Inhibition of bacterial iron oxidation by addition of an aliquot of the "*L. ferriphilum*-extract". Substrate conversion characterized by iron (III) ion concentration [mg/L] with or without addition of 0.2% (v/v) of "*L. ferriphilum*-extract" dissolved in hexane. Incubation at 37°C under shaking (120 rpm) in MAC medium (including 4 g/L iron (II) ions) and 5% inoculum of *L. ferriphilum* (blue), *At. ferrooxidans* (orange), *Am. ferrooxidans* (purple), *A. ferrivorans* (green) and *S. thermosulfidooxidans* (red). For each bacterium a control with and without addition of pure hexane is also shown. Standard error (n= 3) < 5%.

Furthermore, the *Leptospirillum* strains L3, L4 and L7 (L3 and L4 originate from Mina IIba, Romania, and L7 from the Rammelsberg, Germany) were also tested for an inhibition of iron (II) ion oxidation. After growth in MAC medium containing 4 g/L iron (II) ions 0.2% (v/v) of the in hexane dissolved "*L. ferriphilum*-extract" was added. Again, iron-oxidation of all cultures became inhibited (Figure 28). Controls indicated no inhibitory effect of the pure solvent. Finally, to test whether other *Leptospirillum* strains might also produce an inhibitory compound, the supernatants of these three *Leptospirillum* strains were tested also for their effect on the cultures. In Figure 28 these results are displayed. They indicate that these extracts had also an inhibiting effect on iron-oxidation of strains from the *Leptospirillum* family.

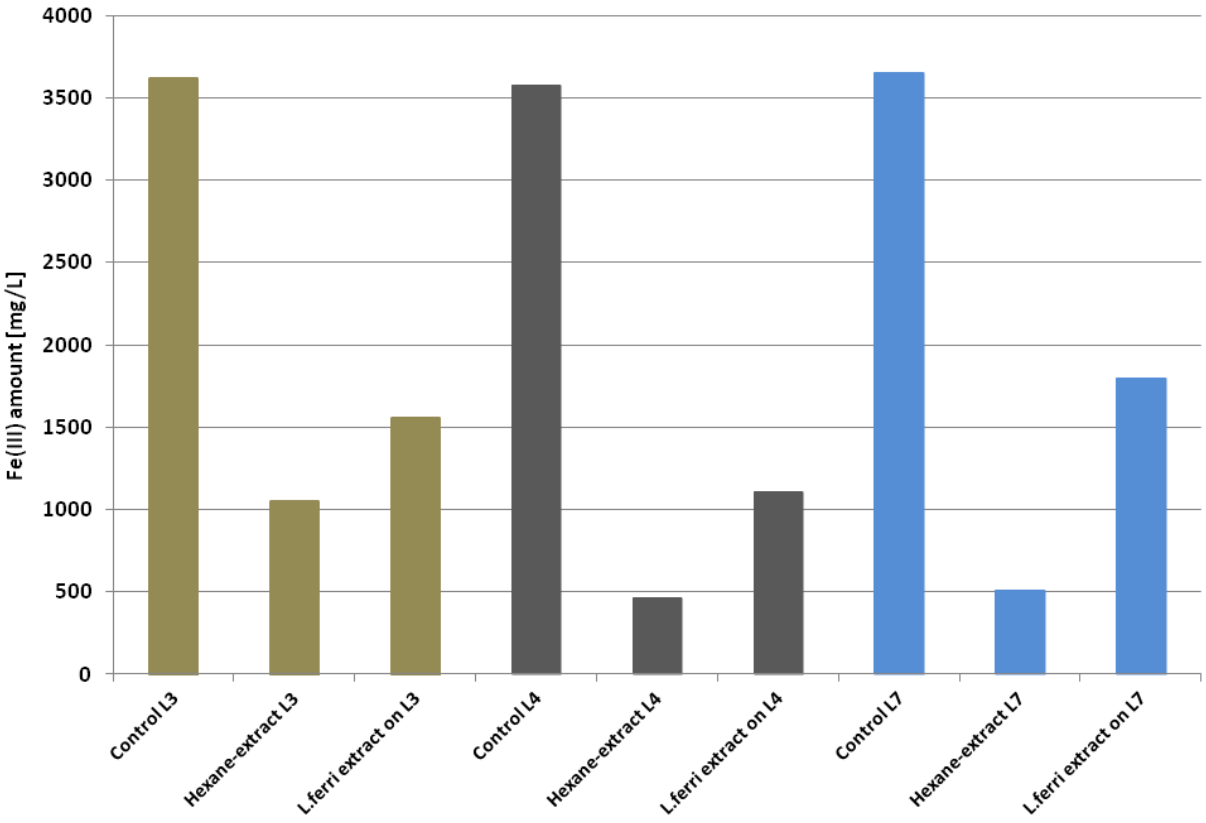

Figure 28: "*L. ferriphilum*-extracts"-inhibit iron oxidation of *L. ferrooxidans* strains
Substrate conversion characterized by iron (III) ion concentration in [mg/L] with addition of 0.2% (v/v) of "*L. ferriphilum*-extract" dissolved in hexane. Also extracts from the *L. ferrooxidans* (L3, L4 and L7) cultures were added at 0.2% (v/v) (labeled as hexane-extract L3, L4, L7) to study their "self inhibition" properties. Incubation was done at 28°C with shaking (120 rpm) in MAC medium (including 4 g/L iron (II) ions) and 5% inoculum of each strain. For every organism, a control with (control L3, L4, L7) addition of hexane was done. Standard error (n= 3) < 6%.

Effect of an addition of the "*L. ferriphilum*-extract " on biofilm formation by cultures of *L. ferriphilum*

The influence of the "*L. ferriphilum*-extract" on *L. ferriphilum* biofilm formation on floating polycarbonate filters was investigated. Experiments were carried out as described above and 0.02 or 0.2% (v/v) of the hexane-dissolved extract was added to the medium. Results are shown in Figure 29. Cells were incubated on floating filters for 5 days and then stained with Syto9 and the lectin ConA. The ConA signal was not detectable for biofilms/ cells on control filters (Figure 29, A2). However, when the "*L. ferriphilum*-extract" had been added, a strong lectin signal became detectable (Fig. 29, B2+C2). Furthermore, the Syto9 signal increased in strength (Figure 29, C1) and the cells changed their morphology in case of an addition of

0.2% (v/v) of the hexane-dissolved extract. The cells seem to be "bloated". Determination of iron (II) ion oxidation exhibited an inhibition in these assays (data not shown).

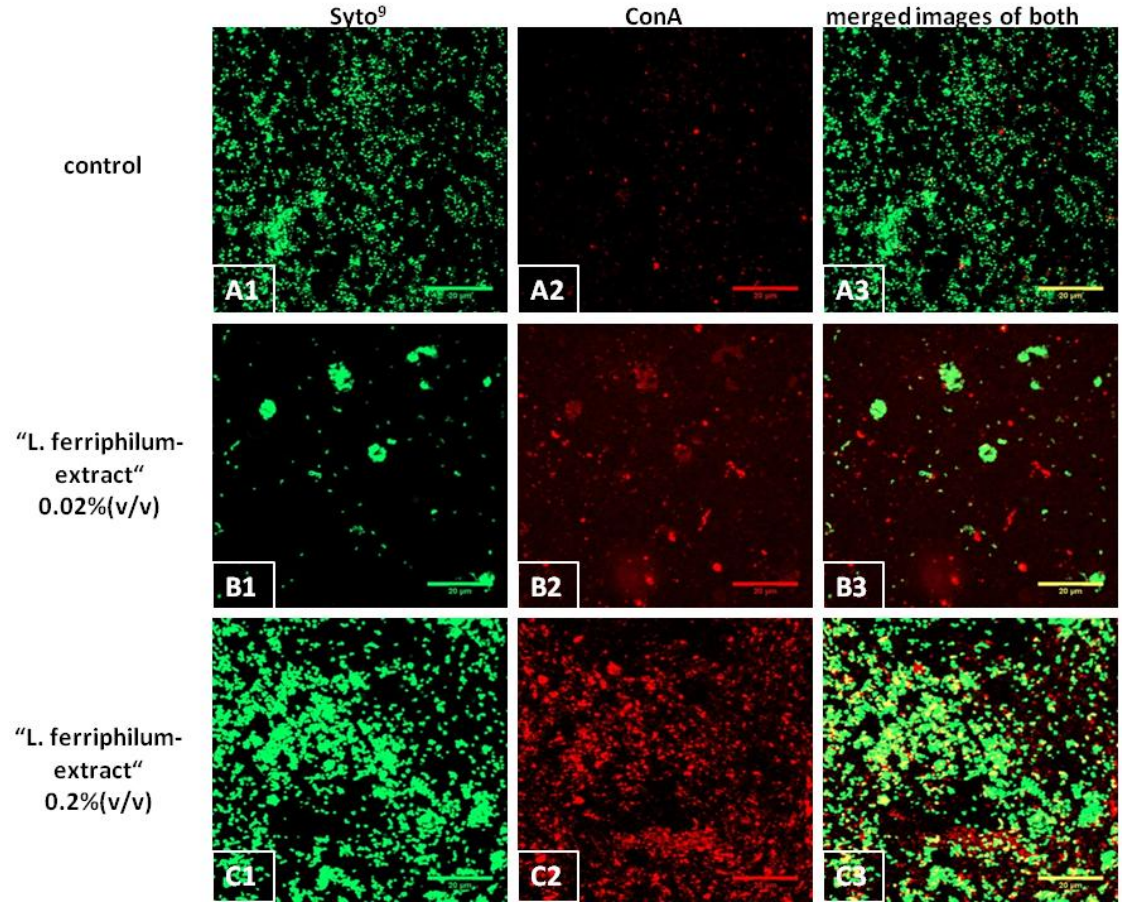

Figure 29: Addition of "*L. ferriphilum*-extract" induced changes of *L. ferriphilum* biofilm formation on floating filters.
Cells of *L. ferriphilum* grown on floating polycarbonate filters without or with the addition of 0.02% (v/v) or 0.2% (v/v) of "*L. ferriphilum*-extract" dissolved in hexane; initial cell number was $1x10^7$ cells/mL. Filters were incubated in 50 mL MAC medium (with 4 g/L iron (II) ions) for 5 days at 37°C without shaking. Filter were double stained with Syto^9(green) and lectin ConA (red); Columns (1-3) show Syto^9, ConA or merged images from both signals, respectively. Rows show images from the control experiment (A1-A3), images from addition of 0.02% (v/v) and images from 0.2% (v/v) of "*L. ferriphilum*-extract" are shown in (B1-B3) and (C1- C3), respectively.

Biosensor tests of *Janthinobacterium* with addition of the "*L. ferriphilum*-extract"

A biosensor test with the organism *Janthinobacterium* sp. HH01 was done to test the influence of the "*L. ferriphilum*-extract" on violacein production. This test is indicative of the presence of a molecule similar to a α-hydroxyketone, which are signal molecules for QS found in *L. pneumophila* and *V. cholerae* (Spirig et al., 2008, Tiaden et al., 2010). Previous studies of Hornung et al. (2013) had described that *Janthinobacterium* encodes a homologue of the *Legionella*- like autoinducer *lqsA*, which produces α-hydroxyketones. For these tests

the *L. ferriphilum*-extract was dissolved in 400 µl DMSO and added in different dilutions (1:10, 1:1000) to the culture of *Janthinobacterium* . Violacein production of the *Janthinobacterium* was measured (Hornung et al., 2013). The data indicated a strong increase, when the "*L. ferriphilum*-extract" was present in the assays (Figure 30). An increase of 100% over the control of violacein production was detected for the 1:1000 dilution. The 1:10 dilution showed only a 50% increase.

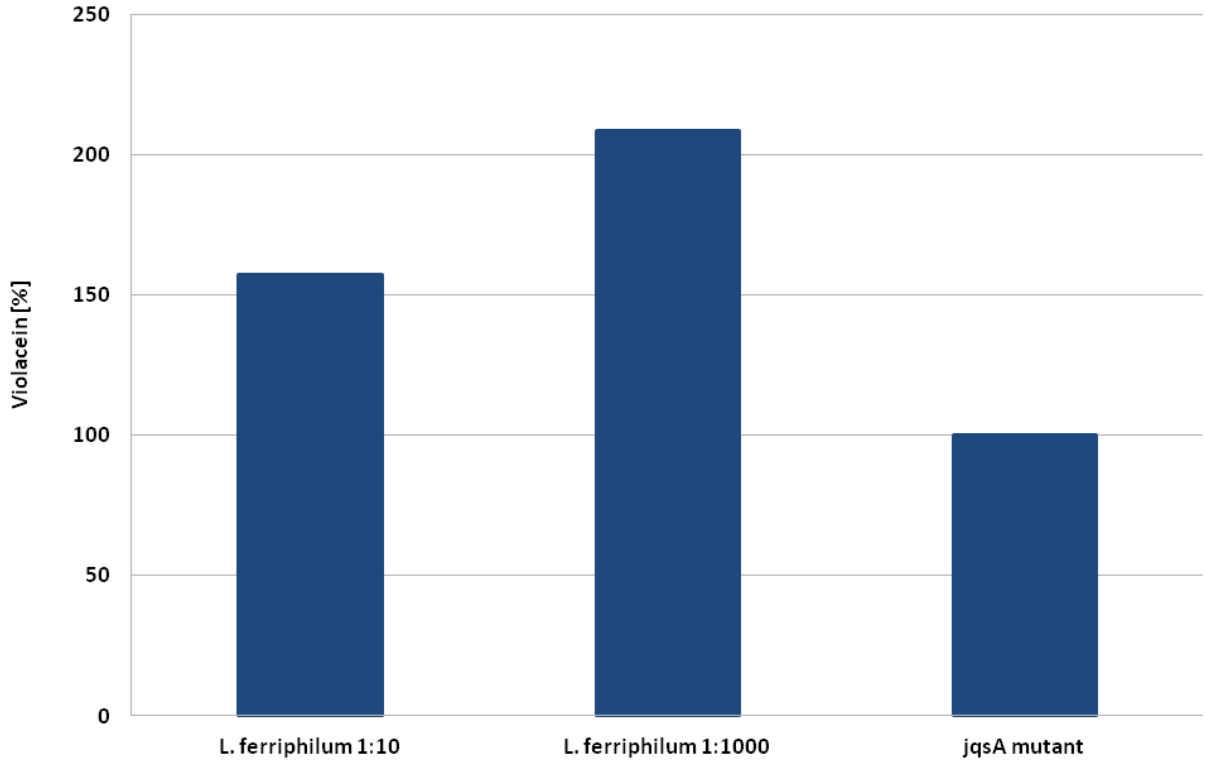

Figure 30: "*L. ferriphilum*-extract" influences violacein production by the biosensor bacterium *Janthinobacterium* HH01.
The "*L. ferriphilum*-extract"- influenced the violacein production of the HH01 biosensor including the *jqsA* mutation. The dried powder of the "*L. ferriphilum*-extract" was dissolved in 400 µl DMSO and added in different dilutions (1:10, 1:100) to the culture. Incubation was carried out at 22°C for 4 8h in 5 mL of R2A medium.

Bioinformatic search of *jqsA/jqsS* genes in the genomes of *Leptospirillum* spp.

Because of the positive results with the *Janthinobacterium* biosensor test a bioinformatic research was done to clarify, whether the *L. ferriphilum* genome includes also homologues of the *jqsA/jqsS* gene sequences. BlastP search was done to compare the genomes of *Janthinobacterium* with the one of *L. ferriphilum* to find possible identities for genes involved in QS. Results of BlastP search are shown in (table 10).

Table 10: BlastP search for the genes jqsA/jqsS screening the genome data of *Janthinobacterium* against the ones of *L. ferriphilum*

ORGANISM	ANNOTATED PROTEIN FUNCTION	LOCUS_TAQ	MAX. IDENTITY BY BLASTP	CORRESPON-DING GENE
L. ferriphilum	7-keto-8-aminopelargonate synthetase	LFML04_1510	27%	*jqsA, Janthinobacterium*
L. ferriphilum	sensor signal transduction histidine kinase	LFML04_0349	28%	*jqsS, Janthinobacterium*

Interestingly, BlastP search was in two cases successful. Two proteins exhibited between 27-28% maximal identities for the ones encoded in *jqsA/jqsS* genes. Thus, a possible presence of homologous genes in the *L. ferriphilum* genome may explain the previous results.

It was also of interest whether these genes are only present in *L. ferriphilum* or also in other species of *Leptospirillum*. BlastP search was done for strains from different species. The cognation of the used *Leptospirillum* strains was investigated before and they were ordered into different species (Mitchell, 2002). Broader experiments and studies confirmed the classification of this species (Tyson et al., 2005) shown in Table 11.

Table 11: Overview of the *Leptospirillum* strains used and the corresponding species adapted from Mitchell (2002)

SUBGROUP/ SPECIES	ORGANISM	ORIGIN	REFERENCE
1	*L. ferrooxidans*, L3	Mine Ilba, Romania	Zenneck, 1987
1	*L. ferrooxidans*, L4	Mine Ilba, Romania	Zenneck, 1987
2	*L. ferriphilum*	Enrichment culture, Peru	Coram and Rawlings (2002)
3	*L. ferrodiazotrophum*, L7	Rammelsberg, Goslar, Germany	Schröter, 1993; Mitchell 2002; Tyson et al, 2005

Interestingly, BlastP search detected that the gene analogues for *jqsA/jqsS* are present in all species of *Leptospirillum* (Table 12). Additionally, the genome of *L. rubarum* (subgroup II) was also screened by BlastP search, even though extracts of this strain have not been used until now in the biotests (see Figure 28).

Results of the BlastP search indicated an maximal identity between 24-29% for the gene analogues of *jqsA/jqsS* of the *Janthinobacterium* genome compared to the genome sequence of three different *Leptospirillum* species.

Table 12: BlastP search for the genes jqsA/jqsS of *Janthinobacterium* against the genomes of three *Leptospirillum* species.

ORGANISM	ANNOTATED PROTEIN FUNCTION	LOCUS_TAQ	MAX. IDENTITY BY BLASTP	CORRESPON-DING GENE
L. ferrooxidans	8-amino-7-oxononanoate synthase	Lferr_0419	27%	*jqsA, Janthinobacterium*
L. rubarum	8-amino-7-oxononanoate synthase	UBAL2_86920025	27%	*jqsA, Janthinobacterium*
L. ferrodiazotrophum	8-amino-7-oxononanoate synthase	UBAL3_80420040	29%	*jqsA, Janthinobacterium*
L. ferrooxidans	multisensor signal transduction histidine kinase	LFE_0034	27%	*jqsS, Janthinobacterium*
L. rubarum	putative multi-sensor signal transduction histidine kinase	UBAL2_79310198	24%	*jqsS, Janthinobacterium*
L. ferrodiazotrophum	integral membrane sensor signal transduction histidine kinase	UBAL3_95450148	29%	*jqsS, Janthinobacterium*

Further bioinformatic analysis (Multiple Sequence Alignment (MSA)) was applied to indicate functional, structural and/or evolutionary relationships between these biological sequences. The software "ClustalW2" is a general purpose multiple sequence alignment program for DNA or proteins and was used for this study. Via the "boxshade-server" publication quality output was generated and the result is shown in Figure 31. 8-amino-7-oxononanoate synthase is the gene which is the homologue of the *jqsA* gene in the sequence of *Janthinobacterium*. Results indicate that the protein "8-amino-7-oxononanoate synthase" is present in all genomes of the *Leptospirillum* strains and has identity with the gene analogues of *Janthinobacterium*. Identity is highlighted in black.

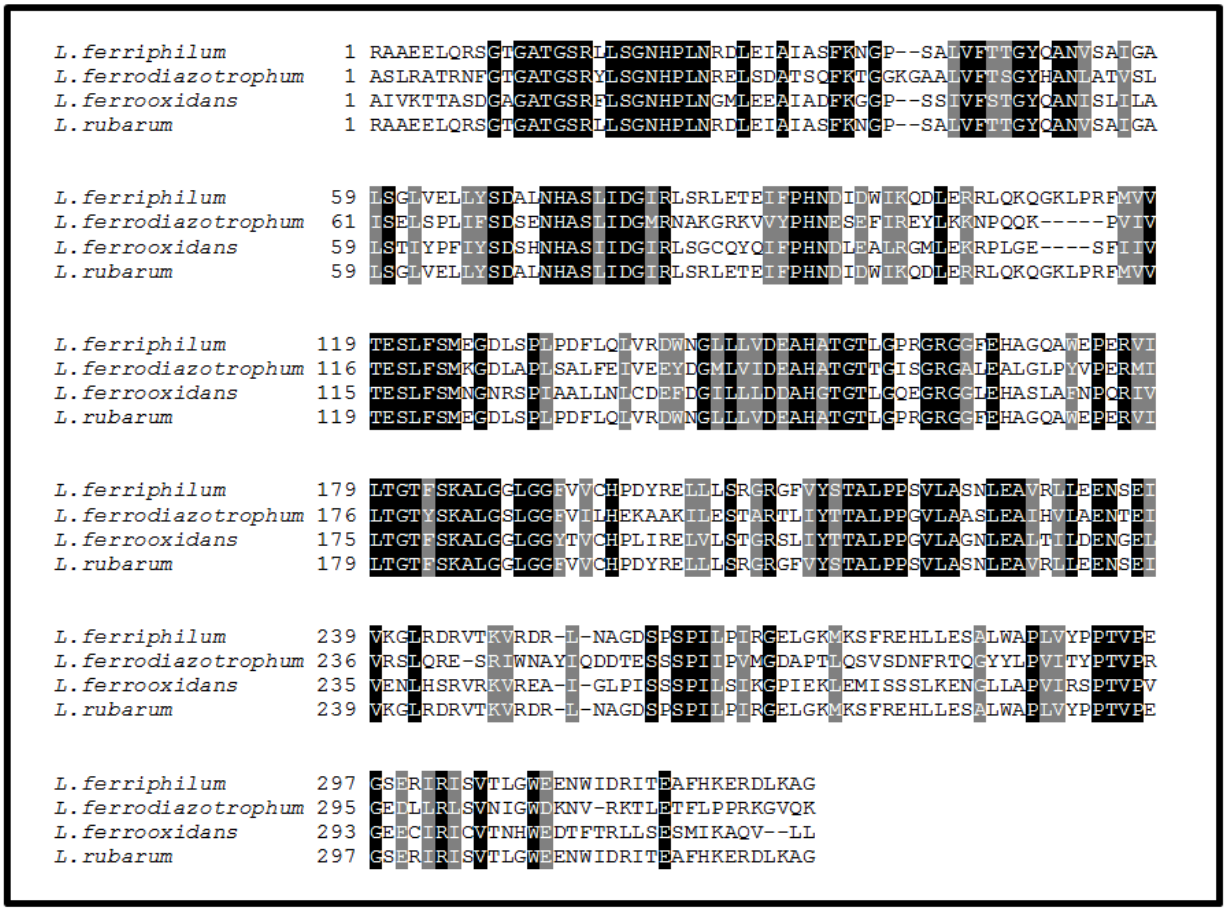

Figure 31: Results of the Multiple Sequence Alignment for the proteins "8-amino-7-oxononanoate synthase" of the *Leptospirillum* species generated via the software "ClustalW" and "boxshade". Proteins found via BlastP search were chosen to apply the multiple sequence alignment between these sequences. Black boxes represent conserved aminoacidic residues.

The same bioinformatic search was done for the gene *jqsS* of *Janthinobacterium* which seem to be the homologue of the "signal transduction histidine kinase", which was detected in the various strains of *Leptospirillum*. Results of the multiple sequence alignment showed that "signal transduction histidine kinase" had less identity to *jqsS* compared with the results obtained for the gene *jqsA* and the "8-amino-7-oxononanoate synthase" (data not shown; further explanations see 5.5). Summarizing, the bioinformatic research demonstrated, that the homologues of the *jqsA* genes are present in all *Leptospirillum* spp.

5. Discussion

Acidophilic bacteria and archaea have an important role in industrial and environmental bioleaching systems. The industrial applications have a significant impact on heavy metal recovery by bioleaching. Thus, this process has to be optimized for industrial applications or prevention of ARD/AMD. In bioreactors or constructed ore heaps, MS such as pyrite are oxidized and heat in form of energy is released during this process. Temperatures between up to 100°C are common for such operations (Okibe & Johnson, 2004). Therefore, between 45-50°C moderately thermophilic microorganisms are of interest. In this thesis four microorganisms of this group, *L. ferriphilum*, *At. caldus*, *Am. ferrooxidans* and *F. acidophilum*, were chosen, because they are known to be widely distributed in bioleaching processes operating at this temperatures (Dopson & Lindström, 2004). For a better understanding of the bioleaching process at moderately thermophilic conditions, it is necessary to investigate attachment and biofilm formation in pure and mixed cultures. It also required to understand their communication mechanisms that can be relevant for interactions in such consortia. Part I of this thesis was focused to answer the following two questions:

- Which member of a defined mixed consortium is the first colonizer of a pyrite surface?
- Which member needs a precolonization by other bacteria to be able to attach or to establish itself in a biofilm?

The investigations about the first colonizer on a pyrite surface and possible need of precolonization were divided in attachment to and leaching experiments on pyrite coupons and grains.

5.1 Attachment to and leaching experiments on pyrite grains

A standardized attachment test had been established previously (Harneit et al., 2006) and was used in this study to investigate initial attachment to pyrite grains. The possibility of errors in total cell count determinations related to cell division during the experimental period can be neglected due to the longer generation times of these bacteria. Also attachment to the glass was quantified and proven to be negligible. Besides the attachment tests, standardized leaching experiments were carried out to quantify the oxidation activities for pyrite.

The correlation between the results of attachment to and leaching experiments on pyrite grains indicate that *L. ferriphilum* had the highest attachment and leaching. Previous reports have also described this organism to be the dominant one in industrial biooxidation tanks (Rawlings et al., 1999; Coram & Rawlings, 2002). Own experiments using mixed cultures of *L. ferriphilum* with especially the sulfur-oxidizing organism *At. caldus* indicated also that in this combination attachment and leaching are strongly increased. These leaching bacteria seem to complement each other. *L. ferriphilum* oxidizes iron (II) ions to iron (III) ions providing the oxidizing agent. RISCs are released by the attack of the iron (III) ions and can be oxidized further by *At. caldus.* This results in an increase of acidity. *At. caldus* has been described as the dominant sulfur-oxidizing bacterium in bioleaching reactors at 45° (Fouchera et al., 2003; Okibe et al., 2003). Furthermore, it was described that *At. caldus* removed solid sulfur precipitates on a mineral surface by production of surface-active agents. These mobilize the sulfur and allow the continued access of Fe (III) ions to the MS surface (Dopson & Lindström, 1999; Semenza et al., 2002). In leaching communities it is often described that the iron- and sulfur- oxidizers in a mixed consortium led to an enhancement of MS dissolution (Johnson, 1998).

In contrast, in case of the combination of *L. ferriphilum* with other iron-oxidizers less favorable results for attachment and leaching were obtained. Attachment of a mixed culture with *L. ferriphilum* and *F. acidophilum* was lower than theoretically calculated. Nevertheless, leaching of pyrite yielded still 3.2 g/L iron (II) ions. This seems to indicate that (at least in shaken flask experiments) reduced attachment values (to pyrite grains) do not necessarily mean that low pyrite dissolution values are obtained.

Interestingly the other combination, *L. ferriphilum* and *Am. ferrooxidans,* showed in both attachment and leaching experiments decreased results. In mixed cultures cells attached about 20% less than theoretically expected. These two iron-oxidizers seem to compete with each other. Both use iron (II) ions as substrate. Competition is well known in bioleaching environments. Under mesophilic temperature conditions it has been described for the two iron-oxidizers *L. ferrooxidans* and *At. ferrooxidans* (Norris et al., 1988; Rawlings et al., 1999). The basis for the competition is unknown. One mechanism may be based on QS-molecules., which will be discussed in a later section (see 5.5).

The fourth combination of *L. ferriphilum* with *At. caldus* and a mixotrophic organisms such as *F. acidiphilum* or *Am. ferrooxidans* exhibited an increase in leaching efficiency. Especially for mixtures with *L. ferriphilum*, *At. caldus* and *F. acidiphilum,* it became obvious, that they

complement each other. In this mixture, the same amount of iron (III) ions was detected compared to mixtures of *L. ferriphilum* with *At. caldus*. The role of *Ferroplasma* spp. in such consortia is not well investigated. This organism is often described to be present under moderately thermophilic conditions. Edwards et al. showed that *Ferroplasma* was dominant in an acid-leaching biofilm in an iron-mountain side (Edwards et al., 2000). Golyshina et al. isolated the type strain of *Ferroplasma* from a pilot plant bioreactor (Tula, Russia) for biooxidation of gold-bearing arsenopyrite-pyrite concentrates (Golyshina et al., 2000).

Okibe & Johnson described in previous studies a positive leaching effect for a mixed culture of *L. ferriphilum* MT6, *At. caldus* KU and *Ferroplasma* M17, too. Facultative mutualism was their possible explanation (Okibe & Johnson, 2004) and is defined as a relationship between different species in which each species benefits (Bronstein, 2001). A mixed culture containing both autotrophic and mixotrophic bacteria seems to be optimized for pyrite dissolution (Okibe & Johnson, 2004). The presence of mixo-/heterotrophic bacteria may help to avoid the accumulation of organic material which may accumulate and inhibit autotrophic iron-oxidizers (Bacela-Nicolau & Johnson, 1998). Possibly, *Ferroplasma* benefits from this niche and uses organic material. It seems to be more resistant against low pH (Golyshina et al., 2000) than the competitor *Am. ferrooxidans*, which also utilizes organic material. Clum described a pH of 2 as optimal growth condition for *Am. ferrooxidans* (Clum et al., 2009), whereas growth of *Ferroplasma* spp. could also be detected at a pH of 1.3 (Golyshina et al., 2000).

Mixed cultures of *At. caldus* with *Am. ferrooxidans* also complement each other. Mixtures of both organisms exhibited a slight but not significant increase of attached cells. However, pyrite dissolution was furthered. In mixed cultures 2.9 g/L iron (III) ions were detected, while pure cultures of *Am. ferrooxidans* reached only 2.4 g/L. But, compared to mixtures of *L. ferriphilum* with *At. caldus* (4 g/L iron(III) ions) the increase is negligible.

Summarizing, mixed cultures of moderately thermophilic microorganisms attach in higher numbers and are more efficient for pyrite leaching than pure cultures. *L. ferriphilum* is probably the most important organism in this temperature range and industrial processes should be optimized for this bacterium. The most useful combinations of mixed consortia are on the one hand *L. ferriphilum* with *At. caldus* or on the other hand *L. ferriphilum* with *At. caldus* and *F. acidophilum*. Combinations of the strains *At. caldus, Am. ferrooxidans* and *F. acidophilum* are less efficient. Mixed cultures of *At. caldus* and *F. acidophilum* or *L. ferriphilum* and *Am. ferrooxidans* should be avoided.

5.2 Attachment to and biofilm formation on pyrite coupons

Attachment to and biofilm formation on pyrite coupons was investigated with pure and mixed cultures of *L. ferriphilum*, *At. caldus* and *Am. ferrooxidans*. *F. acidophilum* was not further investigated. A detailed analysis of the role of this bacterium in attachment and leaching are being part of another PhD thesis of our group.

The highest attachment to pyrite coupons was observed for cells of *L. ferriphilum* followed by those of *Am. ferrooxidans*. *At. caldus* attached in very low numbers. Lectin staining was used for visualization of parts of a formed biofilm on the MS surface. ConA has an affinity for α-D-mannosyl and α-D-glucosyl residues of branched α-mannosidic structures (Marchalonis, 1987) and was already used for staining biofilms of moderately thermophilic leaching bacteria attached to pyrite (Holuscha, 2010). It was further used in biofilm studies with *At. ferrooxidans* (Bellenberg et al., 2012). Cells of *L. ferriphilum* had after 3 days of incubation a strong ConA signal. Tthe high amount of attached *L. ferriphilum* cells and the corresponding strong lectin signal complicated the visualization of cells. Strong EPS formation has already been described in case of AFM and EFM studies of *Leptospirillum spp.* (Noel, 2008; Florian et al., 2011; Florian, 2012). Cells are surrounded by EPS and the tendency to form cluster was described. However, it remained unclear, whether cells attach as single cells or as cell clusters, because previous studies had shown that *Leptospirillum spp.* formed already clusters in solution (Norris 1990, Hallmann et al. 1992). The strong lectin signal suggests that a high amount of EPS is formed by *L. ferriphilum* cells. Gehrke et al. (1998) analyzed the EPS of *At. ferrooxidans* and *L. ferrooxidans* cells grown on pyrite. Sugars such as glucose, rhamnose, fucose, xylose and mannose were part of the EPS (Gehrke et al., 1998; Sand & Gehrke, 2006). It is likely that the EPS of *L. ferriphilum* contain ConA reacting sugars.

Am. ferrooxidans did not show a strong lectin signal, although attached cells were visible after 7 days. Possible explanations may be that ConA was not a useful lectin. Fife et al. described an experiment in which a wheat germ agglutinin (WGA) based staining technique was optimized for the staining of acidophilic, Gram-positive bacteria. WGA binds to the exposed n-acetylglucosamine residues of the peptidoglycan layer of Gram-positive bacteria. Gram-negative bacteria were not stained, because the outer membrane of cells prevents the lectin binding to the peptidoglycan layer (Fife et al., 2000). Another explanation could be that *Am. ferrooxidans* produced only a low amount of ConA binding sites on the EPS under these conditions. Further experiments, especially an EPS analysis of *Am. ferrooxidans*, are

necessary to clarify these findings. In addition, WGA should be tested as a lectin for staining and thus, visualizing of occuring sugars in the EPS of *Am. ferrooxidans*.

Also, mixed cultures of *L. ferriphilum* and *At. caldus* were tested for attachment to and biofilm formation on pyrite coupons. This experiment was focused on this special mixture, because these two organisms had been shown to complement each other and reached the highest values in previous attachment and leaching experiments. On the pyrite surface increased aggregate and biofilm formation was noted. Increased aggregate formation was also described for mixtures of *L. ferriphilum* with *At. caldus* as planktonic or biofilm cells. AFM and EFM studies showed that a higher amount of EPS was present if both organism were mixed compared to pure cultures (Noël, 2008).

Besides EPS production is an important aspect, also Chemotaxis is fundamental for attachment to and biofilm formation on MS surfaces. Edwards et al. described oriented cell attachment on MS surfaces by *At. caldus* cells, suggesting that this bacterium chemotactically select optimal sites for growth on the MS surface (Edwards et al., 2000). A chemotatic response towards metal ions and other compounds has been demonstrated for cells of *At. ferrooxidans* and for *L. ferrooxidans* (Jerez, 2000; Meyer et al., 2002). Furthermore, in cells of *L. ferriphilum* different chemotactic sensory transducers occur dispersed in the genome (Mi et al., 2011). Consequently, bacteria of this leaching consortium could use chemotaxis to detect favorable attachment sites for adherence MS oxidation.

5.3 Precolonization experiments

Precolonization experiments of surfaces of pyrite coupons were carried out to investigate, which organism is the first colonizer and which organism needs a precolonization of a mineral by other bacteria. The combinations of cells of *Am. ferrooxidans* and *At. caldus* were not influenced by precolonization of one of these two bacteria, respectively. However, an increase of aggregate formation was detected for mixed cultures of *L. ferriphilum* and *At. caldus*. Attachment and biofilm formation were not increased with *At. caldus* as the first colonizer. In contrast, when *L. ferriphilum* was the first colonizer, attachment of *At. caldus* seemed to be increased. To clarify this finding, precolonization experiments with pyrite grains were done. It was investigated, whether *At. caldus* needs the presence of EPS from *L. ferriphilum* on the mineral surface or whether the presence of an active biofilm of *L. ferriphilum* cells is necessary for attachment. The biofilm maybe found to be the source of QS molecules. These

may then cause an increased attachment of *At. caldus* cells to MS surface. On the one hand, the importance of EPS for attachment to MS by leaching bacteria was described previously (Gehrke et al. 2001, Harneit et al. 2006, Sand and Gehrke 2006). On the other hand, also an influence of QS molecules such as AHLs has been mentioned to influence attachment and biofilm formation on MS (Ruiz et al., 2007; Gonzalez et al., 2012).

Both hypothesizes may possibly explain an increased attachment of *At. caldus* to MS. To clarify this question, pyrite grains were precolonized with *L. ferriphilum* cells and were heat-inactivated later on. A temperature of 80°C was chosen for the heat inactivation of cells to prevent changes in the EPS composition. Attachment of cells of *At. caldus* was not influenced in case of inactivated *L. ferriphilum* cells or only their EPS present on the MS surfaces. However, active *L. ferriphilum* cells attached on pyrite grains caused an increase of attachment of cells of *At. caldus* by 25%. Thus, the idea of an effect of surface bound EPS of *L. ferriphilum* cells on an attachment of cells of *At. caldus* is invalid. These findings seem to influence a possible excretion of QS- molecules or a chemotactic response of *At. caldus* to RISC compounds on the pyrite surface after *L. ferriphilum* action. As described (see 5.2) *At. caldus* is able to use chemotaxis to search for optimal sites for growth on sulfur-containing surfaces. If active *L. ferriphilum* cells oxidize iron compounds of a MS and release RISCs *At. caldus* is may be able to detect such RISC-rich sites and attach to those. As a consequence, it needs active *L. ferriphilum* cells to start the process of leaching so that RISC-rich sites on the MS became available for *At. caldus*. The possibility of a QS-signal related response by cells of *At. caldus* was analyzed in the following part of this thesis. Here the focus was to answer following questions:

- Is there an effect of QS signal molecules on cultures of moderately thermophilic leaching bacteria?
- Do these microorganisms produce QS signal molecules?
- Do these signal molecules effect attachment to and biofilm formation on pyrite in cultures of moderately thermophilic leaching bacteria?
- Does the utilization of such QS signal molecules influence bioleaching at moderately thermophilic conditions?

5.4 Effect of N-acetyl-homoserine-lactones and other signal molecules

N-acetyl-homoserine-lactones

Recently, it has been shown that the external addition of synthetic AHLs enhanced attachment of *At. ferrooxidans* to pyrite and sulfur surfaces. This enhancement was accompanied by a concomitant increase of EPS (Gonzalez et al., 2012). Also Jerez proposed the modulation of attachment of leaching bacteria to MS through an interference with their QS response as a new way to control metal extraction (Jerez, 2009). It has been described that EPS production and biofilm formation are controlled via QS (Riedel et al., 2001; Decho et al., 2011). Therefore, QS may possibly used even to manipulate biotechnological processes (Choudhary and Schmidt-Dannert, 2010).

The effect of AHLs on moderately thermophilic leaching bacteria was not been investigated until now. Therefore, different types of AHLs were tested with cultures of *At. caldus* and *L. ferriphilum*. C14-, 3-oxo-C14- and 3-hydroxy-C14-AHL decreased the attachment to pyrite coupons for cells in pure cultures of *L. ferriphilum* (Figure 16) with 3-hydroxy-C14-AHL showing the strongest inhibition. Pure cultures of *At. caldus* exhibited an increased amount of attached cells, if C8-, 3-oxo-C8- and 3-hydroxy-C8-AHLs had been added to the growth medium (Figure 17). Leaching with mixed cultures of both organisms was increased, if C8- or 3-hydroxy-C8-AHL were present. The addition of 3-hydroxy-C14-AHL led to a leaching inhibition of 37.5% for pure cultures of *L. ferriphilum* and for mixed cultures of both organisms (Figure 18).

Interestingly, these C14-AHLs were described to enhance attachment by cells of *At. ferrooxidans* to sulfur and pyrite surfaces (Gonzalez et al., 2012). Indigenous *At. ferrooxidans* cells, originating from a semi-industrial reactor, grown on pyrite coupons showed the same effect in the presence of the C14-AHLs. However, this reactor community also contained cells of *L. ferriphilum*, which indicates a decrease of planktonic *L. ferriphilum* cells in the reactor when C14-AHLs were present. They suggested that attachment of *L. ferriphilum* was enhanced but this effect was not shown in this study. Comparing both studies, it became obvious that different conditions such as temperature and consortia (defined or undefined consortia) were tested. The effect of the addition of 3-hydroxy-C14-AHL to a mixture of *At. ferrooxidans* and *L. ferriphilum* has not been studied until now. It would be of interest especially since Gonzalez et al. (2012) described that a cross-communication between

the members of a bioleaching community could be possible. Also the fact that 3-hydroxy-C14-AHL is excreted by *At. ferrooxidans* (Farah et al., 2005) is of interest.

A bioinformatic search (Table 8) indicated that the QS-regulatory gene of *At. ferrooxidans* , *afeR* (Farah et al., 2005), is also present in the genome of *L. ferriphilum* and other *Leptospirillum* spp.. The search for analogues of the gene *afeR* in the genome of *L. ferriphilum* indicated 36% identity via BlastP search between both genomes, the one of *At. ferrooxidans* and *L. ferriphilum*. The genomes of the strains from the *Leptospirillum* family showed similar identities (between 30-40%) for AfeR compared to the genome of *At. ferrooxidans*. Thus, the necessary regulatory protein for the QS mechanism described in *At. ferrooxidans* is also present in *Leptospirillum* spp. It has still to be tested via q-PCR experiments or high throughput studies, whether this gene is activated in cells of *L. ferriphilum* or not and under which conditions.

Similarities in attachment characteristics for mineral adapted cells of *At. ferrooxidans* and *L. ferriphilum* mineral- adapted cells were described by Africa et al. (2012), which indicated that both organisms are competing for the same attachment sites of a mineral. But the question, whether *L. ferriphilum* could possibly be influenced by specific AHLs excreted by *At. ferrooxidans* in a community, is still open and need to be answered. The fact that a cross-communication between *At. ferrooxidans* and *L. ferriphilum* may be possible, will be discussed later on.

BlastP search for AfeR in the genome of *At. caldus* indicated the absence of its encoding gene. It is still unclear, how *At. caldus* is influenced by the presence of the set of C8-AHLs. This seem to make a crosstalk via an AHL autoinducer system regulated via AfeI/R in mixtures of *L. ferriphilum* and *At. caldus* unlikely. Especially the fact that genomes of both organisms do not contain AfeI, the synthase protein in *At. ferrooxidans* (Farah et al., 2005), indicates that AHLs are not produced, at least not via the known pathways. Furthermore, it is confirmed that AfeI/R genes are only present in *At. ferrooxidans* and not in other *Acidithiobacillus* spp. such as *At. caldus* or *At. thiooxidans* (Prof. Dr. N. Guiliani, personal communication).

Recently, new LuxI/R- type QS systems have been described (Hao et al. 2010). Three cosmids (QS6-1, QS10-1 and QS10-2) were identified from environmental samples encoding a novel LuxI family AHL synthase and a LuxR family transcriptional regulator. These Lux protein homologues were able to synthesize multiple AHLs with different chain lengths (Hao et al., 2010). This finding suggests that AHLs could also be synthesized and detected via other

homologues of Lux-type proteins. Consequently, both, *At. caldus* and *L. ferriphilum*, might use different hitherto unknown ways for cell-cell communication. At least it may be possible that both organisms detect or respond to AHLs. In any case, an own production of AHLs seems to be non-existent. In extracts of both cultures via TLC coupled with biosensors for AHLs or by GC-MS AHLs could not be detected. In summary of these results *At. caldus* and *L. ferriphilum* are not able to produce AHLs under our tested conditions.

Nevertheless, it has to be mentioned that TLC and the application of a biosensor or GC or HPLC are limited methods for the detection of AHLs. On the one hand, if a negative result for AHLs is indicated by a biosensor, it is not possible to conclude from the nonappearance of a signal that this organism does not produce AHLs (Steindler & Venturi, 2007). Furthermore, extracts might contain novel structures of AHLs, which are not detectable by the used biosensor. In this study two biosensors for the detection of AHLs were used, which respond to a wide range of AHLs (Steindler & Venturi, 2007), as shown in Table 13. Especially, *A. tumefaciens* is described to detect a broad range of AHLs and to possess the highest sensitivity for such compounds even at very low concentrations (Farrand et al., 2002; Zhu et al, 2003). *C. violaceum* is specific for short chain AHLs (Steindler & Venturi, 2007). Still, not all known AHLs are detected by these reporter strains.

Table 13: AHL biosensors as adapted from Steindler & Venturi (2007)

REPORTER STRAIN	REPORTER SYSTEM	RESPONDS TO	REFERENCE
Chromobacterium violaceum	Violacein pigment	C4 to C8-AHLs	Mc Clean et al., (1997);
Agrobacterium tumefaciens pNTL4	ß- galactosidase	All 3-oxo-AHLs; C6 to C14-AHLs; C6 to C10-3-hydroxy-AHLs	Farrand et al., (2002); Zhu et al., (1998; 2003)

Moreover, the threshold of sensitivity of the biosensor for a detection of AHLs is important. If the tested organisms produce just low concentrations of AHLs the biosensor may show negative results (Steindler & Venturi, 2007). The time of AHL extraction is also significant, as it is known that AHL QS systems are regulated by specific environmental conditions and have to be active (Venturi, 2006).

As a confirmation of the TLC- biosensor results and for quantitative data, chromatographic methods coupled with mass spectroscopy such as GC-MS or LC-MS should be considered. The detection limit of the chromatographic method is important, because it is known that

these compounds are produced at very low concentrations (Cataldi et al., 2004). Another critical point is the possibility of a high background noise which may be caused by the spent medium extract, given that the application of dichlormethane is an unspecific way of extraction for organic substances. GC, HPLC or LC coupled with a mass spectrometer are frequently used for the detection of AHLs (Li et al., 2006). Fragmentation and protonation of the lactone ring lead to a characteristic m/z 102 value, which is useful for screening of AHLs in unknown samples (Li et al., 2006). As mentioned before until now, no AHL signals could be detected in extracts of cells of *At. caldus* or *L. ferriphilum* with the use of this techniques.

Biofilm formation on polycarbonate filters was also influenced with cultures of *L. ferriphilum* by addition of C14- or 3-hydroxy-C14-AHL. In these cases, ConA staining as visualization technique of parts of a biofilm indicated a stronger signal as compared to control cultures and to the addition of 3-oxo-C14-AHL. Gonzalez et al. (2012) discussed that AHLs operate as mediator for CPS/EPS production and, subsequently, for the colonization of a MS. They found, that CPS formation was increased after an AHL addition to cultures of *At. ferrooxidans*. An increase of biofilm formation by *At. ferrooxidans* on pyrite surfaces is attributed to two outer membrane polysaccharide export proteins, AFE_1339 and AFE_2975 (Bellenberg et al., 2012). Expression levels of AFE_2975 were enhanced in biofilm and in planktonic cells of *At. ferrooxidans* subpopulated on pyrite (Bellenberg et al., 2011). This gene was also upregulated after 24h incubation of cells of *At. ferrooxidans* grown on pyrite (Vera et al., 2011). AFE_ 1339 and its neighboring genes were not detectable by PCR, nor by high troughput proteomic studies of the type strain of *At. ferrooxidans* ATTC 23270 (Dr. Mario Vera, personal communication). Reasons have been rearrangements and changes of the corresponding loci in the genome of the type strain due to of the long storage in strain collections.

Based on the information of these genes, BlastP search was done for genes of *At. ferrooxidans* suggested to be involved in EPS and biofilm formation in comparison with the genome of *L. ferriphilum* (Table 9). The respective genes of *L. ferriphilum* showed between 22- 46% maximal identity by BlastP to the genes of *At. ferrooxidans*. However, it remains to test via Q-PCR or high troughput proteomics whether these genes are up- or downregulated by an addition of AHLs during biofilm formation in *L. ferriphilum*.

In this thesis we have shown that, the addition of 3-hydroxy-C14-AHL inhibited leaching of pyrite and iron-oxidation of *L. ferriphilum* cells. If a cross-talk between *At. ferrooxidans* and

L.ferriphilum would be possible, one could assume, that *At. ferrooxidans* excretes this AHL to inhibit cells of *L. ferriphilum*. The increase of EPS and biofilm formation by cells of *L. ferriphilum* may be explained as a stress response to the *At. ferrooxidans*- signal. It has been suggested, that AHL- signals are not only used for cell-density sensors, but also for intergeneric communication between two different species (Riedel et al., 2001). These authors investigated, whether the two species *Pseudomonas aeruginosa* and *Burkholderia cepacia,* are able to communicate via AHLs. Both species utilize AHLs to regulate biofilm formation concomitant with the expression of pathogenic traits (Davies et al., 1998; Riedel et al., 2001). Riedel et al., (2001) described that *B. cepacia* reacted to AHLs signals produced by *P. aeruginosa*, while the latter one did not respond.

Summarizing, the findings for a possible presence of AHLs in cultures of *At. caldus* or *L. ferriphilum* suggest, that both strains do not produce AHLs. However, a reaction to AHLs, which are present in the surrounding environment, might be possible. At least in the genome of *L. ferriphilum* a gene for a protein with similarity to the necessary transcriptional regulator, AfeR, seems to be exist. Attachment to and leaching of pyrite was influenced by addition of synthetic AHLs to pure or mixed cultures of *L. ferriphilum* and *At. caldus*. Biofilm experiments on floating filters with *L. ferriphilum* cells showed an increased EPS formation as a consequence of an addition of AHLs. A cross-communication between leaching bacteria is suggested but still needs to be proven in mixed cultures at the molecular level. Especially, the use of AHLs in the biomining industry seems to be promising, because it could be shown that a modulation of biofilm formation was possible. This may allow to develop new strategies to improve bioleaching for metal recovery. Furthermore, *L. ferriphilum* could be influenced in a way that leaching was inhibited. This opens up new possibilities for a control of environmental problems like AMD/ARD. However, still future requirements are necessary regarding to the degradability of AHLs in the environment of mixed consortia. Many species are able to consume AHLs as carbon or nitrogen source (Horswill et al., 2006). This trait is not dependent on the ability of the bacterium to produce AHLs. Degradation of AHLs occurs via two different enzymatic activities: the breakdown of the homoserine lactone ring via lactonases or the cleavage of the amide bond linking the acyl side chain to the homoserine lactone ring via acylases (Horswill et al., 2007). Especially, in species-rich environments such as in soil, a biological degradation of AHLs should be taken into account, if an addition of AHLs is considered.

Furanone

Besides AHLs, also the effect of an addition of a furanone, (5Z)-4-bromo-5-(bromomethylene)2(5H)-furanone, was tested with cultures of moderately thermophilic microorganisms. This furanone is produced by the red alga *Delisea pulchra* and it is a known inhibitor of QS systems (Givskov et al., 1996; Manefield et al., 1999; Ren et al., 2001). Changes in QS mediated bacterial properties such as swarming, bioluminescence or different phenotypes have been reported after addition of furanones to bacterial cultures (Givskov et al., 1996; Manefield et al., 1999). Furthermore, furanones are described to have antimicrobial and antifouling properties (Givskov et al., 1996).

Growth experiments with cultures of *L. ferriphilum*, *At. caldus* and *Am. ferrooxidans* resulted in the finding that already an addition of 0.2 µM furanone caused an inhibition of iron- or sulfur-oxidation, respectively. Inhibition increased with higher concentrations of the furanone (2 µM). Also, leaching experiments showed a strong inhibition by the use of 2 µM furanone in cultures of *L. ferriphilum*, *At. caldus* and *Am. ferrooxidans*. Floating filter experiments of cultures of *L. ferriphilum* indicated an inhibition of biofilm formation on floating polycarbonate filters.

Summarizing, the furanone inhibit growth, biofilm formation and leaching of cultures of *L. ferriphilum*, *At. caldus* and *Am. ferrooxidans.*

Furanones are from their chemical structure similar to AHLs (Givskov et al., 1996) and a competition between furanone and bacterial AHL for the LuxR protein is possible and was suggested by Pierson et al. (1998). As shown previously, a reaction to an AHL addition by cultures of *L. ferriphilum* and *At. caldus* was detectable. Accordingly, a receptor protein for AHLs has to be present in cells of *L. ferriphilum* and *At. caldus*. Consequently, and an antagonistic effect of the furanone to the AHLs could be one explanation. Nevertheless, also general toxicity could have caused an inhibition.

The inhibiting effect of the furanone occurred also with cultures of *Am. ferrooxidans*. No QS related studies are known until now for this bacterium. Since *Am. ferrooxidans* is a Gram-positive bacterium, an AHL dependant QS system is unlikely to exist because these are absent in Gram-positive bacteria (Federle & Bassler, 2003). However, the use of an AI-2 dependant QS system has been described for Gram-positive bacteria (Federle & Bassler, 2003) and also an inhibiting effect of furanones has been described. Lönn- Stensrud et al. (2007) described that (Z)-5-bromomethylene-2-(5H)-furanone inhibited biofilm formation and bioluminescence induction in *Streptococcus* spp. They suggested that this furanone interferes with the AI-2

signaling pathway. This could explain the inhibition by an addition of that furanone to cultures of *Am. ferrooxidans*. Further analysis has to be done to clarify, which QS system is present in cells of *Am. ferrooxidans* and which genes become active or blocked in the presence of a furanone.

After finishing the furanone experiments, cells were washed and subcultured by growth in fresh medium. In all cases cells grew again. For *L. ferriphilum* obviously the furanone competes with the AHLs for the binding site of the LuxR protein. Manfield et al., (2002) described that the half-life of the protein is reduced up to 100-fold (as measured by Western blot analysis) if furanones are present in cultures of *V. fischeri*. The furanone destabilized the LuxR protein which is not further accessible for AHLs and this changed the expression of AHL- dependant phenotypes or growth of bacteria. This is a reversible process. If the furanone is not present anymore, cells grow again in the known way in fresh media. Givskov et al. (1996) also showed that the inhibitory effect of furanones on cells in cultures of *Salmonella liquefaciens* was reversible and that a competition for the binding site of the regulatory protein was responsible (Givskov et al. 1996).

Concluding the effect of furanones, it is known that furanones interfere with AHL-driven systems or prevent bacterial growth. The effects make furanones interesting for biotechnological industries (Givskov et al., 1996). However, the brominated groups of furanones may cause environmental problems due to their toxicity. Thus, an application of furanones may be difficult, also in biomining. These problems may be overcome by structural modifications of AHL- and furanone- analogues. Estephane et al. (2008) and Sabbah et al. (2013) published studies were structural modifications of AHL- and furanone- analogues were described and tested these analogues for their ability to modulate LuxR- dependant bacterial QS. AHL analogues exhibited antagonistic as well as agonistic activity (Sabbah et al., 2012). In further studies they demonstrated the importance of the methylene group of furanones, either brominated or non-brominated, in LuxR- dependent QS inhibition. Interestingly, the non-brominated furanone exhibited also a strong inhibitory effect (Sabbah et al., 2013). The way to modulate such untoxic antagonists of AHLs gives future perspectives for their use in biotechnological industries.

5.5 Investigations of (a) unknown QS-molecule(s) in cultures of *L. ferriphilum*

The third part of this study deals with the research on a possible novel QS molecule/s excreted by cells of *L. ferriphilum*. During the search for possible AHLs in extracts of cultures of *At. caldus* and *L. ferriphilum*, extracts of these were tested for effects on other leaching bacteria. The investigations showed that the extract originating from cultures of *L. ferriphilum* had a strong inhibiting effect for several iron- oxidizing bacteria. Sulfur oxidizers as well as reference bacteria such as *E. coli* and *B. subtilis* were not influenced by an addition of the "*L. ferriphilum*-extract". The "*L. ferriphilum*-extracts" dissolved in hexane, a strong hydrophobic solvent, achieved the strongest inhibitory effect with cultures of *L. ferriphilum*. This seems to indicate that a lipophilic substance(s) is/are involved. The findings, that the inhibitory effect of the extract was stronger when extracted from pyrite-grown *L. ferriphilum* cultures plus that extracts of older pyrite-grown cultures compared to younger ones were stronger inhibiting, suggests a biofilm related connection. Obviously, pyrite-grown cultures of *L. ferriphilum* produce more of the unknown substance than planktonic *L. ferriphilum* cells. A longer incubation time before extraction give the bacteria the time to establish a biofilm on pyrite before they excrete the unknown substance(s).

Another correlation to biofilm formation was observed by the results with floating filters and cultures of *L. ferriphilum* plus an addition of the "*L. ferriphilum*-extract". With addition of the extract, a stronger ConA signal was detectable for *L. ferriphilum* cells on floating filters as compared to the control assays (Figure 29). Thus, biofilm formation on floating filters with *L. ferriphilum* cells was increased with addition of the "*L. ferriphilum*- extract". An addition of 0.02% (v/v) of the "*L. ferriphilum*- extract" resulted also in a decrease of the number of cells on the filter surface. However, iron-oxidation was inhibited by addition of the extract. This may be a result by a QS- regulated stress response. Cells excrete or respond to the unknown substance by changes of their growth and increase biofilm formation to survive suboptimal conditions.

It is often described that biofilm and EPS formation are controlled via QS (Rives et al., 2005; Valenzuela et al., 2006). Thus, cells respond to changes in cell population densities via excretion of chemical signaling molecules (Hooshangi et al., 2008).

Our efforts to identify possible AHLs in extracts of *L. ferriphilum* cultures via bacterial biosensors and GC-MS experiments ended up with negative results. By a cooperation with the lab of Prof. W. Streit (Biozentrum Klein-Flottbeck, Universität Hamburg) it was possible to

test the "*L. ferriphilum*-extract" with a novel biosensor. Hornung et al. (2013) investigated the genome of the *Janthinobacterium* HH01 and exhibited that this bacterium lacks the general AHL- or AI-2- dependant QS systems. Homologuos genes of the *V. cholerae* CqsA/CqsS and the *L. pneumophila* LqsA/LqsS QS system were detected. On this basis, a *Janthinobacterium*-based biosensor test including violacein measurements to detect possible autoinducers in bacterial extracts has been developed. Testing the "*L. ferriphilum*-extract" with this biosensor gave a positive result. Violacein production of the biosensor strain was strongly increased in the presence of the "*L. ferriphilum*-extract" (Figure 30). This results seem to indicate that an autoinducer similar to the one of the *Janthinobacterium* is present in the "*L. ferriphilum*-extract".

Tiaden et al. (2010) reviewed the QS systems of *V. cholerae* and *L. pneumophila* and described the autoinducers for both bacteria. These organisms use α-hydroxyketones as AIs for their signalling pathways. *Legionella* utilizes 3-hydroxypentadecan-4-one and *Vibrio* uses 3-hydroxytridecan-4-one as autoinducer (Tiaden et al., 2010). Hornung et al. (2013) did not detect the full structure of the autoinducer used by the *Janthinobacteria* until now. But it is highly likely, that it is also an α-hydroxyketone, because of the similarities between the proteins JqsA/JqsS and CqsA/CqsS or LqsA/LqsS. However, it needs to be considered that *L. ferriphilum* is an acidophilic bacterium growing at pH 0 up to pH3. Consequently, the active compound/s must be a derivative/s of an α-hydroxyketone, because the latter one might not stable at low pH (Dr. Yves Queneau, personal communication).

Bioinformatic research was done to clarify whether the *L. ferriphilum* genome includes also homologues of the *jqsA/jqsS* genes. BlastP search was done to compare the genomes of *Janthinobacterium* with the one of *L. ferriphilum* (Table 10). The results of this search indicated between 27-28% maximal identity for *jqsA/jqsS* genes in *L. ferriphilum* compared to the ones in *Janthinobacterium.* Thus, there is a considerable likelihood of a presence of such genes in *L. ferriphilum*.

Summarizing the results of this work there is QS-related function of the unknown compound(s) in the "*L. ferriphilum* extract" and it has/have a lipophilic character. Also a biofilm-related effect was detectable. Together with the positive results of the *Janthinobacterium*-biosensor test and the BlastP search it is highly likely that the unknown compounds/s in the "*L. ferriphilum*-extract" is an autoinducer of the α-hydroxyketone-type. First attempts to identify the novel autoinducer via GC-MS failed, because the background noise of the extract is too high to allow a characterization of the compound. Different types of

alkanes (chain length between tridecan and octadecan) were already determined (data not shown) in the "*L. ferriphilum-extract*", but which compound influence the inhibitory effect is still unclear. Further experiments will be needed using HPLC-MS coupled with a fraction collector to enrich a fraction containing the unknown QS- molecule.

Besides the identification of the unknown QS-compound, it was tested whether a similar compound is only present in cultures of the species *L. ferriphilum* or also in other species of *Leptospirillum*. Therefore, extracts of different *Leptospirillum* strains from various species were extracted and used for inhibition tests in growth experiments with different cultures of *Leptospirillum* spp. The extracts of all *Leptospirillum* strains caused an inhibitory effect similar to the one shown for the "*L. ferriphilum*- extract" on iron-oxidation (Figure 28).

Biosensor tests with *Janthinobacterium* would be the next step to confirm the presence of the yet unknown autoinducer in extracts of the different *Leptospirillum* spp.. BlastP search exhibited that potential homologues of the genes j*qsA/jqsS* are encoded in genomes of strains of *Leptospirillum* (Table 12). The genome of *L. rubarum* (subgroup II) was also included in the BlastP search, even though extracts of this strain have not been available and tested until now. Further bioinformatic analysis (Multiple Sequence Alignment –MSA-) was applied to indicate functional, structural and/or evolutionary relationships between these gene sequences (Figure 31). Results indicate that the protein "8-amino-7-oxononanoate synthase" is present in all genomes of the tested *Leptospirillum*. It can be concluded that this protein is the homologue of the *jqsA* gene of *Janthinobacterium*. The same bioinformatic search was done for the "signal transduction histidine kinase" of the diverse *Leptospirillum* strains, the possible homologue gene of *jqsS* (*Janthinobacterium*). However, results of the MSA indicated a low identity as compared to values for the *jqsA* gene. This suggests that the present genes encode for proteins of the members of the histidine kinase family, but maybe with a function different from the *jqsS* gene.

There is high evidence that a novel QS system is present in strains of *Leptospirillum* spp. which encodes a homologue of the *Janthinobacterium*-, *Legionella*- and *Vibrio*-like QS system. Further analysis has to be done to verify that these novel autoinducers are the reason for the inhibition observed for iron oxidation in *Leptospirillum* spp., *Acidithiobacillus ferrooxidans, Am. ferrooxidans, At. ferrivorans* and *S. thermosulfidooxidans*. It still remains possible that a combination of unknown compounds has lead to the inhibitory effect. As a proof, α-hydroxyketones could be synthesized and used in growth experiments with different iron-oxidizers.

A novel QS molecule excreted by *L. ferriphilum* could explain also the observed effects in mixed cultures with *L. ferriphilum* and *Am. ferrooxidans*. If a cross-communication is possible between the different species, the decrease of leaching in mixed cultures of *L. ferriphilum* with *Am. ferrooxidans* may be explained by the presence of these QS-molecules. *Am. ferrooxidans* as a competitor of *L. ferriphilum* would become disadvantaged. Consequently, *L. ferriphilum* would excrete QS-molecules as a survival strategy. Furthermore, it has to be tested, whether the unknown QS-molecule is also the compound which leads to an increased attachment of *At. caldus* cells. Until now we know only, that sulfur oxidation of this organism is not inhibited by the presence of the "*L. ferriphilum*-extract".

5.6 Conclusions

- *L. ferriphilum* is the first colonizer of a pyrite surfaces in the moderately thermophilic temperature range
- Mixed cultures are more effective than pure cultures for attachment and leaching at moderately thermophilic conditions
- *At. caldus* needs precolonization of active *L. ferriphilum* cells to establish itself in (mixed) biofilms on pyrite surfaces
- Attachment and biofilm formation of *L. ferriphilum* and *At. caldus* are influenced by addition of specific AHLs. Consequently, leaching, at least for cells of *L. ferriphilum* cells, becomes manipulable.
- *L. ferriphilum* and *At. caldus* do not produce AHLs, but they are able to respond to their presence (external addition).
- (5Z)-4-bromo-5-(bromomethylene)2(5H)-furanone inhibits growth, biofilm formation and leaching of moderately thermophilic leaching bacteria. The inhibition is reversible.
- Extracts of *L. ferriphilum* cultures strongly inhibit iron-oxidation in *Leptospirillum* spp., *At. ferrooxidans*, *Am. ferrooxidans*, *At. ferrivorans* and *S. thermosulfidooxidans*. A correlation with a novel autoinducer is possible, but needs further proof.
- A novel QS- related autoinducer molecule was found in cultures of *L. ferriphilum*. It is highly likely that this autoinducer is an α-hydroxyketone.
- The inhibitory effect on iron-oxidation was also detectable for extracts from several *Leptospirillum* species.
- Cross-communication via AHLs or the novel autoinducer is likely to occur in bioleaching bacteria.

6. Literature

Acuña, J., Rojas, J., Amaro, A., Toledo, H., Jerez, C. (1992). Chemotaxis of *Leptospirillum ferrooxidans* and other acidophilic chemolithotrophs: comparison with the *Escherichia coli* chemosensory system. FEMS Microbiol. Lett., 96, pp. 37 - 42.

Acuña, J., Peirano, I., Jerez, C. (1986). In vivo and in vitro methylation of proteins from chemolithotrophic microorganisms: a possible role in chemotaxis. Biotechnol. Appl. Biochem. 8, pp. 309–317.

Africa, C.J., van Hille, R.P., Harrison, S. (2012). Attachment of *Acidithiobacillus ferrooxidans* and *Leptospirillum ferriphilum* cultured under varying conditions to pyrite, chalcopyrite, low-grade ore and quartz in a packed column reactor. Appl. Microbiol. Biotechnol., doi:10.1007/s00253-012-3939-x.

Aljanabi, S.M. and Martinez, I. (1997). Universal and rapid salt-extraction of high quality genomic DNA for PCR-based techniques. *Nucl. Acids Res*. 25, pp. 4692-4693.

Amann, R., Fuchs, B., Behrens, S. (2001). The identification of microorganisms by fluorescence *in situ* hybridisation. Curr. Opin. Biotechnol., 12, pp. 231 - 236.

Amann, R., Ludwig, W., Schleifer, K.-H. (1995). Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. Microbiol. Rev., 59, pp. 143 - 169.

Andrews, G. (1988). The selective adsorption of *Thiobacilli* to dislocation sites on pyrite surfaces. Biotechnol. Bioeng., 31, pp. 378 - 381.

Bacela-Nicolau, P., Johnson, D. (1999). Leaching of pyrite by acidophilic heterotrophic iron-oxidizing bacteria in pure and mixed cultures. Appl. Environ. Microbiol., 65, pp. 585 - 590.

Baker, M.D., Wolanin, P.M., Stock, J.B. (2005). Signal transduction in bacterial Chemotaxis. BioEssays 28, pp. 9-22.

Bellenberg, S., Leon-Morales, C.F., Sand, W. and Vera, M. (2012). Visualization of capsular polysaccharide induction in *Acidithiobacillus ferrooxidans*. Hydromet. 129-130, pp. 82-89.

Bellenberg, S., Vera, M., Sand, W. (2011). Transcriptomic studies of capsular polysaccharide export systems involved in biofilm formation of *Acidithiobacillus ferrooxidans*. Biohydromet.: Biotech key to unlock mineral resources value. Proceedings of the 19th international biohydrometallurgy symposium. Central south university press, Changsha, China.

Bosecker, K. (1997). Bioleaching: metal solubilization by microorganisms. FEMS Microbiol. Rev., 20, pp. 591 - 604.

Boyer, M., Wisniewski-Dye, F. (2009). Cell-cell signaling in bacteria: not simply a matter of quorum. FEMS Microbiol. Ecol., 70, pp. 1-19.

Brandl, H. (2001). Microbial leaching of metals. In: Rehm, H.J., (ed.) Biotech. 10, Wiley-VCH, Weinheim, pp. 191-224.

Bronstein, J.L. (2001). The exploitation of mutualisms. Ecology Letters, 4, pp. 277-287.

Camilli, A., Bassler, B.-L. (2006). Bacterial small-molecule signaling pathways. Science, 311, pp. 1133-1116.

Cataldi, T.R.I., Bianco, G., Frommberger, M., Schmitt-Kopllin, Ph. (2004). Direct analysis of selected N-acyl-L-homoserine lactones by gas chromatography/mass spectrometry. Rapid Commun. Mass Spectrom., 18, pp. 1341-1344.

Chakraborty, R. and Roy, P. (1992). Chemotaxis of chemolithotrophic *Thiobacillus ferrooxidans* towards thiosulfate. FEMS Microbiol. Lett. 98, pp.9-12.

Choudhary, S., Schmidt-Dannert, C. (2010). Applications of quorum sensing in biotechnology. Appl Microbiol Biotechnol 86, pp. 1267–1279.

Clark, D.A. and Norris, P.R. (1996). *Acidimicrobium ferrooxidans* gen. nov. sp. Nov.: Mixed culture ferrous iron oxidation with *Sulfobacillus* species. Microbiol. 142, pp. 785-790.

Clum, A., Nolan, M., Lang, E., DelRio, T.G., Tice, H., Copeland, A., Cheng, J.F., Lucas, S., Chen, F., Bruce, D., Goodwin, L., Pitluck, S. and Ivanova, N. (2009). Complete genome sequence of *Acidmicrobium ferrooxidans* type strain ICP. Stand. Genomic. Sci. 1, pp. 38-45.

Coram, N. and Rawlings, D. (2002). Molecular relationship between two groups of the genus *Leptospirillum* and the findings that *Leptospirillum ferriphilum* sp.nov. dominates south African commercial biooxidation tanks that operate at 40°C. Appl. Environ. Microbiol. 68, pp.838-845.

Crundwell, F.K. (2003). How do bacteria interact with minerals? Hydromet. 71, pp. 75-81.

Davis, R.A., Welty, A.T., Borrego, J., Morales, J.A., Pendon, J.G., Ryan, J.G., (2000). Rio Tinto estuary (Spain) 5000 years of pollution. Environ. Geol. 39, pp.1107–1116.

Davies, G.D., Parsek, M.R., Pearson, J.P., Iglewski, B.H., Costeron, J.W., Greenberg, E.P. (1998). The involvement of cell-to-cell signals in the development of a bacterial biofilm. Science, 280, pp. 295-298.

De Bruyn, J., Boogerd, F., Bos, P., Kuenen, J. (1990). Floating filters, a novel technique for isolation and enumeration of fastidious, acidophilic, iron-oxidizing, autotrophic bacteria. Appl. Environ. Microbiol. 56, 2891- 2894.

Decho, A.W., Frey, R.L., Ferry, J.L. (2011). Chemical challenges to bacterial AHL signalling in the environment. Chem Rev 111:86–99.

Delgado, M., Toledo, H., Jerez, C.A. (1998). Molecular cloning sequencing, and expression of a chemoreceptor gene from *Leptospirillum ferrooxidans*. Appl. Environ. Microbiol. 64, pp. 2380-2385.

Deutsches Einheitsverfahren zur Wasser-, Abwasser- und Schlammuntersuchung (1984). Bestimmung von Eisen, E1. , DIN 38406-I, Weinheim, Germany: Verlag Chemie.

DiSpirito, A., Silver, M., Voss, L., Tuovinen, O. (1982). Flagella and pili of iron-oxidizing *Thiobacilli* isolated from an uranium mine in northern Ontario, Canada. Appl. Environ. Mirobiol., 43, pp. 1196 - 1200.

Dopson, M., Lindström, E. (2004). Analysis of community composition during moderately thermophilic bioleaching of pyrite. Microb. Ecol., 48, pp. 19 - 28.

Dopson, M., Baker-Austin, C., Hind, A., Bowman, J.P., Bond P.L. (2004). Characterization of *Ferroplasma* isolates and *Ferroplasma acidarmanus* sp. nov., extreme acidophiles from

acid mine drainage and industrial bioleaching environments. Appl. Environ. Mirobiol., 70, pp. 2079 – 2088.

Doyle, R. J. (1994). 1. Lectins. 2. Microbial polysaccharides. In: (Eds.) Doyle, R J., Slifkin, M.; (1994). Lectin- Microorganism Interactions, pp. 3-65.

Edwards, K., Rutenberg, A. (2001). Microbial response to surface microtopography: The role of metabolism in localized mineral dissolution. Chem. Geol., 180, pp. 19 - 32.

Edwards, K., Bond, P.L., Gihring, T.M., Banfield, J.F. (2000). An archaeal iron-oxidizing extreme acidophile important in acid mine drainage. Science, 287, pp. 1796-1799.

Ehrhardt, C., Banas, E., Janik, J. (1968). Application of spherically curved chrystals for X-ray fluorescence. Appl. Spectrosc., 22, pp. 730 - 735.

Estephane, J., Dauvergne, J., Soulere, L., Reverchon, S., Queneau, Y., Doutheau, A. (2008). N-acyl-3-amino-5H-furanone derivatives as new inhibitors of LuxR- dependant quorum sensing: synthesis, biological evaluation and binding mode study. Bioorg. Med. Chem. Lett., 18, pp. 4321-4324.

Farah, C., Vera, M., Morin, D., Haras, D., Jerez, C., Guiliani, N. (2005). Evidence for a functional quorum-sensing type Al-1 system in the extremophilic bacterium *Acidithiobacillus ferrooxidans*. Appl. Environ. Microbiol., 71, pp. 7033 - 7040.

Farrand, S.K., Quin, Y., Oger, P. (2002). Quorum-sensing system of *Agrobacterium* plasmids: analysis and utility. Methods Enzymol., 358, pp. 452-484.

Federle, M. and Bassler, B.L. (2003). Interspecies communication in bacteria. JCI, 112, pp. 1291-1299.

Fife, D.J., Bruhn, D.F., Miller, K.F., Stoner, D.L. (2000). Evaluation of a fluorescent lectin-based staining technique for some acidophilic mining bacteria. Appl. Environ. Microbiol., 66, pp. 2208- 2210.

Florian, B. (2012). Investigation of initial attachment and biofilm formation of mesophilic leaching bacteria in pure and mixed cultures and their efficiency of pyrite dissolution. Dissertation, Aquatische Biotechnologie, Biofilm Centre, Universität Duisburg-Essen.

Florian, B., Noël, N., Thyssen, C., Felschau, I., Sand, W. (2011). Some quantitative data on bacterial attachment to pyrite. Miner. Eng., 24, pp. 1132 - 1138.

Fouchera, S., Bruneta, F.B., D'Huguesa, P., Clarensb, M., Godonc, J.J., Morin, D. (2003). Evolution of the bacterial population during the batch bioleaching of a cobaltiferous pyrite in a suspend solids bubble column and comparison with a mechanically agitated reactor. Hydromet., 71, pp. 5-12.

Fuqua, C. and Winans, S.C. (1996). Conserved cis-acting promoter elements are required for density-dependent transcription of *A. tumefaciens* conjugal transfer genes. J. Bacteriol. 178, pp. 435-440.

Gehrke, T., Telegdi, J., Thierry, D., Sand, W. (1998). Importance of extracellular polymeric substances from *Thiobacillus ferrooxidans* for bioleaching. Appl. Environ. Microbiol., 64, pp. 2743 - 2747.

Givskov, M., de Nys., R., Manefield, M., Gram, L., Maximilien, R., Eberl, L., Molin, S., Steinberg, P., Kjelleberg, S. (1996). Eukaryotic interference with homoserine lactone-mediated prokaryotic signalling. J. Bacteriol., 178, pp. 6618-6622.

Golyshina, O.V., Pivovarova, T.A., Karavaiko, G.I., Kondraleva, T.F., Moore, E.R., Abraham, W.R., Lüsndorf, H., Timmis, K.N., Yakimov, M.M. and Golyshin, P.N. (2000). *Ferroplasma acidiphilum* gen. nov., sp. nov., an acidophilic, autotrophic, ferrous-iron-oxidizing, cell-wall-lacking, mesophilic member of the *Ferroplasmaceae* fam. nov., comprising a distinct lineage of the *Archaea*. IJSEM 50, pp. 999-1006.

Golyshina, O.V. and Timmis, K.N. (2005). *Ferroplasma* and relatives recently discovered cell wall-lacking archaea making a living in extremely acid, heavy metal rich environments. Environ. Microbiol. 7, pp. 1277-1288.

Gonzalez, A., Bellenberg, S., Mamani, S., Ruiz, L., Echeverria, A., Soulere, L., Doutheau, A., Demergesso, C., Sand, W., Queneau, Y., Vera, M., Guiliani, N. (2012). AHL signaling molecule with a large acyl chain enhance biofilm formation on sulfur and metal sulfides by the bioleaching bacterium *Acidithiobacillus ferrooxidans*. Appl. Microbiol. Biotechnol. 97, pp. 3729-3737.

Gonzalez, J.-E., Keshavan, N.-D. (2006). Messing with bacterial quorum sensing. Microbiol. Mol. Biol. Rev., 70, pp. 859- 875.

González-Toril, E., Gómez, F., Malki, M., Amils, R. (2006). The isolation and study of acidophilic microorganisms. In F. Rainey, A. Oren, Extremeophiles- Methods in Microbiology (Vol. 35, pp. 471 - 510). Amsterdam, Netherlands: Elsevier/Academic Press.

Gray, N. (1996). Environmental impact and remediation of acid mine drainage: a management problem. Environ. Geol., 30, pp. 62 - 71.

Hallberg, K. B., Dopson, M. and Lindström, E. B. (1996). Reduced sulfur compound oxidation in *Thiobacillus caldus*. J. Bacteriol. 178, pp. 6-11.

Hallberg, K. B. and Lindström, E. B. (1994). Characterization of *Thiobacillus caldus* sp. nov., a moderately thermophilic acidophiles. *Microbiol.* 140, pp. 3451-3456.

Hallmann, R., Friedrich, A., Koops, H.P., Pommereninger-Roser, A., Rohde, K., Zenneck, C., Sand, W. (1992). Physiological characteristics of *Thiobacillus ferrooxidans* and *Leptospirillum ferrooxidans* and physicochemical factors influence microbial metal leaching. Geomicrobiol. 10, pp. 193-206.

Hao, Y., Winans, S.C., Glick, B.R., Charles, T.C. (2010). Identification and characterization of new LuxR/LuxI-type quorum sensing systems from metagenomic libraries. Environ Microbiol., 12, pp. 105-117.

Harneit, K., Göksel, A., Kock, D., Klock, J., Gehrke, T., Sand, W. (2006). Adhesion to metal sulfide surfaces by cells of *Acidithiobacillus ferrooxidans, Acidithiobacillus thiooxidans* and *Leptospirillum ferrooxidans*. Hydrometallurgy, 83, pp. 245 - 254.

Harrison, A. (1981). *Acidiphilium cryptum* gen. nov., sp. nov., heterotrophic bacterium from acidic mineral environments. Int. J. Syst. Bacteriol., 31, pp. 327 - 332.

Henke, J.-M., Bassler, B.-L (2004). Bacterial social engagements. Trends Cell Biol., 14, pp. 648-656.

Holuscha, D. (2010). Visualization of extracellular polymeric substances during attachment to metal sulfides by acidophilic microorganisms. Master Thesis, Universität Duisburg-Essen .

Hornung, C., Poehlein, A., Haack, F., Schmidt, M., Dierking, K., Pohlen, A., Schulenberg, H., Blokesch, M., Plener, L., Jung, K., Bonge, A., Krohn-Molt, I., Utpatel, C., Timmermann, G., Spieck, E., Pommerening-Röser, A., Bode, E., Bode, H., Daniel, R., Schmeisser, C. and Streit, W. (2013). The *Janthinobacterium* sp. HH01 genome encodes a homologue of the *V.cholerae* CqsA and *L.pneumophila* Lqsa autoinducer synthases. PLOS ONE 8, pp. 1-15.

Horswill, A.R., Stoodley, P., Stewart, P.S., Parsek, M.R. (2007). The effect of the chemical, biological, and physical environment on quorum sensing in structured microbial communities. Anal. Bioanal. Chem., 387, pp. 371- 380.

Hooshangi, S., Bentley, W.E. (2008). From unicellular properties to multicellular behavior: bacteria quorum sensing circuitry and applications. Curr. Opin. Biotechnol.,19, pp. 550-555.

Janosch, C. (2013). Sulfur oxidation in moderately thermophilic bacteria. Dissertation, Aquatische Biotechnologie, Biofilm Centre, Universität Duisburg-Essen.

Jerez, C.A. (2009). Metal extraction and biomining. Encyclopedia of microbiology, Oxford, Elsevier, pp. 407-420.

Jerez, C.A. (2007). Proteomics and metaproteomics applied to biomining microorganisms. In E. Donati, W. Sand, Microbial Processing of Metal Sulfides (pp. 35 - 58). Dordrecht, Netherlands: Springer.

Jerez, C.A. (2001). Chemotactic transduction in biomining microorganisms. Hydromet. 59, pp. 347-356.

Johnson, D.B. and Hallberg, K.B. (2009). Carbon, iron and Sulfur Metabolism in Acidophilic Miro-Organisms. Adv. Microbiol. Physiol. 54, pp. 202-255

Johnson, D.B., Bacelar- Nicolau, P., Okibe, N., Yahya, A. and Hallberg, K.B. (2001). Role of pure and mixed Gram-positive eubacteria in mineral leaching. In: Ciminelli VST,Garcia, Jr. O. Biohydromet. Fundamentals, Technology and sustainable development, Elsevier Science BV, Amsterdam, Part A, pp. 461-470.

Johnson, D.B. (1998). Biodiversity and ecology of acidophilic microorganisms. FEMS Microbiol. Ecol. 27, pp. 307-317.

Johnson, D.B. and Roberto, F.F. (1997). Heterotrophic acidophiles and their roles in the bioleaching of sulfide minerals. In: Biomining: Theory, microbes and industrial processes (Rawlings, D.E., Ed.), Springer-Verlag, Georgetown, pp. 259-280.

Kelly, D., Wood, A. (2000). Reclassification of some species of *Thiobacillus* to the newly designated genera *Acidithiobacillus* gen. nov., *Halothiobacillus* gen. nov. and *Thermithiobacillus* gen. nov. Int. J. Syst. Evol. Microbiol., 50, pp. 511 - 516.

Kimmel, K.E., Maier, S. (1969). Effect of cultural conditions on synthesis of violacein in mesophilic and psychrophilic strains of *Chromobacterium*. Can. J. Microbiol.15, pp.111–221.

Kinzler, K., Gehrke, T., Telegdi, J., Sand, W. (2003). Bioleaching - a result of interfacial processes caused by extracellular polymeric substances (EPS). Hydrometallurgy, 71, pp. 83 - 88.

Kock, D. (2003). Untersuchung zur Anheftung von *Acidithiobacillus ferrooxidans* an verschiedenen Mineralen. Diplomarbeit. Abteilung Mikrobiologie, Institut für Allgemeine Botanik, Universität Hamburg.

Li, X., Fekete, A., Englmann, M., Götz, C., Rothballer, M., Frommberger, M. Buddrus, K., Fekete. J., Cai, C., Schröder, P., Hartmann, A., Chen, G., Schmitt-Kopplin P. (2006). Development and application of a method for the analysis of *N*-acylhomoserine lactones by solid-phase extraction and ultra high pressure liquid chromatography. J. Chromatogr. , 1134, pp. 186–193.

Levican, G., Ugalde, J.A., Ehrenfeld, N., Maass, A., Parada, P. (2008). Comparative genomic analysis of carbon and nitrogen assimilation mechanisms in three indigenous bioleaching bacteria: predictions and validations. BMC genomics 9, pp. 1-19.

Llamas, I., Keshavan, N., Gonzalez, J.E. (2004). Use of *Sinorhizobium meliloti* as an indicator for specific detection of long-chain N-acyl homoserine lactones. Appl. Environ. Microbiol., 70, pp. 3715-3723.

Lönn-Stensrud, J., Peterson, F.C., Benneche, T., Scheie, A. (2007). Synthetic bromated furanone inhibits autoinducer-2-mdeiated communication and biofilm formation in oral streptococci. Oral Microbiol. Immun., 22, pp. 340-346.

Loosdrecht, M., Lyklema, J., Norde, W., Zehnder, J. (1990). Influence of interfaces on microbial activity. Microbiol. Rev., 54, pp. 75 - 87.

Mackintosh, M. (1978). Nitrogen fixation by *Thiobacillus ferrooxidans*. J. Gen. Microbiol., 105, pp. 215 - 218.

Manfield, M. de Nys, R., Naresh, K., Roger, R., Givskov, M., Peter, S., Kjelleberg, S. (1999). Evidence that halogenated furanones from *Delisea pulchra* inhibit acylated homoserine lactone (AHL) mediated gene expression by displacing the AHL signal from its receptor protein. Microbiol., 145, pp. 283-291.

Marchalonis, J.J. (1987). Lectins in the isolation of receptors on lymphocytes. Meth. Enzymol. 150, pp. 463-477.

Mc Clean, K.H., Winson, M.K., Fish, L. (1997). Quorum sensing and *Chromabacterium violaceum*: exploitation of violacein production and inhibition for the detection of N-acylhomoserine lactones. Microbiology, 143, pp. 3703-3711.

Mi, S., Song, J., Lin, J., Che, Y., Zheng, H. and Lin, J. (2011). Complete genome of *Leptopirillum ferrooxidans* ML-04 provides insight into its physiology and environmental adaptation. J. Microbiol., 49, pp. 890-901.

Mitchell, Daniell (2002). Untersuchungen zum Verwandtschaftsverhältnis verschiedener Stämme von *Acidithiobacillus thiooxidans* und *Leptospirillum ferrooxidans*. Diplomarbeit, Institut für allgemeine Botanik, Abteilung Mikrobiologie, Universität Hamburg.

Miller, M.B.; Bassler, B.L. (2001). Quorum sensing in bacteria. Ann. Rev. Microbiol., 55, pp. 165-199.

Meyer, G., Schneider-Merck, T., Böhme, S., Sand, W. (2002). A simple method for investigation in the chemotaxis of *Acidthiobacillus ferrooxidans* and *Desulfovibrio vulgaris*. Acta Biotechnol. 22, pp. 391- 399.

Moses, C., Nordstrom, D., Herman, J., Mills, A. (1987). Aqueous pyrite oxidation by dissolved oxygen and ferric iron. Geochim. Cosmochim. Acta., 51, pp. 1561 - 1571.

NG, W.L, Bassler, B.L. (2009). Bacterial Quorum-Sensing network architectures, Ann. Rev. Genet., 43, pp. 197-222.

Noël, N., Florian, B., Sand, W. (2010). AFM EFM study on attachment of acidophilic leaching organisms. Hydrometallurgy, 104, pp. 370 - 375.

Noël, N. (2008). Comparative study of planktonic and sessile cells in cultures of *Leptospirillum ferriphilum* and *Acidithiobacillus caldus* on pyrite. Master thesis. Aquatische Biotechnologie, Universität Duisburg-Essen, Duisburg.

Norris, P. (1990). Acidophilic bacteria and their activity in mineral sulfide oxidation. In H. Ehrlich, C. Brierley (Eds.), Microbial mineral recovery (pp. 3 - 27). New York: McGraw-Hill.

Norris, P., Barr, D., Hinson, D. (1988). Iron and mineral oxidation by acidophilic bacteria: affinities for iron and attachment to pyrite. In P. Norris, D. Kelly (Eds.). In: Biohydrometallurgy, proceedings of the international symposium (pp. 43 – 59). Warwick, Antony Rowe Ltd. Chippenham, Wiltshire, UK.

Okibe, N. and Johnson, D. B. (2004). Biooxidation of pyrite by defined mixed cultures of moderately thermophilic acidophiles in pH-controlled bioreactors: the significance of microbial interactions. Biotechnol Bioeng. 87, pp. 574–583.

Okibe, N., Gericke, M., Hallberg, K., Johnson, D. (2003). Enumeration and characterization of acidophilic microorganisms isolated from pilot plant stirred-tank bioleaching operation. Appl. Environ. Microbiol., 69, pp. 1936 - 1943.

Ohmura, N., Tsugita, J.I., Koizumi, H., Saiki, H. (1996). Sulfur-binding protein of flagella of *Thiobacillus ferrooxidans*. J. Bacteriol. 178, pp. 5776-5780.

Persson , T., Givskov, M., Nielson, J. (2005). Quorum sensing inhibition: Targeting chemical communication in Gram-negative bacteria. Curr. Med. Chem., 12, pp. 3103-3115.

Pierson, L.S., Wood, D.W., Peirson, E.A. (1998). Homoserine lactone-mediated gene regulation in plant-associated bacteria. Ann. Rev. Phytopathol., 36, pp. 207-225.

Rawlings, D., Tributsch, H., Hansford, G. (1999). Reason why '*Leptospirillum*' -like species rather than *Thiobacillus ferrooxidans* are the dominant iron-oxidizing bacteria in many commercial processes for the biooxidation of pyrite and related ores. Microbiology , 145, pp. 5 - 13.

Ren, D., Sims, J.J., Wood, T.K. (2001). Inhibition of biofilm formation and swarming of *Escherichia coli* by (5Z)-4-bromo-5-(bromomethylene)-3-butyl-2(5H)-furanone. Environ. Microbiol., 3, pp. 731-736.

Riedel, K., Hentzer, M., Geisenberger, O., Huber, B., Steidle, A., Wu, H., Givskov, M., Molin, S., Eberl, L. (2001). N-Acylhomoserine-lactone-mediated communication between *Pseudomonas aeruginosa* and *Burkholderia cepacia* in mixed biofilms. Microbiology, 147, pp. 3249-3262.

Rivas, M., Seeger, M., Holmes, D.S. and Jedlicki, E. (2005). A lux-like quorum-sensing system in the extreme acidophile *Acidithiobacillus ferrooxidans*. Biol. Res. 38, pp. 283-297.

Rohwerder, T., Sand, W. (2007). Mechanisms and biochemical fundamentals of bacterial metal sulfide oxidation. In E. Donati, W. Sand, Microbial Processing of Metal Sulfides (pp. 35 - 58). Dordrecht, Netherlands: Springer.

Rohwerder, T., Gehrke, T., Kinzler, K., Sand, W. (2003). Bioleaching review part A: Progress in bioleaching: fundamentals and mechanisms of bacterial metal sulfide oxidation. Appl. Microbiol. Biotechnol., 63, pp. 239 - 248.

Rohwerder, T., Josza, P.G., Gehrke, T., Sand, W. (2002): Bioleaching, G. Bitton (ed.) Encyclopedia of environmental microbiology, 2, John Wiley & Sons NY, pp. 632-41.

Rojas-Chapana, J., Tributsch, H. (2004). Interfacial activity and leaching patterns of *Leptospirillum ferrooxidans* on pyrite. FEMS Microbiol. Ecol., 47, pp. 19 - 29.

Ruiz, L., Valenzuela, S., Castro, M., Gonzalez, A., Frezza, M., Soulere, L., Rohwerder, T., Queneau, Y., Doutheau, A., Sand, W., Jerez, C., Guiliani, N. (2008). AHL communication is a widespread phenomenon in biomining bacteria and seems to be involved in mineral-adhesion efficiency. Hydromet., 94, pp. 133-137.

Sabbah, M., Bernollin, M., Doutheau, A., Soulere, L., Queneau, I. (2013). A new route towards fimbrolide analogues: importance of the exomethylene motif in LuxR dependant quorum sensing inhibition. Med. Chem. Comm., 4, pp. 363-366.

Sabbah, M., Fontaine, F., Grand, L., Boukraa, M., Efrit, M.L., Doutheau, A., Soulere, L., Queneau, I. (2012). Synthesis and biological evaluation of new N-acyl-homoserine-lactone analogues, based on triazole and tetrazole scaffolds, acting as LuxR-dependent quorum sensing modulators. Bioorg. & Med. Chem., 20, pp. 4727-4736.

Sand, W., Gehrke, T. (2006). Extracellular polymeric substances mediate bioleaching/biocorrosion via interfacial processes involving iron(III) ions and acidophilic bacteria. Res. in Microbiol., 157, pp.49-56.

Sand, W., Gehrke, T., Hallmann, R., Schippers, A. (1995). Sulfur chemistry, biofilm, and the (in)direct attack mechanism - a critical evaluation of bacterial leaching. Appl. Microbiol. Biotechnol., 43, pp. 961 - 966.

Sand, W., Rohde, K., Sobotke, B., Zenneck, C. (1992). Evaluation of *Leptospirillum ferrooxidans* for leaching. Appl. Environ. Microb., 58, pp. 85 - 92.

Shaw, P.D., Ping, G., Daly, S.L., Cha, C., Cronan, J.E., Rinehart, K.L. and Farrand, S.K. (1997). Detecting and characterizing N-acyl-homoserine lactone signal molecules by thin-layer chromatography. Proc. Natl. Acad. Sci USA 94, pp. 6036-6041.

Schippers, A. (2007). Microorganisms involved in bioleaching and nucleic acid-based molecular methods for their identification and quantification. In E. Donati, W. Sand, Microbial Processing of Metal Sulfides (pp. 3 - 34). Dordrecht, Netherlands: Springer.

Schippers, A., Sand, W. (1999). Bacterial leaching of metal sulfides proceeds by two indirect mechanisms via thiosulfate or via polysulfides and sulfur. Appl. Environ. Microbiol., 65, pp. 319 - 321.

Schippers, A. (1998). Untersuchungen zur Schwefelchemie der biologischen Laugung von Metallsulfiden. Dissertation. Hamburg.

Schröter, (1993). Mikrokalorimetrische Untersuchungen an chemolitotrohen Eisen (II) und Thiosulfat oxidierenden Bakterien. Dissertation Universität Hamburg.

Semenza, M., Viera, M., Curutchet, G. and Donati, E. (2002). The role of *Acidithiobacillus caldus* in the bioleaching of metal sulfides. Lat. Am. appl. Res. 32, pp. 302-306.

Silverman, M.P. (1967). Mechanism of Bacterial Pyrite Oxidation. Journal of Bacteriology, pp. 1046-1051

Spirig, T., Tiaden, A., Kiefer, P., Buchrieser, C., Vorholt, J.A., Hilbi, H. (2008). The *Legionella* autoinducer synthase LqsA produces an α-hydroxyketone signaling molecule. J. Biol. Chem., 283, pp.18113-18123.

Stein, S. (2004). Anheftung von *Acidithiobacillus ferrooxidans* an Mineraloberflächen nach Anzucht auf unterschiedlichen Substraten. Diplomarbeit. Biozentrum Klein Flottbek und Botanischer Garten, Universität Hamburg.

Steindler, L. and Venturi, V. (2007). Detection of quorum-sensing N-acyl homoserine lactone signal molecules by bacterial biosensors. FEMS Microbiol. Lett. 266, pp. 1-9.

Tiaden, A., Spirig, T., Hilbi, H. (2010). Bacterial gene regulation by α-hydroxyketone signaling. Trends in Micriobio., 18, pp. 288-297.

Tyson, G.W., Lo, I., Baker, B.J., Allen, E.E., Hugenholtz, P., Banfield, J.F. (2005). Genome-Directed Isolation of the Key Nitrogen Fixer *Leptospirillum ferrodiazotrophum* sp. nov. from an Acidophilic Microbial Community. Appl. Environ. Micriobiol., 71, pp. 6319- 6324.

Valenzuela, S., Banderas, A., Jerez, C.-A., Guiliani, N. (2010). Cell-cell communication in bacteria. In E. Donati, W. Sand, Microbial Processing of Metal Sulfides (pp. 253 - 264). Dordrecht, Netherlands: Springer

Valenzuela, L., Chi, A., Beard, S., Orell, A., Guiliani, N., Shabanowitz, J., Hunt, D.F., Jerez, C.A. (2006). Genomics, metagenomics and proteomics in biomining microorganisms. Biotechnol. Adv., 24, pp. 197-211.

Vera, M., Krok, B., Bellenberg, S., Sand, W., Poetsch, A. (2013). Shotgun proteomics study of early biofilm formation process of Acidithiobacillus ferrooxidans ATCC 23270 on pyrite. Proteomics, 1-12.

Vera, M., Krok, B., Sand, W., Poetsch, A. (2011). High throughput comparison of data for planktonic and biofilm-forming cells of *Acidithiobacillus ferrooxidans* ATCC 23270. Biohydromet.: Biotech key to unlock mineral resources value. Proceedings of the 19th international biohydrometallurgy symposium. Central south university press, Changsha, China.

Waters, C.-M., Bassler, B.-L. (2005). Quorum sensing: Cell-cell communication in bacteria. Ann. Rev. Cell Dev. Biol., 21, pp. 319-346.

Watnick, P. and Kolter, R. (2000). Biofilm, city of microbes. J. Bacteriol. 182, pp. 2675-2679.

Yee, N., Fein, J.B. and Daughney, C.J. (2000). Experimental study of pH, ionic strength and reversibility behaviour of bacteria-mineral adsorption. Geochimica et Cosmochimica acta 64, pp. 609-617.

Zenneck, C. (1988). Mikrobiologische Bestandsaufnahme in zwei rumänischen Erzbergwerken und Charakterisierung der litoautotrophen Flora. Diplomarbeit, Universität Hamburg.

Zhou, H.B., Liu, X., Fu, B., Qiu, G.Z., Huo, Q., Zeng, W.M., Liu, J.S., Chen, X.H. (2007). Isolation and characterization of *Acidithiobacillus caldus* from several typical environments in China. J. Cent. South Univ. Technol. 14, pp. 163-169.

Zhu, J., Beaber, J.W., More, M.I., Fuqua, C., Eberhard, A., Winans, S.C. (1998). Analogs of the autoinducer 3-oxooctanoyl-homoserine lactone strongly inhibit activity of the TraR protein of *Agrobacterium tumefaciens*. J. Bacteriol., 180, pp. 5398-5405.

Zhu, J., Chai, Y., Zhang, Z., Lis, S., Winans, S.C. (2003). *Agrobacterium* bioassay strain for ultrasensitive detection of N-acylhomoserine lactone- type quorum sensing molecules: detection of autoinducers in *Mesorhizobium huakuii*. Appl. Environ. Microbiol., 69, pp. 6949-6953.

Curriculum vitae **Nanni Noël**

Personal data:

Date of birth:	02.12.1983
Place of birth:	Oberhausen
Address:	Graßhofstraße 208, 46147 Oberhausen, 0208/6262525
	nanninoel@aol.com
Family status:	unmarried
Nationality:	German

Education:

08.1994 – 06.2003	secondary school
	Gymnasium-Gustav-Heinemann-Schulzentrum,
	Dinslaken-Hiesfeld
	Graduation: Abitur (2,8)

Tertiary education:

10.2003- 08.2006	International Bachelor of Science curriculum: Water
	Science- chemistry, analytics, microbiology
	University Duisburg-Essen, Campus Duisburg
	Graduation: Bachelor of Science (2,7)
10.2006- 09.2008	International Master- of Science curriculum: Water
	Science- chemistry, analytics, microbiology
	University Duisburg-Essen, Campus Duisburg
	Graduation: Master of Science (2,0)
10.2008- 09.2013	Curriculum Water Science: Promotion “Attachment of
	acidophilic bacteria to solid substrata”

Professional Career:

12.2008-31.03.2014	Employment as research associate, Biofilm Centre, Aquatic Biotechnology, University Duisburg-Essen, Campus Duisburg

Work experience abroad:

04.2006- 07.2006	Bachelor thesis "Study on the changes in microbiology for anaerobic digestion" Thames Water, Research and Development, Reading, Great britain
01.2013-02.2013	Cooperation Facultad de Ciencias, Universidad de Chile, Santiago de Chile, focus "molecular biology"

Additional Qualification:

30.11.2005	Efficient attendance in a sampling technique seminar at the IWW Rheinisches Institut für Wasser, Mühlheim an der Ruhr:"Extraction of drinking water samples for official analysis within inspections"
18.05.2006	Acquisition of „comprehensive expert knowledge according to §5Chemikalienverbotsverordnung"
11.2007- 09.2008	Research assistant, Biofilm Centre, Aquatic Biotechnology- Prof. Dr.W. Sand, University Duisburg-Essen, Campus Duisburg

Publications

Poster:

→ISME 2008: "Initial colonization and biofilm formation of leaching bacteria on metal sulfides studied in pure and mixed cultures"
B.Florian, N. Noel, S. Bellenberg, T. Rohwerder and W. Sand

→VAAM 2009: "Attachment of leaching bacteria to metal sulfides elucidated by a combination of AFM and EFM"
B.Florian, N. Noel, S. Bellenberg, T. Rohwerder and W. Sand

→Biofilms IV, Winchester 2010: Control of bioleaching or acid mine drainage by understanding biofilm formation
B.Florian, N. Noel, and W. Sand

→ Cancun, Mexico 2009: "AFM and EFM-study on attachment of acidophilic thermophiles"
N. Noel, B.Florian, and W. Sand

→Cancun, Mexico 2009: "Biofilm formation in bioleaching- how to optimize the process?"
B.Florian, N. Noel, and W. Sand

Paper:

→Florian, B., Noel, N., Thyssen, C., Felschau. I., Sand, W. (2011). Some quantitative data on bacterial attachment to pyrite. Min. Eng., 24, pp. 1132-1138.

→Florian, B., Noel, N., Sand, W. (2010). Visualization of initial attachment of bioleaching bacteria using combined atomic force microscopy and epifluorescence microscopy
Min. Eng., 23, pp. 532-535.

→Noel, N., Florian, B., Sand, W. (2010) AFM and EFM study on attachment of acidophilic leaching organism. Hydromet., 104, pp 370-375.

→Sand**,** W., Florian, B., Noël. N. (2009). Mechanisms of bioleaching and the visualization of these by combined AFM & EFM. Adv. Mat. Res.,71-73, pp. 297-302.

→Florian, B., Noël, N., Bellenberg, S., Huergo, J., Rohwerder, T., Sand, W. (2009). Attachment behavior of leaching bacteria to metal sulfides elucidated by combined atomic force and epifluorescence microscopy. Adv. Mat. Res., 71-73, pp. 337-340.

Presentations:

→Biofilms 4 Winchester 2010: "Effects of different acidophilic, moderately thermophilic microorganisms on attachment and biofilm formation on pyrite"
N. Noel, M.Vera and W.Sand

→BioMicroWorld 2011: "Effects of different quorum sensing molecules on attachment and biofilm formation of acidophilic, moderately thermophilic microorganisms"
N. Noël , M. Vera and W.Sand

Awards:

→Sachtleben Water Award

Hiermit versichere ich, dass ich die vorliegende Arbeit mit dem Titel

„Attachment of acidophilic bacteria to solid substrata“

selbst verfasst und keine außer den angegebenen Hilfsmitteln und Quellen benutzt habe, und dass die Arbeit in dieser oder ähnlicher Form noch bei keiner anderen Universität eingereicht wurde.

Essen, im September 2013