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OPTIMIZING THE DETACHMENT OF ORE AGGLOMERATE BOUND MICROORGANISMS

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ABSTRACT

Erkki Halme: Optimizing the detachment of ore agglomerate bound microorganisms
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By bioleaching metals can be economically recovered from low-grade ores and waste ore, which would not be possible traditional mining methods. In bioleaching, dissolution of metals from sulfide ores is catalyzed by chemolithoautotrophic microorganisms that can survive in the extremely acidic mining environment. In heap leaching as much as 99% of microorganisms responsible for bioleaching are attached to the ore surface. However, the microbial abundance and diversity analyses are typically performed from leach liquor samples and, thus may result in limited or even biased view of microbial communities within the heaps. Although various techniques have been used for cell detachment generally optimized methods do not exist. The aim of this study was to optimize a method for the detachment of microorganisms from ore surfaces.

Optimization was done using model organisms *Acidithiobacillus ferrooxidans, Sulfolobus metallicus* and a mesophilic enrichment culture. Microbes for the enrichment originated from heap leach liquor samples from Terrafame mine located in Sotkamo, Finland. The experiments were conducted using agglomerate of polymetallic black schist ore which also originated from the Terrafame mine. Microorganisms were attached onto the ore surface by recirculating culture solution (180 mL) through a column containing 200 g ore agglomerate for 24 h. For detachment, homogenized 15-g subsamples were taken from the columns and subjected to sonication procedures. Bacterial and archaeal cell abundances were determined using quantitative PCR. Cell counts based on DAPI staining and microscopy were also performed, however, the reliability of the microscopy results was compromised by non-cellular particles after detachment procedures. The recovery percentages of microbes were compared using commercial soil DNA extraction kit with and without sonication pre-treatment.

With *At. ferrooxidans, S. metallicus* and enrichment culture over 94%, 99% and 95% of the cells were attached, respectively. Using commercial soil DNA extraction kit the percent recoveries of *At. ferrooxidans* and *S. metallicus* were 24±9 and 66±65, respectively. For *At. ferrooxidans* detachment with sonication pre-treatment was always more effective than without sonication pre-treatment. For *S. metallicus* detachment without sonication pre-treatment was more effective most of the time. Nevertheless, by using the optimized sonication pre-treatment percent recoveries up to 100% were achieved with both model organisms. With mixed culture 3.5±5% of the bacteria and 4.7±4% of the archaea were recovered without the sonication pre-treatment and the sonication did not improve the % recovery.

The attachment method used in this work demonstrated the fast attachment of bioleaching microorganisms to agglomerated ore matrix. Archaea attached to the ore agglomerate more effectively than bacteria and to our knowledge this is first time the difference in attachment to the ore surface between bacteria and archaea has been compared quantitatively. This work also demonstrated that by using the developed microorganism detachment method, it is possible to recover microorganisms from the ore agglomerate matrix and that qPCR combined with quantitative DNA retrieval and extraction is a good method for determining microorganism abundance in bioleaching environment. The developed detachment method should be usable with different kinds of minerals.

Keywords: Heap leaching, Agglomerate, Microorganisms, Cell attachment and detachment, *Acidithiobacillus ferrooxidans*, *Sulfolobus metallicus*

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TIIVISTELMÄ

Erkki Halme: Agglomeraatin pinnalle kiinnittyneiden mikro-organismien irrotuksen optimointi
Diplomityö
Tampereen yliopisto
Biotekniikka
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Bioliuotuksella on mahdollista kannattavasti liuottaa metalleja köyhistä malmeista ja kaivosoperaatiosta jäljelle jääneistä ”jäte malmeista”, joista metallien talteenotto perinteisin kaivosalan menetelmin ei ole kannattavaa. Bioliuotuksessa kemolitoautotrofiset mikro-organismit, jotka kykenevät selviytymään äärimmäisen happopitoisissa kaivosolosuhteissa, katalysoivat metallien liuotuksen sulfidimalmeista. Kasaliuotuksessa jopa 99% bioliuotusmikrobeista on kiinnittyneenä malmin pintaan. Tästä huolimatta valtaosa bioliuotusmikro-organismien määrään ja monimuotoisuuteen liittyvästä tutkimuksesta tehdään nestenäytteistä, mikä voi antaa rajoitetun ja vääristyneen kuvan kasoilla olevista mikrobipopulaatiosta. Vaikka useita tekniikoita on käytetty mikrobien irrottamiseen malmin pinnalta, mutta yleisesti optimoitua menetelmää ei ole. Tämän diplomityön tavoitteena oli kehittää optimisoitu malmin pinnalle kiinnittyneiden mikro-organismien irrotusmenetelmä.

Optimisointi tehtiin käyttämällä kahta malliorganismia *Acidithiobacillus ferrooxidans* ja *Sulfolobus metallicus* sekä mesofiilistä rikastusviljelmää, jonka mikrobit olivat peräisin Sotkamossa sijaitsevan Terrafamen kaivoksen bioliuotuskasalta otetuista nestenäytteistä. Kokeissa käytettiin monimetallista agglomeroitua mustaliuskemalmia, joka myös oli peräisin Terrafamen kaivokselta. Mikro-organismit kiinnitettiin malmiin kierrättämällä kasvatusliuosta (180 ml) 24 tuntia läpi kolonnin, jonka sisällä oli 200 g agglomeroitua malmia. Solujen malmista irrottamista varten kolonnista otettiin 15 g homogenisoidut malminäytteet, joille tehtiin sonikointikäsittely. Bakteeri- ja arkkisolujen määrät määritettiin kvantitatiivisella PCR:llä. DAPI värjäykseen sekä mikroskopointiin perustuvaa solulaskentaa tehtiin myös, mutta näytteissä olevat malmihiukkaset tekivät solujen laskemisesta paikoin mahdotonta. Irrotusmenetelmän saantoprosentteja verrattiin käyttämällä kaupallista DNA-eristyskittiä ilman sekä sonikointikäsittelyn kanssa.

Tulosten perusteella kiinnittymisprosentit olivat yli 94% *At. ferrooxidans:lle,* yli 99% *S. metallicus:lle* ja yli 95% mesofiiliselle rikastusviljelmälle. Kaupallisella DNA-eristyskitillä saantoprosentit olivat 24±9% *At. ferrooxidans:lle* ja 66±65% *S. metallicus:lle. At. ferrooxidans:in* tapauksessa solujen irrottaminen sonikointikäsittelyn kanssa oli aina tehokkaampaa kuin ilman sonikointikäsittelyä. *S. metallicus:in* tapauksessa irrotus ilman sonikointikäsittelyä oli tehokkaampi valtaosassa kokeita. Tästä huolimatta, sonikointikäsittelyä käyttämällä saavutettiin parhaimmillaan 100% saannot molemmille malliorganismeille. Mesofiilisellä rikastusviljelmällä 3.5±5% bakteereista ja 4.7±4% arkeoneista saatiin irrotettua ilman sonikointikäsittelyä ja sonikoimalla saantoprosentit eivät kasvaneet.

Tässä työssä käytetty mikro-organismien kiinnittämismenelmä osoittaa bioliuotusmikrobien nopean kiinnittymisen agglomeraatin pintaan. Arkeonit kiinnittyvät malmiin tehokkaammin kuin bakteerit ja tietojemme perusteella tämä on ensimmäinen kerta, kun arkeonien ja bakteerien kiinnittymistä malmiin on verrattu kvantitatiivisesti. Tämä työ osoittaa myös, että kehitetyllä mikro-organismien irrotusmenetelmällä on mahdollista irrottaa soluja agglomeraatista ja että kvantitatiivisella PCR yhdessä kvantitatiivisen DNA-eristyksen kanssa on hyvä metodi mikro-organismien määrän tutkimiseen bioliuotus ympäristössä. Kehitetyn irrotusmenetelmän pitäisi soveltua kaiken tyyppisille malmeille,

Avainsanat: Kasa bioliuotus, Agglomeraatti, Mikro-organismit, Solujen kiinnittäminen ja irrottaminen, *Acidithiobacillus ferrooxidans*, *Sulfolobus metallicus*

PREFACE

All the experiments related to this master's thesis were carried out the in Laboratory of Chemistry and Bioengineering of Tampere University's Hervanta Campus. I wish to thank Terrafame Ltd. for funding this work and giving me opportunity to write my thesis about truly interesting topic.

I want to thank my supervisors university lecturer, docent Johanna Rinta-Kanto and professor Jaakko Puhakka for their endless patience and guidance through all the phases of this work. I want to thank Sarita Ahoranta for being my contact to the Terrafame mine and taking care that required samples were sent the lab whenever needed.

I also want to thank Reka Hejdu-Rahkama, Hannele Auvinen, and everyone who helped me in the labs. They made it possible for me to be able to work independently of which I am truly grateful.

And last, I want to thank my family and friends for keeping me sane during this work.

Writing this thesis took a few months longer than I was hoping for, but at least I was able to complete this task that at times felt herculean. This work as a whole, first the experiment phase in the lab and then finally writing this thesis have been invaluable learning experience that will surely help me in my future endeavors.

Tampere, 15 July 2019

Erkki Halme

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LIST OF SYMBOLS AND ABBREVIATIONS

°C	Celsius degree
µm	micrometer
AMD	acid mine drainage
C	counting area dependent conversion factor
C_T	cycle threshold
$CoCl_2$ x 6 H_2O	cobalt(II) chloride dihydrate
$CoSO_4$ x 7 H_2O	cobalt(II) sulfate heptahydrate
$CuCl_2$ x 2 H_2O	copper(II) chloride dihydrate
d	day
DAPI	4′,6-diamidino-2-phenylindole
D_f	cell count dilution factor
DNA	deoxyribonucleic acid
dsDNA	dual-stranded deoxyribonucleic acid
EGTA	ethylene glycol tetraacetic Acid
EPS	extracellular polymeric substances
Fe^{2+}	ferrous iron
Fe^{3+}	ferric iron
FeS_2	pyrite
$FeCl_3$ x 6 H_2O	ferric chloride hexahydrate
$FeSO_4$ x 7 H_2O	ferrous sulfate heptahydrate
g	gram
H^+	proton
H_3BO_3	boric acid
HCl	hydrochloric acid
H_20	water molecule
H_2S_n	polysulfide
H_2SO_4	sulfuric acid
K_a	average cell number in cell counting
KH_2PO_4	potassium dihydrogen phosphate
K_2HPO_4	dipotassium hydrogen phosphate
KCl	potassium chloride
l	liter
Me^{2+}	metal ion
mg	milligram
$MgCl_2$ x 6 H_2O	magnesium chloride hexahydrate
$MgSO_4$ x 7 H_2O	magnesium sulfate heptahydrate min minute
ml	milliliter
mM	millimolar
$MnCl_2$ x 2 H_2O	manganese(II) chloride dihydrate
$MnCl_2$ x 4 H_2O	manganese(II) chloride tetrahydrate
$Na_2B_4O_7$ x 10 H_2O	sodium borate decahydrate
NaCl	sodium chloride
Na_2HPO_4	disodium phosphate
NaH_2PO_4	sodium dihydrogen phosphate
Na_2MoO_4	sodium molybdate
$(NH_4)_2SO_4$	ammonium sulfate MoS_2 molybdenite
O_2	oxygen molecule
PbS	galena
PCR	polymerase chain reaction
PLS	pregnant leach solution
qPCR	quantitative polymerase chain reaction

rpm	revolutions per minute
rRNA	ribosomal ribonucleic acid
S	sulfur
s.d.	standard deviation
$S_20_3^{2-}$	thiosulfate
SO_4^{2-}	sulfate
ssDNA	dual-stranded deoxyribonucleic acid
v/v	volume/volume
$VOSO_4$ x 2 H_2O	vanadium(IV) oxide sulfate dihydrate
V_s	cell count sample volume
w/v	weight/volume¨
W	watt
WS_2	tungstenite
(Zn,Fe)S	sphalerite
$ZnCl_2$	zinc chloride
$ZnSO_4$ x 7 H_2O	zinc sulfate heptahydrate

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1. INTRODUCTION

The continuous increasing of world's population has created a growing need for constructions and novel technologies which then again demand resources such as metals. Environmental concerns for example have led to increased demand for production of electric vehicle and wind turbines both of which need copper and other metals as raw materials (Bennett, 2019). This puts a huge strain on metal industries to meet the demand but as of now global copper market is already under supplied (Mining.com, 2019).

Unfortunately, same time as metal demand increases ore grades keep dropping and discoveries of new high-grade or large low-grade ore deposits are not enough to keep up with the demand (Prior *et al.*, 2012; Watling, 2015). One option to help reach the increasing demand for metals is to invest into circular economy and metal recycling. The other option is to retrieve metals from waste ores, mine tailings and in general from low-grade ores. However, metal recovery from these sources is not economically viable by traditional mining methods. Fortunately, metals can be recovered using the native microorganisms residing in the acidic mining environment by process called bioleaching.

The aim of the study was to develop and optimize a method for detachment and analysis of the ore surface attached microorganisms. A few studies have detached bioleaching microorganisms from ore matrices (Zeng *et al.*, 2010; Halinen *et al.*, 2012; Lizama *et al.*, 2012) but the efficiency of the detachment has not been reported. Development of quantitative microorganism detachment method could increase the knowledge on bioleaching microorganism communities and even lead to discoveries of novel microbe species.

2. LITERATURE REVIEW

Bioleaching means harnessing the natural ability of microbes to extract metals from ores. Microbes have naturally participated in making heavy metals soluble in Earth's crust and so the bioleaching process has been used by people much longer than the role of microorganism in it has been understood (Rawlings, 2002).

Bioleaching has several advantages when compared to conventional physicochemical mining. The microbe mediated leaching processes do not require the high amount of energy that is consumed in roasting and smelting in the traditional metal extraction. Bioleaching also does not produce environmentally harmful gas emissions such as sulfur dioxide. Biomining tailings and wastes are less chemically active than their physicochemical counterparts which can reduce unwanted acid and metal pollution. But maybe the most important advantage of bioleaching is usable with low-grade ores. Metals like copper can be recovered from low-grade ore and even from dumps left from previous mining operations. Many of the metals in these low-grade ores and dumps cannot be economically recovered with nonbiological mining methods. Furthermore, depending on ore-type and geological location bioleaching solution and metal recovery can be done in-situ, which further gives economical advantage. (Rawlings, 2002) Bioleaching is generally perceived as more environmentally friendly mining method (Johnson, 2014) and bioleaching in overall has the potential to decrease the harm done to the environment especially when considering the air pollution caused by the smelters. But using bioleaching also requires care to avoid letting the highly acidic and high metal concentration containing leaching liquor from getting to the environment

2.1 Bioleaching principles and applications

2.1.1 Process design and engineering

Bioleaching processes can be categorized into two main types: irrigation and stirred tank-type processes. In irrigation-type processes acidic leaching solution is circulated through ore in dumps, heaps or columns and it can even be used in situ without removing ore from the ore body (Rawlings, 2002). Irrigation type leaching is mainly used with low grade ores not suitable for traditional mining or flotation concentration (Shiers *et al.*, 2016). In stirred tank-type leaching processes ore concentrate of high value metal is used in continuous operations that has much more finely controlled environment when compared to the irrigation-type.

Compared to other commercial fermentation processes the bioleaching has one differentiating feature: there is no need worry about sterility of the process or microbial contaminations. The bioleaching microorganism create an environment in which other microorganisms are not able to survive. (Rawlings, 2002)

After mining operations run-of-mine ore is stored dumps that can contain billions of tons of ore and be hundreds of meters high. In dump leaching aim is to recover the remaining metals from this low-grade waste ore. The dump is irrigated using iron- and

sulfate-rich mining wastewater or raffinate. Microorganisms growing in the heap catalyze a reaction that turns the remaining metal sulfides in the ore into soluble metal sulfates. Metal sulphates flow in so called pregnant leach solution (PLS) towards the bottom of the dump where it is collected in collections pools and the metal of interest is harvested, for example, by solvent extraction and electrowinning. Leaching a dump with all required preparation lasts years. (Rawlings, 2002) When planning on dump leaching many things need to be considered, including permeability of the dump, grade of the ore and possibility of the leakage (Wu *et al.*, 2009). Dump leaching was first started at Kennecott Copper mine in Utah in 1960s with largest dumps containing over four billion tons of low-grade copper ore (Brierley and Le Roux, 1978).

Heap leaching is similar to the dump leaching expect emphasis is put on making the process more efficient. The ore is crushed, acidified using sulfuric acid and can even be agglomerated to coat the coarser ore particles with finer particles. Heaps are usually piled 2-10 m high and solvent irrigation is done using pads on the top of the heap. Aeration pipes are also usually included to further improve the leaching process. Inorganic nutrients are added to the raffinate before irrigation. The optimizations made for heap leaching can decrease the time to complete the leaching from years to months. (Rawlings, 2002) Even though copper is the most common metal heap leaching can also be used for recovering nickel (Puhakka *et al.*, 2007) and for pretreatment of refractory gold (Logan *et al.*, 2007).

With in situ leaching the ore is leached without bringing it to the surface. Underground ore bodies are fractured for example using explosives and then mine is deliberately flooded with acid mine drainage (AMD), the outflowing acidic water from mines. Using perforated pipes AMD inside the mine is aerated and the leaching liquor recovered to the surface. In situ leaching requires suitable geology so that fluid losses can be kept in check and to have the leaching solutions gather in a known place from which it can be pumped. (Rawlings, 2002; Johnson, 2014) In situ leaching has been used for recovering copper (Schnell, 1997) and uranium (McCready and Gould, 1989).

The three irrigation-type leaching processes are visualized in Figure 1.

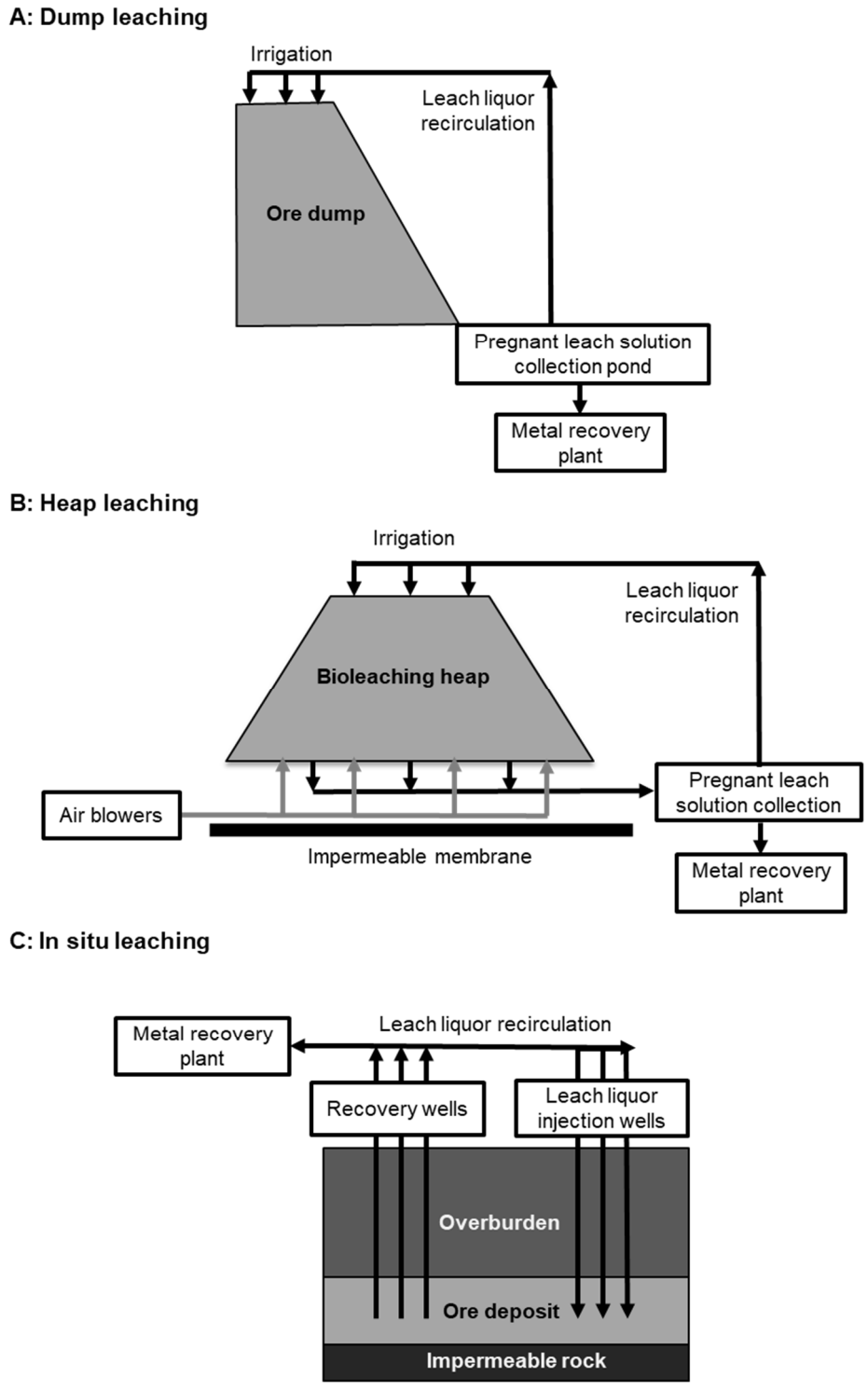

Figure 1. *Irrigation-type bioleaching processes.*

Stirred tank-type process are significantly faster and more efficient when compared to irrigation-type processes. Use of tank leaching is restricted to high-value ore concentrates due to high operating costs. Ore concentrate for the process is prepared for example by flotation. Tank leaching is usually operated at continuous flow and reactors are arranged in series. Concentrate feed is added into the first reactor and from there it flows to the following reactor until it reaches the end of the process. The primary aeration tanks are usually arranged in parallel in order to have higher hydraulic retention time for the concentrate than what is the cell doubling time of the leaching microorganisms. This is done to assure that the microorganism cell numbers reach the high steady-state levels without being washed-out. The contents of the bioreactors are constantly aerated and agitated. pH is also continuously adjusted and due to biooxidation being exothermic constant cooling is also necessary. At the end of the process metal of interest is retrieved from the settling tank. In BIOX process, the most common tank-type process used for gold recovery called, total residence time of gold concentrate is 4-6 days considerably less than duration of any irrigation-type leaching process (Van Aswegen *et al.*, 2007).

Tank leaching is mainly used as pretreatment for recovering gold from recalcitrant arsenopyrite concentrate for which the usual method of making gold soluble, cyanidation, cannot be used easily. Stirred tank-type processes are relatively niche technology that is used almost exclusively for gold leaching by BIOX Process (Van Aswegen *et al.*, 2007; Johnson, 2014) but there are also other applications (Morin and d'Hugues, 2007). The stirred tank-type process is visualized in Figure 2.

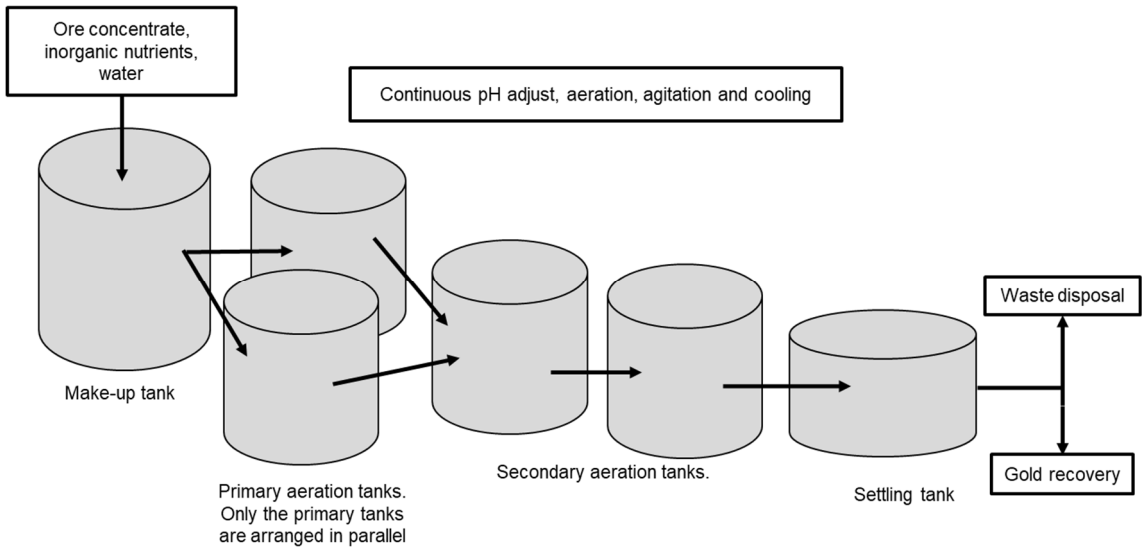

Figure 2. *Stirred tank-type bioleaching based on BIOX gold recovery process.*

Due to the huge size of irrigation-type leaching processes managing them is a great challenge. In the huge heap various physicochemical gradients are unavoidable and basically only things that can be used for managing the gradients are aeration and changing the compositions of leach liquor. Stirred tank-operations on the other hand are relatively small and therefor system is can be kept homogenous and parameters like pH are easily

adjustable. Capacity is also naturally much smaller when compared to irrigation type. Comparison of heap leaching and tank leaching is shown Table 1.

Table 1: *Comparison of heap leaching and tank leaching*

Parameter	Heap leaching	Tank leaching
Substrate	Fine ore	Ore concentrate
Treatment capacity scale (tons/d)	10^3-10^4 [1]	10^1-10^2 [1] [2]
Optimization	Aeration	Completely controlled environment
Environment	A lot of different gradients	Homogenous
Leaching duration	Months or years	Days
Metals extracted	Copper, Nickel, Uranium	Gold (Copper)

[1] (Olson *et al*., 2003)
[2] (Brierley and Brierley, 2013)

2.1.2 Bioleaching applications

Copper heap leaching is the most common application of bioleaching. According to Brierley and Brierley (2013) there were 25 copper heap leaching operations and as of writing the review 18 of those plants were still in operation. Almost half of the operations have been in Chile, but operations are also located all over the world from Australia to China and United States. Only one of the copper heap leaching operations reported by Brierley and Brierley has failed, the other are still running or the operations has ended due to ore depletion.

As mentioned earlier, stirred tank leaching is reserved for high value ore concentrates so it is natural that main application for tank leaching is gold leaching. Brierley and Brierley (2013) report on stirred tank leaching plants for gold concentrates. As of writing of the article there were 17 tank leaching plants of which 13 used the BIOX gold recovery process. Five of the operations have been in Australia but in general tank leaching operation have been located everywhere in the world.

In addition to metals with huge demand like copper and gold there is also need to meet the demand for trace and rare metals. Watling (2015) suggest that bioleaching of multiple metals from polymetallic minerals and broadening the view of what elements are worth extracting could be an important step for meeting the demands of lower demand metals. Watling lists 25 polymetallic ore deposits from all over the world that could potentially be suitable for bioleaching. Some of the mineral in the ore deposits contain over 10 elements worth extracting. This fact together with all the waste ore and mine tailings left from previous mining operations would suggest great potential for bioleaching.

2.1.3 Bioleaching microbes and microbial communities

Bioleaching microorganisms are very diverse. According to Rohwerder *et al.* (2003) microorganism from at least 11 different putative prokaryotic divisions have been found from AMD sites. The leaching microorganisms are both bacteria and archaea. Different bioleaching species, their growth conditions and activities during bioleaching are presented in Table 2. The example species were selected using two different review articles (Rohwerder *et al.*, 2003; Shiers *et al.*, 2016). The species were selected to have represent many different genera that are known for their bioleaching potential.

Table 2: *Temperature and pH dependency and oxidation capabilities of bioleaching microorganisms. The microorganisms that were used in the experiments are described in more detail in body text.*

Species	Optimal temperature range (°C) (Range for growth)	Optimal pH range (Range for growth)	Oxidation activity (Fe^{2+} and/or S) [1]	Reference(s)
Bacteria				
Acidiphilium acidophilum	25-30 (10-35)	3.0-3.5 (1.5-6.0)	S	(Hiraishi *et al.*, 1998)
Acidimicrobium ferrooxidans	45-50	2.0 (1.4-3.0)	Fe^{2+} [2]	(Clark and Norris, 1996)
Acidithiobacillus ferrooxidans	30-35 (10-37)	2.5 (1.3-4.5)	Fe^{2+} and S	(Kelly and Wood, 2000)
Acidithiobacillus thiooxidans	28-30 (10-37)	2.0-3.0 (0.5-5.5)	S	(Kelly and Wood, 2000)
Ferrimicrobium acidiphilum	35	2.0	Fe^{2+}	(Johnson *et al.*, 2009)
Leptospirillum ferrooxidans	30-37 (10-45)	1.5-1.7	Fe^{2+} and S	(Johnson, 2001)
Sulfobacillus thermosulfidooxidans	50 (28-60)	1.9-2.4 (1.9-3.0)	Fe^{2+} and S	(Robbins, 2000)
Archaea				
Acidianus Brierley	1.5-2.0 (1.0-6.0)	70 (45-75)	Fe^{2+} and S	(Huber and Stetter, 2001)
Metallosphaera prunae	75 (55-80)	2 (1.0-4.5)	S	(Fuchs *et al.*, 1995)
Sulfolobus metallicus	65-70 (50-75)	2 (1-4.5)	Fe^{2+} and S	(Huber and Stetter, 1991; Albers and Siebers, 2014)

[1] Shiers *et al.* (2016) was used as reference in addition to the references already mentioned.

[2] Requires yeast extract

Even though biomining organism are diverse, they have common characteristics. All the known bioleaching microorganisms are all chemolithoautotrophic that can use ferrous iron or reduced inorganic sulfur compounds as electron donors. Because sulfur oxidization produces sulfuric acid as a by-product the organisms are acidophilic even if they only oxidize iron. Even though the microorganisms have similarities in optimal growth pH range the optimal growth temperatures range from mesophilic to thermophilic. The biomining microorganisms may have the ability to use electron acceptors other than oxygen, for example ferric iron, but in general they prefer aerobic growth conditions. (Rawlings, 2002)

2.1.3.1 Characteristics of model bioleaching microbes

In the next paragraphs the model microorganism used in this work a described in more detail.

Acidithiobacillus ferrooxidans (name before reclassification *Thiobacillus ferrooxidans*) is the most studied of the bioleaching microorganisms. The species was originally located at acidic and ferrous iron rich environment of mine drainage water (Temple and Colmer, 1951). *At. ferrooxidans* is small rod-shaped gram-negative acidophilic and mesophilic bacterium that belongs into γ-subclass of proteobacteria. The optimal growth conditions for the species are at 30-35 °C and at pH 2.5 but it can survive in much broader temperature and pH range (Table 2) *At. ferrooxidans* is obligately chemolithoautotrophic and gets its energy from oxidization of ferrous iron and reduced sulfur compounds. The species can even survive with ferrous iron as its only energy substrate. (Robertson and Kuenen, 2006; Shiers *et al.*, 2016) *At. ferrooxidans* has six copies of 16S ribosomal RNA (rRNA)-sequence (NCBI, 2019a).

Sulfolobus metallicus is thermophilic archaea. The species was originally retrieved from water- and mudholes of solfataric fields in Iceland. The cells appear as irregular lobed cocci (of about 1.5 µm diameter) but they can resemble discs or pyramids. Optimal growth conditions for *S. metallicus* are at 65-70°C and at pH 2.0 but as is the case with the *At. ferrooxidans* it can survive in much broader temperature and pH range (Table 2) The species grows only in aerobic conditions and can get its energy by oxidizing elemental sulfur or single sulfidic ores. (Huber and Stetter, 1991; Albers and Siebers, 2014) *S. metallicus* has four copies of 16S rRNA-sequence. (NCBI, 2019b)

2.1.3.2 Change and differences in microbe population within bioleaching system

Bioleaching microorganism population composition changes overtime in bioleaching processes. There are even spatial temporal changes in microbe population in the bioleaching heaps. Many studies have focused on researching the bioleaching consortia in irrigation- and stirred tank-type bioleaching processes. Examples of the microbe population in bioleaching processes are presented the studies.

Halinen *et al.* (2012) studied community dynamics in demonstration-scale heap used for leaching of complex sulfide ore. Enrichment culture used as inoculum for the heap

contained species *At. ferrooxidans, Acidithiobacillus thiooxidans, Acidithiobacillus caldus, Leptospirillum ferrooxidans, Alicyclobacillus acidocaldarius, Alicyclobacillus tolerans and Ferrimicrobium acidiphilum.* During the first six months the communities in the leach liquor were diverse with *At. ferrooxidans* as dominant species and from previously cultured species *Sulfobacillus thermosulfidooxidans* was also detected often. After six months *L. ferrooxidans* was observed in all the following samples. Overall, the microbial diversity decreased over time and *At. ferrooxidans* remained dominant species.

Lizama *et al.* (2012) studied the leaching communities in zinc sulfide ore leaching test heap. The aim of their study was to identify differences in microbe population on surface of ore at different depths within the heaps and at same time effect of temperature was also monitored. Heap was 6 m high and operation temperature was within mesophilic range. Ore samples were taken during from three different sections of the heap corresponding to top, middle and top third of the heap. Mesophilic microorganisms were always present at all sections. Moderate thermophiles were only present when average temperature of the heap reached 40°C and even then, they were never found at the top third of the heap. After the heap started to cool down moderate thermopiles disappeared. Overall, nine different bioleaching species were identified and the most dominant species in the heap were mesophilic sulfur oxidizers *At. thiooxidans* and *Acidithiobacillus albertensis* and mesophilic iron oxidizers *At. ferrooxidans* and *L. ferriphilum*.

Shiers *et al.* (2016) studied the effect of pH on mesophilic microbial communities in polymetallic black shale in columns. Column leaching experiments were done at three different pH (1.2, 1.6 and 2.0) at 35°C and 50°C and leaching communities were examined after 102 days of leaching. For experiments at 35°C inoculum contained species from *Leptospirillum*, *Thermogymnomonas*, *Ferroplasma* and *Acidithiobacillus* genera. The first two genera from the inoculum in addition to the *Ferrimicrobium* native to the ore dominated the population at all three pH values. *Ferroplasma* genera was present at all three pH to lesser extent and *Acidithiobacillus* was only present at pH 2.0. For experiments at 50°C inoculum contained species from *Leptospirillum*, *Thermogymnomonas*, *Ferroplasma, Acidithiobacillus, Acidimicrobium* and *Metallosphaera* genera. At this temperature the first four genera from the inoculum in addition to the *Ferrimicrobium* native to the ore dominated the population at all three pH values. *Acidimicrobium* was not present in any of the columns when examined and *Metallosphaera* appeared at pH 1.6 and 2.0. At pH 1.2 *Leptospirillum* was dominant genera and at pH 1.6 and at pH 2.0 it was dominant together with *Metallosphaera*.

Even with stirred tank-type reactors with rather homogenous more easily adjustable conditions microbe population changes over time. Zeng *et al.* (2010) studied moderately thermophilic microbial community structure in chalcopyrite concentrate stirred tank leaching process. *At. caldus, L. ferriphilum*, *Ferroplasma thermophilum* and few *Sulfobacillus* species were present during the process. *At. caldus* was dominant leaching species at the start of the process but by the end of the process *L. ferriphilum* and *F. thermophilum* overtook it. Percentage of *Sulfobacillus* species increased at first but by the end they had completely disappeared.

2.1.4 Attachment of bioleaching microorganisms onto ore surface

Some of the bioleaching organisms always remain suspended in the leaching solution but most of them grow attached to sulfide ore surfaces (Rohwerder *et al.*, 2003). Attachment of bioleaching microorganisms onto ore surface happens mainly via extracellular polymeric substances (EPS) and the attachment in itself stimulates EPS production (Vandevivere and Kirchman, 1993). In the case of attachment of *At. ferrooxidans* to the pyrite attachment occurs mainly due to electrostatic interaction: the cells are positively charged and metal sulfides negatively charged. *At. ferrooxidans* is also able to modify the EPS composition and amount depending on the substrate and therefore the attachment surface. For example, in the planktonic state the cells use soluble substrates like ferrous iron and produce almost no EPS. (Rohwerder *et al.*, 2003) Activity of bioleaching microbe cells within EPS is illustrated in Figure 3.

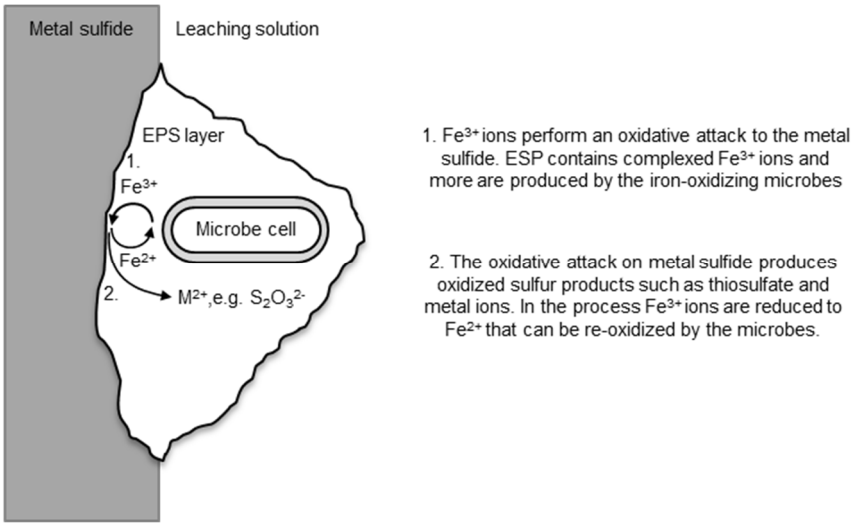

Figure 3. *Microbial activity within extracellular polymeric substances.*

2.1.5 Mechanism of bioleaching

Bioleaching microorganisms are the biocatalysts that make bioleaching possible. The microorganisms turn insoluble metals into soluble form which usually happens by oxidizing metal sulfides into metal sulfates. This results in the metal to be extracted into water for harvestable form. (Rawlings, 2002)

Traditionally bioleaching mechanisms used to be characterized as direct leaching and indirect leaching (Bosecker, 1997; Tributsch, 2001). Direct mechanism assumes that the attached microorganism enzymatically oxidizes the mineral producing sulphate and metal cations (Sand *et al.*, 2001). However, this suggested direct leaching of metal sulfides has not been demonstrated experimentally and does not seem to exist (Vera *et al.*, 2013). Instead, leaching seems to only occur via the indirect mechanism.

In the indirect leaching ferric iron and protons are the only oxidizing agents that dissolve metal sulfides and the microorganisms or their enzymes are not directly degrading or oxidizing the sulfide mineral. The microorganisms on the metal sulfide surface are able to regenerate these oxidizing agents and concentrate them to the needed location to improve the leaching process. (Sand *et al.*, 2001)

The indirect leaching can be categorized into non-contact, contact and cooperative leaching. Non-contact leaching planktonic cells oxidize ferrous iron into ferric iron and that together with protons oxidize the metal sulfides. Contact leaching is driven by bioleaching microorganisms that are attached to the sulfide mineral via EPS. EPS works as a reaction space for both iron and sulfur-oxidizing microorganism. With iron-oxidizing microorganisms leaching mechanism is same as in non-contact leaching expect for increased reaction rates due to changes in pH, ferric iron concentration and redox potential within the EPS. The sulfur-oxidizing microorganisms use cysteine-containing carrier proteins to break bonds within sulfur compounds and produce sulfur colloids and other sulfur intermediates. In cooperative leaching the sulfur colloids and intermediates produced in contact leaching are used by planktonic iron- and sulfur-oxidizing microorganisms to produce ferric iron ions and protons. These oxidizing agents are then used in non-contact leaching (Rawlings, 2002). The three types of leaching are further illustrated in Figure 4.

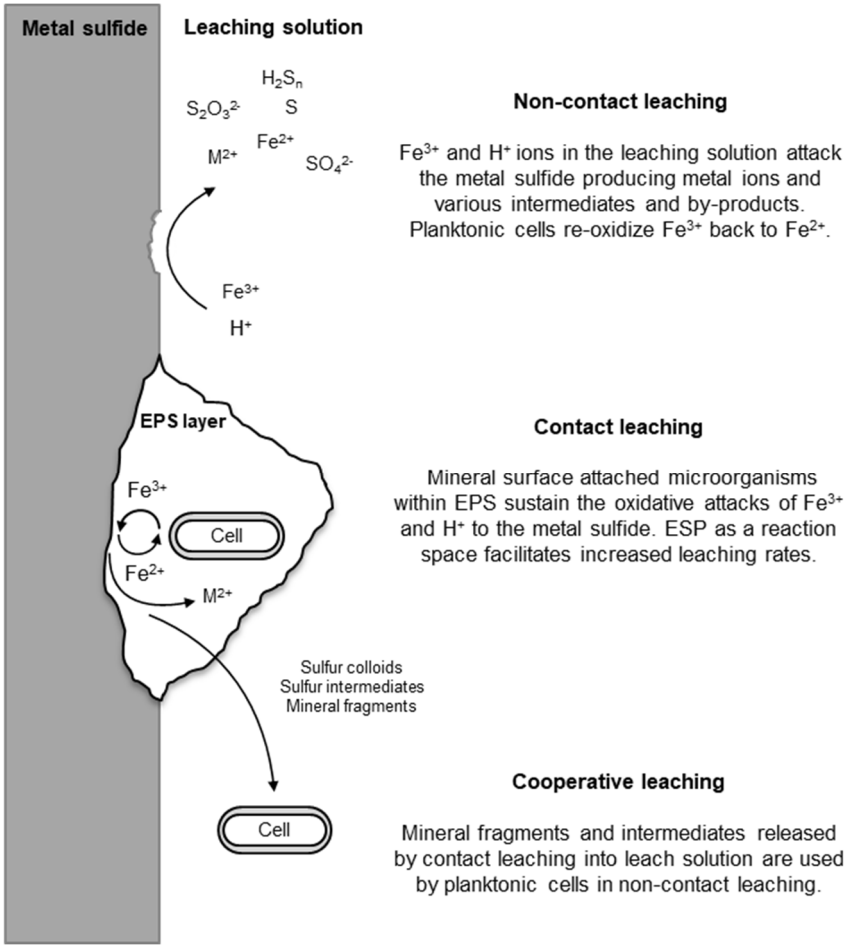

Figure 4. *The three indirect bioleaching types.*

The indirect leaching mechanism occurs through two different pathways: the thiosulfate mechanism and the polysulfide mechanism. Both reactions occur via different sulfur intermediates. The thiosulfate mechanism is based on oxidative attack of ferric iron ions on the insoluble metal sulfides. This mechanism occurs with sulfide minerals like pyrite (FeS_2), molybdenite (MoS_2) and tungstenite (WS_2). The main sulfur intermediate of the reactions is thiosulfate. (Schippers and Sand, 1999) Example reaction using FeS_2 is presented in Formula 1 and 2.

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

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

In the polysulfide method the insoluble metal sulfides are attacked by ferric iron ions, protons or both. This mechanism occurs, for example, with sulfide minerals like sphalerite ((Zn,Fe)S) and galena (PbS). The main sulfur intermediate of the reactions is polysulfide. (Schippers and Sand, 1999) Example reaction for general metal sulfide is presented in Formula 3, 4 and 5.

$$MeS + Fe^{3+} + H^+ \rightarrow Me^{2+} + \frac{1}{2}\,H_2S_n + 6\,Fe^{2+} \quad (n \geq 2) \quad (3)$$

$$\frac{1}{2}\,H_2S_n + Fe^{3+} \rightarrow \frac{1}{8}\,S_8 + Fe^{2+} + H^+ \quad (4)$$

$$\frac{1}{8}\,S_8 + \frac{3}{2}\,O_2 + H_2O \rightarrow SO_4^{2-} + 2\,H^+ \quad (5)$$

The two different dissolving pathways are further illustrated in Figure 5.

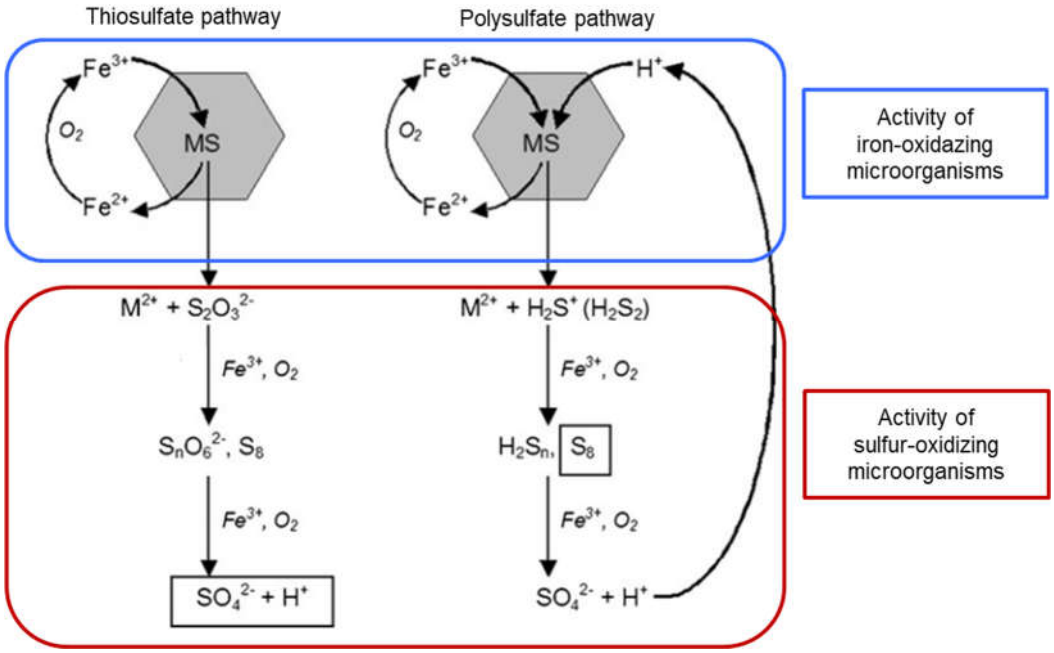

Figure 5. *Thiosulfate and polysulfide pathways of metal sulfide dissolving. (modified from Vera* et al., *2013)*

2.2 Monitoring of bioleaching communities

Various monitoring methods used with bioleaching communities are presented in this chapter. The analytical methods used in this work are explained in detail.

2.2.1 Quantification of microorganisms

Cell counting is a method that can be used to determine microorganism abundance from liquid samples. Cell counts can be performed by plating the culture of interest or by counting the cells by using hemocytometer and light microscopy. The microbial culture density can also be used as an indicator of cell number. One of the methods that is used for bioleaching systems is by **fluorescence microscopy.** Cell counting is an easy to use method for monitoring of cell numbers from liquid samples but method's use for samples that contain solids is limited.

Fluorescence microscopy is based on the use of fluorescent dyes. The fluorescent dyes contain fluorochrome molecule capable of emitting light. When fluorescent dye is exposed to specific wavelength light fluorochrome molecule absorbs a proton which elevates orbital electron to exited state. When the orbital electron returns to original state the fluorochrome emits light that is longer wavelength than the original light used in excitation of the fluorescent dye. Wavelengths of the light used for excitation and the light emitted differ based on the fluorescent dye. (Alberts *et al.*, 2014)

One commonly used fluorescent dye is 4′,6-diamidino-2-phenylindole (DAPI) that was first synthesized in 1970s. DAPI is a DNA-specific fluorescent dye that attaches to adenine-thymine rich regions of deoxyribonucleic acid (DNA) forming a fluorescent complex. (Kapuscinski, 1995) DAPI complex absorbs ultraviolet light and emits blue 460 nm wavelength light (Alberts *et al.*, 2014). Other than cell counting DAPI can be for example used for DNA visualization in various different applications (Kapuscinski, 1995).

The light emitted by fluorescence dye is visualized using fluorescence microscope which has one key difference when compared to traditional light microscope. The illuminating light in fluorescence microscope, originating from high intensity light source, is passed through two different filters: first filter selects the correct wavelength for the fluorescent dye in the sample and the second filter to filter out the unwanted noise from the emitted light (Nelson *et al.*, 2013). The function of fluorescence microscope is illustrated and explained in Figure 6.

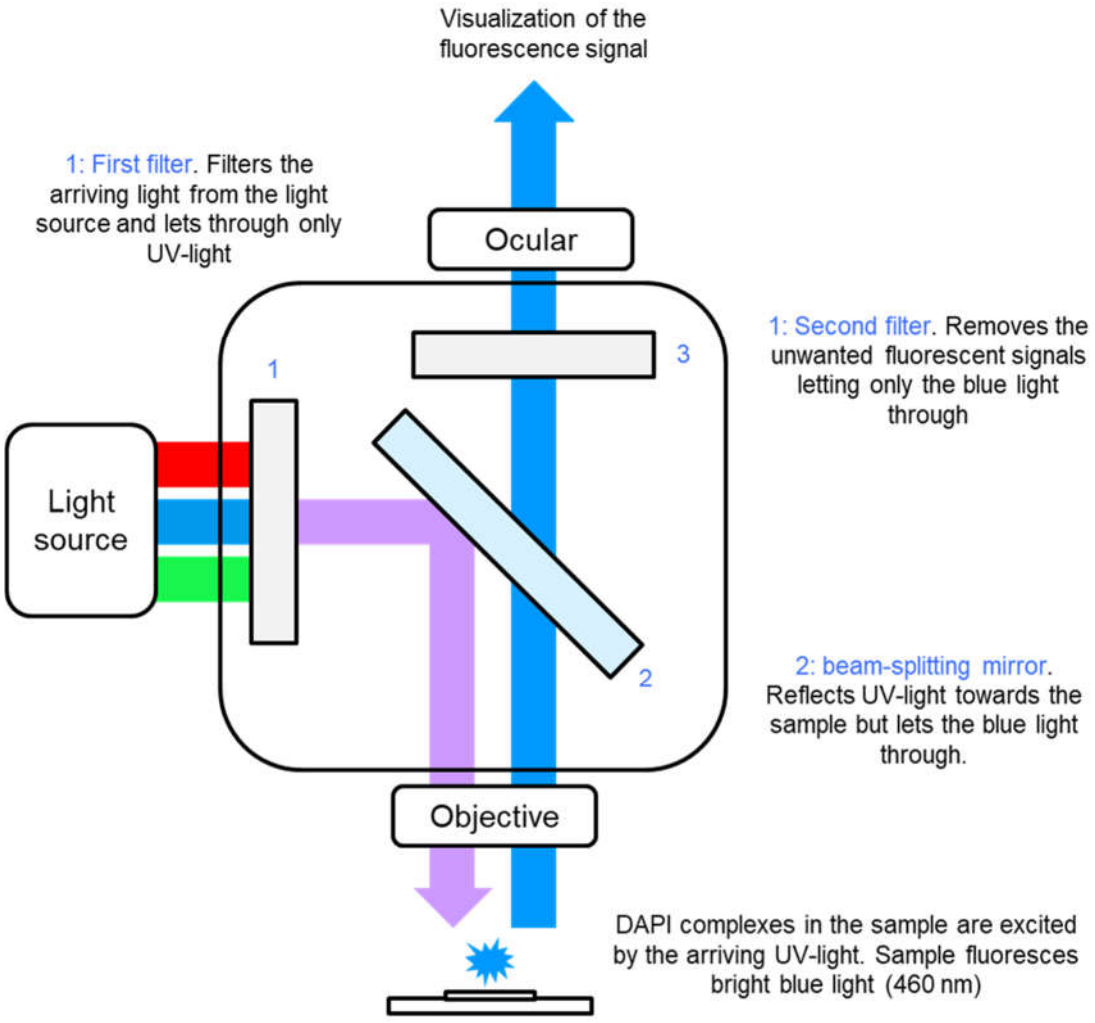

Figure 6. *Fluorescence microscopy with DAPI stained samples. The cells that were stained using DAPI are visible against dark background and can be counted.*

Polymerase chain reaction (PCR) has been used to amplify the amount of DNA since the method invented by Kary Mullis in 1983 (Nelson *et al.*, 2013). The principle of PCR is based on the natural ability of denaturation and renaturation of DNA and use of DNA polymerase to create new DNA.

PCR is a cyclical process that is done in three steps: denaturation, annealing and elongation. In denaturation step dual stranded DNA (dsDNA) separates into two single stranded DNA molecules (ssDNA) due to increased temperate (+90°C). In annealing step oligonucleotide primers attach to the ssDNA strands at the 5'-end of the target DNA sequence. In extension step thermostable DNA polymerase synthesis complementary strands for the ssDNA to 5'- 3'-direction starting from 3'-ends of the primers. The three step cycle is repeated 25-30 times and amount of DNA molecules is doubled after every cycle (Nelson *et al.*, 2013). PCR cycle is visualized in Figure 7.

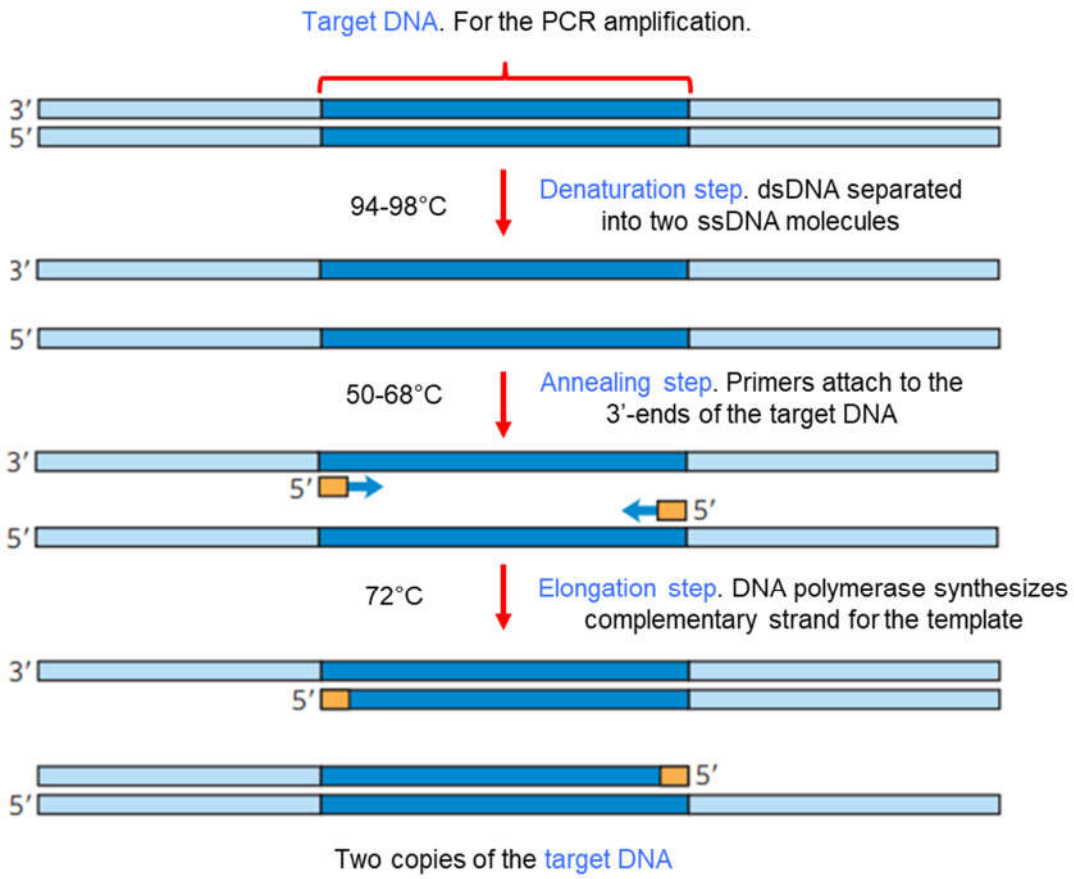

Figure 7. *Polymerase chain reaction PCR. Temperature values of the steps vary depending on the target DNA. (modified from Nelson et al., 2013)*

PCR is very sensitive method as it can detect as little as one DNA molecule and amplify it. This high sensitivity also makes it very susceptible to contaminations (Nelson *et al.*, 2013).

In most cases when using PCR only goal is just to synthesize the DNA template of interest to have sufficient amount of product without caring about the exact amount of copies produced. However, PCR can be made quantitative analysis method that is used to estimate relative copy numbers of DNA sequences in samples (Nelson *et al.*, 2013).

Quantitative PCR (**qPCR**) or real time PCR is a method used in determining the copy numbers of specific gene sequence in a sample. qPCR assays are done with presence of fluorescent probe that binds to the DNA amplicons. Originally dual-labeled oligonucleotide probes (for example TaqMan) that binds to the target DNA sequence between the primers were used (Heid *et al.*, 1996). Dual-labeled probes contains a fluorophore and also a quenching molecule that dampens the fluorescence signal when the probe is not attached to the target DNA (Heid *et al.*, 1996; Nelson *et al.*, 2013). Function of a dual-labelled probe is shown in Figure 8.

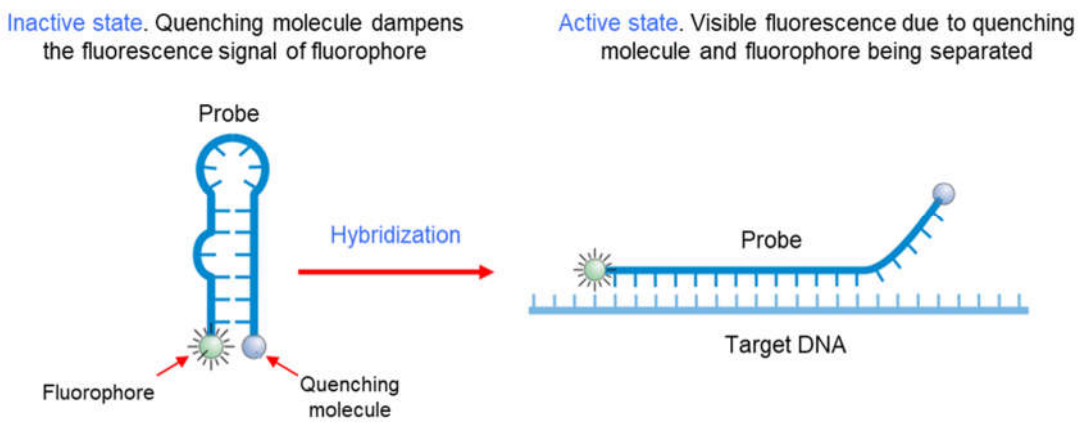

Figure 8. *Function of a dual-labeled oligonucleotide probe. When probe is not attached to the target DNA the probe is self-hybridized (inactive state) where fluorophore and quenching molecule are close to each other which dampens the fluorescence. While is hybridized to the target DNA (active state) quenching molecule is separated from fluorophore and fluorescence is visible. (modified from Nelson et al., 2013)*

As dual-labelled probes are relatively expensive so use of inexpensive, less target DNA specific nucleotide stain SYBR-Green I has become more common in qPCR (Ponchel *et al.*, 2003; Liu *et al.*, 2006). However, both fluorescence methods lead to same result: after every PCR cycle more amplicons are produced meaning that stronger fluorescent signal is also produced (Nelson *et al.*, 2013).

In addition to the use of fluorescence probes the other differentiating factor in qPCR when compared to regular PCR is that annealing and elongation in the PCR cycle can be combined into one step. This is due to the fact that qPCR amplicons are normally relatively short (<200 base pairs) and DNA polymerase has sufficient activity to perform the elongation without needing a separate step (Merck, 2019).

The fluorescence signals in the qPCR assay are monitored real time and the signal strength level is known at point of each PCR cycle. The exact copy number can be determined based on the how fast the fluorescence signal reaches the predetermined threshold level. Intersection of fluorescence signal and the threshold is called cycle threshold (C_T) (Nelson *et al.*, 2013). The exact copy number of target DNA can be determined by preparing standards that have a known copy number of the DNA sequence of interest. The standards are prepared by dilution series from plasmid containing the DNA sequence of interest, for example bacterial 16S rRNA sequence. Fluorescence signals of the standards at C_T are compared to the sample and original copy number of the target DNA can be determined. Process of comparing the fluorescence values is illustrated in Figure 9.

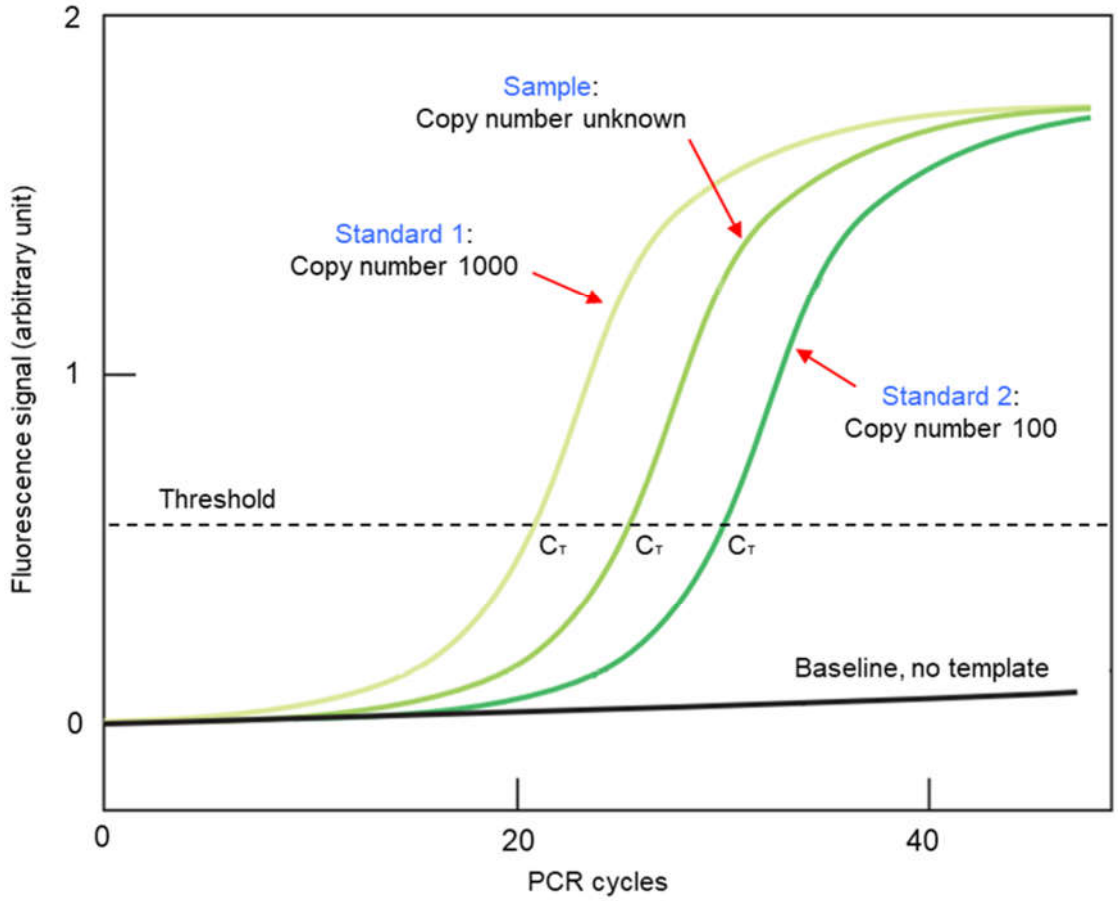

Figure 9. *qPCR. Fluorescence signals of standards and sample reach the cycle thresholds (CT) at different times. More copies of the target DNA there are at the start of the qPCR more quickly the signal strength reaches the threshold. Threshold is placed on the linear, exponential area of the curves. Standard curve is prepared from the fluorescence values of the standards and copy number in sample is determined. (modified from Nelson et al., 2013)*

The results of qPCR results can be validated by inspecting a melt curve which can be produced at the end of the qPCR run. qPCR system increases temperature gradually and the fluorescence signal is monitored continuously. A peak that should form in the specific position of the curve that depends on the melting point of the qPCR product. Only one peak should form for each type of amplicon. For example, if there are multiple peaks present in sample that should contain only one type of microorganism species, there is a great change that the sample was contaminated.

Examples of microorganism quantification techniques used in acidic bioleaching environment are presented in *Table 3*.

Table 3. *Microorganism quantification techniques used in acidic bioleaching environment.*

Technique	Sample origin	Reference
Cell counts (DAPI)	Leaching liquor [1]	(Halinen *et al.*, 2009a)
qPCR	Laboratory culture in liquid media	(Liu *et al.*, 2006)
Most probable number	Leaching liquor [1]	(Halinen *et al.*, 2009a)
Immunological assay	Laboratory culture in liquid media	(Amaro *et al.*, 1994)
Double-layer plating	Mine drainage stream, reactor leach liquor	(Johnson, 1995; Okibe *et al.*, 2003)

[1] Also done for leach residue after detaching and resuspending the cells

Traditional quantification like plating are not a real option for cell quantity determination due to the difficulty of the matrix and specific growth conditions of the leaching microorganisms (Takai and Horikoshi, 2000) and as mentioned before cell counting has only limited use for samples that contain solids. DNA extraction and qPCR has been used for determining microorganism abundance in bioleaching environment (Table 3). However, most of the bioleaching microorganism abundance reports are based on cell counting from liquid phase samples. Some analysis has been done for solid phase samples in bioleaching environment, but the cells were first detached into washing solution and then counted by cell counting methods (Halinen et al., 2009a). For the analysis of microorganism abundance on ore surfaces a good direction would be to move away from detachment combined with the outdated and cumbersome cell counting based methods and move towards quantitative DNA retrieval method combined modern microorganism quantification method of qPCR.

2.2.2 Microbial community profiling

Some methods used for profiling microorganisms of bioleaching communities are presented in Table 4.

Table 4. *Community profiling techniques used in acidic bioleaching environment.*

Technique	Sample origin	Reference
Denaturing gradient gel electrophoresis	Leach liquor [1]	(Halinen *et al.*, 2009b)
Fluorescence in situ hydrolysis	Leach liquor and AMD submerged sediment	(Peccia *et al.*, 2000; Bond and Banfield, 2001)
Amplified ribosomal DNA restriction analysis	Column and stirred tank reactor solution and residues	(Zeng *et al.*, 2010; Qiu *et al.*, 2011)
Phospholipid fatty acid profiling	FBR (Acidic metal containing wastewater) carrier material	(Kaksonen, Plumb, Robertson, *et al.*, 2004)
Clone library analysis	FBR (Acidic metal containing wastewater) carrier material	(Kaksonen, Plumb, Franzmann, *et al.*, 2004)
Community transcriptomics	AMD environment biofilms	(Goltsman *et al.*, 2015)
Single-strand conformation polymorphism	Liquid and Solid phase from bioleaching pulp	(Battaglia-Brunet *et al.*, 2002)

[1] Also done for leach residue after detaching and resuspending the cells

Several community profiling techniques have been used in characterizing biomining communities but as was the case with quantification, community profiling of bioleaching microorganisms has mainly been done for liquid samples.

These methods usually have some draw backs. For example, with denaturing gradient gel electrophoresis can give indication of from what species the extracted 16S rRNA sequences originate but to confirm the results the samples from the gel still need to be sequenced. Also, techniques like phospholipid fatty acid profiling and fluorescence in situ hydrolysis are described as time consuming and labour intensive (Liu *et al.*, 2006) and some methods are specific without ability to target broader microorganism population (Amaro *et al.*, 1994).

These conventional techniques are in general being complemented or replaced by more modern and quantitative methods. One state-of-the-art method for characterization of any DNA sample is deep sequencing (or next generation sequencing). There are multiple different deep sequencing methods (Liu *et al.*, 2012) but in general all deep sequencing methods are based on fragmenting the DNA in the sample in short oligonucleotides, anchoring the oligonucleotides and then sequencing all the oligonucleotide fragment simultaneously. Some of the newer deep sequencing techniques do not even require PCR amplification of the DNA sample of interest (Metzker, 2010). Deep sequencing produces enormous amount of information as all the DNA in the sample is sequenced and bioinformatic methods for analysis are need for. But this would also potentially yield important information that would increase the understanding of the bioleaching communities.

Using deep sequencing for community profiling could be correct direction where research should aim towards. But as with microorganism abundance analysis proper quantitative DNA recovery method would be necessary.

2.3 Occurrence of microorganisms in solution and on solid surfaces

Microorganisms tend to inhabit various surfaces and try to choose most favorable for their activities. Surfaces often provide nutrients and protection for microorganisms. Possible flow across the surface where microorganisms colonize further increases the transport of nutrients to the colonies. Furthermore, microorganisms can produce substances like biofilms that help the microorganisms to stay on an optimal surface, provide self-defense and the let the cells to interact with each other more easily. (Madigan *et al.*, 2019)

The tendency for microorganisms to attach to the surface of their choosing is also true with bioleaching microorganisms. As mentioned earlier most of the bioleaching organisms grow attached to sulfide ore surfaces in EPS (Figure 3; Rohwerder *et al.*, 2003) Harneit *et al.*, (2006) report that over 80% of the *At. ferrooxidans cells* can attach to the ore within an hour. More specific comparisons between bioleaching consortia in solution and on ore surface have been reported.

Halinen *et al.* (2009a) studied effect on pH to microbe population in pH-controlled column with black schist ore. They reported that around 99% of the cells in the inoculum attached to the agglomerated ore. Also, DGGE analysis revealed that there were bacterium species present in leach residue samples that were not found in the leach liquor.

In other study Halinen *et al.* (2012) researched microbial community in demonstration scale black schist ore bio heap. By roughly estimating the number of microorganisms in leach liquor and in the heap, they report that more than 98% of the microorganisms are attached to the ore agglomerate surface. The number of microorganisms was determined by DAPI staining and cell count.

Zeng *et al.* (2010) studied microbe population structure in chalcopyrite concentrate stirred tank leaching process. They compared the populations of free microbes in the leaching solution to the cells attached to the ore. The number of microbes attached to the ore reached their maximum in 16 days when the number of free microbes reached theirs in 24 days. They also used 16S sequence qPCR method to determine amounts *At. caldus, L. ferriphilum* and *F. thermophilum* in both solution and ore surface. All species were present relative quantities varied at lot between the matrix.

When taking account these reports monitoring only the liquid phase of bioleaching environment would most likely give unrepresentative picture of microbe communities. Thus, in order to study microorganism communities various techniques have been developed for cell detachment prior to analysis. Examples of detachment methods are presented in Table 5.

Table 5: *Previously used microorganism detachment methods from solid surfaces.*

Detachment technique	Microorganism recovery (cells/g)	Attachment surface	Reference
Sonication in Zwittergent washing solution	***10^3 - 10^4***	Granulated activated carbon	(Camper *et al.*, 1985)
5 min sonication 0.9% saline solution	**10^5 cells/ml**	Biofilm on stainless steel pipe	(Soini *et al.*, 2002)
5 times 1 min sonication in Zwittergent washing solution	***10^7 - 10^8***	Black schist leach residue	(Halinen *et al.*, 2009a)
Concentrate pellet suspended in MQ-water vortexed with presence of glass beads	***10^8 - 10^9***	Chalcopyrite concentrate	(Zeng *et al.*, 2010)
UltraClean Soil DNA Kit mega Prep (Mo Bio laboratories)	***N/A***	Zinc sulfide ore	(Lizama *et al.*, 2012)

Use of Zwittergent wash solution and sonication for cell detachment was first used in test with granulated activated carbon (Camper *et al.*, 1985). The method has later been applied for heap leaching environments and detaching microorganisms from ore surfaces (Halinen *et al.*, 2009a). Direct DNA extraction from the heap by commercial soil kit has also been done (Lizama *et al.*, 2012) and for stirred tank-leaching pulp vortexing based cell detachment has been used (Zeng *et al.*, 2010). In general, if commercial DNA soil kit was not used the cell detachment consisted of using some sort of buffer solution combined with powerful agitation method like sonication.

Although these various techniques have been used successfully for cell detachment in bioleaching environment no generally qualified, optimized means exist for analyzing microbial communities for bioleaching systems. The aim of our study was to develop and optimize a method for detachment and analysis of the ore surface attached microorganisms. The sonication-based methods previously used in heap leaching environment was selected as the starting point for method development. Challenges of our study included development of quantitative DNA extraction procedure and finding the most optimal sonication intensity for microorganism detachment.

3. METHODS

All experiments were started with the cell attachment to ore agglomerate matrix followed by cell detachment. Sampling was done three times during the experiment. The further details are explained in the following sections. The general workflow of the experiment is presented in Figure 10.

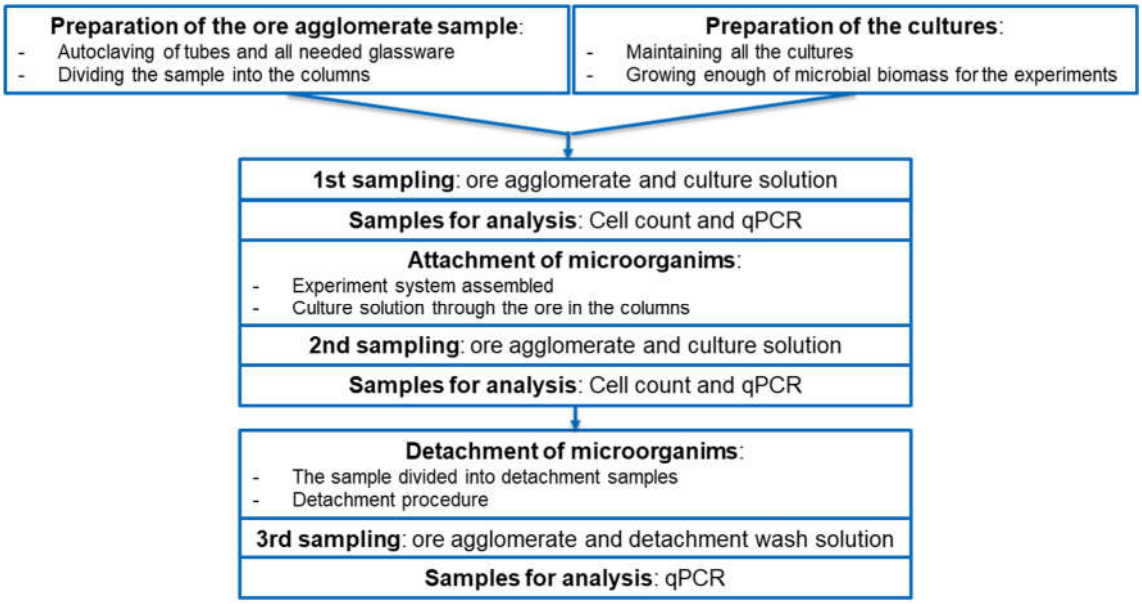

Figure 10. *The workflow of the experiments. Preparation phase was started a week prior with inoculation of the cultures and preparation of the laboratory equipment was done at least one day before the start of the experiment. The experiment from first sampling to the third took place in about 30-hour period.*

The experiments were done in two phases. **Experiment 1** focused on testing the attachment of the microorganism and preliminary detachment were done on the side. **Experiment 2 and 3** focused on optimization of detachment with the model organisms. In **Experiment 4** knowledge from the previous experiments was used in testing detachment of mixed culture.

3.1 Column design and experiments conducted

For the detachment experiments microorganisms were first attached to the polymetallic black schist ore agglomerate originating from Terrafame mine located in Sotkamo, Finland. First, the ore agglomerate was divided into ~200g subsamples according to the Finnish Standard SFS-EN 932-2. (SFS, 1999) and placed into the columns. The used sub-sampling method is presented in **Error! Reference source not found.**.

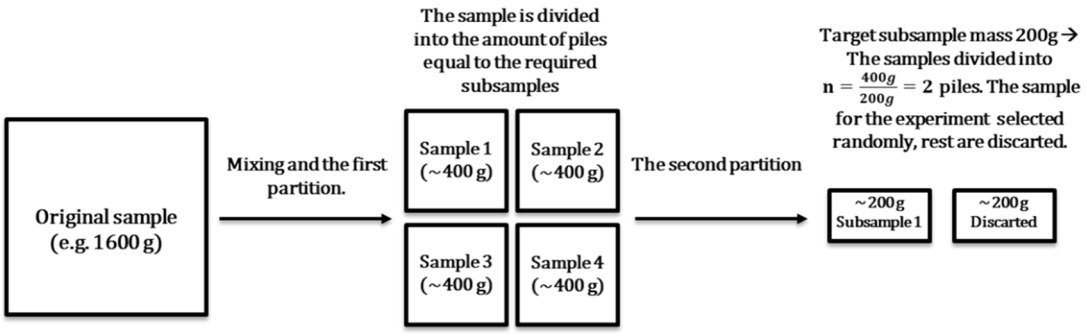

Figure 11 *Pre-processing of ore agglomerate sample and sub-sampling by fractional shovelling (SFS, 1999). Mass of the original ore agglomerate sample was measured and then divided according the procedure shown in the figure. The amount of piles in the second partitions were adjusted depending on the original ore agglomerate sample mass and the target subsample mass.*

To attach the microorganisms, 180ml of the culture solution of interest was circulated through the ore agglomerate (200g) filled 250ml glass column (Laborexin, Finland) for 24 hours. The culture solution was circulated using a Masterflex L/S peristaltic pump (Cole-Parmer, United States) at the rate of 5 ml/min. The goal for the culture solution circulation was to have the culture solution spread into ore agglomerate evenly without saturating the system and at this flow rate this was achieved. The experiments were done at 25°C and all the glassware and tubes were sterilized by autoclaving prior to each experiment. Experiment setup is shown in Figure 12 and Figure 13.

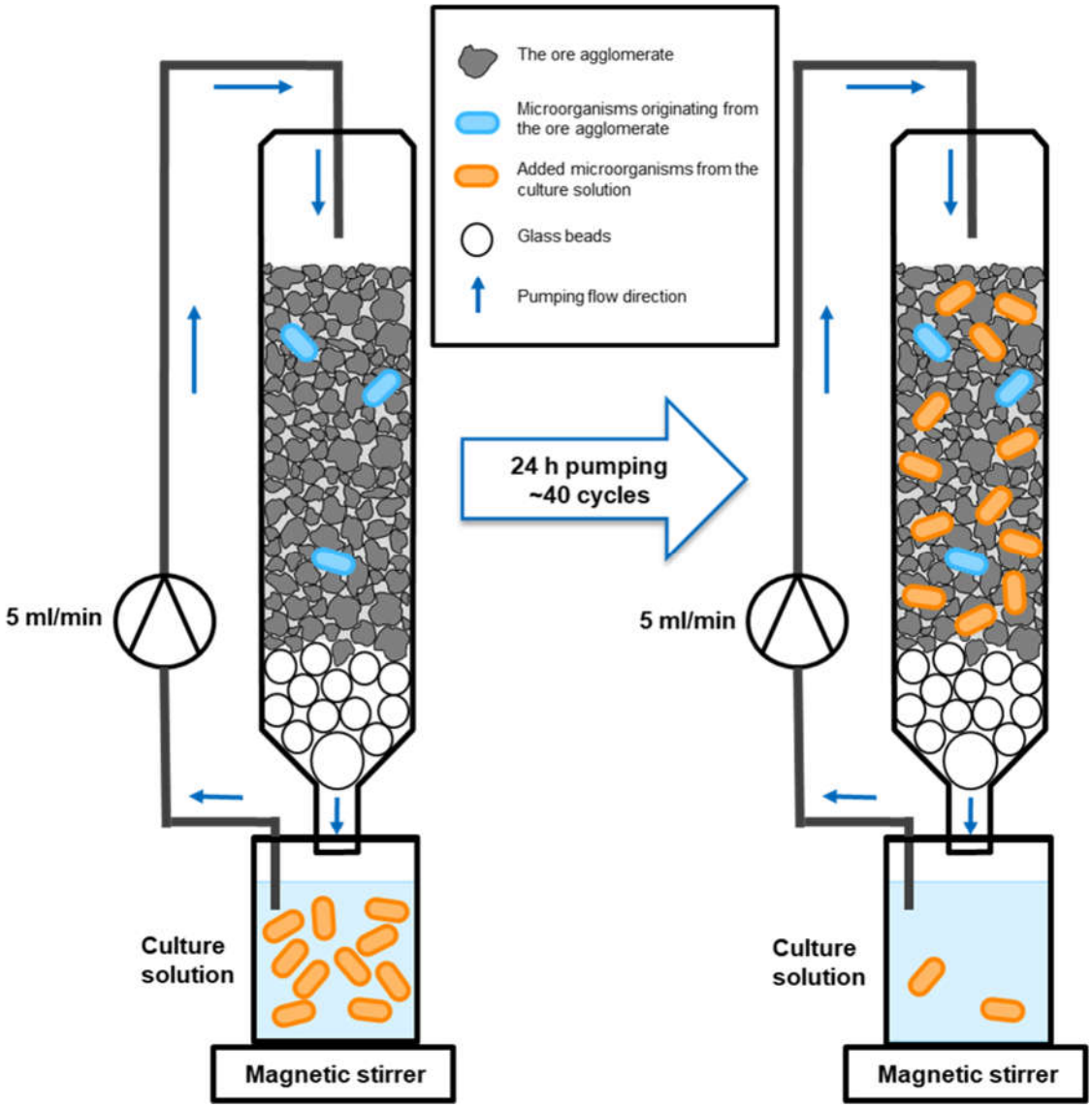

Figure 12: *Principle of attaching microorganisms onto the ore agglomerate surface. Ore agglomerate was added after glass beads were placed on the bottom of the column. The glass beads kept the agglomerate in the column while simultaneously allowing culture solution to flow through the column. Circulation of culture solution made the microorganisms of interest (orange rods) to attach onto agglomerate. The ore agglomerate samples contained microorganisms that were already attached to its surface (blue rods).*

***Figure 13**: Experimental setup for attachment and detachment of microorganisms from ore agglomerate. All the experiments were conducted in an incubation room at 25°C.*

In Experiment 1 Masterflex Tygon Fuel & Lubricant L/S 16 tubing (Cole-Parmer) was used. Masterflex Nonprene L/S 16 tubes (Cole-Parmer) were used in the Experiments 2-4, because this material withstood autoclaving better that the other tubing material. Problem with the autoclaving was noticed during Experiment 1 but tubing was used through the whole experiment for the sake of consistency. Other than tubing used parameters were kept constant between the experiments. The only variable in the experiments was the cell culture used.

3.2 Model microorganisms and enrichment cultures

Four different microbial cultures were grown and maintained for the experiments including two pure cultures and two enrichment cultures. The model microorganisms for pure cultures, ***Acidithiobacillus ferrooxidans*** and ***Sulfolobus metallicus***, were ordered from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Germany). The media for the cultures was prepared according the DSMZ instructions (Appendix A, DSMZ, 2015, 2018). *At. ferrooxidans* was incubated in an incubation room at 25°C statically and *S. metallicus* at 60°C in New Brunswick Innova 44 incubator (Eppendorf, Germany), set at 150 rpm.

The two enrichment cultures were obtained by culturing microbes from water samples obtained from the Terrafame mine. The four water samples originated from four different

triangular notch weirs at the mining site. 2.5 ml subsamples were taken from each of the four water samples and the subsamples were combined into 10 ml (10% v/v) inoculum for the enrichment culture. The medium for the enrichment cultures was prepared according to Halinen *et al.* (2009) (Appendix A). The Enrichment culture 1 was incubated at 25°C on a shaker, set at 150 rpm and Enrichment culture 2 at 60°C in New Brunswick Innova 44 incubator (Eppendorf), set at 150 rpm.

Microbial activity in the cultures was monitored by pH and redox potential. Microbial growth was monitored by cell counts by DAPI staining. At the start of culture monitoring measurements were done at least three times a week and later about biweekly. The amount of ferrous iron was measured by Phenanthroline method (APHA, 1992) in the enrichment cultures and in the pure culture of *At. ferrooxidans*. The amount of sulfate was measured in the enrichment cultures and the pure culture of *S. metallicus*. Also, visual changes in the culture media were monitored. During the culture maintenance media was replaced every 3 to 4 weeks in enrichment cultures, every 7 to 10 days in *At. ferrooxidans* cultures and every 2 to 4 weeks. For the experiments 1 to 2 weeks old culture was used to have a similarly fresh culture for all the experiments. Summary of the cultures and their growth conditions are in the Table 6.

***Table 6*:** Summary of microbial cultures used in the experiments.

Culture	Incubation condition	Media	Main substrate	Media transfer frequency (d)
Enrichment culture 1	25°C 150 RPM	Acidic basal salt medium (Halinen *et al.*, 2009a)	Black schist ore powder 1% (w/v) (ø < 0.2 mm)	21-28
Enrichment culture 2	60°C 150 RPM	Acidic basal salt medium (Halinen *et al.*, 2009a)	Black schist ore powder 1% (w/v) (ø < 0.2 mm)	21-28
Acidithiobacillus. ferrooxidans	25°C, Static	Leptospirillum (HH) medium (DSMZ, 2018)	Iron sulfate 20 g/l	7-10
Sulfolobus metallicus	60°C 150 RPM	Sulfolobus medium (DSMZ, 2015)	Elemental sulfur 5.0 g/l	14-28

3.3 Detachment of microorganism

The attached microorganisms were detached by sonicating the ore agglomerate which was submerged in detachment wash solution (Table 7). The wash solution was sterilized prior to use by filtering it through 0.2 µm pore size sterile mixed cellulose ester membranes (Whatman, United Kingdom).

Table 7: *Composition of the wash solution* (Halinen *et al.*, 2009a).

Compound	Concentration
EGTA, Ethylene glycol tetraacetic Acid (g/l)	0.38
Zwittergent (mg/l)	0.335
KCl (g/l)	3,73
2M HCl	pH 2.5 ~1,5ml/l

After the attachment, the ore agglomerate was removed from the columns, mixed and divided into detachment samples according to the Finnish standard (Anon, 1999). The same procedure was used for dividing the ore agglomerate into the columns (Figure 11). The agglomerate from the columns was emptied on a tray made of laminated polyethylene layer bench protector paper (VWR, United States). The surface of the trey was first sterilized by wiping it with 70% ethanol (v/v) and with MQ-water. The spatulas and other metal equipment used in taking the samples were sterilized by flaming. Approximately 15 g of the ore agglomerate samples were placed into autoclaved 100 ml glass storage bottles and 40 ml of the detachment wash solution was added into the bottles just prior to the sonication. Each detachment was done using three technical replicates per column or sonication amplitude. Control detachment samples were taken prior to attachment using the same procedure.

In Experiment 1 detachment tests were done with all four cultures using FinnSonic M03 waterbed sonicator (FinnSonic/Finland). The sonicator operated at a single pre-set ultrasonic power of 150 W. For sonication, four to six samples were placed in the sonicator water bath at the same time. After the sonication the detachment samples were allowed to settle for 30 min before sampling to allow settling for most of the ore particles remaining in the liquid phase after sonication. Settling was used in the first half of the attachment experiments, but it was replaced by a low rpm centrifugation to speed up the process. For the centrifugation all of the wash solution was transferred by pipetting from the storage bottle into a 50 ml sterile polypropylene centrifuge tube (Thermo Fisher, United States) and samples were centrifuged at 1000rpm (~260 G) for 2min in a Sigma 4K15 centrifuge (Sigma, Germany).

In Experiments 2-4 detachment tests were done using a Soniprep 150 Plus probe sonicator and 9.5 mm diameter probe (MSE, United Kingdom). This sonicator had adjustable sonication amplitude: the amplitude more intense the sonication becomes. At first, detachment tests were done for *At. ferrooxidans* and *S. metallicus* cultures (in separate experiments) using sonication amplitudes of 15.8±0.1 µm, 11.8±0.1 µm and 7.9±0.1 µm. These amplitudes corresponded to 100%, 75% and 50% of the device's maximum amplitude. Based on the results with the first three sonication amplitudes detachment tests were done using 9.5±0.1 µm, 6.3±0.1 µm and 3.2±0.1 µm which corresponded to 60%, 40% and 20% of the maximum. After combining the results of the detachment tests with six different amplitude values, the final detachment test was done

using the Enrichment culture 1 at 7.9±0.1 µm (50%) sonication amplitude. Summary of the specifications of the detachment experiments were as shown in the Table 8.

Table 8: *Summary of detachment test done during the experiments.*

Experiment	Cultures	Sonicator type	Sonication amplitude (µm)	Sonication interval	Settling method
Experiment 1	All	Waterbed sonicator	- (150 W)	5 x 1min, 30s breaks	Settling (30min) or Centrifugation
Experiment 2	Model organisms At. *ferrooxidans* and *S. metallicus*	Probe sonicator	15.8, 11.8, 7.9	5 x 1min, 30s breaks	Centrifugation (1000rpm)
Experiment 3	Model organisms *At. ferrooxidans* and *S. metallicus*	Probe sonicator	9.5, 6.3 ,3.2	5 x 1min, 30s breaks	Centrifugation (1000rpm)
Experiment 4	Enrichment culture 1	Probe sonicator	7.9	5 x 1min, 30s breaks	Centrifugation (1000rpm)

3.4 Sampling

During the experiments samples were taken from the culture solution, detachment wash solution and the ore agglomerate. All the samples were used for DNA-extraction for qPCR. The culture solution samples were also used DAPI-staining for the cell counts. Sampling points during the experiments are shown earlier in this section in Figure 10.

3.4.1 Cell count samples

Samples for microscopic cell counts originated from culture solution before and after attachment. Before the staining dilution of the samples was necessary to obtain appropriate cell density for counting. Dilutions for the samples were done according the information about cell numbers accumulated during culture monitoring. The dilutions were done using sterile MQ water and ranged from 10-fold to 1000-fold.

The filter equipment was washed using warm tap water, 70% ethanol (v/v) and MQ water. The tweezers used for moving the filters were sterilized using ethanol and flame. 5-9ml of the dilutions were filtered onto 0.2µm polycarbonate membrane filter (Whatman) and stained using 1 ml of 1 mg/l DAPI solution for 5 minutes. After the staining the filters were let dry on microscopic glass in dark for at least 15 min before placing a cover glass with a drop Citifluor glycerol PBS solution (Electron Microscopy Sciences, United States). The cell count samples were prepared in duplicates and stored in dark at room temperature.

3.4.2 DNA samples

The DNA-extraction samples originated from culture solution, detachment wash solution and ore agglomerate. Liquid samples were filtered onto 47 mm diameter 0.45 µm pore size sterile mixed cellulose ester membranes (Whatman, United Kingdom). The 0.45 µm pore size filters were used to prevent the ore particles from blocking the membranes.

To improve the DNA yield, some of the filtered samples were exposed to two-step metal removal procedure. Before filtering each sample, the filters were rinsed to remove inhibiting metals by adding 0.9% NaCl (pH 1.8) solution onto the filter with no suction. The solution was let to stand on the filter for 1 min before turning the suction on. Then the suction was cut off and the filter was neutralized by adding 40 mM Na-EDTA in Phosphate buffer saline (NaCl 130 mM, Na_2HPO_4 5 mM, NaH_2PO_4 5 mM at pH 7,2) onto the filter. The solution was let to stand on the filter for 1min before turning on the suction. At first, the metal removal was done only for the detachment wash solution originated samples. However, it was noticed that the metal removal was also needed for the culture solution samples. The filters were folded and placed into either 1.5 ml or 2.0 ml UltraClear polypropylene tubes (VWR) tubes for storage. The samples were stored at -20°C or -80°C until further processing.

The ore agglomerate originated DNA samples were taken at the same time as the ore agglomerate was being divided into the columns or detachment samples. Samples that after sonication were taken after removing excess detachment wash solution. The ore samples were taken using a spatula sterilized using ethanol and flame. The ore agglomerate samples were taken avoiding larger ore particles to have bigger potential ore surface area for the DNA-extraction. At minimum of 0.25 g of agglomerate was placed into either 1.5 ml or 2.0 ml UltraClear polypropylene tubes (VWR). The samples were stored at -20°C or -80°C until further processing

3.5 Analytical methods

3.5.1 Cell counts

Cell counts for the DAPI samples were done using Axioskop 2 light microscope (Zeiss, Germany) fitted with a DAPI filter and a 10x10 square grid in the ocular. From each filter, 20 separate fields were counted. In each field the counting area was determined as the area on the grid in which 20 to 50 cells were seen (10X10, 5X5, 3X3, 2X2 or 1X1 squares on the grid). Depending on the chosen counting area, different conversion factors were used to determine the actual cell number. The formula for counting cell numbers per ml was as shown in Formula 6:

$$Cells/ml = \frac{K_a\, C}{V_s\, D_f}, \tag{6}$$

in which K_a is average cell number of the 20 count fields C is counting area dependent conversion factor, V_s is sample volume (ml) and D_f is dilution factor (for example 10-fold dilutions equals 1/10). The conversion factors were as shown in Table 9.

Table 9. *Cell count conversion factors.*

Counting area	Conversion factor K_a
1X1	1,46E+06
2X2	3,65E+05
3X3	1,62E+05
5X5	5,84E+04
10X10	1,46E+04

3.5.2 DNA extraction

DNA was extracted from the cells contained in the culture solution samples, detachment wash solution samples and the ore agglomerate samples using DNeasy PowerSoil Kit (Qiagen, Germany) under an ultraviolet light hood. During the Experiment 1 DNA extraction was done according to the kit instructions and all three type of DNA samples were transferred into to the Power Bead Tubes. Filters, onto which the cells from culture solution and detachment wash solution were collected, were transferred from storage tubes into Power Bead Tubes using sterilized tweezers, while trying to expose as much of the filter as possible to the reagents inside the tube. About 0,25 g of the ore agglomerate samples were transferred into the tube by flame sterilized spatula.

After Experiment 1 changes were made to the standard protocol to improve the DNA yields and to make the DNA extraction more quantitative. The more quantitative DNA extraction process can be seen in Figure 14.

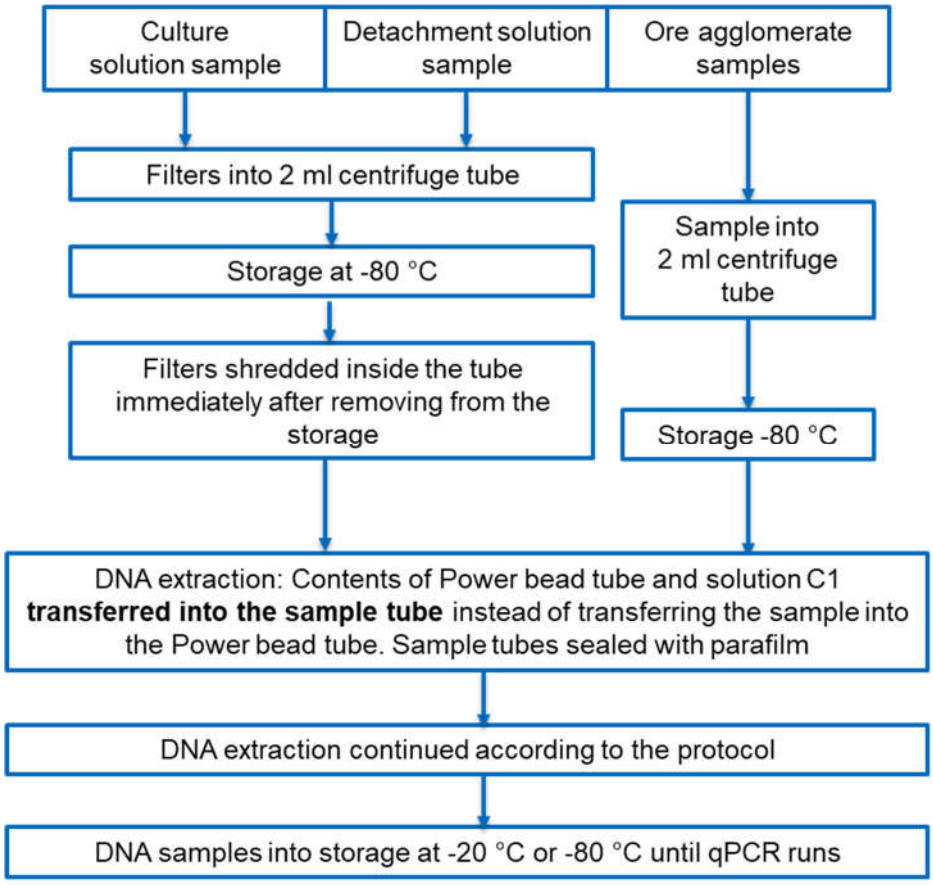

Figure 14. *Quantitative DNA extraction method.*

The DNA samples were stored in the larger 2.0 ml Eppendorf tubes. The filters were shredded inside the tube using a flame sterilized spatula. The contents of the Power Bead tube and other needed reagents were added into the tube. Accordingly, the DNA extraction reagents were also transferred into the sample Eppendorf tube with ore agglomerate samples. The Eppendorf tubes containing the sample and the reagents were sealed using parafilm and vortexed using Vortex adapter. The rest of the DNA extraction process was done according to the kit instructions. Extracted DNA was stored at either -20°C or -80°C.

3.5.3 qPCR

Two different types of qPCR runs were used for the analysis. Eub-qPCR (Eub338F/Eub518R) was used to target bacterial 16S-sequence and it was used for *At. ferrooxidans* pure culture and both enrichment cultures. Archean qPCR (Arch349F/Arch539R) was used to target archaea 16S-sequence and it was used for *S. metallicus* pure culture and both enrichment cultures. Both types of qPCR-runs were run at 1-step procedure using StepOnePlus Real-time PCR system (Applied Biosystems, United States). qPCR thermal cycle parameters are in Table 10, qPCR reagents in Table 11 and used qPCR primers in Table 12.

Table 10. *Thermal cycling parameters for qPCR.*

Step	Bacteria qPCR [1] [2]		Archean qPCR [2]	
	Temperature (°C)	Time	Temperature (°C)	Time
Polymerase activation	95	10 min	95	7 min
Denaturation	95	15 s	95	10 s
Annealing/ Elongation	62	1 min	60	30 s
Melt curve	60-95	+0.3 °C increments every 30s	60-95	+0.3 °C increments every 30s

[1] (Rinta-Kanto *et al.*, 2016)
[2] (Rinta-Kanto *et al.*, 2018)

Table 11. *qPCR reaction mixes.*

Reagent	Bacteria 16S gDNA	Archaea 16S gDNA
Master mix (μl)	12.5 [1]	10 [2]
Forward primer (μM)	0.3	0.5
Reverse primer (μM)	0.3	0.5
Water (μl)	6	3
DNA template (μl)	3	5
Total reaction volume (μl)	25	20

[1] Maxima master mix (Thermo Fisher Scientific, United States)
[2] Dynamo master mix (Thermo Fisher Scientific, United States)

Table 12. *qPCR primers.*

qPCR	Primer pair	Primer sequence (5' -> 3')	Reference
Bacteria 16S gDNA	Eub338F	ACTCCTACGGGAGGCAGCAG	(Fierer *et al.*, 2005)
	Eub518R	ATTACCGCGGCTGCTGG	(Fierer *et al.*, 2005)
Archaea 16S gDNA	Arch349F	GYGCASCAGKCGMGAAW	(Takai and Horikoshi, 2000)
	Arch539R [1]	GCBGGTDTTACCGCGGCGGCTGRCA	(Takai and Horikoshi, 2000)

[1] Reverse Complement of Arch516F

16S-sequences of *S. thermosulfidooxidans* and *S. metallicus* were used as standards for the qPCR runs. Standards were prepared according to Applied Biosystems (2003) guidelines for using plasmid DNA template. Plasmids containing the 16S rRNA-sequences of interest were ordered from GenScript (United States) and standard series with 300 000, 30 000, 3000, 300 and 30 copies of the plasmid were prepared. DNA-samples were diluted to have a copy number on the higher end of the standard curve. The qPCR runs for each DNA sample were done in triplicates.

3.6 Presentation of results

Attachment percentages using both cell counts and qPCR copy numbers were calculated using Formula 7:

$$\%-Attachment = \frac{X_1 - \frac{V_2}{V_1} X_f}{X_2} * 100\%, \quad (7)$$

in which X_1 is cell count or qPCR copy number before attachment, X_2 is cell count or qPCR copy number after attachment, V_1 is the volume of culture solution before attachment and V_2 is the volume of culture solution after attachment

qPCR copy numbers of detachment solution samples and ore agglomerate samples taken after sonication were compared to the calculated average amount of cells attached from culture solution to the ore agglomerate. The comparison is illustrated in Figure 15.

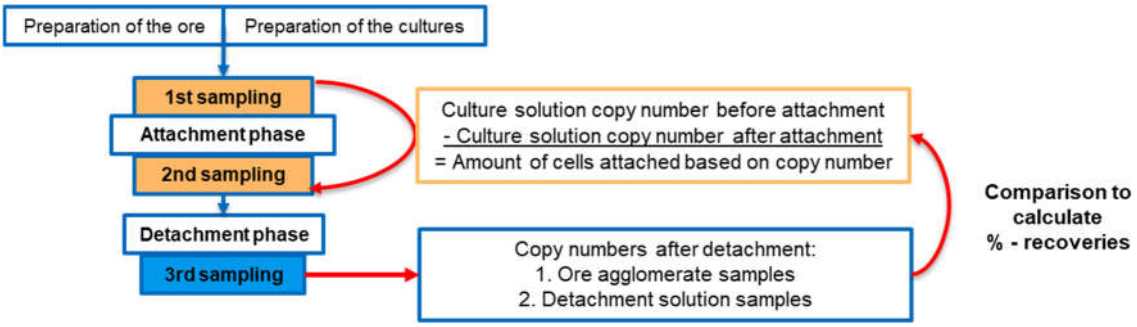

Figure 15. *Comparison of detachment sample copy number to the number of microorganisms attached from the culture solution to the ore agglomerate.*

Detachment results were presented in a bar chart in logarithmic scale. Explanation of the bar chart is shown in Figure 16.

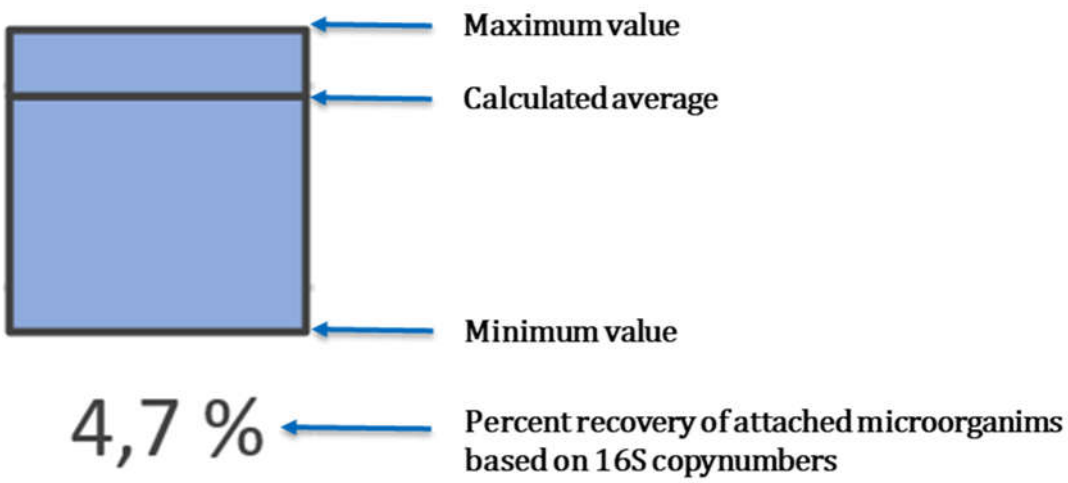

Figure 16. *Explanation of bar chart used for detachment result. Attachment results are presented in similar way but without the percentage value.*

4. RESULTS

In this chapter experimental results are presented.

4.1 Attachment of microorganisms onto ore agglomerate

The percentage of attached microorganisms in the attachment experiments were determined using direct cell counts (Table 13) and qPCR (Table 14).

Table 13. *Percent attachment of cells from Enrichment cultures and pure cultures in Experiment 1 based on cell counts.*

Culture	Average percent attachment (±s.d.)
Enrichment culture 1	89 (±1)
Enrichment culture 2	41 (±21)
Acidithiobacillus ferrooxidans	84 (±7)
Sulfolobus metallicus	94 (±4)

Table 14. *Percent attachment of cells from Enrichment cultures and pure cultures in Experiment 1 based on qPCR copy numbers.*

Culture		Average percent attachment (±s.d.)
Enrichment culture 1	**Bacteria**	58 (±15)
	Archaea	78 (±22)
Enrichment culture 2	**Bacteria**	68 (±25)
	Archaea	70 (±13)
Acidithiobacillus ferrooxidans		90 (±11)
Sulfolobus metallicus		100 (±0,01)

In Experiment 1 average percent attachment ranged from 41±21% to 94±4% with cell count results and from 58±15% to 100±0.01% with qPCR results. For enrichment culture 1 cell count results suggest percent attachment of 89±1% and the qPCR results were 58±15% for bacteria and 78±22% for archaea. For Enrichment culture 2 percent attachment was 41±21 % by cell count and the qPCR results were 58±15 % for bacteria and 78±22 % for archaea. For *At. ferrooxidans* percent attachment was 84±7% by cell counts

and 90±11% by qPCR. For *S. metallicus* percent attachment was 94±4% by cell count and 100±0.01% by qPCR.

The percent attachment of microorganisms in the Experiments 2-4 were determined through qPCR (Table 15 and Table 16).

Table 15. *Attachment percentages of cells from pure cultures during the Experiment 2 and 3 based on qPCR results.*

Culture		**Average percent attachment (±s.d.)**
At. ferrooxidans	**Experiment 2**	60 (±31)
	Experiment 3	94 (±3)
S. metallicus	**Experiment 2**	99.8 (±0.01)
	Experiment 3	99.8 (±0.005)

Table 16. *Attachment percentage of cells from Enrichment culture 1 for the Experiment 4 based on qPCR results.*

Culture		**Average attachment percentage (±s.d.) (%)**
Enrichment culture 1	**Bacteria**	95 (±1)
	Archaea	99 (±1)

*For At. ferrooxidans percent attachment*s were 60±31% in Experiment 2 and 94±3% in Experiment 3. For *S. metallicus percent attachments were* 99.8±0.01% in Experiment 2 and 99.8±0.005% in Experiment 3. For Enrichment culture 1 in Experiment 4 attachment percentages were 95±1% of bacteria and 99±1% of archaea.

4.2 Detachment results in Experiment 1

Experiment 1 detachment results for enrichment cultures and pure cultures are presented in Figure 17.

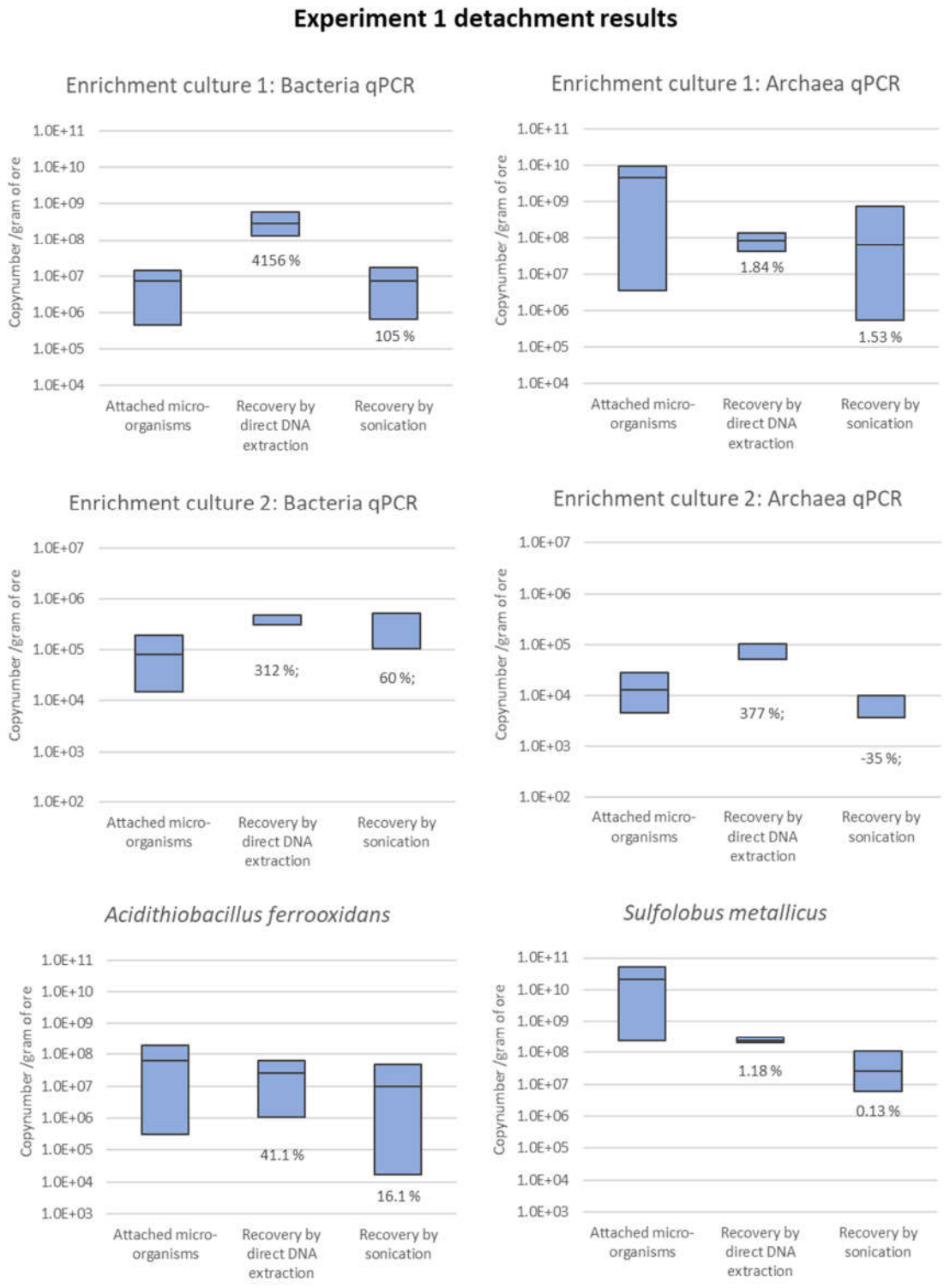

Figure 17. *Detachment results of Experiment 1 for enrichment cultures and pure cultures.*

For Enrichment culture 1 in recovery percentages for bacteria were up to 100% (4156±2818%) without the sonication protocol and up to 100% (105±80%) with the sonication protocol. For archaea the results were 1.84±0.88% without the sonication protocol and 1.53±4.52% with the sonication protocol.

For Enrichment culture 2 the recovery percentages were up to 100% (312±270%) without the sonication protocol and 60±202% with the sonication protocol. For archaea the results up to 100% (377±331%) without the sonication protocol and negative 35±22% with the sonication protocol.

For *At. ferrooxidans* the recovery percentages were 41±45% without the sonication protocol and 16±28% with the sonication protocol. For *S. metallicus* the recovery percentages were 1.2±0.21% without the sonication protocol and 0.13±0.17% with the sonication protocol.

4.3 Detachment results in Experiments 2-4

Modification of the sonication protocol affected the detachment of the cells. Results of the detachment optimization experiments are shown in Figure 18.

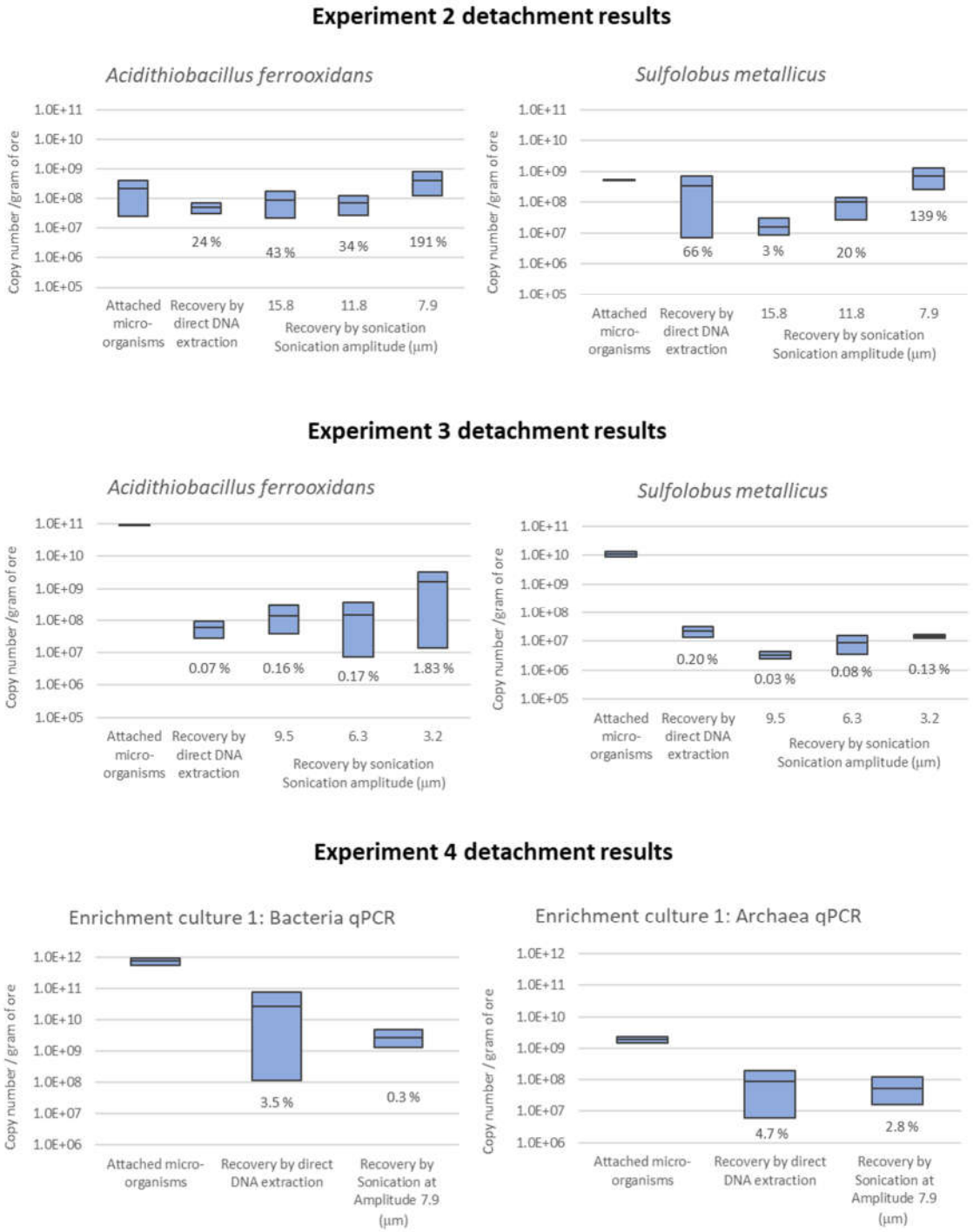

Figure 18. *Detachment results of Experiment 2-4 for Enrichment culture 1 and pure cultures.*

For *At. ferrooxidans* in Experiment 2 the recovery percentages were 24±9% without the sonication protocol and from 34±19% up to 100% (191±134%) with the sonication protocol. In Experiment 3 the results were 0.07±0.01% without the sonication protocol and from 0.16±0.14% up to 1.83±1.82% with the sonication protocol.

For *S. metallicus* in Experiment 2 the recovery percentages were 66±65% without the sonication protocol and from 3.1±1.9% up to 100% (139±78%) with the sonication protocol. In Experiment 3 the results were 0.20±0.10% without the sonication protocol and from 0.029±0.0069% up to 0.13±0.015% with the sonication protocol.

For Enrichment culture 1 in Experiment 4 recovery percentages for bacteria were 3.5±5% without the sonication protocol and 0.3±0.2% with the sonication protocol. For archaea the results were 4.7±4% without the sonication protocol and 2.8±2.6% with the sonication protocol.

5. DISCUSSION

5.1 Attachment of the cells

Cell count results (Table 13) show that with pure cultures and Enrichment culture 1 large fraction of the microorganisms attached from the culture solution onto the ore agglomerate surface and the results seem to be reliable due to low standard deviation. However, with Enrichment culture 2 attachment percentage was considerably lower than with the other three cultures and the standard deviation was much higher than with the other cultures. Enrichment culture 2 had relatively low amounts of cells that made the counting challenging which could be the reason for the different result. Unfortunately, cell count attachment results are reported only for Experiment 1. The cell count results for *At. ferrooxidans* in Experiment 3 (80%) and Enrichment culture 1 in Experiment 4 (80%) were done successfully and both supported the fact that majority of cells attached to the ore agglomerate. Mistakes during cell staining and problems with microscope resulted in loss of most of the cell count samples later experiments.

Similarly to the cell counts qPCR results (Table 14, Table 15 and Table 16) indicate that the majority of the microorganisms attached to the ore agglomerate. The first qPCR results (Table 14) had high standard deviations which shows the higher variability when compared to cell count results. These first results were obtained before the DNA extraction method was optimized and there were large differences in percent attachment results between replicants. Also, during the sampling of Enrichment culture 1 and *At. ferrooxidans* the metal removal procedure described in the methods was not done, which may have further affected the results. After the optimizations were made and the metal removal was added for all the samples, standard deviations of the qPCR results (Table 15 and Table 16) dropped significantly suggesting that the results became much more reliable. Only exception to this is the result for *At. ferrooxidans* but this seems like an outlier due to it being the only one with very high standard deviation.

The results are in line with previous studies that report on attachment of bioleaching microorganisms. Harneit *et al.* (2006) report that at highest 80 to 90% of *At. ferrooxidans* can attach to ore surface within an hour. In their study attachment experiments were done using pyrite, chalcopyrite, sphalerite or galena ores. The microorganism attachment was determined by cell count using a counting chamber. The percent attachment varied greatly between different ore types. The highest attachment was achieved only with pyrite and with the other minerals only 60 to 75% of *At. ferrooxidans* cells attached to the ore surface. In our study agglomerated black schist ore was used for attachment. Our attachment results based on cell count ranged from 80 to 84% so they were in the same range as the highest percent attachments reported by Harneit *et al*.. But our qPCR attachment results after the optimization were a little higher at 94%. With *At. ferrooxidans* our attachment results qPCR seemed to be more reliable. The little higher attachment percentage is most likely a result of the extra 23 h time used for the attachment when

compared the setup of Harneit *et al.* (2006) as well as the extra attachment surface area of ore agglomerate.

Halinen *et al.* (2009a, 2012) studied microbial communities in agglomerated black schist ore bioleaching environments. They report that 99% of the bioleaching microorganisms in columns attached to the ore agglomerate and that 98% of the microorganisms were found attached rather than in the leach liquor. Our qPCR attachment results after the optimization ranged from 94 to 99% when ignoring the assumed outlier so they were well in line with reports of Halinen *et al.*. Our cell count attachment results on the other hand were smaller ranging from 84 to 94%.

With Enrichment culture 1 the copy number of attached bacteria was 10^{11} per gram of ore and the same value for archaea was 10^{9}. With pure cultures there were surprising differences in number of microorganisms attached between experiments. *At. ferrooxidans* has six copies of 16S rRNA gene (NCBI, 2019a) which translates the number of cells attached to 10^{7} per gram of ore in Experiment 2 and 10^{10} in Experiment 3. *S. metallicus* has four copies of 16S rRNA gene (NCBI, 2019b) which translates the number of cells attached to in to 10^{8} per gram of ore in Experiment 2 and 10^{9} in Experiment 3. The difference is surprising because cultures were prepared same way same way each time before experiments and the optimized DNA retrieval method was used in both experiments.

Comparison of the results from this study show, that archaea attach to the ore agglomerate more than bacteria. This was the case with both enrichment cultures even if with Enrichment culture 2 the difference between bacteria and archaea is small. Only qPCR results are used for comparison because it is impossible to differentiate between bacteria and archaea visually under a microscope. The same phenomenon is observed when comparing the pure cultures. Both cell count and qPCR results show that larger fraction of the *S. metallicus* cells attach to the ore agglomerate when compared to the *At. ferrooxidans*. *S. metallicus* results in general show exceptionally high attachment onto the ore agglomerate. The experiments were conducted at 25°C which is not in the growth range of the *S. metallicus* (Table 2). The low temperature should not outright kill the cells, but it might have driven the *S. metallicus* cells to attach onto ore agglomerate more strongly. The temperature might also have affected the archaea and bacteria species in Enrichment culture 2.

To our knowledge this is first time the difference in attachment to the ore surface between bacteria and archaea has been compared quantitatively. Making the comparison was possible due to using modern microorganism quantification method qPCR. Distinguishing bacteria from archaea with traditional cell counting methods is not feasible.

Even though other studies have had success using cell counting in bioleaching environments there were a lot of problems with cell counting in this study. Even with up to 1000-fold dilutions ore particles would sometimes end up onto the filter and prevent focusing the microscope, which made counting difficult or even impossible. Our attachment results indicate that quantitative DNA extraction followed by qPCR seems to be a superior method to traditional cell counting with added benefit of recovering representative DNA sample of the microorganism community.

5.2 Detachment of the cells

Our results show that microorganisms were detached from the ore particles successfully with sonication, but with mixed results. When detachment with a waterbed sonicator (Figure 17) was compared with direct DNA-extraction from the ore agglomerate, the commercial DNA-extraction kit was always more effective. Results were also inconsistent with present such as detachment percentages greatly over 100% results or even negative percent recoveries. With Enrichment culture 2 yields from the control samples exceeded those of the final detachment samples which made the percent recoveries negative which together with attachment results made us neglect the Enrichment culture 2 in further experiments. Standard deviations of the results were high, which is expected when dealing with biological systems. The ore agglomerate used for detachment experiments had a rather heterogenous grain size which, in addition to the sub-sampling method used, made differences between samples inevitable.

The inconsistent results, when using the waterbed sonicator for detachment, are due to unoptimized procedures: DNA extraction was not as quantitative as it could be, sonication intensity of the waterbed sonicator was too low and settling method for the detachment samples was still unoptimized. Main takeaway from the detachment results before optimization is that the DNA of the attached microbes can be retrieved with and without sonication protocol. The results once again highlighted the importance of quantitative DNA extraction procedure and the need for consistency in general. After starting the use of the probe sonicator and quantitative DNA extraction procedure, the detachment results become much more reasonable. The standard deviations of DNA yields were still high but as mentioned earlier this was expected due to the nature of the ore agglomerate samples and the sub-sampling method used.

The detachment results with a probe sonicator (Figure 18) show that it is possible to recover microorganisms using the optimized detachment method and reach higher recoveries compared to when only using DNA extraction kit with the ore. Optimal sonication amplitude was 7.9 μm, 50% of the maximum, as by using it the highest percent recoveries were achieved with both pure cultures. In general, the detachment of cells with sonication protocol was most effective for *At. ferrooxidans* and with every sonication amplitude used recoveries with sonication protocol were higher than when only using the DNA extraction kit. For *S. metallicus* high recovery percentages were reached with the sonication protocol but most of the time higher recoveries were obtained when using only the DNA extraction kit.

Our results show that percent recoveries for Enrichment culture 1 did not improve with the use of the sonication protocol even when using the probe sonicator. When comparing the average values of percent recoveries for bacteria were 3.5% without the sonication protocol and 0.3% with the sonication protocol. For archaea the difference was smaller, 4.7% versus 2.8%, but the detachment with only using the DNA extraction kit was still more efficient.

When considering the 16S rRNA gene copy numbers of *At. ferrooxidans* and *S. metallicus* in one cell (NCBI, 2019b, 2019a) the average detached copy numbers were in the range of 10^7 cells per gram of ore for *At. ferrooxidans* and in the range 10^6 – 10^7 cells per gram of ore for *S. metallicus*. For Enrichment culture 1 copy number of detached bacteria was 10^9 per gram of ore. Even if all the bacteria belong to *At. ferrooxidans,* one

of the most abundant bacteria species in Terrafame mine leach liquors (Halinen *et al.*, 2012) from where water samples used as inoculum for enrichment cultures originated, would the cell number still be range of 10^8 cells per gram of ore. For enrichment culture copy number of detached archaea was 10^7 per gram of ore. The difference in yields between bacteria and archaea for Enrichment culture 1 can be explained by the difference in number of both types of cells attachment to the ore agglomerate.

Microorganism yields have been reported in previous studies when detaching ore surface attached microorganisms. The method used by Halinen *et al.* (2009a, 2012) was the basis that development of our method was built on. By using this sonication-based detachment method Halinen *et al*. retrieved 10^8 cell per gram of ore from leach residues in the column and on average 10^6 cells per gram of ore from heap samples. Cell counts were used on determining the detached cell number. Our detachment yields for pure cultures are in the same range or order of magnitude higher than yields from heap reported by Halinen et al. and lower than the yields from leach residues. Our detachment yields of 10^9 for bacteria in Enrichment culture 1 are potentially at least one order of magnitude higher than yields from the heap reported by Halinen et al. Our archaea yields of 10^7 were an order of magnitude lower.

Zeng *et al.* (2010) used a microorganism detachment method based on vortexing. The method was used on chalcopyrite concentrate in a stirred tank reactor and recovery up to 10^9 cells per gram of ore was reported. The number of detached cells was determined using qPCR on the 16S rRNA gene. The number of cells retrieved is higher than most of our detachment yields but the nature of stirred-tank leaching is different to that of heap leaching and the environment is also very different compared to heap or column leaching.

Lizama *et al.* (2012) used commercial soil kits to retrieve DNA from leached zinc sulphide ore residues. They do not report on the cell yields, but they mentioned that DNA was retrieved from 36 of the 45 samples. Lizama *et al*. did not report how many cells were recovered with the DNA extraction kit and the failed DNA retrievals could indicate low microbial biomass in the heap at the sampling location or that some pre-treatment of ore could help improve the DNA recovery.

The most important differentiator when comparing our study to the other presented studies is that only our study determined how many microorganisms really are attached to the ore surface and compared that number to the number of microorganisms detached. The other studies only compared the liquid phase to the solid matrix to give rough estimate of how many microorganisms were attached.

Even though Halinen *et al.* (2009a, 2012) reported on using the DAPI staining and microscopy to determine cell counts from the detachment samples, there were notable problems in our experiment. The problems with direct cell counts mentioned earlier when discussing the attachment samples further intensified with the increased amount fine ore particles that detached from the agglomerate. Also, there was visible interference in the samples when trying to count the stained cells using a microscope which was most likely caused by the detachment wash solution. Because of these problems the DAPI staining and cell counting was not used with detachment samples and qPCR became the sole method for determining the percent detachment. The additional problems with the detachment cell count samples further highlight the superiority of qPCR in microorganism quantification

Bioleaching microorganisms prefer to stay stationary on the surface of solid matrix next to the suitable energy sources rather than wasting energy by searching energy sources in planktonic state (Madigan *et al.*, 2019). Bioleaching microorganisms produce extracellular polymeric substances (EPS) on the surface of sulfide minerals to stay near to their preferred energy source and to create efficient microenvironment for the leaching (Vandevivere and Kirchman, 1993; Rohwerder *et al.*, 2003). In our experiments microorganisms were attachment during 24-hour period and detachment was performed right after. This means that there was very little or no time for the microorganisms to create EPS layers onto the ore agglomerate surface and so the results do not represent those of a real heap. Sonication-based detachment has been used successfully in pilot-scale heaps (Halinen *et al.*, 2012) where EPS have surely been produced by the bioleaching microorganisms. The next step for the method development would be to use the method with real heaps with established EPS residues and compare the result with commercial soil kits and other type of detachment methods.

6. CONCLUSIONS AND RECOMMENDATIONS

After this study following conclusions and recommendations can be made:

1. The attachment method used in this work demonstrates the fast attachment of bioleaching microorganisms to agglomerated ore matrix.

2. To our knowledge this is first time the difference in attachment to the ore surface between bacteria and archaea has been compared quantitatively. Over 94% *Acidithiobacillus ferrooxidans* and over 99% of Sulfolobus metallicus attached to the ore agglomerate matrix within 24 hours. With mesophilic bioleaching microorganism enrichment culture 95% of bacteria and over 99% of archaea attached to the matrix.

3. Using the developed microorganism detachment method, it is possible to recover microorganisms from the ore agglomerate matrix. The method should be usable with different sort of minerals.

4. qPCR combined with quantitative DNA retrieval and extraction is a good method for determining microorganism abundance in bioleaching environment.

5. Further development of the method requires tests with samples from operating bioleaching heaps with established microbial communities.

6. The retrieved DNA samples from this study and further tests should be deep sequenced. There is possibility to get further understanding on the heap microorganism communities and even find novel bioleaching species.

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APPENDIX A: CULTURE MEDIA

***Acidithiobacillus ferrooxidans*: 882. Leptospirillum (HH) medium (pH 1.8) (DSMZ, 2018)**

Material	Amount
Mineral salt medium (950 ml)	
$(NH_4)_2SO_4$ (mg)	132.0
$MgCl_2$ x 6 H_2O (mg)	53.0
KH_2PO_4 (mg)	27.0
$CaCl_2$ x 2 H_2O (mg)	147.0
Distilled water (ml)	950
pH adjusted to 1.8 with 10 N H_2SO_4. Solution sterilized by autoclaving at 121°C for 20 min.	
FeSO4 solution (50 ml)	
$FeSO_4$ x 7 H_2O (g)	
0.25 N H_2SO_4 (ml)	
pH of the solutions should be 1.2. Solution sterilized by autoclaving at 112°C for 30 min.	
Trace element solution (1 ml)	
$MnCl_2$ x 2 H_2O (mg)	**62.0**
$ZnCl_2$ (mg)	**68.0**
$CoCl_2$ x 6 H_2O (mg)	**64.0**
H_3BO_3 (mg)	**31.0**
Na_2MoO_4 (mg)	**10.0**
$CuCl_2$ x 2 H_2O (mg)	**67.0**
Distilled water (ml)	**1000**
Solution sterilized by autoclaving at 121°C for 20 min.	

***Sulfolobus metallicus*: 88. Sulfolobus medium (pH 2.0) (DSMZ, 2015)**

Material	Amount
Mineral salt medium (1000 ml)	
$(NH_4)_2SO_4$ (g)	1.30
KH_2PO_4 (g)	0.28
$MgSO_4$ x 7 H_2O (g)	0.25
$CaCl_2$ x 2 H_2O (g)	0.07
$FeCl_3$ x 6 H_2O (g)	0.02
Yeast extract (g/l)	0.20
Elemental sulfur (g/l) [1]	5.0
Distilled water (ml)	950
pH adjusted to 2.0 with 1 N HCl. Solution sterilized by autoclaving at 121°C for 15 min.	
Yeast extract stock 10% (w/v)	
Yeast extract (g)	10
Distilled water (ml)	50
Yeast extract should be at natural pH. Solution sterilized by autoclaving at 121°C for 15 min.	
Allen's trace element solution (1 ml)	
$MnCl_2$ x 4 H_2O (mg)	180.000
$Na_2B_4O_7$ x 10 H_2O (mg)	450.00
$ZnSO_4$ x 7 H2O (mg)	22.00
$CuCl_2$ x 2 H_2O (mg)	5.00
Na_2MoO_4 (mg)	3.00
$VOSO_4$ x 2 H_2O (mg)	3.00
$CoSO_4$ x 7 H_2O (mg)	1.00
Distilled water (ml)	1000
pH adjusted to 2.0 with 1 N HCl. Solution sterilized by autoclaving at 121°C for 15 min.	

[1] Elemental sulfur sterilized by keeping it in 105°C oven overnight.

Enrichment cultures: Acidic basal salt medium (pH 1.8) (Halinen *et al.*, 2009a)

Material	Amount
Acidic basal salt medium	
K_2HP_4 (g/l)	0.40
$(NH_4)_2SO_4$ (g/l)	0.40
$MgSO_4$ x 7 H_2O (g/l)	0.40
Black schist ore powder (g/l)	1.0

pH adjusted to 2.0 with concentrated H_2SO_4. Solution sterilized by autoclaving at 121°C for 30 min.

APPENDIX B: CHEMICAL LIST

Chemical	Manufacturer
$CaCl_2$ x 2 H_2O	Riedel-de Haën, Germany
$CoCl_2$ x 6 H_2O	Merck, Germany
$CoSO_4$ x 7 H_2O	Merck, Germany
$CuCl_2$ x 2 H_2O	Merck, Germany
EGTA, Ethylene glycol tetraacetic Acid	Sigma-Aldrich, USA
$FeCl_3$ x 6 H_2O	Merck, Germany
$FeSO_4$ x 7 H_2O	WVR, Belgium
H_3BO_3	Merck, Germany
HCl (37%)	WVR, Belgium
H_2SO_4 (95-97%)	Fisher Chemical, USA
KCl	Sigma-Aldrich, USA
KH_2PO_4	WVR, Belgium
K_2HPO_4	J.T. Baker, Netherlands
$MgCl_2$ x 6 H_2O	Merck, Germany
$MgSO_4$ x 7 H_2O	Merck, Germany
$MnCl_2$ x 2 H_2O	Merck, Germany
$MnCl_2$ x 4 H_2O	Merck, Germany
$(NH_4)_2SO_4$	Sigma-Aldrich, USA
$Na_2B_4O_7$ x 10 H_2O	WVR, Belgium
NaCl	Merck, Germany
Na_2HPO_4	WVR, Belgium
NaH_2PO_4	WVR, Belgium
Na_2MoO_4	J.T. Baker, Netherlands
S	WVR, Belgium
$VOSO_4$ x 2 H_2O	Sigma-Aldrich, USA
$ZnCl_2$	Merck, Germany
$ZnSO_4$ x 7 H2O	WVR, Belgium
Yeast extract	Neogen, USA
Zwittergent	Calbiochem, USA