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BIOOXIDATION OF IRON AND SULPHUR IN BIOHEAP LEACH LIQUORS

Master of Science Thesis

Examiners: Professor Jaakko Puhakka and Doctor Minna Peltola
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ABSTRACT

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Ferrous iron oxidation and sulphur oxidation are two of the main metabolic functions in acidophilic microorganisms present in bioleaching environments. Metals recovery from sulphide ores is based on oxidation of an insoluble metal sulphide to a water-soluble and leachable metal sulphate. Biological oxidation reactions produce oxidising agents such as ferric ions (Fe^{3+}) and protons, after which the metal is leached chemically. The activity of iron- and sulphur-oxidisers is affected by several microbiological, physicochemical, mineral, and processing parameters. In this thesis, several bioheap leach liquors originating from a mine site (Talvivaara Mining Company Plc, Sotkamo, Finland) were examined. The aim was to study the process liquors that were inhibiting biooxidation processes, and explore the microbiological and physicochemical factors responsible for their impaired growth conditions for the indigenous microorganisms.

The indigenous microorganisms present in leach liquors were enriched by supplementing the cultures with nutrients and either ferrous iron (Fe^{2+}; enrichment of iron-oxidisers) or elemental sulphur (S^0; enrichment of sulphur-oxidisers). The enrichment cultures were then used as inoculants in shake flask experiments (100 mL, 150 rpm, pH 1.7, 27 °C) performed to study Fe^{2+} resistance, aluminium (Al^{3+}) toxicity, and nutrient availability in the leaching environment. Different process liquors (PL1 - PL6) were also compared in terms of their bacterial community composition and growth conditions for the microorganisms.

Incubation of the process liquors (95 % v/v) with iron- or sulphur-oxidising microorganisms (5 % v/v) showed that PL4 limited both oxidation mechanisms more than other liquors. This was the case even though the indigenous bacterial community in PL4 was diverse, containing iron-oxidisers (*Leptospirillum ferrodiazotrophum*), sulphur-oxidisers (*Acidithiobacillus thiooxidans*), and bacteria capable of both metabolic functions (*Acidithiobacillus ferrooxidans, Acidiferrobacter thiooxydans, Sulfobacillus acidophilus*). A dilution series of PL4 in mineral salts medium (MSM) demonstrated that 100 % (v/v) of the liquor had a very low iron oxidation rate (27 mg/L/h), whereas addition of 4 % (v/v) of MSM and 1 % (v/v) of TES (trace elements solution) distinctly increased the oxidation activity (to 156 mg/L/h). It was concluded that PL4 was either

lacking essential nutrients, or contained inhibiting components. Compared to other process liquors, it was found that PL4 contained the highest concentrations of several liquor components and in addition, nutrient concentrations in all the process liquors were largely unknown. Thus, further examination was required.

Inoculated PL4 was incubated with nutrients, iron-oxidising microorganisms, and increasing Fe^{2+} concentrations (5, 8, 12, and 16 g/L) in order to study its Fe^{2+} tolerance. High Fe^{2+} content in the culture did not lengthen the oxidation lag phase and increasing iron concentrations even enhanced oxidation rates, demonstrating that the iron-oxidising microorganisms in PL4 were well adapted to high Fe^{2+} concentrations. This result was expected, as some of the other process liquors with effective oxidation activities had a distinctly higher Fe^{2+} content than in PL4. The results of a similar aluminium toxicity experiment with different Al^{3+} concentrations (3, 6, 9, and 12 g/L) revealed that increasing Al^{3+} concentrations slightly lengthened the lag phase and decreased iron oxidation rates. However, as the iron oxidation rate in a culture containing 12 g/L of Al^{3+} (almost double the concentration of PL4) remained quite high (119 mg/L/h), it was concluded that aluminium inhibition was not responsible for the impaired growth conditions in PL4.

Incubation of non-inoculated PL4 (50 % v/v) with MSM (49 % v/v) and TES (1 % v/v) resulted in a high iron oxidation rate (175 mg/L/h). However, without MSM or TES the oxidation rate was significantly lower (18 mg/L/h), suggesting that nutrient deficiency was inhibiting the activity of bioleaching microorganisms. Iron oxidation in PL4 supplemented with sodium (Na), potassium (K), phosphorus (P), boron (B), molybdenum (Mo), or selenium (Se), or with a combination of some of these nutrients, was very slow with oxidation rates in the range of 11 - 17 mg/L/h. However, addition of 3900 mg/L of nitrogen (N; a concentration present in 49 % v/v of MSM) resulted in a distinctly higher oxidation activity, with an iron oxidation rate of 154 mg/L/h. Total organic and inorganic N determination performed for PL4 showed that the liquor's N content was under the detection limit (1 mg/L). It was concluded that the iron oxidation activity in PL4 was N limited. As previously shown in the dilution series, PL4 supplemented with 4 % (v/v) of MSM had a high iron oxidation activity, indicating that PL4 required 319 mg/L or less of N.

This thesis work demonstrated that nitrogen supplementation to PL4 would increase iron oxidation rates and leaching efficiencies in bioheaps. In future studies, the N concentration required by the indigenous microorganisms in PL4 should be examined. Nutrient deficiency rather than high ion concentration was demonstrated to be the limiting factor for microbial activity. The limiting concentrations of some of the most high-content anions and cations in the prosess liquors, such as that of sulphate (SO_4^{2-}), remain to be determined.

TIIVISTELMÄ

TAMPEREEN TEKNILLINEN YLIOPISTO
Luonnontieteiden tiedekunta, Kemian ja biotekniikan laitos
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Perinteisiin metallien erotusmenetelmiin verrattuna bioliuotus on yksinkertainen, halpa ja ympäristöystävällinen prosessi, johon tarvittavia mikrobeja esiintyy synnynnäisesti kaivosmaaperässä. Asidofiiliset mikro-organismit osallistuvat bioliuotusprosesseihin joko muuntamalla liukenemattoman metallisulfidin liukoiseksi -sulfaatiksi, tai muuttamalla mineraalin rakennetta jolloin sen kemiallinen liuotus tehostuu. Prosessiin vaaditaan mikrobien lisäksi vain vettä ja ilmaa. Bioliuotettavan mineraalin tulee joko sisältää rautaa tai pelkistettyä rikkiä, tai olla suorassa yhteydessä näihin komponentteihin. Erilaisia bioliuotusmenetelmiä on useita, joista kasaliuotusta on käytetty maailmanlaajuisesti mm. kuparin liuottamiseen köyhistä sulfidimalmeista. Kasan olosuhteiden pitäminen happamina ja hapellisina voi kuitenkin olla haasteellista. Lisäksi kasaliuotuksen liuotusajat ovat pitkiä ja happamien jätevesien käsittely ongelmallista.

Ferroraudan (Fe^{2+}) ja rikin hapetus ovat kaksi tärkeintä metabolista toimintoa bioliuottavissa mikrobeissa. Hapetusreaktioiden johdosta metalli vapautuu liuokseen, josta se voidaan ottaa talteen. Raudanhapettajabakteerien tärkein tehtävä on muuttaa ferrorauta ferriraudaksi (Fe^{3+}). Ferrirauta on vahva hapetin, joka osallistuu useiden eri sulfidimineraalien liuotukseen ilman hapen tai mikrobien läsnäoloa. Metallisulfidien ominaisuuksista riippuen mineraalit hapetetaan joko tiosulfaatti- (happoon liukenemattomat sulfidit) tai polysulfidimekanismin (happoon liukenevat sulfidit) avulla. Jälkimmäisessä myös protonit osallistuvat mineraalin liuotukseen ferriraudan lisäksi. Tiosulfaatti- ja polysulfidimekanismit tuottavat erilaisia pelkistyneitä rikkiyhdisteitä, jotka rikinhapettajabakteerit hapettavat sulfaatiksi ja protoneiksi. Näistä syntyvä rikkihappo (H_2SO_4) pitää bioliuotusympäristön happamana ja samalla raudan liukoisessa muodossa, kun taas protonit osallistuvat liuotusreaktioihin.

Kaivosten jätevedet voivat aiheuttaa ympäristöongelmia niiden korkeiden metalli- ja sulfaattipitoisuuksien sekä matalan pH:n takia. Erilaisia biohydrometallurgisia tekniikoita kuten bioremediaatio, biosorptio ja bioakkumulaatio on kehitetty vesien epäpuhtauksien poistamiseksi. Fysikaalisiin käsittelymenetelmiin lukeutuvat mm. sedimentaatio

sekä laskeutus ja kemiallisiin menetelmiin sorptio, adsorptio, saostus ja metallien hapetus.

Raudan- ja rikinhapettajamikrobit ovat useimmiten aerobisia, asidofiilisia ja kemolitoautotrofisia bakteereja ja arkkeja, käyttäen ferrorautaa tai pelkistettyjä epäorgaanisia rikkiyhdisteitä (kemiallisen sulfidihapetuksen tuotteita) elektronien luovuttajana, happea elektronien vastaanottajana ja hiilidioksidia hiilen lähteenä. Useimmat bioliuotusbakteerit ovat joko mesofiileja tai termofiileja eli ne kasvavat mieluiten 15 - 60 °C lämpötiloissa. Osa mikrobeista hapettaa sekä ferrorautaa että rikkiyhdisteitä (*Acidithiobacillus ferrooxidans, Acidiferrobacter thiooxydans, Sulfobacillus acidophilus*), osa on pelkkiä raudan- (*Acidimicrobium ferrooxidans, Leptospirillum ferriphilum*) tai rikinhapettajia (*Acidiphilium cryptum, Acidithiobacillus thiooxidans*). Näiden lisäksi sekundääriset bioliuotusmikrobit hyödyntävät orgaanisia yhdisteitä, jotka saattaisivat olla haitallisia autotrofeille. Asidofiiliset raudan- ja rikinhapettajat ovat kehittäneet erilaisia mekanismeja suojautuakseen bioliuotusympäristöjen korkeita metallipitoisuuksia vastaan. Näihin mekanismeihin kuuluvat mm. metallien entsymaattinen konversio ja toksisen metallin pumppaus ulos solusta.

Monet fysikaalis-kemialliset, mikrobiologiset, mineralogiset ja prosessitekijät vaikuttavat mineraalien biologiseen hapetukseen. Fysikaalis-kemiallisista tekijöistä tärkeimpiä ovat liuoksen pH, redox-potentiaali, lämpötila, happipitoisuus, hiilidioksidin määrä, rauta- ja rikkiyhdisteet, ravinteiden saatavuus, erilaiset ionit sekä raskasmetallien esiintyvyys. Liuoksen pH (1.5 - 3.0) on tärkeä sekä hapettajabakteerien toiminnan että ferriraudan ja metallien liukoisuuden kannalta. Bioliuotusympäristön liian korkea pH voi johtaa ferriraudan saostumiseen jarosiittina ja mineraalin passivoitumiseen. Myös liian suuri alkuainerikin pitoisuus voi aiheuttaa mineraalin passivoitumista. Mikrobisolujen kasvun kannalta tärkeimmät ravinteet ovat hiili (ilman hiilidioksidista), typpi ja fosfori (lannoitteista). Lämpötila taas vaikuttaa mikrobiyhteisön rakenteeseen ja kemiallisten reaktioiden nopeuteen.

Tässä työssä tutkittiin Talvivaaran Kaivososakeyhtiö Oyj:n Sotkamon biokasaliuotuksesta peräisin olevia prosessivesiä ja niiden vaikutusta kaivoksen luontaisen mikrobiyhteisön aktiivisuuden laskuun. Aluksi prosessivesien raudan- ja rikinhapettajabakteereja rikastettiin lisäämällä liuoksiin substraatiksi joko ferrorautaa tai alkuainerikkiä. Näitä rikastusviljelmiä käytettiin myöhemmissä 100 ml ravistelupullokokeissa, jotka suoritettiin 27 °C lämpötilassa, pH:ssa 1,7 ja 150 rpm sekoitusnopeudella. Ravistelupullokokeilla tutkittiin biokasabakteerien käyttäytymistä kasvavissa ferrorauta- ja alumiinipitoisuuksissa (Al^{3+}) sekä tarkasteltiin ravinteiden riittävyyttä prosessivesissä. Lisäksi haluttiin tutkia korkeiden sulfaattipitoisuuksien (SO_4^{2-}) vaikutusta bakteerien raudanhapetusaktiivisuuteen. Eri prosessivesiä (PL1 - PL6) myös vertailtiin niiden kasvuolosuhteiden ja bakteeriyhteisöjen osalta.

Prosessivesien (95 % v/v) inkubointi raudan- tai rikinhapettajaviljelmien (5 % v/v) kanssa osoitti, että PL4-liuoksen kasvuolosuhteet olivat molemmille hapetusmekanismeille epäsuotuisammat kuin muiden prosessivesien. PL4 rajoitti hapettajabakteerien toimintaa siitäkin huolimatta, että liuoksen bakteeristo oli moninaisin koostuen sekä raudanhapettajista (*Leptospirillum ferrodiazotrophum*), rikinhapettajista (*Acidithiobacillus thiooxidans*) että molempia substraatteja hyödyntävistä bakteereista (*Acidithiobacillus ferrooxidans, Acidiferrobacter thiooxydans, Sulfobacillus acidophilus*). PL4-liuoksen laimennossarja mineraalisuolaliuoksessa (MSM; mineral salts medium) osoitti, että 100 % (v/v) prosessiveden raudanhapetusnopeus oli hyvin alhainen (27 mg/L/h). Ravistelupullossa, jossa 95 %:iin (v/v) PL4-liuosta lisättiin 4 % (v/v) MSM-liuosta ja 1 % (v/v) hivenaineliuosta (TES; trace elements solution) raudanhapetus kuitenkin selvästi tehostui saavuttaen 156 mg/L/h hapetusnopeuden. Koe osoitti, että joko PL4-liuoksen ravinnepitoisuus oli riittämätön rautaa hapettaville bakteereille tai prosessivesi sisälsi bakteereja inhiboivia aineosia. Muihin prosessivesiin verrattuna PL4 sisälsi suuren pitoisuuden mm. alumiinia, klooria (Cl), sulfaattia ja useita liuotettavia metalleja. Prosessivesien ravinnepitoisuuksia ei tunnettu. Tästä syystä inhibition taustalla olevaa tekijää ei voitu suoraan päätellä, vaan lisäkokeet olivat välttämättömiä.

PL4-liuosta inkuboitiin rautaa hapettavan bakteeriviljelmän, ravinteiden (MSM- ja TES-liuokset) ja kasvavan ferrorautapitoisuuden (5, 8, 12 ja 16 g/l) kanssa rautapitoisuuden vaikutuksen määrittämiseksi raudanhapetusaktiivisuuteen. Ravistelupullokoe osoitti, että kasvava ferrorautapitoisuus nopeutti raudanhapetusta. Koska raudanhapetuksen lag-vaihe ei pidentynyt pitoisuuden kasvaessa, PL4-liuoksen bakteeristo sieti biokasojen korkeita ferrorautapitoisuuksia. Tulos oli odotettu, koska osassa prosessivesistä oli PL4-liuosta selvästi korkeammat ferrorautapitoisuudet, mutta paremmat olosuhteet raudanhapettajille.

Alumiinin toksisuutta tutkittiin ravistelupullokokein käyttäen alumiinipitoisuuksia 3, 6, 9 ja 12 g/l. Kasvava alumiinipitoisuus pidensi hieman raudanhapetuksen lag-vaihetta ja laski raudanhapetusnopeuksia, osoittaen että PL4-liuoksen bakteerikanta ei ollut sopeutunut korkeisiin alumiinipitoisuuksiin yhtä hyvin kuin ferroraudan tapauksessa. Raudanhapetusnopeus oli melko suuri (119 mg/l/h) 12 g/l alumiinipitoisuudessa. PL4 sisälsi 6,1 g/l alumiinia, joka ei inhiboinut liuoksen raudanhapettajia.

PL4-liuoksen sulfaattipitoisuus oli yli 130 g/l ja ioni on mikrobi-inhibiittori. Sulfaatin toksisuutta aiottiin tutkia lisäämällä prosessiveteen eri määriä bariumkloridia ($BaCl_2 \cdot 2\ H_2O$) ja siten pienentämällä liukoisen sulfaatin pitoisuutta saostamalla sitä bariumsulfaattina. Koska kloridi-ioni (Cl^-) inhiboi raudanhapetusta jo pienissä pitoisuuksissa (yli 5 g/l), koetta ei voitu suorittaa. Muita harkittuja pullokoevaihtoehtoja olivat sulfaatin lisääminen pulloihin joko ammonium- tai natriumsulfaattina tai sulfaatin lisäys rikkihappona, jolloin pH olisi säädetty 1,7:ään NaOH:lla tai KOH:lla. Näissä koejärjestelyis-

sä ongelmaksi muodostui kationien konsentroituminen liuokseen ja sitä kautta raudanhapettajabakteerien inhiboituminen.

Ravistelupullokokeessa, jossa 50 % (v/v) PL4-liuosta inkuboitiin 49 % (v/v) MSM-liuoksen ja 1 % (v/v) TES:in kanssa, raudanhapetusnopeus oli suuri (175 mg/l/h). Tämä osoitti, että prosessiveden bakteerit olivat aktiivisia. Samalla PL4-pitoisuudella, mutta ilman ravinnelisäystä hapetusnopeus kuitenkin laski merkittävästi (18 mg/l/h) osoittaen, että ravinteiden puute rajoitti PL4-liuoksen bakteerien aktiivisuutta. Kun liuokseen lisättiin selektiivisesti natriumia (Na), kaliumia (K), fosforia (P), booria (B), molybdeenia (Mo), seleeniä (Se) tai näiden yhdistelmiä, raudanhapetusnopeudet olivat pieniä (11 - 17 mg/l/h). Lisäämällä PL4-liuokseen 3900 mg/l typpeä (N; pitoisuus 49 %:ssa v/v MSM-liuosta), raudanhapetusnopeus kasvoi merkittävästi (154 mg/l/h). Orgaanisen ja epäorgaanisen kokonaistypen määritys osoitti, että kaikkien prosessivesien typpipitoisuudet olivat alhaiset (alle 3 mg/l) ja PL4-liuoksen pitoisuus oli alle määritysraja-arvon (1 mg/l). Tämä osoitti, että PL4-liuoksessa tapahtuva raudanhapetus on typpirajoittunutta. PL4-liuoksen laimennossarjan perusteella prosessiveteen tarvitaan 4 % (v/v) MSM-liuosta (< 319 mg/l typpeä).

Tässä diplomityössä osoitettiin, että typen lisäys biokasaliuotusprosessiin tehostaisi prosessivesien bakteeriyhteisön raudanhapetusta ja sitä kautta metallien liuottamista. Prosessivesien typpipitoisuudet olivat alhaiset. Tulevissa kokeissa olisi syytä selvittää, kuinka paljon PL4-liuoksen mikrobit tarvitsevat typpeä toimiakseen aktiivisesti. Jatkossa typpipitoisuudet tulee määrittää tuoreista näytteistä. Alhaisissa ravinnepitoisuuksissa suuret metalli- ja ionikonsentraatiot (esimerkiksi sulfaatti) saattavat laskea hapetusnopeuksia.

PREFACE

This Master of Science thesis concentrates on the microbial oxidation of iron and sulphur in bioheap leach liquors originating from Sotkamo, Finland. Research work was done at Tampere University of Technology, in the Department of Chemistry and Bioengineering. I wish to thank Talvivaara Mining Company Plc for providing and funding this study.

I would like to express my gratitude to Professor Jaakko Puhakka for his excellent guidance and endless encouragement throughout the making of this thesis, and for giving me the opportunity to work in the department. I also sincerely want to thank Dr. Minna Peltola for proofreading my research plans and finally this thesis, and for being such an inspirational and supportive supervisor. I wish to thank Lic. Marja Riekkola-Vanhanen and Dr. Pauliina Saari at Talvivaara Mining Company Plc for their cooperation and valuable opinions. I also want to thank Dr. Kathryn Wakeman, M.Sc. Toni Jaatinen, and M.Sc. Hanna Hynynen for their knowledge and guidance at the beginning of my laboratory work. The help with DGGE by Dr. Aino-Maija Lakaniemi and with nitrogen determination by Chief Laboratory Technician Tarja Ylijoki-Kaiste is highly appreciated. Antti, thanks for your friendship. Finally I wish to thank everyone working in the department for the wonderfully helpful and cosy atmosphere.

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Tampere, 18th January 2013

Sarita Ahoranta

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ABBREVIATIONS

AMD	acid mine drainage
APHA	American Public Health Association
BLAST	basic local alignment search tool
CDF	cation diffusion facilitator
DGGE	denaturing gradient gel electrophoresis
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
EPS	extracellular polymeric substance
FBR	fluidised-bed reactor
IC	ion chromatography
MSM	mineral salts medium
NCBI	National Center for Biotechnology Information
ORP	oxidation reduction potential
PCR	polymerase chain reaction
PL	process liquor
PLS	pregnant leach solution
RISC	reduced inorganic sulphur compound
RNA	ribonucleic acid
RND	resistance, nodulation, cell division
SFS	Suomen standardisoimisliitto (Finnish Standard Association)
SRB	sulphate-reducing bacteria
TES	trace elements solution
v/v	volume/volume
w/v	weight/volume

1 INTRODUCTION

In bioleaching, components readily present in the environment (water, air, and microorganisms) are used to solubilise and recover metals from sulphide ores (reviewed by Shagufta 2007). Microorganisms such as bacteria and fungi catalyse the bioleaching processes either by converting insoluble metal sulphides or oxides to water-soluble sulphates, or by changing the mineral structure and thus enhancing its chemical solubilisation (for a review, see Rawlings 2005). Copper, cobalt, nickel, zinc, and uranium are extracted from insoluble ores using bioleaching methods, whereas ores containing silver and gold are merely pretreated with biooxidation before chemical solubilisation (reviewed by Rohwerder et al. 2003). Bioleaching is possible for minerals that either contain iron and reduced sulphur, or are in close contact with this kind of mineral (reviewed by Rawlings 2005). Ferrous iron oxidation, sulphur oxidation, and the fixation of carbon dioxide are the main metabolic functions in microorganisms used for bioleaching (reviewed by Shagufta 2007).

Bioleaching has many significant advantages compared to traditional ways of metal extraction. The bioleaching process is simple to expand, operate, and maintain: it uses naturally occurring components and does not require high pressure or temperature (reviewed by Shagufta 2007). Harmful gaseous emissions, such as sulphur dioxide, typically present in physico-chemical mining processes are absent in bioleaching applications (for a review, see Rawlings et al. 2003). The extraction of metals from minerals by the use of microorganisms is also beneficial due to the vast variety of useful iron- and sulphur-oxidisers, a strong positive selection towards the most efficient microorganisms, and the fact that process acepticity is not required (reviewed by Rawlings 2007). Commercial biomining applications in base metal leaching, gold extraction, and copper extraction are well established (reviewed by Brierley 2008).

Dump bioleaching, heap bioleaching, and stirred tank bioleaching are different methods commercially applied to base metal biomining. Heap bioleaching, the focus of this thesis, is used worldwide for instance to extract copper from sulphide ores. Crushed and agglomerated ore is stacked, and the conditions inside the heap are maintained acidic and aerated for a successful growth of indigenous bioleaching bacteria. The heap temperature is increased and decreased simultaneously with mineral oxidation and depletion, respectively (reviewed by Brierley 2008). Heap bioreactors are especially useful in the leaching of low-grade ores, as they are cheap to build and operate (reviewed by Rawlings 2005). However, although appearing to be a simple process, heap bioleaching

has several sub-processes such as flows of solution, gas, and heat (for a review, see Ghorbani et al. 2011). Thus, maintaining the conditions inside the heap favourable for microorganisms can be a challenge, with insufficient aeration and gradients of pH or nutrients being problematic (reviewed by Rawlings 2005). Other disadvantages include low recoveries, lengthy pilot tests and leaching cycles, large footprint, and the possible release of pregnant leach solution (PLS) into the environment (reviewed by Ghorbani et al. 2011).

There are several physicochemical, microbiological, mineral, and processing factors affecting the microbial mineral oxidation (reviewed by Shagufta 2007). The microbial communities present in bioleaching processes are affected and modified by temperature, pH, Fe^{3+}/Fe^{2+} ratio, dissolved oxygen, carbon dioxide, and concentrations of sulphate and metal ions. Consequently, the changes in microbiology have an effect on leaching efficiency (Bowei et al. 2009).

In this thesis, microbial oxidation in heap leaching liquors originating from Talvivaara Mining Company Plc mine site (in Sotkamo, Finland) was studied. Different physico-chemical factors affecting iron and sulphur oxidation such as nutrient sufficiency, heavy metal inhibition, and the impact of several ions were explored using shake flasks. The experiments were performed at low pH (1.7) to simulate the microbial growth conditions at the mine site liquors, and at a temperature of 27 °C. Additionally, microbial community compositions in the process liquors were examined in order to compare the liquors' growth conditions. The aim of this work was to reveal the factors affecting decreased oxidation activity in the bioheaps, and to offer suggestions for its improvement.

2 IRON AND SULPHUR OXIDATION IN BI-OLEACHING

Microorganisms mobilise metals by either forming organic or inorganic acids, through oxidation and reduction reactions, or by excreting complexing agents that increase metal solubilisation (reviewed by Mishra et al. 2005). Metals recovery from sulphide-containing ores using the bioleaching process is based on microbial oxidation of a bivalent insoluble metal sulphide to a water-soluble and thus leachable metal sulphate (for a review, see Mohapatra 2006). Bioleaching processes involve both chemical and biological reactions. The metal is leached chemically, although ferric iron (Fe^{3+}) and protons needed in the process are produced by microorganisms. Ferric ions play an important role as oxidising agents, and oxidation of sulphur compounds maintains the bioleaching environment acidic (Bhatti et al. 2012b). Additionally, the leaching space is generated by microorganisms (reviewed by Rawlings 2005). Leached into a solution, the metal is ready for extraction (for a review, see Rawlings et al. 2003).

2.1 Iron oxidation

In bioleaching systems, Fe^{2+} oxidation is an acid-consuming step, while sulphur oxidation decreases the culture pH (Fu et al. 2008). For years, it was believed that as an alternative to an "indirect mechanism", bioleaching processes may comprise of a so-called "direct mechanism": enzymatic oxidation of the sulphur content in metal sulphides. In the direct leaching mechanism, microorganisms are thought to interact with the mineral and oxidise metal sulphides by obtaining electrons directly from the reduced mineral (for a review, see Bosecker 1997). There are almost as many ways to present the mechanism as there are articles about the theory of it; however, reviews by Mishra et al. (2005) and Sand et al. (2001) both explained the direct leaching of pyrite (FeS_2) using the following equations:

$$FeS_2 + 3.5O_2 + H_2O \rightarrow Fe^{2+} + 2H^+ + 2SO_4^{2-} \quad (1)$$

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

The direct leaching mechanism is independent of the indirect mechanism and requires physical contact between microorganisms and the sulphide. Leaching of non-iron metal sulphides, such as covellite (CuS) and sphalerite (ZnS), to their respective sulphates is possible through direct contact leaching. However, there has been a lot of debate and

research about whether the direct mechanism does exist, and nowadays the indirect mechanism is widely accepted as the only plausible alternative (for reviews, see Rohwerder et al. 2003; Sand & Gehrke 2006). Indirect leaching involves the ferric-ferrous cycle, consisting of microbial oxidation of ferrous ions to ferric ions (Breed & Hansford 1999). Using pyrite as an example, the indirect leaching mechanism may be presented using the following equations (Bosecker 1997; Sand et al. 2001):

$$FeS_2 + 14Fe^{3+} + 8H_2O \rightarrow 15Fe^{2+} + 16H^+ + 2SO_4^{2-} \quad (3)$$

$$MS + 2Fe^{3+} \rightarrow M^{2+} + S^0 + 2Fe^{2+} \quad (4)$$

Elemental sulphur (S^0) produced in indirect leaching is further oxidised to protons and sulphate (sulphuric acid, H_2SO_4) by sulphur-oxidising microorganisms (for reviews, see Mishra et al. 2005; Sand et al. 2001). In the indirect leaching mechanism, no physical contact between the microbes and mineral surface is required: the microorganisms catalytically accelerate the reoxidation of Fe^{2+} to Fe^{3+} ions, a reaction that chemically is very slow (Breed & Hansford 1999; for a review, see Bosecker 1997). As a strong oxidising agent, ferric ion is capable of dissolving a variety of different metal sulphide minerals in the absence of microorganisms and oxygen (Córdoba et al. 2008c). The role of iron-oxidising microorganisms is thus to produce more ferric iron than is consumed in the leaching reactions (reviewed by du Plessis et al. 2007). However, increased Fe^{3+} ion concentrations produce chemical instability and favour precipitation, consequently passivating the mineral (Córdoba et al. 2008c).

Indirect leaching is often divided into two sub-mechanisms: “contact” and “non-contact” mechanism. The non-contact mechanism comprises of Fe^{2+} to Fe^{3+} oxidation by planktonic bacteria, after which the ferric ions are reduced in contact with the ore surface and re-introduced into the iron cycle. In the contact mechanism, mineral dissolution takes place in an extracellular polymeric substance (EPS) or slime between bacterial cell walls and the mineral surface (reviewed by Sand & Gehrke 2006).

There are two different groups of metal sulphides: acid-insoluble and acid-soluble. Thus, two different (indirect) oxidation mechanisms have been proposed (Figure 2.1; for a review, see Schippers 2007). When oxidising acid-insoluble metal sulphides such as pyrite, molybdenite (MoS_2), or tungstenite (WS_2), the mineral dissolution proceeds via thiosulphate mechanism. The mechanism is named after its main intermediate, $S_2O_3^{2-}$, which is the first free sulphur compound released after many electron-removal steps that finally break the chemical bond between the metal and sulphur. The metal sulphide (in this case, pyrite) is solubilised by the attack of ferric iron, not by the attack of protons (Schippers & Sand 1999):

$$FeS_2 + 6Fe^{3+} + 3H_2O \rightarrow S_2O_3^{2-} + 7Fe^{2+} + 6H^+ \quad (5)$$

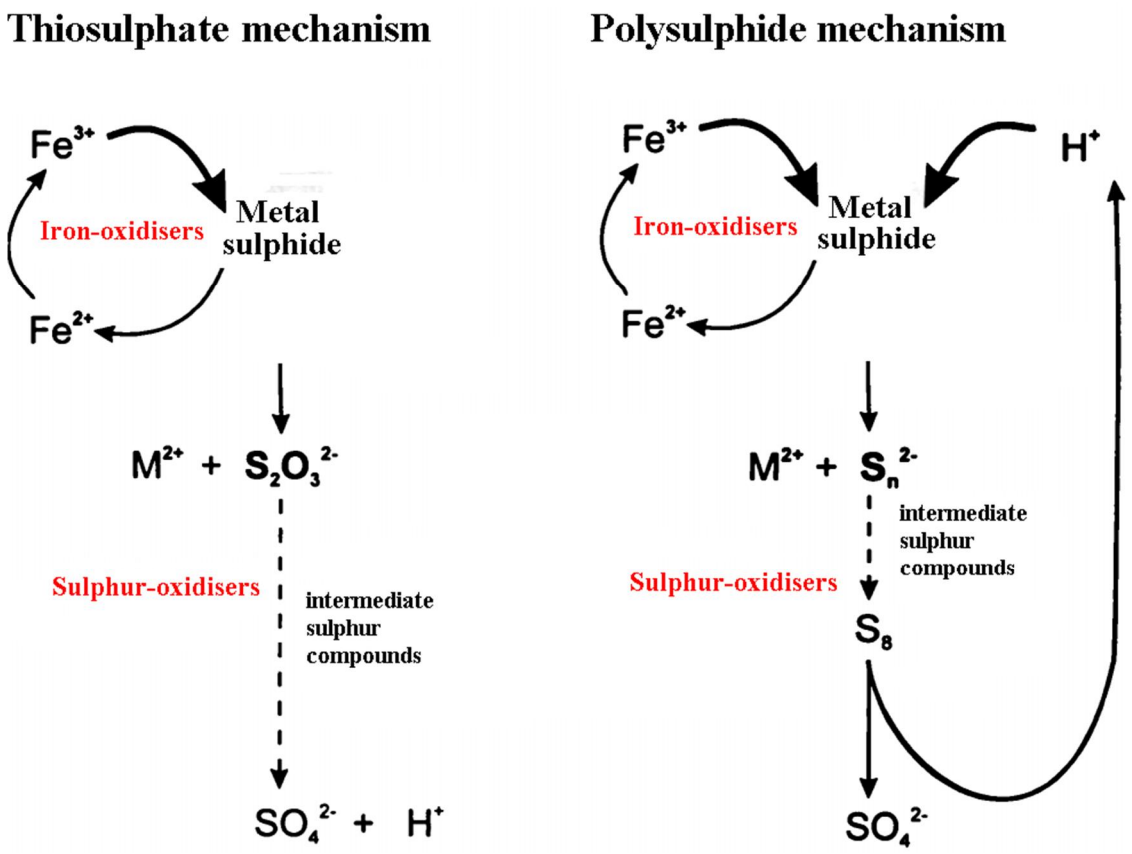

Figure 2.1. Thiosulphate and polysulphide mechanisms. The properties of metal sulphides define which indirect bioleaching mechanism is used: acid-insoluble metal sulphides are oxidised via thiosulphate mechanism and acid-soluble sulphides via polysulphide mechanism. (Modified from Schippers & Sand 1999.)

As thiosulphate is not stable in acidic environments, it is oxidised by sulphur-oxidising bacteria and Fe^{3+} ions to its main end-product sulphate, via intermediates such as tetrathionate and other polythionates (Rawlings 2007):

$$S_2O_3^{2-} + 8Fe^{3+} + 5H_2O \rightarrow 2SO_4^{2-} + 8Fe^{2+} + 10H^+ \quad (6)$$

The absence of sulphur-oxidisers may result to the accumulation of elemental sulphur via intermediates as follows (Rawlings 2007):

$$8S_2O_3^{2-} \rightarrow S_8 + 8SO_3^{2-} \quad (7)$$

Acid-soluble metal sulphides such as sphalerite and chalcopyrite ($CuFeS_2$), on the other hand, are oxidised via polysulphide mechanism. In this case, the metal sulphide is attacked by both ferric iron and protons. Firstly, the binding of two protons releases hydrogen sulphide and breaks the chemical bond between sulphur and the metal. Secondly, in the presence of ferric ions, the sulphur moiety is oxidised via higher polysulphides

to elemental sulphur (for reviews, see Rawlings 2007; Rawlings 2005). The reactions may be presented as follows (Schippers & Sand 1999):

$$MS + Fe^{3+} + H^{+} \rightarrow M^{2+} + 0.5H_2S_n + Fe^{2+} \qquad (n \geq 2) \qquad (8)$$

$$0.5H_2S_n + Fe^{3+} \rightarrow 0.125S_8 + Fe^{2+} + H^{+} \qquad (9)$$

Elemental sulphur is the main intermediate of the polysulphide mechanism, and although quite stable, it can be further oxidised to sulphate by sulphur-oxidising microorganisms (reviewed by Rawlings et al. 2003). Ferrous iron produced in the reactions can be reoxidised to ferric iron by iron-oxidising microorganisms (for reviews, see Holmes & Bonnefoy 2007; Rawlings 2005).

2.2 Sulphur oxidation

In the lithosphere, sulphur is concentrated in metal sulphide ores, coal deposits (up to 20 % by weight), or in other parts of the crust. Sulphur can exist in various oxidation states ranging from -II (for example in hydrogen sulphide), to +VI in sulphate (for reviews, see Dopson & Johnson 2012; Holmes & Bonnefoy 2007). It can be utilised by acidophilic bacteria and archaea in dissimilatory metabolic processes, where elemental sulphur and inorganic sulphur compounds serve as electron donors and acceptors in various redox transformations (Figure 2.2).

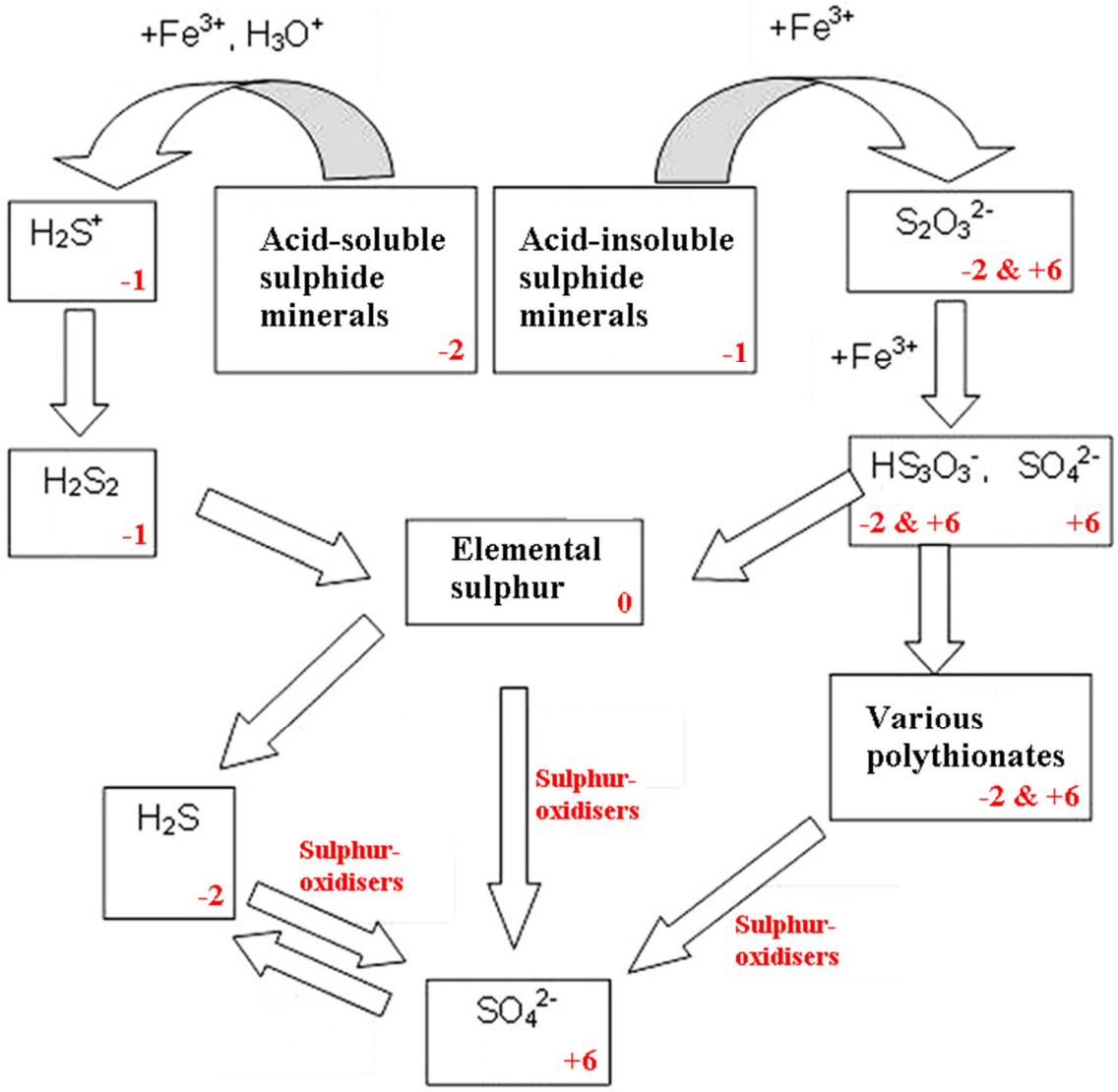

Figure 2.2. Graph of sulphur transformations in acidic environments. Sulphur oxidation states in different compounds and minerals are shown in red numbers. Reactions catalysed by acidophilic sulphur-oxidising prokaryotes are indicated in red; other reactions are catalysed for example by sulphur-reducing prokaryotes. (Modified from Dopson & Johnson 2012.)

Thiosulphate and polysulphide pathways generate a wide range of inorganic sulphur compounds. Elemental sulphur, main intermediate of the polysulphide mechanism, requires bioleaching conditions to be oxidised by microorganisms (reviewed by Schippers 2007). S^0, as well as other reduced forms of sulphur such as H_2S, can be converted to sulphate and protons by sulphur-oxidising acidophiles (Chen & Lin 2004b; for a review, see Rawlings 2005). The reaction can be presented as follows (Fu et al. 2008):

$$S^0 + 1.5O_2 + H_2O \rightarrow SO_4^{2-} + 2H^+ \qquad (10)$$

Removal of built-up S^0 through oxidation also increases leaching efficiency by allowing microbial and Fe^{3+} access to the mineral surface (Dopson & Lindström 1999). Sulphuric

acid (H_2SO_4) produced by sulphur-oxidising microorganisms may also bring forth the polysulphide mechanism in the absence of Fe^{3+} ions (reviewed by Rawlings 2007). H_2SO_4 is the most common inorganic acid found in bioleaching environments (reviewed by Shagufta 2007). It maintains the bioleaching environment acidic and takes part in metal sulphide and oxide mineral leaching by providing protons to the process (Bhatti et al. 2012b). Oxidation of sulphide moiety in minerals is the main acid-generating phase, although protons are also produced through jarosite ([K, Na, NH_4] $Fe_3(SO_4)_2(OH)_6$) formation and the hydrolysis of ferric iron generated in the process (Fu et al. 2008). Pyrite, being acid-insoluble, is an exception and can only be dissolved by ferric ion attack. The leaching of tenorite (CuO) and uranium-trioxide (UO_3), on the other hand, happens via proton attack and can be presented as follows (Schippers 2007):

$$CuO + 2H_2SO_4 \rightarrow CuSO_4 + H_2O \quad (11)$$

$$UO_3 + 3H_2SO_4 \rightarrow UO_2(SO_4)_{4-3} + H_2O + 4H^+ \quad (12)$$

As an example, the oxidation of reduced pyrite to an oxy-hydroxide mineral, schwertmannite, is presented as follows (Dopson & Johnson 2012):

$$8FeS_2 + 30O_2 + 18H_2O \rightarrow Fe_8O_8(OH)_6SO_4 + 30H^+ + 15SO_4^{2-} \quad (13)$$

Unless a sufficient amount of basic minerals is present, the acidic waste produced through sulphur oxidation is enriched with metal cations, thus generating acid mine drainage (AMD) (reviewed by Dopson & Johnson 2012).

In summary, the microbial generation of sulphuric acid is important in bioleaching operations for two reasons: proton attack, and to keep iron in an oxidised state (reviewed by Rawlings 2005). Conversion of sulphur to sulphate is also the most important heat-generating reaction in heap bioleaching (reviewed by du Plessis et al. 2007).

2.3 Iron- and sulphur-oxidising microorganisms

Bioleaching microorganisms oxidise the products of chemical metal sulphide oxidation, including Fe^{2+} ions and different sulphur compounds. The microbes also provide Fe^{3+} ions and protons for sulphide attack, and keep the leaching environment acidic by producing sulphuric acid. This low pH environment keeps iron in solution (for reviews, see Rawlings et al. 2003; Schippers 2007). As the temperature inside a bioheap changes with mineral oxidation and depletion, different groups of indigenous microorganisms activate and deactivate in succession (reviewed by Brierley 2008). The most common bioleaching microorganisms are presented in Table 2.1.

2.3.1 Diversity and different species

Bacteria and archaea oxidising Fe^{2+} ions and intermediate sulphur compounds are aerobic, generally strict or moderate acidophiles, and usually either obligatory or facultative chemolithotrophs (Bowei et al. 2009; for reviews, see Holmes & Bonnefoy 2007; Schippers 2007). Chemolithotrophs use ferrous iron, reduced inorganic sulphur compounds, or both as an electron donor, and oxygen as the terminal electron acceptor (reviewed by Rawlings et al. 2003). Microorganisms capable of both iron and sulphur oxidation have shown to be able to recover their Fe^{2+} oxidation ability when exposed to the substrate, even after months of having S^0 as the sole energy source (Córdoba et al. 2008c).

While some iron- and sulphur-oxidising microorganisms are obligate aerobes (*Acidithiobacillus* [*At.*] *thiooxidans*, *At. caldus*), others are able to use Fe^{3+} as an electron acceptor instead of oxygen and thus, grow in anaerobic environments. These microorganisms are called facultative anaerobes and include bacteria such as *At. ferrooxidans* (reviewed by Dopson & Johnson 2012). Mineral-oxidising microorganisms are mostly capable of CO_2 fixation (autotrophs such as *Acidithiobacillus* and *Leptospirillum* spp.), although obligate heterotrophs require organic carbon for growth. Some bioleaching prokaryotes are able to use both organic and inorganic carbon as a carbon source (reviewed by Johnson & Hallberg 2007). This group of facultative auto- or heterotrophs contains microorganisms such as *Sulfobacillus* spp. and *Acidiphilium acidophilum* (for a review, see Schippers 2007).

Most metal leaching microbes are mesophilic or moderately thermophilic bacteria, with optimal growth temperatures of 15 - 40 and 40 - 60 °C, respectively. Bioleaching archaea are mainly extreme thermophiles (optimal temperature for growth being 60 - 80 °C; for reviews, see Plumb et al. 2007; Schippers 2007). Psychrophilic (T_{opt} < 15 °C) bioleaching acidophiles have not been identified (reviewed by Plumb et al. 2007), although psychrotolerant mixed and pure cultures have successfully been used in bioleaching (Dopson et al. 2007). Hyperthermophilic (T_{opt} > 80 °C) metal leaching microbes are rare (for reviews, see Plumb et al. 2007; Schippers 2007). It should however be noted that some acidophiles can grow at temperatures higher or lower than their optimal temperature for growth, and the ranges for each temperature-based grouping are varying (for reviews, see Johnson & Hallberg 2007; Norris 2007). Especially with heap bioleaching, time of the year during sampling is important as dominating bacteria in summer greatly differ from winter-dominating communities (Bowei et al. 2009). Substrate availability and increasing concentrations of inhibitory ions, such as sulphate, also affect the community composition (Demergasso et al. 2005).

So called secondary bioleaching microorganisms interact with iron- and sulphur-oxidising microorganisms and thus, have an impact on the overall bioleaching process.

Some of these mostly heterotrophic prokaryotes or eukaryotes may also directly impact the mineral oxidation. Many *Acidiphilium* species, for instance, are capable of reduced sulphur compound oxidation but unlike autotrophic prokaryotes, require organic carbon (reviewed by Johnson & Hallberg 2007). Organic compounds produced in oxidation processes may be harmful to the autotrophic microorganisms. Heterotrophic *Ferrimicrobium acidiphilum*, *Ferroplasma acidiphilum*, and *Sulfobacillus* spp. utilise and consequently eliminate the toxicity of organic matter produced by autotrophs such as *Acidithiobacillus* and *Leptospirillum* spp. These species are also capable of anaerobic ferric iron reduction. Thus, the heterotrophs provide electron donors (Fe^{2+}) for the autotrophic iron-oxidising microorganisms (Bowei et al. 2009).

Table 2.1. Main characteristics of some iron- and sulphur-oxidising microorganisms. **Note**: Autotrophs use photo- or chemosynthesis to produce organic molecules from inorganic sources. Heterotrophs require organic energy source e.g yeast extract. Mixotrophs assimilate organic compounds as carbon sources and inorganic compounds as electron donors. Diazotrophs are able to grow without external fixed nitrogen sources. (F = facultative; O = obligate; A = autotroph; M = mixotroph; H = heterotroph; D = diazotroph; a = aerobe; aa = anaerobe)

Species	**Phylum**	**T (°C) growth range (optimum)**	**pH growth range (optimum)**	**Metabolism**	**Respiration**	**Reference**
Iron- and sulphur-oxidising microorganisms						
Acidianus brierleyi	Crenarchaeota	45-75 (70)	1.0-6.0 (1.5-2.5)	FA	Faa	Bosecker 1997; Johnson 1998; Karavaiko et al. 2006; Watling 2006
Acidianus infernus	Crenarchaeota	65-96 (~90)	1.0-5.5 (~2)	OA	Faa	Schippers 2007
Acidiferrobacter thiooxydans	Proteobacteria	<5-47 (38)	1.2-na (~2)	FD	Faa	Hallberg et al. 2011
Acidithiobacillus ferrivorans	Proteobacteria	4-37 (28-33)	1.9-3.4 (2.5)	OA	Faa	Hallberg et al. 2010
Acidithiobacillus ferrooxidans	Proteobacteria	10-37 (30-35)	1.3-4.5 (2.5)	OA	Faa	Breed & Hansford 1999; Hallberg et al. 2010; Johnson 1998
Alicyclobacillus disulfidooxidans	Firmicutes	4-40 (35)	0.5-6.0 (1.5-2.5)	FA	Faa	Schippers 2007; Watling 2006
Alicyclobacillus tolerans	Firmicutes	<20-55 (37-42)	1.5-5.0 (2.5-2.7)	M	Faa	Schippers 2007; Watling 2006
Metallosphaera prunae	Crenarchaeota	55-80 (~75)	1.0-4.5 (2.0-3.0)	FA	a	Dopson & Johnson 2012; Karavaiko et al. 2006; Schippers 2007
Metallosphaera sedula	Crenarchaeota	50-80 (75)	1.0-4.5 (2.0-3.0)	FA	Oa	Dopson & Johnson 2012; Johnson 1998; Karavaiko et al. 2006; Schippers 2007

Species	Phylum	T (°C) growth range (optimum)	pH growth range (optimum)	Metabolism	Respiration	Reference
Sulfobacillus acidophilus	Firmicutes	<30-55 (45-50)	(~2.0)	FA	Faa	Dopson & Johnson 2012; Karavaiko et al. 2006; Schippers 2007
Sulfobacillus sibiricus	Firmicutes	17-60 (55)	1.1-2.6 (2.0)	FA	a	Dopson & Johnson 2012; Karavaiko et al. 2006
Sulfobacillus thermosulfidooxidans	Firmicutes	20-60 (50-55)	1.1-2.4 (1.7-1.8)	FA	Faa	Dopson & Johnson 2012; Karavaiko et al. 2006; Schippers 2007
Sulfobacillus thermotolerans	Firmicutes	20-60 (40-42)	1.2-2.4 (2.0)	M	a	Karavaiko et al. 2006; Schippers 2007
Sulfolobus metallicus	Crenarchaeota	50-75 (70)	1.0-4.5 (2.0-3.0)	OA	a	Karavaiko et al. 2006; Schippers 2007
Sulfolobus tokodaii	Crenarchaeota	70-85 (80)	2.0-5.0 (2.5-3.0)	FA	a	Bosecker 1997; Schippers 2007
Sulfurococcus yellowstonensis	Crenarchaeota	40-80 (60-65)	1.0-5.5 (2.0-2.4)	FA	a	Karavaiko et al. 2006; Schippers 2007
Thiobacillus prosperus	Proteobacteria	23-41 (33-37)	1.0-4.5 (2.0)	OA	a	Schippers 2007; Watling 2006
Iron-oxidising microorganisms						
Acidimicrobium ferrooxidans	Actinobacteria	25-55 (~45-50)	1.5-5.0 (2.0)	FA/M	Faa	Karavaiko et al. 2006; Norris 2007; Schippers 2007
Ferrimicrobium acidiphilum	Actinobacteria	na-37 (35)	1.4-na (2)	OH	a	Johnson et al. 2009
Ferroplasma acidarmanus	Euryarchaeota	32-51 (42)	0.2-2.5 (1.2)	H/M	a/aa	Dopson et al. 2004; Karavaiko et al. 2006; Schippers 2007
Ferroplasma acidiphilum	Euryarchaeota	15-47 (35-42)	1.3-2.2 (1.7)	A/H/M	a/aa	Karavaiko et al. 2006; Schippers 2007; Dopson et al. 2004
Leptospirillum ferriphilum	Nitrospira	na-45 (30-37)	(1.3-1.8)	OA	a	Karavaiko et al. 2006; Schippers 2007

Species	Phylum	T (°C) growth range (optimum)	pH growth range (optimum)	Metabolism	Respiration	Reference
Leptospirillum ferrooxidans	Nitrospira	2-37 (28-30)	1.1-2.5 (2.0)	OA	a	Bosecker 1997; Karavaiko et al. 2006
Leptospirillum thermoferrooxidans	Nitrospira	30-55 (45-50)	1.3-na (1.65-1.90)	OA	a	Karavaiko et al. 2006; Schippers 2007
Sulphur-oxidising microorganisms						
Acidianus ambivalens	Crenarchaeota	na-87 (80)	1.0-3.5 (2.5)	OA	Fa/Faa	Fuchs et al. 1996; Schippers 2007
Acidicaldus organivorans	Proteobacteria	na-65 (50-55)	1.8-3.0 (2.5-3.0)	H	Faa	Schippers 2007
Acidiphilium acidophilum	Proteobacteria	<25-37 (27-30)	1.5-6.5 (2.5-3)	FA/M	a	Dopson & Johnson 2012; Hiraishi et al. 1998; Schippers 2007
Acidiphilium cryptum	Proteobacteria	20-41 (35-41)	1.9-5.9 (3.0)	H	a	Borole et al. 2008; Schippers 2007
Acidithiobacillus albertensis	Proteobacteria	(25-30)	2.0-4.5 (3.5-4.0)	A	a	Karavaiko et al. 2006; Kelly & Wood 2000
Acidithiobacillus caldus	Proteobacteria	32-52 (45)	1.0-3.5 (2.0-2.5)	A/M	Oa	Dopson & Johnson 2012; Hallberg et al. 2010; Schippers 2007
Acidithiobacillus thiooxidans	Proteobacteria	10-37 (28-30)	0.5-5.5 (2.0-3.0)	OA	Oa/Faa	Dopson & Johnson 2012; Hallberg et al. 2010; Watling 2006
Hydrogenobaculum acidophilum	Aquificae	na-~70 (65)	2.0-na (3.0-4.0)	A	a	Schippers 2007
Sulfolobus shibatae	Crenarchaeota	50-86 (81)	1.0-4.0 (3.0)	M	a	Johnson 1998; Karavaiko et al. 2006
Thiomonas cuprina	Proteobacteria	20-45 (30-36)	1.5-7.2 (3.0-4.0)	FA/M	a	Johnson 1998; Schippers 2007; Watling 2006

At. ferrooxidans, previously known as *Thiobacillus ferrooxidans*, is a chemolithoautotrophic and mesophilic bioleaching microorganism and the most widely studied acidophilic bacterium. It uses inorganic substrates as energy source, is able to fix carbon, and leaches metals from both oxide and sulphide ores (reviewed by Harvey & Bath 2007). *At. ferrooxidans* derives its energy for growth from oxidative respiration (Ohmura et al. 2002) and is able to oxidise both ferrous iron to ferric iron, and sulphur compounds to sulphuric acid (Chen et al. 2011). The bacterium is also capable of anaerobic iron respiration with Fe^{3+}, S^0, or reduced sulphur compounds as electron acceptors and H_2 as an electron donor (Bowei et al. 2009; Ohmura et al. 2002). *At. ferrooxidans* is widely used in bioleaching processes as it has been found to oxidise chalcopyrite at a higher rate than many other metal leaching microorganisms, such as *Leptospirillum (L.) ferrooxidans* or *At. thiooxidans* (Akcil et al. 2007).

As *At. ferrooxidans* is easy to cultivate, it was previously believed that sulphidic mineral environments were mainly occupied by this bacterium. However, due to the use of molecular phylogenetic techniques (16S rRNA gene amplification, fluorescent *in situ* hybridisation), the importance of other bacterial species has been discovered (reviewed by Dopson et al. 2003). Several other bacteria and archaea have been identified especially at higher process temperatures of 45 - 80 °C (reviewed by Holmes & Bonnefoy 2007). Other microorganisms known to be involved in bioleaching operations are the mesophilic bacteria *At. thiooxidans* and *L. ferrooxidans*, as well as thermophilic archaea such as *Sulpholobus*, *Ferroplasma*, and *Acidianus* species (Bowei et al. 2009; for a review, see Harvey & Bath 2007). However, their oxidation rates as pure cultures are usually quite low. Mixed microbial cultures in general have shown higher oxidations activities than pure cultures (Akcil et al. 2007).

Sulphur-oxidising mesophiles and moderate thermophiles are exclusively bacteria, while the extreme thermophiles are solely crenarchaeotes with the exception of *Hydrogenobaculum acidophilum*, an autotrophic sulphur-oxidising bacterium. Although none of the acidophilic microorganisms oxidising merely sulphur compounds are psychrophiles, acidophiles capable of oxidising both iron and sulphur such as *At. ferrivorans*, *At. ferrooxidans*, and *Alicyclobacillus disulfidooxidans* grow at temperatures of 10 °C or lower (reviewed by Dopson & Johnson 2012). *At. thiooxidans* is a Gram-negative, strictly acidophilic proteobacterium and an obligately autotrophic sulphur-oxidiser (reviewed by Holmes & Bonnefoy 2007). Although *At. ferrooxidans* is capable of oxidising elemental sulphur to sulphuric acid, *At. thiooxidans* converts it much faster. Simultaneously, it creates acidity and favourable conditions for iron-oxidising bacteria such as *At. ferrooxidans* and *L. ferrooxidans* (reviewed by Bosecker 1997). Another sulphur-oxidiser of the *Acidithiobacillus* genus is *At. caldus*, which has a higher thermotolerance than *At. thiooxidans* and has been shown to have a more important role in sulphur oxidation than previously thought (Bouchez et al. 2006; for a review, see Norris 2007).

2.3.2 Resistance towards high metal concentrations

Although many metals have important biochemical roles as catalysts, enzyme co-factors, and stabilisers, they can become toxic via constitutively expressed transport systems that result in metal accumulation inside the cells (reviewed by Dopson et al. 2003). By growing in bioleaching environments, acidophilic microorganisms have developed several mechanisms to resist high metal ion concentrations. This is due to the fact that many metal ions are released in environments that contain ferric iron and a low pH (for reviews, see Dopson et al. 2003; Rawlings 2007). It has been found that bacteria resistant to one metal may also decrease the toxic effect of other metals by exploiting their existing resistance mechanisms (Ahemad & Malik 2011). Metals such as silver, aluminium, cadmium, gold, lead, and mercury have no function in cells at any concentrations (for a review, see Bruins et al. 2000) and thus, resistance towards these metals is important in bioleaching microorganisms. The heavy metal resistance in different bacterial species has been widely explored, an example being the resistance mechanisms in *Ralstonia metallidurans* reviewed by Nies (2003).

Heavy metal ions need to enter the cell before they can have any physiological or toxic effect (reviewed by Nies 1999). Microorganisms use different chromosomal, transposon, and plasmid-mediated systems to resist high metal concentrations by decreasing sensitivity to them. Metal uptake mechanisms, metabolic role of the metal, and gene location all have an effect on microbial metal resistance (reviewed by Bruins et al. 2000). There are five different mechanisms that increase the metal resistance of acidophilic microorganisms: efflux, or active transport, of the toxic metal out of the cell or microorganism; enzymatic conversion of the metal to a less toxic form; intra- or extracellular binding of the metal; exclusion of the metal by a permeability barrier; and reduction of sensitivity in a cellular component (Figure 2.3; reviewed by Dopson et al. 2003). Intra- and extracellular sequestration are sometimes separated into two mechanisms (reviewed by Bruins et al. 2000). Different metals are excluded through different mechanisms. As an example, mercury is resisted through enzymatic conversion, arsenic through efflux, and copper via cellular binding (for a review, see Dopson et al. 2003).

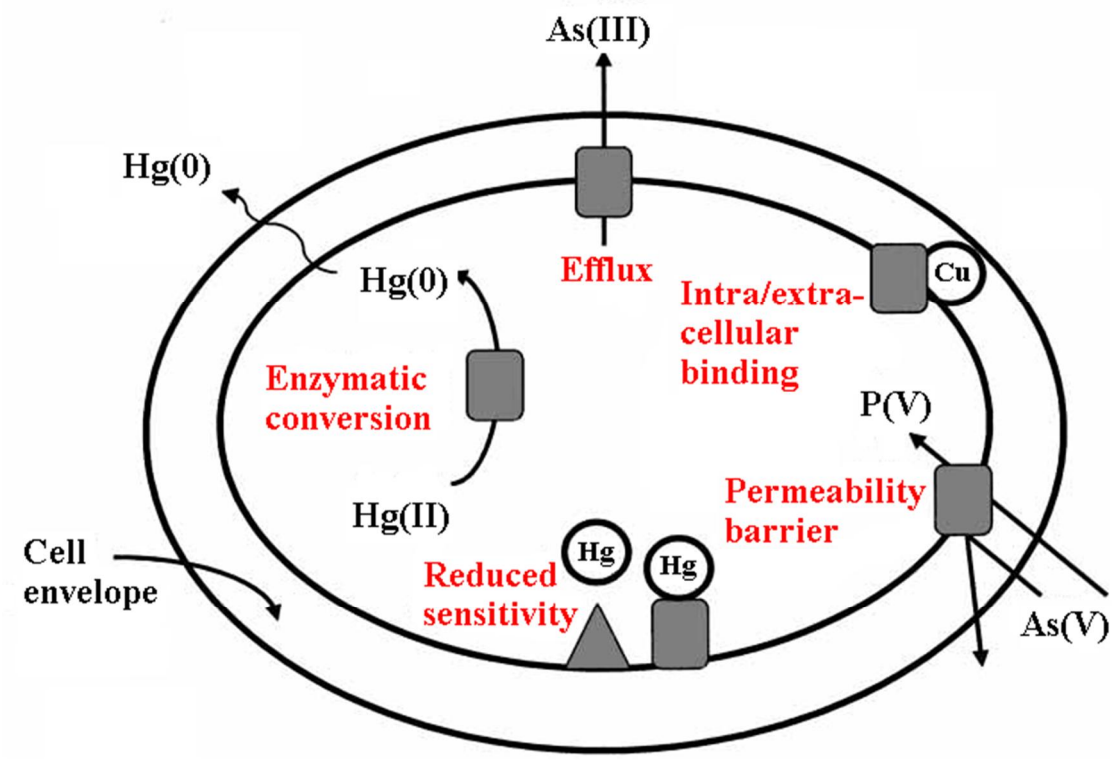

Figure 2.3. Metal resistance mechanisms in acidophilic microorganisms. Clockwise from the top: efflux of the toxic metal out of the cell; reduction of the toxic effect via intra- or extracellular binding; exclusion of metal via a permeability barrier; reduction of sensitivity to the metal through cellular component alteration; enzymatic conversion of the metal to a less toxic form. Examples of toxic metals resisted with each mechanism are shown in the figure. (Modified from Dopson et al. 2003.)

Microorganisms use metal exclusion by permeability barrier when protecting sensitive cellular components (reviewed by Bruins et al. 2000). Alterations in membrane channel proteins (Rouch et al. 1995), as well as nonspecific membrane or envelope binding (Hoyle & Beveridge 1983), are two examples of metal exclusion. Efflux systems, on the other hand, transport essential and nonessential metals away from the microbial cytoplasm through plasmid-encoded or chromosomal mechanisms. Arsenate, cadmium, and copper are examples of metals that are actively transported out of the cells. Intracellular and extracellular accumulation of metals occurs in the cytoplasm or outside the cell, respectively, and prevents metal exposure by binding it into a complex (reviewed by Bruins et al. 2000). Mercury resistance in microorganisms is a good example of enzymatic detoxification. Some Gram-positive and -negative bacteria are able to form an operon that detoxifies Hg^{2+} ions (Misra 1992; Ji & Silver 1995). There are seven known genes behind the mercury resistance operon, with different functions such as coding for transport proteins, mercuric ion reductase enzyme, and organomercurial lyase enzyme (Misra 1992). Sensitivity reducing mutations in, or an increased production of, essential

cellular components are ways of some microorganisms to adapt to high toxic metal concentrations (Rouch et al. 1995; for a review, see Bruins et al. 2000).

2.3.3 Cell growth and its effect on bioleaching

Effective mineral degradation is dependent on the activity of iron- and sulphur-oxidising microorganisms (Akcil et al. 2007). The activity and cell growth of iron- and sulphur-oxidising chemolithotrophs is mainly controlled by the availability of three factors: iron and reduced sulphur (substrates), oxygen (electron acceptor), and carbon dioxide (carbon source). In addition, a wide range of other parameters such as macro- and micronutrients affect the microbial growth (reviewed by du Plessis et al. 2007). Since redox transformations are important in mineral leaching environments, the activity of microorganisms can be detected by measuring oxidation and reduction rates of iron and sulphur elements. Oxygen consumption is also associated with oxidation reactions (for a review, see Johnson & Hallberg 2007). Many physicochemical parameters also affect microbial cell growth. For instance an optimal solution pH results in increased microbial growth rates, even though it appears not to increase the biomass concentration (Breed & Hansford 1999). Physicochemical parameters affecting iron and sulphur oxidation will be discussed in more detail in Chapter 3.

3 PHYSICOCHEMICAL PARAMETERS AFFECTING THE ACTIVITY OF OXIDATION PROCESSES

Bacterial mineral oxidation and metal mobilisation are affected by several factors and parameters. In addition to microbiological, mineral, and processing parameters, physicochemical factors play an important role in the rate and efficiency of oxidation processes (for a review, see Brandl 2001).

3.1 Solution pH and redox potential

A suitable pH value is necessary for the growth and activity of bioleaching microorganisms, as well as for the solubilisation of metals (reviewed by Bosecker 1997). Although some acidophilic archaea are able to grow at pH 0, most mineral-oxidising bacteria require a pH value higher than 1. Extreme and moderate acidophiles grow optimally at pH < 3 and pH 3 - 5, respectively (reviewed by Johnson & Hallberg 2007). Chemical and biological oxidation of metal sulphides requires an acidic environment in order to keep a sufficient amount of metal ions in solution. The most important one is ferric iron, as its solubility is required for the ion to be readily available in bioleaching reactions. A low pH environment is thus required for a successful iron cycle (Bhatti et al. 2012a). As iron precipitation may also prevent the leaching agent from getting in contact with the mineral, a suitable pH controls the overall process of metal solubilisation (Ahonen & Tuovinen 1995). Fe^{3+} solubility is beneficial especially in heaps where oxygen deficiency might occur, as some bacteria are capable of anaerobic respiration with Fe^{3+} as an electron acceptor and hydrogen as an electron donor (Bowei et al. 2009; Ohmura et al. 2002). Solution pH also largely affects whether chemical or biological oxidation is the dominating mechanism, and consequently the leaching efficiency since bacterial leaching has been found to be clearly faster than chemical leaching (Ahonen & Tuovinen 1995). In a study by Meruane & Vargas (2003), chemical Fe^{2+} oxidation dominated at pH over 5.0 whereas below pH 5.0, bacterial oxidation was relevant. Microbial growth and oxidation of minerals however mostly take place at a pH value of 1.5 - 2.0 (Dorado et al. 2012). Highest microbial cell counts have also been obtained in that pH range (Ahonen & Tuovinen 1995). Each bioleaching process should be optimised based on the sulphide ore, as sensitivity to acidity has been shown to be highly metal-dependent (Cameron et al. 2009).

Microbial bioleaching is based on redox reactions, where ferrous ions (Fe^{2+}) and reduced sulphur compounds are electron donors and molecular oxygen serves as the final electron acceptor in the overall bioleaching process (for reviews, see Belzile et al. 2004; Schippers 2007). The Fe^{2+}/Fe^{3+} couple has a relatively high redox potential in low-pH liquors (+ 0.77 V), meaning that in order to maintain the growth of Fe^{2+}-oxidising microorganisms, large amounts of ferrous iron need to be oxidised and consequently, many electrons have to be transferred to oxygen (reviewed by Holmes & Bonnefoy 2007). Due to this very positive standard electrode potential, only oxygen may be used as an electron acceptor. Additionally, since Fe^{2+}/Fe^{3+} and O_2/H_2O redox couples have similar redox potentials, relatively little cell mass is produced by ferrous iron oxidation (reviewed by Rawlings 2007). Redox potential is affected by oxidation reactions and increases due to Fe^{2+} oxidation (Bhatti et al. 2012a; Xia et al. 2008), sulphur oxidation, or when the process is aerated (Chen & Lin 2004b). Increased ORP is a result of decreased free electron concentration in the system (Couillard et al. 1994). The effect of redox potential on bioleaching kinetics is ore- and metal-dependent (Ahonen & Tuovinen 1995; Gericke & Govender 2011). For instance, copper leaching from chalcopyrite is most effective at low redox potential levels of 400 - 430 mV (Ahmadi et al. 2011; Gericke & Govender 2011), whereas efficient Cu dissolution from pentlandite requires a high 600 mV redox potential (Gericke & Govender 2011). Intermediate redox potential of 500 mV has been shown to result in highest cell counts (Ahonen & Tuovinen 1995). Similar to process pH, redox potential is responsible for iron solubility (Ahonen & Tuovinen 1995) as high initial ORP (oxidation reduction potential) favours ferric ion precipitation and thus mineral passivation in chalcopyrite leaching (Córdoba et al. 2008b). Formation of passive layers may thus be prevented by decreasing ORP (Ahmadi et al. 2011).

3.2 Temperature

Biological oxidation of mineral sulphides produces free energy and generates heat. While chemical reaction rates increase along with temperature and can be described using the Arrhenius equation (Peleg et al. 2012), the relationship of temperature and microbial growth follows different kinetics (reviewed by Plumb et al. 2007). Ratkowsky et al. (1983) described the bacterial growth rate of any strain throughout a temperature range in Kelvin degrees:

$$\sqrt{r} = b(T - T_{\min})(1 - e^{c(T - T_{\max})}) \tag{14}$$

In the equation, T_{min} and T_{max} define the minimum and maximum temperatures, respectively, at which no cell growth occurs. Parameter r is a growth rate constant, b a regression coefficient, c an additional parameter which enables the use of temperatures higher than optimal, and T the absolute temperature.

It has been stated that a 10 °C increase in temperature doubles the rate of chemical reactions (reviewed by Rawlings et al. 2003). d'Hugues et al. (2008) found that with microorganisms, no significant effect on bioleaching activity was discovered as the process temperature was increased from 35 to 45 °C. Different minerals can however have quite varying optimal temperatures for fastest decomposition. Since passivating oxidation products such as elemental sulphur are reduced in higher temperatures, metal yields may consequently improve (reviewed by Rawlings et al. 2003). Microbial response of pure cultures to temperature changes is well studied in laboratory conditions; however, a better understanding of the effects of temperature in heap environments is required (reviewed by Plumb et al. 2007). It has been stated that temperature has no significant effect on the rate of ferrous iron oxidation (Abdel-Fattah & Abdel-Fattah 2002), although low temperatures have been shown to decrease leaching rates (Ahonen & Tuovinen 1995). This is debatable, as low temperatures have also been shown to decrease Fe^{3+} precipitation (Dopson et al. 2007), resulting in increased availability of the ion for mineral oxidation.

Following temperature changes in the process, different microbial populations take over (Bowei et al. 2009). Microorganisms have been grouped according to their optimal temperatures for growth: psychrophiles, mesophiles, moderate thermophiles, thermophiles, and hyperthermophiles grow optimally at temperatures of < 15, 15 - 40, 40 - 60, 60 - 80, and > 80 °C, respectively. Acidophilic microorganisms are mostly mesophiles, moderate thermophiles, and thermophiles, although some hyperthermophilic acidophiles have also been found (reviewed by Plumb et al. 2007).

3.3 Dissolved oxygen and carbon dioxide

Although some of the metal leaching microbes can tolerate anaerobic conditions, the cells require dissolved oxygen (O_2) for active growth (reviewed by Mohapatra 2006). O_2 acts as an electron acceptor in bioleaching reactions (reviewed by Ojumu et al. 2006). In the solution phase, O_2 demand is generated by different microbial and chemical oxidation reactions involved in mineral dissolution (for a review, see du Plessis et al. 2007). Sufficient O_2 supply in bioleaching processes is usually achieved by aeration, stirring, sparging, or shaking (for reviews, see Bosecker 1997; du Plessis et al. 2007). Temperature of the leaching environment affects the gas mass transfer (reviewed by du Plessis et al. 2007), with higher temperatures requiring more effective and energy-consuming aeration techniques due to the reduced solubility of O_2 (for a review, see Brierley 2008).

Iron- and sulphur-oxidizing microorganisms present in bioleaching processes are obligate autotrophs: they use carbon dioxide (CO_2) as a sole carbon source and do not require organic substrates (reviewed by Mohapatra 2006). There are several different CO_2 fixation mechanisms used by bioleaching microbes (Bryan et al. 2012). Microbial cul-

tures obtain CO_2 either from air (contains 0.033 % of CO_2 by volume) or from carbonate-containing mineral concentrates that release the gas in acidic environments (reviewed by du Plessis et al. 2007). CO_2 concentration of normal air has been found to be more than enough for the optimal cell growth of iron-oxidising microorganisms. However, mass transfer gradients within heap bioleaching operations create a challenge in aeration (Bryan et al. 2012). CO_2 insufficiency has been shown to limit biomass formation and therefore might have a more inhibiting impact than oxygen deficiency (d'Hugues et al. 2008).

3.4 Substrates and residues

Iron and sulphur serve as electron donors for the iron- and sulphur-oxidising microorganisms during metal leaching processes. Fe^{2+} ion acts as an electron donor when oxidised to Fe^{3+} during aerobic respiration (reviewed by Rawlings 2005). Fe^{2+} ions also slow down Fe^{3+} precipitation and nucleation, thus delaying the passivation of the sulphide mineral (Córdoba et al. 2008a). At high concentrations, Fe^{2+} ions may however inhibit biological iron oxidation (reviewed by Nemati et al. 1998). Fe^{3+} on the other hand is a chemical solvent of sulphide minerals (Ahonen & Tuovinen 1995). Its presence chemically oxidises elemental sulphur and metal sulphides (Chen et al. 2003b). Bacterial generation of Fe^{3+} ensures high redox potential and thus, fast leaching rates (Ahonen & Tuovinen 1995). High concentrations of Fe^{3+} ions may inhibit biological iron oxidation through Fe^{3+} precipitation as jarosite and by, consequently, covering the ore surface (Córdoba et al. 2008c; Harahuc et al. 2000; for reviews, see Brandl 2001; Nemati et al. 1998). Iron- and sulphur-oxidisers favour jarosite formation by producing ferric and sulphate ions, respectively (Córdoba et al. 2008c). It has been indicated that when additional Fe^{3+} is added to a copper recovery process, sulphur oxidation rate becomes the limiting bioleaching step (Dorado et al. 2012). Simultaneously it was found that microorganisms re-oxidised iron faster than Fe^{3+} ions oxidised sulphur, concluding that Fe^{3+} regeneration is not limiting the bioleaching process.

Reduced inorganic sulphur compounds (RISCs) serve as electron donors during biological sulphur oxidation, producing sulphuric acid responsible for low pH environment (Bouchez et al. 2006; for a review, see Rawlings 2005). Oxygen usually acts as the electron acceptor, being energetically the most favourable one (reviewed by Rawlings 2005). Sulphur availability depends on ore mineralogy and the properties of the mineral particles (deportment, physical characteristics). Availability of sulphur for microbial oxidation is process-wise more important than its total content in the ore (reviewed by du Plessis et al. 2007). Rather than its particle size, the number of microspores on S^0 particles defines the substrate's specific surface area. The form of sulphur (i.e. pastilles, powder) also affects the bioleaching performance (Chen et al. 2003a). Bacteria oxidise sulphur by attaching on the S^0 particles via adsorption, indicating that a high substrate concentration favours S^0 oxidation and consequently metal leaching (Chen & Lin 2001).

Similarly to jarosite, another secondary solid phase of bioleaching, high concentrations of S^0 may however passivate minerals (Bhatti et al. 2012a; reviewed by Rawlings et al. 2003). On the other hand, it has been found that the porosity of S^0 may prevent it from forming a passivating layer on the mineral surface (Córdoba et al. 2008b). As not all of S^0 is utilised in bioleaching processes, the remaining substrate may cause further acidification (Chen & Lin 2010).

3.5 Nutrients

Microorganisms responsible for metal sulphide leaching are chemolithoautotrophs. Thus, they require only inorganic compounds for growth and acquire mineral nutrients from the bioleaching environment (reviewed by Bosecker 1997). Sulphide ores usually contain adequate amounts of micronutrients; however, macronutrients such as ammonium (NH_4^+) and potassium need to be supplied (reviewed by du Plessis et al. 2007). After carbon, nitrogen is the second most important element for biomass formation and the demand for it depends on the quality of cell growth (reviewed by Rawlings 2007). Nitrogen source has an effect on Fe^{3+} precipitation which, as has been shown, may have a role in cell attachment to the mineral surface (d'Hugues et al. 2008). Assuring that enough nitrogen is present for iron- and sulphur-oxidisers is problematic for two reasons: firstly, oxygen inhibits nitrogen fixation (although in heaps aeration is usually heterogenous), and secondly, even addition of nitrogen-containing fertiliser to the vast heaps is difficult (reviewed by Rawlings 2007). NH_4^+ limitation has clearly been shown to inhibit bacterial growth and bioleaching efficiency, the extent of the negative effects being dependent on the microbial community composition. For example, *L. ferriphilum* is incapable of nitrogen fixation from air (d'Hugues et al. 2008). As traces of NH_4^+ may be absorbed into the bioleaching solution from air due to the ion's high solubility in acidic environments, the estimation of nitrogen requirement is difficult. Nitrogen, both organic and inorganic, may also inhibit iron oxidation in high concentrations (reviewed by Rawlings 2007). Especially nitrate (NO_3^-) has been shown to have a negative effect on biooxidation at high concentrations (Harahuc et al. 2000; Sarcheshmehpour et al. 2009; Suzuki et al. 1999).

Unlike with nitrogen and carbon, bioleaching microorganisms cannot acquire phosphate (PO_4^{3-}) or trace elements from air; therefore these nutrients need to be added as fertilisers (for a review, see Rawlings 2007). Low PO_4^{3-} concentrations (10 mM) are usually sufficient for maximal iron and sulphur oxidation, although iron-oxidisers are more inhibited by high PO_4^{3-} concentrations than sulphur-oxidising microorganisms (Harahuc et al. 2000). Trace metals are potentially toxic to bioleaching microorganisms at high concentrations, although at reasonable levels they enhance oxidation rates (Abdel-Fattah & Abdel-Fattah 2002). Other nutrients such as potassium and calcium have been shown to have a significant effect on ferrous iron oxidation (Abdel-Fattah & Abdel-Fattah 2002; Sarcheshmehpour et al. 2009). The main function of magnesium in microorgan-

isms is to activate enzymes and sustain the membrane stability. However, when Mg^{2+} concentration becomes excessive, osmotic pressure in the cells increases and consequently eliminates microbial growth (Zhen et al. 2009).

3.6 Anions and cations

Anions that inhibit the growth of bioleaching microorganisms, such as chloride (Cl^-), nitrate (NO_3^-), and fluoride (F^-), may be derived from the ore minerals, or be present in the process waters (reviewed by du Plessis et al. 2007). Salinity has an adverse effect on biological metal extraction and the extent of the negative effect is dependent on Cl^- concentration and microbial strain. However, the solubilisation of some metals, such as that of lead, may increase with increasing Cl^- levels due to the formation of complexes (Deveci 2002). At low concentrations (below 0.1 M), addition of salts has been shown to increase sulphur oxidation activity by neutralising the surface charge of colloid particles. Salt concentrations of 0.2 M, however, lengthen the oxidation lag phase and inhibit microorganisms due to increased osmotic pressure (Suzuki et al. 1999). Changes in osmotic pressure affect sulphur-oxidisers more than iron-oxidising microorganisms. This is demonstrated in a study by Harahuc et al. (2000), in which increased concentrations (over 0.2 M) of sulphate slightly inhibited iron oxidation while drastically decreasing sulphur oxidation activity, and in studies by Gómez et al. (1999) and Harahuc et al. (2000) which showed that Cl^- ions inhibit iron oxidation more than sulphur oxidation. The inhibiting effect of F^- ions is highly dependent on the bioleaching conditions. For instance jarosite is able to include small amounts of F^- by exchanging hydroxide ions with fluoride (Gunneriusson et al. 2009).

It has been discussed that interaction of some ions, such as that of molybdate or sodium (Na^+), with non-toxic ions of nutritional value may result in inhibited oxidation activity (Abdel-Fattah & Abdel-Fattah 2002). Na^+ and other monovalent cations may also promote the formation of harmful ferric precipitates (Deveci 2002; Deveci et al. 2004). During copper leaching, chloride (as NaCl) has been shown to inhibit the cell growth of *S. rivotincti* even at low concentrations (0.1 M NaCl). However, the simultaneous pH increase probably accelerated bacterial death (Gómez et al. 1999). Bioleaching in the presence of several metallic cations such as aluminium (Al^{3+}), at concentrations below their previously detected toxicity thresholds (Ballester et al. 1992; Mier et al. 1995; Mier et al. 1996), has also been studied in order to unveil the possible positive interchange between the cations and bioleaching substrates (Gómez et al. 1999).

3.7 Heavy metals

Metals that have a density of 6 g/cm^3 or above are called heavy metals (reviewed by Akpor & Muchie 2010). Due to their incomplete d-orbitals, heavy metal cations are capable of forming both redox-active and redox-inactive complexes and thus, have an

important role in biochemical reactions or as trace elements. However, if the intracellular heavy metal concentrations become too high, unspecific and toxic complexes are formed (reviewed by Nies 1999). Heavy metals are also very persistent in the environment and therefore can affect oxidation processes for lengthened periods (for a review, see Akpor & Muchie 2010). Heavy metals Sn, Ce, Ga, Zr, and Th have a very low solubility and thus, have little influence on microbial oxidation (reviewed by Nies 1999). Others are reviewed here.

Iron (Fe), molybdenum (Mo), and manganese (Mn) are important trace elements and have a low toxic effect towards microorganisms. Fe is the only heavy metal cation with a macro-bioelement status. Thus, it is biologically the most important of heavy metals and a crucial growth requirement (reviewed by Nies 1999). Fe takes part in redox processes, as do other essential transition metals (for a review, see Bruins et al. 2000). The bioavailability of Fe in aerobic conditions is usually very low due to the chemical oxidation of soluble Fe^{2+} to quite insoluble Fe^{3+}. However, the solubility of Fe^{3+} increases in acidic environments (reviewed by Dopson et al. 2003). Being similar to Mg^{2+} ion, Fe^{2+} is transported and accumulated in bacterial cells through the same transport system as magnesium. This uptake system is especially important to bacteria living in anaerobic environments (reviewed by Nies 1999). Acidophiles are usually more tolerant to Fe than microorganisms living in environments of higher pH, but the resistance mechanisms are unknown (for a review, see Dopson et al. 2003).

Mo is biologically the most important heavy metal oxyanion. It occurs primarily as molybdate, Mo(VI). Mo is capable of oxyanion catalysis and acts as an essential trace element (reviewed by Nies 1999). However, interaction of Mo(VI) with non-toxic ions of nutritional value may result in inhibited oxidation activity (Abdel-Fattah & Abdel-Fattah 2002). Mn is an essential heavy metal for oxygen production during photosynthesis. It has a very low toxic effect compared to other heavy metals. Although the cation Mn^{2+} is the most common state of manganese, the metal can exist in various oxidation states. Mn may be able to perform as a substitute for magnesium since it forms complexes that are fairly inert as redox compounds (reviewed by Nies 1999).

Zinc (Zn), nickel (Ni), copper (Cu), vanadium (V), cobalt (Co), tungsten (W), and chromium (Cr) are heavy metals that have toxic effects towards microorganisms, but also moderate to high importance as trace elements. Zn has a similar toxicity to Cu, Ni, and Co (reviewed by Nies 1999), although bacteria resistant to Zn have also been shown to resist cadmium (Ahemad & Malik 2011). Zn is transported through the cell membrane via several different protein families. One example of Zn detoxification system in neutrophilic bacteria is P-type efflux ATPase; however, the genetics of acidophile zinc resistance are widely unknown. Zinc cation is not capable of redox changes under biological conditions due to its completely filled d orbitals. Despite this, Zn is present in various enzymes as the divalent cation Zn^{2+}. The heavy metal has a very important

function as a component in enzymes and DNA-binding proteins, as well as in forming complex polypeptide chains. The ability of Zn^{2+} to form complexes with cellular components is also the reason for its toxicity (for a review, see Nies 1999).

The toxicity of Ni is quite similar to that of Co. As Ni^{3+} is very unstable, free nickel is mainly found as Ni^{2+} (for a review, see Nies 1999). Ni is used in a few important reactions in the cells, such as in forming complexes with polypeptide chains (reviewed by Dopson et al. 2003). The metal is also similar to transition metals Fe and Cu, which are all involved in cellular redox processes (reviewed by Bruins et al. 2000). Complex rearrangements may be catalysed by Ni in a process where the cation binds small molecules and then splits, or by fusing small molecules or atoms together. Enzymes such as hydrogenase and urease may have Ni bound in their active sites. Cells presumably uptake Ni by chemiosmotic gradient, and the heavy metal is detoxified by sequestration and/or transport (reviewed by Nies 1999).

Copper is required by many microorganisms. It is an essential transition metal that takes part in redox processes in cells (reviewed by Bruins et al. 2000). Cu has a radical character, making it easily reactive with other radicals such as molecular oxygen and thus, the metal can be very toxic. The Cu^{2+}/Cu^{+} pair has a standard electrochemical potential of - 268 mV, making Cu^{2+} the main ionic form of copper (reviewed by Nies 1999). Acidophilic microorganisms are usually more resistant to high Cu^{2+} concentrations than other microbes (Dew et al. 1999; Johnson et al. 1992). Cu acts in terminal oxygen acceptors such as cytochrome-*c* oxidase and thus, has a function in the respiratory chain of many organisms (for reviews, see Dopson et al. 2003; Nies 1999). Another important function is the formation of copper/zinc superoxide dismutases (reviewed by Nies 1999).

Vanadium is mostly toxic to microorganisms. It exists mainly as the trivalent oxyanion vanadate, V(V), a structure similar to phosphate (PO_4^{3-}). Microorganisms are very rarely able to use V for their advantage, and microbial V(V) resistance mechanisms are widely unknown. Co has medium toxicity towards microorganisms. As Co^{3+} is stable in complexes only, Co^{2+} is the main form of the heavy metal. Co takes part in several catalyses as the cofactor B_{12} (for a review, see Nies 1999). Gram-negative bacteria resist high Co concentrations by transenvelope efflux through a RND (resistance, nodulation, cell division) transporter (Saier et al. 1994). Eukaryotes and gram-positive bacteria detoxify Co^{2+} by CDF (cation diffusion facilitator) transporters (Paulsen & Saier 1997). W is the heaviest beneficial element. As WS is more available in anaerobic environments than MoS, anaerobic microorganisms such as bacteria and archaea have tungsten-containing enzymes. The heavy metal may also act as a component of a tungsten cofactor, much like with Mo (reviewed by Nies 1999). Chromium is not usually beneficial for microorganisms. It occurs mainly as two biologically important ions, the divalent oxyanion chromate (Cr(VI), and the trivalent cation, Cr(III). Cr(III) has less toxic effects than

Cr(VI). Structurally Cr(VI) resembles PO_4^{3-}, SO_4^{2-}, and arsenate (As(V)). It has been suspected that the chromate resistance mechanisms in microorganisms are based on the interaction of Cr(VI) reduction and efflux (reviewed by Nies 1999).

Heavy metals that are toxic with limited beneficial function for microorganisms include arsenic (As), silver (Ag), antimony (Sb), cadmium (Cd), mercury (Hg), lead (Pb), and uranium (U) (reviewed by Nies 1999). Arsenic is often found in acidic environments due to its presence in minerals such as arsenical pyrite (reviewed by Dopson et al. 2003). It exists in bioleaching solutions mainly as arsenate, AsO_4^{3-} (As(V)), and arsenite, AsO_2^{-} (As(III)) (for review, see Dopson et al. 2003; Nies 1999). Due to the very similar structures of As(V) and PO_4^{3-}, arsenate may interfere with the metabolism of phosphorus by replacing it in cellular processes and thus, become toxic to the cell (reviewed by Nies 1999). Although acidophilic microbes have proved to be more tolerant towards As than other microorganisms (Suzuki et al. 1997; Harvey & Crundwell 1996), studies have shown that especially As(III) inhibits the growth of bioleaching microbes (reviewed by Dopson et al. 2003). Its toxic effect may occur via formation of extracellular sulphur, as has been noticed with *At. caldus* (Hallberg et al. 1996).

The main ionic form of silver is the monovalent cation Ag^+ due to the standard electrochemical potential of Ag^{2+}/Ag^+ pair (1.56 V at pH 7) (reviewed by Nies 1999). Oxidation of refractory sulphide ores releases the fine heavy metal particles from the mineral matrix (for a review, see Dopson et al. 2003). Ag resistance mechanism in gram-negative bacteria may be based on RND-driven transenvelope efflux, while gram-positive bacteria resist the heavy metal using efflux by P-type ATPases (Gupta et al. 1999). Resistance may also be based on additional complexation by intracellular compounds (reviewed by Nies 1999). It has been shown that neutrophilic microorganisms resist Ag^+ through efflux, while the mechanisms in acidophiles are more unknown (for a review, see Dopson et al. 2003).

The toxic effects of cadmium towards microorganisms have been studied and its toxicity has been demonstrated, but few specific mechanisms have been found (reviewed by Nies 1999). It is however known that Cd is more toxic to *At. ferrooxidans* than zinc (Ragan & Mast 1990) and copper (Mahapatra et al. 2002). The inhibiting effect of Cd^{2+} may be due to interaction with calcium and zinc metabolism, membrane damage, protein denaturation, binding to thiol groups, or loss of a protective function (reviewed by Dopson et al. 2003). Microorganisms use Cd efflux to resist the heavy metal, and RNA- and P-type transport systems may play an important role in cyanobacteria resistance. Mercury has a very high affinity of Hg^{2+} to thiol groups (stronger than the affinity of Cd^{2+} to sulphide). Therefore, it has the highest toxicity of all heavy metals and no beneficial effects for microorganisms (reviewed by Nies 1999). The cation Hg^{2+} is produced along with RISCs by oxidation of cinnabar, HgS (for a review, see Dopson et al. 2003). Gram-negative bacteria transport Hg^{2+} via specific uptake systems and consequently

prevent its toxicity. Lead is not very soluble and thus, has a low toxic effect on microorganisms due to its decreased availability. Metal ion efflux has been considered as the main mechanism of Pb resistance. The toxicity of uranium for bacteria is low as U(VI) (as it occurs in UO_2^{2-}) (reviewed by Nies 1999). Because the heavy metal is capable of forming various ionic forms, it may be used in anaerobic respiration as a substrate (Lovley et al. 1991). Otherwise, U has not proven biologically beneficial (reviewed by Nies 1999).

4 CONTROL OF INHIBITING FACTORS

Acid mine drainage (AMD) may cause environmental problems due to its high soluble metal concentrations, high sulphate content, and low pH. Remediation of AMD contaminated waters concentrates on all of these factors (Aelion et al. 2009). The term biohydrometallurgy contains several technologies where microbes and metals interact with each other. In addition to bioleaching - the dissolution of metals from their mineral sources - biohydrometallurgical technologies include bioremediation, biosorption, bioaccumulation, and other ways of reducing contaminants in mine waste waters (reviewed by Mishra et al. 2005). Of physical contaminant removal, settling and sedimentation are two examples. Chemical removal processes include sorption and adsorption, precipitation, metal oxidation and hydrolysis, and carbonate or sulphide formation (reviewed by Sheoran & Sheoran 2006).

4.1 Filtration

Filtration is often used prior to, combined with, or after a more selective remediation method such as adsorption, precipitation, coagulation, and oxidation. With filtration, any particulate matter that might plug, deactivate, or otherwise disturb the purification process is removed from the contaminated water (reviewed by Malik et al. 2009). The remediation efficiency of filtration techniques is highly dependent on the size of contaminant-bearing particles (reviewed by Hashim et al. 2011). For example Leupin & Hug (2005) studied arsenic removal combining iron filing (oxidant) and quartz sand (filtrate) to repeatedly pass contaminated water through. Membrane and filtration technologies concentrate the contaminants in waste water, thus reducing the volume of water to be treated. Electrodialytic, liquid, polymer, ultrafiltration, and nanofibre membranes are a few membrane types used in remediation (reviewed by Hashim et al. 2011). Microfiltration and ultrafiltration are membrane processes that have been used for water purification from contaminants such as arsenic. Cu(II), Cr(VI), and As(V) have successfully been removed from waste water using metal-complexation followed by ultrafiltration (Clifford 1986). Nano-filtration membranes have been effective in removing dissolved contaminants (for a review, see Hashim et al. 2011). Membrane filtration techniques are not used as often as other remediation methods (precipitation, adsorption, ion exchange) due to their sensitivity, high volumes of formed residuals, and costs (reviewed by Malik et al. 2009).

4.2 Ion exchange

Ion exchange is an adsorption method that can be used to remove specific cations or anions from waste water. In this remediation technique, an ion in waste water is exchanged to another ion that has a similar charge (for reviews, see Akpor & Muchie 2010; Zhuang 2009). An ion exchange resin can be regenerated in situ (reviewed by Malik et al. 2009). Compared to precipitation, electrolytic remediation, evaporation, and adsorption, ion exchange is very efficient and inexpensive. Ion exchange also produces cleaner and a lower volume of sludge than chemical precipitation, and the process recovers metal value (Rengaraj et al. 2001). Ion exchange, as well as adsorption, is often used when only one contaminant (such as arsenic) requires treatment. Dissolved solids, sulphates, and nitrates may disturb the adsorption efficiency. The ion exchange resin may also become saturated with the contaminant (reviewed by Malik et al. 2009). The efficiency of an ion exchange resin is pH-independent. As an example, removal of nickel is optimal at pH 4, whereas zinc is most efficiently removed at a higher pH of 6. The adsorption rates of different metals are also varying (Alyüz & Veli 2009).

4.3 Biological treatment

The use of microorganisms in the treatment of metal-contaminated waters is cost-effective, and new technologies such as biosorption, biological oxidation, and biological reduction have been applied (reviewed by Zhuang 2009). Advantages of microbial remediation include environmental safety, absence of waste, and self-sustainability. However, the suitability of microbial remediation requires a biodegradable contaminant, and the processes are slow and highly specific (reviewed by Akpor & Muchie 2010).

Biosorption, as well as bioaccumulation, is used in environments where heavy metals accumulate (reviewed by Mishra et al. 2005). In biosorption, the various functional groups of living or nonliving biomass (carboxyl-, hydroxyl-, sulphate-, phosphate-, and amino-groups) bind dissolved metals through reversible physicochemical mechanisms. The application of biopolymers as an adsorbent has several advantages compared to conventional organic and inorganic adsorbents, such as lower costs, non-hazardous materials and wastes, and specificity (for a review, see Zhuang 2009). Chemically modified plants, such as *Ficus carica*, have been shown to adsorb metals from aqueous systems, but the presence of some ions (i.e. Ca^{2+}, Mg^{2+}) may reduce the removal efficiency (Gupta et al. 2012). In addition, zinc resistant bacteria have been shown to accumulate zinc and copper and the mechanism could be applied to heavy metal removal from contaminated soils (Ahemad & Malik 2011).

Biological oxidation in waste water treatment is based on microbial formation of insoluble metal hydroxides and carbonates, such as MnO_2 or $Fe(OH)_3$ (reviewed by Zhuang 2009). Iron and sulphate have for instance been precipitated from bioleaching waste

streams by combining an iron-oxidising fluidised-bed reactor (FBR) with a gravity settler (Nurmi et al. 2010). Biological reduction, on the other hand, applies sulphate to sulphide reduction (reviewed by Zhuang 2009). Sulphate-reducing biofilm and suspension processes for the treatment of sulphate-, zinc-, and iron-containing waste waters have been studied (Kaksonen et al. 2003), concluding that SRB (sulphate-reducing bacteria) treatment is a promising alternative for chemical remediation methods, precipitating the metals as sulphides and neutralising the acidity of the waste water. Wakeman et al. (2010) studied the use of SRB in the treatment of metals-containing waste water further, demonstrating that silage can be used as an inexpensive carbon and electron source for sulphate reduction. As a conclusion: biological oxidation and reduction form same kind of products as chemical hydroxide and sulphide precipitation (see Chapter 4.4), but without the use of high temperatures or commercial, possibly expensive or harmful chemicals.

Phytoremediation, the use of plants in removal of selected contaminants from soil, sludge, sediment, or water, may also be categorised as biological treatment. Metals can be removed from waste water using phytoextraction (absorption, translocation, and storage of contaminants in the plant roots), rhizofiltration (breakdown of contaminants using the microbes in plant rhizosphere), or phytostabilisation (limiting metal mobility and availability by absorption, accumulation, adsorption, or precipitation by plant roots) (reviewed by Akpor & Muchie 2010). Zinc, arsenic, mercury, silver, iron, manganese, molybdenum, magnesium, copper, nickel, chromium, cadmium, and lead removal are a few examples of successful phytoremediation (reviewed by Brar et al. 2006).

4.4 Chemical precipitation

Dissolved heavy metals in waste waters have primarily been removed by chemical precipitation. Precipitation methods such as hydroxide-, sulphide-, carbonate-, and dithiocarbamate-precipitation as well as sodium borohydride (SBH) treatment have been used (for a review, see Zhuang 2009). In chemical precipitation, dissolved contaminants are transformed into insoluble solids using coagulants and flocculants, therefore making them easier to remove. Water pH and alkalinity define the chemical concentration needed, and pH also affects the solubility of precipitates and the process kinetics (reviewed by Akpor & Muchie 2010). After the process, the effluent has lower concentrations of metals and inorganics due to further coagulation, flocculation, settlement, clarification, or filtering (reviewed by Zhuang 2009). Chemical precipitation may be combined with other remediation techniques; for instance, precipitation followed by ion exchange has been proven effective (Feng et al. 2000).

Hydroxide precipitation is a simple and inexpensive method for metal removal. Caustic soda, hydrated lime, and magnesium hydroxide are the most common precipitating agents, lime neutralisation being the most often used method (low costs, removes acidi-

ty). The pH is an important factor in metal hydroxide precipitation, dissolution, and sludge stability, depending on the precipitating agent used. Hydroxide precipitation is usually involved in other precipitation methods as well, due to the ability of heavy metals to form hydroxide precipitates (for a review, see Zhuang 2009). Fe^{3+}, Cr^{3+}, and Al^{3+} may be precipitated as hydroxides (reviewed by Neculita et al. 2007). However, hydroxide precipitation requires time for effective settling and filtration, and the leaching of hydroxide concentrates consumes chemicals and energy (Pakarinen & Paatero 2011).

Carbonate precipitation using soda ash (Na_2CO_3) or sodium bicarbonate ($NaHCO_3$) may be performed instead of hydroxide precipitation; however the process is slower than when using lime or caustic soda. The resulting sludge is also difficult to handle (reviewed by Zhuang 2009). Fe^{2+} and Mn^{2+} metal cations are two examples precipitated as carbonates (for a review, see Neculita et al. 2007).

Sulphide precipitation is an efficient and selective precipitation method (Akpor & Muchie 2010). It is usually performed near neutral pH by adding soluble salts such as Na_2S, NaHS, or FeS into the waste water (for a review, see Zhuang 2009). Metal cations that can be removed using sulphide precipitation are Pb^{2+}, Co^{2+}, Cd^{2+}, Cu^{2+}, Ni^{2+}, Fe^{2+}, and Zn^{2+} (reviewed by Neculita et al. 2007). Because they are not stable in oxidising environments, the metal sulphides require further stabilisation (reviewed by Zhuang 2009). Precipitation using sodium sulphide salts may also release toxic compounds in acidic environments (reviewed by Akpor & Muchie 2010). As with hydroxide precipitation, sulphide precipitation requires a long residence time and the concentrate leaching is energy consuming (Pakarinen & Paatero 2011).

SBH reduction is also a selective precipitation treatment resulting in a low sludge volume and therefore easier waste disposal (reviewed by Zhuang 2009). Precipitation using sodium dithiocarbamate is based on metal complex formation as insoluble ionic colloids, resulting in high metal removal efficiency. It is a good alternative for sulphide precipitation (for reviews, see Akpor & Muchie 2010; Zhuang 2009). For the precipitation of manganese and iron from sulphate solutions, soda ash is an effective and inexpensive alternative (Pakarinen & Paatero 2011). Pakarinen & Paatero also found that by combining air oxidation and iron precipitation with limestone, simultaneous Mn recovery and Fe separation is cost effective.

The sludge produced in chemical precipitation usually requires further stabilisation for instance by using phosphate (due to the low solubility of most metal phosphates), different flocculants (for instance ferric sulphate), or adsorbents. Stabilisation reduces the possibility of metal leaching back into solution (reviewed by Zhuang 2009).

4.5 Reverse osmosis

Reverse osmosis has generally been applied in desalination, but the process is also useful in removing pollutants, minerals, and microorganisms from water. The opposite of osmosis, reverse osmosis is based on forced water flow through a semi-permeable membrane from high-solute region to a region of lower solute concentration. The process requires high pressure to overcome the natural osmotic pressure in the solution (reviewed by Rao 2007). Reverse osmosis is an effective method to remove ionic contents from solution. In the process, contaminated water is passed through a membrane, leaving dissolved and particulate minerals behind. However, production and operation of membranes is sensitive and expensive due to the required high pressure (reviewed by Akpor & Muchie 2010).

4.6 Electrodialysis

Contaminants present in low hydraulic permeability soils and sediments may be difficult to remove using primary remediation techniques. Electrodialysis, the application of low-level direct current through electrodes into the contaminated soil, may however become useful. Anode and cathode reactions produce H^+ and OH^- ions, respectively, making pH control an important process parameter. Electromigration (according to the electrical charge of ion contaminants) and electro-osmotic flow are responsible for pollutant movement in the soil. The distribution and association of metals with soil components (through ion exchange, adsorption, precipitation, or complexation) affect the success of electrokinetic remediation (for a review, see Peng et al. 2009). Once the metal anions and cations have accumulated at the anode and cathode, respectively, they are extracted using electroplating, precipitation, pumped water, or complexing with ion exchange (Krishna et al. 2001). In-situ chromium (VI) removal from soil using electrodialysis has been studied, combining electromigration with ion exchange membranes to selectively control the migration of certain ions (Nieto Castillo et al. 2008).

5 MATERIALS AND METHODS

5.1 Process liquors from the mine site

Talvivaara Mining Company Plc provided and sampled six (1 - 6) different process liquors (PL) for this thesis. The process liquors were collected from several parts of the heap leaching process and thus their component concentrations were varying. The samples were stored at 4 °C and used in the experiments unsterilised. PL4 was studied most thoroughly and its chemical composition at three different sampling dates was as presented in Table 5.1 (not shown). The analyses were performed by Talvivaara Mining Company Plc unless otherwise stated.

Process liquors 1, 2, 3, 5, and 6 were sampled only once. All six process liquors and their chemical compositions on the 3rd sampling date are presented in Table 6.1 (not shown).

5.2 Growth media

5.2.1 MSM and TES

Basic culture medium contained 10 % (v/v) of mineral salts medium (MSM) and 1 % (v/v) of trace elements solution (TES). However, in some shake flask experiments the percentages of these solutions varied (see Chapter 5.6). MSM consisted of 37.5 g/L $(NH_4)_2SO_4$, 18.75 g/L $Na_2SO_4 \cdot 10\ H_2O$, 1.25 g/L KCl, 0.625 g/L K_2HPO_4, 6.25 g/L $MgSO_4 \cdot 7\ H_2O$, and 0.175 g/L $Ca(NO_3)_2 \cdot 4\ H_2O$. Manufacturers of the chemicals used in this thesis are presented in Appendix 1.

TES was prepared by adding 1.375 g/L $FeCl_3 \cdot 6\ H_2O$, 0.0625 g/L $CuSO_4 \cdot 5\ H_2O$, 0.25 g/L H_3BO_3, 0.319 g/L $MnSO_4 \cdot 4\ H_2O$, 0.1 g/L $Na_2MoO_4 \cdot 2\ H_2O$, 0.075 g/L $CoCl_2 \cdot 6\ H_2O$, 0.1125 g/L $ZnSO_4 \cdot 7\ H_2O$, and 0.1125 g/L Na_2SeO_4. MSM was adjusted to pH 1.8 and TES to pH 1.5 by addition of concentrated H_2SO_4 or NaOH.

The final volume of each enrichment culture and shake flask experiment was filled up to 100 mL with deionised Milli-Q water. MSM, TES, and Milli-Q were sterilised in an autoclave at 121 °C for 20 minutes.

5.2.2 Substrates

Ferrous iron concentration used in iron oxidation experiments was 5 g/L or more. If iron-oxidising cultures did not contain Fe^{2+}-rich process liquors, they were supplemented with ferrous iron. The iron stock solution containing 22.5 g/L of ferrous iron was prepared by weighing 112 g of $FeSO_4 \cdot 7\ H_2O$ per 1000 mL of Milli-Q. The solution pH was adjusted to 1.8 with concentrated H_2SO_4 in order to prevent Fe^{2+} oxidation to Fe^{3+} ions. Finally, the iron stock solution was sterile-filtered through 0.2 μm polyethersulfone membrane filters (Whatman, UK), protected from light, and stored at 4 °C.

Sulphur-oxidising cultures were supplemented with 10 g/L of elemental sulphur. Sulphur was sterilised by heating at 105 °C over night and stored at room temperature. Both iron stock solution and elemental sulphur were added into the growth media after autoclaving.

5.3 Enrichment cultures

5.3.1 Iron-oxidising enrichment cultures

Iron-oxidising enrichment culture 1

This iron-oxidising culture was previously enriched from PL4 (1st sampling) given by Talvivaara Mining Company Plc. The culture was supplemented with ferrous iron in order to enrich indigenous iron-oxidising microorganisms from the mine site sample. Iron-oxidising enrichment culture 1 was used as an inoculant in the preliminary shake flask experiments.

Iron-oxidising enrichment culture 2

Iron-oxidising enrichment culture 2 was subcultured from an experiment consisting of 10 % (v/v) MSM, 1 % (v/v) TES, and 15 % (v/v) PL4 (from 2nd sampling) inoculated with 5 % (v/v) of iron-oxidising enrichment culture 1 (see Chapter 5.6.5). The aim was to enrich the culture with fresh microorganisms from the mine site. Enrichment culture 2 was used as an inoculant in most of the experiments.

Iron-oxidising enrichment culture 3

The third iron-oxidising culture consisted entirely of the indigenous microorganisms of PL4 on the 2nd sampling date. It was subcultured from a shake flask experiment containing 40 % (v/v) of PL4, 59 % (v/v) of MSM, and 1 % (v/v) of TES (see Chapter 5.6.1). Iron-oxidising enrichment culture 3 was used in some of the shake flask experiments combined with enrichment culture 2.

Enrichment cultures were incubated at 27 °C and shaken at 150 rpm. All of the iron-oxidising enrichment cultures were maintained by subculturing once a week into a fresh growth media and consisted of 10 % (v/v) MSM, 1 % (v/v) TES, 25 % (v/v) iron stock solution (5.6 g/L ferrous iron), and 5 % (v/v) inoculant. If iron oxidation rates in the enrichment cultures decreased, 15 % (v/v) of PL4 was added to the media in order to refresh it with new microorganisms.

5.3.2 Sulphur-oxidising enrichment culture

Indigenous sulphur-oxidising microorganisms were enriched from PL4 (2^{nd} sampling) by supplementing it with MSM, TES, and elemental sulphur (S^0), according to Table 5.2. In order to prevent cell replication in the control flask, a solution containing 2 % (w/v) of thymol (5-methyl-2-isopropylphenol) in methanol was prepared. 5 % (v/v) of this stock solution was added to the control flask after autoclaving.

Table 5.1. Enrichment of sulphur-oxidising microorganisms from PL4. The indigenous heap leaching microbes were supplemented with nutrients (MSM and TES) and elemental sulphur.

MSM (% v/v)	**TES (% v/v)**	**PL4 (% v/v)**	**S^0 (g/L)**	**Starting pH**	**Replicates**	**Controls**
10	1	10	10	1.7	3	With thymol

Sulphur oxidation was observed by following the increase in sulphate concentration by liquid ion chromatography (IC). Since sulphate formation decreases pH, NaOH was added to maintain the culture pH around 1.7. After 42 days, before sulphate concentration stopped increasing and elemental sulphur became limiting, the enrichment cultures were subcultured. Subculturing was done every two weeks into a fresh growth media containing 10 % (v/v) of MSM, 1 % (v/v) of TES, 10 g/L of elemental sulphur, and 5 % (v/v) of inoculant. Like iron-oxidising enrichment cultures, sulphur-oxidisers were shaken at 150 rpm and incubated at 27 °C.

5.4 Analytical solutions

For the determination of ferrous iron concentration, ammonium acetate buffer and phenantroline solution were prepared. Ammonium acetate buffer was made by diluting 250 g of $NH_4C_2H_3O_2$ in 180 mL of Milli-Q water, after which 700 mL of glacial acetic acid (CH_3CO_2H) was added to the solution. For the preparation of phenantroline solution, 5 g of 1,10-phenantroline monohydrate ($C_{12}H_8N_2 \cdot H2O$) was dissolved in about 400 mL of Milli-Q with the help of a few drops of 37 % HCl. The solution was then diluted to 500 mL. Both solutions were prepared and stored (4 °C) in acid washed containers.

The eluent used in ion chromatography (sulphate analysis) consisted of 0.5 M $NaHCO_3$ and 0.5 M Na_2CO_3.

TRIS-buffer for culture filtration (performed before DNA extraction) was prepared by diluting 6.1 g of tris(hydroxymethyl)aminomethane ($C_4H_{11}NO_3$) in 500 mL of Milli-Q water and adjusting the pH to 8.0 with 37 % HCl.

5.5 Analytical methods

5.5.1 Measurement of pH and redox

The pH values were measured with either pH 330 or pH 340 meter (WTW, Germany) and pH-electrode SenTix 41 (WTW, Germany). The pH electrode was calibrated with standard solutions (pH 2 and pH 4) just before measurement. Redox potential was determined with pH 340 meter (WTW, Germany) equipped with redox-electrode BlueLine 31 Rx (Schott Instruments, Germany). Redox potential and pH were measured from non-filtered samples.

5.5.2 Ferrous iron concentration

Iron oxidation was monitored using 3500-Fe ortho-phenantroline method (American Public Health Association, APHA, 1992). Before analysis, the samples were filtered through 0.45 µm polyethersulfone membrane filters (Pall Corporation, USA). 1 mL of sample was prepared by addition of 2 mL phenantroline solution, 1 mL ammonium acetate buffer, 0.1 mL 37 % HCl, and 0.9 mL Milli-Q water. 0.07 M HNO_3 was used to dilute the samples (when necessary) and to prepare the zero sample (1 mL of 0.07 M HNO_3 added instead of the sample). Absorbance at 510 nm was measured from the gently mixed sample within 5 minutes using a Shimadzu UV-1601 spectrophotometer and a quartz-cuvette.

5.5.3 Sulphate analysis

A Dionex DX-120 ion chromatograph (Dionex Corporation, USA) was used to determine sulphate concentrations in the sulphur-oxidising enrichment culture. The IC was equipped with IonPac AG23 (4 x 50 mm) guard column, IonPac AS23 (4 x 250 mm) anion exchange column, and Dionex AS40 autosampler. Before analysis, the samples were filtered through 0.45 µm polyethersulfone membrane filters (Pall Corporation, USA) and diluted with Milli-Q water. Samples were prepared according to the standard SFS-EN ISO 10304-1 (2009). Prior to sulphate analysis, IC eluent was degassed using underpressure whereas during analysis, helium overpressure prevented gas dissolution to the eluent.

5.5.4 Total cell count

To measure the cell number of different process liquors and in selected shake flask experiments, 1 mL of sample was filtered through a 0.2 µm GTBP membrane filter (Millipore, USA). When necessary, samples were diluted using sterile Milli-Q. After filtration, the sample was stained with 1 mL of DAPI solution (1 mg/L 4',6-diamidino-2-phenylindole) and stored in dark until observation by epifluorescence microscopy. Plan-Neofluar objective (100x/1.30 Oil, Carl Zeiss, Germany) was used to count the cells and images were taken using AxioVision 4.8 program (Carl Zeiss, Germany). Cell density was determined using the equation

$$cells/mL = \frac{K_A \cdot C}{V_S}, \tag{15}$$

where K_A is the average cell amount of 20 random counting points, C is a conversion factor and V_S is the sample volume (mL). Coefficient C is dependent on the counting area according to Table 5.3. The area selected for counting depended on cell density.

Table 5.2. Conversion factor *C* in DAPI-stained epifluorescence microscopy based on the selected counting area.

Area	Conversion factor (*C*)
1 x 1	$1.46045 \cdot 10^6$
2 x 2	$3.65113 \cdot 10^5$
3 x 3	$1.62272 \cdot 10^5$
5 x 5	$5.84180 \cdot 10^4$
10 x 10	$1.46045 \cdot 10^4$

5.5.5 Bacterial community analysis

A well-mixed aliquot (20 - 30 mL) of each enrichment culture and heap leaching liquor was filtered through a 0.2 µm polycarbonate filter (Whatman, UK). The cells on the filter were then washed with Milli-Q water (pH adjusted to 2.0 with concentrated H_2SO_4), followed by TRIS-buffer of pH 8.0. The filters were stored on a Petri dish at -20 °C until total DNA extraction with PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Inc., USA).

Partial bacterial 16S rRNA genes from the extracted DNA were amplified by polymerase chain reaction (PCR) using primer pair Ba357F-GC (5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CAG-3') and Un907R (5'-CCG TCA ATT CMT TTG AGT TT-3'). PCR was performed as described by Kolehmainen et al. (2007), using 0.8 U of Dreamtaq™ DNA polymerase (Thermo Fisher Scientific, USA) and a T3000 Thermocycler (Biometra, Germany).

DGGE was performed with denaturing gradient from 30 to 70 % and the gel was prepared and run (100 V for 21.5 h) as described by Koskinen et al. (2006). Sequence data produced by Macrogen Inc., Korea were analysed with Trev (version 1.9-r2852M Staden Package, copyright © Medical Research Council, Laboratory of Molecular Biology, 1995 - 2001). Bacteria were identified using MegaBLAST (Basic Local Alignment Search Tool), optimised to search for highly similar sequences in the National Center for Biotechnology Information (NCBI) database.

5.6 Shake flask experiments

All shake flask experiments were carried out in sterile conditions and incubated at 27 °C, shaken at 150 rpm (Figure 5.1). Culture pH was adjusted before autoclaving by addition of concentrated H_2SO_4 or NaOH. The experiments were mainly performed in duplicate. Each shake flask experiment is described in Table 5.4 using the following variables: nutrient content (MSM, TES, and other selected nutrients), addition of process liquors, starting pH, number of replicates, control flasks, used inoculum, and possible addition of other compounds. The experiments are described in more detail in chapters 5.6.1 - 5.6.5.

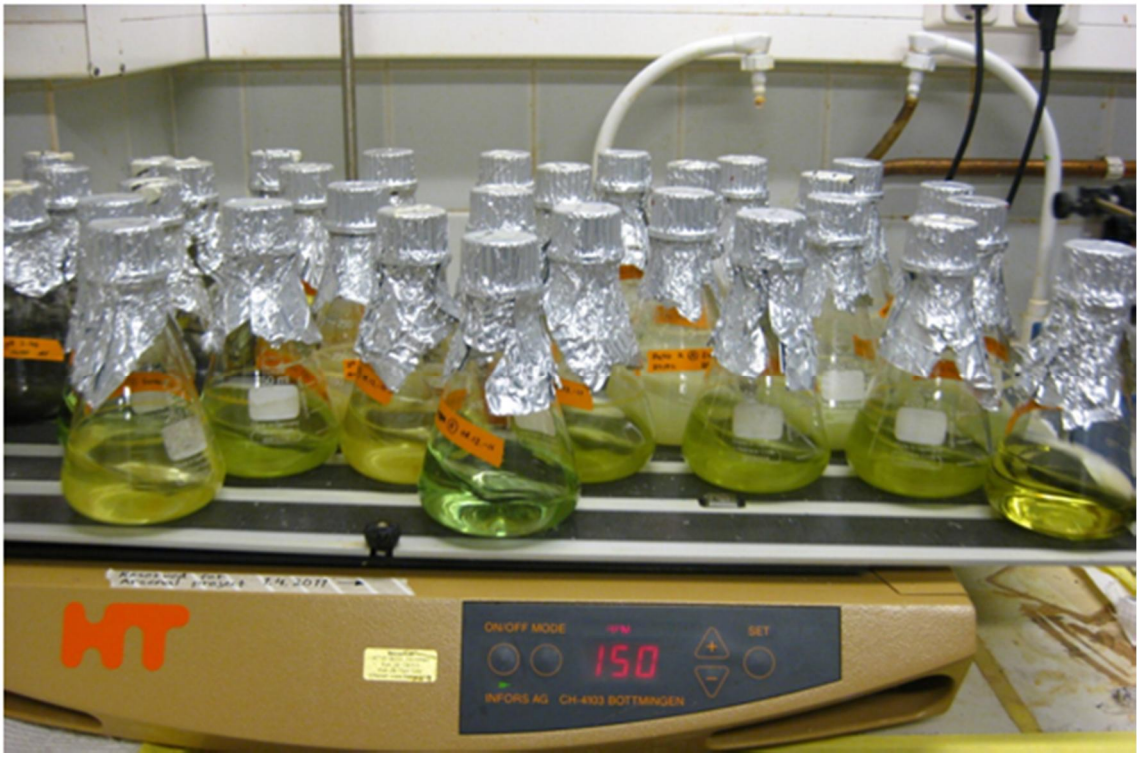

Figure 5.1. Shake flask experiment at 27 °C, shaken at 150 rpm. The green colour of the liquid is due to high ferrous iron concentration. Following iron oxidation (from Fe^{2+} to Fe^{3+} ions), the liquids changed to a reddish colour.

Table 5.3. Experimental parameters for shake flask experiments.

Experiment		**Variable factor(s) (g/L)**	**Inoculum (5 % v/v)**	**MSM (% v/v)**	**TES (% v/v)**	**PL4 (% v/v)**	**Starting pH**	**Replicates**	**Control**
5.6.1	Comparison of different process liquors	-	-	59, 39, 19, 14, 9, 4, and 0	1 (0 in 100 % v/v PL4)	40, 60, 80, 85, 90, 95, and 100	1.7	2	Inoculated with iron-oxidising enrichment culture 2, no PL4
		PL 1 - 6 (95 % v/v each)	Iron-oxidising enrichment culture 2 / Sulphur-oxidising enrichment culture (containing 10 g/L S^0)	-	-	-	-	2	-
5.6.2	Ferrous iron resistance	Ferrous iron (5, 8, 12, and 16)	Iron-oxidising enrichment culture 2	10	1	10	1.7	2	-
5.6.3	Sulphate inhibition	Chloride (3, 5, 10, 20, and 30)	Iron-oxidising enrichment culture 2 + 3	10	1	50	1.7	2	1. No PL4 2. No added chloride
5.6.4	Toxicity of aluminium	Aluminium (3, 6, 9, and 12)	Iron-oxidising enrichment culture 2 + 3	10	1	10	1.7	2	No added aluminium

Experiment			Variable factor(s) (mg/L)	Inoculum	MSM (% v/v)	TES (% v/v)	PL4 (% v/v)	Starting pH	Replicates	Control
5.6.5	Nutrient availability	**A**	MSM and TES	Iron-oxidising enrichment culture 1	10	1	15	1.7	3	1. With thymol 2. Without MSM or TES
		B	MSM and TES *(Washed cells)*	Iron-oxidising enrichment culture 2	-	-	15	1.7	2	-
		C	MSM and TES	-	49	1	50	1.7	2	Without MSM or TES
		D	MSM	-	49	-	50	1.7	2	Experiment **C**
			P (54)	-	-	-	50	1.7	2	Experiments **C** and **D**
			N (3900)	-	-	-	50	1.7	2	
			Na (1300)	-	-	-	50	1.7	2	
			Na and K (1300 and 460, respec-tively)	-	-	-	50	1.7	2	
		E	TES	-	-	1	50	1.7	2	Experiment **C**
			B (0.44)	-	-	-	50	1.7	2	Experiments **C** and **E**
			Mo (0.40)	-	-	-	50	1.7	2	
			Se (0.47)	-	-	-	50	1.7	2	
			B, Mo, and Se (0.44, 0.40, and 0.47, respectively)	-	-	-	50	1.7	2	

5.6.1 Comparison of different process liquors

The process liquors originated from different parts of the bioleaching process, therefore their component concentrations and physicochemical properties varied significantly. Since the plausible inhibitory effects of PL4 were of most interest, iron oxidation in a non-inoculated dilution series of this liquor in MSM was studied. The experiments were carried out according to Table 5.4, containing 40 - 100 % (v/v) of PL4, 1 % (v/v) of TES, and MSM so that the final culture volume was 100 mL. To define the best possible iron oxidation rate, an inoculated control without PL4 addition was made.

The possible inhibitory effects of different process liquors (1 - 6) towards any microorganisms they might contain were studied by incubating 95 % (v/v) of each process liquor with 5 % (v/v) of either iron-oxidising enrichment culture 2 (to study iron oxidation), or the sulphur-oxidising enrichment culture (Table 5.4). Sulphur-oxidising cultures were also supplemented with 10 g/L of elemental sulphur. The activity of sulphur-oxidisers was observed by measuring pH, which decreases following sulphate formation. Total cell counts were carried out from both raw process liquor samples at the start and end of the experiment. The oxidation end point for sulphur-oxidisers was after 2 - 3 days of unchanged pH.

5.6.2 Ferrous iron resistance

To study the resistance of iron-oxidising microorganisms towards high ferrous iron concentrations, PL4 was inoculated with iron-oxidisers, and incubated with nutrients and different Fe^{2+} concentrations (Table 5.4). Ferrous iron concentration of 5 g/L was normally used in iron-oxidising control flasks, so no additional control flask was made. Fe^{2+} concentrations in Talvivaara leach liquors have usually been under 16 g/L.

5.6.3 Sulphate inhibition

Due to high sulphate concentrations in bioleaching liquors (especially in liquors 3 and 4), the possible inhibitive effect of SO_4^{2-} ions towards iron-oxidising microorganisms was considered. Process liquors 3 and 4 contained over 120 g/L of sulphate, whereas liquors 1, 2, 5, and 6 contained less than 80 g/L.

One method to study the effects of different SO_4^{2-} concentrations was to precipitate sulphate ions in PL4 with barium chloride ($BaCl_2 \cdot 2\ H_2O$). However, in order to reach a concentration of 70 g/L SO_4^{2-} (a concentration unlikely to be toxic), chloride ion content in PL4 would increase to 30 g/L due to barium chloride addition. Therefore to study how chloride resistant the enriched iron-oxidisers and the indigenous microorganisms of PL4 were, they were incubated with nutrients and Cl^- concentrations up to 30 g/L (Table 5.4). Chloride was added to the culture before sterilisation as a sodium chloride stock

solution containing 144 g NaCl per liter of Milli-Q (87 g/L Cl^-). Two controls were carried out; one without PL4 (30 g/L of chloride) and one without added chloride.

Other deliberated ways to study sulphate toxicity were addition of different volumes of SO_4^{2-} stock solution (with $(NH_4)_2SO_4$ and $Na_2SO_4 \cdot 10\ H_2O$), or addition of sulphate as H_2SO_4 (after which culture pH was adjusted to 1.7 with NaOH or KOH). The problem with these techniques was the highly increased concentration of cations.

5.6.4 Toxicity of aluminium

Aluminium concentrations of different process liquors ranged between 71 and 9000 mg/L (around 6100 mg/L in PL4). Aluminium toxicity was studied by inoculating and incubating PL4 with different Al^{3+} concentrations (according to Table 5.4). No aluminium was added to the control flask. Aluminium stock solution was prepared by dissolving 216 g of $Al_2(SO_4)_3 \cdot \sim 14\ H_2O$ into 1000 mL of Milli-Q, resulting in an Al^{3+} concentration of approximately 20 g/L. The solution was adjusted to pH 1.7 with H_2SO_4 and sterile-filtered using polyethersulfone membrane filters (0.2 µm, Whatman, UK). Aluminium stock solution was added to the culture after autoclave sterilisation.

5.6.5 Nutrient availability

Nutrient availability in PL4 was studied by incubating it with and without nutrients (MSM and TES). In experiment **A** (Table 5.4), 15 % (v/v) of PL4 was inoculated with iron-oxidising enrichment culture 1 and incubated with nutrients. Two controls were used: one with 5 % (v/v) of thymol solution (2 % w/v in methanol; to prevent cell replication), and another without MSM or TES. A third control flask was later prepared using washed iron-oxidising enrichment culture without nutrients (experiment **B**). The cells were washed thrice before inoculation with acidic Milli-Q (pH adjusted to 2.0 with concentrated H_2SO_4) to prevent any nutrient transfer from the enrichment culture. Washing was carried out by centrifuging the cells at 5000 rcf for 10 minutes, after which the supernatant was removed and exactly the same amount of Milli-Q was added.

Since experiments **A** and **B** were both inoculated, experiment **C** was made to rule out possible nutrient remains from the enrichment cultures. Thus, 50 % (v/v) of PL4 was incubated with 49 % (v/v) of MSM and 1 % (v/v) of TES, in addition to a control flask containing only PL4 and Milli-Q (Table 5.4).

Nutrients available in MSM but possibly lacking from PL4 (concentrations either very low or not measured) were phosphorus (P), nitrogen (N), sodium (Na), and potassium (K). In experiment **D** (Table 5.4), 50 % (v/v) of PL4 was incubated with 49 % (v/v) of MSM. In the absence of TES, the nutritional effects of MSM and TES could be compared. In addition, one or more of the selected nutrients were incubated with 50 % (v/v)

of PL4 according to Table 5.4. The concentration of each nutrient used was similar to its concentration in 49 % (v/v) of MSM.

TES was studied in a similar way (experiment **E** in Table 5.4). Nutrients available in TES but potentially lacking from PL4 were boron (B), molybdenum (Mo), and selenium (Se). The selected nutrients were added to PL4 according to Table 5.4, at same concentrations as were present in 1 % (v/v) of TES. Nutrients were added as compounds normally present in MSM and TES (see Chapter 5.2.1).

Total inorganic and organic nitrogen was determined from heap leaching liquors 1 - 6 with modified Kjeldahl method according to standard SFS 5505 (Suomen standardisoimisliitto, SFS, 1988). The device used for sample distillation was a Kjeltec™ 2100 (FOSS Analytical, Denmark).

5.7 Sampling

To measure ferrous iron concentration, iron-oxidising shake flask experiments were usually sampled once a day during the lag phase of iron oxidation, and twice a day during the exponential growth phase. Sample volume (1 mL) was replaced with sterile Milli-Q water to maintain the culture volume at around 100 mL. Ferrous iron concentration was determined within 60 minutes.

Sulphur-oxidising enrichment cultures were sampled in two different ways. To determine sulphate concentration by ion chromatography, 1 mL of culture was sampled 3 - 5 times a week and stored at - 20 °C until filtration and analysis. For pH measurement, 2 mL of culture was sampled three times a week and the pH value was immediately determined. As with iron-oxidising cultures, sample volume was replaced with sterile Milli-Q.

Process liquors 1 - 6 were sampled shortly after delivery to determine pH and redox potential. 2 mL of each liquor was sampled and immediately analysed. Unlike with iron- and sulphur-oxidising cultures, the sample volume was not replaced with Milli-Q water.

In addition, process liquors and selected experimental flasks were sampled (1 mL) for analysis of total cell number. To determine bacterial community composition, enrichment cultures and process liquors were sampled once (20 - 30 mL). All iron- and sulphur-oxidising cultures and different process liquors were sampled in a laminar flow cabinet to minimise contamination.

6 RESULTS

6.1 Enrichment of sulphur-oxidisers

In experiments regarding iron oxidation, an enrichment culture was ready to use. Indigenous sulphur-oxidisers were enriched from PL4 by incubating the liquor with nutrients (MSM and TES) and 10 g/L of elemental sulphur. Sulphur oxidation was monitored by measuring sulphate concentration in the culture, and culture pH was maintained around 1.7 to ensure suitable conditions for cell growth. The increase in sulphate concentration was compared to a control flask, where cell replication was prevented by thymol addition (Figure 6.1).

As seen in Figure 6.1, sulphur oxidation was slow during the first 10 days of incubation, with a sulphate formation rate of 800 mg/L/d (from a linear part of the curve). Following this lag phase, SO_4^{2-} formation more than doubled, reaching a rate of 1900 mg/L/d. Sulphur oxidation then remained steady for three weeks, until after 31 day cultivation the microorganisms started to generate sulphate at a higher rate. After this point, SO_4^{2-} formation rate remained at 4700 mg/L/d until the end of the experiment. Figure 6.1 shows that the sulphur-oxidising microorganisms of PL4 required a few weeks to adjust to new culture conditions, after which their sulphur oxidation activity distinctly increased.

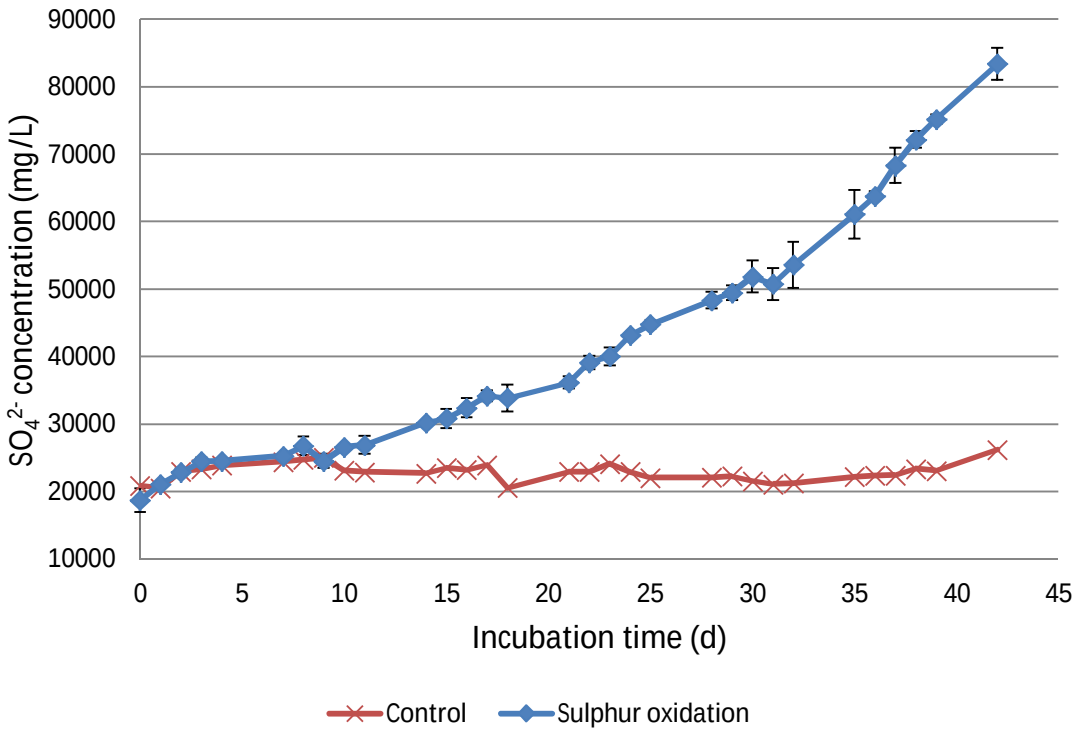

Figure 6.1. Enrichment of sulphur-oxidising microorganisms from PL4. Sulphur oxidation was monitored by measuring sulphate-formation in the culture (blue), and compared to a control where cell replication was inhibited with thymol (red). The cells were subcultured after 42 days of incubation. Error bars show the standard deviation (n = 3).

The aim was to enrich active sulphur-oxidising microorganisms to be used as an inoculum in the following experiments. The cultures were subcultured after 42 days; at this point, sulphur oxidation was still fast but the substrate (elemental sulphur) had not yet become limiting. At the highest oxidation rate (4700 mg/L/d SO_4^{2-}), utilisation of 10 g/L S^0 would take around 14 days, assuming that nearly all of the substrate was oxidised during the 42 days of incubation (Figure 6.1). Thus, two weeks was chosen as an incubation period for future subcultures.

6.2 Inhibition and toxicity

6.2.1 Different process liquors

Process liquors 1 - 6 were compared using total cell count and by incubating the liquors with either iron- or sulphur-oxidising enrichment cultures. The experiments were carried out to reveal the liquors that provide beneficial conditions for bioleaching, and the liquors that might inhibit iron and sulphur oxidation. Since the chemical compositions of the process liquors were known (Table 6.1; not shown), comparison of the liquors could provide information of what nutrients were lacking from the heap leaching liquors, and what metals were limiting oxidation activity in the heaps.

Total cell number of the liquors (Figure 6.2) was examined by epifluorescence microscope shortly after receiving the samples from Talvivaara Mining Company Plc. The liquors had similar cell concentrations, the highest cell count being in PL2 ($8.3 \cdot 10^6$ cells/mL) and the lowest in PL6 ($2.9 \cdot 10^6$ cells/mL). Process liquors 1, 3, 4, and 5 had cell concentrations between $3.5 \cdot 10^6$ and $5.7 \cdot 10^6$ cells/mL. The liquors contained a relatively high cell concentration, indicating that their conditions for cell growth were not considerably inhibiting or toxic.

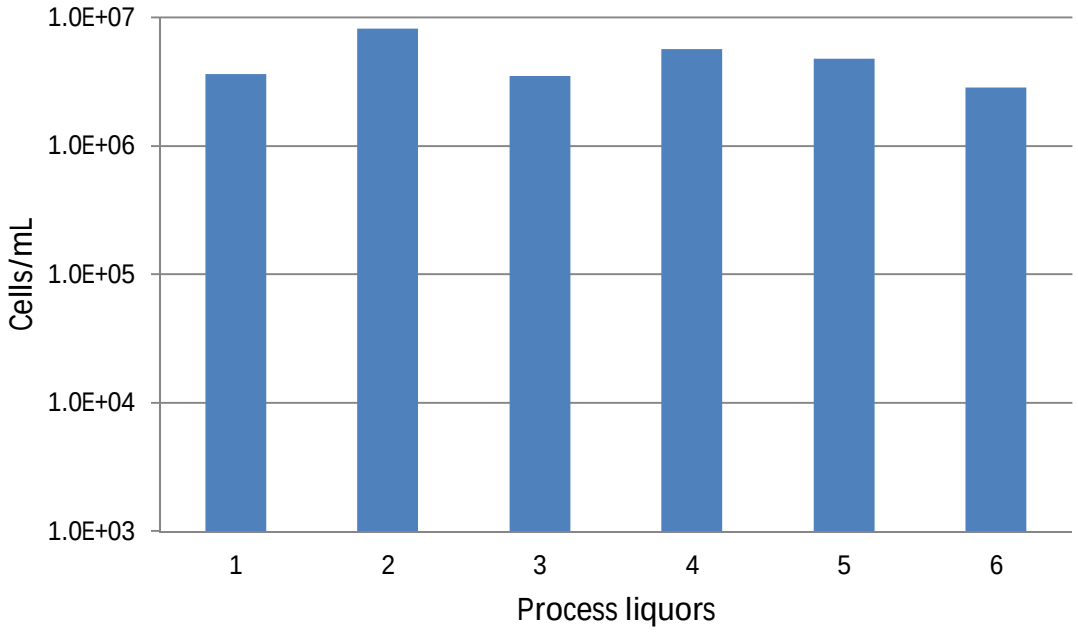

Figure 6.2. Total cell number of process liquors 1 - 6. The cell concentration was determined by DAPI staining and epifluorescence microscopy.

Figure 6.3 presents ferrous iron oxidation in process liquors 1 - 6 inoculated with an iron-oxidising enrichment culture. The aim of the experiment was to examine which liquors had the most desirable conditions for iron oxidation. Iron oxidation was monitored by measuring ferrous iron concentration in the cultures at regular intervals, and iron oxidation rates were calculated from the linear part of the oxidation curve.

Iron oxidation in PL6 was most effective, with an iron oxidation rate of 183 mg/L/h (Figure 6.3). Process liquors 1, 2, and 5 followed closely by, with iron oxidation rates between 150 and 169 mg/L/h. Heap leaching liquors 3 and 4 on the other hand considerably limited iron oxidation, their oxidation rates being 81 and 64 mg/L/h, respectively. Iron oxidation rates in process liquors 1, 2, 5, and 6 were thus more than double the oxidation rate of PL3, and nearly three times the rate of PL4.

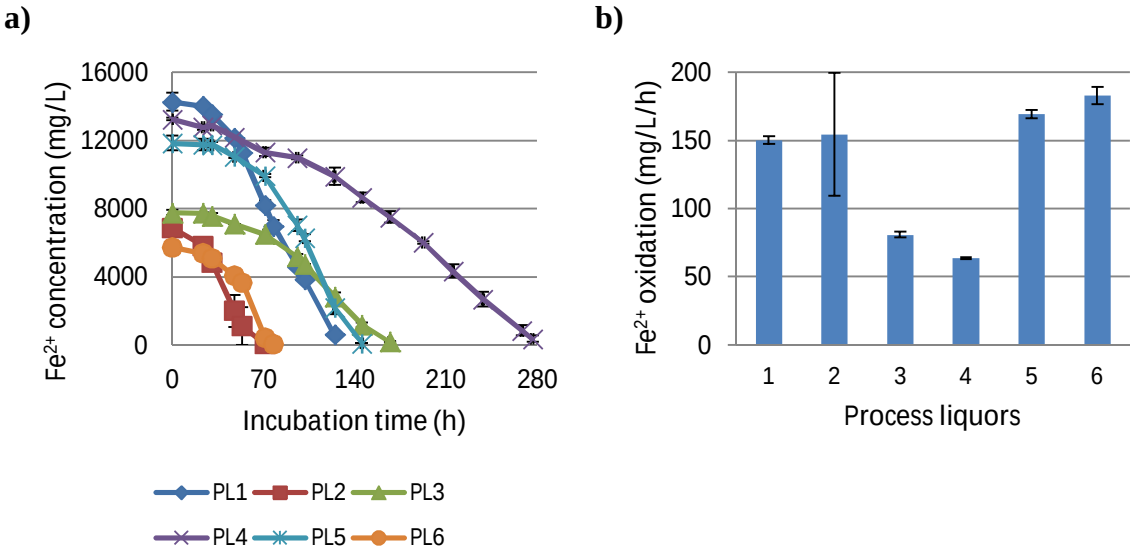

Figure 6.3. Iron oxidation activity in process liquors 1 - 6 incubated with an iron-oxidising enrichment culture. **a)** Iron oxidation was monitored by measuring ferrous iron concentration in the cultures. **b)** Oxidation rates were calculated from the linear part of the oxidation curve. Error bars show the standard deviation (n = 2).

Sulphur oxidation in different process liquors was studied in a similar way, except sulphur-oxidising enrichment culture was used as an inoculant and elemental sulphur was added as a substrate. Changes in culture pH were used to monitor the activity of sulphur-oxidising microorganisms, because sulphate formation decreases pH. Each culture was monitored until sulphur oxidation had ceased, that is until the culture pH had been stable for a week (Figure 6.4).

Culture pH in process liquors 2, 3, and 5 continuously decreased until the experiment was stopped after 44 days (Figure 6.4), indicating sulphur oxidation. PL1 and PL4 on the other hand ceased sulphur oxidation after 16 and 19 days of incubation, respectively, even though the culture pH (1.9 - 2) was still suitable for cell growth. Sulphur-oxidisers incubated in PL6 also finished sulphur oxidation already after 22 days of cultivation. However, compared to other liquors the initial culture pH (1.6) of PL6 was considerably lower and during incubation the pH value decreased to 1.0. Sulphur oxidation in process liquors 2, 3, 5, and 6 ceased several times, however the oxidation activity was usually resumed after a few days of incubation. This indicates that the sulphur-oxidising microorganisms were constantly adapting.

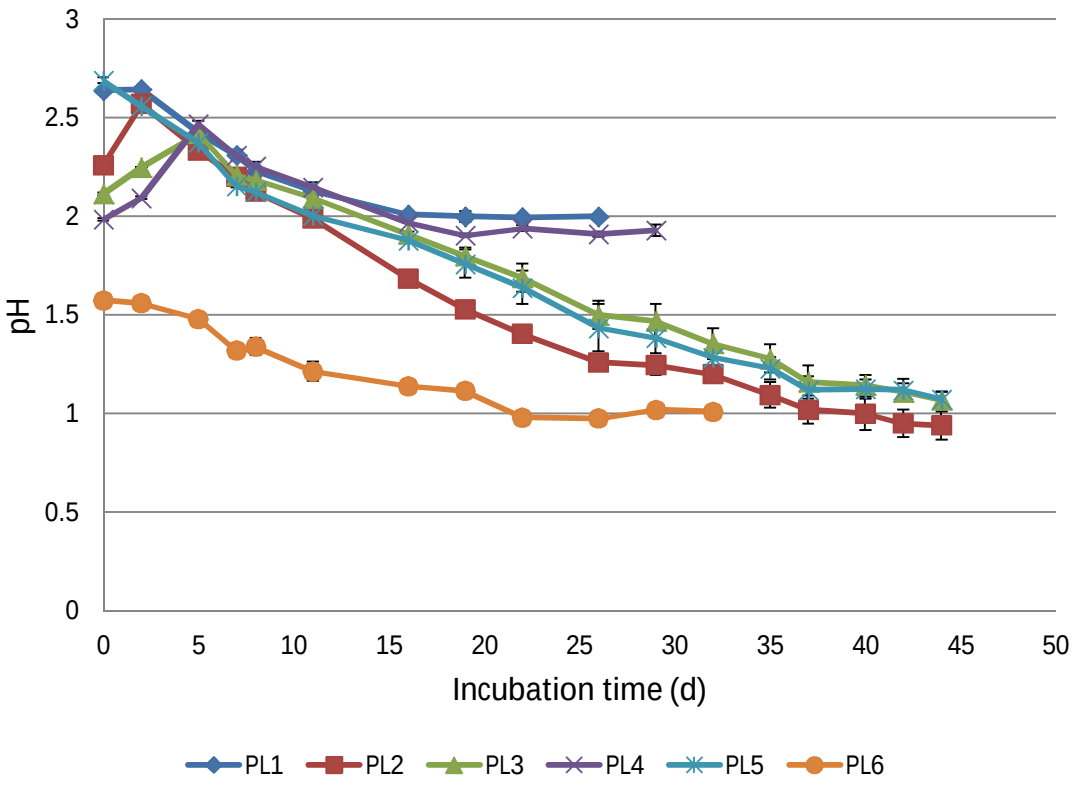

Figure 6.4. Sulphur oxidation activity in process liquors 1 - 6 incubated with a sulphur-oxidising enrichment culture. Sulphur oxidation was monitored by measuring the decrease in culture pH until sulphur oxidation had ceased (pH was stabilised). Error bars show the standard deviation (n = 2).

Total cell counts were carried out for both iron- and sulphur-oxidisers at the start and end of the experiments (Figure 6.5). At the start of the experiments, the cell number was determined immediately after the process liquors had been inoculated. The end point was defined as when the ferrous iron concentration reached its minimum level. With sulphur oxidation, final cell number was determined after the culture pH had been stable for 2 - 3 days. The intention of total cell counting was to compare the conditions for cell growth in process liquors 1 - 6, and to further investigate the differences between iron and sulphur oxidation.

As shown in Figure 6.5, the initial cell count of iron-oxidising experiment varied between $3.8 \cdot 10^6$ cells/mL in PL5, to $1.1 \cdot 10^7$ cells/mL in PL2. However, according to Figure 6.2 non-inoculated PL5 had a cell concentration of $4.8 \cdot 10^6$ cells/mL, indicating a sampling error. Process liquors 1, 3, 4, and 6 had similar cell numbers. Sulphur-oxidisers had similar initial cell numbers ranging from $2.3 \cdot 10^7$ cells/mL in PL1, to $4.0 \cdot 10^7$ cells/mL in PL5. In both iron- and sulphur-oxidising experiments, same process liquors were used and thus fluctuation of initial cell number was due to different inoculants (higher cell number in sulphur-oxidising enrichment culture).

During incubation, the number of microorganisms in both iron- and sulphur-oxidising experiments increased distinctly (Figure 6.5). Studying iron oxidation, the number of cells increased most when incubating the enrichment culture with PL5 (concentration at the end 37 times the initial cell number), PL1 (20 times), and PL6 (15 times). It should however be taken into account that the initial cell number of PL5 was unclear. The final cell concentrations of liquors PL2, PL3, and PL4 were 9, 7, and 6 times their initial cell number, respectively.

When comparing process liquors inoculated with sulphur-oxidising microorganisms, the cell number of PL6 increased the most (final cell number being 12 times the initial), whereas in PL2 cell growth was not so evident (concentration at the end 2 times the initial cell number). The final concentrations of liquors 1, 3, 4, and 5 inoculated with sulphur-oxidisers were 6, 8, 5, and 4 times their initial cell concentration, respectively. However, studying sulphur oxidation the final cell numbers were indistinct: total cell counts were performed after 2 - 3 days of invariable culture pH, not immediately after sulphur oxidation had ceased. Therefore the actual final cell numbers might have been higher.

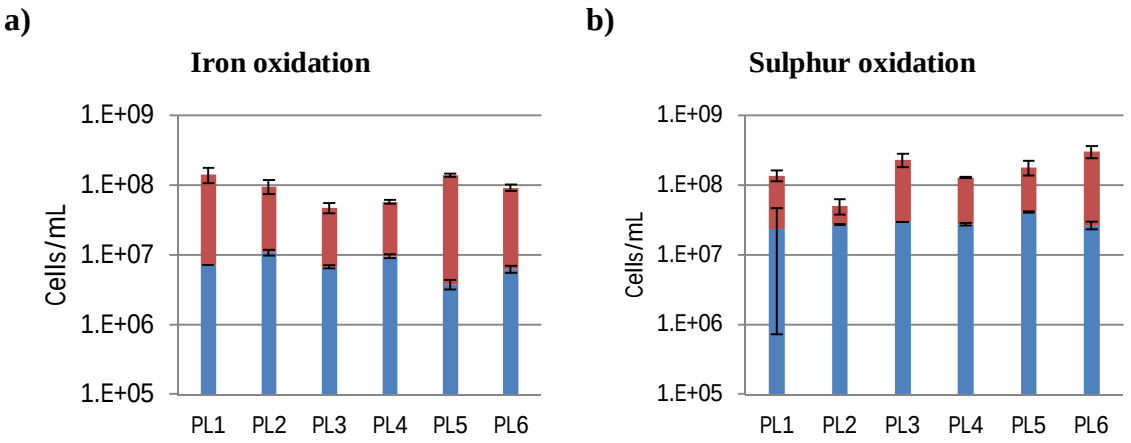

Figure 6.5. Initial and final cell number in process liquors 1 - 6 incubated with (**a**) iron- and (**b**) sulphur-oxidising enrichment cultures. The initial cell concentrations were determined by epifluorescence microscopy immediately after inoculation (blue). Studying iron oxidation, final cell counts (red) were performed after the ferrous iron concentration had reached its minimum level. The final cell number of sulphur-oxidising microorganisms was determined after 2 - 3 days of stable culture pH. Error bars show the standard deviation (n = 2).

In summary, process liquors 3 and 4 inhibited iron oxidation the most when observing both iron oxidation rates and total cell counts. Comparing their composition to other process liquors (Table 6.1; not shown), PL3 and PL4 contained the highest concentrations of several liquor components. These include aluminium (Al), arsenic (As), cadmium (Cd), chlorine (Cl), cobalt (Co), chromium (Cr), copper (Cu), lithium (Li), magnesium (Mg), nickel (Ni), scandium (Sc), sulphate (SO_4^{2-}), thorium (Th), thallium (Tl), va-

nadium (V), zinc (Zn), and zirconium (Zr). Therefore further experiments were required to examine which liquor component was limiting iron oxidation in PL3 and PL4.

Examining sulphur-oxidising microorganisms incubated with different process liquors, PL1 and PL4 offered the least favourable conditions for sulphur oxidation. Based on total cell counts, PL2 limited cell growth the most. However, the final cell numbers of the sulphur-oxidising experiment were indistinct and thus, comparison of process liquors 1 - 6 based on cell counts should be cautious.

6.2.2 Process liquor 4

PL4 was limiting both iron and sulphur oxidation, therefore a dilution series of the non-inoculated liquor in MSM was carried out (Figure 6.6). The aim of the experiment was to study iron oxidation in different concentrations of PL4, and to compare the oxidation rates to an inoculated control containing only ferrous iron and nutrients (giving maximal oxidation rate). In addition to PL4 and MSM, the cultures were supplemented with similar amounts of TES. An exception was the culture containing only PL4 (100 % v/v). Consequently, an approximation could be made about the concentration of inhibiting factors, and the amount of nutrients potentially lacking from the process liquor.

As the concentration of PL4 increased from 40 to 90 % (v/v), iron oxidation rates increased gradually from 83 to 168 mg/L/h, respectively (Figure 6.6). Liquor concentrations of 85, 90, and 95 % (v/v) had similar iron oxidation rates (156, 168, and 156 mg/L/h, respectively) to the control flask (157 mg/L/h). Lag phases were slightly lengthened compared to the control. While PL4 concentration increased from 40 to 95 % (v/v), the amount of MSM distinctly decreased (from 59 to 4 % v/v) and the concentration of TES remained constant. Lower MSM concentration had no inhibiting effect on iron oxidation activity, but to the contrary increased oxidation rates. However, when PL4 was incubated on its own, ferrous iron oxidation was limited with an iron oxidation rate of 27 mg/L/h.

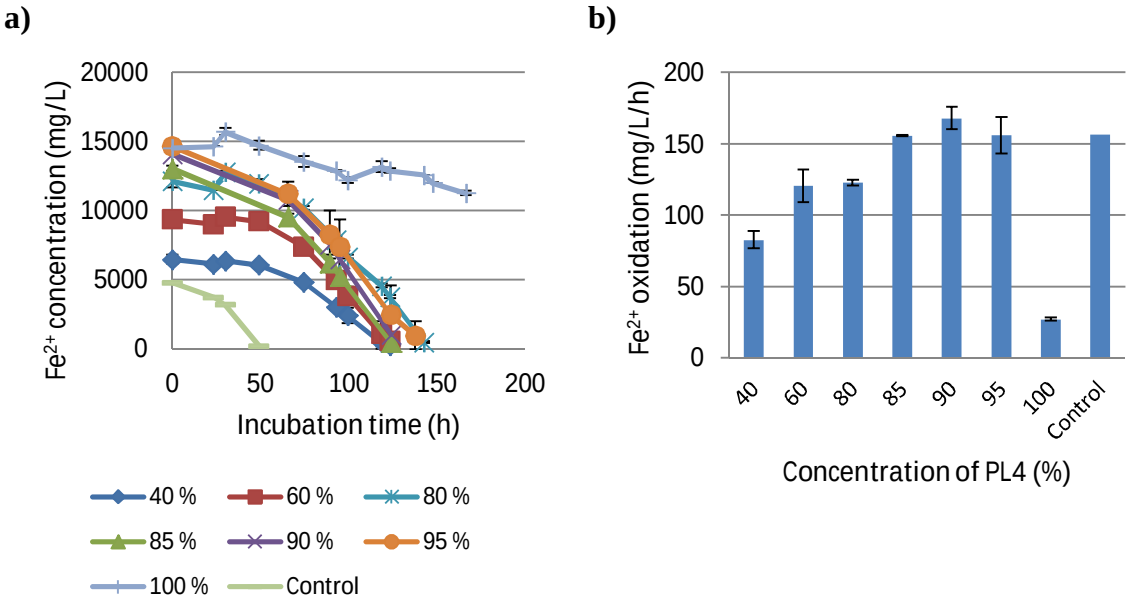

Figure 6.6. Iron oxidation activity in a non-inoculated dilutions series of PL4 in MSM, with 1 % (v/v) of TES added to all cultures except 100 % (v/v) of PL4. Oxidation activity was compared to an inoculated control supplemented with ferrous iron and nutrients (optimal conditions). Iron oxidation was monitored by measuring ferrous iron concentration (**a**), and oxidation rates were determined from the linear part of the oxidation curve (**b**). Error bars show the standard deviation (n = 2).

It is evident from Figure 6.6 that PL4 was either lacking essential nutrients, or contained components that inhibited its iron oxidation activity. Transition from 95 to 100 % (v/v) of PL4 may have led to an inhibiting concentration of one or more of the liquor components. PL4 might also have required trace elements that 1 % (v/v) of TES contained, or nutrients that were present in 4 % (v/v) of MSM.

6.2.3 Ferrous iron

Since heap leaching liquors generally contain high ferrous iron concentrations, it was important to study the tolerance of iron-oxidisers towards Fe^{2+} ions. Thus, PL4 and iron-oxidising enrichment culture were incubated with different concentrations of ferrous iron (Figure 6.7). Ferrous iron concentration of 5000 mg/L (5 g/L) was considered a control, because the concentration was commonly used in iron oxidation experiments.
Iron oxidation rates increased, when the initial ferrous iron concentration was raised (Figure 6.7). With initial ferrous iron concentrations of 5000, 8000, 12000, and 16000 mg/L, the iron oxidation rates were 110, 172, 189, and 211 mg/L/h, respectively. Increased ferrous iron concentrations did not lengthen the lag phase, indicating that the iron-oxidising microorganisms in PL4 had adapted to high Fe^{2+} concentrations.

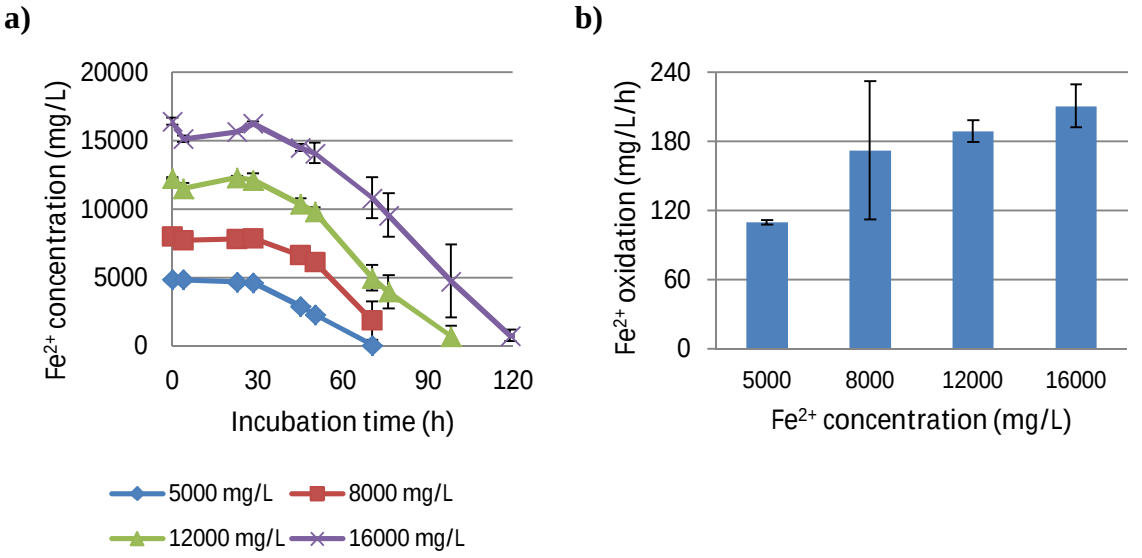

Figure 6.7. PL4 incubated with increasing concentrations of ferrous iron. **a)** Iron oxidation was monitored by measuring the decrease in initial Fe^{2+} concentration. **b)** Oxidation rates were calculated from the linear part of the oxidation curve. Error bars show the standard deviation (n = 2).

Process liquors 1-6 contained around 15000, 7200, 8400, 14000, 13000, and 6100 mg/L of ferrous iron, respectively (Table 6.1; not shown). Thus, PL1 had the highest ferrous iron concentration, followed closely by PL4 and PL5. Considering Figure 6.3, PL1 offered good conditions for iron oxidation whereas PL3, containing only 8400 mg/L of ferrous iron, limited oxidation activity. Based on these results, ferrous iron was unlikely to inhibit iron oxidation activity in the heap leaching liquors.

6.2.4 Sulphate

The heap leaching liquors, especially PL3 and PL4, contained high concentrations of sulphate ions. In order to study sulphate precipitation with barium chloride, the tolerance of iron-oxidising microorganisms towards chloride ions was first examined. Therefore, enriched iron-oxidisers and PL4 were exposed to increasing concentrations of chloride ions (Figure 6.8). Obtained iron oxidation rates were compared to two different controls: one without PL4 (control 1; to study the cooperative action of PL4 and 30 g/L of chloride), and the other without added chloride (control 2).

At increasing chloride concentrations of 3, 5, and 10 g/L, ferrous iron oxidation rates were 135, 81, and 39 mg/L/h, respectively (Figure 6.8). In the cultures containing 20 or 30 g/L of chloride, biological iron oxidation was inhibited entirely with an oxidation rate of 4 mg/L/h. However, addition of 10 g/L chloride already considerably lengthened the lag phase compared to lower Cl^- concentrations. In control 1, iron oxidation was also limited and ferrous iron was oxidised at 3 mg/L/h. Thus, a chloride concentration of 20 g/L alone was responsible for inhibiting iron oxidation.

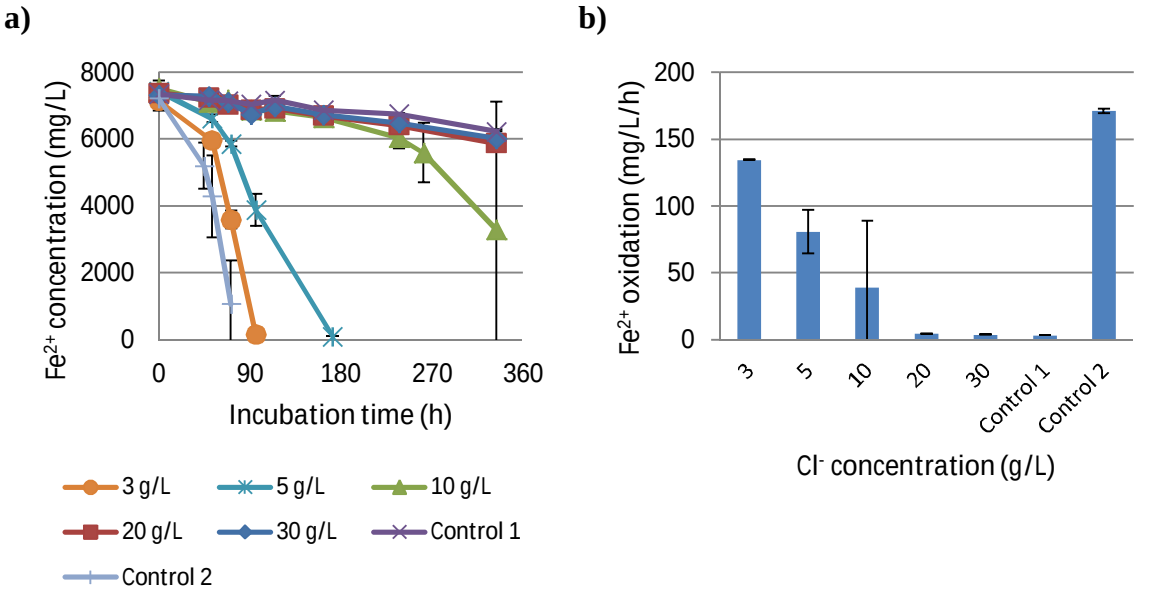

Figure 6.8. PL4 incubated with increasing concentrations of chloride ions. Changes in ferrous iron concentration (**a**) and oxidation rate (from the linear part of the oxidation curve, **b**) were used to examine the effects on iron oxidation activity. Control 1 contained a high Cl^- concentration (30 g/L), but no PL4. Control 2 had no added chloride. Error bars show the standard deviation (n = 2).

In order to precipitate the sulphate concentration of PL4 down to 70 g/L (presumably not inhibiting to iron oxidation) with barium chloride, chloride content would increase to 30 g/L. Since even 3 g/L of Cl^- ions limited iron oxidation slightly (Figure 6.8 **b**), this approach would not result in disclosing the possible sulphate inhibition.

6.2.5 Aluminium

One of the possible inhibiting factors in heap leaching liquors (particularly PL3 and PL4) was high aluminium concentration. Aluminium concentrations in process liquors 1 - 6 were around 1.4, 3.6, 9.0, 6.1, 3.1, and 0.071 g/L, respectively (Table 6.1; not shown). The effect of high aluminium concentration towards iron-oxidising microorganisms was studied by incubating inoculated PL4 with nutrients and different aluminium concentrations (Figure 6.9). The control had no added aluminium.

Even though increased aluminium concentrations slightly lengthened the lag phase and decreased iron oxidation rates, there was no clear inhibiting effect on oxidation activity (Figure 6.9). For aluminium concentrations of 3, 6, 9, and 12 g/L, ferrous iron oxidation rates were 179, 162, 134, and 119 mg/L/h, respectively, whereas the control had an iron oxidation rate of 140 mg/L/h. It can be concluded that at concentrations below 9 g/L, Al^{3+} ions may increase iron oxidation activity.

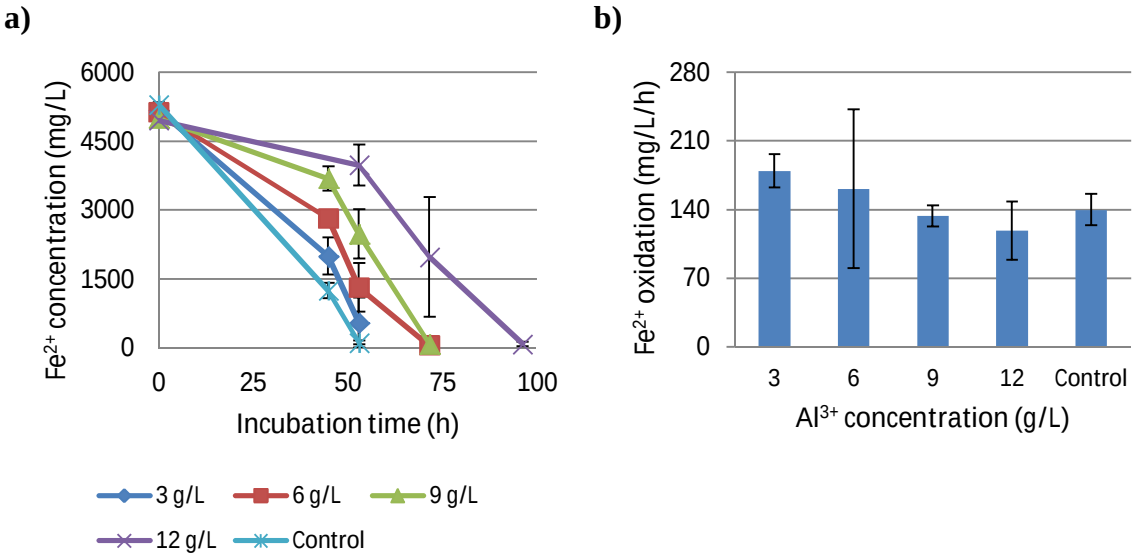

Figure 6.9. The effect of increasing aluminium concentration towards the iron oxidation activity of PL4. Oxidation activity was compared to a control with no added Al^{3+}. **a)** Iron oxidation was monitored by measuring ferrous iron concentration in the culture. **b)** Oxidation rates were calculated from the linear part of the oxidation curve. Error bars show the standard deviation (n = 2).

It can be concluded that iron-oxidising microorganisms tolerated aluminium concentrations present in process liquors 1 - 6 (Table 6.1; not shown).

6.3 Nutrient availability

6.3.1 Process liquor 4

In addition to studying possible inhibiting and toxic compounds present in heap leaching liquors, the availability of essential nutrients in PL4 was examined. To investigate whether the liquor had enough nutrients for iron-oxidising microorganisms, inoculated PL4 was incubated with and without MSM and TES (Figure 6.10 **a**). A control with thymol addition was used to prevent cell growth. Potential nutrient transfer from the iron-oxidising enrichment culture was ruled out by washing the cells before inoculation. Iron oxidation rates were similar both with and without nutrient supplementation. Washing the enrichment culture before inoculation lengthened the lag phase, although it did not affect oxidation effectivity.

Figure 6.10 **b** presents iron oxidation in non-inoculated cultures, where PL4 was supplemented with nutrients (MSM and TES) or without nutrients (Milli-Q). Here, the differences in iron oxidation activity were evident. Although having quite a long lag phase, the culture supplemented with nutrients oxidised ferrous iron faster than PL4 in Milli-Q.

Iron oxidation rates of both experiments were compared in Figure 6.10 **c**. Due to variations in culture conditions and initial ferrous iron concentrations (iron content 3 - 4

times higher in experiment **b** than in experiment **c**), the results are not directly comparable. However, iron oxidation rate was distinctly increased in 50 % (v/v) of non-inoculated PL4 supplemented with nutrients (175 mg/L/h), compared to without nutrients (18 mg/L/h) which was nearly as low as in the control (3 mg/L/h). On the other hand, iron oxidation rates in inoculated PL4 supplemented with and without nutrients were similar, 59 mg/L/h. Washing of the enriched cells before inoculation did not affect iron oxidation activity, with a ferrous iron oxidation rate of 58 mg/L/h.

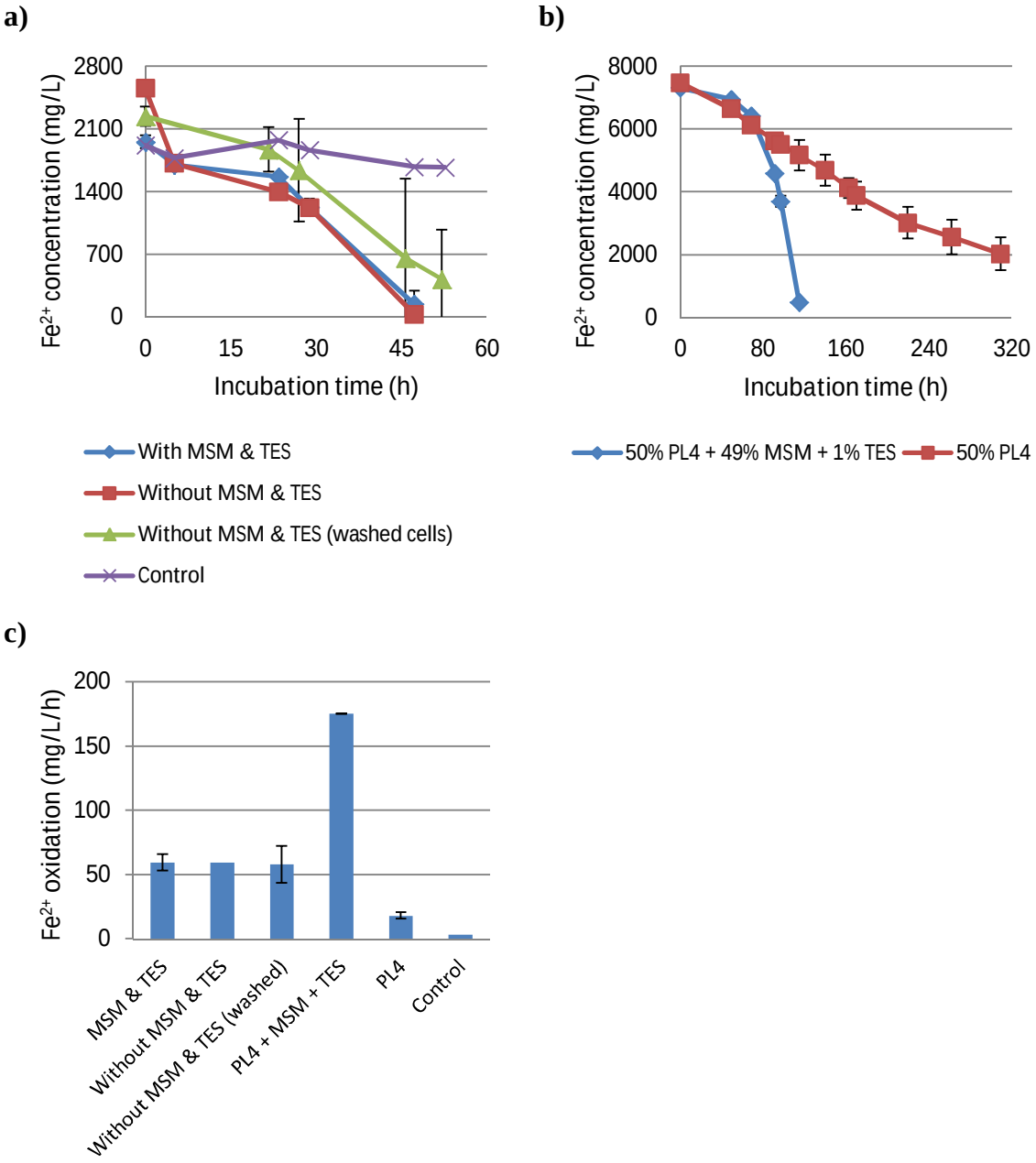

Figure 6.10. In experiment **a)**, inoculated PL4 was incubated with (blue, n = 3) and without (red) nutrients. The green curve (n = 2) represents iron oxidation without nutrients, incubated with a washed enrichment culture (to prevent nutrient transfer). Thymol was added to the control to prevent cell replication. Experiment **b)** presents iron oxidation in a non-inoculated PL4 incubated with (blue) and without (red) nutrient supplementation (n = 2). **c)** Ferrous iron oxidation rates in (from left): inoculated PL4 supplemented with nutrients; inoculated PL4; PL4 inoculated with washed iron-oxidisers; non-inoculated PL4 supplemented with nutrients; non-inoculated PL4; control. Iron oxidation rates were calculated from the linear parts of the oxidation curves (**a** and **b**). Error bars show the standard deviation.

As a conclusion, the iron-oxidising microorganisms growing in PL4 were lacking one or more of the mineral salts and trace elements present in MSM and TES. In Table 6.2, the components of MSM and TES and their concentrations in PL4 are compared. For example, the concentrations of nitrogen (N), potassium (K), phosphorus (P), and boron (B)

in PL4 were unknown and thus, it was necessary to examine their effect on iron oxidation activity. Components that were notably scarce in PL4 were sodium (Na), molybdenum (Mo), and selenium (Se). In theory, PL4 could also be slightly short of chloride (Cl^-).

Table 6.1. Components present in MSM and TES and their concentrations in PL4.

	Component concentration (mg/L)		
	MSM	**TES**	**PL4**
N	7971	-	-
S	11779	66	44528
Na	2676	46	58
K	936	-	-
Cl	594	563	434
P	111	-	-
Mg	616	-	9398
Ca	30	-	632
Fe	-	284	16240
Cu	-	16	89
B	-	44	-
Mn	-	79	6448
Mo	-	40	111 µg/L
Co	-	19	47
Zn	-	26	6036
Se	-	47	< 200 µg/L

Based on the presented iron oxidation rates (Figures 6.6 and 6.10 **c)** and taking into account the component concentrations of different solutions (Table 6.2), the nutrients selected for further examination were nitrogen, sodium, potassium, phosphorus, boron, molybdenum, and selenium.

6.3.2 Phosphorus, nitrogen, sodium, and potassium

PL4 was supplemented with 54 mg/L of phosphorus, 3900 mg/L of nitrogen, 1300 mg/L of sodium, and 460 mg/L of potassium to study if it was lacking nutrients present in MSM (Figure 6.11). Iron oxidation rates were compared to the experiment presented in Figure 6.10 **b**.

Iron oxidation rate in PL4 supplemented with both MSM and TES (175 mg/L/h) was similar to the one containing only MSM (162 mg/L/h), indicating that one or more of the components present in MSM were absent in the liquor. Iron oxidation in PL4 supplemented with phosphorus, sodium, and both sodium and potassium was very slow, with iron oxidation rates of 14, 14, and 11 mg/L/h, respectively. Thus, addition of these

nutrients did not increase iron oxidation activity compared to PL4 incubated with only Milli-Q (18 mg/L/h). However, PL4 supplemented with nitrogen had a distinctly higher oxidation activity, with an iron oxidation rate of 154 mg/L/h.

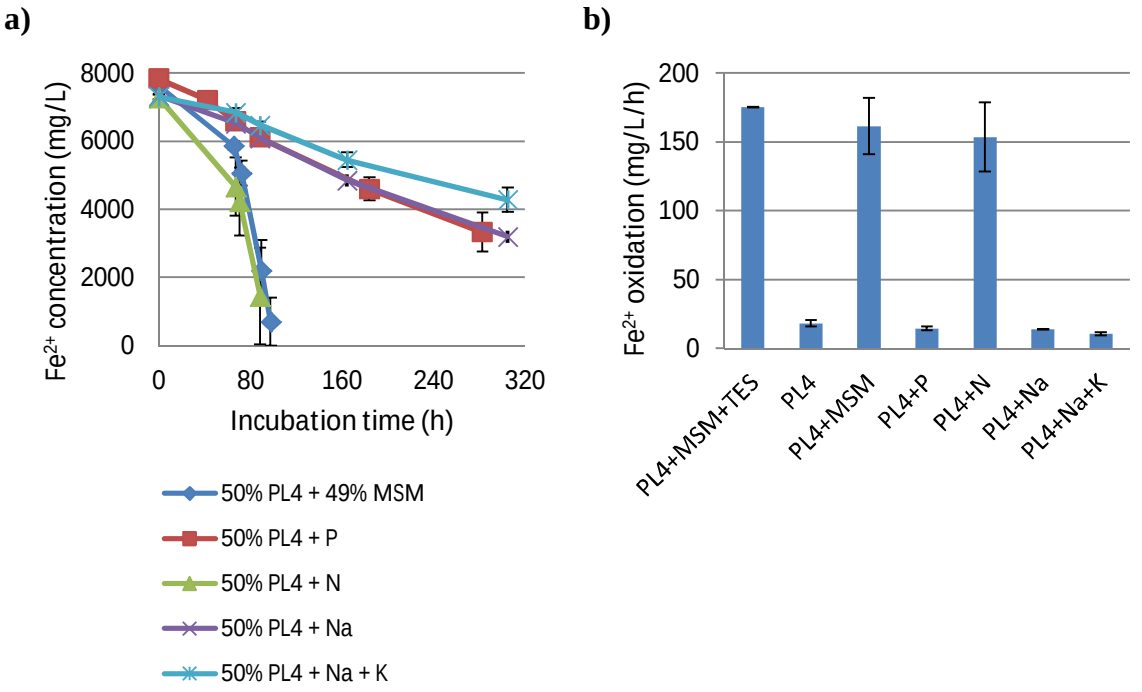

Figure 6.11. a) Iron oxidation activity in PL4 supplemented with MSM, phosphorus, nitrogen, sodium, and both sodium and potassium. **b)** Iron oxidation rates were calculated from the linear part of the oxidation curve, and compared to the experiment presented in Figure 6.10 **b**. Error bars show the standard deviation (n = 2).

Addition of nitrogen considerably increased the oxidation activity of indigenous iron-oxidisers in PL4. Therefore process liquors 1 - 6 were analysed for total organic and inorganic nitrogen (Table 6.3). Nitrogen concentrations in liquors 2, 4, and 6 were below the limit for determination (1 mg/L). PL1, PL3, and PL5 had a total nitrogen content of 2.6, 1.5, and 1.0 mg/L, respectively.

Table 6.2. Concentration of total organic and inorganic nitrogen in process liquors 1 - 6.

	Process liquor					
	1	**2**	**3**	**4**	**5**	**6**
Nitrogen concentration (mg/L)	2.6	< 1.0	1.5	< 1.0	1.0	< 1.0

In summary, neither phosphorus, sodium, nor potassium addition enhanced the ferrous iron oxidation in PL4. Nitrogen, on the other hand, was distinctly absent in the liquor and thus limiting oxidation activity. Comparing different process liquors, total nitrogen concentrations were very low (less than 3 mg/L in all liquors). PL4 supplemented with

3900 mg/L of N oxidised ferrous iron nearly as fast as the culture incubated with all components of MSM and TES, and significantly faster than PL4 incubated without any nutrients. In 95 % (v/v) of PL4 incubated with 4 % (v/v) of MSM and 1 % (v/v) of TES (PL4 dilution series; Figure 6.6), iron oxidation rate was high with a nitrogen concentration of 319 mg/L. Thus, PL4 requires 319 mg/L or less of nitrogen to enhance the iron oxidation activity of its indigenous microorganisms.

6.3.3 Boron, molybdenum, and selenium

Figure 6.12 presents iron oxidation in PL4 supplemented with boron, molybdenum, and selenium (nutrients present in TES). The liquor was supplemented with each of the selected nutrients both separately and together, as well as with 1 % (v/v) of TES to examine their effects on oxidation activity.

The results show that the decreased oxidation activity of PL4 was not due to any of the trace elements present in TES (Figure 6.12). When examining the selected nutrients, iron oxidation rates in PL4 supplemented with boron, molybdenum, selenium, and all the three components together remained at 13, 17, 15, and 15 mg/L/h, respectively. Addition of TES did not enhance iron oxidation either compared to PL4 supplemented with only Milli-Q, with iron oxidation rates of 12 and 18 mg/L/h, respectively. Oxidation activity was thus clearly decreased compared to inoculation with both MSM and TES (175 mg/L/h).

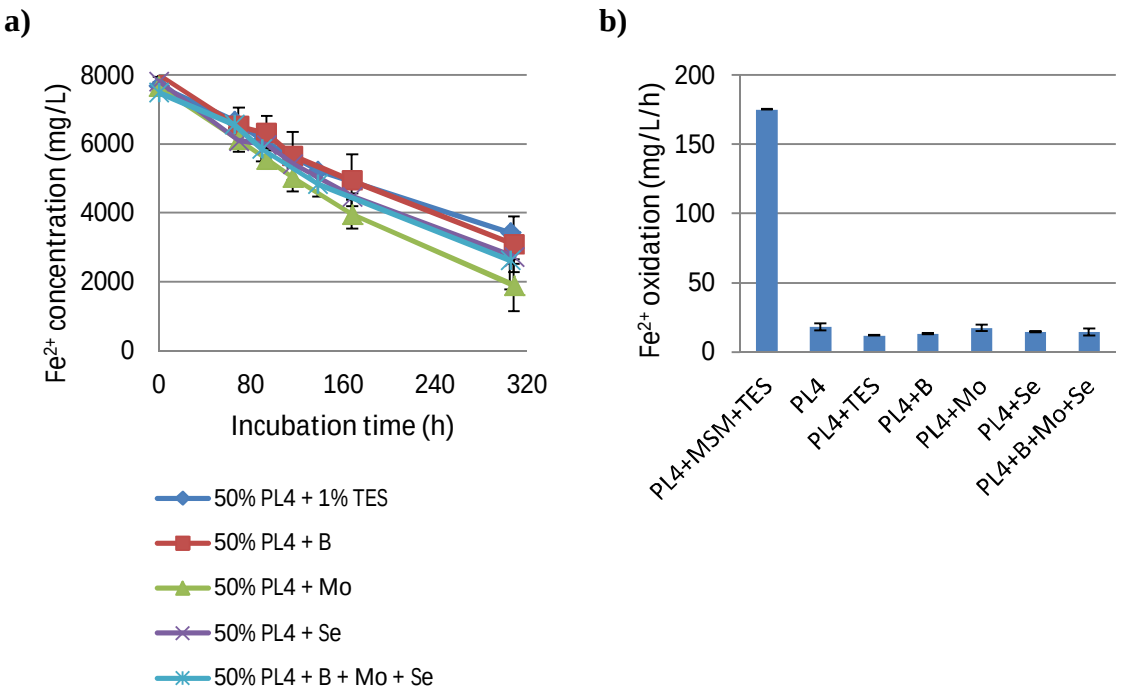

Figure 6.12. a) Iron oxidation activity in PL4 supplemented with TES, boron, molybdenum, selenium, and all three selected nutrients together. **b)** Iron oxidation rates were calculated from the linear part of the oxidation curve, and compared to the experiment presented in Figure 6.10 **b**. Error bars show the standard deviation (n = 2).

As a conclusion, PL4 did not contain enough nitrogen for iron-oxidising microorganisms. Addition of other solution components present in MSM and TES (including boron, molybdenum, and selenium) did not enhance iron oxidation in PL4, demonstrating that nitrogen deficiency was the main reason inhibiting iron oxidation activity.

6.4 Bacterial community composition

PL4 was studied by DAPI-staining and epifluorescence microscopy (Figure 6.13) to examine the cell count and morphology of its indigenous microorganisms. The microbial community consisted mainly of bacillus-shaped microbes, but other cell forms such as filamentous microorganisms were also present.

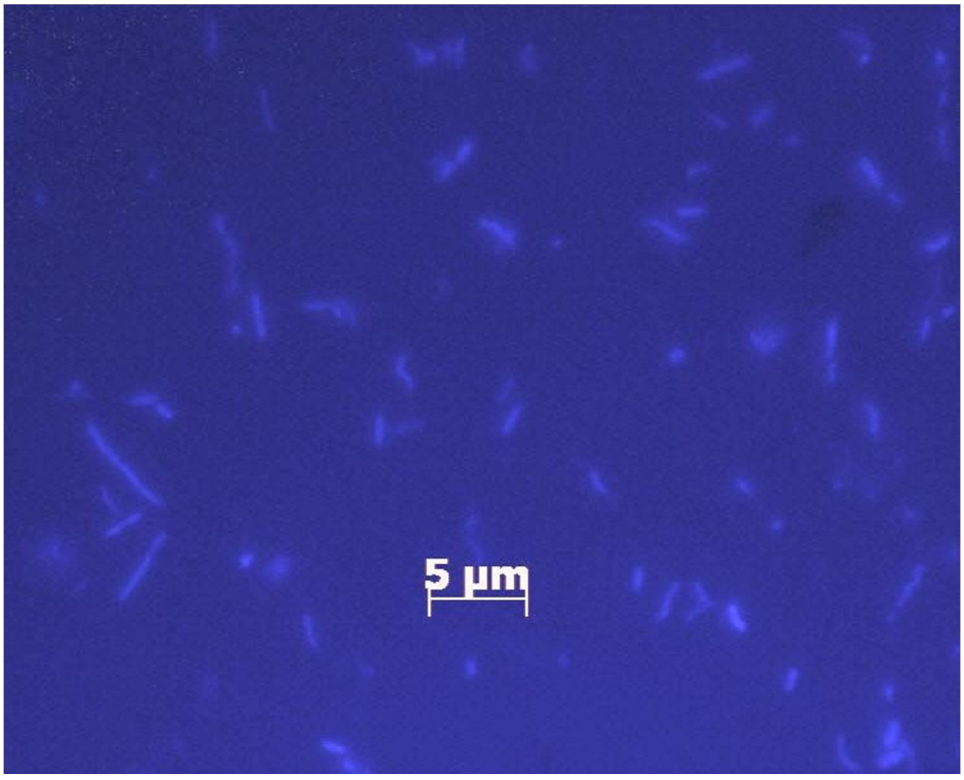

Figure 6.13. DAPI-stained PL4 studied under epifluorescence microscopy.

In an attempt to examine bacterial community at the mining site, process liquors 1 - 6 and different iron- and sulphur-oxidising enrichment cultures were analysed by PCR-DGGE (Figure 6.14). Letters A - J in Figure 6.14 represent the different samples examined by microbial analysis (as presented in Table 6.4).

Table 6.3. Samples analysed by PCR-DGGE and the letters representing them in Figure 6.14.

Letter in Figure 6.14	Sample
A	Process liquor 4
B	Sulphur-oxidising enrichment culture
C	Iron-oxidising enrichment culture 1
D	Iron-oxidising enrichment culture 2
E	Process liquor 1
F	Process liquor 2
G	Process liquor 3
H	Process liquor 5
I	Process liquor 6
J	Iron-oxidising enrichment culture 3

The mine site samples had a diverse bacterial community (Figure 6.14). Comparing process liquors 1 - 6, PL6 (I) had the distinctly lowest microbial diversity. Process liquors 1 - 5 on the other hand consisted of highly similar bacterial communities. Although iron- and sulphur-oxidising enrichment cultures were originally enriched from PL4 (A) associated with several types of bacteria, several weeks of subculturing significantly decreased their microbial diversity. Thus, the oldest iron-oxidising enrichment culture (C) contained only one distinguishable DNA fragment whereas the more recent enrichment cultures (D and J) had several.

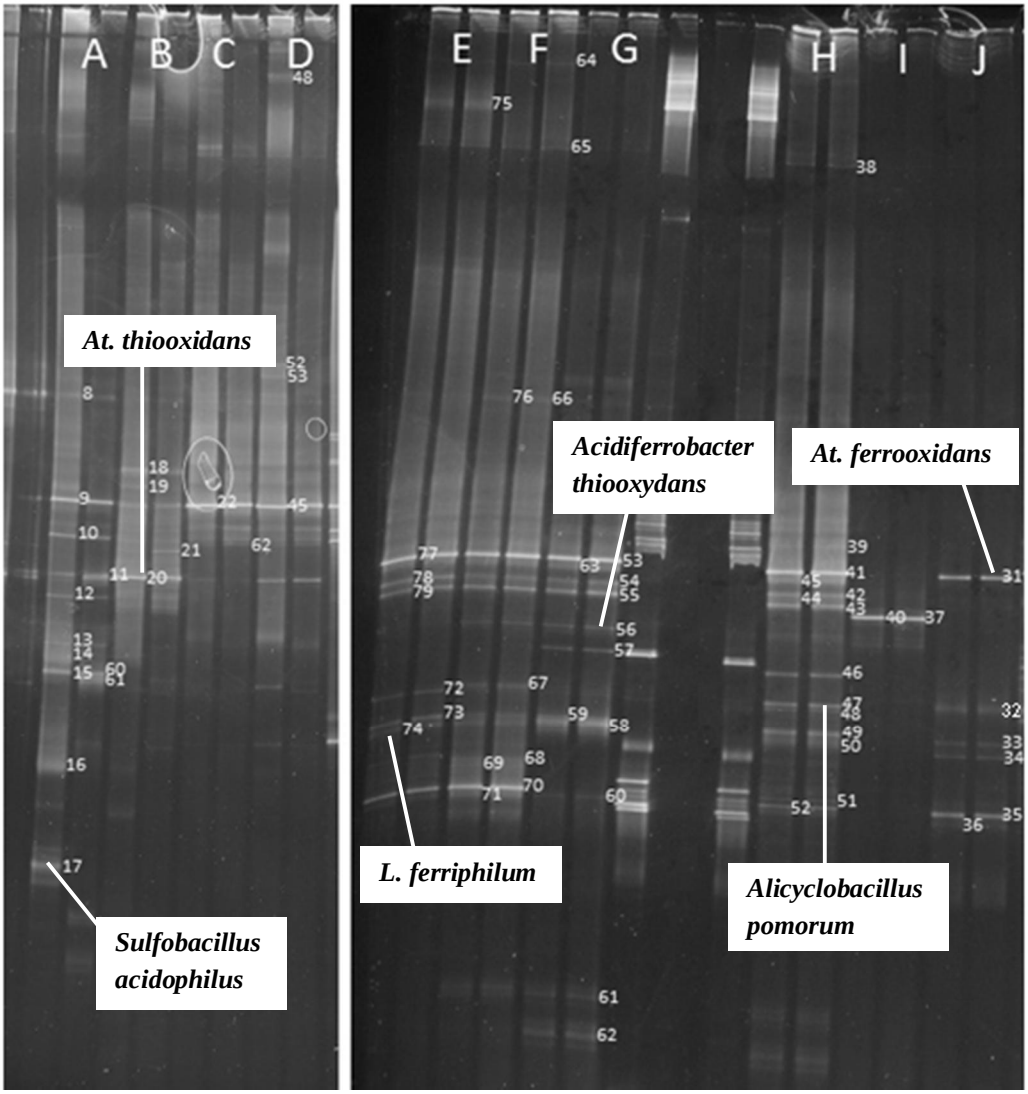

Figure 6.14. Microbial community analysis of process liquors 1 - 6 and iron- and sulphur-oxidising enrichment cultures. Digital image of the DGGE gel with PL4 (A), sulphur-oxidising enrichment culture (B), iron-oxidising enrichment cultures 1 and 2 (C and D, respectively), process liquors 1 - 3 (E-G, respectively), PL5 (H), PL6 (I), and iron-oxidising enrichment culture 3 (J). Generally each band on the gel represents one type of bacteria.

Sequence data from the excised DNA fragments was as presented in Appendix 2. The similarity percentage of most DGGE bands and their closest relatives listed in NCBI was high (> 95 %). *Acidithiobacillus ferrooxidans* (for example DNA fragments **A9**, **C22**, **D45**, **E75**, **G55**, and **J31** in Figure 6.14) was the most common bacterial species found, being present in all process liquors and enrichment cultures except PL6 (which contained solely *Acidithiobacillus thiooxidans*, **I37**). *A. thiooxidans* was also found in PL4, sulphur-oxidising enrichment culture, and iron-oxidising enrichment culture 2. Other common bacterial species associated with the process liquors were *Acidiferrobacter thiooxydans* (in process liquors 2 - 4; **A13**, **G56**), *Leptospirillum ferrodiazotrophum* (in liquors 1 and 4; **E73**), *Leptospirillum ferriphilum* (in liquors 2 - 3; **G58**), and *Sulfobacillus acidophilus* (in liquors 3 and 4; **A17**, **G61**). A bacterium exhibiting a 91 %

identity with *L. ferriphilum* was also present in PL1. Less common bacterial species were *Sulfobacillus benefaciens* present in PL3 (**G62**), and *Alicyclobacillus pomorum* found in PL5 (**H47**).

Some strains remained unrecognised, for instance bacteria similar to *Acidiphilium* species in iron-oxidising enrichment cultures 2 (91 %) and 3 (100 %), and strains similar to *Leptospirillum* species in PL4 (94 - 99 %), PL5 (88 %), and iron-oxidising enrichment culture 3 (99 %). Many of the excised DNA fragments undoubtedly originated from overlapping sequences.

7 DISCUSSION

Based on DAPI-staining and epifluorescence microscopy, process liquors 1 - 6 contained relatively high cell amounts. With cell concentrations ranging between $2.9 \cdot 10^6$ and $8.3 \cdot 10^6$ cells/mL, none of the liquors distinctly differed from the others concerning cell replication. Halinen et al. (2012) studied the microbial community of a low grade nickel ore bioheap and found similar bacterial cell numbers of 10^5 - 10^7 cells/mL. However, when inoculating different liquors with an iron-oxidising enrichment culture, PL3 and PL4 had 51 and 61 % lower oxidation rates than the other process liquors, respectively. A similar experiment with sulphur-oxidising microorganisms showed that PL1 and PL4 offered the least favourable environment for sulphur oxidation. Taking into account that pH measurement was used to monitor sulphur oxidation, the initial pH values of the process liquors may have affected the results. For instance, the pH value of PL6 stopped decreasing quite early (indicating that sulphur oxidation had ceased), but due to the liquor's low initial (1.6) and final (1.0) pH, the activity of sulphur-oxidising microorganisms in that specific liquor was difficult to predict. It has however been reported that for *At. thiooxidans*, the only bacterium present in PL6, the lowest pH after growth on sulphur compounds is 0.5 - 0.8 (Kelly & Wood 2000). The bacterium has also been shown to oxidise sulphur optimally at pH 1.5 - 2.0 and be completely inhibited at pH 0.5 (Plumb et al. 2008). This indicates that sulphur oxidation in PL6 was possibly inhibited by something other than pH. It was however clear that PL4 inhibited both iron and sulphur oxidation more than other process liquors.

The work presented in this thesis focused on finding the factor/factors in PL4 that were inhibiting the oxidation processes. A dilution series performed in nutrient solution showed that with 40 - 95 % (v/v) of PL4, ferrous iron oxidation rate gradually increased and became steady at around 160 mg/L/h (similar to the control flask). However, in 100 % (v/v) of PL4 the oxidation rate drastically decreased by 83 %. Two possible reasons for low oxidation activity were 1) inhibitory ion or metal concentrations, and/or 2) nutrient insufficiency.

7.1 Physicochemical parameters affecting biooxidation

7.1.1 Substrate appearance and dosage

In the present work, substrate concentrations for iron- and sulphur-oxidising enrichment cultures were 5.6 g/L of Fe^{2+} and 10 g/L of S^0, respectively. Several microorganisms, such as *At. ferrooxidans*, are able to utilise both substrates simultaneously (Chen et al.

2011); however, the enrichment of heap microorganisms growing on both Fe^{2+} and S^0 and its effect on the enriched microbial community was not explored in the present study. It has been demonstrated that iron-oxidising bacteria often solubilise metals faster than sulphur-oxidisers, although sulphur-oxidising microorganisms may achieve higher metal leaching percentages due to their higher acid generation capacity (Chan et al. 2003). In the cultures growing on Fe^{2+}, a 24-hour oxidation lag phase was usually detected (with a slight Fe^{2+} decrease due to chemical hydrolysis) before a sharp decrease in Fe^{2+} concentration. The lag phase was 1 day shorter than detected by Chen et al. (2008) in similar culture conditions. Complete oxidation was achieved during 2 to 3 days. The threshold Fe^{2+} concentration (after which no further oxidation occurred) in the present study was found to be around 1 % of the initial Fe^{2+} concentration. This finding is comparable to a study by Özkaya et al. (2007), which demonstrated that the threshold concentration linearly increases with increasing Fe^{2+} concentrations.

Chen & Lin (2001) found that the activity of sulphur-oxidisers was enhanced with increasing S^0 concentrations until above 0.5 % (w/v; 5 g/L), after which the substrate concentration became inhibiting for sulphur oxidation. In 2004a Chen & Lin demonstrated that while increasing the concentration from 0.5 to 5 g/L of S^0, acidification and SO_4^{2-} production increased but were not improved after 3 g/L of S^0. A later study by Chen & Lin (2010) optimised pH reduction and SO_4^{2-} production rates and concluded that in the range of 0.1 - 1.0 % (w/v; 1 - 10 g/L) S^0, sulphur oxidation was enhanced with increasing substrate dosage. As sulphur is insoluble, bacteria adsorb and grow on its surface and thus, sulphur is oxidised. Therefore, increase in sulphur concentration (increase in available surface area) is favourable for oxidation and consequently, for metal solubilisation (Chen & Lin 2001). However, Liu et al. (2008) studied the effect of quite high S^0 concentrations (0.5, 1, 2, 3, and 5 %; 5, 10, 20, 30, and 50 g/L) on heavy metal remobilisation by indigenous sulphur-oxidisers and concluded that a substrate concentration of 20 g/L was indicated to be the best for bacterial activity under the experimental conditions (28 °C, 180 rpm). These different studies indicate that an optimal S^0 concentration for sulphur oxidation is difficult to predict and is largely dependent on the experimental conditions. It may however be deduced that the S^0 concentration used in the present study (10 g/L) should have been adequate for the sulphur-oxidising microorganisms. A study by Suzuki et al. (1999) demonstrated that the type of sulphur is important, and found that sulphur dissolved in DMSO (dimethyl sulphoxide) made the microbial cells more responsive to various effects. In this thesis, S^0 was added as non-dissolved powder. S^0 oxidation is also important for producing acidic conditions, and Bouchez et al. (2006) demonstrated that a low S^0 concentration (0.2 %; 2 g/L) might not be enough to decrease the solution pH. As the culture pH in sulphur-oxidising experiments presented in this thesis decreased efficiently, the S^0 in the cultures was not too low. Chen et al. (2003a) found that the specific surface area available for bacterial adsorption does not necessarily depend on the particle size, but rather on the number of micropores in the

sulphur particles. The porosity of S^0 has also been demonstrated to impact the formation of passivating S^0 layer on the mineral surface (Córdoba et al. 2008b).

7.1.2 Inhibitory anions, cations, and metals

PL4 (along with PL3, which also inhibited iron oxidation) contained higher Al, As, Cd, Cl, Co, Cr, Cu, Li, Mg, Ni, Sc, SO_4^{2-}, Th, Tl, V, Zn, and Zr concentrations than the other process liquors. Although ferrous iron concentration in PL4 was not higher than in other liquors, the effect of increasing Fe^{2+} concentrations on iron oxidation was interesting owing to the fact that bioleaching environments often contain high iron concentrations. In the present work, Fe^{2+} concentrations of 5 - 16 g/L did not inhibit iron oxidation; in fact, increasing iron concentrations enhanced iron oxidation rates. Özkaya et al. (2007) studied the effect of different Fe^{2+} concentrations and found that 2 g/L of Fe^{2+} resulted in the highest specific iron oxidation rate, and at high concentrations the ion inhibited its own oxidation. These results are inconsistent with the work presented in this thesis. One possible reason is that the resistance towards Fe^{2+} ions is highly dependent on the microbial strains present in the bioleaching systems. Nemati & Harrison (2000) showed that the growth of *Acidianus brierley* was inhited at Fe^{2+} concentrations higher than 7.5 kg/m^3 (7.5 g/L; non-growing cells were still able to utilise the substrate), whereas the growth of *At. ferrooxidans* was not affected by Fe^{2+} concentrations lower than 30 kg/m^3 (30 g/L) and its iron oxidation rate was distinctly higher than that of *Acidianus brierley*. Metal leaching is usually improved by increasing Fe^{2+}, and subsequently increasing Fe^{3+}, concentrations (Ahonen & Tuovinen 1995; Chen et al. 2008; Córdoba et al. 2008b). However, it has been shown that a high concentration of Fe^{3+} ions also creates instability in the bioleaching system and thus, favours precipitation and consequently mineral passivation (Córdoba et al. 2008c). At a higher pH (2.5) than studied in this thesis, ferric oxide precipitation occurred and inhibited bacterial activity through adsorption on the cells (Meruane & Vargas 2003). Therefore at pH higher than 2.5, low Fe^{2+} concentrations are preferred. However, as dissolved Fe^{3+} concentrations were not measured in the present work, the possible effects of ferric iron cannot be further discussed.

Due to its extremely high concentration in PL4, sulphate inhibition was discussed. Harahuc et al. (2000) selectively inhibited the oxidation of either Fe^{2+} or S^0 by *At. ferrooxidans* at pH 3 and found that at a concentration of 0.2 M (20 g/L), sulphate did not inhibit iron or sulphur oxidation. At higher concentrations sulphate had little effect on iron oxidation, but the activity of sulphur-oxidising microorganisms was distinctly decreased, possibly due to changes in osmotic pressure. Several sulphate removal methods have been tested, such as barium carbonate precipitation (only if SO_4^{2-} is associated with calcium; Hlabela et al. 2007) and pH-adjusted precipitation as jarosite or gypsum (Nurmi et al. 2010).

In the present study, the toxicity of chloride ions to iron-oxidising microorganisms was studied as a preliminary test before sulphate toxicity experiment. However, Cl^- ions inhibited iron oxidation at such low concentrations (slight inhibition at 3 g/L, total inhibition at 20 g/L) that the sulphate experiment could not be performed as planned. Microorganisms involved in bioleaching are often sensitive to Cl^- ions (Sarcheshmehpour et al. 2009). A concentration of 5 g/L NaCl (3 g/L Cl^-) has been found not to inhibit iron or sulphur oxidation (Vestola et al. 2010), although Harahuc et al. (2000) demonstrated that even 10 mM of chloride (0.4 g/L) inhibited iron oxidation. Sulphur oxidation on the other hand was prevented only at 0.4 M (14 g/L). These results indicate that iron-oxidisers are more affected by high chloride concentrations than sulphur-oxidisers, although with sulphate inhibition the results were contrary (Harahuc et al. 2000). In an experiment performed by Deveci et al. (2008), extreme thermophiles were efficient in ore bioleaching in the range of 10 - 40 g/L of Cl^- whereas mesophiles (mostly present in bioheaps) were inhibited by salinity. This might explain the sensitivity of iron-oxidisers towards Cl^- ions presented in this thesis. Mesophilic bioleaching microorganisms have however been found to resist higher Cl^- concentrations (8 - 10 g/L) than moderate thermophiles (2 g/L) (Deveci 2002). Addition of high NaCl concentrations, as in this thesis, rapidly increases culture pH and consequently accelerates microbial death (Gómez et al. 1999). The presence of certain ions such as Na^+ may also enhance the production of harmful ferric precipitates (Deveci 2002). In the future, it would be reasonable to study the inhibitory effect of SO_4^{2-} towards the indigenous microorganisms present in Talvivaara bioheaps.

Aluminium inhibition was selected for further studies due to its trivalent nature. Al^{3+} concentrations up to 12 g/L were not toxic to iron-oxidising microorganisms; however, increased Al^{3+} concentrations lengthened the oxidation lag phase and slightly decreased oxidation rates. It was also demonstrated that below 9 g/L, Al^{3+} may enhance iron oxidation. Gómez et al. (1999) studied the effect of Al on the bacterial growth of *Sulfolobus rivotincti* and found that at a non-toxic concentration, Al increased cell growth. The presence of Al has also been proven to overcome fluoride (F) toxicity in low-grade copper ores through complexation (Sicupira et al. 2011; Veloso et al. 2012). As PL4 had high concentrations of several ions and metals, complexation might explain why low Al^{3+} concentrations had a positive effect on iron oxidation.

7.1.3 Nutrient sufficiency

In terms of nutrient inadequacy, major nutrients nitrogen, potassium, and phosphorus, as well as minor nutrient boron required further investigation due to their unknown concentrations in PL4. Minor nutrients sodium, molybdenum, and selenium were also examined due to their very low concentrations in the process liquor. The experiments presented in this thesis showed that K, P, B, Na, Mo, and Se concentrations in PL4 were adequate to support microbial growth. Harahuc et al. (2000) demonstrated that a low phosphate (PO_4^{3-}) concentration (10 mM; 0.3 g/L of phosphorus) was enough to achieve

maximal iron and sulphur oxidation in an *At. ferrooxidans* culture. At 50 mM (2 g/L of P), PO_4^{3-} decreased Fe^{2+} oxidation but increased S^0 oxidation activity. At even higher concentration (100 mM; 3 g/L of P), the inhibitory effect of PO_4^{3-} on iron oxidation was significant. Although the PO_4^{3-} content of PL4 was not studied in the present study, the nutrient's inhibitory effect even at 50 mM suggests that supplementing the bioheap leaching process with PO_4^{3-} probably wouldn't enhance the process. A limiting inorganic phosphate concentration for the activity of *At. thiooxidans* has been shown to be 6 mg/L (Zheng & Zhou 2011).

Gómez et al. (1999) studied the effect of Mo on the bacterial growth of *Sulfolobus rivotincti* and found that at a non-toxic concentration, Mo distinctly decreased cell growth. Trace metal solution (containing Na, Ca, Fe, Mn, Zn, Co, Mo, and Cu) and potassium chloride (KCl) have proved to be negative parameters and therefore inhibiting to iron oxidation by *At. ferrooxidans* at high concentrations (Abdel-Fattah & Abdel-Fattah 2002). These results indicate that trace elements should be added to bioleaching processes with care. As potassium has been shown to have a positive effect on copper ore bioleaching (Sarcheshmehpour et al. 2009), the inhibitory effect of KCl was most definitely due to Cl^- ions. However, trace metal solution at low levels enhanced oxidation rates (Abdel-Fattah & Abdel-Fattah 2002).

Nitrogen supplementation to PL4 distinctly increased iron oxidation rates in an experiment presented in this study, and based on PL4 dilution series the process liquor required 319 mg/L or less of N. Sarcheshmehpour et al. (2009) studied the nutrient content of four low-grade copper ore samples and found that concentrations of N, P, K, Ca, Mg, and S were higher in an acidic environment (pH 1.8) than at natural pH. For instance N concentrations at natural pH were in the range of 1.05 - 1.15 mg/L, whereas at pH 1.8 the concentrations were between 1.75 and 2.6 mg/L. This demonstrated higher solubility of the nutrients under acidic conditions. In comparison, process liquors PL1 - PL6 studied in the present work contained N concentrations in the range of < 1.0 - 2.6 mg/L, which, according to Sarcheshmehpour et al. (2009), were not adequate to support bacterial growth. It should however be noted that in freshly sampled process liquors, N concentrations might be higher. As other process liquors providing better biooxidation conditions than PL4 also contained very low N concentrations, it may be hypothesised that in addition to the low nutrient content in the process liquor, other factors might also be affecting the oxidation activity in PL4. The effect of different ions should be taken into account especially since iron oxidation in PL3 containing high SO_4^{2-} and metal concentrations was also limited.

It has been shown that the form of nitrogen has a large impact on the growth of bioleaching microorganisms. Examining different growth media, Sarcheshmehpour et al. found that $(NH_4)_2SO_4$ and $(NH_4)_2HPO_4$ enhanced Fe^{2+} oxidation, whereas nitrate (NO_3^-) had a negative effect on bacterial activity. A study by Harahuc et al. (2000) reached

similar results, indicating that increased NO_3^- concentrations totally inhibited iron oxidation by *At. ferrooxidans*. Sulphur-oxidising microorganisms proved to be more resistant to high NO_3^- concentrations than iron-oxidisers; however, Suzuki et al. (1999) demonstrated that sulphur oxidation by *At. thiooxidans* was almost totally inhibited by NO_3^- at 100 mM (6 g/L). The nutrient was also found to be more inhibitory at low pH (2.3) than at more alkaline conditions (pH 3; Suzuki et al. 1999), probably due to its increased solubility. Nitrogen should therefore be supplemented to the bioheap leach process as ammonium (NH_4^+). Even though NH_4^+ may react with Fe^{3+} to produce jarosite precipitates (d'Hugues et al. 2002), its increased availability for microbial growth enhances the overall process. For example, decreasing the NH_4^+ concentration in nutrient medium from 1000 to 270 mg/L significantly decreased bacterial growth and substrate oxidation in a pyrite bioleaching process (d'Hugues et al. 2008). Different microorganisms were also differently affected by changing NH_4^+ concentrations.

7.2 Microorganisms present in heap bioleaching

Experiments performed with different process liquors showed that iron- and sulphur-oxidising microorganisms were present in all mining waters, and that they could be enriched. Iron- and sulphur-oxidising enrichment cultures originating from PL4 lost a lot of their initial microbial diversity after weeks of subculturing. Iron-oxidisers initially belonged to *Acidithiobacillus* (*At. ferrooxidans*, *At. thiooxidans*), *Acidiphilium*, and *Leptospirillum* species, but over time *At. ferrooxidans* become dominant. Halinen et al. (2012) studied the microbial community of a low grade nickel ore bioheap over a period of three years in Talvivaara, Finland, and found a similar pattern. In addition, Johnson and Hallberg (2007) reported that in a media containing ferrous sulphate (as in the present work), *At. ferrooxidans* rather than *Leptospirillum* spp. is enriched due to the bacterium's faster growth on Fe^{2+}. Sulphur-oxidising enrichment culture consisted solely of *At. ferrooxidans* and *At. thiooxidans*. Johnson & Hallberg (2007) reported that at 30 °C, sulphur-based media enriches *At. thiooxidans*, whereas at higher temperatures (45 °C) *At. caldus* is enriched. As the sulphur-oxidising enrichment culture was incubated at 27 °C, enrichment of *At. thiooxidans* was expected.

In addition to PL4, *At. ferrooxidans* was present in all process liquors except PL6. The iron- and sulphur-oxidising bacterium is often present in bioheaps, as studied by Demergasso et al. (2005), Goebel & Stackebrandt (1994), Halinen et al. (2012), and Halinen et al. (2009a). At high heap temperatures, *Leptospirillum* species might become dominating over *At. ferrooxidans* (Bowei et al. 2009; Halinen et al. 2009b). *At. thiooxidans* was the only bacterial species found in the very acidic (pH 1.6) PL6, and the bacterium has been found to have very limited bioleaching capabilities as a pure culture (Akcil et al. 2007). *At. caldus*, *Acidiferrobacter thiooxydans*, *Leptospirillum* species (*L. ferrodiazotrophum*, *L. ferriphilum*), *Sulfobacillus* species (*S. acidophilus*, *S. benefaciens*), and *Alicyclobacillus pomorum* were also found in different process liquors. *At.*

caldus has, for example, been extensively found in a sulphur-incubated sludge bioleaching process at a pH range from 1 to 2, indicating that *At. thiooxidans* may not be the key microorganism in sulphur oxidation (Bouchez et al. 2006). Similar microbial bioheap communities have been reported in several studies (Bowei et al. 2009; Goebel and Stackebrandt 1994; Halinen et al. 2012; Halinen et al. 2009a; He et al. 2008). Zhou et al. (2010) studied a low pH (1.8), high SO_4^{2-} (52 g/L), high Fe (82 g/L), and high arsenide (21 g/L) AMD sample and found that it was dominated by *At. ferrooxidans* and *L. ferrooxidans*. Demergasso et al. (2005) found variation in dominant species during different heap operation periods, indicating that the microbial community in Talvivaara leaching process might be different if sampled at several time intervals.

Despite the demanding growth conditions (low pH, high SO_4^{2-} and Fe^{2+} concentrations), the microbial community in PL4 was diverse. Several studies have shown that mixed cultures (two or more different bacterial cultures) are more efficient at leaching than pure cultures (Akcil et al. 2007; Fu et al. 2008; Xia et al. 2008; Zhang et al. 2008). However, Fu et al. (2008) found that mixed cultures of moderate thermophiles (*L. ferriphilum* and *At. caldus*) were more effective in leaching than pure or mixed cultures of mesophilic *At. ferrooxidans*. Effective mineral degradation requires active sulphur oxidation in addition to iron-oxidising activities (Akcil et al. 2007; Fu et al. 2008; Zhang et al. 2008). This is due to the fact that iron-oxidisers, such as *At. ferrooxidans*, are responsible for Fe^{3+} production and maintaining high redox potential, whereas sulphur oxidising microorganisms (i.e. *At. thiooxidans*) eliminate the passivation of elemental sulphur on mineral surface and provide the process with protons (Xia et al. 2008). Recent studies (Zhou et al. 2009; Zhu et al. 2011) have demonstrated that the formation of jarosite or $PbSO_4$ on mineral surface is the main passivation component, not elemental sulphur. The formation of leaching products is nonetheless accelerated and thus, microbial activity and leaching rates are increased (Zhu et al. 2011). Since several iron- and sulphur-oxidising microorganisms were indigenously present in Talvivaara heap leaching process, it can be hypothesised that by modifying their growth conditions, the bioleaching process could be improved.

8 CONCLUSIONS

The following conclusions can be drawn from this study:

1. Iron oxidation in PL4 is nitrogen limited and thus, nitrogen supplementation to PL4 will increase iron oxidation rates and leaching efficiencies in bioheaps. Based on PL4 dilution series, the liquor needs 319 mg/L or less of N.

2. In the presence of 40 - 95 % (v/v) PL4 in MSM, high-rate iron oxidation is achieved.

3. Ferrous iron concentrations up to 16 g/L are not inhibitory for the iron-oxidising bacteria in PL4.

4. Chloride inhibits iron oxidation at concentrations higher than 5 g/L.

5. Increasing Al^{3+} concentrations decrease iron oxidation rates slightly, but even 12 g/L of Al^{3+} does not inhibit iron oxidation.

6. PL3 and PL4 limit iron oxidation more than other process liquors.

7. PL1, PL4, and PL6 limit sulphur oxidation the most, while PL3 and PL5 offer good conditions for sulphur oxidation.

8. Most of the process liquors have diverse bacterial communities. PL4 consists mainly of *At. ferrooxidans, At. thiooxidans, Acidiferrobacter thiooxydans, L. ferrodiazotrophum,* and *Sulfobacillus acidophilus*. Other process liquors also contain *L. ferriphilum, Sulfobacillus benefaciens,* and *Alicyclobacillus pomorum*. PL6 contains only *At. thiooxidans.*

9. Iron-enriched cultures initially consist of *At. ferrooxidans, At. thiooxidans,* and *Acidiphilium* and *Leptospirillum species*. Over time, *At. ferrooxidans* becomes the main species present. *At. ferrooxidans* and *At. thiooxidans* become enriched when the medium is supplemented with elemental sulphur.

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APPENDIX 1: CHEMICALS

Chemical	Manufacturer
$Al_2(SO_4)_3 \cdot \sim 14\ H_2O$	VWR, Belgium
$BaCl_2 \cdot 2\ H_2O$	J.T.Baker, Holland
$Ca(NO_3)_2 \cdot 4\ H_2O$	Merck, Germany
CH_3CO_2H	Merck, Germany
$C_{12}H_8N_2 \cdot H_2O$	Merck, Germany
$C_4H_{11}NO_3$	Sigma-Aldrich, Germany
$CoCl_2 \cdot 6\ H_2O$	Merck, Germany
$CuSO_4 \cdot 5\ H_2O$	Merck, Germany
DAPI	Sigma-Aldrich, Germany
$FeCl_3 \cdot 6\ H_2O$	Merck, Germany
$FeSO_4 \cdot 7\ H_2O$	VWR, Belgium
H_3BO_3	Merck, Germany
HCl	VWR, Belgium
HNO_3 (69 %)	BDH, England
H_2SO_4 (95-97 %)	Merck, Germany
KCl	Merck, Germany
K_2HPO_4	VWR, Belgium
KOH	Merck, Germany
$MgSO_4 \cdot 7\ H_2O$	Merck, Germany
$MnSO_4 \cdot 4\ H_2O$	Merck, Germany
NaCl	J.T.Baker, Holland
Na_2CO_3	J.T.Baker, Holland
$NaHCO_3$	VWR, Belgium
$Na_2MoO_4 \cdot 2\ H_2O$	J.T.Baker, Holland
NaOH	Merck, Germany
Na_2SeO_4	Sigma-Aldrich, Germany
$Na_2SO_4 \cdot 10\ H_2O$	Merck, Germany
$NH_4C_2H_3O_2$	VWR, Belgium
$(NH_4)_2SO_4$	Merck, Germany
S^0	VWR, Belgium
Thymol	Sigma-Aldrich, Germany
$ZnSO_4 \cdot 7\ H_2O$	Merck, Germany

APPENDIX 2: BACTERIAL COMMUNITY ANALYSIS

[a]	Sample	Band[b] (sequence length)	Closest relatives[c] (accession number)	Similarity[d] (%)
A	Process liquor 4	**9** (520), **12** (472)	*Acidithiobacillus ferrooxidans* (JF815498.1)	100, 96
		10 (496)	*Acidithiobacillus ferrooxidans* (JN224813.1)	100
		11 (466)	*Acidithiobacillus thiooxidans* (DQ676508.1)	99
			Acidithiobacillus albertensis (GQ254658.1)	
			Acidithiobacillus ferrooxidans (FJ194542.1)	
			Thiobacillus sp. (X98210.1)	
		13 (480), **14** (512)	*Acidiferrobacter thiooxydans* (AF387301.2)	95, 99
		15 (419), **60** (491)	*Uncultured bacterium clone* (HQ322958.1)	99
			Leptospirillum ferrodiazotrophum (JN007036.1)	96, 95
		16 (387)	*Leptospirillum ferriphilum* (JF895819.1)	99
			Leptospirillum ferrooxidans (EF015576.1)	
		17 (489)	*Sulfobacillus acidophilus* (AF507964.1)	96
		61 (480)	*Leptospirillum ferriphilum* (EF025342.1)	94
			Leptospirillum ferrooxidans (X72852.1)	
B	Sulphur-oxidising enrich-ment culture	**20** (520)	*Acidithiobacillus thiooxidans* (DQ676508.1)	100
		21 (464)	*Acidithiobacillus thiooxidans* (DQ676508.1)	99
			Acidithiobacillus albertensis (GQ254658.1)	
			Acidithiobacillus ferrooxidans (FJ194542.1)	
			Thiobacillus sp. (X98210.1)	
C	Iron-oxidising enrichment culture 1	**22** (520), **62** (496)	*Acidithiobacillus ferrooxidans* (JF815498.1)	100
D	Iron-oxidising enrichment culture 2	**45** (520)	*Acidithiobacillus ferrooxidans* (JF815498.1)	100
		48 (460)	*Acidiphilium multivorum* (AP012035.1)	91

[a]	Sample	Band[b] (sequence length)	Closest relatives[c] (accession number)	Similarity[d] (%)
			Acidiphilium cryptum (FJ915152.1)	
			Acidiphilium organovorum (NR 025853.1)	
		52 (496), **53** (512)	*Acidithiobacillus ferrooxidans* (JF815498.1)	99, 100
E	Process liquor 1	**72** (439)	*Acidithiobacillus caldus* (CP002573.1)	99
			Thiobacillus caldus (AF137369.1)	
			Thermus sp. (EU368984.1)	
		73 (403)	*Uncultured Nitrospirae bacterium* (GU120604.1)	99
			Leptospirillum ferrodiazotrophum (JN007036.1)	96
		74 (451)	*Leptospirillum ferriphilum* (DQ646517.1)	91
		75 (488), **78** (473)	*Acidithiobacillus ferrooxidans* (FJ913262.1)	99, 97
		77 (462)	*Acidithiobacillus ferrivorans* (CP002985.1)	99
			Acidithiobacillus ferrooxidans (FN686788.1)	
		79 (452)	*Acidithiobacillus ferrooxidans* (JN224813.1)	99
F	Process liquor 2	**65** (463)	*Acidithiobacillus ferrivorans* (CP002985.1)	99
			Acidithiobacillus ferrooxidans (FN686788.1)	
		67 (457)	*Leptospirillum ferrooxidans* (GU168016.1)	98
			Leptospirillum ferriphilum (GU168013.1)	
		68 (417), **69** (455), **70** (493),	*Uncultured Alicyclobacillaceae bacterium* (FN870323.1)	95, 96, 96,
		71 (449)		96
G	Process liquor 3	**53** (509), **54** (464), **63** (464)	*Acidithiobacillus ferrivorans* (CP002985.1)	99
			Acidithiobacillus ferrooxidans (FN686788.1)	
		55 (462)	*Acidithiobacillus ferrooxidans* (JN224813.1)	100
		56 (441)	*Acidiferrobacter thiooxydans* (AF387301.2)	99
		57 (450)	*Acidithiobacillus ferrooxidans* (AM502930.1)	99
		58 (462)	*Leptospirillum ferriphilum* (EF025342.1)	100
		61 (461)	*Sulfobacillus sp.* (AJ306701.1)	97

[a]	Sample	Band[b] (sequence length)	Closest relatives[c] (accession number)	Similarity[d] (%)
			Sulfobacillus acidophilus (AF507964.1)	95
		62 (464)	*Sulfobacillus benefaciens* (EU495236.1)	99
H	Process liquor 5	**38** (472), **39** (475), **41** (517), **42** (474), **43** (476), **44** (471), **45** (497)	*Acidithiobacillus ferrivorans* (CP002985.1) *Acidithiobacillus ferrooxidans* (FN686788.1)	99, 99, 99, 99, 98, 99, 99
		47 (456)	*Alicyclobacillus pomorum* (AB681267.1)	98
		48 (414)	*Alicyclobacillus pomorum* (AB681267.1) *Alicyclobacillus ferrooxydans* (NR 044413.1)	97
		50 (451)	*Leptospirillum ferriphilum* (DQ646517.1) *Leptospirillum ferrooxidans* (X72852.1)	88
		51 (438)	*Uncultured bacterium* (FR683053.1)	95
I	Process liquor 6	**37** (463)	*Acidithiobacillus thiooxidans* (JQ034367.1)	100
		40 (505)	*Acidithiobacillus thiooxidans* (JQ034367.1) *Acidithiobacillus albertensis* (GQ254658.1) *Acidithiobacillus ferrooxidans* (FJ194542.1) *Thiobacillus sp.* (X98210.1)	99
J	Iron-oxidising enrichment culture 3	**31** (498)	*Acidithiobacillus ferrooxidans* (JF815498.1)	100
		32 (442)	*Acidiphilium multivorum* (AP012035.1) *Acidiphilium cryptum* (FJ915152.1) *Acidiphilium organovorum* (NR 025853.1) *Acidithiobacillus sp.* (HM769772.1)	100
		33 (430)	*Leptospirillum ferriphilum* (JF895819.1) *Leptospirillum ferrooxidans* (EF015576.1)	99
		34 (463)	*Uncultured Sinobacteraceae bacterium* (HQ730615.1)	99
		35 (484), **36** (437)	*Actinobacterium sp.* (EF441939.1) *Acidimicrobium sp.* (EF612360.1)	100

[a]	Sample	Band[b] (sequence length)	Closest relatives[c] (accession number)	Similarity[d] (%)
			Ferromicrobium acidophilum (AJ517364.1)	
			Ferrimicrobium acidiphilum (FN870326.1)	

[a]) Letter in Figure 6.14.

[b]) DGGE-separated DNA fragment.

[c]) NCBI MegaBLAST search results for closest relatives of DGGE-separated DNA fragments.

[d]) Similarity of the DGGE-separated DNA fragment and its closest relatives.