Tampere University

Linda Määttä

EFFECTS OF BATTERY CHEMICAL PROCESS LIQUORS ON HEAP BIO-LEACHING MICROORGANISMS

Master of Science Thesis
Faculty of Engineering and Natural Sciences
Examiners: Professor Jaakko Puhakka
& M.Sc. (Tech.) Réka Hajdu-Rahkama
June 2021

ABSTRACT

Linda Määttä: Effects of Battery Chemical Process Liquors on Heap Bioleaching Microorganisms
Master of Science Thesis
Tampere University
Master's Degree Program in Environmental and Energy Engineering
June 2021

During coming thirty years, the quantity of electric vehicles will increase by eight-fold according to International Energy Agency. For the batteries of electric vehicles, metal-based battery chemicals, such as nickel and cobalt sulfate, are used as a raw material, therefore, increasing quantities of these chemicals will be needed in near future. In nickel and cobalt sulfate production process, ammonium sulfate is formed as a side product when ammonium-rich process liquors, containing ammonium sulfate, are associated with the process. These ammonium-rich side streams are a potent source of nitrogen for heap bioleaching. Therefore, in this thesis, possible negative and positive effects of ammonium sulfate (AS) bleed and feed (ammonium-rich side streams, originating from the battery chemical production process) and their constituents on biological iron and sulfur oxidation was under investigation.

The experiments of this study were performed as small-scale batch studies (shake flask). The enriched indigenous acidophilic microorganisms, present in the irrigating leach liquor obtained from a heap bioleaching plant of Terrafame Oy., were used as an inoculum. The effects of battery chemical process liquors: AS bleed and feed, and their constituents (ammonium sulfate, carboxylic acid (neodecanoid acid), Nessol D100, Cyanex 272, and Baysolvex D2EHPA), on biological iron and sulfur oxidation were investigated separately. In addition to these experiments, possible stimulatory effect of ammonium-rich AS bleed and feed, as nitrogen supplement, on biological iron oxidation was also studied.

Both AS bleed and feed process liquors negatively affected the biological iron oxidation. The process liquors resulted in a lag phase in biological iron oxidation and decrease of iron oxidation rate at concentrations higher than 2% (v/v), whereas at AS bleed and feed concentrations of 20% (v/v) and 50% (v/v), respectively, the biological iron oxidation was completely and irreversibly inhibited. With AS bleed, it was recognized that AS bleed enhanced biological sulfur oxidation at concentration of 8% (v/v) and below. The highest sulfate production yield and rate of 27% and 0.38 g/L/d, respectively, were achieved with AS bleed concentration of 4% (v/v). Ammonium sulfate concentrations higher than 20 g/L resulted in a lag phase in biological iron oxidation, whereas 250 g/L resulted in full and irreversible inhibition. Of the organic solvents (neodecanoid acid, Nessol D100, Cyanex 272, and Baysolvex D2EHPA) only neodecanoid acid affected biological iron oxidation. Neodecanoid acid resulted in a lag phase in iron oxidation with concentration of 2.5% of its water solubility (6.3 mg/L) or higher. Organic solvents Nessol D100, Cyanex 272, and Baysolvex D2EHPA were nonbioavailable for the microbial culture in experimental conditions of this study and, therefore, did not affect biological iron oxidation. In ammonium deficiency experiment, 0.079 g/L ammonium supplementation with 0.1% (v/v) AS feed, and 0.11 g/L ammonium supplementation with 0.09% (v/v) AS bleed enhanced biological iron oxidation. With 1% (v/v) AS bleed and feed concentration, the iron oxidation rate was not significantly increased. Iron oxidation rate of iron oxidizing culture, incubated in ammonium deficit medium, was 2.0 g/L/d, whereas iron oxidation rates of cultures supplemented with AS bleed or feed were over 2.3 g/L/d.

In conclusion, ammonium-rich process liquors have a potential to enhance bioleaching, however, potential nitrogen deficiency in the heap bioleaching liquors (of Terrafame) should be studied to estimate the actual demand for nitrogen supplementation. Furthermore, the battery chemical process liquors and some of their constituents may potentially inhibit heap bioleaching process. Ammonium and organic solvents, present in the process liquors, can result in drastic harmful effects in recipient waters, therefore, their release to the environment must be prevented.

Keywords: battery chemical, bioleaching microorganism, heap bioleaching, inhibition, iron oxidation, nitrogen supplement, organic solvent, sulfur oxidation

The originality of this thesis has been checked using the Turnitin OriginalityCheck service.

TIIVISTELMÄ

Linda Määttä: Akkukemikaaliprosessiliuosten vaikutukset bioliuotusmikrobeihin
Diplomityö
Tampereen yliopisto
Ympäristö- ja energiatekniikan Di-tutkinto-ohjelma
Kesäkuu 2021

Kansainvälisen energiajärjestön (IEA) mukaan sähköautojen määrä kahdeksankertaistuu tulevan kolmenkymmenen vuoden aikana. Sähköautoakkujen raaka-aineisiin lukeutuvat esimerkiksi nikkeli- ja kobalttisulfaatti, jonka vuoksi lähitulevaisuudessa tullaan tarvitsemaan yhä suurempia määriä kyseisiä kemikaaleja. Nikkeli- ja kobalttisulfaatin valmistusprosessissa ammoniumsulfaattia muodostuu sivutuotteena, jolloin prosessiin liittyy korkeita ammoniumpitoisuuksia sisältäviä prosessiliuoksia. Nämä sivuvirrat ovat potentiaalinen typenlähde kasabioliuotukseen. Tämän vuoksi työssä tutkittiin Terrafame Oy:n akkukemikaalitehtaan prosessista peräisin olevien ammoniumsulfaattisivuvirtojen (AS bleedin ja feedin) mahdollisia kielteisiä ja myönteisiä vaikutuksia biologiseen raudan- ja rikinhapetukseen.

Tämän työn kokeellinen osuus suoritettiin laboratoriomittakaavan panoskokeina (ravistelupullo). Kokeissa käytettiin Terrafamen kasabioliuotusalueen kastelunesteestä rikastettuja raudan- ja rikinhapetusbakteerien viljelmiä. Työssä tutkittiin akkukemikaalitehtaan prosessiliuoksien (AS bleed ja feed), ja niiden sisältämien yhdisteiden: ammoniumsulfaatin, karboksyylihapon (neodekanoidihapon), Nessol D100:n, Cyanex 272:n ja Baysolvex D2EHPAn vaikutuksia biologiseen raudan- ja rikinhapetukseen. Työssä tutkittiin myös biologisen raudanhapetuksen mahdollista tehostamista runsaasti ammoniumioneja sisältävien AS bleed ja feed -liuoksia käyttäen.

AS bleed ja feed -liuokset vaikuttivat negatiivisesti biologiseen raudanhapetukseen. Molemmat prosessiliuokset aiheuttivat viiveen raudanhapetuksen käynnistämisessä ja alensivat raudanhapetusnopeutta yli 2 % (v/v) pitoisuuksissa, kun taas 20 % (v/v) AS bleed ja 50 % (v/v) AS feed pitoisuudet inhiboivat raudanhapetuksen kokonaan ja peruuttamattomasti. AS bleedin kanssa toteutetussa rikinhapetuskokeessa havaittiin, että 8 % (v/v) ja sitä alemmilla pitoisuuksilla AS bleed tehosti biologista rikinhapetusta. Korkein sulfaatin saanto (27 %) ja tuottonopeus (0,38 g/L/d) saavutettiin 4 % (v/v) AS bleed-pitoisuudella. Yli 20 g/L ammoniumsulfaattipitoisuus aiheutti viiveen raudanhapetuksessa, kun taas 250 g/L pitoisuus inhiboi mikrobien raudanhapetusaktiivisuuden kokonaan ja peruuttamattomasti. Orgaanisista uuttoliuoksista (neodekanoidihapon, Nessol D100:n, Cyanex 272:n ja Baysolvex D2EHPAn) neodekanoidihappo oli ainoa, joka vaikutti biologiseen raudanhapetukseen. Neodekanoidihappo aiheutti viiveen raudanhapetuksessa 6,3 mg/L pitoisuudessa (2,5 % vesiliukoisuudesta). Tämän työn tutkimusolosuhteissa orgaanisista uuttoliuoksista Nessol D100, Cyanex 272 ja Baysolvex D2EHPA eivät olleet biologisesti saatavilla, eivätkä vaikuttaneet biologiseen raudanhapetukseen. Ammoniumrajoitteisella raudanhapetusviljelmällä biologinen raudanhapetusaktiivisuus tehostui 0,1 % (v/v) AS feed lisäyksellä (ammoniumpitoisuus 0,079 g/L), ja 0,09 % (v/v) AS bleed lisäyksellä (ammoniumpitoisuus 0,11 g/L). AS bleed tai feed -pitoisuuden nostaminen 1 % (v/v) ei parantanut merkittävästi raudanhapetusnopeutta. Ammoniumrajoitteisen raudanhapetusviljelmän raudanhapetusnopeus oli 2,0 g/L/d ja AS bleed ja feed -liuoksia sisältävissä viljelmissä raudanhapetusnopeus oli yli 2,3 g/L/d.

Työn johtopäätöksenä runsaasti ammoniumioneja sisältävät prosessiliuokset voivat tehostaa bioliuotusta. Jatkotutkimuksissa tulee selvittää Terrafamen bioliuotuskasojen prosessivesien typpipitoisuudet, prosessin typpirajoitteisuuden vahvistamiseksi. Akkukemikaaliprosessiliuokset ja niiden sisältämät yhdisteet voivat myös inhiboida kasabioliuotusprosessia. Prosessiliuosten sisältämät ammoniumionit ja orgaaniset uuttoliuokset voivat aiheuttaa vakavia ympäristöhaittoja purkuvesistöissä ja niiden päästäminen ympäristöön tulee estää.

Avainsanat: akkukemikaali, bioliuotusmikrobi, inhibitio, kasabioliuotus, orgaaninen uuttoliuos, raudan hapetus, rikin hapetus, typpilisä

Tämän julkaisun alkuperäisyys on tarkastettu Turnitin OriginalityCheck –ohjelmalla.

PREFACE

The experimental part of this Master of Science thesis was carried out in the Laboratory of Chemistry and Bioengineering at Hervanta campus of Tampere University. I wish to thank multi-metal company Terrafame Oy for funding and providing this work.

I would like to express my deepest appreciation for my supervisors Professor Jaakko Puhakka and M.Sc. (Tech.) Réka Hajdu-Rahkama. Thanks for Jaakko for giving me the opportunity to work in this truly interesting project and giving me excellent advice throughout this project. I want to thank Réka for endlessly guiding and supporting me throughout this project and especially helping me with all the possible problems and difficulties that I encountered during this project.

I want to sincerely thank Dr. Carita Oinonen from Terrafame for effortless and pleasant cooperation and being always available for all the possible questions. I also want to thank staff of the Laboratory of Terrafame for analyzing the DOC samples and especially Department Manager of the Laboratory Jonna Jokiniemi for answering all my questions.

Then for last but not least I want to thank my family and friends for supporting me throughout this process, and especially my boyfriend for extra support and showing me the way of being more laid-back version of myself also during this huge project.

Tampere, 11 June 2021

Linda Määttä

CONTENTS

1. INTRODUCTION 1
2. BIOLEACHING PRINCIPLES AND APPLICATIONS 3
   2.1 Bioleaching mechanisms of sulfide minerals 3
   2.2 Microorganisms associated with bioleaching 5
   2.3 Heap bioleaching 7
   2.4 Mining and metal recovery process of Terrafame 9
3. SUBSTANCES INFLUENCING BIOLEACHING 10
   3.1 Inhibition of bioleaching by organic solvents 10
   3.2 Enhancement of bioleaching by chemical additives 14
      3.2.1 Nitrogen requirement of bioleaching microorganisms 14
      3.2.2 Nitrogenous nutrient additives 15
      3.2.3 Polymeric extracts 19
      3.2.4 Surfactants 23
      3.2.5 EPS inducing additives 41
      3.2.6 Other bioleaching enhancing additives 45
4. MATERIALS AND METHODS 48
   4.1 Microbial cultures 48
      4.1.1 Iron oxidizing cultures 49
      4.1.2 Sulfur oxidizing enrichment culture 51
   4.2 Growth medium 51
   4.3 Process liquors and their constituents 52
      4.3.1 Process liquors of the battery chemical production plant 52
      4.3.2 Constituents of the process liquors 54
   4.4 Experimentation 55
      4.4.1 Experiments with AS bleed 60
      4.4.2 Experiment with AS Feed 60
      4.4.3 Experiments with AS 60
      4.4.4 Experiments with neodecanoid acid 61
      4.4.5 Experiments with Nessol D100 62
      4.4.6 Experiment with Cyanex 272 63
      4.4.7 Experiment with Baysolvex D2EHPA 63
      4.4.8 Ammonium Deficiency Experiment 64
   4.5 Sampling 64
   4.6 Analytical methods 67
      4.6.1 Measurement of pH and redox 67
      4.6.2 Measurement of Fe(II) concentration 68
      4.6.3 Ammonium analysis 68
      4.6.4 Sulfate analysis 69
      4.6.5 DOC analysis 69
5. RESULTS 71
   5.1 AS bleed experiments 71

5.1.1 AS bleed 1st inhibition experiment 71
5.1.2 AS bleed 2nd inhibition experiment 73
5.1.3 AS bleed inhibition experiment with sulfur oxidizing culture 75
5.2 AS feed inhibition experiment 78
5.3 AS experiments 80
5.3.1 AS 1st inhibition experiment 80
5.3.2 AS 2nd inhibition experiment 82
5.4 Neodecanoid acid experiments 85
5.4.1 Neodecanoid acid 1st inhibition experiment 85
5.4.2 Neodecanoid acid 2nd inhibition experiment 89
5.4.3 Neodecanoid acid kinetics experiment 91
5.5 Nessol D100 experiments 93
5.5.1 Nessol D100 1st inhibition experiment 93
5.5.2 Nessol D100 2nd inhibition experiment 95
5.6 Cyanex 272 inhibition experiment 97
5.7 Baysolvex D2EHPA inhibition experiment 99
5.8 Ammonium deficiency experiment with AS bleed and feed 101
6. DISCUSSION 107
6.1 Influence of AS bleed and feed on iron and sulfur oxidation 107
6.2 Inhibitory effects of organic compounds on bioleaching process 109
6.3 Potential of enhancing bioleaching by ammonium-rich process liquors 111
6.4 Environmental effects of residual nitrogen and organic solvents 114
7. CONCLUSIONS 117
REFERENCES 120
APPENDIX 1: CHEMICALS 130

LIST OF FIGURES

Figure 1. *Indirect leaching sub-mechanisms. Top-down sub-mechanisms: non-contact, contact, and cooperative mechanism. (modified from Tributsch 2001)* *5*

Figure 2. *Rod-shaped bacteria, observed from the (a) iron and (b) sulfur oxidizing enrichment cultures. The rod-shaped bacteria are showed in a red circle. Pictures were taken with Axioskop 2 microscope (ZEISS Group, Germany) equipped with N-Achroplan 100x/1.25 oil pH 3 M27 objective and digitalized camera system, magnification 1000x.* *6*

Figure 3. *Terrafame overall mining and metal recovery process (modified from Oinonen 2020).* *9*

Figure 4. *Chemical structures of the studied anionic surfactants, drawn with ChemSketch (Flick 1993; Pubchem 2021A; Sigma-Aldrich 2021A; Thermo Fischer Scientific 2021).* *26*

Figure 5. *Chemical structures of the known cationic surfactants, drawn with ChemSketch (Sigma-Aldrich 2003; Pubchem 2021B).* *30*

Figure 6. *Chemical structures of the studied nonionic surfactants, drawn with ChemSketch (BASF 2003; BASF 2008; Johnson 2013; Sigma-Aldrich 2021B; Sigma-Aldrich 2021C; Sigma-Aldrich 2021D).* *35*

Figure 7. *Various microbial processes affected by extracellular polymeric substances (EPS). Spotted layer covering the microbial cell presents EPS. (modified from Tourney & Ngwenya 2014)* *41*

Figure 8. *Chemical structure of ETU, drawn with ChemSketch (Ren et al. 2020).* *46*

Figure 9. *Diagram of the microbial cultures used in the experiments. The arrows indicate the source of the inoculum. AD in the iron oxidizing AD culture 1 and 2, is an abbreviation for ammonium deficit.* *49*

Figure 10. *Schematic diagram of the battery chemical production plant process (modified from Terrafame 2018).* *53*

Figure 11. *Chemical structures of the studied solvents used in the Terrafame's metal extraction processes, drawn with ChemSketch (ExxonMobil 2018; LANXESS 2018; Neste 2019; Solvay 2019).* *54*

Figure 12. *Two incubators used during the experiments: (a) KS 4000 i Control incubator (IKA, Germany) with a plastic hood and local exhaust ventilation, and (b) larger Innova 44 incubator (New Brunswick Scientific, US) without a plastic hood.* *56*

Figure 13. *Effect of AS bleed concentration (0.1–50% (v/v)) on biological iron oxidation by iron oxidizing enrichment culture. (a) Fe(II) concentration, (b) redox potential, and (c) pH. The standard deviations are presented with the error bars (n=2).* *72*

Figure 14. *Effect of AS bleed 10% (v/v) concentration on biological iron oxidation during first and second incubation. The second incubation was performed with the enrichment culture obtained from the first incubation. The standard deviations are presented with the error bars (n=2).* *73*

Figure 15. *Influence of AS bleed concentration (4–40% (v/v)) on (a) Fe(II) concentration, (b) redox potential, and (c) pH during biological iron oxidation by iron oxidizing enrichment culture. The standard deviations are presented with the error bars (n=2).* *74*

Figure 16. *Effect of AS bleed concentration (4–40% (v/v)) on ammonium concentration and utilization during iron oxidation by the iron oxidizing enrichment culture. Last sampling days: day 3 with AS*

bleed 4%, day 4 with AS bleed 8%, and day 11 with AS bleed 20–40%. The standard deviations are presented with the error bars (n=2). *75*

Figure 17. *Sulfate production from elemental sulfur during the enrichment of the sulfur oxidizing culture. (a) initial culture and (b) culture after the first transfer to fresh medium. The standard deviations are presented with the error bars (n=2).* *76*

Figure 18. *Effect of AS bleed on biological sulfur oxidation by sulfur oxidizing enrichment culture. (a) pH, (b) sulfate concentration and (c) overall sulfate production rate and the highest sulfate production yield. The overall sulfate production rates were calculated from the slope of the linear regression line of the overall sulfate production curves. The standard deviations are presented with the error bars (n=2).* *77*

Figure 19. *Effect of AS feed concentration (0.1–50% (v/v)) on (a) Fe(II) concentration, (b) redox potential, and (c) pH, during iron oxidation by iron oxidizing enrichment culture. Standard deviations are presented with the error bars (n=2).* *79*

Figure 20. *Influence of AS feed concentration (0.1–50% (v/v)) on ammonium concentration and utilization during iron oxidation by the iron oxidizing enrichment culture. Last sampling days: day 3 with AS feed 4–10%, and day 14 with AS feed 50%. The standard deviations are presented with the error bars (n=2).* *80*

Figure 21. *Influence of ammonium sulfate (AS) concentration (4.3–250 g/L) on biological iron oxidation by iron oxidizing enrichment culture. (a) Fe(II) concentration, (b) redox potential, and (c) pH. The standard deviations are presented with the error bars (n=2).* *81*

Figure 22. *Influence of ammonium sulfate (AS) concentration (4.3–250 g/L) on ammonium concentration and utilization during iron oxidation by the iron oxidizing enrichment culture. Last sampling days: day 3 with 4.3–15 g/L AS, day 14 with 59 g/L AS, and day 18 with 250 g/L AS. The standard deviations are presented with the error bars (n=2).* *82*

Figure 23. *Impact of ammonium sulfate (AS) concentration (9.3–53 g/L) on (a) Fe(II) concentration, (b) redox potential, and (c) pH, during biological iron oxidation by iron oxidizing enrichment culture. The standard deviations are presented with the error bars (n=2).* *84*

Figure 24. *Effect of ammonium sulfate (AS) concentration (9.3–53 g/L) on ammonium concentration and utilization during iron oxidation by the iron oxidizing enrichment culture. Last sampling days: day 2 with 20 g/L AS, day 3 with 9.3 g/L and 31 g/L AS, day 7 with 42 g/L AS, and day 10 with 53 g/L AS. The standard deviations are presented with the error bars (n=2).* *85*

Figure 25. *Effect of neodecanoid acid concentration (5–100% of its water solubility) on biological iron oxidation by the iron oxidizing enrichment culture. (a) Fe(II) concentration, (b) redox potential, and (c) pH. The standard deviations are presented with the error bars (n=2).* *87*

Figure 26. *Effect of neodecanoid acid concentration of 100% of its water solubility on biological iron oxidation during incubation without neodecanoid acid. The incubation was performed with the enrichment culture obtained from the first incubation in neodecanoid acid 1st inhibition experiment. Samples were taken daily from the positive control. Standard deviations are presented with the error bars (n=2).* *88*

Figure 27. *Influence of neodecanoid acid concentration (1–5% of its water solubility) on (a) Fe(II) concentration, (b) redox potential, and (c) pH, during biological iron oxidation by the iron oxidizing enrichment culture. The standard deviations are presented with the error bars (n=2).* *90*

Figure 28. *Influence of neodecanoid acid concentration (1–5% of its water solubility) on DOC concentration, during biological iron oxidation by the iron oxidizing enrichment culture. The standard deviations are presented with the error bars (n=2).* *91*

Figure 29. *Effect of neodecanoid acid concentration of 50% of its water solubility on biological iron oxidation by the iron oxidizing enrichment culture. (a) Fe(II) concentration, (b) redox potential, and (c) pH. The error bars present the standard deviation (n=4 on day 0, 1, 25, and 28, n=2 on other days).* *92*

Figure 30. *Influence of neodecanoid acid concentration of 50% of its water solubility on DOC concentration, during iron oxidation by the iron oxidizing enrichment culture. The standard deviations are presented with the error bars (n=2).* *93*

Figure 31. *Influence of Nessol D100 concentration (1–15% of its water solubility) on (a) Fe(II) concentration, (b) redox potential, and (c) pH, during the biological iron oxidation by the iron oxidizing enrichment culture. The standard deviations are presented with the error bars (n=2).* *94*

Figure 32. *The separate liquid phase on top of the Nessol D100 B 15% of its water solubility medium in experimental day of 2. The separate liquid phase is shown with red arrows.* *95*

Figure 33. *Effect of Nessol D100 concentrations of 30% and 100% of its water solubility on during the biological iron oxidation by the iron oxidizing enrichment culture. (a) Fe(II) concentration, (b) redox potential, and (c) pH. The standard deviations are presented with the error bars (n=2).* *96*

Figure 34. *(a) Fe(II) concentration, (b) redox potential, and (pH), during biological iron oxidation by the iron oxidizing enrichment culture, when different Cyanex 272 concentration (5–100% of its water solubility) were present. The standard deviations are presented with the error bars (n=2).* *98*

Figure 35. *White precipitate on top of the Cyanex 272 A 40% of its water solubility medium in experimental day 0. The white precipitate is shown with red arrows.* *99*

Figure 36. *Influence of various Baysolvex D2EHPA concentration (5–100% of its water solubility) on (a) Fe(II) concentration, (b) redox potential, and (c) pH, during the iron oxidation by the iron oxidizing enrichment culture. The standard deviations are presented with the error bars (n=2).* *100*

Figure 37. *White precipitate on top of the Baysolvex D2EHPA A 10% of its water solubility medium in experimental day of 0.* *101*

Figure 38. *Influence of ammonium deficiency on iron oxidation by the iron oxidizing ammonium deficit (AD) culture 2, during (a) pre-cultivation, (b) 1st incubation, (c) 2nd incubation, (d) 3rd incubation, (e) 4th incubation, and (f) 5th incubation. The standard deviations are presented with the error bars (n=2).* *103*

Figure 39. *Effect of AS bleed on (a) Fe(II) concentration, (b) redox potential, and (c) pH, and AS feed on (d) Fe(II) concentration, (e) redox potential, and (f) pH, during iron oxidation of the iron oxidizing ammonium deficit (AD) culture. Inoculum used in positive control,*

was iron oxidizing culture supplemented with ammonium (iron oxidizing AD culture 1). Inoculum used with other controls and experiment flasks was iron oxidizing culture without ammonium supplementation (iron oxidizing AD culture 2). The standard deviations are presented with the error bars (n=2). *105*

Figure 40. *Influence of (a) AS bleed concentration (0.09–1% (v/v)) and (b) AS feed concentration (0.1–1% (v/v)) on ammonium concentration during iron oxidation by iron oxidizing ammonium deficit (AD) culture. Inoculum used in positive control, was iron oxidizing culture supplemented with ammonium (iron oxidizing AD culture 1). Inoculum used with other controls and experiment flasks was iron oxidizing culture without ammonium supplementation (iron oxidizing AD culture 2). The last sampling days: day 3 with positive control, 0.11 g/L ammonium control and AS bleed 0.9%, and day 4 with AD control, AS bleed 0.1% and 1%, and AS feed 0.1% and 1%). The standard deviations are presented with the error bars (n=2).* *106*

Figure 41. *Influence of AS bleed and feed concentration (0.1–50% (v/v)) on biological iron oxidation rate. The iron oxidation rates were calculated from the slope of the linear regression line of the exponential part of the Fe(II) oxidation curves (see figures 13 and 19). The standard deviations are presented with the error bars (n=2).* *107*

Figure 42. *Influence of (a) AS bleed concentration (0.09–1.0 % (v/v)) and (b) AS feed concentration (0.1–1% (v/v)) on iron oxidation rate during iron oxidation by the iron oxidizing ammonium deficit (AD) culture. Iron oxidizing AD culture was cultivated without ammonium supplementation. Iron oxidation rates were calculated from the slope of the linear regression line of the exponential part of the Fe(II) oxidation curves (see figure 39). The standard deviations are presented with the error bars (n=2).* *112*

LIST OF SYMBOLS AND ABBREVIATIONS

AD	ammonium deficit (without any source of ammonium)
AS	ammonium sulfate
ConA	concanavalin A
CLSM	confocal laser scanning microscopy
CMBR	ceramic membrane bioreactor
CMC	critical micelle concentration
CPS	capsular polysaccharides
CUR	carbon dioxide uptake rate
CTMAB	cetyltrimethylammonium bromide
DAP	diamidophosphate
DOC	dissolved organic carbon
EPS	extracellular polymeric substances
ETU	ethylene thiourea
HRT	hydraulic retention time
IC	ion chromatography
MSM	mineral salts medium
MGV	mean gray value
NDIR	non-dispersive infrared
NOEC	no observed effect concentration
NPOC	non-purgeable organic carbon
OPD	O-phenylenediamine
ORP	oxidation-reduction potential
PEG	polyethylene glycol
PLS	pregnant leach solution
SDBS	sodium dodecylbenzenesulfonate
SIBX	sodium isobutyl-xanthate
TES	trace elements solution
TIC	total inorganic carbon
TOC	total organic carbon
TSB	tryptone soya broth
TU	thiourea
UV-Vis	ultraviolet-visible
VOC	volatile organic compound
v/v	volume/volume
w/v	weight/volume

1. INTRODUCTION

Electric vehicles have been invented in the early 1900s, however, only in recent years they have begun to get more attention as a promising alternative way of transportation. This increasing interest of using electric vehicles instead of vehicles that use fossil fuel is mainly due to their positive impact in controlling global warming. Therefore, it is probable that the quantity of the electric vehicles will significantly increase in the future, especially when the production and driving costs will be reduced and ranges increased. (Ajanovic 2015) For the production of the electric vehicle batteries, metal-based chemicals, such as nickel and cobalt sulfate, are required. In nickel and cobalt sulfate production process of battery chemical production plant, ammonium sulfate (AS), is produced as a side product. (Terrafame 2018)

The battery chemicals can be produced from the mineral processing product of nickel cobalt sulfide. Therefore, it would be beneficial to place the battery chemical production plant next to the metal recovery area of the mineral processing plant. From the economically viable metal recovery options, heap bioleaching is one of the competent alternatives, used also by a Finnish multi-metal company Terrafame Oy at their mine site, located in Sotkamo, Finland (Petersen 2016; Terrafame 2018). In mining industry, bioleaching is a specialized bio-hydrometallurgical process utilizing microorganisms, such as bacteria, archea, and fungi for solubilizing and recovering metals, such as nickel and copper, mainly from low-grade sulfide ores (Rawlings 2007). In heap bioleaching, the leaching by microorganisms occurs in large ore piles, called as bioheaps. The bioheaps are constructed by piling crushed and generally agglomerated ore material (du Plessis *et al.* 2007; Petersen 2016). Since physical composition, such as pore sizes, in bioheaps vary, various gradients, such as O_2 and pH gradients, are present in bioheaps, leading to heterogenous bioleaching efficiency. (du Plessis *et al.* 2007; Petersen & Dixon 2007)

Although heap bioleaching has various advantages, such as simple operation and low costs, compared to the conventional metal recovery processes, one of the major disadvantages of the heap bioleaching is that the duration of the metal extraction is generally long, from months to couple of years (Petersen & Dixon 2007; Petersen 2016). This leaching period can even be longer, if the optimal bioleaching conditions, such as sufficient macronutrient nitrogen availability, are not met. Nitrogen availability in the heap

leaching environment of the sulfide ores is generally insufficient for supporting optimal microbial growth and thereby optimal bioleaching efficiency. Therefore nitrogen has to be artificially supplied, for example as ammonium, to achieve more desirable bioleaching conditions (du Plessis *et al.* 2007; Petersen 2016) Since in the AS crystallization process of the battery chemical production plant, ammonium-rich side streams are formed during the start-up and the operational stage of the unit process, these side stream process liquors could be possible source of ammonium supplement, and thereby enhancing the heap bioleaching process (Niemelä *et al.* 1994; du Plessis *et al.* 2007; Terrafame 2018).

In this Thesis, possible negative and positive effects of AS bleed and feed (side stream process liquors), originated from AS crystallization process of Terrafame battery chemical production plant, on biological iron and sulfur oxidation is studied. Possible inhibition by the process liquors and their constituents, including AS and organic extraction solvents, on biological iron and sulfur oxidation was investigated. In addition to the possible inhibitory effect, possible enhancement of iron oxidation by the process liquors was studied. All the experiments were performed as small-scale batch studies (shake flask) at pH of 2 and temperature of 27± 2 °C. A mixed microbial culture, originating from the irrigation leach solution, provided by Terrafame, was used as an inoculum in the experiments. The aim of the study was to see if the process liquors could be used as an ammonium supplement for the bioleaching microorganisms of the Terrafame metal recovery area.

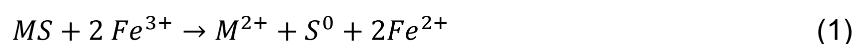
2. BIOLEACHING PRINCIPLES AND APPLICATIONS

Biological metal recovery from the sulfide ores, is based on microbial oxidation of a solid sulfide mineral to a water-soluble metal sulfate by iron and sulfur oxidizing bacteria (Schippers 2007). Oxidation of ferrous iron (Fe(II)) and reduced sulfurous compounds oxidation are the main metabolic functions of the bioleaching process, therefore, bioleaching occurs only with ores that either contain Fe(II) and reduced sulfurous compounds or are closely contacted with some other source of them. (du Plessis *et al.* 2007; Rawlings 2007) From Fe(II), iron oxidizing bacteria form important oxidizing agent: ferric iron (Fe(III)), through its oxidation. Through oxidation of sulfurous compounds, the acidic conditions in the bioleaching environment are maintained. (Rawlings 2007; Schippers 2007)

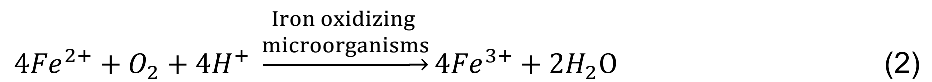
2.1 Bioleaching mechanisms of sulfide minerals

Both chemical and biological reactions are associated with bioleaching. The leaching of sulfide mineral is based on chemical reactions, while formation of Fe(III) and acids (inorganic and organic), required in the bioleaching process, is based on biologically catalyzed reactions. (Rawlings 2007; Schippers 2007) It has been previously proposed that mobilization and leaching of metals from sulfide minerals can occur through direct or indirect mechanisms. In direct mechanism, microorganisms have to be in direct contact with the sulfide mineral to be able to oxidize sulfide to sulfate through enzymatic reactions, whereas in indirect mechanisms, sulfide mineral oxidation occurs via strong oxidizing agent (Fe(III)). Even though, it has been previously proposed that bioleaching can happen either through direct or indirect mechanism, currently, indirect mechanism is the bioleaching mechanism widely accepted. (Kinzler *et al.* 2003; Sand & Gehrke 2006; Schippers 2007)

In indirect mechanism, strong oxidizing agent of Fe(III), oxidizes reduced sulfide minerals (MS), when water-soluble metals (M^{2+}) and elemental sulfur (S^0) are formed. At the same time Fe(III) is reduced to Fe(II). The regenerated Fe(II) is then used again as energy source for iron oxidizing microorganisms. (Rawlings 2007) Oxidation of a sulfide mineral with Fe(III) and biological oxidation of Fe(II) to Fe(III) can be described by using the following equations (Rawlings 2007; Schippers 2007):

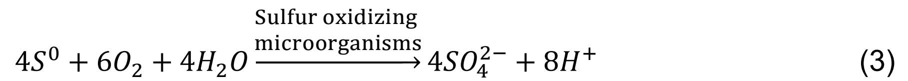


$$MS + 2\,Fe^{3+} \rightarrow M^{2+} + S^0 + 2Fe^{2+} \quad (1)$$



$$4Fe^{2+} + O_2 + 4H^+ \xrightarrow[]{\text{Iron oxidizing microorganisms}} 4Fe^{3+} + 2H_2O \quad (2)$$

Formed elemental sulfur (S^0) in the equation 1, is further oxidized to protons and sulfate by sulfur oxidizing microorganisms, according to the following equation:



$$4S^0 + 6O_2 + 4H_2O \xrightarrow[]{\text{Sulfur oxidizing microorganisms}} 4SO_4^{2-} + 8H^+ \quad (3)$$

The indirect mechanism is generally subdivided into three mechanisms: contact-, non-contact, and cooperative mechanisms (Figure 1) (Tributsch 2001). In the contact mechanism, the bioleaching microorganisms attach to the surface of the sulfide mineral and the sulfide mineral is degraded by Fe(III). During the contact, Fe(III) is reduced back to Fe(II) due to sulfide mineral oxidation, and then re-introduced into the iron (Fe(II)-Fe(III)) cycle. (Tributsch 2001; Kinzler *et al.* 2003; Sand & Gehrke 2006) The dissolution of sulfide mineral by electrochemical reactions occurs in the interface between the bacterial cell wall and the surface of the sulfide mineral. (Sand & Gehrke 2006) In the non-contact bioleaching mechanisms, planktonic microorganisms oxidize Fe(II) to Fe(III), which then comes in contact with a surface of sulfide mineral. (Tributsch 2001; Kinzler *et al.* 2003; Sand & Gehrke 2006) In cooperative mechanisms, microorganisms attached to the surface of the mineral and planktonic microorganisms cooperate. During this cooperation, microorganisms attached to the mineral surface, dissolve mineral and thereby liberate energy carrying compounds, such as sulfur intermediates, which are used to feed planktonic microorganisms in suspension around. (Tributsch 2001)

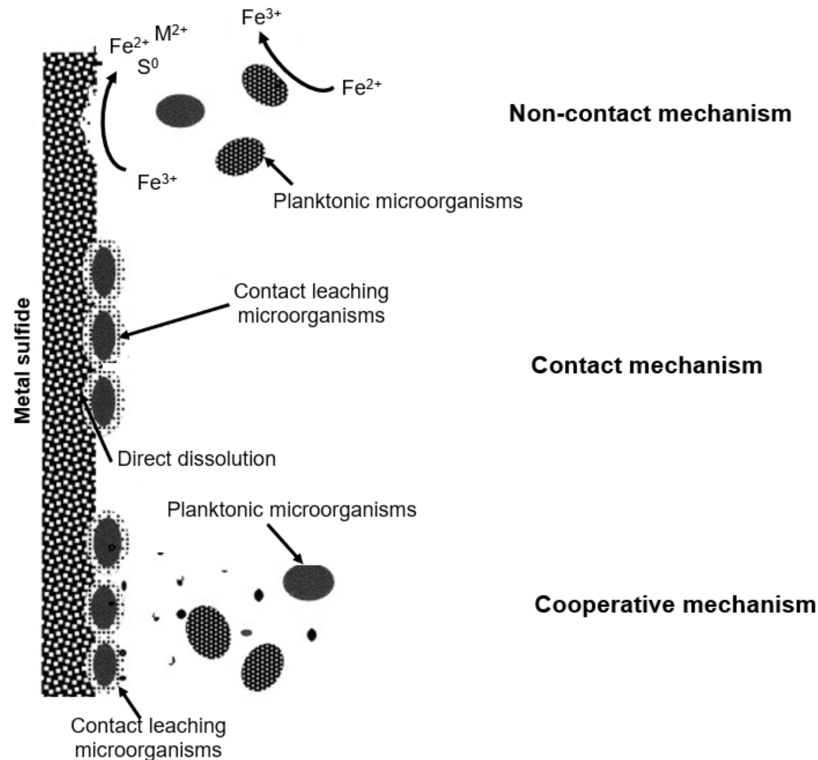


Figure 1. *Indirect leaching sub-mechanisms. Top-down sub-mechanisms: non-contact, contact, and cooperative mechanism. (modified from Tributsch 2001)*

2.2 Microorganisms associated with bioleaching

Microorganisms accountable for the oxidation of Fe(II) and reduced sulfurous compounds, during bioleaching, are chemolithoautotrophs, meaning that they use inorganic compounds as source of energy. In bioleaching environments, indigenous chemolithoautotrophs, include microorganisms from both prokaryote domains: Bacteria and Archaea. (Norris 2007) These iron- and sulfur oxidizing microorganisms contain, for example, rod-shaped *Acidithiobacillus (A.) ferrooxidans* and *A. thiooxidans* bacteria (Johnson 1998). The rod-shaped bacteria were also observed from the original culture, used to enrich iron and sulfur oxidizing microorganisms during the experimental part of this thesis (Figure 2).

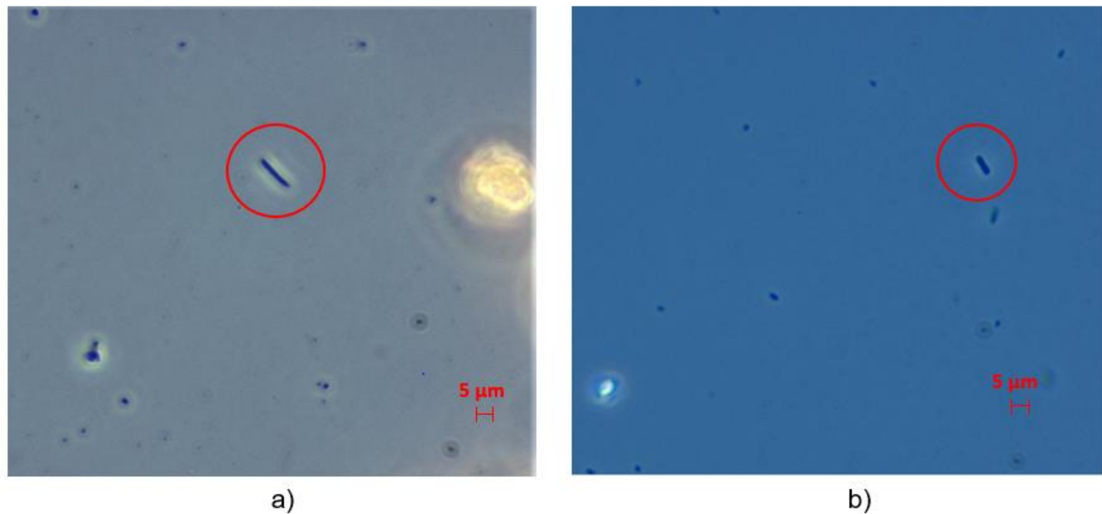


Figure 2. *Rod-shaped bacteria, observed from the (a) iron and (b) sulfur oxidizing enrichment cultures. The rod-shaped bacteria are showed in a red circle. Pictures were taken with Axioskop 2 microscope (ZEISS Group, Germany) equipped with N-Achroplan 100x/1.25 oil pH 3 M27 objective and digitalized camera system, magnification 1000x.*

During the oxidation activities, chemolithoautotrophs use inorganic reduced elements or compounds, such as Fe(II), as an electron donor, and generally oxygen as an electron acceptor. Some microbial species, such as *A. ferrooxidans*, can, however, also use Fe(III) as an electron acceptor. As a carbon source, chemolithoautotrophs are using inorganic carbon dioxide (CO_2), which they can also fix from the atmosphere. (Watling 2006; Schippers 2007)

In addition to the actual bioleaching microorganisms, chemolithoautotrophs, also other microorganisms: chemomixotrophs and heterotrophs, are indigenously present in bioleaching environments. Chemomixotrophs can use various energy and carbon sources, whereas heterotrophs require organic carbon as a carbon source. These chemomixotrophic and heterotrophic microorganisms include, for example, *Sulfobacillus* and *Acidiphilium* species. (Watling 2006; Schippers 2007)

Even though chemomixotrophs and heterotrophs, do not primarily participate in iron and sulfur oxidation activities, it is reported that the presence of these microorganisms enhances metal dissolution from sulfide ores. Bioleaching enhancing effect of these microorganisms, can be due to, for example, utilization of organic carbon present in bioleaching environment. Since organic carbon can be harmful to chemolithoautotrophs, the presence of the chemomixotrophs and heterotrophs, can help to detoxify the growth environment of the chemolithoautotrophs (Johnson & Roberto 1997; Schippers 2007; Li e*t al.* 2011).

All bioleaching microorganisms present in the acidic bioleaching environments, are acidophiles. They can be either strict or moderate acidophiles, which generally prefer pH range below 3. However, the optimal pH range varies by microbial species. (Watling 2006; Norris 2007) The other environmental parameter significantly affecting the bioleaching microorganisms and the microbial composition is temperature. Temperature varies along with the bioleaching process, and therefore also the microbial composition varies. (Watling 2006; Norris 2007) The bioleaching microorganisms can be mesophilic, moderately thermophilic, or thermophilic, which optimal growth temperature vary between 15–40 °C, 40–60 °C, and >60 °C, respectively. From the bioleaching microorganisms, for example, *A. ferrooxidans* are mesophilic, *Sulfobacillus (Sb.) thermosulfidooxidans* are moderately thermophilic, and *Sulfolobus (S.) metallicus* are thermophilic. (Norris 2007; Schippers 2007) Even though all the bioleaching microorganism species have their own optimal growth temperature, they can still grow at temperatures higher or lower compared to their optimal growth temperature range (Norris 2007).

2.3 Heap bioleaching

Bioleaching techniques can be separated into irrigation-type and stirred tank-type bioleaching. Dump, heap reactor and *in situ* bioleaching, in addition to heap bioleaching, are included in the irrigation type bioleaching techniques. Stirred tank-type bioleaching is based on use of stirred tank reactors. In irrigation-type bioleaching processes, acidic leach solution is used to irrigate dumps, heaps, columns or at the mining site as *in situ*, without removing ore from the bedrock. In the stirred-tank type process, bioleaching occurs in stirred tanks, when growth environment of the bioleaching microorganisms can be more carefully controlled. (Rawling 2002)

One of the irrigation-type techniques, heap bioleaching, is currently used worldwide in commercial scale, for leaching of valuable metals from low-grade sulfide ores or as a pre-treatment method of a refractory gold. (Watling 2006; Lan *et al.* 2009). In heap bioleaching, crushed and generally agglomerated ore material is stacked to form typically 4–10 m height heaps, called bioheaps. During agglomeration, finer ore particles attach to the surface of larger ones. (du Plessis *et al.* 2007; Petersen 2016)

Since the bioleaching microorganisms require oxygen, CO_2, and acidic growth environment, the optimal pH and atmosphere conditions for the bioleaching microorganisms are maintained in bioheaps by adding sulfuric acid into the irrigating solution and aerating the heaps. (Norris 2007; Watling 2006; Petersen 2016) Water-soluble metal sulfates, formed during bioleaching, are collected from the bioheaps in pregnant leach solution (PLS), which flows towards the bottom of the heap. In the bottom of the heap, PLS is

collected and transferred to PLS storage pools, and metals of interest are then recovered. (Petersen 2016) Temperature inside the bioheaps increases and decreases simultaneously with the biological oxidation of sulfide ores (Rawlings 2007).

In bioheaps, the diversity of microorganisms is generally greater compared to the stirred tank reactors (Watling 2006; Halinen *et al.* 2012; Ahoranta *et al.* 2017). Typically, bioleaching microorganisms grow naturally in bioheap environments, however, diverse, and dynamic bioleaching microbial community can be possibly achieved through inoculation of heaps, when microorganisms are introduced into the bioheap environments. The inoculation can be executed during the agglomeration. (Watling 2006; Petersen 2016)

The key advantage of heap bioleaching is that it is the only economically viable technique for extraction of valuable metals from low-grade ores. Even though the greatest advantage of heap bioleaching is its economic viability, it has also other technical advantages compared to the conventional bioleaching methods. (Petersen & Dixon 2007; Petersen 2016) Heap bioleaching is a simple leaching method, which is straightforward to operate and maintain, because the process is occurred at ambient temperature and atmospheric pressure. Heap bioleaching process is also simple to expand. In the heap bioleaching process, there is no harmful gas emissions, such as SO_2, present unlike in the conventional physico-chemical mining processes. (Petersen & Dixon 2007; Rawlings 2007; Shagufta 2007) The extracted metal yields of heap bioleaching process, are also generally reasonable, compared to the investment and operational costs of it (Watling 2006; Petersen 2016).

The most prominent challenge, associated with heap bioleaching, is however, a slow metal recovery rate. The slow metal recovery rate is, for example, due to passivation layer, formed onto the mineral surface in standard heap bioleaching conditions. This passivation layer formation is a special challenge with copper sulfide, chalcopyrite. (Ren *et al.* 2020) Because physico-chemical composition inside the bioheaps vary, temperature, pH, O_2 and irrigation gradients are always formed during the bioleaching process. Gradient formation can be affected, for example, by the changing permeability of the heap and by the bioleaching stage. (du Plessis *et al.* 2007; Petersen & Dixon 2007) Even though harmful gaseous emissions are not a problem in heap bioleaching, potentially toxic aqueous emissions are produced during the process. These potentially toxic chemical solutions can cause environmental pollution when released to environment without appropriate treatment. This can occur, for example, when the solutions are leaking through the cracks of piping system or storage pools. (Petersen 2016)

2.4 Mining and metal recovery process of Terrafame

Terrafame's complex polymetallic black schist ore deposit is located in Sotkamo, Finland. The deposit contains two different polymetallic ore bodies of Kuusilampi and Kolmisoppi, which are hosted by a polymetallic black schist ore. The polymetallic black schist ore is a composition of nickel (0.23%), zinc (0.51%), copper (0.13%), and cobalt (0.02%). (Riekkola-Vanhanen 2010)

In the Terrafame mine site, the mining method is an open-pit mining. From the open-pit mine, the ore material is delivered to the crushing and screening process, which covers four separate crushing and screening stages, followed by agglomeration (Figure 3). (Riekkola-Vanhanen 2010) From the agglomeration process, the agglomerated ore material is conveyed and stacked onto the primary bioheaps, in which they are then bioleached for 15 months (Terrafame 2020). The over 8 m height primary bioheaps are aerated and irrigated with a raffinate. After the primary bioleaching stage, the leached ore is collected from the primary bioheaps, conveyed and re-stacked onto secondary heaps. In secondary bioheaps, the ore material is further bioleached. The dissolved metals are collected in PLS, which is then treated with hydrogen sulfide (H_2S) to precipitate the dissolved metals: nickel, zinc, copper, and cobalt. The precipitated metals are filtered, resulting in metal products of copper sulfide, zinc sulfide, and nickel cobalt sulfide. (Riekkola-Vanhanen 2010) In the near future, also uranium recovery from the ore will start in the Terrafame metal recovery plant, and therefore it is also showed in Figure 3 (Terrafame 2020).

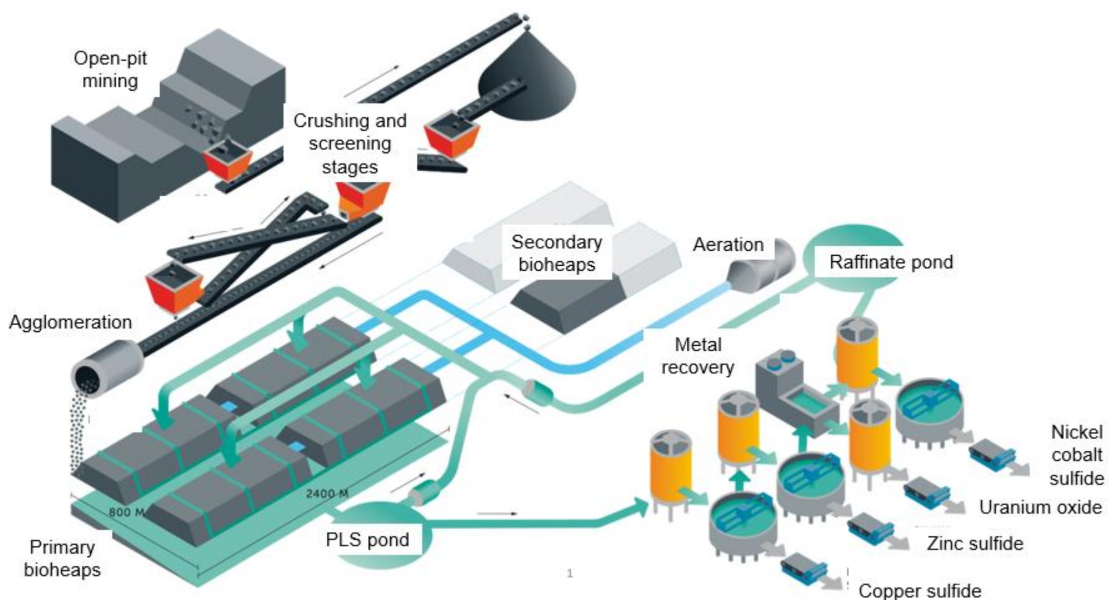


Figure 3. *Terrafame overall mining and metal recovery process (modified from Oinonen 2020).*

3. SUBSTANCES INFLUENCING BIOLEACHING

Bioleaching efficiency and bacterial growth can be affected by various mineral processing related chemical factors. These possible chemical factors include, for example, organic compounds from solvent extraction, which may possibly inhibit the bioleaching process. Various organics are used in the mineral extraction processes, therefore, when the process liquors from these processes are contacted with the bioleaching acidophilic microorganisms, the possible inhibitory effects of the solvents on bioleaching must be known. In Chapter 3.1, solvent extraction organics and their effects on bioleaching, are presented in more detail. In addition to the organic solvents, various chemical additives, can also affect the bioleaching process. In Chapter 3.2, the chemical additives, which can possibly enhance biological heap leaching are described in more detail.

3.1 Inhibition of bioleaching by organic solvents

After bioleaching, all extracted metals, such as nickel and cobalt, are present in the aqueous leach liquor. To result pure or mixed metal products, these dissolved metals must be removed separately from the leach liquor, for example, by solvent extraction and crystallization. (Torma & Itzkovitch 1976; Chen *et al.* 2015) In Terrafame battery chemical production plant, the battery chemical recovery process includes solvent extraction and crystallization (See Chapter 4.3.1) (Terrafame 2018). In the extraction process, organic solvents are used for removing metals from the aqueous solution, and therefore, they are present also in the process liquors of the following crystallization processes. The amount of these residual organics in the process liquors, depends on the water solubility of the organic compound used (Torma & Itzkovitch 1976; Terrafame 2018; Chen *et al.* 2015) If these crystallization process liquors get into contact with the bioleaching microorganism, for example, when the process liquors are recycled back to the bioleaching process, possible inhibitory effect towards bioleaching of these organic solvents must be considered. (Torma & Itzkovitch 1976; Chen *et al.* 2015)

Throughout this chapter, organic solvents used by the Terrafame battery chemical production plant or compounds which are similar to them are presented. These organic compounds include carboxylic acid (neodecanoid acid), Cyanex 272, Baysolvex D2EHPA, and Nessol D100. Chemical information about the organic solvents, such as molecular formulas and chemical structures, are presented in Chapter 4.3.1. Information

about previous studies on the organic solvents, used in Terrafame battery chemical production plants, are presented in Table 1.

Table 1. *Information about the studies on the influence of the organic solvents used by Terrafame battery chemical production plant.*

Organic solvent	Experimental design	Microorganism(s)	Experimental conditions: Temperature/ pH/aeration	Minimum inhibitory concentration (g/L)	Results	Reference
Carboxylic acid (hexanoic acid)	Culture tubes (iron oxidation), shake flask (sulfur oxidation)	*A. ferrooxidans*	35 °C (iron oxidation), 25 °C (sulfur oxidation)/ initial pH 3.1–3.7/ capillary tube aerator (iron oxidation) and by shaking (sulfur oxidation and growth)	0.09	Hexanoic acid concentration of 0.09 g/L leaded to 43% reduction of iron oxidation, whereas hexanoic acid concentration of 0.9 g/L resulted to 78% reduction of iron oxidation. Hexanoic acid concentration of 0.9 g/L, inhibited sulfur oxidation completely.	Tuttle & Dugan 1976
Cyanex 272	Shake flask	Mixed culture (mainly consisted of *A. ferrooxidans* and *Leptospirillum (L.) ferriphilum)*	30 °C/ initial pH 1.8/ by shaking	N.R.*	Saturation concentration of Cyanex 272 in the aqueous phase had no effect on biological iron oxidation or cell growth of the studied microorganisms.	Chen *et al.* 2015
Baysolvex D2EHPA	Manometric technique	*A. ferrooxidans*	35°C/ initial pH 2.3/ air enrichment to 0.2% CO_2	0.264 (of TOC**) (one studied concentration)	D2EHPA concentration of 0.264 TOC g/L decreased specific oxygen uptake rate to 9.71 µL of O_2/h per mg of protein, compared to 30.47 µL of O_2/h per mg of protein of the biological control.	Torma & Itzkovitch 1976
Nessol D100	No study found about the effect of Nessol D100 or other aliphatic hydrocarbons containing solvents on bioleaching.					

*N.R.: not reported

**TOC: total organic carbon

From the studied organic extractants, the effect of neodecanoid acid on bioleaching microorganisms have not yet been studied. However, the influence of other various carboxylic acids on iron and sulfur oxidation by *A. ferrooxidans* have been studied by Tuttle & Dugan (1976). From the studied monocarboxylic acids, hexanoic acid has been closest to neodecanoid acid with its molecular structure. Therefore, hexanoic acid is considered as monocarboxylic acid example, and inhibition results of hexanoic acid are presented in more detail.

In the study of Tuttle & Dugan (1976), the influence of simple organic compounds, including hexanoic acid, on biological iron and sulfur oxidation, respectively, was studied. The results of their study showed that hexanoic acid was inhibitory to iron and sulfur oxidation, as seen in Table 1. The results of the study indicated that the significant factor affecting inhibition of biological iron oxidation was the relative electronegativity of the organic compound. The results also suggested that organic compounds could affect the bioleaching microorganisms, for example, by directly affecting the iron oxidizing enzyme system of the microorganism, reacting with Fe(II) in the environment, and non-selectively disturbing the operation of cell envelope or membrane.

Chen *et al.* (2015) studied the influence of saturation concentration (in 9K medium) of various metal extractants on biological iron oxidation, cell growth, and community structure of acidophilic microorganisms. The results of the study showed, as seen in Table 1, that Cyanex 272 had no effect on biological iron oxidation or cell growth, compared to the biological control. However, presence of Cyanex 272 during the cultivation decreased the quantity of *L. ferriphilum* and *A. ferrooxidans* cell mass, compared to the biological control, when heterotrophic *Acidiphilium cryptum* became dominant.

In the study of Torma & Itzkovitch (1976), the influence of different copper and uranium extractants, such as Baysolvex D2EHPA, on the specific oxygen uptake rate by *A. ferrooxidans* was studied. The studied Baysolvex D2EHPA concentration was its saturation concentration in 9K medium (264 total organic carbon (TOC) in mg/L). The results of the experiment showed, as seen in Table 1, that saturation D2EHPA concentration inhibited biological uranium extraction, possibly due to preventing intimate contact of bacterial cells with the mineral surface, modifying the growth environment of the bacteria, or influencing some nutritive or growth processes of the bacteria.

3.2 Enhancement of bioleaching by chemical additives

Leaching of metals from the low-grade sulfide ores can be challenging even by processing the ores in heap bioleaching, which is generally considered to be an economically viable method for this purpose (Duncan *et al.* 1964; Petersen 2016; Ghadiri *et al.* 2019). Extraction of metals is considered to be especially challenging from chalcopyrite, which has also the highest concentration of valuable metal copper, from all the mineral resources of the world. Therefore, it has been found necessary to try to seek different kinds of chemical additives, which can potentially enhance heap bioleaching of ores, for making the heap bioleaching process more technically and economically efficient. (Lan *et al.* 2009; Zhang *et al.* 2019; Ren *et al.* 2020)

One of the key factors influencing on the efficiency of bioleaching, is nutrient requirement of the bioleaching microorganisms. One of the most essential nutrients for the bioleaching microorganisms is nitrogen, which is also present in the process liquors: AS bleed and feed, of Terrafame battery chemical production plant. (du Plessis *et al.* 2007; Rawlings 2007) Therefore, nitrogen requirement of bioleaching microorganisms and possible nitrogenous nutrient additives are discussed during the following two chapters. During Chapters 3.2.3–3.2.6, other different types of additives as possible heap bioleaching enhancing substances, are reviewed.

3.2.1 Nitrogen requirement of bioleaching microorganisms

Iron and sulfur oxidizing bacteria are chemolithoautotrophs, therefore they use inorganic nitrogen and phosphorus compounds, for their cell growth (du Plessis *et al.* 2007; Rawlings 2007). Nitrogen is the most important nutrient for the formation of new cell biomass and its requirement depends on the quantity of the cell mass. (Rawlings 2007) Sulfide ores, such as polymetallic black schist ore of Terrafame, generally contain sufficient quantities of micronutrients, such as potassium and zinc, for the cell growth, but the amount of macronutrients, such as nitrogen, is too low in the sulfide ores, so it need to be supplemented (du Plessis *et al.* 2007). For example, in the study of Ahoranta *et al.* (2017), nitrogen concentration of the process liquors, collected from the primary heaps of Talvivaara Mining Company Plc (currently Terrafame), varied between <1.0–2.6 mg/L, which was reported to be insufficient amount in Sarcheshmehpour *et al.* (2009) study.

Microorganisms responsible of the genuine bioleaching process, such as *A. ferrooxidans*, are generally able to fix nitrogen from atmosphere (Mackintosh 1978; Norris *et al.* 1995). However, nitrogenase enzyme, an enzyme which catalyzes the nitrogen fixation,

is inhibited by oxygen. Because oxygen is usually present during the bioleaching process, also heterogeneously in heap bioleaching, nitrogen fixation cannot meet alone the full nitrogen requirement of the bioleaching biomass, so supplementing nitrogen is necessary. (Rawlings 2007)

The exact nitrogen demand of the bioleaching is difficult to estimate because the form of the nitrogen affect how well it can be utilized by the bioleaching microorganisms. Also, the quantity of nitrogen in the bioleaching environment varies due to environmental conditions. (du Plessis *et al.* 2007; Rawlings 2007) From the various nitrogen forms, for example, ammonium can be absorbed readily from air into the acidic bioleaching solution, due to its high solubility in acidic solutions (Rawlings 2007).

Nitrogen limitation has been reported to have negative effect on the cell growth of bioleaching microorganisms and lowering the bioleaching efficiency. It is assumed that the lowered bioleaching efficiency is due to the decreased Fe(III) precipitate formation, which then reduces bacterial attachment to the solid surface of the ore. These negative effects have been shown to be dependent on the constitution of the microbial culture. (Krafft & Hallberg 1993; d'Hugues *et al.* 1997)

Even though nitrogen has been shown to be an essential nutrient for efficient bioleaching, it has also been reported that depending on its form, it can inhibit iron and sulfur oxidation. It has been reported that from the various nitrogen forms, nitrate can especially inhibit iron and sulfur oxidation, also with moderate concentrations. (Niemelä *et al.* 1994; Suzuki *et al.* 1999; Harahuc *et al.* 2000) Therefore, it is critical to be aware which form of nitrogen can be used and in which concentration range, when optimal bioleaching nitrogenous nutrient conditions want to be obtained.

3.2.2 Nitrogenous nutrient additives

Information about the various nitrogenous nutrient additives and previous studies on them, are presented in Table 2.

Table 2. *Information about the studies on the influence of various nitrogenous nutrient additives. (**Note:** In the "Ability to enhance bioleaching" column, + implies positive impact of the additive on bioleaching, +/- implies possible impact of the additive on bioleaching stayed unknown, and - implies negative impact on bioleaching.)*

Nitrogenous additive	Experimental design	Microorganism(s)	Experimental conditions: Temperature/ pH/aeration	Optimal enhancing concentration (g/L)	Minimum inhibitory concentration (g/L)	Results	Ability to enhance bioleaching (+/-)	Reference
	Shake flask	Mixed culture (mainly consisted of *A. ferrooxidans)*	room temperature/ initial pH 2.0/ by shaking	0.027	N.R.	Ammonium addition increased leached zinc amount, after 53 days of incubation, from 27% to 35% (Kristineberg ore), and from 7% to 68% (Saxbergel ore), compared to the biological control.	+	Krafft & Hallberg 1993
Ammonium	Shake flask	Mixed culture (mainly consisted of *A. ferrooxidans)*	30 and 35 °C/ initial pH 1.5/ by shaking	0.11 (single studied concentration)	N.R.	Ammonium supplementation of 0.11 g/L elevated Fe(III) concentration, during 10 days of incubation, from about 2.3 g/L to 3.5 g/L, compared to the biological control.	+	Niemelä *et al.* 1994
	Continuous laboratory-scale unit, containing four stirred reactors	Culture consisted of *A. ferrooxidans*, *A. thiooxidans* and *Leptospirillum*-like bacteria	35 °C/ 1.3–1.5/ dissolved oxygen concentration of the reactors varied between 1.6–4.9 mg/L	0.80 (of N)	N.R.	Studied ammonium concentration increased dissolved cobalt yield, after 4 days of retention, from about 65% to about 78%, compared to the urea and DAP supplemented culture.	+	d'Hugues *et al.* 1997

	Shake flask	Iron oxidizing enrichment culture, enriched from a mixed microbial sample	27 °C/ initial pH 1.7/ by shaking	0.32 (of N)	N.R.	Ammonium supplementation of 0.32 N g/L increased the iron oxidation rate from 0.02 g/L/h to 0.16 g/L/h, compared to the biological control, which was not supplemented with nutrients.	+	Ahoranta *et al.* 2017
Urea (with DAP*)	Continuous laboratory-scale unit, containing four stirred reactors	Culture consisted of *A. ferrooxidans*, *A. thiooxidans* and *Leptospirillum*-like bacteria	35 °C/ 1.3–1.5/ dissolved oxygen concentration of the reactors varied between 1.6–4.9 mg/L	0.80 (of N)	N.R.	Dissolved cobalt yield was more than 10% lower, after 4 days of retention, with urea supplemented culture than with ammonium supplemented culture.	+/-	d'Hugues *et al.* 1997
Yeast extract							See Chapter 3.2.2	
TSB**							See Chapter 3.2.2	
	Shake flask	Mixed culture (mainly consisted of *A. ferrooxidans)*	30 and 35 °C/ initial pH 1.5/ by shaking	N.R.	0.38	Nitrate additions of 0.38 g/L and 0.75 g/L decreased Fe(III) concentration, during 10 days of incubation at 30 °C, from about 2.3 g/L to 0.20–0.30 g/L, compared to the biological control.	-	Niemelä *et al.* 1994
Nitrate	Shake flask	*A. thiooxidans*	28 °C/ 2.3, 4.5, and 7.0/ by shaking	N.R.	0.62	Nitrate addition of 6.2 g/L (as $NaNO_3$), at pH 2.3, inhibited sulfur oxidation almost completely. At lower concentrations of 0.62 g/L and 3.1 g/L, partial inhibition was observed.	-	Suzuki *et al.* 1999
	Shake flask	*A. ferrooxidans*	25–28 °C/ 3.0/ by shaking	N.R.	0.62 (with iron oxidation) 6.2 (with sulfur oxidation)	Nitrate concentration of ≥0.62 g/L inhibited iron oxidation, whereas inhibition of sulfur oxidation began with nitrate concentration of 6.2 g/L.	-	Harahuc *et al.* 2000

*DAP: diaminophosphate

**TSB: tryptone soya broth

In the study of Krafft & Hallberg (1993), the influence of ammonium on bioleaching of two Swedish zinc sulfide ores: Kristineberg and Saxbergel, was investigated. In their study it was demonstrated that 0.027 g/L ammonium supplementation stimulated bioleaching of zinc sulfide ore, as seen in Table 2. In the study of Niemelä *et al.* (1994), effect of 0.11 g/L (0.006 mol/L) ammonium addition, on bioleaching of a black schist ore was studied. The results of the study showed that ammonium supplementation enhanced Fe(II) oxidation from the black schist ore, as seen in Table 2.

d'Hugues *et al.* (1997) studied the effect of ammonium and urea on bioleaching. Ammonium concentration of 0.80 N g/L increased dissolved cobalt yield, compared to the culture, which was supplemented with urea and diamidophosphate (DAP) (Table 2). The results of the study showed that nitrogen in the form of ammonium enhanced bioleaching of cobaltiferrous pyrite compared to urea. Influence of ammonium on biological iron oxidation have been also studied by Ahoranta *et al.* (2017). In their study, different nitrogen concentrations, from 0.32 g/L to 4.7 g/L, were investigated. The results of the study showed that nitrogen supplementation increased iron oxidation, as seen in Table 2, and that nitrogen concentration over 0.32 g/L did not significantly change the iron oxidation rates.

Effect of urea and ammonium on bioleaching of cobaltferrous pyrite, have been studied by d'Hugues *et al.* (1997). In their study, the microbial culture was supplemented with urea (1.28 g/L) and DAP (1.08 g/L), when nitrogen level of the medium was same as with ammonium (Table 2). Because cobalt yield was over 10% lower with urea supplemented culture than with ammonium supplemented culture, it can be concluded that urea was not as efficient nitrogen supplement than ammonium. Because there was no biological control present during their study, the information, whether urea enhanced bioleaching, stayed unknown.

Influence of nitrate on biological iron and sulfur oxidation and on bioleaching have been studied by Niemelä *et al.* (1994), Suzuki *et al.* (1999) and Harahuc *et al.* (2000). Niemelä *et al.* (1994) studied effect of nitrate on bioleaching of a black schist ore. During their study, 0.38 g/L (0.0061 mol/L) and 0.75 g/L (0.0121 mol/L) concentration of nitrate was used. The results of the study showed that studied nitrate concentrations had a negative influence of bioleaching of a black schist ore, as seen in Table 2. In the study of Suzuku *et al.* (1999), biological sulfur oxidation in presence of nitrate (potassium nitrate and sodium nitrate) and other possible inhibitors was investigated. At low pH of 2.3, both nitrate compounds inhibited sulfur oxidation, whereas, at pH 4.5 and 7.0 the inhibitory effect of nitrate disappeared (Table 2). This study showed that nitrate inhibited sulfur oxidation at

pH of 2.3. The results of this study also indicated that inhibition of nitrate at lower pH range (pH 2.3) was due to increased nitrate permeability of the cells. Harahuc *et al.* (2000) studied biological iron and sulfur oxidation in presence of nitrate and other various inhibitors, such as anions. Harahuc *et al.* (2000) reported that nitrate inhibited both iron and sulfur oxidation, and that iron oxidation was more sensitive to inhibition of nitrate than sulfur oxidation at concentrations below 6.2 g/L (0.10 mol/L), as seen in Table 2.

3.2.3 Polymeric extracts

Organic compounds can cause partial or full inhibition of bioleaching by chemolithoautotrophs. Therefore, heterotrophs present in the growth environment can enhance bioleaching by degrading organic matter and thereby detoxifying the growth environment for chemolithoautotrophs. (Johnson & Roberto 1997; Schippers 2007; Li e*t al.* 2011) However, because organic polymeric extracts, such as yeast extract, can be used to support on the growth of heterotrophs and chemomixotrophs, and form growth environment detoxifying metal-complexes, polymeric extracts can have two-way effect on bioleaching, by inhibiting or enhancing it. (Tuovinen e*t al.* 1985; Johnson & Roberto 1997; Schippers 2007; Li e*t al.* 2011)

With microbial cultures, the polymeric extracts are functioning as nutrient source, such as nitrogen and phosphorus source, along with organic carbon source. Because bioleaching environment is generally macronutrient, such as nitrogen, limited, addition of organic macronutrient source can therefore increase the cell growth and microbial activity, and thereby enhance the bioleaching process. (du Plessis *et al.* 2007; Rawlings 2007; van Hille *et al.* 2009)

From the organic polymeric extracts, yeast extract has been commonly used, as typical polymeric extract, in the bioleaching studies, during the last forty years (Puhakka & Tuovinen 1987; Rowe & Johnson 2008; Li *et al.* 2011; Zhang e*t al.* 2015). In addition to yeast extract, influence of TSB, with other varying factors, on the composition of the mixed iron oxidizing acidophilic bacteria culture, have been also studied by Demir *et al.* (2020). Information about studies on the influence of polymeric extracts on microbial composition and bioleaching, are presented in Table 3.

Table 3. *Information about studies on the influence of different polymeric extracts. (**Note:** (+) in "Ability to enhance bioleaching" column implies that positive impact on bioleaching is possible, and +/- implies that both positive and negative impact on bioleaching was observed, when experimental conditions varied.)*

Polymeric extract	Experimental design	Microorganism(s)	Experimental conditions: Temperature/ pH/aeration	Optimal enhancing concentration (g/L)	Minimum inhibitory concentration (g/L)	Results	Ability to enhance bioleaching (+/-)	Reference
Yeast extract	Shake flask	Pure culture of *A. ferrooxidans* and three mixed cultures of acidophiles	28 °C/ initial pH 3.2–3.6/ by shaking	0.22	N.R.	The metal recoveries, when 0.22 g/L of yeast extract was present, were 80% Ni, 81%–85% Zn, 32–34% Cu and 69–73% Co, whereas the metal recoveries, when 0.02 g/L of yeast extract was present, were 47–51% Ni, 27–29% Zn, 2% Cu and 23–25% Co.	+	Puhakka & Tuovinen 1987
	Shake flask	Mixed culture	30 to 60 °C/ 1.5/ by shaking	0.1 (single studied concentration)	N.R.	Yeast extract decreased the bioleaching rate at temperature of 30°C, whereas yeast extract increased the bioleaching rate about 8% at temperature of 40 and 50°C. At temperature of 60°C, yeast extract did not affect the bioleaching rate.	+/-	Li *et al.* 2011
	Shake flask	*Sb. thermosulfidooxidans*	45 °C/ initial pH 1.8/ by shaking	0.5	N.R.	Iron oxidation was increased by about 30, 60 and 90%, when 0.1, 0.2, and 0.5 g/L of yeast extract was present, respectively. When ≤ 11.7 g/L of NaCl was added, cell growth and iron oxidation were enhanced, when yeast extract was added. 0.5 g/L addition	(+)	Huynh *et al.* 2020

						of yeast extract leaded to the highest cell number, about 5 times higher than without yeast extract.		
TSB	CMBR*	Mixed culture of iron oxidizing aci-dophilic bacteria	23.5 ± 2.8 °C/ pH of the feed 1.5–2.5/ dis-solved oxygen concen-tration of the reactor ≥ 3 mg/L	N.R.	N.R.	*Acidiphilium cryptum* was dominant bacteria when concentration of TSB was 0.500 g/L and HRT** was 24 h, whereas *Alicyclobacil-lus cycloheptanicus* was dominant, when TSB con-centration and HRT re-duced to 0.250 g/L and 6 h, respectively.	N.R.	Demir *et al.* 2020

*CMBR: ceramic membrane bioreactor

**HRT: hydraulic retention time

In the study of Puhakka & Tuovinen (1987), the influence of yeast extract on bioleaching of a complex sulfide ore material was studied. The results of their study showed that yeast extract concentration of ≥0.22 g/L, enhanced bioleaching with all the studied microbial cultures, as seen in Table 3. The metal recoveries were between 2 to 17 times higher, when 0.22 g/L of yeast extract was present, compared to the groups containing 0.02 g/L of yeast extract. It was reported that yeast extract-to-sulfide ore ratio was a significant factor for enhancing bioleaching.

Li et *al.* (2011) studied the influence of yeast extract and temperature, on bioleaching of chalcopyrite and on the composition of the microbial community. At temperature of 30 °C, yeast extract decreased the bioleaching rate possibly due to reduced quantity of significant chemolithoautotrophs, such as *A. ferrooxidans* and *A. thiooxidans*. At temperature of 40 and 50 °C, yeast extract addition increased the bioleaching rate, as seen in Table 3. The results of the study indicated that presence of yeast extract enhanced the bioleaching rate, possibly due to increased proportion of *Ferroplasma thermophilum*, when temperature was 40 °C, and increased proportion of *Acidianus brierleyi* at temperature of 50 °C. At temperature of 60 °C, presence of yeast extract did not affect the bioleaching rate. Huynh *et al.* (2020) studied the influence of different initial growth conditions of bacteria on biological iron oxidation and to stress response. This study showed that yeast extract increased biological iron oxidation and relieved the stress response of bacteria to NaCl, as seen in Table 3. The stress response was studied by monitoring biological iron oxidation and cell growth.

In addition to these above-mentioned studies, also Rowe & Johnson (2008) and Zhang *et al.* (2015) studied the influence of yeast extract on bacterial cell growth and biological iron-oxidation. Rowe and Johnson (2008) reported that the cell density of obligately heterotrophic *Ferrimicrobium*-like isolate was increased in the presence of yeast extract, resulted in higher initial biological iron oxidation. In the study of Zhang *et al.* (2015), the influence of yeast extract on the cell growth was investigated. They reported that the presence of 0.2 g/L of yeast extract caused minor inhibition on the growth of *L. ferriphilum*, and 0.4 g/L yeast extract concentration completely inhibited the growth. Whereas *Ferroplasma thermophilum* seemed to require yeast extract for optimal cell growth when ferrous iron was the substrate. Therefore, it was suggested that inhibitory effect of yeast extract on obligately chemolithoautotrophic *L. ferriphilum* can be mitigated when using mixed microbial culture, including chemomixotrophic *F. thermophilum*, for bioleaching.

Influence of TSB, with other factors, on the composition of the mixed iron oxidizing acidophilic bacteria culture, have been studied by Demir *et al.* (2020). Because the purpose of the study was to observe how variable factors: hydraulic retention time (HRT), pH, and Fe(II) concentration, affect to the performance of ceramic membrane bioreactor (CMBR) and to the composition of microbial culture present, optimal bioleaching enhancing concentration of TSB was not studied during the experiment. The results of this study showed that the composition of the microbial culture varied during the operation of the CMBR, as seen in Table 3. From these results, it was suggested that HRT and concentration of TSB were able to affect to the composition of the microbial community, and thereby possibly affect the bioleaching performance.

3.2.4 Surfactants

Surfactants are surface active agents used to reduce the interfacial or surface tension of various materials. Because the general working mechanism of the surfactants can be utilized with various materials, they are widely used in various industrial sectors, such as in pharmaceutical, food, and cosmetics sectors. (Schramm e*t al.* 2003; Ghadiri *et al.* 2019) Surfactants typically consist of a hydrophobic alkyl chain group (8-20 carbons), which has the ability to repel water, and a hydrophilic functional group, which is attracted to water. Due to these qualities of the surfactants, they can modify the surface or the interfacial tension of various materials (Schramm *et al.* 2003; Lan *et al.* 2009).

The surfactants that potentially enhance bioleaching can be distributed into three categories based on their ionic character: anionic, cationic, and non-ionic surfactants (Duncan e*t al.* 1964; Lan e*t al.* 2009; Pan *et al.* 2020). Difference between these three surfactants is in their hydrophilic head. Anionic surfactants contain a negatively charged functional group in their hydrophilic head, while cationic surfactants contain a positively charged hydrophilic head, and non-ionic surfactants have functional group which carry no charge (Schramm *et al.* 2003; Lan *et al.* 2009) Because the chemical structures and the charges of the hydrophilic heads of these different surfactant types vary, also their ability to enhance the bioleaching process varies and is affected for example by the type of mineral leached (Duncan *et al.* 1964; Schramm *et al.* 2003; Lan *et al.* 2009).

In bioleaching process, surfactants can be used as additives which are able to enhance the bioleaching rate by changing the wettability and charge of the mineral surface, lowering the surface tension of a bioleaching solution phase, and lowering the interfacial tension between a solution phase and a biological phase. Therefore, improving the bioleaching solution flow penetration into pores and cracks of the minerals and hastening

bacteria for reaching equilibrium on the surface of the mineral. (Kingma & Silver 1979; Sandoval *et al.* 1990; Zhang e*t al.* 2008; Lan e*t al.* 2009; Pan *et al.* 2020)

Even though the surfactants can potentially enhance the bioleaching process, they can also negatively influence the bioleaching by damaging the cells of the microorganisms at high concentrations. This negative effect has been reported with anionic, cationic, and non-ionic surfactants. (Harrison 1991; Harrison *et al.* 1991) One possible factor which may affect the performance of the surfactants, is surfactant specific critical micelle concentration (CMC). When the surfactant concentration is above CMC, it begins to form thermodynamically stable micelles. It has been reported that toxicity of the surfactants can be dependent on CMC of the surfactant. The toxic effects have been reported when the concentration of the surfactants has been close or higher than CMC. (Inácio *et al.* 2011; Ríos *et al.* 2017) Therefore, it is crucial not only to determine the optimal bioleaching enhancing the surfactant concentration, but also define the bioleaching inhibitory concentration.

Information about the studies, concerning the influence of the various surfactants on bioleaching, is presented in Table 4, 5, and 6. In the tables, the surfactants are distributed into three categories by their ionic character. Because the working mechanisms of the different surfactants are generally similar, these are not presented in the table, but discussed in the text.

Anionic surfactants

Information about the different anionic surfactants studied earlier, are presented in Table 4.

Table 4. *Information about studies on the influence of anionic surfactants. (**Note:** (+) in "Ability to enhance bioleaching" column implies that positive impact on bioleaching is possible and - implies no impact or negative impact on bioleaching.)*

Surfactant	Experimental design	Microorganism(s)	Experimental conditions: Temperature/ pH/ aeration	Optimal enhancing concentration (%, v/v)	Minimum inhibitory concentration (%, v/v)	Results	Ability to enhance bioleaching (+/-)	Reference
Duponol 80	Shake flask	*A. ferrooxidans*	35 °C/ initial pH 2.5/ by shaking	0.01 (maximum noninhibitory conc.)	>0.01	Copper concentration after 28 days of incubation was similar to the biological control (0.97 g/L with Duponol 80 and 1.1 g/L without).	-	Duncan *et al.* 1964
Petrowet R	Shake flask	*A. ferrooxidans*	35 °C/ initial pH 2.5/ by shaking	0.005 (maximum noninhibitory conc.)	>0.005	Copper concentration after 28 days of incubation was similar to the biological control (0.95 g/L with Petrowet R and 1.1 g/L without).	-	Duncan *et al.* 1964
Petrowet WN	Shake flask	*A. ferrooxidans*	35 °C/ initial pH 2.5/ by shaking	0.01 (maximum noninhibitory conc.)	>0.01	Copper concentration after 28 days of incubation was similar to the biological control (0.81 g/L with Petrowet WN and 1.1 g/L without).	-	Duncan *et al.* 1964
SDBS*	Shake flask	*A. acidophilus*	30 °C/ initial pH 2.0/ by shaking	N.R.	0.10	All studied concentrations (0.10, 0.15 and 0.18 g/L) lowered the surface desulfurization rate.	-	Pan *et al.* 2020
SIBX**	Shake flask	*A. albertensis* BY-05	30 °C/ initial pH 3.8/ by shaking	10^{-4}–10^{-8} ***	10^{-2} ***	SIBX concentrations of 10^{-4}–10^{-8} g/L elevated the cell number about $2*10^{7}$, when cell number of the biological control was about $34*10^{7}$. SIBX concentration of 10^{-4} g/L also accelerated SO_4^{2-} production.	(+)	Zhang *et al.* 2008

*SDBS: sodium dodecylbenzenesulfonate

**SIBX: sodium isobutyl-xanthate

*** g/L

As shown in Table 4, previously studied anionic surfactants include Duponol 80 (sodium n-octyl sulfate), Petrowet R (sodium alkyl sulfonate), Petrowet WN (sodium alkyl sulfate), sodium dodecylbenzenesulfonate (SDSB), and sodium isobutyl-xanthate (SIBX). Chemical structures of the studied anionic surfactants are shown in Figure 4.

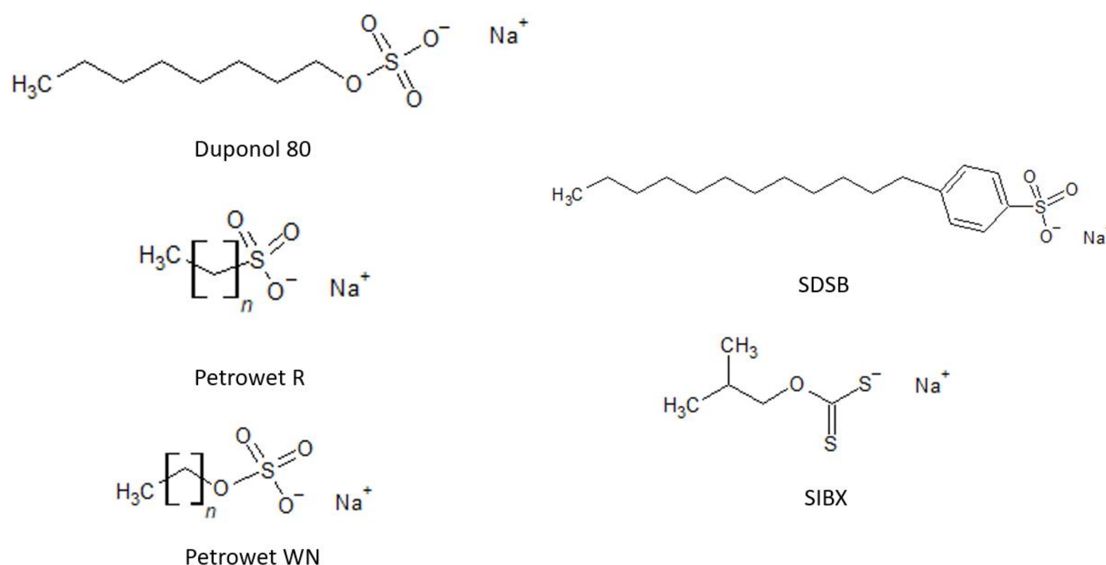


Figure 4. *Chemical structures of the studied anionic surfactants, drawn with ChemSketch (Flick 1993; Pubchem 2021A; Sigma-Aldrich 2021A; Thermo Fischer Scientific 2021).*

Duncan *et al.* (1964) studied the impact of different anionic surfactants: Duponol 80, Petrowet R, and Petrowet WN, on bioleaching of museum-grade chalcopyrite ($CuFeS_2$). As seen in Table 4, the results of the study showed that none of the three different anionic surfactants had an impact on bioleaching. However, any possible reasons for the poor performance of the anionic surfactants were not introduced in the study. (Duncan *et al.*, 1964)

In the study of Pan e*t al.* (2020), the influence of SDBS on bacterial desulfurization effect and on the surface tension of the bioleaching solution was investigated. From the results of their study, it was observed that SDBS lowered the bacterial desulfurization effect of the high-sulfide ore compared to the biological control (Table 4). It was also reported that when SDSB was present, the surface tension of the bioleaching solution became similar to the biological control after five experimental days. High surface tension indicated that the surfactant concentration was very low referring that most of the surfactant was destroyed. According to these above-mentioned results, it seems that SDBS does not have

a positive impact on bioleaching process and durability of it can be poor in certain leaching conditions.

From all the studied anionic surfactants presented in Table 4, surfactant SIBX is the only surfactant that has shown some positive signs for potentially enhancing bioleaching, by improving the growth and sulfur oxidizing activities of a microbial culture of *A. albertensis* BY-05. Zhang *et al.* (2008) reported that the optimal concentration range of SIBX was 10^{-4}–10^{-8} g/L, when the cell concentration was in its highest level, as seen in Table 4. Also, from their results, it was concluded that the studied surfactants were able to alter the surface characteristics of the minerals and enhance the connection between bacterial cells and elemental sulfur particles.

Cationic surfactants

Information about the different cationic surfactants studied earlier, are presented in Table 5.

Table 5. *Information about studies on the influence of cationic surfactants. (**Note:** (+) in "Ability to enhance bioleaching" column implies that positive impact on bioleaching is possible.)*

Surfactant	Experimental design	Microorganism(s)	Experimental conditions: Temperature/ pH/aeration	Optimal enhancing concentration (%, v/v)	Minimum inhibitory concentration (%, v/v)	Results	Ability to enhance bioleaching (+/-)	Reference
Armour 461	Shake flask	*A. ferrooxidans*	35 °C/initial pH 2.5/ by shaking	0.01 (maximum noninhibitory conc.)	>0.01	Copper concentration after 19 days of incubation was similar to the biological control (0.70 g/L with Armour 461 and 0.66 g/L without).	-	Duncan *et al.* 1964
Hyamine 2389	Shake flask	*A. ferrooxidans*	35 °C/ initial pH 2.5/ by shaking	0.001 (maximum noninhibitory conc.)	>0.001	Copper concentration after 26 days of incubation was 51% higher with Hyamine 2389, compared to the biological control.	+	Duncan *et al.* 1964
Nopco CVT	Shake flask	*A. ferrooxidans*	35 °C/ initial pH 2.5/ by shaking	0.001 (maximum noninhibitory conc.)	>0.001	Copper concentration after 19 days of incubation was similar to the biological control (0.78 g/L with Nopco CVT and 0.66 g/L without)	-	Duncan *et al.* 1964
Quaker TT5386	Shake flask	*A. ferrooxidans*	35 °C/ initial pH 2.5/ by shaking	0.005 (maximum noninhibitory conc.)	>0.005	Copper concentration after 19 days of incubation was 50% higher compared to the biological control.	+	Duncan *et al.* 1964
Quaker TT5518	Shake flask	*A. ferrooxidans*	35 °C/ initial pH 2.5/ by shaking	-	0.005	Copper concentration after 19 days of incubation was about two times lower compared to the biological control.	-	Duncan *et al.* 1964
Ultrawet 40	Shake flask	*A. ferrooxidans*	35 °C/ initial pH 2.5/ by shaking	0.005	0.005	Copper concentration after 19 days of incubation was similar to the biological control.	-	Duncan *et al.* 1964

CTMAB*	Shake flask	*A. acidophilus*	30 °C/ initial pH 2.0/ by shaking	0.010**	N.R.	Surface desulfurization rate was 2.9%, 1.6%, and 0.87% higher with 0.010, 0.050 and 0.090 g/L CTMAB concentrations, respectively, compared to the biological control. However, during the 5-day surface tension study, the surface tension of the solution became similar to the biological control.	(+)	Pan *et al.* 2020

* CTMAB: cetyltrimethylammonium bromide

** g/L

As shown in Table 5, previously studied cationic surfactants include Armour 461 (systematic name unknown), Hyamine 2389 (methyldodecylbenzyl trimethyl ammonium chloride), Nopco CVT (systematic name unknown), Quaker TT5386 (systematic name unknown), Quaker TT5518 (systematic name unknown), Ultrawet 40 (systematic name unknown), and cationic cetyltrimethylammonium bromide (CTMAB). Chemical structures of the cationic surfactants, of which systematic names are known, are presented in Figure 5.

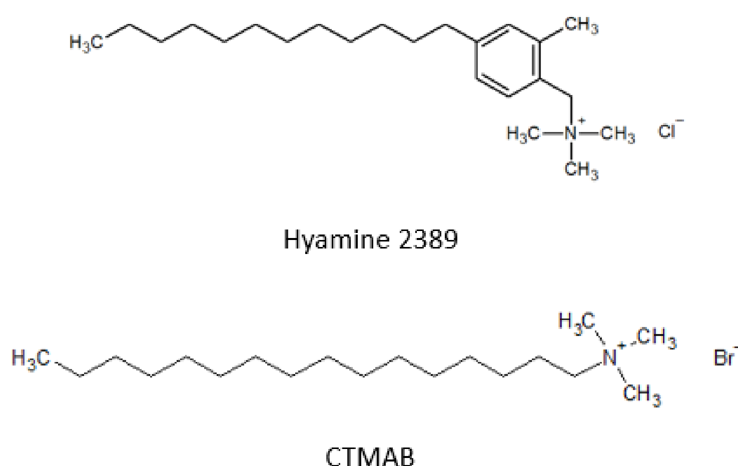


Figure 5. *Chemical structures of the known cationic surfactants, drawn with ChemSketch (Sigma-Aldrich 2003; Pubchem 2021B).*

In the study of Duncan *et al.* (1964), the influence of the cationic surfactants: Armour 461, Hyamine 2389, Nopco CVT, Quaker TT5386, Quaker TT5518, and Ultrawet 40, on bioleaching of museum-grade chalcopyrite was studied. As seen in Table 5, their results showed that Armour 461, Nopco CVT, and Ultrawet 40 had no impact on bioleaching, whereas the single studied Quaker TT5518 concentration of 0.005% (v/v) was inhibitory towards bioleaching. Their results also showed that Hyamine 2389 and Quaker TT5386, enhanced bioleaching, but they were not as efficient surfactants as the most efficient non-ionic surfactant of the study (Tween 20).

Pan *et al.* (2020) studied the influence of CTMAB on bacterial desulfurization effect and on the surface tension of the bioleaching solution. The results of the study showed that CTMAB had a slight positive effect on the surface desulfurization of the high-sulfide ore, as seen in Table 5. The surface tension results showed that after five experiment days, the surface tension of the bioleaching solution became similar to the biological control

as in case of anionic surfactant SDBS. However, CTMAB lowered the surface tension more in the beginning of the 5-day study, before desulfurization, than SDBS, but then increased to the similar level with SDBS. High surface tension of CTMAD addition group indicated that most of the surfactant was destroyed.

Non-ionic surfactants

Information about the different non-ionic surfactants studied earlier, are presented in Table 6.

Table 6. *Information about studies on the influence of non-ionic surfactants. (**Note:** (+) in "Ability to enhance bioleaching" column implies that positive impact on bioleaching is possible.)*

Surfactant	Experimental design	Microorganism(s)	Experimental conditions: Temperature/ pH/aeration	Optimal enhancing concentration (%, v/v)	Minimum inhibitory concentration (%, v/v)	Results	Ability to enhance bioleaching (+/-)	Reference
Igepal CO-630	Shake flask	*A. ferrooxidans*	35 °C/ initial pH 2.5/ by shaking	N.R.	0.01	Copper concentration after 28 days of incubation was 30% lower compared to the biological control.	-	Duncan *et al.* 1964
Triton X-100	Shake flask	*A. ferrooxidans*	35 °C/ initial pH 2.5/ by shaking	0.003–0.004	>0.005	Copper concentration after 18 days of incubation was about two times higher, with optimal concentration range, compared to the biological control.	+	Duncan *et al.* 1964
	Shake flask	*A. ferrooxidans*	30 °C/ initial pH 2.0/ by shaking	0.030*	>0.120*	Copper yield after 24 days of incubation was 42% higher compared to the biological control.	+	Zhang *et al.* 2019
Tween 20	Shake flask	*A. ferrooxidans*	35 °C/ initial pH 2.5/ by shaking	0.001–0.004	>0.01	Copper concentrations after 18 days of incubation were about three times higher, with optimal concentration range, compared to the biological control. Copper yields after 24 days of incubation were about 2.1–2.3 times higher compared to the biological control.	+	Duncan *et al.* 1964

	Shake flask	Mixed thermophilic culture	65 °C/ initial pH 1.3 and 1.1/ by shaking	0.010*	0.020*	Copper recovery was similar to the biological control, but Tween 20 addition decreased the initial leaching lag phase.	-	Ghadiri *et al.* 2019
Tween 40	Shake flask	*A. ferrooxidans*	35 °C/ initial pH 2.5/ by shaking	0.003–0.005	>0.005	Copper yields after 24 days of incubation were about 1.7–2.0 times higher compared to the biological control.	+	Duncan *et al.* 1964
Tween 60	Shake flask	*A. ferrooxidans*	35 °C/ initial pH 2.5/ by shaking	0.001	>0.005	Copper yield after 24 days of incubation was about 1.9 times higher compared to the biological control.	+	Duncan *et al.* 1964
Tween 80	Shake flask	*A. ferrooxidans*	35 °C/ initial pH 2.5/ by shaking	0.001–0.003	>0.005	Copper yields after 24 days of incubation were about 1.9–2.0 times higher compared to the biological control.	+	Duncan *et al.* 1964
	Shake flask	*A. albertensis* BY-05	30 °C/ initial pH 3.8/ by shaking	$<10^{-8}$ *	$>10^{-2}$*	Tween concentration of 10^{-8} g/L elevated the cell number about $2*10^{7}$, when cell number of the biological control was about $30*10^{7}$. Tween 80 concentration of 10^{-8} g/L also slightly accelerated SO_4^{2-} production.	(+)	Zhang *et al.* 2008
	Shake flask	Mixed thermophilic culture	65 °C/ initial pH 1.3 and 1.1/ by shaking	N.R.	0.010*	With the highest studied Tween 80 concentration of 0.02 g/L, copper recovery was decreased by 12.6%.	-	Ghadiri *et al.* 2019
	Shake flask	*A. acidophilus*	30 °C/ initial pH 2.0/ by shaking	0.1	>0.5	Surface desulfurization rate was 24% higher with 0.1% (v/v) concentration of Tween 80 compared to the biological control. With the same concentration, also the change rate of the surface tension was lowest (about 6%).	+	Pan *et al.* 2020

Plurafac LF 120	Shake flask	Mixed thermophilic culture	65 °C/ initial pH 1.3 and 1.1/ by shaking	N.R.	0.010*	Copper recovery was similar to the biological control.	-	Ghadiri *et al.* 2019
Plurafac LF 600	Shake flask	Mixed thermophilic culture	65 °C/ initial pH 1.3 and 1.1/ by shaking	N.R.	0.005*	Copper recovery was about 30% lower with 0.005 g/L Plurafac LF 600 concentration compared to the biological control.	-	Ghadiri *et al.* 2019
Lutensol XL 90	Shake flask	Mixed thermophilic culture	65 °C/ initial pH 1.3 and 1.1/ by shaking	N.R.	N.R.	Copper recovery was similar to the biological control.	-	Ghadiri *et al.* 2019
OPD**	Shake flask	Mixed cultures of *A. ferrooxidans*, *A. thiooxidans* and *L. ferrooxidans*	30 °C/ 2.0/ by shaking	0.01–0.05*	N.R.	Compared to the biological control, zinc extraction rate was 5% and 10% higher, using 0.01 and 0.05 g/L OPD, respectively.	+	Lan *et al.* 2009
PEG*** 2000	Shake flask	*A. ferrooxidans*	30 °C/ initial pH 2.0/ by shaking	0.090*	N.R.	PEG assisted more efficiently chalcopyrite dissolution between experiment days 3–21. In the final day of the experiment (day 21), copper concentration was more than two times higher compared to the biological control.	+	Zhang *et al.* 2016

* g/L

**OPD: O-phenylenediamine

***PEG: polyethylene glycol

Chemical structures of the studied non-ionic surfactants, shown in Table 6, are presented in Figure 6.

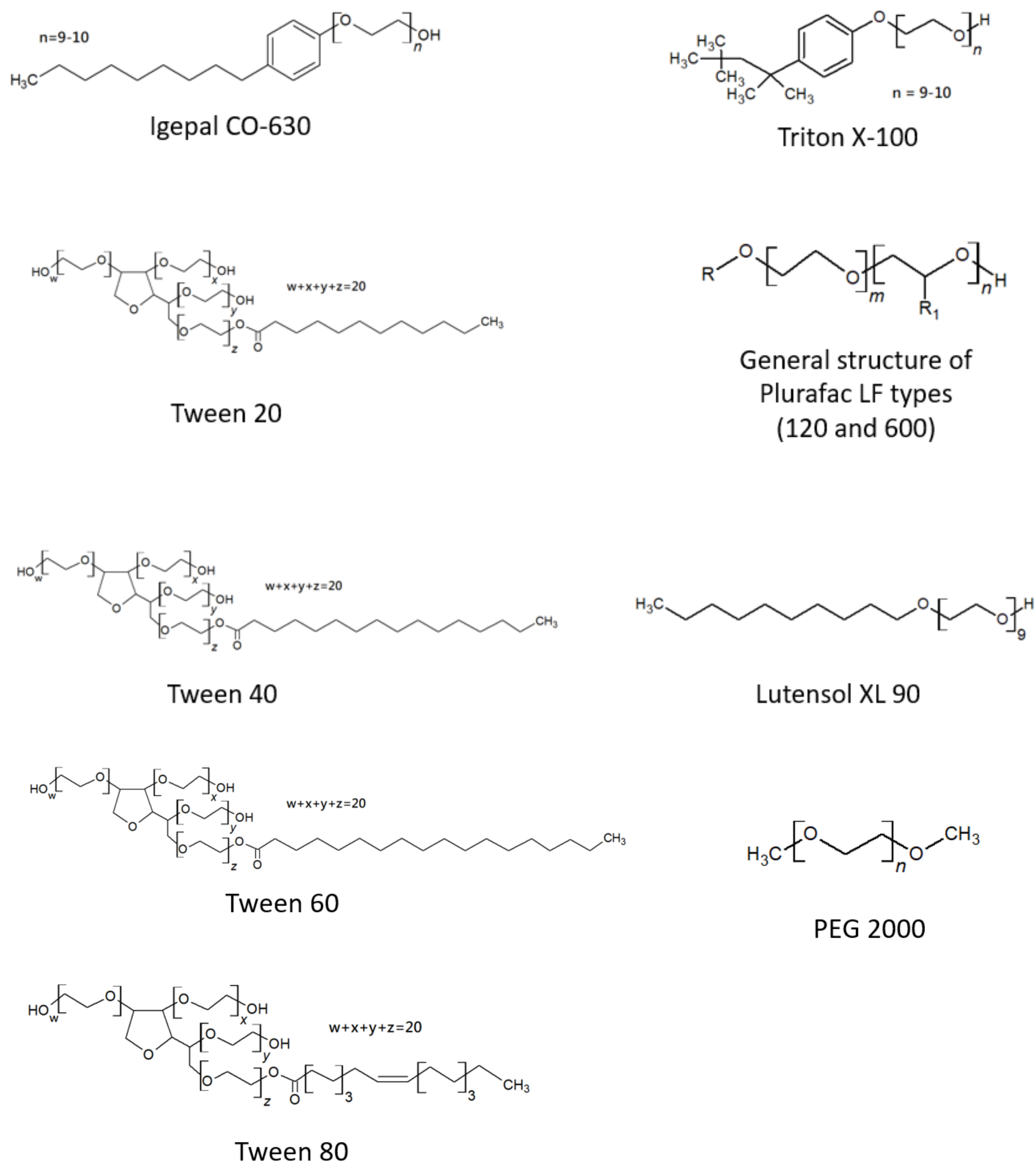


Figure 6. *Chemical structures of the studied nonionic surfactants, drawn with ChemSketch (BASF 2003; BASF 2008; Johnson 2013; Sigma-Aldrich 2021B; Sigma-Aldrich 2021C; Sigma-Aldrich 2021D).*

Duncan *et al.* (1964) studied the influence of the maximum non-inhibitory concentration of non-ionic surfactants, on bioleaching of chalcopyrite. Their study was executed by using six different non-ionic surfactants: Igepal CO-630 (polyoxyethylene (9) nonylphenyl

ether), Triton X-100 (*t*-octylphenoxypolyethoxyethanol), Tween 20 (polyethylene glycol sorbitan monolaurate), Tween 40 (polyoxyethylene sorbitan monopalmitate), Tween 60 (polyoxyethylene sorbitan monostearate) and Tween 80 (polyethylene glycol sorbitan monooleate). During their study, it was noticed that from the non-ionic surfactants, Igepal CO-630 lowered the bioleaching rate of copper, whereas other non-ionic surfactants enhanced the bioleaching process, as seen in Table 6. Their results showed that from all the studied surfactants, Tween 20 was the most effective surfactant. When Tween compounds (Tween 20, 40, 60, and 80) were more closely studied it was also reported that Tween-to-chalcopyrite ratio was more essential factor for enhancing bioleaching than Tween-to-medium ratio, and no individual constituent of Tween molecule was accountable for the enhanced bioleaching.

Influence of Triton X-100 have been studied by Zhang *et al.* (2019), in addition to Duncan *et al.* (1964). In their study, the influence of Triton X-100 on bioleaching of highly pure chalcopyrite was investigated. During their study, it was noticed that the bioleaching efficiency slightly decreased initially under the stress of Triton X-100, but then started rapidly increase, resulting in higher copper yield (Table 6). The results of their study also indicated that Triton X-100 induced bioleaching by enhancing the bioavailability of sulfur by destroying the passivation layer structure of chalcopyrite.

In the study of Ghadiri *et al.* (2019), influence on bioleaching of chalcopyrite concentrate (71.3 wt%) was studied with five different non-ionic surfactants: Tween 20, Tween 80, Plurafac LF 120 and LF 600 (consist of alkoxylated predominantly unbranched fatty alcohols, higher alkene oxides, and ethylene oxide), and Lutensol XL 90 (alkyl polyethylene glycol ethers made from a C10-Guerbet alcohol and ethyleneoxide with alkylene oxides). Their results showed that none of the studied surfactants increased the copper recovery from chalcopyrite at thermophilic temperature (65 °C) (Table 6). However, it was reported that 10 mg/L of Tween 20 decreased the initial leaching lag phase.

Influence of Tween 80 on bioleaching has been studied by Zhang *et al.* (2008) and Pan *et al.* (2020), in addition to Duncan *et al.* (1964) and Ghadiri *et al.* (2019). In the study of Zhang *et al.* (2008), the influence of Tween 80 on growth and sulfur oxidizing activity of *A. albertensis* was investigated. They reported that Tween 80 enhanced the growth and the sulfur oxidizing activity of the bacteria in 10^{-8} g/L or lower concentrations, as seen in Table 6. Pan *et al.* (2020) studied the influence of Tween 80 on bioleaching of a high-sulfide ore. Their results showed that 0.1% (v/v) Tween 80 concentration increased surface desulfurization rate, whereas concentrations higher than 0.5% (v/v) inhibited it.

In addition of the above-mentioned non-ionic surfactants, the influence of non-ionic surfactants o-phenylenediamine (OPD) (Lan *et al.* 2009) and polyethylene glycol (PEG) 2000 (Zhang *et al.* 2016) on bioleaching have also been studied. Lan *et al.* (2009) reported that OPD in low concentrations (0.01–0.05 g/L) enhanced biological zinc extraction from marmatite (Table 6). They reported that zinc was distinguishingly bioleached over iron. At the same time OPD in low concentrations seemed to favour the biological sulfur oxidation, which was possibly due the ability of the surfactant improving the interface conditions between the bacteria and elemental sulfur. During their study, OPD also seemed to eliminate sulfur product layer of marmatite, which functions as an obstacle towards efficient bioleaching. Zhang *et al.* (2016) reported that PEG 2000 accelerated chalcopyrite dissolution and increased the copper extraction, as seen in Table 6. Their results also showed that PEG accelerated the biological oxidation of sulfur by *A. ferrooxidans* by increasing bacterial attachment to the surface of the mineral and eliminating inhibitory elemental sulfur layer of chalcopyrite.

Factors affecting the performance of the surfactants

The efficiency of the surfactant can be affected by charge interactions between the surfactant and the mineral surface, and between the surfactant and the microorganisms. Therefore, it is beneficial not only to concentrate on the charges of the different surfactants, but also be aware of the charges of the bacterial cell surfaces and the mineral surfaces. (Schramm *et al.* 2003; Ghadiri *et al.* 2019; Pan *et al.* 2020)

The charge of the bacterial cell surfaces differs by the species of bacteria. Generally, the charge of the bacterial cell surface is negative, however, the charge of the bacterial cell surface varies due to the characteristics of the bacterial cell surface and environmental conditions. (Abbaszadegan *et al.* 2015) Depending on the cell surface structure, bacteria can be gram-positive or gram-negative. The cell surface of the gram-positive bacteria is formed from thick layers, which mainly consist of long anionic polymers, peptidoglycans, while the cell surface of the gram-negative bacteria is formed from an external surface layer of lipopolysaccharide and a thin layer of peptidoglycans. (Chen *et al.* 2008; Vilinska & Rao 2011; Abbaszadegan *et al.* 2015)

The cell surface charge of the bacteria can also be affected by extracellular polymeric substances (EPS), which is synthesized by various Bacteria and Archaea species. EPS consist primarily of polysaccharides, proteins, lipids, and nucleic acids, and the composition of it varies only slightly, for example, between gram-positive and gram-negative bacteria. The production of EPS is primarily provoked by the signals of the harsh growth

environment conditions, such as high metal concentrations. (Tourney & Ngwenya 2014; Flemming *et al.* 2016) Charge of EPS is determined by the type and the protonation and the deprotonation state of the functional groups present in EPS, such as carboxylic and amino groups. To the protonation and the deprotonation state of the functional groups are highly affected by the pH of the solution. Even though EPS are generally negatively charged, it has been reported that in acidic solution (pH below 2.5), EPS is positively charged (Wang 2012; Tourney & Ngwenya 2014)

It has been demonstrated that positively charged silver nanoparticles have been more bactericidal active against gram-negative bacteria, suggesting that gram-negative bacteria have usually more negatively charged cell surfaces. However, it has been reported that silver nanoparticles have been less bactericidal active against gram-negative bacteria *Escherichia coli*, suggesting that charge of the bacterial cell surface varies plenty, due to the bacterial species. (Abbaszadegan *et al.* 2015) In the study of Vilinska & Rao (2011), it has been reported that gram-negative iron oxidizing bacteria *A. ferrooxidans* has negative surface charge in the entire pH range, when the bacteria have been growing without high metal concentrations. However, when *A. ferrooxidans* is adapted to high zinc or copper concentration, it has a positive surface charge at pH range below 3.3. Decreased cell surface charge is due to the accelerated production of EPS. (Vilinska & Rao 2011) Same kind of cell surface charge behaviour has also been reported with *A. caldus* (Chen *et al.* 2008). Therefore, it can be concluded that gram-characteristics of the bacteria can generally affect to the charge of the bacterial cell surface, however, the cell surface charge also varies a lot according to the species of the bacteria and EPS formation. (Vilinska & Rao 2011; Abbaszadegan *et al.* 2015)

Previously, the diverse microbial community of the Terrafame's bioleaching heaps has been studied by Halinen *et al.* (2012). In their study, microbial composition, and dynamics of a pilot-scale bioleaching heap was investigated. In their study, the most prominent microorganisms observed in the pilot-scale heap included five species of *Acidithiobacillus*: *A.* ferrooxidans, A*. ferrivorans, A. ferridurans, A. caldus* and *A. thiooxidans*, bacterium related to clone H70, related to *Moorella* sp., *L. ferrooxidans*, *Sb. thermosulfidooxidans*, and archaeon ant b7, closest known species *Thermoplasma acidophilum*. Information about the most prominent microorganisms present in the pilot-scale bioheap and their cell surface properties are presented in Table 7.

Table 7. *Information about the most prominent microbial genus/species, presented in Terrafame pilot-scale bioheap, and their cell surface properties (Halinen et al. 2012).*

Microbial genus / species	Gram-positive/ -negative	Charge of the cell surface	Reference
Acidithiobacillus • *A. ferrooxidans* • *A. ferrivorans* • *A. ferridurans* • *A. caldus* • *A. thiooxidans*	Gram-negative	Cell surface charge is generally negatively charged, however, when pH is low (≤3.5) and high concentration of metals is present, cell surface of the bacteria is positively charged due the formation of EPS.	Vilinska & Rao, 2011, Chen *et al.* 2008 & Abbaszadegan *et al.* 2015
Bacterium related to clone H70, related to *Moorella* sp.	Gram-positive (*Moorella* sp.)	Negatively charged cell wall. No information available about EPS formation.	Slobodkin *et al.* 1997
L. ferrooxidans	Gram-positive	Negatively charged cell wall. In bioleaching conditions, forms positively charged EPS.	Bleeze *et al.* 2018
Sb. thermosulfidooxidans	Gram-positive	Negatively charged cell wall. In bioleaching conditions forms positively charged EPS.	Vardanyan *et al.* 2019
Archaeon ant b7, closest known species *Thermoplasma acidophilum*	Gram-negative (*Thermoplasma*)	Constant negative charge of the cell surface when pH is 2–5. No information available about EPS formation.	Hsung & Haug, 1977

Performance of the surfactants can be affected, in addition to the cell surface charges of the bacteria, by surface charge of the ore. Surface charge of the ore is mainly influenced by the mineral composition of the ore, and pH of the solution, when the ore is present in colloidal system. (Tourney & Ngwenya 2014; Bleeze *et al.* 2018) Generally sulfide ores, such as Terrafame polymetallic black schist ore, form a negatively charged surface layer, when the ore particles are present in acidic aqueous solution. Surface charge of the sulfides can, however, change according to the mineral composition of the sulfide, pH of the solution, and stage of the bioleaching. (Bleeze *et al.* 2018; Chen *et al.* 2008) Terrafame polymetallic black schist ore is mainly formed from zinc, nickel, iron, copper, and cobalt sulfide. Information about surface charges of these various sulfide minerals, present in Terrafame black schist ore, are shown in Table 8.

Table 8. *Information about surface charges of the various sulfide minerals, present in ore of Terrafame.*

Sulfide mineral	Surface charge	Reference
Zinc sulfide	Neutral	Wang *et al.* 2011
Nickel sulfide	Negative	Li *et al.* 2014
Iron sulfide (pyrite)	Negative	Bleeze *et al.* 2018
Copper sulfide (chalcopyrite)	Chalcopyrite has a negatively charged surface. Surface charge of the passivation layer, which have been proposed to form, for example, from jarosite and different sulfur species, can vary between neutral, positive, and negative.	Sadowski *et al.* 2001 & Chen *et al.* 2008
Cobalt sulfide	Negative	Li *et al.* 2014

From the possible sulfide minerals present in Terrafame ore, zinc sulfide (ZnS) has been reported to have neutral charge, when pH has been below ~ 3. However, it has also been reported that the elemental composition of zinc sulfide affects to the surface charge. For example, higher sulfide concentration of the mineral lead to a more negative charge (Wang *et al.* 2011). Surface properties of nickel and cobalt sulfide have been investigated by Li *et al.* (2014) in their bioleaching experiment of four different thermophiles. They reported that nickel and cobalt sulfide have a negative surface charge at acidic conditions. From the iron sulfides, for example, surface charge of pyrite (FeS_2), have been reported to be negative in acidic bioleaching conditions. Negative charge of pyrite has been reported, for example, to have a positive effect to the attachment of bacteria, which have formed positively charged EPS in bioleaching conditions (Bleeze *et al.* 2018).

From the different sulfide species, copper sulfide, chalcopyrite, and its surface, has been studied by Chen *et al.* (2008), during the bioleaching process. They have been reported that surface charge of chalcopyrite is negatively charged in acidic bioleaching conditions, when bacterial cells, such as *A. caldus*, have been presented. However, passivation layer formed onto the surface of the chalcopyrite, during the bioleaching process, is possibly able to change the surface charge of the mineral. It is proposed that the passivation layer is partially formed from jarosite, which has been reported to be positively charged mineral below pH 3.9. (Sadowski *et al.* 2001; Ren *et al.* 2020) In addition to jarosite, it has been also proposed that passivation layer of chalcopyrite is formed from different sulfur species, such as elemental sulfur (S^0) and insoluble sulfate (SO_4^{2-}), which can alter

the surface charge of the chalcopyrite to neutral direction or more negative direction. (Chen *et al.* 2008; Zhang *et al.* 2016; Ren *et al.* 2020)

3.2.5 EPS inducing additives

As seen in previous chapter, various bioleaching microorganisms are able to synthesize EPS, when metal concentrations are high in growth environment. Because at low pH formed EPS is positively charged, it is possible that it can enhance the attachment of the bioleaching microorganisms onto the surface of negatively charged ore material, such as sulfide ores. (Sadowski *et al.* 2001; Wang *et al.* 2011; Wang 2012; Li *et al.* 2014; Tourney & Ngwenya 2014; Bleeze *et al.* 2018). Formed EPS can function in various fields of microbial activity, for example, by forming an actively working and absorbing surface area, serving a space for oxidation, enhancing microbial adhesion, functioning as a protective layer against various inhibitors (i.e., metals), and therefore potentially enhance the bioleaching efficiency of the bacteria (Figure 7) (Yu *et al.* 2013; Tourney & Ngwenya 2014; Flemming *et al.* 2016).

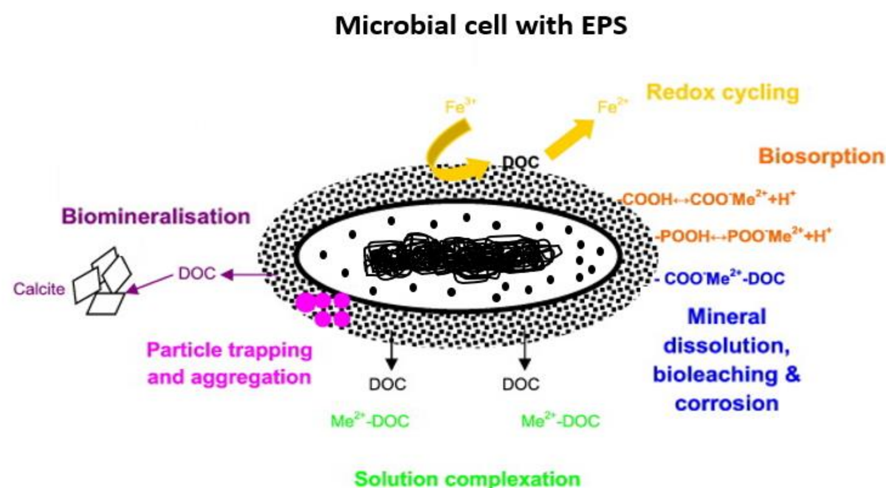


Figure 7. *Various microbial processes affected by extracellular polymeric substances (EPS). Spotted layer covering the microbial cell presents EPS. (modified from Tourney & Ngwenya 2014)*

The formation of EPS can also be potentially enhanced by using organic EPS inducing additives. The studied additives have been mainly monosaccharides. Although these organic compounds have been reported to have inducing effect on EPS, it is also possible that using these additives with unadapted chemolithotrophs bacteria can result inhibition. (Flemming *et al.* 2016; Bellenberg *et al.* 2012; Saavedra *et al.* 2020) In mining conditions,

it has been reported that the bioleaching microorganisms are complex, and consist of chemolithotrophs, chemomixotrophs and heterotrophs. Therefore, it is probable that chemolithotrophs from the mining conditions have already been contacted with carbohydrates (Kinzler *et al.* 2003; Petersen 2016).

Information about the studies on EPS inducing additives, are presented in Table 9.

Table 9. *Information about the studies on the different EPS inducing additives. (**Note:** (+) in "Ability to enhance bioleaching" column implies that positive impact on bioleaching is possible, and - no impact or negative impact on bioleaching.)*

EPS inducing additive	Experimental design	Microorganism(s)	Experimental conditions: Temperature/ pH/aeration	Optimal enhancing concentration (g/L)	Minimum inhibitory concentration (g/L)	Results	Ability to enhance bioleaching (+/-)	Reference
Galactose	Shake flask	*A. ferrooxidans*	28 °C/ initial pH 1.8/ by shaking	2.5 (single studied concentration)	N.R.	Galactose concentration of 2.5 g/L increased ConA* signal from *A. ferrooxidans* about 2.6 times (with galactose: 200±8 MGV**, without galactose: 45±4 MGV). Pyrite dissolution rate was similar, compared to the biological control.	-	Bellenberg *et al.* 2012
	Shake flask	*L. ferrooxidans*	30 °C/ initial pH 1.8/ by shaking	2.5	N.R.	EPS production was 0.63 g/g cell dry weigh, when 2.5 g/L of galactose was present during pre-cultivation, while EPS production without galactose was 0.044 g/g cell dry weight.	(+)	Aguirre *et al.* 2018
	Bioreactor	*A. ferrooxidans*	30 °C/ 1.8/ 0.5 L/min air flow	3.5	0.25 (when cell density was low)	Microbial cultures cultivated in the presence of 3.5 g/L of D-galactose, reached about 25% higher tolerance against ferric iron (with galactose: 48±1.9 g/L, without galactose: 39±0.47 g/L).	(+)	Saavedra *et al.* 2020
Glucose	Shake flask	*A. ferrooxidans*	28 °C/ initial pH 1.8/ by shaking	2.5 (single studied concentration)	N.R.	Glucose concentration of 2.5 g/L increased ConA signal from *A. ferrooxidans* about 2.6 times (with glucose: 120±13 MGV, without glucose: 45±4.0 MGV). Pyrite dissolution rate was similar, compared to the biological control.	-	Bellenberg *et al.* 2012

Casamino acids	Shake flask	*A. ferrooxi-dans*	28 °C/ initial pH 1.8/ by shaking	N.R.	2.5	ConA signal was similar to the biological control (with casamino acids: 57±4.0 MGV, without casamino acids: 45±4.0 MGV). Pyrite dissolution rate was lower compared to the biological control.	-	Bellenberg *et al.* 2012

*ConA: concanavalin A

**MGV: mean gray value

In the study of Bellenberg *et al.* (2012), the influence of D-galactose on the production of capsular polysaccharides (CPS) of the cell was studied. CPS is one of the possible forms of ESP, in addition to more loosely contacted slime polysaccharides. They reported that D-galactose increased CSP amount of the *A ferrooxidans* cells, when the cells were grown as biofilms, as seen in Table 9. The CPS amount was visualized by using fluorescently labelled lectin Concanavalin A (ConA) signal and confocal laser scanning microscopy (CLSM). In their study, leaching of pyrite was also investigated by observing planktonic cell numbers and concentration of sulfate. Their results showed that galactose did not enhance bioleaching of pyrite when *A. ferrooxidans* were present. (Bellenberg *et al.* 2012)

Aguirre *et al.* (2018) reported that EPS amount increased, and the chemical composition of ESP changed, when galactose concentrations increased (Table 9). During their study, it was also noticed that galactose enhanced the attachment of the microbial cells. The highest attachment rate was reported, when 2.5 g/L of galactose was present. According to these above-mentioned results, it was suggested that galactose may enhance the biological oxidation processes of the studied bacteria (*L. ferrooxidans*).

Saavedra *et al.* (2020) studied the influence of galactose on the formation of EPS, by evaluating the biological oxidation activity of *A. ferrooxidans* and its tolerance against high concentrations of Fe(III). They reported that D-galactose inhibited the cell growth, when the cell density was low, but this inhibitory effect was not present with higher cell densities. The results of their study also showed that microbial cultures cultivated with D-galactose reached higher EPS formation and tolerance against Fe(III), as seen in Table 9, suggesting that the greater volume of EPS on cells, leaded to enhanced tolerance of the bacteria against Fe(III).

Bellenberg e*t al.* (2012) reported, in addition to D-galactose, also the influence of glucose and casamino acids on CPS formation. The results of their study showed that glucose increased CPS formation similarly to galactose (Table 9). However, this same CPS formation promoting effect as galactose and glucose was not reported with casamino acids.

3.2.6 Other bioleaching enhancing additives

In addition to these previously mentioned additives, organic compound of ethylene thiourea (ETU), has also shown signs of enhancement of bioleaching (Ren *et al.* 2020). ETU is an organosulfur compound, and an environmental degradation product of ethylene bisdithiocarbamate fungicides (Figure 8) (Engst & Schnaak 1974). Ren *et al.* (2020) studied the catalytic influence of ETU on bioleaching of chalcopyrite by *A. ferrooxidans*, by using stirred tank reactors. The experiment was conducted at room temperature with

initial pH of 1.7 of the leaching solution. The results of their study showed that 0.010, 0.050 and 0.10 g/L concentrations of ETU were able to increase the copper extraction yield by 32%, 86%, and 86%, respectively, compared to the biological control. During their study, it was also observed that oxidation-reduction potential (ORP) plateau was lower compared to the biological control, when 0.050 and 0.10 g/L of ETU was present in solution, because of the impact of ETU on the ORP of the bioleaching solution. ORP plateau phase indicates that all Fe(II) is oxidized into Fe(III) form.

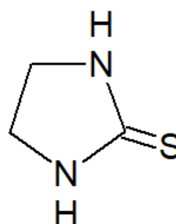


Figure 8. *Chemical structure of ETU, drawn with ChemSketch (Ren et al. 2020).*

According to these above-mentioned results it seems that ETU may have a significant role in enhancing chalcopyrite bioleaching. (Ren *et al.* 2020) It is believed that the catalytic effect of ETU, is due to its thiocarbonyl functional group. Thiocarbonyl functional group forms metal-ligand complexes with transitional metals, which have partly or fully occupied d-orbitals, such as Cu(I) and Zn(II) (Bombicz *et al.* 2004). During the leaching, half bound surface metal ions, in the solid-liquid interface of the sulfide ore, are bonded to the metal-ligand complex. In the metal-ligand complex, synergic bonding, between the metal and the ligand, relieves electrons to the ligand, when enhanced stability of the complex is achieved. This complex forming ability can destabilize the surface structure of the chalcopyrite and accelerate the oxidation of the Cu-S compound (Ren *et al.* 2020).

The enhancing effect of compounds, having thiocarbonyl functional group, on leaching of sulfide minerals have also been studied further and patented. The patent application regards leaching of metals enhanced by contacting the sulfide minerals with an acidic sulfate solution, containing ferric sulfate, and a reagent having thiocarbonyl group, such as ETU and thiourea (TU). In the patent, enhancing influence of reagent TU on leaching of the common sulfide minerals, which contain partly or fully occupied d-orbital transitional metals, such as Cu(I), Cu(II), Ni(II), Co(II) and Cd(II), has been used as an example for the functionality of the thiocarbonyl group in leaching process. In the patent, it is reported that TU has been able to increase mineral extraction about 1.3–10 times, depending on the mineral composition. Because both ETU and TU contain thiocarbonyl functional group, it is probable that also ETU can increase the extraction rate of the sulfide

minerals, which contain partly or fully occupied d-orbital transitional metals, similarly to TU. (WO2018072029A1)

Other possible bioleaching enhancing additives, in addition to ETU, are activated carbon and cysteine, which have been studied, for example, by Nakazawa *et al.* (1998) and Rojas-Chapana & Tributsch (2000), respectively. In the study of Nakazawa *et al.* (1998), the influence of activated carbon on bioleaching of chalcopyrite by *A. ferrooxidans* was studied. The results showed that activated carbon enhanced the extraction of copper from chalcopyrite and the recovery of copper increased with increasing quantity and/or decreasing particle size of the activated carbon. Even though activated carbon enhanced bioleaching of chalcopyrite, it is not included as one of the potential bioleaching enhancing additives used in heap bioleaching, because it is generally used as carrier material for biofilm in bioreactor operation and not to enhance heap bioleaching.

Rojas-Chapana & Tributsch (2000) have been studied the influence of amino acid cysteine on bioleaching of pyrite. They reported that small concentration of cysteine contacted with pyrite tripled the leaching rate of pyrite, compared to the biological control. It was also reported that cysteine oxidized pyrite without bacteria, with leaching rate similar to the bioleaching in normal leaching conditions. Even though cysteine seemed to enhance bioleaching of pyrite, it is not included either as potential bioleaching enhancing additives used in heap bioleaching, because it is not economically viable in large scale.

In addition to these above-mentioned additives, inorganic chloride ions are also known for enhancing the chemical leaching of chalcopyrite. However, the same enhancing effect on bioleaching have varied. (Kinnunen & Puhakka 2004; Bevilaqua *et al.* 2013) In the study of Bevilaqua e*t al.* (2013), it was demonstrated that 5.8 g/L (100 mmol/L) of NaCl enhanced bioleaching of chalcopyrite by *A. ferrooxidans*, whereas, in the study of Kinnunen & Puhakka (2004), same enhancing effect of NaCl was not observed on bioleaching of chalcopyrite, when the iron oxidizing culture was dominated by *L. ferriphilum*. Because the effects of chloride ions on bioleaching have varied with the studied microorganisms and they have not been studied with the mixed microbial culture originating Terrafame heap bioleaching area, chloride ions are not considered as a potential bioleaching enhancing additive for the case study.

4. MATERIALS AND METHODS

The experiments of the study were performed in four parts. In the first part, possible inhibition of two process liquors of the Terrafame battery chemical production plant: AS bleed and feed, on biological iron oxidation was studied. In the second part, possible inhibition of various constituents of the process liquors: carboxylic acid (neodecanoid acid), Cyanex 272, Baysolvex D2EHPA, Nessol D100, and AS, on biological iron oxidation was investigated. In the third part, iron oxidation enhancing effect of ammonium, present in the process liquors, AS bleed and feed, was studied. In the fourth part, possible inhibition effect of AS bleed towards sulfur oxidation, was investigated.

4.1 Microbial cultures

In all the experiments, a mixed microbial culture, originating from an irrigating leach liquor, which is a recirculated leaching solution used for irrigation of the agglomerated ores, was used as an inoculum. The irrigating leach liquor sample was provided by multi-metal company Terrafame Oy. The mixed microbial culture was enriched for iron oxidation. This iron oxidizing culture was used as a stock culture for other cultures, used in the study, according to Figure 9. The stock culture was used to enrich sulfur oxidizing microorganisms, which were used in the AS bleed inhibition experiment with sulfur oxidizing enrichment culture. In addition to the sulfur oxidizing enrichment culture, the stock culture was also used as an inoculum for iron oxidizing ammonium deficit (AD) culture 1 and 2, which were used as an inoculum in the ammonium deficiency experiment. During ammonium deficiency, microbial culture has a shortage of macronutrient nitrogen (ammonium), which negatively effects on the cell growth, and thereby reduces the iron oxidation rate of the microbial culture (Niemelä *et al.* 1994; Rawlings 2007).

Figure 9. *Diagram of the microbial cultures used in the experiments. The arrows indicate the source of the inoculum. AD in the iron oxidizing AD culture 1 and 2, is an abbreviation for ammonium deficit.*

4.1.1 Iron oxidizing cultures

In the iron oxidizing experiments, two different types of iron oxidizing cultures were used as an inoculum.

Iron oxidizing enrichment culture (stock culture)

The iron oxidizing enrichment culture was enriched from the irrigating leach liquor, and pre-cultivated prior to the experiments to provide a high bacterial density for the experiment. The iron oxidizing enrichment cultures were transferred into fresh growth medium on every seventh day. All iron oxidizing cultures were supplemented with Fe(II) stock, mineral salts medium (MSM), and trace elements solution (TES), according to Table 10, for enrichment indigenous iron oxidizing microorganisms. The total working volume of the iron oxidizing culture was 100 mL and initial pH ~2.0 of the culture was not separately adjusted to.

Table 10. *The constituents of the iron oxidizing enrichment culture.*

Culture	Inoculum (%, v/v)	MSM (%, v/v)	TES (%, v/v)	Fe(II) stock (%, v/v)	Milli-Q water (%, v/v)
Iron oxidizing enrichment culture	10	10	1	25	54

The iron oxidizing enrichment culture was incubated with the orbital shaking incubator Certomat R (Sartorius, Germany), which operated at 150 rpm and 27 ± 2 °C. The culture was weekly transferred into a fresh growth medium, in order to provide active inoculum for the experiments. The iron oxidizing enrichment culture was used as an inoculum for the other cultures and in most of the iron oxidation experiments.

Iron oxidizing AD cultures

Both iron oxidizing AD cultures were enriched from the iron oxidizing enrichment culture. Iron oxidizing AD culture 1 was supplemented with ammonium, in form of $(NH_4)SO_4$, whereas iron oxidizing AD culture 2 was cultivated without ammonium. The iron oxidizing AD culture 1 was used as an inoculum for the positive controls, whereas the iron oxidizing AD culture 2 was used as inoculum for the other shake flasks in the ammonium deficiency experiments. The iron oxidizing AD cultures were supplemented with Fe(II) stock, MSM, and TES, according to Table 11. The cultures were prepared similarly to iron oxidizing enrichment culture, but the inoculum volume was only 1% (v/v), and MSM, used with the iron oxidizing AD culture 2, was prepared without AS ($(NH_4)_2SO_4$). The volume of inoculum was lower with these cultures, in order to remove all ammonium from AD culture 2 prior to the ammonium deficiency experiment. Initial pH of the cultures was adjusted to 2.0 with concentrated H_2SO_4 (95–97%) or 40 g/L (1 mol/L) NaOH.

Table 11. *The constituents of the iron oxidizing AD cultures.*

Culture	Inoculum (%, v/v)	MSM (%, v/v)	MSM without $(NH_4)_2SO_4$ (%, v/v)	TES (%, v/v)	Fe(II) stock (%, v/v)	Milli-Q water (%, v/v)
Iron oxidizing AD culture 1	1	10		1	25	63
Iron oxidizing AD culture 2	1		10	1	25	63

The iron oxidizing AD cultures were transferred into fresh growth medium on every seventh day. Before starting the ammonium deficiency experiment, ammonium concentration of the cultures was monitored and iron oxidation was observed, by following pH, redox and Fe(II) concentration. The purpose of the observation was to see when the iron oxidizing AD culture 2 would be suitable for the ammonium deficiency experiment. Iron oxidizing AD culture 2 was estimated to be suitable for the experiment, when no ammonium was present in the culture medium and iron oxidation rate was decreased, compared to the iron oxidizing AD culture 1. During the observation, samples were taken every weekday. The sampling volume was 4 mL of the first and last samples, and 2 mL on other days. The iron oxidizing AD cultures were incubated with the orbital shaking incubator Certomat R (Sartorius, Germany), which operated at 150 rpm and 27 ± 2 °C.

4.1.2 Sulfur oxidizing enrichment culture

The sulfur oxidizing enrichment culture was enriched from the iron oxidizing enrichment culture. The culture was supplemented with elemental sulfur (S^0), MSM, and TES, according to Table 12. The initial pH of the culture was adjusted to 2.0 with concentrated H_2SO_4 (95–97%) or 40 g/L (1 mol/L) NaOH.

Table 12. *The constituents of the sulfur oxidizing enrichment culture.*

Culture	Inoculum (%, v/v)	MSM (%, v/v)	TES (%, v/v)	S^0 (g/L)	Milli-Q water (%, v/v)
Sulfur oxidizing enrichment culture	10	10	1	10	79

The sulfur oxidizing enrichment culture was pre-cultivated for four weeks before the sulfur oxidizing AS bleed experiment, for providing a dense sulfur oxidizing microbial inoculum for the experiment. During the pre-cultivation, sulfur oxidation was observed by following pH and sulfate concentration. Samples were taken on Mondays and Fridays. The sampling volume was 4 mL. After three weeks of pre-cultivation, the culture was transferred into a fresh growth medium, which was then used as an inoculum for the AS bleed experiment after seven days. During the sulfur oxidizing AS bleed experiment, culture was transferred into fresh growth medium every two weeks. Similarly, as the iron oxidizing cultures, the sulfur oxidizing culture was incubated at 150 rpm and 27 ± 2 °C.

4.2 Growth medium

The microbial growth medium, used in the experiments, consisted of 10% v/v of MSM, and 1% v/v of TES. The basic MSM was made of 37.5 g/L $(NH_4)_2SO_4$, 18.75 g/L $Na_2SO_2 \cdot 10\ H_2O$, 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 $Ca(NO_3)_2 \cdot 4\ H_2O$. However, MSM, used with the iron oxidizing AD culture 2 was similarly prepared as the basic MSM, but did not contain any $(NH_4)_2SO_4$. TES consisted of 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. The pH of MSM and TES was adjusted to 1.8 and 1.5, respectively, with concentrated H_2SO_4 (95–97%). Manufacturers of the chemicals used in these experiments are presented in Appendix 1.

The working volumes of the iron and sulfur oxidizing cultures were 100 mL, which was accomplished by adding required volume of deionised Milli-Q water. Cultivation of the cultures were executed by using 250 mL shake flasks. Growth medium was sterilized each time by autoclaving MSM, TES, and Milli-Q containing shake flasks at 121 °C for 20 minutes.

With the iron oxidizing cultures, Fe(II) stock was used as a substrate. Fe(II) concentration used with the iron oxidizing cultures was 5.6 g/L, which was achieved by adding 25 mL of Fe(II) stock, containing 22.5 g/L Fe(II). Fe(II) stock consisted of 112 g/L $FeSO_4 \cdot 7\ H_2O$. $FeSO_4 \cdot 7\ H_2O$ for analysis was used as a substrate, during cultivation, and technical grade $FeSO_4 \cdot 7\ H_2O$ was used in the experiments. The pH of the Fe(II) stock was adjusted to 1.7, with concentrated H_2SO_4 (95–97%), before and during the chemical addition, for preventing the chemical oxidation of Fe(II) into Fe(III). The Fe(II) stock was then sterile-filtered through 0.2 µm sterile polyethersulfone membrane filter (VWR International, US) in laminar, and stored in a cold room at 4 °C with parafilm seal, protected from light.

With the sulfur oxidizing culture, 10 g/L elemental sulfur was used as a substrate. Elemental sulfur was sterilised and dehydrated by keeping the sulfur in the oven at 105 °C over night or over weekend and stored in a desiccator at room temperature. Both Fe(II) stock and elemental sulfur were added into the growth medium after the autoclave of the shake flasks.

4.3 Process liquors and their constituents

4.3.1 Process liquors of the battery chemical production plant

The two studied process liquors (AS bleed and feed) of the battery chemical production were provided by multi-metal company Terrafame Oy. Because during the experiments the battery chemical production plant was at construction stage, AS bleed and feed, studied during the experiments, were collected from the pilot-scale AS crystallization process. AS feed is functioning as a feed of the AS crystallization process, whereas, AS bleed is a mother liquor, a residual solution resulting from the AS crystallization. When the battery chemical plant will be in operational stage (Figure 10), nickel cobalt sulfide, from the metal recovery area, will function as feed of the overall process. Before the AS crystallization process, nickel cobalt sulfide is first treated with pressure dissolution, and then iron precipitate is removed from the process liquor by settling. After the settling, impurities, cobalt, and nickel are extracted in separate stages, by using various organic

solvents, and then crystallized into cobalt sulfate, nickel sulfate, and AS, as shown in Figure 10. (Terrafame 2018)

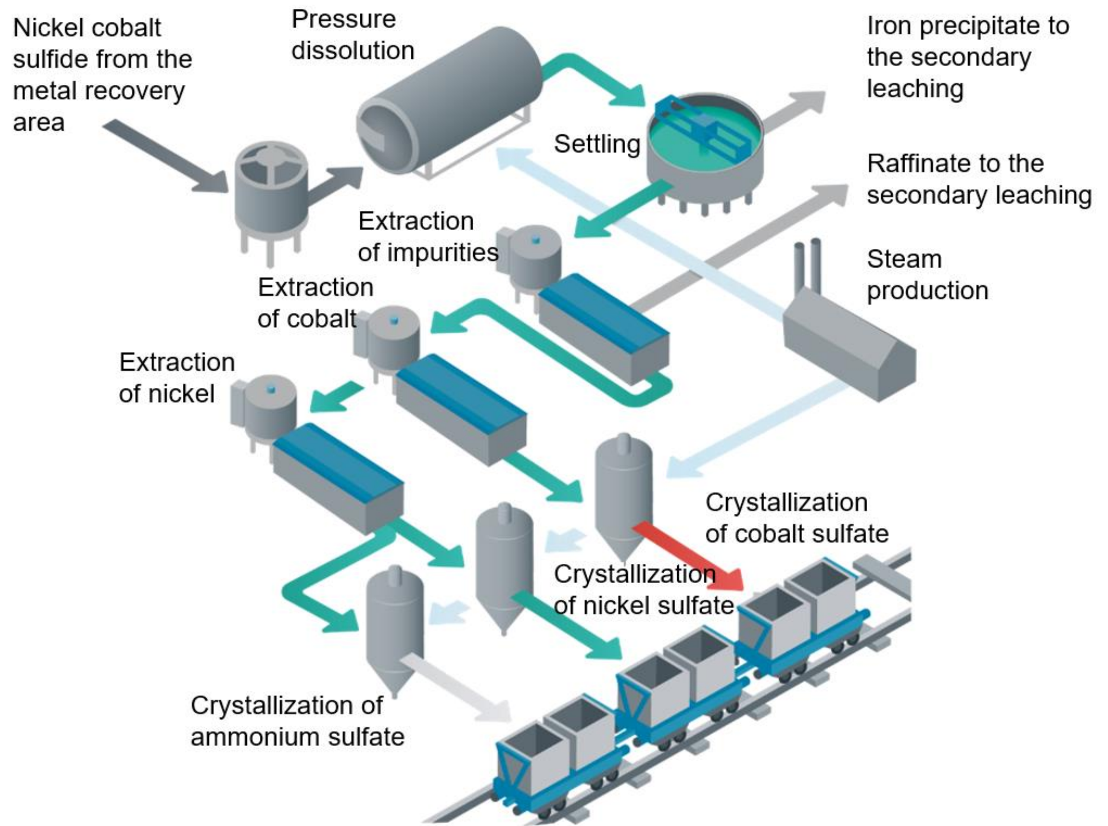


Figure 10. *Schematic diagram of the battery chemical production plant process (modified from Terrafame 2018).*

During the operational stage, both process liquors can contain approximately 150 g/L of ammonium (550 g/L of AS), which may enhance the bioleaching process, when the bioleaching acidophilic microorganisms are supplemented with it in low concentrations (Niemelä *et al.* 1994; Ahoranta *et al.* 2017). However, the ammonium concentration of the solutions was slightly lower because AS bleed and feed used during the experiments, were collected from the pilot-scale AS crystallization process. Average ammonium concentration of AS bleed and feed was 117 g/L (428 g/L of AS) and 79 g/L (288 g/L of AS), respectively.

Because the process liquors are associated with the metal extraction, they contain organic solvent residues which can possibly be toxic towards bioleaching microorganisms (Torma & Itzkovitch 1976; Tuttle & Dugan 1976; Chen *et al.* 2015). It is estimated that TOC concentrations of AS bleed and feed are 300 mg/L and 180 mg/L, respectively. This TOC contains mainly residues of carboxylic acids and Nessol D100 type of solvents but

can also consists of the other organic solvents used in Terrafame's metal extraction process (Cyanex 272 and Baysolvent D2EHPA type of extractants).

4.3.2 Constituents of the process liquors

Various chemicals of AS, neodecanoid acid, Nessol D100, Cyanex 272, and Baysolvex D2EHPA, which will be present in the process liquors, AS bleed and feed, were studied in the separate experiments. In the AS experiments, 450 g/L AS stock, made in the laboratory, was used instead of 550 g/L AS stock, which ammonium concentration would have been similar as in AS bleed and feed during the operational stage. 450 g/L AS stock was used since it was the maximum concentration of AS in Milli-Q water, which was able to be made in the laboratory conditions. The AS stock was prepared by adding 450 g/L of solid $(NH_4)_2SO_4$ into deionised Milli-Q water, its pH was adjusted to 2.0 with concentrated H_2SO_4 (95–97%), and finally the solution was autoclaved at 121 °C for 20 minutes. The AS stock was stored at room temperature.

The studied organic solvents, used in the Terrafame's metal extraction processes, consists of neodecanoid acid, Nessol D100, Cyanex 272, and Baysolvex D2EHPA. The chemical structures of the solvents are presented in Figure 11 (ExxonMobil 2018; LANXESS 2018; Neste 2019; Solvay 2019).

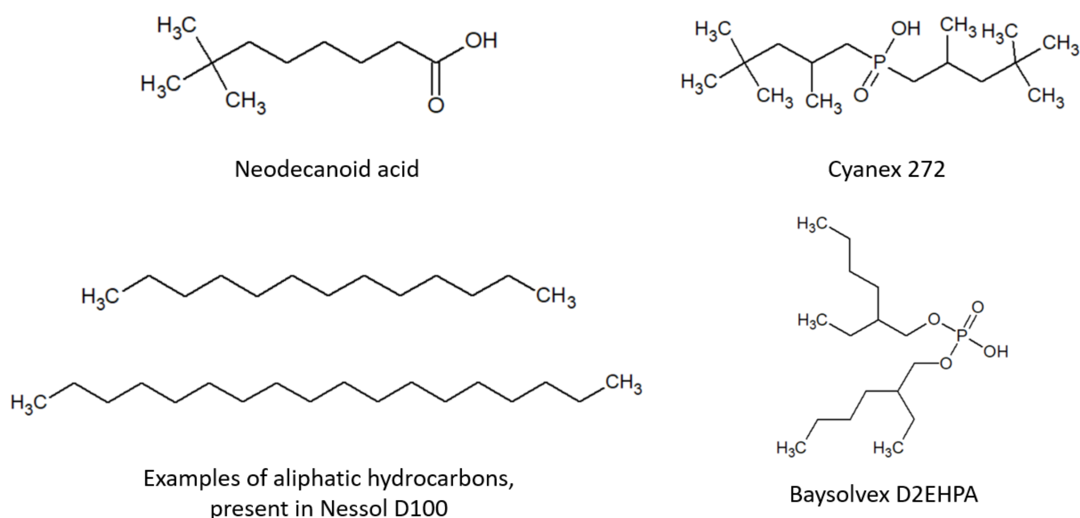


Figure 11. *Chemical structures of the studied solvents used in the Terrafame's metal extraction processes, drawn with ChemSketch (ExxonMobil 2018; LANXESS 2018; Neste 2019; Solvay 2019).*

Neodecanoid acid is a carboxylic acid, which molecular formula is $C_{10}H_{19}O_2H$ (ExxonMobil 2018). It is used in various fields of industry, primarily as an intermediate when producing other chemical products. In the mineral processing field, neodecanoid acid can be used as a solvent for extracting non-ferrous metals. (Gotfryd *et al.* 2015) Neodecanoid acid used in the experiments was 100% concentrated neodecanoid acid, supplied by ExxonMobil Chemical Company, US. (ExxonMobil 2018) Nessol D100, manufactured and supplied by Neste Corporation, Finland, is a trade name for a mixture of aliphatic hydrocarbons (C_{13}–C_{18}). Nessol D100 is used in various industry sectors, such as in surface coatings, fuels, and as a solvent in mineral processing sector. Nessol D100 used in the experiments was supplied by Neste Corporation, Finland. (Neste 2019)

Cyanex 272 is a dialkyl phosphinic liquid extractant, commonly used for separating cobalt from nickel. Cyanex 272 used in the experiments was 100% solution, supplied by Solvay Business Services Latvia SIA, Latvia. (Solvay 2019) Baysolvex D2EHPA (Bis(2-ethylhexyl) hydrogen phosphate) is an alkyl hydrogen phosphate. Baysolvex D2EHPA is used as a versatile extractant in various fields of industry, such as in mineral processing. Baysolvex D2EHPA used in the experiments was 90–100% (w/w) solution, supplied by LANXESS Deutschland GmbH, Germany. (LANXESS 2018)

4.4 Experimentation

All experiments were performed in 250 mL shake flasks (100 mL working volume). During the experiments, the shake flasks were incubated in orbital shaking incubators, which operated at 150 rpm and 27 °C (± 2 °C). The shake flasks containing more concentrated process liquors or their constituents, KS 4000 i Control incubator (IKA, Germany) with a plastic hood and local exhaust ventilation over was used, whereas the shake flasks with more diluted process liquors and their constituents were incubated in larger Innova 44 incubator (New Brunswick Scientific, US) without a plastic hood (Figure 12).

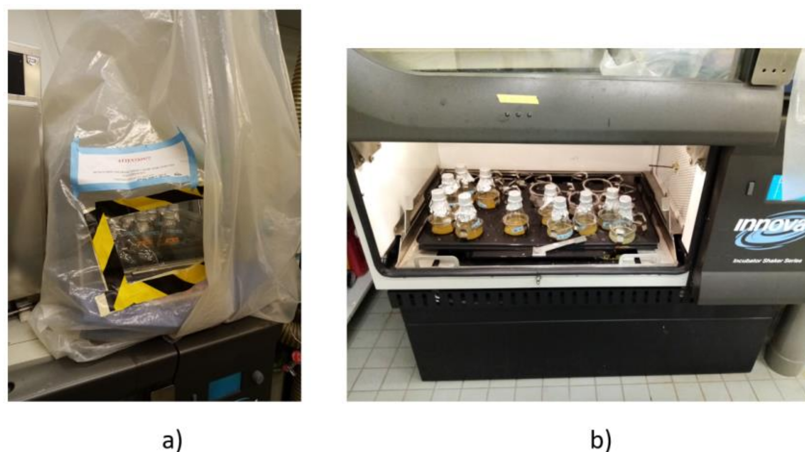


Figure 12. *Two incubators used during the experiments: (a) KS 4000 i Control incubator (IKA, Germany) with a plastic hood and local exhaust ventilation, and (b) larger Innova 44 incubator (New Brunswick Scientific, US) without a plastic hood.*

All experiments, except the neodecanoid acid 50% kinetics experiment, were performed in duplicates. The kinetic experiment was implemented with four shake flasks. The growth medium was sterilized each time by autoclaving MSM, TES, and Milli-Q containing shake flasks at 121 °C for 20 minutes. During every experiment, the initial pH of the culture was adjusted to 2.0, after autoclaving and Fe(II) stock addition. The pH adjustment generally performed after the addition of the studied solution, however, with the concentrated organic solvent solutions, pH adjustment occurred before the solution and inoculum addition. With the sulfur oxidizing culture, elemental sulfur was added after pH adjustment and AS bleed addition, and before inoculum. More information about the performed experiments is presented in Table 13. More detailed information about the experiments is presented in Chapters 4.1.1– 4.1.8.

Table 13. *Detailed information about the experimental parameters and conditions used during the different experiments.*

Experiment	Studied solution(s) and concentrations	Inoculum	MSM (%, v/v)	TES (%, v/v)	Fe(II) stock (%, v/v)	S^0 (g/L)	Initial pH	Control(s)
AS bleed experiments								
AS bleed 1st inhibition experiment	AS bleed (0.1, 1, 2, 10, and 50% (v/v))	10% (v/v) of 4-days old iron oxidizing enrichment culture	10	1	25		2.0	Positive control (without AS bleed, inoculated with the iron oxidizing enrichment culture)
AS bleed 2nd inhibition experiment	AS bleed (4, 6, 8, 20, and 30%(v/v))	10% (v/v) of 4-days old iron oxidizing enrichment culture	10	1	25		2.0	Positive control (without AS bleed, inoculated with the iron oxidizing enrichment culture)
AS bleed inhibition experiment with sulfur oxidizing culture	AS bleed (0.1, 1, 2, 4, and 8% (v/v))	10% (v/v) of 7-days old sulfur oxidizing enrichment culture	10	1		10	2.0	Positive control (without AS bleed, inoculated with the sulfur oxidizing enrichment culture)
AS feed experiment								
AS feed inhibition experiment	AS feed (0.1, 1, 2, 10, and 50% (v/v))	10% (v/v) of 4-days old iron oxidizing enrichment culture	10	1	25		2.0	Positive control (without AS feed, inoculated with the iron oxidizing enrichment culture)
AS experiments								
AS 1st inhibition experiment	AS solution (4.3, 9.3, 15, 59, and 250 g/L)	10% (v/v) of 4-days old iron oxidizing enrichment culture	10	1	25		2.0	Positive control (without additional AS stock, inoculated with the iron oxidizing enrichment culture)
AS 2nd inhibition experiment	AS solution (9.3, 20, 31, 42, and 53 g/L)	10% (v/v) of 4-days old iron oxidizing enrichment culture	10	1	25		2.0	Positive control (without additional AS stock, inoculated with the iron oxidizing enrichment culture)
Neodecanoid acid experiments								

Neodecanoid acid 1st inhibition experiment	Neodecanoid acid (5, 10, 50, and 100% of its water solubility)	10% (v/v) of 4-days old iron oxidizing enrichment culture	10	1	25	2.0	Positive control (without neodecanoid acid, inoculated with the iron oxidizing enrichment culture)
Neodecanoid acid 2nd inhibition experiment	Neodecanoid acid (1, 2.5, 3.8, and 5% of its water solubility)	10% (v/v) of 4-days old iron oxidizing enrichment culture	10	1	25	2.0	Positive control (without neodecanoid acid, inoculated with the iron oxidizing enrichment culture)
Neodecanoid acid kinetics experiment	Neodecanoid acid (50% of its water solubility)	10% (v/v) of 4-days old iron oxidizing enrichment culture	10	1	25	2.0	- Positive control (without neodecanoid acid, inoculated with the iron oxidizing enrichment culture) - Negative control (with neodecanoid acid concentration of 50% of its water solubility)
Nessol D100 experiments							
Nessol D100 1st inhibition experiment	Nessol D100 (1, 5, 10, and 15% of its water solubility)	10% (v/v) of 4-days old iron oxidizing enrichment culture	10	1	25	2.0	Positive control (without Nessol D100, inoculated with the iron oxidizing enrichment culture)
Nessol D100 2nd inhibition experiment	Nessol D100 (30, and 100% of its water solubility)	10% (v/v) of 4-days old iron oxidizing enrichment culture	10	1	25	2.0	Positive control (without Nessol D100, inoculated with the iron oxidizing enrichment culture)
Cyanex 272 experiment							
Cyanex 272 inhibition experiment	Cyanex 272 (5, 10, 40, and 100% of its water solubility)	10% (v/v) of 4-days old iron oxidizing enrichment culture	10	1	25	2.0	Positive control (without Cyanex 272, inoculated with the iron oxidizing enrichment culture)
Baysolvex D2EHPA experiment							
Baysolvex D2EHPA inhibition experiment	Baysolvex D2EHPA (5, 10, 50, and 100% of its water solubility)	10% (v/v) of 4-days old iron oxidizing enrichment culture	10	1	25	2.0	Positive control (without Baysolvex D2EHPA, inoculated with the iron oxidizing enrichment culture)

Ammonium deficiency experiment						
Ammonium deficiency experiment with AS bleed and feed	- AS bleed (0.09, 0.1, and 1% (v/v)) - AS feed (0.1, and 1% (v/v))	- 1% (v/v) of 7-days old iron oxidizing AD culture 1 with positive control - 1% (v/v) of 7-days old iron oxidizing AD culture 2 with other controls and shake flasks	- 10% v/v MSM with $(NH_4)_2SO_4$ used with the positive control - 10% v/v MSM without $(NH_4)_2SO_4$ used with other controls and experiment flasks	1	25	2.0	- Positive control (without AS bleed or feed, inoculated with the iron oxidizing AD culture 1) - AD control (without $(NH_4)_2SO_4$, AS bleed or feed, inoculated with the iron oxidizing AD culture 2) - 0.11 g/L ammonium control (without AS bleed or feed, with 0.11 g/L of ammonium, inoculated with the iron oxidizing AD culture 2)

4.4.1 Experiments with AS bleed

The three different experiments, implemented with AS Bleed, were AS Bleed 1st and 2nd inhibition experiments and AS Bleed inhibition experiment with sulfur oxidizing culture. The aim of the experiments was to study possible inhibition of AS bleed on biological iron and sulfur oxidation, and to determine the lowest inhibitory concentration of the AS bleed. The experiments were performed according to Table 13. During the experiments, five different AS bleed concentrations: 0.1–50% (v/v) in AS bleed 1st, 4–30% (v/v) in AS bleed 2nd, and 0.1–8% (v/v) in AS bleed inhibition experiment with sulfur oxidizing culture, were studied with one positive control. Positive (biological) control did not contain any AS bleed and was similarly prepared as the stock culture. In AS bleed 2nd inhibition experiment and in AS bleed inhibition experiment with sulfur oxidizing culture, AS bleed concentration range was narrower, since in these experiments the aim was to determine more exact inhibitory concentration.

In AS bleed 1st and 2nd inhibition experiments, iron oxidation activity of the culture was monitored by measuring pH, redox, and Fe(II) concentration. In AS bleed inhibition experiment with sulfur oxidizing culture, sulfur oxidation activity of the sulfur oxidizing culture was observed by measuring pH, and sulfate concentration.

4.4.2 Experiment with AS Feed

With AS feed one inhibition experiment with the iron oxidizing enrichment culture was performed. The aim of the experiment was similar as in AS bleed 1st and 2nd inhibition experiments. The purpose of the experiment was to study the possible inhibitory effect of AS feed on biological iron oxidation, and to determine the minimum inhibitory concentration. The experiment was carried out according to Table 13. During the experiment, five different AS feed concentrations 0.1–50% (v/v) were studied with one positive control. Positive (biological) control did not contain any AS feed and was similarly prepared as the stock culture. In addition to iron oxidation, ammonium utilization of the iron oxidizing culture, was also monitored.

4.4.3 Experiments with AS

Since both AS bleed and feed will include high concentration of AS (~550 g/L), in operational stage of the battery chemical production plant, possible inhibition of AS on biological iron oxidation was studied in AS 1st and 2nd inhibition experiments. During these experiments, five different concentrations of AS between 4.3–250 g/L and 9.3–53 g/L,

respectively, were studied with a one positive control. The AS concentrations, in the AS 1st inhibition experiment, correspond to AS concentration of 0.1, 1, 2, 10, and 44% (v/v) AS bleed/feed, in the operational stage, added to the general 3.75 g/L AS concentration of the stock culture. The AS concentrations, in the AS 2nd inhibition experiment, correspond to AS concentration of 1, 3, 5, 7, and 9% (v/v) AS bleed/feed, in the operational stage, added to the general 3.75 g/L AS concentration of the stock culture. Positive (biological) control did not contain any additional AS stock and was similarly prepared as the stock culture. AS concentration of the positive control was 3.75 g/L. In the AS 1st inhibition experiment, preliminary minimum inhibitory concentration of AS was determined, whereas in the AS 2nd inhibition experiment, the minimum inhibitory concentration was specified. In the AS inhibition experiments, 450 g/L AS stock was used as an additional AS solution. Iron oxidation and ammonium utilization of the culture was monitored, during the experiments.

4.4.4 Experiments with neodecanoid acid

There were three different experiments implemented with carboxylic acid of neodecanoid acid: neodecanoid acid 1st and 2nd inhibition experiment, and neodecanoid acid kinetics experiment. In the inhibition experiments, inhibition of neodecanoid acid on iron oxidation was studied, and the lowest inhibitory concentration of neodecanoid acid was determined. In neodecanoid acid kinetics experiment, the fate and transformation kinetics of neodecanoid acid, present in the iron oxidizing culture, was studied. The experiments were carried out according to Table 13. In neodecanoid acid 1st and 2nd inhibition experiments, four different neodecanoid acid concentrations in the range of 5–100% of its water solubility (250 mg/L at 25 °C) and 1–5% of its water solubility with a one positive control, respectively, were studied (Pubchem 2021C). Positive (biological) control did not contain any neodecanoid acid and was similarly prepared as the stock culture. In neodecanoid acid 1st inhibition experiment, the lowest preliminary inhibitory concentration of neodecanoid acid was first determined, whereas in neodecanoid acid 2nd inhibition experiment, more exact lowest inhibitory concentration was specified.

In neodecanoid acid 2nd inhibition experiment, 3.8% of its water solubility (10 mg/L) neodecanoid acid concentration was selected, because this 3.8% concentration corresponds to the minimum neodecanoid acid concentration of the process liquors after the removal processes of the organic solvents. The separate removal processes of the organic solvents will be applied during the operational stage of the battery chemical production plant.

In neodecanoid acid kinetics experiment, the experiment was implemented with nine shake flasks. These shake flasks contained four experiment flasks of 50% of its water solubility of neodecanoid acid concentration (A, B, C, and D), four chemical controls, containing same amount of neodecanoid acid, but no inoculum, and a one positive control. Positive control was used as control for monitoring iron oxidation, whereas the original function of the chemical controls was to be used as controls for dissolved organic carbon (DOC) investigation of neodecanoid acid.

During the neodecanoid acid experiments, 100% neodecanoid acid was pipetted on top of the medium as last step, with concentration of 5% of its water solubility and higher. With the lower concentrations, neodecanoid acid stock solution of 10% of its water solubility, made in the laboratory, was used. The 10% stock contained 3 µL of neodecanoid acid in 100 mL of autoclaved Milli-Q water, which was 10% of its water solubility of neodecanoid acid. Room temperature neodecanoid acid was diluted in Milli-Q water (~27 °C), by shaking the flask roughly for couple of minutes. The 10% neodecanoid acid stock was made on the same day of starting the experiments.

From the shake flasks of the neodecanoid acid inhibition experiments, from the neodecanoid acid kinetics experiment flasks and from the positive control, iron oxidation of the cultures was monitored, during the experiments. From the neodecanoid acid 2nd inhibition experiment and the neodecanoid acid kinetics experiment shake flasks, the fate and kinetics of neodecanoid acid in iron oxidizing culture, was investigated by measuring DOC concentration.

4.4.5 Experiments with Nessol D100

From the organic solvents, possible inhibition of Nessol D100 on biological iron oxidation was studied in two experiments: Nessol D100 1st inhibition experiment and Nessol D100 2nd inhibition experiment. The experiments were performed as shown in Table 13. In Nessol 1st inhibition experiment, four Nessol D100 concentrations between 1.0–15% of its water solubility (10 mg/L, temperature not mentioned) with a one positive control were studied (Neste 2019). In Nessol 2nd inhibition experiment, two Nessol D100 concentrations of 30% and 100% of its water solubility were studied. During the Nessol D100 1st inhibition experiment, possible inhibition of aliphatic Nessol D100 was first studied in lower concentrations, whereas in Nessol D100 2nd inhibition experiment, possible inhibition of Nessol D100 was studied in higher concentrations. Possible influence of Nessol D100 on iron oxidation, was monitored during the experiments.

Since in the Nessol D100 experiments, volumes of the solution were too low for pipetting with other concentrations than 100% of its water solubility, Nessol D100 stock, made in

the laboratory, was used with other concentrations. In laboratory conditions, the highest concentration of Nessol D100 stock, which was able to be made was 60% of its water solubility. Therefore, Nessol D100 stock of 60% was used in Nessol D100 2nd inhibition experiment with the lower concentration, whereas Nessol D100 50% stock was used in the Nessol D100 1st inhibition experiment. Nessol D100 stocks were made in the same day of starting the experiments. With both stock solutions, room temperature Nessol D100 was added into 200 mL of autoclaved Milli Q-water (~27 °C). Then the solution was thoroughly mixed for couple of minutes.

4.4.6 Experiment with Cyanex 272

Possible inhibition of Cyanex 272 on biological iron oxidation was studied in Cyanex 272 inhibition experiment. The experiment was performed as shown in Table 13. During the experiment, four concentrations between 5–100% of its water solubility (16 mg/L at 20 °C) with a one positive control were studied (Solvay 2019). Possible inhibition of Cyanex 272 on iron oxidation, was monitored by measuring pH, redox, and Fe(II) concentration.

In Cyanex 272 inhibition experiment, 100% Cyanex 272 solution was used with the highest 100% concentration, whereas 80% of its water solubility Cyanex 272 stock, made in the laboratory, was used with the other concentrations. 80% Cyanex 272 stock was used, because the volumes were too low for pipetting. 80% of its water solubility was the highest concentration, which was able to be made in the laboratory, therefore 80% Cyanex 272 stock solution was used with the other concentrations except Cyanex 272 concentration of 100% of its water solubility. 80% Cyanex 272 stock was made at the same day than the experiment was starting. The stock was similarly prepared as 10% neodecanoid acid and Nessol D100 stocks.

4.4.7 Experiment with Baysolvex D2EHPA

Possible inhibition of Baysolvex D2EHPA on biological iron oxidation was investigated in Baysolvex D2EHPA inhibition experiment. The experiment was carried out according to Table 13. The possible inhibition of Baysolvex D2EHPA was studied with four different concentrations between 5–100% of its water solubility (182 mg/L, temperature not mentioned), and with a one positive control (LANXESS 2018). In the experiment, 90–100% (w/w) Baysolvex D2EHPA solution was pipetted directly on top the medium, and the possible inhibition of the solution on iron oxidation, was monitored.

4.4.8 Ammonium Deficiency Experiment

Possible iron oxidation enhancing effect of AS bleed and feed was investigated during the ammonium deficiency experiment. In the experiment three different concentrations: 0.09%, 0.1%, and 1% (v/v), of AS bleed, and two different concentrations: 0.1% and 1% (v/v), of AS feed were studied with three different controls. 0.09% AS bleed concentration was studied since the ammonium concentration of it was same as ammonium concentration of 0.11 g/L ammonium control. The other AS bleed and feed concentrations were decided based on the results of the AS bleed and feed inhibition experiments.

The controls used during the experiments contained one positive control, AD control, and 0.11 g/L ammonium control. In 0.11 g/L ammonium control, ammonium concentration was same than in Niemelä *et al.* (1994) study, in which 0.110 g/L (6 mmol/L) ammonium supplementation had been reported to have an enhancing effect on bioleaching of a black schist ore. 0.11 g/L ammonium control was made with 3.96 mL of 10 g/L AS stock. The AS stock was prepared similarly to 450 g/L AS stock.

From the controls, the positive control was supplemented with 10% (v/v) of MSM with $(NH_4)_2SO_4$, and 1% (v/v) inoculum, used with the control, was 7-days old iron oxidizing AD culture 1. 10% (v/v) of MSM without $(NH_4)_2SO_4$ was added into the other controls and experiment flasks and used 1% (v/v) inoculum was 7-days old iron oxidizing AD culture 2. Iron oxidation and ammonium utilization of the cultures were monitored during the experiment.

4.5 Sampling

Sampling from shake flasks, containing iron oxidizing culture (iron oxidizing enrichment culture, iron oxidizing AD culture 1 or 2), occurred generally once every weekday. Only exception to this sampling rate was made with neodecanoid acid experiments, when neodecanoid concentration (50% and 100% of its water solubility) leaded to delayed Fe(II) oxidation. With neodecanoid acid concentrations of 50% and 100% of its water solubility, samples were taken generally every other weekday, during the lag phase, and every weekday, during the exponential growth phase. The sampling frequency and the analysis done are presented in Table 14.

Table 14. *The sampling schedule and the analysis made from the samples of the performed experiments.*

Experiment	Sampling frequency	pH analysis	Redox analysis	Fe(II) analysis	Ammonium analysis	Sulfate analysis	DOC analysis
AS bleed experiments							
AS bleed 1st inhibition experiment	Every weekday	X (from all samples)	X (from all samples)	X (from all samples)			
AS bleed 2nd inhibition experiment	Every weekday	X (from all samples)	X (from all samples)	X (from all samples)	X (from the first and the last samples)		
AS bleed inhibition experiment with sulfur oxidizing culture	- 1st week: every weekday - 2nd week: every other weekday - 3rd and 4th week: on Mondays and Fridays	X (from all samples)				X (from all samples)	
AS feed experiment							
AS feed inhibition experiment	Every weekday	X (from all samples)	X (from all samples)	X (from all samples)	X (from the first and the last samples)		
AS experiments							
AS 1st inhibition experiment	Every weekday	X (from all samples)	X (from all samples)	X (from all samples)	X (from the first and the last samples)		
AS 2nd inhibition experiment	Every weekday	X (from all samples)	X (from all samples)	X (from all samples)	X (from the first and the last samples)		
Neodecanoid acid experiments							
Neodecanoid acid 1st inhibition experiment	- Every weekday during the first week - Every other weekday, during the lag phase	X (from all samples)	X (from all samples)	X (from all samples)			

	- Every weekday, during the exponential growth phase					
Neodecanoid acid 2nd inhibition experiment	Every weekday	X (from all samples)	X (from all samples)	X (from all samples)		X (from the first and the last samples)
Neodecanoid acid kinetics experiment	Every other weekday	X (from all samples)	X (from all samples)	X (from all samples)		X (from A and B samples)
Nessol experiments						
Nessol D100 1st inhibition experiment	Every weekday	X (from all samples)	X (from all samples)	X (from all samples)		
Nessol D100 2nd inhibition experiment	Every weekday	X (from all samples)	X (from all samples)	X (from all samples)		
Cyanex 272 experiment						
Cyanex 272 inhibition experiment	Every weekday	X (from all samples)	X (from all samples)	X (from all samples)		
Baysolvex D2EHPA experiment						
Baysolvex D2EHPA inhibition experiment	Every weekday	X (from all samples)	X (from all samples)	X (from all samples)		
Ammonium deficiency experiments						
Ammonium deficiency experiment with AS bleed and feed	Every weekday	X (from all samples)	X (from all samples)	X (from all samples)	X (from the first and the last samples)	

From the AS bleed, feed, AS inhibition, and ammonium deficiency experimental shake flasks, 4 mL samples were taken on the first and last sampling days, and 2 mL samples on the rest of the experimental days. Sampling was discontinued when all Fe(II) was completely oxidized into Fe(III). During the AS bleed experiment with sulfur oxidizing culture, 4 mL samples were taken at first and last sampling days, and 2 mL samples were taken on the other days of the experiment. From the neodecanoid acid, Nessol D100, Cyanex 272 and Baysolvex 272 inhibition experiment shake flasks, 6 mL sample was taken at first sampling day, 30 mL sample was taken at last sampling day, and 2 mL samples were taken on the other days of the experiment.

All the samples, except the chemical control samples, of the neodecanoid acid kinetics experiment, and 30 mL samples, were filtrated after pH and redox measurement with 0.45 µm polyester filter, Chromafil® Xtra PET-45/25 (Macherey-Nagel, Germany). With the iron oxidizing cultures, the Fe(II) concentration was measured from the fresh filtrated samples. After Fe(II) measurement, the iron oxidizing culture samples were stored at -20°C until ammonium or DOC analysis, depending on the sample. With sulfur oxidizing culture, after filtration samples were stored at -20°C until sulfate analysis.

4.6 Analytical methods

Iron oxidization activity of the iron oxidizing cultures was observed by measuring pH, redox, and Fe(II) concentration from the samples. Sulfur oxidizing activity of the sulfur oxidizing culture was monitored by measuring pH, and sulfate concentration from the samples. From the samples of AS bleed, AS feed, AS, and ammonium deficiency experiment, ammonium utilization was monitored by measuring ammonium concentration of the samples. From the neodecanoid acid 2nd inhibition experiment and the neodecanoid acid kinetics experiment samples, the fate and kinetics of neodecanoid acid in iron oxidizing culture, was observed by measuring DOC concentration.

4.6.1 Measurement of pH and redox

The pH and redox were measured right after sampling from non-filtered samples. The pH of the samples was measured with either pH 3210 meter (WTW, Germany), equipped with pH electrode SenTix 81 (WTW, Germany) or pH 330i meter (WTW, Germany), equipped with pH electrode SlimTrode (Hamilton, US). Redox potential was measured with pH 315i meter (WTW, Germany), equipped with redox electrode BlueLine 31 Rx (SI Analytics, Germany)

4.6.2 Measurement of Fe(II) concentration

Fe(II) concentration, during iron oxidation, was measured by using 3500-Fe ortho-phenantroline method (APHA 1992). The concentration was measured from filtered (0.45 µm) samples. Prior to the analysis, the samples were diluted with 4.41 mg/L (0.07 mmol/L) HNO_3.

Fe(II) concentration was analyzed with ultraviolet-visible (UV-Vis) spectrophotometry. UV-Vis spectrophotometry is a quantitative measurement technique, in which light absorbance of the sample is measured across ultraviolet and visible light regions of the electromagnetic spectrum, and then converted to concentrations, such as Fe(II) concentrations, by using a standard curve (Germer *et al.* 2014). From the samples, absorbance was measured at 510 nm by using a UV-1900i UV-Vis Spectrophotometer (Shimadzu, Japan) with a quartz cuvette. Absorbance results of the samples were converted to Fe(II) concentrations by using Fe(II) concentration standard curve. Standard curve, containing Fe(II) concentrations of 0.25, 0.5, 1.0, 2.5, 5.0, 10, and 20 mg/L, was prepared by using the Fe(II) stock solution.

4.6.3 Ammonium analysis

Ammonium utilization of the iron oxidizing cultures from AS bleed, feed and AS solution was observed by measuring ammonium concentration from the filtrated samples. The concentrations were measured with cationic IC. IC is an analytical method, which separates, identifies, and quantifies ionic and polar constituents of the analytical solution. Separation of the constituents is based upon their interactions with the stationary phase (resin of the column) and the liquid phase (eluent/mobile phase). In cationic IC, a cation column, which attracts specific type of cations, is used as a stationary phase. (Khan & Ali 2018)

During the cationic IC run, pressurized eluent carries the analyte, such as ammonium, through the analytical column, in which specific types of cations, depending on the column, are interacting with the resin causing a slower movement of them. The retention time of the specific cations varies, according to their affinity towards the specific resin. The specific cations, which have been interacting with the column, are measured with an electrical conductivity detector, which produces a peak into a chromatogram. The peak area size is then used to determine the concentration of the specific cation in the analysis solution. (Khan & Ali 2018)

The IC used to determine the ammonium concentration of the samples of this study was Dionex DX-120 IC (Thermo Fischer Scientific, US), which was equipped with Dionex

IonPac CG12A (4 x 50 mm) guard column, IonPac CS12A (4 x 250 mm) analytical cation exchange column, and Dionex AS40 autosampler. Before the analysis, the filtrated samples were diluted with Milli-Q water to attain ammonium concentration below 100 mg/L. Methanesulfonic acid of 1.92 mg/L (0.02 mmol/L) was used as eluent. Prior to the analysis, the eluent was degassed with helium. IC peak area results of the samples were converted to ammonium concentrations by using ammonium concentration standard curve. Standard curve, containing ammonium concentrations of 0.2, 1.0, 5.0, 10, 20, and 50 mg/L, was made by using 1000 mg/L ammonium stock solution.

4.6.4 Sulfate analysis

Sulfate concentration was measured with anionic IC, which operating principals are similar to cationic IC. The main difference is that its column, attracts specific anions, instead of cations. The IC used during this study, was Dionex IC-1600 (Thermo Fischer Scientific, US), which was equipped with IonPac AG42-SC (4 x 50 mm) guard column, IonPac AS4A-SC (4 x 250 mm) analytical anion exchange column, and Dionex AS-DV autosampler. Prior to the analysis, filtrated samples were diluted with Milli-Q water for obtaining sulfate concentration under 100 mg/L. The eluent used during the analysis, consisted of 509 mg/L (4.8 mmol/L) of Na_2CO_3 and 84 mg/L (1.0 mmol/L) of $NaHCO_3$. Before the analysis the eluent, without Na_2CO_3 and $NaHCO_3$, was degassed. The results of IC peak area were converted to sulfate concentrations by using sulfate concentration standard curve. This curve was nine-point standard curve with 1.0, 2.5, 5.0, 7.5, 10, 25, 50, 75, and 100 mg/L of sulfate concentration prepared from 1000 mg/L sulfate stock solution.

4.6.5 DOC analysis

The fate and kinetics of neodecanoid acid in iron oxidizing culture, was observed from the neodecanoid acid 2nd inhibition experiment and neodecanoid acid kinetics experiment samples, by measuring DOC concentration. DOC concentration was measured from the first and the last neodecanoid acid 2nd experiment samples, whereas in neodecanoid acid kinetics experiment, DOC concentration was measured from samples, collected from two 50% of its water solubility neodecanoid acid (neodecanoid acid 50% A and B) shake flasks, during the iron oxidation. DOC concentration was measured by using total organic carbon analyzer and SFS-EN 1484 TOC and DOC standard protocol. The methods, used in DOC measurement, were non-purgeable organic carbon (NPOC) with the neodecanoid acid 2nd inhibition experiment samples and total organic carbon (TOC) (total carbon (TC) – total inorganic carbon (TIC)) with neodecanoid acid kinetics experiment samples. Used standard solution was 1000 mgC/L potassium hydrogen

phthalate in the PNOC method. In TOC method, used standard solutions were 1000 mgC/L potassium hydrogen phthalate in TC analysis, and 1000 mgC/L sodium carbonate and sodium hydrogen carbonate in the TIC analysis. (SFS-EN 1484)

In PNOC method, DOC is measured once, whereas in TOC (TC-TIC) method, TC and TIC are measured separately, and TIC result is then subtracted from TC result. In PNOC and TC analysis, samples are acidified, sparged with carrier air, and then combusted at high temperature of 680 °C for breaking all organic carbon into carbon dioxide. The carbon dioxide of the sample is detected with non-dispersive infrared (NDIR) gas analyzer. In TIC analysis, samples are acidified, for driving TIC equilibria to carbon dioxide, and then sparged with carrier air. Formed carbon dioxide is then detected with NDIR. (Shimadzu Corporation 2007)

DOC concentrations of neodecanoid acid 2nd inhibition experiment samples were measured at the laboratory of Tampere University, while the concentration, from neodecanoid acid kinetics experiment samples, were measured at the laboratory of Terrafame Oy. At the laboratory of Terrafame Oy, samples were filtered with sterile 0.2 µm polyethersulfone filter (VWR, US), before the analysis. TOC analyzer, used with neodecanoid acid 2nd inhibition experiment samples, was TOC-VCPH/CPN (Shimadzu, Japan). TOC analyzer, used with neodecanoid acid kinetics experiment samples, was high-sensitive TOC-L (Shimadzu, Japan).

5. RESULTS

5.1 AS bleed experiments

During this study, three AS bleed inhibition experiments were performed with the iron oxidizing culture, enriched from the irrigating leach liquor of the heap bioleaching area, and with the sulfur oxidizing culture, enriched from the iron oxidizing enrichment culture.

5.1.1 AS bleed 1st inhibition experiment

The effect of AS bleed on biological iron oxidation was studied to determine the lowest inhibitory concentration. With 0.1–2% AS bleed concentrations, Fe(II) was oxidized, and pH and redox potential increased in a similar manner as in positive control (Figure 13). All Fe(II) was completely oxidized in the positive control and at 0.1–2% AS bleed concentrations in less than 3 days. Iron oxidation increased redox potential from 350–400 mV to 600–650 mV, and pH from 2.0–2.1 to 2.4–2.6. At 10% AS bleed concentration, iron oxidation was slower during the first three days than at lower concentrations. After day 3, Fe(II) oxidation rate increased together with pH and redox potential increases but was slower than in the positive control. Fe(II) oxidation from the AS bleed 10% was completed by day 7. With 50% AS bleed concentration, Fe(II) concentration, pH, and redox potential remained stable.

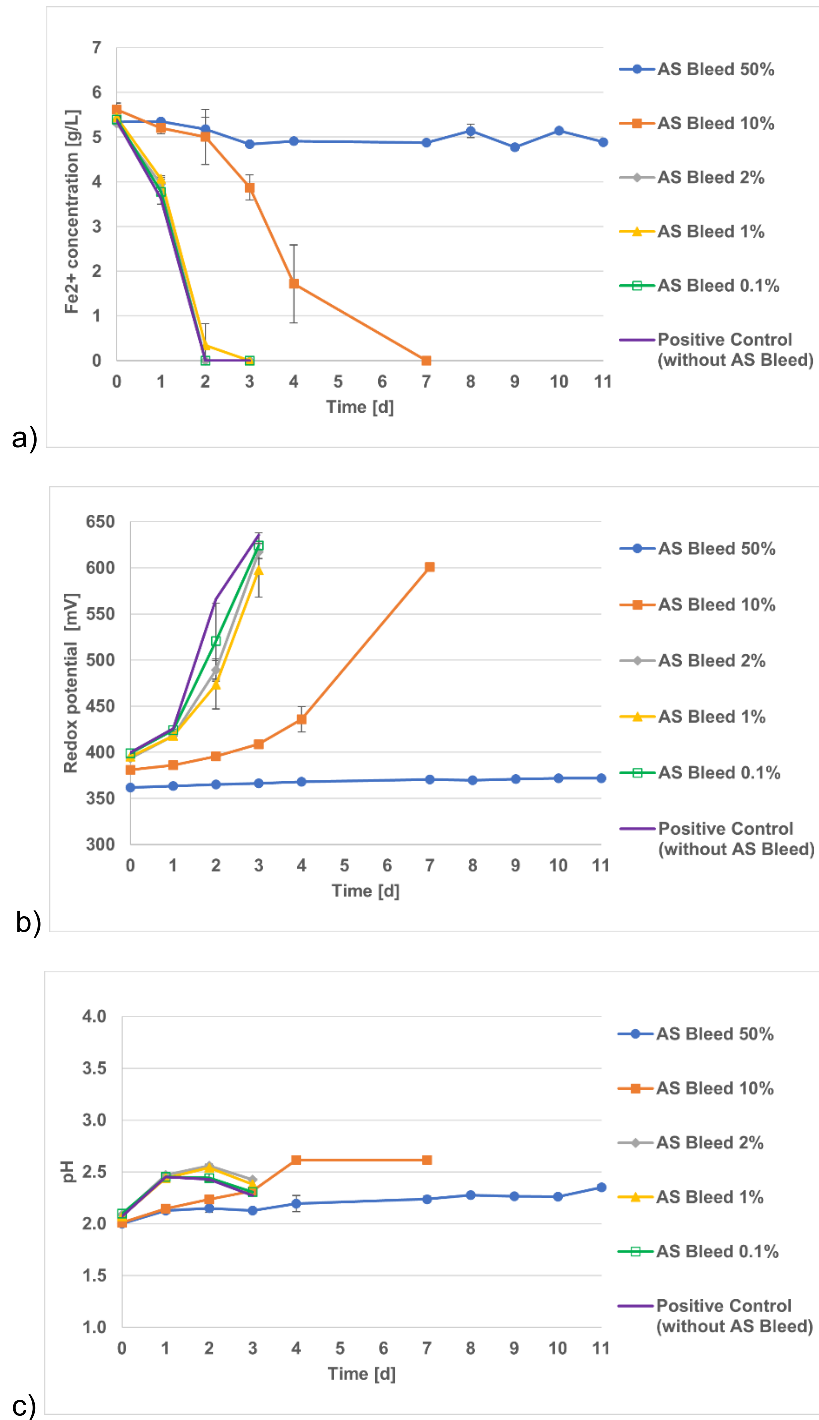


Figure 13. *Effect of AS bleed concentration (0.1–50% (v/v)) on biological iron oxidation by iron oxidizing enrichment culture. (a) Fe(II) concentration, (b) redox potential, and (c) pH. The standard deviations are presented with the error bars (n=2).*

The results show that AS bleed had no effect on iron oxidation at concentrations below 2%. When AS bleed concentration was increased above 2%, iron oxidation was delayed, and the rate decreased. However, with 10% AS bleed concentration, the iron oxidation rate increased after the lag phase (Figure 13). The results of the second incubation in

the presence of 10 % AS bleed (Figure 14) show that the iron oxidizing enrichment culture adapted 10% AS bleed concentration, while at above 10% AS bleed concentration, iron oxidation was inhibited. With AS bleed 50% concentration, the inhibition of iron oxidation was irreversible.

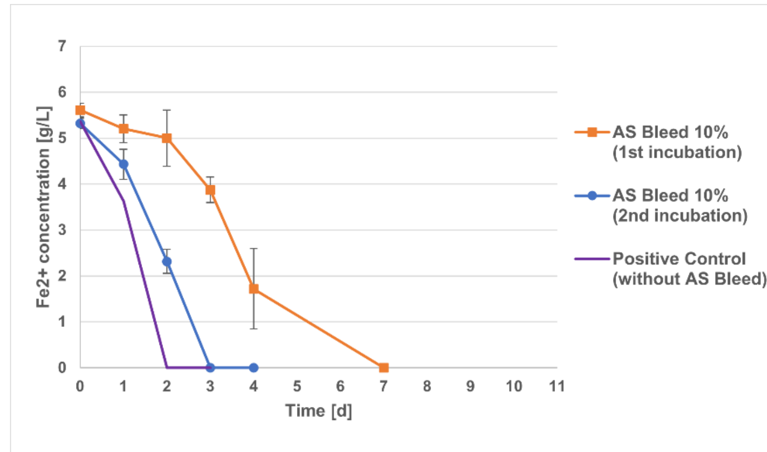


Figure 14. *Effect of AS bleed 10% (v/v) concentration on biological iron oxidation during first and second incubation. The second incubation was performed with the enrichment culture obtained from the first incubation. The standard deviations are presented with the error bars (n=2).*

5.1.2 AS bleed 2nd inhibition experiment

The impact of AS bleed on biological iron oxidation was studied to define the minimum inhibitory concentration in detail. During the iron oxidation, ammonium utilization by the iron oxidizing enrichment culture, was also studied. With 4% AS bleed concentration, iron oxidation was slower during the first day (Figure 15). After day 1, iron oxidation rate increased together with redox potential and pH increases in a similar manner as in positive control. All Fe(II) was oxidized before day 3. At AS bleed concentration of 8%, iron oxidation was slower during the first two days. After day 2, iron oxidation rate increased at the same time with redox potential and pH increases similarly to the positive control. Fe(II) oxidation was completed by day 4. Biological iron oxidation increased pH from 2.1 to 2.5 and redox potential from 350–400 mV to 560–610 mV. With AS bleed concentrations of 20–40%, Fe(II) concentration, pH, and redox potential remained stable.

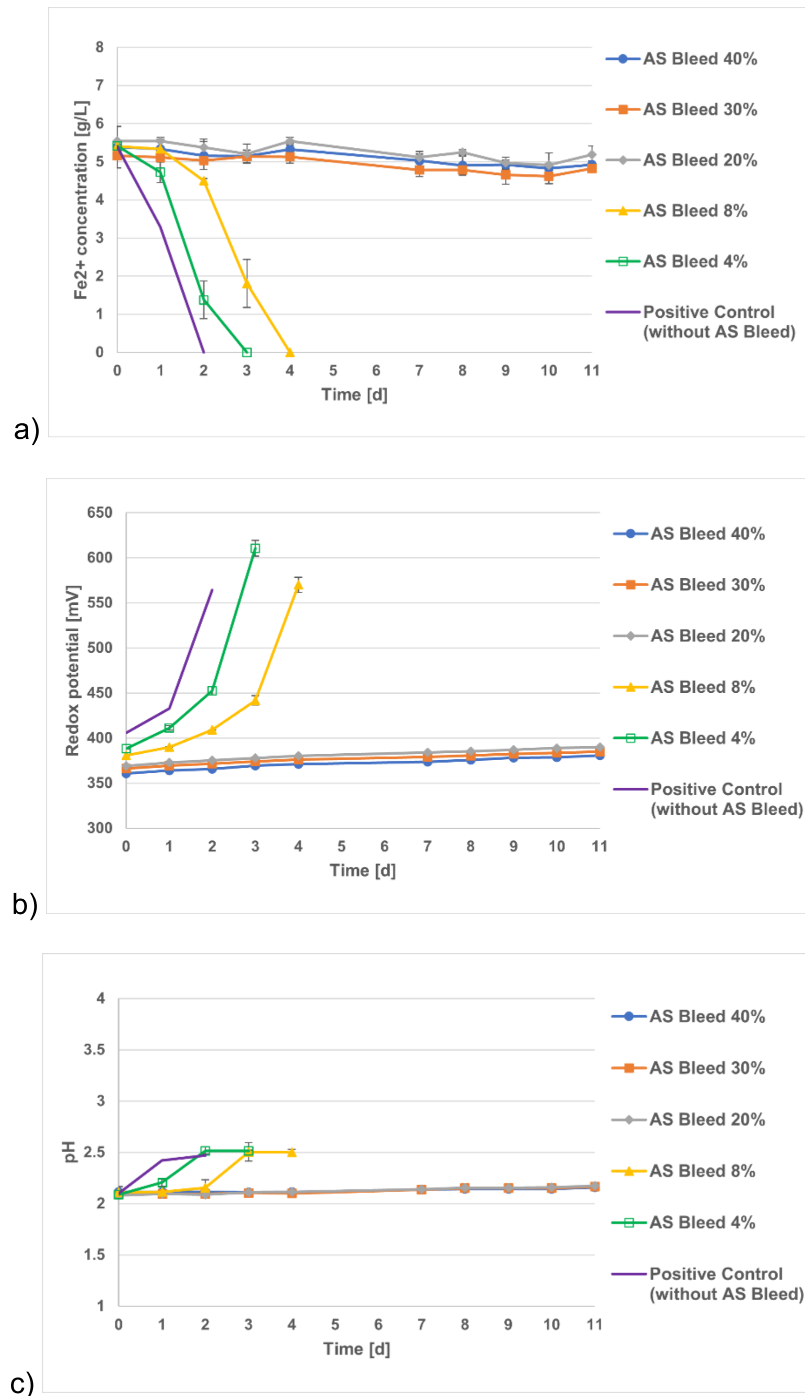


Figure 15. *Influence of AS bleed concentration (4–40% (v/v)) on (a) Fe(II) concentration, (b) redox potential, and (c) pH during biological iron oxidation by iron oxidizing enrichment culture. The standard deviations are presented with the error bars (n=2).*

With the studied AS bleed concentrations, initial ammonium concentration varied between 5–50 g/L (Figure 16). The ammonium concentration increased with the AS bleed concentration. At last sampling day (day 3 with 4% AS bleed, day 4 with 8% AS bleed,

and day 11 with 20–40% AS bleed), ammonium concentration had decreased by 0.2–1.5 g/L with 4–30% AS bleed concentrations, and by about 5 g/L with 40% AS bleed concentration.

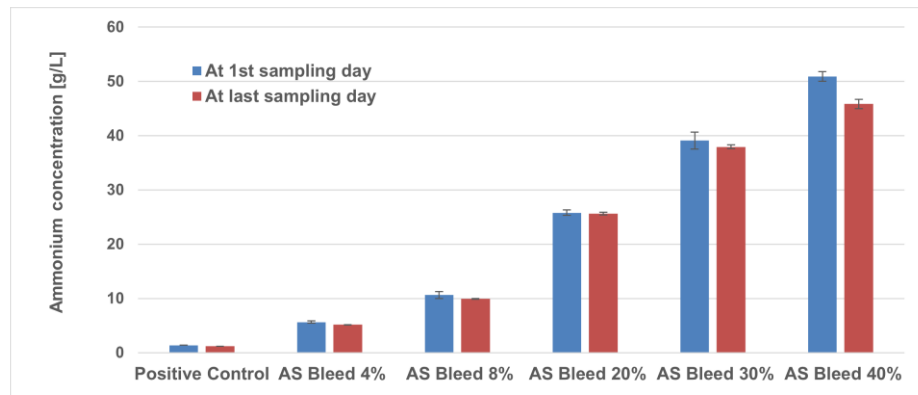


Figure 16. *Effect of AS bleed concentration (4–40% (v/v)) on ammonium concentration and utilization during iron oxidation by the iron oxidizing enrichment culture. Last sampling days: day 3 with AS bleed 4%, day 4 with AS bleed 8%, and day 11 with AS bleed 20–40%. The standard deviations are presented with the error bars (n=2).*

The results show that all studied AS bleed concentrations affected negatively biological iron oxidation. When AS bleed concentration was between 4–8%, iron oxidation delayed, but after the lag phase, the iron oxidation rate was similar to the positive control. At over 8% AS bleed concentration, iron oxidation was inhibited. With AS bleed concentration of 20% and above, the inhibition of iron oxidation was irreversible. Ammonium utilization of the iron oxidizing enrichment culture varied from 0.2 g/L to 5 g/L with all the AS bleed concentrations. Most of the ammonium was not used by the iron oxidizing enrichment culture and remained in the culture medium.

5.1.3 AS bleed inhibition experiment with sulfur oxidizing culture

The effect of AS bleed on biological sulfur oxidation by sulfur oxidizing enrichment culture was studied to determine the lowest inhibitory concentration. Before the AS bleed inhibition experiment, the sulfur oxidizing culture was enriched from the iron oxidizing culture, and sulfur oxidation of the culture was monitored. With the initial culture, sulfate concentration increased from 2.4 g/L to 5.4 g/L (Figure 17). During the first seven days, sulfur oxidation was slow, but after day 7, it speeded up therefore, the sulfate production rate increased. During the second enrichment (culture after first transfer to fresh medium), sulfate concentration increased steadily from 3.2 g/L to 5.3 g/L.

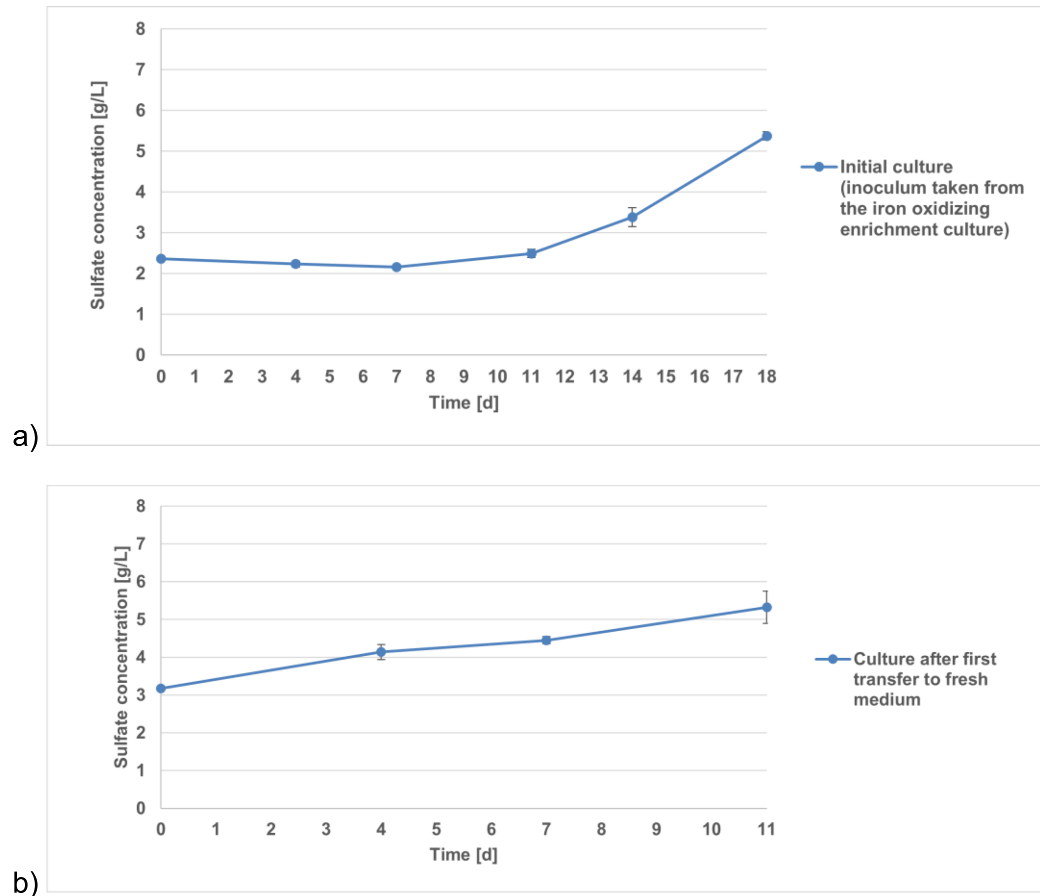


Figure 17. *Sulfate production from elemental sulfur during the enrichment of the sulfur oxidizing culture. (a) initial culture and (b) culture after the first transfer to fresh medium. The standard deviations are presented with the error bars (n=2).*

With AS Bleed concentration of 4% and below, pH reduced at similar rate as in positive control, during 25 days of incubation (Figure 18). The pH decreased from 1.9–2.0 to 1.2–1.4. At 8% AS bleed concentration pH decrease was slower than with positive control. The pH of 8% AS bleed concentration decreased from 2.0 to 1.7. During the first five days, sulfur oxidation was slow with all the AS bleed concentrations and the positive control, but after day 4 sulfur oxidation rate increased (Figure 18). With 0.1% AS Bleed concentration, 4.4 g/L sulfate concentration increase, was slightly lower than the sulfate concentration increase (5.0 g/L) in the positive control. With AS bleed concentrations of 1–4%, sulfate concentration increased by 7.4–8.2 g/L. At AS bleed concentration of 8%, sulfate concentration increased by 6.0 g/L.

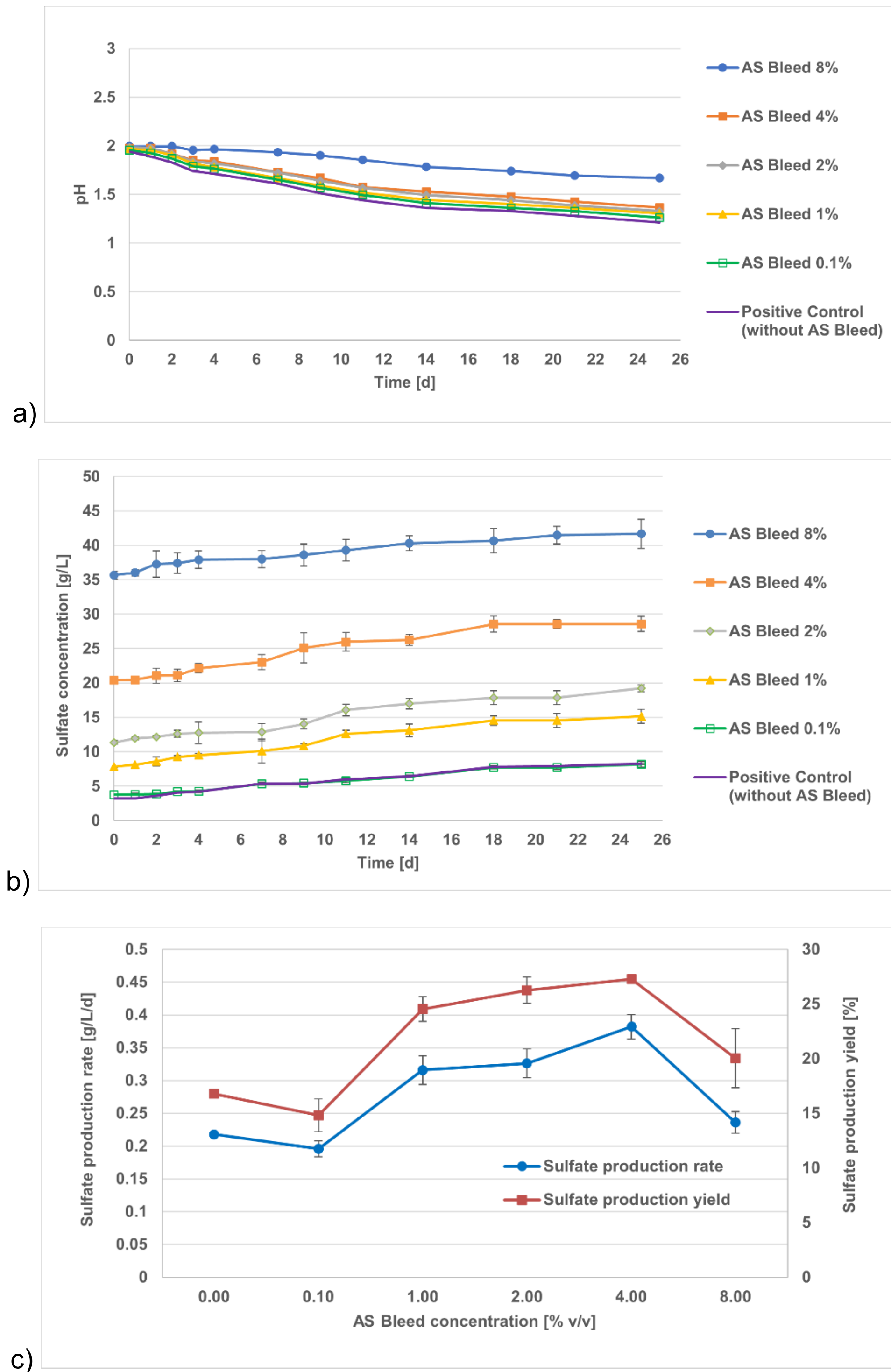


Figure 18. *Effect of AS bleed on biological sulfur oxidation by sulfur oxidizing enrichment culture. (a) pH, (b) sulfate concentration and (c) overall sulfate production rate and the highest sulfate production yield. The overall sulfate production rates were calculated from the slope of the linear regression line of the overall sulfate production curves. The standard deviations are presented with the error bars (n=2).*

The results show that AS bleed did not affect the decrease of pH at below 4%, whereas AS bleed concentration of 8% slowed down the pH decrease. From the sulfate production results, overall sulfate production rate and the highest sulfate production yield were obtained. Overall sulfate production rate was calculated from the slope of the linear regression line of the overall sulfate production curves, when R^2 values of all concentrations were above 0.89. The results show that AS bleed increased the sulfate production rate and yield, except with 0.1% AS Bleed concentration. Below 4% AS bleed concentration, sulfate production rate and yield increased together with AS bleed concentration. When AS bleed concentration was above 4%, sulfate production rate and yield started to decrease. However, below 8% AS bleed concentration, sulfate production rate and yield remained higher compared to the positive control.

5.2 AS feed inhibition experiment

During this study, one AS feed experiment was performed with the iron oxidizing enrichment culture. The influence of AS feed on biological iron oxidation was studied to determine the lowest inhibitory concentration. During the experiment ammonium utilization of the iron oxidizing enrichment culture, was also studied. With AS feed concentrations of 0.1–2%, Fe(II) was oxidized, and pH and redox potential increased in a similar way as in positive control (Figure 19). All Fe(II) was completely oxidized before day 3. Biological iron oxidation increased pH from 2.1 to ~2.5 and redox potential from 350–400 mV to ~600 mV. With 10% AS feed concentration, iron oxidation was slower during the first day, but then iron oxidation rate increased together with redox potential and pH increases similarly to the positive control. Iron oxidation was completed before day 3. At 50% AS feed concentration, Fe(II) concentration, pH, and redox potential remained stable.

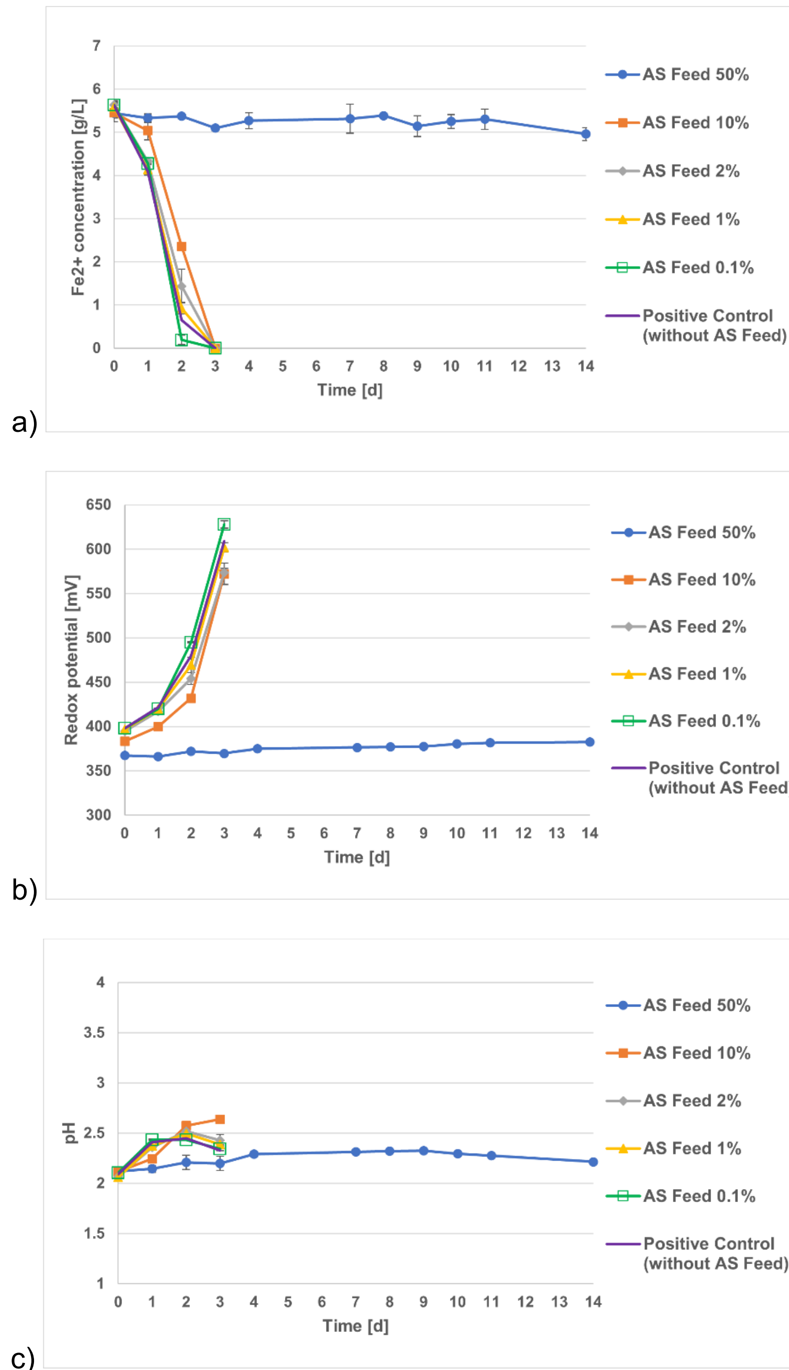


Figure 19. *Effect of AS feed concentration (0.1–50% (v/v)) on (a) Fe(II) concentration, (b) redox potential, and (c) pH, during iron oxidation by iron oxidizing enrichment culture. Standard deviations are presented with the error bars (n=2).*

With the studied AS feed concentrations, initial ammonium concentration varied between 1.2–44 g/L (Figure 20). The ammonium concentration increased with the AS feed concentration. At last sampling day (day 3 with 4–10% AS feed, and day 14 with 50% AS feed), ammonium concentration had decreased by 0.02–0.07 g/L with 0.1–2% AS feed

concentrations, 1.6 g/L with 10% AS feed concentration, and 5.9 g/L with 50% AS feed concentration.

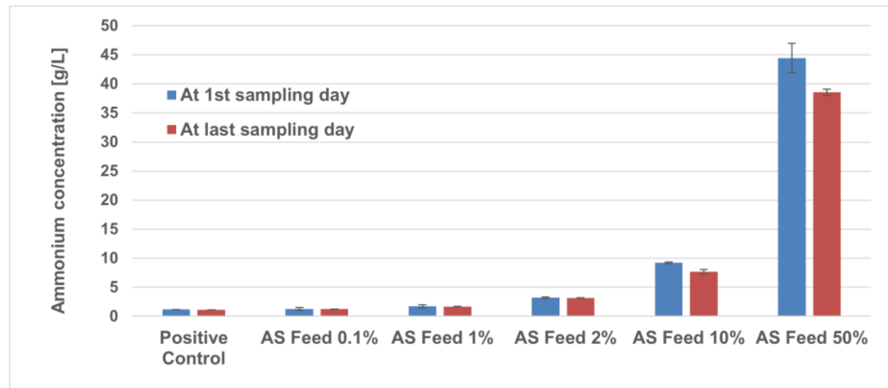


Figure 20. *Influence of AS feed concentration (0.1–50% (v/v)) on ammonium concentration and utilization during iron oxidation by the iron oxidizing enrichment culture. Last sampling days: day 3 with AS feed 4–10%, and day 14 with AS feed 50%. The standard deviations are presented with the error bars (n=2).*

The results show that AS feed had no impact on iron oxidation at concentrations below 2%. When AS feed concentration increased to 10%, iron oxidation delayed. After the lag phase, the iron oxidation rate was similar to the positive control. At above 10% AS feed concentration, iron oxidation was inhibited. With AS feed concentration of 50%, the inhibition towards iron oxidation was irreversible. Ammonium utilization of the iron oxidizing enrichment culture varied from 0.02 g/L to 5.9 g/L with all the AS feed concentrations. Most of the ammonium was not utilized by the iron oxidizing enrichment culture and remained in the culture medium.

5.3 AS experiments

During this study, two AS inhibition experiment were performed with the iron oxidizing enrichment culture.

5.3.1 AS 1st inhibition experiment

The influence of AS solution on biological iron oxidation was studied to determine the lowest inhibitory concentration. In addition to iron oxidation, ammonium utilization of the iron oxidizing enrichment culture was also studied. With AS concentrations of 4.3–15 g/L, Fe(II) was oxidized, and redox potential and pH was increased in a similar way as in positive control (Figure 21). All iron was oxidized within 3 days. Biological iron oxidation increased pH from 2.0–2.1 to 2.4–2.5 and redox potential from 350–400 mV to above 550 mV. With AS concentration of 59 g/L, iron oxidation was slower during the first nine

days. After experimental day 9, iron oxidation rate with pH and redox potential started to increase. All iron was completely oxidized before day 14. At the highest studied AS concentration of 250 g/L, Fe(II) concentration, pH, and redox potential remained stable.

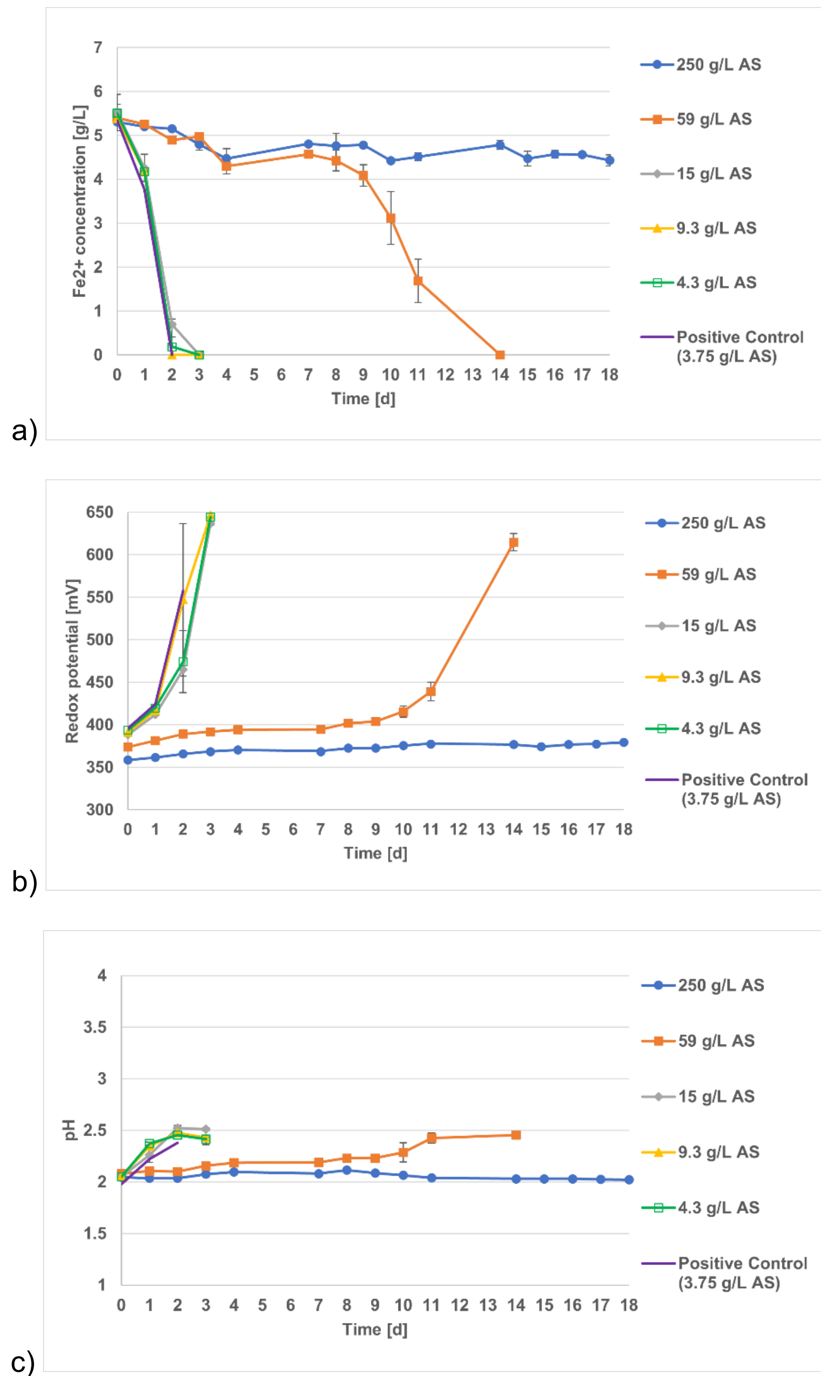


Figure 21. *Influence of ammonium sulfate (AS) concentration (4.3–250 g/L) on biological iron oxidation by iron oxidizing enrichment culture. (a) Fe(II) concentration, (b) redox potential, and (c) pH. The standard deviations are presented with the error bars (n=2).*

With the studied AS concentrations, initial ammonium concentration varied between 1.5–62 g/L (Figure 22). The ammonium concentration increased with AS solution concentration. At last sampling day (day 3 with 4.3–15 g/L, day 14 with 59 g/L, and day 18 with 250 g/L), ammonium concentration had decreased by 0.09–0.17 g/L with 4.3–59 g/L AS concentrations, and by 2.1 g/L with 250 g/L AS concentration.

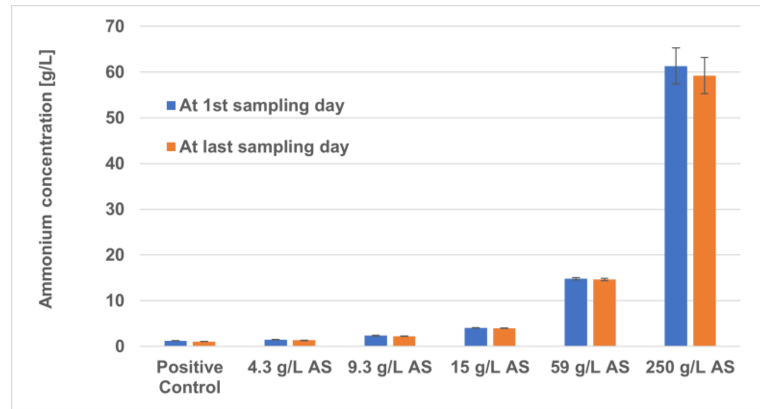


Figure 22. *Influence of ammonium sulfate (AS) concentration (4.3–250 g/L) on ammonium concentration and utilization during iron oxidation by the iron oxidizing enrichment culture. Last sampling days: day 3 with 4.3–15 g/L AS, day 14 with 59 g/L AS, and day 18 with 250 g/L AS. The standard deviations are presented with the error bars (n=2).*

The results show that excessive AS addition did not affect biological iron oxidation, when AS concentration was below 15 g/L. When AS concentration increased to above 15 g/L, iron oxidation was first delayed, and the iron oxidation rate decreased. After the lag phase, iron oxidation rate increased, but was still slightly slower, compared to the positive control. When AS concentration was increased above 59 g/L, biological iron oxidation was inhibited. With AS concentration of 250 g/L, the inhibitory effect was irreversible. Most of the ammonium in the culture medium, was not used by the iron oxidizing enrichment culture but remained in the culture medium.

5.3.2 AS 2nd inhibition experiment

The aim of the experiment was to determine the minimum inhibitory concentration of AS in detail and monitor ammonium utilization by the iron oxidizing enrichment culture. With AS concentrations of 9.3–20 g/L, iron oxidation with redox potential and pH increased similarly to positive control (Figure 23). All iron was completely oxidized before day 3. When AS concentration increased to 31 g/L, iron oxidation was slower during the first two days. After day 2, Fe(II) oxidation rate increased together with pH and redox potential increases in a similar manner as in the positive control. All iron was completely oxidized before day 3. At AS concentration of 42 g/L, Fe(II) oxidation was slower during the first

four days. After day 4, Fe(II) oxidation rate increased together with pH and redox potential increases, and all iron was oxidized before day 7. Since sampling occurred only on weekdays, more precise oxidation rate of 42 g/L AS concentration, between days 4–7, is unknown. When AS concentration increased to 53 g/L, iron oxidation was slower during the first four days. From day 4 forward, the iron oxidation rate increased together with pH and redox potential increases but was slower compared to the positive control. Iron oxidation was completed by day 10. Biological iron oxidation increased redox potential to 550–650 mV and pH from 2.0–2.1 to 2.5.

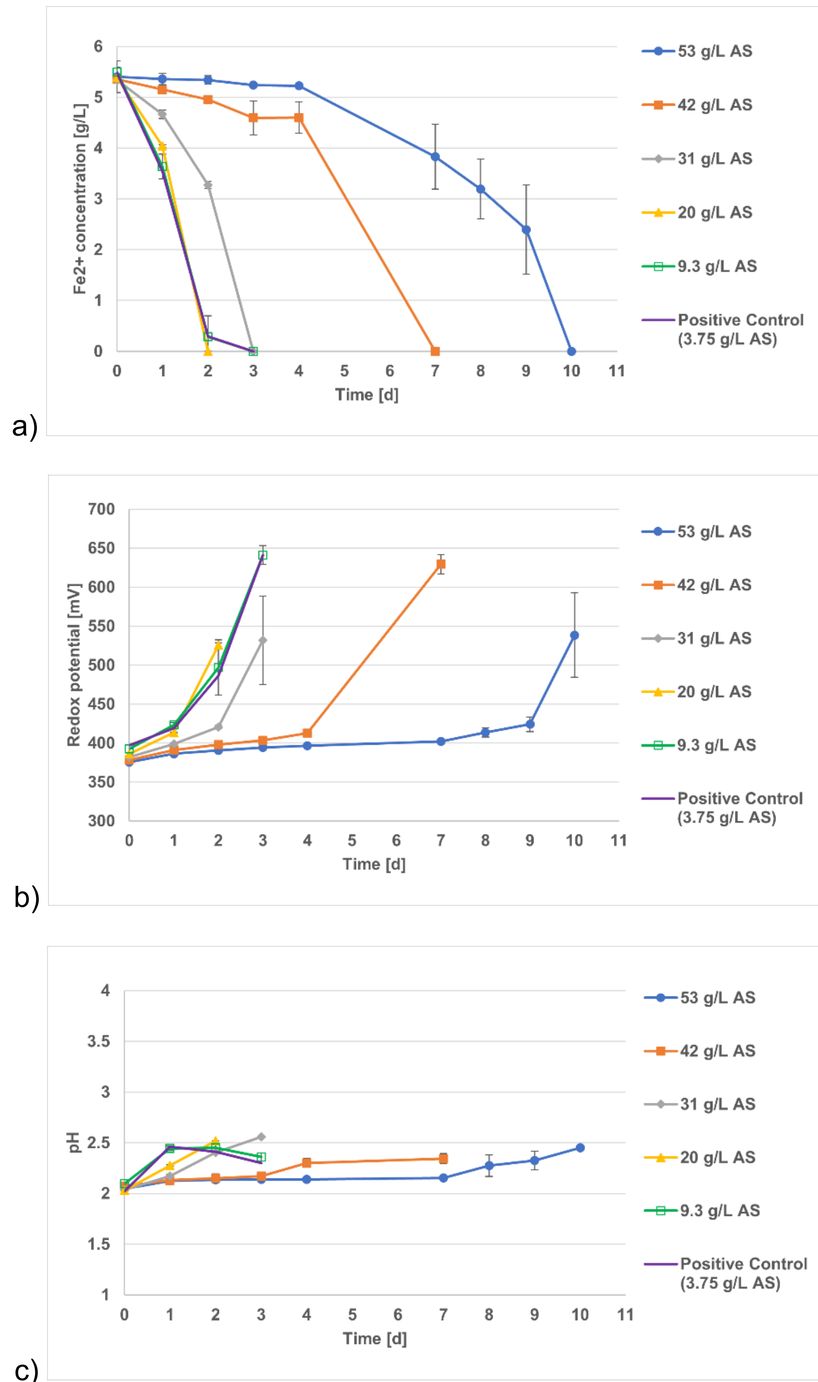


Figure 23. *Impact of ammonium sulfate (AS) concentration (9.3–53 g/L) on (a) Fe(II) concentration, (b) redox potential, and (c) pH, during biological iron oxidation by iron oxidizing enrichment culture. The standard deviations are presented with the error bars (n=2).*

Initial ammonium concentration increased with AS concentration and varied between 2.3–13 g/L (Figure 24). At last sampling day (day 2 with 20 g/L, day 3 with 9.3 g/L and

31 g/L, day 7 with 42 g/L, and day 10 with 53 g/L), ammonium concentration had decreased by 0.02–0.04 g/L with 9.3–20 g/L AS concentrations, and by 0.35–0.64 g/L with AS concentrations of 31–53 g/L.

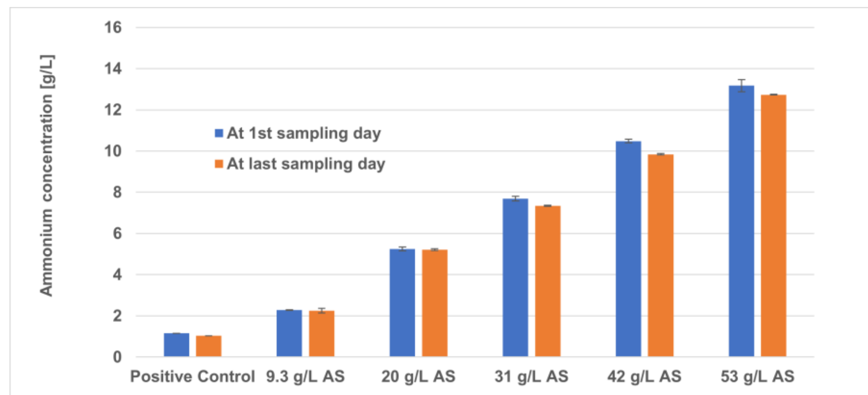


Figure 24. *Effect of ammonium sulfate (AS) concentration (9.3–53 g/L) on ammonium concentration and utilization during iron oxidation by the iron oxidizing enrichment culture. Last sampling days: day 2 with 20 g/L AS, day 3 with 9.3 g/L and 31 g/L AS, day 7 with 42 g/L AS, and day 10 with 53 g/L AS. The standard deviations are presented with the error bars (n=2).*

The results show that excessive AS addition did not have an impact towards iron oxidation, when AS concentration was below 20 g/L. When AS concentration increased over 20 g/L, iron oxidation was first delayed, and the rate decreased. After the lag phase, iron oxidation rate became more similar to the positive control. When AS concentration increased above 31 g/L, iron oxidation lag phase became longer, and iron oxidation rate after the lag phase was slower compared to the positive control. Ammonium utilization by the iron oxidizing enrichment culture was quite low with all the studied AS concentrations. Most of the ammonium was not utilized by the microorganisms but remained in the culture medium.

5.4 Neodecanoid acid experiments

During this study, three neodecanoid acid experiments were executed with the iron oxidizing enrichment culture.

5.4.1 Neodecanoid acid 1st inhibition experiment

The influence of neodecanoid acid on biological iron oxidation was studied to determine the lowest inhibitory concentration. With all studied neodecanoid acid concentrations of 5–100% of its water solubility, iron oxidation was slower compared to positive control (Figure 25). With 5% concentration of its water solubility, iron oxidation was slower during

the first three days. After day 3, Fe(II) oxidation rate increased together with pH and redox potential increases in similar manner as in the positive control. All Fe(II) was completely oxidized before day 7. With neodecanoid acid concentration of 10% of its water solubility, there were difference observed in iron oxidation in A and B experiment flasks, therefore separate lines present them in Figure 25. With 10% A, iron oxidation was slower during the first ten days. After day 10, iron oxidation increased with pH and redox potential increases similarly to the positive control. All iron was oxidized before day 14. With 10% B, iron oxidation was slower during the first four days. After day 4, iron oxidation increased with pH and redox potential elevations similarly to the positive control. All iron was oxidized before day 8.

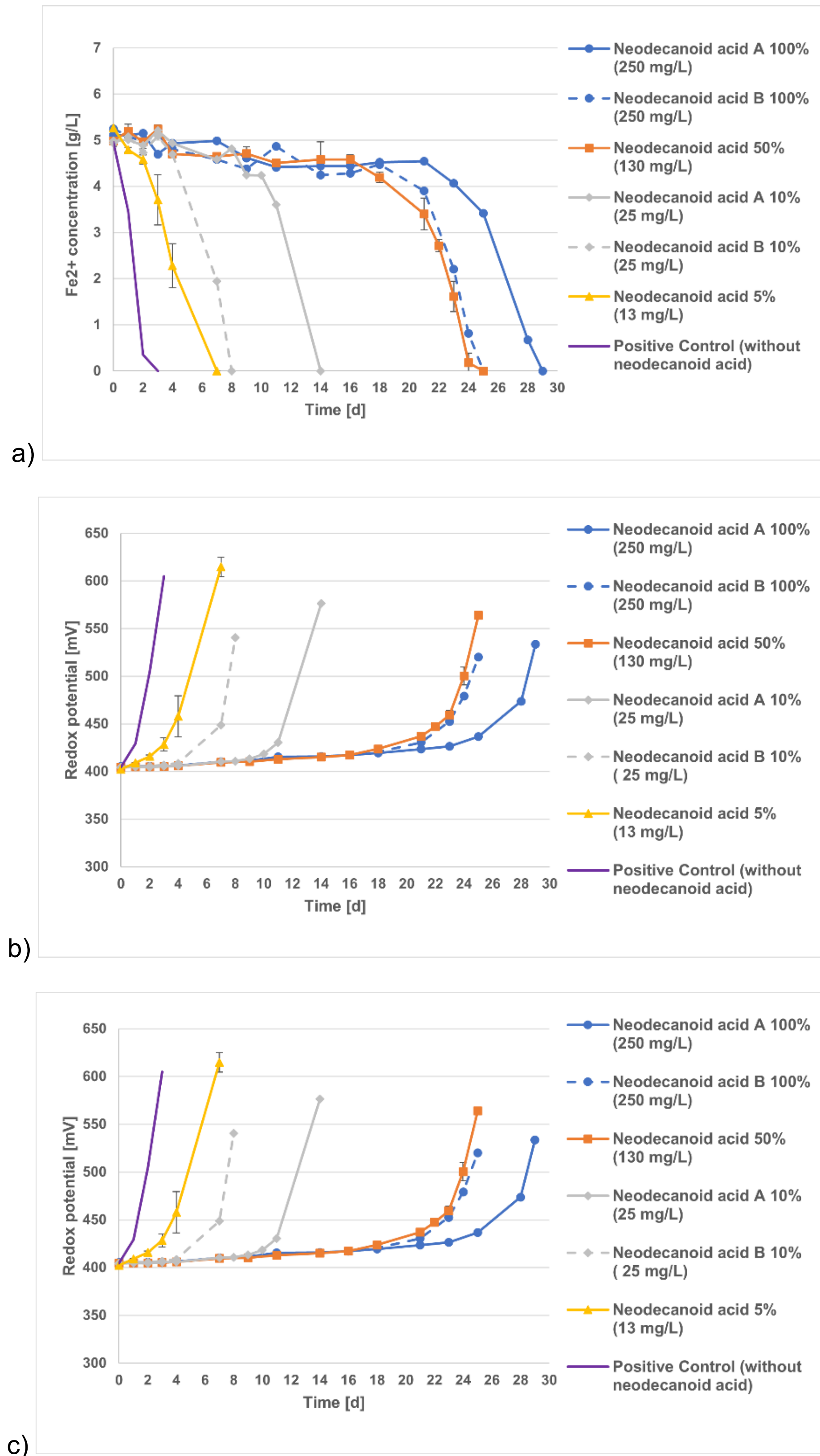


Figure 25. *Effect of neodecanoid acid concentration (5–100% of its water solubility) on biological iron oxidation by the iron oxidizing enrichment culture. (a) Fe(II) concentration, (b) redox potential, and (c) pH. The standard deviations are presented with the error bars (n=2).*

At neodecanoid acid concentration of 50% of its water solubility, iron oxidation was slower during the first 18 days. After day 18, iron oxidation rate increased with pH and redox potential increases but was slower compared to the positive control. All iron was completely oxidized before day 25. With neodecanoid acid concentration of 100% of its water solubility, difference in iron oxidation between experiment flasks A and B was also observed, therefore separate lines present them in Figure 25. With 100% A, iron oxidation was slower during the first 23 days. From day 23 forward, Fe(II) was oxidized and pH and redox potential increased, but the change was slower compared to the positive control. All Fe(II) was oxidized before day 29. With 100% B, iron oxidation was slower during the first 21 days. After day 21, iron oxidation rate increased with pH and redox potential elevations but was slower compared to the positive control. Biological iron oxidation increased pH from 2.1–2.2 to 2.4–2.5 and redox potential from ~400 mV to above 500 mV.

The results of the experiment show that all neodecanoid acid concentrations had a negative effect on biological iron oxidation. All studied neodecanoid acid concentrations delayed iron oxidation during the first 3–23 days, but after the lag phase, iron oxidation rate increased, but stayed slower compared to the positive control. However, this inhibitory effect was reversible. In neodecanoid acid inhibition experiment with concentration of 100% of its water solubility, all Fe(II) was completely oxidized before day 4 (with 100% B) and day 7 (with 100% A) (Figure 26).

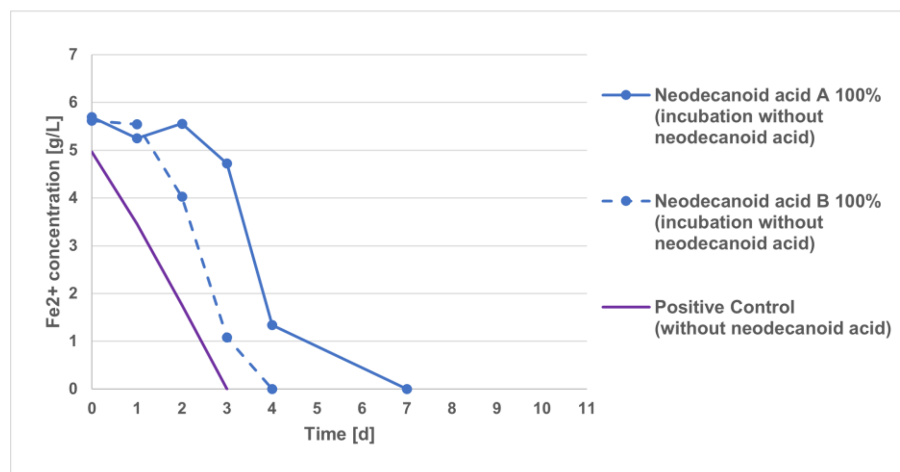


Figure 26. *Effect of neodecanoid acid concentration of 100% of its water solubility on biological iron oxidation during incubation without neodecanoid acid. The incubation was performed with the enrichment culture obtained from the first incubation in neodecanoid acid 1st inhibition experiment. Samples were taken daily from the positive control. Standard deviations are presented with the error bars (n=2).*

5.4.2 Neodecanoid acid 2nd inhibition experiment

The aim of the neodecanoid acid 2nd inhibition experiment was to study the effect of neodecanoid acid on biological iron oxidation and to determine the lower inhibitory concentration in detail. During the experiment, DOC concentration was also monitored. With neodecanoid acid concentration of 1% of its water solubility, Fe(II) was oxidized, and pH and redox potential increased in a similar manner as in positive control (Figure 27). All Fe(II) was oxidized less than 2 days. With neodecanoid acid concentration of 2.5% of its water solubility, Fe(II) was oxidized, and pH and redox potential increased slightly slower compared to the positive control. All Fe(II) was oxidized before day 3. With neodecanoid acid concentrations of 3.8% and 5%, iron oxidation was slower during the first day. After day 1, iron oxidation rate increased with pH and redox potential increases but was slightly slower compared to the positive control. All iron was oxidized before day 3, with 3.8%, and day 4, with 5%. Biological iron oxidation increased pH from 2.1 to 2.3–2.4 and redox potential from ~400 mV to above 550 mV.

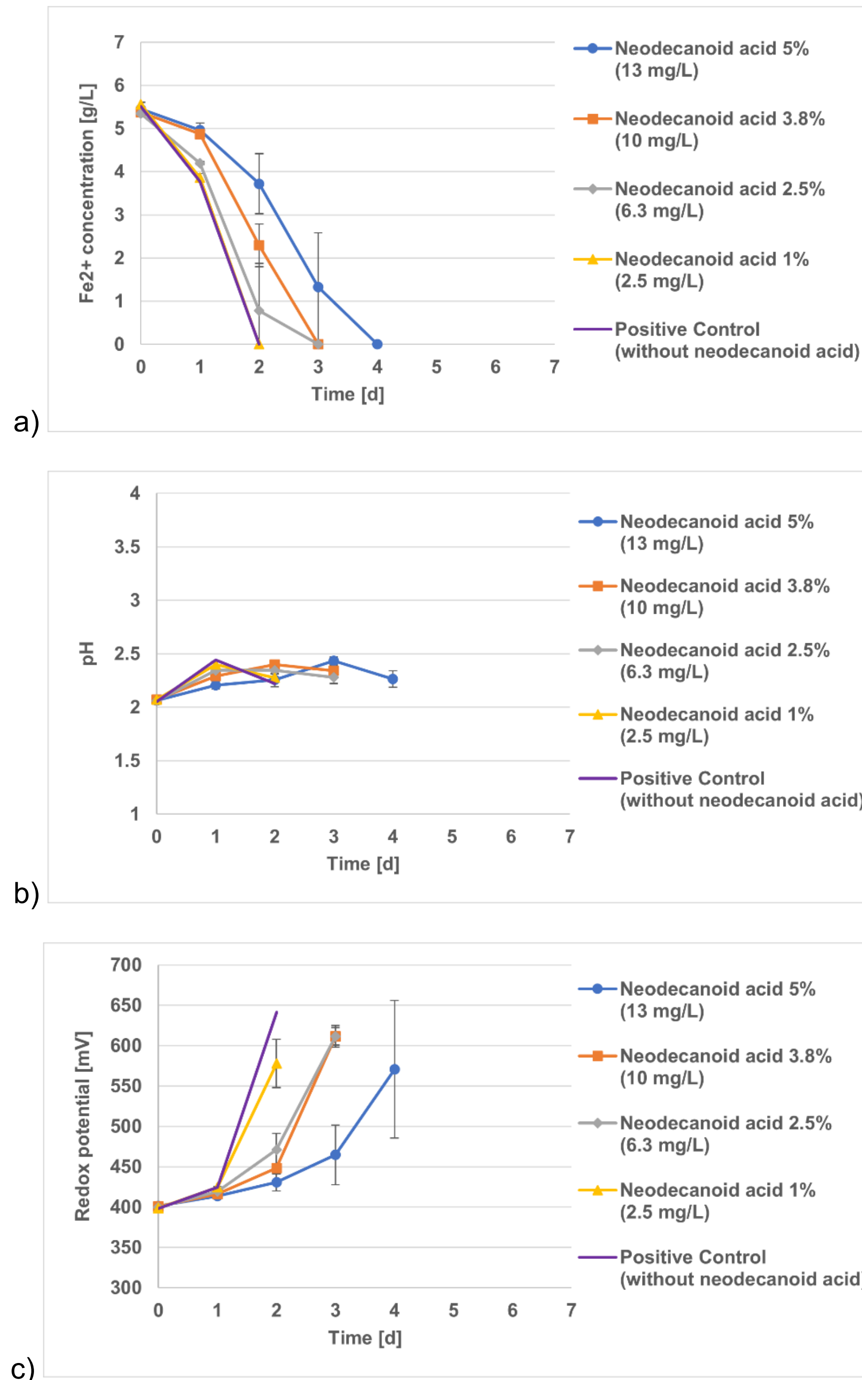


Figure 27. *Influence of neodecanoid acid concentration (1–5% of its water solubility) on (a) Fe(II) concentration, (b) redox potential, and (c) pH, during biological iron oxidation by the iron oxidizing enrichment culture. The standard deviations are presented with the error bars (n=2).*

With the studied neodecanoid acid concentrations, DOC concentration in the beginning varied between 9–14 mg/L and was similar to DOC concentration (12 mg/L) of the positive control (Figure 28). The DOC concentration did not increase with the neodecanoid acid concentration indicating that it was masked by the background DOC concentration

or neodecanoid acid was not present or was only partially present in the aqueous phase of the samples. The DOC concentration in the end of the experiment was lower compared to the beginning, with neodecanoid acid concentrations of 2.5–5% of its water solubility, whereas DOC concentrations of the positive control and neodecanoid acid concentration of 1% of its water solubility were higher in the end than in the beginning of the experiment.

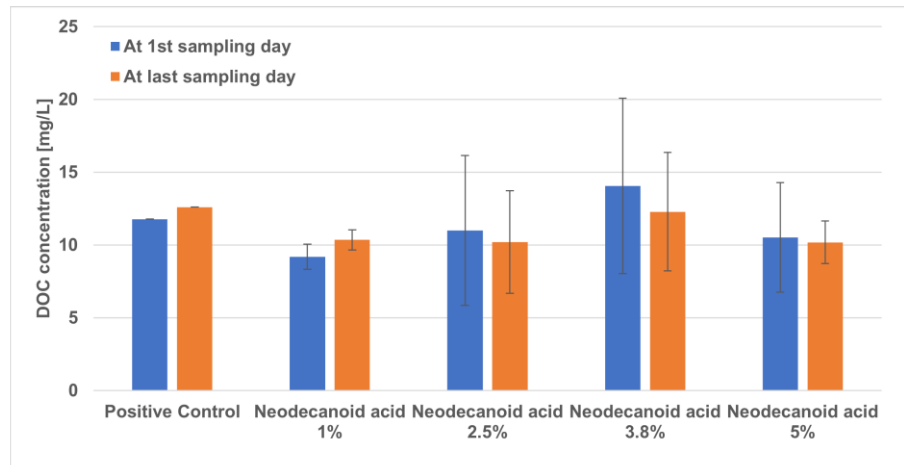


Figure 28. *Influence of neodecanoid acid concentration (1–5% of its water solubility) on DOC concentration, during biological iron oxidation by the iron oxidizing enrichment culture. The standard deviations are presented with the error bars (n=2).*

The iron oxidation results show that neodecanoid acid concentration of 1% of its water solubility did not affect to the biological iron oxidation, whereas with 2.5% concentration the negative effect was very modest. With 3.8% and 5% concentrations, iron oxidation was delayed during the first day, but after day 1, faster iron oxidation began to occur. Iron oxidation rate stayed still slightly lower level compared to the positive control. Since the DOC concentration results seemed to be random, no conclusions can be drawn from these results.

5.4.3 Neodecanoid acid kinetics experiment

The aim of the experiment was to determine the fate and the transformation kinetics of neodecanoid acid concentration of 50% of its water solubility in iron oxidizing culture, by monitoring biological iron oxidation and the change of DOC concentration. With the studied concentration, iron oxidation was slower during the first 16 days (Figure 29)). After day 16, iron oxidation increased with pH and redox potential elevations, but was slower compared to the positive control. All iron was completely oxidized before day 28. Biological iron oxidation increased pH from 2.1 to 2.4 and redox potential above 550 mV.

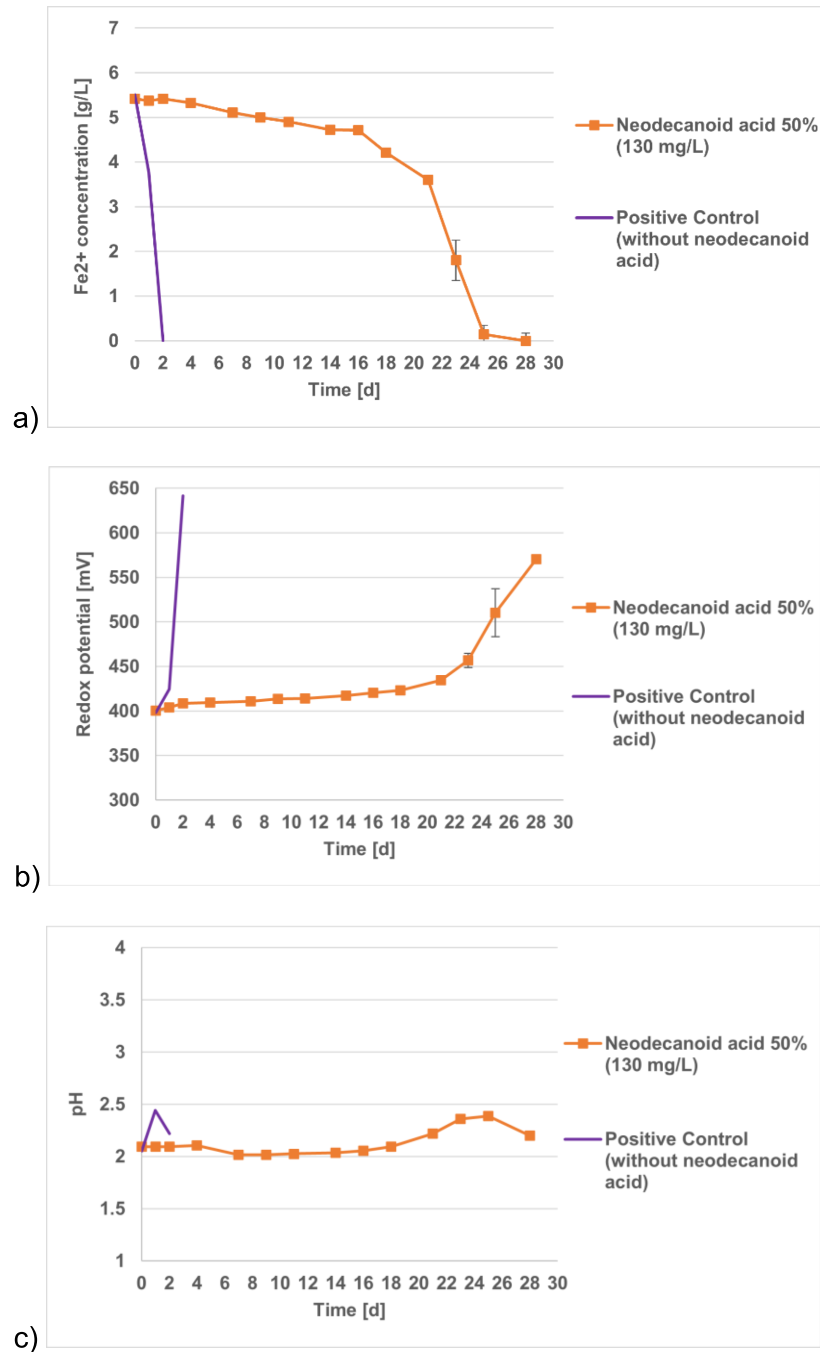


Figure 29. *Effect of neodecanoid acid concentration of 50% of its water solubility on biological iron oxidation by the iron oxidizing enrichment culture. (a) Fe(II) concentration, (b) redox potential, and (c) pH. The error bars present the standard deviation (n=4 on day 0, 1, 25, and 28, n=2 on other days).*

DOC concentrations were measured from samples collected from one (neodecanoid acid 50% A) shake flask or two of the shake flasks (neodecanoid acid 50% A and B). Chemical controls were not used as controls for DOC analysis, according to the original experimental plan, since neodecanoid acid did not dissolve into the aqueous phase of

the chemical controls in any point of the experiment, and during the sampling the chemical controls were contaminated. As seen in Figure 30, DOC concentration increased during the first three days (day 0–2) from 26 mg/L to 35.09 mg/L. During experimental days 7–11, DOC concentration remained stable, and then increased to 70 mg/L on day 14. After day 14, DOC concentration decreased back to 30–35 mg/L for the next ten days (days 16–25), indicating that DOC concentration result on day 14 was not representative. In last sampling day (day 28), DOC concentration increased to 47 mg/L.

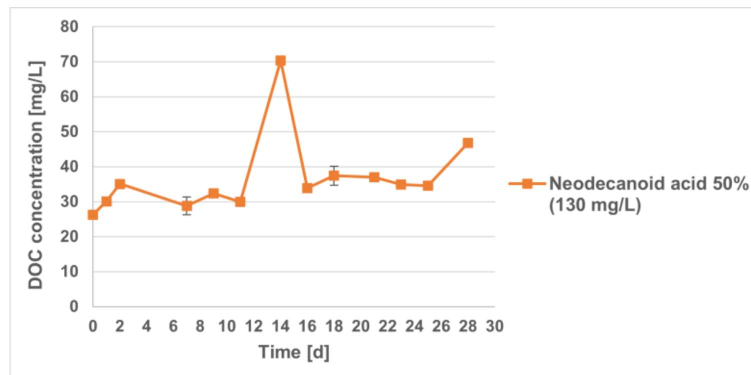


Figure 30. *Influence of neodecanoid acid concentration of 50% of its water solubility on DOC concentration, during iron oxidation by the iron oxidizing enrichment culture. The standard deviations are presented with the error bars (n=2).*

The iron oxidation results show that 50% neodecanoid acid concentration delayed iron oxidation during the first 18 days. After the lag phase, iron oxidation rate increased, but stayed still slightly lower level compared to the positive control. The DOC results show that DOC concentration remained quite stable during the iron oxidation. Therefore, no conclusions about the neodecanoid acid degradation in the iron oxidizing culture can be drawn from these results.

5.5 Nessol D100 experiments

Two Nessol D100 inhibition experiments were performed with the iron oxidizing enrichment culture.

5.5.1 Nessol D100 1st inhibition experiment

The influence of Nessol D100 on biological iron oxidation was studied for determining the lowest inhibitory concentration. With all the studied Nessol D100 concentrations of 1–15% of its water solubility, Fe(II) was oxidized and pH and redox increased in a similar way as in positive control (Figure 31). All Fe(II) was completely oxidized at all studied

Nessol D100 concentrations and in the positive control before day 3. The biological iron oxidation elevated redox potential from about 400 mV to about 650 mV, and pH from 2.1 to 2.4.

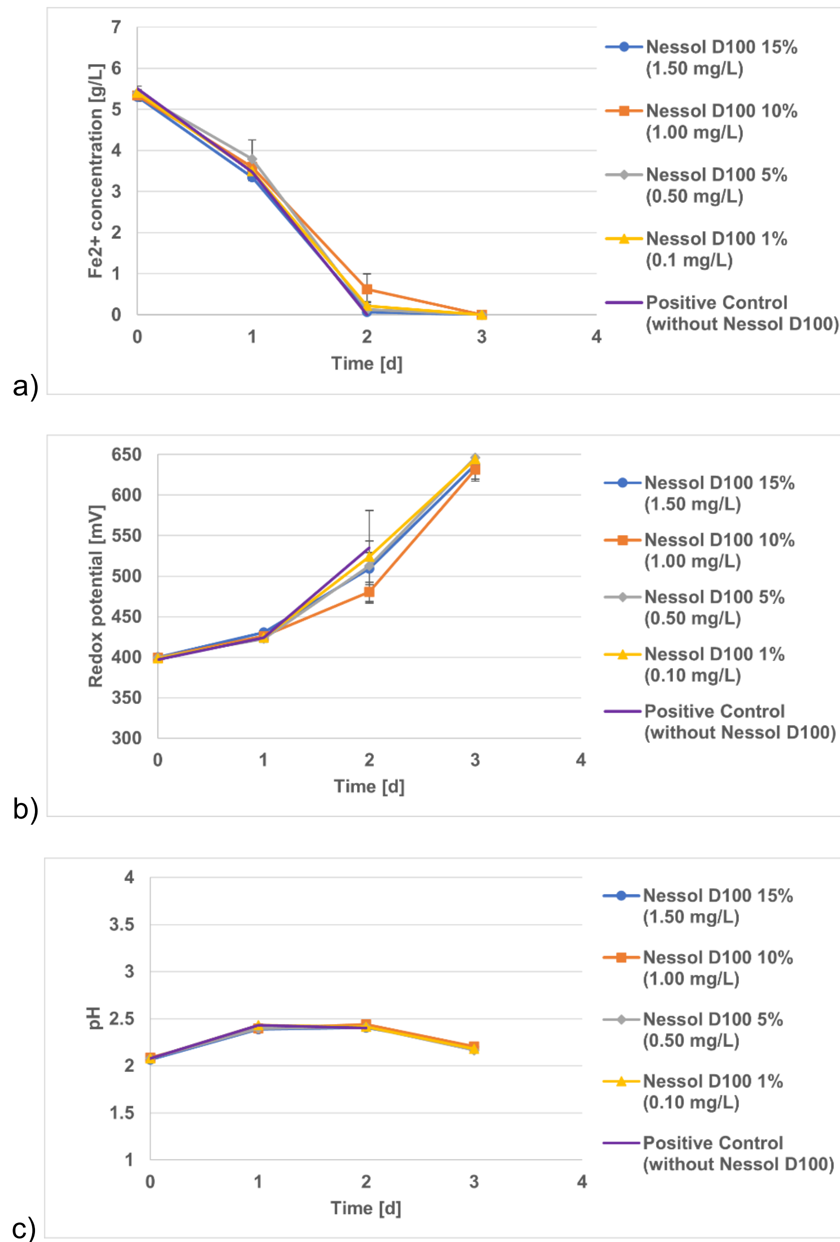


Figure 31. *Influence of Nessol D100 concentration (1–15% of its water solubility) on (a) Fe(II) concentration, (b) redox potential, and (c) pH, during the biological iron oxidation by the iron oxidizing enrichment culture. The standard deviations are presented with the error bars (n=2).*

The results show that Nessol D100 had no effect on iron oxidation at lower concentrations of 1–15% of its water solubility. During the experiment, a very slight liquid phase, which included small precipitate particles (probably Fe(III) precipitates) was recognized

from the surface of the culture medium (Figure 32). This indicates that Nessol D100 did not dissolve in water phase and, thus, did not inhibit biological iron oxidation.

Figure 32. *The separate liquid phase on top of the Nessol D100 B 15% of its water solubility medium in experimental day of 2. The separate liquid phase is shown with red arrows.*

5.5.2 Nessol D100 2nd inhibition experiment

In the Nessol D100 2nd inhibition experiment, the influence of Nessol D100 on biological iron oxidation was studied with higher concentrations than in the first experiment. With all the studied Nessol D100 concentrations (30% and 100% of its water solubility) Fe(II) was oxidized, and pH increased similarly compared to positive control (Figure 33). All iron was oxidized in less than 2 days. Biological iron oxidation increased pH from 2.1 to 2.4. During the first day, redox potential of the studied concentrations increased in similar way as in the positive control, but day 1 forward, redox potential of the studied concentrations increased from about 400 mV to about 500 mV, which was lower compared to 630 mV redox potential of the positive control.

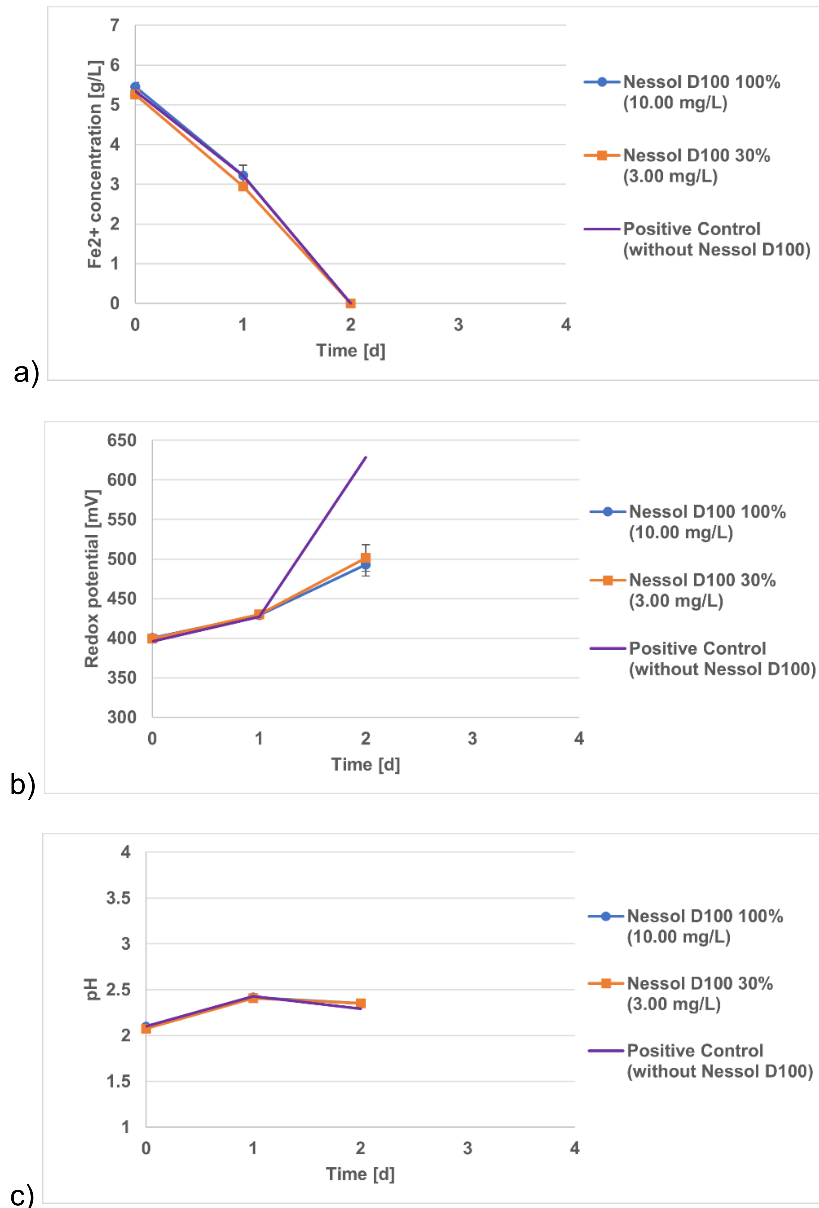


Figure 33. *Effect of Nessol D100 concentrations of 30% and 100% of its water solubility on during the biological iron oxidation by the iron oxidizing enrichment culture. (a) Fe(II) concentration, (b) redox potential, and (c) pH. The standard deviations are presented with the error bars (n=2).*

The results show that Nessol D100 had no effect on iron oxidation either at higher concentrations of 30% and 100% of its water solubility. During this study, a very slight liquid phase, similar to the liquid phase recognized in the Nessol D100 1st inhibition experiment, was also noticed (see Figure 32). This indicates that Nessol D100 did not dissolve in water phase and, thus, did not inhibit biological iron oxidation.

5.6 Cyanex 272 inhibition experiment

During this study, one Cyanex 272 inhibition experiment was executed with the iron oxidizing enrichment culture. The effect of Cyanex 272 on biological iron oxidation was studied for defining the lowest inhibitory concentration. Iron oxidation elevation together with pH and redox potential increases, was similar to positive control with all the studied Cyanex 272 concentrations (5–100% of its water solubility) (Figure 34). All iron was completely oxidized before day 3. During biological iron oxidation, pH was increased from 2.0–2.1 to 2.4, and redox potential from about 400 mV to above 600 mV.

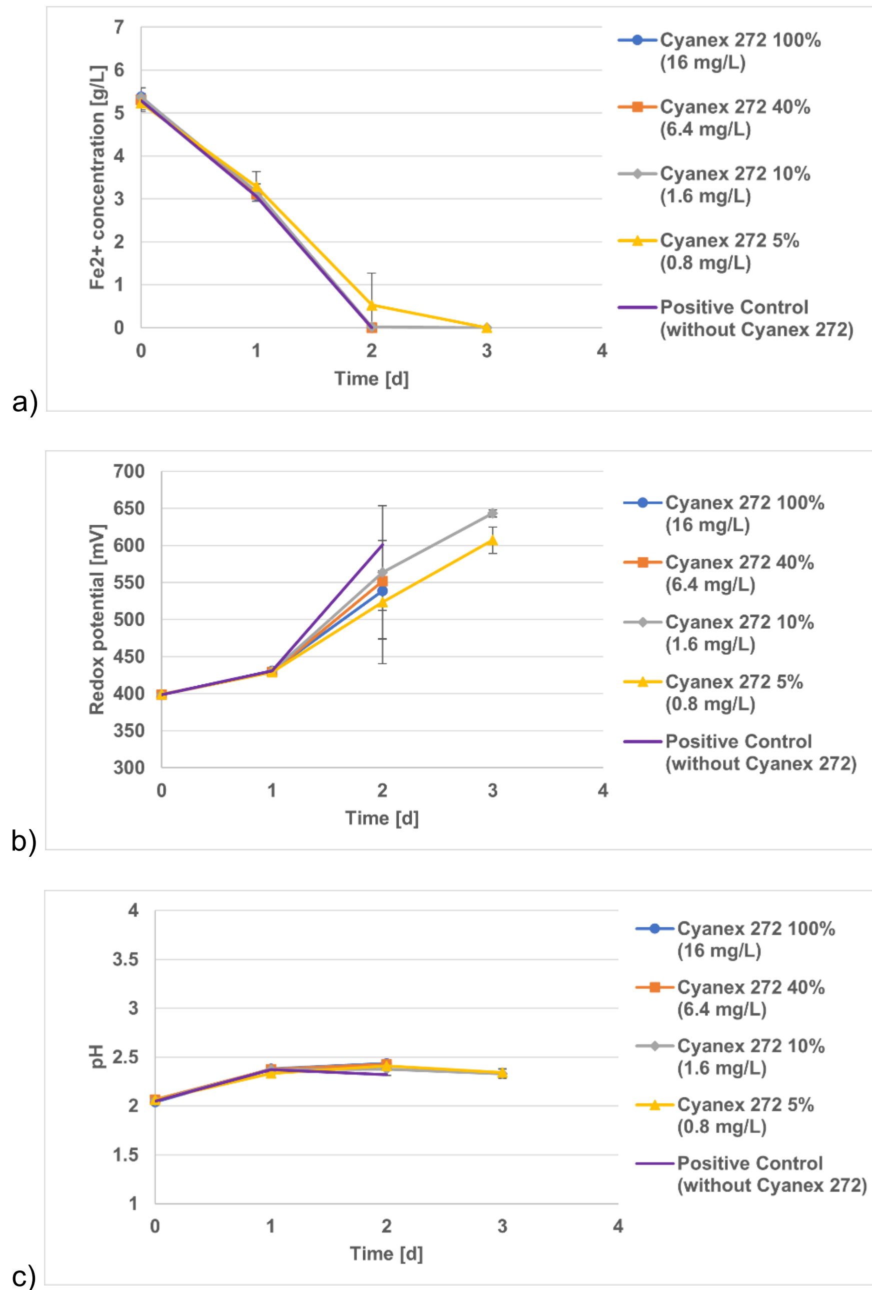


Figure 34. *(a) Fe(II) concentration, (b) redox potential, and (pH), during biological iron oxidation by the iron oxidizing enrichment culture, when different Cyanex 272 concentration (5–100% of its water solubility) were present. The standard deviations are presented with the error bars (n=2).*

The results show that Cyanex 272 did not affect biological iron oxidation below concentration of 100% of its water solubility. In the beginning of the experiment, small white precipitate particles were formed on top of the culture medium, when Cyanex 272 was added into it (Figure 35). This white precipitate remained on top of the medium during the whole experiment. This indicates that Cyanex 272 did not dissolve in water phase and, thus, did not inhibit biological iron oxidation. In precipitation experiments, it was

recognized that Cyanex 272 was reacting with some component of TES and formed this white precipitate.

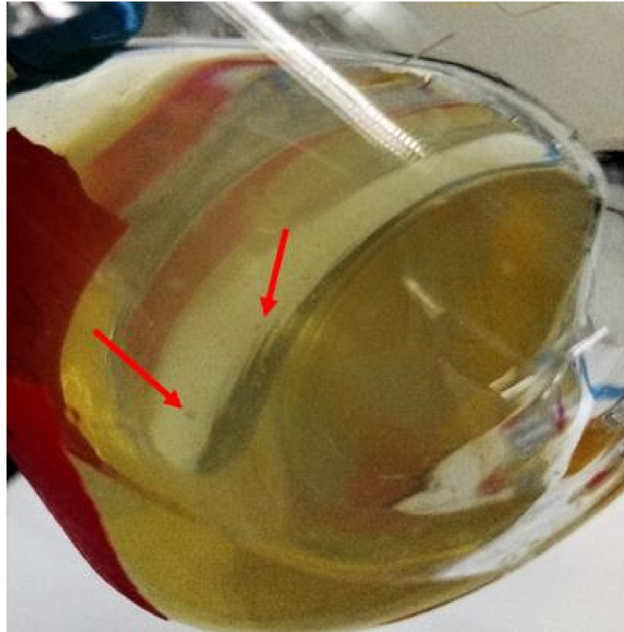


Figure 35. *White precipitate on top of the Cyanex 272 A 40% of its water solubility medium in experimental day 0. The white precipitate is shown with red arrows.*

5.7 Baysolvex D2EHPA inhibition experiment

During this study, one inhibition experiment was performed with Baysolvex D2EHPA solution. The influence of Baysolvex D2EHPA on biological iron oxidation by the iron oxidizing enrichment culture was studied to determine the minimum inhibitory concentration of the D2EHPA solution. With all the studied concentrations (5–100% of its water solubility), iron oxidation rate and pH and redox potential increase was similar to positive control (Figure 36). Fe(II) oxidation was completed by day 3. Redox potential increased from about 400 mV to 550–650 mV, and pH increased from 2.0 to 2.4, during the biological iron oxidation.

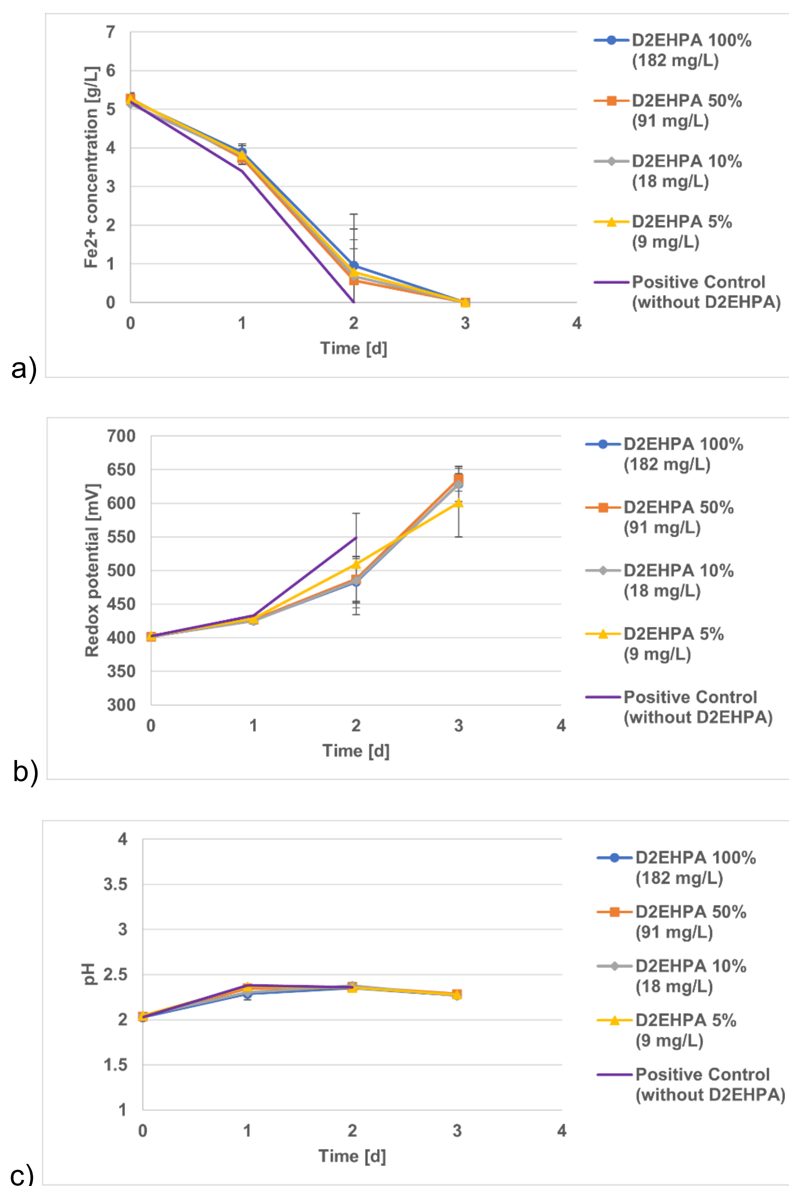


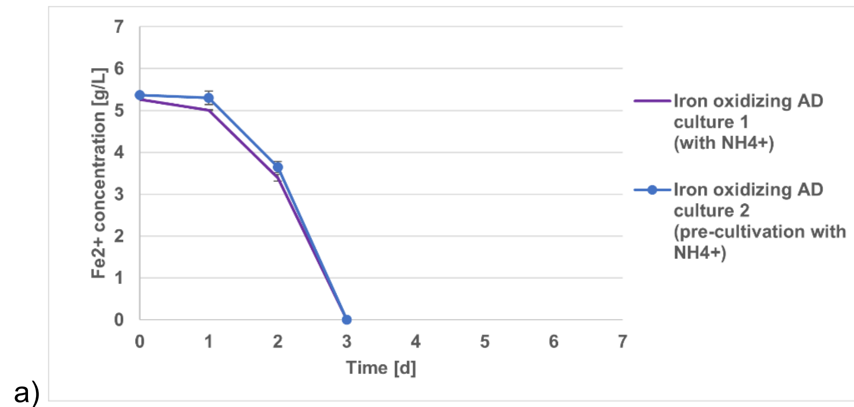
Figure 36. *Influence of various Baysolvex D2EHPA concentration (5–100% of its water solubility) on (a) Fe(II) concentration, (b) redox potential, and (c) pH, during the iron oxidation by the iron oxidizing enrichment culture. The standard deviations are presented with the error bars (n=2).*

The results of the experiment show that Baysolvex D2EHPA did not affect biological iron oxidation with concentrations below 100% of its water solubility. Such as with Cyanex 272, also Baysolvex D2EHPA formed white precipitate on top of the culture medium immediately after the D2EHPA addition (Figure 37). The precipitate particles stayed on top of the medium during the entire experiment. In the precipitation experiment, it was recognized that Baysolvex D2EHPA was reacting with inoculum and forming the similar looking white precipitate. Since inoculum was the only constituent which included Fe(III), it was probable that D2EHPA was reacting with Fe(III).

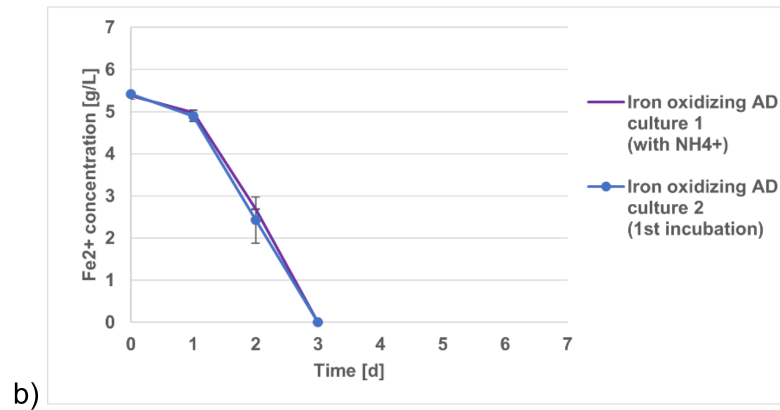
Figure 37. *White precipitate on top of the Baysolvex D2EHPA A 10% of its water solubility medium in experimental day of 0.*

5.8 Ammonium deficiency experiment with AS bleed and feed

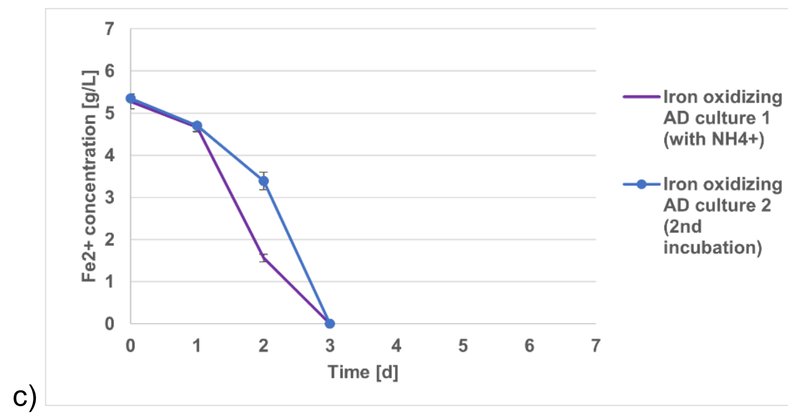
During this study, one ammonium deficiency experiment was performed with AS bleed and feed. The aim of the experiment was to determine if ammonium containing AS bleed and feed can have a positive effect on iron oxidation by iron oxidizing culture that has been grown in ammonium limited conditions. During the iron oxidation, ammonium utilization of the iron oxidizing AD cultures, was also studied. Before the ammonium deficiency experiment, iron oxidation and ammonium concentration were monitored from the iron oxidizing AD cultures. During the pre-cultivation and the 1st cultivation, iron oxidation of the iron oxidizing AD culture 2 was similar to the iron oxidizing AD culture 2, which was supplemented with ammonium (Figure 38). After 1st incubation, iron oxidation of the iron oxidizing AD culture 2 was slowed, compared to the iron oxidizing AD culture 1, due to influence of ammonium deficiency. After the 1st incubation, no ammonium was detected from the iron oxidizing AD culture 2.



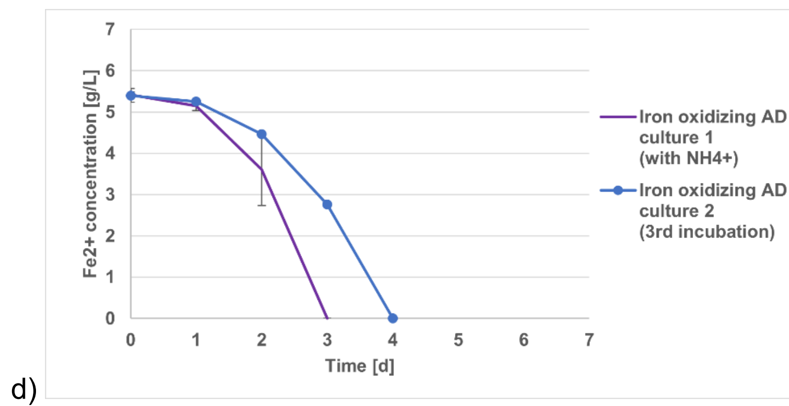
a)



b)



c)



d)

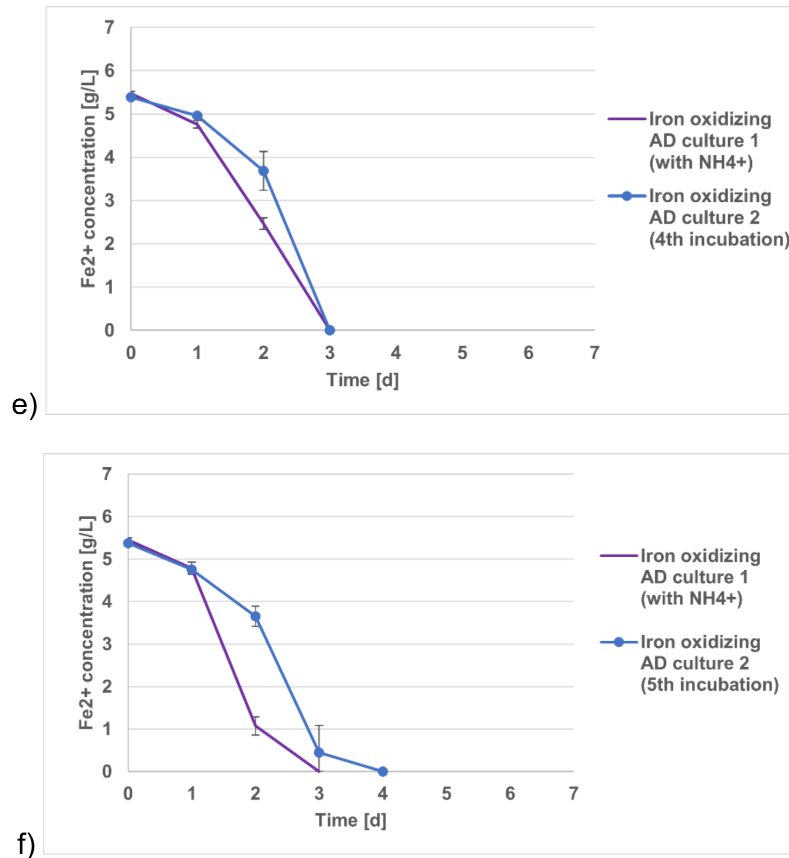
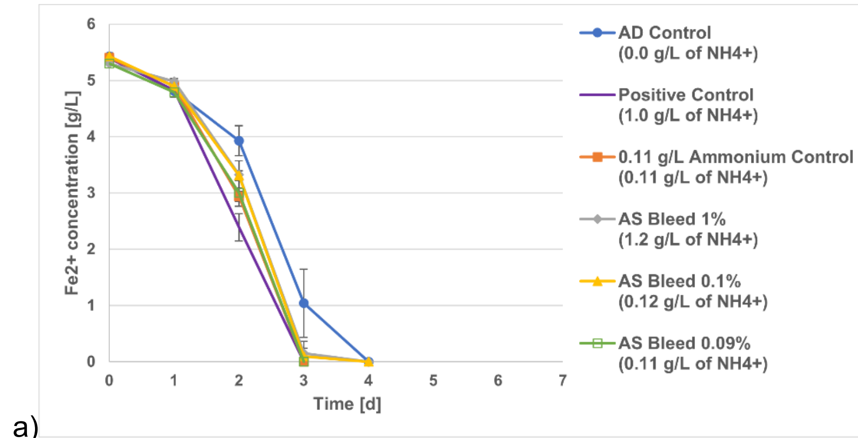
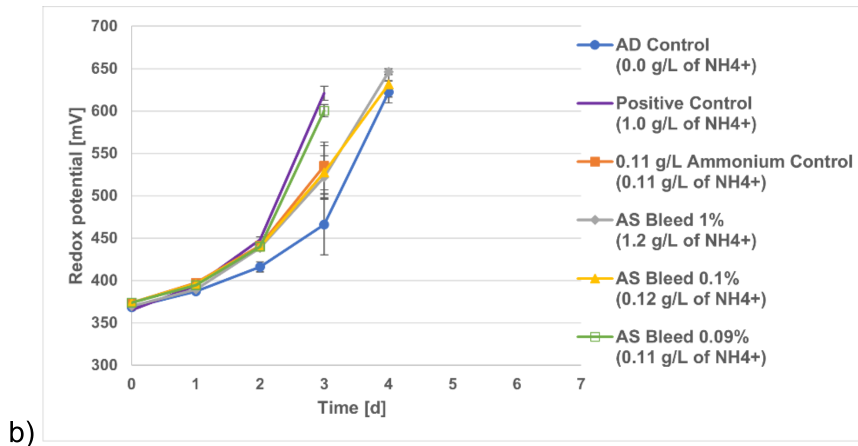


Figure 38. *Influence of ammonium deficiency on iron oxidation by the iron oxidizing ammonium deficit (AD) culture 2, during (a) pre-cultivation, (b) 1st incubation, (c) 2nd incubation, (d) 3rd incubation, (e) 4th incubation, and (f) 5th incubation. The standard deviations are presented with the error bars (n=2).*

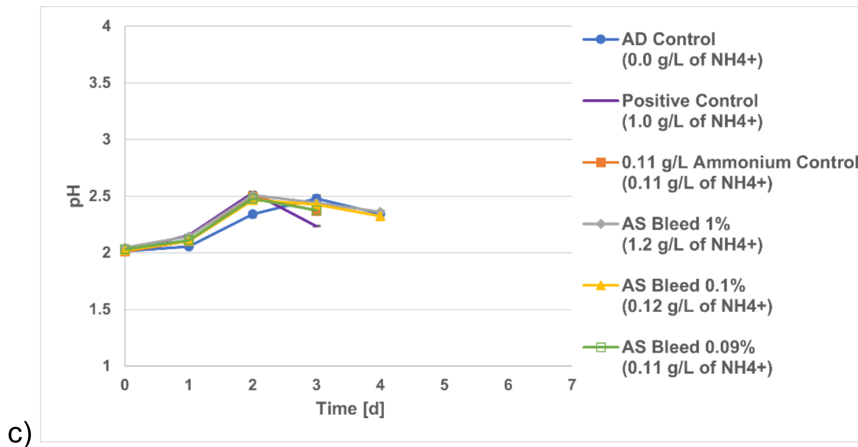
In ammonium deficiency experiment, with all studied AS bleed and feed concentrations, Fe(II) was oxidized, and pH and redox potential increased similarly to positive control and 0.11 g/L ammonium control (Figure 39). All iron was completely oxidized in less than 4 days. With AD control, iron oxidation rate together with redox potential and pH increases, was similar as in the positive control and 0.11 g/L ammonium control, during the first day. After day 1, iron oxidation rate with redox potential and pH increases was slightly slower with AD control compared to the positive control and 0.11 g/L ammonium control. All iron was oxidized completely before day 4. Biological iron oxidation increased redox potential from about 370 mV to 500–650 mV, and pH from 2.0 to 2.5.



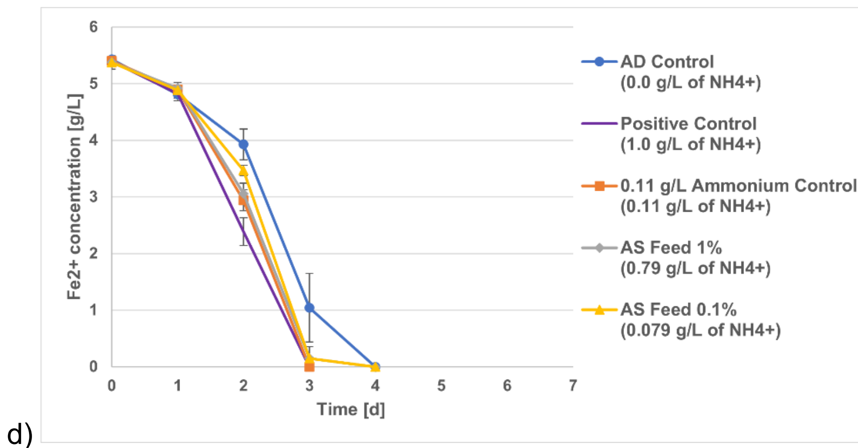
a)



b)



c)



d)

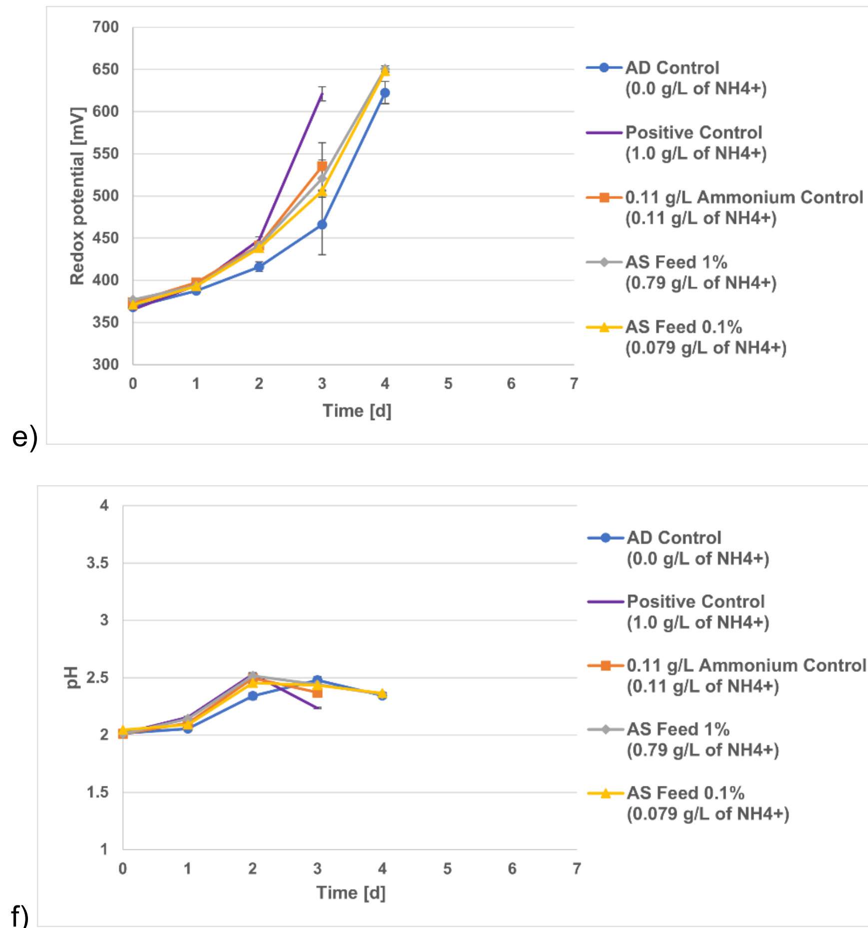


Figure 39. *Effect of AS bleed on (a) Fe(II) concentration, (b) redox potential, and (c) pH, and AS feed on (d) Fe(II) concentration, (e) redox potential, and (f) pH, during iron oxidation of the iron oxidizing ammonium deficit (AD) culture. Inoculum used in positive control, was iron oxidizing culture supplemented with ammonium (iron oxidizing AD culture 1). Inoculum used with other controls and experiment flasks was iron oxidizing culture without ammonium supplementation (iron oxidizing AD culture 2). The standard deviations are presented with the error bars (n=2).*

With the studied AS bleed and feed concentrations, initial ammonium concentration varied between 0.11–1.3 g/L with AS bleed and between 0.09–0.84 g/L with AS feed (Figure 40). The ammonium concentration corresponded with AS bleed and feed concentration increases. At last sampling day (day 3 with positive control, 0.11 g/L ammonium control and AS bleed 0.9%, and day 4 with AD control, AS bleed 0.1% and 1%, and AS feed 0.1% and 1%), ammonium concentration had decreased by 0.004–0.09 g/L with AS bleed concentrations, and by 0.002–0.04 g/L with AS feed concentrations.

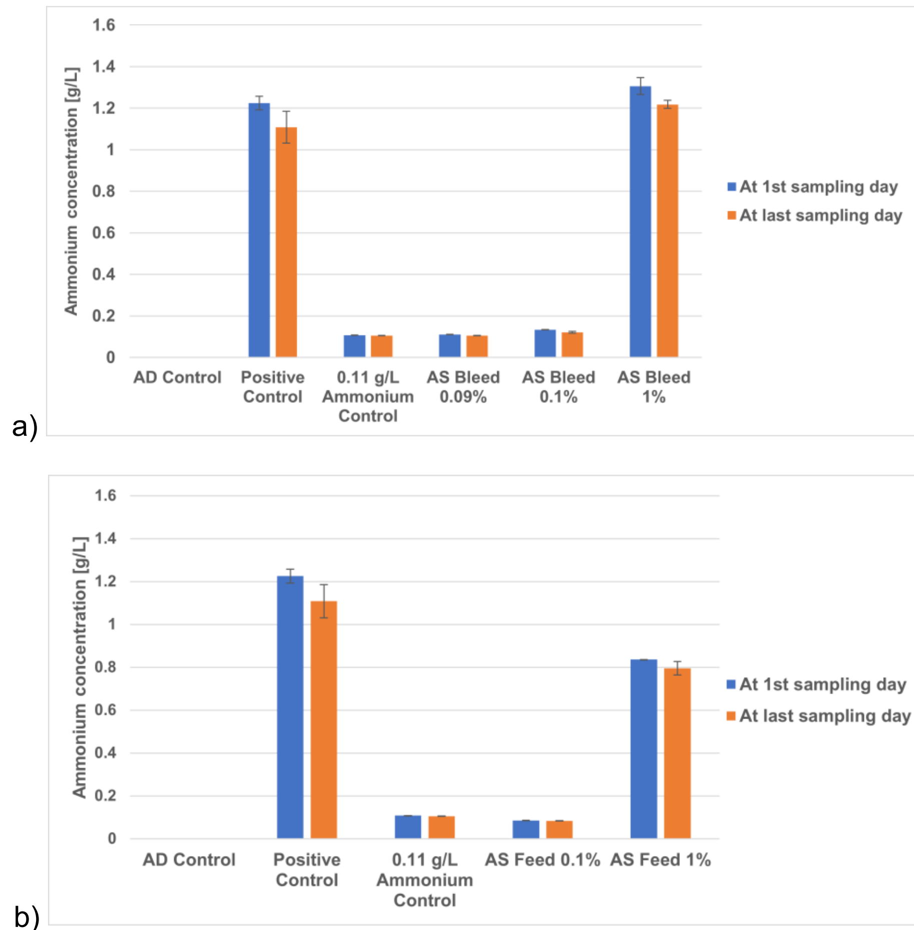


Figure 40. *Influence of (a) AS bleed concentration (0.09–1% (v/v)) and (b) AS feed concentration (0.1–1% (v/v)) on ammonium concentration during iron oxidation by iron oxidizing ammonium deficit (AD) culture. Inoculum used in positive control, was iron oxidizing culture supplemented with ammonium (iron oxidizing AD culture 1). Inoculum used with other controls and experiment flasks was iron oxidizing culture without ammonium supplementation (iron oxidizing AD culture 2). The last sampling days: day 3 with positive control, 0.11 g/L ammonium control and AS bleed 0.9%, and day 4 with AD control, AS bleed 0.1% and 1%, and AS feed 0.1% and 1%). The standard deviations are presented with the error bars (n=2).*

The results show that AS bleed and feed concentration below 1% enhanced biological iron oxidation. Ammonium utilization by the iron oxidizing culture was moderate with all the studied AS bleed and feed concentrations. Most of the ammonium was not utilized but remained in the culture medium.

6. DISCUSSION

The results of this study showed that AS bleed and feed process liquors and some of their constituents (AS and neodecanoid acid) negatively affected biological iron oxidation. It was also demonstrated that with low concentrations (<1% (v/v)) these ammonium-rich AS bleed and feed stimulated biological iron oxidation. These results and possible mechanisms behind them are discussed in more detail in the following three chapters with the results of earlier studies. Moreover, the environmental effects of nitrogen and organic solvents, present in AS bleed and feed, are discussed in the las chapter of the discussion (Chapter 6.4).

6.1 Influence of AS bleed and feed on iron and sulfur oxidation

The results of this study showed minor differences between the effect of AS bleed and feed on the biological iron oxidation rates (Figure 41), as calculated from the slope of the linear regression line of the exponential part of the Fe(II) oxidation curves (R^2 values were above 0.92, except with AS feed 50% (R^2=0.47)). Therefore, the effects of AS bleed and feed of iron oxidizing microorganisms can be considered to be similar.

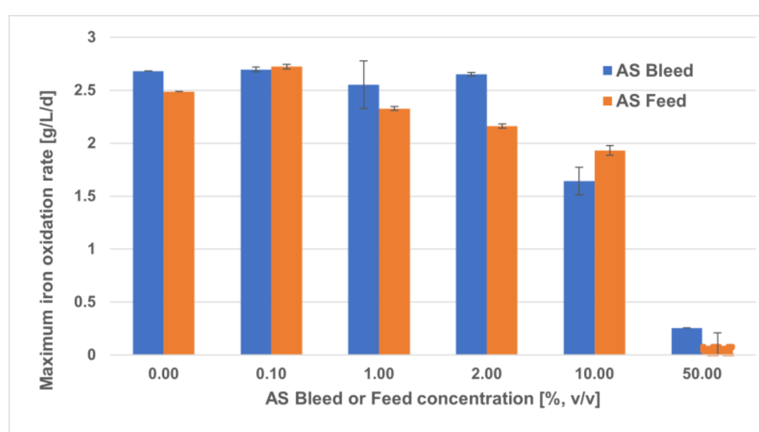


Figure 41. *Influence of AS bleed and feed concentration (0.1–50% (v/v)) on biological iron oxidation rate. The iron oxidation rates were calculated from the slope of the linear regression line of the exponential part of the Fe(II) oxidation curves (see figures 13 and 19). The standard deviations are presented with the error bars (n=2).*

The influence of recycled process liquors of solvent extraction on growth or bioleaching activity of acidophilic bioleaching microorganisms has not been previously reported.

Therefore, direct comparisons to the literature could not be made. However, AS and TOC concentrations of AS bleed and feed were different and may have resulted in the slight differences between these two process liquors towards the iron oxidation. AS bleed contained approximately twice as much AS than the AS feed (see Chapter 4.3.1). According to Terrafame Oy, AS bleed contains almost twice as much TOC than the AS feed (see Chapter 4.3.1).

The AS inhibition results showed that AS delayed the onset of biological iron oxidation at concentration above 20 g/L. The results indicate that AS concentration of AS bleed and feed process liquors is one of the factors negatively affecting iron oxidation and causing the observed differences in iron oxidation rates between these two process liquors. Previous studies have focused on the possible enhancing effect of ammonium containing AS on iron oxidation and bioleaching (Krafft & Hallber 1993; Niemelä *et al.* 1994; d'Hugues *et al.* 1997; Ahoranta *et al.* 2017), whereas no studies have reported about the possible inhibition by AS at higher concentrations.

Organic compounds are inhibitory to autotrophic iron oxidizing microorganisms, and therefore TOC of AS bleed and feed, consisting of multiple organic solvents, is another factor, that negatively affects biological iron oxidation and can result in observed differences in iron oxidation rates between the process liquors (Johnson & Roberto 1997; Schippers 2007; Li e*t al.* 2011). In the following chapter, the influence of organic solvents on bioleaching, iron and sulfur oxidation is discussed in more detail.

The AS bleed did not inhibit biological sulfur oxidation but enhanced the sulfate production with AS bleed concentration of 8% and below (Figure 18). At AS bleed concentration above 4%, the enhancing effect on iron oxidation decreased. This may indicate that increasing the AS bleed concentration further, could negatively affect the sulfur oxidation. As the negative effect of AS bleed on iron oxidation was apparent with concentrations above 2%, it can be concluded that sulfur oxidation was less sensitive to AS bleed. The factors resulting this difference were not investigated in this study and thus remain to be investigated. Therefore, further studies to determine the lowest inhibitory concentration of AS bleed on sulfur oxidation and to investigate the possible reasons for the difference between iron oxidation and sulfur oxidation are recommended.

One possible mechanism for the difference between iron and sulfur oxidation is that some of the constituents of AS bleed would modify the surface of elemental sulfur and make it more bioavailable, similarly as surfactants and ETU (Duncan *et al.* 1964; Ghadiri *et al.* 2019; Ren *et al.* 2020). Other possible explanation can be that sulfur oxidizing enrichment culture was more resistant towards the possible toxic compounds, such as

organic compounds, of AS bleed. Fang & Zhou (2006) reported that sulfur oxidizing *A. thiooxidans* was more resistant towards inhibition by organic compounds than *A. ferrooxidans*. Therefore, it is possible that the enriched sulfur oxidizing culture of this study contained more microbial species, that were more resistant towards organic compounds of AS bleed. However, this should be further investigated using microbial community analysis.

6.2 Inhibitory effects of organic compounds on bioleaching process

From the studied organic solvents, only neodecanoid acid was bioavailable to the microorganisms and therefore, affected biological iron oxidation. Neodecanoid acid delayed the onset of iron oxidation at concentrations higher than 2.5% of its water solubility (6.3 mg/L). During the experiments with Nessol D100, Nessol D100 remained as separate phase on top of the culture medium, while Baysolvex D2EHPA and Cyanex 272, reacted with some of the constituent(-s) of inoculum and TES, respectively, and formed nonbioavailable precipitates.

Torma & Itzkovitch (1976) and Chen *et al.* (2015) reported, influence of various organic solvents, including Baysolvex D2EHPA and Cyanex 272, on bioleaching and iron oxidation. Torma & Itzkovitch (1976) showed that Baysolvex D2EHPA was inhibitory to bioleaching at saturation concentration (0.264 TOC g/L). The results of this study, however, were different from those reported by Torma & Itzkovitch (1976) as no inhibition was observed with Baysolvex D2EHPA. In this study D2EHPA probably reacted with Fe(III) supplied with the inoculum, and no sulfide ore was used. These differences between with the experimental conditions likely explains these different results.

Chen *et al.* (2015) reported no effect of Cyanex 272 on biological iron oxidation, which was similar to this study. In their study no precipitate formation was observed. The growth medium (9K medium) of Chen *et al.* (2015) differed from the growth medium used in this study. In this study, Cyanex 272 reacted with constituent(-s) of TES, which included, for example, cobalt ($CoCl_2 \cdot 6\ H_2O$), that is not present in 9K medium and is separated from nickel with Cyanex 272. Therefore, it is possible that no precipitation occurred during the experiment of Chen *et al.* (2015), and Cyanex 272 does not inhibit biological iron oxidation at saturation concentration.

Tuttle & Dugan (1976) reported the influence of various organic compounds, including carboxylic acids, on growth and iron and sulfur oxidation. On the basis of their results, it was suggested that relative electronegativity of the organic compounds was the most significant factor affecting the ability of the compound to inhibit iron and sulfur oxidation.

Since the experimental design of their study differed from this, the same conclusions about the effect of electronegativity cannot be directly drawn.

Based on the chemical structure, relative electronegativity of neodecanoid acid is lower than, for example, electronegativity of alpha ketoglutaric acid, which was also studied by Tuttle & Dugan (1976). However, inhibitory effect of neodecanoid acid was observed already at much lower concentrations (> 6.3 mg/L), compared to the lowest studied alpha ketoglutaric acid concentration (90 mg/L) of the study of Tuttle & Dugan (1976), which resulted 46% reduction in iron oxidation. Therefore, it is possible that some other factor than just the relative electronegativity of the organic compound can affect toxicity. Based on the pKa value of neodecanoid acid (5.17) and pH of the culture medium (~2), the neodecanoid acid did not dissolve well and was only partially present in the aqueous phase. Therefore, the actual neodecanoid acid concentration of the culture medium could have been even lower than the added amount would suggest (DeRuiter 2011; ECHA 2020).

In addition to the studied organic solvents, the inhibitory effects, and mechanisms on bioleaching microorganisms of the other organic compounds have been investigated in numerous studies. Fang & Zhou (2006) reported that from the studied low molecular weight monocarboxylic acids (formic, acetic, propionic, and butyric acid), formic acid was the most inhibitory to both biological iron and sulfur oxidation. The results of this study showed 50% inhibition of iron oxidation and 70% inhibition of sulfur oxidation at 15 mg/L (0.33 mmol/L) concentration, and both iron and sulfur oxidation was completely inhibited at concentration of 77 mg/L (1.67 mmol/L). Tuttle & Dugan (1976) reported that from the studied carboxylic acids, formic acid was the most inhibitory to biological iron oxidation. Frattini *et al.* (2000) reported that different bacterial strains (*A. ferrooxidans*), had a different response to inhibitory organic compounds. In their study, inhibitory concentrations of citric acid and glucose varied between 9.6–25 g/L (50–130 mmol/L) and 13–50 (70–280 mmol/L), respectively, depending on the strain.

It has been proposed that inhibitory mechanisms of organic acids, would be associated with the dissociation of the acids (Alexander *et al.* 1987; Frattini *et al.* 2000). At low pH (< 2.5), most of the organic acids are in their undissociated form, and thus more easily diffused into the microbial cell through its cytoplasmic membrane (Alexander *et al.* 1987). In close to neutral pH (~6.5) of the cytoplasm, organic acids are dissociated, leading to acidification of cytosol and dispersal of transmembrane pH gradient, which is mandatory for the growth of microorganisms living in acidic bioleaching environments. It has also been proposed that the inhibitory effect of organic compounds, could be due to elevated anion concentration in cytoplasm, formed from dissociation of the organic compounds,

which results in osmotic damage of the microbial cell. (Alexander *et al.* 1987; Frattini *et al.* 2000) Alexander *et al.* (1987) also proposed that inhibitory effect of organic compounds could be explained by using Henderson-Hasselback equation, i.e., organic compounds with lower pKa values would be more inhibitory to bioleaching microorganisms.

Tuttle & Dugan (1976) proposed that possible inhibitory mechanisms of the organic compounds on bioleaching microorganisms would also include direct impact on iron oxidizing enzyme system and reaction with Fe(II), present in bioleaching environment. When organic compounds react abiologically with Fe(II), Fe(II) is no longer a bioavailable substrate for the iron oxidizing bacteria.

The possible inhibitory effects of organic compounds are, however, reduced in mixed acidophilic bioleaching cultures. Mixotrophic and heterotrophic microorganisms, present in bioleaching environment, degrade organic matter, and thereby detoxify the growth environment for the iron and sulfur oxidizing microorganisms. (Johnson & Roberto 1997; Schippers 2007; Li e*t al.* 2011) Since it has been recognized that different bacterial strains respond differently to organic compounds, the inhibitory effect of organics can also be possibly reduced by enrichment of resistant bacterial strains into the bioleaching environment (Frattini *et al.* 2000) In heap bioleaching a diverse and dynamic bioleaching microbial community naturally exists in bioheaps, however, if bioheaps are inoculated, the initially introduced bacterial species and strains can possibly be selected more closely. (Frattini *et al.* 2000; Watling 2006; Halinen *et al.* 2012)

6.3 Potential of enhancing bioleaching by ammonium-rich process liquors

The results of this study showed that both AS bleed and feed enhanced biological iron oxidation at concentrations of 1% (v/v) and below (Figure 42). The iron oxidation rate of AD control, which was intentionally incubated without ammonium (ammonium deficit medium), was 2.0 g/L/d, whereas the iron oxidation rates with 0.11 g/L ammonium control and AS bleed and feed concentrations were above 2.3 g/L/d. When AS bleed and AS feed concentration were above 0.09% (v/v) and 0.1% (v/v), respectively, iron oxidation rate did not increase significantly. The iron oxidation rates were calculated from the slope of the linear regression line of the exponential part of the Fe(II) oxidation curves, with R^2 values above 0.93, except for AD control (R^2=0.86).

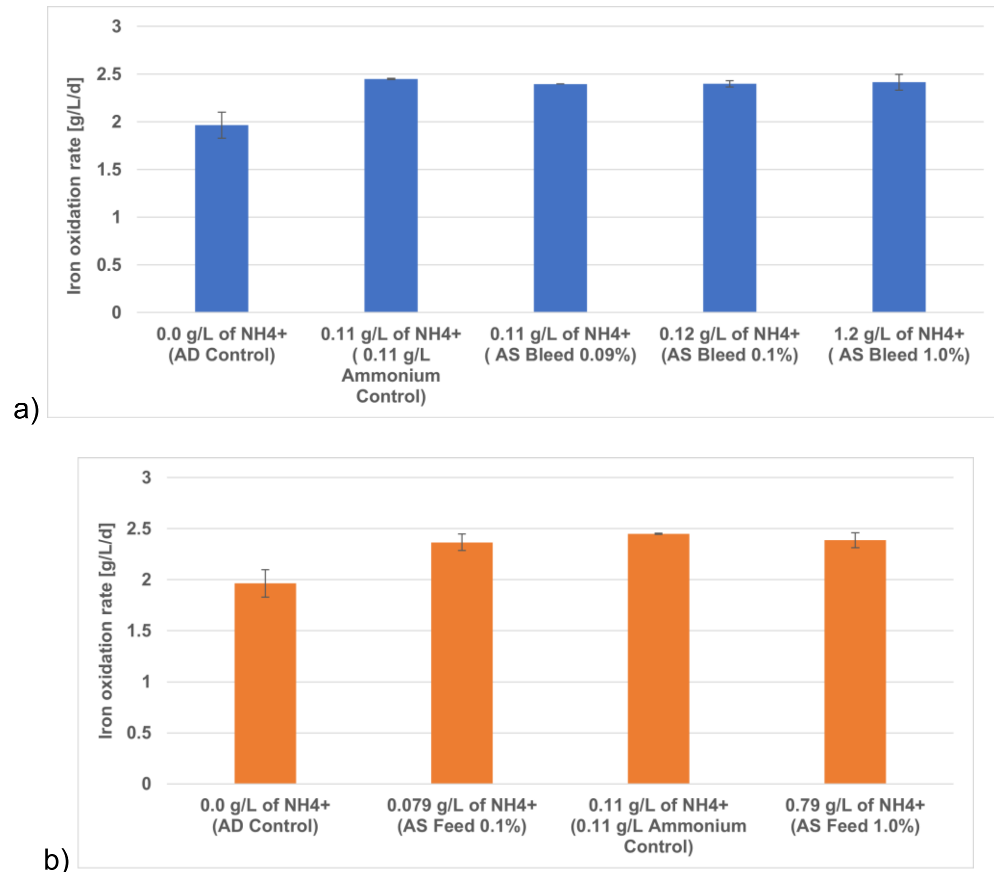


Figure 42. *Influence of (a) AS bleed concentration (0.09–1.0 % (v/v)) and (b) AS feed concentration (0.1–1% (v/v)) on iron oxidation rate during iron oxidation by the iron oxidizing ammonium deficit (AD) culture. Iron oxidizing AD culture was cultivated without ammonium supplementation. Iron oxidation rates were calculated from the slope of the linear regression line of the exponential part of the Fe(II) oxidation curves (see figure 39). The standard deviations are presented with the error bars (n=2).*

Earlier studies have showed that ammonium supplementation could enhance biological iron oxidation and bioleaching (Krafft & Hallberg 1993; Niemelä *et al.* 1994; d'Hugues *et al.* 1997; Ahoranta *et al.* 2017). Krafft & Hallberg (1993) reported that already 0.027 g/L ammonium addition enhanced zinc extraction from the zinc sulfide ores by approximately 5–60%, compared to the microbial culture without ammonium supplementation.

Niemelä *et al.* (1994), d'Hugues *et al.* (1997) and Ahoranta *et al.* (2017) studied influences of higher ammonium concentrations on bioleaching and iron oxidation. Niemelä *et al.* (1994) showed that 0.11 g/L ammonium supplementation enhanced biological iron oxidation by a mixed bacterial culture. This study reported similar results with 0.11 g/L ammonium concentration, when ammonium was supplemented with AS stock and with AS bleed. d'Hugues *et al.* (1997) and Ahoranta *et al.* (2017) reported that 1.03 g/L (0.80 g/L of N) and 0.41 g/L (0.32 g/L of N) ammonium concentration was sufficient to enhance

bioleaching and biological iron oxidation. Since these concentrations were the lowest studied concentrations, it remains unknown whether even lower concentrations would enhance iron oxidation or bioleaching. Ahoranta *et al.* (2017) also reported that the increase of ammonium concentration above 0.41 g/L did not significantly increase the biological iron oxidation, while in this study, ammonium concentration already above 0.079 g/l and 0.11 g/L did not significantly increase the biological iron oxidation.

In addition to ammonium, the influence of nitrogenous organic compounds on bioleaching and biological iron oxidation have been studied by Puhakka & Tuovinen (1987), d'Hugues *et al.* (1997), Li *et al.* (2011), and Huynh *et al.* (2020). Puhakka & Tuovinen (1987) reported that 0.22 g/L yeast extract concentration increased nickel, zinc, copper, and cobalt recovery approximately by 30%, 55%, 30%, and 50%, respectively. The results of Li *et al.* (2011) showed that yeast extract decreased bioleaching rate at temperature of 30 °C, whereas at temperatures of 40 and 50 °C, bioleaching was increased. Huyhn *et al.* (2020) reported that yeast extract increased cell number of *Sb. thermosulfidooxidans* approximately 5 times higher, compared to the culture without yeast extract. D'Hugues *et al.* (1997) studied the difference between the influence of ammonium and urea supplementation to bioleaching. The results of their study showed that inorganic ammonium was more efficient nitrogen source compared to organic urea. Therefore, it can be concluded that nitrogen containing organic compounds can possibly enhance bioleaching and biological iron oxidation.

Previous nitrogen supplementation studies showed that the form of nitrogen has a great influence on the growth and bioleaching activity of bioleaching microorganisms. It has been recognized that ammonium enhances biological iron oxidation and bioleaching, whereas nitrate has been reported to have a negative impact on bioleaching activity of microorganisms. (Krafft & Hallberg 1993; Niemelä *et al.* 1994; d'Hugues *et al.* 1997; Suzuki *et al.* 1999; Harahuc *et al.* 2000; Ahoranta *et al.* 2017) Niemelä *et al.* (1994) reported that nitrate concentration of 0.38 g/L inhibited biological iron oxidation, whereas in Suzuki *et al.* (1999) and Harahuc *et al.* (2000) studies, 0.62 g/L nitrate concentration inhibited biological sulfur oxidation and iron oxidation, respectively. The results of the study of d'Hugues *et al.* (1997) showed that nitrogen supplemented in form of organic compounds: urea and DAP, did not enhance bioleaching as efficiently as ammonium. Therefore, it can be concluded that ammonium is the most suitable source of nitrogen, and that ammonium-rich process liquors can be used as source of ammonium supplement in low concentrations (<1% (v/v)).

6.4 Environmental effects of residual nitrogen and organic solvents

When supplementation of full-scale bioleaching process with the ammonium-rich AS bleed and feed is planned, possible negative environmental effects of residual nitrogen and organic solvents (TOC) must be considered. It is possible that some of the nitrogenous and organic compounds may also be present in the wastewater, and thus, result in environmental pollution. In aquatic environments, nitrogenous compounds result in oxygen depletion and eutrophication, while organic compounds can result in floating organic phases, oxygen depletion, and toxic effects on aquatic life (Nuñez 2001; EPA 2002; Schindler 2006; Keeney & Hatfield 2008; Mullholland & Lowas 2008). Therefore, the release of any ammonium or organic compounds into the environment must be prevented.

Based on the results of this study, when using 0.1% (v/v) AS bleed as a nitrogen supplement, residual ammonium concentration was approximately 10 mg/L. Therefore, large quantities of ammonium ions (tens of thousands of kilograms of ammonium) could be released yearly into the environment, based on the wastewater volumes of Terrafame (approximately 8.0 million cubic meters in year 2020) (Eurofins Ahma Oy 2021). Nitrogen load of the mining industry is mainly originated from the mining explosives and cyanide used in the gold mineral processing. It has been estimated that 2 000 t N/a of nitrogen is released into environment through mining activity in Finland. (Jermakka *et al.* 2015)

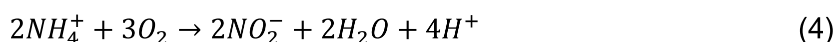
For comparison, in pulp and paper mills nitrogenous discharges are controlled. The overall nitrogen load of the pulp and paper industry in Finland is approximately 2 000 t/a (Ympäristö.fi 2019). In Äänekoski bioproduct plant (of Metsä Fibre and board Oy), ammonium limit value is set to 4 mg/L in the environmental permit, when ammonium load to the recipient waters is approximately 73 000 kg/a (Länsi- ja Sisä-Suomen aluehallintovirasto 2015). In pulp, chemi-thermomechanical pulp, and cardboard plant of Stora Enso Oulu Oy, ammonium limit in the wastewater effluent is 2 mg/L, with the ammonium load of approximately 45 000 kg/a (Pohjois-Suomen aluehallintovirasto 2020).

Most wastewater effluents of Terrafame are released in Nuasjärvi. From Nuasjärvi, water goes to Oulujärvi. (Eurofins Ahma Oy 2021) A large municipal wastewater treatment plant of Peuraniemi also releases its effluent into Oulujärvi. In environmental permit terms of Peuraniemi, there is no limit values for nitrogen or ammonium. However, in Peuraniemi, nitrogen and ammonium load and concentration in wastewater effluent is monitored yearly. In Spring of 2021, ammonium concentration of released wastewater was approximately 26 mg/L and ammonium load was approximately 79 000 kg/a. (AFRY Finland 2021) It is, however, possible that in future nitrogen and ammonium limit values

will be set to Peuraniemi and other separate wastewater treatment plants of the area due to tightened environmental regulations. Generally, nitrification requirement, and in some cases also denitrification requirement, has been set to the inland municipal wastewater treatment plants in Finland (Laitinen *et al.* 2014). For example, in other inland cities of Finland (Karkkila and Jyväskylä) ammonium limit value of the municipal wastewater treatment plants is set to 4 mg/L in the environmental permits (City of Karkkila 2011; JS-Puhdistamo 2013).

In aquatic environments, excess anthropogenic ammonium can result in eutrophication, by supporting growth of excess phytoplankton and vascular plant (Schindler 2006). The excess phytoplankton and plant growth can result in various drastic harmful effects in the waterbodies. Eutrophication can reduce water clarity, decrease plant and animal diversity, and harm water quality. Eutrophication can also produce unpleasant smell, biological fouling and prevent recreational and industrial uses of the surface water. (Schindler 2006; Keeney & Hatfield 2008) When the excess phytoplankton and vascular plant mass is decomposed, dissolved oxygen of the waterbody is consumed at higher rate than it is replenished, leading to hypoxic or anoxic zones, called dead zones, especially in bottom water layers. In hypoxic or anoxic zones, the oxygen level is insufficient for the most aquatic organisms, leading to unhabitable water layers. (Keeney & Hatfield 2008)

The main oxygen consuming mechanism associated with ammonium is nitrification in the recipient water. In nitrification, ammonium is oxidized first into nitrite and then into nitrate by nitrifying bacteria, according to the following equations:



$$2NH_4^+ + 3O_2 \rightarrow 2NO_2^- + 2H_2O + 4H^+ \quad (4)$$

$$2NO_2^- + O_2 \rightarrow 2NO_3^- \quad (5)$$

Ammonium is first oxidized to nitrite (Eq. 4), and four protons are released. Nitrite is further oxidized into nitrate (Eq 5). (Sayavedra-Soto & Arp 2011; Starkenburg *et al.* 2011) Nitrification consumes oxygen, and therefore, it reduces dissolved oxygen in water. In nitrification process, the water quality of the aquatic environment can also be reduced due to decreased alkalinity, pH, and promoted bacterial regrowth. (EPA 2002; Sayavedra-Soto & Arp 2011) Decreased pH and alkalinity of the water can lead to negative ecological effects, such as fish die-offs, when metals are in more bioavailable form in water, and dissolution of the shells and exoskeletons of the aquatic organisms. (EPA 2002; Mullholland & Lowas 2008)

To prevent the negative environmental effects of nitrogen, municipal wastewater treatment plants in Finland generally have a nitrification requirement (Laitinen *et al.* 2014). In

nitrification, ammonium-nitrogen is oxidized into nitrate, and thereby the excess oxygen consumption in the recipient waters is controlled (EPA 2002; Laitinen *et al.* 2014). Preventing the nutrient load of nitrogen, denitrification process, is used in many municipal wastewater treatment plants. In denitrification process, nitrate-nitrogen is reduced to N_2 gas. (Laitinen *et al.* 2014) Suitability of these methods for reducing the nitrogen load of mining wastewaters has been studied in Finland (Papirio *et al.* 2014; Jermakka *et al.* 2015; Zou *et al.* 2015).

In addition of ammonium load, also the TOC concentration of the wastewater must be considered. The main negative environmental effect of organic compounds is oxygen consumption. In biodegradation of organic compounds, oxygen is consumed and result in reduced level of dissolved oxygen in recipient waters, such as in eutrophication and nitrification. (Nuñez 2001; Roy 2001; Schawarzenbach *et al.* 2016)

Organic solvents are harmful to environment, however, this can vary considerably by the organic compound (Nuñez 2001). In aquatic environments, organic compounds can reduce water quality, prevent recreational and industrial uses of the water, and produce unpleasant smell (Nuñez 2001; Roy 2001; Schawarzenbach *et al.* 2016). Organic compounds can also form floating phases on top of water. Aquatic organisms are then exposed to the organic compounds when moving near the water surface. In addition to organic phase, organic compounds can also be partly dissolved in water or volatized into atmosphere. (Roy 2001; Schawarzenbach *et al.* 2016)

Some organic solvents can be toxic or carcinogenic to various species, and thereby affect biotic community of the recipient waters by various ways and reduce aquatic life diversity (Nuñez 2001; Roy 2001). All the studied organic solvents used in Terrafame, except Nessol D100, are classified as hazardous for environment, if they are not treated or disposed appropriately (ExxonMobil 2018; LANXESS 2018; Neste 2019; Solvay 2019). From the organic solvents, Cyanex 272 and Baysolvex D2EHPA are also classified to be toxic to aquatic life (LANXESS 2018; Solvay 2019). Government of Australia has set the no observed effect concentration (NOEC) of 2.9 mg/L Cyanex 272 on *Daphnia magna* (water flea), which is lower than the TOC concentration in the recycled process liquors (10 mg/L) (Worksafe Australia 1996).

Almost all organic solvents are also volatile organic compounds (VOCs), which can form ground level ozone and other oxidants, when participating in photochemical reactions at lower atmosphere. Ground level ozone and other oxidants can have a negative effect on lungs of various species. (Nuñez 2001)

7. CONCLUSIONS

The following conclusions can be drawn based on the experiments of the laboratory-scale study:

- Both AS bleed and feed negatively affect biological iron oxidation. Both delay the onset of biological iron oxidation at concentrations higher than 2% (v/v). With AS bleed and feed concentrations of 20% (v/v) and 50% (v/v), respectively, the inhibition of iron oxidation is irreversible. At concentration 2% (v/v) and below, AS bleed and feed do not affect biological iron oxidation.
- Under ammonium limited growth conditions, 0.079 g/L ammonium supplementation with 0.1% (v/v) AS feed, and 0.11 g/L ammonium supplementation with 0.09% (v/v) AS bleed enhance biological iron oxidation, and therefore also potentially enhance efficiencies of heap bioleaching. When increasing the AS bleed and feed concentration to 1% (v/v), iron oxidation rate is not further significantly increased.
- AS delays the onset of biological iron oxidation at concentrations higher than 20 g/L. AS inhibits iron oxidation irreversibly at concentration of 250 g/L and above.
- AS bleed enhances biological sulfur oxidation at concentration of 8% (v/v) and below.
- Neodecanoid acid delays the onset of iron oxidation at 2.5% of its water solubility (6.3 mg/L) or higher.
- Nessol D100, Baysolvex D2EHPA and Cyanex 272 are nonbioavailable to the bioleaching microorganisms, and, therefore, do not affect biological iron oxidation.
- The results of this study are valid only for laboratory-scale (batch assays) biological iron and sulfur oxidation. Prior to application of the additives in real-scale heap bioleaching, bioleaching experiments with Terrafame's ore must be performed. For example, these experiments could be implemented first as batch assays and in columns (semi-continuous and/or continuously fed) and then followed by pilot-scale heap bioleaching.

Recommendations for the possible upcoming experiments:

- To determine the full potential of nitrogen supplementation with AS bleed and feed, nitrogen limitation of the bioleaching microorganisms in bioheaps of Terrafame should be verified.
- For enhancement of heap bioleaching process, the uses of possible bioleaching additives, other than nitrogen, could be performed.
- Further studies to determine the lowest inhibitory concentration of AS bleed on biological sulfur oxidation and of AS feed on biological iron oxidation, are recommended.
- To study the possible biodegradation of neodecanoid acid, experiments with heterotrophic bioleaching microorganisms should be implemented. Heterotrophic/mixotrophic bioleaching microorganisms should be enriched by using neodecanoid acid as a substrate.

Conclusions and recommendations for the Terrafame battery chemical production plant process:

- Since most of ammonium present in AS bleed and feed, is not utilized by the bioleaching microorganisms, treatment of the residual ammonium, in the wastewater of the metal recovery area, must be considered when supplementation of the full-scale heap bioleaching process is considered with AS bleed and feed, for preventing excess ammonium load and negative environmental effects result of it. When using 0.1% (v/v) AS bleed as a nitrogen supplement, tens of thousands of kilograms of ammonium could be released yearly into the environment, based on the wastewater volumes of Terrafame. This ammonium load could potentially result in harmful environmental effects in recipient waters which should be further studied.
- When the studied organic solvents are present in the aqueous process liquors as separate phase, their physico-chemical effects on the battery chemical production process and process liquor cycle, between the battery chemical production plant and bioheaps, must be considered. The separate organic solvent phase must be removed from the process liquors and treated in order to prevent economic losses and environmental pollution. Since all the studied organic solvents

used in Terrafame, except Nessol D100, are classified as hazardous for environment, and Cyanex 272 and Baysolvex D2EHPA classified as toxic to aquatic life, possible harmful environmental impacts of them in the recipient waters must be considered.

REFERENCES

Abbaszadegan, A., Ghahramani, Y., Gholami, A., Hemmateenejad, B., Dorostkar, S., Nabavizadeh, M. & Sharghi, H. (2015). The effect of charge at the surface of silver nanoparticles on antimicrobial activity against gram-positive and gram-negative bacteria: A preliminary study, Journal of Nanomaterials, Vol. 2015, Article number: 720654.

AFRY Finland Oy. (2021). Peuraniemi – Jätevesitarkkailun yhteenveto, Available at (1.6.2021): https://www.kajaaninvesi.fi/meidan-vetemme/jatevesi/.

Aguirre, P., Guerrero, K., Sanchez-Rodriguez, A., Gentina, J.C. & Schippers, A. (2018). Making sticky cells: effect of galactose and ferrous iron on the attachment of *Leptospirillum ferrooxidans* to mineral surfaces, Research in microbiology, Vol. 169(10), pp. 569-575.

Ahoranta, S.H., Peltola, M.K., Lakaniemi, A.-. & Puhakka, J.A. (2017). Enhancing the activity of iron oxidising bacteria: A case study with process liquors from heap bioleaching of a complex sulphide ore, Hydrometallurgy, Vol. 167 pp. 163-172.

Ajanovic, A. (2015). The future of electric vehicles: Prospects and impediments, Wiley Interdisciplinary Reviews: Energy and Environment, Vol. 4(6), pp. 521-536.

Alexander, B., Leach, S. & Indigledew, W. J. (1987). The relationship between chemiosmotic parameters and sensitivity to anions and organic acids in the acidophile Thiobacillus ferrooxidans, Microbiology, Vol. 133, pp. 1171-1179.

American Public Health Association, APHA. (1992). 3500-Fe ortho-phenantroline method, Standard methods for the examination of water and wastewater, American Public Health Association and Water Environment Federation, Washington, DC, US, 1100 p.

BASF. (2003). Lutensol® XL types, In Technical Information.

BASF. (2008). Plurafac® LF types, In Technical Information.

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

Bevilaqua, D., Lahti, H., Suegama, P.H., Garcia Jr., O., Benedetti, A.V., Puhakka, J.A. & Tuovinen, O.H. (2013). Effect of Na-chloride on the bioleaching of a chalcopyrite concentrate in shake flasks and stirred tank bioreactors, Hydrometallurgy, Vol. 138, pp. 1-13.

Bleeze, B., Zhao, J. & Harmer, S.L. (2018). Selective attachment of *Leptospirillum ferrooxidans* for separation of chalcopyrite and pyrite through bio-flotation, Minerals, Vol. 8(3), Article number 86.

Bombicz, P., Mutikainen, I., Krunks, M., Leskela, T., Madarasz, J. & Niinisto, L. (2004). Synthesis, vibrational spectra and X-ray structures of copper(I) thiourea complexes, Inorg Chim. Acta. Vol. 357(2), pp. 513–525.

Chen, M., Zhang, L., Gu, G., Hu, Y. & Su, L. (2008). Effects of microorganisms on surface properties of chalcopyrite and bioleaching, Transactions of Nonferrous Metals Society of China (English Edition), Vol. 18(6), pp. 1421-1426.

Chen, B., Li, W., Zhou, G., Liu, X. & Wen, J. (2015). Effect of Different Solvent Extractants on the Activity and Community Structure of Acidophilic Microorganisms, Advanced Materials Research, Vol. 1130, pp. 222-225.

City of Karkkila. (2011) Karkkilan kaupungin jätevedenpuhdistamon jaksoraportti 3/2011, Länsi-Uudenmaan vesi ja ympäristö ry.

Demir, E.K., Yaman, B.N., Çelik, P.A. & Sahinkaya, E. (2020). Iron oxidation in a ceramic membrane bioreactor using acidophilic bacteria isolated from an acid mine drainage, Journal of Water Process Engineering, Vol. 38, Article number 101610.

D'Hugues, P., Cezac, P., Cabral, T., Battaglia, F., Truong-Meyer, X.M. & Morin, D. (1997). Bioleaching of a cobaltiferous pyrite: A continuous laboratory-scale study at high solids concentration, Minerals Engineering, Vol. 10(5), pp. 507-527.

deRuiter, J. (2011). Carboxylic Acid Structure and Chemistry: Part 2, in: Evers, A., Maze, M. & Kharasch, E. (Eds.), Principles of Drug Action, Cambridge University Press, Cambridge, UK.

Du Plessis, C. A., Batty, J. D. & Dew, D. W. (2007). Commercial Applications of Thermophile Bioleaching, in: Rawlings D. E. & Johnson, D. B. (Eds.), Biomining, Springer-Verlag Berlin Heidelberg, Berlin, Germany, p. 75.

Duncan, D.W., Trussel, P.C. & Walden, C.C. (1964). Leaching of Chalcopyrite with *Thiobacillus ferrooxidans*: Effect of Surfactants and Shaking, Applied Microbiology, Vol. 12, pp. 122-126.

Engst, R. & Schnaak, W. (1974). Residue of dithiocarbamate fungicides and metabolites on plant foods, Residue Rev., Vol. 52, pp 45-67.

Eurofins Ahma Oy. (2021). Terrafame Oy – Vesipäästöjen tarkkailu 2020, Available at (31.5.2021): https://www.terrafame.fi/ymparisto/ymparisto-vesien-hallinta/ymparistotarkkailuraportit.html.

European Chemicals Agency. ECHA. (2020). Neodecanoid acid: Dissociation constant. Available at (18.5.2021): https://echa.europa.eu/fi/registration-dossier/-/registered-dossier/15692/4/22.

ExxonMobil. (2018). Safety Data Sheet: Neodecanoid acid, prime.

Fang, D. & Zhou, L.X. (2006). Effect of sludge dissolved organic matter on oxidation of ferrous iron and sulfur by *Acidithiobacillus ferrooxidans* and *Acidithiobacillus thiooxidans*, Water, air, and soil pollution, Vol. 171(1-4), pp. 81-94.

Flemming, H.C., Wingender, J., Szewzyk, U., Steinberg, P., Rice, S.A., & Kjelleberg, S. (2016). Biofilms: an emergent form of bacterial life, Nat. Rev. Microbiol., Vol. 14, pp. 563–575.

Flick, E. W. (1993). Industrial Surfactants, Second Edition, Noyes Publications, Park Ridge, New Jersey, US.

Frattini, C.J., Leduc, L.G. & Ferroni, G.D. (2000). Strain variability and the effects of organic compounds on the growth of the chemolithotrophic bacterium *Thiobacillus ferrooxidans*, Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology, Vol. 77(1), pp. 57-64.

Germer, T., Zwinkels, J. & Tsai, B. (2014). Spectrophotometry: Accurate Measurement of Optical Properties of Materials, Vol. 46, Experimental Methods in Physical Sciences, Academic Press, Cambridge, Massachusetts, US, Chapter 2.1.

Ghadiri, M., Harrison, S.T.L. & Fagan-Endres, M.A. (2019). Effect of surfactant on the growth and activity of microorganisms in a heap bioleaching system, Minerals Engineering, Vol. 138, pp. 43-51.

Gotfryd, L., Pietek, G., Szołomicki, Z., Becker, K. & Piwowonska, J. (2015). Neodecanoic acid as extractant of selected non-ferrous metals, Physicochemical Problems of Mineral Processing, Vol. 51(2), pp. 435-445.

Halinen, A.K., Beecroft, N. J., Määttä, K., Nurmi, P., Laukkanen, K., Kaksonen, A. H., Riekkola-Vanhanen, M., Puhakka, J. A. (2012). Microbial community dynamics during a demonstration-scale bioheap leaching operation, Hydrometallurgy Vol. 125-126, pp. 34-41.

Harahuc, L., Lizama, H.M. & Suzuki, I. (2000). Selective inhibition of the oxidation of ferrous iron or sulfur in *Thiobacillus ferrooxidans*, Applied and Environmental Microbiology, Vol. 66(3), pp. 1031-1037.

Harrison, S.T.L. (1991). Bacterial cell disruption: A key unit operation in the recovery of intracellular products, Biotechnology Advances, Vol. 9(2), pp. 217-240.

Harrison, S.T., Dennis, J.S. & Chase, H.A. (1991). Combined chemical and mechanical processes for the disruption of bacteria. Bioseparation, Vol. 2(2), pp. 95-105.

Hsung, J.C. & Haug, A. (1977). ζ-Potential and surface charge of *Thermoplasma acidophila*, BBA - Bioenergetics, Vol. 461(1), pp. 124-130.

Huynh, D., Kaschabek, S.R. & Schlömann, M. (2020). Effect of inoculum history, growth substrates and yeast extract addition on inhibition of *Sulfobacillus thermosulfidooxidans* by NaCl, Research in microbiology, Vol. 171(7), pp. 252-259.

Inácio, A.S., Mesquita, K.A., Baptista, M., Ramalho-Santos, J., Vaz, W.L.C. & Vieira, O.V. (2011). In vitro surfactant structure-toxicity relationships: Implications for surfactant use in sexually transmitted infection prophylaxis and contraception, PLoS ONE, Vol. 6(5). Article number e19850.

Jermakka, J., Merta, E., Mroueh, U.M., Arkkola, H., Eskonniemi, S., Wendling, L., Laine-Ylijoki, J., Sohlberg, E., Heinonen, H., Kaartinen, T., Puhakka, J., Peltola, M., Papirio, S., Lakaniemi, A.M., Zou, G., Ylinen, A., di Capua, F., Neitola, R., Gustafsson, H., Korhonen, T., Karlsson, T., Kauppila, T., Laakso, J. & Mörsky, P. (2015). Solutions for control of nitrogen discharges at mines and quarries – Miniman project final report, Teknologian tutkimuskeskus VTT Oy, Espoo.

Johnson, D.B. & Roberto, F.F. (1997). Heterotrophic acidophiles and their roles in the bioleaching of sulfide minerals. In: Rawlings, D.E., Johnson, B.D. (Eds.), Biomining, Springer, Berlin, Heidelberg, pp. 259–279.

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

Johnson, M. (2013). Detergents: Triton X-100, Tween-20, and More, Materials and Methods, Vol. 3, Article number 2163.

JS-Puhdistamo, (2013). Jyväskylän Seudun Puhdistamo Oy vuosikertomus 2013. Available at: https://www.jyvaskyla.fi/sites/default/files/atoms/files/tilinpaatos2013_puhdistamo.pdf.

Keeney, D.R. & Hatfield, J. L. (2008). The Nitrogen Cycle, Historical Perspectives, and Current and Potential Future Concerns, in: Hatfield, J.L. & Follet, R. F. (Eds.) Nitrogen in the Environment: Sources, Problems, and Management, Academic Press, Cambridge, Massachusetts, US, pp. 7-12.

Khan, S. & Ali, J. (2018). Chemical analysis of air and water, in: Bioassays: Advanced Methods and Applications, Häder, D.P. & Erzinger, G., Elsevier, Amsterdam, the Netherlands, Chapter 2.3.2.

Kingma, Jr., J.G. & Silver, M. (1979). Autotrophic growth of *Thiobacillus acidophilus* in the presence of a surface-active agent, Tween 80, Applied and Environmental Microbiology, Vol. 38(5), pp. 795-799.

Kinnunen, P.H.-. & Puhakka, J.A. (2004). Chloride-promoted leaching of chalcopyrite concentrate by biologically-produced ferric sulfate, Journal of Chemical Technology and Biotechnology, Vol. 79(8), pp. 830-834.

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

Krafft, C. & Hallberg, R.O. (1993). Bacterial leaching of two Swedish zinc sulfide ores, FEMS microbiology reviews, Vol. 11(1-3), pp. 121-127.

Laitinen, J., Nieminen, J., Saarinen, R. & Toivikko, A. (2014). Paras käyttökelpoinen tekniikka (BAT) – Yhdyskuntien jätevedenpuhdistamot, Suomen ympäristö 3/2014, Ympäristöministeriö, Helsinki, pp. 43-44 & 56-57.

Lan, Z., Hu, Y. & Qin, W. (2009). Effect of surfactant OPD on the bioleaching of marmatite, Minerals Engineering, Vol. 22(1), pp. 10-13.

LANXESS. (2018). Käyttöturvallisuustiedote: Baysolvex D2EHPA.

Li, Q., Tian, Y., Fu, X., Yin, H., Zhou, Z., Liang, Y., Qiu, G., Liu, J., Liu, H., Liang, Y., Shen, L., Cong, J. & Liu, X. (2011). The community dynamics of major bioleaching microorganisms during chalcopyrite leaching under the effect of organics, Current microbiology, Vol. 63(2), pp. 164-172.

Li, S., Zhong, H., Hu, Y., Zhao, J., He, Z. & Gu, G. (2014). Bioleaching of a low-grade nickel-copper sulfide by mixture of four thermophiles, Bioresource technology, Vol. 153 pp. 300-306.

Länsi- ja Sisä-Suomen aluehallintovirasto. (2015). Äänekosken tehdasintegraatin ympäristölupapäätös, Dnro: LSSAVI/4652/2014, Available at (31.5.2021): https://ylupa.avi.fi/fi-FI.

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

Mullholland, M. & Lowas, M. (2008). Nitrogen Uptake and Assimilation, in: Carpenter, E., Capone, D., Bronk, D. & Mullholland, M., Nitrogen in the Marine Environment, 2nd Edition, Academic Press, New York, US.

Nakazawa, H., Fujisawa, H. & Sato, H. (1998). Effect of activated carbon on the bioleaching of chalcopyrite concentrate, International Journal of Mineral Processing, Vol. 55(2), pp. 87-94.

Neste. (2019). Käyttöturvallisuustiedote: Nessol D100. Available at: https://www.neste.fi/static/ktt/10528_fin.pdf.

Niemelä, S.I., Riekkola-Vanhanen, M., Sivelä, C., Viguera, F. & Tuovinen, O.H. (1994). Nutrient effect on the biological leaching of a black-schist ore, Applied and Environmental Microbiology, Vol. 60(4), pp. 1287-1291.

Norris, P. R., Murrel, J. C. & Hinson, D. (1995). The potential for diazotrophy in iron and sulfur oxidizing acidophilic bacteria, Arch. Microbiol., Vol. 164, pp. 294-300.

Norris, P. R. (2007). Acidophile Diversity in Mineral Sulfide Oxidation, in: Donati, E.R. & Sand, W. (Eds.), Microbial Processing of Sulfide minerals, Springer Dordrecht, The Netherlands, pp. 199–216.

Nuñez, C. (2001). Regulations, in: Wypych, G. (Ed.), Handbook of Organic Solvents, ChemTec Publishing, William Andrew Publishing, Toronto, New York, US.

Oinonen, C. (2020). Terrafamen ja akkukemikaalitehtaan prosessikuvaus, Personal email 16.12.2020. Receiver: Linda Määttä.

Pan, W., Jin, H., Liu, Z., Tang, J. & Cheng, S. (2020). Experimental and theoretical study on strengthening leaching of sulfide ores by surfactants, Process Safety and Environmental Protection, Vol. 137, pp. 289-299.

Papirio, S., Zou, G., Ylinen, A., Di Capua, F., Pirozzi, F. & Puhakka, J.A. (2014). Effect of arsenic on nitrification of simulated mining water, Bioresource Technology, Vol. 164, pp. 149–154.

Petersen, J. & Dixon, D. (2007). Modeling and Optimization of Heap Bioleach Processes, in: Rawlings D. E. & Johnson, D. B. (Eds.), Biomining, Springer-Verlag Berlin Heidelberg, Berlin, Germany, pp. 153-159.

Petersen, J. (2016). Heap leaching as a key technology for recovery of values from low-grade ores – A brief overview, Hydrometallurgy, Vol. 165, pp. 206-212.

Pohjois-Suomen aluehallintovirasto. (2020). Sellu-, CTMP-ja kartonkitehtaan ympäristölupa, Oulu, Dnro: PSAVI/2638/2019, Available at: https://ylupa.avi.fi/fi-FI.

Pubchem. (2021A). Sodium O-isobutyl dithiocarbonate. Available at (4.1.2021): https://pubchem.ncbi.nlm.nih.gov/compound/Sodium-O-isobutyl-dithiocarbonate.

Pubchem. (2021B). Methyldodecylbenzyl trimethyl ammonium chloride. Available at (4.1.2021): https://pubchem.ncbi.nlm.nih.gov/substance/375968805.

Pubchem. (2021C). Neodecanoic acid. Available at (7.6.2021): https://pubchem.ncbi.nlm.nih.gov/compound/Neodecanoic-acid.

Puhakka, J. & Tuovinen, O.H. (1987). Effect of organic compounds on the microbiological leaching of a complex sulphide ore material, World J Microbiol. Biotechnol., Vol. 3, pp. 429–436.

Rawlings, D. E. (2007). Relevance of Cell Physiology and Genetic Adaptability of Biomining Microorganisms to Industrial Processes, in: Rawlings D. E. & Johnson, D. B. (Eds.), Biomining, Springer-Verlag Berlin Heidelberg, Berlin, Germany, pp.186-187.

Ren, Z., Krishnamoorthy, P., Sanchez, P.Z., Asselin, E., Dixon, D.G. & Mora, N. (2020). Catalytic effect of ethylene thiourea on the leaching of chalcopyrite, Hydrometallurgy, Vol. 196, Article number 105410.

Riekkola-Vanhanen, M. (2010). Talvivaara Sotkamo Mine – Bioleaching of a Polymetallic Nickel Ore in Subarctic Climate, Nova Biotechnologica, Vol. 10, pp. 7-14.

Ríos, F., Fernández-Arteaga, A., Lechuga, M. & Fernández-Serrano, M. (2017). Ecotoxicological characterization of polyoxyethylene glycerol ester non-ionic surfactants and their mixtures with anionic and non-ionic surfactants, Environmental Science and Pollution Research, Vol. 24(11), pp. 10121-10130.

Rojas-Chapana, J.A. & Tributsch, H. (2000). Bioleaching of pyrite accelerated by cysteine, Process Biochemistry, Vol. 35(8), pp. 815-824.

Rowe, O.F. & Johnson, D.B. (2008). Comparison of ferric iron generation by different species of acidophilic bacteria immobilized in packed-bed reactors, Systematic and applied microbiology, Vol. 31(1), pp. 68-77.

Roy, W. (2001). The Environmental Impact Fate and Movement of Organic Solvents in Water, Soil, and Air, in: Wypych, G. (Ed.), Handbook of Organic Solvents, ChemTec Publishing, William Andrew Publishing, Toronto, New York, US.

Saavedra, A., Aguirre, P. & Gentina, J.C. (2020). Biooxidation of Iron by *Acidithiobacillus ferrooxidans* in the Presence of D-Galactose: Understanding Its Influence on the Production of EPS and Cell Tolerance to High Concentrations of Iron, Frontiers in Microbiology, Vol. 11, Article number 759.

Sadowski, Z., Polowczyk, I., Farbiszewska, T., & Farbiszewkska-Kiczma, T. (2001). Adhesion of Jarosite Particles to the Mineral Surface. Prace Naukowe Instytutu Gornictwa Politechniki Wroclawskiej, Vol. 95, pp. 95-103.

Sand, W. & Gehrke, T. (2006). Extracellular polymeric substances mediate bioleaching/

biocorrosion via interfacial processes involving iron (III) ions and acidophilic bacteria, Research in Microbiology, Vol. 157, pp. 49-56.

Sandoval, S.P., Pool, D.L. & Schultze, L.E. (1990). Effect of nonionic surfactants on chalcopyrite leaching under dump chemical conditions. Report of Investigations 9311, Reno Research Center, U.S. Bureau of Mines, Reno, NY., pp. 1-12.
Sarcheshmehpour, Z., Lakzian, A., Fotovat, A., Berenji, A.R., Haghnia, G.H. & Bagheri, S.A.S. (2009). Possibility of using chemical fertilizers instead of 9K medium in bioleaching process of low-grade sulfide copper ores, Hydrometallurgy, Vol. 96, pp. 264–267.

Sayavedra-Soto, L. & Arp, D. (2011). Ammonia-oxidizing bacteria: their biochemistry and molecular biology, in: Ward, B., Arp, D. & Klotz, M. (Eds.), Nitrification, ASM Press, Washington D.C., US, p. 14.

Schawarzenbach, R., Gschwend, P. & Imboden, D. (2016). Environmental Organic Chemistry, 3rd Edition, John Wiley & Sons, Inc., Hoboken, New Jersey, US, pp. 2-7, 53-56.

Schindler, D. W. (2006) Recent advances in the understanding and management of eutrophication, Limnology and Oceanography, Vol. 51, pp. 356-363.

Schippers, A. (2007). Microorganisms involved in bioleaching and nucleic acid-base-molecular methods for their identification and quantification, in: Donati, E.R. & Sand, W. (Eds.), Microbial Processing of Sulfide minerals, Springer Dordrecht, The Netherlands, pp. 3–33.

Schramm, L.L., Stasiuk, E.N. & Marangoni, D.G. (2003). Surfactants and their applications, Annual Reports on the Progress of Chemistry - Section C, Vol. 99, pp. 3-48.

SFS-EN 1484. (1997). Water Analysis. Guidelines for the Determination of Total Organic Carbon (TOC) and Dissolved Organic Carbon (DOC), Finnish Standards Association, Helsinki, Finland.

Shagufta, C. J. (2007). Environmental biotechnology, APH Publishing Corporation, New Delhi, India, p. 280.

Shimadzu Corporation. (2007). Total Organic Carbon Analyzer: TOC-VCPH/CPN, User Manual (For TOC-Control V Ver.2).

Sigma-Aldrich. (2003) Hexadecyltrimethylammonium bromide – Product Information. Available at: https://www.sigmaaldrich.com/content/dam/sigma-aldrich/docs/Sigma/Product_Information_Sheet/2/h6269pis.pdf.

Sigma-Aldrich. (2021A). Sodium decylbenzenesulfonate. Available at (4.1.2021): https://www.sigmaaldrich.com/catalog/substance/sodiumdodecylbenzenesulfonate348482515530011?lang=fi®ion=FI.

Sigma-Aldrich. (2021B). Igepal CO-630. Available at (13.1.2021): https://www.sigmaaldrich.com/catalog/product/aldrich/542334?lang=fi®ion=FI.

Sigma-Aldrich. (2021C). O-phenylenediamine. Available at (15.1.2021): https://www.sigmaaldrich.com/catalog/substance/ophenylenediamine108149554511?lang=fi®ion=FI.

Sigma-Aldrich. (2021D). Polyethylene glycol 2000. Available at (15.1.2021): https://www.sigmaaldrich.com/catalog/product/mm/821037?lang=fi®ion=FI.

Slobodkin, A., Reysenbach, A.-., Mayer, F. & Wiegel, J. (1997). Isolation and characterization of the homoacetogenic thermophilic bacterium *Moorella glycerini* sp. nov. International Journal of Systematic Bacteriology, Vol. 47(4), pp. 969-974.

Solvay. (2019). Käyttöturvallisuustiedote: Cyanex® 272 extractant.

Starkenburg, S., Spieck, E. & Bottomley, P. (2011). Metabolism and genomics of nitrite-oxidizing bacteria: emphasis on studies of pure cultures and of *Nitrobacter* species, in: Ward, B., Arp, D. & Klotz, M. (Eds.), Nitrification, ASM Press, Washington D.C., US, pp. 278-279.

Suzuki, I., Lee, D., Mackay, B., Harahuc, L. & Oh, J.K. (1999). Effect of various ions, pH, and osmotic pressure on oxidation of elemental sulfur by *Thiobacillus thiooxidans*, Applied and Environmental Microbiology, Vol. 65(11), pp. 5163-5168.

Terrafame. (2018). Akkukemikaalitehdas etenee toteutussuunnitteluun, Suurhankeinfo: 23.5.2018. Available at (25.3.2021): https://docplayer.fi/105732090-Akkukemikaalitehdas-etenee.html.

Terrafame. (2020). Tuotantomenetelmämme. Available at (3.5.2021): https://www.terrafame.fi/tuotantomenetelmamme.

Thermo Fischer Scientific. (2021). Alfa Aesar™ Sodium n-octyl sulfate, 99%. Available at (4.1.2021): https://www.fishersci.no/shop/products/sodium-n-octyl-sulfate-99-3/p-7081707.

Torma, A & Itzkovitch, I. (1976). Influence of Organic Solvents on Chalcopyrite Oxidation Ability of *Thiobacillus ferrooxidans*, Applied and Environmental Microbiology, July 1976, pp. 102-107.

Tourney, J. & Ngwenya, B.T. (2014). The role of bacterial extracellular polymeric substances in geomicrobiology, Chemical Geology, Vol. 386, pp. 115-132.

Tributsch, H. (2001). Direct versus indirect bioleaching, Hydrometallurgy, Vol. 59(2-3), pp. 177-185.

Tuovinen, O. H., Puhakka, J., Hiltunen, P. & Dolan, K. M. (1985). Silver toxicity to ferrous iron and pyrite oxidation and its alleviation by yeast extract in cultures of *Thiobacillus ferrooxidans*, Biotechnology Letters, Vol. 7, pp. 389-394.

Tuttle, J. & Dugan, P. (1976). Inhibition of growth, iron, and sulfur oxidation in *Thiobacillus ferrooxidans* by simple organic compounds, Canadian Journal of Microbiology, Vol. 22(5), pp. 719-730.

United States Environmental Protection Agency, EPA. (2002). Nitrification, Distribution System Issue Paper, Office of Groundwater and Drinking Water, Standards and Risk Management Division, Washington DC, US.

Van Hille, R., Bromfield, S., Botha, S., Jones, G., van Zyl, A. & Harrison, S. (2009). The effect of nutrient supplementation on growth and leaching performance of bioleaching bacteria, Advanced Materials Research, Vols. 71-73, pp. 413-416.

Vardanyan, A., Vardanyan, N., Khachatryan, A., Zhang, R. & Sand, W. (2019). Adhesion to mineral surfaces by cells of *Leptospirillum, acidithiobacillus* and *sulfobacillus* from armenian sulfide ores, Minerals, Vol. 9(2), Article number 69.

Vilinska, A. & Rao, K.H. (2011). Surface characterization of *Acidithiobacillus ferrooxidans* adapted to high copper and zinc ions concentration, Geomicrobiology Journal, Vol. 28(3), pp. 221-228.

Wang, M., Zhang, Q., Hao, W. & Sun, Z.-X. (2011). Surface stoichiometry of zinc sulfide and its effect on the adsorption behaviors of xanthate, Chemistry Central Journal 2011, Vol. 5, Article number 73.

Wang, L., Wang, L., Ren, X., Ye, X., Li, W., Yuan, S., Sun, M., Sheng, G., Yu, H. & Wang, X. (2012). PH dependence of structure and surface properties of microbial EPS, Environmental Science and Technology, Vol. 46(2), pp. 737-744.

Watling, H.R. (2006). The bioleaching of sulphide minerals with emphasis on copper sulphides - A review, Hydrometallurgy, Vol. 84(1-2), pp. 81-108.

Worksafe Australia. (1996). National Industrial Chemicals Notification and Assessment Scheme: Bis (2,4,4-trimethylpentyl) phosphinic acid. Available at: https://www.industrialchemicals.gov.au/sites/default/files/NA291%20Public%20Report%20PDF.pdf.

WO2018072029A1. Process of leaching metal sulphides with reagents having thiocarbonyl functional groups, The University of British Columbia, Vancouver, CA & Jetti Resources, Grand Cayman, KY. Dixon, D., Asselin, E., Ren, Z. & Mora Huertas, N., Application PCT/CA2017/051250, Filed at 2017/10/19, Published at 2018/04/28.

Ympäristöhallinnon yhteinen verkkopalvelu. Ympäristö.fi. (2019). Teollisuuden päästöt vesistöön 2017. Available at (7.6.2021): https://www.ymparisto.fi/fi-FI/Kartat_ja_tilastot/Vesistojen_kuormitus_ja_luonnon_huuhtouma/Teollisuuden_vesistokuormitus/Teollisuuden_paastot_vesistoon_vuonna_20(50076).

Yu, R., Liu, J., Chen, A., Zhong, D., Li, Q., Qin, W., Qiu, G. & Gu, G. (2013). Interaction mechanism of Cu2+, Fe3+ ions and extracellular polymeric substances during bioleaching chalcopyrite by *Acidithiobacillus ferrooxidans* ATCC2370, Transactions of Nonferrous Metals Society of China (English Edition), Vol. 23(1), pp. 231-236.

Zhang, C., Xia, J., Zhang, R., Peng, A., NIie, Z. & Qiu, G. (2008). Comparative study on effects of Tween-80 and sodium isobutyl-xanthate on growth and sulfur oxidizing activities of *Acidithiobacillus albertensis* BY-05, Transactions of Nonferrous Metals Society of China (English Edition), Vol. 18(4), pp. 1003-1007.

Zhang, L., Zhou, W., Li, K., Mao, F., Wan, L., Chen, X., Zhou, H. & Qiu, G. (2015). Synergetic effects of *Ferroplasma thermophilum* in enhancement of copper concentrate bioleaching by *Acidithiobacillus caldus* and *Leptospirillum ferriphilum*, Biochemical engineering journal, Vol. 93, pp. 142-150.

Zhang, R., Wei, D., Shen, Y., Liu, W., Lu, T. & Han, C. (2016). Catalytic effect of polyethylene glycol on sulfur oxidation in chalcopyrite bioleaching by *Acidithiobacillus ferrooxidans*, Minerals Engineering, Vol. 95, pp. 74-78.

Zhang, R., Sun, C., Kou, J., Zhao, H., Wei, D. & Xing, Y. (2019). Enhancing the leaching of chalcopyrite using *Acidithiobacillus ferrooxidans* under the induction of surfactant triton X-100, Minerals, Vol. 9(1), Article number 11.

Zou, G., Papirio, S., Van Hullebusch, E. & Puhakka, J. (2015). Fluidized-bed denitrification of mining water tolerates high nickel concentrations, Bioresource Technology Vol. 179, pp. 284-290.

APPENDIX 1: CHEMICALS

Chemical	Manufacturer
Baysolvex D2EHPA (90–100%, w/w)	LANXESS Deutschland GmbH, Germany
$Ca(NO_3)_2 \cdot 4\ H_2O$	Merck, Germany
CH_3CO_2H	Merck, Germany
$C_2H_7NO_2$	Lab Honeywell, Germany
$C_8H_5KO_4$	Merck, Germany
$C_{10}H_{19}O_2H$ (100%)	ExxonMobil Chemical Company, US
$C_{12}H_8N_2 \cdot H_2O$	Merck, Germany
$CoCl_2 \cdot 7\ H_2O$	Merck, Germany
$CuSO_4 \cdot 5\ H_2O$	Merck, Germany
Cyanex 272 (100%)	Solvay Business Services Latvia SIA, Latvia
$FeCl_3 \cdot 6\ H_2O$	Merck, Germany
$FeSO_4 \cdot 7\ H_2O$ (for analysis)	ACROS Organics, the Netherlands
$FeSO_4 \cdot 7\ H_2O$ (technical grade)	VWR, Belgium
H_3BO_3	Alfa Aesar, Germany
HCl (37%)	VWR, France
H_2SO_4 (95–97%)	Merck, Germany
KCl	Merck, Germany
K_2HPO_4	J. T. Baker, the Netherlands
$MgSO_4 \cdot 7\ H_2O$	Merck, Germany
$MnSO_4 \cdot 4\ H_2O$	Merck, Germany
Nessol D100	Neste Corporation, Finland
$(NH_4)_2SO_4$	ACROS Organics, the Netherlands
NaOH (solid)	VWR, Belgium
$Na_2MoO_4 \cdot 2\ H_2O$	J. T. Baker, the Netherlands
Na_2SeO_4	ACROS Organics, the Netherlands
Na_2SO_4	Merck, Germany
$Na_2SO_4 \cdot 10\ H_2O$	Merck, Germany
S^0 (technical grade)	VWR, Belgium
$ZnSO_4 \cdot 7\ H_2O$	VWR, Belgium