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ABSTRACT

The bioleaching of metal sulphide ore has developed into an important industrial process to recover valuable base metals from low-grade ores, because high grade ore resources are depleting. The Talvivaara deposits in Finland have been known for decades, but have not been utilized until now, because of the low nickel concentration. The aim of this work was to study the bioleaching process of a Finnish complex multimetal black schist ore in boreal conditions. The effects of pH and leaching temperature on the dissolution of valuable metals and gangue minerals were studied. The effect of low temperature on iron oxidation and mineral bioleaching was investigated. Microbial community development at different pH values and temperatures was tested in laboratory-scale bioleaching columns and finally the community dynamics were studied in a demonstration-scale bioheap over a period of three years in Talvivaara Finland.

The experiments were carried out using laboratory-scale columns containing about 9 kg of agglomerated ore. The columns were loaded with the ore, irrigated with pregnant leaching solution (PLS) by recycling and aerated from the bottom. The tested pH range was from 1.5 to 3.0 at 21 ºC and temperature range was from 7 to 50 ºC at pH 2.5. The particle size (d_{80}) of the ore was 7.6 mm. Surface water taken from lake near the Sotkamo deposit (slightly affected by acid mine drainage) supplemented with nutrients was used for irrigation. Aeration was provided through a diffuser inserted at the base of the column. The iron- and sulphur-oxidizing bacterial culture used in inoculation of the columns, was enriched from surface water samples (pH 4.5-6.9) obtained from the ore deposit. The pH of irrigation solution was maintained with continuous titration with H_2SO_4. The ore was acid consuming in all tested conditions. The actual pH of the irrigation solutions after 140 days were 0.1-0.5 units over the target values in all columns. Leaching at low pH resulted in increased acid consumption of 160 and 38 H_2SO_4 g kg^{-1} ore at pH 1.5 and 2.0 after 140 days. Temperature, at pH 2.5, had also effect on acid consumption. At 50 ºC acid consumption was highest and lowest at 21 ºC, being 29 and 8 H_2SO_4 g kg^{-1} ore, respectively.

The pH of the irrigation solution clearly affected to the dissolution of nickel and zinc. Nickel solubilization rate was 3.3 times higher at pH 1.5 than at pH 3.0, being 0.42 and 0.13 % (Ni) d^{-1}, respectively. At pH 1.5 valuable metals yields were 59 % for Ni, 52 % for Zn, 13 % for Cu and 16 % for Co, whereas at pH 3.0 yields were 15 % for Ni, 10 % for Zn, 0.5 % for Cu and 6 % for Co after 140 days of bioleaching. No significant bioleaching happened after that at pH 1.5, 2.5 or 3.0. At pH 2.0 the maximum yields were achieved after 230 days of bioleaching. Nickel and zinc leaching rates and yields decreased nearly linearly as pH increased. Copper did not bioleach at high pH (2.5-3.0). After the beginning, no further cobolt dissolution happened at pH 3.0. Decrease in leaching rates may be due to a lack of dissolved ferric iron, serving as a leaching agent, or metal dissolution barriers created by precipitates on the ore surfaces. The ferric iron concentration in PLS increased all the time at pH 1.5, being 36 g l^{-1} after 140 days. At pH 2.0 the ferric iron concentrations varied, being highest 3.8 g l^{-1} after 97 days. At 2.5 and 3.0 no ferric iron was present in PLS and iron concentration remained low, being 15 mg l^{-1}.

After 60 days of bioleaching the leach liquor at pH 1.5 became jelly-like due to solubilization of Si from the ore, which contained 42 % (w w^{-1}) of SiO_2. Quartz, phlogopite, and feldspars (anorthite and microcline) were the main Si-containing phases. After 110 days the Si concentration reached 2.96 g L^{-1} at pH 1.5. Soluble Si increases the solution viscosity and thus hinders leach liquor percolation trough the heap, lowers the oxygen transfer rate, and complicates subsequent metal extraction. Although, dissolved Si did not affect the solubilization of valuable metals, the pH value of the PLS must be kept at over 1.5 to slow down Si-containing mineral dissolution. At pH 2.5 less than 200 mg L^{-1} Si was solubilized and different temperatures had no effect on Si dissolution at that pH.

Based on an optimisation between the maximum valuable metal yields, leaching rates, the acid consumption, and the low dissolution of cations (Si, Al, Ca, Mg and Mn), the leaching solution pH of 2.0 was recommended for a bioheap application. At pH 2.0, the maximum leaching yields were achieved after 230 days, being 54 % for Ni, 37 % for Zn, 13 % for Cu and 12 % for Co.

Temperature strongly affected the valuable metal yields at pH 2.5. Leaching at low temperature (7 ºC) resulted in yields of 24 % for Ni, 17 % for Zn, 2 % for Cu and 6 % for Co after 496 days. The Cu leaching increased all the time during the experiment at 7 ºC, while at other temperatures it slowed down after 100 days. The highest yields were obtained at 21 ºC (26 % for Ni, 18 % for Zn, 0.5 % for Cu and 6 % for Co) after 153 days. After re-inoculation (day 65) with a thermophilic *Sulfolobus* culture, leaching at 50 ºC accelerated but slowed down soon and resulted in 18 % for Ni, 11 for Zn, 0.3% for Cu and 2% for Co (after 140 days). In the column leaching study, after the maximum yields, longer leaching time did not result more metals in solutions.

The redox increased during the first two months at 7 ºC and reflected the start of ferrous iron oxidation and microbial activity. The concentration of ferric iron was around 400 mg L^{-1} after two months. After that ferric iron was present all the time at 7 ºC and this demonstrated that more ferric iron was available for the oxidation of the mineral sulphide than at other temperatures. The leach liquor redox potential stabilized to 500-600 mV (Ag^0/AgCl reference) at 7 ºC after 40 days and at 21 ºC right after beginning, whereas at 35 ºC and at 50 ºC it varied between 300-500 mV. At 50 ºC, all dissolved iron was in ferrous form inspite the variation of redox. After 50 days Fe^{2+} and Fe_{tot} were both 350 mg L^{-1} indicating that iron oxidation and precipitation occurred at the same time. Brown precipitates accumulated to the surfaces of the agglomerated ore in columns from 7 ºC to 50 ºC. Additionally, bright yellow precipitates were formed indicating elemental sulphur or Na-jarosite accumulation at 7 ºC and 21 ºC.

After 50 days of bioleaching, at 7 ºC leach liquor total cell counts (10^8-10^9 cells mL^{-1}) were significantly higher than at other temperatures (10^6-10^7 cells mL^{-1}). Cell counts remained that high troughout the column study. At the end of the experiment, total cell counts in the leach residues were studied. At 7, 21, 35 and 50 ºC cell counts of the leach residues were $3.4 \cdot 10^8$, $2.3 \cdot 10^8$, $1.1 \cdot 10^7$ and $8.7 \cdot 10^6$ cells ore g^{-1}, respectively. The pH did not affect at 21 ºC the numbers of microorganisms in the PLS and cell counts remained at 10^6-10^8 cell mL^{-1} throughout the study and the leach residues contained about 10^8 cells g ore^{-1}.

The microbial community composition and dynamics was by investigated by DNA extraction PCR-DGGE-sequencing approach. The microbial community were not affected by pH. In contrast, temperature affected the microbial populations. After the first months, *Acidithiobacillus ferrooxidans* AP 310 (96-99% sequence similarity, accession DQ35518) was the only species detected at 7 ºC and was also present at other temperatures. After the data of this study was published (2007), two new *Acidithiobacillus* species were described, *A. ferrivorans* and *A. ferridurans*. Genetically these species are very near each other. The 16S rRNA gene sequences of the bands that corresponded 99% of *A. ferrooxidans* AP310 (DQ35518) were identified again in 2015 using the basic local alignment search tool (BLAST). The 16S rRNA gene sequences of *A. ferrooxidans* at temperatures of 7 and 21 ºC corresponded 99% as *A. ferrivorans* SS3 (CP002985). One of the 16S rRNA gene sequences of *A. ferroxidans* strains at 35 ºC corresponded 99% as *A. ferridurans* ATCC 3302 (NR_117036). At 50 ºC, no proper *A. ferroxidans* 16S rRNA gene sequences were gained with the used methods. The presence of *A. ferroxidans* at 50 ºC was concluded based on the fact that the DGGE band was in the same place as the other *A. ferrooxidans* bands. The 16S rRNA gene sequences of *Acidithiobacillus ferrooxidans* strains in pH between 1.5 and 3.0, at 21 ºC, corresponded also 99% as *A. ferrivorans* SS3 (CP002985). In the light of increased knowledge, these species cannot be separated with the denaturing gradient from 40 to 70% that were used in the DGGE. *A. ferrooxidans*, *A. ferrivorans* and *A. ferridurans* are able to oxidize both iron and sulphur compounds.

Leptospirillum ferrooxidans DSM 2705 (98-100%, X86776) and *Sulfobacillus thermotolerans* KR-1 (99%, DQ124681) were mainly detected at 21 ºC and 35 ºC. *Sb. thermotolerans* was present at 50 ºC. *L. ferriphilum* D1 (99 %, DQ665909) appeared after 300 days of bioleaching and was present in every leach residue, except at 7 ºC and pH 3.0. *L. ferrooxidans* and *L. ferriphilum* are able to oxidize only iron. *Sb. thermotolerans* is able to oxidize both iron and sulphur compounds.

Archaeal species were analyzed two times from leach liquors and three species were detected. A species related to an uncultured archaeon clone ant b7 (99%, DQ303249), nearest known species *Thermoplasma acidiphilum* DSM1728 (91%, AL445067) was present in all of the leach liquors except at pH 1.5. Archaea related to *Sulfolobus metallicus* DSM 6482 (98%, SM16SRRN1) were present at pH values 2.5 and 3.0 and in all other temperatures, except at 7 ºC. *Sulfolobus metallicus* is able to oxidize both iron and sulphur compounds. *Ferroplasma acidiphilum* DR1 (98%, AY222042) that can oxidize only iron, was present at pH 2.5 and 2.0, and in all temperatures, expect at 35 ºC.

The mixed iron- and sulphur-oxidizing culture in the recirculation solution at 7 ºC was used in the experiments where Fe^{2+}-oxidation rate and optimum temperature were determined over a temperature range of 2-40 ºC. Two temperature optima of 22.4 ºC and 32.4 ºC were observed. This indicated the presence of both psychrotolerant and/ or mesophilic microorganisms in the culture. This supports the suggestion that *A. ferrooxidans* was actually *A. ferrivorans*, or both species were present. The specific oxidation rates for the culture were similar, with $13.5 \cdot 10^{-8}$ and $12.8 \cdot 10^{-8}$ mg Fe^{2+} $cell^{-1}$ h^{-1} for 22.4 ºC and 32.4 ºC, respectively.

The two demonstration-scale bioheaps (17 000 t) at the Talvivaara mine site were operated and monitored by Talvivaara Mining Company for 30 months. After the start-up of heap irrigation, oxidation of pyrrhotite and pyrite increased the heap temperature in central locations up to 90 ºC. In the second winter temperatures inside the heaps decreased being still 80 ºC at the hottest spots. Leach liquor temperatures varied between 60 ºC and 15 ºC over the whole operation period. The target pH of the PLS was 2.0. Inspite of continuous titration pH varied during the 10 months between 3.5 and 3.0 and after that between 3.0 and 2.5.

The bacterial community composition on the heaps was monitored over time from manholes and the leach liquor collection ponds. At the end of the primary bioleach phase (18 months) cell counts were around 10^6 cells mL^{-1}. Large temperature gradients resulted in the simultaneous presence of mesophilic and thermophilic iron- and sulphur-oxidisers in the heap. In the beginning diversity was broad, but decreased with time. *A. ferrooxidans/ ferrivorans* SS3 (99%, CP002985) was the dominant bacterium and an unknown bacterium related to clone H70 (91%, DQ328625) was present. After six months of bioheap operation *L. ferrooxidans* DSM 2705 (98%, X86776) was observed for the first time and it was present thereafter in nearly all samples. Archaea were analysed during the primary leaching phase from leach liquors. Two novel archaea and one archaea related to *Thermoplasma acidophilum* strain 122-1B2 (91-93%, NR_028235) were detected.

Several ore samples were drilled from the primary bioheaps after one year of bioheap operation. *A. ferrooxidans/ A. ferrivorans* SS3 (99%, CP002985) was present in nearly all samples. The novel bacterium related to clone H70 (91%, DQ328625) and *A. caldus* related bacteria (95%, AY427958) was detected from the areas of wide temperature variation. *Sb. thermosulfidooxidans* strain YN22 (99%, DQ650351) was found from the high temperature zones of the heap. *Ferrimicrobium acidiphilum* T23 (99%, AF251436) was present in the areas where temperature varied between 20 and 35 ºC. After 18 months of demonstration-scale heap operation, the heaps were reclaimed and restacked to the secondary bioheap. At the secondary leaching phase the community remained steady. *A. ferrooxidans/ ferrivorans* SS3 (99%, CP002985) dominated and the novel bacterium related to a clone

H70 (91%, DQ328625) and *L. ferrooxidans* DSM 2705 (98-100%, X86776) were present in the leach liquors of secondary phase bioheaps.

TIIVISTELMÄ

Bioliuotusta käytetään yhä yleisemmin metallien erottamiseen köyhistä sulfidimalmeista, koska korkealaatuiset malmiesiintymät ovat ehtymässä. Tässä työssä tutkittiin kompleksisen monimetallisen mustaliuskemalmin soveltuvuutta bioliuotukseen pohjoisissa olosuhteissa. Talvivaaran malmiesiintymät ovat olleet tiedossa jo vuosikymmeniä, mutta niitä ei ole hyödynnetty aiemmin alhaisen nikkelipitoisuuden vuoksi. Laboratoriomittakaavan kolonneissa tutkittiin kasteluliuoksen pH:n ja liuotuslämpötilan vaikutusta arvometallien ja sivukiven liukenemiseen sekä mikrobiyhteisön kehitykseen. Lisäksi tarkasteltiin raudan vaikutusta arvometallien liukenemiseen. Lopuksi mikrobiyhteisön dynamiikkaa seurattiin kolmen vuoden ajan pilot-mittakaavan bioliuotuskasalla, joka sijaitsi Talvivaarassa.

Kokeissa käytetyissä kolonneissa oli n. 9 kg malmia, jonka raekoko oli 7.6 mm. Kolonneja ilmastettiin pohjasta ja kasteltiin päältä kierrätysliuoksella. Kierrätysliuos oli Sotkamosta toimitettua järvivettä, joka oli lievästi hapanta. Veteen lisättiin pieniä määriä ravinteita. Kolonnikokeen testattavat pH:t olivat 1,5; 2,0; 2,5 ja 3,0 (21 asteessa) sekä lämpötilat 7, 21, 35 ja 50 ºC (pH:ssa 2,5). Kolonnit ympättiin rautaa ja rikkiä hapettavalla mikrobiviljelmällä, joka oli rikastettu malmiesiintymän pintavesistä (pH 4,5-6,9). Kierrätysliuoksen pH:ta säädettiin jatkuvasti titraamalla liuosta rikkihapolla. Malmi kulutti happoa kaikissa testatuissa olosuhteissa. Kasteluliuoksen pH oli 140 päivän jälkeen 0,1-0,5 yksikköä korkeampi kuin tavoite pH kaikissa kolonneisssa. Haponkulutus oli suurinta pH:ssa 1,5. Haponkulutus oli 140 päivän jälkeen 160 H_2SO_4 g/kg malmia. Vastaava haponkulutuksen määrä pH:ssa 2,0 oli 38 H_2SO_4 g/kg malmia. Lämpötilalla oli myös vaikutusta haponkulutukseen pH:ssa 2,5. 50 asteessa haponkulutus oli suurinta (29 H_2SO_4 g/kg malmia) ja pienentä 21 asteessa (8 H_2SO_4 g/kg malmia).

Kierrätysliuoksen pH vaikutti merkittävästi nikkelin ja sinkin liukenemiseen. Nikkelin liukeneminen oli 3,3 kertaa nopeampaa pH:ssa 1,5 kuin 3,0:ssa. Liukenemisnopeus pH:ssa 1,5 oli 0,42 % (Ni) /d ja pH:ssa 3,0 liukenemisnopeus oli 0,13 % (Ni) /d. 140 päivän bioliuotuksen jälkeen nikkelisaanto pH:ssa 1,5 oli 59 prosenttia. Muista arvometalleista oli liuennut 52 % sinkkiä, 13 % kuparia ja 16 % kobolttia. Kasteluliuoksen pH:n ollessa kolme, vastaavat arvot olivat 15 % nikkeliä, 10 % sinkkiä, 0,5 % kuparia ja 6 % kobolttia. Merkittävää arvometallien liukenemista ei tapahtunut enää 140 päivän jälkeen pH:ssa 1,5; 2,5 tai 3,0. Suurimmat metallisaannot pH:ssa 2,0 saavutettiin 230 päivän jälkeen. Nikkelin ja sinkin liukenemisnopeus väheni lähes lineaarisesti pH:n noustessa. Kupari ei liuennut korkeimmissa pH-arvoissa (2,5-3,0). Alun jälkeen koboltti ei liuennut pH:ssa 3,0. Liukenemisnopeuksien laskuun voi olla syynä liuenneen ferriraudan puute kierrätysliuoksessa tai saostumien muodostuminen malmin pinnalle. Ferriraudan määrä pH 1,5 kierrätysliuoksessa lisääntyi koko ajan, ollen 36 g/l 140 päivän jälkeen. Ferriraudan konsentraatio vaihteli pH:ssa 2,0; ollen korkeimmillaan 3,8 g/l 97 päivän jälkeen. Ferrirautaa ei ollut kierrätysliuoksissa, joiden tavoite pH oli 2,5-3,0. Kokonaisraudan määrä oli tällöin myös alhainen (15 mg/l).

60 päivän bioliuotuksen jälkeen kasteluliuos pH:ssa 1,5 muuttui hyytelömäiseksi johtuen piin (Si) liukenemisesta sivukivestä. Malmi sisälsi 42 % (w/w) silikaattia (SiO_2). Kvartsi, flogopiitti ja maasälvät (anortiitti ja mikrokliini) sisälsivät pääosan piistä. Liuenneen piin konsentraatio oli 110 päivän jälkeen 2,96 g/l pH:ssa 1,5. Liuennut pii ei vaikuttanut arvometallien liukenemiseen. Kierrätysliuoksen viskositeetti kuitenkin lisääntyi. Se hidastaa virtausta kasan läpi, alentaa hapen kulkeutumista ja vaikeuttaa metallien talteenottoa. Tutkimus osoitti, että pH tulee pitää yli 1,5 silikaattimineraalien liukenemisen hidastamiseksi. Piitä liukeni pH:ssa 2,5 vähemmän kuin 200 mg/l ja lämpötilalla ei ollut vaikutusta silikaattien liukenemiseen tässä pH:ssa.

Perustuen arvometallien liukenemisnopeuteen, maksimisaantoihin, hapon kulutukseen ja kationien (Si, Al, Ca, Mg ja Mn) liukenemiseen, pH:ta 2,0 suositeltiin käytettäväksi kompleksisen sulfidimalmin biokasaliuotuksessa. Arvometallien saannot 230 päivän bioliuotuksen jälkeen pH:ssa 2,0 olivat 54 %

nikkeliä, 37 % sinkkiä, 13 % kuparia ja 12 % kobolttia.

Lämpötila vaikutti merkittävästi arvokkaiden metallien liukenemiseen. Metalleista liukeni seitsemässä asteessa 24 % nikkeliä, 17 % sinkkiä, 2 % kuparia ja 6 % kobolttia 496 päivässä. Kuparin liukeneminen jatkui koko kokeen ajan 7 ºC:ssa, kun muissa lämpötiloissa kupari ei liuennut 100 päivän jälkeen. Eniten arvokkaita metalleja liukeni 21 ºC:ssa (26 % nikkeliä, 18 % sinkkiä, 0,5 % kuparia ja 6 % kobolttia, 153 päivän jälkeen). Kolonni, joka oli 50 ºC:ssa, ympättiin uudelleen 65 päivän jälkeen termofiilisellä *Sulfolobus*-viljelmällä. Bioliuotus nopeutui hetkellisesti ja saannot olivat parhaimmillaan 140 päivän jälkeen (18 % Ni, 11 % Zn, 0,3 % Cu ja 2 % Co). Maksimisaantojen jälkeen, kolonnikokeen jatkuessa metallien konsentraatio liuoksissa ei kasvanut.

Raudan hapettuminen ja mikrobitoiminta alkoi 7 ºC:ssa kahden kuukauden viiveen jälkeen, jolloin redox-potentiaali lähti nousemaan. Ferriraudan pitoisuus oli tämän jälkeen kierrätysliuoksessa noin 400 mg/l. Ferriraudan, jota tarvitaan sulfidisidoksen katkaisemiseen, pitoisuus oli muissa lämpötiloissa vähäisempi. Kierrätysliuoksen redox-potentiaali stabiloitui 7 ºC:ssa 40 päivän jälkeen ja 21 ºC:ssa heti alun jälkeen 500-600 millivolttiin (Ag^0/AgCl referenssielektrodi). 35 ja 50 ºC:ssa redox-potentiaali vaihteli välillä 300-500 mV. Kaikki liukoinen rauta oli 50 ºC:ssa ferromuodossa, huolimatta redox-potentiaalin vaihtelusta. Kokonaisraudan konsentraatio oli 50 päivän jälkeen 350 mg/l. Tämä osoitti, että raudan hapetus ja saostuminen tapahtuivat samanaikaisesti. Ruskean värisiä saostumia kertyi myös muiden lämpötila-kolonnien (7-50 ºC) malmin pinnalle. Lisäksi kirkkaan keltaisia rikki tai Na-jarosiitti saostumia muodostui 7 ja 21 ºC:ssa oleviin kolonneihin.

50 päivän jälkeen kierrätysliuoksen solumäärä oli 7 ºC:ssa (10^8-10^9 solua/ml) merkittävästi suurempi kuin muissa lämpötiloissa (10^6-10^7 solua/ml). Solumäärät pysyivät kolonnien kierrätysliuoksessa samassa tasossa koko tutkimuksen ajan. Liuotusjäännöksien solumäärät 7 ja 21 ºC:ssa olivat n. 10^8 solua/g malmia. Lämpötiloissa 35 ja 50 ºC liuotusjäännöksen solumäärät olivat $1{,}1 \cdot 10^7$ ja $8{,}7 \cdot 10^6$ solua/g malmia. Kierrätysliuoksen pH ei vaikuttanut huoneen lämpötilassa (21 ºC) olevien kolonnien solumääriin. Solumäärät olivat 10^6-10^8 solua/ml kierrätysliuoksessa ja noin 10^8 solua/g malmia liuotusjäännöksessä.

Mikrobiyhteisön rakennetta ja sen muutoksia tutkiittin ensin uuttamalla DNA ja monistamalla 16S rRNA-geeni polymeraasiketjureaktiolla (PCR). Sen jälkeen eri lajien 16S rRNA-geenit erotetiin denaturoivalla gradientti geeli elektroforeesilla (DGGE). Kasteluliuoksen pH ei vaikuttanut mikrobiyhteisön rakenteeseen. Sen sijaan lämpötilalla oli huomattava vaikutus. Ensimmäisien kuukausien jälkeen *Acidithiobacillus ferrooxidans* AP 310 (96-99% vastaavuus, paikka DQ35518) oli ainoa mikrobi 7 ºC:ssa. *A. ferrooxidans* esiintyi myös muissa lämpötiloissa. Tutkimuksen tietojen julkaisun jälkeen on tunnistettu kaksi uutta *Acidithiobacillus*-lajia, *A. ferrivorans* ja *A. ferridurans*. Näytteiden, joiden 16S rRNA-geenin DNA-sekvenssit vastasivat *A. ferrooxidans* -lajia, DNA-sekvenssit analysoitiin uudelleen v. 2015 käyttäen apua BLAST-hakutyökalua. Ohjelma etsii hakusekvenssin kanssa samankaltaisia sekvenssejä ja rinnastaa ne. DNA-sekvenssit 7 ja 21 ºC:ssä vastasivat 99 prosenttisesti *A. ferrivorans* SS3 -kantaa (CP002985). Yksi 35 asteen 16S rRNA-geenin DNA-sekvenssi vastasi 99% *A. ferridurans* ATCC 3302 -kantaa (NR_117036). 50 asteessa olevasta kierrätysliuoksesta ei käytetyillä menetelmillä saatu riittävän hyvää *A. ferrooxidans* -lajin 16S rRNA-geenin DNA-sekvenssiä. 50 asteen kolonnissa oletettiin esiintyvän sama *A. ferrooxidans,* kuin muissakin kolonneissa, joiden DNA-sekvenssin bändi oli DGGE-kuvassa samassa paikassa. Myös pH-kolonnien *A. ferrooxidans* -lajin 16S rRNA-geenin DNA-sekvenssit vastasivat 99% *A. ferrivorans* SS3 -kantaa (CP002985). Denaturoivalla gradientilla 40-70%, jota tutkimuksen DGGE:ssä käytettiin, ei pysty erottamaan näitä *Acidithiobacillus*-lajeja, jotka ovat geneettisesti hyvin lähellä toisiaan. *A. ferrivorans*, *A. ferridurans* ja *A. ferrooxidans* voivat hapettaa rautaa ja rikkiyhdisteitä.

Leptospirillum ferrooxidans DSM 2705 (98-100%, X867769) ja *Sulfobacillus thermotolerans* KR-1

(99%, DQ124681) esiintyivät huoneenlämmössä ja 35 ºC:ssä. *Sb. thermotolerans* oli läsnä 50 ºC:ssa. *L. ferriphilum* D1 (99%, DQ665909) ilmestyi kierrätysliuoksiin 300 päivän jälkeen ja se havaittiin myös jokaisesta liuotusjäännöksestä, paitsi 7 ºC:sta ja pH:sta 3,0. *L. ferrooxidans* ja *L. ferriphilum* pystyvät hapettamaan vain rautaa. *Sb. thermotolerans* pystyy hapettamaan rautaa ja rikkiyhdisteitä.

Kolonnikokeen aikana kierrätysliuoksista analysoitiin kahdesti arkkeja, lajeja löydettiin kolme. Laji, joka vastasi 91%:sti *Thermoplasma acidophilumia* DSM1728 (AL445067), löydettiin kaikista muista kierrätysliuoksista, paitsi pH:sta 1,5. *Sulfolobus metallicus* DSM 6482 (98%, SM16SRRN1) oli läsnä pH:ssa 2,5 ja 3,0 sekä kaikissa lämpötiloissa, paitsi 7 asteessa. *Sulfolobus metallicus* pystyy hapettamaan sekä rautaa, että rikkiyhdisteitä. *Ferroplasma acidophilum* DR1 (98%, AY22042) esiintyi pH:ssa 2,0 ja 2,5 sekä lämpötiloissa 7, 21 ja 50 ºC.

Kolonnin, joka oli 7 ºC:ssa, kierrätysliuoksesta otettiin ymppi kokeeseen, jossa määritettiin raudan hapettumisen nopeus ja viljelmän lämpötilaoptimi. Testattu lämpötilaväli oli 2-40 ºC. Lämpötilaoptimeita löytyi kaksi; 22,4 ja 32,4 ºC. Tämä osoitti, että viljelmässä oli psykrotolerantteja ja/ tai mesofiilisiä mikrobeja. Tulokset tukevat myös tulkintaa, jonka mukaan *A. ferrooxidans* olikin *A. ferrivorans* tai molemmat lajit olivat läsnä. Raudan hapettumisnopeudet olivat 22,4 ºC:ssa $13{,}5 \cdot 10^{-8}$ mg Fe^{2+}/solua·h ja 32,4 ºC:ssa $12{,}8 \cdot 10^{-8}$ mg Fe^{2+}/solua·h.

Talvivaaran kaivosyhtiö operoi pilot-mittakaavan bioliuotus kasoja (17 000 t) Talvivaarassa 30 kuukautta. Käynnistysvaiheen jälkeen magneetti- ja rikkikiisun hapettuminen nostivat kasan keskustan lämpötilan jopa 90 ºC:seen. Toisena talvena, kasan sisälämpötila oli edelleen kuumimmissa kohdisssa 80 ºC. Kierrätysliuoksen lämpötila vaihteli 60 ja 15 ºC:teen välillä. Kierrätysliuoksen tavoite pH oli 2,0. Huolimatta jatkuvasta pH:n säädöstä, pH vaihteli molemissa kasoissa 3,0 ja 3,5:den välillä ensimmäiset kymmenen kuukautta. Tämän jälkeen se vaihteli välillä 2,5 ja 3,0.

Bioliuotuskasojen mikrobiyhteisöä tarkkailtiin kaivoista ja kierrätysliuoksen keräysaltaista. Solumäärä oli n. 10^6 solua/ml primaarivaiheen (18 kuukautta) jälkeen. Suurten lämpötilagradienttien vuoksi kasassa oli läsnä molempia meso- ja termofiilisiä raudan ja rikin hapettajia. Kierrätysliuoksen mikrobidiversiteetti oli alussa laaja, mutta erilaisten mikrobien määrä väheni liuotuksen edistyessä. Hallitsevana bakteerina oli *A. ferrooxidans/ ferrivorans* SS3 (99%, CP002985) ja tuntematon bakteeri, joka vastasi kloonia H70 (91%, DQ328625) esiintyi usein näytteissä. *L. ferrooxidans* DSM 2705 (98-100%, X86776) havaittiin ensimmäistä kertaa kuuden kuukauden jälkeen. Sen jälkeen se esiintyi lähes kaikissa näytteissä. Primaarivaiheen aikana kierrätysliuoksessa löydettiin kolme arkkia, *Thermoplasma acidophilum* (91-93%, AL445067) sekä kaksi tuntematonta arkkia.

Kasoista porattiin useita malminäytteitä vuoden bioliuotuksen jälkeen. *A. ferrooxidans/ ferrivorans* SS3 (99%, CP002985) esiintyi suuresta lämpötilavaihtelusta (20-90 ºC) huolimatta lähes kaikissa näytteissä. Lisäksi *A. caldus* (96-99%, AY427958) ja tuntematon bakteeri, joka oli sukua kloonille H70 (91%, DQ3286259) olivat läsnä. *Sb. thermosulfidooxidans* YN22 (99%, DQ650351) esiintyi kasan kuumilla alueilla. *Ferrimicrobium acidiphilum* T23 (99%, AF251436) esiintyi alueella, joissa lämpötila vaihteli välillä 20 ja 35 ºC. Bioliuotus kasat putettiin ja kasattiin 18 kuukauden jälkeen uudelleen. Mikrobiyhteisö säilyi toisen liuotusvaiheen samankaltaisena. *A. ferrooxidans*/ *ferrivorans* SS3 (99%) oli hallitseva bakteeri kierrätysliuoksessa, jossa esiintyi myös tuntematon bakteeri, joka vastasi kloonia H70 (91%), ja *L. ferrooxidans* DSM 2705 (98-100%).

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PREFACE AND ACKNOWLEDGEMENTS

This thesis is based on the work carried out at the Department of Chemistry and Bioengineering, Tampere University of Technology, Tampere, Finland. The experimental work was conducted for Talvivaara Mining Company Plc and partly for BioMinE project of the 6th European Framework Programme.

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Anna-Kaisa Halinen

LIST OF ORIGINAL PAPERS

This thesis is based on the following original papers referred to, in this thesis, by the roman numerals:

I Halinen A-K, Rahunen N, Kaksonen AH and Puhakka JA. 2007. Heap bioleaching of a complex sulfide ore Part I: Effect of pH on metal extraction and microbial composition in pH controlled columns. Hydrometallurgy 98: 92-100.

II Halinen AK, Rahunen N, Kaksonen AH and Puhakka JA. 2007. Heap bioleaching of a complex sulfide ore: Part II. Effect of temperature on base metal extraction and bacterial compositions. Hydrometallurgy 98: 101-107.

III Dopson M, Halinen AK, Rahunen N, Özkaya B, Sahinkaya E, Kaksonen AH, Lindström EB and Puhakka JA. 2007. Mineral and iron oxidation at low temperatures by pure and mixed cultures of acidophilic microorganisms. 2007. Biotechnology and Bioengineering 97: 1205-1215.

IV Dopson M, Halinen AK, Rahunen N, Boström D, Sundkvist JE, Riekkola-Vanhanen M, Kaksonen AH and Puhakka JA. 2007. Silicate mineral dissolution during heap bioleaching. Biotechnology and Bioengineering. 99: 811-820.

V Halinen AK, Beecroft NJ, Määttä K, Nurmi P, Laukkanen K, Kaksonen AH, Riekkola-Vanhanen M and Puhakka JA. 2012. Microbial community dynamics during a demonstration-scale bioheap leaching operation. Hydrometallurgy 125-126: 34-41.

AUTHOR'S CONTRIBUTION

Paper I:
Anna-Kaisa Halinen wrote the paper and is the corresponding author. She performed the experimental work with Nelli Rahunen. Anna-Kaisa Halinen interpreted the results.

Paper II:
Anna-Kaisa Halinen wrote the paper and is the corresponding author. She performed the experimental work with Nelli Rahunen. Anna-Kaisa Halinen interpreted the results.

Paper III:
Anna-Kaisa Halinen attented paper writing. She performed the experimental work done with the columns with Nelli Rahunen. N Özkaya and B Sahinkaya performed the temperature gradient incubation test.

Paper IV:
Anna-Kaisa Halinen attented paper writing. She performed the experimental work done with the columns with Nelli Rahunen.

Paper V:
Anna-Kaisa Halinen wrote the paper and is corresponding author. She performed the experimental work with Pauliina Nurmi. Anna-Kaisa Halinen interpreted the results.

The experimental work was carried out under the supervision of Prof. JA Puhakka and Docent AH Kaksonen.

ABBREVIATIONS

AAS	Atomic absorption spectrometry
AMD	Acid mine drainage
BLAST	Basic local alignment search tool
DAPI	4', 6-diamidino-2-phenylindole
DGGE	Denaturing gradient gel electrophoresis
DO	Dissolved oxygen
ICP-AES	Inductively coupled plasma atomic emission spectroscopy
ISL	*In situ* leaching
MPN-Fe	Most probable number technique-Fe
rRNA	Ribosomal RNA
PAM	Procaryotic acidophile microarray
PCR	Polymerase chain reaction
RISC	Reduced inorganic sulphur compound
SEM	Scanning electron microscopy
SX-EW	Solvent extraction - electrowinning process
XRD	X-ray diffraction

1. INTRODUCTION AND BACKROUND

1.1 NICKEL ORE RESOURCES

To meet the growing global nickel demand, mining and metal industry must develop technologies for utilization of low grade nickel ore resources. The growth of stainless steel industry, the primary user of nickel has increased quite steadily during the 40 years. Japan, China and the countries of the European Union are the largest Ni consumers. After 2011 the world nickel consumption has been over 1.5 million tons per year. Althought metal prices have been fluctuating since summer 2011 investments in industry have been made. Figure 1 presents world nickel production from 1900s to present. Nickel is a finite natural resource and sustainable principles to mining are challenging as there is global pressure to reduce energy consumption and gaseous emissions. (Kuck 2011). Recycled nickel accounts approximately 42 % of the nickel used in stainless steel production (Nickel Institute 2015). For economic and technical reasons low grade ore resources are not amenable to high energy demanding pyrometallurgical recovery. Biohydrometallurgy has the potential of increasing resource utilization.

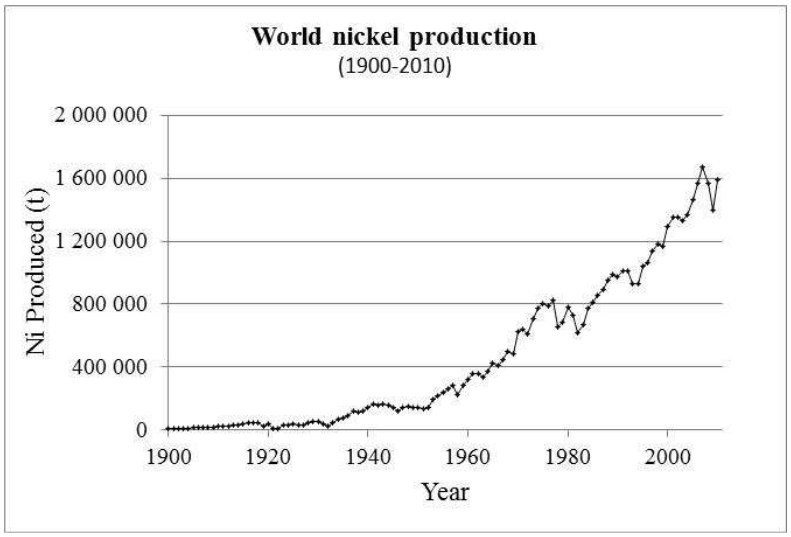


Figure 1. World nickel production (data derived from Anonymous 2014a).

The known reserves of nickel ore in the world are approximately 23 billion tons with an average grade 0.97 %. Nickel is an important metal in modern infrastructure. In addition to stainless steel, it is used in demanding corrosion- and heat-resistant applications, and in areas such as information technology, coinage, batteries, and magnetic superconductors.

Nickel is the earth´s fifth most abundant element by weight comprising about 3 % of the earth's total composition and mostly present in earth's core. Almost 200 hundred nickel-bearing minerals have been identified, but relatively few are abundant enough to be industrially significant. The mined nickel ores are divided into sulphide ores and oxide ores called laterites. The world nickel supply has been predominantly from sulphide deposits. Nickel occurs mainly as pentlandite [$(Ni, Fe)_9S_8$] in association with large amounts of pyrrhotite ($Fe_{n-1}S_n$). (Kelly and Matos 2014).

World largest nickel sulphide deposits are located in Sudbury, Ontario, Canada; in Norilsk, Siberia, Russia; in the Kola Peninsula bordering Finland; in Western Australia and in South Africa. Figure 2 presents largest nickel deposits in the world. Sulphide ores are generally found in areas where glacial action has removed much of the overburden of weathered rock (Hoatson et al. 2006). Sulphide ores can

be concentrated by using mechanical means, such as magnetic separation or flotation. The nickel grade of sulphide ore typically ranges from 0.5 - 4 % (w w^{-1}). Economic exploitation usually depends on the recovery of valuable by-products (Berger et al. 2011). Bioleaching is usually applied when ore contains below 0.5 % (w w^{-1}) metals, otherwise pyrometallurgical application can be used. (Mudd 2009, Mizzi 1987). For nickel no full-scale operations were in use at the time of Talvivaara pilot-scale bioheap in 2007. In China, Jinchuan Group Ltd owns about 400 Mt of low grade nickel sulfide mineral ore deposit. Bioleaching column tests are done by Zhen et al. (2008 and 2009). Several columns were filled with 200 kg of ore and bioleached 252 days.

Nickel is also found in manganese crusts and nodules covering large areas of the ocean floor. The long-term decline in discovery of new sulfide deposits in traditional mining districts has forced exploration teams to shift to more challenging locations like east-central Africa and the Subarctic. (U.S. Geological Survey 2013).

About 70 % of world nickel resources are lateritic. Laterite ores are not readily amenable to pyrometallurgical recovery and alternative technologies are required. Unlike sulphide metals, the valuable metals in laterites are present within the structures of host minerals. (du Plessis et al. 2011). Nickel recovery from laterites requires more energy per ton than from sulphides. Intensive ammonia leach technology or more complex high pressure acid leach processing combined with solvent extraction and electrowinning have been used. (Hoatson et al. 2006). Laterite deposits tend to be lower in grade but larger in total size compared to sulphides. In future laterite projects will have significant impact on the nickel supply. (Hoatson et al. 2006). Major new laterite projects are being developed e.g. in Australia and New Caledonia. (Jessup and Mudd 2014). The potential of bio-process laterites is under research. The process called Ferredox is targeted to tropical limonite, specially goethite [FeO(OH)]. The reductive leaching results the release of valuable metals (Ni, Co and Cu). The process has been demonstrated in anaerobic, acidic conditions (pH 1.5-2) at atmospheric pressure and ambient temperature (25-30 ºC). *A. ferroxidans* has been used in a process, as it can use ferric iron as an electron acceptor, as an alternative to oxygen. In that case an electron donor is required e.g. sulphur. (Hallberg 2011, du Plessis 2011).

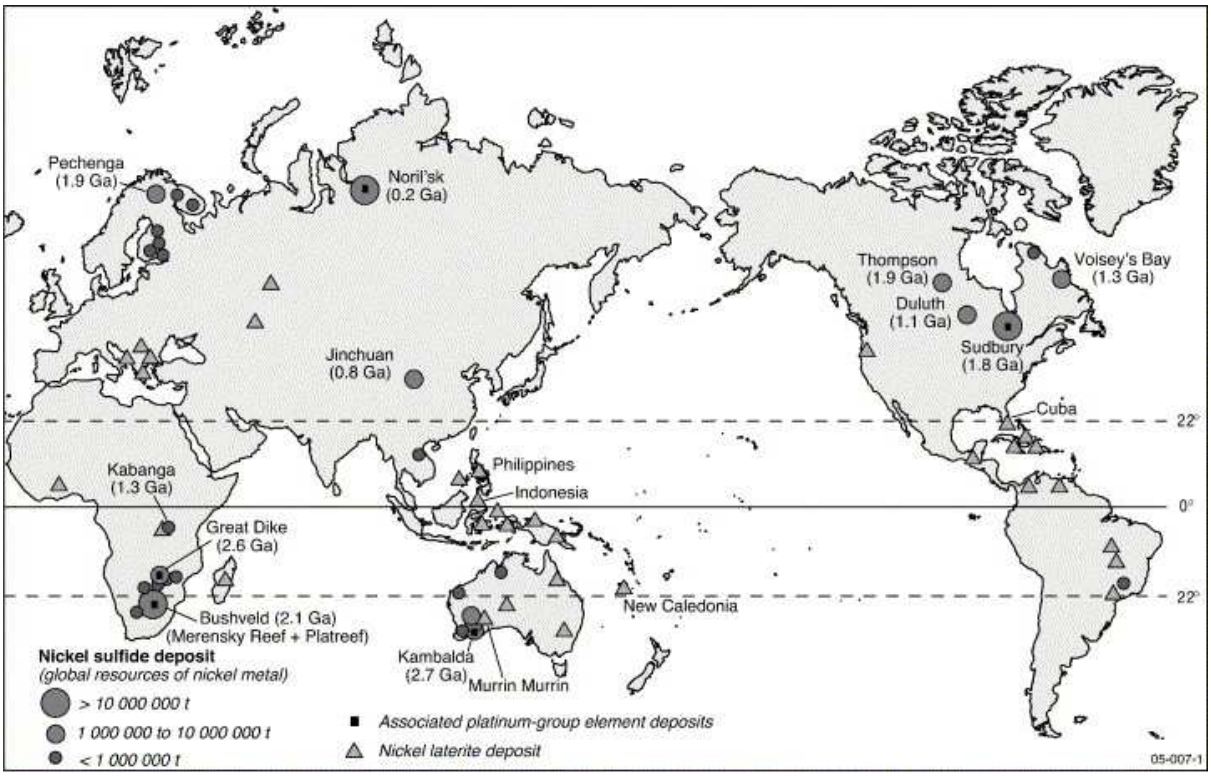


Figure 2. Largest nickel deposits in the world by Hoatson et al. 2006.

1.1.1. Global nickel mining companies

The global nickel marked is led by a number of major producers (Table 1) followed by a range of moderate to minor producers. Russia is the largest nickel-producing nation in the world, followed by Philippines and Indonesia. In 2011, Russia produced 280 000 t of nickel. Philippines and Indonesia both around 230 000 t, Canada 200 000 t and Australia 180 000 t. (Anonymous 2013).

Table 1. The world's ten largest refined nickel producers in 2011. (Derived from anonymous 2014b).

Operation	Produced Ni in 2011 (Mt)	Largest mines
Norilsk Nickel Ltd	286	Polar division and Kola MMC* in Russia (Cu, Ni)
Vale SA	206	Creighton mine in Canada (Ni)
Jinchuan Group Ltd	127	Jin Chuan mines in China (Ni)
Xstrata	106	Nickel Rim South mine (Ni, Cu, Pt)
BHP Billiton	83	Nickel West in Australia (Ni)
Sumimoto Metal Mining Co	65	Hishikari in Japan (Au)
Eramet SA	54	Moanda Mine in Congo (Mn)
Anglo American Plc	48	Minas-Rio in Brazil (Fe)

*the Kola Mining and Metallurgical Company

In Russia, nickel production is pre-dominantly by MMC Norilsk Nickel Ltd, from the Kola region of north-western Russia and the Taimyr Peninsula in north-central Russia (Siperia), with all mines extracting Ni-Cu sulphide ores. Norilsk Nickel is the world's largest producer of nickel and palladium and one of the largest of platinum and copper. Norilsk Nickel has production facilities also located in four other countries: Australia, Botswana, Finland and South Africa. In 2012, Norilsk Nickel produced

300 000 tons of nickel and 364 000 tons of copper (Norilsk Nickel 2014). In Finland, Norilsk Nickel operations include a nickel refining plant in Harjavalta.

In Canada, nickel production is dominated by two companies, Vale SA and Xstrata. Vale operates at large Ni-Cu sulphide deposits at Sudbury in Ontario and at Thompson in Manitoba. Xstrata Nickel Ltd operates also in the Sudbury district, as well as at Raglan in Quebec. All Canadian projects are based on Ni-Cu sulphide ores.

In Australia, nickel production is dominated by BHP Billiton (2014) operating three major Ni sulphide mines at Mt Keith, Leinster and Kambalda. There are some nickel laterite projects, as an example in Murrin Murrin and Cawse. (Hoatson et al. 2006).

1.2 BIOLEACHING APPLICATIONS

Biohydrometallurgy is the processing of metal containing sulphidic ores and ore concentrates using micro-organisms. Bioleaching is a process where an insoluble mineral, usually a metal sulphide, is oxidized into a soluble form in water in a process catalysed by microorganisms. There are several applications where bioleaching is used. Copper and other base metals are recovered from sulphides such as covellite (CuS), chalcocite (Cu_2S), bornite (Cu_5FeS_4), chalcopyrite ($CuFeS_2$), pentlandite ($(Ni,Fe)_9S_8$, millerite (NiS), sphalerite (ZnS) and galena (PbS). These occur usually with pyrrhotite ($Fe_{n-1}S_n$) and pyrite (FeS). In the case of refractory gold ores microorganism are used to oxidize sulphide minerals, which encapsulate gold particles. By dissolving these sulphide minerals, the gold particles can be recovered by further treatments. In gold recovery, the term biooxidation is usually used. (Brandl 2001, Ehrlich 2001).

1.2.1 History of bioleaching

Microorganisms have participated in the oxidation of sulphidic ores in the earth's crust since geologically ancient times. In 1800^{th} century the copper mine in Rio Tinto in Spain was the first large-scale operation where microorganism played a major role (Brierley 1982). At that time there was no understanding of the leaching process or the role of microorganisms. In 1947 bacteria belonging to the genus *Thiobacillus* (later *Acidithiobacillus*) were described in acid mine waters by Colmer and Hinkle (1947), and their role in metal oxidation was demonstrated. (For the reviews, see Brierley and Brierley 2001, Morin et al. 2006, Mousavi et al. 2006).

In the period of 1950-1980 bioleaching was used for the recovery of copper and other metals from waste material and low-grade ores in dumps (Gentina and Acevedo 1985). In the beginning, the dumps were piles of different sizes of minerals and no effort was made to optimize metal dissolution or activate microorganisms. While understanding of bioleaching evolved and metal consumption increased, hydrometallurgical processes developed. The first international biohydrometallurgy meeting was held in 1977 in Braunschweig, Germany. Minera Pudahuel (Lo Aquirre) copper mine in Chile is usually considered as the first heap bioleaching plant. Pudahuel's mine was started in 1980 and closed in 1996 due to ore depletion. First commercial refractory gold biooxidation plant started in 1986 in Fairview in South Africa. (Brierley and Brierley 2001, Morin et al. 2006, Mousavi et al. 2006).

In 1995, work with bioleaching of chalcopyrite concentrate developed and evaluated on commercial scale at the Chuquicamata Mine in Chile, with a design production rate of 20 000 t copper per annum. (Batty and Rorke 2006).

Today, many industrial-scale hydrometallurgical processes are used for recovery of gold and copper. Table 2 lists examples of commercial heap and dump bioleaching plants, all recovering copper. 20 % of the annual demand of copper production is produced via hydrometallurgical processes (Watling 2006). The applications continue to develop further and expand for lower grade nickel, zinc and cobalt ores. Future mining applications will be directed more on lower-grade, lower-quality and complex ores. Developing countries share over 50 % of the world copper production (Pradhan et al. 2008) and their role continues to increase with the diminishinging ore resources. (Dew and Miller 1997, Rawlings 2002, Watling 2008).

Table 2. Examples of commercial, copper recovering heap and dump bioleaching plants.

Plant	**Country**	**Ore processed (t /day)**	**Years in operation**	**References**
Cerro Verde	Peru	32000	1977	[1], [2]
Lo Aquirre	Chile	16000	1980-1996	[1], [2]
Lince II	Chile	3000	1991	[1], [2]
Mt Leyshon	Australia	1300	1992-1997	[1], [2]
Cerro Colorado	Chile	16000	1993	[1], [2]
Ivan Zar	Chile	1500	1994	[1], [2]
Quebrada Blanca	Chile	17300	1994	[1], [2]
Punta del Cobre	Chile	3300	1994	[1], [2], [3]
Andacollo Cobre	Chile	15000	1996	[1], [2]
Dos Amigos	Chile	3000	1996	[1], [2]
Phoenix deposit	Cyprus	3000	1996	[1], [3]
Zaldivar	Chile	20000	1998	[1], [2]
Lomas Bayas	Chile	36000	1998	[1], [2]
Nifty Copper	Australia	5000	1998	[1], [2]
Escondida	Chile	110000	2006	[1], [3]
Monywa	Myanmar	18000	1998	[1], [2]
Toquepala & Cuajone	Peru	128500		[1], [3]
Morenci	Arizona	75000	2001	[1], [2]
Girilambone	Australia	2000	1993-2003	[1], [2]
Zijinshan Copper Mine	China	8400	2008	[3], [4]
Whim Creek and Mons Cupri	Australia	1700	2006	[1], [2]
Lisbon Valley	USA, Utah	18300	2006	[2],[3]
Jinchuan Copper	China		2006	[2]
Spence	Chile	50000	2007	[2],[3]
Talvivaara	Finland	72000	2008	[3]

*Talvivaara heap bioleaching plant in Finland produces nickel, zinc, copper and cobalt.

[1] = Watling 2006
[2] = Brierley 2008
[3] = Neale et al. 2011
[4] = Xing-yu et al. 2008

1.3 MICROBICALLY CATALYSED METAL SOLUBILISATION

Metals are leached from sulphide minerals by the attack of ferric iron (Fe^{3+}) and hydrogen ion (H^+) on the metal sulphide bond. Fe^{3+} and H^+ are formed as a consequence of microbial oxidation. The mechanism includes the following reactions (Sand et al. 1995, Rawlings et al. 2003, Daoud and Karamanev 2005):

$MS + 2\ Fe^{3+} \rightarrow M^{2+} + S^0 + 2\ Fe^{2+}$	(1)	(chemically catalyzed reaction)
$S^0 + 1.5\ O_2 + H_2O \rightarrow SO_4^{2-} + 2\ H^+$	(2)	(bacterially catalyzed reaction)
$4\ Fe^{2+} + O_2 + 4\ H^+ \rightarrow 4\ Fe^{3+} + 2\ H_2O$	(3)	(bacterially catalyzed reaction)
$MS + 2\ O_2 \rightarrow M^{2+} + SO_4^{2-}$	(4)	(chemically/ bacterially catalyzed reaction)

M is metal.

The electron acceptors (ferric iron and oxygen) and the activity of hydrogen ions are the driving forces of metal solubilisation. The leach kinetics depend on pH (the activity of H^+) and the oxidation potential (measured as redox). Ferric iron is a strong oxidizing agent and redox potential is used to tell the ratio of ferric to ferrous ions in the solution. Because microorganisms are efficient at oxidizing ferrous iron to ferric state, the bioleaching conditions typically have a high redox potential (> 500 mV). Usual level with chemical oxidation is around 200 - 400 mV. Thus lowering the pH increases the redox potential via the increasing amount on soluble ferric iron in the solution and that increases the metal solubilisation. (Ahonen and Tuovinen 1995).

Table 3 presents sulphide mineral leaching reactions, their elemental composition and end products. Reactions represented are highly simplified and no intermediates or reaction mechanisms are presented. (For the reviews, see Watling 2008 and Schippers and Sand 1999). Bioleaching mechanisms (indirect or direct) and oxidation pathways are discussed by Rohwerder and Sand (2007) and by Rohwerder et al. (2003).

Table 3. Most common bioleaching reactions. Reactions are highly simplified and no intermediates or reaction mechanisms are presented. Reactions happen in aquaphase in the present of microorganisms and air.

Mineral	Reactants	Products	Reference
Pentlandite	$(Ni,Fe)_9S_8$	$Ni^{2+} + Fe^{2+} + S$	Watling 2008.
Millerite	$NiS + H_2SO_4$	$Ni^{2+} + SO_4^{2-} + H_2S$	Watling 2008.
	$NiS + Fe^{3+}$	$Ni^{2+} + Fe^{2+} + S$	Watling 2008.
Violarite	$FeNi_2S_4$	$Ni^{2+} + Fe^{2+} + S$	Watling 2008.
Pyrrhotite	$Fe_{1-x}S + H_2SO_4$	$Fe^{2+} + SO_4^{2-} + H_2S$	Schippers and Sand 1999.
Pyrite	$FeS_2 + Fe^{3+} + H_2O$	$Fe^{2+} + S_2O_3^{2-} + H^+$	Schippers and Sand 1999.
Sphalerite	$ZnS + Fe^{3+}$	$Zn^{2+} + Fe^{2+} + S$	Fowler and Grundwell 1998.
Chalcopyrite	$CuFeS_2 + Fe^{3+}$	$Cu^{2+} + Fe^{2+} + S$	Erlich 1997.
Chalcocite	$Cu_2S + Fe^{3+}$	$Cu^{2+} + Fe^{2+} + S$	Erlich 1997.
	$Cu_2S + O_2 + H^+$	$CuS + H^+ + Cu^{2+} + H_2O$	Erlich 1997.
Sulphur reactions	$S + H_2O + O_2$	$SO_4^{2-} + H^+$	Watling 2008.
	$H_2S + O_2$	$S + H_2O$	Watling 2008.

As in the case of pyrite and pyrrhotite the reaction products are ferrous iron and reduced inorganic sulphur compounds (RISCs). Microorganisms then reoxidize the ferrous iron back to ferric state. RISCs are metabolized by sulphur-oxidizers. Oxidation of RISCs yields more energy than oxidation of ferrous iron. Therefore, microorganisms that are able to oxidize iron and sulphuric compounds may be perceived to dominate on bioleaching environments, unless there are other factors, such as temperature or pH, with overriding effects. (Sand et al. 1995, Rawlings 2002). Sulphur oxidation is the most

significant acid-generating process in bioleaching (Wakeman et al. 2008). By transforming sulphur compounds to sulphuric acid they reduce accumulation of elemental sulphur. (Ahonen and Tuovinen 1992, Crundwell, 2003, Rohwerder et al. 2003). Combination of iron- and sulphur- oxidizing microbes has been considered effective in bioleaching processes. (Rawlings 2005).

Pyrrhotite and pyrite comprise over 80% of the complex sulphide ore deposits and therefore play a significant role in bioleaching. Pyrrhotite and pyrite oxidation delivers ferric ions into the leaching solution. However, high ferric ion concentrations may be inhibitory, especially to *A. ferrooxidans*. This changes the microbial population when leaching progresses. Precipitated iron products can cover the ore surfaces and thus decrease leaching rates. (Watling 2008). Iron control strategies are therefore needed in downstream processing. (Nurmi et al. 2010). Pyrrhotite oxidation generates significant amounts of heat. Metal dissolution rates are usually increased at higher temperatures, unless, there are no active thermophilic microorganisms. (Watling 2008).

1.4 TYPICAL GANGUE MINERALS

The mineralogical composition of the is of the primary importance. Physical and chemical properties of the ore impact the technique to be chosen and the metal recovery rate. The development of bioleaching consortia is affected on the ore mineralogy. The type and composition affect the need of acid and pH. (Watling 2006). Many gangue materials e.g. carbonates and some silicates (e.g. mica, feldspar) are acid-consuming (Table 4, Rawlings et al. 2003, Strömberg and Banwart 1999). The term 'gangue' is used to describe the valueless minerals in an ore deposit. The leaching rate of silicate ores is lower than that of carbonate ores (Ahonen and Tuovinen 1995). The dissolution of silicates results in gelling of the leach liquor. Ganque minerals can also form alteration (weathering) products. (Rimstidt 1997, Rawlings et al. 2003, Strömberg and Banwart 1999). In aluminosilicates ruptures of Si-O and Al-O bonds are common. (Ehrlich 2001, Pietrobon et al. 1997). Silicate minerals are subject to microbially enhanced solubilisation mediated by ferric iron and sulphuric acid. (Puhakka and Tuovinen, 1986a). The total dissolution of minerals generally consumes more acid than weathering and the acid demand is difficult to predict. (Jansen and Taylor 2014). Dissolution of gangue minerals may also release metals that are toxic to bioleaching (Paper IV, reviewed by du Plessis et al. 2007).

Table 4. Examples of acid consuming gangue reactions. Adapted from Jansen and Taylor (2014). (Reactions are not balanced).

	Start products	**End products**
Silicate breakdown		
K feldspar	$KAlSi_3O_8 + H^+$	$K^+ + Al^{3+} + H_4SiO_4$
Na feldspar	$NaAlSi_3O_8 + H^+$	$Na^+ + Al^{3+} + H_4SiO_4$
Ca plagioclase	$CaAlSi_3O_8 + H^+$	$Ca^{2+} + Al^{3+} + H_4SiO_4$
Carbonate minerals		
Calcite	$CaCO_3 + H^+$	$Ca^{2+} + H_2O + CO_2$
Alteration		
Plagioclase	$H_4SiO_4\ CaAlSi_3O_8 + H_4SiO_4$	$CaSO_4 2H_2O + HAl(Si_3O)_2 + H_2O$
Biotite	$(H,K)_2(Mg,Fe)_2Al_2(SiO4)_3 + H^+ + O_2 + Fe^{3+}\ SO_4^{2-}$	$Mg_2Al_2Si_3O_{10}(OH)_2 + KFe_3(SO_4)_2(OH)_6 + SiO_2$
Limonite breakdown		
Hematite	$Fe_2O_3 + H^+$	$Fe^{3+} + H_2O$
Goethite	$FeO(OH) + H^+$	$Fe^{3+} + H_2O$

1.5 THE pH CONTROL

In the beginning of bioleaching, acid consumption is often high since initial acid consuming reactions occur. Highly reactive gangue minerals (e.g. carbonates) react in short time according to Table 4. In the beginning, agglomeration of the ore with sulphuric acid reduces pH changes and the occurrence of pH gradients. (Rawlings et al. 2003, Strömberg and Banwart 1999).

In spite of breakdown and alteration reactions, formation of solid products is common in leaching environments. Table 5 presents examples of precipitation reactions. Jarosite [$MFe_3(OH)_6(SO_4)_2$] and gypsum ($CaSO_4 \cdot 2H_2O$) precipitations are common. Elemental sulphur can also accumulate and form a rim around the ore particles (Ahonen and Tuovinen 1992, Watling et al. 2006). Precipitates affect metal recovery rates by forming diffusion barriers on mineral surfaces. Precipitates accumulate also in pipelines, pumps and valves making maintenance and repair challenging. Precipitation is pH and temperature dependent. (Ahonen and Tuovinen 1995, Bhatti et al. 2012a and b).

Jarosite is formed in conditions where pH is less than 3 and above 1.5 in temperature range between 4 and 35 ºC (Baron and Palmer 1996, Das et al. 1996). The concentration of ferric iron and sulphate (e.g. continuous addition of acid to the heaps) affects the amount of precipitated jarosite (Ahonen and Tuovinen 1995, Bhatti et al. 2012b). Monovalent alkali cation is also needed. (Watling 2006). The dissolution of silicates leads to a constant supply of cations and increase jarosite precipitations. If solution pH increases, jarosite will precipitate releasing H^+ (lowering pH), but consuming Fe^{3+} (lowering Eh). Jarosite formation reaction is presented in Table 5. The formation of jarosite can be also seen as a proof of microbial activity and mineral dissolution. (Jansen and Taylor 2014, Bhatti et al. 2012 a and b, Tuovinen and Bhatti 1999, Baron and Palmer 1996). When pH rises, jarosite is transformed to various FeO(OH) compounds, including goethite or hematite, depending on temperature. (For a review see Das et al. 1996). In most ores, acid consuming silicate minerals result in Eh-pH conditions within the jarosite field. (Nazari et al. 2014).

Formation of amorphous, gelatinous silicate precipitates may cause solution flow barriers on the surface of minerals that hinder the dissolution of metals, increase the viscosity of the leach liquor, lower the gas transfer rates and inhibit the leach liquor percolation through the heap. Dissolution of silicate minerals increases acid consumption (Brierley 2001, Rawlings et al. 2003, Strömberg and Banwart 1999, Bhatti 2012b). In this study, the dissolution of silicate minerals was evident at pH 1.5. This increased the viscosity of PLS and gelatinous precipitates were formed. In extreme cases, this can clog the flow of solution. (Rawlings et al. 2003, Strömberg and Banwart 1999, Bhatti 2012b).

Table 5. Examples of most common precipitation reactions. Adapted from Jansen and Taylor (2014). (Reactions are not balanced).

Start products	End products	
$Fe^{3+} + M^+ + SO_4^{2-} + H_2O$	$MFe_3(SO_4)_2(OH)_6 + H^+$	Jarosite
H_4SiO_4	$SiO_2 + H_2O$	Silica
$Ca^{2+} + SO_4^{2-} + H_2O$	$CaSO_4 2H_2O$	Gypsum

Where M = K^+, Na^+, NH^{4+}, Ag^+, Pb^+, or H_3O^+

Changes in mineralogical composition of the multimetal black schist ore from Talvivaara mine site were monitored in the study of Bhatti et al. (2010). The transformation of main mica mineral, phlogopite [$KMg_3(AlSi_3O_{10})(F,OH)_2$] to vermiculite ($Mg^{2+}$, Fe^{2+}, Fe^{3+})$_3$[$(AlSi)_4O_{10}$]$(OH)_2 4H_2O$ was complete. That alteration was mainly dependent of the pH of the leach solution and the formation of K-jarosite. Table 6 summarizes gangue silicate leaching reactions arranged by reaction type. Reaction products are classified as precipitation, alteration or solution product.

Table 6. Silicate mineral leaching reactions. Adapted from Jansen and Taylor (2014). (Reactions are not balanced).

Reaction type	Feed mineral	Solution reactants	Precipitation products	Alteration product	Solution products
Breakdown	K/ Na feldspar	$H^+ + SO_4^{2-}$			$H_4SiO_4 + Al^{3+} + Na/K^+$
	Ca plagioclase	$H^+ + SO_4^{2-}$			$H_4SiO_4 + Al^{3+} + Ca^{2+}$
	Biotite	$H^+ + SO_4^{2-}$			$H_4SiO_4 + Al^{3+} + Ca^{2+} + Fe^{2+} + Mg^{2+}$
Precipitation		$SO_4^{2-} + K^+ + Fe^{3+}$	Jarosite		$H^+ + SO_4^{2-}$
		H_4SiO_4	Silica		
		$SO_4^{2-} + K^+ + Al^{3+}$	Alunite		$H^+ + SO_4^{2-}$
		$SO_4^{2-} + Ca^{2+}$	Gypsum		
		$H_4SiO_4 + Al^{3+}$	Pyrophyllite/ Kaoline		$H^+ + SO_4^{2-}$
		$H_4SiO_4 + Al^{3+} + Mg^{2+}$	Vermicullite		
Alteration	Plagioclase	$H^+ + SO_4^{2-}$	Gypsum	Pyrophyllite	$H^+ + SO_4^{2-}$
	Biotite	$H^+ + SO_4^{2-} + Fe^{3+}$	Jarosite, silica	Vermiculite	

Due to a concurrent dissolution, precipitation, oxidation and reduction reactions the net acid consumption or acid production prevails in bioleaching systems (Ahonen and Tuovinen 1995). It is difficult to maintain the solution pH within the desired range without constant pH adjustment (Rawlings et al. 2003). Sulphuric acid consumption can, therefore, become a major operating cost (Watling 2006).

1.6 TECHNIQUES USED IN BIOLEACHING

To bioleach valuable metals, different engineering approaches have been developed, including dump, heap, reactor and *in situ* bioleaching techniques. The process selection depends on the grade of the ore. (For the reviews see, Bosecker 1997, Brandl 2001, Rawlings 2002, Johnson 2008 and Watling 2008).

1.6.1 *In situ* leaching

In situ leaching (ISL) has been used in sites where the ore body is inaccessible or rather poor. ISL is used in production of uranium and copper. (Rawlings 2004). The ore is not brought to the surface in this process. The visual environmental impact is lower than in open pit infrastructures. Leaching solution is injected through an array of wells into the mineral deposit. The liquor gravitates through the ore and is collected in a centrally placed well where it is pumped to the surface (Figure 3) (Sand et al. 1993). The ore body can be artificically fractured, for example, by blasting, to improve the permeability before leaching (Wadden and Gallant 1985). Extensive knowledge of the hydrology and geology of the area is needed so that the process solutions do not migrate away from the mining area. (Kinnunen and Puhakka 2004, Nurmi et al. 2009). In many trial projects the use of ISL has been considered problematic or mining has not been approved by authorities. (Wadden and Gallant 1985, Tuovinen and Bhatti 2001, Mudd 2001a). Table 7 lists commercial uranium and copper mines.

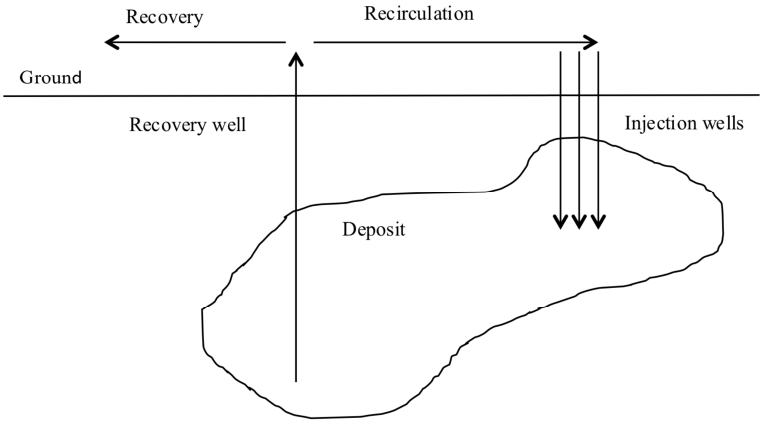


Figure 3. Schematic diagram of *In situ* leaching.

Uranium

According to World Nuclear News (2014) 45 % of uranium is produced by ISL and the production is increasing together with diminishing high grade ore resources. Many ISL uranium mines have operated in the area of former Soviet Union and Eastern Europe. Kazakhstan became the world's leading uranium producer in 2009, with close to 28 % of world production and 38 % in 2013. (Mudd 2001b, World Nuclear Association 2014). In Kazakhstan the ISL technology displaced the conventional techniques in uranium production by 1990s. (Tuovinen and Bhatti 2001). There are several operating uranium mines (Table 7). Kazatomprom, owned by Cameco (2014) is one of the newest operation in Kazakhstan.

Copper

Beside the uranium, ISL mining has been applied succesfully to recover copper, especially in the area of the Arizona copper belt in the US. The Miami unit of Pinto Valley is an open cut copper and gold mine. The Gunnison copper ISL project is located at the same area and is predicted to produce 50 000 t of copper for the first 14 years. (Excelsiormining Corporate 2014).

Table 7. Commercial uranium and copper ISL mines.

Country	**Mine**	**Production (t/a)**	**Product**	**Reference www-page**
Australia	Beverley	453	U_3O_8	world-nuclear.org
Australia	Honeymoon Na	124	U_3O_8	world-nuclear.org
Kazakhstan	JV Inkai Mine	2 000	U_3O_8	cameco.com/mining/inkai/jv_inkai
USA	Mc Arthur River	8 200	U_3O_8	cameco.com/mining/mcarthur_river
USA	Smith Ranch-Highland	772	U_3O_8	cameco.com/mining/highland_smith
USA	Crow Butte	320	U_3O_8	cameco.com/mining/crow_butte
USA	Pinto Valley and Miami Unit	26 800	Cu	capstonemining.com/s/pinto.asp?ReportID=606362
USA	Pinto Valley and Miami Unit	49 kg	Au	bhpbilliton.com
USA	Florenze	starting	Cu	florencecopper.com/s/Home.asp

Gold

In situ bioleahing applications for low grade and refractory gold ores have been evaluated by The Commonwealth Scientific and Industrial Research Organisation (CSIRO). ISL is a potential

pretreatment method to remove pyrite and elemental sulphur before syanidisation. It is expected to enhance gold leaching with chemical lixiviants and to decrease the lixiviant consumption. (Kaksonen 2014a and b).

1.6.2 Reactor-based techniques

The higher value minerals or ore concentrates may be bioprocessed using reactor based techniques. The times required for mineral processing to be completed are usually days and thereby shortest compared to other techniques. Stirred tank reactors are used commonly in practice compared to other configurations such as bubbling columns, airlift columns, percolation columns, Pachuca tanks and rotary reactors. (Rossi 2001). Usually in commercial bioleach plants finely milled mineral concentrate or ore is feed as batches or continuously to two or three primary reactors in parallel, feeding two to three secondary reactors in series. The reactors are aerated, pH- and temperature- controlled. (Atkins et al. 1986, Dew et al. 1997, Rossi 2001, Acevedo and Gentina 2007).

The first commercial bioleching application was the BIOX® process owned by Gencor at that time. Development of the process started in the late 1970s at Gencor Process Research, in Johannesburg, South Africa. The pilot plant was commisioned in 1986 to treat 10 t per day refractory gold-bearing sulphide concentrates with mesophilic microorganisms. Gencor operated the first demonstration plant in Faiview mine, then at Ashanti Shansu and nowadays there are more plants e.g. in Australia and in South America. A typical BIOX® plant operates at 40-45 ºC about an 18 % w v^{-1} solids concentration with a total solids retention time of around 4 days. (van Aswegen et al. 2007).

An alternative to the BIOX® process is the process developed by BacTech. The BacTech and BIOX® processes use similar highly aerated stirred-tanks. The major difference is that the BacTech process is operated at close to 50 ºC with moderately thermophilic bacteria and thus, less cooling is required. One disadvantage is that the solubility of oxygen and carbon dioxide is lower at the higher temperatures. (Neale et al. 2000).

Billiton (which bought Gencor) continued to develop bioleaching to low-grade nickel ores, and the followed process was termed the BioNIC®. The success inspired Billiton to continue to develop bioleaching process to treat copper minerals, such as chalcopyrite. The process was named BioCOP™. Mesophilic microorganisms were not effective to bioleach primary copper minerals, especially chalcopyrite. The work continued and improved copper extraction was achieved with thermophilic microorganisms. The development of the BioCOP™ Process continued from pilot-scale to full-scale commercial demonstration at the Chuquicamata Mine in Chile, with a design production rate of 20 000 t copper per annum. BioCOP™ process has six equal size continuos fed reactors. The first three reactors are in parallel and last three reactors in a series. 50-70 % of the metal dissolution occurs in the primary reactors. Each of the reactors is aerated and agitated. High rate of iron oxidation is achieved with the resulting redox potential of 700 mV. Limestone is used to maintain the pH and to provide carbon dioxide for bacterial growth. SX-EW process (described in Chapter 1.6.6) is used to recover metal from pregnant leaching solution (PLS). Typical BioCOP™ process values are given in Table 8. (Clark et al. 2006).

Table 8. Typical BioCOP™ thermophilic tank bioleaching operating conditions. Adapted from du Plessis et al. (2007).

Operating condition	Value
Temperature (ºC)	78
Pulp density of concentrate feed % (w w^{-1})	12
Primary reactor residence time (h)	48
Overall residence time (h)	96
pH	1.5
DO (mg l^{-1})	1-4
Microbial cell concentration in solution (cells ml^{-1})	10^9
CO_2 supplementation (% of total gas flow)	1
Copper recovery (%)	> 98
Microbial population	*Sulfolobus* spp., *Metallospaera* spp., *Acidianus* spp.
Redox potential (mV Ag^0/AgCl)	700

Biological extraction of cobalt from pyritic concentrates was commercialized in 1999 by the Bureau de Recherches Géologiques et Minières (BRGM) at Kasese Project at the Kilembe mine in Uganda. A 1.1 million ton stockpile of pyrite concentrate contains 1.38 % cobalt. Continuously operated stirred-tank recovers about 92 % of that cobalt. Mesophilic iron-oxidizing bacteria in this process grow optimally at 37 ºC. (Rawlings 2002).

Today several reactor-based technologies are commercialized and patented for base metal recovery and for biooxidation of refractory gold ores. (Pradhan et al. 2008, Watling 2008). Figure 4 presents bioleachingreactors in series. Reactor waste products are usually neutralized e.g. with limestone before final disposal (Johnson 2003).

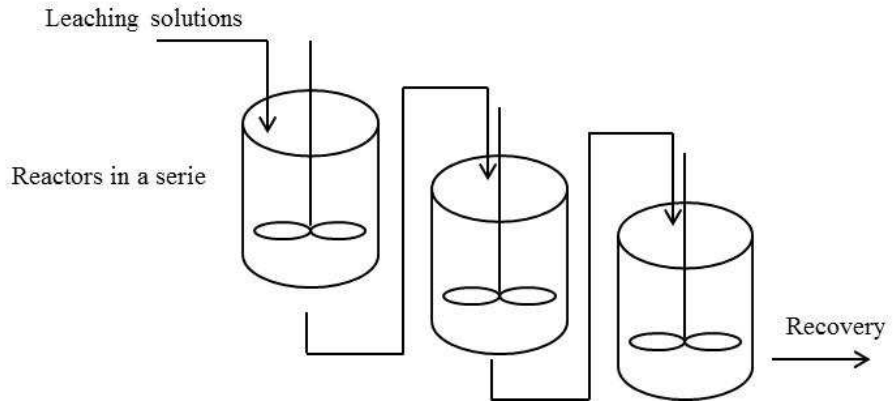


Figure 4. Schematic diagram presenting bioleaching reactors in a series.

1.6.3 Design parameters

The kinetic and engineering fundamentals of the design of reactors for bioleaching have been addressed by several authors (Gormely and Brannion 1989, Harvey et al. 1999, Acevedo 2000, Rossi 2001, Acevedo and Gentina 2007). The key design factors and their typical values are presented in Table 9.

Table 9. Important design factors of bioleaching reactors and their typical values.

Design parameter	Typical value	Ref.
Hydraulic retention time (HRT)	11-17 h	Crundwell 2001
Pulp density	< 20 %	Dew et al. 1995
Particle size	< 75 µm	Ahonen and Tuovinen 1995
Dissolved oxygen (DO)	1.5-4.1 mg l^{-1}	Kock et al. 2004
pH	1.5-2.5	Nemati et al. 1998
Carbon dioxide (CO_2)	5-8 %	Niemelä et al. 1994, Mason and Rice 2002
Possible nutrients	N, P, K	Dew et al. 1997
Temperature control	Depends on the process	Plumb et al. 2008

Bioleaching environments are highly acidic and corrosive. Materials of construction are usually rubber-lined mild steel or stainless steel. The selection of appropriate equipment affect the costs of care and maintenance. Precipitates accumulate in pipelines, valves and pumps. Availability of skilled human resources, energy and water costs, delivery of reactants and spare parts must be considered.

1.6.4 Dump leaching

Dump leaching has been typically used to leach copper from low-grade or run-of-mine material (0.1-0.5 % Cu) with minimal ore preparation. The dump is irrigated from the top with acidified water, the leach liquor. Leach liquor then percolates through the dump and pregnant leaching solution (PLS) is collected from the bottom and recycled again to the dump. When the desired metal concentration is achieved, PLS is collected and replaced or small side flow is taken continuously for metal recovery e.g. to a solvent extraction - electrowinning (SX-EW) process (described in chapter 1.6.6). Raffinate from the circuit is usually recycled to the top of the dump (Brierley 2001). Dump leaching is relatively inefficient because of large particle sizes whilst small particles block solution flows and impede aeration. (Watling 2006). In 2006, BHP Billiton started the largest dumpleaching operation in the world in Escondida Mine in Chile. It is expected to produce 180 000 - 200 000 t of copper per year over the 40 years. (Gentina and Acevedo 2013).

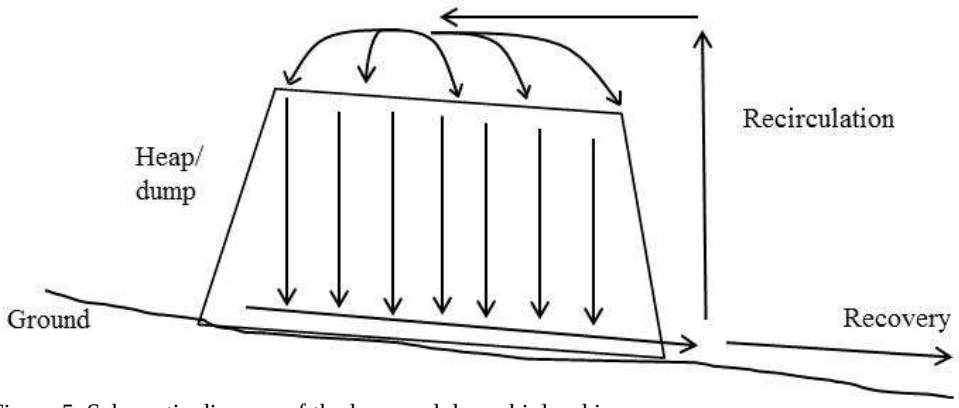


Figure 5. Schematic diagram of the heap and dump bioleaching.

1.6.5 Heap bioleaching

Heap bioleaching principle is rather similar to dump leaching. Heaps are better designed than dumps and efforts are made to enhance leaching rates. The process has been optimized successfully during the last thirty years. (Brierley and Brierley 2001). Heap leaching offers a number of advantages as compared to dumpleaching, including rather low investment and operation cost with reasonable yields over a period of recirculation. Metal recovery times are usually months rather than years. Suitable ore particle size, access of oxygen and geometry are engineered particularly for the processed mineral and are often based on laboratory or pilot-scale tests. However, even the most carefully engineered, bioheaps always form gradients of temperature, pH, DO and irrigation. Typical heap design parameters are presented in Table 10. The variability of microorganisms is much greater in heaps than in strirred tanks. Heaps can be inoculated, but in general, bioleaching micro-organisms grow naturally in heap environments. Inoculation can be done by including the microorganisms into the agglomeration solution. (Watling 2006, Gentina and Acevedo 2013, Strömberg and Banwart 1999, Riekkola-Vanhanen et al. 2001, this study).

Agglomeration

Ore particles are agglomerated in rotating drums with acidified water and piled to 6-10 m high heaps, called pads, on an impermeable ground e.g. high-density polyethylene. Agglomeration of the ore attaches the fine particles to the surfaces of the larger particles. This improves the permeability of the heap, minimises the channelling and reduces acid consumption in the beginning thus, providing better conditions for microorganisms (Acevedo et al. 1993). Microorganisms are distributed evenly, which may speed-up the start of the bioleaching operation. (Walsh et al. 1997).

Irrigation

The heap is irrigated with the leach liquor from the top of the heap. PLS is collected from the bottom and recycled again to the top of the heap. Leach liquor pH can be adjusted before irrigation. Irrigation can be continuos or discontinuous. Discontinuos irrigation is considered more effective. (Lizama et al. 2005). During irrigation, the capillary forces draw the liquid inside the ore particles. Porosity of the ore allows the leaching solution to penetrate more in to the ore. When irrigation stops, the liquid drains out from the capillary and remains on the surfaces. New irrigation carries it with the dissolved metals and the process begins again. The ionic diffusion though a state capillary full of fluid is considerably slower. The frequency of irrigation cycle is determined by the rate of evaporation and the concentration of the metal in the exiting liquid phase. (Lizama et al. 2005). Typical irrigation rate is 5-10 $l\ h^{-1}m^{-2}$. The heap surface degrades or can compact easily as an effect of irrigation with sprinklers and the percolation rate can drop significantly decreasing the metal yields. Heap top can be ripped if solution forms ponds on the top of the heap. (Lizama et al. 2005, Pradhan et al. 2008).


Figure 6. Photographs of the irrigation lines from Talvivaara bioheap. Photos: Marja Riekkola-Vanhanen.

Aeration

In bioleching systems sufficient supply of O_2 and CO_2 are important. Most of the bioleaching bacteria are aerobic and chemolithotrops. Oxygen is the electron acceptor of ferrous iron oxidation. CO_2 serves as the carbon source for biomass generation. As oxygen is required for oxidative metabolism, its depletion has a rate limiting effect. Reactors are easier to aerate than heaps and dumps, in which oxygen gradients may prevail. Constant O_2 concentration through the heap can not be attained (Jensen and Webb 1995). Natural convection occurs when temperature inside the heap is greater than that of outside. As the aeration rate is increased, the heat rises upward (Dixon 2000). Air is also dissolved to the leach liquor. In large heaps, natural convection does not provide enough oxygen deep within the heap for the microorganisms as it is consumed before it reaches the middle parts (Leahy et al. 2006, Gentina and Acevedo 2013).

Air is blown to the process by compressors via network of pipes installed at the bottom of the heap. Distribution network may include e.g. 500 mm headers and 50 mm diameter laterals at 2 m spacing. The density of the holes is dependent of the size of the heap. (Pradhan 2008) The depletion of oxygen may produce H_2S gas catalysed by acidophilic sulphate reducing bacteria (SRB) or pyrrhotite can directly dissolve under acidic conditions forming H_2S and Fe^{2+} (Sen and Johnson 1999, Gunsinger et al. 2006). Sulphate reducing bacteria grows usually in neutral pH, but some acidophilic SRBs have been detected (Sen and Johnson 1999). H_2S may react with metals and form insoluble sulphates (Dvorak et al. 1992).

Table 10. Typical heap design parameters. (du Plessis et al. 2007, Brierley 2001).

Parameter	Typical value
Height (m)	4-10
Leaching perioid (d)	300-450
Air-flow rate (N m^3 t^{-1} h^{-1})	0.02-0.08
Irrigation (L m^2 h^{-1})	4-18

Temperature effects on bioleaching

In heaps and dumps temperature depends on the climatic conditions, ore chemistry and process design. Temperatures are affected by the composition and concentration of the sulphidic minerals due to their exothermic oxidation reactions. (Dixon 2000). The outer layers of a bioheap are affected by climatic conditions. (van Aswegen et al. 2007, Leahy et al. 2005). The relationship between the chemical and microbial reaction rates and temperature are described in Chapter 1.8.1.

Heap bioleaching at low temperatures faces several challenges. At low temperatures all physical and chemical reactions are slowed down. The relationship between the chemical reaction rate and temperature is as described by Arrhenius equation (Franzmann et al. 2005):

$$lnk = \frac{-E_a}{RT} + lnA \quad \text{, where} \qquad (5)$$

ln*k* = the natural log of the first-order rate coeffient or similar rate measure for any temperature *T* (in Kelvin)
E_a = the activation energy, R is the gas constant (8.314 J K^{-1} mol^{-1})
A = the pre-exponential factor

For example, the activation energy for mesophilic bioleaching of chalcocite is about 98 kJ mol^{-1}, so that for each 10 increase in temperature the reaction rate increases 3 times. (Franzmann et al. 2005).

The relationship between microbial growth and temperature is as described by the Ratkowsky equation (Ratkowsky et al. 1983):

$$\sqrt{\frac{1}{Time}} = b \cdot (T - T_{min}) \cdot (1 - e^{(c \cdot (T - T_{max}))}) \quad \text{, where} \qquad (6)$$

T = temperature (ºC)
B and c = fitting parameters

Potential inhibitors of bioleaching organisms

Bioleaching microbes are usually adapted to high metal concentrations. Various strains may tolerate up to 50 g L^{-1} Ni, 55 g L^{-1} Cu and 112 g L^{-1} of Zn. (Bosecker 1997). Recirculation of PLS back to the process may lead to the accumulation of high concentrations of ions. The accumulation can lead to toxic concentrations and inhibite microbial growth and activity. If the consentration is enough high, the osmotic stress can also cause plasmolysis to micro-organisms, instead of metal toxicity. (Hedrich and Johnson 2013). Nickel toxicity varies greatly between species and strains. Subculturing and adaptation enhance tolerance to higher metal concentrations. (Rawlings 2005, Watling 2008). Arsenic, uranium, chloride, nitrate and fluoride inhibit microbial growth. (Hallberg et al. 1996b and Leduc et al. 1997, Dopson et al. 2003). In Paper IV fluoride (F^-) was released from the chalcopyrite in the concentrations of 15.2 and 5.8 mM and that inhibited microbial activity. Table 11 lists product and ferrous iron concentrations reported to inhibite *A. ferrooxidans* and *L. ferriphilum*. In the study of Nurmi et al. (2009) with *L. ferriphilum* Fe^{2+} oxidation proceed at the tested maximum Fe^{2+} 20 g L^{-1} and Fe^{3+} 60 g L^{-1} concentrations, althought Fe^{2+} oxidation rate decreased at above Fe^{2+} 4 g L^{-1} and Fe^{3+} 5 g L^{-1} indicating substrate and product inhibition. With *A. ferrooxidans* concentration of 20 g L^{-1} of ferrous iron has been found to completely inhibit the oxidation of ferrous iron (Barron and Lueking 1990).

Table 11. Product and ferrousiron inhibition concentrations reported to *A. ferrooxidans* and *L. ferriphilum*.

Metal	Concentration ($g\ L^{-1}$)	Strain	Reference
Ni^{2+}	31, 9.7, 60	*A. ferroxidans*	Carbera et al. 2005, Nemati et al. 1998, Dopson et al. 2003
Zn^{2+}	31	*A. ferroxidans*	Carbera et al. 2005
Cu^{2+}	11	*A. ferroxidans*	Carbera et al. 2005
Fe^{2+}	20	*A. ferroxidans*	Barron and Lueking 1990
Fe^{3+}	5-10 *	*A. ferroxidans*	Das et al. 1997
Fe^{2+}	30, >20	*L. ferriphilum*	Kinnunen and Puhakka 2005, Nurmi et al. 2009
Fe^{3+}	>20, >60	*L. ferriphilum*	Kinnunen and Puhakka 2005, Nurmi et al. 2009
Fe^{2+} + Ni^{2+}	40 + 10	*L. ferriphilum*	Nurmi et al. 2009
Fe^{2+} + Zn^{2+}	30 + 40	*L. ferriphilum*	Nurmi et al. 2009
Zn^{2+} + Ni^{2+}	60 + 10	*L. ferriphilum*	Nurmi et al. 2009
Ni^{2+}	>60	*L. ferriphilum*	Nurmi et al. 2009
Zn^{2+}	>60	*L. ferriphilum*	Nurmi et al. 2009

* partial inhibition

Most of bioleaching microorganisms are sensitive to organic material, notably *Leptospirillum* spp., and the growth can be inhibited (Johnson 1995 and 2001). Organic acids, like humic acid from the water used as PLS, cell lysates of bioleaching microorganisms and organic solvents used in downstream processing, might also lead to inhibition problems. (Mazuelos et al. 1999). Heterotrophic microorganisms do not participate in actual bioleaching reactions, but rather oxidize organic carbon and thus are part of the bioleaching ecosystem. (Johnson 1998, Frattini et al. 2000, Matlakowska and Sklodowska 2011).

Monitoring

The extent of the bioheap monitoring varies and may change with the process of bioleaching. The PLS is usually analysed for pH, redox potential, temperature and dissolved oxygen, total and ferrous iron concentrations and different metal concentrations. These analyses provide information on mineral dissolution and the activity of the iron-oxidizing bacteria. (Brierley 2001). Samples of the leached ore are analysed for residual metals. Temperatures are measured at various depths and locations troughout the bioheap. Oxygen measurements indicate whether the aeration is sufficient. (Brierley 2001).

Bacterial counts and molecular techniques together with chemical and physical tests give valuable information on the performance of heap operation. (Brierley 2001).

1.6.6 Iron removal

Iron plays a key role when valuable metals are dissolved from sulphide ores (Please see Section 1.3). Before the valuable metal recovery process, iron needs to be removed by precipitation. Being inhibitory at high concentrations (Please see Table 11) part of the iron can be removed also before recycling the effluent. Iron is commonly removed through hydroxide precipitation by adding lime or limestone to increase the pH approxmately to 3. In addition to chemicals, the pH can be increased also with the help alkaline producing microorganisms (Kaksonen and Puhakka 2007). (For the reviews, see Johnson 2003 and 2006).

Iron-oxidizing microorganisms can also be used in a separate bioreactor to oxidize ferrous iron to ferric state. That allows optimization of the conditions e.g. temperature for both stages. (Rawlings and Johnson 2007). High-rate iron oxidation has been achieved with bioreactors using immobilized iron-oxidizing microorganisms. In the study of Nurmi et al. (2010) PLS after the recovery of target metals

from Talvivaara was used in a fluidized bed reactor (FBR) for biological oxidation of ferrous iron by a *L. ferriphilum*. After oxidation iron was precipitated with or without pH adjustment and settled with gravitation in a subsequent settling tank. The pH was adjusted with $CaCO_3$ or KOH. When pH was increased to 3.5 with KOH, the maximum oxidation rate 3.7 g Fe^{2+} $L^{-1}h^{-1}$ was achieved. Without chemical addition the iron precipitate as jarosite and pH increased. When pH was increased with KOH or $CaCO_3$, the formation of goethite or gypsum was also observed.

Produced sludge must be finally disposed. Jarosite precipitation is a common iron removal method especially in zinc industry. Downside is that other metals may co-precipitate. (Cunha et al. 2008, Ismael and Carvalho 2003, Puhakka and Tuovinen 1986a). Iron can be precipitated also as goethite or hematite. Precipitated iron should have good settling properties. The end product is strongly dependent on pH. Many neutralizing materials can be used, e.g KOH or $CaCO_3$. (Nurmi et al. 2010). Hematite is the most stable form, which can be sold or stored without special precautions. The jarosite and goethite products are less stable and contain heavy metals that are easily released into the environment, resulting in the requirement of strict and costly final disposal systems. (Ismael and Carvalho 2003, Wang et al. 2007). Besides iron effluents contain high concentrations of sulphate. Also sulphate can be precipitated as a stable endproduct. Biological and chemical iron and sulphate removal systems have been examined especially for acid mine drainage (AMD). (Chapter 1.6.7.).

1.6.7 Metal recovery processes

In the beginning of hydrometallurgical solvent extraction - electrowinning process (SX-EW) metal rich solution is mixed with organic solvent (SX). Organic solvent selectively removes metals from original solution. The organic solvent is then separated and metals are stripped from it with a fresh acidic solution. That solution is led to electrowinning (EW), where metal-rich solution is filtered to remove entrained organics, heated, and passed through a series of electrolytic cells where metals are precipitated to form high quality cathodes. (Bartos 2002).

1.6.8 Environmental control

One of the major concerns arising from bioleaching operations is the potential long-term environmental impact. Bioleaching effluents can cause severe problems if they are released to the environment. Effluents are acidic and contain metals in addition to sulphate and iron. (Gray 1997). Acid mine drainage (AMD) can also form in natural conditions where sulphide minerals are exposed to oxidizing conditions (Johnson and Hallberg 2003). The regeneration of ferric iron is the key reaction that accelerates the AMD production. AMD can be prevented by excluding water or oxygen from the mineral. One way of doing this is to isolate minerals eg. by using layers of sediment (Johnson 2000, Johnson and Hallberg 2003). Another way is to change the top soil and replace it with vegetation. In practice, the AMD cannot be prevented totally and remediation applications are needed. (For a review see, Johnson and Hallberg 2005).

AMD and effluents from bioleaching operations are chemically neutralized or precipitated. The used techniques include neutralization and precipitation with alkaline material. Various neutralising reagents have been used, including lime (CaO), Ca- and Na-carbonates ($CaCO_3$, Na_2CO_3), Na- and Mg-hydroxides (NaOH, $Mg(OH)_2$). (Banks et al. 1997). Effectiveness and cost of these chemicals vary. Problem is that many of the metals co-precipitate. The result is iron-rich sludge that contains various other metals. The controlled final disposal increases the costs of the process. (For a review see, Johnson and Hallberg 2005).

Biological remediation is based to microorganisms that are able to generate alkality and thereby immobilise metals. Microbiological processes that generate alkality include denitrification, ammonification, methanogenesis, sulphate-, Fe- and Mn-reduction. Treatment systems vary from actively monitored reactor types to passive wetlands. (Figure 7). Passive treatment applications are relatively low cost and easier to maintain compared to active treatment methods. (For a review see, Johnson and Hallberg 2005).

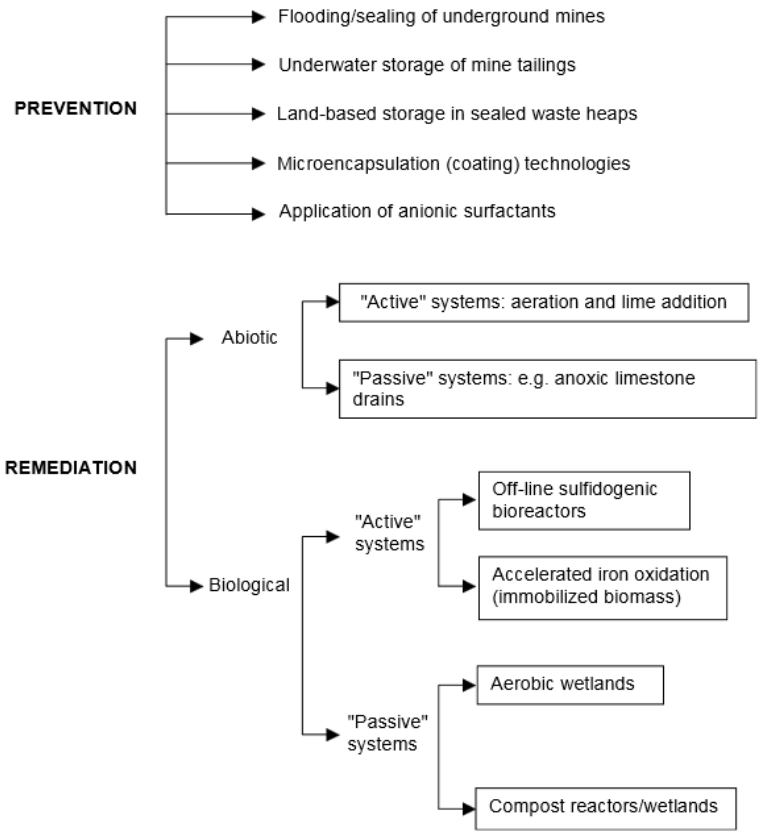


Figure 7. Options for preventing the formation and remediation applications of AMD (adapted from Johnson and Hallberg 2005).

1.7 THE COMPLEX MULTI-METAL BLACK SCHIST ORE DEPOSIT

Talvivaara complex multi-metal black schist sulphide ore deposit is located in north-eastern Finland with 1550 million ton of classified resources. The mineralogy and geochemistry of the deposit have been characterized in the literature (Airo and Loukola-Ruskeenniemi 2004, Loukola-Ruskeenniemi 1996). The Talvivaara deposits comprise two different polymetallic ore bodies hosted by a black schist, Kuusilampi and Kolmisoppi. The deposits are relatively easy to mine as an open pit. The nickel deposit has been known for decades, but it is not been used until now, because of the low nickel concentration.

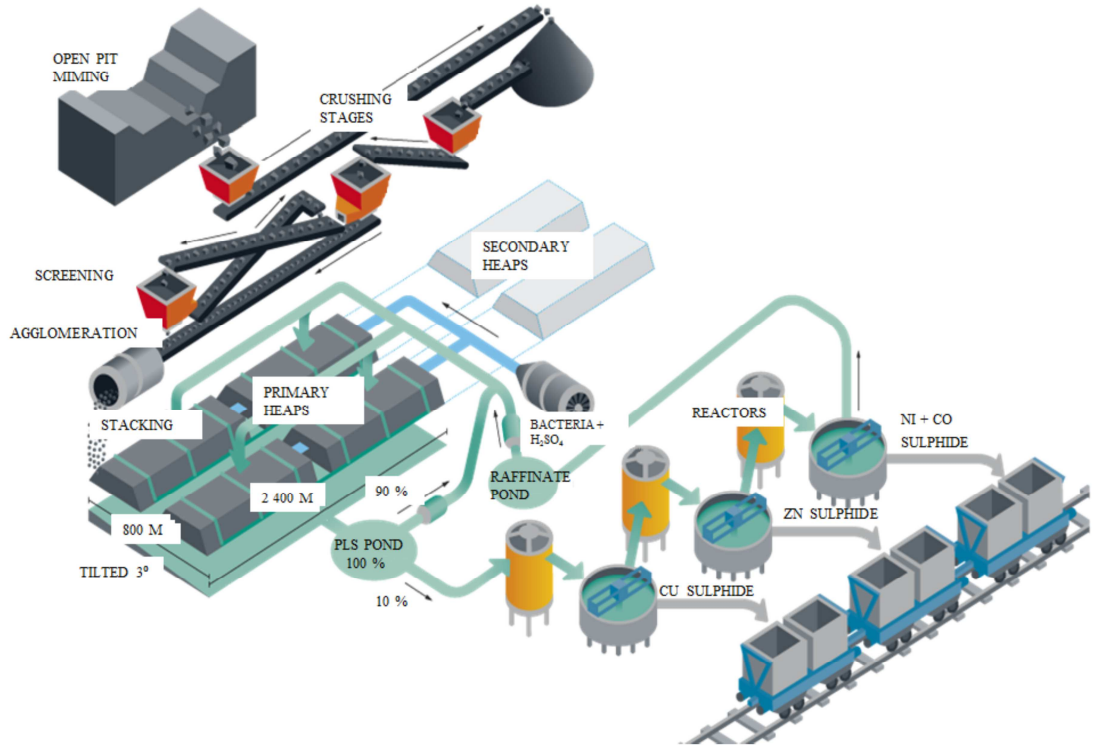
The Geological Survey of Finland carried out a detailed exploration in the Talvivaara area from 1977 to 1983 and continued the geological work in the late 1980's and early 1990's. The resource was found to be large but of very low grade. The Talvivaara deposits remained unexploited until the Talvivaara Project acquired the rights to the deposits in February 2004 and continued the geological work by focusing on sampling for processin purposes. (Riekkola-Vanhanen 2010, 2013). The mineral composition of the sulfide component of the ore was 61.2 % pyrrhotite (FeS), 24.3 % pyrite (FeS_2), 5 % pentlandite [$(Fe_x/Ni_{9-x})_9S_8$], 6.5 % alabandite (MnS) and 2.4 % chalcopyrite ($CuFeS_2$).

In the ore, pentlandite contains between 75-88 % of the contained nickel and pyrrhotite is the second most important mineral in terms of nickel content. Pyrite contains the main share (between 67-90 %) of contained cobalt while chalcopyrite is carrying copper and sphalerite zinc. The mineral resources have been classified with 0.07 % Ni cut-off at 1004 million tons, containing 0.23 % of nickel, 0.51 % of zinc, 0.13 % of copper and 0.02 % of cobalt (Riekkola-Vanhanen 2010, 2013). Si-containing minerals are anorthite ($CaAl_2Si_2O_8$), biotite ($K(Mg,Fe)_3[AlSi_3O_{10}(OH,F)_2]$), microline ($KAlSi_3O_8$), phlogopite [$KMg_3(Si_3Al)O_{10}(F,OH)_2$], plagioclase [$(Na,Ca)(Si,Al)_4O_8$] and quartz ($SiO_2$). (Bhatti et al. 2012b).

During the summer of 2005, a 17 000 t demonstration plant was constructed at the Talvivaara mine site. Results were encouracing and the building of a full-scale commercial plant was started. Commercial bioleaching process was in operation in April 2008 and first nickelsulphide product was delivered in February 2009 to Norilsk Nickel Harjavalta plant. (Riekkola-Vanhanen, personal communication 2015).

Figure 8 presents the overall process from mining to metal recovery. The process involves four crushing stages, followed by agglomeration with PLS. After 13-14 months of bioleaching on the primary pad, the leached ore is reclaimed, conveyed and re-stacked onto the secondary heap pad. Ongoing metals recovery takes place on the secondary pads for a further three and a half years. At the conclusion of this phase, the barren ore remains on the secondary pads permanently. In metal recovery, nickel, copper, zinc and cobalt are precipitated using hydrogen sulphide from PLS and filtered. After the metals are removed, the solution is purified and returned to the heaps. The resulting products, copper-, zinc sulphides and mixed nickel cobalt sulphides are transported to customers. (Talvivaara 2014). The Talvivaara heap bioleaching has been described by Riekkola-Vanhanen (2010).

a)



b)

c)

Figure 8. a) Schematic diagram of the Talvivaara process from mining to metal recovery. b) Photograph of the mine site. c) Heap stacking. Photos: Marja Riekkola-Vanhanen.

In November 2012, the gypsum pond leaked and 1.2 million cubic meters of water containing metals and sulphate were released to the mining area and outside the area of around 240 000 m^3. The gypsum pond was used as storage for too much PLS caused by the rains. The released water was neutralized with limestone in order to reduce its acidity and to precipitate its metal content. However, the effects of the discharge were seen as temporarily increased metal concentrations in the nearby waters. The recovery plant was shut down at 4th of the November and further started at 21th of November 2012, after the permission from the Kainuu Centre for Economic Development, Transport and the Environment. Leakage resumed in April 2013, but the built safety dam hindered the leakage outside the mining area. After these leakages, safety and risk management were improved. (Riekkola-Vanhanen 2015, personal communication).

1.8 MICROORGANISMS INVOLVED IN BIOLEACHING

1.8.1 Microbial ecology in heaps

Microbial ecology involves the study of the relationship of microorganisms to their environment and to each other (Johnson 2001). Microorganisms growing optimally at pH < 3 are defined acidophiles (Norris and Johnson 1998). Mineral processing technologies have developed extremely acidic, metal-rich environments that are quite young when compared to natural occurring sites. However, metal mining has a long history in some regions of the world such as RioTinto Spain (Lopez-Archilla and Amils 1999). This has facilitated the emergence of acidophilic communities at these sites. These naturally occurring communities are often more complex and diverse compared to man-made reactors. (Johnson 2001).

Iron- and sulphur-oxidizing chemolithotrophic microorganisms in the heaps are indigenous. Heaps or dumps have rarely been inoculated. The temperature and acidity gradients in bioleaching environments support a wide diversity of microorganisms. Survival of the species depends on adaptability and diversity to function in varying conditions (Brandl 2001). Temperature, pH and aeration efficiency vary creating different kind of microenvironments where microorganisms habit. Conditions change during the time of stacking and leaching. Heaps heat up due to exothermic bioleaching reactions and ferric iron concentrations tend to increase after start-up.

Acidophilic microorganisms can be divided into the groups with respect to their growth temperatures. As temperature increases to more than 40 ºC mesophiles are displaced by the moderately thermophilic and thermophilic microorganisms. Mesophiles have an optimum temperature around 20-40 ºC. Extreme thermophile grow optimally at temperatures higher than 60 ºC. Mesophilic acidophiles are dominantly rod-shaped, Gram-negative eubacteria. Moderate thermophiles include archaea and eubacteria, the majority of which being Gram-positive. In contrast, extreme thermophiles are exclusively archaea. (Johnson 1998, 2008). Thermophiles generally have higher growth rates and faster substrate utilization rates than mesophiles (Brandl 2001). Metal leaching at low temperatures has also been reported and cold tolerant acidophiles have been identified (Ahonen and Tuovinen 1992). Studies of low-temperature bioleaching microorganisms have been reported by Johnson et al. 2001 and Hallberg et al. 2010. Psychrophiles, grow optimally at temperatures below 15 ºC, whilst psychrotolerant microorganisms preferentially grow at higher temperatures but are also able to grow at temperatures below 10 ºC. Bioheaps that are located in cold areas may benefit inoculation of thermophilic microorganisms since the temperatures inside the heap may reach temperatures near 90 ºC. (Rawling and Johnson 2007).

Althought microbes in leaching environments survive at high metal concentrations and can adapt to physico-chemical changes to some extent, there are limits to which this may occur. Product and substrate inhibition has been discussed in Section 1.6.5.

Most acidic environments, including surface water in the Sotkamo deposit, contain dissolved organic carbon in low concentrations (< 20 mg L^{-1}), and are therefore considered as oligotrophic. Many of the autotrophic acidophiles are sensitive to organic matter. Therefore, heterotrophic acidophiles found in leaching environments are important in consuming organic material and thereby, detoxify the local environment. (Brandl 2001, Johnson 1998, 2008, Robbins 2000, Matlakowska and Sklodowska 2011).

1.8.2 Bioleaching bacteria

Iron- and sulphur-oxidizing chemolithotrophic microorganisms are the most important mineral-oxidizing microbes. They grow autotrophically by fixing CO_2 from the atmosphere and obtain their energy by using ferrous iron (Fe^{2+}) or reduced inorganic sulphur compounds (RISCs), or both, as an electron donor. (For the reviews see, Johnson 1998, Rawlings 2002).

The first bioleaching bacteria *Thiobacillus* (later *Acidithiobacillus*) were described in acid mine waters by Colmer and Hinkle (1947). It was long considered to be the most important bioleaching microorganisms. It can use ferrous iron, sulphur and pyrite as a substrate, that were usually used in enrichment cultures for purpose to isolate acidophilic microorganisms. In the light of nowadays knowledge this may select for a relatively narrow range of acidophiles and give a false impression of the population *in situ*. Besides the key players, a variety of micro-organisms are detected in bioleaching environments. (Rawlings 2002, 2005, Brierley 2001). Characteristics of most studied iron- and sulphur-oxidizing chemolithotrops are presented in Table 12. Occurrence of them in bioleaching heaps are listed in Table 14.

Table 12. Characteristics of some most studied iron- and sulphur-oxidizing chemolithotrops.

Genus	**Species**	**Oxidation Fe/S**	**Temperature**	pH	Hetero-/ autotroph	Reference
Bacteria						
Mesophiles						
Acidithiobacillus	*ferrooxidans*	Fe, S	10-37, 30-35*	1.3-4.5, 2.5*	A	Kelly and Wood 2000, Valdes et al. 2008
Acidithiobacillus	*thiooxidans*	S	10-37, 28-30*	0.5-5.5, 2.0-3.0*	A	Kelly and Wood 2000
Acidithiobacillus	*ferrivorans***	Fe, S	4-37, 27-32*	1.9-3.4, 2.5*	A	Hallberg et al. 2010
Leptospirillum	*ferrooxidans*	Fe, S	<10-45, 30-37*	>1.1, 1.3-2.0*	A	Johnson 2001a
Thermophiles						
Acidithiobacillus	*caldus*	S	32-52, 45*	1.0-3.5, 2.0-2.5*	A	Hallberg et al. 1996, Kelly and Wood 2000
Leptospirillum	*ferriphilum*	Fe	30-45, 30-37*	1.4-1.8	A	Coram and Rawlings 2002
Leptospirillum	*thermoferrooxidans*	Fe	30-55, 45-50*	>1.3, 1.65-1.90*	A	Golovacheva et al. 1992, Johnson 2001
Sulfobacillus	*acidophilus*	Fe, S	45-50*	2.0*	A and H	Norris et al. 1996
Sulfobacillus	*thermosulfidooxidans*	Fe, S	28-60, 50*	1.9-3.0, 1.9-2.4*	A and H	Brandl 2001, Robbins 2000
Archaea						
Acidianus	*brierley*	Fe, S	45-75, 70*	1-6, 1.5-2*	A and H	Huber and Stetter 2001
Ferroplasma	*acidiphilum*	Fe	15-45, 35*	1.3-2.2	A/ mixotroph	Golyshina et al. 2000
Sulfolobus	*acidocaldarius*	S	55-85, 70-75*	2-3*, 1-6	A and H	Brock et al. 1972, Chen et al. 2005
Sulfolobus	*metallicus*	Fe, S	50-75, 65*	1-4.5, 1.3-1.7*	A	Huber and Stetter 2001

*optimum, **psychrotolerant

Acidithiobacillus

The first described acidophilic iron- and sulphur-oxidizing bacterium was *Acidithiobacillus ferrooxidans* (formerly *Thiobacillus*, Kelly and Wood 2000). For many years it was considered to be the most important bioleaching microorganism in environments at 40 ºC or less (for a review, see Brierley 1982). *A. ferrooxidans* grows optimally at 30-35 ºC and at temperature range from 10 to 37 ºC. Many bioleaching operations favor *A. ferrooxidans* at the beginning when little ferric iron is released. This situation also prevails natural environments where leaching solutions are not circulated. However, in commercial bioleaching plants, leach solutions are circulated and steady-state conditions are achieved. Usually this results in accumulation of ferric iron, which decreases the role and occurrence of *A. ferrooxidans*. *A. ferrooxidans* is able to use ferrous iron, hydrogen, citric acid and sulphur as electron donors. (Johnson Hallberg et al. 2010). It is facultative anaerobe, being able to grow trough ferric iron respiration in anoxic environment (Pronk et al. 1992).

The species of *Acidithiobacillus* consists of many strains and it has been a question whether these strains actually comprise different species (Ni et al. 2008, Amouric et al. 2011). In 2010 Hallberg et al. described *A. ferrivorans* that was previously considered to a cold-tolerant strain of *A. ferrooxidans* by Johnson et al. 2001. *A. ferrivorans* grows in the range of 5-37 ºC (Table 12). Both bacteria have similar optimum temperature (Hallberg et al. 2010). Mykytczuk et al. (2010 and 2011) reported also psychrotolerant *A. ferrooxidans* strains. However, according to phylogenetic analysis it belongs to *A. ferrooxidans* group II.

The genus *Acidithiobacillus* includes *A. thiooxidans* (Kelly and Wood 2000) and *A. caldus* (Hallberg et al. 1996a). *A. thiooxidans* and *A. caldus* are incapable of pyrite oxidation, but they can utilize the sulphide moiety of the mineral when it is first released by the action of iron-oxidizing bacteria like *A. ferrooxidans* (Hallberg et al. 1996a). *A. caldus* reflects its thermotolerance with growth rate that exceeds that of *A. thiooxidans* at temperatures over 30 ºC. *A. caldus* dominates at temperatures around 50 ºC (Norris et al. 1996).

A. ferridurans was described in 2013 by Hedrich and Johnson. For the strain isolated from drainage water at a uranium mine in Japan, the pH and temperature optima were 2.1 and 29 ºC, respectfully. *A. ferridurans* tolerates higher iron concentrations than *A. ferrooxidans*. *A. albertensis* is the least studied species in the genus and the affiliation remains uncertain. It has an optimum growth temperature of about 30 ºC and grows between 10-35 ºC, although lower temperatures have not been tested. (Kelly and Wood 2000, Xia et al. 2007).

Leptospirillum

In 1972 Markosyan described *L. ferrooxidans* isolated from mine water of the Alaverda copper deposit in Armenia. At least three major groups of leptospirilli exist (Goltsman et al. 2013). Leptospirilli have been positioned within division of *Nitrospira* group. *L. ferrooxidans* has been increasingly studied since its capacity to grow more successfully in certain circumstances than *A. ferrooxidans* (For a review, see Rawlings et al. 1999). Leptospirilli are the primary iron-oxidizers in several industrial continuous-flow biooxidation tanks (Coram and Rawlings 2002). The reason for this bacteria's domination in tanks is most likely due to the fact that the high ferric-ferrous iron ratio inhibits other species but not *Leptospirillum*. *L. ferrooxidans* has inhibition constant (K_i) of 42.8 mM, while, for example, *A. ferrooxidans* has K_i of 3.10 mM. The growth temperature of *L. ferrooxidans* is also wider compared to *A. ferrooxidans* being <10-45 ºC and <10-37 ºC for *A. ferrooxidans* (Kelly and Wood 2000, Johnson 2001). *Leptospirillum ferriphilum* is a thermotolerant mesophile that dominates in tank bioleaching operations at 35-50 ºC (Coram and Rawlings 2002). Moderately thermophilic *L. thermoferrooxidans* grows solely with ferrous iron but not with sulphide minerals on growth range 30-55 ºC and optimal temperature 45-50 ºC (Golovacheva et al. 1992, Hippe 2000). Unfortunately *L. thermoferrooxidans* has been lost (Coram and Rawlings 2002). Two rather new species "*L.*

ferrodiazotrophum" and "*L. rubarum*" have been isolated from the Richmond Mine in Canada (Tyson et al. 2005, Goltsman et al. 2009, 2013).

Sulfobacillus

The genus *Sulfobacillus* includes Gram-positive, endospore forming acidophilic bacteria that grew autotrophically and mixotrophically on ferrous iron, on elemental sulphur in the presence of yeast extract and heterotrophically on yeast extract. (Bogdanova et al. 2006, Watling et al. 2008). The genus was first described in 1978 by Golovacheva and Karavaiko. Several species of the genus *Sulfobacillus* have been isolated from various mine sites and they are mostly moderately thermophilic. The taxonomy of the genus *Sulfobacillus* has suffered from incomplete description of species and thus they taxonomy has not been properly validated (Johnson et al. 2003, 2005). *Sb. acidophilus* and *Sb. thermosulfidooxidans* are acidophilic, moderately thermophilic Gram-positive rods. (Norris et al. 1996) Optimal conditions for growth of *Sb. acidophilus* are 45-50 ºC and pH of 2. *Sb. thermosulfidooxidans* grows optimally at 50 ºC (Brandl 2001) and at pH 1.9-2.4 (Robbins 2000). The ability of *Sb. thermotolerans* to form endospores is advantageous for survival of bacteria during low temperature periods in heaps, where high seasonal variation in temperature occurs. The optimum temperature of *Sb. thermotolerans* is 40 ºC and the growth range 20-60 ºC (Bogdanova et al. 2006). *Sb. sibiricus* has an pH optimum of 1.5, the growth range of 1.1-2.4 and T_{opt} 52 ºC with the unusual wide growth range of 16-62 ºC (Watling et al. 2008, Melamud et al. 2003).

Alicyclobacillus

The first *Alicyclobacillus* was isolated in 1982 and was originally thought to be strictly limited to thermophilic and acidic environments. After that, the genus has gained more attention in beverage industry due to its ability to survive commercial pasteurization processes and produce off-flavors in fruit juices. Several strains of *Bacillus* and some *Sulfobacillus* strains have been reclassified to genus *Alicyclobacillus* (Wisotkey et al. 1992, Karavaiko et al. 2005) including over 20 species in the genus.

Alicyclobacillus disulfidooxidans (formerly *Sb. disulfidooxidans*, Karavaiko et al. 2005) is a mesophilic aerobic bacterium, originally isolated from wastewater sludge (Dufresne et al. 1996). The optimal pH of growth is between 1.5 and 2.5 and the growth temperature range from 4 to 40 ºC, with an optimum at 35 ºC.

Archaea

The role of archaea in the biomining community has been considered to rather scavenge the organic material than leach minerals. As actual bioleaching species, the use of thermophilic archaea e.g. *Acidianus brierleyi* and *Sulfolobus metallicus* are gaining attention. (Johnson 1998, 2001).

Acidianus

Acidianus brierleyi is the first discovered iron- and sulfur-oxidizing archaea (1965). It was found from acidic hot springs of Yellowstone National Park, USA (Brierley and Brierley 1973). *A. brierleyi* was initially placed in the genus *Sulfolobus* but after further investigation of its metabolic properties, it was reaffiliated to the genus *Acidianus* (Segerer et al. 1986). *A. brierleyi* can both oxidize and reduce sulfur depending on the availability of oxygen. It grows between 45 and 75 ºC and pH from 1 to 6; optimum growth occurs at 70 ºC and pH 1.5 to 2.0. *A. brierleyi* grows heterotrophically on yeast extract and autotrophically on carbon dioxide. (Segerer et al. 1986).

Sulfolobus

The genus *Sulfolobus* contains microorganisms that live in acidothermophilic environments. Members of this genus grow aerobically at pH range between 0.9 and 5.8 and at high temperatures in the presence of elemental sulphur. This genus was the first acidothermophilic genus of archaea described (Brock et al. 1972). Several species have been described e.g. *S. metallicus* (Norris et al. 1996, Huber

and Stetter 2001) and *S. acidocaldarius* (Brock et al. 1973 and Chen et al. 2005). *S. acidocaldarius* grows on sulphur or on a variety of simple organic compounds. *S. metallicus*, instead, is obligately chemolithoautotrophic elemental sulphur and ferrous iron-oxidizer. *S. metallicus* grows at temperatures of 50-75˚C with a growth optimum at 65˚C (Huber and Stetter 2001).

Ferroplasma

Currently three species of *Ferroplasma* are recognized. *F. acidarmanus*, *F. acidiphilum* and *F. thermophilum* (Dopson et al. 2004, Golyshina et al. 2000, Okibe et al. 2003, Zhou et al. 2008). They are extremely acidophilic, with a pH optimum below 2.0 grow as low as pH 0 (Dopson et al. 2004). Usually, they grow mixotrophically on ferrous iron and an organic substrate, such as yeast extract, even though *F. acidiphilum* has the ability to grow autotrophically. The growth temperature for *F. acidiphilum* is 15-45 ºC and its pH range is from 1.3 to 2.2. The optimal temperature is 35-36 ºC and the optimal pH is 1.7. (Golyshina et al. 2000).

Heterotrophic microorganisms

Acidophilic heterotrophic microorganisms have been found in bioleaching operations e.g. *Pseudomonas* spp., *Bacillus* spp. and some fungi like *Penicillium* and *Aspergillus* (Johnson et al. 2001, Xie et al. 2007, Matlakowska and Sklodowska 2011). They are considered important for the bioleaching activity because they remove inhibitory organic compounds. (Matlakowska and Sklodowska 2011). Commonly they do not directly assist in the solubilization of metals from sulphide mineral ores. (Johnson 1998 and 2001, Matlakowska and Sklodowska 2011). Although, they have been reported to be able to leach metals by producing organic acids. (Burgstaller and Schinner 1993, Rezza et al. 2001).

1.9 MICROBIAL COMMUNITIES IN MINING ENVIRONMENTS

Understanding the microbial aspects of bioleaching facilitates heap design and operation. Different techniques have been developed, althought investigation of microorganisms inhabiting bioleaching environments exhibits some challenges. The limited number of microorganisms that have been discovered may be a consequence of the methods used to identify microorganimsms (Watling 2006). Common microbiological methods based on incubation are usually not amenable to these microorganisms. Normal agar or agarose is hard to solidify at low pH and chemolithotrophs are sensitive to organic material. The most successful approach has been the use of a double-layer plating technique with an alternative gelling agent. (Johnson 1995 and Okibe et al. 2003).

A breakthrough was achieved when molecular techniques (gene libraries, DGGE, fluorescence in situ hybridizations) could be applied for samples from mining environments. No growth of the microorganisms are required. PCR-based technique was first used successfully with bioleaching microbes in 1994 by Stackebrandt and Goebel. They investigated the bacteria present in laboratory-scale batch and continuous-flow bioreactors treating a mixed zinc-lead ore at 35-40 ºC.

The DNA approaches used in these analyses are based in the detection and amplification of one specific gene, mainly 16S rRNA gene, indicating the presence of the microorganism containing that gene (Johnson and Hallberg 2007). However, some of the microorganisms may be latent or even dead and still retain stable DNA. Instead, experiences with pure cultures have shown that cells with significant ribosome content are living and metabolically active (Schippers et al. 2005). To obtain information on the active microorganisms, RNA-based analysis should be performed. In recent years important advancement has been the use of oligonucleotide arrays (Yin et al. 2007, Remonsellez et al. 2009). The use of quantitative real-time PCR is an approach to quantitatively describe the community composition (Kock and Schippers 2006, 2008; Remonsellez et al. 2009). Liu et al. (2006) developed a

SybrGreen real-time PCR assay of DNA isolated from representative strains of *A. brierleyi, Sulfolobus* spp., *Sb. thermosulfidooxidans, Sb. acidophilus, A. caldus,* and *L. ferrooxidans.* Different techniques to assess microbial community structure, function and dynamics in the bioleching environments have been used (Table 13).

Table 13. Techniques to assess microbial community structure, function and dynamics in the bioleaching environment.

TECHNIQUE		**Reference**
Double-layer plating technique		Johnson 1995
Genetic Fingerprinting Techniques:		
DGGE	Denaturing Grandient Gel Electrophoresis	Muyzer 1999
TGGE	Temperature Grandient Gel Electrophoresis	Muyzer and Smalla 1998, Muyzer 1999, Mikkelsen 2009
SSCP	Single-Strand Conformation Polymorphism	Hayashi 1991, Battaglia-Brunet et al. 2002
RAPD	Random Amplified Polymophic DNA	Williams et al. 1990, Novo et al. 1996
ANDRA	Amplified Ribosomal DNA Restriction Analysis	Smit et al. 1997, Qiu et al. 2011
T-RFLP	Terminal Restriction Fragment Length Polymorphism	Thies 2007, Bryan et al. 2005
LH-PCR	Lenght Heterogeneity PCR	Mills et al. 2007
RISA	Ribosomal Intergenic Spacer Analysis	Ranjard et al. 2001, Espejo and Romero 1997
DNA Microarrays		Gentry et al. 2006, Yin et al. 2007
Q-PCR	Quantitative PCR (or real-time PCR)	Heid et al. 1996, Liu et al. 2006
FISH	Fluorecence in Situ Hybridization	Amann et al. 1995, Bouchez et al. 2006
Microbial lipid analysis		Banowetz et al. 2006, Ben-David et al. 2003
G+C	Guanine plus Cytosine Content Fractionation	Nusslein and Tjedje 1999

Microbial community structures and dynamics have mainly been studied microorganisms in PLS. Representative ore samples are challenging to obtain as the enormous bioheaps include a huge variety of microenvironments. Attached microorganisms are difficult to release from solid ore particles. According to Remonsellez (2009), the comparison between the microbial communities in associated mineral and solutions of industrial and laboratory samples show enough similarity to be considered as indicator of the community inside the heap. Community structures and dynamics of attached microorganisms during bioleaching operation have been studied by Diaby et al. (2007), Zeng et al. (2010) and by Lizama et al. (2012). Microbial diversities in heap and dump bioleaching operations are summarized in Table 14.

Lizama et al. (2012) studied microorganisms in a zinc bioheapleaching plant. Two 6 m high heaps were sampled from different dephts and at different stages of the leaching cycle. Nine bioleaching microorganisms were identified several times including: *A. ferrooxidans, A. thiooxidans, A. alberttensis, Thiomonas* sp., *Ferroplasma acidophilum, L. ferriphilum, A. caldus, Sulfobacillus* sp. and *Sb. thermosulfidooxidans.* Eight other microorganisms were also present, but not considered to have a role in the actual bioleaching. The highest measured temperature was at the bottom of the heap, being 60 ºC and no extreme thermophiles were found. Suprisingly, *Leptospirillum* species were almost missing. Heaps were inoculated in the beginning with *A. caldus and Sb. thermosulfidooxidans*. However, these species did not present in bioheap profiles and *A. caldus* was detected only once.

Remonsellez et al. (2009) reported a quantitative description of the dynamics of active communities in an industrial bioleaching heap from Escondida Mine, Chile. *A. ferrooxidans* was the most abundant during the first part of the leaching cycle, whilst the abundance of *L. ferriphilum* and *Ferroplasma acidiphilum* increased with the age of the heap. *A. thiooxidans* remained constant throughout the

leaching cycle and Firmicutes group showed a low and patchy distribution in the heap. By prokaryotic acidophile microarray (PAM) Alphaproteobacteria were found in all samples and *Sulfobacillus* genus in older samples. Actinobacteria and Acidobacteria were also detected by PAM. *A. ferrooxidans* phylotypes reached its highest abundance when pH values were over 2 and the ferric and total iron concentrations were less than 1.2 g l^{-1}. *Leptospirillum* species reached their highest abundance when the pH was below 2 and high ferric ion concentration prevailed. The tolerance to high redox potential and ferric ion concentration (Rawlings et al. 1999) could be the reason for their dominance as also described previously (Demergasso et al. 2005). The presence of archaea like *Thermoplasma* and *Ferroplasma* has been related to high amount of total iron and low pH values. (Remonsellez et al. 2009, Xiao et al. 2008).

Table 14. Microbial diversities in large-scale bioheap operations.

Heap type	**Location**	***A. ferrooxidans***	***L. ferrooxidans/ ferriphilum***	***Acidiphilium* spp.**	***Sulfobacillus* spp.**	***A. caldus/ thiooxidans/ alberttensis***	***Ferroplasma* spp.**	**Non-bioleaching microorganisms or unknow species**	**Reference**
Chalcopyrite overburden	Australia	X	X	X		X			Goebel and Stackebrandt 1994
Copper sulphide/ oxide dump	Southwest USA	X	X	X					Bruhn et al. 1999
Chalcocite heap	Australia		X		X	X	X	Proteobacteria	Hawkes et al. 2006
Low-grade Cu sulphide heap	Chile, Escondida Mine	X	X		X	X	X	Firmicutes, *Acidiphilum*-like, *Alicyclobacillus* spp.	Remonsellez et al. 2009
Low-grade Cu sulphide test heap	Chile, Escondida Mine	X	X		X		X	Chrenarchaeota, *Sulfurisphaera* spp.	Demergasso et al. 2005
Copper leaching plant	Chile, Lo Aguirre	X	X			X		Several	Espejo and Romero 1997
Copper and iron bioheap	China, Tong Shankou	X	X	X				Several	Xie et al. 2007
Lead-Zn mine	China, Yinshan mine		X		X	X	X	Several	He et al. 2008
Copper mine	China, Dongxiang	X	X	X		X		Several	Xiao et al. 2008
Zinc sulphide bioheap	USA, Alaska	X	X		X	X	X	Several	Lizama et al. 2012

2. AIMS OF THE PRESENT WORK

Biohydrometallurgy enables the use of low-grade ore resources to recover valuable metals. Bioleaching is based on activity of a wide variety of mesophilic and thermophilic iron- and sulphur- oxidizing microorganisms. Bioleaching technology is site-specific and technology needs to be developed, including laboratory tests complemented with demonstration plant. Full-scale heap leaching depends on the amenability of ore resources for bioleaching, the environmental conditions, the amenability of the leach liquors to metal extraction and finally the overall economic and environmental analysis of the process.

Bioleaching of a complex Finnish multimetal black schist ore was studied. The diversity and dynamics of the bioleaching communities were studied over time in different experimental and demonstration systems. The specific aims of this study were as follows:

- To assess the effects of pH and leaching temperature on the dissolution of valuable metals.
- To reveal the microbial community diversity and development at different pH values and temperatures.
- To monitor the microbial community composition dynamics during a demonstration-scale bioheap over a period of three years.

3. MATERIALS AND METHODS

3.1 SOURCE OF MICROORGANISMS FOR COLUMN LEACHING EXPERIMENTS

The enrichment culture used for the inoculation of the bioleaching columns was obtained by combining several acidic (pH 4.5-6.9) water samples from the Sotkamo ore deposit. The cultures were first enriched in shake flasks at 25 ºC on three different acidic media. All the media contained basal salts [0.4 g L^{-1} each K_2HPO_4, $(NH_4)_2SO_4$, $MgSO_4 \cdot 7\ H_2O$], supplemented with either ferrous iron (4.5 g L^{-1}), elemental sulphur (1 % wt vol^{-1}) or black schist ore powder (1 % wt vol^{-1}) from the Sotkamo deposit. Basal salts were diluted with the surface water originating from the deposit (containing about 20 mg L^{-1} dissolved organic matter, pH 6.9) and solutions were adjusted to pH 1.8 with sulfuric acid. After inoculation (10 % vol vol^{-1}), the suspensions were incubated in orbital shakers at 180 rpm at 25 ºC. After one month of incubation the enrichments cultures were combined to a medium containing basal salts, ferrous iron (4.5 g L^{-1}), sulphur (1 % wt vol^{-1}) and black schist ore powder (1 % wt vol^{-1}). The enrichment culture was subcultured three times with this medium prior transfer to the bioleaching columns. Microbial growth in media was monitored by phase contrast microscopy (Zeiss Axioskop 2).

3.2 BIOLEACHING COLUMN EXPERIMENTS

Seven columns at different target pH values (1.5, 2.0, 2.5 and 3.0 at 21 ºC) and temperature (7 ºC, RT, 35 ºC, 50 ºC at pH 2.5) values containing about 9 kg of agglomerated ore were set up and inoculated (Papers I and II). For attempting to increase the bioleaching rate at 50 ºC, the column was re-inoculated with a *Sulfolobus* culture (Salo-Zieman et al. 2006) on day 65.

The mineral composition of the sulphide ore was 61.2 % pyrrhotite [$(Fe_{1-x})(S_2)$, where X = 0.7–0.9], 24.3 % pyrite (FeS), 5 % pentlandite [$(Fe,Ni,Co)_9S_8$], 6.5 % alabandite (MnS), 2.4 % chalcopyrite ($CuFeS_2$) and 1% sphalerite [(Zn,Fe)S]. Valuable metal contents were as follows: 0.27 % Ni, 0.56 % Zn, 0.14 % Cu and 0.02 % Co (for detailed description see Riekkola-Vanhanen 2007). Si-containing minerals are anorthite ($CaAl_2Si_2O_8$), biotite ($K(Mg,Fe)_3[AlSi_3O_{10}(OH,F)_2]$), microline ($KAlSi_3O_8$), phlogopite [$KMg_3(Si_3Al)O_{10}(F,OH)_2$], plagioclase [$(Na,Ca)(Si,Al)_4O_8$] and quartz ($SiO_2$). The total amount of SiO_2 of the ore was 42 % (w w^{-1}). (Bhatti et al. 2012b).

The columns had a volume of 7.9 L, a height of 100 cm and an inner diameter of 10 cm. The column at 7 ºC was placed in a refridgerator and had a volume of 6.7 L, a height of 85 cm and an inner diameter of 10 cm. The leach liquor containers had a volume of 5 L and were provided with magnetic stirrers. In order to maintain temperatures at 35 ºC and 50 ºC, the columns were equipped with a water jacket connected to a heating thermostat. The solution container at 35 ºC had a heating fabric and the container at 50 ºC was placed in a water bath. A perforated plate and a filter cloth were inserted at the bottom of each column. Aeration was provided through a diffuser inserted at the base of the column at the rate of (8-11) $m^3\ m^{-2}\ h^{-1}$. Ore was irrigated at a rate of 10 L $m^{-2}\ h^{-1}$ by liquid recirculation. A titration apparatus connected to a PC was used to control the pH. Glass beads with a total bulk-volume of 270 ml were placed in the upper part of the column to enable even distribution of the leach liquor. A paraffin paper was set on the top of each column to prevent evaporation. A schematic diagram of a bioleaching column (not drawn to scale) is given in Figure 9 and a photo in Figure 10.

Once the nickel concentration reached 2.50 g L^{-1} in the leach liquor was replaced with fresh liquor in order to decrease possible toxic effects of metals. After 110 and 140 days at pH 1.5, Ni concentration of PLS exceeded 2.5 g L^{-1} and on both times 1 L was replaced with fresh solution. At pH 2.0 that was done after 237 days. Columns at pH 2.0 (after 82 d), pH 2.5 (RT) and 35 ºC (after 117d) were blocked

at the bottom during the bioleaching. The ore was placed back to the columns and the experiment was continued. Surprisingly, in each column, about 200g of ore did not fit back to the column. The mixed sulphur- and iron-oxidizing culture was drawn from the recirculation solution at 7 ºC and used in the experiments where the Fe^{2+} oxidation rate and optimum temperature were determined over a temperature range of 2-40 ºC (Paper III).

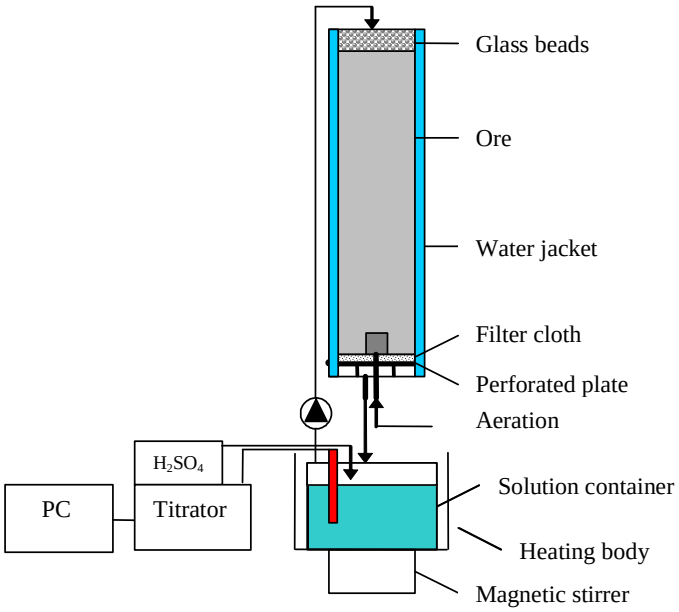


Figure 9. A schematic diagram of a bioleaching column (not drawn to scale).

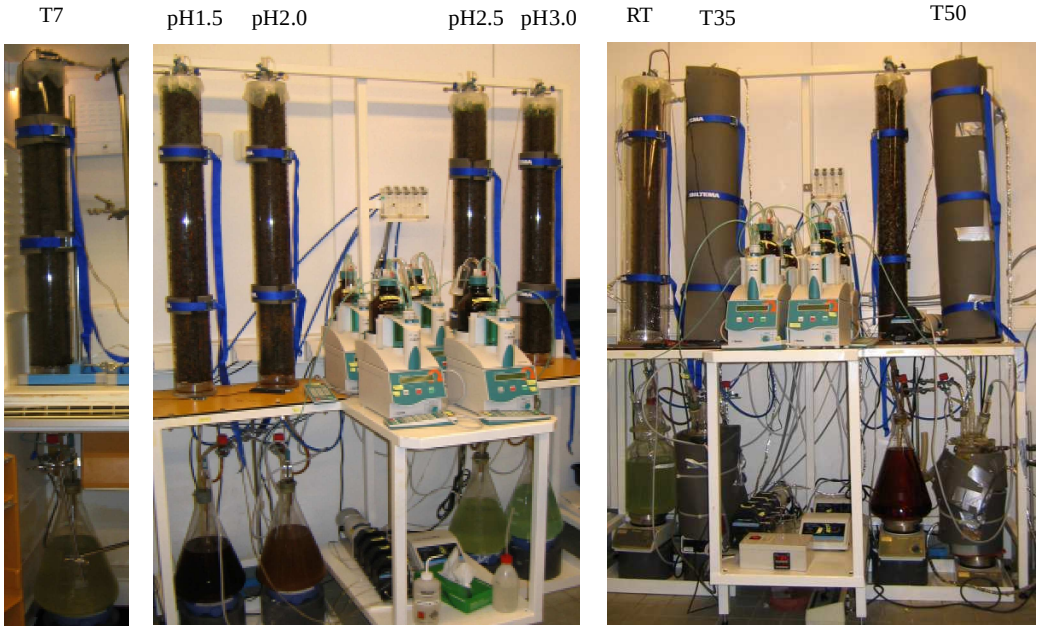


Figure 10. Photograph of the bioleaching columns used in Papers I-IV.

3.3 DEMONSTRATION-SCALE BIOHEAP

In the summer of 2005, a 17 000 t demonstration plant was constructed at the Sotkamo mine site by Talvivaara Mining Company. A representative ore sample was mined, crushed to 80 % -8 mm, agglomerated and stacked in a two-part heap (8 m high, 30 × 120 m). Heap 1 was agglomerated with sulphuric acid solution (pH 1.8) including inoculum (Paper V). Heap 2 was agglomerated with sulphuric acid solution only. Irrigation water was taken from the drilled well on the area (pH 6) and irrigation of the heaps was started in August 2005. The irrigation flow rate was 10 L m^{-2} h^{-1} in the beginning on Heap 1 and 20 L m^{-2} h^{-1} on Heap 2. Irrigation was decreased later to 5 L m^{-2} h^{-1} on both heaps. The mineral composition of the sulphides used in the demonstration-scale bioheaps was as described in Chapter 1.7.

Leach liquors were collected by subsurface drains below the heaps and directed to manholes. From the manholes liquors flowed to PLS ponds and back to irrigation. Both heaps had separate liquid circulations. The operational volumes of ponds 1 and 2 were 175 m^3 and 136 m^3, respectively. Heaps were 8 m high, 30 m wide and 60 m long. The amount of the ore of Heap 1 and 2 were 10 255 t and 6 703 t, respectively. Figure 11 shows the sampling points (manhole 1 and 2, pond 1 and 2) with the direction of the liquid flow in tubes marked with arrows.

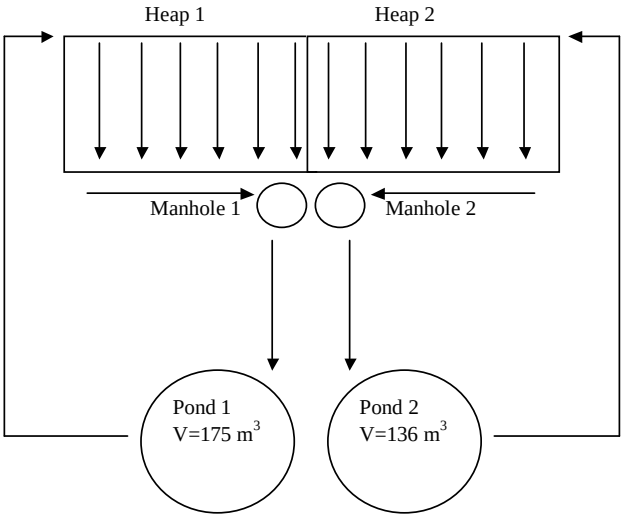


Figure 11. Schematic diagram of the sampling points of the demonstration-scale bioheaps with the direction of the liquid flow marked with arrows.

Ten percent side stream of the leach liquor was continuously removed for metal recovery and replaced with surface water. After the start-up of irrigation, the oxidation of pyrrhotite and pyrite increased the heap temperature up to 90 ºC. Heaps were covered with plastic lining to minimize evaporation and to prevent pipelines from freezing during winter time. Leach liquor temperatures remained always above 15 ºC during the operation period, even during the boreal winter. Figure 12 presents three photograps of the bioheaps. The description of demonstration-scale bioheap is provided by Riekkola-Vanhanen 2007.

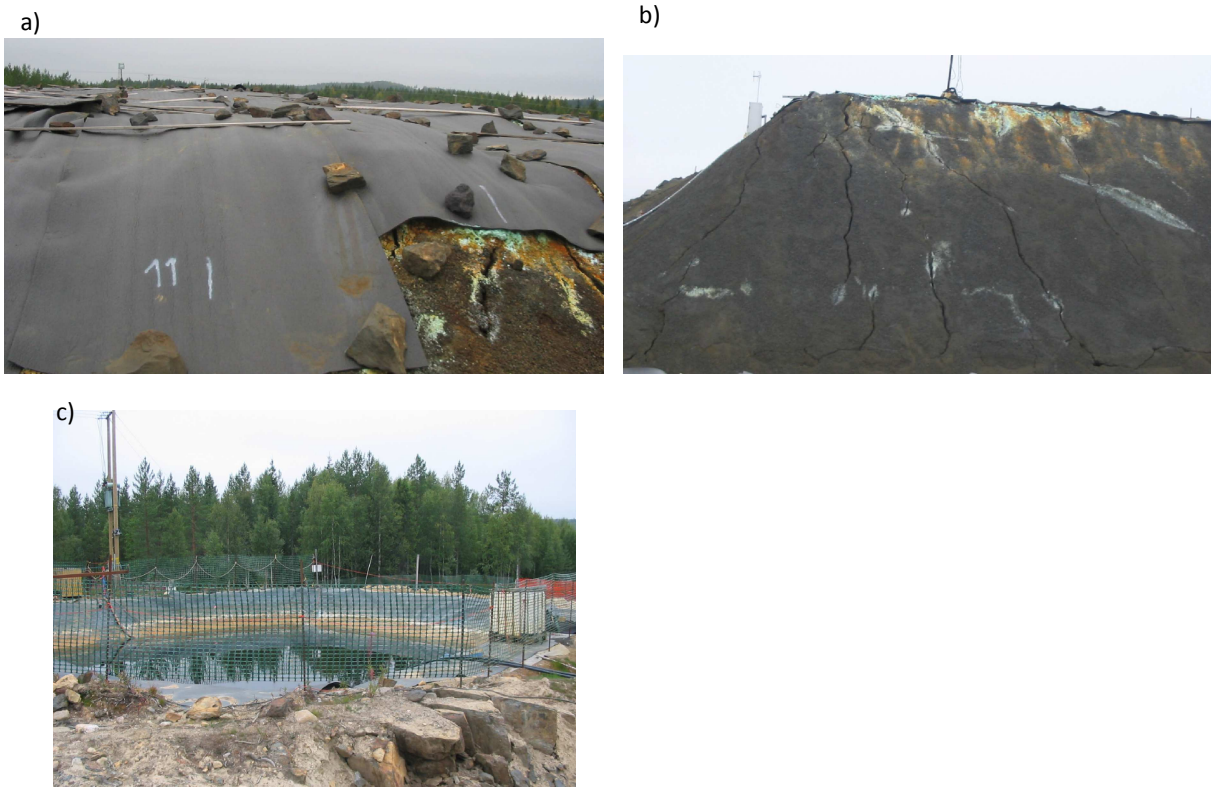


Figure 12. Photographs of demonstration-scale bioheap. a) Top of the heap covered with the plastic lining. b) Side view of the heap. c) Pond of the leach liquors. Photos: Kirsi Määttä.

Secondary bioheaps

After 18 months of demonstration-scale heap operation (February 2007), the heaps were reclaimed and restacked to the secondary bioheap. In secondary heap irrigation rate was 2 L m^{-2} h^{-1}. No aeration was provided.

3.4 ANALYTICAL TECHNIQUES

The frequency of analyses used laboratory-scale columns tests was as summarised in the Table 15. The modified most probable number (MPN) technique is described in Paper I.

Table 15. Summary of the analyses used in the study.

Physicochemical	Method	Interval	Reference
pH	Electrode	Continuously	Papers I-V
Redox potential	Electrode (mV Ag^0/AgCl)	Once a day	Papers I-V
Dissolved Oxygen	Electrode	Once a week	Papers I-V
Temperature	Digital thermometer	Once a day	Papers I-V
Fe^{2+}	AAS or ICP-AES	Once a week	Anon. 1992
Total Fe	AAS or ICP-AES	Once a week	SFS 1980a, b
Ni, Zn, Cu, Co,	AAS or ICP-AES	Once a week	SFS 1980a, b
Al, Ca, Mg, Mn, Si	AAS or ICP-AES	Once a month	Papers I-V
Identification of minerals	XRD	At the time of column blockage	Paper IV
Microbial analyses			
Enumeration	DAPI, MPN-Fe	Once a month	Papers I-V, SFS 4447
Diversity and identification	PCR, DGGE, 16S rRNA sequencing	Once a month	Muyzer et al. 1996

The following computer programs were used in the microbial community profiling: BLAST (Altshul et al. 1990), BioEdit (www.mbio.ncsu.edu/BioEdit/bioedit.html) and phylogenetic analyse (Dereeper et al. 2008 and 2010).

3.4.1 Microbial community analysis

Microbial communities were investigated by DNA extraction and Polymerase Chain Reaction (PCR) - Denaturing Gradient Gel Electrophoresis (DGGE); followed by partial sequencing of 16S rRNA gene. For DNA extraction, a 15-20 mL sample was filtered. The filters were rinsed with NaCl at pH 1.8 to remove the excess metals and then neutralized with Na-EDTA. The filters were stored at -20 ºC prior to nucleic acid extraction. DNA was extracted from preserved filter samples with a DNA isolation kit. The crude DNA was used as a template for PCR. Partial 16S rRNA genes (550 bp) were amplified. The PCR products were checked with agarose gel electrophoresis prior to DGGE. Archaea were characterised using nested PCR approach.

DGGE was performed with the INGENYphorU2×2-system (Ingeny International BV, The Netherlands) as described in Paper I. The denaturing gradient range first from 10 to 80 % and after from 40 to 70 %. Individual bands were excised from the gel, eluted and then stored at -20 ºC. PCR was performed from aliquots (1-2 µL) of the eluate. Before being sequenced, each PCR product was run in an agarose gel to confirm the size and the concentration of the product. The PCR products were purified and sequenced at the DNA Sequencing Facility, Institute of Biotechnology, Helsinki University. To identify the microorganisms, the sequence data was compared with 16S rRNA gene sequences in the GenBank database using the basic local alignment search tool (BLAST, Altschul et al. 1997).

The ore samples (15 g each) were taken according to the Finnish standard SFS-EN 932-2 (Anon. 1997). The sample was mixed with the washing solution as described in Paper I. The mixture was shaken and

sonicated 5×1 min in order to detach microorganisms from ore particles. Thereafter, the sample was allowed to settle. Supernatant (15-20 ml) was filtered for DNA extraction. Microbial numbers were counted from supernatant in order to estimate the amount of attached cells. If no respectable PCR product was gained, a nested PCR approach was used. (Paper I). Otherwise the method was same as with PLS samples. Figure 13 summarizes the microbial community profiling procedure.

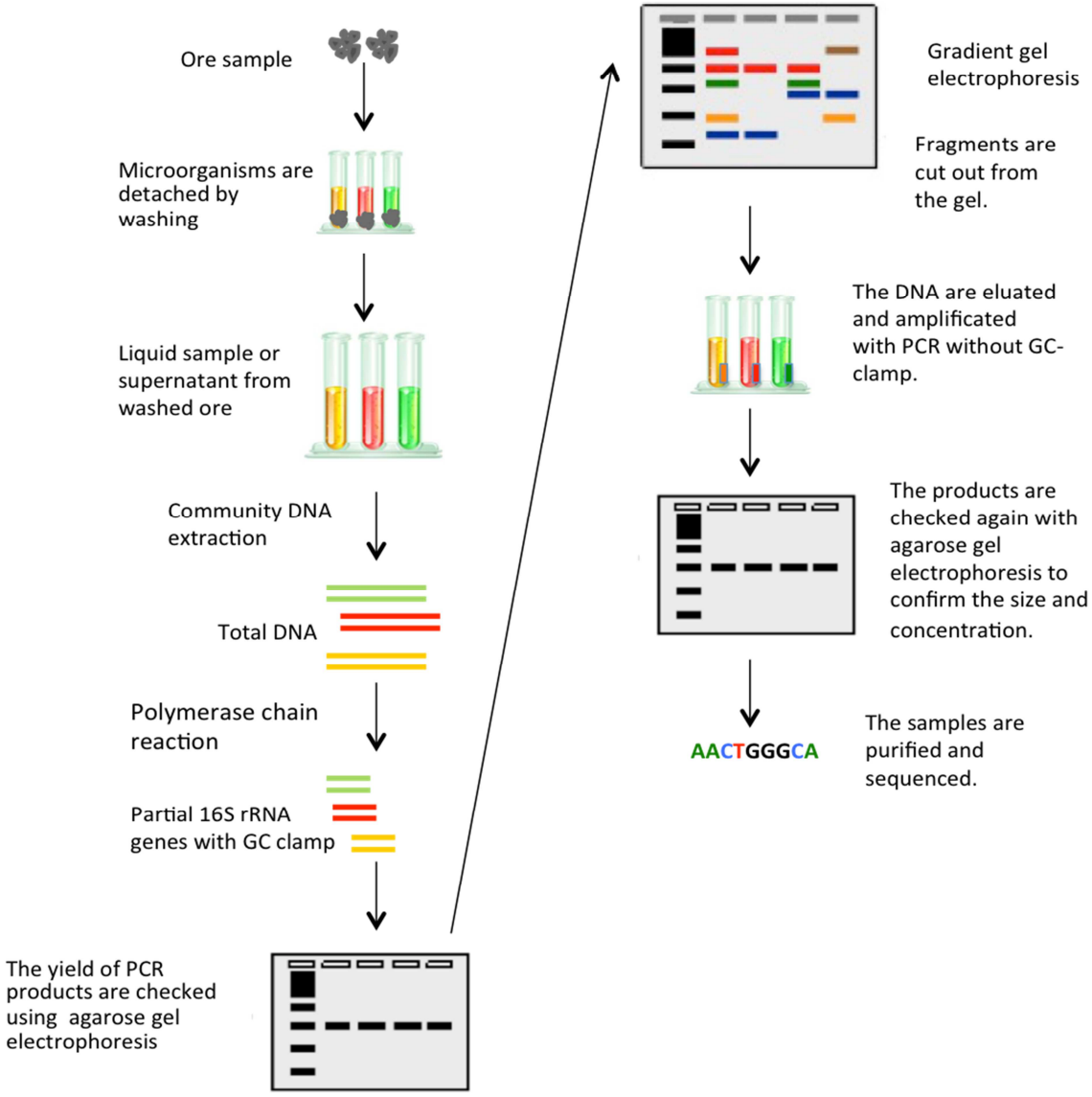


Figure 13. Analysis of the microbial diversity of the ore samples or PLS by denaturing gradient gel electrophoresis (DGGE) and polymerase chain reaction (PCR) - amplified 16S rRNA genes.

4. RESULTS AND DISCUSSION

4.1 SOLUBILISATION OF METALS FROM LOW-GRADE ORE

Starting from the early studies by Puhakka and Tuovinen (1986a, b, c), several laboratories have tested the amenability of the ore to bioleaching (Niemelä et al. 1994, Riekkola-Vanhanen and Heimala 1999, Wakeman et al. 2008, this study and Bhatti et al. 2010, 2012a, b). The results from the studies are summarized in Table 17 and 18. The effect of different factors such as pH, particle size, pulp density, bioleaching method, nutrient additions and temperature has been tested with different acidophilic inocula.

In the present study seven columns with different pH of the PLS (1.5, 2.0, 2.5 and 3.0, at 21 ºC) and different temperatures (7, 21, 35 and 50 ºC, at pH 2.5) were set up. The actual pH of the PLS after 140 days were 0.1-0.5 units over the target values despite the continuous titration. Metal dissolution of valuable metals during the bioleaching experiment at different target pH values are presented in Figure 14.

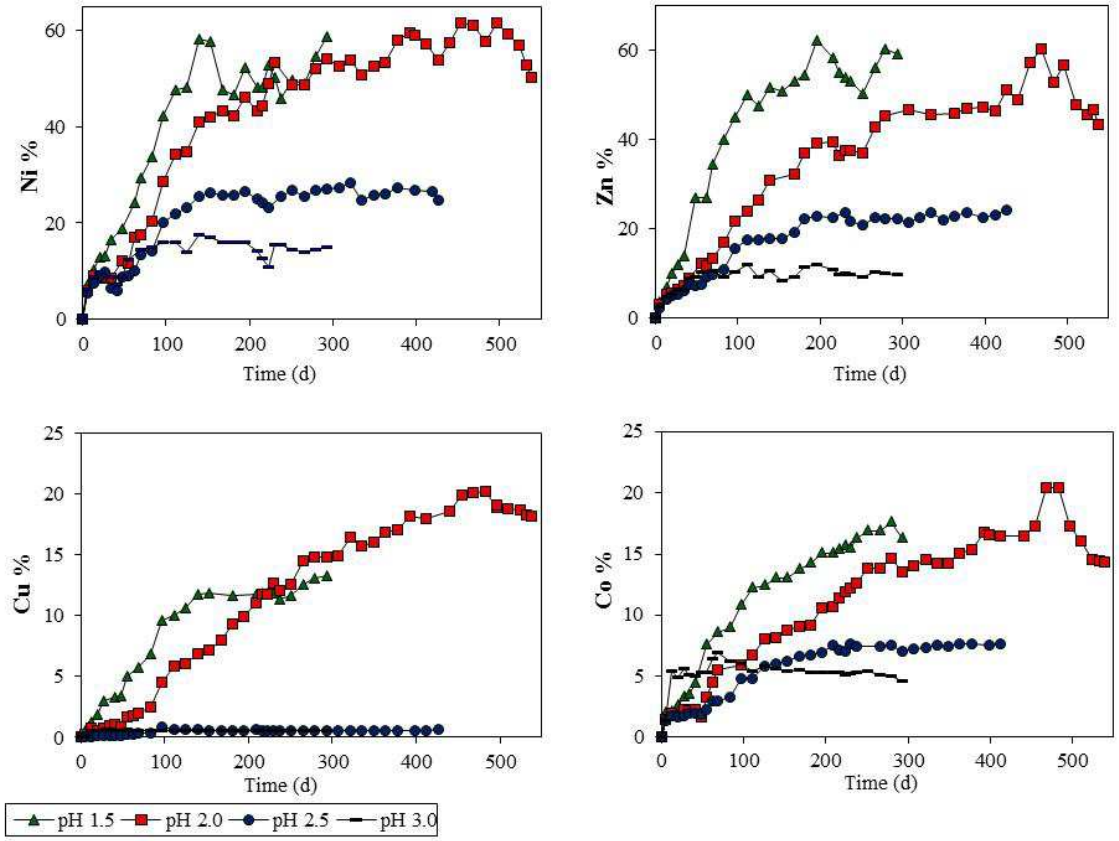


Figure 14. Dissolution of valuable metals during the experiment of column bioleaching of the ore at different target pH values at 21 ºC. (Paper I).

The pH was the most significant factor affecting the dissolution of nickel and zinc. Nickel solubilization was 3.3 times faster at pH 1.5 than at pH 3.0. The leaching rates for nickel, after 140

days of bioleaching were 0.42, 0.29, 0.19, 0.13 % (Ni) d^{-1}, corresponding target pH values 1.5, 2.0, 2.5 and 3.0, respectively. The metal leaching rates are presented in Figure 15.

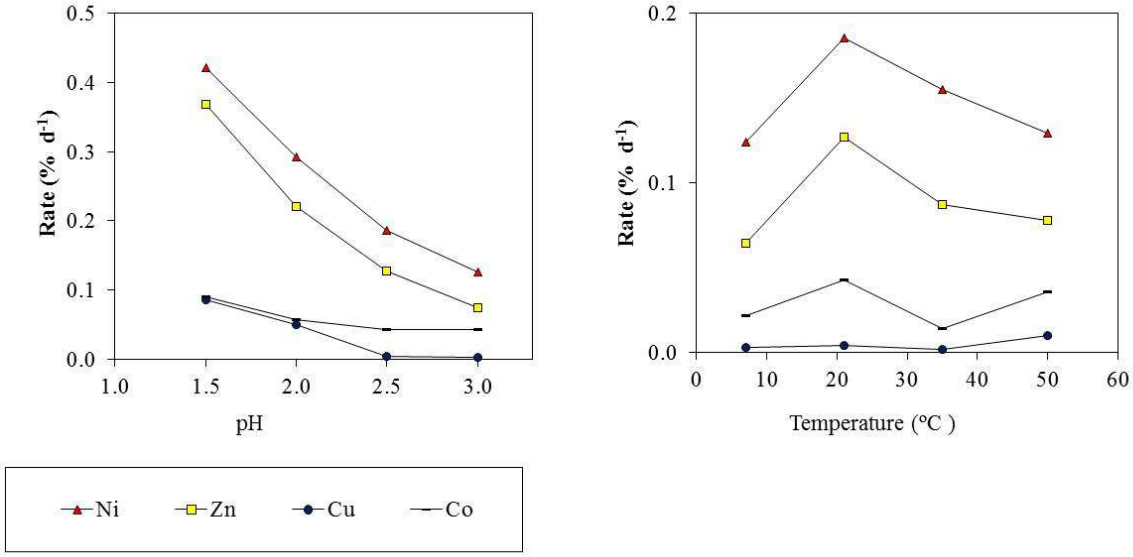


Figure 15. Metal leaching rates of the ore at target pH values 1.5, 2.0, 2.5 and 3.0 at 21 ºC (Paper I) and at temperatures between 7 and 50 ºC at pH 2.5 (Paper II), calculated after 140 days.

After 140 days at pH 1.5, the extracted metal rates were 59 % for Ni, 52 % for Zn, 13 % for Cu and 16 % for Co. After 230 days of bioleaching, the extraction of nickel at pH 2.0 reached nearly the same yield % as at pH 1.5, with no significant dissolution thereafter. The maximum yields are presented in Table 16. At pH 2.5 and 3.0 no further extraction of metals occurred after 150 days. That was probably due to the formed precipitates. Brown precipitates increased from pH 1.5 to pH 3.0 on the surfaces of the ore.

Table 16. The maximum metal yields of Talvivaara complex multimetal black schist ore at different target pH values at 21 ºC and at temperatures between 7 and 50 ºC at pH 2.5.

		Max yield (%)			
pH	**Time (d)**	**Ni**	**Zn**	**Cu**	**Co**
1.5	140	59	52	13	16
2.0	230	54	37	13	12
2.5	153	26	18	0.5	6
3.0	140	15	10	0.5	6
T (ºC)					
7	496	24	17	2	6
21	153	26	18	0.5	6
35	140	22	12	0.3	3.5
50	140	18	11	0	2

Similar bioleaching behavior was seen in the study of Bhatti et al. (2012a and b). The dissolved Ni and Zn decreased with increasing pH. But in contrast to our study, the reverse pH effect was seen in the bioleaching of Cu. Cu concentrations were lower at pH 1.5 than at higher pH values. They suggest that is due to the passivation that is caused to elemental sulfur accumulation of chalcopyrite surfaces.

At all temperatures, the dissolution of nickel was similar over the first 90 days. Following that period, the highest bioleaching of valuable metals took place at 21 ºC. Decrease in leaching rates, especially at temperatures of 35 and 50 ºC, may have been due to the lack of dissolved ferric iron serving as a leaching agent, or barriers created by precipitates. On the other hand, Riekkola-Vanhanen and Heimala (1999) have concluded that the iron precipitation did not interfere bioleaching of a black schist ore. Brown precipitates were observed to accumulate on the surfaces of the ore material in columns from 7 ºC to 50 ºC. Additionally, bright yellow precipitates were formed indicating elemental sulfur or Na-jarosite accumulation at 7 ºC and 21 ºC as in the study of Ahonen and Tuovinen 1990. Bioleaching at 7 ºC continued until 500 days, while the maximum yields at other temperatures were achieved near 150 days of bioleaching.

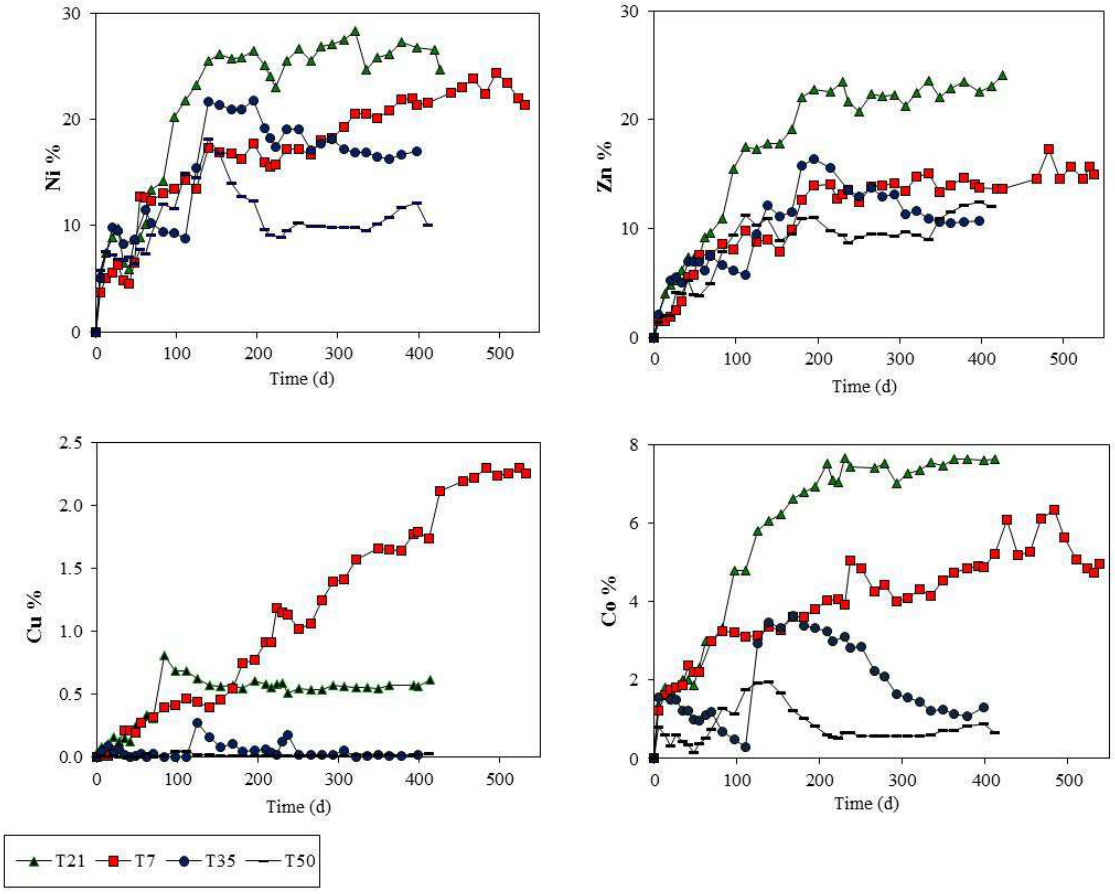


Figure 16. Dissolution of valuable metals during the experiment of column bioleaching of the ore at temperatures between 7 and 50 ºC at target pH 2.5 (Paper II).

In bioheaps gradients of pH and temperatures are always formed. Thus, the bioleaching rates will change at different parts of the heap.

Table 17. Summary of the studies done with the Talvivaara complex multimetal black schist ore. RT = Room temperature.

Reference	Method	Particle size	T (ºC)	pH	Suspension or amount	Leaching time (d)	Yield % Ni	Zn	Cu	Co
Puhakka and Tuovinen 1986a	Aerated column	90%-200 mesh	RT	2.0*	20% (1 500 ml)	76	53	50	39	57
			RT	2.0*	30% (1 500 ml)	97	81	55	27	30
Puhakka and Tuovinen 1986b	Shake flasks	90%-200 mesh	28	3.0*	50 g L^{-1}	28	100	100	30	57
	Air-lift percolation	50%- 16 mesh	23		800 g / 800 ml	180	100	78	18	30
	Airlift reactor	90%-200 mesh	23		100 g / 1 000 ml	100	>84	>91	31-39	65-79
	Aerated column	90%-200 mesh	23		100 g / 1 000 ml	90	**			
Puhakka and Tuovinen 1986c	Percolation system (see Table 18)	50%-16 mesh	23	3.0*	800 g / 800 ml	Half year	100	78	18	47
		< 1 mm	RT				22	14	2	9
							31	19	2	11
							28	19	1	10
							44	25	8	18
Niemelä et al. 1994	Shake flask	0-59 µm	30 or 35	1.5*	5 % wt vol^{-1}	10 or 15	Not measured			
Examined the influence of inorganic nitrogen and phosphate on bioleaching. Phosphate, ammonium or nitrate amendment did not enhance Fe^{2+} oxidation.										
Paper I-V	Aerated column, inoculated	< 8 mm	RT	1.5	9 kg	140	59	52	13	16
			RT	2.0			41	31	7	8
			RT	2.5			26	18	0.6	6
			RT	3.0			15	10	0.5	6
			7	2.5			17	9	0.4	3
			21	2.5			26	18	0.6	6
			35	2.5			22	12	0.2	2
			50	2.5			18	11	1.4	5

* not controlled thereafter
** additional ore added

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Reference	Method	Particle size	T (ºC)	pH	Suspension or amount	Leaching time (d)	Yield Ni	Zn	Cu	Co
Wakeman et al. 2008	Shake flask column bioreactor column bioreactor	0-2 mm 2-6.5 mm 6.5-12 mm	37	2.0*	50g/ 500ml 3 kg 3 kg	6 weeks 40 weeks 40 weeks	80 22 8	68 8 7	20 0 0	nm
Bhatti et al. 2012a	Shake flask	0-59 µm	22	1.5 and over	5 g	30 d	20-30	20-30	40-60	80

* not controlled thereafter, nm = not measured

4.2 LEACHING OF NON-VALUABLE MINERALS AND pH CONTROL

Dissolution of aluminum, calcium, magnesium, manganese and silicon was highest at pH 1.5. After 50 days of bioleaching, the leach liquor at pH 1.5 became viscous and difficult to filter trough the 0.45 µm pore size membrane filter and indicated possible problems for heap leaching. After 110 days the Si concentration peaked 2.96 g L^{-1} and with the formation of jelly with prevented membrane filtration (Table 18). However, dissolved Si did not influence the solubilization of valuable metals. After 140 days, the dissolved concentrations Si were 1 730, 811, 181 and 152 mg L^{-1} at pH values 1.5, 2.0, 2.5 and 3.0, respectively. The study showed that the pH of the PLS needs to be maintained over 1.5 to prevent jelly formation and problems in downstream processing. Temperature did not significantly affect the silicate leaching at pH 2.5. At all temperatures, leach liquors became saturated with dissolved calcium and manganese during the first 100 days but the aluminium concentration rose linearly over this period. The figures of aluminum, calcium, magnesium, manganese and silicon concentrations in leach liquors during the bioleaching at different pH values and temperatures are presented in Papers I and II.

Leaching at low pH values resulted increased acid consumption, being after 140 days 160, 38, 8 and 3 H_2SO_4 g kg^{-1} ore at pH 1.5, 2.0, 2.5 and 3.0, respectively. Temperature, at pH 2.5, had also effect on acid consumption. At 50 ºC acid consumption was highest and lowest at 21 ºC, being 29 and 8 H_2SO_4 g kg^{-1} ore, respectively. In the study of Ahonen and Tuovinen (1995), the acid consumption of the black schist ore from Keretti mine Finland, was at pH 2.5 around 50 H_2SO_4 g kg^{-1} ore after same time of column bioleaching. In their study, the acid production took place after beginning, but the net process remained acid consuming. Acid consumption depends on the amount of gangue minerals and activity of sulphur oxidizers. If minerals dissolve completely, they will consume more acid than during weathering. Complete dissolution is likely to occur at pH values lower than 2. At higher pH values, clay minerals, that are expansible, are likely formed. (Jansen and Taylor 2014). After column blockages, (pH 2.0 and pH 2.5 after 82d, and 35 ºC after 117d) about 200g of the ore did not fit back to each of the column. Blockages are easily managed in laboratory conditions, but in real heaps, it causes severe problems in aeration, irrigation and overall bioheap operation.

The study of Bhatti et al. (2012a and b), with the Talvivaara ore, at pH 1.5, mica was mostly solubilized. If the leaching solution was not pH controled, mica was converted to vermiculite. Gypsum was present most extensively at pH 1.5 than at higher pHs, presumably due to higher sulphate levels and extensive dissolution of Ca-containing phases. Leaching of Si and Al were almost an inverse function of pH as in our study. They suggested that probably it was only one type of silicate that was the source of Si and Al. In their study, at pH values of 3.3 and 3.4, the dissolved Si and Al concentrations were 5-7 times lower than at pH 1.5. In our study, the concentrations were over 20 times lower at pH 3.0 than at 1.5. They suggested that the proton attack caused the massive silicate dissolution. Protons were from sulphuric acid or produced by sulphur oxidation.

Table 18. Silicate mineral studies done with the Talvivaara complex multimetal black schist ore.

Reference	Method	Particle size	T (°C)	pH	Suspension or amount	Leaching time (d)	Si (mg L^{-1})	Al (mg L^{-1})
Paper IV	Aerated column, inoculated	< 8 mm	RT RT RT RT 7 21 35 50	1.5 2.0 2.5 3.0 2.5 2.5 2.5 2.5	9 kg	140	1 730 811 181 152 212 181 183 164	10 300 4 210 704 437 979 704 842 1 160
Bhatti et al. 2012b	Shake flask, inoculated	0-59 µm	22 22	~1.5 2.3	5 g	30	~800 ~700	~800- 1000 ~800
Bhatti et al. 2012b	Shake flask, not inoculated	0-59 µm	22 22	~1.5 3.4	5 g	30	~600-800 ~200	~800- 1000 ~100
							Al_2O_3 (mg L^{-1})	
Puhakka and Tuovinen 1986a	Aerated column	90%-200 mesh	RT RT	2.0* 2.0*	20% (1 500 ml) 30% (1 500 ml)	76 97	~2 200 ~1 000	
Puhakka and Tuovinen 1986b	Shake flasks Air-lift percolation Airlift reactor Aerated column	90%-200 mesh 50%- 16 mesh 90%-200 mesh 90%-200 mesh	28 23 23 23	3.0*	50 g L^{-1} 800 g / 800 ml 100 g / 1 000 ml 100 g / 1 000 ml	28 180 100 90	>600 >2 000 >2 000 >3 200	
Puhakka and Tuovinen 1986c	Percolation system	< 1 mm	RT	3.0*	800 g / 800 ml	~180	~650 ~300 ~250 ~550	continuous recirculation, ore material was not flooded 2h d^{-1} recirculation, ore material was flooded 8h d^{-1} recirculation, ore material was flooded continuous recirculation, ore material was flooded

* not controlled thereafter

4.3 FERROUS IRON OXIDATION AND REDOX POTENTIALS

The redox potentials in the column leach liquors at pH values between 1.5 and 3.0 varied between 515-580 mV (Pt electrode against an Ag^0/AgCl reference), being highest at pH 2.0 and lowest at pH 1.5. The concentrations of ferric and total dissolved iron increased with the decrease in pH. The concentration of dissolved ferrous and ferric iron remained low throughout the experiment at pH 3.0 due to the precipitations. Figures of redox potentials and the concentrations of total dissolved iron and ferrous ion in leach liquors at different pH values are presented in Paper I. Also, Ahonen and Tuovinen (1995) and Nemati et al. (1998) have reported negligible amounts of ferric iron in leach liquors above pH 2.5. The results demonstrated that highest metal yields are achieved at low pH values at high redox conditions where ferric iron remains in solutions, as also reported earlier by Ahonen and Tuovinen (1995).

At 7 ºC the redox increased during the first two months and reflected the start of ferrous iron oxidation and microbial activity. The leach liquor redox potential stabilized to 500-600 mV at 7 ºC and at 21 ºC, whereas at 35 ºC and at 50 ºC it varied between 300-500 mV. According to Brierley (2003) the thermophilic bacteria require greater concentrations of ferrous iron which results in a higher ferrous to ferric iron ratio and low redox potential. Figures of the redox potentials and the concentrations of total dissolved iron and ferrous iron, in leach liquors during the bioleaching of the ore, at different pH values and temperatures, are presented in Paper I and II. Low redox conditions reflect high Fe^{2+}/Fe^{3+} ratio (Ahonen and Tuovinen 1992, Brierley 2003). In the study of Ahonen and Tuovinen the lag period at 4 ºC was 110 days. In our study, after 60 days of bioleaching, total iron and Fe_{tot}/Fe^{2+} ratio was higher in the 7 ºC column leach liquor than at other temperatures where ferrous iron concentration was approximately 50 mg L^{-1} and Fe_{tot} approximately 700 mg L^{-1}. Ferric iron remained in solution at 7 ºC. The decreased ferric iron precipitation at low temperatures has been previously observed (Leduc et al. 1993). At 50 ºC, all dissolved iron was in ferrous form indicating that iron oxidation and precipitation happened at the same time or there weren't enough active iron-oxidizers. Probably therefore, the leaching rates of valuable metals were low. Also, due to lower solubility of oxygen and carbon at high temperatures, the gas-liquid transfer limitation could have negative impact on bioleaching efficiency.

Higher redox potentials, 800 mV, have been achieved with shake flask experiments with fine-grain ore and metal yields have been significantly higher. (Puhakka and Tuovinen 1986a, b and c, Wakeman et al. 2008). Shake flask experiments simulate more reactor leaching than conditions in heap leaching.

4.4 MICROBIAL COMMUNITY OF BIOLEACHING COLUMNS

Cell counts

Bioleaching columns were inoculated as described in Paper I. Cell counts from the study are presented in Table 19. At 7 ºC leach liquor the total counts (10^8-10^9 cells mL^{-1}) were significantly higher than at other temperatures (10^6-10^7 cells mL^{-1}). In pilot-scale bioheap the cell counts were to some extent lower (10^6 cells mL^{-1}, Table 20). In the study of Wakeman et al. (2008) cell count in leach liquors were in average 10^6-10^7 cell mL^{-1} after 40 weeks of bioleaching.

The cell counts decreased slightly in all leach liquors during the leaching. This was likely due to attachment of cells to the agglomerated ore and to the formed precipitates. Increasing attachment with incubation time of the acidophilic bacterial cells to pyrite ore and ferric hydroxysulphates was shown by Ghauri et al. (2007) though the attachment of *A. ferrooxidans* was slower than the attachment of *L. ferrooxidans*. The cell numbers of ferrous iron-oxidizers in column leach liquors, determined by MPN-Fe, were nearly the same than the total cell count determined by 4',6-diamidino-2-phenylindole (DAPI) - method. In leach residues the amount of total cell counts were 10^3 than that of ferrous iron oxidizers.

At pH 3 and temperature of 50 ºC the difference was 10^5. That probably reflects to high ferrous iron concentration and low leaching rates.

Total cell counts in the leach residues at pH columns at room temperature were about 10^8 cells g ore^{-1}. At 7, 35 and 50 ºC the total cell counts of the leach residues were $3.4 \cdot 10^8$, $1.1 \cdot 10^7$ and $8.7 \cdot 10^6$ cells ore g^{-1}. In the study of Bruhn et al. (1999), cell numbers varied from 0-10^6 cell ore g^{-1} in the mixed copper oxide/sulfide dump leach operation. The decreased amount of cell counts at 50 ºC is in line with leaching rates. In the study of Ahonen and Tuovinen (1992), the column leaching expreriment at 46 ºC was discontinued due to the lack of bacterial activity. It might be that cultures derived from borealic conditions are not active in thermophilic conditions. After re-inoculation (day 65) with a thermophilic *Sulfolobus* culture, leaching at 50 ºC accelerated but slowed down soon. *Sulfolobus* was detected on both times when archaea were analysed with DGGE after re-inoculation day. It was also present at room temperature and at 35 ºC. Columns might have been transmitted or the ore or inoculum contained another *Sulfolobus* strain.

Table 19. Total cell counts as an average from the columns during the bioleaching of the ore at different target pH values at 21 ºC and at temperatures between 7 and 50 ºC at pH 2.5.

	COLUMNS		
pH	**PLS average**	stdev	**Leach residue**
1.5	3.0 x 10^7	5.4 x 10^7	1.5 x 10^8
2.0	4.7 x 10^7	5.1 x 10^7	8.4 x 10^7
2.5	4.0 x 10^7	4.6 x 10^7	2.3 x 10^8
3.0	5.3 x 10^7	3.1 x 10^7	1.2 x 10^8
T (°C)			
7	3.6 x 10^8	2.0 x 10^8	3.9 x 10^8
21	4.0 x 10^7	4.6 x 10^7	2.3 x 10^8
35	7.6 x 10^6	3.0 x 10^6	1.1 x 10^7
50	4.3 x 10^6	2.4 x 10^6	8.7 x 10^6

Table 20. Total cell counts as an average the pilot-scale bioheap during the bioleaching. MH = Man hole, P = Pond, IR = Irrigation, stdev = standard deviation. Samples from ponds were taken from primary heaps, irrigation samples from secondary pilot-scale bioheaps.

PILOT							
Primary heaps			**Secondary heaps**		**Ore Samples**	**Depth**	
PLS	**average**	stdev	**average**	stdev	**T (ºC)**	**(m)**	**average**
MH1	8.1 x 10^5	4.0 x 10^5	8.1 x 10^8	2.0 x 10^5	80-90	1-2	7.8 x 10^5
MH2	8.1 x 10^5	2.6 x 10^6	1.7 x 10^7	3.2 x 10^7	80-90	3-4	9.1 x 10^6
P1	1.1 x 10^6	8.7 x 10^5			65-75	1-2	2.5 x 10^6
P2	4.8 x 10^6	3.3 x 10^6			65-75	4-5	1.5 x 10^6
IR1			2.7 x 10^6	2.1 x 10^6	20-35	0-1	1.9 x 10^7
IR2			8.0 x 10^6	7.1 x 10^6	20-35	4-5	9.7 x 10^6

4.5 COMPOSITION AND DYNAMICS OF MICROBIAL COMMUNITIES

Studies done with the black schist ore harboured a diverse microbial population that consisted of well known acidophilic microorganisms, and a few species that are not closely related to existing GenBank sequences, and may possibly be novel species (Figure 17). Microorganisms that were present in the columns or in the pilot-scale bioheap application more than two times are presented in Table 21 and 22. Microorganisms that were detected only in the beginning, or occasionally only one or two times are not presented. The study shows that they are not the key iron-oxidizers in this bioheap application.

After the data of this study was published (2007), two new species of Acidithiobacilli were described, *A. ferrivorans* (Hallberg et al. 2010) and *A. ferridurans* (Hedrich and Johnson 2013). Genetically these species are very near each other. The 16S rRNA gene sequences of the bands that corresponded 99% of *A. ferrooxidans* AP310 (DQ35518) were identified again in 2015 using the basic local alignment search tool (BLAST). The 16S rRNA gene sequences of *A. ferrooxidans* at temperatures of 7 and 21 ºC corresponded 99% as *A. ferrivorans* SS3 (CP002985). One of the 16S rRNA gene sequences of *A. ferroxidans* strains at 35 ºC corresponded 99% as *A. ferridurans* ATCC 3302 (NR_117036). At 50 ºC, no proper *A. ferroxidans* 16S rRNA gene sequences were gained. The presence of *A. ferroxidans* at 50 ºC was concluded based on the fact that the DGGE band was in the same place as the other bands of *A. ferrooxidans.*

The 16S rRNA gene sequences of *Acidithiobacillus ferrooxidans* strains in pH between 1.5 and 3.0, at 21 ºC, corresponded also 99% as *A. ferrivorans* SS3 (CP002985). The pH range for growth is reported to be 1.9-3.4 (Hallberg et al. 2010). In the light of increased knowledge, these species cannot be separated with the denaturing gradient from 40 to 70% that were used in the DGGE. *A. ferrooxidans, A. ferrivorans* and *A. ferridurans* are able to oxidize both iron and sulphur compounds.

Mixed cultures are often more effective in accelerating metal dissolution than pure cultures (Okibe and Johnson 2004). Community changes seem to be related to dynamics of the main substrate such as ferrous iron availability. The most prominent microorganisms in communities were Gammaproteobacteria (*A. ferrivorans/ A. ferrooxidans* and *A. caldus*) and member of phylym Nitrospira (*L. ferrooxidans*). There is no conclusive explanation why *A. thiooxidans* disappeared after beginning from the column leach liquors. The shift in predominance of *Acidithiobacillus* to *Leptospirillum* coincides with decreasing concentrations of ferrous iron in the leach liquor. Leptospirilli were not detected at 7 ºC.

Temperature gradient incubation revealed that ferrous iron oxidation by the 7 ºC enrichment culture had temperature optima of 22.4 ºC and 32.4 ºC (Paper III). This indicated the presence of both psychrotolerant and/ or mesophilic microorganisms in the culture. This supports the suggestion that *A. ferrooxidans* was actually *A. ferrivorans*, or both species were present. The specific oxidation rates for the culture were similar, with $13.5 \cdot 10^{-8}$ and $12.8 \cdot 10^{-8}$ mg Fe^{2+} $cell^{-1}$ h^{-1} for 22.4 ºC and 32.4 ºC, respectively. By far, *A. ferrivorans* is the only described psychrotolerant acidophilic bioleaching bacteria (Hallberg et al. 2010). *A. ferrivorans* SS3 (99%, CP002985)*/ A. ferrooxidans* AP 310 (96-100%, DQ355183) was the dominant bacteria of the pilot-scale bioheap in primary and secondary leaching phase. These species are genetically very near each other and probably both existed in the bioheap application.

At the end of the column experiment (around 350 d), a bacterium related to *L. ferriphilum* D1 (99 %, DQ665909) was seen at pH from 1.5 to 2.5. It has been previously detected in bioleaching tank reactors with high ferric iron concentrations (Coram and Rawlings, 2002). Leptospirilli have generally been described to dominate in high redox potential and ferric iron concentration (Rawlings et al. 1999,

Demergasso et al. 2005). *L. ferriphilum* dominated the microbial consortium at 37 ºC with Talvivaara ore for the greater part during the bioleaching in the study of Wakeman et al. (2008).

Sb. thermotolerans KR-1 (99%, DQ124681) was the major species at 50 ºC during the bioleaching and were detected in the leach liquors at pH values from 1.5 to 3.0. The reported optimum temperature of *Sb. thermotolerans* is 40 ºC and growth range 20-60 ºC (Bogdanova et al. 2006). *Sb. thermosulfidooxidans* N19-50-01 (99-100%, EU499919) was found from the high temperature zones of the pilot-scale heap. *Sulfobacillus* is commonly present in bioheap operations but is not considered to be the main player. In the study of Cameron et al. (2010) *Sulfobacillus* spp. were dominant in the bioreactor at 45 ºC. In pilot-scale bioheap a novel bacterium related to clone H70 (91%, DQ328625) was present nearly in all samples. The DGGE band was cut out, DNA isolated, PCR amplified and sequenced and submitted to GenBank (accession JQ941953). The phylogenetic analysis (Figure 17) revealed that the novel bacterium belonged to the Firmicutes. The role is possibly important and that would need future research.

Detectable archaea in the column leaching study (Table 21) were *Ferroplasma acidiphilum* DR1 (98%, AY222042), a species related to an uncultured archaeon clone ant b7 (99%, DQ303249, nearest known species *Thermoplasma acidophilum* DSM1728, 91%, AL445067) and *Sulfolobus metallicus* DSM 6482 (98%, SM16SRRN1). Surprisingly, *S. metallicus* was present at room temperature. The growth temperature for *S. metallicus* has been reported by Huber and Stetter (2001) of 50-75 ºC, with a growth optimum at 65 ºC. For attempting to increase the bioleaching rate at 50 ºC, the column was re-inoculated with a *Sulfolobus* culture on day 65. The growth temperature of that culture was reported to be 35-76 ºC. (Salo-Zieman et al. 2006). No mesophilic *Sulfolobus* spp. has been described (Salo-Zieman et al. 2006). Columns at 21, 35 ºC and at pH 3.0 might have been transmitted from the re-inoculation of the column at 50 ºC, or the ore or the original inoculum contained another *Sulfolobus* strain. The uncultured archaeon clone ant b7 (99%, DQ303249) was present in all of the leach liquors except at pH 1.5. *Ferroplasma acidiphilum* DR1 (98%, AY222042) that can oxidize only iron, was present at pH 2.5 and 2.0, and in all temperatures, expect at 35 ºC. In the pilot-scale bioheap the archaeal species present were related to uncultivated species, from which, one was related to *Thermoplasma acidophilum* (91-93%).

Table 21. Micro-organisms detected from the columns during the bioleaching of the ore at different target pH at 21 ºC and at temperatures between 7 and 50 ºC at pH 2.5. RT = Room temperature, LR = Leach residue.

Time (months)	Sample/ Species	*Acidithiobacillus ferrooxidans* AP 310 (96-100%) / *A. ferrivorans* SS 3 (99%)	*Acidithiobacillus ferrooxidans* AP 310 (96-100%) / *A. ferridurans* ATCC 3302 (99%)	*A. caldus* MTH-04 (96-99%) or *A. thiooxidans* ORC8 (99 -100%)	*Sulfobacillus thermotolerans* KR-1 (99%) or *Sb. thermosulfidooxidans* N19-50-01 (100%)	Bacterium related to clone H70 (91%)	*Leptospirillum ferrooxidans* DSM 2705 (98-100%)	*Ferrimicrobium acidiphilum* T23 (98%)	*Leptospirillum ferriphilum* D1 (99%)	Gram-positive iron-oxidizing acidophile G1 (99%)	*Ferroplasma acidiphilum* DR1(98%)	Uncultured archeon ant b7 99%, nearest known species *Thermoplasma acidophilum* DSM 1728 (91%)	*Sulfolobus metallicus* DSM 6482 (98%)
1	**pH 1.5, 2.0, 2.5, 3.0 and RT (columns)**	++		+	+		++						
2		++			+		++						
4		+					++				++	++	++
6		+					++						
8											+	+	
10		++					++		++				
14		++					++			+			
18		++					++	+		+			
LR		++					++			+			
1	**7 ºC (column)**	++		++									
4		++									+	++	
8												+	
10		++											
14		++											
18		++											
LR		++						++		++			
1	**35 and 50 ºC (columns)**		+	++			++						
4			+				++				+	++	+
8													
10					+		+		++			+	+
14					+		+		++				
LR			++		+				++				

Table 22. Micro-organisms detected from the pilot-scale bioheap during the bioleaching. MH = Man hole, P = Pond, IR = Irrigation. Samples from ponds were taken from primary heaps, irrigation samples from secondary bioheaps.

Time (months)	Sample/ Species	*Acidithiobacillus ferrooxidans* AP 310 (96-100%) / *A. ferrivorans* SS 3 (99%)	*Acidithiobacillus ferrooxidans* AP 310 (96-100%) / *A. ferridurans* ATCC 3302 (99%)	*A. caldus* MTH-04 (96-99%)	*A. thiooxidans* ORC8 (99 -100%)	*Sulfobacillus thermotolerans* KR-1 (99%) or *Sb. thermosulfidooxidans* N19-50-01 (100%)	Bacterium related to clone H70 (91%)	*Leptospirillum ferrooxidans* DSM 2705 (98-100%)	*Alicyclobacillus acidocaldarius* (98 %) or *Ab. tolerans* (100 %)	Uncultured crenarchaeote clone JG36-GR-88 (100%)	Uncultured archeon ant b7 99%, nearest known species *Thermoplasma acidophilum* DSM 1728 (91%)	Uncultured archaeon SAGMA-X (99%)
1	**MH1 and MH2 (pilot)**	++		+		++			+			
4		+		+		++	+					
5		++		+		++	++				++	
6		++				++	++	++				
7		+					+					
8		++					+					
9		++					+	+				
10		++					+	++				
12		++				+		+				
15		++		+			+	++				
16		++					++	+				
17		++					++	+				
4	**P1 and P2 (pilot)**	++		+		++	++		+	++		++
5		++				++	+			++	++	++
6		++				++	+	++				
7		++				+	+					
8		++					+	+				
9		++					+	++				
10		++					+	++				
ORE	**80-90 ºC (pilot)**	+			+		+			*na		
ORE	**65-75 ºC (pilot)**	+		+	+							
ORE	**20-35 ºC (pilot)**	+		+			+		+			
12	**IR1 and IR2 (pilot)**	+				+				*na		
15		++		+			++	+				
16		++		+			++	+				
17		++		+			++	+				

*na= not analyzed

4.5.1 Community structure analysis

To determine the phylogenetic diversity, a phylogenetic tree was generated. *A. brierleyi* was used as outgroup to root the tree. The scale bar represents 0.2 nucleotide substitutions per nucleotides. 16S rRNA gene sequences of bacteria were distributed into four main phyla Firmicutes, Actinobacteria, Proteobacteria and Nitrospira. Microorganisms that were detected several times during the study (columns and pilot-scale bioheap) are presented.

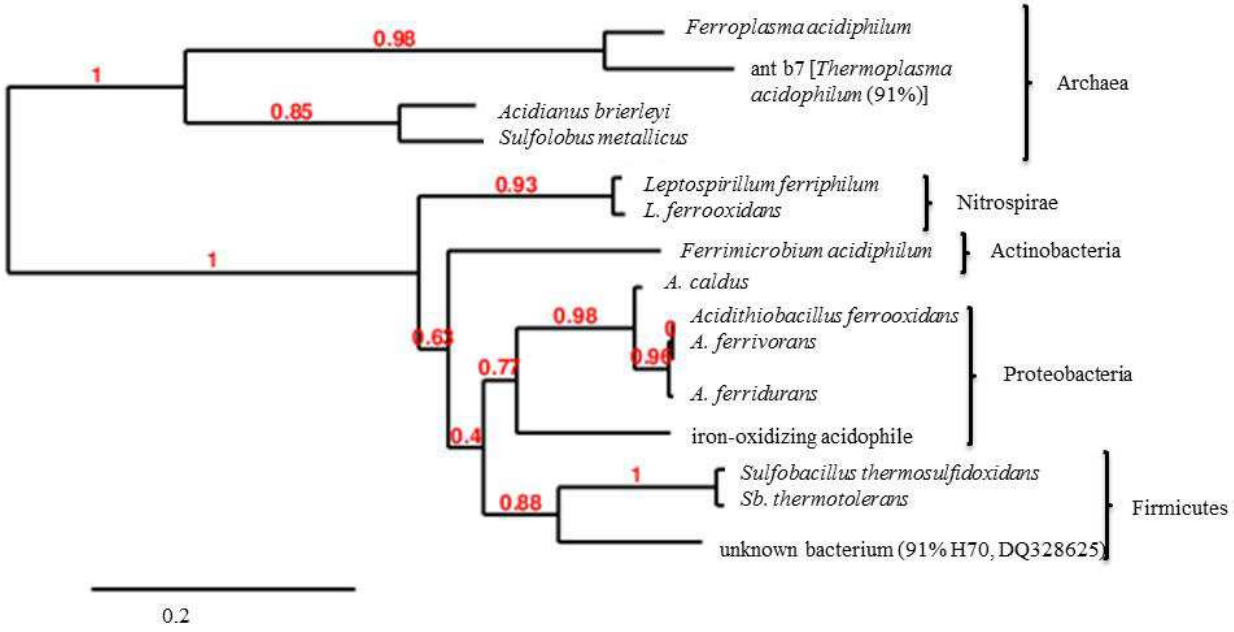

Figure 17. The phylogenetic tree generated using distance matrix and neighbour joining methods based on the 16S rRNA. *A. brierley* was used as outgroup. Numbers at nodes represent bootsrap values based on 1 000 samplings. The scale bar indicates the estimated number of base changes per nucleotide sequence position.

5. CONCLUSIONS

The bioleaching of the complex multimetal black schist ore containing mainly pyrrhotite and pyrite together with minor pentlandite and chalcopyrite originating from the Sotkamo deposit, Finland was studied.

Dissolution of nickel and zinc were mostly affected by the pH value of the irrigation solution. The fastest bioleaching and greatest yields of valuable metals were achieved at pH 1.5. Nickel and zinc leaching rates and yields decreased nearly linearly as pH increased. Nickel solubilization was 3-4 times faster at pH 1.5 than at pH 3.0, being 0.42 and 0.13 % (Ni) d^{-1}, respectively. The maximum metal yields were achieved after 140 days at pH 1.5, and were 59 for % Ni, 52 % for Zn, 13 % for Cu and 16 % for Co. At pH 2.0 maximum yields were achieved over 230 days (54 % for Ni, 37 % for Zn, 13 % for Cu and 12 % for Co). Copper did not bioleach at high pH (2.5-3.0). That was probably due to the passivation of chalcopyrite. After the beginning, no further cobolt dissolution happened at pH 3.0. Low concentrations of ferrous iron demonstrated the activity of iron-oxidizers at all room temperature columns (pH 1.5-3.0). The concentration of ferric iron in solution increased significantly with the decrease in pH. At pH 1.5, ferric iron concentration increased all the time, being 36 g l^{-1} after 140 days. At pH 2.0 the ferric iron concentrations varied, being highest 3.8 g l^{-1} after 97 days. At pH 2.5 and 3.0 the total dissolved iron remained low throughout the experiment due to iron precipitation. The redox potential (Pt electrode against an Ag^0/AgCl reference) varied between 515 and 580 mV, being highest at pH 2.0. The study confirms that the ferric iron and the activity of hydrogen ions were the driving forces of metal solubilisation of the complex multimetal black schist ore. Leaching was acid consuming in all conditions. The acid consumption of the ore overdrive the production of hydrogen ions by sulphur-oxidizers. Leaching at low pH resulted in increased acid consumption of 160 and 38 H_2SO_4 g kg^{-1} ore at pH 1.5 and 2.0 after 140 days. Temperature, at pH 2.5, had also effect on acid consumption. At 50 ºC acid consumption was highest and lowest at 21 ºC, being 29 and 8 H_2SO_4 g kg^{-1} ore, respectively.

Temperature affected significantly to the metal yields (at pH 2.5). The highest yields were obtained at room temperature (21 ºC). After 153 days the maximum yields were 26 % for Ni, 18 % for Zn, 1 % for Cu and 6 % for Co. The redox increased during the first two months at 7 ºC and reflected the start of ferrous iron oxidation and microbial activity. After that ferric iron was present all the time at 7 ºC and this demonstrated that more ferric iron was available for the oxidation of the mineral sulphide than at other temperatures. The leach liquor redox potential stabilized to 500-600 mV (Ag^0/AgCl reference) at 7 ºC after 40 days and at 21 ºC right after beginning. Leaching at low temperature (7 ºC) resulted in yields of 24 % for Ni, 17 % for Zn, 2 % for Cu and 6 % for Co after 496 days. The Cu leaching increased all the time during the experiment at 7 ºC, while at other temperatures it slowed down after 100 days. The leach liquor redox potential at 35 ºC and at 50 ºC varied between 300-500 mV. At 50 ºC, after 50 days Fe^{2+} and Fe_{tot} were both 350 mg L^{-1}. The lack of soluble ferric ion, i.e. presence of iron oxide precipitates, slowed down the bioleaching of valuable metals. After re-inoculation (day 65) with a thermophilic *Sulfolobus* culture, leaching at 50 ºC accelerated but slowed down soon and resulted in maximum yields 18 % for Ni, 11 % for Zn, 0.3 % for Cu and 2 % for Co (after 140 days). In the column leaching study, after the maximum yields, longer leaching time did not result more metals in solutions.

The evaluation of metal solubilisation at higher temperatures than tested, cannot be done with the used inocula that included mostly mesophilic microorganisms. In the real bioheap microorganisms are often indigenous and may include thermophilic bacteria as in the pilot-scale bioheap.

Dissolution of gangue minerals was highest at lowest pH. Large amounts of aluminium, manganese and silicon were leached. After 60 days of bioleaching, the leach liquor at pH 1.5 became jelly like and after 110 days Si reached the concentration of 2.96 g L^{-1}. Amorphous precipitates have the potential to interfere liquid flow in heap leaching and subsequent recovery of base metals. Althought, Si did not affect the solubilization of valuable metals, the pH value of the PLS needs to be kept above 1.5, to prevent these problems. The dissolution of silicate minerals also increases the pH and thus, sulphuric acid consumption can be the major cost. At pH 2.5 less than 200 mg L^{-1} Si was solubilized and different temperatures had no effect on Si dissolution at that pH. After the column blockages, (columns at pH 2.0 and pH 2.5 after 82d, and column at 35 ºC after 117d) about 200g of the ore did not fit back to each of the column. Clay minerals, that are expansible, were likely formed. Blockages are easily managed in laboratory conditions. In real heaps, blockages cause severe problems in aeration, irrigation and overall bioheap operation.

The pH value of the column leach liquor did not affect significantly to cell numbers. At 7 ºC leach liquor the total counts (10^8-10^9 cells mL^{-1}) were significantly higher than at other temperatures (10^6-10^7 cells mL^{-1}). In pilot-scale bioheap, the cell counts were to some extent lower (10^6 cells mL^{-1}). The cell counts decreased slightly in all column leach liquors during the leaching. This was likely due to attachment of cells to the agglomerated ore and to the formed precipitates. Total cell counts in the leach residues at room temperature were about 10^8 cells g ore^{-1}. At 7, 35 and 50 ºC the total cell counts of the leach residues were $3.4 \cdot 10^8$, $1.1 \cdot 10^7$ and $8.7 \cdot 10^6$ cells ore g^{-1}, respectfully.

In conclusion leaching rates of valuable metals were increased, when ferric iron was in solution, the amount of active iron-oxidizers were suffient and dissolution of Si were moderate. Leaching solution pH of 2.0 was recommended for a bioheap application. The two demonstration-scale bioheaps (17 000 t) at the Talvivaara mine site were operated and monitored by Talvivaara Mining Company for 30 months. After the start-up of heap irrigation, oxidation of pyrrhotite and pyrite increased the heap temperature in central locations up to 90 ºC. In the second winter temperatures inside the heaps decreased being still 80 ºC at the hottest spots. Leach liquor temperatures varied between 60 ºC and 15 ºC over the whole operation period. The target pH of the PLS was 2.0. Inspite of continuous titration pH varied during the 10 months between 3.5 and 3.0 and after that between 3.0 and 2.5.

Bioleaching with the black schist ore (column bioleaching and pilot-scale bioheap) harboured diverse microbial population that consisted well known acidophilic microorganisms, and few species that were not closely related to existing GenBank sequences, and may possibly be novel species. Community changes seem to be related to the main substrate such as ferrous iron availability and temperature. The most prominent microorganisms in communities were Gammaproteobacteria, *A. ferrivorans* (99%)*/ A.ferrooxidans* (99%) and *A. caldus* (95-99%), and member of phylym Nitrospira, *L. ferrooxidans* (98-100%). After the data of this study was published (2007), two new *Acidithiobacillus* species of were described, *A. ferrivorans* and *A. ferridurans*. Genetically these species are very near each other. The 16S rRNA gene sequences of the bands that corresponded 99% of *A. ferrooxidans* AP310 (DQ35518) were identified again in 2015 using the basic local alignment search tool (BLAST). Most of the 16S rRNA gene sequences of *A. ferrooxidans* corresponded 99% also as *A. ferrivorans* SS3 (CP002985). In the light of increased knowledge, these species cannot be separated with the denaturing gradient from 40 to 70 % that were used in the DGGE.

In general, in column leaching study, temperature affected the microbial community structure more than pH did. The dominant species at 7 ºC were unique, whereas at room temperature microbial community exhibited similarity. Also higher temperatures (35 and 50 ºC) showed similarities. *A. ferrivorans* (99%)*/ ferrooxidans* (99%) was the dominant microorganisms at 7 ºC. The shift in predominance of *Acidithiobacillus* to *Leptospirillum* coincides with decreasing concentrations of ferrous iron in the leach liquor. At the end of the column experiment (around 350 d), a bacterium

related to *L. ferriphilum* (99%) was seen at pH from 1.5 to 2.5. Leptospirilli have generally been described to dominate in high redox potential and ferric iron concentration. *Sb. thermotolerans* (99%) was the major species at 50 ºC during the leaching and was also detected in the leach liquors at pH from 1.5 to 3.0. *Sb. thermosulfidooxidans* (100%) was found from the high temperature zones of the pilot-scale heap. *Sulfobacillus* is often present in bioheap operations but is not considered to be the main player.

In pilot-scale bioheap, large temperature gradients resulted in the simultaneous presence of mesophilic and thermophilic iron- and sulphur-oxidisers. During the first six months the microbial communities of the leach liquors were diverse and dominated by *A. ferrivorans* (99%)*/ A. ferrooxidans* (99%), *Sb. thermosulfidooxidans* (100%) and a novel bacterium related to clone H70 (91%). After 6 months of bioheap operation *L. ferrooxidans* (100%) was first observed and it was present thereafter in nearly all samples. The microbial diversity in both heaps varied and decreased with time, with *A. ferrivorans* (99%)*/ A. ferrooxidans* remaining as the dominant bacterium and the novel bacterium related to clone H70 (91%) being present. The role of that novel bacterium is probably important and would need future research. In the secondary leaching phase, it was present with *A. ferrivorans* (99%)*/ ferrooxidans* (99%) and *L. ferrooxidans* (98%).

Detectable archaea in the column leaching study were *Ferroplasma acidiphilum* (98%), a novel species that was related to an uncultured archaeon clone ant b7 (nearest known species *Thermoplasma acidophilum*, 91-93%) and *Sulfolobus metallicus* (98%). *Sulfolobus* was present at 21, 35 and at 50 ºC. No mesophilic *Sulfolobus* spp. has been described (Salo-Zieman et al. 2006). Columns might have been transmitted from the re-inoculation of the column at 50 ºC, or the ore or inoculum contained another *Sulfolobus* strain. In the pilot-scale bioheap the archaeal species present were related to uncultivated species, from which, one was related to *Thermoplasma acidophilum* (91-93%).

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Heap bioleaching of a complex sulfide ore Part I: Effect of pH on metal extraction and microbial composition in pH controlled columns

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Heap bioleaching of a complex sulfide ore
Part I: Effect of pH on metal extraction and microbial composition in pH controlled columns

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ABSTRACT

The effect of pH on the bioleaching of a low-grade, black schist ore from Finland containing pyrrhotite, pyrite, pentlandite, chalcopyrite and other mineral sulfides was studied using columns containing 9.0 kg of agglomerated ore that was irrigated with nutrient supplemented surface water from the deposit at ambient temperature. Iron and sulfur-oxidizing enriched culture was used to inoculate the columns. Iron oxidation and metal leaching increased with decreasing pH. At pH 1.5, 59% Ni was bioleached after 140 days together with 59% Zn, 13% Cu and 16% Co. In comparison, at pH 3.0 only 15% Ni, 10% Zn, 5% Cu and 0.5% Co were leached; while at pH 2, 53% Ni was bioleached after 230 days. Based on an optimization between the leaching of metals, the acid consumption, the concentration of soluble iron and the dissolution of other gangue minerals, leaching at pH of 2.0 were recommended for this heap bioleaching application.
The microbial composition as determined by a combination of Polymerase Chain Reaction (PCR)–Denaturing Gradient Gel Electrophoresis (DGGE)-sequencing approach was not significantly affected by pH. *Acidithiobacillus ferrooxidans* and *Leptospirillum ferrooxidans* were the dominant species in all the leach liquors. In addition, *L. ferriphilum* was detected for the first time in extracted leach residue liquor after 300 days of bioleaching. *Sulfobacillus thermotolerans*, *A. caldus*, *A. thiooxidans* and some unknown species were found to lesser extent. Archaeal species were also present in all leach liquors.

1. Introduction

Biohydrometallurgical processes are widely used for the recovery of gold and copper (Brierley and Brierley, 2001; Morin et al., 2006; Watling 2006) and similar techniques for low-grade nickel sulfide ores are under development (Dew and Miller, 1997; Rawlings, 2002; Watling, 2008). The bioleaching applications involve the presence of acidophilic microorganisms that grow between pH 0 and 3 and oxidize ferrous ion to ferric ion to generate solutions with relatively high redox potential that leach the sulfide minerals. However, ferric ion easily hydrolyzes and forms iron(III) hydroxides or jarosite. Precipitation of jarosite has been reported even in the pH range of 1.35–1.5 (Boon, 2000).

Due to a concurrent dissolution, precipitation, oxidation and reduction reactions, both acid consumption and acid production prevails in bioleaching processes (Riekkola-Vanhanen et al., 2001). Many gangue minerals such as chlorite, potassium- and calcium-feldspar are acid-consuming and agglomeration of the ore with sulfuric acid reduces major pH changes at the beginning of leaching (Rawlings et al., 2003; Strömberg and Banwart, 1999). In practice, it is difficult to maintain the solution pH within the desired range without constant pH adjustment and sulfuric acid consumption can be a major operating cost (Watling, 2006).

Bioleaching of nickel bearing complex black schist sulfide ore from Sotkamo deposit, Finland was demonstrated in the early studies of Puhakka and Tuovinen (1986a,b,c). In later studies, heap bioleaching was simulated in columns where the leach liquor percolated through the ore material by gravity (Riekkola-Vanhanen and Heimala, 1999; Wakeman et al., 2008). The aim of the present work was to study the effects of pH (1.5–3.0) on bioleaching this ore using bench scale columns and to compare the dynamics of the microbial community structure over time.

2. Materials and methods

2.1. Ore

The mineral composition of the sulfide component of the ore was 61.2% pyrrhotite (FeS), 24.3% pyrite (FeS_2), 5% pentlandite [$(Fe_x/Ni_{9-x})_9S_8$], 6.5% alabandite (MnS) and 2.4% chalcopyrite ($CuFeS_2$). Valuable metal contents were 0.29% Ni, 0.53% Zn, 0.20% Cu and 0.035% Co. The mineral with the highest nickel content was pentlandite that

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contained about 80% of the total nickel present in the ore. The rest of the nickel was present in pyrrhotite and pyrite. Zinc was present as sphalerite [(Zn,Fe)S], copper as chalcopyrite and cobalt was found in pyrite, pyrrhotite and pentlandite. The crushed ore (approximately 17% 8–12 mm, 38% 3.15–8 mm and the rest 0.020–3.15 mm) was agglomerated using dilute sulfuric acid (pH 1.5), in order to bind fine particles to the surfaces of the coarser particles. The agglomerated ore was kindly provided by the Geological Survey of Finland.

2.2. Enrichment culture

The enrichment culture used for the inoculation of the bioleaching columns was obtained by combining several acidic water samples from the Sotkamo ore deposit. The pH of the water samples varied between 3 and 4 and samples contained red–brown iron(III) precipitates. The cultures were first enriched in shake flasks at 25 °C in three different acidic media. All the media contained basal salts (0.4 g L^{-1} each K_2HPO_4, $(NH_4)_2SO_4$, $MgSO_4 \cdot 7H_2O$), supplemented with either ferrous ion (4.5 g L^{-1}), elemental sulfur (1% wt/vol) or black schist ore powder (1% w/v) from the Sotkamo deposit. Basal salts were diluted with the surface water originating from the deposit containing about 20 mg L^{-1} dissolved organic matter and solutions were acidified to pH 1.8 with concentrated sulfuric acid. Basal salts media were sterilized by autoclaving (30 min, 121 °C). Sulfur was sterilized by heating at 105 °C for at least 24 h. For addition of ferrous ion, the stock solution of 112 g L^{-1} $FeSO_4 \cdot 7H_2O$ at pH 1.8 was prepared and sterilized by filtration through 0.2 μm polyvinylidene fluoride membrane (Whatman). Sulfur and the ferrous ion solution were aseptically added to the medium. Ore powder was sterilized with the basal salts. After inoculation (10% v/v), the suspensions were incubated at 180 rpm min^{-1} at 25 °C.

After one month of incubation the enrichment cultures were combined with the medium. The enrichment culture was sub-cultured three times on this medium prior to transferring to the bioleaching columns. Microbial growth was monitored by phase contrast microscopy (Zeiss Axioskop 2).

2.3. Bioleaching column and start-up

Four columns at different target pH values (1.5, 2.0, 2.5 and 3.0) containing about 9 kg of agglomerated ore were set up. The description of the columns used in the study is given in Halinen et al. (2009-this issue). The columns were inoculated with 200 mL of the microbial solution and the percolated solutions were collected from the bottom of the column. The cells were counted before and after inoculation by 4′, 6-diamidino-2-phenylindole (DAPI) staining and Most Probable Number (MPN) technique adapted for mesophilic iron oxidizers. After inoculation, containers equipped with pH electrodes and magnetic stirrers were placed below the columns and leach liquor (5 L), consisting of surface water from the Sotkamo deposit supplemented with basal salts, was used in each column. The pH was adjusted by continuous titration (Metrohm 719 S Titrino) with concentrated sulfuric acid. The ore in the column was irrigated at the rate of 10 L m^{-2} h^{-1} by liquid recirculation. Aeration was provided through a diffuser inserted at the base of the column blown at the rate of (8–11) m^3 m^{-2} h^{-1}.

2.4. Monitoring metal concentrations and cell counts

The recycle of the leach liquors were initially monitored on a weekly basis and later every second week for pH, redox (Pt electrode Ag^0/AgCl reference), dissolved oxygen (WTW CellOx 325), ferrous ion and soluble Fe, Zn, Ni, Co and Cu. The column and leach liquor temperatures, sulfuric acid consumption and leach liquor pH values were recorded five times per week. The leach liquors of the columns were sampled once a month to estimate the total numbers of microorganisms and every second month to characterize the microbial communities. Samples of leach liquor were replaced with equal volumes of sterile basal salt solution. Losses due to evaporation were estimated on a volumetric basis and compensated by adding distilled water on a day before sampling.

Soluble metals were determined according to the Finnish Standard Methods SFS 3044 and SFS 3047 (Anon., 1980a,b) by atomic absorption spectrophotometer AAS (Perkin Elmer 1100B) and once a month with inductively coupled plasma ICP-AES (Thermo Elemental, USA). Ferrous ion concentrations were determined using the UV 1601 spectrophotometer (Shimadzu Europa GmbH) by the colorimetric *ortho*-phenanthroline method, according to Standard Methods Method no. 3500-Fe (Anon., 1992) modified as follows: 100 μL of concentrated HCl, 900 μL MQ-water, 2 mL of 1,10-phenanthroline (10 g L^{-1}) and 1 mL of ammonium acetate buffer were added to 1 mL of sample.

Total cell counts were estimated with 4′, 6-diamidino-2-phenylindole (DAPI) staining technique using epifluorescence microscopy. Viable counts of mesophilic iron-oxidizing bacteria in the sample were estimated with Most Probable Number technique (MPN) modified from the Finnish Standard Methods SFS 4447 (Anon., 1979). The samples were diluted 10^{-1}–10^{-8}-fold with basal salts solution and 20 μL of the diluted sample was added to a well containing 180 μL of the growth medium on a multiple well plate. Each dilution was analyzed in five parallel wells. Plates were incubated for four weeks at 25 °C in a box moisturized with wet hand towels to prevent wells from drying.

2.5. Microbial community analysis

The microbial communities were investigated by Polymerase Chain Reaction (PCR)–Denaturing Gradient Gel Electrophoresis (DGGE); followed by partial sequencing of 16S rRNA gene. For DNA extraction, a 15–20 mL sample was taken from column liquor and filtered immediately on a 0.2 μm pore size polycarbonate filter (Cyclopore Track Etched Membrane, Whatman). The filters were rinsed with 0.9% (w/v) NaCl at pH 1.8 for 1 min to remove the excess metals and then neutralized with 40 mM Na–EDTA in phosphate buffered saline (PBS; 130 mM NaCl, 5 mM Na_2HPO_4, and 5 mM NaH_2PO_4 adjusted to pH 7.2) for 1 min. The filters were stored at −20 °C prior to nucleic acid extraction. DNA was extracted from preserved filter samples with a Power Soil DNA isolation kit or Ultra Clean Soil DNA Isolation Kit (MoBio Laboratories, Inc.) according to the manufacturer's instructions for DNA yield maximization.

The crude DNA was used as a template for PCR. Partial 16S rRNA genes (550 bp) were amplified using bacterial-specific forward primer 357F 5′-CCT ACG GGA GGC AGC AG-3′ (*E. coli* 16S rRNA gene positions 341–357, Muyzer et al., 1993) and universal reverse primer 907R 5′-CCG TCA ATT CMT TTG AGT TT-3′ (*E. coli* 16S rRNA positions 907–926, Muyzer et al., 1996). The forward primer had a GC-clamp in the 5′-end (5′-GCG CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G-3′) to stabilize the melting behavior of the DNA fragments in the DGGE (Muyzer et al., 1993).

PCR reaction mixtures contained 5 μL of 10×PCR buffer IV (200 mM $(NH_4)_2SO_4$, 750 mM Tris–HCl, and 0.1% (vol/vol) Tween, pH 8.8), 2 mM $MgCl_2$, 100 μM deoxynucleoside triphosphates, 0.5 μM of each primer, 0.8 U of Dynazyme II DNA polymerase, 400 ng bovine serum albumin (BSA) μL^{-1}, and sterile ddH_2O water to a final volume of 50 μL, to which 1–2 μL of template was added. PCR was performed using the following temperature program: initial denaturation at 95 °C for 5 min, 31 cycles of denaturation at 94 °C for 0.5 min, annealing at 50 °C for 1 min and extension at 72 °C for 2 min, followed by final extension at 72 °C for 10 min. The PCR products were checked with 1% (wt/vol) agarose gel electrophoresis using ethidium bromide staining (final concentration 1 μg L^{-1}) prior to DGGE.

Archaea were characterized using nested PCR approach. First partial 16S rRNA gene (933 bp) was amplified using the *Archaea*-specific primers (Jurgens et al., 2000) ArUn4F 5′-TCY GGT TGA TCC TGC CRG-3′ (*E. coli* 16S rRNA positions 8–25, Hershberger et al., 1996)

and Ar958R 5′-YCC GGC GTT GAV TCC ATT T-3′ (*E. coli* 16S rRNA positions 958–976, Delong, 1992). The inner-PCR (601 bp) was conducted using the product from the former, outer-PCR as a template and *Archaea*-specific primers GC-ArchV3F 5′-CCC TAC GGG GYG CAS CAG-3′ (*E. coli* 16S rRNA positions 340–357, Øvreås et al., 1997) and Ar958R. The forward primer included a GC-clamp. Otherwise, the PCR mixture was the same as for bacterial PCR. The outer-PCR was performed using the following temperature program: initial denaturation at 95 °C for 4 min, 40 cycles of denaturation at 92 °C for 1 min, annealing at 57 °C for 1 min and extension at 72 °C for 2 min, followed by final extension at 72 °C for 10 min. The inner-PCR was performed as the outer-PCR, but initial denaturation was at 94 °C for 1 min. Annealing was at 61 °C and number of cycles 32. The PCR products were checked as described above. DGGE was performed with the INGENYphorU2×2-system (Ingeny International BV, The Netherlands) as previously described (Dopson et al., 2007b). Individual bands were excised from the gel with a sterile scalpel and eluted in 20 μL of DNase and RNase free water overnight at 4 °C and then stored at −20 °C. Aliquots (1–2 μL) of the eluate were used as templates in bacterial PCR with primers 357F (without GC-clamp) and 907R. Archaeal templates were amplified with ArchV3F and Ar958R. The PCR mixture (BSA replaced with sterile water) and the PCR programs described above were used. Before being sequenced, each PCR product was run in a 1% (w/v) agarose gel stained with ethidium bromide to confirm the size and the concentration of the product. The PCR products were purified and sequenced at the DNA Sequencing Facility, Institute of Biotechnology, Helsinki University. To identify the microbes, the sequence data was compared with 16S rRNA gene sequences in the GenBank database using the basic local alignment search tool (BLAST; http://www.ncbi.nlm.nih.gov/blast/, Altschul et al., 1997).

2.6. Microbial analysis of the leach residue

Bacterial species of the leach residue were analyzed at the end of the experiment. Circulation of the leach liquor was stopped one day prior to ore removal from the column. The ore samples (15 g each) were taken by squaring according to the Finnish Standard SFS-EN 932-2 (Anon., 1997). The sample was mixed with 40 mL of sterile Zwittergent-washing solution (0.38 g L^{-1} EGTA, 3.35^{-4} g L^{-1} Zwittergent, 3.73 g L^{-1} KCl, pH adjusted to 2.5 with 2 M HCl). The mixture was shaken and sonicated 5×1 min in order to detach microorganisms from ore particles. Thereafter, the sample was allowed to settle for about 30 min to prevent the small ore particles from interfering with DAPI staining and DNA extraction. 15–20 mL of supernatant was filtered for DNA extraction. Microbial numbers were counted from supernatant in order to estimate the amount of attached cells. If no respectable PCR product was gained using primers 357F and 907R, a nested PCR approach was used. The forward bacterial-specific primer 27F 5′-AGA GTT TGA TCM TGG CTC AG-3′ (*E. coli* 16S rRNA positions 8–27, Lane, 1991) and the reverse universal primer 1099R 5′-GGG TTG CGC TCG TTG-3′ (*E. coli* 16S rRNA positions 1099–1114, Lane, 1991) were used to perform outer-PCR. The outer-PCR was done using the following touchdown temperature program: initial denaturation at 95 °C for 15 min, 30 cycles of denaturation at 94 °C for 1.0 min, annealing starting at 53 °C for 0.5 min and extension at 72 °C for 2 min. Annealing temperature was decreased 0.1 °C/s to 48 °C, followed by final extension at 72 °C for 10 min. The inner-PCR primers (357F with GC-clamp and 907R) and the PCR program were as described above.

2.7. Leach residue analysis

The solid residues of the ore from columns at pH 2.0 and 2.5 were studied after 80–90 days of bioleaching after the columns became blocked at the bottom. Circulation of the leach liquors was stopped and the solutions were allowed to flow out of the columns. The mineralogical compositions of solid samples were examined by X-ray diffraction (XRD) (Siemens AG D500) and Scanning Electron Microscopy (SEM) (Stereoscan 360, Cambridge Instruments) coupled with an energy dispersive spectroscope (EDS) (INCA-energy ISIS-300, Oxford Instruments) at Outokumpu Research Oy.

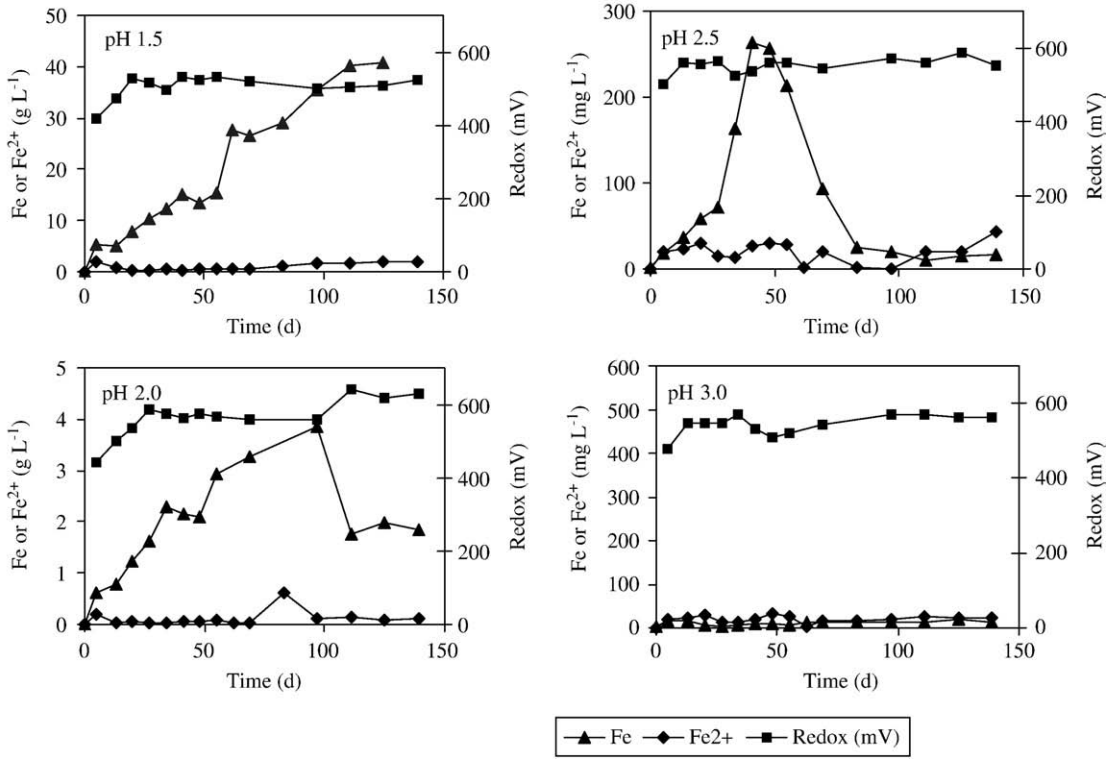

Fig. 1. Redox potentials and the concentrations of total dissolved iron and ferrous ion in leach liquors during the bioleaching of the ore at different pH values.

3. Results and discussion

3.1. H_2SO_4 consumption during bioleaching

The acid consumptions to obtain desired pH levels of 1.5, 2.0, 2.5 and 3.0 were determined for the black schist ore. Acid was steadily consumed in all columns and the pH initially climbed to pH 4–5 before slowly falling. After 140 days of bioleaching leach liquor pH values were 1.5, 2.2, 3.2 and 3.5 after continuous titration of the leach liquor and the cumulative acid consumptions were 160, 38, 8 and 3 g kg^{-1} ore at the respective pH values. Considering the heap bioleaching application, the sulfuric acid consumption at pH 1.5 is too clearly high for the process to be economical. In the study of Hallberg et al. (2007), the pH was adjusted to 1.8 entering the 110 ton-scale column filled with the agglomerated black schist ore and averaged pH 2.7 when leaving the column.

3.2. Redox potential and the fate of iron

Redox potentials and iron concentrations during the bioleaching are presented in Fig. 1. The redox potentials in the column leach liquors varied between 515 mV at pH 1.5 and 580 mV (Ag^0/AgCl reference). The concentrations of ferric and total dissolved iron increased steadily at pH 1.5 and 2.0. At pH 1.5, the average concentration of ferrous ion after 140 days of bioleaching was 800 mg L^{-1} while ferric ion was 20.6 g L^{-1}, which may cause problems in metal recovery process. At pH 2, the concentration of total iron was significantly lower (2.1 g L^{-1}, including 140 mg L^{-1} Fe(II)), while at pH 3, total iron remained below 25 mg L^{-1} throughout the experiment due to brown iron(III) oxide precipitates on the surfaces of the ore material. However, Riekkola-Vanhanen and Heimala (1999) concluded that iron precipitation did not interfere with the bioleaching of a black schist ore.

3.3. Bioleaching of metals

The bioleaching of Ni, Cu, Zn and Co from the ore are presented in Fig. 2. After 140 days at pH 1.5, the concentrations in solution were 2.64 g L^{-1} Ni, 4.20 g L^{-1} Zn, 0.37 g L^{-1} Cu and 0.08 g L^{-1} Co corresponding to 59% Ni, 52% Zn, 12% Cu and 13% Co extraction. The rate of bioleaching nickel and zinc was dependent upon pH and was 3–4 times faster at pH 1.5 than at pH 3. Once the nickel concentration reached 2.50 g L^{-1}, 20% of the leach liquor was replaced with fresh liquor in order to decrease possible toxic effects of high metal concentrations.

After 290 days of bioleaching, the extraction of base metals at pH 2.0 reached the same % level as at pH 1.5, and thereafter remained steady. However, at pH 2.5 and 3.0, there was no further extraction of metals after 150 days, possibly due to metal adsorption onto the iron oxide precipitate. The results demonstrate that the highest leaching of base metals is achieved at low pH values under high redox conditions where ferric ion remains in solutions. Riekkola-Vanhanen and Heimala (1999) achieved 40% bioleaching of nickel in 200 days when leaching black schist ore in column tests at pH 1.8–2.7; while the Talvivaara demonstration plant achieved 94% Ni extraction in 16 months with low pH acid irrigation (Riekkola-Vanhanen, 2007).

The dissolution of gangue minerals at different pH values during column leaching brings aluminum, calcium, magnesium, manganese and silicon into solution as presented in Fig. 3. The highest concentration of these elements occurred at pH 1.5 resulting in 10.3 g L^{-1} Al, 0.57 g L^{-1} Ca, 1.2 g L^{-1} Mg, 15.6 g L^{-1} Mn and 1.7 g L^{-1} Si after 140 days of bioleaching. Dissolved manganese and silica did not affect the bioleaching of valuable metals, but at pH 1.5 it increased acid consumption and it became difficult to filter the leach liquor samples due to amorphous gelatinous silica. This may create solution flow barriers hindering oxygen transfer and liquor percolation the leaching of valuable metals from ore. Dissolution of gangue minerals are furher discussed in the study of Dopson et al., 2007a.

Only a few studies report the effects of different metals on microorganisms and bioleaching rates. In high concentrations, metals

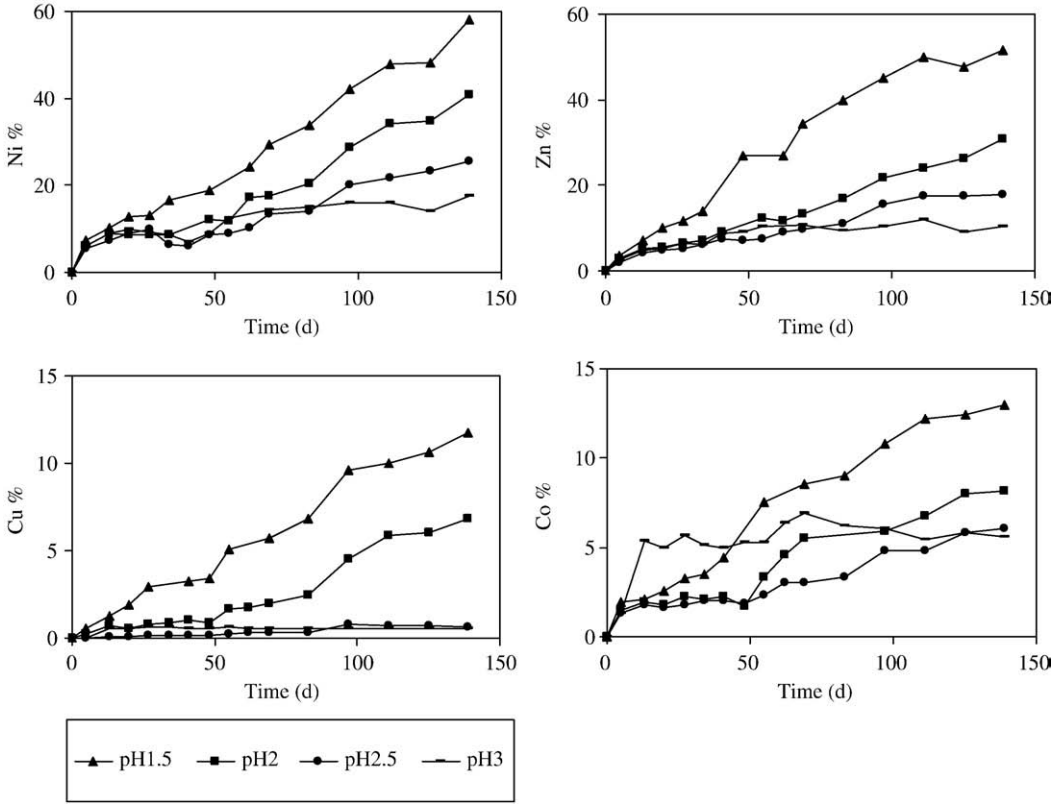

Fig. 2. Bioleaching of base metals during bioleaching of the ore at different pH values.

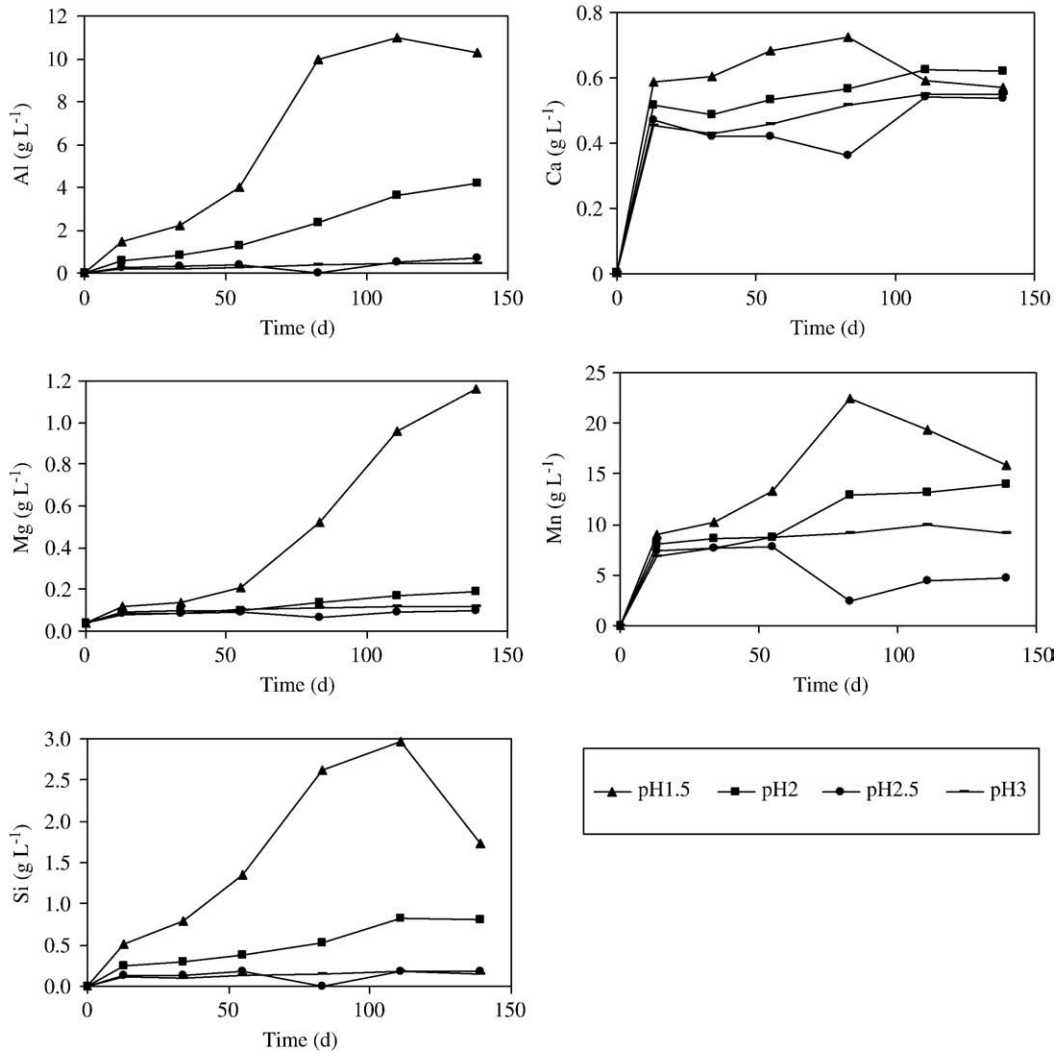

Fig. 3. Aluminum, calcium, magnesium, manganese and silicon concentrations in leach liquors during bioleaching of the ore at different pH values. (Decrease in metal concentrations at pH 2.5 due to leach liquor loss and replacement after 57 days.)

may interfere with microbial ferrous ion oxidation. High concentrations of Mg (Li and Ke, 2001) and Al (Ojumu et al., 2007) have been reported to inhibit bioleaching microbes. Thus, adjusting the pH to 2.0 is recommended for heap bioleaching application to minimize gangue mineral dissolution.

3.4. Total and viable counts

Total cell counts in leach liquors and residues were determined once a month by DAPI staining technique and the viable counts of mesophilic iron oxidizers by MPN technique. The results are presented in Table 1. The enrichment culture used as inoculum contained approximately 10^8 cells mL^{-1} counted by DAPI and MPN. About 99% of the cells of the inoculum became attached to the agglomerated ore corresponding to 10^{10}–10^{11} cells in the column. The pH did not affect the numbers of microorganism in the leach liquor and cell counts (DAPI) remained at 10^6–10^8 cell mL^{-1} throughout the study. The cell counts decreased slightly in all leach liquors during the leaching. This was likely due to attachment of cells to the agglomerated ore and to the formed precipitates. Increasing attachment with incubation time of the acidophilic bacterial cells to pyrite ore and ferric hydroxysulfates was shown by Ghauri et al. (2006) though the attachment of *A. ferrooxidans* was slower than the attachment of *Leptospirillum ferrooxidans*. Jarosites were shown to play an important role in retaining iron oxidizers in a ferric ion-generating fluidized-bed reactor (Kinnunen and Puhakka, 2004; Van der Meer et al., 2007).

The number of mesophilic iron oxidizers in the leach liquor was 10^7–10^8 cells mL^{-1} at the start of bioleaching and decreased during the bioleaching to 10^4–10^6 cells mL^{-1}. Total cell counts in the leach residue were about 10^8 cells g ore^{-1}.

3.5. Microbial composition by PCR-DGGE

Bacterial 16S rRNA gene fragments obtained by PCR amplification of DNA extracted from the leach liquor were analyzed by DGGE. Duplicate DGGE profiles (from 10–80% of denaturing gradient) of the original enrichment and after 27 days of leaching are shown in Fig. 4. Several bands were observed in the samples of all pH columns. The sequence data showed the presence of bacteria in the inoculum and most of the leach liquors related to *A. ferrooxidans* AP310 (99%

Table 1
The average cell counts.

pH	Cell count			
	Leach liquor		Leach residue	
	DAPI	MPN-Fe	DAPI	MPN-Fe
1.5	3.0×10^7	8.0×10^7	1.5×10^8	3.0×10^5
2.0	4.7×10^7	3.1×10^7	8.4×10^7	4.0×10^4
2.5	4.0×10^7	1.4×10^7	2.3×10^8	6.5×10^5
3.0	5.3×10^7	1.2×10^7	1.2×10^8	3.5×10^3

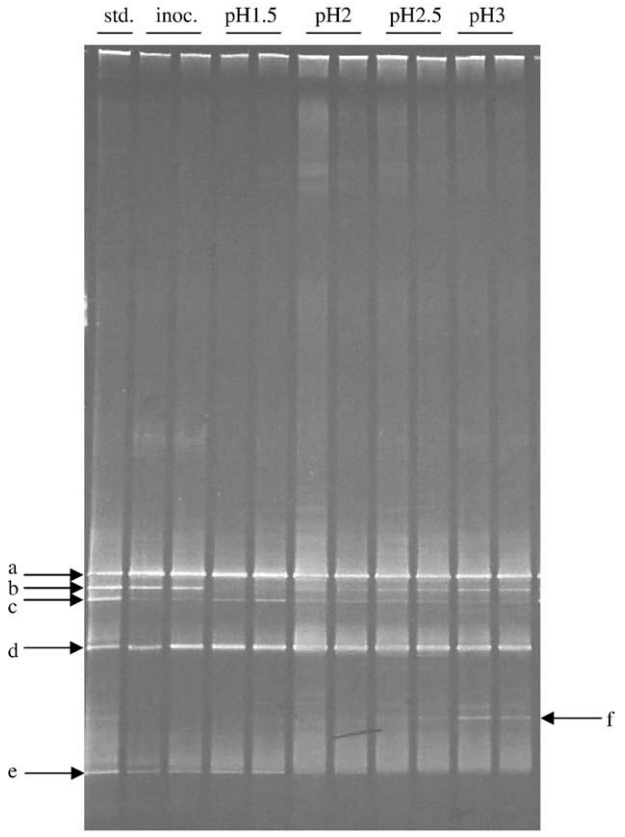

Fig. 4. Bacterial DGGE profiles of partial 16S rRNA gene fragments retrieved from the inoculum and the leach liquor of the columns operated at pH 1.5–3.0 after 27 days. Std = standard. Identity of DGGE bands: a) *Acidithiobacillus ferrooxidans* AP310; b) *A. caldus* MTH-04; c) *A. thiooxidans* ORCS8; d) *Leptospirillum ferrooxidans* DSM 2705; e) *Sulfobacillus thermotolerans* KR-1.

sequence similarity; accession DQ355183), *A. caldus* MTH-04 (96%, AY427958), *A. thiooxidans* ORCS8 (100%, AY830900), *L. ferrooxidans* DSM 2705 (98%, X86776) and *Sulfobacillus thermotolerans* KR-1 (99%, DQ124681). *A. ferrooxidans* grows at pH between 1.3 and 4.5 (Kelly and Wood, 2000).

All the detected bacteria from the leach liquors and leach residues are presented in Tables 2–5. where + corresponds to a weak band on DGGE and ++ corresponds to a strong band on DGGE. *A. ferrooxidans* and *L. ferrooxidans* were the predominant species in leach liquors during the bioleaching at all pH values. Both were found from all of the leach residues, except that *L. ferrooxidans* was not observed in leach residue at pH 3 (Fig. 5). *L. ferrooxidans* oxidizes only ferrous ion and

Table 2
Detected bacterial species from column at pH 1.5.

Microorganism days	27	56	111	197	302	Leach residue
Acidithibacillus ferrooxidans AP310 (99%, DQ355183)	++	++	+	+	++	++
A. caldus MTH-04 (96%, AY427958)	+					
A. thiooxidans ORCS8 (100%, AY830900)	+					
L. ferrooxidans DSM 2705 (98%, X86776)	++	++	+		+	+
Sulfobacillus thermotolerans KR-1 (99%, DQ124681)	+	+				+
Ferrimicrobium acidophilum T23 (98%, AF 251436)			+			
L. ferriphilum D1 (99%, DQ665909)					++	
Ferroplasma acidiphilum DR1 (98%, AY222042)				++		

Table 3
Detected bacterial species from column at pH 2.0.

Microorganism days	27	111	197	302	426	531	Leach residue
A. ferrooxidans AP310 (99%, DQ355183)	++	++	+	++	++	++	++
A. caldus MTH-04 (96%, AY427958)	+						
A. thiooxidans ORCS8 (100%, AY830900)	+						
L. ferrooxidans DSM 2705 (98%, X86776)	++	++	++	++	+	++	++
Sulfobacillus thermotolerans KR-1 (99%, DQ124681)	+						
L. ferriphilum D1 (99%, DQ665909)							
Gram-positive iron-oxidizing acidophile G1 (99%, AY529492)					+	+	+

the optimum pH range is between 1.3 and 2.0 according to Johnson (2001). Coram and Rawlings (2002) reported a pH range of 1.6 to 2.0 for *L. ferrooxidans*.

At the beginning of the experiment, *A. caldus*, *A. thiooxidans* and a species closely related to *S. thermotolerans* were detected in the leach liquors. *A. caldus* was found at pH>1.5, which is consistent with the pH optimum of 2.0–2.5 reported by Kelly and Wood (2000). *A. caldus* uses only S as electron donor (Kelly and Wood, 2000). *A. thiooxidans* and *S. thermotolerans* were present to lesser extent at pH 3.0. These findings are consistent with earlier studies on bioleaching operations, in which iron-oxidizing leptospirilli are generally reported to coexist with a sulfur-oxidizing bacterium such as *A. caldus* or *A. thiooxidans* (for review, see Rawlings, 2002). After one month of bioleaching *A. thiooxidans* was no longer detected and *A. caldus* only once after that (pH 3.0, after 246 days). The absence of these sulfur oxidizers contributes to the high sulfuric acid consumption as suggested by Wakeman et al. (2008). Maeda et al. (1996) showed that 300 mg L^{-1} Ni was inhibitory to *A. thiooxidans*.

Bogdanova et al. (2006) reported the pH range of 1.2–2.4 or pH 2.0–5.0 for the growth of *S. thermotolerans* on media containing ferrous ion or S^0, respectively. At the end of the experiment (around 350 days) a bacterium related to *L. ferriphilum* D1 (99%, DQ665909) was seen in the leach liquor at pH 1.5 and 2.0 and also from leach residue at pH 2.5. Coram and Rawlings (2002) reported a pH range of 1.4 to 1.8 for *L. ferriphilum*.

Archaea were analyzed with DGGE after 111 days (Fig. 6, Table 6) and after 246 days of bioleaching. Archaea related to *Ferroplasma acidiphilum* DR1 (98%, AY222042) were found from leach liquors at pH 1.5 and pH 2.0. Currently two species of *Ferroplasma* are recognized, the other being *F. acidarmanus* (Dopson et al., 2004; Golyshina et al., 2000; Okibe et al., 2003). *F. acidiphilum* oxidizes ferrous ion as the sole energy source and fixes inorganic carbon as the sole carbon source and has a growth range of pH 1.3 and 2.2 (Golyshina et al., 2000). The

Table 4
Detected bacterial species from column at pH 2.5.

Microorganism days	27	56	111	197	302	426	Leach residue
A. ferrooxidans AP310 (99%, DQ35518)	++	++	+	+	+	+	++
A. caldus MTH-04 (96%, AY427958)	+						
A. thiooxidans ORCS8 (100%, AY830900)	+						
L. ferrooxidans DSM 2705 (98%, X86776)	++	++	+	+	+	+	+
Sulfobacillus thermotolerans KR-1 (99%, DQ124681)	+						
Uncultured bacterium clone DX30 (99%, DQ458028)[a]					+		
L. ferriphilum D1 (99%, DQ665909)							
Uncultured bacterium clone QBS9 (99%, DQ840470)[a]						+	
Alicyclobacillus tolerans DSM 16297 (99%, AB222265)							+

[a] No nearest known species.

Table 5
Detected bacterial species from column at pH 3.0.

Microorganism days	27	56	111	197	302	Leach residue
Acidithibacillus ferrooxidans AP310 (99%, DQ355183)	++	++	+	++	++	++
A. caldus MTH-04 (96% AY427958)	+					
A. thiooxidans ORCS8 (100%, AY830900)	+					
L. ferrooxidans DSM 2705 (98%, X86776)	++	++	++	++	+	+

pH of the leach liquor from column at pH 2 was slightly over the optimal pH range for this organism. *Ferroplasma* was also detected intermittently after PCR performed with bacterial-specific forward primer 357F (Muyzer et al., 1993) and universal reverse primer 907R (Muyzer et al., 1996), which can be a result of unspecific amplification.

A species related to an uncultured archaeon clone ant b7 (99%, DQ303249, nearest known species *Thermoplasma acidiphilum* DSM1728, 91%, AL445067) was present in all of the leach liquors except at pH 1.5. Surprisingly, at higher pH values (2.5–3.0), archaea related to *Sulfolobus metallicus* DSM 6482 (98%, SM16SRRN1) were present. The growth temperature for *S. metallicus* has been reported by Huber and Stetter (2001) to be 50–75 °C, with a growth optimum at 65 °C in a pH range of 1.0–4.5. The growth temperature of thermophilic culture VS2, dominated by a *Sulfolobus* sp. was reported to be 35–76 °C. No mesophilic *Sulfolobus* sp. has been described (Salo-Zieman et al., 2006).

3.6. Leach residue analysis

The solid residues of the agglomerate were studied after 80–90 days, when the columns at pH 2.0 and 2.5 became blocked from the bottom. The main sulfides were pyrrhotite, pyrite, sphalerite, chalcopyrite and violarite. Jarosite and goethite were found on the surfaces. However, there was no evidence of gypsum or gelatinous silica acid. Bright yellow precipitates, possibly elemental sulfur, were observed at pH 1.5 and 2.0.

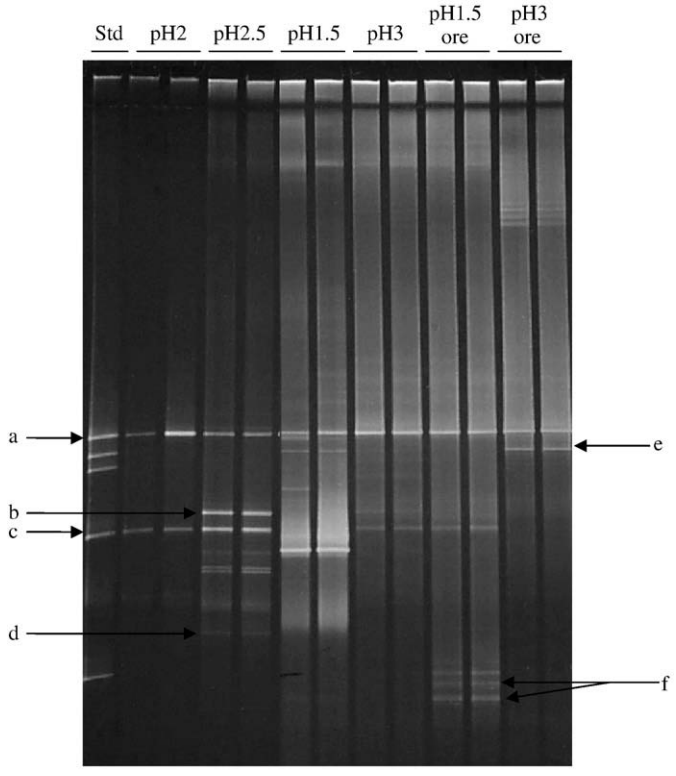

Fig. 5. Bacterial DGGE profiles of partial 16S rRNA gene fragments of the leach liquors and leach residues at pH 1.5 and pH 3.0 after 300 days. Std = standard. Identity of DGGE bands: a) and e) *Acidithiobacillus ferrooxidans* AP310; b) Uncultured bacterium clone DX30; c) *Leptospirillum ferrooxidans* DSM 2705; d) *Leptospirillum ferriphilum* DI; f) *Sulfobacillus thermotolerans* KR-1.

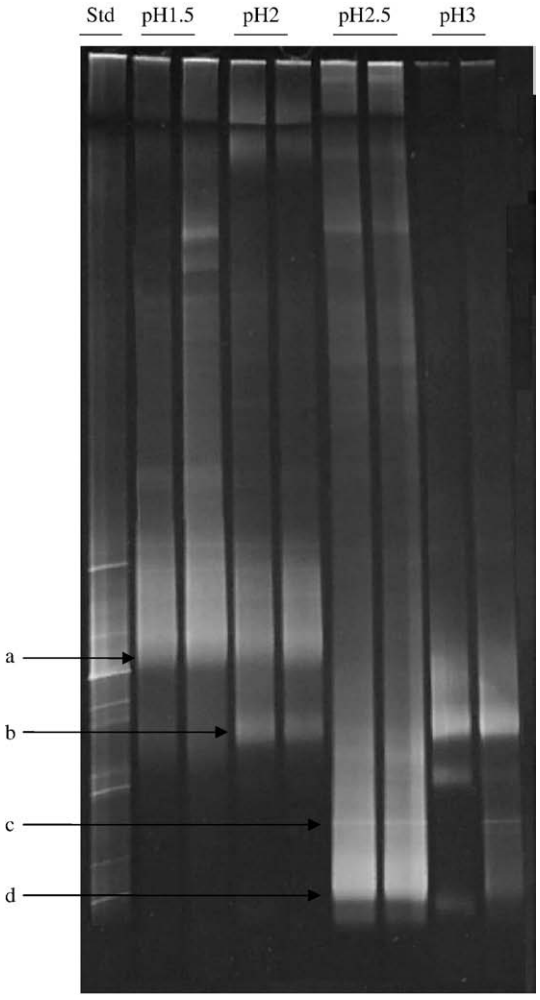

Fig. 6. Archaeal DGGE profiles of partial 16S rRNA gene fragments from the leach liquor of the columns operated at pH 1.5–3.0 after 110 days. Std = standard. Identity of DGGE bands: a) *Ferroplasma acidiphilum* DR1; b) and c) uncultured archeon ant b7; *Thermoplasma acidiphilum* DSM1728, d) *Sulfolobus metallicus* DSM 6482.

Table 6
Detected archaeal species from columns at pH 1.5–3.0.

	Time (d)	
	111	246
Archaea from column at pH 1.5		
Ferroplasma acidiphilum DR1 (98%, AY222042)	++	+
Archaea from column at pH 2.0		
Ferroplasma acidiphilum DR1 (98%, AY222042)	++	+
Uncultured archeon ant b7 (99%, DQ303249, nearest species *Thermoplasma acidiphilum* DSM 1728 (91%, AL445067)	++	+
Archaea from column at pH 2.5		
Uncultured archeon ant b7 (99%, DQ303249, nearest species *Thermoplasma acidiphilum* DSM 1728 (91%, AL445067)	++	
Sulfolobus metallicus DSM 6482 (98%, SM16SRRN1)	++	
Archaea from column at pH 3.0		
Uncultured archeon ant b7 (99%, DQ303249, nearest species *Thermoplasma acidiphilum* DSM 1728 (91%, AL445067)	++	
Sulfolobus metallicus DSM 6482 (98%, SM16SRRN1)	++	

4. Summary

The effects of pH on bioleaching of a complex sulfide ore originating from the Sotkamo deposit, Finland were as follows:

1) The sulfuric acid consumption increased with decreasing pH. At pH 1.5 the cumulative sulfuric acid consumption was 160 g kg^{-1} ore, while for pH 2.0 the acid consumption was 38 g kg^{-1} ore.
2) The redox potential (ref. Ag/AgCl) was highest at pH 2 (580 mV) and lowest at pH 1.5 (515 mV). Low concentrations of Fe(II) demonstrated the activity of ferrous ion oxidizers at all pH values. The concentration of Fe(III) increased significantly with the decrease in pH, but at pH 2.5 and 3.0 the total dissolved iron remained low throughout the experiment due to iron(III) oxide precipitation.
3) The highest bioleaching of base metals were achieved at pH 1.5 where the ferric concentration remained high. After 140 days, 59% Ni, 52% Zn, 12% Cu and 13% Co were extracted. At pH 2.0 similar extractions were achieved over 220 days. The rate of bioleaching of nickel and zinc was 3–4 times faster at pH 1.5 than at pH 3.0.
4) The lack of dissolved ferric ion and diffusion barriers created by iron(III) oxide precipitates slowed down metal leaching at pH 2.5 and 3.0.
5) Dissolution of gangue minerals was significant at pH 1.5 giving high concentrations of aluminium, manganese and amorphous silica which have the potential of interfering with liquid flow in heap leaching and subsequent recovery of base metals.
6) The numbers of microorganism in the leach liquors were 10^6–10^8 cell mL^{-1} and the total cell counts in the leach residues were about 10^8 cells g ore^{-1} throughout the pH range. *A. ferrooxidans* and *L. ferrooxidans* were the dominant species throughout the study.
7) Based on an optimization between the bioleaching of base metals, acid consumption, the concentration of soluble iron and the leaching of undesired cations (Si, Al, Ca, Mg and Mn), a leaching solution of pH 2.0 is recommended for the heap bioleaching application.

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II

Heap bioleaching of a complex sulfide ore: Part II. Effect of temperature on base metal extraction and bacterial compositions

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Heap bioleaching of a complex sulfide ore: Part II. Effect of temperature on base metal extraction and bacterial compositions

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ABSTRACT

The effect of low to moderate temperatures (7 to 50 °C) on the bioleaching of a low-grade, multi-metal black schist ore from Finland in which pentlandite was the main valuable mineral, was studied using columns at set temperatures. The iron and sulfur-oxidizing microbial culture used were enriched from the ore deposit water samples. At 7 °C and 21 °C, the leach liquor redox potential stabilized to 500–600 mV, whereas at 35 °C and at 50 °C it varied between 300 and 500 mV. Microbial iron oxidation started after a lag phase of 20 days at 7 °C and after 60 days of operation, total iron and Fe_{tot}/Fe^{2+}-ratio were higher in the 7 °C column leach liquor than at other temperatures. At 50 °C, all dissolved iron remained in ferrous form and did not indicate microbial activity. Highest bioleaching recoveries of Ni (26%), Zn (18%) and Co (6%) were obtained after 140 days at 21 °C. At 50 °C, bioleaching decreased due to the lack of ferric ion. The microbial composition, as measured by Polymerase Chain Reaction (PCR)–Denaturing Gradient Gel Electrophoresis (DGGE)-sequencing approach, was affected by temperature. *Acidithiobacillus ferrooxidans* was the most common species in the leach liquor at 7 °C; while at 35 °C *Leptospirillum ferrooxidans* dominated and at 50 °C, *Sulfobacillus thermotolerans* was the most common organism. Total liquid-phase cell counts were higher at 7 °C than at other temperatures. Thus the boreal ore deposit enrichment culture was composed of microorganisms capable of being active over a wide temperature range.

1. Introduction

Temperature affects the organisms that inhabit the bioleaching systems. At 40 °C or below, mesophilic Gram-negative bacteria *Acidithiobacillus ferrooxidans* and *Leptospirillum ferrooxidans* dominate, while moderately thermophilic *A. caldus* and *Sulfobacillus thermosulfidooxidans* dominate at temperatures around 40–60 °C (Kelly and Wood 2000; Brandl, 2001). Extreme thermophiles, like *Sulfolobus metallicus*, grow optimally at temperatures higher than 60 °C (Huber and Stetter, 2001). Moderate thermophiles include archaea and eubacteria, the majority of which are Gram-positive. Furthermore, archaea prevail at higher temperatures (up to 90 °C) (Johnson, 1998). In some bioleaching operations the temperature rise due to the exothermal oxidation reactions is above the maximum growth temperature of microorganisms. In particular, the oxidation of pyrrhotite in the presence of moisture and oxygen is characterized by significant heat generation (van Aswegen et al., 2007). If no thermophilic micro-organisms are present, high temperatures may be detrimental to the biological process (Franzmann et al., 2005). Since large temperature gradients can prevail in heaps, the presence of microorganisms active in a range of different temperatures is beneficial (Brierley, 2003).

Microbial iron oxidation is one of the key features of bioleaching (Ahonen and Tuovinen, 1995; Deveci et al., 2004). According to Dopson et al. (2007) at low temperatures, chemical oxidation of the sulfide minerals by ferric iron is the rate limiting step in bioleaching rather than the biological ferrous ion oxidation. Ferric ion easily hydrolyzes in acidic conditions and forms a variety of precipitates including hydroxides and hydroxysulfates e.g. schwertmannite [$Fe_8O_8(OH)_6SO_4$], jarosite [$MFe_3(SO_4)_2(OH)_6$] and goethite (α-FeOOH). Precipitation may cause diffusion barriers on mineral surfaces and thereby hinder bioleaching. Temperature significantly affects precipitation rates. In the study of Wang et al. (2006) two months was required for jarosite to form at room temperature whereas it took only 7 days at 36 °C. (Daoud and Karamanev, 2005; Wang et al., 2006).

The aim of the present work was to study the effects of temperature (7–50 °C) on heap bioleaching of a black schist ore from the Sotkamo deposit, Finland, using bench scale columns and on understanding the development of corresponding bioleaching communities. In Finland, heaps are subject to extreme climatic conditions and large temperature variations.

2. Materials and methods

2.1. Column configuration

This study was performed in parallel with the study of Halinen et al. (2009-this volume) on the effect of pH, except that four columns

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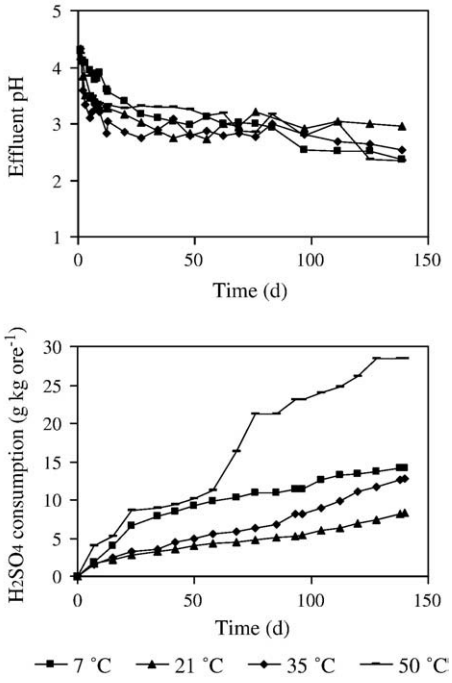

Fig. 1. Leach liquor pH and sulfuric acid consumption during the bioleaching of the ore at different temperatures.

(100 cm × 10 cm) at different temperatures (7, 21, 35 and 50 °C) were used with a total volume of 7.9 L. The column at 7 °C was refrigerated, whilst the columns at 35 °C and 50 °C were water jacketed and connected to a heating thermostat. A perforated plate and a filter cloth were inserted at the bottom of each column. Aeration was provided through a diffuser inserted at the base of the column and blown at the rate of (8–11) $m^3\ m^{-2}\ h^{-1}$. Desired leach liquor pH was 2.5 in all columns and the ore was irrigated at the rate of 10 $L\ m^{-2}\ h^{-1}$ by liquid recirculation. A titration apparatus connected to PC was used to control the pH. Glass beads with the total bulk-volume of 270 mL were placed on the upper part of the column to enable even distribution of the leach liquor. Paraffin paper was set on the top of each column to prevent evaporation.

2.2. Operation of the bioleaching columns

The sulfide component of the ore was mainly pyrrhotite and pyrite with minor pentlandite, alabandite (MnS) and chalcopyrite and contained 0.29% Ni, 0.53% Zn, 0.20% Cu and 0.035% Co. The mineral composition, agglomeration, inoculation procedure, operation of the columns and monitoring were as reported in Halinen et al. (2009-this volume). The short description of operation is given as follows. The enrichment culture used to inoculate columns was derived from the acidic water samples from the ore deposit and initially grown on S^0, Fe^{2+} and ore powder at pH 1.8. The inoculum was poured onto the top of the each column and allowed to percolate through the column. The solutions were collected at the bottom of the columns and the leach liquor circulations were started. In addition thermophilic culture VS2 dominated by *Sulfolobus* spp. (Salo-Zieman et al., 2006) was inoculated the column at 50 °C on day 65.

The recycle of the leach liquors was initially monitored on a weekly basis and later every second week for pH, redox ($Pt/Ag^0/AgCl$ reference), dissolved oxygen, ferrous ion and soluble Fe, Zn, Ni, Co and Cu. The column and leach liquor temperatures, sulfuric acid consumption and leach liquor pH values were recorded five times per week. The leach liquors of the columns were sampled once a month to

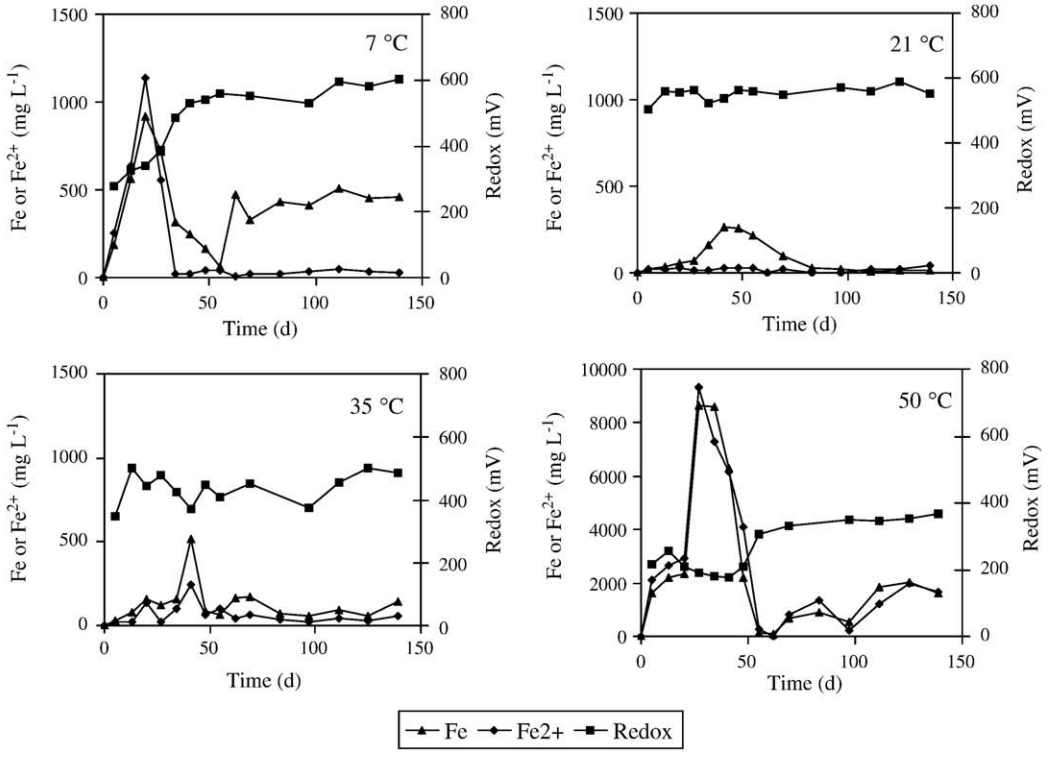

Fig. 2. Redox potentials and the concentrations of total dissolved iron and ferrous ion in bioleach liquors at different temperatures.

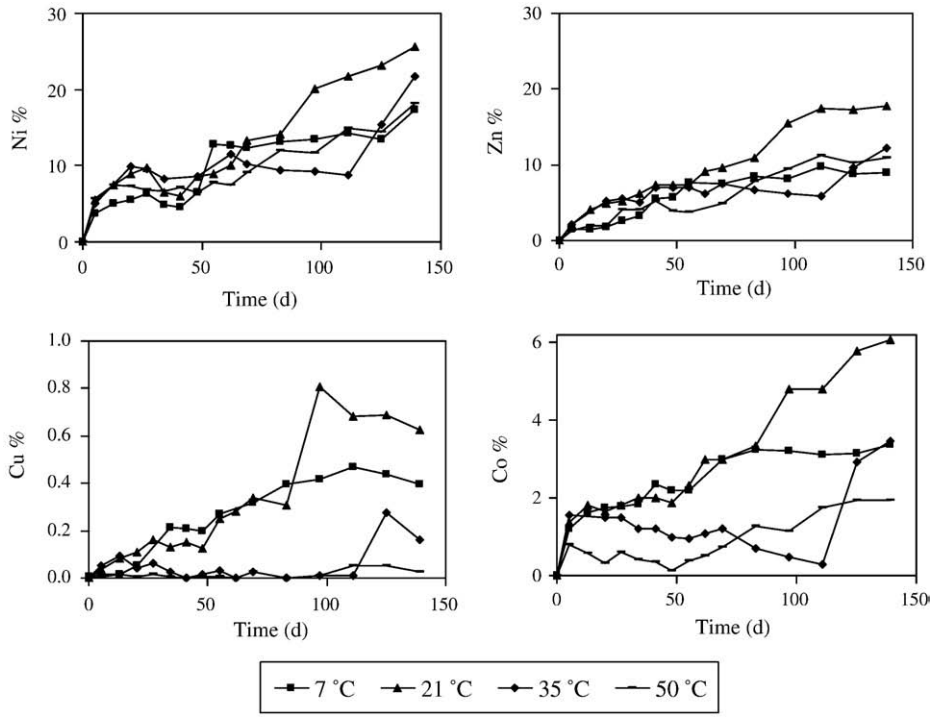

Fig. 3. Bioleaching of base metals after 140 days at different temperatures.

estimate the total numbers of micro-organisms and every second month to characterize the microbial communities.

3. Results and discussion

3.1. H_2SO_4 consumption during bioleaching

The acid consumption during bioleaching at the different temperatures was determined for the ore. Leach liquor pH was continuously titrated and the process was acid-consuming in all tested temperatures as presented in Fig. 1. After 30 days of bioleaching the pH remained below 3 at temperatures of 7 °C, 21 °C and 35 °C. Highest sulfuric acid consumption was 29 g kg^{-1} of ore after 140 days of bioleaching at 50 °C. At temperatures of 7, 21 and 35 °C acid consumptions were 14, 8 and 13 g kg^{-1} ore, respectively. The effect of pH on bioleaching the same black schist ore is reported in Halinen et al. (2009-this volume).

Wakeman et al. (2008) studied the same black schist ore and three columns filled with 3 kg of ore were set-up and operated as a "plug-flow". The pH of leaching solution was adjusted to 2.0 and changed every third week. Without further acid additions pH dropped to 3.5–4.0 after 40 weeks of bioleaching.

3.2. Redox potential and the fate of iron

During column leaching, the redox potentials, ferrous and ferric ion in leach liquors were monitored. At 7 °C and at 21 °C, the leach liquor redox potential stabilized to 500–600 mV, whereas at 35 °C and at 50 °C it varied between 300 and 500 mV (Fig. 2). Low redox conditions reflect a high Fe^{2+}/Fe^{3+} ratio (Brierley, 2003). The total dissolved iron and ferrous ion concentrations at different temperatures are presented in Fig. 2. The redox increase during the first two months of bioleaching at 7 °C reflected the start of ferrous ion oxidation and microbial activity. After 60 days of bioleaching, total iron (700 mg L^{-1}) and Fe_{tot}/Fe^{2+} ratio (14:1) were higher in the 7 °C column leach liquor than at other temperatures. Ferrous ion was oxidized and ferric ion precipitated in leach liquors at 21 and 35 °C. At 35 °C, ferrous ion concentration was 130 mg L^{-1} and Fe_{tot} 220 mg L^{-1} whilst at 50 °C, all the dissolved iron after 50 days (350 mg L^{-1}) was in the ferrous form due to the fast chemical reaction of Fe(III) with the sulfide minerals. Brown precipitates were observed to accumulate on the surfaces of the ore material in all columns from 7 °C to 50 °C. Additionally, bright yellow precipitates were formed indicating elemental sulfur accumulation at 7 °C and 21 °C.

In the study of Dopson et al. (2007) the Fe^{2+} oxidation rates of the culture derived from column leach liquor at 7 °C were tested over the temperature range 2–40 °C. Temperature optima of 22.4 °C and 32.4 °C indicated the presence of both psycho-tolerant and mesophilic microorganisms. However, only *A. ferrooxidans* was found from the column leach liquor in this work (Table 2). Ferric ion precipitation linearly increased with temperature, the maximum amount at 40 °C. The decreased Fe^{3+} precipitation at low temperature results in a greater availability of Fe^{3+} for the mineral sulfide oxidation and thus increased rates of metal dissolution (Dopson et al., 2007).

3.3. Bioleaching of valuable metals

The bioleaching of Ni, Cu, Zn and Co was affected by temperature as presented in Fig. 3. Over the first 90 days bioleaching of nickel was similar at all temperatures. Following that period the highest bioleaching of the metals took place at 21 °C and after 140 days, 715 mg L^{-1} Ni, 800 mg L^{-1} Zn, 11 mg L^{-1} Cu, and 20 mg L^{-1} Co were leached corresponding to 26% Ni, 18% Zn, 1% Cu, and 6% Co extraction.

Table 1
The average cell counts.

T (°C)	Cell count			
	Leach liquor		Leach residue	
	DAPI	MPN-Fe	DAPI	MPN-Fe
7	3.6×10^{8}	2.2×10^{8}	3.9×10^{8}	1.3×10^{5}
21	4.0×10^{7}	1.4×10^{7}	2.3×10^{8}	6.5×10^{5}
35	7.6×10^{6}	1.5×10^{6}	1.1×10^{7}	2.3×10^{6}
50	4.3×10^{6}	1.1×10^{5}	8.7×10^{6}	$<1.0\times10^{2}$

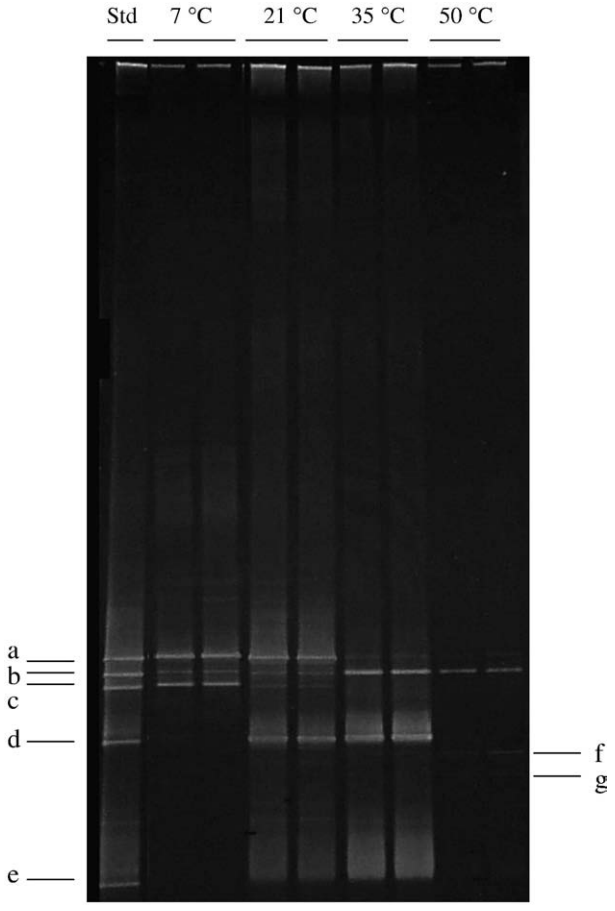

Fig. 4. Bacterial DGGE profiles of partial 16S rRNA gene fragments retrieved from the inoculum (Inoc.) of bioleaching columns and the leach liquor of the columns operated at 7, 21, 35 and 50 °C after 27 days. Std = Standard. Identity of DGGE bands: a) *Acidithiobacillus ferrooxidans* AP310; b) *A. caldus* MTH-04; c) *A. thiooxidans* ORCS8; d) *Leptospirillum ferrooxidans* DSM 2705; e) *Sulfobacillus thermotolerans* KR-1; f) *Thiomonas cuprina* DSM 5498; g) *Alicyclobacillus tolerans* K1.

At 7 °C, the corresponding percentage extractions were 17, 9, 0.4 and 3.4%, respectively. Not surprisingly, the bioleaching of metals increased when soluble ferric ion was present in the leach liquors. Hence at 50 °C, the extraction was similar to that at 7 °C (18% Ni, 11% Zn, 0% Cu and 2% Co) due to the precipitation of dissolved ferric ion (Fig. 2). At 50 °C bioleaching of Ni, Zn and Co accelerated after inoculation with *Sulfolobus* culture on day 65 (Salo-Zieman et al., 2006), but slowed soon after as the pH increased.

The dissolution of gangue minerals producing aluminium, calcium, magnesium, manganese and silicon in solution was also measured at different temperatures during 140 days of bioleaching. Dissolution of these cations were most significant at 50 °C resulting in 1160 mg L^{-1} Al, 570 mg L^{-1} Ca, 240 mg L^{-1} Mg, 7.4 g L^{-1} Mn, and 165 mg L^{-1} Si after 140 days. At all temperatures, leach liquors became saturated with dissolved calcium and manganese during the first 100 days but the aluminium concentration rose linearly over this period. Temperature did not significantly affect silicate leaching.

Table 2
Detected bacteria from column at 7 °C.

Microorganism days	27	161	302	426	531	Leach residue
A. ferrooxidans AP310 (99%, DQ35518)	++	++	++	++	++	++
A. caldus MTH-04 (96%, AY427958)	+					
A. thiooxidans ORCS8 (100%, AY830900)	++					
Ferrimicrobium acidiphilum T23 (98%, AF 251436)						++
Gram-positive iron-oxidizing acidophile G1 (99%,AY529492)						++

Table 3
Detected bacteria from column at 21 °C.

Microorganism days	27	161	246	302	426	Leach residue
A. ferrooxidans AP310 (99%, DQ35518)	++	+	+	++	+	++
A. caldus MTH-04 (96%, AY427958)	+					
A. thiooxidans ORCS8 (100%, AY830900)	+					
L. ferrooxidans DSM 2705 (98%, X86776)	++	+	+	++	++	+
L. ferriphilum D1 (99%, DQ665909)						+
Alicyclobacillus tolerans K1 (100%, AF137502)						+
Sulfobacillus thermotolerans KR-1 (99%, DQ124681)	+					
Uncultured bacterium clone QBS9 (99%, DQ840470), no near known species				++	++	

3.4. Total and viable counts

Total cell counts were determined once a month by 4′, 6-diamidino-2-phenylindole (DAPI) staining and Most Probable Number (MPN) technique adapted for mesophilic iron oxidizers. Average cell counts are presented in Table 1. The liquid-phase total counts (10^8–10^9 cells mL^{-1}) were significantly higher at 7 °C leach liquor than at other temperatures (10^6–10^7 cells mL^{-1}). The viable counts of mesophilic iron oxidizers varied between 10^5 and 10^6 cells mL^{-1} at 21 °C and 35 °C, while at 7 °C the count was 10^8–10^9 cells mL^{-1}. At 50 °C the number of mesophilic iron oxidizers was considerably lower. Overall, as the temperature increased the total cell counts of the leach residues decreased. In the study of Wakeman et al. (2008), with the same black schist ore, the total cell counts present in leach liquors from the column reactors (3 kg $column^{-1}$) were around 10^6–10^7 cells mL^{-1} at 37 °C.

3.5. Microbial composition by PCR-DGGE

Bacterial 16S rRNA gene fragments obtained by PCR amplification of DNA extracted from the leach liquor and leach residues communities were separated by DGGE. Duplicate DGGE profiles of the inoculum after 27 days of leaching are shown in Fig. 4. Bands excised from the DGGE gel and the DNA fragments obtained by PCR amplification were sequenced. The sequence data showed the presence of bacteria in the inoculum related to *A. ferrooxidans* AP310 (99% sequence similarity; accession DQ355183), *A. caldus* MTH-04 (96%, AY427958), *A. thiooxidans* ORCS8 (100%, AY830900), *L. ferrooxidans* DSM 2705 (98%, X86776) and *Sulfobacillus thermotolerans* KR-1 (99%, DQ124681).

During the study, the leaching temperature changed the dominance of liquid-phase microorganisms in the columns as presented in Tables 2–6— where + corresponds to a weak band on DGGE and ++ corresponds to a strong band on DGGE. At 7 °C *A. ferrooxidans*, *A. thiooxidans* and *A. caldus* were initially present, whereas *L. ferrooxidans* and *S. thermotolerans* were not observed. After the first months, *A. ferrooxidans* was the only species detected at 7 °C. In the earlier studies, only the activity of *Acidithiobacillus*-like bacteria has been observed at low temperatures down to 0 °C (Elberling et al., 2000; Kupka et al., 2007; Langdahl and Ingvorsen, 1997; Sand et al., 1992). Finally, after 500 days of leaching, *A. ferrooxidans*, Gram-

Table 4
Detected bacteria from column at 35 °C.

Microorganism days	27	111	197	302	400	Leach residue
A. ferrooxidans AP310 (99%, DQ35518)	+		+			++
A. caldus MTH-04 (96%, AY427958)	++					
L. ferrooxidans DSM 2705 (98%, X86776)	++	++	+	+	+	
L. ferriphilum D1 (99%, DQ665909)				++	++	++

Table 5
Detected bacteria from column at 50 °C.

Microorganism days	27	111	246	302	419	Leach residue
A. ferrooxidans AP310 (99%, DQ35518)	+	+				
A. caldus MTH-04 (96%, AY427958)	++					
L. ferrooxidans DSM 2705 (98%, X86776)			+			
Sulfobacillus thermotolerans KR-1 (99%, DQ124681)			+	+	+	+
L. ferriphilum D1 (99%, DQ665909)				+	+	+
Alicyclobacillus acidocaldarius DSM 455 (98%, AB059665)	+	+				
Thiomonas cuprina DSM 5498 (99%, AB331954)	+					
Alicyclobacillus tolerans K1 (100%, AF137502)			+			

positive iron-oxidizing acidophile G1-like bacterium (99% AY529492), nearest known species *Alicyclobacillus disulfidooxidans* (90%, AB089843) and *Ferromicrobium acidophilum* T23 (98%, AF251436) were found in the leach residue. These species were not detected in the leach liquor, which may be due to the attachment of most bioleaching microbes onto the ore particles (Rohwerder et al., 2003). The role of *F. acidophilum* has been suggested to be the removal of organic compounds that can be toxic for autotrophic bacteria that are mainly responsible for leaching reactions (Johnson, 1995).

At room temperature *A. ferrooxidans* and *L. ferrooxidans* were the dominant species throughout the study. In addition to these *A. caldus*, *A. thiooxidans* and *S. thermotolerans* were detected at the beginning. During the study, an uncultured bacterium similar to the clone QBS9 from acid mine drainage from the Qibaoshan copper mine (99%, DQ840470; no nearest known species) was detected in the leach liquor several times. *L. ferriphilum* and *Alicyclobacillus tolerans* K1 (100%, AF137502) were detected in the leach residue in addition to *A. ferrooxidans* and *L. ferrooxidans*. In the study of Hallberg et al. (2007) at least two strains of *A. ferrooxidans* were present in the leach liquor of a 110 ton scale bioleaching column with the same ore. When the temperature of that column was increased from ~20 to 35 °C, the relative abundance of *A. ferrooxidans*-like bacteria decreased while the abundance of unidentified bacteria (Gram-positive iron-oxidizing acidophile G1, AY529492) increased. Total cell counts did not change considerably after the temperature change. Gram-positive iron-oxidizing acidophile G1 was also detected in our study at pH 2.0 from column leach liquor and residue (Halinen et al., 2009-this volume) and in the study of Wakeman et al. (2008).

At 35 °C, the microbial community included *A. ferrooxidans*, *L. ferrooxidans* as the dominating species. *A. caldus* was detected at the beginning and later *L. ferriphilum* species was present. *A. ferrooxidans* and *L. ferrooxidans* were also found in the leach residue. The growth of *L. ferrooxidans* has been reported at temperatures below 45 °C (Coram and Rawlings, 2002). Furthermore, leptospirilli are rarely detectable at temperatures below 20 °C (Coram and Rawlings 2002). Unlike with *A. ferrooxidans*, the ability of *L. ferrooxidans* to oxidize ferrous iron is not inhibited by ferric iron (Rawlings, 2002). In the work on this ore by Wakeman et al. (2008) using 24 different acidophilic microorganisms from different sources at 37 °C, *A. ferrooxidans* dominated the early phase of the experiment, while *L. ferriphilum* dominated the microbial consortium for the greater part of the experiment. *L. ferrooxidans* and *A. caldus* were detected in relatively small proportions. As in our study, some microorganisms were found in the leach liquor that did not correspond to any of the microorganisms present in the inoculum.

By contrast, *S. thermotolerans* was the major species during leaching at 50 °C, although a PCR product was not gained every month. *S. thermotolerans* was also detected in leach residue with *L. ferriphilum*. The ability of *S. thermotolerans* to form endospores would

Table 6
Detected archaeal species from columns at 7–50 °C.

Archaea from column at 7 °C	Time (d)	
	111	246
Ferroplasma acidiphilum DR1 (98%, AY222042)	+	
Uncultured archaeon ant b7 (99%, DQ303249, nearest known species *Thermoplasma acidiphilum* DSM 1728 (91%, AL445067)	++	++
Archaea from column at 21 °C	**Time (d)**	
	111	246
Ferroplasma acidiphilum DR1 (98%, AY222042)	+	
Uncultured archaeon ant b7 (99%, DQ303249, nearest known species *Thermoplasma acidiphilum* DSM 1728 (91%, AL445067)	++	
Sulfolobus metallicus DSM 6482 (98%, SM16SRRN1)	+	
Archaea from column at 35 °C	**Time (d)**	
	111	246
Uncultured archaeon ant b7 (99%, DQ303249, nearest known species *Thermoplasma acidiphilum* DSM 1728 (91%, AL445067)	+	
Sulfolobus metallicus DSM 6482 (98%, SM16SRRN1)	++	++
Archaea from column at 50 °C	**Time (d)**	
	111	246
Ferroplasma acidiphilum DR1 (98%, AY222042)	+	
Uncultured archaeon ant b7 (99%, DQ303249, nearest known species *Thermoplasma acidiphilum* DSM 1728 (91%, AL445067)	++	++
Sulfolobus metallicus DSM 6482 (98%, SM16SRRN1)	+	+

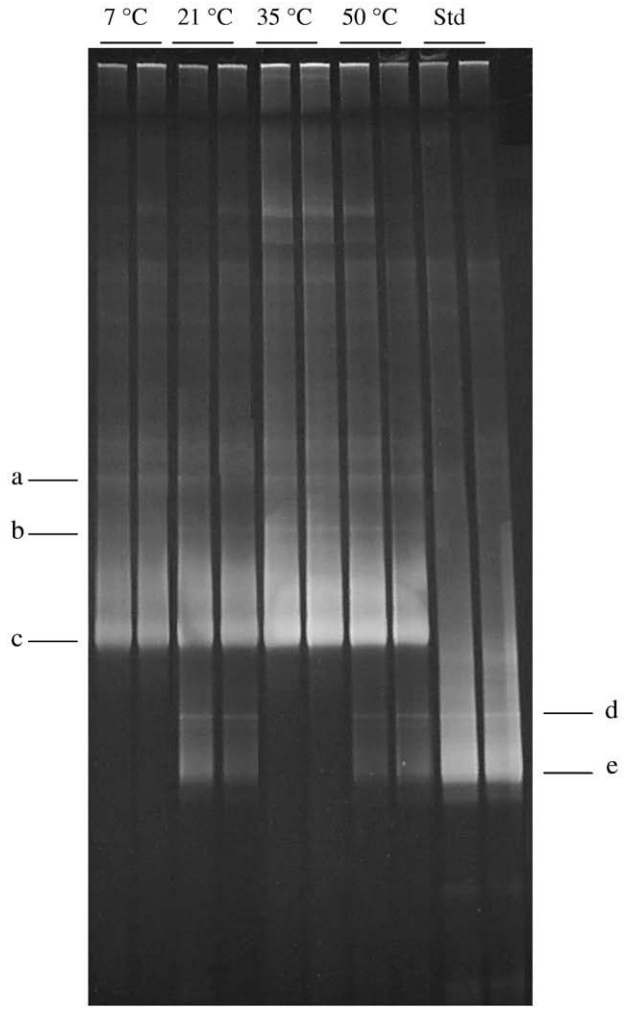

Fig. 5. Archaeal DGGE profiles of partial 16S rRNA gene fragments from the leach liquor of the columns operated at 7, 21, 35 and 50 °C after 110 days. Std = Standard. Identity of DGGE bands: a) and b) *Ferroplasma acidiphilum* DR1; c) uncultured archaeon clone ant b7; d) and e) *Sulfolobus metallicus* DSM 6482.

be advantageous for survival of bacteria during low temperature periods in a heap operated in boreal conditions, where high seasonal variation in temperature occurs. The reported optimum temperature of *S. thermotolerans* is 40 °C and growth range 20–60 °C (Bogdanova et al., 2006). *A. tolerans* K1 (100%, AF137502) and *Thiomonas cuprina* DSM 5498 (99%, AB331954) were also detected at 50 °C after a month, although the DGGE bands were weak. A bacterium related to thermoacidophilic Gram-positive *Alicyclobacillus acidocaldarius* DSM 455 (98%, AB059665) (previously *Bacillus acidocaldarius*, Wisotzkey et al., 1992) was also detected at the beginning.

A. acidocaldarius has been reported to grow at temperatures from 45–70 °C, with an optimum of 60–65 °C and in a quite wide pH range from 2 to 6 (optimum pH 3–4) (Darland and Brock, 1971). *A. caldus* has been found to dominate at temperatures around 50 °C (Norris et al., 1996), but it was detected in leach liquor only at the beginning of the experiment. After re-inoculating with *Sulfolobus* dominated culture on a day 65, the leaching activity at 50 °C accelerated, but decreased soon as did the cell counts.

Archaea were analyzed two times during bioleaching as presented in Table 5. Fig. 5 presents the first archaeal DGGE after 111 d of bioleaching. Besides the uncultured archaeon clone ant b7 (99%, DQ303249, nearest known species *Thermoplasma acidiphilum*, 91%, AL445067), the species *Ferroplasma acidiphilum* DR1 (98%, AY222042) was found at all temperatures. *S. metallicus* – a related species – was surprisingly found in column leach liquor at 21 °C. *Sulfolobus* species have been reported earlier to grow at higher temperatures. Salo-Zieman et al. (2006) reported the growth range to be 34–76 °C for their strain of *Sulfolobus* whilst Plumb et al. (2002) and Zillig et al. (1980) have reported growth at temperatures ranging from 50 to 90 °C.

The absence of sulfur-oxidizing bacteria, mainly *A. caldus* and *A. thiooxidans*, could explain the continuous acid-consumption as Wakeman et al. (2008) suggested. The conditions in the bioheaps are never completely uniform and large temperature gradients may prevail. Therefore different microorganisms with different optimum temperatures were beneficial. In heap bioleaching applications the control of microorganisms is usually not worthwhile to try as it is an open environment. Adaptation of certain individual strains has been shown possible, but no large differences can be attained. Mixed cultures from native conditions tend to give better mineral leaching results than pure cultures (Brierley, 2001).

In summary, the microbial enrichment culture and the possible microorganisms from the ore from boreal minesite samples contained a wide variety of microorganisms ranging from psychrotrophs to thermophiles.

4. Conclusions

In the present work the effect of temperature (7 to 50 °C) on bioleaching of a low-grade, multi-metal black schist ore was studied containing mainly pyrrhotite and pyrite together with minor pentlandite and chalcopyrite originating from the Sotkamo deposit, Finland. The following conclusions can be drawn:

1) The redox potential during bioleaching was highest (500–600 mV) at 21 °C and lowest at 50 °C where ferrous ion predominated. At 7 °C, the redox increases with time reflecting ferrous ion oxidation is faster than ferric ion leaching.
2) The acid consumption was lowest at 21 °C where significant Fe(III) is precipitated and highest at 50 °C.
3) Highest bioleaching was obtained at 21 °C after 140 days with 26% Ni extracted together with 18% Zn, 1% Cu and 6% Co.
4) Dissolution of non-valuable metals was considerable at 50 °C resulting in 1160 mg L^{-1} Al, 570 mg L^{-1} Ca, 240 mg L^{-1} Mg, 7.4 g L^{-1} Mn, and 165 mg L^{-1} Si after 140 days.
5) The lack of ferric ion, or presence of iron oxide precipitates, appears to hinder the bioleaching of base metals at 50 °C.
6) The microbial composition and dynamics in the columns were affected by temperature. The cell counts (10^8–10^9 cells mL^{-1}) were significantly higher at 7 °C in the leach liquor than at other temperatures (10^6–10^7 cells mL^{-1}).
7) The dominant species were *A. ferrooxidans*, *A. ferrooxidans* and *L. ferrooxidans*, *L. ferrooxidans* and *S. thermotolerans* at corresponding temperatures of 7, 21, 35 and 50 °C. In heap bioleaching applications, large temperature gradients are likely resulting in corresponding microbial activities at different parts of the heap.

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III

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IV

Silicate mineral dissolution during heap bioleaching

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V

Microbial community dynamics during a demonstration-scale bioheap leaching operation

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Microbial community dynamics during a demonstration-scale bioheap leaching operation

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ABSTRACT

In the present work the microbial community of a low grade nickel ore demonstration-scale bioheap was examined under varying weather (outside air temperature between +30 and −39 °C) and operational conditions over a period of three years in Talvivaara, Finland. After the start-up of heap irrigation, oxidation of pyrrhotite and pyrite increased the heap temperature up to 90 °C. Leach liquor temperatures varied between 60 and 15 °C over the operation period, affecting the progress of sulfide ore oxidation.
The microbial communities were profiled by polymerase chain reaction (PCR) — denaturing gradient gel electrophoresis (DGGE) followed by partial sequencing of 16S rRNA gene. Large temperature gradients prevailed resulting in the simultaneous presence of active mesophilic and thermophilic iron- and/or sulfur-oxidisers in the heap. As mineral oxidation progressed microbial diversity decreased and *Acidithiobacillus ferrooxidans* became increasingly dominant. The number of bacteria in the leach liquors was in the range of 10^5–10^7 cells mL^{-1}. After one year of bioheap operation several ore samples were drilled from the heap and *A. ferrooxidans*, *Acidithiobacillus caldus,* an uncultured bacterium clone H70 related organism, *Ferrimicrobium acidiphilum* and a bacterium related to *Sulfobacillus thermosulfidooxidans* were found. Cell counts from the ore samples varied between 10^5 and 10^7 cells g^{-1} ore sample. The archaeal species present in leach liquors were novel and related to uncultivated species. During the secondary leaching phase the leaching community remained steady. *A. ferrooxidans* dominated, and an uncultured bacterium clone H70-related organism and *Leptospirillum ferrooxidans* were present.

1. Introduction

Heap bioleaching of low-grade sulfide ores has become an important process for metal recovery (for reviews, see: Rawlings, 2002; Watling, 2008). During the last twenty years the process has been optimized successfully including ore crushing, agglomeration, aeration, leach liquor distribution and stacking stages (Brierley and Brierley, 2001). Leach liquor pH can be adjusted before irrigation. Temperatures are affected by the composition and concentration of the sulfidic minerals because of exothermic oxidation reactions. Aeration and irrigation rates affect evaporation and heat dissipation (Ehrlich, 2001; Rawlings, 2002; Watling, 2006).

Microorganisms present in bioheaps are mainly ferrous iron- and sulfur-oxidizing chemolithotrophs, although some heterotrophs have been reported (Hallberg and Johnson, 2001). The regeneration of ferric iron (Fe^{3+}) and proton release (H^+) are essential for metal sulfide oxidation and dissolution of valuable metals. As sulfuric acid is produced by the oxidation of sulfur, these organisms generate an acidic growth environment. Many of the chemolithotrophic acidophiles are sensitive to organic matter and thus heterotrophic acidophiles detoxify the bioleaching environment. A small fraction of the bioleaching microorganisms is found in the leach liquor, while most of the microorganisms adhere to the mineral surfaces (Rohwerder et al., 2003; Crundwell, 1996). Studies of microbial communities inhabiting commercial reactor based, bioleaching processes have been successfully carried out in recent years (Pradhan et al., 2008). However, microorganisms inhabiting industrial bioheaps and dumps have gained less attention (Demergasso et al., 2005).

The aim of the present work was to study the microbial community structures and their dynamics during a demonstration-scale complex sulfide ore (17 000 tons) bioheap leaching operation. Spatial and temporal changes in microbial communities were monitored and included strong fluctuations.

1.1 . Talvivaara ore deposit

Talvivaara complex multi-metal black schist ore deposit is located in central-eastern Finland with 1550 million tons of classified

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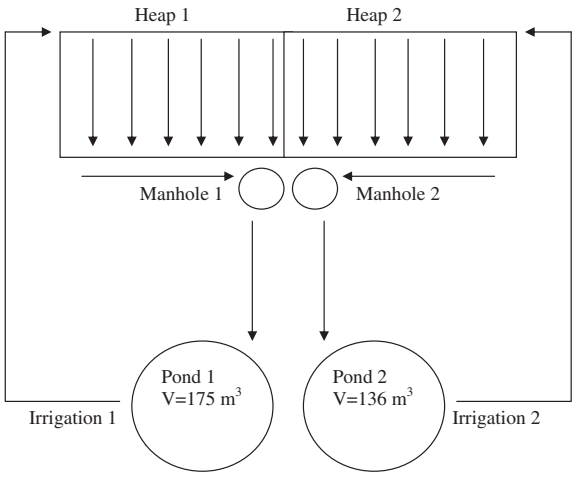

Fig. 1. Diagram of the sampling points of the Talvivaara bioheaps with the direction of the liquid flow marked with arrows. Each heap had its own liquid circulation. The amount of the ore of Heap 1 was 10 255 tons and for Heap 26 703 tons, respectively.

resources (Talvivaara, 2012). The mineral composition of the sulfides used in the demonstration-scale bioheaps was 61.2% pyrrhotite [$(Fe_{1-x})(S_2)$, where X = 0.7–0.9], 24.3% pyrite (FeS), 5% pentlandite [$(Fe,Ni,Co)_9S_8$], 6.5% alabandite (MnS), 2.4% chalcopyrite ($CuFeS_2$) and 1% sphalerite [(Zn,Fe)S]. Valuable metal contents were as follows: 0.27% Ni, 0.56% Zn, 0.14% Cu and 0.02% Co (for detailed description see Riekkola-Vanhanen, 2007). Prior to the bioheap demonstration, laboratory scale studies had demonstrated the amenability of Talvivaara ore to bioleaching (e.g. Puhakka and Tuovinen, 1986a, b, c; Riekkola-Vanhanen and Heimala, 1999; Wakeman et al., 2008; Halinen et al., 2009a, b). After a year of bioleaching 65% of nickel and 60% of zinc were leached. After 48 months, 99% of nickel and zinc were leached.

2. Materials and methods

2.1. Design and start-up of the demonstration heaps

During summer 2005, a 17 000 ton demonstration plant was constructed at the Talvivaara mine site (Fig. 1). A representative ore sample was mined, crushed to 80% −8 mm, agglomerated and stacked in a two-part heap (8 m high, 30 × 120 m). Heap 1 was agglomerated with sulfuric acid solution (pH 1.8) including inoculum (described below). Heap 2 was agglomerated with sulfuric acid solution only. Irrigation of the heaps was started in August 2005. The irrigation flow rate was at the beginning 10 L m^{-2} h^{-1} on Heap 1 and 20 L m^{-2} h^{-1} on Heap 2. It was decreased later to 5 L m^{-2} h^{-1} on both heaps.

Leach liquors were collected by subsurface drains below the heaps and directed to manholes. From the manholes liquors flowed to pregnant leach solution (PLS) ponds and back to irrigation (Fig. 1). The operational volumes of ponds 1 and 2 were 175 m^3 and 136 m^3, respectively. Ten percent side stream was removed continuously for metal recovery and replaced with well water. After the start-up of irrigation, the oxidation of pyrrhotite and pyrite increased the heap temperature up to 90 °C. Leach liquor temperatures remained always at above 15 °C over the operation period, even during the boreal winter.

2.2. Inoculation of Heap 1

The iron and sulfur-oxidizing enrichment culture was originally enriched from mine site water samples on Fe^{2+}, S^0 and Talvivaara ore powder at pH 1.8 (Halinen et al., 2009a). The enrichment culture was grown in laboratory to the volume of 4.5 m^3 (Geological Survey of Finland (GTK), Outokumpu). It was transported to the mine site and pumped into a microbial pond (MP) with initial water volume of 40 m^3. Most of the water used originated from on-site drilled well (temperature 5 °C). Liquid pH in the pond was adjusted to 1.8 with sulfuric acid and pulp concentration (w v^{-1}) was set to 1% prior to inoculation. After the inoculation ammonium sulfate concentration was increased stepwise to 0.4 g L^{-1} using 25% (v v^{-1}) stock solution and 500 kg of elemental sulfur was added. No liquid heating or cover was used. The volume of 40 m^3 was increased to 150 m^3 with well water. Inoculation of Heap 1 was accomplished during agglomeration and by irrigating the heap by acidic microbial solution, total inoculum volume being 99 m^3. Heap 2 was not inoculated.

2.3. Secondary bioheaps

On February 2007 after 18 months of operation, the heaps were reclaimed and restacked to the secondary bioheap. Irrigation rate was 2 L m^{-2} h^{-1}. No aeration was provided. Bioleaching of copper and cobalt was continued (data not shown). Minor amounts of nickel and zinc were bioleached, probably from the parts that were not reached during the primary phase.

2.4. Sampling

First samples for microbiological analyses were taken from the microbial pond (MP), where the inoculum was grown, and from the manholes (MH 1 and 2) that collected the irrigation and rain water that percolated trough the heaps. Next samples were taken after 3 months of bioleaching. Samples (50 mL) from manholes and ponds (P 1 and 2) were collected thereafter every month. In July 2006 pond samples were changed to irrigation samples (IR 1 and 2). Sampling was continued when primary bioheaps were reclaimed to the secondary bioheaps. Fig. 1 shows the sampling points and the sampling and analysis program was as presented in Fig. 2.

2.5. Cell counts

Total cell counts were estimated from the samples with 4′, 6-diamidino-2-phenylindole (DAPI) staining technique using epifluorescence microscopy. Microbes were detached from the ore samples according to methods described in Halinen et al. (2009a). 15 g of the ore sample was mixed with 40 mL of sterile Zwittergent-washing solution (0.38 g L^{-1} ethylene glycol tetraacetic acid, 3.35^{-4} g L^{-1} Zwittergent, 3.73 g L^{-1} KCl, pH adjusted to 2.5 with 2 M HCl). The mixture was shaken and sonicated 5 × 1 min in order to detach microorganisms from ore particles. Thereafter, the sample was allowed to settle for about 30 min to prevent the small ore

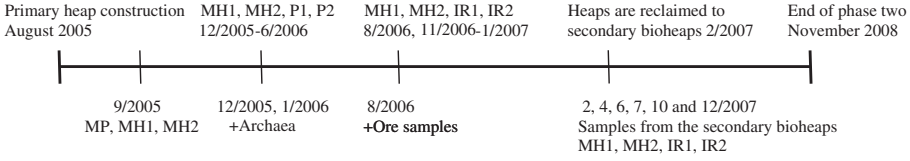

Fig. 2. Timescale of sampling. MP = microbial pond, MH1 = manhole 1, MH2 = manhole 2, P1 = pond 1, P2 = pond 2, IR1 = irrigation 1, IR2 = irrigation 2.

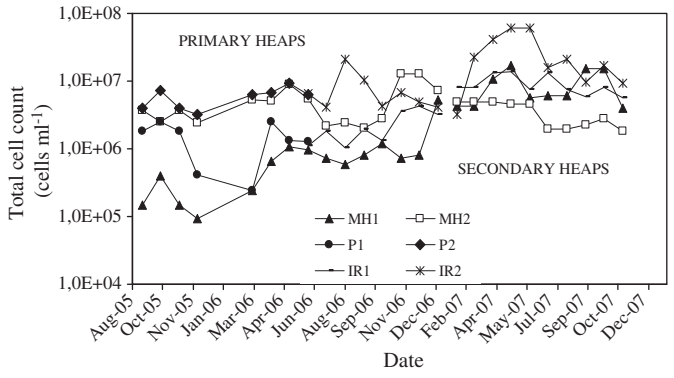

Fig. 3. The cell counts (DAPI) from leach liquors during the bioleaching. MH1 = manhole 1, MH2 = manhole 2, P1 = pond 1, P2 = pond 2, IR1 = irrigation 1, IR2 = irrigation 2.

particles from interfering with DAPI staining. Microbial numbers were counted from supernatant to account for attached cells.

2.6. Microbial community analyses

The microbial communities were investigated during the bioleaching from the leach liquors. Samples were sent to Tampere University of Technology and 15 mL duplicates of every sample were filtered on a 0.2 μm pore size polycarbonate filter prior to concentrating the microorganisms for DNA extraction (Cyclopore Track Etched Membrane, Whatman). The filters were rinsed with 5 mL of 0.9% (wt vol^{-1}) NaCl at pH 1.8 for 1 min to remove the excess metals and then neutralized with 5 mL of 40 mM Na-EDTA in phosphate buffered saline (PBS; 130 mM NaCl, 5 mM Na_2HPO_4, and 5 mM NaH_2PO_4 adjusted to pH 7.2) for 1 min. The filters were stored at −20 °C prior to nucleic acid extraction. The microbial communities were investigated by polymerase chain reaction (PCR) — denaturing gradient gel electrophoresis (DGGE) followed by partial sequencing of 16S rRNA gene as described previously (Halinen et al., 2009a).

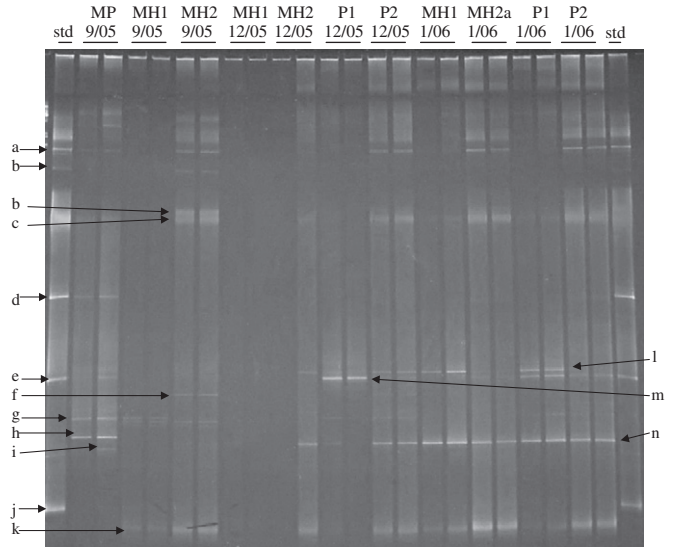

Fig. 4. Bacterial DGGE profiles of partial 16S rRNA gene fragments retrieved from leach liquors after 4 months of bioleaching. Samples were from microbial pond (MP), manhole 1 (MH1), manhole 2 (MH2), pond 1 (P1), pond 2 (P2). Identity of DGGE bands: a) and c) *Acidithiobacillus ferrooxidans*, b) *A. caldus*, d) *A. thiooxidans*, e) *Leptospirillum ferrooxidans*, f) *Thiomonas arsenivorans*, g) *Alicyclobacillus acidocaldarius*, h) and m) *Alicyclobacillus tolerans* i), *Ferrimicrobium acidiphilum*, j and k) *Sulfobacillus thermosulfidooxidans*, l) *A. caldus*, n) an uncultured *Firmicutes* bacterium clone H70 related bacterium that is related to *Moorella* sp. Please see Table 1 that presents the percentages of 16S rRNA gene similarities and data base designations.

In October 2006 several ore samples were obtained by drilling from the heaps. The first samples were drilled from a part of Heap 1 where the temperature was 80–90 °C at the depths of 1–2 m and 3–4 m. Next samples were taken from Heap 1, from the depth of 1–2 m and 4–5 m in area where the temperature was 65–75 °C. Last samples were drilled from Heap 2 from area where the temperature was 20–35 °C, from the depth of 0–1 m and 4–5 m (Fig. 5). Characteristic leach liquor at the time when ore samples were drilled from Heap 1 (PLS1) were as follows: temperature (T) 46.5 °C, dissolved oxygen (DO) 2.2 mg L^{-1}, pH 2.75, redox 331 mV (Pt electrode against an Ag^0/AgCl reference), soluble Fe^{2+} 12.6 g L^{-1}, soluble Fe_{tot} 13.7 g L^{-1} and from the Heap 2: T 35.1 °C, DO 2.1 2 mg L^{-1}, pH 2.67, redox 392 mV, soluble Fe^{2+} 6.2 g L^{-1}, soluble Fe_{tot} 7.4 g L^{-1}.

3. Results and discussion

3.1. Bioleach conditions in heaps

Ferrous iron concentrations were high during the first half year of bioleaching, being mainly between 20 and 35 g L^{-1} and between 10 and 20 g^{-1} L^{-1} in Heaps 1 and 2, respectively. After 6 months ferrous iron concentrations started to decrease steadily, being between 10 and 20 g^{-1} L^{-1} in both heaps. Ferric iron concentration remained low in both heaps (data not shown). Leach liquor pH values of both heaps were quite similar varying during the 10 months between 3.5 and 3.0 and after that between 3.0 and 2.5. The pH was maintained by continuous adjustment by dosing sulfuric acid. Redox potentials varied between 200 and 400 mV in both heaps increasing toward 400 mV as leaching progressed. At the beginning (autumn 2005) leach liquor temperatures were between 25 and 55 °C in both heaps. During the first year, temperatures varied between 20 and 50 °C in Heap 1 and between 20 and 40 °C in Heap 2. In the second winter leach liquor temperatures were between 20 and 40 °C in Heap 1 and between 15 and 25 °C in Heap 2. Temperatures inside the heaps varied greatly, being between 15 and 90 °C during the first year. In the second winter temperatures inside the heaps started to drop being still 80 °C in the hottest zone.

3.2. Cell counts

Total cell counts (DAPI staining) in leach liquors from the primary and secondary phase were as presented in Fig. 3. Liquid volumes

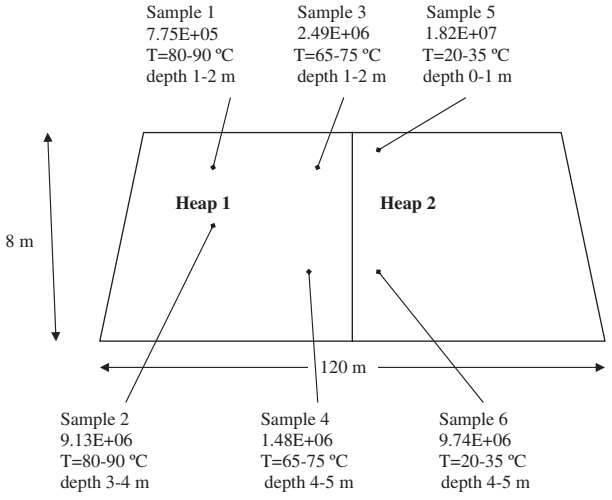

Fig. 5. The average cell counts from the drilled ore samples from Talvivaara bioheaps. The locations and temperatures of the sample sites are shown in a side view of the heaps.

Table 1
Bacteria present in demonstration-scale bioheaps for complex sulfide ore during the primary leaching phase. MP = microbial pond, MH1 = manhole 1, MH2 = manhole 2, P1 = pond 1, P2 = pond 2, IR1 = irrigation line 1, IR2 = irrigation line 2. + corresponds weak band on DGGE and ++ corresponds strong band on DGGE, empty cell = not detected.

Date	Time (months)	Sample\ species	*Acidithiobacillus ferrooxidans* AP310 (99%, DQ355183)	*A. caldus* MTH-04 (99–96%, AY427958)	*A. thiooxidans* ORCS8 (100%, AY830900)	*Thiomonas arsenivorans* (100%, AY950676)	*Sulfobacillus thermosulfidooxidans* N19-50-01 (100%, EU499919)	Bacterium related to clone H70 (91%, DQ328625)	*Leptospirillum ferrooxidans* DSM 2705 (98%, X86776)	*Alicyclobacillus acidocaldarius* (99%, AB059665)	*Alicyclobacillus tolerans* (100%, AF137502)	*Ferrimicrobium acidiphilum* T23 (100%, AF251436)
8/2005	0	MP	++	+	++				+	++	++	+
9/2005	1	MH 1		+			+			+		
12/2005	4											
1/2006	5			+			+	++				
2/2006	6		+				+	+	+			
3/2006	7		+					+				
4/2006	8		++					+				
5/2006	9		++					+	+			
6/2006	10		++					+	+			
8/2006	12		+				+		+			
11/2006	15		++	+				+	++			
12/2006	16		++					++	+			
1/2007	17		++					++	+			
9/2005	1	MH 2	++	+		+	++			+		
12/2005	4		+	+			++	+				
1/2006	5		++				++	+				
2/2006	6		++				++	++	++			
3/2006	7		+									
4/2006	8		++					+				
5/2006	9		++					+	+			
6/2006	10		++					+	++			
8/2006	12		++						+			
11/2006	15		++					+	+			
12/2006	16		++					++	+			
1/2007	17		++					+	+			
12/2005	4	P1		+			+	++		+		
1/2006	5		+				+	+				
2/2006	6							+			+	
3/2006	7		++					+			+	
4/2006	8		++					+	+			
5/2006	9		++					+	++			
6/2007	10		++					+	++			
12/2005	4	P2	++	+			++	++		+		
1/2006	5		++				++	++				
2/2006	6		++				++	+	++			
3/2006	7		+									
4/2006	8		++					+	+			
5/2006	9		++					+	+			
6/2007	10		++					+	+			
8/2006	12	IR1	+				+					
11/2006	15		++	+				++	+			
12/2006	16		++					++	+			
1/2007	17		++	+				++	+			
8/2006	12	IR2	+									
11/2006	15		++					++	+			
12/2006	16		++	+				+	+			
1/2007	17		++	+				++	+			

Table 2
Archaea present in demonstration-scale bioheaps for complex sulfide ore during the primary leaching phase. MH1 = manhole 1, MH2 = manhole 2, P1 = pond 1, P2 = pond 2. + corresponds weak band on DGGE and ++ corresponds strong band on DGGE, empty cell = not detected.

Date	Time (months)	Species\sample	Uncultured crenarchaeote clone JG36-GR-88 (100%, AJ535129)	Thermoplasma acidiphilum strain 122-1B2 (93%, NR_028235)	Uncultured archaeon SAGMA-X (99%, AB050229)
12/2005	4	MH1			
1/2006	5			++	
12/2005	4	MH2			
1/2006	5			++	
12/2005	4	P1	++		++
1/2006	5		++		++
12/2005	4	P2		++	
1/2006	5			++	

increased drastically during the first four months resulting in dilution of leach liquors. During the primary phase cell counts were higher in Heap 2 leach liquors than in Heap 1 even though Heap 2 was not inoculated. The high ferrous iron concentrations in Heap 1 may have affected the growth of microorganisms.

At the end of the primary bioleach phase, cell counts were quite similar in all samples being between 3.2×10^6 mL^{-1} and 7.2×10^6 cells mL^{-1}. Cell counts increased most in manhole 1 being 1.5×10^5 mL^{-1} at the beginning and 5.2×10^6 cells mL^{-1} in the end on primary phase. In the secondary phase, cell counts varied between 1.8×10^6 and 6.0×10^7 cells mL^{-1}, being higher than in primary phase, likely resulting from the stabilized growth conditions. All cell counts decreased toward the end of the secondary phase. After restacking of the ore, the oxygen supply likely improved and decreased again toward the end.

The total cell count in the drilled ore samples averaged 10^6 cells ore g^{-1}. Highest cell counts (1.8×10^7 cell ore g^{-1}) were detected from Heap 2 near the surface where the temperature varied between 20 and 35 °C (Fig. 5). A rough estimate based on liquid and solid sample analysis and associated volumes gives a total of 2×10^{16} cells in the heap, while the liquid phase estimate is 3×10^{14} cells. This shows that more than 98% microorganisms were attached.

3.3. Microbial community characteristics

3.3.1. Start-up phase

Duplicate DGGE profiles after 4 months of bioleaching were as shown in Fig. 4. The enrichment culture used in Heap 1 inoculation contained *Acidithiobacillus ferrooxidans* (99% 16S rRNA gene sequence similarity), *Acidithiobacillus thiooxidans* (100%), *Leptospirillum. ferrooxidans* (98%), *Sulfobacillus thermotolerans* (99%) and a species related to *Acidithiobacillus caldus* (96%) (Halinen et al., 2009a). When the inoculums was grown in the microbial pond (MP) *A. ferrooxidans* (100%), *A. caldus* (99%), *A. thiooxidans* (100%), *L. ferrooxidans* (98%), *Alicyclobacillus acidocaldarius* (99%), *Alicyclobacillus tolerans* (100%) and *Ferrimicrobium acidiphilum* (100%) were present. Several acidophilic microorganisms were also detected at the beginning from the manhole samples. *A. ferrooxidans* (99%), *A. caldus* (96%), *A. acidocaldarius* (99%), *Thiomonas arsenivorans* (100%) and *S. thermosulfidooxidans* (100%) were present (Fig. 4). During the first six months the microbial communities of the leach liquors were diverse and dominated by *A. ferrooxidans* (99%). *S. thermosulfidooxidans* (100%) and a bacterium related to *Firmicutes* clone H70 (91%) were also detected frequently. The DGGE band of the novel bacterium related to clone H70 was cut out, DNA isolated, PCR amplified and sequenced and submitted to GenBank (accession JQ941953).

Table 3
Bacteria present in demonstration-scale bioheaps for complex sulfide ore during the secondary leaching phase. MH1 = manhole 1, MH2 = manhole 2, IR1 = irrigation line 1, IR2 = irrigation line 2. + corresponds weak band on DGGE and ++ corresponds strong band on DGGE, empty cell = not detected.

Date	Time (months)	Sample\species	*Acidithiobacillus ferrooxidans* AP310 (100%, DQ355183)	Bacterium related to clone H70 (91%, DQ328625)	*Leptospirillum ferrooxidans* DSM 2705 (100%, X86776)
2/2007	0	MH1	++	++	+
4/2007	2		++	++	
6/2007	4		++	++	+
7/2007	5		++	++	
10/2007	8		++	++	+
12/2007	9		++	++	
2/2007	0	MH2	++	++	+
4/2007	2		++	++	+
6/2007	4		++	++	+
7/2007	5		++	++	
10/2007	8		++	++	+
12/2007	9		++	++	++
2/2007	0	IR1	++	+	+
4/2007	2		++	+	
6/2007	4		++	++	+
7/2007	5		++	+	
10/2007	8		++	+	
12/2007	9		++	+	++
2/2007	0	IR2	++	++	+
4/2007	2		++		
6/2007	4		++	++	+
7/2007	5		++	+	+
10/2007	8		++	+	
12/2007	9		++	+	+

3.3.2. Temporal dynamics of microbial communities

The bacterial community composition was monitored over time from manholes (MH) and ponds (P) that collected the leach liquor. Bacterial species detected throughout the primary leaching phase were as presented in Table 1. After 6 months of bioheap operation *L. ferrooxidans* (100%) was first observed and it was present thereafter in nearly all samples. The microbial diversity in both heaps varied and decreased with time, with *A. ferrooxidans* remaining as the dominant bacterium and the bacterium related to clone H70 (91%) being present.

Archaea were analyzed after 133 and 163 days of bioleaching from leach liquors of primary heaps and three uncultured species were found (Table 2). One species was related to an uncultured *Crenarchaeote* (100%) retrieved from uranium mining waste from the pond 1 on both days. From the same sample a species related to other uncultured archaeon *SAGMA-X* (99%), also found in deep South African gold mines, was detected. This species is a crenarchaeotic phylotype (Takai et al., 2001). In Pond 2 on day 133 one species related to a clone with the nearest known species of *Thermoplasma acidophilum* (93%) was detected. This species was also present on day 163 from samples MH1, MH2 and P2.

At the secondary leaching phase the leaching community remained steady. *A. ferrooxidans* dominated and the bacterium related to clone H70 and *L. ferrooxidans* were present (Table 3).

3.3.3. Microbial communities on mineral surfaces

Several ore samples were drilled from the primary bioheaps in October 2006 and their microbial communities were as shown in Table 4. *A. ferrooxidans* (99%) was present in nearly all samples. The bacterium related to clone H70 (90%) and *A. caldus* was detected from the areas of wide temperature variation. *S. thermosulfidooxidans* (99%) was found from the high temperature zones of the heap. *F. acidiphilum* (99%) was present in the areas where temperature varied between 20 and 35 °C.

3.3.4. Mesophiles present in demonstration scale bioheaps

Temperatures of the leach liquors were mainly between 20 and 50 °C. Microorganisms were therefore mainly mesophilic and moderately thermophilic. Genus *Acidithiobacillus* (formerly *Thiobacillus*) includes e.g., *A. ferrooxidans, A. thiooxidans* and *A. caldus* that were detected at the primary phase. *A. ferrooxidans* was the dominating micro-organisms during the operation time. *A. ferrooxidans* grows optimally at 30–35 °C and at temperature range of 10–37 °C (Kelly and Wood, 2000). *A. ferrooxidans* was also detected from the ore samples from the high temperature area (80–90 °C), even though the temperature greatly exceeded their growth temperature. *A. ferrooxidans* is able to oxidize reduced inorganic sulfur compounds (RISCs), ferrous iron molecular hydrogen, formic acid and some other metal ions (Rohwerder et al., 2003). It can also grow anaerobically with H_2 or S^0 as an electron donor and S^0 or Fe^{3+} as an electron acceptor (Ohmura et al., 2002).

A. thiooxidans grows in a temperature range of 10–37 °C and has optimum temperature at 28–30 °C. *A. caldus* has a growth rate that exceeds that of *A. thiooxidans* at temperatures over 30 °C (Norris et al., 1986). It has been reported as the dominant sulfur-oxidizing bacterium in bioleaching reactors at temperatures between 40 and 50 °C (Dopson and Lindström, 1999; Okibe et al., 2003). *A. caldus* has a growth temperature range of 32–52 °C and optimum temperature of 45 °C (Kelly and Wood, 2000). *A. thiooxidans* and *A. caldus* that are incapable of pyrite degradation utilize the sulfide moiety of the mineral when it is first released by the action of iron-oxidizing bacteria like *A. ferrooxidans*.

L. ferrooxidans was detected in leach liquors for the first time after 6 months of bioleaching even though it was present in the inoculum. Competition between *A. ferrooxidans* and *Leptospirillum* species has been reviewed (Rawlings et al., 1999; Coram and Rawlings, 2002). *Leptospirillum* species dominate in environments with greater concentrations of ferric iron. The environments classified by high ferrous iron concentration ($>5\ g\ L^{-1}$) seem to select for *A. ferrooxidans* (Pizarro et al., 1996). *A. ferrooxidans* and *Sulfobacillus* spp. are able to oxidize both ferrous iron and RISCs and might exploit the leaching environment more effectively than *A. caldus* or *L. ferrooxidans* that are specialized to oxidize iron or sulfur oxidizers. However, growth ranges and other factors may have overriding effects. The growth temperature range of *L. ferrooxidans* is wider (<10–45 °C) compared to that of *A. ferrooxidans* (Johnson, 2001).

Other mesophiles detected were *F. acidiphilum* and *Thiobacillus arsenivorans*. *F. acidiphilum* has been found in several acidophilic environments (Johnson et al., 2009). It is mesophilic iron-oxidizing obligate heterotroph and grows below 37 °C with the optimum pH 2. *T. arsenivorans* has been originally isolated from a disused mine site by growth using arsenite [As(III)] as energy source. Optimum growth occurred at temperatures between 20 and 30 °C, and at pH between 4.0 and 7.5 (Battaglia-Brunet et al., 2006).

3.3.5. Thermophiles present in demonstration scale bioheaps

At the beginning of bioleaching, temperatures of the Talvivaara demonstration-scale heaps were high and thermophilic and thermotolerant microorganisms were present in leach liquors. *S. thermosulfidooxidans* was detected from leach liquors several times during the first six months of bioleaching. It was also present in ore samples where temperatures were between 65 °C and 90 °C. The genus *Sulfobacillus* includes Gram-positive rods that obtain energy by oxidizing both ferrous iron and elemental sulfur (Norris et al., 1996). *S. thermosulfidooxidans* grows optimally at 50 °C and at pH 1.9–2.4 (Brandl, 2001; Robbins, 2000). The optimum temperature of *S. thermotolerans* is 40 °C and growth range 20–60 °C (Bogdanova et al., 2006). The ability to form endospores is advantageous for survival of bacteria during low temperature periods in a heap operated in boreal conditions, where high seasonal variation in temperature occurs. *Sulfobacillus* species and their occurrence in acidic and bioleaching environments have been reviewed by Watling et al. (2008).

The genus *Alicyclobacillus* was also detected at the beginning of the bioleaching. It was reclassified from genus *Bacillus* by Wisotzkey et al. (1992). They are heterotrophic, aerobic or facultative aerobic, gram-positive or gram variable, spore-forming bacteria and grow at temperatures between 25 and 70 °C and pH values of 2.5 to 6.0.

Table 4
Bacteria present in the drilled ore samples from the demonstration-scale bioheaps for complex sulfide ore during the primary leaching phase. First sample was drilled in a part of the Heap 1 where temperature was 80–90 °C in the depth of 1–2 m. Second was taken in the same place in the depth of 4–5 m. Next samples were taken from Heap 1 in the area where temperature was 65–75 °C. Last samples were drilled from the Heap 2 from the area were temperature was 20–35 °C in the depths of 0–1 m and 4–5 m, respectively.

Species\sample	80–90 °C		65–75 °C		20–35 °C	
	1–2 m	3–4 m	1–2 m	4–5 m	0–1 m	4–5 m
Acidithiobacillus ferrooxidans AP310 (99%, DQ355183)	+	+	+		+	+
A. caldus related bacterium (95%, AY427958)			+			+
Bacterium related to clone H70 (90%, DQ328625)	+	+			+	+
Sulfobacillus thermosulfidooxidans strain YN22 (99%, DQ650351)	+	+	+	+		
Ferrimicrobium acidiphilum T23 (99%, AF251436)					+	+

The novel bacterium that was related to uncultured *Firmicutes* clone H70 (91%) was present during the whole bioleaching time, except in the enrichment culture. Clone H70 was detected in acidic hot springs (T = 55 °C) in North America (Wilson et al., 2008).

The use of extremely thermophilic archaea (e.g., *Sulfolobus* and *Acidianus*) in bioleaching processes is getting more attention. The role of archaea in the biomining community has been considered to rather scavenge the organic material than leach minerals (Johnson, 1998, 2001) but their activity in mineral sulfide oxidation of the bioheap cannot be ruled out. The archaeal species present in the Talvivaara demonstration-scale bioheap leach liquors were related to uncultivated species.

4. Conclusion

The following conclusions can be drawn from the microbial characterization in demonstration-scale bioheap leaching of a complex sulfide ore:

1. The temperature conditions and profiles varied over a wide range (15–90 °C) during the 30 months of operation of the bioheaps.
2. The temperature increased due to exothermic biologically catalyzed oxidation of the sulfidic materials and especially that of pyrrhotite.
3. The total numbers of microbial cells in the heap were estimated to be approximately 2×10^{16} with over 98% of cells being on the ore surfaces.
4. When the enrichment culture was grown in the microbial pond *A. ferrooxidans*, *A. caldus*, *A. thiooxidans*, *L. ferrooxidans*, *A. acidocaldarius*, *A. tolerans* and *F. acidiphilum* were present.
5. During the first six months the microbial communities of the leach liquors were diverse and dominated by *A. ferrooxidans* (99%). An uncultured bacterium related to *Firmicutes* clone H70 (91%) and *S. thermosulfidooxidans* (100%) were also detected frequently.
6. *L. ferrooxidans* was first observed after 6 months of bioheap operation and in all subsequent samples. The microbial diversity in both heaps varied and decreased with time, *A. ferrooxidans* remaining the dominating bacterium.
7. At the secondary leaching phase the leaching community remained steady. *A. ferrooxidans* dominated and the bacterium related to an uncultured clone H70 and *L. ferrooxidans* were present.

In conclusion the multi-metal, low-grade nickel bioheap harbored a diverse microbial community that underwent spatial and temporal changes during leaching. It should be pointed out that DNA based community profiling indicates the presence of a microorganism but not it's activity in a given sample. Microorganisms having different growth temperatures were considered beneficial to the bioheap leaching.

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