Microbial population dynamics of inoculated low-grade chalcopyrite bioleaching columns

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Abstract

This study investigates the effect of temperature on the population dynamics of microorganisms in bioleaching columns charged with a low-grade chalcopyrite ore. A mixed culture containing ten known bioleaching microorganisms was used to inoculate four bioleaching columns operated at 60, 50, 40 or 30ºC. Subsequently, Terminal Restriction Fragment Length Polymorphism (T-RFLP) was used to examine the diversity of bacterial and archaeal populations in the leachates and ores of the four columns. Similar results from samples collected from different locations in the columns give confidence in the reproducibility of the methods used.

Of the 10 microbial inoculants, only *Acidithiobacillus caldus*, *Leptospirillum ferriphilum* and *Ferroplasma acidiphilum* were identified from the leachate and the column solids. However, adventitious growth of a number of other species resulted in different microbial populations in the leachate and on the ore. The results bring into question the effectiveness of heap inoculation, a strategy proposed to overcome the paucity of thermophilic organisms occurring naturally, even in very long-term sulfide-leaching operations. The anticipated impact of temperature on the leachate population was ameliorated by the solution management regime used for the columns, which was chosen to imitate heap leach practice.

Keywords: Bioleaching; Inoculation, T-RFLP; Heap leaching

1. Introduction

As high-grade ore reserves are processed and in the absence of discoveries of new high-grade deposits, it is increasingly necessary to process ores of lower grade to meet demand. Mineral bioleaching is a method by which some low-grade ores can be processed economically. Metal extraction from low-grade sulfide ores and concentrates can be achieved with the assistance of acidophilic chemolithotrophic iron- and sulfur-oxidizing microorganisms (Bosecker, 1997; Watling, 2006).

A wealth of knowledge exists regarding bioleaching microorganisms grown in pure and mixed culture, in chemically defined media (e.g., Franzmann et al., 2005; Johnson, 1998). However, little is known about the dynamics of such microorganisms in the mixed populations of mineral extraction systems. A limited number of studies provide a qualitative description of bacterial populations associated with commercial bioleaching systems (He et al., 2008; Xie et al., 2007). However, only one study has been found (Plumb *et al.*, 2008) in which the growth and activity of acidophilic microorganisms provided with a low-grade chalcopyrite ($CuFeS_2$) energy source is examined. These authors showed that in pure culture, the selected acidophiles were all capable of growth on the sterilised low-grade chalcopyrite ore, albeit at different rates, but that in mixed culture certain strains became dominant.

Microbial population dynamics alter with changing environmental temperature. Microorganisms such as *Acidithiobacillus ferrooxidans, Acidithiobacillus thiooxidans, Leptospirillum ferrooxidans* and *Leptospirillum ferriphilum* dominate mesophilic (15 - 40ºC) bioleaching environments while *Acidithiobacillus caldus, Sulfobacillus thermosulfidooxidans* and

Acidimicrobium ferrooxidans dominate moderately thermophilic (40 – 60ºC) bioleaching environments (Hallberg and Johnson, 2001; Watling, 2006). Archaea of the genera *Acidianus*, *Sulfolobus* and *Metallosphaera* are the dominant mineral sulfide oxidisers in engineered thermophilic (>60ºC) environments rather than bacterial species (Johnson, 1998).

Moderate thermophiles and thermophiles can be exploited in the higher-temperature oxidation of chalcopyrite (Fu et al., 2008; Marhual et al., 2008). However, optimising the temperature for thermophilic activity and/or creating growth conditions that generate the greatest cell numbers do not necessarily result in the greatest copper yields (Vilcáez et al., 2008). High copper extraction is the result of a combination of factors, including microbiological factors.

As in other environments, it may be assumed that acidophiles exist in various symbiotic associations, thus they assist bioleaching more ably when present in mixed cultures. The proportions of various microorganisms in mixed cultures depends on mineralogy and leaching conditions (Pradhan et al., 2008). Sulfide ore heaps represent heterogeneous microbiological habitats with gradients of pH, O_2, CO_2 and nutrients. Therefore, spatial variations in microbial populations within these systems would be anticipated.

Due to the difficulties of collecting solids materials from sulfide heaps during operation, most studies on the microbial diversity have been undertaken by analysing the leachate that exits the base of the heap. However, the formation of biofilms encasing microorganisms attached to the mineral surfaces is a well recognised phenomenon. Therefore, in order to

obtain a complete picture of the biodiversity of a heap, it is necessary also to examine the microbial populations associated with the ore.

The purpose of this study was to investigate the impact of temperature on the survival of inoculant strains (both bacterial and archaeal) in both the leachate and ores of bioleaching columns charged with a low-grade chalcopyrite ore and operated at 60, 50, 40 or 30ºC. The adventitious growth of native microbial populations in the columns contributed further insights to the study.

2. Materials and methods

2.1. Bioleaching columns

Four experimental stainless steel columns were acid conditioned to leach any acid-soluble components arising from the internal acid-resistant coating. They were then charged with a non-sterile, low-grade chalcopyrite ore with approximately 0.5% copper and 0.4% sulfide contents. Chalcopyrite was the main copper-bearing phase with minor bornite (Cu_5FeS_4) also present. The ore had an iron content of about 3% but the pyrite content was <0.5%. The ore was acid conditioned (agglomerated) with 20 kg/t concentrated sulfuric acid. The mass of ore in each column was approximately 36 kg.

Each column was operated separately at the selected temperature using a heated water jacket. Solution management was via closed-cycle, drip feed irrigation (approximately 1.7 mL/min) from an ambient-temperature 17-litre reservoir to the top of the columns. The initial feed solution contained 500

mg/L Fe^{2+} and 500 mg/L Fe^{3+}. Solution residence time within the column was about 1.25 days and six days in the reservoir. Airflow was 500 mL/min from the column base. Periodic addition of concentrated acid to the reservoir maintained the feed to the desired pH 1.5 throughout the experiment.

2.2. Microbial inoculants

Each experimental column was inoculated with a consortium of ten microorganisms, seven bacterial species (*Acidimicrobium ferrooxidans* DSM 10331^T, *Acidithiobacillus caldus* DSM 8584^T, *Acidithiobacillus ferrooxidans* DSM 584^T, *Acidithiobacillus thiooxidans* DSM 14887^T, *Leptospirillum ferriphilum* DSM 14647^T, *Leptospirillum ferrooxidans* DSM 2705^T, *Sulfobacillus thermosulfidooxidans* DSM 9293^T) and three archaeal species (*Acidianus brierleyi* DSM 1651^T, *Ferroplasma acidiphilum* DSM 12658^T, *Metallosphaera hakonensis* DSM 7519^T).

Prior to inoculation each strain was cultured at its optimum temperature and pH using an appropriate iron (II), sulfur or heterotrophic medium to achieve high cell numbers (Franzmann et al., 2005; Plumb et al., 2008). Strains were not adapted to the ore, as sufficiently high cell densities could not be achieved during growth on the ore. Cells from individual cultures were harvested using centrifugation (20 min at 48 000 × g) and re-suspended in 9K medium to achieve the desired cell density (2.1 x 10^8 cells/mL). Equal volumes of each single-strain culture were then mixed to form the inoculum. One litre of mixed inoculum was added to each column to give a cell count of 5.9 x 10^8 cells/kg ore.

2.3. Sampling of leachate

Column leachate (2 L) was collected aseptically from the column discharge after 275 days of operation. Leachates were examined by phase contrast microscopy to ascertain that viable cells were present. The microorganisms present in the leachate were harvested by centrifugation (1 h at 48 000 × g) and then viewed again by phase contrast microscopy. Total DNA was extracted from the leachate, as described in Zammit et al., (2008).

2.4. Sampling of ores

Replicate ore samples were collected aseptically after 414 days at four depths in each column; top (0- 300 mm), middle top (300-600 mm), middle bottom (600-900 mm) and bottom (900-1200 mm). Samples were dissected into approx 1 g (wet weight) subsamples and total DNA extracted using the FastDNA® SPIN Kit for Soil and the FastPrep® Instrument (MP Biomedicals, Santa Ana, CA, USA) as per manufacturers' instructions.

2.5. Clone library of amplified bacterial and archaeal 16S rRNA genes from leachate

Bacterial and archaeal 16S rRNA gene clone libraries were constructed using DNA extracted from the leachate samples for each of the four columns, giving eight libraries in total. PCR amplification was performed as described

by Watkin et al., (2009) and the product ligated into the pGEM-T Easy vector system (Promega, Australia) as per manufacturer's instructions. Positive bacterial and archaeal clones containing the full length DNA insert were subjected to restriction fragment length polymorphism (RFLP) analysis using restriction enzymes *Msp*I and *Rsa*I respectively (Promega, Australia).

Representative clones of each of the dominant PCR-RFLP patterns were prepared for sequencing as described in Watkin et al., (2009). Contigs were checked and assembled using ContigExpress (Vector NTI Advanced 10.3.0, Invitrogen). The sequences were then compared to the sequences lodged in the GenBank database using BLAST. Sequence data have been submitted to the GenBank database and assigned accession numbers FJ216433 to FJ216449.

2.6. Terminal restriction fragment length polymorphism analysis of extracted DNA from leachate and ore.

The diversity of microbes present in the leachate and ore at the end of the bioleaching experiment and their inferred approximate relative abundances was determined by T-RFLP analysis, as described in Hallberg et al., (2006) with the following exceptions. PCR amplification of the 16S rRNA gene was performed using bacterial (fluorescently labelled WellRED D2 (Sigma-Genosys, Australia) 27F and unlabelled 1492R) and archaeal primers (fluorescently labelled WellRED D3 (Sigma-Genosys, Australia) 20F and unlabelled 1392Gr). Restriction digests were performed on successfully

amplified products (5 µl) using *Alu*I, *Cfo*I, *Hae*III for both bacterial and archaeal products and *Msp*I for only bacterial products.

Terminal restriction fragments (T-RF's) were determined by comparison of their mobilities with those of the size standard. The relative abundance of each T-RF was calculated using the peak areas for each T-RF relative to the total peak area.

T-Align (Smith et al., 2005) was used for comparisons of replicate T-RFLP profiles in order to generate consensus profiles to identify shared and unique T-RF's between leachate and ores of the four columns. Identification of microbial species using the individual T-RF's from the columns was deduced by comparing with the clones identified in this study and those T-RF's in a database held at Bangor University, UK (Johnson et al., 2008; Wakeman et al., 2008).

3. Results

3.1. Leachate analysis

The physiochemical composition of the leachate at the time of sampling (day 275 and day 414) from each of the four columns are shown in Table 1. Copper extraction (%) increased with column temperature, which is consistent with the known temperature-dependence of chalcopyrite oxidation using ferric ions (Watling, 2006). Microbial activity was high in all columns, evidenced by the high redox potentials and negligible ferrous ion concentrations in the

leachates, which were otherwise typical in their elemental compositions for the dissolution of this ore at different temperatures.

3.2. Column leachate microbial diversity

The leachates, when viewed under 100 × phase contrast microscopy, contained archaea (cocci morphology) and some bacterial cells (rod morphology) (approx. 2 - 17 $\times$ 10^6 cells/mL) before the initial centrifugation step.

General bacterial and archaeal 16S rDNA PCR products were successfully amplified from each of the leachates and clone libraries were then generated from the PCR products. The distinct bacterial clones sequenced were used to identify novel terminal restriction fragments (T-RF's) that could not be matched with those from the inoculum or the Bangor University (UK) database (Johnson et al., 2008; Wakeman et al., 2008).

Terminal fragment analysis of the leachate bacterial 16S rRNA gene, revealed greatest diversity when digested with *Msp*I (Fig 1). Identification of the following T-RF's are listed in Table 2 and in parenthesis as follows. T-RF's nt. 493 (*Acidithiobacillus caldus*) is observed in the 40ºC and 50ºC columns. T-RF nt. 70 (Isolate P22) is observed in the 50ºC and 60ºC column while T-RF nt. 497 (*Serratia marcescens*) is only absent in the 50ºC column. T-RF's nt. 77 (*Leptospirillum ferriphilum* strain Fairview), nt.172 (*Leptospirillum ferriphilum* strain BRGM1) and nt. 445 (uncultured bacterium clones SX1-107 and D3-77) are observed in all four columns. While T-RF nt. 190 is also present in all four columns, no match to the 16S rDNA clones or database

was observed. *Leptospirillum ferriphilum* (nt. 77 and nt.172) was dominant in leachate samples (> 47%) except for the 50ºC column which had a significant proportion (26%) of *Acidithiobacillus caldus* (nt. 493).

Terminal fragment analysis of the archaeal 16S rRNA gene revealed greatest diversity when digested with *Cfo*I (data not shown). The leachate DNA sample from the 30ºC column did not amplify with fluorescent primers despite repeated attempts. T-RF nt. 324 established as *Ferroplasma acidiphilum* based on the Bangor University database, is dominant in the leachate of the 40ºC and 50ºC columns and present in the 60ºC column. T-RF nt. 58 is the dominant fragment in the 60ºC column leachate. No other T-RF's were present.

3.3. Column ore microbial diversity

Terminal fragment analysis of the ore bacterial 16S rRNA gene again revealed greatest diversity when digested with *Msp*I (Fig 2). Identification of the following T-RF's are listed in Table 2 and in parenthesis as follows. T-RF's nt. 59 (no match), nt. 65 (*Acidisphaera rubrifaciens*), nt. 493 (*Acidithiobacillus caldus*) and nt. 605 (*Acidithiobacillus* sp. CC2) are observed in all four columns. *Acidithiobacillus caldus* (nt.493) clearly dominates (> 42%) each column at all four temperatures. The other T-RF's are observed randomly over the four columns. T-RF's nt. 148 (no match) and nt. 151 (*Bosea* sp. RM1) were observed in all but the 30ºC column, T-RF's nt. 74 (no match) and nt. 77 (*Leptospirillum ferriphilum* strain Fairview) were observed in all but the

50ºC column while T-RF nt. 118 (*Sulfobacillus acidophilus* YTF1) was observed in the 40ºC and 50ºC columns.

Terminal fragment analysis of the archaeal 16S rRNA gene revealed greatest diversity when digested with *Cfo*I (data not shown). As was seen in the leachate, T-RF nt. 324 (*Ferroplasma acidiphilum*) is dominant in all columns.

Bacterial T-RF nt. 493 (*Acidithiobacillus caldus*) was dominant at each of the four locations (top, mid-top, mid-bottom and bottom) in all columns except for the mid-top location of the 40ºC column (data not shown). T-RF nt. 118 (*Sulfobacillus acidophilus* YTF1) was dominant at this location. Archaeal T-RF nt. 324 (*Ferroplasma acidiphilum)* was dominant at all depths within all columns (data not shown).

With the exception of T-RF nt. 74 (no match), nt. 77 (*Leptospirillum ferriphilum*) and nt. 493 (*Acidithiobacillus caldus*) the bacterial population for the ores differed from those of the leachates at all temperatures (Fig 3 a-d).

4. Discussion

In this study, the microbial communities in leachates and ore from four bioleaching columns operated at different temperatures were examined 275 and 414 days, respectively, after inoculation with a microbial consortium of seven bacteria and three archaea. The conditions for column operation were selected to imitate a heap environment and generate data on the bio-assisted extraction of copper from chalcopyrite in the ore for a range of temperatures. At typical heap leach operations, the leachate is recycled through ambient temperature ponds before being dripped or sprayed onto the surfaces of the

heaps (Watling, 2006). This was imitated by maintaining the reservoir at room temperature before recycling the leachate back into the system.

The ore used in this study was neither pre-treated nor sterilised before being packed into the columns, nor were the columns themselves treated in any way other than the acid resistant coating. Microbial species present in the leachate and ores but not introduced via the inoculum may have been introduced into the experimental system via the columns or ore itself at the on-set of the experiment.

4.1. Fate of inoculant species and value of inoculation

Two bacterial inoculants, *Acidithiobacillus caldus* and *Leptospirillum ferriphilum*, and one archaeal inoculant, *Ferroplasma acidiphilum*, colonised the columns. The first species has the ability to oxidise reduced inorganic sulfur compounds (RISC) such as elemental sulfur or polythionates but not to oxidise iron(II) while the latter two species can oxidise iron(II) but not RISC.

Acidithiobacillus caldus (T-RF's nt. 493; 99% similarity to inoculant type strain) was the dominant bacterial species observed in the ores but was only detected in the leachate at 40 and 50ºC. These results are similar to those of Plumb et al., (2008) who identified *Acidithiobacillus caldus* in mixed cultures after 98 days growth on the chalcopyrite ore at 28, 35 and 45ºC. *Acidithiobacillus caldus* has been enriched from hot springs (pH ~3) at 33ºC and 48ºC (Burton and Norris, 2000) and found to be the dominant sulfur oxidiser in bioleaching tanks operated at 40 to 55ºC (Rawlings et al., 1999a).

Leachate samples were dominated by T-RF's related to *Leptospirillum ferriphilum* (nt. 77; Fairview and nt.172; BRGM1) for all columns. *Leptospirillum ferriphilum* strain Fairview and Leptospirillum ferriphilum strain BRGM1 show 99% and 97% similarity, respectively, to the *Leptospirillum ferriphilum* type strain DSM14647 used in the inoculum. It is therefore likely that T-RF's nt. 77 were derived from the inoculum while T-RF's nt. 172 were native to the ore. The importance of *Leptospirillum ferriphilum* has only recently been recognised. It has been shown to dominate the microbial population in some mineral processing bioreactors (Chen et al., 2007; Okibe et al., 2003) and growth has been reported at temperatures above 55ºC (Coram and Rawlings, 2002).

The only archaeal inoculant to be identified in the column leachate and ore was *Ferroplasma acidiphilum*. It was the dominant archaeal T-RF (nt. 324) for the 30, 40 and 50ºC columns, but was also detected in the 60ºC column. Franzmann et al., (2005) reported the T_{OPT} for *Ferroplasma acidiphilum* (DSM 12658^{T}) as 39.6ºC and the notional T_{MAX} as 47.2ºC but higher-temperature strains have been reported (Burton and Norris, 2000).

The remaining seven inoculants were not detected in any column leachates or ores. *Acidithiobacillus ferrooxidans*, *At. thiooxidans and L. ferrooxidans,* all mesophiles, were expected to be present in the 30ºC and perhaps 40ºC column leachates. While the reasons for the absence of these microorganisms are not clear, the results are consistent with those of Plumb et al. (2008). In that study, growth of the three microorganisms was demonstrated in pure culture on ground ore (P_{80} 75 µm) but no growth was seen in mixed cultures grown on the ore at 28, 35 or 45ºC. It was assumed

that, at this grind size, a large proportion of the ~0.8 % sulfide in the ore was exposed to the leachate and bacteria in those tests. Wakeman et al. (2008) identified differing microbial composition and dynamics of bioleaching microorganisms depending on the particle size of the ore. Those authors compared leaching of ultra-fine ground ore (<2 um) with particles in the ranges 2-6.5 mm and 6.5-12 mm and reported that the cultures grown on ultra-fine ground ore were dominated by *L. ferriphilum* with smaller numbers of *At. caldus* and *L. ferrooxidans*. However in their columns, while *At. ferrooxidans* dominated initially it was later outgrown. For the columns the leaching results confirmed that occlusion of sulfide grains within gangue minerals hindered metals extraction, as expected. For the present study, it can be assumed that particle size has an affect on metal extraction. It was estimated that as little as 25% of the contained sulfide was exposed to the leachate and bacteria (i.e. ~0.2%), a content that was apparently insufficient to support the growth of these microorganisms when in competition. The *Acidithiobacillus* species were once thought to be the most important in bioleaching systems (Bosecker, 1997). However this study, together with those of Rawlings et al. (1999b) and Plumb et al. (2008) tend to contradict that earlier assumption.

It was also anticipated that *Acidimicrobium ferrooxidans* would be found in the 30, 40 and 50ºC and possibly the 60ºC columns, as it is a moderate thermophile with T_{OPT} 49ºC (Franzmann et al., 2005) and has been shown to oxidise iron(II) in the temperature range 25 to 59ºC (Keeling et al., 2005). *Acidimicrobium ferrooxidans* grows well on ferrous ion but RISC are poorly oxidised, if at all (Clark and Norris, 1996). In our laboratories, it was found to

grow poorly on mineral sulfides and only under pH-controlled conditions (unpublished data) and it was not very tolerant of copper (Watkin *et al.*, 2009). The chalcopyrite ore used in the present study was acid consuming over a prolonged period and, as such, may not have provided conditions conducive to the growth of *Acidimicrobium ferrooxidans*, particularly in the early stages of leaching.

In retrospect, the absence of clones or T-RF's of *Sulfobacillus thermosulfidooxidans* type strain in any of the column samples is not entirely surprising. While this species has been shown to enhance the oxidation of chalcopyrite concentrate (Stott et al., 2003) and also to grow, but poorly, on the chalcopyrite ore in this study, it does have a preference for mixotrophic growth, a condition not well met by this chalcopyrite ore. A further contributing factor may be the difficulty encountered in lysing *Sulfobacillus thermosulfidooxidans* cells in order to extract the DNA (Zammit *et al.*, 2009). This is an extremely important consideration when determining microbial diversity in mixed environmental samples, as the basis for species or strain detection relies upon reproducible and representative extraction of the DNA (Wintzingerode et al., 1997).

Acidianus brierleyi and *Metallosphaera hakonensis* were not identified in the column samples from the four bioleaching columns. The highest temperature column, at 60ºC, was 10 degrees below the T_{OPT} (70^{o}C) of both microorganisms. *Acidianus brierleyi* has been shown to be a poor iron oxidiser at temperatures below its T_{OPT} (Franzmann et al., 2005). Their preferred habitats are solfatara fields or geothermally-heated acidic springs and they grow well at pH 1 or less, that is at higher acidity than many of the bioleaching

bacteria (Segerer *et al.*, 1986; Takayanagi *et al.*, 1996; Kurosawa *et al.*, 2003; Plumb *et al.*, 2008). In the present study, the pH tended to be higher due to the acid consuming properties of the ore. This, together with the maximum temperature experienced being considerably lower than the preferred growth temperatures for the two species and the relatively long residence times of the leachates in ambient-temperature reservoirs, probably did not provide an environment conducive to growth.

The question of inoculation in the context of thermophilic heap leaching of low-grade ores arises quite often, prompting such studies as described here. The target of such studies is the heap bioleaching of chalcopyrite, the most abundant but refractory copper sulfide which oxidises faster at higher temperatures. Heap inoculation at copper operations tends to be a relatively passive process of applying mixed cultures (containing mesophiles and some moderate thermophiles) deliberately enriched from the relevant ore in ponds or tanks, or raffinate solution being recycled from the SX plant and containing representative species from the heap, during acid conditioning, stacking and/or heap irrigation. The results obtained here and in two previous studies (this laboratory, not published) suggest that successful inoculation of low-grade ores with extreme thermophiles is difficult, the organisms being adversely affected by key factors such as prevailing temperature and acidity but also by sulfide (or sulfur) content. Sulfide is in short supply in low-grade ores but present in abundance in stirred tank reactors processing concentrates and where inoculation with, and exploitation of, extreme thermophiles has been demonstrated successfully (du Plessis *et al.*, 2007). It is worth noting that no report of extreme thermophiles being isolated from

commercial heaps has been found, even where the heaps reached high temperatures as a result of sulfide oxidation, except where those species were deliberately inoculated. However, culture-independent analysis of Escondida heap ore has revealed sequences related to archaeal *Sulfolobus* species (Demergasso *et al.*, 2005). Thus the way to achieve the goal of thermophilic heap bioleaching for chalcopyrite may depend upon the development of a successful inoculation protocol that delivers extreme thermophiles to the ore when conditions are conducive to colonisation and growth.

4.2. Biodiversity of ore and leachate

Microorganisms that were observed in the columns, and were identified in both the leachate and ore fractions, were generally representative of the optimum temperature (T_{OPT}) at which each species was expected to survive. Different bacterial populations were clearly observed in the leachate and ore samples (Fig 3 a-d) for all four columns.

While the operation of the columns at four different temperatures impacted as expected on copper leaching efficiency (Table 1), the solution management method adopted for the columns buffered the influence of column temperature on the microbial populations in leachates (Fig 1). The detection of mesophilic and/or moderately thermophilic strains in the leachates of nominally high-temperature columns is one of the consequences of solution recycle through, and prolonged residence in, an ambient-temperature reservoir.

In contrast to the leachates, the ores were maintained at temperature by means of the temperature-controlled water jackets surrounding each column. Regardless of location in each of the columns, the ores were dominated by the same bacterial and archaeal species (nt. 493; *Acidithiobacillus caldus* and nt. 324; *Ferroplasma acidiphilum*) except the mid-top region of the 40ºC column which was dominated by the bacterial T-RF nt. 118 (*Sulfobacillus acidophilus*). It is concluded that the height of the ore bed was insufficient to develop different microbial populations at different depths. Thus the subtle differences observed in microbial populations in the ores with respect to the different columns can be attributed to temperature (Fig 2). Some lower-temperature strains were detected in column discharge solutions immediately following passage through the heated ore bed. From this it is deduced that these strains are more resilient than might be inferred from their temperature-dependent growth characteristics (e.g. Franzmann *et al.*, 2005). The ores offer unique microenvironments for the survival of the microorganisms. They can either be protected in small spaces between the ore particles or more significantly encased within the extracellular polysaccharide (EPS) matrix of biofilms which may shield them from harsher conditions to which they might otherwise be exposed.

4.3. Adventitious colonisation of columns

In most cases at heap leaching operations it is assumed that the ore contains suitable organisms which become active once moisture and air (carbon dioxide and oxygen) are supplied. Heaps become colonised by iron(II) and sulfur oxidising organisms to the extent supported by the exposed

sulfide minerals. A diverse heterotrophic population develops somewhat later, reliant on the organic content of the ore. These are circumstances well known in acid mine drainage, which is essentially the same oxidation system without the management regime. The ore used in the columns was expected to contain within it a variety of chemolithotrophs and heterotrophs which might be augmented by organisms present in the laboratory setting. Some of these have been detected during the microbial analysis of column leachates and ores (Table 2). Apart from a small amount of yeast extract added as part of the inoculum at the start of the experiment, no additional organic compounds were added to the columns. It is surmised that the relatively low presence of the heterotrophic species in some column samples was the result of growth on lysed cells and cell exudates from other microorganisms.

An *Acidithiobacillus* T-RF, nt. 605 (*Acidithiobacillus* sp. NO-37) was observed albeit it in low proportions in ores from the four columns. This species, originally isolated from mine drainage from an abandoned copper mine (Johnson et al., 2001), shows 98% similarity to the *Acidithiobacillus ferrooxidans* type strain. It is therefore likely to be a native microorganism.

T-RF nt. 70 (*Acidimicrobium* sp Y0018) was detected in the leachate of both the 50 and 60 ºC columns. This species of *Acidimicrobium* (Y0018) shows 95% similarity to the *Acidimicrobium ferrooxidans* type strain in the inoculum. While it does indicate that *Acidimicrobium* is present in the system, the strain is more probably a native organism of the ore. The failure to detect the *Acidimicrobium ferrooxidans* type strain in the columns at any temperature is probably due to a combination of physiological attributes and mineralogical factors. As mentioned previously, this species has a strong capacity to oxidise

ferrous ion and also grows heterotrophically (Clark and Norris, 1996), but growth on mineral sulfides such as pyrite or chalcopyrite is poor, especially when there is poor pH control.

The only *Sulfobacillus*-like T-RF (nt. 118; *Sulfobacillus acidophilus* YTF1; 90% similarity to inoculant *Sulfobacillus thermosulfidooxidans*) was detected in the ore of the 40 and 50ºC columns. The known *Sulfobacillus* species have in common that they grow in extreme sulfur-rich environments with a broad geographical distribution. Specifically, *Sulfobacillus acidophilus* has been identified in samples from Europe, North America, Africa and Asia-Pacific (Watling *et al.*, 2008) and is probably ubiquitous in sulfidic and sulfurous locations as well as bioleaching reactors (heaps and tanks) of appropriate temperature and acidity. Physiologically, it is closely aligned with *Sb. thermosulfidooxidans*, sharing a T_{OPT} of 51^{o}C and a preferred pH of about 1.7 and capable of oxidising both iron(II) and reduced inorganic sulfur compounds and of spore formation which may confer additional protection against hostile environments.

A number of acidophilic heterotrophs were also detected in the columns (Table 2). *Acidisphaera rubrifaciens* and two uncultured bacterial clones (SX1-107 and D3-77) (He *et al.* 2008), sequence similarity 93% to *Acidiphilium cryptum* sp., were detected in all columns. *Acidisphaera rubrifaciens* and *Acidiphilium cryptum* sp. have been found in a range of acidic environments such as hot springs and mine drainage (Burton and Norris, 2000; He et al., 2008; Garcia-Moyano et al., 2007; Hiraishi et al., 2000; Bridge and Johnson, 2000.

Other species identified in low proportions by either T-RFLP or 16S rRNA gene clone sequencing in all columns but not normally associated with mineral bioleaching include *Serratia marcescens* and *Bosea*. *Serratia* spp. isolated from contaminated sites have shown arsenic (Turpeinen et al., 2004), zinc (Bhadra et al., 2007) and nickel resistance (Abin et al., 2002; Marrero et al., 2007), the last strain being isolated from a nickel laterite deposit. *Bosea* sp. RM1 has been isolated from Mn(II) contaminated waters (Mariner et al., 2008) while *Bosea thiooxidans* which is 99% similar has the ability to oxidise thiosulfate (Das et al., 1996).

The identity of a number of T-RF's (nt. 59, 74, 148 and 190) could not be established by either the Bangor Database or 16S clone library sequencing.

5. Summary

There is both a dominant bacterial and a dominant archaeal species in the leachates collected from all of the bioleaching columns. While generally different microbial populations were identified in the leachate and the ores, the columns were still dominated by two of the bacterial inoculants; *Leptospirillum ferriphilum,* in the leachate, *Acidithiobacillus caldus* in the ore and one of the archaeal inoculants, *Ferroplasma acidiphilum,* in both the leachates and ores.

A significant proportion of sequenced clone types and/or terminal restriction fragments were detected which were not components of the initial consortium inoculated into the columns. These are attributed to microbes persisting in the non-sterile chalcopyrite ore used in the experiments. The columns were open to the environment and operated with a drip feed and solution recycle. This type of continuous flow system will select for those microorganisms, not

necessarily the inoculants, which are able to grow most efficiently within the specific environment. The columns were inoculated only once, at the beginning of the study. Survival of the inoculant strains was not monitored during the progress of the trial. In subsequent research it would be interesting to track the inoculant strains as a function of time.

5. Acknowledgments

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Fig 1. T-RFLP analysis of the bacterial diversity in the column leachates, showing the relative abundance of each terminal restriction fragment of amplified 16S rRNA genes digested with *Msp*I as a percentage of the total peak area of all terminal restriction fragments.

Fig 2. T-RFLP analysis of bacterial diversity in the column ore showing the relative abundance of each terminal restriction fragment of amplified 16S rRNA genes digested with *Msp*I as a percentage of the total peak area of all terminal restriction fragments.

Fig 3. T-RFLP analysis of bacterial diversity in all four columns of the leachate and ore showing the relative abundance of each terminal restriction fragment of amplified 16S rRNA genes digested with *Msp*I as a percentage of the total peak area of all terminal restriction fragments.

Table 1. Physiochemical data for the leachates collected at day 275 and 414 respectively from four experimental leaching columns.

Parameters								
Temp (ºC)	30		40		50		60	
Time (d)	275	414	275	414	275	414	275	414
pH	1.59	1.31	1.72	1.38	1.63	1.43	1.70	1.38
E_H (mV)	686	688	672	550	661	628	625	587
Fe^{2+} [mg/L]	16	23	1	5	1	5	8	4
Cu [mg/L]	659	925	1171	634	996	1185	888	1168
Fe [mg/L]	4391	6604	1193	2003	316	667	332	553
S_{SOL} [mg/L]*	25569	38062	22212	36293	17515	30828	21986	33071
Mg [mg/L]	7819	11673	7597	12727	6245	11127	8493	12468
Al [mg/L]	5362	7776	4896	8429	3872	7201	4835	7642
Si [mg/L]	239	470	188	290	142	186	134	128
K [mg/L]	1940	3394	1084	5185	559	3835	813	4117
Ca [mg/L]	600	601	580	623	534	604	577	555
Na [mg/L]	76	83	65	87	57	79	73	92
Cu (% leached)	12	17	31	38	55	64	62	73

* Predominantly sulfate anion, associated with significant Fe, Mg, Al, K and Ca cations

Table 2. Bacterial terminal fragment (T-RF) length identification

T-RF Length (nt)	Leachate	Ore	Proposed Bacterial Identity*	% similarity to inoculant type strain[#]
59		✓	No match	
65		✓	*Acidosphaera rubrifaciens* (Mirete et al., 2007)	
70	✓		Isolate P22 (Bangor isolate) and *Acidimicrobium* sp Y0018 (Johnson et al., 2003)	95 (*Am fx*)
74		✓	No match	
77	✓	✓	*Leptospirillum ferriphilum* strain Fairview (Coram and Rawlings, 2002)	99 (L fp)
118		✓	*Sulfobacillus acidophilus* YTF-1 (Bridge and Johnson, 1998)	90 (S th)
148		✓	No match	
151		✓	*Bosea* sp. RM1 (Mariner et al., 2008)	
172	✓		*Leptospirillum ferriphilum* strain BRGM1 (Battaglia-Brunet et al., 2002)	97 (L fp)
190	✓		No match	
445	✓		Uncultured bacterium clones SX1-	93

			107 and D3-77 (He et al., 2008)	(Acidiphilum cryptum)
493	✓	✓	*Acidithiobacillus caldus* strains N39-30-02 and N39-45-02 (Watkin et al., 2009)	99 (At c)
497	✓		*Serratia marcescens* strain L1 (Zhu et al 2007)	
605		✓	*Acidithiobacillus* sp. CC2 (Bangor isolate) and NO-37 (Johnson et al., 2001)	98 (At fx)

* Identity based on 16S rRNA gene clone sequences and Bangor University database

[#] unless otherwise stated: Am fx, *Acidimicrobium ferrooxidans*: L fp, *Leptospirillum ferriphilum*: S th, *Sulfobacillus thermosulfidooxidans*: At c, *Acidithiobacillus caldus*: At fx, *Acidithiobacillus ferrooxidans*

Fig 1

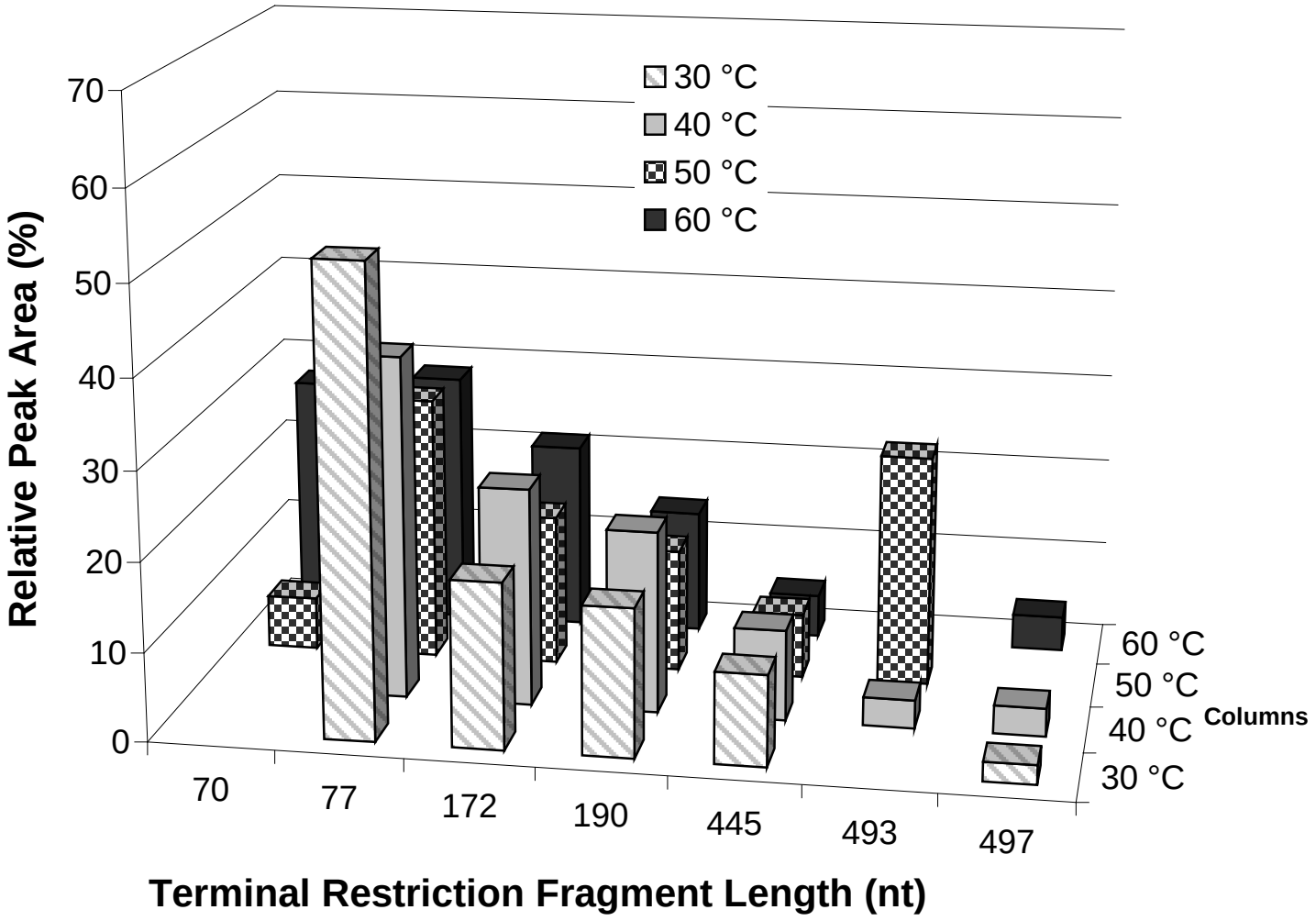

30 °C
40 °C
50 °C
60 °C
Relative Peak Area (%)
0
10
20
30
40
50
60
70
70
77
172
190
445
493
497
Terminal Restriction Fragment Length (nt)
60 °C
50 °C
40 °C
30 °C
Columns

Fig 2

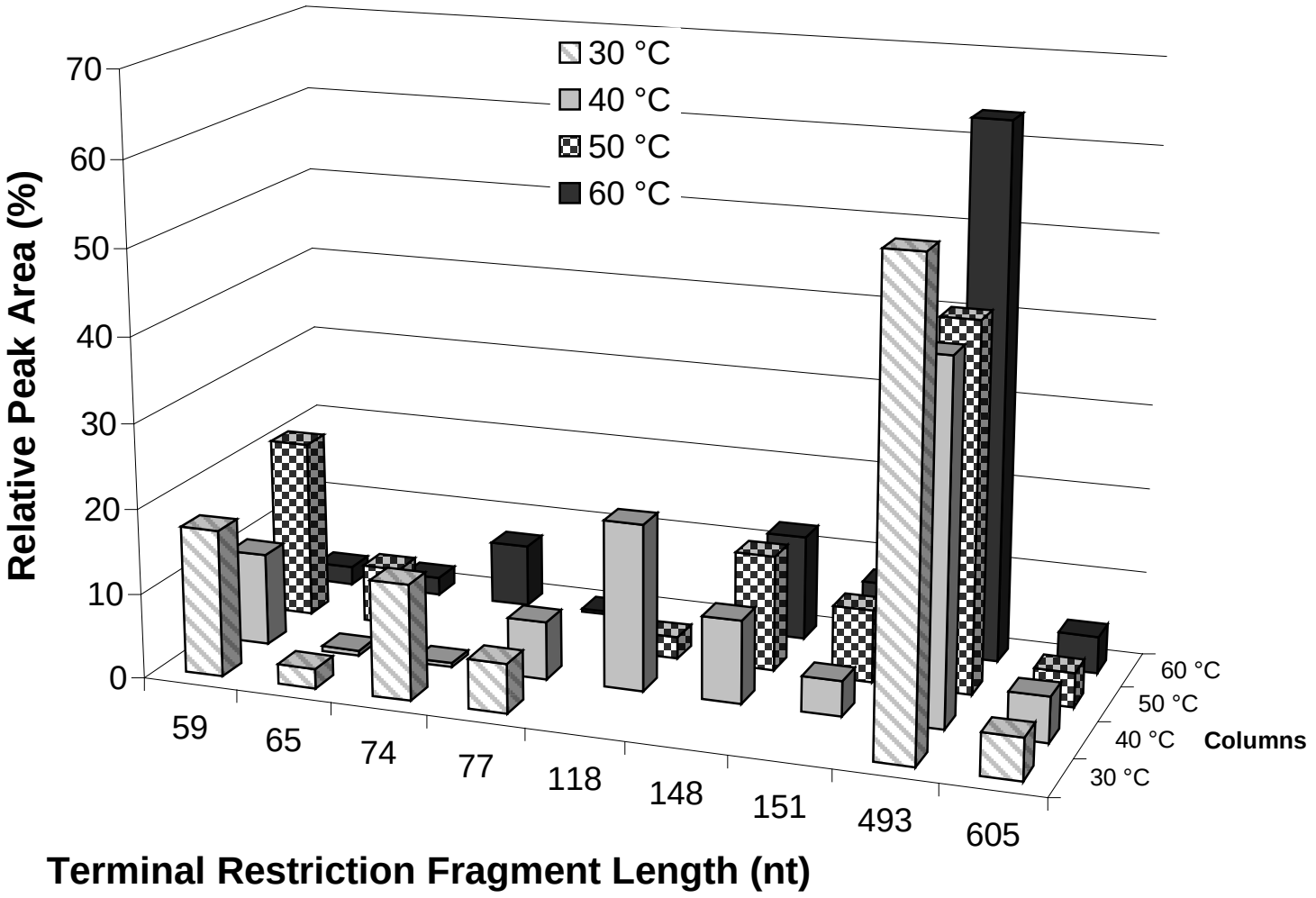
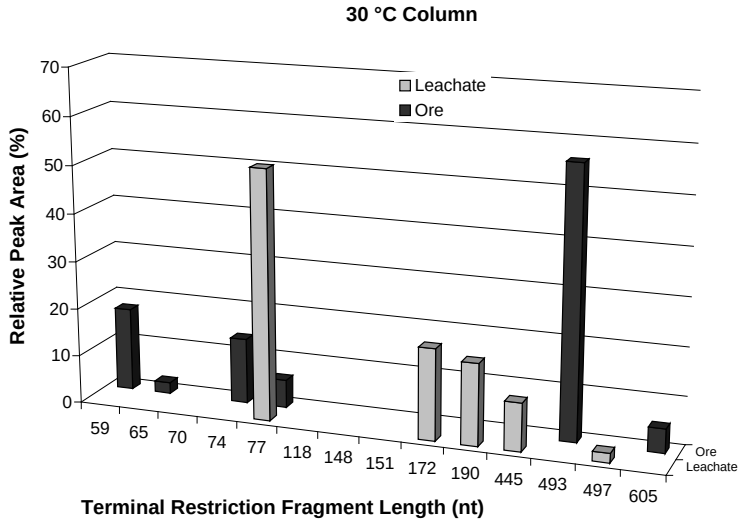

30 °C
40 °C
50 °C
60 °C
Relative Peak Area (%)
70
60
50
40
30
20
10
0
59
65
74
77
118
148
151
493
605
Terminal Restriction Fragment Length (nt)
60 °C
50 °C
40 °C
30 °C
Columns

Fig 3


30 °C Column
Leachate
Ore
Relative Peak Area (%)
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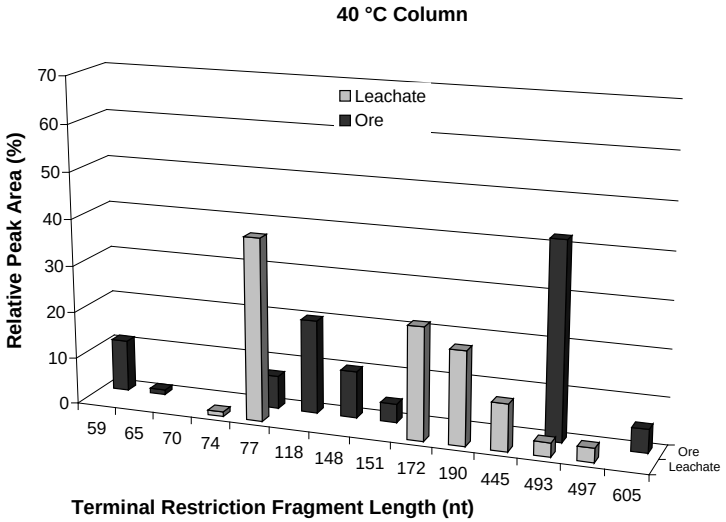
10
20
30
40
50
60
70
59
65
70
74
77
118
148
151
172
190
445
493
497
605
Ore
Leachate
Terminal Restriction Fragment Length (nt)

a


40 °C Column
Leachate
Ore
Relative Peak Area (%)
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20
30
40
50
60
70
59
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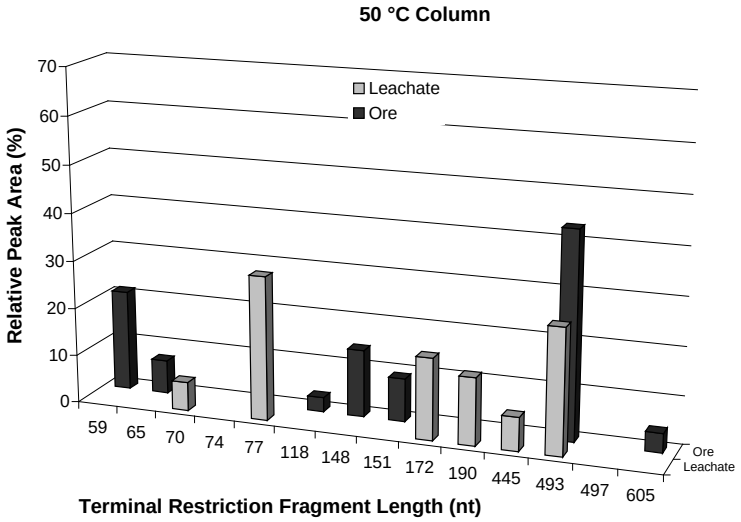
118
148
151
172
190
445
493
497
605
Ore
Leachate
Terminal Restriction Fragment Length (nt)

b


50 °C Column
Leachate
Ore
Relative Peak Area (%)
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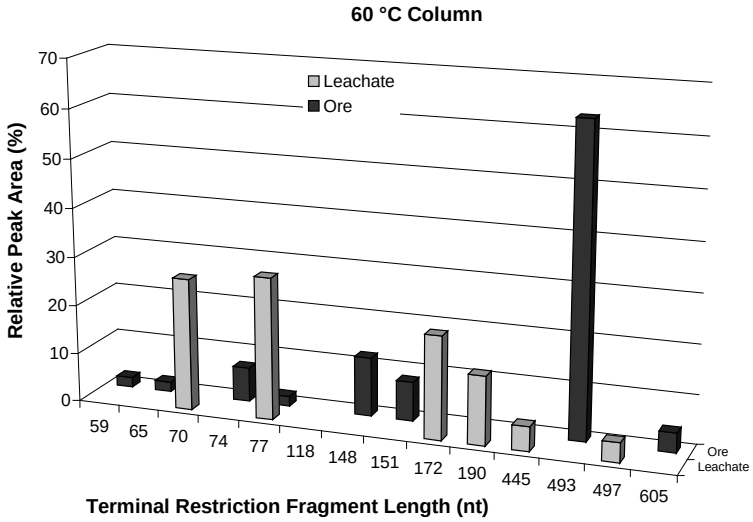
20
30
40
50
60
70
59
65
70
74
77
118
148
151
172
190
445
493
497
605
Ore
Leachate
Terminal Restriction Fragment Length (nt)

c


60 °C Column
Leachate
Ore
Relative Peak Area (%)
0
10
20
30
40
50
60
70
59
65
70
74
77
118
148
151
172
190
445
493
497
605
Ore
Leachate
Terminal Restriction Fragment Length (nt)

d