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Microbial Ecology Assessment of a Mixed Copper Oxide/Sulfide Dump Leach Operation

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Microbial consortia composed of complex mixtures of autotrophic and heterotrophic bacteria are responsible for the dissolution of metals from sulfide minerals. Thus, an efficient copper bioleaching operation depends on the microbial ecology of the system. A microbial ecology study of a mixed oxide/sulfide copper leaching operation was conducted using an "overlay" plating technique to differentiate and identify various bacterial consortium members of the genera *Thiobacillus, "Leptospirillum", "Ferromicrobium"*, and *Acidiphilium.* Two temperatures (30°C and 45°C) were used to select for mesophilic and moderately thermophilic bacteria. Cell numbers varied from $0-10^6$ cells/g dry ore, depending on the sample location and depth. After acid curing for oxide leaching, no viable bacteria were recovered, although inoculation of cells from raffinate re-established a microbial population after three months. Due to low the pH of the operation, very few non-iron-oxidizing acidophilic heterotrophs were recovered. Moderate thermophiles were isolated from the ore samples. Pregnant liquor solutions (PLS) and raffinate both contained a diversity of bacteria. In addition, an intermittently applied waste stream that contained high levels of arsenic and fluoride was tested for toxicity. Twenty vol% waste stream in PLS killed 100% of the cells in 48 hours, indicating substantial toxicity and/or growth inhibition. The data indicate that bacteria populations can recover after acid curing, and that application of the waste stream to the dump should be avoided. Monitoring the microbial ecology of the leaching operation provided significant information that improved copper recovery.

1. INTRODUCTION

Complex microbial consortia of autotrophic and heterotrophic bacteria are known to be responsible for the dissolution of metals from sulfide minerals (1). The number and composition of the bacteria responsible for this oxidation vary within the heap and with time. Dumps present diverse and changing conditions affecting bacterial activity and survival, including temperature, pH, Eh and solute concentration (2). Therefore, in order to optimize a copper bioleaching operation and determine whether the process is perturbed, it is important to assess the microbial ecology of the system. Several techniques have been used to evaluate the microbial diversity in the leaching environment. Some of these include Most Probable

Number (MPN), 16S rRNA gene analysis, spacers between the 16S and 23S rRNA genes, immunofluorescence, fatty acid methyl esters, and plating (1,3-6). Most of these techniques suffer from limitations caused by the extremely acidic environment and either do not give complete species profiles or accurate counts of each species.
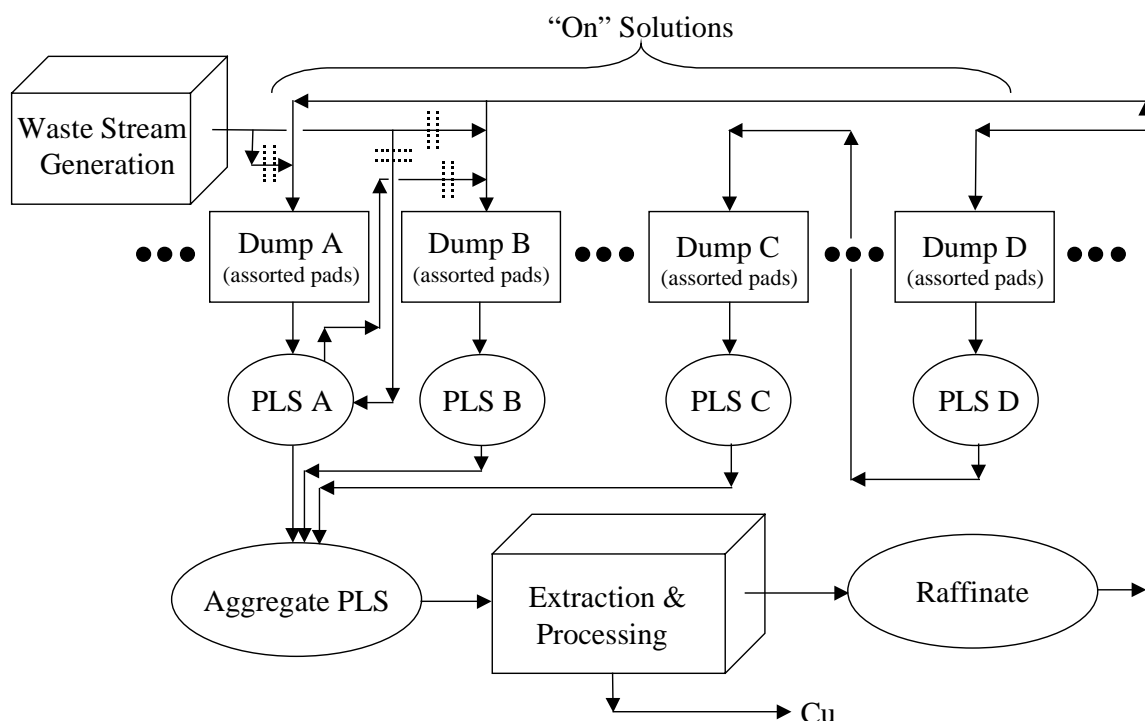
In this study, the microbial ecology of an oxide/sulfide copper leaching operation was assessed using an "overlay" selective plating technique in which an acidophilic heterotrophic bacterium is incorporated into the underlayer of a two-layer gel (7). This medium, which includes tetrathionate, ferrous iron, and tryptone soya broth, has been used successfully to culture all known iron-oxidizing mesophilic, and moderately thermophilic bacteria. The medium also allows differentiation and preliminary identification of isolates by colony morphology. Bacteria were recovered from surface ore, ore samples from depth, pregnant liquor solution, raffinate, "On" solution (solution applied to the surface of the dumps) and makeup water. Samples were screened for commonly occurring iron- and sulfur-oxidizing mesophilic and thermophilic organisms as described above. Several minimum inhibitory concentration studies were also done to assess the potential toxicity of solutions added in the leaching process to the microorganisms.

2. MATERIAL AND METHODS

Ore and liquid samples were collected from a large oxide/sulfide copper mine in the Southwestern U.S. The ore was composed of copper oxides, chalcocite, chrysocolla and trace amounts of chalcopyrite. At least 4 dumps, designated A-D, were included in the leaching system, each with several leach pads designated, for example, A(1) - A(n) for Dump A; see Figure 1. The dumps were acid-cured for 3-5 days with 200g/L sulfuric acid, rested for 3 days and then rinsed with raffinate for ~120 days. PLS from Dump D was used as "On" solution for Dumps C(1) and C(2). A waste stream mixed with raffinate was used as "On" solution for Dump A. "On" solution for Dump B consisted of raffinate, raffinate intermittently mixed with variable amounts of a waste stream, or with PLS from Dump A.

Several water chemistry measurements, including pH, Eh, total iron, ferrous iron, total copper, nitrates and phosphates were taken using standard techniques. Surface ore samples were taken from a total of six pads on two dumps, at various locations within the pads, and at various stages of the acid-curing process. These included samples from Dump C(2) (100 days after curing), Dump C(1) (recently cured), and Dumps B(1-3) (time since curing unknown). Three months later, samples were taken from Dump B(4) (immediately after curing), and Dump C(1). In addition, a tractor with a backhoe was used to dig two holes on Dump C(1) and two samples were taken at depth from each hole. Ore samples were taken with a shovel that was sterilized with a 1% bleach solution.

Ore samples were wet sieved to 6-10 mesh. Bacteria were washed from five grams of sieved ore in sterile 100 mL serum vials containing 50 mL of a pH 2.5 minimal salts solution containing 0.25 g/L tryptone soya broth, 1.25 g/L $(NH_4)_2SO_4$, and 0.5 g/L $MgSO_4 \cdot 7H_2O$. The sample was placed on a wrist shaker for one hour at top speed and plated within one minute of removal from the shaker to avoid bacterial reattachment to the ore. Dilutions were performed in a minimal salts medium, and on-site water samples were plated within four to six hours. Bacteria were plated on an overlay medium (7) at 30°C to grow iron- and sulfur-oxidizing bacteria such as *T. ferrooxidans, T. thiooxidans, "L. ferrooxidans"* and "*F. acidophilus*".

Figure 1: Flowchart showing the basic leaching operation and the interrelationships between the various leach solutions and ore samples. Squares represent dumps, ovals represent ponds, and cubes represent processing steps. The dashed pairs of lines near the outlet of the Waste Stream Generation indicate variable, intermittent flows.

Incubation of overlay plates at 45°C allowed screening for moderately thermophilic organisms including iron-oxidizing bacteria such as *Sulfobacillus* and/or *Acidimicrobium*, sulfur-oxidizing species such as *T. caldus,* and acidophilic heterotrophic bacteria such as *Alicyclobacillus*. Cycloheximide was incorporated into all media at 50 μg/mL to prevent fungal growth; cycloheximide was shown to be nontoxic to iron-oxidizing bacteria at this concentration (data not shown). Bacterial colonies were counted on all plates after 14 to 21 days.

A minimal inhibitory concentration (MIC) study was done on the waste stream that was applied to Dump B, which contained 250-1000 ppm arsenic and 400-500 ppm fluoride. The waste stream MIC study was done using PLS, which has higher cell numbers than the raffinate, at 1:30, 1:20, 1:10, and 1:5 (3.3, 5.0, 10, and 20 vol% waste stream in PLS, respectively). Samples were taken at 0, 4, 24, and 48 hours into the run, and plated on overlay plates at 30°C.

3. RESULTS

Water pH ranged from 1.2-1.8 for all liquid samples except for the makeup water which was pH 2.5 (Table 1). Eh values ranged from +400 to +600 mV. Total iron, which was predominantly ferrous iron, was about 2000 ppm in the pregnant liquor solution (PLS) and raffinate and slightly lower, at about 1200 ppm, in the "On" solutions. Nitrates and phosphates were present in all samples.

Table 1: Water chemistry results for various samples.

Sample	pH	Eh (mV)	Total Fe (ppm)	Fe^{2+} (ppm)	Total Cu (ppm)	Nitrate N (ppm)	Phosphate P (ppm)
Aggregate PLS Pond	1.8	+413	1800	1900	2400	120	1.6
Dump C "On" Solution	1.5	+438	1300	1500	470	70	0.77
PLS Pond A	1.2	+398		1300		4.0	0.23
PLS Pond B	1.8	+410	1900	2400	2000	50	17
PLS Pond C	1.8	+418	1900	1600	2800	110	120
Dump B "On" Solution	1.4	+406	1200	1500	1600	4.0	0.43
Raffinate Pond	1.7	+418	1800	1800	170	25	100
Makeup Water	2.5	+536	17	56	100	9.0	0.00

Three surface ore samples were taken from Dump C(2), which was acid-cured about 100 days earlier. Mesophilic iron- and sulfur-oxidizing bacterial cell counts ranged from 5.7×10^5 to 1.1×10^6 bacteria/g dry ore. There was some species diversity in these samples although no *"L. ferrooxidans"* were detected (Table 2). *Acidiphilium*-like heterotrophs were detected in only one sample.

Dump C(1) was sampled from one hole at 0, 0.8 and 1.7 m depths, and from another at 0, 0.9 and 2.6 m. Mesophilic iron and sulfur oxidizers counts ranged from 0 to 6.6×10^4 bacteria/g dry ore (Table 2). Bacterial diversity was low, with mostly sulfur oxidizers comprised of either *T. ferrooxidans* or *T. thiooxidans*. Probably due to recent acid-curing, bacterial counts were low on Dump C(1) as compared to Dump C(2). Therefore, to determine whether the bacteria would adapt over time, additional samples were analyzed three months later. These included a sample from the recently acid-cured Dump B(4), and a new sample from Dump C(1). No viable bacteria were recovered from the Dump B(4) sample. The new sample from Dump C(1)-35, taken approximately 3 months after its initial acid-curing, now contained 1.4×10^6 bacteria/g dry ore, although diversity was still low with detection of *T. ferrooxidans* only (Table 2).

Moderate thermophiles were recovered from one hole on Dump C(1) and from all three samples taken on Dump C(2). Cell counts ranged from 1.0×10^4 to 3.8×10^5 moderate thermophiles/g dry ore (Table 2). All were iron oxidizers with the exception of sample C(2)-9, which contained 4.3×10^3 sulfur oxidizers, and sample C(2)-11 which contained *Acidiphilium*-like heterotrophs.

Table 2: Cell count data for ore samples from Dumps C and B at 30 and 45°C. Data presented are the original data divided by 10^3.

Location[a]	Depth (m)[b]	Total Mesophilic Cells	Mesophilic Sulfur Oxidizers[c]	T. f.	F. a.	T. t.	Total Thermophilic Cells
Dump C							
C(2)-9	0	570	220	130	220	0	10
C(2)-10	0	1100	890	120	89	0	44
C(2)-11	0	1100	630	330	96	0	380
C(1)-1	0	0	0	0	0	0	14
C(1)-2	0.9	0	0	0	0	0	280
C(1)-3	2.6	0.19	0	0.097	0.097	0	0
C(1)-6	0	46	42	[c]	4.1	[c]	0
C(1)-7	0.8	66	64	[c]	1.9	[c]	0
C(1)-8	1.7	8.5	6.3	0	2.2	0	0
C(1)-35	0	1200	0	1200	0	0	
C(1)-35	0	1600	0	1600	0	0	
Dump B							
B(4)-34	0	0	0	0	0	0	
B(4)-34	0	0	0	0	0	0	
B(1)-12	0	1300	1300	[c]	0	[c]	0
B(2)-13	0	96	96	[c]	0	[c]	1.1
B(3)-14	0	0	0	[c]	0	[c]	0
B(1)-27	0	0	0	[c]	0	[c]	0.097
B(2)-28	0	0	0	[c]	0	[c]	0
B(3)-29	0	420	420	[c]	0	[c]	0

The header row "Cell Numbers[d] (cells/g dry ore)" spans the data columns.

a Samples are listed as Dump(Pad)-Sample Number, ie. C(i)-1 through C(i)-n.
b Depth scale is zero at dump surface.
c Sulfur oxidizers may include *T. ferrooxidans* and/or *T. thiooxidans* not already listed under those headings due to difficulty in differentiation.
d *T. f.* = *T. ferrooxidans*, *L. f.* = "*L. ferrooxidans*", *F. a.* = "*F. acidophilus*", *T. t.* = *T. thiooxidans*. Samples containing no culturable bacteria were shown as "0" but assumed to contain less than 10 cells/g ore.

Results from Dump B surface samples, which received intermittent application of the waste stream, were varied. Three samples contained no culturable mesophilic iron- and/or sulfur-oxidizing bacteria. The three remaining samples ranged from 9.6×10^4 to 1.3×10^6 cells/g of ore, which were all sulfur-oxidizing *T. ferrooxidans* or *T. thiooxidans*. Two

Table 3: Cell count data for liquid samples at 30 and 45ºC. Data presented are the original data divided by 10^2.

Sample	Cell Numbers[a] (cells/mL)						
	Total Mesophiles	*T. f.*	*T. t.*	*L. f.*	*F. a.*	Hets.	Total Moderate Thermophiles
Dump B "On" Sol'n	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0
Dump A PLS	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0.050 ±0.070
Dump B PLS	210 ±29	210 ±2.8	0 ±0	0 ±0	0 ±0	0.70 ±0.85	140 ±21
Dump C "On" Sol'n	37 ±2.5	11 ±12	4.0 ±5.7	1.5 ±2.1	1.4 ±2	19 ±0	1.0 ±1.4
Dump C PLS	62 ±56	12 ±10	1.6 ±2	13 ±11	36 ±33	0 ±0	100 ±71
Aggregate PLS Pond	73 ±18	12 ±12	1.0 ±1.4	9.5 ±13	41 ±31	9.5 ±13	42 ±11
Raffinate Pond	14 ±11	2.7 ±2.1	0.45 ±0.35	2.7 ±3.3	8.5 ±6.4	0 ±0	0 ±0
Makeup H_2O	530 ±36	0.55 ±0.21	0 ±0	0 ±0	2.9 ±0.92	530 ±35	32 ±23

a *T. f.* = *T. ferrooxidans*, *L. f.* = "*L. ferrooxidans*", *F. a.* = "*F. acidophilus*", *T. t.* = *T. thio-oxidans*, and Hets. = *Acidiphilium*-like Heterotrophs. Samples containing no culturable bacteria were shown as "0" but assumed to contain less than 10 cells/g ore.

samples also contained moderate levels of iron-oxidizing thermophiles, ranging from 97 to 1100 cells/g of ore (Table 2).
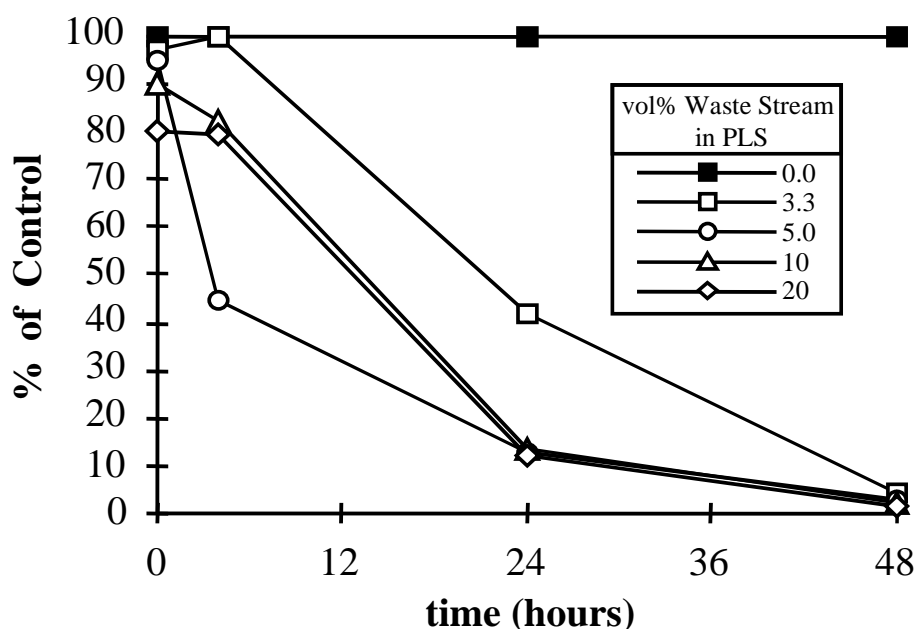
Liquid samples incubated at 30°C (Table 3) indicated that PLS Pond A, which is mixed with raffinate and the waste stream and sprayed onto Dump B, contained no viable acidophilic bacteria. The "On" solution collected from Dump B also contained no culturable bacteria. PLS Pond B contained 2.1×10^4 cells/mL, which were comprised mainly of *T. ferrooxidans* and a small number of *Acidiphilium*-like heterotrophs. PLS from Dump C contained almost an order of magnitude less cells than PLS Pond B, with only 6.2×10^3

cells/mL representing all four types of iron and sulfur oxidizers (*T. ferrooxidans, T. thiooxidans, "L. ferrooxidans"* and *"F. acidophilus"*). The Aggregate PLS Pond contained 7.3×10^3 cells/mL, again with all four types of iron and sulfur oxidizers represented.

"On" solution from Dump C(1) contained 3.7×10^3 cells/mL, while the raffinate pond contained 1.4×10^3 cells/mL All four types of iron and sulfur oxidizers were represented in both samples. The "On" solution for Dump C(1) also contained *Acidiphilium*-like heterotrophs. The predominant population in the make-up water (higher pH) was acidophilic heterotrophs, probably of the genus *Acidiphilium*, with only a few *T. ferrooxidans* and *"F. acidophilus"*.

"On" solution for Dump B and the raffinate pond samples incubated at 45°C (Table 2) contained no culturable moderate thermophiles. PLS Pond A samples incubated at 45°C contained only a few iron-oxidizing moderate thermophiles, and the "On" solution from Dump C also contained some iron-oxidizing moderate thermophiles. PLS Ponds B and C both contained approximately 1×10^4 iron-oxidizing moderate thermophiles per mL, which is higher than the 0-100 cells/mL present in the "On" solutions. PLS Pond B also contained a few moderately thermophilic heterotrophs as did the Aggregate PLS Pond. The Aggregate PLS Pond contained about one half the iron-oxidizing moderate thermophiles seen in PLS Ponds B and C. In addition, sulfur-oxidizing moderate thermophiles were seen in the Aggregate PLS Pond. The make up water also contained iron-oxidizing and heterotrophic moderate thermophiles.

Toxicity results for the MIC study of the waste stream in PLS are summarized in Figure 2. The waste stream was toxic to the cells, even at 3.3 vol% in the PLS. By 48 hours of exposure, fewer than 10 % of the initial cells remained at all exposure levels, relative to the

Figure 2: MIC results for waste stream toxicity to cells present in the PLS. Cell counts are presented as percents of the control at each time point, over 48 hours of exposure to the waste stream.

control. The 20 vol% waste stream in PLS samples had the highest kill rate, as expected, with cells lagging 50, 90, and 100 % behind the control after 4, 24, and 48 hours, respectively, indicating some toxicity and strong growth inhibition.

4. DISCUSSION

While the best leaching solution for oxidized minerals is sulfuric acid, the best for sulfide minerals is acidified ferric sulfate (8).   Azurite and malachite are rapidly leached in sulfuric acid solutions, and chrysocolla leaches slightly more slowly. Chalcocite is soluble in acidified ferric sulfate and dissolves in two stages, the first being much more rapid than the second. The ferric sulfate reacts with chalcocite to form sulfuric acid and ferrous sulfate. The ferrous sulfate is then reoxidized by iron-oxidizing bacteria to produce ferric sulfate and the cycle is repeated.   Thus, efficient leaching of a mixed oxide/sulfide ore favors diverse mixtures of iron- and sulfur-oxidizing bacteria capable of ferric regeneration, including mesophiles, moderate thermophiles, and heterotrophs.

All cell counts from Dump C(1), which was recently acid-cured, were one or more orders of magnitude lower than from Dump C(2) (leached for about 100 days).   After additional samples were obtained to investigate the possibility that acid curing was detrimental to the microbial populations, no viable cells were seen in the sample taken from the newly acid-cured Dump B(4).   This indicates that acid curing killed the microbial populations. The sample from Dump C(1) collected three months later had 1.4×10^6 cells /g ore, which is comparable to what was seen earlier on Dump C(2). The number of bacteria on Dump C(2) and C(1) after three months of leaching were high enough to support bioleaching and ferric regeneration, but species diversity was low, also a potential result of acid curing. Most bacteria recovered on the overlay plates oxidized only sulfur. When these organisms were subcultured, some were able to oxidize iron, and one colony type's rRNA sequence was investigated and was found to be similar to *T. ferrooxidans.* This observation may also be an indication of the stress on the microbes from acid curing.

The PLS sample from Dump C contained 10^4 culturable leaching bacteria, with more species diversity than was seen in any of the ore samples.   It is clear that ore was in general reinoculated with leaching bacteria from raffinate in the "On" solutions.   However, PLS populations were more diverse than raffinate populations, suggesting potential toxicity carryover from the extraction process. Cell numbers were an order of magnitude higher in the PLS (except for PLS A) than in "On" solutions, indicating that sulfide ores were present in the dumps and that the bacteria were actively growing.   Typically, when the dumps were operating well the ferric iron leaving the dump was 50% of the total iron. Thus, the lack of ferric iron in the PLS may indicate low microbial activity near the bottom of the dumps. Another explanation could be that iron oxidation was limited by the availability of oxygen in the lower reaches of the dump. Thus solutions percolating through would have been reduced by the chemical reaction between ferric iron and sulfide minerals, producing ferrous iron.

Moderate thermophiles would be expected in areas where the temperature is above 40°C, generally at subsurface depths where dump temperatures have increased due to exothermic activity from biotic and abiotic sources. Moderate thermophiles were present in low numbers in about half of the ore samples, although they were absent from the other half. Since the only subsurface samples taken were from an area that had been recently acid-cured and all cell numbers were low in that area, low numbers of moderate thermophiles were expected.   Water samples contained moderate thermophiles in comparable numbers to

mesophilic bacteria in all samples except the raffinate pond. This indicates that, as expected, the moderate thermophiles were present in the dumps at depth since the numbers were higher in PLS compared to raffinate.

About 400 *Acidiphilium*-like heterotrophs were present in one gram of C(2)-9 ore. C(2)-11 ore contained 96 moderately thermophilic acidophilic heterotrophs per gram. *Acidiphilium*-like heterotrophs were present only in Dump B PLS, Dump C "On" solution, and the Aggregate PLS Pond. Moderately thermophilic heterotrophs were present in Dump B PLS and in the Aggregate PLS Pond. In addition, the makeup water contained 5.3×10^4 *Acidiphilium*-like heterotrophs and 1.1×10^3 moderate thermophilic heterotrophs per mL, up to 4 orders of magnitude more non-iron-oxidizing heterotrophic bacteria than seen in the other water samples (0 to 2000 cells/mL). This effect was probably due to the low pH of most water samples compared to the makeup water. *Acidiphilium*-like heterotrophs, which are among the least pH-tolerant acidophiles, have an optimum pH around 3 and most strains cannot tolerate a pH below 2. *Acidiphilium*-like heterotrophs are important in metal mobilization due to their use of organic materials that are inhibitory to iron-oxidizing autotrophs such as *T. ferrooxidans* and *"L. ferrooxidans"*. The lack of *Acidiphilium*-like heterotrophs could thus decrease leaching efficiency.

The waste stream was highly toxic and/or inhibitory to cells in PLS at all concentrations tested. The cells appeared unhealthy on the plates after exposure to the waste stream, showing limited ability to oxidize iron, while the control cells increased in number by two orders of magnitude. This effect was likely due to the low pH and to the presence of toxic arsenic and fluoride in high concentrations. Because of this, potential problems were indicated for Dump B. PLS Pond A, which contained a mixture of PLS from Dump A and the waste stream, was mixed intermittently with "On" solution and sprayed onto Dump B. This could explain the variable cell numbers seen on Dump B, since some areas could have received more frequent or concentrated applications of the waste stream. There was also a decrease in the diversity of the organisms present on Dump B, possibly due to differing tolerances to the waste stream. Thus, leaching of mixed oxide/sulfide dumps could be improved by minimizing or eliminating recycle of the waste stream onto dumps containing sulfide ore, and/or by recycling only to dumps containing oxide ore.

This study identified several problems with the microbial ecology at the dump leach operation tested, using the overlay plating technique. The ability to look at cell diversity and numbers was beneficial to this end, since other available techniques may not have presented as clear a picture of the problems with the leaching operation. For example, the use of MPN with ferrous sulfate would not have differentiated between *T. ferrooxidans* and *"L. ferrooxidans"*. *"Ferromicrobium acidophilus"*, which requires yeast extract and ferrous iron, would not have been detected. The use of 16S rRNA gene analysis or 16S-23S rRNA gene spacers would have required more labor and time than plating, and would only indicate which species were present; no information on the numbers of each species would have been obtained. In addition, information on the presence of moderate thermophiles may not have been obtained, since these bacteria are more difficult to lyse than mesophilic bacteria. Immunofluorescence probes would only work for organisms to which probes have been developed, and would miss any other species that might be present. Finally, fatty acid methyl esters would not give as much information on species present and their numbers. Thus, the overlay plating method is more versatile as a screening technique for characterizing the microbial ecology of leaching operations.

5. CONCLUSIONS

Efficient copper bioleaching depends on the microbial ecology of the system. The majority of techniques available for studying the microbial ecology of natural systems are good for identifying bacterial species but are generally unsuited for enumeration of the species, or vice versa. The overlay plating technique used here is a useful tool for microbial ecology studies of mining environments, since it overcomes these problems. This technique was used to determine the effect of acid curing and of recycle of a toxic, acidic waste on the microbial ecology of a combined copper oxide/sulfide dump leach operation. Acid curing eliminated culturable bacteria in the dump, although inoculation from the "On" solution reestablished a less diverse microbial community after 3 months or so. In addition, results were used to show that the waste stream was not only highly toxic, but it also decreased the diversity in the surviving population. Decreased diversity in the leaching population can adversely affect copper recovery, and so is an important economic concern.

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