The Genetic Diversity of Microbes in an Biochemical Reactor Treating Metal-rich Landfill Leachate: What This Tells Us About Treatment Mechanisms.

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Abstract
Seepage from land-filled mineral processing waste containing arsenic, zinc and other metals plus elevated concentrations of sulphate is treated in a sub-surface, vertical flow biochemical reactor (BCR). The water percolates through a mixture of pulp and paper mill biosolids, limestone and sand. One of the main postulated treatment mechanisms is the reduction of sulphate by sulphate-reducing bacteria (SRB), which are obligate anaerobic microbes that use low molecular weight carbon sources for food and energy. Since the biosolids are mostly composed of complex and recalcitrant carbohydrates, other bacteria need to hydrolyze and ferment the material to produce carbon sources that the SRB can use. SRB are important since they produce sulphide that combines with metal ions and removes them from solution as sparingly soluble precipitates. Variable success rates with these types of treatment systems limits their widespread adoption. We have used genomics tools to characterize the whole microbial community of an operating BCR in Trail, British Columbia, over the period of one year. Although the overall community diversity is uniform spatially and temporally, the prevalence of different groups correlates with their specific environmental conditions.

Key Words: genomics, bioremediation, mining, phylogeny, methanogens, sulphate-reducing bacteria

Introduction
A passive treatment system near Trail, British Columbia, based on a series of sub-surface flow and surface flow wetlands has been successfully treating landfill seepage containing arsenic, zinc, cadmium and high concentrations of sulphate for over 10 years (Mattes *et al.* 2010). The first two steps in this process consist of biochemical reactors (BCRs), which contain a mixture of pulp mill biosolids (from the close-by Celgar pulp and paper mill in Castlegar), iron-coated sand and limestone sandwiched between borrow-pit sand layers and geotechnical liners. The water flows through the organic layer from the bottom to the top. Chemical data collected by the operators showed that the average influent concentrations of arsenic and zinc were 35.2 mg/L total and 5.4 mg/L dissolved, and 72.4 mg/L total and 39.6 mg/L dissolved, respectively, during the study period (July 2008 – June 2009). In the first BCR, where the approximate hydraulic retention time is one month, 48% and 34.2% of the dissolved As and Zn, respectively, were removed. Sulphate reduction through the treatment system was very variable; low initially, then increasing throughout the study period with an average of 34% removal (influent concentrations varied from 500-800 mg/L). Not much is known about the mechanisms of metal and sulphate removal, although they are believed to involve microbial processes. Thus, an intensive sampling program was carried out from July 2008 to October 2009 to collect solid and liquid samples from the first BCR, which were then analyzed for microbial species and certain chemical parameters.

Passive treatment systems at mine sites rely on natural biological and geochemical processes to remove metals from process wastewater, drainage and seepage streams that exceed the regulated limits for aquatic ecosystems. One of the most important postulated mechanisms for metal removal is through metal sulphide precipitation (Jalali and Baldwin 2000). Metal sulphides have a lower solubility product than metal hydroxides and are thus more stable. The sulphide is provided by the activity of sulphate-reducing

bacteria (SRB) that are obligate anaerobes respiring on sulphate. These microbes require an oxygen-free environment and low molecular weight organic acids or alcohols as carbon sources and for energy (Postgate 1984). Because complex organic materials are used in passive treatment systems, such as the composted pulp mill sludge used in the Trail BCR, SRB are reliant on other microbes that decompose carbohydrate and protein polymers into simple sugars and amino acids that are fermented into organic acids and alcohols by the same or other organisms (Schmidtova and Baldwin 2011). Sulphate-reducing bacteria face competition from other groups of microbes, such as methanogens and iron-reducing bacteria, that also use acetate, CO_2 and H_2 for energy, but it is thought that SRB outcompete these in environments that are sulphate-rich. Thus, passive treatment relies on a consortium of microbes for effective metal removal. However, due to their passive nature, these treatment systems are subject to the vagaries of the microbial community, which changes in composition depending on the environment and over time as the carbon sources are degraded away. For the most part it is almost impossible to determine exactly what is going on in a passive treatment system except by extrapolation from chemical concentration data collected at the effluent. The active chemical processes can be inferred from release of products such as sulphide or methane in the field or by measuring microbial activity in samples taken to the laboratory. However, metabolites may disappear quickly thereby evading detection and only very few microbes can be cultivated outside of their natural environment. Therefore, in this project, we tested the use of genomics technologies for determining what microbes are present in the Trail BCR and to see if we could find correlations between the types of microbes present, their chemical environment and the performance of the treatment system. The technologies that we investigated use genes (i.e. deoxyribonucleic acids (DNA)) collected from the environment to infer microbial community composition. In some cases, if we are able to collect enough genetic material, we can assemble the genes into putative genomes for some of the microbes present in our system. But the first step is to survey the phylogeny, which describes the types of microbes present based on their classification into taxonomic groups. This is done by selectively amplifying the small sub unit ribosomal ribonucleic acid (SSU rRNA) from DNA extracted from an environmental sample (so called eDNA). This SSU rRNA is highly conserved for specific microbial species and by comparing the SSU rRNA from our eDNA sample to those in public databases for known microbes we can discover what microbial species likely exist in the environment that we are studying. Thus, samples were removed from the Trail BCR over the duration of the study and used for three different types of genomics analyses. Firstly, almost the full-length SSU rDNA genes (long reads of ~1,400 bases long) were sequenced using standard cloning and sequencing technology. Then, next generation sequencing was used to obtain many more albeit shorter reads (400-500 bases) from the same samples. And finally, so-called DNA microarrays containing all the SSU rRNA genes from a public database were used for a quantitative comparison of microbial species numbers from sample to sample. In this paper, we describe how these genomic technologies were used, how the information was interpreted and what the results told us about this treatment system.

Materials and Methods

Sampling the study site

The first BCR of the Trail wetland covers an area of about 90 m^2 and is up to 3 m deep. The water-saturated organic layer where treatment occurs lies below a dry cap of borrow pit sand and gravel though which we needed to drill. Core samples were taken from three holes and at three different time points so as to obtain a thorough genetic diversity survey. At each sampling, a light-weight hydraulic drill was used to extract 2 inch diameter core samples, which were cut into shorter sections that were capped, and frozen with liquid N_2 within a couple of hours. The three seasons sampled were July 2008 (Summer), April 2009 (Spring) and October 2009 (Fall). Three cores were removed in each season for a total of 9 cores of 30 – 90 cm in length each. At the same time, pore water was analyzed for sulphide, ferrous ion, ammonium, using Chemets Kits (Chemtech International Inc. Media, PA, U.S.A.), and pH, oxidation reduction potential (ORP), dissolved oxygen (DO) and temperature using probes (6-series probes on a sonde, YSI, Yellow Spring, Ohio, U.S.A.) in the field. Water samples were sent off for metals analysis using inductively coupled plasma mass spectrometry (ICP-MS) plus nitrate, nitrite, sulphate and phosphate

concentrations using Hach kits (Hach Company, Loveland, Colorado, U.S.A.) at the Teck analytical laboratory on the Trail smelter site. Some holes revealed an environment deemed more suitable for SRB; i.e. with circum-neutral pH, no DO detected and Eh less than -100mV, than others where the pH was lower, some DO was detected and ORP was mildly oxidizing. Throughout the sampling period, samples were taken from the influent and effluent and analyzed for the same chemical parameters as were measured in the pore water by the Teck analytical laboratories.

Genomic techniques for microbial community analysis

Genomic DNA isolation for clone library construction

So that we can examine the vertical partitioning of microbial niches, the cores were sectioned into 5 cm intervals. The core sections were kept at -80 °C until DNA extraction. We extracted environmental genomic DNA from 5 g of each homogenized soil section using the Power Max TM kit (Mo Bio Laboratories Inc., Carlsbad CA) following the manufacturer's instruction. For this particular study we used only the mid-section of each core, that is the 20-30 cm section (distance measured from the top).

Clone library construction

Small subunit ribosomal RNA gene (SSU rRNA) libraries of bacteria and archaea were constructed from nine samples: The mid-section of each core. The polymerase chain reaction (PCR) was performed with 50 ng of template environmental genomic DNA using Herculase® II fusion DNA polymerase (Stratagene, La Jolla, CA). The amplification conditions were 95°C, 10 min: 95°C, 20s: 60°C (for archaea and bacteria) or 62°C (for eukarya), 20s: 72°C 1min; 30 cycles/ 72°C 3min. The primer pairs used for archaea, bacteria and eukarya were 4Fa/958R, 27F/1492R, and EukF/1391R respectively. The sequences of the primers are as follows; 4Fa: 5'- TCCGGTTGATCCTGCCRG-3', 958R: 5′-YCCGGCGTTGAMTCCAATT-3', 27F: 5'-AGAGTTTGATCCTGGCTCAG-3', 1492R: 5'-GGTTACCTTGTTACGACTT-3', EukF: 5'-AACCTGGTTGATCCTGCCAGT-3', 1391R: 5'-GACGGGCRGTGWGTRCA-3'. The PCR products were purified using QIAquick Gel Extraction Kit (Qiagen Inc., Valencia, CA), cloned using Zero Blunt Cloning Kit (Invitrogen, Burlington, ON) and transformed into α-Select Gold Efficiency Cells (Bioline, Taunton, MA). In total, 9 libraries were produced each containing clones representing 96 archaea and 192 bacteria. The Genome Sciences Centre of the University of British Columbia sequenced these SSU clones using Sanger technology (we will refer to them as clone library sequences).

Preparation of DNA for DNA microarray analysis by the Lawrence Berkeley National Laboratory Phylochip and for next generation sequencing with the Roche 454 GS-FLX System Using Titanium Chemistry.

We extracted genomic DNA from all 88 samples using a modified method described by Miller *et al.* (1999). The use of specific extraction buffers was combined with a bead-beating procedure for most efficient extraction of DNA and maximum diversity applying the Bio 101 Fast Prep 120 machine for 45 sec and 6.7 m/s following a procedure described by DeSantis *et al.* (2007). Obtained purified environmental DNA was used to amplify the phylogenetic marker SSU rRNA for bacteria and archaea. Gradient PCR for maximal diversity was performed in 50 µL reactions on an 8-temp. gradient (48° - 58° C, 25 cycles) for bacteria and archaea. An aliquot of all temperature products from each sample was run on a 0.8 % agarose gel to verify PCR worked and then pooled and purified with a PCR purification kit. For the Phylochip hybridization, PCR products of two sections of each core were pooled to reduce the amount of samples. This resulted in a total of 41 samples. Samples for hybridization to the Berkeley Phylochip version G3 were sent to Berkeley, California.

Sample preparation for next generation sequencing with the Roche 454 GS-FLX System using titanium chemistry

DNA prepared previously was PCR amplified using primers specific for the V6 variable region of the SSU rRNA gene: forward primer 454T-FB 5' CTATGCGCCTTGCCAGCCCGCTCAG and reverse

primer 5’ CGTATCGCCTCCCTCGCGCCATCAG. Attached also to the primers were barcodes and linker-adapters for the LibA chemistry used for sequencing. The resulting so-called pyrotags were sequenced by Centre d’innovation Génome Québec et Université McGill (Montréal, Québec).

Bioinformatics

The Sanger sequences from the clone libraries were assembled and quality checked using the computer program Sequencher (Gene Codes Corporation, Ann Arbor, MI, U.S.A.) and processed into operational taxonomic units (OTUs) using the MOTHUR suite of programs (Schloss *et al.* 2009). The pyrotag sequences were screened to eliminate poor quality reads and assembled into OTUs using Qiime python scripts (Caporaso *et al.* 2010). The OTUs were assigned to taxonomic groups by comparing their representative sequences to the current Ribosomal Database Project (RDP) Release 10 SSU rRNA database. Some OTUs could be assigned to specific species, but many were novel and could only be assigned to broader taxonomic groups, such as Family, Order, Class or Phylum, depending on their degree of novelty.

Results

The first set of sequences that we obtained came from the clone libraries for each core sample. Sequencing costs are based on per read and since Sanger sequencing, the standard method until before the next generation sequencing technologies became available, is expensive only two 96 well plates were sequenced for bacteria and one 96 well plate for archaea per sample. The advantage of Sanger reads is that they are long. The two ends of the SSU rRNA molecule are sequenced (700-800 bases long) and assembled into a read of ~1,400 nucleotides. Initial tests of the samples with general primers for all bacteria and all archaea revealed a stronger signal for archaea, suggesting that organisms belonging to this Domain dominate the Trail BCR. To identify what species of archaea are likely present in the Trail wetland, we compared the Sanger sequences from our clone libraries to those in the National Center for Biotechnology Information (NCBI) database and found that most of the Trail archaea sequences clustered with three methanogen species. In Figure 1, the labels in bold, 78ar2F03, 109ar3D10 and 109ar3C08, are representative sequences from the archaea groups found in the Trail BCR. A phylogenetic analysis found that these were closely related to *Methanocorpusculum labreanum*, *Methanospirilum hungatei* and *Methanosarcina vaculata*. Looking at the number of reads that we obtained for each one of these groups allowed us to get an idea of their relative abundance in each one of the core samples (Figure 2). The distribution of these methanogens was random with no patterns emerging. In the heat map shown in Figure 2, the intensity of the green colour is proportional to the percentage of methanogen-related reads in that sample that were assigned to each methanogen group. In addition, the samples and methanogen groups have been clustered using an hclust algorithm leading to the dendograms shown in Figure 2 (R version 2.11.0 (2010-04-22), http://www.r-project.org/). Some of the samples were dominated by a particular methanogen, such as the *Methanosarcina* species in the April hole 1 and the July hole 3. The *Methanocorpusculum* species were the most prevalent methanogens in two samples from April and one from October. The other four samples contained mostly *Methanocorpusculum* and *Methanospirilum* species. However, most species were found to some extent in most samples. The clustering exercise did not reveal any trends with respect to season.

Twice as many reads were obtained per sample for bacteria, since we expected there to be a wider variety of bacteria, than archaea, species present. All of the sequences were clustered into OTUs and after filtering out the OTUs represented by only a small number (1 or 2) of sequences, we found the main OTUs shown in Figure 3. Along the x-axis in this Figure are the OTUs each one named for its genus affiliation. In some cases more than one OTU was identified for the same genus. According to the taxonomic classification, indications are that most of the bacteria in the Trail wetland are from the *Bacteroidetes* and *Firmicutes* phyla that contain microbial species known to degrade complex organic matter. Possible cellulose

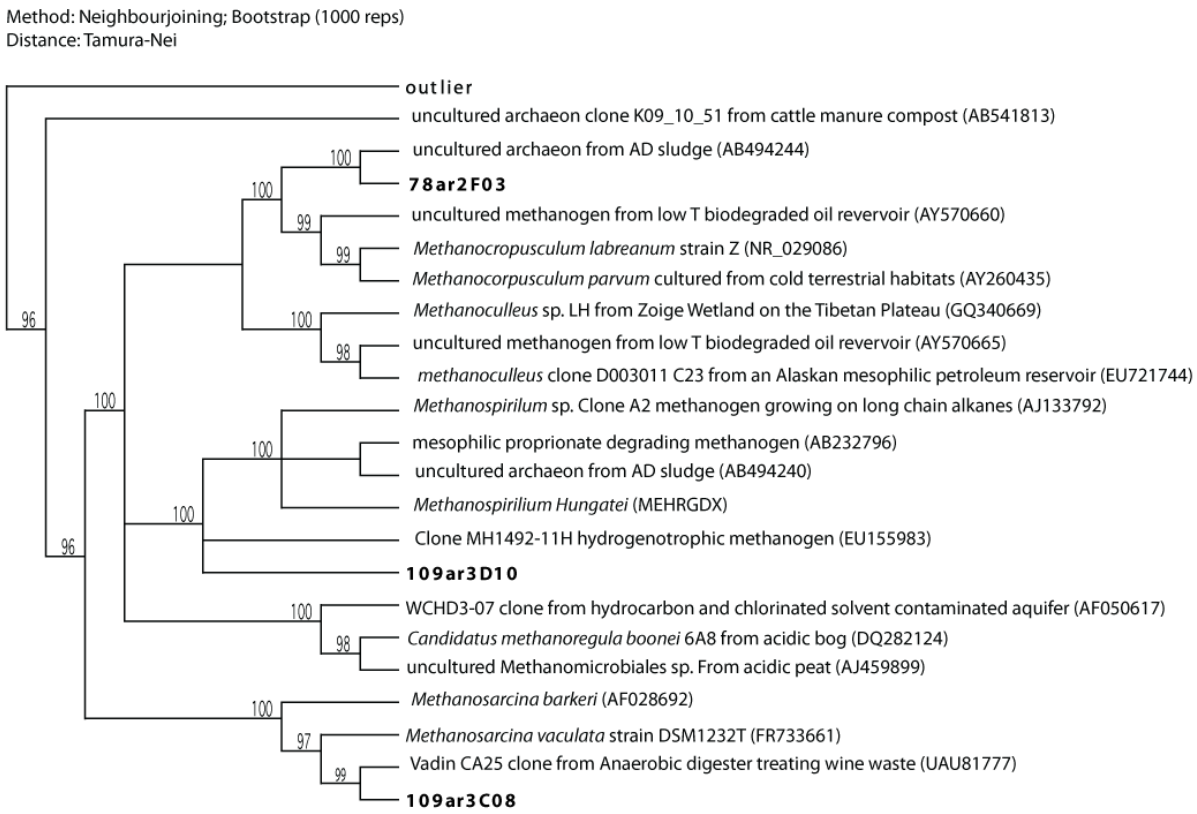

Figure 1. Phylogenetic tree showing the relationships between the three representative methanogen Trail clones and cultured species and sequences for other environmental clones.

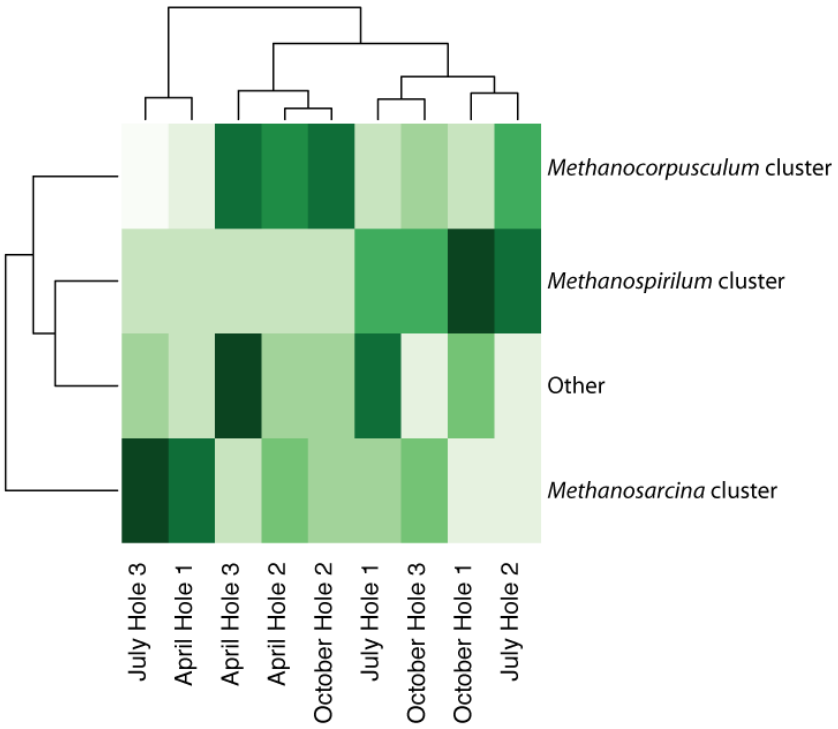

Figure 2. Heatmap and dendogram showing the distribution of methanogen-related sequences among the three major groups in each one of the core samples.

degrading bacteria include the OTUs related to *Clostridium* sp. (Feinberg *et al.*2011), *Bacteroides, Paludibacter*, *Fibrobacter* (Suen *et al.* 2011), *Acetivibrio* and *Ruminococcus*. Many anaerobic microbes that degrade cellulose can also ferment sugars into organic acids as well (Leschine 1995) showing that it is not possible to assign a specific role to any of these OTUs. Some of the Trail sequences were assigned to microbes that breakdown proteins and ferment amino acids to organic acids such as *Acidoaminococcus*, *Proteiniphilum*, *Lutispora* and *Cloacibacillus*. Many of the other OTUs were related to fermenters such as *Prolixibacter* (Holmes *et al.* 2007), *Parabacteroides*, *Petrimonas*, *Anaerophaga* and *Ethanoligenens*. Cellulolytic microbes are often syntrophic with fermenters (Murray 1986). A more thorough phylogenetic analysis the groups of bacteria found in the Trail wetland is underway and will be included in future publications.

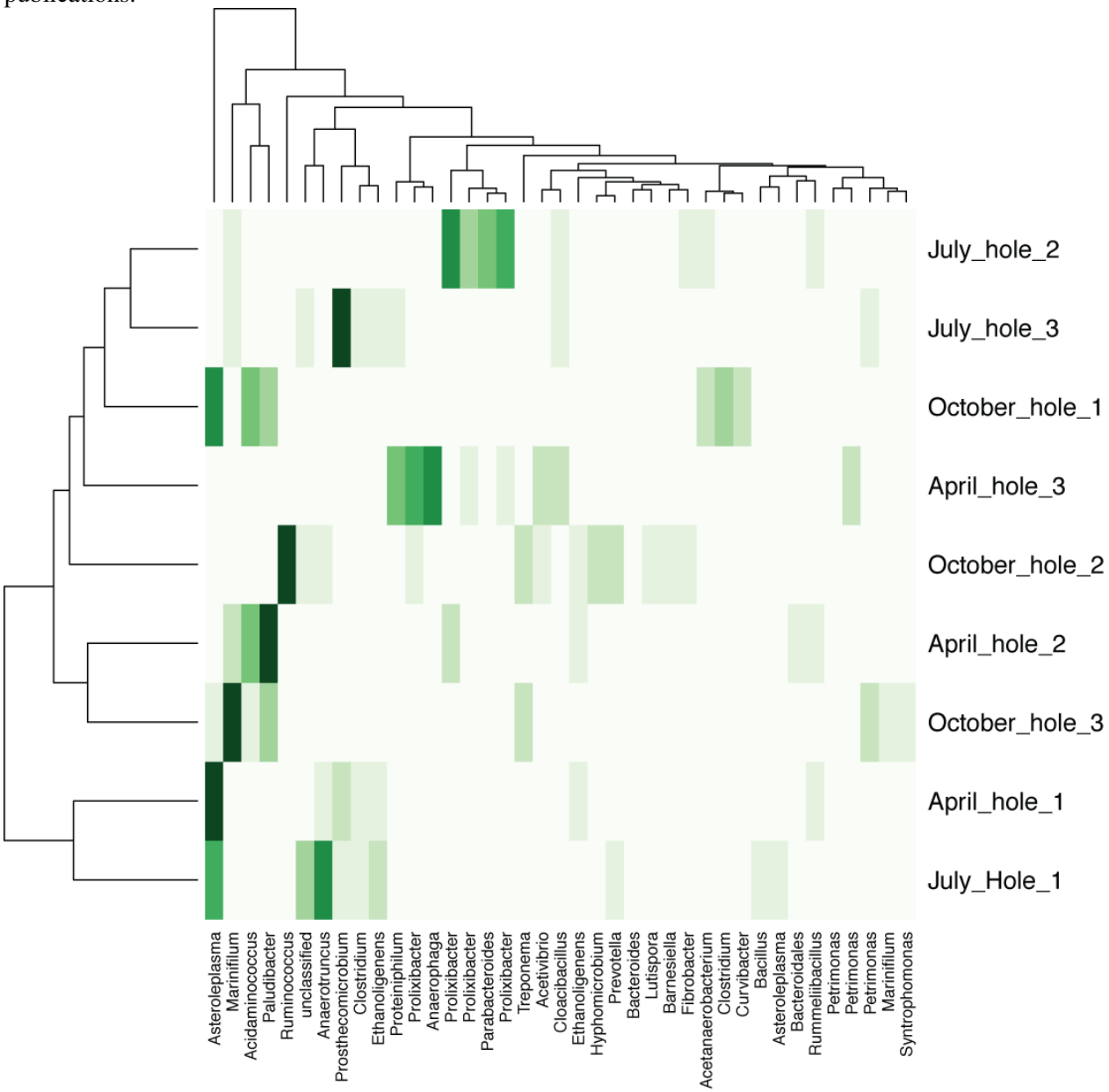

Figure 3. Main OTUs found by sequencing SSU rRNA clones from the Trail core samples. Their taxonomic classifications are given on the horizontal axix. The intensity of the green colour indicates the percentage of sequences in each sample assigned to each of the OTUs.. Darker shades of green indicate greater prevalence of that OTU in the sample.

The relative abundances of reads grouped in these OTUs for each of the samples is shown by the heat map in Figure 3. Again the intensity of the green colour is proportional to the percentage of sequences in that sample assigned to each OTU. For example, the July hole 1 and April hole 1, which were most similar to each other, contained many sequences related to a *Tenericutes* phylum genus called *Asteroleplasma*, but the confidence in this taxonomic assignment for this OTU was only 75%, indicating that it may be a novel organism only distantly related to those in the database. Like the archaea, although all phylotypes were found to some extent in all samples, their relative abundances are random and there were no clear groupings of phylotypes with each other nor any clustering of microbial community structures with season.

In summary, these sequences tell us what bacteria are likely present in the Trail wetland and the sequences themselves can be used as probes to monitor the abundance of these species. The results show that the Trail BCR is heterogeneous with respect to the distribution of numbers of these bacteria. When assessing the diversity of an environment, it is important to take multiple samples from different locations and in different seasons. The Trail BCR likely consists of many micro-niches each with their own mixture of microbes. But, the diversity in the Trail wetland is not that great compared with other terrestrial environments, in that the same phylotypes consistently appear in each hole. This is due to the nature of the substrate, pulp mill biosolids, as well as the chemical composition of the water that select for specific microbes most adapted to this environment. The fact that no patterns were discerned with respect to season we can take to mean that temperature does not greatly affect the relative amounts and types of bacteria present.

Another statistical method was used to see if chemical parameters measured in the pore water (Table 1) could explain grouping of the samples, or of the bacteria types. The numerical data from Figure 3 were imported into the R statistical package and subjected to detrended correspondence analysis (DCA), an approach to clustering developed specifically for ecology. Attempting to fit the DCA differences to chemistry data in Table 1 revealed no significant correlations. Variations in the particular chemical parameters in Table 1 do not drive changes in microbial community structure in the Trail BCR cores, but other unique overall features of this site may. It should be noted that pore water chemistry is very difficult to measure accurately especially DO and ORP. Although these were measured the day after core removal, the bore hole may have allowed some oxygen diffusion into the area from which the core was removed. ORP is known to be strongly influenced by the presence of high concentrations of dissolved organic compounds. A comparison of the Trail BCR microbial community with other anaerobic bioreactors is underway and when completed may reveal more about environmental drivers of microbial ecology at metal remediation sites. It is interesting though that, although oxygen was detected at some sites, hardly any aerobic microbes were detected.

Table 1. Chemistry of the Trail core pore water.

	DO (mg/L)	pH	ORP (mV)	T (oC)	Sulphide (mg/L)
July Hole 1	0	5.6	-132.5	17.6	1.1
July Hole 2	0	5.56	-112.1	15.9	2.6
July Hole 3	0	6.9	-238.1	19.4	3.45
April Hole 1	1.5	6.9	-337	7.6	0
April Hole 2	1.05	6.93	-337	6.5	0
April Hole 3	0.74	7.03	-337	7.1	0
October Hole 1	1.24	6.71	-117.5	11.2	0
October Hole 2	1.25	6.22	-244.2	11.5	0
October Hole 3	1.25	7.54	-130.3	11.2	0

Since many studies reveal that microbial diversity is far greater than we think, deeper sequencing is needed to capture all the relevant phylotypes. Microbes that are important for the performance of the Trail wetland, such as sulphate-reducing bacteria (SRB), are not necessarily those that are most abundant. Therefore, we used so-called next generation sequencing to obtain more reads from the Trail core eDNA samples. With Roche 454 GS-FLX sequencing we obtained between 5,349 and 7,750 reads per core sample, compared with 55 – 169 reads per sample obtained from Sanger sequencing of the clone libraries. The next generation reads are shorter and averaged 492 bases compared with ~1,400 bases for Sanger clone library sequences.

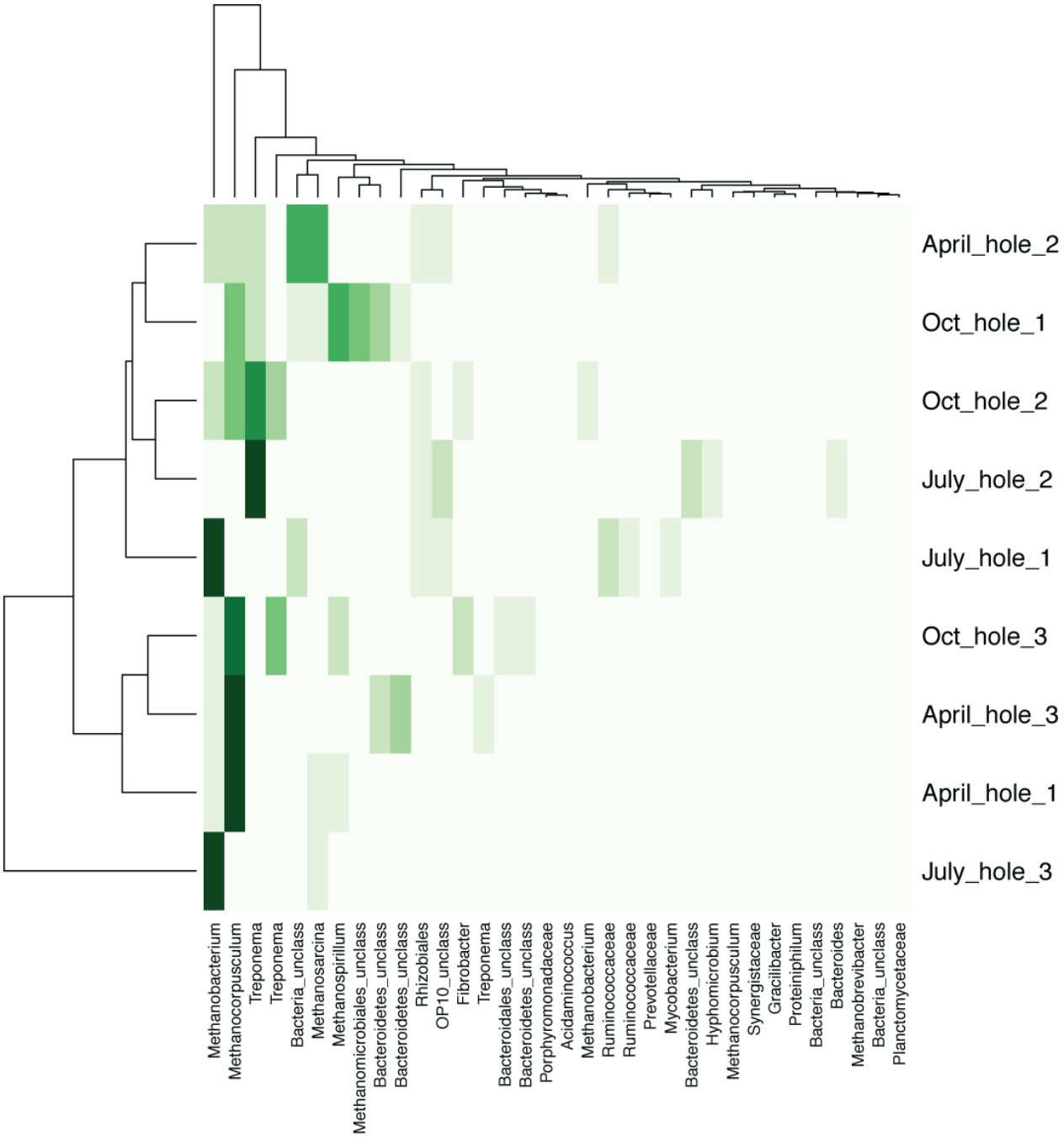

Figure 4. Main OTUs found by sequencing pyrotags from the Trail core eDNA samples.

When doing phylogenetic studies with shorter reads, specific variable regions within the SSU rRNA gene are targeted. Because of the PCR step for amplifying these regions some bias is introduced in that some microbes are less likely to be amplified than others, depending on their SSU rRNA sequences. Nevertheless the much larger number of reads allows us to perform a deeper analysis of microbial diversity. Also, with the newer technique, both archaea and bacteria are amplified at the same time and their relative abundances can be assessed together. To screen out the low abundance species, we only included OTUs with more than 200 sequences in the whole library. This revealed 34 predominant OTUs in the Trail samples indicating that diversity of predominant phylotypes is low even with the much-increased depth of sequencing (Figure 4). The results confirmed that methanogens were the most represented microbes in the Trail BCR by far, with the same three genera detected as before plus additional OTUs related to *Methanobacterium* and *Methanobrevibacter* indicating a diverse methanogen community. The methanogens were so predominant in the July hole 3 and April hole 2 cores that they dominated most of the reads in these samples. In fact, enough genomic information was obtained from the eDNA from one of these samples in a parallel metagenomic analysis that the genome for the *Methanocorpusculum labreanum* species found in the Trail BCR can be assembled from the eDNA collected (data not shown).

Of the bacteria, a *Spirochaetes* related to the *Trepomena* genus was most prevalent. This is likely a fermentative bacterium that may be syntrophic with the methanogens. Other of the most predominant bacteria were related to cellulose degrading and fermenting genera as was seen in the clone libraries. The biggest advantage of the large number of reads obtained with pyrotag sequencing is that we can obtain information about the low abundance microbes, especially the ones that are important for metal removal. Although the methanogens, cellulose degraders and fermenters are dominant, there are other microbes present that are involved in metal precipitation. We found sequences related to microbes known to be involved in sulphur reduction in both the clone library sequences and the pyrotag reads. The clone library sequences were related to nine different genera of sulphur reducers (Figure 5).

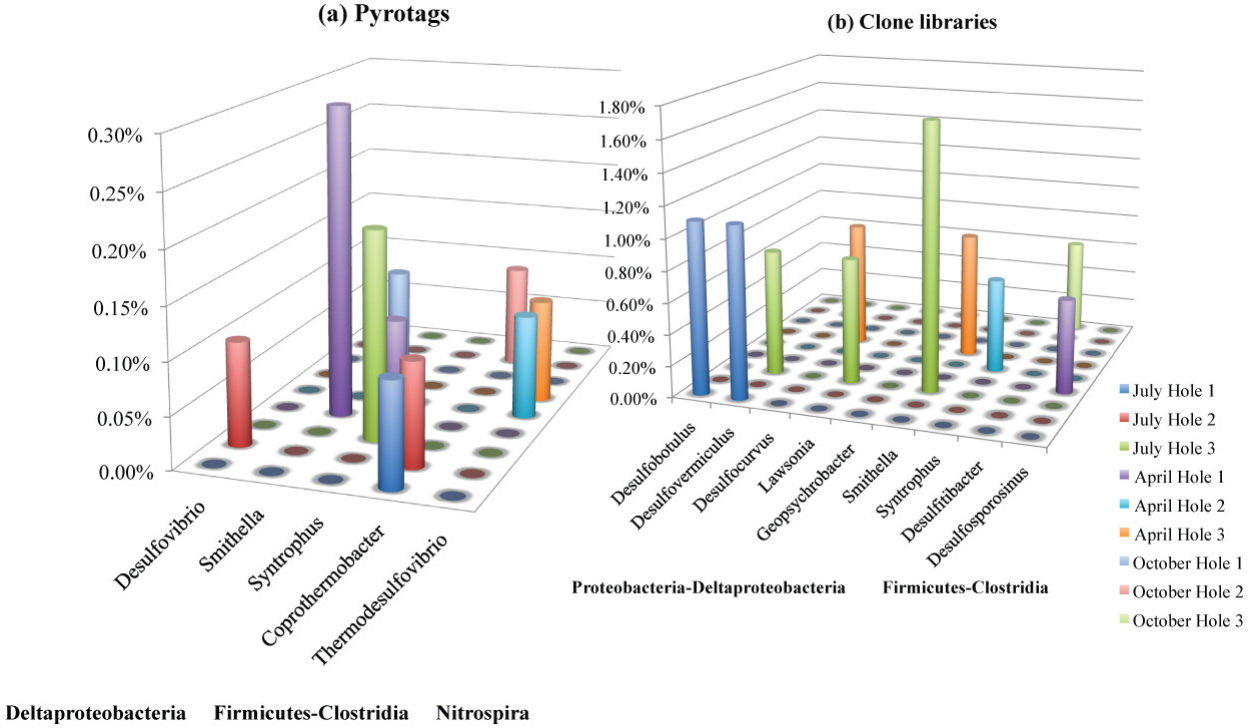

Figure 5: Relative abundance (percentage reads in each sample) of pyrotag (a) and clone (b) sequences related to sulphur-reducing bacteria.

Of these, seven were found in the *Delta-proteobacteria* class where most SRBs are classified. Other sulphate reducers, that are spore forming, are classified in the *Firmicutes* phylum and the Trail clone sequences were related to two genera of this group. Surprisingly, the pyrotag sequences, although there were many more of them, contained fewer SRB groups that were somewhat different from those found in the clone libraries (Figure 5). In addition to the *Delta-proteobacteria* and *Clostridia* sulphate-reducers, *Nitrospira* related sequences were found in the pyrotags. The *Nitrospira* related sequence detected in the Trail BCR is related to *Thermodesulfovibrio* species, which are obligate, thermophilic sulphate-reducing microbes previously isolated from thermal sulphur springs and methanogenic sludge (Sekiguchi *et al.* 2008). In summary, evidence for sulphur-reducers was found in the Trail BCR according to both the clone library and the pyrotag sequencing methods, but the methods differ in the types of sulphur reducers detected. The low numbers of sequences assigned to sulphate-reducers indicate that sulphate reduction does not play a significant role in terminal organic matter degradation in this system, which is mostly carried out by methanogens. Nevertheless, there may be enough sulphate reduction for some of the metals to be removed as sulphides.

Both the methods described above were useful for identifying what types of microbes are present in the Trail treatment system and they indicate that carbon degradation and methanogenesis are the main processes taking place. Although, the number of reads (sequences) assigned to each OTU gives some indication of the relative numbers of these microbes in the sample, both procedures are not definitive quantitative methods. Thus, we used a DNA microarray, the Berkeley Phylochip, for a quantitative comparison of the relative numbers of certain groups of bacteria between the samples. The DNA microarray was designed to compare species abundance across samples and was used previously to show that the oil plume in the 2010 Gulf Oil spill was enriched for petroleum-degrading bacteria (Hazen *et al.* 2010). The microarray is capable of detecting about 60,000 different species clusters according to all known high-quality 16S rRNA gene sequences from a database (greengenes.lbl.gov), that include known cultured microbes as well as sequences that have been found in different environments. The Phylochip identified four *Delta-proteobacterial* sulphate-reducing bacteria families in the Trail BCR sample: *Desulfobaccaceae*, *Desulfobacteraceae*, *Desulfobacterium* and *Desulfomonile*. The Phylochip data revealed that the SRB were present in their lowest amounts in the April hole 2 and July hole 3 cores (Figure 6). These SRB were found in their largest numbers in the April hole 3 and October hole 1 cores.

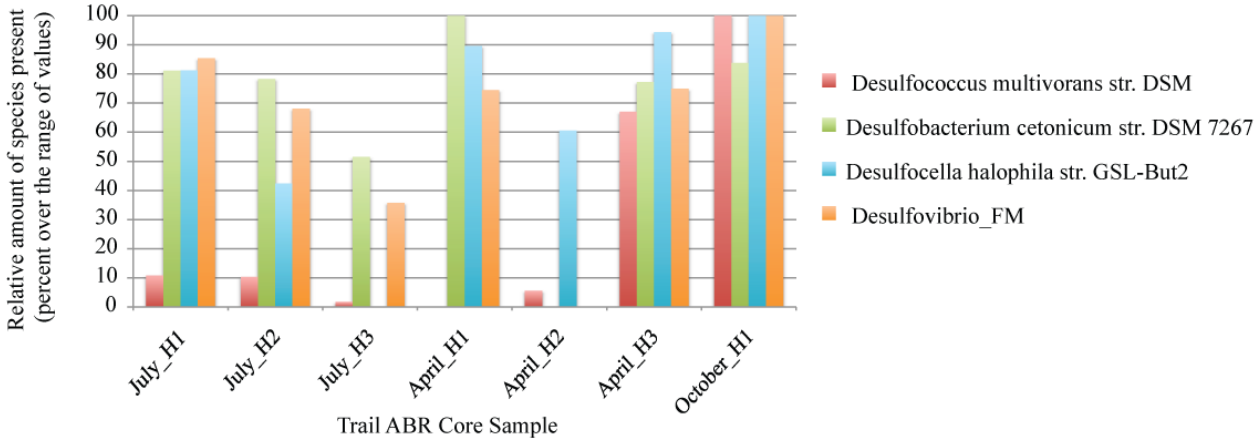

Figure 6: Relative abundances of selected sulphate-reducing species found in the Trail BCR by the LBNL Phylochip.

The Phylochip data can be used to compare the relative abundances of other lineages to the patterns seen for SRB. This was done for select methanogen species, *Methanocorpusculum* sp. and *Methanosarcina* sp. In all of the July hole samples, the Phylochip revealed that *Methanocorpusculum* sp. were present at their lowest abundance, which is consistent with the results from the pyrotags. In April hole 2 where SRB were

in their lowest abundance, the methanogen *Methanosarcina* sp. was at its highest abundance. It could be that this methanogen outcompetes the SRB in April hole 2. Although the *Delta-proteobacteria* SRB detected by the Phylochip were in low abundance in the July hole 3, this was the hole where the highest sulphide concentrations were measured in the pore water. As shown in Figure 5, there are microbes in other phylogenetic groups that are sulphur reducers, such as *Coprothermobacter* that reduce thiosulphate to sulphide, and these were present in their highest amounts in the July hole 3 sample according to the Phylochip. Some *Syntrophobacter* grow on propionate and sulphate, and sequences related to this group were detected in the July hole 3 pyrotags.

Conclusions

In this study, three genomics methods were used to discover what microbes were present in a passive treatment sub-surface flow wetland treating metal and sulphate rich seepage. Very surprisingly we found that methanogens were the most prevalent microbes at this site. Perhaps due to the age of the site, which was reaching its expected lifetime for a passive treatment system, methanogens had out-competed SRB and were the main consumers of acetate and H_2. We do not believe that the high metal concentrations were inhibitory to SRB since we found many SRB in other types of organic materials that were immersed in this system in a separate study (Schmidtova and Baldwin 2011). In future passive treatment systems operators need to take into account that methanogens may have a tendency to take over these systems especially as the conditions become more reducing. As metal concentrations were decreasing through the BCR during the study period, the mechanisms for their removal may involve processes other than sulphate reduction. A separate metagenomic study of these Trail BCR samples is underway to determine what these mechanisms are. The sequences in this study provide a starting point for studying the relationships between microbes in this treatment system. By studying the trends we can identify which species co-occur with each other and we can correlate community composition with more detailed chemistry data taken from the site. The sequences themselves can be used to design quantitative probes, either PCR based or microscopy based using florescence in situ hybridization.

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Acronyms

BCR	Biochemical reactor
DNA	Deoxyribonucleic acids
eDNA	environmental DNA
ORP	Oxidation reduction potential
PCR	Polymerase chain reaction
SRB	Sulphate-reducing bacteria
DCA	Detrended correspondence analysis
DO	Dissolved oxygen
ICP-MS	Inductively coupled plasma mass spectroscopy
OTU	Operational taxonomic unit
RDP	Ribosomal database project
SSU rRNA	small sub-unit ribosomal ribonucleic acids