KINETICS AND COMMUNITY PROFILING OF SULFATE-REDUCING BACTERIA IN ORGANIC CARBON TREATED MINE TAILINGS

by

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ABSTRACT

Acid rock drainage (ARD) poses a significant health and environmental hazard worldwide via the discharge of highly acidic waters and potentially toxic levels of mobile metals. This is a result of weathering and microbial oxidation of pyretic minerals present in mine tailings. Sulfate reducing bacteria (SRB), which are often indigenous to mine tailings, have demonstrated promising potential in metabolically raising effluent pH and immobilizing metals through precipitation and biomineralization. The addition of an organic carbon source has the potential of stimulating the SRB and reducing ARD at its source. Often the success of a process based on implementing endemic microbial consortia for in situ bioremediation is highly dependent on an understanding of the community structure and potential activity of microbial community members when provided a specific substrate.

The goal of this research was to identify viable methodologies that can be used to select and monitor successful bioremediation treatments. Differences in microbial community structure and activity of batch cultures inoculated with tailings were observed for independent treatments of whey and lactate as carbon sources. Community response to whey treatment of bench-scale columns was also observed. Development and optimization of DNA extraction and purification methods was required for the highly contaminated tailing samples. Microbial community structure and phylogeny were identified using denaturing gradient gel electrophoresis (DGGE) and automated sequencing.

The methods used in this paper were successful at identifying pre- and post-treatment community structure of endemic microbial populations. Shifts in community structure were observed in treated columns and treated batch cultures. Sulfate reduction in treated batch cultures was highly variable between samples, suggesting microheterogeneities in community structure of sampled tailings. Selection for specific phylogenies was evident with respect to carbon source treatment, culturing conditions, and sampled inocula. Variability in community structure was roughly correlated to sulfate reduction in individual organic carbon treatments. Resulting community profiles were highly dependent on methods used in obtaining, amplifying, and isolating community DNA of phylogenetically distinct populations. The success of implementing molecular techniques to observe and optimize bioremediation is ultimately dependent on the methodology used.

CHAPTER 1

INTRODUCTION

Background

Acid Rock Drainage (ARD)

Over the last century worldwide industrial activity has seen exponential growth, supporting both technological advancement and environmental destruction. Surface and underground mining have contributed significantly to international environmental contamination. In all but the most arid environments, mining operations of metal ores and coal results in the contamination of surface and/or ground waters (Johnson and Hallberg, 2003). The nature and extent of the contamination is dependent on such factors as the composition of the ore body and associated geologic strata, local climate, method of mining and ore extraction, and enforced governmental mining regulations. As such, the resulting environmental impact and nature of the contamination can be highly variable from site to site.

Processing of the ore body results in mine tailings, often rich in sulfide minerals. Oxidative dissolution of sulfide minerals is a natural process that occurs in the presence of water and air at a relatively slow rate. This process is accelerated and the resulting contamination is dramatically increased by mining

activity and the resulting exposure of highly reactive sulfide minerals. Increased mineral surface area and the bacterial catalysis of the oxidizing reaction lead to a rapid acidification of pore water. The resulting effluent is often referred to as “acid mine drainage” (AMD) or “acid rock drainage” (ARD). Heavy metals exposed to the acidic solution can be dissolved, resulting in a low pH effluent with high concentrations of sulfates and heavy metals that can be discharged to surrounding surface waters.

Vertical transport of acidic drainage through the tailings can also occur, and resulting flow-through can migrate into the nearest aquifer and ultimately discharge into oxygenated surface waters. Soils and riparian areas exposed to ARD can be impacted for miles as toxic levels of soluble heavy metals, metal precipitates and acidic waters decimate local floral and faunal populations. Since the character of the waste will to a large extent determine its impact on the surrounding environment, physical, chemical, mineralogical and microbiological aspects of the waste have to be considered (Ledin and Pederson, 1996).

Over the last few decades, the potential threats of ARD have been recognized and treatment processes developed to reduce the potential hazards. A number of procedures employing chemical and/or biological reactions are used to mitigate environmental contamination of ARD. Conventional hydroxide precipitation and heavy metal confinement techniques based on sulfide precipitation are the most predominant procedures for treatment. Sulfide precipitation is often favored over hydroxide precipitation because of the higher

degree of metal removal at low pH (pH 3-6). In addition, sludge characteristics are greatly improved as sulfides are chemically more stable, denser and less voluminous. However, chemical sulfide precipitation is still an expensive process producing a heavy metal contaminated sludge that must be treated and disposed of (Cocos et al., 2002).

More recently, biologically mediated sulfide precipitation has been identified as an effective and economic process for removing contaminants from and neutralizing ARD by directing flow of ARD through microbiologically rich wetlands or biobarriers (Ledin and Peterson, 1996; Cocos et al., 2002; Christensen et al., 1996; Kim et al., 1999; Fortin and Beveridge, 1997; Jong and Parry, 2003; Chang et al., 2000). The microbial communities, specifically sulfate reducing bacteria (SRB), catalzye a biochemical reaction that converts sulfate to hydrogen sulfide (H_2S) (Equation 1) in the presence of an organic carbon source, H_2S then reacts with divalent metals to form metal sulfide precipitates (Equation 2).

$$2CH_2O + SO_4^{2-} \rightarrow H_2S + 2HCO_3^- \quad \textbf{(1)}$$

$$Me^{2+} + H_2S \rightarrow MeS + 2H^+ \quad \textbf{(2)}$$

The method of directing effluent ARD through a treatment structure (ex situ treatment) has been the predominant method of interest, with little mention or interest in direct treatment of the mine tailings (in situ treatment). Like most environments on the planet, mine tailings support a stratified ecology of microbial

organisms, each suited and contributing to their local niche. The most well recognized and identifiable strata in both terrestrial and aquatic environments are the oxic and anoxic zones. This is also true of mine tailings and of significant importance when considering in situ treatment of ARD contaminants. The uppermost portion of the tailings is subject to a constant supply of atmospheric oxygen rarely limited by diffusion. This is termed the oxic zone, in which Fe- and S-oxidizing bacteria catalyze the oxidation of pyrite. Deeper in the tailings oxygen is depleted, a condition facilitated by increasing pore water saturation with depth. Sulfate reduction, mediated by anaerobic SRB and most commonly limited by low organic carbon concentrations, begins to occur in this oxygen-deprived anoxic zone. By increasing available organic carbon to endemic SRB, effluent ARD could be mitigated before it even left the tailings pile.

Microbial Activity

Although the sulfate reducing bacteria were first discovered in 1895 by Beijerinck, it wasn't until the late 1970s that a basic understanding of the phylogenetic and metabolic diversity of the SRB began to be realized. Postgate (1984) is often credited for sparking the explosive "revolution" in contemporary SRB research. Postgate himself recognized as recently as the early 1990s that the "growth of knowledge of sulphate-reducing bacteria is still in its exponential phase" and that recent advancements in molecular genetics has opened our view to untouched possibilities within the field. The biochemical capacity of the SRB is

so far removed from any other grouping of organisms, it has made them a model for conceivable extraterrestrial biota by leading SRB specialists with specific reference to Mars, well known for it sulfate rich strata (Postgate, 1984). Although sulfate reduction is limited to a relatively strict ecological niche, SRB can be found in almost all environments on the planet: soils, fresh, marine and brackish waters, hot springs and geothermal areas, oil and natural gas wells, sulfur deposits, estuarine mud, sewage, and salt pans.

When Postgate (1984) referred to the sulfate reducing bacteria as the "penultimate stage of a grossly polluted environment" most viewed the SRB as the result and potential cause of widespread environmental contamination and economic distress. Over the last two decades, since the Comprehensive Environmental Response Compensation and Liability Act (CERCLA) was enacted in the United States, considerable interest has been placed on identifying their effectiveness in the remediation of contaminated soils and aquifers. Of significant interest is the wide array of organic compounds that can be used as substrates by SRB for dissimilatory sulfate reduction. Almost 100 electron donors have been described (Hansen, 1993). Sulfate-reducing bacteria have also been investigated for their ability to remove metals and radionuclides from contaminated soils, sediments and groundwater (Kovacova and Sturdik, 2002).

Hao et al. (1996) presented a wide array of growth factors that influence SRB metabolism, including carbon, sulfur source, ecology, metals, and sulfide. Other

than the general ratio of approximately 2:1 representing the reduction of sulfate and oxidation of organic matter, SRB growth stoichiometry is expected to be community specific. Furthermore, SRB activity varies with exposure to different organic carbon sources or environmental conditions. In fact, the specific populations constituting the consortia can have a significantly different overall activity in the presence or absence of other populations when exposed to otherwise constant growth conditions. For example, growth of ceratin methanogenic species can inhibit SRB in the presence of a specific organic carbon source, whereas in the absence of these methanogens, the same SRB populations would flourish. Other methanogens can stimulate SRB growth via fermentation and production of organic acids that are readily consumed by SRB. Thus, the presence of a particular group of organisms can have significant implications when treating a site containing potentially active populations of SRB. It is important then to identify not only the populations of SRB in the consortia, but also other populations that can affect SRB activity subsequent to ARD mitigation.

The preferred carbon sources for SRB are low-molecular weight compounds such as organic acids (e.g. lactate), volatile acids (e.g. acetate), and alcohols (e.g. methanol) (Hao et al., 1996), nearly all of which are products of the anaerobic degradation of carbohydrates, proteins and lipids. Some SRB do not utilize one or more of the listed organics. For example, some members of *Desulfovibrio* converts lactate to acetate, but do not utilize acetate. These are

commonly referred to as incomplete SRB. While some SRB can grow autotrophically with energy derived from sulfite disproportionation, most strains are heterotrophic. In untreated tailing piles, iron and sulfur oxidizing bacteria (IOB and SOB, respectively) are believed to be the major source of organic carbon percolating into the anoxic zone, sustaining small SRB communities. Endemic heterotrophs must also be present to provide the low-molecular weight, metabolic by-products necessary for SRB survival.

A portion of the existing ARD remediation technologies which employ SRB are based on the addition of a readily consumable organic carbon source to stimulate existing communities and accelerate their metabolic activity. Little investigation has been done to identify prominent species of SRB communities present in tailings, as well as potential competitor species. A better understanding of the species present, their metabolic requirements, potential inhibition and rate of activity is an obvious necessity in optimizing carbon source selection and treatment application. Although a fair amount of research has been done to quantify simple consortia of SRB in pure culture and their activity relative to organic carbon mixtures, little research has been done to identify SRB community structure and the activity of specific communities in response to a specific organic carbon treatment. In fact, as Jackson et al. (2001) point out, few researchers have even attempted comprehensive surveys of the microbial communities present in AMD generating systems or tried to link structural patterns to biogeochemical processes.

Monitoring Community Structure and Dynamics

Populations of organisms, and their associated degradative activities responsible for contaminant reduction can be identified and monitored throughout the bioremediation process. Sayler and Layton (1990) first proposed the use of molecular gene probes in monitoring endemic organisms during contaminant degradation. Eight years later Sayler and his colleagues provided a listing of viable approaches, using nucleic acid analysis, to assess and characterize contaminated sites and the potential use of endemic populations for bioremediation of site-specific contaminants (Stapleton et al. 1998). A number of other publications (Dojka et al., 1998; Power et al., 1998; White et al., 1998) proposed the use of molecular-based methods to aid in assessing bioremediation strategies.

In 1994, the U.S. Department of Energy (DOE) initiated the Microbial Genome Program (MGP) to aid in carrying out several of its most challenging missions, including environmental-waste cleanup, carbon sequestration, and biotechnology. Some of the potential microbial applications identified by the DOE include: (i) cleanup of toxic waste sites, (ii) production of chemical catalysts, reagents, and enzymes to improve efficiency of industrial processing, and (iii) use of genetically altered bacteria as living sensors (biosensors) to detect harmful chemicals in soil, air, and water. Any one of these potential applications could ultimately be applied by industry for the reduction, monitoring, and treatment of contaminant wastes. Some complementary programs associated

with the MGP include the DOE's Biological and Environmental Research (BER) Program and the Natural and Accelerated Bioremediation Research (NABIR) Program. The latest BER program, called Genomics:GTL (Genomes to Life), combines completed microbial DNA sequences with new high-throughput technologies to develop a set of comprehensive models of how living systems function, and is directly tied to NABIR. Some of these sequences include those of organisms associated with the mining industry (e.g. *Acidithiobacillus ferroxidans*, *Desulfovibrio* spp., *Ferroplasma acidarmanus*). The list also includes several microbial consortia identified from anaerobic bioreactors, wide ranging environmental soils, and acid mine drainage (Iron Mountian, Calif.).

The desire and need for the identification of microbes and microbial consortia and subsequently applying their potential activities to future technologies is evident. The first steps often include (i) identifying what organisms exist at the site of interest, (ii) identifying what is occurring with respect to the contaminant (e.g.. degradation), and (iii) correlating microbial activity, and thus gene expression, to shifts in the contaminant concentration or speciation. Genetic fingerprinting is a broad molecular technique that encompasses a number of methods for identifying and monitoring microbial communities and their activities within environmental samples. This approach allows us to effectively identify these organisms in a manner that is far less selective or biased than the traditional culturing techniques. When these methods are used in conjunction with laboratory and field studies of contaminant degradation, possible

correlations can be made with the observed microbial consortia and/or their activities.

Ribosomal intergenic spacer analysis (RISA), terminal restriction fragment length polymorphism (T-RFLP), amplified ribosomal DNA restriction analysis (ARDRA), in situ hybridization (ISH), denaturant gradient gel electrophoresis (DGGE), plasmid cloning, and automated sequencing are the most noted nucleic acid techniques for analyzing microbial community diversity, structure and dynamics. Each of the listed techniques relies on the basic premise that the nucleic acid sequence of each class, species, and strain of microbe varies to a certain degree within specific regions of the molecular genome.

Research Needs Summary

Of the limited research that has been done with respect to ARD remediation, little has focused on the in situ treatment of mine tailings to mitigate effluent ARD before leaving the tailings pile. Laboratory and field application of direct tailings treatment is needed to test the feasibility of this method and to address the scaling effects imposed by the biases of standard laboratory experiments. When using bioremediation technologies little has been done to optimize conditions by tailoring treatments to endemic microbial communities. This would require the identification of specific phylogenies inhabiting ARD tailings and targeting optimal communities of SRB and other anaerobic heterotrophs through specific organic carbon treatments, thus promoting increased sulfate reduction or decreased fe-

and S-oxidation. Monitoring temporal shifts of microbial consortia in response to organic carbon treatment and environmental perturbations (e.g. metal toxicity) is also needed to identify necessary adjustments to treatment application.

Purpose

Goal

The goal of this research is to identify viable methodologies that can be used to select and monitor successful bioremediation treatments in an array of environments. This thesis will focus on the microbiologically mediated mitigation of acid rock drainage via direct application of an organic carbon source to pyritic mine tailings. Through the identification of endemic microbial consortia structure and their relative activities in response to treatments, specific treatment technologies and carbon sources could potentially be identified to achieve maximum rates of remediation. Another aspect of this research will be to address the impact of scaling effects with respect to microbial activity and selection of laboratory growth medium. The following objectives address this goal statement:

Objective 1

Select and optimize molecular methods that will provide representative phylogenies of endemic microbial consortia within sampled mine tailings.

* Develop methodology for obtaining representative DNA extract of the microbial community.
* Select PCR primers to target a wide variety of organisms and provide the most diverse community profile.
* Optimize PCR conditions to reduce artifacts and preferential template amplification, maintaining a community profile that is truly representative of the sampled consortia.
* Effectively separate community DNA into phylogenetically distinct groups for the identification of predominant populations constituting the microbial consortia.

Objective 2

Measure the kinetic response of indigenous SRB populations from sampled tailings to treatment application.

* Construct anaerobic microcosms inoculated with sampled mine tailings treated with a carbon source.
* Monitor microbial response to treatment via measurements of sulfate reduction, hydrogen sulfide production, pH, and metal sulfide production.

* Quantify sulfate reduction rates in response to treatment.
* Compare microcosm sulfate reduction rates to previously observed sulfate reduction rates in bench-scale columns to ascertain the impact of scaling effects and experimental setup.

Objective 3

Determine the dynamics of community structure and identify specific phylogenies resulting from treatment application.

* Extract, purify, and amplify community 16S rDNA using previously optimized molecular techniques from tailings inocula and treated microcosms.
* Construct representative community profiles of pre- and post-treatment samples using denaturant gradient gel electrophoresis (DGGE).
* Identified predominant phylogenies using automated sequencing of amplified template DNA isolated by DGGE.
* Compare shifts in phylogenies resulting from microcosm and bench-scale treatments

Organization

This thesis is organized into three chapters, each addressing the objectives presented above. Chapter 2 describes the methods used obtain representative

community profiles from sampled mine tailings. The primary areas of interest include: (i) DNA extraction and purification, (ii) primer selection and template amplification (PCR) conditions, and (iii) community profiling via denaturant gradient gel electrophoresis (DGGE). Chapter 3 is a description of microbiologically mediated sulfate reduction and hydrogen sulfide production kinetics, and the observable shifts in microbial consortia during this process. Relative community responses to treatment were determined based on the extent of changes in measured sulfate and hydrogen sulfide concentrations, pH, and iron sulfide formation. Sulfate reduction rates observed in microcosms and bench-scale columns were compared to ascertain the relative effects of culturing methods and scaling effects. Optimal molecular methods determined in Chapter 2 were used to construct community profiles of the predominant phylogenies present in pre- and post-treatment microbial consortia. Some of the predominant phylogenies in each of the samples were classified through NCBI-BLAST searches of distinct sequences isolated with DGGE and identified by automated sequencing. Shifts in predominant phylogenies resulting from treatment application were determined through side-by-side comparisons of community profiles and identified phylogenies. Chapter 4 reviews the conclusions drawn from results obtained in the previously described analyses, as well as suggestions for future research.

CHAPTER 2

MOLECULAR METHODS DEVELOPMENT

Background

Prior Research

Microbial techniques that have been used in the past (i.e. media based cultivation and isolation) are selective for culturable organisms, and the majority of soil-based microorganisms are not detected. It is estimated that only 1 % of all microbial populations can be cultivated using standard techniques (Ward et al. 1990). Comparisons of the percentage of culturable bacteria with total cell counts from different habitats have shown enormous discrepancies (Amann et al. 1995). This is may be due to specific nutritive or environmental requirements that are either unknown or impossible to provide given current techniques. Extraction amplification and isolation of bacterial nucleic acids from natural environments through various molecular techniques has become a useful tool to detect nonculturable bacteria (Ward et al., 1990).

Molecular analyses employing genetic fingerprinting techniques are becoming more and more popular in the identification of microbial community structure, activity, interaction and role in ecosystem cycling and maintenance. A number of molecular techniques have been used for such purposes in a wide variety of

environmental habitats (Yu and Mohn, 2001; Heuer et al., 1997; Meithling et al., 2000; Chang et al., 2000; Ranjard et al., 2000 and 2001; Borneman and Triplett, 1997; von Canstein et al., 2002; Erikson et al., 2001; Massol-Deya et al., 1997; Zhou et al., 1996). To date few molecular techniques have been used to identify the microbial communities in iron- and sulfate-rich sediments, such as ARD generating mine tailings (Bond et al. 2000; Peccia et al., 2000; Hao et al. 2002). This can be attributed to the inherent difficulties involved with current molecular based techniques. These include incomplete extraction of representative nucleic acids, sample contamination, and subsequent PCR inhibition. The method of sample handling, isolation and purification, choice of primers used in PCR amplification, PCR amplification conditions, and the fingerprinting method used in analyzing a single environmental sample can generate considerably different community profiles.

Obtaining Representative DNA

The validity of using molecular techniques in environmental studies depends primarily on obtaining representative extracts of nucleic acids from entire microbial communities (Miller et al., 1999). Environmental compounds (heavy metals), conditions (pH, buffering capacity), cellular constituents (cellular debris, nucleases, proteins, polysaccharides), and the physical structure and location of bacterial cells and communities can all inhibit complete extraction and amplification of representative DNA or RNA (Hao et al., 2002; Miller et al., 1999;

Wilson, 1997). Most direct extraction methods have been tested on a limited number of soil types, with only one having been tested on sediments from ARD (Bond et al. 2001), but not specific to processed mine tailings. These methods of extraction vary widely within published works, but rely on individual component steps (i.e. cell lysis, nucleic acid extraction and nucleic acid purification) each having specific inefficiencies.

A primary attribute of bacteria inhabiting ARD generating mine tailings is their resilience to such hostile environments. This resilience can be attributed to a number of physical and physiological characteristics. Biomineralization of elements to form an “armored” coating on their cell wall and close associations with the particulate substrate are the primary limiting factors in the complete extraction of clean nucleic acids. Several studies have revealed that relatively large quantities of heavy metal cations are adsorbed and complexed by bacteria and fungi (Miller et al., 1999; Liu and Fang, 2002; Chen et al., 2000; Hughes and Pool, 1989). Southam and Beveridge (1992) found that mineralized bacteria were encased in secondary mineral aggregates not normally present in the original tailings. Fortin et al. (1996) found numerous mineralized bacteria, showing amorphous Fe-oxides on their cell walls, within tailings particles. Fe-oxides also coat and cement tailings particles, producing microenvironments in which SRB could survive the low pH conditions of the tailings. One genera of SRB, *Desulfotomaculum*, have exhibited extensive precipitation of FeS on its cell wall when grown in the presence of Fe(II) (Fortin et al. 1995).

The issue of extracting a representative sample of DNA or RNA of the microbial community through lysing attached and mineralized cells without shearing nucleic acids is of primary concern in mine tailings and has proven to be a difficult task. Another inefficiency in the use of molecular techniques is the presence of inhibitory substances that are coextracted. Inhibitory substance of particular interest to mine tailings are heavy metals and constituents of bacterial and fungal cells, specifically proteins and polysaccharides, which can give rise to misleading results. In addition to PCR-reaction failure, sample contamination may be manifested as spurious background bands during amplification-based DNA fingerprinting (Wilson 1997). The presence of extensive and diverse inhibitory substances requires highly effective means of sample purification. A considerable amount of research has also been devoted to optimizing extraction and purification techniques from samples of various environmental habitats (Jackson et al., 1997; Miller et al., 1999; Selenska-Pobell, 1995; Wilson, 1997; Zhou, 1996). Bond et al (2000) developed a method for washing microbial biofilms inhabiting an extreme acid mine drainage site, which also proved to be effective for the extraction of nucleic acids from sediment submerged in the AMD (Bond and Banfield, 2001). Unfortunately no published work has been done to optimize a method for the extraction and purification of nucleic acids directly from ARD generating mine tailings.

Obtaining and extract that is representative of in situ communities can also be impacted by sample handling prior to nucleic acid extraction. According to

Posgate (1984) examinations should begin as soon as possible, preferably within 24 hours, with samples being kept cool to minimize metabolic activity. Rochelle et al. (1994) found that freezing within two hours of sampling should always be employed when sediment samples are to be used to assess bacterial diversity by molecular methods, particularly with respect to anaerobic samples. Freezing will retard any shift in community diversity that may be due to changes in the microenvironment of the sample.

Genetic Fingerprinting

Genetic fingerprinting techniques use variations in the molecular genome to provide a pattern or profile of genetic diversity within a microbial community (Muyzer and Smalla, 1998). Genetic fingerprinting techniques have been used to observe microbial community structure and consortial shifts in a wide range of environments, including metal contaminated soils. To date, no published work has been conducted the use of genetic fingerprinting to identify communities specifically within mine tailings.

Denaturant gradient gel electrophoresis (DGGE), a method of genetic fingerprinting that has gained a significant amount of interest, utilizes the PCR-amplified product of environmentally sampled 16S rDNA fragments. First used to profile community complexity of a microbial mat and bacterial biofilms by Muyzer et al. (1993), it has proven to be an effective method for identifying community diversity across a wide spectrum of environmental samples. By selectively

amplifying regions of DNA that contain nucleic sequences of high variability, phylogenetically distinct sequences representing separate microbial populations can be isolated and identified. Like all methodologies, however, limitations and biases exist. Only an integrated approach which combines multiple molecular techniques, new isolation strategies and physiological characterizations of specific microbes will reveal the role of microbial diversity in the biogeochemical cycling of elements in ARD generating tailings.

The genetic fingerprinting of microbial communities can be specialized for specific organisms of interest. This is of particular use when monitoring temporal changes in specific microbial populations. The basis of this strategy is the amplification of DNA fragments obtained with group-specific primers (e.g. *Archea*, *Bacteria*). Furthermore, the banding patterns of complex microbial communities generated by genetic fingerprinting techniques can be simplified by using PCR primers for functional genes, which are only present in particular microbial populations or species (Wawer and Muyzer, 1995; Iwamoto et al., 2000), such as the use of APS reductase gene to target sulfate reducing bacteria and Archaea (Deplancke et al., 2000). According to Muyzer (1999) the sequencing of 16S rRNA encoding genes is possibly the most powerful tool to explore microbial diversity and to analyze community structure. Figure 1 presents a flow diagram of the different steps involved in identifying the presence and activity of predominant constituents in microbial assemblages using DGGE fingerprinting techniques.

Potential Problems in Template Amplification

Much of the methodology developed in the field of molecular biology has been built on the foundation of the polymerase chain reaction (PCR). It is this cycling of temperature over time that allows the molecular biologist to select regions of DNA and exponentially produce millions of copies for future analysis. The potential utility of this tool has been so well received that the inherent biases and inefficiency are often overlooked or regarded as insignificant. The PCR process is perfectly simple having only three steps and a handful of necessary components. Double helix denaturation, primer annealing, and template extension (duplication) are sequentially repeated with specific temperatures applied over specific periods of time. The basic reaction mixture contains template DNA, primers targeting the region of interest, free nucleotides to build the replicate DNA, polymerase for physically binding the nucleotides in the right sequence, and magnesium to buffer the reaction. Though this list is short, slight variations in any component (i.e. temperature, time, template concentration or size, magnesium…) can have a significant effect on the success of the PCR.

Frequently, in the PCR amplification of target gene sequences, mispriming by one or both of the oligonucleotide primers can lead to spurious bands in the product spectrum of a community sample. This is in part due to the fact that shorter misprimed products have a greater chance of being completely replicated than longer correct products. This problem is compounded over an increasing number of cycles and more likely to occur when template DNA is present in only

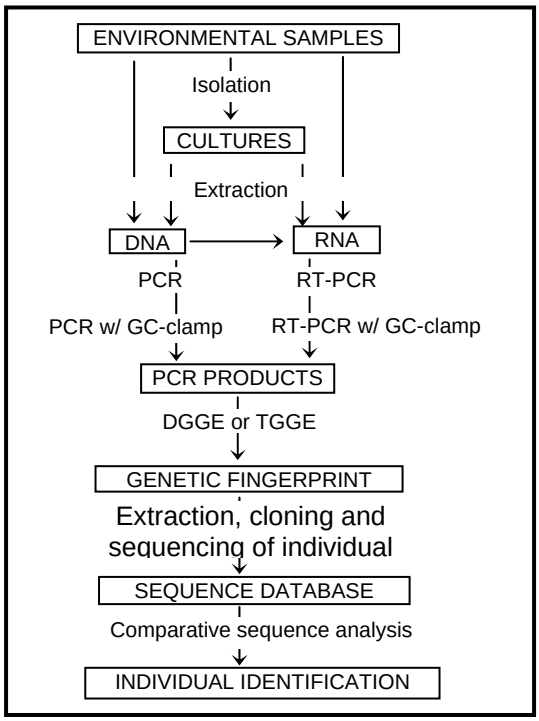


Figure 1 Flow diagram of the different steps involved in the process of identifying primary constituents of a microbial community using DGGE and TGGE molecular techniques. Genetic fingerprinting by DGGE or TGGE is the central core of the strategy to identify the presence (DNA) and activity (RNA) of microbial populations in a complex system. Individual identification of community members is also possible with the excision and sequencing of individual bands present in the gels. These techniques can also be used to ensure isolation of bacteria in pure cultures, identify strains of individual bacteria, and screen clone libraries for redundancy. Hybridization analysis can also be employed to observe taxon-specific population changes in the genetic fingerprints of discrete environmental samples. Modeled after Muyzer and Smalla, 1998.

small amounts, as is encountered in the biologically limited mine tailings. Potentially longer misprimed products could also be amplified during the PCR process if conditions are relatively lenient with respect to extension time and annealing temperature. There are three methods that can presumably remedy this problem of nonspecific amplification, or mispriming. The first two include systematically adjusting the Mg^{2+} concentration or the annealing temperature of the PCR. These methods would be effective assuming that the spurious

interactions are sufficiently less stable than the specific interactions as a result of sequence mismatch. It is known that decreases in temperature and increases in Mg^{2+} concentrations increase the stability of hybrids containing mismatched base pairs, contributing directly to the formation of non-specific PCR products (Giovanannoni, 1991). The third method employs an annealing temperature that steadily decreases over the duration of the PCR, and is appropriately termed 'touchdown' PCR (Don et al., 1991). The initial annealing temperature is established at or above the expected annealing temperature. As the reaction progresses the annealing temperature is steadily decreased, for example $1^{o}C$ every, two cycles, until a 'touchdown' temperature is achieved at which point 10 additional cycles are carried out. Often the difference in initial and 'touchdown' annealing temperatures is 10^{o} C, with the highest temperature being 10^{o} C above the expected annealing temperature of the primers used, identified as the T_m. Touchdown PCR (TD-PCR) is considered a useful technique for avoiding the amplification of spurious DNA fragments, such as non-rDNA fragments and fragments of improper size. It should be noted that a bias does exist against species with improper primer matching and they could be entirely excluded from the community profile. Watanabe et al. (2001) suggest using TD-PCR only when a given sample generates spurious products under standard conditions.

Additional bands that are not representative of the community can also be generated by the formation of chimeric sequences assembled from the simultaneous sequencing of multiple species. Two main factors increase the

likelihood of chimera formation: (i) the availability of partial length fragments present in low-molecular-weight genome DNA or generated by the premature termination of elongation during PCR and (ii) the percentage of highly conserved stretches along the primary structure of the rDNA, where single strands originating from different species can anneal in these regions after denaturing (Amann et al., 1995). If the community is constructed of similar species the length of these complimentary stretches increase, thus increasing the probability and stability of chimera formation. This is of significant concern with respect to this research. The diversity of extremophiles and the stimulated anaerobic community that may be present in the tailing samples is presumably quite small and of close phylogenetic relation. The length of complimentary regions is in turn presumably greater and thus a concern with respect to chimera formation.

Primer purification can also have a significant effect on the overall genetic fingerprint of the community sampled, in both band number and intensity. Villadas et al. (2002) found that oligonucleotides used in PCR-TGGE (temperature gradient gel electrophoresis) analysis of microbial communities yielded far better results when purified via high-performance liquid chromatography (HPLC). It is reasonable to assume that the ultimate effects of primer purification on the community profile result from the high efficacy purification of just the +GC primer. This assumption is based on the fact that oligonucleotides containing long stretches of guanine (G) have a tendency to knot up on themselves forming quadruplexes. This could potentially cause

inefficient amplification of template DNA, as well as significant background and banding artifacts in the resulting genetic fingerprint.

There is significant difficulty in preparing genomic DNA free of contaminant DNA, and subsequent amplification of the contaminant through PCR, particularly with respect to samples containing low biomass. Tanner et al. (1998) correlated several organisms that were commonly identified from physically and chemically distinct environments with those identified in a survey of 16S rRNA gene sequences obtained from negative extraction controls that did not contain template extract. This is not to say that those organisms identified as molecular contaminants were not present in the previously sequenced environmental samples. However, it does support the need to run control samples, which do not contain sampled DNA, throughout the entire processes of extraction, amplification and subsequent analyses. This provides a means of identifying and disregarding banded phylogenies not present in the sampled community.

Theoretical and Practical Aspects of DGGE

Denaturing Gradient Gel Electrophoresis (DGGE) is an electrophoretic method to identify single base changes in a segment of DNA. In theory, double-stranded DNA fragments of equal length are subjected to an increasing chemically derived denaturant environment and will melt in discrete segments as the fragments migrate through a polyacrylamide gel. Each segment or “melting domain” has a specific melting temperature. As a domain reaches its specific melting

temperature the helical DNA segment is partially melted and looses its relative electrophoretic mobility. Triple bonds of guanine-cytosine pairs are more resistant to melting than the double bonds of adenine-thiamine pairs. As such, the melting temperature of individual domains is sequence specific. DNA fragments containing a larger concentration of guanine and/or cytosine will migrate further through the gel into higher melting domains before halting in the gel.

With DGGE, 50% of sequence variants can be detected from DNA fragments up to 500 base pairs (bp) (Muyzer et al., 1996). That number can be increased to nearly 100% of sequence variants with the attachment of a GC-rich sequence, or GC-clamp, to one side of the DNA fragment. The GC-clamp consists of a specific sequence of guanine (G) and cytosine (C) which is added to the 5'-end of one of the PCR primers, coamplified and introduced into the amplified DNA fragment. Optimal resolution is achieved with the 30-40 base pair GC-clamp by insuring that the region screened is in the lowest melting domain and that the DNA will remain partially double stranded. An alternative to using GC-clamps is the use of PCR ChemiClamp primers. These primers covalently link the two DNA strands at one end. It should be noted however that the use of PCR ChemiClamp primers inhibits reamplification and sequencing of the fragments isolated from a gel.

Melting domains represent predominant populations (when DNA is used) or most physiologically active members (when RNA is used) of the environmental

sample being analyzed. Nucleic acid fragments from individual domains, isolated on gradient gels, can be reamplified and sequenced to identify individual constituents of the DNA or RNA extracted from environmental samples.

DGGE profiles can also be blotted onto nylon membranes and hybridized with group-specific radioactively-labeled oligonucleotide probes. These probes are designed for specific types of bacteria, such as sulfate-reducing bacteria. Some probes are, however, specific to particular genetic sequences, or genes, which must be known prior to probe design. This can be achieved from prior DGGE band sequencing. Probes can then be used to monitor changes of specific microbial populations. Another strategy employs polynucleotide probes flanking the V6 region of the 16S rRNA, applied under stringent hybridization conditions (Heuer et al., 1997). The advantage of this strategy is that no sequence information is required to produce the probes.

DGGE can be a rapid, highly repeatable technique for the identification of microbial populations present in highly variable environments. It can be used to identify consortial shifts over time and/or in response to environmental perturbations. In the case of AMD amendment, changes in microbial populations, physiological activity, and community structure can be observed over the course of amendment applications. Amendments can then be selected and optimized to achieve the desired response.

Limitations of DGGE

The separation of only relatively small fragments up to 500 basepairs can limit the amount of sequence information necessary for phylogenetic inference and species identification. It can also limit the specificity of probes designed for hybridization analysis. Ideally, the melting domains of sequences varying in as little as one basepair can be separated using DGGE. However, it has been demonstrated that it is not always possible to separate DNA fragments with little (2 – 3 bp) sequence variation (Vallaeys et al., 1997; BuchholzCleven et al., 1997). The use of different regions of the 16S rDNA and different DGGE conditions could result in different resolutions of separation. It is also advisable to clone the amplified product identified as an individual band prior to sequencing. This ensures a clean, legible sequence of at least one organism present at a single melting domain. Another alternative is to excise the band, reamplify, and run the resulting product on a narrower gradient DGGE gel to increase band seperation.

Co-migration of DNA fragments can have various repercussions in subsequent analyses, inhibiting the sequencing of what appear to be individual bands and underestimating the community diversity of the sample. As mentioned above, a smaller range in the gradient of denaturant could possibly separate individual species that would otherwise appear as a single species.

Conversely, DGGE can expose sequence microheterogeneity in individual bacteria. When looking at community diversity, sequence heterogeneity of a

single species could lead to an overestimation of the number of bacteria within natural communities. Overestimation of community diversity can also result from the double bands in DGGE and TGGE patterns produced by the use of degenerate primers in PCR reactions (Kowalchuk et al., 1997) or the formation of chimeric rRNA sequences during the PCR amplification of community DNA (Amman et al., 1995).

In addition, there is a limit to the number of different DNA fragments that can be separated by DGGE. Torsvik et al. (1990) found that there might be as many as 10^4 different genomes present in soil samples. This may be of minor concern in the present study as the diversity of lithotrophic organisms present in mine tailings is presumably lower then would be expected in a contaminant-free soil. In general, these molecular techniques will only display the rDNA fragments obtained from predominant species present. Muyzer et al. (1993; 1996) revealed that bacterial populations that make up only 1% or more of the total community should be detected by DGGE. Others argue that one can be confident in assuming a more conservative value of 10%. The use of RNA in DGGE can provide considerably different banding patters than would be seen from those generated with DNA. As mentioned, PCR amplified RNA is representative of those populations which are most physiologically active. Thus, with the use of RNA, it is possible that smaller active populations, making up less than 1% of the total community, could be detected by DGGE, resulting in an entirely different genetic fingerprint.

Purpose

Molecular applications used in identifying microbial structure and function provide unique insights into the uncultured microbial communities of both soils and water because they avoid the biases inherent in traditional culture-based microbial methods. However, the validity of applying molecular techniques, particularly genetic fingerprinting techniques, is dependent on obtaining representative extracts of nucleic acids from the entire microbial community and amplifying those extracts without interference from methodological biases.

The success of obtaining representative extracts is complicated by the inefficiencies of nucleic acid extraction (more specifically cell lysis), possible DNA sorption to soil surfaces, coextraction of PCR inhibitors, and shearing of nucleic acids (Miller et al., 1999; Wilson, 1997; Zhou et al., 1996). Amplification of DNA extract can be greatly effected by the presence of contaminants, resulting in failed amplification, preferential amplification and even false amplicons. The methods of sample handling, nucleic acid extraction, purification, and template amplification can lead to distinctly different PCR-DGGE profiles representing different microbial consortia due to their inherent biases (Neimi et al., 2001). The limitations and inherent biases of genetic fingerprinting techniques employed must also be acknowledged.

The purpose of this research was to systematically develop and test methodologies to achieve optimal conditions necessary to minimize the influence of these biases on the resulting genetic fingerprint of the microbial community.

Materials and Methods

Overview

A variety of traditional and recently published molecular methods were tested to evaluate their effectiveness in developing representative community profiles of sampled mine tailings. Some modifications were made to these methodologies with the intent of optimizing them to the tailing samples. The first issue of concern was to identify a method of DNA extraction that would provide a representative community profile and a method of purification that would allow for unhindered PCR amplification and minimized artifacts in the genetic fingerprint. Seven different methods were tested to identify which would provide the best, repeatable results. Repeatable results were defined as a consistent amount and quality of DNA extract and nearly identical community profiles from multiple subsamples of a single homogeneous sample.

The second step in optimizing the molecular techniques was to select primers that would amplify regions of interest containing multiple hypervariable regions, providing adequate separation of genetically distinct populations into defined melting domains. These melting domains should in turn contain a single phylogeny that could be successfully sequenced and identified. This also required optimization of PCR conditions specific to each set of primers. Comparisons of amplified template obtained from variations in fixed annealing temperatures and touchdown conditions were conducted to identify a PCR

protocol that would yield a clean, diverse community profile containing minimal artifacts. Oligonucleotide purification was also tested to evaluate the increased efficacy of HPLC purification over standard desalt purification as proposed by Villadas et al. (2002).

The third step was to optimize the DGGE protocol for community profiling. Though it is a relatively standardized method of community analysis, DGGE can be modified in several ways to achieve the best results possible. Gradient concentrations, gel construction, buffer quality, staining and handling of the gel, DNA isolation methods, and isolate DNA preparation for sequencing were systematically considered and tested to determine which method combinations provided the best possible community profiles.

The following is a description of the methods that were tested and evaluated to overcome the possible biases and inefficiencies resulting from the use of published methodologies on inherently difficult samples (i.e. mine tailings).

Sampling

Tailings were sampled from laboratory columns that were used to test the effects of carbon source treatment over a period of years. The tailings were originally collected from the tailings impoundment of the Fox Lake Mine in Manitoba. Each of three columns consisted of 12 inch diameter PVC pipe with the top exposed to atmospheric conditions, capped at the bottom and having a single, external port for sampling approximately 3 inches from the base. A thin

layer of pea gravel was placed on bottom of the columns with approximately 36 inches of tailings packed on top. Additional sampling ports for oxygen and carbon dioxide concentrations were place at 6, 18 and 30 inches. All three columns had been watered on a weekly basis for approximately 1700 days to accelerate weathering and facilitate weekly effluent microbial, pH, ORP, sulfate, and metals analysis. Two of the columns, TC1 and TC2, had been exposed to repeated treatments of whey, molasses, and methanol. Treatments occurred every six months on average. The third column, TCC, was used as a control to monitor the effects of accelerated weathering and oxidation of pyrite minerals.

Ranjard et al. (2003) suggested that the sampling strategy should be different according to the objectives. A rather large sample (> 1 g) should be used for a global description of the community genetic structure, where as a large number of smaller samples are best for a more complete inventory of the microbial diversity. Due to the limited surface area of the laboratory columns containing the tailings and the increased affects of multiple sampling on columns, particularly with respect to increased channeling and aeration of the anaerobic zones, a single core sample was taken for each sampling period. Approximately 10 g of tailing sample could be obtained from a 6 inch long core sample. Samples could then be homogenized to nullify any stratified community distribution that may have been present within the portion of tailings sampled. Once samples were removed from the columns they were immediately packed into a 15 ml sample tube (Falcon) to minimize headspace and exclude air.

Samples were immediately frozen at –70^{0}C and maintained at that temperature for a minimum of 24 hours until community DNA could be extracted. Samples taken from microcosms were also handled in the same manner.

DNA Extraction and Purification

A variety of extraction and purification methods were evaluated to test the efficacy of each method in obtaining nucleic acids that were free of contaminants, of significant size (~23 kb), and readily amplified under standard PCR conditions. To test quality and quantity of nucleic acid extract, 10 ul of suspended extract was visualized on a 1.5 % agarose gel stained with ethidium bromide. A sufficient extract was determined by the presence of a well-defined band: approximately 23,000 base pairs in size. Size and concentration were determined using a 100 bp DNA ladder (Promega). To minimize the potential for contaminant DNA, extraction and PCR amplification preparations were performed using sterilized equipment in either a laminar flow hood or near a flame. All solutions were prepared with reverse osmosis H_2O, and autoclaved prior to use. Homogenized tailing samples were removed from the –70^{0}C freezer and thawed immediately prior to extraction. The remaining sample was refrozen for future extractions.

Extraction Method 1 (EM1) was taken from Zhou et al. (1996) and was developed for obtaining extract from soils of diverse composition. Five grams of tailing sample was added to a sterile 50 ml polypropylene conical tube (Falcon),

to which 13.5 ml extraction buffer (100 mM Tric-HCL [pH8.0], 100 mM sodium EDTA (ethylenediaminetetraacetic acid) [pH 8.0], 100 mM sodium phosphate [pH 8.0], 1.5 M NaCl, 1% CTAB (hexadecyltrimethylammonium bromide)) and 100 ul Protienase-K (10 mg/ml) were added. Tubes were shaken horizontally for 30 minutes at 225 rpm and 37^0 C. A volume of 1.5 ml of 20% SDS (sodium dodecyl sulfate) (w/v) was then added to the mixture. Samples were incubated at 65^0 C for 2 hours with gentle end-over-end inversions every 15 minutes. Sample tubes were subsequently centrifuged at 6,000 x g for 10 minutes at room temperature. Supernatant was collected and placed in sterile 50 ml tubes. To the remaining tailings, 4.5 ml extraction buffer and 0.5 ml 20% SDS were added, vortexed for 10 seconds, incubated at 65^0 C for 10 minutes and centrifuged as previously described. Supernatant was collected and added to that obtained in the previous step. This re-extraction step was repeated a third time, for a total of three volumes of pooled supernatant. Extract was purified by mixing with an equal volume of chloroform-isoamyl alcohol (24:1 v/v). Tubes were centrifuged at 6,000 x g for 10 minutes at room temperature. The aqueous phase was collected and placed in a sterile 50 ml tube. Nucleic acids were precipitated with 60% volume of isopropanol at room temperature for 1 hour. Tubes were centrifuged at 16,000 x g for 20 min to pellet precipitate. Pellets were then washed with cold 70% ethanol (4^0 C), and resuspended in 100 ul sterile water.

In an attempt to increase extract yield, EM1 was slightly modified to include an additional cell lysis step (EM2). To 5 grams of sample, 13.5 ml of extraction

buffer and 1.5 ml 20% SDS were added. Samples were repeatedly frozen (3 times) at –70^0 C for 45 minutes and thawed at 70^0 C for 5 minutes. After the third thaw cycle 100 ul of Protienase-K (10 mg/ml) was added to the sample followed by horizontal shaking at 225 rpm for 30 minutes at 37^0 C. Samples were then incubated at 60^0 C for 2 hours with gentle end-over-end inversions every 15 minutes. Tubes were centrifuged at 6,000 x g for 10 minutes at room temperature. Subsequent steps were identical to those presented in EM1.

To minimize the potential shearing of extracted nucleic acids and increase purification, EM2 was slightly modified. Additional steps were modeled after those presented by Yeats et al. (1995) and will be identified hereafter as EM3. Tailing samples (5 grams) were added to sterile 50 ml tubes along with 14.5 ml extraction buffer and 0.5 ml 20% SDS (w/v). Tubes were briefly vortexed to ensure complete mixing of sample particles and buffer. Samples were repeatedly (3 times) frozen at –70^0 C for 45 min and thawed at 60^0 C for 10 min, with brief vortexing prior to each freeze cycle. This was followed by the addition of 100 ul of Protienase-K (10 mg/ml) to each sample, moderate mixing (150 rpm) and incubation for 1 hour at 37^0 C. Tubes were then incubated at 60^0 C for 1 hour followed by centifugation at 6,000 x g for 10 minutes at room temperature. Supernatants were collected and placed in fresh 50 ml tubes. One half volume of polyethylene glycol (30%)/sodium chloride (1.6 M) buffer was added to samples, incubated at room temperature for 2 hours and centrifuged for 20 min at 10,000 x g (room temperature). The pellet was resuspended in 5 ml TE buffer

(10 mM Tris-base [pH 8.5], 1 mM EDTA, 10 mM NaCl), to which an equal volume of potassium acetate (1 M) was added. Samples were immediately transferred to ice for 5 minutes. Proteins and polysaccharides were precipitated by centrifugation at 16,000 x g for 30 seconds at a temperature of 4^0 C. The supernatant was transferred to a fresh 50 ml tube and an equal volume of chloroform/isoamyl alcohol (24:1) was added. Tubes were centrifuged (6,000 x g) at room temperature for 10 minutes. Aqueous phase was collected and placed in a fresh tube. Nucelic acids were precipitated with a 60% volume of isopropanol at room temperature for 2 hours. Samples were centrifuged at room temperature and 10,000 x g for 30 minutes. Pelleted extract was washed with cold (4^0 C) 70% ethanol and resuspended in 100 ul TE buffer.

In an attempt to increase extract yield EM3 was slightly modified (EM4) by rapid freezing of samples. Rather than freezing samples at -70^0C for 45 minutes, samples were submerged in liquid nitrogen for 5 minutes. This is a commonly used method, in conjunction with physical disruption, to lyse the cell walls of plant tissue. Tubes were vented by piercing the caps of the sample tubes prior to submersion. Samples were then thawed at room temperature followed by brief vortexing. The freeze-thaw cycle was repeated for a total of three cycles.

Nucelic acids extracted via EM4 were purified with an additional step beyond the purification applied in the extraction method (polyethylene glycol/sodium chloride, potassium acetate, and chloroform/isoamyl alcohol). Extract was further purified using a silica-binding matrix and guanidine thiocyanate wash

provided in the BIO101 FASTDNA SpinKit for Soil (Q-BIOgene). Sample purification was performed as per the protocol provided by Q-BIOgene.

The fifth extraction method (EM5) employed the BIO101 FastDNA SPIN Kit for Soil (Q-BIOgene). This extraction method uses bead beating and surfactants for cell lysis, a protein precipitation buffer, and guandine thiocyanate for sample purification. The method uses prepared reagents along with provided tubes and filters, is relatively quick and straightforward.

Due to failures and inconsistencies in the previously mentioned methods, the final methods (EM6 & EM7) were developed. The two methods are based on the same premise and utilize the FastDNA SPIN Kit for community DNA extraction. Prior to bead beating, samples were washed to remove heavy metal precipitates and to increase the pH of the tailing samples. Extraction Method 6 employed three wash steps; precipitate dissolution, metal chelation, pH neutralization, and sample rinse. Precipitates were dissolved by adding 1 ml of a 0.3 N sulfuric acid solution to 0.5 g of tailing sample. Tubes were inverted for 5 minutes then centrifuged for 10 minutes at 10,000 x g. Supernatant was discarded and tailings were washed twice with 1 ml 0.25 M EDTA (pH 8.0). The final rinse involved adding 1 ml of TE buffer to samples. Between each step, samples were inverted for 5 minutes prior to 10 minutes of centrifugation at 10,000 x g. The resulting supernatant was discarded. Subsequent extraction steps were followed as presented in the BIO 101 protocol for the FastDNA SPIN Kit for Soil. A slight

modification to EM6, identified as EM6s, included 2 minutes of sonication in a water bath during the sulfuric acid wash.

Extraction Method 7 also employed two wash steps to dissolve metal precipitates and increase sample pH. The following protocol is a slight variation of that provided by Bond et al. (2000), which was initially developed for preparing biofilm streamers collected from AMD for DNA extraction. A volume of 1.0 ml of PBS (pH 1.8) was added to 0.5 g of sample, inverted for 5 minutes and centrifuged at 10,000 x g for 10 minutes. The resulting supernatant was discarded and 1.0 ml of a solution containing one part Buffer A (200 mM Tris [pH 1.2 – 1.8], 50 mM EDTA, 200 mM NaCl, 2 mM Sodium Citrate, 10 mM $CaCl_2$) and one part 50% glycerol was added to the samples. Sample tubes were again inverted for 5 minutes and centrifuged at 10,000 x g for 10 minutes. Subsequent extraction steps were followed as presented in the BIO 101 protocol for the FastDNA SPIN Kit for Soil.

Template DNA from any of the listed extraction methods that was not amplified under standard PCR (Appendix A) conditions with the addition of 1 ul template were diluted in series and subjected to an additional round of PCR. Though this series dilution reduces the amount of template in the reaction, it also reduces the concentration of contaminants in the extract elution. This attempt at reducing contaminants below an inhibitory concentration also increases the likelihood of some populations not being amplified and subsequently identified in the

community profile. However, predominant populations should persist as strong bands in the DGGE profile.

Primer Selection and PCR Optimization

To obtain distinct phylogenetic sequences of community populations, highly variable regions of 16S rDNA were targeted with universal and bacterial-specific primers. Muyzer et al. (1996) identified Bac341f (5'-CCTACGGGAGGCAGCAG-3') and Univ907r (5'-CCCCGTCCATTCCTTTGAGTTT-3') as highly effective for analyzing total community structure, specifically when used in conjunction with DGGE. The primers effectively bracket three hypervariable regions (Figure 2.2) over a length of approximately 550 bp. It has been reported however, that the 16S rDNA sequences of some newly described groups are so diverse that mismatches to some of the accepted bacterial primers (e.g. Bac341f) exist (Dojka et al., 1998; von Wintzingerode, 2000). This consequently suggests that a portion of organisms present would be poorly represented in the community profile since a mismatch between template DNA and primers greatly reduce amplification efficiency. These primers were nonetheless chosen for DGGE analysis of the tailings based on their acceptance within the scientific community and published success.

Ferris et al. (1996) first used Bac1070f (5'-ATGGCTGTCGTCAGCT-3') and Univ1392r (5'-ACGGGCGGTGTGTAC-3') for DGGE community analysis of a hot spring microbial mat community. The primers were developed from regions

initially identified by Amann et al. (1995) as conserved among the domain *Bacteria* (Bac1070f) and universally conserved among multiple domains (Univ1392r). These primers bracket two hypervariable regions (Figure 2), providing less phylogenetic information than the previously mentioned primer set. Ferris et al. (1996) found that populations detected from cloning were not represented in the DGGE profile. This suggests the presence of specific phylogenies that are not effectively amplified with these primers. However, they were effective at targeting a large number of expected and unexpected populations. This is the second set of primers that were used to develop community profiles.

The third set of primers that were used is a combination of the previous two sets, Bac341f and Univ1392r. By selecting this primer pairing, six hypervariable regions (Figure 2) could potentially be amplified and sequenced simultaneously, increasing the certainty of phylogenetic identification. However, these primers also bracket relatively long conserved regions targeted by 907r and 1070f, increasing the possibility of chimera formation. This is yet another bias to be wary of, but it does not overwhelm the potential gain of increased sequence variability. An additional point of concern is the product size limitation of DGGE. As stated previously, DGGE effectively separates sequence variants from DNA fragments up to 500 base pairs (Myers et al., 1985). This primer set produces fragments of approximately 1000 bp, a segment length that may not be effectively separated by DGGE.

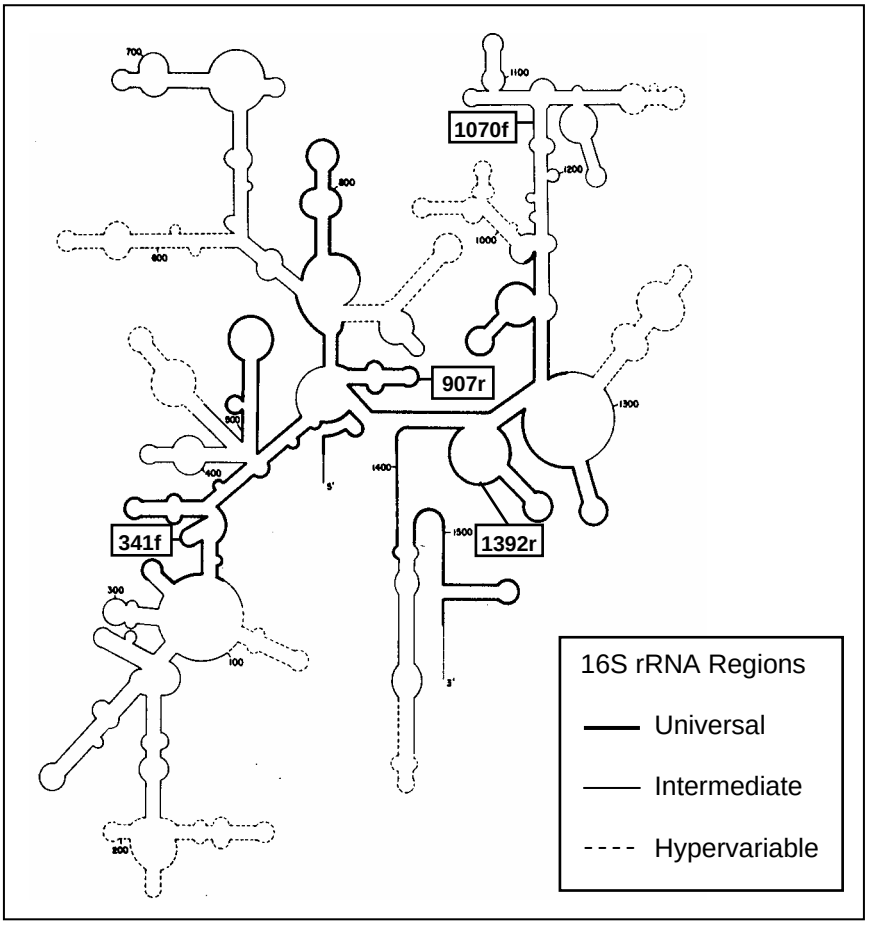


Figure 2 16S rRNA structure and regions of variability (*E. coli*). Primers and their respective annealing locations are presented. Dashed lines represent hypervariable regions amplified with specific primers.

Template amplification was initially conducted using the primers presented above. Prior to running a DGGE, aliquots from the initial amplification product were used as template in a second round of PCR. In the second round of PCR one of the two oligonucleoutides had a GC-clamp (5'-CGCCCGCCGCGCGCG GCGGGCGGGGCGGGGGCACGGGGGG-3') attached (Muyzer et al., 1995). The product of this PCR reaction was then used for DGGE analysis.

All primers were purified using a standard desalt method by the oligonucleotide manufacturer (IDT-Integrated DNA Technologies), except for 1392r with the GC-clamp attached (1392r+GC). This primer was purified using high-performance liquid chromatography (HPLC) as suggested by Villadas et al. (2002). The efficacy of primer purification was tested (see below) by comparing DGGE profiles generated from identical samples using the HPLC purified 1392r+GC and standard desalt purified 1070f against the standard desalt purified 1070f+GC and 1392r.

Fixed Annealing Temperature and Touchdown PCR

In hopes of optimizing the PCR conditions, a comparative analysis of traditional PCR using a fixed annealing temperature and TD-PCR using varied annealing temperatures was conducted on samples collected from the columns. Because the initial concentration of nucleic acid extract from the tailing samples was low, TD_PCR was expected to offer advantages over traditional PCR in minimizing the amplification of nonspecific template DNA. Initially, optimal conditions were determined for each of the PCR trials by varying the fixed annealing temperature, as well as the TD-PCR range and 'touchdown' annealing temperatures. Initial conditions were based on the T_m of the oligonucleotides provided by the manufacturer (IDT). Optimal conditions were identified as those yielding the brightest and cleanest amplified product as viewed on a 1.5% agarose gel stained with ethidium bromide. To further analyze optimization of PCR and TD-

PCR conditions, the amplified products were compared using DGGE. The final, "optimal" conditions were based on the sharpness, definition and diversity of bands in the genetic fingerprint.

Template DNA obtained from sample extraction was amplified in two separate reactions. Samples were initially amplified in 25 ul volumes using 1 ul of extracted DNA template. Extract was amplified over 25 cycles at a range of fixed annealing temperatures. Annealing temperatures started at the manufacturer provided T_m and increased by 2^0C. The annealing temperature yielding the best product, as determined by visualization on 1.5% agarose gels stained with ethidium bromide, was that used in the first round of PCR. The determined optimum annealing temperature varied depending on the primer set used. For primers 1070f (5'-ATGGCTGTCGTCAGCT-3') and 1392r (5'-ACGGGCGGTGTG TAC-3') the amplification sequence consisted of 5 min at 94^0C, 25 cycles of 45 s at 94^0C, 45 s at an annealing temperature of 60^0C, and 1 min at 72^0C, finishing with 5 min at 72^0C. For primers 341f (5'-CCTACGGGAGGCAGCAG-3') and 1392r the amplification sequence consisted of 10 min at 94^0C, 25 cycles of 1 min at 94^0C, 45 s at an annealing temperature of 62^0C, and 2 min at 72^0C, finishing with 10 min at 72^0C. Five microliters of Taq-&GO Mastermix 5 x C (Q-BIO Gene) was added per 25 ul of reaction mixture. The second round of PCR was done using the same primers with a GC-clamp attached to the 5' end of the 1392r primer. Touchdown PCR (TD-PCR) was employed in the second round of amplification to decrease mispriming and nonrepresentative product. For the

primer set 1070f-1392r+GC the optimal amplification sequence consisted of 5 min at 94^{0}C, 20 cycles of 45 s at 94^{0}C, 45 s starting at 68^{0}C and decreasing by 0.5^{0}C/cycle, and 1 min at 72^{0}C, plus 10 cycles of 45 s at 94^{0}C, 45 s at 58^{0}C, and 1 min at 72^{0}C, finishing with 5 min at 72^{0}C. The optimal TD-PCR amplification sequence for primers 341f-1392r+GC consisted of 10 min at 94^{0}C, 20 cycles of 1 min at 94^{0}C, 45 s starting at 70^{0}C and decreasing by 0.5^{0}C/cycle, and 2min at 72^{0}C, plus 10 cycles of 1 min at 94^{0}C, 45 s at 60^{0}C, and 2 min at 72^{0}C, finishing with 10 min at 72^{0}C. The second PCR sequence was done using a final reaction volume of 25 ul to which a 1 ul aliquot of the previous PCR product was added as template. Five microliters of Taq-&GO Mastermix was added per 25 ul of reaction mixture. Negative control reactions were carried out in both the first and second rounds of PCR, with the first negative control being treated as a sample in the second PCR. All reactions were carried out using a Mastercycler epGradient Thermal Cycler (Eppendorf). Samples were immediately frozen (-20^0 C) after PCR was complete. Amplified product was checked against low mass ladders or 100bp DNA ladders (Promega) on a 1.5 % agarose gel stained with ethidium bromide. Stained bands were visualized using the FluorChem 8800 Imaging System and AlphaEaseFC software (Alpha Innotech). Contrast, brightness, and in some cases gray scale inversion, were the only modifications done to the images using Adobe Photoshop Elements 2.0.

Primer Purification

In an attempt to identify the effectiveness of HPLC purification of primers containing the GC-clamp on community profiles, a side-by-side comparison of primers was conducted. Initial tests were carried out on post-treatment column communities from varying depths. Each of the community profiles was run on individual gels, so a side-by-side comparison of specific bands was difficult. However, the overall banding pattern, number of bands, band intensity, and background noise (i.e. smearing) could be compared. Samples were initially amplified using the 1070f and 1392r 16S rDNA primers with a fixed annealing temperature PCR (Appendix A). Amplified template from the initial reaction was then used for each of the two subsequent PCR reactions. Amplification conditions were the same as the previous amplification, with the exception of varied primers: 1) 1070f+GC - 1392r purified using a standard desalt (SD) protocol and 2) 1070f – 1392r+GC with HPLC purification applied to the 1392r+GC primer and a standard desalt applied to the 1070f primer. The same experiment was done for a side-by-side comparison of newly extracted tailings from the anaerobic zone (30” – 36”). Amplification conditions and primers were identical to those stated above.

DGGE

DGGE was performed at 60^0 C with a D-Code Universal Mutation Detection System (Bio-Rad Laboratories). Initially six and eight percent (w/v) acrylamide gels with denaturant gradients of 40 to 70% (Muyzer et al., 1996) were used for analyzing fragments amplified using 341fGC – 907r and 341f – 1392rGC, and 1070f – 1392rGC respectively. However, based on observed results from 341f-1392r an 8 to 12% acrylamide gradient (Girvan et al., 2003), in conjunction with the 40 to 70% denaturant gradient, was also used. A total volume of 25 ml was used to pour the gels, which were allowed to polymerize prior to pouring a 0% denaturant stacking gel for the loading wells. Electrophoresis was performed for 16 hours at 60 V. Gels were subsequently stained with SYBR Green I (Cambrex Bio Science) and gel images were obtained using a FluorChem 8800 Imaging system and AlphaEase FC software (Alpha Innotech). Major bands were excised from the gel using a razor blade. DNA was extracted from the polyacrylamide gel slices using the QIAEX II Gel Extraction Kit (Qiagen) and the protocol provided. Eluted DNA was reamplified, visualized, prepared for sequencing, and sequenced. Contents and conditions for making DGGE reagents are presented in Appendix B.

Sequencing preparation was performed as suggested by Mary Bateson (personal communication), whom performed the automated sequencing of excised bands. Positive PCR product of eluted DNA was reamplified using BigDye version 3.1 (ABI) and 5X buffer. The sequencing reaction set up

included 4 ul BigDye, 2ul 5X buffer, 5 pmole of one primer, DNA (~10 ng) and sterile water to a final volume of 20 ul. Reactions were carried under the following conditions: 96^0 C for 1 min, 25 cycles of: 96^0 C for 10 sec, 50^0 C for 5 sec, 60^0 C for 4 min; and hold at 4^0 C. Product was then purified to remove unincorported dyes using an ethanol/EDTA precipitation method. To the 20 ul reaction volume 5 ul of 125 mM EDTA was added making sure the EDTA reached the bottom of the reaction tube. Sixty microliters of 100% ethanol was then added to the tubes, which were then capped and mixed by inverting. Samples were left at room temperature for 15 minutes prior to centrifugation at 2250 rfu for 30 minutes. Tubes were immediately removed, uncapped and inverted onto a paper towel to remove supernatant. Additional supernatant was removed by brief centrifugation at 185 rfu. Remaining pellets were rinsed by adding 70 ul of 70 % ethanol to each tube. Tubes were then centrifuged for 15 minutes at 1650 rfu. Supernatant was removed as previously mentioned with centrifugation occurring for 1 minute. Purified samples were sequenced by Mary Bateson at Montana State University - Bozeman using an ABI Prism 310 Genetic Analyzer.

Phylogenies of predominant populations were identified based on the success of matching resulting sequences to phylogenetically known sequences stored in the NCBI-GenBank database using the Basic Local Alignment Search Tool (BLAST), publicly available on the web (http://www.ncbi.nlm.nih.gov/BLAST/). Nucleotide-nucleotide searches were conducted using the standard blastn

program, using an 11-base contiguous word to initiate extensions. “Expect values”, indicating the validity of the match, were used to determine the likelihood of a real match over that of a chance match. The smaller the expect value, the more likely that the match is phylogenetically correct.

Results

Summary

The results provided in this expansive analysis of optimizing molecular methods to obtain representative community profiles clearly indicate the inefficiencies of standardized methods. The establishment of an effective and repeatable extraction method proved very difficult, with sample conditions inhibiting essential steps in the isolation and purification extracted nucleic acids. Primer selection, primer purification, PCR, and DGGE conditions each had a significant effect on the resulting community profile. Combining the many potential possibilities in each of these steps to determine the most effective protocols and representative community profiles requires an exhaustive matrix of analytical tests. The following sections provide results yielded from a sequential analysis of the many possible conditions in each of the listed categories. This was done to identify the best performing methods in each of these categories, which in combination would provide the most representative profile of the

microbial community sampled. Some of these results contradict established conditions set forth by others in published literature.

DNA Extraction and Purification

Extraction Method 1 (EM1) initially yielded very little extract. This can be attributed to two possible conditions; (i) cell lysis was insufficient and for (ii) extract was bound to contaminants that were precipitated and discarded during the purification process. To further test EM1, extraction of DNA was attempted two more times, proving poor repeatability and little success at nucleic acid extraction (Figure 3). To increase nucleic acid yield, EM2 was developed to include an additional freeze-thaw cell lysis step. Freeze-thaw methods of cell lysis have had mixed reviews in the literature, primarily due to the fact that it is a gentle method of cell membrane destruction that minimizes shearing of nucleic acids and yet provides a moderate amount of extract. The extract from EM2 was considerably better then EM1, but the amount of extract was still quite low.

Extraction method three (EM3) yielded more DNA, of significant size and quality, when compared to EM2 (Figure 4). Initial extract from TC2 using EM3 was practically nonexistent. Extract from TCC, the sample containing a presumably lower concentration of cell mass since it was not fed organic carbon, yielded a large amount of both DNA and RNA extract. Notice also in Figure 4 that a large amount of sheared extract resulted from EM3 of the TC1 sample. Inhibition of PCR amplification occurred in all sampled extracts obtained from

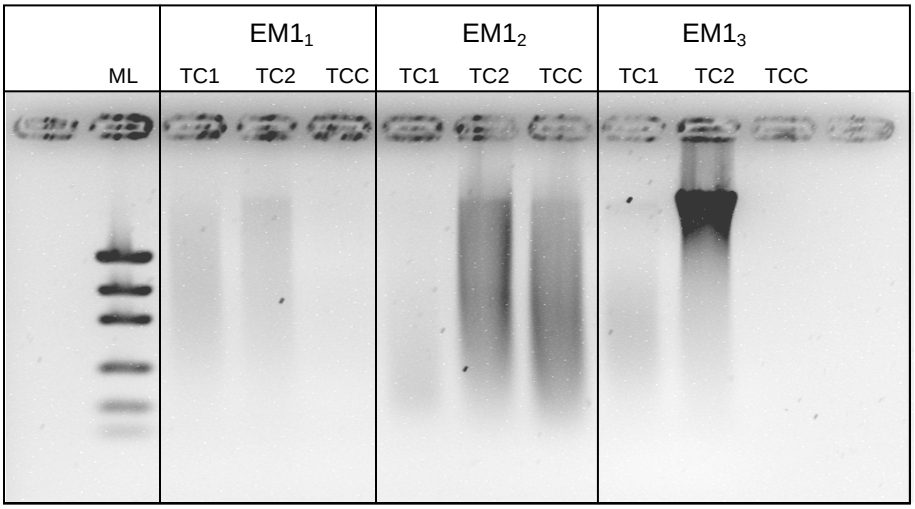


Figure 3 Comparison of Extraction Method 1 conducted in triplicate (subscripts). Extract from the three samples in each trial of EM1 compared on 1.5 % agarose gel with low mass ladder (ML). Samples collected from treated (TC1 and TC2) and untreated (TCC) tailing columns.

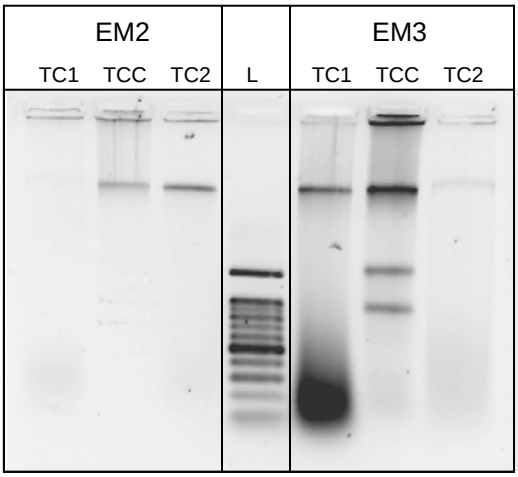


Figure 4 Comparison of Extraction Method 2 and Extraction Method 3. Extract from EM2 and EM3, respectively with 100 bp DNA ladder (L). Samples collected from treated (TC1 and TC2) and untreated (TCC) tailing columns.

EM1, EM2, and EM3 (data not shown). It is believed that coextraction of contaminants was to blame for PCR failure based on the obvious presence of DNA extract and the successful amplification of a positive control.

EM4 was then tested on TC1, TC2 and TCC and amplified using standard PCR conditions with a fixed annealing temperature (Appendix A) in an attempt to obtain a greater amount of extract. Unfortunately, sample contamination resulted in the inhibition of template amplification (data not shown). Purification of extract was attempted using the silica-binding matrix and guanidine thiocyanate wash provided by QBIOgene. The resulting samples were reamplified under the same conditions yielding amplified product of only the TCC extract. Figure 5 represents raw nucleic acid extract using EM4, guanidine thiocyanate purification product and PCR amplified template. Each column sample was mixed to decrease possible heterogeneities in cell distribution prior to each extraction, thus decreasing the possibility of obtaining differences in cell concentration in any subsample used for extraction. However, extract from each of the previously tested methods yielded variations in extract concentration from the three subsamples (TC1, TC2, and TCC), suggesting poor repeatability and efficacy of the extraction methods.

Successful extraction and purification of DNA from tailing samples was highly variable when EM5 was employed, with the majority of extractions being unsuccessful. Due to several factors, extract could not be visualized on an agarose gel. This could be attributed to low population density in the samples and that this method uses a minimal amount of sample (0.5 g) for extraction. To check for purified extract, PCR amplification of template DNA was conducted to determine the effectiveness of EM5. Amplification of extract yielded few results

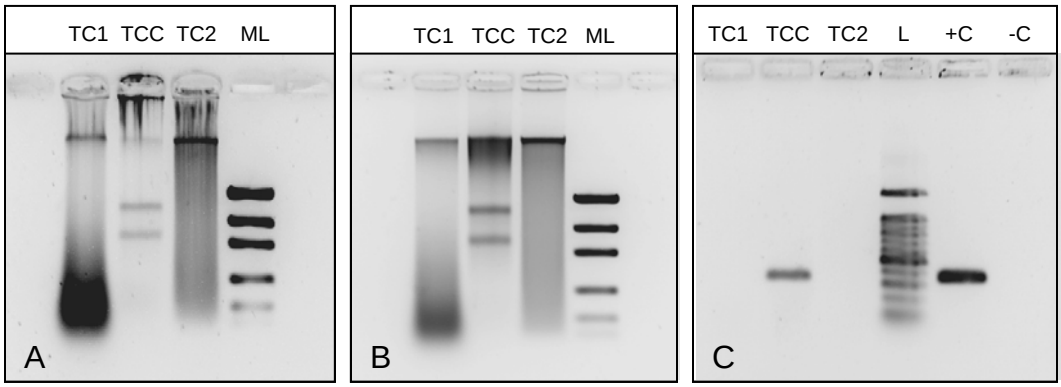


Figure 5 Evaluation of Extraction Method 4 and guanidine thiocyanate purification. EM4 extract (A), guanidine thiocyanate purification product (B), and PCR product (C) with positive (+) and negative (-) controls. Size and concentration were evaluated using a low mass ladder (ML) and a 100 bp DNA ladder (L). Samples collected from treated (TC1 and TC2) and untreated (TCC) tailing columns. Negative (-C) and positive (+C) PCR controls were run to identify contaminant DNA and successful template amplification, respectively.

when using optimum fixed annealing temperature PCR for primers 1070f-1392r (Appendix A). After multiple attempts, repeatability and universal success of this method could not be achieved (data not shown).

Based on the fact that the only samples that could be successfully extracted, time and again, were those from the 36 inch depth in TC1 and TC2 where conditions were less hostile (e.g. higher pH, less free iron and other metals), it was apparent that although the extraction method might be effective at lysing cells, it clearly failed at isolating and purifying extracted DNA. The efficiency of DNA binding to the silica matrix is dependent on maintaining a buffer pH below 7. To test the potential affects of sample pH on binding matrix efficacy, extraction was carried out on samples collected from TCC and TC2 at varying depths. During the purification step, pH of silica/guanine thiocyanate buffer was monitored. Previous extractions not having initial wash steps maintained a pH

value between 5 and 6, whereas those having initial wash steps had a pH value of approximately 6.5. The initial wash steps had neither deleterious nor beneficial affects on the binding efficiency of the silica, with respect to buffer pH. The success of binding could, however, be affected by dissolved metals (i.e. iron) not removed in previous steps. Extracted DNA can bind to metals and other inhibitory coextractants, greatly reducing the concentration of DNA that is free to bind to the silica matrix. Bound DNA could potentially be lost during rinsing of the silica matrix, or be eluted along with clean DNA into the final sample, leading to the inhibition of PCR amplification. A lower sample pH could also potentially have negative affects on the extraction buffer and protein precipitation steps prior to purification. An initial wash treatment, such as that proposed by Bond (2000), using an acidic buffer to remove free and weakly bound iron, and a basic wash buffer to raise pH could potentially yield a sample that was clean enough for extraction. The acid wash (EM6) and Bond wash (EM7) treatments were based on this premise (Figure 6).

Further practice and refining of Methods EM6 and EM7, as well as the optimization of PCR conditions and effective primer selection, yielded better results than those presented in Figure 6. Extract and amplified product was obtained from samples collected at the lower stratum (36”) of the tailing columns using EM7 and EM6 (Figure 7). Figure 8 presents is a side-by-side comparison of community profiles of samples collected from various depths within the columns using EM7 and EM6. Profiles of extract amplified under identical

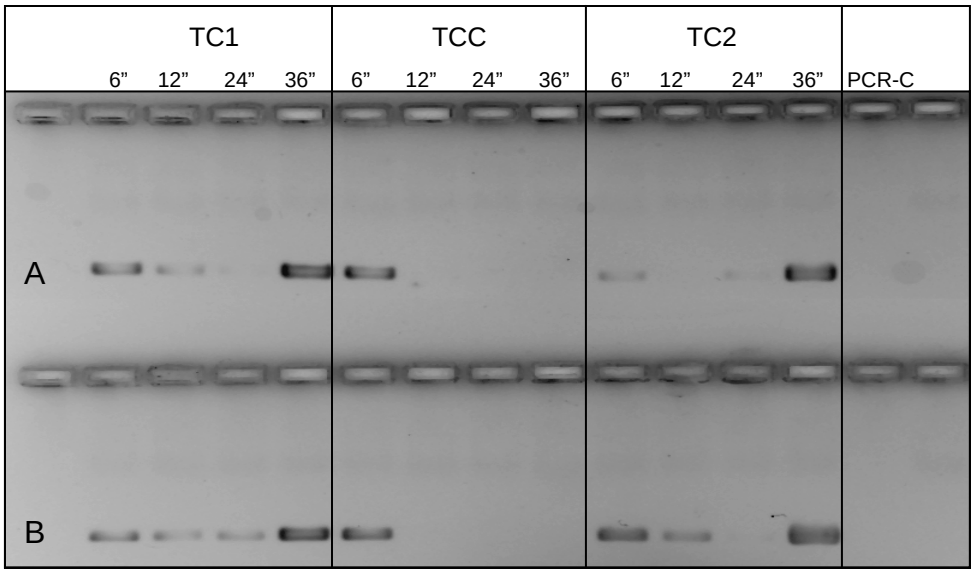


Figure 6 Comparison of amplified template from Extraction Methods 6 (A) and 7 (B). Eluted extract was amplified using 341f-907r. Samples collected at various depths from previously treated (TC1 and TC2) and untreated (TCC) tailing columns. PCR controls (PCR-C) containing no extract template were run to identify contaminant bands.

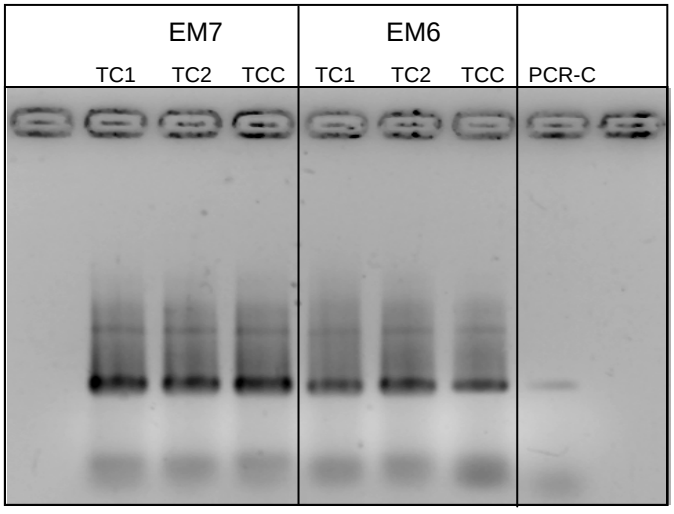


Figure 7 Amplified extract from modified Extraction Method 6 compared with Extraction Method 7. Extract from each method was amplified with 1070f-1392r. Samples collected from treated (TC1 and TC2) and untreated (TCC) tailing columns. PCR controls (PCR-C) containing no extract template were run to identify contaminant bands.

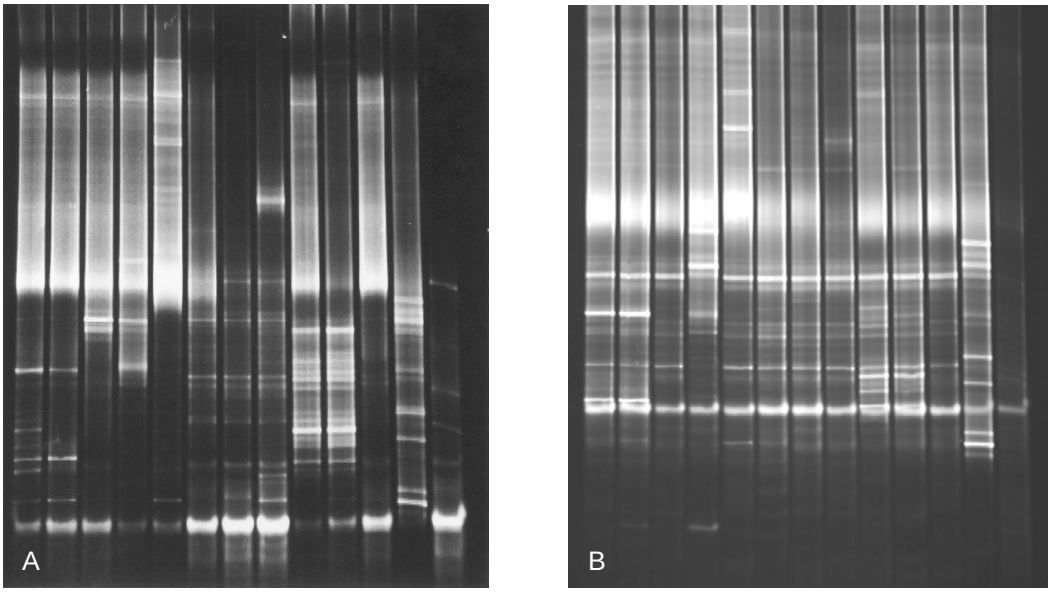


Figure 8 DGGE comparison of Extraction Methods 6 and 7. A) EM6 and B) EM7 yielded relatively similar community profiles when identical samples were amplified with 1070f+GC-1392r under identical PCR conditions.

conditions are quite similar with a few obvious differences in banding pattern.

The profiles in Figure 8 are far from ideal for critical profile comparison, primarily due to primer purification discussed later in this section. However, due to the repeated success, relative similarity of community profiles, and simplicity of application, EM7 was determined to be the best extraction method over the other methods presented here. Three additional extractions of homogenized sample collected from 36 inches in TCC were amplified and run as a side-by-side comparison on a DGGE to test the repeatability of EM7 and the effectiveness of sample homogenization. The extracts were amplified using either an SD or HPLC purified GC-primer along with negative PCR controls (PCR-C). Figure 9 illustrates the repeatability of EM7 and the potential for misidentification of bands that are contaminants of the PCR reaction mixture. Bands present in the PCR-C

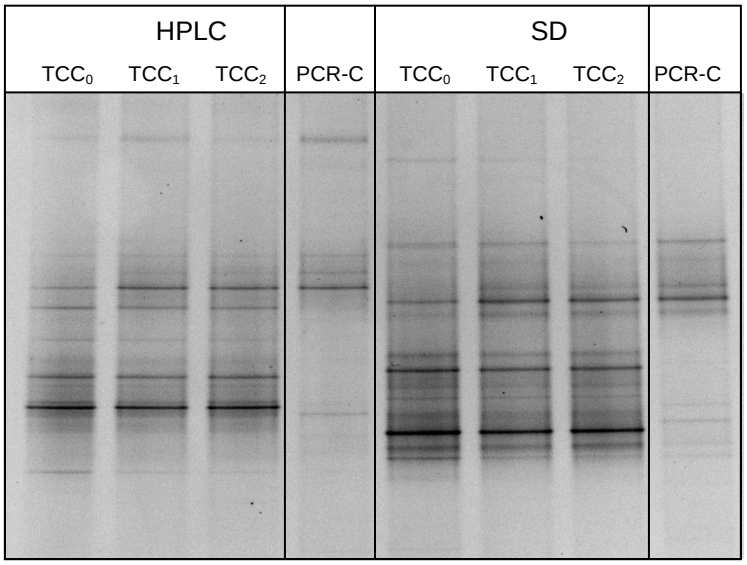


Figure 9 Repeatability of extraction method (EM7) and resulting DGGE community profile. Sample TCC, was extracted in triplicate, amplified under a fixed annealing temperature using HPLC and standard desalt (SD) purified primers (1070-1392), respectively. Samples collected from untreated (TCC) tailing columns. PCR controls (PCR-C) containing no extract template were run to identify contaminant bands. Subscripts denote replicate extraction.

lanes are PCR contaminants also present in all of the amplified extracts. Once those bands are eliminated it is apparent that the extraction method is successful at obtaining an extract that yields repeatable results. The quality of extract and similarity in banding patterns of the three subsamples taken from TCC is evident. There is a slight variation in band intensities of those found in TCC_0, whereas TCC_1 and TCC_2 remain nearly identical. This raises some concern due to the inference of population density relative to others based on variations of band intensity. A shift in intensity might be construed as a selection for or against a particular population, but the fact that the band is present with the intensity it has is supportive of a repeatable extraction and purification method. An explanation

of the differences seen in banding pattern as a result of primer purification will be provided later in this chapter.

Primer Selection and PCR Optimization

Extract amplification was conducted with the three sets of primers previously mentioned, each under a range of reaction conditions. The first primer set, 341f-907r, is by far the most widely reported in the literature when amplifying 16S rRNA coding regions for DGGE analysis. Unfortunately, the resulting product rarely yielded the best community profile with respect to band definition and background smearing. In an attempt to increase primer specificity and reduce background smearing, a small range in touchdown PCR annealing temperatures was tested. A 2^0C increase in the touchdown annealing temperature provided a more defined community profile, with reduced smearing and sharper, well-defined bands (Figure 10). Unfortunately, some bands were reduced in intensity while some were completely excluded from the community profile. This is of no surprise since an increase in annealing temperature raises the selection pressure for exact pairing of primes and template DNA. DGGE profiles shown in Figure 10 were extracted from tailings treated with different carbon sources in 100 ml microcosms.

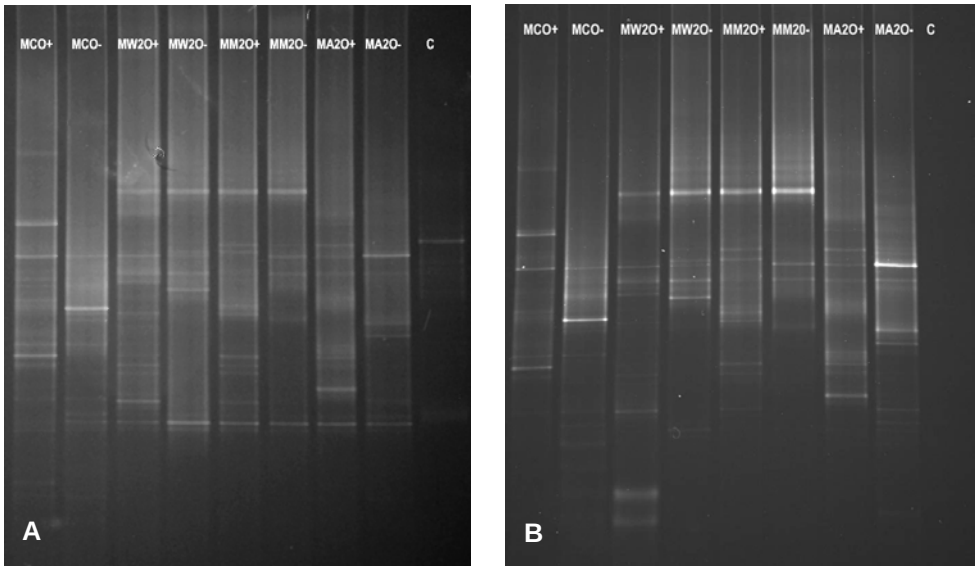


Figure 10 DGGE profiles amplified under differing touchdown PCR conditions. Community DNA from microcosms inoculated with tailings from Mammoth Mine, Montana amplified with 341f-907r under varied touchdown annealing conditions of (A) 64^0C-58^0C and (B) 66^0C-60^0C.

It should be noted that in this test, touchdown PCR varied slightly from that presented in the methods section of this chapter. Initial touchdown PCR tests were conducted with annealing gradient of 6^0C. Later TD-PCR reactions were conducted over a range of 10^0C. It is also important to note that all primers in this pairing (i.e. 341f, 907r, and 907r+GC) were purified with a standard desalt procedure. Unlike 1392r+GC, the 907r+GC primer was not HPLC purified. The method of purification undoubtedly has beneficial results with respect to better banding and reduced background, as will be discussed later.

When compared to the resulting DGGE of samples amplified with 341f-907r, the1070f-1392r primer set yielded far better profiles with increased banding diversity, increased definition and reduced smearing. Figure 11 presents the DGGE comparison of these primer sets. The same sample template that was

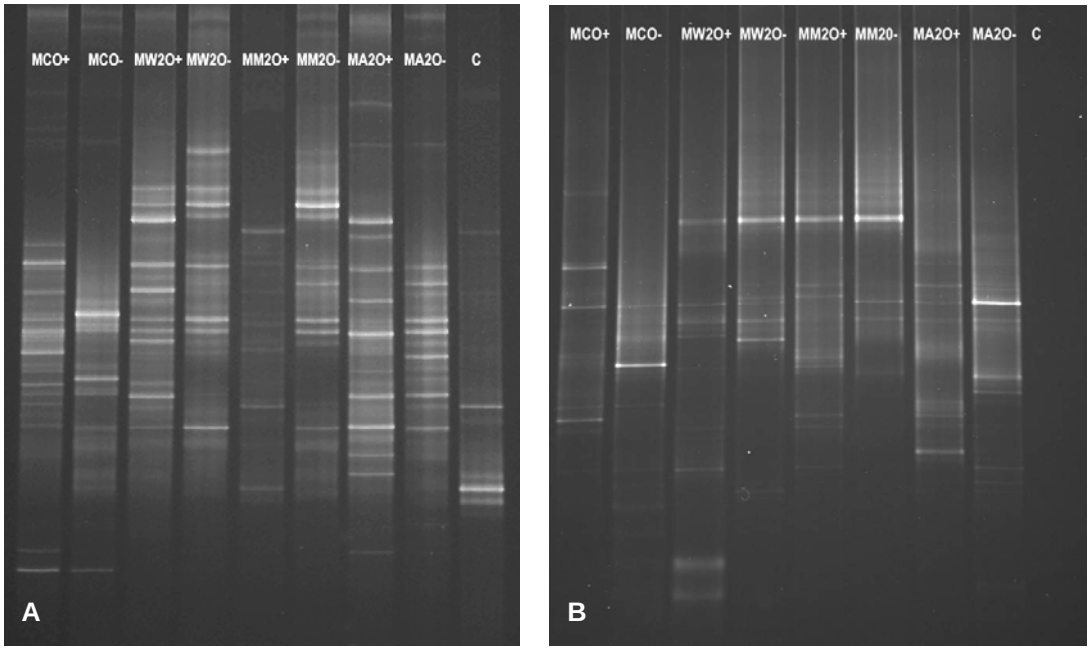


Figure 11 DGGE profiles amplified with different oligonucleotides under optimized PCR conditions. Extract from treated microcosms inoculated with Mammoth Mine tailings, amplified with (A) 1070f-1392r at an annealing temperature of 64^0C-58^0C and (B) 341f-907r at an annealing temperature of 66^0C-60^0C (TD-PCR). All primers were purified with the standard desalt method.

used in Figure 10 was amplified with 1070f+GC-1392r under similar TD-PCR conditions. The resulting banding pattern is far more diverse, with an increase in band number and intensity. The increased band numbers is somewhat contradictory to predicted results based on the regions to which they anneal. Recall the location of these primers in the 16S rRNA structure (Figure 2). Primers 341f, 907r, and 1392r fall in regions of universal variability, where as 1070f is within a region of intermediate variability. Based on this placement of primers it could be assumed that the 341f-907r primer set would be specific to a wider variety of organisms having maintained this region of reduced variability through their evolutionary history. In contrast, 1070f anneals to a region with

increased variability between known species, and should target a narrower range of microbes under stringent PCR conditions. One possible prediction that could explain these unexpected results is that the hypervariable regions amplified with 1070f-1392r have an increased variability over those amplified with the 341f-907r primer set. The only real argument to this explanation is the fact that 341f-907r brackets three hypervariable regions, thus increasing the likelihood of distinct sequence variability over the two hypervariable regions bracketed by 1070f-1392r. However, from these profiles it is apparent that 1070f-1392r amplifies template with increased variability and reduced artifacts or smearing.

The third primer set was used in an attempt to target the six hypervariable regions falling within the 341f and 1392r priming sites. The results were far better than expected considering the previously predicted limitations of DGGE. Separation of individual phylogenies defined by the 1000 bp fragment was successful, though only under specific DGGE conditions, contradictory to those presented in published literature. Conditions and resulting profiles are presented in the following section of DGGE results, as well as Figures 18 and 19. By amplifying fragments containing six hypervariable regions, an increase in distinct sequences (i.e. varied GC content) is observed, as presented by the increased number of bands. It is possible however that the brighter bands observed in the1070f-1392r amplified fragment contain more than one phylogenetically distinct sequence, which is easily determined by the success or failure of sequencing those excised bands.

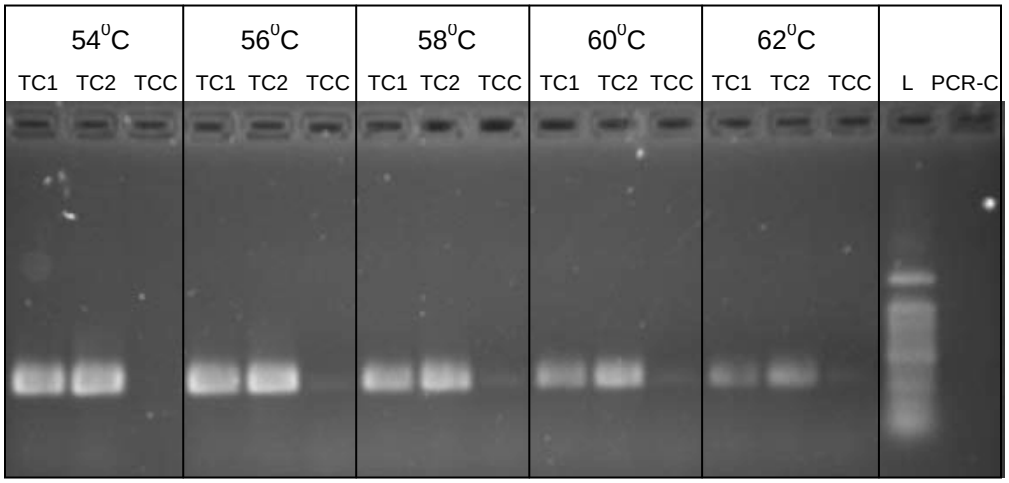


Figure 12 Fixed annealing temperature gradient test on 1070f-1392r primer set. Intensity of bands, and thus concentration of product, gradually decreases as annealing temperature is increased. No additional bands, other than the target length of ~300bp is evident in gel. Product from TCC is very weak, but visible, at all annealing temperatures. Samples collected from treated (TC1 and TC2) and untreated (TCC) tailing columns. PCR controls (PCR-C) containing no extract template were run to identify contaminant bands. 100 bp DNA ladder (L) identifies amplified fragment size.

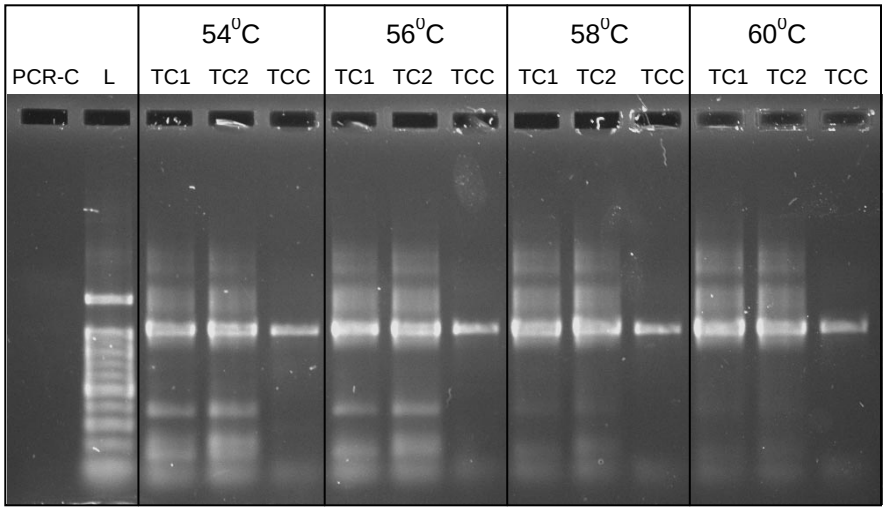


Figure 13 Fixed annealing temperature amplification test with 341f-1392r. Note that the brightest band is just larger than 1000bp and that weak, smaller bands are present at lower annealing temperatures. Increased annealing specificity occurs at higher temperatures, reducing smaller fragments formed from mispriming. Samples collected from treated (TC1 and TC2) and untreated (TCC) tailing columns. PCR controls (PCR-C) containing no extract template were run to identify contaminant bands. 100 bp DNA ladder (L) identifies amplified fragment size.

Optimization of the PCR annealing temperature for each of the primer sets was performed to minimize the formation of non-representative fragments. These fragments were recognized as spurious bands that were of a larger or smaller size than the expected fragment length: ~300 bp (1070f-1392r), ~600 bp (341f-1392r), and ~1000 bp (341f-1392r). Initial tests were done using fixed annealing temperatures over 25 cycles. Images of the resulting PCR products amplified with 1070f-1392r and 341f-1392r are presented in Figure 12 and Figure 13, respectively. Figure 12 presented no spurious bands and minimal smearing. Samples did however reduce in intensity as annealing temperature was increased. This was due to either increased specificity of the primer to target sequences or a reduction in the efficacy of the Taq polymerase at prolonged high temperatures. Figure 13 is a prime example of increased priming specificity resulting from higher annealing temperatures, above the T_m. Temperatures yielding bright product with insignificant spurious bands or smearing were set as the touchdown temperatures for subsequent TD-PCR reactions: 58^0C (1070f-1392r) and 60^0C (341f-1392r).

Primer Purification

Initial tests on primer purification were consistent with the results reported in Villadas et al. (2002). The HPLC purified primer yielded a much cleaner profile with less background staining or blurring of bands (Figure 14). The ratio of well-defined bands to blurred bands was much higher in the HPLC profile than the SD

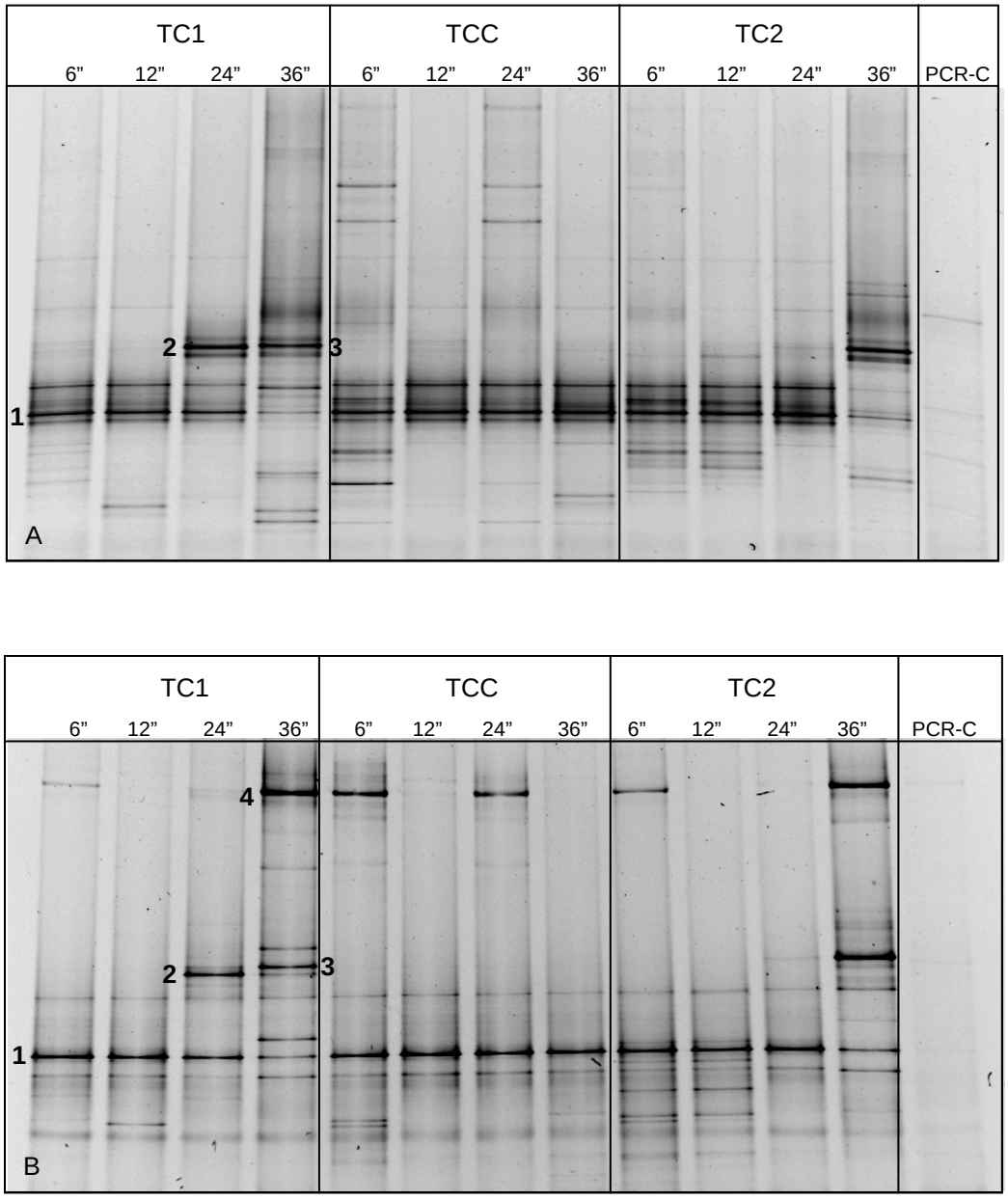


Figure 14 Comparison of (A) 1070f+GC-1392r with standard desalt (SD) purified versus (B) 1070f-1392r+GC with HPLC purification of oligonucleotides using post-treatment community samples. Samples collected at various depths from previously treated (TC1 and TC2) and untreated (TCC) tailing columns. PCR controls (PCR-C) containing no extract template were run to identify contaminant bands.

profile. It is apparent from these results that use of HPLC purified primers greatly reduces background noise and questionable, blurred bands. It should also be noted that the banding pattern was slightly different. Most notable is the change in banding pattern around the lower, more predominant band [1] present in nearly all samples, specifically in samples TC1-36", TCC-6", TC2-6", and TC2-12". Below band [1] in TC1-36" two obvious bands were lost when using the HPLC purified primer. The band that remained just below band [1] was also apparent in all of the samples, but was not present in the PCR control, removing the possibility of the band being a contaminant. Contrary to a loss of bands in TC1-36", TC2-6" and TC2-12" saw an increase in the number of bands below band [1]. Though it is difficult to make a comparison of profiles 14A and 14B without having run the samples side by side, it is apparent that the band number and intensity of the bands in the profile are influenced by the purification of the +GC primer. A good example of this is the sudden presence and varied intensities of band [4] in a number of the samples (Figure 14B). The fact that the band is not present in all of the samples, including the PCR control, eliminates the possibility of contamination.

Further evaluation of Figures 14A and 14B reveals the bands [2] and [3] appear to be slightly shifted from one another representing a small difference in the GC content of the two bands (Figure 14A). In Figure 14B it is apparent that the two bands do in fact have different melting domains, with band [3] being slightly, though obviously higher than band [2]. More important is the fact that band [2] is

present in TC1-36", though at a significantly lighter intensity. Using SD purified primers, the separation in melting domains was so minimal between the two bands that they converged into what appeared to be a single band. This apparent band separation is clarified by the HPLC purified primer profile.

The increase in band separation can possibly be explained by the fact that the GC clamp was placed on opposing primers, inadvertently shifting the progression of the sample through the acrylamide gel and denaturant gradient. Assuming that the DNA fragment had a proportionally larger percentage of guanine and cytosine toward the 1392r priming sequence and the GC-clamp was present on the 1070f primer it would migrate further through the gel before reaching its specific melting domain. Alternatively, if the same conditions were true, but the GC-clamp were attached to the 1392r primer, the 1070f region would denature more rapidly as it progressed through the denaturant gradient, thus increasing the ultimate melting domain of individual samples. Note that the 1070f primer contains a GC value of 56%, where as the 1392r primer contains 67% (Appendix A). Thus, it would be a good prediction that a sample amplified with a primer set having the GC-clamp on the 1070f primer would migrate further into the gel than if the GC-clamp was attached to the 1392r primer. This would in turn delay the effects of the denaturant on the remaining nucleotides in the sample, thus limiting separation of discrete bands. In fact, this was observed when samples were run on the same gel (Figure 16).

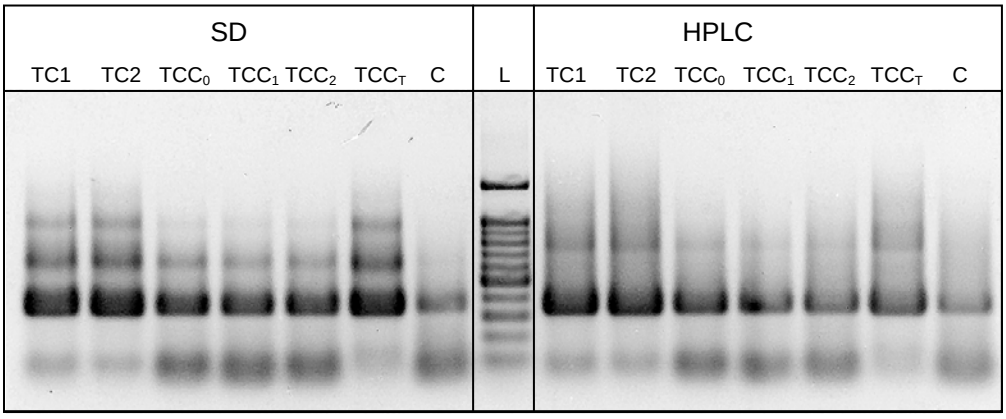


Figure 15 PCR product of primer purification comparison. 1070f+GC-1392r with standard desalt (SD) versus 1070f-1392r+GC with HPLC, using samples collected from anaerobic zone of columns visualized on 1.5% agarose gel. Control (C) and 100 bp ladder (L). Samples collected from previously treated (TC1 and TC2) and untreated (TCC) tailing columns. PCR controls (PCR-C) containing no extract template were run to identify contaminant bands. Subscripts identify replicate extractions.

Samples collected from the lower region (36") of the columns were used to run a side-by-side comparison of the purified primers. Initially, amplified product was run on a 1.5% agarose gel to check the quality, quantity and size of the PCR product (Figure 15). Note that the brightest product or band in all of the samples is of the desired size of approximately 300 base pairs (bp). Of significant interest is the presence of two larger bands (~ 600 bp and ~1000 bp) in the samples amplified with the SD primer. Although they are considerably lower in quantity, determined by their relatively weak intensity, there is the potential that these samples will either appear as discrete bands or cause significant background in the final community profile. In contrast, the samples amplified with the HPLC purified primer are free of these larger bands. There is a slight band appearing

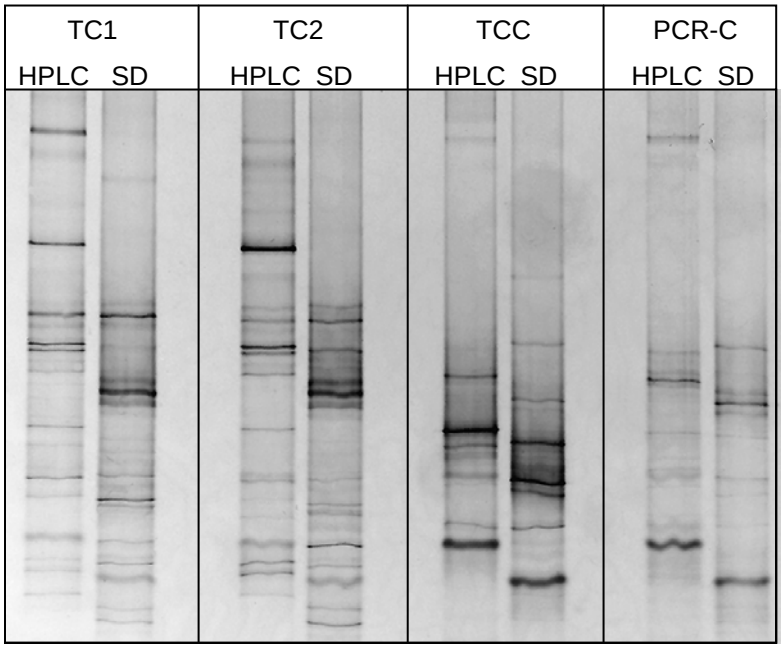


Figure 16 DGGE of primer purification comparison. Anaerobic samples amplified with HPLC purified 1070f-1392r+GC and standard desalt (SD) purified 1070f+GC-1392r. Samples collected from previously treated (TC1 and TC2) and untreated (TCC) tailing columns. PCR controls (PCR-C) containing no extract template were run to identify contaminant bands.

at a size of approximately 750 bp, but the intensity is so weak that the threat of PCR artifacts appearing as bands or background is minimal.

The first three PCR products from each of the two treatments shown in Figure 15 were compared on a 40 to 60% denaturant gradient DGGE (Figure 16). Again, in analyzing the gel it is important to check for the presence of bands in the PCR control (PCR-C). Aside from the negative control bands it is evident that the shift in banding pattern does occur depending upon the primer to which the GC-clamp is attached. The 1070f+GC primer does migrate further into the gel than does the 1392r+GC primer, just as predicted. More important is the definition and separation of bands in samples amplified with the HPLC purified

GC-primer. Using negative control bands as a reference of migration, it is apparent that the shift in band migration is minimal, eliminating the possibility that bands merely migrated off the gel or outside of view (a potential problem that is addressed in the following section).

DGGE

The practice of employing DGGE for microbial community analysis is as much an art as it is a science. Small differences gel formulation, mixing, and pouring can have large effects on the resulting profiles. Proven protocols for both PCR and DGGE, specific to samples of interest, must be developed to obtain representative and repeatable profiles containing well-defined bands that will yield clean sequences. One of the first DGGE gels produced in this research is presented in Figure 17. Samples were extracted from various depths within the columns and were amplified using 341f-907r. Conditions for setting up a DGGE were exactly those presented in the methods section of this chapter, but without the use of a stacking gel. The PCR product (not shown) was riddled with misprimed fragments, sheared DNA and PCR contaminants, evident in the resulting DGGE as blurred bands and background smearing. This gel demonstrates the importance of primer selection and PCR optimization.

DGGE is still a relatively new technology, open to manipulation and experimental optimization. It has been shown previously in this chapter that the

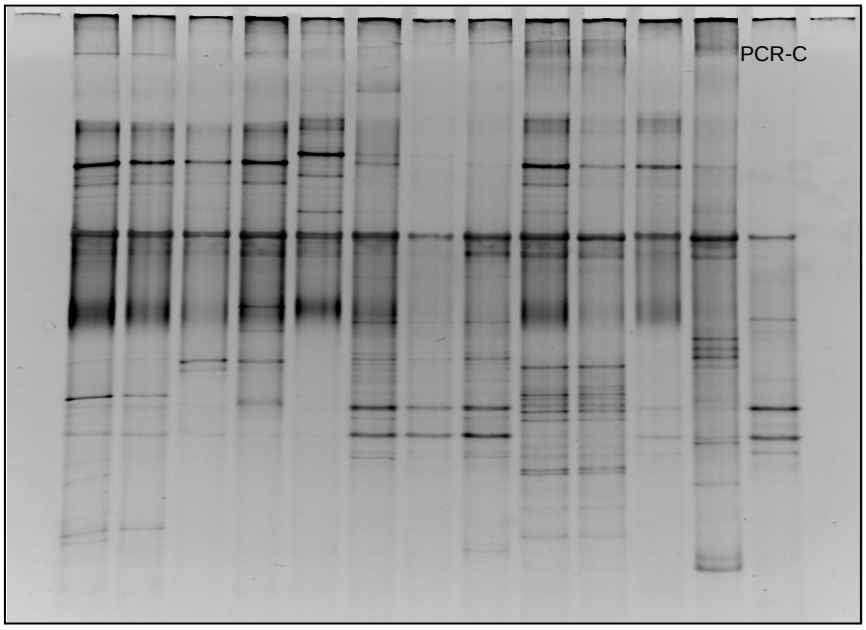


Figure 17 DGGE of direct extract amplified with 341f-907r primers. This is a poor quality gel riddled with artifacts and contaminants.

method of oligonucleotide purification can have a significant effect on the resulting profile of amplified product. Another concern not well stated in the available literature is the fact that the melting domains are not well-defined positions within the acrylamide gel at specific denaturant concentrations. Because a fragment may completely denature at a specific melting domain does not mean that its migration will completely halt in the gel. The GC-clamp merely acts to inhibit complete denaturation of the fragment into single stranded segments. By doing so, a 300 bp fragment will progressively increase in size until it has reached its melting domain, at which point its size will be equivalent to approximately 600 bp. Since the acrylamide provides structure and maintains the properties of a tortuous filter the concentration of acrylamide has a direct effect on the migration rate of fragments. Amplified fragments of a particular size

migrate more slowly in a gel having a greater acrylamide concentration under steady voltage. Fragment size also influences the rate of migation with respect to acrylamide concentration, with larger fragments migrating more slowly than smaller fragments in the same acrylamide gel. As fragments denature their migration through the gel progressively slows to a constant rate, at which point the denatured fragment has reached its melting domain. The fragment will continue to migrate, though at a much slower rate than partially denatured fragments having higher GC content.

The fact that the migration of completely denatured fragments is not completely halted raises concern over the potential loss of shorter fragments containing a higher GC content. It also contests the established protocol first identified by Muyzer (1998) and maintained by manufacturers of DGGE equipment. According to the BIORAD Manual provided with the electrophoretic device used for DGGE, a 6% acrylamide gel should be used for fragment lengths of 300 to 1000 bp, an 8% gel for fragment lengths of 200 to 400 bp, and a 10% gel for fragments of 100 to 300 bp. However, 1000 bp fragments produced a far better, more distinct and inclusive community profile when run on a 10% acrylamide gel compared to those run on the suggested 6% acrylamide gel (Figure 18). This higher concentration provided good band separation and minimized sample loss out of the gel via migration. The 6% acrylamide DGGE had a denaturant gradient of 50 to 70% where as the other two (8 and 10%) had gradients of 40 to 70% denaturant. This explains the higher location of the grouped bands

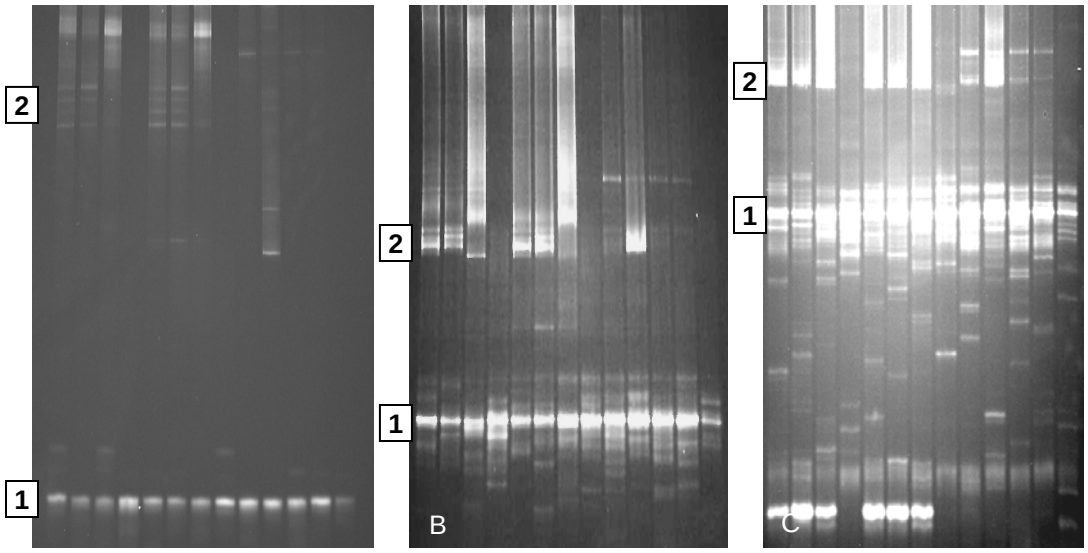


Figure 18 Comparison of DGGE profiles in gels of varied acrylamide concentration. Denaturant gradient gels containing A) 6%, B) 8%, and C) 10% acrylamide. Note the shift in dominant bands, also present in the negative PCR control, as their migration is decreased in the gel under greater acrylamide concentrations, independent of denaturant concentration; A) 50 - 70%, B) and C) 40 – 70%.

identified as [2] in Figure 18. Note also that this separation of grouped bands becomes limited as the acrylamide concentration increases, with the group appearing as a single intense band in the 10% acrylamide gel.

In an attempt to utilize the migration inhibition properties of both the 8 and 10% acrylamide concentrations, gels containing gradients of both denaturant and acrylamide were tested. Fragments amplified with 1070f-1392r+GC and 341f-1392r+GC were run on an acrylamide gradient gel of 8 to 12% with a denaturant gradient of 40 to 70%. Comparisons of the acrylamide gradient gels and fixed acrylamide gels for each of the amplified fragments are presented in Figure 19 and Figure 20.

Longer fragments amplified with 341f-1392r primers benefited little from the acrylamide gradient gel. Overall, bands were sharper and more defined when run on a fixed 10% acrylamide gel, however a few bands (particularly 1 and 2; Figure 2.19B) did appear sharper in the gradient acrylamide gel. Community profiles generated from shorter fragments were considerably different between acrylamide gel concentrations. Phylogentic diversity was greater and bands more defined when the 300 bp fragments were run on the 8 to 12% acrylamide gradient.

Figure 20 yielded results with differences in banding patterns similar to those seen in the primer purification analysis (Figure 16). However, both samples were amplified with the HPLC purified 1392r+GC primer under identical PCR conditions with the only major variation being that of the acrylamide concentration of the DGGE gels. Band separation was significantly better in the 8 to 12% acrylamide gradient gel than those seen in the 8% acrylamide gel, Figure 20B and 20A, respectively. The fact that bands were separating rather than increasing in number is supported by identical PCR conditions and failure in attempts to sequence bands excised from the 8% acrylamide gel. Automated sequencing yielded chromatograms indicative of isolate contamination. Based on the presence of two clean chromatograms out of the twelve sampled, method of sample preparation did not introduce universal contamination. Another, though minor, difference in samples was the age of the 1392r+GC primer used. Samples run on the 8% acrylamide gel were amplified using a primer stock that

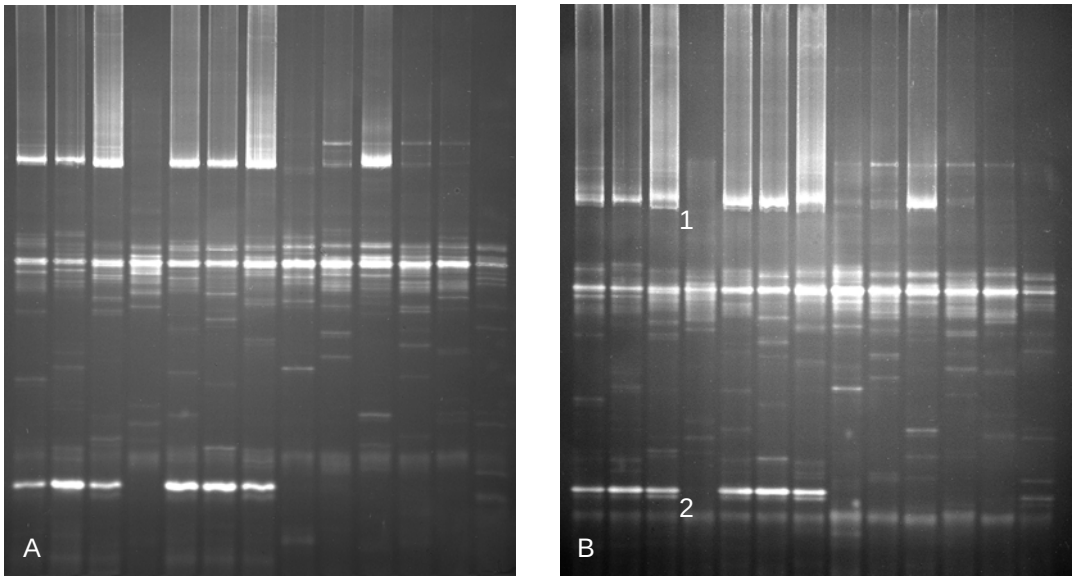


Figure 19 DGGE comparison of 341f-1392r+GC amplified fragments on fixed and gradient acrylamide gels. Identical products run on A) 10% and B) 8 to 12% acrylamide gels with 40 to 70% denaturant gradients.

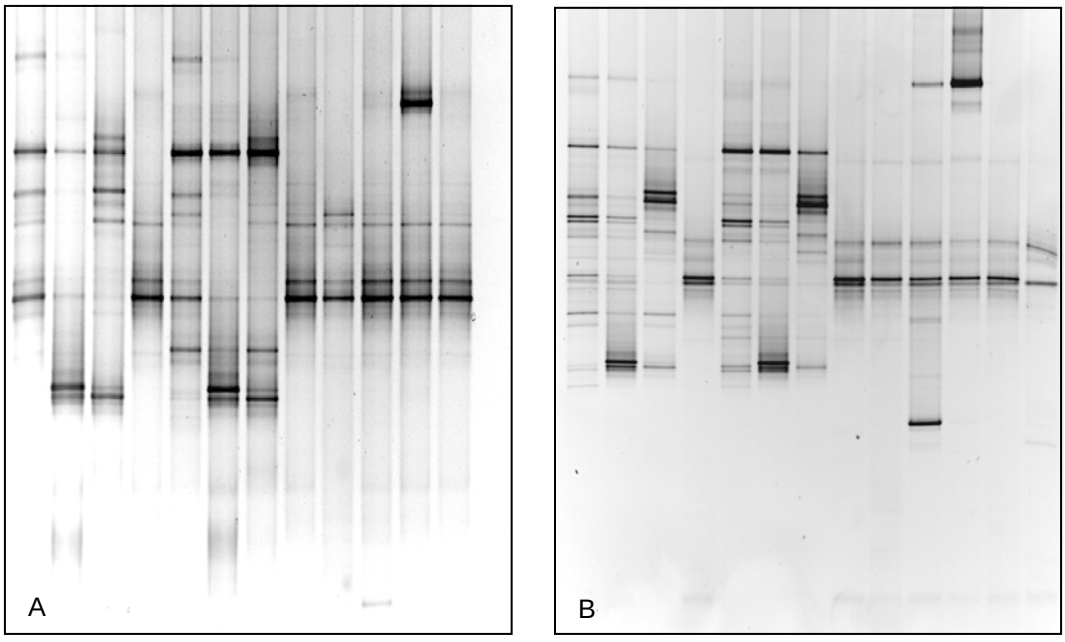


Figure 20 DGGE comparison of 1070f-1392r+GC amplified fragments on fixed and gradient acrylamide gels. Identical products run on A) 8% and B) 8 to 12% acrylamide gels with 40 to 70% denaturant gradients.

was approximately four months old, where as the other sample set was amplified with fresh primer stock received the day prior to amplification. All samples are stored in 20 ul aliquots at -20^0C, greatly reducing the potential for primer degradation. It is difficult to believe that this small variation would yield such diverse results, however it is something that needs to be addressed in a more systematic manner to negate its potential for causing differences in community profiles.

To test the effects of biases introduced by culturing on community profiling, comparisons were made of nucleic acids extracted directly from tailing samples and cultures inoculated with those samples. The first test was a comparison of direct extraction from tailings collected at a depth of 6 inches and populations grown on a medium selective for iron oxidizing bacteria (IOB) (Figure 21A). Banding patterns were almost completely different between samples. It should be noted that these samples were not run with a negative PCR control, so it is not possible to eliminate reaction contaminants. However, it does provide some insight into the structure of predominant populations; specifically that individual IOB populations make up less than 1 to 10% of the total microbial community. Recall that DGGE should detect populations constituting 1% or more of the total microbial population, but that 10% is a far more conservative number. The second test was a comparison of extract from tailings and consortia cultured in serum vials containing Postgate B medium for sulfate reducing bacteria (SRB) (Figure 21B). After eliminating the band present in the PCR-C it is evident that

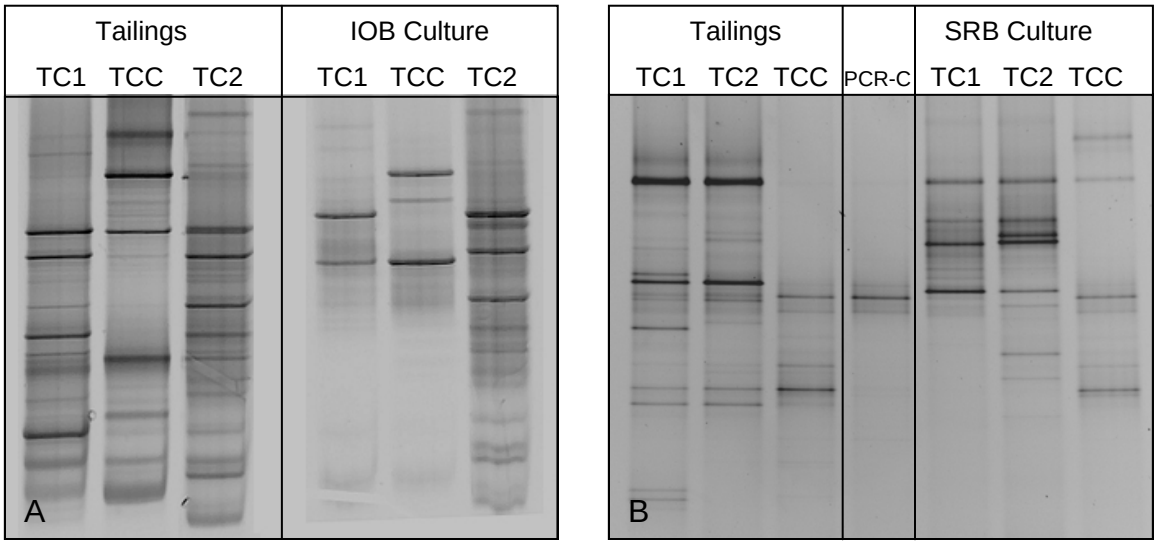


Figure 21 DGGE profile comparison of natural and cultured microbial communities. Microbial communities present in tailing samples and cultures inoculated with tailing samples were compared. Cultures selective for A) iron oxidizing bacteria (IOB) and B) sulfate reducing bacteria (SRB) were inoculated with the tailing samples collected from A) 6 inches and B) 36 inches and run for comparison.

the Postgate medium stimulated the growth of populations both present and absent from community profiles of the tailings inocula. It is apparent that the serum vial treatment leads to selective exclusion of some populations that are evidently out-competed by populations more suited to the experimental conditions. Culturing conditions specific for iron-oxidizing bacteria result in higher selection pressures for populations that are not apparent in tailings inocula, than those observed in SRB specific cultures.

Discussion

Though appearing exhaustive in nature, this experimental analysis did not attempt to produce a matrix of experimental conditions necessary to truly identify the best protocol for obtaining a representative community profile. Identifying a repeatable and efficient method of nucleic acid extraction was the greatest obstacle in this methods development. The two successful extraction methods, EM6 and EM7, provided consistently similar results. EM7 was determined to be the best choice due to the reduction in steps, and thus a reduction in potential for error or bias in the resulting extract, and the clarity of the DGGE profile. The three primer sets chosen in this analysis and their respective PCR conditions yielded varied results. Clean profiles and the potential information that could be gleaned from samples amplified with 341f-1392r and 1070f-1392r determined their use in further analyses. Additional testing could have also been done with 341f-907r, including HPLC purification of the 907r+GC primer and PCR annealing conditions. Due to a lack of time and an interest in the potential success of bracketing six hypervariable regions with 341f-1392r, as well as the exceptional results provided by 1070f-1392r, the highly accepted primer set was abandoned in subsequent testing. Successful implementation of any molecular method is highly dependent on the concurrent use of multiple techniques and tools, including the selective conditions introduced with a suite of primers. As such, future research would benefit from the determination and implementation of optimal conditions to successfully employ the 341f-907r primer set.

Of significant interest was the effect of acrylamide concentration and primer purification on DGGE profiles. Contrary to popular belief, gels containing high acrylamide concentrations (10%) and acrylamide gradients (8 to 12%) yielded superior DGGE profiles from both short (300 bp) and long (1000 bp) fragments. The bulk of the literature, including manuals provided by the manufacturer of the D-Code system suggest an 8% gel for most fragments, and a 6% gel for fragments as large as 1000 bp. Results obtained in this analysis contradicted these established protocols for DGGE-based community profiling. Primers containing the GC clamp should always be purified under the most stringent conditions, such as high-performance liquid chromatography. Though this may appear a logical choice, it is rarely identified as having been used in the published literature.

Some applied methods, such as PCR amplification and acrylamide concentration, can be optimized and established as standard protocols for given parameters (i.e. specific HPLC purified primer sets) and be used in the analysis of any environmental sample. Other methodologies, such as extraction, purification, and primer selection, can be highly dependent upon the characteristics of the environmental sample to be analyzed. In the case of mine tailings, pre-extraction wash treatments and quanidine thiocyanate purification provided the best results. Because of this, standard protocols pertaining to each of these methods must be met with caution and be tested for their individual success on the sample of interest.

CHAPTER 3

KINETICS AND MICROBIAL COMMUNITY ANALYSIS OF PYRITIC MINE TAILINGS

Background

Prior Research

At the birth of the industrial age mining for precious metals grew exponentially throughout the world. Mechanical and chemical methods of metal extraction and isolation have generated mountains of processed waste ores, often stockpiled with complete disregard of the environmental impact. Decades of this activity has lead to millions of cubic feet of mine tailings; deposits of processed ore containing trace amounts of metals and often large amounts of sulfide minerals commonly associated with precious ores like gold, copper and silver. Over time these mine tailings have generated volumes of acidic effluent, termed acid rock drainage (ARD), through geochemical and biochemical processes, decimating fragile riparian ecosystems. This ARD can have a pH value of near 1 and often contains high concentrations of dissolved metals.

Remediation of ARD generated from abandoned and active repositories remains a significant challenge. Many methods of remediation and mitigation have been proposed and employed, both experimentally and industrially. For the

sake of clarity, future reference to remediation and mitigation will refer to ex situ and in situ treatment of ARD generating mine tailings, respectively. A potential alternative technique for the remediation and mitigation of ARD is the stimulated activity of sulfate-reducing bacteria. Under anaerobic conditions sulfate-reducing bacteria (SRB) oxidize simple organic compounds by utilizing sulfate as an electron acceptor. This results in the production of hydrogen sulfide (H_2S) and bicarbonate ions (Equation. 3)

$$3SO_4^{2-} + 2CH_3CH(OH)COOH \rightarrow 3H_2S + 6HCO_3^- \quad \textbf{(3)}$$

The buffering capacity of the bicarbonate ions produced during the SRB reaction stabilizes solution pH and can cause some metal ions to precipitate as insoluble hydroxides. The H_2S can also react with heavy metal ions to form insoluble metal sulfides. The increase in pH and buffering capacity, as well as metal sulfide formation, develops an environment favoring SRB growth. SRB activity in mine tailings is often limited by organic carbon availability. Sulfate concentrations are often high, the result of geochemical and biologically mediated oxidation of sulfide minerals (Equations 4 and 5).

$$FeS_{2(s)} + \tfrac{7}{2}O_{2(aq)} + H_2O_{(l)} \rightarrow Fe^{2+}{}_{(aq)} + 2SO_4^{2-}{}_{(aq)} + 2H^+{}_{(aq)} \quad \textbf{(4)}$$

$$FeS_{2(s)} + 14Fe^{3+}(aq) + 8H_2O_{(l)} \rightarrow 15Fe^{2+}{}_{(aq)} + 2SO_4^{2-}{}_{(aq)} + 16H^{+}{}_{(aq)} \quad \textbf{(5)}$$

Reaction 5 generates 16 moles of protons per mole of pyrite oxidized, acidifying effluent pH and mobilizing heavy metals from tailings and waste rock. Under saturated conditions the oxidation reaction is limited by the low diffusion coefficient of oxygen in water. This often leads to anoxic zones at depth. It is within this anoxic region of the tailings that SRB activity occurs.

To date, a number of studies have used SRB for the ex situ treatment of ARD. Treatment schemes vary widely, employing packed bed reactors filled with sand and crushed stone (Jong and Parry, 2003; Foucher et al., 2001) or solid waste materials (Chang et al., 2000), membrane bioreactors (Tabak and Govind, 2003), reactive walls or biobarriers (Cocos et al., 2002), and constructed wetlands. All of these systems treat ARD after it has left the tailings pile. These methods often require additional area for ARD detention and treatment structures, extensive monitoring and control expenses, as well as the cost of the organic carbon source.

A more economical method, requiring far less process design, is the in situ treatment of the tailings themselves prior to ARD discharge. Few studies have been done to test this potential method of mitigation, however. One such method of ARD mitigation incorporates woodchip or pulp waste into the tailings pile (Hulshof et al., 2003). Due to the chemical nature of the treatment source, sulfate reduction rates rapidly drop as the readily degradable organic carbon is

consumed, leaving more complex materials not readily consumed by SRB. In addition, this method requires repeated mixing of fresh organic carbon sources into tailings. Another proposed method of in situ ARD mitigation is the addition of a dissolved carbon source to the surface of the tailings (Kim et al., 1999; Sturman, 2004). The organic carbon treatment is allowed to percolate through the tailings to the saturated anoxic zone stimulating SRB growth and sulfate reduction. This method lends itself to a simple, repeatable application of a potentially low cost organic carbon treatment. The efficacy of this method is dependent on the amount and type of organic carbon, retention time of the solute, and the microbial populations constituting endemic aerobic and anaerobic consortia.

In oligotrophic mine tailings, naturally occurring SRB depend on the activity of other organisms to provide them with simple organics necessary for their metabolism. Prior to 1977, a limited number of compounds were considered to be suitable energy substrates for SRB; including hydrogen, lactate, formate, pyruvate, malate, and glycerol. Likewise, only a limited number of genera of sulfate-reducers had been characterized. More recent studies under carefully controlled culturing conditions have uncovered the metabolic diversity of SRB, defining a diverse ecophysiological group.

Based on the known metabolic requirements of SRB, Kim et al. (1999) treated laboratory columns with a treatment medium containing lactate. The idea was to provide a source of organic carbon that is widely known as a substrate for SRB

activity. Sturman (2004), however, treated laboratory columns with whey, an inexpensive product of the dairy industry. When adding a complex carbon source, such as whey, the resulting SRB populations are dependent on the products of hetertrophic metabolism. The direct addition of a simple carbon source, such as lactate or methanol, might prove successful in targeting a specific consortium of SRB and their resulting activity, but such refined carbon sources are expensive and limit the potential activity of other, metabolically distinct SRB. The addition of whey can utilize the metabolic processes of hetertrophs and fungi, endemic to the tailings, to degrade the whey into a variety of products more readily metabolized by a wide array of SRB. An added benefit to the addition of a relatively complex organic carbon source is that it has the potential of raising the anoxic zone within the tailings. The biological oxygen demand of the treatment addition would exceed the oxygen available in the tailings, exhausting the oxygen supply higher in the tailings than would occur under normal conditions. The remaining organic compounds can then be utilized by the SRB for growth and sulfate reduction.

The survival and activity of sulfate reducing bacteria is well documented in tailings and ARD. Fortin et al. (1996, 1997) conducted landmark research on the role of iron oxidizing and sulfate-reducing bacteria in acidic mine tailings. A framework for biochemical cycling, physiological activity, and survival mechanisms of bacteria inhabiting ARD generating mine tailings was established. Utgikar et al. (2002) have also addressed the survival tactics

employed by SRB in ARD and the potential for activity inhibition. Sulfate reduction rates of SRB have also been investigated to identify potential activity of SRB in bioremediation (Cocos et al., 2002; Christensen et al., 1996; Elliot et al., 1998; Jong and Parry, 2003; Foucher et al., 2001; Tabak and Govind, 2003) and mitigation of ARD (Blekinsopp et al., 1992; Hulsof et al., 2003; Kim et al., 1999; Sturman 2004). Of these studies only Sturman (2004) monitored endemic SRB rates in response to soluble organic carbon treatment of tailings. Sturman's is also the only study to have employed molecular techniques to identify community structure and dynamics within tailings prior to and after treatment application.

To date, every published study pertaining to SRB diversity in mine tailings has relied on cultivation and isolation techniques. Though these techniques are the foundation on which all microbiological systematics is based, their extreme selective bias, a shortcoming also identified by this research, has been exposed by the recently popular technique of genetic sequencing. Environmental samples can now be screened to identify nearly all endemic microbes present and active in nearly any habitat, independent of culturing. These genetic fingerprinting and cloning techniques have been used for the identification of SRB diversity in a number of environmental samples (Korestsky et al., 2003; Fortin et al., 2000; Teske et al., 1998; Santegoeds et al., 1999; Sahm et al., 1999; Cifuentes et al., 2003), including organisms present in AMD of abandoned mine shafts (Bond et al., 2000a; Bond and Banfield, 2001; Takai et al., 2001; Baker et al., 2003; Edwards et al.,1999;Leveille et al., 2001). Current literature is void of any

research pertaining to the identification of anaerobic community structure within tailings via molecular methods. Nor is there any published research that has attempted to correlate anaerobic community structure with SRB activity, specifically sulfate reduction rates, in response to organic carbon treatment.

Integration of molecular biology and microbial ecology has assisted the bioremediation of highly recalcitrant compounds, where distinct organisms are employed to safely remove and transform environmental contaminants (Stapleton et al., 1998). Under some circumstances, for bioremediation to reach full potential, it is necessary to determine the activity of specific microbial populations. Molecular methods can be used to ensure that the intrinsic community necessary for degradation is present (Romantschuk et al., 2000). After treatment, impact analysis, via molecular and metabolic profiling can be employed to detect shifts in community structure and function. These methods also have the potential to correlate results of pilot to the successful implementation of large-scale, commercial applications (van Eslas et al., 1998). This is of particular interest with respect to the stringent culturing conditions necessary for SRB growth in the laboratory. Selective pressures introduced in laboratory experiments can alter SRB population structure and activity, a different population than would be observed in the field.

Purpose

The purpose of the research present here is to employ genetic fingerprinting techniques to identify shifts in anaerobic microbial community structure resulting from soluble organic carbon treatment of mine tailings. Molecular analysis of microbial communities stimulated in microcosm treatments will be used to identify possible correlations between observed sulfate reduction rates and microbial community structure. Sulfate reduction rates and active consortia observed in microcosms will be compared to those observed in bench scale columns to assess potential experimental biases.

Materials and Methods

Overview

Anaerobic tailing samples were collected from laboratory columns containing treated and untreated tailings described in Chapter 2 of this thesis. Tailings were used to inoculate two separate microcosm experiments. The first experimental setup consisted of 10 ml serum vials containing 1 gram of tailings and exposed to three treatment conditions, including whey as the sole organic carbon source. Sulfate concentrations were monitored periodically over a period of approximately 800 hours. The second experiment consisted of 25 ml microcosms containing 5 grams of tailings and 15 ml of Postgate B medium, with

lactate as the dominant organic carbon source. Initial and final sulfate concentrations were monitored, as well as daily hydrogen sulfide production.

Microbial community structure was identified for pre- and post-treatment samples using DGGE and automated sequencing. Shifts in dominant phylogenies were identified in DGGE community profiles and compared across treatments. Comparisons of sulfate reduction rates and community profiles were conducted to determine the relevance of applying molecular techniques in targeting specific phylogenies and predicting endemic SRB response to specific organic carbon treatments.

Column Sampling

Stainless steel tubing 48 inches long and 3/8 inch in diameter was used to obtain a core sample of column tailings. Every 6 inches tubing was removed and the subsequent core discarded until a clean coring could be extracted from the maximum column depth of 30 to 36 inches. The pH of the core at 30 and 36 inches was measured using a standard silver/silver chloride pH electrode and meter (Fisher Scientific, Accumet Portable). A small slice of saturated sample from the 36 inch core was placed on the electrode and the pH was measured. The pH value obtained was relatively consistent with the pH obtained using pH indicator paper (Whatman, pH 0-14). A small amount of tailings was placed on the paper, left for approximately one minute and removed by shaking off and lightly rinsing the paper with RO water. The remaining core sample was

immediately placed in a 15 ml tube (Falcon) and capped. Each of the tubes was filled to the top to minimize any bias introduced by the presence of oxygen. Samples were immediately refrigerated at 4^0C prior to use in microcosms.

Samples were collected in July 2004 for serum vial experiments and in October 2004 for respirometer experiments. Shortly after sampling for serum vial microcosms, all three of the bench-scale columns were treated with whey, including TCC. TC1 and TC2 columns were last treated (whey) in January 2004. A noticeable increase in effluent sulfate was observed over a two month period prior to sampling in July 2004 (Sturman, 2004).

Microcosm Construction

Serum Vial Experiment

The first microcosm experiment, using serum vials, was carried out in triplicate with each tailing sample (TC1, TC2, and TCC) being treated under three varied conditions. The conditions to which they were subjected are listed in Table 1.

Table 1 Serum vial microcosm treatment conditions

Condition	Tailings	Postgate B	Whey	Sterilization
Abiotic (A)	✓	✓	✓	✓
Whey (W)	✓	✓	✓	
Control (C)	✓	✓		

Postgate B medium (0.5 g KH_2PO_4, 1 g NH_4Cl, 1 g $CaSO_4$, 2 g $MgSO_4$-$7H_2O$, 0.5 g $FeSO_4$-$7H_2O$, 1 L dH_2O) containing no organic carbon was distributed to six

flasks, two for each column sample. Media pH was adjusted (HCl) to that observed in the tailing samples (pH 4 for TC1 and TC2, and pH 2 for TCC). 3 g/ L dissolved whey was added to one flask of each column set. Media was boiled under a nitrogen head to remove dissolved oxygen and a nitrogen head was applied to the 10 ml glass vials (Fisher Scientific) to which 9 ml of medium was added. Vials were sealed with rubber septa and aluminum caps. Nine vials for each column sample were constructed, three for each experimental condition. Microcosms were subsequently autoclaved for 45 minutes at 123^0C and 41 psig.

A minimum of 10 g of tailing sample was collected from the Fox Lake columns at a depth of 30 to 36 inches. The samples were homogenized to remove potential heterogeneities in the sampled microbial community. Previously autoclaved and cooled microcosms were uncapped under a nitrogen head in a sterilized laminar flow hood. 1 g of tailing sample was added to each of the microcosms. The microcosms were then capped. Control vials were autoclaved under the previously listed conditions.

Using a simplified stoichiometric equation for sulfate reduction:

$$2CH_2O + SO_4^{2-} \rightarrow H_2S + 2HCO_3^- \qquad \textbf{(6)}$$

60 g of carbohydrate is consumed to remove 96 g sulfate. The organic carbon content of the whey was previously determined to be 35% by weight. Based on

the amount of sulfate in the Postgate medium (1659 mg/L), 3 g/L whey was necessary to consume all sulfate added to the system.

Respirometer Experiment

The second microcosm experiment, using the respirometer, was carried out in triplicate with each tailing sample (TC1, TC2, and TCC) being treated with a slightly modified Postgate B medium (0.5 g/L KH_2PO_4; 1 g/L NH_4Cl; 1 g/L $CaSO_4$; 2 g/L $MgSO_4$-$7H_2O$; 3.5 g/L sodium lactate (lactic acid (60%)); 1 g/L yeast extract; 0.1 g/L ascorbic acid; 0.025 g/L $FeSO_4$-$7H_20$; 0.1 g/L thioglycollic acid; 2 g/L sodium citrate-$2H_2O$; 1 L dH_2O; pH 5.5). Sodium citrate was added to Postgate's original medium for its reported property of holding iron and possibly other trace nutrients in solution (Postgate, 1984), thus minimizing iron sulfide formation. A minimum of 25 g of tailing sample was collected from the Fox Lake columns at a depth of 30 to 36 inches, approximately three months after sampling for the serum vial experiment. Samples were immediately taken to the lab and homogenized, by manual mixing with a sterile pipette tip, to remove potential heterogeneities in the sampled microbial community.

Four vials for each column sample were constructed: three for active communities and one as a sterile control. 5 g of tailing sample was added to each of the 25 ml glass, screw cap microcosms. Control vials were autoclaved for 45 minutes at 123^0C and 41 psig. Postgate medium, with iron sulfate absent, was sterilized by autoclaving for 30 minutes. After sterilization iron sulfate was

filter sterilized (0.2 um) and added to the medium prior to boiling under a nitrogen head to remove traces of dissolved oxygen. A nitrogen head was also applied to the 25 ml glass, screw cap vials to which 15 ml of medium was added. Caps were replaced and microcosms were immediately attached to the respirometer (Columbus Instruments). All microcosm components were autoclaved for sterilization.

Microcosm Kinetics

Sulfate Reduction

Serum vials were covered and incubated. The 27 microcosms were periodically sampled, beginning the first day of operation. Sampling involved brief mixing of the microcosm sample, removal of 100 ul aqueous solution with a sterile syringe and needle through the rubber septa, placement of the sampled solution in a 0.2 ml polypropylene microfuge tube, and immediate freezing at -70^0C. Two standards were also frozen during each sampling event. The two standards included 100 ul of a 10 mg/l S-SO_4^- standard from reagent grade $NaSO_4^-$ and 100 ul nanopure water, to evaluate possible adsorption and dissolution of sulfate from the tubes, respectively.

Samples were stored for no longer than 3 days prior to analysis, at which time they were thawed to room temperature, mixed, and diluted 1:100 in reverse osmosis water. Standard solutions were generated from reagent grade $NaSO_4^-$

in concentrations of 1, 5, 10 and 25 mg/l. Samples were filtered using IC Acrodisc 0.2 um Super (PES) membrane filters (Gelman Laboratory) prior to being loaded into test vials (Poly Vial, Dionex). Loaded vials were then analyzed using the DX500 Ion Chromatograph Unit with a pulse electrochemical detector using a Dionex IonPac AS4A-SC Analytical Column (Dionex). Calculated peak areas of standards were used to generate a second order polynomial trendline, from which sulfate concentrations of samples were determined using PeakNet 5.2 Software (Dionex). Every tenth sample was a 10 mg/l SO_4^-.

Sulfate concentrations of the respirometer microcosms were measured at the beginning and end of the experiment. Initial sulfate samples were taken from influent Postgate B medium. From each of the 12 microcosms, 100 ul was removed using a sterile syringe and needle to obtain final sulfate concentrations. Samples were prepared and analyzed identically to those in the serum vial experiment, with a 10 mg/l SO4- standard check performed after the twelve microcosm samples.

Hydrogen Sulfide Formation

Respirometry microcosm headspace was monitored for H_2S, CO_2 and O_2 concentrations using Columbus Instruments sensors. Sensor calibrations, experimental setup, and data collection was performed with Micro-Oxymax version 6.06e. Data was collected every 24 hours for a period of 22 days. Data files were transferred from the DOS-based Micro-Oxymax to Microsoft Excel for

analysis (Appendix D). Assuming equilibrium between gaseous and aqueous hydrogen sulfide had been reached at the time of sampling, Henry's Law was used to calculate aqueous H_2S concentrations. Using stoichiometry presented in Equation 2, sulfate consumption was determined based on hydrogen sulfide formation. This calculation is compromised by the presence of iron sulfide precipitate and at an effluent pH at or above the pK_{a1} of H_2S (7.04).

Molecular Analysis

DNA Extraction

Microbial DNA was extracted from frozen inocula and treated tailings at the end of microcosm experiments. Samples were prepared using a modified version of the method previously described by Bond et al. (2000). Samples were thawed at room temperature and 0.5 g of homogeneous sample was placed in a lysing tube provided in the FastDNA SPIN Kit. Samples were washed using a modified method of Bond et al. (2000). A volume of 1.0 ml of PBS (pH 1.8) was added to 0.5 g sample, inverted for 5 minutes and centrifuged at 10,000 x g for 10 minutes. The resulting supernatant was discarded and 1.0 ml of a solution containing one part buffer A (200 mM Tris [pH 1.2 – 1.8], 50 mM EDTA, 200 mM NaCl, 2 mM sodium citrate, 10 mM $CaCl_2$) and one part 50% glycerol was added to the samples. Sample tubes were again inverted for 5 minutes and centrifuged at 10,000 x g for 10 minutes. Following sample preparation, community DNA

was extracted using FastDNA SPIN Kit for Soil (Q-BIO Gene), employing bead beating and surfactants for cell lysis and genome isolation, and guanidine thiocyanate for sample purification. Subsequent extraction steps were followed as presented in the BIO 101 protocol. DNA extraction and PCR amplification preparations were performed in a laminar flow hood to minimize aerial contamination. All solutions were prepared with reverse osmosis H_2O, and autoclaved prior to use.

PCR Amplification

Template DNA obtained from sample extraction was amplified in two separate reactions. Samples were initially amplified in 25 ul volumes using 1 ul extracted DNA template. Extract was initially amplified over 25 cycles at a fixed annealing temperature. For primers 1070f (5'-ATGGCTGTCGTCAGCT-3') and 1392r (5'-ACGGGCGGTGTG TAC-3') the amplification sequence consisted of 5 min at 94^0C, 25 cycles of 45 s at 94^0C, 45 s at an annealing temperature of 60^0C, and 1 min at 72^0C, finishing with 5 min at 72^0C. For primers 341f (5'-CCTACGG GAGGCAGCAG-3') and 1392r the amplification sequence consisted of 10 min at 94^0C, 25 cycles of 1 min at 94^0C, 45 s at an annealing temperature of 62^0C, and 2 min at 72^0C, finishing with 10 min at 72^0C. Five ul Taq-&GO Mastermix 5xC (Q-BIO Gene) was added per 25 ul reaction mixture. The second round of PCR was done using the same primers with a GC-clamp (5'-CGCCCGCCGCGCGCGGCGGGC GGGGCGGGGGCACG GGGGG-3')

attached to the 5' end of the 1392r primer. Touchdown PCR (TD-PCR) was employed in the second round of amplification to decrease mispriming and nonrepresentative product. For the primer set 1070f-1392r+GC the amplification sequence consisted of 5 min at 94^0C, 20 cycles of 45 s at 94^0C, 45 s starting at 68^0C and decreasing by 0.5^0C/cycle, and 1 min at 72^0C, plus 10 cycles of 45 s at 94^0C, 45 s at 58^0C, and 1 min at 72^0C, finishing with 5 min at 72^0C. The TD-PCR amplification sequence for primers 341f-1392r+GC consisted of 10 min at 94^0C, 20 cycles of 1 min at 94^0C, 45 s starting at 70^0C and decreasing by 0.5^0C/cycle, and 2min at 72^0C, plus 10 cycles of 1 min at 94^0C, 45 s at 60^0C, and 2 min at 72^0C, finishing with 10 min at 72^0C. The second PCR sequence was conducted using a final reaction volume of 25 ul to which a 1 ul aliquot of the previous PCR product was added as template. Five ul Taq-&GO Mastermix was added per 25 ul of reaction mixture. Negative control reactions were carried out in both the first and second rounds of PCR, with the first negative control being treated as a sample in the second PCR. All primers were purified using a standard desalt method (except for the 1392r+GC primer which was HPLC purified) and were performed by the oligonucleotide supplier (Integrated DNA Technologies). All reactions were carried out using a Mastercycler epGradient Thermal Cycler (Eppendorf). Samples were immediately frozen (-20^0 C) after PCR was complete. Amplified product was checked against Low Mass Ladders or 100bp DNA Ladders (Promega) on a 1.5 % agarose gel stained with ethidium bromide. Stained bands were visualized using the FluorChem 8800 Imaging System and

AlphaEaseFC software (Alpha Innotech). Contrast, brightness, and in some cases gray scale inversion, were the only modifications done to the images using Adobe Photoshop Elements 2.0.

DGGE

Community profiles of pre- and post-treatment tailing samples were compared to identify shifts in predominant phylogenies in response to experimental conditions. DGGE was performed to isolate predominant phylogenetic populations in the amplified template of community DNA extract. Extract amplified with 1070f-1392r+GC and 341f-1392r+GC were run in 8 to 12% gradient and fixed 10% acrylamide gels, respectively, containing a 40 to 70% denaturant gradient. Gels were subjected to an electrophoretic charge of 60 volts at a constant temperature of 60^0C for 16 hours in a D-Code Universal Mutation Detection System (Bio-Rad Laboratories). A total volume of 25 ml was used to pour the 1 mm thick gels, which were allowed to polymerize prior to pouring a 6% acrylamide stacking gel used to form loading wells. Gels were subsequently stained with SYBR Green I (Cambrex Bio Science) and gel images were obtained using a FluorChem 8800 Imaging System and AlphaEase FC software (Alpha Innotech). Major bands were excised from the gel using sterilized razor blades. Excised bands were briefly rinsed to remove residual external DNA. Selected fragments contained in the gel were isolated from the polyacrylamide using the QIAEX II Gel Extraction Kit (Qiagen) and protocol

provided. Eluted DNA was reamplified, visualized on a 1.5% agarose gel, and sequenced. Contaminant bands were not excised and similar bands in multiple samples were excised only once, with the exception of bands that did not clearly share the same melting domain. Contents and conditions for making DGGE reagents are presented in Appendix B.

16S rRNA Sequencing

Sample preparation and sequencing were performed using the methods presented in Chapter 2 of this thesis.

Phylogenetic Analysis

Phylogenies of predominant populations were identified using the method presented in Chapter 2 of this thesis.

Results

Summary

Sulfate reduction was obvious in all three replicates of whey treated TC1 and TC2 serum vial microcosms. Iron oxidation was observed in abiotic treatments of samples TC1 and TC2. No visible changes occurred in TCC. Sulfate

reduction rates were higher in TC2 than in TC1, with no significant reduction occurring in TCC. Lactate treated microcosms run on the respirometer yielded no significant numerical data for calculation of sulfate reduction rates. However, increases in pH, measured hydrogen sulfide, and obvious iron sulfide precipitate formation suggest sulfate reduction occurred in response to lactate treatment. TC2 was the most responsive with the greatest increase in pH, through out all three replicates. Community profiles revealed observable shifts in community structure as a result of culturing, both with and without organic carbon treatment. Phylogenetic analysis provides evidence of the selection for sulfate-reducing bacteria, endemic to pre-treatment communities, from organic carbon treatments. Bands were only excised from 1070f-1392r amplified profiles. These profiles suggested significant similarities between samples, whereas 341f-1392r profiles revealed a noticeable increase in phylogenetic diversity between samples.

Serum Vial Experiment

Whey treatments promoted increased rates of sulfate reduction in previously treated tailings. Visible signs of sulfate reduction were not apparent until nearly 400 hours of operation. At this point, iron sulfide formation (black precipitate) was observable in whey-treated serum vials inoculated with TC1 and TC2 tailings. Figure 22 is a digital image of TC1 and TCC serum vials at 597 hours. TC2 was omitted from the figure because of its distinct similarity to TC1. TCC inoculated serum vials formed no visible FeS precipitate throughout the

experiment. An orange precipitatewas present at the gas-liquid interface in the abiotic, heat-treated vials inoculated with TC1 and TC2. This appeared at approximately the same time as the iron sulfides in whey-treated vials, and was likely oxidized iron.

The serum vial experiment was terminated after 815 hours of operation. After freezing, solid phase samples were removed from the vials for molecular analysis. Agglomerations were present in the whey-treated replicates of samples TC1 and TC2, the result of possible biofilm or FeS. All other treatments and replicates lacked this noticeable aggregation of sediment particles. Community DNA extract was far more concentrated in whey treated TC1 and TC2 samples, suggesting a pronounced increase in cell mass over that of other treatments. The lowest concentration of extract was from abiotic treatments. In the abiotic treatments positive amplified product could be due to either the presence of spore-forming species or bound fragments of DNA unaffected by the sterilization treatment. However, because sulfate concentration did not change in the abiotic and control samples over the duration of the experiment, and no visual signs of biological activity were present, the sterilization process was likely effective.

Sulfate Reduction

Sulfate reduction occurred most rapidly over the 479 to 597 hour sampling period (Appendix C; Figure 23) in whey treated TC1 and TC2 replicates. Average reduction rates (Table 2) were greatest in TC2, with a maximum

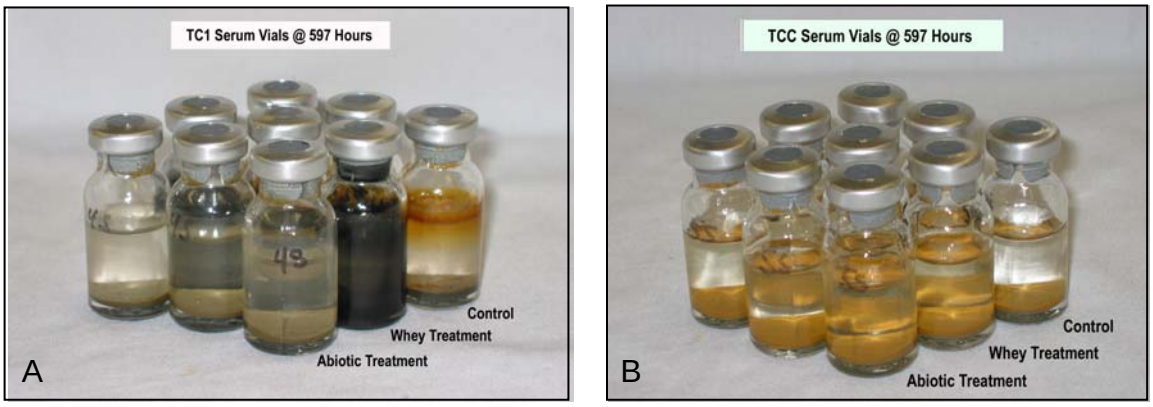


Figure 22 Serum vial microcosms 597 hours after treatment. TC1 (A) and TC2 (not shown) microcosms were visually identical with obvious microbial activity occurring in whey and control treatments. TCC (B) microcosms presented no visual differences between treatments.

replicate rate of 73 mg L^{-1} d^{-1} and an average rate of 53 mg L^{-1} d^{-1}. The TC2 average compares relatively well with the maximum reduction rate observed in whey-treated TC1 replicates at 57 mg L^{-1} d^{-1}, which had an average rate of 27 mg L^{-1} d^{-1}. The variability in replicate sulfate reduction rates was quite high, particularly in TC1 samples, as presented by standard deviation (Table 2). The variation was less pronounced in TC2 replicates, with two rates being within 5% of one another. The remaining third replicate was far less (16 mg L^{-1} d^{-1}). This could be due to poor sample homogenization prior to vial inoculation. All replicates were grouped prior to molecular analysis in this experiment, so community profile differences cannot be used to evaluate the cause of observed differences in reduction rates.

Maximum sulfate reduction rates in TC1 and TC2 serum vials were quite similar to those observed in the effluent of bench-scale columns (Sturman, 2004). Effluent sulfate reduction rates in whey treated TC1 and TC2 columns were

Table 2 Average sulfate reduction rates in response to whey treatment. Rates are specific to replicates of serum vial microcosms over the 479 to 597 hour sampling period. Standard deviation provided in brackets below rates.

Average Sulfate Reduction Rates (mg L^{-1} d^{-1})

	WHEY	CONTROL	ABIOTIC
TC1	27.39 (25.64)	3.19 (8.34)	8.14 (5.71)
TC2	53.29 (32.09)	25.36 (6.84)	0.88 (4.82)
TCC	10.64 (4.75)	16.54 (4.12)	2.78 (17.29)

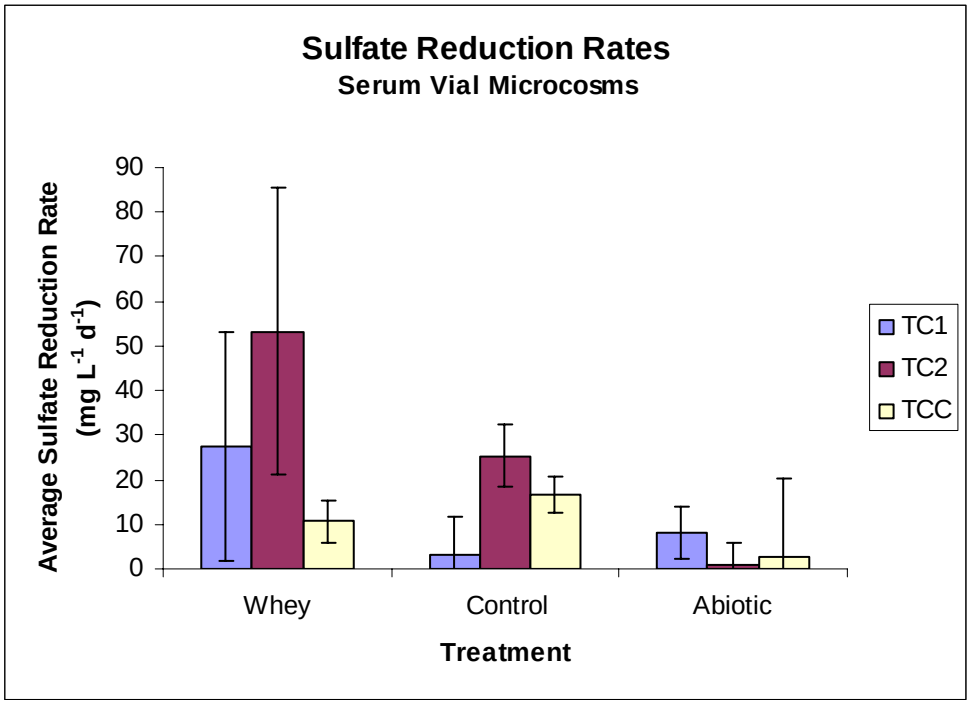


Figure 23 Average sulfate reduction rates observed in whey treated serum vials. Rates are specific to replicates of serum vial microcosms over the 479 to 597 hour sampling period. Standard deviation represented as error bars.

roughly 59 and 65 mg L^{-1} d^{-1}, respectively, assuming that sulfate production in the upper strata is constant over time. Sulfate concentration was actually increasing over this period of monitoring in the control column, which might translate to treated columns having a higher sulfate reduction rate than presented above.

A steep rise and fall in sulfate concentrations was observed over the 233 to 307 and 307 to 479 hour sampling periods, respectively (Figure 24). This peak in sulfate concentration, halfway through the experiment, was most pronounced in control treatments, with the sharpest rise occurring in TCC samples where sterile and control treatments mirrored one another Aside from the noticeable spike in the sulfate concentration, abiotic sulfate concentrations remained relatively stable over the 479 to 597 hour sampling period, and well within the allowable 10% error of the IC equipment. Control samples did have an observable rate of sulfate reduction during the sampling period of interest, possibly due to the presence and consumption of residual carbon and dead cell mass in sampled tailings.

Total sulfate concentration of the medium, according to the protocol, was 1659 mg/L. After the addition of tailings to their respective media, dissolved sulfate concentrations, as determined by ion chromatography, were approximately 1000 mg/L in TC1 and TC2 and 1500 mg/L in TCC. This is of no surprise considering the differences in pH (pH 4 and 2, respectively) of media. The pH would presumably have an effect on dissolved sulfate concentration. However, within

the first 24 hours a rise in sulfate concentration occurred, with the steepest rise occurring in TCC samples. The rise was less pronounced in TC2 and far less so in TC1. Considering the spread in data (i.e. error bars) at the 24 hour sampling point, this increase in sulfate concentration is insignificant in TC1 and TC2. However, this is not the case in TCC, which averaged a sulfate increase of 34%, well outside the range of experimental error and spread in observed concentrations. Due to the similarities in phenomena occurring over such short periods of time, the rate of sulfate dissolution relative to media pH, and the chemistry of the samples, this occurrence is likely chemical, rather then biological. Over the first 90 hour period it is likely that the electrochemical conditions of the vials approached an equilibrium. The redox potential (E_h) of the microcosm environment must produce reducing conditions (less than –100 mV) for sulfate reducing activity (SRA) to occur. Postgate (1984) determined that a Postgate B medium containing no redox-poising agent (such as the recommended thioglycolic acid) would have a redox potential near +200mV. Thioglycolic acid was not used in the medium because it is a potential source of organic carbon. Until the redox potential and pH became equilibrated, the presence and absence of certain sulfate species would potentially be observed.

The later spike in sulfate concentration is potentially the result of microbial activity. Several chemomixotrophic organisms (e.g. *Acidithiobacillus*, *Sulfolobus*, *Desulfobulbus*) are known to produce sulfate under anaerobic conditions (Lovely and Phillips, 1994). Considering the pH and the likely presence of elemental

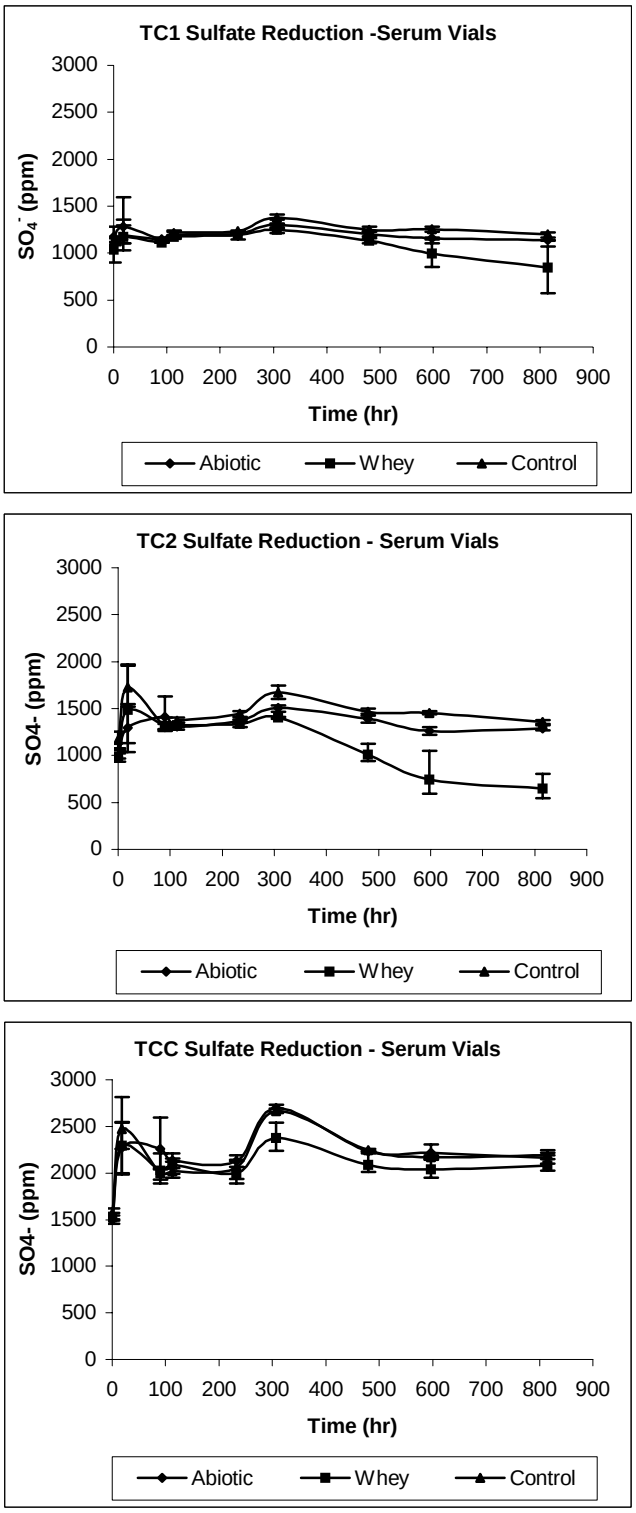


Figure 24 Serum vial sulfate concentrations with respect to time. Maximum and minimum error bars represent maximum and minimum values observed in treatment replicates. Average sulfate concentrations at each time point were used to construct graph.

sulfur, Fe(III), and Mn(IV) oxides, this process could result in the observed rise of sulfate concentration.

Termination of the serum vial experiment was initiated with the plateau in sulfate concentration observed in TC2. Sulfate reduction was still occurring in TC1, but at a slightly reduced rate. The observed plateau could have been due to several conditions, including; (i) hydrogen sulfide toxicity inhibiting SRB activity in the closed system, (ii) iron sulfide formation limiting SRB activity by the sequestration of Fe(II), (iii) applied carbon sources were consumed, and (iv) a shift in phylogeny was occurring. The first two possibilities are most likely to have occurred. A stoichiometric estimate of carbon consumption for mineralization of lactate to CO_2 by a mixed population of both lactate oxidizing and acetate oxidizing organisms is shown in Equation 7 (Kim et al., 1999).

$$CH_3CHOHCOOH + 1.33H_2SO_4 + 0.066NH_3 \rightarrow 0.34CH_{1.4}N_{0.2}O_{0.4} + 1.33H_2S + 2.66CO_2 + 2.88H_2O \quad \textbf{(7)}$$

Using the fastest sulfate-reduction rate observed (replicate three of the TC2 whey treatment) over the 307 to 815 hour sampling period, 796 mg (8.3 mmol) SO_4^{2-} was consumed. Based on the stoichiometry of Equation 7 this would have consumed 993 mg lactate; one third of the whey mass added to the vial. It is likely that carbon became limiting in the vials, assuming that complete oxidation of lactate occurred and two thirds of the original carbon was consumed by other

anaerobic heterotrophs. A shift in phylogeny is yet another possibility. The observed lag period of 9 days after sulfate reduction ceased is a significant amount of time, reducing the possible occurrence of a shift in metabolically distinct SRB populations.

DGGE

Community profiles generated from 1070f-1392r and 341f-1392r amplified products (Figures 25 and 26) provided an excellent view of selection, for and against, individual phylogenies across treatments, where community structure changed as a direct result of culturing and whey treatment. Although it is not possible to correlate consortia structure with replicate sulfate reduction rates, pooled DNA profiles do provide information on the response of intrinsic populations in response to whey-treatment. Appearance of bands in treated populations that were absent from, or only slightly visible in, tailing profiles suggests that minor intrinsic populations are stimulated under experimental conditions.

1070f-1392r Amplified Profiles

Community profiles provided by 1070f-1392r reveal that shifts in consortia structure result from both carbon source treatment and experimental conditions (Figure 25). Control treatments were extremely helpful in determining the effect of experimental conditions, devoid of carbon, on community structure. The most

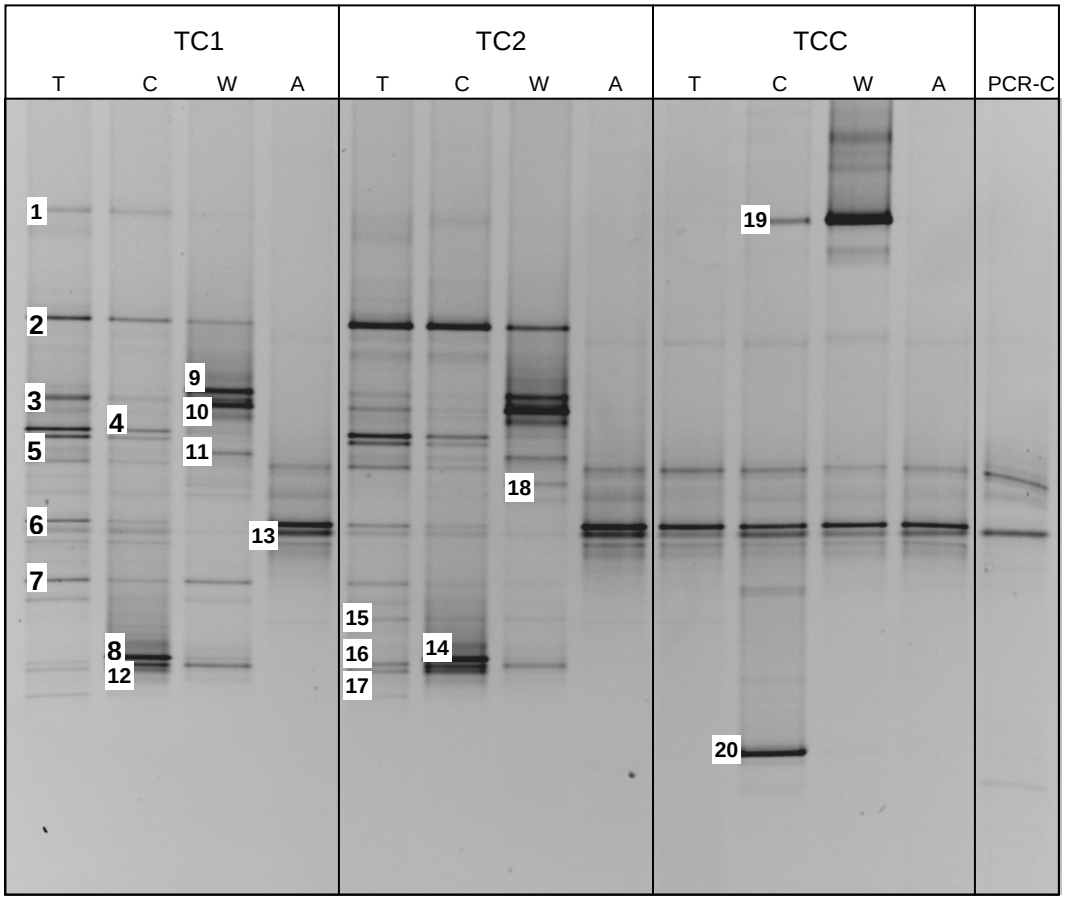


Figure 25 Community profile of serum vial and tailings extract amplified with 1070f-1392r+GC. Numbered bands were excised, amplified, and sequenced to obtain a phylogenetic identification of prominent populations. Only numbers in boxes yielded clean sequences. Tailings inoculum (T), control without whey (C), whey treatment (W), heat-treated with whey (A), PCR reaction control (PCR-C)

obvious example of this is the appearance of Bands 8, 14, 19, and 20. These bands were stimulated specifically by experimental conditions, presumably components of the buffer solution.

Community diversity of previously treated tailings inocula (TC1 and TC2) were similar to one another and far more diverse than untreated tailings (TCC). Whey treatment had a noticeable effect on the resulting microbial community structure

in all samples. Some bands present in the tailing profiles (e.g. bands 9 and 10) proliferated in the presence of whey, while others were lost or unaffected. Minor populations appeared (e.g. bands 11 and 18) that were absent from the tailing profiles prior to treatment. The fact that bands stimulated in control treatments were no longer present in whey treatments supports the direct affect of whey, and not other experimental conditions, on community structure dynamics.

The presence of band 6 in abiotic and all TCC treatments suggests either the presence of a spore-forming organism in these samples or the possibility of contaminant DNA in extraction buffers. However, this is unlikely given the fact that negative control extractions yielded no profile. The intensity of band 13 in abiotic treatments of TC1 and TC2 suggests the stimulation of an additional population different from that identicfied in the PCR control.

341f-1392r Amplified Profiles

Aside from the strongest bands present in 341f-1392r amplified profiles (1, 2 and 3) there is little similarity in community structure between tailings, abiotic, control and whey treatments, suggesting far more diversity between treatments than is presented by 1070f-1392r amplified profiles (Figure 26). Bands were not excised for sequencing and resulting phylogenetic identification is unavailable for distinct bands. The most significant result from this profile is the increased number of bands present in the TCC tailings profile relative to that identified by the 1070f-1392r product. Differences in bands present in TC1 and TC2 abiotic

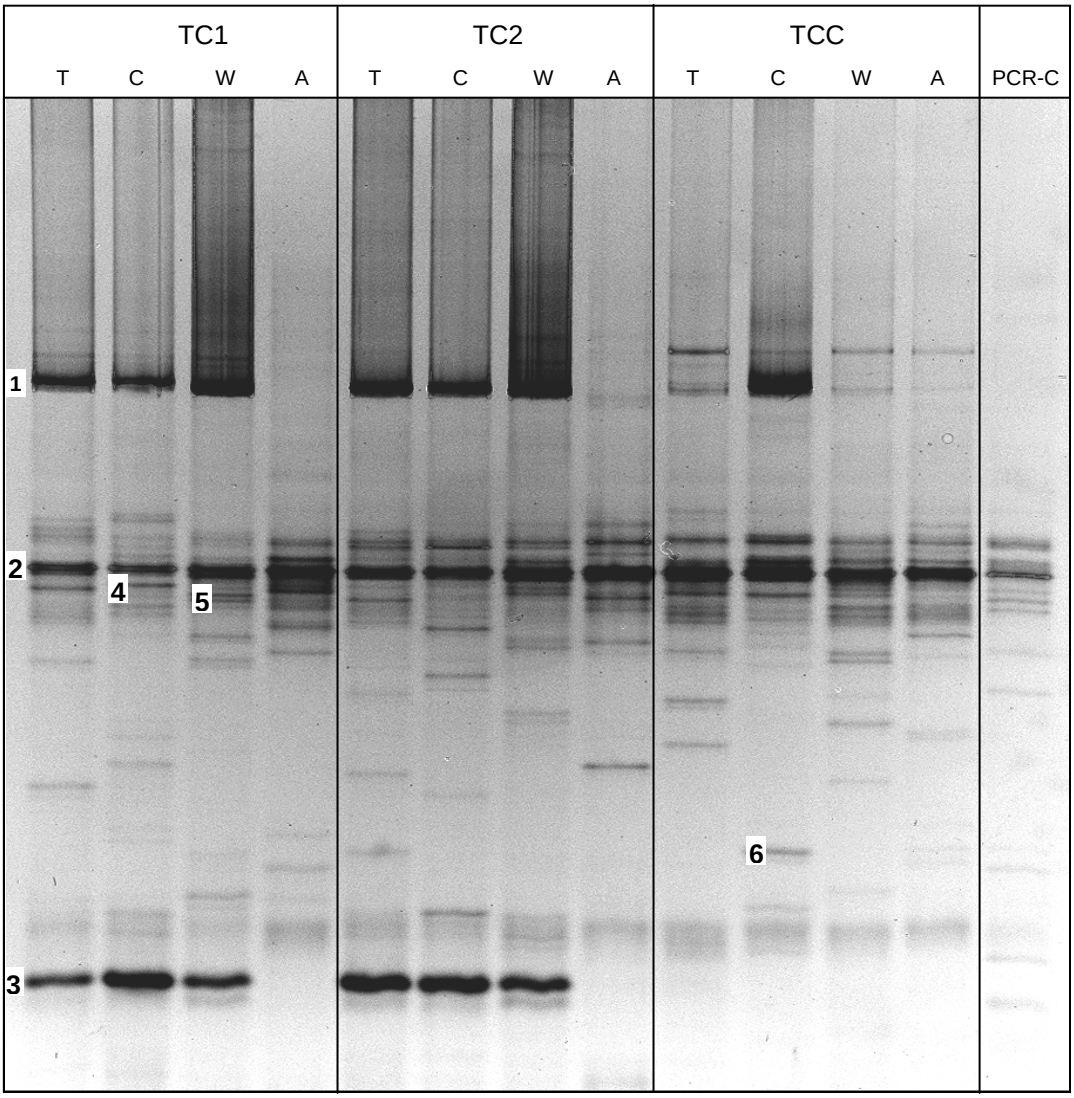


Figure 26 Community profile of serum vial and tailings extract amplified with 341f-1392r+GC. Tailings inoculum (T), control without whey (C), whey treatment (W), heat-treated with whey (A), PCR control (PCR-C)

treatments are also significant, suggesting an increased number of different spore-forming phylogenies between the three columns. Increased intensity of these bands relative to those in the PCR-C product, reduces the possibility of these distinct bands being contaminants. Another insight provided by this profile

is the increased diversity in banding patterns of tailing samples in TC1, TC2, and TCC. Tailing profiles for TC1 and TC2 in Figure 25 suggest significant similarities in populations present in the two columns. Bands 1, 2 and 3 are the only bands present in both TC1 and TC2 profiles amplified with this primer set. The banding pattern appeared more diverse to the eye than in the image, revealing limitations in the FluorChem 8800 Imaging System.

Phylogenetic Analysis

Based on DGGE profiles of community structure whey treatment stimulated growth of *Clostridium* sp. This genera has a wide range of known metabolic activity, including sulfite reduction (Prescott et al., 1996), and are commonly associated with SRB communities (Muthumbi et al., 2001; Spear et al., 2000). Angeles-Chavez (2001) identified a new strain of anaerobic bacteria closely linked (97% phylogenetic similarity) with *Clostridium sphenoides*, isolated from a medium rich in lactate and sulfate, and identified it is a new species of SRB. Sulfite was never observed during IC analysis of samples, though the column used for the sulfate analysis was capable of identifying sulfite.

Sequencing of excised bands was only successful on 11 of 20 bands (Table 3). Resulting sequences of the remaining 9 bands were either too long or produced a chromatograph that was too weak for analysis. Long sequences ranged from 600 to 1300 bp suggesting contamination or multiple phylogenies occurring at the same relative melting domain. Autoclaved abiotic treatments of TC1 and TC2,

Table 3 Phylogenetic identity of selected 1070f-1392r amplified bands. Automated sequences used as queries to search via NCBI using blastn search program. Rows lacking information, designated with "M", were not successfully sequenced

Band	Organism	Accession Number	Identities (% Match)
1	*Ferroplasma acidiphilum*	AJ224936	97
2	Uncultured bacterium clone BIgi18	AJ318136	95
3	*Desulfosporosinus* sp.	AF076245	88
4	M		
5	*Desulfosporosinus* sp.	AF076247	91
6	*Desulfosporosinus* sp.	AJ302078	97
7	*Acidithiobacillus ferrooxidans*	AJ621559	98
8	M		
9	*Clostridium* sp.	X68179	99
10	*Clostridium* sp.	X68179	99
11	M		
12	M		
13	M		
14	M		
15	M		
16	M		
17	M		
18	M		
19	*Ferroplasma acidiphilum*	AJ224936	96
20	Uncultured *Sulfobacillus* sp.	AF460985	98

and all TCC samples were predominated by *Desulfosporosinus sp.* (Band 6). Though multiple spore-forming phylogenies (*Desulfosporosinus*, *Clostridium*, and *Sulfobacillus*) were present in samples, this particular isolate proliferated after heat sterilization. Band 13 did not provide a readable sequence, possibly due to its location with respect to a PCR-C contaminant band.

The presence of *Ferroplasma sp*. (Band 19) is of little surprise, considering this iron oxidizing Archaeon has been identified from mine drainage streamers with

growth observed at pH 0 (Edwards et al. 2000). *Ferroplasma* isolates have recently been identified as facultative anaerobes, coupling chemoorganotrophic growth on yeast extract to the reduction of ferric iron (Dopson et al. 2004). This study supports this anaerobic chemoorganotrophism. Band 19 dominated in whey-treated TCC samples, at an initial pH 2, and was only slightly visible in TCC control and TC1.

Whey treated TC1 and TC2 tailings were dominated by *Clostridium sp*. (Bands 9 and 10). Uncultured bacterium clone BIgi18 was identified from Band 2, which was present in TC1 and TC2 tailings and control treatments. This clone was initially identified from a diverse waste gas-degrading community in an industrial biofilter. The presence of *Acidithiobacillus ferrooxidans* (band 7) in whey treated TC1 might provide reason for smaller sulfate reduction rates observed in TC1 vials relative to TC2. *Acidithiobacillus ferrooxidans* are facultative anaerobes, capable of producing SO_4^{2-} from elemental sulfur under anoxic conditions.

Due to the fact that a number of bands (identified by an “M” in Table 3) provided no readable sequences, a full analysis of sample phylogeny and response to treatment could not be achieved. Alternative methods, such as rerunning samples on DGGE gels prior to sequencing to confirm positive product and isolation, are necessary to provide a full understanding of community dynamics.

Respirometer Experiment

Kinetics

Sulfate concentrations were measured at the beginning and end of the experiment, while H2S was measured daily over a period of 22 days. The intent of this study was to perform a kinetic analysis based on $H_2S_{(g)}$ production rates, while using changes in SO_4^{2-} to check total sulfate removal. Sulfate reduction calculated from gaseous hydrogen sulfide production measured in the headspace yielded results several orders of magnitude below that observed in columns and serum vial microcosms. A rise in pH (above pK_{a1} = 7.04), could explain these results. Once above pH 7, sulfide is predominantly in the form of the highly soluble and highly reactive hydrogen sulfide ion (HS^-). Measured pH values in vials containing reduced iron sulfides were near or above pH 7 (Table 4). Metal sulfide formation became evident, as a black precipitate, within 24 hours of the first hydrogen sulfide reading in nearly all cases, resulting in the removal of hydrogen sulfide from the headspace, with inconsistent H_2S production subsequently observed (Figure 27; TC1 @ t=456, TC2 @ t=192). Sulfate reduction rates obtained from initial buffer and final microcosm effluent sulfate concentrations were negative in nearly all cases; potentially the result of rapid sulfate release into the system as observed in serum vial microcosms, and possible contributions from the anaerobic production of sulfate.

Table 4 Final effluent pH values of lactate treated microcosm. Italicized pH values correspond to microcosms having obvious iron sulfide formation.

Final Microcosm Effluent pH Values - Lactate

	Replicate			Control
	1	2	3	
TC1	5.15	*6.38*	*6.18*	5.09
TC2	*8.29*	*7.93*	*7.82*	*8.13*
TCC	5.72	6.03	5.72	5.27

Twenty-four hours prior to measured hydrogen sulfide formation, bubbles began to form and rise from the tailing sediments in TC2 microcosms. This was not observed in TC1. Bubble formation corresponded to a rapid increase in measured carbon dioxide beyond the limits of the sensor, suggesting significant metabolic activity. Noticeable bubble formation continued for 72 hours after initial iron sulfide formation and subsequent hydrogen sulfide disappearance. Increased formation of black precipitate, in conjunction with bubble formation, over the 72-hour period suggests that metabolic activity, including sulfate reduction, did not cease after the disappearance of hydrogen sulfide from the headspace.

Significant increases in media pH (from pH 5.5 to values presented in Table 4) of microcosms having black precipitate (underlined values in Table 4) suggest active sulfate reduction beyond that suggested by gaseous hydrogen sulfide data. Based on pH alone, TC2 was far more active in sulfate reduction than that observed in TC1 or TCC, including the TC2 control.

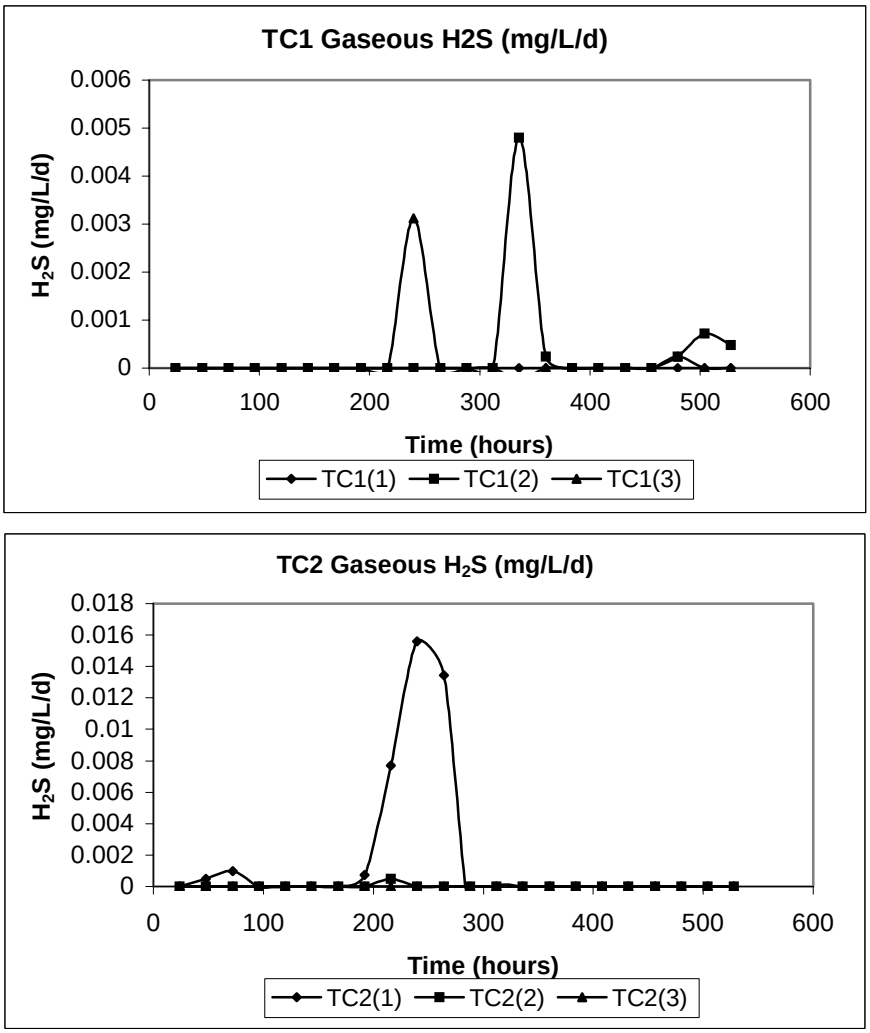


Figure 27 Gaseous hydrogen sulfide production rates over 22 day sampling period in response to lactate treatment. Samples are from previously treated tailings inocula (TC1 & TC2).

Heat treatment was apparently ineffective in eliminating active sulfate reducing bacteria. Spore-forming SRB are well known and suspect in an extreme environment such as mine tailings. Due to potentially rapid population shifts occurring in response to sampling, repeated sterilization or extended sterilization (i.e. pasteurization) could not be performed. All samples were needed, including

controls, to start experimental analyses as rapidly as possible to maintain endemic consortia structure. The period of sterilization used (45 minutes at 123^0C and 41 psig), was obviously ineffective in killing all organisms present in tailings.

DGGE

Lactate treatment yielded greater diversity than was observed in whey-treated microcosms. The most pronounced difference was the emergence and increased intensity of multiple bands in TC1 and TC2 treated replicates. Increased diversity was also observed in all pre-treatment tailings, including TCC, a likely response to recent feeding of whey to all three columns. Spore-forming bands identified in heat-treated serum vial microcosms were also observed in lactate-treated microcosms. Significant differences were observed between TC1 and TC2 lactate treated tailings, as well as within treatment replicates of TC1.

1070f-1392r Amplified Profile

Lactate treatment of microcosm cultures resulted in a definitive shift of community structure. Banding patterns differed significantly between TC1 and TC2 lactate-treated tailings. Bands 26, 12, and 13 dominated TC1 replicates, while bands 25 and 2 dominated TC2 replicates. Significant differences were

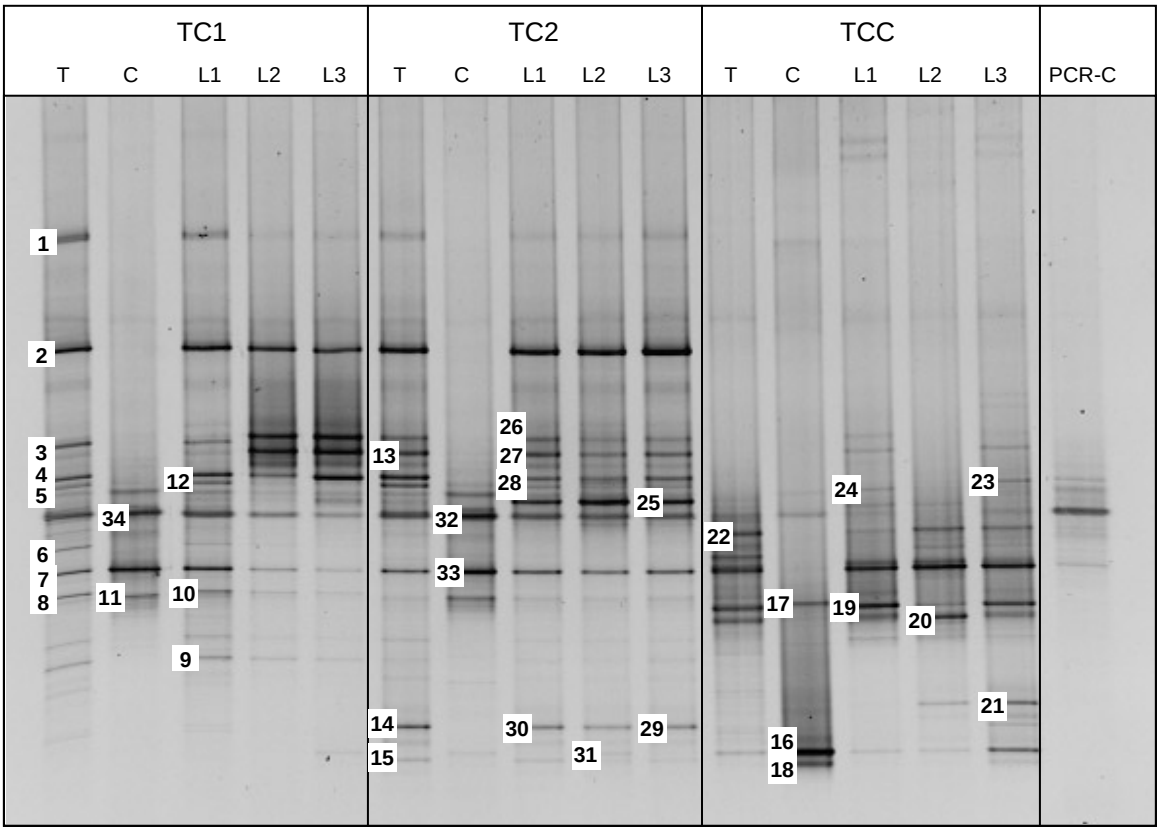


Figure 28 Community profile of lactate treatment and tailings extract amplified with 1070f-1392r+GC. Numbered bands were excised, amplified, and sequenced to obtain a phylogenetic identification of prominent populations. Only numbers in boxes yielded clean sequences. Tailings inoculum (T), heat-treated with lactate (C), lactate treated replicate (L#).

also observed within different TC1 replicates, with bands 26 and 13 dominating TC1(2) and TC1(3). The presence of these bands correlate well with observed sulfate reduction activity in these replicates, and bands 26 and 13 were only slightly visible in TC1(1), which had no observable sulfate reduction. Bands from lactate-treated TC2 replicates were identical, with the appearance and dominance of band 25. This band appears at a low intensity in pre-treatment extract (T), indicating that lactate stimulated phylogenies are present in pre-

treatment samples. Sulfate reducing activity was observed in TC2 control and not in TC1 control, however community profiles of these extracts suggest identical community structure and thus similar metabolic potential.

Community profiles in TCC were far more diverse than observed in serum vial treatments, although treatment replicates were very similar to pre-treatment extract profiles, with only slight intensity differences between them. Some variation was obvious between replicates with an increased intensity of Bands 16 and 21 in TCC(L3). Band 20 was also distinct to TCC(L2), which had the only significant increase in pH (pH 5.5 to 6.03) of treated TCC samples.

341f-1392r Amplified Profiles

Much like the results observed in serum vial community profiles amplified with 341f-1392r, obvious differences occur between nearly all samples (Figure 29), suggesting greater diversity than is represented by 1070f-1392r amplified profiles. The resulting profile is difficult to analyze due to extremely low intensity bands, however, it is the dim bands that support observable differences between replicate community structure and activity. Low intensity bands are highlighted in Figure 29. Bands were not excised from 341f-1392r profiles for sequencing. Future work could attempt this, however sequencing of some well defined bands from serum vial tailings was not successful, therefore the potential for positive results is questionable.

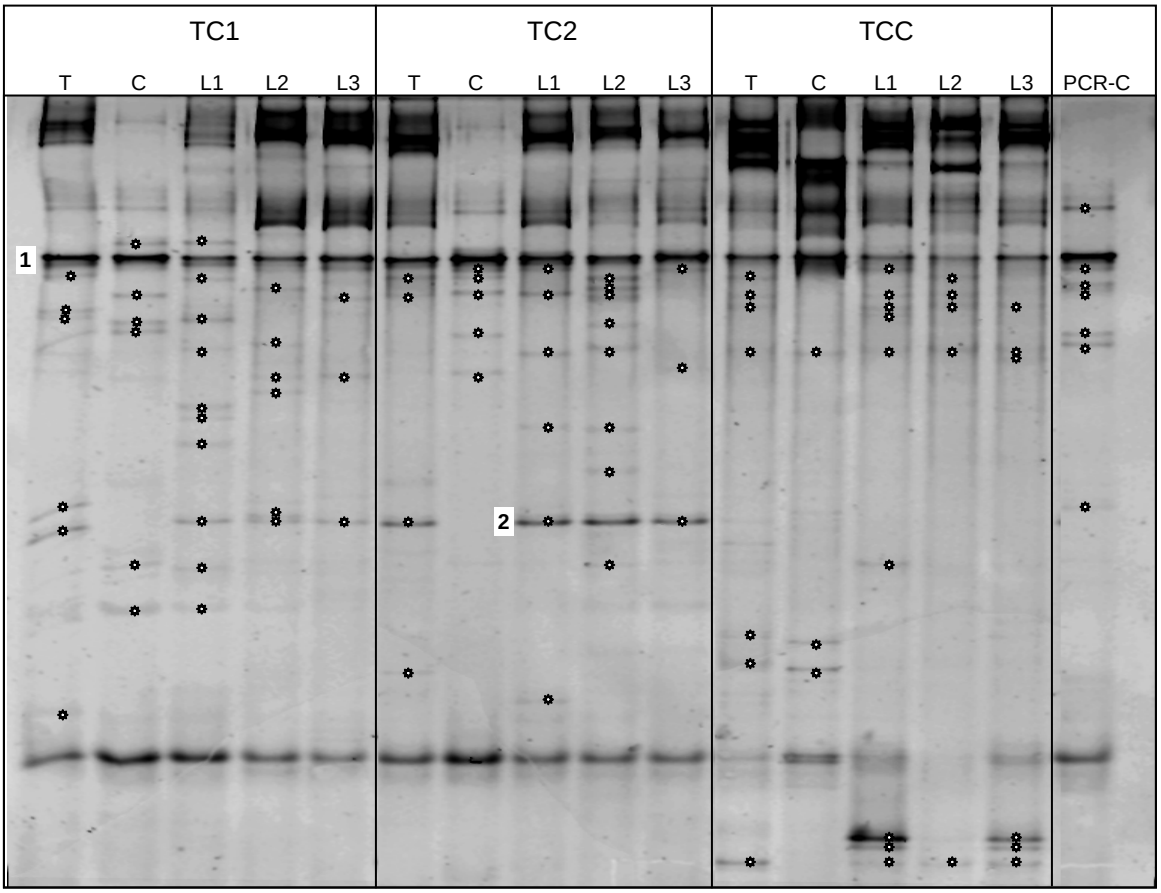


Figure 29 Community profile of lactate treatment and tailings extract amplified with 341f-1392r+GC. Dots are located at dim, but visually distinct, bands. Tailings inoculum (T), heat-treated (+) lactate (C), lactate treated replicate (L#).

Phylogenetic Analysis

Lactate treatment of tailing samples resulted in the selection and stimulation of known sulfate-reducing bacteria (i.e. *Desulfosporosinus* and *Desulfitobacterium*). Successful sequencing of selected bands (from 1070f-1392r) proved again, to be quite difficult, with only 13 of 34 bands providing strong matches (>94%) (Table

Table 5 Phylogenetic identity of selected 1070f-1392r amplified bands from lactate treated microcosms. Automated sequences used as queries to search via NCBI using blastn search program. Rows containing ("M") had extended sequences, suggesting multiple phylogenies, or poor sequences resulting in a negative BLAST search.

Band	Organism	Accession Number	Identities (% match)
1	*Ferroplasma acidiphilum*	AJ224936	93
2	Uncultured bacterium, clone BIgi18	AJ318136	97
3	*Desulfosporosinus* sp.	AY007667	98
4	*Desulfitobacterium metallireducens*	AF297871	97
5	M		
6	M		
7	*Desulfosporosinus* sp.	AJ302078	98
8	M		
9	M		
10	M		
11	M		
12	M		
13	*Clostridium diolis*	AJ458418	98
14	*Cellulomonadaceae* str. K12	AB078824	99
15	*Thermoactinomyces* sp. H0165	AB088364	94
16	*Sulfobacillus* sp. Y0017	AY140239	98
17	*Rhodococcus* sp.	AY785730	100
18	M		
19	M		
20	M		
21	M		
22	M		
23	M		
24	M		
25	*Desulfosporosinus orientis*	AJ493052	98
26	M		
27	M		
28	M		
29	*Cellulomonadaceae* str. K12	AB078824	98
30	M		
31	M		
32	M		
33	M		
34	M		

5). Some of the most significant bands were, unfortunately, those that were unsuccessfully sequenced (Bands 12, 19, 21, 32, and 34). However, due to redundant sampling, positive identification of similar bands can be achieved with confidence. For example, *Desulfitobacterium metallireducens* (Band 4) is likely to be the phylogenetic identity of Band 12 based on its similar location within the gel. Uncultured bacterium clone BIgi18 (Band 2) corroborates the same identification from serum vial samples. Bands 32 and 34 are very likely spore-forming organisms, due to their strong presence in heat-treated TC1 and TC2 controls. Like Band 13 in whey treated profiles of TC1 and TC2, sequencing failure of Bands 32 and 34 is likely the result of the close proximity of the contaminant band present in PCR-C. This occurred in several samples, as noted in Table 5.

Similar phylogenies were identified in both whey-treated and lactate-treated tailing samples (*Desulfosporosinus*, *Clostridium*, *Sulfobacillus*, BIgi18 clone). Several new phylogenies were identified from lactate-treated samples (*Cellulomonadaceae*, *Thermoactinomyces*, and *Rhodococcus*) that were not previously identified in the whey-treatment experiment. The appearance of these phylogenies is likely the result of column treatment, rather than culturing medium, due to their presence in pre-lactate-treated tailings (T). Due to a slight upward shift of Band 17 in TCC(C), it is difficult to say whether or not *Rhodococcus* was actually present in pre-treatment tailings, but Band 19 is certainly present in pre-treatment TCC samples and entirely absent from serum

vial TCC profiles. *Cellulomonadaceae* str. K12 was successfully identified from Bands 14 and 29 and is distinct to TC2 samples. *Thermoactinomyces* sp. H0165 (AB088364) was initially identified and associated with an anaerobic thermal composting process. Some Rhodococci are well known for their ability to degrade chlorinated hydrocarbons. The identified *Rhodococcus* sp. (AY785730) from sampled tailings was originally identified in a PCB-degrading site.

Phylogenetic analysis has revealed organisms that have previously been found in ARD and similarly acidic environments. These are organisms capable of anaerobic activity persisting in extreme and contaminated environments. Past analyses of column tailings has also revealed an abundance of Alicyclobacilli. These anaerobes are common to mine tailings (Bond et al., 2000), but were not identified in this or the whey treated community analysis. Considering the fact that not all bands were successfully sequenced, it is not unlikely that these organisms are still present and active in these communities.

Discussion

This chapter employed the molecular methods optimized in Chapter 2 to identify the potential stimulation of SRB, endemic to mine tailings, by varied carbon source treatments. Results presented in this chapter support the following conclusions:

- Sulfate-reducing activity increased in direct response to whey treatment.

- Lactate treatment stimulated sulfate reduction.
- DGGE community profiles identify significant shifts in community structure resulting from cultured treatments.
- DGGE identified preferential stimulation of known sulfate-reducing bacteria in lactate treated microcosms.
- Observed increases in sulfate reduction could be roughly correlated with specific SRB phylogenies in lactate treated microcosms.
- Whey treatment of bench-scale columns resulted in increased microbial diversity and selection (Figure 30).

Mine tailings have been touted as relatively simple systems, with limited diversity and metabolic ability. Tailings, and most other extreme environments, are in fact very complex systems, with an ever-increasing understanding of community and physiological complexity. Biogeochemical activity and geochemistry are interdependent, and redox potential and pH both control and respond to these reactions. The interplay of microbial activity and geochemical reactions complicate respirometric quantification of sulfate reducing activity, as was apparent in this study. Sulfate reduction rates are clearly more easily quantified via analysis of effluent, dissolved sulfate concentrations.

Microheterogeneities in community structure can contribute significantly to variations in observed rate data. Even in a system that is considered quite simple, attempts at homogenizing samples proved difficult. Agglomerate integrity was maintained to a certain extent, in hopes of reducing sampling affects on

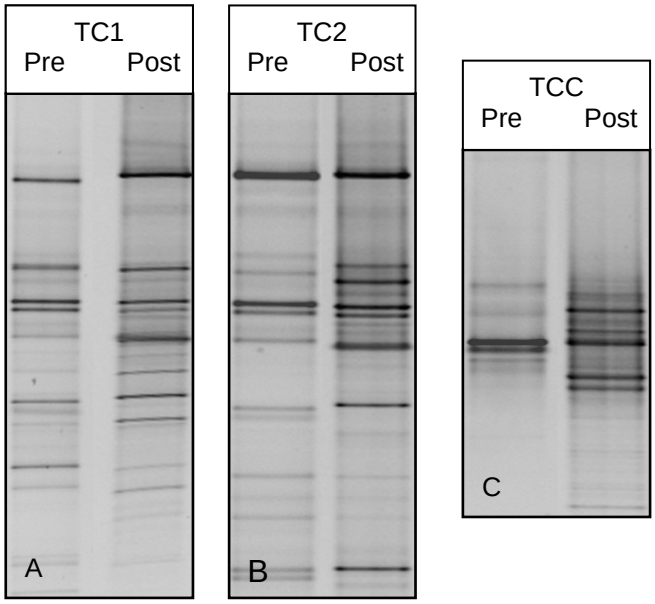


Figure 30 DGGE comparison of pre- and post-whey-treatment of bench-scale columns. Previously treated (TC1(A) and TC2(B)) and untreated (TCC(C)) columns.

endemic community structure. Previous tests, presented in Chapter 2, revealed strong similarity of community DNA extracted from individual subsamples. This was done using samples from TCC where community diversity is expected to be relatively small and homogenous throughout the tailings, unlike that of TC1 and TC2. Reduced variability in sulfate concentration observed between replicates used in TCC serum vial treatments, with respect to TC1 and TC2 data, supports these expectations and the potential for poor sample homogenization. Replicates from serum vial treatments were pooled prior to extraction and community profiling, thus providing a limited view of possible differences in community structure between replicate treatments. Future research would

benefit from community analysis of individual replicates as was done with lactate-treated tailings.

Resulting community profiles do not always support observed differences in microbial activity. Control lactate-treatments of TC1 and TC2 yielded the same community structure (according to 1070f-1392r amplified profiles) and considerably different levels of activity. It was apparent that sulfate reduction was occurring in TC2 and was absent in TC1, yet community structure was identical. Multiple primers, and additional methods, are needed to gain a more complete view of community dynamics in response to treatment and with respect to activity.

Whey treatment of column tailings between experimental sampling resulted in the stimulation of specific anaerobic populations in all columns, particularly TCC. This is made evident by the increase in number and intensity of bands of tailings extract. Lactate treatment yielded far more diversity in community structure than did whey-treated samples. This is most likely a response to differences in experimental media and recent treatment of tailings inocula. Increased medium pH, carbon source and micronutrient diversity likely contributed to a more diverse and active community. Community profiles revealed similarities in tailings inocula, and obvious differences between post-lactate-treatment TC1 and TC2 communities, suggesting community response to treatment application is difficult to predict, yet DGGE can measure their differences.

Time of sampling, nutrient medium used, and method of sample monitoring can all have significant affects on resulting sulfate reduction rates and community structure. However, the ability to observe changes in community structure and relate dominant phylogenies to observed activity is available with molecular methods such as DGGE. Practical limitations of DGGE do not allow for complete confirmation of target phylogenies present in endemic communities prior to treatment. Nor does DGGE alone predict community response to treatment based on profiled community structure. Additional methods must be used to increase confidence in predicted results of specific treatment applications.

DGGE is an effective method for observing shifts in microbial populations of consortia. Levels of observable diversity however, are quite dependent on oligonucleaotides used in extract amplification. Subsequent identification of isolate bands via sequencing has proven to be quite difficult, yielding poor success rates. Multiple phylogenies having the same melting domain, the possible existence of artifacts, and the overall difficulty of obtaining product free of contaminant DNA can all contribute to failure in phylogenetic identification. Cloning could potentially be used in conjunction with DGGE profiling to circumvent the difficulties of sequencing excised bands, as well as minimizing the laborious nature of screening clone libraries. Once distinct clones are phylogenetically identified, their resulting banding profiles could be used as markers in the identification and tracking of temporal population shifts in response to treatment application.

The work that was done here supports the use of DGGE in identifying and monitoring dynamic consortia structure. While it was not possible to identify all community members in the experiments conducted, the technique nonetheless offers valuable insight into the effects of organic carbon treatment on mine tailings microbiota. The use of multiple methods, such as DGGE, cloning and culturing, could enhance the performance of this method through the comparison of combined clones with direct community DGGE profiles of pre- and post-treatment communities.

It is important to understand that community profiles obtained in this and future work are merely snapshots of the microbial community at a single time. With respect to this research, profiles represent the structure of treated communities at two specific time points, revealing a limited view of successional shifts in community populations over the duration of treatment. A more representative model of community dynamics in response to treatment may require a timeline of community changes, which could in turn be correlated to specific events in community activity (e.g. sulfate spike at approximately 300 hours observed in serum vial microcosms).

CHAPTER 4

CONCLUSION

The purpose of this research was to evaluate the potential use of molecular methodologies for the monitoring of microbial community shifts in response to addition of an organic carbon source and in relation to community activity. This was accomplished by observing genetic profiles of community DNA extract, using denaturant gradient gel electrophoresis (DGGE), collected from pre- and post-treatment communities. Batch cultures inoculated with acid generating mine tailings were independently treated with whey and lactate. Sulfate reduction and hydrogen sulfide production were measured for each treatment, respectively. Predominant phylogenies isolated in the genetic profile were sequenced in an attempt to identify those phylogenies.

This research required the development and optimization of molecular methods necessary in obtaining representative community profiles. Sample chemistry proved problematic in obtaining complete and purified DNA extract. Template amplification and electrophoretic conditions of primers selected for DGGE analysis of were optimized to produce well defined, representative community profiles. These methods were than applied to observe temporal shifts of community structure in response to carbon source treatment. Differences in community structure were compared to observed differences in activity, including rates of sulfate reduction.

A listing of each objective presented in Chapter 1 and the success in achieving those objectives in subsequent chapters is presented below:

* Select and optimize molecular methods that will provide representative phylogenies of endemic microbial consortia within sampled mine tailings.

Throughout the optimization and development of the molecular methods (Chapter 2) it was evident that community profiles are highly dependent on the methods used, including sample preparation, DNA extraction and purification, primer selection, PCR conditions, and DGGE gel matrix composition. The optimized methods used in Chapter 3 were successful in identifying representative community profiles of endemic microbial consortia in pre- and post-treatment mine tailings. They were not, however, always successful in identifying the presence of stimulated populations prior to organic carbon treatment. This does not suggest a limitation of DGGE analysis, but the potential effect of treatment on numerically minor populations.

* Measure the kinetic response of indigenous SRB populations from sampled tailings to organic carbon treatment application.

The addition of both whey and lactate resulted in the reduction of sulfate. Experimental design limited rate measurements to whey treated microcosms.

Variable sulfate reduction rates were observed between replicates suggesting heterogeneity in sampled SRB population structure and activity. Increased effluent pH, the presence of H_2S gas, the precipitation of black FeS, and stimulation of known SRB resulted in lactate treatment of tailings inocula. Independent of strong differences in structure, similar sulfate reduction rates were observed in whey treated serum vial microcosms and bench-scale column effluent.

* Determine the dynamics of community structure and identify specific phylogenies resulting from treatment application.

Shifts in community structure in response to organic carbon treatment were observed in all samples, including whey treatment of bench-scale columns. Both whey and lactate treatment resulted in the selection of specific phylogenies evident in tailings inocula. Lactate treatment resulted in selection of known SRB phylogenies, whereas DGGE profiles of whey treatments suggest the selection of *Clostridium* species. Limited correlations between community structure and sulfate reducing activity were apparent. Stimulated community structure can be identified from pre-treatment inocula with some limitation.

This paper provides strong evidence supporting the application of DGGE as a community profiling technique to monitor shifts of endemic microbial communities

in response to changing environmental conditions. Methods were optimized to obtain representative community profiles of the microbiota endemic to mine tailings, an extremely hostile environment and difficult medium from which to extract useful DNA. This supports an increased confidence in the use of these techniques to monitor microbial community structure in nearly any environment. Though limitations and biases are exist, a thorough understanding of the processes and a combination of multiple methods, each with their own limitations, can lead to a representative understanding of community dynamics.

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APPENDICES

APPENDIX A

POLYMERASE CHAIN REACTION (PCR) AMPLIFICATION

PRIMERS AND CONDITIONS

16s rDNA Primers

Primer	Sequence (5' –> 3')		
Bac341f	CCT ACG GGA GGC AGC AG	(Bacteria)	Muyzer et al. 1995
Univ907r	CCC CGT CCA TTC CTT TGA GTT T	(Universal)	Muyzer et al. 1995
Bac1070f	ATG GCT GTC GTC AGC T	(Bacteria)	Ferris et al. 1996
Univ1392r	ACG GGC GGT GTG TAC	(Universal)	Ferris et al. 1996
GC-clamp	CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G		Muyzer et al. 1995

PCR Conditions

Primers: 341f - 907r
Program: Fixed Temp.

Temperature (^{0}C)	Time
94^0	5 minutes
25 cycles	
94^0	1 minute
62^0	45 seconds
72^0	1.5 minutes
72^0	10 minutes
4^0	-

Primers: 341f - 907r
Program: Touchdown

Temperature (^{0}C)	Time	
94^0	5 minutes	
20 cycles		
94^0	1 minute	
70^0	45 seconds	Reduce 0.5^0 / cycle
72^0	1.5 minutes	
10 cycles		
94^0	1 minute	
60^0	45 seconds	
72^0	1.5 minutes	
72^0	10 minutes	
4^0	-	

Primers: 1070f - 1392r
Program: Fixed Temp.

Temperature (^{0}C)	Time
94^0	3 minutes
25 cycles	
94^0	45 seconds
60^0	45 seconds
72^0	1.5 minutes
72^0	7 minutes
4^0	-

Primers: 1070f - 1392r
Program: Touchdown

Temperature (^{0}C)	Time	
94^0	3 minutes	
20 cycles		
94^0	45 seconds	
68^0	45 seconds	Reduce 0.5^0 / cycles
72^0	1.5 minutes	
10 cycles		
94^0	45 seconds	
58^0	45 seconds	
72^0	1.5 minutes	
72^0	7 minutes	
4^0	-	

Primers: 341f - 1392r
Program: Fixed Temp.

Temperature (^{0}C)	Time
94^0	10 minutes
25 cycles	
94^0	1 minute
62^0	45 seconds
72^0	2 minutes
72^0	10 minutes
4^0	-

Primers: 341f - 1392r
Program: Touchdown

Temperature (^{0}C)	Time	
94^0	10 minutes	
20 cycles		
94^0	1 minute	
70^0	45 seconds	Reduce 0.5^0 / cycle
72^0	2 minutes	
10 cycles		
94^0	1 minute	
60^0	45 seconds	
72^0	2 minutes	
72^0	10 minutes	
4^0	-	

APPENDIX B

DENATURANT GRADIENT GEL ELECTROPHORESIS (DGGE)

REAGENT PROTOCOLS

DGGE Reagents

40% Acrylamide/Bis (37.5:1)

Reagent	Amount
Acrylamide	38.93 g
Bis-acrylamide	1.07 g
dH2O	to 100.0 ml

Filter through 0.45 um filter and store at 4^0C

50X TAE Buffer

Reagent	Amount	Final Concentration
Tris Base	242.0 g	2 M
Acetic acid, glacial	57.1 ml	1 M
0.5 M EDTA, pH 8.0	100.0 ml	50 mM
dH2O	to 1,000.0 ml	

Mix. Autoclave for 20-30 minutes. Store at room temperature.

40% Denaturing Stock Solution

Reagent	8%	10%
40 % Acrylamide/Bis	20 ml	25 ml
50x TAE buffer	2 ml	2 ml
Formamide (deionized)	16 ml	16 ml
Urea	16.8 g	16.8 g
dH2O	to 100 ml	to 100 ml

Degas for 10 – 15 minutes. Filter through 0.45 um filter. Store at 4^0C in a brown bottle for approximately one month.

70% Denaturing Stock Solution

Reagent	10%	12%
40 % Acrylamide/Bis	25 ml	30 ml
50x TAE buffer	2 ml	2 ml
Formamide (deionized)	28 ml	28 ml
Urea	29.4 g	29.4 g
dH2O	to 100 ml	to 100 ml

Degas for 10 – 15 minutes. Filter through 0.45 um filter. Store at 4^0C in a brown bottle for approximately one month. Place in warm bath and stir to re-dissolve any crystals that may have formed.

10% Ammonium Persulfate

Reagent	Amount
Ammonium persulfate	0.1 g
dH2O	1.0 ml

Store at -20^0C for about a week.

2X Gel Loading Dye

Reagent	Amount	Final Concentration
2% Bromophenol blue	0.25 ml	0.05%
2% Xylene cyanol	0.25 ml	0.05%
100% Glycerol	7.0 ml	70%
dH2O	2.5 ml	
Total Volume	10.0 ml	

Store at room temperature.

1X TAE Running Buffer

Reagent	Amount
50x TAE buffer	140 ml
dH2O	6,860 ml
Total volume	7,000 ml

10% Acrylamide Gel Solution (DGGE)

	Denaturant Concentration	
Reagent	40%	70%
10% Acrylamide stock solution	12.5 ml	12.5 ml
10% Ammonium persulfate	100 ul	100 ul
TEMED	10 ul	10 ul

Add ammonium persulfate and TEMED immediately prior to casting gel.

8 to 12 % Gradient Acrylamide Gel Solution (DGGE)

	Denaturant Concentration	
Reagent	40%	70%
8% Acrylamide stock solution	12.5 ml	-
12% Acrylamide stock solution	-	12.5 ml
10% Ammonium persulfate	100 ul	100 ul
TEMED	10 ul	10 ul

Add ammonium persulfate and TEMED immediately prior to casting gel.

6% Acrylamide Stacking Gel

Reagent	Amount
40 % Acrylamide/Bis	300 ul
1x TAE buffer	1.7 ml
10% Ammonium persulfate	15 ul
TEMED	2 ul
Total Volume	2 ml

Add ammonium persulfate and TEMED immediately prior to casting gel.

APPENDIX C

WHEY TREATEMENT - SERUM VIAL MICROCOSM DATA

Serum Vial Sulfate Reduction Data

Sample: TC1

S =Tails w/ Whev & Autoclaved
W =Tails w/ Whev
C =Tails w/o Whev

0 hrs ppm: SO4-

S1	1282
S2	1121
S3	1121
W1	1161
W2	903
W3	1042
C1	1173
C2	1087
C3	1123

	Average	SD	CV (%)	+	-
S	1175	92.95339	7.913172	107	54
W	1035	129.1291	12.47223	126	132
A	1128	43.1895	3.829989	45	41

S-10 ppm 9.19

18 hrs ppm: SO4-

S1	1103
S2	1148
S3	1596
W1	1360
W2	1032
W3	1130
C1	1106
C2	1158
C3	1296

	Average	SD	CV (%)	+	-
S	1282	272.5735	21.25606	314	179
W	1174	168.3686	14.34145	186	142
A	1187	98.19029	8.274463	109	81

S-10 ppm 9.19

90 hrs ppm: SO4-

S1	1167
S2	1139
S3	1121
W1	1111
W2	1111
W3	1113
C1	1162
C2	1139
C3	1137

	Average	SD	CV (%)	+	-
S	1142	23.18045	2.02922	25	21
W	1112	1.154701	0.103871	1	1
A	1146	13.89244	1.212255	16	9

S-10 ppm 10.24

113 hrs ppm: SO4-

S1	1175
S2	1185
S3	1225
W1	
W2	1196
W3	1153
C1	1188
C2	1239
C3	1213

	Average	SD	CV (%)	+	-
S	1195	26.45751	2.214018	30	20
W	1175	830.7752	70.73437	22	22
A	1213	25.50163	2.101783	26	25

S-10 ppm 10.43

233 hrs ppm: SO4-

S1	1215
S2	1204
S3	1209
W1	1213
W2	1216
W3	1148
C1	1226
C2	1241
C3	1241

	Average	SD	CV (%)	+	-
S	1209	5.507571	0.455422	6	5
W	1192	38.42308	3.222512	24	44
A	1236	8.660254	0.700668	5	10

S-10 ppm 10.58

307 hrs ppm: SO4-

S1	1315
S2	1278
S3	1315
W1	1261
W2	1216
W3	1260
C1	1361
C2	1412
C3	1360

	Average	SD	CV (%)	+	-
S	1303	21.36196	1.639864	12	25
W	1246	25.69695	2.062908	15	30
A	1378	29.73774	2.158559	34	18

S-10 ppm 11.47

479 hrs ppm: SO4-

S1	1180
S2	1234
S3	1189
W1	1162
W2	1106
W3	1131
C1	1240
C2	1282
C3	1227

	Average	SD	CV (%)	+	-
S	1201	28.93095	2.408905	33	21
W	1133	28.05352	2.476039	29	27
A	1250	28.74601	2.300295	32	23

S-10 ppm 10.38

597 hrs ppm: SO4-

S1	1169
S2	1167
S3	1147
W1	1107
W2	1037
W3	851
C1	1225
C2	1281
C3	1252

	Average	SD	CV (%)	+	-
S	1161	12.16553	1.047849	8	14
W	998	132.3077	13.25286	109	147
A	1253	28.00595	2.235707	28	28

S-10 ppm 11.08

815 hrs ppm: SO4-

S1	1135
S2	1139
S3	1134
W1	1070
W2	894
W3	573
C1	1164
C2	1215
C3	1219

	Average	SD	CV (%)	+	-
S	1136	2.645751	0.232901	3	2
W	846	252.0007	29.79905	224	273
A	1199	30.66486	2.556825	20	35

S-10 ppm 11.02

Time (hr)	S	+	-	W	+	-	C	+	-
0	1175	107	54	1035	126	132	1128	45	41
18	1282	314	179	1174	186	142	1187	109	81
90	1142	25	21	1112	1	1	1146	16	9
113	1195	30	20	1175	22	22	1213	26	25
233	1209	6	5	1192	24	44	1236	5	10
307	1303	12	25	1246	15	30	1378	34	18
479	1201	33	21	1133	29	27	1250	32	23
597	1161	8	14	998	109	147	1253	28	28
815	1136	3	2	846	224	273	1199	20	35

Sulfate reduction rates obserevd between 479 and 597 hours

Sterile			Whev			Control		
r_1=	2.2	mg L^{-1} d^{-1}	r_1=	11.2	mg L^{-1} d^{-1}	r_1=	3.1	mg L^{-1} d^{-1}
r_2=	13.6	mg L^{-1} d^{-1}	r_2=	14.0	mg L^{-1} d^{-1}	r_2=	11.6	mg L^{-1} d^{-1}
r_3=	8.5	mg L^{-1} d^{-1}	r_3=	57.0	mg L^{-1} d^{-1}	r_3=	-5.1	mg L^{-1} d^{-1}

Sample: TC2

S =Tails w/ Whev & Autoclaved
W =Tails w/ Whev
C =Tails w/o Whev

0 hrs ppm: SO4-

S1	978
S2	965
S3	1021
W1	987
W2	934
W3	1054
C1	1256
C2	1156
C3	1078

	Average	SD	CV (%)	+	-
S	988	29.309	2.9665	33	23
W	992	60.136	6.0641	62	58
A	1163	89.226	7.6699	93	85

S-10 ppm 9.4

18 hrs ppm: SO4-

S1	1323
S2	1033
S3	1523
W1	1364
W2	1131
W3	1954
C1	1972
C2	1656
C3	1548

	Average	SD	CV (%)	+	-
S	1293	246.37	19.054	230	260
W	1483	424.21	28.605	471	352
A	1725	220.34	12.771	247	177

S-10 ppm 9.4

90 hrs ppm: SO4-

S1	1628
S2	1277
S3	1331
W1	1402
W2	1261
W3	1277
C1	1335
C2	1280
C3	1319

	Average	SD	CV (%)	+	-
S	1412	189	13.385	216	135
W	1313	77.203	5.8784	89	52
A	1311	28.29	2.1574	24	31

S-10 ppm 9.91

113 hrs ppm: SO4-

S1	1352
S2	1273
S3	1299
W1	1305
W2	1317
W3	1347
C1	1406
C2	1388
C3	1318

	Average	SD	CV (%)	+	-
S	1308	40.262	3.0781	44	35
W	1323	21.633	1.6352	24	18
A	1371	46.49	3.3918	35	53

S-10 ppm 10.39

233 hrs ppm: SO4-

S1	1353
S2	1366
S3	1384
W1	1363
W2	1300
W3	1352
C1	1471
C2	1451
C3	1409

	Average	SD	CV (%)	+	-
S	1368	15.567	1.1382	16	15
W	1338	33.65	2.5143	25	38
A	1444	31.644	2.1919	27	35

S-10 ppm 10.67

307 hrs ppm: SO4-

S1	1512
S2	1468
S3	1535
W1	1402
W2	1414
W3	1394
C1	1748
C2	1668
C3	1605

	Average	SD	CV (%)	+	-
S	1505	34.044	2.2621	30	37
W	1403	10.066	0.7173	11	9
A	1674	71.668	4.2821	74	69

S-10 ppm 11.43

479 hrs ppm: SO4-

S1	1351
S2	1387
S3	1425
W1	939
W2	1128
W3	958
C1	1501
C2	1454
C3	1448

	Average	SD	CV (%)	+	-
S	1388	37.005	2.6667	37	37
W	1008	104.07	10.321	120	69
A	1468	29.023	1.9775	33	20

S-10 ppm 10.7

597 hrs ppm: SO4-

S1	1219
S2	1299
S3	1271
W1	593
W2	1048
W3	598
C1	1475
C2	1452
C3	1440

	Average	SD	CV (%)	+	-
S	1263	40.596	3.2142	36	44
W	746	261.26	35.006	302	153
A	1456	17.786	1.2218	19	16

S-10 ppm 11.11

815 hrs ppm: SO4-

S1	1275
S2	1271
S3	1326
W1	581
W2	807
W3	545
C1	1338
C2	1376
C3	1352

	Average	SD	CV (%)	+	-
S	1291	30.665	2.3759	35	20
W	644	142.02	22.041	163	99
A	1355	19.218	1.418	21	17

S-10 ppm 11.21

Time (hr)	6A	+	-	6W	+	-	6S	+	-
0	988	33	23	992	62	58	1163	93	85
18	1293	230	260	1483	471	352	1725	247	177
90	1412	216	135	1313	89	52	1311	24	31
113	1308	44	35	1323	24	18	1371	35	53
233	1368	16	15	1338	25	38	1444	27	35
307	1505	30	37	1403	11	9	1674	74	69
479	1388	37	37	1008	120	69	1468	33	20
597	1263	36	44	746	302	153	1456	19	16
815	1291	35	20	644	163	99	1355	21	17

Sulfate reduction rates obserevd between 479 and 597 hours

Sterile			Whev			Control		
r_1=	5.3	mg L^{-1} d^{-1}	r_1=	16.3	mg L^{-1} d^{-1}	r_1=	26.9	mg L^{-1} d^{-1}
r_2=	-4.3	mg L^{-1} d^{-1}	r_2=	70.4	mg L^{-1} d^{-1}	r_2=	17.9	mg L^{-1} d^{-1}
r_3=	1.6	mg L^{-1} d^{-1}	r_3=	73.2	mg L^{-1} d^{-1}	r_3=	31.3	mg L^{-1} d^{-1}

Sample: TCC

S =Tails w/ Whev & Autoclaved
W =Tails w/ Whev
C =Tails w/o Whev

0 hrs ppm: SO4-

S1	1545
S2	1452
S3	1490
W1	1487
W2	1516
W3	1573
C1	1506
C2	1623
C3	1573

	Average	SD	CV (%)	+	-
S	1496	46.75824	3.126248	49	44
W	1525	43.7531	2.868428	48	38
A	1567	58.70548	3.745564	56	61

S-10 ppm 9.46

18 hrs ppm: SO4-

S1	1997
S2	2538
S3	2273
W1	1985
W2	2546
W3	2345
C1	2257
C2	2812
C3	2351

	Average	SD	CV (%)	+	-
S	2269	270.5186	11.92062	269	272
W	2292	284.2305	12.40098	254	307
A	2473	297.0359	12.00954	339	216

S-10 ppm 9.46

90 hrs ppm: SO4-

S1	2050
S2	2132
S3	2597
W1	2209
W2	1982
W3	1887
C1	2029
C2	1931
C3	2041

	Average	SD	CV (%)	+	-
S	2260	295.0023	13.05512	337	210
W	2026	165.4479	8.166233	183	139
A	2000	60.34346	3.01667	41	69

S-10 ppm 10.23

113 hrs ppm: SO4-

S1	2128
S2	2157
S3	2124
W1	2105
W2	2210
W3	1948
C1	2008
C2	1993
C3	2050

	Average	SD	CV (%)	+	-
S	2136	18.00926	0.842998	21	12
W	2088	131.8572	6.316011	122	140
A	2017	29.54657	1.464877	33	24

S-10 ppm 10.42

233 hrs ppm: SO4-

S1	2065
S2	2189
S3	2132
W1	1891
W2	1948
W3	2144
C1	2129
C2	2114
C3	1935

	Average	SD	CV (%)	+	-
S	2129	62.06717	2.915777	60	64
W	1994	132.7115	6.654428	150	103
A	2059	107.9367	5.241342	70	124

S-10 ppm 10.65

307 hrs ppm: SO4-

S1	2734
S2	2654
S3	2715
W1	2347
W2	2237
W3	2542
C1	2655
C2	2643
C3	2692

S-10 ppm 11.45

	Average	SD	CV (%)	+	-
S	2701	41.79713	1.547469	33	47
W	2375	154.4614	6.502726	167	138
A	2663	25.54082	0.958979	29	20

479 hrs ppm: SO4-

S1	2241
S2	2210
S3	2235
W1	2009
W2	2027
W3	2228
C1	2256
C2	2227
C3	2253

S-10 ppm 10.88

	Average	SD	CV (%)	+	-
S	2229	16.44182	0.737742	12	19
W	2088	121.5771	5.82266	140	79
A	2245	15.94783	0.710266	11	18

597 hrs ppm: SO4-

S1	2143
S2	2195
S3	2307
W1	1952
W2	2000
W3	2155
C1	2152
C2	2162
C3	2188

S-10 ppm 11.7

	Average	SD	CV (%)	+	-
S	2215	83.80931	3.783716	92	72
W	2036	106.0959	5.211849	119	84
A	2167	18.58315	0.85742	21	15

815 hrs ppm: SO4-

S1	2165
S2	2214
S3	2103
W1	2026
W2	2023
W3	2192
C1	2147
C2	2187
C3	2245

S-10 ppm 11.55

	Average	SD	CV (%)	+	-
S	2161	55.62673	2.574517	53	58
W	2080	96.7178	4.649149	112	57
A	2193	49.27474	2.24691	52	46

Time (hr)	5S	+	-	5W	+	-	5C	+	-
0	1496	49	44	1525	48	38	1567	56	61
18	2269	269	272	2292	254	307	2473	339	216
90	2260	337	210	2026	183	139	2000	41	69
113	2136	21	12	2088	122	140	2017	33	24
233	2129	60	64	1994	150	103	2059	70	124
307	2701	33	47	2375	167	138	2663	29	20
479	2229	12	19	2088	140	79	2245	11	18
597	2215	92	72	2036	119	84	2167	21	15
815	2161	53	58	2080	112	57	2193	52	46

Sulfate reduction rates observed between 479 and 597 hours

Sterile			Whey			Control		
r_1=	19.9	mg L^{-1} d^{-1}	r_1=	11.6	mg L^{-1} d^{-1}	r_1=	21.2	mg L^{-1} d^{-1}
r_2=	3.1	mg L^{-1} d^{-1}	r_2=	5.5	mg L^{-1} d^{-1}	r_2=	15.3	mg L^{-1} d^{-1}
r_3=	-14.6	mg L^{-1} d^{-1}	r_3=	14.8	mg L^{-1} d^{-1}	r_3=	13.2	mg L^{-1} d^{-1}

APPENDIX D

LACTATE TREATMENT – RESPIROMETER MICROCOSM DATA

pH / Sulfate Data

Postgate B Medium pH = 5.5

Microcosm Effluent Post-treatment pH

	Replicate			Control
	1	2	3	
TC1	5.15	6.38	6.18	5.09
TC2	7.93	8.29	7.82	8.13
TCC	5.715	6.03	5.72	5.27

Postgate Sulfate Concentration (mg/L) = 1343

Microcosm Sulfate Concentration (mg/L)

	Replicate			Control
	1	2	3	
TC1	2022	1746	1417	1358
TC2	1265	851	1459	950
TCC	2308	1990	2112	2220

Microcosm Sulfate Reduction Rates (mg/L/d)

	Replicate			Control
	1	2	3	
TC1	-30.9	-18.3	-3.4	-0.7
TC2	3.5	22.4	-5.3	17.9
TCC	-43.9	-29.4	-35.0	-39.9

Example Calculation for Sulfate Reduction from Hydrogen Sulfide Formation

Equations:

$$mg\ H_2S_{(g)} = mg/L\ H_2S_{(g)} * (V_{hs}/1000)$$
$$mg/L\ H_2S_{(aq)} = mg/L\ H_2S_{(g)} * 2.77$$
$$mg\ H_2S_{(aq)} = mg/L\ H_2S_{(aq)} * (V_m/1000)$$
$$mg\ dH_2S_{(aq)} = mg\ H_2S_{(aq)_t} + mg\ H_2S_{(g)t} - mg\ H_2S_{(aq)t-1}$$
$$mg\ dSO_{4(aq)} = mg\ dH_2S_{(aq)} * (-MW_{SO4}/MW_{H2S})$$
$$mg/L/d\ SO_4 = mg\ dSO_{4(aq)}/(V_m/1000)$$

Measured Values:

0 mg/L/h = H_2S(g) production rate (t = 13)
0.0002 mg/L/h = H_2S(g) production rate (t = 14)
36 ml = Headspace Volume
15 ml = Effluent Volume
34 g/mole = Hydrogen Sulfide Molecular Weight
96 g/mole = Sulfate Molecular Weight
2.77 g/g = Henry's Law Constant; volumetric (Metcalf and Eddy, 2003)

Solution:

$$0.0002\ mg/L/h * 24\ h = 0.0048\ mg/L\ H_2S_{(g)}$$
$$0.0048\ mg/L * \left(\frac{36\ mL}{1000\ mL/L}\right) = 0.0001728\ mg\ H_2S_{(g)}$$
$$0.0048\ mg/L\ H_2S_{(g)} * 2.77\ \frac{mg/L(aq)}{mg/L(g)} = 0.013296\ mg/L\ H_2S_{(aq)}$$
$$0.013296\ mg/L\ H_2S_{(aq)} * \left(\frac{15\ mL}{1000\ mL/L}\right) = 0.00019944\ mg\ H_2S_{(aq)}$$
$$0.00019944\ mg\ H_2S_{(aq)_t} + 0.0001728\ mg\ H_2S_{(g)_t} - 0\ mg\ H_2S_{(g)_{t-1}} = 0.00037224\ mg\ dH_2S_{(aq)}$$
$$0.00037224\ mg\ dH_2S_{(aq)} * \left(-\frac{96\ g/mole}{34\ g/mole}\right) = -0.001051\ mg\ SO_{4\ (aq)}^{-}$$
$$-0.0010151\ mg\ SO_{4\ (aq)}^{-} * \left(\frac{1000\ mL/L}{15\ mL}\right) = -0.07\ mg/L/d\ SO_{4\ (aq)}^{-}$$

Respirometry Data

Columbus Instruments Micro-Oxymax v6.06e
Experiment File: C:\MICRO6\MCB-H2S.DAT
Start of Experiment: Fri Oct 22 09:59:43 2004

EXPERIMENT PARAMETERS:

Channels: Channels in Experiment (1 - 20) Start: 1 End: 13
File : Path Name for experiment file C:\MICRO6\
: File Name for experiment file (XXXXXXXX.DAT) MCB-H2S
Timing : Sample interval for experiment (HHH:MM:SS) 024:00:00
: Duration of experiment in minutes 0000
Purge : Purge sensors between measurements Y
Refresh : Refresh threshold (% O2 or CO2) 0.00
: Refresh experiment after this many samples (2 - 99) 0
: Refresh window (seconds) 60
Temps : Starting chamber to use auxiliary temperature probe 0
Volumes : Automatically measure chamber volumes in this experiment Y
: Sensor Volume remeasurement interval (0=Do not use) 0
Data : 0=Point Decimal 1=Comma Decimal 0
Units : Gas Measurement units (1=ul 2=ml 3=mg 4=ug 5=uM) 3
: Time units (1=minutes 2=hours) 2
: Normalize units (0=No normalize 1=gm 2=kg 3=ml 4=l) 4
: Measure O2 consumption as a positive number Y
Yield : Calculate % of Theoretical Maximum Yield N
: Starting Channel used for control 0 = not used 0
: Number of channels to use for control (0-4) 0
Multi Experiment Mode. Drier: Switched.
Barometric Pressure: 607 mmHg Sensor Pressure: 670 mmHg

MEASURED CHAMBER DATA:

Chamber	Headspace (L)
1	35
2	36
3	34
4	34
5	32
6	32
7	47
8	33
9	1
10	31
11	32
12	33
13	10039

Respirometer Data

Intv	Cham	Time	Temp	RER	OvrPres	% O2	mg/l/hr O2	mg/l O2	OvrRng	% CO2	mg/l/hr CO2	mg/l CO2	OvrRng	% H2S	mg/l/hr H2S	mg/l H2S	OvrRng	mg/l H2S(g)	mg H2S (g)	mg/l H2S(aq)	mg H2S (aq)	mg dH2S	mg dSO4	mg/l/d dSO4
1	TC1(1)	10/23/2004 10:38	22.2	-0.2		0.771	-0.01414	-0.3487		0.044	0.00293	0.0723		0	0	0		0	0	0	0	0	0	0
2	TC1(1)	10/24/2004 10:38	22.2	0		0.414	-0.03006	-1.0702		0.046	0.00006	0.0737		0	0	0		0	0	0	0	0	0	0
3	TC1(1)	10/25/2004 10:38	22.2	0.02		0.371	-0.03224	-1.844		0.043	-0.00106	0.0483		0	0	0		0	0	0	0	0	0	0
4	TC1(1)	10/26/2004 10:38	22.2	0.05		0.365	-0.03094	-2.5867		0.046	-0.00214	-0.0029		0	0	0		0	0	0	0	0	0	0
5	TC1(1)	10/27/2004 10:38	22.4	0.04		0.365	-0.03229	-3.3617		0.048	-0.00178	-0.0457		0	0	0		0	0	0	0	0	0	0
6	TC1(1)	10/28/2004 10:38	22	0.06		0.364	-0.031	-4.1058		0.043	-0.00265	-0.1092		0	0	0		0	0	0	0	0	0	0
7	TC1(1)	10/29/2004 10:38	22.1	0.08	*	0.317	-0.02718	-4.7582		0.039	-0.00298	-0.1807		0	0	0		0	0	0	0	0	0	0
8	TC1(1)	10/30/2004 10:38	22.3	0.07		0.37	-0.03277	-5.5446		0.033	-0.00299	-0.2526		0	0	0		0	0	0	0	0	0	0
9	TC1(1)	10/31/2004 11:50	22.2	0.03		0.421	-0.03536	-6.471		0.032	-0.00152	-0.2923		0	0	0		0	0	0	0	0	0	0
10	TC1(1)	11/1/2004 9:38	22.2	0.02	*	0.813	-0.08243	-8.268		0.031	-0.00256	-0.3481		0	0	0		0	0	0	0	0	0	0
11	TC1(1)	11/2/2004 9:38	22.3	0		0.45	-0.03491	-9.1058		0.027	0.00001	-0.3478		0	0	0		0	0	0	0	0	0	0
12	TC1(1)	11/3/2004 9:38	22.7	0.01		0.4	-0.03366	-9.9136		0.026	-0.00053	-0.3604		0	0	0		0	0	0	0	0	0	0
13	TC1(1)	11/4/2004 9:38	22.5	0		0.378	-0.03322	-10.711		0.028	0.00014	-0.3571		0	0	0		0	0	0	0	0	0	0
14	TC1(1)	11/5/2004 9:38	22.4	0	*	0.343	-0.02868	-11.3993		0.027	0	-0.3571		0	0	0		0	0	0	0	0	0	0
15	TC1(1)	11/6/2004 9:38	22.3	0		0.393	-0.03591	-12.2612		0.027	0.00047	-0.3459		0	0	0		0	0	0	0	0	0	0
16	TC1(1)	11/7/2004 9:38	22.2	0	*	0.341	-0.02808	-12.9351		0.024	0.00005	-0.3448		0	0	0		0	0	0	0	0	0	0
17	TC1(1)	11/8/2004 9:38	22.2	0	*	0.357	-0.03195	-13.702		0.026	0.00066	-0.329		0	0	0		0	0	0	0	0	0	0
18	TC1(1)	11/9/2004 9:38	22.4	0		0.39	-0.03409	-14.5202		0.028	0.0008	-0.3097		0	0	0		0	0	0	0	0	0	0
19	TC1(1)	11/10/2004 9:38	22.4	0		0.379	-0.03338	-15.3213		0.034	0.00167	-0.2696		0	0	0		0	0	0	0	0	0	0
20	TC1(1)	11/11/2004 9:38	22.2	0		0.373	-0.03158	-16.0792		0.039	0.00203	-0.2208		0	0	0		0	0	0	0	0	0	0
21	TC1(1)	11/12/2004 9:38	22.2	-0.1		0.366	-0.03214	-16.8507		0.05	0.00358	-0.1348		0	0	0		0	0	0	0	0	0	0
22	TC1(1)	11/13/2004 9:38	22.2	-0.1		0.349	-0.02923	-17.5522		0.071	0.00591	0.0072		0	0	0		0	0	0	0	0	0	0
1	TC1(2)	10/23/2004 10:49	22.2	-0.3		0.551	-0.01045	-0.2596		0.036	0.00478	0.1186		0	0	0		0	0	0	0	0	0	0
2	TC1(2)	10/24/2004 10:49	22.2	-0.2	*	0.243	-0.01348	-0.5832		0.036	0.00407	0.2163		0	0	0		0	0	0	0	0	0	0
3	TC1(2)	10/25/2004 10:49	22.2	-0.6		0.189	-0.01344	-0.9057		0.085	0.01176	0.4985		0	0	0		0	0	0	0	0	0	0
4	TC1(2)	10/26/2004 10:49	22.2	-4.6		0.095	-0.00369	-0.9942		0.173	0.02322	1.0558		0	0	0		0	0	0	0	0	0	0
5	TC1(2)	10/27/2004 10:49	22.4	-14	*	0.058	-0.00134	-1.0265		0.203	0.0267	1.6967		0	0	0		0	0	0	0	0	0	0
6	TC1(2)	10/28/2004 10:49	22	-4.6		0.081	-0.0043	-1.1296		0.215	0.02688	2.3418		0	0	0		0	0	0	0	0	0	0
7	TC1(2)	10/29/2004 10:49	22.2	36.5	*	0.036	0.00055	-1.1164		0.214	0.02756	3.0033		0	0	0		0	0	0	0	0	0	0
8	TC1(2)	10/30/2004 10:49	22.4	-29	*	0.045	-0.00066	-1.1323		0.214	0.02663	3.6424		0	0	0		0	0	0	0	0	0	0
9	TC1(2)	10/31/2004 13:32	22.2	-2.4		0.128	-0.00827	-1.3614		0.239	0.02718	4.3955		0	0	0		0	0	0	0	0	0	0
10	TC1(2)	11/1/2004 9:49	22.4	-4.1	*	0.116	-0.00495	-1.4618		0.196	0.0278	4.9595		0	0	0		0	0	0	0	0	0	0
11	TC1(2)	11/2/2004 9:49	22.2	-22	*	0.063	-0.00097	-1.4852		0.223	0.02936	5.664		0	0	0		0	0	0	0	0	0	0
12	TC1(2)	11/3/2004 9:49	22.5	-3.9		0.092	-0.00511	-1.6078		0.222	0.02762	6.3269		0	0	0		0	0	0	0	0	0	0
13	TC1(2)	11/4/2004 9:49	22.5	-29	*	0.054	-0.00082	-1.6275		0.247	0.03253	7.1078		0	0	0		0	0	0	0	0	0	0
14	TC1(2)	11/5/2004 9:49	22.5	-77	*	0.051	-0.00127	-1.658		0.949	0.13526	10.354	X	0.0018	0.0002	18		0.0048	0.0001728	0.013296	0.00019944	0.0003722	0.00105103	0.070068706
15	TC1(2)	11/6/2004 9:49	22.3	-54	*	0.052	-0.00195	-1.7048		0.95	0.14517	13.838	X	0.0001	0.00001	1		0.00024	0.00000792	0.0006648	0.000009972	-0.000182	-0.00051261	-0.034173741
16	TC1(2)	11/7/2004 9:49	22.2	-19		0.072	-0.00423	-1.8064		0.762	0.11283	16.5458		0	0	0		0	0	0	0	-9.97E-06	-2.8156E-05	-0.001877082
17	TC1(2)	11/8/2004 9:49	22.2	113	*	0.038	0.00042	-1.7962		0.541	0.06556	18.1192		0	0	0		0	0	0	0			
18	TC1(2)	11/9/2004 9:49	22.4	-9.4		0.08	-0.00429	-1.8992		0.456	0.05533	19.4472		0	0	0		0	0	0	0	0	0	0
19	TC1(2)	11/10/2004 9:49	22.4	-59	*	0.051	-0.00074	-1.9171		0.461	0.06002	20.8877		0	0	0		0	0	0	0	0	0	0
20	TC1(2)	11/11/2004 9:49	22.2	-16		0.07	-0.00314	-1.9924		0.528	0.06772	22.513		0.0001	0.00001	1		0.00024	0.00000792	0.0006648	0.000009972	1.789E-05	5.0519E-05	0.003367906
21	TC1(2)	11/12/2004 9:49	22.2	-49	*	0.054	-0.00132	-2.0241		0.661	0.08892	24.647		0.0002	0.00003	2		0.00072	0.00002376	0.0019944	0.000029916	4.37E-05	0.0001234	0.008226635
22	TC1(2)	11/13/2004 9:49	22.2	-20		0.07	-0.00323	-2.1017		0.704	0.08928	26.7896		0.0003	0.00002	3		0.00048	0.00001584	0.0013296	0.000019944	5.868E-06	1.6568E-05	0.001104565

Intv	Cham	Time	Temp	RER	OvrPres	% O2	mg/l/hr O2	mg/l O2	OvrRng	% CO2	mg/l/hr CO2	mg/l CO2	OvrRng	% H2S	mg/l/hr H2S	mg/l H2S	OvrRng	mg/l H2S(g)	mg H2S (g)	mg/l H2S(aq)	mg H2S (aq)	mg dH2S	mg dSO4	mg/l/d dSO4
1	TC1(3)	10/23/2004 11:00	22.2	-0.1		0.715	-0.03487	-0.8724		0.035	0.00469	0.1172		0	0	0		0	0	0	0			
2	TC1(3)	10/24/2004 11:00	22.2	-0.1		0.528	-0.04287	-1.9013		0.034	0.00398	0.2126		0	0	0		0	0	0	0	0	0	0
3	TC1(3)	10/25/2004 11:00	22.2	-0.2		0.472	-0.0415	-2.8974		0.069	0.009	0.4287		0	0	0		0	0	0	0	0	0	0
4	TC1(3)	10/26/2004 11:00	22.1	-1.1		0.308	-0.0239	-3.471		0.258	0.03501	1.2691		0	0	0		0	0	0	0	0	0	0
5	TC1(3)	10/27/2004 11:00	22.4	-4.4		0.161	-0.01141	-3.7447		0.497	0.06822	2.9063		0	0	0		0	0	0	0	0	0	0
6	TC1(3)	10/28/2004 11:00	22.2	-5.2		0.131	-0.00981	-3.9802		0.563	0.07059	4.6006		0	0	0		0	0	0	0	0	0	0
7	TC1(3)	10/29/2004 11:00	22.2	-5.9		0.122	-0.00989	-4.2176		0.615	0.07968	6.5129		0	0	0		0	0	0	0	0	0	0
8	TC1(3)	10/30/2004 11:00	22.3	-5.9		0.117	-0.00907	-4.4353		0.596	0.07299	8.2646		0	0	0		0	0	0	0	0	0	0
9	TC1(3)	10/31/2004 15:14	22.2	-4.2		0.193	-0.01435	-4.8545		0.751	0.08188	10.6576		0	0	0		0	0	0	0	0	0	0
10	TC1(3)	11/1/2004 10:00	22.2	-10		0.129	-0.01142	-5.069		0.949	0.1568	13.6017	X	0.0009	0.00013	9		0.00312	0.00010608	0.0086424	0.000129636	0.0002357	0.00066555	0.044370071
11	TC1(3)	11/2/2004 10:00	22.2	-8.7		0.124	-0.01188	-5.3542		0.949	0.14247	17.0209	X	0	0	0		0	0	0	0	-0.00013	-0.00036603	-0.024402071
12	TC1(3)	11/3/2004 10:00	22.4	-8.1		0.133	-0.01226	-5.6486		0.943	0.13714	20.3122	X	0	0	0		0	0	0	0	0	0	0
13	TC1(3)	11/4/2004 10:00	22.5	-6.7		0.124	-0.01194	-5.9352		0.74	0.11012	22.9551		0	0	0		0	0	0	0	0	0	0
14	TC1(3)	11/5/2004 10:00	22.4	-6.3		0.123	-0.00973	-6.1687		0.728	0.0838	24.9664		0	-0.00001	0		-0.00024	-0.00000816	-0.0006648	-0.000009972	-1.81E-05	-5.1196E-05	-0.003413082
15	TC1(3)	11/6/2004 10:00	22.2	-5.6		0.126	-0.01033	-6.4167		0.675	0.07916	26.8663		0	0	0		0	0	0	0	9.972E-06	2.8156E-05	0.001877082
16	TC1(3)	11/7/2004 10:00	22.2	-6		0.122	-0.00939	-6.6422		0.667	0.07761	28.7289		0	0	0		0	0	0	0	0	0	0
17	TC1(3)	11/8/2004 10:00	22.3	-7.1		0.112	-0.00896	-6.8572		0.696	0.08711	30.8196		0	0	0		0	0	0	0	0	0	0
18	TC1(3)	11/9/2004 10:00	22.3	-7.7		0.124	-0.00985	-7.0937		0.831	0.10408	33.3175		0	0	0		0	0	0	0	0	0	0
19	TC1(3)	11/10/2004 10:00	22.5	-8.9		0.123	-0.0101	-7.3362		0.949	0.1231	36.272	X	0	0	0		0	0	0	0	0	0	0
20	TC1(3)	11/11/2004 10:00	22.3	-9.2		0.117	-0.01075	-7.5942		0.949	0.13548	39.5237	X	0.0001	0.00001	1		0.00024	0.00000816	0.0006648	0.000009972	1.813E-05	5.1196E-05	0.003413082
21	TC1(3)	11/12/2004 10:00	22.2	-9.2		0.116	-0.01101	-7.8585		0.949	0.1387	42.8524	X	0	0	0		0	0	0	0	-9.97E-06	-2.8156E-05	-0.001877082
22	TC1(3)	11/13/2004 10:00	22.2	-8.5		0.124	-0.01142	-8.1326		0.95	0.13405	46.0695	X	0	0	0		0	0	0	0	0	0	0
1	TCC(1)	10/23/2004 11:11	22.2	-0.3		0.537	-0.01291	-0.3253		0.036	0.0047	0.1185		0	0	0		0	0	0	0	0	0	0
2	TCC(1)	10/24/2004 11:11	22.4	-0.2		0.259	-0.01494	-0.6839		0.033	0.00376	0.2087		0	0	0		0	0	0	0	0	0	0
3	TCC(1)	10/25/2004 11:11	22.2	-0.2		0.225	-0.01659	-1.0822		0.032	0.0036	0.2951		0	0	0		0	0	0	0	0	0	0
4	TCC(1)	10/26/2004 11:11	22	-0.2	*	0.18	-0.01264	-1.3855		0.038	0.00267	0.3592		0	0	0		0	0	0	0	0	0	0
5	TCC(1)	10/27/2004 11:11	22.4	-0.1		0.197	-0.0166	-1.7839		0.049	0.00236	0.4158		0	0	0		0	0	0	0	0	0	0
6	TCC(1)	10/28/2004 11:11	22.2	-0.1		0.192	-0.01538	-2.1531		0.049	0.00135	0.4482		0	0	0		0	0	0	0	0	0	0
7	TCC(1)	10/29/2004 11:11	22.2	-0.1	*	0.157	-0.0122	-2.4458		0.049	0.0012	0.4771		0	0	0		0	0	0	0	0	0	0
8	TCC(1)	10/30/2004 11:11	22.4	0	*	0.158	-0.01243	-2.7441		0.046	0.00064	0.4925		0	0	0		0	0	0	0	0	0	0
9	TCC(1)	10/31/2004 16:56	22.2	-0.1		0.274	-0.01967	-3.3486		0.061	0.00149	0.5382		0	0	0		0	0	0	0	0	0	0
10	TCC(1)	11/1/2004 10:11	22.1	-0.2	*	0.17	-0.01656	-3.6345		0.091	0.00564	0.6355		0	-0.00001	0		-0.00024	-0.00000792	-0.0006648	-0.000009972	-1.79E-05	-5.0519E-05	-0.003367906
11	TCC(1)	11/2/2004 10:11	22.2	-0.1	*	0.173	-0.01433	-3.9785		0.081	0.00234	0.6918		0	0	0		0	0	0	0	0	0	0
12	TCC(1)	11/3/2004 10:11	22.3	0		0.208	-0.01738	-4.3955		0.065	-0.00006	0.6902		0	0	0		0	0	0	0	0	0	0
13	TCC(1)	11/4/2004 10:11	22.5	-0.1	*	0.173	-0.01371	-4.7246		0.06	0.00139	0.7236		0	0	0		0	0	0	0	0	0	0
14	TCC(1)	11/5/2004 10:11	22.3	-0.1		0.191	-0.01569	-5.1011		0.065	0.00214	0.7749		0	0	0		0	0	0	0	0	0	0
15	TCC(1)	11/6/2004 10:11	22.3	-0.2	*	0.17	-0.01365	-5.4289		0.077	0.00456	0.8845		0	0	0		0	0	0	0	0	0	0
16	TCC(1)	11/7/2004 10:11	22.2	-0.6	*	0.164	-0.01291	-5.7386		0.12	0.01029	1.1315		0	0	0		0	0	0	0	0	0	0
17	TCC(1)	11/8/2004 10:11	22.3	-0.9	*	0.156	-0.01265	-6.0423		0.162	0.01604	1.5164		0	0	0		0	0	0	0	0	0	0
18	TCC(1)	11/9/2004 10:11	22.4	-0.7		0.195	-0.01633	-6.4343		0.178	0.01572	1.8938		0	0	0		0	0	0	0	0	0	0
19	TCC(1)	11/10/2004 10:11	22.5	-0.9	*	0.167	-0.01335	-6.7546		0.189	0.01685	2.2982		0	0	0		0	0	0	0	0	0	0
20	TCC(1)	11/11/2004 10:11	22.4	-0.8		0.192	-0.01598	-7.138		0.207	0.01847	2.7414		0	0	0		0	0	0	0	0	0	0
21	TCC(1)	11/12/2004 10:11	22.2	-0.8		0.19	-0.01589	-7.5195		0.2	0.01793	3.1716		0	0	0		0	0	0	0	0	0	0
22	TCC(1)	11/13/2004 10:11	22.3	-0.8	*	0.156	-0.01173	-7.8011		0.173	0.01322	3.4888		0	0	0		0	0	0	0	0	0	0
1	TCC(2)	10/23/2004 11:22	22.2	-0.2		0.527	-0.0168	-0.4266		0.035	0.00456	0.1159		0	0	0		0	0	0	0	0	0	0
2	TCC(2)	10/24/2004 11:22	22.3	-0.1		0.285	-0.01975	-0.9005		0.032	0.00367	0.2039		0	0	0		0	0	0	0	0	0	0

Intv	Cham	Time	Temp	RER	OvrPres	% O2	mg/l/hr O2	mg/l O2	OvrRng	% CO2	mg/l/hr CO2	mg/l CO2	OvrRng	% H2S	mg/l/hr H2S	mg/l H2S	OvrRng	mg/l H2S(g)	mg H2S (g)	mg/l H2S(aq)	mg H2S (aq)	mg dH2S	mg dSO4	mg/l/d dSO4
3	TCC(2)	10/25/2004 11:22	22.2	-0.1		0.264	-0.02202	-1.4291		0.028	0.00337	0.2848		0	0	0		0	0	0	0	0	0	0
4	TCC(2)	10/26/2004 11:22	22	-0.1		0.252	-0.02048	-1.9205		0.025	0.00281	0.3522		0	0	0		0	0	0	0	0	0	0
5	TCC(2)	10/27/2004 11:22	22.4	-0.1		0.256	-0.02175	-2.4424		0.024	0.00271	0.4172		0	0	0		0	0	0	0	0	0	0
6	TCC(2)	10/28/2004 11:22	22.3	-0.1		0.243	-0.01954	-2.9113		0.021	0.00221	0.4702		0	0	0		0	0	0	0	0	0	0
7	TCC(2)	10/29/2004 11:22	22.2	-0.1		0.235	-0.01983	-3.3872		0.018	0.00192	0.5164		0	0	0		0	0	0	0	0	0	0
8	TCC(2)	10/30/2004 11:22	22.4	-0.1		0.233	-0.019	-3.8431		0.016	0.00157	0.5541		0	0	0		0	0	0	0	0	0	0
9	TCC(2)	10/31/2004 18:37	22.2	-0.1		0.345	-0.0233	-4.5944		0.027	0.0024	0.6314		0	0	0		0	0	0	0	0	0	0
10	TCC(2)	11/1/2004 10:22	22.1	-0.1	*	0.199	-0.02101	-4.9254		0.017	0.00191	0.6615		0	0	0		0	0	0	0	0	0	0
11	TCC(2)	11/2/2004 10:22	22.2	-0.1		0.27	-0.02415	-5.5049		0.018	0.00171	0.7026		0	0	0		0	0	0	0	0	0	0
12	TCC(2)	11/3/2004 10:22	22.4	-0.1		0.27	-0.02217	-6.037		0.021	0.00216	0.7545		0	0	0		0	0	0	0	0	0	0
13	TCC(2)	11/4/2004 10:22	22.5	-0.1		0.261	-0.02218	-6.5692		0.03	0.00366	0.8422		0	0	0		0	0	0	0	0	0	0
14	TCC(2)	11/5/2004 10:22	22.2	-0.3	*	0.229	-0.01806	-7.0027		0.058	0.00727	1.0167		0	0	0		0	0	0	0	0	0	0
15	TCC(2)	11/6/2004 10:22	22.3	-0.4		0.264	-0.02319	-7.5592		0.096	0.01261	1.3194		0	0	0		0	0	0	0	0	0	0
16	TCC(2)	11/7/2004 10:22	22.2	-0.6		0.247	-0.02003	-8.04		0.14	0.0175	1.7394		0	0	0		0	0	0	0	0	0	0
17	TCC(2)	11/8/2004 10:22	22.3	-0.9	*	0.209	-0.01699	-8.4476		0.171	0.02151	2.2556		0	0	0		0	0	0	0	0	0	0
18	TCC(2)	11/9/2004 10:22	22.4	-0.7		0.258	-0.02183	-8.9716		0.171	0.02001	2.7358		0	0	0		0	0	0	0	0	0	0
19	TCC(2)	11/10/2004 10:22	22.5	-0.8		0.247	-0.02088	-9.4727		0.195	0.02437	3.3206		0	0	0		0	0	0	0	0	0	0
20	TCC(2)	11/11/2004 10:22	22.4	-1.3	*	0.216	-0.0169	-9.8783		0.239	0.02913	4.0198		0	0	0		0	0	0	0	0	0	0
21	TCC(2)	11/12/2004 10:22	22.1	-1.4		0.254	-0.02217	-10.4103		0.322	0.04197	5.0272		0	0	0		0	0	0	0	0	0	0
22	TCC(2)	11/13/2004 10:22	22.3	-2.1		0.233	-0.01883	-10.8622		0.431	0.05497	6.3465		0	0	0		0	0	0	0	0	0	0
1	TCC(3)	10/23/2004 11:33	22.2	-0.1		0.639	-0.02916	-0.7457		0.038	0.00487	0.1245		0	0	0		0	0	0	0	0	0	0
2	TCC(3)	10/24/2004 11:33	22.2	-0.1		0.439	-0.03439	-1.571		0.035	0.00405	0.2217		0	0	0		0	0	0	0	0	0	0
3	TCC(3)	10/25/2004 11:33	22.3	-0.1		0.407	-0.03521	-2.4159		0.031	0.00377	0.3123		0	0	0		0	0	0	0	0	0	0
4	TCC(3)	10/26/2004 11:33	22.1	-0.1		0.401	-0.03374	-3.2258		0.026	0.00304	0.3852		0	0	0		0	0	0	0	0	0	0
5	TCC(3)	10/27/2004 11:33	22.4	-0.1		0.405	-0.0356	-4.0801		0.024	0.00288	0.4543		0	0	0		0	0	0	0	0	0	0
6	TCC(3)	10/28/2004 11:33	22.3	0		0.392	-0.03289	-4.8696		0.02	0.00225	0.5082		0	0	0		0	0	0	0	0	0	0
7	TCC(3)	10/29/2004 11:33	22.2	0	*	0.366	-0.03158	-5.6276		0.017	0.00203	0.5568		0	0	0		0	0	0	0	0	0	0
8	TCC(3)	10/30/2004 11:33	22.4	0	*	0.391	-0.03344	-6.4303		0.015	0.00172	0.5981		0	0	0		0	0	0	0	0	0	0
9	TCC(3)	10/31/2004 20:19	22.2	0		0.582	-0.03877	-7.7393		0.026	0.00238	0.6786		0	0	0		0	0	0	0	0	0	0
10	TCC(3)	11/1/2004 10:33	22.2	0		0.298	-0.03489	-8.2362		0.012	0.00179	0.704		0	0	0		0	0	0	0	0	0	0
11	TCC(3)	11/2/2004 10:33	22.2	0	*	0.359	-0.0319	-9.0018		0.014	0.00179	0.747		0	0	0		0	0	0	0	0	0	0
12	TCC(3)	11/3/2004 10:33	22.4	0		0.436	-0.03811	-9.9165		0.017	0.00197	0.7944		0	0	0		0	0	0	0	0	0	0
13	TCC(3)	11/4/2004 10:33	22.5	-0.1	*	0.384	-0.03272	-10.7017		0.019	0.00234	0.8504		0	0	0		0	0	0	0	0	0	0
14	TCC(3)	11/5/2004 10:33	22.4	-0.1		0.427	-0.03698	-11.5891		0.024	0.00261	0.9131		0	0	0		0	0	0	0	0	0	0
15	TCC(3)	11/6/2004 10:33	22.4	-0.1		0.392	-0.03377	-12.3996		0.03	0.00323	0.9905		0	0	0		0	0	0	0	0	0	0
16	TCC(3)	11/7/2004 10:33	22.2	-0.1	*	0.357	-0.02937	-13.1045		0.041	0.00422	1.0918		0	0	0		0	0	0	0	0	0	0
17	TCC(3)	11/8/2004 10:33	22.4	-0.2	*	0.386	-0.03442	-13.9306		0.075	0.00901	1.3081		0	0	0		0	0	0	0	0	0	0
18	TCC(3)	11/9/2004 10:33	22.5	-0.4		0.411	-0.0352	-14.7754		0.153	0.01934	1.7722		0	0	0		0	0	0	0	0	0	0
19	TCC(3)	11/10/2004 10:33	22.5	-0.5		0.39	-0.03391	-15.5892		0.198	0.0251	2.3746		0	0	0		0	0	0	0	0	0	0
20	TCC(3)	11/11/2004 10:33	22.4	-0.5		0.365	-0.03047	-16.3205		0.197	0.0228	2.9217		0	0	0		0	0	0	0	0	0	0
21	TCC(3)	11/12/2004 10:33	22.2	-0.5		0.381	-0.0335	-17.1246		0.184	0.02101	3.4259		0	0	0		0	0	0	0	0	0	0
22	TCC(3)	11/13/2004 10:33	22.2	-0.5		0.366	-0.03065	-17.8602		0.196	0.02124	3.9358		0	0	0		0	0	0	0	0	0	0
1	Empty	10/23/2004 11:44	22.2	0		0.888	-0.0201	-0.5176		0.003	0.00005	0.0013		0	0	0		0	0	0	0	0	0	0
2	Empty	10/24/2004 11:44	22.2	0		0.377	-0.01997	-0.9968		0.003	0.00011	0.0039		0	0	0		0	0	0	0	0	0	0
3	Empty	10/25/2004 11:44	22.2	0		0.284	-0.02113	-1.5039		0.003	0.00014	0.0073		0	0	0		0	0	0	0	0	0	0
4	Empty	10/26/2004 11:44	22	0		0.264	-0.01996	-1.9829		0.003	0.00014	0.0107		0	0	0		0	0	0	0	0	0	0

Intv	Cham	Time	Temp	RER	OvrPres	% O2	mg/l/hr O2	mg/l O2	OvrRng	% CO2	mg/l/hr CO2	mg/l CO2	OvrRng	% H2S	mg/l/hr H2S	mg/l H2S	OvrRng	mg/l H2S(g)	mg H2S (g)	mg/l H2S(aq)	mg H2S (aq)	mg dH2S	mg dSO4	mg/l/d dSO4
5	Empty	10/27/2004 11:44	22.4	0		0.26	-0.02078	-2.4816		0.003	0.00024	0.0164		0	0	0		0	0	0	0	0	0	0
6	Empty	10/28/2004 11:44	22.4	0		0.257	-0.01972	-2.9549		0.003	0.0002	0.0213		0	0	0		0	0	0	0	0	0	0
7	Empty	10/29/2004 11:44	22.2	0		0.243	-0.01914	-3.4142		0.003	0.0002	0.0261		0	0	0		0	0	0	0	0	0	0
8	Empty	10/30/2004 11:44	22.3	0		0.244	-0.01867	-3.8622		0.002	0.00014	0.0295		0	0	0		0	0	0	0	0	0	0
9	Empty	10/31/2004 22:01	22.2	0		0.363	-0.02171	-4.6281		0.005	0.00031	0.0402		0	0	0		0	0	0	0	0	0	0
10	Empty	11/1/2004 10:44	22.1	0	*	0.188	-0.01981	-4.8802		0.003	0.00037	0.045		0	0	0		0	0	0	0	0	0	0
11	Empty	11/2/2004 10:44	22.4	0		0.253	-0.02177	-5.4027		0.003	0.00023	0.0506		0	0	0		0	0	0	0	0	0	0
12	Empty	11/3/2004 10:44	22.4	0		0.266	-0.02056	-5.8962		0.003	0.00025	0.0566		0	0	0		0	0	0	0	0	0	0
13	Empty	11/4/2004 10:44	22.4	0		0.257	-0.02051	-6.3884		0.003	0.00022	0.0619		0	0	0		0	0	0	0	0	0	0
14	Empty	11/5/2004 10:44	22.4	0		0.259	-0.01979	-6.8632		0.003	0.00026	0.0682		0	0	0		0	0	0	0	0	0	0
15	Empty	11/6/2004 10:44	22.3	0	*	0.229	-0.01735	-7.2795		0.004	0.00025	0.0741		0	0	0		0	0	0	0	0	0	0
16	Empty	11/7/2004 10:44	22.2	0		0.25	-0.01982	-7.7552		0.004	0.00013	0.0773		0	0	0		0	0	0	0	0	0	0
17	Empty	11/8/2004 10:44	22.3	0		0.248	-0.01975	-8.2292		0.005	0.0001	0.0796		0	0	0		0	0	0	0	0	0	0
18	Empty	11/9/2004 10:44	22.5	0		0.256	-0.01977	-8.7036		0.01	0.0001	0.0819		0	0	0		0	0	0	0	0	0	0
19	Empty	11/10/2004 10:44	22.5	0		0.257	-0.02066	-9.1995		0.014	0.00025	0.0879		0	0	0		0	0	0	0	0	0	0
20	Empty	11/11/2004 10:44	22.3	0		0.254	-0.01963	-9.6706		0.014	0.00004	0.0888		0	0	0		0	0	0	0	0	0	0
21	Empty	11/12/2004 10:44	22.2	0		0.256	-0.02064	-10.166		0.014	0.00028	0.0956		0	0	0		0	0	0	0	0	0	0
22	Empty	11/13/2004 10:44	22.2	0		0.254	-0.01965	-10.6376		0.014	0.00005	0.0967		0	0	0		0	0	0	0	0	0	0
1	TC2(1)	10/23/2004 11:55	22.2	2.46		0.158	0.02572	0.6669		0.663	0.08695	2.2551		0	0	0		0	0	0	0	0	0	0
2	TC2(1)	10/24/2004 11:55	22.3	-38		0.078	-0.00239	0.6095		0.95	0.126	5.2791	X	0.0002	0.00002	2		0.00048	0.00001584	0.0013296	0.000019944	3.578E-05	0.00010104	0.006735812
3	TC2(1)	10/25/2004 11:55	22.2	-23		0.071	-0.00463	0.4983		0.95	0.14371	8.7282	X	0.0003	0.00004	3		0.00096	0.00003168	0.0026592	0.000039888	5.162E-05	0.00014576	0.009717459
4	TC2(1)	10/26/2004 11:55	22.1	-21		0.073	-0.00483	0.3825		0.95	0.13938	12.0733	X	0	0	0		0	0	0	0	-3.99E-05	-0.00011262	-0.007508329
5	TC2(1)	10/27/2004 11:55	22.4	-21		0.068	-0.00447	0.2751		0.87	0.13148	15.2288		0	0	0		0	0	0	0	0	0	0
6	TC2(1)	10/28/2004 11:55	22.4	-21		0.068	-0.00338	0.194		0.778	0.09644	17.5434		0	0	0		0	0	0	0	0	0	0
7	TC2(1)	10/29/2004 11:55	22.2	-999	*	0.035	-0.00004	0.193		0.672	0.08573	19.6008		0	0	0		0	0	0	0	0	0	0
8	TC2(1)	10/30/2004 11:55	22.3	-121	*	0.037	-0.00059	0.1787		0.769	0.09912	21.9798		0.0003	0.00003	3		0.00072	0.00002376	0.0019944	0.000029916	5.368E-05	0.00015156	0.010103718
9	TC2(1)	10/31/2004 23:42	22.4	-9.5		0.126	-0.0064	-0.0565		0.95	0.0837	25.0586	X	0.0043	0.00032	43		0.00768	0.00025344	0.0212736	0.000319104	0.0005426	0.00153213	0.102141741
10	TC2(1)	11/1/2004 10:55	22.1	-39	*	0.037	-0.00295	-0.0896		0.506	0.15805	26.8311		0.0027	0.00065	27		0.0156	0.0005148	0.043212	0.00064818	0.0008439	0.00238271	0.158847247
11	TC2(1)	11/2/2004 10:55	22.4	-45	*	0.045	-0.0015	-0.1257		0.682	0.09269	29.0557		0.0051	0.00056	51		0.01344	0.00044352	0.0372288	0.000558432	0.0003538	0.00099889	0.066592376
12	TC2(1)	11/3/2004 10:55	22.4	-11		0.081	-0.00505	-0.2469		0.614	0.07604	30.8807		0	-0.00008	0		-0.00192	-0.00006336	-0.0053184	-0.000079776	-0.000702	-0.0019809	-0.132059859
13	TC2(1)	11/4/2004 10:55	22.3	-19		0.059	-0.00238	-0.3041		0.492	0.06185	32.365		0	0	0		0	0	0	0	7.978E-05	0.00022525	0.015016659
14	TC2(1)	11/5/2004 10:55	22.4	-53	*	0.041	-0.00064	-0.3195		0.386	0.04639	33.4783		0	0	0		0	0	0	0	0	0	0
15	TC2(1)	11/6/2004 10:55	22.3	-7.3		0.071	-0.00446	-0.4265		0.347	0.04444	34.545		0	0	0		0	0	0	0	0	0	0
16	TC2(1)	11/7/2004 10:55	22.2	-12		0.057	-0.00219	-0.4791		0.296	0.03613	35.412		0	0	0		0	0	0	0	0	0	0
17	TC2(1)	11/8/2004 10:55	22.3	79.3	*	0.031	0.00033	-0.4713		0.277	0.03561	36.2667		0	0	0		0	0	0	0	0	0	0
18	TC2(1)	11/9/2004 10:55	22.5	-5.2		0.075	-0.00463	-0.5823		0.263	0.03278	37.0534		0	0	0		0	0	0	0	0	0	0
19	TC2(1)	11/10/2004 10:55	22.5	-6.6		0.071	-0.00374	-0.672		0.262	0.03406	37.8708		0	0	0		0	0	0	0	0	0	0
20	TC2(1)	11/11/2004 10:55	22.4	-7.5		0.066	-0.00308	-0.7459		0.256	0.03194	38.6374		0	0	0		0	0	0	0	0	0	0
21	TC2(1)	11/12/2004 10:55	22.3	-7.4		0.067	-0.00347	-0.8292		0.268	0.03507	39.4792		0	0	0		0	0	0	0	0	0	0
22	TC2(1)	11/13/2004 10:55	22.2	19.5	*	0.026	0.00111	-0.8026		0.242	0.02972	40.1925		0	0	0		0	0	0	0	0	0	0
1	TC2(2)	10/23/2004 12:06	22.2	-0.5		1.697	-0.1617	-4.2235		0.95	0.11166	2.9164	X	0	0	0		0	0	0	0	0	0	0
2	TC2(2)	10/24/2004 12:06	22.3	-0.5		1.908	-0.17797	-8.4947		0.931	0.11257	5.6181		0	0	0		0	0	0	0	0	0	0
3	TC2(2)	10/25/2004 12:06	22.2	-0.4		2.033	-0.19609	-13.2009		0.95	0.11908	8.4759	X	0	0	0		0	0	0	0	0	0	0
4	TC2(2)	10/26/2004 12:06	22.1	-0.5		1.904	-0.17763	-17.4641		0.95	0.11505	11.237	X	0	0	0		0	0	0	0	0	0	0
5	TC2(2)	10/27/2004 12:06	22.4	-0.5		1.82	-0.17578	-21.683		0.949	0.11999	14.1169	X	0	0	0		0	0	0	0	0	0	0
6	TC2(2)	10/28/2004 12:06	22.4	-0.6		1.6	-0.14861	-25.2496		0.949	0.11608	16.9027	X	0	0	0		0	0	0	0	0	0	0

Intv	Cham	Time	Temp	RER	OvrPres	% O2	mg/l/hr O2	mg/l O2	OvrRng	% CO2	mg/l/hr CO2	mg/l CO2	OvrRng	% H2S	mg/l/hr H2S	mg/l H2S	OvrRng	mg/l H2S(g)	mg H2S (g)	mg/l H2S(aq)	mg H2S (aq)	mg dH2S	mg dSO4	mg/l/d dSO4
7	TC2(2)	10/29/2004 12:06	22.2	-0.6		1.646	-0.15872	-29.0589		0.95	0.12151	19.819	X	0	0	0		0	0	0	0	0	0	0
8	TC2(2)	10/30/2004 12:06	22.3	-0.6		1.571	-0.146	-32.5628		0.95	0.11617	22.6071	X	0	0	0		0	0	0	0	0	0	0
9	TC2(2)	11/1/2004 1:24	22.4	-0.1		9.968	-0.66372	-57.981		0.95	0.0821	25.7514	X	0.0002	0.00002	2		0.00048	0.00001584	0.0013296	0.000019944	3.578E-05	0.00010104	0.006735812
10	TC2(2)	11/1/2004 11:06	22.1	-0.5		1.845	-0.42609	-62.1154		0.949	0.29354	28.5996	X	0	-0.00004	0		0	0	0	0	-1.99E-05	-5.6312E-05	-0.003754165
11	TC2(2)	11/2/2004 11:06	22.4	-0.5		1.728	-0.16681	-66.1188		0.95	0.12155	31.5167	X	0	-0.00003	0		0	0	0	0	0	0	0
12	TC2(2)	11/3/2004 11:06	22.3	-0.5		1.831	-0.17065	-70.2144		0.949	0.11771	34.3418	X	0	0	0		0	0	0	0	0	0	0
13	TC2(2)	11/4/2004 11:06	22.2	-0.5		1.854	-0.17922	-74.5156		0.949	0.12322	37.299	X	0	0	0		0	0	0	0	0	0	0
14	TC2(2)	11/5/2004 11:06	22.4	-0.3		2.148	-0.2009	-79.3372		0.698	0.08718	39.3913		0	0	0		0	0	0	0	0	0	0
15	TC2(2)	11/6/2004 11:06	22.2	-0.2		2.177	-0.20943	-84.3634		0.495	0.06306	40.9048		0	0	0		0	0	0	0	0	0	0
16	TC2(2)	11/7/2004 11:06	22.2	-0.2		2.182	-0.20242	-89.2215		0.382	0.04663	42.024		0	0	0		0	0	0	0	0	0	0
17	TC2(2)	11/8/2004 11:06	22.3	-0.1		2.234	-0.21504	-94.3824		0.327	0.04118	43.0123		0	0	0		0	0	0	0	0	0	0
18	TC2(2)	11/9/2004 11:06	22.5	-0.1		2.314	-0.21482	-99.5381		0.278	0.03357	43.818		0	0	0		0	0	0	0	0	0	0
19	TC2(2)	11/10/2004 11:06	22.5	-0.1		2.485	-0.2396	-105.2885		0.258	0.03218	44.5903		0	0	0		0	0	0	0	0	0	0
20	TC2(2)	11/11/2004 11:06	22.2	-0.1		2.399	-0.22278	-110.6352		0.231	0.0275	45.2504		0	0	0		0	0	0	0	0	0	0
21	TC2(2)	11/12/2004 11:06	22.2	-0.1		2.508	-0.24182	-116.4387		0.21	0.02578	45.8691		0	0	0		0	0	0	0	0	0	0
22	TC2(2)	11/13/2004 11:06	22.2	-0.1		2.699	-0.25198	-122.4862		0.187	0.0221	46.3995		0	0	0		0	0	0	0	0	0	0
1	TC2(3)	10/23/2004 12:17	22.2	2.47		0.172	0.02342	0.6159		0.69	0.07965	2.095		0	0	0		0	0	0	0	0	0	0
2	TC2(3)	10/24/2004 12:17	22.2	-47		0.159	-0.00142	0.5818		0.779	0.09227	4.3094		0	0	0		0	0	0	0	0	0	0
3	TC2(3)	10/25/2004 12:17	22.2	-45		0.164	-0.00193	0.5355		0.95	0.11971	7.1824	X	0	0	0		0	0	0	0	0	0	0
4	TC2(3)	10/26/2004 12:17	22.1	-24		0.161	-0.00393	0.4412		0.95	0.13011	10.3051	X	0	0	0		0	0	0	0	0	0	0
5	TC2(3)	10/27/2004 12:17	22.4	-24		0.154	-0.00415	0.3417		0.949	0.13441	13.531	X	0	0	0		0	0	0	0	0	0	0
6	TC2(3)	10/28/2004 12:17	22.3	-30		0.136	-0.00314	0.2665		0.949	0.13005	16.6522	X	0	0	0		0	0	0	0	0	0	0
7	TC2(3)	10/29/2004 12:17	22.2	264	*	0.103	0.00032	0.2741		0.828	0.11603	19.4369		0	0	0		0	0	0	0	0	0	0
8	TC2(3)	10/30/2004 12:17	22.4	-52		0.127	-0.00109	0.2479		0.697	0.07743	21.2952		0	0	0		0	0	0	0	0	0	0
9	TC2(3)	11/1/2004 3:06	22.2	-3.6		0.753	-0.01069	-0.1776		0.731	0.05332	23.418		0	0	0		0	0	0	0	0	0	0
10	TC2(3)	11/1/2004 11:17	22.2	3.46	*	0.142	0.02327	0.013		0.412	0.11075	24.3252		0	0	0		0	0	0	0	0	0	0
11	TC2(3)	11/2/2004 11:17	22.3	-24		0.142	-0.00155	-0.0241		0.452	0.05173	25.5668		0	0	0		0	0	0	0	0	0	0
12	TC2(3)	11/3/2004 11:17	22.3	-17		0.156	-0.00195	-0.071		0.422	0.0446	26.6373		0	0	0		0	0	0	0	0	0	0
13	TC2(3)	11/4/2004 11:17	22.4	-54		0.142	-0.00059	-0.0853		0.404	0.04434	27.7014		0	0	0		0	0	0	0	0	0	0
14	TC2(3)	11/5/2004 11:17	22.5	14.6	*	0.138	0.00192	-0.0393		0.36	0.03855	28.6267		0	0	0		0	0	0	0	0	0	0
15	TC2(3)	11/6/2004 11:17	22.3	-18		0.168	-0.00167	-0.0794		0.346	0.04063	29.6018		0	0	0		0	0	0	0	0	0	0
16	TC2(3)	11/7/2004 11:17	22.2	10.9	*	0.138	0.00249	-0.0196		0.325	0.03721	30.4948		0	0	0		0	0	0	0	0	0	0
17	TC2(3)	11/8/2004 11:17	22.3	18.7	*	0.142	0.00157	0.0179		0.33	0.04027	31.4613		0	0	0		0	0	0	0	0	0	0
18	TC2(3)	11/9/2004 11:17	22.5	-25		0.177	-0.00119	-0.0105		0.337	0.0402	32.426		0	0	0		0	0	0	0	0	0	0
19	TC2(3)	11/10/2004 11:17	22.5	-23		0.188	-0.00141	-0.0444		0.355	0.04442	33.4921		0	0	0		0	0	0	0	0	0	0
20	TC2(3)	11/11/2004 11:17	22.4	-84		0.18	-0.0004	-0.054		0.379	0.04615	34.5997		0	0	0		0	0	0	0	0	0	0
21	TC2(3)	11/12/2004 11:17	22.2	-28		0.189	-0.00134	-0.0862		0.403	0.05124	35.8295		0	0	0		0	0	0	0	0	0	0
22	TC2(3)	11/13/2004 11:17	22.4	-116		0.197	-0.00032	-0.0939		0.42	0.05155	37.0669		0	0	0		0	0	0	0	0	0	0
1	TC1(C)	10/23/2004 12:28	22.2	-0.1		0.827	-0.02952	-0.7819		0.039	0.00486	0.1288		0	0	0		0	0	0	0	0	0	0
2	TC1(C)	10/24/2004 12:28	22.3	-0.1		0.588	-0.03604	-1.6469		0.035	0.00434	0.233		0	0	0		0	0	0	0	0	0	0
3	TC1(C)	10/25/2004 12:28	22.2	-0.1		0.54	-0.03638	-2.5202		0.042	0.0055	0.365		0	0	0		0	0	0	0	0	0	0
4	TC1(C)	10/26/2004 12:28	22.1	-0.1		0.526	-0.03344	-3.3227		0.041	0.00509	0.4873		0	0	0		0	0	0	0	0	0	0
5	TC1(C)	10/27/2004 12:28	22.4	-0.1		0.527	-0.03566	-4.1785		0.039	0.00501	0.6076		0	0	0		0	0	0	0	0	0	0
6	TC1(C)	10/28/2004 12:28	22.4	-0.1		0.516	-0.03236	-4.9553		0.037	0.0045	0.7157		0	0	0		0	0	0	0	0	0	0
7	TC1(C)	10/29/2004 12:28	22.2	-0.1		0.512	-0.03447	-5.7825		0.025	0.00303	0.7884		0	0	0		0	0	0	0	0	0	0
8	TC1(C)	10/30/2004 12:28	22.4	-0.1	*	0.472	-0.02825	-6.4605		0.028	0.0035	0.8725		0	0	0		0	0	0	0	0	0	0

Intv	Cham	Time	Temp	RER	OvrPres	% O2	mg/l/hr O2	mg/l O2	OvrRng	% CO2	mg/l/hr CO2	mg/l CO2	OvrRng	% H2S	mg/l/hr H2S	mg/l H2S	OvrRng	mg/l H2S(g)	mg H2S (g)	mg/l H2S(aq)	mg H2S (aq)	mg dH2S	mg dSO4	mg/l/d dSO4
9	TC1(C)	11/1/2004 4:48	22.2	0		0	0.54892	16.2214		0.028	0.00187	0.9499		0	-0.00001	0		0	0	0	0	0	0	0
10	TC1(C)	11/1/2004 11:28	22.2	0	*	0.972	-0.77111	11.071		0.02	0.00602	0.9901		0	0.00001	0		0	0	0	0	0	0	0
11	TC1(C)	11/2/2004 11:28	22.2	-0.1	*	0.596	-0.03578	10.2124		0.024	0.00292	1.0601		0	0	0		0	0	0	0	0	0	0
12	TC1(C)	11/3/2004 11:28	22.5	0		0.58	-0.0386	9.2859		0.023	0.00231	1.1155		0	0	0		0	0	0	0	0	0	0
13	TC1(C)	11/4/2004 11:28	22.4	0	*	0.523	-0.03347	8.4825		0.023	0.00209	1.1655		0	0	0		0	0	0	0	0	0	0
14	TC1(C)	11/5/2004 11:28	22.4	0		0.565	-0.03784	7.5744		0.02	0.00119	1.1942		0	0	0		0	0	0	0	0	0	0
15	TC1(C)	11/6/2004 11:28	22.3	0	*	0.509	-0.0337	6.7657		0.021	0.00119	1.2227		0	0	0		0	0	0	0	0	0	0
16	TC1(C)	11/7/2004 11:28	22.2	0		0.567	-0.03876	5.8356		0.017	0.00037	1.2317		0	0	0		0	0	0	0	0	0	0
17	TC1(C)	11/8/2004 11:28	22.3	0	*	0.498	-0.03234	5.0595		0.02	-0.00015	1.2281		0	0	0		0	0	0	0	0	0	0
18	TC1(C)	11/9/2004 11:28	22.5	0.01		0.556	-0.03982	4.1038		0.022	-0.00044	1.2175		0	0	0		0	0	0	0	0	0	0
19	TC1(C)	11/10/2004 11:28	22.4	0.01		0.544	-0.03851	3.1796		0.026	-0.00043	1.2072		0	0	0		0	0	0	0	0	0	0
20	TC1(C)	11/11/2004 11:28	22.4	0.01		0.51	-0.03259	2.3974		0.025	-0.00056	1.1938		0	0	0		0	0	0	0	0	0	0
21	TC1(C)	11/12/2004 11:28	22.2	0		0.513	-0.035	1.5573		0.026	0.00069	1.2104		0	0	0		0	0	0	0	0	0	0
22	TC1(C)	11/13/2004 11:29	22.3	0		0.496	-0.03034	0.8289		0.022	0.00074	1.2282		0	0	0		0	0	0	0	0	0	0
1	TC2(C)	10/23/2004 12:39	22.2	0		0.724	-0.03383	-0.9021		0.02	0.00216	0.0575		0	0	0		0	0	0	0	0	0	0
2	TC2(C)	10/24/2004 12:39	22.3	0		0.524	-0.04016	-1.866		0.016	0.0016	0.0958		0	0	0		0	0	0	0	0	0	0
3	TC2(C)	10/25/2004 12:39	22.2	0		0.489	-0.04129	-2.8569		0.016	0.00168	0.1362		0	0	0		0	0	0	0	0	0	0
4	TC2(C)	10/26/2004 12:39	22.1	0		0.487	-0.04006	-3.8184		0.014	0.00133	0.1682		0	0	0		0	0	0	0	0	0	0
5	TC2(C)	10/27/2004 12:39	22.4	0		0.49	-0.04199	-4.8263		0.013	0.00133	0.2001		0	0	0		0	0	0	0	0	0	0
6	TC2(C)	10/28/2004 12:39	22.4	0		0.481	-0.03943	-5.7726		0.011	0.00107	0.2257		0	0	0		0	0	0	0	0	0	0
7	TC2(C)	10/29/2004 12:39	22.2	0	*	0.454	-0.03826	-6.6909		0.01	0.00101	0.2499		0	0	0		0	0	0	0	0	0	0
8	TC2(C)	10/30/2004 12:39	22.4	0		0.51	-0.04339	-7.7323		0.008	0.0007	0.2668		0	0	0		0	0	0	0	0	0	0
9	TC2(C)	11/1/2004 6:29	22.2	0		0	0.19389	0.5725		0.014	0.00091	0.306		0.0003	0.00002	3		3	0.099	8.31	0.12465	0.22365	0.63148235	42.09882353
10	TC2(C)	11/1/2004 11:39	22.2	0		2.166	-1.30912	-6.1917		0.011	0.00577	0.3358		0	-0.00002	0		0	0	0	0	-0.12465	-0.35195294	-23.46352941
11	TC2(C)	11/2/2004 11:39	22.4	0		0.687	-0.03882	-7.1232		0.01	0.00111	0.3623		0	0	0		0	0	0	0	0	0	0
12	TC2(C)	11/3/2004 11:39	22.5	0		0.526	-0.04104	-8.1081		0.009	0.00091	0.3841		0	0	0		0	0	0	0	0	0	0
13	TC2(C)	11/4/2004 11:39	22.4	0		0.51	-0.04365	-9.1557		0.008	0.00082	0.4038		0	0	0		0	0	0	0	0	0	0
14	TC2(C)	11/5/2004 11:39	22.5	0		0.489	-0.03977	-10.1101		0.007	0.00077	0.4223		0	0	0		0	0	0	0	0	0	0
15	TC2(C)	11/6/2004 11:39	22.4	0		0.49	-0.04209	-11.1202		0.007	0.00068	0.4387		0	0	0		0	0	0	0	0	0	0
16	TC2(C)	11/7/2004 11:39	22.2	0	*	0.468	-0.03773	-12.0257		0.006	0.00066	0.4545		0	0	0		0	0	0	0	0	0	0
17	TC2(C)	11/8/2004 11:39	22.4	0		0.524	-0.04614	-13.133		0.007	0.00074	0.4723		0	0	0		0	0	0	0	0	0	0
18	TC2(C)	11/9/2004 11:39	22.5	0		0.49	-0.03971	-14.086		0.007	0.00067	0.4885		0	0	0		0	0	0	0	0	0	0
19	TC2(C)	11/10/2004 11:39	22.5	0		0.511	-0.04415	-15.1457		0.008	0.00081	0.508		0	0	0		0	0	0	0	0	0	0
20	TC2(C)	11/11/2004 11:39	22.4	0		0.493	-0.04044	-16.1161		0.007	0.00067	0.5241		0	0	0		0	0	0	0	0	0	0
21	TC2(C)	11/12/2004 11:39	22.2	0		0.499	-0.04292	-17.1463		0.008	0.00087	0.545		0	0	0		0	0	0	0	0	0	0
22	TC2(C)	11/13/2004 11:40	22.2	0		0.493	-0.04077	-18.1251		0.007	0.00067	0.561		0	0	0		0	0	0	0	0	0	0
1	TCC(C)	10/23/2004 12:50	22.2	0.01		2.15	0.25682	6.8961		0.007	0.00335	0.0899		0	0	0		0	0	0	0	0	0	0
2	TCC(C)	10/24/2004 12:50	22.3	0.03		2.099	0.03811	7.8109		0.007	0.00164	0.1292		0	0	0		0	0	0	0	0	0	0
3	TCC(C)	10/25/2004 12:50	22.2	0		2.153	-0.44772	-2.9343		0.008	0.00874	0.339		0	0	0		0	0	0	0	0	0	0
4	TCC(C)	10/26/2004 12:50	22.1	0		2.123	-0.05598	-4.2778		0.008	-0.0002	0.3342		0	0	0		0	0	0	0	0	0	0
5	TCC(C)	10/27/2004 12:50	22.4	0		2.188	-0.50176	-16.3202		0.008	0.00354	0.4191		0	0	0		0	0	0	0	0	0	0
6	TCC(C)	10/28/2004 12:50	22.3	0		2.062	0.3805	-7.1884		0.008	-0.00126	0.389		0	0	0		0	0	0	0	0	0	0
7	TCC(C)	10/29/2004 12:50	22.2	0.01		2.141	-0.55984	-20.6245		0.007	-0.00783	0.201		0	0	0		0	0	0	0	0	0	0
8	TCC(C)	10/30/2004 12:50	22.3	0.01		1.946	0.70201	-3.776		0.007	0.00555	0.3342		0	0	0		0	0	0	0	0	0	0
9	TCC(C)	11/1/2004 7:59	22.2	-0.1		3.197	-3.28396	-148.7408		0.085	0.26538	12.0489		0	-0.0002	0		0	0	0	0	0	0	0
10	TCC(C)	11/1/2004 11:50	22.2	0		2.302	23.91291	-56.5166		0.042	-1.59803	5.8858		0	0.00219	0		0	0	0	0	0	0	0

Intv	Cham	Time	Temp	RER	OvrPres	% O2	mg/l/hr O2	mg/l O2	OvrRng	% CO2	mg/l/hr CO2	mg/l CO2	OvrRng	% H2S	mg/l/hr H2S	mg/l H2S	OvrRng	mg/l H2S(g)	mg H2S (g)	mg/l H2S(aq)	mg H2S (aq)	mg dH2S	mg dSO4	mg/l/d dSO4
11	TCC(C)	11/2/2004 11:50	22.4	0.23		2.119	0.62194	-41.5904		0.073	0.194	10.5418		0	0	0		0	0	0	0	0	0	0
12	TCC(C)	11/3/2004 11:50	22.5	-0.2		2.275	-0.91484	-63.5466		0.105	0.20857	15.5476		0	0	0		0	0	0	0	0	0	0
13	TCC(C)	11/4/2004 11:50	22.4	0.7		2.151	0.34845	-55.1838		0.157	0.33708	23.6374		0	0	0		0	0	0	0	0	0	0
14	TCC(C)	11/5/2004 11:50	22.4	0.35		2.031	0.35426	-46.6815		0.182	0.17131	27.7489		0	0	0		0	0	0	0	0	0	0
15	TCC(C)	11/6/2004 11:50	22.3	-0.3		2.066	-0.35664	-55.2409		0.204	0.1609	31.6106		0	0	0		0	0	0	0	0	0	0
16	TCC(C)	11/7/2004 11:50	22.2	-5.5		2.045	-0.10529	-57.7677		0.327	0.79338	50.6517		0	0	0		0	0	0	0	0	0	0
17	TCC(C)	11/8/2004 11:50	22.2	0.26		1.731	1.23962	-28.0168		0.393	0.444	61.3078		0	0	0		0	0	0	0	0	0	0
18	TCC(C)	11/9/2004 11:50	22.5	-0.4		1.882	-0.85212	-48.4677		0.454	0.43116	71.6556		0	0	0		0	0	0	0	0	0	0
19	TCC(C)	11/10/2004 11:50	22.4	0		2.008	-0.75733	-66.6437		0.445	0.01234	71.9516		0	0	0		0	0	0	0	0	0	0
20	TCC(C)	11/11/2004 11:50	22.4	2.25		2.018	-0.21671	-71.8447		0.328	-0.66924	55.8899		0	0	0		0	0	0	0	0	0	0
21	TCC(C)	11/12/2004 11:50	22.2	0.39		2.202	-1.02822	-96.5223		0.231	-0.55574	42.552		0	0	0		0	0	0	0	0	0	0
22	TCC(C)	11/13/2004 11:51	22.2	1.54		2.206	-0.21135	-101.5962		0.154	-0.44867	31.781		0	0	0		0	0	0	0	0	0	0