Linking microbial populations and geochemical processes in soils, mine tailings, and geothermal environments
by Richard Eugene Macur

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Land Resources and Environmental Sciences MONTANA STATE UNIVERSITY
Bozeman, Montana
Montana State University

Abstract:
The primary goal of this work was to identify and characterize the microbial populations responsible for transformations of As and 2,4-D in soils and waters. Chemical, spectroscopic, and microscopic techniques were used to characterize the aqueous and solid phase geochemistry of soils, mine tailings, and a geothermal spring. The role of specific microbial populations in these systems was examined using cultivation-independent molecular methods [total DNA extraction, 16S rDNA amplification, denaturing gradient gel electrophoresis (DGGE), and sequence analysis] coupled with either characterization of microorganisms isolated from the same systems, or inference of physiological characteristics from (i) closely related (16S rDNA sequence) cultured microorganisms and (ii) the geochemical environments in which they were detected.

The microbial reduction of As(V) to As(III) and the subsequent effects on As mobilization in contaminated mine tailings was examined under transport conditions. Enhanced elution of As from mine tailings apparently resulted from the enrichment of aerobic As(V)-reducing Caulobacter leidyi, Sphingomonas yanoikuyae, and Rhizobium loti -like populations after liming.

Arsenite was rapidly oxidized to As(V) via microbial activity in unsaturated Madison River Valley soil columns. Eight aerobic heterotrophic bacteria with varying As redox phenotypes were isolated from these columns. Three isolates, identified as Agrobacterium tumefaciens, Pseudomonas fluorescens, and Variovorax paradoxus -like organisms, were As(III) oxidizers and all were apparently important members of the soil microbial community responsible for net As(III) oxidation.

Successional changes in microbial communities colonizing an As-rich acid-sulfate-chloride geothermal spring stream channel in Norris Geyser Basin of Yellowstone National Park were examined. Enhanced As(III) oxidation correlated in time and space with the appearance of three Hydrogenobaculum -like populations. The formation of an As(V)-rich hydrous-ferric-oxide mat correlated with the detection of Thiomonas, Acidimicrobium, and Metallosphaera —like populations whose nearest cultivated relatives (based on 16S rDNA sequence) were Fe-oxidizers.

Fingerprints of microbial communities (DGGE) established under increasing concentrations of 2,4-D (0 - 500 mg kg'1) in batch soil microcosms showed that at least 100 mg kg'1 2,4-D was required to obtain apparent shifts in community structure. The microbial community selected at high 2,4-D concentrations was predominantly composed of Burkholderia -like populations, which harbored homologs of tfdA genes.

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Richard Eugene Macur

This dissertation has been read by each member of the dissertation committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

William P. Inskeep, Ph.D. William P. Inskeep (Signature) 1/13/04 Date

Approved for the Department Land Resources and Environmental Sciences

Jon M. Wraith, Ph.D. Jon M. Wraith (Signature) 1-14-04 Date

Approved for the College of Graduate Studies

Bruce R. McLeod, Ph.D. Bruce R. McLeod (Signature) 1-14-04 Date

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ABSTRACT

The primary goal of this work was to identify and characterize the microbial populations responsible for transformations of As and 2,4-D in soils and waters. Chemical, spectroscopic, and microscopic techniques were used to characterize the aqueous and solid phase geochemistry of soils, mine tailings, and a geothermal spring. The role of specific microbial populations in these systems was examined using cultivation-independent molecular methods [total DNA extraction, 16S rDNA amplification, denaturing gradient gel electrophoresis (DGGE), and sequence analysis] coupled with either characterization of microorganisms isolated from the same systems, or inference of physiological characteristics from (i) closely related (16S rDNA sequence) cultured microorganisms and (ii) the geochemical environments in which they were detected.

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CHAPTER 1

INTRODUCTION

Geochemical processes are often inextricably linked with the activity of microbial communities in soil and water environments. The geochemical environment controls which microorganisms may grow or survive by defining the bioavailability of specific chemical constituents including essential nutrients and toxic elements. Conversely, microorganisms alter their surrounding chemical environment as they carry out metabolic and physiologic functions necessary for growth and survival. At a minimum, microbial activity involves the uptake of nutrients, the release of cellular by-products, and the exchange of compounds associated with energy conservation. Microorganisms influence their microenvironment in many other significant ways including the release of siderophores that mobilize metals (e.g., *1*), detoxification reactions which result in transformation of toxic compounds (e.g., *2*), exudation of organic chemicals including biofilm formation (e.g., *3*) and release of protons. In essence, the geochemical environment is a primary factor controlling microbial species distribution while simultaneously the resident microorganisms are important mediators of the geochemical environment.

The role of specific microbial populations must be considered to accurately predict speciation and fate of many chemical constituents in soils and waters. The use of traditional cultivation methods to identify dominant microbial populations has been problematic since cultivation approaches may not capture the organisms important in the

actual physical-chemical environments (*4*). With the advent of molecular techniques (e.g., amplification and sequencing of 16S rRNA genes), it is now possible to assess the predominant microbial populations in natural samples without cultivation bias. Coupling traditional cultivation with molecular approaches can be used to more accurately describe the distribution and function of microorganisms in soils and natural waters. In some cases, 16S rDNA sequence analysis of environmental samples can be used to suggest the function of uncultured organisms by inferring physiological attributes from phylogenetically related characterized organisms. However, examples exist where such phylogenetic inference is inadequate for defining the physiology of naturally occurring microorganisms. For example, closely related *Thiomonas* strains exhibit different arsenic (As) transforming capabilities under the same conditions (*5*). Moreover, numerous 16S rDNA sequences identified in soil-water systems do not have closely related cultivated organisms from which to infer microbial properties (*6*, *7*, also see Chapter 4, Novel Organisms section).

The primary goal of the research conducted during my Ph.D. program was to identify microbial populations responsible for observed chemical transformations of contaminants in soils, mine tailings, and geothermal springs using a combination of molecular, cultivation, and chemical characterization tools. The function of specific populations detected using cultivation-independent 16S rDNA sequence analysis was determined by cultivating microorganisms with identical 16S rDNA sequences and characterizing their chemical transforming properties. In the geothermal spring study, function of populations detected with 16S analysis was inferred from the co-occurrence

of specific geochemical processes with the appearance of specific microbial populations. The use of these strategies to identify microbial populations associated with chemical processes occurring *in situ* has provided an important methodological approach for unraveling the complex biological interactions that govern the distribution of important chemical species in soil-water environments.

The research presented in this thesis demonstrates the application of this conceptual approach to understanding several microbially mediated chemical processes in soil and water systems. Specifically, this work was focused on the behavior of arsenic (As) and 2,4-dichlorophenoxyacetic acid (2,4-D) in soil-water systems, two very different, yet important priority environmental pollutants. Arsenic is a highly toxic metalloid, ubiquitous in the environment and often concentrated in mine tailings and geothermal systems. Potentially harmful levels have also been detected in many drinking water sources throughout the world. Currently, the largest mass poisoning in human history is taking place in Bangladesh and surrounding areas, where As laden ground waters are being consumed by millions of people (*8*). 2,4-D is a chlorinated organic herbicide utilized extensively for broadleaf control throughout the world. The Montana Department of Agriculture and the U.S. Geological Survey have conducted several monitoring programs and have found significant amounts of 2,4-D in ground waters throughout the U.S., including Montana (*9*, *10*). Both As and 2,4-D are subject to microbial interactions that mediate their chemical speciation and subsequent behavior in soils and waters. Understanding processes that control the fate and transport of these important types of contaminants is critical for successfully managing impacted

ecosystems. In addition, understanding the behavior of these compounds may help us to predict the behavior of other inorganic and chlorinated aromatic contaminants in the environment.

Increased interest in the biogeochemical pathways controlling As distribution has resulted from epidemiological studies indicating that relatively low levels of As can impair human health (*11*). These findings have caused the U.S. EPA to decrease the maximum contaminant level in drinking water from 50 ppb to 10 ppb. The role of microorganisms in As cycling in the environment has received considerable attention because it is thought that microbially mediated transformations of As may be the most important factor controlling As speciation. The speciation of As is important because different As species exhibit variation in solubility, mobility, bioavailability, and toxicity. Chapters two, three, and four of this dissertation describe three separate studies focused largely on the microbial populations important in As oxidation-reduction reactions in three different geochemical environments: mine tailings, unsaturated agricultural soils, and geothermal springs. Although each individual study has provided meaningful insight about microbial processes important in As-cycling, observations across different environments provides necessary data for exploring similarities and patterns useful for predictive purposes.

My initial research effort, fully described in chapter two, was to characterize the As redox activity of aerobic microbial populations residing in mine tailings. This was accomplished by utilizing both molecular and traditional isolation techniques to identify dominant microbial populations present in the mine tailings, and then, correlating these

populations to observed As transformations. The results demonstrated that microbially mediated arsenate [As(V)] reduction was important under aerobic conditions and that this process enhanced the leaching potential of As. In the study presented in chapter three, I examined the diversity and relevance of As-transforming microbial populations resident in an As-contaminated agricultural soil. This work showed that bacteria capable of either oxidizing arsenite [As(III)] or reducing As(V) for apparent detoxification purposes coexist in soils, and that identification of microorganisms based solely on 16S rDNA sequence may not be sufficient for predicting the As transforming capabilities of specific bacterial populations.

The goal of the study described in chapter four was to monitor changes in aqueous and solid phase geochemistry, and microbial community structure associated with initial colonization of an acid-sulfate-chloride geothermal spring in Norris Geyser Basin, Yellowstone National Park. The co-occurrence of specific geochemical processes with the appearance of specific microbial populations was used to infer the physiology and ecological role of microorganisms detected using molecular methods. Dr. Heiko Langner, a postdoctoral fellow in Dr. Inskeep's laboratory, initiated this project and led much of the effort to collect aqueous chemical field data. During this time, my work focused on molecular characterization of the spring. With his departure after the field season, I assumed the responsibilities of leading and completing the project. The objectives of this study were to; (i) correlate the distribution of bacterial and archaeal sequences in time and space with changes in temperature and geochemical energy gradients, (ii) identify microorganisms responsible for rapid rates of *in situ* As(III) oxidation, and (iii) determine

the microorganisms and processes responsible for the formation of As-rich hydrous ferric oxide (HFO) mats. Understanding microbial processes associated with the formation of HFO phases is important primarily due to their extremely high capacity to sequester As, and the fact that HFO phases are important in controlling the mobility of As in many natural water systems. The suite of complementary analyses included the 16S rDNA molecular fingerprinting technique, denaturing gradient gel electrophoresis (DGGE) coupled with sequence analysis, and a variety of chemical, microscopic, and spectroscopic techniques.

Microorganisms also play a crucial role in the degradation of chlorinated organic compounds in soils and waters. In the study presented in chapter five, I examined the effects of 2,4-D application rate and hydrodynamic environment on the diversity of microbial populations associated with 2,4-D degradation in an agricultural soil. Molecular techniques were used in combination with traditional isolation and characterization to describe the activity of the dominant 2,4-D degrading microbial populations. The role of specific functional genes associated with 2,4-D degradation in these organisms was also investigated.

These studies serve as significant contributions to our understanding of microbial processes controlling the chemical speciation of important contaminants in various environments. Coupling 16S rDNA based molecular techniques with traditional methods was shown to be effective in most cases for elucidating the role of specific microbial populations involved in geochemical transformations of As and the degradation of 2,4-D.

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CHAPTER 2

MICROBIAL POPULATIONS ASSOCIATED WITH THE REDUCTION AND ENHANCED MOBILIZATION OF ARSENIC IN MINE TAILINGS

Introduction

Microorganisms possess a variety of mechanisms for reducing As(V) and oxidizing As(III) (*1-4*). Transformation of As by microorganisms has important environmental implications because As(V) and As(III) have different sorption and toxicological characteristics; As(III) is often considered the more mobile and toxic species and thus more problematic regarding contamination of natural waters (*5-7*). Reduction of As(V) to As(III) in anoxic environments is thought to occur primarily by dissimilatory reduction where microorganisms utilize As(V) as a terminal electron acceptor for anaerobic respiration (*8, 9*). To date, dissimilatory reduction has been characterized in at least seven bacteria; *Sulfurospirillum barnesii, Bacillus arsenicoselenatis, B. selenitireducens, S. arsenophilum, Desulfotomaculum auripigmentum, Chrysiogenes arsenatis, and Desulfomicrobium* strain Ben-RB (*1, 10, 11*), which represent genera scattered throughout the bacterial domain. In addition, dissimilatory reduction of As(V) has been observed in two hyperthermophilic archaea; *Pyrobaculum arsenaticum* and *P. aerophilum* (*12*). Microorganisms may also possess reduction mechanisms that are not coupled to respiration, but instead are thought to impart As resistance (*2, 13, 14*). Enzymes involved in the detoxification pathway are transcribed by the *ars* operon. Homologues of the *ars* operon have been discovered in the *Pseudomonas, Bacillus, Klebsiella, Enterobacter, Citrobacter, Staphylococcus,*

Salmonella, Thiobacillus, Yersinia and *Escherichia* genera (*13-18*); genotypes that also are scattered throughout the bacterial domain. Microorganisms which express As resistance genes are able to withstand higher concentrations of As through the intracellular reduction of As(V) and the subsequent excretion of As(III) into the surrounding media. It is thought that this pathway may function in aerobic as well as anaerobic environments (*10, 11*), and may contribute to apparent nonequilibrium conditions where As(III) has been often observed in oxic surface waters (*19, 20*). Although it is likely that bacteria in soil, especially at As-contaminated sites, possess As detoxification pathways, to our knowledge, the effect of aerobic As(V) reducing bacteria on As behavior in soils has not yet been presented in the literature.

Liming of acidic mine tailings is recognized as an effective method for immobilizing trace metals and promoting plant establishment (*21*). However, as demonstrated by Jones et al. (*22*), liming may also result in enhanced As mobilization due to the pH dependence of As sorption reactions. The work presented here is an extension of the study conducted by Jones et al. (*22*), wherein our primary objective was to examine the effect of microbial processes on As behavior in mine tailings. We hypothesized that microbial reduction of As(V) may enhance As mobilization in mine tailings and that liming may impact the populations and activities of As(V)-reducing microorganisms. These hypotheses were tested by: (i) examining the affect of microbial As(V) reduction on mobilization of As in mine tailings under column transport conditions, and by (ii) using molecular and traditional cultivation techniques to identify microorganisms responsible for As(V) reduction in the mine tailings. Column transport experiments were designed to observe relationships among pH (limed vs unlimed

treatments), redox potential (Pt-electrode), dissolved As species, and corresponding shifts in microbial populations. Several microbial populations enriched after liming were identified using molecular and phylogenetic analysis of 16S rDNA fragments, and through traditional methods of bacterial cultivation. Several isolates matching 16S fragments observed in the environmental samples were shown to reduce As(V) under aerobic conditions, suggesting a potential role of aerobic heterotrophs in As cycling.

Materials and Methods

Sample Collection and Chemical Characterization

Arsenic contaminated reprocessed mine tailings (RT) were collected near an abandoned copper smelter in Anaconda, Montana (EPA Superfund Site # MTD093291656). Extensive characterization of this sample by Jones et al. (*22*) included sequential extractions of As fractions, scanning electron microscopy (SEM)/energy dispersive analysis of X-rays (EDAX), and total metal concentrations as determined by x-ray fluorescence. Briefly, Fe represented 15-21 % (w/w) of the RT, primarily in the form of Fe-oxyhydroxides. Total As accounted for approximately 0.3 % (w/w) of the RT, with about 32 % of the As sorbed to the Fe-oxyhydroxide phases (quantified by the 0.1 M NaOH sequential extraction step which has been correlated with As surficially bound to Fe-oxyhydroxide). The largest fraction of As, approximately 60 %, was in the "nonlabile" pool, extracted only after a four acid heat treatment. Extensive analysis of the RT with SEM-EDAX revealed no discrete solid phases concentrated in As.

Column Experiments

Mobilization of As under transport conditions was studied using polycarbonate columns (length = 54 mm, diam. = 32 mm) packed with a mixture of 20 % RT and 80 % acid-purified, autoclaved quartz sand (50-70 mesh, Sigma Chemical, St. Louis, MO) for a total mass of 62 g (bulk density ~ 1.43 g cm^{-3}). Limed treatments included a mix of 60 % $CaCO_3$/40 % $Ca(OH)_2$ at a concentration of 25.6 g kg^{-1} (*23*). The column apparatus was either autoclaved or fumigated with chloroform prior to packing. Sterilized treatments used RT that was either autoclaved (12 g RT portions for 1 h x 2) or exposed to chloroform (~ 5 mm layer RT in a vacuum evacuated dessicator with chloroform for ~ 250 h). The alternate chloroform sterilization treatment was used to circumvent potential alteration of solid phases during autoclaving, however, our results showed no apparent differences between the two sterilization methods (e.g., steady-state values of pH, Fe and As measured in the effluent of chloroform-treated limed columns bracketed values for the autoclaved columns). Autoclaved influent was supplied to the bottom of the columns with a continuous flow pump set to deliver 0.88 mL h^{-1} (1.1 pore volume d^{-1}, pore water velocity = 0.24 cm h^{-1}). The influent, formulated to simulate a "typical" soil solution (SSE) was modified from Angle et al. (*24*) and contained NH_4NO_3 (1.25 mM), $CaSO_4$ (2 mM), $MgCl_2$ (2 mM), KH_2PO_4 (10 μM), KOH (1.25 mM), $FeCl_2$ (5 μM), supplemented with 100 μL L^{-1} of micronutrient solution (*25*). The pH of the influent was adjusted with HCl to pH 3.6 for unlimed columns and with NaOH to pH 7.0 for limed columns. Effluent pH values in the limed treatments near 7.7 were due to liming amendments as opposed to affects of the unbuffered, pH-adjusted influent solution. Because organic amendments and topsoils rich in organic C are routinely added to mine tailings in

reclamation efforts, an additional treatment simulating these high C environments included 0.5 mM glucose and 1.0 mM lactate in the influent. For several specific columns, air was continuously pumped through a port in the bottom end cap at a rate of 10 mL min^{-1} to insure that oxic conditions were present and to disrupt potential redox gradients.

An effluent collection system was devised to minimize microbial and abiotic transformations of As mobilized from the columns. Solution exiting the top of the columns was plumbed directly into 50 mL glass syringes whose pistons were allowed to freely extend as effluent flowed in. The syringes were housed within a N_2(g)-purged temperature-controlled chamber set at 2 ± 1 °C. To allow for expulsion of air from aerated columns, tubing exiting the top endcap was open to the atmosphere and effluent flowing from the tube was collected with a fraction collector rather than syringes. During the column transport experiments, samples were removed periodically, filtered (0.22 μm) and analyzed for As(V), As(Total), Fe(II), Fe(Total), sulfide and pH. The method used for quantifying As species was modified from Masscheleyn et al. (*26*). Specifically, 5 mL aliquots of effluent or standard were added to each of two 15 mL polyethylene bottles and analyzed separately for total As and As(V), with As(III) determined by difference. Arsenite was liberated from one sample by selectively reducing As(III) to arsine gas and subsequent purging of the arsine gas. This was accomplished by adding 1 mL of 0.25 M NaOH and 0.79 M $NaBH_4$ (over a period of 3 min) to a sample buffered with 1 mL of 2 M Tris (pH 6.5) while sparging with N_2(g). The sample was then sparged for 7 additional min. Total As was analyzed with hydride generation-atomic absorption spectrophotometry as described in Jones et al. (*22*). The phenanthroline method was used

to determine Fe(II) and Fe(total) concentrations, and sulfide was measured colorimetrically using methylene blue (*27*). Redox potential within the columns was measured using a Pt-wire inserted into the center of the column midway between the top and bottom endcaps and sealed prior to starting the experiments (surface area = 0.5 cm^2), and a reference electrode connected to the top endcap. The Pt-electrodes were interfaced to a computer that collected voltage data at prescribed intervals. Prior to the experiments, the Pt-electrodes were calibrated in accordance with ASTM method D1498-76 (*28*) using Fe(II)/Fe(III) reference solutions. All column transport experiments were conducted in triplicate unless otherwise noted. At the conclusion of the experiments, columns were dismantled and the RT/sand mixture was used for isolation of As(V) reducing microorganisms and for molecular analysis.

As(V) Reducing Isolates

Bacteria were isolated by adding 1 g of post-experimental RT-sand mixture to 10 mM NaCl and shaking @ 100 cycles min^{-1} for 5 min. The slurry was serially diluted and 0.1 mL aliquots of each dilution were plated onto various media designed to culture aerobic and anaerobic bacteria, and specifically, bacteria capable of anaerobic As(V) respiration. Bacteria were isolated using yeast-extract peptone-glucose (YEPG) agar media and SSE agar media supplemented with 1 mM glucose and 2 mM lactate (SSE+C). Both media also contained 13 μM As(V). These plates were incubated under both aerobic and anaerobic conditions. To specifically isolate As(V) respiring bacteria, SSE agar media was prepared with 500 μM As(V) and 250 μM cysteine; NH_4NO_3 was replaced with 2.5 mM NH_4Cl. Anaerobic plates were degassed for several days, inoculated, then

incubated in a chamber containing a GasPak Plus generator (Becton Dickinson, Sparks, MD). Isolated colonies were restreaked several times to obtain pure cultures.

Isolates obtained from the post-experimental RT/sand were screened for their ability to reduce As(V) in serum bottles containing 50 mL SSE+C media and 13 μM As(V). Aerobic treatments were maintained by continuously purging the solution with filter-sterilized air (> 5 mL min^{-1}). Anaerobic treatments were conducted using $N_{2(g)}$-purged serum bottles containing 13 μM As(V). The potential for isolates to respire on As(V) was tested in $N_{2(g)}$-purged serum bottles containing SSE media supplemented with 500 μM As(V) and 250 μM cysteine; NH_4NO_3 was replaced with 2.5 mM NH_4Cl. Aerobic isolates that were capable of reducing As(V) were further characterized using serum bottles containing SSE media plus 5 mM MOPS buffer, 20 mM glucose, 50 μM NaH_2PO_4, and 200 μM Na_2HAsO_4. Serum bottle experiments with isolates obtained from the C supplemented columns utilized the same media with exception of 10 μM P and 1.4 or 156 μM As(V). Serum bottles were inoculated to attain an initial cell density of 10^6 cells mL^{-1}, as determined using an empirically-developed relationship between cell enumeration with epifluorescence microscopy of DAPI stained cells and optical density (OD) measurements (A_{500}) of cell suspensions. At each sampling interval, 3.5 mL of suspension was removed for determination of OD (A_{500}), As(V) and As(Total) concentrations as described above.

DNA Extraction and Purification

Total sample DNA was extracted using the FastDNA SPIN Kit for Soil (Bio 101, Vista, CA) following the manufacturer's instructions. The extracted DNA was

electrophoresed in a 1 % SeaKem GTG agarose gel (FMC BioProducts, Rockland, ME), and stained with ethidium bromide.

Partial 16S rDNA Amplification and Denaturing Gradient Gel Electrophoresis (DGGE)

DNA extracts were used as template for polymerase chain reaction (PCR) that targeted a 322 bp region within the 16S rRNA gene. The 1070 forward primer (Integrated DNA Technologies, Coralville, IA) targeted the domain *Bacteria* (*E. coli* positions 1055-1070) and the 1392 reverse-GC primer targeted a universally conserved region (*E. coli* positions 1392-1406). The reverse primer contained a 40 bp GC-rich clamp used in DGGE (5'-CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC CAC GGG CGG TGT GTA C-3'; *29*). PCR reaction mixtures (50 µL) contained 1-5 µL template DNA (2-20 ng), 2 mM Tris-HCl (pH 8), 10 mM KCl, 10 µM EDTA, 2.5 mM $MgCl_2$, 800 µM dNTP's, 0.5 µM of each primer, and 1.25 U *Taq* DNA polymerase (Promega, Madison, WI). PCR reactions were run on a 9700 GeneAmp PCR System (Perkin-Elmer, Foster City, CA). The protocol was 94 °C for 4 min, 30 cycles of 94 °C, 55 °C and 72 °C each for 45 sec, and a final 7 min extension period at 72 °C. DNA was quantified by electrophoresis on a 3 % SeaKem GTG agarose gel (FMC BioProducts, Rockland, ME) run with a Low DNA Mass Ladder (Gibco BRL, Grand Island, NY) and stained with ethidium bromide.

PCR products were separated and visualized using DGGE as described by Muyzer et al. (*30*) with the following modifications. A DCode Universal Mutation Detection System (Bio-Rad, Hercules, CA) was used to resolve the PCR products. The gel consisted of 8 % acrylamide and a 35-70 % gradient of urea/formamide increasing in

the direction of electrophoresis (running buffer = 1X TAE [40 mM Tris, 20 mM acetic acid, and 2 mM EDTA at pH 8.5]; 60 V at 60 °C for 17 h). DGGE gels were stained with SYBR Green II (Molecular Probes, Eugene, OR) in 1X TAE for 30 min and photographed using UV transillumination. DGGE bands of interest were stabbed with a sterile pipet tip, rinsed in sterile molecular biology grade water and used as template for PCR amplification and subsequent sequencing reactions.

PCR Amplification and DNA Sequencing of DGGE Bands

Template for sequencing of DGGE bands was amplified using primers 1114 forward and 1392 reverse (without the GC clamp; *31*) as described above. The product was purified with a QIAquick PCR Purification Kit (Qiagen Inc., Valencia, CA) and the sequencing reaction was carried out using an ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit as described by the manufacturer (Perkin-Elmer). The samples were processed on an ABI Prism 310 capillary sequencer (Perkin-Elmer) and the resultant sequences were aligned using Sequencher 3.1.1 software (Gene Codes Corporation, Ann Arbor, MI). Phylogenetic information was obtained by using BLAST to compare the sequences with sequences found in the GenBank database (*32*).

Full Length 16S rDNA Amplification and Sequencing

DNA from each of the isolates was amplified using primers that amplify nearly the entire 16S rRNA gene. Template for the reactions was obtained by scraping several colonies with a sterile pipet tip and swirling the tip in 50 µL of DNase free water. The suspension was heated at 98 °C for 10 min. and 1.0 or 5.0 µL was used as template for

PCR. Primers for the initial PCR consisted of the *Bacteria*-specific primer Bac8 forward (5'-AGAGTTTGATCCTGGCTCAG-3') and the universal primer Univ1492 reverse (5'-GGTTACCTTGTTACGACTT-3'). The products were purified with a QIAquick PCR Purification Kit. All primers for the full-length sequence reactions were derived from the probes described by Amann et al. (*33*). Sequencing conditions and sequence analysis were conducted as described above.

Results and Discussion

Column Experiments

The mean steady-state effluent pH increased from 3.85 in unlimed treatments to 7.75 after liming (Table 2.1), consistent with lime requirement calculations for this acid mine soil (*23*). Iron eluted from unlimed columns was predominantly Fe(II), which is consistent with thermodynamic predictions of Fe speciation at pH < 4 and E_H < 530 mV (Table 2.1; *34*). As expected, the pH increase after liming resulted in undetectable concentrations of Fe in the effluent (< 0.8 μM), and thus confirmed the use of liming as an effective method for immobilizing Fe in RT. No detectable sulfide (> 3.0 μM) was released from either unlimed or limed columns. Estimates of redox potential using Pt-electrodes suggested that limed columns not supplemented with C were too oxidized to support significant concentrations of As(III) (Figure 2.1; Table 2.1). At pH 7.75, thermodynamic predictions suggest that redox potential values must be less than -23 mV to support significant concentrations of As(III) (> 1 % of total; *35*); observed Pt-E_H values at steady-state were never less than 380 mV (Table 2.1). In limed columns that

Table 2.1. Mean steady-state pH values and Fe and As concentrations in effluent from reprocessed tailing (RT) columns. Influent for these treatments was not supplemented with C.

Treatments	pH	E_H	Fe(II)	Fe(Total)	As(III)	As(Total)
		(mV)	(μM)		(nM)	
Unlimed Sterile[a]	3.9 (0.1)[b]	473.2 (12.3)	158.6 (9.9)	162.5 (12.4)	8.3 (5.0)	16.7 (8.4)
Unlimed Nonsterile	3.8 (0.03)	522.5 (19.6)	78.3 (42.5)	76.4 (43.7)	16.3 (7.9)	24.5 (5.9)
Limed Sterile	7.8 (0.07)	418.3 (38.6)	0.0 (0.0)	0.0 (0.0)	3.0 (3.0)	99.3 (14.3)
Limed Nonsterile	7.7 (0.06)	412.9 (17.4)	0.0 (0.0)	0.0 (0.0)	252.1 (52.8)	300.3 (61.4)
Limed-Aerated-Nonsterile	7.8	393	0.0	0.0	299.5	340.0

[a]Values for sterilized treatments are means of three replicate columns experiments; two of the experiments used RT pretreated with chloroform, and the third used RT that was autoclaved.
[b]Standard errors of three replicate column experiments in parenthesis.

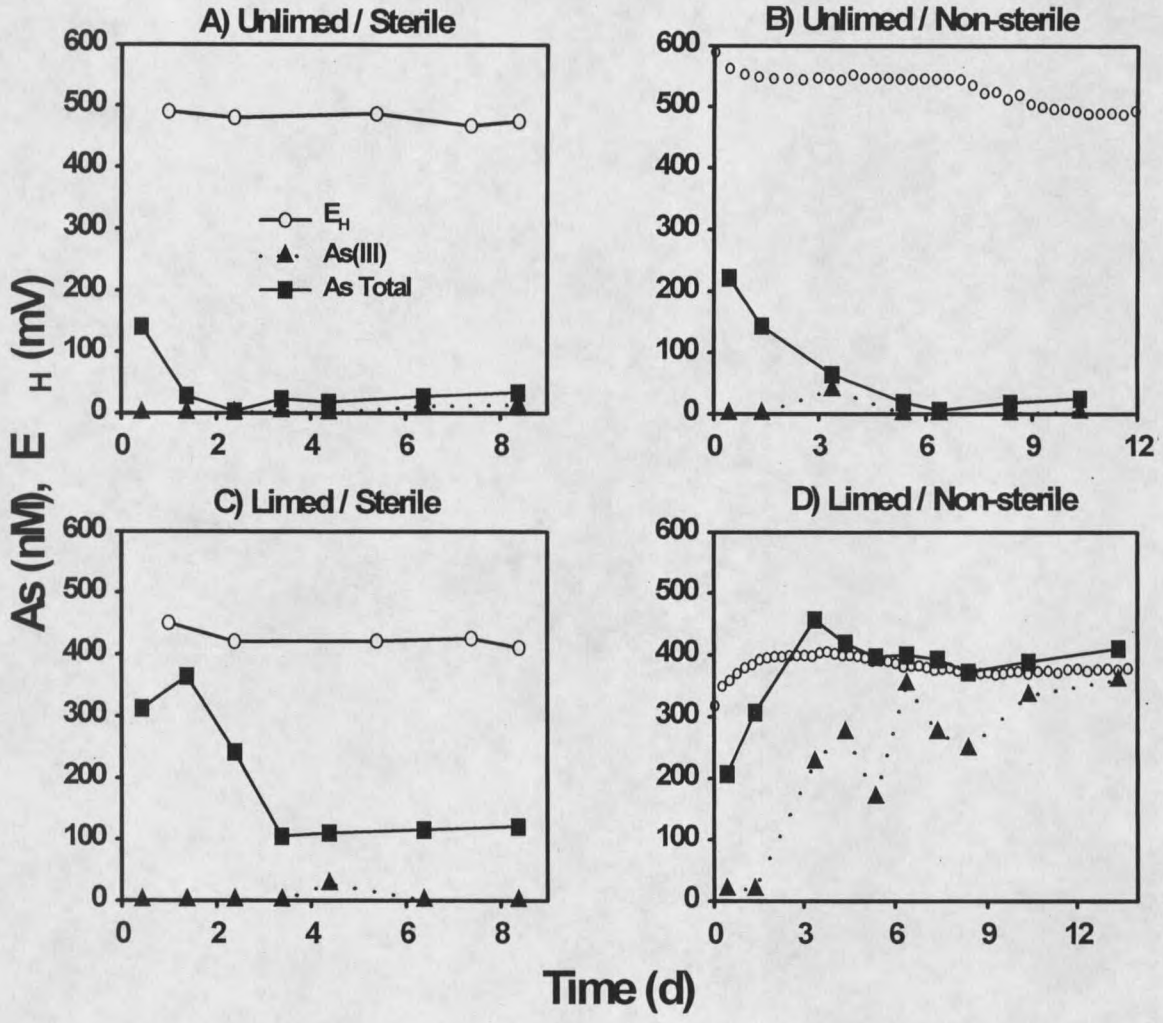

Figure 2.1. Total As and As(III) concentrations in the effluent from unlimed and limed, sterile (autoclaved) and non-sterile RT columns. Influent was not supplemented with C. The As(V) concentration is the difference between total As and As(III) concentrations. The E_H was measured with a Pt-electrode positioned in the center of the columns.

received influent containing 0.5 mM glucose and 1.0 mM lactate, redox potential values that theoretically favored the presence of As(III) were attained after 5 d (Figure 2.2).

In unlimed columns, both the sterilized and nonsterilized treatments released an initial pulse of As which ranged from 100 to 250 nM, then gradually declined to approximately 20 nM As after 1 to 5 d (Figure 2.1A, B). After liming, steady-state concentrations of total As mobilized from sterile columns increased significantly to about 100 nM (Figure 2.1C; Table 2.1). In nonsterile treatments, mean steady-state As concentrations increased further to approximately 300 nM (Figure 2.1D, Table 2.1). In the absence of microbial activity, As(V) was the predominant species mobilized after liming. Conversely, in the limed, nonsterile treatments, As(III) was the predominant species mobilized after 3 d, and the increased mobilization of As was associated with the reduction of As(V) to As(III). To verify that As(V) reduction and enhanced As mobilization occurred under oxic conditions, an additional experiment was conducted during which air was continuously pumped through a limed nonsterile column. Steady-state effluent As(III) and total As concentrations for the aerated treatment were essentially identical to concentrations in the nonaerated-limed treatments (Table 2.1), supporting the conclusion that microbial As(V) reduction within these limed columns occurred under redox conditions considered oxic as determined using Pt-electrode measurements and Fe^{2+} concentrations (*34*). Limed columns that received influent supplemented with C released about 450 nM total As after 3 d; nearly all as As(III) (Figure 2.2).