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Microbial Community Succession during Lactate Amendment and Electron-acceptor Limitation Reveals a Predominance of Metal-reducing *Pelosinus* spp.

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Short Title: Microbial Community Dynamics during Stimulation

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Abstract

Determining the success of *in-situ* bioremediation strategies is complex. By using controlled laboratory conditions, the influence of individual variables such as U(VI), Cr(VI) and electron donor and acceptors on community structure, dynamics, and the metal-reducing potential can be studied. Triplicate anaerobic, continuous-flow reactors were inoculated with Cr(VI) contaminated groundwater from the Hanford, Washington 100-H area, amended with lactate and incubated for 95 days to obtain stable, enriched communities. The reactors were kept anaerobic with N_2 gas (9ml/min) flushing the headspace and were fed a defined medium amended with 30 mM lactate and 0.05 mM sulfate with a 48 hr generation time. The resultant diversity decreased from 63 genera within 12 phyla to 11 bacterial genera (from 3 phyla) and 2 archaeal genera (from 1 phylum). Final communities were dominated by *Pelosinus* spp. and to a lesser degree, *Acetobacterium* spp. with small levels of other organisms including methanogens. Four new strains of *Pelosinus* were isolated with 3 strains being capable of Cr(VI)-reduction while one also reduced U(VI). Under limited sulfate, it appeared that the sulfate-reducers, including *Desulfovibrio* spp., were outcompeted. These results suggest that during times of electron-acceptor limitation *in-situ*, organisms such as *Pelosinus* spp., may outcompete the more well-studied organisms while maintaining overall metal-reduction rates and extents. Finally, lab-scale simulations can test new strategies on a smaller scale while facilitating community member isolation so a deeper understanding of community metabolism can be revealed.

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61 **Introduction**

62 Microbial community structure and function are controlled by many physicochemical factors
63 including pH, temperature, electron donors and acceptors, and hydrology (15, 21, 25). Altering
64 these conditions may shift community composition and select for members that can adapt to, and
65 outcompete, other organisms under differing parameters (35, 53). Anthropogenic contamination
66 adds an additional influence (27). Although communities at historically contaminated sites have
67 exhibited lower biomass and diversity (25), specific electron donors can increase the microbial
68 biomass and activity (3, 7, 64), but the specific interplay of these events is not well understood
69 due to difficulties with *in-situ* assessment and the lack of replicated stimulations (11).

70 Uranium (U(VI)) and chromium (Cr(VI)) are common metal contaminants which pose a
71 smaller human health risk when reduced to U(IV) (58) and Cr(III) (13). A wide diversity of
72 metal-reducing bacteria have been isolated in pure and mixed cultures (4, 63) with reduction via
73 direct enzymatic processes or indirectly through metabolic by-products such as Fe(II) or sulfide
74 (4, 31, 36). In metal contaminated sites with an adequate carbon and electron source, subsurface
75 communities exhibit a preference in processes where nitrate-reduction tends to predominate (46),
76 followed by Fe(III)- and sulfate-reduction (8).

77 Selection of specific compounds for stimulation of groundwater microbial communities can
78 selectively affect community structure (3). Various electron donors have been used to stimulate
79 specific biochemical activities at contaminated sites ranging from ethanol, methanol, glycerol
80 and lactate to more complex substrates as glycerol polylactate and humic acids (1, 6, 9, 14, 41,
81 54). Stimulation of *in-situ* anaerobic microbial communities with lactate increased metal-
82 reduction rates (3, 5, 14) with the enrichment of *Acidobacteria, Firmicutes, Deltaproteobacteria*
83 and *Betaproteobacteria* (5). The *Deltaproteobacteria* are the most well recognized metal-

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reducers; the sulfate- reducing bacteria (SRB) and Fe(III)-reducing bacteria (IRB) ((19, 33-34, 51)), while *Clostridium* sp. within the *Firmicutes* also reduce U(VI) (17). Most laboratory studies use ample exogenous electron-acceptor. However, *in-situ* experiments rely on groundwater electron-acceptor concentrations which are occassionally depleted due to increased microbial activity or dilution from rainfall (13). The current work was performed to address which populations within a subsurface community contaminated with Cr(VI) would persist with nearly depleted sulfate concentrations in the absence of other electron-acceptors to examine their metal-reduction potential. Lactate was chosen not only because it has been used *in-situ* at Hanford 100-H (13), but also because acetate would likely be generated to support a greater percentage of the initial community. Organisms such as *Geobacter* spp. and SRB might persist with low sulfate levels, or via fermentation. Under low nitrate and sulfate conditions, fermentors such as the firmicutes may be more likely to outcompete these organisms but their capacity for metal-reduction is unknown. If firmicutes exhibit the capacity to reduce metals such as Cr(VI) and U(VI), then an underappreciated portion of the subsurface community capable of accomplishing these activities during times of depleted electron-acceptor may have been revealed.

Methods

Sampling and cultivation

Groundwater samples were collected from well H-100 on the Department of Energy's Hanford Site (Washington, USA) (14). Samples (600 ml) were sealed under N_2, placed on ice and shipped to Oak Ridge National Laboratory. Upon arrival, 150 ml was removed as a reference, and immediately frozen at -80°C. The remaining groundwater (450 ml) was inoculated into triplicate custom-built, anaerobic glass fermentation vessels as described (37)) (Allen Glass,

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Boulder, CO), each receiving 150 ml, with working volumes of ~650 ml (Supplemental Figure 1). The reactors were supplied with medium from a single 19 L carboy (10 L medium) via a peristaltic pump at a flow rate of 0.22-0.23 ml/min for a dilution rate/media turnover of 0.487 d^{-1}. The carboy was kept anaerobic via constant purging with filter sterilized N_2 gas.

The lactate-enriched CCM1 medium (57) was modified to not contain exogenous electron-acceptors and was constantly stirred. Anaerobic conditions were maintained with N_2 gas (7-9 ml min^{-1}) flushing through the medium inlet drip-tube substantially decreasing biofilm development. Vessel temperature was maintained at 30°C ± 2°C by a recirculating water bath. Spent culture fluid and gas drained out of the vessel overflow vents into a closed collection vessel to maintain a constant volume. Exit gas passed through a Zn-acetate solution (1% w/v) to remove H_2S before being vented into a chemical fume hood (Supplemental Figure 1). Gas samples were taken with needles and syringes through vessel top ports sealed with butyl rubber stoppers. Liquid samples were taken bi-weekly throughout the 95-day experiment via syringe and a stainless steel cannula inserted through one of the stoppers.

Cell counts

Microscopic cell counts were performed using Live/dead bacteria viability kit (Baclight, Invitrogen, Eugene, OR) (23) and a Petroff Hausser Counting Chamber on a Zeiss Axioskop 2 plus microscope (Carl Zeiss Light Microscopy, Germany). For each temporal sample, 16 fields of view were counted and the average and standard deviation calculated. Samples from the first two weeks of the experiment were concentrated via centrifugation and resuspended due to low cell counts (< $1x10^7$ cells/ml).

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Metabolite Analysis

Filtered supernatants were acidified with 200 mM H_2SO_4 (5 mM final concentration) before injection into a Hitachi Lachrom Elite HPLC system (Hitachi High Technologies, USA). Metabolites were separated on an Aminex HPX-87H column (BioRad Laboratories) under isocratic temperature (40ºC) and flow (0.5 ml/min), then passed through a refractive index (RI) detector (Hitachi L-2490). Metabolite identification used retention time comparison to known standards and quantification was calculated against linear standard curves. All standards were prepared in fresh culture medium to account for the interference of salts in the RI detector.

Fermenter gases were collected via sterilized Hamilton gas-tight syringes and injected into an Agilent 6850 GC (Agilent Technologies, USA) equipped with a thermal conductivity detector (TCD) for CO_2 quantification. Analytes were separated on an HP-PLOT U column (30m x 0.32 mm x 0.10 um film, J&W Scientific, Agilent Technologies, USA). Two HP-PLOT U columns were joined together for a total length of 60m for optimized separation. Samples were injected into a 185ºC split-splitless injector with a split ratio of 3:1 and an isocratic oven (70ºC) with He carrier flow (7.0 ml min^{-1}). The detector had 10 ml/min helium makeup flow at 185ºC, with the detector filament set for positive polarity.

Samples to detect CH_4 concentrations were injected into an Agilent 6890 gas chromatograph equipped with a flame ionization detector (FID). Samples were separated on a DB-FFAP column (30m x 0.32 mm x 0.5 um film, J&W Scientific, Agilent Technologies, USA) after passing through a 230ºC split-splitless injector with the split ratio set to 3:1 and isocratic oven (50ºC) and helium carrier flow (1.5 ml min^{-1}). The FID had a hydrogen flow of 40 ml min^{-1}, air of 450 ml min^{-1} and helium makeup flow at 45 ml min^{-1}. The detector temperature was set at

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230ºC. Peak identifications were performed by comparison with known standards and compound quantification was calculated against individual linear standard curves.

DNA extraction and pyrosequencing of the bacterial and archaeal 16S rRNA genes

For pyrosequencing analysis, 13 ml samples were collected every two weeks from reactor outflows, centrifuged and stored at -80°C until analysis. Selected samples were analyzed at the conclusion of the experiment. Total community genomic DNA (cgDNA) was extracted using the PowerSoil™ DNA Isolation Kit (Mo Bio Labs, Inc., Carlsbad, CA). Pyrosequencing was conducted using the barcode tagging method described at the Ribosomal Database Project (RDP) Pyrosequencing Pipeline (http://pyro.cme.msu.edu/index.jsp) and primers designed for the hypervariable V4 region (~200-210 bp) of the 16S rRNA gene for GS 454 FLX pyrosequencing (Roche Inc.) as described (55) using 50 µl PCR reactions with high fidelity AccuPrime™ *Pfx* DNA polymerase (Invitrogen, Carlsbad, CA). The PCR amplicons were purified using the Agencourt AMPure solid-phase paramagnetic bead technology (Agencourt Bioscience Corporation, Beverly, MA). The PCR amplicon purity, concentration and size were estimated using DNA 1000 reagents and an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Waldbronn, Germany). The reactions were paired according to DNA quantity and quality prior to performing emulsion reactions for sequencing on a 454 Life Sciences Genome Sequencer FLX (Roche Diagnostics, Indianapolis, IN) using the unidirection amplicon library sequencing protocol with emPCR Kit II (Roche). Primary processing of the raw 454 FLX data (~100 Mb for bacteria) was conducted through the RDP Pyrosequencing Pipeline (10). Sequences were sorted by tag sequence, and the 16S rRNA gene primers with low-quality sequences were removed. A total of 68,481 high quality (99% cutoff) sequences of 200-250 bp were obtained for 19 samples.

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175 Archaeal sequences were analyzed similarly except that amplification included ~300 bp
176 of the 16S rRNA Archaea gene with forward primer nucleotides containing modified U519F
177 primer (49) fused to variable key tags for multiplexing (10) and to the 454 FLX sequencing
178 primer A (5'GCCTCCCTCGCGCCATCAGxxxxxx**CAGYMGCCRCGGKAAHACC**, where
179 the x region represents the various key tags and the 16S rRNA primer is bold). The reverse
180 primer was a fusion of the 454 FLX sequencing primer B and a modified Arch806R primer (50)
181 (5'-GCCTTGCCAGCCCG**CTCAGGGACTACNSGGGTMTCTAAT**, where the 16S rRNA
182 region is bold). Reactions were sequenced on the 454 FLX. Raw data (~36 Mb for archaea) was
183 processed as above with 20,923 high quality sequences of 290-300 bp obtained for 19 samples.
184
185 *Quantitative PCR Analyses*
186
187 SYBR green quantification of the 16S rRNA gene copy number was performed in a Bio-
188 Rad CFX96™ (Hercules, CA) thermal cycler on DNA extractions prepared as above in
189 duplicate. Both general archaeal and bacterial assays were performed in empirically-optimized,
190 20 µL reactions. Archaeal assays used primers arc 915f and arc1059r (Eurofins MWG Operon;
191 Huntsville, AL) at 350 nM each, iQ Supermix at 1× with 2 µL of cgDNA. Amplification used
192 45 cycles and then a fluorescence reading. Following amplification, products were denatured
193 (95°C, 10 sec), and a melt curve determined (60-95°C). Standard curves used *Methanococcus*
194 *maripaludis* S2 gDNA diluted from 10^7-10^2 16S rRNA gene copies per reaction (43).
195 Bacterial assays used primers Eub338 and Eub558 (Integrated DNA Technologies;
196 Coralville, IA) at 500 nM each, iQ Supermix at 1× and 2 µL of cgDNA. Amplification again
197 used 45 cycles and a fluorescence reading. Following amplification, products were denatured
198 (95°C, 10 sec), and a melt curve determined (50-95°C). Standard curves were constructed using

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199 *Escherichia coli* gDNA diluted from 10^8-10^3 16S rRNA gene copies per reaction (16).

200

201 *Phylogenetic analyses*

202 Bacterial and archaeal 16S rRNA sequences were assigned to a set of hierarchical taxa
203 using a Naïve Bayesian rRNA classifier version 2.0 with confidence threshold of 80%
204 (http://rdp.cme.msu.edu/classifier/classifier.jsp) (61). Sequences from this study were
205 subsequently aligned using the fast, secondary-structure aware Infernal aligner (39) and clustered
206 by the complete-linkage clustering method available at the RDP's Pyrosequencing Pipeline.

207 Further exploration into the shifts in the archaeal community present in the reactors was
208 performed by clustering the sequences from each genus at 97% confidence level. Clusters
209 containing 10 or fewer sequences were eliminated due to possible sequence error or artifacts.
210 Heat maps were constructed using relative abundance for each cluster from the total number of
211 sequences using Genesis version 1.7.6 software (Graz University of Technology, Graz, Austria).

212

213 *Sequence accession numbers*

214 Sequences from this study were deposited in the GenBank Short Read Archive database
215 under accession number SRP003881.2.

216

217 *Statistical analyses*

218 Constrained ordination techniques were utilized to identify patterns of sequence variation
219 between reactors, sampling date, sequence relative abundance and metabolite concentrations.
220 Bacterial and archaeal sequences were combined in order to observe the overall increasing
221 similarity during the reduced diversity of reactor communities. Although the pyrosequencing and

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amplification reactions of bacteria and archaea were amplified and analyzed separately, ratios of the gene copy numbers obtained from qPCR analyses of the gDNA were used to determine the percentage that each domain contributed to the overall sample DNA concentration. Accordingly, the data could be combined based upon relative abundance to perform constrained ordination statistics. Sequence abundances for each genus were converted into weight percentages by dividing by the total abundance per sample; weight percentage values were natural log transformed (ln + 1). Relative abundances of bacterial and archaeal data were combined to bacteria:archaea as determined by qPCR. Detrended correspondence analysis (DCA), an indirect gradient analysis based on segment length, was performed to determine the modality of the sequence data. The analyses resulted in short (<2.0) segment lengths indicating linear datasets. Therefore, redundancy analysis (RDA) was performed (CANOCO 4.5, Microcomputer Power). The RDA identified variation patterns among genera present in each reactor and correlated those patterns to predictor variables. Sequence data were used as response variables, and predictor variables used were the measured metabolite data and cell counts. Forward selection of the predictor variables followed by Monte Carlo permutation tests were used to prevent artificial inflation of variation due to autocorrelation in the constrained ordination model (30).

Isolates

At the conclusion of the experiment, isolates were obtained by either fluorescence-activated cell sorting (FACS) (20) or by serial dilution to extinction. Isolates obtained through FACS were cultured using the CCM1 medium. A fresh fermentation vessel sample (7.5 ml) was vigorously shaken and then diluted 1:100 in anaerobic, ice cold PBS solution. Cells were sorted via forward/side scatter into the 48 well plate, one cell per well using an InFlux Flow Cytometer

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(Cytopeia, Seattle, WA) and the plates were returned to the anaerobic glovebag. After 5 days of incubation, individual wells were screened for growth via adding bromothymol blue (final concentration 150 µg/ml) to indicate a drop in pH followed by microscopy. Wells displaying growth were marked as putative isolates and transferred to Balch tubes containing CCM1 medium and incubated at 30º C until visible growth occurred. FACS isolates were also grown with exogenous sulfate to determine if growth could be spurred by sulfate.

Attempts to obtain isolates were also carried out using serial dilutions and plating for sulfate- and Fe(III)- reducing bacteria as well as methanogens. The same medium was used with the following modifications; 1) Fe(III)-reducing bacteria were enriched with 30mM acetate (rather than lactate) and 10 mM fumarate, 2) SRB medium contained 10 mM sodium sulfate, and 3) tubes for methanogens used 30 mM acetate with 14 µM choline chloride. A second set of methanogen tubes were pressurized (3 psi) with 80%:20% H_2:CO_2 gas. The headspace of all other tubes contained 80%:20% N_2:CO_2 gas at 3 psi. The Balch tubes were sealed using butyl stoppers and aluminum crimp seals and made anaerobic by three cycles of vacuum to -20 psi and pressurizing with the appropriate gas to 3 psi. All tubes were then autoclaved at 121°C for 20 min and allowed to cool before receiving post-autoclave amendments as described above.

Serial dilutions (10 fold) were made from 10^{-1} -10^{-8} in triplicate and incubated (30°C). After growth occurred, samples were taken from the 10^{-5}-10^{-8} tubes and inoculated into fresh medium (giving dilutions of 10^{-6}-10^{-9}). Samples (100µl) were plated with the same medium plus agar. Isolated colonies were collected and inoculated into Balch tubes and incubated (30°C). One more series of dilutions were made into fresh media tubes to ensure the isolation of single organisms. Microscopy was used to verify that only one morphotype was present for each isolate.

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269 *Sequencing of Isolates*

270 Genomic DNA was extracted from 100 µl of isolate culture using a PowerSoil™ DNA
271 Isolation Kit (Mo Bio Labs, Inc., Carlsbad, CA) or a liquid N_2 grinding process (26). For
272 bacterial isolates, 16S rRNA genes were amplified using universal bacterial primers 8F and
273 1492R (20)Methanogen isolate 16S rRNA genes were amplified using universal archaeal primers
274 Ar21F and Ar958R. A separate amplification using bacterial primers was performed on the
275 putative methanogen cultures to ensure that the cultures were devoid of bacteria.

276 16S rRNA amplification was performed using 10 ng gDNA template, 0.25 µM primers,
277 250 µM dNTPs, and 1 unit *Pfu* DNA polymerase in 1 × *Pfu* DNA polymerase reaction buffer [20
278 mM Tris•HCl (pH 8.8), 2 mM $MgSO_4$, 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 0.1 $mgml^{-1}$ BSA, and
279 0.1% Triton X-100]. Universal primer 1100R was used for single pass sequencing reactions of
280 each bacterial isolate and Ar958R was used to generate a single pass sequencing product from
281 the methanogen isolates. DNA sequences were determined using BigDye™ terminator chemistry
282 (Applied Biosystems, Foster City, CA) according to manufacturer recommendations and
283 resolved using a 3730 DNA analyzer at a 5:1 dilution. Sequences were compared to known
284 organisms using the Basic Local Alignment Search Tool (2)through the NCBI database.

285

286 *Metal reduction assays*

287 Cultures of each isolate and *Pelosinus fermentans* strain R7 (DSM 17108) (44) were
288 grown in lactate-enriched CCM1 media in duplicate 1 L pyrex bottles sealed with a rubber
289 stopper under N_2 headspace at 30ºC. All manipulations were conducted in an anaerobic glove
290 bag unless otherwise noted. Cultures were grown to log phase and centrifuged (8000 rpm, 8

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minutes at 4ºC) and washed 3 times with 30 mM lactate/30 mM $NaHCO_3$ buffer (pH 6.8) and finally resuspended to 7.5 ml (12). Each metal reduction assay (detailed below) was performed in duplicate serum vials (sterile, degassed vials containing 30 mM lactate/30 mM $NaHCO_3$ buffer) and contained 4 ml of the washed cells. Separate "no cell" and heat-killed controls (*Shewanella oneidensis* MR1 and *P. fermentans* R7) were employed while S. *oneidensis* MR-1 acted as the positive control (24, 40). Samples were taken at 0, 180 and 480 minutes.

Assays for soluble (FeIII) and solid iron (FeOOH) reduction contained 10mM $FeCl_3 \cdot 6H_2O$ and 20 mM FeOOH, respectively and used the ferrozine method (32). Chromate reduction assays used 60 µM potassium chromate and 60 µM potassium dichromate, individually with the diphenlycarbazide method (65). U(VI) reduction assays used 0.5 mM uranyl acetate and 0.25 ml sample was added to 2.25 ml of 0.1 M HNO_3, mixed with Uraplex (Chemchek Instruments Inc.), removed from the anaerobic chamber and analyzed on a Kinetic Phosphorescence Analyzer (KPA, Chemcheck Instruments, Inc.) (52).

Results

Cell counts and qPCR quantification

Direct cell counts from the initial groundwater sample numbered 8.0 x 10^5 cells/ml and increased by day 7 in all three reactors to 1.08-1.28 x 10^6 cells/ml (Figure 1A). By day 37, all three reactors reached cell densities greater than 1 x 10^9 cells/ml that were maintained throughout the experiment. Similar trends and values were determined with duplicate qPCR analysis and yielded a ratio of archaeal vs. bacterial gene copies (Archaea: slope -3.461, reaction efficiency 94.5%, R^2 0.999; Bacteria: slope -3.419; reaction efficiency 96.1%, R^2 0.998; Figure 1B). Overall, the gene copies per reactor were highly similar except for archaea that decreased in

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reactor 1 for days 20-40, but then became similar to the other reactors by day 50. This similarity was sustained throughout the remainder of the experiment, indicating a steady state had been achieved for the reactors, though not necessarily for individual populations.

Metabolite analysis

Lactate concentrations in each reactor decreased from the initial 30 mM present on day 3 to as low as 19.7 mM in reactor 1 and 25.0 mM in reactors 2 and 3 (Figure 2A) but then returned to 30 mM by day 7. This was followed by a steady decrease until lactate was below detection by day 32 in reactors 2 and 3 and day 39 in reactor 1 (Figure 2A) suggesting electron donor and carbon limitation. Acetate concentrations steadily increased to 38.4-44.5 mM by day 24 and to stoichiometric amounts during the rest of the cultivation. Other organic acids, specifically formate and pyruvate, were intermittent but always below 0.1 mM (data not shown).

Carbon dioxide was not detected until day 7 when concentrations ranged from 0.1-0.13 mM and fluctuated throughout the experiment (Figure 2B). For example, at day 24, a divergence was observed with reactor 1 (2.49 mM) while reactors 2 and 3 showed concentrations from 0.7-1.0 mM, perhaps suggesting an increase in the fermentor population in reactor 1. However, by day 32, reactors 2 and 3 attained similar peak concentrations of 2.73 and 2.55 mM, respectively, while concentrations in reactor 1 had decreased to 0.64 mM. Methane was also initially detected on day 7 (0.01-0.02 mM) and appeared intermittently from days 9 through 37 (Figure 2C). By day 43, methane ranged from 0.012-0.05 mM. Hydrogen in headspace was below detection limits throughout the entire experiment (< 0.5 μM).

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Microbial community composition of initial groundwater sample

Pyrosequencing analysis of the initial groundwater sample yielded 2,351 bacterial sequences and 83 archaeal sequences identified through the RDP classifier at the 80% confidence threshold. Of the bacteria, 309 (13.1%) sequences were unclassified bacteria at the domain level. The remainder of the bacterial community was classified within 11 phyla with 661 (28.1%) sequences classified within 55 genera, while the remaining 1,381 (58.7%) sequences were grouped as unclassified classes, orders or families within distinct phyla. The most abundant sequences grouped as "unclassified *Clostridiales*" (19.1%) (Figure 3). While these sequences were classified as members of the phylum *Clostridia* and order *Clostridiales* at confidence of greater than 99%, the sequences were not comparable to other known sequences in the RDP database at a more specific level of classification. The next most abundant groups were the unclassified *Betaproteobacteria* (16.3%) and the unclassified *Gammaproteobacteria* (12.7%). With respect to more specific classifications, the most abundant genus was *Pelosinus* (8.9%), followed by genus *Syntrophomonas* (2.2%) and *Pseudomonas* (1.8%) (Figure 3).

All 83 archaeal sequences in the initial groundwater samples were classified within the phylum *Euryarchaeota*. Members of the genus *Methanosarcina* comprised the majority of the sequences (47.0%) followed by unclassified *Methanomicrobiaceae* (15.7%). While these identifications were performed at a confidence level of >99%, determining the classification at a more specific level was not possible. The genera *Methanocella* and *Methanosphaerula* each comprised 9.64% of the archaeal sequences, while *Thermoplasmatales* contributed 7.23%. The remaining groups, unclassified *Euryarchaeota*, *Methanobacterium*, *Thermogymnomonas* and *Ferroplasma* each possessed <5% of the sequences (Figure 4A).

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359 *Enriched microbial community composition*

360 Throughout the course of the cultivation, the diversity of the microbial community was
361 reduced and a few distinct genera emerged as the dominant populations within the enriched
362 community. By day 20, the bacterial community was dominated by *Acetobacterium* (18.6-
363 72.3%), *Sporumosa* (15.6-36%), *Pelosinus* (4.4-30.8%) and unclassified *Enterobacteriaceae*
364 (6.2-13.6%). By day 49 and throughout the remainder of the experiment, the community was
365 dominated by *Pelosinus* spp. (48.4-97.2%) and to a lesser extent *Acetobacterium* spp. (1.0-8.3%)
366 (Figure 3). Although the triplicate reactors received the same medium and gas feed, there were
367 temporal variations in the community compositions. Most notably was the community shift
368 observed in reactor 2 on day 63 associated with increases in *Rhizobium*, *Brevundimonas*,
369 *Aeromonas*, unclassified *Rhizobiales* and unclassified *Enterobacteriaceae* which, in turn,
370 decreased by day 95 and yielded a community very similar to reactors 1 and 3. However, by day
371 95, all 3 bacterial communities were highly similar in the percentage of the community that each
372 genera represented as shown by *Pelosinus* spp. (89.0-91.7%), *Acetobacterium* spp. (5.0-8.3%),
373 Unclassified *Clostridiales* spp. (1.1-1.3%) and unclassified *Veillonellaceae* spp. (0.3-0.4%).

374 Only two archaeal genera, the acetoclastic *Methanosarcina* and the hydrogenophagic
375 *Methanobacterium* (within the *Methanomicrobiaceae*), maintained substantial populations but
376 had notable temporal fluctuations between the reactors Early on, only a few archaeal sequences
377 were detected, but by day 29 *Methanosarcina* was the dominant genera (93.7-99.0 %). By day
378 37, *Methanobacterium* (21.3-99%) abundances increased while *Methanosarcina* decreased (1-
379 78.7%) (Figure 4B). For the remainder of the experiment, the archaeal proportions remained
380 relatively stable in reactors 2 and 3, while reactor 1 showed greater variation resulting in the
381 highest concentration of *Methanobacterium* (83.8%).

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382 Due to these continued fluctuations, the archaeal compositions were further investigated by
383 cluster analysis of all the archaeal OTU sequences and their temporal relative abundances. The
384 sequences grouped into 13 clusters and three clades at the 97% confidence level and the temporal
385 abundance and sequence distribution from each cluster is displayed (Figure 4B,C). The majority
386 of sequences in the initial sample were found in clusters C2, C5 and C8 (22-35%) and to a lesser
387 degree C1, C3 and C9 (1.6-3.2%). Clusters C4, C6, C7, and C10-C13 were initially below
388 detection limits using the 97% cutoff value, but abundance variations were observed over time.
389 For example, C4 was originally below detection, but increased over time in reactors 1 and 2
390 (Figure 4B). Similarly, while originally detectable, cluster C5 showed an even more dramatic
391 increase late in the experiment in reactor 3. The other clusters (C6, C7, C10-13) were rare or
392 below detection limits throughout the course of the experiment.

393 RDA analysis was utilized to examine patterns of relative sequence abundance variation to
394 measured descriptor variables (i.e. metabolites, cell counts and gas concentrations) (Figure 5).
395 RDA axes 1 and 2 described 67.1% of the variation in microbial composition from each reactor
396 ($F=19.98$; $p=0.002$). Samples taken from the reactors on day 20 grouped together in the triplot
397 according to higher abundances of *Hydrogenophaga*, *Sporomusa*, *Sulfurospirillum*,
398 *Stenotrophomonas*, *Acetobacterium* and unclassified *Enterobacteriaceae*, and were correlated
399 with high concentrations of lactate ($r=0.7385$). As the experiment progressed and cell counts
400 ($r=0.8949$), methane ($r=0.6403$) and acetate ($r=0.4644$) concentrations increased, these correlated
401 to greater relative abundances of *Pelosinus*, *Methanobacterium*, *Methanosarcina*, unclassified
402 *Clostridiales*, and *Pseudoxanthomonas*. It was also notable that over the course of the
403 experiment, the variation in the community composition within the triplicate reactors decreased
404 as shown by the decreasing distance from the origin of the sampling day values for each reactor

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405 (e.g., the closer grouping of the day 37 and later samples versus the day 20 and 29 samples)
406 along the Y-axis.

407

408 *Isolates*

409 A total of 16 isolates were obtained from FACS sorting and identified as being 99-100%
410 identical to the 16S rRNA gene of *Pelosinus fermentans* strain R7 (DSM 17108) through the
411 NCBI database (44). Although these isolates possessed similar 16S rRNA gene sequences to the
412 type strain, they displayed varying metabolic characteristics. All strains tested reduced soluble Fe
413 (III), including *Pelosinus fermentans* strain R7 (Table 1). No strain reduced solid iron (FeOOH).
414 FACS strain A11 also reduced U (VI), monochromate and dichromate. FACS strain B3 and
415 *Pelosinus fermentans* strain R7 reduced monochromate and dichromate while, FACS strain B4
416 only reduced monochromate. All strains were tested for the ability to reduce sulfate but none
417 were capable of sulfidogenesis (data not shown). The enrichment cultures via serial dilutions for
418 sulfate- and Fe(III)- reducing bacteria were co-cultures after repreated streaking and serial
419 dilutions. Further efforts to attain pure cultures are ongoing. Methanogens were isolated that
420 appear closely related (greater than 98% similarity) to *Methanosarcina barkerii* and uncultured
421 members of *Methanosarcina* spp. (data not shown). Although unlikely to be new species, they
422 may be among the first methanogenic isolates from these Hanford groundwaters.

423

424 **Discussion**

425 After *in-situ* lactate amendments in the presence of abundant acceptors, U(VI)- and Cr(VI)-
426 reduction typically coincides with increased sulfate- and Fe(III)- reducing populations (13, 58,

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427 62). According to phylochip and other supporting data (13), lactate utilizing SRB are followed
428 by acetate utilizing SRB. However, the selective pressure of lactate with low (50μM) sulfate
429 resulted in a shift in the *in-situ* microbial community structure where the extensively studied
430 *Desulfovibrio* spp. and *Geobacter* spp. (18, 59) decreased from their initial 1.51% and 0.33%,
431 respectively to an average of 0.13% and 0.14%, respectively. It was instead the less studied
432 *Pelosinus* spp. becoming dominant followed by *Acetobacterium* spp. Within the archaea, both
433 *Methanobacterium* and *Methanosarcina* genera appeared to out-compete others. This suggested
434 that although there was ample lactate and acetate, sulfate concentrations were not sufficient to
435 allow *Desulfovibrionales* to thrive. H_2 was routinely near detection limits and methanogenic
436 populations were present as potential terminal electron acceptors throughout the 95 days.
437 However, coupling of SRB with methanogens, though well documented, (48, 56) did not appear
438 to predominate in these reactors. Rather, SRB and *Geobacter* spp. were unable to compete and
439 were displaced by a *Pelosinus* spp. and *Acetobacter* spp. dominated community.

440 Previous sediment based experiments utilizing lactate amended, U(VI) contaminated
441 microcosms produced different results where *Pelosinus spp*. and *Geothrix spp*. became
442 predominant (5). Long-term lactate enrichments using flow-through contaminated sediment
443 columns and microcosms resulted in increased *Geobacter* spp. and *Desulfovibrio* spp. (3, 45)
444 while another study using lactate-enriched sediments from the same site observed *Pelosinus*
445 *fermentans* to be dominant with various *Deltaproteobacteria* showing increased abundance (22).
446 The latter also reported that in acetate-based Fe(III)-reducing enrichments of saturated
447 sediments, *Desulfovibrio* and *Desulfomicrobium* spp. dominated and not *Geobacter* spp. while
448 *Pelosinus* spp. were predominant in nutrient poor saturated, unsaturated and acidic sediments.
449 These observations may suggest a competitive advantage for *Pelosinus* spp. via metabolic

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450 flexibility with respect to nutrient poor, unsaturated or lower pH conditions (22). In each of these
451 cases, ample electron-acceptor was available. In direct competition experiments between
452 *Acetobacterium*, *Desulfovibrio* and *Veillonella* with L-lactate, *Desulfovibrio* outcompeted
453 *Actetobacterium* and *Veillonella* under lactate-limited conditions while sufficient sulfate and
454 Fe(III) were present (29). Neither electron-acceptor was present in appreciable concentrations in
455 the present study, but *Desulfovibrio* spp. has been successful during lactate limitation. Both
456 *Geobacter* spp. and *Desulfovibrio* spp. were outcompeted here, suggesting that adequate
457 electron-acceptors may be important for the dominance of *Geobacter* spp. and *Desulfovibrio*
458 spp., perhaps being more critical than electron donors and carbon sources. These direct
459 competition results suggest that *Desulfovibrio* outcompeted *Acetobacterium* in lactate limited
460 conditions and *Pelosinus* outcompeted *Desulfovibrio* here. Accordingly, *Pelosinus* outcompeting
461 *Acetobacterium* under electron-acceptor limitation, as occurred here, should not be surprising.

462 One reason that *Pelosinus spp.* may have overwhelmed the community by becoming
463 90.0±1.5% of the final consortia is that it comprised ~9% of the initial groundwater community;
464 ~6 times greater than *Desulfovibrio* spp. and ~30 times than *Geobacter* spp. However, this also
465 suggests that *Pelosinus* spp. displayed a fitness under the conditions tested and perhaps also *in-*
466 *situ*. Further, *Acetobacterium* spp. were a mere 0.09% of the community initially, but became the
467 second most abundant population at 6.9±1.7%. It is worthy to note that *Acetobacterium* increased
468 to 72% of the community by day 29 when lactate became limiting and then diminished, perhaps
469 being outcompeted for lactate by *Pelosinus*.

470 The closest known organism to the *Pelosinus* strains isolated in this study is *Pelosinus*
471 *fermentans*, which is to date one of only three cultured strains within this genus and is capable of
472 fermenting lactate and coupling its oxidation to Fe(III)-reduction (44). The second species, P.

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473 *defluvii* cannot utilize lactate, but can reduce Fe(III) but not sulfate (38) while *P. fermentans*
474 strain UFO1, isolated from Oak Ridge sediments, can consume lactate and reduce Fe(III) as well
475 as U(VI) (42). While Fe(III)- and U(VI)- reduction have been observed in *Pelosinus* isolates,
476 none have been shown to reduce Cr. Although the isolates obtained in this study were a >99%
477 match to the *P. fermentans* type strain according to the 16S rRNA sequences, they demonstrated
478 broad metal-reducing characteristics. Strain A12 can only reduce Fe(III) as opposed to strains
479 A11, B3 and the type strain that can reduce both mono- and di- chromate while strain B4 can
480 only reduce monochromate. Not only is strain A11 capable of the above activities, but it can also
481 reduce U(VI) similar to strain UFO1. Hence, not only did the genus *Pelosinus* become dominant
482 under electron-accepting conditions over the more extensively studied metal-reducers during
483 electron-acceptor limitation, but the characterization of metal-reducing capabilities for four
484 different strains now allows for an appreciation of the diversity of metal-reduction within this
485 genus. Given this information, further characterization of the functional and genomic capabilities
486 of these isolates will be pursued.

487 With respect to the archaeal populations, several OTU's belonging to two methanogenic
488 genera maintained populations throughout the experiment; the acetoclastic *Methanosarcina* spp.
489 and the hydrogenotrophic *Methanobacterium* spp. suggesting multiple routes of carbon
490 mineralization from fermentation by-products *in-situ*. However, there was no obvious correlation
491 between temporal species abundances and volatile fatty acid or gas concentrations, similar to
492 studies of anaerobic sludge and food waste reactors (28, 60). However, further investigation into
493 archaeal community revealed that several OTU's, while not initially detectable in the original or
494 early temporal samples, were able to maintain low level populations despite numerous reactor
495 turnovers and becoming robust in later time periods. Such organisms may be part of the "rare

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biosphere" that were not readily culturable or detected, but can grow and become dominant if the proper micro-environmental conditions are met (47) such as low gas and liquid flow rates.

In summary, comprehensive investigations such as these allow for the study of consequential succession in microbial communities from contaminated environments with the ability to determine the relative importance of particular community populations via alteration of selected, imposed perturbations. The designed system for continuous steady state enrichment over many generations followed by FACS or other isolation methods can facilitate these studies on a more discreet level than is possible *in-situ*. It also presents certain advantages for the cultivation of organisms that have been traditionally difficult to isolate, but whose presence is routinely indicated by pyrosequencing. In the present case, the limitation of available electron-acceptor on a community that has been shown to transiently increase and decrease in sulfate- and Fe(III)- reducing organisms with concomitant Cr(VI)-reduction *in-situ* (13) was explored. While the abundance of *Desulfovibrio* spp. and *Geobacter* spp. was expected to be lower than *in-situ*, it was unknown whether their numbers could increase via fermentation or if fermenting organisms would instead become dominant. With respect to metal-reduction, it is encouraging that although the population matrix was substantially different during electron-acceptor limitation, the resultant dominating species were capable of the complete reduction of *in-situ* Cr(VI) levels as well as U(VI). This suggests that whether the contaminated areas are electron-acceptor rich or depleted, the native microbial community may be capable of reducing and immobilizing oxidized metals and radionuclides, whether they result from plume movement into the area or from re-oxidation of previously reduced Cr and U pools.

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776 **Figure Legends**
777
778 Figure 1. (A) Cell counts and (B) qPCR quantification data for microbial consortium in triplicate
779 anaerobic continuous flow reactors inoculated with groundwater from Hanford well H-100.
780 Average values with standard error bars are presented.
781
782 Figure 2. (A) Actetate (closed symbols) and lactate (open symbols), (B) CO_2 and (C) CH_4
783 concentrations from the triplicate anaerobic continuous flow reactors inoculated with
784 groundwater from Hanford well H-100. Squares (F1), Diamonds (F2), Circles (F3).
785
786 Figure 3. The original and temporal changes in the bacterial community composition according
787 to pyrosequencing analysis from selected dates from triplicate continuous flow bioreactors of
788 lactate-enriched Hanford well H-100 groundwater sample where Pelosinus (green) and
789 Acetobacterium (orange) became dominant.
790
791 Figure 4. (A) The original archaeal groundwater community and (B) temporal changes in
792 archaeal OTU abundance from selected dates from triplicate continuous flow bioreactors of
793 lactate-enriched Hanford well H-100 groundwater sample. *Methanosarcina* OTU are dotted and
794 *Methanobacteria* OTU's are solid. (C) A distance tree of sequence representatives from the
795 individual archaeal clusters (97% level).
796
797 Figure 5. Triplot of redundancy analysis (RDA) of the relative abundances of microbial genera
798 determined by pyrosequencing analysis of selected dates from triplicate continuous flow reactor
799 experiment of lactate-enriched Hanford well H-100 groundwater sample. Dashed arrows (blue)
800 indicate genera associated with the variation in microbial community composition. Solid (black)
801 arrows indicate metabolite data significantly associated with the variation.
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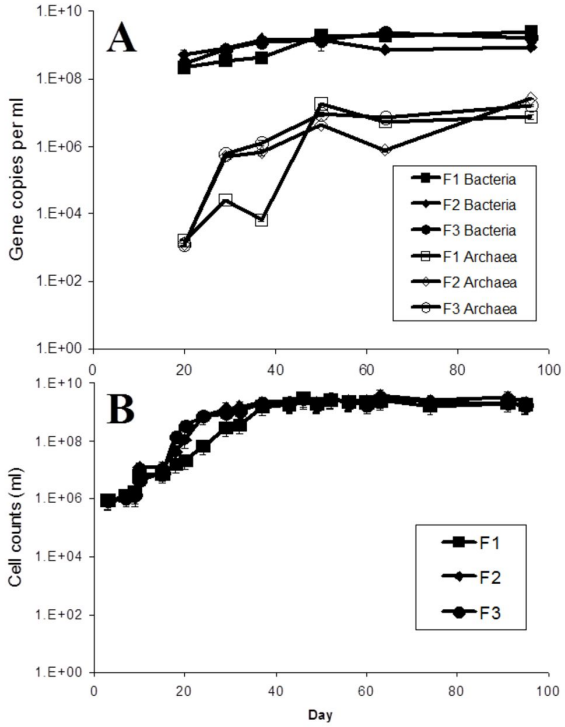

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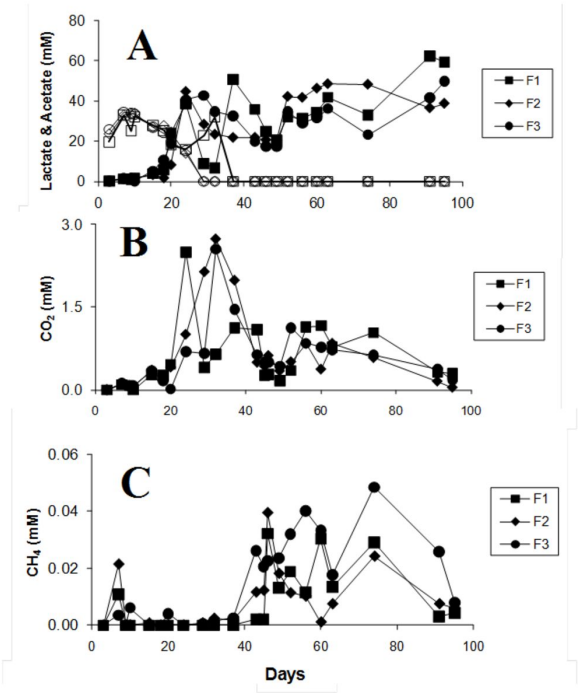

Figure 2

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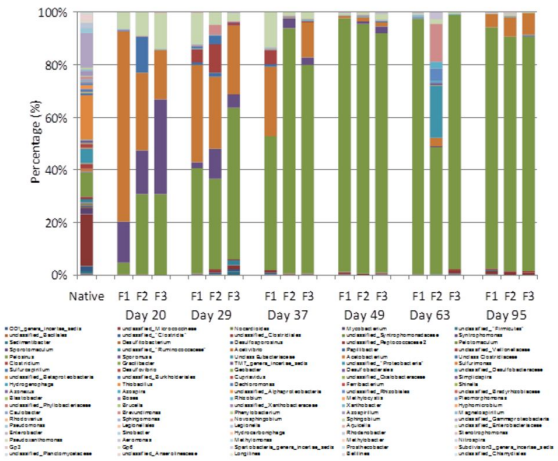

Figure 3

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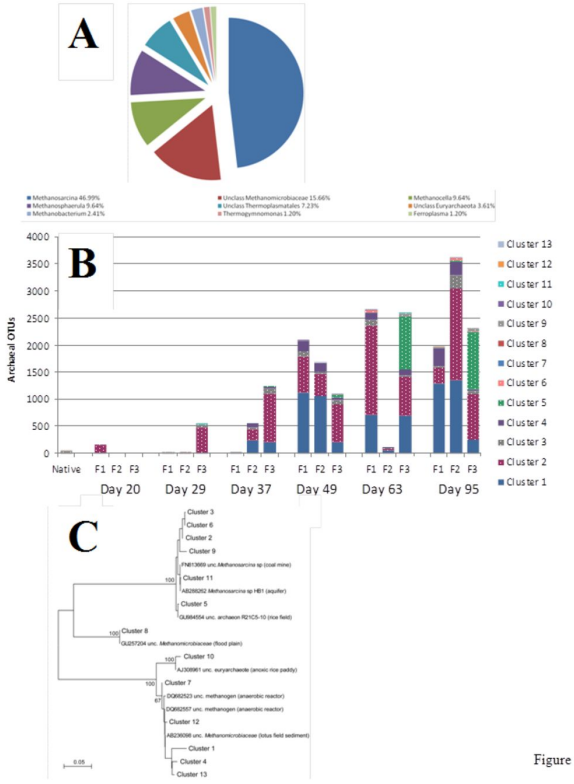

Figure 4

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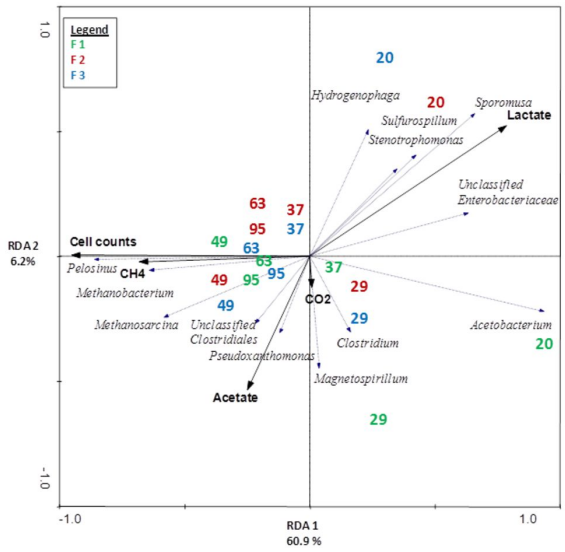

Figure 5

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Table 1: Metal reduction assays for *Pelosinus fermentans* type strain and Hanford isolates

Metal Reduction Assays					
Isolate	**Iron (III)**	**Solid Iron (FeOOH)**	**Monochromate**	**Dichromate**	**Uranium**
Pelosinus fermentans strain A11	Yes	No	Yes	Yes	Yes
Pelosinus fermentans strain A12	Yes	No	No	No	No
Pelosinus fermentans strain B3	Yes	No	Yes	Yes	No
Pelosinus fermentans strain B4	Yes	No	Yes	No	No
Pelosinus fermentans strain R7*	Yes	No	Yes	Yes	No

* - indicates type strain.