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Phase Preference by Active, Acetate-Utilizing Bacteria at the Rifle, CO Integrated Field Research Challenge Site

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ABSTRACT. Previous experiments at the Rifle, Colorado Integrated Field Research Challenge (IFRC) site demonstrated that field-scale addition of acetate to groundwater reduced the ambient soluble uranium concentration. In this report, sediment samples collected before and after acetate field addition were used to assess the active microbes via ¹³C acetate stable isotope probing on 3 phases [coarse sand, fines (8-approximately 150 micron), groundwater (0.2-8 micron)] over a 24 -day time frame. TRFLP results generally indicated a stronger signal in ¹³C-DNA in the “fines” fraction compared to the sand and groundwater. Before the field-scale acetate addition, a *Geobacter-like* group primarily synthesized ¹³C-DNA in the groundwater phase, an alpha Proteobacterium primarily grew on the fines/sands, and an *Acinetobacter sp.* and *Dechloromonas-like* OTU utilized much of the ¹³C acetate in both groundwater and particle-associated phases. At the termination of the field-scale acetate addition, the *Geobacter-like* species was active on the solid phases rather than the groundwater while the other bacterial groups had very reduced newly synthesized DNA signal. These findings will help to delineate the acetate utilization patterns of bacteria in the field and can lead to improved methods for stimulating distinct microbial populations *in situ*.

KEYWORDS. uranium reduction; stable isotope probing; active bacteria; sediment-associated microbes

Introduction:

Research at the Integrated Field-Scale Subsurface Research Challenge Site (IFRC) at Rifle, Colorado addresses knowledge gaps in understanding the mechanisms and rates of U(VI) bioreduction in alluvial sediments. For example, a 3-month bio-stimulation experiment was initiated in 2002 that stimulated *in situ* uranium reduction in the field (1). Characterization of the bacteria during this experiment from groundwater indicated *Geobacter*-like microbes dominated up to 80% of the clones in SSU libraries during the first 20 days of stimulation. These *Geobacter*-like clones were found to diminish as the system approached sulfate reduction during continued acetate amendment (1). Likewise, a similar injection experiment in 2003, replicated uranium reduction during biostimulation with enrichment of *Geobacter*-like clones in groundwater (approx 75%) and sediment samples (<10%), suggesting differences between microbial populations in groundwater and soil particles (2). These reports indicated that *Geobacter*-like microbes responded positively to acetate utilization and may be important in uranium reduction. However, the presence/absence of a particular SSU rRNA gene sequence in an environmental sample does not conclusively demonstrate that the particular microorganism is active or crucial in any biogeochemical process at the time of sampling. Furthermore, multiple questions remain including: A) which microorganism(s) are consuming this acetate at Rifle, B) can changes in activity patterns in sediment associated bacteria be observed after field-scale acetate addition, and C) do active microorganisms demonstrate a preference for phase (either sand associated, fines associated, or planktonic in the groundwater)?

In order to address these issues and elucidate the active bacteria involved in acetate utilization at Rifle, stable isotope probing (SIP) methods were applied to determine those microbe(s) which could use ¹³C acetate to replicate their genomes in microcosms incubated from 1-24 days. For example, stable isotope incorporation into macromolecules from environmental samples has been used to target metabolically active bacteria by monitoring phospholipids (3, 4, 5) This principle of purifying isotopically-labeled nucleic acids and coupling to molecular ecology techniques was presented by

Radajewski *et al.*, using ^{13}C -methanol to identify the active degraders in soil samples (6). Similar studies using the SIP techniques have been applied to a variety of natural systems using substrates ranging from methanol, methyl bromide, phenol, naphthalene, phenanthrene, benzoate, and salicylate (7, 8, 9).

During the summer of 2007, another acetate field-scale amendment was initiated at Rifle and found to significantly reduce uranium groundwater concentrations to below the regulatory drinking water standards ($<0.137\ \mu\text{M}$). This field study (aka. the Winchester experiment) was designed to replicate the dominance by *Geobacter* sp. in groundwater from prior studies during biostimulation (10). Therefore, SIP microcosm experiments were initiated with freshly drilled alluvial soil samples both before and after field biostimulation with acetate to determine if changes in active bacterial profiles could be observed. In order to ascertain if the acetate-utilizing bacteria demonstrated a preference for being sediment-associated or free-living, 3 different phases were operationally defined to determine whether particles harbored different ^{13}C labeled bacterial DNA than groundwater. The fractions (coarse sand, fines, and planktonic) were obtained by settling or filtration onto 8.0 or 0.2 micron filters. The findings indicated multiple groups of bacteria were using the carbon from acetate initially, that specific bacteria prefer being planktonic or particle attached for growth, that changes in bacterial behavior are observed during the field scale addition of electron donor, and that the “fines” contained the largest ^{13}C DNA signal. Ultimately, these types of studies, promoting natural and enhanced uranium bioremediation in the field, should lead to an improved understanding of geomicrobiological systems and predictive reactive transport models which can aid DOE site managers in determining policy to protect watersheds and human health.

Material and Methods:

Field site. For the 2007 Winchester field experiment (10) an injection/monitoring gallery of 22 wells (2-4 inch) was established in June of 2007 (Figure 1). This gallery configuration was designed to provide uniform acetate concentrations ($<10\ \text{mM}$) *in situ* for a period of 30-35 days (Aug 1-Sept 1,

2007) by cross-well mixing (11). Acetate was added in a double pulse (separated by an unamended groundwater addition) and groundwater samples were collected from all monitoring/upstream wells in the gallery twice per week and analyzed for electron donor concentration, U(IV) (labile and total), Fe(II), as described in (1, 10). The before-field addition sediment samples for SIP were collected during injection gallery/well installation on June, 2007 from wells DO2, DO3. The post-acetate amendment samples were collected in October, 2007 from wells P101 and P102 (Figure 1). The alluvial sediments used in these experiments came from 2 depths for each core (i.e. 4, 5, or 6 m). Unfortunately, the cores are generally filled with cobbles and it was often difficult to obtain material that could be placed in serum vials to conduct activity experiments from all depths for any one particular core.

Activity Determination. SIP experiments were performed by filter sterilizing upstream groundwater and overfilling 60 ml serum vials to minimize oxygen contamination. A sample of freshly collected sediment (5-6 g) from wells DO2, DO3, P101 and P102 was then added with 1mM ^{13}C acetate. The vials were capped, shaken, and allowed to incubate at room temperature for 1, 5, and 24 days. Control additions included 1mM ^{12}C acetate. After the incubation, each vial was shaken again and destructively sampled. Sand and coarse particles were collected within the SIP incubation vials by allowing a 1-hour settling period after shaking. Fines and silts were obtained by collecting this supernatant after settling with a pipette and concentrating on an 8.0 μm filter. Planktonic bacteria within the vials were collected from the < 8.0 μm filtrate using a 0.2 μm filter. This approach was necessary since traditional particle size fractionation methods would contaminate the SIP incubations with oxygen and generate artifacts during collection.

DNA purification and SIP Gradients. Genomic DNA from each size fraction was purified by phenol/chloroform methods (12). Separation of ^{12}C and ^{13}C DNA utilized CsCl gradients with archaeal carrier DNA (100 ng of SIP experimental DNA, 300 ng of ^{12}C and ^{13}C -archaeal carrier DNA) as described in (13). This approach yields 2 bands, i.e. a ^{12}C band containing genomic DNA from the resident bacteria present in the original sample and a ^{13}C band containing newly-synthesized DNA from

bacteria utilizing carbon from the ^{13}C acetate. Small subunit rRNA genes were then PCR amplified using the universal forward primer 27F (labeled with 6-FAM) and the bacterial-specific primer 1100R on a standard volume of ^{12}C or ^{13}C band for TRFLP analysis. A total of 107 profiles were generated during this study (including ^{12}C -acetate and no amendment controls) with 133 OTUs detected in all samples. Cloning was accomplished with a TA cloning kit (Invitrogen) on ^{13}C bacterial SSU amplicons. Specific colonies corresponding to specific TRF peaks were identified in the clone library. The double stranded sequence from these SSU genes was determined by dye terminator chemistry. The reactions were run on a Perkin-Elmer ABI 310 genetic analyzer (ABI, Foster City, CA). Accession numbers for the various TRFLP peaks are GU943742-45. Control incubations using ^{12}C acetate did not detect any amplifiable bacterial DNA in the ^{13}C carrier band using our priming sets and conditions, demonstrating contamination by ^{12}C bacterial DNA in our ^{13}C carrier bands was below our PCR detection limits (supplemental figure 1).

Results and Discussion:

Field Site Biogeochemistry. During acetate addition, the down gradient wells of the injection gallery exhibited increases in dissolved Fe(II) concentrations from 20-120 μM when experiencing acetate amendment, consistent with iron reduction as a terminal electron sink (Fig. 2). The highest concentrations of dissolved Fe(II) were in the first D01-D04 wells, nearest the injection gallery (data not shown). The spike in dissolved Fe lasted approximately 40 days. During this time, the dissolved uranium concentration in many of the monitoring wells dropped from 1.0 μM to below 0.2 μM , with the lowest concentrations below the drinking water standard of 0.137 μM , suggesting iron reducing bacteria may also be using uranium as a terminal electron acceptor. Unfortunately, there is no direct evidence as to which bacteria are utilizing uranium, since respiration is a dissimilatory process. Therefore, our efforts have focused on elucidating the microbes that are assimilating acetate since acetate loss is associated with decreases in uranium concentration in the field (Fig. 2).

SIP Measurements. Stable isotope probing experiments were used to ascertain the bacteria

responsible for the acetate uptake at Rifle, on different particle size fractions. The 3 size fractions produced high molecular weight genomic DNA when extracted from the 1-24 day incubations, indicating there was a migration of bacteria from the soil/fines to the aqueous phase in each microcosm, since the groundwater had been filter sterilized. However, the planktonic phase generally had the lowest concentration of bacterial genomic DNA while the “fines” had the highest concentration (data not shown). When this genomic DNA was placed in a CsCl gradient with ^{13}C archaeal carrier DNA, it was possible to profile the bacterial SSU genes that were present (^{12}C labeled) and active (^{13}C labeled). An example of SIP-TRFLP profiles is shown in figure 3, containing biological replicates for each phase at each time point. Each biological replicate is an independent ^{13}C incubation, extraction, amplification, and profile. Excellent agreement was observed between the active profiles from all biological replicates. The fingerprints indicate specific enrichment in particular peaks in the active fractions compared to the resident fraction in most profiles. For example, the active planktonic (0.2-8.0 μm) fraction had higher 187 and 277 peaks, the active fines (>8.0 μm) demonstrated an increase in peak area for the 59, 163, 164, and 212 bp peaks, and active coarse sand profiles illustrated a near complete elimination of the 176, 187, 210, 212, and 273 bp peaks compared with the planktonic and fines fraction and an increase in the 235 and 277 bp peak areas. The terminal fragment 277 was present in all active and resident fractions from the various phases. When observing a single time point in the incubation, the ^{12}C -resident populations were highly similar in all phases collected from the microcosms (supplemental figure 3). For example, all resident profiles from these samples contained the 166, 206, 273, and 277 bp peaks that dominated the overall community fingerprint. In contrast, the active profiles demonstrated differences in the microorganisms utilizing acetate in the various phases.

Phylogenetic Identification of Active Bacteria. To identify the various TRFLP peaks within the active fraction, clone library analysis of SSU genes and phylogenetic tree re-construction were performed. The results are presented in figure 4. Many of the TRFLP peaks (173, 191, 187, 206, 210 bp) were *Geobacter*-like clones. While the 212 bp TRF matched *Geobacter bemedjiensis*. The SSU

gene associated with the 166 bp peak was an α -Proteobacteria with closest matches to environmental clones from a deep coal seam in Japan (accession number AB294270), and a North Sea oil field seawater injection system (EU330917). The ribosomal RNA gene associated with the 235 bp TRFLP peak was closely related to *Dechloromonas sp. JJ* (99% identity) and the 277 bp peak matched *Acinetobacter sp. ND12*. Finally, clones corresponding to the 164 and 273 bp peaks were not represented in the SSU library when screening over 200 colonies.

Bacterial Activity Associated with Different Phases. To assess whether these various bacteria have a preference for being particle associated or planktonic during DNA synthesis using ^{13}C acetate, the relative proportion of the specific groups to the overall profiles was calculated (figure 5). This was accomplished by first normalizing all profiles to a single total area, adjusting the area for each peak (TRF), and then determining the contribution of those specific TRFs to the overall community profile. It can be seen from this analysis that *Geobacter*-like ^{13}C signal is generally higher in the planktonic phase and lower in the fines or the coarse sands for the pre-acetate field treatment samples (DO2 and DO3). In contrast, the α -Proteobactium, 166 bp, appears to be more active on solid surfaces when compared to the groundwater phase. The *Acinetobacter sp.* was active on all fractions, but seems to have the greatest response in the fines. The *Dechloromonas* was predominantly seen in the later time points and had the highest signal on sediments. In the post- -stimulation samples (figure 6), there was no detectable α -Proteobactium ^{13}C acetate associated DNA synthesis activity in either P101 or P102. A strong *Geobacter*-like signal was observed in the fines and sand phase from the shallow P101 sample. However, the deeper P101 sample indicated virtually no activity from any of the groups active in pre-stimulation samples. The P102 samples were generally less active for *Geobacter* and *Acinetobacter* when compared with the pre-stimulation samples, with the exception of the fines fraction from the deepest sample for the longest time point. For the P101 and 102 samples, most of the ^{13}C DNA signal is seen in peaks 149, 176, 273, 294 (data now shown). These TRF peaks were not represented in the clone library created from the pre-acetate field injection SIP incubations and presumably are related to sulfate-

reducing populations since levels of FeII generally decline after 20 days of acetate amendment (Fig. 2).

Relevance to other studies of uranium contaminated sites. The concept that DNA containing different stable isotopes could be separated on a cesium chloride gradient had been demonstrated in early research on the mechanism of DNA replication (14). Other investigators have utilized stable isotopic methods to identify active bacteria in a field setting experiencing uranium reduction. For example, Chang et al. amended Bio-Sep beads with ^{13}C -acetate at the Rifle site and incubated for 30-days to detect different bacteria in iron supplemented and control samples by DGGE (15). The authors present different banding patterns for wells samples M03, M08, and M13 with a slight increase in band intensity for a *Desulfobacter* and a *Geobacter*-like OTU in ^{13}C amended samples. However, many of the DGGE bands were also present in the controls and this result casts doubt on the specific enrichment on ^{13}C acetate after the prolonged incubations due to cross feeding issues. Furthermore, the authors report some samples did not have visible ^{13}C bands in their cesium gradients with portions of the gradient collected at the same height as laboratory control gradient. Although this has been a common practice for many SIP incubations, it has been shown that ethidium bromide:DNA ratio can profoundly influence buoyant density (16) and there is no guarantee that an invisible environmental ^{13}C band will sediment at the same position as a visible laboratory ^{13}C control band with a much higher DNA concentration. In a comparable study, Burkhold *et al.*, investigated ^{13}C -ethanol and ^{13}C -lactate incorporation in soil from Thuringia, Germany (17). In this report, incubations lasted 14 and 34 days and ^{13}C incorporation was assessed by collecting gradient fractions. The authors describe a single DNA peak at a density range of 1.52-1.62 (rather than 2 peaks) and it appears that 12 fractions were collected. TRFLP and clone library analysis was performed on half of these fractions and the results indicated detection of PCR product in fractions 6 and 7 for the 2 week incubation and in nearly all fractions after a 1 month incubation for both electron donors. Although the direction of ^{13}C enrichment is indicated in the TRFLP profiles, it is unclear which fraction corresponds to the peak in DNA concentration. Furthermore, there is no mention of ^{12}C ethanol or ^{12}C lactate controls to ensure that any PCR product

detected in the gradients actually correspond to ^{13}C substrate addition.

In contrast to these other studies of uranium contaminated sites, our gradients always have clearly defined ^{12}C and ^{13}C bands due to the use of archaeal carrier DNA. In addition, we performed ^{12}C acetate amendment controls to ensure that any PCR detected in the ^{13}C carrier band only resulted from ^{13}C acetate addition. This approach allows for the unambiguous identification of the ^{12}C and ^{13}C fractions, ensures the SIP procedure is detecting heavy labeled DNA, and significantly shortens the time necessary to detect a ^{13}C signal from the incubations (13). The TRFLP analysis indicates a strong fluorescent signal is observed in all our profiles (Figure 3) and a distinct enrichment is observed for specific peaks when compared with the ^{12}C [resident] bacterial community. For example, the 187 and 277 peaks (*Geobacter*-like) are nearly undetectable in the resident population for the planktonic and fines fraction, but are greatly enhanced in the overall active profile. Selective enrichment along with a persistence and increase of specific OTUs in the ^{13}C fraction during time course incubations has been proposed as sufficient evidence for specific labeling of community members by ^{13}C substrates in SIP incubation (18).

Overall, our Winchester findings replicated the earlier results of *Geobacter*-like sequences being enriched in the groundwater phase while uranium reduction occurred (1, 2). However, different *Geobacter*-like SSU genes were identified with respect to the prior studies and most of the initial acetate utilization by *Geobacter*-like SSU genes appears to be concentrated on the “planktonic” fraction. This result is surprising, given that nearly identical microbial populations exist on the sand, in the water, and on the fines and presumably acetate is (Fig. 3). One explanation for this data would be that many of the bacteria are capable of transporting acetate into their cells, but only those bacteria associated with terminal electron acceptors are capable of ATP formation, DNA synthesis, and growth.

Another finding from the initial SIP experiments on pre-stimulation samples was the smaller peak area in the ^{13}C band by the *Geobacter*-like organisms as compared to the α -Proteobactium (166 bp) and the *Acinetobacter sp.* peaks in the earliest time points or the shallower samples. Interestingly, many of

the ^{12}C profiles indicate that the *Geobacter*-like organisms are present and in high abundance in the many of size fractions. This finding suggests that in our pre-stimulation samples, microorganisms that use high energy, soluble electron acceptors (such as nitrate) may be able to process the carbon from acetate into newly synthesized DNA faster than the bacteria that require solid electron acceptors (such as iron) or lower energy soluble electron acceptors (such as sulfate or uranium). Presumably, after the oxygen/nitrate become depleted, the *Geobacter*-like microbes persist in the system and later come to dominate the acetate utilization signal. This is exemplified by the 121, 187, 206, and 210 TRFLP peaks, which take up a significant amount of ^{13}C and are all *Geobacter*-like sequences. It is also possible the *Geobacter*-like microorganisms may leave the solid phase when saturating conditions are encountered, accumulate electron donor such as acetate, but have to return to the solid phase to complete electron transfer and commence DNA synthesis. Additionally, this competition for acetate as an electron donor may be what is leading to the approximately 20-day lag in uranium reduction that is seen at the Rifle site (Fig. 2).

However, after the in-field acetate addition, the *a-Proteobacterial* peak disappears from the SIP-TRFLP profiles and the *Geobacter*-like peaks increase in area on the solid surfaces. Likewise, the *Acinetobacter* sp., and the *Dechloromonas* sp. JJ persist on the solid phases as well, indicating that these surfaces are providing the only remaining electron acceptors for these groups in the microcosm enrichments. It would be interesting to see whether the “fines” or coarse sand size fraction contains the goethite and phyllosilicates that are implicated in iron reduction by bacteria at various Dept. of Energy sites (19, 20) after field biostimulation. In conclusion, numerous bacteria are capable of acetate utilization at the Rifle, CO IFC site. This finding will be important for addressing uncertainties in reactive/transport modeling at the Rifle field site with respect to the microorganisms assumed to be consuming most of the injected acetate and to improve the conceptualization of transitions between iron-reducing and sulfate-reducing microorganisms (21). Initially, non- *Geobacter*-like microbes are responsible for most of the acetate uptake signal. However, with time, *Geobacter*-like organisms come

to dominate the groundwater during acetate stimulation *in situ* and become a major acetate uptake signal on the solid phase fractions (figures 5 and 6). Perhaps there are other electron donors that can provide a quicker stimulus to these microorganisms or other specific bacterial populations and induce uranium reduction in a faster manner.

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FIGURES.

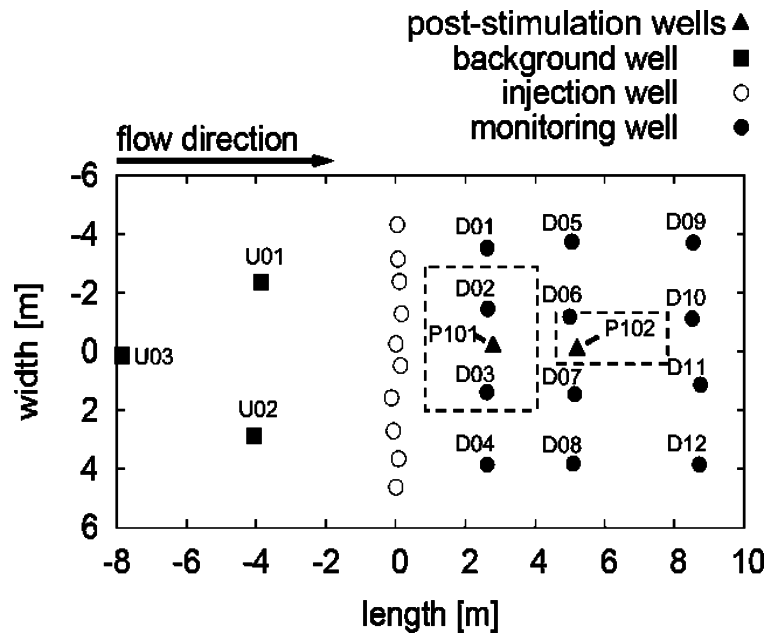


Figure 1. Schematic of Winchester gallery showing the location/spacing of the wells. The dashed boxes indicate those wells sampled for SIP measurements.

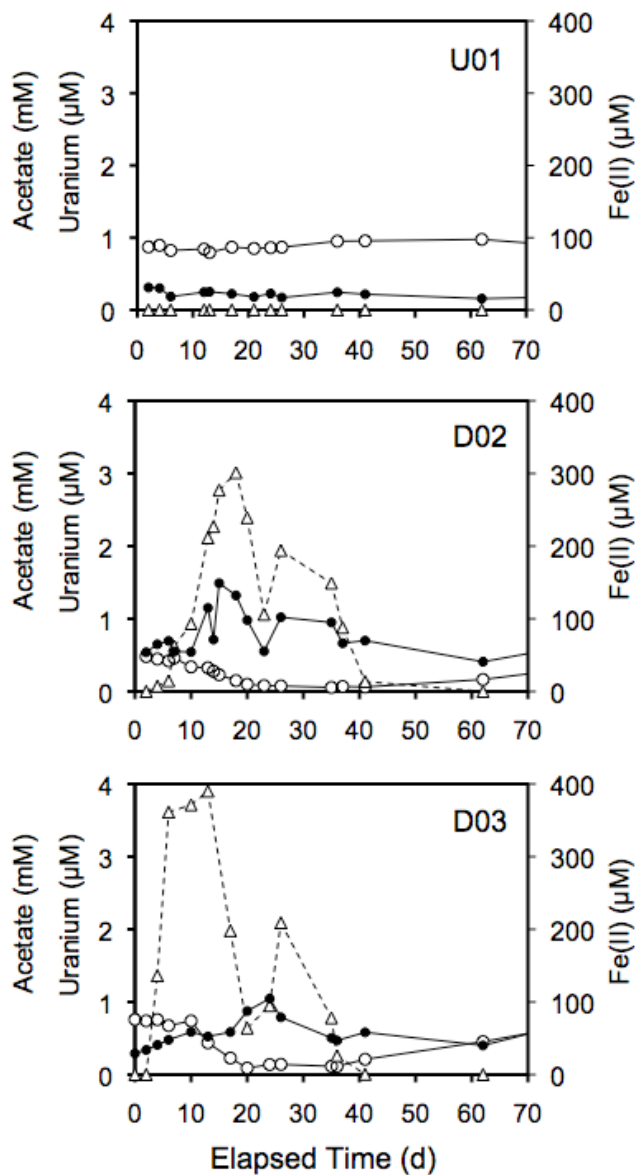


Figure 2. Time course of acetate (open triangles), Fe II (closed circles), and uranium concentrations (open circles) during field addition of 10mM acetate.

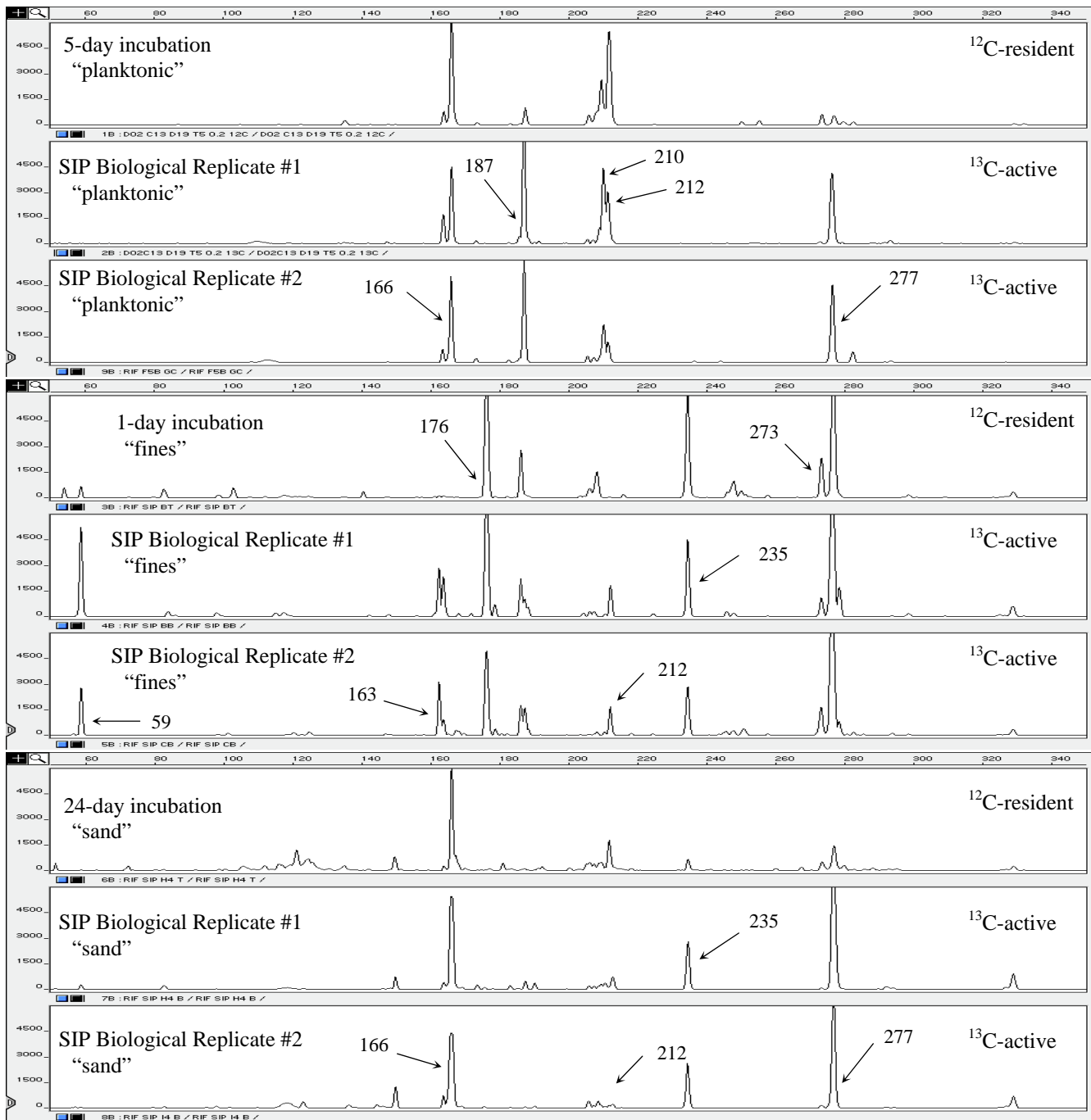


Figure 3. SIP-TRFLP fingerprints demonstrating biological replicates at 1, 5, and 24 day time points with the resident (^{12}C) and active (^{13}C) bacterial populations in the planktonic, fines, and sand phases indicated.

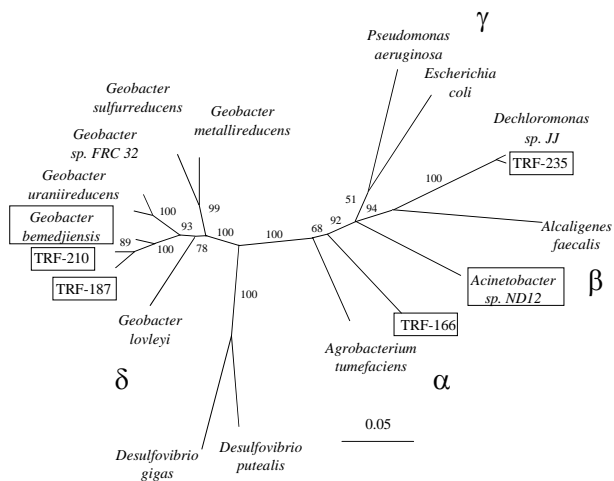


Figure 4. Phylogenetic tree reconstruction of SSU clones related to major peaks in the SIP profiles (Genbank accession # GU943742-45). Both *Geobacter bemedjiensis*(TRF-212) and *Acinetobacter sp.* ND12 (TRF-277) were also detected. Major subdivisions of the Proteobacteria are indicated.

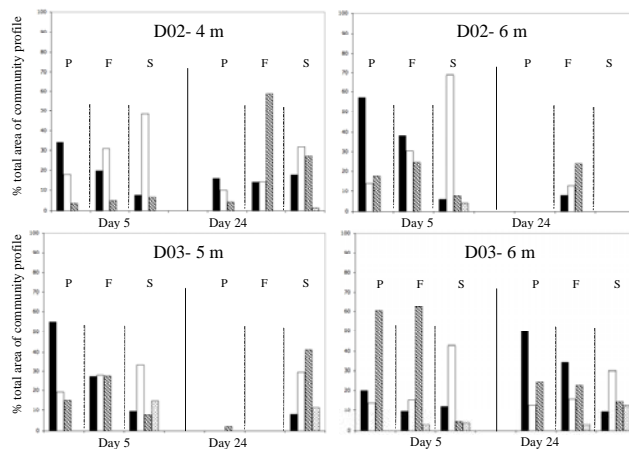


Figure 5. Summary graphs indicating relative proportion of ^{13}C profiles for the major TRFLP peaks before field-scale acetate addition [DO2, DO3]: *Geobacter*-like group (black), 166 bp (white), *Acinetobacter* sp. (diagonal), *Dechloromonas* sp. (hashed).

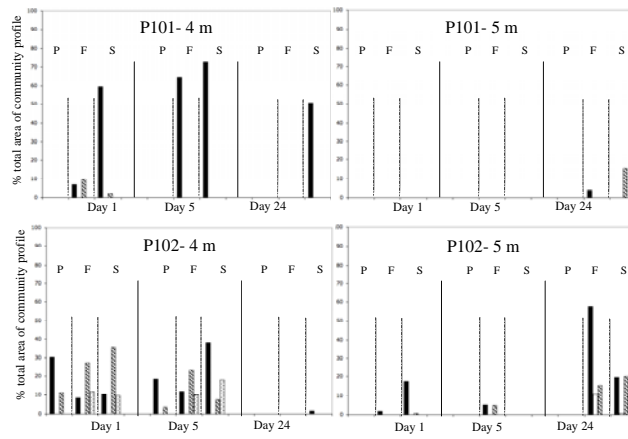


Figure 6. Summary graphs indicating relative proportion of ^{13}C profiles for the major TRFLP peaks after field-scale acetate addition [P101, P102]: *Geobacter*-like group (black), 166 bp (white), *Acinetobacter* sp. (diagonal), *Dechloromonas* sp. (hashed).

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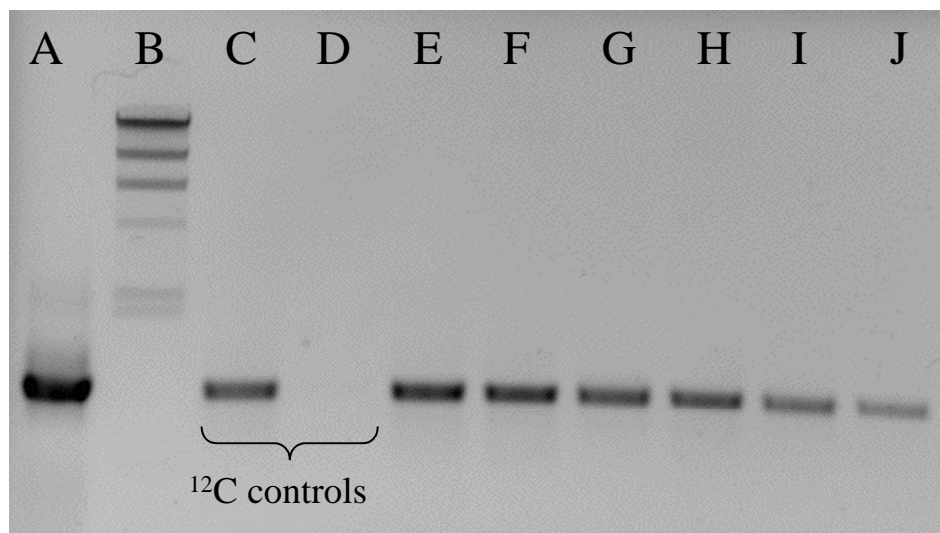
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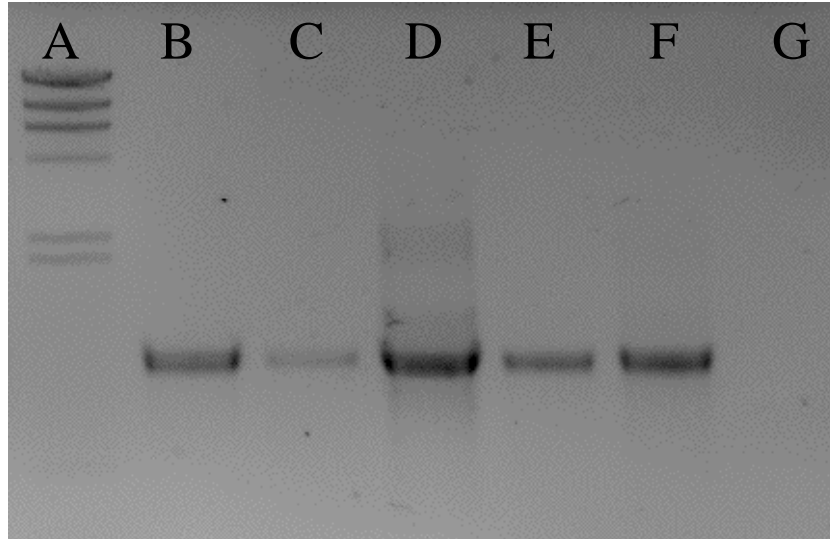
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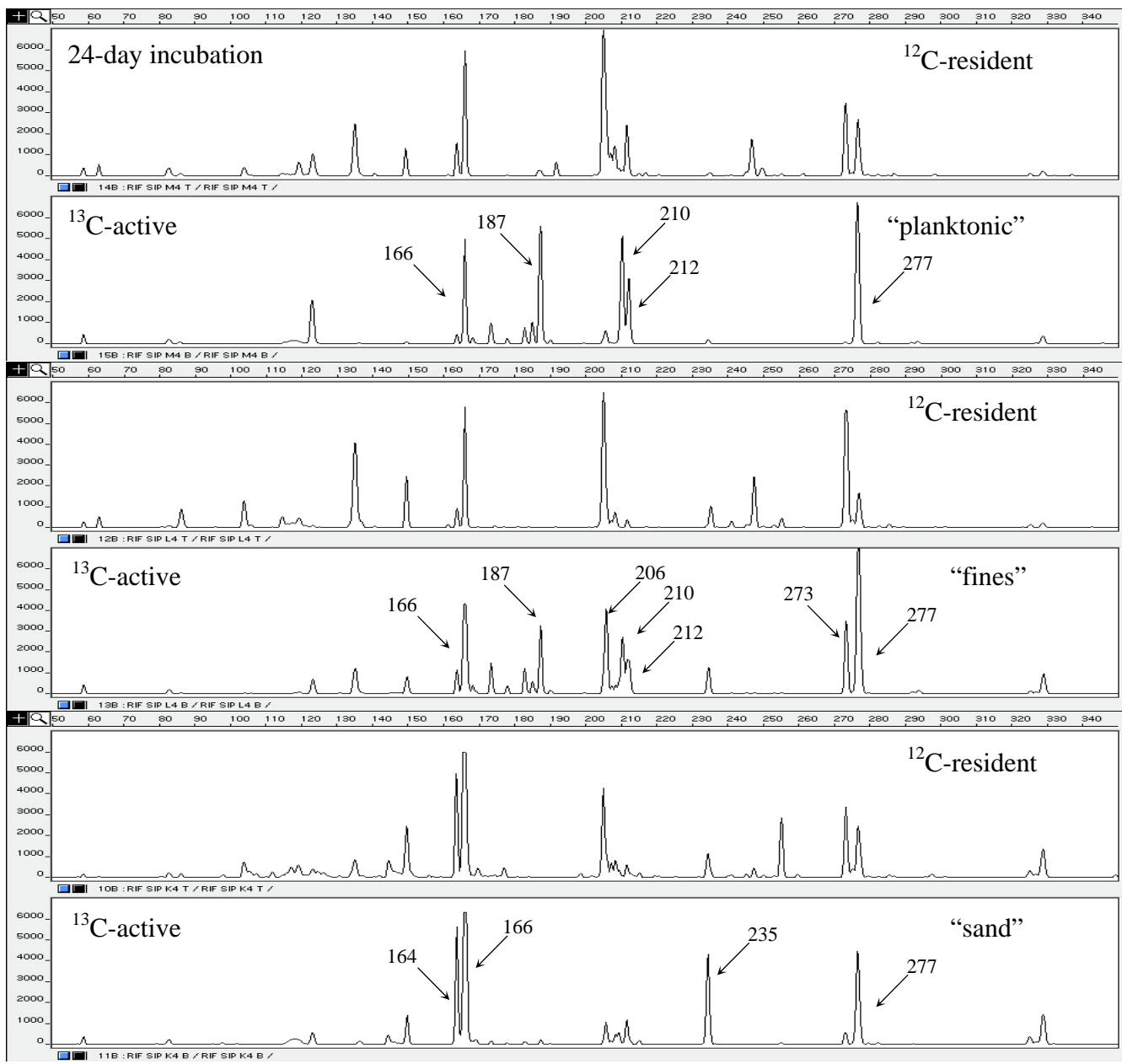
SYNOPSIS TOC: Soil-associated microorganisms display a preference when utilizing ^{13}C acetate for being in groundwater or different particulate phases at the Rifle, CO, Integrated Research Site.



Supplemental Figure 1. PCR amplification of ^{12}C (resident) and ^{13}C (active) bacteria from well DO3 (6m) after 24 d incubation. Lanes are: A) PCR + control B) Lambda HindIII marker C) ^{12}C acetate amended planktonic fraction resident D) ^{12}C acetate amended planktonic fraction active E) ^{13}C acetate amended planktonic fraction resident F) ^{13}C acetate amended planktonic fraction active G) ^{13}C acetate amended fines fraction resident H) ^{13}C acetate amended fines fraction active I) ^{13}C acetate amended sand fraction resident J) ^{13}C acetate amended fines fraction active.



Supplemental Figure 2. PCR amplification of ^{12}C (resident) and ^{13}C (active) bacteria from well P102 (6m) after 1 d incubation. Lanes are: A) Lambda HindIII marker B) ^{13}C acetate amended planktonic fraction resident C) ^{13}C acetate amended planktonic fraction active D) ^{13}C acetate amended fines fraction resident E) ^{13}C acetate amended fines fraction active F) ^{13}C acetate amended sand fraction resident G) ^{13}C acetate amended fines fraction active.



Supplemental Figure 3. Example of TRFLP fingerprints from SIP experiment from 24 day incubation showing both the resident (^{12}C) and active (^{13}C) bacterial population in the planktonic, fines, and sand phases.

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