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**In Situ Biostimulation of Uranium Reducing Microorganisms at
the Old Rifle UMTRA Site**

A Dissertation

Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

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ABSTRACT

Bioremediation is a promising strategy for cleaning up heavy metal and radionuclide contamination. Nutrient or electron donor amendment is an increasingly accepted practice used to stimulate the growth of microorganisms capable of immobilizing dissolved uranium *in situ*, but there is scant understanding of the systematic effects of nutrient addition on indigenous microbial populations or the progress of the bioremediation. Successful implementation of metal and radionuclide bioremediation in heterogeneous environments requires an understanding of the complex microbial and geochemical interactions that influence the redox speciation and mobility of toxic metals. The major challenge in microbial ecology and biogeochemistry is to connect observed biogeochemical processes to the microbial populations responsible for carrying them out. This thesis thus investigated the effects of electron donor addition to indigenous microbial populations actively involved in uranium bioremediation. Stable Isotope Probing (SIP) technique for environmental application was developed and established. A microcosm study was designed in parallel to a field biostimulation test at the Old Rifle, UMTRA site.

In the microcosm study that simulated Rifle *in situ* biostimulation of uranium reducing organisms, the microbial community dynamics were analyzed quantitatively and qualitatively using Phospholipid Fatty-acid Analysis (PLFA) and Denaturing Gradient Gel Electrophoresis (DGGE) analysis combined with SIP, which was modified to accommodate low biomass environmental samples. The microcosms consisted of sediment and groundwater from the Rifle, Colorado UMTRA site and activated carbon

bead microbial traps (Biosep beads). ^{13}C labeled acetate amended and non-amended microcosms were compared. Lipid analyses showed a significant biomass increase with acetate amendment, specifically monounsaturated PLFA. The data also demonstrated a community shift in acetate-amended microcosms, mirroring the observation of DGGE analysis. The bacterial community in non-amended microcosms showed notable differences from those amended with acetate. *β -proteobacterial* sequences dominated the non-amended community. Furthermore, ^{13}C DNA analysis indicated that acetate treatment encouraged the growth of Gram-negative microorganisms such as *Pseudomonas*, *Geobacter*, and sulfate reducing bacteria (SRB). PLFA extracted from beads and sediment also showed uptake of the ^{13}C -acetate, mainly in 14:0, 16:1 ω 7c, 16:1 ω 5c, 16:0, cy17:0 and 18:1 ω 7c, supporting the DNA results. *Geobacter* and SRB sequences were not detected until day 20, while *Pseudomonas* sequences were prevalent by day 5 and continued to be one of the dominant sequences retrieved. The dominance of *Geobacter* was much more pronounced in bead samples than in sediments. GC-IRMS analysis also demonstrated the ^{13}C enrichment in fatty acids of i15:0, i17:0, 17:0 and 18:0 extracted from beads samples, which might be indicators of *Geobacter*, SRB or Gram+. The SIP technique enabled an evaluation of the taxonomic and metabolic diversity of key groups of microbes actively involved in biostimulation. The microbial monitoring in microcosms can elucidate the bacterial populations responsible for uranium reduction and may indicate that SIP using ^{13}C -acetate added to microbial traps can provide important data on ecosystem function in the field.

At Rifle, Colorado, a field-scale acetate amendment experiment was performed to stimulate *in situ* microbial reduction of U (VI) in groundwater. Geochemical measurements indicated reduction of iron, uranium, and sulfate, which were stimulated by acetate injection. The PCR-DGGE analysis of 16S rRNA genes revealed 15 major lineages in the bacterial domain, enriched during biostimulation. A temporal (T1, T2, and T3, T4) and spatial (B-02, M-03, M-08 and M-13) distribution of the bacterial community structure was demonstrated. The background well showed no significant community shift throughout the experiment, and was dominated by *β-proteobacteria* with no *Geobacteraceae* detected. The down-gradient monitoring wells, on the other hand, shared similar community structure with background wells before acetate injection, but exhibited significant enrichment of *Geobacter* and *Desulfuromonas* sequences during the injection. This enrichment disappeared after the injection of electron donor ceased and was replaced by sequences originating from organisms of *Sulfuricurvum*, SRB within *δ-proteobacteria*, and gram positives closely related to either *Desulfotomaculum*, or *Clostridium*. Consistent with the reducing activities determined by geochemical analysis, well M-13, furthest away from the injection gallery, appeared to be less similar with other down gradient monitoring wells in community composition. PLFA analysis indicated a similar trend in community shift and displayed an increase in monounsaturated PLFAs (indicative of Gram-negative bacteria), as well as terminally branched saturated PLFAs (indicative of anaerobic sulfate reducing bacteria) relative to the background well. The data presented demonstrates the effects of biostimulation and bioreduction by addition of acetate, and lead to the conclusion that *Geobacteraceae* was initially responsible for enzymatic uranium reduction, but had no role afterwards. The sulfate

reducers played an important role in reducing uranium and also maintaining the low concentration of uranium at the Old Rifle site. Nitrate reducers such as *Sulfuricurvum* bacteria may also had important part in maintaining the stability of reduced uranium by removing the subsurface nitrate.

To evaluate the microorganisms responsible for uranium microbial reduction during the field experiment, Biosep beads baited with ^{13}C labeled acetate were deployed into well boreholes and sampled when groundwater chemistry indicated metal and or sulfate reduction. Incorporation of the ^{13}C into cellular DNA and PLFA biomarkers was examined. The ^{13}C labeled DNA fraction demonstrated an enrichment of *Geobacteraceae* sequences in down gradient monitoring wells. *Geobacter* sequences dominated in wells approximately 3.7 meters from the injection gallery. Further down gradient, sequences belonging to *Desulfuromonas* increased. *Pseudomonas* sequence was also found to be stimulated. PLFA profiling of activated carbon beads suspended in the monitoring wells showed the incorporation of ^{13}C into the bacterial cellular lipids, particularly the 16:1 ω 7c.

A comparison among groundwater, sediment, and biotraps was performed, which indicated that the biotraps captivated the key populations of both groundwater and sediment but are probably more representative of the groundwater.

The research presented in this thesis demonstrates the importance of metal reduction and sulfate reduction in stimulated uranium immobilization, also expands our knowledge of quantitatively important iron and sulfate reducing bacteria in uranium contaminated

subsurface environment. The direct introduction of ^{13}C labeled substrates into ecosystems, coupled with DNA and PLFA analyses, which combine detailed taxonomic description with a quantitative measure of metabolic diversity allowed detection and definition of the metabolically active subset of the microbial community.

This study provides an effective technique and experimental model to identify particular microbial populations involved in a desired process. Future research may explore whether the sediment or groundwater has even greater diversity of uranium reducing populations than those we have identified. More focused study on sulfate reducers are needed to shed light on their involvement in uranium reduction, either biotic or abiotic, or both.

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Chapter 1. Introduction and Literature Review

Heavy Metal Contamination

The term heavy metal refers to any metallic chemical element that has a relatively high density and is toxic or poisonous at low concentrations. Heavy metals are natural components of the Earth's crust. Examples of heavy metals include mercury (Hg), cadmium (Cd), uranium (U), chromium (Cr), thallium (Tl), and lead (Pb), etc. all of which pose risks for human health and the environment. Environmental contamination from heavy metals is of serious concern because they cause damages consistent with those caused by persistent toxic chemicals. The toxicity of most organic contaminants can be reduced with biotransformation and/or biodegradation, but metals cannot be degraded or destroyed and they exhibit long lasting toxic effects (Clark, 1992). While the contamination of heavy metals can increase their concentrations in biota through bioaccumulation, they also have toxic effects at very low concentrations (Davey *et al*, 1973).

At many sites around the nation, heavy metals have been mined, smelted, or used in other industrial and military processes. The waste (tailings, smelter slag, etc.) left behind has polluted large areas of surface and ground water locally and via leaching. The presence of heavy metals in natural water systems, soil and subsurface sediment has in some instances caused significant ecosystem degradation.

Traditional cleanup (or remediation) technologies for reducing the harmful effects at heavy metal-contaminated sites typically involve excavation, decimation or burial of the contaminated materials, which is called active remediation. An innovative clean up

strategy called natural attenuation, that relies on naturally occurring processes that stabilize or remove the metals in the environment, such as dilution, volatilization, adsorption, and the use of some microorganisms to transform or remove heavy metals from contaminated subsurface is bioremediation, as defined by the American Academy of Microbiology, that is "the use of living organisms to reduce or eliminate environmental hazards resulting from accumulations of toxic chemicals or other hazardous wastes" (Gibson and Sayler, 1992). The accelerated bioremediation refers to the use of indigenous microorganisms with manipulative enhancement, which is gaining increasing attention from the scientific community. The use of growing plants to stop the spread of contamination or to extract the metals from the soil are termed as phytoremediation. Bioremediation can be used for sequestration of metals and radionuclides through microbially mediated transformation processes, and for remediating large plumes of dilute contaminants that are broadly dispersed in the environment. Thus, it has the potential to be one of the most cost-effective technologies for dealing with environmental remediation problems.

The research reported here focuses on *in situ* biostimulation and bioreduction, with uranium as the primary contaminant of concern. Uranium is the most common radionuclide and heavy metal pollutant found within the U.S. nuclear weapon complexes managed by the U.S. Department of Energy.

General Properties of Uranium

In order to deal with the problem caused by environmental uranium contamination, it is essential to have some knowledge of the environmental chemistry of Uranium. Uranium

is a very heavy (dense) metal, which can be used as an abundant source of concentrated energy. It was discovered in 1789 by Martin Klaproth, a German chemist, in the mineral called pitchblende. It was named after the planet Uranus, which had been discovered eight years earlier (Rocchietta, 1976).

The uranium occurs in most rocks in concentrations of 2 to 4 parts per million. Its melting point is 1132°C (Yoo et al., 1993). On a scale arranged according to the increasing mass of their nuclei, uranium is the heaviest of all the naturally occurring elements, while hydrogen is the lightest.

Uranium is a member of the actinide series in the periodic table, which has an atomic number of 92 and ten radioactive isotopes, but ^{238}U (99.27%), ^{235}U (0.72%) and ^{234}U (0.0055%) are the three most abundant. The dominant isotope by mass, ^{238}U , has a physical half-life of 4.5×10^9 years, while ^{235}U and ^{234}U have half-life of 7×10^8 years and 2.5×10^5 years respectively (Sheppard, 1980; 2003). ^{235}U is the only naturally occurring nuclear fission fuel and has gained attention with the development of practical uses of nuclear energy.

Although U has oxidation states of (+III) to (+VI), the two major oxidation states are U (+VI) and U (+IV). The U (+IV) are insoluble and readily oxidized to soluble U (+VI) as UO_2^{2+} . The uranyl ion, UO_2^{2+} , is the most stable U species in an oxidizing solution and the most prevalent form in the environment (Ribera et al., 1996; Sheppard, 1980; Maynard, 1983).

Uranium Toxicity and Environmental Behavior

Uranium is both chemically and radiologically toxic nuclide that poses environmental and human health hazards (Sheppard et al., 2005; Milvy et al., 1990). There is no known biological function reported for uranium and it is toxic to cells at very low concentrations --- it can be 20 to 40 times more toxic than copper or nickel (LeDuc et al, 1997). The toxicity of uranium is primarily derived from its chemical properties rather than from its radioactivity (Sheppard, 2003).

Radiological toxicity of U is primarily manifest in bone sarcomas, whereas chemical and combined chemical and radiological effects are reported as nephrotoxicity. In general, ^{235}U and ^{234}U pose a greater radiological health risk than ^{238}U because they have much shorter half-lives, decay more quickly, and are thus "more radioactive" (Sheppard, 2003). Because all uranium isotopes are primarily alpha emitters, they are only hazardous if ingested or inhaled. However, because several of the radioactive uranium decay products are gamma emitters, workers in the vicinity of large quantities of uranium in storage or in a processing facility can also be exposed to low levels of external radiation. Chemical ecotoxicity of U is dependent on several environmental parameters. The most important are carbonate content, because of the formation of soluble carbonate complexes, and divalent cation content (Ca^{2+} and Mg^{2+}), because of their competitive interaction with the uranyl ion (UO_2^{2+}).

A by-product of the uranium enrichment process is called depleted uranium (DU), which has a lower content of fissionable material than natural uranium. In addition to being radioactive, DU is also a chemically toxic metal--much like lead. It has been reported widely in the press that numerous Persian Gulf War veterans have become ill with Gulf

War Syndrome. Studies have suggested that depleted uranium used in the war may have caused their illnesses and genetic defects in their children conceived and born after the war (Ehrlich, 1996; Scott, 2003).

U (VI) is highly soluble and thus easily transported in the environment. Uranium (IV) is rather insoluble, and can be complexed in only small amounts by various inorganic ligands such as fluoride, sulfate and phosphate (Langmuir, 1978). Unlike many contaminant metals, uranium is not easily immobilized by neutralizing the acidic waste at a site. Although uranium forms insoluble solids or adsorbs to mineral surfaces at alkaline pH, it also forms soluble carbonate complexes that markedly increase its solubility, availability, and mobility. The carbonate is the most important complexing agent for uranium (Duff et al., 1998; Sheppard et al., 1983). In long term investigation on the fate of uranium in soil applied with phosphate fertilizer, results showed that most of the uranium was associated with organic matter, indicating the importance of the organic matter for the long-term behavior of uranium (Rothbaum et al., 1979). In addition, uranium mobility in soil can be affected by the sorption to or metabolism by microorganisms—microbial reduction (Lovley et al., 1992a), though U (VI) associated with solid phase such as iron oxides/hydroxides is not microbially reducible (Ortiz-Bernad et al., 2004). Furthermore Suzuki et al. (2002) showed that uraninite (UO_2) particles formed from uranium in sediments by bacterial reduction can be typically less than 2 nanometers across and that the small size has important implications for uraninite reactivity and fate. Because these tiny particles may still be transported in an aqueous environment, precipitation of uranium as insoluble uraninite cannot be presumed to immobilize it. Overall, the chemical fate of uranium released from uraninite in a natural

system is governed by precipitation, adsorption, and formation of solution complexes (Langmuir, 1978). Knowledge about those interactions in the environment is of utmost interest to protect environment and human health. Ribera et al. (1996) have provided a detailed description of the behavior, transfer and fate of U in both terrestrial and aquatic environments.

Uranium Contamination

Uranium contamination is a legacy of uranium mining and nuclear weapon production. As a result, large amounts of toxic and radioactive wastes have contaminated over 7200 km² of soils and groundwater surrounding many U.S. Department of Energy research and laboratory sites (DOE, 1997). The most predominant radionuclide contamination found in these areas is uranium. Mining and milling activities also have created uranium contamination with the soil, sediment and water locally and, via leaching, over a widespread area. Remediation of subsurface contamination at 24 abandoned tailings piles across the United States is a major problem facing the U.S. Department of Energy's Uranium Mill Tailings Remedial Action (UMTRA) project (Groelsma, 1982). It has been estimated that tailings in the United States attributed to abandoned mills contain more than 25 million metric tons, while currently active mills are generating 170 million metric tons of contaminated materials every year. Similarly, uranium mill sites throughout the world have produced vast quantities of tailings that are contaminating soils, sediments and groundwater. The GULF war syndrome further highlights the increasing concerns over the fate and environmental health impact of uranium in

contaminated areas, particularly subsurface sediment and groundwater (Milvy et al., 1990; Scott, 2003; Bem et al., 2004; Tournier et al., 2002).

Uranium Remediation Strategies

Federal law (UMTRCA, 1978) required the U.S. Department of Energy to clean-up many uranium contaminated sites and thus, intensive research has been directed at uranium remediation strategies, which encompass either *ex-situ* (e.g., pump-and-treat) or *in situ* (e.g., bioremediation or flow barriers) methods.

The traditional approach of excavation, packaging, transportation, and burial at an approved disposal site can be costly and sometimes ineffective. Chemical approaches are available for metal remediation, such as engineering biosorption-- the metabolism-independent sorption of heavy metal or radionuclides to biomass (Gadd, 2000; Lloyd et al., 2000a), coprecipitation through biodegradation of associated organic compounds (Thomas et al., 2000; Satroutdinov et al., 2000; Inoue et al., 2000), ion exchange resins (Reid et al., 1985; Gu et al., 2004) and newly developed zero-valent iron approach (Morrison et al., 2002). Some of those do warrant attention, but are often expensive to apply and lack the specificity required to treat target metals against a background of competing ions. In addition, such approaches are not applicable to cost-effective remediation of large-scale subsurface contamination *in situ*.

The innovative "natural" alternatives are becoming increasingly accepted as methods for cleaning up contaminated soils and groundwater. Among them, bioremediation strategy has been intensively studied and has exhibited great promises and potentials (Lovley, 2001; Lloyd et al., 2000a, 2000b). In nature, uranium generally exists in either the U (VI)

or the U (IV) oxidation state. The oxidation-reduction and acid-base chemistry of uranium depict that under oxidizing conditions U (VI) is present as the uranyl ion UO_2^{2+} which is soluble in water and, therefore, mobile in the aqueous environment. Under reducing conditions insoluble U (IV) is stable and presents a smaller threat to water resources. This knowledge of uranium chemistry forms the basis of the bioremediation strategy for contaminated ground water associated with uranium.

Enzymatic reduction of soluble U (VI) (primarily the uranyl ion, UO_2^{2+} , and its complexes) to solid U (IV) (uraninite) is an important mechanism for the precipitation and immobilization of uranium from contaminated groundwater. This approach is operationally flexible and can be used both *in situ* and *ex situ*.

One of the more promising strategies for the *in situ* bioremediation of U (VI) waste involves the “biostimulation” of U (VI) reducing organisms. Biostimulation is defined as addition of nutrients (carbon and other nutrient mineral sources) that serve to increase the number or activity of indigenous microorganisms available for bioremediation activity. Microbial reduction of uranium is the process that takes electrons from hydrogen or an organic compound and transfers them to the uranium, thereby reducing the uranium to the insoluble and less mobile/toxic form. Therefore providing abundant organic compound or electron donor can in theory accelerate the reducing activity. Previous laboratory and field studies with uranium contaminated sediment and groundwater have demonstrated that indigenous metal-reducing microorganisms can be stimulated and effectively used for microbially mediated uranium reduction (Abdelouas et al., 1998; 2000; Finneran et al., 2002; Francis, 1998; Francis et al., 1994; Kashefi and Lovley, 2000), and therefore immobilize the uranium in subsurface, preventing its further migration within the

groundwater. The *in situ* stimulation of indigenous microorganisms capable of immobilizing dissolved uranium has been proposed as a potential cost-effective remediation strategy to restore contaminated environments inexpensively yet effectively (Lovley, 1995, 2001; Istok et al., 2004).

During the last two decades, the use of nutrient addition as cost-effective bioremediation strategy has received much attention (Abdelouas et al., 2000; Kauffman et al. 1986, and Lovley and Phillips, 1992b). Acetate addition to the subsurface at the Old Rifle UMTRA site stimulated the enrichment of *Geobacter* sp., and the loss of soluble U (VI) from the groundwater (Anderson et al., 2003). Through a series of injections of ethanol, acetate or glucose, a recent field research project also demonstrated that bio-immobilization is a viable remediation strategy at a site contaminated with mixtures of U (VI) and Tc (VII) (Istok et al., 2004). Nutrient or electron donor amendment is now increasingly accepted as a practice in uranium contamination remediation, but there is scant understanding of the systematic effects of nutrient addition on indigenous microbial populations or the progress of the bioremediation. The mechanisms by which uranium was reduced and the microbial species responsible remain as topic for further investigation.

Microbial Potential for Uranium Reduction

Successful implementation of stimulated uranium bioremediation requires detailed knowledge of uranium behavior during proposed remediation procedures. An extensive understanding of metal-reducing microorganisms and the environmental parameters controlling their activities is required.

It has been reported that bacteria capable of reducing U (VI) to U (IV) are ubiquitous in nature. The ability to reduce U (VI) to U (IV) occurs in phylogenetically diverse organisms, including archae (Kashefi and Lovley, 2000), Fe (III) reducing organisms (Coates et al., 1998, 2001; Ganesh et al., 1997) sulfate reducing organisms (Lovley et al., 1992a, 1993a; Beyenal et al., 2004), fermentative *clostridium* bacteria (Francis et al., 1994), and denitrifying *Pseudomonas* (Barton et al., 1996), etc.

Members of the *Geobacteraceae* family are well known Fe (III) reducing organisms and have been well studied. They are readily isolated from a diversity of marine, estuarine, and freshwater sedimentary environments (Coates et al., 1996; Lovley, 2000a) and can effectively reduce soluble U (VI) (Lovley, 1991, 1995). Both lab incubation (Holmes et al., 2002) and field trial (Anderson et al., 2003) have suggested that indigenous iron-reducing bacteria belonging to the *Geobacteraceae* family are likely responsible for acetate stimulated uranium bioreduction. Some SRB such as *Desulfovibrio sp.* has been well studied for their ability to enzymatically reduce uranium (Lovley et al., 1992a, 1993a). *Desulfovibrio desulfuricans*, important in metabolizing sulfates, is able to convert uranium in solution to uraninite, a crystalline precipitate that can be removed and disposed of easily (Lovley et al., 1992a; Payne et al., 2002). Neal et al (2004) demonstrated uranium complex formation at hematite surfaces colonized by sulfate reducing bacteria. Suzuki et al., (2003) reported that organisms closely related to *Desulfosporosinus* spp. will reduce U (VI) and sulfate in the natural sediment when it becomes anaerobic and during bioremediation. Previous research also showed a correlation between uranium conc. and occurrence of *Desulfotomaculum*-like sequences at a contaminated milling site (Chang et al., 2001), suggestive of potential *in situ* uranium

biotransformation by sulfate-reducing bacteria. A *desulfotomaculum* species isolated from marine sediments was indeed shown to reduce U (VI) with butyrate as the electron donor (Tebo et al., 1998). Francis et al. (1994) showed U (VI) reduction by a *Clostridium* sp. Thus, it is inferred that organisms related to *Clostridium* sp. are partially responsible for U (VI) reduction and removal of uranium from solution. This result implies that U (VI) can be reduced in natural and contaminated anaerobic sediments during fermentation, before the onset of dissimilatory Fe (III) or sulfate reduction with H₂ and short-chain fatty acids produced by fermentation. *Pseudomonas* also displayed ability to reduce uranium in the presence of sulfate (Barton et al., 1996).

All of which suggest that the capacity for uranium reduction is likely to be spread throughout the domain *Bacteria*. Among them, sulfate reducing and Fe (III)-reducing bacteria are so far the two major groups of microorganisms capable of U (VI) reduction (Lovley, 1995, 2000a) and have been demonstrated to effectively catalyze the reduction and immobilization of uranium (VI) from contaminated subsurface environment.

Techniques for Analyzing Microbial Community

To gain full understanding of the metal reducers potentially important for *in situ* uranium bioremediation, it is essential to first analyze the microbial community and identify indigenous organisms which are stimulated and actively involved in bioremediation process, and to link the biogeochemical functions to the taxonomic identity of those organisms.

The identification of organisms has traditionally been achieved by cultivation techniques. However, because more than 99% of natural microorganisms are not culturable by standard

cultivation techniques (Amann et al., 1995; Streit et al., 2004), alternate methods have been developed to describe the community composition. Among them, one approach that revolutionized our understanding of microbial diversity is through the use of rRNA based, molecular phylogenetic techniques (Owen, 2004; Amann et al., 2000; Ludwig et al., 1994). In this approach, rRNA genes are obtained directly from environmental DNA, commonly through PCR, cloning, and sequencing (Amann et al., 1995; Lane et al., 1985). Comparative analyses of the rRNA sequences reveal the phylogenetic types of organisms that comprise the community. The 16S rRNA gene sequence analysis avoided the bias imposed by culturing and led to the discovery of vast new lineages of microbial life. Functional gene probes were subsequently developed to study key enzymes involved in specific metabolism of interest (Taroncher-Oldenburg et al., 2003; Baxter et al., 2002). Other alternatives evolved include single-strand conformation polymorphism (SSCP) (Delbes et al., 2001), terminal restriction fragment length polymorphism (T-RFLP) (Osborn et al., 2000), amplified rDNA restriction analysis (ARDRA) (Dijkshoorn et al., 1998), amplified ribosomal intergenic spacer analysis (ARISA) (Cardinale et al., 2004) and cloning. Methods rely on making clone libraries, sorting the clones into operational taxonomic units (OTUs) via RFLP and sequencing representatives to determine their identity has been proven effective in determining populations in the environment, but this is also time consuming and labor intensive. A simplified method uses PCR in conjunction with denaturation gradient gel electrophoresis (DGGE), that utilizes sequence variations in DNA fragments of identical length and separates them based on their different melting property in polyacrylamide gels of increasing denaturant gradient (Muyzer et al., 1993).

The primary advantage of DGGE over conventional PCR for the detection of bacteria is that the relative abundance of all the numerically dominant bacteria can be assessed simultaneously by analysis of a single PCR reaction with a single set of primers. The intensities of the recovered bands in DGGE provide a measure of changes in the relative abundance of the major eubacterial species present. Therefore it is ideally suited for community analysis of a spatial or temporal sample series and can effectively handle a large number of samples.

DGGE has been successfully used to study the diversity of bacteria in various environments (Phung et al., 2004; Gonzalez et al., 2004) and to monitor the shift in community structure, e.g. following an oil spill bioremediation (MacNaughton et al., 1999), or *in situ* biostimulation (Iwamoto et al., 2000). Parameters have been developed and established to profile the functional genes such as those of ammonia oxidizers or methanotroph (Kowalchuk et al., 2000; Henckel et al., 2000). Later it has been extended to study the communities of archaea, fungi etc. (Watanabe et al., 2004; Anderson et al., 2004) and proved to be highly effective and efficient, compared to cloning approach. However, there are also limitations with this technique. It is likely that any given species must compose at least 1% of the total target organisms in a sample to remain above the background level of numerically minor bacterial amplification products (Muyzer et al., 1998; Heuer and Smalla, 1997). Therefore, this technique can only detect very pronounced changes in the microbial community. Subtle changes in species composition cannot be observed without the use of group specific PCR primers (eg. Kowalchuk et al., 1997).

In recent years, the metagenomics, also referred to as environmental and community genomics, is employed as a means of systematically investigating the genetic potential of a sample irrespective of the cultivability of the microorganisms. It is the genomic analysis of microorganisms by direct extraction and cloning of DNA from an assemblage of microorganisms, which has potential to answer fundamental questions in microbial ecology (Handelsman, 2004). The rRNA gene studies yielded only a phylogenetic description of community membership, providing little insight into the genetics, physiology, and biochemistry of the members, while metagenomics provides a second tier of technical innovation that facilitates study of the physiology and ecology of environmental microorganisms.

Signature lipid biomarker (SLB) analysis is another powerful molecular tool that has been proven very useful in studying the microbial community (White, 2002), such as monitoring changes in response to environmental contamination (Findlay et al., 1985; Frostegård, et al., 1996; MacNaughton et al., 1999). The SLB method is based on extraction of “signature” lipid biomarkers from the cell membranes and walls of microorganism (Findlay et al, 1983; White et al., 1979a, 1979b). All intact cells contain polar lipids, which are primarily phospholipids. With cell death, exogenous and endogenous phospholipases rapidly transform the polar lipids in the cell membranes to nonpolar neutral lipid diglycerides by removing polar phosphate-containing head groups (White et al., 1969). Determination of phospholipid ester-linked fatty acid provides a quantitative measure of viable microbial biomass. Information obtained from the lipid analysis also provides quantitative profiles of community structure by identifying signature biomarkers indicative of different eukaryotic and prokaryotic taxa, though it

may not provide definitive information in specific microbial groups. With lipid analysis, it is also possible to assess the physiological status of the microbial community. Many subsets of the microbial community respond to specific conditions in their microenvironment with shifts in lipid composition. Specific patterns of PLFA also change in response to environmental stress. Respiratory quinone structure indicates the degree of aerobic activity in gram-negative heterotrophic facultative bacteria (Hedrick and White, 1986; Geyer et al., 2004). The PLFA for all above described analysis was modified by White et al. (1979a, 1979b) from an extraction system of Bligh and Dyer (1954).

The application of above molecular methods has provided considerable information regarding the microbial community dynamics in the environment studied, however it did not reveal any information regarding the function of communities associated with the specific environmental process. In another words, which functions are attributable to which microorganisms in the environment? This remains as one of the fundamental questions in microbial ecology that needs to be addressed.

Linking the Identity to Function---Stable Isotope Probing

Direct links between microbial identity and biogeochemical processes are currently being determined by several culture independent techniques. Among them, stable isotope probing (SIP) provides powerful approach.

Meselson & Stahl (1958) first demonstrated the SIP principle with *E.coli* grown on $^{15}\text{NH}_4^+$, DNA synthesized during microbial growth on the substrate enriched with the

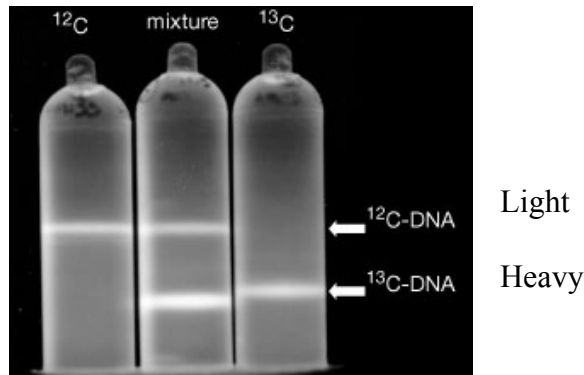


Figure 1-1. The separation of “heavy” and “light” fraction of DNA by CsCl gradient ultracentrifugation

(Photo Credit: from Fig. 1a---- Radajewski S, Ineson P, Parekh NR, Murrell JC. 2000. Stable-isotope probing as a tool in microbial ecology. *Nature*. 10; 403(6770): 646-9.)

stable isotope becomes labeled sufficiently and heavy enough to be resolved from unlabeled DNA by density-gradient centrifugation. The current SIP technique was developed based on this principle: it uses isotopically enriched atoms (e.g. 99% ^{13}C) when compared with their natural abundance (e.g. 1.1% ^{13}C), which enables the fate of a compound to be followed under different desired conditions. Furthermore, by following incorporation of the enriched-isotope into microbial cells or cellular biomarkers, the distinct microbial groups that actively incorporate a substrate can be identified directly. DNA-SIP exploits the density difference between the isotopes, e.g. ^{12}C and ^{13}C , by using CsCl/Ethidium bromide density gradient centrifugation to isolate the ‘heavy’ DNA of microorganisms that assimilated an isotope enriched (e.g. ^{13}C -enriched) substrate (Fig. 1-1) (Sambroock et al., 1989). Although the buoyant density of DNA varies with its G-C content, the incorporation of a high proportion of a naturally rare, stable isotope (^2H , ^{15}N

or ^{13}C) into DNA enhances greatly the density difference between labeled and unlabeled DNA fractions (Rolfe et al., 1959; Vinograd, 1963). Since an entire copy of the genome of microorganisms involved in substrate metabolism is isolated, both phylogenetic and functional gene probes can be used to analyze the diversity of the functionally active community. PLFA-SIP measures stable isotope ratio in an individual PLFA (Burke et al., 2003). The technique primarily uses gas chromatography-combustion-isotope ratio mass spectrometry (GC-c-IRMS) to separate and quantify PLFA, and then determine the isotopic composition of respective PLFAs. It comprises a GC equipped with a capillary column that is used to separate the compounds of interest at high resolution. The outlet of the column is attached to a miniature oxidation reactor where the organic molecules are combusted to CO_2 , N_2 or pure gas contained within a carrier gas. A reduction reactor is included for ^{15}N analysis to convert oxidized nitrogen species to N_2 gas. The purified CO_2 and N_2 are led into an isotope ratio mass spectrometer (IRMS). Because of its design, an IRMS measures the isotopic ratios between the heavy and light isotopes (e.g. $^{13}\text{C}/^{12}\text{C}$ for carbon and $^{15}\text{N}/^{14}\text{N}$ for nitrogen) and results are always calibrated against an international standard or derived reference material (Goodman et al., 1992; Bluck et al., 2002; Docherty et al., 2001).

The combination of substrate addition and isotopic analysis of biomarkers provides unique and powerful method to directly link microbial identity (biomarkers, e.g. DNA, PLFA), microbial abundance (quantity of the biomarker) and microbial activity (isotope incorporation) to environmental processes (geochemical parameters).

The full potential of SIP technique in microbial ecology has just begun to be explored within last few years. Radajewski et al. (2000, 2002, 2003) successfully used the DNA-

SIP technique to identify active methanotrophs in different environmental processes; Stable carbon isotope analysis of biomass and analyses of phospholipid fatty acids (PLFA), glycolipid fatty acids (GLFA), and mycolic acids enabled characterization of mixed-substrate utilization by *Mycobacterium frederiksbergense* LB501T under various substrate regimens (Wick et al., 2003); Arao (1999) reported success with *in situ* detection of changes in soil bacterial and fungal activities by measuring ^{13}C substrate incorporation into membrane phospholipid fatty acids; Fang et al. (2004) was able to link toluene degradation to specific microorganisms by PLFA-SIP analysis. The application of the technique is steadily increasing!

Bio-sep Beads Sampler – “bug trap” or “bio-trap”

In spite of the success of SIP-based studies for the analysis of microbial community (Lin et al., 2004; Hutchens et al., 2004; Hanson et al., 1999; Arao, 1999), this approach has seldom been applied to field scale experiment. The fact that ^{13}C labeled substrate will be greatly diluted in an unconfined natural environment has limited its implementation in field scale investigation. Method that couples ^{13}C label with Bio-Sep beads thus was developed to conquer the challenge.

Standard Bio-Sep beads are fabricated by dissolving Nomex in dimethylacetamide (DMAc) and blending with powdered activated carbon (PAC). This suspension is then forced under pressure through a nozzle with droplets falling into an aqueous quenching solution. Since Nomex is insoluble in water and DMAc is freely miscible with water the Nomex precipitates as beads entrapping the PAC in the internal films that make up the



Figure 1-2. Cross section of BioSep® beads, 2-3 mm in diameter, autoclavable. 74% porosity, 600 m² of surface area/g, 25 % Nomex, 75% Powdered Activated Carbon [PAC], Surrounded by ultrafiltration-like membrane with 1-10 micron holes, Cleaned of contaminating biomarkers by heating to 300 °C 5 hrs.

(Photo Credit: Fig. 3 from----White, D. C., J. S. Gouffon, A. D. Peacock, R. Geyer, A. Biernacki, G. A. Davis, M. Pryor, M. B. Tabacco, and K. L. Sublette. 2003. Forensic Analysis by Comprehensive Rapid Detection of Pathogens and Contamination Concentrated in Biofilms in Drinking Water Systems for Water Resource Protection and Management, *Environmental Forensics* 4(1): 63-74.)

interior structure of the beads. Much of the PAC surface is still accessible and available to interact with soluble material that diffuses into the beads.

Bio-Sep beads have been shown to be rapidly colonized in a variety of aqueous environments from drinking water distribution lines to contaminated aquifers (PCE, BTEX, MTBE) subsequently yielding information as to viable biomass diversity and community structure of the more active fraction of subsurface microbes (Sublette et al., 2003; Jenneman et al., 2004; White et al., 2003; Peacock et al., 2003, 2004). The very large internal surface area coupled with the adsorptive surface very likely explains the rapid formation of biofilms in these beads when exposed to an aqueous environment (Fig. 1-2). In some environments the PAC may serve to concentrate limiting nutrients inside

the beads. The PAC may also aid in the rapid formation of pre-conditioning films that are a prerequisite of biofilm formation.

Potential bioremediation amendments such as ^{13}C -acetate can be incorporated into the Bio-Sep[®] beads during fabrication by entrapment or post-fabrication by adsorption onto the PAC component of the beads, thus provide slow releasing and undiluted ^{13}C -labeled substrate. The availability of such amendment in or near the beads has been shown to alter the community structure of biofilms formed in the beads (Sublette et al., 2003). This approach did show promises (Geyer et al., 2005), though it is still in its early exploration stage.

Chapter 2. Objectives and Experimental Design

Research Objectives

Microbially mediated uranium reduction is an important process that may influence uranium biogeochemistry in a variety of subsurface environment and offers potential for *in situ* bioremediation of uranium contaminated groundwater (Anderson et al., 2002; Lloyd et al., 2000b; Abdelouas et al., 2000; Lovley et al., 1991; Finneran et al., 2002). The engineered uranium bioremediation thus aims at stimulating uranium reduction by promoting the growth and maintenance of active indigenous microbial community capable of immobilizing dissolved uranium (VI) (Lovley et al., 1995, 2001; Wronkiewicz et al., 1999; Suzuki et al., 2003).

Stimulating microbial reduction of soluble U(VI) to insoluble U(IV) shows substantial promise as a strategy for *in situ* bioremediation of uranium-contaminated subsurface environments. Both field (Anderson, et al. 2003; North, et al., 2004; Peacock, et al., 2004) and laboratory studies (Finneran, et al. 2002; Holmes, et al. 2002) have demonstrated that addition of acetate to promote the growth and activity of dissimilatory Fe (III)-reducing microorganisms can lead to removal of soluble U (VI) from contaminated groundwater to levels below the prescribed regulatory level of 44 ug/L (Uranium Mill Tailings Remedial Action, UMTRA, DOE-NABIR standard http://www.gjo.doe.gov/lm/documents/rifle_o/GCAP/oldGCAP2001.pdf). U (VI) removal in most of the above studies was associated with the stimulation of Fe (III) reduction and growth of microorganisms in the family *Geobacteraceae* which became the

predominant organisms, accounting for almost half or more of the total microbial community (Holmes, et al. 2002; Anderson, et al. 2003; North, et al., 2004). However, continued acetate addition to the subsurface of a uranium-contaminated aquifer at Rifle, CO resulted in a shift in the dominant terminal electron accepting process (TEAP) to sulfate reduction, this resulted in complete degradation of the injected acetate under sulfate-reducing conditions and an apparent cessation in the removal of soluble U (VI) (Anderson, et al. 2003). These results probably stressed the importance of maintaining metal-reducing conditions within the subsurface and highlighted the need to understand the dependence of stimulated U (VI) removal on shifts in microbial communities in the subsurface. These results also hinted that increases in the amount of added electron donor, over the short term, would prolong stimulated metal reduction within down-gradient portions of the flowing subsurface. Therefore, the overall objectives of this study were to better understand and characterize the microbial ecology that is associated with a stimulated uranium bioremediation activity, and to link the microbial identity to the functional geochemical processes. The specific objectives of this thesis are as follows:

- To modify the stable isotope probing technique available from literatures to be applicable to the current environmental bioremediation study
- To determine the impact of acetate biostimulation on indigenous microbial community both in field and laboratory studies
- To determine microbial response upon nutrient addition via Bio-Sep beads, both temporally and spatially at the site

- To exam the microbial activities reflective of successive shifts in terminal electron acceptors (TEAP), and explore the stability of reduced uranium in subsurface
- To identify active populations stimulated by electron donor addition and link the identity to geochemical function

These research objectives were designed such that the outcome of the research would provide a more advanced understanding of how and what bacterial populations responded to biostimulation. The results will help in achieving the goal of immobilizing soluble uranium in contaminated environment. More specifically, this research was designed to provide insight into the uranium bioremediation under acetate stimulation and link functions to identities. It was also anticipated that this research would contribute to development of flexible field SIP application.

Experimental Design

In order to meet the objectives of this study, the research described herein consisted of the following task areas:

(1) Microcosm study: In addition to the field sampling activities, parallel incubations of Old Rifle aquifer sediments were performed in the laboratory and amended with ^{13}C -acetate under both Fe (III)- and U (VI)-reducing conditions. The anaerobic microbial populations that were actively involved in acetate biostimulation and uranium bioreduction were investigated. The ^{13}C techniques modified and established was first tested and applied to the microcosm experiment.

The goal of this experiment was to analyze changes in anaerobic microbial community dynamics in response to induced bioreduction of uranium, to identify active microbes involved in the process, and to confirm the application of the modified ^{13}C SIP technique.

(2) Field study of temporal and spatial distribution: At Rifle, Colorado, uranium contamination in a shallow aquifer provides opportunity for field-scale electron donor amendment experiments. A temporal and spatial investigation of the microbial communities prior, during and after *in situ* biostimulation, in association with geochemical parameters was conducted.

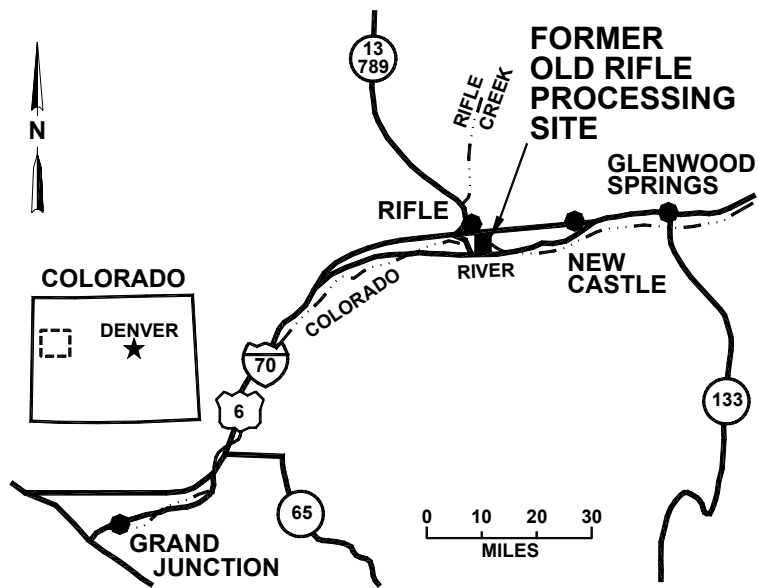
(3) Field SIP application: To detect microbial incorporation of ^{13}C labeled acetate at the field scale, activated powdered Bio-sep beads samplers infused with ^{13}C acetate were deployed down-well in the field. The communities recovered from beads were examined for ^{13}C incorporation.

(4) Comparison of microbial communities recovered from sediment, groundwater, and bio-traps: to examine whether the bio-traps were representative of the subsurface community.

The Old Rifle --- UMTRA Site

The field experiment described in this thesis was conducted at the Old Rifle site.

The Old Rifle Uranium Mill Tailings Remedial Action (UMTRA) Project site is a former ore-processing facility located approximately 0.3 mile east of the city of Rifle in Garfield County, Colorado (Fig. 2–1). The site is situated on a relatively low-lying alluvial terrace created by a flood-plain meander of the Colorado River. The terrace is bounded on the south side by a steep slope that abruptly descends to the river and on the other sides by



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Figure 2-1. Location of the Old Rifle Site

steeply ascending slopes of the more resistant sedimentary rocks of the Wasatch Formation.

The U.S. Department of Energy (DOE) completed surface remediation of abandoned uranium mill tailings and other contaminated surface radioactive material associated with the former milling operation at the site by relocating the contaminated materials to the Estes Gulch disposal cell approximately 9 miles north of Rifle. Surface remedial action began in 1992 and was completed in 1996. The former processing site is currently covered and regarded with clean fill material (including a relatively impermeable clay layer) and reseeded with range grasses. Groundwater contamination remains.

Uranium is the most prevalent site-related contaminant occurring in the alluvial ground water. The U concentrations up to 0.17 milligrams per liter (mg/L) present beneath the

site exceed the UMTRA maximum contaminant level (MCL) of 0.044 mg/L, but steadily decrease to background levels near the down gradient edge of the site. Similarly, selenium concentrations exceeding the 0.01 mg/L UMTRA MCL are present up to 0.06 mg/L near the center of the former tailings pile footprint and also decrease to background levels near the down gradient edge of the site. No groundwater standards have been established for vanadium, however, concentrations up to 0.87 mg/L are present near the former tailings pile footprint, which exceed the 0.33 mg/L human health risk-based concentration for a residential setting (EPA, 2000). Arsenic concentrations in groundwater are less than the UMTRA MCL of 0.05 mg/L, but exceed maximum acceptable levels for human health risk at a single location near the center of the former tailings pile footprint.

New Strategy Based on Previous Experiment

Previous *in situ* field experiments conducted at the Old Rifle UMTRA site in Rifle, CO (USA) demonstrated a potential for removing soluble uranium from groundwater by stimulating the activity of naturally occurring anaerobic bacteria found within the subsurface. Acetate addition to the Old Rifle aquifer stimulated a decrease in soluble U (VI) concentrations to levels at or below the UMTRA maximum contaminant limit in some wells within the test plot coincident with the stimulation of Fe (III) reduction and an enrichment of known Fe (III)- and U (VI)-reducing *Geobacteraceae* within the aquifer. These observations are consistent with the known ability of *Geobacter* species to enzymatic reduce soluble U (VI) to the insoluble mineral, uraninite (UO₂). These findings

showed the potential for a novel and economical bioremediation technique to rapidly remove uranium from groundwater at a wide variety of uranium-contaminated sites. However, sustained *in situ* uranium removal from groundwater was not observed during this initial field test. Continued acetate addition to the Old Rifle aquifer ultimately stimulated sulfate reduction within the subsurface. Removal of soluble U (VI) appeared to be less efficient under stimulated sulfate-reducing conditions than under Fe (III)-reducing conditions and U (VI) concentrations in the groundwater actually increased during this period. These observations stress the importance of maintaining the activity of Fe (III)- and U (VI)-reducing *Geobacteraceae* in the subsurface in order to sustain the removal of soluble U (VI) to low levels in groundwater over longer time periods. One strategy for limiting the impact of sulfate reduction and sustaining metal reduction in the subsurface is to increase the concentration of acetate delivered to the subsurface to a level greater than the ambient sulfate concentration, which creates an “electron acceptor limiting” condition with respect to sulfate. Therefore, even if all available sulfates is depleted due to stimulated sulfate reduction, there will still be a net export of acetate to the down-gradient areas. In this new field study at the Old Rifle UMTRA site in Rifle, CO, an increased concentration of acetate (approx. 3 millimolar) was injected as an electron donor into the subsurface to create anaerobic conditions that will stimulate growth of metal reducing bacteria capable of reducing soluble U (VI) to insoluble U (IV). The subsurface microbial communities captivated on bio-traps were evaluated in terms of structure and biomass both quantitatively and qualitatively. BioSep beads baited with C¹³ was incubated in the

groundwater in order to assess active bacterial community, in addition both sediments and groundwater were sampled to provide comparison.

Research Hypotheses

The hypotheses are:

- Amendment of “bug traps” with ^{13}C label can effectively enrich ^{13}C incorporating populations--sufficient for DNA and PLFA analysis, and hence provide insight into the biological responses to stimulated uranium bioremediation, and to link active populations to uranium bioremediation
- Injected higher concentration of acetate may prolong the iron and uranium reducing conditions.
- The composition of the microbial community in the sediments mirrors results obtained from BioSep bead samples, indicating an enrichment of metal reducers, that are potential uranium reducers.
- The laboratory biostimulation will reflect the field biostimulation, but exhibit differences.
- Active microbial communities reflect the stimulated geochemical activity *in situ*, which can be determined by measuring geochemical parameters.

Chapter 3. Stable Isotope Probing of Biostimulation in Sediment Microcosms

Introduction

Nutrient or electron donor amendment is now increasingly accepted as practice in uranium contaminated sites, but there is scant understanding of the systematic effects of nutrient addition on indigenous microbial populations or the progress of uranium bioremediation.

In a contaminated aquifer, native or added organic carbon is used as an electron donor, and dissolved oxygen (DO) is used first as the prime electron acceptor. After the DO is consumed, anaerobic respiration became the major alternative and microorganisms typically use additional electron acceptors such as nitrate, ferric iron, and sulfate etc. Anaerobic respiration has been shown to be an effective process in bioremediation (Lovley, 2001; Guha et al., 2001; Fredrickson et al., 1996), however studies have indicated that this effectiveness is not consistent at different sites or different time (Lovley, 2000b; Lee, 1998; Fennell et al., 2001; Anderson et al., 2003). It is unclear whether this is the result of heterogeneity of anaerobe distribution or the influence of different environmental factors controlling the microbial respiration. Further for those anaerobic processes which are effective in uranium bioremediation, the mechanisms by which uranium was reduced and the microbial species responsible remain as topic of further investigation. Dissimilatory iron-reducing bacteria (DIRB) and sulfate reducing bacteria are two major groups known to be capable of reducing soluble uranium (Lovley et al., 1992b). A previous study indicated that stimulated enzymatic uranium reduction is concomitant with iron reduction and the enrichment of *Geobacter* (Anderson et al.,

2003). In an X-ray absorption near-edge structure (XANES) analysis, Suzuki et al (2003) confirmed that without microbial activity, no U (IV) was detected, indicating that U (VI) reduction requires microbial activity. Nevertheless, uranium can also be reduced abiotically: indirect reduction by sulfide produced by sulfate-reducing bacteria plays a major role in uranium sulfide immobilization (Beyenal et al., 2004; Reynolds et al., 1983).

For nutrient addition or electron donor amendment to be a useful strategy and practice in bioremediation, it clearly requires knowledge of the microbial populations involved in the process. Previous studies using PCR-DGGE and PLFA analysis have resulted in good qualitative and quantitative measurement of bacterial biomass and community structure shifts (Macnaughton et al., 1999; White, 2002; Villanueva et al., 2004), however they provided little information as to relations between identity and biogeochemical functions, largely due to the methodological constraints. Recent development of SIP technique provides powerful approach to link the microbial identity to biogeochemical functions (Boschker and Middelburg, 2002; Radajewski et al., 2003). In this study, I attempt to identify active microorganisms involved in microcosm biostimulation using Stable Isotope Probing (SIP). The DNA-SIP technique was modified to adapt to low biomass environmental samples. The aquifer sediment microcosms consisted of sediment and groundwater samples from Old Rifle field undergoing *in situ* biostimulation. The laboratory incubation of the microcosms with ^{13}C acetate amendment was performed to investigate anaerobic microbial populations that were actively involved in acetate utilization and possibly uranium bioreduction.

The goal of this part study was to establish and evaluate the ^{13}C DNA/PLFA-SIP technique in microcosm, and to analyze changes in anaerobic microbial community dynamics in response to induced biostimulation, and to identify active microbes involved in the process. At the same time as a prelude to the field test, it is also aimed to verify the feasibility of applying ^{13}C SIP technique to field scale experiments. The PCR-DGGE and PLFA lipid analyses on microcosm reveal the relative degrees of importance of phenotypic and taxonomic changes in response to acetate addition. The techniques coupled with SIP further allow monitoring of changes in structure that are linked to function of bacterial assemblages during biostimulation and bioreduction. The results indicated a significant biostimulation effect resulted from acetate anaerobic incubation and a successful application of ^{13}C DNA-SIP coupled with ^{13}C PLFA-SIP.

Materials and Methods

Sediment and Groundwater: Both sediments and groundwater were collected from the old Rifle UMTRA field site, where *in situ* acetate injection was performed. P11-15 was the sediment core 14.75 ft (4.5 m) upgradient from the injection gallery, P13-15 and P15-15 were the sediment cores downgradient 9.2 ft (2.8 m), and 27.2 ft (8.3 m) from the injection gallery respectively. Groundwater was collected from down-gradient monitoring well of M-08, on Dec. 4, 2003, which was pumped from multi level sampling cartridge after purging 12 Liters. More detailed description of the Rifle experiment and site information has been published by Anderson et al. (2003) and can be found in Chapter 4 of this thesis. Both sediments and groundwater were stored at 4°C until microcosm set-up.

Anaerobic Microcosms: Microcosms consisted of 50-ml anaerobic roll tube (Bellco, Inc) with No. 4 butyl rubber stopper (VWR, International), containing 15 g sediments from cores of P11-15, P13-15, P15-15 respectively, and 30-ml groundwater from well M-08 of the Old Rifle UMTRA site. One biotrap made from nylon net 10 cm x 1.8 cm, receiving about 0.60 gram biosep beads (roughly 100 beads) was included in each microcosm (Fig.3-1). The traps were soaked in sterile water and autoclaved, then blow under nitrogen before use.

Microcosms containing sediments P13-15 or P15-15 (Microcosm P13 or microcosm P15) received the groundwater containing 10 mM Acetic Acid- ^{13}C , sodium salt (minimum 99% atom ^{13}C , hygroscopic, ISOTEC) and were used for observing biostimulation and bioreduction. Microcosms containing sediments P11-15 (Microcosm P11) received the groundwater without acetic acid- ^{13}C addition and served as background controls. Two additional experimental controls include one with 10 mM sodium acetate groundwater and trap, no sediment, the other with sterile water (that was used to hydrate the traps) and sterilized trap only.

The microcosms were anaerobically incubated in the dark at 22-25 °C. Duplicated ^{13}C acetate amended and non-amended microcosms were sacrificed at 5 and 20 days for analysis. Both sediment and bio-trap samples were frozen at -70°C until ready for DNA and PLFA analysis. The pHs of the groundwater were determined immediately.

Aqueous pH Measurement and Observations for Reducing Activity: The pH value was measured using digital pH/milli volt meter 611 (ORION Research) at room temperature. Black precipitation was observed as sign of sulfate reduction, which

Negative
control

P11

P13

P15



Figure 3-1. The laboratory incubation of Old Rifle sediments with BioSep bead samplers. The microcosms P13 and P15 were amended with ^{13}C acetate to stimulate both Fe (III)- and U (VI)-reducing conditions and microcosm P11 received no acetate.

indicated the occurrence of iron and U reduction also, due to thermodynamic reasons.

DNA Extraction and CsCl Gradient Separation: Nucleic acid was extracted directly from the sediments or bio-trap beads using FastDNA Spin Kit (for soil, BIO101) following manufacture's instruction. DNA was eluted into 2 x 50 ul sterilized deionized water and several extracts were pooled together (10 for sediment samples, 4 for bead samples) for the following CsCl gradient separation. Then CsCl of 1.0 g was added to 1ml DNA extract in TE buffer containing 10 ul ethidium bromide (10 mg/ml), which was then transferred to a polyallomer Bell-top ultracentrifuge tube (11 x 32 mm; Beckman). Following centrifugation at 70000 r.p.m. using a Beckman TL-100.2 rotor (TL-100 Beckman Ultracentrifuge) for 22 hours at 20 °C in a CsCl density gradient (Sambroock *et al.*, 1989), DNA fractions were resolved and visualized with UV light (365 nm). The heavy fraction of DNA from each ^{13}C acetate amended microcosm sample was withdrawn gently from the gradients using a 1 ml syringe and hypodermic needle (18 gauge). Care was taken during collection of the ^{13}C DNA fraction to avoid co-extraction of the ^{12}C -DNA band.

The ^{12}C and ^{13}C DNA standards were extracted from *E. coli* grown overnight on basic cultivation medium with addition of normal D-Glucose and D-Glucose- ^{13}C 6 (99 ATOM% ^{13}C , Sigma-Aldrich) respectively. A mixture of 10 ug ^{12}C and ^{13}C DNA standards were run as a control in each CsCl ultra centrifugation separation and in the situation where ^{13}C DNA band was too weak to be visible, the standard control tube was aligned with the sample tube and ^{13}C DNA was withdrawn from the position where the standard indicated.

Ethidium bromide was removed by extracting the DNA with an equal volume of n-butanol saturated with deionized water. Following three extractions, the DNA was dialysed against a large volume of deionized water using Tube-O-Dialyzer (Geno Technology Inc. St. Louis, MO).

PCR-DGGE and Sequence Analysis: PCR amplification of both regular and ^{13}C fraction 16S rDNA fragments prior to DGGE was performed as described by Muyzer et al. (1993). The primers targeted eubacterial 16S V3 regions corresponding to *E. coli* positions 341-534 (Brosius et al. 1981). The sequences of the primers were (forward, G-C clamp underlined) CGCCCGCCGCGCGCGGGCGGGGCGGGGGCACGGGGGG CCTACGGGAGGCAGCAG, and (reverse) ATTACCGCGGCTGCTGG.

Thermocycling consisted of 35 cycles of 94°C, 60s, 55°C, 60s and 68°C, 45s, using 1.25 units of Expand HF polymerase (Boehringer, Indianapolis, IN), 100 pmol each of the primers in a total volume of 25 uL performed on a “Robocycler™” PCR block (Stratagene, La Jolla, CA). The final extension period was performed for 7 min at 68°C.

DGGE was performed using a D-Code 16/16 cm gel system (BioRad, Hercules, CA) as referenced in (Chang et al. 1999). The buffer chamber of DGGE apparatus contained 6.5 L of 0.5 x TAE buffer (20 mM tris-acetate, 0.5 mM EDTA, pH 8.0) and was maintained a constant temperature of 60°C. Gradients were formed between 25 and 65% denaturant (100% denaturant defined as 7 M urea plus 40 % v/v formamide) and the gels were run at 35 voltage for 16 hr. Gels were then stained in purified water (Milli-Ro™, Millipore, Bedford, MA) containing ethidium bromide at 0.5 mg per liter, and de-stained twice in 0.5 x TAE. Images were captured using Gel Doc-It Imaging System (UVP, Inc. Upland,

CA). The central 1 mm² portions of DGGE bands of interest were excised using a razor blade (American Safety Razor Company, Verona, VA) and soaked in 36 ul of purified water (Millipore) overnight. A portion (5 ul) was removed and used as the template in a PCR reaction as above except that the forward primer lacked the GC clamp (Muyzer et al., 1993). The products were purified by glass-milk extraction (Gene-Clean™ kit; BIO-101), then subject to sequencing using the primer 516r (GWATTACCGCGGCKGCTG; W= A or T, K= G or T: Lane et al., 1985) and an ABI-Prism model 373 automatic sequencer with dye-terminators (Perkin-Elmer, Foster City, CA). Sequences were assembled using “SeqPup Version 0.6” (Gilbert 1996) and compared to the available GenBank database by use of the BLASTN facility of the National Center for Biotechnology Information to determine their approximate phylogenetic affiliations. Partial sequences were then compiled in SeqPup 0.6 and aligned with rRNA sequences from the Ribosomal Database Project (SSU_rep_Prok, RDP) by use of the “on-line analysis” facility (Maidak et al., 1999; Cole et al., 2005), then further aligned in ARB software environment (Aligner v2.0, Strunk and Ludwig 1997). Phylogenetic trees were constructed by applying the Jukes and Cantor correction, and the neighbor-joining method, using the ARB software package (<http://www.mikro.biologie.tu-muenchen.de>, Strunk and Ludwig, 1996). To evaluate the tree topology, phylogenies were reconstructed by using evolutionary distance, maximum parsimony and maximum likelihood analyses of the aligned partial sequences (Ludwig et al., 1998). As topologies obtained from the different analyses were generally similar. The trees presented in this thesis are based on the neighbor-joining analysis with the confidence of branch bootstrap values determined using the algorithm of SEQBOOT -- also operated within the ARB.

Phospholipid Fatty Acid (PLFA) Analysis: PLFA analysis was performed using previously reported precautions (White and Ringelberg, 1998a). All solvents used were of GC grade and were obtained from Fisher Scientific (Pittsburgh, PA). The biosep beads were extracted with the single-phase chloroform-methanol-buffer system of Bligh & Dyer (1954), as modified (White et al., 1979b). The total lipid extract was fractionated into neutral lipids, glycolipids, and polar lipids by silicic acid column chromatography (Guckert, et al., 1985). The polar lipids were transesterified to the fatty acid methyl esters (FAMES) by a mild alkaline methanolysis (Guckert, et al., 1985), with the Mayberry and Lane (1993) method to protect cyclopropane PLFA and release plasmalogen ethers as dimethylacetals. The FAMES were analyzed and identified by gas chromatography/mass spectroscopy using an Agilent 6890 series gas chromatograph interfaced to an Agilent 5973 mass selective detector with a 50 m non-polar column (0.2 mm I.D., 0.11 μ m film thickness) with a temperature program of 100°C initial temperature, 10°/min to 150°C for a minute, 3°/min to 282°C for 5 min with injector temperature at 270°C and detector at 290°C. Total analysis time was 55 min.

GC-Isotopic Ratio Mass Spectrometry (IRMS): Stable carbon isotope ratios ($^{13}\text{C}/^{12}\text{C}$) were measured by a Finnigan (Austin, TX, USA) Delta Plus isotope ratio mass spectrometer with a GC-III combustion interface and an Agilent 6890 gas chromatograph (Palo Alto, CA, USA). The fatty acid methyl esters were separated on an HP-1 column (Dimensions: 50m x 200 μ m) with constant pressure at 43.7 psi. The oven (column) temperature was programmed from 60°C to 150°C at 10°C/min, then to 312°C at 3°C/min. A standard containing known concentrations of ~40 fatty acids was analyzed on

the GC/IRMS along with to ensure reproducibility (variation $\leq 10\%$) and peak identification. The methanol used to prepare methyl esters had a $\delta^{13}\text{C}$ value of -48‰ .

Notation. The standard notation for expressing high-precision gas isotope ratio MS results in δ being defined as follows:

$$\delta(\text{‰}) = [(R_{\text{FAME}}/R_{\text{PDB}}) - 1] \times 10^3$$

where R_{FAME} and R_{PDB} are the $^{13}\text{C}/^{12}\text{C}$ isotope ratios corresponding respectively to the sample and to the international internal standard Pee Dee Belemnite. The more ^{13}C a compound contains, the higher $\delta^{13}\text{C}$ becomes.

The ^{13}C atom percent in the compound was calculated as:

$$\text{At \% } ^{13}\text{C} = \{^{13}\text{C}/(^{13}\text{C} + ^{12}\text{C} + ^{14}\text{C})\} 100 \text{ At \%}$$

$$\begin{aligned} &= \frac{\{(\delta\text{‰}/1000 + 1) \times R_{\text{PDB}}\} \times 100}{\{(\delta\text{‰}/1000 + 1) \times R_{\text{PDB}}\} + 1} \\ &= R_{\text{FAME}} / (1 + R_{\text{FAME}}) \quad (100 \text{ At\%}) \end{aligned}$$

The amount of naturally present ^{13}C including the ^{13}C atoms introduced from the methyl moiety that came from the derivatization process was considered as background. A compound with At % exceeding the background was considered to be enriched.

Statistical Analysis: Results were expressed per gram dry weight of the sediment or per bead. Phospholipid fatty acids were analyzed both as picomole per gram sediment or per bead and as mole percents. Analysis of variance (ANOVA) was used to determine whether there were significant differences among lipid biomarker data obtained for the 3 treatments over time. 2-4 replications were sampled at each sampling point. Differences or correlations were taken to be significant at $P < 0.05$. To detect patterns in the variation

of the community composition measured as PLFA, a hierarchical cluster analysis (ward method) based on Euclidean distance was conducted on the transformed data. ANOVA, and hierarchical analysis were performed using the statistical package JMP Version 5.1.2 for Windows (JMP, SAS Institute. Inc., Gary, NC).

Nucleotide Sequence Accession Numbers: Partial 16S rDNA sequences recovered from sediment were submitted to GenBank as with accession numbers AY994811 to AY994825 and AY994848 to AY994874. Sequences recovered from beads were submitted as AY994826 to AY994847, and AY994875 to AY994913.

Results

Bioreduction and pH Response: Throughout the incubation, the pH values showed a decreasing trend, the control microcosms maintained slightly higher pH values than the microcosms received acetate (Fig. 3-2).

At first sampling event of day 5, the sediments in the microcosms did not show color change. At the second sampling event of day 20, P11 sediments remained brown, P13 sediments exhibited noticeable black precipitation (Fig. 3-1), indicating sulfate reduction, metal reduction and formation of mineral sulfide. Sporadic black precipitation was also observed with microcosm P15.

Biomass: The bacterial biomass was determined to include the total PLFA minus the normal saturates over 18 carbons in length and the polyenoic PLFA. Both of these are generally associated with eukaryote biomass (White et al., 1979a, 1998a; Bossio et al.,

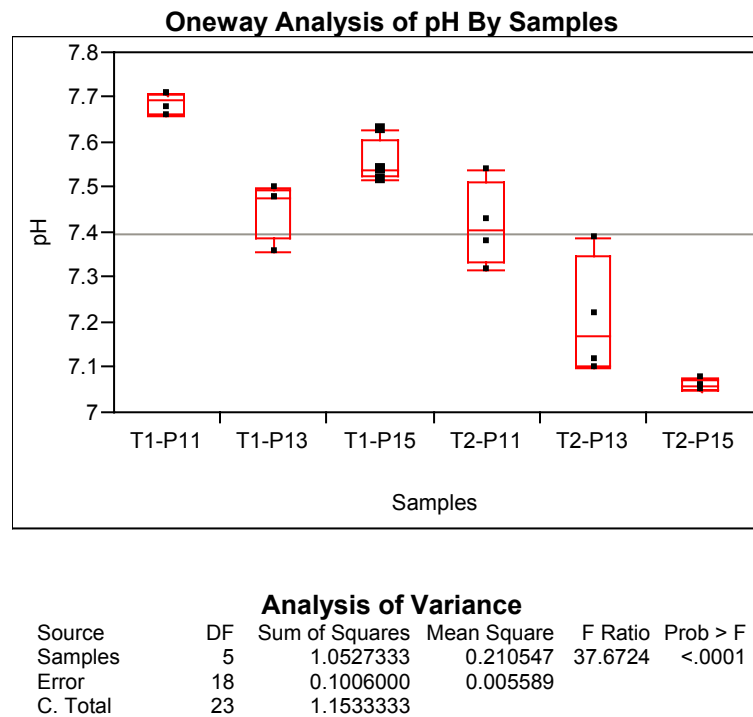


Figure 3-2. Whiskers box plots for the microcosm aqueous pH values, showing a decreasing trend. (n = 4). Nomenclature: T1 and T2 represent incubation time day 5 and day 20 respectively; P11, P13 and P15 indicate microcosm type.

1998), although there are exceptions. The trace quantities of PLFA of unknown structure were also excluded from the prokaryote biomass.

All microcosm sediments showed varied increase in bacterial biomass after incubation. Noticeably the biomass of sediments that received acetate amendment was significantly higher than that of sediments taken from unamended microcosms (ANOVA, $P < 0.05$, Fig. 3-3). Biomass between day 5 and day 20 was insignificantly different. The highest amount of biomass (10537 pmol/g) was observed with P13 sediment after 5 day incubation, the lowest biomass was also found with P13 microcosm, but at day zero (168

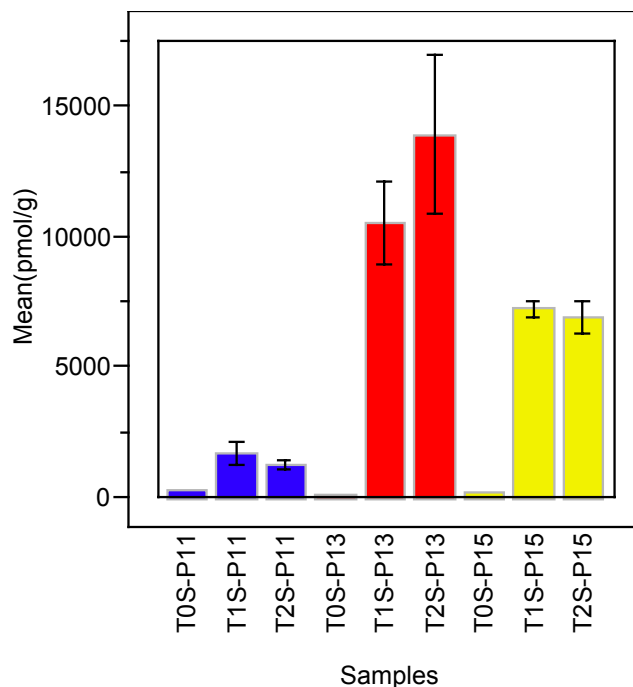


Figure. 3-3. The bacterial biomass from microcosm sediment as measured by total bacterial PLFA (pmol/g), which shows significant increase in ^{13}C acetate amended microcosms relative to control microcosms. Nomenclature: The prefix TnS is followed by microcosm type (P11, P13 and P15). T represents incubation time, S indicates sediment. (T0—day 0, T1—day 5, T2—day 20)

pmol/g). Bio-trap samples showed similar biomass increase, but no significant biomass difference among amended and unamended samples was observed until after 20 days incubation (ANOVA, $p < 0.05$, Fig. 3-4). The biomass ranged from lowest of 21 pmol/bead with sample T1-P13 to the highest of 153 pmol/bead with sample T2-P15. Interestingly, microcosm P13 sediments showed significantly higher biomass than P15 sediment, which is the opposite for respective beads samples (Fig. 3-4).

PCR-DGGE Analysis of Bacterial Community: PCR-DGGE analysis of bacterial community structure was carried out on all three type microcosms (P11 Background,

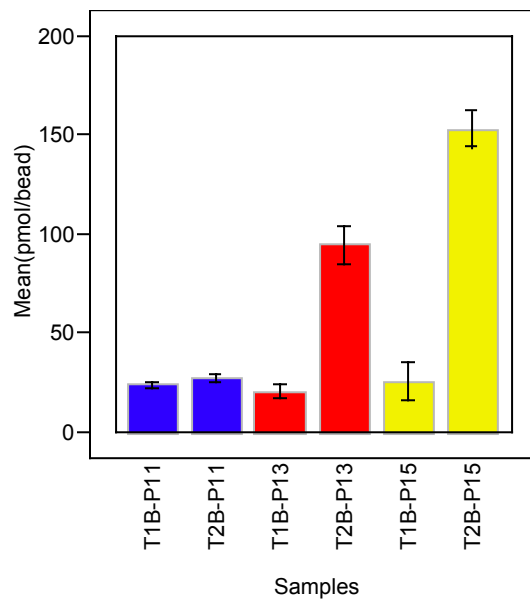


Figure 3-4. The bacterial biomass from microcosm bio-traps as measured by total bacterial PLFA (pmol/bead), that shows significant increase in ^{13}C acetate amended bio-traps over control microcosm traps. Nomenclature: The prefix TnB is followed by microcosm type (P11, P13 and P15). T represents incubation time, B indicates biotrap beads. (T1—day 5, T2—day 20)

P13 and P15 acetate amendment microcosms), each with two replications at different incubation time. Sufficient PCR products for DGGE analysis were obtained from all genomic DNA extracts with the exception of the background microcosm samples at day 5 (T1S-P11), that had weak amplification. To overcome this, two extra PCR products from T1S-P11 were pooled for DGGE analysis. Banding patterns are shown in Figure 3-5.

The communities of sediment or bio-traps from two replicates were most similar to one another, indicating reliable experimental set-up and sample processing.

The sediment microbial community displayed most similar structure with bead bacterial community at day 5 (Fig.3-5A). Then at day 20, differences between sediment and bead microbial communities evolved, largely due to the rise of *Geobacter*, and SRB, as

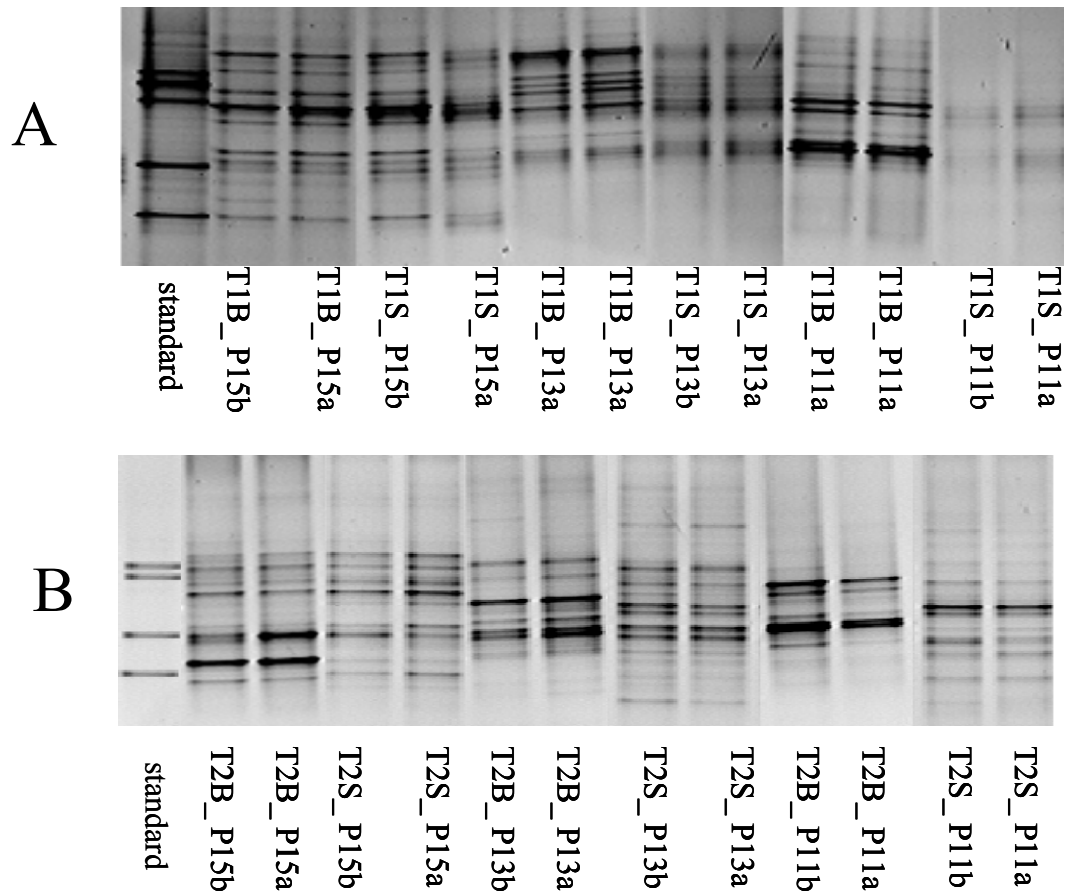


Figure 3-5. DGGE analysis of microcosm bacterial community in both beads and sediment samples at sampling time day 5 (A) and day 20 (B). Amplified 16S rDNAs gene fragments were separated on a gradient of 20 % to 65 % denaturant. Nomenclature: TnB or TnS are followed by microcosm type (P11, P13 and P15), then sample replication a or b. T represents incubation time, S indicates sediment, and B represents biotrap beads. (T1 – day 5, T2 – day 20)

indicated by sequence analysis. Fig. 3-6 and Fig.3-7 are the evolutionary distance trees of the bacterial domain showing the distribution of more than 10 bacterial phylotypes recovered from sediment (Fig. 3-6) and bio-trap bead samples (Fig. 3-7). For all sediment or all bead samples, they exhibited some sequence types that was dominant (such as *pseudomonas*, *hydrogenaphaga*, *Gram +*, SRB), as well as some sequence types that occurred only once or twice in a particular sample.

Sediment The microbial community before anaerobic incubation (day 0) in the sediment was rather complex, causing difficulty in resolving the DGGE banding pattern (data not shown). With care, sequence information was obtained for this time point (T=0), which indicated β -*proteobacteria*, *Pseudomonas* and uncultured bacterial sequences were the major constituents. After 5 days incubation, the community showed single sequence dominance: the majority (11 out of 13) of the bacterial sequences recovered from sediment were phylogenetically members of well-recognized *Pseudomonas* genera, though some β -*proteobacteria* were also detected along with. The *Pseudomonas* dominance was less pronounced at day 20 (10 out of 27), particularly with sample P13, in which sequences derived from uncultured *Gram +*, uncultured SRB within δ -*proteobacteria* and *cytophaga* became the major components of the community. As for the P15 microcosms, *Pseudomonas* was still in dominance at day 20, but sequences associated with SRB within δ -*proteobacteria*, *hydrogenophaga*, and uncultured *actinobacteria* were also appreciably detected (Fig. 3-6). Five sequence types had no specific association with any of the known cultivated representatives and were phylogenetically divided among 3 novel divisions. However consistent branching order of divisions in the tree shown in Fig. 3-6 were established by bootstrap resampling.

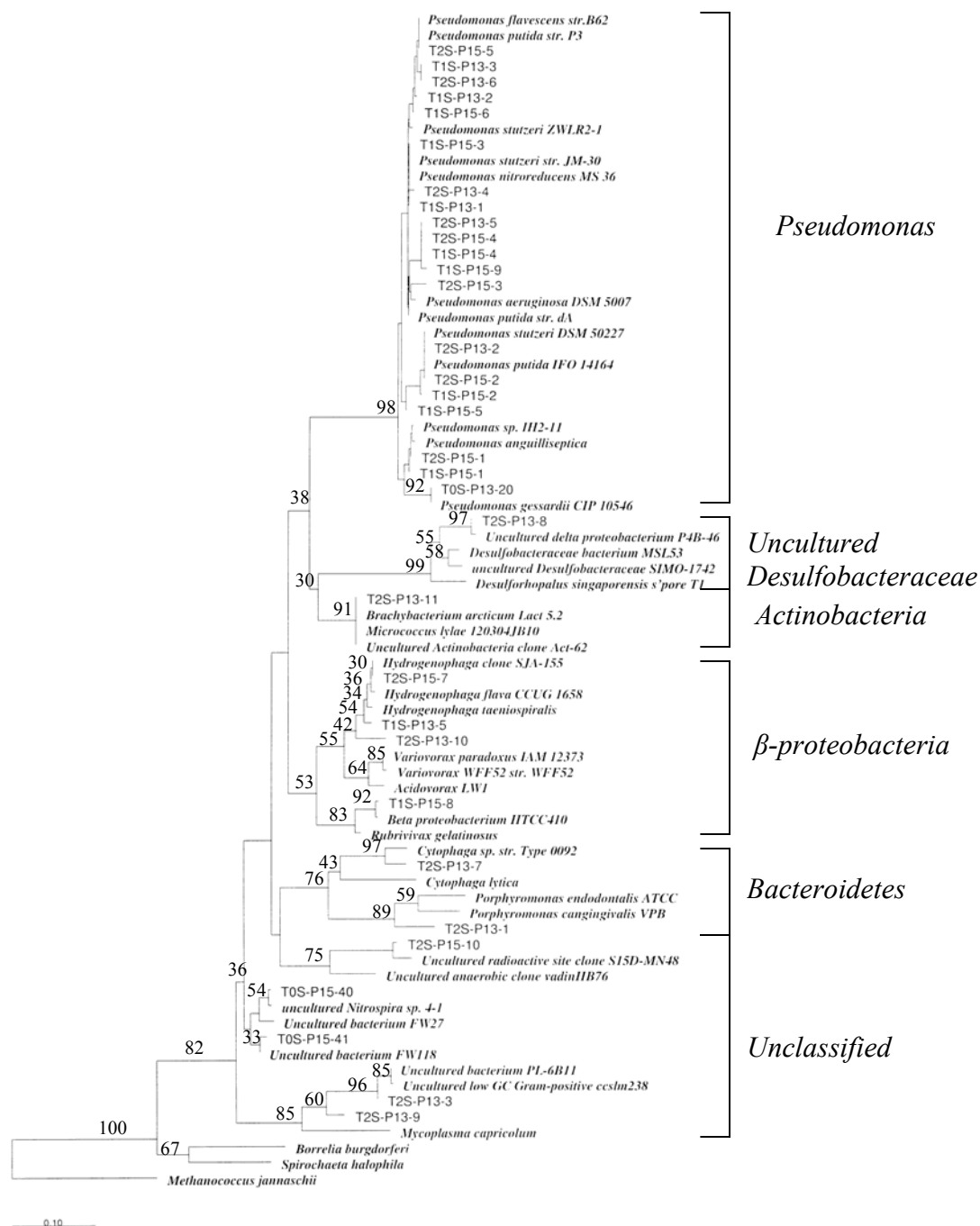


Figure 3-6. Neighbor-joining analysis of 16S rRNA gene sequences from excised DGGE bands, derived from sediment of microcosms P13 and P15. Numbers on the tree refer to bootstrap values on 1000 replicates, only values above 30 are given. Scale bar represents 10 % estimated change. Sequences prefixed “TnS” (n = 0, 1, 2) were generated during this study. Tn represents sampling time point (T0: day zero; T1: day 5; T2: day 20; S indicates sediment samples). The prefix is followed by the microcosm types (P13, or P15), then band number.

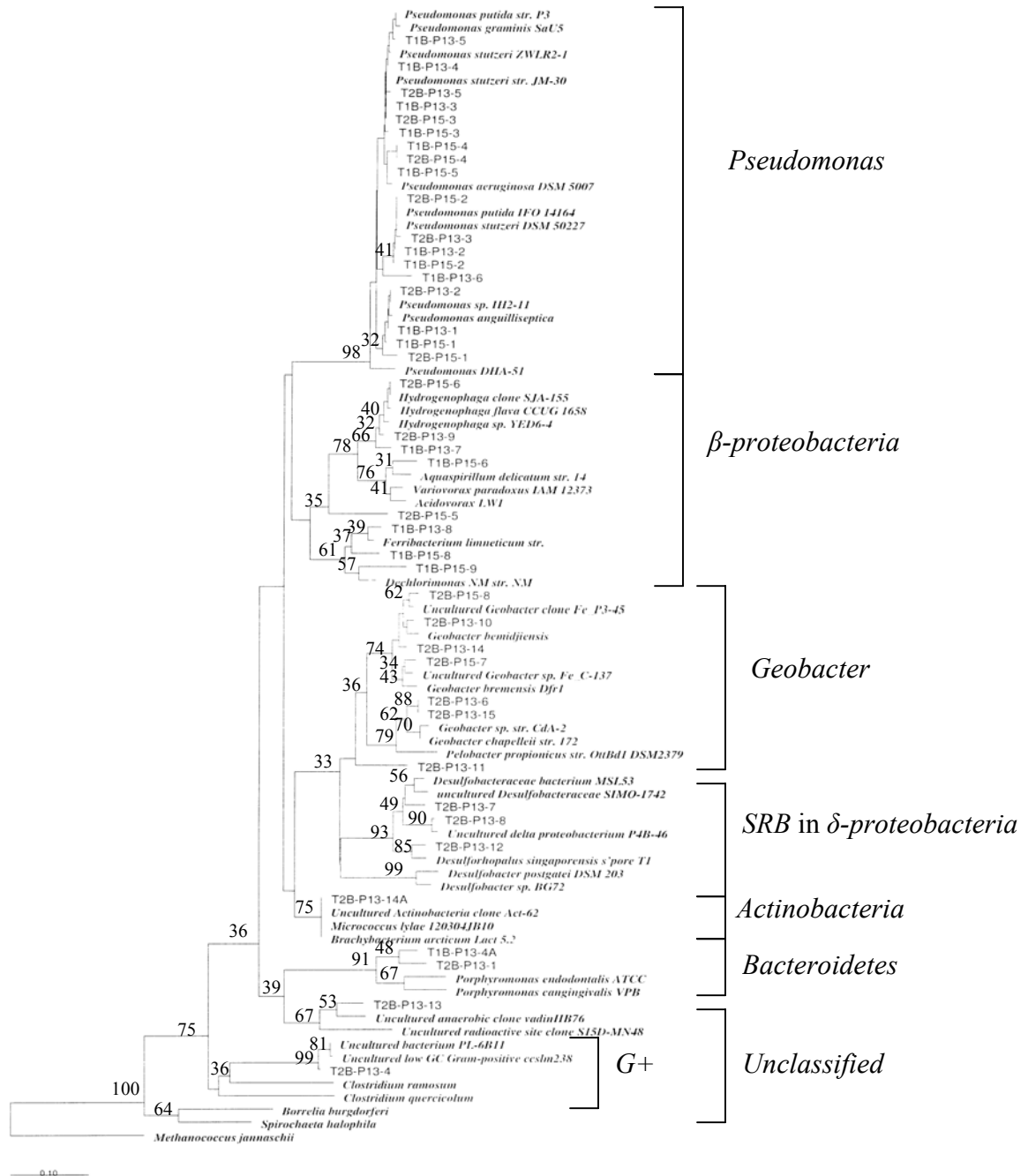


Figure 3-7. Neighbor-joining analysis of 16S rRNA gene sequences from excised DGGE bands, derived from biotrap samples of microcosms P13 and P15. Numbers on the tree refer to bootstrap values on 1000 replicates; only values above 30 are given. Scale bar represents 10% estimated change. Sequences prefixed “TnB” (T1: day 5; T2: day 20; B indicates bio-trap samples) were generated during this study. The prefix is followed by the microcosm types (P13, or P15), then band number.

Background The community structures in the background microcosm were markedly different from those of acetate amended microcosms. Members of β -proteobacteria affiliated with *Hydrogenaphaga*, *Variovorax*, *Aquaspirillum*, *Dechlorimonas* and *Mythylophilus*, etc. dominated the background microcosms. On the contrary to amended microcosms, which displayed various varieties of *Pseudomonas* sequence types, there was only one uncultured γ -proteobacteria sequence belong to *Pseudomonas*, that was detected at both day 5 and day 20 (Fig. 3-8). Throughout the experiment the community within the background microcosms remain distinct from those amended, with β -proteobacteria and γ -proteobacteria (*Pseudomonas*) in dominance.

Bio-trap beads The microbial diversity and community structure from bio-trap samples mirrored what was revealed based on sediment samples. At day 5, *Pseudomonas* also dominated the acetate amended microcosms, coexisting in the environment with a few β -proteobacteria (Fig. 3-6). At day 20, the community showed noticeable shift towards δ -proteobacteria dominance, especially with microcosm P13. Sequences belong to *Geobacter*, and sulfate reducing bacteria related to *Desulfobacteraceae*, *Desulforhopalus* were detected repeatedly (9 out of 17 within P13 microcosms). The uncultured *Geobacter* sequences were detected from P15 microcosms as major component, as well as β -proteobacteria affiliated with *Hydrogenaphaga*, *Ferribacterium* though no SRB sequences were retrieved.

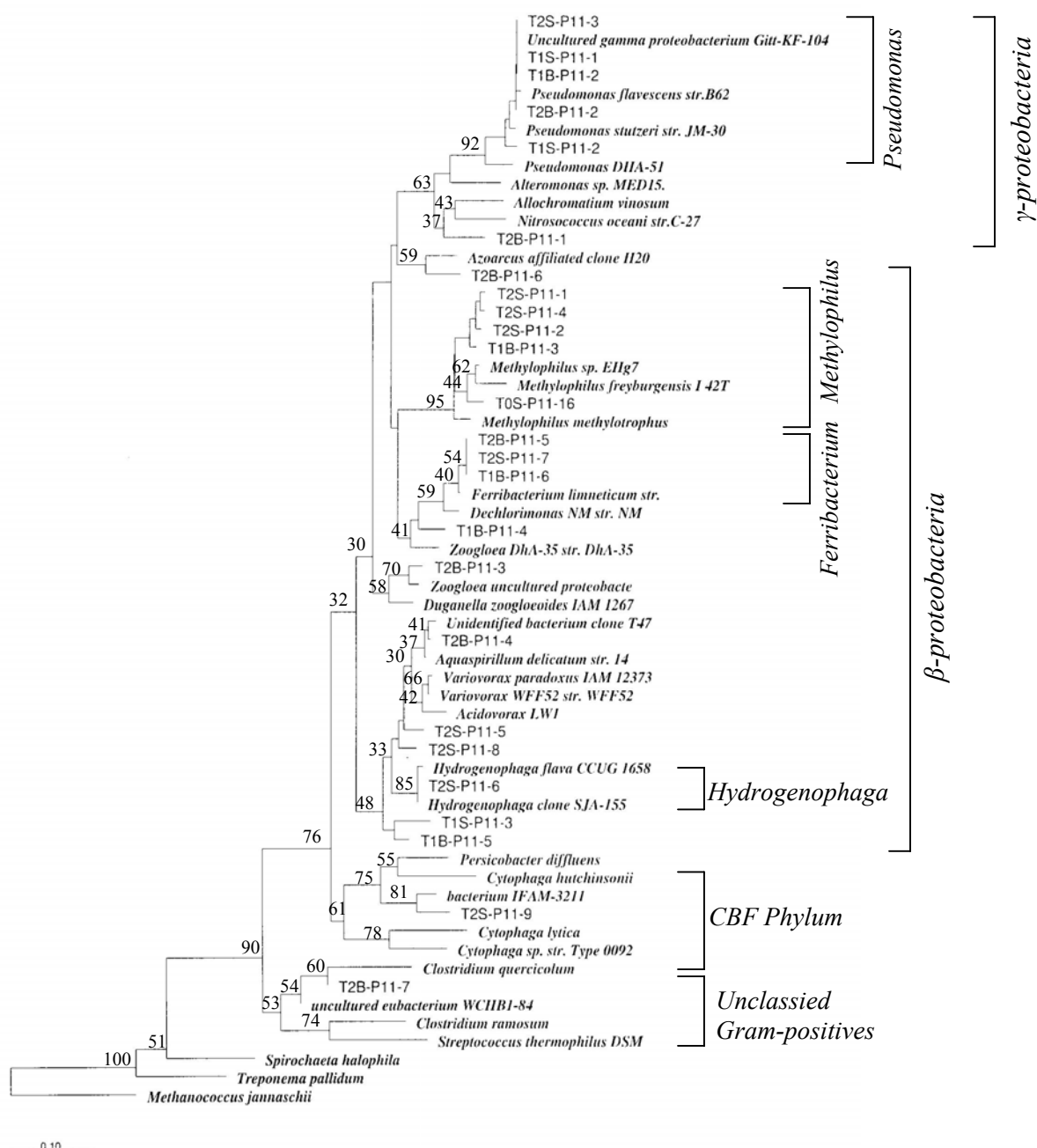


Figure 3-8. Neighbor-joining analysis of 16S rRNA gene sequences from excised DGGE bands, derived from background microcosm P11. Numbers on the tree refer to bootstrap values on 1000 replicates, only values above 30 are given. Scale bar represents 10% estimated change. Sequences prefixed “TnS” or “TnB” (n=0, 1, 2) were generated during this study. The prefix is followed by the microcosm types (P11), then band number (for detailed nomenclature, refer to legend of Fig. 3-6 and Fig.3-7).

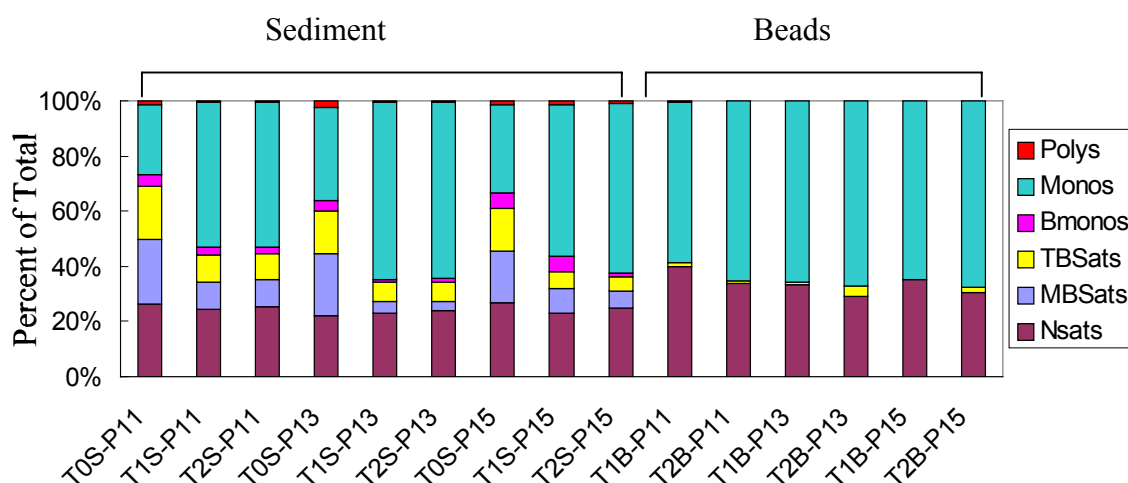


Figure 3-9. PLFA community profiles of microcosm samples. For detailed nomenclature, refer to Figs. 3-3 and 3-4. PLFA abbreviations: Nsats: normal saturates; MBSats: Mid-chain branched saturates; TBSats: Terminally branched saturates; Bmonos: Branched monounsaturates; Monos: Monounsaturates.

Community Structure Revealed by PLFA Analysis: The microbial communities of microcosm samples were determined using the total PLFA structure classes profiles (prokaryote and eukaryote PLFA) and are shown in Figure 3-9.

The community structure of sediment samples, differs from that of the bio-trap samples. The former (sediment) is more complex, containing all 8 groups of fatty acids and diacids, the latter (bio-traps) only had 4 groups. The community also shifted away with different treatment, as well as over the incubation time. In the bio-trap samples, terminally branched saturates, indicative of gram positive and anaerobic sulfate reducing bacterium, showed significant increase at 20 day incubation (ANOVA, $p < 0.05$, Figure 3-10.) compared to that at 5 day incubation. This increase only occurred in acetate amended microcosms, not in background microcosm samples. The shift in terminally branched saturates coincided with the rise of *Geobacter*, sulfate-reducing bacteria sequences at day 20 in the bead samples, which were not detected at day 5 either.

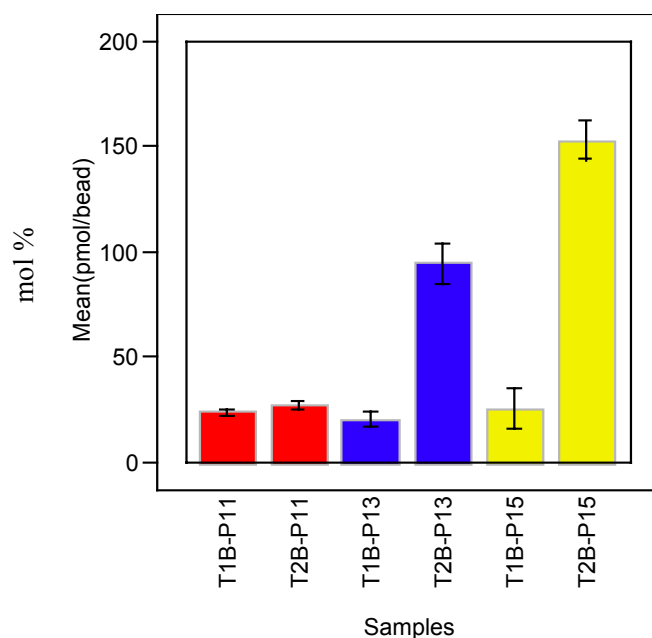


Figure. 3-10. PLFA relative abundance of terminally branched saturates (TBSats, mol %) in bio-trap beads samples. A significant increase was demonstrated at day 20 compared with control microcosm traps. For detailed nomenclature, refer to Figs. 3-3 and 3-4.

The major difference in the sediment PLFA profiles was that the microbial communities in the acetate amended microcosms contained significantly higher proportion of monoenoic unsaturated PLFA (specifically 16:1 ω 7c, and 18:1 ω 7c; all at $P < 0.05$), indicative of Gram-negative bacteria (Tunlid et al., 1992; White et al., 1998b), than did those in the untreated microcosm samples (Figure 3-11.). On the contrary, mid-chain branched saturates, branched monounsaturates and terminally branched saturates all showed significant decreases in acetate amended sediments over unamended samples (Figure 3-12). Shifts over the incubation time within the same treatment were not significant (ANOVA, all at $p > 0.05$, except 16:1 ω 7c, and 18:1 ω 7c). The PLFA indicative of eukaryote, specifically 18:2 ω 6—a biomarker for fungi (Harwood et al., 1984) was

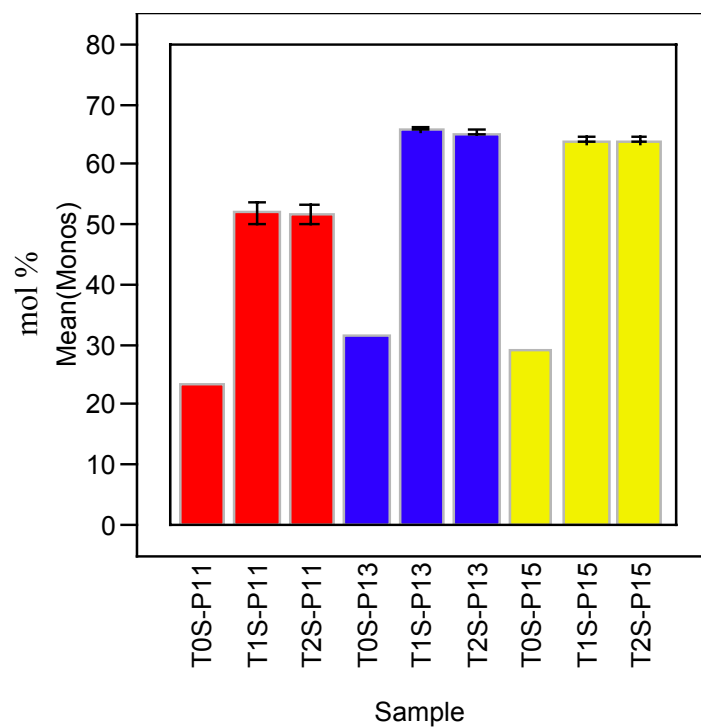


Figure. 3-11. PLFA relative abundance of monounsaturates (Monos, mol %) in microcosm sediment samples. A significant increase of monounsaturates in amended samples over control samples was demonstrated. For detailed nomenclature, refer to Figs. 3-3 and 3-4.

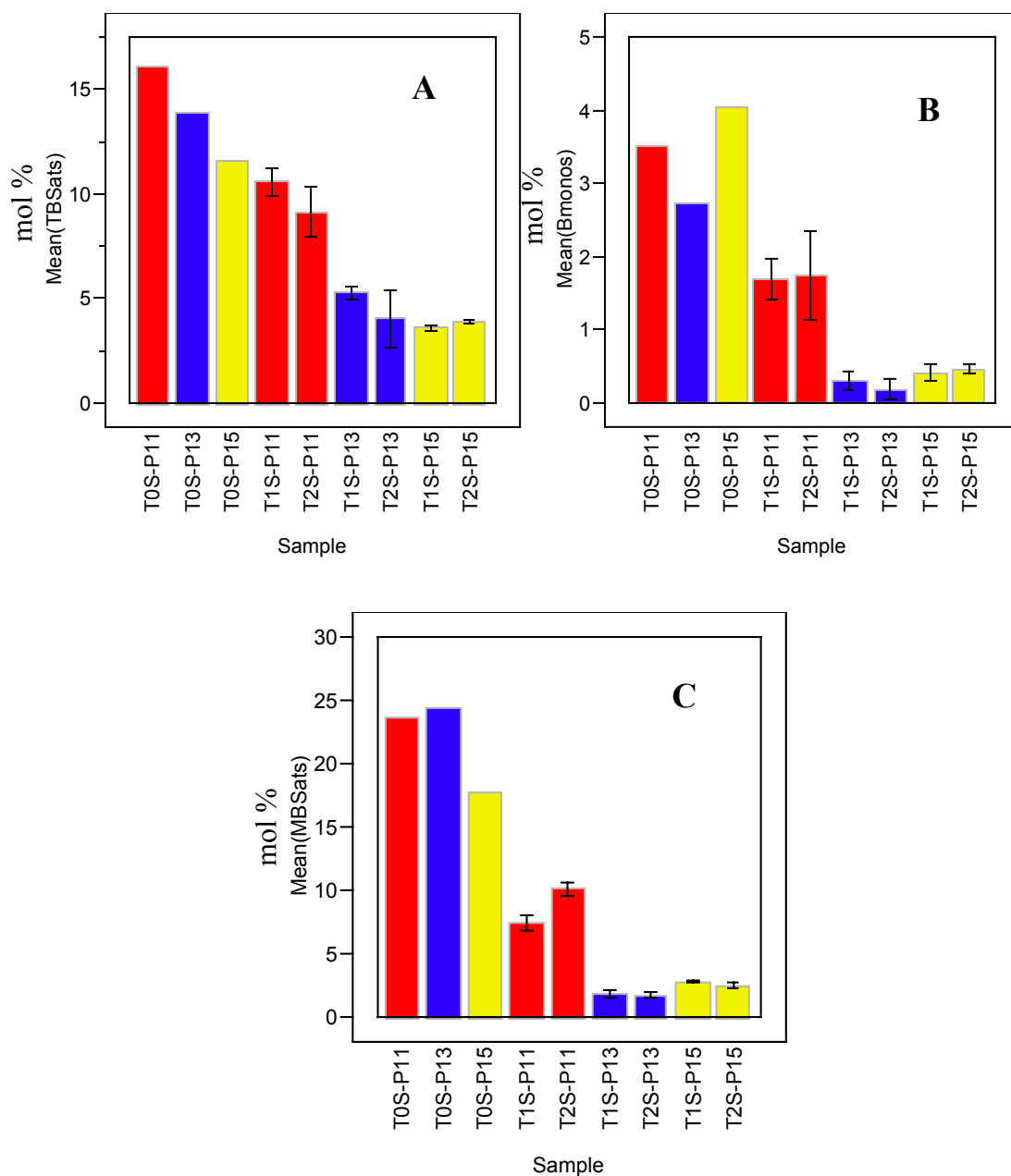


Figure 3-12. PLFA relative abundance (mol %) of terminally branched saturates (TBSats, A), branched monounsaturates (Bmonos, B) and mid-chain branched saturates (MBSats, C) in microcosm sediment samples. After anaerobic microcosm incubation, all above three PLFA groups showed significant decreases in acetate amended samples compared to those unamended. For detailed nomenclature, refer to Figs. 3-3 and 3-4.

detected among amended sediments at both day 5 and day 20, but not detectable in untreated sediments (data not shown).

A hierarchical cluster analysis (HCA) of the PLFA profiles the major trends within the data set (Fig. 3-13). All the sediment samples from time 0 clustered together and the remaining samples clustered according to treatment. The profiles from the bio-trap beads clustered according to time, but were apparently separated from the sediment samples.

Physiological Status: The lipid profiles of microorganisms are a product of the metabolic pathways and consequently reflect the phenotypic response of the organism to its environment and any changes therein (Wilkinson, 1988). Gram-negative bacteria make *trans*-monounsaturated fatty acids as a result of changes in their environment, e.g., exposure to solvent (Heipieper et al., 1992; Pinkart et al., 1996), toxic metals (Frostegard et al., 1996), or starvation (Guckert et al., 1986; Keift et al., 1994). The physiological status of Gram-negative communities can be assessed from the ratios of the *trans/cis* PLFA, with ratios of less than 0.05 shown to be representative of healthy, non-stressed communities (White et al., 1998b). Based on 16:1 ω 7, the calculated *trans/cis* ratios for the sediment samples were significantly higher ($P < 0.05$) than for the biotrap samples (Fig. 3-14.). Irrespective of treatment or incubation time, bio-trap samples showed no significant differences of the *trans/cis* ratio values, while this ratio for acetate amended sediment samples increased significantly at incubation day 20 ($p < 0.05$), indicating less favorable physiological condition in the sediment than that in the traps (Fig. 3-14). Major PLFA shifts, including *trans/cis* ratios are summarized in Table 3-1.

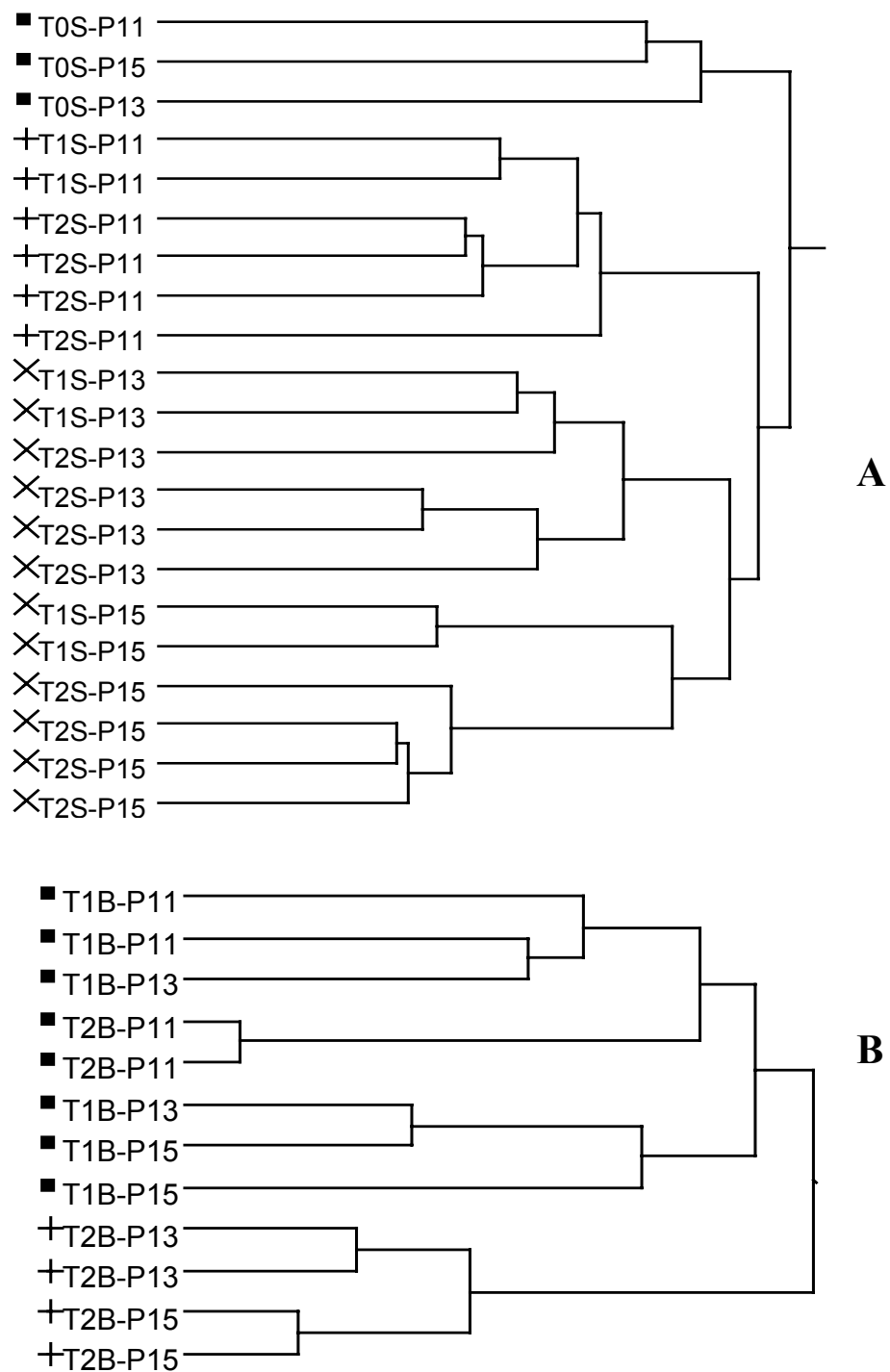


Figure 3-13. Hierarchical clustering analysis of microcosm sediment (A) and bead (B) samples based on PLFA profiles. Clusters are represented by signs of +, ■, and x. Refer to Figs. 3-3 and 3-4 for detailed nomenclature.

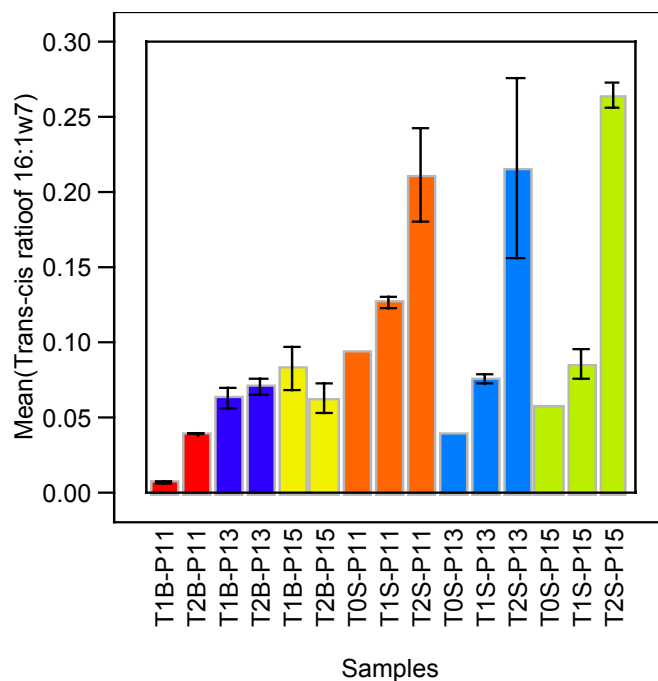


Figure 3-14. *Trans/Cis* ratio calculated based on 16:1w7 fatty acids of all microcosm samples (n = 2~4). Biosep beads samples generally exhibited lower ratios than sediment samples do --- indicating a healthier community. Detailed nomenclature can be found with Figs. 3-3 and 3-4.

Table 3-1. Summary of major shifts indicated by PLFA analysis (n=2~4)

BioSep samples	T1B-P11	T2B-P11	T1B-P13	T2B-P13	T1B-P15	T2B-P15
Biomass (pmol/g)	24.6±2.2	27.6±2.9	21.1±4.7	95.1±13.7	26.2±14.0	153.3±13.1
Terminally branched saturates (mol%)	1.25±0.29	1.03±0.11	1.29±0.25	3.87±0.4	0.18±0.26	1.69±0.09
<i>Trans/Cis</i> Ratio	0.008±0	0.04±0	0.06±0.01	0.07±0.01	0.08±0.02	0.06±0.01

Sediment samples	T0S-P11	T1S-P11	T2S-P11	T0S-P13	T1S-P13	T2S-P13	T0S-P15	T1S-P15	T2S-P15
Biomass (pmol/g)	305	1693±521	1279±336	168	10537±2257	13947±6107	260	7249±425	6929±1196
Mol %									
Monounsaturates	23.5	52.2±2.5	51.8±3.1	31.7	66±0.3	65.4±0.5	29.5	64.2±0.6	64.1±1.2
Terminally branched saturates	16.2	10.6±1	9.2±2.4	13.9	5.3±0.4	4.1±2.8	11.7	3.6±0.2	3.9±0.2
Midchain branched saturates	23.6	7.5±0.9	10.2±1	24.5	1.9±0.5	1.9±0.5	17.8	2.9±0.2	2.6±0.5
Branched mono-unsaturates	3.5	1.7±0.4	1.8±1.2	2.7	0.3±0.2	0.2±0.3	4	0.4±0.1	0.5±0.1
<i>Trans/Cis</i> Ratio	0.09	0.13±0.0	0.21±0.06	0.04	0.08±0.00	0.22±0.12	0.06	0.09±0.01	0.26±0.01

Microbial Incorporation of ^{13}C Acetate Carbon into DNA: The ^{13}C band of DNA resolved from CsCl gradient separation was visualized and extracted from all sediment samples amended with ^{13}C acetate. For bio-trap bead samples, ^{13}C DNA fraction was withdrawn according to standard indication. PCR and DGGE were performed as described after dialysis (Fig. 3-15). Phylogenetic trees were presented in Figures 3-16, 3-17. DGGE analysis revealed almost identical banding pattern between sediment and bio-traps at day 5 (Fig. 3-15A), with *Pseudomonas* in dominance. However the two communities were clearly distinct from one another at day 20 (Fig. 3-15B), sharing in common mostly *Pseudomonace* sequences (Fig. 3-16). *Geobacter* sequences were apparently more enriched within beads than in the sediment. SRB are mostly recovered from P13 microcosms at day 20 only (Fig. 3-17).

To further investigate the involvement of *Pseudomonas* in this biostimulation experiment, a comparison between ^{13}C and ^{12}C communities was performed on selected samples of T1S-P13, which were known to have *Pseudomonas* in predominance. This comparison revealed that majority *Pseudomonas* sequences were actively stimulated and only detected in the ^{13}C fraction (Fig. 3-18), while one *Pseudomonas* type was present in both ^{13}C and ^{12}C fractions, which shares 100% identity with the one recovered from the background microcosms (Fig. 3-17.).

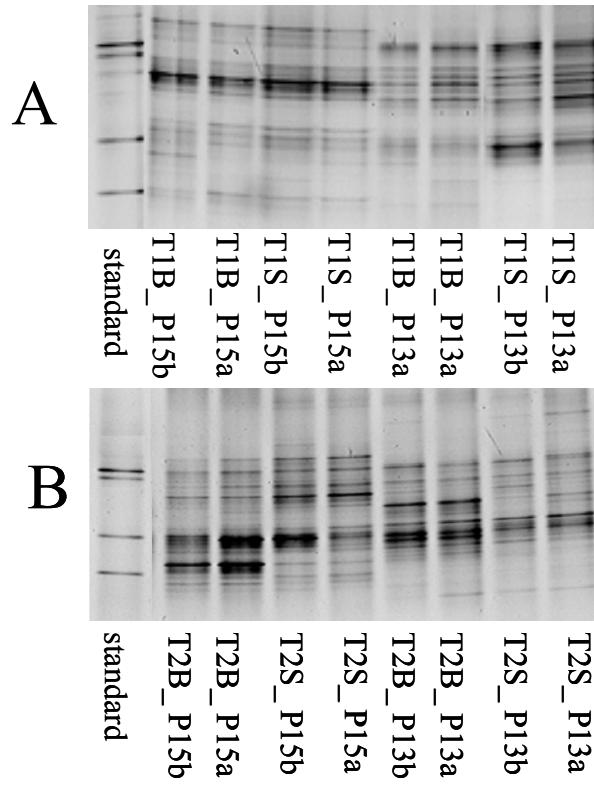


Figure 3-15. DGGE analysis of ^{13}C labeled microcosm bacterial communities recovered from both beads and sediment samples at sampling time day 5 (A) and day 20 (B). Amplified 16S rDNA fragments were separated on a gradient of 20 % to 65 % denaturant. For nomenclature, refer to Figs. 3-5.

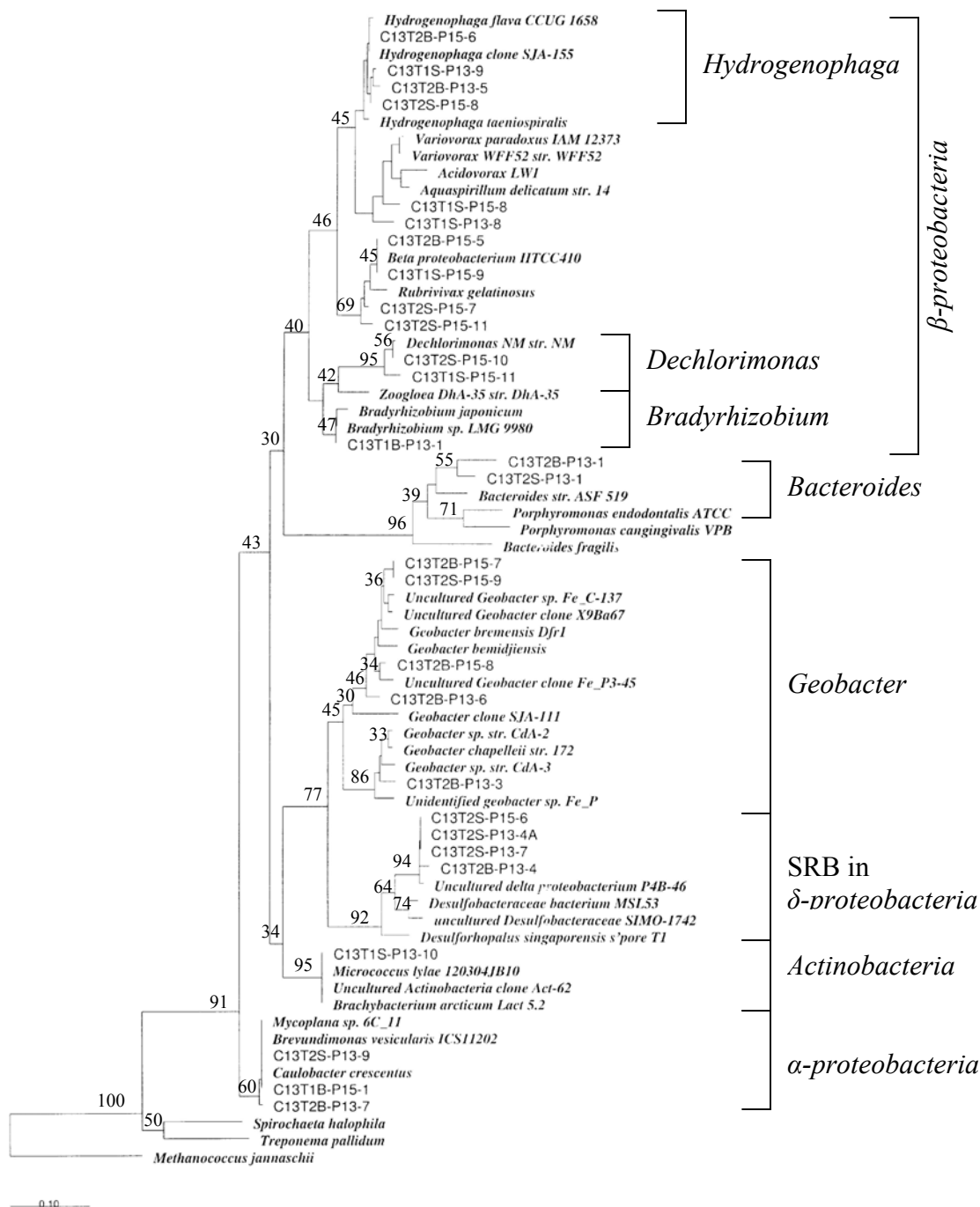


Figure. 3-16. Neighbor-joining analysis of 16S rDNA sequences derived from ^{13}C DNA fractions of the microcosm samples, which shows relationship with reference organisms from β -proteobacteria, δ -proteobacteria, *Bacteroides* and *Actinobacteria* etc. obtained from RDP. Numbers on the tree refer to bootstrap values on 1000 replicates, only values above 30 are given. Scale bar represents 10% estimated change. All sequences recovered from ^{13}C DNA fraction was prefixed with C13. The remaining nomenclature is the same as described in Figs. 3-6 and 3-7.

Nomenclature:

Sequences prefixed “C13TnS, C13TnB, or C12T1S” were generated during this study. Tn represents incubation time point (T0: day zero; T1: day 5; T2: day 20; S indicates sediment samples, B represents bio-trap bead samples). The prefix is followed by the microcosm types (P11, P13, or P15), then band number. All sequences obtained from ^{13}C or ^{12}C labeled fractions were prefixed with C13 or C12.

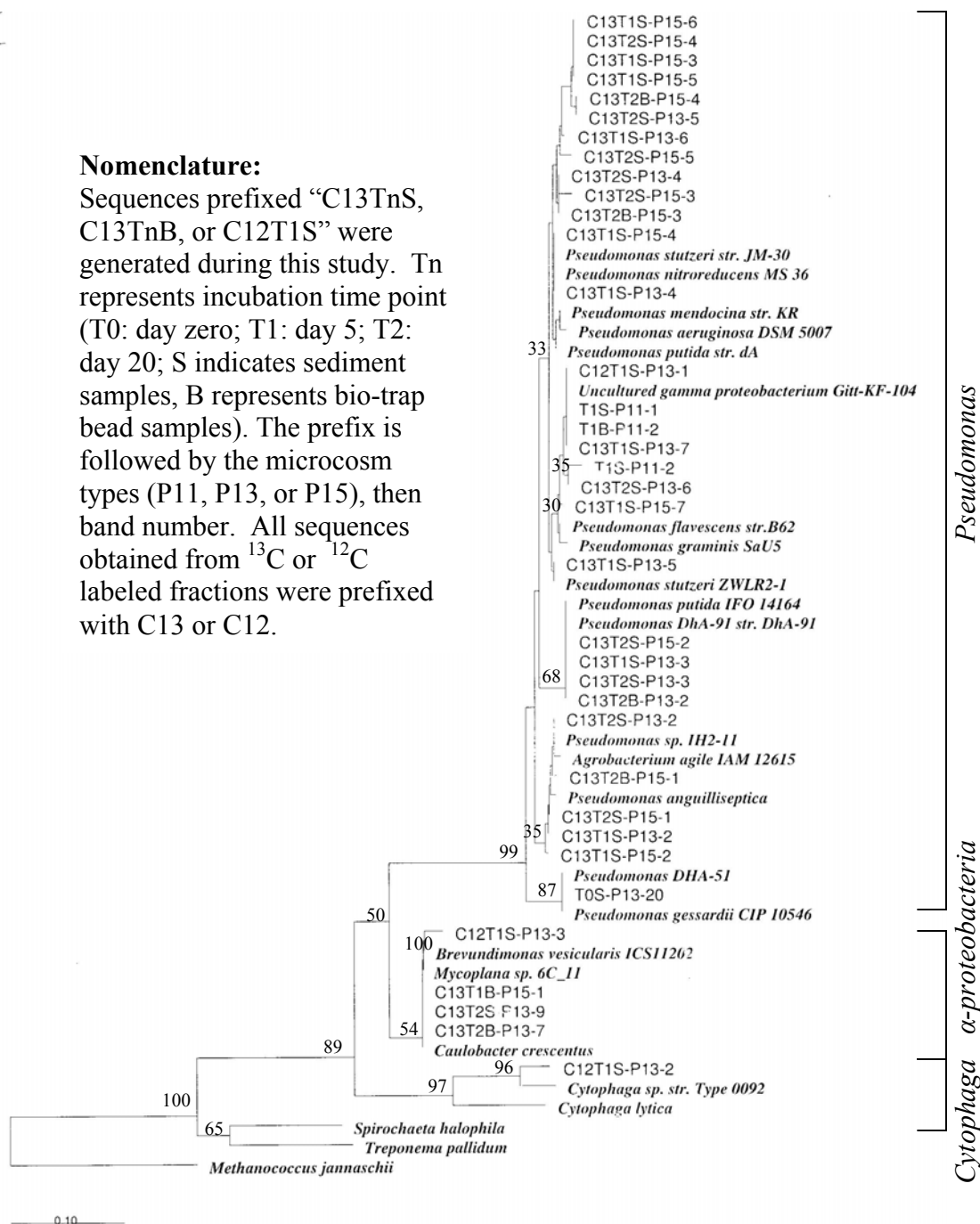


Figure. 3-17. Neighbor-joining analysis of 16S rRNA sequences from excised DGGE bands, derived from this ^{13}C microcosm study. Relationships with reference organisms from *Pseudomonas*, α -proteobacteria and *Cytophaga* obtained from RDP are shown. Numbers on the tree refer to bootstrap values on 1000 replicates, only values above 30 are given. Scale bar represents 10 % estimated change. *Pseudomonas* sequences retrieved from background samples or samples at time zero were included for comparison.

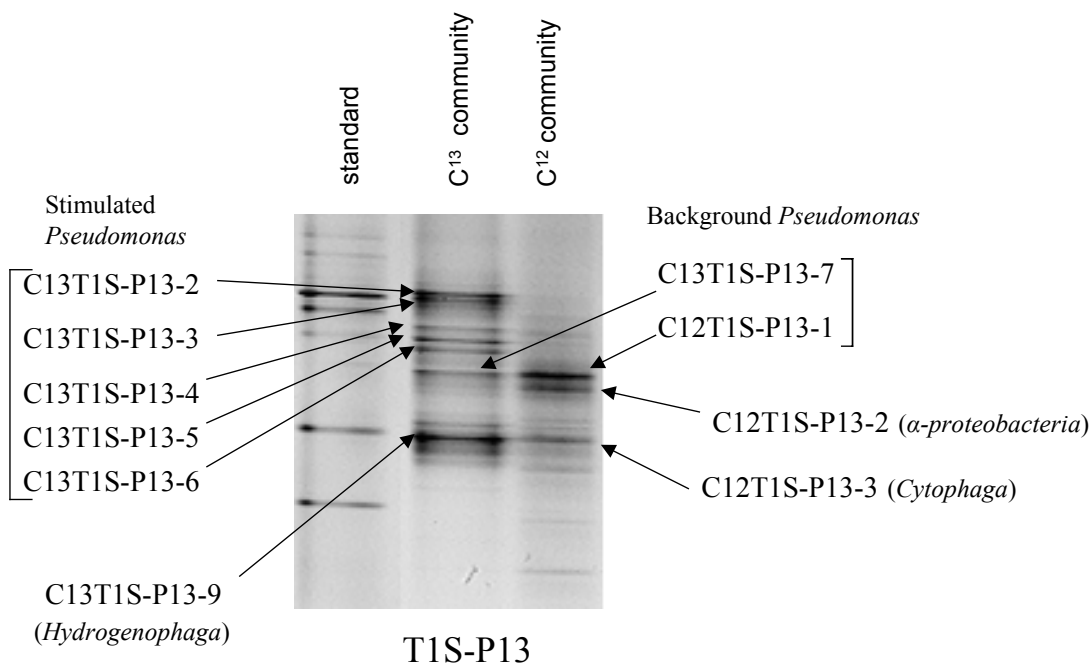


Figure. 3-18. The comparison of DNA profiles between ¹³C fraction and ¹²C fraction, derived from sediment of P13 microcosm at day 5 (T1S-P13). Multiple *Pseudomonas* sequences shown in ¹³C profile were absent from ¹²C profile, indicating that *Pseudomonas* sp. were stimulated and actively utilizing the ¹³C acetate.

Microbial Incorporation of ^{13}C Acetate Carbon into PLFAs: IRMS analysis revealed assimilation status of acetate carbon into microbial membrane of fatty acids. Only those fatty acids between C14 and C19 were used for data analysis. Mean stable carbon isotope compositions ($\delta\text{-}^{13}\text{C}$ per mille relative to that of PDB) of fatty acids in the unlabelled background sediments ranged from $-22.79 \pm 1.830 \text{ ‰}$ for 18:1 ω 7c, and $-29.6542 \pm 0.10731 \text{ ‰}$ for the 19:0 fatty acids. The unlabelled beads samples showed a delta value from $-26.44 \pm 0.78 \text{ ‰}$ for the 14:0 fatty acid to $-36.875 \pm 7.26 \text{ ‰}$ for the 16:0 fatty acid (Appendix A), slightly depleted relative to that of sediment sample carbon. The ^{13}C atom percent in fatty acid of non-labeled samples -- both sediments and beads, remained as $1.193 \pm 0.001 \text{ ‰}$ (n=35), close to natural abundance level throughout the experiment. Substantial ^{13}C enrichment of bacterial PLFA was observed with all ^{13}C acetate amended samples. Due to the high rate of incorporation, saturation with IRMS detectors occurred, which may lead to underestimate of the delta values, however, the trend of relative abundance was clearly demonstrated. Similar ^{13}C incorporation pattern was observed with both sediments and beads, though some differences regarding types of PLFA labeled were noticed. Representative incorporation patterns are shown in Fig. 3-19, derived from microcosm sediments that received acetate amendment. The individual PLFAs in sediments that incorporated the most label were 16:1 ω 7c, 16:1 ω 5c, 16:0, cy17:0 and 18:1 ω 7c. While major PLFAs highly labeled in bead samples include 14:0, 16:1 ω 7c, 16:0, cy17:0, and 18:1 ω 7c. These five FAMES were nearly fully labeled in the beads, which caused difficulty in instrumental interpretation and a reasonable measurement of the delta values were unsuccessful. Other fatty acids that were labeled in the bead samples include

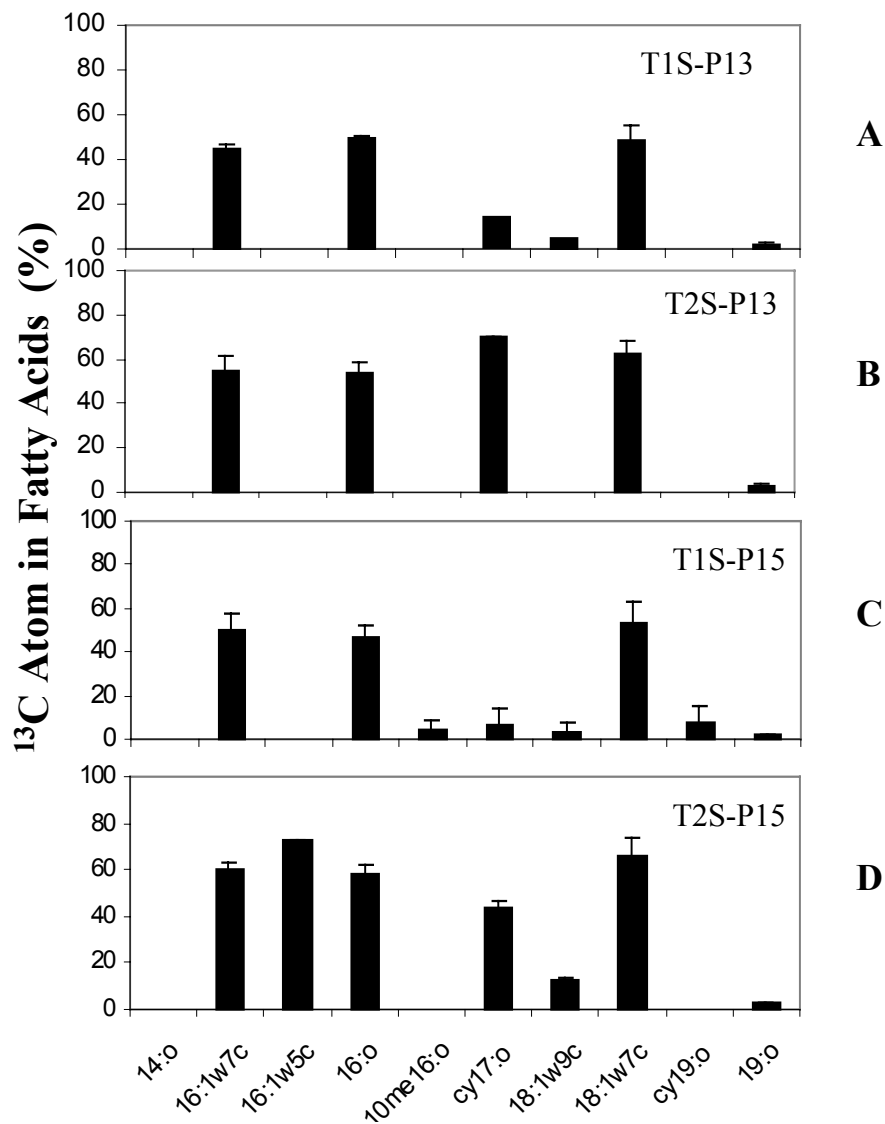


Figure 3-19. Results of the ^{13}C -acetate labeling study in microcosm sediment. The percent of ^{13}C atom in the major enriched PLFAs from P13 (A, B) and P15 microcosm sediment (C, D) at day 5 (A, C) and day 20 (B, D). $n = 2\sim 4$.

Nomenclature: T1 and T2 indicate incubation day 5 and day 20, S represents sediment, P13 and P15 represent microcosms that received acetate amendment.

i15:0, i17:0, 17:0 and 18:0, which clearly indicated an enrichment at day 20 (Appendix A). The incorporation rate was higher at day 20 than at day 5, which applies to both sediment and beads.

Discussion and Conclusion

Microbial Community Analysis: The PCR-DGGE of 16S rRNA coupled with PLFA analysis proved to be valuable in assessing community structure shift under the influence of electron donor amendment. Bacterial biomass determined by PLFA analysis showed significant increase with acetate amendment, which further supports DNA results being ascribed to the effect of biostimulation and bioreduction. The overall trend of the population progressed from communities abundant in sequences belong to facultative anaerobic bacteria of *Pseudomonas* to communities abundant in sequences belong to strict anaerobic bacteria of *Geobacter* and SRB within δ subclass of the *proteobacteria*. The results of PCR-DGGE analysis coupled with sequence analysis were in good agreement with the PLFA analysis as it applied to the bacterial community. DGGE analysis suggested that the bacterial communities of the acetate amended microcosms were dominated by Gram-negative microorganisms of *Pseudomonas*, *Geobacter* and SRB. PLFA of monounsaturates, a biomarker for gram-negative bacterium also indicated a significant increase in the sediments. In the beads samples, the proportion of terminally branched saturates was significantly elevated after 20 day incubation and this may suggest the increase of gram-negative anaerobic SRB in the beads community. DNA results support the lipid evidence by demonstrating an enrichment of *Geobacter* and SRB sequences recovered from the beads at day 20.

The banding patterns revealed by DGGE analysis were almost identical between sediments and bio-traps at day 5 (Fig. 3-15A), with *Pseudomonas* in dominance.

However the two communities were clearly distinct from one another at day 20, sharing in common mostly *Pseudomonace* sequences. These results indicated that bio-trap bead samplers in prolonged incubation may not be capturing the sediment community, rather it was more representative of the active and prevalent populations of both groundwater and sediment.

However, it should be recognized that PCR-DGGE as applied can only detect microorganisms which represent 1- 2 % of the target group (Muyzer et al., 1993; Stephen et al., 1999). Therefore, the method used is incapable of detecting minor community components that may be essential in the bioreduction of uranium.

Stable Isotopic Probing (SIP): To date, DNA or PLFA based SIP has been successfully used to identify active microbes, involved in oxidation of methanol/methane (Hutchens et al., 2004; Radajewski et al., 2000), oxidation of aromatic hydrocarbon compounds (Jeon et al., 2003; Fang et al., 2004; Pombo et al., 2002), oxidation of propionate (Lueders et al., 2004). etc. This thesis presents the first identification of microbes that are actively involved in anaerobic reduction of heavy metal using SIP, and the first combination of SIP-DNA and SIP-PLFA.

Murrell et al. (University of Warwick, U.K., personal communication) and Tillmann et al (2004) all reported that ~15 ug is the minimum quantity of DNA necessary to see a clear band using EtBr in CsCl gradients, an amount, which may be difficult to obtain from natural environments such as soil or water. In the current research, efforts were made to

modify the technique to accommodate to the smaller volume ultracentrifuge tube with a tilted rotor. As a result, the detection sensitivity of the procedure was increased about 30 fold and with a quantity of 500 ng, it allowed visible detection of “heavy” DNA band from its “light” fraction. This improvement facilitated the field application in the following work and paved the way for low biomass environmental sample processing. A low background of unspecific nucleic acids was previously reported in all DNA or even rRNA gradient fractions (Tillmann et al., 2004), false labeling caused by cross-feeding is also possible, which is important for the interpretation of environmental SIP results. Consequently, PLFA analysis of secondary macromolecular biomarker provides a complementary verification and hence higher precision for retrieval of isotopically enriched biomarkers than using DNA gradient fractionation alone. This is an important consideration for tracing microbial populations metabolizing ^{13}C -labeled compounds in natural ecosystems. However, IRMS is designed for detecting natural abundance of stable isotope, which is usually low (1%), while DNA requires sufficient labeling (25%) for a full separation of ‘light’ and ‘heavy’ nucleic acids (Radajewski et al., 2000, 2003; Manefield et al., 2002). In order to gain full advantages of SIP with both DNA and PLFA, care should be taken to optimize the concentration of labeling substrates or the incubation time, Future research may adopt low concentration of labeling substrate with prolonged incubation or high concentration of labeling substance but short period of incubation. Combined SIP-DNA and SIP-PLFA holds promising future and proved to be a new powerful tool in microbial ecology.

Active Microbial Populations: The stable isotopic analysis of ^{13}C labeled rDNA demonstrated that *Pseudomonas*, *Geobacter*, SRB within δ -*proteobacteria* were the most abundant and active populations stimulated by acetate amendment. At day 5, *Pseudomonas* was predominant, accounting for ~84% of the sequences retrieved, which continued to be the major component of the community. *Geobacter* and SRB rise to be detected at day 20 and became the prominent member of the community.

δ - ^{13}C PLFA analysis provided additional information supporting the finding based on ^{13}C DNA analysis. The PLFA profile of *Pseudomonas sp.* was comprised of ~10 different fatty acids, where 16:0, 16:1 ω 7c, cy17:0, 18:1 ω 9c and 18:1 ω 7c represented more than 85% of the total PLFA (Mauclaire et al., 2003, Fang et al., 2004), accordingly, these fatty acids were the major fatty acids shown to be significantly labeled in this study and ^{13}C atom percent was as high as 72.5% (18:1 ω 7c of T2S-P15, Fig. 3-19). The fact that this enrichment pattern remained similar with prolonged incubation, minimized possible cross-feeding of label into community members not involved in acetate biostimulation. Several 17-carbon compounds have been proposed as specific biomarkers for sulfate – reducing bacteria in the large Gram-negative group (White 2002). The fatty acids of i15:0, coupled with 16:0 and 16:1 ω 7c were suggestive of *Geobacteraceae* (Anderson et al., 2003). The GC-IRMS results demonstrated the moderate to high enrichment of the above fatty acids in beads samples at day 20: 16:0 and 16:1 ω 7c were about 100% labeled, i15:0, i17:0 and 17:0 were moderately labeled seen only within T2B-P13 beads samples (Appendix A). In agree with the δ - ^{13}C PLFA results, ^{13}C DNA also indicated the rise of *Geobacter* and SRB at day 20, and *Geobacter* sequences were apparently more enriched

within beads than in the sediment, plus SRB are mostly recovered from P13 microcosms at day 20.

The enrichment of iron reducers (*Geobacter*) and sulfate reducers is not surprising, as sulfate reduction in the microcosm was well under way, indicated by black precipitation and iron reduction is theoretically preceding sulfate reduction because of thermodynamics. These organisms are the major bacterial populations known to be capable of reducing uranium. *Geobacter* and SRB did not reach detection levels until incubation day 20, that is probably because they have a slow rate of growth and substrate turnover due to low energy yield compared to *Pseudomonas*, or they represented only a numerically small fraction of the microbial community at day 5. In all these cases it is likely that only minimum amount of labeled nucleic acids of *Geobacter* or SRB was formed.

The Predominance of *Pseudomonas*: All amended microcosms exhibited a marked rise in the proportion of *Pseudomonas* sp. starting at the first sampling event of day 5. The prominent bands representing *Pseudomonas* sp., that detected in amended microcosm samples differ in sequences from the *Pseudomonas* band recovered in background untreated samples. Therefore it is likely the appearance of the source microorganism(s) of these bands was directly related to the addition of acetate and the process it stimulated, which may have been limiting in the background microcosms, thus precluding their development. This provides evidence that acetate addition brought about a change in the microbial ecology in the amended microcosms, which consequentially caused the significantly higher reducing activities in these microcosms. The rise of γ -subgroup

proteobacterial Pseudomonas species as a major component of the bacterial community precedes the rise of *Geobacters* and SRB, indicating more competitive growth ability of *Pseudomonas* under the condition. Also, the microcosms were not completely anoxic initially, which favored the facultative anaerobic respiration by *Pseudomonas*, while *Geobacter* and SRB are traditionally more strictly anaerobic.

Pseudomonas is also known to be capable of reducing nitrate and nitrate reduction is thermodynamically more favorable than iron, uranium or sulfate reduction. However, without information regarding chemical shifts, particularly, nitrate concentration, it is difficult to speculate whether *Pseudomonas* was there to reduce nitrate or actually involved in uranium reduction. Apparently more investigation is necessary to elucidate the role of *Pseudomonas* sp. in biostimulated uranium bioreduction.

Implications in Bioremediation: In this investigation, SIP technique enabled an evaluation of the taxonomic and metabolic diversity of key groups of microbes actively involved in biostimulation. The microbial monitoring in microcosms can elucidate the bacterial populations responsible for uranium reduction and may indicate that SIP using ¹³C acetate added to microbial traps can provide important data on ecosystem function in the field.

The current strategy of acetate addition has been proved to be successful in stimulating uranium bioreduction both in laboratory and *in situ* (this study and Anderson et al., 2003). However, this approach suffers from specific limitations that potentially confer selectivity via differential treatment of indigenous organisms, therefore overlook other potential for uranium bioremediation. Given that uranium reducers occur in

phylogenetically diverse organisms, such as *Hyperthermophilic archaeon* (Kashefi et al., 2000), *Thermophilic bacterium* (Kieft et al., 1999), mesophilic Fe (III)- and sulfate reducing bacteria (Coates et al., 1998, 2001; Pietzsch et al., 1999; Lovely et al. 1991, 1992a, 1992b, 1993b), and fermentative bacteria *Clostridium* (Francis et al., 1994). Experiments performed under a variety of incubation conditions will explore whether the sediment or groundwater had even greater diversity of uranium reducing populations than those we have identified under our experimental conditions. The current study has provided an effective technique and experimental model to identify particular microbial populations involved in the process desired. Furthermore, laboratory incubations do not necessarily accurately reflect field conditions, and field trials must be conducted to verify findings of laboratory experiments (Swannell et al., 1996; Holmes et al., 2002), which will be the focus of next chapter.

Chapter 4. Temporal and Spatial Distribution of Microbial Communities in Relation to Stimulated *In Situ* Bioremediation of Uranium

Introduction

Although toxic metal and radionuclide contaminants cannot be destroyed, their toxicity and mobility can be dramatically altered by microbial activity (Lloyd and Lovley, 2001). The ability to transform toxic metal --- uranium, occurs in phylogenetically diverse organisms (Coates 1998, 2001; Francis et al., 1994; Kashefi et al., 2000; Kieft et al., 1999; Lovley et al., 1991, 1992b, 1993a; Pietzsch et al., 1999) and bioremediation has proven to be effective method for immobilizing uranium as contaminant in subsurface environment (Anderson et al., 2003; Lovley, 1995).

A key prerequisite for optimizing *in situ* uranium bioremediation strategy includes an ability to predict the activity and *in situ* distribution of microbial populations associated with uranium reduction/immobilization (Lehman, et al. 2001a; 2001b, 2002; Holm, et al. 1992; Lloyd and Lovley, 2001). In addition to toxic metals such as uranium, many contaminated sites contain both iron-containing minerals and co-contaminants such as nitrate (NO_3^-), sulfate, or other heavy metals such as Tc, etc. (NABIR, 2003; Riley et al., 1992). Successful implementation of metal and radionuclide bioremediation in such environments requires an understanding of the complex microbial and geochemical interactions that influence the redox speciation and mobility of toxic metals (Shelobolina et al., 2004; Elias et al., 2003; Maynard, 1983). Studies regarding bacterial population distribution, in relation to geochemical processes are urgently needed. However, due to technology constrains and other limitations, e.g. high cost, dynamic temporal and spatial

studies are difficult to conduct. Most uranium bioremediation studies have focused on laboratory incubation, community composition at contaminated sites, or at most spatial study of the bacteria undergoing uranium bioremediation (Vrionis et al., 2005). At Rifle, Colorado, uranium contamination in a shallow aquifer provides opportunity for field-scale electron donor amendment experiments. The interdisciplinary efforts brought up by DOE with many Universities, research institutes have made it possible to study temporal and spatial shift of the microbial populations over a relatively long term *in situ* field bioremediation experiment.

Acetate (CH_3COO^-) is a major intermediate in the microbial degradation of organic matter, particularly in anaerobic environments where it occupies a central role in the anaerobic microbial food chain. The thermodynamic favorability of redox reactions associated with acetate oxidation has a major influence on the zonation of electron-accepting processes in redox-stratified environments such as aquatic sediments and groundwater flow paths. In order to investigate the response and distribution of microbial communities during uranium bioremediation, acetate was added to the subsurface of the uranium-contaminated aquifer at concentrations greater than the ambient sulfate concentration ($>10\text{mM}$) to stimulate microbial activity and subsequent uranium reduction (refer to chapter 2). Temporal and spatial analysis of aquifer geochemistry over the course of acetate amendment was performed by groundwater sample collection from a series of down-gradient multi-level sampling (MLS) wells, also some pumped groundwater samples. Culture-independent molecular analysis of bio-trap bead samplers was performed before, during and after acetate injection. DNA profiles the community shift at one depth level horizontally over time, while PLFA analysis was performed not

only on samples along groundwater flow path horizontally, but also vertically at two different depths. Molecular techniques for analyzing the bacterial community structure might reveal the relative degrees of importance of phenotypic and taxonomic changes in response to electron donor addition. These techniques also allow monitoring of changes in structure and function of bacterial assemblages during acetate biostimulation. The results documented development of an anaerobic plume, a succession of microbial terminal electron acceptor processes and loss of U (VI) downgradient from the acetate injection gallery. Furthermore, distinct differences in microbial distribution between background and down-gradient monitoring wells were observed particularly for iron reducers and sulfate reducers (SRB), which showed unique patterns of enrichment during the biostimulation and bioreduction. The issue of uranium reoxidation and stability is discussed based on results observed.

Materials and Methods

Site Description and Test Plot Design: The test plot sits within a uranium-contaminated aquifer, which is located at the Old Rifle site, a former uranium ore processing facility in Rifle, Co. The site is now a part of the Uranium Mill Tailings Remedial Action (UMTRA) program of the U.S. Department of Energy (DOE, 1999) (Fig. 2-1). Uranium contamination in groundwater within the plot ranged from 0.88 μM -1.82 μM , exceeding the UMTRA maximum contamination limit of 0.18 μM . Prior to initiation of this study, the site had been under natural flushing without amendments for 8 months. Groundwater analysis over this time showed no significant increase in dissolved oxygen (DO) subsequent to cessation of previous experiment at the site. Trace levels of nitrate (50-300

uM) were observed in some of the monitoring wells, sulfate concentrations ranged from 7.6-9.5 mM.

An injection gallery positioned perpendicular to groundwater flow was installed to inject acetate into the subsurface (Fig. 4-1, and 4-2). To evaluate stimulated changes, down-gradient monitoring wells were installed at distances of 3.7m (M03), 7.3m (M08) and 14.6m (M13) from the injection gallery, roughly corresponding to groundwater travel times of approximately 4, 9, and 18 days. The background monitoring wells placed 3.7m (B02) upstream of the injection gallery to serve as the control well. All monitoring and control wells were installed to the same depth and were screened over the same intervals as the injection wells. Groundwater containing U (VI) flows past the control wells and injection gallery from upgradient sources and out into the monitoring-well field (Fig. 4-1). Native groundwater amended with sodium acetate (and potassium bromide as tracer) was prepared and injected into the subsurface at an appropriate concentration and rate to produce ~10 mM sodium acetate (and ~300 uM potassium bromide) within the aquifer through flow dilution.(Anderson et al. 2003) Groundwater flow at the site was ~ 0.85 meter/day.

Detailed site descriptions, the geochemical properties and test plot design for this *in situ* biostimulation experiment have previously been described (DOE, 1999; Anderson et al. 2003).

Sample Acquisitions: Acetate injection into the aquifer began on 6/27/2003, and was continuous over a 3.5 month period till mid-October. Groundwater samples were

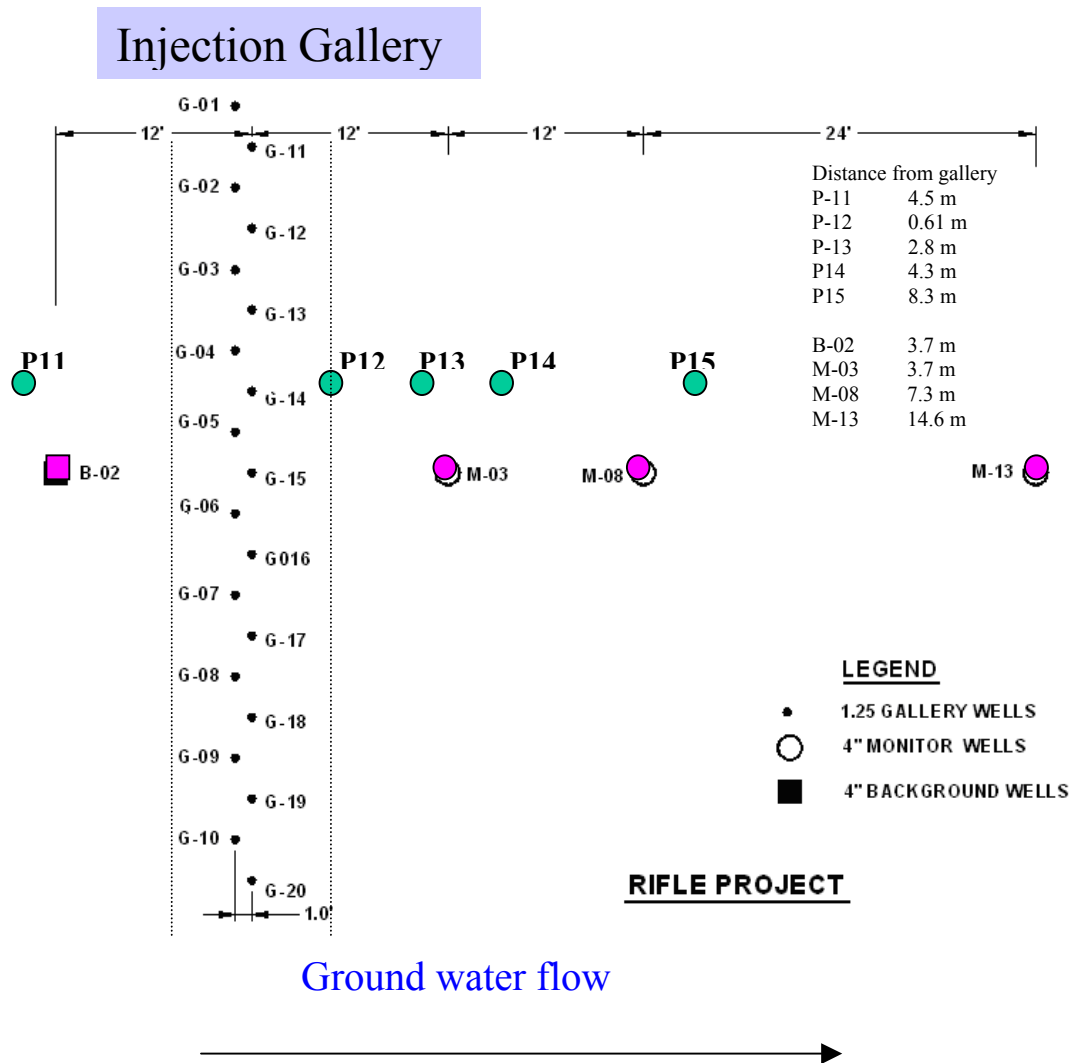


Figure 4-1. The layout of the *in situ* biostimulation test at Old Rifle UMTRA site, showing a schematic diagram of the sampling location and designation of each upgradient background and downgradient monitoring cores and wells.



Figure 4-2. Photograph of injection gallery after installation (From NABIR Biostimulation Field Experiment Test Plan for the Old Rifle UMTRA Site, 2003 Rifle, Colorado, Rev. 1.0 June 2003, by Pacific Northwest National Laboratory Richland, WA; Microbiology Dept. Umass, Amherst, MA; S. M. Stoller Corporation Grand Junction, CO. U.S. Department of Energy, Office of Science---Natural and Accelerated Bioremediation Research Program, In collaboration with the U. S. Department of Energy UMTRA Ground Water Project).

systematically collected at regular intervals from all background (B02) and monitoring wells (M03, M08 and M13) prior to, during and after the injection of acetate.

Groundwater samples: The multi-level samplers (MLS) were set with flow-through sample cartridges at 0.3 m intervals of depth to the confining layer. The MLS flow-through cartridges containing permeable membranes at either end enabling exchange with surrounding groundwater. Sampled cartridges were recovered from the subsurface at depths of ~5 m, and ~6 m and the equilibrated groundwater was filtered (0.2 μm) and placed into 15 mL conical tubes for U (VI) and anion analyses at regular time intervals during acetate injection. Filtered water samples were also collected for Fe (II), bromide, acetate and other parameters. Samples for acetate analysis were collected into scintillation vials and preserved with 1 mL of 0.1N H_2SO_4 . The water samples were shipped to the laboratory via overnight courier and stored at 4°C prior to analysis. After sampling, all cartridges were washed at least twice and then completely filled with distilled water and placed back into their sampling positions on the MLS well insert. The MLS insert was then slowly positioned back into the monitoring well.

The pH, dissolved oxygen (DO), sulfide, ferrous iron (II), and the redox potential were determined from groundwater pumped from 5.43 ± 0.02 m (17.83 ± 0.08 ft) below top of casing using a peristaltic pump (Cole-Palmer, Vernon Hills, IL). All wells were purged ~12 liters until groundwater parameters stabilized. Sulfate, nitrate, acetate and uranium (VI) concentrations were determined from MLS sampling cartridges at respective depths.

Bio-trap samplers: The bio-traps constructed from powdered activated carbon Bio-Sep[®] beads and baited with ferrihydrite were equipped with multi-level samplers (MLS) and deployed down well at depths of 5.88 ± 0.03 m ($17.65 \text{ ft} \pm 0.09 \text{ ft}$) and 5.04 ± 0.03 m

(15.12 ± 0.09 ft) respectively (for simplicity, they are designated as depth 6 m and 5 m (Fig. 4-3). The ferrihydrite traps were the normal standard bio-traps with total of 10-20 mg ferrihydrite added to the bottom and middle of the traps respectively. They were shown to yield higher biomass than standard bio-traps did, but no significant impact on community structure (for details, see chapter 5). Therefore the ferrihydrite traps were used for this temporal and spatial study. The traps were incubated for a period of around 30 days down in the wells as described by Peacock et al. (2004). Sampling was performed from wells B02, M03, M08 and M13 (Fig. 4-1) on 06/30/03, 07/25/03, 08/08/03, 09/05/03, 10/01/03, 01/16/04, 05/11/04, 07/24/04, approximately 3 days, 28 days, 38 days, 70 days, 94 days, 200 days, 315 days, 388 days after acetate injection initiated. Once recovered the bio-trap samplers were frozen onsite with dry ice, shipped to the CBA laboratory, and stored at -80°C prior to lipid and DNA analysis. The ^{13}C infused traps were deployed on 08/08/03 into the wells B02, M03, M08 and M13 (Fig. 4-1), and they were suspended at depth of 5.04 m and recovered on 09/05/03, nearly 70 days since acetate injection initiated. Those ^{13}C baited traps were analyzed to examine active microbial populations that involved in the stimulated process in subsurface and the results are presented and discussed in Chapter 5.

Sediment samples: The sediment cores were collected at varying distances from the injection gallery after 40 days of acetate injection. A background core of P11 was 4.5 m upstream of the injection gallery. Down-gradient cores of P12, P13, P14 and P15, were 0.61 m, 2.8 m, 4.3 m and 8.3 m away from the injection gallery respectively (Figure 4-1). Coring was done using a rotosonic drilling rig (Boart Longyear, Environmental Drilling Division, Little Falls, MN, USA) to a depth of 5.8 - 6.4 m below land surface.

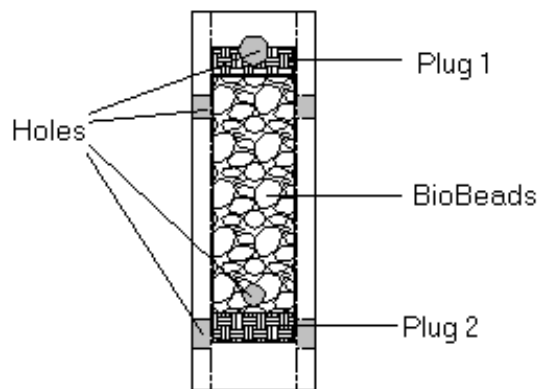


Figure 4-3. Diagram of a Biosep beads sampler (left, also called bio-trap) and multi level samplers (MLS) with bio-traps. Plugs are made of glass wool and the sampler is loaded with Biosep beads.

Core samples were sectioned and logged by depth, then placed on dry ice to be shipped to the laboratory overnight. Sediment samples were also stored at -80°C prior to lipid and DNA analysis. The results will be presented and discussed in Chapter 6.

Geochemical Analysis: Acetate was measured on a Hewlett Packard series 1100 high-pressure liquid chromatograph (Agilent technologies, Albany, NY) using a fast-acid analysis column (Bio-Rad, Hercules, CA) with 0.5 M H_2SO_4 eluent and absorbance detection (210 nm). The bromide concentrations were measured with a Dionex DX-100 ion chromatograph. Sulfate, sulfide and nitrate were determined as components of a suite of anions analyzed using ion chromatography (Dionex Model DX-300; AS-4a column, chemical suppression, and conductivity detection) according to McKinley *et al.* (1997). Samples were quantified against commercial standards that ranged from 0.1 to 100 mg/L. Uranium U (VI) concentrations were determined using a kinetic phosphorescence analyzer (Model KPA-11, Chemchek Instruments, Inc.) according to McKinley, *et al.* (1995). The detection limit for U was 0.3 $\mu\text{g/L}$. Quantitation was against NIST-traceable standards over the standard concentration range of 0.25 $\mu\text{g U L}^{-1}$ to 50 $\mu\text{g U L}^{-1}$ in eleven steps. Samples were treated only by the addition of a phosphorescent complexant, and were run in batch using an autosampler. When necessary, samples were diluted and re-run so that raw results fell within the standard concentration range and yielded acceptable counting statistics. A full suite of standards was run at the beginning and end of each analytical sample set as an internal check on accuracy and precision. Dissolved oxygen (DO) was measured using a flow cell during well purging. Stable (invariant) DO values typically occurred prior to completion of well purging; the minimum observed

concentration was taken as the *in situ* value. The pH was measured by electrode against commercial standards. Fe (II) concentration was determined by previously described spectrophotometric methods (Lovley et al., 1987a).

DNA and PLFA Analysis: Nucleic acid was extracted directly from the bio-trap beads using FastDNA Spin Kit (for soil, BIO101) following manufacture's instruction. Details can be found in Chapter 3. PLFA analysis was performed as described in Chapter 3 also.

Respiratory Quinone Analysis: The neutral lipid fraction of the Bligh and Dyer (1954) extract (modified by White et al., 1979a, 1979b) after fractionation on silicic acid columns was examined for respiratory ubiquinone and menaquinone isoprenologues by high performance liquid chromatography/atmospheric pressure photoionization tandem mass spectrometry (HPLC/APPI/MS/MS) (Lytle et al., 2001a, 2001b; Geyer et al., 2004).

Nucleotide Sequence Accession Numbers: Partial 16S rDNA sequences recovered from this study were submitted to GenBank as with accession numbers AY995015 to AY995113.

Statistics: Phospholipid fatty acids were analyzed both as picomole per bead and as mole percents. The linear regression and correlation analysis between geochemical variables and PLFA biomarkers were performed using the statistical package JMP Version 5.1.2 for Windows (JMP, SAS Institute. Inc., Gary, NC). The hierarchical cluster analysis (ward method) based on Euclidean distance was also conducted with JMP Version 5.1.2.

Results

Chemical Profiling of the Groundwater: The injected acetate provided ample carbon source that was metabolized by microorganisms. This metabolism was coupled to the reduction of terminal electron acceptors available in the environment (O_2 , Fe (III), U (VI), SO_4^{2-} , etc.), resulting in the accumulation of reduced species (Fe (II), H_2S , sulfide minerals, and U (IV)). To evaluate the functional geochemical processes, the changes of those electron acceptors were closely monitored. Measurements of ferrous iron, sulfide, pH, dissolved oxygen and redox potential were made from pumped groundwater samples after purge, which were also representative of biotrap results spanning the appropriate time interval. Measurements of bromide, acetate, sulfate, uranium and nitrate concentrations were performed with MLS sampler cartridges, permitting a finer spatial assessment of the geochemical functions at different depth.

Consistent with bromide measurements (Figure 4-4A, 4-4B), acetate reached all down gradient monitoring wells shortly after injection (Figure.4-4C, 4-4D), with M-08 exhibiting highest amount. Similar acetate plot was obtained at both depths, but the concentration detected at a shallower depth of ~5 m (actually 5.25m) was much lower, approximately 40% of what was detected at a deeper depth of ~ 6 m (6.1m actually) (Figure. 4-4D). Both bromide and acetate showed two phases of concentration increase, one at later July, the other was around later September.

Before the initiation of acetate injection, groundwater from background and down gradient monitoring wells all displayed average redox potentials of 128 ± 33 mV (Appendix B). Within 12 days of the injection, redox potentials in all down gradient

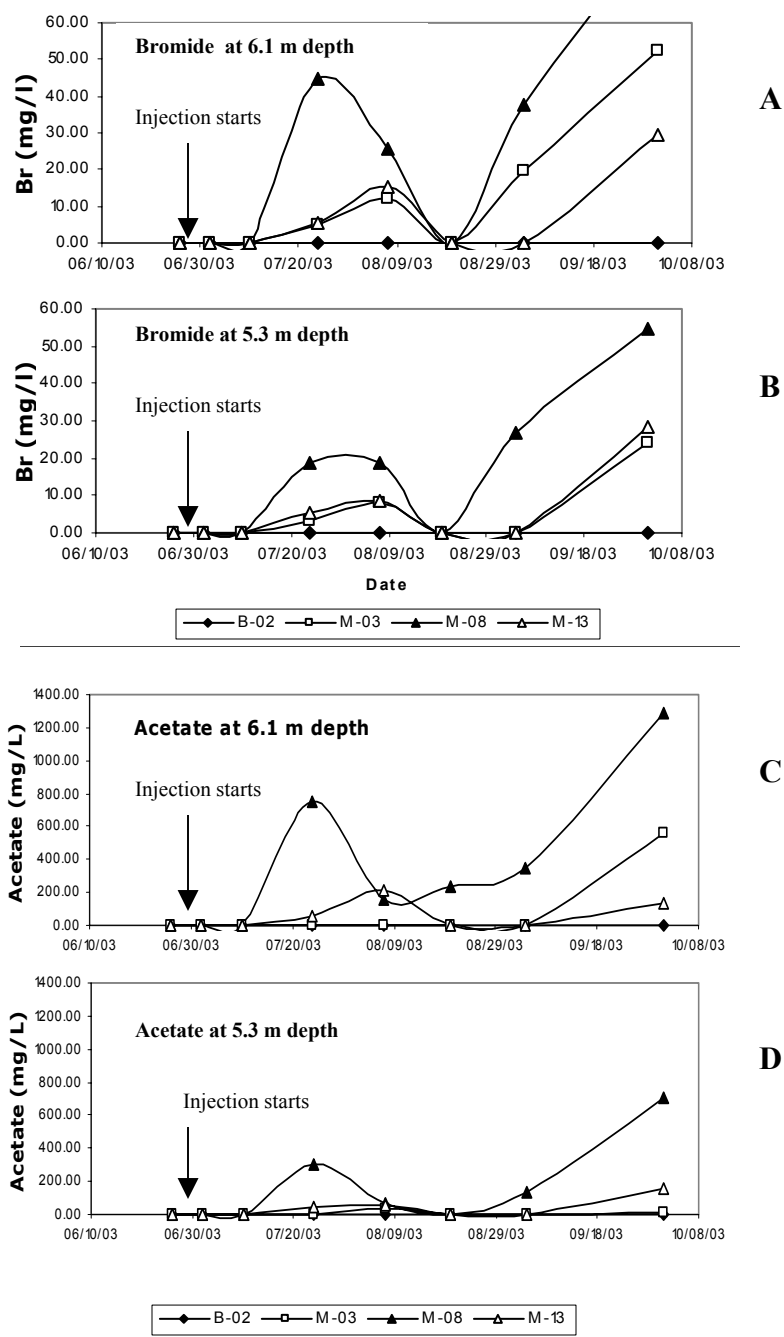


Figure 4-4. Changes in the concentrations of bromide (A, B) and acetate (C, D) in Rifle groundwater, at two different depths during acetate biostimulation experiment. All data points are the means of triplicate measurements. Arrow points to when acetate injection started.

monitoring wells showed a noticeable decrease, particularly with wells M-03 and M-08, in which the redox potential dropped nearly half shortly after 30 days. By day 40, the redox potential of all down gradient monitoring wells were reduced to an intensity of less than 0 mV, which lasted for a duration of more than 3 months (Figure 4-5A). The rate of decrease is apparently more pronounced in wells M-03, M-08 than it is in well M-13. Beginning early November, the redox potential displayed an increasing trend and was brought back close to zero in all three down gradient monitoring wells after December. The background well B-02 did not show any significant redox potential change other than normal fluctuation (Fig. 4-5A), maintaining a more oxidized condition than wells received acetate treatment throughout the experiment. The pH values in all down gradient monitoring wells were moderately elevated compared with background wells (Fig. 4-5C), which is consistent with the alkalinity result of acetate oxidation that generates HCO_3^- . Dissolved oxygen remained approximately $0.13 \text{ mg/L} \pm 0.03$ (n=12) after an initial drop and background had slightly higher DO ($0.16 \text{ mg/L} \pm 0.02$ (n=4)) (Fig. 4-5B). A sudden sharp increase in DO concentration measured with well M13 was distinct from all other DO measurements --- perhaps, it is a result of high heterogeneity of the subsurface environment or event such as rain storm.

In all down-gradient monitoring wells, iron reducing and sulfate reducing activities were evidenced by increases of ferrous iron and sulfide and a decrease of sulfate, beginning roughly 12 days after the injection and the reductions were clearly demonstrated afterwards (Fig.4-6, 4-7). Ferrous iron concentration in well M-03 nearly doubled 27 days after the injection, but gradually decreased to its original values, or even lower. This pattern of rising followed by a decline was observed in wells of M-08 and M-13 also

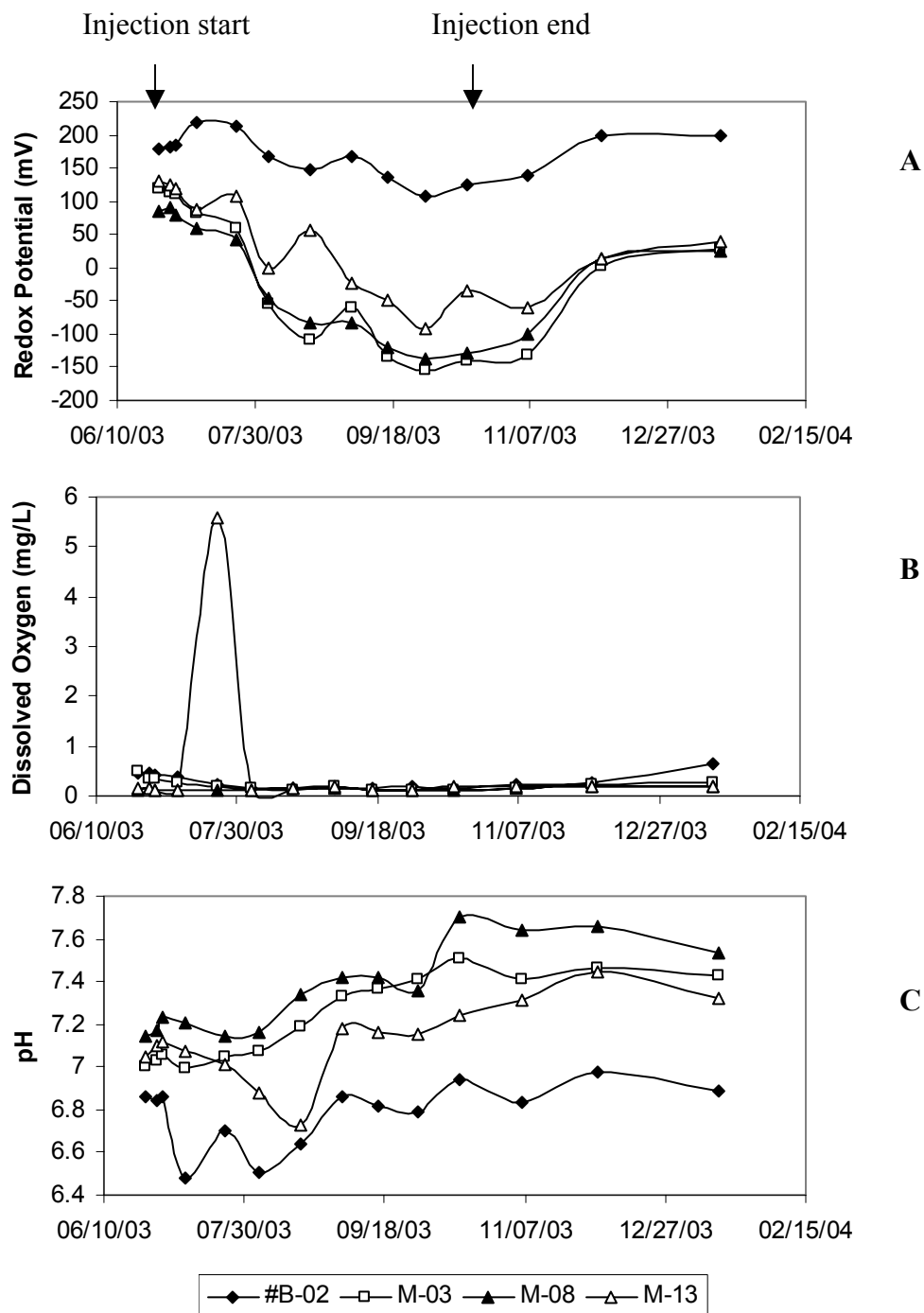


Figure 4-5. Changes in redox potential (A), concentration of dissolved oxygen (B) and the pH (C) in Rifle groundwater during acetate biostimulation experiment. All data points are the means of triplicate measurements. Arrows indicate the start point and the end point of acetate injection.

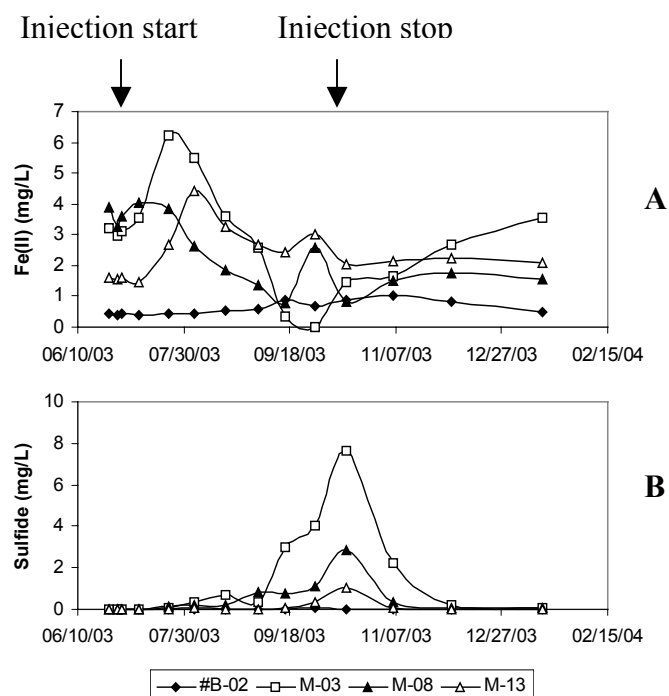


Figure 4-6. Iron and sulfate reduction as measured by Fe (II) production (A) and sulfide production (B). Arrows indicate the start point and the end point of acetate injection.

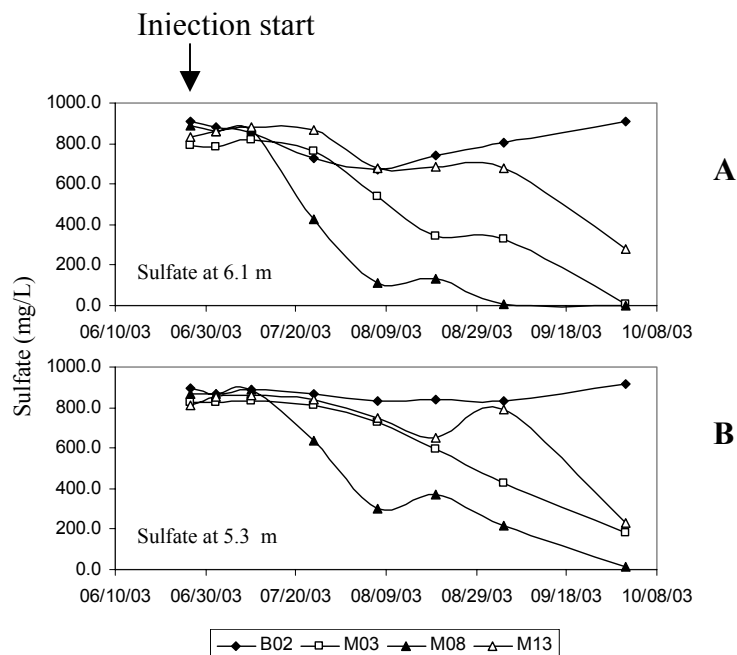


Figure 4-7. Sulfate reduction as measured by loss of sulfate at different depths over time. Arrow indicates the time point when acetate injection started.

(Fig. 4-6A). The sulfate concentration decreased after Fe (II) reduction started (Fig. 4-6, 4-7). About one month after injection, sulfide concentration began to increase and reached its peak in the middle of October, most noticeably in wells M-03 and M-08, then fall back to where it was (Fig. 4-6B). The loss of sulfate was concurrent in wells M-03 and M-08 at both depth of 6.0 meters (Fig.4-7A) and 5.25 m (Fig. 4-7B). A more detailed display of sulfate concentrations in different wells and at different depths were presented in Figure 4-8. Well M-08 displayed the highest rate of sulfate reduction (Fig.4-7), but the lowest iron reduction rate (Fig.4-6A).

Uranium reduction was evidenced by a decrease concurrently with Fe (II) production in wells M-08 and M-13, or shortly after an increase of ferrous iron observed with well M-03 (Fig. 4-9). Uranium concentration in well M-08 showed a relatively high rate of

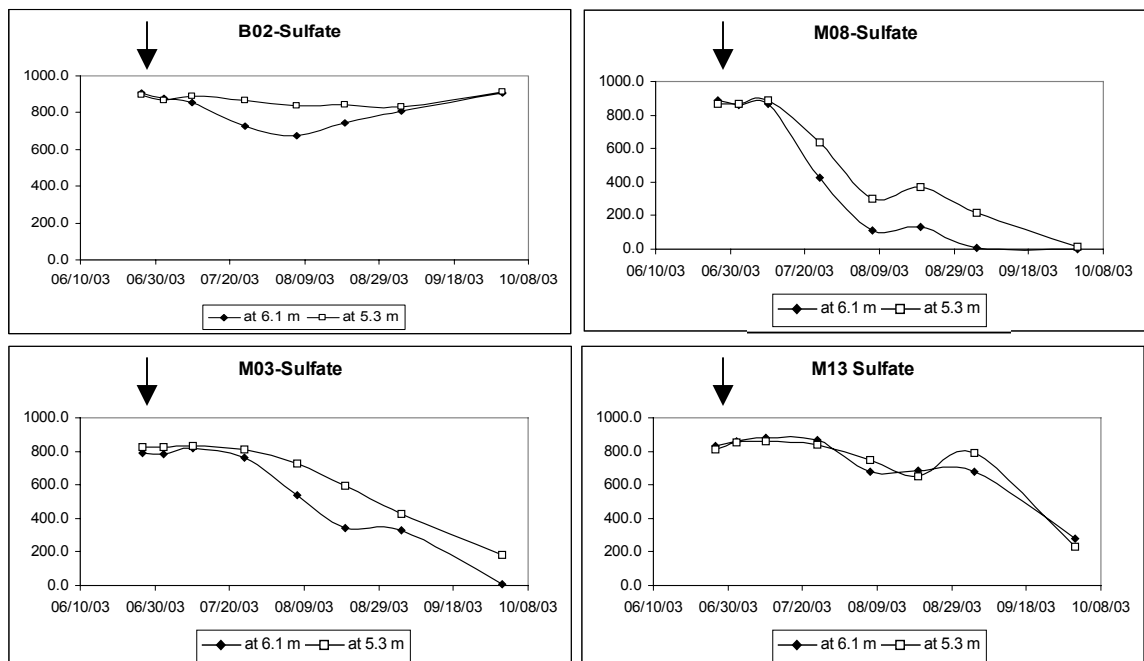


Figure 4-8. Sulfate concentrations (mg/L) measured at depth of 6.1 m (◆) and 5.3 m (□) in different wells. Arrow indicates start point of acetate injection.

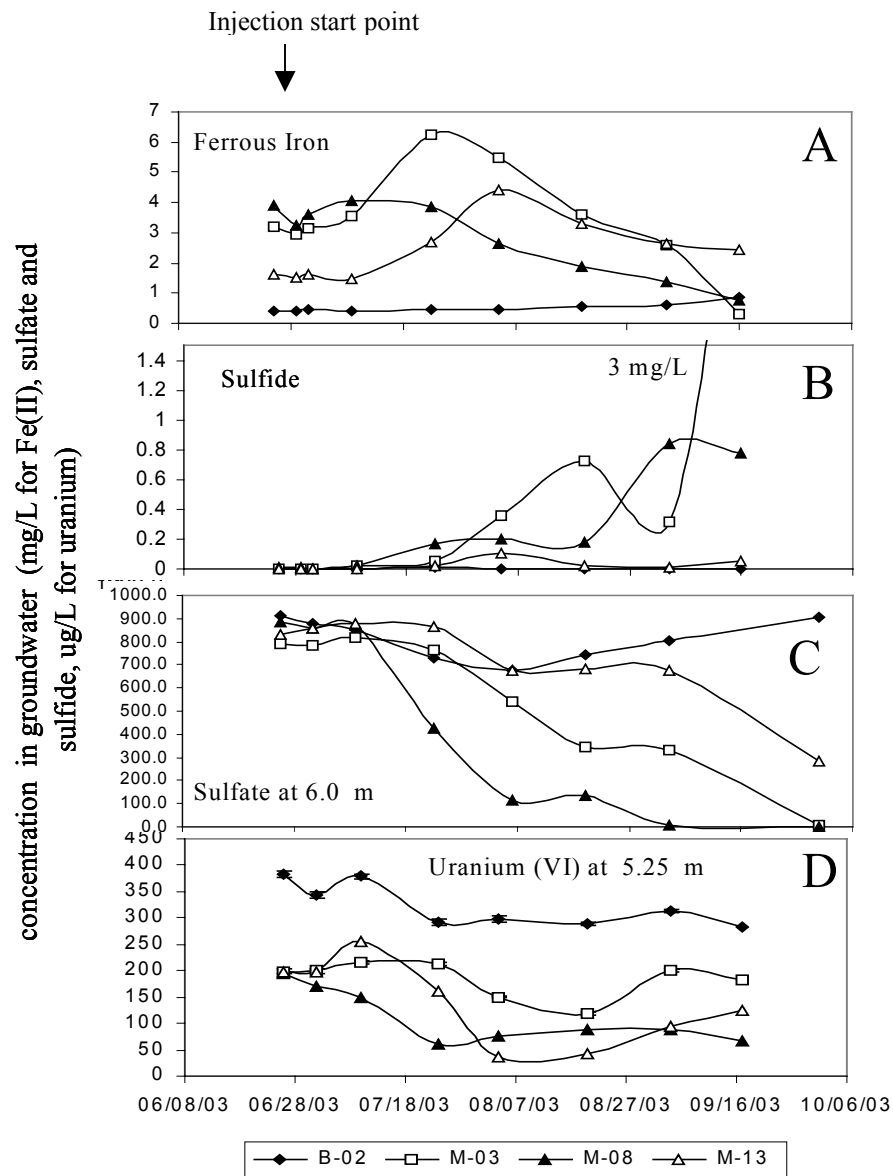


Figure 4-9. Compilation of evidences for iron, sulfate and uranium reduction that shows the relative sequence of the events. Arrow indicates the start point of acetate injection.

reduction (Fig. 4-10, 4-11) and the resulting low level uranium was clearly maintained throughout the experiment, though an increase of uranium was seen in well M-13, occurred from middle of November to middle of February (Fig. 4-11D), also in well M-03 towards the end of the experiment (Fig.4-11B). The reduced uranium concentration showed somewhat high fluctuation in well M-13, but overall it was relatively stable and a low uranium concentration was sustained. Furthermore at a deeper depth (6 m) uranium concentration was consistently lower than that at a shallower depth of 5 m (Fig. 4-11). Background well B02 has the highest level of Uranium contamination, which was relatively stable throughout the experiment. M03 and M08, further downgradient are the least polluted through out the contamination plume after stimulated bioreduction. Nitrate was consistently below the quantification limit (< 0.056 mg/L) in all wells until

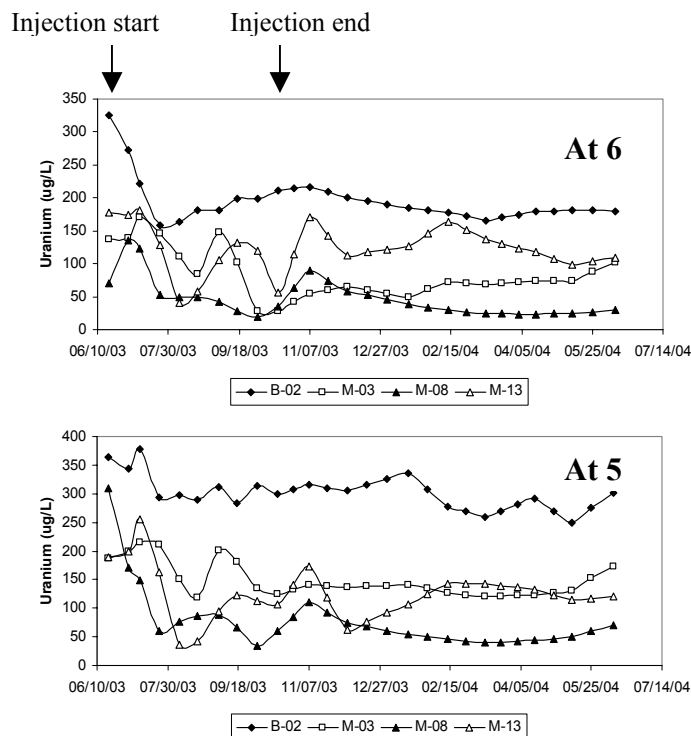


Figure 4-10. Uranium reduction as measured by U (VI) disappearance at different depths over time. The arrows indicate the start point and the end point of acetate injection.

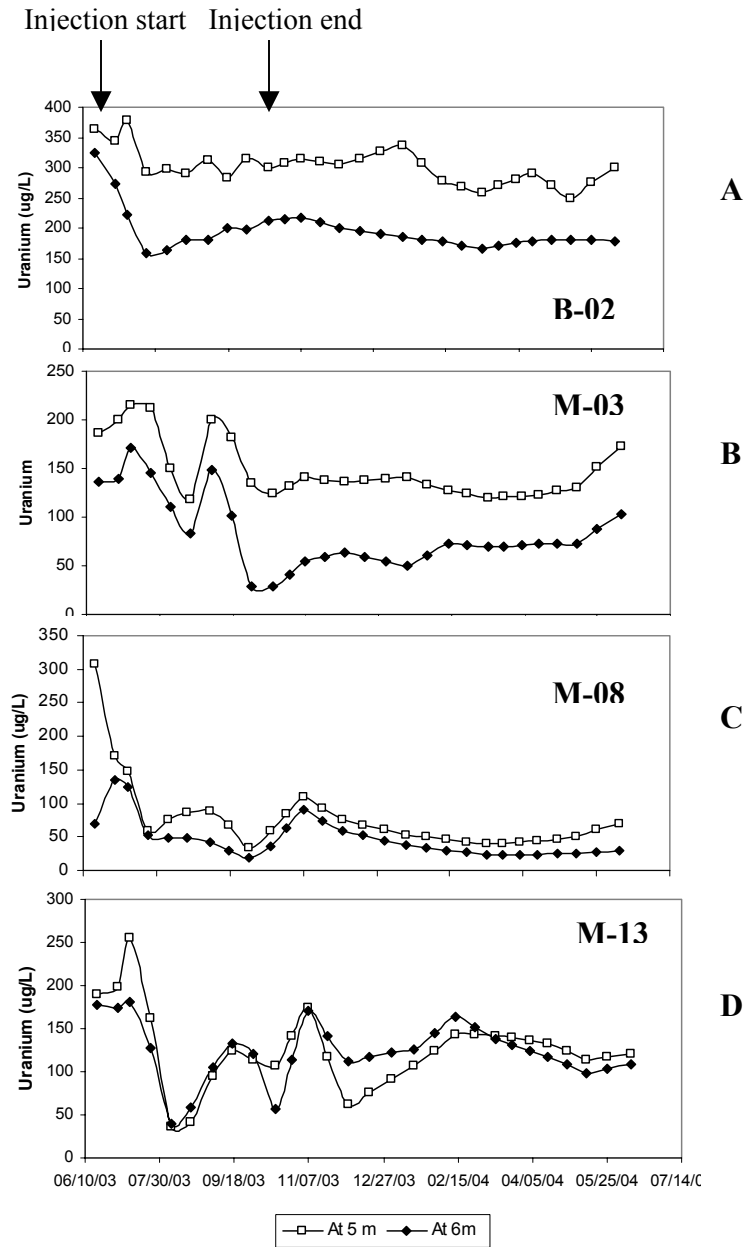


Figure 4-11. Uranium concentrations (ug/L) measured at depth of 6 m (◆) and 5 m (□) in monitoring wells B-02 (A), M-03 (B), M-08 (C) and M-13 (D). Arrows indicate the start point and the end point of acetate injection.

Table 4-1. Nitrate Concentration in groundwater sampled from background well B-02 and monitoring wells M-03, M-08 and M-13.

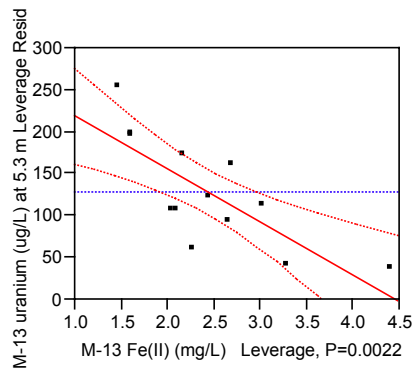
Samples/ Date Collected	Nitrate (mg/L) at 5m				Nitrate (mg/L) at 6m			
	B-02	M-03	M-08	M-13	B-02	M-03	M-08	M-13
06/25/03	<0.056	<0.056	<0.056	<0.056	<0.056	<0.056	<0.056	<0.056
07/01/03	<0.056	<0.056	<0.056	<0.056	<0.056	<0.056	<0.056	<0.056
07/09/03	<0.056	<0.056	<0.056	<0.056	<0.056	<0.056	<0.056	<0.056
07/23/03	<0.056	<0.056	<0.056	<0.056	<0.056	<0.056	<0.056	<0.056
08/06/03	<0.056	<0.056	<0.056	<0.056	<0.056	<0.056	<0.056	<0.056
08/19/03	<0.056	2.10	2.54	1.38	<0.056	3.67	4.88	1.25
09/03/03	<0.056	2.37	4.31	<0.056	<0.056	3.07	10.54	<0.056
09/30/03	<0.056	3.67	9.29	4.49	<0.056	8.82	<0.056	4.68

nearly two months (~52 days) after the injection and the amount measured as 4.1+2.6 mg/L (n=16) still was not significant (Table 4-1).

A fit model for correlation tests were run among all geochemical parameters. Uranium concentration was significantly correlated with Fe (II) concentration in well M13 at both depths (Fig. 4-12). Between uranium and sulfide, the significant correlation was only existed in well M-03 at a deeper depth of 6.1 m (Fig. 4-13). The redox potential showed positive correlation with uranium in wells M-03, M-08 only (Fig. 4-14), where redox potential also showed significant correlation with sulfide (Fig. 4-15), and uranium verse sulfate were also significantly correlated (except at shallower depth of M-03) (Fig. 4-16), suggesting relations between sulfate and uranium reduction in wells M-03 and M-08.

Sulfate concentration in all downgradient wells showed significant correlation with sulfide concentration (Fig.4-17), further confirm the occurrence of sulfate reduction.

Redox potential was significantly correlated with sulfate concentration in three down-gradient monitoring wells only (Fig. 4-18), with the strongest correlation in well M-08

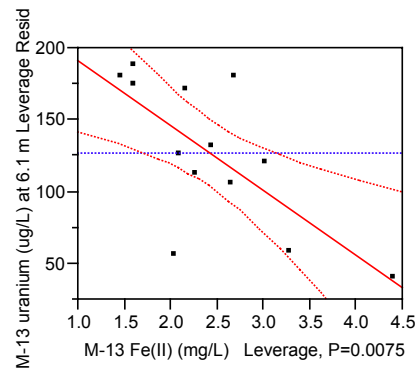


Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	1	30256.101	30256.1	15.7106
Error	11	21184.189	1925.8	Prob > F
C. Total	12	51440.290		0.0022

Correlation of Estimates

Corr	Intercept	M-13 Fe(II) (mg/L)
Intercept	1.0000	-0.9537
M-13 Fe(II) (mg/L)	-0.9537	1.0000



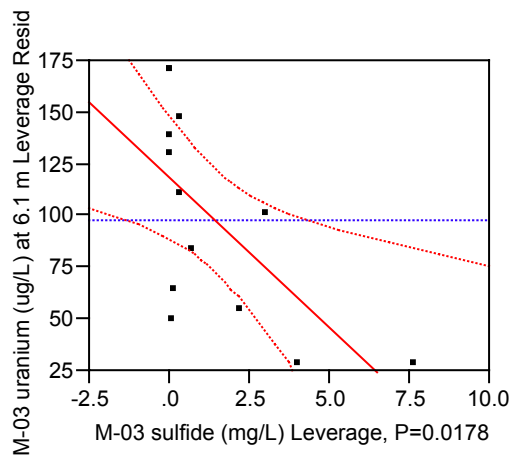
Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	1	15685.214	15685.2	10.6961
Error	11	16130.891	1466.4	Prob > F
C. Total	12	31816.105		0.0075

Correlation of Estimates

Corr	Intercept	M-13 Fe(II) (mg/L)
Intercept	1.0000	-0.9537
M-13 Fe(II) (mg/L)	-0.9537	1.0000

Figure 4-12. The leverage plot showing significant negative correlation between uranium and Fe (II) concentration in well M13 only, at both depths of ~5 m (left) and ~6 m (right).



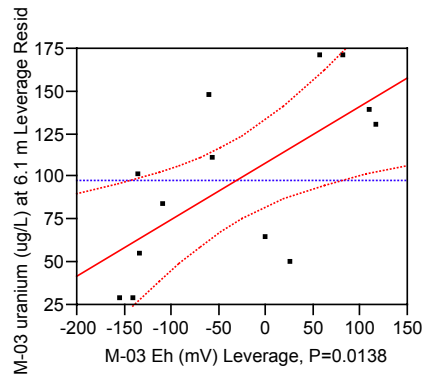
Correlation of Estimates

Corr	Intercept	M-03 sulfide (mg/L)
Intercept	1.0000	-0.5464
M-03 sulfide (mg/L)	-0.5464	1.0000

Analysis of Variance

Source	DF	Sum of Square	Mean Squar	F Ratio
Model	1	12905.252	12905.3	7.7410
Error	11	18338.408	1667.1	Prob > F
C. Total	12	31243.660		0.0178

Figure 4-13. The leverage plot showing significant negative correlations between uranium and sulfide concentration in well M-03 at deeper depth of ~6 m

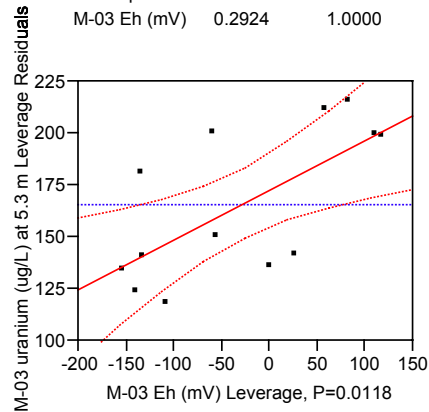


Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	1	13676.964	13677.0	8.5643
Error	11	17566.696	1597.0	Prob > F
C. Total	12	31243.660		0.0138

Correlation of Estimates

Corr	Intercept	M-03 Eh (mV)
Intercept	1.0000	0.2924
M-03 Eh (mV)	0.2924	1.0000

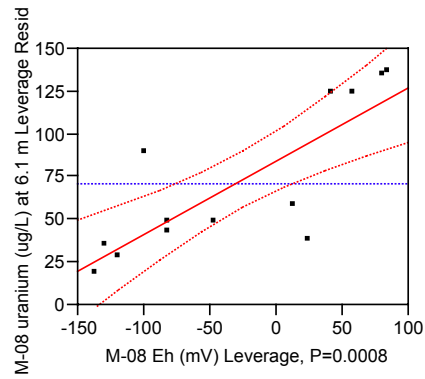


Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	1	7040.996	7041.00	9.0815
Error	11	8528.398	775.31	Prob > F
C. Total	12	15569.394		0.0118

Correlation of Estimates

Corr	Intercept	M-03 Eh (mV)
Intercept	1.0000	0.2924
M-03 Eh (mV)	0.2924	1.0000

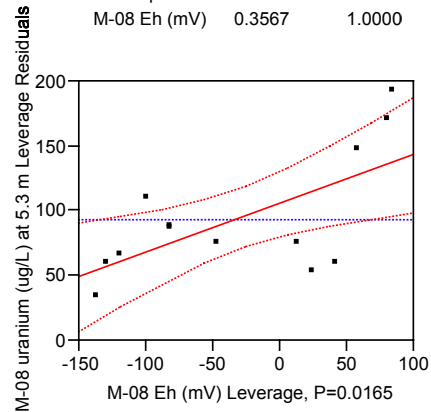


Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	1	15162.281	15162.3	20.6767
Error	11	8066.327	733.3	Prob > F
C. Total	12	23228.608		0.0008

Correlation of Estimates

Corr	Intercept	M-08 Eh (mV)
Intercept	1.0000	0.3567
M-08 Eh (mV)	0.3567	1.0000



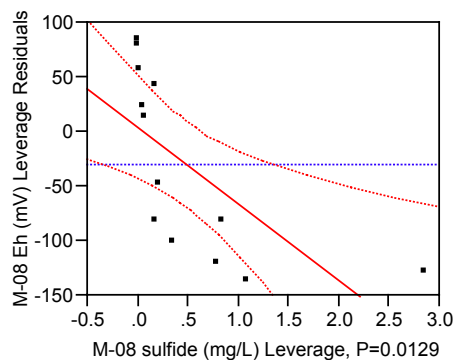
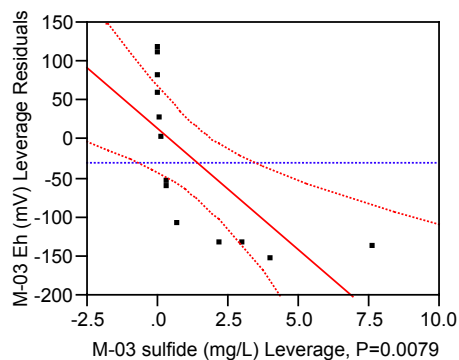
Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	1	11749.042	11749.0	7.9762
Error	11	16203.185	1473.0	Prob > F
C. Total	12	27952.227		0.0165

Correlation of Estimates

Corr	Intercept	M-08 Eh (mV)
Intercept	1.0000	0.3567
M-08 Eh (mV)	0.3567	1.0000

Figure 4-14. The leverage plot showing significant positive correlations between redox potential and uranium concentration in wells M-03 and M-08 at both depths.



Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	1	59773.39	59773.4	10.4700
Error	11	62799.37	5709.0	Prob > F
C. Total	12	122572.77		0.0079

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	1	36703.757	36703.8	8.7714
Error	11	46029.474	4184.5	Prob > F
C. Total	12	82733.231		0.0129

Correlation of Estimates

Corr

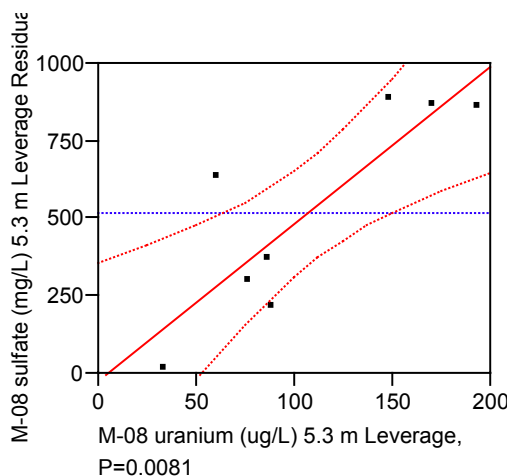
	Intercept	M-03 sulfide (mg/L)
Intercept	1.0000	-0.5464
M-03 sulfide (mg/L)	-0.5464	1.0000

Correlation of Estimates

Corr

	Intercept	M-08 sulfide (mg/L)
Intercept	1.0000	-0.5554
M-08 sulfide (mg/L)	-0.5554	1.0000

Figure 4-15. The leverage plot showing significant negative correlations between redox potential and sulfide concentration in wells M-03 and M-08.

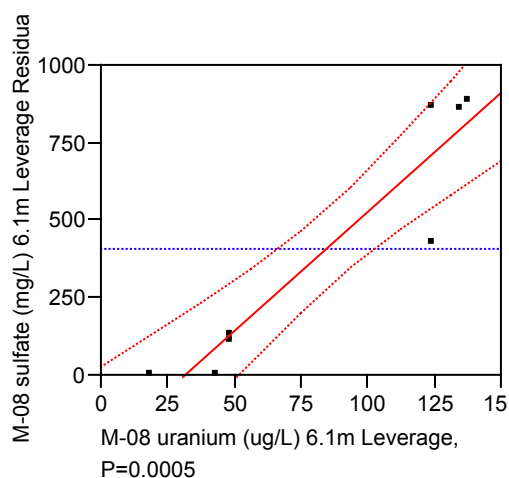


Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	1	575845.67	575846	15.0843
Error	6	229051.42	38175	Prob > F
C. Total	7	804897.09		0.0081

Correlation of Estimates

Corr	Intercept	M-08 uranium (ug/L) 5.3 m
Intercept	1.0000	-0.8967
M-08 uranium (ug/L) 5.3 m	-0.8967	1.0000

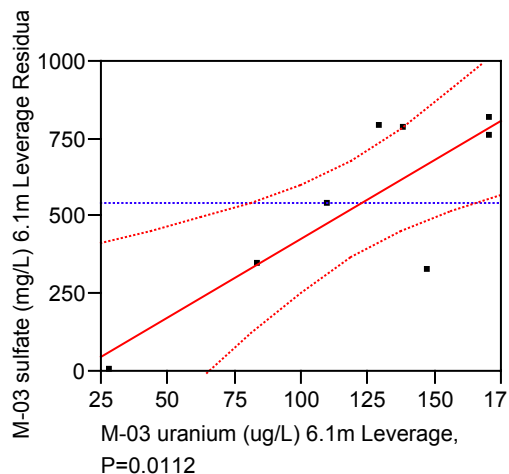


Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	1	1007195.2	1007195	45.7465
Error	6	132101.3	22017	Prob > F
C. Total	7	1139296.5		0.0005

Correlation of Estimates

Corr	Intercept	M-08 uranium (ug/L) 6.1m
Intercept	1.0000	-0.8779
M-08 uranium (ug/L) 6.1m	-0.8779	1.0000



Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	1	420874.38	420874	13.0726
Error	6	193171.22	32195	Prob > F
C. Total	7	614045.61		0.0112

Correlation of Estimates

Corr	Intercept	M-03 uranium (ug/L) 6.1m
Intercept	1.0000	-0.9388
M-03 uranium (ug/L) 6.1m	-0.9388	1.0000

Figure 4-16. The leverage plot showing significant positive correlations between uranium and sulfate concentration in wells M-03 and M-08.

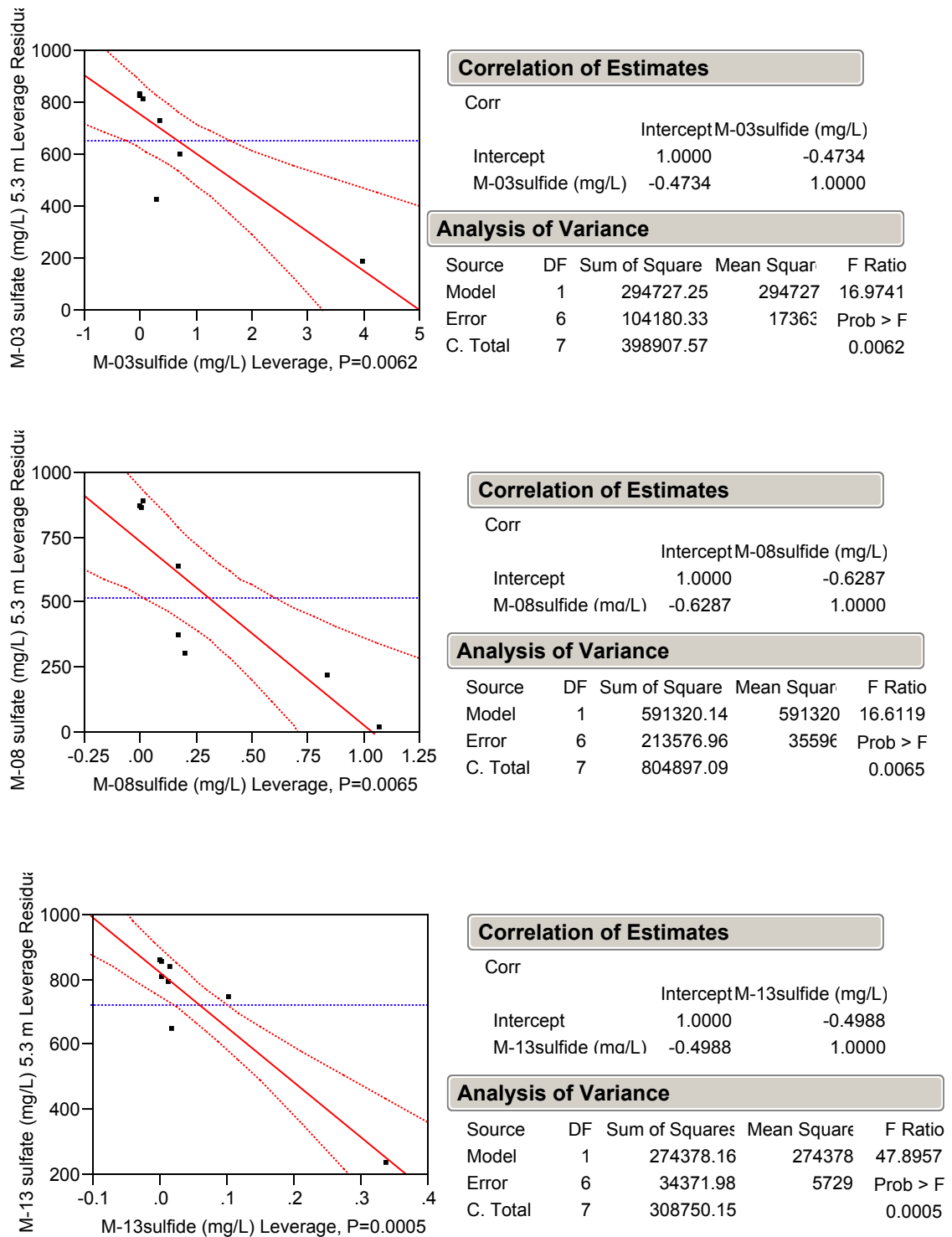
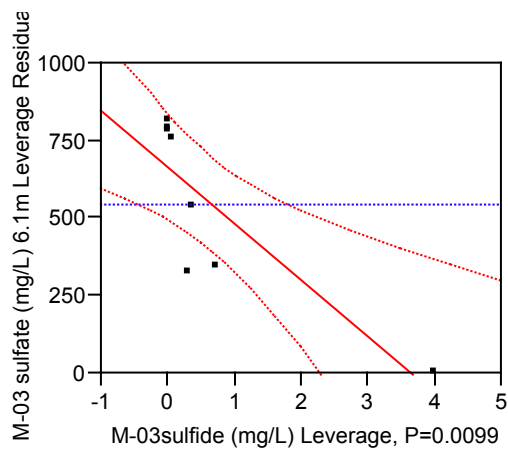


Figure 4-17 (I). At ~5 m (I) and ~6 m(II), sulfate negatively correlates with sulfide in all down-gradient monitoring wells ($p < 0.01$).

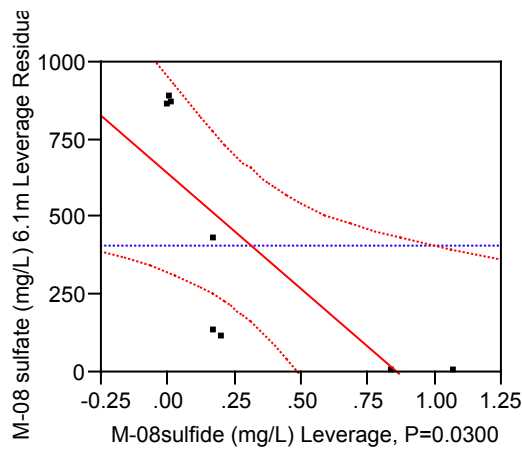


Correlation of Estimates

Corr	Intercept	M-03sulfide (mg/L)
Intercept	1.0000	-0.4734
M-03sulfide (mg/L)	-0.4734	1.0000

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	1	428053.14	428053	13.8087
Error	6	185992.47	30999	Prob > F
C. Total	7	614045.61		0.0099

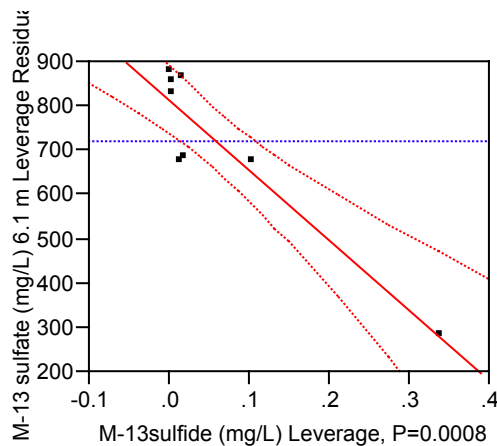


Correlation of Estimates

Corr	Intercept	M-08sulfide (mg/L)
Intercept	1.0000	-0.6287
M-08sulfide (mg/L)	-0.6287	1.0000

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	1	651126.8	651127	8.0029
Error	6	488169.7	81362	Prob > F
C. Total	7	1139296.5		0.0300



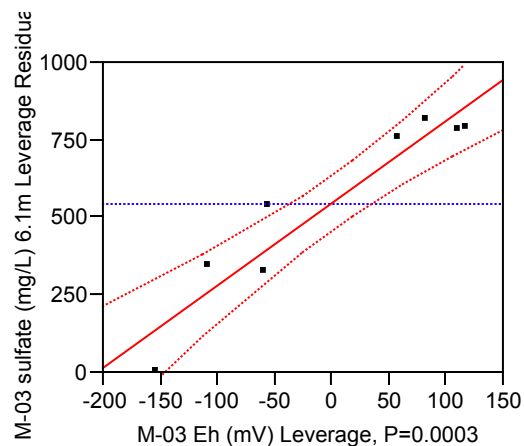
Correlation of Estimates

Corr	Intercept	M-13sulfide (mg/L)
Intercept	1.0000	-0.4988
M-13sulfide (mg/L)	-0.4988	1.0000

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	1	236675.66	236676	38.0982
Error	6	37273.48	6212	Prob > F
C. Total	7	273949.15		0.0008

Figure 4-17 (II). At ~5 m (I) and ~6 m(II), sulfate negatively correlates with sulfide in all down-gradient monitoring wells ($p < 0.01$)

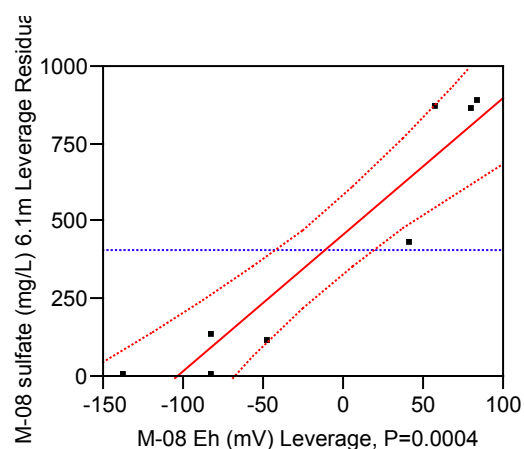


Correlation of Estimates

Corr	InterceptM-03 Eh (mV)	
Intercept	1.0000	0.0101
M-03 Eh (mV)	0.0101	1.0000

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	1	552447.63	552448	53.8116
Error	6	61597.97	10266	Prob > F
C. Total	7	614045.61		0.0003

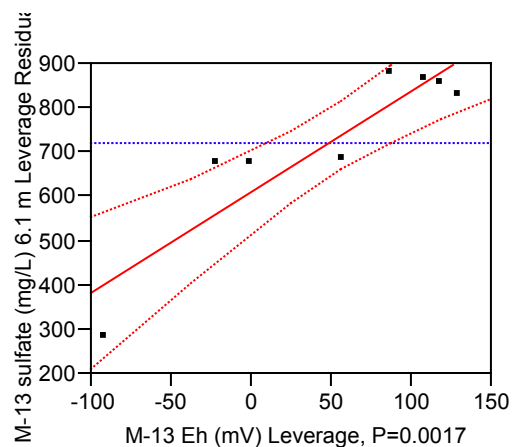


Correlation of Estimates

Corr	InterceptM-08 Eh (mV)	
Intercept	1.0000	0.1290
M-08 Eh (mV)	0.1290	1.0000

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	1	1012716.2	1012716	48.0035
Error	6	126580.3	21097	Prob > F
C. Total	7	1139296.5		0.0004



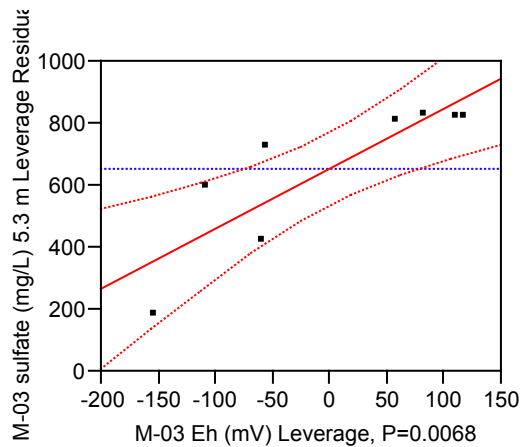
Correlation of Estimates

Corr	InterceptM-13 Eh (mV)	
Intercept	1.0000	-0.5453
M-13 Eh (mV)	-0.5453	1.0000

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	1	226958.12	226958	28.9789
Error	6	46991.02	7832	Prob > F
C. Total	7	273949.15		0.0017

Fig. 4-18 (I). Sulfate concentration positively correlates with the redox potential in all monitoring wells at both depths of ~6 m (I) and 5 m (II).

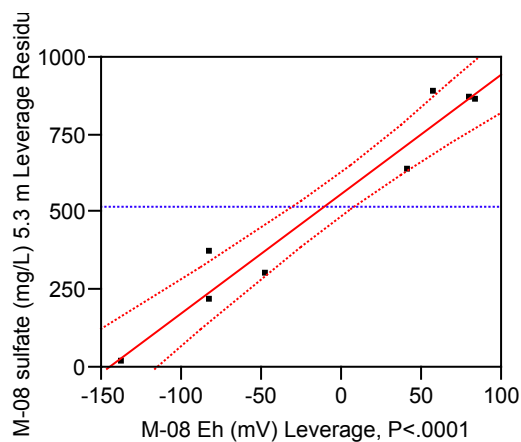


Correlation of Estimates

Corr	Intercept M-03 Eh (mV)	
Intercept	1.0000	0.0101
M-03 Eh (mV)	0.0101	1.0000

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	1	291872.19	291872	16.3613
Error	6	107035.38	17839	Prob > F
C. Total	7	398907.57		0.0068

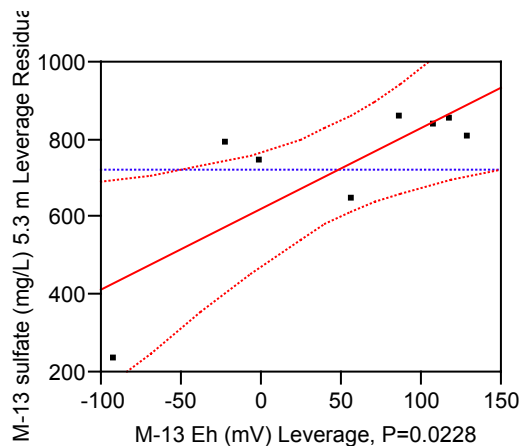


Correlation of Estimates

Corr	Intercept M-08 Eh (mV)	
Intercept	1.0000	0.1290
M-08 Eh (mV)	0.1290	1.0000

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	1	764129.32	764129	112.4608
Error	6	40767.78	6795	Prob > F
C. Total	7	804897.09		<.0001



Correlation of Estimates

Corr	Intercept M-13 Eh (mV)	
Intercept	1.0000	-0.5453
M-13 Eh (mV)	-0.5453	1.0000

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	1	187160.70	187161	9.2357
Error	6	121589.45	20265	Prob > F
C. Total	7	308750.15		0.0228

Figure 4-18 (II). Sulfate concentration positively correlates with the redox potential in all monitoring wells at both depths of ~6 m (I) and 5 m (II).

($r^2 = 0.95$, $P < 0.0001$), the weakest in well M-13 ($r^2 = 0.61$, $P = 0.0228$) (Fig. 4-18 (II)), suggesting sulfate reduction is the overall driving force in altering the subsurface redox potential.

Biomass: The bacterial biomass (measured as pmol PLFA per bead, Figure 4-19) was determined to include the total PLFA minus the normal saturates over 18 carbons in length and the polyenoic PLFA. Both of these are generally associated with eukaryote biomass (White et al., 1998a; Bossio and Scow, 1998), although there are exceptions. The trace quantities of PLFA of unknown structure were also excluded from the prokaryote biomass. The biomass content at the site varied considerably between background and down gradient monitoring wells. The highest amount of biomass nearly 1200 pmol/bead was detected 7.3 meter away from injection gallery, 5 meters down in the bio-trap of well M08. The lowest detectable biomass of 0.19 pmol/bead was from background well B-02 at a depth of ~6 meters. The most variability of biomass was observed at shallower depth of 5 meters, where 44% of the traps from B-02 and M-13 showed biomass below detection limit. Bacterial biomass contents in the traps taken from downgradient monitoring wells M-03, M-08 and well M-13 all displayed an increasing pattern and reached peak at third sampling event (early August), followed by a steady decrease (Fig. 4-19). This pattern was similar at two different depths measured, but absent in the background well. (Fig. 4-20). More interestingly, the monitoring well M-08 generally exhibited highest biomass among all four wells during active biostimulation phase (Fig. 4-19, 4-20), which coincided with the highest sulfate and uranium reductions observed in well M-08.

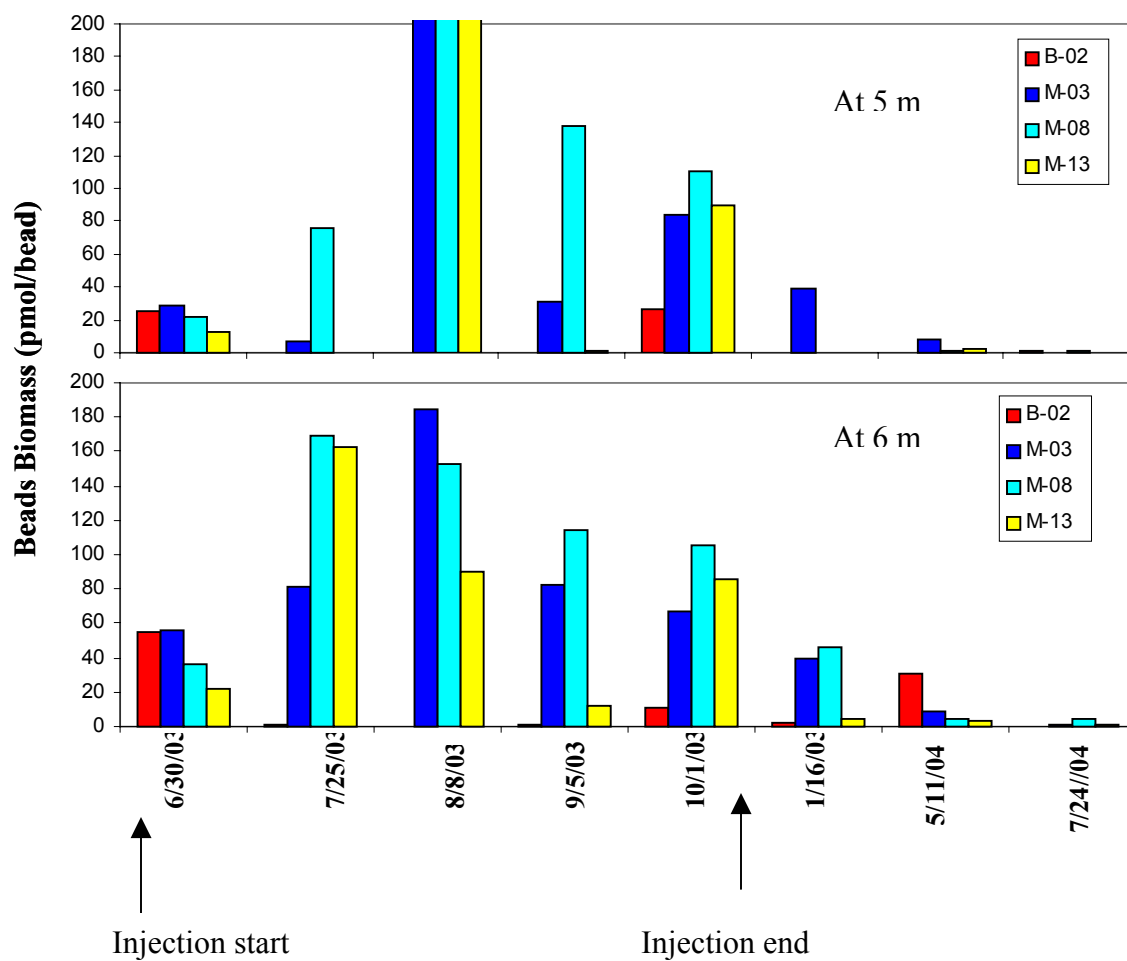


Figure 4-19. Bacterial biomass measured as PLFA per bead at depth of 5 m (top) and 6 m (bottom). A pattern of increase followed by decrease was observed with biomass measurement. Arrows indicate the start point and the end point of acetate injection.

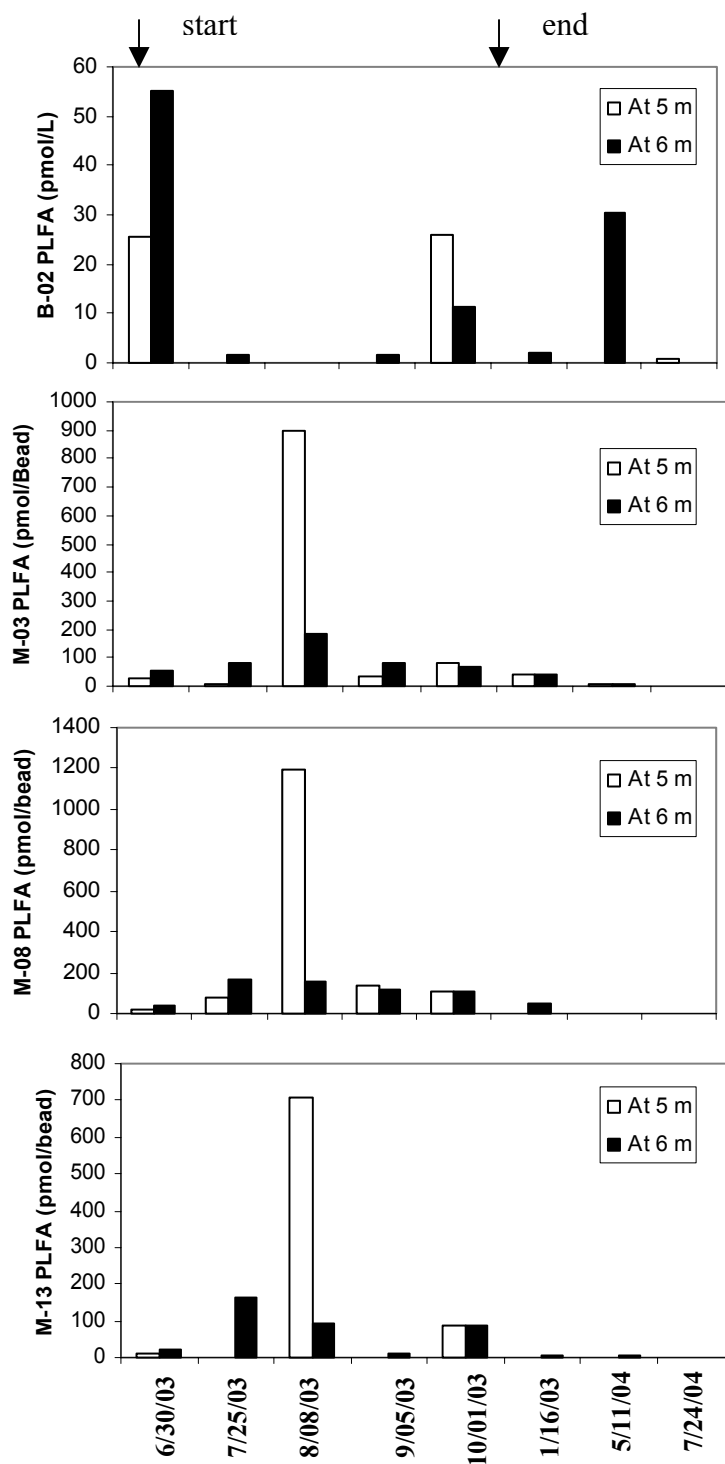


Figure 4-20. Bacterial biomass measured as pmol PLFA per bead in background well B-02 and down-gradient monitoring wells M-03, M-08 and M-13 respectively. A pattern of biomass increase followed by decline was observed in all down-gradient monitoring wells, but not with background well. Arrows indicate the start point and the end point of acetate injection.

Community Structure Based on PLFA Profiles: PLFA analysis revealed a relative simple community structure, mainly consisted of normal saturates, monounsaturates, mid-chain branched saturates and terminally branched saturates. Figure 4-21 shows a comparison of the relative percentage of total PLFA functional groups from the biomass of wells B-02, M-03, M-08 and M13 at different time period. The community structures were similar to each other at different depths examined.

Monounsaturates, indicative of Gram-negative bacteria showed a noticeable increase (nearly doubled or more) in down gradient monitoring wells compared with background well. Samples from well M-03, M-08 and M-13 contained significantly more terminally branched saturates (including i15:0, i17:0 and 10me16:0) than did samples B-02 (mostly below detection). The percent of monounsaturates and terminally branched saturates in the same well displayed an increase followed by decrease over experimental time – a pattern similar to what was seen with biomass. Although most often associated with the presence of Gram-positive bacteria, terminally branched saturates, in particular the i15:0 and i17:0 are often detected in Gram-negative sulfate reducing bacteria (Wilkinson, 1988), 10me16:0 was an indicator of *Desulfobacter* sp. (Dowling *et al.*, 1986). The amount of fatty acids of i15:0, 16:0 and 16:1 ω 7c, considered as *Geobacteracea* indicators (Anderson *et al.*, 2003), were highest in well M-03 and M-08. (Data not shown).

A hierarchical cluster analysis (HCA) of the PLFA characterizes the major trends within the data set (Fig. 4-22). Two major clusters separated according to treatment were clearly demonstrated. 12 out of all 14 background well samples, spanning over a year, grouped with 12 samples retrieved from down gradient monitoring wells when acetate addition had stopped for 7 to 9 months (except two samples from well M-13) and biostimulation

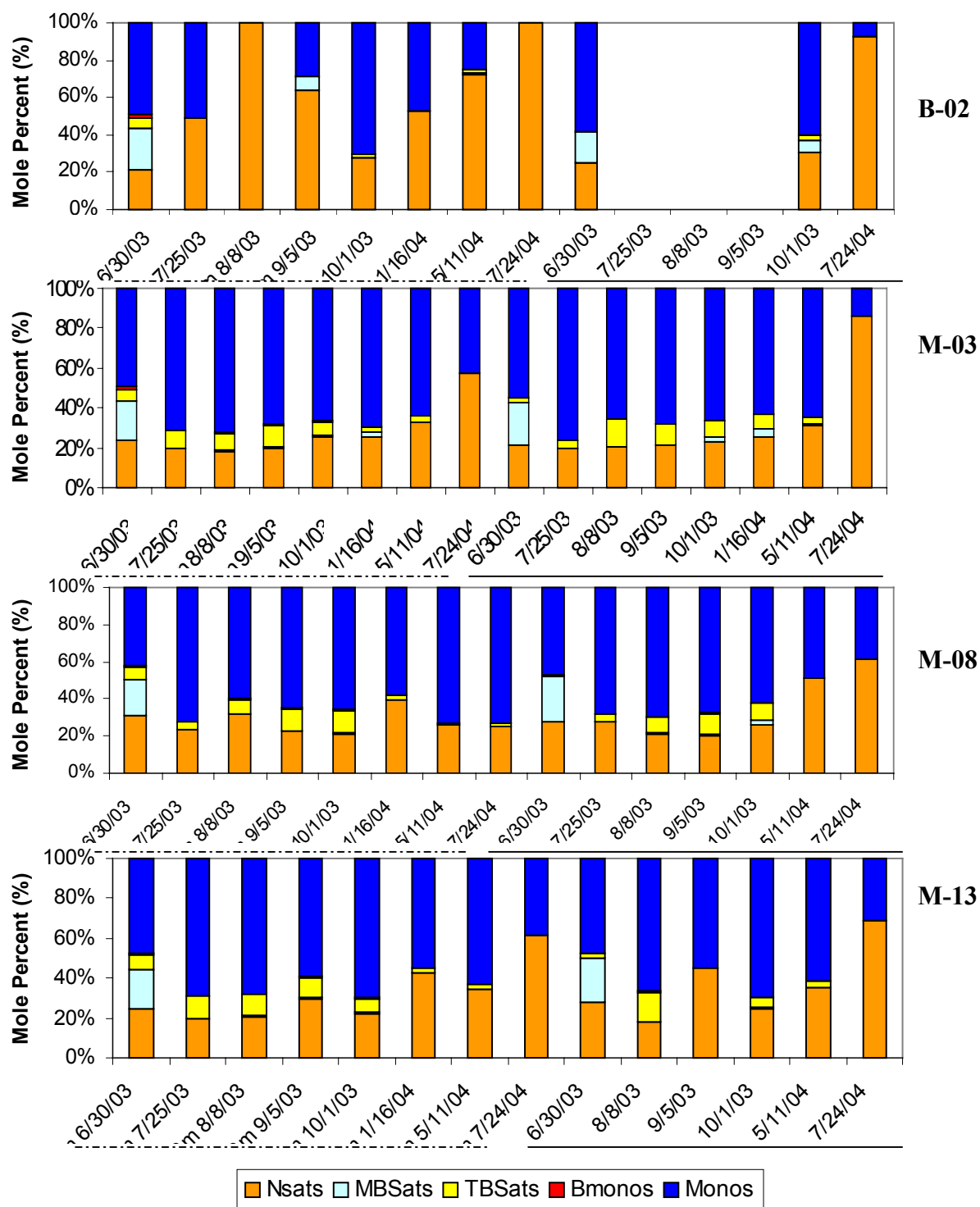


Figure. 4-21 . Microbial community structure in wells B-02, M-03, M-08 and M-13, determined by PLFA profiles recovered from respective bead samplers. Nsats: normal saturates; MBSats: Mid-chain branched saturates; TBSats: Terminally branched saturates; Bmonos: Branched monounsaturates; Monos: Monounsaturates. The dotted and solid lines indicate bead samplers from 6 m and 5 m deep respectively.

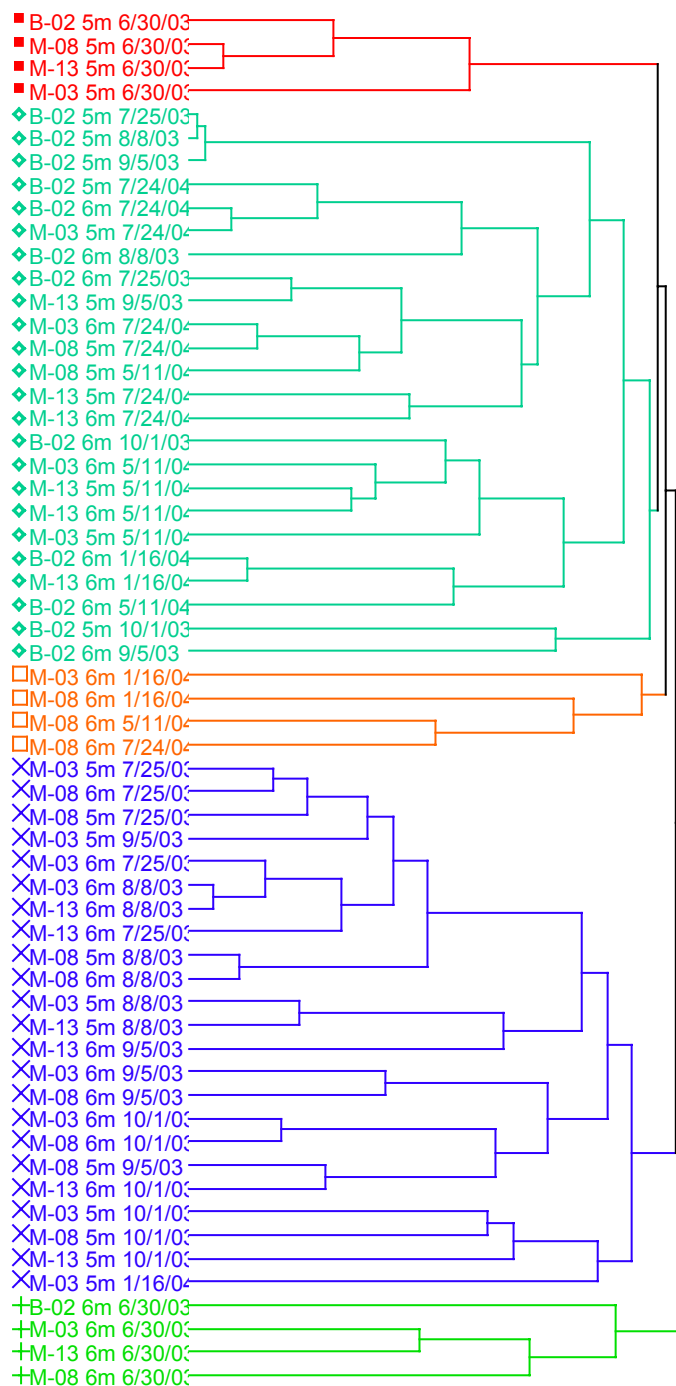


Figure 4-22. Hierarchical clustering analysis of bio-trap sampler's PLFA profiles (Ward method). Different clusters are indicated by signs of +, □, X, ■ and ◆.

effect subsided or diminished. All remaining 23 monitoring well samples, spanning from July to Oct during acetate injection, clustered together. The depth didn't seem to be a factor in grouping the above two major clusters, however it does play certain roles in shaping the subsurface bacterial community. The microbial structures recovered from biotrap harvests on first sampling event of 6/30/03 from 6 m depth were clearly and distantly separated from those at 5 m depth, regardless of background well or monitoring wells. Furthermore 4 samples from wells M-03 and M-08 after the cessation of acetate injection, all at deeper depth (6 m), were separated from any other group and formed their own cluster (Fig. 4-22).

Physiological and Respiratory Status: The lipid composition of microorganisms is a product of metabolic pathways and so reflects the phenotypic responses of the microbe to the environment. Gram-negative bacteria make *trans* fatty acids to modify their cell membranes against environmental stress, as such the physiological status of Gram-negative communities can be assessed by ratios of specific PLFAs. The total *trans* to *cis* isomer ratio for 16:1 ω 7 was smaller than 0.05 (Table 4-2) in nearly all samples measured, indicating a healthy population. The ratio based on 18:1 ω 7 was not presented here because only 3 out of 59 samples had detectable amount of 18:1 ω 7.

The proportion of aerobically respiring organisms indicated by ubiquinone/menaquinone ratio (UQn/MKn) decreased at all monitoring wells, which was not observed with samples taken from background wells. Interestingly, an increase of ubiquinone/menaquinone ratio was noticed at well M-03 preceding the decrease effect (Table 4-3). The decrease of quinone ratios (UQn/MKn) indicated a shift from aerobic

Table 4-2. Summary of physiological status of the bio-trap beads microbial community indicated by *Tran/Cis* ratio calculated based on fatty acids of 16:1 ω 7

Sampling		At 5 meters						
Data	6/30/03	7/25/03	8/8/03	9/5/03	10/1/03	1/16/04	5/11/04	7/24/04
B-02	0.00	#DIV/0!	#DIV/0!	#DIV/0!	0.17			0.00
M-03	0.00	0.00	0.02	0.00	0.04	0.08	0.05	0.00
M-08	0.00	0.00	0.02	0.03	0.05		0.04	0.00
M-13	0.00		0.05	0.00	0.02		0.09	0.00

Sampling		At 6 meters						
Data	6/30/03	7/25/03	8/8/03	9/5/03	10/1/03	1/16/04	5/11/04	7/24/04
B-02	0.16	0.00	#DIV/0!	0.00	0.04	0.00	0.02	#DIV/0!
M-03	0.16	0.02	0.02	0.02	0.02	0.06	0.05	0.00
M-08		0.02	0.02	0.03	0.04	0.05	0.06	0.04
M-13	0.24	0.01	0.00	0.04	0.02	0.00	0.05	0.00

#DIV/0! = A number divided by zero

Table 4-3. Microbial respiration state measured by quinone ratio

Samples/ Date Collected	UQn/MKn Ratio			
	B-02	M-03	M-08	M-13
06/30/03	2.2	1.15	1.06	0.69
07/25/03	MKn=0	9.25	0.57	0.30
08/08/03	MKn=0	0.81	0.40	0.55

to anaerobic respiration, which reached a value less than 1.0 in all monitoring wells (Table 4-3) by early August, suggesting an anaerobic condition with a past history of oxygen availability (Polglase et al. 1966; Geyer et al. 2004).

Bacterial Population Distribution Based on PCR-DGGE Analysis: Samples collected from 5 meter depth at selected time points were subject to 16S rRNA gene analysis. Biotraps recovered on 6/30/03 are expected to mostly harvest bacterial populations before acetate injection and designated as T1. Traps collected on 9/5/03 will likely harvest the populations stimulated during acetate addition and labeled as T2. While traps sampled on 5/11/04 and 7/24/04 will reflect the subsurface community after the biostimulation test and designated as T3 and T4 respectively. The analysis of 16S rDNA gene by DGGE revealed a community shift between samples from background well and down-gradient monitoring wells (Fig. 4-23). This observation applies to both during (Fig. 4-23A) and after (Fig. 4-23B) acetate addition, however, well M-13 displayed least similarity with other two monitoring wells during acetate injection (Fig. 4-23A). The phylogenetic analysis of major bacterial lineages recovered are presented in figures 4-24, 4-25, 4-26, 4-27, and summarized in Table 4-4.

The background well displayed the most stable community structure throughout the experiment, dominated by mostly *β-proteobacteria* (16 out of 25 sequences), such as *Hydrogenophaga*, *Dechloromonas*, *Methylophilus*, *Rhodoferax* etc. (Table 4-4 and Fig. 4-24). Sequences belong to *γ-proteobacteria* and *α-proteobacteria* were recovered less frequently (9 out of 25). The bacterial composition in down-gradient monitoring wells before the injection (T1) shared much similarities with that of background well, with

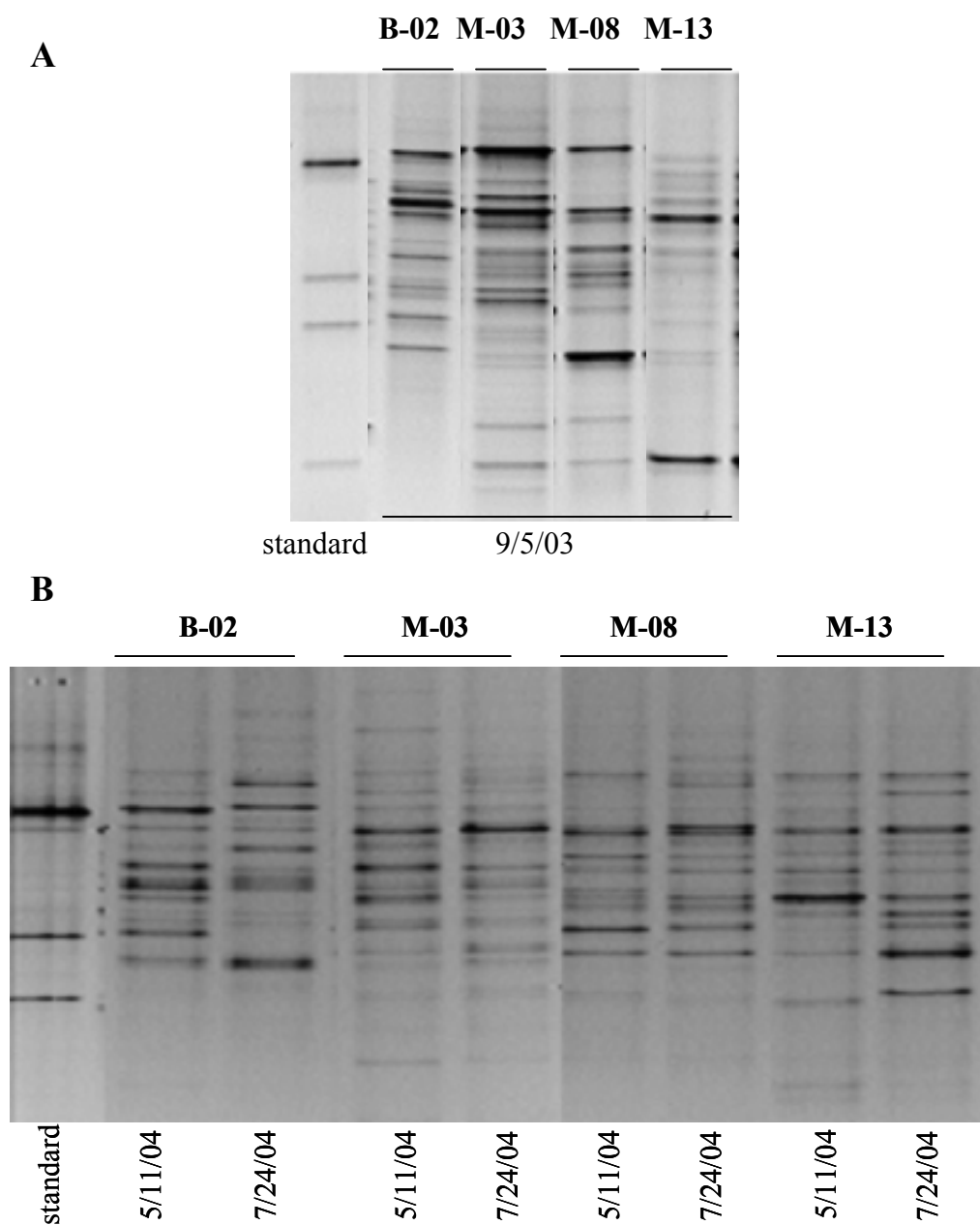


Figure 4-23. DGGE analysis of bacterial community recovered from bio-trap beads suspended in monitoring wells (B-02, M-03, M-08 and M-13) during (A) and after (B) acetate injection experiment. Amplified 16S rDNA fragments were separated on a gradient of 20% to 65 % denaturant. The date indicates time when those bio-traps were sampled.

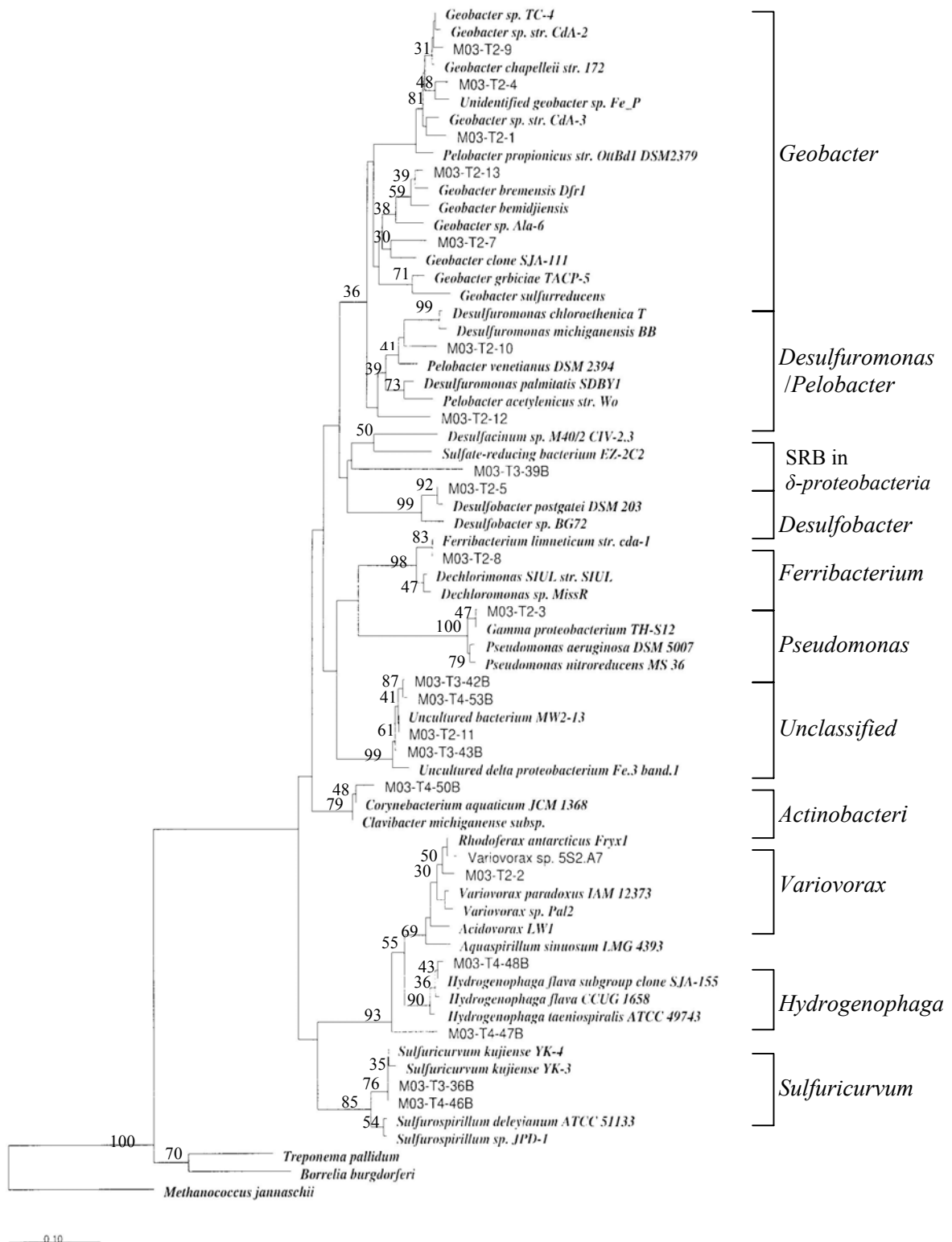


Figure 4-25. Neighbor-joining analysis of 16S rRNA gene sequences recovered from bio-traps suspended in down-gradient well M-03. Numbers on the tree refer to bootstrap values on 1000 replicates; only values above 30 are given. Scale bar represents 10% estimated change. Nomenclature: well name M03 followed by sampling time (T1, T2, T3 and T4, refer to text), then DGGE band number.

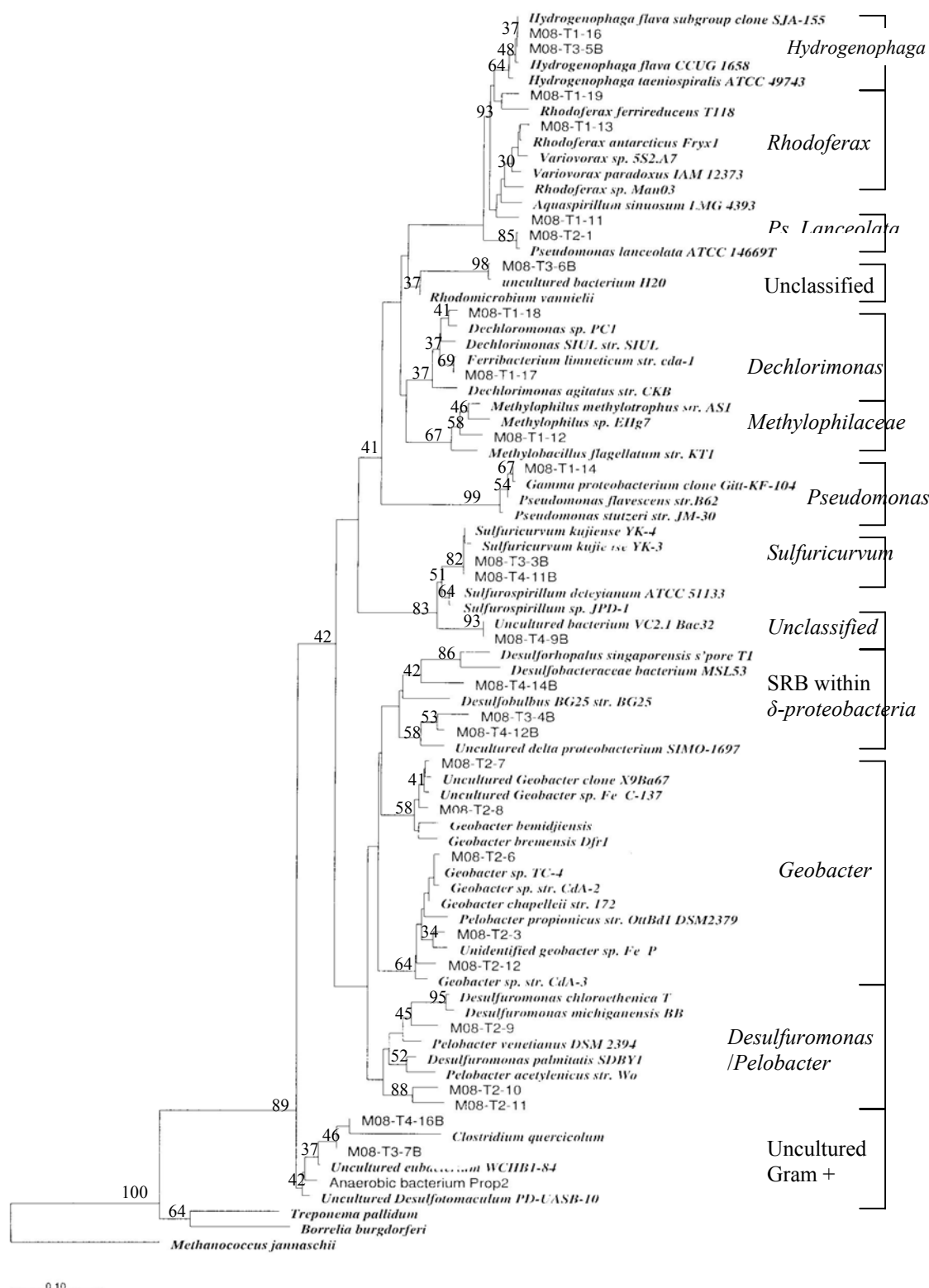


Figure. 4-26. Neighbor-joining analysis of 16S rRNA gene sequences recovered from bio-traps suspended in down gradient monitoring well M-08. Refer to Fig. 4-24 for detailed nomenclature, bootstrap analysis and scale bar information.

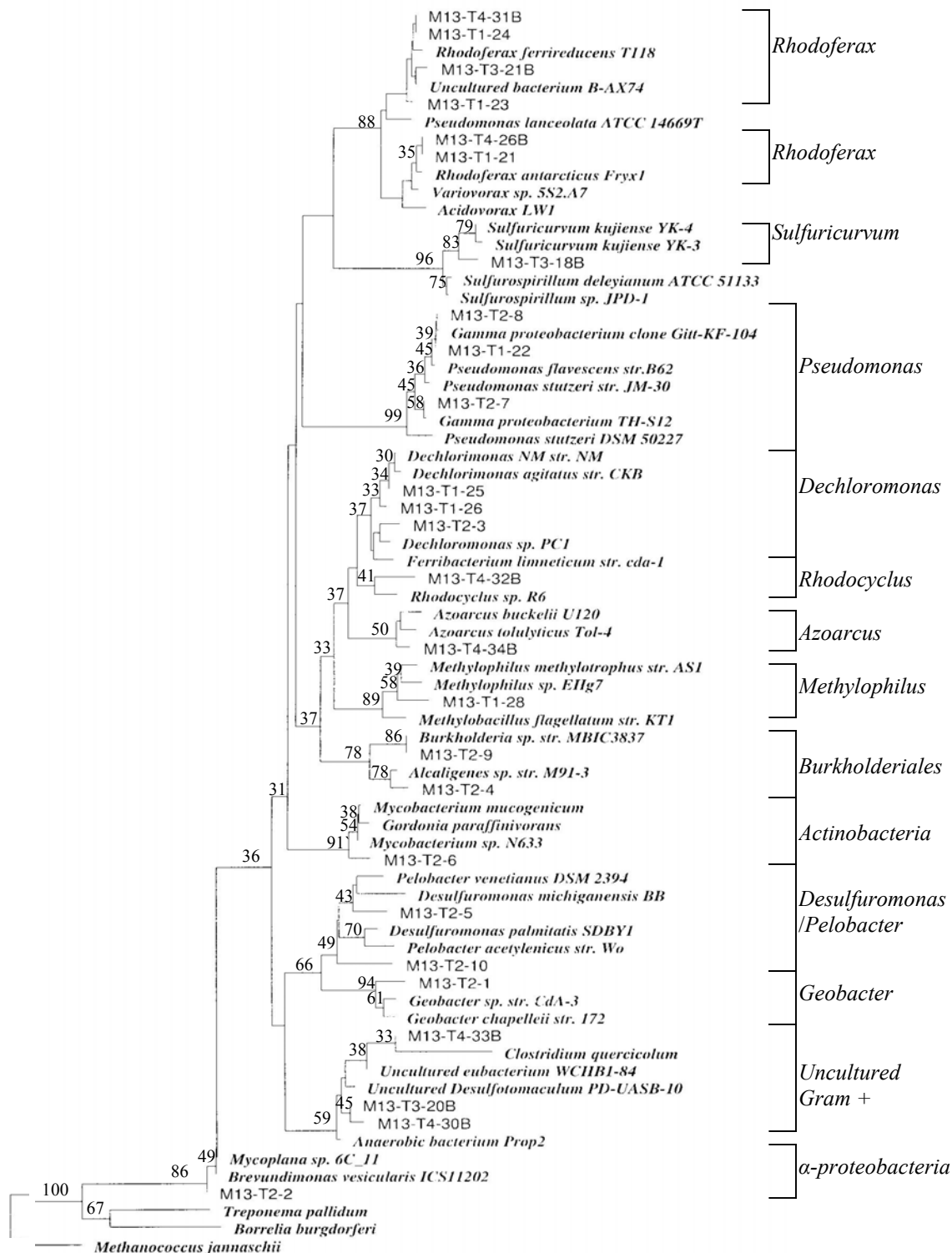


Figure. 4-27. Neighbor-joining analysis of 16S rRNA gene sequences recovered from bio-traps suspended in down gradient monitoring well M-13. Refer to Fig. 4-24 for detailed nomenclature, bootstrap analysis and scale bar information.

Table 4-4 Temporal and spatial distribution of major sequences recovered

	B-02	M-03	M-08	M-13
T1 (Before)			<i>Hydrogenophaga</i> 1 <i>Rhodoferrax</i> 2 <i>Dechloromonas</i> 1 <i>Ferribacterium</i> 1 <i>Methylophilus</i> 1 <i>Pseudomonas</i> 1	<i>Rhodoferrax</i> 3 <i>Dechloromonas</i> 2 <i>Methylophilus</i> 1 <i>Pseudomonas</i> 1
T2 (During)	<i>Hydrogenophaga</i> 1 <i>Rhodoferrax</i> 1 <i>Dechloromonas</i> 2 <i>Ferribacterium</i> 1 <i>Methylophilus</i> 3 <i>Methylobacillus</i> 1 <i>Pseudomonas</i> 2 <i>Rhodocyclus</i> 1 <i>Azoarcus</i> 1	<i>Geobacter</i> 5 <i>Desulfuromonas</i> / <i>Pelobacter</i> 1 SRB in δ -proteobacteria 1 <i>Desulfobacter</i> 1 Uncultured δ -proteobacteria 1 <i>Ferribacterium</i> 1 <i>Pseudomonas</i> 1 <i>Variovorax</i> 1	<i>Geobacter</i> 5 <i>Desulfuromonas</i> / <i>Pelobacter</i> 3 <i>Ps. Lanceolata</i> 1	<i>Geobacter</i> 1 <i>Desulfuromonas</i> / <i>Pelobacter</i> 2 <i>Dechloromonas</i> 1 <i>Pseudomonas</i> 2 <i>Burkholderiales</i> 2 <i>Actinobacteria</i> 1
T3, T4 (After)	<i>Hydrogenophaga</i> 3 <i>Unclassified</i> 1 Uncultured γ -proteobacteria 3 <i>Aquaspirillum</i> 2 <i>Herbaspirillum</i> 1 <i>Rhodopseudomonas</i> 2	<i>Hydrogenophaga</i> 1 <i>Unclassified</i> 2 <i>Sulfuricurvum</i> 2 Uncultured δ -proteobacteria 3 <i>Actinobacteria</i> 1	<i>Hydrogenophaga</i> 1 <i>Unclassified</i> 2 <i>Sulfuricurvum</i> and its relatives 3 SRB in δ -proteobacteria 3 Uncultured Gram + 2	<i>Sulfuricurvum</i> 1 <i>Unclassified</i> α and β -proteobacteria 2 Uncultured Gram + 2 <i>Rhodoferrax</i> 3 <i>Azoarcus</i> 1

Sequences were recovered from background and down-gradient monitoring wells respectively. T1, T2 and T3, T4 represents sampling time before, during and after acetate injection. Number after the organism name denotes its detection frequency --- how many times it was retrieved.

similar β -proteobacteria in dominance, e.g. *Rhodospirillum rubrum*, *Dechloromonas*, *Methylophilus*. After the injection stopped for 7 to 9 months (T3, T4), differences in community structures evolved between background and down-gradient monitoring wells. Sequences belong to *Sulfuricurvum* of *Campylobacteriales*, *Epsilonproteobacteria* were one of the major species occurring in all three down-gradient monitoring wells, SRB or unidentified bacteria within δ -proteobacteria were repeatedly detected in wells M-03 and M-08. Uncultured gram positives closely related to *Clostridium* or *Desulfotomaculum* were dominant in wells M-08 and M-13. All of these were not observed with samples from background well, where the bacterial community exhibited little changes. The most striking responses from subsurface community were noticed in down gradient monitoring wells during acetate injection (T2), when biostimulation was most active. *Geobacter* and *Desulfuromonas*/*Pelobacter* sequences were abundantly detected in wells M-03, M-08, and less frequently recovered in well M-13. Sequences belong to *Desulfobacter*, other SRB or unidentified within δ -proteobacteria were also numerically important in well M-03. Clearly, δ -proteobacteria include *Geobacter* are the major populations responding to acetate injection. Among all 26 sequences belong to δ -proteobacteria retrieved in this study, 23 were obtained from wells M-03 and M-08 (Fig. 4-28), indicating the divergence of wells M-13 from other two monitoring wells. *Geobacter* and *Desulfuromonas* sequences were prevalent only during acetate injection (T2) but fall below detection after the injection stopped (T3, T4) (Fig. 4-28, Table 4-4).

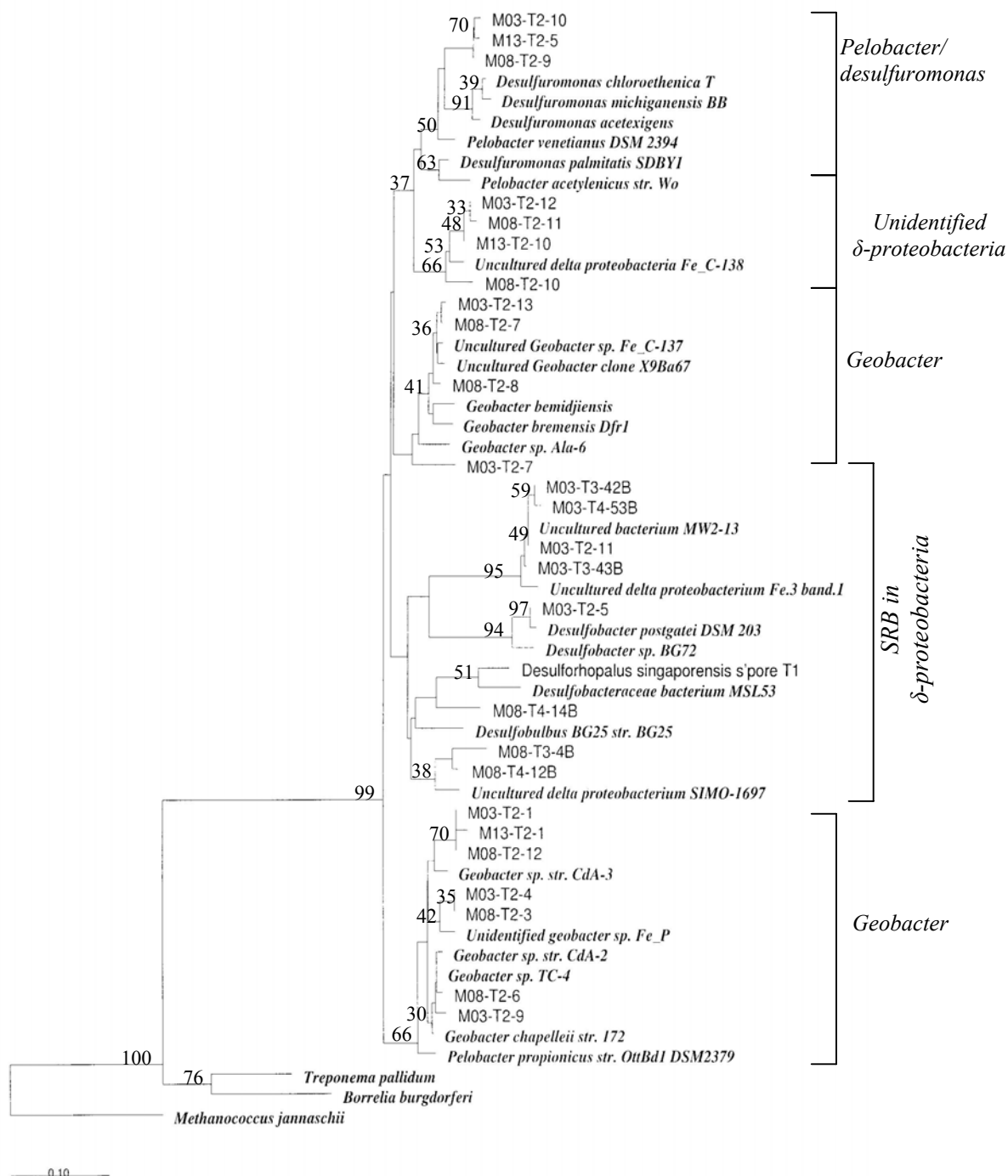


Figure 4-28. Neighbor-joining analysis of δ -proteobacterial 16S rDNA sequences recovered from all bio-trap beads samplers. Relationships with reference organisms from δ subclass of the *proteobacteria*, obtained from RDP. Numbers on the tree refer to bootstrap values on 1000 replicates, only values above 30 are given. Scale bar represents 10% estimated change.

Discussions and Conclusions

Microbial Activities Stimulated by Acetate Injection:

Anaerobic respiration Groundwater chemistry was monitored continuously before, during and after acetate injection, the results showed substantial differences between the background and downgradient monitoring wells, with little chemical shift in the background samples, while strong evidences indicated reducing activities in down-gradient monitoring wells after acetate injection. This differentiates the result of electron donor amendment from the result of natural fluctuation and a biostimulation effect caused by acetate injection is clearly demonstrated.

In all down-gradient monitoring wells, the redox potentials underwent intense reduction for a period of approximately 1+ month subsequent to acetate injection, indicating that there was a lack of oxygen and reducing condition. The significant correlation between redox potential and sulfate concentration (in all down-gradient monitoring wells, Fig.4-18 (I), 4-18 (II)) indicate that the observed reduction is largely caused by the oxidation of the acetate coupled with sulfate reduction.

The quinone ratio also suggested a transition from aerobic to anaerobic respiration after acetate injection, with UQn/MKn dropping below 1 in down gradient monitoring wells roughly one month after injection (Table 4-3), while the ratio of UQn/MKn in background wells remained well above 1, indicating aerobic or partial microaerophilic condition in the presence of Gram-negative bacteria (Polagase et al 1966; Collins, 1981; Hiraishi 1999; Geyer et al. 2004). Clearly, aerobic or microaerophilic condition of the

original subsurface groundwater was altered to anaerobic condition as injected acetate was microbiologically oxidized.

Nitrate reduction For anaerobic respiration, microorganisms utilize electron acceptors other than oxygen. Coupling oxidation of organic acetate compounds with nitrate reduction is more thermodynamically favorable than reduction of other electron acceptors abundantly present in the environment (e.g., Fe (III), U (VI) and sulfate), though there are exceptions, such as that sulfate-reducing bacterial strain UFZ B 490 preferred sulfate as electron acceptor in the presence of both sulfate and nitrate, irrespective of whether it had been precultivated on sulfate or nitrate (Pietzsch and Babel, 2003). Nevertheless, it is generally accepted that nitrate would inhibit the growth of metal reducing organism. But it was not the case in this experiment, nitrate concentration was consistently below detection limit, until later stage when an insignificant amount was detected (Table 4-1), thus they pose little concern for inhibiting metal-reducers in this study.

The slight increase of nitrate occurred in down gradient wells only, suggesting its correlation with microbial activities stimulated by acetate injection.

Sulfate reduction and iron reduction The dissolved ferrous iron and sulfide have been found to be good indicators of reductive processes, which was true in this study. Fe (III) reduction was directly evidenced by the rapid accumulation of soluble Fe (II). Sulfate reduction was supported by a parallel increase of sulfide.

The highest rate of Fe (III) reduction was seen in well M-03 (Fig. 4-6A). However, despite the production of soluble Fe (II) following the acetate application, dissolved ferrous iron concentrations declined afterwards and eventually remained at pre-application levels or even lower, suggesting the formation of minerals or possible iron

reoxidation. The loss of sulfate was accompanied by an accumulation of sulfide and a decrease in soluble Fe (II), which suggests precipitation of iron sulfides more likely. The redox potential stayed below zero during this time, that provides additional support for this explanation.

Sulfate was quantitatively the dominant electron acceptor with an initial concentration of 850 ± 44 mg/L (in all background and down-gradient monitoring wells), while U (VI) initial concentration had an average value of 210 ± 80 ug/L and nitrate was mostly below detection limit. Reduced redox potentials were indicative of the corresponding sulfate reduction that was found to be significant following the acetate injection.

In all down-gradient monitoring wells, sulfate reduction was shown to be persistent with highest rate in well M-08, coinciding with highest biomass in well M-08, which suggests that the rate of reduction may be a function of microbial abundance. Further, the monitoring wells of M-03 and M-08 were noticeably more reduced than well M-13 (Fig. 4-5A), This is likely due to their higher rate of metal and sulfate reduction (Fig. 4-6, 4-7), and could be attributable to both bacterial abundance and composition.

The competitive relationship between microbial Fe and sulfate reduction is presumed to function through concentrations of important common electron donor such as acetate and the availability of respective electron acceptors. When sufficient Fe (III) is available, Fe reduction may deplete the donor concentrations below the threshold of utilization by sulfate reduction (Lovley, 1988; Lovley et al., 1987); When electron donor is sufficiently abundant, sulfate reduction will take over iron reduction eventually, which was the case in this study and Fe (II) production occurred before the onset of sulfate reduction (Fig. 4-

9). The two reducing activities were shown to overlap and coexist afterwards, this is consistent with the heterogeneity nature of the environment.

U reduction and its U stability Uranium reduction was observed in all down gradient monitoring wells. Previous investigations suggested that removal of uranium in natural contaminated environment resulted from reductive microbial precipitation of UO_2 and also other possible mechanisms, such as adsorption to solid surfaces, indirect reduction by sulfide produced by sulfate-reducing bacteria (Liger et al., 1999; Mohagheghi et al., 1985). However, data showing that U (VI) reduction was initiated before sulfate reduction started (Fig. 4-9), which supports the conclusion that uranium reduction in this study occurred via a direct enzymatic pathway.

After the acetate addition stopped, the reduced low level uranium was still maintained in down-gradient monitoring wells, especially with well M-08, where sulfate reduction was most active. It is not understood whether this sustenance is the result of continuing enzymatic reduction or the result of other mechanisms such as a biotic precipitation. The low uranium concentrations resulted from acetate injection showed best stability in wells M-03 and M-08 (Fig. 4-11), this suggests that the biotrap from wells M-03 and M-08 during that period are most likely reflective of uranium reducing populations or conditions, also raised the question of SRB for their role in Uranium reduction and sustenance, as sulfate reduction was dominant process in those wells. Further the consistent lower uranium concentration at deeper depth compared to that at shallower depth imply the involvement of anaerobic microbes. Thus, it is reasonable to hypothesize that the sustenance of low uranium concentration is directly or indirectly related to anaerobic sulfate reducers.

Impact of Biostimulation on Microbial Distribution and its Implications: The

limitation of molecular methods such as cell preferential lyses, DNA extraction efficiency, primer selectivity and coverage, number of PCR cycles, and limitations of DGGE analysis, all of which will distort sequences from their original *in situ* habit in the sampled microbial community. However, all samples were treated equivalently, suggesting inter-sample comparisons should be valid (Von Wintzingerode et al., 1997; Gotelli et al., 2001).

The PCR-DGGE analysis of rRNA gene in this study have resulted in the recovery of over 15 major lineages in the bacterial domain. All of the analyzed sequences were affiliated with recognized bacterial divisions and the majority (95%, 93 out of 98) belongs to known groups with cultured representatives. It is presumed that the distribution of sequence approximately reflects the *in situ* distribution of organisms.

A temporal (T1, T2, and T3, T4) and spatial (B-02, M-03, M-08 and M-13) distribution of the bacterial population was demonstrated and established taxonomically. Over the time course, background well showed no significant community shift, that was consistent with the chemical measurement -- indicating minimum natural fluctuations. The down-gradient monitoring wells, on the other hand, exhibited significant enrichment of *Geobacter*, *Desulfuromonas* and other SRB sequences during the injection, this enrichment disappeared after the injection stopped and was replaced by sequences originated from organisms of *Sulfuricurvum*, SRB within *δ-proteobacteria*, and gram positives related to either *Desulfotomaculum*, or *Clostridium*.

Consistent with the reducing conditions determined by geochemical analysis, well M-13, furthest away from the injection gallery, appeared to be less similar with other down gradient monitoring wells in both community composition and the extent of *Geobacteraceae* sequence enrichment during the injection (T2, Table 4-4). After the injection (T3, T4), the SRB within *δ-proteobacteria* were frequently detected in well M-03 and M-08, but not in well M-13. The above differences likely reflect the subsurface reducing conditions caused by bacterial populations stimulated by acetate injection. The lowest sulfate reduction in well M-13 (Fig. 4-7) with SRB sequences below detection coincide with the uranium fluctuation observed in this well. Clearly, potential exists for the direct or indirect involvement of SRB in Uranium bioreduction and its *in situ* stability. A number of sulfate reducers within delta subdivision of the *proteobacteria* were found to be able to reduce Fe (III) (Coleman, et al., 1993; Lovley et al., 1993a), and uranium (Lovley et al., 1992a; Spear et al., 2000). It was reported in a biofilm study, that U was immobilized both enzymatic ally by SRB and chemically, by reacting with microbially generated H₂S (Beyenal et al., 2004). Previous study also suggested that in some marine sediment, U (VI) might be reduced within the sulfate reducing rather than the Fe (III) reducing zone (Klinkhammer et al., 1991). Barton et al. (1996) reported that the sludge inoculums growing in the presence of higher concentration of sulfate (3.0 g/L) accounted for greater levels of U (VI) reduced than when low level sulfate (0.3 g/L) was available. Further SRB cultures -- both mixed and pure, have exhibited a faster rate of uranium reduction in the presence of sulfate and no lag time until the onset of U reduction in contrast to U alone (Spear et al., 2000).

Clearly sulfate reduction play important role in uranium reduction. Based on observation in this study and previous reports, it is reasonable to hypothesize that sulfate reducing organisms may reduce U (VI) and sulfate in the subsurface groundwater when it became anaerobic during bioremediation. It is the sulfate reducers or sulfate reduction that directly or indirectly maintain the reduced low uranium level in this experiment. However, abiotic mechanism involving sulfate reduction is not excluded, as bacterial biomass declined after initial stimulation, while low level U was relatively stable. As a well-known iron reducer, *Geobacter* has been intensively studied and implicated to be responsible for reducing uranium in a number of environments (Lovley et al., 1991; Gorby et al., 1992; Holmes et al., 2002; Nevin et al., 2003). The enrichment of *Geobacter* in downgradient monitoring wells was one of the most pronounced observations in this study, and the most prominent population that considered being involved in biostimulation and uranium bioreduction. However, those sequences were not detected after acetate injection stopped. Thus the occurrence of significant, lasting changes in uranium concentration in downgradient monitoring wells after the acetate amendment appears to challenge the idea that *Geobacter* is responsible for uranium reduction and immobilization at the Old Rifle site. At least they apparently have no role in maintaining the reduced low uranium concentration.

The significance of detected Gram positives related to *Clostridium* or *Desulfotomaculum* in this investigation is unknown. However, considering the uranium concentration increase occurred in well M-03 towards the end of the experiment (6-9 months after acetate injection stopped), coincidentally, Gram-positives were not retrieved from this well but appreciably detected in M-08 and M-13 during the same time period. This may merit

further study of Gram positives for their potential to reduce uranium. Francis et al. (1994) showed U (VI) reduction by a *Clostridium* sp. and it was inferred that organisms related to *Clostridium* sp. are partially responsible for U (VI) reduction and removal of uranium from solution. Their result implies that U (VI) can be reduced in natural and contaminated anaerobic sediments during fermentation, before the onset of dissimilatory Fe (III) or sulfate reduction with H₂ and short-chain fatty acids produced by fermentation. Also, previous work in this lab demonstrated a correlation between uranium concentration and *Desulfotomaculum* sequence cluster at Shiprock – another UMTRA site, which implying possible involvement of *Desulfotomaculum* in uranium reduction (Chang et al., 2001). The dormant, sporeforming *Desulfosporosinus* and *Clostridium* sp. are ubiquitous even in oxic sediments and soils (Widdel, 1991), these organisms may be stimulated with addition of sulfate and nutrients to remove uranium from groundwater at contaminated sites.

Sulfuricurvum kujiense is a facultative anaerobic, chemolithoautotrophic, sulfur-oxidizing bacterium, strains YK- were isolated from an underground crude-oil storage cavity at Kuji in Iwate, Japan. It utilized sulfide, elemental sulfur, thiosulfate and hydrogen as the electron donors and nitrate as the electron acceptor under anaerobic conditions, but it did not use nitrite. The organism did not grow on sugars, organic acids or hydrocarbons as carbon and energy sources. Considering the nitrate increases in down gradient monitoring wells seen two months after the injection (Table 4-1), the occurrence of *Sulfuricurvum* sequences in all those three wells are likely associated with nitrogen cycle. Before the acetate injection, the low nitrate level was probably maintained by common denitrifiers such as *Pseudomonas*, and many β -proteobacteria native to the environment. During the

injection, *δ-proteobacteria* such as *Geobacter*, SRB etc. were significantly enriched and out compete the native denitrifiers, which caused slight increase in nitrate level. After the injection, the ecology of the groundwater has been altered and favored the growth of *Sulfuricurvum*, which contributed to the *in situ* stability of reduced uranium by keeping the nitrate down, thus promoting the metal-reducing organisms.

It is recognized that the abundance of rRNA genes discussed above does not necessarily reflect the relative abundance of organisms, since the number of rRNA genes varies in different organism (Farrelly et al., 1995). Additionally, PCR potentially skews relative proportions of different sequence type during the amplification cycles (Farrelly et al., 1995; Reysenbach et al., 1992; Suzuki et al., 1996). However, PLFA analysis provides additional line of evidence that supports the results obtained from DNA data. The cluster analysis also suggested grouping among communities from background wells, as well as down-gradient monitoring wells after the injection stopped (Figure 4-22). The PLFA profiles retrieved from downgradient wells during active biostimulation and bioreduction were clearly separated and form a distinct cluster. An increase of terminally branched saturates within the community, indicating an increase of anaerobic sulfate reducer proportion, parallels with an enrichment of *Geobacteraceae* and SRB within the *δ-proteobacteria*. The total bacterial biomass also reflected the biostimulation effect, indicating a significant enrichment of the bacterial biota.

Conclusions: Microbial Fe reduction is an important process in many aquatic types of sediment. In a recent compilation, Fe reduction contributed 22% on average to anaerobic carbon oxidation in 16 different continental margin sediments, with the rest being

primarily coupled to sulfate reduction (Thamdrup 2000). Sulfate reduction is an environmentally significant process, as SRB can be responsible for ~50% of the total carbon mineralization in active coastal sediments (Jørgensen et al., 1982). A detailed understanding of the regulation of Fe and sulfate reduction in subsurface includes knowledge of the quantitatively important Fe (III) and sulfate reducing bacteria. However, the size and composition of the Fe and sulfate reducer microbial community in subsurface is virtually unknown. This study demonstrated the importance of these two processes in stimulated uranium bioreduction, and increased our knowledge of iron reducers and sulfate reducers.

Abdelouas et al. (2000) have shown that bacteria capable of reducing U (VI) to U (IV) are ubiquitous in nature. The uranium reducers are primarily sulfate reducers and are stimulated by adding nutrients to the groundwater, such as ethanol and trimetaphosphate. The data described in this thesis demonstrated the stimulation by adding acetate, and lead to a conclusion that sulfate reducers played important role in reducing uranium and maintaining the low concentration of uranium at Old Rifle site. Iron reducer such as *Geobacter* may have worked sequentially with sulfate reducers in this case, but apparently not involved in *in situ* uranium stability. Denitrifiers and organisms of *Sulfuricurvum* contributed to the reduced uranium stability by keeping the nitrate level down.

Some similarities between field and laboratory experiments were observed (refer to chapter 3) In both cases, a sustained change in bacterial community structures occurred. Furthermore, selection for *Geobacter* and SRB was demonstrated both in field and at laboratory incubation. This validates the usage of laboratory experiment, and provides

convenient experimental model for future bioremediation experiment. However, differences were also observed, such as the changes in pH values exhibited opposite trend between microcosm and field experiment. This is likely due to the differences between an open system and a sealed microenvironment. Additional differences about detection of *Pseudomonas* and *Desulfuromonas* were also noticed. All of these strengthen the importance to verify the laboratory results in actual field investigation.

Chapter 5. Linking the Identity to Function --- a Field Scale Trial

Introduction

A major challenge in microbial ecology and biogeochemistry is to connect observed biogeochemical processes to the bacterial populations responsible for carrying them out, which is particularly difficult at field scale environment.

The application of molecular methods has provided considerable information regarding the microbial populations in the environment studied, but it did not reveal any information regarding the function of communities associated with the specific environmental process. Only within the last few years has the use of stable isotope probe (SIP) coupled with DNA (Radajewski et al., 2000, 2002, 2003) or PLFA (Hanson et al., 1999; Wick et al., 2003; Fang et al., 2004) analysis been explored. Microorganisms that utilize ^{13}C -labelled compound incorporate the label into their macromolecules (lipids, nucleic acids, amino acids, etc.), thus providing direct evidence of their involvement in specific activity. In spite of the success of SIP-based studies for the analysis of microbial population (Lin et al., 2004; Hutchens et al., 2004; Arao, 1999), this approach has seldom been applied to field scale experiment. The factor that ^{13}C labeled substrate will be greatly diluted in an unconfined natural environment has limited its implementation in field scale investigation. The development of Bio-Sep beads provides a practical strategy to harbor the ^{13}C amendment within the beads and provide slow releasing ^{13}C labeling substrate in the beads (Sublette et al., 2003).

To optimize *in situ* uranium bioremediation strategy, it is critically important to first understand indigenous organisms present and to characterize their spatial and temporal

fluctuation under the condition of biostimulation as it was investigated in Chapter 4. Now in this Chapter, the effort is focused on linking the biogeochemical functions to the identity of those indigenous organisms. This chapter documents the detection of microbial incorporation of ^{13}C labeled acetate in an *in situ* uranium bioreduction test at the Old Rifle field. The activated powdered bio-sep beads in bio-trap samplers were infused with 99% ^{13}C acetate (Sublette et al., 2003) and served as a slow releasing C13 labeling source. To my knowledge, this is the first attempt to follow uranium remediation using ^{13}C DNA/PLFA biomarker directly at the field scale in a natural contaminated subsurface environment. The aims of this study have three folds: one to determine the feasibility of detecting ^{13}C -incorporation in DNA/PLFA biomarkers derived from suspended bio-trap samplers; second is to examine active microbial populations stimulated by acetate injection; third is to link the microbial identity to geochemical functions. Bacterial populations were analyzed at a selected time point by DGGE analysis of ^{13}C labeled 16S rRNA gene fragments generated by PCR amplification with universal bacterial primers. Viable biomass, community structures was examined using phospholipid fatty acid analysis (PLFA). The results demonstrated the effectiveness of using ^{13}C labeling to link the microbial populations to biostimulation and uranium reduction by focusing on the members actively involved.

Materials and Methods

Beads Amendment: Bio-Sep beads “baited” with ^{13}C -sodium acetate were fabricated by suspending powdered ^{13}C -sodium acetate (99%, ISOTEC) and PAC in a solution of Nomex in dimethylacetamide (DMAc). This suspension was then forced under pressure

through a nozzle with droplets falling into an aqueous quenching solution. Beads about 3-4 mm in diameter were obtained with ^{13}C -sodium acetate and PAC uniformly distributed throughout the beads as determined by microscopic examination. The final composition of the beads was approximately 20-25% ^{13}C -sodium acetate, 50-55% PAC, and the balance Nomex. The porosity was comparable to standard Bio-Sep beads (about 74%). Bio-traps baited with ^{13}C -sodium acetate were packed with about 100 Bio-Sep beads, which had been fabricated as described above to contain powdered ^{13}C -sodium acetate. In water these beads acted as a slow-release source of labeled sodium acetate (Sublette et al., 2003).

The bio-traps baited with ferrihydrite were the normal standard bio-traps with total of 10-20 mg ferrihydrite added to the bottom and middle of the traps respectively. They were included in the test to examine any possible enrichment effect for uranium reducers.

Bio-trap Sampling: Both standard (ctr) and 2-line ferrihydrite bio-traps (iron) constructed from ^{13}C baited beads, were equipped with multi-level samplers (MLS) and suspended down well at depth of 5.04 ± 0.03 m (15.12 ± 0.09 ft) m. The traps were deployed on 8/8/03 and incubated for a period of around 30 days down in the wells as described by Peacock et al. (2004). These traps were retrieved from wells B02, M03, M08 and M13 (Fig. 4-1) on 9/5/03, nearly 70 days after acetate injection initiated. Once recovered the bio-trap samplers were frozen onsite with dry ice, shipped to the CBA laboratory, and stored at -80°C prior to Lipid and DNA analysis. For detailed test plot and acetate injection plan, refer to chapter 4---Materials and Methods.

Others—DNA, PLFA, IRMS: The measurement of stable carbon isotope ratios ($^{13}\text{C}/^{12}\text{C}$) were attempted by a Finnigan (Austin, TX, USA) Delta Plus isotope ratio mass spectrometer with a GC-III Combustion interface and an Agilent 6890 gas chromatograph (Palo Alto, CA, USA) (refer to chapter 3—Materials and Methods). The detailed methods for DNA and PLFA analysis also can be found in chapter 3.

Nucleotide Sequence Accession Numbers: Partial 16S rDNA sequences recovered from this study were submitted to GenBank as with accession numbers AY994914 to AY994977.

Results

Biomass and Community Structure Based on PLFA: The bacterial biomass (measured as pmol PLFA per bead, Fig. 5-1) was determined to include the total PLFA minus the normal saturates over 18 carbons in length and the polyenoic PLFA. The trace quantities of PLFA of unknown structure were also excluded from the prokaryote biomass. The biomass content at the site varied considerably between background and monitoring wells. The highest amount of biomass 138 pmol/bead was detected 7.3 meter away from injection gallery, with the iron trap in well M08. The lowest detectable biomass of 1.55 pmol/bead was from monitoring well M-13. Bacterial biomass content in the traps taken from the background well B02, and well M-13 (control beads) was below detection limit. Iron traps exhibited a higher biomass compared with control traps. More interestingly, the biomass at monitoring well M-08 was highest among all four wells with both type of

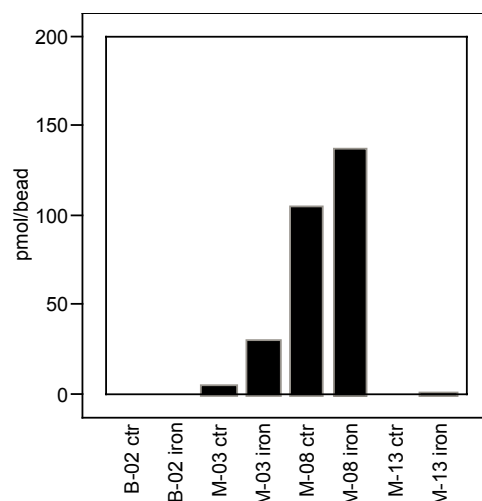


Figure 5-1. Bacterial biomass recovered from iron trap (iron) and control trap (ctr) in background (B-02) and down-gradient monitoring wells (M-03, M-08 and M-13).

traps (Fig. 5-1), which coincided with the highest sulfate and uranium reductions observed in well M-08 (chapter 4).

PLFA analysis also revealed a relative simple community structure, mainly consisted of normal saturates, monounsaturates and terminally branched saturates. Figure 5-2A shows a comparison of the relative percentages of total PLFA functional groups from the biomass. With 3 out of all 8 samples were below the detection limit, reliable statement for community shift was difficult to make. However, combining the results obtained at a different depth and same incubation period (presented in Chapter 4), it was possible to examine the trend of community fluctuation.

Monounsaturates, indicative of Gram-negative bacteria showed a noticeable increase (nearly doubled or more) in monitoring wells compared with background well. Samples from well M-03 and M-08 contained significantly more terminally branched saturates

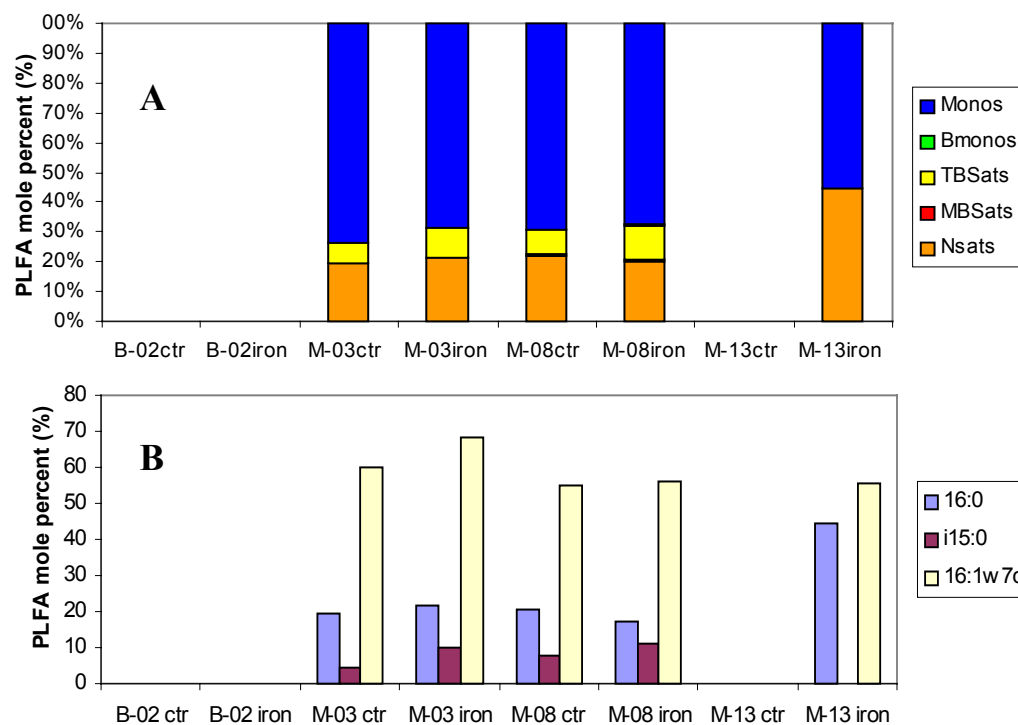


Figure. 5-2. PLFA microbial community profiles (A) and potential signature lipids for *Geobacteraceae* (B) detected from Biosep beads suspended in four selected background and downgradient monitoring wells (B-02, M-03, M-08 and M13). For full name of PLFA abbreviations, refer to Fig. 4-21.

(including i15:0, i17:0 and 10me16:0) than did samples B-02 (below detection), or M-13 (only detected at 6 meter deep). Although most often associated with the presence of Gram-positive bacteria, these terminally branched saturates, and in particular the i15:0 and i17:0 are often detected in Gram-negative sulfate reducing bacteria (Wilkinson 1988), 10me16:0 was an indicator of *Desulfobacter* sp. (Dowling *et al.*, 1986). The amount of fatty acids of i15:0, 16:0 and 16:1w7c, can also potentially indicate *Geobacteraceae* (Anderson *et al.*, 2003), were highest in well M-03 and M-08. (Fig.5-2B)

Microbial Population Incorporating ^{13}C -Acetate: Due to the relatively low biomass, ^{13}C DNA fraction was difficult to be visibly identified after CsCl gradient centrifugation

and thus was withdrawn according to standard indication. Sufficient PCR products for DGGE analysis were obtained from all ^{13}C DNA fractions extracted.

The banding pattern revealed by DGGE analysis indicated community shifts — a markedly different structure for background well, while monitoring wells M-03 and M-08 share most similarities, and well M-13 apparently share little commonality with other two monitoring wells (Fig. 5-3). Phylogenetic trees in Fig.5-4 and 5-5 showed the major bacterial lineages recovered, that incorporated the ^{13}C acetate.

The background wells displayed the highest level of diversity. Sequences retrieved include *α -proteobacteria*, *β -proteobacteria*, *Actinobacteria*, and organisms belong to *Flexibacter-Bacteroides-Cytophaga* division (CBF phylum). Among them, *β -proteobacteria* (6 out of 18, 33%) and *α -proteobacteria* (8 out of 18, 44%) constitute the majority of the sequences, such as *Methylophilus*, *Hypomicrobiaceae* etc. (Fig.5-5)

The monitoring wells of M03 and M08 exhibited a simpler diversity and analysis of ^{13}C 16S rDNA sequences retrieved displayed a predominance of *δ -proteobacterial* phylotypes, represented by four lineages. The a cluster is the largest lineages containing 14 bacterial sequences belong to *Geobacter* and 13 of them were recovered from wells M-03 and M-08. The b cluster includes three sequences obtained from monitoring wells, and it was weakly detected in wells M-03 and M-13, but strong in well M-08.

Comparative sequence analysis revealed that none of the b sequences was identical to 16S rRNA sequences of cultured microorganisms. However they branched within radiation of cultivated representatives of well-known *Pelobacter/Desulfuromonas* genera. The c cluster sequences represented a novel cluster so far described only by an environmental sequence originated from iron reducing landfill leachate-polluted aquifer,

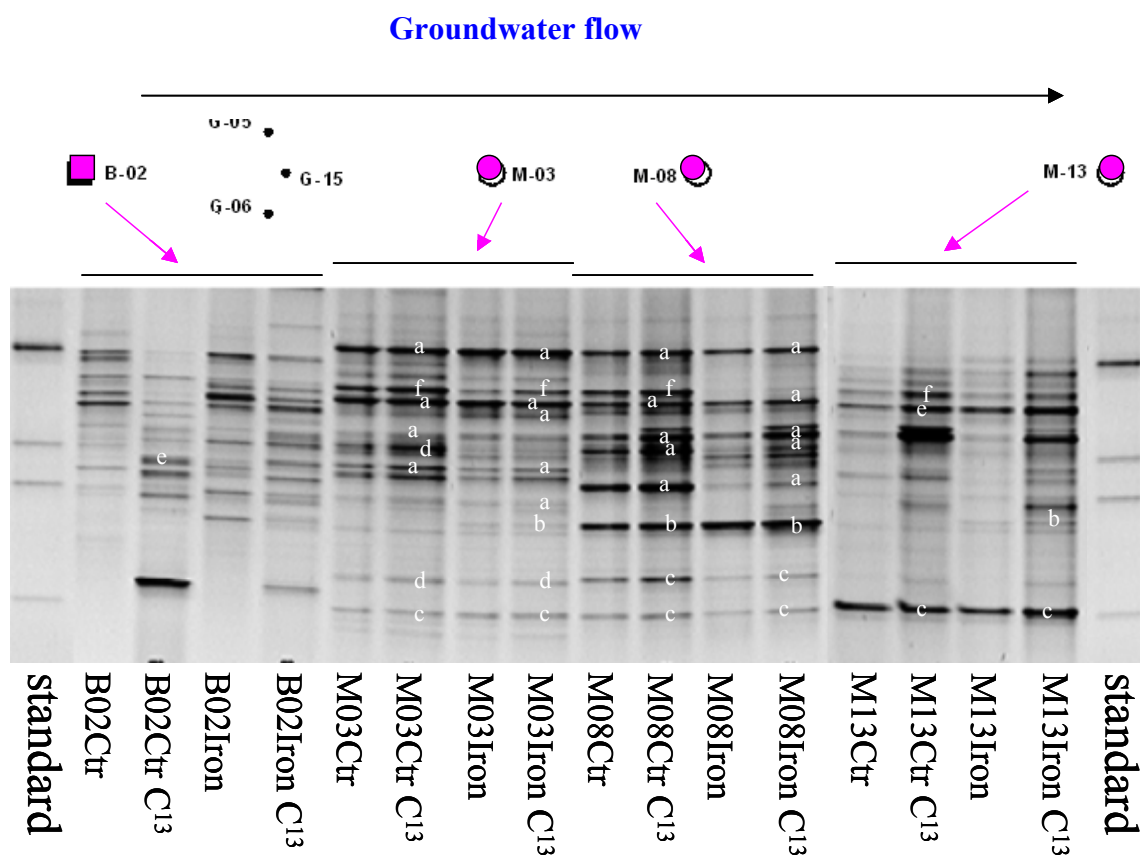


Figure. 5-3. DGGE analysis of microcosm bacterial community recovered from beads. Amplified ¹³C16S rDNAs were separated on a gradient of 20 % to 65 % denaturant, along with its overall mixed DNA profile (eg. M08Iron C¹³ and M08Iron). Ctr. represents the standard control trap samplers, Iron represents the two line ferrihydrite biotrap. Band label: a-*Geobacter*, b-*Pelobacter/Desulfuromonas*, c-unclassified δ -*proteobacteria*, d-*Desulfobacter* and its relatives, e, f-*Pseudomonas*. For more detailed designation information, refer to Fig. 5-1 and Fig.5-2

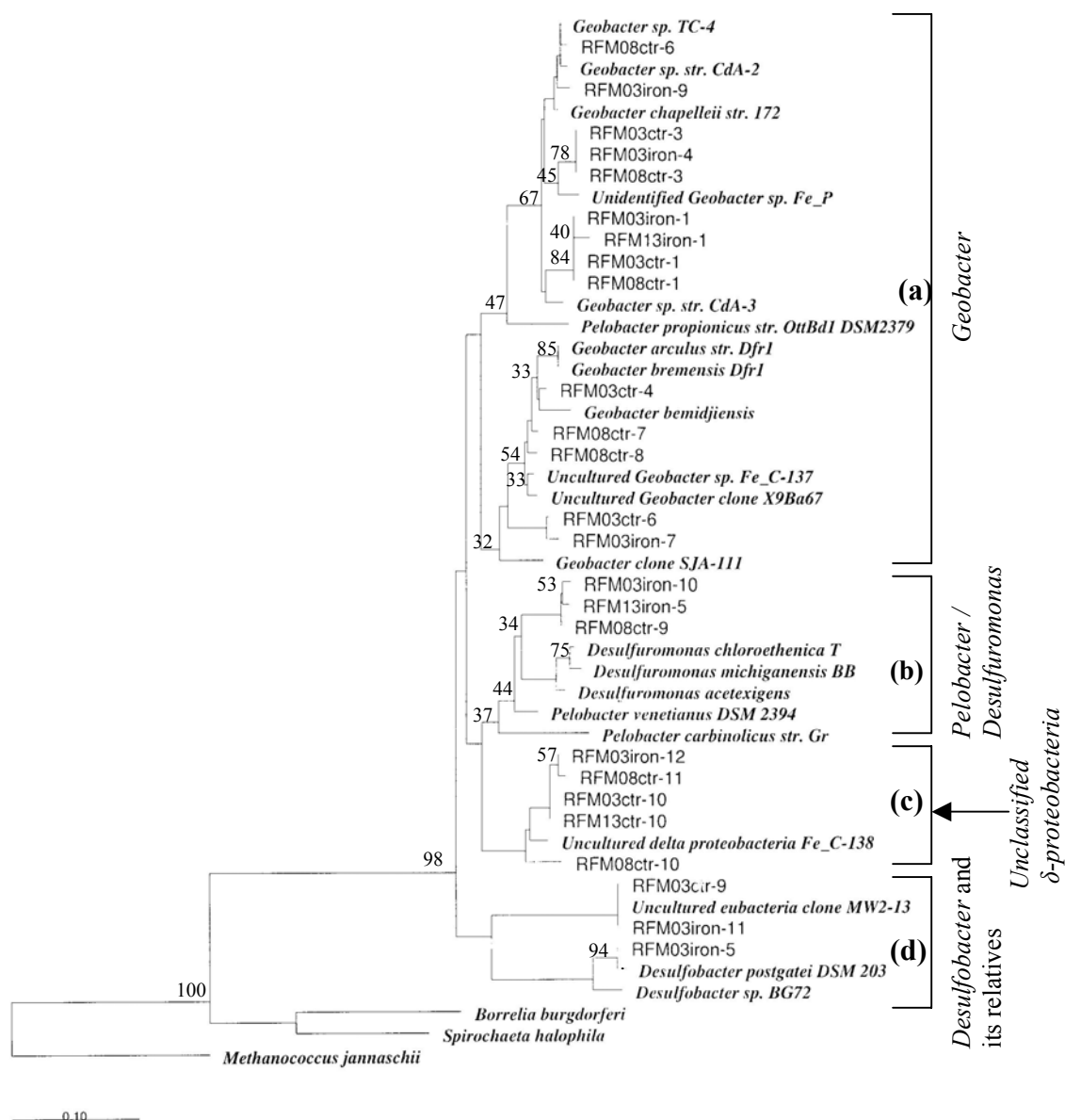


Figure 5-4. Neighbor-joining analysis of ^{13}C 16S rDNA sequences recovered from ^{13}C baited beads that show relationship with reference organisms from delta subdivision of the *proteobacteria*, obtained from RDP. Numbers on the tree refer to bootstrap values on 1000 replicates, only values above 30 are given. Scale bar represents 10% estimated change.

Sequences prefixed “RF” were generated during this study. Nomenclature: The prefix (“RF”) is followed by the well number (B02, M03, M08 and M13), then bead type (ctr. for control or iron) and at the end, band number from original DGGE gel image.

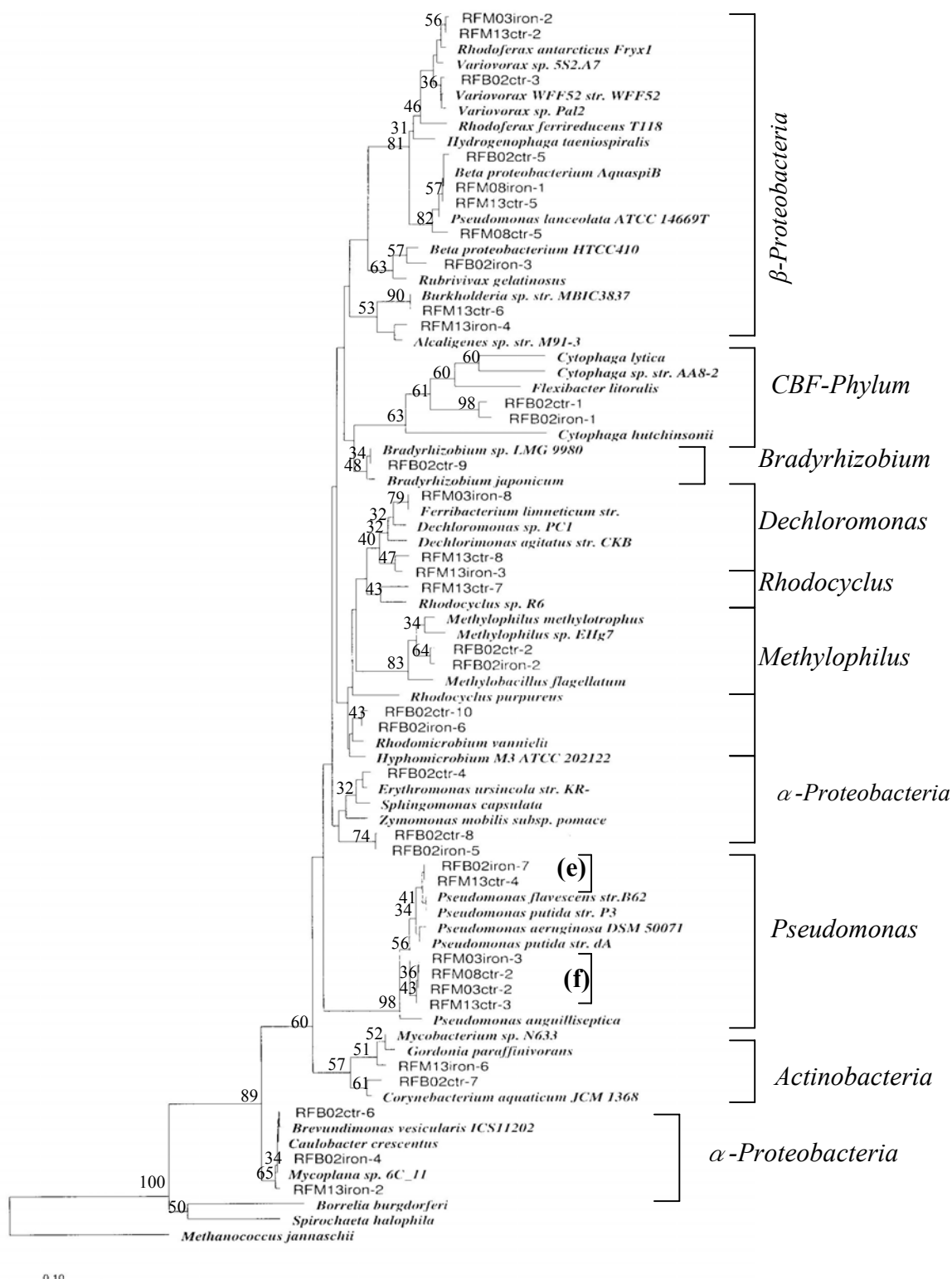


Figure 5-5. Neighbor-joining analysis of ^{13}C 16S rDNA sequences recovered from ^{13}C baited beads, that shows relationship with reference organisms from bacterium other than delta subdivision of *proteobacteria*, which were retrieved from RDP and GeneBank. For nomenclature, boot strap values, refer to Figure 5-4 legend.

similar to this research's environment (AY752784). The c cluster sequence was detected in all three monitoring wells, but much more stronger in well M-13. The d cluster sequence was only retrieved from well M-03, and represented by *Desulfotobacter* and those peripherally related to *Desulfobacter* (Fig. 5-4). *Pseudomonas* sequence of cluster f was also a major component of the community in wells M-03 and M-08, which was different from the *Pseudomonas* seen in background well (cluster e, Fig.5-5).

All *Geobacter* bands remained strong in wells M-03 and M-08, but had disappeared from M-13 and to be replaced by more different banding patterns, sharing in common with the background for some sequences derived from α and β -proteobacteria, *Actinobacteria* and *Pseudomonas*. (Figs. 5-3 and 5-5).

There are minor differences detected between control and iron traps, but they generally exhibited similar pattern. Significant enrichment of one type sequence over another was not observed.

GC-IRMS analysis was unable to analyze all FAME peaks desired, due to unexpected problems occurred with PLFA extraction and instrument sensitivity, but ^{13}C enrichment in the fatty acids was still demonstrated, particularly, the fatty acid of 16:1 ω 7c from samples in well M-03 and M-08 were highly labeled by ^{13}C (data not shown).

Discussions

Anaerobic microorganisms typically use additional electron acceptors in the following order of preference: nitrate, ferric iron, sulfate, and finally carbon dioxide. Evaluation of the distribution of these electron acceptors can provide evidence of where and how

uranium bioreduction is occurring. Based on chapter 4, the subsurface reducing condition has been characterized. The redox potential of all three monitoring wells were reduced to an intensity of less than 0 mV about one month after the acetate injection, which spanned the ^{13}C bio-trap incubation duration and sampling event. Sulfate reduction was evidently active in this phase and iron, uranium reductions were also demonstrated.

Active Populations Involved in the Processes: The analysis of ^{13}C labeled 16S rDNA PCR-DGGE revealed a significant change of microbial populations present in the subsurface upon acetate injection. In wells M-03 and M-08, nearly all bacterial community captured on bio-trap beads was active in ^{13}C acetate utilizations. However, PCR-DGGE migration pattern also proved that rDNA templates present in “heavy” fraction were distinct from the overall unseparated community---most noticeably in wells B-02 and M-13, which is an important prerequisite for the correct interpretation of environmental SIP data.

Reflective of *in situ* microbial activities stimulated by acetate injection, the wells B-02 and M-13 exhibited a different banding pattern from wells M-03 and M-08, with sequences originated predominantly from α - and β -*proteobacteria*. The monitoring wells of M03 and M08 displayed a relatively simple diversity with 6-9 strong bands (as labeled, Fig.5-3). The derived sequences from those bands a, b, c, and d suggested dominance of the community by Gram-negative microorganisms belong to δ -*proteobacterial* phylotypes, represented by four distinct lineages, including *Geobacter*, *Pelobacter/Desulfuromonas*, unclassified δ -*proteobacteria* and *Desulfobacter* and its close relatives (Fig. 5-4).

PLFA analysis provided additional evidence by revealing the dominance of monounsaturates (represented over 65% of the total PLFA) in monitoring wells, which may be an indication of a large population of Gram-negative bacteria within the community (Zelles, 1999; White et al., 1998a). Together with the stimulation of terminally branched saturates in monitoring wells—indicating anaerobic sulfate reducing bacteria, they represented the largest change in the microbial community structure upon acetate injection.

Members of the *Geobacteraceae* family are well known Fe (III) reducing organisms and have been isolated from many sedimentary and groundwater subsurface environments (Lovley, 2000a) and was previously reported to be associated with uranium reduction (Anderson et al., 2003). The predominance of ^{13}C labeled *Geobacter* sequences in the wells M-03, M-08 and the uranium reduction activity suggested by geochemistry are strong indications that these bacteria conducted the acetate oxidation and are quantitatively important in the uranium bioreduction stimulated by acetate injection.

Pelobacter or *Desulfuromonas* is another major component retrieved in monitoring wells, mostly in well M-08. Both *Pelobacter* and *Desulfuromonas* belong to the family of *Geobacteraceae* and are able to reduce Fe (III), while *Pelobacter* are primarily hydrogen-oxidizing Fe (III) reducers and are not able to oxidize organic acids completely, *Desulfuromonas* can oxidize acetate to carbon dioxide (Loneragan et al., 1996; Lovley, 2000a). *Pelobacter* species are phylogenetically interwind among acetate-oxidizing *Geobacteraceae*, and are interspersed among *Desulfuromonas* and *Desulfuromusa* species. Further, given the fact that band b was strongest in well M-08, where highest sulfate reduction occurred, plus the sequence cluster b appear evolutionally more closer

to *Desulfuromonas* lineage (Fig. 5-4), it is reasonable to suspect that these sequences are more likely originated from *Desulfuromonas sp.*, capable of both sulfate reduction and Fe (III) reduction. More sequence information is required to permit refined analysis.

Additional phylotype c, affiliated with the δ -subclass of *proteobacteria*, not grouped with any cultured representatives, that was recovered from all three monitoring wells, with strongest in well M-13. Phylogenetic affiliation makes it likely that they are associated with *Pelobacter/Desulfuromonas*. Furthermore, this cluster share ~96% sequence identity with an uncultured δ -*proteobacteria* Fe_C-138 (AY752784), which was originated from a Banisveld landfill iron-reducing clean part of groundwater. It also share 95% sequence identity with an uncultured iron-reducing bacteria (AY524550) detected from iron (III)-reducer enrichment obtained from acidic subsurface sediment contaminated with uranium (VI). For environmental rDNA sequences with no known cultivated representatives, any physiological interpretations must rely heavily on the environmental conditions of a sample from which the sequence was generated. Based on the groundwater chemistry, the affiliation with other uncultured organisms, we speculate that similar to cluster b, organisms generating c sequences may engage in Fe (III), uranium reducing activity, and capable of sulfate reducing metabolism.

Desulfobacter is a well-known sulfate reducing bacteria and has not demonstrated metal reducing capability. In this study, sequences belong to *Desulfobacter* and its close relatives (cluster d) were detected in well M-03 only and thus may not be directly responsible for uranium reduction.

The ability of bacteria to use Fe (III) or U (VI) as a terminal electron acceptor is not limited to members of the delta subdivision of *proteobacteria*. Another major component

in wells M-03 and M-08 was found to be *Pseudomonas* (band f, Fig. 5-3), and it was different from the *Pseudomonas* detected from background well. Since the nitrate concentration was insignificant, it is not clear whether this organism was responsible for reducing nitrate or participating in removal of uranium.

Method Evaluation and Consideration for Future Research: Molecular techniques for analyzing the bacterial community structure allow monitoring of changes in structure and function of bacterial assemblages during acetate biostimulation. However, studies of microbial community have typically focused either on their structures or their metabolic functions. Linking the structure and function remains a challenge, particularly at field scale. The current research combining ^{13}C baited bio-sep beads and stable isotope probing proved to be a successful attempt in this aspect and holds promises for extended field scale investigation.

The advantage of working with the ^{13}C infused bio-sep beads is the relatively high abundance of biomass that could be recovered, and low cost easier ^{13}C amendment. The very large internal surface area coupled with the adsorptive surface resulted rapid formation of biofilms in these beads when exposed to an aqueous environment. By incorporating the amendments into Bio-Sep[®] beads, incubating the beads in the aquifer to be treated, the effects of the amendments on the microbial ecology of bead biofilms can be evaluated using biomarker analysis. All of which rendered the strategy low cost, easy handling and effective recovery of the community, and that makes it feasible for field application. Furthermore other amendment can be fabricated into the beads for variety of different research purposes.

In all cases, labeling of active populations was achieved by stimulating dissimilation and assimilation of a substrate. However, acetate may in principle be coassimilated by other bacteria that are present and active in the same habitat but that utilize a different type of electron acceptor for dissimilatory purposes. Microbial activities in sediments or groundwater are characterized by complex interactions between different microbial populations, availability and characteristics of electron acceptors, carbon and energy sources, nutrients and geochemical properties of the environment, care should be taken in examining geochemical measurements in order to infer physiological function of the microbial community.

The PCR inherited bias is a well-known phenomenon, it is difficult to assess potential bias introduced during amplification and primer selectivity. The quantitative PLFA analysis complements this PCR defect and the agreement between two analyses supports the conclusion. Furthermore, previous studies have suggested that microbial community analysis with a single copy or low-copy-number genes may be more accurate than analysis with high-copy-number of 16S rRNA gene (Peixoto et al., 2002). Also, 16S rDNA provides no information about potential physiological differences between closely related bacteria, therefore, developing primers targeting at specific function group with low copy number will worth the effort in future research.

The temporal and spatial analysis presented in Chapter 4 provides an initial indication of microbial diversity and confirms the presence of likely U (VI)-reducer organisms (*Geobacter*, *Desulfuromonas* and other SRB within δ -*proteobacteria*). Additional unclassified δ -*proteobacteria* organisms (c cluster) found in the ^{13}C baited bio-traps may have played roles in U (VI) reduction during the acetate injection. Isolation of these

organisms and testing for U (VI) reduction by the isolates are necessary to determine the full range of organisms that may contribute to U (VI) reduction in the Old Rifle site.

Conclusions

Through this study, we gained knowledge of the quantitatively important Fe and sulfate reducing bacteria in a stimulated uranium remediation. The integration of microbiological and biogeochemical approaches proved very useful for investigating the ecology of metal reducing bacteria. Coupling with stable isotope probing and Biosep beads amendment, it provided a practical and promising strategy to link microbial function to its identity in the field scale.

A significant, specific enrichment of microorganisms in the family *Geobacteraceae* associated with the stimulation of Fe (III) and U (VI) reduction and the U (VI) removal from the groundwater was clearly demonstrated in this study. Sulfate reducing bacteria such as *Desulfuromonas* and other unclassified δ -*proteobacteria* are also active and may play important role in reducing uranium (VI) and maintain the reduced U (IV).

Dissimilatory Fe (III)-reducing bacteria are capable of rapid reduction and immobilization of U (VI) and therefore are targeted organisms for uranium bioremediation strategies. However, heterotrophic bacteria are also capable of reducing soluble U (VI) (Barton et al., 1996). Clearly, this is an area that should be pursued and it will be important to expand our studies to other organisms responding to biostimulation in subsurface environment.

Chapter 6. Do Beads Sample the Groundwater or Sediments?

Introduction

Other than added acetate, there is still large endogenous pool of available electron donors in contaminated areas. The electron donors that might favor uranium reduction are largely unknown, but clearly many different types of inorganic or organic contaminants or nutrients are present in groundwater and soil, where the microbial biota distribution will likely reflect the differences of electron donor/acceptor distribution.

The characterization of subsurface microbial populations has mainly focused on microbes recovered from sediment samples (Bhattacharya et al., 2003; Cheung, et al., 2001; Dojka et al., 1998) or groundwater (Kleikemper et al., 2002; Chang et al., 2001). The groundwater is more readily obtained than sediments, but contaminated groundwater poses additional problem of secondary waste generation. Further it has been suggested that those microorganisms most likely to be involved in contaminant transformation are often associated with the surfaces of the geological matrix through which the groundwater percolates (Godsy et al., 1992; Harvey et al., 1984; Hazen et al., 1991; Holm et al., 1992; Kolbel-Boelke et al., 1998). While water-sampling techniques allow for temporal and large-scale spatial sampling of transient or planktonic communities, they may not capture key populations partitioned on the solid phases.

Sediments play a useful role in the assessment of heavy metal contamination (Forstner & Wittmann, 1981). This is because in unperturbed environments, heavy metals are preferentially transferred from the dissolved to the particulate phase and as a result metal concentrations in sediments are generally much higher than in the overlying water and

therefore more easily detected, which results in more pronounced community impact (Bryan & Langston, 1992). Plus, continuous monitoring of water is not all informative, as sediment concentrations indicate contamination loads over longer periods of time (Forstner, 1989). However, subsurface sediment-based diversity studies are often impractical because sediment cores are both expensive and difficult to acquire. Due to these limitations, studies requiring numerous samples (e.g., temporal or spatial studies) are unrealistic with current traditional methods. Developments in bio-samplers such as bio-sep bead traps described in this thesis have helped to overcome this limitation by providing an easily accessible substitute solid surface to which cells can attach and grow (Peacock, Chang et al. 2004; Subtellel et al., 2003). The retrievable traps can contain particles such as hematite with surface properties representative of the natural geological matrix or amendments like acetate to serve as source of added electron donor (Sublette et al., 2003; Reardon et al., 2004). These surfaces serve as sites for colonization by planktonic microbial populations and promote close physical and temporally stable cooperative associations between members of the community. Biosep bead traps thus offer a pragmatic means of recovering populations of microorganisms that form communities optimized to function under a particular set of environmental conditions. However, considering differences between the nature of bio-trap growth supports and the heterogeneous sediments that are the natural environment for indigenous microorganisms, the question needs to be addressed is whether the bio-traps provide a representative view of subsurface communities. This chapter is thus designed to compare the communities retrieved from sediment, groundwater and bio-trap samplers. The investigation indicated that the traps are not a census of subsurface microbial biota, but it

captured key populations under the particular environmental conditions and provides valuable assessment of subsurface microbial communities.

Materials and Methods

The Groundwater: The DNA clone library data for groundwater was obtained by colleagues from the University of Massachusetts (Vrionis et al., 2005). DNA was extracted from groundwater filters, also using the FastDNA SPIN kit (Bio101 Inc. Carlsbad, CA). 16S rDNA was amplified with universal bacterial primers 8F (AGAGTTTGATCMTGGCTCAG) and 907R (CCGTCAATTCMTTTRAGTTT). PCR mixtures contained appropriate volumes of TAQ polymerase and Q buffer (Qiagen, Valencia, CA) to produce a 1x working concentration, 0.5 µg bovine serum albumin, 200 µM deoxyribonucleoside triphosphates (Sigma-Aldrich Co., St. Louis, MO), 25 pmol forward and reverse primers (Sigma Genosys, The Woodlands, Tx) and 1.25 U *Taq* polymerase (Qiagen). PCR tubes containing all reaction components except the template and *Taq* polymerase were UV irradiated for 7 min to ensure sterility. Reactions were performed in a Peltier thermal cycler (PTC 200; MJ Research Inc, Waltham MA) beginning with a 5 minute denaturation at 95°C and then 25 to 30 cycles of 94 °C (30s), 45 °C (1 min), 72 °C (1 min) and a final 10 minute elongation at 72 °C. For most samples, two different reactions with ~5 ng and ~1 ng template DNA were performed. PCR products were isolated by gel extraction (Qiagen), mixed and cloned into the TOPO TA vector pCR 2.1 and introduced into chemically competent *Escherichia coli* TOP10 cells according to manufacturer's instructions (Invitrogen, Carlsbad, CA). Inserts of 16S rDNA from at least 30 clones from each clone library were amplified with M13 forward

and reverse primers (Messing et al., 1982; Vieira et al., 1982). PCR products were cleaned with the QIAquick PCR purification kit (Qiagen) and sequenced with the M13F primer.

Sufficient numbers of clones for each library (a minimum of 30) were analyzed to ensure that coverage of 16S rDNA clone libraries constructed from groundwater samples was 80% or greater. Sequences were compared to those compiled in GenBank with the BLAST suit of programs (<http://www.ncbi.nlm.nih.gov/BLAST/>); Screening for chimeric artifacts was done with the Chimera_check program (<http://rdp.cme.msu.edu/cgis/chimera.cgi?su=SSU>) of the RDP (Ribosomal Database Project) (Maidak et al. 1999).

Alignments using ClustalW and similarity identity matrices were performed in Bioedit (Hall 1999). Representative 16s rDNA sequences for tree construction were obtained from the RDP. Phylogenetic analysis was performed using the Wisconsin Package, 10.2 (Genetic Computer Group, Madison, Wisconsin). Distance analysis was performed using the Jukes-cantor and kimura-2 parameter distance algorithms. Trees were constructed using neighbor joining distance methods with Bootstrap analysis of 100 replicates.

Sediment: The test plot, the sediment sampling were described in chapter 4. Briefly, background core samples P11 were collected 4.5 m upstream of the injection gallery. Down gradient cores, P12, P13, P14, P15, were collected at 0.61m, 2.8 m, 4.3 m and 8.3 m, respectively, from the injection gallery (Figure 4-1). Core samples were sectioned and logged by depth: P15-13 represents core samples from depth of 4-4.6 m, P15-15 represents core sample depth of 4.6-5.2 m, P15-17 refers to sample at depth of 5.2-6.4 m,

P15-19 indicates the core samples at a depth below 6.4 m. This depth designation scheme applies to core samples P11, P12, P13 and P14 also.

Bio-Traps and Others: The biotrap sampling and geochemical, biological analysis were also described in chapter 4. Detailed methods for DNA and PLFA analysis also can be found in Materials and Method section of Chapter 3.

Nucleotide Sequence Accession Numbers: Partial 16S rDNA sequences recovered from this study were submitted to GenBank as with accession numbers AY994978 to AY995014.

Results

Microbial Populations in Groundwater Revealed by 16S Clone Library Analysis: As stated in the Materials and Method section, this part of data was provided by colleagues from the University of Massachusetts (Vrionis et al., 2005). Briefly, *β*-proteobacteria were the predominant (41%) members of the microbial community in groundwater from the background well, where *Actinobacteria*, *γ*-proteobacteria sequences were also observed to an appreciable extent. Other *δ*-proteobacteria comprised 17% of the sequences in background groundwater. The classification/designation other *δ*-proteobacteria was used for sequences that fell into this class of bacteria but did not group with either the DIRB of the *Geobacteraceae* family or the SRB in the three families comprising the order *Desulfobacterales* (*Desulfoarculaceae*, *Desulfobacteraceae* and *Desulfobulbaceae*) or *Desulfovibrio*.

Groundwater from the down gradient monitoring wells at 3.7 m (M03) exhibited enrichment in δ -proteobacteria SRB (~ 8%). An increase in *Geobacteraceae* 16S rDNA gene sequences was exhibited at all down-gradient monitoring wells. The highest percentage (71 %) of *Geobacteraceae* in groundwater was observed in M13, 14.6 m away from injection gallery. Furthermore, a great proportion of *Geobacteraceae* sequences recovered from groundwater (70%) had at least 93% sequence identity to *Desulfuromonas thiophilus*, a member of a portion of the *Desulfuromonas* cluster that contains primarily freshwater microbes. Elevated numbers of *Firmicutes* sequences, specifically of sulfate-reducing *Desulfotomaculum* and *Desulfosporosinus*-like species, were seen in M03 and M08 (18 % and 33 %, respectively).

Sediment Bacterial Biomass: In order to determine bacterial biomass, PLFA generally taken to be indicative of eukaryotes (normal saturates over 18 carbon in length, polyunsaturates) as well as the trace quantities of PLFA of unknown structure were excluded. The biomass was determined as pmol PLFA per gram of lyophilized sediment, which displayed a moderately varied range, from highest as 629 pmol/g with P12 core at depth of 5.2 m (P12-17) to the lowest biomass of 161 pmol/g found with P14-13 (at depth of 4m). Vertically, an increasing trend of biomass was observed with increasing depth. Horizontally a stimulation of the biomass was displayed at the core closest to the injection gallery, which was followed by a decline (Fig. 6-1).

Sediment Microbial Structures Determined by PLFA Analysis: PLFA analysis revealed highly complexed community structure within the sediment environment.

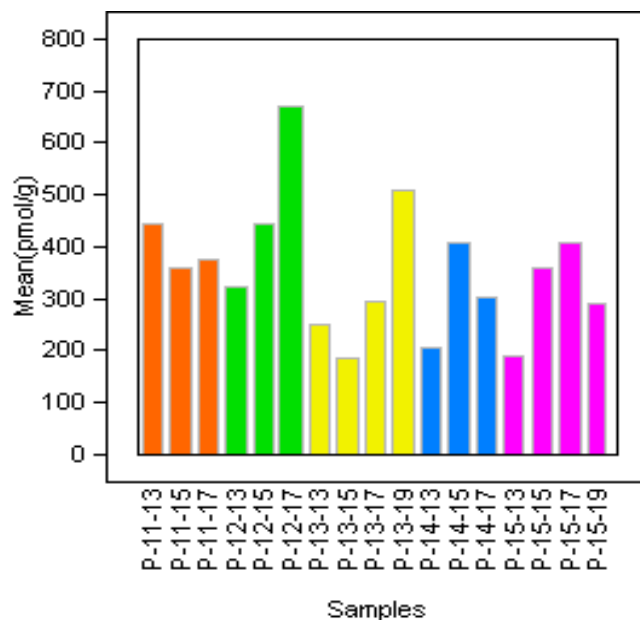


Figure 6-1. Bacterial biomass recovered from sediment core samples at different depths as measured by PLFA analysis (pmol/g sediment). The sediment core (P-11, P-12, P-13, P-14 and P-15) was followed by depths (13, 15, 17 and 19, represents depth of 4 - 4.6m, 4.6 – 5.2 m, 5.2 – 6.4 m and below 6.4m respectively)

Community complexity was estimated from the number of different fatty acids detected (in this case 83), which belong to polyunsaturates, normal saturates, mid-chain branched saturates, branched monounsaturates, monounsaturates, terminally branched saturates and even diacid respectively (Fig. 6-2).

The major difference in the PLFA profiles was that the microbial communities, closest to the injection gallery in the P12 core at all depths, and in the deepest of P13 core, contained significantly more monoenoic PLFA (specifically 16:1 ω 7c, 18:1 ω 7c, 16:1 ω 7t and 18:1 ω 7t), indicative of Gram-negative bacteria (Tunlid et al., 1992; White et al., 1998), than did those in the background and other monitoring core samples. On the contrary, diacids showed a significant decrease in the cores closest to the injection

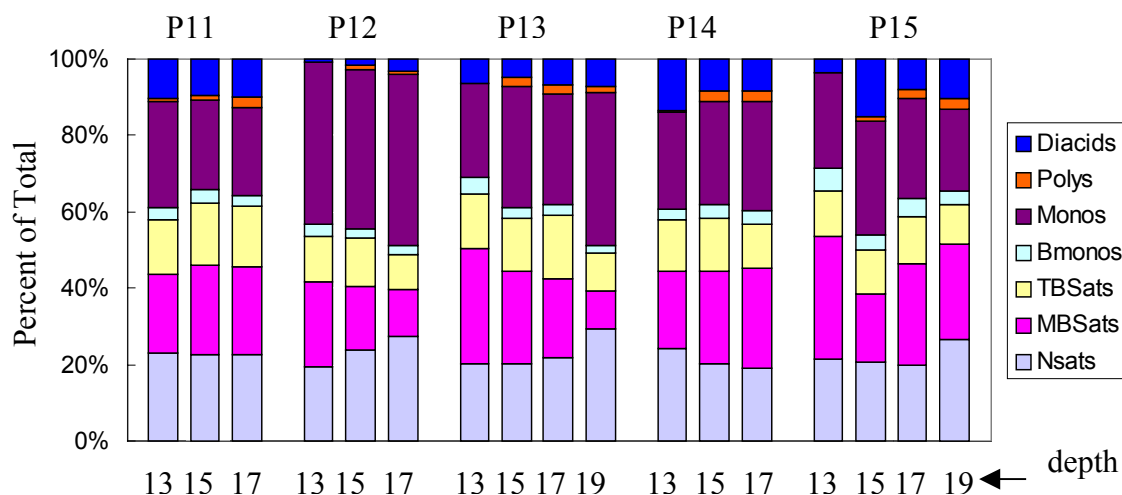


Figure 6-2. PLFA microbial community profiles determined from sediment samples at different depths. Nsats: normal saturates; MBSats: Mid-chain branched saturates; TBSats: Terminally branched saturates; Bmonos: Branched monounsaturates; Monos: Monounsaturates; Polys: Polyunsaturates; Diacids: Dicarboxylic acid. (For depth information, refer to Fig. 6-1)

gallery, then gradually recovered as further away from the gallery (Fig. 6-2).

A hierarchical cluster analysis (HCA) of the PLFA profiles (arc sine transformed mole percent data) showed the major trends within the data set (Fig. 6-3). Interestingly, samples containing the highest proportion of monounsaturates mentioned above clustered together and were separated from all other samples.

Physiological Status of Sediment Bacteria: The physiological status of Gram-negative communities was assessed from the ratios of the *trans/cis* PLFA, with ratios of less than 0.05 shown to be representative of healthy, non-stressed communities (White et al., 1998). Once again, samples from P12 core at all depth and P13-19 displayed significantly lower *trans/cis* ratio with an average of 0.058 ± 0.007 , compared to all remaining samples that had average *trans/cis* ratio of 0.17 ± 0.09 (Table 6-1).

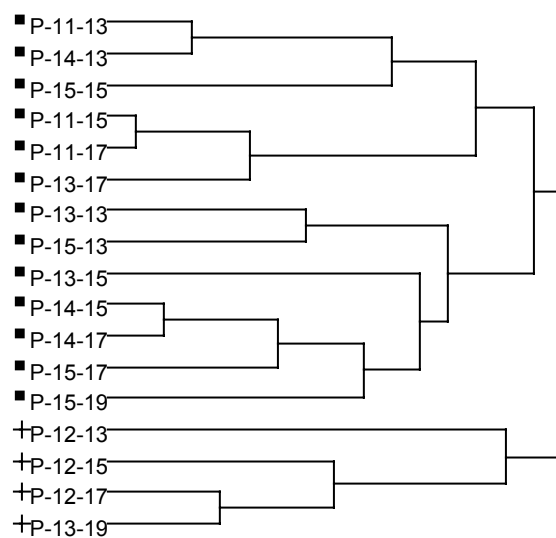


Figure 6-3. Hierarchical clustering analysis of sediment PLFA profiles (Ward method). Different clusters are indicated by signs of + and ■.

Table 6-1. Summary of physiological status of the sediment microbial community indicated by *Tran/Cis* ratio calculated based on fatty acids of 16:1 ω 7 and 18:1 ω 7.

Samples	Ratio	Samples	Ratio	Samples	Ratio
P-11-13	0.14037	P-12-13	0.06624	P-14-13	0.0834
P-11-15	0.12639	P-12-15	0.05347	P-14-15	0.16936
P-11-17	0.16418	P-12-17	0.06231	P-14-17	0.17764
Samples	Ratio	Samples	Ratio		
P-13-13	0.18812	P-15-13	0.29079		
P-13-15	0.10976	P-15-15	0.11137		
P-13-17	0.11088	P-15-17	0.11626		
P-13-19	0.05038	P-15-19	0.42475		

Comparative Sequence Analysis of the Sediment Bacterial Populations: A total of 17 sediment core samples from background and monitoring cores at different depths were subject to PCR-DGGE analysis. The community complexity reflected by banding patterns were highly complexed compared to patterns obtained from biotrap samples. With care, nearly 40 sequences were retrieved, ranging from *Geobacteraceae* family, sulfate reducers within δ -*proteobacteria* and Gram positive phylum, such as *Desulfotobacter* and *Desulfotomaculum* to variety of β -*proteobacteria* and a number of unclassified environmental species. Figure 6-4 is an evolutionary distance tree of the bacterial domain showing the distribution of recovered sediment bacterial sequence types among bacterial division, a rough description of the bacterial biodiversity in the sediment. The sediment community was too diverse for any dominant ribotypes to be identified except *Desulfobacter* sequences. Sequences sharing 95 to 100% identity with *Desulfobacter postgatei* were quantitatively dominant in the core closest to injection gallery (P12-15, P12-17), this sequence type was also prevalent in deepest sediment core of P15. Overall, sulfate reducers were detected more frequently than metal reducers (*Geobacteraceae*) in the sediment. A large portion of the sediment sequences (40%) cannot be placed with any cultured representatives, but form three distinct clusters with relatively high bootstrap values, one of them peripherally related to uncultured δ -*proteobacteria*.

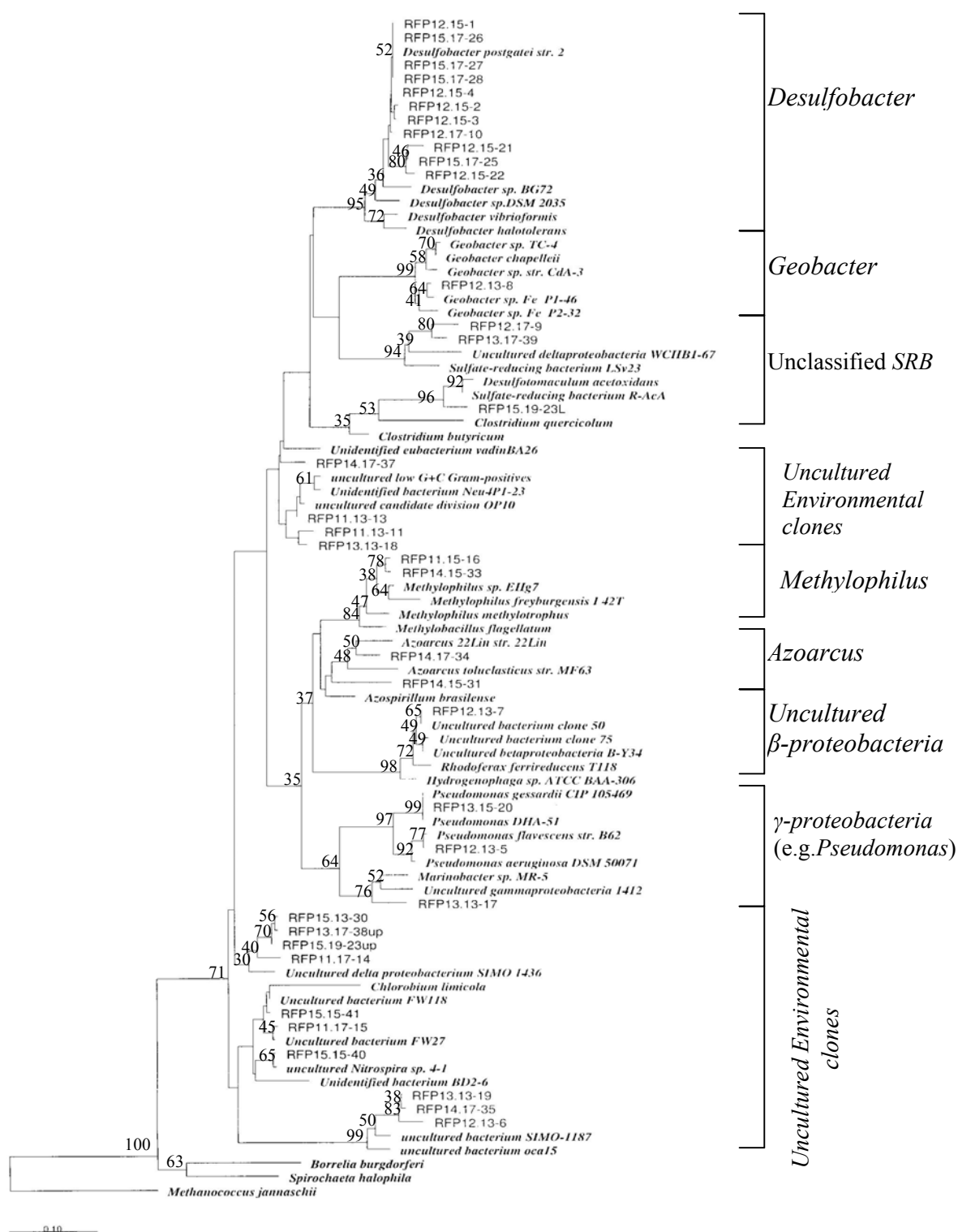


Figure 6-4. Neighbor-joining analysis of 16S rRNA gene sequences from Rifle field sediment samples. Sequences prefixed “RF” were generated during this study, the prefix is followed by sediment core name and depth (e.g. P11.17), then band number (refer to Fig. 6-1 for more detailed nomenclature). Numbers on the tree refer to bootstrap values on 1000 replicates, only values above 30 are given. Scale bar represents 10% estimated change.

Discussion

Comparison Between Communities Retrieved from Sediments, Groundwater and

Biotraps: The sediment was sampled 40 days after the onset of acetate injection (early August), when the subsurface environment entered a stimulated reducing condition, indicated by geochemical analysis (chapter 4). The groundwater clone library analysis was performed on samples from day 38 of acetate injection (early August. Vrionis et al., 2005). The biotraps spanning from August to early September (T2, chapter 4) was also reflective of the microbial community under the active biostimulation phase, though the traps were collected nearly 70 days after the acetate addition. The similarity of the geochemical conditions granted useful comparison between the samples of sediment, groundwater and traps.

In parallel with highly complexed PLFA profile, PCR-DGGE and comparative sequence analysis revealed a broader spectrum of bacterial diversity in the sediment than it is in the bio-trap beads. The high complexity of the bacterial community in sediment yielded significantly more number of DGGE bands, which causing difficulty in retrieving valid sequence information. The results presented in Fig. 6-4 are representative of only a small fraction of the community.

Consistent with our colleague's finding, the dramatic dominance of *Desulfobacter* at the sediment core closest to the injection gallery is noteworthy. PLFA also indicated an increase in monounsaturates --- a marker for Gram-negatives. *Desulfobacter* has been documented as a sulfate reducer in various environment and it was reported to be more prevalent and active in marine sediments than members of other SRB families are

(Rooney-Varga et al., 1997). SRB in sediments use only a limited number of simple organic substrates, predominantly acetate, which are produced during the first fermentative steps in the anaerobic decomposition of organic matter (Sørensen et al., 1981; Parkes et al., 1989). Furthermore under conditions where sulfate reduction is the dominant process, acetate is most likely directly consumed by sulfate-reducing bacteria like *Desulfobacter* sp. and *Desulfotomaculum acetoxidans* (Hansen, 1993; Widdel and Hansen, 1992). The analysis of the biotraps also demonstrated the presence of this *Desulfobacter* sequences in well M-03, closest to injection gallery, but not as quantitatively dominant as it is in sediment. An elevated δ -proteobacteria SRB sequence number was also observed in down-gradient well M-03 groundwater. This dramatic dominance by *Desulfobacter* sequences apparently distinct sediment samples from groundwater and bio-trap samples.

Bio-trap samples exhibited an enrichment of *Desulfuromonas* sequences in all three down-gradient wells, but more strikingly *Geobacter* sequences were overwhelmingly prevalent in bio-trap samples deployed into the monitoring wells M-03 and M-08, this prevalence was not seen in groundwater clone library analysis, which showed an enrichment of *Geobacteraceae* sequences, with majority of them (70%) are closely related to the *Desulfuromonas* cluster. On the other hand, PCR-DGGE analysis of the sediment, indicated an elevation of *Geobacter* sequences. In this aspect, the bio-traps displayed distinct collections of microbial communities, therefore it is believed that bio-traps captured key stimulating populations in sediment—*Geobacter*.

Fe (III) reduction is thermodynamically more favorable than sulfate reduction. However, most oxidized iron in freshwater subsurface sediment is commonly present as insoluble

hydrous ferric oxides (HFO) (Coey et al., 1974), the iron reduction thus may not be a more competitive respiration than reduction of the less oxidized molecules such as sulfate. In a groundwater flow, Fe (III) is more accessible to the bacteria, which perhaps resulted the prevalence of sulfate reducers (*Desulfobacterales*) in sediment and metal reducers (*Geobacteraceae*) in groundwater and biotrap beads. The partition of sulfate reducers and metal reducers in sediment, groundwater and biotrap may indicate the different electron acceptor processes with sulfate reduction more dominant in the sediment than in the groundwater at the time examined. Of course, other factors such as flow path variability, sediment heterogeneity, natural fluctuations, etc., all have influenced the above results in certain way.

In the sediments, there was considerable number (40%) of sequences that could not be unequivocally classified within any known group due to the absence of sufficient similarity to any previously cultured microbial representative. Their frequent isolation from sediment, but only sporadically from bio-traps may have been attributed to the heterogeneity nature of the sediment. Interestingly, the groundwater exhibited even higher percent unclassified sequences, particularly in well M-03. An rRNA sequence in the absence of additional information provides little insight into the physiological nature of the organism that contributed the sequence. More investigation is required in order to elucidate the role of these unclassified organisms in uranium biostimulation.

As previously observed (chapter 4), *β-proteobacteria* were the prevalent (45%) members of the microbial communities in bio-traps from background and down gradient monitoring wells, but these sequences comprised only around 14% of all sediment

sequences recovered. The result of groundwater analysis was consistent with that of biotrap analysis, with *β-proteobacteria* in dominance.

Do Beads Sampled Groundwater or Sediments? The subsurface strata can affect microbial activity, hence microbial diversity in various ways. Sediment and groundwater have been traditionally used to assess the subsurface microbial community diversity and activity. Bio-traps are developed to overcome some of the limitations inherited with groundwater and sediment samples.

A description of the diversity obtained from groundwater and sediments was presented in this chapter and its comparison with diversity from bio-traps (chapter 4) was discussed above. It is recognized that the bio-trap did not approach an accurate census of the organisms at the site because a number of methodological, environmental and biological constraints. However it did capture the key populations in the two environments under the stimulated reducing conditions at the Old Rifle site. Perhaps the bio-traps were more similar to the groundwater than to the sediment in some way.

Although the bacterial communities of these three sampling are phylogenetically divergent, distinct phylogenetic clusters, such as metal reducers, sulfate reducers, are repeatedly detected. These results are in line with the geochemical conditions that indicated sulfate and iron reduction at the site.

The bio-traps collected a relatively simple community, while our colleagues reported considerably greater diversity in the groundwater, possibly because they examined a larger number of clones than numbers of DGGE band were examined in this study.

Although less diverse than sediment habitats, subsurface microbial communities often

support unique microbial species (Pederson, 2001), plus the existence of transient or planktonic community, diversity and heterogeneity was thus expected here within flowing groundwater.

The groundwater showed dominance of *Desufuromonas*, while the traps displayed *Geobacter* sequences as more prevalent. This probably reflected the long term integration effect of the biotrap versus one time point collection of the community in groundwater.

In the sediments, *Geobacter* sequences were also retrieved, but not *Desufuromonas* sequence, this suggests that bio-traps also captured the part of sediment community through extended subsurface incubation, though optimum length of deployment remains unknown. The future research may include determination of incubation time impact on bio-trap communities captured, further it would be more informative to keep all sampling time, locations and sample processing consistent.

Bioremediation Implications: As a new approach to improve our understanding of microbial diversity in subsurface environments, the utilization of Biosep beads bio-traps has proven to be rapid, cost-effective and providing valuable information regarding subsurface microbial communities. While the collection of microbial communities on the surfaces of bio-traps may be more similar to groundwater samples than sediments, it still captured the key populations at this stimulated contaminated subsurface environment, both sediments and groundwater.

The *in situ* biostimulation was shown to effectively stimulate Uranium immobilization in this study, and thus by extension, it could be applied to other heavy metals or nucleotides, such as chromium, cadmium, tritium, etc. other redox-sensitive metals, characteristic of

DOE legacy subsurface wastes. The application and analysis of bio- traps in such cases can effectively provide insight into the engineering of the critical parameters necessary for effective biological immobilization.

The composition of microbial communities reflects physical, chemical, geological and biological characteristics of their habits. Thus, information about key community members can be combined with knowledge of ecosystem history to develop hypotheses about biogeochemical processes occurring in a given habitat. In all of which, bio-trap offers a new convenient, inexpensive methodology to investigating microbial structures in the subsurface.

Nevertheless, caution must be exercised when interpreting the results from such studies.

The communities that formed on the biotrap beads do exhibit differences from those of groundwater or sediment. It has been reported that some microorganisms preferentially attach to different solid substrata (Caccavo et al., 2002; Lower et al., 2001). Furthermore, the length of biotrap incubation under the subsurface also may influence the biodiversity formed on the beads, as it provides a limited time period for successional changes to occur compared to the native situation of the sediments.

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APPENDIX

Appendix A. The ^{13}C delta values measured from microcosm sediments (Top) and beads samples (Bottom)

Samples	FAME ID								
	16:1w7c	16:1w5c	c16:0	10me16:0	cy17:0	18:1w9c	18:1w7c	cy19:0	c19:0
T1S-P11a	-24.512		-24.6		-25.457	-21.114	-21.496	-27.161	-29.516
T1S-P11b	-28.431		-26.395		-27.91	-21.307	-24.084	-30.563	-29.605
T2S-P11a									-29.63
T2S-P11c	-24.608		-25.527		-23.932		-19.461	-24.547	-29.732
T2S-P11d			-25.904				-18.985		-29.788
T1S-P13a	68329.6		80643.5				90718.1		841.806
T1S-P13b	59705.6		77009		11998.4	3405.2	61142.5		1071.41
T2S-P13a	83016		95418.5		83375.1		162191		2115.57
T2S-P13b	85346.4		106879		191372		155133		2516.13
T2S-P13c							141675		1246.42
T2S-P13d	131167		160896						1818.58
T1S-P15a	66994.3		60261.3	2564.1	5109.04	2047.1	69766.1	5690.04	454.898
T1S-P15b	98915.2		82998.2				123770		546.634
T2S-P15a	114049		102752		54872.4	11206.5	115040		1002.86
T2S-P15b	100310	218252	93299.6		67403.2	10910.4	119097		883.025
T2S-P15c	129861		132461				212966		1339.13
T2S-P15d	140064		128484				211181		1008.28

Samples	FAME ID											
	c14:0	i15:0	16:1w7c	c16:0	i17:0	cy17:0	c17:0	18:1w9c	18:1w7c	c18:0	cy19:0	c19:0
T1B-P11a				-26.707					-26.943			-31.014
T1B-P11b				-41.034					-28.541			-24.449
T2B-P11a	-26.992			-36.759					-32.47			-28.694
T2B-P11b	-25.89			-43					-32.555			-28.854
T1B-P13a				-123.17					-51.186			67.834
T1B-P13b	14.224			-873.07					-67.784			192.868
T2B-P13a	-342.95	58.481		-1099.9	760.029	-545.86	-7.636	139.399	-197.85	209.608	-58.786	242.23
T2B-P13b	-374.33	-11.222		-1199.3	775.499	-432.01		87.3	-95.542	138.877		248.171
T1B-P15a	-21.318			-343					-80.904			60.231
T1B-P15b				-654.65					-71.663			226.826
T2B-P15a	-281.35			-2337.1					-102.53			447.395
T2B-P15b				-3287.5					-127.37			
Negative beads control									-26.72			-28.621

Appendix B. Field geochemical measurements made during monitoring well sampling at Old Rifle site for biostimulation experiment

Ferrous iron Fe(II) (mg/L)				
date	B-02	M-03	M-08	M-13
06/25/03	0.42	3.22	3.9	1.61
06/29/03	0.39	2.96	3.26	1.54
07/01/03	0.44	3.12	3.6	1.61
07/09/03	0.4	3.54	4.04	1.47
07/23/03	0.46	6.22	3.86	2.69
08/04/03	0.45	5.48	2.62	4.4
08/19/03	0.55	3.6	1.86	3.28
09/03/03	0.59	2.58	1.35	2.66
09/16/03	0.86	0.32	0.78	2.44
09/30/03	0.67	ND	2.58	3.02
10/15/03	0.86	1.47	0.85	2.04
11/06/03	1.02	1.66	1.53	2.16
12/03/03	0.81	2.67	1.75	2.26
01/15/04	0.51	3.56	1.56	2.1

Sulfide Concentration (mg/L)				
date	B-02	M-03	M-08	M-13
06/25/03	0.002	0.004	0.006	0.005
06/29/03	0.003	0.004	0.006	0.005
07/01/03	0.003	0.004	0.005	0.003
07/09/03	0.002	0.016	0.016	0.002
07/23/03	0.007	0.055	0.173	0.016
08/04/03	0.002	0.36	0.202	0.104
08/19/03	0.003	0.725	0.175	0.02
09/03/03	0.002	0.318	0.838	0.013
09/16/03	0.003	3	0.78	0.054
09/30/03	0.093	4	1.077	0.339
10/15/03	0.003	7.65	2.855	1.02
11/06/03	0.01	2.23	0.35	0.065
12/03/03	0.006	0.187	0.072	0.031
01/15/04	0	0.068	0.048	0.019

Redox potential (mV)				
date	B-02	M-03	M-08	M-13
06/25/03	178	118	84	129
06/29/03	181	113	90	124
07/01/03	184	111	80	118
07/09/03	219	82	58	87
07/23/03	212	58	42	108
08/04/03	167	-55	-47	-1
08/19/03	147	-108	-82	57
09/03/03	167	-60	-82	-22
09/16/03	135	-134	-120	-49
09/30/03	107	-154	-137	-92
10/15/03	124	-139	-129	-36
11/06/03	138	-133	-100	-60
12/03/03	198	1	13	13
01/15/04	198	27	24	40

Dissolved Oxygen (mg/L)				
date	B-02	M-03	M-08	M-13
06/25/03	0.45	0.51	0.12	0.14
06/29/03	0.47	0.33	0.17	0.16
07/01/03	0.42	0.35	0.12	0.13
07/09/03	0.39	0.26	0.11	0.12
07/23/03	0.22	0.18	0.12	5.59
08/04/03	0.15	0.16	0.13	0.11
08/19/03	0.14	0.12	0.14	0.15
09/03/03	0.16	0.2	0.14	0.2
09/16/03	0.15	0.11	0.1	0.11
09/30/03	0.18	0.1	0.1	0.13
10/15/03	0.17	0.12	0.1	0.19
11/06/03	0.23	0.17	0.15	0.18
12/03/03	0.28	0.21	0.2	0.18
01/15/04	0.64	0.28	0.19	0.19

date	pH			
	B-02	M-03	M-08	M-13
06/25/03	6.86	7	7.14	7.05
06/29/03	6.84	7.03	7.17	7.10
07/01/03	6.86	7.06	7.23	7.12
07/09/03	6.48	6.99	7.21	7.07
07/23/03	6.7	7.05	7.14	7.01
08/04/03	6.51	7.07	7.16	6.88
08/19/03	6.64	7.19	7.34	6.73
09/03/03	6.86	7.33	7.42	7.18
09/16/03	6.82	7.37	7.42	7.16
09/30/03	6.79	7.41	7.36	7.15
10/15/03	6.94	7.51	7.7	7.24
11/06/03	6.83	7.41	7.64	7.31
12/03/03	6.98	7.46	7.66	7.45
01/15/04	6.89	7.43	7.53	7.32

Sulfate concentration at 6.1 m (mg/L)				
date	B02	M03	M08	M13
06/25/03	909.5	787.2	886.1	829.9
07/01/03	877.9	781.8	861.3	857.8
07/09/03	851.9	815.5	870.5	879.7
07/23/03	729.0	760.9	428.7	866.7
08/06/03	672.4	537.2	114.7	677.9
08/19/03	744.3	342.4	132.8	684.3
09/03/03	807.3	328.5	3.6	678.3
09/30/03	908.4	5.1	1.0	282.9

Sulfate concentration at 5.25 m (mg/L)				
date	B02	M03	M08	M13
06/25/03	894.6	822.6	863.8	808.9
07/01/03	865.7	822.9	869.1	850.7
07/09/03	888.0	831.4	886.3	860.3
07/23/03	863.7	812.3	635.9	837.3
08/06/03	834.5	727.6	302.2	745.2
08/19/03	841.4	596.2	368.3	647.5
09/03/03	832.0	423.7	214.3	793.3
09/30/03	913.1	181.1	16.7	231.0

Uranium concentration at 5.25 m (ug/L)					Uranium concentration at 6.1 m (ug/L)				
Date	B-02	M-03	M-08	M-13	Date	B-02	M-03	M-08	M-13
06/18/03	363.452	186.904	308.755	189.188	06/18/03	325.569	136.898	70.4915	176.856
07/02/03	344.02	199.318	170.722	198.227	07/02/03	272.529	138.891	134.817	174.756
07/10/03	378.631	215.531	148.255	254.785	07/10/03	221.629	171.048	123.871	180.616
07/24/03	293.022	211.733	60.018	161.992	07/24/03	158.598	145.973	52.0803	127.787
08/07/03	297.815	150.422	76.0299	36.7822	08/07/03	163.503	110.267	48.4242	39.8936
08/20/03	289.255	118.444	86.8577	41.8162	08/20/03	181.459	83.917	48.4076	58.1969
09/04/03	312.494	200.632	88.9471	94.5362	09/04/03	180.389	147.898	42.9481	105.206
09/17/03	282.635	181.131	66.5177	123.57	09/17/03	199.429	101.446	28.8068	131.908
10/01/03	314.007	134.512	33.9317	113.323	10/01/03	198.231	28.2393	18.5387	120.477
10/16/03	300.2	123.875	60.0515	107.36	10/16/03	211.062	28.6138	35.3537	56.2953
10/27/03	307.985	132.468	85.037	140.565	10/27/03	213.856	41.4853	62.5178	113.592
11/07/03	315.77	141.061	110.023	173.769	11/07/03	216.649	54.3569	89.682	170.89
11/20/03	310.235	138.485	92.5979	117.617	11/20/03	208.605	59.2905	74.2569	141.73
12/04/03	304.701	135.91	75.1733	61.465	12/04/03	200.561	64.2241	58.8319	112.57
12/18/03	315.246	137.767	68.0324	76.5798	12/18/03	195.253	59.4635	52.0719	117.092
01/01/04	325.791	139.623	60.8915	91.6947	01/01/04	189.945	54.7029	45.3119	121.615
01/16/04	336.335	141.48	53.7507	106.809	01/16/04	184.637	49.9423	38.5518	126.137
01/30/04	306.99	134.067	50.3969	124.721	01/30/04	180.89	61.2945	33.8189	145.273
02/13/04	277.644	126.654	47.0431	142.634	02/13/04	177.142	72.6467	29.086	164.408
02/26/04	268.366	123.52	43.1479	142.293	02/26/04	171.623	70.8677	26.6245	150.953
03/11/04	259.088	120.385	39.2526	141.952	03/11/04	166.104	69.0888	24.163	137.498
03/22/04	269.799	121.052	40.776	138.905	03/22/04	170.341	70.4455	23.925	130.714
04/03/04	280.511	121.718	42.2994	135.858	04/03/04	174.578	71.8023	23.6869	123.931
04/15/04	291.222	122.385	43.8227	132.812	04/15/04	178.814	73.1591	23.4489	117.147
04/28/04	269.918	126.525	46.761	123.343	04/28/04	180.112	73.1492	24.268	108.026
05/11/04	248.613	130.665	49.6992	113.875	05/11/04	181.409	73.1393	25.087	98.9061
05/25/04	274.651	151.806	60.1561	117.173	05/25/04	180.31	87.9371	27.1455	104.152
06/10/04	300.69	172.948	70.6131	120.472	06/10/04	179.211	102.735	29.2041	109.398

VITA

Yun-juan Chang was born in WuXi, JinagSu Province, China. She grew up in the city of SuZhou, where she received her primary, secondary and high school education. Then she spent her four undergraduate years at QinDao University of Oceanography and graduated with BSc degree. After that, she went abroad and continued to pursue her education at the Dept. of biological sciences., University of Dundee, Scotland, U.K. and received her MSc degree there. Since then her long journey to earn PhD degree began: first she studied at University of Saskatchewan (Canada) on a PhD scholarship, a year later she quitted and enrolled into University of Purdue, U.S.A.. But soon she had to quit once again due to the family situation. She then stayed home for several years to raise her children, she also worked at Dr. White's lab. Finally, nearly three years ago here at the University of Tennessee, she resumed her journey to pursue her PhD. It was not easy to go back to school after what life has brought to her, but she made it, which brings us to today.