GeoChip-based Analysis of Functional Microbial Communities in a Bioreduced Uranium-Contaminated Aquifer during Reoxidation by Oxygen

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Abstract.

A pilot-scale system was established for *in-situ* biostimulation of U(VI) reduction by ethanol addition at the U.S. DOE's Field Research Center (Oak Ridge, TN). After achieving U(VI) reduction, the bioreduced area was further tested to determine the stability of the reduced U under a resting state (no ethanol injection) and during reoxidation [dissolved oxygen (DO) was introduced]. Geochip, a comprehensive 50mer functional gene array involved in N, S, and C cycling, metal resistance, and contaminant degradation, was used to monitor the dynamics of microbial community structure and function in groundwater from two monitoring wells. Detrended correspondence analysis showed a substantial change in community structure after ethanol injections resumed. The communities experienced an increase in the number of genes and an increased diversity. However, while the number of genes detected changed, the relative abundance of most gene groups detected showed little change over the course of the study. An increase in DO did result in a decrease in the relative abundance of *dsr* genes, but no change was detected for cytochrome c genes. During the reoxidaton period, U(VI) increased suggesting a reoxidation of previously reduced U(IV). However, after DO was controlled, U(VI) reduction resumed and returned to pre-reoxidation levels. Cluster analysis of individual gene groups showed an overlap of functional genes demonstrating a functional redundancy within these communities, which may be responsible for the rapid recovery of this community after perturbation.

INTRODUCTION

Uranium (U) is a common groundwater contaminant at U.S. Department of Energy (DOE) sites from cold war era nuclear weapons research and production (Riley et al., 1992). Due to its prevalence and potential toxicity, the DOE is interested in remediation strategies to stabilize the U(VI) and minimize the risk of transport off-site. Remediation of U-contaminants can be achieved by a change in oxidation state of soluble U(VI) to the less soluble U(IV) to limit transport off-site via metal precipitation using dissimulatory metal reduction (Lovley, 1995). Reduction of U (VI) is facilitated by a number of different microorganisms including sulfate- (SRB) (Lovley and Phillips, 1992; Lovley and Phillips, 1994; Tebo and Obraztsova, 1998) and Fe(III)-reducing (FeRB) (Lovley et al., 1991; Wu Q. *et al*., 2006) bacteria as well as other microorganisms, such as *Clostridium* sp. (Frances *et al.*, 2004) and *Deinococcus radiodurans* (Fredrickson et al., 2000). Many of these microorganisms have been found at U contaminated sites, including the Oak Ridge Field Research Center (Oak Ridge, TN; OR-FRC) (North et al., 2004; Nyman et al., 2006; Brodie et al., 2006; Amos et al., 2007; Cardenas et al. 2008) and so could be useful for bioremediation efforts at these sites.

At the DOE's OR-FRC U contamination originated from the S-3 waste disposal ponds. These four unlined ponds collected waste, primarily nitric acid, metals, and radionuclides (U, Tc) until 1983 (Brooks, 2001). The ponds have since been neutralized, denitrified, capped, and are currently covered with an asphalt parking lot. Groundwater in the surrounding area has a low pH (3.4-3.6) and high concentrations of U (50 mg L^{-1}) and nitrate (8-12 g L^{-1}) (Oak Ridge Field Research Center, 2007). A pilot test facility designed to examine the applicability of *in situ* U(VI) bioremediation was constructed adjacent to the former S3 ponds at the OR-FRC (Luo et al., 2006; Wu et al., 2006a, 2006b). Bioreduction and immobilization of U *in situ* has been achieved by intermittently injecting ethanol to the subsurface. The U concentration in the groundwater decreased to below the U.S. Environmental Protection Agency (EPA) maximum contaminant limit (MCL) for drinking water (< 0.03 mg L^{-1}) (Wu et al., 2007) and U speciation in sediment samples showed the reduction of U(VI) to U(IV) (Wu et al., 2007, Kelly et al, 2008).

Examining microbial community structural and functional gene changes during bioremediation and system permutations can improve our understanding of these processes and improve bioremediation strategies. However, one of the major challenges in examining microbial communities is that only an estimated 1% of all microbes are culturable (Whitman et al., 1998). As such, culture-independent molecular tools are necessary to examine these populations. Functional gene arrays (FGA) contain probes for genes that encode enzymes or proteins involved in specific functions of interest (Wu et al., 2001; Gentry et al., 2006; He et al., 2007). The most comprehensive FGA available to date is the GeoChip 2.0, which targets ~10,000 genes involved in the geochemical cycling of N, C, and S, metal reduction and resistance, and in organic contaminate degradation (He et al., 2007). The GeoChip has been used in a variety of studies to examine the functional potential of microbial communities (Wu L. et al., 2006x; He et al., 2007; Yergeau et al., 2007; Zhou et al., 2008; Rodríguez-Martínez et al., 2006; Mason et al., submitted), probe pure culture isolates for specific gene functions (Van Nostrand et al., 2007), and in stable isotope probing experiments (Leigh et al., 2007).

In the current study, the GeoChip was used to examine microbial community changes in the bioreduced subsurface environment of the OR-FRC pilot bioremediation test facility during the resting (no ethanol injection), reoxidation (introduction of DO), and recovery (restoration of reducing conditions by ethanol injection) periods to better understand how changes to the functional microbial community affect U stability. Our results indicated that while the community structure was dramatically altered by introduction of ethanol to the system, the overall functional abilities were not changed.

MATERIAL AND METHODS

Field treatment system. A detailed description of the system setup and design is elsewhere (Luo et al., 2006a; Wu et al., 2006a; Wu et al., 2007). Briefly, the system consists of two injection, two extraction, and three multilevel monitoring wells (MLS) in a nested design (Fig. S1). The outer loop was designed to protect the inner loop from adjacent water flow and stabilize the water flow, minimizing introduction of contaminated groundwater (Luo et al., 2006a; Wu et al., 2006a). Injection of clean water

(nitrate-free, treated groundwater plus tapwater or tapwater alone) to the outer loop maintained a flow field that protects the inner loop from invasion of contaminated groundwater. On day 137 (Jan. 7, 2004), after preconditioning to remove bulk nitrate and Al and increase pH to 5.7-6.1, an ethanol solution, prepared with industrial grade ethanol (88.12% ethanol, 4.65% methanol, w/w), was intermittently (2-3 days per week) injected into the inner loop injection well (FW104) at a concentration measured as the chemical oxygen demand (COD) at 120-150 mg L^{-1} to provide electron donors for U(VI) reduction. The recirculation flow rates between the injection and extraction wells of both the inner and outer loops were 0.45 L min^{-1}. The injection flow rate of clean water was 0.9-1.2 L min^{-1}. DO in the aquifer is naturally low (0-0.3 mg L^{-1}), but, during the initial 638 days of operation, DO penetrated the treatment area via injection of clean water into the outer loop. To prevent DO penetration, Na_2SO_3 (0.9 mM) was added to the clean water in the storage tank to consume DO ($Na_2SO_3+O_2 \rightarrow Na_2SO_4$) from days 638, except for the reoxidation period (days 806-884). Groundwater samples were taken to monitor pH, COD, HCO_3^-, U(VI), sulfate (SO_4^{-2}), sulfide (H_2S), Fe(II), nitrate (NO_3^-), and metals.

Resting state and DO reoxidation periods. From days 713 to 754, ethanol addition ceased in order to study how a resting state affected the system. Ethanol injections were resumed and the system was allowed to recover for 58 days. Over days 810 to 884 air-saturated tap water (9-12 mg L^{-1} DO) was introduced into the outer recirculation loop, allowing an increase in DO to occur within the inner loop (Wu et al., 2007). During this period ethanol injections ceased. This allowed for the examination of the effects of DO on U(VI) reduction, stability of previously reduced U(IV), and re-oxidation of U(IV) in this system. Subsequent control of the DO was restored on day 884 by addition of Na_2SO_3 to the stored tap water and ethanol was again injected to FW104 weekly.

Monitoring wells and groundwater sample collection. Two MLS wells (FW101-2 and FW102-3; 13.7 and 12.2 m below ground, respectively) were selected to monitor changes in the functional potential of the microbial communities based on the results of hydrological tests using Br^- as a tracer. A full description of the hydraulic connection characterization between the injection well (FW104) and the MLS wells was described elsewhere (Luo et al., 2006b, 2007). Both MLS are in the fast flow zone connected to the inner loop injection well FW104. Tracer tests showed that up to 90% of the Br^- was

recovered from these two wells. The mean travel time from FW104 to FW101-2 and FW102-3 was 2.8 and 3.7 h, respectively (Luo et al., 2007).

Samples were taken from the two selected MLS wells on days 746, 754, 810, 850, 887, 901, and 992 (Table 1). Groundwater was pumped into sterilized glass bottles using a peristaltic pump and kept on ice until delivered to the laboratory. The groundwater (2 L) was filtered through a 0.2 μm filter to collect the biomass and the filters were stored at -80°C until ready for DNA extraction.

In this study we examined only the groundwater microbial community. There could be microorganisms attached to the sediment or otherwise inaccessible via the groundwater which may be involved in the processes examined. Previous studies have shown that different environments and sample types have differing numbers of microorganisms for both active and quiescent cells (Haglund et al., 2002, Reardon et al., 2004). However, due to the nature of the system examined, groundwater was a necessary choice as the use of groundwater allows for sampling more frequently and for larger volumes.

Analytical methods. The source and quality of chemicals used in the field test were described previously (Wu et al, 2006a, 2006b). COD was used as an overall indicator to monitor the consumption of electron donors (ethanol, its metabolite acetate and others). COD, sulfide, and Fe(II) were determined using a Hach DR 2000 spectrophotometer (Hach Chemical). DO concentrations in the injection and extraction wells were determined directly using a HATCH Q10 DO meter while DO in the MLS wells was measured above ground by passing groundwater through a glass vial. Anions (including NO_3^-, Br^-, and SO_4^{2-}) were analyzed with an ion chromatograph equipped with an IonPac AS-14 analytical column and an AG-14 guard column (Dionex DX-120). Metals (Al, Ca, Fe, Mn, Mg, U, and K) were determined using an inductively coupled plasma mass spectrometer (ICP-MS) (Perkin Elmer ELAN 6100) as described elsewhere (Wu et al., 2006a). Ethanol and acetate were determined by a HP5890A gas chromatograph equipped with a flame ionization detector and an 80/120% Carbopack BDA column (Supelco Division; Sigma-Aldrich) using He as the carrier gas.

DNA extraction and amplification. Community DNA was extracted by the freeze-grinding method of Zhou et al. (1996). Extracted DNA was stored at -80 °C until needed.

Aliquots (50 ng) of DNA were amplified using the Templiphi kit (GE Healthcare) with the following modifications. Spermidine (0.1 mM) and single-stranded binding protein (267 ng μL^{-1}) were added to improve the amplification efficiency (Wu et al., 2006c). Reactions were scaled down to 10 µL sample and reaction buffers and 0.6 µL enzyme. Precautions described by Zhang et al. (2006) were followed to decrease the amount of background DNA amplified.

Microarray hybridization. Amplified DNA (22 µL) was mixed with 20 µL random primers (octamers, 2.5×; Invitrogen BioPrime DNA Labeling kit), heated to 99 °C for 5 min, and immediately placed on ice. The labeling master mix [8 µL; (2.5 µL dNTP [5 mM dAGC-TP, 2.5 mM dTTP], 1 µL Cy-5 dUTP [25 nM; Amersham], 2 µL Klenow [40 U μL^{-1}], 2.5 µL water)] was added and the samples were incubated at 37 °C for 3 h in a thermocycler. After the addition of Cy5, samples were protected from the light as much as possible. Labeled DNA was cleaned using a QIAquick purification kit (Qiagen) per the manufacturer's instructions and then dried down in a SpeedVac (45 ºC, 45 min; ThermoSavant).

A comprehensive 50mer functional gene array (GeoChip 2.0; He et al., 2007) was used to examine the functional gene diversity of the samples before, during, and after the perturbation periods. The GeoChip contained 24,243 probes for functional genes involved in C, N, and S cycling, metal reduction and resistance, and contaminant degradation (He et al., 2007). Microarray slides (UltraGAPS^{TM} coated slides, Corning) were spotted with 50mer probes as described by Rhee et al. (2004) and cross-linked at 600 mJ (Stratagene UV Stratalinker 2400).

Samples were hybridized overnight (14-16 h) at 42 ºC. Labeled DNA was suspended in hybridization mix (40 µL; 50% formamide, 3× SSC, 0.3% SDS, 0.7 µg μL^{-1} Herring sperm DNA, 0.86 mM DTT) and incubated at 95 ºC for 5 min then maintained at 60 ºC until hybridization. Arrays were assembled and samples processed as described in Van Nostrand et al. (2007). After hybridization, GeoChips were imaged (ScanArray Express Microarray Scanner, Perkin Elmer) and analyzed using the Imagene software (6.0 premium version, Biodiscovery). Raw data from Imagene was analyzed using a GeoChip data analysis pipeline. A signal to noise ratio (SNR) of $\geq$ 1.5 was considered a positive signal. While most studies use an SNR of $\geq$2, a recent study conducted in our lab using a

mixed community culture containing genes complementary to probes on the GeoChip showed than an SNR threshold of 1.2 was required to detect all of the genes expected based on other approaches. In addition, similar relationships of overall community structure were observed for the current study using SNR values of ≥2 and ≥1.5. A positive signal in at least 1/3 of the probes for a particular gene (minimum of 2 probes) was required for a gene to be considered positive. Most of the genes had 3 probes on the array (He et al., 2005, 2007).

Statistical analysis. Statistical analyses were performed using PC-ORD (Version 5.0 MjM Software; McCune and Mefford, 1999) or Canoco. Detrended correspondence analysis (DCA) was performed using Canoco (Version 4.5, Biometris – Plant Research International, The Netherlands) with detrending by segments. Canonical correspondence analysis (CCA) was also preformed with Canoco. Environmental factors included sulfate, sulfide, U, COD, temperature, pH, and Fe. Geochemical results were z transformed to convert all measurements to the same scale ($z = (x_i - \bar{x})/s$; Sokal and Rohlf, 1994) prior to statistical analysis. The analysis was performed with focus on interspecies distance and significance was tested using the Monte Carlo permutation (499 permutations). Hierarchical clustering was done in Cluster 3.0 using uncentered correlations and average linkage for both genes and samples and trees were visualized in TreeView.

RESULTS

Field operation and geochemical changes during the test periods. The microbial community structure was examined over four experimental periods after low U concentrations were achieved (Fig. 1): (i) a resting period during which ethanol injection was halted (days 713-753), (ii) biostimulation by ethanol injection (days 754-803), (iii) a reoxidation period during which DO was added to the subsurface with suspension of ethanol injection (days 806-883), and (iv) a recovery period during which ethanol was injected intermittently to restore reducing conditions (days 884-992).

The electron donor, ethanol, was injected weekly at a rate of 35 g d^1 for two days except days 713-753 (resting period), 804-867, and 869-884 (reoxidation period) (Fig.

1A). Ethanol was chosen as the electron donor based on previous experiments (Wu et al., 2006b) and was monitored using COD. During ethanol injection, the COD in FW104 (inner loop injection well) was 140-150 mg L^{-1} and dropped to <10 mg $L^{-1}$when injection stopped. The COD concentration in FW101-2 was 60-80% of that in FW104 and FW102-3 was 30-50% (data not shown). This is likely due to the longer mean travel time from FW104 to FW102-3 than to FW101-2 and subsequent electron donor consumption. Acetate was the primary COD component (>90%) in FW101-2 and FW102-3 during ethanol injection (data not shown). The DO in the inner loop injection well (FW104) was maintained at <0.15 mg L^{-1} in the MLS except during the reoxidation period. After DO was introduced to the subsurface, DO gradually increased to 2.0 mg L^{-1} in FW101-2, but only to 0.5 mg L^{-1} in FW102-3 (Fig 1B). Sulfide concentrations increased during ethanol injection and decreased when ethanol injection stopped (Fig. 1C). Fe(II) was detected in FW102-3 throughout the test period, but decreased to near zero in FW101-2 during the reoxidation period (Wu et al., 2007, data not shown).

Low U concentrations in the MLS were observed until day 715 when ethanol injection was halted (Fig. 1D). U concentrations began to increase during the resting period due to U from the recirculating groundwater. When ethanol injection resumed, the U concentration decreased. During reoxidation U concentrations increased significantly in FW101-2 due to the reoxidation of U(IV). A similar response occurred in FW102-3, but lower levels of U were remobilized. The U(VI) concentrations returned to below 0.03 mg L^{-1} in FW101-2 on days 971 and on days 1207 in FW102-3 (data not shown). Reduction of Fe(III), sulfate, and U(VI) was stimulated by injection of ethanol on days 867-869 (Fig. 1B, data not shown). Nitrate concentrations were low (<0.2 mg L^{-1} as N) or near zero throughout the period examined (data not shown). The pH was maintained between 6.0-6.2 by occasional K_2CO_3 injection. The temperature of the subsurface varied from 12 to 22 °C during summer and winter, respectively.

Changes in functional gene diversity. Community functional gene diversity, richness, and evenness in the two wells were assessed based on microarray results (Table 2). During the resting period, when no ethanol was added, the diversity and richness in both wells were relatively stable with approximately 100-200 genes detected at each time

point from day 746 to 850. Increased DO levels did not appear to have a profound effect on diversity, although this could be due to a lack of sensitivity of this index. Well FW101-2 showed a decrease in diversity only at the end of the reoxidation period, while diversity was unaffected in well FW102-3. Once ethanol injections resumed, genes numbers and diversity immediately began to increase in well FW102-3 and had increased in well FW101-2 by days 901. Well FW102-3 shows an almost 10-fold increase in the number of genes detected on day 887 as compared to days 746-850. FW101-2 showed an initial decrease in gene number followed by a more modest 4-fold increase in gene number on day 901 compared to days 746-850.

Gene overlap between samples was also calculated. Higher percentages of shared genes were observed between time points having similar DO and ethanol treatments. For example, in well FW101-2, 29.7% of the genes were shared between days 810 and 850, both during the reoxidation period while only 15.75% of the genes were shared between days 754 and 850, during resting and reoxidation periods. Three samples had $\geq$10% unique genes: FW101-2, days 850 and FW102-3, days 901 and 992.

Changes in overall community structure. Detrended correspondence analysis (DCA) of all detected genes was also used to examine overall functional structure changes in the microbial communities (Fig. 2). DCA is an ordination technique that uses detrending to remove the arch effect typical in correspondence analysis (Hill and Gauch, 1980). The total inertia for the model was 1.588. Eigenvalues for the first two axes of the DCA were 0.560 and 0.194, respectively, and explained ~50% of the variance. The wells clustered into two main groups: one during resting and reoxidation and the other after ethanol injections were resumed (Fig. 2). The samples without ethanol addition clustered more tightly than the samples with ethanol. Three points did not fall into these clusters, days 754 (FW101-2 and FW 102-3) and 850 (FW101-2). On day 850, FW101-2 had been exposed to higher levels of oxygen for 40 days, the community was adapting to the increased DO and was less similar to the community on day 810. DCA results indicated that the microbial communities were significantly altered after ethanol addition, as indicated by the separate clusters before and after ethanol injections. This suggests that while recovery from the stress of starvation and oxygen exposure occurred (indicated by

increased gene numbers and decreased U), the recovered communities differed from the original communities.

Relative abundance of functional gene groups. The relative abundance of functional gene groups represented on the GeoChip was compared over the course of these experiments (Fig. 3). Contaminant degradation accounted for approximately 40% of all genes detected at each time point. Another 15-25% of the genes were involved in metal resistance. Carbon cycling genes accounted for 15-20%, with 10-15% associated with carbon degradation, 1-7% with methane oxidation, 0-3% with carbon fixation, and <0.5% for methane production. Methane oxidation genes decreased to 1% in both wells on day 850 and then increased again by day 887. Nitrogen cycling genes accounted for 15-20% of genes detected, with 5-10% associated with ammonification (urease), ammonium assimilation (glutamate dehydrogenase), and nitrification, 1-7% with nitrate and nitrite reduction, 0-6% with nitrogen fixation, and 1-3% with denitrification. The remaining 1-5% was involved in sulfate reduction. Overall, the relative abundance of the various gene categories changed less obviously over time although there were changes in some gene categories. The relative abundance of dissimulatory sulfite reductase genes (*dsrAB*) did decrease during the period of increased DO, with a more pronounced decrease in FW101-2 compared to FW102-3 (Fig. 3 and S2). The relative abundance of metal resistance genes decreased in both wells after ethanol injections were restarted (Fig. 3 and S2).

Changes of individual functional gene categories. Specific gene categories were further analyzed using hierarchical clustering. The *dsr* genes, which are indicative of SRB, a group of microorganisms that have been shown to reduce U(VI) (Lovley and Phillips, 1992, 1994; Lovley et al., 1993; Tebo and Obraztsova, 1998) clustered based on before and after ethanol injections. Interestingly, while the relative abundance of *dsr* genes was reduced during the reoxidation period (Fig. S2), no separate cluster was obvious for the reoxidation period (Fig. 4). The most frequently detected *dsr* genes, other than from uncultured SRB, were those similar to *Desulfovibrio* and *Desulfotomaculum* species. The *dsr* genes were below detectable limits in FW102-3 on day 746.

Metal resistance genes showed a decrease in relative abundance after ethanol injections were resumed (Fig. S2 and S3). Genes for resistance to a variety of metals, including Al, Cd, Cu, As, Cr, and Hg, were detected. The most frequently detected genes were for resistance to As, Cr, Hg, and Te. A gene similar to Te resistance from *Rhodobacter sphaeroides* was detected in both wells at all time points. Genes similar to Cr and Te resistance from *Leuconostoc mesenteroides* and to As resistance from *Campylobacter jejuni* were detected in both wells at most time points. Metal concentrations (maximum concentrations detected in parentheses) were low in both wells, but Al (0.26 mg L^{-1}), As (0.007 mg L^{-1}), Cr (0.002 mg L^{-1}), Zn (0.055 mg L^{-1}), Cu (0.003 mg L^{-1}), Pb (0.002 mg L^{-1}), and Ni (0.003 mg L^{-1}) were detected in both wells. Cd was below detection limits in both wells.

While the relative abundance of carbon degradation genes remained stable over the course of this study, the number of genes detected sharply increased once ethanol injections resumed (Fig. 5). During the periods when no ethanol was being injected, the carbon degradation genes were dominated by cellulase (45%), chitinase (25%), and mannose (20%). Most of these genes were detected throughout the experimental periods. After ethanol injections resumed, carbon degradation was dominated by cellulase (50%), chitinase (21%), polygalacturonase (20%), and laccase (18%). In addition, two acetyl-CoA synthetase (*acsA*) genes derived from *Leptospira interrogans* (gi 46448414) and *Staphylococcus aurea* (gi 14324459) were detected after ethanol injection resumed. Only a few genes involved in methanogenesis were detected (Fig. S4). With the exception of FW101-2 on day 850, these genes were only detected after ethanol injections were resumed and diversity had started to increase (FW101-2 days 850, 901, 992; FW102-3 days 887, 901, and 992. Most of the detected genes were similar to those found in uncultured microorganisms. A gene similar to *mcr* from *Methanococcus vannielii* (gi126868) was the only methanogenic gene detected on day 850. A gene similar to mcrA from *Methanothermobacter thermautotrophicus* (gi7445687) was detected on all days except 850 and was the most abundant strain based on signal intensity.

Similar to the carbon degradation genes, most of the denitrification genes were detected after ethanol injection resumed (Fig. S5). Four denitrification genes, similar to norB from *Nitrosomonas wuropaea* (gi 34391466), nirK from *Pseudomonas chlororaphis*

(gi 287907), nirK from an uncultured bacterium (gi 27125563), and nirK from *Alcaligenes* sp. (gi 29466066) were detected at most time points. Five genes involved in nitrogen fixation were detected across most time points (Fig. S6). These genes were similar to the nifH from unidentified or uncultured microorganisms (gi 3157698, 780713, 29293420, 1255496, 6523533, and 3157512). Three other nifH genes similar to those from unidentified or uncultured microorganisms (gi 3157722, 21586771, and 3157610) were only detected in FW101-2 during time points that did not have ethanol addition. Several nifH genes, including those derived from *Calothrix* sp. (gi 1698856 and1698854) and *Serratia marsescens* (gi 11875063), were only detected in FW102-3.

A variety of organic contaminant degradation genes were detected throughout the course of this study (Fig. S7). Several genes were detected at almost all time points (5-6) in both wells. These include those similar to the genes involved in the degradation of 2,4-D (*Bradyrhizobium* sp., gi 17298107; *Wautersia eutropha*, gi 567073), benzoate (*Acinetobacter* sp., gi 2352826), chloroacrylic acid (*Pseudomonas pavonaceae*, gi 10637971), phenylpropionate (*Nostoc punctiforme*, gi 23126645), protocatechuate (*Rhodobacter sphaeroides*, gi 22975204), vanillin (*Corynebacterium efficiens*, gi 25027190), catechol (*Helicobacter pylori,* gi 15645359), cyclohexanol (*Acinetobacter* sp., gi 9965287), dibenzothiophene (*Thiobacillus* sp., gi 14149089), nitrotoluene (*Burkholderia cepacia*, gi 17942394) and toluene (*Clostridium tetani*, gi 28210648).

Cytochrome c genes, which are involved in electron transport and U(VI) reduction (Lovley et al., 1993), were also examined. Therefore, the average signal intensities for the different genera covered by these probes were calculated (Fig. 13). Most of the cytochrome genes detected were from *Desulfovibrio-, Geobacter-, Rhodopsuedomonas-*, and *Shewanella*-like microorganisms. Cytochromes from two other genera, *Chlamydophila* and *Mycobacterium*, were also detected. No genes from *Anaeromyxobacter* were detected.

Phylogenetic diversity. Although the GeoChip 2.0 does not contain specific phylogenetic markers, some information regarding what microorganisms are present can be obtained. The probes on the GeoChip were designed to be as specific as possible for a particular gene from a particular microorganism (Wu et al., 2001; Rhee et al., 2004; He et

al., 2007). Over all sampling points, genes were detected from 272 genera or microorganisms similar to these genera. Most of the microorganisms detected were present during the recovery period after the functional gene diversity increased. Microorganisms similar to *Desulfovibrio, Pseudomonas, Geobacter, Wautersia,* and *Rhodospirillum* were detected at all time-points in both wells. Several genes from organisms similar to genera shown to be capable of U-reduction, including *Clostridium, Deinococcus, Desulfomicrobium, Desulfosporosinus, Desulfovibrio, Geobacter, Pseudomonas, Pyrobaculum, Salmonella, Shewanella, Thermoanaerobacter,* and *Thermus* (Wall and Krumholz, 2006), were detected. Of these, genes for organisms similar to *Clostridium, Deinococcus, Desulfovibrio, Geobacter, Pseudomonas, Salmonella,* and *Shewanella* were detected in both wells at all or most time points. Genes from a microorganism similar to the Fe(II)-oxidizer *Thiobacillus* and *Clostridium* were also detected at almost all time points. We were particularly interested in *Geobacter* and *Desulfovibrio* sp. as these have been shown to be important in U(VI) reduction. Comparable amounts of genes from organisms similar to both *Desulfovibrio* and *Geobacter* were detected at all time points, based on the average signal intensity of all genes detected (Fig. S8).

Relationships between community structure and environmental variables. To determine the most significant geochemical and environmental variables affecting microbial community structure, canonical correspondence analyses (CCA) of both wells were performed for each of the seven environmental variables (sulfate, sulfide, U, Fe(II), COD, pH, and temperature). Five of these variables were chosen based on the p-values generated during these analyses: temperature ($p=0.072$), COD ($p=0.176$), sulfate ($p=0.102$), Fe(II) ($p=0.152$), and U ($p=0.160$). Both sulfide and pH had p-values above 0.35.

Initial CCA used these five environmental variables and all detected functional genes from both wells. The variance inflation factors (VIF) for sulfate and Fe(II) were greater than 20, indicating there was significant correlation between the environmental variables (multicollinearity) (data not shown). So, Fe(II), which had the highest VIF, was removed and the CCA repeated. The VIFs for the remaining variables decreased to less than 5. In

addition, the wider angles between arrows indicate there is less correlation between these environmental variables. The specified CCA model with four environmental variables (sulfate, COD, U, and temperature) explained 72% of the variation (sum of all eigenvalues, 1.588) and was significant (p=0.002) (Fig. 7; Table 3). The first canonical axis is positively correlated with sulfate concentration and temperature and negatively correlated with COD and U concentration. The second axis is negatively correlated with COD. The length of the arrow in the biplot indicates the importance of each environmental variable (ter Braak et al., 1986). As such, COD appears to be the most important environmental parameter, while U, temperature, and sulfate appeared to have lesser, but equivalent importance to the community structure. FW101-2, day 992, FW102-3, day 754, and both wells on day 901, were positively correlated with COD and negatively correlated with temperature. In contrast, FW101-2, days 746, 754, 850, and 887 and FW102-3, days 746, 850, and 992 were positively correlated with temperature and negatively correlated with COD. Well FW102-3, day 887 was positively correlated with U and FW101-2, days 810 and 992 and FW102-3, days 754 and 810 were positively correlated with sulfate.

Next, variation portioning analysis (VPA) (Økland and Eilertsen, 1994; Ramette and Tiedje, 2007) was done to better understand how much each environmental variable was influencing the functional community structure (Fig. 8). The same variables used for CCA were used for VPA. When geochemistry (sulfate and U) and temperature were held constant, there was a significant correlation between the community structure and the COD (p=0.016). Similarly, when COD and temperature were held constant, geochemistry (U and sulfate) showed a significant correlation (p=0.006) with the community composition. Temperature showed a nearly significant correlationship (p=0.056) with the community structure when COD and geochemistry were held constant. COD was able to independently explain 13.5% of the variation observed while geochemistry explained 25.5%, and temperature explained 9.3%. Interactions between the three variables appeared to have more influence in this system than individual environmental variables (30.4% total). About 1/3 of the variation (35.9%) was unexplained. These results suggest that the variables selected greatly influenced the functional gene composition.

DISCUSSION

Functional gene arrays. Understanding the diversity and functional abilities of microbial communities at contaminated sites is a key step in designing and implementing bioremediation strategies. However, a great challenge in studying microbial communities is that the vast majority of microorganisms in the environment cannot be cultured (Whitman et al., 1998). Microarray technology can overcome this obstacle by allowing for the examination of thousands of genes from microbial communities at one time in a high-throughput, parallel manner. A great deal of progress has been made over the last decade regarding the development and design of FGAs (Wu et al. 2001, 2004, 2006; Adey et al. 2002; Rhee et al. 2004; Liebich et al. 2006; Gao et al. 2007; He et al. 2007). Because FGAs provide information regarding the potential functional abilities of microbial communities, this type of array is ideally suited for linking microbial communities with geochemical processes. FGAs have been used to successfully examine microbial communities from a variety of environments in order to better understand the mechanisms of geochemical cycling, contaminant remediation, and microbial ecology (Wu et al., 2001; Rodríguez-Martínez et al., 2006; Wu et al., 2006; He et al., 2007; Leigh et al., 2007; Yergeau et al., 2007; Wagner et al., 2007; Wu et al., 2008; Zhou et al., 2008; Mason et al., in press). These previous studies have shown GeoChip 2.0 to be a reliable and powerful tool for analyzing microbial communities.

When examining microbial communities array specificity is always of concern, especially since so many of the environmental sequences are unknown. Because of the unknown nature of natural communities, it is important to focus on relative changes across samples rather than specific changes since this will cancel out any cross-hybridization (Wu et al., 2008; Zhou et al., 2008). The general consistency of results in the present study across experimental conditions supports the reliability and usefulness of the GeoChip. First, the number of genes detected was lower at the beginning of this study period, during a phase of no ethanol addition, and then increased once ethanol additions started (Table 2). Next, changes in the relative abundance of several gene groups detected during this study were consistent with expectations. For example, methanogens were only detected after ethanol addition resumed and the microbial

populations increased. In addition, the relative abundance of *dsr* genes decreased during the reoxidation period and then recovered once DO was controlled. These findings support the reliability of the results obtained from GeoChip.

Effect of environmental variables on functional gene structure. A central goal of microbial ecology is to understand the influence of environmental factors on microbial community diversity, structure and function. Because of the comprehensive nature of GeoChips, they can be used to begin understanding these influences on microbial communities by linking microbial communities to geochemical cycling (Zhang et al., 2007; Yergeau et al., 2007). GeoChip derived data can then be combined with environmental and spatial parameters to begin to understand the influence of environmental factors on the microbial community structure (Zhou et al., 2008). VPA has been used to define the amount of influence multiple environmental parameters have on microbial communities (Økland and Eilertsen, 1994; Ramette and Tiedje, 2007). Ramette and Tiedje (2007) were able to explain only 20-30% of the biological variation for presence, abundance, and genotypic similarity of *Burkholderia ambifaria* and 50-70% of the biological variation for abundance of *Burkholderia* spp. using environmental variables, plant diversity, or spatial scale. Zhou et al. (2008) were able to explain less than half of the biological variation using environmental parameters and spatial scaling. In this study CCA results showed that sulfate, COD, U, and temperature were able to explain 72% of the variation observed in the functional gene structure at each timepoint (Fig. 7). Almost 65% of the total variation was explained by using COD, geochemical variables, and temperature (Fig. 8). The higher level of explanation obtained in this study compared to previous studies may be due to this being a much simpler system with lower diversity that would be expected for forest or grassland soil communities.

Effect of operational phase on the microbial community. Having a microbial community that is functionally diverse, stable, and capable of rebounding from adverse conditions is critical in establishing an effective and long-term bioremediation system. Microbial communities play an important role in remediation of contaminated environments and changes in these communities as the result of variations in

environmental conditions or natural fluctuations in the community. Principle component analysis results of a 16S rRNA microarray (Phylochip) showed microbial communities within a soil column from the OR-FRC separated based on experimental phase (original sediment, U reduction, and U oxidation) (Brodie et al., 2006). Earlier studies of the system examined in this paper, revealed that changes in community structure and composition were correlated with specific remediation phases (e.g., active U(VI) reduction) (Wu L. et al., 2006; He et al., 2007). However, by day 745, when the current study period began, U-reduction rates had decreased and U concentrations had fallen to near or below EPA drinking water standards and appeared stable during normal operation (intermittent ethanol injections, DO control) (Wu W. et al., 2006b). As such, changes in the functional community structure observed in this study did not correlate with specific remediation phases. However, changes to the functional gene structure were associated with perturbations to the system (increased DO, cessation of ethanol addition).

COD, which was used to monitor ethanol concentrations, appeared to be one of the most important environmental variables in determining the diversity and structure of the groundwater communities (Fig. 2, 7 and 8). The most obvious effect of ethanol amendment to this system was increased gene number and concomitant increase in diversity. These results are consistent with other studies showing that nutrient limitation reduces microbial activity and diversity and that introducing additional carbon sources increases microbial diversity and activity (Akob et al., 2007; Brodie et al., 2006; Fields et al., 2005; Reardon et al., 2004). The communities in this system appeared to respond quickly to the ethanol injections. An injection of ethanol occurred on day 754, a few hours before samples were taken. By the time sampling occurred, the communities had already started to shift, resulting in communities less similar to the communities at day 746. An alternative explanation is that water flow during the injection period may have caused the temporary shift in functional gene-based community. However, this immediate shift did not occur with the communities from day 887 in well FW101-2, but was observed for FW102-3. This difference may be due to the stress of increased DO in FW101-2, which required additional time to overcome.

While in general, infusion of nutrients increases microbial diversity and numbers, microcosm experiments using sediments from this site showed an initial drop in diversity

when ethanol was added (Luo et al., 2007). This is similar to the initial decrease observed in functional gene diversity in FW101-2 on day 887 (Table 2). A drop in diversity does not, however, necessarily indicate a decrease in function. Brodie et al. (2006) found an increase in microbial activity concomitantly with a decrease in biomass during the U(IV) oxidation phase of a soil column from Area 2 of the OR-FRC. Similarly, the percentage of U(VI) reducing FeRB and SRB increased after *in situ* ethanol biostimulation of a U contaminated site at the FRC (North et al., 2004).

DO appeared to be less important in determining the community structure than ethanol. However, there were some changes that could be linked to the presence of DO. The most obvious affect was a decrease in the relative abundance of *dsr* during the reoxidation period (Fig. 3 and S2). While very low concentrations of O_2 have been shown to inhibit growth of SRB (Marschall et al., 1993), some strains of *Desulfovibrio* are more tolerant to O_2 (Sigalevich et al., 2000), and many have mechanisms to utilize, limit, or escape O_2 exposure (Le Gall and Xavier, 1996; Sass et al., 2002). However, another study at the FRC using artificial neural networks suggested that DO had little effect on *dsr* gene frequencies (Palumbo et al., 2004). An additional study of *dsr* gene diversity across a contaminant gradient at the FRC showed similar *dsr* composition in all wells examined, both contaminated and uncontaminated wells, suggesting a resiliency in the SRB community (Bagwell et al., 2006). The decrease in the relative abundance of *dsr* genes (and presumably SRB) observed in the current study could be the result of a protective response to limit DO exposure. The decrease in the relative abundance of *dsr* genes was less prominent in FW102-3 than FW101-2. This could be explained by differences in the travel time between the injection and monitoring wells. The mean travel time from FW104 to FW102-3 (>3.7 h) was greater than that to FW101-2 (2.8 h) and most of the DO would be consumed before reaching FW102-3, thereby decreasing exposure of this well to DO. This would also explain why communities from FW101-2 and FW102-3 did not cluster together on days 850 and 887. In addition, this could explain why, on day 887, FW102-3 was more similar to the recovered communities than FW101-2. In contrast to the *dsr* genes, the relative abundance of cytochrome c genes, which have previously been shown to be important for U(VI) reduction (Lovley et al., 1993; Shelobolina et al., 2007) did not appear to be affected by the system permutations.

The cytochrome c genes detected during the reoxidation period were primarily from non-SRB (data not shown, Fig. 6) and may not have been as affected by the DO as the SRB.

While we observed changes in the community structure under the different system permutations, the ultimate question is not whether the communities change under various conditions, but rather whether these changes affect their function. Previous studies on this system demonstrated an active U(VI) reducing population (Wu et al., 2006; He et al., 2007) that was capable of reducing U(VI) to below EPA drinking water standards (Wu et al., 2007). However, an increase in U(VI) concentration during the reoxidation period was observed, indicating a reoxidation of previously reduced U(IV) (Fig. 1; Wu et al., 2007). Once DO levels were controlled, U(VI) reduction resumed and returned to pre-oxidation levels at the end of the study period. A similar recovery was noted for sulfate and sulfide concentrations. So, while changes in the overall community occurred, the ability to reduce U(VI) and sulfate did not change.

It is interesting that communities with very low gene numbers and lower diversity functioned in a very similar fashion to communities with 5 to 20 times that number of genes and higher levels of diversity. This could be because due to a very small number of community members being involved in specific functions. If this were the case, then as long as this set of core members survived, then the functional ability would remain. Alternatively, multiple members of the community perform the same function providing functional diversity. Several studies have demonstrated that duplication of function is key to a stably functioning community, even during periods of adverse conditions which results in changes to the community (Andren and Balandreau, 1999; Loreau et al., 2001). Studies have suggested that, because diversity provides a wider range of microorganisms that are able to perform specific functions, highly diverse populations afford communities an increased level of stability compared less diverse populations (Loreau et al., 2001; Girvan et al., 2005). A study of OR-FRC samples by Fields et al. (2006) showed that while diversity based on SSU rRNA sequences was affected by the level of contaminants present (i.e., less diversity with increased contaminants), the functional abilities, based on the presence of the respective functional genes, was similar. In the current study, genes with similar functions but not necessarily from the same microorganism, were observed

at most timepoints, suggesting functional stability was provided by functional redundancy.

U(IV) reoxidation. As mentioned above, an increase in U(VI) concentrations during the reoxidation period was observed in this system, indicating a reoxidation of previously reduced U(IV). This reoxidation was previously attributed to chemical mechanisms rather than microbial ones (Wu et al., 2007). However, *Thiobacillus*, an Fe(II)-oxidizing bactierium that is able to oxidize U(IV) (Ivarson, 1980), was detected in sediment samples from this site (Cardenas et al., 2008) and biological U reduction has been shown to occur (Nyman et al., 2006). Brodie et al. (2006) observed a decline in major/efficient metal reducing bacteria during a U reoxidation phase using a sediment column from FRC sediment, although the authors suggested that reoxidation was not the result of changes in the microbial community in that system. In the current study, the increase in U(VI) during reoxidation corresponded with a decrease in *dsr* genes (Fig. 1, 3, and S2). This presumptive decrease in SRB is most likely due to inhibition by DO. Previously, a significant correlation was observed between U(VI) concentrations and the amount of cytochrome genes detected ($r=0.73$, $p<0.05$) in this system, indicating the importance of this group of microorganisms in U(VI) reduction (He et al. 2007). However, in this study, a decrease was not observed with cytochrome c genes, nor was any correlation between U and cytochrome genes observed. The cytochrome-containing microorganisms detected could be U(IV) oxidizers (DiSpirito and Tuovinen, 1982, Finneran et al., 2002, Senko et al., 2007; Beller et al., 2008). In addition, genes from a microorganism similar to *Thiobacillus* (carbon fixation and contaminant degradation genes) were detected, suggesting the possibility of biological U(IV) oxidation. Another possibility for the U(IV) reoxidation is abiotic factors. This would most likely be from oxidation by the DO. There is some evidence that supports and abiotic reoxidation. For example, in FW102-2 and FW102-3, U(IV) associated with the sediment increased during the reoxidation period (Wu et al., 2007), suggesting that U(VI) reduction was still active. In addition, MLS wells closer to the injection well showed higher levels of U(IV) reoxidation which reflected the DO level in the respective wells (Wu et al., 2007). While

there is support for both biotic and abiotic reoxidation, there is insufficient evidence to determine which processes are most responsible for the reoxidation of U(VI).

Several U(VI) reducers, including *Desulfovibrio*, *Geobacter*, *Anaeromyxobacter*, *Desulfosporosinus*, and *Acidovorax* and FeRB (*Geothrix* and *Ferribacterium*) have been detected previously in FW101-2 and FW102-3 on days 773 using 16S rRNA clone libraries (Cardenas et al., 2008). In addition, high levels of *Geobacter lovleyi* strain SZ were detected at this site after biostimulation (Amos et al., 2007). Previous studies on this system have suggested that SRB, including *Desulfovibrio* spp. may be the primary organisms reducing U (Nyman et al., 2006), suggesting that changes in this group could affect U(VI) reduction. GeoChip detected genes from organisms similar to *Desulfovibrio* and *Geobacter* in this study. Both were detected at all time points examined.

GeoChip results demonstrated that ethanol additions resulted in an increase in diversity and overall gene numbers detected as well as changes to the overall functional microbial community. However, while there were significant changes within the microbial community, no changes in the functional ability of this community were evident. This study demonstrates the ability of GeoChip to examine the functional microbial communities and the affect of environmental parameters on the communities. However, while GeoChip is able to provide information regarding the functional potential of microbial communities, information on active communities is not provided. Additional studies will be required using mRNA-based hybridizations or stable isotope probing to identify the active community members.

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Figure 1. Geochemical measurements taken during the experimental periods. Ethanol (A), DO (B), sulfide (C), and U (D) were monitored over the course of this study. Closed triangles represent FW101-2, open triangles represent FW102-3, open circles represent FW104 (inner loop injection well, DO panel only), and black circles indicate timepoints at which samples were taken.

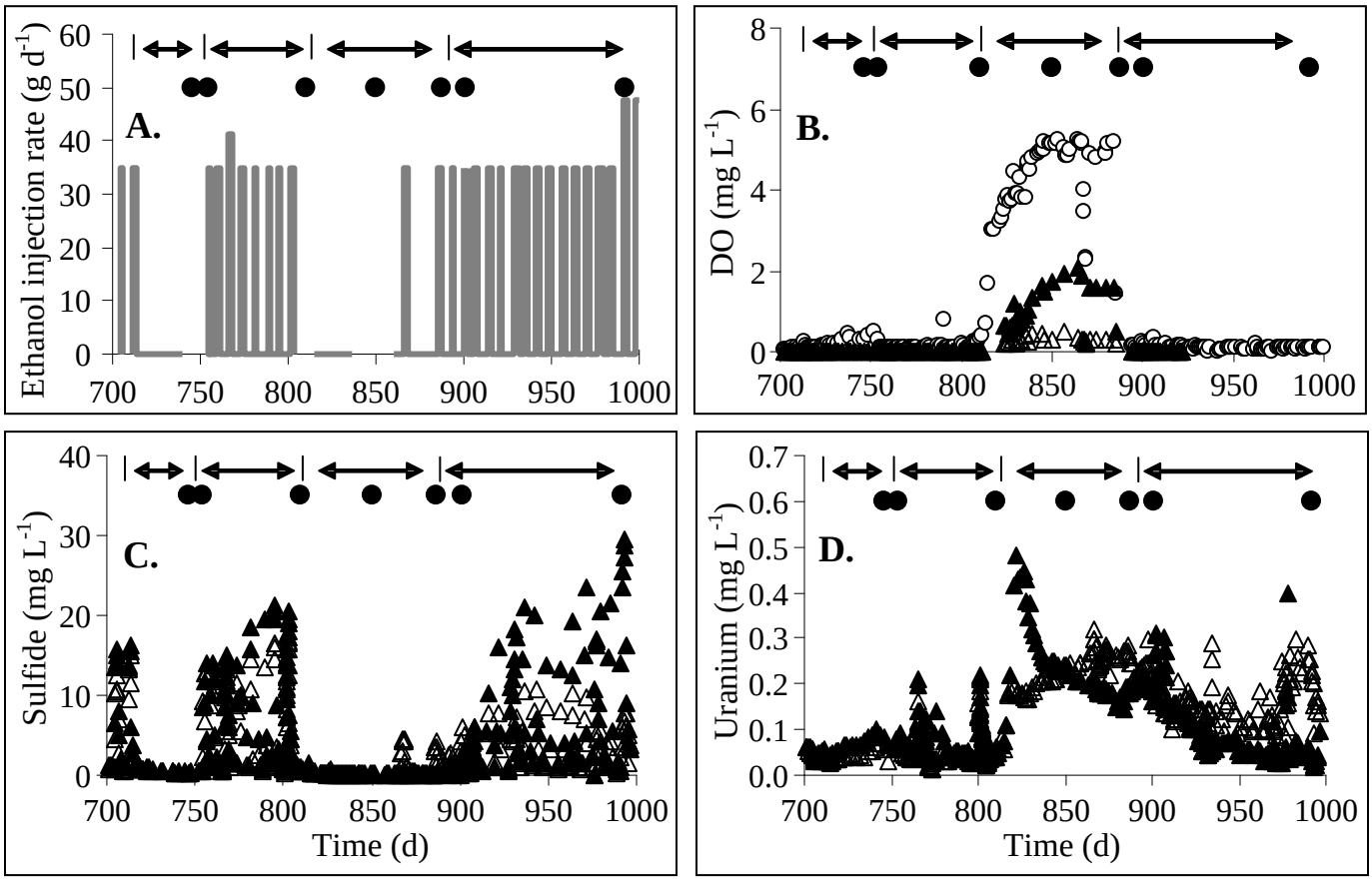

Figure 2. Detrended correspondence analysis of functional genes from FW101-2 and FW102-3. Functional genes detected using the GeoChip 2.0 were used for DCA. The numbers next to each symbol are the operational day corresponding to sample collection. Gray circles represent FW101-2 and black circles represent FW102-3.

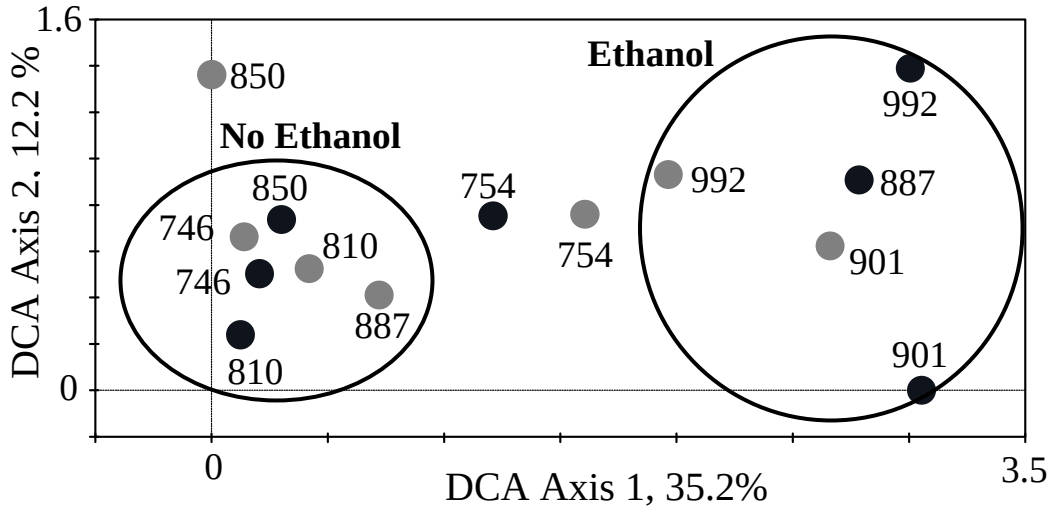

Figure 3. Relative abundance of all functional gene groups detected. The total number of genes detected at each time point was used to calculate the relative abundance of each gene group from FW101-2 (A) and FW102-3 (B). Numbers along x-axis are operational day.

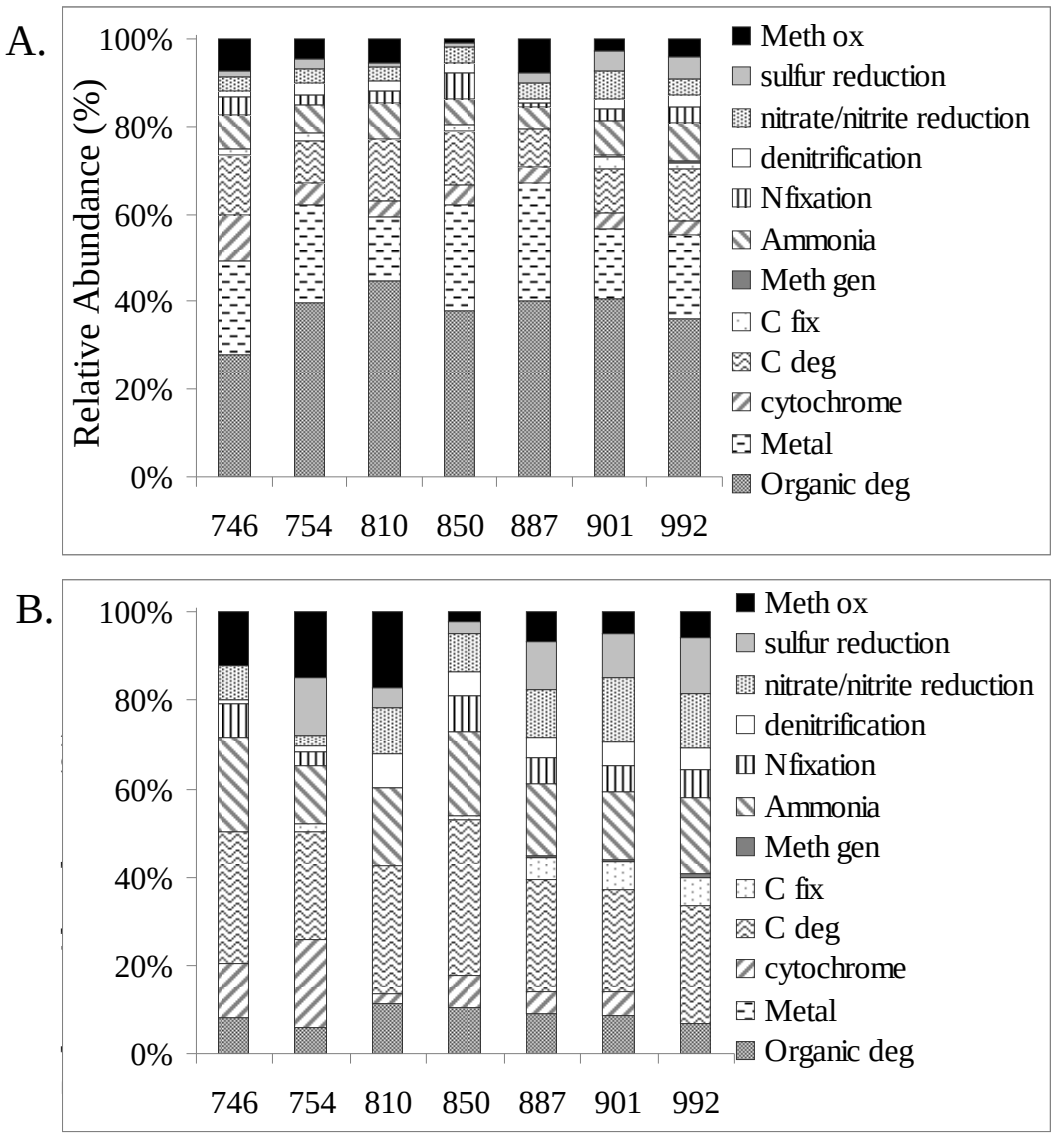

Figure 4. Hierarchical cluster analysis of dissimilatory sulfite reduction genes. Genes that were present in at least 3 timepoints were used for cluster analysis. Results were generated in CLUSTER and visualized using TREEVIEW. Red indicates signal intensities above background while black indicates signal intensities below background. Brighter red coloring indicates higher signal intensities.

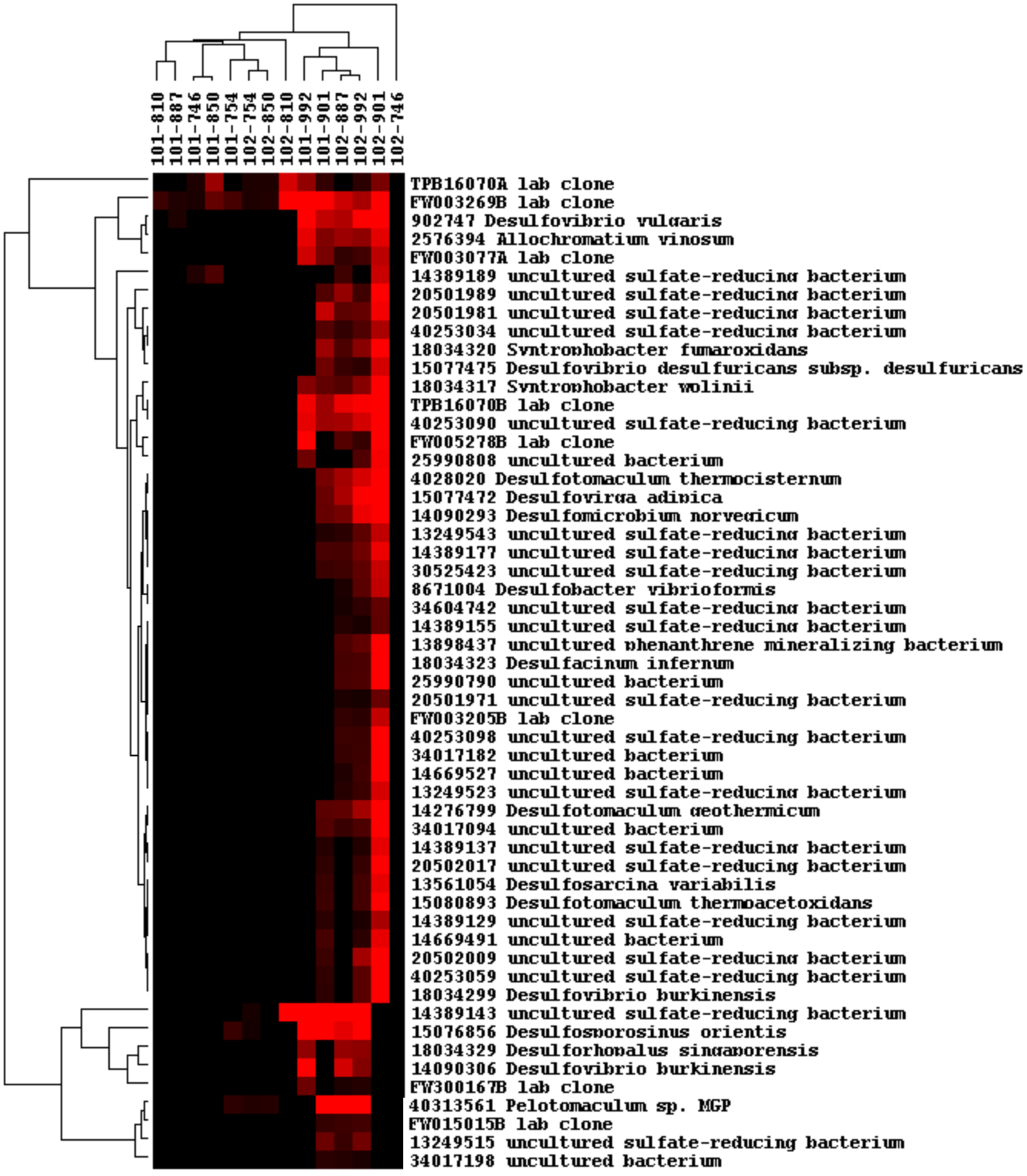

Figure 5. Hierarchical cluster analysis of carbon degradation genes. Genes that were present in at least 4 timepoints were used for cluster analysis. See legend for Fig. 6 for explanation.

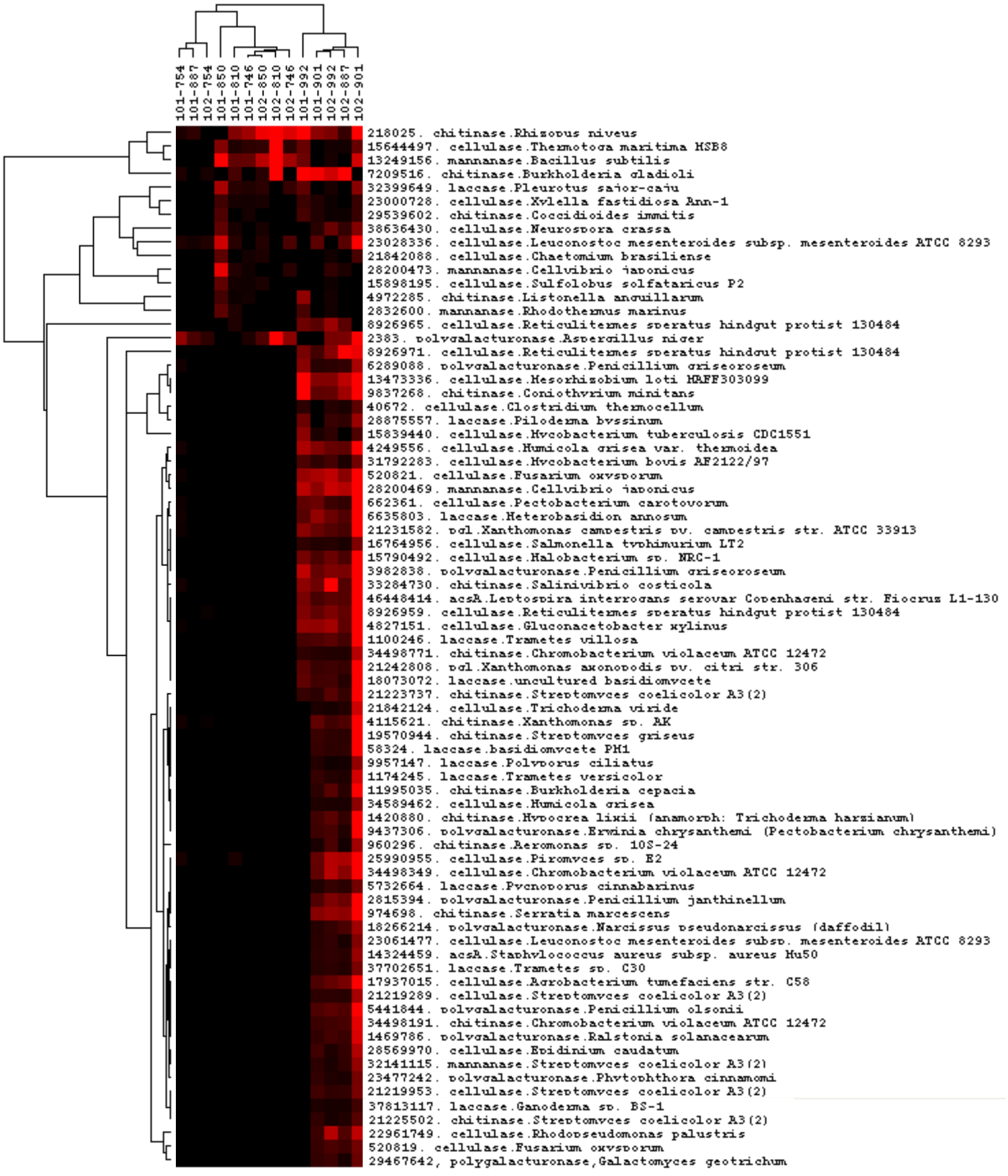

Figure 6. Relative abundance of cytochrome-containing genera. The relative abundance was calculated based on the total signal intensity of all cytochrome genes originating from each genera. 'Other' includes *Chlamydophila* and *Mycobacterium.*

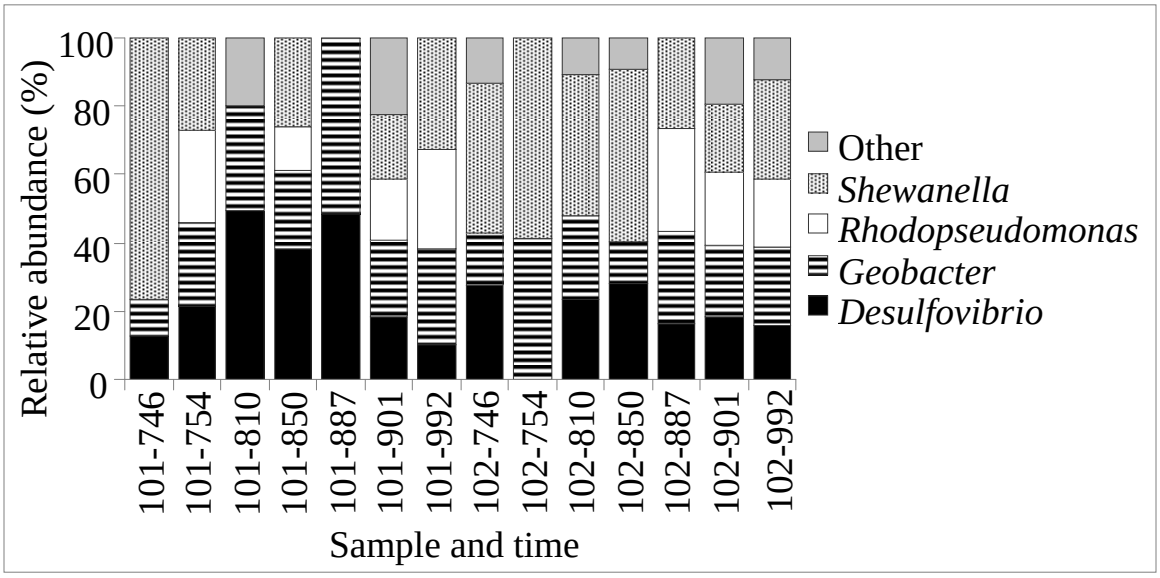

Figure 7. Canonical correspondence analysis for FW101-2 and FW102-3. Analysis was done using all functional genes detected (symbols) and environmental variables (arrows) from FW101-2 and FW102-3. Environmental variables were chosen based on significance calculated from individual CCA results and variance inflation factors calculated during CCA.

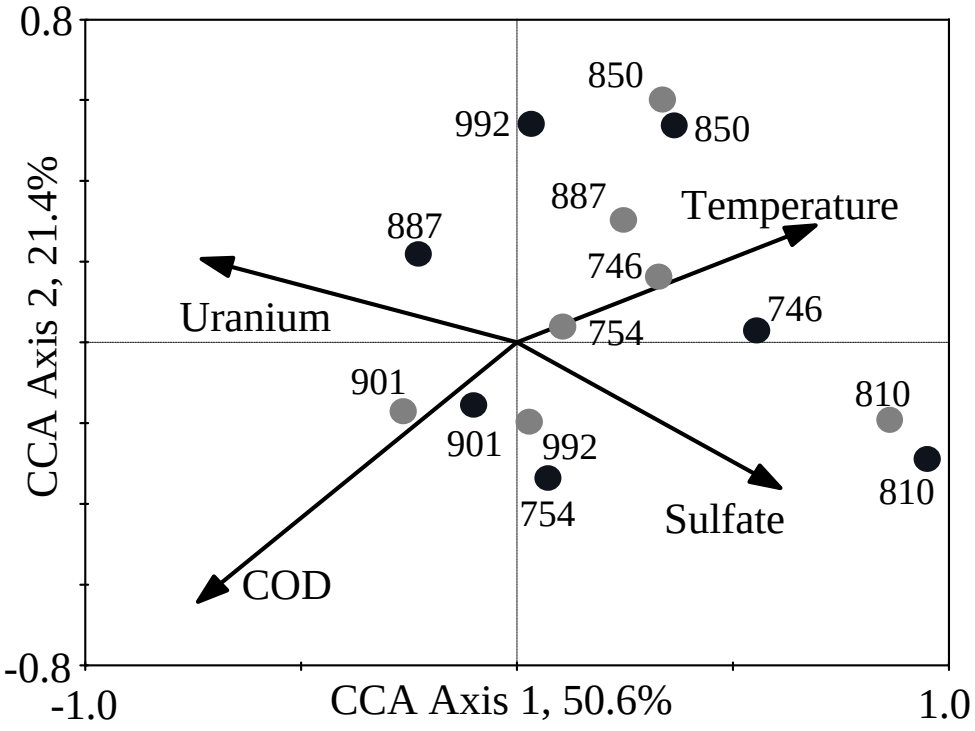

Figure 8. Variance partitioning of environmental variables analyzed by CCA. The diagram represents the relative effects of each variable upon the functional community in FW101-2 and FW102-3. The circles represent the effect of individual variables, by partitioning out the effects of the other variables. The squares between the circles represent the combined effect of the circles on either side of the square. Their was no combined effect of all variables. The square at the bottom of each figure represents the effect that could not be explained by any of the variables tested. Variables used in CCA were used for the VPA. COD, chemical oxygen demand; T, temperature; G, geochemical variables (sulfate and uranium). p-values shown were generated during partial CCA.

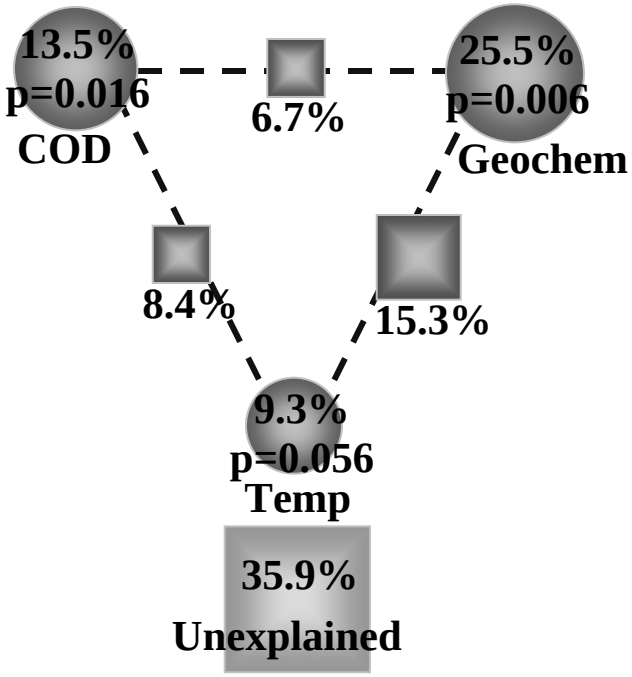

Figure S1. Groundwater recirculation system at OR-FRC. Arrows indicate ground water flow. Circles with an x indicate injection (FW024 and FW104) and extraction wells (FW026 and FW103). Filled circles indicate monitoring wells (FW100, FW102, and FW102).

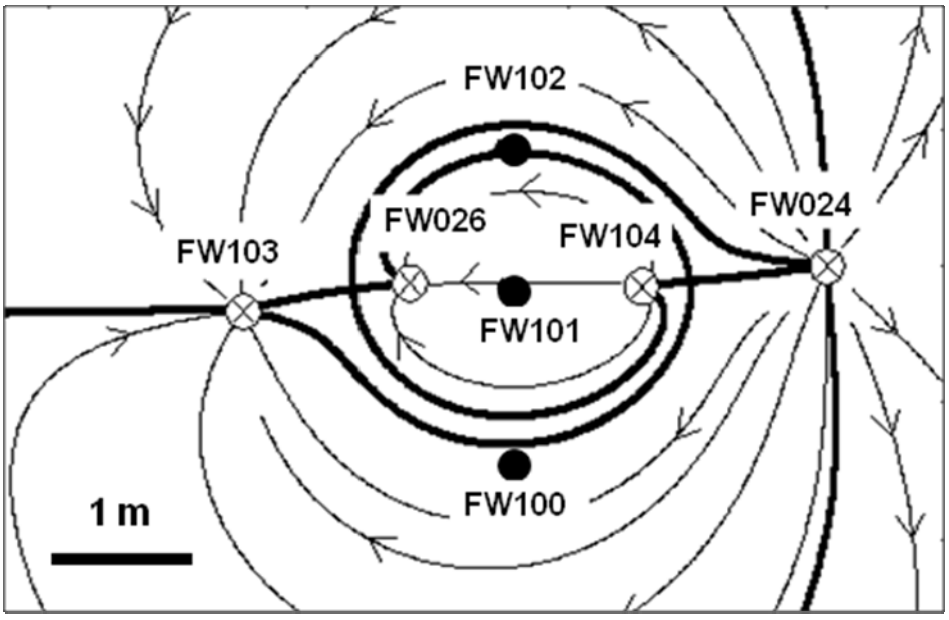

Figure S2. Relative abundance of cytochrome c, *dsr*, and metal resistance genes. The relative abundance of genes was calculated based on the total signal intensity of each gene group. The relative abundance of cytochrome c (A), *dsr* (B) and metal resistance (C) genes in FW101-2 (gray) and FW102-3 (black) are shown.

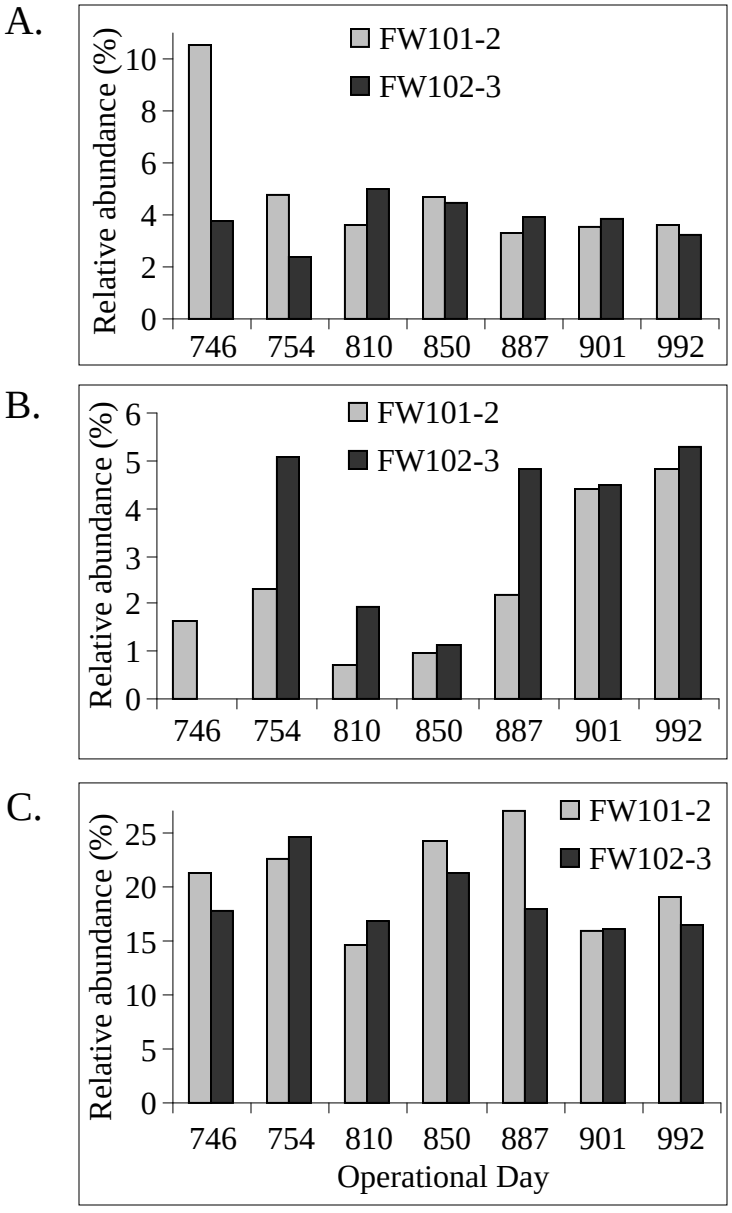

Figure S3. Hierarchical cluster analysis of metal resistance genes. Genes that were present in at least 5timepoints were used for cluster analysis. See legend for Fig. 6 for explanation.

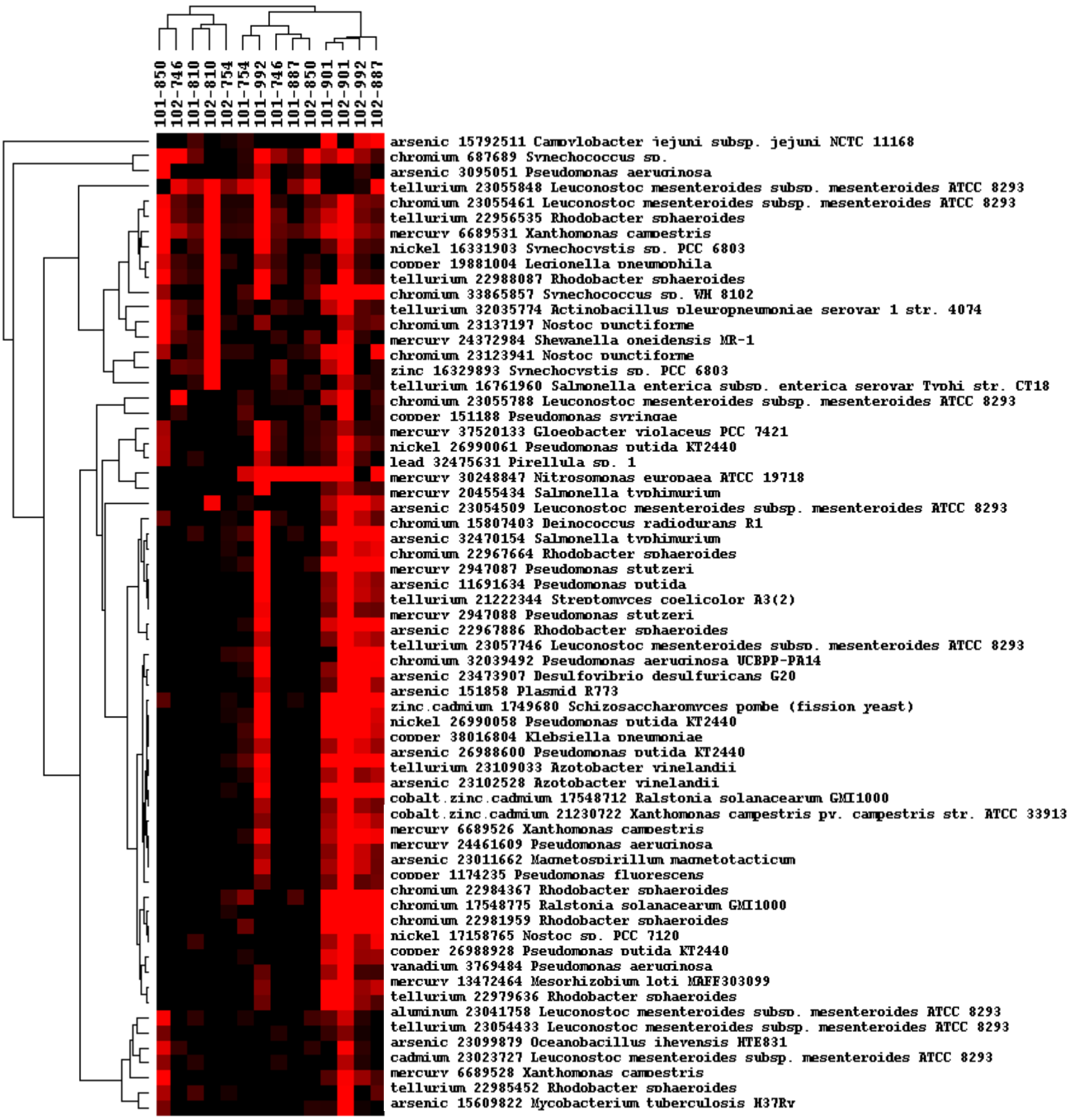

Figure S4. Hierarchical cluster analysis of methanogenensis genes. All genes were used for cluster analysis. Methanogenesis genes were only detected at timepoints shown in the figure. See legend for Fig. 6 for explanation.

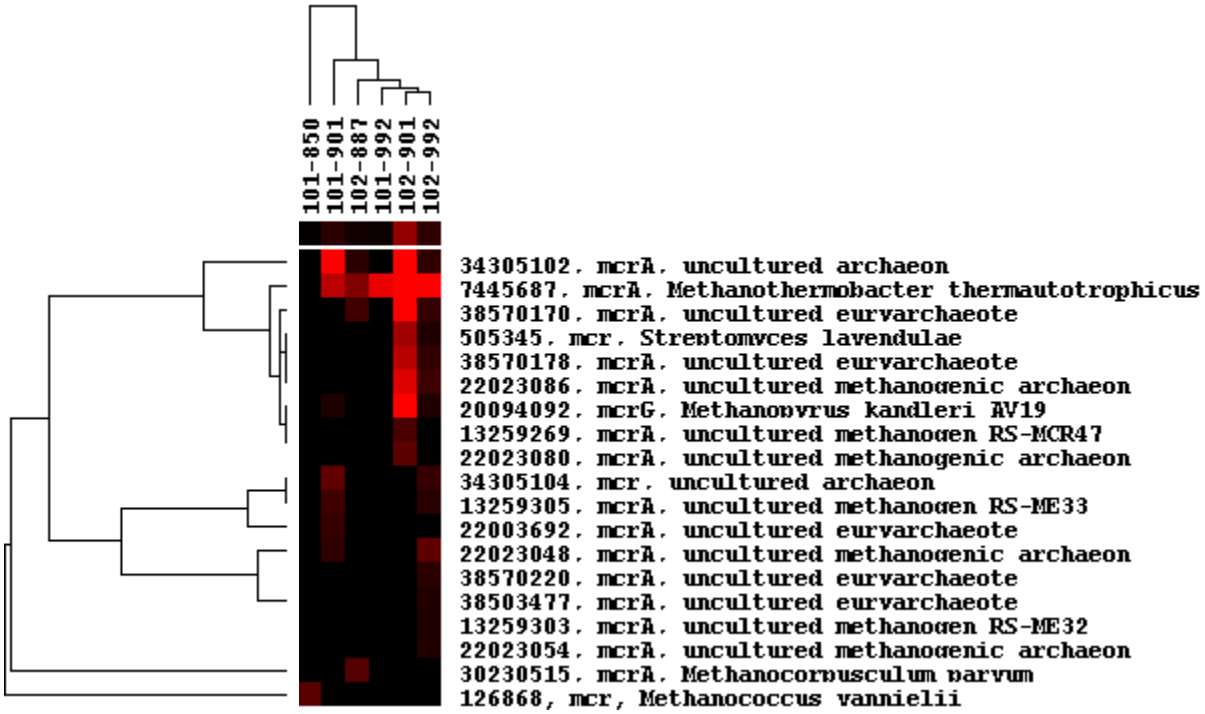

Figure S5. Hierarchical cluster analysis of denitrification genes. Genes that were present in at least 4 timepoints were used for cluster analysis. See legend for Fig. 6 for explanation.

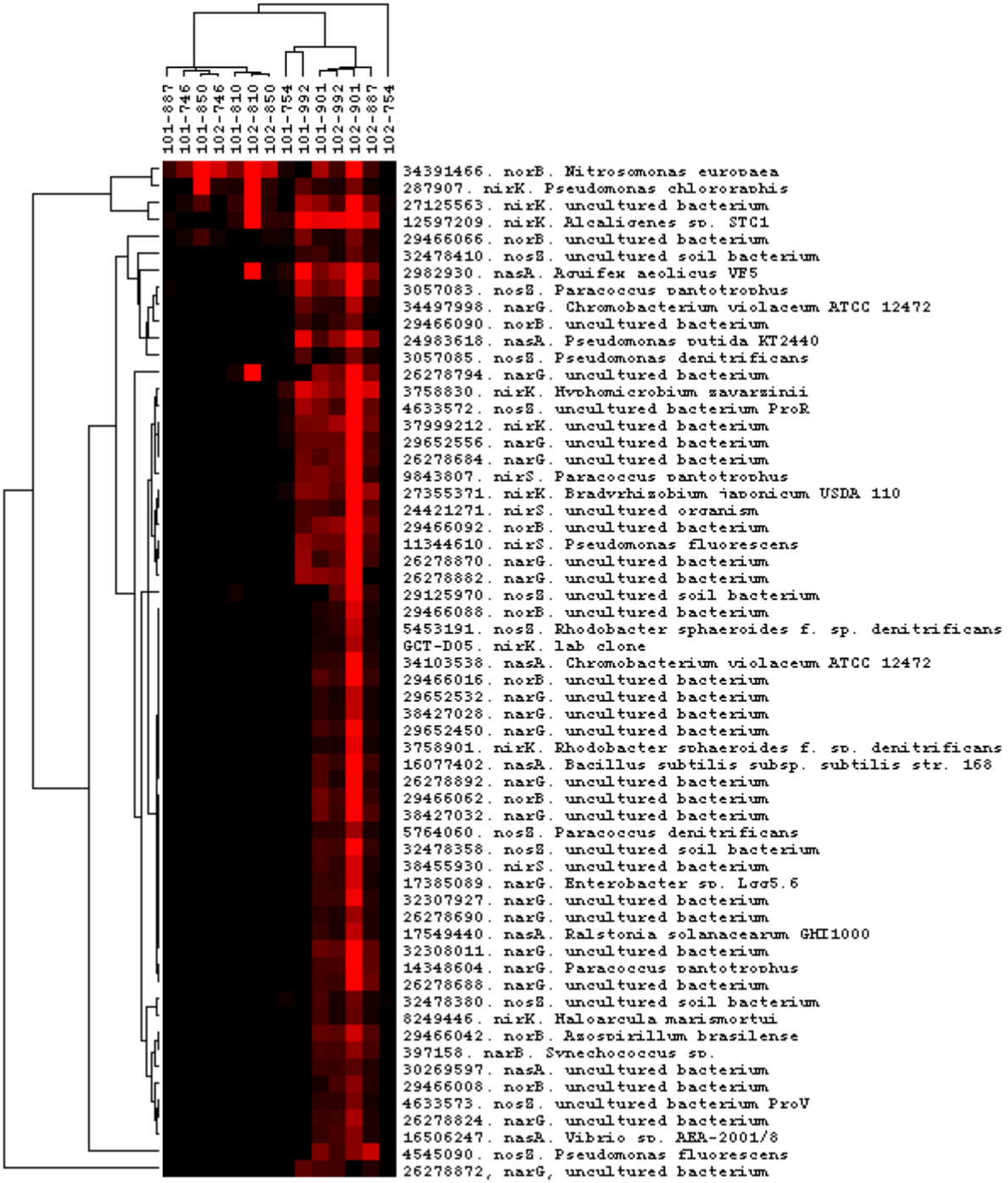

Figure S6. Hierarchical cluster analysis of nitrogen fixation genes. Genes that were present in at least 2 timepoints were used for cluster analysis. See legend for Fig. 6 for explanation.

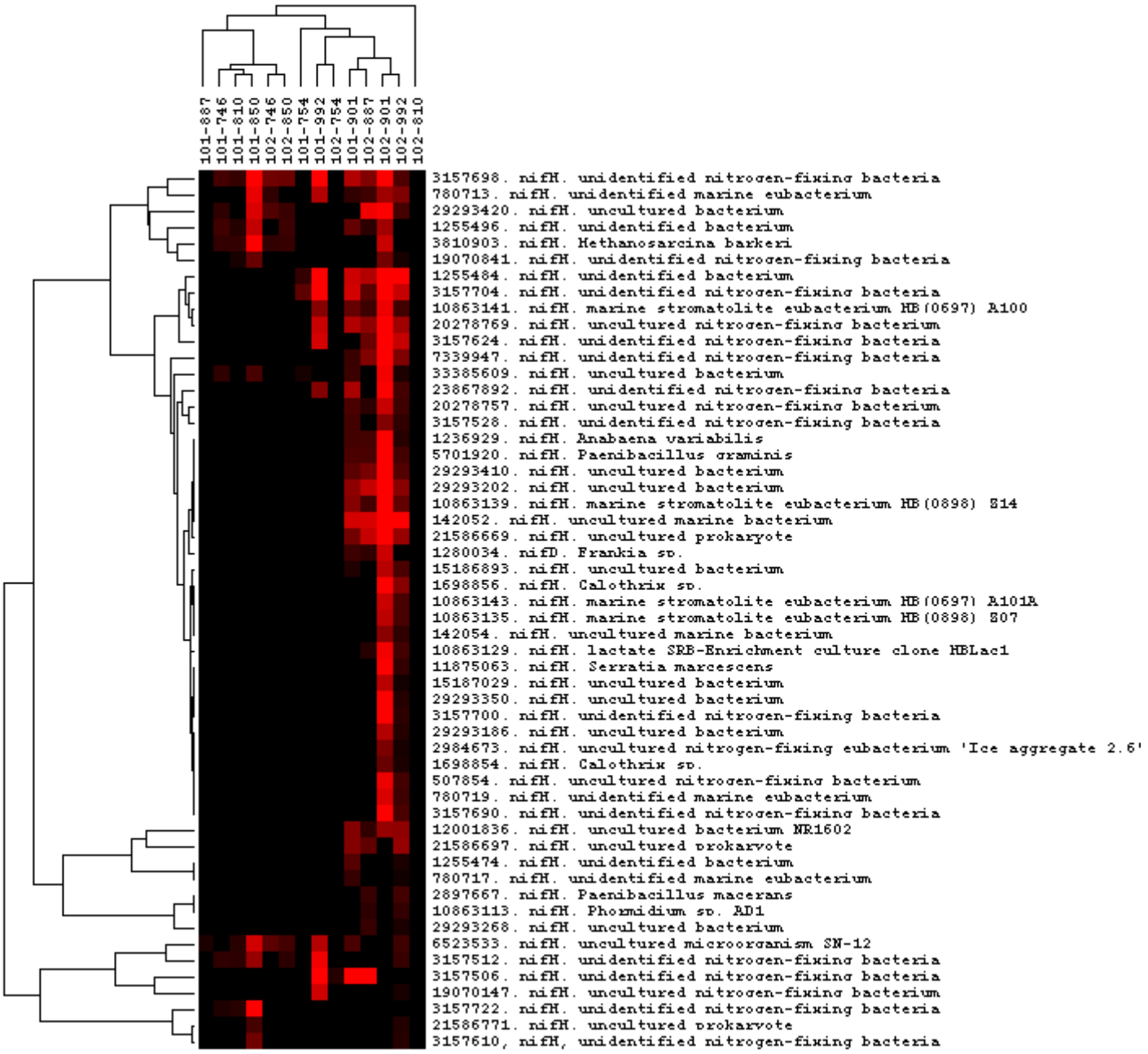

Figure S7. Hierarchical cluster analysis of contaminant degradation genes. Genes that were present in at least 6 timepoints were used for cluster analysis. See legend for Fig. 6 for explanation.

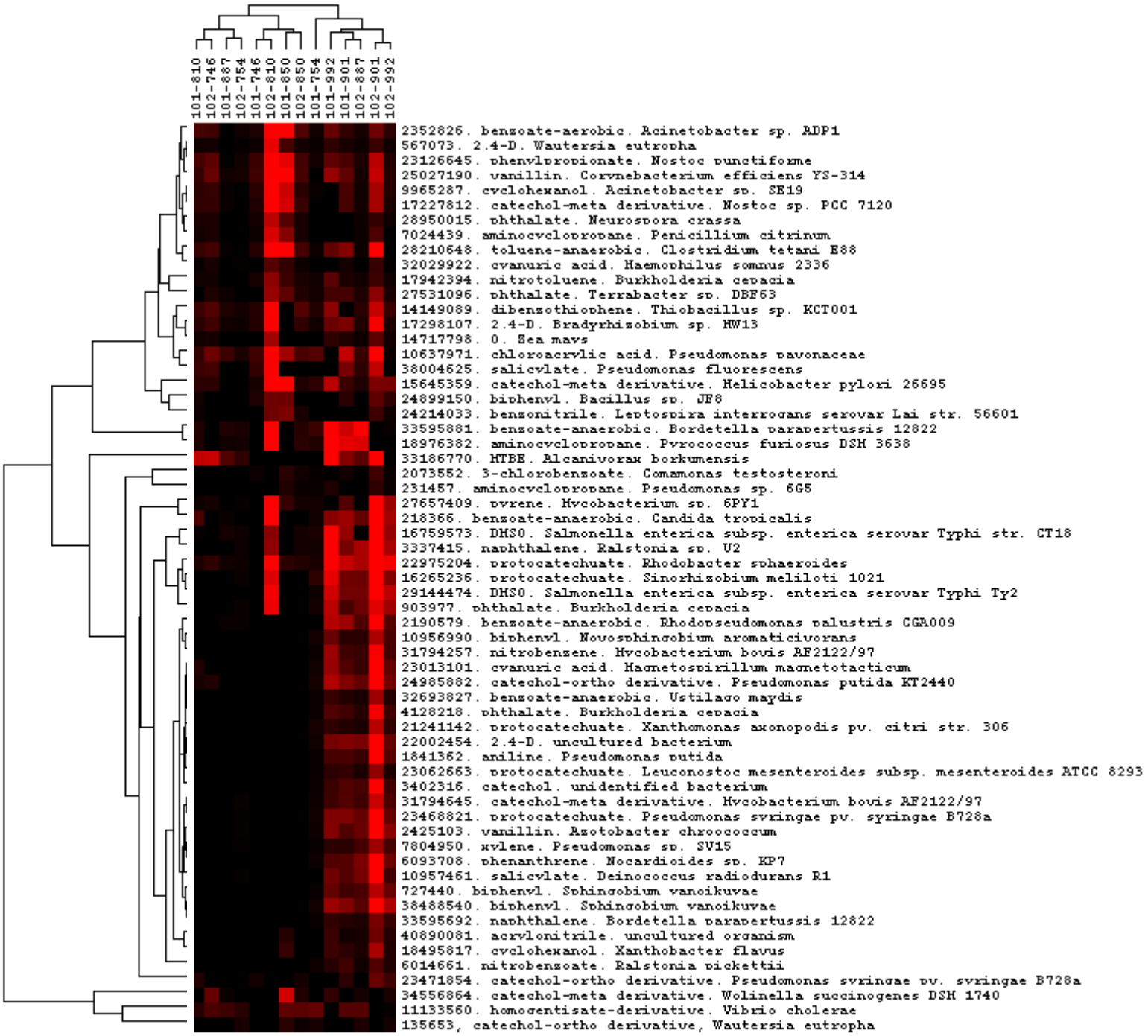

Figure S8. Abundance of all genes from *Desulfovibrio* and *Geobacter*. The average signal intensity of all detected genes derived from *Desulfovibrio* and *Geobacter* at each time point from wells FW101-2 (A) and FW102-3 (B).

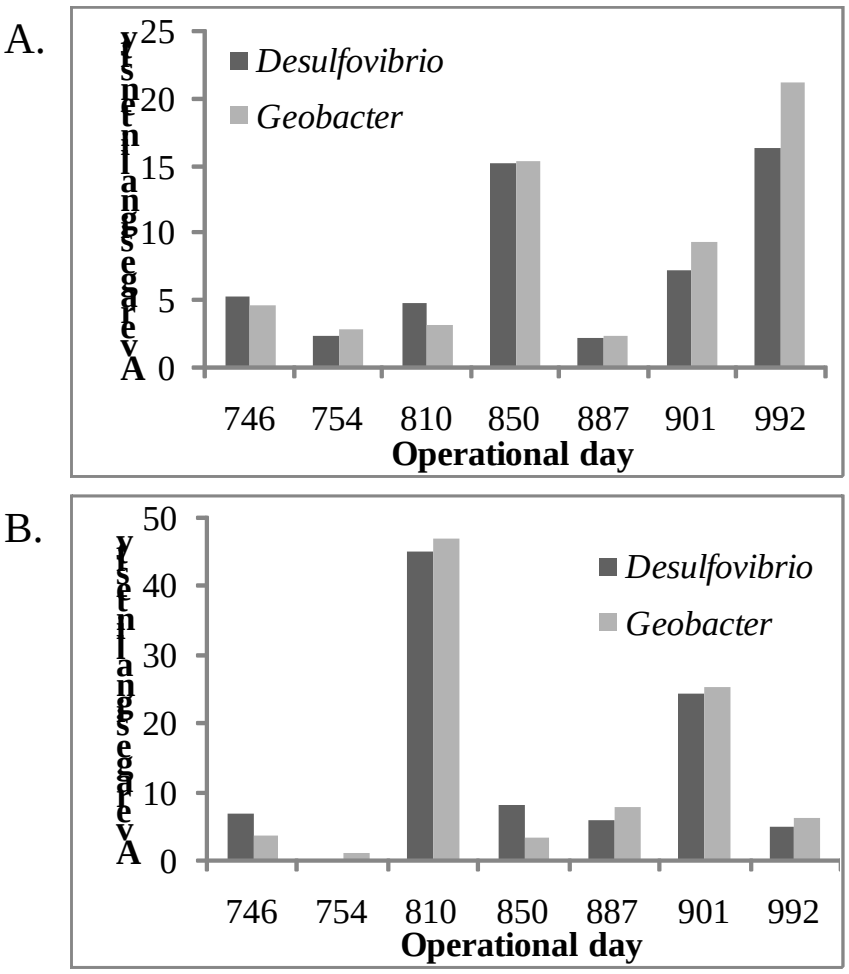

Table 1. Major geochemical concentrations at each timepoint examined

FW101-2							
Operational Day	**746**	**754**	**810**	**850**	**887**	**901**	**992**
Sulfate	111.5	101.4	120.1	56.0	68.8	95.2	41.4
Sulfide	0.3	1.0	0.9	0.02	0.2	1.5	23.5
Uranium	0.1	0.1	0.04	0.2	0.2	0.3	0.04
COD[a]	3	44	3	3	39	126	130
Fe(II)	1.26	1.2	1.33	3.95	3.22	0.82	2.09
pH	6.00	5.98	5.83	6.00	5.97	6.25	6.69
Temperature (°C)	19.7	19.6	17.30	14.3	14.3	13.7	16.1
FW102-3							
Operational Day	**746**	**754**	**810**	**850**	**887**	**901**	**992**
Sulfate	112.5	96.3	136.2	64.4	36.5	67.4	61.5
Sulfide	0.3	2.3	0.4	0.2	3.5	6.0	6.1
Uranium	0.1	0.1	0.1	0.2	0.2	0.2	0.2
COD	7	52	3	3	57	67	15
Fe(II)	2.52	2.5	0.57	4.0	3.45	2.5	3.76
pH	6.00	5.83	5.73	5.63	5.43	5.99	5.85
Temperature (°C)	19.7	19.6	17.30	14.3	14.3	13.7	16.1

[a] COD, chemical oxygen demand. COD was used to monitor ethanol concentration.

Table 2. Gene overlap (italicized), uniqueness (bold), and diversity indices of FRC samples[a]

FW101-2									
Day	**Operational Period**		**746**	**754**	**810**	**850**	**887**	**901**	**992**
	Ethanol	**DO**							
746	N	N	**1.03%**	*16.51%*	*43.66%*	*34.30%*	*16.67%*	*6.07%*	*11.95%*
754	Y	N		**0.64%**	*19.46%*	*15.75%*	*22.49%*	*14.74%*	*28.87%*
810	N	Y			**0.93%**	*29.73%*	*25.60%*	*7.90%*	*13.95%*
850	N	Y				**9.94%**	*12.68%*	*7.65%*	*16.52%*
887	Y	N					**0.00%**	*4.65%*	*8.78%*
901	Y	N						**1.64%**	*28.31%*
992	Y	N							**0.90%**
Total genes detected			93	150	102	174	48	886	316
Shannon-Weaver *H*			4.20	4.79	4.34	4.82	3.58	6.59	5.48
Shannon-Weaver evenness			0.93	0.96	0.94	0.94	0.93	0.97	0.95

FW102-3									
Day	**Operational Period**		**746**	**754**	**810**	**850**	**887**	**901**	**992**
	Ethanol	**DO**							
746	N	N	**0.00%**	*20.45%*	*43.36%*	*41.91%*	*5.93%*	*3.77%*	*2.83%*
754	Y	N		**2.53%**	*28.80%*	*22.29%*	*6.85%*	*3.66%*	*2.91%*
810	N	Y			**2.44%**	*40.29%*	*6.53%*	*4.00%*	*2.91%*
850	N	Y				**0.00%**	*6.84%*	*5.02%*	*4.07%*
887	Y	N					**1.65%**	*45.56%*	*39.46%*
901	Y	N						**14.58%**	*54.13%*
992	Y	N							**29.59%**
Total genes detected			74	76	76	106	993	1737	2321
Shannon-Weaver *H*			4.05	4.14	4.07	4.38	6.64	7.26	7.42
Shannon-Weaver evenness			0.94	0.96	0.94	0.94	0.96	0.97	0.96

[a] Genes were used as "species" and abundance was indicated by the average normalized signal intensity data of each gene from triplicate arrays x 10. DO, dissolved oxygen; N, no; Y, yes.

Table 3. Summary of canonical correspondence analysis results.

Axes	**1**	**2**
Eigenvalues	0.455	0.193
Species-environment correlations	0.929	0.966
Cumulative percentage variance		
Of species data	28.7	40.8
Of species-env relation	50.6	72.0
Sum of all eigenvalues	1.588	
Sum of all canonical eigenvalues	0.900	
Summary of Monte Carlo test		
Test of significance of first canonical axis	0.455	
F-ratio	3.617	
P-value	0.008	
Test of significance of all canonical axes	0.900	
F-ratio	2.940	
P-value	0.002	