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# Phylogenetic and Functional Biomakers as Indicators of Bacterial Community Responses to Mixed-Waste Contamination

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Few studies have demonstrated changes in community structure along a contaminant plume in terms of phylogenetic, functional, and geochemical changes, and such studies are essential to understand how a microbial ecosystem responds to perturbations. Clonal libraries of multiple genes (SSU rDNA, *nir*K, *nir*S, *amo*A, *pmo*A, and *dsr*AB) were analyzed from groundwater samples ($n = 6$) that varied in contaminant levels, and 107 geochemical parameters were measured. Principal components analyses (PCA) were used to compare the relationships among the sites with respect to the biomarker ($n = 785$ for all sequences) distributions and the geochemical variables. A major portion of the geochemical variance measured among the samples could be accounted for by tetrachloroethene, $^{99}Tc$, $NO_3$, $SO_4$, Al, and Th. The PCA based on the distribution of unique biomarkers resulted in different groupings compared to the geochemical analysis, but when the SSU rRNA gene libraries were directly compared ($\Delta C_{xy}$ values) the sites were clustered in a similar fashion compared to geochemical measures. The PCA based upon functional gene distributions each predicted different relationships among the sites, and comparisons of Euclidean distances based upon diversity indices for all functional genes ($n = 432$) grouped the sites by extreme or intermediate contaminant levels. The data suggested that the sites with low and high perturbations were functionally more similar than sites with intermediate conditions, and perhaps captured the overall community structure better than a single phylogenetic biomarker. Moreover, even though the background site was phylogenetically and geochemically distinct from the acidic sites, the extreme conditions of the acidic samples might be more analogous to the limiting nutrient conditions of the background site. An understanding of microbial community-level responses within an ecological framework would provide better insight for restoration strategies at contaminated field sites.

## Introduction

The biodegradation and/or biotransformation of pollutants in the natural environment is a complex process that depends on the quantitative and qualitative aspects of the contaminants, the structure and dynamics of the indigenous microbial community, and the geochemical conditions (*1*). In addition, many disturbed sites contain multiple contaminants that can complicate treatment and/or maintenance. The elucidation of individual and community-wide microbial responses to the combination of mixed wastes and varied geochemical conditions is a crucial aspect for the improvement and implementation of bioremediation strategies.

Yet, due to the inherent difficulties from heterogeneity and multiple interacting variables, studies have examined closed systems with only one or two disturbances. As recently noted by Girvan et al. (*2*), questions dealing with microbial diversity in soil systems may require a functional approach due to complexity. Similar approaches may be needed for aqueous systems, including groundwater. However, it has been difficult to capture a significant portion of all possible variables that likely affect community composition and structure at the microscale. A major question that needs to be addressed is what microbiological measurements provide accurate and reliable indicators of community and population responses in order to provide a basis for predictive ecology in the context of bioremediation.

A major challenge in microbial ecology is to understand a given ecosystem from the type of data that are commonly collected (e.g., gene sequences and geochemistry). The question then arises if the data can be evaluated in the contexts of ecological theory and the principles applied for predictive bioremediation. In the realm of microbial ecology, challenges are manifested by the occurrence of similar genes in different organisms, similar organisms with different genes, and even possibly similar community structures that contain different populations. These challenges are exacerbated by the question of even how a bacterial species is defined (*3*, *4*). Possible relationships between phylogenetic diversity, functional potential, and habitat conditions need to be delineated.

One of the major goals of the Field Research Center (FRC) is to understand biotic and abiotic interactions that can affect bioremediation. The FRC is located within the Y-12 Security Complex near Oak Ridge, TN, and the site includes 243 acres of a previously disturbed contaminated area. The waste ponds at the FRC were four unlined surface impoundments that received nitric acid/uranium bearing wastes for approximately 30 years. The subsurface at the FRC contains one of the highest concentration plumes of mobile uranium located in the United States, and contains various levels of nitrate, heavy metals, and organic contamination (http://www.esd.ornl.gov/nabirfrc/).

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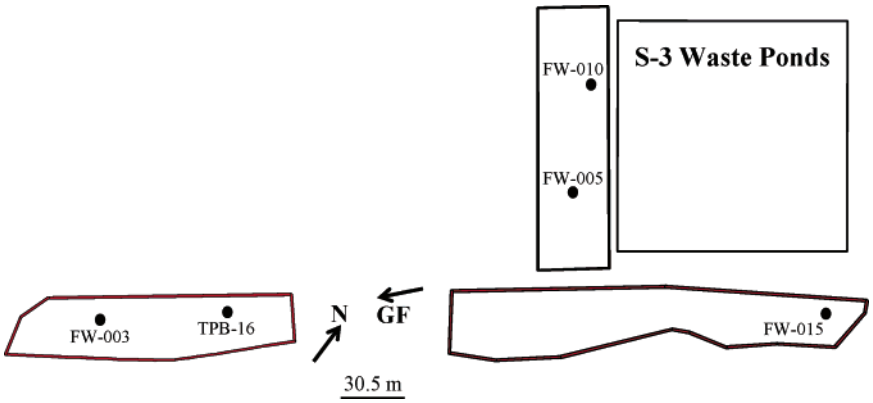


**FIGURE 1. Schematic map of the former S-3 waste ponds at the Field Research Center. Sampled wells are denoted on the map, and the background well (FW-300) is approximately 2 km from the contaminated area and lies directly along the geologic strike of the contaminated area. Background is underlain by nearly identical geology, mineralogy, and structure. The substratum of the sampling sites was similar, and consisted of shale with inner-embedded limestone that has been weathered to clay, silty saprolite. GF, groundwater flow; N, north.**

To determine how varying levels of multiple contaminants have affected microbial community structure at different levels of resolution, the clonal libraries for six genes were analyzed from six sites along a contamination plume. A total of 6127 gene sequences and 107 geochemical measures were combined for the analyses from the six sites, and the large data set was used to make different comparisons between respective sites in the context of bacterial community structure and function. Our results showed that different components of groundwater communities responded differently to disturbances, and based upon the tested biomarkers, low and high contamination sites were functionally similar despite geochemical and phylogenetic differences.

## Materials and Methods

**Sampling and Water Chemistry.** Groundwater samples were collected from six wells at the FRC: FW-300, FW-003, TPB-16, FW-005, FW-010, and FW-015 (Figure 1). Areas 1 and 3 are in close proximity to the waste ponds, and Area 2 is approximately 275 m downgradient from the waste ponds. Well FW-300 is located in the uncontaminated background area, approximately 2 km northwest of the source ponds. The water table was approximately 6 m, and water was collected from a screened interval below the water table at each of the six wells on the same day (December 2000).

All water analytes ($n = 107$) were measured according to EPA methods as reported at the Field Research Center website (http://www.esd.ornl.gov/nabirfrc/). Briefly, a precalibrated YSI XL6000M multi-parameter probe (Yellow Springs Instruments, Yellow Springs, OH) was used for field measurements of conductivity, dissolved oxygen, $E_h$, temperature, and pH, and a Hach method was used for Fe(II) and sulfide. Anions (bromide, chloride, nitrate, phosphate, and sulfate) were measured via ion chromatography and organic carbon with a Shimadzu total organic carbon analyzer (TOC-5000A, Tokyo, Japan) (EPA method 415.1). Elemental analysis was done with an inductively coupled plasma poly-scan spectrometer (method SW846-6010B), $^{241}$Am and $^{237}$Np were measured via method ASO-Y/P65-7206, $^{99}$Tc was measured via method ASO-ACD-160063, and organics were analyzed with gas chromatography−mass spectroscopy (method SW846-8260-UP).

**Microscopy.** Epifluorescence microscopy (Nikon Phase Contrast Optiphot) of acridine-orange stained samples was done as previously described (*5*, *6*). Live/dead analyses (MolecularProbes, Inc.) were done according to the manufacturer's instructions.

**DNA Extraction and Purification.** Groundwater samples (1−2 L) were collected and transported to the laboratory in amber glass bottles. Bacteria were harvested by centrifugation (10 000*g* force, 4 °C for 30 min), and the pellets were stored at −80 °C until used for DNA extraction. The cell pellet was resuspended in a lysis buffer and the cells were disrupted with a previously described grinding method (*7*). DNA was extracted and purified as previously described (*7*, *8*). The same nucleic acid samples were used for the amplification of all gene sequences.

**SSU rRNA Gene Sequences.** The phylogenetic relatedness of the clonal libraries from background (FW-300) and the three acidic sites (FW-005, FW-010, FW-015) were recently described (*9*). The GenBank accession numbers for clonal sequences are AY661934−AY662049. For this study, two additional sites (FW-003 and TPB-16) were selected to represent intermediate sites that had circumneutral pH with relatively low levels of contaminants. The SSU rDNA clonal sequences for FW-003 and TPB-16 were determined and analyzed as previously described (*9*). The primers FD1 (5′-agagtttgatcctggctcag-3′) and 1540R (5′-aaggaggtgatccagcc-3′) were used for amplification from environmental samples as previously described (*9*), and primer 529r was used for sequence determination of the 5′ end (5′-cgcggctgctggcac-3′). The GenBank accession numbers for clonal sequences from TPB-16 and FW-003 are DQ407297−DQ407473.

**Functional Gene Sequences.** The amplification, cloning, and phylogenetic analyses of the *nir*K and *nir*S clonal libraries that correspond to the FRC groundwater samples have been previously described (*10*). The nucleic acid sequence accession numbers in GenBank are as follows: AF548909−AF548940 and AF548942−AF548958 for *nir*K clones, and AF548959−AF549061, AF549063−AF549065, and AF549067−AF549103 for *nir*S. The amplification, cloning, and phylogenetic analyses of the clonal libraries for the *dsr*AB gene sequences (AY885558−AY885427) have been previously described (*11*). The amplification and cloning for the *amo*A/*pmo*A gene sequences were done as described for the *nir* genes (*10*) except with specific primers for *amo*A/*pmo*A, and detailed phylogenetic analyses will be described in a separate manuscript.

**Operational Taxonomic Unit (OTU) Distribution and Statistical Analyses.** The SSU rRNA gene sequences were screened via direct sequence determination of the first 500 nucleotides (*9*, *12*, this study). The functional gene sequences were screened via RFLP analysis, and partial sequences were determined for unique OTUs as described previously (*10*). All unique OTUs were based upon ≤97% sequence identity with other clonal sequences. For each gene dataset, the distribution and overlap of each unique OTU was tabulated as a percentage of the total from each respective site. Alternatively, the distribution of unique OTUs was scored for the presence or absence at each respective site. Factor

analysis, principal components analysis (PCA), and correlative distance matrices were done within the SPSS statistical methods package (version 13.0, SPSS Inc.).

LIBSHUFF (v. 1.2) analysis was used to compare the respective SSU rDNA libraries from the six sites as previously described (*9*, *13*). LIBSHUFF analyses compute the homologous and heterologous coverage within and between clonal libraries, and analysis was performed according to specified directions given at the LIBSHUFF website (http://www.arches.uga.edu/~whitman/libshuff.html). The analysis estimates the similarity between clonal libraries from two different samples based upon evolutionary distances of all sequences. Thus, the sampled diversity of a community can be directly compared to another community. The values are reported over a sequence similarity range or evolutionary distance (*D*) based upon a distance matrix. A $\Delta C_{xy}$ value between two samples can be computed and represents the degree of dissimilarity between the two samples (higher $\Delta C_{xy}$ represents more dissimilarity). A distance matrix was constructed with the $\Delta C_{xy}$ values from LIBSHUF analysis that represented the pairwise comparison of the six samples. UPGMA and neighbor-joining methods were used to construct phylogenetic trees of the six sites based upon distance matrices based upon correlation values or $\Delta C_{xy}$ values with MEGA 2.1 (*14*).

## Results

**Geochemical Analysis.** The chemical characteristics were significantly different among the six tested sites, and the major markers of contamination (pH, nitrate, uranium, nickel, and total organic carbon) have been described previously (*9*, *10*). In total, 107 analytes were used in the current analyses (Table 1, and all data can be obtained at a query-based database (http://www.esd.ornl.gov/nabirfrc/). When six major geochemical measures were used to compare the sites based upon PCA (pH, nitrate, uranium, nickel, sulfate, and Al), the two least contaminated sites were grouped (FW-300 and TPB-16) and FW-003, FW-005, FW-010, and FW-015 were grouped (data not shown). When all analytes were included in the comparison, the sites were grouped in a similar fashion, except more resolution was observed among the four sites with increased nitrate levels (Figure 2a). These results indicated that a smaller number of major contaminants could be selected to represent the association between the sites based upon geochemical characteristics; however, for more similar sites, additional analytes were needed.

Based upon factor analysis, the two sites with lowest contamination, background and TPB-16, were grouped. The circumneutral site, FW-003, was grouped with the acidic sites, and factor analysis suggested that nitrate, Ca, and TOC levels were major determinants. In addition, the three acidic sites (FW-005, FW-010, FW-015) had significant loadings for tetrachloroethene, 1,1,2-trichloro-1,2,2-trifluorethane, nitrate, $^{99}$Tc, sulfate, Al, Nb, Zn, Sb, V, and Th. Nitrate and the radionuclide, U, have been a major concern at the FRC, but the factor analysis and PCA suggested that other metals (e.g., Al, Sb, V, $^{99}$Tc) and some organics were major determinants that differentiated the sites based upon groundwater chemistry.

**Enumeration of Microbial Numbers.** The direct microscopic counts from circumneutral groundwater were similar to the numbers estimated with culture-based methods ($10^7$ vs $10^6$ cells/mL), but direct counts of acidic groundwater were 2−3 log higher than those estimated with cultivation-based methods ($10^5$ vs $10^2$−$10^3$ cells/ml). MR2A was used for cultivable counts (*15*). The results suggested that the number of microorganisms that could be cultivated was decreased in the contaminated groundwater, but direct counts indicated the bacterial numbers in the acidic groundwater were only approximately one log lower compared to background. These results suggested that cells present at the more contaminated sites might be viable but difficult to cultivate.

**TABLE 1. Analytes (Including Organics, Chloro-organics, Elemental, and Miscellaneous) Used for Geochemical Measurements**

| Carbon | Element |
|---|---|
| 1,2-dibromoethane | As |
| 2-butanone | Ba |
| ether | Be |
| 2-hexanone | B |
| 4-methyl-2-pentanone | Cd |
| acetone | Al |
| acrolein | Br |
| benzene | $^{241}$Am |
| bromochloromethane | C |
| bromodichloromethane | Cl |
| bromomethane | Cr |
| carbon disulfide | Co |
| dibromochloromethane | Cu |
| dibromomethane | Fe |
| dichlorodifluoromethane | $Fe^{2+}$ |
| dimethylbenzene | Pb |
| ethanol | Li |
| ethyl methacrylate | Mg |
| ethylbenzene | Mn |
| iodomethane | Mo |
| methylenechloride | $^{237}$Np |
| styrene | Ni |
| toluene | Nb |
| trichlorofluoromethane | P |
| vinyl acetate | K |
| | Se |
| Chloro-organic | Si |
| 1,1,1,2-tetrachloroethane | Ag |
| 1,1,1-trichloroethane | Na |
| 1,1,2,2-tetrachloroethane | Sb |
| 1,1,2-trichloro-1,2,2-trifluoroethane | Sr |
| 1,1,2-trichloroethane | S |
| 1,1-dichloroethane | |
| 1,1-dichloroethene | $^{99}$Tc |
| 1,2,3-trichloropropane | Tl |
| 1,2-dibromo-3-chloropropane | Th |
| 1,2-dichlorobenzene | Ti |
| 1,2-dichloroethane | U |
| 1,2-dichloroethene | V |
| 1,2-dichloropropane | Zn |
| 1,4-dichloro-2-butene | |
| 1,4-dichlorobenzene | Miscellaneous |
| 2-chloroethylvinyl | bromoform |
| carbon tetrachloride | conductivity |
| chlorobenzene | dissolved oxygen |
| chloroethane | $E_h$ |
| chloroform | pH |
| chloromethane | phosphate |
| *cis*-1,2-dichloroethene | sulfate |
| *cis*-1,3-dichloropropene | sulfide |
| tetrachloroethene | temperature |
| *t*−1,2-dichloroethene | acrylonitrile |
| *t*−1,3-dichloropropene | nitrate |
| *t*−1,4-dichloro-2-butene | |
| trichloroethene | |
| vinyl chloride | |

**Site Comparison Based upon SSU rRNA Gene Sequences.** The SSU rRNA sequence serves as a universal phylogenetic marker that can be used to measure all populations that can be sampled. Clonal libraries of the SSU rRNA genes were compared from groundwater samples for the six sites. When the OTU distributions across the six sites were compared with PCA, the three acidic sites were grouped and 76% of the variance could be explained by three components (Figure 2b). The circumneutral (FW-003, TPB-16) and background sites were distinct from the acidic samples and from each other when compared based upon the SSU rRNA gene sequences. The factor loadings suggested that populations

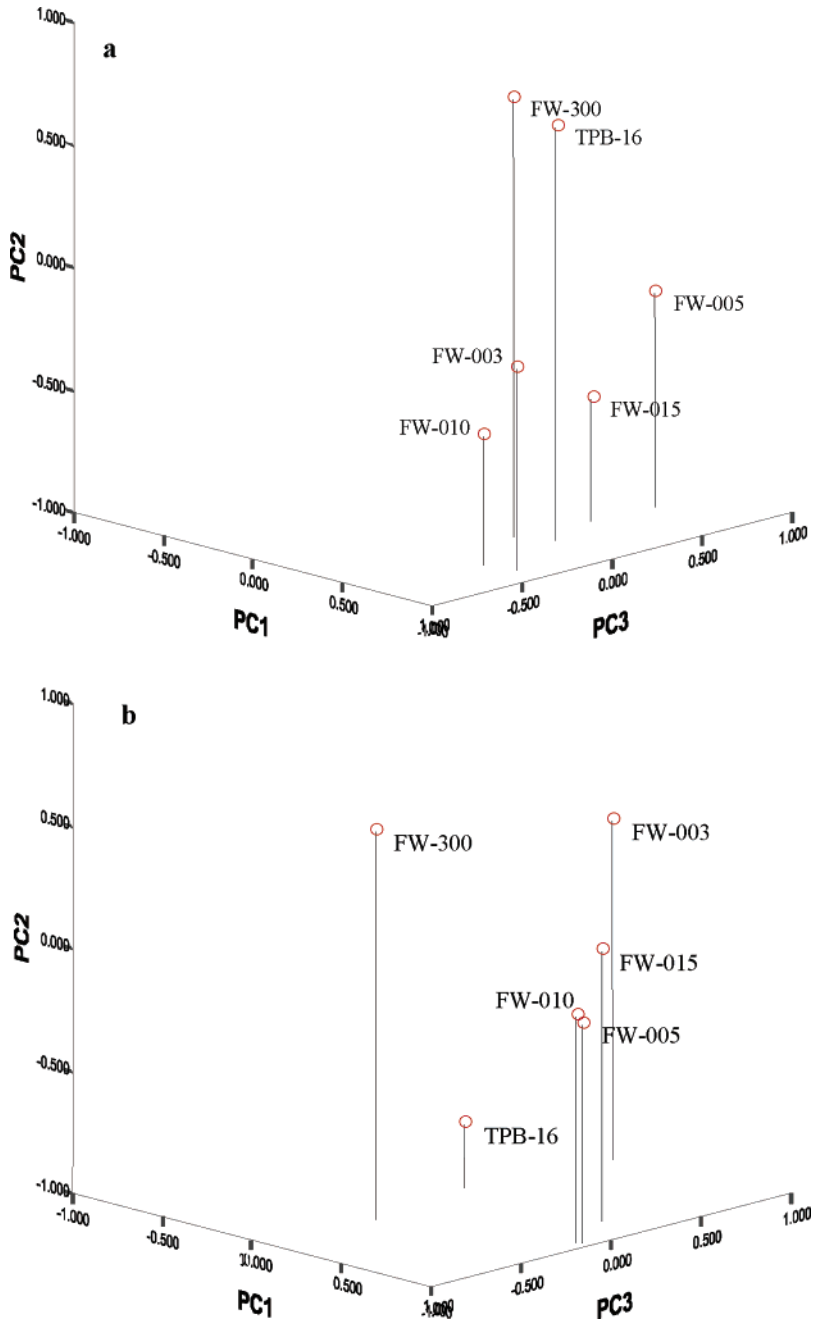


**FIGURE 2. Tripartite principle components analysis of geochemical data (a) and distribution of unique SSU rRNA gene sequences (b) after factor analysis. Each site represents 107 geochemical measures listed in Table 1 and unique sequences were based upon a cutoff of 97% identity (*12*).**

identified as *Azoarcus*, *Pseudomonas*, *Ralstonia* could explain a majority of the variance observed for the acidic, contaminated sites.

The circumneutral site with elevated nitrate levels (FW-003) was distinct, and populations identified as *Rhizobium* and *Diaphorobacter* were major loading factors. The *Rhizobium*-like sequences were most closely related to clonal sequences from trichloroethene-contaminated groundwater (*16*), and *Diaphorobacter* is a recently described denitrifier (*17*). The circumneutral site with the least contamination (TPB-16) had the most diversity of any of the sites and was distinct from the other sites. It should be noted that a subsurface iron barrier was put in place in close proximity to TPB-16 in 1997, and the trench was filled with guar gum (*18*). The presence of additional organic carbon might explain the increased diversity. However, the evenness value was still reduced compared to background (0.80 versus 0.97), and this result suggested competitive exclusion may have been caused by the increase disturbance of organic carbon.

The SSU rRNA gene clonal libraries were compared between the sites with differences between coverage curves (LIBSHUFF analysis, v. 1.2) as previously described (*13*). The acidic samples had the lowest $\Delta C_{xy}$ values when compared to each other (*9*), FW-003 had the lowest $\Delta C_{xy}$ values with the acidic samples, and TPB-16 had the highest $\Delta C_{xy}$ values compared to all sites. When the sites were clustered based upon the $\Delta C_{xy}$ values and UPGMA, the three acidic samples were grouped with FW-003 (Figure 3). In addition, FW-010, FW-015, and FW-003 were more closely clustered compared with the other acidic site, FW-005. The intermediate site, TPB-16, was least similar to any of the sites.

When the $\Delta C_{xy}$ tree was compared to the PCA groupings based upon site geochemistry, the associations were very similar. These results indicated that separate analyses of the

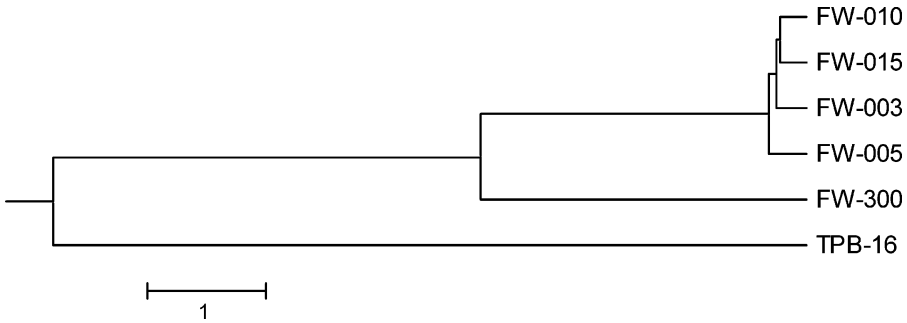


**FIGURE 3. Clustering of the different sites based upon $\Delta C_{xy}$ values determined from LIBSHUFF analysis. Tree was constructed with UPGMA in MEGA2.**

**TABLE 2. Total Number of Unique Operational Taxonomic Units (OTUs) Per Number of Screened Clones for Each Sampling Site**

| | unique OTUs/clones |
|---|---|
| SSUrRNA | 353/1630 |
| *nir*K | 48/958 |
| *nir*S | 144/1162 |
| *amo*A | 63/539 |
| *pmo*A | 14/26 |
| *dsr*AB | 163/1812 |

biotic and abiotic realms of the habitat estimated similar relationships between the sites along the contamination plume. Analysis based upon $\Delta C_{xy}$ values takes advantage of the full array of sequence data, and might provide a more detailed analysis of community structure compared to simple distributions of unique OTUs. Moreover, the OTU distributions were based upon relative abundances in PCR clonal libraries, and clonal libraries might contain inherent biases. Therefore, the OTU distributions were simply scored for the presence or absence of each unique OTU, and Euclidean distances were used to construct a UPGMA tree. The cluster of sites was very similar to that observed based upon the $\Delta C_{xy}$ tree (data not shown). These results indicated that the SSU rDNA PCR clonal libraries could give meaningful results in the context of environmental conditions.

**Site Comparison Based upon Functional Gene Sequences.** The *nir* genes represent the nitrite reductase, a biomarker for the denitrification process. The *amo* and *pmo* genes serve as biomarkers for monooxygenases that can be involved in ammonia oxidation, and the *dsr*AB genes represent the sulfite reductase, a biomarker for sulfate reduction. In total, 4497 functional gene sequences were analyzed in addition to 1630 SSU rDNA sequences (Table ). Thus, the total number of unique OTUs for functional genes used in the analysis was 432 and included genes involved in denitrification, ammonification, and sulfate reduction. The data suggested that FW-003, TPB-16, and FW-015 contained unique populations with respect to the functional genes, and that the background site was most similar to the more heavily contaminated sites for each analyzed functional gene (data not shown).

When the OTU distributions were combined for PCA ($n$ = 432), the two circumneutral sites were distinct from a group that contained the background and acidic sites (data not shown). When only the presence or absence of the functional gene OTUs were analyzed, similar results were observed (data not shown). The presence/absence profile of the combined data set was used to generate a Euclidean distance matrix, and a tree was constructed with UPGMA. The background site was closely related to the FW-010, an acidic site with the highest nitrate levels (Figure 4). The site FW-005 was also clustered with FW-010 and FW-300, and TPB-16 was the least similar to any of the sites.

When the individual diversity indices for the respective functional gene libraries were compared via a Euclidean distance matrix, the UPGMA tree clustered FW-015, FW-010, and TPB-16, and FW-003 and FW-005 were more similar to each other than to the other sites (Figure 5). Interestingly, FW-015 and FW-010 were sites with some of the highest contamination and TPB-16 had low contaminant levels but elevated sulfate levels. The sites FW-003 and FW-005 had intermediate levels of contamination in terms of $NO_3$, Al, and Ni. The results based upon community-level functionality suggested that the sites with intermediate levels of contamination were more closely related and formed a cluster distinct from the sites with high or low levels of contamination.

## Discussion

Previous studies have shown that bacterial community structure changes with respect to environmental conditions, including contamination (*19*−*21*). Our results indicated that different gene sequences estimated different relationships between populations within the microbial communities. The diversity and versatility of microbial communities, not only at the population level but also how those populations interact, contribute to the difficulties in interpretation. PCR-based cloning approaches are commonly used to assess diversity and community structure, and can be an informative tool for environmental studies. PCR-based approaches do have inherent biases, and protocols were followed to minimize PCR amplification-induced artifacts as described previously (*9*, *22*). In addition, when the presence or absence of unique OTUs was scored instead of relative abundances, the relationships between the sites were similar (data not shown). Absence versus presence would further minimize the presence of any preferential over-amplification that may occur during PCR, but still take advantage of PCR sensitivity.

It should also be noted that the gene sequence and geochemical data set represents one time point studied in depth, but this is the first report of extensive coverage of both biotic (785 unique gene sequences) and abiotic (107 analytes) parameters along a contamination gradient. Factor analysis can be used for data reduction to identify a small number of factors that explain most of the variance observed in a much larger number of manifest variables, and thus, a subset of major factors can be selected for subsequent analyses. All relationships will not be captured, but the major associations should be represented and allow for future hypothesis testing in the context of structure/function relationships in light of additional parameters (e.g., time, season, etc.).

The intermediate-disturbance hypothesis typically holds true for disturbances that disrupt community and/or population structure, and previous laboratory and field studies have indicated that this idea can be applied to bacteria (*23*, references therein). The intermediate-disturbance theory states that a community will display the greatest diversity at

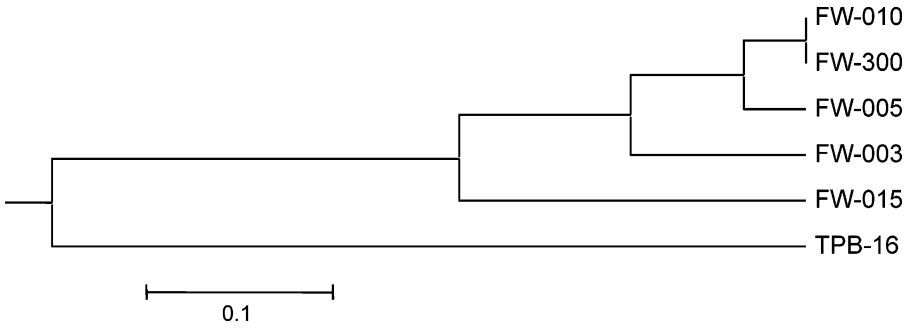


**FIGURE 4. Clustering of the different sites based upon correlative distance matrix (Euclidean) of absence/presence data for unique functional genes (*nir*K, *nir*S, *amo*A, *pmo*A, *dsr*AB). Tree was constructed with UPGMA in MEGA2.**

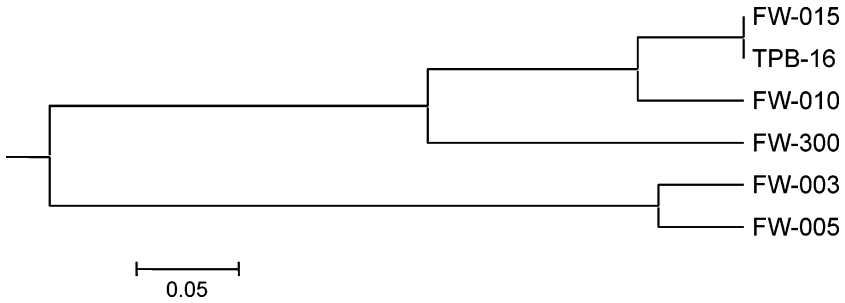


**FIGURE 5. Clustering of the different sites based upon correlative distance matrix (Euclidean) of diversity index (H′) for functional genes (*nir*K, *nir*S, *amo*A, *pmo*A, *dsr*AB). Tree was constructed with UPGMA in MEGA2.**

relatively intermediate levels of disturbance (i.e., contaminants, nutrients, etc.), and community diversity will be lowest at relatively low and high disturbances (*24*). Previous data suggested a relationship between bacterial diversity and perturbations, but most of the previous studies have characterized responses to one or two disturbances and not the combination of multiple factors. In addition, genetic diversity is often measured in different ways, and a systematic examination of the possible relationships to phylogenetic, genomic, and functional diversity is needed to better understand responses of bacterial communities. As pointed out by Horner-Devine et al. (*23*), diversity at different scales of resolution and the study of bacterial diversity along gradients of disturbance would prove fruitful for the characterization of diversity-structure relationships. Our data along a multiple-contaminant gradient suggested that despite geochemical and phylogenetic differences, low and high contamination sites were functionally similar (based upon sequence data). Future work will be needed to investigate the relationships between phylogenetic and functional diversity with biochemical capacity, and further test the application of the intermediate-disturbance theory to microbial communities.

Previous studies have shown that even though diversity was reduced in soil communities contaminated with Cu, benzene, Hg, or tylosin that different measures of functionality did not change (*2*, *19*). These results suggested that changes in community structure could be uncoupled from functionality due to functional redundancy, and that community structure would be better measured in terms of functionality and/or activity and not necessarily phylogeny. However, functionality can be difficult to measure due to constraints of high-throughput methodology and the determination of importance in the context of heterogeneity and multiple variables. The question remains if gene sequences associated with particular functions can be used as markers for functional robustness (i.e., biochemical capacity), and our results suggested that functionality in terms of gene sequences might provide informative measures for responsiveness to environmental changes by broadening the view of the community.

One measure that has been used in the past for plant and animal systems is primary productivity (*25*). Various studies have reported positive quadratic relationships between productivity (represented by chlorophyll levels) and diversity in which diversity typically peaks at intermediate levels of productivity. Horner-Devine et al. (*25*) noted that productivity of a microbial aquatic system effected taxonomic groups of bacteria differently. Some groups peaked in diversity at intermediate levels of productivity while other groups had highest diversity at low and high productivity.

In our study, when the diversity of individual functional genes from groundwater was compared to overall species richness for each respective site, the functional gene populations responded differently. The *nir*S and *amo*A/*pmo*A comparisons were quadratic, and the highest community diversity was at intermediate levels of phylogenetic diversity for the respective functional genes. In contrast, the *nir*K populations appeared to slightly increase as the community diversity increased, and the *dsr*AB comparison suggested that *dsr*AB diversity was highest at low and high overall diversity.

In relation to contaminated field sites, functional activity can be difficult to assess due to several limitations: multiple biochemical activities could be of interest, in situ enzymatic activities can be difficult to measure due to multiple contributors, and low biomass. The present study used gene sequences as indicators for functional diversity, and the relationships were similar to those of recent studies that compared productivity and diversity (*25*). Our analyses showed that different components of a groundwater community (as measured via functional genes) also responded differently to disturbances. The data suggested the importance of functional redundancy and diversity within microbial communities and the possible repercussions for stability and resiliency in terms of biochemical capacity, but there is lack of complete understanding of how community structure (phylogenetic or functional) impacts system-level activity. Further work is needed to understand what types of functional populations should be promoted for bioremediation under

various field conditions, and how community structure impacts activities of interest. A fundamental understanding of structure/function relationships will greatly assist in environmental restoration strategies.

Functional groups that respond differently under a particular perturbation should have different patterns of diversity along the contaminant gradient in relation to growth and competitive displacement, and the data supported this hypothesis. The present work has helped in data reduction for the selection of key variables that can be analyzed in more samples over time for more detailed analyses. It also remains to be seen how closely groundwater analyses track sediment analyses, especially at low biomass sites (*26*). Until faster and cheaper methods of sediment sampling can be developed we will have to rely upon more rigorous groundwater analyses. Further work is needed at contaminated field sites to better understand the relationship between structure and function in microbial communities in the context of functionality, stability, and resiliency to aid predictive ecology for bioremediation. The majority of biochemical activities in situ are most likely a combination of multiple populations that are interdependent, and better methods are needed to account for these variables.

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