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ORIGINAL ARTICLE

Subsurface ecosystem resilience: long-term attenuation of subsurface contaminants supports a dynamic microbial community

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The propensity for groundwater ecosystems to recover from contamination by organic chemicals (in this case, coal-tar waste) is of vital concern for scientists and engineers who manage polluted sites. The microbially mediated cleanup processes are also of interest to ecologists because they are an important mechanism for the resilience of ecosystems. In this study we establish the long-term dynamic nature of a coal-tar waste-contaminated site and its microbial community. We present 16 years of chemical monitoring data, tracking responses of a groundwater ecosystem to organic contamination (naphthalene, xylenes, toluene, 2-methyl naphthalene and acenaphthylene) associated with coal-tar waste. In addition, we analyzed small-subunit (SSU) ribosomal RNA (rRNA) genes from two contaminated wells at multiple time points over a 2-year period. Principle component analysis of community rRNA fingerprints (terminal-restriction fragment length polymorphism (T-RFLP)) showed that the composition of native microbial communities varied temporally, yet remained distinctive from well to well. After screening and analysis of 1178 cloned SSU rRNA genes from Bacteria, Archaea and Eukarya, we discovered that the site supports a robust variety of eukaryotes (for example, alveolates (especially anaerobic and predatory ciliates), stramenopiles, fungi, even the small metazoan flatworm, Suomina) that are absent from an uncontaminated control well. This study links the dynamic microbial composition of a contaminated site with the long-term attenuation of its subsurface contaminants.

The ISME Journal (2010) **4**, 131–143; doi:10.1038/ismej.2009.101; published online 24 September 2009 **Subject Category:** microbial ecology and functional diversity of natural habitats **Keywords:** biodegradation; natural attenuation; *Nitrosopumilus*; protozoa; microeukayotes; predation

Introduction

The growing effect of human activities on ecosystems is well recognized (Poliakoff *et al.*, 2002; Dietz *et al.*, 2003; Palmer *et al.*, 2004; Liu *et al.*, 2007). Ecosystem resilience has been variously conceptualized as 'the magnitude of disturbance that a system can experience before it shifts into a different state' (Holling, 1973) and 'the capacity of a system to absorb disturbance and reorganize' (Folke *et al.*, 2004). Resilience is considered a fundamental property of ecosystems useful in predicting their response to the anthropogenic change (Dietz *et al.*,

2003; Folke et al., 2004), yet the microbiological processes affecting ecosystem resilience are often unrecognized or poorly defined (Allison and Martiny, 2008; Falkowski et al., 2008; Madsen, 2008). In the classic view of aquatic ecosystem resilience (reviewed in Folke et al., 2004), an excess of anthropogenically released materials (for example, phosphorus) may trigger a series of events that convert a clear-water lake (that is, oligotrophic, high oxygen, low plant biomass and dominated by game fish) to a turbid eutrophic water body (that is, low oxygen, high plant biomass and with game fish absent). The capacity of the lake system to receive phosphorus inputs while remaining in the clearwater state is defined as its resilience. In this example, two key processes mediate the transition from oligotrophic to eutrophic states: photosynthesis (primary production by plants and phototropic microorganisms, no longer limited by phosphorus)

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and aerobic respiration (by heterotrophic microorganisms, supported by accumulation of deceased phototrophic biomass). The oxygen depletion, driven by microbiological processes, may exceed the threshold of system resilience, leading to a stable shift to a new state of habitat colonization (Folke *et al.*, 2004). In contrast to lakes, groundwater ecosystems harbor few complex fauna and are, correspondingly, microbially dominated (Madsen and Ghiorse, 1993; Chapelle, 2003; Steube *et al.*, 2009). When groundwater habitats are affected by organic contaminants, microbiological reactions can destroy contaminants, contributing directly to system resilience (Botton *et al.*, 2006).

The widespread problem of ecosystem disturbance by industrial chemical waste (NRC, 2000; Schwarzenbach et al., 2006) has advanced the need for understanding the response of naturally occurring microbial communities (Alexander, 1999; Diaz, 2008). The capabilities of naturally occurring microbial communities to enzymatically attack (biodegrade) and grow upon many contaminant compounds (Alexander, 1999; Madsen, 2008) is the basis for a site cleanup technology, termed 'Intrinsic Bioremediation' and 'Monitored Natural Attenuation', by the US Environmental Protection Agency and the US National Academy of Sciences (USEPA, 1997; NRC, 2000). Microbially mediated ecosystem resilience underlies monitored natural attenuation (Botton et al., 2006). Although many field studies of monitored natural attenuation for a variety of chemical pollutants have been previously reported (for example, Dojka et al., 1998; Bekins et al., 2001; Cozzarelli et al., 2001; Kleikemper et al., 2002; Suarez and Rifai, 2002; Ulrich et al., 2003; Griebler et al., 2004; Hunkeler et al., 2005; Takahata et al., 2006), to our knowledge, none have comprehensively integrated (1) the long-term field evidence for contaminant elimination, (2) temporal variability in microbial community composition and (3) the composition of an elaborate eukaryotic microbial food chain responding to the contamination.

Site timeline and previous investigations

In the 1960s, coal-tar waste from a manufactured gas plant was buried in South Glens Falls, New York, resulting in a contaminant plume of polycyclic aromatic hydrocarbons and other monocyclic constituents dispersed by the groundwater flow (Figure 1; Madsen *et al.*, 1991; Murarka *et al.*, 1992; EPRI, 1996; Nelson *et al.*, 1996; Neuhauser *et al.*, 2009). In situ biodegradation of contaminants was shown by the enhanced metabolism of naphthalene and phenanthrene in the sediment samples from inside the plume, but not from outside (Madsen *et al.*, 1991). Furthermore, the high biomass of protozoa that occurred only in the contaminated zone proved that the bacterial prey were actively growing on the contaminants (Madsen *et al.*,



Figure 1 Plan view of the coal-tar waste-contaminated site in Glens Falls, New York, showing locations of the monitoring wells.

1991). In 1991, \sim 7000 m³ of sediment containing the main mass of coal-tar waste was excavated from the site and the cavity was back filled with clean sand (Murarka et al., 1992), followed by natural attenuation (through solubilization, transport, sorption, dilution and microbial metabolism) of aromatic hydrocarbons that had been transported to downgradient sediments (USEPA, 1997; NRC, 2000). Detection of both the transient intermediary metabolite, 1,2-dihydroxy-1-2-dihydronaphthalene (Wilson and Madsen, 1996), and mRNA transcripts of the naphthalene dioxygenase gene (nahAc) (Wilson et al., 1999; Bakermans and Madsen, 2002a), and the stable-isotope probing analyses (Jeon et al., 2003) confirmed the on-going aerobic microbial metabolism of naphthalene, the major soluble component of the coal-tar waste (Nelson et al., 1996). However, oxygen depletion in the contaminated zone and the clear evidence for methanogenesis and sulfate reduction (strictly anaerobic processes incompatible with aerobic naphthalene degradation) (Bakermans et al., 2002; Bakermans and Madsen, 2002b; Yagi et al., 2009) signify that the on-site metabolism is limited by the influx of geochemical oxidants, especially naturally occurring nitrate, sulfate and dissolved oxygen delivered largely by flow from uncontaminated groundwater upgradient into the system (Bakermans et al., 2002b). A 16S ribosomal RNA (rRNA)-based clone library (100 clones from each of the four wells across the site's contamination gradient) provided an initial characterization of the on-site bacterial community (Bakermans and Madsen, 2002b).

Materials and methods

Site and groundwater sampling

The contaminated site is a rural area in South Glens Falls, New York, where coal-tar waste was buried in the early 1960s (Figure 1; Madsen *et al.*,

Groundwater samples were collected from two monitoring wells located along the groundwater flow path and a background control well upgradient of the source contamination. Two commercial firms (GEI, Inc., Glastonbury, CT, USA and META Environmental, Inc., Watertown, MA, USA) conducted annual sampling and analysis of the well waters for oxygen and organic contaminants from 1989 to 2005, as previously described (Murarka *et al.*, 1992; Neuhauser *et al.*, 2009).

For molecular and microbiological analyses, groundwater was collected between 2005 and 2007, as described by Bakermans and Madsen (2002b). In brief, water from the monitoring wells 12 and 36 and from the background well was gathered at a flow rate of $300 \text{ ml} \text{min}^{-1}$ using a Geopump-2 (Geotech Environmental, Denver, CO, USA). For each sample, a minimum of three well volumes were pumped before filtering cells from \sim 31 of water through 0.22-µm pore size, 142-mmdiameter Durapore membranes (Millipore Corp., Bedford, MA, USA) that were then immediately frozen in sterile Whirlpak bags (NASCO, Modesto, CA, USA) on site (dry ice/ethanol). The filters were stored at -80 °C preceding the analyses described below.

Nucleic acid extraction

For DNA and RNA extraction from groundwater biomass, we followed the previously described procedures (Wilson et al., 1999; Bakermans and Madsen, 2002b; Jeon *et al.*, 2003; Liou *et al.*, 2008) with minor modifications. In brief, for DNA extraction, frozen filters were crushed and extracted twice each by boiling (5 min) with 5 ml pre-heated extraction buffer (1% sodium dodecyl sulfate, 0.1 M NaCl, 10 mM Tris, 1 mM ethylenediaminetetraacetic acid, pH 8.0). Decanted extracts were added to an equal volume of phenol (pH 8.0) and mixed vigorously followed by centrifugation at 4 °C (15 min at 10000 g). The upper aqueous layer was extracted with an equal volume of phenol and chloroform (1:1), followed by centrifugation as above and a final extraction with an equal volume of chloroform and isoamyl alcohol (24:1) and centrifugation. DNA was precipitated from the final aqueous layer at -80 °C with sodium acetate (0.3 M) and an equal volume of isopropanol. After centrifugation (30 min at 15000g), the DNA pellets were washed in 70% ethanol and reprecipitated. Pellets were then air-dried and carefully resuspended in 50 µl TE [10 mM Tris (pH 8.0), 1 mM EDTA].

For RNA processing, all reagents and labware were either certified ribonuclease free, baked overnight at 200 °C and treated using RNase ZAP (Ambion, Austin, TX, USA) or were diethyl pyrocarbonate treated for inactivation of ribonuclease activity, as appropriate. RNA was extracted from frozen filters using a modified version of the DNA extraction protocol described above. In brief, crushed filter pieces were processed using acid extraction buffer (pH 5.1), and acidic phenol (pH 4) was used for phenol and for phenol-chloroformisoamyl alcohol (125:25:1) extraction. RNA was precipitated from aqueous extracts with glycogen as a coprecipitant $(2 \mu g m l^{-1})$ at $-80 \degree C$ using sodium acetate (2.5 M) and 2 volumes of ethanol. Purified RNA pellets were recovered using centrifugation (30 min at 15 000 g), and were air-dried and resuspended in 50 µl ribonuclease-free water. DNA was removed from RNA extracts using DNase I treatment (Invitrogen, Carlsbad, CA, USA), and RNA was reverse-transcribed to complementary DNA using SuperScript III reverse transcriptase (Invitrogen) and random hexamers according to the manufacturer's instructions. No-reverse transcriptase control PCR reactions were performed using primers and PCR conditions described below to ensure complete removal of contaminating DNA.

Ribosomal RNA (rRNA) gene clone libraries

Representative DNA clone libraries were constructed from selected groundwater extracts (see Results). PCR amplification of prokaryotic and eukaryotic small-subunit (SSU) rRNA used the following primer pairs: 27f/1492r targeting Bacteria (Lane, 1991), 21Fa/1492R targeting Archaea (De-Long, 1992) and both 3Fphp/1749Rphp (Richards et al., 2005) and 360Fe/1391Re (Dawson and Pace, 2002) targeting Eukarya. PCR amplification was performed using ThermoStart DNA polymerase (ABgene, Rockford, IL, USA) on a PTC-200 DNA Engine thermocycler (MJ Research, Watertown, MA, USA). Duplicate reactions were performed for each clone library using 0.5-25 ng DNA template in $25 \,\mu$ l volumes and the previously described cycling conditions (DeLong, 1992; Dawson and Pace, 2002; Richards et al., 2005; Liou et al., 2008). Pooled PCR products were examined by agarose gel electrophoresis, purified using the QIAquick gel extraction kit (Qiagen, Santa Clarita, CA, USA) and ligated into the vector pCR2.1 (TOPO-TA cloning, Invitrogen) or pSC-A (StrataClone PCR cloning, Stratagene, La Jolla, CA, USA), following the manufacturers' recommended protocols. After transformation of plasmids into host cells and blue-white screening, for each library inserts in 80-130 randomly picked colonies were verified using PCR with vectorspecific primers that flanked the cloning regions. The amplicons were digested with HaeIII and HhaI and sorted by restriction-fragment length polymorphism (RFLP) patterns on 3% MetaPhor agarose gels (BioWhittaker; Molecular Applications, Rockland, ME, USA). The clones containing unique RFLP patterns were selected for sequencing, grown overnight in 3 ml of Luria-Bertani broth with kanamycin (50 μ g μ l⁻¹), pelleted and the plasmids were purified (QiaPrep spin miniprep kit; Qiagen). Sequencing (Cornell University Life Sciences Core Laboratories Center) was conducted routinely with M13 forward and M13 reverse primers. Sequences were manually checked for quality and edited using 4Peaks software (available at http://www.mekentosj. com/4peaks/). The SSU rRNA sequences were checked for chimeras using Bellerophon with the Huber–Hugenholtz correction and a 300-bp window size (Huber *et al.*, 2004) and Pintail (Ashelford *et al.*, 2005). The chimeras, vector sequences and sequences of poor quality were excluded from further phylogenetic analyses. 16S and 18S gene sequences were aligned using the ARB FastAligner tool to the most recent GreenGenes (DeSantis et al., 2006) and SILVA (Pruesse et al., 2007) databases, respectively, running locally in the ARB package (Ludwig et al., 2004). A 50% base frequency filter was used to omit highly variable regions of the SSU rRNA. Alignments were manually edited with ARB Edit4. Sequences were also compared with the GenBank nucleotide database library by BLAST online searches (http://ncbi.nlm.nih.gov/BLAST) and the closest BLAST matches were included in phylogenetic comparisons. Phylogenetic trees were constructed in ARB using a neighbor-joining algorithm and an Olsen-corrected distance matrix.

Terminal-restriction fragment length polymorphism (T-RFLP) analyses

Small-subunit rRNA genes samples were amplified from groundwater DNA or complementary DNA as described above, except that PCR primer 27F-FAM (5 end labeled with phosphoramidite fluorochrome 5-carboxy-fluorescein) with reverse primer 1492R was used to target 16S rRNA genes, and similarly, 3FphpF-FAM with reverse primer 1749Rphp was used to amplify 18S rRNA genes. The fluorescently labeled PCR products were digested overnight with MspI (New England Biolabs, Ipswich, MA, USA), purified using the QIAquick PCR purification kit (Qiagen, Valencia, CA, USA), and electrophoretically separated in an Applied Biosystems 3730 DNA analyzer (Applied Biosystems, Foster City, CA, USA) as described previously (Liou *et al.*, 2008). The electrophoretic data were analyzed using Genemapper and/or PeakScanner software (Applied Biosystems) using a threshold value of 50 relative fluorescence units. Representative duplicate DNA extractions from filters collected on the same day were processed to verify reproducibility. T-RF peak heights were used in principal component analysis to compare and group community profiles (that is, T-RFLP patterns) along the axes (principal components) on the basis of the fragment patterns alone (Jongman et al., 1995). Principal component analysis plots were generated using R statistical software version 2.4.1 (http://www.r-project.org/) and the Vegan package version 1.8–8 (http://cc.oulu.fi/~jarioksa/; Dixon, 2003). For comparison of T-RF profiles with dominant TRFs derived from the environmental groundwater samples, T-RFLP analysis was performed

as above on PCR amplicons from clones representing dominant phylotypes from the 16S rRNA gene clone libraries (described above).

Nucleotide sequence accession numbers

The nucleotide sequence data reported here have been submitted to GenBank under the accession numbers FJ810524 to FJ810621.

Results

A 16-year record of contaminant attenuation in groundwater

Well-water concentrations (1989-2005) of five coaltar-waste constituents (naphthalene, xvlenes. toluene, 2-methylnaphthalene and acenaphthylene) and oxygen are depicted in Figure 2. The changes in well-water contamination since the removal of the source of coal-tar waste reflect both the excavationrelated physical mobilization of chemicals and the natural attenuation processes (USEPA, 1997; NRC, 2000). Well 12 is farthest downgradient from the source material (Figure 1) and featured the lowest contaminant concentrations. Well 36, though further downgradient than well 8 (Figure 1) featured the highest contaminant concentrations, reflecting the presence of clay lenses that may bind coal-tar-waste constituents and resist both groundwater flushing and oxygen infiltration (Murarka et al., 1992; Bakermans et al., 2002). Thus, subsurface matrix effects. especially sorptive surfaces and hydraulic conductivity, seem to have a major role in the persistence of the groundwater contaminants. Naphthalene concentrations rose sharply in all three wells after source excavation (to ~ 1600 p.p.b. in 1991 and 1992) and then gradually diminished through 2005 (Figure 2). The rates of naphthalene attenuation were most rapid for well 12 and slowest for well 36, in which the 2005 residual level was still \sim 380 p.p.b. The patterns in the attenuation of xylenes, toluene, 2-methylnaphthalene and acenaphthylene (middle panels, Figure 2) were similar to those of naphthalene: diminishing concentrations with time, rapid attenuation in well 12 and slowest attenuation in well 36.

Oxygen depletion associated with contaminantinduced microbial biodegradation processes is widely recognized (NRC, 2000). In 1988 and 1989, the average (n=4) level of O_2 in uncontaminated water from an upgradient control well was 30% of the saturated levels (~3.3 mg O_2 per liter at 10 °C). Routine monitoring of well-water oxygen, beginning in 1992 and 1993, showed that for all the three contaminated wells, oxygen was far below the level of the background well ($\leq 6\%$ saturation; Figure 2). Such low values are often indistinguishable from zero, given the likely intrusion of atmospheric oxygen during on-site pumping and analysis. The fluctuating oxygen concentrations in all wells through to 2005 suggest that the system is geochemically highly

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Figure 2 Historical trends of the five site contaminants (naphthalene, xylenes, toluene, 2-methyl naphthalene and acenaphthylene), and oxygen in the groundwater of well 12 (a), well 8 (b) and well 36 (c) from 1989 to 2005.

Year

2000

1992

dynamic. Replenishment of atmospheric O_2 into the groundwater (through gaseous diffusion and both vertical and horizontal influx of non-depleted water) occurred most markedly in well 12 (lowest contamination, during 1994 to 1998) and least markedly in well 36 (highest contamination).

1992

Year

2000

Dynamic native communities

а

qd 800

1600

1200

400

0 800

600

200

0 300

200

100

0 24

16

8

0

qdd

% Saturation

qd 400

The on-site fluctuations in chemical groundwater constituents (Figure 2, see also Yagi *et al.*, 2009) led us to hypothesize that the native microbial communities were causing and/or responding to the geochemical changes, and that this would be reflected by dynamic fluctuations in the microbial populations. The bacterial community profiles from wells 12 and 36, generated using T-RFLP analysis of DNA (total community; panels a, c and e of Figure 3), show time-dependent individual fragments (for example, 186, 201, 222 and 276 only in August 2006) and periodicity (for example, 162, 430 and 488, shared by November 2005 and 2006, but absent in August 2006). This trend was reinforced by examining reverse-transcribed RNA (metabolically active

1992

2000

Year



Figure 3 Dynamic changes in the native bacterial communities in well 36. DNA fingerprints (terminal-restriction fragment lengths, T-RFLs) generated from ribosomal RNA (rRNA) gene amplicons for groundwater microbial communities illustrate shifts in native bacterial communities in well 36. The x axis and the numbers above the peaks indicate major fragment sizes (phylotypes) in DNA (a) and complementary DNA (cDNA) (b) from November 2005, DNA (c) and cDNA (d) from August 2006 and DNA (e) from samples taken in November 2006. The y axis shows the intensity of the peaks in relative fluorescence units.

bacterial community, panels b and d of Figure 3) that showed many fragments (for example, 108, 119, 132, 136, 148, 162, 435, 437, 438, 470 and 484) that were not shared by the profiles of November 2005 and August 2006. These data clearly show the dynamic nature of the groundwater microbial communities, given that the independently processed replicate samples gathered on a single day did not show such variations (Supplementary Figure S1).

Principal component analysis of T-RFLP patterns for bacterial and eukarval domains reveals another aspect of the variability of site microbial communities (Figure 4). During multiple site visits (spanning November 2005, August 2006, November 2006, December 2006 and May 2007), seven 16S and six 18S rRNA analyses were completed for wells 12 and 13. Over this period, considerable on-site fluctuations in the geochemical (see Figure 2 and Yagi et al., 2009) and population (Figure 3) parameters occurred. Despite these compositional variations in time, the microbial populations of the wells remained coherent relative to one another (Figure 4). Thus, despite evidence for temporal variability (Figure 3), the site-specific habitat characteristics associated with wells 12 and 36 seemed to be the dominant factors that determined the overall community composition.

Microbial community composition: enriched eukaryotic food chain

To identify the representatives of all three domains (*Bacteria, Eukarya* and *Archaea*) present in the site well waters, we prepared clone libraries from genes encoding SSU rRNA. Using RFLP patterns, we screened 1178 cloned DNA sequences encoding SSU rRNA genes (234 bacterial, 442 archaeal and 502 eukaryal) and analyzed 57 representative sequences (Table 1 and Figure 5).

Bacteria. The sequences of Bacteria fell into 12 established classes (β -, δ -, ϵ -, γ -proteobacteria, Acidobacteria, Actinobacteria, D-BACT lineage, Firmicutes, Bacteroidetes/Chlorobi, Candidate Division OP3, Chloroflexi and Unclassified; Figure 5a). The distribution of the field site's dominant bacterial clones between wells 12 and 36 is shown in Table 2. Despite the proximity of the two wells (Figure 1) and the hydrologic continuity, the absence of phylogenetic overlap between the wells is striking (compare columns 4 and 5 of Table 2). The array of site *Bacteria* (Figure 5a and Table 2) represents a diverse phylogeny, including close relatives of cultured microorganisms and clones derived from the subsurface, marine and lake habitats. Chlorobia ($\geq 32\%$), Syntrophus ($\geq 20\%$)

and β -proteobacteria ($\geq 8\%$) dominated the clone library from well 36. The dominant well-characterized taxa in the library from well 12 were



Figure 4 Principal components analysis showing the spatial differentiation of microbial community composition (terminalrestriction fragment length (T-RFLP) fingerprints of small-subunit RNA genes). Analyses for *Bacteria* (a) and *Eukarya* (b) were obtained from wells 36 and 12 (September 2005; August, November and December 2006; and May 2007). The ordination points for the profiles from well 36 are indicated by diamonds, and the triangles represent samples from well 12.

β-proteobacteria ($\geq 21\%$) and δ-proteobacteria (in particular, *Geobacter psychrophilus*-like sequences; $\geq 18\%$). Two clades particularly relevant to site processes (see Yagi *et al.*, 2009) were observed: Chlorobia, recently associated with oxidation of elemental sulfur and sulfide to sulfate, a process likely to occur in the study site when aerobic waters infiltrate zones of previous sulfate reduction (Hose *et al.*, 2000; Engel *et al.*, 2003); and an unclassified group aligning with taxa responsible for anaerobic oxidation of methane using nitrate as an electron acceptor (lineage associated with uncultured bacterium clone D-BACT in Figure 5a; Raghoebarsing *et al.*, 2006).

Archaea. Both Euryarchaeota and Crenarchaeota were represented in the archaeal clone libraries (Figure 5b and Table 1). Group I.1a Crenarchaeota (most closely related to environmental clones derived from subsurface and freshwater sediments) comprised a major proportion of the clones from well 12 (\geq 47%) (see also Supplementary Table S1 for the distribution of the archaeal clones between wells). There were two prominent characteristics of the archaeal sequences: (1) the presence of sequences closely related to the marine ammonia oxidizer, Nitrosopumilus (Konneke *et al.*, 2005) and (2) the absence of sequences related to the common methanogenic orders, Methanobacteriales and Methanomicrobiales.

Eukarya. While preparing our clone libraries for *Eukarya*, it was intriguing that no PCR amplicon of the 18S rRNA gene was obtained from the background control well. This indication of very low eukaryal biomass outside of the contaminated zone is consistent with our previous report of > 300-fold enrichment of predatory protozoa in the contaminated

Table 1 Characteristics of Bacteria, Archaea and Eukarya SSU rRNA clone libraries from the site well waters

Domain	Source well	PCR primer set	No. of clones screened by RFLP		No. of OTUs (RFLP per well), (no. of distinctive patterns) ^a
			Aug 06	Nov 06	
Bacteria	Well 36	27F/1492R		106	31
	Well 12			128	33
			Total clones $= 234$		Total = 64 (59)
Archaea	Well 36	21Fa/1492R	113		30
				106	42
	Well 12		102		36
				121	34
			Total clones $=$ 442		Total = 142 (79)
Eukarya	Well 36	3Fphp/1749Rphp	32	89	15
	Well 12		33	88	19
			Total clones $= 242$		Total = 34 (24)
	Well 36	360Fe/1391Re		131	18
	Well 12			129	44
			Total clones $= 260$		Total = 62 (58)

Abbreviations: rRNA, ribosomal RNA; SSU, small subunit; T-RFLP, terminal-restriction fragment length polymorphism. ^aThe total of operational taxonomic units (OTUs) shows a simple sum from each well, and in parentheses, a lower number of distinctive OTUs caused by patterns shared between wells. JM Yagi et al

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Figure 5 Phylogenetic trees of relationships of bacterial 16S (a), archaeal 16S (b) and eukaryal 18S (c) representing the diversity of ribosomal RNA (rRNA) sequences derived from wells 36 and 12 (indicated by boldface type). Reference sequences are followed by Genbank accession numbers. Sequence nomenclature specifies the targeted domain (for example, B = Bacteria), and an arbitrary clone number. Notable bacterial and eukaryal lineages are described in the text and their distribution between wells 36 and 12 are shown in Table 2 and Supplementary Table S1. Archaeal sequences clustered with Group I.1a Crenarchaeota and the deep-sea hydrothermal vent Euryarchaeota (DHVE) group II lineages DHVE4 and DHVE5. Trees were determined using neighbor-joining analysis with Olsen correction implemented in the ARB analysis package using a 50% base frequency filter to omit highly variable regions. Bootstrap values $\geq 50\%$ from 100 replicates are indicated next to the nodes. The scale bar represents the expected number of changes per nucleotide position.

zone (Madsen *et al.*, 1991). We used two different sets of PCR primers to obtain eukaryal sequences, leading to the recovery of a broad diversity spanning alveolates, stramenopiles, fungi and small metazoa (Figure 5c and Table 1) comparable to previous studies (Dawson and Pace, 2002). The distribution of the field site's dominant eukaryal clones revealed only moderate phylogenetic overlap between wells 12 and 36 (Supplementary Table S1). The particularly striking trends in the on-site eukaryal phylogeny are the prevalence of sequences closely related to: (1) the anaerobic ciliates (for example, *Paruroleptus*), (2) predatory ciliates (for example, *Lemba*- *dion*) and (3) the fresh interstitial-water flatworm, *Suomina*, a metazoan.

Discussion

Before interpreting the results of this study, we must acknowledge the implicit limitations in the methodologies. First, though widely accepted as indicative of the degree of contamination, aqueousphase sampling of well waters within aquifer materials does not completely account for contaminant constituents strongly associated with aquifer

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Phylogenetic affiliation	Clone sequence	No. of related clones			
	1	Total	Well 36	Well 12	
Acidobacteria	B-1225	8	0	8	
	B-1201	5	0	5	
	B-1222	3	0	3	
Actinobacteria	B-3607	2	2	0	
Bacteroidetes/chlorobi	B-3683	5	5	0	
	B-1259	3	0	3	
	B-1226	2	0	2	
	B-3629	2	2	0	
	B-3665	27	27	0	
β-Proteobacteria	B-1253	4	0	4	
	B-3664	3	3	0	
	B-1202	17	1	16	
	B-3667	3	3	0	
	B-1217	5	0	5	
	B-3672	3	1	2	
Candidate Div. OP3	B-3678	2	2	0	
	B-1223	2	0	2	
	B-3647	2	2	0	
Chloroflexi	B-3604	2	2	0	
2	B-1235	2	0	2	
	B-3698	2	2	0	
D-BACT lineage	B-1212	4	0	4	
δ-Proteobacteria	B-3680	5	5	0	
	B-3669	2	2	0	
	B-1215	4	0	4	
	B-1203	12	0	12	
	B-3609	25	18	7	
	B-3676	3	3	0	
	B-3675	2	1	1	
Firmicutes	B-1229	4	0	4	
γ-Proteobacteria	B-1218	2	0	2	
Unclassified	B-3632	4	1	3	
	B-3610	3	1	2	
	B-1207	21	0	21	
	B-3617	12	12	0	
	B-1205	5	0	5	

Table 2 Summary of representative SSU rRNA gene sequencesfrom Bacteria clone libraries from the wells 12 and 36 (November2006)

Abbreviations: rRNA, ribosomal RNA; SSU, small subunit.

sediments. Similarly, planktonic microbial cells (drawn from a ~3l aquifer volume) are not necessarily fully representative of the microbial community occupying the mineral matrix. Another key methodological consideration is that the clone-library approach, which we used to characterize microbial populations, has potential biases (von Wintzingerode *et al.*, 1997) and provides an incomplete census for any microbial community (for example, Tringe *et al.*, 2005; Elshahed *et al.*, 2008). Despite the above caveats, the procedures used enabled successful integration of information on the long-term natural attenuation of organic chemicals with microbial community dynamics in the subsurface contaminated study site (see also Yagi *et al.*, 2009).

A variety of studies have made progress in showing relationships between the composition of subsurface microbial communities and both site geochemistry and the presence of specific pollutant compounds (for example, Haack and Bekins, 2000; Röling et al., 2000; Pickup et al., 2001; Haack et al., 2004). In our previous study, Bakermans and Madsen (2002b) used sequencing of 16S rRNA clone libraries to describe bacterial communities in four site wells. Clones representing the most abundant amplified ribosomal DNA restriction analysis patterns were sequenced (31 total). Sequences related to the aerobic bacteria (for example, Nitrospira, Methylomonas and Gallionella) predominated among those that were retrieved from the uncontaminated area of the site, whereas sequences related to the facultatively aerobic and anaerobic bacteria (for example, Azoarcus, Syntrophus and Desulfomaculum) predominated among those that were retrieved from the contaminated areas of the site (wells 8, 12 and 36). In this study (~ 5 years later), we selected two of the previously characterized wells (12 and 36) and focused on the temporal variations and both broader (all three domains of life) and deeper and more extensive characterization through the cloning and sequencing of SSU rRNA genes. In agreement with the previous study, we found evidence that the composition of the microbial communities of the wells 12 and 36 were distinctive, likely reflecting wellspecific geochemical selective pressures. In addition, bacterial phylotypes grouping with aerobic nitrite-oxidizing Nitrospira and with the Gammaproteobacteria comprised a relatively small fraction of the sequenced bacterial populations. However, in contrast to the previous study (Bakermans and Madsen, 2002b) in which the most abundant amplified ribosomal DNA restriction analysis patterns from well 36 grouped with the low guanine and cytosine Gram-positive organisms and with the *Betaproteobacteria*, the predominant phylotypes for well 36 in this study grouped with Bacteriodetes/ Chlorobi, Deltaproteobacteria and unclassified lineages (Figure 5a). For well 12, the majority of clones in the current study grouped with the Deltaproteobacteria, Betaproteobacteria and unclassified lineages (Figure 5a). The likely reason for differences between the results of the two studies is geochemical habitat alterations (hence, change in selective pressures) over the intervening years, although incomplete clone coverage in both studies and/or inconsistencies in PCR amplification outcomes may also have contributed to the apparent discrepancies.

Other notable findings reported here include confirmation of Bakermans and Madsen's (2002b) discovery of sequences representing the D-BACT lineage (Figure 5a) that subsequently were shown by Raghoebarsing *et al.* (2006) to anaerobically oxidize methane using nitrate as an electron acceptor. In addition, sequences closely aligned with *Nitrosopumilus* within the archaeal Group I.1a (Figure 5b), known to carry out ammonia oxidation (Konneke *et al.*, 2005), provide support for the likely onsite redox cycling of ammonia, as verified by Yagi *et al.*, 2009.

Studies on the temporal dynamics of naturally occurring microbial communities, completed for a variety of habitats, have used molecular fingerprinting procedures ranging from denaturing gradient gel electrophoresis to T-RFLP to automated ribosomal intergenic spacer analysis. The key habitats examined for temporal microbial dynamics have included the mid-Atlantic bight (Nelson et al., 2008), the Cariaco Basin (Lin et al., 2008), epilithic biofilms in a freshwater stream (Anderson-Glenna et al., 2008), landfill leachate (Sundberg et al., 2007), an estuarine harbor (Kan et al., 2006), the Mediterranean Sea (Ghiglione et al., 2005) and the coastal California sea water (Fuhrman et al., 2006). Each community fingerprinting approach has its own strengths and weaknesses. We chose to utilize T-RFLP in this study because it can be consistently applied to pools of DNA and RNA and reveal spatial and temporal variations in community composition (Schutte et al., 2008). Under the best scenario, community composition surveys can be correlated to information about habitat characteristics, leading to predictable, mechanistic relationships between site geochemical conditions and the microbial community composition (for example, Fuhrman et al., 2006). The T-RFLP analyses completed in this study sought to explore the spatial and temporal variations in the site well waters. We found that microbial communities in the subsurface site were, in fact, dynamic in time (Figure 3), but were also constrained by local geochemical conditions (Figure 4). Slight temporal variability in subsurface microbial community composition was reported by Hendrickx et al. (2005) using denaturing gradient gel electrophoresis profiles to monitor colonization of aquifer material placed in contaminated and uncontaminated wells over a 122-day period. More pronounced changes in subsurface sediment community composition were found to be associated with aquifer recharge events over a 9-month period using community amplified ribosomal DNA restriction analysis, denaturing gradient gel electrophoresis and multivariate statistical analyses (Haack et al., 2004). Similarly, Simon et al. (2001) found in a karst aguifer that bacterial cell density, hydrolytic activity and respiration were influenced by the water-flow events.

The pioneering molecular phylogenetic survey of eukaryotic rRNA gene sequences by Dawson and Pace (2002) revealed unanticipated diversity in three anoxic sediments, including seven previously unknown kingdom-level lineages. Since that time, additional nonculture-based studies describing eukaryotic diversity in a variety of ecological settings have emerged. Among these habitats are: a multipond saltern (Casamayor *et al.*, 2002), rice soil (Murase *et al.*, 2006), an anoxic Norwegian fjord (Behnke *et al.*, 2006), Mediterranean sea water (Diez *et al.*, 2001), a sulfide-rich freshwater spring (Luo *et al.*, 2005), a seafloor hydrothermal vent field (López-García *et al.*, 2007) and an anaerobic aquifer polluted with landfill leachate (Brad *et al.*, 2008). The latter study, most relevant to data presented here, used Eukarya-specific PCR primers (Diez et al., 2001) to amplify 18S rRNA genes and analyze them through denaturing gradient gel electrophoresis profiling and sequence analysis. Brad et al. (2008) were surprised to observe that the microeukaryotic community in their anaerobic sandy landfill aquifer site was dominated by fungi (Basidiomycota yeasts), and also included both Chlorophyta and the bacterial predatory nanoflagellate, Heteromita, globosa (within the Cercozoa), but groundwater mesofauna were not found. Similar to Brad et al. (2008), we found evidence for predatory protozoa only in the contaminated zones in our site (Figure 5c)—this makes it clear that a microbial food chain based on the bacterial metabolism of pollutants exists at the site. Although Madsen et al. (1991) established in 1991 at this coal-tar waste-contaminated site that the high numbers of culturable predatory protozoa were an important indicator for in situ pollutant metabolism, the extensive diversity of the food chain had not been examined until now. Not only did we find evidence for a variety of fungi, stramenopiles and alveolates, but we also detected sequences related to the free-living turbellarian flatworm, Suomina, known to consume bacteria and protozoa (Kolasa, 2000). Thus, the contaminant-stimulated trophic community at our study site is both elaborate and has endured for a decade and a half.

The role of microeukaryotes (protists) in contributing to the function of microbially based food chains has been emphasized in several previous literature reviews (Novarino et al., 1997; Finlay and Esteban, 1998; Corliss, 2002; Foissner, 2006; Euringer and Lueders, 2008). Both by feeding on growing prokaryotic biomass and/or by adapting a heterotrophic lifestyle, protists have roles in ecosystems that include remineralization of nutrients, cycling of nutrients to higher trophic levels, excretion of growth factors and alteration of hydrologic flow and contaminant bioavailability (for example, Mattison and Haravama, 2001; Mattison *et al.*, 2002). Kinner et al. (2002) showed that, in a carbon-limited aquifer, contaminant biodegradation may be enhanced by protistan predation and that the rate of carbon uptake per unit bacterial biomass can be increased. Consistent with the findings of Kinner et al. (2002) and Madsen et al. (1991), both Sinclair et al. (1993) and Zarda et al. (1998) also found high subsurface protozoan biomass associated with aromatic-hydrocarbon-contaminated subsurface sediments.

Data describing loss of contaminants from the site groundwater (Figure 2) unequivocally documented the recovery of the site through natural attenuation. The previous and current reports (Madsen *et al.*, 1991; Wilson *et al.*, 1999; Jeon *et al.*, 2003; Yagi *et al.*, 2009), taken together, firmly indicate that the key contaminant-attenuating agents are the native microbial populations actively metabolizing the organic contaminant compounds *in situ*. Resilience has been identified as an ecosystem property useful in predicting responses to the ever-increasing anthropogenic influence on the biosphere (Holling, 1973; Folke *et al.*, 2004; Botton *et al.*, 2006). The microbial biogeochemical processes, such as those described here and by Yagi *et al.*, 2009, may govern ecosystem resilience and increasingly need to be rigorously defined, understood and managed.

Conflict of interest

The authors declare no conflict of interest.

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