ENVIRONMENTAL ENGINEERING SCIENCE
Volume 24, Number 8, 2007
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DOI: 10.1089/ees.2007.0016

Microbial Characterization of Groundwater Undergoing Treatment with a Permeable Reactive Iron Barrier

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ABSTRACT

Phylogenetic analyses of micro-organisms in groundwater samples from within and around a zero-valent iron (ZVI) permeable reactive barrier (PRB) identified several bacteria that could utilize H_2 produced during anaerobic ZVI corrosion and residual guar biopolymer used during PRB installation. Some of these bacteria are likely contributing to the removal of some groundwater constituents (i.e., sulfate). Bacteria concentrations increased from $\sim 10^1$cells mL^{-1} at 2 m upgradient to $\sim 10^2$ cells mL^{-1} within the PRB and $\sim 10^4$ cells mL^{-1} at 2 to 6 m downgradient. This trend possibly reflects increased substrate availability through the PRB, although a corrosion-induced increase in pH beyond optimum levels within the iron layer (from pH 7 to 9.8) may have limited microbial colonization. Micro-organisms that were detected using quantitative PCR include (iron reducing) *Geobacter* sp. (putative methanogenic) *Archaea*, and (sulfate reducing) δ-proteobacteria such as *Desulfuromonadales* sp. Sequencing of DGGE bands also revealed the presence of uncultured dissimilatory metal reducers and *Clostridia* sp., which was dominant in a sample collected within the ZVI-PRB. These results suggest that indigenous microbial communities are likely to experience population shifts when ZVI-PRBs are installed to exploit several metabolic niches that evolve when ZVI corrodes. Whether such population shifts enhance ZVI-PRB performance requires further investigation.

Key words: phylogenetic analyses; groundwater samples; zero-valent iron; permeable reactive barrier

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INTRODUCTION

PERMEABLE REACTIVE IRON BARRIERS (PRBs) frequently consist of a granular zero-valent iron layer (ZVI) buried in the path of a contaminant plume to intercept and chemically reduce a wide variety of groundwater pollutants [e.g., chlorinated solvents, nitrate, explosives, Cr(VI) and radionuclides] (for review, see Scherer *et al.*, 2000). In addition to direct contaminant reduction at the iron surface, ZVI–PRBs can stimulate microbial growth and reductive biotransformations through the production of water-derived H_2 during anaerobic ZVI corrosion (Weathers *et al.*, 1995; Novak at al., 1998; Till *et al.*, 1998; Oh *et al.*, 2001):

$$Fe^0 + 2H_2O \rightarrow Fe^{2+} + 2OH^- + H_2 \qquad (1)$$

Furthermore, if guar biopolymer is used during PRB installation, its breakdown products can serve as a substrate for indigenous microorganisms (Phillips *et al.*, 2003; Savoie et al, 2003). Several studies have found microbial colonization of iron PRBs (Alvarez *et al.*, 1999; Gu *et al.*, 2002). However, little is known about the microbial metabolic niches that evolve in and around PRBs and their role in the treatment process.

In this work, quantitative PCR and denaturing gradient gel electrophoresis (DGGE) were used to identify and quantify the concentration of various micro-organisms present in groundwater samples collected from within and around a ZVI–PRB. The phylogenetic information obtained provides insight into the microbial ecology of PRBs and serves as a basis to postulate several metabolic niches associated with ZVI–PRB corrosion that may influence the treatment process.

MATERIALS AND METHODS

Site information and sample collection

Groundwater samples were obtained from a site near Grand Island, Nebraska, contaminated with 2,4,6-trinitrotoluene (TNT), 2-amino-4,6-dinitrotoluene (ADNT), and hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) (Table 1). Groundwater at the site also contained high concentrations of sulfate. A ZVI–PRB was installed at the site in November 2003. Details of the installation are given in Johnson *et al.* (2007). Briefly, a 15-m long by 4.5-m deep by 0.9-m wide (50 × 15 × 3 foot) thick trench containing 30% by weight granular iron (Peerless Abrasives and Metals, Detroit, MI) in local sand was emplaced in a trench perpendicular to groundwater flow using guar gum slurry to maintain the trench during installation. The linear groundwater flow velocity at the site was ~0.2–0.3 m day^{-1}, and the water table was between 5 and 6 m below ground surface.

A network of groundwater monitoring locations was installed upgradient, within and downgradient of the PRB. Each location consisted of a multilevel sampler with four or five sampling depths, each depth screened over a 0.6-m interval. Groundwater samples (600 to 1,500 mL) for microbial analyses were collected 2 m upgradient of the PRB,

Table 1. Groundwater chemistry at the site (7.5 m deep) after 2 years of operation.

Parameter	*−2 m (upgradient)*	*Within PRB*	*2 m*	*4 m*	*6 m*
2,4,6-trinitrotoluene (TNT) ($\mu g\ L^{-1}$)	30–200	nd	nd	nd	nd
hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) ($\mu g\ L^{-1}$)	1–2	nd	nd	nd	nd
2-amino-4,6-dinitrotoluene (DNT) ($\mu g\ L^{-1}$)	10–50	nd	nd	nd	nd
Sulfate (mg L^{-1})	161	2	28	117	79
pH	7.2	9.8	7.8	7.0	6.9
O_2 (mg L^{-1})	<0.1	<0.1	<0.1	<0.1	<0.1
Eh (mV)	−48	−280	−134	−100	−98
Hydrogen (% sat)	nd[a]	1	nd	nd	nd
Methane (% sat)	nd	nd	nd	28	41
Conductivity (μS)	1,007	456	266	580	510
Alkalinity (mg L^{-1} as $CaCO_3$)	310	170	120	210	160
Ca^{2+} (mg/L)	48	nd	22	98	65

[a]nd, not detected.

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within the PRB and at 2, 4, and 6 m downgradient of the PRB and at 7.5 m below ground surface. Samples were taken 20 months after installment of the barrier.

Microbial analyses of the groundwater samples were conducted using molecular microbial ecology techniques as described below. Solid PRB and aquifer material samples were not obtained due to core collection difficulties, including poor sample recovery from the PRB and redistribution of fluids during core sampling. Since most subsurface micro-organisms are attached to surfaces rather than present in groundwater (Lehman *et a*l., 2001), our microbial characterization was not a comprehensive effort. Nevertheless, the identification and quantification of micro-organisms in groundwater samples yielded valuable insight into the microbial processes that may develop in and around ZVI–PRBs.

DNA extraction

Groundwater samples were filtered by vacuum using a 0.22-μm filter (Osmonics Inc., Minnetonka, MN). The filter was used as a matrix for DNA extraction using the MoBio Power Soil™ kit (Carlsbad, CA) according to the manufacturer's protocol. A 2-μL aliquot of bacteriophage λ DNA (500 bp) (Sigma-Aldrich, St. Louis, MO) was spiked in each sample prior to DNA extraction and used as an internal standard for the determination of DNA efficiency recovery (Beller *et al.*, 2002).

Real-time quantitative PCR (RTQ-PCR)

RTQ-PCR was used to quantify total *Bacteria*, *Archaea*, sulfate-reducing bacteria (SRBs) (*δ-Proteobacteria* class, such as some members of the *Geobacter* and *Peloobacter* genera), *Desulfovibrio*, *Desulfomicrobium*, *Desulfuromosa*, and *Desulfuromonas*, and iron-reducing bacteria of the *Geobacter* genus. The concentration of total bacteria was measured using the universal primers BACT1369F and PROK1492R (Table 2) (Beller *et al.*, 2002). The concentration of *Archaea* was measured using primers ARCH1-1369F, ARCH2-1369F, and PROK1541R (Suzuki *et al.*, 2000). *Geobacter* spp., which are generally capable of reducing iron (III), were targeted using the primers 361F, and 685R with the GBC1 probe (Stults *et al.*, 2001). Sulfate-reducing bacteria were quantified using the broad-spectrum detector probe EUB1 with the primers 361F and 685R (Stults *et al.*, 2001). All primers and probes were obtained from Integrated DNA Technologies (Coralville, IA) (Table 2). The number of bacteria in each sample was estimated as described by Da Silva and Alvarez (2004). Calibration curves (10^1 to 10^8 gene copies mL^{-1}) were prepared for all genes under consideration, yielding r^2 values $\geq$0.99. The detection limit of each assay was about 5 cell/mL^{-1}.

To date, no primers are available for detection of nitrate-reducing bacteria using RTQ-PCR. Thus, concentration of nitrate-reducing bacteria was estimated by targeting the heme-(*nir*S) nitrite reductase (Braker *et al.*, 1998) using MPN-PCR (Rose *et al.*, 1997).

Denaturing gradient gel electrophoresis (DGGE)

DGGE analyses were conducted to determine the phylogenetic association of bacteria colonizing the PRB. PCR-based DGGE was conducted as described by Ferris *et al.* (1996). Gene sequencing of the dominant bands was performed by Lone Star Labs, Inc. (Houston, TX). Phylogenetic affiliations were determined by comparing

Table 2. Maximum concentration of bacteria measured by PCR.

Target	*Source of gene sequence*	*Maximum concentration detected (cell mL^{-1})*
Bacteria	Beller *et al*., 2002	$5.1 \pm 1.0 \times 10^4$
Archaea (e.g., Methanogens)	Suzuki *et al*., 2000	$3.7 \pm 0.3 \times 10^6$
Geobacter spp. (e.g., iron-reducers)	Stults *et al*., 2001	$4.5 \pm 0.12 \times 10^2$
δ-Proteobacteria (e.g., sulfate-reducers)	Stults *et al*., 2001	$1.9 \pm 0.63 \times 10^2$
Nitrite-reductase (e.g., nitrate-reducers)	Braker *et al*., 1998	10^2
Clostridium sp.	(NCBI accession AY756140) Kjeldsen *et al*., 2004	[a]
Metal-reducing bacterium (clone P3OU-7)	(NCBI accession AY756140) Suzuki *et al*., 2003	[a]

[a]Detected using DGGE and gene sequence analyses.

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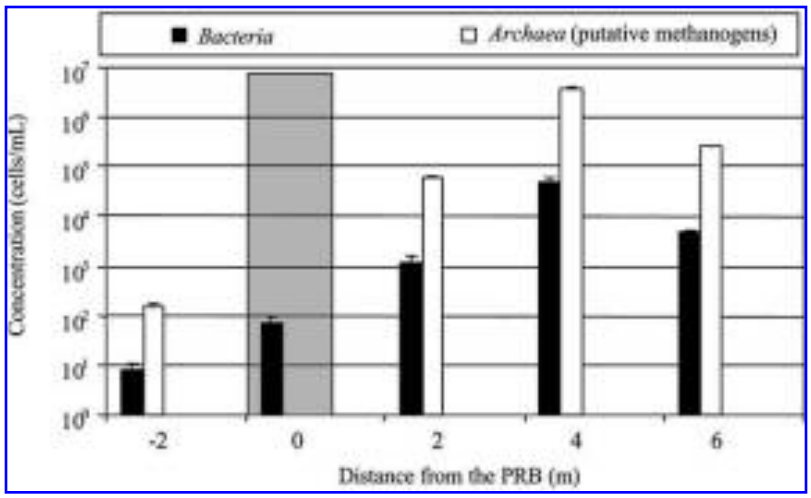

Figure 1. Microbial concentration profile upgradient and downgradient from the PRB. Samples were collected at a depth of 7.5 m. The PRB location is represented as the shadowed box. Error bars depict one standard deviation from triplicate measurements.

the DNA sequences retrieved to known bacterial sequences in the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/) and Ribosomal Database Project II (http://rdp.cme.msu.edu/index.jsp).

RESULTS AND DISCUSSION

The ZVI–PRB had a significant effect on groundwater geochemistry, similar to that observed at other sites (O'Hannesin and Gillham, 1998; Phillips *et al.*, 2000; Wilkin *et al.*, 2002; Savoie *et al.*, 2003). Specifically, ZVI decreased the oxidation potential and increased the pH and Fe(II) concentration downgradient of the barrier (Table 1). Alkalinity decreased due to the precipitation of carbonates at the higher pH, and sulfate concentrations were substantially reduced in the vicinity of the PRB.

The total bacteria concentration measured in groundwater samples collected 2 m upgradient from the PRB was relatively low (8 ± 1 cells/mL^{-1}) (Fig. 1). Bacteria concentrations in samples obtained within the PRB were one order of magnitude higher (70 ± 21 cells/mL^{-1}). Apparently, some bacteria adapted to the low Eh and high pH environment within the PRB (Table 1). The microbial concentration increased in downgradient samples, with the highest concentration ($5.1 \pm 1 \times 10^4$ cells/mL^{-1}) measured 4 m downgradient of the PRB (Fig. 1). The concentration of *Archaea* also increased downgradient of the PRB (from $1.6 \pm 0.2 \times 10^2$ to $3.7 \pm 0.3 \times 10^6$ cells/mL^{-1}), and their presence is consistent with the increased methane concentrations in the downgradient monitoring wells (Table 1) because all known methanogens belong to the *Archaea* domain. The general increase in microbial concentrations in the downgradient direction (Fig. 1) possibly reflects increased substrate availability through the PRB, although a corrosion-induced increase in pH beyond optimum levels within the

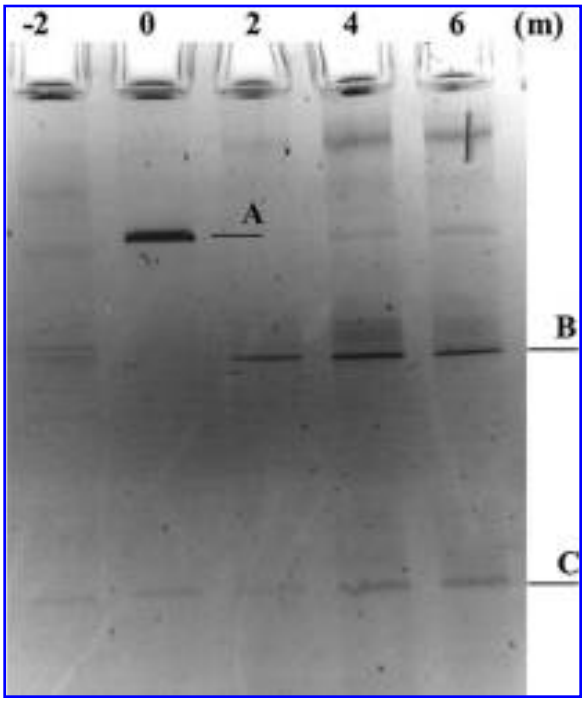

Figure 2. Picture of gel used for DGGE analysis. Gene sequencing of the two bands observed in the sample collected within the PRB showed 92% sequence similarity (SI) with *Clostridia* (**A**), 96% SI with a metal-reducing uncultured bacterium (**B**), and 91% SI with *Desulfuromonadales* bacterium JN18_A94_J (**C**). Column numbers correspond to the distance from the PRB in meters.

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iron layer (from pH 7 to 9.8) may have limited microbial colonization. No methanogens were detected within the PRB.

The detection of various target genotypes (Table 2) corroborates a recent study by Gu *et al.* (2002) that examined the microbiological characteristics of the Y-12 barrier in Oak Ridge, TN, and found that significant numbers of sulfate reducing bacteria and denitrifiers were colonizing the barrier. The presence of *Geobacter* spp. and other metal reducers is a novel observation that is noteworthy because it suggests that iron-reducing bacteria might colonize areas under the influence of ZVI–PRB corrosion to exploit iron(III) reduction as a metabolic niche.

The thickest DGGE bands identified in groundwater samples from within the PRB were not associated with nitrate-, sulfate- or iron-reducing bacteria (Fig. 2). Analysis of band (A) showed the presence of an uncultured low G+C Gram-positive bacterium (similarity index 92%), division *Firmicutes* (SI 43%), class *Clostridia* (SI 40%) (Ribosomal Database Project II database). This gene sequence was also compared with the NCBI database, showing a 92% sequence identity with a *Clostridium* sp. that was first observed in a biofilm grown on metal surfaces in an alkaline district heating system (NCBI accession AY756140). The second band (B), observed in all samples, showed 96% sequence similarity with an uncultured bacterium clone P3OU-7 (NCBI accession AF414578) previously identified in a microbial community undergoing Uranium (VI) reduction (Suzuki *et al.*, 2003). This potential metal-reducing bacterium (not associated with *Geobacter*, as determined by our RTQ-PCR analyses) was present upgradient and downgadient of the PRB. The third band (C) observed in all the samples, showed 91% sequence similarity (NCBI accession DQ168651) with a δ-proteobacterium, *Desulfuromonadales bacterium* (JN18_A94_J) (Bedard *et al.*, 2006). The presence of this putative sulfate-reducing bacterium in samples from the PRB would explain the observed removal of sulfate (Table 1), which was likely a biological process because chemical reduction of sulfate by ZVI is generally insignificant under natural conditions (Shokes and Moller, 1999; Fernandez-Sanchez *et al.*, 2003).

Although the PRB-related metabolic niches filled by these micro-organisms cannot not be discerned based on their phylogenetic identity, these results support the notion that multiple bacterial species could influence contaminant removal within and around ZVI–PRBs. In addition to bacteria that serve as polishers by metabolizing potential byproducts of contaminant reduction by ZVI (Oh *et al.*, 2001), *Geobacter* spp. and other metal-reducers might enhance PRB reactivity by reductive dissolution of iron oxides that occlude reactive iron surfaces, and by generating reactive iron(II) species that contribute to contaminant reduction, as shown in previous lab studies (Gerlach *et al.*, 2000; Gregory *et al.*, 2001; McCormick *et al.*, 2002). Furthermore, some members of the *Clostridium* genus are homoacetogens and produce acetate and propionate from CO_2 and H_2 (Boga *et al.*, 2003). Although it is unknown whether the *Clostridium* sp. detected in this work (using nonculturable techniques) can produce acetate using cathodic H_2 [Equation (1)], it is tempting to speculate syllogistically that ZVI corrosion might support primary productivity (e.g., $4Fe^0 + 2CO_2 + 5H_2O \rightarrow CH_3COO^- + 4Fe^{+2} + 7OH^-$), which would stimulate heterotrophic activity in PRBs where assimilable organic carbon is scarce.

Overall, this work suggests that some biogeochemical interactions that develop in and around ZVI–PRBs may influence contaminant fate and transport, and indicates that further research on the microbial ecology of these niches might lead to enhancements in barrier performance and longevity.

ACKNOWLEDGMENT

The Environmental Security Technology Certification Program (ESTCP, Project number CU-0223) is gratefully acknowledged for its support of this work.

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