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

Marcio L.B. Da Silva, Richard L. Johnson, and Pedro J.J. Alvarez

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 H₂ 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 \( \sim 10^4 \) cells mL\(^{-1} \) at 2 m upgradient to \( \sim 10^5 \) cells mL\(^{-1} \) within the PRB and \( \sim 10^9 \) 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
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 \( \text{H}_2 \) during anaerobic ZVI corrosion (Weathers et al., 1995; Novak et al., 1998; Till et al., 1998; Oh et al., 2001):

\[
\text{Fe}^0 + 2\text{H}_2\text{O} \rightarrow \text{Fe}^{2+} + 2\text{OH}^- + \text{H}_2 \quad (1)
\]

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 \( \approx 0.2–0.3 \) 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>
<thead>
<tr>
<th>Parameter</th>
<th>(-2 \text{ m (upgradient)})</th>
<th>Within PRB</th>
<th>2 m</th>
<th>4 m</th>
<th>6 m</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,4,6-trinitrotoluene (TNT) ((\mu\text{g L}^{-1}))</td>
<td>30–200</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) ((\mu\text{g L}^{-1}))</td>
<td>1–2</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>2-amino-4,6-dinitrotoluene (DNT) ((\mu\text{g L}^{-1}))</td>
<td>10–50</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Sulfate (mg L(^{-1}))</td>
<td>161</td>
<td>2</td>
<td>28</td>
<td>117</td>
<td>79</td>
</tr>
<tr>
<td>pH</td>
<td>7.2</td>
<td>9.8</td>
<td>7.8</td>
<td>7.0</td>
<td>6.9</td>
</tr>
<tr>
<td>(\text{O}_2) (mg L(^{-1}))</td>
<td>(&lt;0.1)</td>
<td>(&lt;0.1)</td>
<td>(&lt;0.1)</td>
<td>(&lt;0.1)</td>
<td>(&lt;0.1)</td>
</tr>
<tr>
<td>Eh (mV)</td>
<td>(-48)</td>
<td>(-280)</td>
<td>(-134)</td>
<td>(-100)</td>
<td>(-98)</td>
</tr>
<tr>
<td>Hydrogen (% sat)</td>
<td>nd(^a)</td>
<td>1</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Methane (% sat)</td>
<td>nd</td>
<td>nd</td>
<td>28</td>
<td>41</td>
<td></td>
</tr>
<tr>
<td>Conductivity ((\mu\text{S}))</td>
<td>1,007</td>
<td>456</td>
<td>266</td>
<td>580</td>
<td>510</td>
</tr>
<tr>
<td>Alkalinity</td>
<td>310</td>
<td>170</td>
<td>120</td>
<td>210</td>
<td>160</td>
</tr>
<tr>
<td>(\text{Ca}^{2+}) (mg/L)</td>
<td>48</td>
<td>nd</td>
<td>22</td>
<td>98</td>
<td>65</td>
</tr>
</tbody>
</table>

\(^a\text{nd, not detected.}\)
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 al., 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*, *Archaemia*, sulfate-reducing bacteria (SRBs) (*δ*-Proteobacteria class, such as some members of the *Geobacter* and *Pelobacter* genera), *Desulfovibrio*, *Desulfovibrio*, *Desulfuromonas*, and *Desulfuroma*, 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 *Archaemia* 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 (2002). Calibration curves (10¹ to 10⁸ gene copies mL⁻¹) were prepared for all genes under consideration, yielding r² values ≥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-(nirS) 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

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**Table 2. Maximum concentration of bacteria measured by PCR.**

<table>
<thead>
<tr>
<th>Target</th>
<th>Source of gene sequence</th>
<th>Maximum concentration detected (cell mL⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteria</td>
<td>Beller et al., 2002</td>
<td>5.1 ± 1.0 x 10⁴</td>
</tr>
<tr>
<td><em>Archaemia</em> (e.g., Methanogens)</td>
<td>Suzuki et al., 2000</td>
<td>3.7 ± 0.3 x 10⁶</td>
</tr>
<tr>
<td><em>Geobacter</em> spp. (e.g., iron-reducers)</td>
<td>Stults et al., 2001</td>
<td>4.5 ± 0.12 x 10²</td>
</tr>
<tr>
<td><em>δ</em>-Proteobacteria (e.g., sulfate-reducers)</td>
<td>Stults et al., 2001</td>
<td>1.9 ± 0.63 x 10²</td>
</tr>
<tr>
<td>Nitrite-reductase (e.g., nitrate-reducers)</td>
<td>Braker et al., 1998</td>
<td>10²</td>
</tr>
<tr>
<td><em>Clostridium</em> sp. (clone P3OU-7)</td>
<td>(NCBI accession AY756140)</td>
<td>a</td>
</tr>
<tr>
<td>Metal-reducing bacterium</td>
<td>(NCBI accession AY756140)</td>
<td>a</td>
</tr>
</tbody>
</table>

*Detected using DGGE and gene sequence analyses.*

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) (Fig. 1). Bacteria concentrations in samples obtained within the PRB were one order of magnitude higher (70 ± 21 cells/mL). 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 ± 1 × 10⁴ cells/mL) measured 4 m downgradient of the PRB (Fig. 1). The concentration of Archaea also increased downgradient of the PRB (from 1.6 ± 0.2 × 10² to 3.7 ± 0.3 × 10⁶ cells/mL), 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 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.

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.
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 and Banfield, 2003). This potential metal-reducing bacterium (not associated with Geobacter, as determined by our RTQ-PCR analyses) was present upgradient and downgradient 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 (U18_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 CO2 and H2 (Boga et al., 2003). Although it is unknown whether the Clostridium sp. detected in this work (using nonculturable techniques) can produce acetate using cathodic H2 [Equation (1)], it is tempting to speculate syllogistically that ZVI corrosion might support primary productivity (e.g., 4Fe0 + 2CO2 + 5H2O → CH3COO− + 4Fe2+ + 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

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REFERENCES


bacteria in environmental samples. Appl. Environ. Microbiol. 64(10), 3769.


DA SILVA

AU1
Phone and fax numbers for corr. au?

AU2
No Da Silva and Alvarez 2002 in list.

AU3
No Suzuki and Banfield, 2003 in list.

AU4
Please clarify all last names and initials in Johnson ref.