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Feasibility of Enhanced Biodegradation of Petroleum Compounds in Groundwater Under Denitrifying Conditions

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Abstract Groundwater was collected from a petroleum hydrocarbon contaminated site and characterized for microbial and physiochemical properties to assess the feasibility of enhanced natural attenuation. Results demonstrate the depletion of nitrate and dominance of denitrifying bacteria in the groundwater. Microcosm studies of amending nitrate and nutrients were attempted to enhanced biodegradation of petroleum compounds under denitrifying condition. Results show that 75% of petroleum compounds was degraded within 152-day in microcosms amended with nitrate, compared to 25% removal in the non-amended controls. Data indicate that nitrate amendment to groundwater may offer a viable remedy for enhanced natural attenuation of petroleum compounds.

Keywords Biodegradation · Denitrification · Groundwater · Petroleum hydrocarbons

Bioremediation of hydrocarbon-contaminated groundwater and soils has been evaluated as a cost-effective and environmentally sound treatment method (Margesin and Schinner 2001). In situ enhancement of both aerobic and anaerobic biodegradation of hydrocarbon compounds has been successfully demonstrated in field-scale applications and become an established remedial technology (USEPA 2001). Anaerobic hydrocarbon-degrading microorganisms mainly include denitrifying bacteria (Lei et al. 2005), iron-reducing bacteria (Jahn et al. 2005), sulfate-reducing bacteria (Cravo-Laureau et al. 2005), and methanogenic consortia (Kleikemper et al. 2005). Generally, denitrifying bacteria and sulfate-reducing bacteria (SRB) are sought after for enhanced anaerobic biodegradation, known for their degradative capabilities of a wide range of petroleum hydrocarbons among anaerobic microorganisms (Caldwell et al. 1998; Van Hamme et al. 2003). Enhanced biodegradation typically involves the supplementation of nutrients and/or electron acceptors.

Due to the lack of microbial data for the study site, microbial characterizations were conducted to determine if capable microbial populations exist and could be enhanced. Our effort started from a baseline characterization of chemical and microbiological parameters, followed by testing nitrate and nutrient amendments in the laboratory to assess the feasibility of enhanced biodegradation of the remaining Extractable Petroleum Hydrocarbon (EPH) under denitrifying conditions.

Materials and Methods

The confidential site under study is located in northwestern United States. It had a history of various industrial activities, including refinery, gas station, and timber treatment. Historical spillage of petroleum hydrocarbons and pentachlorophenol (PCP) have caused severe environmental disturbance in this area and posed a substantial risk to

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human health. Lead and PCP were previous removed by remedial actions taken during the past decades; however, petroleum contaminants, monitored as EPH and dominated by diesel range hydrocarbons (C_{10}–C_{18}), remained onsite. Groundwater monitoring showed no observable natural attenuation of EPH.

Groundwater was collected from up-gradient background well (MW-1) and a "worst case" well (MW-4) containing substantial level of EPH based on previous monitoring. Groundwater was sampled by using a low-flow purging and sampling method. A water-interface probe was used to determine the depth of water table and the middle point from which was drawn with a peristaltic pump at 400 mL/min. Temperature, conductivity, and pH were measured at 3-min intervals until the readings stabilized and the values were within ±10% of each other, at which point water samples were collected. Anion and cation samples were collected into plastic bottles with minimal headspace and stored at 4°C. Dissolved metals samples were collected, and filtered (0.45 μm filter) into plastic bottles preserved with HNO_3 (pH ~ 2) and stored at 4°C. For organic constituents and microorganisms, samples were collected into 5-gal plastic containers without preservatives and headspace.

The conductivity of water samples was measured in the field with an Orion Thermo model 150 conductivity meter (ThermoElectron, Beverly, MA) calibrated with a 1,417-S/cm Traceable One-Shot conductivity standard. The pH of water samples was measured in the field with an Orion Thermo model 720A+ pH meter equipped with an Orion Ag/AgCl combination electrode calibrated with Orion pH 4 and 7 buffers. The dissolved oxygen (DO) of water samples was measured using an YSI model 95 DO meter calibrated in the air. The total organic carbon (TOC) was analyzed on a Shimazdu TOC-5000A total organic carbon analyzer. Extractable petroleum hydrocarbons were determined by following the January 1998 Massachusetts method. Ammonium was determined colorimetrically by a Shimadzu UV Mini 1240 UV–vis spectrophotometer. Sulfide was determined colorimetrically by light spectroscopy according to EPA Method 376.2. Anions were analyzed on a DIONEX DX-100 ion chromatograph (IC) equipped with a 4-mm AS-14 anion exchange column (Dionex, Sunnyvale, CA). Base cations were analyzed on a flame atomic absorption spectrophotometer.

Total bacteria enumeration was conducted by using acridine orange staining and a fluorescent microscope equipped with an oil lens and a micrometer eye-piece. The most probable number (MPN) technique was used to assay four populations of microorganisms: Aerobic bacteria, sulfate-reducing bacteria (SRB), denitrifying bacteria, and iron-reducing bacteria (IRB; APHA 1998a, b; Tiedje 1982; Gould et al. 2005).

Water samples (100 mL) were vacuum-filtered through 0.2-μm membrane filters (Fisher Scientific) and DNA was extracted from the biological material on the filters with an AquaPure Genomic DNA extraction kit (Bio-Rad, Hercules, CA) to isolate and enumerate methanogens. Real-time polymerase chain reaction (RT-PCR) analysis was used to identify and quantify methanogens. Specific DNA sequences (primers) were used to identify individual bacterial species, since these sequences have been identified specific and conservative to corresponding species. Universal small subunit (SSU) rDNA was used as a positive control for all bacteria and archaea in the groundwater. The nucleotide sequence that is universal to bacteria was used. The forward primer was 5′TGACTGACTGAGTGCCAGCMGCCGCG G3′, and the sequence for the reverse primer was 5′TGAC TGACTGAGGYTACCTTGTTACGACTT3′ (Lane 1991). The nucleotide sequence used for the methanogen forward primer was 5′TAYGAYCARATHTGGYT3′ and the sequence for the reverse primer was 5′ACRTTCATNG CRTARTT3′ (Erkel et al. 2005).

The microcosm study was initiated to determine the feasibility of enhanced biodegradation under denitrifying conditions. Anaerobic microcosms were established in triplicate in 125-mL serum bottles using various nutrient and inhibitory amendments with groundwater from well MW-4 (Table 1). Groundwater sample was decanted into each corresponding microcosm serum bottle containing no headspace. All microcosms were prepared in an anaerobic chamber (glove box) with N_2 atmosphere (O_2-free), under sterile conditions, at room temperature (~20°C), and stored in the dark. All nutrient amendment ratios and inhibitor amendment amounts remained constant for microcosms within corresponding groups. Ammonium chloride (Fisher Scientific, Fair Lawn, NJ) and potassium phosphate dibasic (Fisher Scientific) were used as sources of nitrogen and phosphorus. The nutrients were added based on a molar C:N:P ratio of 100:30:3. Carbon (C) concentration was based on the TOC concentration determined through baseline chemical characterizations (Table 2). Potassium nitrate (J.T. Baker, Phillipsburg, NJ) was also added to corresponding treatments based on the molar C:N ratio of 100:30. Calcium carbide was added (CaC_2; Sigma–Aldrich) to corresponding treatments at a concentration of 2.0 mM to form acetylene (produced when CaC_2 reacts with water), which inhibits denitrification, sulfate reduction, and methanogenesis (Oremland and Capone 1988). Microcosm serum bottles were sealed with a Teflon septum and aluminum cap to ensure anaerobic conditions.

Results and Discussion

Microbial degradation is one of the major mechanisms for natural attenuation and remediation of hydrocarbons in

Table 1 Experimental treatments of groundwater sample collected from the monitoring well MW-4

Treatment	Amendments[a]	Inhibitors[b]
C1	–	–
E1	N, P	–
E2	NO_3^-	–
E3	NO_3^-, P	–
E4	NO_3^-	Acetylene
E5	NO_3^-, P	Acetylene

–, No addition

[a] N from ammonium chloride, P from potassium phosphate dibasic, and NO_3^- from potassium nitrate

[b] Acetylene inhibits denitrification, sulfate reduction, and methanogenesis

Table 2 Baseline water quality for groundwater samples collected from monitoring wells MW-1 and MW-4

Parameters	Measurement units	Minitoring wells	
		MW-1	MW-4
pH	°C	7.51	6.79
Temperature	dS/m	7.2	6.1
EC	mg/L	2.09	2.58
DO	mg/L	0.25	0.32
TOC	mg/L	16.3	89.1
NO_3^-	mg/L	84.2	BDL
S^{2-}	mg/L	0.05	0.11
SO_4^{2-}	mg/L	25.2	104.1
Cl^-	mg/L	11.0	6.0
NH_4^+	mg/L	0.34	2.36
Ca	mg/L	90.6	136.8
EPH	µg/L	–	3,500

–, Not analyzed; EPH, extractable petroleum hydrocarbons

soils and groundwater (Leahy and Colwell 1990). However, direct measurement of in situ biodegradation is difficult. Degradation is usually indexed by the measurements of geochemical and microbial parameters (Borden et al. 1995).

Results from the groundwater characterization are listed in Table 2. Groundwater from well MW-4 was measured with an EPH concentration at 3,500 µg/L. Dissolved oxygen in the groundwater was measured at a range of 0.25–0.37 mg/L, showing a generally reduced condition that may favor denitrification. The pH of the groundwater samples was in the range of 6.8–7.5, which is also favorable to majority of microbial activities. Concentrations of nitrate, which serves as both a source of nitrogen and an alternate electron acceptor for microbial growth, were detected in the background well MW-1 (84.2 mg/L) but depleted in MW-4 (Table 2), which suggests that intrinsic biodegradation using nitrate as an electron acceptor is occurring. Sulfate concentrations were detected at 25.2 and 104.1 mg/L in samples from MW-1 and MW-4, respectively. Concentrations of sulfide were measured at 0.02 and 0.11 mg/L, respectively, in samples collected from MW-1 and MW-4.

Table 3 Microbial populations in groundwater samples collected from monitoring wells MW-1 and MW-4

Parameters	Monitoring wells	
	MW-1 (cells/mL)	MW-4 (cells/mL)
Total bacteria	19,935	54,661
Aerobes	ND	50
Denitrifiers	11,000	35,000
Iron reducing	90	350
Sulfate reducing	30	110
Fermentors	ND	30
Methanogens	ND	ND

ND, none detected

Bacterial populations detected in groundwater samples collected from the background well MW-1 and contaminated well MW-4 were summarized in Table 3. Higher populations were measured in groundwater from contaminated sites than from the reference site, with total bacteria populations of 2.0×10^4 and 5.5×10^4 cells/mL, respectively in samples collected from MW-1 and MW-4. MPN analyses for aerobes, denitrifiers, IRB, and SRB yielded a range of population densities as shown in Table 3. Denitrifiers dominated in MW-4 with a population of 3.5×10^4 cells/mL, while the number of IRB and SRB in MW-4 were negligible. The dominance in denitrifying population indicated that that denitrification was probably the main metabolic pathway in vicinity of MW-4.

However, the depletion of nitrate might have stopped further denitrifying activities, resulting in the accumulation of EPH in MW-4. Therefore, amending nitrate as the electron acceptor in MW-4 is expected to resume and enhance the biodegradation of EPH under denitrifying conditions. Total bacterial enumeration of each treatment for MW-4 after 125 days is summarized in Fig. 1.

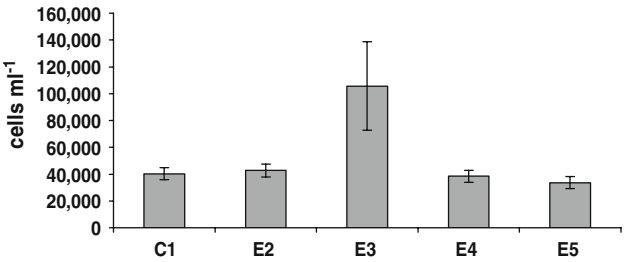

Fig. 1 Total bacteria enumeration of microcosms after 125 days, groundwater collected from monitoring well MW-4

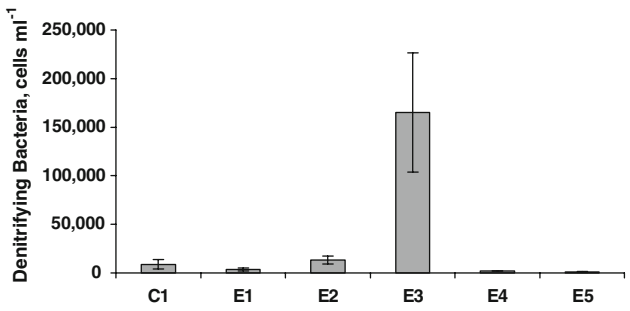

Fig. 2 Denitrifying bacteria MPN in microcosms containing groundwater collected from monitoring well MW-4 (152-day incubation)

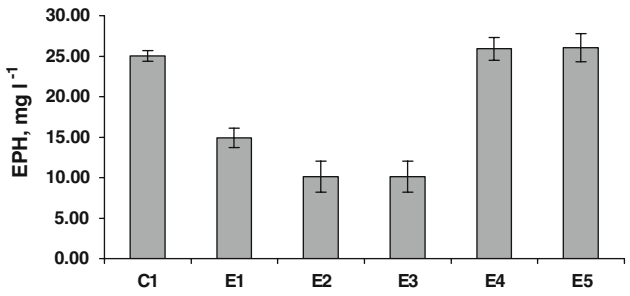

Fig. 3 EPH concentrations in microcosms containing groundwater collected from monitoring well MW-4 (152-day incubation)

Treatment E3, which amended with nitrate and P, showed the most substantial increase in bacterial populations (1.1×10^5 cells/mL). No substantial increase in microbial population was observed in other treatments.

After 152 days of incubation, denitrifying bacteria were enumerated by using selective medium and MPN methods in treatments of groundwater collected from MW-4. Treatment E3 (amended with nitrate and P) contained the highest denitrifying population at 1.6×10^5 cells/mL (Fig. 2), dominating the total population. Comparatively, denitrifying populations in other treatments (E1, E2) and controls (C1, E4 and E5) were insignificant and appear to be minor in the total bacterial population.

Diesel concentrations in EPH were monitored in the microcosms containing groundwater collected from MW-4 and presented in Fig. 3. After 152 days of incubation, treatments amended with nitrate demonstrated the most significant enhancement (>60%) in EPH degradation (E2 and E3). The degradation rate was determined to be 10.10–11.75 mg/(l day). Treatment E1 that received nutrient amendments also showed an enhanced EPH degradation (40%) when compared to the background control (C1), with a degradation rate at 0.07–0.08 mg/(l day). Inhibited controls (E4 and E5) showed the similar EPH concentrations as in the background control of C1. Results show that 75% of petroleum compounds as EPH was degraded within 152-day in microcosms with nitrate amendment, compared to 25% background degradation in the non-amended controls during the same period. Amendment of nitrate and nutrients (if needed), instead of air sparging other aeration actions, may enhance denitrifying activity that can achieve satisfactory degradation of petroleum compounds in groundwater that is depleted of oxygen and other electron acceptors.

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