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Monitoring of petroleum hydrocarbon degradative potential of indigenous microorganisms in ozonated soil

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Abstract

This study was performed to investigate the petroleum hydrocarbon (PH) degradative potential of indigenous microorganisms in ozonated soil to better develop combined pre-ozonation/bioremediation technology. Diesel-contaminated soils were ozonated for 0–900 min. PH and microbial concentrations in the soils decreased with increased ozonation time. The greatest reduction of total PH (TPH, 47.6%) and aromatics (11.3%) was observed in 900-min ozonated soil. The number of total viable heterotrophic bacteria decreased by three orders of magnitude in the soil. Ozonated soils were incubated for 9 weeks for bioremediation. The number of microorganisms in the soils increased during the incubation period, as monitored by culture- and nonculture-based methods. The soils showed additional PH-removal during incubation, supporting the presence of PH-degraders in the soils. The highest removal (25.4%) of TPH was observed during the incubation of 180-min ozonated soil during the incubation while a negligible removal was shown in 900-min ozonated soil. This negligible removal could be explained by the existence of relatively few or undetected PH-degraders in 900-min ozonated soil. After a 9-week incubation of the ozonated soils, 180-min ozonated soil showed the lowest TPH concentration, suggesting that appropriate ozonation and indigenous microorganisms survived ozonation could enhance remediation of PH-contaminated soil. Microbial community composition in 9-week incubated soils revealed a slight difference between 900-min ozonated and unozonated soils, as analyzed by whole cell hybridization. Taken together, this study provided insight into indigenous microbial potential to degrade PH in ozonated soils.

Introduction

Many studies have shown that ozonation is effective in removing contaminants such as petroleum hydrocarbons (PH) from soil (Hus & Masten 1997; Lim et al. 2002; Nelson & Brown 1994; Stehr et al. 2001; Sung & Huang 2002). Molecular ozone or its decomposition products (e.g., hydroxyl radicals) react with organic compounds to convert them into oxidized products. Oxidized products resulted from ozonation can be more water-soluble and/or more bioavailable than parental compounds, which leads to better biodegradation (Gilbert et al. 1983, 1987; Legube et al. 1981). Although the strong oxidizing activity of ozonation can remove PH effectively, ozonation is an expensive process since ozone generation requires a high-voltage electric discharge to oxygen molecules.

Studies on pre-ozonation and subsequent biodegradation have been performed to enhance remediation of soils contaminated with PH components, such as polycyclic aromatic hydrocarbons

(PAHs), and the cleanup technology was shown to be promising (Nam & Kukor 2000; Stehr et al. 2001). The studies employed artificially short-term contaminated soils and externally inoculated one or more known members of bacterial consortia for biodegradation in ozonated soils (Nam & Kukor 2000; Stehr et al. 2001). External introduction of microorganisms into soil often yielded inconsistent or disappointing results, caused mainly by a rapid decline of the number and/or activity of inoculated cells (van Veen et al. 1997). Therefore, employing already-acclimated indigenous microorganisms could be an alternative to achieve successful remediation based on pre-ozonation and subsequent biodegradation.

Most studies on soil remediation by ozonation mainly focused on the removal efficiency of chemicals (Choi et al. 2002; Lim et al. 2002; Masten & Davies 1997). Few studies have paid attention to indigenous microorganisms in ozonated soils (Lute et al. 1998). This lack of insight into indigenous microorganisms has hampered development of a combined ozonation and subsequent biodegradation strategy. Due to the strong-oxidizing abilities of ozone and its decomposition products, ozonation is used to disinfect microorganisms in medical and food products. Molecular ozone or its decomposition products inactivate or destroy microorganisms rapidly by reacting with cell components such as proteins, nucleic acids, cell envelope materials, etc. (Khadre et al. 2001; Kowalski et al. 1998). Lute et al. (1998) showed sterilizing effect of ozonation on the total number of indigenous soil microorganisms based on culture-based methods. However, information on target compound degraders in ozonated soil has not been reported.

PH degraders are frequently isolated from PH-contaminated soils (Ahn et al. 1999; Cerniglia 1993; Foght et al. 1990). When ozonation is applied to the contaminated soils to remove PH, indigenous PH-degraders are exposed to ozonation as well. Therefore, considering the fact that ozonation can cause microbicidal action against indigenous microorganisms, it is important to balance the two ideas of enhancing chemical removal by ozonation and maintaining microbial activity for subsequent biodegradation in a remediation strategy based on pre-ozonation and biodegradation by indigenous microorganisms. Quantitative information on both contaminants and their degraders in ozonated soil is necessary to better develop a remediation strategy for contaminated soil.

This study was undertaken to monitor PH-degradative potential and community composition of indigenous microorganisms in the ozonated soil that was previously contaminated with diesel. The microbial potential was monitored based on microbial abundance, as estimated by conventional culture-based methods (plate count and phenanthrene spray plate assay) and by nonculture-based molecular methods (direct soil DNA extraction and catabolic gene probing). Microbial community composition was analyzed using whole cell hybridization that employed group-specific rRNA-targeted oligonucleotide probes. Results of this study provide quantitative data showing that appropriate ozonation for contaminated site can enhance remediation of PH-contaminated soil. To the best of our knowledge, this study is the first to monitor the potential of indigenous microorganisms to degrade PH in ozonated soil.

Materials and methods

Site description and core sampling

The soil was obtained from a diesel-contaminated site in Ilsan, Kyungki province, Korea. The site had been contaminated for more than 5 years due to a leaking underground storage tank at a nearby gas station. A core sampler was used to collect a soil sample approximately 5 m below ground surface. The soil sample was kept on ice and transported to Gwangju Institute of Science and Technology. Soils were homogenized with sterile spatula and immediately used for total viable counts, the spray plate assay, and ozonation. Soil samples for molecular and chemical analyses were maintained at -20 °C until use.

The soil used in this study consisted of 16.5% sand, 37.5% silt, and 46.0% clay. Soil organic matter (SOM) and water content of the soil were 4.99% (w/w) and 20.5% (w/w), respectively. The soil showed a pH of 5.2 in distilled water (1:1, w/v). Soil analyses were performed according to the methods described by Soil Science Society of America (1986). Unless otherwise specified, all experiments were performed at room temperature (21 °C).

Soil ozonation and incubation

Glass columns (2.5 cm i.d. × 20 cm height) were employed as the soil columns. Seven soil columns were prepared using the columns. Each soil column contained a total of 160 g soil packed evenly into the column by shaking. Each soil column was placed in a batch column reactor system (Figure 1). Ozone was generated from oxygen (>99.99%) with an ozone generator (Model GL-1; PCI-WEDECO Environmental Technologies, Inc., West Caldwell, NJ). Gaseous ozone was moisturized by passing through a gas-washing bottle and then injected into a UV/VIS spectrophotometer (Model Smart Plus 1900; Youngwoo Instrument Co., Seoul, Korea) at a gas flow rate of 300 ml min^{-1} by using a mass flow controller (Model F201C-FAC-22-V; Bronkhorst Hi-Tec, Ruurlo, the Netherlands) to determine ozone concentration. Once ozone concentration was stabilized at 30 mg l^{-1}, gaseous ozone was introduced into the bottom of the soil column. Seven different periods of ozonation were employed in this study: the seven soil columns were ozonated for 0, 10, 30, 60, 180, 300, and 900 min, respectively. Residual ozone in the columns was purged with nitrogen gas for 1 min at the end of ozonation, and the soil columns were then sacrificed.

After the soils were ozonated for different times (0–900 min), they were subjected to incubation. Ozonated soil (60 g) was layered into a 500-ml beaker to a thickness of less than 2 cm, as described by Song & Bartha (1990). The beakers, each containing soil ozonated for a different amount of time, were covered with polyethylene film and then incubated in the dark at room temperature for 9 weeks. During incubation, the beakers were aerated every 2 or 3 days with a stainless steel wire. The loss of moisture was less than 3% during the incubation period.

Chemical extraction and analysis

Pressurized solvent extraction (PSE, Applied Separations, Inc., Allentown, PA) was employed to extract diesel components from soil according to the manufacturer's instructions. PSE is comparable with Method 3545 (pressurized fluid extraction) in SW-846 (US EPA 1996a). Soil sample (10 g) was added into the extraction vessel of PSE and extraction solvent was pumped into the vessel. The extraction solvent was a mixture (1:1, v/v) of acetone and methylene chloride. Temperature and pressure was increased to 100 °C and 100 bar, respectively, and this condition was maintained for 10 min. The solvent was discharged from the vessel and then the vessel was rinsed with fresh extraction solvent. Extract was analyzed for TPH by a 5890 series gas chromatograph (Hewlett-Packard, Palo Alto, CA) equipped with a flame ionization detector (FID), an autosampler, and an Alltech Econo-Cap EC-1capillary column (Deerfield, IL; 30 m length × 0.32 mm i.d. × 0.25 μm film thickness). The carrier gas was helium, at a flow rate of 10 cm s^{-1}. The temperatures of the injection port and detector were 270 and 280 °C, respectively. The oven temperature was programmed at 40 °C for 10 min followed by a linear increase of 8 °C min^{-1} to 280 °C, and the temperature was held for 10 min. TPH standards were

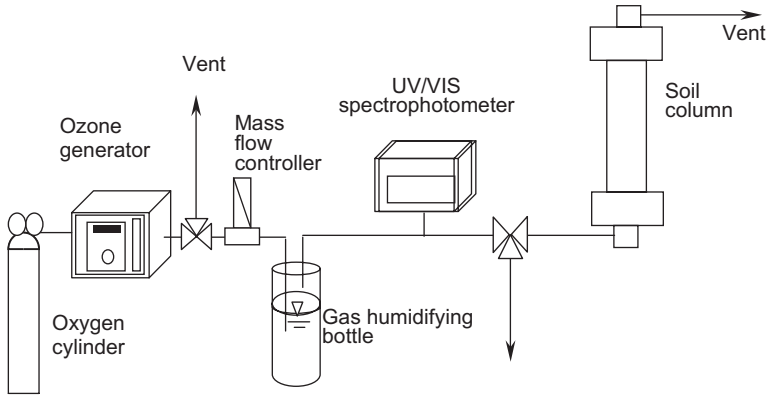

Figure 1. Schematic of the batch column reactor system used to ozonate soil.

prepared using commercial diesel fuel that was weathered for more than 2 week at room temperature. The extraction procedure showed 98 ± 4% recovery efficiency of 2-fluorobiphenyl (surrogate) spiked soil.

The extract was fractionated on a silica gel column according to Method 3630C (US EPA 1996b) as a cleanup procedure. Briefly, the extraction solvent was exchanged for cyclohexane. A slurry of activated silica gel (10 g) in methylene chloride was placed into a glass column (10 mm i.d.) and the solvent was eluted. Anhydrous sodium sulfate was added to the top of the silica gel and the column was pre-eluted with 40 ml of pentane. The cyclohexane sample extract was transferred onto the column and the column was eluted by the subsequent addition of two solvent systems: pentane and methylene chloride/pentane (2:3, v/v). Pentane eluate was discarded and methylene chloride/pentane eluate was collected and concentrated using a rotary evaporator (Model RE200; Yamato Scientific Co., Tokyo, Japan). The eluate was analyzed for aromatics using the GC-FID as described above. Reference standard for aromatics was a mix of polynuclear aromatic hydrocarbons (Supelco, Bellefonte, PA) that consisted of 16 PAHs in methylene chloride:benzene (50:50, v/v).

Plate count and phenanthrene spray plate assay

One gram of each soil sample was suspended in 9 ml of 0.1% (w/v) sodium pyrophosphate buffer (pH 7.2) and was vortexed vigorously for 1 min to release cells from soil particles (Ripp et al. 2000). Appropriate dilutions were prepared in phosphate buffered saline (PBS; 0.13 M NaCl, 10 mM sodium phosphate buffer, pH 7.2) and plated in triplicate on YEPG medium or hexadecane-containing minimal medium (Ahn et al. 1999; Sayler et al. 1999). YEPG medium was employed to enumerate total viable heterotrophic microorganisms. Colonies of hexadecane-degraders were counted using minimal medium containing filter-sterilized hexadecane (1%, v/v) as the sole carbon source. Plates were incubated in the dark at room temperature. Colonies of heterotrophic and hexadecane-degrading microorganisms were enumerated on days 4 and 14 of incubation, respectively. The phenanthrene spray plate assay was performed as described by Ahn et al. (1999) to enumerate phenanthrene-degrading colonies. Phenanthrene-degraders were counted on day 7, after spraying phenanthrene dissolved in acetone onto colonies pregrown on YEPG plates

DNA extraction and slot blot hybridization

Twenty grams of soil was used to extract DNA using a soil DNA isolation kit (Mo Bio Lab. Inc., Solana Beach, CA) according to the manufacturer's instructions, except that soil and bead mixture was vortexed for 5 min (ten 30 s pulses with a 30 s interval between pulses) after addition of a sodium dodecyl sulfate containing solution, and then spiked with 100 ng of an internal standard DNA to determine DNA recovery efficiency (%). The internal standard was a 3.5 kb *Sal*I-linearized DNA consisting of the 0.5 kb *lambda* phage DNA fragment (representing positions 7131–7630, Sanger et al. 1982) inserted into 3 kb pGEM®-T Easy vector (Promega, Madison, WI). Plasmid DNA was prepared as previously described (Ausubel et al. 1989). DNA concentration was determined using a Lambda 12 spectrophotometer (Perkin Elmer, Foster city, CA).

DNA extracts were treated with DNAse-free RNAse I (Roche Diagnostics GmbH, Mannheim, Germany) at 37 °C for 30 min and blotted onto a nylon membrane (Roche Diagnostics GmbH) using a slot blotting apparatus (Bio-Rad, Richmond, CA) in accordance with the manufacturer's protocols. The blotted DNA was subjected to DNA hybridization using Digoxigenin-labeled probes and hybridized target genes were detected with a DIG nucleic acid detection kit (Roche Diagnostics GmbH) according to the manufacturer's instructions. Prehybridization, hybridization, and washing of blotted DNA were performed at 65 °C for all gene probes except universal 16S oligonucleotide probe that was used at 42 °C instead (Stapleton & Sayler 1998).

DNA standards and probe preparation

Gene probes (Table 1) were prepared by polymerase chain reaction (PCR) using a PCR kit (Takara Shuzo Co., Ltd., Shiga, Japan). PCR conditions to amplify the probes consisted of 30 cycles: *alkB* probe, 94 °C for 30 s, 42 °C for 30 s, and 72 °C for 2 min; *todC* probe, 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 2 min; *lambda* probe,

Table 1. DNA probes used in slot blot hybridization

Probe	Size	Source organism	Target gene	Reference
alkB	1.2 kb	*Pseudomonas oleovorans*	Alkane hydroxylase (alkane 1-monooxygenase)	Kok et al. (1989)
nahA	1.0 kb	*P. putida* PpG7	Naphthalene dioxygenase	Simon et al. (1993)
todC	1.0 kb	*P. putida* F1	Toluene dioxygenase	Zylstra & Gibson (1989)
Lambda	0.5 kb	*Lambda* phage	Internal standard for DNA recovery during soil DNA extraction	Sanger et al. (1982)
Universal 16S	15 bp	*Escherichia coli*	16S rRNA gene	Stahl et al. (1988)

94 °C for 30 s and 68 °C for 30 s. Before the 30 cycles, preheating at 94 °C for 10 min was employed. Probe *nahA* was amplified as described in Ahn et al. (1999). The amplified products were detected by 1% (w/v) agarose gel electrophoresis. PCR products were used as templates for re-amplification under the same PCR conditions. Re-amplified PCR products were used as templates in PCR to obtain Digoxigenin-labeled probes using a PCR DIG labeling mix (Roche Diagnostics GmbH). Universal 16S rDNA oligonucleotide probe was 3′-end labeled with a Dig oligonucleotide 3′-end labeling kit (Roche Diagnostics GmbH).

DNA standards (known concentrations of genes in Table 1) were obtained by PCR as described for gene probe preparation, except that unlabeled PCR products were prepared. The DNA standard for 16S rDNA was 1.5 kb DNA fragment PCR-amplified using primers 27f and 1492r and *Escherichia coli* genomic DNA (Lane 1991). PCR products were purified with a purification system (Promega) before used for slot blotting. DNA standards were used to generate standard curves for the quantitative analysis of genes in soil DNA extracts.

Quantitative analysis of genes in soil DNA extracts using hybridization signal intensity analysis

The amount (g) of genes in soil samples was determined by an image analysis (SigmaGel; Jandal Scientific, San Rafael, CA) of hybridization signal intensities. Standard curves were generated based on hybridization signal intensities of DNA standards that were blotted along with sample DNA at each instance of vacuum blotting. The following equation was used to calculate the number (Y) of cells containing target genes per g soil (Applegate et al. 1995; Sanseverino et al. 1993)

$$Y = \left(\frac{X_{\text{hybridized}}}{v}\right) \times \left(\frac{1}{X_{\text{target}}}\right) \times \left(\frac{V_{\text{total}}}{X_{\text{soil}}}\right) \times \left(\frac{1}{C_{\text{target}}}\right) \times \left(\frac{100}{E_{\text{recovery}}}\right)$$

where $X_{\text{hybridized}}$ is the amount (g) of hybridized target genes in soil DNA extract applied to the slot blot; v is the volume (ml) of soil DNA aliquot applied to the slot blot; X_{target} is the mass (g) of one target gene in the DNA standards applied to the slot blot; V_{total} is the total volume (ml) of soil DNA suspension; X_{soil} is the mass (g) of soil used for DNA extraction, C_{target} is the copy number of target genes; and E_{recovery} is DNA recovery efficiency of the soil DNA extraction method used in this study. Calculations were performed based on following assumptions; one copy of each catabolic gene (*nahA*, *todC*, or *alkB*) per cell and five copies of 16S rRNA gene (rDNA) per cell (Applegate et al. 1995; Klappenbach et al. 2001; Stapleton & Sayler 1998). All cells in soil were also assumed to have 100% lysis efficiency in the soil DNA extraction method employed in this study.

Whole cell hybridization and DAPI staining

Cells released from soil particles were rinsed with PBS and fixed with 4% paraformaldehyde in PBS at 4 °C for 1 h. Fixed cells were rinsed and then suspended in PBS. The suspended cells were mixed with equal volume of 96% ethanol and stored at −20 °C and used within a week for whole cell hybridization and DAPI (4′,6-diamidino-2-phenylindole) staining. For whole cell hybridization with probe HGC69a, cells were ethanol fixed as

Table 2. Oligonucleotide probes used in whole cell hybridization

Probe	Target		Reference
	rRNA	Organisms	
EUB338	16S	Domain bacteria	Amann et al. (1990)
ALF1b	16S	α-subgroup *Proteobacteria*	Manz et al. (1992)
BET42a	23S	β-subgroup *Proteobacteria*	Manz et al. (1992)
GAM42a	23S	γ-subgroup *Proteobacteria*	Manz et al. (1992)
SRB385Db	16S	Most members of *Desulfobacteriaceae* in δ-subgroup *Proteobacteria*	Rabus et al. (1996)
HGC69a	23S	Gram (+) bacteria with high G + C content	Roller et al. (1994)

described by Roller et al. (1994). Whole cell hybridization was performed as described by Manz et al. (1992). The fluorescently labeled probes used in this study (Table 2) were specific for rRNA of bacteria belonging to the α-, β-, γ-, and δ-*Proteobacteria* and to Gram-positive bacteria with high G + C content. Probes 5′-end labeled with either fluorescin or TAMRA (tetramethyl rhodamine) and purified by reverse-phase high-pressure liquid chromatography were obtained from ThermoHybaid GmbH (Ulm, Germany). DAPI staining was performed after hybridization. DAPI-stained cells were washed briefly with distilled water and were air dried at room temperature before epifluorescence microscopy.

Cells hybridized or stained were visualized using a Zeiss Axiolab (Jena, Germany) with a 50 W-mercury lamp. Images were obtained with a digital camera (Model Coolpix 995; Nikon, Tokyo, Japan) mounted on the microscope. The cell counting was done with at least 10 random microscopic fields. MS Excel was used for statistical analysis.

Results and discussion

Chemical analysis

In ozonation, removal efficiency of chemicals depends on soil characteristics such as soil texture, moisture content, and SOM content (Choi et al. 2002; Sung & Huang 2002). When different ozonation times were applied to the homogenized soil in this study, the initial amounts (kg soil^{-1}) of TPH (2651.5 mg) and aromatics (278.6 mg) in the soil decreased with increased ozonation time (Figure 2) and initial fast removal and subsequent slow removal patterns were observed. The greatest reduction of TPH (47.6%) and aromatics (11.3%) was observed in 900-min ozonated soil.

Ozonated soils were incubated for 9 weeks and chemical analyses were performed after the incubation to investigate if contaminants were further removed during the incubation period. Chemical analyses showed additional removal of PH. Compared to TPH removal, removal of aromatics was very little during incubation, showing that aromatics were removed from the soils mainly by the ozonation. During incubation, the highest removals of TPH (25.4%) and aromatics (4.7%) were observed in 180- and 0-min ozonated soils, respectively. However, 900-min ozonated soil showed a negligible reduction of 3.6% TPH and 0.5% aromatics during the incubation period.

Comparative analysis of PH concentrations in the incubated soils revealed that 180- and 300-min ozonated soils showed the lowest TPH concentrations. Considering the total removal of TPH observed after both the ozonation and subsequent 9-week incubation together, the initial amount of TPH decreased most in 180- and 300-min ozonated soils. This suggests that 180-min ozonation followed by a 9-week incubation appears to be a more effective treatment for TPH removal than either 300-min or 900-min ozonation alone in this study. Results of this study show effectiveness of pre-ozonation and biodegradation by indigenous microorganisms to remediate PH-contaminated soil.

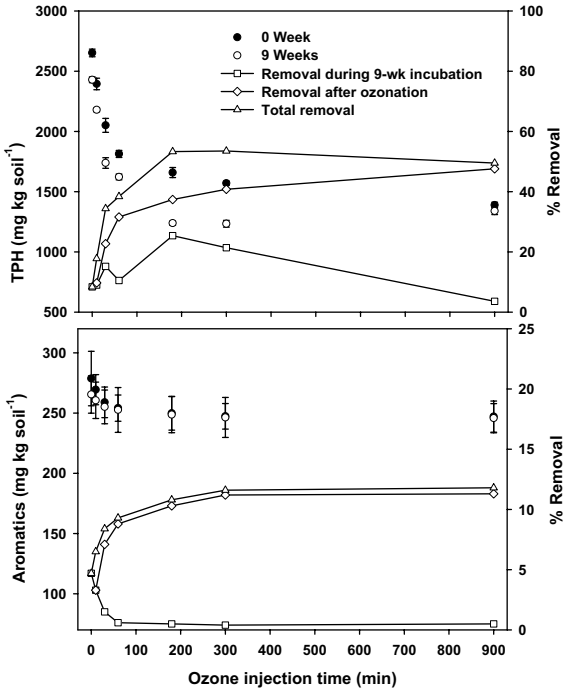

Figure 2. Concentrations of TPH (top) and aromatics (bottom) in soils subjected to different ozonation times. Zero and 9 weeks indicate the times when chemical analyses were performed during incubation of the soils. Zero week also indicates the time right after ozonation. Removals after ozonation and during the 9-week incubation represent the percentage of TPH and aromatics removed from the soil after ozonation and during the 9-week incubation, respectively. Total removal represents % of TPH or aromatics removed from soil by ozonation and subsequent 9-week incubation. Error bars represent standard deviation of the mean ($n = 3$) for TPH and standard uncertainty (type A) of the mean ($n = 3$) for aromatics. Standard uncertainty (type A) was calculated using the equation: $\frac{\sigma}{\sqrt{n}}$.

Effectiveness of pre-ozonation and biodegradation was previously reported using microorganisms introduced into pre-ozonated soils (Nam & Kukor 2000; Stehr et al. 2001). Different from the remediation strategy using introduced microorganisms, pre-ozonation and biodegradation employing indigenous microorganisms requires optimal dosage of ozonation to achieve chemical removal by pre-ozonation and maintain microbial activity for subsequent biodegradation. Appropriate pre-ozonation could reduce high initial concentration of PH to delay or prevent groundwater contamination that might be caused by leaching of PH. Indigenous microorganisms survived ozonation could degrade remained PH in soil after ozonation. Such remediation strategy as combined pre-ozonation and biodegradation by indigenous microorganisms is relatively ecologically sound, compared to soil remediation employing ozonation alone.

The large error range (standard uncertainty type A of the mean, $n = 3$) shown in the lower graph of Figure 2 could be caused by a heterogeneous distribution of aromatics in the soils. Heterogeneous distribution of aromatics is typical in a contaminated site and the soil used for ozonation in this study was obtained from an actual, contaminated site. Heterogeneity of soil must also be taken into account.

Quantification of bacterial populations based on culture-based methods

Aliphatic and aromatic hydrocarbons are major components of PH. As model compounds of the components, hexadecane (C16) and phenanthrene were commonly employed to study microorganisms to degrade PH (Ahn et al. 1999; Foght et al. 1990; Sotsky et al. 1994). Because hexadecane shows low volatility and the greatest abundance (22.2%, w/w) among alkanes (C10–C24) of diesel range organics, and phenanthrene has relatively high water-solubility, high biodegradability, and low volatility among aromatic hydrocarbons. Alkane or aromatic hydrocarbon degraders are frequently isolated from soils contaminated with PH (Ahn et al. 1999; Cerniglia 1993; Foght et al. 1990), which is probably due to enrichment by selective pressure.

A longer period of ozonation decreased concentrations of TPH and aromatic hydrocarbons in soils (Figure 2). Likewise, an increased ozonation time sharply decreased the colony-forming unit (CFU) of total viable heterotrophs and phenanthrene- and hexadecane-degraders in soils (Figure 3), showing a pattern of initial fast decrease and subsequent slow decrease of CFU. Initial CFU in unozonated soil (g^{-1}) were as follows: total heterotrophic bacteria, 1.6×10^8; phenanthrene degraders, 1.2×10^6; and hexadecane degraders, 1.3×10^7. The initial number of heterotrophic bacteria decreased by the following orders of magnitude in ozonated soils: 0.5, 10-min ozonated soil; 1, 30-min ozonated soil; 2, 60- and 180-ozonated soils; and 3, 300- and 900-min ozonated soils. The initial number of hexadecane degraders also decreased in the similar mode to

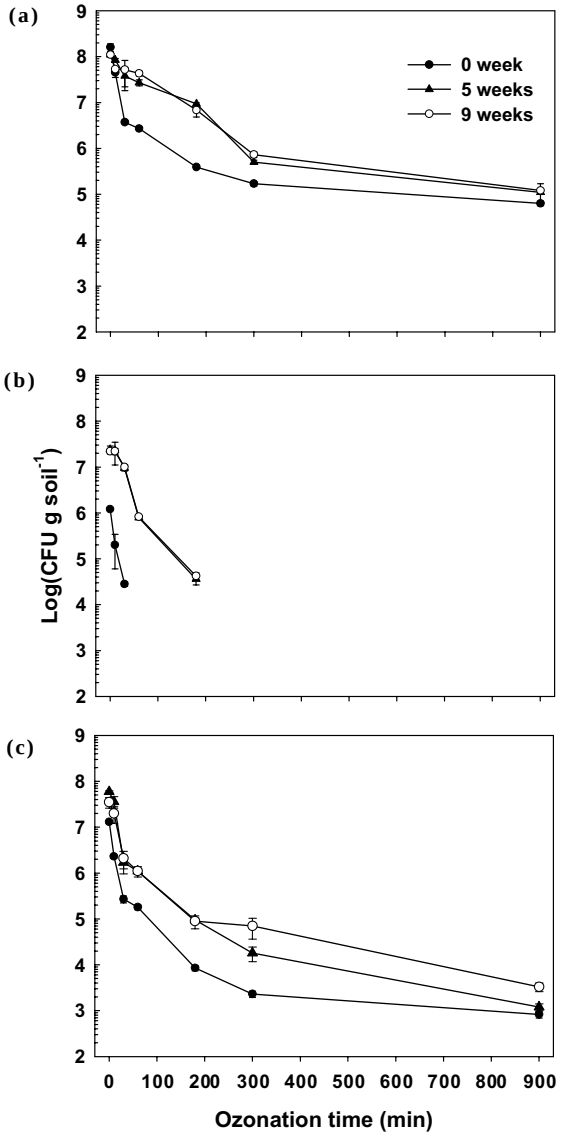

Figure 3. Abundance of indigenous microorganisms in 0–900-min ozonated soils as determined by culture-based methods: (a) total viable heterotrophic populations; (b) phenanthrene-degraders; and (c) hexadecane-degraders. Plate count was used for a and c while the phenanthrene spray plate assay was employed for (b) Zero, 5, and 9 weeks indicate the times when microbiological analysis was performed during the incubation of the soils. Samples analyzed were 0-, 10-, 30-, 60-, 180-, 300-, and 900-min ozonated soils. CFU data not shown in the graph are under the detection limit. Error bars represent standard deviation of the mean ($n = 3$).

heterotrophic bacteria with increased ozonation. However, phenanthrene degraders showed more sensitivity to increased ozonation. They were not detected in the soils ozonated for 60-min or longer.

Indigenous microorganisms survived ozonation caused increase in CFU during 9-week incubation period (Figure 3). Heterotrophic microbial populations in ozonated soils increased for 5 weeks and remained constant afterward. An increase of phenanthrene- and hexadecane-degraders was more evident than an increase of heterotrophic populations in ozonated soils. Initially, phenanthrene degraders (CFU) in soils (g^{-1}) ozonated for 60-min or longer were not detected by the spread method used in this study, but after a 9-week incubation period, they were detected in the range of $8.2 \times 10^5 - 4.2 \times 10^4$ CFU per g of 60- and 180-min ozonated soils. However, phenanthrene degraders in 300-min or longer ozonated soils were not detected even after a 9-week incubation. Hexadecane degraders detected in 300- and 900-min ozonated soils ranged from 7.0×10^4 to 3.3×10^3 CFU g $soil^{-1}$. Theoretical detection limits for the methods employed in this study to enumerate phenanthrene- and hexadecane-degraders were 1.2×10^2 and 7.5×10^2 CFU g^{-1} soil, respectively. Less than 4% reduction in aromatics was observed during the 9-week incubation period (Figure 2), which could be partly explained by the sharp reduction in the number of phenanthrene degraders upon ozonation and their relatively low detection after 9 weeks of incubation.

Phenanthrene- and hexadecane-degraders in unozonated soil showed an increase of up to one order of magnitude after a 9-week incubation, while the heterotrophic bacterial population in the soil remained relatively constant during the period. The increased numbers of phenanthrene- and hexadecane-degraders observed in both unozonated and ozonated soils were probably due to enhanced aeration caused by periodic tilling of the soils during the incubation, since bacterial catabolism of PH generally requires oxygen to initiate degradation of the compounds (Harayama et al. 1992; Kok et al. 1989; Simon et al. 1993; Zylstra & Gibson 1989).

Quantification of microorganisms based on soil DNA analysis

Low culturability of soil microorganisms limits the enumeration of relevant degraders using conventional culture-based methods such as the plate count and phenanthrene spray plate assay employed in this study. Therefore, a nonculture-

based molecular method was employed as well to better enumerate relevant microorganisms in soil. DNA extracted from the soils was subjected to DNA hybridization to detect genes encoding degradative enzymes for aromatic hydrocarbons and alkanes that are major components of diesel. Microbial abundance in each soil sample was estimated based on the amount of genes detected: total microorganisms, 16S rDNA; microorganisms with aromatic hydrocarbon catabolic genes, *nahA* and *todC*; microorganisms with alkane catabolic gene, *alkB* (Table 1). Although there is a diversity of genes responsible for aromatic hydrocarbon or alkane degradation (Ahn et al. 1999; Vomberg & Klinner 2000), the catabolic genes used in this study have been frequently used to monitor microbiological potential to degrade PH in contaminated soils due to the abundance of isolates containing the genes and accumulated knowledge of the genes (Ahn et al. 1999; Sanseverino et al. 1993; Sotsky et al. 1994).

Quantitative soil DNA analysis was performed using soils incubated for 9 weeks (Figure 4). Compared to culture-based estimates, cell estimates based on detected genes showed higher population abundance by 1–2 orders of magnitude. Soils ozonated for 0- to 180-min showed cell number (g soil^{-1}) estimates in the following ranges: total cell numbers, 4.9×10^9–8.1×10^7; cells containing *nahA*, 9.9×10^7–5.9×10^6; cells containing *todC*, 5.7×10^7 to under the detection limit; and cells containing *alkB*, 4.6×10^8–9.2×10^6. However, those cells containing *nahA* or *todC* were under the detection limit in soils ozonated for 300 min or longer, whereas cells with the *alkB* genotype were estimated at 4.9×10^6 cells g^{-1} soil ozonated for 300 min. Cells containing *alkB* were under the detection limit in 900-min ozonated soil. The detection limits of soil DNA analysis used in this study were 0.1 ng for 16S rDNA and 0.01 ng for the catabolic genes in soil. The detection limits are based on the lowest amount of standard DNA used to quantify soil DNA samples. The average efficiency of DNA recovery was 82 ± 10% in the soil DNA extraction method used in this study. Microbial abundance estimated by direct soil DNA extraction and catabolic gene probing further supported the relative abundance of microorganisms in the soils, as observed by culture-based methods. Comparative analyses of cell estimates based on culture-based methods and molecular analysis supported previous reports that culture-based methods may underestimate population abundance by 1–2 orders of magnitude (Amann et al. 1995; Sanseverino et al. 1993).

Composition of soil microbial community

Microbial community composition in 9-week incubated soils was analyzed using whole cell hybridization that employed group-specific fluorescently labeled, rRNA-targeted oligonucleotides. Whole cell hybridization results revealed a slightly different composition of the microbial community in ozonated soil than that in unozonated soil (Figure 5). Especially, the γ-subgroup *Proteobacteria* showed a decreased fraction with ozonation. Its fractions in 0-, 300- and 900-min ozonated soils were 17.5 ± 5.3, 9.5 ± 1.1 and 7.0 ± 3.1% of DAPI cell counts (corresponding to total cell counts), respectively. However, the eubacterial fraction in ozonated soils was relatively constant in the range of 70.8–66.0%, although total cell numbers decreased with ozonation time (Figures 3 and 4). Unozonated soil showed very similar composition of microbial community before (data not shown) and after the 9-week incubation.

Various microorganisms have been reported to degrade aromatic or alkane compounds. Most of those reported so far belong to either *Proteobacteria* or Gram-positive bacteria with high G+C content in DNA. They are exampled by microorganisms belonging to the genera *Sphingomonas*, *Burkholderia*, *Pseudomonas*, *Rhodococcus*, and *Mycobacterium* (Cerniglia 1993; Kok et al. 1989; Vomberg & Klinner 2000; Zylstra & Gibson 1989). The first three genera belong to α-, β-, and γ-*Proteobacteria*, respectively while the latter two genera belong to Gram-positive bacteria with high G + C content in DNA. *Gamma Proteobacteria* are important since they are associated with the majority of isolates able to degrade aromatic or alkane compounds despite the phylogenetic diversity of the degraders (Ahn et al. 1999; Cerniglia 1993; Kok et al. 1989; Vomberg & Klinner 2000).

Most microorganisms in soil habitat occur as microcolonies on the surfaces of soil particles (Casida 1971; Madigan et al. 2000). Sensitivity of indigenous soil microorganisms to in situ ozonation has not been reported, although bacterial spores show less sensitivity to ozone than vegetative

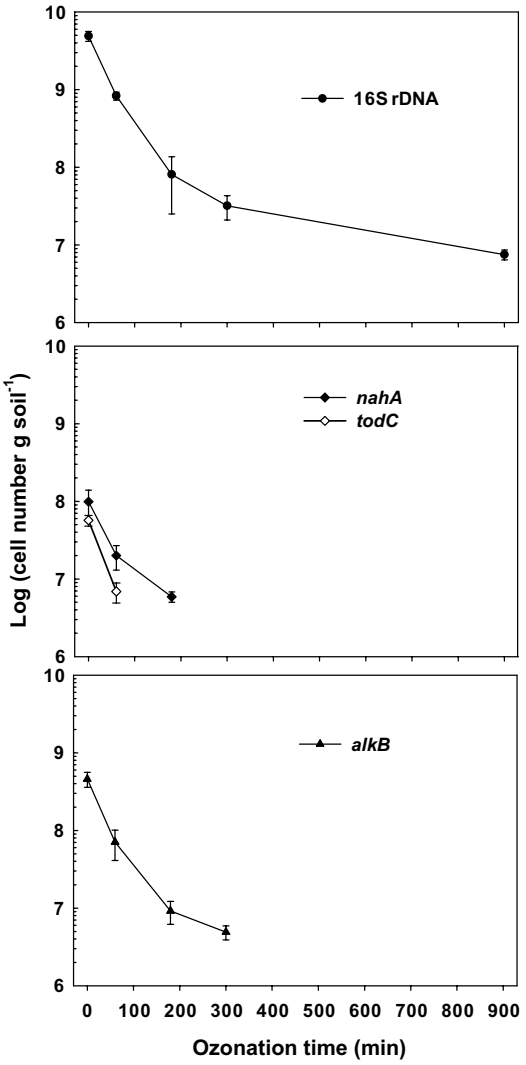

Figure 4. Abundance of indigenous microorganisms in 0- to 900-min ozonated soils as determined by quantitative analysis of soil DNA using gene probes. Soils incubated for 9 weeks were used for the analysis. Cell number estimates were based on the amount of genes (16S rDNA, *nahA*, *todC*, and *alkB*) detected in each soil sample as described in the Materials and Methods. Samples analyzed were 0-, 60-, 180-, 300-, and 900-min ozonated soils. Data not shown in the graph are under the detection limits. Error bars represent standard deviation of the mean ($n = 2$).

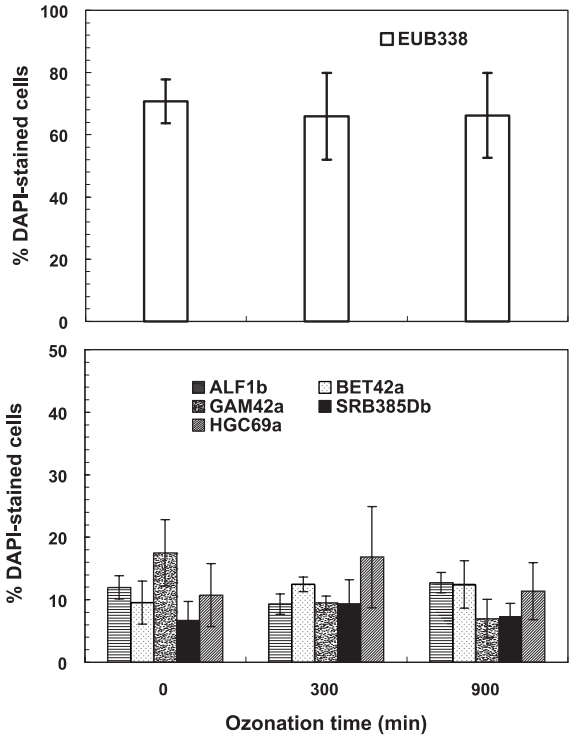

Figure 5. Microbial community composition in 9-week incubated soils as determined by whole cell hybridization using rRNA-targeted group-specific oligonucleotide probes. Error bars represent standard deviation of the mean ($n = 2$).

bacterial cells in aqueous suspension (Khadre et al. 2001). Sensitivity of soil microorganisms to ozonation might vary depending on their soil niche characteristics such as SOM and water contents, like chemical removal efficacy of ozonation depends on such soil characteristics (Choi et al. 2002; Lim et al. 2002). Microorganisms associated with ozone-demanding compounds were shown to be less sensitive to ozone than unassociated or purified ones (Khadre et al. 2001).

Conclusions

Diesel-contaminated soils underwent different periods (0–900 min) of ozonation. Increased period of ozonation decreased not only the concentration of PH but also the number of microorganisms in the soils. Nine hundred minutes ozonated soil showed greatest reduction in PH concentration and in total number of viable heterotrophic bacteria. Ozonated soils were incubated for 9 weeks and changes in microbial number and PH concentration were monitored. Microorganisms in the ozonated soils increased during incubation as monitored by culture- and nonculture-based methods. Higher (1–2 orders of magnitude) cell numbers were observed by a quantitative analysis of soil DNA using probes detecting genes encoding 16S

rRNA (*rrn*), naphthalene dioxygenase (*nahA*), toluene dioxygenase (*todC*), and alkane hydroxylase (*alkB*), than the microbial abundance estimated by culture-based methods. Such PH-degraders were relatively few or were under the detection limit in 900-min ozonated soil. Additional PH-removal was observed during the incubation period. This supported the presence of PH-degraders in the ozonated soils. The highest reduction (25.4%) of TPH was observed in 180-min ozonated soil while a negligible reduction was shown in 900-min ozonated soil during the incubation period; so among the incubated soils, 180-min ozonated soil showed the lowest TPH-concentration. Microbial community composition in 9-week incubated soils revealed slight difference between 900-min ozonated and unozonated soils as analyzed by whole cell hybridization using group-specific rRNA-targeted oligonucleotides. The results of this study suggested that strategy of appropriate ozonation and subsequent biodegradation by indigenous microorganisms could enhance remediation of PH-contaminated soil. Such remediation strategy is relatively ecologically sound, compared to soil remediation employing ozonation alone.

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