한국지하수토양환경학회 추계학술발표회
2003년 9월 26~27일 제 주 대 학 교

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

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Diesel-contaminated soils were ozonated for different times (0 - 900 min) and incubated for 9 wk to monitor petroleum hydrocarbons (PH)-degradative potential of indigenous microorganisms in the soils. Increased ozonation time decreased not only concentration of PH but also number of microorganisms in the soils. Microorganisms in the ozonated soils increased during 9-wk incubation as monitored by culture- and nonculture-based methods. Higher (1-2 orders of magnitude) cell number was observed by quantitative analysis of soil DNA using probes detecting genes encoding 16S rRNA (*rrn*), naphthalene dioxygenase (*nahA*), toluene dioxygenase (*todC*), and alkane hydroxylase (*alkB*) than microbial abundance estimated by culture-based methods. Such PH-degraders were relatively a few or under detection limit in 900-min ozonated soil. Further PH-removal observed during the incubation period supported the presence of PH-degraders in ozonated soils. Highest reduction (25.4%) of total PH (TPH) was observed in 180-min ozonated soil while negligible reduction was shown in 900-min ozonated soil during the period, resulting in lowest TPH-concentration in 180-min ozonated soil among the ozonated soils. Microbial community composition in 9-wk incubated soils revealed slight difference between 900-min ozonated and unozonated soils as analyzed by whole cell hybridization using group-specific rRNA-targeted oligonucleotides. Results of this study suggest that appropriate ozonation and subsequent biodegradation by indigenous microorganisms may be a cost-effective and successful remediation strategy for PH-contaminated soils.

key word : biodegradation, gene probe, ozonation, petroleum hydrocarbon, remediation, soil

1. Introduction

Many studies showed that ozonation is effective in removing contaminants such as PH in soil (1). Molecular ozone or its decomposition products (e.g., hydroxyl radicals) react with any organic compound and convert to its oxidized products. Despite the fact that strong oxidizing activity of ozonation can remove PH effectively, ozonation is an expensive process since ozone generation requires high-voltage electric discharge to oxygen molecules. Due to nonspecific strong-oxidizing abilities of ozone and its decomposition products, ozonation inactivates or destroys microorganisms rapidly by reacting with cell components (2).

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 estimated by culture-based conventional methods (plate count and phenanthrene spray plate assay) and nonculture-based molecular methods (direct soil DNA extraction and catabolic gene probing). Microbial community composition was analyzed using whole cell hybridization using group-specific rRNA-targeted oligonucleotide probes. Results of this study provide quantitative data that appropriate ozonation for contaminated site could accommodate not only cost-effectiveness but also bioremediation by indigenous microbes that have survived in situ ozonation.

2. Materials and methods

Soil sample was obtained from a diesel-contaminated site in Ilsan, Kyungki province, Korea. The site was contaminated for more than five years and source of the contamination was a leaking underground storage tank at a gas station nearby. The soil used in this study consisted of 16.5% sand, 37.5% silt, and 46.0% clay. Soil organic matter and water content of the soil were 4.99% (w/w) and 20.5% (w/w), respectively.

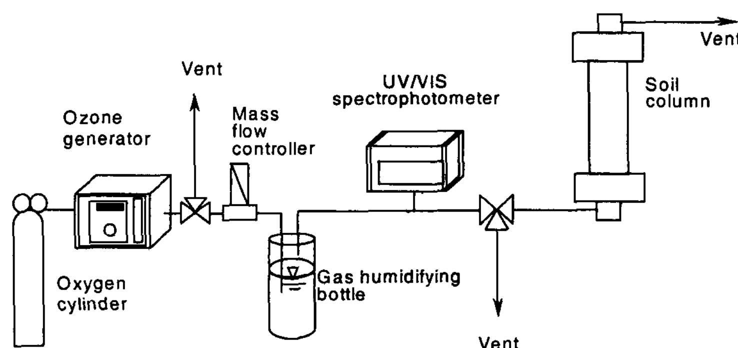

Figure 1. Schematic of the batch column reactor system used to ozonate soil.

Soil was packed evenly into a glass column (2.5 cm i.d. x 20 cm height) placed in a batch column reactor system (Fig. 1). Ozone was generated from oxygen by an ozone generator (Model GL-1; PCI-WEDECO Environ. Technologies, Inc.). Gaseous ozone (30 mg l^{-1}) was introduced into the bottom of each soil column for different times. Soil samples were layered into 500 ml beakers and then incubated at room temperature for 9 wk (3). Pressurized solvent extraction method was employed to extract diesel components (TPH) from soil and aromatics were separated from TPH extract using Method 3630C (4). TPH and aromatics were analyzed by a 5890 series gas chromatograph (HP).

YEPG medium was employed to enumerate total viable heterotrophic microorganisms (5). Colonies of hexadecane-degraders were counted using minimal medium containing filter-sterilized hexadecane (3%, v/v) as a sole carbon source (5, 6). The phenanthrene spray plate assay was performed to enumerate phenanthrene-degraders (5).

Soil DNA was extracted using a soil DNA isolation kit (Mo Bio Lab. Inc.). DNA blotting and hybridization were performed as previously described (7). Probes (Table 1) were labeled with

Digoxigenin and hybridized target genes were detected by a Dig nucleic acid detection kit (Roche). 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 (7). DNA standards were used to generate standard curves for the quantitative analysis of genes in soil DNA extracts. The amount (g) of genes in soil samples determined by image analysis of hybridization signal intensities. Standard curves were generated based on hybridization signal intensities of DNA standards that were blotted along with sample DNA on each event of vacuum blotting. Number of cells containing target genes per g soil was calculated using the equation described previously (7).

Whole cell hybridization was performed to analyze community composition (8). Cells hybridized or stained were visualized by epifluorescence microscopy.

3. Result

Diesel-contaminated soils were ozonated for 0 to 900 min and used for chemical and microbial analyses to monitor degradative potential of indigenous microorganisms in the soils. PH in soil decreased with increased ozonation time. Greatest reduction of total PH (TPH, 47.6%) and aromatics (11.3%) were observed in 900-min ozonated soil (Fig. 2). Soils showed further PH-removal (TPH, 3.6-25.4%; aromatics, 0.4-4.7%) during 9 wk of incubation.

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

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

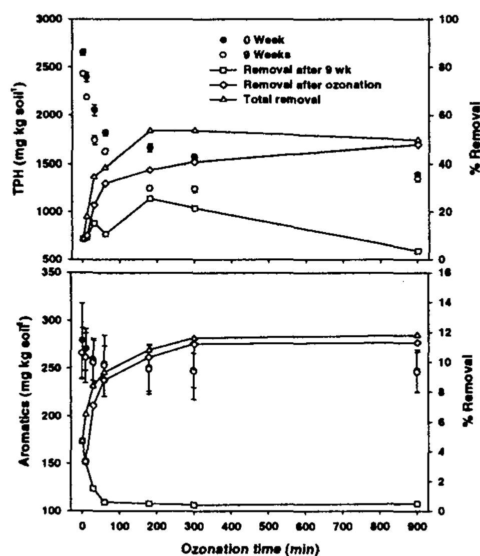

Fig. 2. Concentrations of TPH (top) and aromatics (bottom) in soil samples subjected to different ozonation times. Zero and 9 weeks indicate the times when chemical analyses were performed during incubation of the soils. Removals after 9 wk and ozonation represent percentage of removed TPH or aromatics from each soil sample after 9-wk incubation and ozonation, respectively. Total removal represents % of total removed TPH or aromatics from each soil sample after ozonation and 9-wk incubation. Error bars represent standard deviations of the mean (n=3).

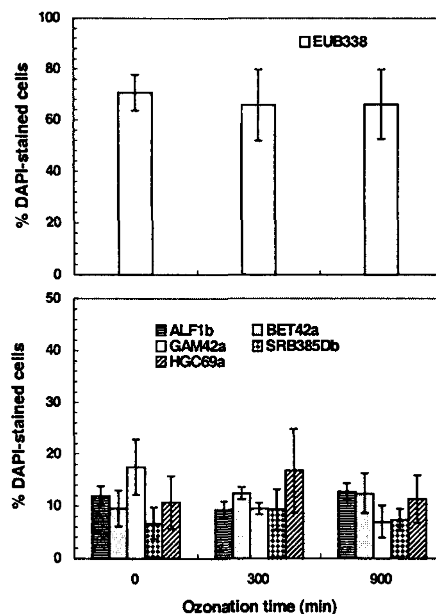

Fig. 2. Microbial community composition in 9-wk incubated soils as determined by whole cell hybridization using rRNA-targeted group-specific oligonucleotide probes.

The lowest TPH concentrations were observed in 180- and 300-min ozonated soils after the incubation, suggesting that 180-min ozonation and following 9-wk incubation appears to be most effective and economic treatment in this study. Increased ozonation time decreased number of microorganisms in the soil as well (Fig. 3). Microorganisms in the ozonated soils increased during 9-wk incubation as monitored by culture- and nonculture-based methods (Fig. 3, 4). The presence of PH-degraders in ozonated soils supported further removal of PH observed during the incubation period. PH-degrading cells were relatively a few or under detection limit in 900-min ozonated soils. This could explain the negligible reduction of TPH observed in 900-min ozonated soil during the period. Microbial community composition in 9-wk incubated soils revealed slight difference between 900-min ozonated and unozonated soils as analyzed by whole cell hybridization using group-specific rRNA-targeted probes (Fig. 5). Taken together, this study provided insight into indigenous microbial potential to degrade PH in ozonated soil. Information obtained in this study will aid to develop a cost-effective remediation strategy, appropriate ozonation and following biodegradation by indigenous microorganisms, for PH-contaminated soils.

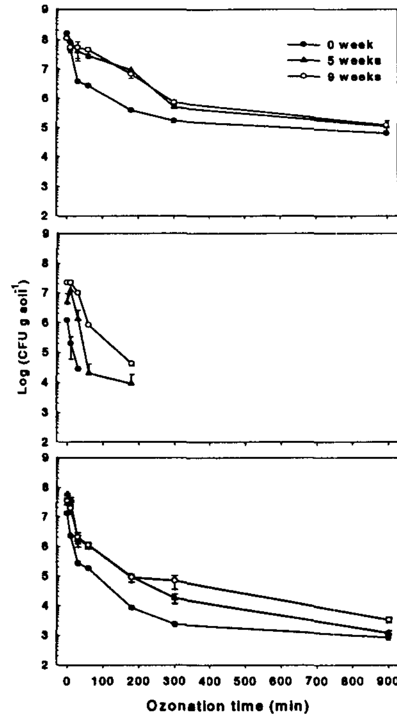

Fig. 3. Abundance of microorganisms in 0- to 900-min ozonated soils as determined by culture-based methods. A), total viable heterotrophic populations; B), phenanthrene-degraders; C), and hexadecane-degraders. Zero, 5, and 9 weeks indicate the times when microbiological analysis was performed during 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 detection limit. Error bars represent standard deviations of the mean (n=3).

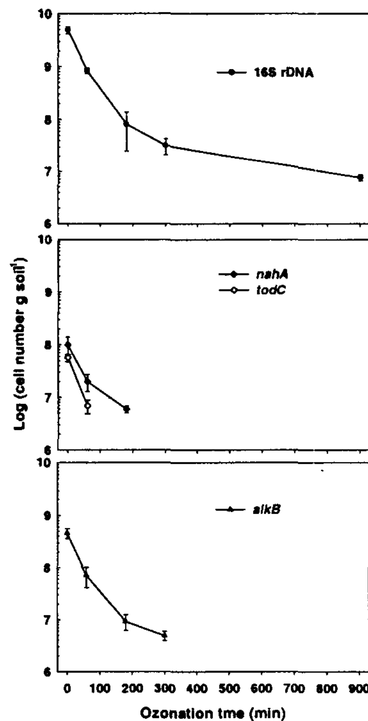

Fig. 4. Abundance of microorganisms in 0- to 900-min ozonated soils as determined by quantitative analysis of soil DNA using gene probes. Soil samples incubated for 9 wk were used for the analysis. Cell number estimates were based on the amount of genes (16S rDNA, nahA, todC, and alkB) detected in each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

Acknowledgements

This work was sponsored in part by the Korea Science and Engineering Foundation through the ADvanced Environmental Monitoring Research Center and in part by grant No. R08-2003-000-11084-0 (2003) from Basic Research Program of KOSEF.

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