Bull. Environ. Contam. Toxicol. (2005) 74:356–364

DOI: 10.1007/s00128-004-0592-9

Bulletin of Environmental Contamination and Toxicology

Natural Biodegradation of MTBE Under Different Environmental Conditions: Microcosm and Microbial Identification Studies

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Received: 12 July 2004/Accepted: 3 November 2004

Oxygenates are commonly added to gasoline to enhance octane index and decrease emissions of carbon monoxide (CO) and ozone (O_3). Among fuel oxygenates, methyl tert-butyl ether (MTBE) is the most commonly used oxygenate now due to its low cost, convenience of transfer, and ease of blending and production. Because of its wide usage, MTBE has been detected in groundwater, surface water, storm runoff, air, and even in drinking water derived from groundwater (Squillace et al. 1997; US EPA 1997). MTBE is less biodegradable due to the tertiary butyl group and ether linkage on it. In addition, MTBE is a highly water-soluble compound and it has a low retardation value. Thus, it always migrates a longer distance than other gasoline components [(e.g., benzene, toluene, ethylbenzene, xylenes (BTEX)] in gasoline-contaminated groundwater (Squillace et al. 1997; Prince 2000). Currently, MTBE is temporarily classified by the US Environmental Protection Agency (US EPA) as a possible human carcinogen and the US EPA has set its advisory level for drinking water at 20-40 μg/L. This advisory range is also for the purpose to avoid unpleasant taste and odor in drinking water that can protect sensitive individuals (US EPA 1997).

Many available literatures reported that MTBE could be aerobically biodegraded. In 1994, Salanitro et al. isolated a mixed bacterial culture (BC-1) from activated sludge of a chemical plant bioreactor, which was the first culture able to use MTBE as sole carbon and energy sources under aerobic condition (Salanitro et al. 1994). Lately, more laboratory and field studies support that MTBE can be biodegraded under both aerobic and cometabolic conditions (Prince 2000). Although it seems that MTBE is more recalcitrant under anaerobic condition, there have been some reports, which demonstrate that MTBE can also be degraded anaerobically. Results of these researches reveal that MTBE can be partially or completely biodegraded by sediments or aquifer materials under denitrifying, sulfate reducing, iron reducing, and methanogenic conditions (Mormlle et al. 1994; Yeh and Novak 1994; Prince 2000; Bradley et al. 2001; Finneran and Lovley 2001; Somsamak et al. 2001). Recently, natural bioremediation or enhanced bioremediation is an attractive remediation option for contaminated site due to its economic benefit and environmental friendly. In general, contaminated sites contain indigenous microorganisms, which play an important role in the removal of pollutants. However, the biodegrading rate might

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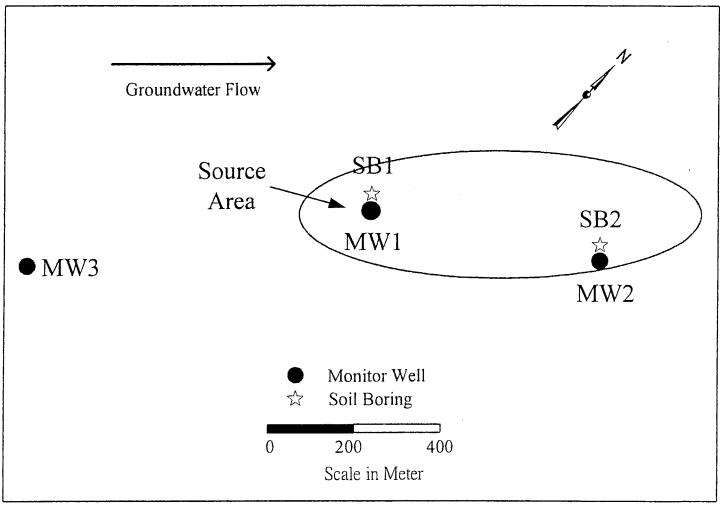

Figure 1. Site map showing the contaminant source area, groundwater flow direction, and the soil and groundwater sampling locations.

decrease if the nutritional and physiological requirements are not met. Thus, it is important to assess the biodegradability of natural microorganisms under various site conditions to obtain optimal remediation strategies. In this study, an MTBE-contaminated site was selected to assess the feasibility of applying bioremediation as the remedial alternative. Results from previous studies suggest that indigenous microorganisms are responsible for the decrease of contaminants, however, the dominant biodegrading mechanism at this site is not clear. For this reason, aquifer materials collected from this site were applied as the source of microorganisms for microcosm study (Chen et al. 2003). The main objectives of this study were to (1) evaluate MTBE biodegradability under different redox conditions with the addition of various carbon sources and (2) determine the dominant native microorganisms from the contaminated site for further application.

The selected site in this study is an oil-refining facility located in southern Taiwan, which produces gasoline, diesel, jet fuel, kerosene, and lubricating oils. Groundwater at this site is contaminated due to inappropriate operation and storage of the products. Soils at the site consist of silty sand, silt, and clay, and the main component of site soils is silty sand. The water table is generally found at depths ranging from 2 to 7 m below ground surface (bgs). A thickness of 5-10 m weak clay layer is located at 40 m bgs. Groundwater within this aquifer flows as a velocity of 0.2-1.4 m/day from southwest to northeast. The average hydraulic conductivity of the host geological material is 0.1 cm/sec and the gradient is approximately 0.25% according to the results from hydrogeologic tests. Figure 1 presents the site map showing the contaminant source area, groundwater flow direction, and the soil and groundwater sampling locations used in this study.

Results from previous studies reveal that the contaminants resulted in an approximately 900 m long and 300 m wide plume from source area to downgradient. The concentrations for MTBE and total BTEX compounds were about 200 μg/L and 200 mg/L, respectively, in collected groundwater samples from highly contaminated zone. In the downgradient monitoring well, MTBE concentrations was lower than 10 μg/L and BTEX concentrations were approximately 1.2 mg/L in collected groundwater samples.

Overall, contaminants were decreased along the groundwater flow direction from upgradient to downgradient area. Dissolved oxygen (DO) in groundwater samples was low (about 1 mg/L or lower) and carbon dioxide (CO_2) in groundwater samples was high (100-280 mg/L). Moreover, the byproduct of MTBE biodegradation, tert-butyl alcohol (TBA) was also detected at concentrations of 1.0 mg/L and 10 μg/L at upgradient and downgradient area, individually (Chen et al. 2003). This indicates that significant microbial activity and intrinsic bioremediation occurred in this area. In addition, methane was also observed in sampled groundwater. Therefore, methanogenesis might be also occurring within the contaminated zone. Based on the above discussion, native microorganisms at this site are active which might contribute on the attenuation of contaminants.

MATERIALS AND METHODS

In order to assess the potential of MTBE biodegradation by native microorganisms under various site conditions, microcosm experiments were conducted to examine the feasibility of MTBE biodegradation under different redox conditions (aerobic, denitrifying, iron reducing, and methanogenic conditions). Aquifer sediments collected from the MTBE-contaminated site were applied as the source of microorganisms. Microcosm experiments were constructed with 20 g of aquifer sediments (SB2), 35 mL of in situ groundwater, and 50 mg/L of MTBE in 70 or 125-mL of serum bottles sealed with thick butyl rubber septa. Control bottles contained 250 mg/L of $HgCl_2$ and 500 mg/L of NaN_3, and inocula used for the control groups were autoclaved before use. In the aerobic microcosms, different initial dissolved oxygen concentrations (2 mg/L, 5 mg/L, and 8 mg/L of oxygen) were used to evaluation the effect of oxygen on MTBE biodegradation. Anaerobic microcosms were prepared in an anaerobic glovebox to preclude intrusion of oxygen. A redox indicator (0.0002% of resazurin), and 250 mg/L of L-cysteine and 250 mg/L of sodium sulfide were added to iron reductive and methanogenic microcosms as reducing agent, respectively. In the methanogenic microcosms, propane and ethanol were added to enhance the biodegradation rate of MTBE. In addition, 100 mg/L of NO_3-N and 0.2 M of Fe(III) (ferric iron) were applied to denitrifying and iron reducing microcosms, individually, to enhance denitrification and iron reduction. Table 1 lists the characteristics and components of each microcosm.

On day 0, 85 and 267, total bacterial DNAs from 1 g of soil samples were extracted with Wizard DNA clean-up kit (Promega, Madison, WI.) for detecting the community dynamics in the process of MTBE degradation. Bacterial 200-bp

Table 1. Characteristics and components of microcosms.

Microcosm & Control	Treatment	Inocula	Constituents
A1 & A1-C[1]	Aerobic biodegradation	aquifer sediments	sediments 20 g + in situ groundwater 35 mL + MTBE 50 mg/L with 8 mg/L initial concentration of DO and 80 mL headspace
A2 & A2-C	Aerobic biodegradation	aquifer sediments	sediments 20 g + in situ groundwater 35 mL + MTBE 50 mg/L with 5 mg/L initial concentration of DO
A3 & A3-C	Aerobic biodegradation	aquifer sediments	sediments 20 g + in situ groundwater 35 mL + MTBE 50 mg/L with 2 mg/L initial concentration of DO
D1 & D1-C	Denitrification	aquifer sediments	sediments 20 g + in situ groundwater 35 mL + MTBE 50 mg/L + NO_3-N 100 mg/L
I1 & I1-C	Iron reduction	aquifer sediments	sediments 20 g + in situ groundwater 35 mL + MTBE 50 mg/L + 0.2 M Fe(III)
M1 & M1-C	Methanogenesis	aquifer sediments	sediments 20 g + in situ groundwater 35 mL + MTBE 50 mg/L
M2 & M2-C	Methanogenesis	aquifer sediments	sediments 20 g + in situ groundwater 35 mL + MTBE 50 mg/L + propane 1 mL
M3 & M3-C	Methanogenesis	aquifer sediments	sediments 20 g + in situ groundwater 35 mL + MTBE 50 mg/L + ethanol 100 mg/L

[1]Control group, $HgCl_2$ + NaN_3 were added into each control microcosm.

fragments of 16S rDNA V3 region for subsequent denaturing gradient gel electrophoresis (DGGE) analysis were obtained with the primer combination of 341f with a GC clamp (40-nucleotide GC-rich sequence, 5-CCTACGGGAGGCAGCA G-3) and 534r (5-ATTACCGCGGCTGCTGG-3). The polymerase chain reaction (PCR) reacted mixtures contained 10 ng of DNA extract, 4 pmol of each primer, and 5 U of *Taq* polymerase (Takara, Shiga, Japan) in final concentrations of 2.5 mM of $MgCl_2$ and 0.12 mM of deoxyribonucleoside triphosphates in PCR buffer. The PCR amplification was conducted for 35 cycles: denaturation at 94℃ for 1 min, annealing at 55℃ for 1 min, and extension at 72 ℃ for 2 min. The equal concentration of each amplified PCR products (2,500 ng) was furthermore performed with DGGE using a Bio-Rad DCode system (Bio-Rad, Hercules, CA, USA), as described by the manufacturer. The 10% polyacrylamide gel with a 30-60% denaturant gradient was used and electrophoresis was performed at 60℃ and 70 V for 14 h. The gels were then stained with SybrGreen I and photographed. The relative intensity of amplified bands in gels was analyzed with Phoretix 1D software (Nonlinear Dynamics, Newcastle upon Tyne, NE1, UK). The PCR-amplified products were eletro-eluted from gel and then sequenced by provider (MdBio, Taipei, Taiwan). Those sequences were evaluated by using the basic local alignment search tool (BLAST) to determine the closest relatives in the GenBank databases (http://www.ncbi.nlm.nih.gov). Alignment of nucleotide sequences of PCR-amplified products generated a matrix of similarity coefficients with Neighbor-Joining method (Saitou and Nei, 1987). The dendrogram based on these similarity coefficients was plotted with UPGMA (unweighted pair-group method with arithmetic mean) method for clustering (Felsenstein, 1993).

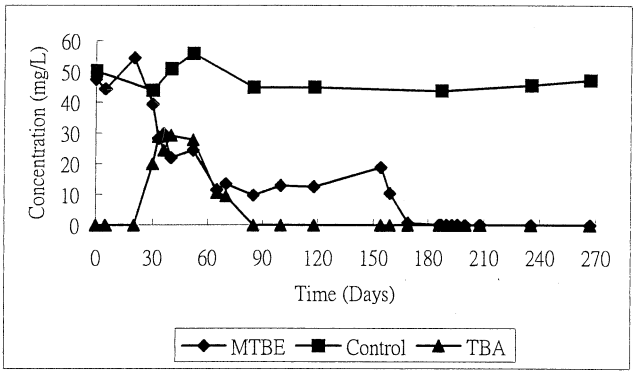

Figure 2. MTBE biodegradation results in Microcosms A1.

RESULTS AND DISCUSSION

Under aerobic conditions, a total of three groups (A1 to A3) of microcosms with different initial oxygen concentrations were prepared. The initial MTBE concentrations in microcosm bottles were approximately 50 mg/L. Figure 2 shows the MTBE biodegradation results in Microcosm A1. The concentration of MTBE dropped to 10.6 mg/L after 65 days of incubation with a 20-day lag period. However, no further MTBE biodegradation was observed from day 65 to day 154 in A1 and the measured oxygen concentrations were less than 1 mg/L during this period. Thus, microcosm bottles of A1 were reaerated on day 154. After the reaeration process, DO increased to 8 mg/L, and MTBE was biodegraded to less than 0.8 mg/L on day 168. The concentrations of MTBE were not further decreased until microcosm A1 was reaerated again on day 235. Complete MTBE depletion by indigenous microorganisms was observed after 267 days of incubation. The accumulation of TBA was also detected and was then biodegraded by the end of the experiment. Results from microcosm A1 reveal that oxygen might be the major limiting factor of MTBE biodegradation at this site. In addition, no MTBE removal was observed in microcosms A2 and A3 due to low initial oxygen concentrations (< 5 mg/L) in the microcosms (data not shown). This indicates that higher initial DO concentrations are required to activate the biodegradation mechanisms of MTBE. No MTBE removal was observed in any of the D1, I1, M1, M2, and M3 microcosms (data not shown). This reveals that MTBE could not be biodegraded under denitrifying, iron reductive, and methanogenic conditions during the 300-day incubation period with or without the supplement of primary substrates. Results from microcosm study indicate that aerobic biodegradation was the dominant process at this site and more studies need to be performed to further evaluate the anaerobic MTBE biodegradation for this site. Furthermore, field investigation revealed that there were small amount of residual DO, and significant concentrations of CO_2 and TBA in the monitoring wells. Based on the results of microcosm study and field investigation, aerobic biodegradation might be the major mechanism for the consumption of MTBE at this site.

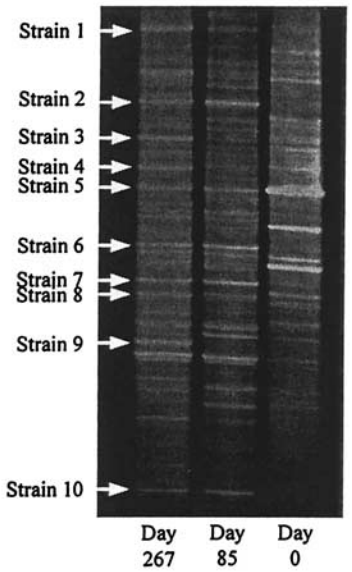

Figure 3. DGGE profiles of the PCR-amplified 16S rDNA for soils collected from microcosm A1 on days 0, 85, and 267.

PCR amplification of 16S rDNA and DGGE analysis were performed to determine the dominant microorganisms on MTBE biodegradation. Figure 3 shows the DGGE profiles of the PCR-amplified 16S rDNA for soils collected from microcosm A1 on days 0, 85, and 267. As shown in Figure 3, the profile of DGGE for detecting soil samples showed that 10 of microorganisms were predominant during the removal of MTBE. Table 2 shows the variations in the intensities of the 10 strains during the experiment. On day 0, both strains 2 and 6 were negligible but significantly increased on day 85. After 267 days of incubation, eight strains were increased (Figure 3). Results also show that four of those eight strains were indistinct at the beginning (strains 2, 6, 9, and 10).

Table 2. Variations in the intensities of the selected 10 strains versus time.

Strain	Intensity		
	Day 0	Day 83	Day 267
1	9600	8614	22064
2	0	33230	29204
3	38584	13239	34663
4	23565	7983	25230
5	70701	24139	14358
6	0	41139	24385
7	5530	24270	12582
8	14151	12259	15408
9	0	0	69931
10	0	8369	5663

Although strains 3 and 5 were decreased on day 267, their intensities were still noticeable during the experimental period. Overall, the intensities of all microorganisms were located within an intensive range except for strain 9 (highest intensity) and strain 10 (lowest intensity) during the incubation period (Table 2). Results indicate that the mixed bacterial consortia might be responsible for the biodegradation of MTBE in this study. Consequently, these dominant native microorganisms could be further isolated and enriched to enhance the efficiency of the bioremediation process. Moreover, strains 2, 5, 6, and 7 were the four most significant microbes appeared on day 85 when the MTBE dropped to 10 mg/L. Furthermore, strain 9 was the most significant microbe appeared on day 267 after the depletion of MTBE. These microorganisms might be employed as indicators to evaluate the occurrence and completion of the bioremediation process of MTBE at this contaminated site.

Table 3. Possibilities of bacterial species from isolates.

Strain	Microorganisms	Similarity (%)
1	*Acinetobacter sp.*	97
	Glacial ice bacterium	97
	Gamma proteobacterium	95
	Some unculturable or unidentified bacterial species	97
2	*Clostridium thermosuccinogene*	90
3	Some anaerobic strains	65
4	*Nocardiopsis sp.*	94
	Some unculturable or unidentified bacterial species	95
5	*Stenotrophomonas sp.*	98
	Pseudomonas sp.	97
	Xanthomonas sp.	97
	Some unculturable or unidentified bacterial species	97
6	*Nannocystis sp.*	93
	Agricultural soil bacterium clone	93
	Myxobacterium sp.	93
	Clostridium glycolicum	92
	Some unculturable or unidentified bacterial species	93
7	*Desulfovibrio sp*	95
	Mesorhizobium tianshanense	94
	Myxobacterium sp.	94
	Clostridium sp.	93
	Nocardioides sp.	93
	Some unculturable or unidentified bacterial species	93
8	Some unculturable or unidentified bacterial species	93
9	*Bacillus sp.*	93
	Chlorobium sp.	93
	Paenibacillus sp.	93
10	*Brachymonas sp.*	95
	Nocardiopsis sp.	94
	Acidovorax sp.	94
	Variovorax sp.	94
	Xylophilus sp.	94
	Aquaspirillum sp.	94
	Pseudomonas sp.	94
	Some unculturable or unidentified bacterial species	94

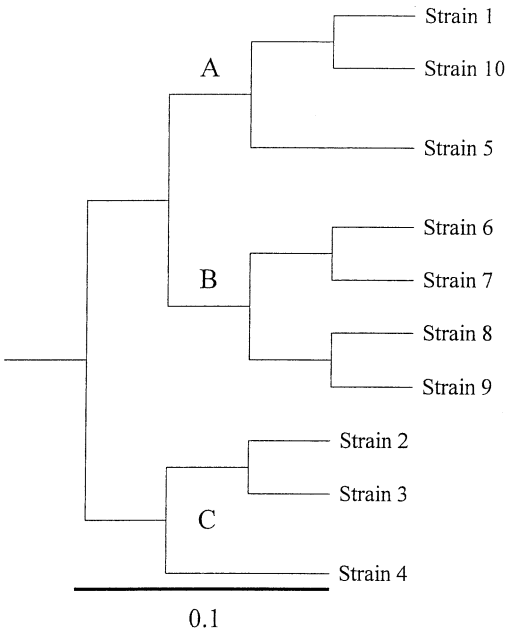

Figure 4. The UPGMA dendrogram for illustrating relationships among 10 of specific microorganisms.

The nucleotide sequences of 16S rDNA variable V3 regions obtained from PCR-amplified products of 10 specific microorganisms were compared with the database from GeneBank (Table 3). The strain 3 was only 65% of identities to some unidentified anaerobic bacteria, which published on GeneBank, comparing with nucleic acid databases. This indicates that a novel microorganism, which might be sensitive to MTBE and capable of degrading MTBE exists in this ecosystem. Using the similarity coefficients in 16S cDNA gene sequences, an UPGAM dendrogram allocated 10 specific microorganisms in this population into three separate phylogenetic clusters (Figure 4). *Pseudomonas sp.*, the possible species for strain 5 and strain 10, and *Xanthomonas sp.*, the possible species for strain 5, have been reported that they could biodegrade MTBE under aerobic conditions (Prince 2000; Fiorenza and Rifai 2003). However, the biodegradability on MTBE for other microorganisms listed in Table 3 has not been reported. If some microbes have similar genetic background in DNA and are closely related to some clusters, there is a possibility that they could utilize the same xenobiotic compounds.

In this study, MTBE biodegradability under different redox conditions with the addition of various carbon sources were evaluated to determine the dominant biodegradation process and native microorganisms at this site. Results indicate that aerobic biodegradation was the dominant degradation process and aerobic bioremediation might be a more appropriate option for the site remediation.

MTBE biodegradation under anaerobic conditions at this site is uncertain and needs to be further evaluated. According to the results from GeneBank, two microorganisms, *Pseudomonas sp.* and *Xanthomonas sp.*, which can biodegrade MTBE under aerobic conditions, might exist at this site. Results also reveal that DGGE and nucleotide sequence techniques provide a guide for further microbial isolation and identification. Results from this study provide us insight into the characteristics of intrinsic biodegradation of MTBE. Knowledge and comprehension obtained in this study will be helpful in designing a practical system for the bioremediation of MTBE-contaminated site.

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