Journal of Hazardous Materials 198 (2011) 323–330

Contents lists available at SciVerse ScienceDirect

Journal of Hazardous Materials

journal homepage: www.elsevier.com/locate/jhazmat

# Use of specific gene analysis to assess the effectiveness of surfactant-enhanced trichloroethylene cometabolism

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ARTICLE INFO

*Article history:*
Received 2 May 2011
Received in revised form 9 October 2011
Accepted 17 October 2011
Available online 20 October 2011

*Keywords:*
Aerobic cometabolism
Groundwater contamination
TCE-degrading enzyme
Trichloroethylene

ABSTRACT

The objective of this study was to evaluate the effectiveness of in situ bioremediation of trichloroethylene (TCE)-contaminated groundwater using specific gene analyses under the following conditions: (1) pretreatment with biodegradable surfactants [Simple Green™ (SG) and soya lecithin (SL)] to enhance TCE desorption and dissolution, and (2) supplementation with SG, SL, and cane molasses as primary substrates to enhance the aerobic cometabolism of TCE. Polymerase chain reaction (PCR), denaturing gradient gel electrophoresis (DGGE), and nucleotide sequence analysis were applied to monitor the variations in specific activity-dependent enzymes and dominant microorganisms. Results show that TCE-degrading enzymes, including toluene monooxygenase, toluene dioxygenase, and phenol monooxygenase, were identified from sediment samples collected from a TCE-spill site. Results from the microcosm study show that addition of SG, SL, or cane molasses can enhance the aerobic cometabolism of TCE. The TCE degradation rates were highest in microcosms with added SL, the second highest in microcosms containing SG, and lowest in microcosms containing cane molasses. This indicates that SG and SL can serve as TCE dissolution agents and act as primary substrates for indigenous microorganisms. Four dominant microorganisms (*Rhodobacter* sp., *Methyloversatilis* sp., *Beta proteobacterium* sp., and *Hydrogenophaga pseudoflava*) observed in microcosms might be able to produce TCE-degrading enzymes for TCE cometabolic processes.



## 1. Introduction

Groundwater at many existing and former industrial sites and disposal areas is contaminated by halogenated organic compounds that were released into the environment. Chloroethenes are ubiquitous pollutants found at many priority cleanup sites, and they are known or suspected carcinogens [1–3]. Among the various chloroethenes, trichloroethylene (TCE) is among the most ubiquitous chlorinated compounds found in groundwater contamination. One cost-effective approach for the remediation of contaminated aquifers that is attracting increased attention is the application of enhanced bioremediation for contaminant degradation. Because the biodegradation of TCE and its daughter compounds [e.g., dichloroethenes (DCEs) and vinyl chloride (VC)] is generally more efficient under aerobic conditions, introduction of dissolved oxygen (DO) into the TCE plume will increase the TCE biodegradation (cometabolism) rate and significantly reduce the TCE mass flux if sufficient bioavailable primary substrates are present [3–6]. Although microbial cultures are not able to utilize TCE as a growth substrate under aerobic conditions, some aerobic bacterial cultures containing oxygenase enzymes (e.g., methane, toluene, phenol, propane and ammonia oxidizers) could degrade TCE cometabolically [7–10]. The induced enzymes are able to create enzyme active sites to catalyze the degradation of non-growth substrates such as TCE [3]. Several aerobic microorganisms or microbial communities have the ability to synthesize oxygenase enzymes [e.g., toluene oxygenase, phenol oxygenase, particulate methane monooxygenase (pMMO), toluene dioxygenase] that catalyze the initial step in the oxidation of their respective primary or growth substrates, and these enzymes have the potential to initiate the oxidation of TCE and other chlorinated aliphatic hydrocarbons [11–14]. These groups of aerobic bacteria include oxidizers of the following compounds: methane, propane, ethylene, toluene, phenol, acetic acid, propionic acid, cresol, ammonia, and isoprene [7,10]. Among these bacteria, methane oxidizers, phenol degraders, and toluene degraders are the main bacteria that are able to perform the aerobic cometabolic process of TCE [6,7].

Based on the above discussion, in situ aerobic bioremediation is a feasible technology to clean up TCE-contaminated sites if oxygen and biodegradable primary substrates can be provided efficiently to the subsurface. Cane molasses is waste from the sugar industry. It is a good candidate for use as a primary substrate because it is

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doi:10.1016/j.jhazmat.2011.10.050

relatively inexpensive and rich in bioavailable carbon [12]. Surfactants can increase the solubility of TCE by partitioning it into the hydrophobic cores of surfactant micelles to levels above the critical micelle concentration (CMC). Thus, surfactants are able to improve the mass-transfer of TCE from the solid to aqueous phases and, consequently, the microbial remediation of TCE in groundwater [15–18]. Surfactants are useful for the biodegradation of contaminates because they are able to enhance desorption and increase the solubility of hydrophobic compounds. Some studies have been conducted to enhance the biodegradation of TCE by employing surfactants in contaminated soil and groundwater [19,20]. However, little information is available concerning the effects of surfactants on the enhancement of aerobic TCE cometabolism and the impacts of surfactant application on the subsurface environment. Soya lecithin (SL), a rhizosphere phytogenic and nonionic surfactant that consists of a mixture of phospholipids and quillaya saponin, has been studied in the aerobic biodegradation of phenanthrene and fluoranthene in shake batch cultures of three polycyclic aromatic hydrocarbon (PAH)-degrading bacteria [21,22]. SL was found to have higher PAH and polychlorinated biphenyl (PCB)-solubilizing activity and a lower bacterial toxicity [23,24]. Simple Green (SG), a nonionic surfactant, can be used as a soil washing agent and applied in non-aqueous phase liquid (NAPL) flushing [25,26]. In the current study, the feasibility of using SL and SG for the enhancement of aerobic TCE cometabolism was evaluated.

Recently, molecular biology has been used in site remediation studies to confirm the effectiveness of the bioremediation and identify the bacterial species that are critical for the biodegradation of contaminants of concern [27,28]. Results from other studies reveal that polymerase chain reaction (PCR), denaturing gradient gel electrophoresis (DGGE), and nucleotide sequence analysis techniques provide a direct evaluation of bacteria that actively contribute to contaminant biodegradation. In addition, these techniques can be used to understand the microbial ecology at the site, which can be used to identify trends in the biodegradation process [12,27]. The main objectives of this study were the following: (1) to develop a specific gene analytical method to confirm the existence of TCE-degrading enzymes at a TCE-contaminated site, (2) to evaluate the effectiveness of aerobic cometabolism of TCE-contaminated groundwater using specific gene analyses in the presence of cane molasses and biodegradable surfactants (SG and SL) for the enhancement of TCE desorption and dissolution in a microcosm study, and (3) to determine the dominant microorganisms responsible for aerobic cometabolism of TCE in microcosms using a series of molecular biology techniques, including DNA extraction, PCR amplification, DGGE, and DNA analysis. Total bacterial DNAs and DNA amplification were used to verify the presence of phenol, methane, and toluene-degrading enzymes in microcosms and field soil samples that had been subjected to TCE degradation. PCR, DGGE, and nucleotide sequence analysis were applied to monitor the variations in activity-dependent microbial diversity and dominant native microorganisms.

## 2. Site description

An industrial park site located in southern Taiwan was chosen as the site at which to perform the present study. At this site, a TCE storage tank had leaked and contaminated the groundwater with TCE. Soil and groundwater samples from monitor wells were collected and analyzed to determine the local hydrogeology and delineate the TCE plume during a previous site investigation study. Site investigation results showed that the components of the site soils are consistent with a sandy loam texture (48% sand, 43% silt, and 9% clay). The water table is generally found at depths ranging from 6 to 7 m below ground surface. The site groundwater flows to the northeast at a velocity of 7.2 cm/day and with a hydraulic conductivity of 0.006 cm/s. Fig. 1 presents the site map that shows the groundwater flow direction, estimated plume boundary, and one groundwater and four soil sampling locations chosen for this study.

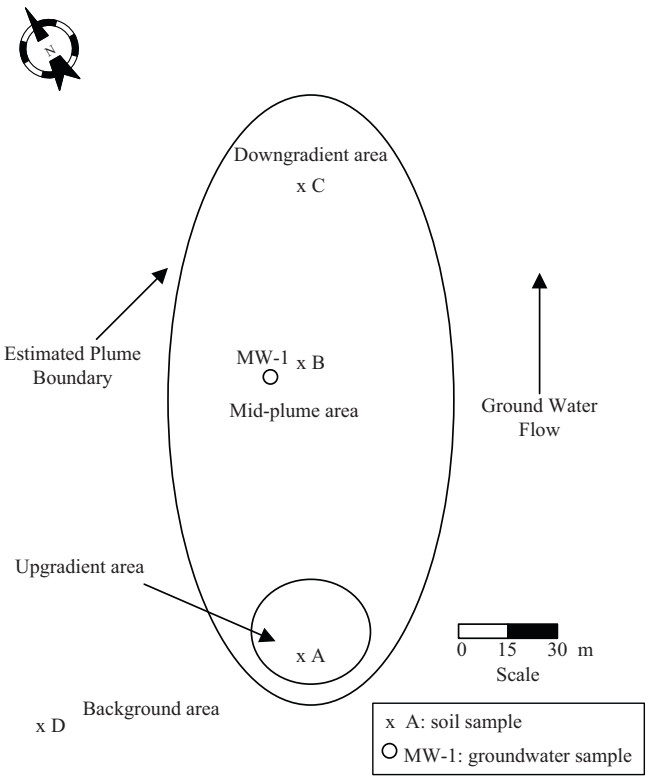


**Fig. 1.** Site map showing the groundwater flow direction, estimated plume boundary, and one groundwater and four soil sampling locations chosen for this study.

## 3. Materials and methods

### 3.1. Chemicals

All chemicals used in this study were analytical grade and purchased from Wako Chemical (Kyoto, Japan). The TCE was purchased from Fisher Chemical (Fair Lawn, NJ) (99.99%) and used as received. This study employed the nonionic surfactants SL and SG, which were supplied by Prodotti Gianni Spa (Italy) and Sunshine Makers, Inc. (USA). Ninety-six percent (w/w) of SL (CENTROLEX-E322) is made up of phospholipids, of which 73% are pure phospholipids (23% phosphatidylcholine, 20% phosphatidylethanolamine, 14% phosphatidylinositol, 8% phosphatide acid, and 8% other phospholipids), 15% glycolipids, 8% carbohydrates, and 3% neutral lipids. SG has an average molecular weight of 106 and a molecular formula of $HOCH_2H_2O$-$(CH_2)_3CH_3$. Cane molasses was purchased from Taiwan Sugar Corp., Ltd. (Taiwan) and contained 78.6 g/L of glucose, 79.7 g/L of fructose and galactose, 170.9 g/L of sucrose, 10.2 g/L of raffinose, and 339.4 g/L of fermentable sugar.

### 3.2. Collection of sediment and groundwater samples

Aquifer sediments (collected from locations A to D) and groundwater (collected from monitor well MW-1) were collected from the TCE-spill site, and it was analyzed for the presence of specific TCE-degrading genes and for groundwater quality (Fig. 1). Groundwater samples were analyzed for TCE and geochemical indicators including pH, oxidation–reduction potential (ORP), and DO. TCE

analysis was performed in accordance with U.S. EPA Method 602, using a Tekmer Purge-and-Trap Model LSC 2000 with a Perkin-Elmer Model 9000 Auto System Gas Chromatograph (GC). DO, ORP, and pH were measured in the field. An Accumet 1003 pH/Eh meter (Fisher Scientific) was used for pH and Eh measurements; an Orion DO meter (Model 840) was used for DO measurements. Analytical procedures of the site groundwater are described in Standard Methods [29].

### 3.3. Microcosm study

Microcosm experiments were conducted to examine the feasibility of TCE biodegradation in the presence of surfactants and cane molasses under aerobic cometabolic conditions. Each microcosm was constructed with 100 mL of site groundwater, 10 g of aquifer sediments, 5 mL of TCE solution, and 0.1 g of surfactant or cane molasses in a 160-mL bottle sealed with Teflon-lined rubber septa. Groundwater and sediment samples were collected from MW-1 and Location B in the mid-plume area, respectively. The collected groundwater and sediment samples were purged before use to remove volatile organic compounds. The initial TCE concentration in each microcosm bottle was approximately 2.7 mg/L. Five groups of microcosms (kill control, live control, SG, SL, and cane molasses) were constructed. Control microcosms contained 250 mg/L $HgCl_2$ and 500 mg/L $NaN_3$. Groundwater and aquifer sediments used for the kill control microcosms were autoclaved before use. No primary substrate was added in the live control microcosms. Aerobic conditions were maintained by purging the groundwater solution and bottles with air during the preparation. Triplicates of the microcosms were scarified at each time point during the analyses. Water samples were periodically collected and analyzed for TCE, total viable bacteria, DO, pH, ORP, and chemical oxygen demand (COD). Results were recorded as TCE concentration or total bacteria versus time. The spread-plate technique was used for microbial enumeration (total viable bacteria) following the procedures described in Standard Methods [29]. COD measurements were used to evaluate the variations in primary substrate (e.g., SL, SG, or cane molasses) concentrations and find out if the supplied substrates are sufficient to enhance the aerobic cometabolism. COD measurement was conducted in accordance with the dichromate reflux method described in Standard Methods [29].

### 3.4. DNA extraction, PCR amplification, and TCE-degrading gene analyses

Sediment samples collected from four different locations (labeled as A to D) from the TCE-spill site were analyzed for the specific genes of TCE-degrading enzymes. Locations A to D were located in an upgradient area, mid-plume area, downgradient area, and background area, respectively (Fig. 1). DNA extraction for each sediment sample was performed using a Power Soil™ extraction kit (MoBiol, USA) for the detection of specific TCE-degrading enzymes. In the microcosm study, 1 g of sediments was also extracted with a Power Soil™ extraction kit (MoBiol, USA) for the detection of dominant microbial species and specific TCE-degrading enzymes after 30 days of operation. The PCR primers for aromatic oxygenase genes and PCR protocols were described by Baldwin et al. [30]. The individual primer sets were allowed to amplify the fragments of toluene dioxygenase, toluene monooxygenase, pMMO, and phenol hydroxylase [7,14,31]. These primer sets are listed in Table 1. Annealing temperatures, the amounts of DNA, and the concentrations of $MgCl_2$ and primers were optimized based on the protocols described by Baldwin et al. [30]. Conventional PCR was performed with the following temperature program: (1) 10 min at 95 °C; (2) 30 cycles with 1 cycle consisting of 1 min at 95 °C, 1 min at the optimum annealing temperature, and 2 min at 72 °C; and (3) a final extension step of 10 min at 72 °C. PCR products were routinely visualized by running 10 μL of the PCR mixture on 1% agarose gels in 1× Tris–acetate–EDTA (TAE) buffer stained with ethidium bromide. Reproducibility was confirmed by performing PCR at least three times with positive control DNA.

### 3.5. DGGE analyses

Total bacterial DNA from 1 g of collected sediment samples was extracted with a Power Soil™ extraction kit (MoBiol, USA). Before DGGE analysis, the V3 region of 16S rDNA was amplified with the primer sets (341f, forward: 5′-CCTACGGGAGGCAGCAG-3′ containing a GC clamp of 40-nucleotide GC-rich sequence; 534r, reversed: 5′-ATTACCGCGGCTGCTGG-3′). DGGE was performed with D-Code™ universal mutation detection system (BioRad Lab., USA). PCR products were loaded on 8% (w/v) polyacrylamide gels with a denaturing gradient of 30–60% (100% denaturant based on 7 M urea plus 40% formamide in 1× TAE buffer) and were run for 7 h at 150 V and at 60 °C in 1× TAE buffer (pH 7.4) [31]. After electrophoresis, the gels were stained with SYBE Safe™ (Invitrogen, UK) and then were put in a shaker at 150 rpm for 10 min, followed by excision of the bands from the gel with a pipette tip under UV illumination. The excised bands were directly placed in 1.5 mL microcentrifugation tubes containing 50 μL ultrapure DNase/RNase-free sterile water and incubated at 4 °C for overnight to elute DNA. Ten microliters of the supernatants was used as a template for the re-amplification (as described above). The resulting amplicons were again electrophoresed on a DGGE gel to verify the position of the original band. Subsequently, the amplicons were purified using the Gel/PCR DNA fragments extraction kit (Geneaid, Taiwan). The PCR products were then cloned into pGEMR – T Vector System (Promega, USA). Clones were screened according to the manufacturer's instructions. Recombinant plasmids were isolated from overnight cultures by alkaline lysis, and the inserts were detected by PCR amplification with a M13 primer set. Subsequently, the 16S rDNA gene fragments were sequenced by Mission Biotech. Co., Taiwan. All sequences were compared to those in the GenBank database (http://www.ncbi.nlm.nih.gov).

## 4. Results and discussion

Figs. 2 and 3 present the variations in total viable bacterial counts and TCE concentrations versus time in five groups of microcosms, respectively. Table 2 presents the observed DO, pH, ORP, and COD values on days 0, 15, and 30 for all five microcosms. Increases in microbial populations correspond to decreases in TCE concentrations during the operational period in microcosm groups SL, SG, cane molasses, and a live control. In the live control

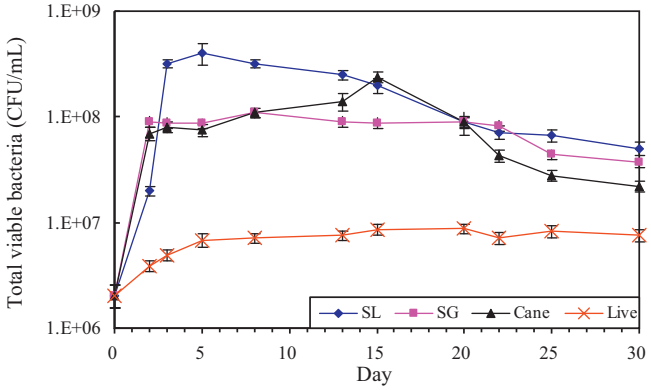


**Fig. 2.** Total bacterial counts in various microcosm experiments with SG, SL, cane molasses and live control.

**Table 1**
Primer sets of four TCE-degrading enzymes.

| Primer[a] | Target | Sequence | Reference |
|---|---|---|---|
| PHE-F<br>PHE-R | Phenol monooxygenase | F: 5′-GTGCTGAC(C/G)AA(C/T)CTG(C/T)TGTTC<br>R: 5′-CGCCAGAACCA(C/T)TT(A/G)TC | Lee et al. [7] |
| RMO-F<br>RMO-R | Toluene monooxygenase | F: 5′-TCTC(A/C/G)AGCAT(C/T)CAGAC(A/C/G)GACG<br>R: 5′-TT(G/T)TCGATGAT(C/G/T)AC(A/G)TCCCA | Hazen et al. [14] |
| A-189<br>A-682 | Particulate methane monooxygenase | F: 5′-GGNGACTGGGACTTCTGG<br>R: 5′-GAASGCNGAGAAGAASGC | Erwin et al. [31] |
| TOD-F<br>TOD-R | Toluene dioxygenase | F: 5′-TGAGGCTGAAACTTTACGTAGA<br>R: 5′-CTCACCTGGAGTTGCGTAC | Lee et al. [7] |

[a] Forward (-F) and reverse (-R) primers are indicated.

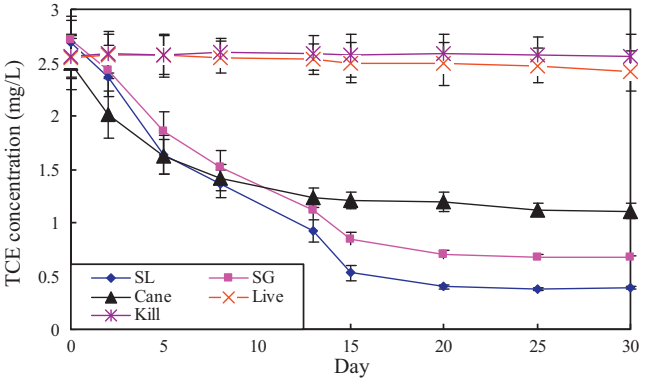


**Fig. 3.** Aerobic cometabolism TCE biodegradation with the addition of various carbon sources (SL, SG, cane molasses, and TCE only).

microcosms, the total bacterial counts slightly increased from $2.05 \times 10^6$ CFU/mL on day 0 to $8.2 \times 10^6$ CFU/mL on day 30. Compared to the live control microcosms, more significant increases in microbial populations (one to two orders of magnitude higher) were observed in surfactant and substrate addition groups (SL, SG, and cane molasses groups) during the operational period (Fig. 2). The total bacterial counts reached the highest microbial populations after approximately 5 days. This indicates that surfactants (SG and SL) and cane molasses could be served as carbon and energy sources by indigenous bacteria, which caused a significant increase in their populations during the early incubation period (0–5 days). Although SC and SL are surfactants, they are biodegradable and can be directed used by indigenous heterotrophic microorganisms as carbon and energy sources and also enhance the aerobic cometabolic mechanisms for TCE biodegradation. This surfactant-enhanced process provides a more thorough TCE remediation due to the increased contact between TCE and TCE-degrading enzymes. The results also indicate that both SG and SL are biodegradable and are not microbial inhibitors, and thus, they would not have an ecological adverse impact after use. However, the growth of microbial populations remained almost stagnant after 5 days of incubation and slight reductions in microbial populations was observed in SL, SG, and cane molasses groups after approximately 20 days of operation. This could be due to the fact the microbial growth reached the stationary phase after 5 days of incubation, and thus, the growth curve was leveled off after 5 days. Moreover, the drop in pH and DO concentrations (Table 2) also inhibited the growth of aerobic bacteria. Due to the existence of abundant carbon sources in SL, SG, and cane molasses microcosms, anoxic bacteria became predominant after the significant drops of DO and ORP in microcosms. Thus, high microbial populations were still observed although DO and ORP reached the anoxic levels after 15 days.

Although the initial TCE concentrations in microcosm bottles were prepared to be 2.7 mg/L, lower TCE concentrations were observed in control and cane molasses microcosms (Fig. 3). This might be due to the sorption of TCE onto soil particles after TCE addition. Compared to the control and cane molasses microcosms, slightly higher TCE concentrations were observed in SL and SG microcosms. This indicates that the addition of biodegradable surfactants increased the dissolution of TCE in the microcosms. Thus, the bioremediation efficiency was enhanced because of the increased TCE concentration in the water phase. In live control microcosms, TCE concentrations dropped from approximately 2.54 to 2.42 mg/L after 30 days of incubation. This reveals that intrinsic TCE attenuation was insignificant if a primary substrate was not supplied. In SL microcosms, up to 86% of TCE was removed (a

**Table 2**
Calculated TCE degradation rates and pH, DO, ORP, and COD after 15 and 30 days of operation.

| Microcosm | Day 0 pH | Day 15 pH | Day 30 pH | Day 0 DO (mg/L) | Day 15 DO (mg/L) | Day 30 DO (mg/L) | Day 0 ORP (mV) | Day 15 ORP (mV) | Day 30 ORP (mV) |
|---|---|---|---|---|---|---|---|---|---|
| SL | 7.41 ± 0.12[a] | 5.91 ± 0.32 | 5.61 ± 0.30 | 6.45 ± 0.25 | 0.35 ± 0.14 | 0.35 ± 0.17 | 428 ± 33 | −58 ± 31 | −87 ± 45 |
| SG | 7.42 ± 0.11 | 6.03 ± 0.27 | 5.86 ± 0.28 | 6.40 ± 0.32 | 0.22 ± 0.16 | 0.22 ± 0.22 | 415 ± 27 | −35 ± 26 | −59 ± 39 |
| Cane molasses | 7.37 ± 0.15 | 5.52 ± 0.18 | 5.17 ± 0.31 | 6.31 ± 0.14 | 0.19 ± 0.22 | 0.19 ± 0.28 | 402 ± 24 | −83 ± 38 | −103 ± 48 |
| Live control | 7.43 ± 0.13 | 7.41 ± 0.24 | 7.21 ± 0.22 | 6.47 ± 0.18 | 5.35 ± 0.11 | 5.11 ± 0.21 | 429 ± 19 | 230 ± 19 | 213 ± 33 |
| Kill control | 7.44 ± 0.10 | 7.45 ± 0.14 | 7.37 ± 0.19 | 6.45 ± 0.20 | 6.42 ± 0.09 | 6.23 ± 0.15 | 424 ± 21 | 418 ± 13 | 389 ± 26 |

| Microcosm | Day 0 COD (mg/L) | Day 15 COD (mg/L) | Day 30 COD (mg/L) | Degradation rate (mg/day/g)[b] |
|---|---|---|---|---|
| SL | 2520 ± 67 | 2374 ± 89 | 2259 ± 105 | 0.144 |
| SG | 276 ± 19 | 162 ± 26 | 87 ± 23 | 0.124 |
| Cane molasses | 965 ± 26 | 782 ± 32 | 649 ± 36 | 0.087 |
| Live control | 8.4 ± 0.6 | 6.2 ± 0.8 | 5.7 ± 0.9 | –[c] |
| Kill control | 8.1 ± 0.7 | 7.5 ± 0.9 | 7.1 ± 0.7 | – |

[a] Mean ± standard deviation.
[b] (Initial concentration – final concentration)/reaction period/g of substrate (reaction period: from day 0 to day 15).
[c] Not available.

decrease from 2.69 to 0.39 mg/L) after 30 days of operation without a lag period. Similar results were observed in SG microcosms. In SG microcosms, TCE concentrations dropped from 2.71 to 0.67 mg/L after 30 days of incubation without any delay. This indicates that both SG and SL can act as primary substrates for indigenous bacteria and induce TCE-degrading enzymes to remove TCE. However, TCE removal leveled off after approximately 15 days of incubation in SL, SG, and cane molasses groups.

The measured DO, pH, and ORP for site groundwater in MW-1 were approximately 1.2 mg/L, 7.3, and 119 mv, respectively (Table 2). After groundwater purging, DO and ORP increased from 1.2 mg/L and 119 mv to approximately 7.4 mg/L and 418 mv; these values were used as the initial DO and ORP values on day 0. Results show that significant decreases in DO, ORP, and pH values in both SG and SL microcosms were observed on days 15 and 30 (Table 2). This indicates that the anoxic conditions in both microcosms did not favor the aerobic cometabolic mechanisms. The pH drop would also inhibit the activity of the TCE-degrading enzymes and decrease the efficiency of TCE removal. In cane molasses microcosms, TCE concentrations dropped from approximately 2.5 to 1.1 mg/L after 30 days of operation (Fig. 3). However, no further TCE biodegradation was observed after 15 days of incubation. As in SL and SG microcosms, significant decreases in DO, ORP, and pH values in cane molasses microcosms were observed on days 15 and 30 (Table 2). This reveals that anoxic and acidic conditions caused the inhibition of TCE-degrading enzymes in cane molasses microcosms. Because each microcosm was operated in a sealed bottle, oxygen was supplied in the headspace, and no additional oxygen was applied during the operation. Thus, microcosms became anoxic after oxygen was depleted. In the field application, oxygen can be supplied through the biosparging or air sparging wells. Thus, oxygen will not become a limiting factor in the practical application. Moreover, acid was produced during the biodegradation processes and caused the solutions to acidify. This caused the pH to drop from the initial value of 7.4 to the final values of 5.61, 5.86, and 5.17 in SL, SG, and cane molasses microcosms, respectively. In practical field applications, soil and groundwater systems have a natural buffer and dilution capacity, so significant decreases in pH value would not be observed in field remediation. Furthermore, biospraging wells can be installed in the treatment zone to supplement oxygen and maintain aerobic conditions for aerobic cometabolism to occur. Thus, a significant drop in DO in the treatment zone can be prevented. No significant TCE removal was observed in either live and kill control microcosms. This indicates that the supplementation of primary carbon and energy sources (SG, SL, and cane molasses) was necessary to enhance the aerobic TCE cometabolism, and TCE could not be used as the growth substrate by microorganisms. The slight TCE decreases in live and kill control microcosms might be due to volatilization and sorption mechanisms.

Results from COD analyses show that the initial COD in SL, SG, and cane molasses microcosms were 2520, 276, and 965 mg/L, respectively. This indicates that significant amounts of COD could be released from those substrates to activate the aerobic cometabolic process of TCE. After 15 days of operation, approximately 5.8, 41.3, and 19% of COD drops were observed in SL, SG, and cane molasses microcosms, respectively. Approximately 146, 114, and 183 mg/L of COD were removed in SL, SG, and cane molasses microcosms, respectively, which caused 2.16, 1.86, and 1.19 mg/L of TCE removal during the 15-day incubation period. Thus, it would require 1 mg of COD of SL, SG, and cane molasses to cometabolize 0.015, 0.016, and 0.007 mg of TCE, respectively. Results also indicate that 1 g of SL, SG, and cane molasses has the potential to cometabolize 373, 45, and 68 mg of TCE, respectively. Results reveal that the degradation of the released organic materials from the substrates caused the decreases in COD measurements and TCE cometabolism.

In this microcosm study, TCE degradation rates are presented in Table 2. The degradation rate indicates the total TCE removed during the reaction period enhanced by the certain amounts of added substrate (0.1 g for SL, SG, or cane molasses). Because the TCE degradation trend leveled off after 15 days of incubation, the reaction period used in the degradation rate calculation was from day 0 to day 15. The calculated TCE degradation rates for SL, SG, and cane molasses were 0.15, 0.13, and 0.09 mg/day/g of substrate, respectively. The calculated TCE degradation rates were in the order of SG > SL > cane molasses. Results show that the TCE degradation rates in SG and SL groups were higher than the rate in the cane molasses group. Although cane molasses has been used as the primary substrate to enhance TCE cometabolism in other studies [12,16], higher TCE removal rates were observed when SG and SL were used as substrates. This indicates that SG and SL can be used and can replace cane molasses as the primary substrate for specific microorganisms that synthesize oxygenase enzymes necessary to cometabolize TCE. Moreover, the addition of primary substrates (cane molasses, SG, and SL) caused an increase in microbial populations in microcosms and enhanced the TCE aerobic cometabolism.

Results from this study also indicate that the supplied surfactants (SG and SL) were able to desorb and increase the solubility of TCE in microcosms. This phenomenon would enhance the TCE solubility and subsequent TCE cometabolism due to increased contact between TCE and TCE-degrading enzymes. Results from Fig. 3 indicate that the initial TCE concentrations increased from 2.5 mg/L in cane molasses microcosm to 2.69 and 2.71 mg/L in SL and SG microcosms, respectively. This indicates that the addition of 0.1 g of SL and SG caused dissolution of 0.019 and 0.021 mg of TCE in solution, respectively. Thus, the TCE dissolution ratios are 0.19 and 0.21 mg when 1 g of SL and SG are applied to enhance TCE desorption and dissolution. Thus, the application of biodegradable surfactants (SG and SL) would result in a more complete and thorough removal of TCE.

In situ anaerobic bioremediation has been proposed for the remediation of a variety of groundwater contaminants [32,33]. Soybean oil and other food-grade edible oils can provide an effective, long-lasting substrate for enhancing anaerobic biodegradation processes. Oil-in-water emulsions can be prepared using only food-grade materials, which makes regulatory approval more likely [34]. Thus, the biodegradable surfactants SL and SG can be used for the design of an in situ biobarrier system for practical applications. SL and SG, which are food-grade emulsifiers, could be applied to generate stable soybean oil-in-water emulsions. Moreover, SL and SG are soluble compounds, and they can be biodegraded effectively. Thus, they can create an anaerobic environment after injection if oxygen is not supplied. This indicates that SL and SG might also be applied to enhance the in situ reductive dechlorination of some chlorinated compounds (e.g., tetrachloroethylene) that cannot be biodegraded under aerobic cometabolic conditions.

Fig. 4 presents the gel showing the PCR-amplified fragments (206 bp) produced using the PHE primer on DNA extracted from the sediments of the TCE-spill site. Results indicate that all four sediments (A: upgradient area, B: mid-plume area, C: downgradient area, and D: background area) contained phenol monooxygenase, which was able to cause the degradation of TCE. Fig. 5 presents the gel showing the PCR-amplified fragments (466 bp) produced using the RMO primer on DNA extracted from the sediments of the TCE-spill site. Results indicate that only two sediments (A and B) contained toluene monooxygenase. This indicates that toluene monooxygenase was only detected in areas contaminated with higher TCE concentrations (>100 μg/L). Results also indicate that the toluene monooxygenase was not evenly distributed in the entire site. Variations in environmental conditions (e.g., substrate concentration, contaminant concentration, oxidation–reduction trend) would cause the change in the appearance of specific

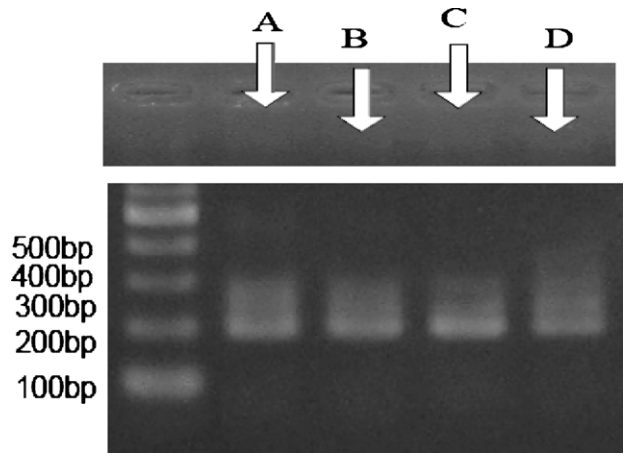


**Fig. 4.** Gel showing the PCR-amplified fragments (206 bp) produced using the PHE primer on DNA extracted from the sediments of the TCE-spill site. This analysis detects the presence of phenol monooxygenase (A: upgradient area, B: mid-plume area, C: downgradient area, and D: background area).

TCE-degrading enzymes. Toluene dioxygenase was analyzed for the produced PCR-amplified fragments (757 bp) using the TOD primer on DNA extracted from the sediments. Results indicate that none of the four sediments (A to D) contained significant amounts of toluene dioxygenase (data not shown). This indicates that toluene dioxygenase was not significant at this TCE-spill site. This might be due to the fact that site sediments contained fewer microbial populations that could produce toluene dioxygenase. With the supplement of primary substrates, the production of specific microbial species and their associated TCE-degrading enzymes might occur. The pMMO was analyzed for the produced PCR-amplified fragments (525 bp) using the A primer on DNA extracted from the sediments. Results indicate that none of the four sediments (A to D) contained significant amounts of pMMO (data not shown). Jiang et al. [35] reported that the production of pMMO was positively related to methane concentrations. Because the site groundwater was under aerobic conditions, no methane was detected in groundwater samples (data not shown), which caused insignificant pMMO production at this site. Because the detected phenol monooxygenase and toluene monooxygenase are able to activate the aerobic cometabolism of TCE, the observed phenol monooxygenase and toluene monooxygenase at this site imply that in situ enhanced bioremediation is a feasible technology for site groundwater remediation.

Table 3 presents the results of specific TCE-degrading enzyme analyses for all five microcosms after 30 days of incubation. Results show that phenol monooxygenase, toluene monooxygenase, and toluene dioxygenase genes were detected in SL and SG microcosms.

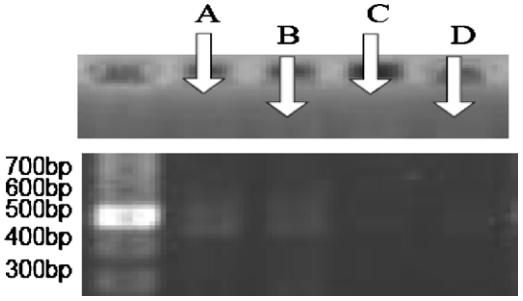


**Fig. 5.** Gel showing the PCR-amplified fragments (466 bp) produced using the RMO primer on DNA extracted from the sediments of the TCE-spill site. This analysis detects the presence of toluene monooxygenase (A: upgradient area, B: mid-plume area, C: downgradient area, and D: background area).

**Table 3**
The detected TCE-degrading enzymes (phenol monooxygenase, toluene monooxygenase, particulate methane monooxygenase, and toluene dioxygenase) in four aerobic microcosms after 30 days of operation.

| | Live control | SL | SG | Cane molasses |
|---|---|---|---|---|
| Phenol monooxygenase | +[a] | + | + | + |
| Toluene monooxygenase | + | + | + | – |
| Particulate methane monooxygenase | –[b] | – | – | – |
| Toluene dioxygenase | – | + | + | + |

[a] Detected.
[b] Not detected.

However, only phenol monooxygenase and toluene dioxygenase genes were detected in cane molasses microcosms. No significant genes of TCE-degrading enzymes were detected in live and kill control microcosms. The pMMO enzyme was not detected in any of the five groups of microcosms. Although toluene dioxygenase was not observed in the field samples, it was still observed in microcosms with SG, SL, or cane molasses addition. Moreover, toluene monooxygenase was not observed in the cane molasses microcosm, although it was observed in field sediment samples. Results indicate the detection of TCE-degrading enzymes corresponded with significant decreases in TCE concentrations. The specific aerobic microbial communities were able to degrade primary substrates (SG, SL, and cane molasses) and synthesize TCE-degrading enzymes for subsequent TCE cometabolism. Results also indicate that the enhanced bioremediation process would alter the dominant microorganisms in the ecosystem, which would cause the variations in produced enzymes.

PCR amplification of 16S rDNA and DGGE analyses were performed to determine the variations in microbial community patterns and in the dominant microorganisms involved in TCE degradation in the microcosm study. Fig. 6 shows the DGGE profiles of the PCR-amplified 16S rDNA for soils and groundwater mixture collected from Location B and four microcosms (1: Location B; 2: live control; 3: SL; 4: SG; 5: cane molasses) on day 30. Results indicate that significant variations in microbial diversity are observed among five sediments collected from Location B and different groups of microcosms. Furthermore, the results suggest that the addition of substrate caused the increase in the microbial diversity and changed the dominant microbial species. As shown in Fig. 6, the profiles of DGGE show that six microorganisms

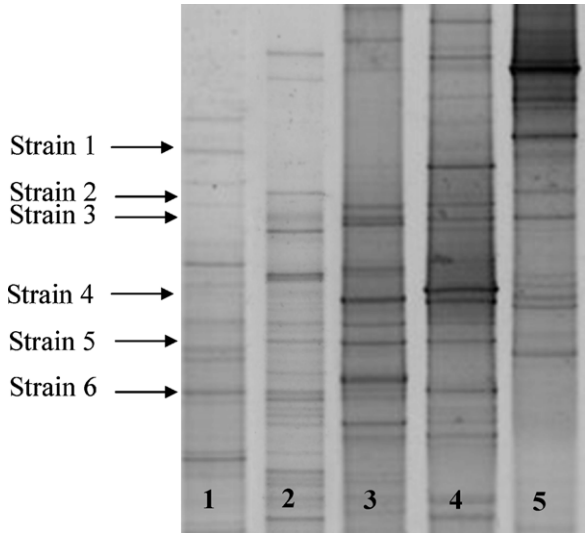


**Fig. 6.** DGGE profiles of the PCR-amplified 16S rDNA for sediments collected from Location B and different microcosms on day 30 (1: Location B; 2: live control; 3: SL; 4: SG; 5: cane molasses).

**Table 4**
Comparison of the nucleotide sequences of 16S rDNA of six specific microorganisms with the database from GenBank.

| Strain | Microorganisms | Similarity (%) |
|---|---|---|
| 1 | *Alpha proteobacterium* JAMSTEC38 | 97 |
| | *Alpha proteobacterium* 34649 | 97 |
| | *Alpha proteobacterium* SK50-21 | 97 |
| | Some unculturable or unidentified bacterial species | 97 |
| 2 | *Rhodobacter* sp. JPB-1.18 | 97 |
| | *Rhodobacter* sp. EMB 174 | 97 |
| | *Rhodobacter* gluconicum | 97 |
| | *Rhodobacter* sp. oral taxon C30 strain WC014 | 97 |
| | *Rhodobacter* sp. XJ-1 | 97 |
| | *Rhodobacter* sp. NP25b | 97 |
| | Some unculturable or unidentified bacterial species | 97 |
| 3 | *Beta proteobacterium* BAC48 | 99 |
| | *Beta proteobacterium* CDB21 | 99 |
| | *Beta proteobacterium* HTCC379 | 99 |
| | Some unculturable or unidentified bacterial species | 99 |
| 4 | *Rhodospirillales bacterium* TP418 | 98 |
| | *Rhodospirillaceae bacterium* 232 | 95 |
| | *Rhodospirillaceae bacterium* 237 | 95 |
| | Some unculturable or unidentified bacterial species | 98 |
| 5 | *Hydrogenophaga pseudoflava* | 99 |
| | *Hydrogenophaga flava* strain 2 | 99 |
| | *Hydrogenophaga flava* | 99 |
| | *Hydrogenophaga* sp. C0015(2010) | 99 |
| | *Hydrogenophaga* sp. YED6-4 | 99 |
| | *Hydrogenophaga* sp. YED1-18 | 99 |
| | *Hydrogenophaga pseudoflava* strain GA3 | 99 |
| | Some unculturable or unidentified bacterial species | 98 |
| 6 | *Iron-reducing bacterium* | 96 |
| | *Methyloversatilis* sp. | 95 |
| | Some unculturable or unidentified bacterial species | 96 |

were predominant during the TCE cometabolic processes. The intensities for strains 1–6 were within intensive ranges during the incubation period. It is possible that the mixed bacterial consortia might be responsible for aerobic cometabolism of TCE in this study. Strains 1–4 were the most significant microbes and appeared on day 30. Although strains 5 and 6 were not significant in the cane molasses group, their intensities were still noticeable during the experimental period. Consequently, these dominant indigenous microorganisms could be further isolated and enriched to enhance the efficiency of the bioremediation process.

Table 4 shows the comparison of the nucleotide sequences of 16S rDNA of six specific microorganisms with the database from GenBank. As shown in Table 4, the identities of the nucleotide sequences of six dominant bands are shown to be in a range of 95–99% of specific microorganisms as compared to database of GenBank (Table 4). This indicates that novel microorganisms, which might be sensitive to TCE and capable of synthesizing TCE-degrading enzymes, exist in this ecosystem. *Rhodobacter* sp., the possible species for strain 2, and *Methyloversatilis* sp., the possible species for strain 4, have been reported to be able to biodegrade organic contaminants under different experimental conditions [36–38]. *Beta proteobacterium* sp., the possible species for strain 3, has been reported to be able to biodegrade and synthesize enzymes for MTBE, *cis*-dichloroethene, and TCE aerobic cometabolism [8,39,40]. *Hydrogenophaga pseudoflava*, the possible species for strain 5, has been reported to be able to biodegrade and synthesize enzymes for TCE aerobic cometabolism [41]. According to the results from GenBank, four microorganisms, *Rhodobacter* sp., *Methyloversatilis* sp., *B. proteobacterium* sp., and *H. pseudoflava*, might be able to produce specific enzymes to activate the aerobic cometabolism of TCE that could exist in site sediments and groundwater. Results also reveal that DGGE and nucleotide sequence techniques provide a guide for further microbial isolation and identification.

In the future field application, the applied substrates will be injected into the subsurface directly. Thus, in this microcosm study, sterilization of the substrates was not conducted. Because SL and SG are surfactants, microorganisms usually would not grow on them directly. Cane molasses is the waste product from the sugar industry, it might contain some microorganisms. Because the six dominant microorganisms are observed in sediments from either Location B or live control microcosms, they were not brought into the system through substrate addition. Addition of substrates could enhance their activities, and thus, their intensities can be enhanced.

## 5. Conclusions

In this study, the effectiveness of aerobic TCE cometabolism with the addition of SG, SL, and cane molasses was evaluated using specific gene analyses. PCR, DGGE, and nucleotide sequence analysis were applied to monitor the variations in specific activity-dependent enzymes and dominant microorganisms in field and microcosm sediments. Conclusions of this study are as follows:

(1) TCE-degrading enzymes, including toluene monooxygenase and phenol monooxygenase, were identified in field sediment samples. This indicates that in situ enhanced bioremediation is a feasible technology for site groundwater remediation.
(2) The application of gene analysis for the screening of TCE-degrading enzymes is a necessity before in situ bioremediation is applied to site remediation. Gene analysis would be helpful in determining if the TCE-degrading enzymes exist at TCE-contaminated sites.
(3) The addition of SG, SL, and cane molasses can significantly enhance the efficiency of TCE biodegradation through the aerobic cometabolic process. Moreover, the addition of primary substrates would cause significant increases in microbial populations and variations in TCE-degrading enzymes.
(4) Results from the microcosm study reveal that substrate addition caused increases in microbial diversity and changed the dominant microbial species. Four dominant microorganisms (*Rhodobacter* sp., *Methyloversatilis* sp., *B. proteobacterium* sp., and *H. pseudoflava*) might be able to produce TCE-degrading enzymes for the TCE cometabolic process.
(5) Positive control microcosm experiments (with real microorganisms) would provide more information for future field system design. Thus, the positive control experiment could be performed in the future study.
(6) SG and SL can serve as not only TCE dissolution agents but also primary substrates (carbon and energy sources) for indigenous microorganisms. Thus, SG and SL can be directed used by indigenous heterotrophic microorganisms as carbon and energy sources and also enhance the aerobic cometabolic mechanisms for TCE biodegradation. This surfactant-enhanced process provides a more thorough TCE remediation due to the increased contact between TCE and TCE-degrading enzymes. Thus, the application of biodegradable surfactants (SG and SL) would result in a more complete and thorough TCE removal and reach the remedial goal more efficiently. Moreover, SG and SL can create anaerobic conditions after injection if oxygen is not supplied. Thus, SL and SG can also be applied to enhance the in situ reductive dechlorination of some chlorinated compounds.

## Acknowledgements

This project was funded by National Science Council in Taiwan. Additional thanks are extended to the personnel at Taiwan Ministry of Economic Affairs and Nan-Zih Industrial Park and Guan Cheng

Environment Technology Protection Co., Ltd. for their assistance and support throughout this project.

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