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Application of enhanced bioremediation for TCE-contaminated groundwater: a pilot-scale study

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ABSTRACT

The industrial solvent trichloroethylene (TCE) is among the most ubiquitous chlorinated compounds found in groundwater contamination. The objective of this pilot-scale study was to apply the combined biosparging and enhanced *in situ* bioremediation technology to remediate TCE-contaminated groundwater at a TCE-spill site. A biosparging well was installed inside the TCE plume for oxygen supplement. Primary substrate (cane molasses) was injected into the TCE plume through the biosparging well to enhance the rate of TCE co-metabolism. Three monitoring wells were installed in series downgradient of the biosparging well along the groundwater flow path. Results of polymerase chain reaction and nucleotide sequence analysis revealed that no appropriate TCE-degradation enzymes were observed in site groundwater. Thus, aerobic-activated sludge containing TCE-degraders collected from an industrial wastewater treatment plant were injected into the biosparging and three monitoring wells as an inoculum to provide microbial consortia for TCE biodegradation. After sludge injection, TCE-degraders (type I and II methanotrophs) and TCE-degrading enzymes (e.g. toluene monooxygenase, phenol monooxygenase) were detected in the injection and downgradient monitoring wells and remained in the aquifer during the 140-day pilot-scale study. Results indicate that significant TCE removal was observed (with TCE concentration dropped from 210 to 18 μg/L in substrate injection well). This reveals that appropriate substrates and inocula are required to effectively enhance the aerobic co-metabolic rate of TCE. Results from this study indicate that the enhanced *in situ* bioremediation is a promising technology to remediate TCE-contaminated groundwater.

Keywords: Bioremediation; Co-metabolism; Groundwater contamination; Trichloroethylene

1. Introduction

Groundwater, in many existing and former industrial sites and disposal areas is contaminated by halogenated organic compounds. The chlorinated solvent trichloroethylene (TCE) is one of the most ubiquitous compounds. One cost-effective approach for the remediation of contaminated aquifers that attract increasing attention is the application of enhanced bioremediation for contaminant biodegradation [1,2].

Bioremediation is an attractive remediation option because of its economic benefit. Recently, intrinsic

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bioremediation has been considered as one of the potential methods for the cleanup of petroleum-hydrocarbon contaminated sites. If the intrinsic bioremediation rate is limited by *in situ* environmental factors (e.g. oxygen, nutrients, microbial consortia, and electron acceptors), enhanced *in situ* bioremediation can be applied to stimulate contaminants' biodegradation. Current evidence suggests that TCE can be degraded under aerobic co-metabolic conditions or under anaerobic reductive dechlorinating conditions by supplying an alternate primary substrate [3–5]. As the biodegradation of TCE is generally faster under aerobic conditions, introduction of dissolved oxygen (DO) into the plume will increase the TCE biodegradation (cometabolism) rate and significantly reduce the TCE mass flux, if bioavailable primary substrates are sufficient.

Moreover, due to the recalcitrant characteristics of the chlorinated solvents, unlike the petroleum-hydrocarbon contaminated sites, most of the TCE-contaminated site groundwater is under oxic conditions. Therefore, *in situ* aerobic bioremediation is a feasible technology to clean up TCE-contaminated aquifers if oxygen can be provided to the subsurface economically. Several aerobic microorganisms or microbial communities have the ability to synthesize oxygenase enzyme systems that catalyze the initial step in the oxidation of their respective primary or growth substrates and have the potential for initiating the oxidation of TCE and other chlorinated aliphatic hydrocarbons [6–9]. The groups of aerobic bacteria include oxidizers of the following compounds: methane, propane, ethylene, toluene, phenol, acetic acid, propionic acid, cresol, ammonia, and isoprene [4]. Methane oxidizer, phenol degrader, and toluene degrader are the main bacteria, which are able to perform the aerobic co-metabolic process of TCE [10–12]. Based on the above discussion, *in situ* bioremediation is a feasible technology to clean up TCE-contaminated sites if oxygen and biodegradable primary substrates can be provided to the subsurface efficiently.

Cane molasses is the waste from sugar industry. It has the following characteristics that make it a good candidate for using as the primary substrate: (1) it is rich in carbon, an essential energy source for biodegradation; (2) it has the potential to exhibit sufficient carbon bioavailability for aerobic cometabolism to occur; and (3) it is relatively inexpensive. The above discussion suggests that a cane molasses injection system installed upgradient of the plume is a practical method to enhance TCE biodegradation. Soluble organic hydrocarbons released from the biobarrier will enhance the reductive dechlorination of TCE. Biosparging is an effective mechanism for removal of volatile organic compounds (VOCs) including TCE under aerobic biodegradation mechanism [13–16]. Biosparging functions by injecting air at a low rate into the aquifer below the zone of contamination. The injected air promotes oxygenation of the aquifer as necessary to promote aerobic biodegradation. In this study, the purpose of the biosparging system is to stimulate aerobic co-metabolism of TCE. Thus, the development of an aerobic co-metabolic reactive zone containing both sufficient oxygen and primary substrates would be a feasible alternative to enhance *in situ* bioremediation of TCE cost-effectively.

Recently, molecular biology technologies have been applied in site remediation studies to confirm the effectiveness of the bioremediation [4,17,18]. Results from other studies reveal that polymerase chain reaction (PCR) and nucleotide sequence analysis techniques provide a guide for microbial ecology, which can be used as an indication of the trend of biodegradation process [4,16,19]. Thus, total bacterial DNAs of representative groundwater samples were extracted in this study for detecting the community dynamics in the process of TCE degradation [18,20]. In this study, the dominant microorganisms and existence of bacteria responsible for aerobic co-metabolism of TCE were verified using a series of molecular biology techniques including DNA extraction, PCR amplification, and DNA analysis. The objectives of this pilot-scale study were to: (1) evaluate the effectiveness of the combined biosparging and enhanced *in situ* aerobic co-metabolism technology on the remediation of TCE-contaminated groundwater and (2) evaluate the feasibility of using activated sludge as the inoculum to enhance the *in situ* aerobic co-metabolic of TCE.

2. Materials and methods

2.1. Site description

A government-owned industrial park site located in southern Taiwan was selected for this *in situ* bioremediation study. In early 2000, leakage from a TCE storage tank resulted in the groundwater contamination by TCE. During the following four-year investigation period, more than 230 soil samples were collected, and 25 monitoring wells were installed for site characterization and TCE-plume delineation. On-site borings encountered up to 22 m of mostly brownish and fine to medium sand loam. The average groundwater elevation within the shallow aquifer is approximately 6–7 m below land surface. Groundwater in the unconfined aquifer, according to the groundwater elevation in monitoring wells, flows towards northwest. The measured effective porosity is 0.31, and the average hydraulic conductivity for the

underlying, unconfined aquifer is 0.006 cm/s. The calculated site groundwater flow velocity is 9.1 cm/d. The measured groundwater temperature in the underlying aquifer varies from 19 to 27°C. The preliminary site investigation results indicate that the TCE contamination has become a diffuse pollution, which has resulted in a much dispersed TCE plume in the site. Since 2007, a pilot-scale study has been applied within the TCE plume to evaluate the effectiveness of applying *in situ* bioremediation technology for plume control and TCE removal.

2.2. System design

In this study, a biosparging well (labeled as IW) was installed in the upgradient area for air and primary substrate (cane molasses) injection and groundwater monitoring. Three monitoring wells (labeled as MW-1, MW-2, and MW-3) were installed in series downgradient of the biosparging well along the groundwater flow path for groundwater sampling and analyses. A background monitoring well (labelled as BGW) located in the uncontaminated area was used for the background sample collection. Three monitoring wells MW-1, MW-2, and MW-3 were located 3, 6, and 9 m downgradient of the biosparging well (IW), respectively. All wells were screened from 7 to 17 m below ground surface (bgs). Fig. 1 presents the site map showing the locations of representative monitoring wells and groundwater flow direction. The biosparging system consisted of a biosparging well (injection points) (well screen at 8–10 m bgs), air compressor, flow indicator, inline regulator, and pressure gage. For the biosparging well, the air flow was approximately 0.06–0.17 m/min so that the TCE volatilization can be minimized. In this study, air and substrate (4 kg of cane molasses for each injection) were injected during the 109-day operational period (from day 31 to day 140). Biosparging system was operated daily and substrate injection was performed monthly during the operation. Results from the preliminary study show that the site groundwater did not contain significant amounts of TCE-degradation enzymes. Thus, aerobic activated sludge containing TCE-degraders collected from an industrial wastewater treatment plant was injected into the biosparging and three monitoring wells. In each well, 10 L of sludge (as inoculum) was dissolved in 100 L of groundwater to provide microbial consortia for TCE biodegradation.

2.3. PCR analysis and microbial identification

In the field study, groundwater samples collected from injection and monitoring wells were used for the

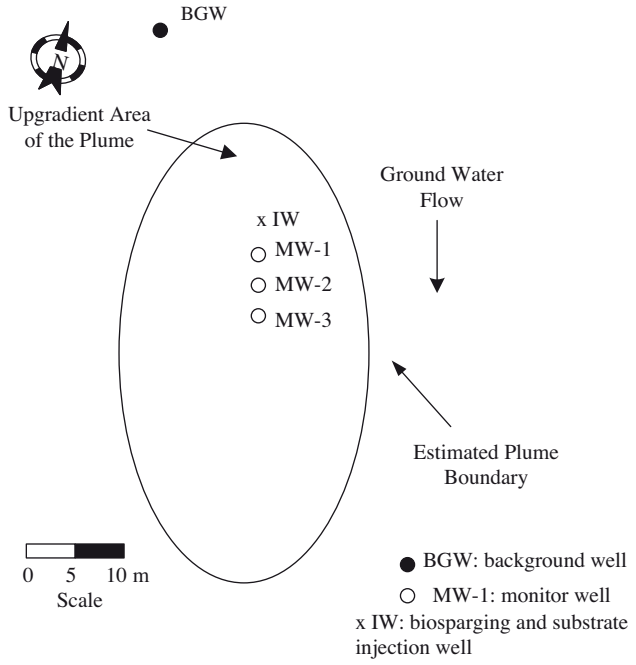

Fig. 1. Site map showing the locations of representative three monitoring wells, the biosparging and substrate injection well, and groundwater flow direction.

PCR analysis to evaluate the occurrence of genes responsible for the aerobic cometabolism of TCE before and after bioremediation process. Total bacterial DNAs from 4 L of each well were extracted with DNA purification kits (GeneMark Co., Taiwan) for detecting the community dynamics. Bacterial 200-bp fragments of 16S rDNA V3 region were amplified with the primer sets (341f, forward: 5′-CCTACGGGAGGCA GCAG-3′ containing a GC clamp of 40-nucleotide GC-rich sequence; 534r, reversed: 5′-ATTACCGCGGCTGCTGG-3′) [17]. The individual primer sets were allowed to amplify fragments of toluene monooxygenase, phenol monooxygenase, and type I and type II methanotrophs [11,19,21,22]. These primer sets were listed in Table 1. The mixtures of PCR contained 10 ng of DNA extract, 4 pmol of each primer, and 5 U of *Taq* polymerase (Takara, Shiga, Japan) in the 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°C for 1 min, annealing temperature was initially 65.8°C, and it was decreased by 1°C per cycle until it was 55.8°C, after which 25 additional cycles were carried out at 55.8°C; and extension at 72°C for 2 min. The 10% polyacrylamide gel with a 30–60% denaturant gradient was used and electrophoresis was performed at 60°C and 70 V for 14 h. The gels were then stained with SybrGreen I and photographed. The PCR-amplified products were electro-

eluted from gel and then sequenced by MdBio, Inc. in 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).

2.4. Groundwater sample analyses

Groundwater samples were collected and analyzed for organic compounds and geochemical indicators including TCE, CH_4, CO_2, inorganic nutrients (ammonia, nitrate, and phosphate), Fe(II), pH, oxidation-reduction potential (ORP), DO, and chemical oxygen demand (COD). Organic compound analyses were performed in accordance with US EPA Method 602, using a Tekmer Purge-and-Trap Model LSC 2000 with a Perkin-Elmer Model 9000 Auto System Gas Chromatograph (GC). Methane was analyzed on a Shimadzu GC-9A GC using headspace techniques. Ion chromatography (Dionex) was used for inorganic nutrients and anions analyses. COD measurements were conducted in accordance with the dichromate reflux method described in Standard Methods [22]. DO, ORP, pH, CO_2, and temperature were measured in the field. An Accumet 1003 pH/ORP meter (Fisher Scientific) was used for pH and ORP measurements, an Orion DO meter (Model 840) was used for DO and temperature measurements, and a Hach digital titrator cartridge was used for CO_2 measurements. Perkin-Elmer Plasma II Inductively Coupled Plasma-Argon Emission Spectrometer (ICP-AES) was used for Fe(II) analyses. The analytical procedures for groundwater analyses are described in Standard Methods [23].

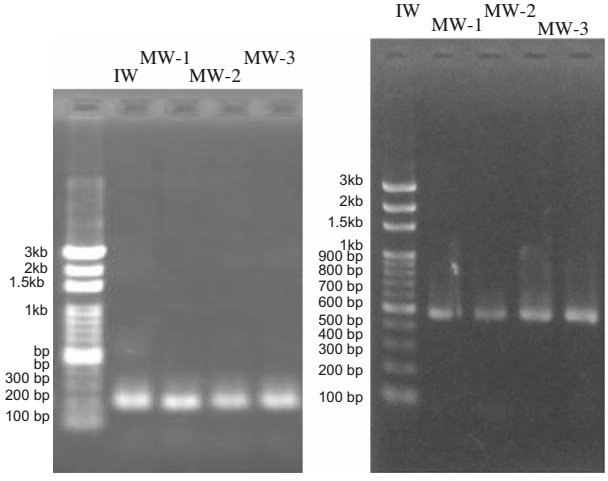

Fig. 2. Two gels showing the PCR-amplified fragments (206 and 466 bp).

3. Results and discussion

Results from our preliminary study through PCR and nucleotide sequence analysis show that the site groundwater did not contain significant amounts of TCE-degradation enzymes. Thus, previous *in situ* aerobic co-metabolism of TCE was not significant even though air and substrate were injected. Results from previous study show that less than 38% of TCE removal was obtained, probably due to the lack of TCE-degradation enzymes (data not shown). Thus, aerobic-activated sludge containing TCE-degraders collected from an industrial wastewater treatment plant containing TCE in the influent was injected in to the biosparging and three monitoring wells as an inoculum to provide microbial consortia for TCE biodegradation. Groundwater samples were collected for TCE-degrading enzymes analyses on day 30 after sludge injection to confirm the existence of TCE-degrading enzymes in the subsurface. Fig. 2 presents the gel showing the PCR-amplified fragments (206 and 466 bp) produced using the dedicated primer on phenol monooxygenase and toluene monooxygenase DNA extracted from the groundwater samples of the TCE-spill site (Table 1). Fig. 3 presents the gel showing the PCR-amplified fragments (920 and 950 bp) produced using the dedicated primer on type I and type II methanotrophs DNA extracted from the

Table 1
Primer sets of four TCE-degrading enzymes

Target	Primer sequence	Reference
Phenol monooxygenase	[a]F:5′-GTGCTGAC(C/G)AA(C/T)CTG(C/T)TGTTC R:5′-CGCCAGAACCA(C/T)TT(A/G)TC	[11]
Toluene monooxygenase	F:5′-TCTC(A/C/G)AGCAT(C/T)CAGAC(A/C/G)GACG R:5′-TT(G/T)TCGATGAT(C/G/T)AC(A/G)TCCCA	[19]
Type I methanotrophs	F:5′-CCTTCGGGMGCYGACGAGT R:5′-GATTCYMTGSATGTCAAGG	[21]
Type II methanotrophs	F:5′-GAGTTTGATCMTGGCTCAG R:5′-CATCTCTGRCSAYCATACCGG	[21]

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

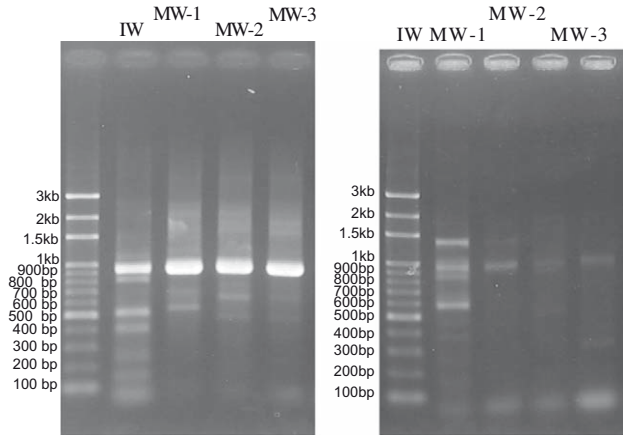

Fig. 3. Two gels showing the PCR-amplified fragments (920 and 950 bp).

groundwater samples. Results indicate that all three groundwater samples (IW, MW-1, and MW-2) contained toluene phenol monooxygenase, toluene monooxygenase, type I methanotroph, and type II methanotroph in site groundwater. As the detected phenol monooxygenase, toluene monooxygenase, and type I and type II methanotrophs are able to activate the aerobic co-metabolism of TCE, the observed specific enzymes at this site imply that *in situ* enhanced bioremediation is a feasible technology for site groundwater remediation.

As the detected TCE-degrading enzymes are able to activate the aerobic co-metabolism of TCE, the observed specific enzymes at this site imply that *in situ* enhanced bioremediation is a feasible technology for site groundwater remediation. The results indicate that a thorough gene analysis is necessary before *in situ* bioremediation is applied to site remediation. Gene analysis would be helpful in determining if the TCE-degrading enzymes exist at the site and if *in situ* bioremediation is a potential remedial option.

The pilot-scale study was carried out to evaluate the effectiveness of enhanced aerobic cometabolism for TCE biodegradation after the air, substrate, and

Table 2
Averages of analytical results for BGW, IW, and monitoring wells during the operational period

Parameters	pH	DO (mg/L)	ORP (mV)	Sulfate (mg/L)	CO_2 (mg/L)	Fe^{2+} (mg/L)	Nitrate (mg/L)	Ammonia (mg/L)	Phosphate (mg/L)	COD (mg/L)	Bacterial count (CFU/mL)	TCE (μg/L)
BGW	7.2	1.7	133	23	132	<0.1	1.4	2.1	0.06	5	3.2×10^3	BDL
IW	6.7	3.2	237	44	256	<0.1	1.1	9.8	1.5	223	4.0×10^8	18
MW-1	6.8	2.3	176	36	203	<0.1	1.3	4.7	1.2	61	5.3×10^6	56
MW-2	6.9	1.8	144	27	177	<0.1	1.2	3.1	0.6	14	5.1×10^5	109
MW-3	7.1	1.2	135	22	168	<0.1	1.4	2.2	0.2	8	4.2×10^4	127

Notes: Methane < 0.01 mg/L in all five wells.
BDL: below detection limit.

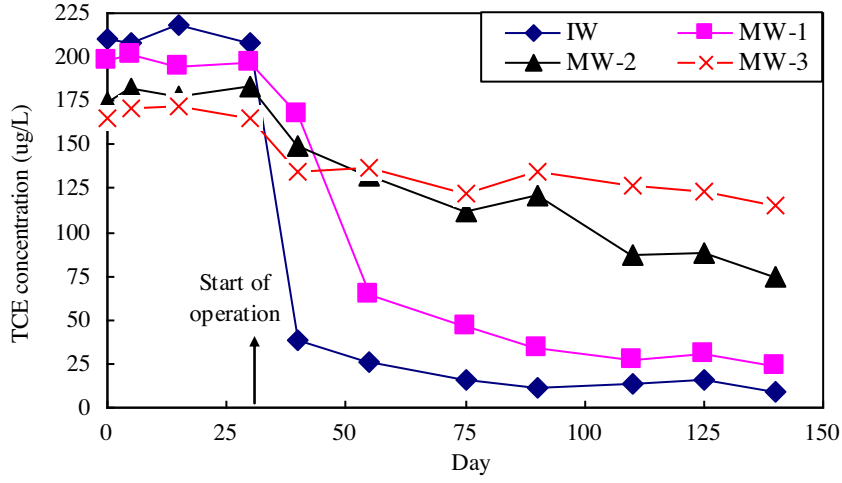

Fig. 4. Variations in TCE concentrations in IW and three monitoring wells (MW-1–MW-3) during the operational period.

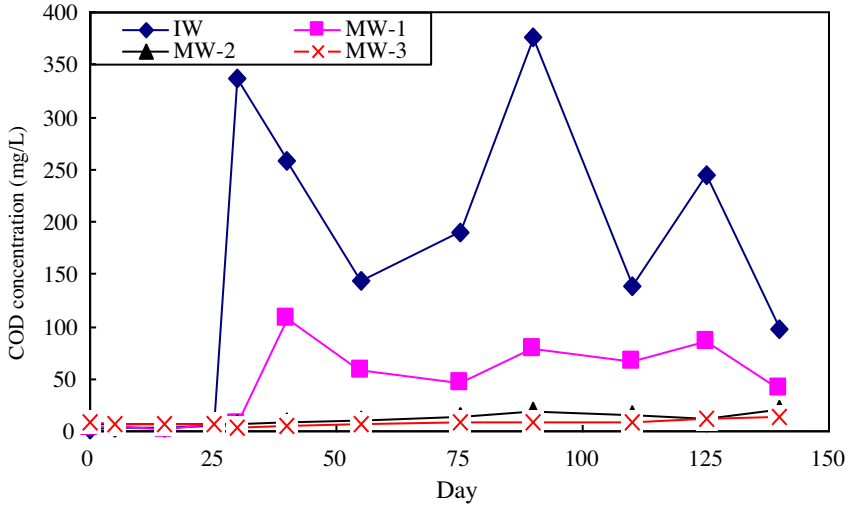

Fig. 5. Variations in COD concentrations in IW and three monitoring wells (MW-1–MW-3) during the operational period.

activated sludge injection. In this study, cane molasses (4 kg for each injection) was injected monthly into IW for primary substrate supplement, and air was supplied through IW. Table 2 shows the averages of groundwater analytical results during the operational period. Results show that the pH, DO, and ORP varied from 6.7 to 7.1, 1.2 to 3.2 mg/L, and 135 to 237 mV, respectively, within the TCE plume. Compared to the pH, DO, and ORP values in BGW, field results reveal that the groundwater was in oxidative conditions in both contaminated and uncontaminated areas. Moreover, a slight decrease in pH and increase in DO and ORP were observed in IW and downgradient wells. This reveals that the occurrence of aerobic biodegradation caused the drop of pH due to the production of CO_2 after the biodegradation process. Air sparging process also caused an increase in DO and ORP. No significant variations in nitrate, sulfate, methane, and ferrous iron concentrations were observed when compared to the BGW well. This might be due to the fact that aerobic biodegradation process was the dominant degradation process, and no anaerobic biodegradation occurred during the operational period.

The variations in TCE concentrations, COD concentrations, and total viable bacterial counts in IW and three monitoring wells (MW-1 to MW-3) during the operational period are presented in Figs. 4–6,

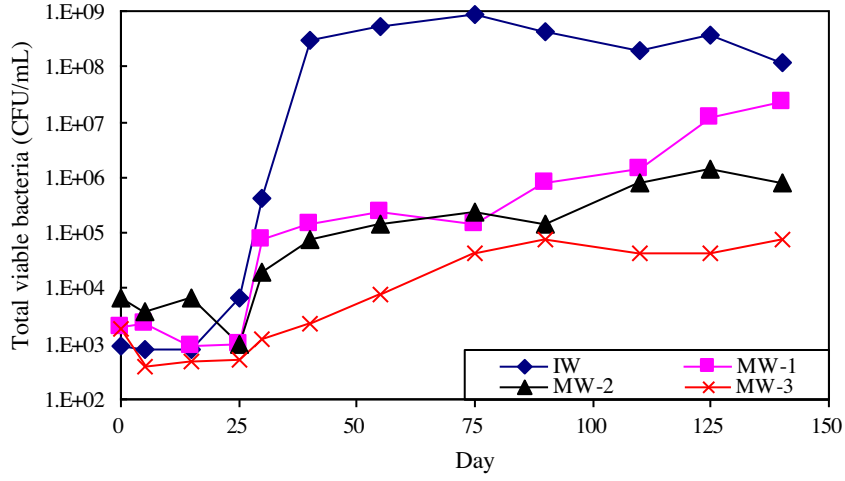

Fig. 6. Variations in total viable bacterial counts in IW and three monitoring wells (MW-1–MW-3) during the operational period.

respectively. Results from Fig. 4 and Table 2 show that the averaged TCE concentrations in IW, MW-1, MW-2, and MW-3 were 18, 56, 109, and 127 μg/L, respectively, during the operational period. The averaged COD concentrations in IW, MW-1, MW-2, and MW-3 were 223, 61, 14, and 8 mg/L, respectively, during the operational period. In IW, the TCE concentrations dropped to 38 μg/L after 9 days of operation. The COD concentrations in IW samples jumped up to 258 mg/L after 9 days of operation (Fig. 5). This indicates that the injection of cane molasses caused an increase in COD concentrations and the added cane molasses was used as the primary substrate and energy source for the enhancement of TCE co-metabolism, which resulted in TCE biodegradation. In BGW, no significant variations in COD concentrations were observed. The averaged total viable bacterial counts in IW, MW-1, MW-2, and MW-3 were approximately 4×10^8, 5.3×10^6, 5.1×10^5, and 4.2×10^4 CFU/mL quantities, respectively (Fig. 6). The CO_2 produced in IW and MW-1 were approximately 256 and 203 mg/L (measured with the Hach digital titrator cartridge), respectively. The high total bacterial counts and CO_2 production in IW and MW-1 indicate that the injected carbon and air sources activate the microbial activity and increase the microbial population, which enhanced the aerobic co-metabolism of TCE around the injection area.

Results show that significant TCE removal was observed in IW indicating that high concentrations of COD and DO played important roles in TCE co-metabolism. However, less TCE removal efficiency was observed in MW-3. This could be due to the lower COD concentrations, which could not cause significant proliferation of microbial populations for subsequent TCE-degrading enzymes. The aerobic TCE cometabolism could be confirmed by the following investigations: (1) decrease in TCE concentrations along the plume travel path from IW to MW-3; (2) increase in COD concentrations and microbial populations along the plume travel path from IW to MW-3; and (3) production of CO_2 and decrease in pH value in IW and monitoring wells. In the future, field application, substrate, oxygen, and inoculum injection can be used to form the downgradient biobarrier system to enhance the biodegradation of groundwater contaminants migrating into the treatment zone. The injected substrates and inocula would enhance the aerobic co-metabolism of TCE *in situ*.

4. Conclusions

In this study, the effectiveness of aerobic TCE co-metabolism on TCE concentration reduction with the injection of air, cane molasses, and sludge was evaluated at a TCE-spill site. Conclusions of this pilot-scale study include the following:

(1) Aerobic cometabolism was the major cause of the decrease in TCE concentrations in groundwater in this study. Without air, primary substrate, and inoculum supplement, aerobic cometabolic mechanism could not occur.
(2) The effectiveness of biosparging and substrate injection and occurrence of aerobic TCE cometabolism could be confirmed by the following investigations within the plume: (a) significant decrease in TCE concentrations; (b) increase in COD concentrations and microbial populations; (c) increase in DO and ORP in the biosparging well; (d) significant depletion of DO and decrease in ORP within the TCE plume after the supplement of cane molasses; (e) production of CO_2 and decrease in pH value, and (f) detected specific TCE-degrading enzymes after sludge injection. Field results reveal that the operation of biosparging caused the shifting of low oxygen conditions inside the plume to aerobic conditions.
(3) TCE-degrading enzymes, including toluene monooxygenase, phenol monooxygenase, and type I and type II monooxygenase were identified in field sediment samples. This indicates that *in situ* enhanced bioremediation is a feasible technology for site groundwater remediation.
(4) Identification of TCE-degrading enzymes using gene analysis is a useful tool to evaluate the feasibility of applying enhanced *in situ* bioremediation for TCE removal. Appropriate inoculum injection is a necessity to enhance the TCE biodegradation efficiency.

Acknowledgments

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