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A permeable reactive barrier for the bioremediation of BTEX-contaminated groundwater: Microbial community distribution and removal efficiencies

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ABSTRACT

This study was conducted with column experiments, batch experiments, and bench-scale permeable reactive barrier (PRB) for monitoring the PRB in the relation between BTEX (benzene, toluene, ethylbenzene, and *p*-xylene) decomposition efficiency and the distribution of a microbial community. To obtain the greatest amount of dissolved oxygen from oxygen-releasing compounds (ORCs), 20-d column tests were conducted, the results of which showed that the highest average amount of dissolved oxygen (DO) of 5.08 mg l^{-1} (0.25 mg-O_2 d^{-1} g^{-1}-ORC) was achieved at a 40% level of CaO_2. In the batch experiments, the highest concentrations of benzene and toluene in which these compounds could be completely degraded were assumed to be 80 mg l^{-1}. Long-term monitoring for a PRB indicated that ORCs made with the oxygen-releasing rate of 0.25 mg-O_2 d^{-1} g^{-1}-ORC were applicable for use in the PRB because these ORCs have a long-term effect and adequately meet the oxygen demand of bacteria. The results from the DGGE of 16S rDNAs and real-time PCR of catechol 2,3-dioxygenase gene revealed the harmful effects of shock-loading on the microbial community and reduction in the removal efficiencies of BTEX. However, the efficiencies in the BTEX decomposition were improved and the microbial activities could be recovered thereafter as evidenced by the DGGE results.

1. Introduction

Benzene, toluene, ethylbenzene and xylenes, commonly referred to as BTEX, are critical monoaromatic environmental contaminants worldwide, of which the major aromatic contaminants in gasoline pose serious environmental health problems because these compounds are frequently found together at hazardous waste sites. Leaking tanks or ruptured pipelines pollute soil and groundwater with these compounds. In recent years, several studies on the treatment of these compounds have been conducted to improve the respective removal efficiencies at contaminated sites [1–3]. The most popular removal methods have been bioremediation, advanced oxidation technologies, photocatalysis, sonolysis and radiolysis [4,5]. Among these, bioremediation has received more attention due to its generally nontoxic attributes in comparison with the others, which tend to form byproducts having higher toxicity than the original contaminants [6]. In biodegradation, decomposition can proceed by various pathways, involving either an activation of the aromatic ring by dioxygenation or monooxygenation reactions and catalysis of the dioxygenolytic cleavage of the aromatic ring, or by processing the side chains [7,8].

Permeable reactive barrier (PRB) technology is suitable for contaminated groundwater treatment, having received increased attention in the literature [9–11]. There are studies published reporting results of full-scale installed PRB for *in situ* remediation of groundwater contaminated with organic compounds (BTEX, among others) [12,13]. In a PRB, polluted groundwater transversely flows through a reactive material under natural hydraulic gradients wherein the contaminants are physically adsorbed, chemically or biologically degraded [14]. One of the main advantages of a PRB is the lower cost accrued by using the natural flow to bring the contaminants in contact with the reactive materials without installing any above-ground facilities or energy inputs. For groundwater *in situ* bioremediation, it should be more suitable when the technology is applied for increasing aerobic metabolism than for anaerobic. However, the dissolved oxygen (DO) content is usually very poor within the groundwater (below 3 mg l^{-1}) [15]. The deficiency of DO in groundwater can be surmounted by using oxygen-releasing materials, which renders the environmental aerobic condition and overcomes the reduction state caused by an anaerobic condition, thereby obtaining higher degradation efficiency. Oxygen-releasing materials, consisting of mixtures of CaO_2 or MgO_2, cement, sand and other materials of certain proportions, have been investigated

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and applied to contaminated groundwater studies and remediation projects [9,16,17]. The long-term efficiency of these substances can be extended six months or even one year [18].

In reactive barrier technology, oxygen-releasing compounds (ORCs) are applied to enhance aerobic reactions [16]. In conjunction with biotreatment, ORCs provide a supplemental source of dissolved *in situ* oxygen. Calcium peroxide releases oxygen slowly in the presence of water, its chemical reaction being $2CaO_2 + 2H_2O \rightarrow O_2 + 2Ca(OH)_2$. Use of CaO_2 instead of MgO_2 to provide oxygen for contaminant degradation has been described in the literature [19]. In this process, the $Ca(OH)_2$ produced can result in a significant rise in the pH of the solution, which in turn may reduce the enzymatic activity of the microbes [9,20]. Therefore, the pH was effectively regulated by using buffers such as K_2HPO_4 and KH_2PO_4.

The factors affecting the effectiveness of bioremediation are closely related to microbial distribution and growth conditions [21]. Therefore, it is important to examine the relationships among the contaminants, environmental factors and microbial communities. Microbial data were collected by molecular biochemistry techniques, which are more useful than traditional ones [22]. In recent years, monitoring the dynamics of microbial populations has been implemented by polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) [23,24]. In our study, real-time quantitative polymerase chain reaction (real-time PCR) [25,26] and DGGE techniques were used to investigate the BTEX-degrading microbial community distribution in a bench-scale PRB system.

2. Materials and methods

2.1. Sources of microorganisms

The mixed cultures were obtained from two sources, one from an organics-contaminated groundwater site in central Taiwan, the other from industrial wastewater-treatment sludge in the Mailiao Industrial Park, Yunlin County, Taiwan. The mixture from the two sources was further defined as '*in situ* microorganisms'.

2.2. Column experiment for justification of using ORC

Column tests were conducted to investigate the effectiveness and the respective efficiencies in removing contaminants by addition of various amounts of CaO_2. The column was constructed in three sections using a transparent cylindrical plastic acrylic glass column (10 cm in diameter and 60 cm in height). The top and bottom sections of the column were packed with Ottawa standard sands (E-315, Geotest, USA) providing a total bed depth of approximately 50 cm (top 40 cm; bottom 10 cm). A 5–10 cm bed depth in the middle section was filled with 100 g of ORC consisting of cement, sand, H_2O, KH_2PO_4, K_2HPO_4, $NaNO_3$, and 10–50% CaO_2. Perforated stainless steel plates located at the bottom and between sections supported the packing media. The column system was operated at inlet liquid flow-rate of 3.45 ml min^{-1} upward.

For forming ORC beads, the reactive mixture was obtained by adding K_2HPO_4, KH_2PO_4, cement and sand to CaO_2. The pH was effectively regulated by using K_2HPO_4 and KH_2PO_4, which could be used as nutrient components in the medium. Various amounts of CaO_2 were used to investigate their effectiveness as well as their respective efficiencies in removing contaminated substances. A sufficient amount of water was added to this mixture, followed by the casting.

2.3. Batch experiment and oxygen demand

The objectives of batch experiments were to investigate the inhibitory concentrations of benzene and toluene, and to estimate the required DO for tentative use in the PRB system. In addition, results (i.e., amounts of functional gene and biomass) from batch tests were used to develop the protocol for real-time PCR.

Five millilitre of inoculum was introduced into screw cap amber glass bottles of 250 ml volume, each containing 100 ml of an autoclaved phosphate-buffered mineral salts solution, sealed with Teflon Mininert® valves. Initial biomass was determined by sampling five millilitre of solution from the bottles before the introduction of benzene and toluene. Benzene and toluene (20, 40, 80, 120, 160, 240 and 320 mg l^{-1}) were then injected into each bottle by using a stainless steel needle fitted to a gas-tight syringe, the bottles were then sealed and kept in the dark under constant shaking at 150 rpm and a temperature of $30 \pm 1\,^{\circ}C$. The final biomass was measured when the changes of benzene and toluene concentrations were less than 5%. The biomass was measured spectrophotometrically. Growth was monitored with optical measurement at 600 nm by using a Hitachi UV spectrophotometer. The biomass concentration was estimated from a correlation optical density to the dry weight.

To estimate the required DO in the batch experiment for tentative use in the PRB system, biochemical oxygen demand (BOD) analysis was conducted. The BOD analysis method was based on BOD_5 testing guidelines specified in National Institute Environmental Analysis (NIEA) document W510.54B published by the Environmental Protection Administration, Taiwan [27].

2.4. PRB system

The bench-scale PRB system built and used in the experiments reported here is shown schematically in Fig. 1. Its major component was a transparently double-jacketed plastic acrylic glass tank. The tank was packed with 25 kg Ottawa standard sands (E-315, Geotest, USA) providing a total bed depth of 20 cm and a total packed-bed volume of approximately 18 l. The ORC section (2 cm in thickness) situated 20 cm from the water inlet was filled with 800 g of ORC consisting of cement, sand, 40% CaO_2, KH_2PO_4, K_2HPO_4, $NaNO_3$, and H_2O. Three perforated stainless steel plates were located at the both sides of the ORC section to cage the ORC beads (1.1 cm inside diameter; 2.5 cm in length), and at the outlet region to prevent the channeling. Four rows of twelve monitoring wells (inside diameter of 2 cm; length of 15 cm) were located in the PRB tank to monitor the DO, CFU, pH, oxidation–reduction potential (ORP) and BTEX concentrations along the tank. The PRB tank was maintained at a temperature of 23–25 °C by using temperature-controlled circulating water outside the tank. The PRB tank was completely closed; therefore, losing of BTEX in analytical process was restricted.

Synthetic water was used for the experiments. The inflow was set at 3.45 ml min^{-1} (flow velocity of 50 cm d^{-1}; hydraulic residence time of 3.3 d; hydraulic conductivity of 2.9×10^{-2} cm s^{-1}). BTEX were continuously injected by a syringe pump (Kd Scientific, model 100) into the influent. The inlet concentrations of benzene, toluene, ethylbenzene and *p*-xylene being maintained each at approximately 30 mg l^{-1}. Twice of shock-loadings (spiked with higher BTEX concentrations) were also applied to test the stability and the recovery capability of the PRB system, when the direction of flow changed or channeling occurred during pumping activity leading to the unexpected contaminants leaking or spike in remediation sites. According to oxygen-releasing rate estimated in the column experiments, the initial amounts of ORCs were determined. With the rapid decline in DO, ORCs were replaced as need. Water samples were collected for BTEX analysis daily, and for DO, CFU, pH and ORP analysis weekly.

Two phases of bioremediation were performed in the PRB system, the first being biostimulated with the addition of ORC and the

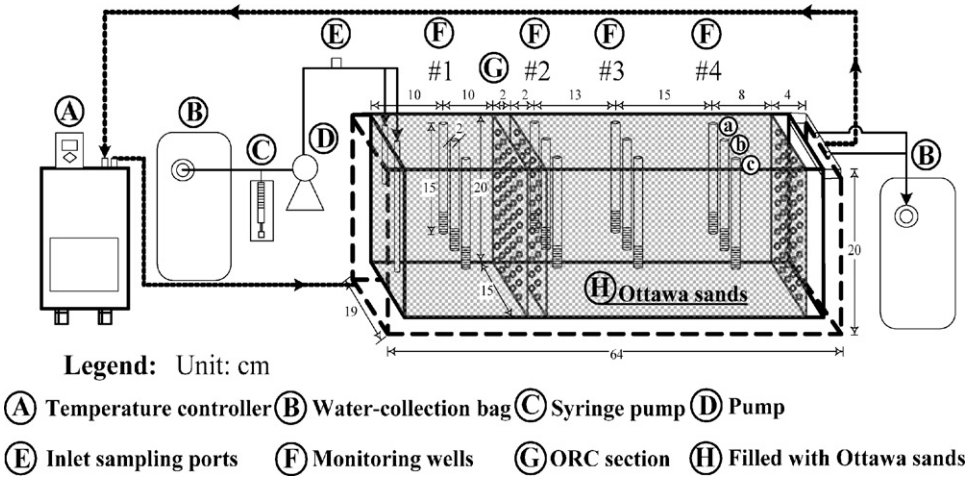

Fig. 1. Schematic diagram of an oxygen-releasing reactive barrier system.

implantation of 3000 ml *in situ* microorganisms (4.62×10^8 colony-forming units [CFUs] ml^{-1}), the second being a continuation of the first, with bioaugmentation (BTEX-degrading strains were added using a peristaltic pump through locations #1, #2, and #3; 200 ml per well; 8.49×10^9 CFU ml^{-1}). After a 15–20-d start-up period, the system remained normally stable, with a difference in removal efficiency of less than 5% in the first phase and continuing in the second.

2.5. Synthetic groundwater analysis

Water samples from the PRB system were collected by using needles and a peristaltic pump. The equivalent volumes of 2–3 times those of the wells were first removed at locations #4-a, #4-b, and #4-c. Subsequently, 10 ml samples were collected from the wells to await water quality analysis. Moreover, 3 ml of samples was taken by the needles, after which 1 ml of a subsample was sealed in a 3 ml glass bottle to await further analysis. Gaseous samples of BTEX were obtained from the headspace of a 3 ml glass bottle by 250 μl gas-tight syringes equipped with Teflon Mininert valve fittings. The samples were then analyzed by using a GC-FID (gas chromatograph, model GC-14B, Shimadzu, Japan). Similarly, water samples from locations #1 to #4 were collected and analyzed for DO, CFU, pH, and oxidation reduction potential (ORP). Fifteen-millilitre water sample obtained by the needles was used for water quality analysis, and an additional 1.5 ml water sample was collected by using needles, sealed in a micro-centrifuge tube and preserved at −20 °C for DNA extraction.

2.6. DNA extraction and PCR amplification

DNA extraction from the synthetic groundwater samples was performed with an AxyPrep™ Bacterial Genomic DNA Miniprep Kit (Axygen Biosciences, Union City, CA, USA) from 2 ml synthetic groundwater per sample, according to the manufacturer's instructions.

PCR amplification reaction mixture (final volume 50 μl) contained 0.55 μl template DNA and 1.25 U Taq DNA polymerase (ABgene, Epsom, Surrey, UK), 1.5 mM $MgCl_2$, 200 μM dNTP, and 0.5 μM primers in 1× reaction buffer IV. PCR was performed with periods of 30 s at 94 °C for melting, 30 s for annealing at 55 °C, and 30 s at 72 °C for synthesis, for 30 cycles, in MiniCycler (MJ Research, Waltham, MA, USA).

2.7. Functional genes for aromatic hydrocarbons

Gene-encoding catechol 2,3-dioxygenases (C23O; EC 1.3.11.2), as key enzymes in various aerobic aromatic degradation pathways, were used as functional targets to assess the catabolic gene diversity in the different BTEX contaminants by real-time PCR [8,28]. The C23O, belonging to the extradiol dioxygenase family, catalyzes the ring cleavage of catechol and chloro-, methyl-, and ethyl-substituted catechols in a *meta* fashion [29]. In this study, the primer pairs were used as part of the aromatic hydrocarbons of specific primers, cpC23O-R (5′-CTCGTTGCGGTTGCCG CTSGGGTCGTCG AAGAAGT-3′) and cpC23O-F (5′-ATCGAGGCCTGGGGTGTG AAGAC-CACCATGC T-3′), respectively.

2.8. DGGE assay and real-time PCR

The microbial communities were analyzed by using the PCR-DGGE method described by Ercolini [30] and Hendrickx et al. [31], with certain modifications (polyacrylamide gel concentration; denaturing gradient; electrophoresis voltage and time). DGGE was performed on 6–10% polyacrylamide gels with a denaturing gradient of 20–60% (where 100% denaturant gels contain 7 M urea and 40% formamide). Electrophoresis was performed at a constant voltage of 200 V for 3–5 h with 1× Tris–acetate–EDTA buffer (TAE) at 60 °C in a DCode™ universal mutation detection system (Bio-Rad Laboratories, Hercules, CA, USA). After electrophoresis, the gels were stained with silver nitrate solution according to Radojkovic and Kusic [32]. The analysis of the DGGE gels was done with the LabWorks 4.5 software (UVP, Inc., Upload, CA, USA).

Real-time PCR reactions were achieved on a LightCycler® carousel-based system (Roche Molecular Biochemicals, Indianapolis, IN), with a SybrGreen master mix used according to the manufacturer's instructions (FastStart DNA Master SYBR Green I kit, Roche). The microorganisms in the BTEX-degradation amplification mixture contained 10 μl of distilled H_2O (ddH_2O), 2 μl of 25 mM $MgCl_2$, 0.5 μl of a 20 μM cpC23O-F and cpC23O-R solution, respectively, and 2.0 μl of FastStart DNA Master SYBR Green I. This mixture was loaded into precooked LightCycler glass capillary tubes followed by 5 μl of template DNA (all 2.5 ng l^{-1}) to a final volume of 20 μl. The following real-time PCR program was executed on the LightCycler: the reactions were run for 45 cycles; the denaturation was 95 °C for 10 s; annealing, 57–59 °C for 5 s; and extension, 72 °C for 19 s. An initial 10-min denaturing step at 95 °C was used. The cycle threshold (C_t), or PCR cycle where fluorescence

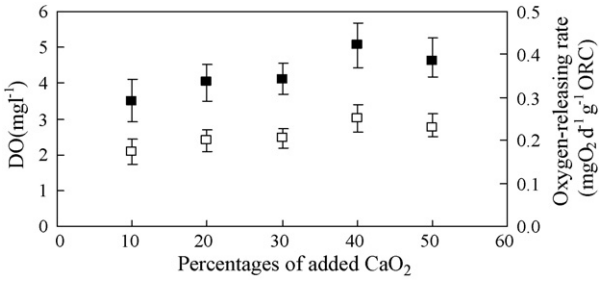

Fig. 2. Oxygen-releasing rates (□) from ORCs. (DO: ■).

first occurred, was automatically determined by using sequence detection software (version 3.5, LightCycler Software, Roche).

3. Results and discussion

3.1. Preliminary column tests for ORC bead and DO

Eq. (1) was used to estimate the oxygen-releasing rate of the ORC bead. The oxygen-releasing rate is equivalent to the ORC release rate in units of weight (mg-O_2 d^{-1} g^{-1}-ORC); DO, the average dissolved oxygen content (mg l^{-1}); Q, the inlet flow-rate (ml min^{-1}); ORC, the filling amount in grams.

$$\text{Oxygen-releasing rate} = \frac{\text{DO} \times Q}{\text{ORC}} \quad (1)$$

Fig. 2 shows the proportion of CaO_2 in correlation with the oxygen-releasing rates for 20 days, when the inlet flow-rate was maintained at 3.45 ml min^{-1} during filling with 100 g of ORC. The average amounts of DO were increased from approximately 3.51 to 5.08 mg l^{-1} with an increase in CaO_2 from 10% to 50%. By solving Eq. (1), the oxygen-releasing rates in the units of weight were 0.17, 0.20, 0.20, 0.25 and 0.23 mg-O_2 d^{-1} g^{-1}-ORC, respectively, to the aforementioned amounts of CaO_2 at 10, 20, 30, 40, and 50%. At 40% CaO_2, the oxygen-releasing rate was highest (0.25 mg-O_2 d^{-1} g^{-1}-ORC) compared to other percentages.

3.2. Efficiencies in removal of benzene and toluene via batch experiments

Table 1 lists the biomass variations and percentages of remaining benzene and toluene (added at initial concentrations of 20, 40, 80, 120, 160, 240 and 320 mg l^{-1}) in batch experiments at various concentrations for '*in situ* microorganisms'. When the concentrations were at 20, 40 and 80 mg l^{-1}, these contaminants could be decomposed to a residual amount not exceeding 5% within the short times of 12, 20 and 26 h, respectively. In these cases, the bacteria used these compounds as the carbon sources to build their own biomass. The biomass measured by OD was the highest at 80 mg l^{-1} concentrations of benzene and toluene but decreased when their concentrations were increased because, at high concentrations (320 mg l^{-1}) of both compounds, these substrates become inhibitory factors. Therefore, almost 90% of the benzene and toluene remained.

Table 1
Variations in biomass and percentages of remaining benzene and toluene.

Substrate concentration (ppm)	Remaining (%)		Biomass (optical density)	
	Benzene	Toluene	Initial	Final
20	5	5	0.1	0.16
40	5	5	0.07	0.22
80	5	5	0.1	0.37
120	40	10	0.12	0.3
160	60	40	0.1	0.25
240	65	55	0.07	0.17
320	90	90	0.08	0.1

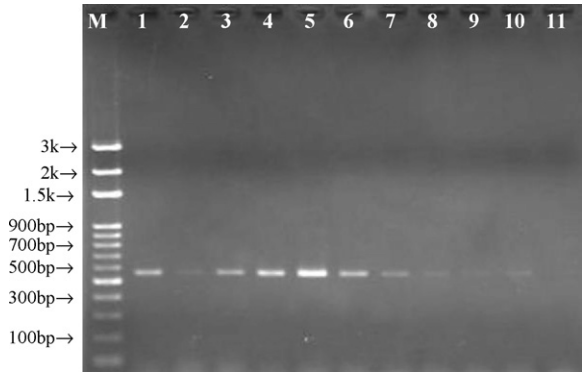

Fig. 3. PCR-amplified 16S rDNA gene fragments obtained from bacterial DNA, using primer pairs EUB1 and UNV2. Lane M: marker (Gen 100 DNA ladder, GM-1000, AB-gene); lane 1: no dilution of microorganisms (original); lane 2: dilution of microorganisms and placement into PRB system; lanes 3–9: concentrations of BTEX (20, 40, 80, 120, 160, 240 and 320 mg l^{-1}, respectively); lane 10: positive control; lane 11: negative control.

Fig. 3, depicting the agarose gel electrophoresis for PCR products, shows a consistent increase in PCR product concentrations (i.e., higher brightness of band) in the respective lanes for benzene and toluene at 20, 40, 80 mg l^{-1}, and a decrease in PCR product concentrations at 120, 160, 240 and 320 mg l^{-1}. It appears that the benzene- and toluene-degrading microbial communities were largest at 80 mg l^{-1} substrate condition and the growth of microbes was inhibited by higher substrate concentrations. This finding is consistent with the results listed in Table 1, wherein the final optical density at the levels of 160, 240 and 320 mg l^{-1} was lower than at 80 mg l^{-1}.

The real-time PCR method monitors the amount of PCR products as they are modified in real-time conditions. The initial concentration of catechol 2,3-dioxygenase gene in different substrate concentrations can be estimated from the change of PCR products throughout the amplified cycles. The results from a real-time PCR revealed that the concentration of functional gene increases progressively before 80 mg l^{-1} of substrate and decreases when substrate concentration increases (Table 2). This conversion conforms to the change of microbial biomass. The highest amount of functional gene was appeared at the 80 mg l^{-1} of benzene and toluene. This achievement again strengthened the

Table 2
Biomass in batch tests using real-time PCR.

Sample no.	Test conditions	Calculated concentration (copies per μl)	C_t[b]
1	SD[a] 10^0	1.449×10^0	28.85
2	SD[a] 10^{-1}	6.623×10^{-2}	28.52
3	SD[a] 10^{-2}	6.763×10^{-3}	30.50
4	SD[a] 10^{-3}	1.489×10^{-3}	31.82
5	20 ppm	1.123×10^{-2}	30.06
6	40 ppm	2.574×10^{-2}	29.34
7	80 ppm	1.380×10^{-1}	27.89
8	120 ppm	4.537×10^{-3}	30.85
9	240 ppm	5.870×10^{-3}	30.63
10	320 ppm	4.372×10^{-3}	30.88
11	NTC[c]	–	–

[a] SD (standard): a sample of known concentration used to construct a standard curve by running standards of varying concentrations.
[b] C_t (threshold cycle): fractional cycle number at which fluorescence passes the fixed threshold.
[c] NTC (no template control): a sample not containing a template, used to verify amplification quality.

Table 3
Series dilution rate of DO_0 and DO_5.

Dilution rate (iterations)	DO_0 (mg l^{-1})	DO_5 (mg l^{-1})	$DO_0 - DO_5$ (mg l^{-1})	Condition 1[b]	Condition 2[c]
Control (0)	7.4	7.3	0.1[a]	–	–
6	7.4	0.2	7.2	N	Y
10	7.4	2.8	4.6	Y	Y
30	7.4	6.2	1.2	Y	N
60	7.4	7.0	0.4	Y	N

[a] For a control sample, the value of '$DO_0 - DO_5$' should be less than 0.2 mg l^{-1} (testing guidelines specified in NIEA W510.54B).
[b] Condition 1: remaining dissolved oxygen should be higher than 1 mg l^{-1}.
[c] Condition 2: consumption of dissolved oxygen should be higher than 2 mg l^{-1}.

results shown in Table 1 and Fig. 3. It should be noted that the benzene- and toluene-degraders can degrade substrates only within the 20–80 mg l^{-1} range. However, benzene and toluene become inhibitory compounds for bacteria at high concentrations. Melting curve experiments indicated that all of these PCR products consisted of the same DNA fragment, indicating good specificity and efficiency of the amplification primers and reactions (data not shown).

3.3. Oxygen production

To ascertain that ORC beads can provide sufficient oxygen for bacterial activities, three water samples were collected from locations # 3-a, # 3-b and # 3-c after seven days' application of ORCs in a PRB system. These three samples were then mixed and used as a bacterial source for biochemical oxygen demand (BOD) evaluation. The mixed sample was further diluted 0, 6, 10, 30 and 60 times and kept at a constant temperature of 20 °C. After five days of incubation, the changes in DO content in these samples (i.e., DO_5), were 7.3, 0.2, 2.8, 6.2 and 7.0 mg l^{-1}, respectively, as listed in Table 3. According to the BOD_5 testing guidelines [27], the DO consumption of a blank sample cannot exceed 0.2 mg l^{-1}. Moreover, the DO consumption should be higher than 2 mg l^{-1} and the remaining DO should be higher than 1 mg l^{-1}.

On the basis of the testing guidelines (NIEA W510.54B) and the DO data listed in Table 3, only the 10-iteration dilution rate meets the requirement. With this ratio of dilution, BOD_5 was calculated as 45.8 mg l^{-1}, thereby indicating that the DO consumption was 9.2 mg l^{-1} d^{-1}. For 7.94 l water content in the void of sands in PRB, the DO demand was 73 mg-O_2 d^{-1}. When using 800 g of ORC containing 40% CaO_2, the oxygen-releasing rate was 0.25 mg-O_2 d^{-1} g^{-1}-ORC (data from column tests). In the entire PRB system, oxygen was thus released at a rate of 200 mg-O_2 d^{-1}. This value (200 mg-O_2 d^{-1}) is thus 2.7 times higher than the DO demand (73 mg-O_2 d^{-1}) in the BOD_5 test. Compared to the DO consumption while using BTEX as substrates, the DO consumption is further higher in BOD_5 test which is using rich substrates such as glucose. These data indicate that the amount of ORC used can provide sufficient oxygen for the bacterial demands, offer an aerobic environmental condition and increase the efficiency in decomposing the pollutants.

3.4. Efficiencies in BTEX removal from a PRB system

Fig. 4 plots the efficiencies in removing BTEX from a PRB system during a 100-d operation. For the first 43 days, the average efficiencies were in the following ascending order: ethylbenzene > toluene> *p*-xylene > benzene. On days 44 and 52, shock-loadings were exercised with BTEX concentrations increasing from 30 mg l^{-1} to 60 mg l^{-1} for four hours, after which the concentrations of these compounds were reduced to the original 30 mg l^{-1} level. In the first shock-loading (solid line), there was no obviously downward trend in the removal of BTEX, a phenomenon thought to be due to the acclimation of bacteria with the environmental conditions inside the PRB system. After the second shock-loading (vertical dotted line), the efficiencies in removing benzene, toluene, ethylbenzene and *p*-xylene from this system were clearly reduced to minimums of 32, 44, 75 and 75%, respectively. Compared to the pre-shock-loading, these declined by approximately 10–21% but subsequently and slowly increased to a stable level.

3.5. Dissolved oxygen analysis

Fig. 5 shows the variations in DO distribution in samples from monitored wells in a PRB system during a 100-d operation, indicating that the DO content was the highest at location #2, and followed by locations # 3, # 4 and # 1. Because location #2 is downgradient and most close to ORC barrier, the elevated DO content was observed. However, the DO decreasing rate at location #2 was the largest as shown in Fig. 5. It is believed that microbial activity and biomass growth occurred when oxygen, microbes and BTEX first meet at location #2. The DO content was the lowest at location #1, a result attributable to the lower diffusion rate of the oxygen released from the ORC than that of the flow-rate of the groundwa-

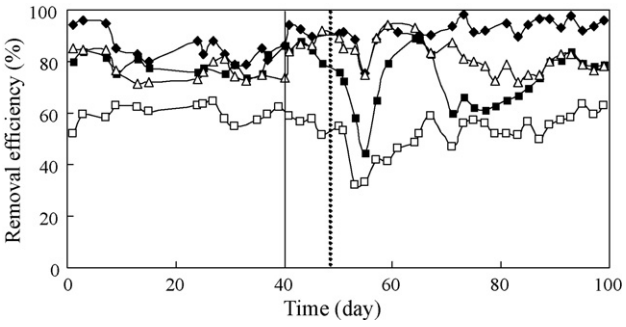

Fig. 4. BTEX removal efficiencies in a long-term study. Vertical solid line (–) first shock-loading; vertical dotted line (- - -): second shock-loading; (□) benzene; (■) toluene; (♦) ethylbenzene; (△) *p*-xylene.

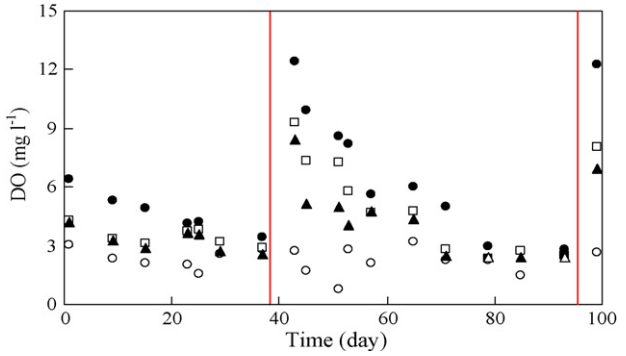

Fig. 5. Variations in release of dissolved oxygen from ORC in a long-term study. (○) #1 (−5 cm); (●) #2 (5 cm); (□) #3 (15 cm); (▲) #4 (30 cm); ( ) replacement of ORC

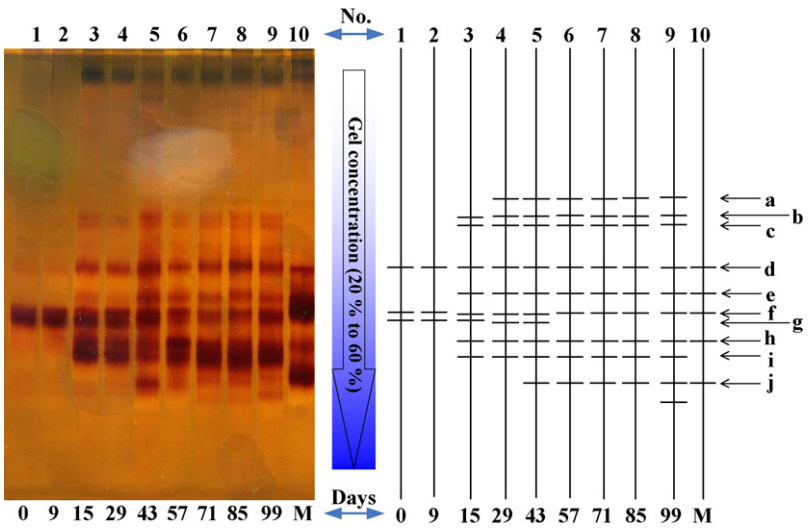

Fig. 6. DGGE profiles of 16S rDNA-defined bacterial populations for groundwater samples at site #3 in a PRB system.

ter. Along with the flow direction, the DO content was gradually decreased, probably due to oxygen consumption by the microorganism downstream. The DO content during the first 37 days was significantly reduced at location #2, especially from days 29 to 37, the difference in DO being lower than 1 mg l^{-1} between locations #1 and #2. To provide sufficient oxygen, the ORC was replaced on day 38. The DO content at location #2 on day 43 was 12.46 mg l^{-1}, being stable (5.03–5.62 mg l^{-1}) from days 57 to 71, then gradually declining. The ORC was again replaced on day 94, the DO content being 12.24 mg l^{-1} on day 99. We therefore concluded that an ORC can be used for approximately 40 days, thus ensuring a DO release sufficient to maintain microbial growth.

3.6. Microbial community dynamic

Fig. 6 illustrates the microbial community distribution in the PRB system from the water samples taken at location #3 on days 0, 9, 15, 29, 43, 57, 71, 85 and 99, profiled by DGGE as numbers 1–9, respectively. The PCR-DGGE patterns in this figure indicate that, in the earlier days (lanes 1 and 2), the dominant bacteria were those represented by bands d, f and g. Until day 43, the number of species (at least ten in the system) abundantly increased (lane 5). On day 57, band g disappeared after duplicate shock-loading. Lane 6 showed only nine bands, each representing a strain of bacteria for degrading the BTEX compounds. The disappearance of band g is attributable to the shock-loading, which tends to cause partial disappearance of microorganisms. On days 71, 85 and 99 (lanes 7–9), the existing bands were a, b, c, e, f, h, i and j, thus indicating that the microbial community had stabilized. The marker (lane 10) included bands d, e, f, h and j, representing the known standard strains. When the PRB system was operated in a long-term for 30 mg l^{-1} BTEX, the microbial community did not show the change of diversity in a short period of shock-loading as compared with Figs. 4 and 6. However, when a shock-loading at higher concentration of BTEX is exercised, the microbes may be inhibited, thereby reducing the BTEX removal efficiency.

The real-time PCR results listed in Table 4 reveal the changes in the biomass of the sampled bacteria over time. On days 37, 43, 45 and 51, the increases in the biomass were 9.711×10^{-3}, 8.166×10^{-2}, 1.289×10^{-1} and 1.611×10^{-1} copies per μl, respectively. However after a shock-loading, a certain number of bacteria could not survive and were therefore washed out from the system. The biomass then decreased to 1.264×10^{-1}, 6.756×10^{-2} and 2.601×10^{-2} copies per μl on days 53, 57 and 65, respectively. On day 80, the microorganisms were able to adapt to their surroundings; hence, the number of copies was increased to 3.197×10^{-2} per μl. This finding is consistent with the results obtained from BTEX removal.

Table 4
Biomass in a PRB system using real-time PCR.

Sample no.	Test conditions	Calculated concentration (copies per μl)	C_t[b]
1	SD[a] 10^0	1.362×10^0	24.80
2	SD[a] 10^{-1}	7.347×10^{-2}	28.81
3	SD[a] 10^{-2}	3.784×10^{-3}	39.72
4	SD[a] $10^{-2.5}$	9.012×10^{-3}	31.68
5	SD[a] 10^{-3}	7.325×10^{-2}	31.97
6	Day 37	9.711×10^{-3}	31.58
7	Day 43	8.166×10^{-2}	28.66
8	Day 45	1.289×10^{-1}	28.02
9	Day 51	1.611×10^{-1}	27.73
10	Day 53	1.264×10^{-1}	28.06
11	Day 57	6.756×10^{-2}	28.92
12	Day 65	2.601×10^{-2}	30.23
13	Day 79	3.197×10^{-2}	29.95
14	NTC[c]	–	–

[a] SD (standard): a sample of known concentration used to construct a standard curve by running standards of varying concentrations.
[b] C_t (threshold cycle): fractional cycle number at which fluorescence passes fixed threshold.
[c] NTC (no template control): a sample not containing template, used to verify amplification quality.

4. Summary

This study has shown the microbial community dynamics in a PRB using a real-time PCR technique. The recommended ratio of CaO_2 in the ORC was 40% (w/w), obtained from column tests. In this research, the BTEX removal efficiencies were high, being consistent with the results obtained by Thiruvenkatachari et al. [11]. The removal efficiencies for these compounds were as follows: ethylbenzene > toluene > *p*-xylene > benzene and *p*-xylene > ethylbenzene > benzene > toluene before and after shock-loading, respectively, in ascending order. In this PRB system, the occurrence of aerobic degradation was verified by the consumption of BTEX and the differences in DO levels in the effluent compared to the influent. Moreover, the changes in the microbial community structure were ascertained by agarose gel electrophoresis, DGGE and real-time PCR. The results from this

study can be useful in designing a PRB system for field remediation of petroleum hydrocarbons in contaminated groundwater.

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References

[1] L. Yerushalmi, M.F. Manuel, S.R. Guiot, Biodegradation of gasoline and BTEX in a microaerophilic biobarrier, Biodegradation 10 (1999) 341–352.
[2] S. Saponaro, M. Negri, E. Sezenna, L. Bonomo, C. Sorlini, Groundwater remediation by an *in situ* biobarrier: a bench scale feasibility test for methyl tert-butyl ether and other gasoline compounds, J. Hazard. Mater. 167 (2009) 545–552.
[3] L. Mater, R.M. Sperb, L.A.S. Madureira, A.P. Rosin, A.X.R. Correa, C.M. Radetski, Proposal of a sequential treatment methodology for the safe reuse of oil sludge-contaminated soil, J. Hazard. Mater. 136 (2006) 967–971.
[4] G. Mascolo, R. Ciannarella, L. Balest, A. Lopez, Effectiveness of UV-based advanced oxidation processes for the remediation of hydrocarbon pollution in the groundwater: a laboratory investigation, J. Hazard. Mater. 152 (2008) 1138–1145.
[5] T. Garoma, M. Gurol, O. Osibodu, L. Thotakura, Treatment of groundwater contaminated with gasoline components by an ozone/UV process, Chemosphere 73 (2008) 825–831.
[6] Y. Zang, R. Farnood, Effects of hydrogen peroxide concentration and ultraviolet light intensity on methyl tert-butyl ether degradation kinetics, Chem. Eng. Sci. 60 (2005) 1641–1648.
[7] S. Dagley, Microbial catabolism, the carbon cycle and environmental pollution, Naturwissenschaften 65 (1978) 85–95.
[8] H. Junca, D.H. Pieper, Functional gene diversity analysis in BTEX contaminated soils by means of PCR-SSCP DNA fingerprinting: comparative diversity assessment against bacterial isolates and PCR-DNA clone libraries, Environ. Microbiol. 6 (2004) 95–110.
[9] S.J. Liu, B. Jiang, G.Q. Huang, X.G. Li, Laboratory column study for remediation of MTBE-contaminated groundwater using a biological two-layer permeable barrier, Water Res. 40 (2006) 3401–3408.
[10] O. Gibert, S. Pomierny, I. Rowe, R. Kalin, Selection of organic substrates as potential reactive materials for use in a denitrification permeable reactive barrier (PRB), Bioresour. Technol. 99 (2008) 7587–7596.
[11] R. Thiruvenkatachari, S. Vigneswaran, R. Naidu, Groundwater protection from cadmium contamination by permeable reactive barriers, J. Hazard. Mater. 160 (2008) 428–434.
[12] A.S. Ferguson, W.E. Huang, K.A. Lawson, R. Doherty, O. Gibert, K.W. Dickson, A.S. Whiteley, L.A. Kulakov, I.P. Thompson, R.M. Kalin, M.J. Larkin, Microbial analysis of soil and groundwater from a gasworks site and comparison with a sequenced biological reactive barrier remediation process, J. Appl. Microbiol. 102 (2007) 1227–1238.
[13] O. Gibert, A.S. Ferguson, R.M. Kalin, R. Doherty, K.W. Dickson, K.L. McGeough, J. Robinson, R. Thomas, Performance of a sequential reactive barrier for bioremediation of coal tar contaminated groundwater, Environ. Sci. Technol. 41 (2007) 6795–6801.
[14] A.R. Gavaskar, Design and construction techniques for permeable reactive barriers, J. Hazard. Mater. 68 (1999) 41–71.
[15] R.T. Wilkin, R.D. Ludwig, R.G. Ford, Workshop on monitoring oxidation-reduction processes for ground-water restoration, Workshop Summary, April 25–27, 2000, Dallas, Texas, United States Environmental Protection Agency.
[16] G.C. Bianchi-Mosquera, R.M. Allen-King, D.M. Mackay, Enhanced degradation of dissolved benzene and toluene using a solid oxygen-releasing compound, Ground Water Monit. R. 14 (1994) 120–128.
[17] C.M. Kao, S.C. Chen, J.Y. Wang, Y.L. Chen, S.Z. Lee, Remediation of PCE-contaminated aquifer by an *in situ* two-layer biobarrier: laboratory batch and column studies, Water Res. 37 (2003) 27–38.
[18] L. Vezzulli, C. Pruzzo, M. Fabiano, Response of the bacterial community to *in situ* bioremediation of organic-rich sediments, Mar. Pollut. Bull. 49 (2004) 740–751.
[19] D.P. Cassidy, R.L. Irvine, Use of calcium peroxide to provide oxygen for the contaminant biodegradation in a saturated soil, J. Hazard. Mater. 69 (1999) 25–39.
[20] J. Dong, Y. Zhao, W. Zhang, M. Hong, Laboratory study on sequenced permeable reactive barrier remediation for landfill leachate-contaminated groundwater, J. Hazard. Mater. 161 (2009) 224–230.
[21] C.W. Lin, C.Y. Lai, L.H. Chen, W.F. Chiang, Microbial community structure during oxygen-stimulated bioremediation in phenol-contaminated groundwater, J. Hazard. Mater. 140 (2007) 221–229.
[22] C.M. Kao, C.Y. Chen, S.C. Chen, H.Y. Chien, Y.L. Chen, Application of *in situ* biosparging to remediate a petroleum-hydrocarbon spill site: field and microbial evaluation, Chemosphere 70 (2008) 1492–1499.
[23] S.B. Wang, Q. Li, W.J. Liang, Y. Jiang, S.W. Jiang, PCR-DGGE analysis of nematode diversity in Cu-contaminated soil, Pedosphere 18 (2008) 545–553.
[24] P. Hu, G. Zhou, X. Xu, C. Li, Y. Han, Characterization of the predominant spoilage bacteria in sliced vacuum-packed cooked ham based on 16S rDNA-DGGE, Food Control 20 (2009) 99–104.
[25] M. Filion, M. St-Arnaud, S.H. Jabaji-Hare, Direct quantification of fungal DNA from soil substrate using real-time PCR, J. Microbiol. Methods 53 (2003) 67–76.
[26] B. Andersen, J. Smedsgaard, I. Jorring, P. Skouboeb, L.H. Pedersen, Real-time PCR quantification of the AM-toxin gene and HPLC qualification of toxigenic metabolites from Alternaria species from apples, Int. J. Food Microbiol. 111 (2006) 105–111.
[27] NIEA, National Institute of Environmental Analysis, Environmental Analysis Laboratory.http://www.niea.gov.tw/niea/WATER/W51054B.htm, Executive Yuan, Taiwan, ROC.
[28] D. Kim, J.C. Chae, J.Y. Jang, G.J. Zylstra, Y.M. Kim, B.S. Kang, E. Kim, Functional characterization and molecular modeling of methylcatechol 2,3-dioxygenase from o-xylene-degrading *Rhodococcus* sp. strain DK17, Biochem. Biophys. Res. Commun. 326 (2005) 880–886.
[29] J.C. Chae, E. Kim, E. Bini, G.J. Zylstra, Comparative analysis of the catechol 2,3-dioxygenase gene locus in thermoacid- ophilic archaeon *Sulfolobus solfataricus* strain 98/2, Biochem. Biophys. Res. Commun. 357 (2007) 815–819.
[30] D. Ercolini, PCR-DGGE fingerprinting: novel strategies for detection of microbes in food, J. Microbiol. Methods 56 (2004) 297–314.
[31] B. Hendrickx, W. Dejonghe, F. Faber, W. Boënne, L. Bastiaens, W. Verstraete, E.M. Top, D. Springael, PCR-DGGE method to assess the diversity of BTEX mono-oxygenase genes at contaminated sites, FEMS Microbiol. Ecol. 55 (2006) 262–273.
[32] D. Radojkovic, J. Kusic, Silver staining of denaturing gradient gel electrophoresis gels, Clin. Chem. 46 (2000) 883–884.