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Semicontinuous microcosm study of aerobic cometabolism of trichloroethylene using toluene

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Abstract

A semicontinuous slurry-microcosm method was applied to mimic trichloroethylene (TCE) cometabolic biodegradation field results at the Que–Jen in-situ pilot study. The microcosm study confirmed the process of aerobic cometabolism of TCE using toluene as the primary substrate. Based on the nucleotide sequence of 16S rRNA genes, the toluene-oxidizing bacteria in microcosms were identified, i.e. *Ralstonia* sp. P-10 and *Pseudomonas putida*. The first-order constant of TCE-degradation rate was 0.5 day^{-1} for both *Ralstonia* sp. P-10 and *P. putida*. The TCE cometabolic-biodegradation efficiency measured from the slurry microcosms was 46%, which appeared pessimistic compared to over 90% observed from the in-situ pilot study. The difference in the TCE cometabolic-biodegradation efficiency was likely due to the reactor configurations and the effective time duration of toluene presence in laboratory microcosms (1 days) versus in-situ pilot study (3 days). The results of microcosm experiments using different toluene-injection schedules supported the hypothesis. With a given amount of toluene injection, it is recommended to maximize the effective time duration of toluene presence in reactor design for TCE cometabolic degradation.

Keywords: Microcosm; In-situ; Trichloroethylene; Toluene; Aerobic cometabolism

1. Introduction

Trichloroethylene (TCE) was widely used as a solvent in the later part of the 20th century. As a result of improper disposal, TCE caused contaminated groundwater and soil. Various treatment techniques, such as air sparging [1–3], chemical oxidation [4,5], and biodegradation [6,7], have been developed to remediate TCE-contaminated groundwater. TCE is resistant to biodegradation because it cannot be used as an energy and growth substrate by organisms [8,9]. Wilson and Wilson [8] first found that TCE might be susceptible to cometabolism by soil communities fed natural gas. Cometabolism is a process that microorganisms growing on the primary substrate produce enzyme and fortuitously transform a secondary substrate, from which microorganisms cannot obtain energy for growth [9]. Effective cometabolic transformations of TCE has been demonstrated by aerobic microorganisms growing on methane [10,11], phenol [12–15], and toluene [6,7,14–16]. The toluene-grown TCE-degrading bacteria such as, *Pseudomonas putida* [17], *Burkholderia cepacia* G4, *Ralstonia pickettii* PKO1, and *Pseudomonas mendocina* KR1 [18], are reported in the literature.

TCE in groundwater was efficiently biodegraded by aerobic cometabolism in the presence of toluene at the Que–Jen in-situ pilot study [7]. The pilot was constructed with a 30 cm wide, 12 m long, and 1 m deep trench. The pilot was then filled with clean coarse river sand and followed with a layer of silt about 15 cm thick and a concrete cover about 5 cm thick (Fig. 1). An indigenous toluene-utilizing biological population was developed in the presence of TCE via pulse-injection for toluene-vapor and air. Over 90% removal of TCE was achieved with primary substrate (toluene) degraded to a concentration below 10 μg/L.

Using the same clean river sand employed for aquifer material at the Que–Jen in-situ pilot study without any pretreatment as an inoculum, a semicontinuous slurry microcosm method was studied to: (1) mimic TCE cometabolic biodegradation field results at the Que–Jen in-situ pilot study, (2) compare the

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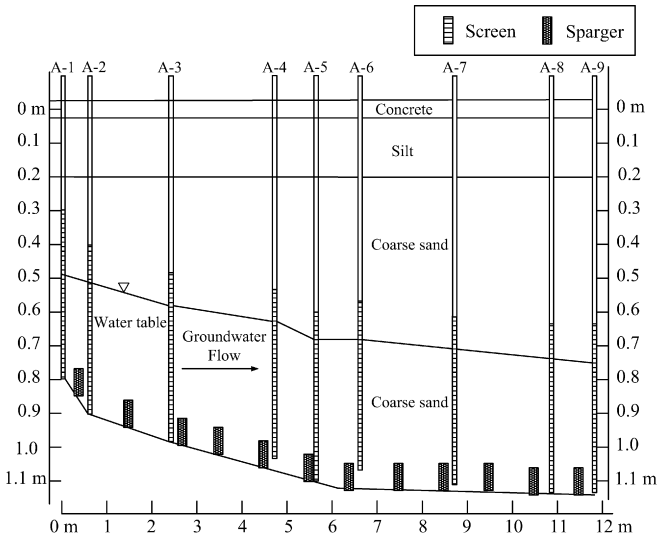

Fig. 1. Schematic diagram of Que–Jen in-situ pilot (adapted from Kuo et al. [7]).

TCE removals measured from the microcosm experiments to those estimated from the pilot study, (3) identify the toluene-grown TCE-degrading bacteria using molecular methods based on the nucleotide sequence of 16S rRNA genes and compare the results with the literature information, and (4) conduct microcosm experiments using various toluene-injection schedules to interpret the difference in the TCE cometabolic-biodegradation efficiency observed between the slurry microcosms and the in-situ pilot study.

2. Materials and methods

2.1. Slurry microcosms

To evaluate TCE cometabolic biodegradation, a modified method of semicontinuous slurry microcosms described by Jenal-Wanner and McCarty [15] was adopted. The same clean river sand employed for aquifer material at the Que–Jen in-situ pilot study [7] without any pretreatment was used as an inoculum in microcosms. Fig. 1 shows the schematic diagram for the Que–Jen in-situ pilot and the steady-state water-table profile maintained by an injection well (A-1) and an extraction well (A-9) located 11.84 m apart. Air and toluene-vapor were distributed into the groundwater through spargers uniformly spaced at the bottom of the pilot to establish a toluene-degrading consortium in the pilot. Seven wells were used to monitor the TCE cometabolic degradation in the ground water.

Sixteen sterile, 64 mL screw cap glass bottles were first added with aquifer material (23 g/bottle, dry weight) and then filled without headspace using sterile oxygen-containing (≈32 mg/L) nutrient solution (55 mL/bottle). Pure oxygen was bubbled through a sterile nutrient solution to achieve oxygen saturation (≈32 mg/L). The high initial DO concentration was used for maintaining aerobic environment for microcosms. The composition of the nutrient solution was as follows (mg/L): $(NH_4)_2SO_4$, 4.7; K_2HPO_4, 1.23; Na_2HPO_4, 3.57; $MgSO_4{\cdot}7H_2O$, 0.62; $CaSO_4{\cdot}2H_2O$, 0.0031; $FeSO_4{\cdot}7H_2O$, 0.0062; $ZnSO_4{\cdot}7H_2O$, 0.0062; $MnSO_4{\cdot}H_2O$, 0.0031; $CuSO_4{\cdot}5H_2O$, 0.0062; $Na_2MoO_4{\cdot}2H_2O$, 0.0062. The microcosms were capped with Teflon-lined silicon septa and screw caps.

2.2. Incubation of microcosms

Microcosms were incubated inverted in the dark at aquifer temperature of the pilot study (≈25 °C) on a shaker at 150 rpm. The incubation periods were 14 days for the first incubation, and alternating 4 and 3 days of incubations thereafter. No TCE was added in the first two incubations. Fluid and chemicals were periodically replenished as follows. Microcosms were settled for 30 min, 40 mL of clear supernatant was removed and replaced with oxygen-containing (≈32 mg/L) nutrient solution, respiked with the primary substrate (toluene) and TCE, and reincubated as described above.

A total of sixteen microcosms were fed toluene as the primary substrate in the first 11 incubations. After the 11th incubation, the feeding of toluene was terminated to four microcosms which were used as the control to evaluate the TCE loss due to leakage and adsorption. The 12 replicate microcosms were toluene-fed so that a kinetic study can be conducted to evaluate the TCE biodegradation rate during a given incubation period when steady-state TCE-removal phase is attained. The experimental design for the non-toluene-fed controls in this study is different from Jenal-Wanner and McCarty [15]. The non-toluene-fed controls in Jenal-Wanner and McCarty method [15] started from the first incubation. The non-toluene-fed controls in this study started from the 11th incubation when a toluene-utilizing consortium was developed in all the microcosms. The non-toluene-fed controls in this study were used to: (1) evaluate the TCE loss due to leakage and adsorption, and (2) estimate the cometabolic biodegradation of TCE by the toluene-utilizing bacteria by comparing the difference between the toluene-fed microcosms and non-toluene-fed controls.

With each replenishment, toluene (1380 μL of a 400 mg/L toluene stock solution) was added to the microcosms. In order to avoid possible substrate toxicity, with each replenishment, toluene was added in three separate pulses within the first 12 h of an incubation period, i.e. 3 × 460 μL of a 400 mg/L toluene stock solution. These additions in effect resulted in an initial concentration in the toluene-fed microcosms at 3.7 ± 0.3 mg/L toluene for each of the three pulses. The concentration errors are ±1 standard deviation after simple averaging of samples. The high initial DO concentration at 27 ± 1 mg/L was used for maintaining aerobic environment for microcosms. The limiting factor for the biological process in both the pilot study and laboratory microcosms was toluene.

TCE was added to the microcosms from the third incubation on. At each replenishment, 200 μL of a 132 mg/L TCE stock water solution were added to the microcosms in one single pulse resulting in an average initial TCE concentration in the microcosms at 546 ± 69 μg/L, which was close to the TCE influent concentration used in the pilot study (430 ± 119 μg/L).

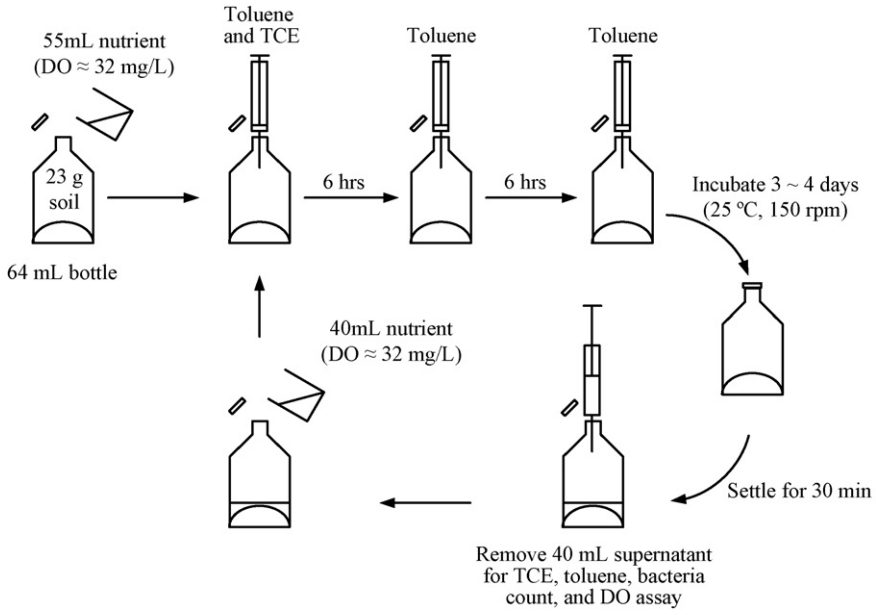

Fig. 2. Schematic diagram of semicontinuous slurry microcosm method used for a typical toluene-fed microcosm in one incubation period.

Fig. 2 shows the schematic diagram of semicontinuous slurry microcosm method used in this study for a typical toluene-fed-microcosm in one incubation period. The semicontinuous liquid exchange and reincubation noted above was repeated over 41 times. Steady state in terms of TCE-removal was achieved after the 20th incubation.

2.3. Microcosm sampling

Microcosms were sampled for DO measurements, toluene and TCE analyses, and the enumeration of the active microbial population at the end of each incubation period (40 mL). Since the culture DO concentration was above air saturation, and TCE and toluene are volatile, efforts were made to accomplish sampling as quickly as possible when the screw caps were removed from microcosms. Immediately after sampling, volumes removed were carefully replaced with sterile oxygen-containing nutrient solution in order to prevent formation of a head space.

2.4. Chemical analytical methods

Dissolved oxygen was measured by a DO probe (YSI 5100). TCE and toluene were measured using a gas chromatograph (HP 6890 GC) with a J&W Scientific DB1, 60 m, 0.32 mm i.d. capillary column, and mass selective detector (HP 5973 MSD). Zero-grade helium carrier gas flow rate was 25 mL/min at a head pressure of 4.24psi g. The temperatures of injector and detector were 200 and 265 °C, respectively. A purge and trap (Tekmar Velocity XPT with a Tekmar Purge Trap K) was used. Samples of 5 mL were added to sampling tubes and purged for 11 min at 35 °C. Desorption preheat was at 265 °C and desorption time was 4 min at 270 °C. The trap lines and valves were kept constant at 150 °C and the injection port was 265 °C. The detection limits for TCE and toluene were 2.6 μg/L and 1.1 μg/L, respectively.

2.5. Microbiology methods

The spread plate method was used for the quantification of the active microbial population on the nutrient broth (NB) agar plates [19] at 37 °C for 48 h. A total of 11 colonies of pure cultures were obtained by streak plate method and they were identified by 16S rRNA molecular methods.

The genomic DNA of isolated strains was extracted from the culture samples by the Miller method [20]. PCR amplification was used EX-Taq (TAKARA) and performed using RoboCycler Gradient 96 (Stratagene). The universal PCR primer set 11F (5′-GTTTGATCCTGGCTCAG-3′) and 1512R (5′-GGY(T/C)TACCTTGTTACGACTT-3′) was used to amplify the bacterial 16S rRNA gene [21]. The bacterial PCR was started with an initial denaturation for 3 min at 95 °C. A total of 28 cycles, each including 90 s at 95 °C, 45 s at 52 °C, and 120 s at 72 °C, was followed by a final extension step of 3 min at 72 °C. PCR products (16S rRNA) were construed by tT&A cloning kit (Yeastern Biotech Co.). The clones were digested with *Hap* II in a total volume of 10 μL containing 100–200 ng amplified DNA, 10 U of enzyme, and 1 μL of 10× digestion buffer recommended by the manufacturer; incubation was at 37 °C for 3 h. The nucleotide sequence data were obtained from the Automated Sequencing Core Laboratory at the National Cheng Kung University and have been deposited in the NCBI nucleotide sequence database (National Center for Biotechnology Information; www.ncbi.nlm.nih.gov).

2.6. Evaluation of toluene-oxidizing bacteria using pure culture

Each isolated pure culture was stocked into a 2-mL eppendorf with the 20% glycerol at –20 °C. A re-streak with pure culture stock was performed on NB plate at 37 °C for isolation and activation. Isolated pure culture was acclimated and

enriched in the nutrient solution containing 10 mg/L toluene and 32 mg/L dissolved oxygen. Each acclimated pure culture was inoculated into microcosms without the soils to evaluate the TCE removal efficiency and biodegradation rate. Two types of microcosm experiments were conducted to confirm that each toluene-oxidizing bacterium could biodegrade TCE individually by aerobic cometabolism. In one type of experiments, triplicate microcosms for each individual acclimated toluene-oxidizing bacterium were used to evaluate TCE and toluene removals at the end of 7 days. In another type of experiments, a total of 16 microcosms for each individual acclimated toluene-oxidizing bacterium were analyzed at various times to evaluate the biodegradation rates and cometabolic kinetics of TCE and toluene during an incubation period of 3 days. TCE leakage controls were used to evaluate the loss of TCE due to leakage.

3. Results and discussion

3.1. Oxygen demand for toluene biodegradation

Previous studies showed that the availability of oxygen was a major concern for the cometabolism of TCE using toluene as the primary substrate [15,22]. An overall stoichiometry equation can be written for toluene oxidation and biosynthesis reactions, assuming that an empirical cell formula of $C_5H_7O_2N$ [23] applies:

$$C_6H_5CH_3 + 9f_eO_2 + \frac{9f_s}{5}(HCO_3^- + NH_4^+) \rightarrow \frac{9f_s}{5}C_5H_7O_2N + \left(7 - \frac{36f_s}{5}\right)CO_2 + \left(18f_e + \frac{81f_s}{5} - 14\right)H_2O \quad (1)$$

where f_e represents the fraction of toluene oxidized and f_s is the fraction synthesized and equals $1-f_e$. Estimates of oxygen consumption at 6.0 ± 0.1 and 4.5 mole-oxygen/mole-toluene were reported by Jenal-Wanner and McCarty [15] and Lu and Lee [24], respectively. During steady state operation, oxygen consumption was determined as 5.1 ± 0.6 mole-oxygen/mole-toluene in this study. Since 9 mol of oxygen are required for complete oxidation, f_e here equals 0.5. The stoichiometry of toluene biodegradation using ammonium as the nitrogen source and oxygen as the electron acceptor can be written as follows.

$$C_6H_5CH_3 + 5.10_2 + 0.8(HCO_3^- + NH_4^+) \rightarrow 0.8C_5H_7O_2N + 3.9CO_2 + 3.2H_20 \quad (2)$$

The above empirical stoichiometry equation indicates that the oxygen demand for the toluene removal would be 1.79 mg-oxygen/mg-toluene. The amount of oxygen present in each replenishment solution was 1.28 mg-O_2 (40 mL × 32 mg/L). The amount of toluene present in each replenishment solution was 0.552 mg-toluene (1380 μL × 400 mg/L). The oxygen demand for biodegrading 0.552 mg-toluene was 0.99 mg-O_2. Therefore, the amount of oxygen present in each replenishment solution was quantitatively large enough for the biodegradation of toluene. Oxygen supply should not be the limiting factor for the biological process in the microcosms.

3.2. TCE removal in slurry microcosms

Laboratory microcosms at the steady state of TCE removal were used to measure TCE removal efficiency. Fig. 3a shows the TCE removal efficiency obtained from laboratory microcosms for 41 successive incubations which totals 143 days. The difference in TCE removal between toluene-fed microcosms and non-toluene-fed-controls confirmed the process of cometabolic biodegradation. Fig. 3a and Table 1 show that during the steady state of TCE removal, the difference in the TCE-removal between toluene-fed microcosms and non-toluene-fed controls was 46%, which is the TCE removal efficiency due to cometabolic biodegradation. When the feeding of toluene was terminated to non-toluene-fed-controls, the toluene-utilizing bacteria could not effectively cometabolize TCE. The 15% TCE removal observed in non-toluene-fed controls might be due to adsorption on sand matrix and biomass or due to possible leakage.

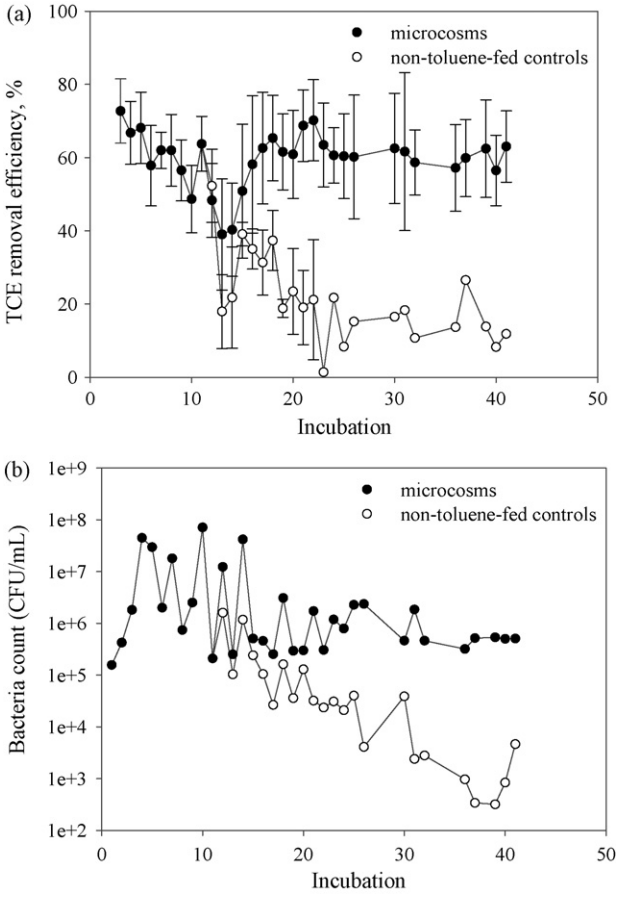

Fig. 3. (a) TCE percentage removal in toluene-fed microcosms and non-toluene-fed controls. The error bars indicate ±1 standard deviation after simple averaging of samples. (b) Biomass in toluene-fed microcosms and non-toluene-fed controls.

Table 1
Summary of TCE and toluene removals observed in slurry microcosms[a] and in-situ pilot study[b]

	In-situ[b]	Toluene-fed microcosms[c]	Non-toluene-fed controls[c]
Initial TCE concentration (μg/L)	430 ± 119	546 ± 69	641 ± 70
TCE removal (%)	>90	62 ± 4	15 ± 7
Initial toluene concentration (mg/L)	9.2 ± 1.0	11.2 ± 1.0	0
Toluene removal (%)	100	100	NA[d]
Toluene utilization efficiency (μg-TCE/μg-toluene)	0.042	0.023	NA
TCE-degradation rate coefficient (day^{-1})	2	0.6	NA
Bacteria count (CFU/mL)	10^6–10^8	10^5–10^6	10^2–10^3

[a] The same river sand employed for aquifer material at the Que–Jen in-situ pilot study was used as an inoculum in microcosms.
[b] Results are average and standard deviations over 92 days and 76 days for TCE and toluene, respectively (Kuo et al. [7]).
[c] Results are averages and standard deviations over 15 incubations during the steady state TCE-removal phase.
[d] Not applicable.

Biomass estimation was evaluated by bacteria enumeration of water samples using the spread plate method for all microcosms. The bacteria counts of water samples from toluene-fed microcosms ranged from 1×10^5 to 1×10^6 CFU/mL during the steady-state operation (after the 20th incubation). Fig. 3b also showed that the active biomass started to decline after the termination of toluene feeding; and the activity of toluene oxygenase in the toluene-utilizing bacteria could not be continuously maintained to cometabolize TCE. The bacteria counts in non-toluene-fed controls declined to about 1×10^2 CFU/mL near the 40th incubation.

3.3. TCE biodegradation rate in slurry microcosms

Under steady state conditions, the 38th and 44th incubation were chosen randomly to study TCE-removal and toluene-degradation rates. Each data point on Fig. 4a and b was obtained from the analysis of a single microcosm at a given time. Fig. 4a and b showed the variation of TCE and toluene concentrations versus time for the 38th and 44th incubation, respectively. Fig. 4b suggested that TCE removal appeared to be more significant when toluene was available. After the depletion of toluene, TCE was continuously cometabolized by toluene-utilizing bacteria at a lower rate, as long as active oxygenase remained available. In the 38th incubation, activity of remaining toluene oxygenase in the toluene-utilizing bacteria in phase 2 appeared higher than in the 44th. The entire incubation period consisted of two phases. During phase 1, the primary substrate existed and completely degraded toward the end of phase 1. Without the presence of toluene, oxygenase in toluene-utilizing bacteria could not be continuously maintained to cometabolize TCE [22,24]. As a result, the TCE transformation rate declined in phase 2.

Rate coefficients for TCE degradation during phase 1 are useful for mathematical modeling and predictions of field results. Monod model [25] was used to express the rate of cometabolism as follows [15]:

$$-\frac{dS}{dt} = \frac{k_s S}{K_s + S} X_a \quad (3)$$

$$-\frac{dC}{dt} = \frac{k_c C}{K_{sc} + C} X_a \quad (4)$$

where S (mg/L) and C (μg/L) are the aqueous concentrations of primary substrate (toluene) and contaminant (TCE); X_a (mg/L) is the the aqueous concentration of active biomass; k_s (day^{-1}) and k_c (day^{-1}) represent the maximum rate of the primary substrate and contaminant consumption per unit of active biomass per day; and K_s (mg/L) and K_{sc} (μg/L) are the affinity constants for primary substrate and contaminant, respectively. When the contaminant (TCE) concentration is low, Eq. (4) can be

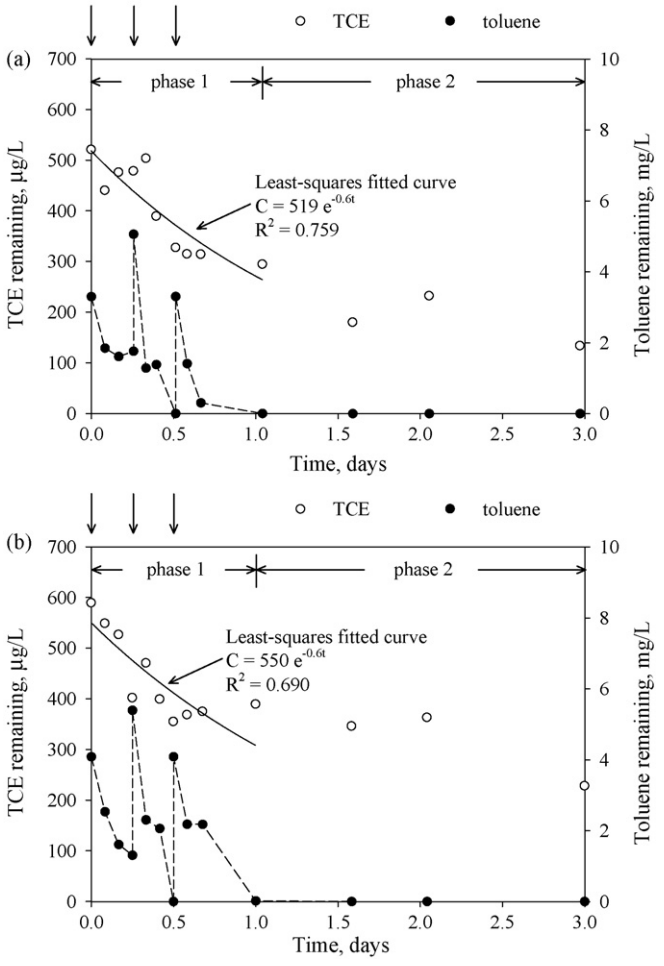

Fig. 4. TCE and toluene remaining in microcosms during an incubation period under steady state conditions. Arrows represent toluene pulses. Each data point refers to a single microcosm. (a) Incubation 38 and (b) Incubation 44.

approximated as follows:

$$-\frac{dC}{dt} \approx \frac{k_c}{K_{sc}} X_a C = k' X_a C \quad \text{when} \quad C \ll K_{sc} \tag{5}$$

If X_a is uniformly distributed in the microcosms and constant over time, Eq. (5) can be integrated for TCE removal over an incubation period as follows:

$$C = C_i e^{-k' X_a t} \tag{6}$$

where C_i is the initial aqueous concentration of TCE (μg/L) and C is the effluent concentration of TCE (μg/L) from the slurry microcosms at a reaction time t (day).

For incubations under steady state conditions, the biodegradation of TCE was the dominant process in slurry microcosms (Fig. 3). To apply Eq. (6) to the slurry microcosms under steady state conditions, both the adsorption and desorption of TCE on the aquifer material were assumed negligible. Under this assumption, the first-order constant of TCE-degradation rate determined with slurry microcosms is an apparent rate constant.

In the presence of toluene and TCE, competitive inhibition of TCE degradation probably occurred. Both the inhibition by toluene to TCE degradation and vice versa were assumed negligible in this study. Lu et al. [22] observed no inhibition by toluene to TCE degradation for an initial toluene concentration up to 5 mg/L. Lee and Liu [28] reported the inhibition by toluene to TCE degradation for an initial toluene concentration at 30 mg/L. For an initial toluene concentration at 3.7 ± 0.3 mg/L for each of the three pulses, competitive inhibition of TCE degradation was unlikely to occur in the microcosms in this study. Due to the low initial concentration of TCE (546 ± 69 μg/L), the inhibition by TCE to toluene utilization was also unlikely to occur in the microcosms in this study.

Fig. 5a shows that toluene was absent most of the time in microcosms for an incubation period of 3 days. Phase 1 in microcosms only lasted for 1 day. Fig. 5a also shows the least-squares fitted curve for TCE concentration versus reaction time with Eq. (6) during phase 1 as follows:

$$C = 517 e^{-0.6t} \tag{7}$$

A correlation coefficient of $R^2 = 0.654$ suggests that the first-order kinetic model is acceptable for the rate equation of cometabolic degradation. This corresponds to the first-order rate coefficient of TCE cometabolic degradation during phase 1, $k'X_a = 0.6\ \text{day}^{-1}$ for the operating condition in microcosms with the bacteria counts of water samples at about 1×10^6 CFU/mL.

3.4. Comparison of TCE removals in slurry microcosms and in-situ pilot

The groundwater flow in the Que–Jen pilot study shown in Fig. 1 was visualized as a plug-flow reactor [7]. Fig. 5b shows the least-squares fitted curve for TCE concentration profile obtained in the Que–Jen in-situ pilot versus residence time with Eq. (6). This corresponds to the first-order rate constant of TCE cometabolic degradation, $k'X_a = 2\ \text{day}^{-1}$ for the operating condition in the Que–Jen in-situ pilot with the bacteria counts

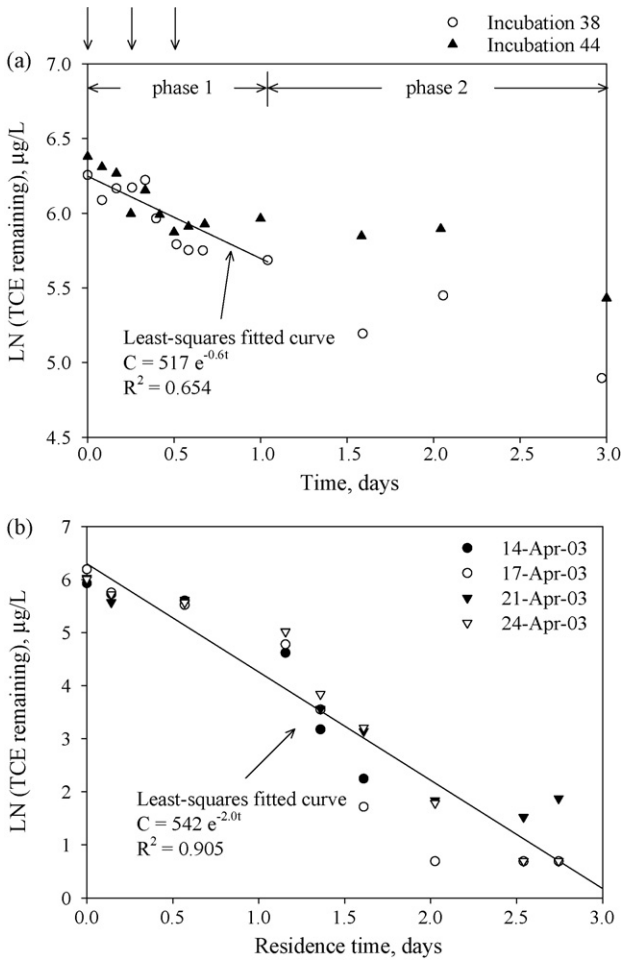

Fig. 5. (a) TCE remaining in microcosms during an incubation period under steady state conditions. Arrows represent toluene pulses. Each data point refers to a single microcosm. (b) Steady-state TCE profile vs. residence time for Que–Jen in-situ pilot (adapted from Kuo et al. [7]).

of groundwater samples at about 1×10^7 CFU/mL [7]. Fig. 5b shows that phase 1 lasted for all the residence time (3 days) in the plug flow reactor because toluene was distributed uniformly along the flow direction in the Que–Jen in-situ pilot.

The toluene utilization efficiency is defined as the ratio of the total mass of TCE cometabolized to the total mass of toluene utilized. Table 1 showed that the toluene utilization efficiency was 0.042 and 0.023 μg-TCE/μg-toluene for slurry microcosms and in-situ pilot study, respectively. Table 1 also showed that the TCE cometabolic-degradation efficiency measured from the laboratory microcosms was 46%, which appeared pessimistic compared to over 90% measured from the Que–Jen in-situ pilot study. It could be partly due to that the active biomass concentration in the laboratory microcosms was lower than that in the Que–Jen in-situ pilot. Also the reactor configuration could play a part in the decrease rate. The microcosms are virtually batch reactors while the Que–Jen in-situ pilot can be visualized as a continuous plug-flow reactor. The key to achieve over 90% TCE removal in the Que–Jen in-situ pilot was the uniform distribution of toluene along the flow direction of the plug flow reactor [7]. Toluene was present for all the residence time (3 days)

Table 2
Summary of TCE and toluene removals in HYL-QT1 (*Ralstonia* sp. P-10) and HYL-QT2 (*Pseudomonas putida*) microcosms[a]

	HYL-QT1 (*Ralstonia* sp. P-10)	HYL-QT2 (*Pseudomonas putida*)	TCE leakage controls
TCE remaining (μg/L)	498 ± 27	448 ± 27	654 ± 17
Toluene remaining (mg/L)	ND[b]	ND	NA[c]
TCE-degradation rate coefficient (day^{-1})	0.5	0.5	NA

Initial TCE and toluene concentrations were 662 ± 23 μg/L and 10.7 ± 0.3 mg/L, respectively
[a] Results are averages and standard deviations of triplicates.
[b] Not detectable.
[c] Not applicable.

in the Que–Jen in-situ pilot. With the presence of toluene, the toluene-utilizing bacteria could effectively cometabolize TCE. While in slurry microcosms, toluene was only present for 1 day. This was likely the reason for the low TCE cometabolic-degradation efficiency observed in the laboratory microcosms.

3.5. Identification of toluene-oxidizing bacteria

Water samples from the toluene-fed microcosms during the steady-state operation were taken for identifying bacteria in the slurry microcosms. Total of 11 bacterial colonies on nutrient agar plates were selected for DNA extraction and PCR amplification.

Restriction site analysis of the PCR products were analyzed by *Hap* II and the results showed that there are two different banding patterns in these eleven bacterial colonies. After sequencing, two different isolates HYL-QT1 and HYL-QT2 were named. HYL-QT1 were 99% identical to the sequence of *Ralstonia* sp. P-10, and HYL-QT2 were 100% identical to the sequence of *P. putida* by NCBI BLAST software at the GeneBank website (GeneBank, National Center for Biotechnology Information; www.ncbi.nlm.nih.gov). The nucleotide sequence data obtained in this study have been deposited in the NCBI nucleotide sequence database under accession numbers DQ910800 and DQ910801.

3.6. Evaluation of toluene-oxidizing bacteria

Table 2 shows the results of microcosm experiments for the individual toluene-oxidizing bacteria, HYL-QT1 (*Ralstonia* sp. P-10) and HYL-QT2 (*P. putida*). The initial OD_{605} level and equivalent bacteria count of the microcosms were 0.089 and 1×10^6 CFU/mL, respectively. The initial concentration for toluene and TCE was 10.7 ± 0.3 mg/L and 662 ± 23 μg/L, respectively. After an incubation period of 7 days, the TCE removal was 25 ± 4% and 32 ± 4% for HYL-QT1 (*Ralstonia* sp. P-10) and HYL-QT2 (*P. putida*), respectively. Toluene was completely degraded. The difference between TCE concentrations remaining in toluene-fed microcosms and non-toluene-fed-controls shown in Table 2 confirmed the function of TCE cometabolic biodegradation for HYL-QT1 (*Ralstonia* sp. P-10) and HYL-QT2 (*P. putida*). Literature information has also reported that *P. putida* is capable to degrade TCE using toluene as the primary substrate [17]. Previous studies have shown that *Ralstonia* sp. with different strains is capable of degrading TCE using phenol as the primary substrate [26,27]. According to the microcosm experiments in this study, HYL-QT1 (*Ralstonia* sp. P-10) is also able to biodegrade TCE using toluene as the primary substrate.

The biodegradation rates of TCE and toluene during an incubation period were also evaluated for each individual toluene-oxidizing bacterium. The results are illustrated in Fig. 6a and b for HYL-QT1 (*Ralstonia* sp. P-10) and HYL-QT2 (*P. putida*), respectively. Each data point on Fig. 6a and b was obtained from the analysis of a single microcosm at a given time. In Fig. 6a and b, a total of 16 microcosms for each individual

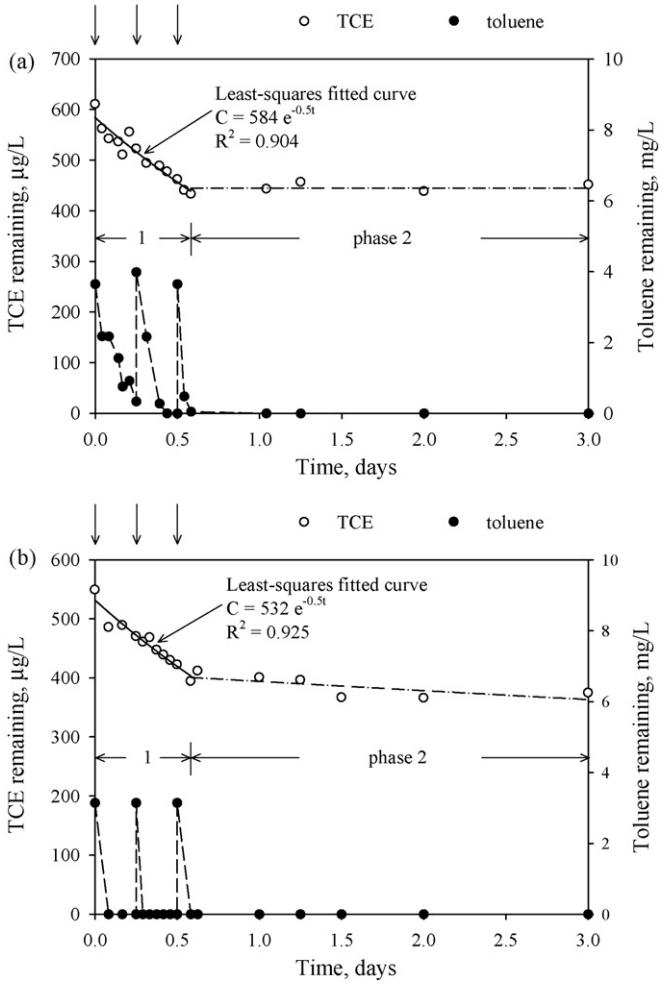

Fig. 6. TCE and toluene remaining in batch microcosms of (a) HYL-QT1 (*Ralstonia* sp. P-10) and (b) HYL-QT2 (*Pseudomonas putida*). Arrows represent toluene pulses. Each data point refers to a single microcosm.

Table 3
Summary of TCE and toluene removals for four toluene-injection schedules in HYL-QT2 (*Pseudomonas putida*) microcosms[a]

	Toluene injection schedule				TCE leakage controls
	A	B	C	D	
TCE remaining (μg/L)	416 ± 20	384 ± 10	357 ± 5	326 ± 5	458 ± 12
Toluene remaining (mg/L)	ND[b]	ND	ND	ND	NA[c]

Initial TCE concentration was 465 ± 2 μg/L. Total toluene injection per microcosms was 0.64 mg.
[a] Results are averages and standard deviations of triplicates.
[b] Not detectable.
[c] Not applicable.

acclimated toluene-oxidizing bacterium were analyzed at various times during an incubation period of 3 days to evaluate the biodegradation rates of TCE and toluene. Fig. 6a and b also show that the whole incubation period consisted of two phases for pure cultures (HYL-QT1 and HYL-QT2). Toluene was biodegraded rapidly and completely in phase 1. Fig. 6a and b suggested that TCE removal was more significant during phase 1 when toluene was still available. The cometabolism rate of TCE was much slower in phase 2 when toluene was absent.

Fig. 6a and b also showed the least-squares fitted curve with Eq. (6) for TCE concentration versus reaction time during phase 1. The first-order rate coefficients of TCE cometabolic degradation during phase 1 were determined as 0.5 day^{-1} for both HYL-QT1 and HYL-QT2. Lu et al. [22] reported the initial TCE removal rate at 24 μg-TCE/L-hr for toluene-utilizing microorganisms with initial TCE and toluene concentrations at 207 and 3 mg/L, respectively. The equivalent rate coefficient of first-order TCE cometabolic degradation determined by Lu et al. [22] was 2.8 day^{-1} for toluene-utilizing mixed cultures, which was higher than those determined for pure cultures (HYL-QT1 and HYL-QT2).

3.7. Effect of toluene-injection schedules on TCE removal

The short duration of toluene presence in the laboratory microcosms was likely the reason for the low TCE removal observed. To support our hypothesis, microcosm experiments were conducted to compare the TCE removals with four different toluene-injection schedules for a given amount of toluene injection. Each microcosm was filled with with 64 mL sterile oxygen-containing (≈32 mg/L) nutrient solution containing acclimated toluene-utilizing bacteria (HYL-QT2, *P. putida*). The initial TCE concentration was 465 ± 20 μg/L. The initial bacteria count in the microcosms was 8×10^6 CFU/mL. For each microcosm, toluene injection totaled 0.64 mg. For schedule A, toluene was fed in one pulse at the beginning of the experiment (1 × 1800 μL of a 362 mg/L toluene stock solution). For schedule B, toluene was injected in three separate pulses within the first 12 h (3 × 600 μL of a 362 mg/L toluene stock solution); for schedule C, six separate pulses in the first two days (6 × 300 μL of a 362 mg/L toluene stock solution); for schedule D, nine separate pulses in three days (9 × 200 μL of a 362 mg/L toluene stock solution). The duration of toluene injection is longest for schedule D and is shortest for schedule A. After an incubation period of 4 days, the efficiency of TCE cometabolic degradation was 11 ± 4%, 17 ± 2%, 23 ± 1% and 30 ± 1% for toluene-injection schedules A, B, C and D, respectively. Schedule D, with the longest duration of toluene injection, measured highest in TCE cometabolic degradation efficiency. Table 3 supports that efficiency of TCE cometabolic degradation can be enhanced by increasing the duration of toluene injection. With a given amount of toluene injection, it is recommended to maximize the duration of toluene injection and presence for more efficient TCE cometabolic degradation.

4. Conclusions

The following conclusions can be drawn from this study.

The modified method of semicontinuous slurry microcosms adopted from Jenal-Wanner and McCarty [15] proves that TCE can be cometabolically degraded by the indigeneous soil bacteria. The non-toluene-fed controls in this study were prepared with a toluene-utilizing consortium developed in the microcosms. The modified method manifests the effect of toluene injected. Upon termination of toluene feeding, the active biomass started to decline. The activity of toluene oxygenase in the toluene-utilizing bacteria could not be continuously maintained to cometabolize TCE in the non-toluene-fed controls.

Based on the nucleotide sequence of 16S rRNA genes, the toluene-oxidizing bacteria in microcosms were identified, i.e., *Ralstonia* sp. P-10 and *P. putida*. According to the microcosm experiments in this study, HYL-QT1 (*Ralstonia* sp. P-10) and HYL-QT2 (*P. putida*) are capable to biodegrade TCE using toluene as the primary substrate. Previous studies have shown that *Ralstonia* sp. with different strains is capable of degrading TCE in the presence of phenol as the primary substrate [26,27].

TCE removal and toluene degradation were evaluated in both slurry and pure-culture microcosms. An incubation period was marked by a rapid and complete removal of toluene followed by a slow and incomplete removal of TCE. TCE removal appeared to be more significant when toluene was still available. After the depletion of toluene, TCE was continuously cometabolized by toluene-utilizing bacteria at a lower rate, as long as active oxygenase remained available.

The toluene utilization efficiency was 0.042 and 0.023 μg-TCE/μg-toluene for slurry microcosms and in-situ pilot study, respectively. The TCE cometabolic-degradation efficiency measured from the slurry microcosms was 46%, which appeared pessimistic compared to over 90% measured from the Que–Jen in-situ pilot study. The high TCE removal in the Que–Jen in-situ

pilot is a result of the uniform distribution of toluene along the flow direction of the plug flow reactor [7]. We hypothesized that the short duration of toluene presence in the laboratory microcosms was likely the reason for the low TCE removal observed. The results of microcosm experiments using different toluene-injection schedules supported the hypothesis. With a given amount of toluene injection, maximizing the effective time duration of toluene presence in reactor design increases TCE cometabolic degradation.

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