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Cometabolic biodegradation of chlorinated ethenes with methanotrophs in anaerobic/aerobic simulated aquifer

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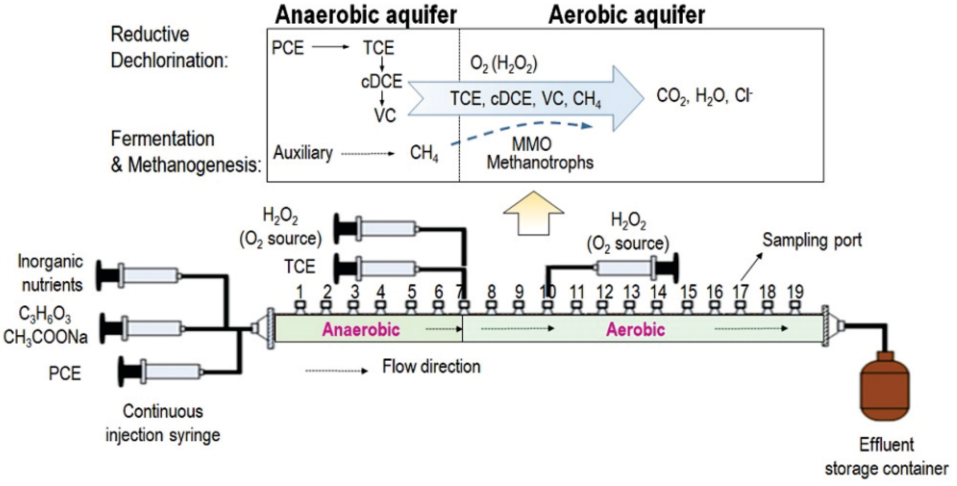
Abstract

Aim: This study explores anaerobic/aerobic biodegradation efficiencies of aerobic cometabolism with methanotrophs when contaminants trichloroethylene (TCE) and cis-1,2-dichloroethylene (cDCE) are present individually or in tandem.

Methodology: Batch tests and an anaerobic/aerobic column system were used to simulate saturated, contaminated aquifers. A brown glass bottle with an effective volume of 44 m l^{-1} was prepared for the batch test. An integrated one-dimensional sequential anaerobic/aerobic column system was used to simulate the accumulative intermediates such as TCE, cDCE and VC caused by incomplete degradation of PCE during the upgradient anaerobic stage in the saturated aquifer. In the downgradient aquifer, aerobic cometabolism was employed to degrade the intermediates. Methanotrophs in the aerobic aquifer were inoculated to degrade the by-products of incomplete degradation of PCE by aerobic cometabolism.

Results: In the batch test, biodegradation of TCE was significantly inhibited by cDCE. However, biodegradation of cDCE was not significantly inhibited by TCE. In the simulated aquifer test, aerobic cometabolism completely degraded intermediates (TCE, cDCE, and VC) produced by incomplete anaerobic degradation of tetrachloroethylene (PCE). The results showed that methane, a by-product of anaerobic reductive dechorination of PCE, was used as a primary substrate for aerobic degradation, at a utilization rate of almost 100%.



Interpretation: Biodegradation of TCE was significantly inhibited by cDCE. Bioremediation should have sufficient oxygen and methane at aerobic stage to ensure that chlorinated ethenes fully mineralize.

Keywords: Aerobic cometabolism, Anaerobic/aerobic biodegradation, *cis*-1,2-dichloroethylene, Methanotrophs, Trichloroethylene

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Introduction

Chlorinated ethenes (such as tetrachloroethylene, trichloroethylene, etc.) are volatile organic solvents widely used as cleaning agents, have biological toxicity (Spencer *et al.*, 2006),and are classified as carcinogens by the International Agency for Research on Cancer (Purdue *et al.*, 2017). Chlorinated ethenes (CEs) often contaminate groundwater due to improper use or storage (Moran *et al.*, 2007). Tetrachloroethylene (PCE) and trichloroethylene (TCE) have been proved to completely degrade through anaerobic reductive dechlorination, to either ethylene or ethane (McCarty, 1997). However, in most anaerobic conditions, incomplete dechlorination often occurs, resulting in the accumulation of *cis*-1, 2-dichloroethylene (cDCE) or vinyl chloride (VC) (Tarnawski *et al.*, 2016; Cupples *et al.*,2004).These intermediates, especially VC, have been confirmed to be toxic and carcinogenic (Kielhorn *et al.*, 2000). Aerobic biodegradation provides a way to completely mineralize and degrade these contaminants and intermediates (Schmidt *et al.*, 2014). The less toxic CEs, such as cDCE, can not only rapidly oxidize and degrade under aerobic condition, but also avoid conversion into more toxic intermediate product, VC, under anaerobic conditions (Shukla *et al.*, 2014). Several studies have shown that the combination of anaerobic and aerobic systems (anaerobic/aerobic) can successfully degrade CEs (Chang *et al.*, 2017; Liu *et al.*, 2017; Yoshikawa *et al.*, 2017). An electron donor provided in an anaerobic condition can dechlorinate PCE to TCE and cDCE. By then converting the system to an aerobic condition, aerobic microorganisms such as phenol decomposing bacteria or methane oxidizing bacteria (methanotrophs) could completely mineralize TCE and cDCE to carbon dioxide and chloride (Lontoh and Semrau, 1998).The main advantage of this approach is that it can prevent the accumulation of toxic and stable metabolites, it requires less of the highly sensitive *Dehalo coccoides* genus, and it reduces the auxiliary substrates that provide electron donors (Tiehm and Schmidt, 2011). The combination of anaerobic and aerobic technologies has previously been applied to *in situ* bioremediation (Conrad *et al.*, 2010).Therefore, the combination of anaerobic and aerobic microbial degradation for complete mineralization of CEs may become a novel technology and trend in bioremediation (Azubuike *et al.*, 2016).

TCE, cDCE and VC can be cometabolized and degraded by non-specific oxidation enzymes of aliphatic and aromatic degrading bacteria with methane, propane, toluene, and phenol as primary substrates (Elango *et al.*, 2011; Findlay *et al.*, 2016; Li *et al.*, 2014). The cost of this type of bioremediation technology is competitive with traditional chemistry/physics processes (Frascari *et al.*, 2015), and biodegradation is one of the safer and environmentally friendly methods (Gnanasekaran *et al.*, 2019).However, in most cases, due to the provisions of laws and regulations, there are still many doubts on adding the aforementioned substrates into the groundwater, since they may cause secondary pollution, which provides a challenge to *in situ* bioremediation. Methanotrophs are widely distributed in nature, as long as there is an environment where methane and air coexist. They can survive in seawater, freshwater, soil, sediment, ponds, etc., even in high or low pH and at temperatures exceeding 55°C (Pol *et al.*, 2007; van Teeseling *et al.*, 2014). Aerobic biodegradation of TCE and other chlorinated solvents by methanotrophic bacteria can potentially occur even in acidic aquifers (Shao *et al.*, 2019), and can be explored for *in situ* bioremediation of a wide range of pollutants (Benner *et al.*, 2015; Pandey *et al.*, 2014).Because methane is almost ubiquitous in nature, the use of methane cometabolism for biodegradation of recalcitrant pollutants is becoming increasingly important and requires a more comprehensive understanding (Wang *et al.*, 2019). Methane is a greenhouse gas (Hanson and Hanson, 1996). If the oxidation of methane can be used to cometabolize and remove CEs in groundwater, not only it improves groundwater pollution, but it also reduces the emission of greenhouse gases in the atmosphere at the same time. By combining anaerobic/aerobic bioremediation technologies, an auxiliary substrate does not necessarily need to be added in the aerobic stage, and methane oxidizing bacteria can be used to reduce the methane produced in the anaerobic stage, which is a benefit to the environment. In the cometabolism process, a variety of intermediates often co-exist, and the competition and inhibition of these intermediates in the metabolic process should be further understood to facilitate future *in situ* bioremediation projects. Combining anaerobic and aerobic technologies is an effective way of removing CEs completely. However, the best timing for anaerobic-aerobic conversion is still not fully understood. If it occurs too early will it cause incomplete degradation? If it occurs too slowly will it cause accumulation of more toxic VC? All of these problems need to be further clarified. The purpose of this study is mainly to explore the anaerobic/aerobic biodegradation efficiency of aerobic cometabolism with methanotrophs when TCE and cDCE are present individually or in tandem.

Materials and Methods

Batch tests: A brown glass bottle with an effective volume of 44 ml was prepared for the batch test. For carrying out batch test, 2.5 ml of inorganic nutrients and 15 ml of methane oxidizing bacteria (isolated in a previous study conducted by Chen *et al.*, in 2020) was added to the bottle. After adding the inorganic nutrients and bacteria solution, the bottle was sealed with a switch cap covered with a Teflon gasket. Then, 10 mg l^{-1} of methane, carbon and energy sources for the growth of methanotrophs was injected into the brown bottle with a gas-tight syringe, and the target pollutants cDCE and TCE were also injected into the brown bottle. To measure the concentrations of cDCE, TCE and methane, a gas-tight needle was used to draw 0.5 ml from the headspace of brown bottle for the gas chromatography analysis. Additionally,

considering the volatilization loss of CEs and the adsorption loss of bacterial cells, a control experiment was performed on a blank and sterilized bacteria group. The blank was supplemented with 2.5 ml of inorganic nutrient solution and 15 ml of ultrapure water, while the sterilized bacteria group was supplemented with 2.5 ml of inorganic nutrients solution and 15 ml of bacteria solution sterilized in a sterilizer and adjusting the absorbance.

Simulated aquifer tests: This study used bioaugmentation and biostimulation to simulate the biodegradation of CEs in aquifers. In the experiment, an integrated one-dimensional sequential anaerobic/aerobic column system was used to simulate the accumulative intermediates such as TCE, cDCE and VC caused by incomplete degradation of PCE during the upgradient anaerobic stage in the saturated aquifer. In the down gradient aquifer, aerobic cometabolism was employed to degrade the intermediates. Methanotrophs in the aerobic aquifer were inoculated to degrade the by-products of incomplete degradation of PCE by aerobic cometabolism. The microbial population and biodegradation rate of aerobic bacteria were observed to evaluate the effectiveness of bioremediation. The column length was 200 cm and had an inner diameter of 3 cm, and a sampling port was set every 10 cm, for a total of 19 sampling holes (Fig. 1).The column was packed with sand of particle size 0.25 to 0.59 mm. The density of sand was 2.6 g cm^{-3}, the porosity of the column after filling was 34.6%, the cross-section area of pores was 2.5 cm^2, and the overall density was about 1.7 g cm^{-3}. During operation, the pore volume was 489 cm^3, the inflow rate simulating groundwater flow was controlled at 3.6 ml hr^{-1}, and the pore velocity was 1.5 cm hr^{-1}. The actual hydraulic retention time was 0.99 days and 1.83 days for anaerobic stage and aerobic stage, respectively. The column system was closed to facilitate the control of anaerobic or aerobic states during the experiment. At the beginning, the column was maintained in an anaerobic condition. Once the system was stabilized, hydrogen peroxide was added to the Port 7 to make the simulated aquifer operate as anaerobic in its upgradient and aerobic in its downgradient. In order to ensure sufficient dissolved oxygen (DO) in the aerobic simulated aquifer, the Port 10 was reserved as a spare injection port for hydrogen peroxide.

Anaerobic simulated aquifer: Anaerobic simulated aquifer was continuously fed the target contaminant PCE at 10 µM (1.7 mg l^{-1}), along with auxiliary substrates and nutrients. The C:N:P:micronutrient stoichiometric ratio of the nutrients was 100:10:1:0.01.The concentration of each component was as follows: fermentation substrate (60 µM $C_3H_6O_3$, 55 µM CH_3COONa, 5.5 µM $NaHCO_3$), nitrogen source (15.4 µM NH_4Cl), phosphorus source (0.9 µM K_2HPO_4, 0.7 µM KH_2PO_4, 0.3 µM $(NaPO_3)_6$), mineral (0.4 µM $MgSO_4$ $7H_2O$, 0.16 µM $CoCl_2$ $6H_2O$, 0.15 µM $CaCl_2$ $2H_2O$, 0.14 µM $FeCl_2$ $4H_2O$), and micronutrient (0.03 µM Cysteine, 0.01 µM H_3BO_3, 0.01 µM NH_4VO_3, 0.01 µM Na_2MoO_4 $2H_2O$, 0.01µM Na_2SeO_3 $2H_2O$, 0.01 µM Na_2WO_4 $2H_2O$).The velocity of anaerobic simulated aquifer was 35 cm d^{-1}, and the flow rate was controlled at 86 ml d^{-1}. The mixed cultures (*Aquitalea magnusonii, Curvi bactergracilis*, *Dechloromona* ssp. EMB 50, *Desulfovibrio butyratiphilus, Desulfo vibrio* sp.BL-157, *Smithella propionica*, and *Dehalococcoides* sp.,etc.) were used as microbial sources for the anaerobic simulated aquifer. These anaerobic microorganisms were acclimated within a chemostat and isolated in a previous study (Chang *et al.*, 2018).

Aerobic simulated aquifer: After stabilizing the anaerobic simulated aquifer, 50 mg l^{-1} of hydrogen peroxide was continuously injected into the aquifer, at Port 7, by a syringe pump. The H_2O_2 was used as a oxygen source, and the DO concentration was kept above 2 mg l^{-1} to maintain an aerobic environment in the simulated aquifer. The influent concentration was kept at a constant value; however, the total influent flow rate was increased to 130 ml d^{-1}. The aerobic microorganisms (*Gammaproteobacteria, Thiohalospira halophila*s train HL23, *Opitutaceae*, *Methylococcaceae, Verrucomicrobiaceae* bacterium DC2c-37, *Pseudomonas aeruginosa* strain PC29, and *Methylosarcina fibrata* strain AML-C10, etc.) were inoculated into the aerobic simulated aquifer. These microorganisms were isolated in a previous study (Chen *et al.*, 2020), where they were extracted from the effluent of petrochemical plants and rice fields, and then acclimated within a chemostat. In order to explore the competition and inhibition effects of TCE and DCE in tandem, an extra 7µM TCE was added to Port 7 of the aerobic simulated aquifer.

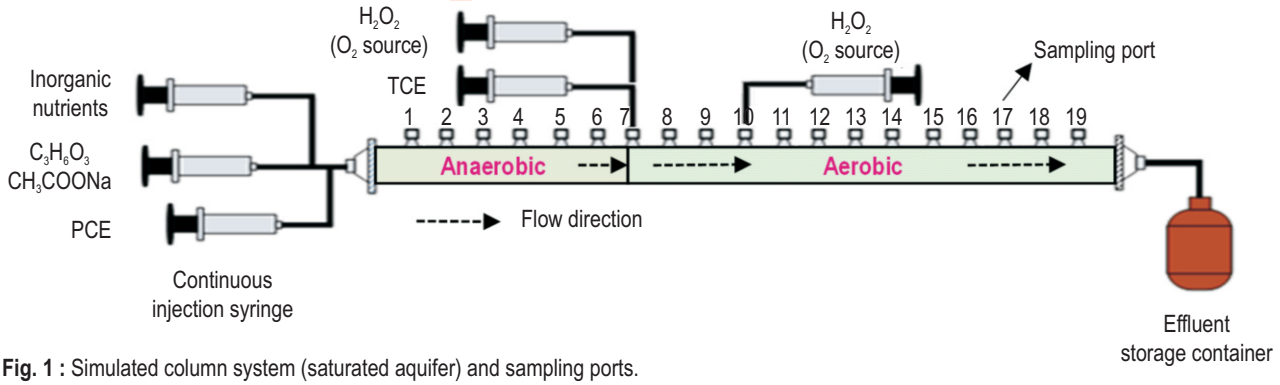

Fig. 1 : Simulated column system (saturated aquifer) and sampling ports.

Analyses of methane, ethylene and chlorinated ethenes: A gas chromatograph, GC/FID (Agilent 7890A), was used in the experiment to analyze the contaminant concentrations of CEs and ethylene in water (aquifer) samples, and the concentrations of methane in gas samples. A 4 ml serum bottle was used to collect the water samples at 25°C at a constant temperature for 1 hr. A gas-tight syringe was then used to extract 500 µl of gas sample from the upper space of serum bottle and injected into GC/FID to analyze the concentrations of different contaminants. The inlet and detector temperatures were 200°C and 250°C, respectively. The carrier gas and supplementary gas used in the analysis were high-purity nitrogen (99.99%). The flow rate of carrier gas and supplementary gas was 10 ml $min^{-1}$and 20 ml min^{-1}, respectively. The oven temperature was 35°C at the beginning and heated to 140°C at a rate of 10°C min^{-1}. The final temperature was maintained for 5 min. The calibration curve of each contaminant was established in this experiment, and the retention time of PCE, TCE, cDCE, ethylene, and methane was 14.8, 12.3, 6.3, 1.4 and 0.9 min, respectively.

Analysis of sMMO of methanotrophs: The activity of soluble methane monooxygenase (sMMO) was measured with naphthalene (Brusseau *et al.*, 1990). One ml of bacterial solution (sterilized for 30 min) and 1ml of naphthalene saturated solution was collected, put into a brown bottle and sealed with a Teflon gasket, and shaken at 100 rpm for 2 hrs in a 30°C water bath so that sMMO could oxidize naphthalene to naphthol. After adding 100 µl of tetrazotized-o-dianisidine solution and allowing it to react with naphthol, the solution turned purple-red, and the absorbance of the solution was read on a spectrophotometer at 530 nm wavelength at 30°C.

Bacterial analysis: DNA was extracted from the water sample collected from the simulated aquifer sampling port, using a standard DNA commercial kit (Power Soil DNA Kit, MO BIO Laboratories, Inc.). The extracted DNA was amplified by PCR, and specific primers were selected according to different target strains. The 730f and 1350r primer sets were used to amplify all 16S rDNA of *Dehalococcoides* sp. (He *et al.*, 2003). The 958F-gc and 1392R primers were used to amplify the16S rDNA of eubacteria (Ferris *et al.*, 1996).The A571F and UA1204R primer sets were used to amplify the 16S rDNA of archaea (Baker *et al.*, 2003). In addition, moxF f1003 and moxF r1561 primers were used to amplify the 16S rRNA of methanotrophs that produces MMO (McDonald *et al.*, 1995). Gradient former (BIO-RAD) was used in this study to prepare denaturing gradient gel electrophoresis (DGGE). After completing DGGE and imaging with UV light, the bright band was purified and recovered by acrylamide/Bis colloid. The purified DNA sequencing was done by Mission Biotech, Taiwan, and the Nucleotide BLAST service provided by the National Center for Biotechnology Information (NCBI) was used to compare the sequencing results with the known sequences in the database. By analyzing the changes of bacterial populations in the simulated aquifer, the main microorganisms involved in the biodegradation of target contaminants could be identified.

Statistical methods: The batch tests consisted of three replicates. All related graphs and data tables are depicted as mean value ± S.D. based on the experimental results.

Results and Discussion

Previous studies have shown that on cometabolizing CEs with methane as primary substrate, the optimal concentration of methane was approximately 10 mg l^{-1} (Chen *et al.*, 2020). In order to understand the cometabolic efficiency of methanotrophs for cDCE and TCE, a series of sealed batch tests were performed with methane concentrations of 10 mg l^{-1} (in air) and with cDCE, TCE, and both cDCE and TCE concentrations (in aqueous) of 5 µM, 10 µM, 20 µM, 50 µM, 100 µM, 500 µM, and 1000 µM. When cDCE was an individual contaminant, the cometabolism removal efficiencies of cDCE ranged between 10 to 67%, after 7 days of operation (Fig. 2). When cDCE and TCE were dual contaminants, the cometabolism removal efficiencies of cDCE ranged between 12 to 69%, after 7 days of operation. The results indicate that when cDCE concentrations were in the range of 5 to 1000 µM, the presence of TCE at concentrations below 1000 µM did not inhibit the cometabolic removal of cDCE. In other words, when cDCE and TCE were dual contaminants, the cometabolic degradation efficiencies of cDCE were not affected.

When TCE concentrations were in the range of 5 to 1000 µM, the cometabolic removal efficiencies after 7 days of operation was approximately 71%, 72%, 79%, 69%, 62%, 29%, and 15%, respectively (Fig. 3). When TCE and cDCE were dual contaminants, the cometabolic removal efficiencies of TCE after 7 days of operation was approximately 59%, 43%, 47%, 47%, 25%, 13%, and 13%, respectively. When TCE and cDCE were dual contaminants, the cometabolic degradation efficiencies of TCE were significantly reduced in the presence of cDCE. At lower concentrations of TCE, it has been shown that cDCE would prefer to compete with methane monooxygenase over TCE, therefore resulting in a better cometabolic degradation efficiency of TCE. However, compared to the individual presence of cDCE or TCE, the cometabolic degradation efficiency of TCE was better than that of cDCE if its concentration was higher than 500 µM. This means that at higher concentrations of TCE, the cometabolic degradation efficiency of TCE was also higher.

In addition, the results of different CEs concentrations and methane utilization rates showed that as the concentration of cDCE increased, the methane utilization rate decreased from 93% to 44% (Table 1). In the cometabolism process, where TCE was individual contaminant, the utilization rate of methane was maintained approximately at 87 to 93% (Table 2). However, in the cometabolism process where cDCE and TCE were dual

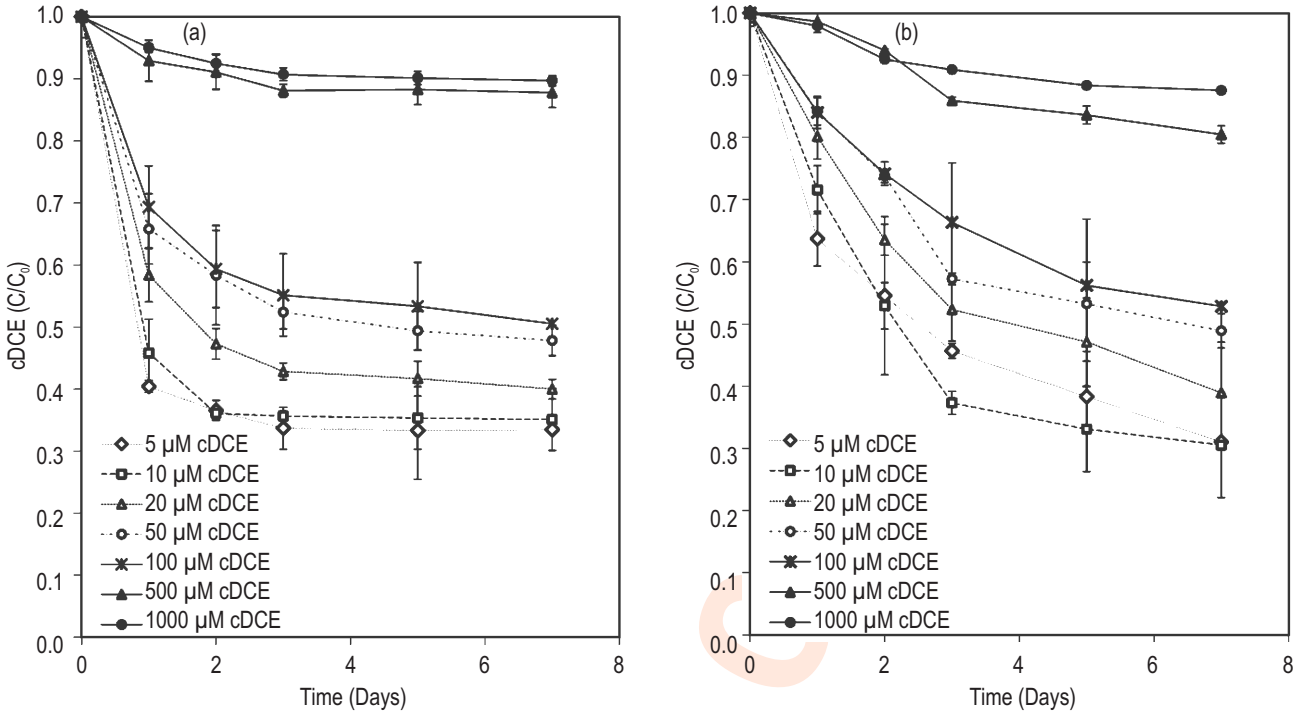

Fig. 2 : Comparison of the efficiency of aerobic cometabolism of cDCE with methane under different concentrations and conditions (a) as an individual contaminant (cDCE); (b) in coexistence with TCE (dual contaminants, both cDCE and TCE).

Table 1 : Aerobic cometabolic degradation rate of different concentrations of cDCE with methane in batch tests

Microbial species	CH_4 ($mg\ l^{-1}$)	cDCE (µM)	CH_4 utilization rate (%)	Degradation rate ($\mu M\ d^{-1}$)[a]		sMMO ($\mu molhr^{-1}\ mg^{-1}$ of protein)
				Mean	SD[b]	
Methanotrophs	10	5	89	1.12	0.05	1.8
		10	89	2.20	0.02	1.4
		20	93	3.95	0.36	1.5
		50	90	8.20	0.48	1.5
		100	90	15.32	2.03	2.0
		500	58	20.47	1.75	2.4
		1000	44	31.96	3.48	1.5

a: Aerobic cometabolic degradation rate during first three days ; b: Standard deviation

contaminants, the methane utilization rate was reduced from 98% to 12% as the concentration of cDCE increased (Table 3).These results showed that compared to TCE, cDCE was competing more for soluble methane monooxygenases (sMMOs).Therefore, an increase in the concentration of cDCE significantly reduced the utilization rate of methane. At the same time, when cDCE was present during the cometabolism process, the concentration of sMMOs per mg of protein was lower than that of single contaminant TCE. Previous studies have also indicated that high concentrations of cDCE compete with methane (growth substrate) for sMMOs and produce unstable epoxides or toxic substances (Dolinova *et al.*, 2017; van Hylckama Vlieg *et al.*, 1997; van Hylckama Vlieg and Janssen, 2001), which in turn reduce cometabolism and affect enzymatic viability and microbial activity.

The kinetics of cometabolic degradation reaction can be explicated by a first-order equation model or a complex multi-substrate, mixed-order equation model (Alvarez-Cohen and Speitel, 2001; Chen *et al.*, 2008; Jesus *et al.*, 2016). The most commonly used method is the simple first-order Michaelis-Menten/Monod equation model, as shown below (Bradley and Chapelle, 1998):

$$V = \frac{V_{max}\ S}{K_s + S} \quad (1)$$

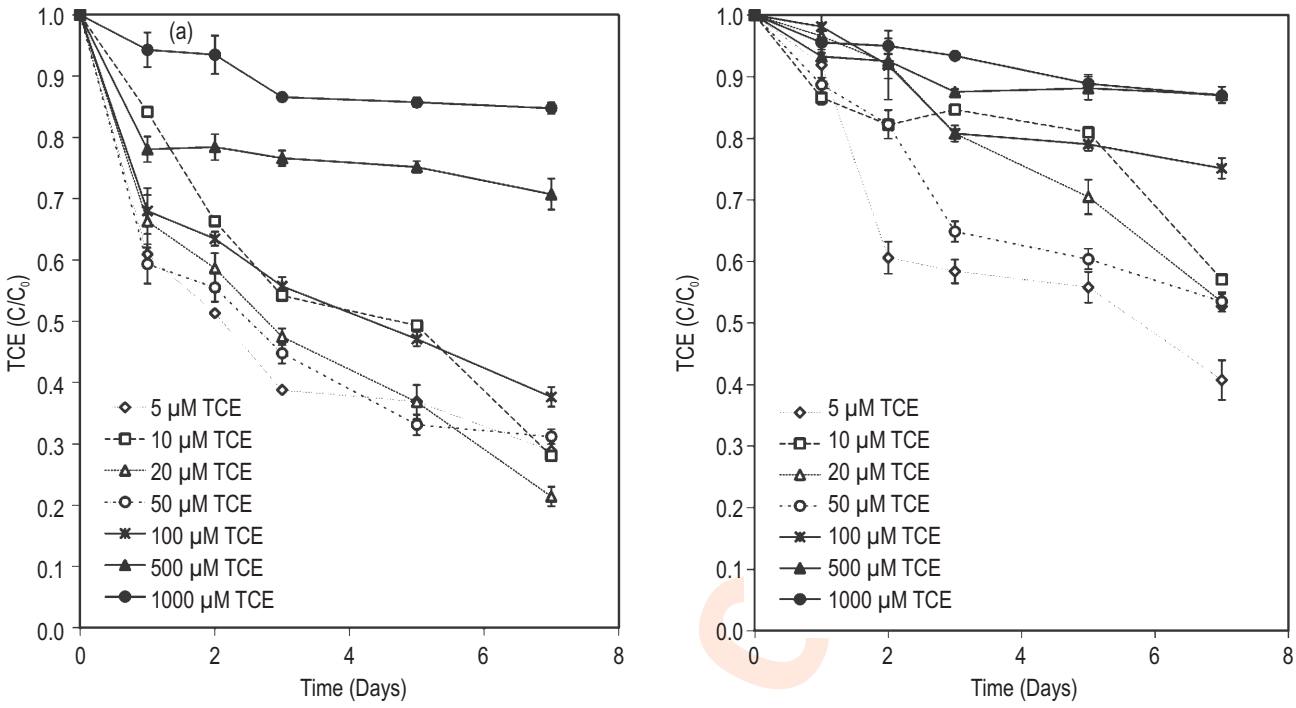

Fig. 3 : Comparison of the efficiency of aerobic cometabolism of TCE with methane under different concentrations and conditions (a) as an individual contaminant (TCE); (b) in coexistence with cDCE (dual contaminants, both cDCE and TCE).

Table 2 : Aerobic cometabolic degradation rate of different concentrations of TCE with methane in batch tests

Microbial species	CH_4 (mg l^{-1})	TCE (μM)	CH_4 utilization rate (%)	Degradation rate (μM d^{-1})[a]		sMMO (μmol hr^{-1} mg^{-1} of protein)
				Mean	SD[b]	
Methanotrophs	10	5	87	1.02	0.02	1.9
		10	90	1.53	0.13	2.3
		20	91	3.50	0.09	1.8
		50	91	9.20	0.28	2.9
		100	92	14.76	0.51	2.4
		500	92	38.97	2.07	2.8
		1000	93	44.72	1.72	2.8

a: Aerobic cometabolic degradation rate in the first three days; b: Standard deviation

Invert and multiply by S, and the following equation can be obtained:

$$\frac{S}{V} = \frac{K_s}{V_{max}} + \frac{1}{V_{max}} S \quad (2)$$

where, V is the specific degradation rate (μM d^{-1}), V_{max} is the maximum specific degradation rate (μM d^{-1}), S is the contaminant initial concentration (μM), and K_s (μM) is the contaminant half-velocity constant. Equation (1) can be rearranged to get equation (2), as shown in Fig. 4, Table 1,2 and 3,where a Hanes-Woolf plot regression analysis was performed in the linear form of the Michaelis–Menten/Monod equation (Oldenhuis *et al.*, 1991), and a straight line with the equation y = mx + c was generated. Its slope m is equal to $1/V_{max}$, and the intercept c in the figure represents K_s/V_{max}.Therefore, V_{max} and K_s can be obtained separately (Table4).

From the Michaelis-Menten/Monod equation of aerobic cometabolism of cDCE in Fig. 5, it was determined that there was no significant difference in the cometabolic degradation of cDCE in the batch test in the presence or absence of TCE. According to the kinetic parameters in Table 4, when cDCE was an individual contaminant, its degradation rate and K_s values were 35μM d^{-1} and 167μM, respectively. These values were 36 μM d^{-1} and 199 μM, in the presence of both cDCE and TCE. The K_s value implies the substrate concentration required for biodegradation to reach half of the maximum degradation rate V_{max}. In the case of dual contaminants, the maximum degradation rate of cDCE did not decrease, indicating that when TCE was present, the biodegradation of cDCE under aerobic cometabolism with

Table 3 : Aerobic cometabolic degradation rate of different concentrations of cDCE and TCE with methane in batch tests

Microbial species	cDCE (μM)	TCE (μM)	CH_4utilization rate (%)	cDCE Degradation rate (μMd^{-1})[a]		TCE degradation rate (μMd^{-1})[a]		sMMO (μmol hr^{-1} mg^{-1} of protein)
				Mean	SD[b]	Mean	SD[b]	
Methanotrophs	5	5	92	0.91	0.21	0.69	0.03	1.5
	10	10	98	2.09	0.06	0.51	0.05	1.8
	20	20	90	3.18	0.34	1.28	0.26	1.4
	50	50	85	7.12	0.16	5.86	0.19	1.6
	100	100	79	11.22	0.63	6.40	0.21	1.6
	500	500	35	23.47	0.94	20.78	0.81	1.9
	1000	1000	12	30.32	0.51	21.94	1.07	1.8

a: Aerobic cometabolic degradation rate during first three days; b: Standard deviation

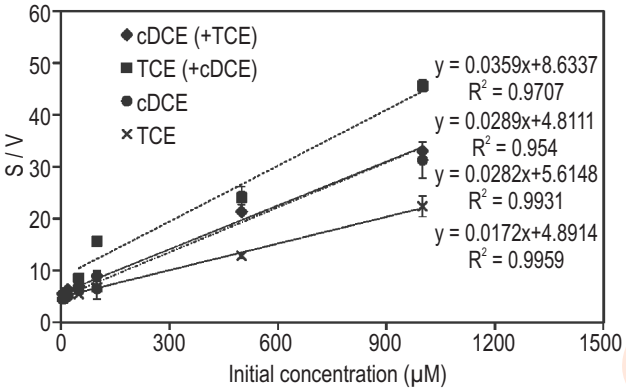

Fig. 4 : Regression analysis of aerobic cometabolic degradation rate of cDCE and TCE.

methane oxidizing bacteria was more competitive than that of TCE. The results indicated that cDCE was more competitive for sMMOs when sMMOs were in the presence of both cDCE and TCE. However, TCE was still competitive for sMMOs. Therefore, cDCE concentrations were relatively higher (167-199 μM) to reach their maximum biodegradation rate as a dual contaminant (both cDCE and TCE) than when cDCE was the sole contaminant.

As cDCE concentrations increased as shown in Fig.5, the inhibition of TCE cometabolic biodegradation became more significant. The results from the kinetic parameters of Table 4 indicate that the maximum degradation rate of TCE as individual contaminant was 58 μM d^{-1} and the K_s was 284 μM. However, the V_{max} of TCE as a dual contaminant with cDCE was 28 μM d^{-1}, and the K_s was 241 μM. Although, the K_s value of TCE as a dual contaminant was lower than that of TCE as a single contaminant, the V_{max} of TCE as a dual contaminant was about half of that of TCE as an individual contaminant. When present as a dual contaminant, the cometabolic efficiencies of TCE decreased. From the result in Fig. 5, the higher the concentration of cDCE, the more obvious the inhibition of TCE cometabolic efficiencies.Therefore, to cometabolize TCE and cDCE together, the lower the concentration of cDCE, the less the aerobic cometabolic biodegradation reaction is inhibited.

From Fig. 6, it can be observed that the redox potential of anaerobic simulated aquifer in this test was close to -200 mV, showing that the anaerobic simulated aquifer was under an absolute anaerobic condition, which is conducive to the occurrence of reductive dechlorination. The anaerobic reduction and dechlorination of PCE produced intermediates TCE, cDCE and VC. Following the anaerobic stage, biodegradation in the aerobic stage formed carbon dioxide, which affected the downgradient pH value of the system. The pH value of the downgradient aerobic simulated aquifer dropped to between 6.1 and 6.5, and the redox potential in the aerobic stage was above 50 mV.

In the upgradient (Port 1, 10 cm) of the anaerobic simulated aquifer, PCE was quickly converted into cDCE and VC, and there was almost no residual PCE in the downgradient (Port 3, 30 cm).The degradation efficiency of PCE reached up to 94%. In the simulated aquifer test, TCE that formed in the upgradient anaerobic stage did not accumulate significantly, rather it was effectively converted to cDCE and VC. The concentration of TCE was about 0.0 to 0.7μM, however, there was accumulation of cDCE and VC, the by-products of the degradation of PCE and TCE.The anaerobic simulated aquifer also saw a large amount of methane production (3.6 to 11.4 μM). Since the fermenters, methanogens, and dechlorination bacteria all competed for hydrogen at the same time (Patil *et al.*, 2014; Ziv-El *et al.*, 2012;Wen *et al.*, 2015), the dechlorination ability of the dechlorination bacteria was reduced, so PCE could only be dechlorinated to cDCE and VC.Many studies have also shown that the anaerobic biodegradation of PCE usually results in the accumulation of cDCE and VC and is accompanied by the formation of a large amount of methane (Anam *et al.*,2019; Major *et al.*, 2002).Some studies have found that TCE, cDCE and VC can be cometabolically degraded by using methane as a

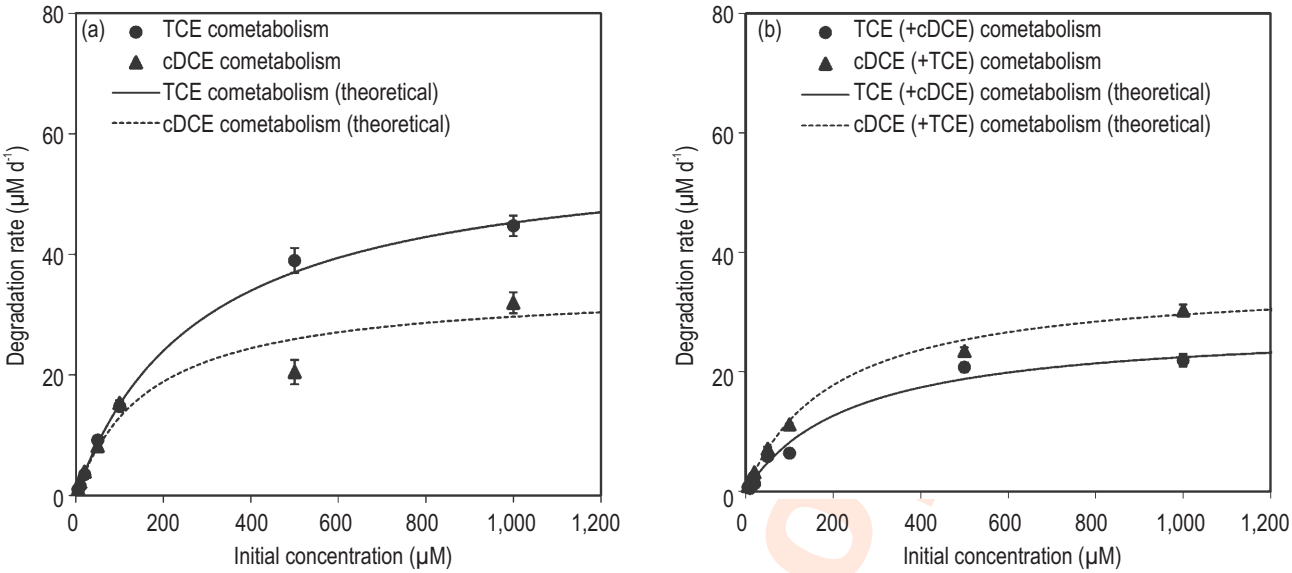

Fig. 5 : Michaelis-Menten/Monod equation for aerobic cometabolism of cDCE and TCE (a) assingle contaminants (b) as dual contaminants.

Table 4 : Kinetics parameters of aerobic cometabolism of cDCE and TCE under individual and coexisting states

kinetics parameter	Individual contaminant				Dual contaminants			
	cDCE		TCE		cDCE		TCE	
	Mean	SD[a]	Mean	SD[a]	Mean	SD[a]	Mean	SD[a]
V_{max} (μMd^{-1})	35	4	58	3	36	1	28	2
K_s (μM)	167	18	284	12	199	12	241	39

a: Standard deviation

substrate under aerobic conditions (Fogel *et al*., 1986; Němeček *et al*., 2020; Wymore *et al*., 2007). Therefore, this test inoculated acclimated methanotrophs in the following aerobic simulated aquifer, using methane generated in the anaerobic simulated aquifer as the carbon source and energy source of the methanotrophs, to produce methane monooxygenases. Methane formed in the upgradient side of the simulated aquifer was employed to cometabolically biodegrade the residual TCE, and the major intermediates cDCE and VC (Weatherill *et al*., 2018). This integrated anaerobic and aerobic simulated aquifer system could result in the complete mineralization of PCE and its biodegradation intermediates. In order to understand the cometabolic biodegradation of methanotrophs under aerobic conditions when TCE and cDCE are present at the same time, 7μM TCE were purposely and continuously added at the end of anaerobic aquifer (Port 7, 70 cm) so that both cDCE and the added extra TCE migrated to the following aerobic aquifer.

Fig. 7 shows that by 5th day of operation, PCE was almost completely degraded to form cDCE and VC at anaerobic stage. At aerobic stage, after an extra 7μM of TCE was continuously added, the TCE, cDCE and VC were cometabolically degraded. The overall degradation efficiency of CEs reached 95%, the average degradation efficiencies of PCE, TCE, cDCE, and VC were about 100%, 97%, 88% and 98%, respectively. The concentration of ethylene during the operation period was lower than 0.1μM, and the effect during the biodegradation process was minimal. On 15th day of operation, in the upgradient portion of aerobic simulated aquifer (Port 7 (70 cm) and Port 11 (110 cm)), cDCE was not completely degraded by cometabolism, and still continued to accumulate. Due to the presence of dechlorinating bacteria at anaerobic to aerobic conversion stage, the continuously added TCE could have still been degraded to form more cDCE and VC through dechlorination, resulting in the accumulation of cDCE. The addition of extra TCE at influent-side of aerobic aquifer (Port 5) resulted in incomplete removal of cDCE. However, without adding extra TCE, the formed cDCE from the anaerobic stage of the aquifer could not have been almost completely cometabolized in the aerobic side of the aquifer. This phenomenon of cDCE accumulation may occur during *in situ* bioremediation, therefore it needs further attention and understanding. Since methane concentration in the downgradient side of the aerobic aquifer was

Table 5 : Combined anaerobic/aerobic simulated aquifer conditions and utilized methane during cometabolic biodegradation of CEs

Sampling ports		Anaerobic column				Aerobic column							
Conc. (μM)		1st (10 cm)		5th (50 cm)		7th (70 cm)		11th (110 cm)		15th (150 cm)		19th (190 cm)	
Analysis period (days)		mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
PCE	1~20	4.97	1.11	0.64	0.07	0.79	0.12	0.39	0.36	0.42	0.65	0.05	0.03
	21~45[a]	1.53	1.39	0.39	0.36	0.52	0.29	0.23	0.32	0.23	0.32	0.00	0.00
TCE	1~20	0.13	0.15	3.26	0.29	6.37	2.35	0.62	0.57	0.31	0.34	0.30	0.25
	21~45[a]	0.56	0.67	0.06	0.13	5.69	0.61	1.13	0.23	0.51	0.71	0.53	0.83
DCE	1~20	3.24	2.33	3.26	1.66	4.11	3.94	1.71	1.47	1.05	0.88	1.23	1.09
	21~45[a]	11.02	4.60	11.77	5.07	4.90	1.81	6.97	3.14	5.23	1.80	4.03	1.05
VC	1~20	6.21	2.63	6.30	1.69	3.92	1.16	2.38	0.90	0.30	0.25	0.07	0.02
	21~45[a]	2.71	1.06	5.25	2.18	3.33	1.47	2.60	1.42	0.07	0.08	0.03	0.01
CH_4	1~20	9.22	0.82	8.81	2.10	5.06	0.96	0.14	0.12	0.07	0.14	0.06	0.10
	21~45[a]	5.38	0.75	7.24	1.10	4.52	1.46	0.62	0.58	0.34	0.23	0.35	0.25

a:Starting on day 21, a small amount of methane was added to the aerobic simulated aquifer

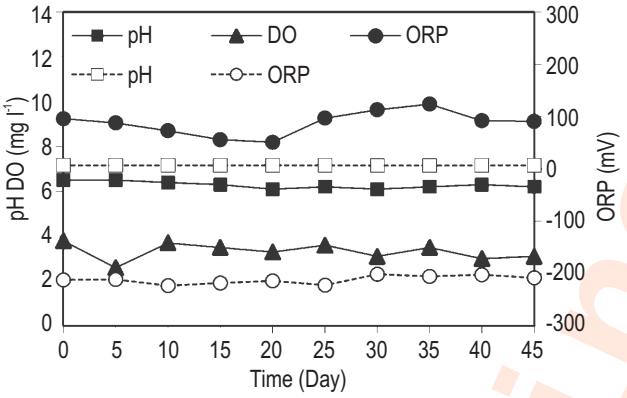

Fig. 6 : Changes in operating conditions over time of pH, ORP and DO in an anaerobic/aerobic simulated aquifer: anaerobic aquifer (dashed line), aerobic aquifer (solid line).

nearly zero, on 21st day, a small amount of methane was added at Port 10(100 cm) to enhance the activity of methanotrophs. The results showed that although the cumulative concentration of cDCE reached its highest concentration on 35th day in the aerobic aquifer, the cumulative concentration of cDCE decreased significantly by 45th day, due to improvement in cometabolic degradation ability caused by extra addition of methane. Therefore, in order to ensure that CEs can be fully mineralized, anaerobic/aerobic bioremediation should have sufficient oxygen supply and methane at aerobic stage. This prevents premature conversion of anaerobic stage into aerobic stage, which is important because continuous reductive dechlorination could still be ongoing, resulting in the potential hazards of cDCE and VC residues in the groundwater.

Table 5 shows that average degradation efficiencies of PCE, TCE, cDCE and VC were about 100%, 100%, 70, and 99 in the first 20 days of operation, respectively, and 100%, 92%, 66%, and 99% on 21st to 45th day of operation, respectively. Related research indicate that the degradation efficiencies of aerobic cometabolism were VC >cDCE> TCE (Semprini *et al.*, 1991; Suttinun *et al.*, 2013). Theoretically, the concentration of residual undegraded contaminants would be TCE >cDCE> VC. In this study, due to the addition of extra TCE at upgradient end of the aerobic aquifer, there was a subsequent reductive dechlorination reaction in which TCE degraded to cDCE and VC during operation. VC was easier to cometabolically degrade than cDCE, so the decomposition rate of VC in the simulated aerobic aquifer was faster than that of cDCE. Therefore, some undecomposed cDCE remained in the aerobic aquifer, resulting in lower overall degradation efficiency of cDCE (van Hylckama Vlieg *et al.*, 1997).

After operating the simulated aquifer for 20 days, Port 3 was used as a representation of anaerobic environment and Ports 9 and 16 were used as representations of aerobic environment. Samples were collected from these sampling ports for microorganism analysis. The PCR-DGGE map of microbial population in the simulated aquifer is shown in Fig. 8.The bright red band was identified as *Dehalococcoides ethenogenes* strain 195, the bright blue band was mixed flora of acclimated methanotrophs, and the bright green band was *Methanolinea* sp. The identification of *Dehalococcoides* in the anaerobic aquifer showed that the system had the ability for reductive dechlorination (Hellal *et al.*, 2021; McCarty *et al.*, 2020). In addition, the anaerobic aquifer contained methanogens (*Methanolinea* sp.), which could convert the added auxiliary substrate into methane (Vítězová *et al.*, 2020).Therefore, during the process of reductive dechlorination, a large amount of methane was also produced .*Dehalococcoides* was found in both Port 3 and Port 9 in the aquifer, indicating that the reductive

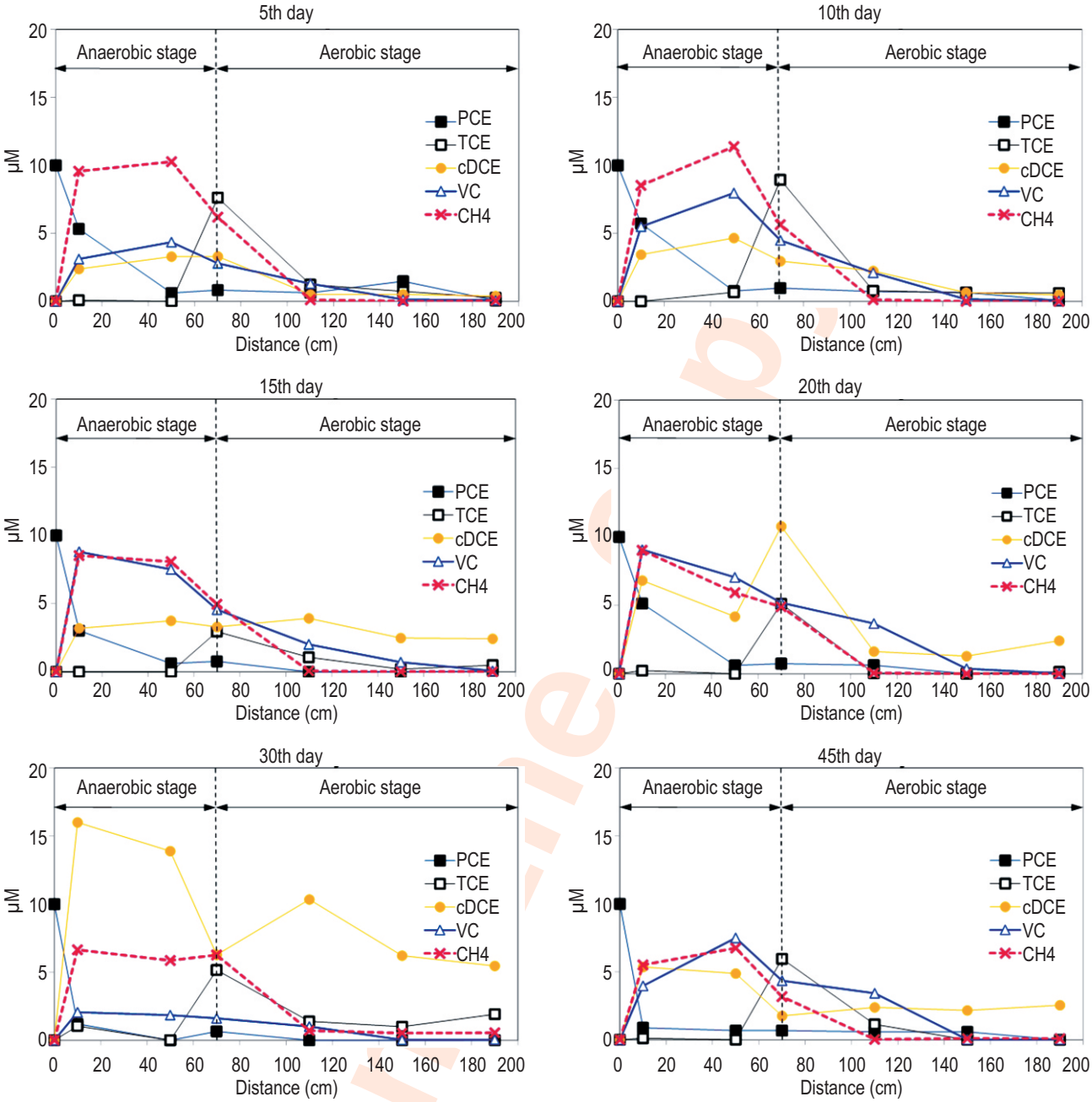

Fig. 7 : Changes of CEs and methane concentration (in the aqueous solution) in anaerobic/aerobic simulated aquifer over distance and time (extra TCE was purposely added to Port 7 of the column).

dechlorination reaction could continue upgradient of aerobic stage. This also explained that after adding extra 7µM TCE at aerobic stage, cDCE could not be completely cometabolically degraded, but only partially converted from TCE.

The aerobic simulated aquifer contained three kinds of methanotrophs: *Methylococcaceae* bacterium, *Methylosarcina fibrata* strain AML-C10, and *Verrucomicrobiaceae* bacterium DC2c-37.This was why in the aerobic stage, methane was almost 100% consumed, producing sMMO degrading enzymes, and cometabolizing TCE, cDCE and VC at the same time. Since methanotrophs are sensitive to inhibition of organic compounds, mixed cultures are more stable and suitable than pure cultures for environmental remediation (Fogel *et al.*, 1986). On 15th day of operation, there was almost no methane in the downgradient of aerobic simulated aquifer (Port 15). The methane concentration

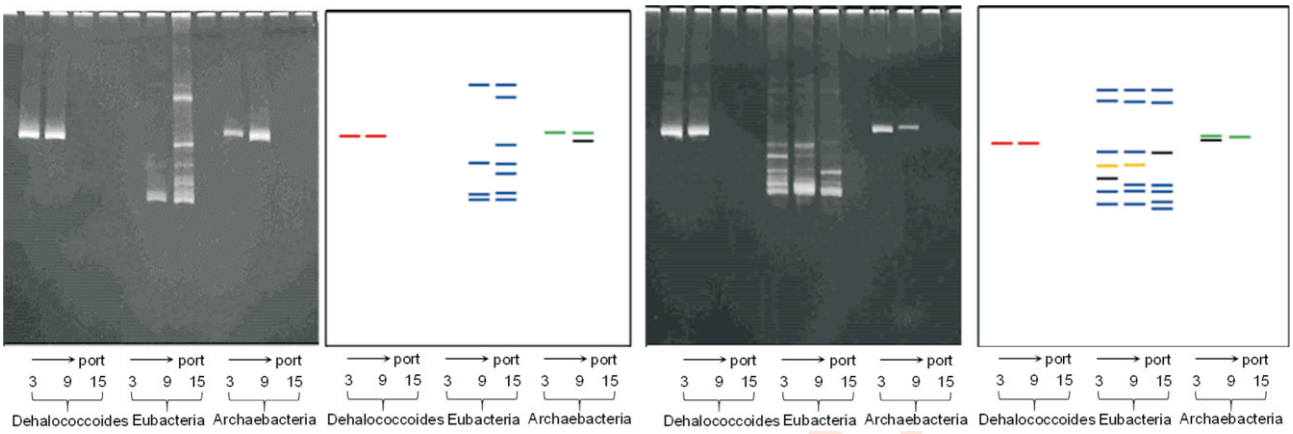

Fig. 8 : Microbial populations in the simulated aquifer.

was too low, which affected the activity of methanotrophs, resulting in insufficient sMMO enzymes and inability to fully execute the cometabolism effect. It was found that in the downgradient of aerobic simulated aquifer, the concentration of cDCE gradually accumulated. In this case, methane can be added to maintain the activity of methanotrophs in this system. Since methane from the front section of aerobic aquifer was almost depleted, a small amount of methane was purposely and continuously added to the aerobic aquifer after 21st day of operation to increase the activity of methanotrophs at aerobic stage. By 40th day, the growth of methanotrophs had stabilized, the cometabolism efficiency had increased again, and the concentration of cDCE in the aerobic aquifer had decreased significantly again (Fig. 7). Microorganisms in the upgradient anaerobic aquifer were also stable and abundant as compared to initial period, and *Nitrospira* sp. strains (bright yellow band) that could degrade CEs were also found in the system. The *Nitrospira* sp., has also been found in the population of aerobic bacteria from 300 remediation sites contaminated by TCE and cDCE across the United States (Guan *et al.*, 2013; Miller *et al.*, 2007).

In conclusion, aerobic cometabolism was used to degrade the intermediates produced by incomplete anaerobic degradation of PCE. A small amount of cDCE might remain in the aerobic stage, therefore, in order to ensure that CEs can be fully mineralized, in the process of anaerobic/aerobic bioremediation should have sufficient oxygen supply and methane in the aerobic stage.

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Add-on Information

Authors' contribution: S.K. Chen: Conducting experiment and manuscript preparation, **Y.L. Chin:** Conducting experiment, **H.Y. Yang:** Data analysis, **C.J. Lu:** Supervising experiment and data analysis, **M.H. Liu:** Data analysis and manuscript editing.

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