J. Microbiol. Biotechnol. (2016), 26(1), 120–129
http://dx.doi.org/10.4014/jmb.1510.10026

jmb

Development and Characterization of PCE-to-Ethene Dechlorinating Microcosms with Contaminated River Sediment

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Received: October 12, 2015
Revised: October 25, 2015
Accepted: October 26, 2015

First published online
October 27, 2015

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Supplementary data for this paper are available on-line only at http://jmb.or.kr.

pISSN 1017-7825, eISSN 1738-8872

An industrial complex in Wonju, contaminated with trichloroethene (TCE), was one of the most problematic sites in Korea. Despite repeated remedial trials for decades, chlorinated ethenes remained as sources of down-gradient groundwater contamination. Recent efforts were being made to remove the contaminants of the area, but knowledge of the indigenous microbial communities and their dechlorination abilities were unknown. Thus, the objectives of the present study were (i) to evaluate the dechlorination abilities of indigenous microbes at the contaminated site, (ii) to characterize which microbes and reductive dehalogenase genes were responsible for the dechlorination reactions, and (iii) to develop a PCE-to-ethene dechlorinating microbial consortium. An enrichment culture that dechlorinates PCE to ethene was obtained from Wonju stream, nearby a trichloroethene (TCE)-contaminated industrial complex. The community profiling revealed that known organohalide-respiring microbes, such as *Geobacter*, *Desulfuromonas*, and *Dehalococcoides* grew during the incubation with chlorinated ethenes. Although *Chloroflexi* populations (*i.e.*, *Longilinea* and *Bellilinea*) were the most enriched in the sediment microcosms, those were not found in the transfer cultures. Based upon the results from pyrosequencing of 16S rRNA gene amplicons and qPCR using TaqMan chemistry, close relatives of *Dehalococcoides mccartyi* strains FL2 and GT seemed to be dominant and responsible for the complete detoxification of chlorinated ethenes in the transfer cultures. This study also demonstrated that the contaminated site harbors indigenous microbes that can convert PCE to ethene, and the developed consortium can be an important resource for future bioremediation efforts.

Keywords: Chlorinated ethenes, reductive dechlorination, *Dehalococcoides mccartyi*, pyrosequencing

Introduction

Tetrachloroethene (PCE) and trichloroethene (TCE) are the most common groundwater contaminants in aquifers of industrial and urban areas [3, 7, 27]. Since PCE and TCE are listed as probable carcinogens by the International Agency for Research on Cancer, and vinyl chloride (VC), an intermediate in reductive dechlorination of PCE/TCE, is proven as a human carcinogen, chlorinated ethenes in groundwater aquifers are prior environmental contaminants threatening human health. During the last two decades, many researchers have demonstrated that PCE and TCE can be transformed under anaerobic conditions to ethene, a nontoxic compound. However, lesser chlorinated intermediates, such as *cis*-1,2-dichloroethene (*cis*-DCE) and VC, can accumulate in the environment owing to their slower degradation rates caused by constraints of the microbial species responsible for their dechlorination [3, 27, 44]. Several bacterial species are known to dechlorinate PCE to TCE or DCEs; however, the ability to dechlorinate DCEs and VC to ethene is restricted to a few known *Dehalococcoides mccartyi* strains [11, 12, 18–20, 30, 31, 34–36,

45, 46, 48]. Previous case studies also showed that reductive dechlorination to ethene could be successfully applied to contaminated sites [26, 32].

In Korea, chlorinated ethenes, especially TCE, are also frequently detected in industrial and urban areas [1, 25]. An industrial complex in Wonju was one of the most problematic sites in Korea, exhibiting TCE concentrations exceeding the South Korean groundwater standard of 0.03 mg/l since 1995. For the last two decades, remedial trials including pump and treat, soil vapor extraction, and bioventing were conducted to remove TCE from the site, with limited success, and chlorinated compounds were still detected in monitoring wells. Remediation failures have been attributed to the hydrogeologic characteristics of the fractured bedrock aquifers and a misjudgment of the contamination source [1]. Reductive dechlorination intermediates, namely *cis*-DCE and VC, were observed downgradient in the groundwater and near the Wonju stream, located nearby the industrial complex [1], suggesting that native dechlorinating microorganisms are present but their ability to convert these intermediates to ethene is not known. Given the potential importance of the microbial reductive dechlorination for site remediation, further investigation into natural attenuation by indigenous bacteria is important for predicting contaminant fate and managing such sites [13]. The Korea Ministry of Environment has reactivated remediation of the area, but knowledge of the indigenous microbial communities and their dechlorination abilities remains limited.

The objectives of the present study were to evaluate the dechlorination abilities of indigenous microbes at the Wonju site, to characterize which microbes and reductive dehalogenase genes were responsible for the dechlorination reactions, and to develop a PCE-to-ethene dechlorinating microbial consortium.

Materials and Methods

Site Characterization and Sampling

The study area is an industrial complex in Wonju, which is located in the north central part of South Korea. A diagram of the industrial complex, groundwater flows, and the sampling location are shown in Fig. S1 (modified from [1]). The dechlorination intermediates (*i.e.*, *cis*-DCE and VC) were observed in the monitoring wells near the stream. Sediment samples were collected from the Wonju stream using 50 ml conical tubes (BD Biosciences, NJ, USA). The sampling location was possibly exposed to chlorinated ethenes according to the previous observations [1]. The tubes were filled with the sediments and sealed to avoid exposure to air. Samples were stored for 10 days at 4°C. Then, samples were homogenized and directly used for microcosm setup in an anoxic chamber (Coy Laboratory Products Inc., Glass Lake, MI, USA).

Microcosm Setup and DNA Extraction

Approximately 10 g of sediment was transferred to 160 ml glass serum bottles containing 90 ml of sterile, anoxic medium (5 mM sodium bicarbonate buffer, pH 7.2) [28]. Triplicate microcosms were amended with 0.1 mM PCE (aqueous phase concentration) or 0.1 mM TCE (aqueous phase concentration). Sterile hydrogen gas (90 μmol) and 5 mM acetate were used as the electron donor and carbon source, respectively. Triplicate heat-treated microcosms (15 min at 121°C) served as controls. All microcosms were incubated stationary, in the dark and at 25 °C, but were sampled periodically to monitor chlorinated ethene concentrations. For the measurements, 100 μl headspace samples were taken and analyzed using a Hewlett-Packard 7890 A GC equipped with a flame ionization detector and a HP-624 column (60 m by 0.32 mm; film thickness, 1.8 μm) (Agilent, CA, USA). All injections were performed with a split ratio of 50:1. Helium was used as the carrier gas with a flow rate of 3 ml/min. The inlet and detector temperatures were 200°C and 300°C, respectively. The initial oven temperature was kept at 60°C for 2 min, increased with a rate of 25°C/min to 200°C. Standard curves were prepared by measuring the headspace samples of vials that contained known masses of PCE, TCE, *cis*-DCE, VC, and ethene. Among nine replicate microcosms, two PCE-amended microcosms were destructively collected for DNA extraction after 30 days (maximum production of *cis*-DCE, M1), 50 days (VC formation, M2), and 80 days (ethene formation, M3). After all chlorinated ethenes were depleted, genomic DNA from the PCE-to-ethene dechlorinating microcosm (WJ-1, 120 days) and the TCE-to-ethene dechlorinating microcosm (WJ-2, 90 days) was extracted using the PowerSoil DNA Isolation Kit (MO Bio, CA, USA) according to the manufacturer's recommendations. Genomic DNA from the initial sediments was also extracted to investigate microbial community shifts during the incubation.

Enrichment of the Dechlorinating Consortium with Serial Transfers and Selective Substrates

After all chlorinated ethenes were completely converted to ethene, the cultures were transferred to fresh medium (2% (v/v)) using disposable syringes and 21-gauge needles. Anoxic mineral salts medium (reduced, 30 mM bicarbonate-buffered, sterilized, pH 7.2–7.3) was prepared in 160 ml serum bottles and the medium was amended with 0.1 mM PCE (aqueous phase) and vitamins [28, 29]. For 1st to 4th transfer cultures, 90 μmol of sterile hydrogen gas and 5 mM acetate were used as the electron donor and carbon source, respectively. Anoxic, filter-sterilized ampicillin solution (1 mg/ml) was added to the 3rd transfer cultures to inhibit growth of susceptible bacteria and selectively enrich *Dehalococcoides*

populations [28]. For the 5th transfer cultures, 5 mM lactate, 5 mM formate, and 5 mM propionate were also tested to discover the optimal substrate for the dechlorinating consortium. To inhibit methanogens, 2 mM of 2-bromoethanesulfonate (BES) was added to the 6th transfer cultures. After the 6th transfer, lactate was used as the electron donor and carbon source. During the transfers, 1 ml of supernatant was collected from each culture and filtered (0.25 μm pore size). Genomic DNA was extracted from the filter using the PowerSoil DNA Isolation Kit as described above.

454 Pyrosequencing

To prepare PCR products of suitable length for pyrosequencing, primer sets targeting the V1 to V3 region of bacterial 16S rRNA genes (533 bp) [8] and the VC reductase (*vcrA*) genes (441 bp) [5, 36] were used. Eight-nucleotide-long barcodes with a common linker (AC for bacterial 16S rRNA genes and CA for *vcrA* genes) were inserted into each primer set to distinguish the samples. PCR amplifications and pyrosequencing were performed by Chunlab (http://www.chunlab.com; Seoul, Korea). The amplification of bacterial 16S rRNA genes for pyrosequencing was performed as previously described [8]. For the amplification of *vcrA* genes, a touchdown PCR was performed to improve the amplification specificity. The reaction mixture (50 μl) contained the same ingredients as the reaction mixture for the 16S rRNA gene amplification, except the addition of 1.5 mM of $MgCl_2$. Amplification used the following conditions: (i) an initial denaturation step of 94°C for 3 min; (ii) 10 cycles of denaturation, annealing, and extension (94°C for 45 sec followed by 64°C to 55°C for 30 sec, with an extension step at 72°C for 1 min); (iii) another set of 25 cycles of denaturation, annealing, and extension (94°C for 45 sec followed by 55°C for 30 sec, with an extension step at 72°C for 1 min); and (iv) the final extension of 72°C for 7 min. During the first 10 cycles, the temperature of each annealing step was decreased by 1°C every cycle. The PCR products were purified using the QIAquick PCR purification kit (Qiagen, CA, USA). The amplicon pyrosequencing was performed using a 454/Roche GS-FLX Titanium Instrument (Roche, NJ, USA).

Sequence Analysis

The obtained sequences (179,716 sequences for sediment microcosms and 2,496 sequences for the 7th transfer cultures) were screened for the sequences with 0 to 2 primer mismatches and 0 ambiguous base calls (Ns), and separated by sample-specific barcodes using the Ribosomal Database Project II (RDP) pyrosequencing pipeline (http://rdp.cme.msu.edu/) [4, 10]. Sequences shorter than 300 nucleotides, with an Average Quality Score of less than 20, were removed. After the quality control, chimeric sequences were removed using the Mothur software package [42]. Bacterial 16S rRNA sequences obtained from pyrosequencing were aligned and clustered (*i.e.*, complete linkage clustering) at 3% sequence dissimilarity cut-off using the RDP pyrosequencing pipeline. Representative sequences for each operational taxonomic unit were classified through Classifier and SeqMatch through the RDP website. Notable genera were selected if their relative abundances were over 1% after incubation. The 16S rRNA gene sequences of *Dehalococcoides* populations were aligned with previously characterized sequences of *D. mccartyi* isolates [6, 18, 19, 35, 36, 45] as reference sequences using MUSCLE [14]. The average length of the aligned and trimmed sequences was 350 bp. Using the sequence alignments, phylogenetic trees were constructed for cultures WJ-1 and WJ-2 with MEGA4 [47]. For the phylogenetic tree inference, a neighbor-joining algorithm and a bootstrapping test (1,000 replicates and 64,238 random seeds) were used.

The *vcrA* gene sequences were aligned and clustered (*i.e.*, complete linkage clustering) at 90% sequence similarity using Mothur [42]. To exclude the sequences containing stop codons, the representative sequences were translated to amino acid sequences using the six-frame translation in the website of BCM search Launcher (http://searchlauncher.bcm.tmc.edu/seq-util/Options/sixframe.html). The selected sequences were subjected to BLAST and a phylogenetic tree was constructed as described above with the known VC dehalogenases [5]. All the 16S rRNA and *vcrA* gene sequences have been deposited in the Short Read Archive, under the accession number SRP007971.

Quantitative Real-Time PCR

Genomic DNA was extracted as above from the original sediments (WJI), the PCE-to-*cis*-DCE-dechlorinating microcosm (M1), the *cis*-DCE-to-VC-dechlorinating microcosm (M2), and the VC-to-ethene-dechlorinating microcosm (M3), and after all chlorinated ethenes were depleted (WJ-1). SYBR Green assays were performed to quantify the total numbers of bacterial and *D. mccartyi* 16S rRNA genes and reductive dehalogenase genes (*i.e.*, *tceA*, *bvcA*, and *vcrA*) using the ViiA 7 Real-Time PCR System (Applied Biosystems, CA, USA). All SYBR Green assays used the Power SYBR Green PCR Master Mix (Applied Biosystems). Each 20 μl reaction mixture contained 1× SYBR Green Master Mix, primers (300 nM each), and 2 μl of template DNA. Amplifications were performed under the following conditions: (i) an initial denaturation step of 94°C for 10 min, (ii) 40 cycles of 15 sec at 94°C and 1 min at 60°C, and (iii) the melting curve analysis (95°C for 15 sec, 60°C for 1 min, a slow ramp of 0.05°C/sec to 95°C, and 95°C for 15 sec). TaqMan assays were also performed to confirm the SYBR Green quantification results. Each 20 μl reaction mixture contained 1× TaqMan Universal PCR Master Mix (Applied Biosystems), primers and probe (300 nM each), and 2 μl of template DNA. Amplifications were performed under the same thermocycling conditions, excluding the melting curve analysis. The quantifications were conducted using the primer and probe sets targeting bacterial 16S rRNA genes [16, 39], *D. mccartyi* 16S rRNA genes [18], and dehalogenase genes [21, 39] (Table S1). Calibration curves were established using 10-fold serial dilutions of plasmid DNA solutions containing a *D. mccartyi* strain FL2 16S

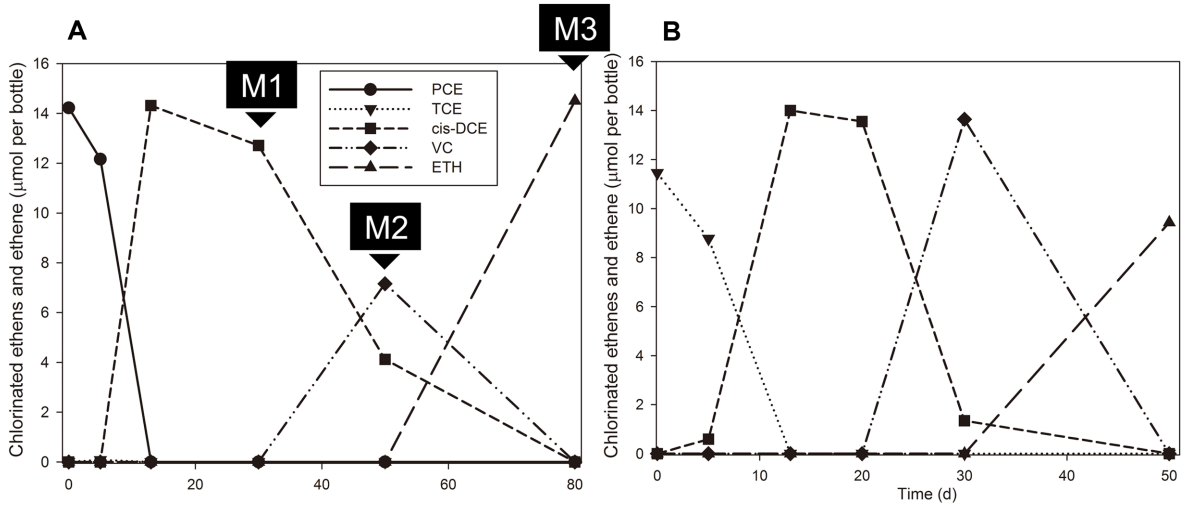

Fig. 1. Dechlorination of chlorinated ethenes in the microcosms using sediment sample.
Culture WJ-1 (**A**) was amended with PCE and culture WJ-2 with TCE (**B**). M1, M2, and M3 indicate the periods, when samples were taken for DNA extraction.

rRNA gene and a *tce*A, *bvc*A, or *vcr*A gene fragment [17]. Gene copy numbers (per gram of sediment) were calculated as previously described [39].

Results

Dechlorination Activities of Sediment Microcosms and Enriched Dechlorinating Consortium

In the sediment microcosms, PCE was completely converted to ethene within 80 days (Fig. 1A; culture WJ-1) and TCE was converted to ethene within 50 days (Fig. 1B; culture WJ-2). An average of 500 µmol/bottle methane was produced in both enrichment cultures (data not shown); however, no inhibitory effects on reductive dechlorination were noted, indicating that the microcosms were not electron donor-limited.

Dechlorinating cultures derived from the WJ-1 microcosm have been transferred every 3–4 months and maintained their dechlorinating abilities for over 3 years. All transfer cultures exhibited 10–14-day lag periods before dechlorination started. During the dechlorination process, TCE and *cis*-DCE were not detected, while VC accumulated. VC was then dechlorinated to ethene and all VC had been consumed after 120 days of incubation. Apparently, the VC-to-ethene step was rate-limiting, as had been observed previously [40]. The substrate test in the 5th transfer cultures demonstrated that the cultures receiving 5 mM lactate had dechlorinated most of the chlorinated ethenes to ethene within 120 days. During the same period, VC was the most abundant product in the cultures with H_2+acetate or formate, whereas TCE accumulated in the cultures amended with propionate (Table 1). Therefore, lactate was selected as the substrate for the further transfers. Methanogenesis did not seem to inhibit the dechlorination processes in the sediment microcosms (*i.e.*, WJ-1 and WJ-2 cultures). However, as methanogens became more abundant through serial transfers, electron donor limitation affected the extent of reductive dechlorination. After methanogens were eliminated using 2 mM BES in the 6th transfer cultures, the production of methane ceased (data not shown).

Table 1. Total mass of chlorinated ethenes that remained in the transfer cultures amended with different substrates.

Substrates	Injected PCE (µmol)	Chlorinated ethenes and ethene after 120 days (µmol)				
		PCE	TCE	*cis*-DCE	VC	ETH
H_2+acetate	43.4	2.0	0	0	25.2	4.4
Lactate	37.1	0	0	0	4.1	23.8
Formate	42.3	4.6	2.4	0.7	32.6	0.3
Propionate	40.7	0	21.6	3.6	1.3	0

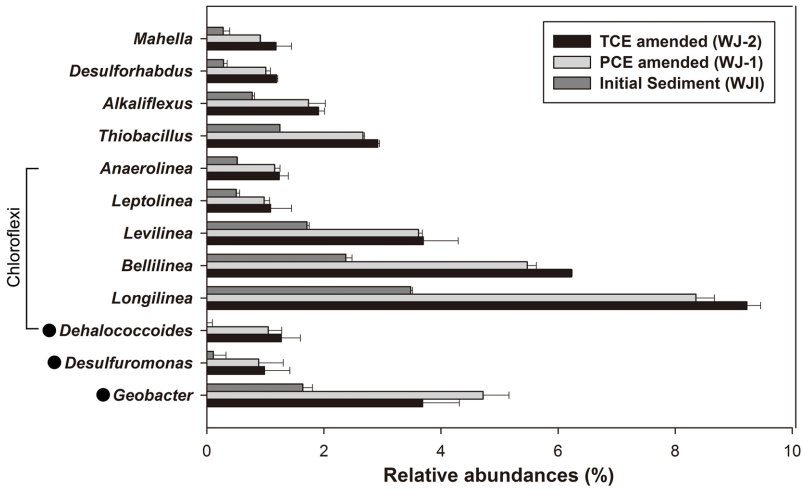

Fig. 2. Changes in relative abundance of major populations in response to the dechlorination enrichment.
The relative abundances are the mean values of duplicate samples. The genera with the dots on the left side are known PCE/TCE dechlorinators from previous studies. Error bars indicate the standard deviations of the relative abundances for the duplicate sample.

Bacterial Community Shifts During the Dechlorination Process

The duplicate DNA samples of the original sediment (WJI) and the dechorinating microcosms WJ-1 and WJ-2 yielded 67,031 16S rRNA gene sequences that passed quality control and chimera removal. The sequence analysis demonstrated that the microbial community of the original WJI sediment was significantly different from the two microcosms WJ-1 and WJ-2 (Fig. 2). Since the microbial communities of the duplicate samples were almost identical to each other, the means of the relative abundances of the duplicate samples were selected as the representative value for each genus. Members of the genera *Geobacter*, *Desulfuromonas*, and *Dehalococcoides* seemed responsible for reductive dechlorination in both WJ-1 and WJ-2 microcosms. In addition, the relative abundances of *Dehalococcoides* populations increased remarkably by 515- and 638-fold in cultures WJ-1 and WJ-2, respectively. Only one *Dehalococcoides* 16S rRNA gene sequence was found in both WJI replicates (0.002% of total sequences), whereas 265 and 134 sequences of *Dehalococcoides* populations were observed in WJ-1 (1.0% of total sequences) and WJ-2 (1.2% of total sequences), respectively. However, the most dominant enriched populations were not the previously characterized dechlorinating groups but two clades of Chloroflexi: the described genera closest to them are *Longilinea* and *Bellilinea*. In addition, three other Chloroflexi populations, relatives of *Levilinea*, *Leptolinea*, and *Aerolinea*, also increased during the incubation. Together they comprised 20% of the total sequences.

Identification and Sequence Analysis of *Dehalococcoides* Populations

Among the obtained *Dehalococcoides* sequences (*i.e.*, 400 sequences from WJI, WJ-1, and WJ-2), a large portion of the *Dehalococcoides* sequences (80.4% for WJ-1 and 89.5% for WJ-2) were highly related to the three *D. mccartyi* strains GT, FL2, and BAV1 (99% rRNA gene sequence identity). However, 19.6% of sequences for WJ-1 and 10.5% for WJ-2 showed 98% or less sequence similarity to the known *D. mccartyi* sequences (Fig. S2), and 11.6% of the *Dehalococcoides* sequences for WJ-1 and 4.2% for WJ-2 were even more dissimilar, with 97% similarity to the known sequences. The sequences fell into three groups: Group 1A contains sequences most similar to those of *D. mccartyi* strain BAV1, Group 1B sequences resembled those of *D. mccartyi* strain GT and strain FL2, and Group 2 sequences formed a novel cluster (95–97% sequence similarity to the known *D. mccartyi* 16S rRNA genes).

Quantification of *Dehalococcoides mccartyi* 16S rRNA and Reductive Dehalogenase Genes

During the *cis*-DCE-to-ethene reductive dechlorination (M2 to WJ-1), quantification using SYBR Green chemistry resulted in 200-fold higher *vcr*A gene (known to be up-regulated in response to TCE, *cis*-DCE, and VC) abundances compared with *D. mccartyi* 16S rRNA gene abundances for the same sample (Table 3). This discrepancy was unexpected, because *D. mccartyi* strains are known to have one 16S rRNA gene and one of *tceA* (in response to TCE and *cis*-DCE), *bvcA* (in response to DCEs and VC), or *vcrA* genes

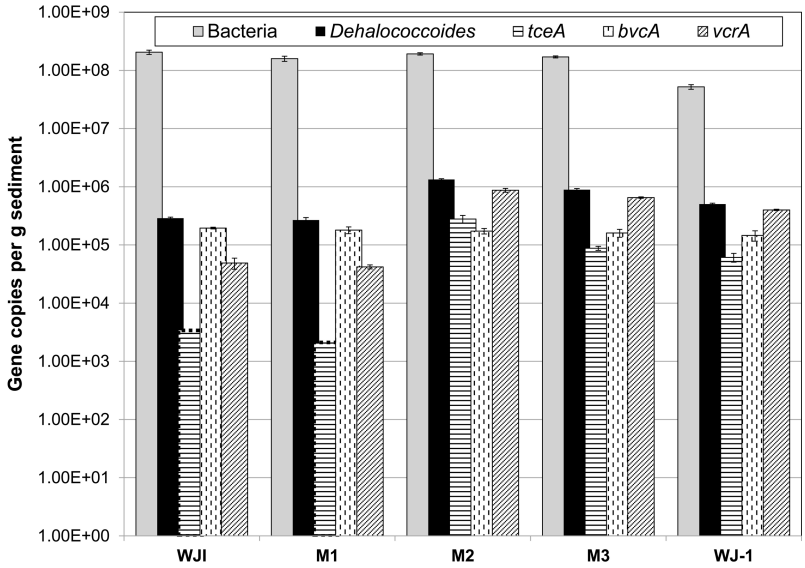

Fig. 3. qPCR results (TaqMan assays) for total bacterial 16S rRNA, *Dehalococcoides mccartyi* 16S rRNA, and dehalogenase genes (*tceA*, *bvcA*, *vcrA*) at each dechlorination stage (WJI, initial sediment; M1, PCE-to-*cis*-DCE dechlorination; M2, *cis*-DCE-to-VC dechlorination; M3, VC-to-ethene dechlorination; WJ-1, after all chlorinated ethenes were depleted).
Error bars indicate the standard deviation of the triplicate amplification. Dotted lines indicate "detected but not quantifiable."

per genome [31, 39, 49]. To validate the results produced by the SYBR Green chemistry, TaqMan assays were performed for the same DNA samples. Both detection methods (*i.e.*, SYBR Green and TaqMan) did not show significant differences (*i.e.*, not exceeding 4-fold) in quantifying bacterial and *D. mccartyi* 16S rRNA genes (Table 3). However, TaqMan chemistry showed the expected 1:1 ratio of dehalogenase (*i.e.*, sum of *tceA*, *bvcA*, and *vcrA*) to *D. mccartyi* 16S rRNA genes for all DNA samples (Fig. 3).

Over the course of PCE-to-ethene reductive dechlorination (M1 to M3), the TaqMan qPCR results demonstrated that there was no significant change in the number of bacterial 16S rRNA genes (1.59×10^8 to 1.70×10^8 copies/g) (Fig. 3). During the same period, the number of *D. mccartyi* 16S rRNA, *tceA*, and *vcrA* genes increased 3-fold (2.67×10^5 to 8.92×10^5 copies/g), 41-fold (2.11×10^3 to 8.76×10^4 copies/g), and 15-fold (4.20×10^4 to 6.53×10^5 copies/g), respectively (Fig. 3). Although *bvcA* genes were more abundant than other reductive dehalogenase genes in the WJI sediment (1.95×10^5 copies/g) and the M1 microcosm (1.80×10^5 copies/g), the number of *bvcA* genes decreased slightly during the incubation period (1.60×10^5 copies/g).

Phylogenetic Analysis of VC Reductase Genes

Since *vcrA* genes were possibly involved in VC-to-ethene dechlorination in the enrichment cultures, those amplicons were sequenced and analyzed. After quality filtering, 1,417 and 1,427 sequences were obtained from WJ-1 and WJ-2, respectively. Among the sequences, 1,410 sequences from WJ-1 sequences were clustered together, but 1,342 sequences from WJ-2 clustered into two clusters, the smaller one comprising 83 sequences. The phylogenetic tree at amino acid level showed that most of the sequences were highly close to previously known reductive dehalogenases (Fig. S3). The BLAST results also showed that the representative *vcrA* gene sequences were identical (99–100% sequence similarity) to the reductive dehalogenase genes from *D. mccartyi* strain GT (YP_003463052), VS (YP_003330719), and KB-1 (ABA64549).

Microbial Community Analysis of 7th Transfer Cultures

Owing to the elimination of methanogens using BES in the 6th transfer cultures, methanogenesis no longer affected the reductive dechlorination process. After complete detoxification of chlorinated ethenes in the subsequent transfer (*i.e.*, 7th transfer cultures), the microbial community of the non-methanogenic dechlorinating consortium was analyzed using 454 pyrosequencing (Table 2). As expected, *Dehalococcoides* populations were predominant in the culture. The overall community compositions between technical replicates (Rep #1 and Rep #2) were similar but the relative abundances differed slightly. Most of the *Dehalococcoides* sequences obtained from pyrosequencing and Sanger sequencing were >99% identical to *D. mccartyi*

Table 2. Bacterial community composition at the genus level, and the relative abundances of major genera (% of total).

454 Pyrosequencing		
Genus	Rep #1 (*N* = 906)	Rep #2 (*N* = 1,174)
Dehalococcoides (Chloroflexi)	62.9	56.2
Treponema (Spirochaetes)	9.1	11.3
Thermovirga (Synergistetes)	5.4	7.0
Pseudoramibacter (Firmicutes)	3.5	3.5
Fulvivirga (Bacteroidetes)	3.2	3.3
Thermodesulfobium (Firmicutes)	0.6	2.0
Nitritalea (Bacteroidetes)	1.5	1.4
Others	13.8	15.2
Sum	100.0	100.0

Pyrosequencing was performed using technical duplicates.

strain GT and strain BAV1.

Discussion

The present study showed that the Wonju River sediment contained bacterial populations that dechlorinate PCE and TCE to ethene, and that the genera comprising known dechlorinators, namely *Geobacter*, *Desulfuromonas*, and *Dehalococcoides*, grew significantly during the incubation, making them candidates responsible for the dechlorination process. In particular, only one of the 16S rRNA gene sequences (0.002% of the total) assigned to *Dehalococcoides* was detected in the amplicon library from the original sediment, but remarkably increased up to 265 sequences (1% of the total) after enrichment with PCE, H_2, and acetate. Through the transfers for over 3 years, a non-methanogenic dechlorinating microbial consortium dominated by *Dehalococcoides* was selectively enriched with the capacity for complete detoxification of chlorinated ethenes.

In the sediment microcosms, Chloroflexi populations (*i.e.*, *Longilinea* and *Bellilinea*) were the most enriched but not found in the transfer cultures. Nevertheless, the enrichment of Chloroflexi populations in the sediment microcosms deserves mention because they were becoming the most dominant members during the incubation. Five Chloroflexi populations grew 2–3 fold, but more importantly, they accounted for 20% of all sequences. Among them, two of the clades, *Longilinea*- and *Bellilinea*-like, showed by far the dominant populations in the microcosms. Both Chloroflexi populations were not found in the transfer cultures, suggesting that their growth in the microcosms could be related to sediment-associated substrates. The transfers under the laboratory condition were not successful to keep the Chloroflexi populations and did not provide evidence to link the growth of Chloroflexi and their actual function. However, investigations on the roles of those populations are still required to get a better understanding of the whole dechlorination processes by complex microbial consortia in the field [22, 23, 37, 43].

There are two notable features in the detected *Dehalococcoides* populations. Although 90% of the sequences were >99% identical to those of strains BAV1, FL2, and GT, 10% seemed to be novel, with 95–98% sequence similarity to any known *D. mccartyi* strains. This possibly means that they are different species, since 16S rRNA cut-off at the species level is <98.5% to previously known groups [9, 15]. However, detailed analysis of these sequences revealed that these sequence dissimilarities were generated by sequencing errors, mostly insertion and deletion errors (Fig. S4). Sequencing errors, caused during either PCR or pyrosequencing itself, have been demonstrated using mock

Table 3. Quantification of bacterial 16S rRNA genes, *Dehalococcoides mccartyi* 16S rRNA genes, and *vcrA* genes using two detection chemistries: TaqMan and SYBR Green.

	Bacterial 16S rRNA genes				*Dehalococcoides mccartyi* 16S rRNA genes				*vcrA* genes			
	TaqMan		SYBR Green		TaqMan		SYBR Green		TaqMan		SYBR Green	
	AVE/g	STD	AVE/g	STD	AVE/g	STD	AVE/g	STD	AVE/g	STD	AVE/g	STD
WJI	2.05E+08	1.79E+07	7.54E+08	4.49E+07	2.89E+05	1.26E+04	1.34E+05	6.52E+03	4.88E+04	1.06E+04	9.61E+04	5.57E+03
M1	1.59E+08	1.63E+07	8.31E+07	2.77E+06	2.67E+05	2.78E+04	5.60E+05	1.71E+04	4.20E+04	3.32E+03	8.61E+04	3.72E+03
M2	1.92E+08	8.99E+06	6.34E+08	2.78E+07	1.34E+06	3.40E+04	2.46E+06	2.42E+05	**8.69E+05**	6.70E+04	**4.67E+08**	5.09E+07
M3	1.70E+08	6.69E+06	1.13E+08	9.31E+06	8.92E+05	4.23E+04	1.18E+06	4.50E+04	**6.53E+05**	1.83E+04	**2.03E+08**	1.16E+07
WJ-1	5.20E+07	4.79E+06	1.89E+08	5.14E+06	5.03E+05	1.75E+04	7.77E+05	3.30E+04	**4.00E+05**	1.02E+04	**1.72E+08**	5.36E+06

There were no significant differences in 16S rRNA gene results as previously reported [17], but notable differences were found in *vcrA* gene results (bold numbers with underlines). Considering that TaqMan assays are more accurate and more sensitive [17], the SYBR Green method overestimated the number of *vcr*A genes.

community studies [2, 38, 41]. Although determining which sequences have errors (especially for substitutions) in thousands of sequences obtained from environmental samples is difficult, it is required in order to exclude suspicious sequences and avoid erroneous interpretations. The second notable feature is that all the recovered *Dehalococcoides* 16S rRNA gene sequences belong to the Pinellas group (*i.e.*, strains BAV1, FL2, and GT), but the number of *bvcA* genes did not increase during the dechlorination processes, although *Dehalococcoides* possessing *bvcA* genes (*i.e.*, strain BAV1 type) were more abundant than other *Dehalococcoides* populations in the initial sediment (WJI). Considering that *D. mccartyi* strain VS (Victoria group) and strain GT (Pinellas group) are known to have one *vcrA* gene in their genomes, it was also interesting that there were no VS-type 16S rRNA gene sequences in the enrichment cultures. This observation suggests that GT-type *vcrA* genes were responsible for the complete detoxification of chlorinated ethenes in the enrichment cultures.

D. mccartyi strains are documented to have one 16S rRNA gene and one of *tceA*, *bvcA*, or *vcrA* genes per genome [31, 39, 49]. In a previous study, qPCR using the SYBR Green method showed that the sum of dehalogenase gene copies was 1–2 orders of magnitude higher than the *D. mccartyi* 16S rRNA genes [33]. Possible explanations that Maphosa *et al*. [33] suggested can be summarized as follows: (i) the specific 16S rRNA primer set could not capture all the responding *Dehalococcoides* populations, (ii) the primer sets targeting *vcrA* genes amplified similar reductive dehalogenase genes in *Dehalococcoides* populations that had many copies in their genome, and these populations grew selectively, (iii) undiscovered dechlorinating populations containing *vcrA* genes might exist, and (iv) *vcrA* genes might be transferred to other microorganisms by horizontal gene transfer and those populations grew more favorably. In the present study, qPCR using SYBR Green also showed similar results; the qPCR results showed the expected 1:1 ratio of dehalogenase (*i.e.*, sum of *tceA*, *bvcA*, and *vcrA*) to 16S rRNA genes in the early, PCE-, and TCE-dechlorinating stages of the study, but at the stage where *cis*-DCE and VC removal occurred, the ratio grew to higher than 100:1. Previously, it was known that the SYBR Green method overestimated the number of *D. mccartyi* 16S rRNA genes in groundwater samples (not in laboratory pure and mixed culture samples) and the TaqMan method was recommended to achieve sensitive and accurate quantifications [17]. Since it was also possible that the SYBR Green method overestimated dehalogenase genes, the TaqMan method was applied for the same DNA samples to compare with the SYBR Green results. The TaqMan assays showed the expected 1:1 ratio in all samples (Table 3). This implies that the unexpected ratio between dehalogenase genes and 16S rRNA genes from SYBR Green assays may not be true and may result in misled interpretations. The accurate results might not be far away from the 1:1 ratio, based upon the current detection technologies.

This study showed that indigenous microbes at the Wonju site were capable of converting PCE to ethene, which is one of the key requirements for bioremediation. The developed dechlorinating consortium can also be an important resource for bioaugmentation. The remaining needs are to show that growth of indigenous dechlorinating populations (and rates) can be enhanced in an economical manner and to evaluate that the physical and chemical conditions of the site are suitable for the enhanced reductive dechlorination.

Acknowledgments

This work was supported by the Yonsei University Research Fund of 2015 (2015-52-0022).

References

1. Baek W, Lee JY. 2011. Source apportionment of trichloroethylene in groundwater of the industrial complex in Wonju, Korea: a 15-year dispute and perspective. *Water Environ. J.* **25:** 336-344.
2. Bakker MG, Tu ZJ, Bradeen JM, Kinkel LL. 2012. Implications of pyrosequencing error correction for biological data interpretation. *PLoS One* **7:** e44357.
3. Bradley PM. 2003. History and ecology of chlororethene biodegradation: a review. *Bioremediat. J.* **7:** 81-109.
4. Cardenas E, Cole JR, Tiedje JM, Park J. 2009. Microbial community analysis using RDP II (Ribosomal Database Project II): methods, tools and new advances. *Environ. Eng. Res.* **14:** 3-9.
5. Carreón-Diazconti C, Santamaría J, Berkompas J, James A, Brusseau ML. 2009. Assessment of in-situ reductive dechlorination using compound-specific stable isotopes, functional-gene PCR, and geochemical data. *Environ. Sci. Technol.* **43:** 4301-4307.
6. Cheng D, He J. 2009. Isolation and characterization of "*Dehalococcoides*" sp. strain MB, which dechlorinates tetrachloroethene to *trans*-1,2-dichloroethene. *Appl. Environ. Microbiol.* **75:** 5910-5918.
7. Christ JA, Ramsburg CA, Abriola LM, Pennell KD, Löffler FE. 2005. Coupling aggressive mass removal with microbial reductive dechlorination for remediation of DNAPL source

zones: a review and assessment. *Environ. Health Perspect.* **113:** 465-477.
8. Chun J, Kim KY, Lee JH, Choi Y. 2010. The analysis of oral microbial communities of wild-type and Toll-like receptor 2-deficient mice using a 454 GS FLX titanium pyrosequencer. *BMC Microbiol.* **10:** 101.
9. Cole JR, Konstantinidis KT, Farris RJ, Tiedje JM. 2010. Microbial diversity and phylogeny: extending from rRNAs to genomes, pp. 1-19. *In* Liu W-T, Jansson JK (eds.). *Environmental Molecular Microbiology*. Caister Academic Press, Wymondham, UK.
10. Cole JR, Wang Q, Cardenas E, Fish J, Chai B, Farris RJ, *et al*. 2009. The Ribosomal Database Project: improved alignments and new tools for rRNA analysis. *Nucleic Acids Res.* **37:** D141-D145.
11. Cupples AM, Spormann AM, McCarty PL. 2003. Growth of a *Dehalococcoides*-like microorganism on vinyl chloride and *cis*-dichloroethene as electron acceptors as determined by competitive PCR. *Appl. Environ. Microbiol.* **69:** 953-959.
12. Cupples AM, Spormann AM, McCarty PL. 2004. Comparative evaluation of chloroethene dechlorination to ethene by *Dehalococcoides*-like microorganisms. *Environ. Sci. Technol.* **38:** 4768-4774.
13. Dowideit K, Scholz-Muramatsu H, Miethling-Graff R, Vigelahn L, Freygang M, Dohrmann AB, Tebbe CC. 2010. Spatial heterogeneity of dechlorinating bacteria and limiting factors for in situ trichloroethene dechlorination revealed by analyses of sediment cores from a polluted field site. *FEMS Microbiol. Ecol.* **71:** 444-459.
14. Edgar RC. 2004. Muscle: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acid Res.* **32:** 1792-1797.
15. Erko S, Ebers J. 2006. Taxonomic parameters revisited: tarnished gold standards. *Microbiol. Today* **33:** 152-155.
16. Harms G, Layton AC, Dionisi HM, Gregory IR, Garrett VM, Hawkins SA, *et al*. 2003. Real-time PCR quantification of nitrifying bacteria in a municipal wastewater treatment plant. *Environ. Sci. Technol.* **37:** 343-351.
17. Hatt JK, Löffler FE. 2012. Quantitative real-time PCR (qPCR) detection chemistries affect enumeration of the *Dehalococcoides* 16S rRNA gene in groundwater. *J. Microbiol. Methods* **88:** 263-270.
18. He J, Ritalahti KM, Aiello MR, Löffler FE. 2003. Complete detoxification of vinyl chloride by an anaerobic enrichment culture and identification of the reductively dechlorinating population as a *Dehalococcoides* species. *Appl. Environ. Microbiol.* **69:** 996-1003.
19. He J, Ritalahti KM, Yang K-L, Koenigsberg SS, Löffler FE. 2003. Detoxification of vinyl chloride to ethene coupled to growth of an anaerobic bacterium. *Nature* **424:** 62-65.
20. Holliger C, Hahn D, Harmsen H, Ludwig W, Schumacher W, Tindall B, *et al*. 1998. *Dehalobacter restrictus* gen. nov. and sp. nov., a strictly anaerobic bacterium that reductively dechlorinates tetra- and trichloroethene in an anaerobic respiration. *Arch. Microbiol.* **169:** 313-321.
21. Johnson DR, Lee PKH, Holmes VF, Alvarez-Cohen L. 2005. An internal reference technique for accurately quantifying specific mRNAs by real-time PCR with application to the *tce*A reductive dehalogenase gene. *Appl. Environ. Microbiol.* **71:** 3866-3871.
22. Kittelmann S, Friedrich MW. 2008. Identification of novel perchloroethene-respiring microorganisms in anoxic river sediment by RNA-based stable isotope probing. *Environ. Microbiol.* **10:** 31-46.
23. Kittelmann S, Friedrich MW. 2008. Novel uncultured Chloroflexi dechlorinate perchloroethene to *trans*-dichloroethene in tidal flat sediments. *Environ. Microbiol.* **10:** 1557-1570.
24. Lee J, Lee TK, Löffler FE, Park J. 2011. Characterization of microbial community structure and population dynamics of tetrachloroethene-dechlorinating tidal mudflat communities. *Biodegradation* **22:** 687-698.
25. Lee JY, Lee KK. 2000. Use of hydrologic time series data for identification of recharge mechanism in a fractured bedrock aquifer system. *J. Hydrol.* **229:** 190-201.
26. Lendvay JM, Löffler FE, Dollhopf M, Aiello MR, Daniels G, Fathepure BZ, *et al*. 2003. Bioreactive barriers: a comparison of bioaugmentation and biostimulation for chlorinated solvent remediation. *Environ. Sci. Technol.* **37:** 1422-1431.
27. Löffler FE, Edwards EA. 2006. Harnessing microbial activities for environmental cleanup. *Curr. Opin. Biotechnol.* **17:** 274-284.
28. Löffler FE, Sanford RA, Ritalahti KM. 2005. Enrichment, cultivation, and detection of reductively dechlorinating bacteria. *Methods Enzymol*. **397:** 77-111.
29. Löffler FE, Tiedje JM, Sanford RA. 1999. Fraction of electrons consumed in electron acceptor reduction and hydrogen thresholds as indicators of halorespiratory physiology. *Appl. Environ. Microbiol.* **65:** 4049-4056.
30. Löffler FE, Yan J, Ritalahti KM, Adrian L, Edwards EA, Konstantinidis KT, *et al*. 2013. *Dehalococcoides mccartyi* gen. nov., sp. nov., obligately organohalide-respiring anaerobic bacteria relevant to halogen cycling and bioremediation, belong to a novel bacterial class, Dehalococcoidia classis nov., order Dehalococcoidales ord. nov. and family Dehalococcoidaceae fam. nov., within the phylum Chloroflexi. *Int. J. Syst. Evol. Microbiol.* **63:** 625-635.
31. Magnuson JK, Romine MF, Burris DR, Kingsley MT. 2000. Trichloroethene reductive dehalogenase from *Dehalococcoides ethenogenes*: sequence of *tceA* and substrate range characterization. *Appl. Environ. Microbiol.* **66:** 5141-5147.
32. Major DW, McMaster ML, Cox EE, Edwards EA, Dworatzek SM, Hendrickson ER, *et al*. 2002. Field demonstration of successful bioaugmentation to achieve dechlorination of tetrachloroethene to ethene. *Environ. Sci. Technol.* **36:** 5106-5116.
33. Maphosa F, Smidt H, De Vos WM, Röling WFM. 2010.

Microbial community and metabolite dynamics of an anoxic dechlorinating bioreactor. *Environ. Sci. Technol.* **44:** 4884-4890.

34. Maymó-Gatell X, Chien Y, Gossett JM, Zinder SH. 1997. Isolation of a bacterium that reductively dechlorinates tetrachloroethene to ethene. *Science* **276:** 1568-1571.
35. Maymó-Gatell X, Nijenhuis I, Zinder SH. 2001. Reductive dechlorination of *cis*-1,2-dichloroethene and vinyl chloride by "*Dehalococcoides ethenogenes*". *Environ. Sci. Technol.* **35:** 516-521.
36. Müller JA, Rosner BM, Von Abendroth G, Meshulam-Simon G, McCarty PL, Spormann AM. 2004. Molecular identification of the catabolic vinyl chloride reductase from *Dehalococcoides* sp. strain VS and its environmental distribution. *Appl. Environ. Microbiol.* **70:** 4880-4888.
37. Narihiro T, Terada T, Ohashi A, Kamagata Y, Nakamura K, Sekiguchi Y. 2012. Quantitative detection of previously characterized syntrophic bacteria in anaerobic wastewater treatment systems by sequence-specific rRNA cleavage method. *Water Res.* **46:** 2167-2175.
38. Pinto AJ, Raskin L. 2012. PCR biases distort bacterial and archaeal community structure in pyrosequencing datasets. *PLoS One* **7:** e43093.
39. Ritalahti KM, Amos BK, Sung Y, Wu Q, Koenigsberg SS, Löffler FE. 2006. Quantitative PCR targeting 16S rRNA and reductive dehalogenase genes simultaneously monitors multiple *Dehalococcoides* strains. *Appl. Environ. Microbiol.* **72:** 2765-2774.
40. Scheutz C, Durant ND, Dennis P, Hansen MH, Jørgensen T, Jakobsen R, *et al*. 2008. Concurrent ethene generation and growth of *Dehalococcoides* containing vinyl chloride reductive dehalogenase genes during an enhanced reductive dechlorination field demonstration. *Environ. Sci. Technol.* **42:** 9302-9309.
41. Schloss PD, Gevers D, Westcott SL. 2011. Reducing the effects of PCR amplification and sequencing artifacts on 16S rRNA-based studies. *PLoS One* **6:** e27310.
42. Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, *et al*. 2009. Introducing Mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl. Environ. Microbiol.* **75:** 7537-7541.
43. Sekiguchi Y. 2006. Yet-to-be cultured microorganisms relevant to methane fermentation processes. *Microbes Environ.* **21:** 1-15.
44. Smidt H, De Vos WM. 2004. Anaerobic microbial dehalogenation. *Annu. Rev. Microbiol.* **58:** 43-73.
45. Sung Y, Ritalahti KM, Apkarian RP, Löffler FE. 2006. Quantitative PCR confirms purity of strain GT, a novel trichloroethene-to-ethene-respiring *Dehalococcoides* isolate. *Appl. Environ. Microbiol.* **72:** 1980-1987.
46. Sung Y, Fletcher KE, Ritalahti KM, Apkarian RP, Ramos-Hernández N, Sanford RA, *et al*. 2006. *Geobacter lovleyi* sp. nov. strain SZ, a novel metal-reducing and tetrachloroethene-dechlorinating bacterium. *Appl. Environ. Microbiol.* **72:** 2775-2782.
47. Tamura K, Dudley J, Nei M, Kumar S. 2007. MEGA4: molecular evolutionary genetics analysis (mega) software version 4.0. *Mol. Biol. Evol.* **24:** 1596-1599.
48. Taş N, Van Eekert MHA, De Vos WM, Smidt H. 2010. The little bacteria that can – diversity, genomics and ecophysiology of "*Dehalococcoides*" spp. in contaminated environments. *Microb. Biotechnol.* **3:** 389-402.
49. Van Der Zaan B, Hannes F, Hoekstra N, Rijnaarts H, De Vos WM, Smidt H, Gerritse J. 2010. Correlation of *Dehalococcoides* 16S rRNA and chloroethene-reductive dehalogenase genes with geochemical conditions in chloroethene-contaminated groundwater. *Appl. Environ. Microbiol.* **76:** 843-850.