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ORIGINAL PAPER

Characterization of microbial community structure and population dynamics of tetrachloroethene-dechlorinating tidal mudflat communities

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Abstract Tetrachloroethene (PCE) and trichloroethene (TCE) are common groundwater contaminants that also impact tidal flats, especially near urban and industrial areas. However, very little is known about dechlorinating microbial communities in tidal flats. Titanium pyrosequencing, 16S rRNA gene clone libraries, and dechlorinator-targeted quantitative real-time PCR (qPCR) characterized reductive dechlorinating activities and populations in tidal flat sediments collected from South Korea's central west coast near Kangwha. In microcosms established with surface sediments, PCE dechlorination to TCE began within 10 days and 100% of the initial amount of PCE was converted to TCE after 37 days. *cis*-1,2-Dichloroethene (*cis*-DCE) was observed as dechlorination end product in microcosms containing sediments collected from deeper zones (i.e., 35–40 cm below ground surface). Pyrosequencing of bacterial 16S rRNA genes and 16S rRNA gene-targeted qPCR results revealed *Desulfuromonas michiganensis*-like populations predominanted in both TCE and *cis*-DCE producing microcosms. Other abundant groups included *Desulfuromonas thiophila* and *Pelobacter acidigallici*-like populations in the surface sediment microcosms, and *Desulfovibrio dechloracetivorans* and *Fusibacter paucivorans*-like populations in the deeper sediment microcosms. *Dehalococcoides* spp. populations were not detected in these sediments before and after incubation with PCE. The results suggest that tidal flats harbor novel, salt-tolerant dechlorinating populations and that titanium pyrosequencing provides more detailed insight into community structure dynamics of the dechlorinating microcosms than conventional 16S rRNA gene sequencing or fingerprinting methods.

Keywords Titanium pyrosequencing · Microbial reductive dechlorination · Tidal flat

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Introduction

The massive use of chlorinated solvents, such as tetrachloroethene (PCE) and trichloroethene (TCE) for dry cleaning, chemical feedstocks, and metal

degreasing, as well as their improper discharge, caused extensive groundwater contamination (Bradley 2003; Christ et al. 2005; Gribble 1994, 2003; Kim and Kweon 2010; Löffler and Edwards 2006; Zinder and Gossett 1995). Under anaerobic conditions chlorinated ethenes can be reductively dechlorinated to lesser chlorinated daughter products including ethene, a nontoxic compound (Bradley 2003; Holliger et al. 1998; Smidt and de Vos 2004). Some specialized microorganisms conserve energy for growth from reductive dechlorination reactions in a process called organohalide respiration (Löffler et al. 1996; Holliger et al. 1998). Due to their toxicity, it is important to examine whether contaminant-receiving environments harbor bacteria capable of reductive dechlorination of chlorinated ethenes. Several bacterial species are known to dechlorinate PCE to TCE or DCEs in terrestrial environments (summarized in Amos et al. 2009). The ability to dechlorinate DCEs and VC to ethene is restricted to some *Dehalococcoides* strains (Christ et al. 2005; Cupples et al. 2003, 2004; Maymo-Gatell et al. 1997, 2001; Müller et al. 2004; Sung et al. 2006a, b; Magnuson et al. 2000; He et al. 2003a, b). Many studies have characterized reductively dechlorinating bacteria from terrestrial sources including contaminated groundwater aquifers (US EPA 2006).

Chlorinated contaminants have also been released into estuarine and marine environments, including tidal flat sediments from urban-area discharge. Contamination of costal sediments (i.e., tidal flats) by chlorinated hydrocarbons can be a serious environmental concern, since tidal flats are environmentally and ecologically important for nutrient cycling, primary productivity and coastal fisheries (Wilms et al. 2006; Koh 2001). Unfortunately, little is known about the presence of chlorinated ethene-dechlorinating bacteria in estuarine and marine environments. In addition to the influx of anthropogenic chloroethenes, natural production of chlorinated hydrocarbons also occurs in marine environments. Marine algae produce halogenated compounds, including highly chlorinated ethenes (Abrahamsson et al. 1995), and subsurface volcanic emission and pyrogenic activity also produce highly chlorinated ethenes (Gribble 1994, 2003). Hence, tidal flats might be a reservoir for marine bacteria capable of dechlorinating chlorinated ethenes. One recent study demonstrated PCE dechlorination to predominantly *trans*-DCE in North Sea tidal flat sediment microcosms. Stable isotope probing implicated a novel Chloroflexi cluster and a *Dehalobium* sp. in the observed dechlorination reactions (Kittelmann and Friedrich 2008). Dechlorination beyond *trans*-DCE was not observed and the sequence identity of the novel Chloroflexi 16S rRNA gene sequences was only 92–94% to known *Dehalococcoides* sequences.

Since the commonality of chlorinated ethene-dechlorinating bacteria in tidal flat sediments and the response of the microbial community to enrichment with PCE is unknown, we explored whether chlorinated ethene dechlorination in microcosms established with tidal flat sediments collected from the west coast of Korea. The west coast of the Korean Peninsula harbors one of the world's most extensive tidal flat ecosystems (4,980 km^2), which has essential ecological and economic functions in these regions receive runoff and discharge from the Seoul region, one of the largest population and industrial areas. We carried out dechlorination assays with in sediment microcosms, and determined population shifts in the community by using 16S rRNA gene based qPCR, clone libraries and titanium pyrosequencing.

Materials and methods

Sample collection and microcosm setup

Tidal flat sediment samples were collected from Yeochari area, Kangwha, South Korea. The sediment samples were collected using clean PVC pipes (50 cm by 10 cm with 0.5 mm thickness) and stored at 4°C for no longer than 30 days. The top (0–5 cm depth) and bottom (35–40 cm depth) sediment layers were used to establish microcosms inside an anoxic chamber (Coy Laboratory Products Inc., Glass Lake, MI). Approximately 2.5 g of sediment was transferred to 20-ml vials with 9 ml of sterile, anoxic saline (3% [w/w] NaCl, 5 mM potassium phosphate buffer, pH 7.2). Triplicate microcosms were amended with 60 μM PCE, and a second set of triplicate microcosms received PCE and 5 mM lactate as electron donor. Heat-treated microcosms (15 min at 121°C) served as negative controls. The following treatments were established: TM; microcosms with the top sediments, BM; microcosms with the bottom sediments, TL; microcosms with the top sediments amended with lactate, and BL; microcosms with the

bottom sediments amended with lactate. Microcosms were incubated at 25°C without shaking for 50 days.

Analytical methods

Gas-tight 250 µl glass syringes (Hamilton, NV, USA) with gas-tight Teflon valves and Luer Lock adapters were used to withdraw 0.1 ml of headspace gas from the microcosms. Volatile chlorinated ethenes were measured using a Hewlett-Packard 6890 GC equipped with a flame ionization detectors and an HP-624 column (60 m by 0.32 mm; film thickness, 1.8 µm). Helium was the carrier gas with a flow rate of 10 ml/min. Temperature was kept at 50°C for 4 min, increased with a rate of 25°C min^{-1} to 250°C, and then held at 250°C for 4 min. Standard curves were prepared by measuring the headspace samples of vessels that contained known masses of chlorinated ethenes (Amos et al. 2007).

PCR amplifications of 16S rRNA genes, cloning and sequencing

Total genomic DNA was extracted from the untreated sediments and from PCE dechlorinating microcosms using a PowerSoil DNA Extraction kit (MO BIO Laboratories, USA) according to the manufacturer's recommendations. Bacterial 16S rRNA genes were PCR amplified using the universal primers 27F and 1492R (Lane 1991). Each PCR reaction contained 1 µl of template DNA, 1× PCR buffer, 2 mM $MgCl_2$, 200 µM of each deoxynucleoside triphosphate (dNTP), 100 pM of each primer, and 1.25 U of *Taq* polymerase (Invitrogen, Inc., WI, USA) in a total volume of 25 µl. Amplification occurred in a C1000TM Thermal Cycler (BIO-RAD, CA, USA) using following conditions: (i) an initial denaturation step of 94°C for 3 min, (ii) 25 cycles of denaturation, annealing and extension (94°C for 1 min followed by 54°C for 30 s, with an extension step at 72°C for 2 min), and (iii) a final extension at 72°C for 5 min. The PCR products were purified using the QIAquick PCR purification kit (Qiagen, CA, USA) before the amplicons were inserted into the pCR4-TOPO cloning vector using a TOPO TA Cloning Kit for Sequencing (Invitrogen, Inc., WI, USA) according to the manufacturer's recommendations. The cloned nearly complete 16S rRNA genes were sequenced by Macrogen Inc. (Seoul, South Korea).

Nested PCR for detecting *Dehalococcoides* 16S rRNA gene sequences in tidal flat sediments

To assess the presence of *Dehalococcoides* populations, a nested PCR approach was applied for the initial sediments (TI; the untreated top sediments and BI; the untreated bottom sediments). Purified amplicons obtained with the universal primer pair were used as template DNA for the second amplification reaction, which contained 1 µl of template DNA, 1× PCR buffer, 2.5 mM $MgCl_2$, 200 µM of each deoxynucleoside triphosphate (dNTP), 100 pM of each primer, and 1.25 U of *Taq* polymerase (Invitrogen, Inc., WI, USA). The *Dehalococcoides* specific primers, 730F and 1350R were used for the nested PCR (Löffler et al. 2000). Purification, cloning, and sequencing procedures were as described above. Distinct sequences have been deposited in the GenBank (accession numbers are HM124529 and HM124530).

Quantitative real-time PCR (qPCR) analysis

The total numbers of 16S rRNA genes of bacteria, *Dehalococcoides* spp., and *Desulfuromonas michiganensis* were quantified to confirm the growth of major dechlorinating populations. The primer sets targeting bacterial 16S rRNA genes (Harms et al. 2003), *Dehalococcoides* spp. (He et al. 2003a, b), and *Desulfuromonas michiganensis* [TCG GGT CCT ACT GTC AT (forward) and TAA TTC CGA ACA ACG CTT (reverse); applied for the first time in this study]. Each 20 µl reaction mixture contained 1× SYBR Master Mix (BIO-RAD, USA), primer sets (300 nM each) and 10-fold-diluted template DNA. qPCR was performed with the iQ5 Real-Time PCR Detection System (BIO-RAD, CA, USA). Calibration curves were established using 10-fold serial dilutions of plasmid DNA carrying cloned *Dehalococcoides* sp. strain BAV1 and *Desulfuromonas michiganenesis* strain BB1 16S rRNA gene fragments. Gene copy numbers (per gram of sediment) were calculated as described (He et al. 2003a, b).

Pyrosequencing

For pyrosequencing, a PCR primer set targeting the V3 region of the 16S rRNA genes, 27F and 518R

(Andersson et al. 2008), was used to prepare amplicons with suitable length. The reaction mixture (25 µl) contained 15–25 ng of template DNA, 10 µM of each primer (BIONEER, Seoul, Korea), 120 ng/µl of bovine serum albumin (New England Biolabs, MA, USA), and 1.25 U of AccuPrime^{TM} *Taq* NA Polymerase High Fidelity (Invitrogen, WI, USA), 50 mM of $MgSO_4$, 1× of PCR buffer. Amplification occurred in a C1000TM Thermal Cycler (BIO-RAD, CA, USA) using following conditions: (i) an initial denaturation step of 94°C for 3 min, (ii) 25 cycles of denaturation, annealing and extension (94°C for 1 min followed by 54°C for 30 s, with an extension step at 72°C for 2 min), and (iii) a final extension at 72°C for 5 min. PCR products were purified using an QIAquick PCR purification kit (Qiagen, CA, USA). Amplicon pyrosequencing was performed using a 454/Roche GS-FLX Titanium Instrument (Roche, NJ, USA). Barcodes were applied to distinguish each sample prior to sequencing at Macrogen. Sequences shorter than 250 nucleotides or with an Average Quality Score of less than 20 were removed through quality filters. Distinct sequences (1,800 sequences) have been deposited in the Sequence Read Archive (GenBank accession number of study is SRP002289).

Community analysis

Sequence alignment and complete linkage clustering from 0 to 10% dissimilarity were performed using the Ribosomal Database Project II (RDP) pyrosequencing pipeline (http://rdp.cme.msu.edu/) (Cole et al. 2009; Cardenas et al. 2009). The number of clusters was considered as Operational Taxonomic Units (OTUs) for generating rarefaction curves analysis and for calculating diversity indices.

The clustering result based upon 97% of sequence identity was used for the preliminary community shift analysis. Representative sequences for each OTU were identified through Classifier and SeqMatch provided through the RDP website. After the genera that increased during the incubation were identified, an additional clustering analysis based upon 100% of sequence similarity was performed for these sequences and their closest related sequences were identified with Greengenes (http://greengenes.lbl.gov/). The sequences that increased during the incubation were aligned with the 16S rRNA sequences from known dechlorinating bacteria and with the sequences capture in the clone libraries using MUSCLE (Edgar 2004). The average size of the aligned and trimmed sequences was 350 bp. Using the multiple-aligned sequences, phylogenetic trees were constructed for the TM and BM communities with MEGA4 (Tamura et al. 2007). For the phylogenetic tree inference, a neighbor-joining algorithm was used, and a bootstrapping test (1,000 replicates and 64238 random seeds) was run. As reference sequences, the partial 16S sequences of previously characterized dechlorinating isolates (Suyama et al. 2001; Löffler et al. 1997; Holliger et al. 1998; Chang et al. 2000; Krumholz et al. 1996; Krumholz 1997; Sung et al. 2003, 2006a, b; Luijten et al. 2003; Neumann et al. 1994; Scholz-Muramatsu et al. 1995; Sharma and McCarty 1996) and tidal flat dechlorinating populations (Kittelmann and Friedrich 2008) were used.

Results

Characterization of PCE dechlorination

Following a lag period of about 1 week, PCE was dechlorinated to lesser chlorinated ethenes in all live microcosms tested (Fig. 1). PCE was dechlorinated to TCE and *cis*-DCE in the TM and BM incubations. Dechlorination beyond *cis*-DCE did not occur, even after 50 days of incubation. The addition of 5 mM of lactate to the TL and BL microcosms did not improve dechlorinating activities; however, methane was produced (data not shown).

Microbial diversity of measurements

The sediment samples TI and BI (i.e., without PCE and lactate addition) and the dechlorinating microcosms without an exogenous electron donor/carbon source (i.e., TM and BM) yielded 48,700 sequences (range 10,045 to 13,441 sequences per sample). Sample TI showed the highest degree of microbial diversity (Shannon index, $H' = 8.02$) (Fig. 2). After the dechlorination, microbial diversity in the top sediment microcosm was significantly reduced ($H' = 6.58$), and in the bottom sediment microcosm was slightly reduced (6.61 to 6.35).

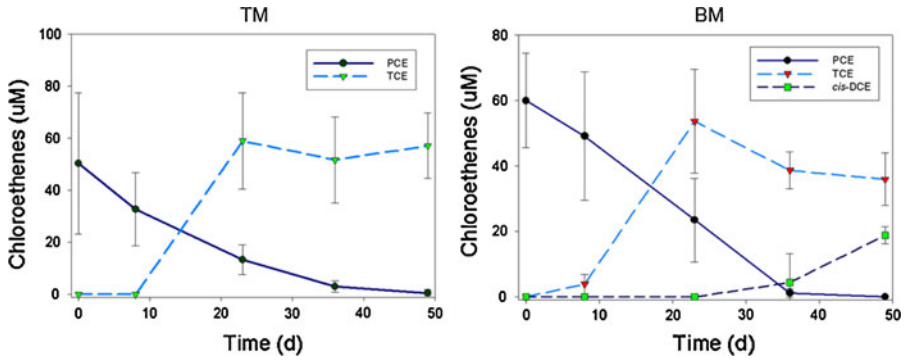


Fig. 1 Reductive dechlorination of PCE (*filled circle*) to TCE (*filled down triangle*) or *cis*-DCE (*filled square*) in the microcosms for top (TM) or bottom (BM) tidal flat sediments. The *Y*-axis error *bar* indicates a standard error from at least two independent microcosm experiments

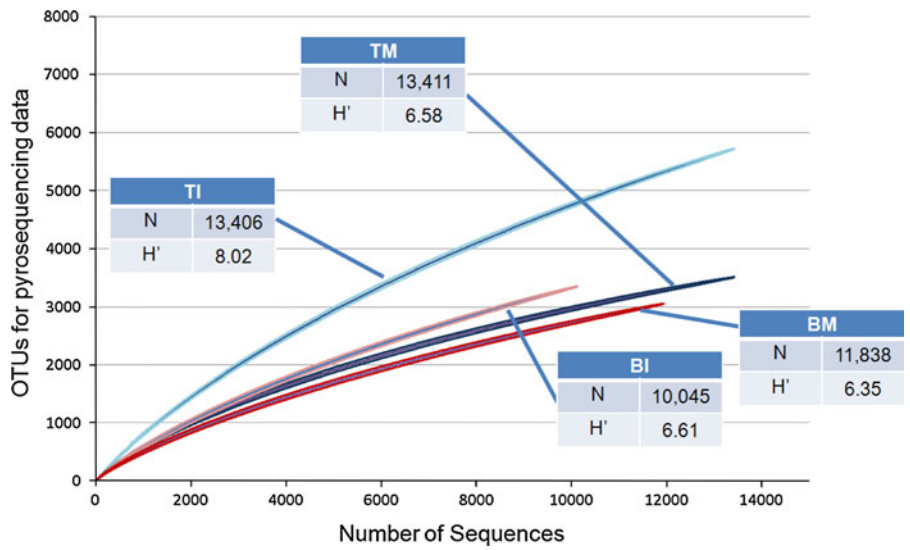


Fig. 2 Rarefaction curve analysis (OTUs at sequence dissimilarity cutoff <3%) and Shannon index as an indicator of microbial diversity. *N* indicates the number of sequences used for the rarefaction curve analysis and calculation for Shannon index

Response of bacterial populations to substrate amendments

After 50 days of incubation, the relative abundances of phylum/sub-class groups were compared between the different treatments to examine shifts in microbial community structures (Table 1). Prior to incubation, the microbial communities of TI and BI were dominated by Gammaproteobacteria (34.7% for TI and 37.0% for BI), followed by Deltaproteobacteria (16.8 and 13.9%), Alphaproteobacteria (7.77 and 10.7%), and Bacteroidetes (9.23 and 9.58%). After incubation, Deltaproteobacteria were predominant (67.3% for TM and 65.2% for BM) while the relative abundance of Gammaproteobacteria was reduced into 9.05% for TM and 4.11% for BM. These changes show that significant community shifts occurred during the incubation period.

We compared our pyrosequencing results to those obtained by Sanger sequencing (89 and 93 sequence reads for TM and BM, respectively). The comparative analysis demonstrated a similar trend to the Titanium results (Table 1). A linear regression analysis showed a good correlation between the Sanger sequencing and the pyrosequencing data ($R^2 = 0.95$). As expected, some rare populations were only detected by the Titanium pyrosequencing, were not detected by Sanger sequencing.

Since Deltaproteobacteria populations increased approximately 4-fold during the 50 day incubation period in the TM and BM microcosms (Table 1), the composition of the Deltaproteobacteria was analyzed

Table 1 Bacterial community composition at the phylum/sub-class level, and the relative abundances of bacterial phylum/sub-class groups (% of total)

Phylum/sub-class	Pyrosequencing		Sanger sequencing		Pyrosequencing		Sanger sequencing	
	TI ($N = 13{,}406$)	TM ($N = 13{,}411$)	TI ($N = 89$)	TM ($N = 89$)	BI ($N = 10{,}045$)	BM ($N = 11{,}838$)	BI ($N = 93$)	BM ($N = 93$)
Acidobacteria	2.09	1.19	3.37	3.37	1.57	1.27	ND	1.08
Actinobacteria	3.05	1.36	1.12	2.25	2.50	1.84	ND	ND
Bacteroidetes	9.23	4.43	21.35	3.37	9.58	3.84	5.38	4.30
Chlamydiae	0.68	0.32	ND	ND	1.88	1.32	ND	ND
Chloroflexi	6.48	5.98	ND	3.37	6.67	5.97	ND	3.23
Cyanobacteria	4.71	1.33	3.37	1.12	1.15	0.64	ND	ND
Deferribacteres	0.58	0.33	ND	1.12	0.91	0.75	2.15	ND
Firmicutes	1.57	1.16	2.25	ND	3.42	6.94	3.23	10.75
Lentisphaerae	2.83	0.81	1.12	ND	1.32	0.85	ND	ND
Nitrospirae	0.80	0.34	1.12	1.12	1.16	0.54	5.38	2.15
Planctomycetes	2.24	1.19	1.12	2.25	1.49	1.08	1.08	2.15
Alphaproteobacteria	7.77	2.37	4.49	1.12	10.68	2.09	6.45	ND
Betaproteobacteria	1.17	0.48	1.12	ND	0.64	0.33	ND	ND
Deltaproteobacteria	16.81	67.38	16.85	67.42	13.89	65.16	12.90	70.96
Gammaproteobacteria	34.86	9.05	34.83	11.25	37.04	4.11	55.90	4.30
Epsilonproteobacteria	0.46	0.28	1.12	ND	3.33	0.73	4.30	1.08
Spirochaetes	0.37	0.24	1.12	1.12	0.47	0.36	3.23	ND
Verrucomicrobia	2.56	0.45	3.37	1.12	0.36	0.22	ND	ND
Others	1.73	1.30	2.24	ND	1.93	1.96	ND	ND
Total	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00

N number of sequences, *TI* top initial sediments, *TM* dechlorinating microcosm with top sediments, *BI* bottom initial sediments, *BM* dechlorinating microcosm with bottom sediments, *ND* not detected

in more detail (Table 2). *Desulfuromonas*-like populations became predominant in both TM and BM enrichments (28.3% for TM and 26.6% for BM; see Table 2). Prior to incubation, *Desulfuromonas*-like populations made contributed only 0.72% for TI and 0.17 for BI. The relative abundances of *Desulfuromonas* significantly increased in the dechlorinating communities, i.e., 39.4 times and 156 times for TM and BM, respectively. In addition, the relative abundances of *Fusibacter* and *Desulfovibrio* groups significantly increased only in the BM microcosms, which dechlorinated PCE-to-*cis*-DCE (Table 2).

The number of the total OTUs at 97% of sequence similarity was 13,277, and only 166 OTUs had a relative abundance of >0.1% and significantly increased during the incubation, i.e. 0.10 to 44.7% for TM and 0.20 to 51.3% for BM (Fig. 3). The identities of the dominant OTUs at 100% of sequence similarity were *Desulfuromonas michiganensis* strain BB1-like populations in both TM and BM, followed by *Desulfuromonas thiophila* and *Pelobacter acidigallici* in TM and *Desulfovibrio dechloracetivorans* and *Fusibacter paucivorans* in BM. The *Fusibacter paucivorans* and *Desulfovibrio dechloracetivorans*-like population grew only in the bottom sediment microcosms, in which *cis*-DCE was produced as the end degradation intermediate.

Phylogenetic analysis: comparison to terrestrial dechlorinating bacteria

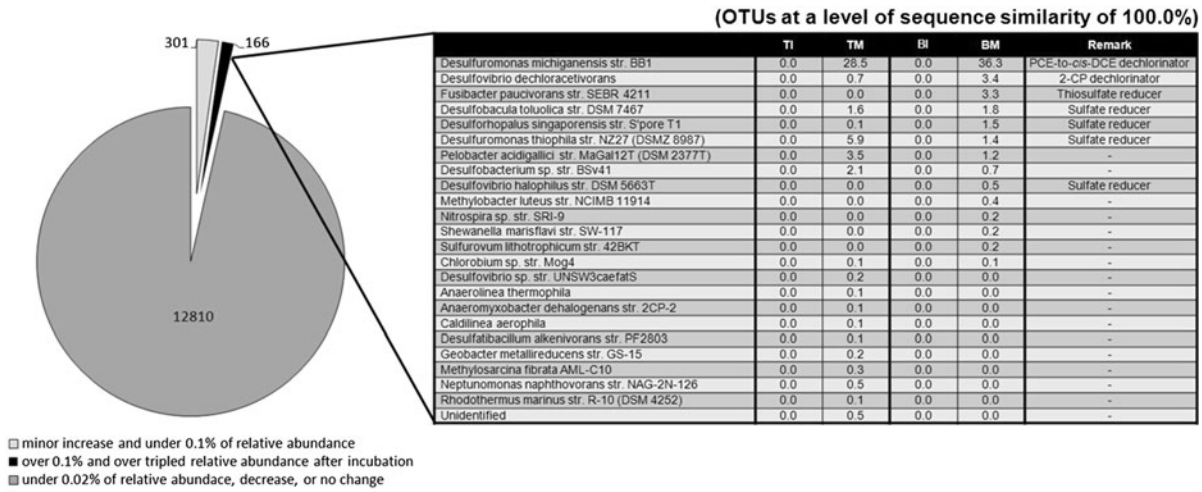
To compare *Desulfuromonas* and *Fusibacter* populations with previously characterized dechlorinating bacteria, a phylogenetic analysis was conducted (Fig. 4). The sequences that significantly increased during the incubations fell into 4 groups; Cluster I to Cluster IV. The *Desulfuromonas* 16S rRNA gene sequences that increased in the bottom sediment

Table 2 Identification at the genus level of the populations that grew and their relative abundances (% of total)

Major genera	TI ($N = 13{,}406$)	TM ($N = 13{,}411$)	Fold increase	BI ($N = 10{,}045$)	BM ($N = 11{,}838$)	Fold increase	Phylum/sub-class
Desulfuromonas	0.72	28.33	39.3	0.17	26.63	156.6	Deltaproteobacteria
Pelobacter	1.43	15.12	10.6	0.36	15.76	43.8	
Geothermobacter	0.92	6.52	7.1	0.44	2.69	6.1	
Geopsychrobacter	0.39	5.97	15.3	0.45	1.64	3.6	
Desulfobacter	0.01	2.37	237.0	ND	2.43	–	
Malonomonas	0.15	1.77	11.8	0.09	0.43	4.8	
Desulfovibrio	0.01	1.12	112.0	0.01	5.1	510.0	
Desulfotalea	0.31	0.59	1.9	0.02	1.41	70.5	
Fusibacter	0.02	0.18	9.0	ND	3.82	–	Firmicutes

Sequences from Titanium pyrosequencing were used

N number of sequences, *TI* top initial sediments, *TM* dechlorinating microcosm with top sediments, *BI* bottom initial sediments, *BM* dechlorinating microcosm with bottom sediments, *ND* not detected



	TI	TM	BI	BM	Remark
Desulfuromonas michiganensis str. BB1	0.0	28.5	0.0	36.3	PCE-to-cis-DCE dechlorinator
Desulfovibrio dechloracetivorans	0.0	0.7	0.0	3.4	2-CP dechlorinator
Fusibacter paucivorans str. SEBR 4211	0.0	0.0	0.0	3.3	Thiosulfate reducer
Desulfobacula toluolica str. DSM 7467	0.0	1.6	0.0	1.8	Sulfate reducer
Desulforhopalus singaporensis str. S'pore T1	0.0	0.1	0.0	1.5	Sulfate reducer
Desulfuromonas thiophila str. NZ27 (DSMZ 8987)	0.0	5.9	0.0	1.4	Sulfate reducer
Pelobacter acidigallici str. MaGal12T (DSM 2377T)	0.0	3.5	0.0	1.2	-
Desulfobacterium sp. str. BSv41	0.0	2.1	0.0	0.7	-
Desulfovibrio halophilus str. DSM 5663T	0.0	0.0	0.0	0.5	Sulfate reducer
Methylobacter luteus str. NCIMB 11914	0.0	0.0	0.0	0.4	-
Nitrospira sp. str. SRI-9	0.0	0.0	0.0	0.2	-
Shewanella marisflavi str. SW-117	0.0	0.0	0.0	0.2	-
Sulfurovum lithotrophicum str. 42BKT	0.0	0.0	0.0	0.2	-
Chlorobium sp. str. Mog4	0.0	0.1	0.0	0.1	-
Desulfovibrio sp. str. UNSW3caefatS	0.0	0.2	0.0	0.0	-
Anaerolinea thermophila	0.0	0.1	0.0	0.0	-
Anaeromyxobacter dehalogenans str. 2CP-2	0.0	0.1	0.0	0.0	-
Caldilinea aerophila	0.0	0.1	0.0	0.0	-
Desulfatibacillum alkenivorans str. PF2803	0.0	0.1	0.0	0.0	-
Geobacter metallireducens str. GS-15	0.0	0.2	0.0	0.0	-
Methylosarcina fibrata AML-C10	0.0	0.3	0.0	0.0	-
Neptunomonas naphthovorans str. NAG-2N-126	0.0	0.5	0.0	0.0	-
Rhodothermus marinus str. R-10 (DSM 4252)	0.0	0.1	0.0	0.0	-
Unidentified	0.0	0.5	0.0	0.0	-

Pattern in population change	TI	TM	BI	BM
under 0.02% of relative abundance, decrease, or no change	97.9 %	51.5 %	96.8 %	36.8 %
minor increase and under 0.1% of relative abundance	2.00 %	3.80 %	3.00 %	11.9 %
over 0.1% and significantly increased during incubation	0.10 %	44.7 %	0.20 %	51.3 %
Total	100 %	100 %	100 %	100 %

Fig. 3 Populations in 166 OTUs among 13,277 of total OTUs significantly increased during the incubations (0.10–44.7% in the top sediments and 0.20–51.3% in the bottom sediments). Based upon 100% of sequence similarity, those sequences in 166 OTUs were identified using Greengenes database. In both TM and BM, the most abundant group was close to *Desulfuromonas michiganensis* str. BB1, however, the following groups were different in TM (i.e., sulfate reducing bacteria) and BM (i.e., 2-CP dechlorinating group)

Clusters I were most closely affiliated with the *Desulfuromonas michiganensis* sequences of isolates strain BRS1 and strain BB1, which are known PCE-to-*cis*-DCE dechlorinating bacteria from freshwater sediments (96–98% sequence similarity). The Deltaproteobacteria sequences recovered from the top sediment groupd into Cluster II and were also affiliated with the genus *Desulfuromonas* with 94–95% sequence similarity to *Desulfuromonas michiganensis*. The bottom sediment Cluster IV was close to *Desulfovibrio dechloracetivorans*. Cluster III included the sequences from the bottom sediments

that were highly similar to *Fusibacter paucivorans* strain SEBR 4211, which is known as a thiosulfate reducer. The PCE-to-*trans*-DCE dechlorinating Chloroflexi groups from German tidal flats (TfC20H clone sequences in Fig. 4) were phylogenetically distant from any sequences from Korean tidal flat bacterial populations (<90% sequence similarity).

Quantification of *Desulfuromonas* populations in dechlorinating communities

Microbial populations declined slightly in the dechlorinating microcosms as the total bacterial 16S rRNA gene copies, as determined by qPCR decreased from in top and bottom sediments microcosms from 3.10–5.55 $\times$ 10^{11} and 3.22–3.25 $\times$ 10^{11} gene copies per gram to 2.47–2.49 $\times$ 10^{11} and 1.61–1.67 $\times$ 10^{11}, respectively. The pyrosequencing results showed that *Desulfuromonas* 16S rRNA sequences predominated the dechlorinating communities (Table 2). qPCR results corroborated this observation and *Desulfuromonas* 16S rRNA gene sequences increased approximately 200-fold in the top sediment microcosms and 70-fold in the bottom sediment microcosms (Fig. 5). After 50 days of incubation, *Desulfuromonas* populations became predominan, and were 55.7 and 70.1% of total 16S rRNA gene copies quantified in TM and BM microcosms, respectively.

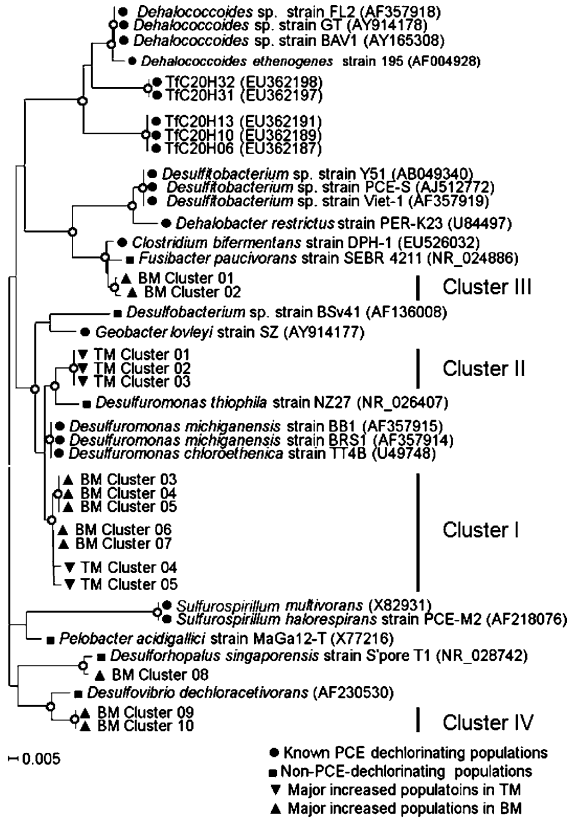


Fig. 4 Distribution of 16S rRNA sequences obtained from the Titanium pyrosequencing. *Desulfuromonas*-like clusters were close relatives to *Desulforumonas michiganensis* strain BB1, and strain BRS1, which were previously characterized PCE-to-*cis*-DCE dechlorinating isolates. Bootstrap values >50 are indicated by *open circle*

Detection and quantification of *Dehalococcoides* 16S rRNA genes

To examine the presence and the potential involvement of *Dehalococcoides* bacteria in the observed dechlorination reactions, nested PCR and qPCR were performed on samples prior to enrichment and after enrichment from both dechlorinating microcosms. According to the qPCR analysis, the *Dehalococcoides* 16S rRNA gene copy numbers were quite low (i.e., less than 3.00 $\times$ 10^5 in all samples) and did not increase during the incubation period (Fig. 5). Amplicons were obtained with the *Dehalococcoides* targeted primers; however, the sequences were only 92–93% similar (over stretch of 650 bp) to those of previously characterized *Dehalococcoides*. The closest match was to a *Dehalococcoides* environmental clone sequence (GQ143778) (Fig. 6). In addition, the Chloroflexi sequences YCTF Chloroflexi 01 and 02 were not close to the Chloroflexi group identified from the German tidal flat. The qPCR and nested PCR results support the findings from the titanium pyrosequencing, i.e., that there was no-growth of Chloroflexi (Table 1) and no-detection of known *Dehalococcoides* 16S rRNA gene sequences (Table 2).

Discussion

Whilst the lag period and the extent of dechlorination observed in the present study were similar to a previous study using German tidal flat sediments (Kittelmann and Friedrich 2008), the end products were different. In German tidal flat microcosm, more *trans*-DCE was produced than *cis*-DCE, while only *cis*-DCE was detected in our study. RNA-based SIP

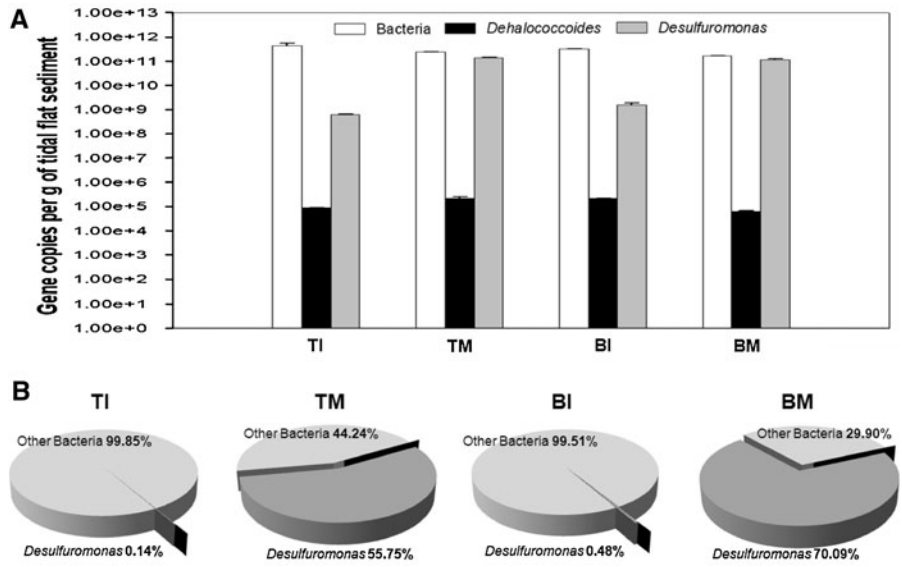


Fig. 5 16S rRNA-based qPCR results for total bacteria, *Desulfuromonas* and *Dehalococcoides* in the tidal mudflat sediments (TI, BI) and their dechlorinating microcosms (TM, BM)

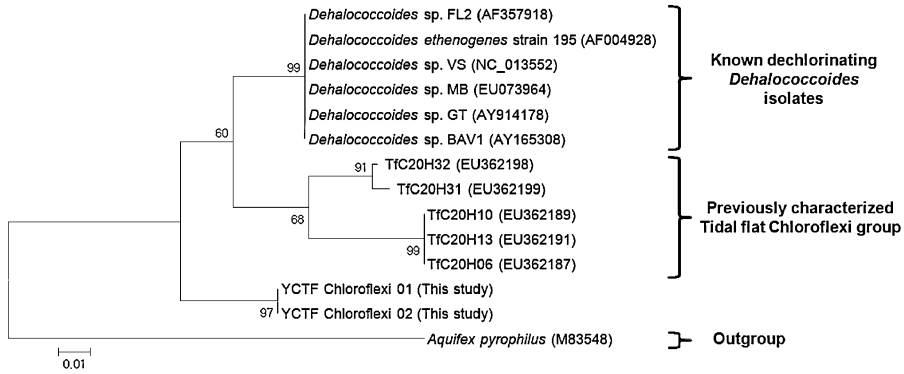


Fig. 6 Phylogenetic placement of Chloroflexi group (YCTF Chloroflexi 01 and 02 from this study) with previously characterized *Dehalococcoides* populations. The *scale bar* represents 1% sequence divergence

suggested that a Chloroflexi group was involved in PCE to *trans*-DCE dechlorinating and that *Desulfuromonas*-like populations were not involved (Kittelmann and Friedrich 2008). In contrast, our results suggest that *Desulfuromonas*-like bacteria were the major dechlorinating populations contributing to PCE to *cis*-DCE dechlorination.

According to studies with terrestrial *Dehalococcoides* isolates (Amos et al. 2009; Cheng and He 2009; He et al. 2003a, b; Löffler et al. 2000; Maymo-Gatell et al. 2001), a PCE-to-*trans*-DCE dechlorinating isolate (MB) and *cis*-DCE producing or respiring isolates (BAV1, FL2, GT, VS, and ethenogenes strain 195) are close relatives (Fig. 6). However, in our and German tidal mudflat sediment communities, PCE-to-DCE dechlorinating microorganisms are phylogenetically distant from the terrestrial *Dechloccoides* populations. Also Chloroflexi populations in the German tidal flat dechlorinating community (PCE-to-*trans*-DCE) were phylogenetically different from those in our tidal flat dechlorinating community (PCE-to-*cis*-DCE). These finding might provide insight into relationship between phylogenetic and biodegradative characteristics of tidal mudflat dechlorinating Chloroflexi populations. This possibility has yet to be examined with further studies.

We also found that a *Fusibacter paucivorans* and *Desulfovibrio dechloracetivorans*-like population increased in abundance during incubation. At present, *Desulfovibrio dechloracetivorans* is known to dechlorinate 2-chlorophenol (2-CP) (Sun et al. 2000) and *Fusibacter paucivorans* is known as a thiosulfate-reducing bacterium (Ravot et al. 1999). Even though there is no evidence that shows the involvement of *Desulfovibrio dechloracetivorans* in PCE to *cis*-DCE dechlorination, the *Desulfovibrio*

dechloracetivorans-like population grew only in the bottom sediment microcosms. We found different *Desulfuromonas*-like populations that grew in the top but not the bottom sediments and vice versa, with those in the bottom more similar to the known PCE-to-*cis*-DCE populations. Hence *Desulfuromonas michiganensis*-like and *Desulfovibrio dechloracetivorans*-like populations in bottom sediment microcosms would be the most likely *cis*-DCE producers in the dechlorinating TL and BL microcosm. The somewhat different groups in the top sediments are the best candidates for producing the TCE formed in these microcosms.

It is known that environmental factors (i.e., sulfate concentrations, salinity, available electron donors, etc.) and the presence of competitors (i.e., methanogens) affect the degree of the reductive dechlorination (Kassenga and Pardue 2006; Aulenta et al. 2008). The amount of electron accepting capacity in PCE vs SO_4^{2-} (60 uM vs 1.31 mM in TM and 60 uM vs 1.56 mM in BM, sulfate reduced) means that PCE had much less of a selective effect on growth than sulfate making it difficult to attribute the dechlorination to any particular population. However, the extensive growth of *Desulfuromonas michiganensis*-like and *Desulfovibrio dechloracetivorans*-like populations, which have known dechlorinating representatives, makes them the best candidates for the observed dechlorination reactions in the microcosms.

The community structure determined by 16S rRNA clone libraries (650 bp reads) and pyrosequencing ($\sim$350 bp reads) were very similar ($R^2 = 0.95$) suggesting the validity of pyrosequencing method and its robustness for bacterial community analysis.

As there is no straightforward physical or chemical treatment to remove pollutants from contaminated costal sediments, natural attenuation and volatilization are the likely means of removal. Although there is still no evidence that Korean tidal flat sediments harbor population that can detoxify PCE to ethene, the finding that PCE is dechlorinated to TCE and *cis*-DCE suggests that marine microbial communities harbor dechlorinating bacteria that attenuate the common contaminants PCE and TCE. The current studies of chlorinated solvent detoxification have focused on freshwater bacteria; however, marine sediments are major recipients of contaminated freshwater. The discovery of PCE dechlorinators in tidal flat microbial communities suggest that tidal flat ecosystems play major roles in attenuating the flux of contaminant from freshwater to the ocean, and more detailed explorations of reductive dechlorination processes in tidal flat environments and warranted.

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