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The reductive dechlorination of 2,3,4,5-tetrachlorobiphenyl in three different sediment cultures: evidence for the involvement of phylogenetically similar *Dehalococcoides*-like bacterial populations

Tao Yan, Timothy M. LaPara, and Paige J. Novak

Department of Civil Engineering, University of Minnesota, Minneapolis, MN, USA

Abstract

Anaerobic cultures capable of reductively dechlorinating 2,3,4,5-tetrachlorobiphenyl (CB) were enriched from three different sediments, one estuarine, one marine and one riverine. Two different electron donors were used in enrichments with the estuarine sediment (elemental iron or a mixture of fatty acids). The removal of doubly flanked *meta* and *para* chlorines to form 2,3,5-CB and 2,4,5-CB was observed in all cultures. Bacterial community analysis of PCR-amplified 16S rRNA gene fragments revealed different communities in these cultures, with the exception of one common population that showed a high phylogentic relatedness to *Dehalococcoides* species. No *Dehalococcoides*-like populations were ever detected in control cultures to which no PCBs were added. In addition, the dynamics of this *Dehalococcoides*-like population were strongly correlated with dechlorination. Subcultures of the estuarine sediment culture demonstrated that the *Dehalococcoides*-like population disappeared when dechlorination was inhibited with 2-bromoethanesulfonate or when 2,3,4,5-CB had been consumed. These results provide evidence that *Dehalococcoides*-like populations were involved in the removal of doubly flanked chlorines from 2,3,4,5-CB. Furthermore, the successful enrichment of these populations from geographically distant and geochemically distinct environments indicates the widespread presence of these PCB-dechlorinating, *Dehalococcoides*-like organisms.

Keywords

reductive dechlorination; polychlorinated biphenyls; *Dehalococcoides*

Introduction

Polychlorinated biphenyls (PCBs) are widespread environmental contaminants (Hutzinger & Veerkamp, 1981). Most PCB contamination is found in anaerobic sediments where reductive dechlorination (Brown *et al.*, 1987; Quensen *et al.*, 1988) is a possible approach for remediation. Little is known, however, about the microbiology of PCB dechlorination and about the diversity of these organisms. To date, there are only two reports in the literature of an isolated strain PCB-dechlorinating organism, *Dehalococcoides ethenogenes* strain 195, which dechlorinates two PCB congeners in the presence of perchloroethene (Fennell *et al.*, 2004) and *Dehalococcoides*-like bacterium, DF-1, capable of respiring PCBs (Miller *et al.*, 2005). Although field observations and experimental results have suggested the presence of multiple

groups of PCB-dechlorinators (Morris *et al.*, 1992;Ye *et al.*, 1992;Brown *et al.*, 1987;Wu & Wiegel, 1997;Cutter *et al.*, 2001;Wu *et al.*, 2002), efforts to categorically identify these microorganisms have not been successful.

Currently, an uncultured anaerobic PCB-dechlorinator, from a marine environment (Baltimore Harbor), has been putatively identified from highly enriched culture using the ribosomal RNA approach (Cutter *et al.*, 2001). Phylogenetic analysis indicated that this organism, *o-17*, is distantly related to known *Dehalococcoides* species (Cutter *et al.*, 2001), such as *Dehalococcoides ethenogenes* strain 195 (Maymó-Gatell *et al.*, 1997) and *Dehalococcoides* spp. strain CBDB1 (Adrian *et al.*, 2000). Although bacteria with identical 16S rRNA gene sequences can express substantially different physiologies (Jaspers & Overmann, 2004), all *Dehalococcoides* isolates studied to date appear to be obligate dehalorespirers, using halogenated aliphatic (Maymó-Gatell *et al.*, 1997;He *et al.*, 2003) or aromatic (Adrian *et al.*, 2000;Bunge *et al.*, 2003;Fennell *et al.*, 2004) compounds as terminal electron acceptors. Therefore, there is evidence that at least three PCB dechlorinators are dehalorespirers and are phylogenetically related to *Dehalococcoides* species.

The identification of additional PCB-dechlorinating bacteria would provide not only more insight about the diversity of these organisms in various environments, but also additional critical information for evaluating remediation systems to treat PCB-contaminated sediments. Molecular-based techniques could be developed to quantify the abundance of PCB-dechlorinating microbes present at contaminated sites. This would help determine the feasibility of biostimulation at a particular site and the effectiveness of the stimulation strategy.

The goal of this research was to identify PCB-dechlorinators in three different sediments. Based on the hypothesis that PCB-dechlorinators would be enriched only when PCBs were present, the bacterial community structures of PCB-amended cultures were compared to the structures of cultures to which no PCBs were amended. It was found that the presence of *Dehalococcoides*-like populations were statistically correlated to the removal of doubly flanked (DF) chlorines from the PCB congener 2,3,4,5-tetrachlorobiphenyl (CB), and despite large differences in sediment characteristics and source, these *Dehalococcoides*-like populations and the concomitant removal of DF chlorines were observed in enrichment cultures derived from three very different sediments. This research should help to develop techniques to enrich for and enumerate these organisms in PCB-contaminated environments.

Materials and methods

Microcosm preparation and incubation

Sediment samples collected from Baltimore Harbor (BH), Palos Verdes (PV), and Hudson River (HR) were transported anaerobically and stored in an anaerobic glovebag (COY labs, Grass Lake, MI). An estuarine medium without sulfate (E-Cl) (Cutter *et al.*, 1998) was prepared for BH and PV cultures and contained (per liter of deionized water): 0.001 g resazurin (redox indicator), 0.1 g MgCl₂·6H₂O, 0.1 g CaCl₂·6H₂O, 3.0 g Na₂CO₃, 0.5 g NH₄Cl, 0.6 g Na₂HPO₄, 8.7 g K₂HPO₄, 10 mL vitamin solution, and 10 mL mineral solution (Wolin *et al.*, 1963). Reduced anaerobic mineral medium (RAMM) was prepared for use with HR cultures as described by Shelton & Tiedje (1984) except that 2 mM L-cysteine was used as a reducing agent instead of Na₂S. The pH of the E-Cl and RAMM media was adjusted to 7.0 with H₃PO₄ or NaOH. The media was sterilized by autoclaving and was transferred into an anaerobic glovebag. The media was reduced immediately prior to use with 20 mM cysteine.

Baltimore Harbor sediment cultures

Baltimore Harbor sediment microcosms were established in triplicate with either a mixture of fatty acids or Fe(0) as the electron donor. Treatments consisted of microcosms to which PCBs were added (PCB-fed) and control microcosms to which no PCBs were added (no-PCB control). Microcosms were set up in 160 mL serum bottles filled with 100 mL E-CI medium and were opened only in the glovebag to avoid exposure to air. For PCB-fed treatments, 1 mL of hexane containing 10 μmol of 2,3,4,5-tetrachlorobiphenyl (2,3,4,5-CB) was added to the media in the bottles and the hexane was allowed to evaporate by leaving the reactors open in the glovebag (gas composition: 4% H_2 and 96% N_2) for about 10 min until the added hexane was no longer visible on top of the medium. Sediment (5 g dry weight equivalent) was added as inoculum to all bottles. A 1 mL mixture of acetate, propionate, and butyrate (20 mM of each, final concentration) was added to the fatty acid-fed bottles (in triplicate) using a pre-reduced (2 g L^{-1} cysteine) stock solution adjusted to a pH of 7.0. Fe(0) (20 mg iron powder, Fisher Scientific, Fairlawn, NJ, unwashed, 99% pure, 100 mesh) was added to the Fe(0)-fed bottles (in triplicate). All of the bottles were then capped with Teflon-lined stoppers and aluminum crimps and removed from the glovebag. The capped and crimped bottles were vigorously shaken on a wrist-action shaker for 2h to promote the homogeneous distribution of 2,3,4,5-CB. Control microcosms (no-PCB control) were set up in the glovebag as described above for both fatty acid-fed and Fe(0)-fed treatments, except that no 2,3,4,5-CB was introduced into the bottles. Hexane was not added to the no-PCB controls. Other than the initial time required to evaporate the added hexane (approximately 10 min), the no-PCB controls were handled identically to the PCB-fed treatments. All of the bottles were incubated in the dark on a rotating shaker (120 r.p.m.) over the course of the experiment. The bottles were sampled in the anaerobic glovebag to prevent contact with oxygen.

Baltimore Harbor sediment subcultures

Subcultures containing sterile BH sediment were established (in duplicate) from the PCB-dechlorinating BH cultures to further study the microbial community. To sterilize the BH sediment, it was first air dried and then autoclaved at 121 °C for 40 min on 2 consecutive days. Sterile sediment (2 g dry weight) was added to four 30 mL serum bottles. The congener 2,3,4,5-CB (2.5 μmol in 0.25 mL hexane) was then added to two of the sediment-containing bottles. The hexane was allowed to fully evaporate by placing the uncapped PCB-containing bottles in the glovebag for approximately 1 h, after which E-CI medium (20 mL) and the mixture of fatty acids (as above) were added. Each of the four subcultures was then inoculated with 2 mL of well-mixed culture from a single actively-dechlorinating BH sediment microcosm (described above). The bottles were sealed, shaken and incubated in the same manner as described for the original BH sediment cultures.

Baltimore Harbor sediment-free subcultures

Sediment-free subcultures of the PCB-dechlorinating BH culture were also established (in triplicate) to investigate the effects of 2-bromoethanesulfonic acid (BESA), an inhibitor of methanogenesis (DiMarco *et al.*, 1990) and dechlorination (Löffler *et al.*, 1997; Ye *et al.*, 1999; Chiu & Lee, 2001) on PCB dechlorination and the bacterial community. E-CI medium (20 mL) and the fatty acid mixture (as above) were added into six 30 mL serum bottles before 2,3,4,5-CB (2.5 μmol in 0.25 mL hexane) was added. The hexane was again allowed to evaporate in the glovebag for approximately 1 h. BESA (3 mM, final concentration) was added to three of the six bottles, using a prerduced stock solution (pH = 7.0). Each of the six bottles was then inoculated with 2 mL of supernatant from one of the actively-dechlorinating BH sediment microcosms (described above). The bottles were then sealed, shaken and incubated in the same manner as described above.

Other sediment cultures

Additional sediment cultures were established as described above to investigate the effect of sediment source on dechlorination pattern and microbial community. These microcosms were set up as described above for the fatty acid-fed BH sediment cultures, except that RAMM medium was used for triplicate HR cultures (E-Cl medium was used for duplicate PV cultures). Again, control microcosms with no added 2,3,4,5-CB were set up.

Sample collection

Samples for PCB and microbial community analysis were collected every 20 to 60 days. Bottles were vigorously hand-shaken for 2 min before they were opened in the glovebag and approximately 1.5 mL of the sediment slurry was withdrawn with a sterile filed-off Pasteur pipette. For PCB analysis, samples were collected in a 20 mL serum vial and the exact weight of the sample was measured for PCB concentration (mg PCBs per kg sediment slurry) determination; samples were extracted immediately (see below). For microbial community analysis, culture samples were collected in sterile 1.6 mL microcentrifuge tubes, centrifuged at 10 000 g for 5 min, and the pellet was stored at -70°C before analysis (see below).

PCB analysis

PCBs were extracted from samples in a manner similar to that described by Quensen *et al.* (1988). Briefly, sediment slurry samples were sequentially extracted, first with acetone (10 mL), then twice with hexane-acetone (1 : 1) (10 mL). The extracts were pooled and then purified in sequential steps with (1) a 2% (v/v) NaCl solution (10 mL), (2) a 30% (v/v) H_2SO_4 solution (4 mL), and (3) a 2% (v/v) NaCl solution (10 mL). The residual water in the extract was removed with anhydrous Na_2SO_4 , and the extract was filtered through a Florisil-copper (25% copper w/w) column. PCBs were analyzed on a gas chromatograph (GC) (Hewlett Packard, Palo Alto, CA; 5890 series) equipped with an electron capture detector (ECD). PCB congeners (2 μL sample size) were separated on a HP-1 capillary column (25 m \times 0.200 mm \times 0.11 μm film thickness) with a carrier gas (He, UHP zero grade) flow rate of 3 mL min^{-1} . Authentic standards (AccuStandard, New Haven, CT) were used to identify 2,3,4,5-CB and its dechlorination products and to construct 7-point external calibration curves for quantification. The method detection limit for 2,3,4,5-CB was 0.01 μM .

Biphenyl was analyzed on a WatersTM (Milford, MA) LC Module 1 Plus high performance liquid chromatograph (HPLC) equipped with a UV detector set at a wavelength of 254 nm. Biphenyl separation was accomplished on a C16 column (Discovery[®] RP Amide, 15 cm \times 4.6 mm, 5 μm , Supelco) using gradient elution with acetonitrile and nanopure water (Millipore, Billerica, MA) as mobile phases (0.5 mL min^{-1}). An external calibration curve for biphenyl was developed using authentic biphenyl standards [AccuStandard, 20 mg L^{-1} in AcCN : THF (1 : 1)] diluted in hexane. The identity of biphenyl was further confirmed in sediment extracts on an Agilent 6890 series GC (Palo Alto, CA) equipped with an Agilent 5973 network mass selective detector (MS). An Rtx-1 (Restek, Bellefonte, PA) column was used for separation (30 m \times 0.32 mm \times 5 μm).

Sediment characterization

Sediment characterization was performed by the Research Analytical Laboratory at the University of Minnesota (Table 1). The total N was measured according to the Dumas method, using a LECO FP-528 Nitrogen Analyzer (St Joseph, MI, USA) (Yeomans & Bremner, 1991). Sulfate was measured based on turbidity measurement using a Klett colorimeter following extraction by $\text{Ca}(\text{H}_2\text{PO}_4)_2 \cdot \text{H}_2\text{O}$ and precipitation with $\text{BaCl}_2 \cdot \text{H}_2\text{O}$ (Missouri Agricultural Experiment Station, 1998). Phosphate was extracted by NaHCO_3 , reduced by ascorbic acid, and the concentration was then determined using a Brinkmann PC 600D probe

colorimeter (Westbury, NY) (Missouri Agricultural Experiment Station, 1998). Nitrate was extracted with CaSO₄ and quantified colorimetrically with an Alpkem Rapid Flow Analyzer (Saskatoon, SK, Canada) (Willis & Gentry, 1987). The total organic carbon was determined by dry combustion and subsequent measurement of CO₂ by IR spectrum absorption using a Skalar Primacs carbon furnace. Total inorganic carbon was measured by converting all of the inorganic carbon to CO₂ with phosphoric acid, and then measuring the quantity of CO₂ by IR spectrum absorption. Elemental analysis was conducted by inductively coupled plasma-atomic emission spectrometry (ICP-AES) (Dahlquist & Knoll, 1978) following extraction from the sediment using diethylenetriaminepentaacetic acid (DTPA) (US EPA, 1992).

Community analysis

A combination of sodium dodecyl sulfate treatment and bead-mill homogenization were adopted for cell lysis (Miller *et al.*, 1999). Briefly, 450 µL phosphate buffer (100 mM, pH = 8) and 450 µL of SDS lysis mixture (100 mM NaCl, 500 mM Tris [pH = 8], 10% [w/v] SDS) were added to the sediment pellets. The samples were then incubated in a water bath at 70 °C for 90 min. Bead-beating and purification were conducted according to the manufacturer's instructions using a FastDNA kit for soil (Q-BIOgene, Irvine, CA, USA).

The procedure for denaturing gradient gel electrophoresis of PCR-amplified 16S rRNA gene fragments (PCR-DGGE) was similar to that described previously (LaPara *et al.*, 2000). Briefly, diluted genomic DNA was amplified by PCR using a primer pair targeting the highly variable V3 region of the domain *Bacteria* (PRBA338F (5'-ACT CCT ACG GGA GGC AGC AG-3') (Lane, 1991) attached to a GC clamp (Muyzer *et al.*, 1993) and PRUN518R (5'-ATT ACC GCC GCT GCT GG-3') (Muyzer *et al.*, 1993). Approximately equal quantities of PCR amplicons were then loaded onto a denaturing gradient gel (30–55%) for electrophoresis. Electrophoresis was performed on a D-Code apparatus (BioRad, Hercules, CA) in 0.5× TAE buffer at 60 °C, initially at 20 V for 20 min followed by 200 V for 180 min. The gel was then stained with SYBR Green I (Molecular Probes, Carlsbad, CA; diluted 1 : 5000 in 0.5× TAE) for 12 min before visualizing on an EC³ Bioimaging System (Ultra-Violet Products, Upland, CA). Bands with densities greater than 3% of the maximum intensity were categorized as prominent.

Prominent bands were excised, incubated overnight in 20 µL of sterile water, and used as template for repeated cycles of PCR-DGGE until only a single band was detectable. The purified excised bands were then reamplified using primers PRBA338F (without the GC clamp) and PRUN518R. These PCR products were purified using a GeneClean Kit (Q-BIOgene) before sequencing. In several cases, vertically comigrating bands were analyzed from different gel lanes for quality assurance.

Nearly complete 16S rRNA genes targeting all members of the domain *Bacteria* were amplified by PCR using primers 27F (5'-AGA GTT TGA TCC TGG CTC AG-3') (Edwards *et al.*, 1989) and 1522R (5'-AAG GAG GTG ATC CAN CCR CA-3') (Johnson, 1994). PCR was also used to amplify 16S rRNA gene fragments from *Dehalococcoides*-like populations using primers 27F and DeR (*E. coli* positions 1406–1422) (Cupples *et al.*, 2003) from the PV samples. PCR products were purified using the GeneClean Kit (Q-BIOgene), ligated into the pGEM-T Easy cloning vector (Promega, Madison, WI), and transformed into competent *E. coli* DH5α cells. Transformants with inserts were selected by the blue/white screening method, and plasmids were extracted by the alkaline lysis method (Sambrook *et al.*, 1989). Clones were initially screened by PCR-DGGE as described above to identify unique inserts; clones with unique inserts were then subjected to nucleotide sequence analysis as described below.

Nucleotide sequences were determined using an ABI 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA; operated by the Advanced Genetic Analysis Center of University

of Minnesota). PCR-DGGE bands were sequenced using primers PRBA338F and PRUN518R. Plasmid inserts were sequenced using primers 27F, 907F, 907R (Lane *et al.*, 1991) and 1522r. Reported nucleotide sequences are the consensus of bi-directional sequence information. DNA sequences were screened for potential chimera using Chimera-Check from the Ribosomal Database Project II (Maidak *et al.*, 2001). Sequences were then compared with sequences in the GenBank database (Benson *et al.*, 2000) using the BLASTn program (Altschul *et al.*, 1997) to search for their closest phylogenetic relatives. Sequences were deposited in the GenBank database under the accession numbers DQ080116 to DQ080206. The 16S rRNA gene sequences of *Dehalococcoides* strains, Bacteria DF-1 and *o*-17, and other dechlorinators were obtained from the Genbank database (Benson *et al.*, 2000). These sequences, along with the *Dehalococcoides*-like and *Chloroflexi* clones of this study, were aligned by using ClustalW (Thompson *et al.*, 1994). A bootstrapped phylogenetic tree (100 replicates, numbers at the nodes show percentage of replication) was constructed using Seqboot, Dnadist, Neighbor, Consensus, and Drawgram programs from the PHYLIP package (Felsenstein, 2002).

Statistical analysis

The correlation between 2,3,4,5-CB dechlorination and the microbial population dynamics was analyzed using the point-biserial correlation coefficient (r_{pb}) (Chen & Popovich, 2002).

$$r_{pb} = \frac{(\bar{X}_1 - \bar{X}_0)\sqrt{p(1-p)}}{S_X}$$

The dynamics of an individual band on a gel was represented by the variable Y , with its value as 1 or 0 when present or absent, respectively, at a certain sampling point. The dechlorination of 2,3,4,5-CB was represented by X , with its value as the average number of chlorines present per biphenyl molecule at a certain sampling point. The values of \bar{X}_1 and \bar{X}_0 are the mean values of X when $Y = 1$ and $Y = 0$, respectively. The value of p is equal to the number of X values when $Y = 1$ divided by the total number of X values (i.e. if there are five X values, and for three of them $Y = 1$ and for two of them $Y = 0$, $p = 3/5$). S_X is the standard deviation of X . The P value was determined using the calculated r_{pb} and corresponding tabulated P values (Chen & Popovich, 2002).

Results

Dechlorination in Baltimore Harbor sediment cultures

The removal of DF chlorines from 2,3,4,5-CB was the most prominent dechlorination activity observed in BH sediment cultures amended with either Fe(0) (Fig. 1a) or a mixture of fatty acids (Fig. 1b). The DF *para* chlorine of 2,3,4,5-CB was preferentially removed, resulting in the formation of 2,3,5-CB as a major dechlorination product. The addition of different electron donors (Fe(0) or a mixture of fatty acids) to the BH sediment cultures resulted in noticeable differences in the lag period prior to dechlorination and in the extent of dechlorination (Fig. 1); nevertheless, the prominent dechlorinating activity observed in the PCB-amended cultures was similar (i.e. DF chlorine removal).

Bacterial community dynamics in Baltimore Harbor sediment cultures

Bacterial community dynamics of the BH sediment cultures, both those to which 2,3,4,5-CB was added and control cultures that received no 2,3,4,5-CB, were investigated over time using PCR-DGGE (Fig. 2). Prominent bands were excised, purified, and sequenced; the phylogenetic affiliations of these bands were determined by comparison with sequences found in the GenBank database (Table 2). The communities in the PCB-amended cultures fed Fe(0) and fatty acids were almost completely different, with the exception of a single band, which was

identical in the two cultures (Band BFE2 and Band BFA3). Nucleotide sequence analysis revealed that these two bands were 100% identical to each other, and to *Dehalococcoides* sp. CBDB1 over the fragment of the 16S rRNA gene that was analyzed (160 base pairs).

Comparisons between PCB-amended and control (no PCB) cultures revealed several differences between the two cultures. In the Fe(0)-fed cultures in which only DF dechlorination of 2,3,4,5-CB occurred, BFE2 was the only population that was present in the PCB-amended cultures but absent in the control cultures. In the fatty acid-fed cultures, in which both DF dechlorination of 2,3,4,5-CB and further dechlorination to 2,5-CB occurred, Bands BFA3, BFA6 and BFA8 were present in the PCB-amended cultures but absent in the control cultures. The selective enrichment of a *Dehalococcoides*-like population (represented by Bands BFE2 and BFA3) in the 2,3,4,5-CB-amended cultures, coincident with the selective enrichment of DF dechlorination, strongly suggests the involvement of this population in 2,3,4,5-CB dechlorination to 2,3,5-CB and 2,4,5-CB. Nevertheless, selective enrichment of two additional populations (represented by Bands BFA6 and BFA8) was also observed in the fatty acid-amended cultures in which dechlorination past 2,3,5-CB and 2,4,5-CB to 2,5-CB occurred. Therefore, the involvement of these two non-*Dehalococcoides*-like populations in dechlorination, or their association with dechlorinators, cannot be precluded.

Population dynamics in the PCB-amended cultures, and their correlation with the dynamics of dechlorination, were also investigated. This correlation was based on the presence or absence of a band at a given time and the number of chlorines removed from 2,3,4,5-CB at that time. Based on the point-biserial correlation test, Band BFE2 ($P = 0.068$), BFA6 ($P = 0.06$), and Bands BFA3 and BFA8 ($P = 1.3 \times 10^{-8}$) were bands in which the population dynamics were most highly correlated with dechlorination (Fig. 2). No other bands were highly correlated with dechlorination ($P \leq 0.10$).

Clone libraries from Baltimore Harbor sediment cultures

Clone libraries were constructed to provide longer 16S rRNA gene sequences for the populations identified in the 2,3,4,5-CB-fed cultures via PCR-DGGE. In the BH sediment cultures, a total of 57 clones were screened and sequenced to obtain 19 unique 16S rRNA gene sequences from one of the fatty acid-amended cultures (Day 294). Thirty clones were screened and sequenced to obtain eight unique 16S rRNA gene sequences from one of the Fe(0)-amended cultures (Day 293). The composition of these bacterial communities as detected by PCR-cloning was largely similar to that revealed by PCR-DGGE. In particular, two clones contained identical sequences to Bands BFE2 and BFA3, confirming the presence of these *Dehalococcoides*-like populations in the enrichment cultures, while simultaneously providing more sequence information. These two clones were identical over 1449 bases and were 99.7% identical to *Dehalococcoides* sp. CBDB1. The phylogenetic relationship between these two clones, representing populations BFE2 and BFA3 (clones BFE-P1 and BFA-K21), to other known *Dehalococcoides* species is shown in Fig. 3.

Baltimore Harbor subcultures

The fatty acid-amended BH dechlorinating culture was subcultured to investigate the effect of 2,3,4,5-CB withdrawal on the bacterial community structure. Positive control subcultures that received additional 2,3,4,5-CB sustained active dechlorination, and after 62 days 8.9 ± 3.7 (average \pm standard deviation) mole percent 2,3,4,5-CB, 68.1 ± 16.3 mole percent 2,3,5-CB, 2.35 ± 0.1 mole percent 2,4,5-CB and 20.7 ± 19.9 mole percent 2,5-CB were present. Based on PCR-DGGE, the *Dehalococcoides*-like population was also present in these subcultures 62 days after the amendment of additional 2,3,4,5-CB (Fig. 4; lanes 1 and 2, indicated by arrows). In the subcultures to which no additional 2,3,4,5-CB was amended, the *Dehalococcoides*-like population, as determined by PCR-DGGE on Day 62, was lost (Fig. 4, lanes 3 and 4). This

further suggests that the presence of 2,3,4,5-CB was important for the enrichment of this *Dehalococcoides*-like population, and this population was responsible for the DF dechlorination of 2,3,4,5-CB.

BESA-inhibition experiments were also performed on subcultures (sediment-free) established from the fatty acid-fed BH sediment cultures. BESA was previously thought to be a specific inhibitor of methanogens (DiMarco *et al* 1990); more recent evidence, however, has shown that it can also inhibit dechlorinating bacteria (Löffler *et al.*, 1997; Ye *et al.*, 1999; Chiu & Lee, 2001). The concentration of the daughter products observed in the sediment-free subcultures (2,3,5-CB and 2,4,5-CB) is shown in Fig. 5a. As expected, the subcultures amended with BESA were inhibited, with no dechlorination of 2,3,4,5-CB (data not shown) and no formation of 2,3,5-CB and 2,4,5-CB (Fig. 5a). Dechlorination in the sediment-free subcultures to which no BESA was added primarily resulted in the formation of 2,3,5-CB (Fig. 5a). The bacterial communities in both treatments (BESA-amended and no-BESA) were finger-printed using PCR-DGGE on Day 194. A *Dehalococcoides*-like population, identical to the one identified in the BH sediment cultures, was detected in all of the dechlorinating replicates to which no BESA was added (Fig. 5b, lanes 1–3). In the BESA-amended treatments (in which dechlorination was inhibited), five populations disappeared (Fig. 5b), one of which was the *Dehalococcoides*-like population.

Dechlorination in other sediment cultures

Dechlorinating cultures were also enriched from HR and PV sediments. The three sediments used as inocula in this study (BH, HR, and PV) had significantly different characteristics (Table 1). BH and PV sediments had much higher sulfate contents than the HR sediment. The nutrient content was also quite different between the three sediments, with BH sediment containing the highest quantity of organic carbon, nitrogen, and trace metals, and HR sediment containing the lowest quantities of these nutrients (Table 1). As observed with BH sediment cultures, dechlorination in the HR and PV sediment cultures also began with the removal of DF chlorines (Fig. 6). The DF *para* chlorine of 2,3,4,5-CB was preferentially removed, resulting in the formation of 2,3,5-CB as a major dechlorination product. Further dechlorination of daughter products, including 2,3,5-CB, 2,4,5-CB and/or 3,4,5-CB, to 2,5-CB, 2,4-CB, 3,5-CB, and/or biphenyl was observed in all of the cultures (Fig. 6), but to different extents depending on the sediment source. PV cultures were capable of dechlorination to the greatest extent (biphenyl was observed in one of the duplicate cultures at concentrations of 0.55 and 28.3 $\mu\text{mol kg}^{-1}$ on Days 297 and 327, respectively), whereas dechlorination in HR sediment cultures proceeded only to the di-chlorinated congener 2,5-CB. Nevertheless, the initial, prominent, and common dechlorination activity observed in all sediment cultures (BH, Fig. 1; PV and HR, Fig. 6) was DF chlorine removal.

Bacterial community dynamics in other sediment cultures

Bacterial community dynamics were also examined in both the PV (Fig. 7a) and HR (Fig. 7b) sediment cultures using PCR-DGGE. Prominent bands were excised from these gels and sequenced to determine the phylogenetic identity of these populations (Table 3). The bacterial populations detected in these microcosms were almost completely different from each other, and were also almost completely different from those identified in the BH sediment cultures (Table 2). Only two bands, PV3 and HR3 in PV and HR sediment cultures, respectively, shared an identical sequence, the closest phylogenetic relative of which was *Dehalococcoides* sp. CBDB1 (100% identity), based on the 16S rRNA gene fragment (160 base pairs) that was investigated. In contrast, no *Dehalococcoides*-like population was detected in the PV and HR control cultures that were not amended with 2,3,4,5-CB (Fig. 7). Other populations absent from the control (no-PCB) cultures included bands PV5, HR7, HR8 and HR10. The possible involvement of these populations in PCB dechlorination requires further investigation. Because

dechlorination patterns other than DF chlorine removal were observed in both sediment cultures, it is possible that multiple dechlorinators, particularly non-*Dehalococcoides*-like organisms, were present in the microcosms amended with 2,3,4,5-CB.

Population dynamics in the PCB-amended cultures and their correlation with the dynamics of dechlorination were also investigated, as with the BH sediment cultures. Based on the point-biserial correlation test, Bands PV3 and PV9 were the ones most correlated with dechlorination in the PV sediment cultures ($P = 7.5 \times 10^{-7}$), with Bands PV1, PV2, PV4, PV5, PV6 (all $P = 0.067$) and PV11 ($P = 0.008$) also correlating with dechlorination, although less strongly. Bands HR3, HR7 and HR11 were the most correlated ($P = 0.001$) with dechlorination in the HR sediment cultures (Fig. 7), with Bands HR5 ($P = 0.04$) and HR10 ($P = 0.006$) also correlating with dechlorination. No other bands were highly correlated with dechlorination ($P \leq 0.10$).

Clone libraries from other sediment cultures

Clone libraries were constructed for both HR and PV cultures to provide longer 16S rRNA gene sequences for the populations identified via PCR-DGGE. For the HR cultures, 39 clones were screened and sequenced to obtain 15 unique 16S rRNA gene sequences (Day 373). For the PV cultures (Day 297), 68 clones were screened and 13 unique sequences were obtained. Because no sequences corresponded to the *Dehalococcoides*-like population (Band PV3), a second clone library (three unique clones out of 10 clones screened) was generated with this sample by targeting *Dehalococcoides*-like populations with a second primer set. The correspondence between the *Dehalococcoides*-like DGGE bands and the *Dehalococcoides*-like clones in both PV and HR cultures was established based on a perfect and exclusive match, indicating that the bands and the clones represented the same populations. The 16S rRNA gene sequences of the *Dehalococcoides*-like clones from PV and HR sediment were different from one another and were different from the *Dehalococcoides*-like clone enriched in the BH sediment microcosms, with 99.2–99.6% and 99.9% similarity between the PV clones and the HR clones to *Dehalococcoides* sp. CBDB1, respectively (based on 1336 and 1421 bases, respectively). The phylogenetic relationship between these four clones, representing populations PV3 (clones DE3, DE5 and DE7) and HR3 (H18), to other known *Dehalococcoides* species is shown in Fig. 3.

Discussion

Despite the potential biases of PCR-based molecular techniques (van Witzengerode *et al.*, 1997; Head *et al.*, 1998), these cultivation-independent methods have the ability to reveal microbial communities and community dynamics in complex environments (Amann *et al.*, 1995). These techniques are particularly useful when the microorganisms of interest are difficult to isolate and when phylogenetic identification may provide critical information, such as when functional groupings correspond to phylogenetic groupings. Although little is known about dehalorespirers in general, a subset of these unique organisms appears to group phylogenetically (the *Dehalococcoides*-like dehalorespirers, Fig. 3). Indeed, 16S rRNA gene-based molecular techniques have been used to profile the microbial communities of PCB-dechlorinating cultures (Holoman *et al.*, 1998), and to identify two putative PCB dechlorinators in highly-enriched cultures (Cutter *et al.*, 2001; Wu *et al.*, 2002). In this study, two PCR-based 16S rRNA gene analysis techniques, cloning and DGGE, were combined to identify putative PCB dechlorinators in cultures derived from different sources of sediments and subjected to different enrichment conditions.

In BH sediment cultures, BH subcultures, and BH sediment-free subcultures, the selective enrichment of a *Dehalococcoides* sp. CBDB1-like population was concomitant with active PCB dechlorination (DF chlorine removal). Indeed, all of the *Dehalococcoides*-like populations in the cultures investigated (BH, PV and HR) were correlated with PCB

dechlorination at a confidence level of $\geq 93\%$, with the *Dehalococcoides*-like species in the fatty acid-fed BH, PV, and HR cultures correlating with dechlorination at a confidence level of $>99\%$. Because PCBs are thought to be used as electron acceptors in energy conservation (Cutter *et al.*, 2001; Wu *et al.*, 2002; Cho *et al.*, 2003), the absence of PCBs should result in the absence of PCB-dechlorinators. No *Dehalococcoides*-like populations developed in any of the control cultures to which no 2,3,4,5-CB was added (BH, PV and HR), presumably because no niche for these organisms existed without the presence of a chlorinated electron acceptor (Figs 2, 7). In addition, the disappearance of the *Dehalococcoides*-like populations occurred in BH subcultures when 2,3,4,5-CB was no longer available (Fig. 4). Finally, the inhibition of PCB dechlorination through the addition of BESA to sediment-free BH subcultures resulted in the disappearance of the *Dehalococcoides*-like population. Taken together, these data provide strong evidence that DF dechlorination of 2,3,4,5-CB was catalyzed by *Dehalococcoides*-like bacterial populations.

It is particularly interesting that functionally and phylogenetically similar *Dehalococcoides*-like organisms were present in geographically distant and geochemically distinct sediments. This finding suggests that these organisms were initially present in the sediment at low concentrations (below the PCR-DGGE detection limit) and enriched over time as a result of the niche provided by amended 2,3,4,5-CB. This finding is unlikely to be an artifact of cross-contamination, as (1) no *Dehalococcoides*-like species were ever detected in the no-PCB controls and these controls were opened in the glovebag with the same frequency as PCB-amended microcosms, (2) slight differences in the 16S rRNA gene sequences were present in the *Dehalococcoides*-like species present in the three different sediments used (BH, PV and HR) (Fig. 3) and (3) the detection of the *Dehalococcoides*-like populations statistically correlated with the onset of PCB dechlorination in these microcosms.

This is particularly exciting because it implies that PCB-dechlorinating *Dehalococcoides*-like organisms may be widespread in the environment. Studies on other chlorinated organic compounds have discovered a variety of *Dehalococcoides*-like organisms capable of dechlorinating a range of xenobiotic compounds. Fennell *et al.* (2004) reported that *Dehalococcoides ethenogenes* strain 195, isolated on tetrachloroethene (Maymó-Gatell *et al.*, 1997), was capable of dechlorinating two PCB congeners when tetrachloroethene was used as a cosubstrate. Other chlorinated compounds that this *Dehalococcoides* strain is able to dechlorinate include 1,2,3,4-tetrachlorodibenzo-*p*-dioxin, 1,2,3,4-tetrachloronaphthalene, and hexachlorobenzene (Fennell *et al.*, 2004). This versatility is not surprising, as 17 putative dehalogenase genes have recently been identified from the genome of *Dehalococcoides ethenogenes* strain 195 (Seshadri *et al.*, 2005). In addition, another *Dehalococcoides* species, CBDB1, is also functionally versatile, dechlorinating both chlorinated benzenes (Adrian *et al.*, 2000) and chlorinated dioxin (Bunge *et al.*, 2003). Based on nearly complete 16S rRNA gene sequences, the phylogenetic relatedness between the *Dehalococcoides*-like populations detected in the three sediments reported herein and *Dehalococcoides* isolates (Maymó-Gatell *et al.*, 1997; Adrian *et al.*, 2000; He *et al.*, 2003) is higher than 99% (Fig. 3). Although 16S rRNA gene-based phylogeny can only be used to infer physiology in very specific cases (Lonergan *et al.*, 1996; Purkhold *et al.*, 2000; Jaspers & Overmann, 2004), all *Dehalococcoides* isolates to date appear to be obligate dechlorinators. This suggests that in addition to other dechlorination processes previously linked to the presence of *Dehalococcoides* species (e.g. tetrachloroethene dechlorination), some PCB dechlorination patterns, notably DF dechlorination, also appear to be linked to the presence of *Dehalococcoides*-like species. With current knowledge of the physiology of *Dehalococcoides* species, the stimulation, enrichment and eventual isolation of *Dehalococcoides*-like PCB dechlorinators should now be more readily accomplished. In addition, the development and use of molecular-based techniques to assay contaminated sites for the presence and number of such organisms should now be feasible.

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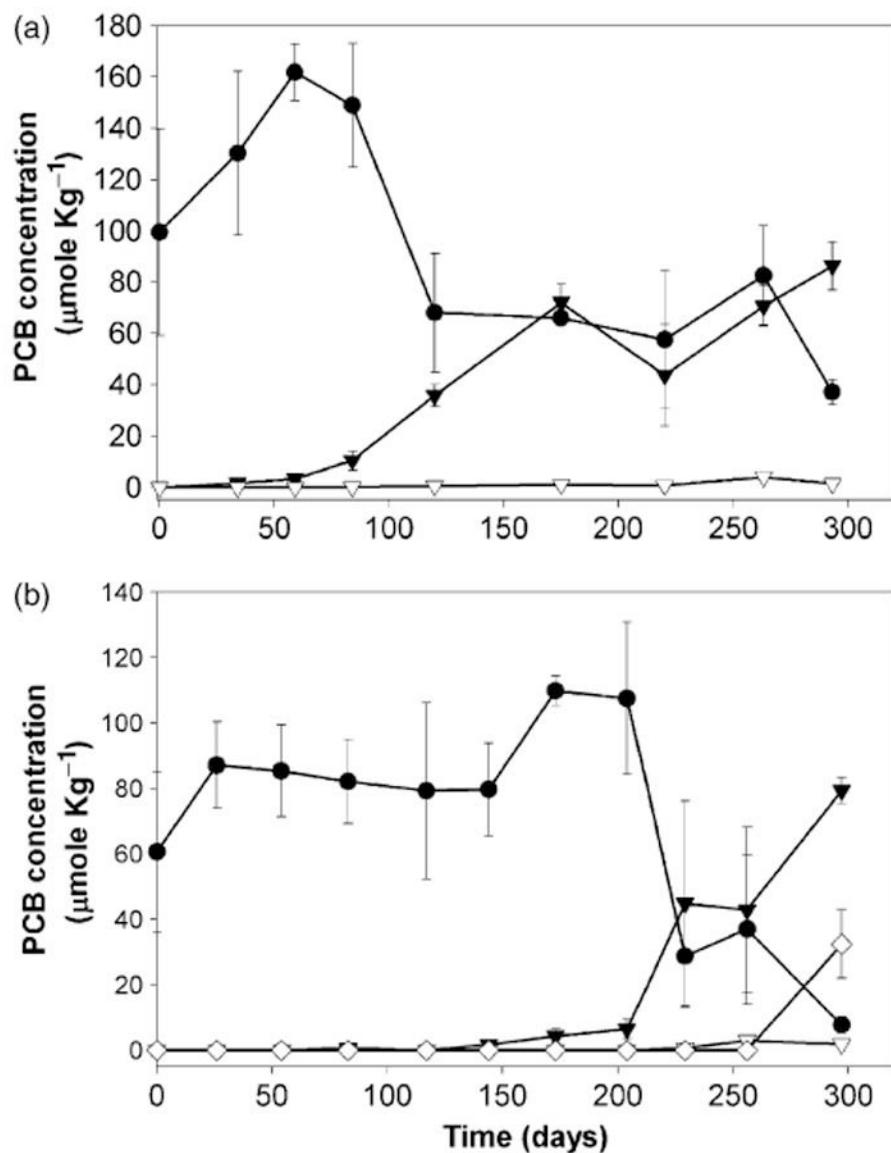


Fig 1. Dechlorination of 2,3,4,5-tetrachlorobiphenyl (CB) in cultures derived from Baltimore Harbor sediment amended with Fe(0) (a) and fatty acids (b). Symbols are 2,3,4,5-CB: black circle, 2,3,5-CB: black triangle, 2,4,5-CB: white triangle, and 2,5-CB: white diamond. The mean values of triplicate microcosms are shown in (a) and the mean values of duplicate microcosms are shown in (b); error bars represent the standard deviation of replicate microcosms.

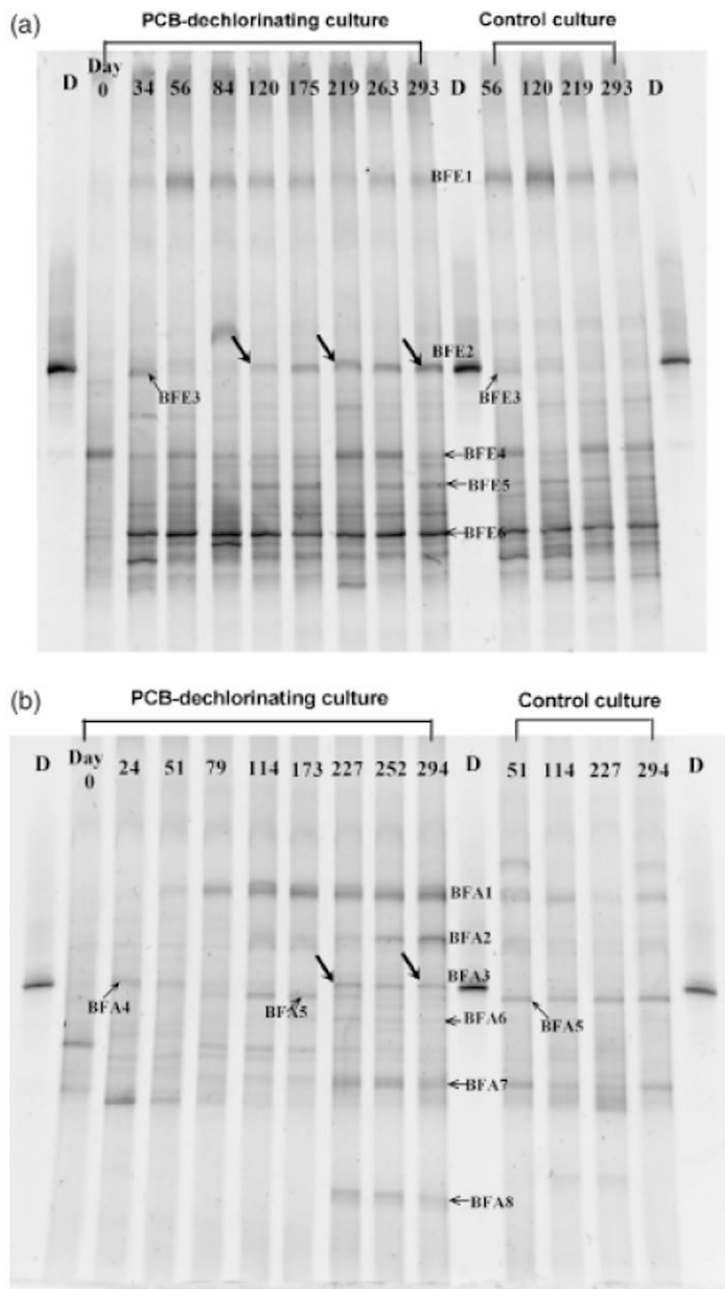


Fig 2. Microbial population dynamics, as determined by PCR-denaturing gradient gel electrophoresis, of 2,3,4,5-tetrachlorobiphenyl (CB)-dechlorinating Baltimore Harbor cultures amended with Fe(0) (a) and fatty acids (b). Lanes labeled D contain marker DNA for *Dehalococcoides ethenogenes*. Control cultures contained no added 2,3,4,5-CB but were otherwise incubated in an identical manner to the dechlorinating cultures. Bands indicated with an arrow (with or without a label) were excised and sequenced.

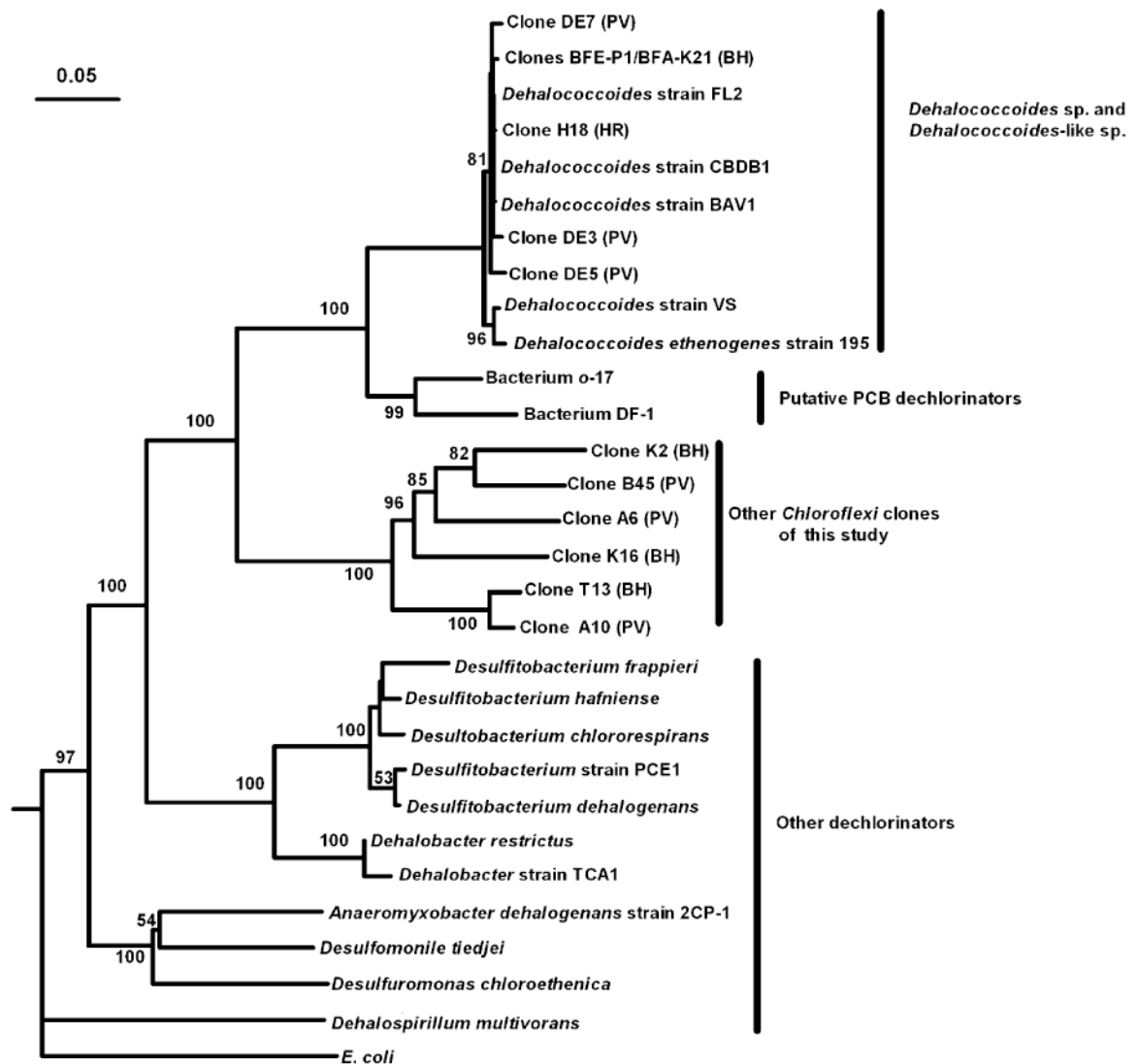


Fig 3. Phylogenetic tree for dechlorinators (adapted from Holliger *et al.*, 1999), *Dehalococcoides* isolates (e.g. *D. ethenogenes* Strain 195 and Strain CBDB1), putative PCB dechlorinators (DF-1 and o-17) and the *Dehalococcoides*-like clones identified in this study. Sediment source is indicated in parentheses.

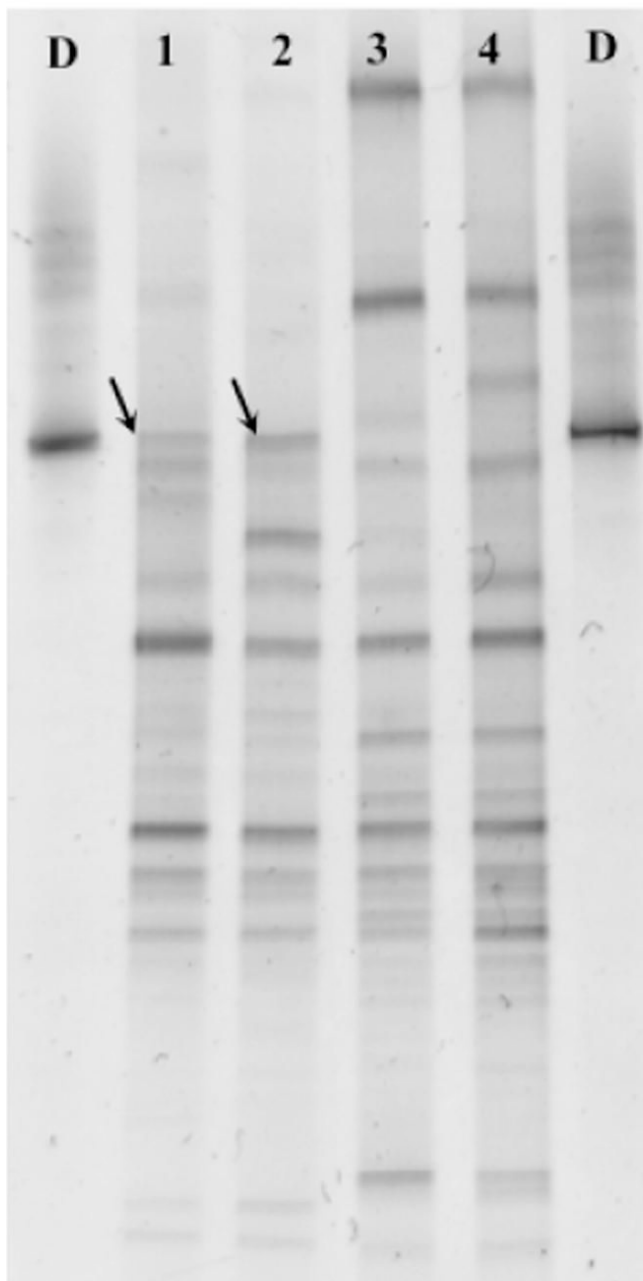


Fig 4. Effect of the addition or removal of 2,3,4,5-tetrachlorobiphenyl (CB) to Baltimore Harbor subcultures on the microbial communities present after 62 days, as determined by denaturing gradient gel electrophoresis. Lanes 1 and 2 represent duplicate subcultures in which additional 2,3,4,5-CB was amended; lanes 3 and 4 represent duplicate subcultures in which no additional 2,3,4,5-CB was amended. Lanes labeled D contain marker DNA for *Dehalococcoides ethenogenes*.

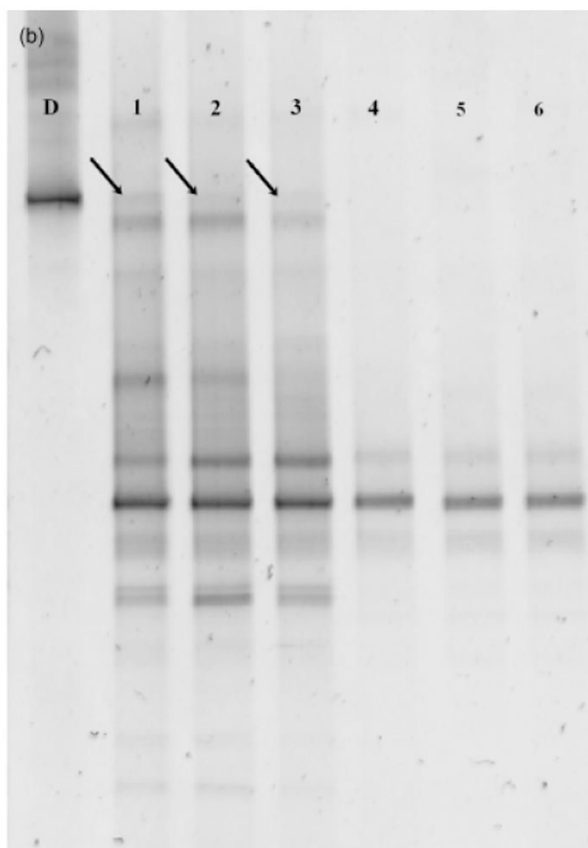
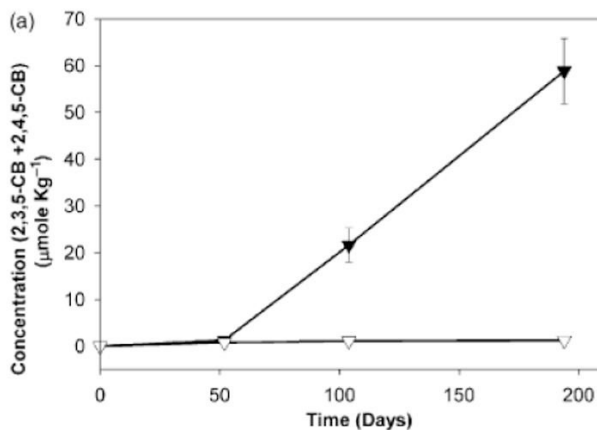


Fig 5. Inhibition of the dechlorination of 2,3,4,5-tetrachlorobiphenyl (CB) in sediment-free subcultures by 2-bromoethanesulfonic acid (BESA) (a) and the denaturing gradient gel electrophoresis pattern showing the bacterial communities in the subcultures on Day 194 (b). Black triangles show subcultures to which no BESA was added and white triangles show subcultures amended with BESA. Lane D contains DNA for *Dehalococcoides ethenogenes* as a marker; lanes 1–3 show the dechlorinating sediment-free cultures to which no BESA was added and lanes 4–6 show the sediment-free cultures to which BESA was added that were not capable of dechlorination. The arrows point to the bands corresponding to the *Dehalococcoides*-like population, which were excised and sequenced.

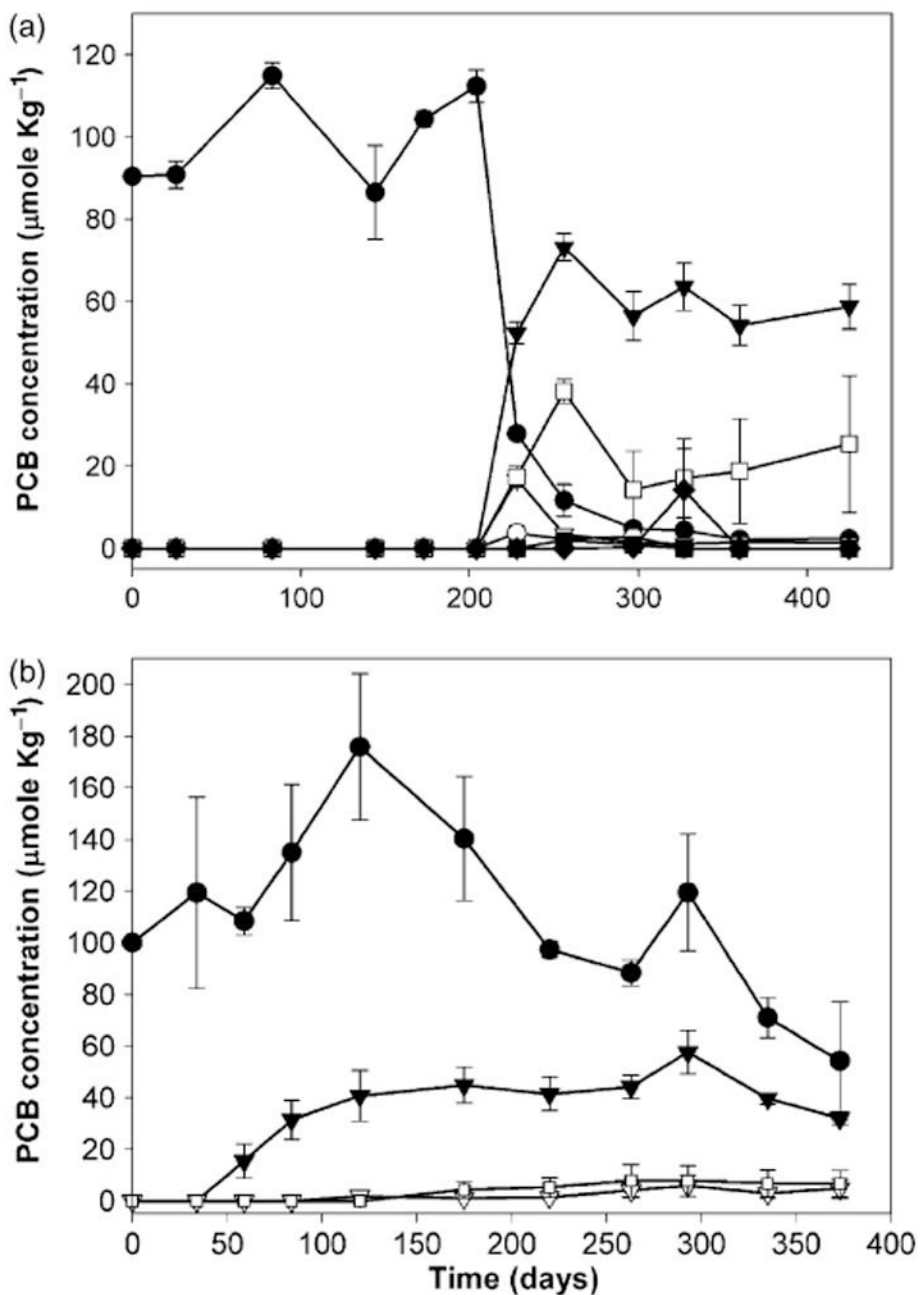


Fig 6. Dechlorination of 2,3,4,5-tetrachlorobiphenyl (CB) in cultures derived from Palos Vedes (a) and Hudson River (b) sediment amended with fatty acids. The mean values of duplicate microcosms are shown in (a) and the mean values of triplicate microcosms are shown in (b); error bars represent the standard deviation of replicate microcosms. Symbols are 2,3,4,5-CB: black circle, 2,3,5-CB: black triangle, 2,4,5-CB: white triangle, 3,4,5-CB: white circle, 3,5-CB: black square, 2,4-CB: white square, and biphenyl: black diamond.

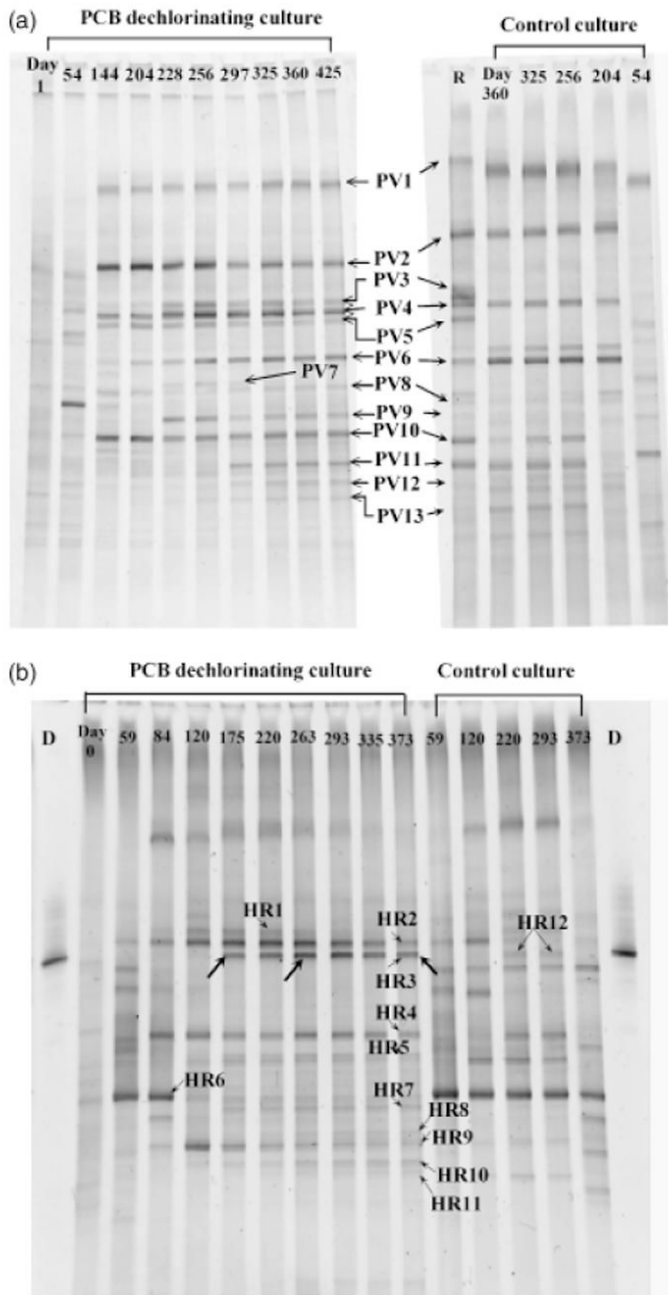


Fig 7. Microbial population dynamics, as determined by PCR-denaturing gradient gel electrophoresis (DGGE), of 2,3,4,5-tetrachlorobiphenyl (CB)-dechlorinating Palos Vedes (PV) cultures (a) and Hudson River cultures (b). Control cultures contained no added 2,3,4,5-CB but were otherwise incubated in an identical manner to the dechlorinating cultures. For PV cultures (a), the DGGE pattern of control culture was on a separate gel; a reference lane R, which is from PCB-dechlorinating culture (Day 297), illustrates the relative locations of bands. Bands indicated with an arrow (with or without a label) were excised and sequenced.

Table 1
Chemical characteristics of Baltimore Harbor (MD), Palos Verdes (CA), and Hudson River (MA) sediments

Parameters	Baltimore Harbor	Hudson River	Palos Verdes
Total N (%)	0.500	0.075	0.155
Water pH	7.7	7.1	8.0
Sulfate (p.p.m.)	141	11	123
Phosphate (p.p.m.)	40.0	10.0	39.5
Nitrate (p.p.m.)	1.7	1.6	1.6
TOC (%)	6.795	2.115	1.60
Inorganic carbon (%)	0.615	≤0.15	1.16
Fe (p.p.b.)	438.990	84.325	193.600
Mn (p.p.b.)	20.814	40.448	3.463
Zn (p.p.b.)	227.450	4.213	31.846
Cu (p.p.b.)	47.725	1.830	8.861
Pb (p.p.b.)	101.360	13.967	12.330
Ni (p.p.b.)	5.547	0.231	1.852
Cd (p.p.b.)	1.155	0.076	1.947
Cr (p.p.b.)	0.241	0.028	0.207

Phylogenetic affiliation of excised denaturing gradient gel electrophoresis bands from the Baltimore Harbor sediment culture as shown in Fig. 2

Table 2

Bands	Sequence length (bp)	Closest phylogenetic relative (accession no.)	Identity (%)	Phyla
BFE1	153	<i>Capnocytophaga sputigena</i> (X67609)	86.3	Bacteroidetes
BFE2	164	<i>Dehalococcoides</i> sp. CBDB1 (AF230641)	100.0	Chloroflexi
BFE3	157	<i>Clostridium lactatifermentans</i> (AY033434)	99.3	Firmicutes
BFE4	159	Uncultured bacterium SHA-15 (AJ249098)	97.5	Unknown
BFE5	156	Uncultured bacterium clone DSBACT72 (AY762633)	90.3	Firmicutes
BFE6	156	<i>Clostridium cellulovorans</i> (X73438)	97.4	Firmicutes
BFA1	163	<i>Epulopiscium</i> sp. morphotype (M99574)	84.7	Firmicutes
BFA2	160	<i>Anaerolinea thermophila</i> (AB046413)	87.0	Chloroflexi
BFA3	141	<i>Dehalococcoides</i> sp. CBDB1 (AF230641)	100.0	Chloroflexi
BFA4	160	<i>Clostridium lactatifermentans</i> (AY033434)	96.3	Firmicutes
BFA5	159	<i>Epulopiscium</i> sp. morphotype (M99574)	84.7	Firmicutes
BFA6	159	<i>Pelospora glutarica</i> (AJ251214)	88.1	Firmicutes
BFA7	159	<i>Syntrophomonas</i> sp. TB-6 (AB098336)	91.0	Firmicutes
BFA8	158	Uncultured <i>Desulfosarcina</i> sp. clone SIMO-1222 (AY710662)	93.7	δ -Proteobacterium

Phylogenetic affiliation of excised denaturing gradient gel electrophoresis bands from the Palos Vedes (PV) and Hudson River (HR) sediment cultures as shown in Fig. 7

Table 3

Bands*	Sequence length (bp)	Closest phylogenetic relative (accession no.)	Identity (%)	Phyla
PV1	181	Uncultured bacterium (AJ306785)	93.3	Ambiguous
PV2	180	Uncultured bacterium TSAC12 (AB186798)	99.4	Ambiguous
PV3	154	<i>Dehalococcoides</i> sp. CBDB1 (AF230641)	100	Chloroflexi
PV4	174	Uncultured bacterium SHA-94 (AJ306738)	94	Ambiguous
PV5	179	<i>Syntrophomonas</i> sp. TB-6 (AB098336)	94	Firmicutes
PV6	153	Uncultured bacterium SHD-245 (AJ278174)	93	Chloroflexi
PV7		Chimeric		
PV8	156	<i>Pelotomaculum</i> sp. FP (AB159558)	94.9	Firmicutes
PV9	178	<i>Enterobacter sakazakii</i> (AY752940)	100	γ -Proteobacteria
PV10	154	Uncultured bacterium SJA-116 (AJ009487)	96.7	Chloroflexi
PV11	179	<i>Olavius algarvensis</i> (AF328857)	91.2	δ -Proteobacteria
PV12	178	Uncultured bacterium DSB-Dsa99-4 (AJ300510)	92.5	δ -Proteobacteria
PV13	172	<i>Cytophaga</i> sp. (AB015525)	93.3	Bacteroidetes
HR1	153	<i>Capnocytophaga cynodegmi</i> (X97245)	91.1	Bacteroidetes
HR2	174	<i>Capnocytophaga cynodegmi</i> (X97245)	92.1	Bacteroidetes
HR3	155	<i>Dehalococcoides</i> sp. CBDB1 (AF230641)	100	Chloroflexi
HR4	179	<i>Syntrophospora bryantii</i> (M26491)	92.1	Firmicutes
HR5	179	<i>Syntrophospora bryantii</i> (M26491)	94.2	Firmicutes
HR6	175	<i>Syntrophus aciditrophicus</i> (U86447)	90.8	γ -Proteobacteria
HR7		Sequence failed		
HR8		Sequence failed		
HR9	157	<i>Clostridium sporogenes</i> (AY442816)	91.2	Firmicutes
HR10	154	Uncultured bacterium TDC-S1:29 (AF447148)	100	Unknown
HR11	173	<i>Flavobacterium mizutaii</i> (AF361550)	91.3	Bacteroidetes
HR12	179	<i>Syntrophomonadaceae</i> genomo sp. P1 (AY341821)	85.3	Firmicutes

* PV indicates bands derived from PV sediment cultures and HR indicates bands derived from HR sediment cultures.