Reductive dehalogenation activity of indigenous microorganism in sediments of the Hackensack River, New Jersey

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Title: Reductive Dehalogenation Activity of Indigenous Microorganism in Sediments of the Hackensack River, New Jersey

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Abstract

Organohalogen pollutants are of concern in many river and estuarine environments, such as the New York-New Jersey Harbor estuary and its tributaries. The Hackensack River is contaminated with various metals, hydrocarbons and halogenated organics, including polychlorinated biphenyls (PCBs) and polychlorinated dibenzo-\textit{p}-dioxins. In order to examine the potential for microbial reductive dechlorination by indigenous microorganisms, sediment samples were collected from five different estuarine locations along the Hackensack River. Hexachlorobenzene (HCB), hexabromobenzene (HBB), and pentachloroaniline (PCA) were selected as model organohalogen pollutants to assess anaerobic dehalogenating potential. Dechlorinating activity of HCB and PCA was observed in sediment microcosms for all sampling sites. HCB was dechlorinated via pentachlorobenzene (PeCB) and trichlorobenzene (TriCB) to dichlorobenzene (DCB). PCA was dechlorinated via tetrachloroaniline (TeCA), trichloroanilines (TriCA), and dichloroanilines (DCA) to monochloroaniline (MCA). No HBB debromination was observed over 12 months of incubation. However, with HCB as a co-substrate slow HBB debromination was observed with production of tetrabromobenzene (TeBB) and tribromobenzene (TriBB).

\textit{Chloroflexi} specific 16S rRNA gene PCR-DGGE followed by sequence analysis detected \textit{Dehalococcoides} species in sediments of the freshwater location, but not in the estuarine site. Analysis targeting 12 putative reductive dehalogenase (\textit{rdh}) genes showed that these were enriched concomitant with HCB or PCA dechlorination in freshwater sediment microcosms.
Keywords: River sediments; microbial reductive dehalogenation; halogenated aromatics;

reductive dehalogenase; *Dehalococcoides*

Capsule: Halogenated aromatic pollutants are reductively dehalogenated by indigenous microorganisms in an estuarine river system.
1. Introduction

Halogenated aromatics have been widely used in industry and in agriculture. Due to their persistence and toxicity, the most problematic compounds have been listed as persistent organic pollutants (POP; Stockholm Convention, 2001; for reviews see Häggblom and Bossert, 2003). In river environments, organohalides typically accumulate in sediments due to their low solubility in water and sorption onto particulate material. However, these compounds dissolve in lipids, thus they can bioaccumulate and biomagnify in the food chain (Lu and Metcalf, 1975; Chiou et al., 1977; Goerke et al., 2004) and are of concern as pollutants in many river and estuarine environments.

The New York-New Jersey Harbor estuary and its tributaries have a long-term history of organohalogen pollution through the combination of direct discharge from several production plants as well as non-point sources (Bopp et al., 1991; Crawford et al., 1995; Huntley et al., 1998; Walker et al., 1999; Wenning et al., 2004; Dimou et al., 2006). The Hackensack River, one of the principal tributaries to Newark Bay, is contaminated with various metals, hydrocarbons and halogenated organics, including PCBs and polychlorinated dibenzo-\(p\)-dioxins (Crawford et al., 1995; Martello et al., 2007). The Hackensack River is approximately 45 miles long, flowing from southeastern New York into Newark Bay, New Jersey. The water quality became worse after a construction of a dam in 1923. In addition, the Hackensack River has been impacted by both municipal and industrial wastewaters since the river is located within the major New York metropolitan area, with a multitude of industrial sites in Elizabeth and Newark, NJ. Furthermore, the lower section of the Hackensack River, the Hackensack Meadowlands had been a hotspot of garbage dumping by factories and municipalities. As a result, the Hackensack River is
contaminated by a variety of pollutants, such as total suspended solid, organic matter, nitrogen and ammonia, metals and chlorinated compounds including PCBs and dibenzo-p-dioxins. Contaminants can be found in water, sediment and even in fish and birds (Crawford et al., 1995; Weis and Ashley, 2007; Tsipoura et al., 2008; Shin et al., 2013). The Hackensack River and its associated wetlands provide an important habitat for migratory birds and sediment and water contaminants are thus of concern.

Microbiologically mediated dehalogenation can reduce the toxicity of many halogenated aromatic compounds, and the dehalogenation products may also be more amenable to subsequent degradation by aerobic bacteria (for reviews, see Häggblom and Bossert, 2003). Understanding the factors that affect the dehalogenation of organohalide pollutants by indigenous microorganisms is thus of interest. Here we studied the dehalogenation of potential of three problematic organohalide compounds: hexachlorobenzene (HCB), hexabromobenzene (HBB), and pentachloroaniline (PCA). HCB has been used as a pesticide and is listed as one of the POPs slated for elimination (Bailey, 2001), HBB is a flame retardant (Alaee, 2003), while PCA is an intermediate in the biodegradation of the fungicide, pentachloronitrobenzene (PCNB) (Tamura et al., 1995; Susarla et al., 1996; Okutman Tas and Pavlostathis, 2005).

The major objective of this work was to understand the distribution and activity of dehalogenating bacteria in Hackensack River sediments collected along an estuarine gradient at five locations along the river (designated H1 to H5). The three main goals were to: determine whether indigenous microorganisms in Hackensack River sediments have dehalogenating activity; identify the key members of the microbial community active in degrading the
halogenated model compounds hexachlorobenzene, hexabromobenzene, and pentachloroaniline through 16S rRNA gene analysis; and discern the reductive dehalogenase (rdh) genes profile for each halogenated model compound by PCR with specially designed 12 rdh gene targeted primer sets.
2. Materials and Methods

2.1. Chemicals and stock solutions

Chlorinated benzenes (hexachlorobenzene, pentachlorobenzene, tetrachlorobenzenes, trichlorobenzenes, dichlorobenzenes), 1,2,3,5-tetrabromobenzene and 1,3,5-tribromobenzene were purchased from Sigma-Aldrich (St. Louis, MO). Chlorinated anilines (pentachloroaniline, tetrachloroanilines, trichloroanilines, dichloroanilines, chloroanilines) were purchased from UltraScientific (North Kingstown, RI). Hexabromobenzene was from TCI America (Portland, OR). Stock solutions of chlorobenzenes and chloroanilines were prepared by dissolving the compounds in hexane to a final concentration of 10 mM. Stock solutions of bromobenzenes were prepared by dissolving the compounds in toluene to a final concentration of 5 mM.

2.2. Microcosm preparation

Sediment samples were collected from five locations along the Hackensack River, New Jersey in July 2012 from North Hackensack to the mouth of the river near Newark Bay, designated H1 to H5 (Fig. 1 and Supplementary Table S1). GPS coordinates for the sampling sites are as follows: H1, 40.85, -74.03; H2, 40.82, -74.04; H3, 40.80, -74.07; H4, 40.78, -74.09; and H5, 40.74, -74.08. The samples were collected using a pole-mounted Ekman grab as described by Janssen et al. (2015). Subsamples were transferred to clean glass jars with a stainless steel scoop, placed in plastic bags and stored on ice immediately after collection and during transport to the laboratory. Sediments were stored in sealed glass jars at 4°C until setup of microcosms.

Sediment slurry microcosms were prepared in 60 mL serum bottles capped with Teflon coated rubber stoppers and crimped with aluminum seals. Sediment slurries (50 mL) contained 10%
(wet weight/volume) sediment and electron donor mixture in anaerobic media. Anaerobic media was made as described by Cole et al. (1994) with some modifications. The medium contained the following (per liter): NaCl, 1.0 g; MgCl\(_2\)•6H\(_2\)O, 0.5 g; KH\(_2\)PO\(_4\), 0.2 g; NH\(_4\)Cl, 0.3 g; KCl, 0.3 g; CaCl\(_2\)•2H\(_2\)O, 0.015 g; resazurin, 1 mg; trace element solution A, 1 mL; trace element solution B, 0.1 mL; Na\(_2\)S•9H\(_2\)O, 0.048 g; cysteine hydrochloride, 0.035 g; NaHCO\(_3\), 2.52 g; and vitamin solution, 1 mL. Trace element solution A contained the following (per liter): FeCl\(_2\)•4H\(_2\)O, 1.5 g; CoCl\(_2\)•6H\(_2\)O, 0.19 g; MnCl\(_2\)•4H\(_2\)O, 0.1 g; ZnCl\(_2\), 70 mg; H\(_3\)BO\(_3\), 6 mg; Na\(_2\)MoO\(_4\)•2H\(_2\)O, 36 mg; NiCl\(_2\)•6H\(_2\)O, 24 mg; and CuCl\(_2\)•2H\(_2\)O, 2 mg. Trace element solution B contained 0.03 g of NaSeO\(_3\) and 0.08 g of Na\(_2\)WO\(_4\) per liter. Vitamin solution contained the following (per 500 mL): D-biotin, 0.01 g; folic acid, 0.01 g; pyridoxine hydrochloride, 0.05 g; thiamin hydrochloride, 0.025 g; riboflavin, 0.025 g; nicotinic acid, 0.025 g; DL-calcium pantothenate, 0.025 g; vitamin B\(_{12}\), 0.005 g; p-aminobenzoic acid, 0.025 g; lipoic acid (thiotic acid), 0.025 g; 1,4-naphthoquinone, 0.02 g; nicotinamide, 0.05 g; and hemin, 0.005 g.

Four treatments were established in triplicate: halogenated electron acceptors hexachlorobenzene (HCB), hexabromobenzene (HBB), or pentachloroaniline (PCA), and a control without organohalide addition. All cultures were amended with a mixture of electron donors containing butyrate and propionate to a concentration of 500 μM each. Duplicate killed controls for each substrate were also prepared by autoclaving of sediment slurries for 30 min at 121 °C on three consecutive days before addition of electron acceptors and donors.

To provide a carrier for the halogenated substrates, approximately 0.2 g of silica was added evenly into each serum bottle, and sterilized. The silica was then spiked with the respective
halobenzene or chloroaniline stock solution to yield a final nominal concentration of 20 μM and
the solvent was allowed to evaporate completely leaving a coating of the halogenated substrates.
The serum bottles were then moved to an anaerobic chamber with a headspace of N\textsubscript{2}:H\textsubscript{2} (97:3)
for the remaining steps. Sediment slurries of each site were prepared in large stock bottles and 50
mL was transferred to each serum bottle. The microcosms were stirred, capped and then
incubated upright without shaking in the dark at room temperature (~21 °C). At each sampling
time point the flasks were vigorously shaken and 0.5 mL samples collected for chemical
analysis. An additional 1 mL sample was collected from each microcosm every 4 weeks for
molecular analysis. Samplings were done in the anaerobic chamber. Samples were stored at -20
°C or extracted immediately after sampling.

Additional microcosms were established to determine whether HCB would stimulate HBB
dehalogenation. Triplicate cultures originating from H1 sediment microcosms previously set up
for monitoring HCB dechlorination were spiked with a combination of HBB and HCB. In these
cultures, previously added HCB was already dechlorinated and the cultures had accumulated
dechlorination products. Culture bottles were supplemented with anaerobic media to
approximately 50 mL and an additional 10 μM of HCB and HBB were provided as electron
acceptors. HCB and HBB were added as a coating on about 0.4 g of silica on the bottom of
autoclaved serum bottles, as described above, and the whole culture was transferred to the new
culture bottle in the anaerobic chamber. Cultures were incubated as described above. Samples
(0.5 mL) were collected every three or four weeks for chemical analysis. After 19 weeks and 39
weeks of incubation, 1 mL samples were collected for molecular analysis.
2.3. Chemical analytical methods

Samples were extracted by shaking at 180 rpm for two hours. For extraction of chlorobenzenes and chloroanilines, 500 μL of hexane was used. For extraction of bromobenzenes or the mixture of chloro- and bromobenzenes, 750 μL of a mixture of toluene and acetone (1:1; v:v) was used. The supernatant was transferred to a GC vial. When solvent and aqueous/sediment phase were not clearly separated, brief sonication in a water bath was applied. Samples were analyzed with an Agilent 6890 gas chromatograph (Agilent Technologies, Inc., Santa Clara, CA) equipped with a HP-5MS capillary column (0.25 mm × 60 m, 0.25 μm film thickness, J&W Scientific, Folsom, CA) and an Agilent 5973 Network Mass Selective Detector with helium as the carrier gas. For analyses of chlorinated benzenes and brominated benzenes, the GC column temperature was first held at 50 °C for 2 min, increased to 300 °C at a rate of 12 °C min⁻¹, and then held for 5 min. For analyses of chlorinated anilines, the GC column temperature was first held at 100 °C for 1 min and then increased to 300 °C at a rate of 20 °C min⁻¹. A calibration mixture containing chlorinated benzenes, chlorinated anilines, or brominated benzenes was analyzed with samples. Each model compound and its dehalogenation products were combined. For example, HCB, PeCB, TeCB, TriCB and DCB were combined and used for calibration of HCB dechlorination products. For quantification, standards of 10, 20, 30, 40, and 50 μM were used. Detection limits were ~0.1 μM for HCB and its intermediates, 0.1~0.5 μM for TeBB and TriBB, 0.5~1 μM for HBB, 0.1~0.5 μM for PCA and its intermediates.

2.4. Molecular analyses

Genomic DNA was extracted from sediment samples collected from HCB and PCA amended cultures from H1 and H5 sites at day 0 and day 112 using the PowerSoil™ DNA Isolation Kit.
Extracted DNA was loaded on an agarose gel or its concentration was measured by a Nanodrop ND-1000 spectrophotometer (Nanodrop technologies, Wilmington, DE) to check quality and quantity before PCR. Nested PCR was performed to amplify the DNA of the putative dechlorinating community following previously developed procedures (Krumins et al., 2009; Park et al., 2011). Resulting PCR products were analyzed by denaturing gradient gel electrophoresis (DGGE). First, DNA samples extracted from microcosms were amplified with primer 338F and Chl1101R targeting a 790 bp region of the Chloroflexi 16S rRNA gene. The resulting PCR products were re-amplified using general bacterial PCR primers 341F-GC and 534R. All PCR reaction mixtures contained 10 mM Tris-HCl (pH 8.3 at 25 °C), 2.5 mM MgCl₂, 0.25 mM deoxynucleotide triphosphates, 10 picomole of each primer, and 1 U of Taq DNA polymerase. The temperature profile for nested PCR was 94 °C for 5 min followed by 30 cycles of 94 °C for 20 sec, 55 °C for 45 sec, and 72 °C for 45 sec. A final extension step was carried out for 7 min at 72 °C, after which the DNA was stored at 4 °C.

PCR products (20 μL) were mixed with 2X DGGE dye and loaded onto 8% polyacrylamide gels in 1X Tris-acetate EDTA (TAE) buffer using DCode™ universal mutation detection system (Bio-Rad Laboratories, Hercules, CA). The polyacrylamide gels were made with a linear denaturing gradient from 40% at the top to 60% denaturant at the bottom. After electrophoresis for 17 h at 60 °C and 40 V, the gels were stained with ethidium bromide and photographed on a UV transilluminator (BioRad Gel Doc™ EZ Imager, Bio-Rad Laboratories, Hercules, CA). Image analysis was conducted with Image Lab program version 5.1 (Bio-Rad Laboratories, Hercules, CA).
DGGE bands of interest were excised and DNA was eluted overnight in 50 μL of MilliQ H₂O at 4 °C. After centrifugation, DNA in the supernatant was amplified using general bacterial primers as described above. The PCR products were analyzed by DGGE to confirm band position and sequenced (GeneWiz, South Plainfield, NJ, USA). Phylogenetic analyses were conducted using MEGA version 6 (Tamura et al., 2013). The sequences of excised bands from PCR-DGGE are provided in the Supplementary Information.

To determine the presence of the chlorobenzene reductive dehalogenase, cbrA, in enrichment cultures, primers were designed to amplify the cbrA gene (1467 bp) (cbrA-F: ATGAGTAACCTTTCAATTCAAC, cbrA-R: TTAATTACCAGGTACAGCCC). The PCR in total reaction mixture volume of 20 μL was performed at 94 °C for 5 min, 30 cycles of 94 °C for 20 sec, 45 °C for 45 sec and 72 °C for 90 sec, final incubation at 72 °C for 7 min, after which the DNA was stored at 4 °C. PCR products were examined as described above, but a 0.7 % agarose gel in 1X TAE was used.

2.5. Reductive dehalogenase gene profile analysis

PCR of extracted DNA was done with 12 sets of primers designed to amplify reductive dehalogenase genes based on rdh gene sequence similarity pairs of Dehalococcoides mccartyi strain 195 and strain CBDB1 (Park et al., 2011). The PCR reaction mixture (20 μL) contained 2X premix (ReadyMix™ Taq PCR Reaction Mix with MgCl₂, Sigma-Aldrich), 10 picomole of each primer and 1 μL template DNA. The premix contained the following: 20 mM Tris-HCl; pH 8.3; 100 mM KCl; 3 mM MgCl₂; 0.002 % gelatin; 0.4 mM dNTP mix (dTTP, dATP, dCTP, dGTP, TTP); stabilizers; and 0.06 units Taq DNA polymerase/μL. The PCR was performed as follows:
initial incubation for 5 min at 94 °C, 35 cycles of 94 °C for 20 sec, 52 °C for 1 min, and 72 °C for 1 min, final extension for 7 min at 72 °C, after which the DNA was stored at 4 °C. Samples of PCR products (5 μL) were examined on 1.5 % agarose gels containing ethidium bromide in 1X TAE. The electrophoresis was run for 35 min at 100 V. After electrophoresis, the gels were photographed on a UV transilluminator (BioRad Gel Doc™ EZ Imager, Bio-Rad Laboratories, Hercules, CA). Image analysis was conducted with Image Lab program version 5.1 (Bio-Rad Laboratories, Hercules, CA).
3. Results

3.1. Dehalogenating activities of sediment microcosms

Dehalogenation of HCB and PCA was observed in Hackensack River sediment microcosms from all five sampling sites (Fig. 2 and Fig. 3). Microcosms set up with sediment from locations H1 to H4 (upper river) showed a start of HCB dechlorination earlier than cultures with sediment from H5 (lower river). TriCB was detected as a dechlorination product on day 28 in the H1 to H4 microcosms. In the H5 microcosms, TriCB appeared later, at day 42. A subsequent dechlorination product, DCB was detected in H2 cultures from day 28, and it was detected in H1, H3 and H4 cultures from day 42. In a consistent trend with TriCB, DCB was detected in H5 cultures much later, at day 77. In some of the cultures, PeCB was also detected as an early intermediate of dechlorination. There was no dechlorination activity in killed controls confirming that activity was microbial. Based on these results, dechlorination of HCB is mediated by indigenous microorganisms of Hackensack River sediments.

Dechlorination of PCA was observed in microcosms of all five sampling sites, with TeCA, TriCA, DCA, and eventually MCA detected as dechlorination intermediates (Fig. 3). H1 microcosms showed an earlier start of PCA dechlorination similar to what was observed for HCB. TeCA and TriCA were detected in H1 cultures at day 14. For the H2 to H4 cultures, dechlorinating products were detected at day 28. The H5 cultures showed a later start for PCA dechlorination, with TriCA and DCA detected as dechlorinating products at day 44. These observations were consistent for all triplicate cultures. Killed controls showed no dechlorination of PCA. Therefore, we can conclude that indigenous bacteria in Hackensack River sediments mediated PCA dechlorination.
No dehalogenation of HBB or detection of intermediates was observed in any of the microcosms (data not shown) after up to 12 months of incubation. PeBB was, however, detected at low levels as a contaminant of the HBB stock, but any increase in the PeBB concentration in microcosms was negligible.

3.2. Dechlorination pathways

HCB dechlorination pathways were established based on GC-MS analysis by comparison of mass spectral data and retention times of intermediates to those of authentic standards (Fig. 4). HCB was converted to PeCB, with subsequent detection of 1,3,5-TriCB and 1,3-DCB. One possible TeCB intermediate is 1,2,3,5-TeCB, since 1,3,5-TriCB would be a likely dechlorination product of 1,2,3,5-TeCB. In addition, a low level of 1,4-DCB was also observed in some cultures. Hence, we could expect that 1,3,4-TriCB is also formed from 1,2,3,5-TeCB or from other intermediates not detected.

PCA dechlorination intermediates were similarly determined by comparison of retention times of intermediates to those of authentic standards. Some intermediates were not observed, but predicted to propose a complete pathway (Fig. 4). Overall, based on detected intermediates, PCA was dechlorinated to 2,3,4,6- and 2,3,5,6-TeCA. 2,3,5,6-TeCA was dechlorinated via 2,3,5-TriCA to 3,5-DCA and 2,5-DCA. 2,3,4,6-TeCA was dechlorinated to 2,4,5-TriCA and 2,4,6-TriCA. 2,4,5-TriCA was dechlorinated to 2,4-DCA and 2,5-DCA. Finally, 3,5-DCA and 2,5-DCA were further dechlorinated to 3-CA (Fig. 4). 2,4-DCA and 2,5-DCA cannot be distinguished by GC-MS since
they have the same retention time. In the proposed dechlorination pathway presented in Fig. 4, it is assumed that both 2,4- and 2,5-DCA are produced.

3.3. Enrichment of a Dehalococcoides community in dechlorinating cultures

In order to follow the dynamics of the active dechlorinating microorganisms, Chloroflexi-specific PCR-DGGE analysis was conducted for DNA extracted from sediment microcosms, focusing on sites H1 and H5 (D0; D112 ED control, HCB, and PCA treated). DNA was extracted from all triplicate cultures. PCR-DGGE analyses indicated that several Dehalococcoides species were enriched in the H1 sediment microcosms amended with HCB or PCA (Fig. 5). Four PCR fragment bands were excised and sequenced from the gels of H1 sediment cultures. Two bands were cut from non-amended (ED) controls from day 112, and two other bands were excised from HCB and PCA treated cultures from day 112, respectively. A second PCR was performed using primers 341F-GC and 534R to confirm the band purity and to increase the DNA amount before sequencing.

Sequence analysis demonstrated that Dehalococcoides species (D. mccartyi) were enriched in the HCB and PCA dechlorinating sediment cultures from site H1. Based on the DGGE migration and phylogenetic analysis (Fig. 6), the putative dechlorinating bacteria had 100% identity with the 16S rRNA gene of the “Pinellas subgroup” (Hendrickson et al., 2002) of Dehalococcoides mccartyi, which includes strain CBDB1 capable of TeCB dechlorination (Adrian et al., 1998). For cultures established with sediment originating from site H5, there was no specific signal for Dehalococcoides after 112 days based on PCR-DGGE analysis (data not shown). While dehalogenating activity was observed in all HCB- and PCA-amended cultures, our PCR-DGGE
analysis only detected *Dehalococcoides* spp. in H1 microcosms. Phylotypes distantly related to *Dehalococcoides* were detected by the Chloroflexi PCR-DGGE at time 0 and in electron donor control cultures, however these bands were not enriched in response to addition of HCB or PCA.

### 3.4. Effect of HCB as a “haloprimer” for HBB dehalogenation

Since we observed no reductive dehalogenation of HBB over 12 months in any of the five sediment microcosms sets, a second set of microcosms were set up in an attempt to stimulate reductive debromination by adding HCB to the microcosms as a co-substrate (“haloprimer”). Triplicate cultures previously shown to dechlorinate HCB were re-spiked with 10 μM HCB and HBB. These microcosms showed reductive dehalogenation of both of HCB and HBB (Fig. 7). However, HCB dechlorination was stalled after 50 days compared to the initial cultures and HCB remained at 50-60% mole fraction for the remainder of the incubation (until 273 days).

1,2,3,5-TeBB and 1,3,5-TriBB were detected as HBB debromination products, although their concentrations were low (less than 0.1 μM).

### 3.5. Reductive dehalogenase gene profile

The relative abundance of twelve selected *rdh* genes was analyzed using a PCR assay (Park et al., 2011). The indigenous bacteria enriched from H1 and H5 sediment had similar dehalogenating activity for HCB and PCA, but they had different *rdh* gene profiles (Table 1). In general, more *rdh* genes were detected in H1 sediment cultures than in H5 sediment cultures. Of the 12 *rdh* genes (designated here as *rdh* 1 to 12) tested 10 (except *rdh* 4 and *rdh* 5) were detected in HCB amended H1 cultures, but only a weak signal for *rdh* 4 and *rdh* 5 was observed in one of the triplicates. In H1 sediments amended with PCA, 10 *rdh* genes (except *rdh* 4 and *rdh* 5) were
detected. Rdh 1, rdh 3, rdh 4, rdh 6, rdh 11, and 12 were detected also in D0 cultures or/and control at D112, but with a weak signal and only detected in one or two of the triplicates. In contrast, in H5 sediment cultures, only three *rdh* genes (rdh 1, rdh 3, and rdh 5) were enriched in PCA amended cultures. However, if we eliminate genes that were also amplified from non-amended controls, only the rdh 5 gene was specific to dehalogenating H5 cultures. In HCB-amended H5 cultures, weak signals of rdh 1 and rdh 3 were detected, but they were detected as well at day 0 and in controls at day 112. All tested *rdh* genes were detected in cultures containing both HCB and HBB as electron acceptors (day 133 and 273, data not shown). Primers targeting the *cbrA* gene was also designed and tested, but no signal was detected from either H1 or H5 sediment.
4. Discussion

In this study, we demonstrate that indigenous bacteria in sediments of the Hackensack River, New Jersey were capable of dehalogenating three persistent halogenated aromatic compounds, hexachlorobenzene (HCB), hexabromobenzene (HBB), and pentachloroaniline (PCA). These compounds were selected for study because they are problematic in the environment, but also provide information that can be applied to understanding the fate of more complex halogenated aromatics, such as polychlorinated biphenyls (PCBs) and polychlorinated dibenzo-\(p\)-dioxins.

Dechlorination of HCB and PCA was observed in most of the Hackensack River sediment microcosms within a few weeks after a short lag period (Fig. 3 and Fig. 4). In contrast, Brahushi et al. (2004) reported HCB dechlorination after a lag period of 2 months in arable soil that had not previously been exposed to HCB. The Hackensack River sediment is contaminated by halogenated aromatics such as PCBs and polychlorinated dibenzo-\(p\)-dioxins (for reviews, see Crawford et al., 1995), which we postulate has resulted in the enrichment of indigenous dehalogenating bacteria, and hence only a short lag period before the onset of dechlorination.

The Hackensack River sediments were collected along an estuarine gradient at five sites, with salinity and sulfate levels increasing closer to the mouth of the river in Newark Bay. The temporal variation in salinity and sulfate concentration in the estuarine section of the river is expected to lead to dynamic and variable redox conditions and microbial activities. Several studies have shown that sulfate impedes the enrichment of dehalogenating communities through interspecific competition for electron donors or through direct inhibition of dehalogenation (for reviews see Zanaroli et al., 2015). In comparison of the dechlorination rates observed in the
different microcosms (see Fig. 1 for different sampling sites) the delayed onset of dechlorination in H5 sediment microcosms was likely caused by the higher sulfate levels in sediments at the site. In our experiments we used the same medium to examine the potential of dehalogenation regardless the difference of the sediment characteristics, which may have influenced the results. Generally, studies have shown that sulfate inhibits reductive dechlorination (Gibson and Suflita, 1986; Alder et al., 1993), even though in some cultures sulfate did not affect dechlorination or only caused partial inhibition (Kohring et al., 1989; Häggblom and Young, 1990; Boyle et al. 1999). Indeed, facultative dehalorespiring bacteria that can also respire sulfate have been isolated from estuarine and marine environments (Zanaroli et al., 2015). The delayed onset of dechlorination in H5 sediments might be explained by salinity as well. Prytula and Pavlostathis (1996) reported that the dechlorination rate of sediment bound HCB was two orders magnitude lower than that of freshly added, bioavailable HCB, with salinity causing increased contaminant hydrophobicity. Microbial reductive dechlorination of the sediment bound contaminants caused the long-term release of less chlorinated and more mobile products. By reducing the bioavailability of contaminants, salinity can inhibit indigenous microorganisms.

HCB was sequentially dechlorinated to PeCB, 1,3,5-TriCB and finally 1,3- and 1,4-DCB (Fig. 4). A similar HCB dechlorination pathway has also been observed in previous studies (Fathepure et al., 1988; Beurskens et al., 1994; Chang et al., 1997; Chen et al., 2000; Pavlostathis and Prytula, 2000; Wu et al., 2002; Brahushi et al., 2004; Fennell et al., 2004; Tas et al., 2011). Based on their data, the most predominant dechlorination pathway follows HCB to 1,2,3,5-TeCB and finally to 1,3,5-TriCB. 1,2,3,5-TeCB was not detected as an intermediate in our HCB amended cultures, likely because it was rapidly dechlorinated further to 1,3,5-TriCB without
substantial accumulation. However, it was a likely dechlorination intermediate since 1,3,5-TriCB could be only formed from 1,2,3,5-TeCB. For *Dehalococcoides mccartyi* strain CBDB1, the major dehalogenation pathway of HCB and PeCB is following to 1,2,4,5-TeCB (~70%) to 1,3,5-TriCB to 1,3- and 1,4-DCB. Only ~30% of TeCB was detected as 1,2,3,5-TeCB and further dechlorinated to 1,3,5-TriCB (Jayachandran et al., 2003). Ramanand et al. (1993) observed another dechlorination pattern of HCB and PeCB to CB via 1,2,3,4-TeCB to 1,2,3- and 1,2,4-TriCB to 1,2- and 1,4-DCB. Dechlorination of TriCB has been observed in previous studies (Bosma et al., 1988; Holliger et al., 1992; Middeldorp et al., 1997). 1,2,3- and 1,3,5-TriCB only dechlorinated to 1,3-DCB (Bosma et al., 1988; Holliger et al., 1992; Wagner et al., 2009). 1,2,4-TriCB dechlorinated to CB via 1,4-DCB (Middeldorp et al., 1997) or 1,3- and 1,4-DCB (Wagner et al., 2009). A presence of 1,4-DCB in the culture suggested that 1,2,4-TriCB was one intermediate of HCB dechlorination in the Hackensack River sediment cultures.

In PCA amended cultures, sequential dechlorination was observed and it appeared more divergent than HCB dechlorination. Based on observed and predicted intermediates, PCA was sequentially dechlorinated to 2,3,5,6- and 2,3,4,6-TeCA, 2,3,5-, 2,4,5-, and 2,4,6- TriCA, 2,4-, 2,5-, 2,6-, and 3,5-DCA, and eventually to 3-chloroaniline (CA). Susarla et al. (1996) observed transformation of PCNB in anaerobic sediment cultures as PCNB reduced nitro group and sequential dechlorinated to CAs via 2,3,4,5-TeCA, 3,4,5-TriCA, and 3,5-DCA. Okutman Tas and Pavlostathis (2005) reported a PCNB degradation pathway under methanogenic conditions proceeding via nitro reduction of PCNB to PCA, followed by sequential dechlorination to 2,3,4,5- and 2,3,5,6-TeCA, 2,4,5- and 2,3,5-TriCA, 2,4-, 2,5-, and 3,5-DCA, and eventually 3- and 4-CA. Liu et al. (2014) reported a similar conversion of PCNB to PCA, followed by
dechlorination to 2,3,4,6- and 2,3,4,5-TeCAs, and then to 2,4,5- and 2,4,6-TriCA. The 2,4,6-
TriCA was the main product after 11 months. TriCAs were accumulated and was slowly
dechlorinated further to 2,4- or 2,5-DiCA (which could not be separated in analysis).

Dehalogenating bacteria indigenous to different geographical locations thus mediate slightly
different dechlorination pathways of PCA.

*Chloroflexi* specific 16S rRNA gene PCR-DGGE followed by sequence analysis detected
members of the “Pinellas subgroup” of *Dehalococcoides mccartyi* in H1 sediment. PCR-DGGE
and reductive dehalogenase gene profile analyses of H1 and H5 cultures showed different
microbial communities enriched from each site. Even though HCB and PCA dehalogenation
activities were observed for both H1 and H5 cultures, their *rdh* gene profiles were dissimilar. H1
cultures had more *rdh* genes enriched than H5 cultures, suggesting that different dehalogenating
communities with different *rdh* genes were enriched. In H1 microcosms, ten *rdh* genes common
to both *D. mccartyi* strains 195 and CBDB1 were amplified clearly, but *rdh* 4 and *rdh* 5 in low
abundance and detected only with weak signal. We recognize that the full suite of *rdh* genes
would not be detected by our 12 tested primer sets. The enrichment of 16S rRNA gene of
*Dehalococcoides* and the enrichment of *rdh* genes detected by primer sets designed from *D.
mccartyi* strain 195 and strain CBDB1 in H1 cultures (Fig. 6 and Table 1) suggests that
*Dehalococcoides* spp. were the likely dehalogenators responsible for the fast onset of
dechlorination. The enrichment of members of the Pinellas subgroup of *Dehalococcoides*, which
includes *D. mccartyi* strain CBDB1 as a member, has been observed in organohalide-amended
sediments from many different geographical locations (Park et al., 2011; Liu et al., 2014;
The abundance of Dehalococcoides spp. has been found to be associated with organohalide contamination and dechlorination activity (Krzmarzick et al., 2011).

Debromination of HBB was not observed in initial microcosms during incubation for up to 12 months (data not shown). It is possible that hexabromobenzene does not fit in the active site of the reductive dehalogenase of the indigenous microorganisms, or that HBB is poorly available and does not induce dehalogenating activity. When HCB and HBB were added as co-substrates to HCB dechlorinating cultures, slow debromination of HBB (or PeBB; pentabromobenzene) was observed (Fig. 7), with production of TeBB and TriBB after 92 days of incubation (although at low concentration, less than 0.1 μM). Several earlier reports have demonstrated that dehalogenation rates can be improved with amendment of halogenated co-substrates (Ahn et al., 2005, 2007, 2008; Bedard et al., 1998; Kuokka et al., 2014; Liu et al., 2014; Wu et al., 1997). It is not apparent whether this stimulation was due to the growth of a separate debrominating community or the induction of the perquisite dehalogenases. For example, Morris et al. (1992) suggested that different microbial strains were selected for dehalogenation of polybrominated biphenyls (PBB) and PCBs. Furthermore, the debrominating bacterium Desulfoluna spongiphila is not capable of dechlorination (Ahn et al., 2009). The chlorinated contaminants in the Hackensack River sediments may enrich for dehalogenators specifically selected for chlorinated compounds, while debrominating bacteria have not been enriched.
5. Conclusions

In summary, we demonstrate that indigenous microorganisms can be enriched from Hackensack River, NJ sediment and have dehalogenating activities for HCB and PCA. Different dehalogenating communities were enriched on HCB and PCA from H1 and H5 sediments, with *Dehalococcoides* species likely responsible for the earlier onset of dehalogenation in H1 sediment. Putative reductive dehalogenase genes were enriched in low-salinity/sulfate sediment microcosms concomitant with HCB and PCA dechlorination. HBB was recalcitrant, but slow debromination was stimulated by HCB on as a co-substrate. These results suggest that dehalogenation of halogenated aromatic contaminants may be mediated by indigenous microbial populations as a means for *in situ* bioremediation of sediments contaminated with halogenated compounds. More detailed characterization of the microbial communities and *rdh* gene profiles involved in dehalogenation process will aid in the assessment of contamination by persistent halogenated compounds and *in situ* bioremediation.

Acknowledgments

We thank Prof. John Reinfelder (Dept. of Environmental Sciences) for providing the Hackensack River sediment samples, Prof. Lily Young (Dept. of Environmental Sciences) for supplying hexabromobenzene, and Dr. Joong-Wook Park for guidance on DGGE analyses. This work was funded in part by the New Jersey Agricultural Experiment Station and the New Jersey Water Resources Research Institute.
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Ahn, Y.B., Häggblom, M.M., Fennell, D.E., 2005. Co-amendment with halogenated compounds enhances anaerobic microbial dechlorination of 1,2,3,4-tetrachlorodibenzo-p-dioxin and 1, 2, 3, 4-tetrachlorodibenzofuran in estuarine sediments. Environmental Toxicology and Chemistry 24, 2775–2784.


Figure 1. Sampling locations along the Hackensack River, NJ.
Dechlorination of HCB in Hackensack River sediment microcosms. HCB and its dechlorination products are represented as the mole fraction of all chlorobenzenes detected. Data points are the mean ± standard deviation of three replicate microcosms.
Figure 3. Dechlorination of PCA in Hackensack River sediment microcosms. PCA and its dechlorination products are represented as the mole fraction of all chloroanilines detected. Data points are the mean ± standard deviation of three replicate microcosms.
Figure 4. Proposed pathways for HCB and PCA dechlorination based on observed intermediates.

Gray-colored isomers are postulated to make the pathway complete, but were not detected in cultures over the course of dechlorination.
Figure 5. PCR-DGGE analysis of H1 sediment microcosms at day 0 and day 112. The red boxes represent four bands excised for sequence analysis.
Figure 6. Phylogenetic analysis of 16S rRNA gene DGGE band sequences. The tree was constructed using the Maximum Likelihood method with MEGA 6 with 132 unambiguously aligned positions. Excised bands from previously analyzed dehalogenating cultures are indicated with an asterisk. KJ sequences were from Kymijoki River sediment, Finland (Liu et al., 2014). AR sequence was from Anacostia River sediment, DC (Park et al., 2011).
Figure 7. Change of HCB and HBB concentration and their intermediates in spiked Hackensack River sediment enrichment cultures. HCB and HBB are represented with their intermediates in separate plots as the mole fraction. HBB dehalogenation intermediates were represented in a logarithmic scale. Data points are the mean ± standard deviation of three replicate microcosms.
Table 1. Reductive dehalogenase gene profile constructed with 12 rdh genes in H1 sediment (ED; HCB; and PCA) and H5 sediment (ED; HCB; and PCA).

<table>
<thead>
<tr>
<th></th>
<th>H1</th>
<th>H5</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>D0</td>
<td>D112</td>
</tr>
<tr>
<td>Rdh 1</td>
<td>w - -</td>
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<tr>
<td>Rdh 2</td>
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<td>- - -</td>
</tr>
<tr>
<td>Rdh 3</td>
<td>w w -</td>
<td>w w -</td>
</tr>
<tr>
<td>Rdh 4</td>
<td>w - -</td>
<td>w w -</td>
</tr>
<tr>
<td>Rdh 5</td>
<td>- - -</td>
<td>w - -</td>
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<td>- - -</td>
<td>w - -</td>
</tr>
<tr>
<td>Rdh 12</td>
<td>- - -</td>
<td>w - -</td>
</tr>
</tbody>
</table>

+, signal; -, no signal; w, weak signal
Supplementary Data

Reductive Dehalogenation Activity of Indigenous Microorganism in Sediments of the Hackensack River, New Jersey

Seo Yean Sohn and Max M. Häggblom

Table S1. Physicochemical parameters of sediments collected along the Hackensack River, NJ.

<table>
<thead>
<tr>
<th>Sampling station</th>
<th>Distance from the river mouth (km)</th>
<th>Temperature</th>
<th>pH</th>
<th>Organic matter (%)</th>
<th>Organic carbon (%)</th>
<th>Salinity (ppt)</th>
<th>Sulfate (mg/L)</th>
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<tr>
<td>H1</td>
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<td>28.4</td>
<td>7.45</td>
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<td>H2</td>
<td>16.64</td>
<td>28.26</td>
<td>7.65</td>
<td>13.76</td>
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<td>5.79</td>
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<tr>
<td>H3</td>
<td>11.88</td>
<td>27.79</td>
<td>7.66</td>
<td>10.22</td>
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<td>H4</td>
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<td>28.07</td>
<td>7.51</td>
<td>8.62</td>
<td>3.75</td>
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<tr>
<td>H5</td>
<td>1.97</td>
<td>28.38</td>
<td>7.4</td>
<td>9.39</td>
<td>3.89</td>
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</tr>
</tbody>
</table>


Janssen, S.E., and Reinfelder, J.R., Personal Communication
Sequence data of excised bands from PCR-DGGE (Figures 5 and 6).

ED control (1)*
GAAAGCCTGACGCAGCGACTCCGCGTGAGGGATGAAGGCCAtCGGGTTGTATACCCTTTTCCGAGGAAGAAATTATGAcGGTACCCGCAGAAATAAGTCTtGGCTAACTACGTGCCAGCAGCCGCTGTAATA

ED control (3)*
GCAAGCCTGATGCAGCAACGCCGCTGAGGGACGAACGCGCTTCCGGGTCAACCTCTTTTTCTGAGGGACGAAGAAAGGACGGTACCTCAGGAATAAGTCTCGGCTAACTACGTGCCAGCAGCCGCTGTAATA

HCB (1)*
CGAAAGCCTGACCCAGCAACGCCGCTGAGGGATGAAGGCTTTCGGGTTGTAAACCTCTTTTTGATAGGGAAAGAATAATGACGGTACCTGTGGGAATAAGCTCGGCTAACTACGTGCCAGCAGCCGCTGTAATA

PCA (1)*
GCAATGGGCCAGAAAGCCTGACCCAGCAACGCCGCTGAGGGATGAAGGCTTTCGGGTTTGTAACCTCTTTTTGATAGGGAAAGAATAATGACGGTACCTGTGGGAATAAGCTCGGCTAACTACGTGCCAGCAGCCGCTGTAATA