In Situ Enhancement of Anaerobic Microbial Dechlorination of Polychlorinated Dibenzo-*p*-dioxins and Dibenzofurans in Marine and Estuarine Sediments

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List of Acronyms

BP	bromophenol
CDD	chlorodibenzo-*p*-dioxin
CDD/F	chlorodibenzo-*p*-dioxin and chlorodibenzofuran
CDF	chlorodibenzofuran
CP	chlorophenol
DCB	dichlorobenzene
DCDD	dichlorodibenzo-*p*-dioxin
DCDF	dichlorodibenzofuran
DCN	dichloronaphthalene
HMN	heptamethylnonane
HPγCD	hydroxyl-propyl-γ-cyclodextrin
HxCDF	hexachlorodibenzofuran
Lac	lactate
MCDD	monochlorodibenzo-*p*-dioxin
MCDF	monochlorodibenzofuran
OCDD	octachlorodibenzo-*p*-dioxin
OCN	octachloronaphthalene
PCB	polychlorinated biphenyl
PCDD	polychlorinated dibenzo-*p*-dioxin
PCDD/Fs	polychlorinated dibenzo-*p*-dioxins and dibenzofurans
PCDF	polychlorinated dibenzofuran
PCR	polymerase chain reaction
PentaCDF	pentachlorodibenzofuran
Pro	propionate
TeCA	tetrachloroanisole
TeCB	tetrachlorobenzene
TeCBA	tetrachlorobenzoic acid
TeCDD	tetrachlorodibenzo-*p*-dioxin
TeCDF	tetrachlorodibenzofuran
TeCN	tetrachloronaphthalene
TeCP	tetrachlorophenol
TrCAP	trichloroacetophenone
TrCB	trichlorobenzene
TrCDD	trichlorodibenzo-*p*-dioxin
TrCDF	trichlorodibenzofuran
TrCN	trichloronaphthalene
TRFLP	terminal restriction fragment length polymorphism

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Executive Summary:

The management of marine and estuarine sediments contaminated with toxic organic compounds, including polychlorinated dibenzo-*p*-dioxins and dibenzofurans (PCDD/Fs), is a major problem with far-reaching economic and ecological consequences. Enhancement of microbial degradation of PCDD/Fs *in situ* is an attractive remediation alternative that could potentially detoxify sediments, avoid the problematic redistribution of contaminants that is associated with dredging, and decrease the cost of sediment management. Reductive dehalogenation is a promising mechanism for the removal of toxic organohalides from estuarine and marine sediments. Anaerobic dechlorination of PCDD/Fs has been reported in marine and estuarine sediments, however, rates are slow and the activity may be the result of a combination of both respiratory and cometabolic processes. For *in situ* bioremediation, it would be most desirable to stimulate respiratory dechlorination, which is typically associated with higher dechlorination rates.

The current impetus for the use of *in situ* enhancement of native dehalogenating bacteria to remediate freshwater aquifers has not yet been extended to contaminated marine environments. Among the impediments to extension of the technology are the complex nature of the contaminants (PCDD/Fs, PCBs, chlorinated pesticides, etc.); the limitations imposed by the close association of the contaminants to highly organic sediments; and, perhaps most importantly, the lack of basic information about how dehalogenating bacteria perform and compete in these often highly sulfidogenic environments. Anaerobic reductive dechlorination is the first step required for the ultimate complete degradation of highly chlorinated PCDD/F congeners. Therefore, identification of the organisms involved, determination of their dechlorinating potential, characterization of how they compete for reducing equivalents with other members of the community, and development of strategies for enhancing their dehalogenating activities, are all needed. The overarching goal of this project, therefore, was to identify environmental conditions and amendments that enhance and accelerate dechlorination of PCDD/Fs by indigenous microbial populations and to identify the organisms responsible for the dechlorination using biomolecular methods.

Results from this project show that anaerobic dehalogenation of PCDD/Fs was readily promoted in estuarine, marine and freshwater, sediments from several sites. Co-amendment with more soluble halogenated aromatic compounds such as halogenated phenols, chlorinated benzenes, and chlorinated anisoles, as “priming” agents was found to greatly enhance the rates of PCDD/F dechlorination. This stimulation was brought about in the presence of added electron donors lactate and propionate to ensure reduced conditions and adequate reducing equivalents to stimulate dechlorination. In highly organic sediments with adequate reducing power, it is possible that these halogenated additives may be just as effective without additional electron donors. PCDD dechlorination was, in general, more easily stimulated and proceeded to a greater extent in sediments previously contaminated with other chlorinated compounds than in less contaminated or pristine sediments. Our results suggest that halogenated aromatic compounds with structural similarity to PCDD/Fs simulate bacteria with the ability to dechlorinate these contaminants. Of critical importance is that these amendments stimulate the desirable lateral dechlorination of PCDD/Fs (i.e., removal of chlorines at position 2 and 3) that ultimately results

in detoxification of the compounds. A lateral dechlorination pathway dominated in most of the sediments and conditions we examined.

While the process has much potential, a key aspect of the technology – the nature and capability of the intrinsic microbial community – must also be understood at a fundamental level. In 2003, other researchers reported that *Dehalococcoides* strain CBDB1 could dechlorinate selected PCDDs. A result of this project was our finding that *Dehalococcoides ethenogenes* strain 195, originally isolated on tetrachloroethene, has the potential to dechlorinate many different types of chlorinated aromatic compounds, such as PCDD/Fs, PCBs and chlorinated naphthalenes. Strain 195 carried out a lateral, detoxification dechlorination pathway on the PCDD/F congeners that we examined.

Our results with Strain 195 coupled with recent findings that some species of dehalogenating bacteria have multiple dehalogenases and dechlorination capabilities suggests that halogenated compounds other than PCDD/Fs could be used to enrich and isolate organisms that have activity on PCDD/Fs. Within the Chloroflexi, which includes both *Dehalococcoides* spp. and other yet to be described genera, there appears to be several strains with the ability to reductively dechlorinate PCBs and other halogenated compounds as electron acceptors. These findings may explain our results that show the stimulation of PCDD/F dechlorination in a variety of sediments upon addition of simpler halogenated compounds. Identification of naturally-occurring or less toxic halogenated co-amendments that are completely degraded in the sediment environment is desirable for field application, however enrichment of bacteria for bioaugmentation purposes under controlled conditions could use volatile compounds such as tetrachloroethene.

In addition to determining that a well-know dehalorespirer can dechlorinate PCDD/Fs, we used a variety of molecular approaches to identify and characterize and identify the bacteria involved in PCDD/F dechlorination. For example, polymerase chain reaction (PCR) coupled to terminal restriction fragment length polymorphism (TRFLP) analysis was used to explore microbial community differences between different sites exhibiting PCDD dechlorination. PCR using primers specific for dehalogenase genes were also used to delineate the functional genes present in actively dechlorinating communities. Future studies will focus on developing these methods further for identifying and monitoring PCDD/F-dechlorinating bacteria under *in situ* conditions. For example, real-time PCR analysis will be used to quantify total bacteria, *Dehalococcoides ethenogenes* 195 and reductive dehalogenase genes from *Dehalococcoides ethenogenes* 195 in bioaugmented and non-bioaugmented sediments. This basic understanding of microbial processes and the ability to identify and track the activity of specific strains or genes is essential for the design of site-specific solutions for PCDD/F bioremediation and sediment restoration projects.

Data collected from enrichment cultures undergoing different amendment strategies and the accompanying microbial community characterization was used for the development of a biological process model to describe the stimulatory effect of different enhancement methods under different conditions and at different sites. Predictive modeling of San Diego Bay sediments under methanogenic and sulfidogenic conditions suggested that hydrogen concentration was a major factor controlling the activity of dechlorinating bacteria under different redox conditions.

Sediment treatment is currently limited primarily to dredging with *ex situ* treatment or sequestration. New methods are needed for *in situ* containment and degradation of contaminants and could decrease the cost of long-term sediment management. Anaerobic reductive dehalogenation offers a promising approach towards eventual detoxification and complete degradation of halogenated contaminant mixtures. *In situ* bioremediation combined with in-place containment through capping could avoid the problematic redistribution of contaminants that is associated with dredging and, where feasible, offer a more cost effective treatment alternative to dredging. Developing amendment technologies for enhanced microbial dehalogenation and understanding how amendment placement and mixing stimulates dehalogenation and impacts the fate and transport of organohalide mixtures is thus a high priority for the successful management of contaminated sediments. This project has greatly expanded our understanding of the microorganisms that carry out dehalogenation of PCDD/Fs in sediments and has produced a variety of techniques that can be used to enhance this activity. On-going work by our team will allow field verification of the effectiveness of these approaches for remediating contaminated sediments.

Objective:

The management of marine and estuarine sediments contaminated with toxic organic compounds, including polychlorinated dibenzo-*p*-dioxins and dibenzofurans (PCDD/Fs), is a major problem with far-reaching economic and ecological consequences. Enhancement of microbial degradation of PCDD/Fs *in situ* is an attractive remediation alternative that could potentially detoxify sediments, avoid the problematic redistribution of contaminants that is associated with dredging, and decrease the cost of sediment management.

The overall objectives of the proposed research were to identify environmental conditions and amendments that enhance and accelerate dechlorination of PCDD/Fs by indigenous microbial populations and to identify the organisms responsible for the dechlorination using biomolecular methods. The specific objectives were to:

1. Characterize the PCDD/F dechlorinating capability of indigenous dehalogenating bacteria.
2. Identify specific amendments and their combinations that prime and/or accelerate the dechlorination of PCDD/Fs by dehalorespiring or cometabolizing bacteria
3. Characterize the effect of different redox conditions and competitive terminal electron acceptors (e.g. sulfidogenic, iron-reducing, methanogenic) on the dechlorination and further transformation of PCDD/Fs.
4. Identify and characterize specific PCDD/F-dechlorinating bacteria and consortia using molecular techniques.

Background:

Halogenated organic compounds constitute one of the largest and most problematic groups of environmental pollutants. These compounds are integral to a variety of industrial applications, including use as solvents, degreasing agents, hydraulic and heat transfer fluids, intermediates for chemical synthesis, and flame retardants, but may also be industrial/combustion by-products. For example, polychlorinated dibenzo-*p*-dioxins (PCDDs), the class of compounds under study, are tricyclic and planar aromatic compounds produced inadvertently through a number of anthropogenic processes. They are nonpolar, hydrophobic, lipophilic, stable chemicals, and are extremely toxic to human beings (Czuczwa et al. 1984; Alcock and Jones 1996). The majority of the halogenated pollutants are chlorinated, but brominated, fluorinated and iodinated compounds are also used in industrial applications (Häggblom and Bossert 2003). Their production and use has resulted in widespread dissemination and environmental contamination, with estuarine and marine sediments as significant sinks. Consequently, the management of marine and estuarine sediments contaminated with toxic organic compounds, including PCDD/Fs, PCBs, chlorinated pesticides and other organohalogen pollutants is a major problem with far-reaching economic and ecological consequences.

While there are widespread problems with halogenated contaminants in sediments associated with estuarine systems, harbors, and coastal areas, situ treatment has not as yet been pursued as a viable treatment alternative. While the use of *in situ* anaerobic microbial dehalogenation to clean up aquifers contaminated with chlorinated solvents has been studied extensively, similar technology has not yet been extended to contaminated marine environments. Among the impediments to extension of the technology are the existence of complex mixtures of contaminants (PCDD/Fs, PCBs, chlorinated pesticides, etc.); the limitations imposed by the close association of the contaminants to highly organic sediments; and, perhaps most importantly, the lack of basic information about how dehalogenating bacteria perform and compete in these often highly sulfidogenic environments. Identification of organisms involved, determination of their dechlorinating potential, characterization of how they compete for reducing equivalents with other members of the community, and development of strategies for enhancing their dehalogenating activities, are all sorely needed.

Microbial degradation is one of the key factors that determines the ultimate fate of organohalides in the environment, with cleavage of the carbon-halogen bond being one of the critical steps. Microbial degradation of the carbon structure requires the presence of enzymes that cleave this bond under physiological conditions and microorganisms have evolved a variety of metabolic strategies for cleaving the carbon-halogen bond (for reviews, see Häggblom 1992; Häggblom and Bossert 2003; Bossert et al. 2003; Holliger et al. 2003; Löffler et al. 2003). Dehalogenation reactions comprise different strategies, where organohalides serve either as electron donors (and carbon sources) or as electron acceptors. The reductive dehalogenation and subsequent anaerobic degradation of a variety of organohalides is well established (for reviews see Häggblom, 1992; Mohn and Tiedje, 1992; Holliger et al. 2003). Microbial reductive dehalogenation can be categorized as either a respiratory process that is coupled to growth and energy conservation and generation of ATP within the organism (dehalorespiration), or as a

cometabolic process that is not linked to generation of ATP (Mohn and Tiedje, 1992; Holliger and Schraa, 1994). In addition, spontaneous dehalogenation may also occur as result of chemical degradation of unstable primary metabolites. In anaerobic sediments the most important process is dehalorespiration, in which the organohalide serves as an alternate electron acceptor for anaerobic respiration. Reductive dechlorination requires a source of reducing equivalents as an electron donor, with the halogenated compound serving as a concomitant electron acceptor. Anaerobic dehalogenation is influenced by the microbial communities active in different redox zones and the availability of suitable electron donors and acceptors affects the biodegradability of organohalides (Fig 1).

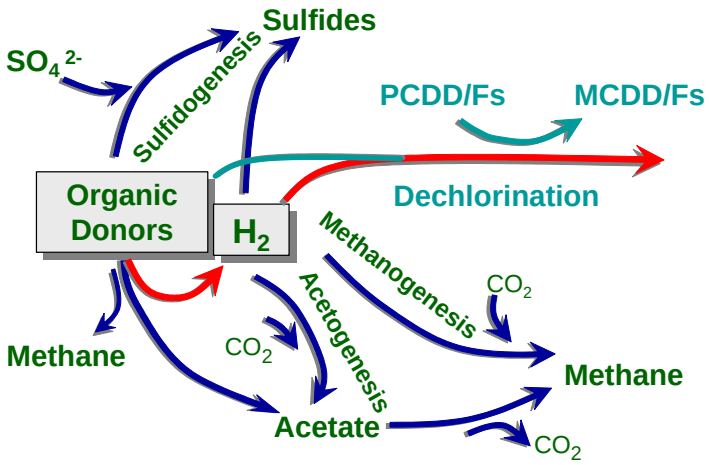

Fig. 1. Competing electron flow pathways in anaerobic sediments.

Microbial reductive dechlorination of PCDD/Fs in sediments is an important environmental process because it has the potential of decreasing the toxicity of PCDD/Fs if lateral chlorines are removed. Dechlorination may also be advantageous since lesser chlorinated congeners are more susceptible to subsequent aerobic degradation (Wittich 1998). A potential drawback of dechlorination is that the preferential removal of chlorines from the 1-, 4-, 6-, and 9-positions from octachlorodibenzo-*p*-dioxin (OCDD) form 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (2,3,7,8-TeCDD) and other 2,3,7,8-substituted congeners that are considered potential human carcinogens (Pohl et al. 2002). While anaerobic dechlorination of PCDD/Fs has been reported in marine and estuarine sediments and is apparently linked to biological activity, rates are slow. Dechlorination of PCDD/Fs in sediments were shown to be the result of a combination of both respiratory and cometabolic microbial processes (Toussaint et al. 1998; Gruden et al. 2003; Adriaens and Grbic-Galic 1994, 1995; Beurskens et al. 1995; Barkovski and Adriaens 1996; Ballerstedt et al. 1997) in addition to a variety of abiotic mechanisms (Gruden et al. 2003; Adriaens et al. 1996; Barkovski and Adriaens 1998; Fu et al. 1999). In most cases, PCDD/F contamination occurs at low concentrations. Low aqueous solubilities and strong sorption of the PCDD/Fs to sediments reduce their availability as substrates for bacteria (Harms 1998).

At the commencement of this project little was known about the nature of the organisms that dechlorinate PCDD/Fs. Enrichment or co-amendment of sediments with alternate halogenated compounds had been shown to enhance dechlorination of PCDDs. Beurskens et al.

(1995) demonstrated dechlorination of 1,2,3,4-tetrachlorodibenzo-*p*-dioxin (1,2,3,4-TeCDD) in a hexachlorobenzene-enriched culture from Rhine River sediment. Albrecht et al. (1999) demonstrated that octachlorodibenzo-*p*-dioxin was dechlorinated to 2-monochlorodibenzo-*p*-dioxin (2-MCDD) after a single amendment with 2-monobromodibenzo-*p*-dioxin and hydrogen. Our group (Vargas et al. 2001) demonstrated that dechlorination of 1,2,3,4-TeCDD was more extensive in heavily contaminated estuarine sediments from the Arthur Kill, an estuarine inlet located between Staten Island, New York and New Jersey, USA, when pre-enrichment on bromophenols was performed. The responsible organisms were not identified.

Identification of the organisms responsible for dehalogenation is an important first step for developing appropriate bioremediation methods for site-specific treatment. However, elucidating the metabolic role of members within complex microbial communities without isolation and testing of individual strains in the laboratory remains a challenge. A variety of biomarkers, including lipids, proteins, and nucleic acids can be used to differentiate the various members of the microbial community. Currently, the simplest, most popular way to identify bacteria is by polymerase chain reaction (PCR) amplification and sequencing of 16S rRNA genes. This characterization of SSU genes has become routine in many labs (for reviews see, Head et al. 1998; Hugenholtz et al. 1998; Amann et al. 1995; Torsvik et al. 1996; Woese 1987). This approach has been used in a variety of studies for detecting microorganisms involved in dehalogenation (Knight et al. 1999; Löffler et al. 2000; Fennell et al. 2001; Richardson et al. 2002; Becker et al. 2001; Holoman et al. 1998; Wu et al. 2001). Most studies have been able to identify the bacteria present within microcosms, bioreactors and in the field, but presence/absence does little to elucidate activity and cannot absolutely determine the microorganism(s) capable of or responsible for the bulk of the dehalogenation. Unfortunately, these DNA-based methods have the drawback that the total community is assayed - whether active or inactive. Recently, small subunit (SSU) ribosome characterization using group specific probes (Becker et al. 1999; de los Reyes et al. 1998) or species-specific approaches (Delbes et al. 2000, 2001; Muttray et al. 1998) have been used to distinguish the active members of a microbial community. This characterization using intact ribosomes offers a more sensitive method of assessing a system since there appears to be a robust relationship between growth and rRNA content for a variety of microorganisms (Kerkhof and Ward 1993). Later work extended the range of growth rates exhibiting a relationship between rRNA content and growth to environmentally realistic doubling times (Kemp et al. 1993; Kerkhof and Ward 1993; Poulsen et al. 1993). Thus, by assaying ribosomal RNA during dehalogenation, the active populations can be identified and monitored.

We developed an assay using a starved consortium (low ribosome content culture) fed a suite of selective substrates and the analysis of the newly synthesized 16S rRNA as a means for assigning a metabolic role to members of a dehalogenating consortium using terminal-restriction fragment length polymorphism (TRFLP) analysis of reverse transcribed rRNA. This system was used because respiratory dehalogenation is a dissimilatory process in which the halogenated compounds are not incorporated directly into microbial biomass. Therefore, radiolabeled compounds cannot be used to track the microorganism(s) that actively transform these compounds. By first starving a consortium, then supplying various substrates; it is possible to identify those microorganisms which are actively growing in the various amendments (i.e. utilizing particular substrates) by characterizing the newly synthesized 16S rRNA. Those starved

cells that do not utilize a particular substrate will not create new ribosomes and are not discernable. Thereby, the active microbial community performing a metabolic function is distinguished from the inactive community.

An alternative to the indirect method using ribosomes to elucidate dehalogenating microorganisms in consortia or natural environments involves the use of functional genes. Recently, several membrane bound reductive dehalogenases (RDH) have been purified and characterized: the tetrachloroethene reductase (*pceA*) gene from *Dehalospirillum multivorans* (Neumann et al. 1998) and a *Desulfitobacterium* sp. (Suyama et al. 2002), the 2-chlorophenol reductase (*cprA*) gene from *Desulfitobacterium dehalogenans* (van de Pas et al. 1999), and the trichloroethene reductase gene (*tceA*) from *Dehalococcoides ethenogenes* (Magnuson et al. 2000). We designed several sets of degenerate PCR primers to amplify RDH gene fragments and putative RDH genes from the PCDD/F enrichments have been cloned and identified.

Understanding the role of anaerobic respiratory processes and the specific microbial communities involved in the dechlorination of PCDD/Fs is essential for developing enhanced *in situ* treatment technologies. Enhancement of microbial dehalogenation is an attractive remediation alternative that could potentially detoxify sediments. Furthermore, remediation of organohalide-contaminated sediments *in situ* could avoid the problematic redistribution of contaminants that is associated with dredging and would decrease the cost of sediment management. However, application of *in situ* bioremediation of organohalide-contaminated marine and estuarine sediments has been limited by lack of fundamental knowledge about the microorganisms responsible for anaerobic dehalorespiration, the first step required for the ultimate complete degradation of highly halogenated compounds. Application of bioremediation to organohalide-contaminated sediments is also limited by the low bioavailability of these compounds and the inability to effectively stimulate biodegradation. Our study was undertaken with the goal of filling these data gaps to allow rational formulation of strategies for remediation of contaminated sediments *in situ*.

Materials and Methods

1. Dechlorination of PCDD/Fs by Indigenous Dehalogenating Bacteria in Estuarine and Marine Sediments

***Sediment samples*.** The Space and Naval Warfare Systems Command in San Diego, CA, USA supplied sediment obtained from San Diego Bay in March and October 2001. Sediments were obtained from Shelter Island, a pleasure craft area, and Paleta Creek and Graving Dock (Pier 5), sites within the Naval Station. We also collected sediment from a pristine estuary near Tuckerton, NJ, USA in September 2001. The watershed impacting this coastal area is non-industrialized and has a light residential population. Sediments were packed into sterile jars, sealed and stored at 4°C until used.

Chemicals. 1,2,3,4-TeCDD, 1,2,3-trichlorodibenzo-*p*-dioxin (1,2,3-TrCDD), 1,2,4-TrCDD, 2,3-dichlorodibenzo-*p*-dioxin (1,2-DCDD), 1,3-DCDD, 1,4-DCDD, 1-monochlorodibenzo-*p*-dioxin (1-MCDD), 2-MCDD, dibenzo-*p*-dioxin and 2,2',5-trichlorobiphenyl (2,2',5-PCB) were obtained from AccuStandard (New Haven, CT, USA). 1,2,3,4-TeCDF, 2,3,4-trichlorodibenzofuran (2,3,4-TrCDF), 2,4-dichlorodibenzofuran (2,4-DCDF), 2-monochlorodibenzofuran (2-MCDF), 4-monochlorodibenzofuran (4-MCDF) and dibenzofuran were obtained from Ultra Scientific (North Kingstown, RI, USA). Tetrachlorobenzene (TeCB), tetrachloroanisole (TeCA), tetrachlorophenol (TeCP), tetrachlorobenzoic acid (TeCBA), trichloroacetophenone (TrCAP), phenol, 2-chlorophenol (2-CP), 3-chlorophenol (3-CP), 4-chlorophenol (4-CP), 2-bromophenol (2-BP), 3-bromophenol (3-BP), 4-bromophenol (4-BP), propionic acid and sodium lactate were obtained from Sigma-Aldrich (St. Louis, MO, USA). The structures of some of the halogenated compounds used in the study are shown in Fig. 2.

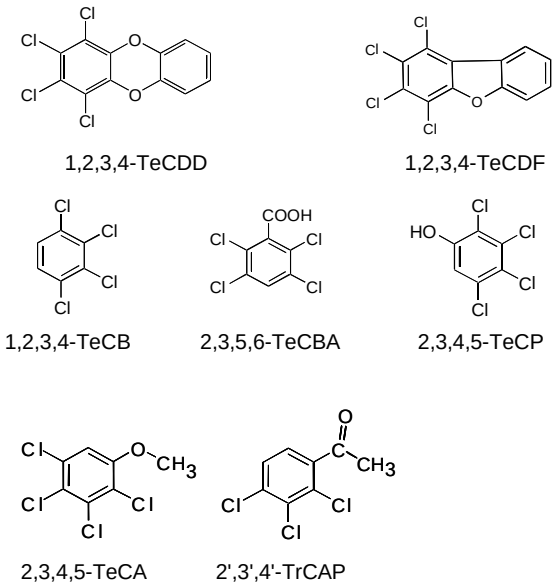

Fig. 2. Halogenated compounds used in this study (TeCDD, tetrachlorodibenzo-*p*-dioxin; TeCDF, tetrachlorodibenzofuran; TeCB, tetrachlorobenzene; TeCA, tetrachloroanisole; TeCP, tetrachlorophenol; TeCBA, tetrachlorobenzoic acid; TrCAP, trichloroacetophenone).

Preparation of sediment slurries, enrichment cultures and microcosms. Sediment slurries were prepared using strict anaerobic technique as described in Vargas et al. (2001) and Ahn et al. (2005). Triplicate bottles containing a 25% (v/v) sediment slurry were established under methanogenic or sulfate-reducing conditions in volumes of 100 mL in 160-mL serum bottles for the 1,2,3,4-TeCDD studies and under methanogenic conditions in volumes of 25 mL in 50-mL serum bottles for the 1,2,3,4-TeCDF studies. 1,2,3,4-TeCDD or 1,2,3,4-TeCDF was added to each serum bottle as a coating on dry, sterile sediment prior to the addition of the sediment slurry inoculum as previously described (Vargas et al. 2001). The resulting nominal concentration of 1,2,3,4-TeCDD was 31 μM based upon the final volume of 100 mL while that of 1,2,3,4-TeCDF was 49 μM based upon the final volume of 25 mL. Halogenated compounds

and other amendments were added from deoxygenated stock solutions as described in Ahn et al. (2005).

Experimental sets. The protocols for the experimental conditions to test stimulation of 1,2,3,4-TeCDD and 1,2,3,4-TeCDF dechlorination by different halogenated co-amendments is described in detail in Ahn et al. (2005). We determined whether 1,2,3,4-TeCDD dechlorination could be stimulated in methanogenic or sulfidogenic Paleta Creek sediment enrichments via co-amendment with 2-BP, a compound we had previously documented to be readily dehalogenated under sulfate reducing and methanogenic conditions in Paleta Creek sediments. No additional electron donor (i.e., lactate/propionate was added).

The effectiveness of the electron donors phenol and lactate/propionate alone, and the halogenated amendments BPs, CPs, TeCB, TeCA, TeCBA, TeCP, or TrCAP with an added electron donor, lactate/propionate, for stimulating 1,2,3,4-TeCDD dechlorination was also compared in Paleta Creek sediments with amendment intervals of either 3 or 6 months (Table 1) under methanogenic conditions as described in detail in Ahn et al. (2005). Finally, the 1,2,3,4-TeCDD dechlorinating activity was compared in sediments from Paleta Creek, Graving Dock, Shelter Island and Tuckerton with amendments of 25 µM TeCB plus 500 µM each lactate/propionate using a 3 month amendment interval.

Analytical methods. The sediment slurries were sampled for CDD/Fs at select time points. The bottles were shaken thoroughly and 2 mL of slurry was withdrawn with a sterile syringe flushed with oxygen-free N_2. Extraction with acetone/toluene and CDD/F analysis was performed as described (Vargas et al. 2001; Ahn et al. 2005). Briefly, samples were separated into aqueous and solid phases by centrifugation. Water was removed from the solid phase by an acetone rinse. The 2,2',5-PCB was added as an internal standard (3.9 µM based on original sample volume) and the solid phase was extracted with toluene:acetone (1:1 volume per volume (v/v)) overnight and then again for 4 hours. The toluene/acetone extracts were pooled with the aqueous phase. Acetone was removed by reverse partition into water and the toluene extract was gently concentrated. Sample clean-up to remove interfering organic compounds was performed in a 2 mL Pasteur pipette plugged with glass wool then filled two-thirds full with 60/100 mesh Florisil (Sigma-Aldrich, St. Louis, MO, USA). Samples were analyzed by gas chromatography mass spectrometry (GC-MS) on a Hewlett Packard 5890 gas chromatograph with a HP 5971 mass-selective detector, using a DB-5MS fused silica column (30 m, 0.25 mm i.d., film thickness 0.2 µm, J & W Scientific, Folsom, CA, USA). The detector was run in selected ion monitoring mode for m/z: 322 (TeCDD); 286 (TrCDD); 252 (DCDD); 218 (MCDD); 184 (dibenzo-*p*-dioxin) and 186 (2,2',5-PCB). TeCB and TeCA and their dehalogenation products were analyzed by GC-MS following the same extraction procedure as for CDD/Fs but without Florisil column cleanup to minimize loss from volatilization. Dechlorination products were identified by comparison of retention times and mass spectra to known standards. The 1,2,3,4-TeCDF dechlorination products were identified by the number of chlorine substituents but not chlorine position, since not all potential dechlorination products were available commercially. Dechlorination products were detected based upon selective ion monitoring of expected major ions for tetra-, tri-, di-, or monochlorinated congeners, m/z: 304 (TeCDF); 270 (TrCDF); 236 (DCDF) and 202 (MCDF).

Table 1. Enrichments established for the comparison of alternate halogenated compounds to stimulate 1,2,3,4-tetrachlorodibenzo-*p*-dioxin (1,2,3,4-TeCDD) dechlorination in Paleta Creek sediments.

Sample Set	[a]Bottle Set	[b]TEAP	[c]Spike Interval (Months)	[d]Halogenated Additive(s) (μM)	Electron Donor (μM)
Sulfate effect	Live	M, S	na	None	None
	2-BP	M, S	3	2-BP (400)	None
[e]Compare mono-halogenated to tetra-halogenated	Killed	M	na	None	None
	Live	M	na	None	None
	Phenol	M	6	None	Phenol (500)
	Lac/Pro	M	6	None	Lac/Pro (500)
	CP	M	6	2-BP, 3-BP, 4-BP (200 each)	Lac/Pro (500)
	BP	M	6	2-CP, 3-CP, 4-CP (200 each)	Lac/Pro (500)
	TeCB	M	6	1,2,3,4-TeCB (25)	Lac/Pro (500)
	TeCA	M	6	2,3,4,5-TeCA (25)	Lac/Pro (500)
[f]Compare tetra-halogenated	Killed	M	na	None	None
	Live	M	na	None	None
	TeCB	M	3	1,2,3,4-TeCB (25)	Lac/Pro (500)
	TeCA	M	3	2,3,4,5-TeCA (25)	Lac/Pro (500)
	TeCP	M	3	2,3,4,5-TeCP (25)	Lac/Pro (500)
	TeCBA	M	3	2,3,5,6-TeCBA (25)	Lac/Pro (500)
	TrCAP	M	3	2',3',4'-TrCAP (25)	Lac/Pro (500)
	Lac/Pro	M	3	None	Lac/Pro (500)

[a] Live, live control; Killed, autoclaved control; Lac/Pro, lactate/propionate; M, methanogenic; S, sulfidogenic; BP, bromophenol; CP, chlorophenol; TeCB; tetrachlorobenzene; TeCA, tetrachloroanisole; TeCP, tetrachlorophenol; TeCBA, tetrachlorobenzoic acid; TrCAP, trichloroacetophenone. [b] TEAP, terminal electron-accepting process. [c] na, not applicable. [d] all sediment slurries were spiked with 31 μM 1,2,3,4-TeCDD. [e] using sediment from sampling in March 2001. [f] using sediment from sampling in September 2001.

Quantification was based on comparison to standard curves comprised of five concentration levels. For unknown 1,2,3,4-TeCDF dechlorination daughter products, quantification was performed against available tri-, di-, and mono-chlorinated dibenzofurans. The results for CDD/Fs are presented by expressing each compound as a mole percent (mol %) of the total concentration of the congers detected at each sampling point. Data are presented as averages of triplicate data points plus/minus one standard deviation. Total CDD/F recovered from each sample varied because of slight differences of aqueous phase/sediment phase volumes sampled at each time point and because of differences in the solubilities of dechlorination products. The extraction efficiency for 1,2,3,4-TeCDD spiked to sediment slurries by the method described was found to be approximately 85% (Vargas et al. 2001) and the detection limit was approximately 0.01 μM (Fennell et al. 2004b). The method of data presentation assumes that no anaerobic degradation of the dibenzo-*p*-dioxin or dibenzofuran structure occurred and that the CDD/Fs underwent no significant reactions other than dechlorination. For purposes of comparison of different treatments, the half-life of 1,2,3,4-TeCDD/F was determined. Time progression data from triplicate sediment slurry treatments were fit to first-order reaction kinetic expressions using a non-linear regression method. First-order rate constants were obtained, from which half-lives (months) and 95% confidence intervals were calculated.

Halogenated phenols and benzoates, phenol, volatile fatty acids, and organic acids were analyzed in aqueous samples by HPLC (LC-10AS, Shimadzu, Kyoto, Japan) with either a C-18 Spheroclone column (Supleco, Bellefonte, PA, USA) with UV detection at 240 nm (aromatics) (Häggblom et al. 1993; Monserrate and Häggblom 1997; Ahn et al. 2003) or an Aminex HPX-87H (300 X 7.8 mm) column (Bio-Rad, Hercules, CA, USA) with UV detection at 210 nm (fatty and organic acids), as described previously (Fennell et al. 1977). The TeCP, TeCBA, and TrCAP were also analyzed by HPLC using the C-18 column. A mobile phase of methanol:water:acetic acid (80:18:2 v/v/v for TeCP and 60:38:2 v/v/v for TeCBA and TrCAP) at a flow rate of 1 mL/min was used.

Sulfate was determined by ion chromatography (Dionex model 120, Sunnyvale, CA, USA) with an IonPac AS 14 column (4 X 250 mm) and conductivity detection. The eluant was 3.5 mM Na_2CO_3 /1 mM $NaHCO_3$ at 1.5 mL/min.

2. Reductive Dechlorination of Diverse Chlorinated Aromatic Pollutants by *Dehalococcoides ethenogenes* strain 195

Strain cultivation. A mixed culture containing *D. ethenogenes* strain 195 was grown at 34°C on PCE and butyric acid as described in Fennell et al. (2004b). The mixed culture contained approximately 16 μg *D. ethenogenes* protein/mL as determined as described in Fennell et al. (2004b) through stoichiometric estimation based upon growth yield values (Maymó-Gatell et al. 1997), a solids retention time of 40 days and an influent PCE concentration of 1100 μM. Pure culture *D. ethenogenes* strain 195 was grown at 34°C as described (Fennell et al. 2004b). The culture was used after dechlorination of approximately 500 μmol/L PCE, which corresponds to approximately 2-4 $x10^8$ cells per mL or 5-10 μg protein per mL as estimated by use of the growth curve of Maymó-Gatell et al. (1997).

Dehalogenation tests. The mixed culture was used to assess the best mode of delivery of 1,2,3,4-TeCDD, 2,3,7,8-TeCDD, 2,3-DCDD, 1,2,3,4-TeCDF, 2,3,4,5,6-PeCB, 1,2,3,4-TeCN to the cultures. We added 0.78 µmol of 1,2,3,4-TeCDD to triplicate bottles of mixed culture via a coating on dry, sterile sediment (Vargas et al. 2001) or by coating on 1.5 mm borosilicate glass beads or the sides and bottom of the serum bottle itself. No other amendment techniques were attempted. The culture tolerated the CDD/F-coated sediment and this mode of delivery yielded the highest dehalogenation activity compared to the other addition methods. In a separate experiment, addition of dry sterile sediment did not interfere with PCE dechlorination by the pure culture. Therefore the pure culture was amended with the halogenated compounds via the sediment method. Briefly, dry, sterile sediment, 0.25 g, was added to a 50 mL serum vial. The vials were sealed with a Teflon-coated gray butyl rubber stopper, crimped with an aluminum crimp cap and autoclaved. Stock solution containing each respective substrate was added to triplicate bottles via a sterile glass syringe (Table 2) as described in detail in Fennell et al. (2004b). The stock solution was allowed to coat the sediment. The solvent was evaporated overnight under sterile, anoxic nitrogen. After the sediment was dry, the bottle was purged an additional 30 minutes with 70% nitrogen/ 30% carbon dioxide. Chlorophenols were added from 0.1 N NaOH solutions via a sterile anoxic syringe. Killed controls amended with 1,2,3,4-TeCDD and PCE were prepared by autoclaving for 30 minutes.

Table 2. Substrates tested for dehalogenation by *Dehalococcoides ethenogenes* strain 195.

Substrate	MW (g/mol)	Stock Solvent	mg/bottle	µmol/bottle	µmol/L culture†	µg/g sediment‡
PCE	165.8	Neat (toluene)*	0.5 to 1.5	2.8 to 8.8	110 to 350	na
Chlorobenzenes	varied	Pentane/ hexadecane	varied by congener	20	2000	na
1,2,3,4-TeCDD	322	toluene	0.25	0.78	31	1000
2,3,7,8-TeCDD	322	toluene	0.01	0.03	1.2	40
1,2,3,4-TeCDF	305.98	toluene	0.1	0.33	13	400
2,3,4,5,6-PeCB	326.4	toluene	0.25	0.77	31	1000
1,2,3,4 TeCN	265.95	toluene	0.2	0.75	30	800
2,3-DCDD	252	isooctane	0.008	0.03	1.2	30
2-, 3-, 4- Chlorophenol	128.56	0.1 N NaOH	0.64 (each)	5 (each)	200 (each)	na

na=not applicable
* toluene was added to dried sediments then evaporated (as for other bottles sets) during the set up of PCE-only controls.
† nominal aqueous-phase concentration neglecting partitioning
‡ assuming 100 % partitioning to the sediment

To test for dechlorination of 1,2,3,4-TeCDD and to compare modes of CDD/F delivery, 25 mL mixed culture was added to the serum bottles using a sterile glass syringe. Butyric acid (440 µM) and pre-fermented yeast extract (4 µL of a 50 g/L solution) were added as electron donor and nutrient source, respectively (Fennell et al. 1997; Fennell 1998). PCE (110 µM) was added at time zero to ensure a successful establishment of the cultures. Butyric acid and pre-

fermented yeast extract were added at time zero and on day 8, 26, 120 and 178. Mixed cultures were agitated inverted at 200 rpm at 34 °C.

Dechlorination of octachlorodibenzo-*p*-dioxin (OCDD) and 1,2,3,4,7,8-hexachlorodibenzofuran (1,2,3,4,7,8-HxCDF) was also examined in the mixed culture containing *D. ethenogenes* strain 195. An individual PCDD/F congener served as sole electron acceptor in one triplicate bottle set while tetrachloroethene (PCE) or 1,2,3,4-tetrachlorobenzene (TeCB) were added as growth co-substrates along with the PCDD/F congener in another two sets of triplicate bottles as described above. The OCDD and 1,2,3,4,7,8-HxCDF were added at 5 µM, PCE and TeCB were added at 25 µM, and butyrate was added periodically at 0.1 mM as a hydrogen source.

D. ethenogenes pure culture was used to test the dehalogenation of 1,2,3,4-TeCDD, 2,3,7,8-TeCDD, 2,3-DCDD, 1,2,3,4-TeCDF, 2,3,4,5,6-PeCB, 1,2,3,4-TeCN and the chlorophenols as described in Fennell et al. (2004b). PCE (350 µM) was added initially and the bottles were pressurized to 5 PSI with pure hydrogen gas. The pure cultures were incubated inverted at 28 °C at 100 rpm. PCE dechlorination in the pure culture exhibited a lag period of approximately two months. This lag may have been caused by residual toluene (40 ± 40 µM) from the delivery of the stock solutions. On day 21, bottle headspaces were purged with 70% nitrogen/30% carbon dioxide for 0.5 hr, reducing the toluene residual by approximately half (19 ± 19 µM) with the highest concentration at 60 µM. After purging, PCE was dehalogenated to a mixture of VC and ethene within five weeks. PCE (175 µM) was added again on day 164 and day 185 and was dechlorinated to VC and ethene within two weeks.

Analytical methods. Chloroethenes and toluene were determined from headspace samples analyzed by gas chromatography with flame ionization detection (GC-FID) (Fennell et al. 2004b). PCDD/Fs and PCBs were analyzed by gas chromatography-mass spectrometry as previously described (Alder et al. 1993; Vargas et al. 2001). PCNs were analyzed using the PCB method. Briefly, the bottles were shaken thoroughly and 1 mL of culture medium-sediment slurry was withdrawn with a sterile syringe flushed with oxygen-free nitrogen. Samples were separated into an aqueous and solid phase by centrifugation. Water was removed from the solid phase by an acetone rinse. 2,2',5-Trichlorobiphenyl was added as an internal standard for PCDD/F analyses and octachloronaphthalene (OCN) was added as an internal standard for PCB and PCN analyses. For PCDD/F analyses, the solid phase was extracted with toluene:acetone (1:1 v/v) overnight and then again for 4 hours. The toluene/acetone extracts were pooled with the aqueous phase. For PCB and PCN analyses, the solid phase was extracted with hexane:acetone (1:1 v/v) overnight and then again for 4 hours. The hexane/acetone extracts were pooled with the aqueous phase. Acetone was removed by reverse partition into water and the toluene or hexane extract was concentrated. Sample clean-up to remove interfering organic compounds was performed using a Florisil column. Samples were analyzed by gas chromatography mass spectrometry (GC-MS) as described in Fennell et al. (2004b). PCBs and PCDDs were identified based on retention times of standards and selective ion monitoring (m/z: TeCDD 322, TrCDD 286, DCDD 252, MCDD 218, PeCB 326, tetrachlorobiphenyl 292, trichlorobiphenyl 256, dichlorobiphenyl 222, OCN 404). We were unable to resolve the 2,4,5,6- and 2,3,5,6-tetrachlorobiphenyl (2,3,4,6-TeCB/2,3,5,6-TeCB) isomers by our method (data not shown). The

extraction efficiency for 1,2,3,4-TeCDD (at 2 μM) was above 85% (30). The detection limit for the different compounds was approximately 0.01 μmol/L.

The 1,2,3,4-TeCDF and 1,2,3,4-TeCN dechlorination products were identified by the number of chlorine substituents, not chlorine position, since not all potential dechlorination products were available commercially. Dechlorination products were detected based upon selective ion monitoring of expected major ions for tetra-, tri- or dichlorinated congeners (m/z: TeCDF 304, TrCDF 270, DCDF 236, OCN 404, TeCN 266, trichloronaphthalene 231, dichloronaphthalene 196).

Results for the chlorinated aromatic compounds are presented by expressing each compound as a mole fraction of the total concentration of the congers detected at each sampling point. Total PCDD/F, PCB, or PCN recovered at each sampling event varied because of the difficulty in sampling the aqueous/sediment slurry in a representative manner and because of the differing aqueous solubilities of the parent and dechlorination daughter compounds. The method of data presentation assumes no anaerobic degradation of the dibenzo-*p*-dioxin, dibenzofuran, biphenyl, or naphthalene molecule (if produced) and that the chlorinated compounds underwent no significant reactions other than dechlorination.

3. Development of Methods for Detection and Identification of Dehalogenating Bacteria

Source of bromophenol dehalogenating culture. A sediment enrichment from the Arthur Kill, an intertidal strait on the New York-New Jersey border, was used as the starting culture for this study (Knight et al. 1999, Fennell et al. 2004a). The enrichment was maintained in a minimal salts medium and enrichment of dehalogenating bacteria performed with lactate as carbon source and the halogenated compound as electron acceptor as described (Fennell et al. 2004a). Methods for chemical analysis are described in detail in (Fennell et al. 2004a). The complementary molecular approaches are outlined in Fig 3.

Dehalogenation experiments. Time course studies with starved enrichments and specific amendments were performed to identify the dehalogenating organism. An enrichment was fed successive doses of 600 μM 2BP, transferred, and subcultured over the course of 1 year to obtain a final working volume of 2 liters. Sequential transfers of the culture into fresh medium diluted the culture to 10^{-4} of the original enrichment. The culture was incubated without feeding for approximately 3 months prior to initiation of the experiments to minimize the background ribosome activity. Two liters of the starved culture was centrifuged at 16,000 x g for 10 min, and the cell pellet was washed twice in 2 liters of sulfate-free medium. The final washed cell pellet was resuspended in 2 liters of sulfate-free medium. All culture manipulations were done in an anaerobic glove box (Coy Laboratory Products, Inc.) with an atmosphere containing 3% H_2 and 97% N_2. Headspaces were subsequently purged with 30% CO_2-70% N_2 to remove the hydrogen. The culture was divided into 100-ml subcultures in 160-ml serum bottles. The following conditions were set up in triplicate: autoclaved control; 2BP (450 μM) plus SO_4^{2-} (20 mM); 2BP (450 μM) plus H_2 (3% [vol/vol] in the headspace or about 120 μmol/bottle); phenol (450 μM) plus SO_4^{2-} 20 mM); and phenol (450 μM). The subcultures were amended with the substrates and

were monitored for substrate disappearance. Samples were removed periodically for nucleic acid extraction analysis after activity was apparent.

Isolation and characterization of dehalogenating bacteria. In an attempt to isolate dehalogenating bacteria, the original sulfidogenic enrichment was washed with and 1% (vol/vol) of the suspension was transferred and spiked with 2BP. After three transfers (total dilution, 10^{-6}), a portion of the culture was serially diluted in agar shake culture tubes and incubated in a dark room at 28°C. After 4 weeks, colonies were picked and transferred to liquid medium and then cultivated to check for dehalogenating activity. We retrieved 10 cultures having 2BP dehalogenation activity. One of these cultures was selected for further study, and colonies were reisolated in agar shake dilution cultures. Characterization of the dehalogenating bacterium included determination of the range of electron acceptors (including halogenated compounds), electron donors and microscopic observation as described in detail (Fennell et al. 2004a). TRFLP analysis and microscopy were used to check the makeup of the purified culture. The peak area in the TRFLP electropherogram was used for relative quantification of the members of the coculture. To use this PCR-TRFLP method for quantification, we needed to verify whether the TRFLP peak area could be used to estimate microbial population size. Details for nucleic acid extraction, purification and analysis are described in Fennell et al. (2004a). All sequences obtained were compared with entries in the GenBank database by using Blast.

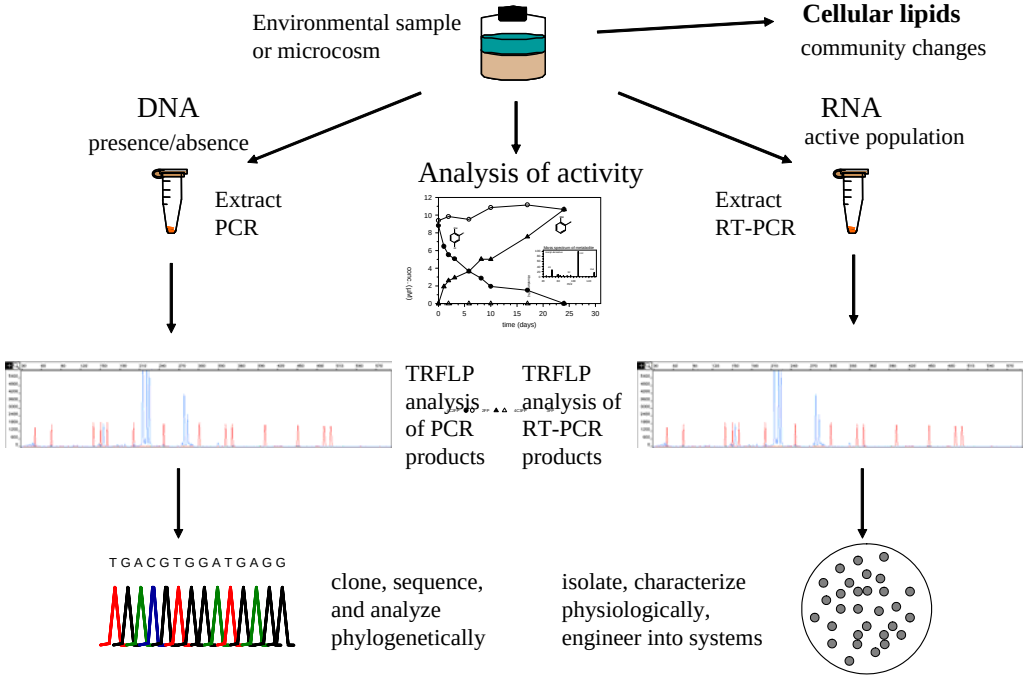

Fig. 3. Complementary approaches for microbial community analysis.

4. Modification of a Dechlorination Model to Simulate PCDD Dechlorination

A model developed to simulate dechlorination in the presence of competing terminal electron-accepting processes (Fennell and Gossett 1998) was modified to include biokinetic expressions for PCDD dechlorination, bromophenol debromination, and hydrogen production from phenol degradation. Preliminary simulations were made for the Paleta Creek sulfate reducing and methanogenic enrichments either with (+2BP) or without (-2BP) the addition of 2-bromophenol (2BP) (see Section 1).

The following simplifying assumptions were made for the model simulations: dehalogenating microorganisms use hydrogen as an electron donor and have similar kinetic properties (high affinity for hydrogen and halogenated compounds) as those reported for known strains, e.g., *Dehalococcoides*; controlling PCDD concentrations are the aqueous solubilities of the congener of interest; lag in dehalogenation reflects growth of a small population of dehalogenating microorganism, not inhibition or acclimation; and all spiked PCDD is bioavailable.

5. Effect of System Heterogeneity on Dechlorination of PCDD

Typical approaches to studying dehalogenation in sediments or cultures spiked with specific PCDD congeners (e.g., 1,2,3,4-TeCDD) include using dry, sterile soil as a carrier (as we have done). Dry sterile sediment is added to a bottle, PCDD dissolved in a solvent is delivered to the bottle to saturate the sediment and then, the solvent is allowed to evaporate, leaving a layer of PCDD on the sediment. Growth media and enrichment culture or fresh, live sediment is then added to the bottle. A similar approach involves delivering PCDD on to the sides and bottom of a glass serum bottle prior to addition of bacteria. Other modes of PCDD addition were examined to determine their usefulness in stimulating faster dechlorination. We tested the biocarrier hydroxypropyl- γ-cyclodextrin (HPγCD) and the non-aqueous phase liquid, heptamethylnonane (HMN).

Two percent transfers of the Arthur Kill culture were established in duplicate 50 mL-aliquots in 160-mL serum bottles (using supernatant from the original, diluted enrichment). The transfers were amended with 10 ppm (nominal concentration) 1,2,3,4-TeCDD using one of the following methods: (1) as a coating on sterile sediment (as described above); (2) predissolved in 1 mL of HMN; (3) predissolved in 1 mL of HMN with HPγCD added to the growth medium; and (4) as a coating on the sides and bottom of the glass serum bottle with HPγCD added to the growth medium. Hydrogen plus acetate was added as the electron donor and the bottles were agitated gently at 28 °C.

Results and Accomplishments:

1. 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

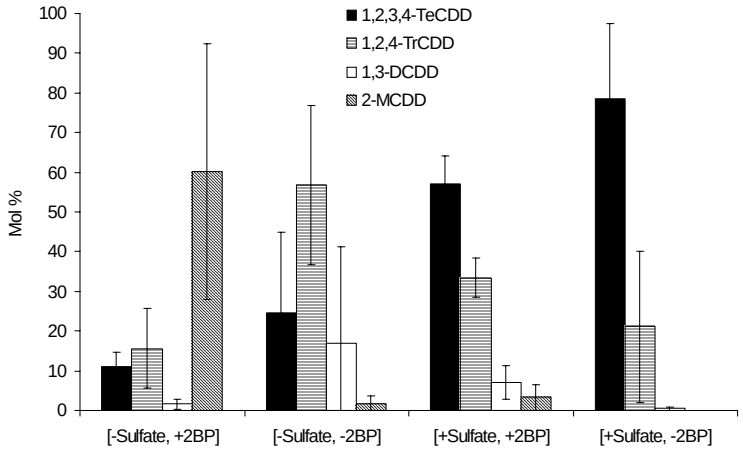

Fig. 4. Mol percent of 1,2,3,4-tetrachlorodibenzo-*p*-dioxin (1,2,3,4-TeCDD) and its dechlorination daughter products in Paleta Creek sediment slurries under methanogenic [- Sulfate] and sulfidogenic [+ Sulfate] conditions after 688 days with [+ 2BP] and without [- 2BP] the addition of 2-bromophenol (2BP). Mol percent is the ratio of moles of a specific chlorinated dibenzo-p-dioxin congener to the total moles of all chlorinated dibenzo-*p*-dioxin congeners detected in the sample. Error bars represent the standard deviation of samples from triplicate bottles.
From Ahn et al. (2005).

Effect of 2-Bromophenol addition on 1,2,3,4-TeCDD dechlorination under methanogenic and sulfate-reducing conditions. 2-Bromophenol (BP) was readily degraded under a variety of redox conditions in Paleta Creek sediments (data not shown). We therefore chose 2-BP as a halogenated additive that would be useful for comparing 1,2,3,4-TeCDD dechlorination under different terminal electron accepting conditions (Ahn et al. 2005). After 688 days (23 months), 1,2,3,4-TeCDD was dechlorinated to a mixture of 1,2,4-TrCDD, 1,3-DCDD, and 2-MCDD (Figs. 4 & 5). 1,2,4-TrCDD was first detected in both methanogenic culture sets after 194 days and in 2-BP-amended sulfate-reducing cultures after 320 days (Fig. 5). 2-BP was depleted within three months under methanogenic and sulfate-reducing conditions during repeated re-amendment and without accumulation of phenol (data not shown). The most extensive dechlorination of 1,2,3,4-TeCDD occurred under methanogenic conditions when 2-BP was added, and 2-MCDD made up 60 mol % of the CDDs detected (Fig. 4). Methanogenic slurries without 2-BP exhibited less extensive dechlorination with accumulation of a mixture of mainly 1,2,4-TrCDD and 1,3-DCDD. Sulfate-reducing sediment slurries exhibited dechlorination of 1,2,3,4-TeCDD to 1,2,4-TrCDD both with and without 2-BP co-amendment, however the onset of dechlorination was about 100 days later in the sulfate-reducing slurries not receiving 2-BP (Fig. 5). In sulfate-reducing slurries co-amended with 2-BP, 1,3-DCDD (7 mol %) and 2-MCDD (3 mol %) were detected by the end of the incubation period.

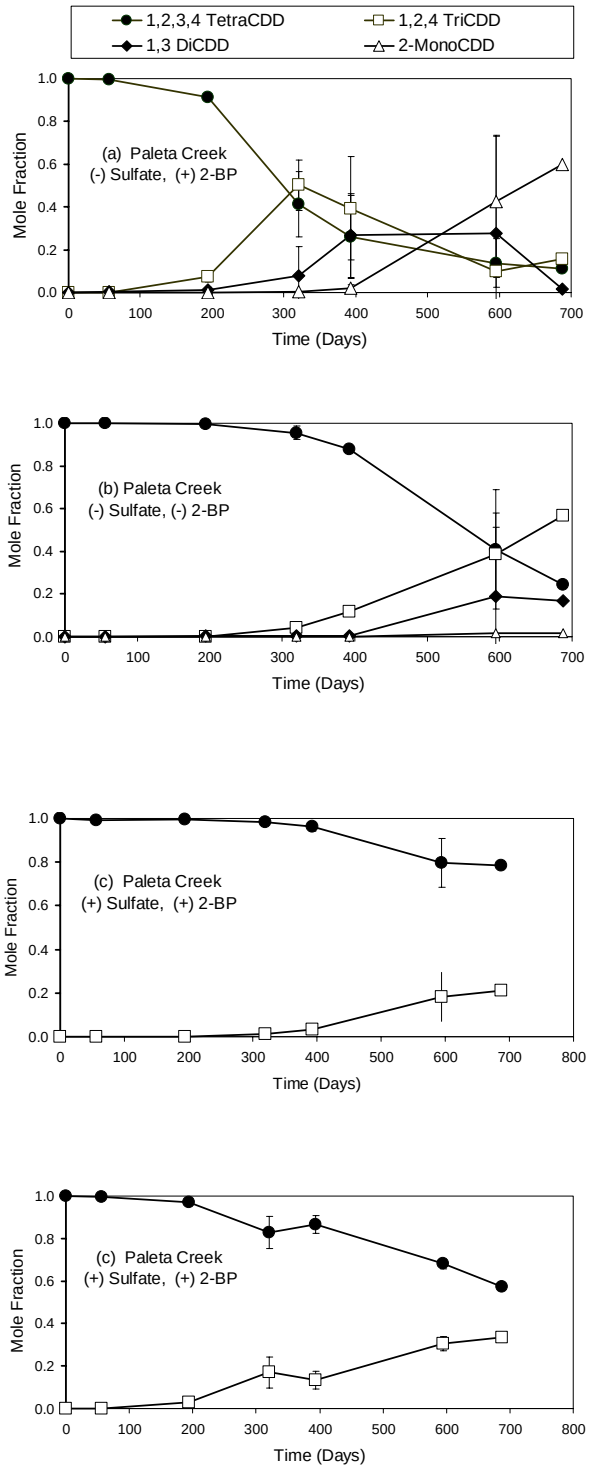

Fig. 5. Time course of 1,2,3,4-tetrachlorodibenzo-*p*-dioxin (1,2,3,4-TeCDD) dechlorination in Paleta Creek sediment slurries under methanogenic (- Sulfate) and sulfidogenic (+ Sulfate) conditions with (+ 2BP) and without (- 2BP) the addition of 2-bromophenol (2BP).

Stimulation of 1,2,3,4-TeCDD dechlorination by halogenated co-amendments under methanogenic conditions in Paleta Creek sediments. Phenol, a lactate/propionate mixture, BPs (plus lactate/propionate), CPs (plus lactate/propionate), TeCB (plus lactate/propionate) and TeCA (plus lactate/propionate) were compared as amendments to stimulate 1,2,3,4-TeCDD dechlorination in Paleta Creek sediments under methanogenic conditions. Fig. 6A shows a comparison of 1,2,3,4-TeCDD dechlorination in all culture sets after 15 months of incubation using an amendment interval of 6 months. The time progression data for two example treatments, BPs (plus lactate/propionate) and TeCB (plus lactate and propionate) are shown in Fig. 6B and 6C, respectively.

No significant dechlorination of 1,2,3,4-TeCDD was observed in either the killed or live controls to which no amendments were added (Fig. 6A). 1,2,3,4-TeCDD dechlorination in sediment slurries receiving CPs (plus lactate/propionate); or with lactate/propionate alone were similar in magnitude after 15 months (9 -14 mol % reduction in 1,2,3,4-TeCDD) (Fig. 5A). BPs (plus lactate/propionate) and phenol alone showed slightly more activity than CPs (plus lactate/propionate). In the cultures amended with phenol 18.5 ± 5.0 mol % of 1,2,4-TrCDD was detected (Fig. 6A). In the slurries amended with a mixture of BPs 19.7 ± 3.7 mol % of 1,2,4-TrCDD was eventually produced (Fig. 6A and 6B) and the half-life of 1,2,3,4-TeCDD was 45 months (95% confidence interval, 36 to 61 months). In TeCB- and TeCA-amended slurries, the half-life of 1,2,3,4-TeCDD was 5.8 months (95% confidence interval, 4.3 to 8.5 months) and 7 months (95% confidence interval, 6.2 to 8 months), respectively. TeCB or TeCA plus lactate/propionate as electron donors stimulated approximately six-fold more 1,2,3,4-TeCDD dechlorination than any of the other treatments. The time progression data for all treatments is shown in Fig. 7.

The first metabolite of 1,2,3,4-TeCDD dechlorination in TeCB- and TeCA-amended cultures, initially detected at 3 months, was identified as 1,2,4-TrCDD, while the second metabolite, detected after 6 months was 1,3-DCDD. No other CDD isomers were detected in appreciable amounts. After 15 months in 1,2,3,4-TeCB amended sediments, 37.8 ± 1.9 mol % of 1,2,4-TrCDD, 8.4 ± 4.9 mol % of 1,3-DCDD, and 8.4 ± 1.1 mol % of 2-MCDD were observed (Fig. 6A and 6C). After 15 months in 1,2,3,4-TeCA amended sediments, 35.6 ± 15.7 mol % of 1,2,4-TrCDD, 18.8 ± 7.3 mol % of 1,3-DCDD, and 2.4 ± 2.7 mol % of 2-MCDD were found (Fig. 6A). 1,2,3,4-TeCDD was thus reductively dechlorinated in the lateral position to 1,2,4-TrCDD, followed by sequential peri-dechlorinations to 1,3-DCDD and 2-MCDD.

The BPs and CPs were dehalogenated and phenol was degraded within 30 days (data not shown). Formation of 1,3-dichlorobenzene (1,3-DCB) from the reductive dechlorination of 1,2,3,4-TeCB was observed within 1 month and by 3 months TeCB had been dehalogenated to a mixture of 1,3-DCB and 1,2-dichlorobenzene (1,2-DCB). No further dechlorination of the 1,2-DCB and 1,3-DCB was observed (data not shown).

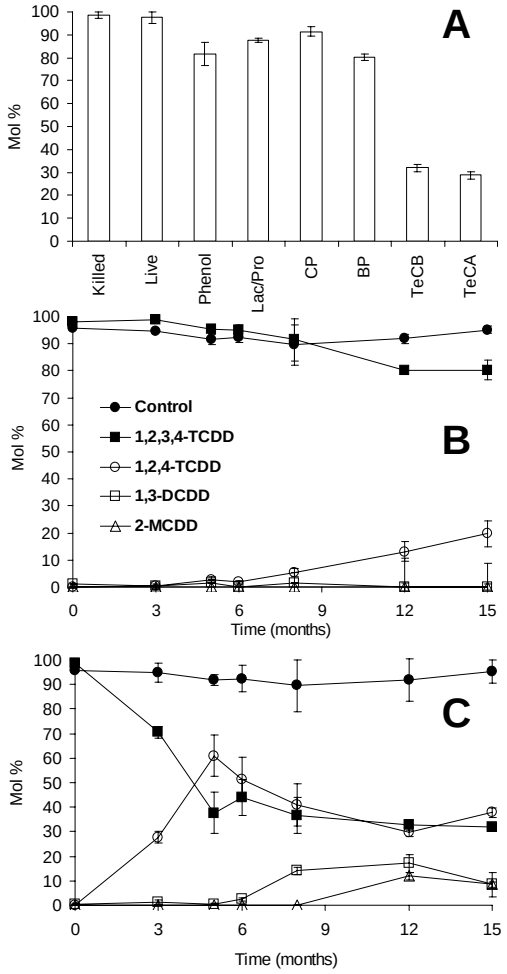

Fig. 6. 1,2,3,4-Tetrachlorodibenzo-*p*-dioxin (1,2,3,4-TeCDD) dechlorination in Paleta Creek sediment slurries under different electron donor (Lac/Pro, lactate/propionate) and halogenated co-amendment (BP, bromophenol; CP, chlorophenol; TeCB, tetrachlorobenzene; TeCA, tetrachloroanisole) conditions after 15 months with a 6-month re-amendment interval.
(A) Mol percent of 1,2,3,4-TeCDD remaining after 15 months: Killed, autoclaved at 121°C for 30 min on three consecutive days; Live, no haloprimer and no electron donor; Phenol, 500 μM phenol; Lac/Pro, no haloprimer and 500 μM each lactate/propionate; CP, 200 μM each 2-, 3-, 4-CP plus 500 μM each lactate/propionate; BP, 200 μM each 2-, 3-, 4-BP plus 500 μM each lactate/propionate; TeCB, 25 μM TeCB plus 500 μM each lactate/propionate; TeCA, 25 μM TeCA plus 500 μM each lactate/propionate. Mol percent is the ratio of moles of a specific chlorinated dibenzo-*p*-dioxin congener to the total moles of all chlorinated dibenzo-*p*-dioxin congeners detected in the sample. Error bars represent the standard deviation of samples from triplicate cultures.
(B) Time progression of 1,2,3,4-TeCDD dechlorination in BP-amended slurries, 200 μM each 2-, 3-, 4-BP plus 500 μM each lactate/propionate. Symbols represent averages of triplicate cultures and error bars represent the standard deviation.
(C) Time progression of 1,2,3,4-TeCDD dechlorination in TeCB-amended slurries, 25 μM TeCB plus 500 μM each lactate/propionate. Symbols represent averages of triplicate cultures and error bars represent the standard deviation.
From Ahn et al. (2005).

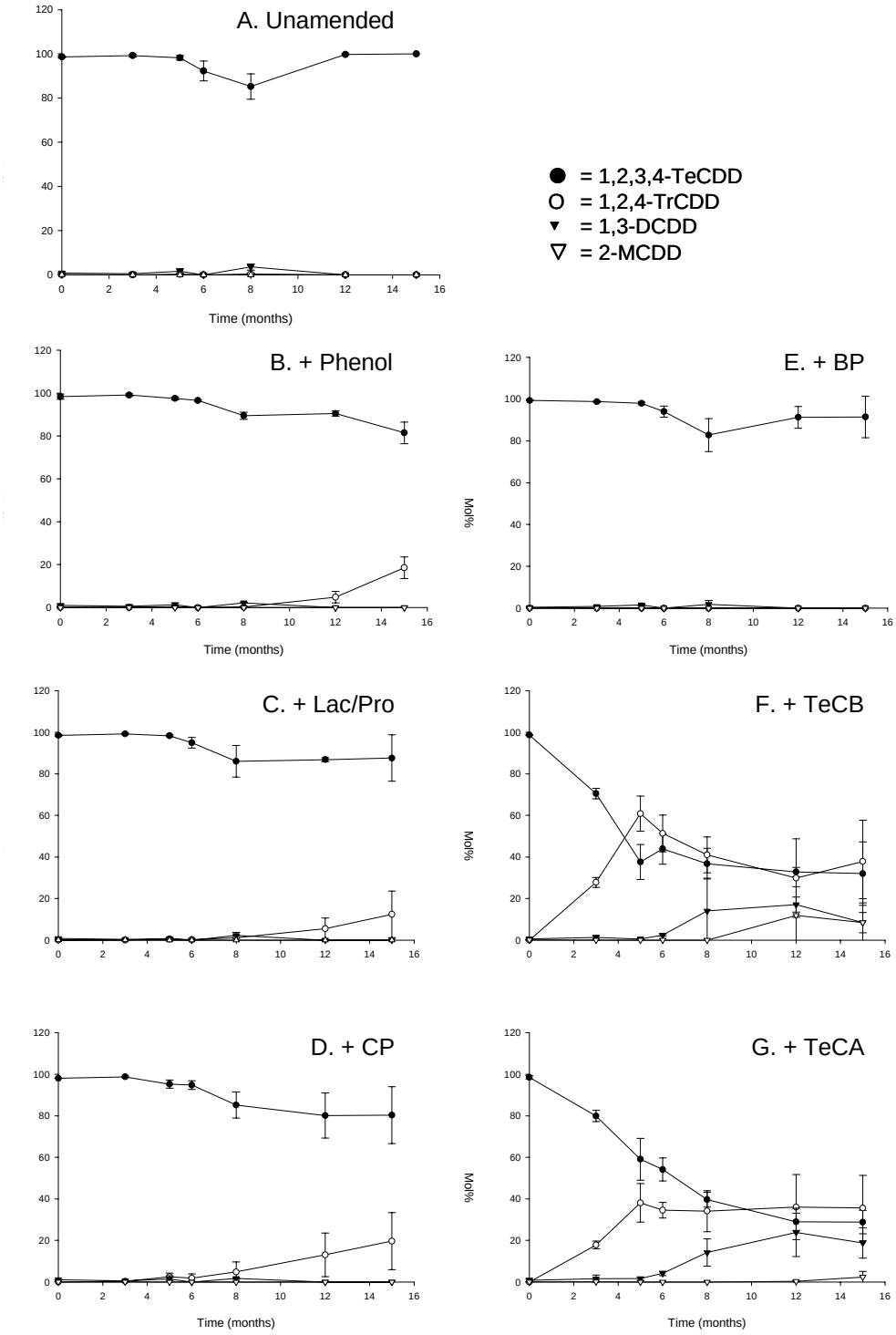

Fig. 7. Time progression of 1,2,3,4-tetrachlorodibenzo-*p*-dioxin (1,2,3,4-TeCDD) dechlorination in Paleta Creek sediment slurries under different electron donor (Lac/Pro, lactate/propionate) and halogenated co-amendment (BP, bromophenol; CP, chlorophenol; TeCB, tetrachlorobenzene; TeCA, tetrachloroanisole) conditions with a 6-month re-amendment interval.

After determining the greater effectiveness of TeCB and TeCA as stimulatory co-amendments over the BPs and CPs, we expanded the study to other structurally similar compounds. Dechlorination of 1,2,3,4-TeCDD was examined in Paleta Creek sediment slurries amended with TeCB, TeCA, TeCP, TeCBA, or TrCAP, plus lactate/propionate as electron donors. Fig. 8A shows a comparison of 1,2,3,4-TeCDD dechlorination in all bottle sets after 9 months of incubation using an amendment interval of 3 months. The time progression data for two example treatments, TeCB (plus lactate and propionate) and TeCBA (plus lactate and propionate) are shown in Fig. 8B and 8C, respectively. The time progression data for all treatments is shown in Fig. 9.

Of the five amendments tested, all but TeCBA significantly enhanced the overall extent of 1,2,3,4-TeCDD dechlorination in Paleta Creek sediment cultures after 9 months (Fig. 8A). The half-lives of 1,2,3,4-TeCDD in the TeCB-, TeCA-, TeCP-, and TrCAP-amended sediments were: TeCB, 4.3 months (95% confidence interval, 2.7 to 10.3 months); TeCA, 3.9 months (95% confidence interval, 3.3 to 4.8 months); TeCP, 4.8 months (95% confidence interval, 3.9 to 6.3 months); and TrCAP, 5.3 months (95% confidence interval, 3.8 to 8.5 months). In contrast, in the TeCBA- and lactate/propionate-amended sediments, the 1,2,3,4-TeCDD half-lives were: TeCBA, 17.6 months (95% confidence interval, 10.8 to 46 months); and lactate/propionate, 12.8 months (95% confidence interval, 7.1 to 64 months).

A total transformation of 63 to 73 mol % of the added 1,2,3,4-TeCDD occurred in the TeCB-, TeCA-, TeCP-, and TrCAP-amended sediment slurries over the 9-month incubation period . In the TeCB-amended sediments, 1,2,3,4-TeCDD was dechlorinated to yield 0.9 ± 1.2 mol % of 1,2,4-TrCDD, 6.6 ± 1.8 mol % of 1,3-DCDD, and 46.5 ± 0.9 mol % of 2-MCDD (Fig. 8B). In TrCAP-amended sediments, 8.3 ± 1.9 mol % of 1,2,4-TrCDD, 1.4 ± 2.5 mol % of 1,3-DCDD, and 26.4 ± 9.3 mol % of 2-MCDD were observed. In TeCA-amended sediments, 1.7 ± 1.5 mol % of 1,2,4-TrCDD, 21.7 ± 23.4 mol % of 1,3-DCDD and 17.0 ± 14.6 mol % of 2-MCDD were detected. In TeCP-amended sediments, 3.2 ± 3.3 mol % of 1,2,4-TrCDD and 61.2 ± 4.5 mol % of 1,3-DCDD were detected. In contrast, in TeCBA-amended bottles, transformation of only 23 mol % of the added 1,2,3,4-TeCDD was observed with 20.0 ± 1.3 mol % of 1,2,4-TrCDD detected (Fig. 8C). The lactate/propionate electron donor control showed slightly less activity than the non-amended live control. Only trace amounts of dechlorination products were detected in the killed control. Lactate, propionate and methanol were depleted and methane was produced in all live treatments. We did not detect TeCA, TeCP, TeCBA, and TrCAP after 90 days of incubation.

1,2,3,4-TeCDD dechlorination in different sediments. TeCB with lactate/propionic acid as electron donors stimulated 1,2,3,4-TeCDD dechlorination in slurries made from Paleta Creek, Graving Dock, Shelter Island, and Tuckerton sediments (Fig. 10). Dechlorination of 1,2,3,4-TeCDD proceeded to a much greater extent in the more contaminated Graving Dock (1,2,3,4-TeCDD half-life, 3.5 months, 95% confidence interval 2.8 to 4.6 months) and Paleta Creek (1,2,3,4-TeCDD half-life, 3.7 months, 95% confidence interval 2.1 to 14 months) sediments than in the less contaminated Shelter Island (1,2,3,4-TeCDD half-life greater than 138 months) and pristine Tuckerton (1,2,3,4-TeCDD half-life, 6 months, 95% confidence interval 4.1 to 10.9 months) sediments. After 6 months, in Graving Dock and Paleta Creek sediments, 1.6 ± 0.3 and

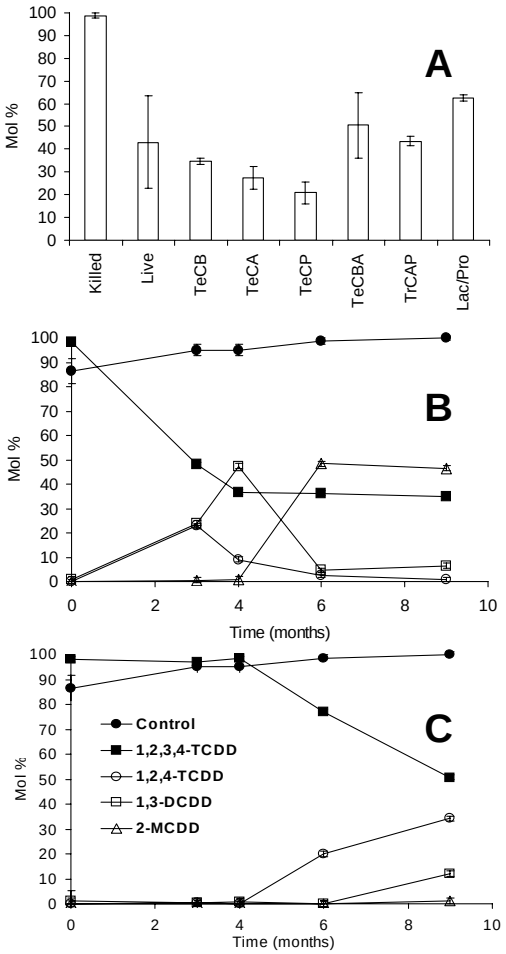

Fig. 8. 1,2,3,4-Tetrachlorodibenzo-*p*-dioxin (1,2,3,4-TeCDD) dechlorination in Paleta Creek sediment slurries under different electron donor (Lac/Pro, lactate/propionate) and halogenated co-amendment (TeCB, tetrachlorobenzene; TeCA, tetrachloroanisole; TeCP, tetrachlorophenol; TeCBA, tetrachlorobenzoic acid; TrCAP, trichloroacetophenone) conditions over 9 months with a 3-month re-amendment interval.

(A) Mol percent of 1,2,3,4-TeCDD remaining after 9 months. Killed, autoclaved at 121°C for 30 min on three consecutive days; Live, no haloprimer and no electron donor; TeCB, 25 μM TeCB plus 500 μM each lactate/propionate; TeCA, 25 μM TeCA plus 500 μM each lactate/propionate; TeCP, 25 μM TeCP plus 500 μM each lactate/propionate; TeCBA, 25 μM TeCBA plus 500 μM each lactate/propionate; TrCAP, 25 μM TrCAP plus 500 μM each lactate/propionate; Lac/Pro, no haloprimer and 500 μM each lactate/propionate. Mol percent is the ratio of moles of a specific chlorinated dibenzo-*p*-dioxin congener to the total moles of all chlorinated dibenzo-p-dioxin congeners detected in the sample. Error bars represent the standard deviation of samples from triplicate cultures.

(B) Time progression of 1,2,3,4-TeCDD dechlorination in TeCB-amended slurries, 25 μM TeCB plus 500 μM each lactate/propionate. Symbols represent averages of triplicate cultures and error bars represent the standard deviation.

(C) Time progression of 1,2,3,4-TeCDD dechlorination in TeCBA-amended slurries, 25 μM TeCBA plus 500 μM each lactate/propionate. Symbols represent averages of triplicate cultures and error bars represent the standard deviation

From Ahn et al. (2005).

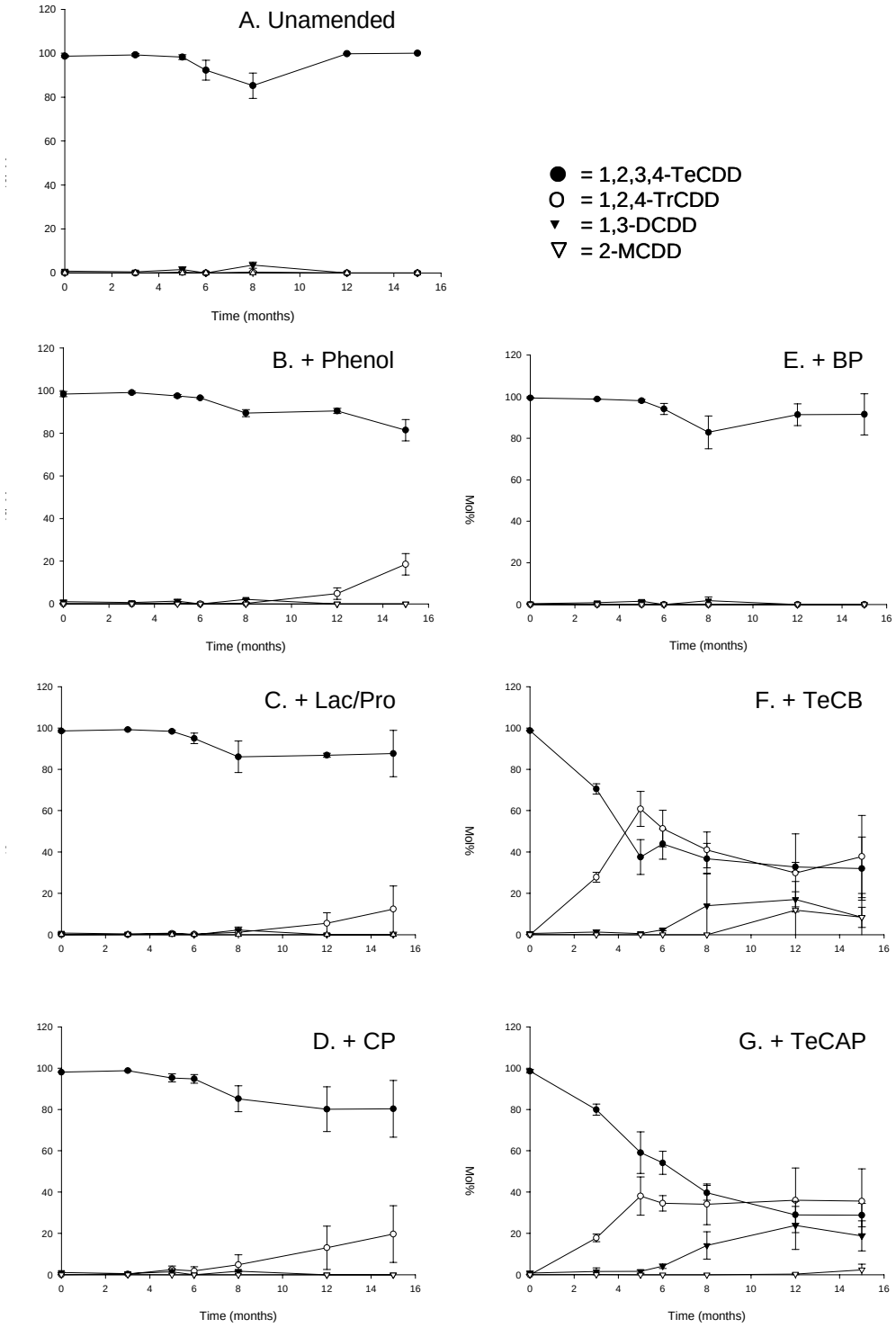

Fig. 9. Time progression of 1,2,3,4-tetrachlorodibenzo-*p*-dioxin (1,2,3,4-TeCDD) dechlorination in Paleta Creek sediment slurries under different electron donor (Lac/Pro, lactate/propionate) and halogenated co-amendment (TeCB, tetrachlorobenzene; TeCA, tetrachloroanisole; TeCP, tetrachlorophenol; TeCBA, tetrachlorobenzoic acid; TrCAP, trichloroacetophenone) with a 3-month re-amendment interval.

2.7 ± 0.8 mol % of 1,2,4-TrCDD, 5.4 ± 1.5 and 4.7 ± 1.1 mol % of 1,3-DCDD and 50 ± 1.2 and 48 ± 1.0 mol % of 2-MCDD were found, respectively. In pristine Tuckerton sediments, 62.0 ± 1.6 mol % of 1,2,4-TrCDD was detected. Shelter Island sediments exhibited the least amount of 1,2,3,4-TeCDD dechlorination with only a trace of 1,2,4-TrCDD detected after 6 months of incubation. A time course of 1,2,3,4-TeCDD dechlorination in the different sediments is shown in Fig. 11.

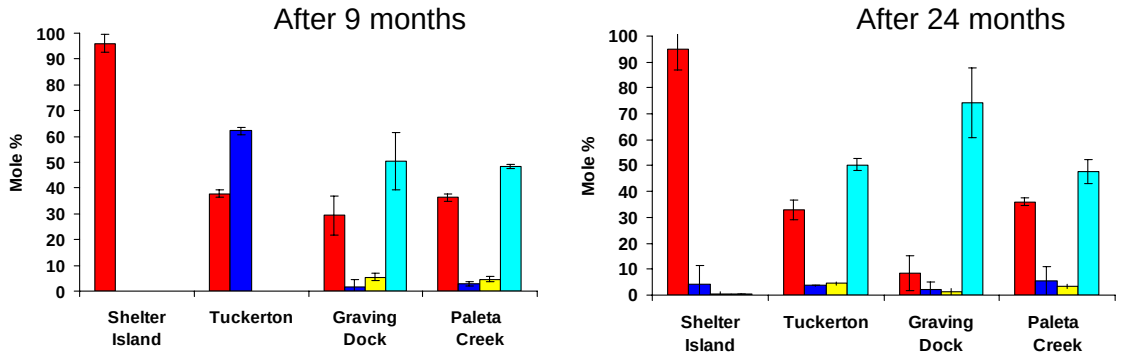

Fig. 10. Mol percent of 1,2,3,4-tetrachlorodibenzo-*p*-dioxin (1,2,3,4-TeCDD) and its dechlorination daughter products in TeCB (tetrachlorobenzene) plus lactate/propionate-amended sediment slurries from different sampling locations after 6 and 24 months (Tuckerton, NJ, USA; Paleta Creek, Graving Dock and Shelter Island, San Diego Bay, CA, USA). Mol percent is the ratio of moles of a specific chlorinated dibenzo-*p*-dioxin congener to the total moles of all chlorinated dibenzo-*p*-dioxin congeners detected in the sample. Error bars represent the standard deviation of samples from triplicate cultures. Adapted from Ahn et al. (2005).

Dechlorination of 1,2,3,4-TeCDD under different re-amendment time intervals. The effect of the haloprimer amendment interval on 1,2,3,4-TeCDD dechlorination was compared in slurries re-amended with either TeCB or TeCA plus the electron donors lactate/propionate at either a 3-month interval or a 6-month interval. Addition of TeCB and TeCA every 3 months resulted in more rapid onset of 1,2,3,4-TeCDD dechlorination than addition every 6 months. In the 3-month amendment interval samples with TeCB, 1,2,4-TrCDD (2.7 mol %), 1,3-DCDD (4.7 mol %), and 2-MCDD (48 mol %) were formed. While those slurries amended with TeCA exhibited 1,2,4-TrCDD (21 mol %), 1,3-DCDD (45 mol %), and 2-MCDD (0.7 mol %). In the 6 month amendment interval samples with TeCB, 1,2,4-TrCDD (51 mol %) and 1,3-DCDD (2.4 mol %) were detected. While in those with TeCA showed 1,2,4-TrCDD (34 mol %) and 1,3-DCDD (4.1 mol %). The reductive dechlorination of TeCB and TeCA were observed within 3 months.

Dechlorination of 1,2,3,4-TeCDF in methanogenic sediment slurries. The dechlorination of 1,2,3,4-TeCDF was compared in Paleta Creek sediment slurries using TeCB, TeCA, TrCAP, TeCP or TeCBA co-amendments plus lactate/propionate as electron donors, re-amended every 6 months. All chlorinated dibenzofuran congeners that were potential daughter products of 1,2,3,4-TeCDF were not commercially available. Therefore, the chlorine substituent

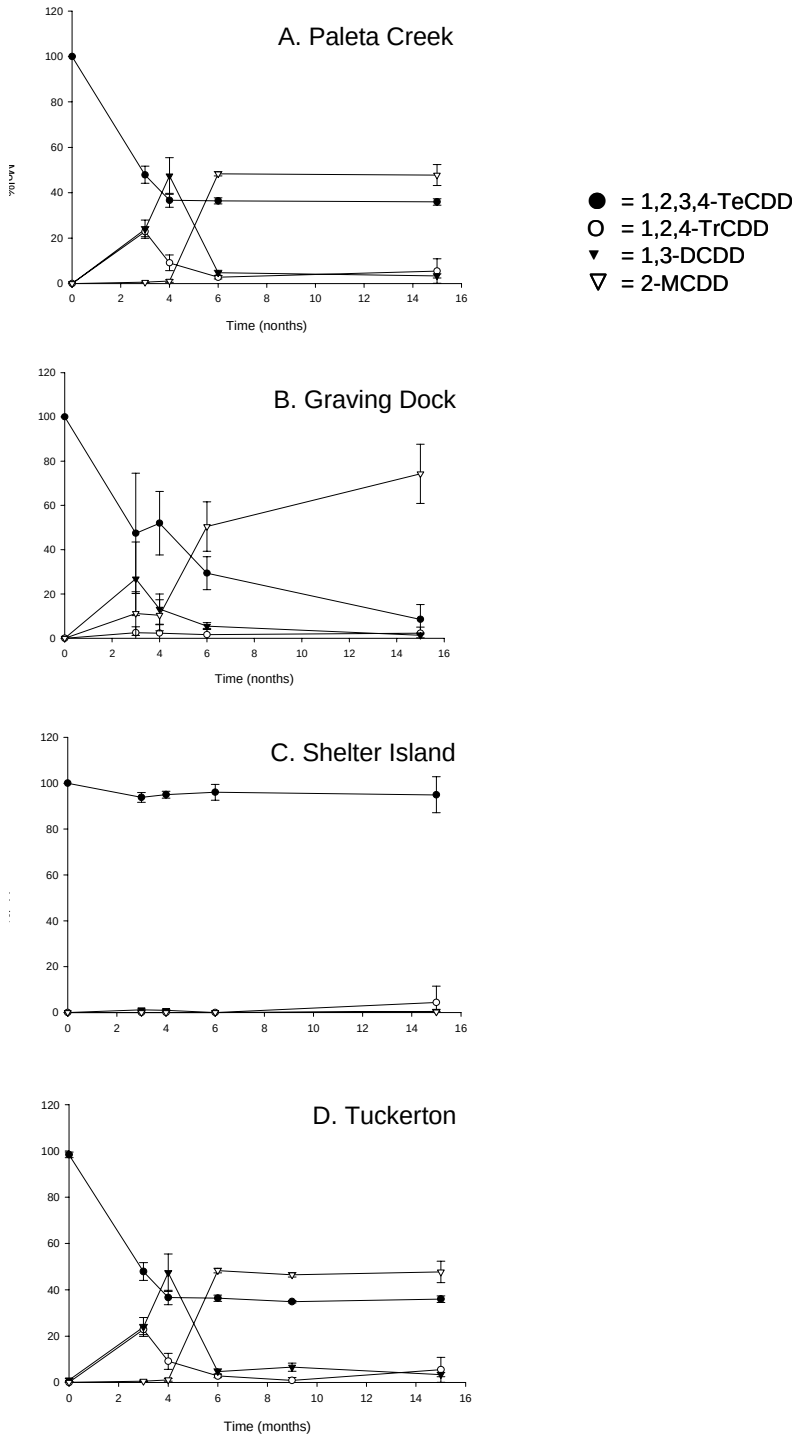

Fig. 11. Time course of 1,2,3,4-tetrachlorodibenzo-*p*-dioxin (1,2,3,4-TeCDD) dechlorination in TeCB (tetrachlorobenzene) plus lactate/propionate-amended sediment slurries from different sampling locations (Tuckerton, NJ, USA; Paleta Creek, Graving Dock and Shelter Island, San Diego Bay, CA, USA).

position and the pathway of PCDF dechlorination could not be determined. All of the five chlorinated co-amendments significantly enhanced the extent of 1,2,3,4-TeCDF dechlorination in comparison to electron donor alone (Fig. 12A, 13). A total transformation of 63 to 81 mol % of the added 1,2,3,4-TeCDF occurred in the TeCB, TeCA, and TrCAP-amended cultures over the 10-month incubation period. The half-lives of 1,2,3,4-TeCDF in TeCB-, TeCA-, and TrCAP-amended sediment slurries were: TeCB, 3.7 months (95% confidence interval, 2.8 to 5.3 months); TeCA, 7.1 months (95% confidence interval, 5.8 to 9.3 months); and TrCAP, 3.6 months (95% confidence interval, 3 to 4.4 months). In the TeCP- (Fig. 12B) and TeCBA-amended cultures, transformation of only 36 to 56 mol % of the added 1,2,3,4-TeCDF was observed. The 1,2,3,4-TeCDF half-life for TeCP amendment was 15.5 months (95% confidence interval, 14.3 to 16.9 months) and for TeCBA amendment was 9.4 months (95% confidence interval, 6.4 to 18 months). In TeCB and TrCAP- (Fig. 12C) amended sediment cultures, 1,2,3,4-TeCDF was dechlorinated to a mixture of trichlorodibenzofuran (TriCDF) (11 to 15 mol %), dichlorodibenzofuran (DCDF) (31 to 37 mol %), and monochlorodibenzofuran (MCDF) (33 mol %) Dechlorination of 1,2,3,4-TeCDF in the lactate/propionate electron donor control was minimal and showed little difference from the non-amended live controls. No dechlorination products were detected in the killed control.

Although previous research has demonstrated that 1,2,3,4-TeCDD is dechlorinated under anaerobic conditions, few investigators have studied dehalogenation of 1,2,3,4-TeCDD/F in estuarine sediments, the differences between sites, or the effect that different electron donors and halogenated electron acceptor co-amendments have on the dechlorination. 1,2,3,4-TeCDD dechlorination was readily established in estuarine sediments from several sites. Co-amendment with halogenated compounds enhanced dechlorination of spiked 1,2,3,4-TeCDD and 1,2,3,4-TCDF in estuarine sediments under a variety of conditions. Dechlorination occurred in the presence of sulfate in Paleta Creek sediments amended with 2-BP or with no amendment. As in methanogenic sediment slurries, 2-MCDD was formed as a final dechlorination product, although it was produced far more slowly and at much lower levels (3 mol %) than in methanogenic slurries amended with 2-BP (60 mol %).

Dechlorination of 1,2,3,4-TeCDD was extensive in slurries established from the heavily polluted Paleta Creek and Graving Dock Naval Station sites of San Diego Bay while comparatively little dehalogenation occurred in Shelter Island sediment slurries. We cannot entirely explain this difference except to note that Shelter Island is dominated primarily by pleasure boating activities, and while it is reported to have PCB contamination, it is at comparatively lower concentrations than the sites near the Naval Station and San Diego, CA, USA urban center (Fairey et al. 1998, McCain et al. 1992). Compared to the Graving Dock and Paleta Creek locations, Shelter Island also has far lower concentrations of the chlorinated pesticide chlordane indicating that overall exposure to halogenated compounds is lower. Only 1,2,4-TrCDD was produced from the dechlorination of 1,2,3,4-TeCDD in the Tuckerton sediment slurries. Tuckerton, NJ, USA is the location of an estuarine sanctuary and has little impact from urban, shipping, or industrial activities. Dechlorination of 1,2,3,4-TeCDD proceeded to greater extent in the less contaminated pristine Tuckerton sediments (62 mol % of 1,2,4-TrCDD) than in the more contaminated Shelter Island sediments in which no 1,2,4-TrCDDwas formed after 6 months. Since Tuckerton supports a healthy estuarine ecosystem, native biota may produce natural halogenated compounds (Fielman et al. 1999) that result in

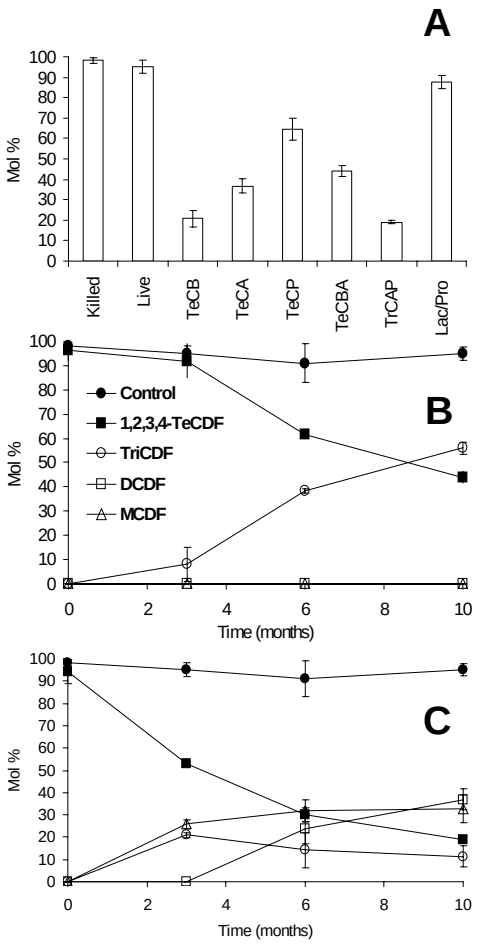

Fig. 12. 1,2,3,4-Tetrachlorodibenzofuran (1,2,3,4-TeCDF) dechlorination in Paleta Creek sediment after 10 months under different electron donor (Lac/Pro, lactate/propionate) and halogenated co-amendment (TeCB; tetrachlorobenzene; TeCA, tetrachloroanisole; TeCP, tetrachlorophenol; TeCBA, tetrachlorobenzoic acid; TrCAP, trichloroacetophenone) conditions with a 6-month re-amendment interval.

(A) Mol percent of 1,2,3,4-TeCDF remaining after 10 months. Killed, autoclaved at 121°C for 30 min on three consecutive days; Live, no haloprimer and no electron donor; TeCB, 25 μM TeCB plus 500 μM each lactate/propionate; TeCA, 25 μM TeCA plus 500 μM each lactate/propionate; TeCP, 25 μM TeCP plus 500 μM each lactate/propionate; TeCBA, 25 μM TeCBA plus 500 μM each lactate/propionate; TrCAP, 25 μM TrCAP plus 500 μM each lactate/propionate; Lac/Pro, no haloprimer and 500 μM each lactate/propionate. Mol percent is the ratio of moles of a specific chlorinated dibenzofuran congener to the total moles of all chlorinated dibenzofuran congeners detected in the sample. Error bars represent the standard deviation of samples from triplicate cultures.

(B) Time progression of 1,2,3,4-TeCDF dechlorination in TeCP-amended slurries, 25 μM TeCP plus 500 μM each lactate/propionate. Symbols represent averages of triplicate cultures and error bars represent the standard deviation.

(C) Time progression of 1,2,3,4-TeCDF dechlorination in TrCAP-amended slurries, 25 μM TrCAP plus 500 μM each lactate/propionate. Symbols represent averages of triplicate cultures and error bars represent the standard deviation.

From Ahn et al. (2005).

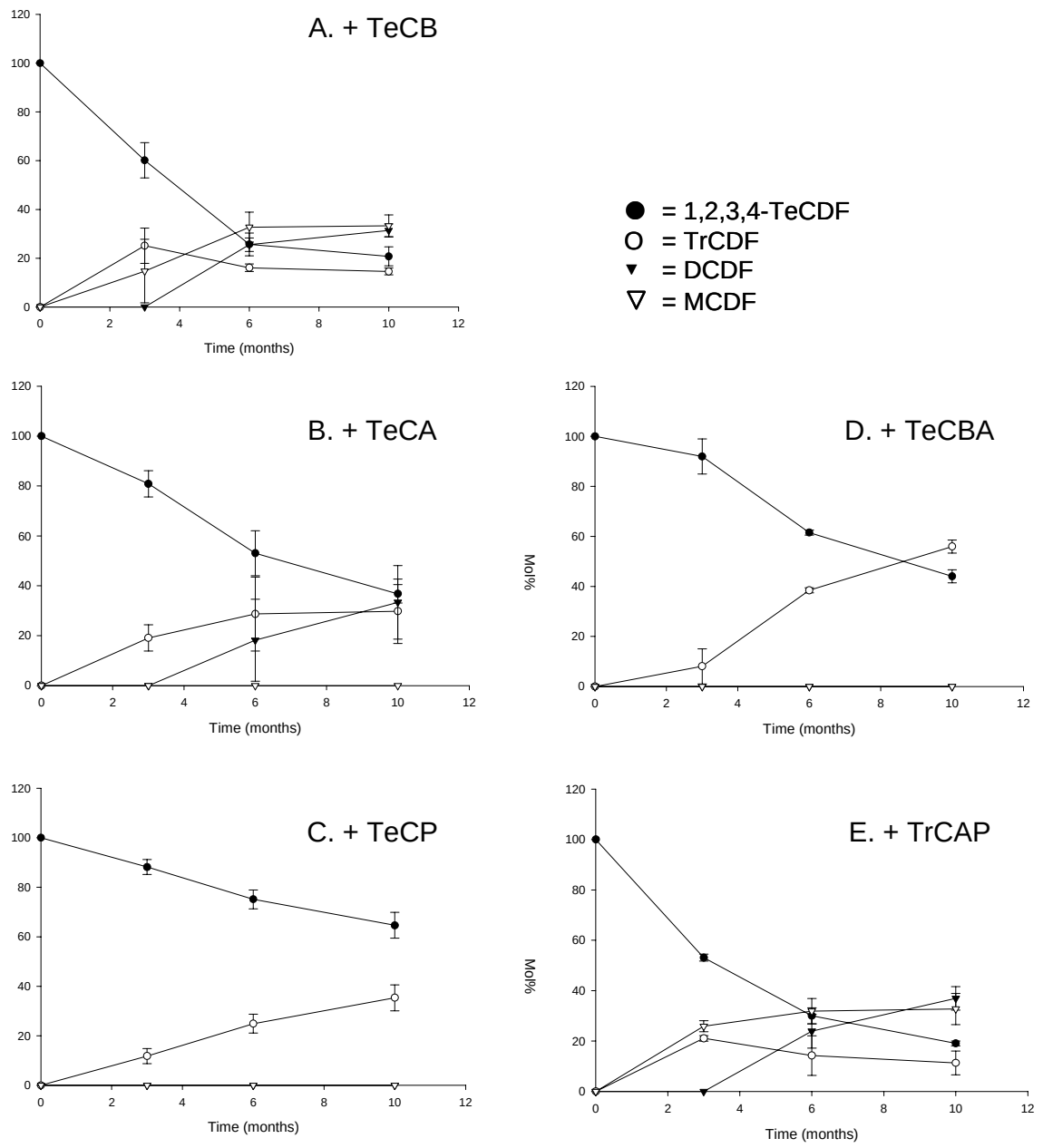

Fig. 13. Time course for 1,2,3,4-tetrachlorodibenzofuran (1,2,3,4-TeCDF) dechlorination in Paleta Creek sediment under different electron donor (Lac/Pro, lactate/propionate) and halogenated co-amendment (TeCB; tetrachlorobenzene; TeCA, tetrachloroanisole; TeCP, tetrachlorophenol; TeCBA, tetrachlorobenzoic acid; TrCAP, trichloroacetophenone) conditions with a 6-month re-amendment interval.

enrichment of dehalogenating bacteria. In Graving Dock and Paleta Creek sediments, 1,2,3,4-TeCB was dehalogenated to a mixture of 1,2-DCB and 1,3-DCB within 3 months. No further dechlorination of the 1,2-DCB and 1,3-DCB was observed. In comparison, Tuckerton sediments showed only trace amounts of 1,2-DCB and 1,3-DCB daughter products. In Shelter Island sediments only 1,2,3-trichlorobenzene (1,2,3-TCB) was formed as a dehalogenation product of TeCB. The relative extents of dechlorination of the TeCB co-amendment was consistent with the amount of dechlorination of the 1,2,3,4-TeCDD by the various sediments.

There were significant differences in the effectiveness of various halogenated co-amendments for stimulating 1,2,3,4-TeCDD dechlorination. The 1,2,3,4-TeCDD was dechlorinated to 1,2,4-TrCDD (19.7 mol %) after 15 months in BP-amended sediments. In TeCB-amended sediments, 1,2,3,4-TeCDD was dechlorinated to 1,3-DCDD (6.6 mol %), and 2-MCDD (46.5 mol %) after 9 months. The bromophenols were a less effective co-amendment perhaps because BP may select for dehalogenating organisms that were not as effective at 1,2,3,4-TeCDD dechlorination. Also, BP is readily degradable in sediments and persisted for a shorter period of time compared to TeCB. This could have affected both the regulation of enzymes and the abundance of microorganisms involved in dechlorination. Cho et al. (2002) showed that the PCB dechlorination enhancement in sediments enriched with chlorobenzoates, chlorophenols and chlorobenzenes was related to the size of the dechlorinating microorganism population.

1,2,3,4-TeCDF was dechlorinated to unidentified TrDCF, DCDF and MCDF congeners and was similarly enhanced by the addition of the halogenated co-amendments TeCB, TeCA, TrCAP, TeCP and TeCBA (Fig 13). Dechlorination of 1,2,3,4-TeCDD/F was enhanced more effectively by electron donors plus TeCB, TeCA, TeCP, TrCAP, and TeCBA than BPs and CPs plus electron donor, or electron donor alone. Although methanol was not added to the electron donor control, the low activity in the TeCBA-amended sediment slurries, which also received methanol, indicates that methanol, by itself, was not responsible for the enhancement of 1,2,3,4-TeCDD dechlorination.

Addition of TeCB and TeCA every 3 months resulted in more rapid onset of 1,2,3,4-CDD dechlorination than addition every 6 months. The stimulatory effect of TeCB and TeCA on 1,2,3,4-TeCDD dechlorination could be associated with growth of dehalogenating populations. Earlier, Beurskens et al. (1995) reported cross-reactivity of a hexachlorobenzene-dechlorinating culture that was able to dechlorinate 1,2,3,4-TeCDD, in addition to PCBs. The ubiquity and diverse nature of the dehalorespiratory bacteria (Löffler et al. 2003) and the apparent diversity of dehalogenases reported to be present in the genome of *Dehalococcoides ethenogenes* strain 195 (Villemur et al. 2002) and other *Dehalococcoides* strains (Hölscher et al. 2004), suggest that dehalogenating bacteria represent a rich natural resource with the ability to transform a variety of halogenated compounds. Bunge et al. (2003) recently reported that *Dehalococcoides sp.* strain CBDB1, originally isolated on trichlorobenzene (Adrian et al. 2000), can also dechlorinate PCDDs and that it can sustain growth from these compounds. *Dehalococcoides ethenogenes* strain 195 exhibited growth on chlorinated benzenes and also dechlorinated 1,2,3,4-TeCDD and 1,2,3,4-TeCDF (Fennell et al. 2004; see section 2) although it is not known if this activity is growth linked. Therefore, the addition of structural analogues of CDD/Fs such as TeCB may

result in the growth or stimulation of 1,2,3,4-TeCDD/F dehalogenating organisms, or perhaps in induction of dehalogenases that can also dechlorinate 1,2,3,4-TeCDD/F.

These experiments demonstrate that the addition of other, more soluble halogenated compounds greatly speeds 1,2,3,4-TeCDD/F dechlorination in estuarine sediments. This stimulation was brought about in the presence of added electron donors lactate and propionate to ensure reduced conditions and adequate reducing equivalents to stimulate dechlorination. The addition of lactate and propionate alone did not result in effective stimulation, suggesting that the primary stimulatory effect is contributed by the halogenated additive. In highly organic sediments with adequate reducing power, it is possible that these halogenated additives would be just as effective when added without additional electron donors; however that would need to be determined separately for individual sites. This study coupled with findings that some species of dehalogenating bacteria have multiple dehalogenases and dechlorination capabilities (Adrian et al. 2000; Bunge et al. 2003; Fennell et al. 2004; Villemur et al. 2002; Hölscher et al. 2004) suggests that halogenated compounds other than CDD/Fs could be used to enrich and isolate organisms that have activity on CDD/Fs. This would provide a faster and easier way to study dechlorinating bacteria with effectiveness on CDD/Fs since enrichment on CDD/Fs is slow and difficult considering the low concentrations that can be provided in enrichments. This study also demonstrates that the addition of halogenated co-amendments may be one tool to enhance dechlorination of CDD/Fs in contaminated sediments. Further work is needed to document the effectiveness of the halogenated amendments to enhance dechlorination of environmentally relevant CDD/Fs in historically contaminated sediments. Identification and further study of CDD/F-dehalogenating bacteria and their physiological capabilities offers the hope of finding non-toxic co-amendments that would be suitable for *in situ* applications.

2. *Dehalococcoides ethenogenes* Strain 195 Reductively Dechlorinates Diverse Chlorinated Aromatic Pollutants

Dechlorination of 1,2,3,4,-TeCDD by mixed culture. A mixed culture containing *D. ethenogenes* strain 195 dechlorinated 1,2,3,4-TeCDD (Fennell et al. 2004b), but with differing efficiencies depending upon whether the mode of addition of the PCDD was via either a coating on sterile glass beads (Figure 14A), the sides and bottom of the serum bottle (Figure 14B) or on dry sterile sediment (Figure 14C). Dechlorination was inefficient when the compounds were amended onto glass beads or serum bottle surfaces possibly due to poor bioavailability under these conditions. The most rapid dechlorination was observed when the PCDD was amended to sediment, when dechlorination proceeded to a mixture of 1,2,4-TrCDD and 1,3-DCDD within approximately 40 days. Dechlorination then ceased with about 10 % of the original substrate remaining despite re-amendment of electron donor on day 8, 26, 120 and 178 (Figure 14C). In the same culture PCE added on Day 0 was dechlorinated to a mixture of VC and ethene within two weeks. No dechlorination was observed in autoclaved controls (Figure 14D).

Dechlorination of 1,2,3,4,-TeCDD by pure culture. *D. ethenogenes* strain 195 in pure culture exhibited a lag period prior to onset of dechlorination activity on PCE or the chloroaromatics. Thereafter, the pure culture dechlorinated PCE to a mixture of VC and ethene. 1,2,3,4-TeCDD was dechlorinated primarily to 1,2,4-TrCDD, with a trace of 1,3-DCDD (Figure

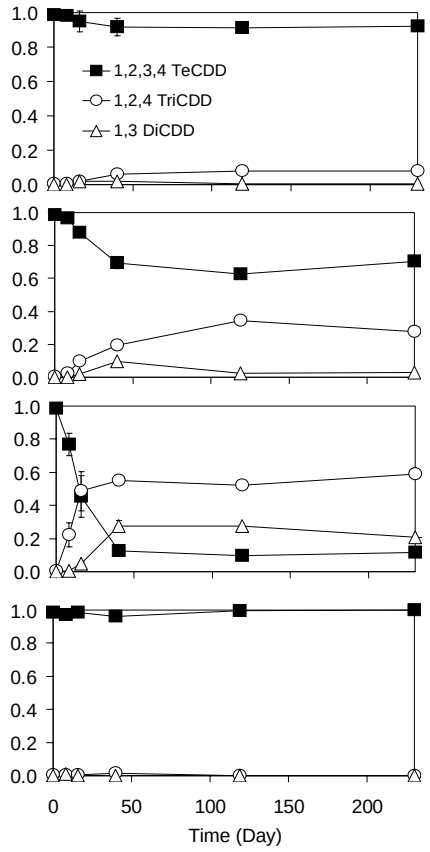

Fig. 14. Dechlorination of 1,2,3,4-tetrachlorodibenzo-*p*-dioxin by a butyrate-PCE enriched mixed culture containing *Dehalococcoides ethenogenes* strain 195 when amended on (A) the serum bottle walls (B) glass beads (C) on sterile sediment and (D) on sterile sediment and autoclaved. PCE added on Day 0. Butyrate added on Days 8, 26, 120 and 178. Symbols are averages of triplicate cultures. Error bars represent standard deviations. Where not visible, error bars are smaller than the symbol. From Fennell et al. (2004a).

15A). 1,2,3,4-TeCDF was dechlorinated to a trichlorinated dibenzo-furan (TrCDF) congener (Figure 15B). 2,3,4,5,6-PeCB was dechlorinated to 2,3,4,6-TeCB and/or 2,3,5,6-TeCB (these congeners could not be resolved) and 2,4,6-trichlorobiphenyl (2,4,6-TrCB) (Figure 15C). 1,2,3,4-TeCN was dechlorinated primarily to an unidentified dichloronaphthalene congener (DCN) after 249 days of incubation. A summary of the dechlorination pathways is shown in Figure 16. The rate of dechlorination of 2,3,4,5,6-PeCB was more rapid and the extent was greater than that of 1,2,3,4-TeCDD/F dechlorination. Between Day 79 and Day 149, slower rates of 1,2,3,4-TeCDD and 1,2,3,4-TeCDF dechlorination were observed. Re-amendment of PCE on day 164 and 185 did not result in apparent concomitant increase in the rate of chloroaromatic dechlorination (Figure 16). We did not detect dechlorination daughter products from the monochlorophenols, 2,3-DCDD or 2,3,7,8-TeCDD after 249 days. In all cultures, the final two PCE additions were dechlorinated to a mixture of VC and ethene within two weeks, demonstrating that the cultures remained viable and active throughout the incubation period. We did not investigate the ability of *D. ethenogenes* to gain energy for growth on chlorinated dibenzo-*p*-dioxins, dibenzofurans,

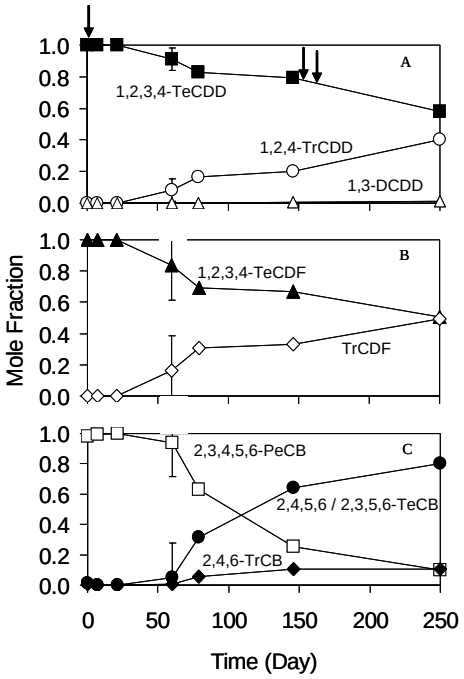

Fig. 15. Dechlorination by *Dehalococcoides ethenogenes* strain 195 of sediment applied (A) 1,2,3,4-tetrachlorodibenzo-*p*-dioxin; (B) 1,2,3,4-tetrachlorodibenzofuran; and (C) 2,3,4,5,6-pentachlorobiphenyl. Arrows show addition of PCE. Symbols are averages of triplicate cultures. Error bars represent standard deviations. Where not visible, error bars are smaller than the symbol. From Fennell et al. (2004b).

biphenyls or naphthalenes. However, we further demonstrated the ability to transfer dechlorination activity with the chlorobenzenes (Fennell et al. 2004b).

These experiments have demonstrated that *D. ethenogenes* strain 195 has the ability to dechlorinate many different types of chlorinated aromatic compounds, in addition to its known chloroethene respiratory electron acceptors. This is significant given the prevalence of *Dehalococcoides*-like organisms in the environment and its relatedness to *Dehalococcoides* sp. strain CBDB1 which was reported to carry out dehalorespiration with selected PCDD congeners (Bunge et al. 2003) and PCB dehalogenating strains DF-1 and *o*-17 (Cutter et al. 2001; Wu et al. 2002a, b). *D. ethenogenes* strain 195 and *Dehalococcoides* sp. strain CBDB1 are close relatives (98% identity over 1422 nucleotides of 16S rRNA gene sequence). Cell extracts of strain CBDB1 were capable of converting hexachlorobenzene to pentachlorobenzene Hölscher et al. (2003). Furthermore, the organism exhibited growth with TeCB, and 1,2,3- or 1,2,4-trichlorobenzen (Bunge et al. 2003; Adrian et al. 2000), however, it converted PCE only to *trans*-1,2-DCE (Adrian 2001). Conversely, strain 195 utilized hexachlorobenzene, pentachlorobenzene, and 1,2,3,4- and 1,2,4,5-TeCB, and converts tetrachloroethene to vinyl chloride and ethene. In the dechlorination of 1,2,3,4-TeCDD, strain 195 and strain CBDB1 differed in that strain 195 produced 1,2,4- TrCDD and 1,3-DCDD while strain CBDB1 produced 2,3-DCDD and 2-MCDD. Although these strains are closely related and were capable of dechlorinating some of the same substrates, their substrate range did not completely overlap.

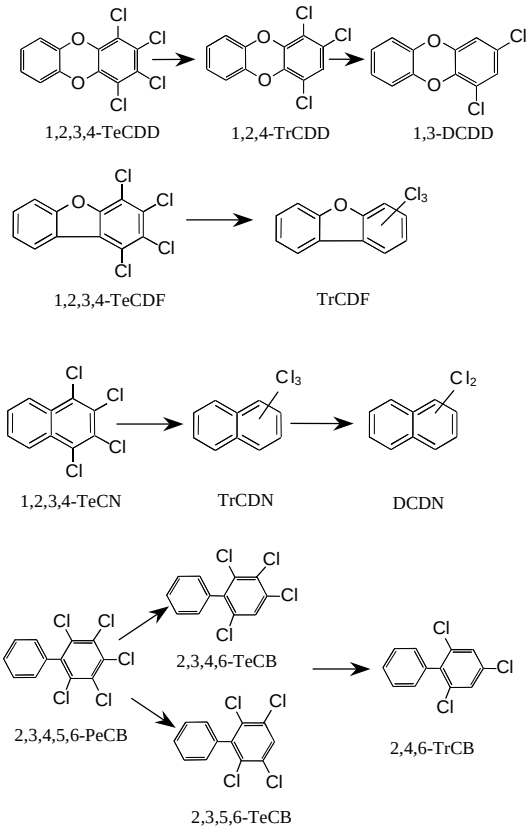

Fig. 16. Observed pathways of reductive dechlorination of chlorinated aromatic compounds by *Dehalococcoides ethenogenes* strain 195.
From Fennell et al. (2004b).

The rate and extent of 2,3,4,5,6-PeCB dechlorination by the pure culture was greater than that of 1,2,3,4-TeCDD/F. Also, the rate and extent of 1,2,3,4-TeCDD dechlorination were less in the pure culture than in the mixed culture. Possible explanations include the higher incubation temperature (34°C) of the mixed culture versus the pure culture (28°C), the slightly higher estimated strain 195 concentration in the mixed culture (16 µg protein/mL) relative to the pure culture (5 to 10 µg protein/mL), the better meeting of the nutritional needs of *D. ethenogenes* in the mixed culture (Maymo-Gatell et al. 1997), or the presence of other dehalogenating organisms in the mixed culture. The mixed culture also dehalogenates vinyl chloride to ethene more efficiently than the pure culture (Maymo-Gatell et al. 1997; Tandoi et al. 1994).

The observed differences in the dechlorination pathways for different Dehalococcoides strains raises many questions about the potential for expression of the multiple putative dehalogenase genes that are present in the *Dehalococcoides* genome (Villemur et al. 2002) and the phylogenetic and phenotypic relatedness of the organisms within this group. We did not determine whether *D. ethenogenes* strain 195 transformed the chlorinated dibenzo-*p*-dioxins, dibenzofurans, biphenyls or naphthalenes only through cometabolism—much as occurs with the transformation of vinyl chloride by *tceA*. Results obtained after re-amendment of cultures with

PCE were inconclusive and we were unable to determine whether on-going PCE dehalorespiration resulted in a concomitant increase in the rate of chloroaromatic dechlorination. We did show that *D. ethenogenes* strain 195 could be transferred and express dechlorination activity with hexa-, penta- or tetra-chlorinatedbenzenes as sole electron acceptors (Fennell et al. 2004), which is suggestive of growth with these compounds.

Dehalococcoides-containing mixed cultures have been utilized successfully for bioaugmentation of chloroethene contaminated aquifers in small scale field tests (Lendway et al. 2003; Harkness et al. 1999; Major et al. 2002). In light of the ability of *D. ethenogenes* and related organisms to utilize diverse chlorinated organic substrates, cultures containing *Dehalococcoides* spp. or possibly recombinant dehalogenase-containing transgenic organisms developed using genetic elements taken from *Dehalococcoides* could be used to treat diverse chlorinated pollutants. In particular, the ability to grow *Dehalococcoides* spp. under more favorable conditions in mixed culture on alternate, more bioavailable substrates such as chloroethenes is a significant advantage for producing larger quantities of cells that could be used for bioaugmentation of sites contaminated with poorly available polyhalogenated aromatic compounds such as PCDD/Fs and PCBs. If it is shown that *Dehalococcoides* spp. can sustain growth and perpetuate themselves with compounds such as PCDD/Fs and PCBs, this could be one approach for enhancing bioremediation of contaminated soils and sediments.

Dechlorination of environmentally relevant PCDD/F congeners. Dechlorination of octachlorodibenzo-*p*-dioxin (OCDD) and 1,2,3,4,7,8-hexachlorodibenzofuran (1,2,3,4,7,8-HxCDF) was examined in a mixed culture containing *D. ethenogenes* strain 195. Dechlorination of 1,2,3,4,7,8-HxCDF resulted in the production of a non-2,3,7,8-substituted penta-CDF congener within 1 month. The penta-CDF was further dechlorinated to two non-2,3,7,8-subsituted tetrachlorodibenzofuran (tetra-CDF) congeners in the set amended with 1,2,3,4-TeCB. One of the two tetra-CDF congeners was tentatively identified as 1,3,7,8-tetraCDF. The environmentally relevant 1,2,3,4,7,8-HxCDF was dechlorinated to less chlorinated and less toxic CDF congeners—an advantageous reaction with potential application to bioremediation.

3. Detection and Characterization of a Dehalogenating Microorganism by Terminal Restriction Length Fragment Length Polymorphism Fingerprinting of 16S rRNA

In order to identify the dehalogenating organisms in anaerobic estuarine and marine sediments we have pursued the characterization of the structure and diversity of microbial food webs that are active during dehalogenation. Thus, by assaying ribosomal RNA during dehalogenation, the active populations can be identified and monitored (Fennell et al. 2004a). In this study, we developed an assay using a starved consortium (low ribosome content culture). The newly synthesized 16S rRNA was then analyzed using terminal-restriction fragment length polymorphism (TRFLP) analysis of reverse transcribed rRNA as a means for assigning a metabolic role to the different members of the dehalogenating consortia. Those organisms that became active after substrate feeding were recognized as distinct peaks in the electropherogram and could be identified based on data from clonal libraries.

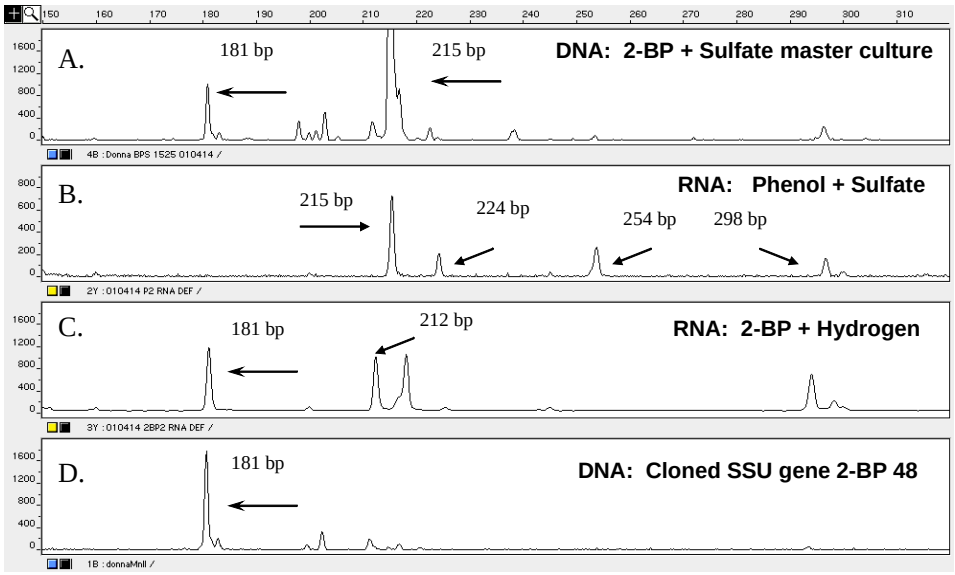

Fig. 17. T-RFLP fingerprints of various samples, obtained using 16S rRNA gene primers with DNA or rRNA as a template. (A) Master enrichment (DNA template; bacteria present in cultures). (B) Amendment with phenol plus sulfate (RNA template; bacteria active during phenol degradation). (C) Amendment with 2-BP plus hydrogen (RNA template; bacteria active during dehalogenation). (D) A cloned 16S rRNA gene corresponding to the 181-bp peak.
From Fennell et al. (2004a).

This method was initially tested using a 2-bromophenol-degrading sulfidogenic consortium as a model (Fennell et al. 2004a). Direct terminal restriction fragment length polymorphism fingerprinting of ribosomes in the various subcultures (fed different substrate combinations) indicated that phylotype 2BP-48 (a *Desulfovibrio*-like sequence reported previously; Knight et al. 1999) was responsible for the dehalogenation of 2-bromophenol (Fig. 17). The entire microbial community of the master enrichment can be discerned from the 16S rRNA gene T-RFLP analysis of genomic DNA (Fig. 17A). This fingerprint shows approximately 12 restriction fragments representing the more numerous bacteria present in the consortium. T-RFLP fingerprints for the various substrate treatments are shown in Fig. 17B and 17C. The organisms represented by the TRFs of 215, 224, 254, and 298 bp were implicated in phenol degradation, although the organism represented by 215 bp was the most significant (as indicated by the peak size) suggesting the largest number of ribosomes produced during degradation of phenol. The organisms represented by the restriction fragment (RF) of 181 and 212 bp appear to be active only in the treatments where dehalogenation occurred (Fig. 17A and 17C) and were therefore implicated in the dehalogenation process. In the 2BP plus hydrogen treatment, there were TRF peaks induced in the enrichment which were undetectable in the master culture, (218 and 294 bp; Fig. 17C). It was deemed likely these microorganisms are induced by the hydrogen amendment and did not play a significant role in 2BP dehalogenation.

A stable co-culture was established containing predominantly 2BP-48 and a second *Desulfovibrio* species capable of mineralizing 2-bromophenol coupled to sulfate reduction. The16S rRNA gene sequence of strain 2BP-48 showed closest homology with the genus *Desulfovibrio* (Fig. 18). Strain 2BP-48 in the co-culture could couple reductive dehalogenation to

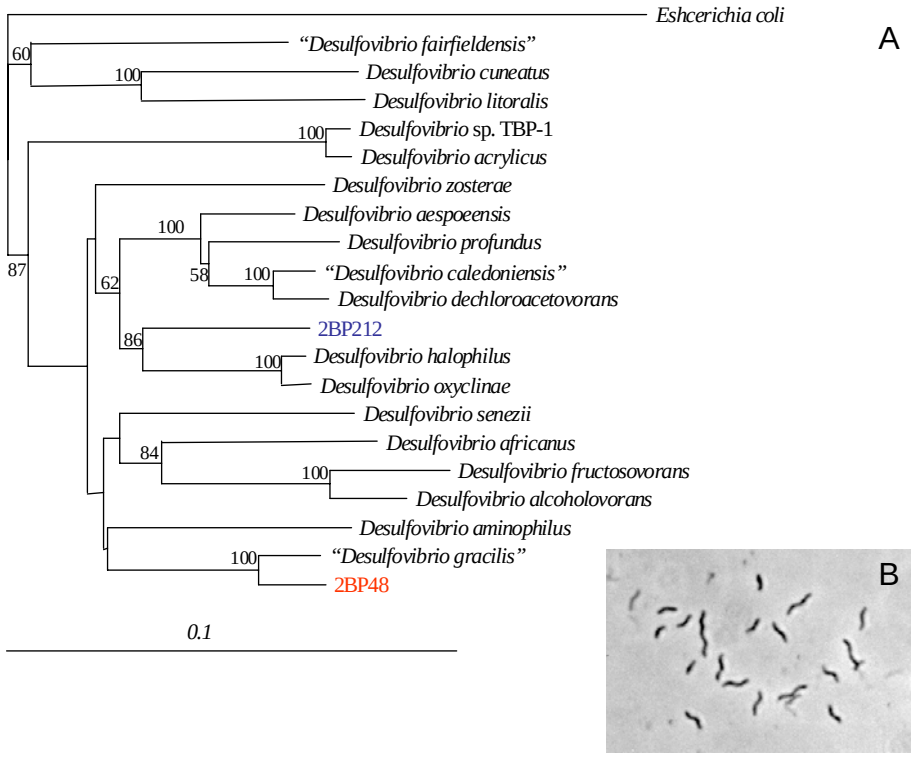

Fig. 18. Phylogenetic tree for strain 2BP-48, strain BP212, and related species based on the 16S rRNA gene sequences. Bootstrap values less than 50% are not indicated at the nodes. Scale bar = 0.1 substitution per 100 positions.
From Fennell et al. (2004a).

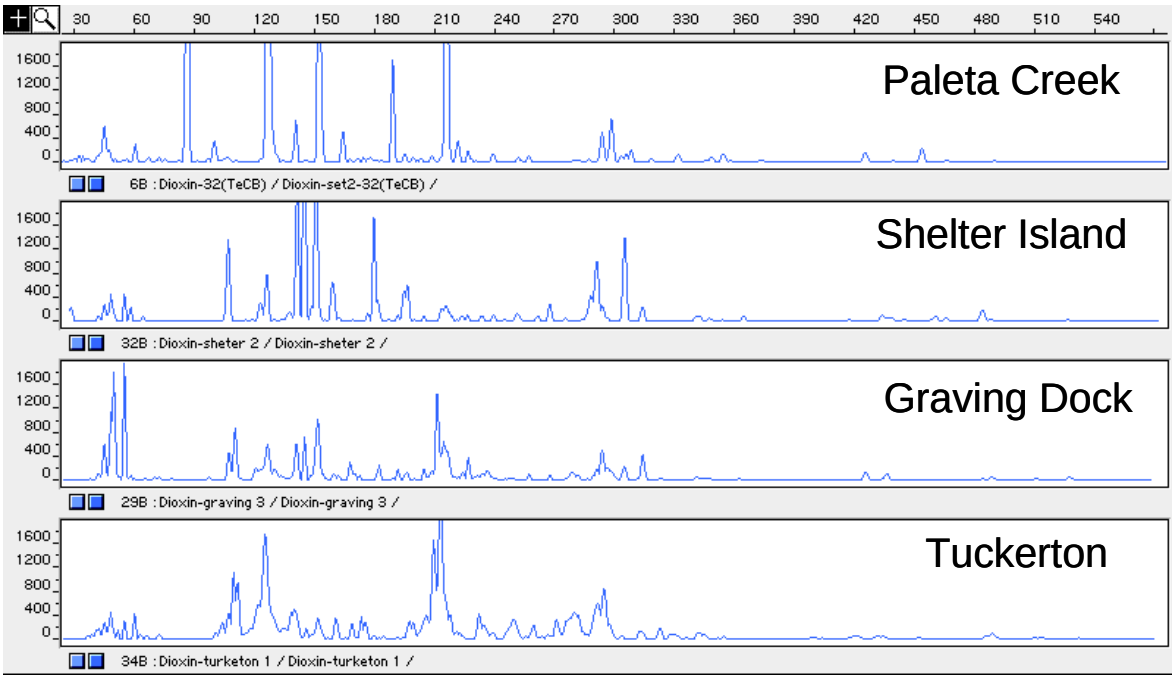

Fig. 19. 16S rRNA gene TRFLP analysis of 1,2,3,4-TeCDD dechlorinating cultures.

growth with 2-bromophenol, 2,6-dibromophenol, or 2-iodophenol with lactate or formate as an electron donor. Interestingly, this strain is capable of simultaneous sulfidogenesis and reductive dehalogenation in the presence of sulfate.

Terminal restriction fragment length polymorphism (TRFLP) analysis of 1,2,3,4-TeCDD dechlorinating cultures is shown in Fig 19. Distinct populations were enriched with each halogenated co-amendment and from historically contaminated versus less contaminated or non-contaminated sediments. The analysis shows that the addition of structural analogues of chlorinated-*p*-dioxins could serve to selectively grow and/or stimulate dehalogenating organisms. TRFLP analysis also suggests the enrichment of *Dehalococcoides*-like populations (Fig. 20), but this needs to be confirmed using other methods, such as quantitative PCR with selective primers.

The use of functional genes (Rhee et al. 2003) to elucidate dehalogenating microorganisms in consortia or natural environments is an alternative to the indirect method using ribosomes. Recently, several membrane bound reductive dehalogenases (RDH) have been purified and characterized (see Holliger et al. 2003). Several sets of degenerate PCR primers to amplify RDH gene fragments have been designed and putative RDH genes have been cloned and identified (e.g. Rhee et al. 2003; Ahn et al. 2003, unpublished; Fig. 21). The amino acid sequences deduced from the C-terminal region of the enzymes were compared and showed high similarity. However, several of these putative RDH genes were linked to transposon-like sequences and may, in fact, be pseudogenes (Rhee et al. 2003). Thus, additional assays must be done at the mRNA level to identify expressed RDH genes in natural samples in order to monitor dehalogenating populations.

Using these combined methods we have found that 16S rRNA genes from the *Chloroflexi* were present in cultures highly enriched with PCDDs and tetrachlorobenzene. This finding supports current evidence that the dechlorinating *Chloroflexi* exhibit a wide range of halogenated electron acceptors and suggests that these organisms are widely distributed in the environment.

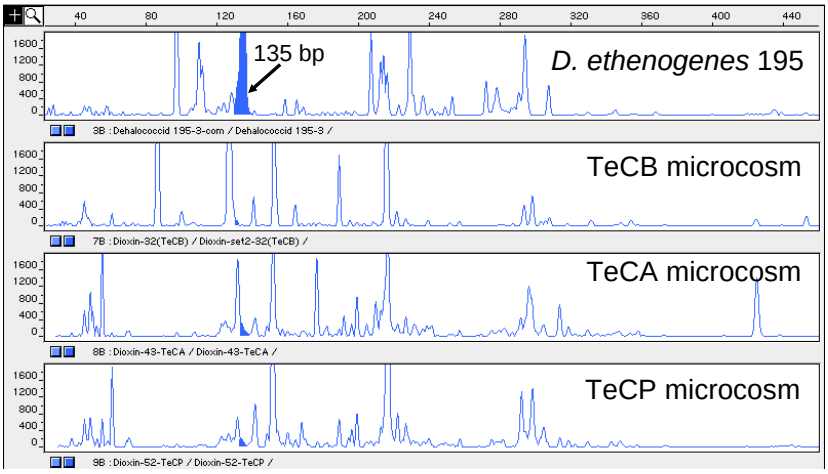

Fig. 20. Comparison of TRFLP fingerprints of TCDD-dechlorinating Paleta Creek cultures enriched with TeCB, TeCA or TeCP as cosubstrates. D. ethenogenes as the predominant member of a mixed culture has a TRF of 135 bp.

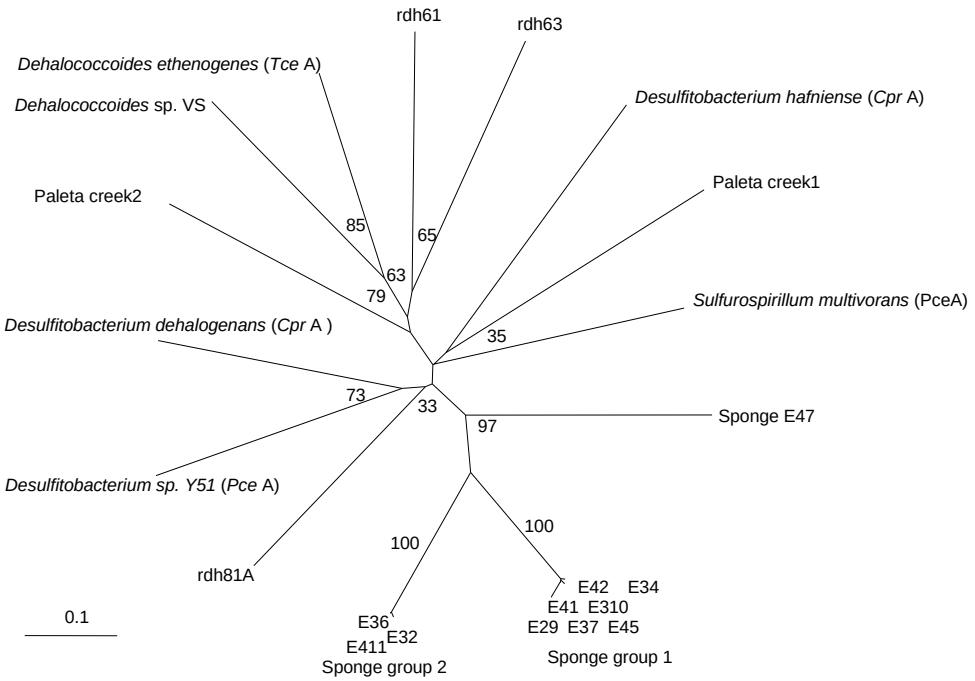

Fig. 21. Phylogenetic tree of deduced partial amino acid sequences of reductive dehalogenases. Reference bar indicates 10 amino acid exchanges per 100 amino acids.

These molecular tools/methodologies are further being refined for rapid monitoring of specific microbial populations responsible for respiratory dehalogenation of organohalide contaminants. The goal is to have a "toolbox" of rapid techniques to detect, enumerate and estimate the activity of specific bacterial populations for extensive bioremediation monitoring.

4. Bioprocess Modeling to Delineate the Probably Role of Alternate Electron Acceptors and Electron Donors on PCDD Dechlorination

Addition of simple bromophenol (BP) compounds was found to stimulate PCDD dechlorination in two different sediments [Arthur Kill (Vargas et al. 2001) and San Diego Bay, Paleta Creek]. As we have now seen from a more extensive analysis of haloprimer effectiveness, halogenated benzenes and anisoles were far more effective when compared to the effectiveness of BPs. Yet, BPs appeared to be slightly more effective than phenol or chlorophenols. BPs may potentially stimulate dechlorination of PCDDs by two potential mechanisms. First, BPs occur naturally in marine systems and these simple compounds may act as a surrogate electron acceptor for dehalogenating bacteria that can also use PCDDs as an electron acceptor. Since BPs may be added at concentrations many times that of PCDD, their addition allows the stimulation and growth of a large dehalogenating population that may then simultaneously dehalogenate the PCDDs. Secondly, BPs are dehalogenated to phenol, which is then degraded to acetate and hydrogen. These ultimate degradation products may then serve as simple electron donors (over and above that supplied by the sediment organic matter) for PCDD dehalogenation.

We used a modeling approach to further characterize the potential mode of action of 2-BP in stimulating PCDD dehalogenation in Paleta Creek sediments. We measured the prevailing

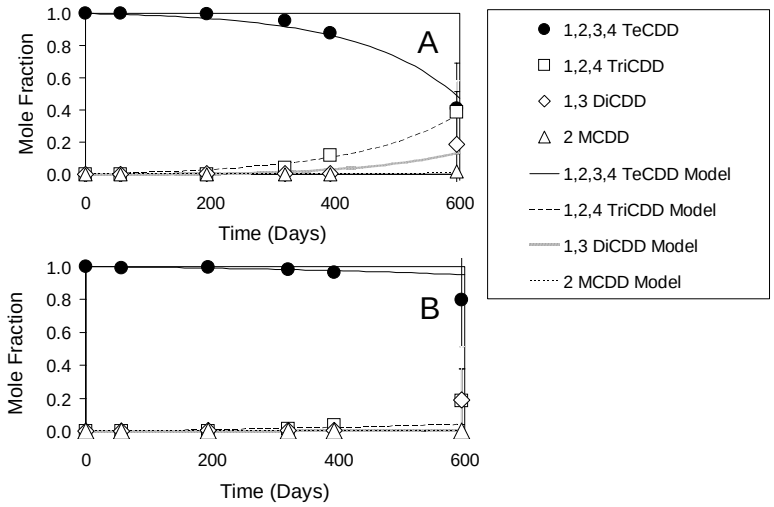

Fig. 22. Model simulations of time course data for dechlorination of 1,2,3,4-TeCDD in Paleta Creek sediments for unamended sediment slurries under (a) methanogenic and (b) sulfate-reducing conditions. Simulations were carried out assuming prevailing hydrogen concentrations for each condition.

hydrogen levels in sulfidogenic and methanogenic enrichments from Paleta Creek. The aqueous hydrogen concentration averaged 5.5 nM in methanogenic enrichments and 1.6 nM in sulfidogenic enrichments. Those hydrogen concentrations are similar to those reported for redox zones dominated by methanogenesis or sulfate reduction, respectively, as the dominant terminal electron-accepting process (see for example Lovley et al. 1994).

Using the assumptions outlined in the methodology section, the model was fit to the (-2BP) methanogenic data set for 1,2,3,4-TeCDD dechlorination (see end point data in Section 1, Fig. 4) using an inverse modeling approach in which the initial dechlorinating microbial biomass content of the sediment was varied to obtain the best fit of the model to the data assuming that the prevailing hydrogen concentration was controlled by methanogenesis at 5.5 nM (Fig. 22a). The same set of kinetic parameters, and biomass concentrations were then used and a new simulation was run wherein the hydrogen level was held at the concentration observed under sulfate reducing conditions, 1.6 nM (Fig. 22b). This exercise, in essence tests whether the prevailing hydrogen level—controlled by either methanogenesis or sulfate reduction—dictates the different observed rates of PCDD dechlorination in methanogenic and sulfidogenic enrichments. The new simulation fit the (-2BP) sulfate reducing data set for 1,2,3,4-TeCDD dechlorination reasonably well (Fig. 22b). This suggests that assumption that hydrogen concentration imparts extensive control over observed rate of PCDD dehalogenation is not unreasonable.

Next, simulations were performed for the (+2BP) methanogenic data set. Two scenarios were explored. In the first, we assumed that the only mode of action of 2BP was the production

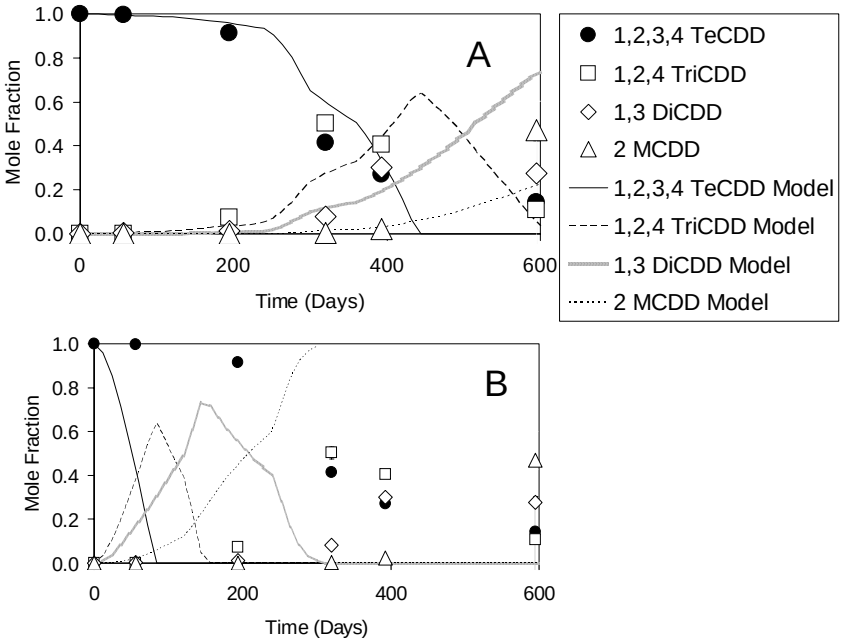

Fig. 23. Model simulations of time course data for dechlorination of 1,2,3,4-TeCDD in Paleta Creek sediments amended with 2BP and assuming (a) 2BP serves only as a hydrogen source and (b) 2BP serves as a growth substrate for PCDD dechlorinators.

of phenol which then became a source of hydrogen (via its fermentation). Thus, the input of 2BP directly resulted in pulse inputs of hydrogen which stimulated the PCDD dechlorinators. The simulation was carried out using the same set of kinetic parameters and starting biomass as for the simulations shown in Fig. 22 and includes only the additional pulse hydrogen inputs. This simulation adequately fit the data reflecting the observed dechlorination of 1,2,3,4-TeCDD over approximately 2 years (Fig. 23a).

In the second simulation scenario for the (+2BP) methanogenic data set, we assumed that not only did 2BP supply hydrogen to stimulate the PCDD dehalogenators, but that some portion of it went to directly support the growth of PCDD dehalogenators as an alternate electron donor. Assumption that just 5 percent of the added 2BP supported PCDD dehalogenator growth, resulted in a vastly overestimated simulation of the observed rate of dechlorination of 1,2,3,4-TeCDD (Fig. 23b).

These modeling explorations also seem to confirm that the *primary* mode of stimulation of PCDD dehalogenation by BPs is as a source of hydrogen (or electron donor) not as an alternate electron acceptor. Furthermore, prevailing hydrogen concentrations, as controlled by the prevailing electron accepting process—methanogenesis or sufidogenesis—appears to exert significant control over the kinetics of PCDD dechlorination.

5. Effect of System Heterogeneity on Dechlorination of PCDD

The slow rate of PCDD dehalogenation is directly related to the hydrophobic nature of the compounds and their very low aqueous solubility (Table 3). The low solubility intrinsically limits both our ability to study PCDD-dehalogenating organisms and limits the application of dehalogenation as a bioremedial technology.

Table 3. Aqueous solubility of selected CDD congeners.

Dioxin Congener	Aqueous Solubility (µg/L)	Aqueous Solubility (nM)
1,2,3,4-tetrachlorodibenzo-*p*-dioxin	0.63	1.96
2,3,7,8-tetrachlorodibenzo-*p*-dioxin	0.2	0.62
2,3-dichlorodibenzo-*p*-dioxin	14.9	52
2-chlorodibenzo-*p*-dioxin	318	1270

Results after 5 months of incubation comparing sediment and non-sediment carrier systems (Fig. 24) showed that the addition of HPγCD did significantly increase the amount of 1,2,3,4-TeCDD associated with the (unfiltered) aqueous phase (treatments c and e in Fig. 25). Furthermore, the amount of PCDD associated with the unfiltered aqueous phase increased over a period of agitated incubation between 5 and 30 days. It should be noted that the observed aqueous-phase associated PCDD levels were already far higher in most cases than the solubilities reported in Table 3. The aqueous phases were not filtered or centrifuged prior to extraction for

Sediment | Glass + Cyclodextrin | HM-nonane | HM-nonane +Cyclodextrin

Fig. 24. Photograph of 160-mL serum bottles containing 1 mg of TeCDD and 50 mL of culture medium: containing 1 g sediment carrying TeCDD; TeCDD applied directly to the glass surface and amended with 5 g/L HPγCD in the medium; TeCDD carried in 1 mL HMN; and TeCDD carried in 1 mL HMN and amended with 5 g/L HPγCD in the medium.

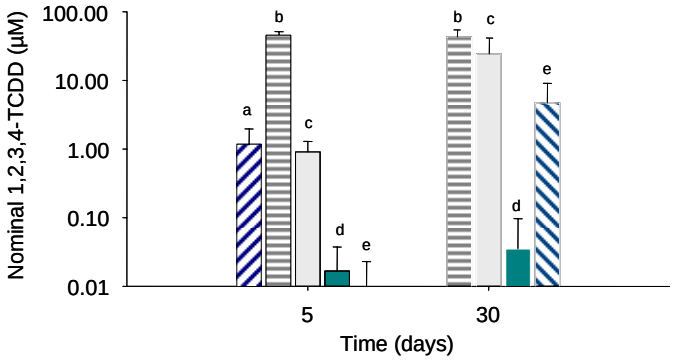

Fig. 25. Concentration of 1,2,3,4-TeCDD in 160-mL serum bottles containing 1 mg of TeCDD and 50 mL of culture medium: (a) the unfiltered aqueous phase of bottles containing 1 g sediment carrying TeCDD (measured only at Day 5); (b) mixed sediment/aqueous phases of bottles containing 1 g sediment carrying TeCDD(expected total conc., 62 µM); (c) the unfiltered aqueous phase of bottles with TeCDD applied directly to the glass surface and amended with 5 g/L HPγCD in the medium; (d) the unfiltered aqueous phase of bottles with TeCDD carried in 1 mL HMN; and (e) the unfiltered aqueous phase of bottles with TeCDD carried in 1 mL HMN and amended with 5 g/L HPγCD in the medium. Data shown are the averages from at least four separate bottles and the error bars represent the standard deviation.

PCDD. The higher apparent concentrations are likely the result of sediment fines, iron-sulfide precipitates and cells, as well as the HPγCD, carrying PCDDs. Dissolution of the 1,2,3,4-tetra-CDD from HMN to the aqueous phase, (in the absence of HPγCD) was not significant (Fig. 25, treatment d).

Unfortunately, dehalogenation of the TeCDD associated with the cyclodextrin was only a trace of that observed in bottles where the TeCDD was added to sediment. 1,2,3,4-TetraCDD was dechlorinated completely to 2-monoCDD within about 5 months in the cultures where TeCDD was added to sterile sediment. However, only a trace of 1,2,4-triCDD was formed in all other treatments within the same time period (data not shown). These finding could indicate that the concentration of HPγCD added (5 g/L) was inhibitory to the dehalogenating organisms, or that the TeCDD associated with the HPγCD was not “bioavailable” to the organisms. Further work is needed to determine whether other biocarrier molecules or surfactants would be useful in enhancing PCDD dehalogenation.

Table 4. Dechlorination after 5 months under system conditions meant to foster enhanced bioavailability.

Dioxin Carrier	Biocarrier	Electron Donor	Inhibitor	PCDD Dehalogenation
Soil (Killed)	-	-	-	-
Soil	-	**Bromophenols**	-	+
Glass	Cyclodextrin	H_2 (+acetate)	-	-
	Cyclodextrin	H_2 (+acetate)	BES	-
	Cyclodextrin	H_2 (+acetate)	BES + Ampicillin	-
Heptamethylnonane	-	H_2 (+acetate)	-	-
	-	H_2 (+acetate)	BES	-
	-	H_2 (+acetate)	BES + Ampicillin	-
Heptamethylnonane	Cyclodextrin	H_2 (+acetate)	-	-
	Cyclodextrin	H_2 (+acetate)	BES	-
	Cyclodextrin	H_2 (+acetate)	BES + Ampicillin	-
Soil	-	**H_2 (+acetate)**	-	+
	-	H_2 (+acetate)	BES	-
	-	H_2 (+acetate)	BES + Ampicillin	-

Conclusions:

Reductive dehalogenation is a promising mechanism for the removal of toxic organohalides from estuarine and marine sediments. Anaerobic dehalogenation of halogenated aromatic contaminants, such as PCDD/Fs, was readily promoted in estuarine and marine sediments from several sites. Co-amendment with more soluble halogenated compounds as "priming" agents greatly speeds PCDD/F dechlorination in anaerobic sediments. This stimulation was brought about in the presence of added electron donors lactate and propionate ensuring reduced conditions and adequate reducing equivalents to stimulate dechlorination. In highly organic sediments with adequate reducing power, it is possible that these halogenated additives may be just as effective without additional electron donors. While the process has much potential, a key aspect of the technology – the nature and capability of the intrinsic microbial community – is often unknown. The use of molecular tools for monitoring microbial activity coupled with the understanding of how redox processes affect dehalogenation may be used as a rapid screening method for determining whether a site is a good candidate for bioremediation and to tailor a bioremediation strategy for that site.

The addition of alternate halogenated compounds as "priming" agents, has previously been shown to enhance dechlorination of PCBs and PCDDs (Beurskens et al. 1995; DeWeerd and Bedard 1999; Albrecht et al. 1999; Cho et al. 2002). For example, brominated compounds stimulated the dechlorination of PCBs in freshwater sediments (Bedard et al. 1998; DeWeerd and Bedard 1999), monobromo-dibenzo-*p*-dioxin stimulated dechlorination of historically contaminated PCDDs (Albrecht et al. 1999), and BPs stimulated dechlorination of PCDDs in estuarine sediments (Vargas et al. 2001). This approach has also been used with some success in small scale field studies (Bedard 2003).

Our results (Vargas et al. 2001; Fennell et al. 2004a; Ahn et al. 2005) suggest that halogenated aromatic compounds with structural similarity to 1,2,3,4-TeCDD/F stimulate bacteria with the ability to dechlorinate chlorinated dibenzo-*p*-dioxin and furans. Of critical importance is that these amendments stimulate the desirable lateral dechlorination of PCDD/Fs (removal of chlorines at position 2 and 3) that ultimately results in detoxification. Also, PCDD dechlorination should be more easily stimulated in sediment previously contaminated with other chlorinated compounds than in pristine sediments. This study coupled with findings that some species of dehalogenating bacteria have multiple dehalogenases and dechlorination capabilities suggests that halogenated compounds other than CDD/Fs could be used to enrich and isolate organisms that have activity on CDD/Fs. Identification of naturally-occurring or less toxic halogenated co-amendments that are completely degraded in the sediment environment is desirable for field application.

Biostimulation may be achieved through the addition of electron donors and/or of halogenated co-amendments that serve as more bioavailable electron acceptors. Hydrogen is used by many dehalogenating bacteria and has also been shown to enhance dechlorination of PCDD residues in contaminated sediments (Albrecht et al. 1999). Hydrogen can be supplied directly or indirectly, at different biologically maintained levels, through the addition of fermentable organic compounds that serve as hydrogen donors (Fennell et al. 1997, 1998;

Fennell and Gossett 2003; Yang and McCarty 1998). In strict competition for hydrogen, dehalogenating bacteria can out-compete sulfate reducers. For example, the hydrogen threshold achieved by tetrachloroethene-dechlorinating populations in estuarine sediments was 0.5 nmol, less than that of the hydrogen level achieved by sulfate reduction in the same system (Mazur and Jones 2001). Löffler et al. (1999) provided clear evidence for the role of hydrogen threshold concentrations in determination of electron accepting processes, also showed that thermodynamic constraints, i.e., uptake and energy yield, favor utilization by dehalorespirers in the presence of organohalide substrates. Clearly, all of these factors are important to consider when attempting to stimulate dehalogenation *in situ*.

We have demonstrated that *D. ethenogenes* strain 195 has the potential to dechlorinate many different types of chlorinated aromatic compounds, in addition to its known chloroethene respiratory electron acceptors (Fennell et al. 2004b). *D. ethenogenes* strain 195 dechlorinated 1,2,3,4-tetrachlorodibenzo-*p*-dioxin to a mixture of 1,2,4-trichlorodibenzo-*p*-dioxin and 1,3-dichlorodibenzo-*p*-dioxin. 2,3,4,5,6-Pentachlorobiphenyl was dechlorinated to 2,3,4,6- and/or 2,3,5,6-tetrachlorobiphenyl and 2,4,6-trichlorobiphenyl. 1,2,3,4-Tetrachloronaphthalene was dechlorinated primarily to an unidentified dichloronaphthalene congener. Results obtained after re-amendment of cultures with PCE suggest that the dehalogenation observed was not necessarily dependent on on-going PCE dehalorespiration since no concomitant increase in the rate of chloroaromatic dechlorination was observed. Dechlorination of 1,2,3,4,7,8-HxCDF, one of the seventeen regulated PCDD/F congeners, by *D. ethenogenes* strain 195 to produced non-2,3,7,8-substituted daughter products is an important finding. Use of this strain to detoxify environmentally relevant PCDD/F congeners suggests that the *Dehalococcoides* species that can be enriched and grown relatively easily on substrates such as tetrachloroethene may have utility under some circumstances for use in bioaugmentation to detoxify aquatic sediments.

Dehalococcoides ethenogenes strain 195 dechlorinates tetrachloroethene to vinyl chloride and ethene and its genome has been found to contain up to 17 dehalogenase gene homologues (Seshadri et al. 2005) suggesting diverse dehalogenation potential. Detection of multiple dehalogenase homologs in other strains (Hölscher et al. 2004) provides further evidence for the metabolic versatility of this phylogenetic group. Within the Chloroflexi, which includes both *Dehalococcoides* spp. and other yet to be described genera, there appears to be several strains with the ability to reductively dechlorinate PCBs and other halogenated compounds as electron acceptors (Wu et al. 2002a, b; Bunge et al. 2003; Watts et al. 2001; Miller et al. 2005).

Our finding that *D. ethenogenes* strain 195 has the ability to dehalogenate such a diverse array of organohalides is significant given the prevalence of *Dehalococcoides*-like organisms in the environment. Bunge et al. (2003) have shown that *Dehalococcoides* sp. strain CBDB1, which was originally cultivated on trichlorobenzene (Adrian et al. 2000), dehalogenates and sustains growth on chlorinated dioxins. Highly enriched cultures containing strains DF-1 and o-17, bacteria distantly related to *Dehalococcoides*, are able to dehalogenate chlorinated biphenyls (Wu et al. 2002a; Cutter et al. 2001) and DF-1 also dechlorinates the chlorinated benzenes (Wu et al. 2002b). Aside from these reports, little is known about the anaerobic micro-organisms that mediate reductive dechlorination of PCDD/Fs, which is required to initiate complete degradation of highly chlorinated congeners (Toussaint et al. 1998; Gruden et al. 2003), or how to enhance the growth of these organisms under field conditions. Microbial reductive dehalogenation in

sediments is an important environmental process because it has the potential of decreasing the toxicity of organohalides such as PCDD/Fs. Dehalogenation may also be advantageous in designing contaminant treatment trains, since lesser chlorinated congeners are more susceptible to subsequent aerobic degradation.

Cyclodextrins are cyclic oligosaccharides that have a hydrophilic shells and toroidal hydrophobic cavities and are capable of including hydrophobic molecules in their cavities (Wang et al. 1998; Ko et al. 1999; Bardi et al. 2000). Cyclodextrins are available with different cavity volumes. Cyclodextrins are used widely in medicine and food science applications because they are considered non-toxic and are biodegradable. HPγCD has an inclusion cavity of 0.51 nm3, which might be expected to accommodate dioxin congeners. Dioxin molecules have molecular volumes ranging from 0.353 nm^3 (monochlorinated) to 0.458 nm^3 (tetrachlorinated) to 0.597 nm^3 (octachlorinated) (Zhang & Gobas 1995).

2,2,4,4,6,8,8-Heptamethylnonane (HMN) has been used as a nonaqueous phase reservoir for poorly soluble or toxic substrates during the enrichment of microorganisms (Holliger et al. 1992). HMN provides a constant flux of substrate into the aqueous phase. We also investigated HMN as a carrier for dioxin both with and without the presence of HPγCD.

Several researchers have reported success using cyclodextrins to increase the biodegradation of aromatic and polyaromatic compounds such as naphthalene (Wang et al. 1998; Ko et al. 1999; Bardi et al. 2000). Other studies have documented an increase in the overall mass of chlorinated solvent DNAPL flushed from contaminated aquifers when cyclodextrins are used as carriers (McCray & Brusseau 1998; Boving et al. 1999). In initial studies, we found that cyclodextrin did not enhance dioxin dechlorination in dilute aqueous enrichments. It is not know whether this was caused by inhibition by cyclodextrin, or whether dioxin associated with the cyclodextrin was not available to the organisms.

Sediment treatment is currently limited primarily to dredging with *ex situ* treatment or sequestration. New methods are needed for *in situ* containment and degradation of contaminants and could decrease the cost of long-term sediment management. Contaminated sediments often contain complex mixtures of pollutants, including PCBs, PCDD/Fs, chlorinated pesticides, halogenated flame retardants, polycyclic aromatic hydrocarbons (PAHs) and various heavy metals and the challenge of remediating the sites is significant. Our fundamental microbial studies are revealing the potential for remedial applications in aquatic systems and providing a foundation for development of integrated microbial and engineered approaches to enhance *in situ* bioremediation. Anaerobic reductive dehalogenation offers the most promising approach towards eventual detoxification and complete degradation of halogenated contaminant mixtures. *In situ* bioremediation combined with in-place containment through capping could avoid the problematic redistribution of contaminants that is associated with dredging and, where feasible, offer a more cost effective treatment alternative to dredging. Developing amendment technologies for enhanced microbial dehalogenation and understanding how amendment placement and mixing stimulates dehalogenation and impacts the fate and transport of organohalide mixtures is thus a high priority for the successful management of contaminated sediments.

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Appendices

List of Technical Publications

1. Articles and Papers in Peer-reviewed Journal

Rhee S-K, Fennell DE, Häggblom MM, Kerkhof LJ (2003) Detection of reductive dehalogenase motifs in PCR fragments from a sulfidogenic 2-bromophenol-degrading consortium enriched from estuarine sediment. FEMS Microbiol. Ecol. **43:**317-324.

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2. Technical Reports

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5. Text Books or Book Chapters

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