

Bioremediation and Detoxification of Polychlorinated Dioxin Contaminated Environments

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Environmental PCDD contamination jeopardizes human health and requires economically feasible remediation that detoxifies the environment. Recent studies have shown the bacterial degradation of toxic PCDDs, indicating that PCDD contamination could be addressed via bioremediation. In order to implement successful bioremediation of PCDDs, three areas of research need to be developed. First, the functions of putative dechlorinases need to be defined, beginning with reductive-dehalogenase-homologous genes. Second, the search for novel PCDD RDases and angular dioxygenases needs to continue with emphasis on molecular isolation techniques. Third, an effective implementation procedure should be developed for soil, water, and flyash environments.

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Keywords

dioxin, PCDD, dechlorinase, reductive dehalogenase, dioxygenase, *Dehalococcoides*, *Sphingomonas*

Abbreviations

PCCD - polychlorinated dibenzo-*p*-dioxin
 CDD - chlorinated dibenzo-*p*-dioxin
 ARD - anaerobic reductive dechlorination
 DDG - dioxin detoxifying gene
 RDase - reductive dechlorinase
 PCB - polychlorinated biphenyl
 PAH - polyaromatic hydrocarbons
 ZVI - zero valence iron
 PCDD congeners - mono-, di-, tri-, tetra-, penta-, hexa-, hepta-, octochloro-dibenzo-*p*-dioxins will be designated as MCDD, DCDD, TrCDD, TCDD, PeCDD, HCDD, HpCDD, and OCDD respectively

Introduction

Environmental contamination of polychlorinated dibenzo-*p*-dioxins (PCDDs), or dioxins, poses “one of the most challenging problems in environmental science and technology” [1] because of their toxicity, persistence, and bio-unavailability [2•]. Dioxin contamination is important because the compound is carcinogenic [3]. PCDDs are released into the environment from a variety of sources including: combustion, incineration, pulp and paper manufacturing, pesticides, and some natural sources [4]. Figure 1 shows the structure of the compound, and many different chlorinated dibenzo-*p*-dioxin (CDD) congeners are defined by differing number of chlorine substituents and location of substitution [5]. Dioxin congeners will be abbreviated as listed above. The release of dioxins into the environment has resulted in an estimated 500,000 tons of contaminated soil that need treatment [6]. Currently, PCDD contaminated sites are remediated only by physical and chemical processes that are very expensive (large remediation projects cost from \$100 to \$500 million) [7]. Studies have demonstrated that these remediation practices are not sustainable, because of high cost and complex logistics related to containment or relocation of PCDDs as compared to the potential benefits of PCDD destruction [7]. Bioremediation has been examined as a technique to degrade or detoxify dioxins at a lower cost.

Microbial degradation of dioxins has been studied extensively, and takes place through anaerobic reductive dechlorination (ARD) or through aerobic dioxygenation [2•, 5, 8•, 9]. ARD takes place in anaerobic microbial environments when a chlorine atom is removed and replaced with a hydrogen atom [10] (see Figure 2A). The chlorinated compound acts as the terminal electron acceptor, and “*Dehalococcoides*” can save energy from the dechlorination process [8•].

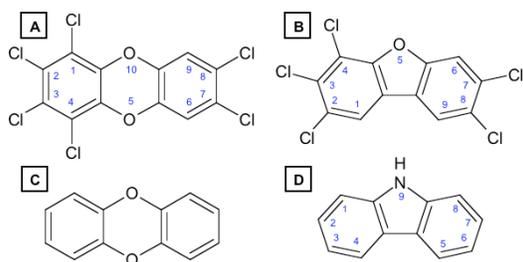


Figure 1. Structures of compounds. A) 1,2,3,4,7,8-hexachlorodibenzo-*p*-dioxin (HCDD); B) 2,3,4,7,8-pentachlorodibenzofuran (PeCDF); C) Dibenzo-*p*-dioxin (DD); D) carbazole (CAR). Different congeners (species) of these compounds are named by listing the locations of the chlorine substitutions. Adapted from: Chang YS, 2008, *J Mol Microbiol Biotechnol*, 15:152-171.

Table 1 presents a summary of observed microbial dechlorination of CDDs. Dechlorination rates decrease with increasing chlorine substitution, and complete dechlorination of the compound by bacteria has not been observed. Recently, it was shown that toxic 1,2,3,4,7,8-HCDD was dechlorinated by “*Dehalococcoides*” *ethenogenes* strain 195 to less toxic congeners [11••]. In aerobic environments, microbes can degrade the compound through aerobic oxidation and subsequent cleavage of the aromatic rings. The oxidation pathway in Figure 2B shows that after both ether bridges are broken, a chloro-catechol and a six-carbon chain are formed. Table 2 presents a summary of observed aerobic oxidation of PCDDs by bacteria. Increased chlorine substitution decreases the rate of oxidation [12]. Recently, toxic 1,2,3,4,7,8-HCDD was shown to be oxidized to less toxic compounds [13]. *Sphingomonas* (especially strain RW1), and *Pseudomonas* are the most efficient PCDD oxidizers [2•].

It is critical that microbial degradation of PCDDs results in a less toxic product. 2,3,7,8-TCDD is the most toxic congener. Other congeners with all four lateral chlorines are also highly toxic. The compound decreases in toxicity when any lateral chlorine is removed [3], or when the aromatic structure is broken [5]. For this reason, lateral dechlorination is of great importance because it results in a detoxified product [11••]. Anaerobic dechlorination and

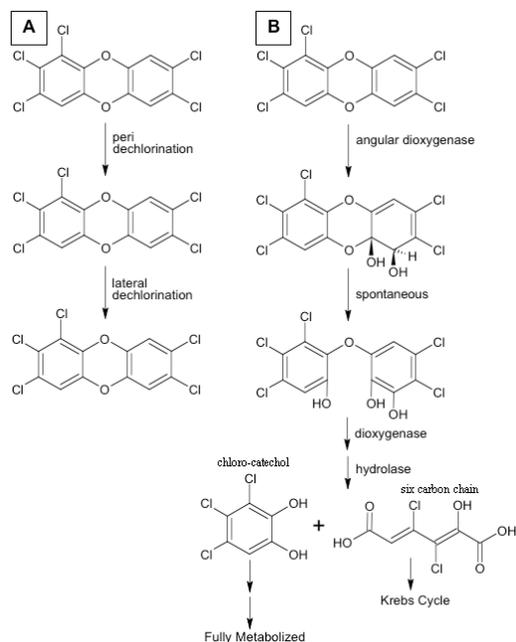


Figure 2: Proposed PCDD dechlorination and oxidation pathways. Pathway A shows *peri*- and lateral-dechlorination of 1,2,3,7,8-PeCDD. Pathway B shows the oxidation of 1,2,3,7,8-PeCDD by the indicated enzymes. These pathways are shown for illustrative purposes, and are not confirmed. Adapted from Field, 2008 [2•]; Wittich, 1998 [9]; and Nam, 2006 [13].

aerobic angular dioxygenation detoxify PCDDs, and the genes involved in these two reactions will be called dioxin detoxification genes (DDGs) for convenience in this paper.

Bioremediation of PCDD

PCDDs are subject to microbial degradation and detoxification as demonstrated in Tables 1 and 2, but much advancement is needed in order to develop a successful PCDD bioremediation strategy. First, putative, or alleged, dechlorinases need to be described. Second, the isolation of novel DDGs should continue with increased emphasis on using molecular techniques. These two steps will describe PCDD degrading enzymes more fully. Then, an effective implementation procedure should be designed for the contaminated environment (soil, water sediment, or flyash) with a plan to promote the expression of PCDD degrading enzymes. When these steps are taken, bioremediation of PCDDs could make the transition from the laboratory bench to the field.

PCDD Compound	Microbial Culture	Degrad. Rate (% remov. / time)	Genes Involved	Products ^d	References of Interest
23-DCDD	<i>Dehalococcoides</i> sp.CBDB1	53% / 28 days	PDGs ^b	2-CDD	Bunge <i>et al.</i> [20●]
123-TrCDD	<i>Dehalococcoides</i> sp.CBDB1 Anaerobic consortium ^c	60% / 57 days	PDGs	23-/ 13-DCDD, 2-MCDD	Bunge <i>et al.</i> [20●] Ballerstedt <i>et al.</i> [28]
124-TrCDD	<i>Dehalococcoides</i> sp.CBDB1 Anaerobic consortium ^c	55% / 57 days	PDGs	13-DCDD, 2-MCDD	Bunge <i>et al.</i> [20●] Ballerstedt <i>et al.</i> [28] Bunge <i>et al.</i> [29●]
1234-TCDD	Anaerobic enrichment ^c Methanogenic enrichment ^c Sulfate reducing <i>Dehalococcoides</i> sp.CBDB1 <i>D. ethanogenes</i> strain 195 ^c	24% / 84 days	PDGs	124-TrCDD 13-/ 23-DCDD 2-MCDD	Bunge <i>et al.</i> [20●]
12378-PeCDD	<i>Dehalococcoides</i> sp.CBDB1	75% / 84 days	PDGs	2378-TCDD, DCDD, 237-TrCDD	Bunge <i>et al.</i> [20●]
123478-HCDD	Mixed culture containing <i>D. ethanogenes</i> 195	10% / 200 days	PDGs	1378-/ 1248-TCDD	Liu [11●●]

^a Adapted from Field and Alvarez [2●].

^b Putative dehalogenase genes. We cannot implicate a specific gene or genes to the dechlorination of PCDD/Fs. Only genes similar to *tceA* or *pceA* have been implicated.

^c The culture was first enriched on another chlorinated electron acceptor

^d General product list and not specific to one culture. Products are listed in decreasing order of rate of formation.

Table 1. Critical studies that have demonstrated the successful dechlorination of PCDDs. The table shows the compound, the organism found to degrade it, the degradation rate, the genes involved, and the formed products. It is important to note that the specific gene(s) relative to PCDD dechlorination have not been isolated, and no study has shown the complete dechlorination of PCDD under anaerobic conditions.

Function of putative dechlorinases

“*Dehalococcoides*” sp. has been identified as the most successful PCDD dechlorinator [8●], but we cannot implicate a specific gene or genes related to PCDD dechlorination [14●●]. Progress is being made to describe these reductive dechlorinases (RDases). The entire genomes of two dioxin dechlorinators “*Dehalococcoides*” *ethanogenes* [15] and “*Dehalococcoides*” sp. strain CBDB1 [16] were both published in 2005. Two tetrachloroethene RDases were characterized and named *pceA* and *tceA*. About 50 unique sequences that shared the characteristic features of these two genes were found among “*Dehalococcoides*” sp. strains CBDB1, FL2, BAV1 and 195 and were termed reductive-dehalogenase-homologous (RDH) genes. The function of these genes has not been described [14●●]. Determining if these genes encode for PCDD RDases would allow us to probe for these genes in PCDD contaminated environments by real-time PCR or microarray, to monitor their expression, and the extent of microbial PCDD dechlorination.

This technique has been used to study polychlorinated biphenyl (PCB) biodegradation. “*Dehalococcoides*” was found to dechlorinate Aroclor 1260, a mixture of PCBs. Quantitative real-time PCR indicated that the “*Dehalococcoides*” population increased by nearly two orders of magnitude in the presence of Aroclor 1260 [17●].

Isolation of novel dioxin detoxification genes (DDGs)

Notwithstanding recent observation of highly chlorinated dioxin detoxification [11●●, 13, 18], the known diversity of DDGs is narrow. Dehalogenating bacteria of all chloro-organic compounds are phylogenetically diverse [8●], but efficient PCDD dechlorinators are limited to the “*Dehalococcoides*” group. Efficient PCDD oxidizers are mainly only *Sphingomonas* and *Pseudomonas* (see Tables 1 and 2). Sipila *et al.* has claimed, “... we are unfortunately only in the beginning of grasping the overwhelming diversity of bacteria involved in biodegradation in soil”

[19••]. PCDD contaminated environments likely harbor many other DDGs that remain unknown.

Multiple factors may contribute to why our knowledge of DDGs is limited. First, PCDD degradation is generally very slow in nature and yields little biomass production, which may contribute to the seemingly elusive nature of DDGs [14••]. Second, most studies have attempted to screen environmental samples to a pure culture that can detoxify PCDDs [20•]. It might be that dioxin detoxifying organisms rely on other organisms to provide substrates, and/or provide enzymes for reactions further down the pathway (biphenyl dioxygenase, hydrolase, chloro-catechol degrading genes), and thus screening to a pure culture may result in a loss of the PCDD degrading cultures. For these reasons it may be necessary to localize DDGs by way of molecular methods, before culturing and enrichment procedures. This type of procedure was followed in the description of RDH genes. Probing PCDD contaminated environments with broad primers from conserved regions of known angular dioxygenases may lead to the discovery of novel angular dioxygenases. Iwai *et al.* used a broad primer set targeting biphenyl/toluene dioxygenases together with pyrosequencing to obtain over 900 unique dioxygenase sequences in historically contaminated environmental samples, demonstrating large biodiversity of dioxygenases in the environment [21•].

Effective implementation procedure

PCDDs are found in surface soils, water sediments, and incinerator flyash, and each environment presents unique challenges in the implementation of PCDD bioremediation. An effective implementation procedure will promote growth of microbes of interest and the expression of DDGs under the environmental conditions of the contaminated site.

Biostimulation and bioaugmentation have been shown to promote the growth of microbes of interest and the expression of

DDGs. Biostimulation is the practice of amending the environment with a necessary growth substrate or co-substrates, and bioaugmentation is the inoculation of the environment with a microorganism, or microbial consortium known to have the desired degrading ability. 1,2,3,4-tetrachlorobenzene has been shown to increase the dechlorination rate of 1,2,3,4-TCDD by native microbial communities compared to samples with only a growth substrate [22]. Ahn *et al.* tested the affect of bioaugmentation (with "*Dehalococcoides*" *ethenogenes* strain 195) and biostimulation (with 1,2,3,4-tetrachlorobenzene, and 2',3',4'-trichloroacetophenone) on the dechlorination of PCDDs, and at the same time monitored community and gene dynamics. They found that heavily contaminated sites harbored more indigenous dechlorinators than less contaminated sites, by denaturing gradient gel electrophoresis (DGGE). Dechlorination at the heavily contaminated sites was not greatly enhanced by biostimulation and bioaugmentation, while at less contaminated sites dechlorination was enhanced by these methods [23].

Surface soils

PCDDs are generally only found in the upper portions of the soil profile because the compound is extremely insoluble in water (water solubility equals 0.019 ppb) [2•], which prevents the compound from leeching or moving with ground water. Surface soils are generally aerobic and converting a large environment of this type to an anaerobic state to promote ARD would be difficult and expensive. Therefore, ARD in surface soils is likely to not occur at a significant rate, but dioxygenation would be the most common mode of dioxin detoxification by bacteria.

Biodegradation of PCDDs with dioxygenation as the only designed mode of detoxification would result in the persistence of a significant fraction of toxic dioxin congeners, because dioxygenation of HpCDD and OCDD has not been observed [2•]. In this situation, it may be helpful to employ a chemical treatment simultaneously. Zero valence iron (ZVI) has been shown to rapidly dechlorinate highly

PCDD Compound	Microbial Culture	Degrad. Rate (% remov/time)	Genes Involved	Products	References of Interest
1-/ 2-MCDD	<i>Rhodococcus, Bacillus, Pseudomonas, Sphingomonas, Burkholderia, Terrabacter, Klebsiella, others</i>	75% / 2 days	CARDO ^b DFDO ^c DDDO ^d	chlorocatechol, chloro acids, hydroxyl-CDD	Habe et al. [30] Wittich et al. [9]
2-/ 3-/ 4-MCDF	<i>Sphingomonas, Terrabacter, Klebsiella, Pseudomonas, Burkholderia, others</i>	88% / 1 day	DFDO	chlorosuccinyl acids, CBPs	
23-/ 27-/ 28-DCDD	<i>Sphingomonas, Pseudomonas, Rhodococcus, Terrabacter, P450 enzyme</i>	42% / 4 days	CARDO DFDO DDDO	chlorocatechols, chloroketone, CDDs	Habe et al. [30] Sulistyaningdhah [31] Hong et al. [32]
123-/ 237-TrCDD	<i>Spingomonas wittichii</i> RW1, <i>Pseudomonas</i> sp. EE41, P450 enzyme	31% / 30 days	CARDO DFDO DDDO	polychlorocatechol, polychloroethers, hydroxy PCDD	Nam et al. [13] Sulistyaningdhah [31] Habe et al. [30]
1234-TCDD	<i>Sphingomonas wittichii</i> RW1, <i>Pseudomonas veronii</i> PH-03	15% / 5 days	DDDO DFDO	polychlorocatechol, polychloroguaiacol	Hong et al. [32]
2378-TCDD	<i>Pseudomonas testosteroni</i> G1036, <i>Bacillus magaterium</i>	60% / 244 days	DFDO	hydroxy-TeCDD, unidentified	
123478-HCDD	<i>Spingomonas wittichii</i> RW1	10% / 5 days	DDDO	tetrachlorocatechol, others	Nam et al. [13]

^a Adapted from Field and Alvarez, (2008). All listed oxidations utilized another compound (dibenzofuran, carbazole, others) as the growth substrate.

^b carbazole 1,9a dioxygenase [30]

^c dibenzofuran 4,4a dioxygenase [30]

^d dibenzo-*p*-dioxygenase [9]

Table 2. Critical studies that demonstrated oxidation of PCDDs with aromatic ring cleavage. The table shows the compound, the organism(s) found to degrade it, the degradation rate (an average of many studies), the genes involved, the products formed, and references. The three genes listed here are angular dioxygenases, and dioxin dioxygenase is the only that directly attacks PCDD. Mono-chloro-dibenzofuran (MCDF) oxidation is listed to show its oxidation properties relative to MCDD.

chlorinated PCDDs, even OCDD. This process was carried out in an anaerobic environment in conjunction with PCDD dechlorinating bacteria [5]. It is not known if this process would function in an aerobic environment, but if it does, highly chlorinated PCDDs could be dechlorinated by ZVI, and the products would be subject to dioxygenation, which may result in a highly detoxified environment.

Another promising technology in surface soils would be the use of phytoremediation and rhizoremediation. Phytoremediation is the removal and/or degradation of pollutants by

plants, and rhizoremediation is the degradation of pollutants by soil microbes that grow very close to plant roots. As of 2007, there was no documentation of dioxin uptake by plants from the soil [24•]. A study by Jou *et al.* reported dioxin uptake by tappa (*Boussonetia papyrifera*) in highly contaminated soils, and by *Physalis angulata* in low contaminated soils [24•]. Dioxins are taken up at lower levels in zucchini cultivars [25] and some other annual and perennial plants [26]. The mechanism of PCDD accumulation was not stated. It is possible PCDDs are more bioavailable in the rhizosphere of these plants, which allows for

its uptake. In a study by Sipila *et al.*, it was determined that the microbial community structure, as determined by terminal restriction fragment length polymorphism (TRFLP), changed with the addition of PAHs and the cultivation of birch trees. The diversity of dioxygenases was greater in the rhizosphere compared to the bulk soil. The PAH degrading microbial community in the bulk soil was 48.5% dissimilar from the PAH degrading microbial community in the rhizosphere. This study outlines an excellent method of determining the diversity of genes that execute the desired function [19••]. Similar studies should be carried out in regards to PCDD dioxygenases of the rhizosphere. Sipila *et al.* also showed an increase in microbial diversity in the rhizosphere [19••]. This may be advantageous to PCDD oxidation, because as indicated in Figure 2B, many different enzymes are required in PCDD oxidation. These enzymes could be supplied by not just one key organism, but a variety of soil bacteria. The rhizosphere may provide a favorable environment for a diverse dioxin detoxifying microbial community.

Water sediments

PCDD contaminated soil particles can be eroded and deposited in rivers and lakes. These PCDD contaminated water sediments sink to the floor of the body of water and remain in an anaerobic environment. Such an environment would facilitate the ARD of PCDDs, but M/DCDD would remain (see Table 1 for the products of ARD reactions). There is no known mechanism to biodegrade M/DCDD further under anaerobic conditions. Thus, the detoxified M/DCDD product may be the best solution to PCDD contaminated sediments [20•].

Flyash

Flyash is a major source of environmental dioxin contamination [4]. PCDDs form chemically when organic matter is burned in the presence of chlorine and flyash is a residue after incineration. Nam *et al.* performed a series of experiments to test the degradability of PCDD in flyash by *Sphingomonas wittichii* strain RW1. The total organic carbon content of the flyash was

$0.0014 \pm 0.004\%$, which is very low compared to soil, indicating that the environment is nutrient depleted and a harse environment for microbial growth. In 2005, they observed 75.5% removal of all PCDD and 83.8% removal of 2,3,7,8-TCDD from flyash by way of degradation and adsorption onto live and dead cell biomass [27]. In 2008, a mix of 4 bacterial and 5 fungal strains were combined to form a dioxin-degrading biocatalyst in flyash. This biocatalyst degraded 68.7% of all PCDD and 66.8% of 2,3,7,8-TCDD substituted congeners. In this study it was shown that fungal strains provided extra cellular non-specific oxidases to degrade highly chlorinated congeners. These fungal oxidases are non-specific, meaning they are not specific to a single substrate, but can degrade an array of compounds, including very stable PCDDs and lignin [18•]. Flyash is the only environment that has shown fungi to be effective degraders of PCDDs *in situ*. This may be because the microbes were in a carbon-depleted environment and PCDDs were available for carbon and energy use. In soil, the total organic carbon content is higher, fungi are not effective PCDD degraders because they preferentially degrade higher energy yielding compounds [2•].

Conclusion

Current physical and chemical remediation of PCDD contaminated sites is not sustainable and bioremediation could be a more favorable alternative. There are two research initiatives that need to be completed before bioremediation is a viable option for PCDD clean-up. First, a higher percentage of toxic PCDD congeners need to be shown to be degraded by bacterial enzymes. Second, these laboratory methods must be successfully implemented in the field. The bacterial degradation of a few highly chlorinated congeners, and many mono-, and di-CDDs has been demonstrated. Recent studies have shown both the bacterial dechlorination and oxidation of 1,2,3,4,7,8-HCDD, which indicates that the bacterial enzymes may exist to degrade other toxic PCDD congeners, but have not yet been discovered, isolated, or characterized. Reductive-dehalogenase-homologous (RHD) genes are suspected to

dechlorinate PCDDs. Their function has not been confirmed, but is a likely area of future research. Use of broad primers from conserved regions of known angular dioxygenases together with molecular methods (real-time PCR, microarrays, pyrosequencing, DGGE, and TRFLP) to detect the expression of DDGs may lead to easier isolation and characterization of novel DDGs, rather than isolation through culturing methods. Once we have isolated a sufficient number of DDGs, *in situ* bioremediation strategies need to be developed. PCDDs exist in surface soils, water sediments and flyash. Aerobic surface soils would allow for aerobic dioxygenation, but would not allow for anaerobic reductive dechlorination (ARD). Rhizoremediation may encourage a greater dioxygenase diversity, and higher PCDD bioavailability to increase bioremediation rates. PCDDs in water sediments may be detoxified through ARD. PCDD contaminated flyash may best be detoxified by a bacterial and fungal biocatalyst, which has already been shown to be successful.

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