In situ remediation options for polychlorinated dibenzo-p-dioxins and dibenzofurans (PCDD/Fs) in Sydney Harbour sediments

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In situ remediation options for polychlorinated dibenzo-*p*-dioxins and dibenzofurans (PCDD/Fs) in Sydney Harbour sediments

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Abstract

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DNA sequencing revealed the presence of bacteria closely related to known PCDD/F degrading microbes belonging to the *Dehalococcoides* genus in the harbour sediments. Anaerobic enrichment cultures supplied with perchloroethene (PCE) as terminal electron acceptor stimulated the reductive dechlorination of the most toxic dioxin 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) to 2,3,7-trichlorodibenzo-p-dioxin (2,3,7-TriCDD) and the most abundant dioxin octachlorodibenzo-p-dioxin (OCDD) to its hepta- and hexa-chlorinated congeners. This is consistent with the existence of microbes capable of reductively dechlorinating PCDD in Sydney Harbour. The reasons for the lack of significant *in situ* PCDD/F biotransformation were considered. With 4500 and 10 times higher than the environmental concentrations, respectively, TCDD and OCDD partially but reversibly inhibited the microbial dechlorination activity. The low aqueous solubility of TCDD and OCDD likely limited the capacity of PCDD/F respiring bacteria dechlorinating these compounds and with the use of a biosurfactant lecithin, the microbial dechlorination of TCDD and OCDD was enhanced. These findings

demonstrated that applying indigenous microorganisms maybe part of the remedy for Sydney Harbour PCDD/F contamination.

Furthermore, this study explored the potential of using a known PCDD dechlorinating bacteria *Dehalococcoides* mccartyi strain CBDB1 and sulfidized nanoscale zerovalent iron (S-nZVI) in addressing PCDD degradation in conditions found in Sydney Harbour. The tolerance of strain CBDB1 to seawater environments was determined, with a view to its deployment into Sydney Harbour's contaminated sediments. Additionally, S-nZVI did not react with TCDD and OCDD, but it was capable of supplying H_2 and conditioning the redox potential necessary for strain CBDB1 organohalide respiration, with the potential to assist strain CBDB1 dechlorinating PCDD in Sydney Harbour.

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Abstract

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Furthermore, this study explored the potential of using a known PCDD dechlorinating bacteria *Dehalococcoides mccartyi* strain CBDB1 and sulfidized nanoscale zerovalent iron (S-nZVI) in addressing PCDD degradation in conditions found in Sydney Harbour. The tolerance

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List of Abbreviations

PCDDs	polychlorinated dibeno-p-dioxins
PCDFs	polychlorinated dibenzofurans
DD	dibenzo-p-dioxin
MCDD/F	monochlorinated dibenzo-p-dioxin/dibenzofuran
DCDD	dichlorinated dibenzo-p-dioxin/dibenzofuran
TriCDD	trichlorinated dibenzo-p-dioxin/dibenzofuran
TCDD	tetrachlorodibenzo-p-dioxin/dibenzofuran
PeCDD	pentachlorinated dibeno-p-dioxin/dibenzofuran
HexCDD	Hexachlorinated dibeno-p-dioxin/dibenzofuran
HepCDD	heptachlorinated dibeno-p-dioxin/dibenzofuran
OCDD	octachlorinated dibeno-p-dioxin/dibenzofuran
PCE	perchloroethene
TCE	trichloroethene
DCE	dichloroethane
VC	vinyl chloride
HCB	hexachlorobenzene
PeCB	pentachlorobenzene
TeCB	tetrachlorobenzene
TCB	trichlorobenzene
DCB	dichlorobenzene
CB	chlorobenzene
PCB	polychlorinated biphenyls
2,4-D	2,4-dichlorophenoxyacetic acid

2,4,5-T	2,4,5-trichlorophenoxyacetic acid
HHB	Homebush Bay
ORB	organohalide respiring bacteria
nZVI	nanoscale zerovalent iron
S-nZVI	sulfidized nanoscale zerovalent iron
WHO-TEQ	World Health Organization toxicity equivalent
TEF	toxicity equivalence factor
CBDB1	*Dehalococcoides mccartyi* strain CBDB1

Chapter One - Introduction

1.1 Overview

Polychlorinated dibenzo-p-dioxins (PCDDs) and dibenzofurans (PCDFs) are highly toxic, recalcitrant and carcinogenic environmental contaminants (Mandal, 2005). Their toxicity to humans and resistance to degradation have raised significant public concern (WHO, 2010; Wittich, 1998). Although the release of PCDD/Fs to the environment has been effectively controlled, their historical legacy persists in many environments, threatening human health and ecosystem function (Kulkarni *et al.*, 2008).

Sydney Harbour is severely contaminated with PCDD/Fs as a result of the frequent production and utilization of organochlorine products in Sydney (Banks *et al.*, 2016; Montoya, 2015). High concentrations of PCDD/Fs were detected in harbour sediments and seafood harvested from Sydney Harbour (Birch *et al.*, 2007; Manning *et al.*, 2017). The presence of PCDD/Fs in sediments and biota has resulted in a fishing ban and restrictions on recreational activity west of Sydney Harbour Bridge. Therefore, a practical and effective strategy for PCDD/F remediation in Sydney harbour is sought.

Bioremediation is considered a viable clean-up option for organohalide contaminated environments, superior to traditional "dig and dump" or "pump and treat technologies" (McCarty, 1993; Frascari *et al*., 2015), and PCDD/Fs are also amenable to biodegradation by microorganisms, suggesting that bioremediation technologies could be a viable solution *in situ* (Kulkarni *et al.*, 2008; Halden and Dwyer, 1997; Field and Sierra-Alvarez, 2008). The primary objective of this research study was to assess the feasibility of bioremediation technologies for PCDD/F detoxification and elimination in the context of Sydney Harbour sediments.

1.2 Polychlorinated dibenzo-p-dioxins and dibenzo-furans (PCDD/Fs)

1.2.1 What are PCDD/Fs?

PCDD/Fs are polychlorinated aromatic hydrocarbons (Figure 1.1) (US EPA, 2010). Their chemical structure consists of two benzene rings, connected with at least one ether bond and substituted with chlorine atoms (Halden and Dwyer, 1997).

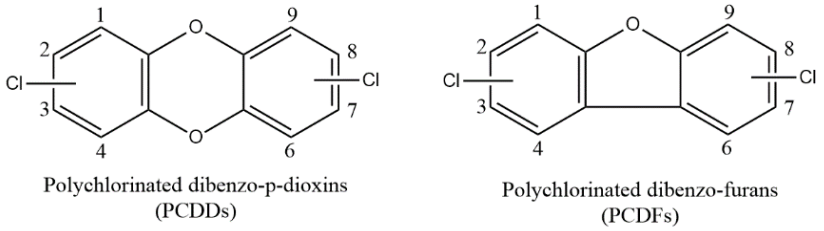

Figure 1.1 Chemical structure of PCDD/Fs (Wittich, 1998).

There are 210 different congeners in total, of which 75 are PCDDs and 135 are PCDFs. However, only congeners with chlorine substitution at 2,3,7,8- (lateral-) positions show dioxin-like toxicity. Therefore, 7 of the 75 PCDDs and 10 of the 135 PCDFs have toxic potential, whereas the toxicity of non-2,3,7,8 substituted congeners are negligible (Mandal, 2005; Rodenburg *et al.*, 2017; Van den Berg *et al.*, 2006). Among these 17 toxic congeners, 2,3,7,8-TCDD (Figure 1.2) is the most toxic, with only 0.6 μg/kg (body weight) required to kill a guinea pig (Pohl et al., 1998). In 1997, 2,3,7,8-TCDD was classified as a Group One 'Human carcinogenic' by the International Agency for Research on Cancer (IARC, 1997).

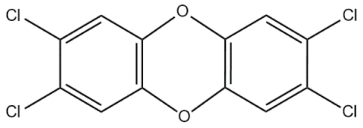

Figure 1.2 Chemical structure of 2,3,7,8-TCDD

PCDD/Fs are physically stable. They are solids at room temperature and their melting points range from 122 to 326 ℃ (Rordorf, 1989; Alessandro *et al.*, 1997; Mackay *et al.*, 2008). They also have very low volatility. Their vapor pressure ranges from 10^{-3} to 10^{-10} Pa at 298K. The vapor pressure of 2,3,7,8-TCDD is approximately 6.2×10^{-6} Pa at 298K (Li *et al.*, 2005).

PCDD/Fs are also stable owing to their chemical structure. The dibenzo-ring structure exerts an inductive effect to withdraw electron density to resist oxidation and hydrolysis (Anders, 1985; Halden and Dwyer, 1997). Therefore, they have very long half-lives and can remain in the environment for decades or even centuries (Montoya, 2015; Milbrath *et al.*, 2009). Aside from biodegradation, photodegradation by sunlight in the atmosphere is the only degradation process known in nature (Hart *et al.*, 1993).

One key feature of PCDD/Fs is their ability to bioaccumulate in organisms (Atkinson, 1991). PCDD/Fs are extremely lipophilic compounds, and readily bioaccumulate in adipose tissue and biomagnify via food-chains (Bokare *et al*., 2013; Zhao et al., 2018). The accumulation of PCDD/Fs in the human body usually leads to a wide range of adverse health effects including cancer. (Kahn *et al.*, 1988; Food Standards Australia New Zealand, 2004; Panteleyev and Bickers, 2006).

1.2.2 The history and source of PCDD/Fs

PCDD/Fs are widely distributed in the ecosystem, and were present even before human history (WHO, 2010). They can be generated in natural events such as volcanic eruptions or forest fires, but only to limited levels (Mandal, 2005). The contamination nowadays is a result of human activities over the last 200 years (Field and Sierra-Alvarez, 2008; Alcock and Jones, 1996). Their primary anthropogenic sources are chemical manufacturing and waste combustion (Kao and Wu, 2000l; Buekens and Zhang, 2016). PCDD/Fs are by-products of chemical manufacturing, particularly in chlorine industries. The utilization of these chemical products and the disposal of chemical wastes are their primary route to entering the environment (Safe, 1994). Another critical source is the emission of fly ash from incineration plants. The combustion of chlorine-containing materials under high temperature catalyses the formation of PCDD/Fs, especially when the waste contains chlorinated aromatic hydrocarbons (Hites, 2011; Frumkin, 2003; McKay, 2002).

The anthropogenic production of PCDD/Fs spans more than 200 years (White and Birnbaum, 2010). In the early 19^{th} century, people started to produce chlorinated products. German chemical factories began to use sodium chloride to produce sodium carbonate for washing soda products (Weber *et al.*, 2008). The manufacturing process resulted in the generation of PCDD/Fs, likely when hydrochloric acid gases reacted with coal tar in the oven (Balzer et al., 2007). An investigation was carried out after the factories were dismantled and found approximately 10^3 pg-TEQ/kg-soil PCDD/Fs at their waste deposit sites (Balzer et al., 2007; White and Birnbaum, 2010).

The environmental levels of PCDD/Fs dramatically increased in the 20^{th} century, particularly during the period of the 1930s to the 1970s, when polychlorinated biphenyls (PCBs) (Figure 1.3) were manufactured commercially at scale. PCBs were used in wood preservatives,

electrical apparatus, flame retardants, pesticides and herbicides, with manufacturing also generating PCDD/Fs (Tindall, 1985; White and Birnbaum, 2010; Rossberg *et al.*, 2006; Taylor, 1979).

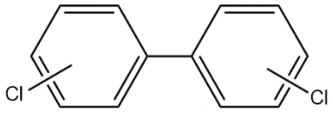

Figure 1.3 Chemical structure of PCBs (Rossberg et al., 2006)

The toxicity of PCDD/Fs was only recognized in the late 1890s, when workers from a German chloro-alkali factory were exposed to their chlorine products and developed painful, cystic and hyperkeratotic skin lesions. Von Bettman (1897) first described this in the literature, and Herxheimer (1899) hypothesized the skin lesions were caused by chlorine exposure and hence named it "Chloracne". Later, several accidents highlighted the danger of PCDD/Fs exposure. In 1947, American cattle developed serious chloracne after exposure to dioxin-like compounds (Bell, 1953). In 1957 in America, millions of chickens died from TCDD-contaminated feeds (Firestone, 1973). After an accident at a 2,4,5-trichlorophenol plant in America, where workers developed serious chloracne after exposure to chemical compounds, Kimmig and Schulz (1957) identified 2,3,7,8-TCDD (a by-product of 2,4,5-T synthesis) as the real chloracnegenic trigger. Other accidents include Yusho disease in 1968 and Yucheng in 1979 caused PCDD/F contaminated rice oil, and serious health problems among Vietnamese people after exposure to Agent Orange, which contains PCDD/Fs.

Through legislative regulations and scientific efforts, the emission of PCDD/Fs has been effectively controlled. In 1970, the manufacturing and the use of PCBs was banned in many countries (Loganathan and Masunaga, 2015). Other PCDD/Fs forming chemicals were sequentially banned in the following years (WHO, 2004 Gupta, 2014). In 1985, the U.S. Environmental Protection Agency (U.S EPA) setup health risk assessments on PCDD/Fs. In 2001, the Stockholm Convention provided the framework for global PCDD/Fs reduction (Stockholm Convention, 2008). Their efforts have prevented further PCDD/Fs emission and have significantly decreased human exposure over the past decades.

1.2.3 The toxicity of PCDD/Fs

PCDD/Fs can cause serious health problems (Coleman and Marks, 1999). Exposure to high concentration of PCDD/Fs causes skin lesions as described above. Long-term exposure to PCDD/Fs can lead to a range of toxicity, including increasing blood lipids, organ system dysfunction, reproductive and developmental defects and mental disability. They also act as carcinogen promoters and triggers (Montoya, 2015; WHO, 2010).

The dioxin toxicological effects mainly mediate via a protein called aryl hydrocarbon receptor (AhR). This protein is a transcriptional protein to regulate dioxin-mediated toxicity expression.(Bock & Köhle, 2009; Mandal, 2005). PCDD/F toxicity is dependent on the different binding affinity to AhR. Increasing chlorination on 2,3,7,8-lateral positions increases the binding affinity with the protein, while increasing chlorination on non-lateral positions weakens the affinity. It was hypothesized that the chlorination on non-lateral sites might result in increasing molecular size, volume and lipophilicity, but decreasing coplanarity and aromatic ring electron density, and thus weakened the binding affinity (Heuvel and Lucier, 1993; Safe, 1986).

In the 1980s, the U.S. EPA introduced the toxicity equivalence factor (TEF) methodology for PCDD/F quantification (US EPA, 2010). As they exist in mixtures, the TEF method can quantify a mixed PCDD/F concentration by choosing 2,3,7,8-TCDD as the index chemical and transfer other congeners into total toxicity equivalence (TEQ). Based on their toxicological data collected from *in vitro* and *in vivo* studies, structure-activity relationship and studies on biochemical changes, toxicity and carcinogenicity, each congener is assigned with a TEF, where the scaling factor of the index chemical 2,3,7,8-TCDD is 1.0. To calculate the total TEQ, individual concentration is multiplied by its TEF and then summed to get the total equivalent concentration of the mixtures. The equation is shown below (Equation 1.1) and the TEF of each congener is shown in Table 1.1:

$$TEQ = \sum_{i=1}^{n}(C_i \times TEF_i) \qquad \text{(Equation 1.1)}$$

Where C_i represents concentration of each congener (ng/kg).

Table 1.1 WHO Toxicological Equivalence Factors for human health risks (US EPA, 2010)

Congener	TEF
2378-TetraD	1
12378-PenCDD	1
123478-HexCDD	0.1
123678-HexCDD	0.1
123789-HexCDD	0.1
1234678-HepCDD	0.01
OctaCDD	0.003
2378-TetraF	0.1
12378-PenCDF	0.03
23478-PenCDF	0.3
123478-HexCDF	0.1
123678-HexCDF	0.1
234678-HexCDF	0.1
123789-HexCDF	0.1
1234678-HepCDF	0.01
1234789-HepCDF	0.01
OctaCDF	0.003

Studies demonstrated that higher chlorinated congeners such as OCDD and HepCDD are more abundant in the environment than less chlorinated congeners (Birch *et al*., 2007; Rodenburg *et al*., 2017; Sundqvist *et al*., 2009). However, these higher chlorinated congeners often contribute limited toxicity. For example, the toxicity of octachlorodibenzo-*p*-dioxin (OCDD) is 3000 times lower than 2,3,7,8-TCDD at the same concentration (US EPA, 2010). Therefore, using the TEF methodology gives a comprehensive and accurate assessment on the toxicity of PCDD/Fs.

1.2.4 Remediation Technologies for PCDD/Fs

Various technologies have been developed for PCDD/F remediation over the last decades. In general, these can be classified into: physical, chemical and biological categories (Frascari et al., 2015; Kao and Wu, 2000; Kulkarni et al., 2008).

Physical methods include land capping, thermal decomposition (Wait and Thomas, 2003), photolytic destruction (Zeng *et al*., 2017) and solvent extraction/adsorption (Freeman and Harris, 1995). Landfill capping is a common *in situ* remediation method achieved by

covering PCDD/F-contaminated soils or sediments with a layer of clean materials to obstruct PCDD/Fs further leaching into the environment (Lichtfouse *et al.*, 2013). This method had been applied to the most contaminated area in Sydney Harbour to cover but not remove the contaminants, so ongoing site management is required. Additionally, this method was too expensive to apply to the whole harbour (Birch *et al.*, 2013; Montoya, 2015). In contrast, thermal decomposition method is a common *ex-situ* method used for highly contaminated sites. In summary, PCDD/F-contaminated waste or soil were excavated from contaminated sites and transported to the thermal treatment plant. In a 600℃ oven, PCDD/Fs are evacuated from the solid-phase and sequentially decomposed to CO_2 and water when the oven temperature increases to 1000℃ (Vogg and Stieglitz, 1986; Lundin and Marklund, 2007). However, this method is not practical for extensively contaminated sites, due to its high cost and energy consumption (Rathna et al., 2018). Since the solar spectrum can cleave the C-Cl and C-O bonds on PCDD/Fs, photolytic destruction is a method that utilizes sunlight or UV light to transform and degrade PCDD/Fs, but it only operated in the laboratory and has not yet been applied in the field (Wu and Ng, 2008; Chittim, 2000). Soil washing is a method using solvents to assist PCDD/F separation and extraction from contaminated soils, which is usually followed by thermal treatment for PCDD/F decomposition (Vogg and Stieglitz, 1986).

Chemical methods are achieved by applying strong chemical reagents to oxidize or reductively dechlorinate PCDD/Fs (Strandberg and Odén, 2011). PCDD/Fs can be oxidized into CO_2 and water by strong oxidants such as hydroxyl radicals and Fenton reagents (Kao and Wu, 2000; Kulkarni et al., 2008). For the latter process, strong reductants such as zerovalent metal particles can reductively dechlorinate higher chlorinated PCDD/Fs into less chlorinated congeners to achieve PCDD/F detoxification (O'Carroll *et al.*, 2013; Hrabák *et al.*, 2016).

Zerovalent metals (ZVMs) have been applied for organohalide remediation for approximately 30 years (Gillham and O'Hannesin, 1994), exploiting the reducing power of ZVMs for the reductive dehalogenation of organohalides (Equation 1. 2). Nano sizing of ZVMs further enhances its dehalogenation rate by increasing its active surface area (Liu et al., 2005; Li et al., 2006). Therefore, nanoscale zerovalent metals (nZVMs) have been considered a remediation strategy for chlorinated aliphatic hydrocarbons (CAHs) (Gillham and O'Hannesin, 1994), chlorophenols (Tso and Shih, 2015; Li *et al.*, 2013), chlorobenzenes (Shih *et al.*, 2011; Zhu and Lim, 2007) and polybrominated diphenyl ethers (PBDEs) (Xu *et al.*, 2014).

$$\text{Metal (0)} + \text{R-X} + H^+ \rightarrow \text{Metal}^+ + \text{R-H} + X^- \quad \text{(Equation 1. 2)}$$

Previous studies have compared the capacity of nanoscale zerovalent iron (nZVI), nanoscale zerovalent zinc (nZVZ) and palladized nZVI (Pd/nZVI) on PCDD dechlorination. nZVZ can rapidly dechlorinate OCDD to 1,2,4,7-TCDD (Bokare et al., 2013) and Pd/nZVI can dechlorinate 1,2,3,4-TCDD to non-chlorinated dioxin (Kim et al., 2008), but nZVI showed no dechlorination in both studies. However, applying zinc and noble metals (such as Pd) for environmental remediation is not feasible due to their expense and toxicity (Lowry and Reinhard, 2000; Fosmire, 1990).

Recent studies have discovered that sulfidized nanoscale zerovalent iron (S-nZVI), encasing nZVI in an iron-sulfide shell, offers higher organohalide dehalogenation efficiency compared with nZVI (Kim et al., 2011; He et al., 2018; Li et al., 2017; Rajajayavel and Ghoshal, 2015). The sulfidation process uses dissociated sulfide (S^{2-}) to dissolve the passive iron hydroxide (FeOOH) outer layer and coat nZVI with the iron-sulfide layer [$Fe(OH)_2 + SH^- \rightarrow FeSH^+ + OH^-$], with conductivity (10-1000 $S \cdot cm^{-1}$) orders of magnitude higher than FeOOH (10^{-5} $S \cdot cm^{-1}$), thus enhancing the efficiency of electron transfer to organohalides (Yepez and Obeyesekere, 2017; Zhang et al., 2013; Xi et al., 2019; Lipczynska-Kochany et al., 1994; Butler and Hayes, 2001; He et al., 2008).

There are drawbacks for physical and chemical techniques. Firstly, *ex-situ* treatments are expensive, particularly the soil/sediment excavation and transportation, and the energy consumption for thermal oxidation. Secondly, using chemical reagents or solvent at contaminated sites may cause secondary contamination. Thirdly, based on economic and technological considerations, they are not suitable for large contaminated sites like Sydney Harbour (Halden and Dwyer, 1997; Kulkarni *et al.*, 2008; Strandberg and Odén, 2011).

Scientists have already identified certain PCDD/F-degrading microorganisms from contaminated sites. In the following section, this literature review focused on the biological transformation of PCDD/Fs.

1.3 PCDD/F biodegradation

Bioremediation of organochlorine is well established particularly for chlorinated aliphatics (Wittich, 1997; Marco-Urrea, 2015). This approach is a competitive alternative to physical/chemical approaches or can be combined effectively with physical /chemcial

approaches. It is cost-effective, sustainable with low energy consumption and has less interference with the environment (Tu et al., 2014).

Studies have shown that PCDD/Fs are also amendable to microbial biodegradation (Field and Sierra-Alvarez, 2008). Such investigations began as early as the 1970s. In 1972, Kearney *et al*. found that in two TCDD-contaminated sites in Hagerstown and Lakeland (U.S.), over 50% of TCDD was removed after one year, and they hypothesized this was caused by biodegradation. Matsumura and Benezet (1973) tested the ability of 100 pesticide-degrading microbial strains to degrade TCDD and found five were successful, including *Trichoderma viride* and *Pseudomonas putida*. Matsumura and Ward (1982) further investigated the degradation pathway of these five TCDD-degrading strains, by using labelled ^{14}C-TCDD and observed the generation of $^{14}CO_2$ by microbial activities. Since then, more and more PCDD/F degrading strains have been identified and isolated.

Under aerobic conditions, PCDD/Fs are amendable to oxidation by microorganisms such as *Sphingomonas* spp., *Pseudomonas* spp. and certain fungi (Field and Sierra-Alvarez, 2008). Scientists originally believed that PCDD/Fs could not be degraded under anaerobic conditions, because investigations on anaerobic sediments had shown insignificant decrease with respect to PCDD/F concentration. In 1983, an observation on the change of PCDD/F congener proportions in anoxic sediments indicated that microorganisms were also able to transform PCDD/Fs under anaerobic conditions, but utilizing different pathways to aerobic microbes, which was later identified as reductive dechlorination (Townsend, 1983; Field and Sierra-Alvarez, 2008).

1.3.1 Aerobic oxidation

As previously described in 1.2.1.1, the two aromatic rings on PCDD/Fs are connected by an ether bond. Certain aerobic microorganisms can catalyze the cleavage of these aryl-ether bonds, generating highly unstable hemiacetal intermediates such as chloro-cis-1,10a,dihydroxy-1-hydro-dibenzo-p-dioxin and chloro-cis-4,4a,dihydroxy-4-hydro-dibenzofuran (A & F, Figure 1.4). These intermediates can be further metabolized or co-metabolized into water and CO_2 (Nojiri and Omori, 2002; Halden and Dwyer, 1997; Wittich, 1998; Field and Sierra-Alvarez, 2008).

Approximately 90% of the known aerobic PCDD/F-degrading microorganisms work via co-metabolism (Field and Sierra-Alvarez, 2008). Several genera have been identified that are able to directly metabolize these compounds including *Sphingomonas* (Kertesz *et al.*, 2018), *Pseudomonas* (Cerniglia *et al*., 1979), *Burkholderia* (Arfmann *et al.*, 1997) and white-rot fungi (Field et al., 1993).

The genu *Sphingomonas* is widely distributed in soil, oceans and the subsurface environment, They are known to utilize hydrocarbon contaminants as their carbon and energy sources (Waigi *et al.*, 2015; Kertesz *et al.*, 2018). Some lineages of *Sphingomonas* can degrade PCDD/Fs via oxidation. For example, *Sphingomonas wittichii* strain RW1, isolated from a dioxin-contaminated river in Germany, can utilize non-, mono- and dichlorinated dibenzo-p-dioxins (DD) and dibenzofurans (DF) as sole carbon and energy sources (Wittich, Wilkes, Sinnwell, Francke, & Fortnagel, 1992; Wilkes, Wittich, Timmis, Fortnagel, & Francke, 1996); *Sphingomonas* sp. strain HH19k can degrade dichlorinated DF (Harms and Zehnder, 1995);

Members of the *Pseudomonas* genus have the capacity for PCDD/F degradation. *Pseudomonas mendocina* strain NSYSU, isolated from pentachlorophenol (PCP) contaminated sites in China, can degrade PCP as well as OCDD and OCDF (Kao et al., 2005; Tu et al., 2014); *Pseudomonas* sp. strain HH69 and HH27 is able to metabolize and co-metabolize DD (Fortnagel et al., 1990); *Pseudomonas* sp. strain PS12 has demonstrated capacity on degrading 2,3,7,8-TCDD and 2,3,7,8-TCDF (Sander et al., 1991).

Some lineages of white-rot fungus also have the capacity for PCDD/F degradation. For example, *Phanerochaete chrysosporium* can degrade 2,3,7,8-TCDD and 2,7-DCDD (Bumpus *et al.*, 1985; Valli *et al.*, 1999); *Phanerochaete sordida* YK-624 degrades tetra- to hexa-chlorinated DD and DF (Takada et al., 1996).

These PCDD/F-degrading microorganisms can generate a kind of regioselective dioxygenases called angular dioxygenase (Halden and Dwyer, 1997). The degradation path ways are described in Figure 1.4. The dioxygenase catalyzes the hydroxylation at the ether bond-carrying carbon and stimulates the formation of highly active phenolic hemiacetal intermediates (A and F) (Fortnagel *et al.*, 1989). These intermediates then spontaneously convert into chlorinated trihydroxydiphenyl ethers (B) or trihydroxydipenyls (G). Other dioxygenases sequentially catalyze the cleavage of their dihydroxylated aryl ring, leaving them susceptible to normal metabolic or co-metabolic degradation (Field and Sierra-Alvarez, 2008).

The angular dioxgenase plays the most vital role in PCDD/F aerobic biodegradation (Fortnagel *et al.*, 1989). The enzyme purified from *Sphingomonas* RW1 revealed the essential composition of this catalytic protein. The molecular mass is between 46.3 to 33.6 KDa, and it consists of a FAD-containing reductase, a [2Fe-2S] cluster and a terminal heterotetrameric oxidase (Armengaud and Timmis, 1997).

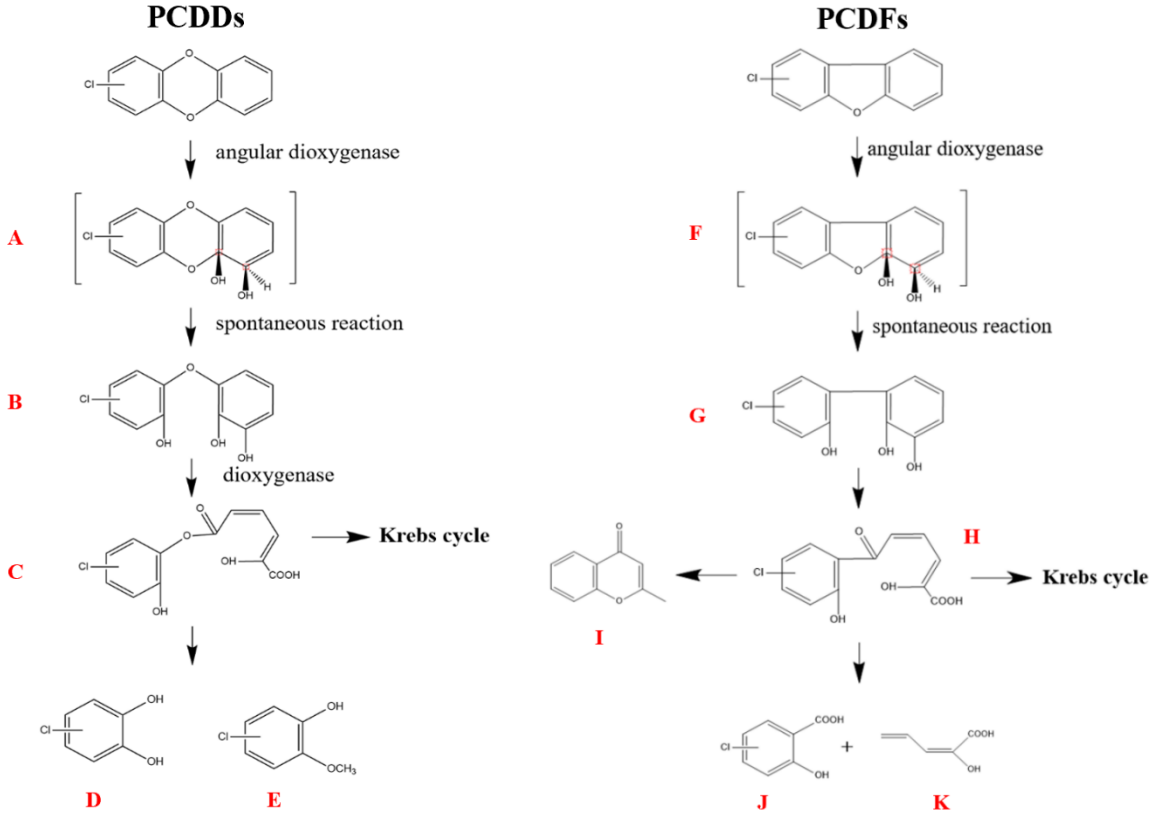

Figure 1.4 Aerobic microbial degradation of PCDD/Fs

A: chloro-cis-1,10a,dihydroxy-1-hydro-dibenzo-p-dioxin; B: chloro-2,2',3-trihydroxydiphenyl ether; C: 2-hydroxy-6-oxo-6-(chloro-2-hydroxyphenoxy-)-2,4-hexadie-noic acid; D: chlorocatechol; E: chloroguaiacol; F: Chloro-cis-4,4a,dihydroxy-4-hydro-dibenzofuran; G: chloro-2,2',3-trihydroxydiphenyl; H: 2-hydroxy-6-oxo-6-(chloro-2-hydroxyphenoxy-)-2,4-hexadienoic-noic acid; I: chloro-2-methyl-4H-chroman-4-one; J: chlorosalicylic acid; K: 2-hydroxypenta-2,4-dienoate (Wittich, 1998; NOJIRI and OMORI, 2002; Hong *et al.*, 2004; Field and Sierra-Alvarez, 2008)

1.3.2 Anaerobic reductive dechlorination

Under anaerobic conditions, organohalide respiring bacteria (ORB) can use organohalide compounds (organohalides) as terminal electron acceptors, and consume H_2, acetate, formate or lactate as electron donors for energy harvesting (DeWeerd et al., 1990).

This process catalyzes the replacement of chlorine substitution with a hydrogen atom on organohalides, which is known as reductive dechlorination (Maphosa *et al.*, 2010). Due to the constant anthropogenic utilization and release of organohalide products, ORB are significantly abundant in the environment, and thus have a high potential for use in bioremediation (Fincker and Spormann, 2017; Field and Sierra-Alvarez, 2008).

ORB are found in numerous genera, which include facultative ORB-*Desulfitobacterium*, *Sulfurospirillum*, *Desulfovibrio* and *Geobacter*, and obligate ORB-*Dehalobacter*, *Dehalogenimonas* and *Dehalococcoides* (Fincker and Spormann, 2017). They can dechlorinate a broad spectrum of chloroalkanes, including tetrachloroethene (PCE) and trichloroethene (TCE), chloroform (CF) and dichloromethane (DCM) and long-chain chlorinated hydrocarbons. For example, *Desulfitobacterium sp.* strain PCE (Gerritse et al., 1996) and *Dehalococcoides mccartyi* strain 195 (Fung et al., 2007), *Dehalococcoides mccartyi* strain VS (Müller et al., 2004) are known to dechlorinate PCE and TCE to less chlorinated ethenes or ethene (Figure 1.5); *Dehalobacter sp.* strain UNSWDHB dechlorinates CF to dichloromethane (Wong et al., 2016).

Aromatic organohalides are also amendable to microbial dechlorination. For example, *Dehalococcoides mccartyi* strain CBDB1 is capable of dechlorinating chlorophenols and chlorobenzenes (Adrian et al., 2007); *Dehalococcoides* strains CG1, CG4 and CG5 can dechlorinate a range of commercial PCBs (Wang et al., 2014); *Dehalobacter sp.* strain TeCB1 can dechlorinate tetra- and trichlorobenzene into chlorobenzene (Figure 1.6) (Alfán-Guzmán et al., 2017).

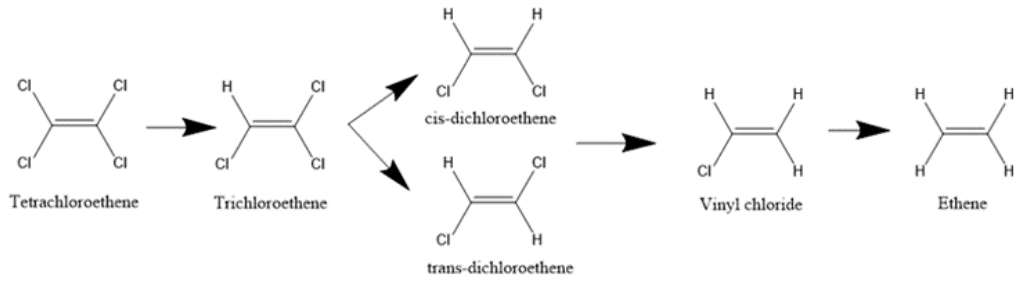

Figure 1.5 The microbial reductive dechlorination of PCE (Aulenta *et al.*, 2006)

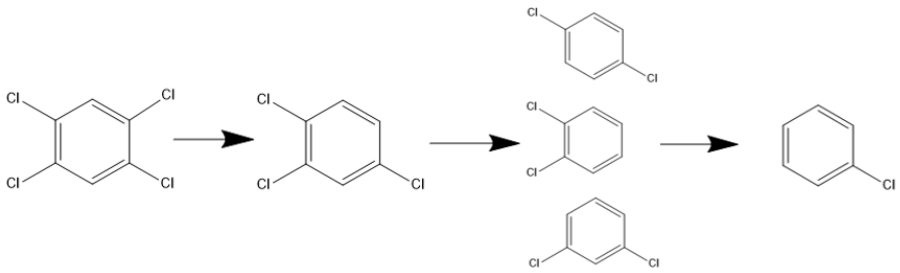

Figure 1.6 Reductive dechlorination of 1,2,4,5-TeCB (Alfán-Guzmán et al., 2017)

ORB cataylze the dehalogenation of organohalides via a group of oxygen-sensitive enzymes called reductive dehalogenases (RDases) (Hug et al., 2013; Parthasarathy et al., 2015). About 254 independent RDases have been identified, including PceA catalyzing the dechlorination of PCE to ethene (Neumann *et al.*, 1998), TceA catalyzing the dechlorination of TCE to vinyl chloride (Magnuson et al., 2000), BvcA and VcrA catalyzing the dechlorination vinyl chloride to ethene (Krajmalnik-Brown et al., 2004), CbrA catalyzing the dechlorination of chlorinated benzenes and chlorophenols (Adrian et al., 2007), TmrA catalyzing the dechlorination of Chloroform (CF) to dichloromethane (DCM) (Jugder et al., 2016) and CfrA catalyzing CF to DCM and 1,1,1-trichloroethane to 1,1-dichloroethane (Tang and Edwards, 2013).

Most of these RDases have similar characteristics (McMurdie *et al.*, 2011; Jugder *et al.*, 2015). Their molecular weights range from 35 to 65 kDa (Bommer et al., 2014). They have two important subunits: generally RdhA and RdhB (Neumann *et al.*, 1998; Jugder *et al.*, 2015). RdhA is a catalytic subunit, responsible for cataylsing electron transfer, whereas RdhB is a membrane-anchoring subunit protein, adjusting RdhA with cytoplasmic membrane (Futagami *et al.*, 2008; Hug et al., 2013). The structure of RdhA involves two Fe-S clusters at the C terminus and a twin-arginine (TAT) signal sequence at the N terminus (Schumacher *et al.*, 1997; Maillard *et al.*, 2003; Berks, 1996). The Fe-S clusters are responsible for electron transfers during dehalogenation (Van De Pas et al., 1999). The TAT signal sequence triggers the translocation of the enzyme across the cytoplasmic membrane (Palmer *et al.*, 2005). RdhA also contains a corrinoid cofactor at the catalytic center (Futagami *et al.*, 2008), a derivative of cyanocobalamin (vitamin B12), which is the redox mediating substance for electron transmissions (Field and Sierra-Alvarez, 2008; Jugder *et al.*, 2015). Other downstream Rdhs,

such as RdhC and RdhT, are involved in the regulation of genes encoding and dehalorespiration reactions (Kube et al., 2005).

Studies have found that PCDD/Fs are also amendable to microbial reductive dechlorination under anaerobic conditions, catalysing higher chlorinated PCDD/Fs to less chlorinated congeners (Townsend, 1983; Beurskens *et al.*, 1995; Field and Sierra-Alvarez, 2008). Further methanisation of these dechlorinated products by methanogenic consortia eventually eliminate PCDD/Fs from the environment (Dwyer and Tiedje, 1986). Several PCDD/F-dechlorinating cultures have been identified, including three pure *Dehalococcoides* strains: *D. mccartyi* strain 195 (Fennell et al., 2004a), *D. mccartyi* strain CBDB1 (Bunge et al., 2003) and *D. mccartyi* strain DCMB5 (Pöritz et al., 2015a).

1) *D. mccartyi* strain 195

D. mccartyi strain 195 has broad dechlorinating capacities, including dechlorination of PCE and TCE into ethene (Maymó-Gatell et al., 1997), as well as many chlorinated aromatic contaminants, such as chloronaphthalene, hexachlorobenzene (HCB) and PCBs (Fennell et al., 2004). Fennell *et al.* (2004) discovered that *D. mccartyi* strain 195 had dechlorinating capacity on 1,2,3,4-TeCDD, of which 1,2,3,4-TeCDD was dechlorinated to 1,2,4-TriCDD and then to 2,4-DiCDD (Figure 1.7). Zhen *et al.* (2014) later found that it was also able to dechlorinate 1,2,3,7,8-PeCDD to 1,3,7,8-TeCDD, terminating at 1,3,7- and 1,3,8-TriCDD, without generating 2,3,7,8-TCDD (Figure 1.8).

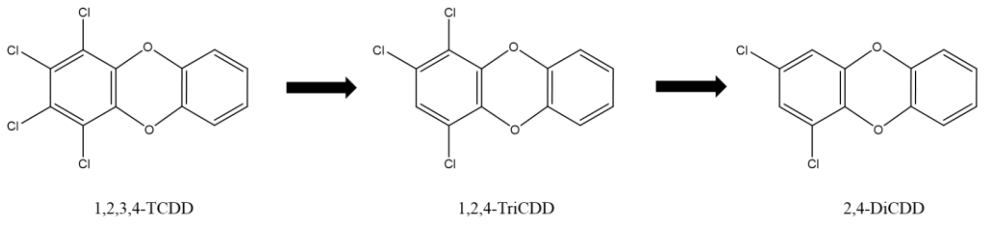

Figure 1.7 Reductive dechlorination of 1,2,3,4-TeCDD by D. *mccartyi* strain 195 (Fennell et al., 2004c)

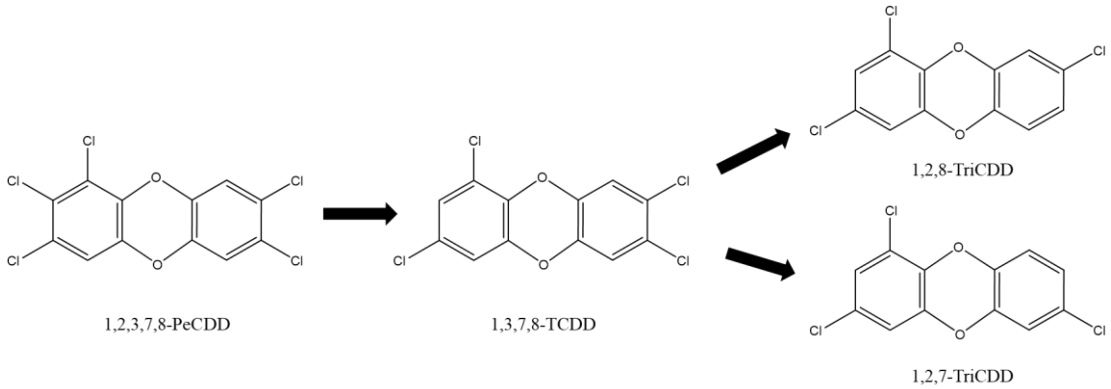

Figure 1.8 Reductive dechlorination of 1,2,3,7,8-PeCDD by D. *mccartyi* strain 195 (Zhen et al., 2014)

2) *Dehalococcoides mccartyi* strain CBDB1

D. mccartyi strain CBDB1 is capable of dechlorinating chlorinated benzenes (Adrian *et al.*, 2000) and chlorophenols (Van De Pas et al., 1999). Bunge et al. (2003) found it could als dechlorinate 1,2,3,4-TeCDD to 1,2,4- and 1,2,3-TriCDD. 1,2,4-TriCDD was further dechlorinated to 1,3-DiCDD and 2-MCDD, while 1,2,3-TriCDD was dechlorinated to 2,3-DiCDD, 1,3-DiCDD and further to 2-MCDD, as well as dechlorinated 1,2,3,7,8-PeCDD to 2,3,7,8-TCDD and 1,3,7,8-TCDD, and then to 2,3,7-TriCDD and 2,7-/2,8-DiCDD (Figure 1.9). Importantly, *D. mccartyi* strain CBDB1 is that it is the only known ORB to dechlorinate 2,3,7,8-TCDD.

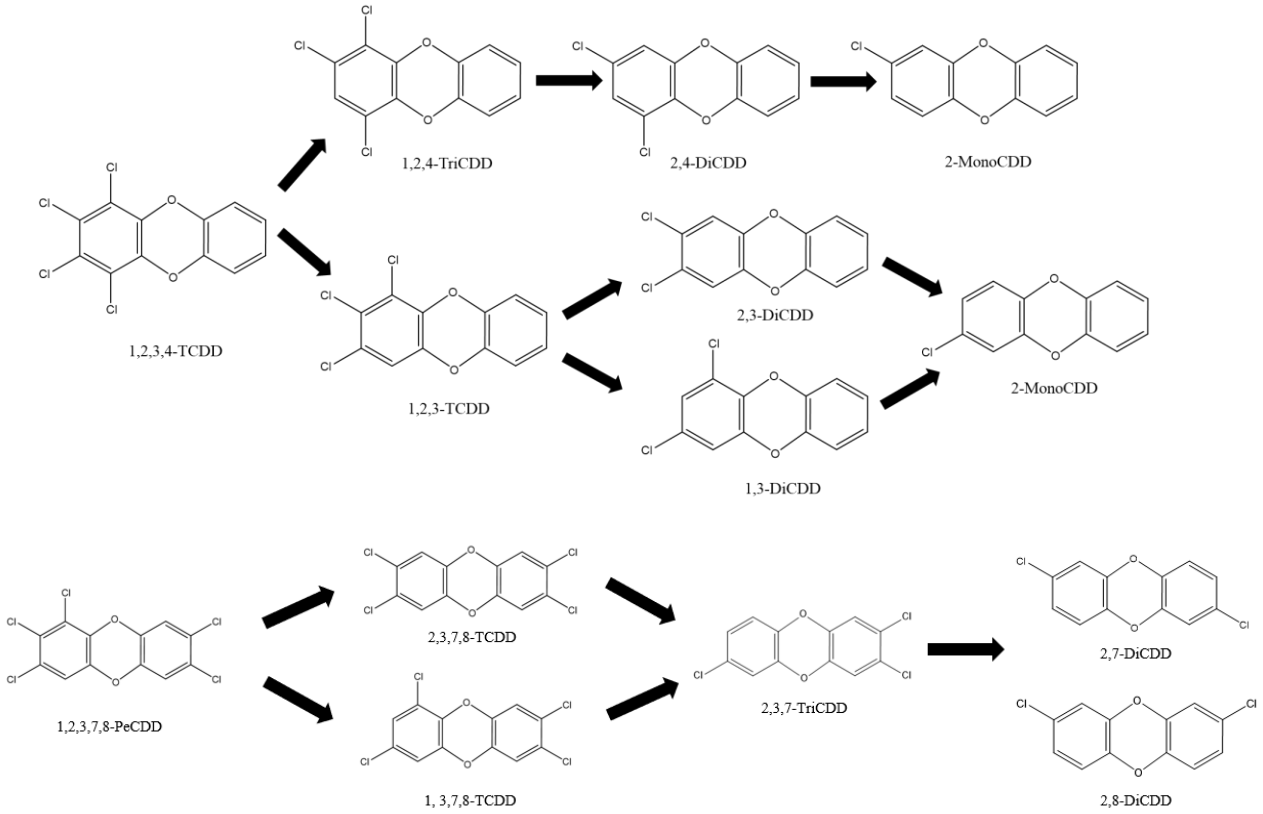

Figure 1.9 Reductive dechlorination of 1,2,3,4-TCDD and 1,2,3,7,8-PeCDD by *Dehalococcoides* CBDB1 (Bunge *et al*., 2003)

3) *Dehalococcoides mccartyi* strain DCMB5

D. mccartyi strain DCMB5 originates from dioxin-polluted river sediments and shows dechlorinating capacity for PCDD/Fs (Figure 1.10). It can dechlorinate 1,2,3,4-TCDD to 1,2,4- and 1,2,3-TriCDD. Trichlorinated congeners can be further dechlorinated to mono- to non-chlorinated dioxins (Pöritz et al., 2015a).

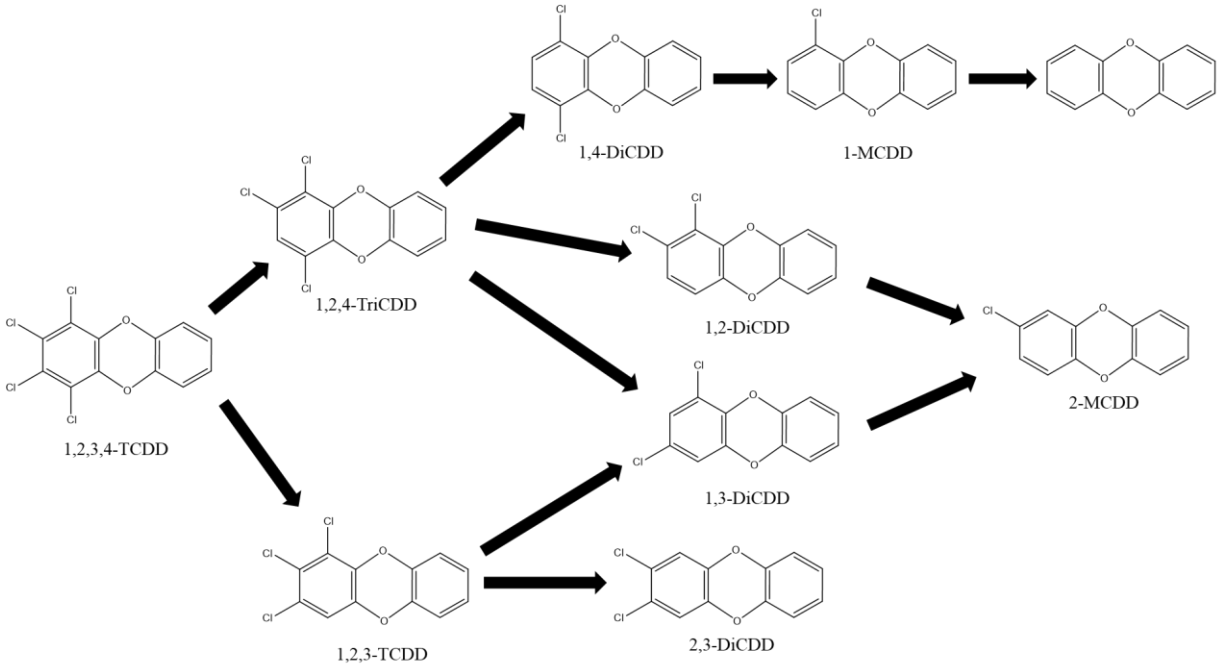

Figure 1.10 Reductive dechlorination of 1,2,3,4-TCDD by *D. mccartyi str.* DCMB5 (Pöritz et al., 2015a)

Reductive dechlorination of PCDD/Fs is achieved via two pathways: lateral- and peri-dechlorination (Barabás *et al.*, 2004; Rodenburg *et al.*, 2017). Lateral-dechlorination substitutes chlorine atoms with hydrogen atoms from the 2,3,7,8 lateral-positions, while peri-dechlorination happens at the 1,4,6,9 peri-positions. Lateral-dechlorination effectively decreases PCDD/F toxicity, whereas peri-dechlorination generates more toxic congeners, particular 2,3,7,8-TCDD, significantly increasing the toxicity (Barabás *et al.*, 2004; Rodenburg *et al.*, 2017; Field and Sierra-Alvarez, 2008). According to the WHO- TEF (Table 1.1), the toxicity become negligible with one chloride substituted from lateral-positions, otherwise, the toxicity equivalents increase 3 to 10-fold with one chloride substituted from the peri-position (US EPA, 2010; Rodenburg *et al.*, 2017).

1.3.3 Challenges of aerobic and anaerobic biodegradation

For aerobic biodegradation of PCDD/Fs, the challenges include: 1) Most aerobic microbes have difficulties oxidizing highly chlorinated congeners (more than four Cl substitutes) (Field and Sierra-Alvarez, 2008); 2) Microbes often require oxygen to support their metabolism or co-metabolism; 3) The oxidation process may result in dead-end products, such as carboxydiphenyl ether and carboxylic acid (Fortnagel et al., 1990; Wittich, 1998); 4)

Approximately 90% of the identified microbes oxidize PCDD/Fs via co-metabolism, so the energy from PCDD/F oxidation cannot support their growth and abundance (Field and Sierra-Alvarez, 2008).

On the other hand, there are challenges for microbial reductive dechlorination. 1) ORB have limited growth rates and biomass yields, which affects their abundance in the natural environment (Jugder et al., 2015); 2) ORB often require extra supplies of nutrients. For example, *Dehalococcoide*s strains are strictly hydrogenotrophic (only able to use H_2 as electron donor), and therefore, depend on exogenous provision of H_2 by fermenting microbes (Holliger et al., 1998); 3) Some ORB are obligate anaerobic microbes, which are only active under certain reducing potentials. Thus, they require reducing agents such as sulfide, iron sulfide and titanium citrate to condition the redox potential necessary for their organohalide respiration; 3) Most ORB are not obligate ORB, resulting in electron competition between organohalides and other electron acceptors such as nitrite and sulfate, limiting the efficiency of dehalogenation (Futagami *et al.*, 2008; Frascari *et al.*, 2015); 4) Dechlorination of PCDD/Fs on peri-positions leads to increasing toxicity, especially the generation of 2,3,7,8-TCDD (Barabás *et al.*, 2004; Rodenburg *et al.*, 2017; Field and Sierra-Alvarez, 2008).

1.4 PCDD/Fs in Sydney Harbour sediments

1.4.1 The history of PCDD/F contamination in Sydney harbour

Since the beginning of the 20th century, organohalide chemical products were frequently used in Australia (Birch et al., 2007a). Homebush Bay, in the upper reaches of Sydney Harbour, was a site for manufacturing these organohalide products, and now it is considered as the primary source of PCDD/F contamination in Sydney Harbour (Montoya, 2015). From 1928 to 1985, Union Carbide manufactured various organohalide products at Homebush Bay (Table 1.2) and PCDD/Fs were the primary by-products generated from manufacturing pentachlorophenol (PCP) used for timber products and Agent Orange-a mixture of 2,4-dichlorophenoxyacetic acid (2,4-D) and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) (Birch et al., 2007).

Table 1.2 Chemical products from Union Carbide (Birch et al., 2007)

Years	Chemical Products
1928-1986	Timber products (including pentachlorophenol)
1932-1985	Xanthates
1940-1961	Aniline and nireobenzene
1942-1971	Phenol
1947-1976	Chlorophenol and chlorobenzenes
1949-1976	2,4,5-trichlorophenoxyacetic acid (2,4,5-T) and 2,4-dichlorophenoxyacetic acid (2,4-D)
1952-1976	Chlorine gas
1955-1968	Tri- and dichlorodipenylthrichloroethane (DDT and DDD)
1960-1976	Disphenol-A (DPP)
1964-1976	Phenol-formaldehyd resins and molding component

PCP was used in wood preservatives and herbicides/pesticides from the 1930s to the 1970s (U.S. EPA, 1978). The manufacturing of PCP readily generates highly chlorinated PCDD/Fs when two PCP dimerize, predominantly producing octa- and hepta-chlorinated DDs and DFs (Figure 1.11) (Johnson, 2017). A study demonstrated that 1 kg of PCP production contains 0.5 to 2 mg-TEQ PCDD/Fs (Hagenmaier, 1987).

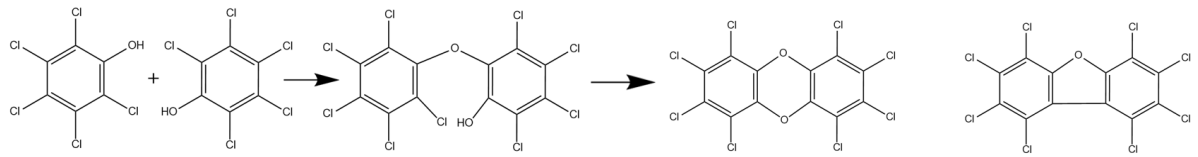

Figure 1.11 The formation reaction of OCDD during the manufacturing of PCP (Plimmer, 1973)

Agent Orange is a 1:1 mixture of 2,4-dichlorophenoxyacetic acid (2,4-D) and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), which was used in the Vietnam War as chemical weapons by the U.S. military to destroy forests and food supplies (Thompson *et al.*, 1992; Schecter and Focus, 2007). From 1949 to 1976, Union Carbide was assigned to manufacture

Agent Orange to support the U.S. military. The manufacturing of 2,4,5-T generates the most toxic dioxin congener 2,3,7,8-TCDD (Figure 1.12) (Hites, 2011). Similar to the formation mechanism of OCDD, the intermediate 2,4,5-trichlorophenol dimerizes, forming 2,3,7,8-TCDD. A study showed that 1 kg of Agent Orange contains approximately 40 mg of 2,3,7,8-TCDD (Schwetz et al., 1973). During the Vietnam War, approximately 77 million litres of Agent Orange were deployed in the south and central Vietnam, and more than 3 million people were exposed to it, developing serious health effects (Schecter and Focus, 2007).

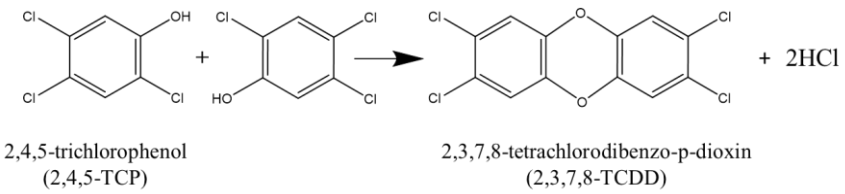

Figure 1.12 The formation reaction of 2,3,7,8-TCDD from 2,4,5-trichlorophenol (Hites, 2011)

In the early 1990s, Union Carbide left Australia, but did not remove its contaminated waste. PCDD/Fs leached into Sydney Harbour via three primary pathways: 1) PCDD/F-contaminated chemical wastes were used as land capping materials and directly poured along the estuary shore of Homebush Bay; 2) Chemical wastewater was directly pumped into the harbour; 3) Combustion of waste containing organohalides also contributed to PCDD/F emissions (Coward, 1988; Birch *et al.*, 2007; Australian Pesticides Map, 2015).

1.4.2 The current situation

A decade ago, Birch *et al.* (2007) carried out an investigation on PCDD/F concentrations in Sydney Harbour sediments, collecting sediment samples from 16 positions across Sydney Harbour (Figure 1.13). They found that Sydney Harbour had one of the highest levels of PCDD/Fs in the world. Sediments at Homebush Bay had the highest concentration, ranging from 667.8 to 4352.5 pg WHO-TEQ/g-dry sediments, with a mean value of 2094 pg WHO-TEQ/g, which was considered as a source zone of PCDD/Fs. Unfortunately, PCDD/Fs had already spread to other parts of Sydney Harbour by tidal current. The concentration ranged from 31.5 to 4352 pg WHO-TEQ/g, with a mean value of 712 pg WHO-TEQ/g.

Seafood, including fish captured from Sydney Harbour, contained PCDD/F levels three times higher than from other parts of Australia (Manning et al., 2017). Blood tests for a

fishermen family showed their PCDD/F levels in serum were four to seven times higher than average people (Rudge et al., 2008). Therefore, a commercial fishing ban is currently in place in the harbour west of Sydney Harbour Bridge (Hedge et al., 2013).

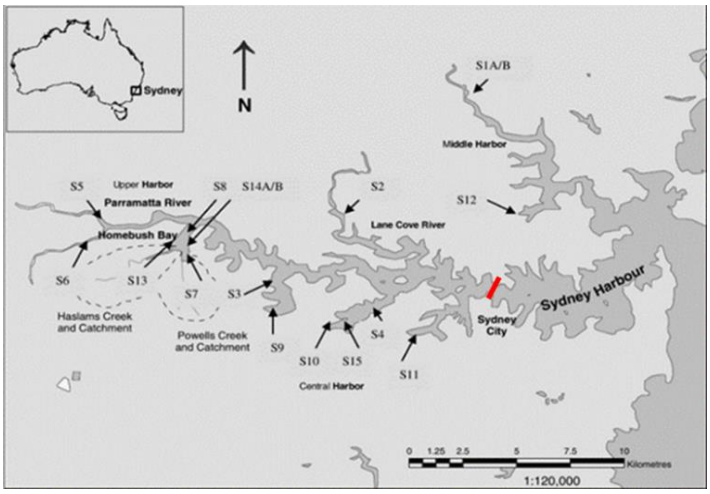

Figure 1.13 Sydney Harbour sample locations in the 2007 investigation (Birch et al., 2007). The red line identifies as the location of Sydney Harbour Bridge.

From 2005 to 2011, a remediation project was conducted by the government to recover the most contaminated area in Homebush Bay (S14 in Figure 1.13). The upper 0.5 m of sediments was dug removed and replaced with clean capping materials. Removed sediments were transported to a thermal treatment plant for decomposition. The total cost was $21 million (Birch et al., 2013; Montoya, 2015). However, current remediation technologies are too expensive for extensive contaminated areas, especially for places like Sydney Harbor. Therefore, a practical and cheaper alternative is highly desirable.

1.5 Research objectives and overview

This literature review has provided an overview of the history of PCDD/Fs in Sydney Harbour and the risks associated with these highly toxic compounds, as well as the potential of utilizing microorganisms to degrade and detoxify PCDD/Fs. The ultimate goal of this study was to survey the feasibility of applying bioremediation technologies for PCDD/F detoxification in Sydney Harbour sediments.

Specifically, the aims addressed in this study are:

1. Assess the ability of microorganisms in Sydney Harbour sediments to transfrom PCDD/Fs to less toxic compounds.

2. Assess the utility of sulfidized nanoscale zerovalent iron (S-nZVI) on transformation of PCDDs.

3. Examine the PCDD/F reducing activity of *Dehalococcoides mccartyi* strain CBDB1 in Sydney Harbour conditions, including surveying its tolerance to seawater and the suitability of S-nZVI to support organohalide respiration.

In Chapter two, the study assessed the ability of indigenous microorganisms in Sydney Harbour sediments to transform TCDD and OCDD. Whilst sediments directly spiked with TCDD and OCDD did not show activity, microbes in the harbour sediments were able to dechlorinate PCE to trichloroethene (TCE) and dichloroethene (DCE). After PCE stimulation, dechlorination activity against TCDD and OCDD was observed, consistent with the existence of PCDD/F dechlorinating microbes in Sydney Harbour sediments. Additionally, the impact of a biosurfactant lecithin on TCDD and OCDD dechlorination by sediment microcosms was tested. The use of lecithin accelerated the desorption of TCDD and OCDD from sediments and enhanced their solubility in the liquid-phase, as well as promoted the microbial dechlorination of TCDD and OCDD.

In Chapter three, the study explored the feasibility of employing other technologies to assist PCDD transformation in Sydney Harbor sediments. S-nZVI was tested for TCDD and OCDD transformation. Even with enhanced TCE dechlorination efficiency and extended longevity, S-nZVI did not tranform TCDD and OCDD after 3 months, suggesting S-nZVI cannot transform TCDD or OCDD. A known PCDD reducing *Dehalococcoides mccartyi* strain (CBDB1) was assessed for its activity in Sydney Harbour sediment conditions. Salt tolerance of strain CBDB1 was tested and the seawater-tolerant cultures were enriched. Additionally, the feasibility of utilizing S-nZVI to support strain CBDB1 for organohalide dehalogenation was examined. S-nZVI was found to be less toxic than nZVI, and can serve as a low redox media conditioning agent and H_2 donor to promote CBDB1 activity, with the potential to be deployed with strain CBDB1 to assist PCDD/F bioremediation in Sydney Harbour sediments.

Chapter Two- Response of indigenous microorganisms in Sydney Harbour sediments to TCDD and OCDD transformation

2.1 Introduction

The last quantification of PCDD/Fs in Sydney Harbour sediments was published more than a decade ago in 2007 (Birch et al., 2007). In 2017, prior to commencement of the study reported herein, sediment samples were collected from 10 locations in Sydney Harbour (Figure 2.1). These sediment cores were divided into the upper layer (upper 25 cm) and the lower layer (25-50 cm) sediments (Figure 2.2). PCDD/Fs in the upper and lower sediment cores were separately extracted and quantified because the upper layer is more susceptible to tidal influence, with potential contaminant dilution with uncontaminated sediments. The concentration of seventeen toxic PCDD/Fs and their WHO toxicity equivalents are presented in Table 2.1 and Table 2.2, respectively.

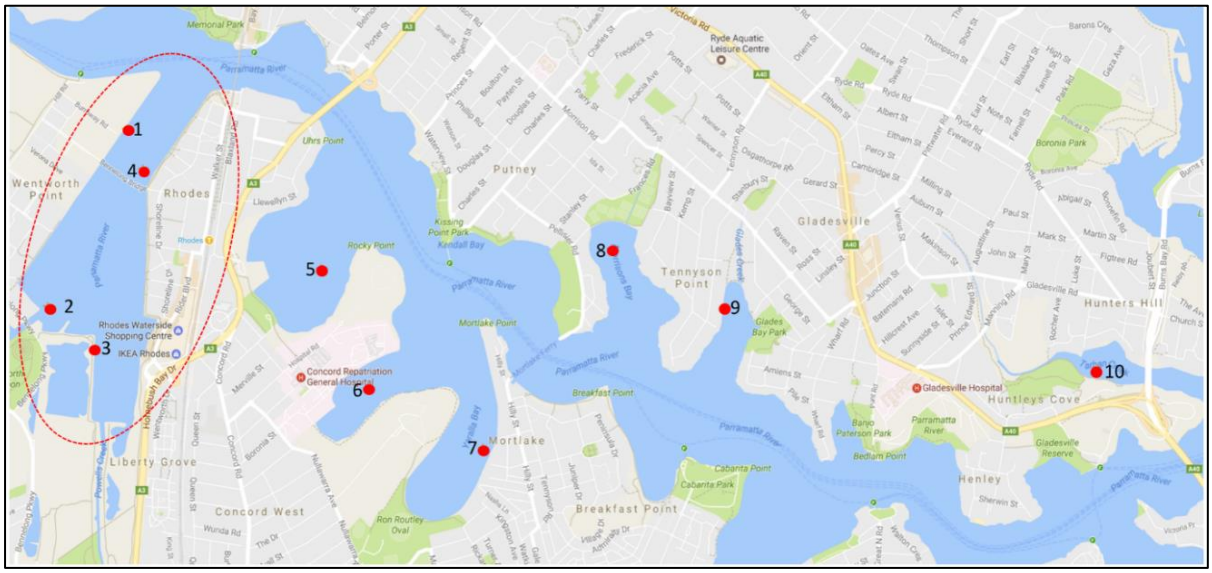

Figure 2.1 Sampling locations of Sydney Harbour sediments in 2017. The red circle identifies Homebush Bay where PCDD/F concentrations are highest.

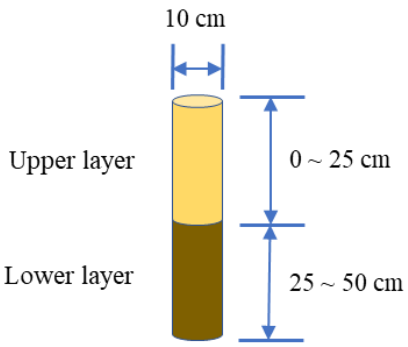

Figure 2.2 The illustration of the sediment cores. (Upper layer: 0-25 cm; Lower layer: 25-50 cm)

Table 2.1 PCDD/F concentrations in sediment samples from 10 locations in Sydney Harbour (pg/g-dry sediment)

Location	1. HHB1		2. HBB2		3. HBB3		4. HBB4		5. Bray's Bay		6. Major Bay		7. Yaralla Bay		8. Morrison's Bay		9. Galdes Bay		10. Tarban Creek	
pg/g-dry sediment	upper	lower	upper	lower	upper	lower	upper	lower	upper	lower	upper	lower	upper	lower	upper	lower	upper	lower	upper	lower
2378-TetraD	142.5	149.4	9.6	0.0	78.9	86.7	330.4	661.1	95.5	0.0	27.1	159.6	110.8	24.9	94.9	117.0	102.9	45.0	30.5	0.0
12378-PCDD	45.0	49.1	0.0	0.0	0.0	41.4	68.2	55.8	44.2	31.8	41.8	0.0	62.2	38.5	0.0	0.0	0.0	0.0	0.0	0.0
123478-HCDD	300.8	0.0	0.0	0.0	0.0	0.0	583.3	1721.7	0.0	23.0	0.0	0.0	0.0	24.5	0.0	0.0	0.0	0.0	0.0	0.0
123678-HCDD	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1736.2	466.9	285.6	309.4	268.8	588.2	119.4	400.8	521.0	420.2	528.0	285.9	315.5
123789-HCDD	0.0	150.0	57.3	45.0	93.4	112.9	199.5	304.4	101.8	45.0	59.4	130.0	142.3	45.1	0.0	91.9	77.7	76.0	61.2	0.0
1234678-HepCDD	11033.9	11205.6	1302.1	942.6	21217.2	10286.4	59616.7	58060.8	9552.6	1637.3	2378.2	17847.5	13534.9	1005.2	5959.0	9669.2	5107.6	11064.9	2082.6	1407.8
OctaD	667663.2	850821.5	81936.4	1473.7	564353.1	578631.3	1761517.9	4108152.3	628779.8	160809.4	237555.5	1350515.3	1100594.8	41249.2	407117.6	709011.4	360315.2	741233.4	178702.4	181073.9
2378-TetraF	39.4	46.0	0.0	0.0	0.0	35.7	57.6	93.6	43.3	0.0	0.0	0.0	0.0	30.3	0.0	0.0	0.0	0.0	0.0	0.0
12378-PCDF	0.0	294.2	0.0	0.0	0.0	0.0	522.0	259.5	0.0	0.0	0.0	0.0	0.0	50.7	0.0	0.0	0.0	0.0	0.0	0.0
23478-PCDF	34.0	0.0	0.0	0.0	41.1	0.0	66.3	2285.8	38.5	0.0	0.0	0.0	315.8	24.4	0.0	0.0	0.0	0.0	0.0	0.0
123478-HCDF	0.0	249.0	37.1	42.8	151.9	128.0	627.3	1027.5	161.1	0.0	73.1	237.8	258.6	43.0	115.4	250.0	192.4	174.3	91.8	0.0
123678-HCDF	0.0	0.0	56.4	0.0	0.0	0.0	554.0	6383.9	0.0	0.0	0.0	0.0	0.0	29.6	0.0	0.0	0.0	0.0	0.0	0.0
234678-HCDF	0.0	0.0	0.0	0.0	0.0	0.0	2160.0	6802.7	0.0	0.0	0.0	0.0	0.0	22.8	0.0	0.0	0.0	0.0	0.0	0.0
123789-HCDF	26.0	0.0	24.7	0.0	31.8	53.0	25.7	72.7	0.0	0.0	0.0	0.0	0.0	22.8	0.0	0.0	0.0	0.0	0.0	0.0
1234678-HepCDF	0.0	0.0	0.0	0.0	0.0	0.0	0.0	5468.1	993.5	352.9	552.8	1538.7	1215.6	135.6	700.3	795.2	650.1	963.5	396.1	386.7
1234789-HepCDF	88.9	43.0	0.0	25.8	176.2	157.1	343.1	542.0	72.8	0.0	0.0	187.7	185.9	29.9	131.7	0.0	79.4	157.3	0.0	48.8
OctaF	57713.7	59868.4	5552.0	43601.8	30821.8	31311.3	144047.3	362636.4	31745.3	4340.0	12529.5	54721.9	40057.1	667.1	18466.1	26808.1	16918.7	31303.0	3561.7	4665.2
Total PCDD	679185.4	862375.6	83305.3	2461.4	585742.5	589158.7	1822316.0	4170692.3	639040.9	162832.2	240371.3	1368921.2	1115033.3	42506.8	413572.4	719410.5	366023.5	752947.3	181162.6	182797.1
Total PCDF	57902.0	60500.6	5670.2	43670.4	31222.8	31685.2	148403.4	385572.2	33054.6	4692.9	13155.5	56686.0	42033.0	1056.3	19413.5	27853.3	17840.6	32598.1	4049.6	5100.7
Total Concentration	737087.4	922876.3	88975.6	46131.7	616965.3	620843.9	1970719.4	4556264.5	672095.4	167525.1	253526.8	1425607.2	1157066.3	43563.0	432985.9	747263.8	383864.0	785545.3	185212.2	187897.9

Table 2.2 WHO-TEQ concentration of PCDD/Fs in sediment samples from 10 locations in Sydney Harbour (WHO-TEQ pg/g-dry sediment)

Location	1. HHB1		2. HBB2		3. HBB3		4. HBB4		5. Bray's Bay		6. Major Bay		7. Yaralla Bay		8. Morrison's Bay		9. Galdes Bay		10. Tarban Creek	
pg/g-dry sediment	upper	lower	upper	lower	upper	lower	upper	lower	upper	lower	upper	lower	upper	lower	upper	lower	upper	lower	upper	lower
2378-TetraD	142.5	149.4	9.6	0.0	78.9	86.7	330.4	661.1	95.5	0.0	27.1	159.6	110.8	24.9	142.5	149.4	9.6	0.0	78.9	86.7
12378-PCDD	45.0	49.1	0.0	0.0	0.0	41.4	68.2	55.8	44.2	31.8	41.8	0.0	62.2	38.5	45.0	49.1	0.0	0.0	0.0	41.4
123478-HCDD	30.1	0.0	0.0	0.0	0.0	0.0	58.3	172.2	0.0	2.3	0.0	0.0	0.0	2.5	30.1	0.0	0.0	0.0	0.0	0.0
123678-HCDD	0.0	0.0	0.0	0.0	0.0	0.0	0.0	173.6	46.7	28.6	30.9	26.9	58.8	11.9	0.0	0.0	0.0	0.0	0.0	0.0
123789-HCDD	0.0	15.0	5.7	4.5	9.3	11.3	20.0	30.4	10.2	4.5	5.9	13.0	14.2	4.5	0.0	15.0	5.7	4.5	9.3	11.3
1234678-HepCDD	110.3	112.1	13.0	9.4	212.2	102.9	596.2	580.6	95.5	16.4	23.8	178.5	135.3	10.1	110.3	112.1	13.0	9.4	212.2	102.9
OctaD	200.3	255.2	24.6	0.4	169.3	173.6	528.5	1232.4	188.6	48.2	71.3	405.2	330.2	12.4	200.3	255.2	24.6	0.4	169.3	173.6
2378-TetraF	3.9	4.6	0.0	0.0	0.0	3.6	5.8	9.4	4.3	0.0	0.0	0.0	0.0	3.0	3.9	4.6	0.0	0.0	0.0	3.6
12378-PCDF	0.0	8.8	0.0	0.0	0.0	0.0	15.7	7.8	0.0	0.0	0.0	0.0	0.0	1.5	0.0	8.8	0.0	0.0	0.0	0.0
23478-PCDF	10.2	0.0	0.0	0.0	12.3	0.0	19.9	685.7	11.6	0.0	0.0	0.0	94.7	7.3	10.2	0.0	0.0	0.0	12.3	0.0
123478-HCDF	0.0	24.9	3.7	4.3	15.2	12.8	62.7	102.7	16.1	0.0	7.3	23.8	25.9	4.3	0.0	24.9	3.7	4.3	15.2	12.8
123678-HCDF	0.0	0.0	5.6	0.0	0.0	0.0	55.4	638.4	0.0	0.0	0.0	0.0	0.0	3.0	0.0	0.0	5.6	0.0	0.0	0.0
234678-HCDF	0.0	0.0	0.0	0.0	0.0	0.0	216.0	680.3	0.0	0.0	0.0	0.0	0.0	2.3	0.0	0.0	0.0	0.0	0.0	0.0
123789-HCDF	2.6	0.0	2.5	0.0	3.2	5.3	2.6	7.3	0.0	0.0	0.0	0.0	0.0	2.3	2.6	0.0	2.5	0.0	3.2	5.3
1234678-HepCDF	0.0	0.0	0.0	0.0	0.0	0.0	0.0	54.7	9.9	3.5	5.5	15.4	12.2	1.4	0.0	0.0	0.0	0.0	0.0	0.0
1234789-HepCDF	0.9	0.4	0.0	0.3	1.8	1.6	3.4	5.4	0.7	0.0	0.0	1.9	1.9	0.3	0.9	0.4	0.0	0.3	1.8	1.6
OctaF	17.3	18.0	1.7	13.1	9.2	9.4	43.2	108.8	9.5	1.3	3.8	16.4	12.0	0.2	17.3	18.0	1.7	13.1	9.2	9.4
Total PCDD (TEQ)	528.2	580.8	52.9	14.4	469.7	415.8	1601.5	2906.2	480.8	131.8	200.8	783.1	711.6	104.7	316.7	487.7	311.8	438.4	139.7	99.9
Total PCDF (TEQ)	35.0	56.7	13.5	17.6	41.7	32.6	424.7	2300.4	52.2	4.8	16.6	57.5	146.6	25.6	25.4	41.0	31.6	38.0	14.2	5.8
Total TEQ concentration	563.1	637.5	66.4	32.0	511.4	448.5	2026.1	5206.7	533.0	136.6	217.4	840.5	858.2	130.2	342.1	528.7	343.4	476.4	153.9	105.7

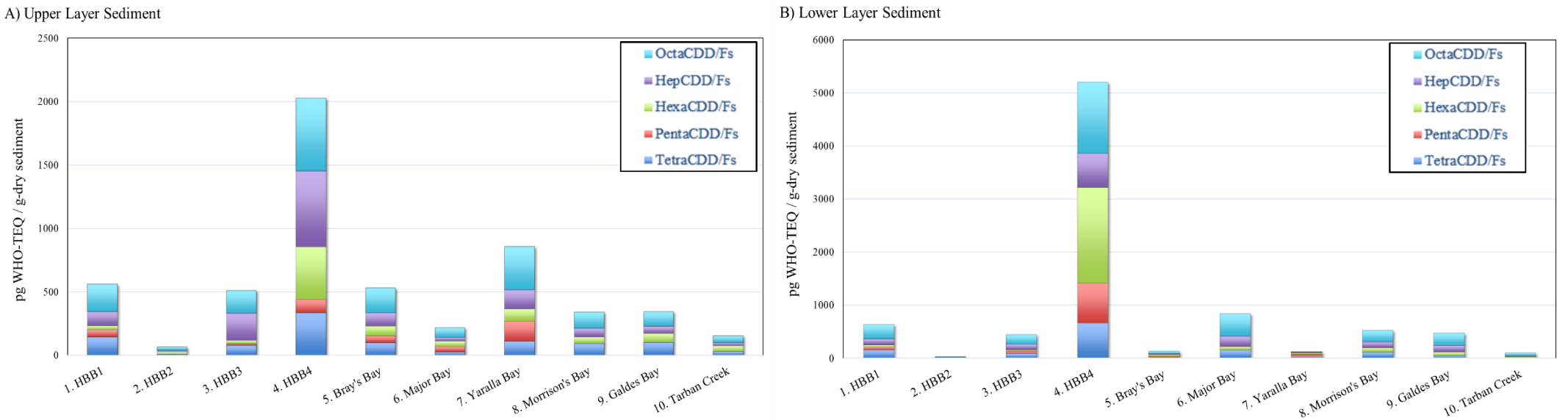

Figure 2.3 Profiles of PCDD/Fs in upper sediment cores (A) and lower sediment cores (B).

Table 2.3 Profiles of tetra- to octa-chlorinated PCDD/Fs in sediment samples

	1. HBB1		2. HBB2		3. HBB3		4. HBB4		5. Bray's Bay		6. Major Bay		7. Yaralla Bay		8. Morrison's Bay		9. Galdes Bay		10. Tarban Creek	
WHO-TEQ pg/g	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower
TetraCDD/Fs	146.5	154.0	9.6	0.0	78.9	90.3	336.2	670.5	99.9	0.0	27.1	159.6	110.8	27.9	94.9	117.0	102.9	45.0	30.5	0.0
PentaCDD/Fs	55.2	57.9	0.0	0.0	12.3	41.4	103.7	749.3	55.8	31.8	41.8	0.0	156.9	47.3	0.0	0.0	0.0	0.0	0.0	0.0
HexaCDD/Fs	32.7	39.9	17.5	8.8	27.7	29.4	415.0	1804.9	73.0	35.4	44.2	63.7	98.9	30.7	51.6	86.3	69.0	77.8	43.9	31.5
HepCDD/Fs	111.2	112.5	13.0	9.7	213.9	104.4	599.6	640.7	106.2	19.9	29.3	195.7	149.4	11.7	67.9	104.6	58.4	121.9	24.8	18.4
OctaCDD/Fs	217.6	273.2	26.2	13.5	178.6	183.0	571.7	1341.2	198.2	49.5	75.0	421.6	342.2	12.6	127.7	220.7	113.2	231.8	54.7	55.7
Total TEQ concentration	563.1	425.6	66.4	32.0	511.4	448.5	2026.1	5206.7	533.0	136.6	217.4	840.5	858.2	130.2	342.1	528.7	343.4	476.4	153.9	105.7

In general, there has been no significant improvement in the contaminant profiles over the last 10 years with respect to toxicity equivalents (Figure 2.3 and Table 2.3). Homebush Bay (HBB) was the most contaminated area. The highest toxicity equivalents were found at HBB4, which was adjacent to the former Union Carbide manufacturing site. The toxicity equivalents in HBB4 upper and lower sediment cores were 2026.1 and 5206.7 pg WHO-TEQ/g-dw, respectively. At HBB1 and HBB3, toxicity equivalentss were approximately 5 to 10-fold less than at HBB4. The toxicity equivalents at HBB2 were the lowest, with 66.4 and 32.0 pg WHO-TEQ/g in the upper and the lower sediment cores, respectively.

Total PCDD/F concentrations in general tended to decrease with distance from HHB4 (source zone) (Figure 2.4), with a few exceptions. Upper core samples at Yaralla Bay (3.5 km from HBB4) and lower cores samples at Major Bay (3 km from HBB4), had PCDD/F concentrations higher than samples collected 2 km (Bray's Bay) from the source. Tarban Creek was the farthest sampling location from HBB4, but PCDD/Fs were still detectable, with 1.8×10^5 and 1.9×10^5 pg/g in the upper and lower sediment cores, respectively.

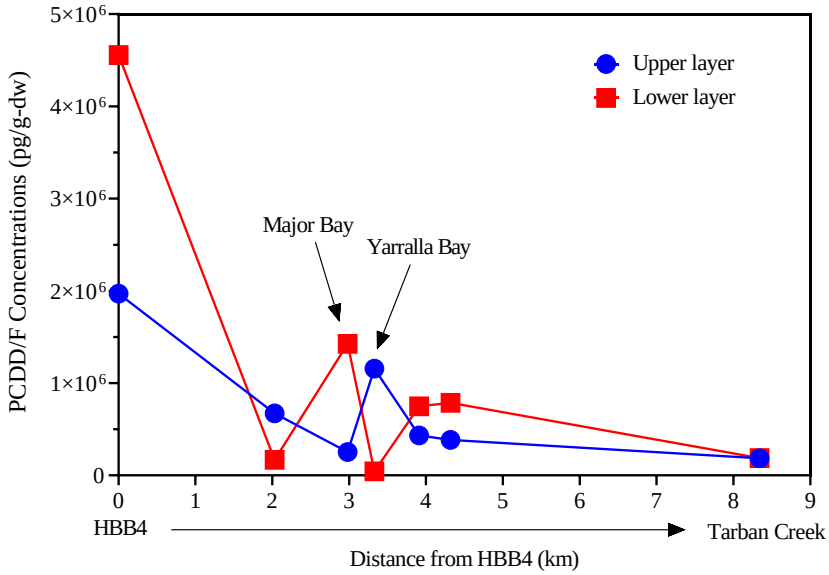

Figure 2.4 PCDD/F sediment concentrations (pg/g) change with the distance from HBB4.

The two most important PCDD congeners in Sydney Harbour sediments were 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and octachlorodibenzo-*p*-dioxin (OCDD). TCDD was the most toxic, making up only 0.01% of the total PCDD/F concentration (pg/g-dw), but contributing 15% of the toxicity equivalents (WHO-TEQ pg/g-dw), while OCDD was the most

abundant, making up 95% of the total PCDD/F concentration and 30% of the toxicity equivalents.

The aim of this chapter was to determine the potential of indigenous microorganisms in Sydney Harbour sediments to transform TCDD and OCDD by organohalide respiration. Harbour sediments directly spiked with TCDD and OCDD showed no transformation on TCDD and OCDD after 2 years (Lee et al, unpublished results). It was hypothesized that the low solubility of TCDD and OCDD might limit the capacity of PCDD/F-dechlorinating microbes respiring these compounds to support their growth, thus the dechlorination was too weak to be observed. As mentioned in Chapter One, PCDD/F-dechlorinating ORB also respire perchloroethene (PCE) as a terminal electron acceptor. For example, *Dehalococcoides mccartyi* strain 195 can dechlorinate PCE to ethene (Maymó-Gatell et al., 1997), and *Dehalococcoides mccartyi* strain CBDB1 can dechlorinate PCE to TCE and DCE (Marco-Urrea et al., 2011). Therefore, an attempt was made to enrich PCDD/F-dechlorinating microbes using PCE as a more water soluble and bioavailable electron acceptor.

2.2 Materials and Methods

2.2.1 Chemicals

Hexane, dichloromethane (DCM), PCDD/Fs, perchloroethene (PCE), trichloroethene (TCE), cis- and trans-dichloroethene (DCE) were purchased from Sigma-Aldrich. Lecithin (food-grade) was purchased from Woolworths supermarket, Australia. Seawater was taken from Coogee Beach, Sydney, Australia.

2.2.2 Quantification of PCDD/Fs in Sydney Harbour sediment

In 2017, sediment cores were collected from 10 locations in Sydney Harbour. The sediment cores were sampled to 0.5 m, except for the HBB4 location. From 2005 to 2011, remediation was carried out at the eastern shore of HBB4, by removing the upper 0.5 m sediment and refilling with clean materials (Birch *et al.*, 2013; Montoya, 2015). Thus, deeper sediment cores (from 1.0 to 1.5 m) were taken from this location.

The moisture content of wet sediments was approximately 50%. Wet sediments were dried in a 50℃ oven for 24 hours. The dried sediments were ground to a fine powder with a mortar and pestle. Dried sediments (20 g) were extracted by a Dionex™ ASE™ 150 Accelerated Solvent Extractor using toluene as the solvent. Seventeen C^{13}-labeled 2,3,7,8-substituted PCDD/Fs were spiked as internal standards for quantification (US EPA, 2010). Sediment cores were extracted without replication, because the extraction protocol is expensive and time-consuming, taking 20 min for each sample. Secondly, the environmental concentrations of PCDD/Fs are extremely low (pg/g), which requires large volume of sediments for extraction and concentration. For example, 20 g dried sediments were required for extraction to reach the detection limit of GC-TQMS.

After being concentrated by nitrogen gas flow, the extracts were reconstituted with 1 ml DCM and applied to a 10 cm x 1 cm column of Florisil to clean up interfering compounds, such as humic acid. The PCDD/Fs were eluted with dichloromethane (50 ml) from Florisil column. The DCM was evaporated under a stream of N_2. The residue was reconstituted in toluene (0.5 ml) and analyzed by an Agilent Technologies 7890A gas chromatograph, equipped with a 7000A triple quadrupole mass spectrum (TQMS) and a DB-5 capillary column (60 m×0.32 mm×0.25 μm; J&W scientific). The inlet temperature was set at 250℃. The oven temperature program was controlled as follows: initially hold at 125℃ for 5 min, increase to 250℃ at a rate of 25℃ per min and hold for 5 min, then increase to 285℃ at a rate of 3℃ per min and hold for 15 min.

The TQMS detector was operated in the Multiple Reaction Monitoring (MRM) mode. The 17 most toxic PCDD/Fs were quantified (US EPA, 2010). Their retention times and MRM settings are listed in **Appendix 1**.

2.2.3 Microbial community characterization

To characterize microbial communities in the harbour sediments, genomic DNA extracts (following Urakawa et al. 2010) from individual sediment samples (5 g wet weight) from each location were subject to amplicon generation and sequencing without replication. In summary, each sediment cores were homogenized and lysed with 2-mercaptoethanol lysis buffer. DNA pellet was extracted by phenol-chloroform-isoamylalcohol, precipitated by

isopropanol, washed twice with 80% ethanol and finally dissolved in 30 µl of DNA-free molecular grade water.

DNA was amplified via polymerase chain reaction (PCR) using universal primers 515F (5'-TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG GTG YCA GCM GCC GCG GTA A -3') and 806R (5'-GTC TCG TGG GCT CGG AGA TGT GAT TAA GAG ACA GGG ACT CAN VGG GTW TCT AAT -3') to obtain their 16S rRNA gene amplicons. The PCR mixture contained 2 µl of DNA template, 0.4 µl of each primer, 20 µl of PCR Master Mix and 17.5 µl of DNA-free water. Thermocycling comprised of denaturation at 94℃ for 3 min, followed by 35 cycles of 45 seconds at 94℃, 1 min at 50℃ and 1.5 min at 72℃, ending with 72℃ for 10 min.

The amplicons were sequenced by Next-Generation Sequencing Facility at Western Sydney University. The raw sequence data was analyzed by QIIME2-2019.7 with the Silva132.99 database.

2.2.4 Enrichment procedure

To enrich ORB in the harbour sediments, sediment samples from the most contaminated site-HBB4 were used as the inoculum sources. The first PCE enrichment was set up in mineral salt medium using Milli-Q water as basal medium. In summary, Milli-Q water was amended with mineral salts (g/L) [NaCl (1.0), $MgCl_2 \cdot H_2O$ (0.5), KH_2PO_4 (0.2), NH_4Cl (0.3), KCl (0.3) and $CaCl_2 \cdot 2H_2O$ (0.015)], 1 ml of trace element stock solution (Table 2.4) and 30 mM of acetate. The pH was adjusted to 7.0.

Table 2.4 100 times concentrated Trace element stock solution (g/l)

Trace element A	
HCl	25% w/w
$FeCl_2 \cdot 4H_2O$	1.5
$CoCl_2 \cdot 6H_2O$	0.19
$MnCl_2 \cdot 4H_2O$	0.1
$ZnCl_2$	0.07
H_3BO_3	0.006
$Na_2MoO_4 \cdot 2H_2O$	0.036
$NiCl_2 \cdot 6H_2O$	0.024
$CuCl_2 \cdot 2H_2O$	0.002

Trace element B	
$Na2SeO_3 \cdot 5H2O$	0.006
$NaWO_4 \cdot 2H2O$	*0.008*
NaOH	0.5

After deoxygenation by nitrogen sparging for 45 min, a 100 ml medium was dispensed into a 120 ml serum bottle containing 1 g sediments, leaving 20 ml of headspace. The flasks were sealed with Teflon coated rubber septa and aluminum crimps. The headspace was flushed with N_2/CO_2 (4:1) for 3 min. The medium was supplied with 1 ml of vitamin stock solution (Table 2.5), 10 mM $NaHCO_3$ as the buffer substance and 0.2 mM of Na_2S as the reducing agent and the nutritional sulfur sources. The cultures were spiked directly with 0.2 mM neat PCE using a 10 µl glass syringe. 0.5 bar of hydrogen gas was supplied as an electron donor. All enrichment cultures were incubated statically at 30℃ in the dark. Even though the concentrations of H_2 and acetate were not monitored, extra 0.5 bar of H_2 and 10 mM of acetate were supplied to these cultures approximately every month.

Table 2.5 1000 times concentrated vitamin stock solution

Chemical	mg/L
4-aminobenzoic acid	20
biotin	5
Nicotinic acid	50
calcium pantothenate	54.5
pyridoxine hydrochloride	91
thiamine hydrochloride	63.5
cyanocobalamin	50

In the second enrichment attempt, filter-sterilized seawater from Coogee Beach, Australia, was used instead of Milli-Q water. The total salt concentration was approximately 36 g/L Detailed compositions of seawater have yet to be characterized, but the suggested ionic composition of marine seawater is listed in Table 2.6 (Byrne et al., 2018). Other compositions in the medium were the same as mineral salt medium. After 90 days, DNA was extracted from one of the triplicates that had the best PCE dechlorinating performance and its 16S rRNA amplicons were sequenced to identify the potential PCE-dechlorinating bacteria. For artificial seawater, mineral salt medium was amended with extra 2% (w/v) NaCl.

Table 2.6 Ion composition of seawater (Byrne et al., 2018)

Ionic constituent	g/L of seawater
Chloride (Cl^-)	19.162
Sodium (Na^+)	10.679
Magnesium (Mg^{2+})	1.278
Sulfate (SO_4^{2-})	2.680
Potassium (K^+)	0.3953
Calcium (Ca^{2+})	0.4096
Inorganic carbon	0.0276
Bromide (Br^-)	0.0663
Boron (B)	0.0044
Strontium (Sr)	0.0079
Fluoride (F^-)	0.0013
Salinity	34.7

2.2.5 Quantitative PCR (qPCR)

qPCR was used to monitor the abundance of the total bacteria and *Dehalococcoides* spp. during PCE enrichment. The qPCR mixtures contained 2 µl of DNA template, 5 µl of 2×SsoFastTM EvaGreen Supermix, 0.1 µl each of forward and reverse primers, 0.1 µl of Bovine Serum Albumin (BSA) solution and 2.7 µl of DNA-free Molecular grade water. Data results were from triplicate cultures with technical replicates (n=3).

Universal bacterial primers Eub1048F (5'-GTGSTGCAYGGYTGTCGTCA-3') and Eub1195R (5'-ACGTCRTCCMCACCTTCCTC-3') were used to quantify the total bacteria concentration (Horz et al., 2005). Thermocycling comprised of 95℃ for 3 min, followed by 40 cycles of denaturation at 95℃ for 20 seconds and annealing at 62℃ for 50 seconds. Following amplification, melting curve analysis was conducted with increments at 0.5℃ per 10 seconds from 60 to 99℃.

Dehalococcoides primers Dehalo505F (5'-GGCGTAAAGTGAGCGTAG-3') and Dehalo686R (5'-GACAACCTAGAAAACCGC-3') were used to quantify *Dehalococcoides* spp (Behrens et al., 2008). Thermocycling comprised of 98℃ for 3 min, followed by 45 cycles of denaturation at 95℃ for 30 seconds and annealing at 58℃ for 50 seconds. Following amplification, melting curve analysis was conducted with increments at 0.5℃ per 5 seconds from 55 to 95℃.

qPCR was also used to identify the reductive dehalogenases (RDases) responsible for PCE dechlorination, including PceA (Neumann *et al.*, 1998) and TceA (Magnuson et al., 2000). The primers and their amplification protocols are list in **Appendix 2**.

2.2.6 Sediment microcosms on TCDD and OCDD transformation

To replicate the existing forms of PCDD/Fs in Sydney Harbour, TCDD and OCDD were adsorbed onto dried sediments for use as stocks, rather than dilution in organic solvents. To prepare the stock sediments, TCDD and OCDD were dissolved into 50 ml hexane and properly mixed with 20 g of dried sediment collected from Tarban Creek, which was the farthest sample location from HBB4. After that, hexane was completely evaporated under nitrogen flow, making TCDD and OCDD adsorb onto sediments. The concentrations of TCDD and OCDD in stock sediments were 3.0 µg/g-dw and 54.0 µg/g-dw, respectively.

To test for TCDD and OCDD biotransformation, PCE-enriched HBB4 sediment microcosms were purged with nitrogen flow for 30 min to evacuate all chloroethenes, and 10% (v/v) inoculated in a 50 ml anaerobic 2% NaCl artificial seawater medium and spiked with 1 g of TCDD or OCDD stock dry-sediments. 0.5 bar H_2 and 30 mM acetate were supplied to the cultures as the electron donors and carbon sources, respectively, and extra H_2 (0.5 bar) and acetate (10 mM) were supplied to the cultures approximately every two months. All enrichment cultures were incubated statistically at 30°C in the dark.

After 14 months of cultivation, DNA was extracted from triplicated TCDD/OCDD cultures (with/without lecithin) and their 16S rRNA amplicons was sequenced to characterize PCDD/F-dechlorinating genera.

2.2.7 Impact of TCDD and OCDD on microbial PCE dechlorination

To initially test for toxicity, TCDD (3.0 µg/g-dw) and OCDD (54.0 µg/g-dw) were added to 10% (v/v) PCE-enriched sediment microcosms described in 2.2.6 and amended with PCE (0.2 mM). The concentration of TCDD and OCDD spiked into the cultures were approximately 4500 and 14 times higher than the environmental concentrations in harbor sediment at HBB4, respectively. Additionally, 1 g/L of lecithin was tested for its ability to

enhance aqueous solubility and hence bioavailability of TCDD and OCDD. The quantification of dechlorination performance was based on the calculation of the theoretical chloride release from the dechlorination of PCE to TCE and DCE (Equation 2.1). For active controls, 10% (v/v) PCE-enriched microcosms were only spiked with (0.2 mM) PCE, but they were not spiked with blank dried sediments amended with hexane.

Chloride released = TCE concentration + 2× DCE concentration (Equation 2.1)

2.2.8 Impact of lecithin on the solubility of TCDD and OCDD

There were concerns that the low bioavailability of PCDD/Fs might prevent microbes respiring these compounds (Field and Sierra-Alvarez, 2008). Therefore, a biosurfactant lecithin was tested for its ability to enhance PCDD dechlorination by sediment microcosms. Lecithin is a rhizosphere phytogenic biosurfactant extracted from soya beans or egg yolk (Linow, 1990), and has been used to enhance microbial degradation of PCE and TCE (Brant and Smith, 2015), polychlorinated biphenyls (PCBs) (Fava and Gioia, 2000), polycyclic aromatic hydrocarbons (PAHs) (Soeder et al., 1996a) and OCDD (Lin et al., 2017). Triplicate PCE-enriched sediment microcosms were amended with 1 g/L of lecithin to estimate its effect on TCDD and OCDD dechlorination.

To estimate whether lecithin can improve the aqueous solubility of TCDD and OCDD, 1 g/L of lecithin was added to a 20 mM NaN_3 sterilized sediment solution and spiked with 1 g/L of TCDD and OCDD stock sediments. Blank controls were the sterilized sediment solution with TCDD and OCDD but without lecithin. All these sediments were shaken in the dark for 7 days and then centrifuged at 4000 rcf for 4 min. The supernatant and the pellet were separately extracted by organic solvent and analyzed by GC-MS/MS (Figure 2.5).

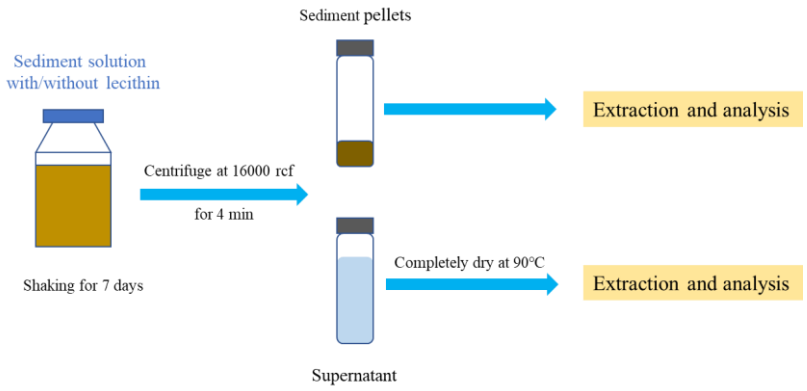

Figure 2.5 Extraction protocols of PCDD sediment with/without lecithin.

2.2.9 Analytical methods

2.2.9.1 Chloroethenes quantification

100 µl of culture headspace gas was manually injected into an Agilent Technologies 7890A gas chromatography equipped with a flame ionization detector (GC-FID) and a GS-Q capillary column (30 m ×0.32 µm; J&W Scientific). The temperature of inlet and detector were set at 250℃. The helium carrier gas flow rate was 3 ml/min. The oven program was initially held at 150℃, increased to 250℃ at a rate of 30℃ per min and held for 2 min. Retention times (minutes) for each compound were as follows: methane (1.30), ethene (1.63), vinyl chloride (1.75), trans-DCE (2.56), cis-DCE (2.81), TCE (3.41) and PCE (4.32).

2.2.9.2 Polychlorodibenzo-*p*-dioxins quantification

2 ml of sediment solution was taken to a 10 ml glass tube and centrifuged at 4000 rcf for 4 min. The supernatant was discarded, and the pellet was extracted by 4 ml of 1:1 methanol/hexane twice. Anhydrous sodium sulfate was used to absorb the residual moisture. The extract was completely evaporated under nitrogen gas flow and reconstituted with 1 ml of dichloromethane (DCM). PCDD/Fs extraction from TCDD and OCDD cultures was from triplicated cultures. The results of PCDD/F concentration were the average values of triplicates.

For TCDD analysis, 2 µl of liquid was injected into an Agilent Technologies 7890A/7000A GC-MS/MS described in 2.2.2. The temperature of inlet was set at 250℃. The oven program was initially held at 120℃ for 5 min, increased to 250℃ at a rate of 25℃ per min and held for 5 min, and then increased to 280℃ at a rate of 3℃ per min and finally held for 2 min. MRM model was used to quantify TCDD and its dechlorinated products: 2,3,7-triCDD, 2,7-/2,8-diCDD, 2,4-diCDD, monoCDD and dibenzo-p-dioxin (DD). Their retention times and MRM settings are listed in **Appendix 1**.

For OCDD analysis, the method was previously described in 2.2.2.

2.2.9.3 Sulphate (SO_4^{2-}) quantification

To monitor the reduction of SO_4^{2-}, 1 ml of culture solution was centrifuged at 16000 rcf for 20 min. The supernatant was analysed by a DIONEX^{TM} ICS-3000 ionic chromatograph (IC), equipped with a DIONEX IonPaC^{TM} AG 18 column and a DIONEX conductivity detector. The sample was eluted with 30 mM of KOH solution at an isocratic flow rate of 1 ml/min. The total running time was 20 min and the retention time of SO_4^{2-} was 5.91 min.

2.3 Results

2.3.1 Microbial community analysis of Sydney Harbour sediments

Microbial communities in harbour sediments were characterized by 16S rRNA gene amplicon sequencing to identify potential doxin degrading bacteria. Chloroflexi was the most abundant phylum, followed by Proteobacteria, Patescibacteria, Acidobacteria and Firmicutes (Figure 2.6). The major microbial communities (>0.5%) assigned to family and genus are presented in Figure 2.7. Under the class of Dehalococcoidia in Chloroflexi (phylum), there were several genera known to facultatively or obligately dehalogenate organohalides (Appendix 5). Especially, the genus *Dehalococcoides*, some lineages of which are known to respire PCDD/Fs, was found in Sydney Harbour sediments, ranging from 0.00 to 0.62% of the total bacteria, with a mean value of 0.135% across the 20 samples (Figure 2.8). The genus *Dehalobium*, some lineages of which are known to respire PCE and PCBs (Cutter et al., 2001; Kittelmann and Friedrich, 2008), was also identified in the sediment samples at relative abundances below 0.1%.

There was no obvious correlation between PCDD/F toxicity equivalents and the relative abundance of *Dehalococcoides* or *Dehalobium* (Figure 2.8) (Pearson correlation, $P_{Dehalococcoides}$ =0.692 and $P_{Dehalobium}$ =0.617). Interestingly, in HBB2 lower sediment cores, which had the lowest toxicity equivalents, the abundance of *Dehalococcoides* strains was the highest, making up 0.62% of the total bacteria. In contrast, in HBB4 lower sediment cores with the highest toxicity equivalents, *Dehalococcoides* strains were not highly abundant and only made up 0.14% of the total bacteria, which was close to the mean value (0.135%).

In addition, the genus *Pseudomonas* and *Sphingomonas*, some lineages of which are known to oxidize PCDD/Fs, were also detected in Sydney Harbour, but their relative

abundances were below 0.1% (data not shown). Since the harbour sediments were primarily under anaerobic conditions, this study only assessed the microbial biotransformation of TCDD and OCDD under anaerobic conditions.

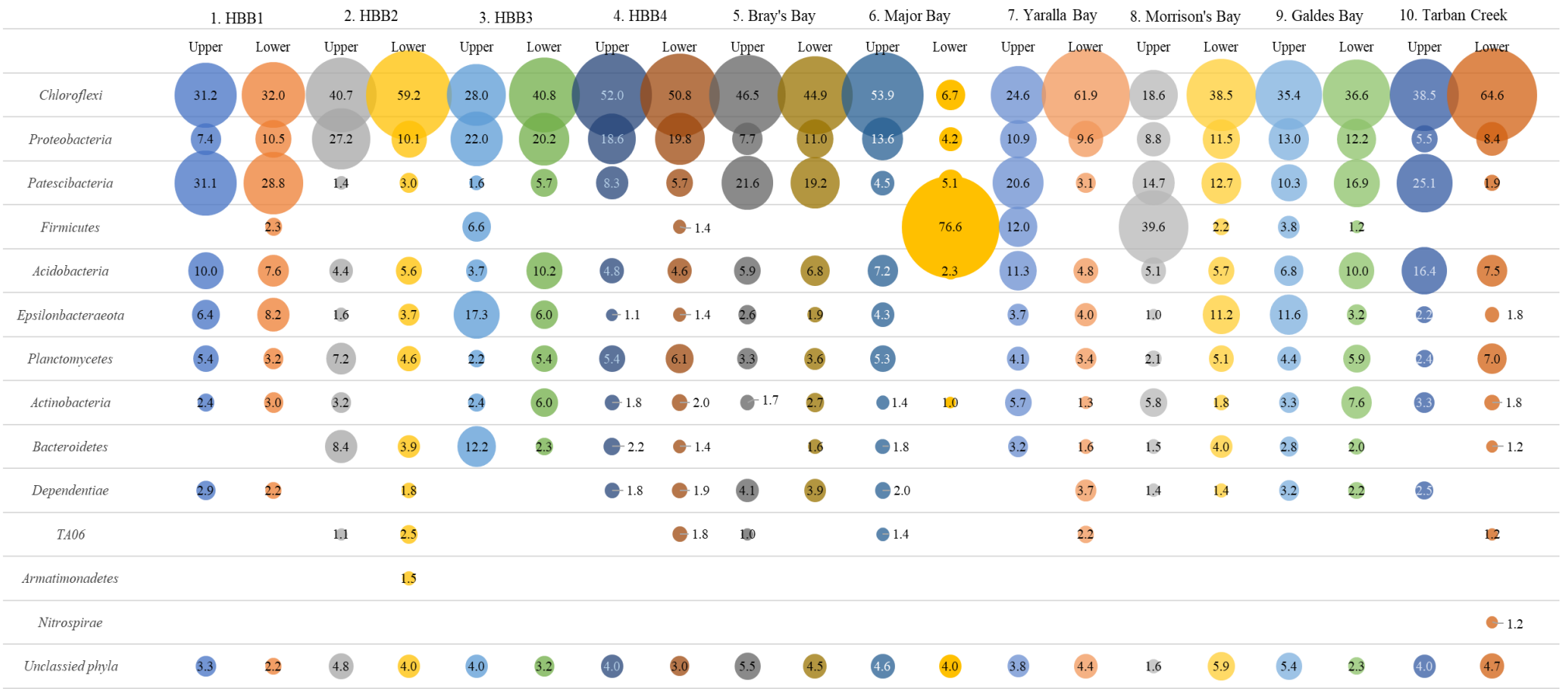

Figure 2.6 Relative abundance of phyla (>0.5%) in Sydney Harbour sediments (sorted by largest to smallest). Bubble sizes and figures represent the relative abundance (%) of bacterial lineages.

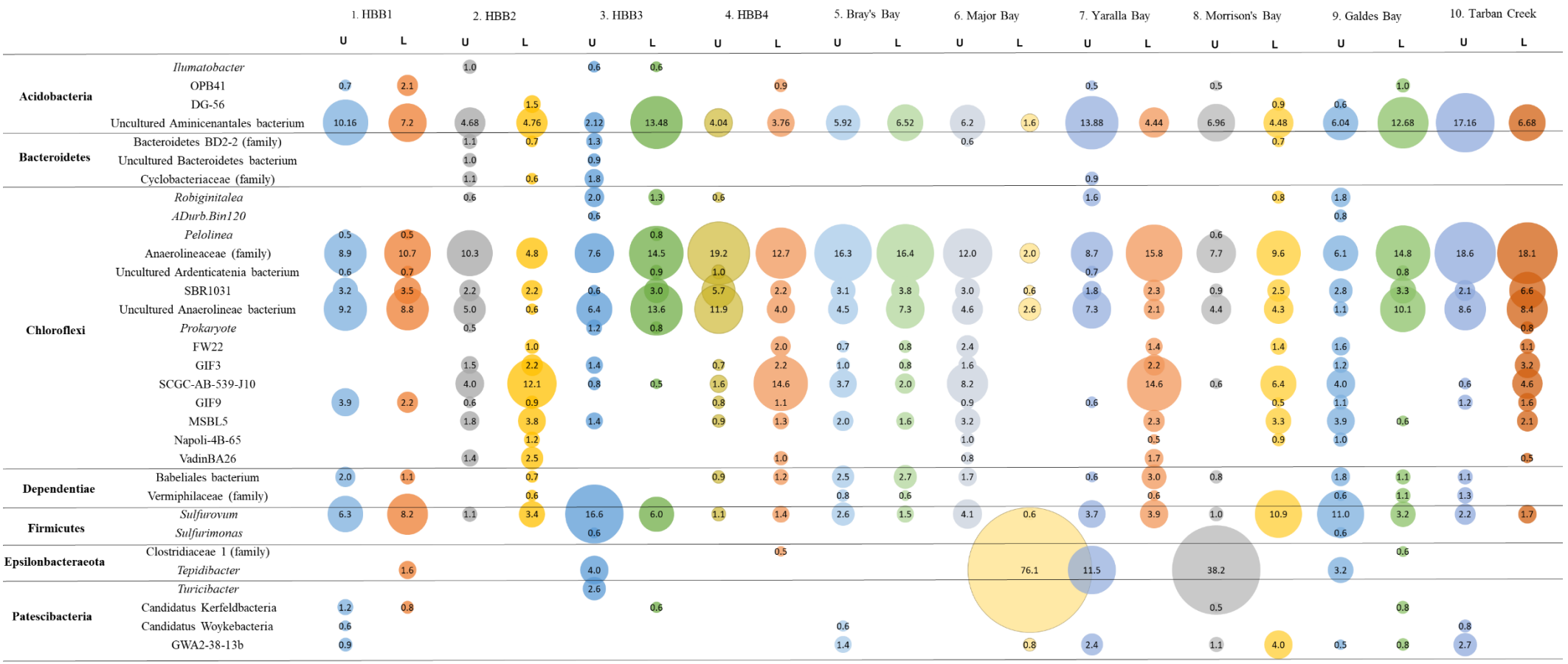

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Figure 2.7 The major microbial communities in Sydney Harbour sediments (>0.5%). These cultures were assigned to family and genus. Bubble sizes and figures represent the relative abundance (%) of bacterial lineage

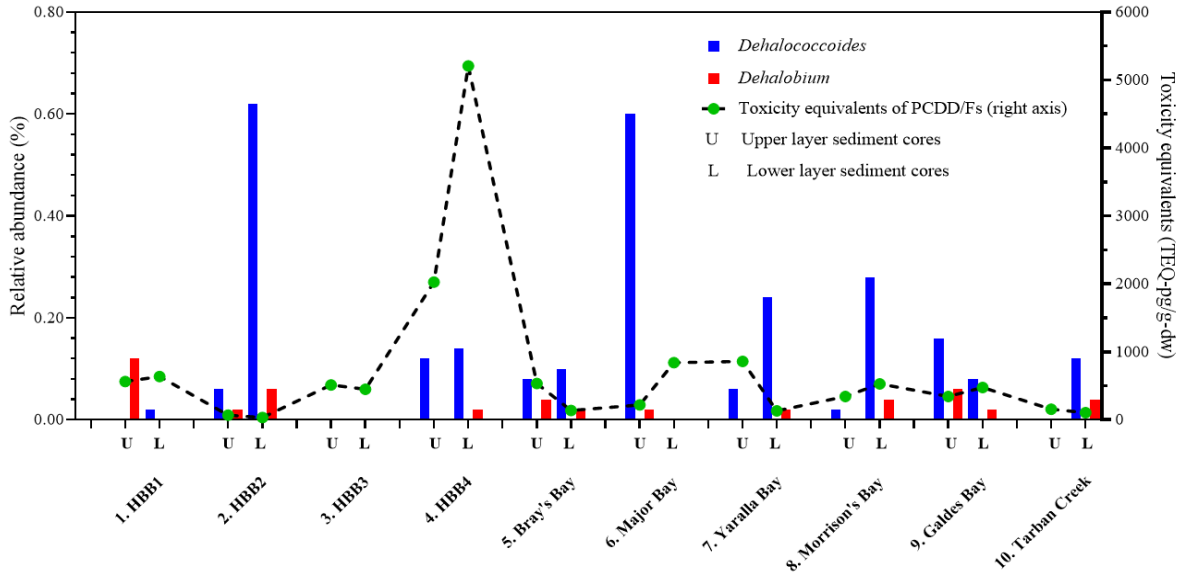

Figure 2.8 Relative abundance (%) of *Dehalococcoides* and *Dehalobium* in Sydney Harbour sediments and their relationship with PCDD/F toxicity equivalents (TEQ-pg/g-dw sediments).

2.3.2 TCDD and OCDD transformation in harbour sediment microcosms

2.3.2.1 Enrichment for ORB activity in harbour sediments

Anaerobic cultures inoculated with Sydney Harbour sediments were directly spiked with TCDD and OCDD stock sediments (1 µg/g), but these enrichments showed no transformation after 2 years of cultivation (Lee et al. unpublished results). Therefore, PCE was proposed as an alternative electron acceptor to enrich endogenous ORB prior to assessing their transformation of TCDD and OCDD.

To test for ORB activity where PCDD/F concentrations were the highest, HBB4 sediments were used to inoculate PCE enrichment cultures. In the first PCE enrichment in mineral salt medium, only one of the triplicate cultures demonstrated dechlorination activity (Figure 2.9). Approximately 85% of PCE (0.14 mM) was dechlorinated to TCE within 7 days, but the reaction stopped for more than 31 days and dechlorinated 0.17 mM PCE to TCE (0.01 mM), cis-DCE (0.1 mM) and trans-DCE (0.03 mM) after 77 days. However, in other two cultures, dechlorination was negligible (Figure 2.10). After 77 days of cultivation, only 6.1% of PCE (approximately 0.01 mM) was dechlorinated to TCE.

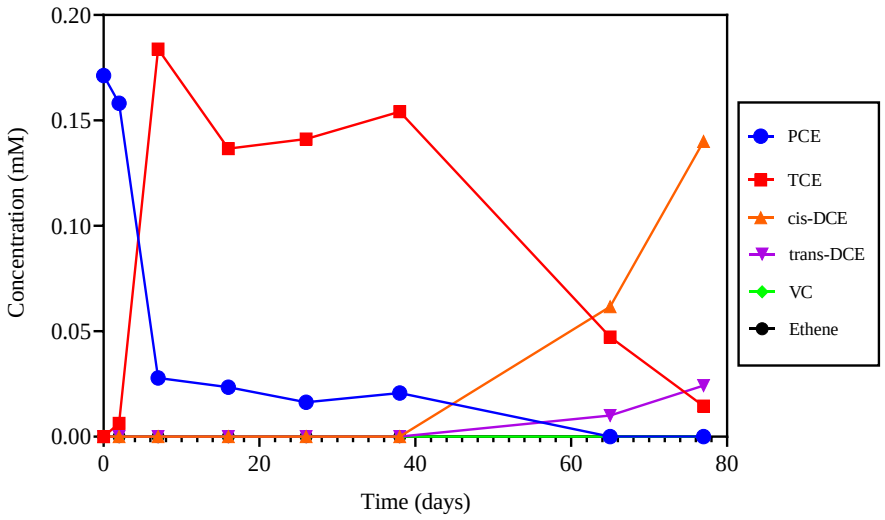

Figure 2.9 PCE transformation by HBB4 sediment microcosms in one of the triplicates in mineral salt medium.

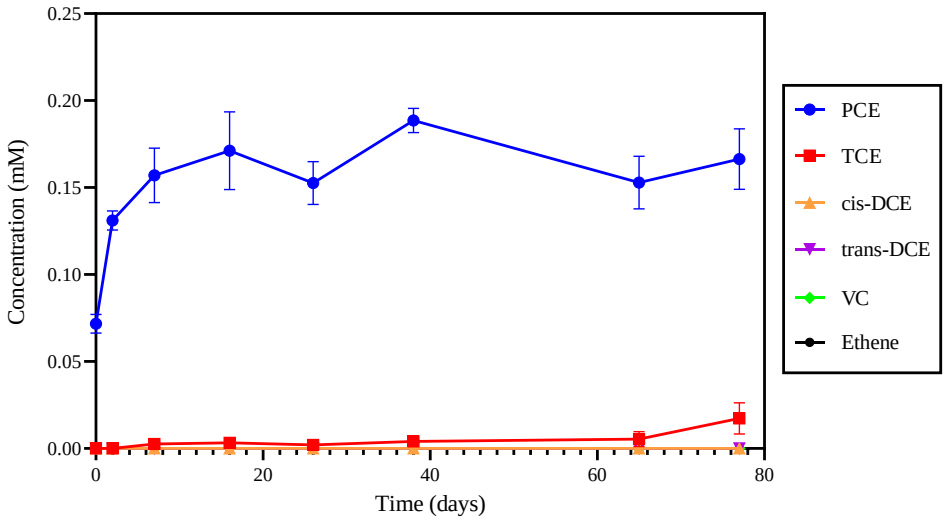

Figure 2.10 PCE transformation by HBB4 sediment microcosms in mineral salt medium in other two cultures. Data points are from duplicate cultures and error bars represent the standard deviation of the duplicates.

In a second PCE enrichment attempt, filter-sterilized seawater replaced Milli-Q water in the mineral salt medium to more faithfully replicate *in situ* conditions. The salinity of seawater was approximately 36 g/L. Enrichment cultures were initially spiked with 0.2 mM PCE. The dechlorination of PCE in the seawater medium was much faster than the first enrichment. Cultures inoculated from upper sediment cores showed faster PCE dechlorination than those from the lower cores (**Appendix 3**). In upper core cultures, within 6 days, 97.2% of

PCE was dechlorinated to TCE (0.10 mM) and DCE (0.06 mM), while no dechlorination was observed in the autoclave-sterilized controls (Figure 2.11). Additional PCE (0.2 to 0.4 mM) was supplied when the initial amount was completely removed. After 85 days cultivation, for example, one of the replicated sediment microcosms in seawater medium had dechlorinated 2.60 mM PCE to TCE (1.41 mM) and DCE (1.19 mM) (Figure 2.12). However, no further dechlorination to vinyl chloride (VC) or ethene was observed. This suggested that the dechlorination pathway was from PCE to TCE, and then to cis- and trans-DCE as end products. Given these results, enrichment in seawater medium were better than enrichment in mineral salt medium. Therefore, the former was used to test for TCDD and OCDD dechlorination.

During the first 46 days, the dechlorination rates demonstrated an increasing trend, but after that, the dechlorination rate tended to decrease (Figure 2.13). This suggested that ORB proliferated with PCE stimulation, but were subsequently inhibited when TCE and DCE isomers accumulated.

After 90 days of cultivation, sulfate was completely depleted from the seawater medium (data not shown), which did not occur in sterilized controls. This suggested that, in addition to chloroethene dechlorinating activity, sulfate-reducing bacteria were also active.

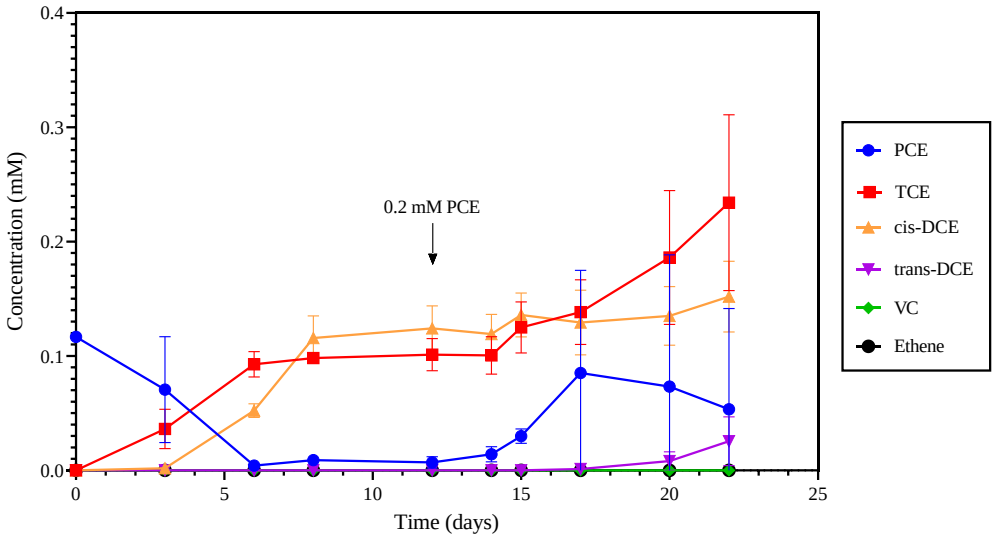

Figure 2.11 PCE transformation by HBB4 upper cores sediment microcosms in seawater medium. Data points are from triplicate cultures and error bars represent the standard deviation of the triplicate. Arrows indicate the time points when PCE was supplied.

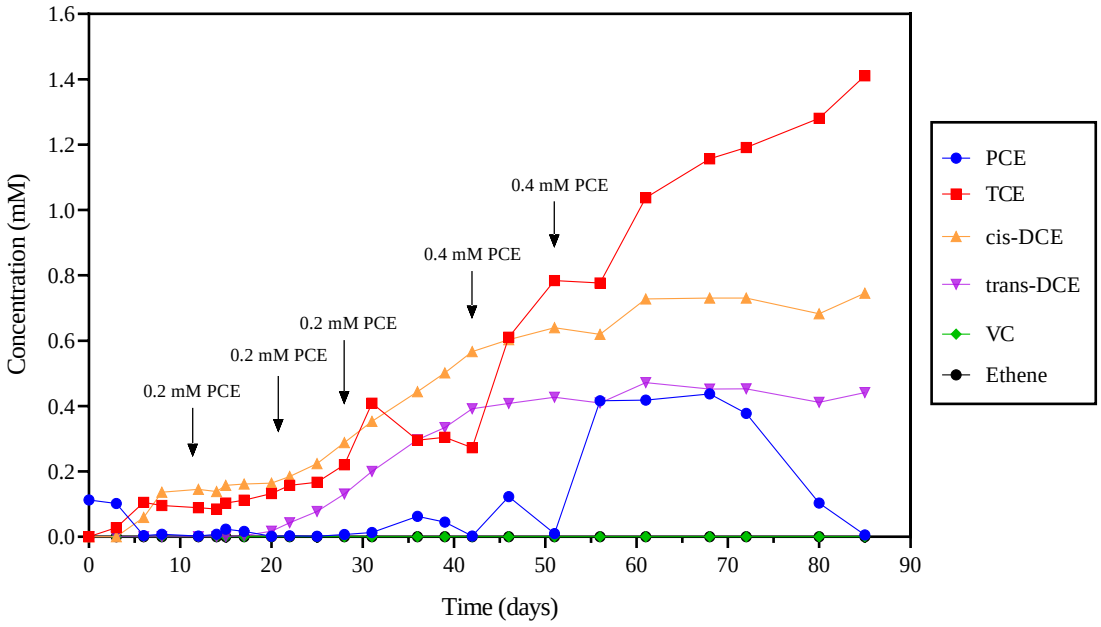

Figure 2.12 PCE transformation by HBB4 upper cores sediment microcosms in seawater medium over 85 days. Data points are from one of the triplicate cultures. Arrows indicate time points when PCE was suppled. Other replicate enrichments are presented in **Appendix 3**.

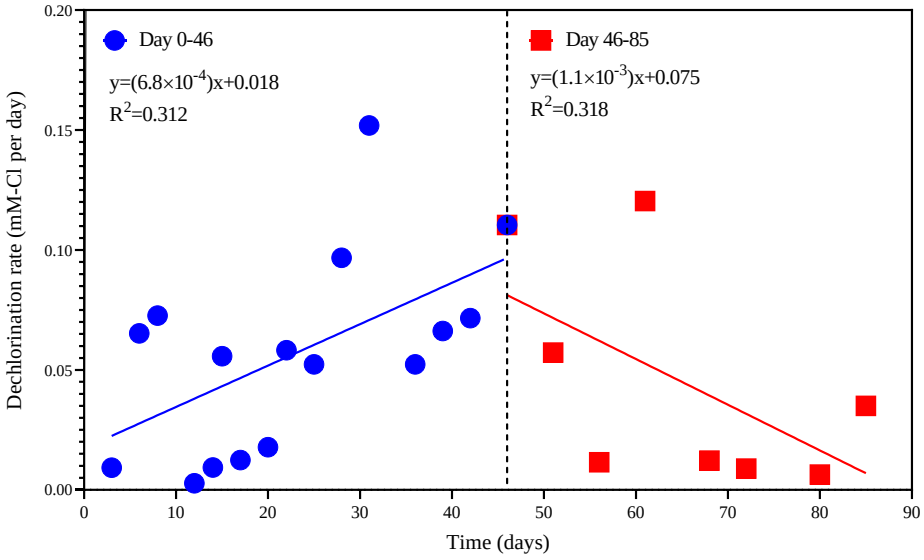

Figure 2.13 PCE dechlorination rates by sediment microcosms varied over 85 days. The calculation was based on the theoretical chloride released from PCE to DCE isomers (chloride released= TCE concentration + 2×DCE isomer concentration) per day. Data points are corresponding to data shown in Figure 2.12.

2.3.2.2 Abundance of *Dehalococcoides* lineages during PCE enrichment

The abundance of total bacteria and *Dehalococcoides* were monitored monthly via 16S rRNA gene quantitative PCR (Figure 2.14). The concentration of total bacteria did not change significantly (t-test, P=0.781), increasing from (1.55 ± 0.29) $\times 10^7$ to (4.46 ± 1.97) $\times 10^7$ copies/ml after 2 months and then decreasing to (1.03 ± 0.32) $\times 10^7$ after 3 months. In contrast, the concentration of *Dehalococcoides* lineages did significantly decrease (t-test, P<0.01) from (2.24 ± 0.72) $\times 10^5$ to (7.20 ± 2.74) $\times 10^3$ copies/ml after PCE enrichment. The relative abundance of *Dehalococcoides* strains decreased from 1.45% to 0.07%.

Quantitative PCR was also used in an attempt to identify respiratory reductive dehalogenases (RDases) responsible for PCE dechlorination. No amplification was observed with primers for PceA and TceA (LOD = 100 copies/ ml), indicating PCE dechlorination was catalysed by other unknown respiratory RDases or was co-metabolic.

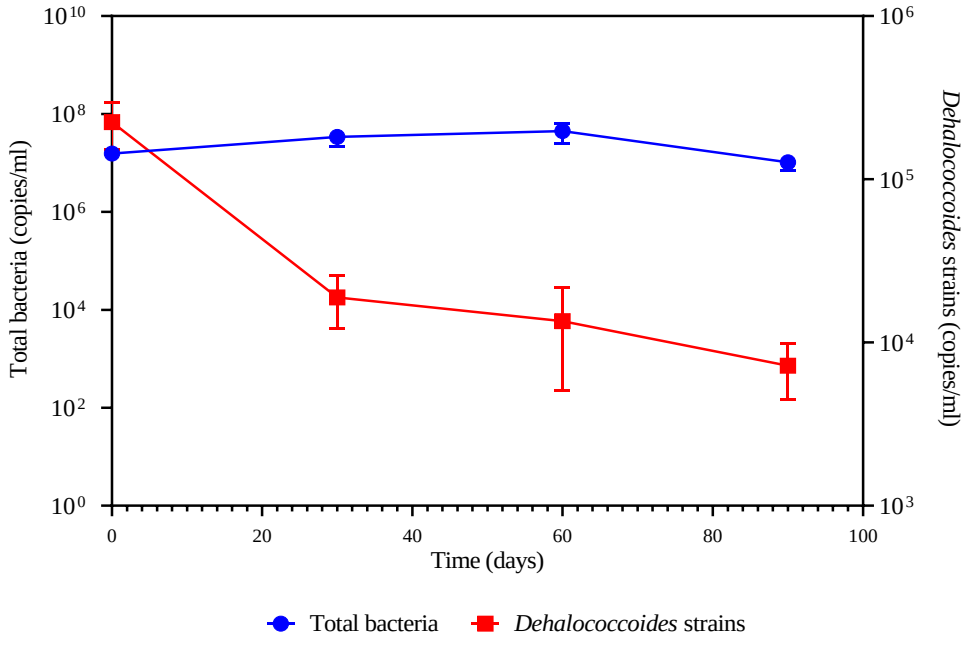

Figure 2.14 Abundance of total bacteria (left y-axis) and *Dehalococcoides* lineages (right y-axis) in PCE amended enrichment cultures. Data points are from triplicate cultures with technical replicates (n=3) and error bars represent the standard deviation of the replicates.

2.3.2.3 Molecular characterization of PCE enrichment cultures

DNA was extracted from PCE-enrichment cultures in seawater medium at Day 0 and Day 90. Derived 16S rRNA amplicons were analyzed by Illumina sequencing (Figure 2.15).

In the inoculum (Day 0), the relative abundance of Dehalococcoidaceae (family) was approximately 1.7%, while the genus *Desulfovibrio* was 0.6%. After PCE enrichment in seawater medium for 90 days, the most abundant bacterial genus was *Desulfovibrio* (44.7%), while *Dehalococcoides* fell below the detection limit after PCE enrichment, which was consistent with the results of quantitative PCR described above.

Additionally, PCE enrichment also stimulated the abundance of unclassified Dehalococcoidia bacteria, accounting for 5.3% of the total bacteria, but their capacity among PCE dechlorination was not clear yet.

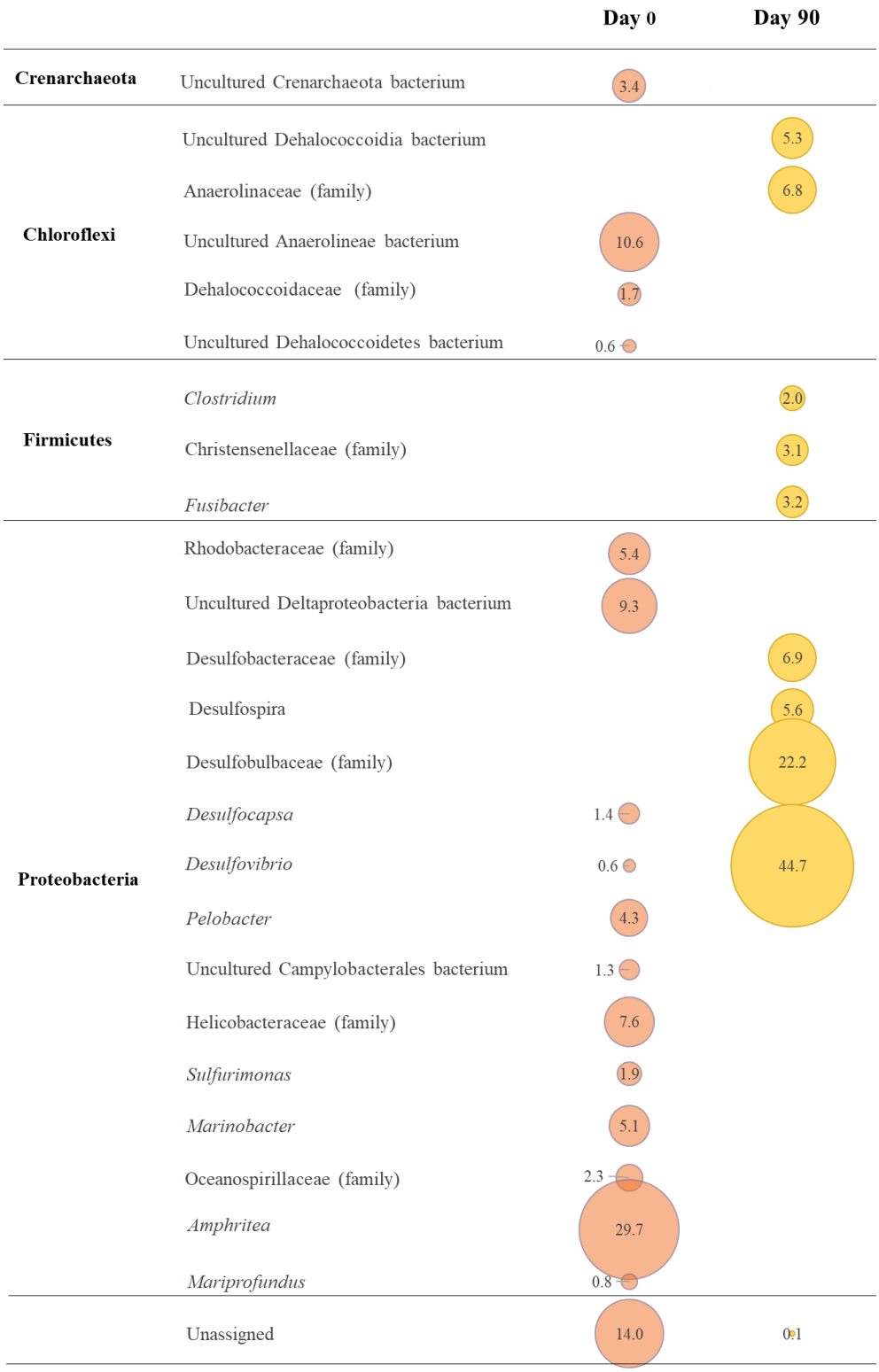

Figure 2.15 Abundance (>0.5%) of bacterial lineages in the PCE amended enrichment community. These cultures were assigned to family and genus. Bubble sizes and figures represent the relative abundance (%) of bacterial lineages. DNA was extracted from one of the triplicate PCE enrichment cultures, and the results of PCE dechlorination is corresponding to data shown in Figure 2.12.

2.3.2.4 PCE-enriched sediment microcosms on TCDD and OCDD transformation

a) Inhibitory effects of TCDD/OCDD on PCE dechlorination

The dechlorination of PCE by cultures spiked with TCDD and OCDD are presented in Figure 2.16 and Figure 2.17, respectively. TCDD and OCDD inhibited PCE dechlorination, especially on Day 11 and Day 19 (t-test with TCDD & OCDD free controls, P_{TCDD} and P_{OCDD} <0.01), but the inhibitory effect was reversible. Dechlorination activity in TCDD and OCDD amended cultures commenced after a lag period, completely dechlorinating 0.2 mM PCE to DCE within 26 days and 33 days, respectively. Contrary to the hypothesis that lecithin would enhance solubility possibly increasing toxicity, lecithin lessened the inhibitory effect on sediment microcosms in the presence of TCDD and OCDD, shortening the lag time before PCE dechlorination.

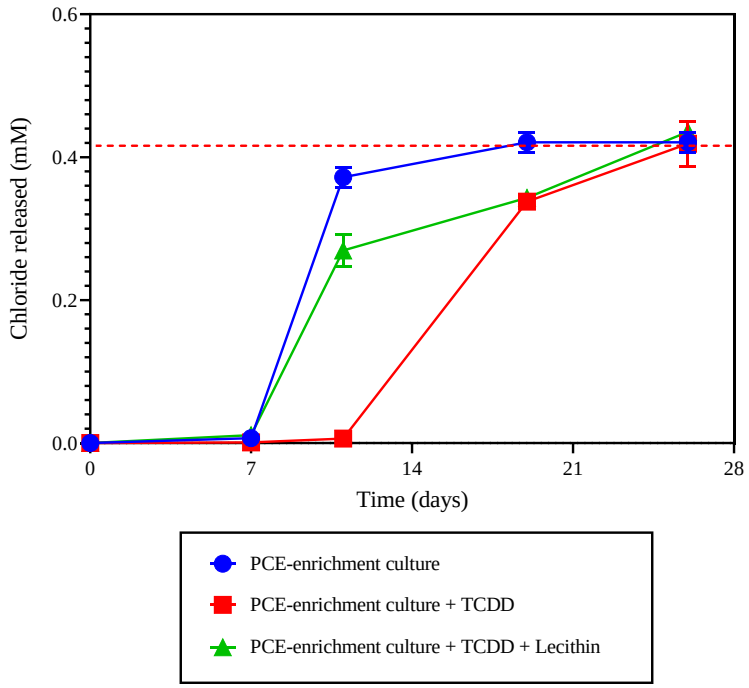

Figure 2.16 The effect of TCDD (3.0 µg/g-dw) on PCE-enriched sediment microcosms dechlorinating PCE. The red dotted line represents the theoretical chlorides released when PCE is completely dechlorinated to DCE. Data points are averages of triplicate cultures and error bars represent the standard deviation of the triplicates.

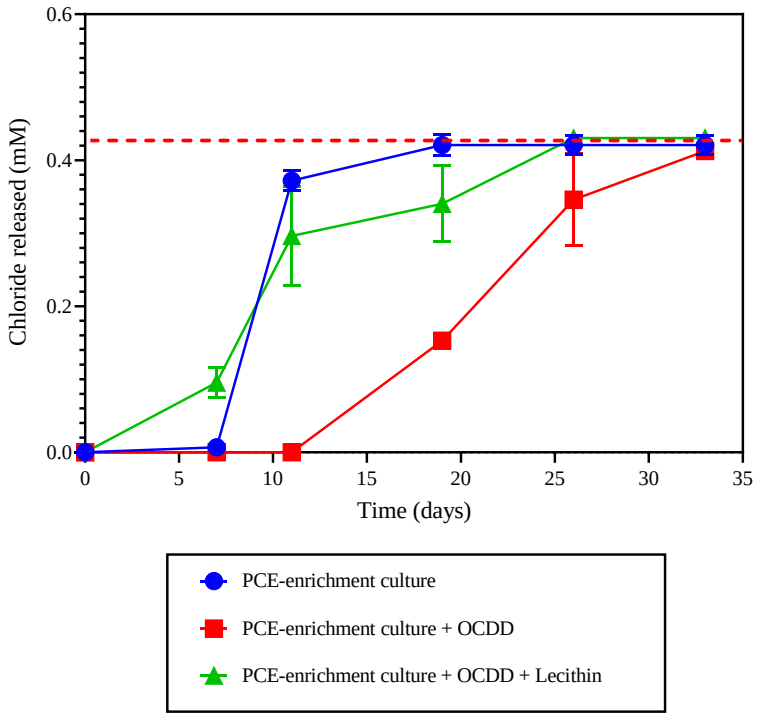

Figure 2.17 The effect of OCDD (54.0 µg/g-dw) on PCE-enriched sediment microcosms dechlorinating PCE. The red dotted line represents the theoretical chlorides released when PCE is completely dechlorinated to DCE. Data points are averages of triplicate cultures and error bars represent the standard deviation of the triplicates.

b) Impact of lecithin on the solubility of TCDD and OCDD

Lecithin significantly enhanced the solubility of TCDD and OCDD (t-test with non-lecithin controls, PTCDD and POCDD<0.01) (Figure 2.18). Without lecithin, TCDD and OCDD in the aqueous phase were not detectable, while with lecithin amendment, the aqueous phase concentrations of TCDD and OCDD were 0.3 µg/L and 0.8 µg/L, respectively. Moreover, in lecithin amended sediment, TCDD and OCDD concentration in the solid-phase were approximately 35% and 21% higher than non-lecithin controls, respectively, likely due to biosurfactants accelerating the desorption of PCDD/Fs from the solid-phase and thus enhancing the solvent extraction performance (Adrion et al., 2017; Song et al., 2007).

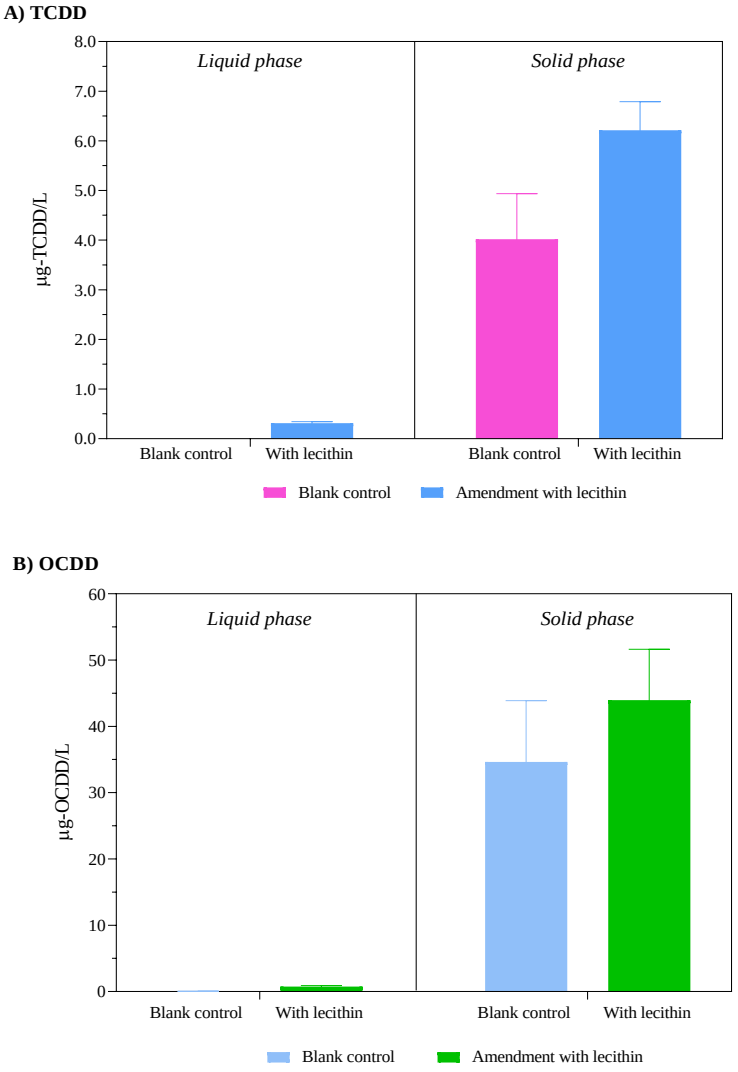

Figure 2.18 Impacts of lecithin on the solubility of TCDD (A) and OCDD (B). Column are the mean values from technical replicated extraction (n=4) and error bars represent the standard deviation (n=4).

c) Sediment microcosms on TCDD and OCDD dechlorination

PCE-enriched microcosms were tested for TCDD (3.0 µg/g-dw) and OCDD (54.0 µg/g-dw) transformation (Table 2.7). After 14 months incubation, 0.85% of the TCDD added had been reductively dechlorinated to 2,3,7-TriCDD (Figure 2.19), while 4.51% of OCDD had been reductively dechlorinated to hepta-chlorinated (3.1 ± 0.9%) and hexa-chlorinated (1.46 ± 0.2%) congeners respectively (Figure 2.20). Additionally, lecithin also enhanced the dechlorination of TCDD 2-fold and OCDD 4-fold. Except one of triplicate OCDD cultures that was slow (2.5% OCDD dechlorinated), other two replicate cultures dechlorinated 18.6 ± 1.0% of OCDD to HepCDD (6.0 ± 0.27%) and HexCDD (12.6 ± 1.3%). No dechlorination was observed in sterile controls, suggesting that the transformation was dependent on microbial activity.

Table 2.7 The dechlorination of TCDD and OCDD by PCE-enrichment sediment microcosms

	5 months		14 months	
	TriCDD (µg/g-dw)	Ratio (%)	TriCDD (µg/g-dw)	Ratio (%)
TCDD	0.00 ± 0.00	0.00 ± 0.00	0.03 ± 0.01	0.85 ± 0.28
TCDD + Lecithin	0.01 ± 0.02	0.34 ± 0.58	0.05 ± 0.03	1.52 ± 0.83

	HepCDD (µg/g-dw)	Ratio (%)	HexCDD (µg/g-dw)	Ratio (%)	HepCDD (µg/g-dw)	Ratio (%)	HexCDD (µg/g-dw)	Ratio (%)
OCDD	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	1.65 ± 0.46	3.05 ± 0.85	0.79 ± 0.12	1.46 ± 0.21
OCDD + Lecithin	0.66 ± 0.61	1.21 ± 1.12	0.00 ± 0.00	0.00 ± 0.00	2.50 ± 1.29	4.63 ± 2.39	4.63 ± 3.76	8.57 ± 6.96

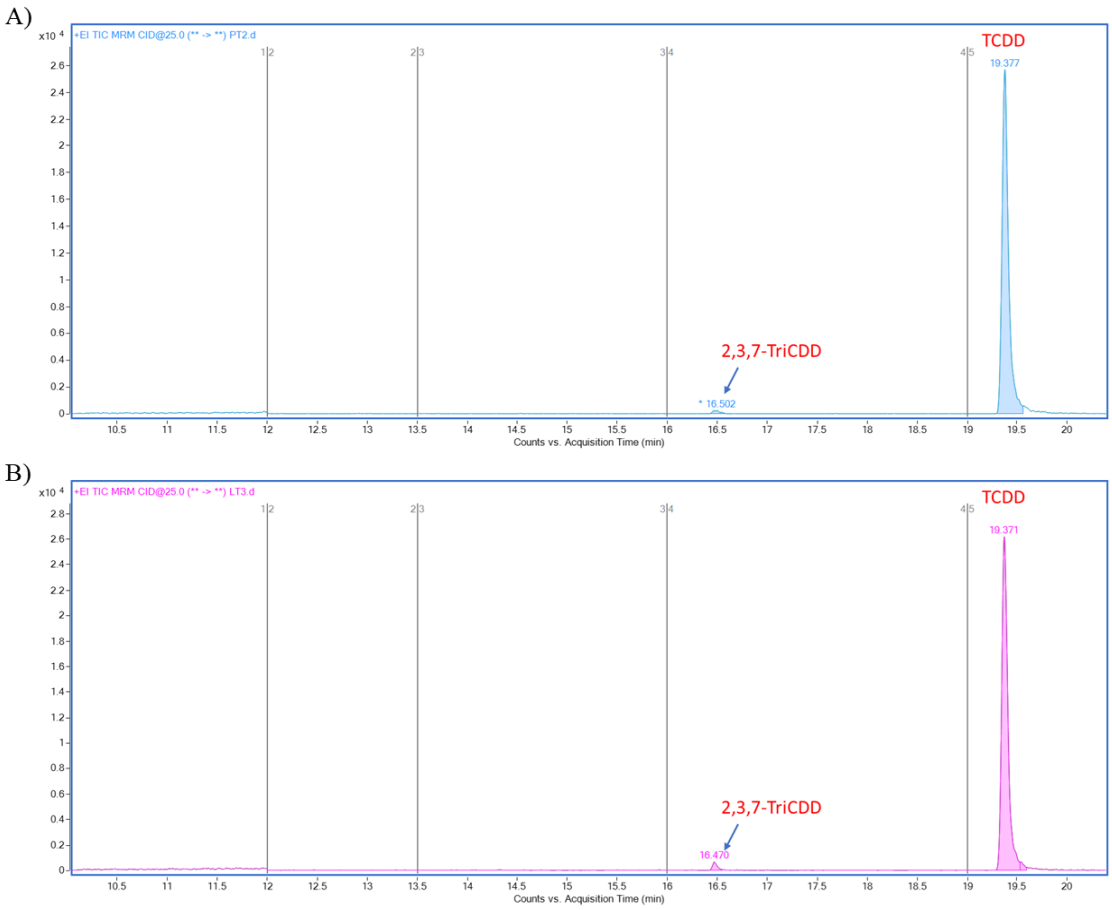

Figure 2.19 The GC-MS/MS chromatogram of TCDD dechlorination with lecithin (A) and without lecithin (B) after 14 months cultivation. The chromatographs are from one of the triplicate cultures. A peak was observed at 16.5 min, which belongs to 2,3,7-TriCDD.

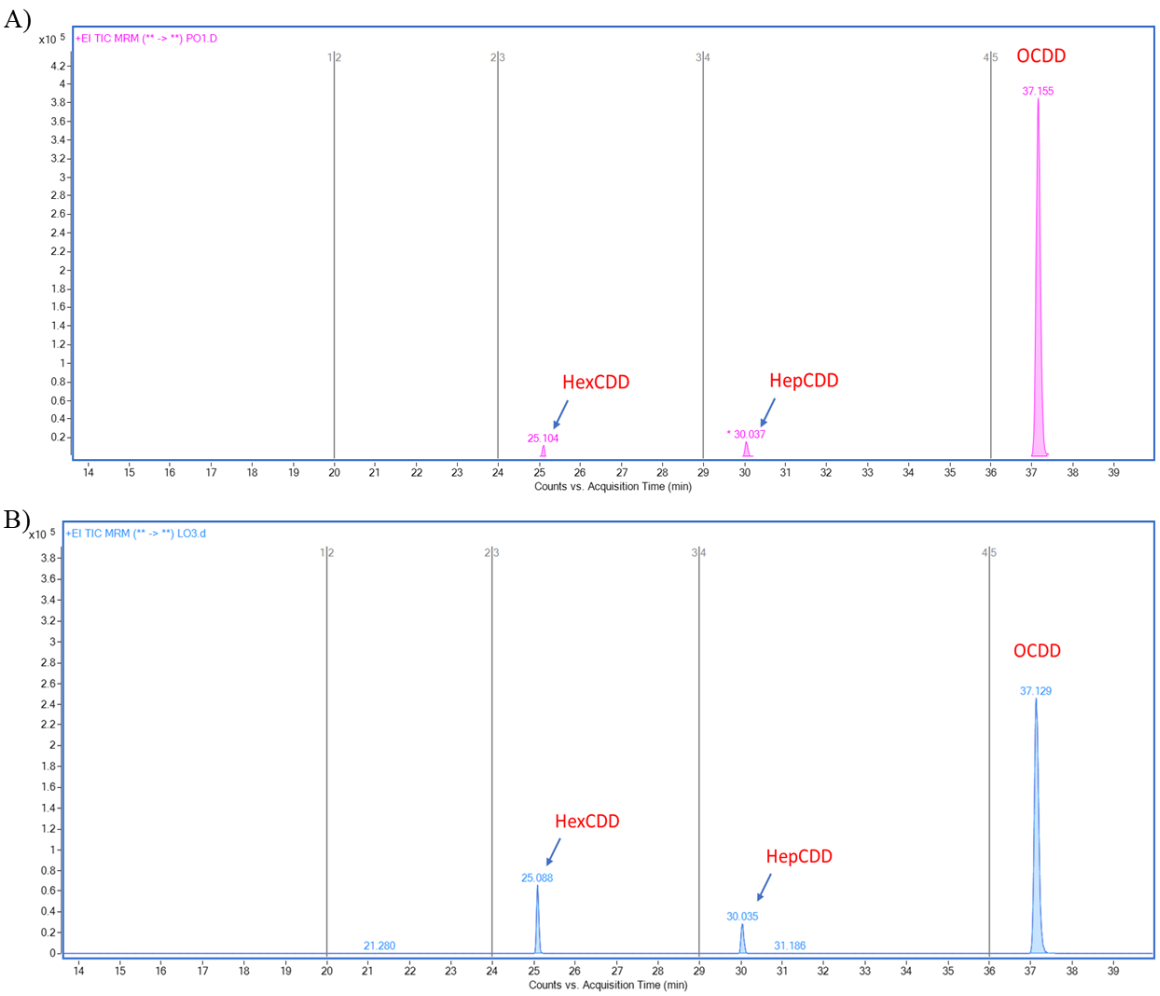

Figure 2.20 The GC-MS/MS chromatogram of OCDD dechlorination with (A) or without (B) lecithin after 14 months cultivation. The chromatographs are from one of the triplicated cultures. Peaks were observed at 30.0 min and 25.0 min, which belonged to HepCDD and HexCDD, respectively.

2.4 Discussion

2.4.1 PCDD/F concentrations in Sydney Harbour

PCDD/F toxicity equivalents in Sydney Harbour sediments have remained more or less the same over the last 10 years. As observed previously, the eastern shore of Homebush Bay was the most contaminated area. Compared with Birch *et al.* (2007), the toxicity equivalents in that area did not decrease, but the congener composition was different (Table 2.8). For example, the TCDD concentration was lower by approximately 3-fold, while the OCDD concentration was higher approximately 100 to 300-fold. It is unclear from the data if this represents a trend

in contaminant profile transformation or a reflection of spatial heterogeneity in PCDD/F distribution at that area, but the duplicated quantification in that area (S14 A/B) by Birch *et al.* (2007) also showed heterogeneity in 1234678-HepCDD, OCDD and OCDF, in which sample S14A were 2 to 4-fold higher than sample S14B. PCDD/F concentrations at other HBB locations were much lower than at HBB4, consistent with HBB4 being the source zone of PCDD/Fs. The concentrations at HBB2 were the lowest, likely caused by sediments being constantly flushed by influent water from Haslams Creek.

Table 2.8 PCDD/F concentrations at the eastern shore of Homebush Bay in 2017 v.s. in 2007.

Concentration (pg/g-dw)	Birch et al. (2007)		This study	
	S14A	S14B	Upper	lower
2378-TetraD	1,500	1,600	330	661
12378-PenCDD	40	110	68	56
123478-HexCDD	0	0	583	1,722
123678-HexCDD	4,000	2300	0	1,736
123789-HexCDD	590	850	200	304
1234678-HepCDD	180,000	67,000	59,617	58,061
OctaCDD	35,000	14,000	1,761,518	4,108,152
2378-TetraF	41	35	58	94
12378-PenCDF	0	0	522	260
23478-PenCDF	85	130	66	2,286
123478-HexCDF	420	530	627	1,027
123678-HexCDF	79	71	554	6,384
234678-HexCDF	75	180	2,160	6,803
123789-HexCDF	0	0	26	73
1234678-HepCDF	5,800	4,300	0	5,468
1234789-HepCDF	370	380	343	542
OctaCDF	3,800,000	950,000	144,047	362,636
Total concentration	4,028,000	1,041,486	1,970,719	4,556,264
WHO-TEQ concentration	4352.5	3151.6	2026.1	5206.7

PCDD/Fs in the upper and lower sediment cores were separately quantified in this study because the upper layer is more susceptible to tidal influence, with potential contaminant dilution with uncontaminated sediments. However, except for HBB4, which was manually capped with clean sediments in a remediation project (Birch *et al.*, 2013; Montoya, 2015),

PCDD/F concentrations in the upper and lower sediment cores did not have significant differences (t-test, P=0.8286). At Bray's Bay and Yaralla Bay, PCDD/Fs in the upper sediment cores were approximately 3-fold higher than in their lower sediment cores. Therefore, the management strategy suggested by Banks et al. (2016) to wait until new sediments cover the contaminated layer is impractical to address the issue in a short to medium-term time scale.

The contaminated areas in Sydney Harbour were extensive and PCDD/Fs were from multiple sources. They could be transported from Homebush Bay by tidal movement (Birch et al., 2007). Additionally, from 1922 to 2002, land reclamation was extensively carried out at Sydney Harbour, including in Yaralla Bay, Parramatta River, Middle Harbour and Lane Cove River. Chemical, building and municipal garbage wastes were used as infilling materials, resulting in heavy metals, PCDD/Fs and other hazardous compounds leaching into the harbour sediments (Mcloughlin, 2000; Birch, 2007). This might explain why PCDD/F concentrations at Major Bay and Yarralla Bay were much higher than upstream.

Given their infamous recalcitrance, it was not surpirising that there was no significant decrease in PCDD/F concentrations over the last decade. This confirms that the risks associated with the contamination will remain into the future, or until a practical remediation strategy is developed.

2.4.2 Harbour sediments on TCDD and OCDD transformation

The aim of this chapter was to survey the ability of indigenous microorganisms in Sydney Harbour sediments to transform TCDD and OCDD by organohalide respiration. DNA sequencing revealed the presence of potential PCDD/F degrading bacteria in Sydney Harbour sediments, including the genus *Dehalococcoides*. In previous studies, three pure *Dehalococcoides* strains are known to dechlorinate PCDD/Fs, and sediment microcosms containing *Dehalococcoides* strains have shown dechlorination of PCDD/Fs (Bunge et al., 2003; Fennell et al., 2004b; Pöritz et al., 2015b; Zhen et al., 2014). For example, Hieke et al. (2016) found that PCDD/F contaminated marine sediments from Texas, U.S. containing *Dehalococcoides* strains reduced 45% of the total TEQ within 24 months in their laboratory experiments. *D. mccartyi* strain DCMB5, known to dechlorinate 1,2,3,4-TCDD, was isolated from PCDD/F contaminated river sediments from Spittelwasser Creek, Germany. Given these

examples, ORB in Sydney Harbour sediments may have the capacity to transform or even detoxify PCDD/Fs.

To test for PCDD transformation, anaerobic incubations were established with harbour sediments and directly spiked with TCDD and OCDD, but they showed no dechlorination activity after two years. We hypothesized the low solubility of TCDD and OCDD might limit the capacity of sediment ORB respiring these compounds to support their growth, as such their dechlorination activity was too weak to be observed. Therefore, PCE was used as an alternative substrate with more water solubility and bioavailability to stimulate the growth of ORB prior to surveying for PCDD dechlorination.

PCE enrichments were active, but activity was much faster when seawater used as the basal medium. Properties of seawater, such as salinity and sulfate concentration, likely affected the microbial activity. These microcosms dechlorinated PCE to TCE, and then to cis- and trans-DCE, but no vinyl chloride or ethene was generated. After 90 days cultivation, sulfate in active PCE enrichments was completely depleted, and approximately 50% of the total bacteria belonged to the *Desulfovibrio* genus, while *Dehalococcoides* was undetected after the enrichment, which was unexpected. *Desulfovibrio* are sulfate-reducing bacteria (Madigan et al., 2008), so sulfate from seawater likely drove the selection. However, *Desulfovibrio* strains are not typical ORB. Only one study found a *Desulfovibrio* strain able to reductively dechlorinate 2-chlorophenol via respiration (Sun et al., 2000). During the enrichment process, the dechlorination rate decreased with the continuous supplementation of PCE, likely due to the incomplete dechlorination of chloroethenes. TCE and DCE accumulation is known to be toxic to microbes (McCarty, 1997). PCE dechlorination may also have been a co-metabolic reaction with sulfate reduction, so the depletion of sulfate slowed down the dechlorination process. Additionally, there was a significant enrichment of unclassified Dehalococcoidia bacteria after PCE fed. These unclassified bacteria might be responsible to PCE dechlorination, however, due to the limitation of sequencing database, details of these unclassified Dehalococcoidia bacteria were not clear yet.

PCE stimulation of sediment microcosms resulted in observation of reductive dechlorination of TCDD and OCDD. Whilst there was an extended lag, the dechlorination products were detected and their concentrations slowly increased over time. In TCDD cultures, 2,3,7-TriCDD was detected as the only daughter product, while HepCDD and HexCDD were

the observed daughter products in OCDD cultures. This observation was made late in the project, with little scope to investigate further.

Due to the unique toxicological characteristics of PCDD/Fs, toxicity is reduced only when dechlorination occurs at lateral-positions (2,3,7,8-positions), otherwise, toxicity will increase 3- to 10-fold with one chloride being substituted with hydrogen atoms at peri-positions (1,4,6,9-positions) (Rodenburg et al., 2017). Thus, the dechlorination of 2,3,7,8-TCDD to 2,3,7-TriCDD is expected to have reduced toxicity. However, whether the dechlorination of OCDD proceeded at peri-positions or lateral-positions is not yet known. Even though the dechlorination of OCDD on lateral-positions is more energetically favourable than on peri-positions (Table 2.9) (Huang et al., 1996; Kim et al., 2009), previous studies still observed microbial dechlorination occurring on peri-positions, leading to the formation of more toxic congeners, including 2,3,7,8-TCDD (Adriaens et al., 1994; Barkovskii et al., 1996). Therefore, future experiments should focus on identifying the dechlorination pathway of OCDD and estimating the change of toxicity equivalents over time.

Table 2.9 Gibbs free energy of reductive dechlorination with H_2 as the electron donor (at 25°C, pH=7)

Substrate	Product	$\Delta G^{0'}$ (kJ/mol)	Reference
OCDD	1,2,3,4,6,7,9-HepCDD (lateral dechlorination)	-171.2	Huang et al., 1996
	1,2,3,4,6,7,8-HepCDD (peri dechlorination)	-159.1	
2,3,7,8-TCDD	2,3,7-TriCDD	-143.2	
PCE	TCE	-55.4	Dolfing et al., 2006
TCE	DCE	-53.1	

From the data generated, it is not possible to conclude that PCE-dechlorinating microbes were responsible for TCDD and OCDD dechlorination. The initial purpose of PCE stimulation was to enrich *Dehalococcoides* strains in the harbour sediment, however, after PCE enrichment, neither *Dehalococcoides* strains nor *Dehalobium* strains was detected, while *Desulfovibrio* strains became highly abundant. Microbial community composition data for the TCDD and OCDD dechlorinating cultures was not available at the time of writing, but it may be possible that microbial communities have changed in the last 14 months in the presence of TCDD and OCDD.

The question remains why dioxin biotransformation does not occur *in situ* in Sydney Harbour sediments. Toxicity of TCDD and OCDD was proven not to be the main reason for this. With TCDD and OCDD concentrations approximately 4500 and 14 times higher than the most contaminated area, respectively, PCE dechlorination by sediment microcosms was only partially inhibited and recovered after a lag period. It was likely that even supplied with excess TCDD and OCDD, due to their low solubility and low bioavailability (Table 2.10), the toxic effect to microbes is limited. Therefore, *in situ* concentrations of TCDD and OCDD, which are lower than the *in-vivo* concentrations, are unlikely to completely inhibit the biological dechlorination activity. However, it is unclear whether the extended lag of TCDD and OCDD dechlorination in PCE enrichment cultures was only caused by an inhibitory effect of TCDD and OCDD, because active controls used for comparison were only spiked with PCE but without blank dried sediments amended with hexane. In those TCDD/OCDD cultures, dried sediment powders and residual hexane might also interfere dechlorination performance, which was unclear.

Table 2.10 Water solubility of chloroethenes and dioxins at 25 ℃

Chemical	Water solubility (mg/L)	Reference
OCDD	4.00×10^{-7}	Ying Shlu et al., 1988
2,3,7,8-TCDD	2.00×10^{-4}	
PCE	15	Horvath et al., 1999
TCE	128	

It was hypothesized that the low solubility of TCDD and OCDD might limit the capacity of bacteria respiring these compounds to support their growth (Table 2.10). Even though the saturated solubility of TCDD (0.2 µg/L) is above the detection limits of GC-MS/MS (LOD_{TCDD} and $LOD_{OCDD} \approx 10^{-2}$ µg/L), without biosurfactant amendment, TCDD and OCDD were not detected in the aqueous phase. This suggested that in the presence of sediments, TCDD and OCDD were more readily adsorbed to the solid-phase rather than dissolved in the aqueous-phase, so their actual solubilities were much lower than the theoretical values (Table 2.10) (Poiger and Schlatter, 1980; Milliken et al., 2004). In this study, TCDD and OCDD were spiked into sediment cultures using stock sediment to more faithfully replicate their existing forms in Sydney Harbour. Interestingly, without lecithin amendment, PCE-enriched sediment microcosms were still able to dechlorinate TCDD and OCDD. This suggested that TCDD and OCDD with low solubility were still accessible to PCDD/F-dechlorinating microbes. On the

other hand, even though the dechlorination of OCDD and TCDD are more energetically favourable than the dechlorination of PCE and TCE (Table 2.10), PCE and TCE dechlorination rates were much faster than the former, likely due to their differences in water solubility (Huang et al., 1996; Dolfing et al., 2006; Ying et al., 1988; Horvath et al., 1999). Given these results, it was hypothesized that the low bioavailability of PCDD/Fs might be one of the key factors slowing the microbial dechlorination, thus limiting the abundance of PCDD/F-dechlorinating microbes *in situ*, as a possible reason to explain why dioxin biotransformation in Sydney Harbour was not significant, and PCE with higher solubility and bioavailability likely stimulated these microbes to higher abundance.

Previous studies have suggested biosurfactants can enhance the solubility of PCDD/Fs and other low soluble contaminants and thus enhance their biodegradation (Lin et al., 2014; Adrion et al., 2017; Fava and Gioia, 2000; Adrion et al., 2017). In this study, a biosurfactant lecithin was used to enhance the water solubility of TCDD and OCDD. Lecithin was studied due to its affordability and lower toxicity than artificial surfactants (Soeder et al., 1996b), and it had been used to successfully enhance the biodegradation of PCE and TCE (Brant and Smith, 2015), polychlorinated biphenyls (PCBs) (Fava and Gioia, 2000), polycyclic aromatic hydrocarbons (PAHs) (Soeder et al., 1996a) and OCDD (Lin et al., 2017). In this study, the use of lecithin significantly enhanced the solubility of TCDD and OCDD in the aqueous phase. The aqueous phase concentration of OCDD (0.8 µg/L) was approximately 2000 times higher than its saturated water solubility (4×10^{-4} µg/L), while the aqueous phase concentration of TCDD (0.3 µg/L) was 1.5 times higher than its theoretical solubility (0.2 µg/L). Interestingly, enhanced solubility of TCDD and OCDD by lecithin did not increase toxicity. Instead, the dechlorination by PCE enrichment cultures increased approximately 2-fold for TCDD and 4-fold for OCDD. Enhancements were also observed in PCE dechlorination in the presence of TCDD and OCDD. One explanation is that lecithin may have accelerated the desorption of PCDD and PCE from sediment and enhanced their solubility in the liquid-phase, making them more accessible to the dechlorinating bacteria (Bustamante et al., 2012). On the other hand, lecithin is abundant with phospholipids, which may sorb the cell surface and cover it with a lipophilic layer to increase microbial adherence to lipophilic contaminants (Bustamante et al., 2014; Singh et al., 2007; Singh et al., 2007; Fava and Gioia, 2000). Additionally, lecithin is a good carbon and energy source or it might be similar to fermented yeast extract, supplying unknown nutrients and growth factors to facilitate the growth of specific bacteria in sediment

microcosms. (Fava and Gioia, 2000; Holliger et al., 1993; Kengen et al., 1999; Fava and Gioia, 2000; Soeder et al., 1996b).

Lack of electron donors in Sydney Harbour sediments might be another reason why dioxin biotransformation was insignificant. However, according to the theoretical hydrogen threshold calculation in Appendix 4, the minimal H_2 concentration required for OCDD dechlorination is approximately 10^{-35} M, which is extremely low and consistent with TCE dechlorination to cis-DCE by *Dehalococcoides mccartyi* strain 195 (Mao et al. 2015). In this study, even though the concentrations of electron donors were not monitored, extra H_2 and acetate were regularly supplied to PCE enrichment cultures and TCDD/OCDD cultures, which should be enough to maintain their dechlorinating respiration. However, in actual conditions such as in Sydney Harbour, other factors might also affect PCDD/F dechlorinating bacteria consuming H_2 and the required H_2 concentration might be much higher that this theoretical value. For example, in marine sediments, nitrate and sulfate reducing bacteria and methanogens compete with ORB for electrons, which might limit their dechlorinating performance (Xu et al., 2018; Löffler et al., 1999; Men et al., 2012). Additionally, PCDD/Fs may be toxic to fermenting bacteria, and as such, H_2 generated by fermenting bacteria is affected.

In conclusion, the existence of microbes in Sydney Harbour sediments capable of reductively dechlorinating TCDD and OCDD has been shown for the first time. Whilst sediment microcosms directly spiked with TCDD and OCDD showed no transformation after 2 years, PCE used as the priming substrate effectively stimulated sediment microcosms to dechlorinate TCDD and OCDD. Toxicity of TCDD and OCDD was not the main reason for the lack of significant dioxin biotransformation *in situ*, but their low solubility and low bioavailability seemed to be a factor for that. Lecithin enhancing TCDD and OCDD dechlorination was another interesting discovery in this study, but its role in the dechlorination is not completely clear, so further investigation of the effect of lecithin on TCDD and OCDD dechlorinating microcosms using DNA sequencing is worth. Future experiments to be carried out, include: 1) monitoring TCDD and OCDD cultures if further dechlorination occurs; 2) identifying the dechlorination pathways of OCDD to predict the change of toxicity and assessing the feasibility of using these reactions for PCDD/F remediation in Sydney Harbour; 2) identifying microbes possibly responsible for TCDD and OCDD dechlorination and isolating these microbes; 3) investigating the roles of lecithin in microbial dechlorination of

TCDD and OCDD; 4) testing the ability of other biosurfactants, such as cyclodextrin, to assist the dechlorination of TCDD and OCDD.

Chapter Three- Impacts of marine salinity and sulfidized nanoscale zerovalent iron (S-nZVI) on dechlorination activity of *Dehalococcoides mccartyi* strain CBDB1

3.1 Introduction

In the previous chapter, data presented was consistent with the existence of PCDD-dechlorinating microbes in Sydney Harbour sediments. The requirement for pre-enrichment with PCE and the duration of the lag before TCDD and OCDD dechlorination commenced, suggests such microbes exist at low abundance in harbour sediments. In this chapter, aspects of the feasibility of applying known PCDD-dechlorinating *Dehalococcoides mccartyi* strain CBDB1 to harbor sediment remediation were explored (Figure 3.1) (Bunge et al., 2003). This included assessing the tolerance of strain CBDB1 to elevated salinity and exploring interactions between sulfidized nanoscale zerovalent iron (S-nZVI) and strain CBDB1.

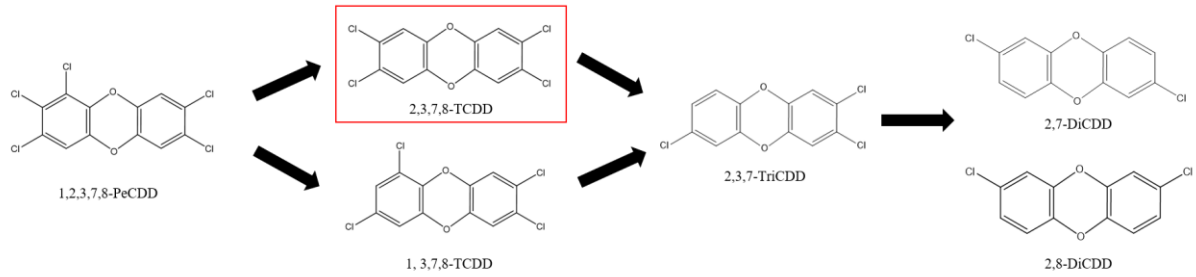

Figure 3.1 Reductive dechlorination of 1,2,3,7,8-PeCDD and 2,3,7,8-TCDD by *Dehalococcoides* CBDB1 (Bunge *et al*., 2003). It is the only known *Dehalococcoides* strain to dechlorinate 2,3,7,8-TCDD.

The water in Sydney Harbour has similar salinity to seawater (3.5%, w/v), however, strain CBDB1 has been maintained in low salinity (0.1% w/v) medium since isolation (Birch and Rochford, 2010; Johnston et al., 2015; Adrian et al., 1998a; Hölscher et al., 2003). Therefore, CBDB1's tolerance to different salinities was examined. Provide studies have shown that microbes have regulation systems to adapt to the high salinity environment. When the environmental salinity suddenly changes, microbes can increase their intracellular osmotic pressure by accumulating ions (such as K^+ and Ca^{2+}) and proteins (such as glutamate and proline) to resist water loss (Tempest et al., 1970; Vreeland, 1987; Vreeland et al., 1983).

Microbes can also secrete extracellular polymeric substances to reduce cell destruction from high osmotic pressure (Johir et al., 2013). Given these findings, strain CBDB1 may also be able to acclimate high salt environment.

Previous studies have shown that S-nZVI provides better reductive dehalogenation performance with organohalides and extended longevity compared with bare nZVI (Kim et al., 2011; He et al., 2018; Li et al., 2017; Rajajayavel and Ghoshal, 2015). Rajasekar and Ghoshal (2015) showed that the sulfidation of nZVI increased the dechlorination rate of trichloroethene (TCE) by up to 40 times. The iron-sulfide layer also suppresses the corrosion of nZVI by water and unnecessary waste of electrons to generate H_2 [$Fe(0) + 2H_2O \rightarrow Fe^{2+} + H_2 + 2OH^-$], thus enhancing its longevity (Yepez and Obeyesekere, 2017; Kim et al., 2011). Given these results, S-nZVI may have potential to dechlorinate PCDDs. Therefore, the abililty of S-nZVI to dechlorinate TCDD and OCDD was assessed.

Dehalococcoides strains are strictly hydrogenotrophic (Holliger et al., 1998; Adrian et al., 2007). Therefore, for bioremediation applications in Sydney Harbour, a sustainable source of H_2 for organohalide respiration is needed. Typically, organic carbon, such as lactate or vegetable oil, is used to feed indigenous fermentative microflora to maintain high hydrogen partial pressure. Whilst nZVI has been shown to be a suitable alternative to supply H_2 (Zemb et al., 2010; Koenig et al., 2016; Xie et al., 2017), there has been no investigation into the potential of S-nZVI to serve as a longer lasting source of H_2 to sustain organohalide respiration.

Strain CBDB1 is also sensitive to oxygen and requires a low redox environment to maintain viability and catalyse reductive dechlorination reactions, especially those organohalides with low midpoint potentials. To achieve these conditions, titanium(III) citrate is used for cultivating pure cultures in the laboratory (Adrian et al., 2007; Ge et al., 2012). However, due to its high cost and tendency to precipitate as $Ti(OH)_3$, titanium(III) citrate is unsuitable for *in situ* applications (Machado and Wallbank, 1990). S-nZVI has the potential to serve as a reductant for conditioning the habitat of strain CBDB1 to facilitate reductive dechlorination based metabolism. It was hypothesised that S-nZVI could generate enough H_2 to support strain CBDB1 respiring organohalides and possibly lower the redox potential sufficiently to facilitate rapid microbial dechlorination. The lower cost of S-nZVI means it could be used as an alternative reducing agent to titanium citrate for *in situ* remediation (Jiang et al., 2018; Li et al., 2017).

In this study, a mixture of 1,2,3- and 1,2,4-trichlorobenzene (TCB) was primarily used as a legitimate dioxin proxy to assess the effects of environmental factors on CBDB1's activity. This decision was based on the shorter incubation times needed for dechlorination by strain CBDB1 to be observed and to avoid health risks associated with handling TCDD.

3.2 Materials and methods

3.2.1 S-nZVI synthesis

The synthesis of nZVI used the wet-chemical method. The reaction is presented in Equation 3.1 (Yuvakkumar et al., 2011). In an anaerobic glove box, ferrous chloride tetrahydrate ($FeCl_2 \cdot 4H_2O$) was dissolved in anoxic Milli-Q water and constantly stirred by a mixer. Sodium borohydride ($NaBH_4$) was separately dissolved in anoxic Milli-Q water and poured in a separatory funnel. The molar ratio of BH_4^-/Fe^{2+} was approximately 1.5:1. BH_4^- solution in the funnel was added dropwise to Fe^{2+} solution at a speed of 1 drop per second, and after a few drops, tiny black particles were formed. After the borohydride solution had been completely added, the mixture was left stirring for another 20 min.

$$2Fe^{2+} + BH_4^- + 3H_2O \rightarrow 2Fe(0) + H_2BO_3^- + 4H^+ + 2H_2 \quad \text{(Equation 3.1)}$$

To obtain nZVI, the iron suspension was transferred to a glass beaker and used a neodymium magnet used to accelerate settling of iron particles. The supernatant was discarded, while the iron particles were washed with anoxic Milli-Q water and repeated settlement for 3 times. Finally, nZVI was reconstituted with anoxic Milli-Q water to a concentration of 50 g/L.

To synthesize S-nZVI, the iron suspension with residual borohydride was added to a sodium sulfide solution. The S/Fe molar ratio was 0.5, which was the optimal ratio for abiotic TCE dechlorination (O'Carroll et al., unpublished results). The mixture was stirred constantly for another 30 min. The iron particles were settled and washed with anoxic Milli-Q water 3 times. Finally, the iron particles were reconstituted with anoxic Milli-Q water to a concentration of 50 g/L. Unless stated otherwise, the concentration of S-nZVI represents the iron concentration.

3.2.2 TCE transformation by S-nZVI

TCE was used to compare S-nZVI and nZVI dechlorination performance. Unless stated otherwise, the experiments were set up in 35 ml serum flasks with 18 ml anoxic Milli-Q water and 17 ml headspace. The flasks were sealed with Teflon coated rubber septa and aluminum crimps. The headspace was flushed with nitrogen flow for 30 min. 1 ml of 50 g/L iron particles stock solution were added to the final iron concentrations of 2 g/L. Reaction vessels were spiked with 1 ml of TCE stock solution (1 g/L) to a final concentration of 0.40 mM. The reaction vessels were placed on a horizonal shaker at 150 rpm at 25℃. The headspace gas monitored regularly by GC-FID described in 3.2.5.1.

3.2.3 S-nZVI on TCDD and OCDD transformation

TCDD and OCDD stock glass beads were prepared similar to stock sediments described in 2.2.6. Weighed TCDD and OCDD were dissolved into 50 ml hexane and properly mixed with 20 g of glass beads. After that, hexane was completely evaporated under nitrogen flow, so that the TCDD and OCDD adsorbed onto the glass beads. The concentrations of TCDD and OCDD on glass beads were 0.3 µg/g-dw and 2.0 µg/g-dw, respectively.

To estimate the ability of S-nZVI to dechlorinate TCDD and OCDD, one gram of TCDD and OCDD stock glass beads were separately spiked into 50 ml of 2 g/L S-nZVI solution. The reaction vessels were placed on a horizonal shaker at 150 rpm at 25℃. The analytical methods for TCDD and OCDD are described in 3.2.5.3.

3.2.4 Anaerobic medium for strain CBDB1

The protocols for anaerobic medium were similar to 2.2.4 described in Chapter two. In summary, Milli-Q water was amended with the appropriate amount of 50x mineral salt solution, 1000x trace element stock solution, 30 mM acetate and 0.2mM sodium sulfide. After deoxygenation by nitrogen sparging for 45 min, 25 ml medium was dispensed into 35 ml serum bottles, leaving 10 ml of headspace. The flasks were sealed with Teflon coated rubber septa and aluminum crimps. The headspace was flushed with N_2/CO_2 (4:1) for 3 min. The medium was autoclaved at 121℃ for 30 mins. After cooling down, cultures were inoculated into the

medium and supplied with 1000x vitamin stock solution, 30 mM of $NaHCO_3$ and 1 mM titanium(III) citrate. All cultures were incubated statically at 30℃ in the dark.

To test for CBDB1 tolerance to different salinities, the anaerobic medium was supplied with 0.1, 0.2, 0.5, 1.0 and 2.0% (w/v) NaCl. For seawater medium, Milli-Q water was replaced with filter-sterilized seawater from Coogee Beach, Sydney, with 3.6% (w/v) salt.

TCB stock solution (1 M) was prepared by dissolving equivalent 1,2,3- and 1,2,4-TCB in methanol. Before autoclaving of anaerobic medium, approximately 1 µL of TCB stock solution was spiked into anaerobic medium using a 10 µl glass syringe. The final concentration of TCB was approximately 40 µM. For HCB cultures, approximately 10 mg HCB crystals were directly added into 50 ml medium before autoclaving, as described by Jayachandran et al. (2003).

3.2.5 Analytical methods

3.2.5.1 Trichloroethene quantification

100 µl of culture headspace gas was manually injected into an Agilent Technologies 7890A gas chromatography equipped with a flame ionization detector (GC-FID) and a GS-Q capillary column (30 m ×0.32 µm; J&W Scientific). The inlet and detector temperature were set at 250℃. The helium carrier gas flow rate was 3 ml/min. To separate acetylene, ethene and ethane, the oven program was initially held at 70℃ for 2 min, and then increase to 250℃ at a rate of 40℃ per min and held for 2 min. Retention times (minutes) for each compound were as follows: methane (1.20), ethene (1.54), acetylene (1.60), ethane (1.71) and TCE (6.52).

3.2.5.2 Chlorobenzenes quantification

0.5 ml of culture liquid was taken to a 10 ml GC vial and sealed with a magnetic steel cap with silicone septa. Chlorobenzenes were analysed by a Shimadzu GC-2010 Plus gas chromatograph, equipped with an FID detector and a DB-5 capillary column (30m × 0.32 µm × 0.25 µm; J&W Scientific). Before injection, samples were pre-heated at 80℃ and shaken for 5 min. A Shimadzu AOC-5000 Plus autosampler injected 250 µl of headspace gas into the GC-FID. The inlet and detector temperature were set at 250℃. The helium carrier gas flow rate

was 3 ml/min. The oven program was initially held at 50 ℃ for 1 min, and then increase to 190 ℃ at a rate of 20 ℃ per min. Due to low volatility of hexa-, hepta- and tetrachlorobenzene, this method only detected tri- to nono-chlorobenzene. Retention time (min) of chlorobenzenes were as follows: 1,3-DCB (5.45), 1,4-DCB (5.55), 1,3,5-TCB (6.53), 1,2,3-TCB (6.95) and 1,2,4-TCB (7.20).

3.2.5.3 Polychlorodibenzo-*p*-dioxins quantification

2 ml of the mixture was taken to a 10 ml glass tube and centrifuged at 4000 rcf for 4 min. The supernatant was discarded, and the pellet was extracted by 4 ml of 1:1 methanol/hexane twice. Anhydrous sodium sulfate was used to absorb residual moisture. The extracts were completely evaporated under nitrogen gas flow and reconstituted with 1 ml of dichloromethane (DCM). The extracts in DCM was measure by GC-MS/MS described in 2.2.8.2 in Chapter Two.

3.2.5.4 Redox potential measurement

The medium redox potential was measured by a HANNA HI-5521 Research Grade Meter equipped with a HANNA HI5312 Ag/AgCl (1 M KCl) reference electrode. In an anaerobic glove box, medium was poured into a 50 ml breaker. The tip of the electrode was submerged in medium approximately 4 cm below the liquid surface until the reading stabilized. The Ag/AgCl referenced ORP values were converted to the standard hydrogen electrode referenced ORP by adding 236 mV (Nordstrom and Wilde, 1998).

3.2.5.5 H_2 quantification

H_2 concentration was measure by a Shimadzu 2010 gas chromatograph equipped with a HP-Molesieve capillary column (30m × 0.32 μm × 0.25 μm; J&W Scientific) and a Pulsed Discharge Detector (PDD). The inlet temperature was 250℃, and the helium carrier gas flow rate was 3 ml/min. 10 μL of headspace gas was manually injected into GC-PDD. The oven program was held at 50 ℃ for 1.3 min. Retention time of H_2 was 1.05 min.

3.2.5.6 Dissolved iron quantification

A 1 ml volume of iron suspension was filtered with a 0.22 µm syringe filter. The filtrate was 10 times diluted with 1% HNO_3 and measure by an Agilent 7700 Inductively Coupled Plasma (ICP), equipped with a mass spectrometry detector.

3.3 Results

3.3.1 The tolerance of *D.* mccartyi strain CBDB1 to seawater salinity

To examine the tolerance of strain CBDB1 to different salinities, strain CBDB1 was cultivated in anaerobic medium with different concentrations of NaCl (0.1 to 2.0%, w/v) and seawater with 3.6%, (w/v) salt (Figure 3.2). There was no significant inhibition of TCB dechlorination by strain CBDB1 when the salt concentration was below 0.5% (ANOVA test, P=0.84). Cultures had longer lag times in 0.5% salt, but still completely transformed approximately 47.9 µM TCB to 1,3- and 1,4-dichlorobenzenes (DCBs) within 33 days. When the salinity reached 1.0%, inhibition became significant (t-test, P=0.03), but after approximately 50 days of lag, TCB dechlorination commenced, and 49.1 µM TCB was completely transformed to DCBs within 127 days. In contrast, only one of three replicate cultures in 2.0% salt medium was active. After a 20-day lag, this active culture completely transformed 48.1 µM of TCB to DCBs within 98 days. CBDB1 inoculated into seawater medium with 3.6% (w/v) salt showed no activity after 98 days in any of the replicate cultures (n=3) (data not shown).

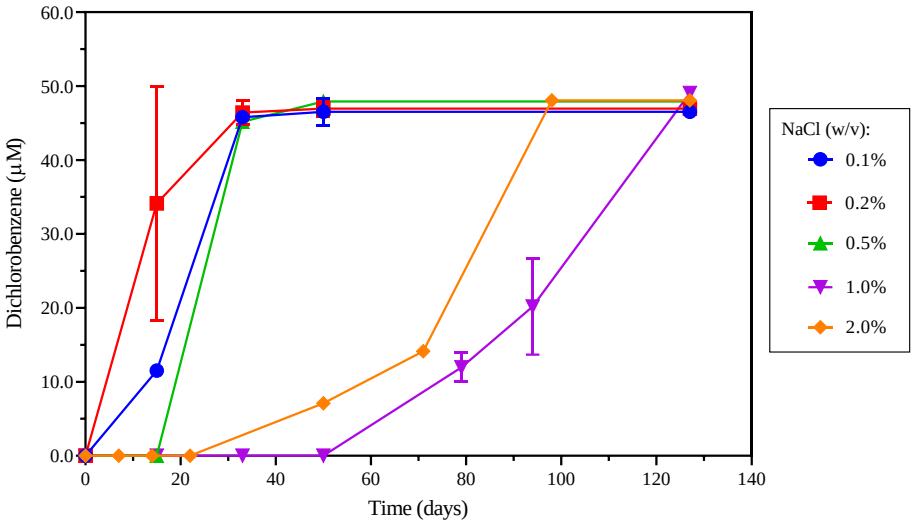

Figure 3.2 TCB transformation by *D. mccartyi* strain CBDB1 in medium with different salt concentration (NaCl: 0.1 to 2.0%, w/v). Except cultures in 2.0% salt medium, data points are the mean values of triplicates and error bars represent the standard deviation (n=3). Only one of the triplicate cultures in 2.0% salt medium transformed TCB to DCB.

To determine if strain CBDB1 could be adapted to higher salt concentrations, the culture active in 2.0% salt medium (described as first generation) was inoculated 5% (v/v) into a new 2.0% salt medium (described as second generation) (Figure 3.3). All replicate (n=3) in second-generation cultures showed dechlorination activity completely transforming 43.3 µM TCB to DCB within 81 days. To determine whether CBDB1 cultures had adapted to seawater after culturing in 2% salt medium, the second-generation cultures were inoculated 5% (v/v) to seawater medium (3.6%, w/v). After approximately 30 days of lag, dechlorination commenced. Even though the standard deviation was large, all replicates (n=3) in seawater medium showed dechlorination activity, completely transforming 45.9 µM TCB to DCB within 100 days. This indicated that after two generations of subculturing in 2.0% salt medium, CBDB1 cultures adapted to salinity levels found in Sydney Harbour sediments.

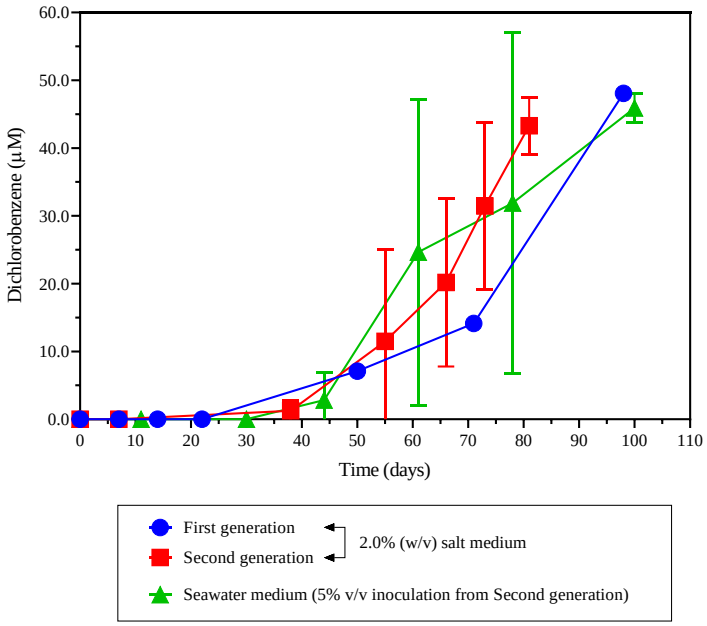

Figure 3.3 TCB transformation by *D. mccartyi* strain CBDB1 in 2.0% salt medium and seawater medium. Except for the first generation, data points are the mean values of triplicate cultures and error bars represent the standard deviation (n=3). The first-generation culture in 2.0% salt medium is also shown in Figure 3.2.

3.3.2 Abiotic S-nZVI on TCDD and OCDD dechlorination

The ability of S-nZVI to dechlorinate TCDD or OCDD was tested by shaking incubation in 2 g/L S-nZVI solution at 25 ˚C. However, no dechlorination of TCDD and OCDD was observed after 75 days (data not shown).

To test whether the lack of activity was owing to the quality of the S-nZVI synthesis, activity was assessed on a substrate (TCE) for which data already exists (Rajajayavel and Ghoshal, 2015). S-nZVI dechlorinated 96.3% of TCE (approximately 0.37 mM) to non-chlorinated alkenes within 24 hours (Figure 3.4). The dechlorinated products were primarily acetylene, followed by ethylene, and approximately 1.6% of TCE (approximately 6.4 µM) was transformed to ethane within 24 hours. The overall mass balance was approximately 97%. After 7 days, acetylene was completely transformed to ethylene (Data not shown). Dichloroethene (DCE) and vinyl chloride (VC) were not detected.

TCE dechlorination by S-nZVI was a *pseudo* first-order reaction. Fifty percent of TCE was transformed to acetylene and ethylene within 3 hours, and a further 46% was transformed

within 24 hours (Figure 3.5). The pseudo first-order reaction rate constant (K_{obs}) was 0.153 hr^{-1}, which was close to the 0.193 hr^{-1} observed by Rajajayavel and Ghoshal (2015). This showed the synthesis of S-nZVI was successful.

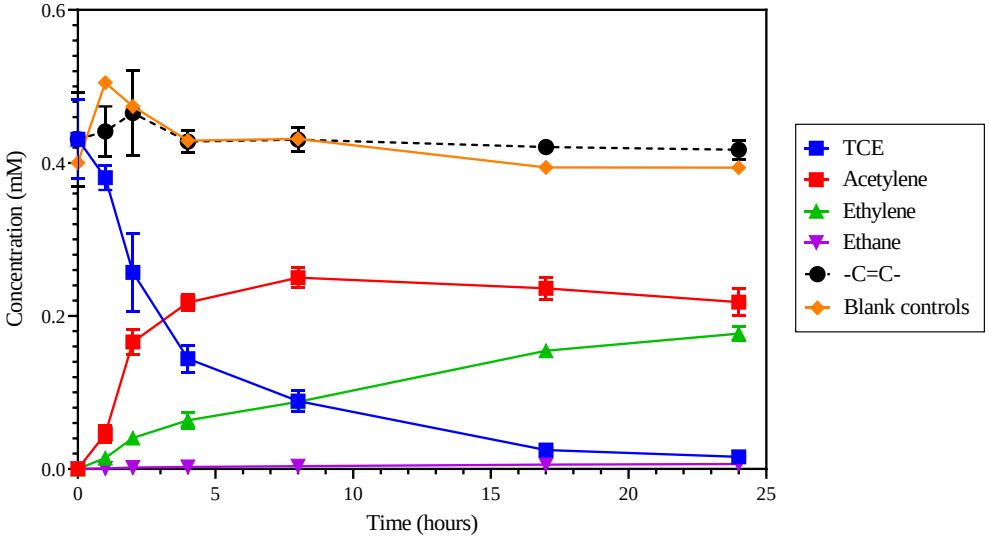

Figure 3.4 TCE transformation by S-nZVI. -c=c- represents the sum concentration of TCE, acetylene, ethylene and ethane. Data points are the mean values of triplicates and error bars represent the standard deviation (n=3).

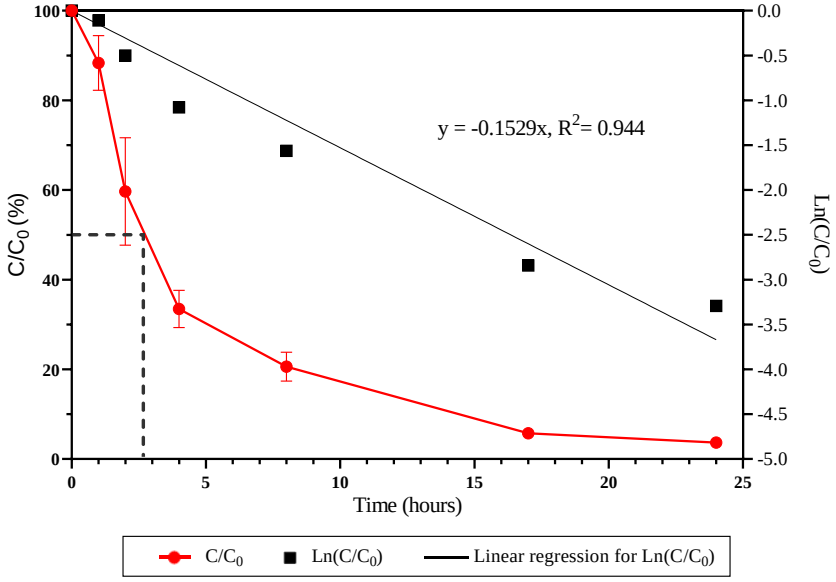

Figure 3.5 The removal of 0.4 mM TCE with time and the linear regression of $Ln(C/C_0)$ to calculate the pseudo first order constant. Data points are the mean values of triplicates and error bars represent the standard deviation (n=3).

To understand the aging effect of S-nZVI in the reaction medium, which might explain why transformation of TCDD and OCDD did not occur, 0.4 mM TCE removal over 2 h was repeatedly tested over 83 days (Figure 3.6). After one month, the dechlorination activity increased by approximately 10% compared with Day 0, while after 2 months, the activity decreased by approximately 50%. After 83 days, S-nZVI was still active, with 42% of the initial activity. This means that S-nZVI was still active on TCE dechlorination after 3 months.

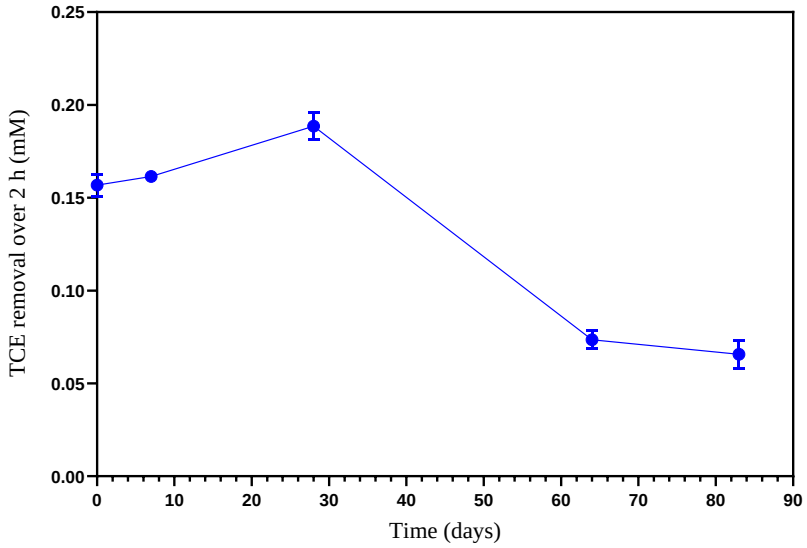

Figure 3.6 The removal rate of 0.4 mM TCE by 2 g/L S-nZVI over 2 hours (mM/2h). Data points are the mean values of triplicates and error bars represent the standard deviation (n=3).

In contrast, fresh nZVI (not sulfidized) was tested on TCE dechlorination as a comparison, but the dechlorination was negligible (Figure 3.7). After 7 days, only 0.6 % of TCE (0.003 mM) was dechlorinated to acetylene and ethene. The concentration of TCE did not decrease and DCE and VC were not detected. The K_{obs} was 2.7×10^{-3} h^{-1}, which was approximate 4.5 times less than the result observed by Rajajayavel and Ghoshal (2015) (1.2×10^{-2} h^{-1}). This result suggested that nZVI was rapidly passivated two hours after synthesis and its TCE dechlorination efficiency was 70-fold lower than S-nZVI.

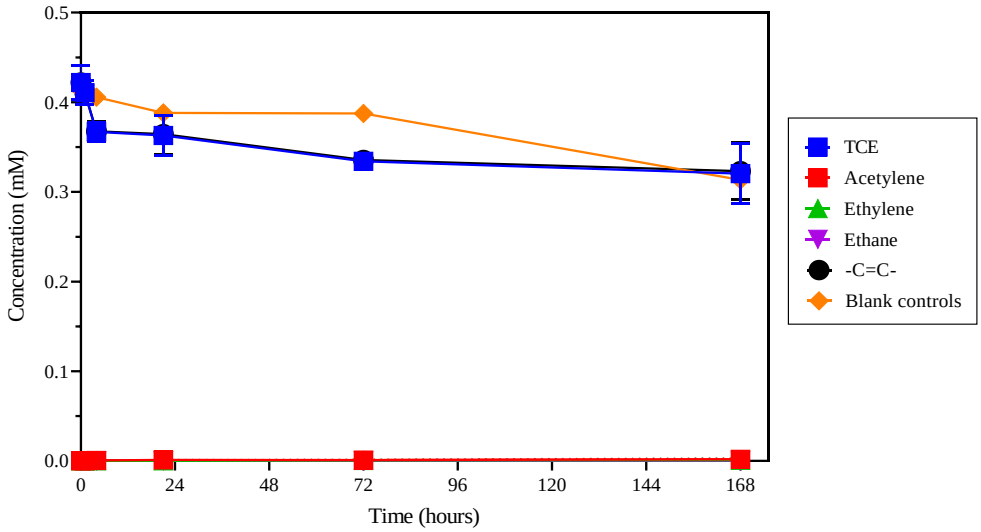

Figure 3.7 TCE transformation by nZVI. -c=c- represents the sum concentration of TCE, acetylene, ethylene and ethane, but it is covered the line of TCE concentration. Data points are the mean values of triplicates and error bars represent the standard deviation (n=3).

3.3.3 S-nZVI assists organohalide respiration by strain CBDB1

3.3.3.1 *D. mccartyi* strain CBDB1 shows tolerance to S-nZVI

To test the tolerance of CBDB1 to S-nZVI, cultures were amended with different concentrations of S-nZVI (0.1 to 5 g/L) and supplied with 40 µM TCB as the electron acceptor for respiration. Previous studies have shown that bare nZVI has high toxicity to microbes and CBDB1 tolerated nZVI below 0.25 g/L (Chaithawiwat et al., 2016; Semerád and Cajthaml, 2016; Xu et al., 2014; Koenig et al. 2016). To determine the effect of sulfidation on nZVI's toxicity, 0.5 g/L nZVI was used for comparison.

Dechlorination rates were slower in the presence of 0.1 to 2 g/L S-nZVI and severely impacted by 5 g/L (Figure 3.8). Without CBDB1 inoculation, S-nZVI did not transform TCB after 34 days, suggesting that TCB dechlorination was microbially mediated. In S-nZVI amended cultures, the dechlorination time for equivalent TCB (40 µM) increased with S-nZVI concentration ranging from 26 days (0.1 g/L) to above 34 days (2 g/L). Interestingly, CBDB1 had a much higher tolerance to S-nZVI than nZVI. Dechlorination by CBDB1 was still observed with 5 g/L S-nZVI and completed dechlorination after two months, but CBDB1 was completely halted by 0.5 g/L nZVI. No further dechlorination to chlorobenzene (CB) was observed in S-nZVI amended cultures, suggesting that S-nZVI also could not transform DCB.

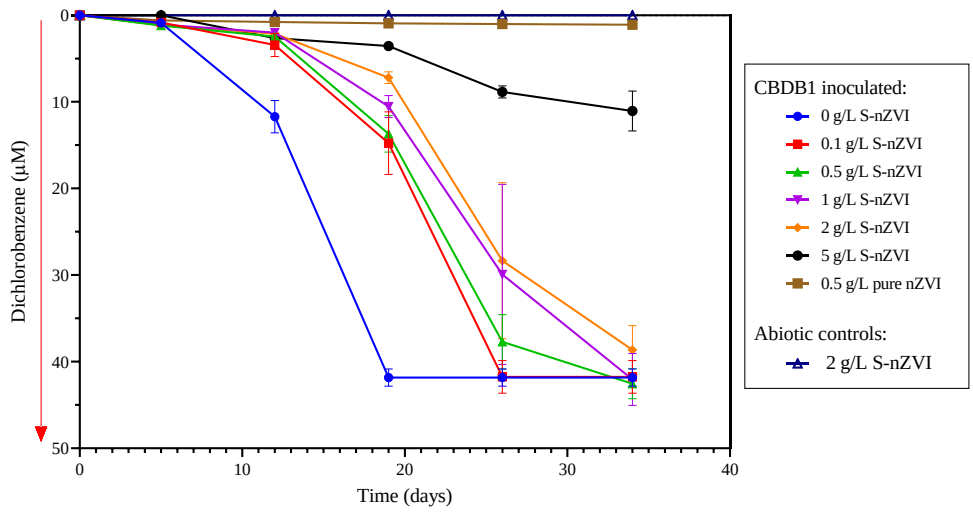

Figure 3.8 *D. mccartyi* strain CBDB1 with different concentrations of S-nZVI (0.1 to 5 g/L) and nZVI (0.5 g/L) on TCBs transformation (n=3). Data points are the mean values of triplicate cultures and error bars represent the standard deviation (n=3). Abiotic controls represent S-nZVI transformation on TCBs without CBDB1 inoculation. The quantification is based on the complete transformation of TCBs to 1,3- and 1,4-DCB.

3.3.3.2 S-nZVI acts as a H_2 donor for CBDB1 organohalide respiration

To determine if S-nZVI could supply H_2 to CBDB1 via iron corrosion and hydrolysis of H_2O, cultures were prepared excluding exogenous H_2 and supplied with S-nZVI (0.1 to 2 g/L) and nZVI (0.5 g/L) (Figure 3.9). The medium was also supplied with 30 mM acetate as carbon source and 1 mM titanium(III) citrate as the reducing agent.

S-nZVI could supply H_2 for CBDB1 respiration with 0.5 to 1 g/L being the optimal concentration. Below 0.5 g/L S-nZVI, the TCB transformation rate increased with increasing S-nZVI concentration. With more than 1 g/L S-nZVI, the transformation rate decreased with increasing S-nZVI concentration. No TCB dechlorination was observed in cultures amended with 0.5 g/L nZVI, which was consistent with the toxicological result described above. Surprisingly, TCBs transformation was also observed in CBDB1 cultures without exogenous or endogenous H_2 supply, however, the dechlorination rate was the slowest. This result was in contrast to the theory by Holliger et al. (1998), and likely caused by H_2 carry over with inoculum.

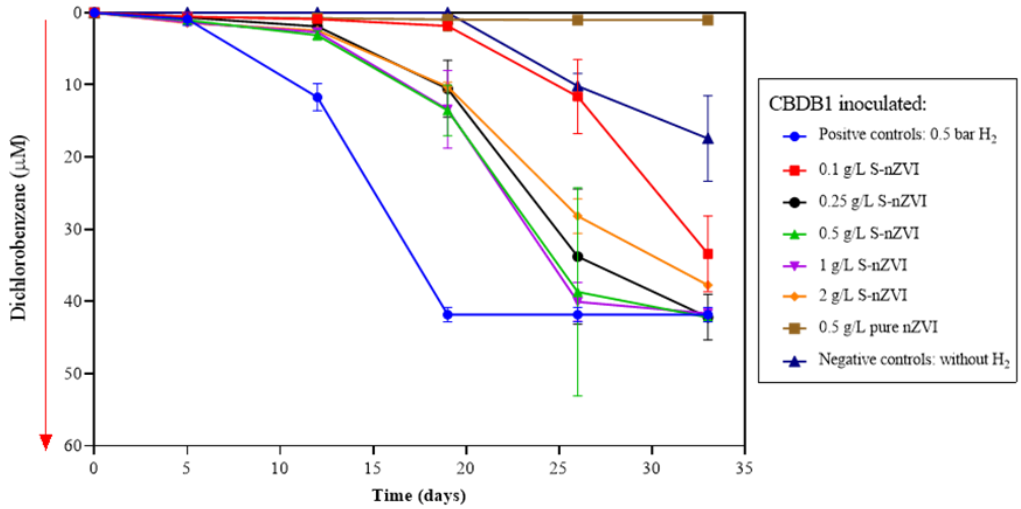

Figure 3.9 S-nZVI (0.1 to 2 g/L) as a H_2 donor for *D. mccartyi* strain CBDB1 on TCB transformation. Data points are the mean values of triplicate cultures and error bars represent the standard deviation (n=3).

To determine the H_2 generation rate by S-nZVI in anaerobic medium, uninoculated medium supplied with S-nZVI and nZVI were monitored over 27 days (Figure 3.10 and Table 3.1). The H_2 generation rate increased with S-nZVI concentration. nZVI generated H_2 at 1.12 mM per day, which was approximately 5 times faster than equivalent iron concentrations of S-nZVI (0.22 mM per day). In media inoculated with CBDB1 but without exogenous H_2, the H_2 concentration increased at a rate of 0.05 mM per day, indicating that H_2 generating reactions were occurring. Media amended with 1 mM FeS was also measured, but no H_2 was detected after 27 days (Data not shown).

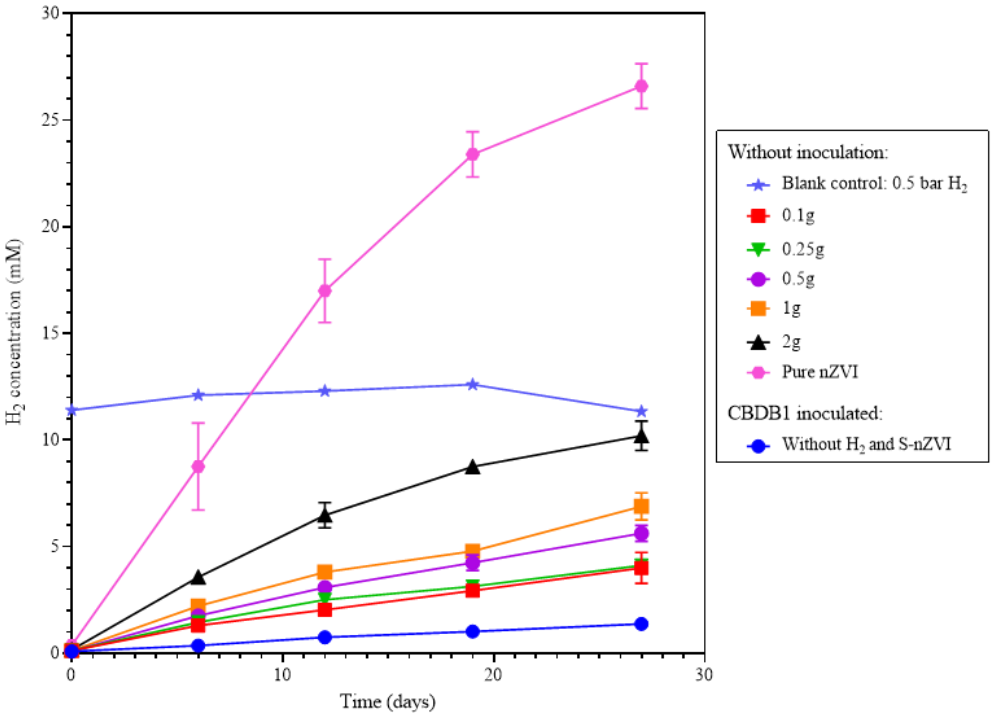

Figure 3.10 H_2 generation by S-nZVI (0.1 to 2 g/L) and nZVI (0.5 g/L). Data points are the mean values of triplicates and error bars represent the standard deviation. Blank controls represent the uninoculated medium supplied with 0.5 bar H_2.

Table 3.1 The H_2 generation rate by S-nZVI (0.1 to 2 g/L) and nZVI (0.5 g/L)

	Iron concentration (g/L)	H_2 generation rate (mM per day)	R^2
S-nZVI	0.1	0.15	0.975
	0.25	0.17	0.935
	0.5	0.22	0.973
	1	0.26	0.965
	2	0.43	0.923
nZVI	0.5	1.12	0.925
Inoculated cultures	0.0	0.05	0.975

3.3.3.3 S-nZVI effectively lowers the redox potential

The redox potentials (ORP vs H_2) of medium amended with different reducing agents were measured (Table 3.2). These included: 1 mM titanium(III) citrate, 1 mM sodium sulphide (Na_2S), 0.5 g/L S-nZVI, 0.5 g/L nZVI and 1 mM iron sulfide (FeS).

Without reducing agents, the initial ORP of anoxic medium was 56.1 mV and increased to 89.4 mV after three months. The initial ORP with Na_2S was the highest (-284.4 mV), followed by nZVI (-317.1 mV). After 3 months, the ORP with nZVI decreased to -440.3 mV, whereas ORP with NaS_2 increased to -251.2 mV. The ORP with titanium(III) citrate was the lowest (-436.3 mV) and slightly decreased to -465.7 mV after 3 months. In contrast, the initial ORP with S-nZVI and FeS were -410.3 mV and -364.6 mV, but both increased after 3 months, respectively.

Table 3.2 Redox potentials (mV vs. H_2) of medium with different reducing agents

Different reducing agents	Day 0	3 months after
Anoxic but unreduced medium	56.1	89.4
1 mM Na_2S	-284.4	-251.2
0.5 g/L nZVI	-317.1	-440.3
1 mM FeS	-364.6	-323
0.5 g/L S-nZVI	-410.3	-388.7
1 mM titanium(III) citrate	-436.3	-465.7

The effect of different reducing agents on CBDB1's ability to dechlorinate TCB was tested (Figure 3.11). All cultures were supplied with 0.5 bar H_2, 30 mM acetate and 0.2 mM Na_2S as the nutritional sulfur source. Except for nZVI, CBDB1 amended with all reducing agents facilitated TCB dechlorination. Cultures amended with titanium(III) citrate and FeS had the fastest dechlorination rate on TCB, completing TCB transformation to DCB within 20 days, which was followed by cultures with S-nZVI. Cultures amended with Na_2S had the longest lag time and the slowest dechlorination rate, but completed the transformation within 27 days. Using different reducing agents did not significantly affect the ratio of 1,3- and 1,4-DCB (Table 3.3) (ANOVA, $P>0.999$), and no further dechlorination to chlorobenzene was observed, suggesting that the use of different reducing agents did not affect the dechlorination pathway.

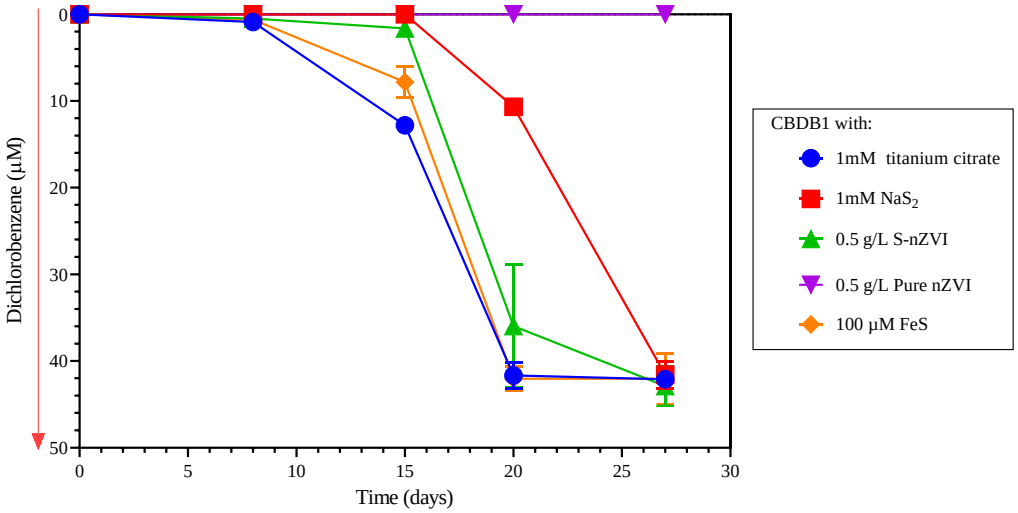

Figure 3.11 TCB transformation by *D. mccartyi* strain CBDB1 amended with different reducing agents. Data points are the mean values of triplicate cultures and error bars represent the standard deviation.

Table 3.3 The ratio (%) of dechlorination products by different reducing agents

Ratio of end products (%)	1,3-DCB	1,4-DCB
Titanium(III) citrate	29.3 ± 0.5	70.7 ± 0.5
S-nZVI	37.4 ± 1.7	62.6 ± 1.7
FeS	41.2 ± 2.6	58.8 ± 2.6
Na_2S	34.2 ± 0.5	65.8 ± 0.5

The impact of different reducing agents on hexachlorobenzene (HCB) transformation by CBDB1 was also tested (Figure 3.12), because higher chlorinated benzenes are more resistant to biodegradation than less chlorinated congeners (Malcolm et al., 2004), and HCB (4.7×10^{-3} mg/L) is less water soluble than TCB (30 to 49 mg/L) but close to TCDD (2.0×10^{-4} mg/L) (Southworth and Keller, 1986; HSDB, 2011; Talian., 1986; Ying Shlu et al., 1988). nZVI was excluded from this experiment due to its toxicity. HCB (10 mg) was added into 50 ml medium in a crystalline form, so the saturated concentration was 4.7×10^{-3} mg/L (HSDB, 2011).

S-nZVI did not dechlorinate HCB abiotically, suggesting that HCB dechlorination was microbially mediated. All reducing agents facilitated HCB dechlorination. CBDB1 cultures amended with titanium(III) citrate and S-nZVI had the fastest dechlorination rate, without statistical differences (t-test, P=0.6429), while FeS amended cultures on HCB dechlorination

was significantly slower than those amended with titanium(III) citrate (t-test, P=0.016). Cultures amended with Na_2S had the longest lag time and the slowest dechlorination rate, which was consistent with TCB dechlorination. Additionally, different reducing agents did not significantly affect the ratio of dechlorination products (ANOVA, P>0.999) (Table 3.4), but Na_2S amended cultures generated higher ratios of 1,3,5-TCB.

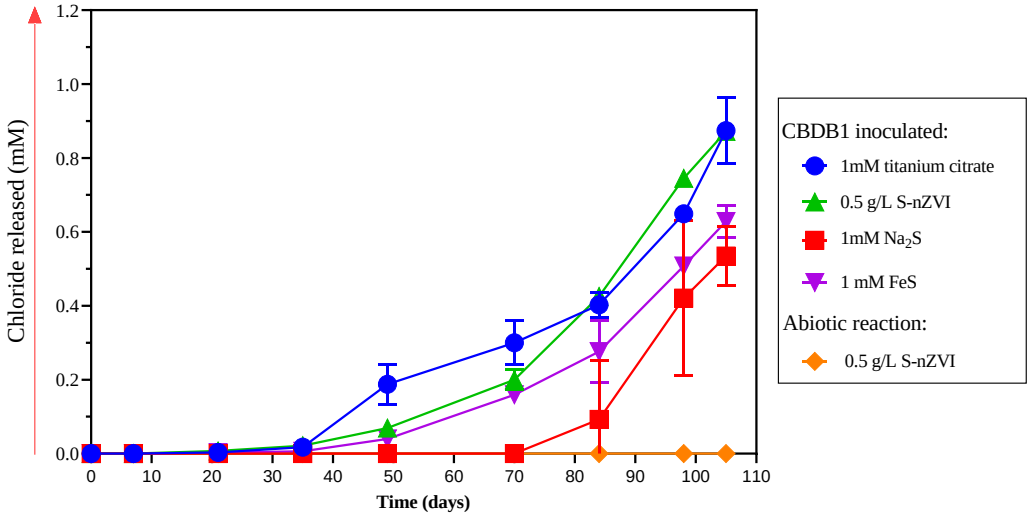

Figure 3.12 *D. mccartyi* strain CBDB1 amended with different reducing agents on HCB transformation. Data points are the mean values of triplicate cultures and error bars represent the standard deviation. The quantification was based on the theoretical chloride release from HCB to 1,3,5-TCB and 1,3- and 1,4-DCB (Chloride released= 3×TCB + 4×DCB).

Table 3.4 The ratio (%) of HCB dechlorination end products by different reducing agents

Ratio of end products (%)	1,3,5-TCB	1,3-DCB	1,4-DCB
Titanium(III) citrate	56.7 ± 3.2	16.7 ± 0.9	26.7 ± 2.2
S-nZVI	56.8 ± 1.6	17.0 ± 0.7	26.2 ± 0.8
FeS	51.1 ± 3.1	17.5 ± 2.9	31.4 ± 0.1
Na_2S	66.9 ± 0.8	14.1 ± 1.1	19.0 ± 1.9

3.4 Discussion

This study ascertained the suitability of *D. mccartyi* strain CBDB1 in its application for PCDD/F remediation in Sydney Harbour, including its tolerance to marine salinity and the

potential of S-nZVI to dechlorinate TCDD and OCDD and to support organohalide respiration through liberation of H_2 as electron donor and lowering of redox potential. TCB was primarily used as a proxy substrate in tests instead of TCDD, because it is more soluble and less toxic than TCDD, which shortened the time scale of experiments and increased analytical output. From a laboratory safety perspective, the use of TCB reduced the risks associated with handling TCDD.

D. mccartyi strain CBDB1 could tolerate salinity equivalent to marine environments. The cultures were significantly inhibited at intermediate salt concentrations (above 1% w/v NaCl), but this inhibitory effect was reversible and the TCB transformation commenced after longer lag time. CBDB1 was initially inactive in seawater with 3.6% (w/v) total salt, but the strain became seawater tolerant after acclimation through two generations of subcultivation in 2% NaCl medium. Till now, there was no previous study that has demonstrated Dehalococcoides strain adaptation to high concentration salt through increasing salt concentration, however, some literatures demonstrated Dehalococcoides strains are capable of surviving under high salt pressure. Empadinhas et al. (2004) discovered that under high osmotic pressure, *Dehalococcoides spp.* synthesizes a compatible solute called mannosylglycerate to regulate osmotic balance and stabilize protein structures inside the cell (Alarico et al., 2007; Borges et al., 2014). This phenomenon might explain why *Dehalococcoides* strains could tolerate high salt concentration and why they have been observed in marine sediments worldwide, including in this study (Hieke et al., 2016; Shiang Fu et al., 2005; Ahn et al., 2007). Given these findings, there is potential for strain CBDB1 to be deployed in harbour sediments for TCDD dechlorination.

It was initially hypothesized that nZVI did not react with PCDDs in previous studies because of the rapid formation of the passive FeOOH layer ($Fe(0) + 2H_2O \rightarrow Fe(OH)_2 + H_2$), leading to extremely short dechlorination rates (Bokare et al., 2013; Kim et al., 2008). However, even with enhanced dechlorination efficiency and extended longevity, S-nZVI was still incapable of dechlorinating TCDD and OCDD at an observable rate. Low concentration of TCDD and OCDD might be a factor limiting their transformation, because S-nZVI mediated dechlorination is a pseudo first-order reaction and its dechlorination rates depend on contaminant concentrations (Butler and Hayes, 2001). In other studies, however, nZVZ (zinc) dechlorinated 20% of 4.6 µg/L OCDD to TCDD within 2 hours (Bokare et al., 2013), and Pt/C rapidly dechlorinated 78% of 1 mg/L OCDD to non-chlorinated dioxin within 5 min (Zhang et

al., 2007), indicating that even with low PCDD concentration, zinc and palladium can still catalyse the dechlorination of PCDD within a short time scale. A possible explanation might be that sulfidation did not change the dechlorination mechanism via dissociative electron transfer, which likely determined its dechlorination capacity, but only offered a more electron-conductive layer on nZVI's surface to facilitate faster electron transfer to organohalides and higher dechlorination efficiency (He et al., 2018). As with TCDD and OCDD, abiotic S-nZVI could not dechlorinate TCB and DCB, which was consistent with Zhu and Lim (2007) using nZVI to transform TCB. These results suggested that sulfidation might be not able to expand the dechlorination capacity to what bare nZVI cannot dechlorinate. On the other hand, it was suggested that the dechlorination mechanism by zinc(0) was also via dissociative electron transfer (Arnold and Lynn Roberts, 1998; Bokare et al., 2013). The reason why zinc(0) can dechlorinate PCDD but iron(0) cannot is not clear yet. Therefore, other factors, such as the activation energy and the difference in electron orbits between iron and zinc, likely determine the commencement of PCDD dechlorination.

Even though S-nZVI did not effectively dechlorinate TCDD and OCDD, this study ascertained that S-nZVI was able to supply H_2 and to maintain a low reducing environment required for CBDB1 organohalide respiration, with the potential to be deployed with strain CBDB1 to assist PCDD bioremediation in Sydney Harbour. CBDB1 had much higher tolerance to S-nZVI than nZVI. Dechlorination by CBDB1 was still observed with 5 g/L S-nZVI, but was completely halted by 0.5 g/L nZVI, indicating that sulfidation significantly reduced nZVI toxicity to CBDB1 (t-test, $P<0.05$). Previous studies have shown that the cytotoxicity of nZVI to cells is primarily via direct contact (Wang et al., 2016; Li et al., 2010). For example, nZVI attachment to cells causes membrane structure disruption, membrane permeability changes and protein and genetic damage (Chaithawiwat et al., 2016; Li et al., 2010; Lee et al., 2008). Additionally, the release of reactive oxygen species (such as ·OH from Fenton reaction) and dissolved iron (II and III) were the other causes of damage and inhibition on cells (Lee et al., 2008; Cheng et al., 2019).

Sulfidation reducing nZVI toxicity could be explained from two aspects. From the physical aspect, sulfidation changed the surface properties of nZVI. A study showed that microbes could tolerate high concentration of amorphous ferrous sulfide due to its insolubility (Brock and O'Dea, 1977), therefore, the iron-sulfide layer likely acted as a protective barrier limiting direct CBDB1 contact with the Fe(0) core. Additionally, the protective effect might be

explained through electrostatic repulsion provided by the iron-sulfide layer. Yepez and Obeyesekere (2017) found that at pH=7.5, the surface charge of iron sulfide was neutral. The surface charge became positive at pH < 7.5, and became negative at pH>7.5. In this study, the pH of the carbonate-buffered medium amended with S-nZVI was between 7.5 to 8.0, so the surface of S-nZVI might be negatively charged. CBDB1 is a Gram-negative bacterium with a negative cell surface charge due to the abundance of phospholipids and lipopolysaccharides on the cell wall (Adrian et al., 2000; Lee et al., 2019). The negatively charged iron-sulfide layer might exert an electrostatic repulsive force on CBDB1, thus prevent their aggregation on the S-nZVI surface in so decreasing the toxic effects. In contrast, the surface of nZVI is positively charged (Li et al., 2006), so its aggregation with CBDB1 might be an explanation for inhibition. Li et al. (2010) used transmission electron microscopy (TEM) to visualize the spatial distribution of *Escherichia coli* with bare and polyelectrolyte-coated nZVI. They found that *E. coli* readily aggregated with nZVI, because the poyelectrolyte coating provided negative charge on the surface nZVI preventing aggregation with cells (Ishihara et al., 2019).

On the other hand, sulfidation has been shown previously to slower the release of ferrous (Fe^{2+}) and ferric (Fe^{3+}) iron from nZVI corrosion (Cheng et al., 2019). In this study, after 30 days cultivation, the concentration of dissolved iron species (Fe^{2+} and Fe^{3+}) from 0.5 g/L S-nZVI (0.41 mg/L) was approximately 5 times less than from the equivalent concentrations of S-nZVI (2.39 mg/L). Previous studies suggested that Fe^{2+} was more toxic than Fe^{3+}, so the release of Fe^{2+} was the main cause for cell inactivation (Lee et al., 2008; Li et al., 2010; Cheng et al., 2019). We did not separately quantify Fe^{2+} and Fe^{3+}, but quantified the total dissolved iron concentration using ICP. Based on the Pourbaix diagram of iron (Figure 3.13) in a low-redox potential (-400 to -500 mV) and neutral pH (7.5) condition, Fe^{2+} is the predominant iron species (Parsons, 1967). Even though CBDB1 tolerance to Fe^{2+} has not been reported yet, the limited release of Fe^{2+} from S-nZVI could be another explanation for reduced toxicity.

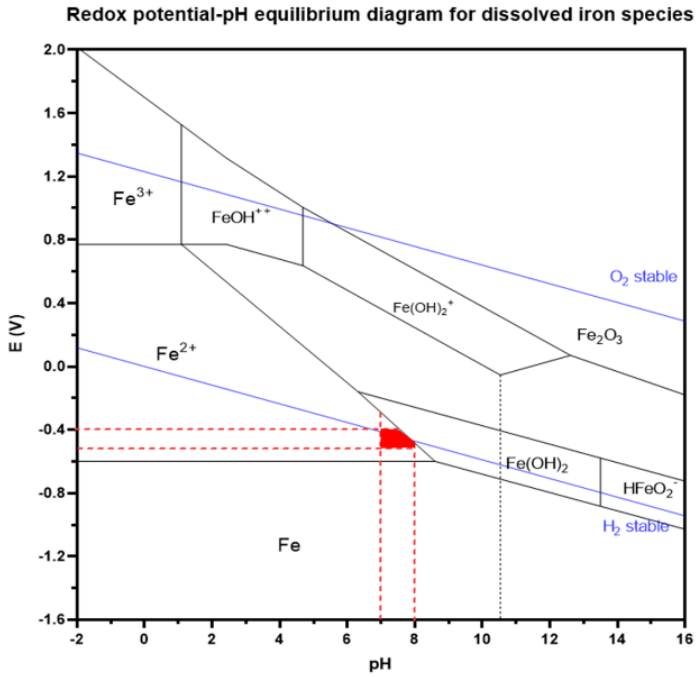

Figure 3.13 Pourbaix diagram of iron at 25°C (Parsons, 1967). The red polygon indicates when pH between 7.5 to 8.0 and ORP between -400 to -500 mV, Fe^{2+} is identified as the predominant iron species.

S-nZVI was able to supply H_2 for CBDB1 organohalide respiration and 0.5 g/L was the optimal dosage. The dechlorination rate by CBDB1 was slower when S-nZVI was below 0.5 g/L, likely due to low H_2 concentrations in the first few days, whereas toxicity was likely responsible for the decreased dechlorination rate when S-nZVI was above 0.5 g/L. nZVI rapidly produced H_2, but it was not suitable to supply H_2, because of toxicity to CBDB1 with complete inhibition at 0.5 g/L. H_2 generation from S-nZVI was approximately 5 times slower than from nZVI, but was enough to support TCB dechlorination, because S-nZVI liberated H_2 past the thresholds for organohalide respiration (Löffler et al. 1999; Luijten et al., 2004; Kassenga et al., 2004; Lu et al., 2001). However, CBDB1 without exogenous H_2 slowly dechlorinated TCB and H_2 concentration in the headspace slowly increased, suggesting that the inoculum might be contaminated by fermenting bacteria that respired acetate and citrate (from titanium(III) citrate) to generate H_2 (Hugenholtz, 1993).

S-nZVI is an effective reducing agent to lower the redox potential for obligate anaerobic microbes. Different reducing agents were compared with S-nZVI in this study. Redox potential (ORP) is a parameter indicating the tendency of acquiring or losing electrons and a more negative redox potential means a stronger electron-donating environment

(Søndergaard, 2009). The ORP of the medium with different reducing agents were sorted as: titanium(III) citrate < S-nZVI < FeS < nZVI < Na_2S< anoxic medium. This indicated that S-nZVI was superior to Na_2S and FeS, and as effective at lowering redox potential as titanium(III) citrate. Previous studies have shown that nZVI could lower the redox potential though the oxidation of Fe(0) and Fe(II) (Wei et al., 2012; Shi et al., 2015; Koenig et al., 2016; Wang et al., 2016). H_2 generated by nZVI can also lower redox potential, with a 1 ppm H_2 increase associated with a 14 mV redox potential decrease (Lee et al., 2019; Shi et al., 2015). In this study, the ORP reduced by nZVI decreased further after three months, indicating that the reducing effect by nZVI was not immediate but gradual via iron oxidation and H_2 generation. In contrast, FeS has a better reducing capacity than nZVI with immediate reducing effect, not only because it can dissociate S^{2-} to reduce O_2 (Meyer et al., 1983), the catalytic effects of Fe^{2+} also accelerated FeS rapidly reacting with oxygen (Brock and O'Dea 1977). S-nZVI inherited the merits from both FeS and nZVI. Dissolved oxygen can be rapidly reduced by Fe(0), Fe^{2+} and S^{2-}. The H_2 generated by S-nZVI also further reduced the redox potential, making S-nZVI superior to FeS.

To estimate the effect of different reducing agents on CBDB1 dechlorination, TCB was used as a proxy substrate in tests due to its water solubility, making it more readily available for dechlorination. Except for nZVI, all the reducing agents facilitated CBDB1 dechlorination with TCB as electron acceptor. Even though nZVI was theoretically sufficient to maintain low enough ORP for TCB dechlorination, its toxicity completely inhibited CBDB1 activity. The dechlorination rate with Na_2S was the slowest and the ORP was the highest, which was consistent with the results reported by Adrian et al. (1998). In contrast, the cultures amended with S-nZVI had a lower ORP than those amended with FeS, but with a lower dechlorination rate than FeS amended cultures, presumably because S-nZVI was more toxic than FeS.

HCB was also used to estimate the effect of different reducing agents on CBDB1 dechlorination, because higher chlorinated benzenes are more resistant to biodegradation than less chlorinated congeners (Malcolm et al., 2004) and the solubility of HCB is much lower than TCB and close to TCDD (Table 3.5). In this study, HCB transformation by abiotic S-nZVI was not observed. However, Shih et al. (2011) previously found that nZVI could dechlorinate HCB to 1,2,4-TCB, but they dissolved 5 mg/L HCB into 1:10 methanol-water (v/v). In this study, HCB crystals were added in excess to CBDB1 cultures without methanol because of potential toxicity to CBDB1. The saturated aqueous-phase concentration of HCB is 4.7×10^{-3} mg/L at

25℃ (HSDB, 2011), approximately 1000-fold lower than Shih et al. (2011). Therefore, at such a low concentration, the reaction of HCB with S-nZVI might have been too slow to detect.

Table 3.5 Solubility of TCBs, HCB and 2,3,7,8-TCDD at 25℃

Chemical	Water solubility (mg/L)	Reference
1,2,4-TCB	49.0	Southworth and Keller, 1986
1,2,3-TCB	30.0	Talian et.al, 1986
HCB	4.7×10^{-3}	HSDB, 2011
2,3,7,8-TCDD	2.00×10^{-4}	Ying Shlu et al., 1988

Except for nZVI that was excluded from the experiment due to its toxicity, all reducing agents facilitated HCB dechlorination by CBDB1. Using titanium(III) citrate and S-nZVI were the fastest without a significant difference (t-test, P=0.643), while the dechlorination rate with FeS was significantly slower than using titanium(III) citrate (t-test, P=0.016), followed by cultures with Na_2S. Several studies have shown that microbial dechlorination rates increase with the decreasing redox potential (Cord-Ruwisch et al., 2009; McCue et al., 1996; Stuart et al., 1999). This trend was evident when observing CBDD1 dechlorination rates of TCB and HCB at varying reduction potentials. However, toxicity of each reducing agent also likely affected the dechlorination rates. For example, in a short to medium term time scale, cultures with FeS had a better dechlorination performance on TCB than the cultures with S-nZVI, but after a lag, cultures with S-nZVI displayed better HCB dechlorination than cultures with FeS. Different reducing agents did not significantly affect the ratio of dechlorination products, and no further dechlorination to less chlorinated congeners was observed.

Ultimately, these data suggested that S-nZVI can be used as an alternative reducing agent to titanium(III) citrate for CBDB1 mediated dechlorination in Sydney Harbour sediment, because it lowered the redox potential to facilitate rapid dechlorination as effectively as titanium(III) citrate. The use of S-nZVI is also cheaper than using titanium(III) citrate (Machado and Wallbank, 1990; Jiang et al., 2018; Li et al., 2017). Additionally, S-nZVI can supply H_2 to support CBDB1 organohalide respiration, which titanium(III) citrate and FeS cannot.

In conclusion, this study found that the CBDB1 was able to function in a marine environment, with the potential to be deployed in Sydney Harbour to assist PCDD remediation. Furthermore, even though S-nZVI did not react with TCDD and OCDD, it was shown to be a

sustainable source of electron donor whilst simultaneously lowering the redox potential required for optimal CBDB1's organohalide respiration. Taken together, these findings provide a proof of concept for bioremediation of PCDD contamination in Sydney Harbour sediments. A trial using strain CBDB1 with S-nZVI for TCDD dechlorination was set up in sealed serum flasks, with controls cultures supplied with exogenous H_2 and titanium(III) citrate. However, after one-month cultivation, dechlorination has yet to commence under either condition. The experiment was set up late in the project, so further monitoring will be carried out.

Chapter Four- General discussion and perspective

4.1 Summary of findings

Sydney Harbour is severely contaminated with polychlorinated dibenzo-p-dioxins and dibenzofurans (PCDD/Fs) (Montoya, 2015). These highly toxic contaminants were released from a chemical manufacturing facility in Homebush Bay and have spread to other parts of Sydney Harbour (Birch et al., 2007). In 2017, PCDD/F concentrations were again quantified in the harbour sediments (Lee et al. unpublished results), with little change observed over the decade. Given their infamous recalcitrance and the risks associated with contamination, a practical remediation strategy for this contamination is sought. Therefore, this study assessed the feasibility of applying biotechnologies for PCDD/F remediation in Sydney Harbour sediments.

In Chapter Two, this study reported the potential of microbes present in Sydney Harbour sediments for PCDD/F biodegradation. Whilst DNA sequencing revealed the presence of bacteria closely related to known PCDD dechlorinating microbes *Dehalococcoides*, enrichment cultures inoculated with harbour sediment and directly spiked with 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and octachlorodibenzo-p-dioxin (OCDD) failed to show biodegradation activity (Lee et al. unpublished results). After an initial enrichment with perchloroethene (PCE) as the alternative electron acceptor, reductive dechlorination of TCDD and OCDD was observed. This observation was made late in the project, with little scope to investigate further. Overall, the data suggests the potential for dioxin biotransformation exists in the harbour sediments, though there is no evidence of this dechlorination process occurring *in situ*. The main discoveries in this chapter included:

1) Sediment microcosms exhibited dechlorination activity with PCE as an electron acceptor using seawater as basal medium. PCE was dechlorinated to trichloroethene (TCE) and to cis- and trans-dichloroethene (DCE) as end products. Sulfate in seawater was completely depleted after the enrichment.

2) The 16S rRNA sequencing identified *Desulfovibrio* strains were the most abundant in the PCE amended enrichment, making up approximately 50% of the total bacteria. In contrast, *Dehalococcoides* strains were below detection after the enrichment. Additionally, there was a significant enrichment of unclassified Dehalococcoidia

bacterium, increasing from 0% to 5.3% OTUs after PCE enrichment, but their functions in PCE dechlorination was unclear.

3) The concentration of TCDD (3.0 µg/g-dw) and OCDD (54.0 µg/g-dw) spiked into PCE-enriched sediment microcosms were approximately 4500 times and 14 times higher than the most contaminated area (HBB4), respectively, but the cultures on PCE dechlorination were only partially inhibited and recovered after a lag phase.

4) PCE stimulation of sediment microcosms effectively triggered the reductive dechlorination of TCDD and OCDD. After 14 months of cultivation, sediment microcosms dechlorinated 0.85% of spiked TCDD to 2,3,7-triCDD and dechlorinated 4.5% of spiked OCDD to HepCDD and HexCDD.

5) Lecithin significantly enhanced the solubility of TCDD and OCDD. Without lecithin, TCDD and OCDD were not detected in the aqueous phase, while with lecithin amendment, the aqueous phase concentration of TCDD and OCDD was approximately 1.5 times and 2000 times higher than their saturated solubilities, respectively.

6) Instead of increasing toxicity of TCDD and OCDD, the use of lecithin lessened the inhibitory effect of TCDD and OCDD on PCE dechlorination, reducing the lag phase duration.

7) Lecithin enhanced the dechlorination of TCDD and OCDD approximately 1.8-fold and 4-fold, respectively.

In Chapter Three, the feasibility of utilising a known 2,3,7,8-TCDD reducing *Dehalococcoides* culture (strain CBDB1) and sulfidized nanoscale zerovalent iron (S-nZVI) to accelerate PCDD/F transformation at seawater salinity was assessed. This included an assessment of the tolerance of the bacterium to marine environments and the potential of using S-nZVI to dechlorinate TCDD and OCDD and to support CBDB1 organohalide respiration through lowering of redox potential and liberation of H_2 as electron donor. Chlorinated benzenes were used as PCDD proxies for strain CBDB1's activity, to increase experimental output and to minimise risks associated with handling TCDD. After adaptation to intermediate salinity, strain CBDB1 showed convincing activity in full strength marine salts media, so there

is potential for strain CBDB1 to be deployed in harbour sediments for TCDD dechlorination. On the other hand, even though S-nZVI did not dechlorinate TCDD and OCDD, it was shown to be a sustainable source of electron and to lower the redox potential to support optimal organohalide respiration by strain CBDB1, which could be used to support CBDB1 dechlorinating PCDD in Sydney Harbour sediments. The main discoveries in this chapter included:

1) High salt concentrations caused reversible inhibition on CBDB1. Whilst no TCB transformation was observed when CBDB1 was directly inoculated in seawater, the cultivation in intermediate (2% w/v) salinity medium assisted the cultures to adapt the high salt pressure and CBDB1 to finally tolerate seawater environment with 3.6% (w/v) salt.

2) S-nZVI had approximately 70 times higher TCE dechlorination efficiency than nZVI, and it was still active after 3 months in anaerobic medium, with 42% of the initial activity. However, even with enhanced dechlorination efficiency and extended longevity compared with nZVI, S-nZVI was incapable of dechlorinating TCDD and OCDD over three months.

3) Strain CBDB1 had higher tolerance to S-nZVI than nZVI, suggesting sulfidation significantly reduce the toxicity of nZVI. The dechlorination activity of CBDB1 was still observed with 5 g/L S-nZVI, but was completely inhibited by 0.5 g/L nZVI.

4) S-nZVI supplied H_2 via iron corrosion for CBDB1 organohalide respiration and 0.5 g/L was the optimal dosage. With equivalent iron concentration, nZVI generated H_2 approximately 5 times faster than S-nZVI, but was not suitable to support CBDB1 respiration due to its toxicity.

5) S-nZVI can be used as an alternative reducing agent to titanium(III) citrate to assist strain CBDB1 dechlorinating PCDD in Sydney Harbour. It lowered the redox potential close to using titanium(III) citrate and facilitated the dechlorination of chlorobenzenes as effectively as titanium(III) citrate, as well as produced H_2 to support CBDB1 respiration, which Na_2S, titanium(III) citrate and FeS could not.

4.2 Environmental significance and perspective

4.2.1 The existence of TCDD and OCDD dechlorinating microbes in Sydney Harbour

This study for the first time generated evidence of the existence of TCDD and OCDD dechlorinating microbes in Sydney Harbour sediments, with future potential to develop low-cost and effective bioremediation for PCDD/Fs in Sydney Harbour sediments. If these microbes effectively dechlorinate TCDD and OCDD in Sydney Harbour sediment, the toxic equivalents of PCDD/Fs are expected to be reduced by approximately 30 - 60%. However, before *in situ* trials, further interrogation of the activity is necessary.

There is no doubt that the dechlorination of TCDD to 2,3,7-triCDD effectively reduced the toxicity, but whether the dechlorination of OCDD happened at peri-positions or lateral-positions is unclear yet, as it may generate a more toxic congener 1,2,3,4,6,7,8-HepCDD. The toxic equivalent of 1,2,3,4,6,7,8-HepCDD is approximately 3 times higher than OCDD. Therefore, it is vitally important to identify the dechlorination pathway of OCDD in future experiments.

The results demonstrated that there was no significant decrease in PCDD/F concentrations in Sydney Harbour sediments over the last ten years, suggesting that dioxin biotransformation does not occur *in situ* at observable rates. TCDD and OCDD were shown not to completely inhibit the dechlorination of PCE, TCDD and OCDD, suggesting that their toxicity is not a key factor, but we hypothesize that the low bioavailability of TCDD and OCDD might be a reason. This study found that only when PCE was used to stimulate ORB in the harbour sediments, could TCDD and OCDD transformation be observed after an extended lag. Previous studies have also demonstrated the efficacy of PCE as a more soluble substrate to stimulate the dechlorination of low soluble organohalides, such as PCBs (Xu et al., 2019; Krumins et al., 2009). TCDD and OCDD *in situ* may not be bioavailable enough to support the concentration of PCDD/F-dechlorinating microbes required to affect dechlorination, but PCE seemed to stimulate the enrichment of PCDD/F-dechlorinating microbes. However, whether PCE-dechlorinating microbes were responsible for TCDD and OCDD dechlorination was not definitively shown and requires DNA sequencing and further cultivation to reveal their relationship.

This study also found that the biosurfactant lecithin effectively accelerated the desorption of TCDD and OCDD from sediments and thus enhanced their solubility in the

liquid-phase. Interestingly, instead of increasing the toxicity, enhanced solubility of TCDD and OCDD also enhanced their microbial dechlorination, likely by making TCDD and OCDD more accessible to the dechlorinating bacteria (Bustamante et al., 2012). The mechanism by which lecithin enhanced TCDD and OCDD dechlorination is not yet clear, but given the success of lecithin enhancing TCDD and OCDD dechlorination, assessing other biosurfactants to assist TCDD and OCDD dechlorination is worthwhile. Cyclodextrin is a possible candidate, because it can enhance the biodegradation of phenanthrene (Wang et al., 1998), polycyclic aromatic hydrocarbons (PAHs) (Allan et al., 2007) and polychlorinated biphenyls (PCBs) (Fava et al., 2003), as well as accelerate the desorption of PCDD/Fs from soil (Cathum et al., 2007).

Heavy metals in the harbour sediments may also inhibit the activity of PCDD/F-dechlorinating microbes in Sydney Harbour sediments. Apart from PCDD/Fs, Sydney Harbour sediments also suffer from heavy metal contamination, with particularly high concentrations of cadmium, lead and zinc (Birch et al., 2013; Montoya, 2015). For example, the concentration of cadmium ranged from 0 to 24.3 mg/kg sediment, with a mean value of 0.8 mg/kg (Birch et al., 2013). Previous studies have shown that organohalide respiring bacteria are sensitive to heavy metals, especially to cadmium, as they can alter the essential cations inside the enzyme and damage DNA structures (Lu et al., 2020; Pardue et al., 1996; Kong, 1998). Given this, we have also proposed to quantify heavy metals in the sediment samples we collected to estimate the possible effect of heavy metals on PCDD/F-dechlorinating microbes in the harbour sediments. This result will also be useful for our proposal to introduce the dioxin dechlorinating bacterium *Dehalococcoides mccartyi* strain CBDB1 for bioremediation in Sydney Harbour sediments.

In addition, purification and isolation of PCDD/F-dechlorinating microbes will be carried out in future experiments to reveal their genomic characteristics and their propensities, in preparation for reintroducing them back to assist PCDD/F bioremediation in Sydney Harbour. Until recently, there is only one 2,3,7,8-TCDD dechlorinating strain isolated (Bunge et al., 2003) and the successful isolation of an OCDD-dechlorinating strain has not previously been reported. For scientific purposes, the isolation of TCDD and OCDD dechlorinating microbes will fill these knowledge gaps and provide more candidates for PCDD/F bioremediation in Australia.

4.2.2 Assessment of *Dehalococcoides mccartyi* strain CBDB1 and S-nZVI to assist PCDD remediation in Sydney Harbour

This study ascertained the suitability of the PCDD/F-dechlorinating bacteria *Dehalococcoides mccartyi* strain CBDB1 in its application for PCDD/Fs remediation in Sydney Harbour. We demonstrated that strain CBDB1 can adapt to seawater environments with dechlorination activity, which can be deployed in marine sediments such as those in Sydney Harbour. As it is known to dechlorinate the most toxic dioxin 2,3,7,8-TCDD (Bunge et al., 2003), this strain has the potential of reducing up to 30% of the toxicity equivalents in Sydney Harbour. On the other hand, even though S-nZVI cannot effectively transform TCDD and OCDD, our study proved that it can be used to support strain CBDB1 organohalide respiration, with potential to be deployed with CBDB1 to assist PCDD/Fs bioremediation in Sydney Harbour. The iron oxidation of S-nZVI liberates enough H_2 to support hydrogenotrophic ORB such as strain CBDB1 respiration, and due to its strong reducing capacity and low cost, S-nZVI can be used as an alternative reducing agent to titanium citrate in future remediation in Sydney Harbour.

It was interesting to observe in this study that sulfidation of nZVI significantly reduced the cytotoxicity. Previous studies have shown the potential of the combination of ORB and nZVI as a promising system for organohalide remediation, since it provides a solution for remedying the contaminations which cannot be achieved by ORB or nZVI alone. For example, the study of Xu et al. (2014) integrated CBDB1 with nZVI, completely transforming decabromodiphenyl ether to non-brominated diphenyl ether. The two agents were complimentary, with nZVI transforming higher brominated diphenyl ether but not lower brominated congeners (Kim et al., 2014; Zhuang et al., 2012), while strain CBDB1 cannot transform highly brominated congeners but can completely debrominate lower brominated congeners to diphenyl ether (Wagner et al., 2012). Several other studies demonstrated enhanced dehalogenation performances by integrating ORB with nZVI. However, the cytotoxicity of nZVI is one of the most important obstacles for their practical application. Previous studies have demonstrated ORB are highly sensitive to nZVI even with low dosage, including this study (Xu et al., 2014; Koenig et al., 2016; Wang et al., 2016; Xiu et al., 2010). Our study proved that strain CBDB1 had much higher tolerance to S-nZVI than nZVI, suggesting that S-nZVI may be a more suitable candidate than nZVI for use in bio-nZVI systems for organohalide remediation. The enhanced dechlorination efficiency and extended longevity also make S-

nZVI more effective and efficient than nZVI for organohalide remediation applications (Kim et al., 2011; He et al., 2018; Li et al., 2017; Rajajayavel and Ghoshal, 2015).

Before *in situ* trials, column trials have been planned. Fresh Homebush Bay sediments in seawater medium and enriched seawater tolerant CBDB1 cultures will be used to replicate *in situ* PCDD/F bioremediation, as well as using S-nZVI for supplying H_2 and lowering the redox potential. This experiment will be useful to better understand 1) the efficacy of strain CBDB1 on detoxifying PCDD/Fs in the harbour sediments, 2) whether PCDD/Fs in the harbour sediments are bioavailable enough for CBDB1 respiration and 3) the effect of introducing CBDB1 and S-nZVI on the indigenous microbial communities.

For the first question above, even though strain CBDB1 is the only known strain to dechlorinate 2,3,7,8-TCDD to 2,7-/2,8-DCDD, it is also known to dechlorinate 1,2,3,7,8-PeCDD primarily to 2,3,7,8-TCDD (Bunge et al., 2003). Our results identified that the 1,2,3,7,8-PeCDD contributed 0 to 30% of toxicity equivalents in Sydney Harbour sediments, with a mean value of 6%. Even though the TEF of 2,3,7,8-TCDD and 1,2,3,7,8-PeCDD are the same, introducing strain CBDB1 into Sydney Harbour may generate more 2,3,7,8-TCDD, therefore, it will be important for future experiments to compare the dechlorination rates of 1,2,3,7,8-PeCDD and 2,3,7,8-TCDD and assess the change of toxicity equivalents over time.

For the second question above, since strain CBDB1 has already been highly enriched, the results will be useful as an analogy to address whether the low abundance of PCDD/F-dechlorinating bacteria was the main reason why dioxin biotransformation did not occur *in situ*. If bioavailability of PCDD/Fs in the harbour sediments is a key issue, whether the use of biosurfactant like lecithin also enhance CBDB1 transforming PCDD/Fs in Sydney Harbour.

For the third question above, the effect of introducing exogenous microbes and chemicals on the indigenous microbial communities will be important for future remediation. For example, S-nZVI may stimulate methane production by methanogens in the harbour sediments, thus reducing the efficiency of H_2 transferring to CBDB1 respiration (Dong et al., 2019; Zanaroli et al., 2015), and strain CBDB1 may not be active when spiked into harbour sediments. Importantly, permission has not been sought for release of strain CBDB1 for in situ remediation in Australia, though other organohalide respiring cultures from overseas have been applied. Column trials will provide useful results to inform the government of the suitability of introducing strain CBDB1 to assist PCDD/F remediation in Sydney Harbour.

4.3 General conclusions

With little improvement on PCDD/F toxicity equivalents being observed over the last decade, this research examined the potential of utilizing bioremediation technology for PCDD/Fs in Sydney Harbour and provided two conceptually viable solutions.

DNA sequencing revealed the presence of known PCDD/F-degrading microbes in the harbour sediment and the use of PCE effectively stimulated the sediment microcosms to reductively dechlorinate 2,3,7,8-TCDD to 2,3,7-TriCDD and OCDD to HepCDD and HexCDD. This suggested that the indigenous microbial population is capable of detoxifying the PCDD/F contamination in Sydney Harbour, but how this can be achieved without adding another priority pollutant (i.e. PCE) for biostimulation remains to be seen. Other factors to be considered are the inhibitory effects of TCDD and OCDD on the indigenous population, as well as the use of biosurfactant that was shown to increase the bioavailability of PCDD/Fs and enhance dechlorination activity.

In case the endogenous population could not be suitably stimulated to address PCDD/F contamination, this study revealed that PCDD-dechlorinating bacteria *Dehalococcoides mccartyi* strain CBDB1 could be used in a bioaugmentation strategy as it was shown to be amenable to seawater conditions found at the contaminated site. Furthermore, this study revealed a novel means by which to sustain strain CBDB1 activity using S-nZVI, as both electron donor and redox conditioning agent. Taken together, these findings have given some credence to the notion of a bioremediation strategy for PCDD/Fs in Sydney Harbour.

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Appendix

1. GC-MS/MS setting parameters for PCDD/Fs analysis

Name	RT (min)	Quantifier			Qualifier			Ion Polarity
		Precursor Ion	Product Ion	CE (ev)	Precursor Ion	Product Ion	CE (ev)	
Dibenzo-p-dioxin	11.8	184.0	128.1	25	184.0	102.1	25	Positive
2-MCDD	13.2	217.9	155.0	25	217.9	127.1	25	Positive
2,7-DCDD	14.8	251.9	189.0	25	251.9	217.0	25	Positive
2,3-DCDD	14.9	251.9	189.0	25	251.9	217.0	25	Positive
2,3,7-TriCDD	17.1	285.8	223.0	25	285.8	251.1	25	Positive
2,3,7,8-TCDD	20.1	321.8	259.0	24	321.8	257.0	24	Positive
1,2,3,7,8-PeCDD	22.6	353.9	292.9	25	353.9	290.9	25	Positive
1,2,3,4,7,8-HexCDD	27.5	389.8	326.9	25	391.8	328.8	25	Positive
1,2,3,6,7,8-HexCDD	27.6	389.8	326.9	25	391.8	328.8	25	Positive
1,2,3,7,8,9-HexCDD	28.0	389.8	326.9	25	391.8	328.8	25	Positive
1,2,3,4,6,7,8-HepCDD	30.5	423.8	360.8	25	425.8	362.8	25	Positive
OCDD	37.8	457.7	394.8	26	459.5	396.8	26	Positive
2378-TetraF	19.9	303.9	240.9	33	305.9	242.9	33	Positive
1,2,3,7,8-PenCDF	22.9	339.9	276.9	35	337.9	274.9	35	Positive
2,3,4,7,8-PenCDF	23.5	339.9	276.9	35	337.9	274.9	35	Positive
1,2,3,4,7,8-HexCDF	26.6	373.8	310.9	35	375.8	312.9	35	Positive
1,2,3,6,7,8-HexCDF	26.7	373.8	310.9	35	375.8	312.9	35	Positive
2,3,4,6,7,8-HexCDF	27.3	373.8	310.9	35	375.8	312.9	35	Positive
1,2,3,7,8,9-HexCDF	28.3	373.8	310.9	35	375.8	312.9	36	Positive
1,2,3,4,6,7,8-HepCDF	32.4	407.8	344.8	36	409.8	346.8	36	Positive
1,2,3,4,7,8,9-HepCDF	30.7	407.8	344.8	36	409.8	346.8	36	Positive
OCDF	38.9	443.7	380.6	35	441.7	378.8	35	Positive

2. qPCR amplification primers and protocols for reductive dehalogenases

Target genes	Primer	Sequence (5`-> 3`)	Reference
PceA	pceA877F pceA976R	ACCGAAACCAGTTACGAACG GACTATTGTTGCCGGCACTT	Behrens *et al.*, 2008
TceA	tceA1270F tceA1336R	ATCCAGATTATGACCCTGGTGAA GCGGCATATATTAGGGCATCTT	Ritalahti *et al.*, 2006

qPCR amplification protocols

PceA: 98°C for 3.00 min, followed by 25 cycles of 94°C for 1 min and annealing 57°C for 1min, 1min at 72°C and final extension of 9 min at 72°C. Following amplification, melting curve analysis was operated with increments at 0.5°C per 5 seconds from 55 to 95°C.

TceA: 98°C for 3.00 min, followed by 45 cycles of 94°C for 30 sec and annealing 55°C for 50 seconds. Following amplification, melting curve analysis was performed with 1.0°C increments every 5 seconds from 55°C to 95°C.

3. PCE enrichments in seawater medium

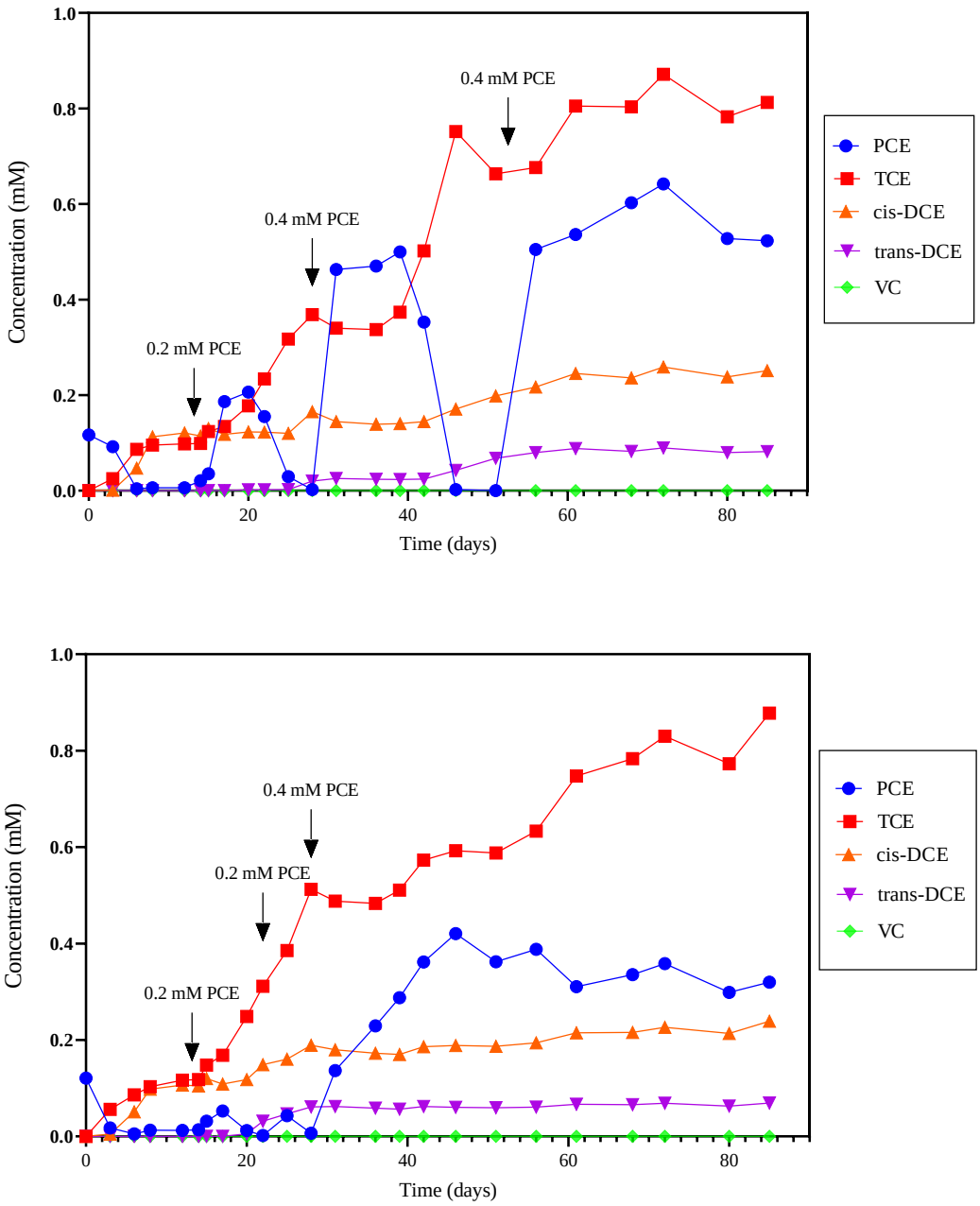

Figure 1 PCE transformation by HBB4 upper cores sediment microcosms in seawater medium over 85 days. Arrows indicate time points when PCE was suppled.

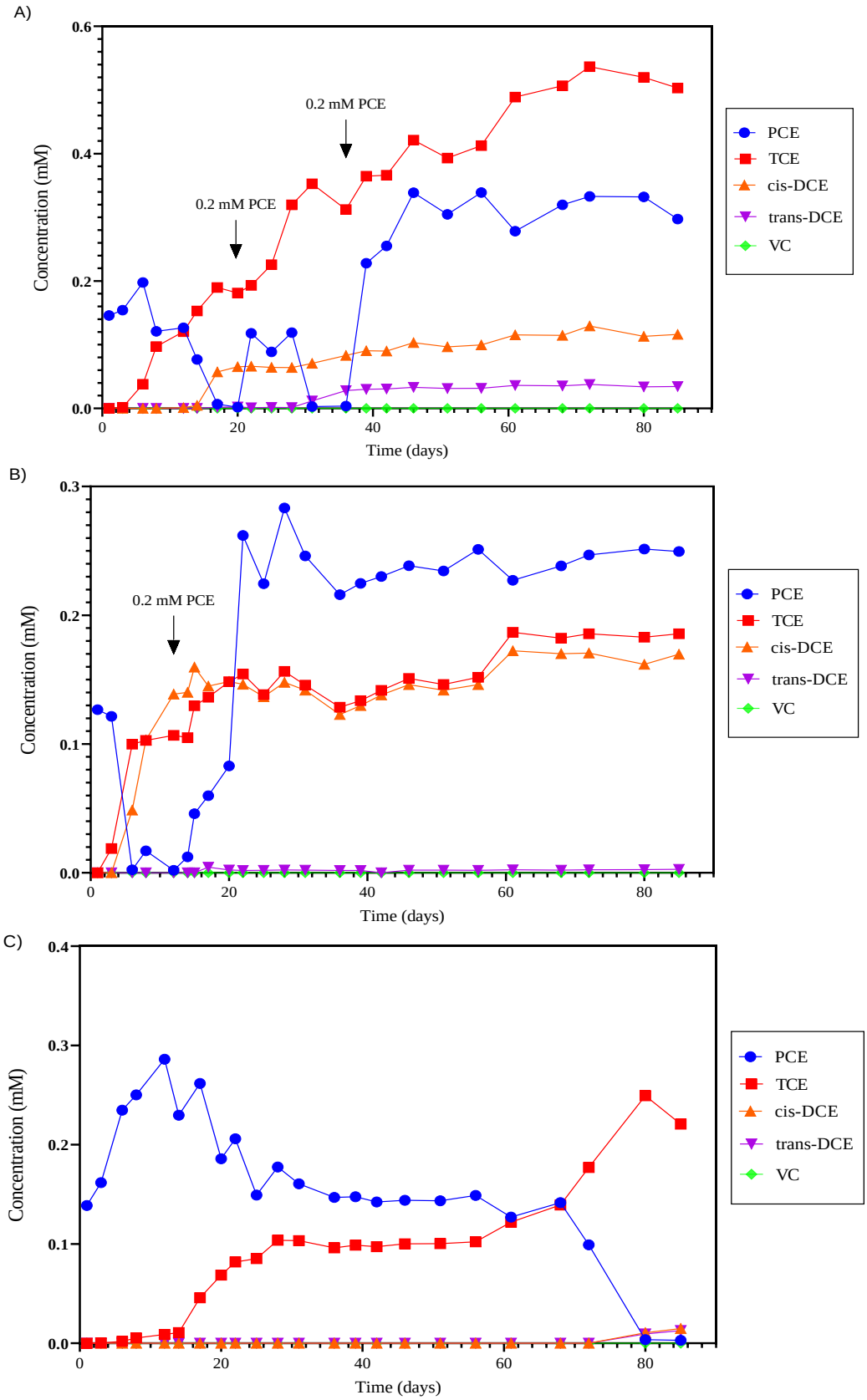

Figure 2 PCE transformation by HBB4 lower cores sediment microcosms in seawater medium over 85 days. Arrows indicate time points when PCE was suppled.

4. **Theoretical hydrogen threshold of OCDD reductive dechlorination**

The formula of OCDD reductive dechlorination to 1,2,3,4,6,7,9-HepCDD is defined as Equation A1:

$$\mathrm{OCDD} + \mathrm{H_2(aq)} \rightarrow \mathrm{HepCDD} + \mathrm{H^+} + \mathrm{Cl^-} \text{ (Eq. A1)}$$

To calculate the theoretical hydrogen threshold, OCDD reductive dechlorination to 1,2,3,4,6,7,9-HepCDD presumably happens in seawater (pH=7.5 and NaCl=0.6 M). OCDD and HepCDD are presumed to be saturated in the medium, so their concentration are 8.7×10^{-13} M and 5.8×10^{-12} M, respectively. The molarity of H_2 is unknown (defined as X). Gibbs fee energies of formation ($\Delta_f G_i^o$) at 298K (25 ℃) and 100 kPa are shown in Table A1.

Table A1 Gibbs fee energies of formation at 298K (25 ℃) and 100 kPa

	$\Delta_f G_i^o$ (aq) (kJ·mol^{-1})	Concentration (M)	Reference
OCDD	-126.7	8.7×10^{-13}	Huang et al., 1996
H_2	0	X	Thauer et al., 1977
1,2,3,4,6,7,9-HepCDD	-124.3	5.8×10^{-12}	Huang et al., 1996
Cl^-	-131.2	0.6	Wagman et al., 1982
H^+	-39.9	$10^{-7.5}$	Thauer et al., 1977

The standard free energy change ($\Delta G°$) for OCDD reductive dechlorination to 1,2,3,4,6,7,9-HepCDD is shown as Equation A2:

$$\Delta \mathrm{G°} = \Sigma \Delta_\mathrm{f} \mathrm{G°(products)} - \Sigma \Delta_\mathrm{f} \mathrm{G°(substrates)}$$

$$= \Delta_\mathrm{f} \mathrm{G°(HepCDD)} + \Delta_\mathrm{f} \mathrm{G°(Cl^-)} + \Delta_\mathrm{f} \mathrm{G°(H^+)} - \Delta_\mathrm{f} \mathrm{G°(OCDD)} - \Delta_\mathrm{f} \mathrm{G°(H_2)}$$

$$= -124.3 \text{ kJ} \cdot \text{mol}^{-1} - 131.2 \text{ kJ} \cdot \text{mol}^{-1} - 39.9 \text{ kJ} \cdot \text{mol}^{-1} + 126.7 \text{ kJ} \cdot \text{mol}^{-1} + 0 \text{ kJ} \cdot \text{mol}^{-1}$$

$$= -168.7 \text{ kJ} \cdot \text{mol}^{-1} \text{ (Eq. A2)}$$

The Gibbs energy for PCDD dechlorinating bacteria reductively dechlorinating OCDD to 1,2,3,4,6,7,9-HepCDD is calculated in Equation A3, where T=298 K and R represents the

universal gas constant 8.314×10^{-3} kJ·mol^{-1}·K^{-1}. To calculate the theoretical hydrogen threshold, the available Gibbs free energy should be close to zero ($\Delta G \approx 0$) (Karadagli and Rittmann, 2007).

$$\Delta G = \Delta G° + RT \cdot ln\frac{[HepCDD][Cl^-][H^+]}{[OCDD][H_2]}$$

$$= -168.7\ kJ \cdot mol^{-1} + 8.314\ \text{kJ} \cdot \text{mol}^{-1} \cdot \text{K}^{-1} \times 298\ K \times ln\frac{5.8 \times 10^{-12}\ \text{M}\times10^{-7.5}\ M\times0.6\ M}{8.7\times10^{-13}\ M\times X} = 0 \text{ (Eq. A3)}$$

Therefore, the calculation of the hydrogen threshold (X) is shown as Equation A4.

$$X = \frac{5.8\ \times\ 10^{-12}\ \text{M} \times 10^{-7.5}\ M \times 0.6\ M}{8.7 \times 10^{-13}\ M \times e^{\frac{168.7\ kJ\cdot mol^{-1}}{8.314\ \text{kJ}\cdot\text{mol}^{-1}\cdot\text{K}^{-1}\times 298\ K}}} = 1.1 \times 10^{-36.5} M$$

Therefore, the theoretical hydrogen threshold of OCDD reductive dechlorination was consistent with TCE reductive dechlorination to cis-DCE obtained by Mao et al. (2015) (approximately 10^{-35} M).

5. **Genera under Dehalococcoidia (class) in Sydney Harbour sediments**

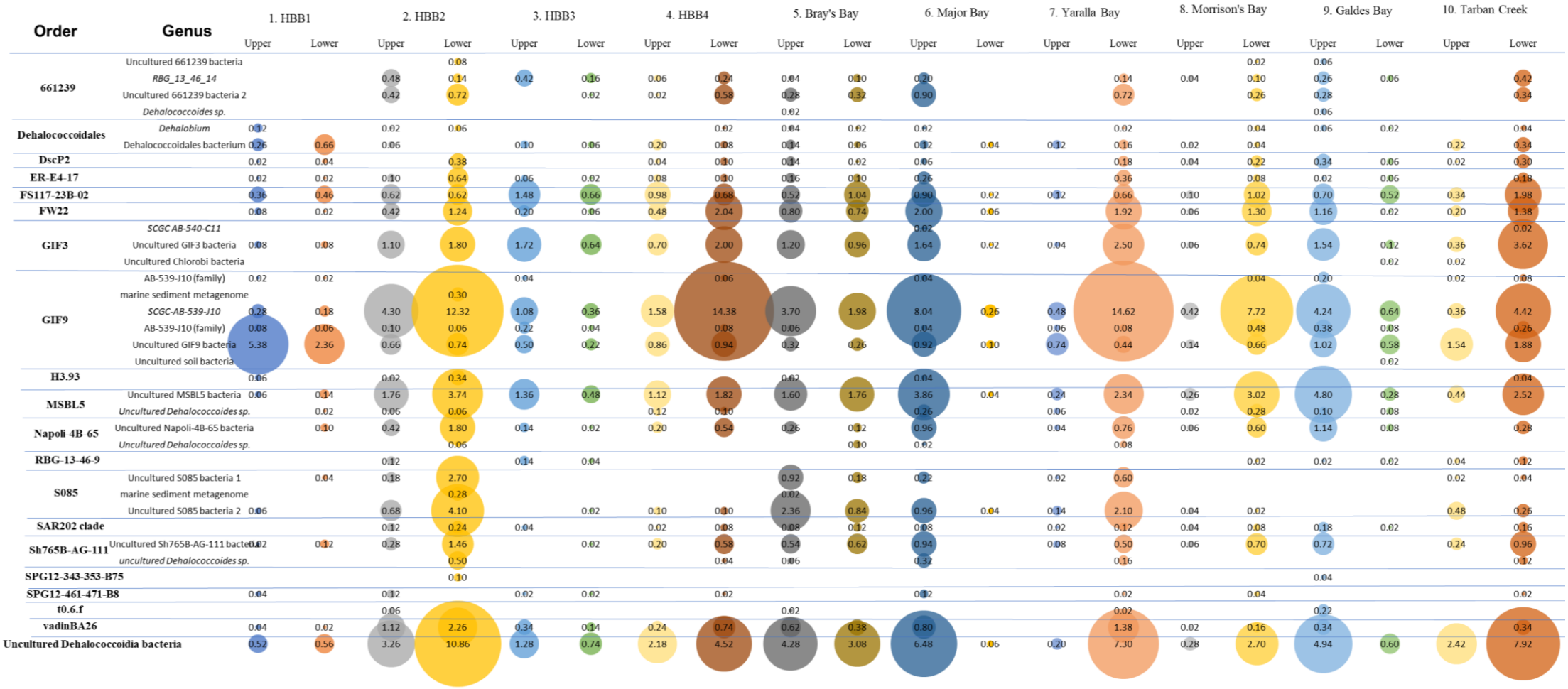

Figure 3 The major Genera under Dehalococcoidia (class) in Sydney Harbour sediments (>0.01%). These cultures were assigned to order, family and genus. Bubble sizes and figures represent the relative abundance (%) of bacterial lineage.