# Exploring Additional Dehalogenation Abilities of DehaloR^2, a Previously Characterized, Trichloroethene-Degrading Microbial Consortium

by

Kylie Kegerreis

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Graduate Supervisory Committee:

Rosa Krajmalnik-Brown, Chair
Rolf Halden
Cesar Torres

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ABSTRACT

DehaloR^2 is a previously characterized, trichloroethene (TCE)-dechlorinating culture and contains bacteria from the known dechlorinating genus, *Dehalococcoides*. DehaloR^2 was exposed to three anthropogenic contaminants, Triclocarban (TCC), tris(2-chloroethyl) phosphate (TCEP), and 1,1,1-trichloroethane (TCA) and two biogenic-like halogenated compounds, 2,6-dibromophenol (2,6-DBP) and 2,6-dichlorophenol (2,6-DCP). The effects on TCE dechlorination ability due to 2,6-DBP and 2,6-DCP exposures were also investigated.

DehaloR^2 did not dechlorinate TCC or TCEP. After initial exposure to TCA, half of the initial TCA was dechlorinated to 1,1-dichloroethane (DCA), however half of the TCA remained by day 100. Subsequent TCA and TCE re-exposure showed no reductive dechlorination activity for both TCA and TCE by 120 days after the re-exposure.

It has been hypothesized that the microbial TCE-dechlorinating ability was developed before TCE became abundant in groundwater. This dechlorinating ability would have existed in the microbial metabolism due to previous exposure to biogenic halogenated compounds. After observing the inability of DehaloR^2 to dechlorinate other anthropogenic compounds, DehaloR^2 was then exposed to two naturally occurring halogenated phenols, 2,6-DBP and 2,6-DCP, in the presence and absence of TCE. DehaloR^2 debrominated 2,6-DBP through the intermediate 2-bromophenol (2-BP) to the end product phenol faster in the

presence of TCE. DehaloR^2 dechlorinated 2,6-DCP to 2-CP in the absence of TCE; however, 2,6-DCP dechlorination was incomplete in the presence of TCE.

Additionally, when 2,6-DBP was present, complete TCE dechlorination to ethene occurred more quickly than when TCE was present without 2,6-DBP. However, when 2,6-DCP was present, TCE dechlorination to ethene had not completed by day 55.

The increased dehalogenation rate of 2,6-DBP and TCE when present together compared to conditions containing only 2,6-DBP or only TCE suggests a possible synergistic relationship between 2,6-DBP and TCE, while the decreased dechlorination rate of 2,6-DCP and TCE when present together compared to conditions containing only 2,6-DCP or only TCE suggests an inhibitory effect.

# DEDICATION

"I can do all things through Christ who strengthens me." Philippians 4:13

To the original Engineer and the Creator of this universe, the One who made all of this possible: thank you just does not seem enough.

To my family: I would not be who I am today without you. Mom and Dad, thank you for teaching me what it means to work hard and to never give up. As I have struggled to find my place in this world you have always believed in me and loved me as only parents can. Thank you for instilling in me a sense of purpose and morality. I am forever indebted to you both. To my sister and brother, Kelsey and Jason, thank you for accepting me for the complete nerd that I am and for loving me anyway. I could not have been blessed with better people to call my siblings and friends. I am so proud to be your big sister. Grandma, thank you for reminding me whom all of this is for, and for providing such a wonderful example of what it means to be a strong, Christian woman.

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# LIST OF ACRONYMS

| Acronym | Definition |
|---|---|
| 2-BP | 2-Bromophenol |
| 2-CP | 2-Chlorophenol |
| 2,6-DBP | 2,6-Dibromophenol |
| 2,4-DCP | 2,4-Dichlorophenol |
| 2,6-DCP | 2,6-Dichlorophenol |
| ATSDR | Agency for Toxic Substances and Disease Registry |
| BCF | Bioconcentration Factor, [concentration in organism per concentration in water] |
| BP | Bromophenol |
| CA | Chloroethane |
| CEC | Contaminants of emerging concern |
| CP | Chlorophenol |
| CT | Carbon tetrachloride |
| DCA | 1,1-Dicholroethane |
| DCC | 4,4’-Dichlorocarbanilide (Diclocarban) |
| DCE | *cis*-Dichloroethene |
| DDT | Dichlorodiphenyltrichloroethane |
| GC-FID | Gas Chromatograpy with Flame Ionization |
| $K_{OC}$ | Soil organic carbon-water partition coefficient, [mass of a chemical absorbed in the soil per mass of organic carbon] |
| $K_{OW}$ | Octanol/water partition coefficient, [concentration in octanol per concentration in aqueous phase] |
| LC-ESI-MS/MS | Liquid Chromatography- Electrospray Ionization Tandem Mass Spectrometry |
| NCC | Non-chlorinated carbanilide |
| NPL | National Priority List |
| OC | Organochlorine insecticide |
| OFR | Organophosphate flame retardant |
| OP | Organophosphorus insecticide |
| PAH | Polycyclic aromatic hydrocarbons |
| PBB | Polybrominated biphenyl |
| PCB | Polychlorinated biphenyl |
| PCDD | Polychlorinated dibenzodioxins |
| PCDF | Polychlorinated dibenzofurans |
| PCE | Tetrachloroethylene (aka Tetrachloroethene and Perchloroethene) |
| POP | Persistent organic pollutants |
| PPCP | Personal Care Product and Pharmaceutical |
| qRT-PCR | Quantitative real-time Polymerase Chain Reaction |
| RDase | Reductive dehalogenase |
| SPE | Solid phase extraction |

| | |
|---|---|
| TCA | 1,1,1-Trichloroethane |
| TCC | 3,4,4'-Trichlorocarbanilide (Triclocarban) |
| TCE | Trichloroethylene (aka Trichloroethene) |
| *tceA* | Trichloroethene reductive dehalogenase encoding gene |
| *tceB* | Trichloroethene reductive dehalogenase associated B protein encoding gene |
| UPLC | Ultra Performance Liquid Chromatography |
| USDHHS | United States Department of Health and Human Services |
| USEPA | United States Environmental Protection Agency |
| VC | Vinyl chloride |
| *vcrA* | Vinyl chloride reductive dehalogenase encoding gene |
| *vcrB* | Vinyl chloride reductive dehalogenase hydrophobic B protein encoding gene |
| *vcrC* | Protein transcriptional regulator similar to the NosR/NirI family |
| VOC | Volatile Organic Compound |

# CHAPTER 1

# INTRODUCTION

## 1.1 INTRODUCTION

Anthropogenic, or man-made, sources of pollution have inevitably existed as long as mankind, but did not occur in such a significant quantity until the Industrial Revolution [1]. Since then, innumerable compounds not found in the natural environment have been created to solve a wide range of natural and man-made dilemmas, often resulting in unforeseen complications. The organochlorine insecticide (OC), dichlorodiphenyltrichloroethane (DDT), was originally seen as a scientific breakthrough and the chemist credited with the discovery and application of DDT, Paul Hermann Müller, even won a Noble Prize, until the negative impacts of the insecticide were realized [2]. However, DDT is still recognized as the synthetic compound that may have saved the most lives in the history of mankind and is still used in developing countries with widespread malaria.

Chlorinated ethenes—or chloroethenes—are compounds created for use as solvents in dry cleaning, metal degreasers, and in various applications in the textile industry [3]. The wide range of uses for chloroethenes combined with past careless disposal has made chloroethenes one of the most common classes of pollutants at hazardous waste sites [4]. Many of these chlorinated ethenes including trichloroethene (TCE) are highly toxic and known or suspected carcinogens [5]. TCE is present at around 60% of the United States Environmental Protection Agency's (USEPA) National Priority List (NPL) or

Superfund sites [6]. Although TCE is naturally produced by marine algae [7], its extensive anthropogenic production and use as a degreasing and cleaning solvent far outweighs the biogenic production.

Anaerobic bioremediation has been established as an efficient means for removing TCE [8-13]. This is not only due to the occurrence of TCE pollution, especially in groundwater where anoxic conditions exist, but also to the difficulty in removing TCE under aerobic conditions [14]. Throughout the various research efforts, many mixed [12, 13, 15-17] and pure [18-21] cultures capable of dechlorinating TCE have been discovered. The bacteria in these cultures use TCE as an electron acceptor and gain energy through dehalorespiration and are sometimes referred to as anaerobic halorespirers [22].

Among these halorespiring bacteria is a unique group known as *Dehalococcoides* spp. Currently, *Dehalococcoides* spp. are the only known bacteria capable of completely dechlorinating PCE and TCE [23]; however, not all strains of *Dehalococcoides* are capable of this complete dechlorination [13]. Some strains incompletely dechlorinate TCE to *trans*- [24, 25] and *cis*-dichloroethene (DCE) [26-28] and vinyl chloride (VC) [29, 30]. Additionally, there are other species of bacteria capable of dechlorinating TCE to the first intermediate product, *cis*-DCE such as *Dehalobacter* [31-33], *Desulfuromonas* [34, 35], and *Geobacter* [35, 36]. *Dehalococcoides* spp. have also shown the ability to dehalogenate other compounds such as chlorinated phenols (chlorophenols, CPs) [37], brominated diphenyl ethers [38], and chlorinated benzenes [20, 39].

In addition to anthropogenic sources of halogens, there are also biogenic sources [40]. These biogenic compounds are produced naturally by species such as ticks [41-46], which produce 2,6-dichlorophenol (2,6-DCP), and marine hemichordates [47, 48], which produce 2,6-dibromophenol (2,6-DBP). Extensive research has been done on CPs and bacteria capable of degrading CPs [37, 49-70] while bromophenols (BPs) have not been studied quite as thoroughly [48, 71-77].

Despite the lack of research, by looking at the periodic table a few hypotheses can be generated: (1) it should take less energy to remove a bromine compared to a chlorine, (2) the bromines should not be bonded as strongly as the chlorines on a halogenated compound, and (3) because bromine and chlorine have such similar chemical properties, an organism capable of reductive dechlorination could perform reductive debromination [38, 39].

Although brominated compounds are not as abundant as chlorinated compounds, studies have shown that polybrominated compounds are just as toxic to humans [78, 79] and animals [80, 81] and recalcitrant in the environment [82] as polychlorinated compounds. Studies examining toxicity of lesser-brominated compounds (such as di- and monobromophenols) are not available [73].

As mentioned previously, *Dehalococcoides* spp. have exhibited a wide range of dehalogenation ability in regards to multiple anthropogenic compounds. However, *Dehalococcoides* has most likely existed in the environment much longer than the discovery and mass production of anthropogenic compounds. The biggest questions here are: (1) How were these bacteria living and thriving before anthropogenic sources of halogenated compounds? And (2) How does

*Dehalococcoides* have the necessary metabolic machinery to dehalogenate compounds given that developing such metabolic machinery through evolution or gene transfer events would theoretically take more than a few centuries?

## 1.2 RESEARCH OBJECTIVES

Using the summary presented in section 1.1, the following objectives for this research were developed. The specific aims were:

(1) To determine if the TCE-dechlorinating culture, DehaloR^2 [13] is capable of dechlorinating three additional chlorinated organics of anthropogenic origin: a chlorinated solvent, 1,1,1-trichloroethane (TCA), an antimicrobial agent, triclocarban (3,4,4'-trichlorocarbanilide, TCC), and an organophosphorus flame retardant, tris(2-chloroethyl) phosphate (TCEP);

(2) To examine the ability of DehaloR^2 to dehalogenate two model biogenic-like compounds: 2,6-dibromophenol (2,6-DBP), which can be produced by a marine hemichordate, and 2,6-dichlorophenol (2,6-DCP), which in nature is a sex pheromone secreted by multiple tick species;

(3) To explore the effects of 2,6-DBP and 2,6-DCP on the TCE-dechlorinating ability of DehaloR^2.

This thesis is divided into six chapters. Important background information regarding the various anthropogenic and biogenic-like compounds and previous research related to bioremediation using the anaerobic bacteria *Dehalococcoides* is presented in Chapter 2. Research findings are presented in Chapters 3, 4, and 5. Chapter 3 examines the results of an initial screening exposing the DehaloR^2

culture to the three anthropogenic compounds: TCA, TCC and TCEP. The results and implications of reductive dehalogenation of the model biogenic-like compounds 2,6-DBP and 2,6-DCP are presented in Chapter 4. In Chapter 5, I investigate the effects of 2,6-DBP and 2,6-DCP on the previously established TCE-dechlorinating ability of DehaloR^2. Finally, the major conclusions from these studies, recommendations for future researchers, and personal next research steps are presented in Chapter 6.

# CHAPTER 2

# BACKGROUND

## 2.1 ANTHROPOGENIC SOURCES OF HALOGENATED COMPOUNDS

Anthropogenic compounds are human-made materials not found in the natural environment [83] and are used in "agriculture, industry, medicine, and military operations" [84]. These compounds can be further categorized into sub-classes such as persistent organic pollutants (POP), polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), and polybrominated biphnyls (PBBs), polychlorinated dibenzodioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), organochlorine insecticides (OCs), organophosphorus insecticides (OPs), volatile organic compounds (VOCs), organophosphate flame retardants (OFRs), and contaminants of emerging concern (CECs) [85]. Many of these anthropogenic compounds are recalcitrant in the environment and harmful to various animals and biota. Three such anthropogenic compounds are triclocarban (TCC), tris(2-chloroethyl) phosphate (TCEP), and 1,1,1-trichloroethane (TCA).

### *2.1.1 Triclocarban (TCC)*

TCC is a polychlorinated phenyl urea often used as an antibacterial and antifungal component in pharmaceuticals and personal care products (PPCPs) [86]. TCC has become a focus for both public attention and the scientific community due to its widespread use [87-90], persistence in the environment [91-98], and its toxicity to some babies [99, 100] and many environmental species [101-107]. TCC is most often found in antimicrobial soaps, and a study performed

by Perencevich (2001) demonstrated TCC in ~29% of bar soaps [87]. As of 2012, TCC is solely an anthropogenic compound and not produced by natural means. The chemical structure of TCC is shown in Figure 2.1. A summary of chemical properties of TCC is shown in Table 2.1.

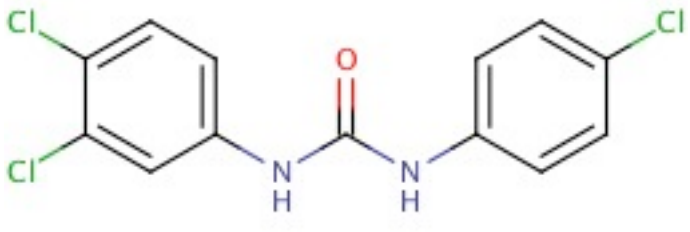


**Figure 2.1** Chemical structure of TCC [108].

**Table 2.1** Chemical properties of TCC ((SciFinder) and [109]).

| ***Property*** | ***TCC*** | ***DCC*** | ***NCC*** | ***Units*** | ***Conditions*** |
|---|---|---|---|---|---|
| **CAS** | 101-20-2 | 1219-99-4 | 102-07-8 | | |
| **Molecular Formula** | $C_{13}H_9Cl_3N_2O$ | $C_{13}H_{10}Cl_2N_2O$ | $C_{13}H_{12}N_2O$ | | |
| **Molecular Weight** | 315.58 | 281.14 | 212.25 | g/mol | |
| **Density** | 1.534±0.06 | 1.450±0.06 | 1.249±0.06 | $g/cm^3$ | at 20°C |
| **Viscosity** | NA (solid) | NA(solid) | NA( solid) | cP | at 25°C |
| **Solubility** | 1.0E-04 | 5.1E-04 | 0.03 | g/L | at 25°C |
| **Vapor Pressure** | 6.67E-05 | 3.64E-04 | 0.0112 | torr | at 25°C |
| **Henry's Law Constant** | < 1E-08 [1] | -- | -- | atm-$m^3$/mol | |
| **Bioconcentration Factor** | 24300 | 8520 | 115 | | at 25°C |
| **$K_{OC}$** | 48000 | 22600 | 1040 | | at 25°C |
| **Log $K_{OW}$** | 4.90[2] | 4.3[3] | | | |

TCC is a hydrophobic compound and sorbs to particulate matter [93, 95, 112-114]. This is evidenced by high $K_{OC}$ and $K_{OW}$ values, and causes it to primarily accumulate in digester sludge and, consequently, biosolids. TCC has also been found in river, wastewater, groundwater and freshwater sediments [98].

[1] Estimated by USEPA using Estimation Programs Interface Suite™ v3.20 [110]
[2] Estimated using KOWINv1.67[111]]
[3] [112]

Table 2.2, summarizes concentrations of TCC previously reported in various matrices in the environment.

**Table 2.2** Concentrations of TCC found in various matrices in the environment.

| **Matrix** | River Water[4] | Wastewater[d] | Groundwater[5] | Dewatered, Digested Sludge[6] | Biosolids[f] |
|---|---|---|---|---|---|
| **Concentration** | 5600 | 6750 | 12 | 51.15±15 | 51,000 |
| **Units** | ng/L | ng/L | ng/L | mg/kg dry wt. | ng/g |

Few toxicological studies have been done on the toxicity of TCC to humans; however, TCC has been shown to bioaccumulate in aquatic species. The most well known health concern regarding TCC is that it causes methemoglobinemia in children [99, 100]. Additionally, studies have shown that TCC interferes with mammalian reproduction [105, 116] and "amplifies transcriptional activity of steroid sex hormones in the estrogen and androgen receptors of humans"[117-119].

Ying et al., observed aerobic dechlorination of TCC [120]. However, to date, anaerobic bacterial species have not been identified which dechlorinate TCC. In 2008, Miller et al. theorized that reductive dechlorination may be responsible for observed degradation by-products of TCC (4,4'-dichlorocarbanilide (DCC) and carbanilide (NCC)) in aged deep sediment [95].

[4] [91]
[5] [115]
[6] [93]

Miller (2010) later discovered *Alcaligenaceae* bacteria in activated sludge which consume TCC and NCC as the sole carbon source[121].

*2.1.2 Tris(2-chloroethyl) phosphate (TCEP)*

TCEP is a colorless to pale yellow liquid with a slight odor mainly used in the production of liquid unsaturated polyester resins as a flame retardant [122]. In 1995, TCEP was placed on the European Commission second priority list [123]:[124]. To date, TCEP is classified as an anthropogenic compound produced by natural means. The chemical structure of TCEP is illustrated in Figure 2.2. A summary of chemical properties for TCEP is provided in Table 2.3.

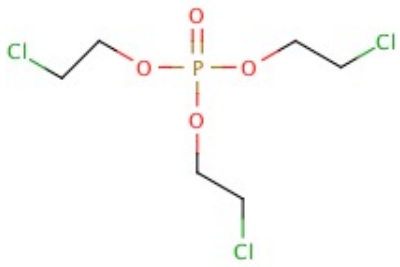


**Figure 2.2** Chemical structure of TCEP [108].

**Table 2.3** Chemical Properties of TCEP (SciFinder) and [122]).

| ***Property*** | ***TCEP*** | ***Units*** | ***Conditions*** |
|---|---|---|---|
| **CAS** | 115-96-8 | | |
| **Molecular Formula** | $C_6H_{12}Cl_3O_4P$ | | |
| **Molecular Weight** | 285.5 | g/mol | |
| **Density** | 1.392±0.06[7] | $g/cm^3$ | at 20°C |
| **Viscosity** | 34[8] | cP | at 25°C |
| **Solubility** | Slightly, 7.4[e] | g/L | at 25°C |
| **Vapor Pressure** | 1.08E-04[d] | torr | at 25°C |
| **Henry's Law Constant** | 3.30E-06[e] | atm-$m^3$/mole | |
| **Bioconcentration Factor** | 7.69[d] | | at 25°C |
| **$K_{OC}$** | 150[d] | | at 25°C |
| **Log $K_{OW}$** | 1.7[e] | | |

[7] SciFinder.org
[8] [122]

TCEP has been found in a wide range of environmental matrices, including indoor [125-132] and outdoor air [130, 133], water [130, 134-142], sediments [143, 144], soils [142, 145], and landfill leachate [146, 147]. A cause for concern is the possibility for long transport [137, 148-150]. Due to this phenomenon, TCEP has been detected in: Antarctica [148], pine needles in the Sierra Nevada Mountains [149], precipitation in remote areas in Ireland, Poland, and Sweden [150], and rainwater in Germany [137]. Table 2.4, summarizes typical concentrations of TCEP found in various matrices in the environment.

**Table 2.4** Concentrations of TCEP found in various environmental matrices [123]

| **Matrix** | Urban Rivers [136] | WWTP Effluent [135] | Marine Sediment [144] | Indoor Air [125] |
|---|---|---|---|---|
| **Concentration** | 0.5 | 30 | 1 | 250 |
| **Units** | μg/L | μg/L | ng/g | $ng/m^3$ |

TCEP contamination is a cause of concern due to the risk posed to the environment and the possible toxicity to humans from exposure to the substance [123], as established from animal studies. Table 2.5 summarizes some key TCEP toxicity studies performed and their findings.

**Table 2.5** Key studies on toxicity effects from TCEP

| Type | Year | Study | Key Findings | Reference |
|---|---|---|---|---|
| Neurotoxicity | 1990 | Rats given 275 mg TCEP/kg body weight | High doses in Rats caused brain lesions, convulsions (within 60-90 minutes), and impaired performance in water maze | [151] |
| | 1993 | 2 year study, focusing on chronic exposure | • Principal toxic effects occurred in the brain and kidney | [152] |
| Reproductive Toxicity | 1991 | Male and female rats exposed to TCEP | • Adversely effects male fertility in rats and mice<br>• Reduced fertility due to TCEP exposure occurs at doses of 175 mg/kg body weight or more | [153] |
| Carcinogenicity | 1989, 1990 | Rats and mice exposed to varying levels of TCEP | Causes benign tumors in rats and mice | [154, 155] |
| | 2006 | Various cultures were dosed with TCEP | Findings suggest non-genotoxic carcinogen. Not cytotoxic, anti-estrogenic | [156] |

Takahashi et al. (2012) recently published an article showing complete detoxification of TCEP using the bacterial strains *Sphingobium* sp. strain TCM1 and *Xanthobacter autotrophicus* strain GJ10 [157]. While Takahashi et al. (2008), reported the mixed culture capable of TCEP degradation, these finds have yet to be reproduced [158, 159].

*2.1.3 1,1,1-Trichloroethane (TCA)*

TCA, an anthropogenic, colorless liquid with a sweet, sharp odor [160] is used as a cleaning solvent and a degreaser. While initially developed as a "safer

alternative" to TCE, TCA was as toxic to the environment as TCE. However, TCA is not as toxic to humans and is not a known human carcinogen [161]. Like TCE, TCA most often appears in groundwater; the daughter products of TCA, 1,1-DCA (DCA) and chloroethane (CA), appear where TCA contamination has occurred due to various natural abiotic and biotic transformations [161]. Figure 2.3 shows the chemical structure of TCA, and Table 2.6 provides a summary of the chemical properties of TCA and its daughter products.


**Figure 2.3** Chemical structure of TCA[108].

**Table 2.6** Chemical Properties for TCA and daughter products DCA and CA (Unless otherwise specified, all properties were obtained from Scifinder [162]).

| *Property* | *TCA* | *DCA* | *CA* | *Units* | *Conditions* |
|---|---|---|---|---|---|
| **CAS** | 71-55-6 | 75-34-3 | 75-00-3 | | |
| **Molecular Formula** | $C_2H_3Cl_3$ | $C_2H_4Cl_2$ | $C_2H_5Cl$ | | |
| **Molecular Weight** | 133.40 | 98.96 | 64.51 | g/mol | |
| **Density** | 1.393±0.06[c] | 1.168±0.06[c] | 0.884±0.06[c] | $g/cm^3$ | at 20°C |
| **Viscosity** | 0.790[9] | 0.464[e] | 0.259[10] | cP | at 25°C |
| **Solubility** | 1.5[11] | 2.1[c] | 3.7[e] | g/L | at 25°C |
| **Vapor Pressure** | 122[c] | 232[c] | 1170[c] | Torr | at 25°C |
| **Henry's Law Constant** | 1.7E-02[12] | 6.2E-03[g] | 1.2E-02[g] | atm-$m^3$/mole | |
| **Bioconcentration Factor** | 35.8[c] | 12.8[c] | 10.1[c] | | at 25°C |
| **$K_{OC}$** | 450[c] | 215[c] | 182[c] | | at 25°C |
| **Log $K_{OW}$** | 2.49[13] | 1.45-1.48[14] | 1.43[15] | | |

TCA has been found in urban, rural, and indoor air, groundwater, wastewater, surface water, sediments, and soil [160]. In 2006, the US Department of Health and Human Services (USDHHS) published a oxicological profile for TCA, which is summarized in Table 2.7.

[9] [163]
[10] [164]
[11] [160]
[12] [165, 166]
[13] [160]
[14] [167]
[15] [168]

**Table 2.7** Concentrations of TCA found in various matrices in the environment [160] (All concentrations are given in ppb unless otherwise specified).

| Matrix | | Concentration |
|---|---|---|
| Air | Urban | 0.1-1 |
| | Indoor | 0.3-0.4 |
| | Outdoor | 0.11-0.92 |
| Water | Ground | 0-18 |
| | Drinking | 0.01-3.5 |
| | Surface | 0-9.7 |
| Sediment and Soil | | 0.01-2500 |

While it is not classified as a carcinogen [76], numerous reports state that TCA causes negative health effects upon exposure. Symptoms of TCA exposure include: "central nervous system depression, hypotension, cardiac arrhythmia, diarrhea and vomiting, mild hepatic effects, and dermal and ocular irritation."

*Dehalobacter* sp. is the only identified dehalorespiring, anaerobic bacteria capable of TCA dechlorination[161, 169, 170]. TCA can also inhibit TCE dechlorination until all TCA is dechlorinated to DCA [170, 171] [172].

## 2.2 BIOGENIC SOURCES OF HALOGENATED COMPOUNDS

Biogenic compounds are naturally occurring materials. Biogenic, halogenated compounds, known as organohalides, are comprised of primarily chlorinated and brominated compounds, with iodinated compounds and fluorinated compounds less prevalent. By 2003, over 3000 organohalides had been identified. Many brominated biogenic compounds are produced by marine biota and may serve as a chemical defense against predators and may inhibit biofouling in sea sponges [40]. Biogenic chlorinated compounds are rarely seen in sponges but are found in many tick species [41-46].

*2.2.1 2,6-Dibromophenol (2,6-DBP)*

2,6-Dibromophenol (2,6-DBP) is a halogenated phenol known more specifically as a bromophenol. 2,6-DBP is naturally produced by various species of marine hemichordates [40, 47, 48] and sponges [72, 77]. Figure 2.4 illustrates the chemical structure, and Table 2.8 provides the chemical properties of 2,6-DBP.

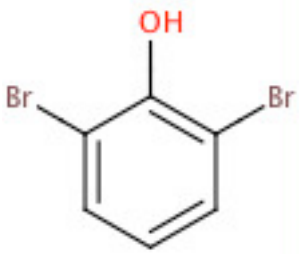


**Figure 2.4** Chemical structure of 2,6-DBP [108]

**Table 2.8** Chemical Properties of 2,6-DBP (Scifinder)

| *Property* | *2,6-DBP* | | *Units* | *Conditions* |
|---|---|---|---|---|
| **CAS** | 608-33-3 | | | |
| **Molecular Formula** | $C_6H_4Br_2O$ | | | |
| **Molecular Weight** | 251.9 | | g/mol | |
| **Density** | 2.095±0.06 | | $g/cm^3$ | at 20°C |
| **Viscosity** | NA | | cP | |
| **Solubility** | 0.76 | | g/L | at 25°C |
| **Vapor Pressure** | 9.49E-03 | | torr | at 25°C |
| **Henry's Law Constant** | 1.202E-05[16] | | atm-$m^3$/mole | at 25°C |
| **Bioconcentration Factor** | Varies based on pH | | | at 25°C |
| | pH | Value | | |
| | 6 | 158 | | |
| | 7 | 78.2 | | |
| | 8 | 13 | | |
| **$K_{OC}$** | Varies based on pH | | | at 25°C |
| | pH | Value | | |
| | 6 | 1260 | | |
| | 7 | 624 | | |
| | 8 | 104 | | |
| **Log $K_{OW}$** | 3.36 | | | at 25°C |

Few investigative studies of 2,6-DBP and its fate in the environment are available. Since 2,6-DBP is produced by marine species, it is present in marine environments.

While studies examining toxicity of 2,6-DBP are not available [73], studies of polybrominated compounds have demonstrated they are as toxic to humans [78, 79] and animals [80, 81] as polychlorinated compounds.

The majority of research into biodegradation of bromophenols (BPs) has focused on higher substituted brominated compounds and polybrominated diphenyls (PBBs) [38, 78, 79, 81, 82, 174-176]. Ahn et al. (2003) reported complete debromination of 2,6-DBP to phenol by bacteria present in a marine

[16] Estimated using Exper. Database match and HENRYWIN v3.10, respectively [173]

sponge [77]. Figure 2.5 shows the experimentally observed and theoretical biodegradation pathways for the debromination of 2,6-DBP.

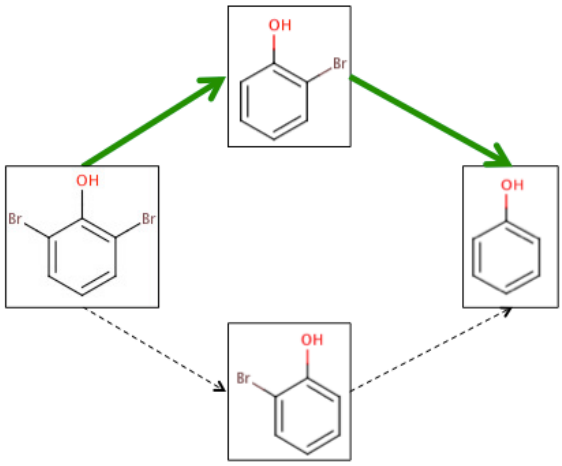


**Figure 2.5** Pathways for reductive debromination of 2,6-DBP [108]. The experimentally observed pathway is represented by the bold, green arrows [77]. The theoretical formation of 6-bromophenol (6-BP) is shown with a dashed, black arrow.

*2.2.2 2,6-Dichlorophenol (2,6-DCP)*

2,6-Dichlorophenol (2,6-DCP) is a halogenated phenol known more commonly known as chlorophenol. 2,6-DCP is a biogenic [41, 177] and, to a lesser extent, anthropogenic source of chlorine [178]. Biogenically, 2,6-DCP is a sex pheromone secreted by numerous tick species including the Lone-Star tick. [40]:[41-46, 68, 179]. Anthropogenically, 2,6-DCP is an intermediate in the production of insecticides, herbicides, preservatives, antiseptics, and disinfectants [178]. The chemical structure of 2,6-DCP is provided in Figure 2.6, and its chemical properties are provided in Table 2.9.

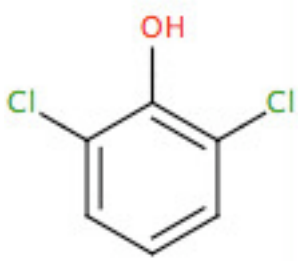


**Figure 2.6** Chemical structure of 2,6-DCP [108].

**Table 2.9** Chemical Properties of 2,6-DCP (Scifinder).

| ***Property*** | ***2,6-DCP*** | | ***Units*** | ***Conditions*** |
|---|---|---|---|---|
| **CAS** | 87-65-0 | | | |
| **Molecular Formula** | $C_6H_4Cl_2O$ | | | |
| **Molecular Weight** | 163 | | g/mol | |
| **Density** | 1.458±0.06 | | $g/cm^3$ | at 20°C |
| **Viscosity** | | | | |
| **Solubility** | 0.52 | | g/L | at 25°C |
| **Vapor Pressure** | 0.0828 | | torr | at 25°C |
| **Henry's Law Constant** | 4.308E-06[17] | | atm-$m^3$/mole | at 25°C |
| **Bioconcentration Factor** | Varies based on pH | | | at 25°C |
| | 6 | 85.5 | | |
| | 7 | 48 | | |
| | 8 | 8.95 | | |
| **$K_{OC}$** | Varies based on pH | | | at 25°C |
| | 6 | 819 | | |
| | 7 | 459 | | |
| | 8 | 85.7 | | |
| **Log $K_{OW}$** | 2.80[q] | | | at 25°C |

Being biogenic, 2,6-DCP is found in many environmental matrices including surface water, groundwater, drinking water, food, and indoor and ambient air. Table 2.10 summarizes reported concentrations of 2,6-DCP in these matrices.

[17] [180] Estimated using HENRYWIN v3.10 and KOWWIN v1.67, respectively

**Table 2.10** Concentrations of 2,6-DCP found in various environmental matrices [68]

| **Matrix** | Surface Water | Groundwater | Drinking Water | Food | Indoor Air | Ambient Air |
|---|---|---|---|---|---|---|
| **Concentration** | 0.1 | ≤27,300 | 0.2-267 | 2-105 | <1-165 | 0.3 |
| **Units** | μg/L | μg/L | μg/L | μg/kg | μg /$m^3$ | μg /$m^3$ |

Although multiple studies have been performed examining toxicity of chlorophenols [68, 181-186], 2,6-DCP has not been included [68]. However, Keith and Telliard (1979) reported another dichlorophenol, 2,4-dichlorophenol (2,4-DCP), and the intermediate 2-chlorophenol (2-CP) were included on the list of priority pollutants in 1979 due to "their toxicity and suspected carcinogenicity" [187].

There have been numerous investigations into the anaerobic and aerobic biodegradation of 2,6-DCP [188-190]. Multiple researchers have observed reductive dechlorination of chlorinated phenols in methanogenic conditions, resulting in methane and carbon dioxide [49, 51, 53, 54, 56, 58, 59, 191, 192] production. Varying results have been reported for DCP reduction when sulfur is present. Häggblom, et al. (1993) [58, 59] observed biodegradation under sulfate-reducing conditions and three other types of reducing conditions, and these findings further supported reports of 2,6-DCP reduction by sulfidogenic cultures [52, 75, 192], including consortia from an anaerobic reactor [49]. However, the chlorophenol degradation decreased when one research group added sulfur to an established 2,6-DCP reducing methanogenic culture[75]. Another well-documented occurrence is that the preferred aromatic chlorine position for these methanogenic cultures is the *ortho* position, compared to the *meta*- and *para*-

positions, where dechlorination is less readily observed [50, 55, 192-194]. Figure 2.7 shows the experimentally observed and theoretical biodegradation pathways for 2,6-DCP. A summary of previously isolated and identified bacterial strains capable of degrading 2,6-DCP is presented in Table 2.11.

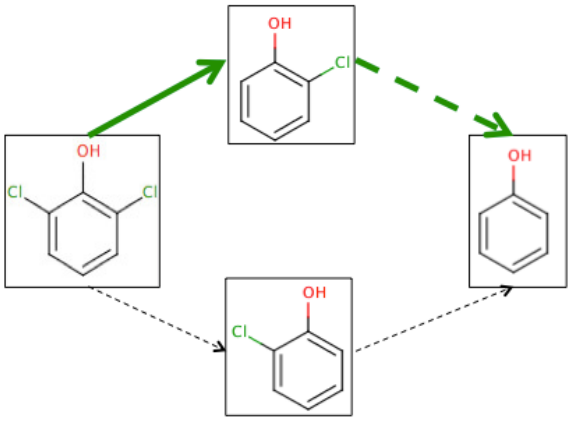


**Figure 2.7** Pathways for reductive dechlorination of 2,6-DCP [108]. The experimentally observed pathway is represented by green arrows. The bold, green arrow represents dechlorination by the strain *Dehalococcoides mccartyi* str. CBDB1 [37], and the dashed, green arrow represents dechlorination by *Anaeromyxobacter dehalogenans* [67, 195, 196]. The theoretical formation of 6-chlorophenol (6-CP) is shown with a dashed, black arrow.

**Table 2.11** Previously isolated and identified bacterial strains capable of degrading 2,6-DCP [69]

| Bacterial Strain | Condition | Cell Yield, g dtw/g | Electron Donor | Products | Reference |
|---|---|---|---|---|---|
| *Anaeromyxobacter dehalogenans* | Anaerobic (Halorespiring) | | Acetate, $H_2$, succinate, pyruvate, formate | Phenol | [67, 195, 196] |
| *Desulfitobacterium chlororespirans* Co23 | Anaerobic (Halorespiring) | | Formate, butyrate, $H_2$, pyruvate, lactate | 2-CP | [195] |
| *Desulfitobacterium dehalogenans* | | | | | [197] |
| *Desulfitobacterium hafniense* PCP-1 | Anaerobic (Halorespiring) | | Pyruvate | 2-CP | [63] |
| *Desulfovibrio dechloracetivorans* SF3 | Anaerobic (Halorespiring) | 0.033 | Acetate, pyruvate, lactate, fumurate, propionate, ethanol | Phenol | [66] |
| *Sphingomonas* sp. P5 | Aerobic | 0.210 | 2,6-DCP | | [198] |
| Unidentified strain 2CP-1 | Anaerobic (Halorespiring) | | Formate, acetate, yeast extract | | [199] |

## 2.3 BIOGENIC AND ANTHROPOGENIC SOURCES OF HALOGENATED COMPOUNDS

As mentioned above, some compounds are both biogenic and anthropogenic sources of halogenated compounds. Additionally, certain biogenic compounds are now produced in such large quantities that they are considered more anthropogenic source than biogenic contamination. Such is the case with

chlorinated solvents such as chlorinated ethenes and ethanes (discussed previously) [200].

At ambient temperature, chlorinated solvents are liquids which are denser than water, resulting in deep penetration into groundwater aquifers [3]. Chlorinated solvents have been used in a wide variety of processes throughout the last century including cleaning and degreasing of clothes, electronic parts, and machinery, intermediates in chemical manufacturing, in textile processing, as adhesives, and in pharmaceuticals [3]. According to Figure 2.8, peak total chlorinated solvent usage in the United States occurred around 1970, with ~3 billion pounds of carbon tetrachloride (CT), 1,1,1-trichloroethane (TCA), tetrachloroethene (PCE) and trichloroethylene (TCE).

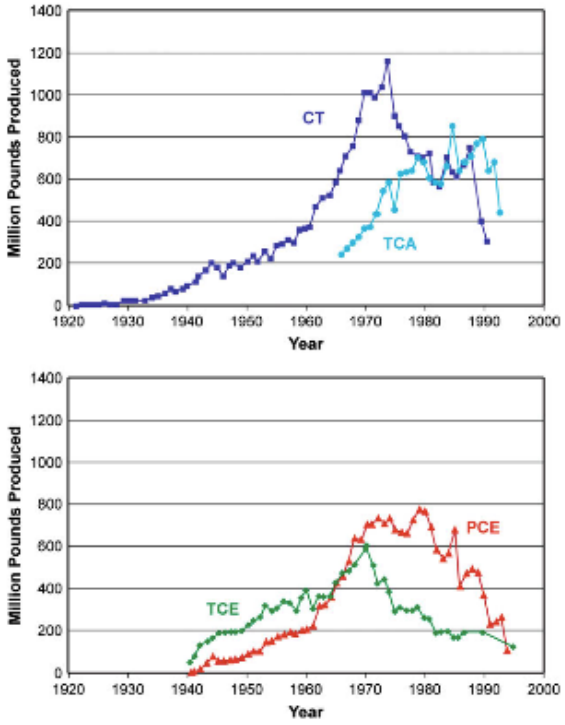


**Figure 2.8** United States yearly production of chlorinated solvents (from [201] and [202], respectively) [3] .

The United States Environmental Protection Agency's (USEPA) Clean Water Act of 1977 resulted in the formation of a priority pollutant list including CT, PCE, TCA, and TCE. By 1980, proposed drinking water quality criteria for these chemicals were published. Today, these four chlorinated solvents are the most widely found contaminants at USEPA Superfund sites [3].

Chlorinated ethenes are composed of two carbon centers joined by a double bond. These two carbons can bond to one or two chlorine or hydrogen substituents on one or both of the carbons. These compounds are slightly water soluble, dense, very stable, and highly volatile [3]. Chlorinated ethenes include, in order of decreasing chlorine substituents, tetrachloroethene (PCE), trichloroethene

(TCE), 1,1-dichloroethene (1,1-DCE), *trans*-dichloroethene (*trans*-DCE), *cis*-dichloroethene (*cis*-DCE), and vinyl chloride (VC). The specific chemical properties of these compounds are presented and discussed later.

Chlorinated ethanes are the single-bonded form of chlorinated ethenes. These compounds have similar chemical characteristics to chlorinated ethenes (i.e. are slightly water soluble, relatively dense, highly volatile, etc.). Chlorinated ethanes include, in order of decreasing chlorine substituents, hexachloroethane (also called perchloroethane), pentachloroethane, tetrachloroethene, trichloroethene, dichloroethane, and chloroethane. The single bond present in chlorinated ethanes allows for a greater number of chlorinated substituents and isomeric arrangements. The specific properties of trichloroethene, dichloroethane, and chloroethane were discussed previously.

*2.3.1 Trichloroethene (TCE)*

Trichloroethene (TCE), also called trichloroethylene, is a chlorinated ethene most often used in industrial dry-cleaning and as a degreasing agent [14]. It is naturally produced by some marine algae [7] but is more widely produced as an anthropogenic compound. TCE is classified as a volatile organic chemical (VOC) that is colorless or blue with a sweet odor similar to chloroform [203]. TCE was used as a general anesthetic prior to 1977 [4, 204]. TCE is used in chemical production as a chemical intermediate and is found in consumer products such as correction fluid, paint removers, adhesives, and spot removers [205]. Peak TCE production in the US occurred in 1970 (280 million kilograms or 616 million pounds) and has declined since then due to regulation [206]. Sites

with TCE contamination usually contain its lesser-chlorinated daughter products of *cis*-dichloroethene (DCE) [28], *trans*-dichloroethene, and vinyl chloride (VC) [29]. Previously, this blend of contaminants is shown to be the product of abiotic [207, 208] and biotic [10, 11, 209] transformations. Figure 2.9 demonstrates the chemical structure of TCE, and Table 2.12 shows the chemical properties of TCE, DCE, and VC.

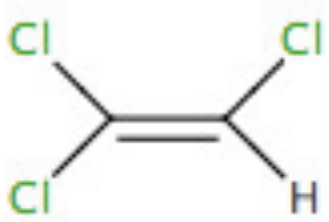


**Figure 2.9** Chemical structure of TCE [108]

**Table 2.12** Chemical Properties of TCE, DCE, and VC (Unless otherwise stated, properties were obtained from, Scifinder [162]).

| ***Property*** | ***TCE*** | ***DCE*** | ***VC*** | ***Units*** | ***Conditions*** |
|---|---|---|---|---|---|
| **CAS** | 79-01-6 | 156-59-2 | 75-01-4 | | |
| **Molecular Formula** | $C_2HCl_3$ | $C_2H_2Cl_2$ | $C_2H_3Cl$ | | |
| **Molecular Weight** | 131.39 | 96.95 | 62.5 | g/mol | |
| **Density** | 1.474±0.06 | 1.243±0.06 | 0.918±0.06 | $g/cm^3$ | at 20°C |
| **Viscosity** | 0.53[18] | 0.48[19] | 0.0107[b] | cP | at 25°C |
| **Solubility** | 0.39 | 1.1 | 3.6 | g/L | at 25°C |
| **Vapor Pressure** | 72.4 | 333 | 2580 | torr | at 25°C |
| **Henry's Law Constant** | 9.9E-03[a] | 3.37E-03[b] | 2.78E-02[20] | atm-$m^3$/mole | |
| **Bioconcentration Factor** | 52.4 | 24.7 | 11.3 | | at 25°C |
| **$K_{OC}$** | 592 | 346 | 198 | | at 25°C |
| **Log $K_{OW}$** | 2.4[21] | 1.86[b] | 1.58[c] | | |

[18] [210] Estimated using PHYSPROP and EPI, respectively
[19] [211]
[20] [212]
[21] [213]

TCE is found almost everywhere in the environment including, surface water [214, 215], groundwater [216-218], drinking water [216, 219, 220], food [216, 221], indoor [222] and ambient air [223] and in the atmosphere [224]. Typical concentrations are listed in Table 2.13.

**Table 2.13** Previously reported environmental concentrations of TCE [206]**.**

| Matrix | Concentration | Units |
|---|---|---|
| Surface Water | 0.1[v] | μg/L |
| Groundwater | ≤27,300[w] | μg/L |
| Drinking Water | 0.2-267[x] | μg/L |
| Food | 2-105[y] | μg/kg |
| Indoor Air | <1-165[z] | μg /$m^3$ |
| Ambient Air | 0.3[aa] | μg /$m^3$ |
| Atmosphere | 3.18[bb] | pptv |

The fate of TCE depends on the method by which it is released to the environment. Its low vapor pressure of 72.4 torr (0.01 atm) results in a portion of the TCE released to surface soils being volatilized in the soil void space rather than being sorbed to particulates. TCE also has a moderate water solubility of 1.1

[v] Median value based on studies done in the United States in 1983 [216]. More recent measurements have shown TCE concentrations in the 0.03-0.04 μg/L range with only three detects in 150 samples [206].

[w] Range was the highest given in the USEPA report and encompasses all other values presented [216-218].

[x] Values summarized from pre-1990 studies [216, 219, 220].

[y] Foods tested included: cheese, butter, nuts, peanut butter, various meats, and various pastries with the highest concentrations found in beef frankfurters [216, 221].

[z] Locations tested included the United States, Canada and Europe [225-228].

[aa] Mean level concentration. [206] In 2006, 258 ambient air monitors recorded means ranging from 0.03 to 7.73 μg /$m^3$ [223].

[bb] "During pollution incidences" [224]

g/L, so TCE released into soils via landfills migrates through the soil and dissolves in groundwater [214]. In the atmosphere, most TCE is present in the vapor phase and can be removed by precipitation (due to the moderate solubility) [206] and by photo-oxidation by hydroxyl radicals [229]. High concentrations of TCE found in indoor air are typically the result of groundwater contamination below the building. A 2007 article on TCE vapor intrusion in Cortlandville, New York, reported groundwater TCE concentrations up to 22 μg/L with corresponding indoor air concentrations up to 34 $\mu g/m^3$ [230].

TCE is a lipophilic compound with oral, dermal and inhalation routes of exposure. The highest concentrations of TCE are typically found in the kidney and liver [206]. With the 2011 release of the USEPA Toxicological Review of Trichloroethylene, TCE was reclassified as "carcinogenic in humans by all routes of exposure" [206]. This reclassification is in accordance with the USEPA *Guidelines for Carcinogenic Risk Assessment* [231] and supported by enumerable studies linking significant health hazards posed to humans to TCE exposure. TCE is associated with numerous incidences of liver cancer [232, 233], kidney cancer [232-264], non-Hodgkin's lymphoma [222, 265-281], prostate cancer [232-235, 237, 240-242, 244-246, 249, 251, 252, 254, 256, 261, 282-286], and multiple myeloma [232, 233, 240, 242, 244-246, 256, 261, 268, 287-290], as well as elevated risks of death from Hodgkin's disease, multiple myeloma, and cervical and liver cancers [291, 292].

TCE has noncancerous acute and chronic effects, including headache, confusing, sleepiness, fatigue, dizziness, nausea, blurred vision, facial numbness

and weakness [5, 293]. Additionally, occupational TCE exposure has shown a reported increase in the incidence of miscarriages in females, and animal studies have reported developmental effects resulting from exposure to TCE and its aerobic metabolites (trichloroacetic acid and dichloroacetic acid) [5, 293-295].

Biodegradation of TCE has been a research topic of increasing interest over the past three decades due to its persistence and prevalence [296]. Three metabolic processes appear to be involved in the biological degradation of chlorinated ethenes: co-metabolism, direct oxidation, and reductive dechlorination [297].

While it may occur aerobically [298], co-metabolism is generally an anaerobic process (i.e., degradation occurs due to biochemical interactions, but does not provide growth or a co-factor benefits to the bacteria) [299]. Co-metabolism is generally a slow process, and the dechlorination of VC is an example of a co-metabolic step. Aerobic and anaerobic direct oxidation utilizes chlorinated ethenes as electron donors [300-302].

Reductive dechlorination is an anaerobic process which occurs when chlorinated ethenes are used by microorganisms as electron acceptors[300, 303]. During reductive dechlorination, microorganisms sequentially remove chlorine atoms from the electron acceptor TCE and replace them with hydrogen provided from the electron donor [304]. As the chlorine atoms are removed, TCE is transformed to primarily *cis*-DCE (with lesser amounts of *trans*-DCE and 1,1-DCE), followed by VC. VC may then be transformed to ethene [305], as illustrated in Figure 2.10.

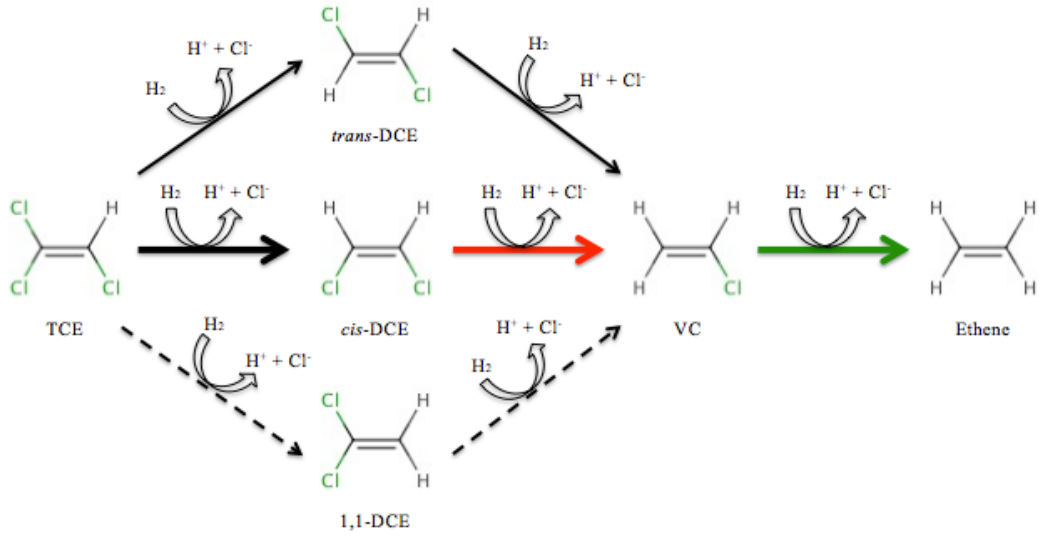


**Figure 2.10** Pathways for the reductive dechlorination of TCE [172, 296, 304, 306]. The experimentally observed pathway is represented by the bold arrows, with the desired final step represented by a green arrow. The undesired, but necessary, second step is represented by a red arrow. Dashed arrows represent the theoretical formation of 1,1-DCE. The minor formation of the by-product *trans*-DCE is shown with a thinner, black arrow.

While anaerobic conditions are required for biological degradation of TCE [19, 26, 307], the rate of reductive dechlorination processes decrease as the number of chlorine atoms on a chlorinated molecule decrease [308]. Consequently, the rate decrease results in accumulation at a contamination site [309-312] of *cis*-DCE, a probable human carcinogen, and VC, a known human carcinogen more toxic than TCE.

Reductive dechlorination is particularly interesting as certain bacteria generate energy for growth from TCE biodegradation. This respiratory metabolic process, known as dehalorespiration [305], uses TCE as an electron acceptor and

can produce a growth yield exceeding $10^8$ rRNA gene copies/μmol $Cl^-$ [30]. This is especially advantageous because greater amounts of TCE are degraded as the bacterial population grows.

*Dehalococcoides* are the only bacterial strains demonstrated to dechlorinate TCE to ethene[13, 23]. Several pure strains of *Dehalococcoides* [18-21] and mixed [12, 13, 15-17] cultures containing *Dehalococcoides* have been characterized, although not all of these pure and mixed cultures can completely dechlorinate TCE to ethene. An example is the newly designated *Dehalococcoides mccartyi* sp., capable of completely dechlorinating tetrachloroethene (PCE) (the fully substituted ethene containing four chlorines) [16, 19, 171, 313-316] and TCE [8, 12, 13, 15, 26, 27, 171, 313, 317-324]. Figure 2.11 shows the isolated strains of *Dehalococcoides* sp. and the chlorinated ethenes they transform.

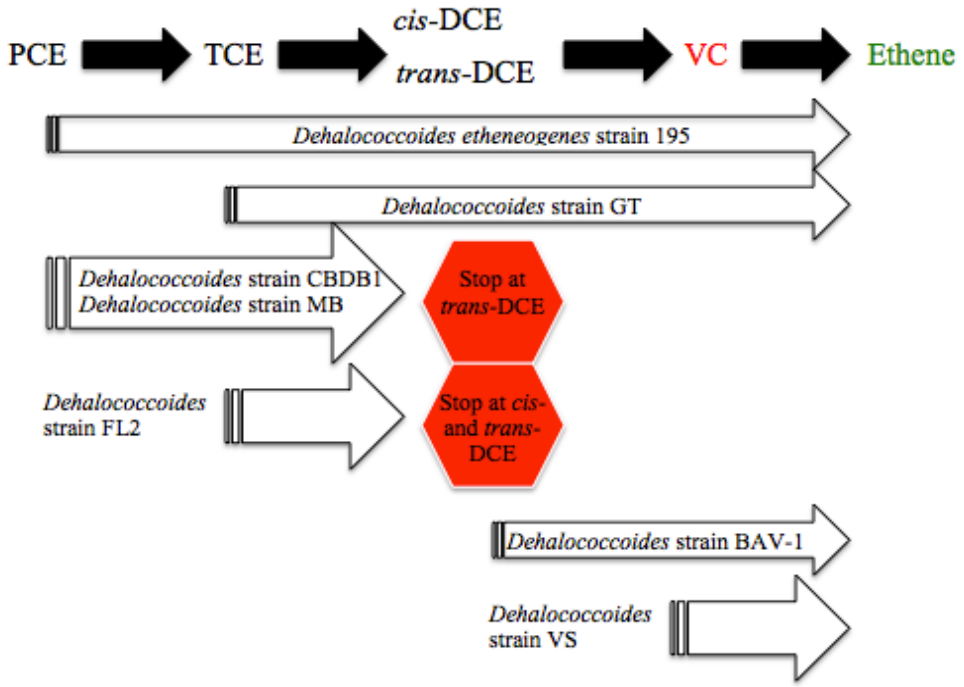


**Figure 2.11** Isolated *Dehalococcoides* spp. and the chlorinated ethenes they transform [23].

A few key gene expressions relating to TCE dechlorination are: TCE reductive dehalogenase encoding gene (*tceA),* which dechlorinates TCE to ethene [325, 326], TCE reductive dehalogenase associated B protein encoding gene (*tceB*) [327, 328], the VC reductive dehalogenase encoding gene (*vcrA*), which dechlorinates VC to ethene and is co-transcribed with *vcrB,* a hydrophobic protein, and *vcrC*, a protein similar to transcriptional regulators of the NosR/NirI family [329].

# CHAPTER 3

# EFFECTS OF SELECT ANTHROPOGENIC COMPOUNDS ON A TRICHLOROETHENE-DEGRADING MIXED CULTURE

## 3.1 INTRODUCTION

DehaloR^2 is a novel anaerobic microbial consortium capable of biotransforming the carcinogenic compound trichloroethylene (TCE) to a non-harmful end product, ethene. This sediment-free culture, maintained in the laboratory for four years, was enriched from sediments of the Back River, a tributary of the Chesapeake Bay [13]. A map showing the location of Chesapeake Bay, as well as, the Back River, is shown in Figure 3.1.

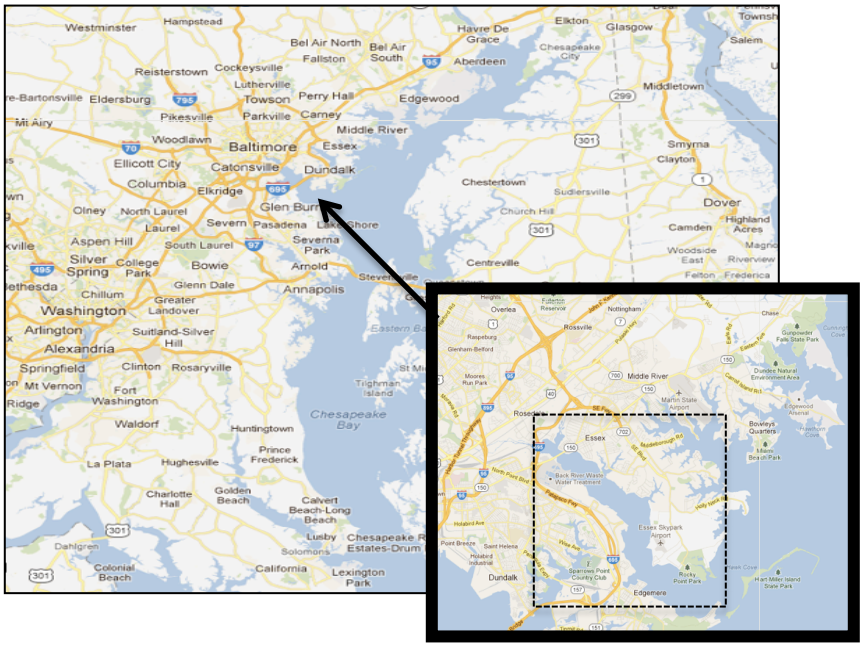


**Figure 3.1** Sediment sampling in Chesapeake Bay near Baltimore, MD. Inset: The Back River Tributary [330].

The sediment sampling location was selected, because it receives effluent from the Back River Wastewater Treatment Facility. The location, as well as, the plant has been detailed previously [95, 331]. Analyzed sediment samples from this location showed the presence of 3,4,4'-trichlorocarbanilide—more commonly known by the trade name Triclocarban and abbreviated TCC—and dichlorocarbanilide (DCC), monochlorocarbanilide (MCC), and the non-chlorinated congener, carbanilide (NCC). The last three compounds are possible products of biological reductive dechlorination [95].

Previous DNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR) has shown the presence of the dechlorinator *Dehalococcoides* in the sediment and subsequent sediment-free culture [13, 332].

Because of the fast rates achieved for TCE dechlorination and previous exposure to TCC and other anthropogenic compounds, one of the main goals of this study was to test the potential of DehaloR^2 for biodegradation of TCC and two additional anthropogenic, halogenated compounds.

The three compounds selected to be tested were TCC, tris(2-chloroethyl)phosphate (TCEP), and 1,1,1-trichloroethane (TCA). TCC is often found in sediments and biosolids. The sediment sampling location contained TCC and its lesser-chlorinated congeners, and the initial purpose of the sediment sampling was to enrich for microorganisms capable of dechlorinating TCC [93, 95]. TCEP has been detected in sediments [142-145] and surface and groundwater [130, 135-138, 140, 141]. TCA is typically a groundwater contaminant and is often a co-contaminant with TCE at Superfund sites [160].

Additionally, these three compounds represent three differing partitioning situations (detailed in Chapter 2) as shown in Figure 3.2. TCA has the highest Henry's law constant (by over three orders of magnitude compared to TCEP and six orders of magnitude compared to TCC) and is the most likely to volatilize. TCEP has the highest solubility and will most likely occur in water. TCC has a very small solubility and Henry's law constant, but has the highest Log $K_{OW}$ value (two orders of magnitude greater than TCA or TCEP) and will appear in solids and sediments.

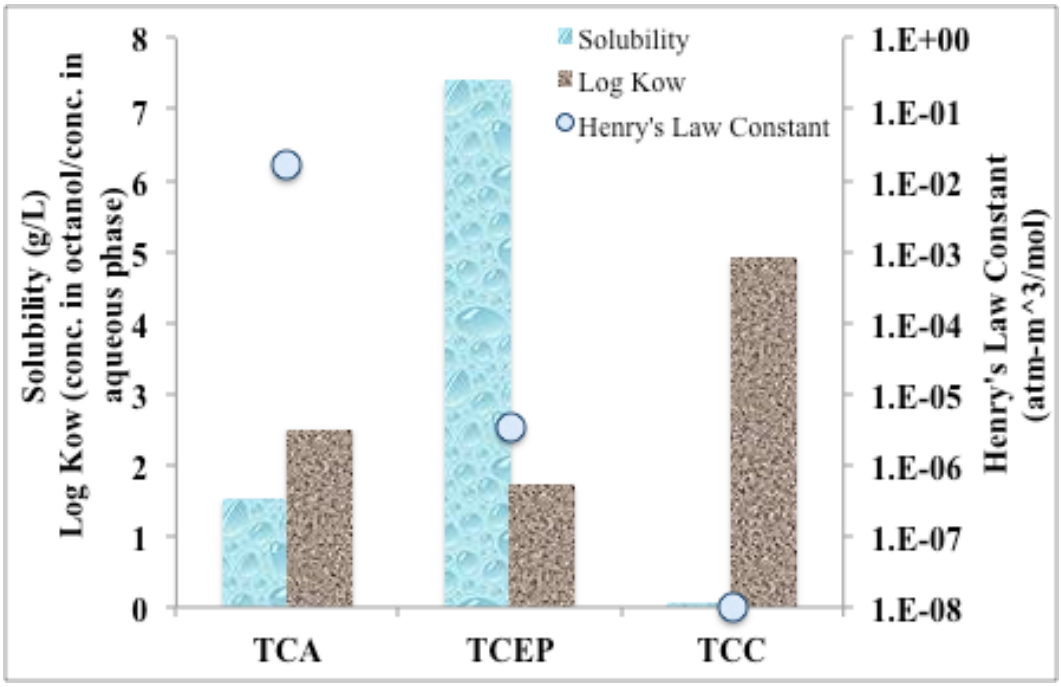


**Figure 3.2** Solubility, Log $K_{OW}$, and Henry's Law Constants for TCA, TCEP, and TCC

The second chlorinated compound selected was TCEP. TCEP is an organophosphate flame retardant. Organophosphate flame retardants (OFRs) are a class of pollutants, which have been at the forefront of remedial research [123,

125, 138, 139, 152, 156]. OFRs have a high consumption volume (around 186,000 tons used annually worldwide was reported in 2001 [123, 129]) and have been observed in multiple environmental samples [123, 125, 135-138, 333, 334]. Within this class of pollutants lies the flame retardant TCEP, a banned substance [335] previously used in polyurethane foams.

The third compound selected was TCA. TCA is a common groundwater contaminant generally found at industrial facilities and waste disposal sites, as well as, at 29% of the active and proposed sites listed on the U.S. EPA National Priorities List (NPL) (calculated by performing a search of the USEPA database in October 2012). Of the 394 sites contaminated with TCA, 80% have TCE as a co-contaminant. As was stated previously, TCA falls under the category of chlorinated solvents and, more specifically, chlorinated ethanes or chloroethanes).

The aim of the initial study was to assess the ability of the DehaloR^2 enriched culture to dechlorinate three additional anthropogenic sources of chlorinated organic compounds.

## 3.2 MATERIALS AND METHODS

### *3.2.1 Chemicals*

Neat TCA and DCA, and TCC (99% purity), DCC, NCC and TCEP (98% purity) were purchased from Sigma-Aldrich Co. (St. Louis, MO).

### *3.2.2 Mixed Cultures Set-Up*

For this study, mixed culture is defined as an anaerobic, airtight serum bottle containing the following: anaerobic media, DehaloR^2 culture, electron donor, chlorinated compound used as the electron acceptor (TCC), and vitamins.

A complete summary of constituents is provided in Appendix A

In duplicate, 10 mL of DehaloR^2 culture previously grown in a 160-mL serum bottle was added to 50 mL anaerobic media (prepared via the Hungate technique and fully defined by Löffler, et al [336], see Appendix B) in 160-mL serum bottles. The bottles were capped with rubber stoppers and crimped with aluminum crimps. The two bottles were injected with a varying volumes of 1 M lactate stock solution, ATTC vitamin mix, vitamin B-12, and stock solutions of electron acceptor (TCC, TCEP, or TCA) in methanol.

*3.2.3 Analytical Methods*

3.2.3.1 Gas Chromatography with Flame Ionization (GC-FID)

A Shimadzu GC-2010 (Columbia, MD) with an RtTM-QSPLOT capillary column (30 mm x 0.32 mm x 10 μm, Restek, Bellefonte, PA) was used to analyze changes in TCA and concentration, as well as, formation of TCA and TCE degradation by-products (DCA and CA and cis-DCE and VC, respectively) and to measure methane for all experiments. A 500-μL gas-tight syringe (Hamilton Company, Reno, NV) was used to withdraw a 200-μL gaseous sample from the headspace of the serum bottle (Figure 3.3).

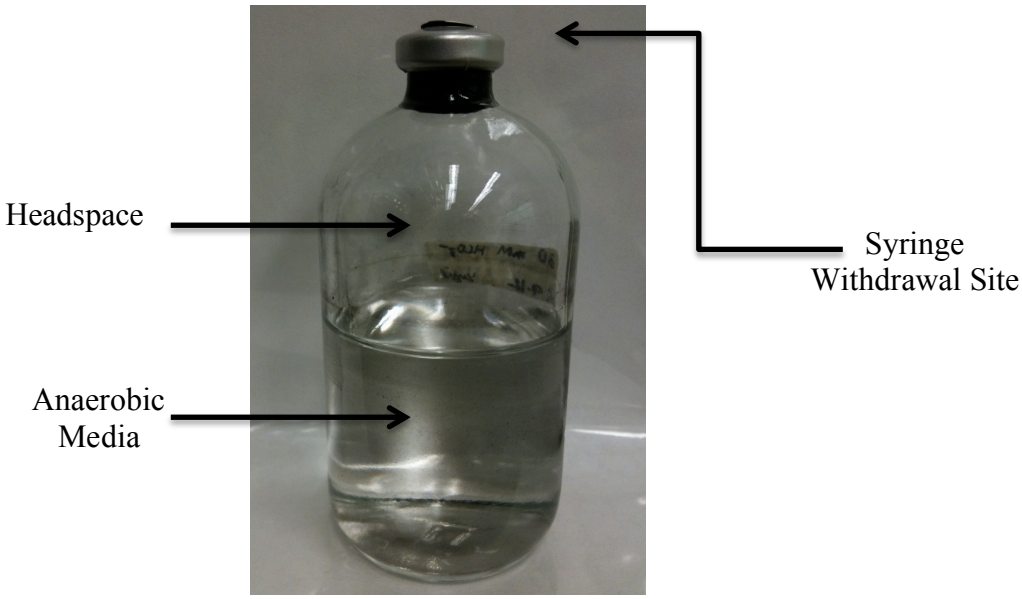


**Figure 3.3** Anaerobic media bottle showing headspace used in GC-FID analysis

The initial oven temperature was 110 °C and was held for one minute, and was then raised with a gradient of 50°C/min to 200 °C. Lastly, the oven temperature was raised with a final gradient of 15°C/min to 240 °C, where it was held for the last 1.5 minutes. Ultra High Purity (UHP) helium was used as the carrier gas and UHP hydrogen and zero grade air were used as the FID gases. The injector and FID temperatures were both 240°C, the pressure was 89.5 kPa, and the linear velocity was 35.1 cm/sec. The column flow was 1.72 mL/min and the total flow was 19.9 mL/min.

Calibration curves for the chlorinated compounds and methane were determined by adding a range of known masses of each compound to 160-mL serum bottle containing 100 mL of anaerobic media. The bottles were shaken overnight. Various volumes (200-µL, 150-µL, and 100-µL) of headspace gas from these serum bottles were injected in triplicates into the GC-FID and the corresponding concentrations were graphed versus signal area. The concentrations

were determined using a solvent partitioning Excel© worksheet and the reported Henry's law constants [337].

The GC-FID calibration parameters and retention times for each constituent of interest are summarized in Table 3.1.

**Table 3.1** Calibration parameters and retention times for GC-FID

| Constituent | $R^2$ | Slope | Intersect | Retention Time (minutes) |
|---|---|---|---|---|
| Methane | 1 | 1.67E-05 | 1.45 | 1.7 |
| Chloroethane | 0.982 | 5E-09 | 0.0023 | 3.69 |
| 1,1-Dichloroethane | 1 | 5E-05 | -0.9074 | 5.17 |
| 1,1,1-Trichloroethane | 0.999 | 3E-05 | 5.7475 | 5.79 |
| Ethene | 0.999 | 1E-05 | 0.253 | 1.8 |
| VC | 0.99995 | 3E-05 | 1.1206 | 3.1 |
| *cis*-Dichloroethene | 0.99994 | 1E-04 | -1.6076 | 5 |
| Trichloroethene | 0.99995 | 5E-05 | -1.9831 | 5.9 |

3.2.3.2 Solid Phase Extraction (SPE)

Solid phase extraction (SPE) was used to separate TCC, DCC, and NCC from sediments and culture. The protocol is included in Appendix C.

3.2.3.3 Liquid Chromatography-Negative Electrospray Ionization Tandem Mass Spectrometry (LC-ESI-MS/MS)

After SPE, an API 4000 mass spectrometer (Applied, Biosystems, Framingham, MA) coupled to a Shimadzu Prominence HPLC (Shimadzu Scientific Instruments, Inc., Columbia, MD), method previously detailed [98], was used to analyze TCC, DCC, and NCC concentrations.

## 3.3 RESULTS AND DISCUSSION

### *3.3.1 TCC Exposure*

Figure 3.4 depicts results for the duplicate serum bottles containing active DehaloR^2 culture and TCC. This DehaloR^2 culture was also used in parallel experiments where TCE dechlorination was observed. The TCC concentration does not decrease and is virtually identical to the concentration of TCC in the abiotic control duplicates.

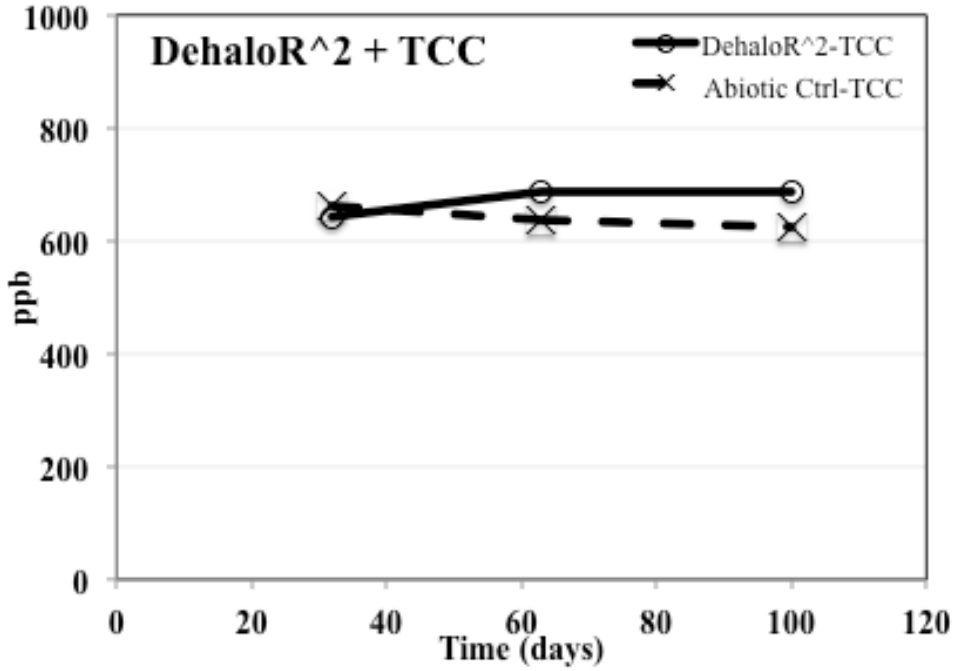


**Figure 3.4** TCC concentration over time in serum bottles containing DehaloR^2 culture.

Previously, Ying et. al. (2007) reported observed TCC degradation via aerobic soil microorganisms, but no degradation under anaerobic conditions [120]. As evidenced by Figure 3.5, TCC dechlorination did not occur in the DehaloR^2 culture bottles over a period of 110 days. Reductive dechlorination did not take place.

*3.3.2 TCEP Exposure*

The second anthropogenic chlorinated compound we tested using DehaloR^2 was TCEP. Figure 3.6, shows the average concentration of TCEP in duplicate DehaloR^2 cultures with TCEP as the only electron acceptor, and in abiotic controls containing TCEP. The concentration of TCEP was nearly identical to the abiotic control and did not change over 92 days.

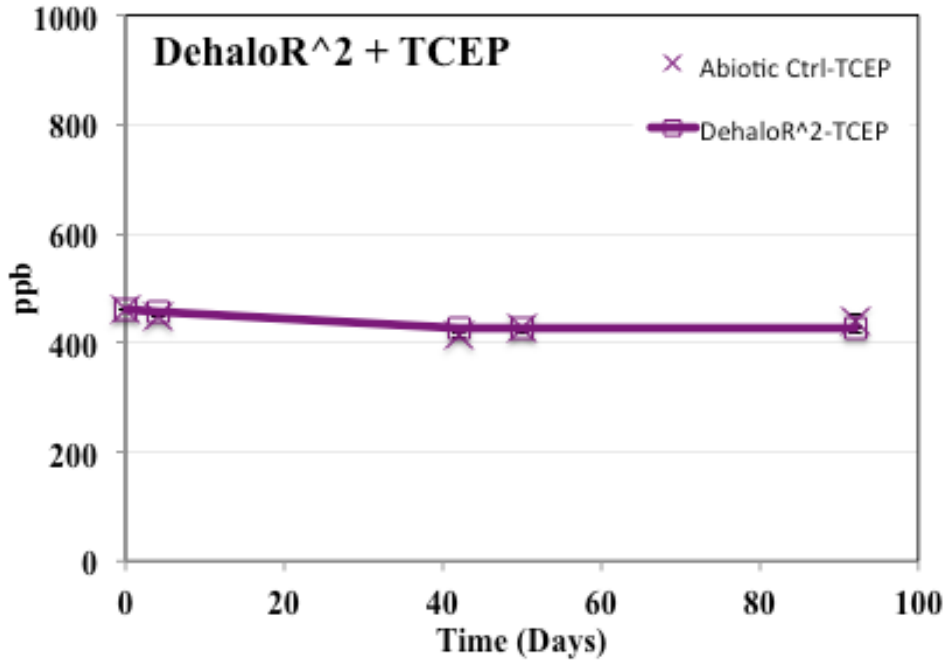


**Figure 3.5** TCEP concentration over time in serum bottles containing DehaloR^2 culture.

Takahashi, et al., isolated two bacterial strains belonging to *Sphingomonas* sp. and *Sphingobium* sp. capable of degrading TCEP [159]. As these bacteria are aerobic, neither are contained in the DehaloR^2 mixed culture. The TCEP concentration in the DehaloR^2 bottles stay nearly identical to the TCEP concentration in the abiotic control bottles. Again, no reductive dechlorination takes place.

*3.3.3 TCA and Subsequent TCE Exposure*

Figure 3.6 shows TCA degradation by DehaloR^2 in duplicate serum bottles. Figure 3.6 shows that TCA is slowly converted to DCA. At day 100, half of the initial TCA is still present and around 0.1 mM of DCA has been formed.

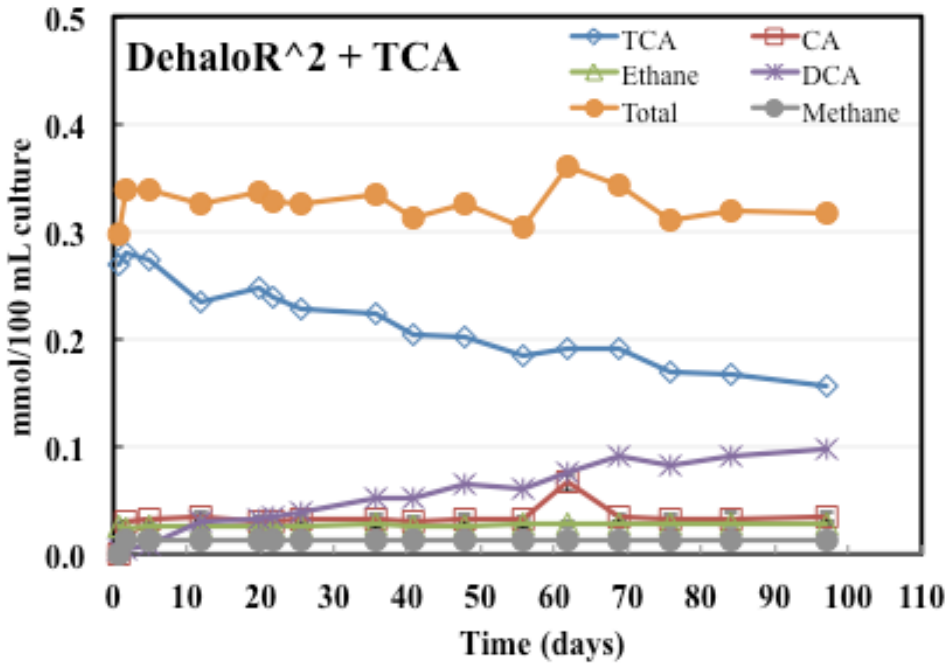


**Figure 3.6** TCA concentration over time in serum bottles containing DehaloR^2 culture.

After day 100, the duplicate bottles were transferred into four more bottles to observe if DehaloR^2 could (1) again dechlorinate TCA to DCA in the same manner and (2) dechlorinate TCE after TCA exposure. We observed that no TCA daughter products had formed after 120 days when the DehaloR^2 culture was transferred to duplicate serum bottles containing fresh media and was then injected with TCA again (results not shown). Additionally, once the TCA-exposed, DehaloR^2 culture was amended with TCE (the electron donor this culture grew on and prefers), the culture could no longer dechlorinate TCE

(results not shown). Therefore, reductive dechlorination initially occurred when DehaloR^2 was first exposed to TCA, but the TCA acted as an inhibitor to subsequent TCE and the initial results could not be replicated.

## 3.4 CONCLUSION

An overview of these studies is provided in Table 3.2. DehaloR^2 was exposed to three anthropogenic sources: TCC, TCEP, and TCA. Our results show that the DehaloR^2 culture, which was grown on, and can reductively dechlorinate TCE, could not reductively dechlorinate the additional chlorinated compounds of anthropogenic origin tested in this study. Furthermore, once we added TCA to a DehaloR^2 culture, it was no longer able to dechlorinate TCE.

TCC inhibition on the DehaloR^2 cultures TCE dechlorinating ability has previously been studied in depth [296, 332]. However, further studies could be done to examine if TCEP causes TCE-dechlorinating inhibition for the DehaloR^2 culture. This would be important if DehaloR^2 was to be used for in situ bioremediation as undetected TCEP contamination could negatively effect any TCE-remediating goals.

**Table 3.2** Summary of studies examining DehaloR^2 and anthropogenic chlorinated compounds

| Constituent | Concentration | Length of Time Studied (days) | Result |
|---|---|---|---|
| TCC | 10 μM | 100 | No reductive dechlorination |
| TCEP | 10 μM | 92 | No reductive dechlorination |
| TCA | 0.5 mM | 100 | Dechlorination of half the initial TCA and formation of 0.1 mM DCA after 100 days |
| | 0.5 mM TCA or 1 mM TCE | 120 | No reductive dechlorination of TCA of TCE |

# CHAPTER 4

# DEHALOGENATION OF 2,6-DIBROMOPHENOL (2,6-DBP) AND 2,6-DICHLOROPHENOL (2,6-DCP) BY A TRICHLOROETHENE-DEGRADING MIXED CULTURE

## 4.1 INTRODUCTION

In Chapter 3, I observed that an anaerobic culture, DehaloR^2, enriched in our laboratory and capable of reductive dechlorination of the priority pollutant trichloroethene (TCE), did not have the capacity to dechlorinate the anthropogenic compounds 3,4,4'-trichlorocarbanalide (TCC), tris(2-chloroethyl)phosphate (TCEP), or 1,1,1-trichloroethane (TCA). Dehalogenating bacteria such as *Dehalococcoides* have been around for a long time, while TCE has been anthropogenically released in increasing quantities into the environment only in the last century. Two important ecological questions are: 1) How were these microorganisms making a living before high concentrations of halogenated solvents were present in the environment due to anthropogenic input? And 2) How do microorganisms such as *Dehalococoides* have the necessary metabolic machinery to dehalogenate halogenated compounds given that developing these metabolic machinery through evolution or gene transfer events would take more than a century?

It has been hypothesized that anaerobic bacteria might have the ability to dechlorinate TCE due exposure to naturally occurring, or biogenic compounds, containing chlorine or bromine. In this chapter I explore the ability of the

DehlaoR^2 culture to dechlorinate and debrominate two biogenic-like compounds: 2,6-dichlorophenol (DCP) and 2,6-dibromophenol (DBP).

## 4.2 MATERIALS AND METHODS

### *4.2.1 Chemicals*

TCE was purchased from Sigma-Aldrich Corporation (St. Louis, MO) and *cis*-DCE was purchased from Supelco Coporation (Bellefonte, PA). Gaseous vinyl chloride was purchased from Fluka Chemical Corporation (Ronkinkoma, NY) and ethene was purchased from Scott Specialty Gases (Durham, NC). Neat 2,6-DBP and 2,6-DCP were purchased from Sigma-Aldrich Corporation (St. Louis, MO), as well as, 2,6-DBP at 99% purity and 2,6-DCP at 99% purity. 2,CP and phenol were purchased from Fluka Chemical Corporation (Ronkinkoma, NY) at 99.9% purity for use as analytical standards. Sodium lactate and vitamin B-12 were purchased from Sigma Aldrich Corporation (St. Louis, MO) and the mixed vitamin solution was obtained from ATCC (Catalog No. MD-VS, Manassas, VA).

### *4.2.2 Culture and Initial Experimental Set-Up*

Reduced anaerobic mineral medium was prepared using the Hungate technique described previously by Löffler et al. [336] and provided in Appendix B. The medium was prepared in a 2-L batch flask and then allocated into 160 mL serum bottles. These bottles were then capped with butyl rubber stoppers and crimped with aluminum crimps as shown in Figure 4.1.

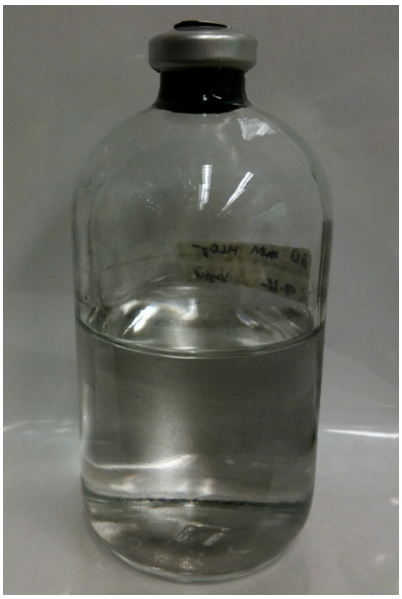

**Figure 4.1** Anaerobic medium in 160 mL serum bottle.

Abiotic controls for this experiment were prepared by adding 10 mL of DehaloR^2 culture to anaerobic media (for a total volume of 100 mL) and autoclaving the serum bottles for 1 hour at 121°C with a sterilization time of 15 minutes. The experimental set-up is summarized in Figure 4.2. All 21 bottles contained the following: 50 µL vitamin B-12 and 1 mL ATCC vitamin mix. All bottles contained 0.5 mL of 1 mM lactate to be used as an electron donor. TCE was delivered to the necessary bottles via a 1000 mM stock solution in methanol. A volume of 50 µL was added for a final concentration of 0.5 mM TCE. 2,6-DBP and 2,6-DCP were delivered to the necessary bottles from 100 mM stock solutions in methanol. A volume of 100 µL was added for a final concentration of 0.1 mM. The methanol provided to the cultures via the stock solutions served as an additional electron donor. The serum bottles were incubated at 30°C, inverted, and shaken at 200 rpm on an orbital shaker. The serum bottle triplicates and their constituents are summarized in Appendix D.

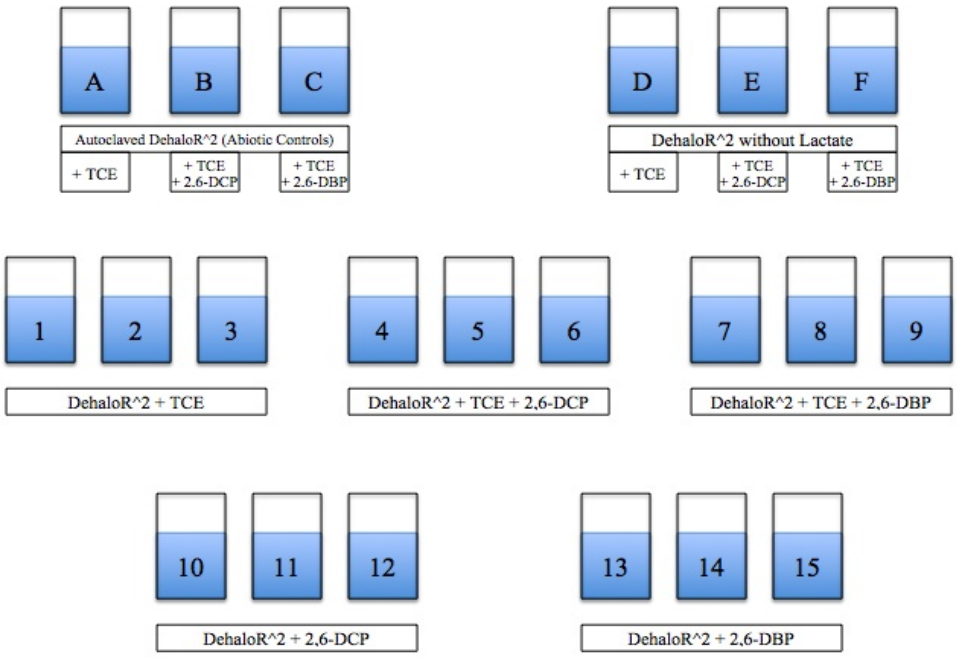


**Figure 4.2** Experimental set-up showing the conditions tested and the number of replicates.

### *4.2.3 Analytical Methods*

#### 4.2.3.1 Ultra High Performance Liquid Chromatography (UPLC)

An Acquity Waters Ultra High Performance Liquid Chromatograph (UPLC) equipped with an Acquity UPLC® BEH Shield $C_{18}$ column (50 mm x 2.1 µm x 1.7 µm, Milford, MA) and an Acquity Waters PDA detector (model number GOTWPD601M, Milford, MA) was used to measure 2,6-dibromophenol (2,6-DBP), 2-bromophenol (2-BP), 2,6-dichlorophenol (2,6-DCP), 2-chlorophenol (2-CP), and phenol.

Liquid samples (0.5 mL volume) were taken from the serum bottles and were passed through a 0.2-µm PVDF, HPLC certified membrane filters (Pall Corporation, Ann Arbor, MI). The samples were then diluted 1:2 with HPLC grade methanol (250 µL of the sample, 250 µL methanol) to ensure a final analyzable volume of 0.5 mL. From the liquid sample, 1-µL was injected into the column. The conditions employed for the UPLC method are summarized in Table 4.1.

**Table 4.1** Acquity UPLC method conditions for detections of halogenated, phenolic compounds.

<table>
<tr><th>Condition</th><th>Value</th><th>Unit</th></tr>
<tr><td>Mobile Phase A</td><td>Acetonitrile</td><td>--</td></tr>
<tr><td>Mobile Phase B</td><td>Water</td><td>--</td></tr>
<tr><td>Gradient</td><td>
<table>
<tr><td>Time</td><td>Flow Rate</td><td colspan="2">Profile</td></tr>
<tr><td>(Mins)</td><td>(mL/min)</td><td>%A</td><td>%B</td></tr>
<tr><td>Initial</td><td>0.5</td><td>50</td><td>50</td></tr>
<tr><td>0.8</td><td>0.350</td><td>50</td><td>50</td></tr>
<tr><td>2</td><td>0.250</td><td>100</td><td>0</td></tr>
</table>
</td><td>--</td></tr>
<tr><td>Temperature</td><td>30</td><td>°C</td></tr>
<tr><td>Detection</td><td>UV @ 272</td><td>nm</td></tr>
</table>

Calibration curves were determined by creating a serial dilution starting with 10 mM stock solutions of 2,6-DCP, 2-CP, phenol, 2,6-DBP, and 2-BP. These stock solutions were diluted to final concentrations of 50 µM, 25 µM, 5 µM, 2.5

μM, 0.5 μM, 0.25 μM, and 0.05 μM. The parameters for the calibration curves are presented in Table 4.2.

**Table 4.2** Calibration curve parameters for phenolic compounds.

| Constituent | $R^2$ | Slope | Intersect | Retention Time (minutes) |
|---|---|---|---|---|
| 2,6-DCP | 0.998 | 0.1761 | -0.497 | 0.69 |
| 2-CP | 0.999 | 0.1371 | 0.0745 | 0.5 |
| 2,6-DBP | 0.997 | 0.1506 | 0.8671 | 0.87 |
| 2-BP | 0.988 | 0.0167 | 0.0038 | 0.54 |
| Phenol | 0.999 | 0.2087 | -0.6955 | 0.44 |

4.2.3.2 DNA Extraction for *Dehalococcoides* and Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

The cultures were shaken vigorously and then 1.5 mL of liquid was removed from each serum bottle with a sterile syringe and dispensed into 2 mL microcentrifuge tubes. The microcentrifuge tubes were then centrifuged at 13,200 rpm for 15 minutes in order to make pellets. The supernatant was removed and the pellets were stored at -20°C until DNA extraction was performed. A Qiagen DNeasy Blood & Tissue Kit with modifications to enhance lysis of *Dehalococcoides* was used for DNA isolation from cultures. The full protocol is provided in Appendix E.

Quantitative real-time polymerase chain reaction (qRT-PCR) was performed to enumerate *Dehalococcoides* 16S rRNA genes using the following constituents: forward primer,, Dhc1200F (5'-CTG GAG CTA ATC CCC AAA GCT-3'), reverse primer, Dhc1271R (5'-CAA CTT CAT GCA GGC GGG-3'),

and probe, Dhc1240Pr (FAM-TCC TCA GTT CGG ATT GCA GGC TGAA/3-BHQ-1) [338].

qRT-PCR was carried out in a spectrofluorimetric thermocycler (Mastercylcer, epgradient S, eppendorf). Plasmids containing *Dehalococcoides* strain BAV1 16S rRNA genes were used as standards to construct a calibration curve. The calibration curve values were performed in triplicate and a linear range of 6 orders or magnitude was obtained. The slope of the calibration curve was -3.568 and the y intercept was 42.89. The thermocycler program was as follows: 2 minutes at 95°C followed by 40 cycles of 15 seconds at 95°C, 20 seconds at 58°C, and 20 seconds at 68°C.

## 4.3 RESULTS AND DISCUSSION

### *4.3.1. Debromination of 2,6-DBP to Phenol by DehaloR^2*

Figure 4.3 shows time course 2,6-DBP reductive debromination by DehaloR^2 culture in the presence of TCE, and Figure 4.4 shows the time course 2,6-DBP reductive debromination with 2,6-DBP by DehaloR^2 culture as the only halogenated electron acceptor. When DehaloR^2 was exposed to 2,6-DBP in the presence of TCE, we observed an increase in the expected debromination products 2-BP and phenol from time zero to day 7 (Figure 4.3). However, when 2,6-DBP was the only halogenated electron acceptor in the culture we observed an initial lag in debromination products appearance from time zero to day 7, despite an observed decrease in 2,6-DBP (Figure 4.4). Then, we observed an increase in 2-BP and phenol from day 7 to day 11 and day 7 to day 15 in the replicates with and without TCE, respectively. At day 15, the 2,6-DBP in the

triplicates with TCE was almost completely transformed to phenol at a concentration of 0.045 mM versus 0.015 mM phenol concentration at day 15 when 2,6-DBP was the sole brominated electron acceptor. At the final measuring point (day 55), we observed only phenol for and neither 2,6-DBP or 2-BP in both triplicate conditions (with and without TCE). Furthermore, we observed no significant loss of 2,6-DBP or formation of degradation products in the abiotic controls. This confirms that the observed activity is biological and not abiotic.

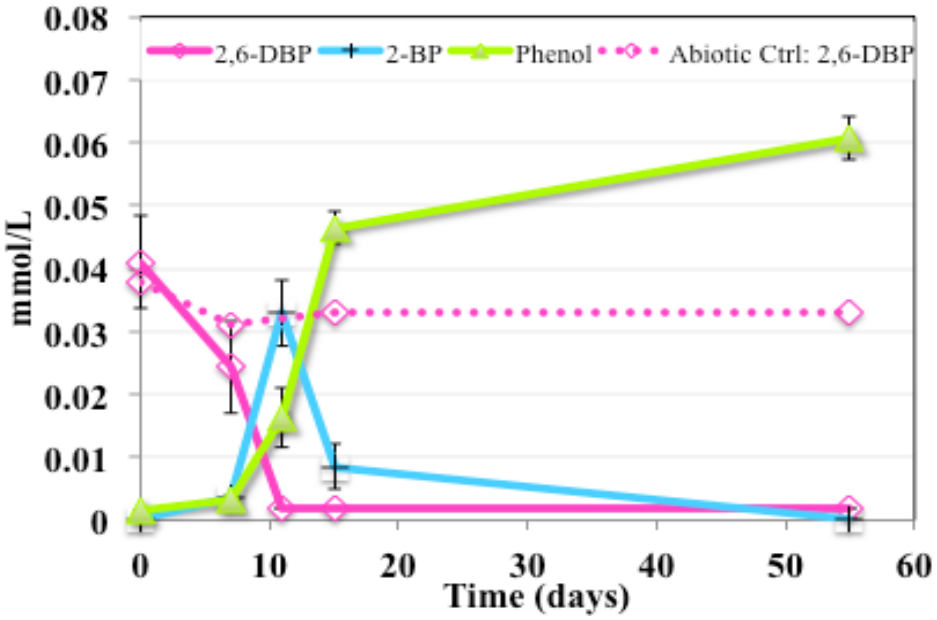


**Figure 4.3** 2,6-DBP debromination in the presence of 0.5 mM TCE. Error bars represent triplicates standard deviation. When error bars are not visible, the standard deviation is either very small or zero. The abiotic control does not contain error bars as only one abiotic control was set-up and analyzed

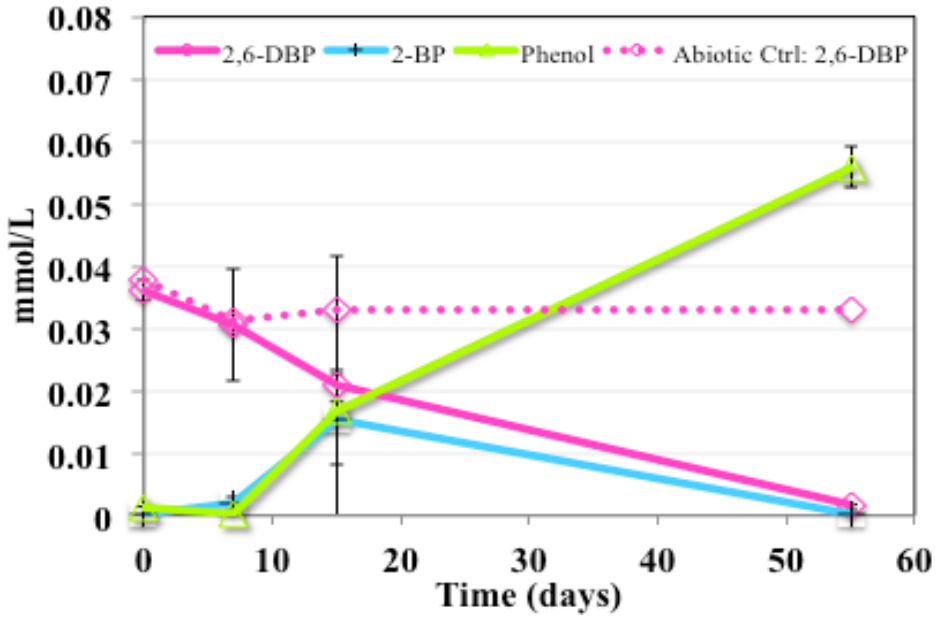


**Figure 4.4** 2,6-DBP debromination. Error bars represent standard deviation. When error bars are not visible, the standard deviation is either very small or zero. The abiotic control does not contain error bars as only one abiotic control was set-up and analyzed.

Ahn et. al., reported complete debromination of 2,6-DBP to phenol and 2-BP to phenol each within 30 days under methanogenic and sulfidogenic conditions by bacteria present in a marine sponge. The bacteria belonged to the phyla *Bacteroidetes*, *Chloroflexi*, and were similar to the species *Desulfovibrio* sp. strain TBP-1, an uncultured TCE-dechlorinating bacterium, and an uncultured DCE-dechlorinating enrichment [77]. Under denitrifying conditions, no debromination occurred, whereas debromination occurred under sulfidogenic and methanogenic conditions, and transformation rates were lower under sulfidogenic compared to methanogenic conditions. Our study took place under methanogenic conditions, and similarly to the cultures used by Ahn et al, DehaloR^2 [13] contains bacteria belonging to the *Bacteroidetes* and *Chloroflexi* phyla. However it is unknown which bacteria present in DehaloR^2 [13] performed the debromination process.

The debromination pathway reported by Ahn et. al. was different to the one observed in DehaloR^2, since the microorganisms present in their culture showed a preference for *ortho*-substituent compared to *para*-substituent removal as their cultures accumulated 4-BP and 4-BP was sequentially debrominated to phenol.

Our results suggest a synergistic relationship occurring when TCE and 2,6-DBP are both present as electron acceptors compared to when 2,6-DBP is the sole electron acceptor. When TCE was present, 2,6-DBP debromination to 2-BP and phenol occurred more rapidly (0.045 mM phenol was present at day 15 in the

TCE and 2,6-DBP triplicates versus 0.015 mM phenol present at day 15 in the 2,6-DBP triplicates).

*4.3.2 Dehalococcoides Growth and Yield Results*

Figure 4.5 shows the growth of *Dehalococcoides* during dechlorination or debromination for the cultures grown with TCE as an electron acceptor only and the culture grown with 2,6-DBP as an electron acceptor only, respectively. The growth was measured using qRT-PCR, hence the results are reported as *Dehalococcoides* (*Dhc*) 16S rRNA gene copies/L. The TCE only and the 2,6-DBP only conditions each showed an increase in the concentration of *Dehalococcoides* (*Dhc*) 16S rRNA gene copies, although the TCE only condition had a larger increase in gene copies (5.81E+11 compared to 1.31E+11 in the 2,6-DBP only serum bottle). This difference in final concentration of gene copies could be a result of differences in the concentrations of TCE and 2,6-DBP provided to the bacteria. The TCE only serum bottles received 0.5 mM of TCE whereas the 2,6-DBP only serum bottles received only 0.1 mM of 2,6-DBP.

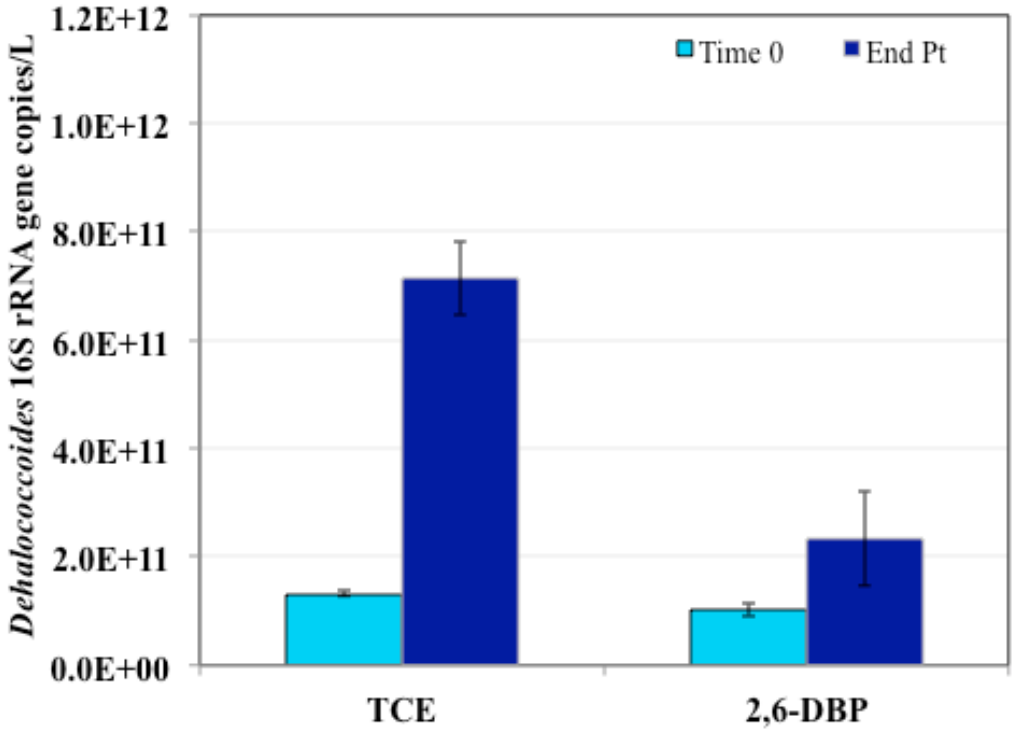


**Figure 4.5** Growth of *Dehalococcoides* during TCE and 2,6-DBP dehalogenation. Error bars represent the standard deviation of triplicate measurements for one DNA extraction. End point is 21 days and 55 days for TCE only and 2,6-DBP only, respectively.

The data presented shows that *Dehalococcoides* sp. grew on 2,6-DBP. A growth experiment starting with low culture densities (around $10^7$ gene copies/L) will provide further evidence that growth is actually occurring and will allow us to establish growth parameters for *Dehalococcoides* grown with 2,6-DBP.

Table 4.3 shows the calculated yield for *Dehalococcoides* grown on TCE only and for *Dehalococcoides* grown on 2,6-DBP only. The calculated yield was higher in the 2,6-DBP only bottles. This is not surprising as a much smaller number of μmoles (80 versus 1440) of halogen were initially available for the

bacteria in the cultures with 2,6-DBP as electron acceptor. These results are exciting because it appears that *Dehalococcoides* still grew on a smaller amount of a new electron acceptor.

**Table 4.3** Calculated yield for TCE and 2,6-DBP

| Condition | Δ *Dhc* 16S rRNA gene copies | µmoles of halogen released | Yield (*Dhc* 16S rRNA gene copies/ µmoles of halogen released) |
|---|---|---|---|
| **TCE Only** | 5.81E+11 | 1440 | 4.04E+08 |
| **2,6-DBP Only** | 1.31E+11 | 80 | 1.73E+09 |

*4.3.3 Dechlorination of 2,6-DCP to 2-CP by DehaloR^2*

Figures 4.6 and 4.7 show the results of 2,6-DCP reductive dechlorination with and without the presence of TCE, respectively by DehaloR^2 culture. We observed an initial lag in dechlorination products from time zero to day 7 (there was an unexplained phenol peak at day 7, which is most likely due to analytical errors). Then, an increase in 2-CP occurred from day 7 to day 15. At the final measuring point (day 55) we observed only 2-CP formation and a non-detect (ND) for either 2,6-DCP or phenol. Additionally, at the final measuring point there was twice as much 2-CP produced in the triplicates without TCE compared to the triplicates containing TCE (0.04 mM and 0.02 mM, respectively). We observed no activity in the abiotic 2.6 DCP controls, providing further evidence that the activity observed is biological.

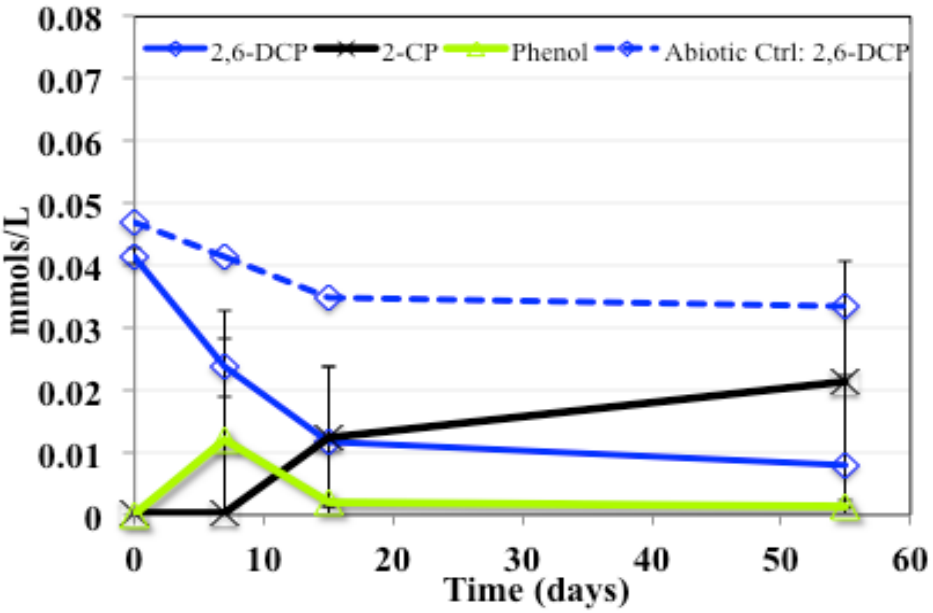


**Figure 4.6** 2,6-DCP dechlorination in the presence of 0.5 mM TCE. Error bars represent triplicates standard deviation. When error bars are not visible, the standard deviation is either very small or zero. The abiotic control does not contain error bars as only one abiotic control was set-up and analyzed.

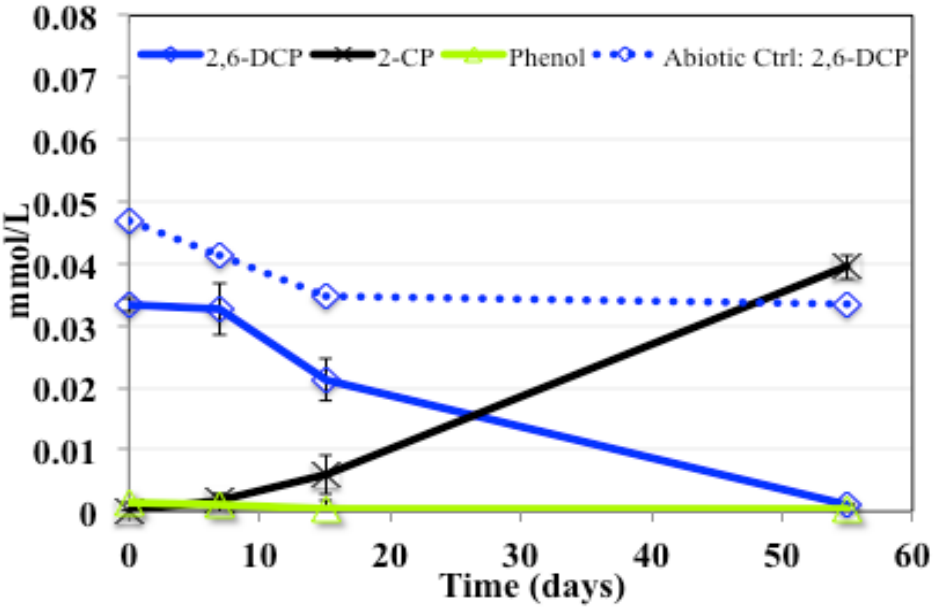


**Figure 4.7** 2,6-DCP dechlorination. Error bars represent standard deviation. When error bars are not visible, the standard deviation is either very small or zero. The abiotic control does not contain error bars as only one abiotic control was set-up and analyzed.

It has been documented that different groups of bacteria can reduce chlorinated phenols. For example, *Anaeromyxobacter dehalogenans* can dechlorinate 2-CP to phenol [67, 195, 196]. This bacterial strain is generally grown using fumarate as an electron donor [67, 196]. Acetate and lactate can also be utilized, but proved to be a poor electron donors [195]. He et al. also found that the dechlorination activity had to be induced and, after induction, 2,6-DCP could be dechlorinated to 2-CP and then phenol [67]. Additionally, *Desulfovibrio dechloracetivorans* SF3 can dechlorinate 2,6-DCP to phenol [66]. Adrian, et al., observed that *Dehalococcoides mccartyi strain* CBDB1 could dechlorinate 2,6-DCP to 2-CP, but at slower rates than other dichlorophenols such as 2,4-DCP and 2,3-DCP. *Dehalococcoides mccartyi* strain 195 did not dechlorinate 2,6-DCP but could dechlorinate other dichlorophenols, though only to 2-CP [37]. Adrian, et al., also observed that dechlorination was fastest for the two *Dehalococcoides* strains when the chlorines were situated at the *ortho* position, but only if the *meta* position was also chlorinated.

Our results are consistent with these previous studies. To our knowledge, DehaloR^2 does not contain *Anaeromyxobacter dehalogenans* or *Desulfovibrio dechloracetivorans* or close relatives to these bacteria, but, it does contain primarily *Dehalococcoides* bacteria which, thus far, have been shown to only partially dechlorinate 2,6-DCP to 2-CP.

Additionally, the results suggest that TCE presence has an inhibitory effect on 2,6-DCP dechlorination. When 2,6-DCP was present as the sole

chlorinated electron acceptor, dechlorination of 2,6-DCP to 2-CP, occurred at faster rates, as exemplified in Figure 4.7.

## 4.4 CONCLUSIONS

An anaerobic, TCE-dechlorinating culture, DehaloR^2, was exposed to two biogenic-like, halogenated phenolic compounds, in the presence and absence of TCE, to examine whether the TCE-dechlorinating culture could also dehalogenate biogenic-like, phenolic compounds. We observed complete debromination of 2,6-DBP to 2,6-BP and, finally, to phenol. We also observed dechlorination of 2,6-DCP to 2-CP. Additionally, we observed faster debromination of 2,6-DBP in the presence of TCE and slower dechlorination of 2,6-DCP in the presence of TCE. For both biogenic-like compounds, no significant degradation and no formation of degradation products occurred in our abiotic controls.

Further research is needed to confirm that *Dehalococcoides* does grow on 2,6-DBP. This can be done with a traditional growth experiment starting with very low culture densities. We should also explore: the effects of varying concentrations of these biogenic-like compounds to determine if a threshold concentration of 2,6-DBP exists, the use of different electron donors to determine if the results are independent of the type of electron donor used, and maximum utilization rates. Additional, qRT-PCR should be performed to analyze changes in the microbial community in the presence and absence of TCE and each biogenic-like compound and to examine whether the dehalogenations of 2,6-DBP and 2,6-DCP are dehalorespiring processes, and to verify a synergistic relationship in the

presence of TCE and 2,6-DBP co-contamination, which could help in further development of the DehaloR^2 culture for in-situ bioremediation.

# CHAPTER 5

# EFFECTS OF TWO BIOGENIC, HALOGENATED COMPOUNDS ON TCE-DECHLORINATION IN A PREVIOUSLY CHARACTERIZED, TRICHLOROETHENE-DEGRADING MIXED CULTURE

## 5.1 INTRODUCTION

In Chapter 4, I observed that an anaerobic culture, DehaloR^2, enriched in our laboratory and capable of reductive dechlorination of the priority pollutant trichloroethene (TCE), had the capacity to dehalogenate two biogenic-like compounds, 2,6-dibromophenol (2,6-DBP) and 2,6-dichlorophenol (2,6-DCP). 2,6-DBP was completely debrominated to phenol, with intermediate production of 2-bromophenol (2-BP). DehaloR^2 was also able to dechlorinate 2,6-dichlorophenol (2,6-DCP), however, dechlorination stopped at the intermediary product, 2-chlorophenol (2-CP). I also observed that TCE increased rates of 2,6-DBP debromination, and slowed down 2,6-DCP dechlorinatio. In this chapter, I explored the effects of, 2,6-dibromophenol (2,6-DBP) and 2,6-dichlorophenol (2,6-DCP) on DehaloR^2 ability to dechlorinate TCE.

## 5.2 MATERIALS AND METHODS

### *5.2.1 Chemicals, Analytical Methods, and Culture Establishment*

Chemicals and analytical methods are previously described in Chapter 4 and Chapter 3, respectively. Culture establishment is also previously described with two adjustments:

(1) Re-spike refers to re-injection of:

   a. 0.5 mL of 1 M lactate

   b. 50 μL of 1 M TCE in the TCE only triplicates and the TCE and a halogenated phenol sets of triplicates

   c. 100 μL of 100 mM 2,6-DBP in the TCE and 2,6-DBP triplicates

   d. 100 μL of 100 mM in the TCE and 2,6-DCP triplicates

(2) Two re-spikes were provided to the triplicate serum bottles at time 28 days and time 55 days. The days were chosen based on when all TCE-containing triplicates were done with TCE dechlorination.

## 5.3 RESULTS AND DISCUSSION

### *5.3.1 2,6-DBP Effect on TCE Dechlorination Ability*

Figures 5.1 and 5.2, show time course TCE dechlorination with and without the presence of 2,6-DBP, respectively, by DehaloR^2 culture initially grown in batch bottles. For both conditions, TCE was no longer detected around day 4.5. The TCE only triplicates showed complete transformation to ethene around day 21, whereas, the cultures with TCE and 2,6-DBP showed complete transformation to ethene around day 10.5.

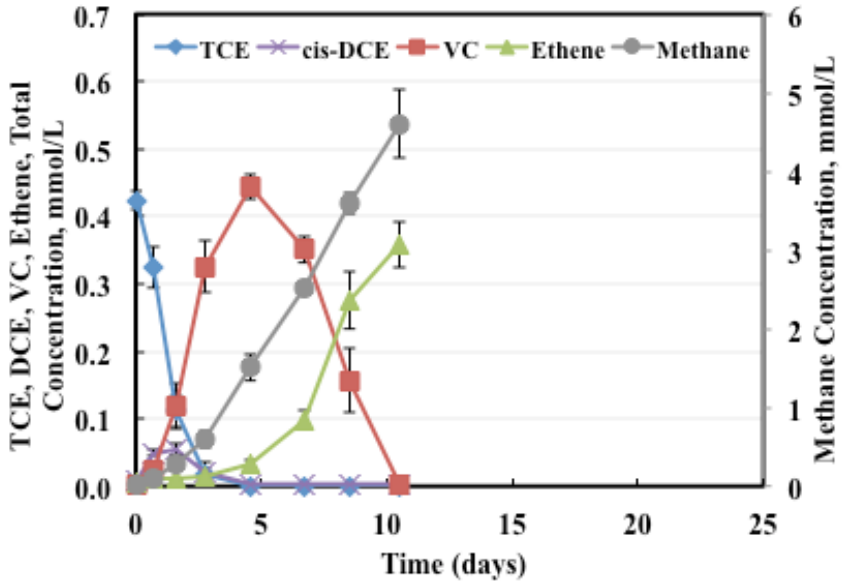


**Figure 5.1** Dechlorination of TCE to ethene in the presence of 0.04 mM 2,6 DBP. Error bars represent standard deviation. When error bars are not visible, the standard deviation is either very small or zero.

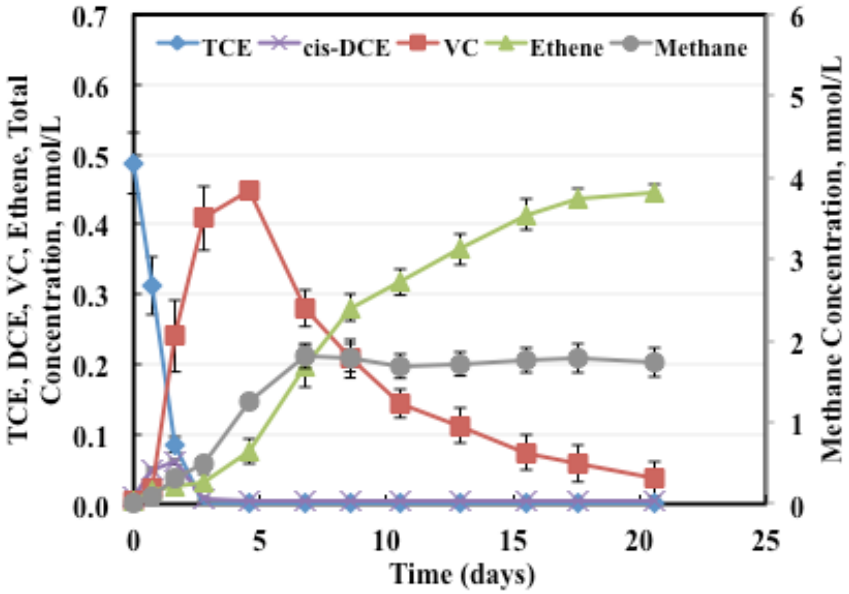


**Figure 5.2** Dechlorination of TCE to ethene. Error bars represent standard deviation. When error bars are not visible, the standard deviation is either very small or zero.

TCE and 2,6-DBP could occur as co-contaminants in a marine environment since both are naturally produced: TCE by marine algae [7] and 2,6-DBP by marine hemichordates [47, 48, 77]. However, after a thorough literature review, no studies examining the effects of TCE and 2,6-DBP co-contamination or the effects of 2,6-DBP on TCE dechlorinating ability were found. Therefore, these results cannot be compared to previous studies and represent the first of their kind.

The triplicates containing 2,6-DBP and TCE completely dechlorinated TCE to ethene by day 10.5 and the triplicates with only TCE completed the same process by day 21. This was the first time DehaloR^2 was intentionally exposed to 2,6-DBP. The increased rate of complete dechlorination from TCE to ethene in the TCE and 2,6-DBP bottles is suggestive of synergistic dehalogenation occurring in the DehaloR^2 when both compounds are present. The TCE dechlorination rates were nearly identical until around day 6.5. At this point, the triplicates with TCE and 2,6-DBP contained half as much ethene as the TCE only triplicates (0.1 mM and 0.2 mM, respectively). However, after 4 more days (10.55 days), the TCE and 2,6-DBP triplicates contained only ethene, whereas, the TCE only triplicates still contained VC. Additionally, around day 6.5 in the TCE triplicates we observed a plateau of methane until day 21 along with slower rates of ethene formation. This plateau is indicative of hydrogen limitation and could have contributed to the slower ethene formation in the positive controls. Hydrogen limitation was not an issue in the triplicates containing TCE and 2,6-DBP because these bottles received twice as much methanol (used as electron

donor) from the two stock solutions (TCE in methanol and 2,6-DBP in methanol) compared to the TCE only triplicates.

*5.3.2 2,6-DCP Effect on TCE Dechlorination Ability*

Figure 5.3 shows TCE dechlorination in the presence of 2,6-DCP, by DehaloR^2 culture. Figure 5.2 can be used as a comparison of TCE dechlorination without the presence of 2,6-DCP. For both conditions, TCE was no longer detected around day 4.5. The TCE only triplicates showed complete transformation to ethene by about day 21, whereas, in the cultures with TCE and 2,6-DCP at day 21 only about half of the TCE had been transformed to ethene and complete transformation to ethene had not occurred at day 55 (data after day 21 not shown in Figure 5.3).

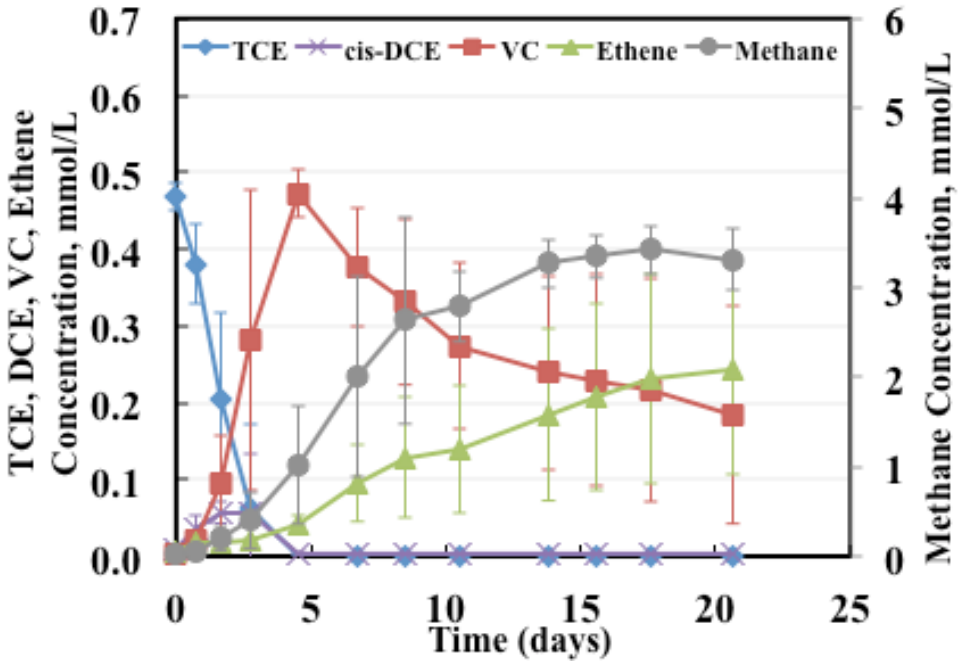


**Figure 5.3** Dechlorination of TCE in the presence of 2,6 DCP. Error bars represent standard deviation. The error bars are representative of varying biological activity among the TCE and 2,6-DCP triplicates. Data shown up to day 21 (when the TCE only triplicates finished dechlorination).

After a thorough literature review, no studies examining the effects of TCE and 2,6-DCP co-contamination or the effects of 2,6-DCP on TCE dechlorinating ability were found. Therefore, these results cannot be compared to previous studies and represent the first of their kind.

This was the first time DehaloR^2 was intentionally exposed to 2,6-DCP. The TCE dechlorination rates were nearly identical until around day 6.5 between the triplicates with TCE and the triplicates with TCE and 2,6-DCP. At day 6.5, the triplicates with 2,6-DCP had about half as much ethene as the TCE only bottles (0.09 mM and 0.20 mM, respectively), and more VC compared to the TCE only

bottles (0.38 mM and 0.28 mM, respectively). The slower VC dechlorination was unexpected due to the initial TCE dechlorination speed.

*5.3.3 Comparing Dehalococcoides Growth and Yield Results For All Three TCE Conditions*

Figure 5.4 shows the *Dehalococcoides* growth results for a TCE only serum bottle, a TCE and 2,6-DBP serum bottle, and a TCE and 2,6-DCP serum bottle at time 0 days and time 7 days. All three conditions showed an increase in *Dhc* 16S rRNA gene copies/L, however, the TCE and 2,6-DBP serum bottle showed the largest increase, and the TCE and 2,6-DCP serum bottle showed the smallest increase. This is well correlated to the dehalogenation rates discussed previously.

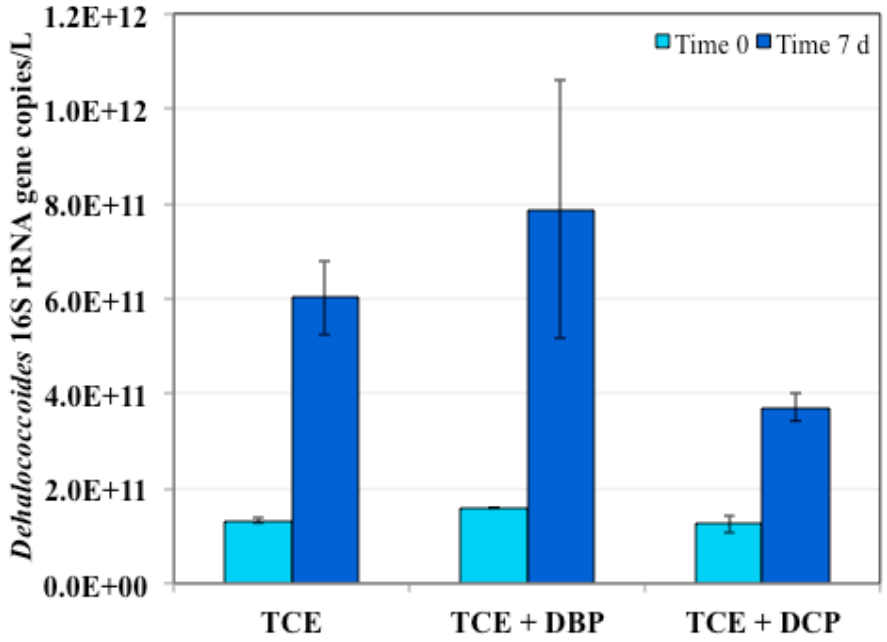


**Figure 5.4** Growth of *Dehalococcoides* during TCE only, TCE and 2,6-DBP, and TCE and 2,6-DCP dehalogenation. Error bars represent the standard deviation of triplicate measurements for one DNA extraction.

It appears that *Dehalococcoides* sp. replicated more in the presence of 2,6-DBP (without statistical significance), again supporting the theory of synergistic growth and less in the presence of 2,6-DCP supporting the theory of some inhibitory process. A growth experiment starting with low culture densities (around $10^7$ gene copies/L) will prove whether or not growth and inhibition are actually occurring.

Table 5.1 shows the calculated yield for TCE only, TCE and 2,6-DBP, and TCE and 2,6-DCP. The yield was again higher in the presence of 2,6-DBP. In the TCE and 2,6-DCP serum bottles, more initial μmoles of halogens are available as electron acceptor for the bacteria (1523 μmoles compared to 1440 μmoles in the TCE only serum bottle and 1392 μmoles in the TCE and DBP only serum bottle), however the yield is almost an order of magnitude lower than the TCE and 2,6-DBP serum bottle and nearly equivalent to the TCE only serum bottle.

**Table 5.1** Calculated yield for TCE only, TCE and 2,6-DBP, and TCE and 2,6-DCP conditions at time 7 days,

| **Condition** | **Δ *Dhc* 16S rRNA gene copies** | **μmoles of halogen released** | **Yield (*Dhc* 16S rRNA gene copies/ μmoles of halogen released)** |
|---|---|---|---|
| **TCE Only** | 4.70E+11 | 1210 | 3.88E+08 |
| **TCE + 2,6-DBP** | 6.28E+11 | 1020 | 1.77E+09 |
| **TCE + 2,6-DCP** | 2.46E+11 | 1040 | 2.37E+08 |

## 5.4 CONCLUSION

DehaloR^2 was exposed to halogenated phenolic compounds to examine the effects these biogenic-like compounds on TCE-dechlorination. The presence of 2,6-DBP resulted in faster TCE dechlorination and the presence of 2,6-DCP resulted in slower TCE dechlorination compared to TCE contamination alone.

Further research is needed to verify if *Dehalococcoides* does grow on 2,6-DBP via a growth experiment starting with very low culture densities, to verify if 2,6-DCP is inhibitory to TCE dechlorination, to examine if expression occurs in the dechlorinating genes (*tceA*, *tceB*, *vcrA*, *vcrB*, and *vcrC*), to explore the effects of varying concentrations of TCE with these biogenic-like compounds to verify if the concentration of each constituent affects TCE dechlorination, the use of different electron donors to determine if the results are dependent on the electron donor used, and to study maximum utilization rates.

# CHAPTER 6

# SUMMARY AND RECOMMENDATIONS

## 6.1 SUMMARY

In this study, a previously characterized, TCE-dechlorinating microbial consortium, DehaloR^2, was exposed to three anthropogenic, halogenated compounds (TCC, TCEP, and TCA), and two biogenic-like halogenated phenols (2,6-DBP and 2,6-DCP). DehaloR^2 did not reductively dehalogenate the three anthropogenic compounds used in this study.

DehaloR^2 was exposed to 2,6-DBP and 2,6-DCP as model naturally produced compounds (biogenic-like), each in the presence of absence of TCE to examine if the culture could dehalogenate the two biogenic-like compounds. DehaloR^2 debrominated 2,6-DBP to phenol in the presence and absence of TCE, but the debromination occurred in a shorter time frame in the presence of TCE. DehaloR^2 dechlorinated 2,6-DCP to 2-CP in the absence of TCE, however, dechlorination to 2-CP had not completed by day 55 in the presence of TCE. These findings suggest a possible synergistic effect occurring when TCE and 2,6-DBP are present together, and an inhibitory effect occurring when TCE and 2,6-DCP are present together.

Additionally, TCE dechlorination by DehaloR^2 was studied in the presence and absence of 2,6-DBP and 2,6-DCP. TCE dechlorination occurred in a shorter time frame in the presence of 2,6-DBP compared to the cultures with TCE only. TCE dechlorination was not completed by day 55 in the TCE and 2,6-DCP cultures compared to a dechlorination end point (to ethene) of 21 days in the

culture with TCE only. Again, these findings provide additional evidence of a synergistic effect occurring when TCE and 2,6-DBP are present together, and an inhibitory effect occurring when TCE and 2,6-DCP are present together.

Lastly, qRT-PCR was used to examine changes in *Dehalococcoides* concentrations using the 16S rRNA gene, and determine if growth was occurring when the halogenated phenols were present as possible electron acceptors. Preliminary results suggest that *Dehalococcoides* did grow when 2,6-DBP was available as the sole electron acceptor. Furthermore, *Dehalococcoides* grew but to a lesser extent when 2,6-DCP was present with TCE compared to TCE only.

## 6.2 RECOMMENDATIONS FOR FUTURE STUDIES

Based on the results from this study, I suggest the following topics should be further researched to better understand the dehalogenating abilities of *Dehalococcoides* and DehaloR^2.

(1) Perform a growth experiment, starting with a diluted culture to determine if *Dehalococcoides* growth is a product of 2,6-DBP debromination.

(2) Determine effects of 2,6-DBP and 2,6-DCP on additional microbial members and study shifts in microbial community.

(3) Investigate effects of various concentrations of 2,6-DBP on TCE dechlorination and vice versa. Also, examine effects of these concentrations on microbial community.

(4) Investigate dehalogenating ability of DehaloR^2 in regards to other brominated, and possibly iodinated and fluorinated, compounds.

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# APPENDIX A

# CONSTITUENTS FOR ANTHROPOGENIC EXPERIMENT

# (TCC, TCEP, AND TCA)

**DehaloR^2 culture + TCC**

| Constituent | Final Conc. | Stock Soln. | 100 mL Solution (160 mL serum bottles) |
|---|---|---|---|
| Anaerobic Media | | | 50 mL |
| DehaloR^2 Culture | | | 10 mL |
| ATCC Vitamins | | | 0.5 mL |
| cyanocobalamine (B12) | 5 mg/L | 10,000 mg/L | 25 µL |
| Lactate | 5 mM | 1 M | 0.25 mL |
| TCC | 10 µM | 10 mM | 50 µL |

**DehaloR^2 culture + TCEP**

| Constituent | Final Conc. | Stock Soln. | 100 mL Solution (160 mL serum bottles) |
|---|---|---|---|
| Anaerobic Media | | | 90 mL |
| DehaloR^2 Culture | | | 10 mL |
| ATCC Vitamins | | | 1 mL |
| cyanocobalamine (B12) | 5 mg/L | 10,000 mg/L | 50 µL |
| Lactate | 5 mM | 1 M | 0.5 mL |
| TCEP | 10 µM | 10 mM | 10 µL |

**DehaloR^2 culture + TCA**

| Constituent | Final Conc. | Stock Soln. | 100 mL Solution (160 mL serum bottles) |
|---|---|---|---|
| Anaerobic Media | | | 90 mL |
| DehaloR^2 Culture | | | 10 mL |
| ATCC Vitamins | | | 1 mL |
| cyanocobalamine (B12) | 5 mg/L | 10,000 mg/L | 50 µL |
| Lactate | 5 mM | 1 M | 0.5 mL |
| TCA | 0.5 mM | (neat) | 5 µL |

# APPENDIX B

# PROCEDURE FOR MAKING MEDIA

(1) Collect ice and fill metal coil cooler next to reflux cooler so that the ice completely covers the wire coil

(2) Clean a 2-L Bulb Flask, rinsing with DI water

(3) Fill the 2-L Bulb Flask with 2 L of DI water

(4) Add the following constituents:

(a) 20 mL of Salt Stock Solution—**Swirl to mix**

(b) 2 mL of Trace Element Solution A—**Swirl to mix**

(c) 2 mL of Trace Element Solution B—**Swirl to mix**

(d) 0.5 mL of 0.1% Resazurin stock solution—**Swirl to mix**

(5) Hook up to reflux cooler

(a) Turn reflux cooler on counterclockwise (gas bubble should begin at the bottom of the cooler and travel to the top)

(b) Turn on gas ($N_2$ or $CO_2/N_2$ mix)

(c) Ensue gas tubing is flushing surface of media with gas

(6) Turn on H gas and light a burner under the Bulb Flask (ensure there is a metal screen to protect the flask from the flame)

(7) Once the liquid has reached a rapid boil, allow boiling for **30 minutes**

(8) Collect additional ice

(9) After boiling, move the flask to half-full bucket of ice (***Using autoclave gloves)*** and cover with remaining bucket of ice. (Continue flushing with gas)

(10) Cool to room temperature

(11) Add the following compounds:

(a) **5.04 g** $NaHCO_3$ (Final Conc.: 30 mM)

(b) **0.096 g** L-cysteine (Final Conc.: 0.2 mM)

(c) **3 mL** of $Na_2S$ x $9H_2O$ stock solution (Final Conc.: 0.2 mM)

(12) Once solids have dissolved, measure on pH meter

(13) Adjust pH to between 7-7.5 (bottle with opposite gas as used during boiling)

(14) Follow the bottling protocol:

(a) Flush 2 bottles with gas for about **30 seconds – 1 minute**

(b) Add 50 mL of media to each bottle

(c) Add 40 mL of media to each bottle

(d) Close with stopper and aluminum crimp immediately following addition of 40 mL of media

(15) Autoclave bottles

*Stock Solutions*

- Salt Stock Solution

  Add the following to 1 L of DI water:

| Salt | Amount (g) |
|---|---|
| NaCl | 100 |
| $MgCl_2$ x $6H_2O$ | 50/5 |
| $KH_2PO_4$ | 20 |
| $NH_4Cl$ | 30 |
| KCl | 30 |
| $CaCl_2$ x $2H_2O$ | 1.5/0.5 |

- Trace Element Solution A

  Add the following to 1 L of DI water:

| Chemical | Amount |
|---|---|
| HCl (25% solution) | 10 mL |
| $FeCl_2 \times 4H_2O$ | 1.5 g |
| $CoCl_2 \times 6H_2O$ | 0.19 g |
| $MnCl_2 \times 4H_2O$ | 0.1 g |
| $ZnCl_2$ | 70 mg |
| $H_3BO_3$ | 6 mg |
| $Na_2MoO_3 \times 2H_2O$ | 36 mg |
| $NiCl_2 \times 6H_2O$ | 24 mg |
| $CuCl_2 \times 2H_2O$ | 2 mg |

- Trace Element Solution B

  Add the following to 1 L of DI water:

| Chemical | Amount |
|---|---|
| $Na_2SeO_3 \times 5H_2O$ | 6 mg |
| $Na_2WO_4 \times 2H_2O$ | 8 mg |
| NaOH | 0.5 g |

# APPENDIX C

## SOLID PHASE EXTRACTION (SPE) PROTOCOL

(1) Use Waters Oasis HLB 60 mg 3 cc cartridges

   (a) Label cartridge and insert into SPE with the label facing out and the effluent opening closed

(2) Adjust waste holder and place waste tubes into holder underneath the cartridge

(3) Condition using 3 mL of MeOH:Acetone/10 mM Acetic Acid—**stop at interface**

(4) Equilibrate using 3 mL MS-grade Water/10 mM Acetic Acid—**stop at interface**

(5) Load 2.4 mL MS-grade Water/10 mM Acetic Acid and add 200 μL of sample in cartridge—**stop at interface**

   (a) When pooling samples divide 200 μL by the number of samples (i.e. 2 samples = 100 μL of each sample)

   (b) 1/17/12: Add 20 μL of 50 ppb* $^{13}C$ TCC standard (*Concentration can change based on expected concentration of TCC in sample)

(6) Rinse cartridge using 3 mL MS-grade water/10 mM Acetic Acid—**do not need to stop at interface**

(7) Dry cartridge for ***1 hour***

   (a) Open cartridges one full turn

   (b) Pressurize SPE to 15" Hg

(8) Remove waste tubes and replace with labeled sample vials

(9) Elute sample using 3 mL MeOH:Acetone/10 mM Acetic Acid

(10) Blow down sample to dryness for ***1 hour***

(a) Place sample vials in Styrofoam holder

(b) Insert/remove needles based on number of samples and re-cap any unused ports

(c) Clean needles using Acetone wash

(d) Insert needles into sample vials near the opening of the vial

(e) Turn on gentle $N_2$ gas stream (automatically regulated, will not exceed 2 psi)

(11) Reconstitute sample in 1 mL Acetonitrile

(12) Vortex briefly and sonicate sample for ***30 minutes*** in the sonication bath using the purple floaters

(13) If necessary, filter sample using a 0.2 μm PTFE filter and PP syringe in a fresh vial

(14) Take 750 μL sample and add 750 μL MS-grade water in labeled LC/MS vial

Final Conc.: 200 μL sample→1 mL acetonitrile →½ dilution with water

→10 x dilution

*Analytes*

(1) Methanol:Acetone (1:1) / 10 mM Acetic Acid—Organic solvent used to clean and condition cartridge

- Equal parts LC/MS grade Methanol and Acetone
- 10 mM LC/MS grade Acetic Acid
- Example: 50 mL Methanol, 50 mL Acetone, 34 μL Acetic Acid

  Total Volume: 100 mL, Final Concentration of Acetic Acid: 10 mM

(2) MS-Grade Water / 10 mM Acetic Acid—Matrix used to equilibrate sample

- 1 L LC/MS grade Water (in storage cabinet)
- 343 μL Acetic Acid
- Total Volume: 1 L, Final Concentration of Acetic Acid: 10 mM

(3) MS-Grade Acetonitrile

# APPENDIX D

## CONSTITUENTS FOR BIOGENIC EXPERIMENT

**TCE Only**

| Constituent | Final Conc. | Stock Soln. | 100 mL Solution (160 mL serum bottles) |
|---|---|---|---|
| Anaerobic Media | | | 90 mL |
| DehaloR^2 Culture | | | 10 mL |
| ATCC Vitamins | | | 1 mL |
| cyanocobalamine (B12) | 5 mg/L | 10,000 mg/L | 50 µL |
| Lactate | 5 mM | 1 M | 0.5 mL |
| TCE | 0.5 mM | 1 M | 50 µL |

**TCE + 2,6-DBP**

| Constituent | Final Conc. | Stock Soln. | 100 mL Solution (160 mL serum bottles) |
|---|---|---|---|
| Anaerobic Media | | | 90 mL |
| DehaloR^2 Culture | | | 10 mL |
| ATCC Vitamins | | | 1 mL |
| cyanocobalamine (B12) | 5 mg/L | 10,000 mg/L | 50 µL |
| Lactate | 5 mM | 1 M | 0.5 mL |
| TCE | 0.5 mM | 1 M | 50 µL |
| 2,6-DBP | 0.1 MM | 100 mM | 100 µL |

**TCE + 2,6-DCP**

| Constituent | Final Conc. | Stock Soln. | 100 mL Solution (160 mL serum bottles) |
|---|---|---|---|
| Anaerobic Media | | | 90 mL |
| DehaloR^2 Culture | | | 10 mL |
| ATCC Vitamins | | | 1 mL |
| cyanocobalamine (B12) | 5 mg/L | 10,000 mg/L | 50 µL |
| Lactate | 5 mM | 1 M | 0.5 mL |
| TCE | 0.5 mM | 1 M | 50 µL |
| 2,6-DCP | 0.1 MM | 100 mM | 100 µL |

**2,6-DBP Only**

| Constituent | Final Conc. | Stock Soln. | 100 mL Solution (160 mL serum bottles) |
|---|---|---|---|
| Anaerobic Media | | | 90 mL |
| DehaloR^2 Culture | | | 10 mL |
| ATCC Vitamins | | | 1 mL |
| cyanocobalamine (B12) | 5 mg/L | 10,000 mg/L | 50 µL |
| Lactate | 5 mM | 1 M | 0.5 mL |
| 2,6-DBP | 0.1 mM | 100 mM | 100 µL |

**2,6-DCP Only**

| Constituent | Final Conc. | Stock Soln. | 100 mL Solution (160 mL serum bottles) |
|---|---|---|---|
| Anaerobic Media | | | 90 mL |
| DehaloR^2 Culture | | | 10 mL |
| ATCC Vitamins | | | 1 mL |
| cyanocobalamine (B12) | 5 mg/L | 10,000 mg/L | 50 µL |
| Lactate | 5 mM | 1 M | 0.5 mL |
| 2,6-DCP | 0.1 mM | 100 mM | 100 µL |

# APPENDIX E

## DNA EXTRACTION FOR *DEHALOCOCCOIDES*

(1) Set the temperature on two incubators or water baths (one at 56°C, one at 37°C)

(2) Make pellets with 10 mL of culture, freeze overnight

(3) Remove all supernatant from solution

(4) Add 180 µL Enzyme lysis buffer, mix by pipetting up and down

(5) Incubate at 37°C for **60 minutes**

Periodically check the incubations and flick tubes if necessary to keep cells in suspension

(a) Thermomyxer: 500 rpm every 3 minutes for 30 seconds

(6) Add SDS to 1.2% v/v and vortex briefly.

(7) Incubate at 56°C for **10 minutes** (The suspension will clarify)

→ ***Begin following Step 4 of the Qiagen-DNeasy Blood & Tissue kit pretreatment protocol for Gram positive bacteria)***

(8) Add the following and vortex briefly (the suspension will further clarify):

(a) 25 µL proteinase K

(b) 200 µL buffer AL (without ethanol)

(9) Incubate at 56°C for **30 minutes**

(10) Spin the lysate at 10,000 x g for **1 minute**

(11) Check for any intact cell material or debris and remove the supernatant to a separate tube

(12) Add 200 µL molecular grade ethanol (96-100%) and mix thoroughly by vortexing

(13) Spin down briefly (**1 minute** at 10,000 x g) to remove lysate from the lid of the microcentrifuge tube

(14) Carefully pipet (avoid bubbling) the entire lysate onto the DNeasy spin column placed in a 2 mL collection tube

(a) Centrifuge at 8,000 x g for **1 minute**

(b) Discard the flow-through and the centrifuge tube

(15) Place the spin column onto a clean collection tube

(a) Add 500 µL buffer AW1

(b) Centrifuge at 8,000 x g for **1 minute**

(c) Discard the flow-through and the centrifuge tube

(16) Place the spin column into a clean collection tube

(a) Add 500 µL AW2

(b) Centrifuge at 17,000 x g for **3 minutes** to dry the DNeasy membrane

(c) Discard the flow-through and the centrifuge tube

(17) Place the spin column into a clean microcentrifuge tube

(a) Add 100 µL buffer AE to the membrane

(b) Let stand for **1 minute**

(c) Centrifuge at 10,000 x g for **1 minute**

(18) Apply the eluent back onto the same spin column and centrifuge again at 10,000 g for **1 minute**

(19) Store DNA at -20°C

*DNA Extraction Solutions*

- Lysis Buffer (Add the following per 1 mL DI water)
    - 20 µL Tris-HCl (Final Conc.: 20 mM)
    - 0.6 mg EDTA (Final Conc.: 2 mM)
    - 0.2 mg Achromopeptidase
    - 20 mg lysozyme
- 10% SDS Solution (7 mL)
    - 0.7 g SDS
    - 7 mL DI water