OPTIMIZATION OF ANAEROBIC DEGRADATION OF 1,1,1-TRICHLORO-2,2-DI(4-CHLOROPHENYL)ETHANE (DDT), 1-CHLORO-4-[2,2-DICHLORO-1-(4-CHLOROPHENYL)EHTYL]BENZENE (DDD) AND 1,1-BIS-(4-CHLOROPHENYL)-2,2-DICHLOROETHENE (DDE) IN ORGANIC MUCK SOILS OF LAKE APOPKA

By

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To my parents

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LIST OF ABBREVIATIONS

ASE	Accelerated solvent extraction
ATSDR	Agency for Toxic Substances and Disease Registry
ATRA	A private company organizing a group of national experts, who analyzed and interpreted information gathered from the NSRA, Apopka
BLAST	Basic local alignment search tool
cDCE	Cis-1,2-dichloroethene
Ce	Equilibrium concentration (µg/ml)
CMC	Critical micelle concentration
Co	Initial concentration (µg/ml)
Cytob	Cytochrome b
DBP	Bis(4-chlorophenyl)methanone
DCA	Dichloroethane
DDA	2,2-bis(4-chlorophenyl)acetic acid
DCB	Dichlorobenzene
DCE	Dichloroethene
DDD	1-chloro-4-[2,2-dichloro-1-(4-chlorophenyl)ethyl]benzene
DDE	1,1-bis-(4-chlorophenyl)-2,2-dichloroethene
DDOH	2,2,2-trichloro-1,1-bis(4-chlorophenyl)ethanol
DDMS	1,1'-(2-chloroethane-1,1-diyl)bis(4-chlorobenzene
DDMU	1,1-di(p-chlorophenyl)-2-chloroethylene
DDNU	1-chloro-4-[1-(4-chlorophenyl)ethenyl]benzene
DDT	1,1,1-trichloro-2,2-di(4-chlorophenyl)ethane
DDx	Sum of DDT, DDD and DDE
DLG	Dehalococcoides-like groups

DOC	Dissolved organic carbon
ECD	Electron capture detector
Eh	Redox potential
EPA	Environmental protection agency
ETC	Electron transport chain
fc	Methanol fraction (mL)
FeRB	Iron (III) reducing bacteria
GC	Gas chromatograph
HOC	Hydrohobic organic compounds
IARC	International Agency for Research on Cancer
Kd	Soil-water distribution coefficient (mL/g)
Koc	Organic carbon distribution coefficient
Kow	Octanol-water distribution coefficients
LB	Luria Bertini
LSC	Liquid scintillation counter
m	Mass of soil (g)
NAPLs	Non aqueous phase liquids
NPL	National priority list
NSRA	North shore restoration area
NRB	Nitrate reducing bacteria
OCP	Organochlorine pesticides
PAHs	Polyaromatic hydrocarbons
PCB	Polychlorinated biphenyl
PCE	Tetrachloroethene
PCPA	(4-chlorophenyl) acetic acid

PMF	Proton motive force
Se	Adsorption to soils
SJRWMD	St. Johns river water management district
SRB	Sulfate reducing bacteria
TCB	Trichlorobenzene
TCE	Trichloroethene
TDTMABr	Tetradecyltrimethylammonium bromide
TEA	Terminal electron acceptor
TeCB	Tetrachlorobenzene
TCC	Tower chemical company
TOC	Total organic carbon
U.S.EPA	U.S. Environmental protection agency
UMBBD	University of Minnesota biocatalysis/biodegradation database
v	final volume (mL)
VC	Vinyl chloride

Abstract of Dissertation Presented to the Graduate School
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OPTIMIZATION OF ANAEROBIC DEGRADATION OF 1,1,1-TRICHLORO-2,2-DI(4-CHLOROPHENYL)ETHANE (DDT), 1-CHLORO-4-[2,2-DICHLORO-1-(4-CHLOROPHENYL)EHTYL]BENZENE (DDD) AND 1,1-BIS-(4-CHLOROPHENYL)-2,2-DICHLOROETHENE (DDE) IN ORGANIC MUCK SOILS OF LAKE APOPKA

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Lake Apopka was one of the largest lakes (12960 ha) in Florida before 1940s; however, during the mid- to late-1940s, approximately 7285 hectares of wetlands around the north shore of Lake Apopka were drained for muck farming. As a result of intense agriculture, substantial quantities of nutrients and pesticides were introduced to this area. In 1996, under the Lake Apopka Improvement and Management Act, a decision was made to buy out the farms of the northern shore of Lake Apopka, thereby creating the North Shore Restoration Area (NSRA). The plan included buying the properties and flooding the farms to restore the wetlands and the lake. This became a major attraction for migratory birds, which fed on fish from the lake. In the winter of 1999-2000, more than 600 birds associated with the lake died. The autopsy results demonstrated that high levels of organochlorine pesticides such as DDT (1,1,1-trichloro-2,2-di(4-chlorophenyl)ethane) ,DDD (1-chloro-4-[2,2-dichloro-1-(4-chlorophenyl)ethyl]benzene), DDE (1,1-bis-(4-chlorophenyl)-2,2-dichloroethene)

(collectively known as DDx), toxaphene and dieldrin were present in some birds (U.S. Fish and Wildlife Service report, 2004).

In this study, we employed different approaches to optimize anaerobic biodegradation of DDx by indigenous microorganisms. To promote selective growth of degrading consortia, soils in laboratory microcosms were amended with different carbon, energy and terminal electron acceptor sources, similar to those found in a natural system. The greatest amount of degradation (approximately 87%) was observed in lactate microcosms. Lactate may have been used directly by the degrading organisms, or may have been fermented to small organic acids such as formate, acetate and hydrogen, which may have been used as electron donors by the degraders. In an effort to scale up the conditions exhibiting the greatest degradation rates, mesocosms were established. During mesocosm incubations, a release phase was observed, in which DDx extractability and measured concentrations increased with a simultaneous increase in dissolved organic carbon (DOC). Increased DDx concentrations were likely mediated by soil organic matter.DOC is known to increase aqueous solubility, bioavailability and resulting in increased biodegradation of DDx. The release phase was followed by a degradation phase, in which the now available DDx were degraded by the lactate fermenting consortia.

One of the major limitations of biodegradation of hydrophobic compounds such as DDx in organic soils is bioavailability. Hydrophobic compounds are strongly adsorbed to organic matter, such that their availability to degrading consortia may be very limited. For this reason, various strategies such as the use of sodium ions and Tween-80 (surfactant listed in the U.S. EPA - January 2008 “List of inert ingredients for use in non

food use pesticide products") were applied to aged DDx contaminated Apopka soils to investigate their potential in increasing biodegradation rates.

In the case of Na^+ microcosms, a positive correlation between Na^+ concentration and degradation was observed. Low concentrations of Na^+ increased degradation of aged DDx, possibly due to dispersal of soil polymers, resulting in increased bioavailability. Another important observation from the Na^+ microcosms was that trace metals and minerals promoted degradation, as they may work as cofactors for degradative enzymes. As before, information gained from laboratory scale microcosms was scaled up to mesocosm levels. No significant effect of Na^+ on degradation was observed in mesocosms, however.

In case of Tween-80® preliminary sorption experiments demonstrate that Tween decreased DDx sorption to soils. But when microcosms were established, no effect of Tween on biodegradation was observed. There could be several reasons for this such as surfactants are known to have detrimental effects on bacteria, they can lyse a cell by destroying the cell wall. Anoxic incubations, Na^+ ions and Tween® may have potential to remediate the DDx contaminated soils but further research is needed before these approaches can be applied to field level.

CHAPTER 1
INTRODUCTION OF DDT, ITS ENVIRONMENTAL AND ECOLOGICAL HAZARDS AT THE SITE, LAKE APOPKA, FL

Defining DDT, Its History and Entry into the Environment

DDT (1,1,1-trichloro-2,2-di(4-chlorophenyl)ethane) is a synthetic organochlorine pesticide. Although its initial synthesis was in 1874 by a German student named Othmar Zeidler, the insecticidal properties of DDT were unraveled much later in 1939 (Smith, 1991; EPA report, 1975). It was first used in the Second World War to protect military forces against diseases, such as malaria and typhus that are transmitted by vectors such as mosquitoes, fleas, and body lice. It later became popularly used by civilians for control of insect mediated disease in crops and livestock, and for domestic use (EPA report, 1975). Manufacturing on a large scale began in 1943, and in 1950s it became the most commonly used insecticide following its price reduction from $1 per pound in 1945 to about 25 cents in 1950s (Dunlap, 1981). One of the major reasons for DDT's popularity was its persistent nature, which reduced the need for reapplication. Low costs, high efficacy, and prolonged persistence lead to making it the world's most widely used insecticide in the 1960s. It drastically improved agricultural production (Attaran and Maharaj, 2000) and use in the U.S. agriculture peaked to 27 million pounds during 1966 (Gianessi and Puffer, 1992).

By the early 1970s the adverse environmental effects of DDT and its metabolites DDD (1-chloro-4-[2,2-dichloro-1-(4-chlorophenyl)ethyl]benzene) and DDE (1,1-*bis*-(4-chlorophenyl)-2,2-dichloroethene) (collectively known as DDx) were becoming evident, which led to its ban in the U.S. in 1972 (WHO, 1979; Turusov, 2002; Ratcliffe, 1967), although emergency public health use was allowed (Meister and Sine 1999). Prior to its ban, a total of approximately 1,350,000,000 pounds of DDT were used (EPA report,

1975). Following the ban in the U.S. in 1972, it was still manufactured for export purposes. Technical grade DDT was composed of about fourteen different chemicals, of which only 65-80% was the active ingredient p,p'- DDT. The remaining portion included o,p'-DDT (15-20%), p,p'- DDD (up to 4%) and 1-(p-chlorophenyl)-2,2,2-trichloroethanol (up to 1.5%) (Worthing, 1979; Metcalf, 1995). The non- p,p'- DDT part was not removed because of potent pesticidal properties. Chemical structures of DDxs are presented in Table 1-1.

Even though it was banned in most of the developed countries in the early 1970s its presence is still ubiquitous. Logetivity in addition to the highly lipophilic nature made DDx to bioaccumulate in tissues of all life forms, starting at low concentrations in lower life forms and magnifying as it travels up the trophic levels (Dearth and Hites, 1991; Tanabe et al., 1983; Gray et al., 1992; Vrecl et al., 1996; Longnecker, 1997; Nataka et al., 2002; Turusov et al., 2002; Jaga and Dharmani, 2003; Kunisue et al., 2004). Later, it was declared to be a probable human carcinogen by the U.S.EPA (ATSDR, 2002), and was included as one of the 12 priority persistent organic pollutants by the Stockholm Convention, 2002. The environmental presence of such persistent organic pollutants (POPs) is of global concern today not only because of its deposition (Dimond and Owen, 1996) but also because of bioaccumulation (Kunisue, 2003).

Some developing countries still continue using DDT because of its low cost and potent effectivity against transmission of diseases such as malaria, leishmaniasis and typhus. The metabolites DDE and DDD are recalcitrant and toxic as well. Both DDD and DDE are highly persistent and have similar chemical and physical properties (ATSDR, 2002). DDD and DDE are formed under either reducing conditions by reductive

dechlorination (Wedemeyer, 1966; Zoro et al., 1974; Baxter, 1990) or under aerobic conditions, respectively (Pfaender and Alexander, 1972).

Importance for DDxs Removal - Health and Environmental Issues

DDx have been found at 305 of the 441 National Priority List (NPL) hazardous waste sites in the U.S. (HazDat, 2002). DDx are persistent and accumulate in the environment because their loss through chemical, physical or biological pathways is very slow. Longetivity is a result of physical parameters such as low aqueous solubility and high affinity for soil organic matter as reflected in their K_{oc} (organic carbon distribution coefficient) values (Tables 1-2 and 1-3). The K_{oc} values suggest that the compounds adsorb strongly to soil organic carbon.

Bioavailability is yet another issue. With time compounds with high K_{oc} become tightly sequestered into the soil organic matrix (Alexander, 1995; 1997). As early as 1975, an EPA report showed DDx's considerable presence throughout the food web, including phytoplankton, fishes, amphibians, aquatic mammals, invertebrates, terrestrial organisms, reptiles, piscivorous birds, and humans (EPA report, July 1975). The order of susceptibility is highest in amphibians, followed by mammals and birds (ATSDR, 2002). DDx causes egg shell thinning, such that it destroys eggs upon hatching (EPA, 1975; Heberer and Dunnbier, 1999; Newton, 1995), but high concentration also may be lethal to birds (Carson, 1962; Fry, 1995; Cooper, 1991). Egg shell thinning results in problems in hatching, leading to a severe decline in bird populations. Researchers include DDx in estrogenic chemicals which suggest that it can affect the sex of a developing embryo in birds (Fry, 1995), Florida panther (Facemire et al., 1995), aquatic mammals (Helle et al., 1976) and reptiles such as alligators (Guillette et al., 1996). DDT is also considered to be an endocrine disrupting chemical; such

chemicals may cause problems in reproduction and development in a wide range of species.

An example of an altered ecosystem is Lake Apopka, FL where a multitude of organochlorine pesticides (OCP) are present in high concentrations. Many studies strongly support that the OCPs present resulted in altered reproductive status and feminization of fish (Gallagher et al., 2001; Kristensen et al., 2007; Toft et al., 2003), turtles (Guillette et al., 1994), alligators (Guillette et al., 1994; 1996), and Florida panthers (Facemire et al., 1995). A 90% decline in the juvenile alligator population occurred from 1981 to 1986 and the primary reason was reproductive failure (Jennings et al., 1988). A decrease in egg viability (Woodward et al., 1993), alterations in sexual differentiation, and feminization of alligators at the lake (Gross et al., 1994; Guillette et al., 1994; Gross, 1997) were observed, which coincides with substantial amounts of OCPs present in alligator egg yolks (Heinz et al., 1991).

Octanol-water distribution coefficients (K_{ow}) of compounds help to predict the extent a contaminant would bioaccumulate in fatty tissues of life forms. The K_{ow} values for DDx (Tables 1-2 and 1-3) indicate that they are highly lipophilic. The lipophility combined with a very long half life (Table 1-4) allows them to bioaccumulate and, hence, biomagnify as they move up the trophic levels. A study conducted by Le Blanc (1995) shows that the DDT concentrations increased as the trophic levels increased.

DDT in humans is stored in all tissues, with the highest concentration in body fat (Smith, 1991). DDT is considered to be a probable human carcinogen according to the International Agency for Research on Cancer (IARC, 2006). Several studies associated DDx (especially DDT and DDE) with early pregnancy loss, fertility loss, pancreatic

cancer, neurodevelopmental deficits, diabetes, breast cancer, sarcoma, non-Hodgkin's lymphoma, and leukemia (Beard, 2006; Chen and Rogan, 2003; Cox et al., 2007; Eriksson and Talts, 2000; Garabrant et al., 1992; Ribas-Fito et al., 2006; Snedeker, 2001; Venners et al., 2005, Dich et al., 1997).

Reasons for DDxs Recalcitrance – Chemical and Physical Properties

Reasons for DDx recalcitrance include physicochemical properties such as hydrophobicity, low volatility, and the presence of chlorine substituents (Tables 1-2 and 1-3). Several factors contribute to its resistance to microbial degradation: (1) aqueous solubilities are very low (Augustijin et al., 1994), which makes them relatively less bioavailable to microbes; (2) DDx are xenobiotics and normally do not resemble any naturally occurring compound, and soil microorganisms may lack the enzyme systems to use them as carbon sources; however, enzyme systems with broader specificities are known to transform DDx (Bumpus and Aust, 1987; 1994); and (3) bioavailability decreases with time because DDxs sequester with the soil organic matrix in a process known as aging (Alexander, 1995; 1998).

DDx are extremely lipophilic as reflected in their octanol water partition coefficients (log K_{ow}) (Tables 1-2 and 1-3). According to Augustijin and collegues (Augustijin et al., 1994) DDT and its metabolites have low aqueous solubilities and high lipophilicities indicating that they probably have relatively limited bioavailability (Robertson and Alexander, 1998). The aromatic chlorines contribute to their recalcitrance. If one of the aromatic chlorines is substituted with a methyl group, it showed a significantly higher degradability (Kapoor et al., 1973). Furthermore, the higher the number of halogen substituents on organic compounds, the higher energy required by microbes to break the carbon-halogen bond (Dobbins, 1995).

Chlorine as a substituent on the aromatic ring makes it less succeptible to ring cleavage via oxidative attacks. Oxidative attack occurs through electrophilic substitution reaction using oxygen as the oxidant. Chlorine is an electronegative substituent that deactivates the aromaticity by altering electron density and resonance across the ring. Electron withdrawing groups such as chlorine make the ring less succeptible to attack by electrophiles by drawing more electron density toward itself. Furthermore the position of chlorine may cause steric hindrances for enzyme mediated attacks (Furukawa., 1986; Sylvestre and Sandossi, 1994).

Chlorinated aromatics are more susceptible to attack by anaerobic bacteria than by aerobes. Since electron acceptors are generally limited in anaerobic environments, the metabolic diversity of anoxic bacteria enables them to use a wide array of substrates as electron acceptors while conserving energy; use of a chlorinated compound as electron acceptor is termed dehalorespiration (Holliger et al., 1999; Smidt and de Vos, 2004). During reductive dehalogenation, the halogen (chlorine) is replaced by hydrogen, and successive reductions would remove more chlorines from aromatic compounds (McEldowney et al., 1993). The dehalogenated compound would then be more susceptible to oxidative attacks or anoxic ring cleavage pathways.

DDX molecules are subject to aging, a process through which the chemical diffuses deep into the organic matrix or becomes tightly bound (Alexander et al., 1995; 1998; Peterson et al., 1971; Robertson and Alexander, 1998). Studies by Boul and coworkers (Boul et al., 1994) suggest that aged DDx are not subject to significant extraction, volatilization, leaching or degradation.

Study Site

The study site for the research described in this dissertation is the North Shore Restoration Area (NSRA) of Lake Apopka, located in central Florida (Figure 1-1). The NSRA was used for muck farming as early as the 1940s. Lake Apopka was one of the largest lakes in Florida (12960 ha) before drainage (Woodward et al., 1993). Approximately 7285 hectares of marsh was drained for muck farming (Woodward et al., 1993Benton et al., 1991), which was continued until the 1980s. The principal source of pesticide in the NSRA was extensive agricultural practices (Huffstutler et al., 1965; Florida Dept. Environ. Reg., 1979).

The primary reasons for pesticide and nutrient loading in the lake included a combination of many events. Farmers would pump excess water into the lake during wet seasons and drain off farms from flooding every two years, a method intended to control nematodes. In addition, seepage into the lake would increase pesticide and nutrient concentrations in the lake. Another independent event occurred in 1980, when a chemical spill from the Tower Chemical Company (TCC) spilled large quantities of DDT and Dicofol into the south east side of the lake. A 90% decline in the juvenile alligator population followed the TCC spill from 1981 to 1986 (Jennings et al., 1988).

During the mid-1940s when agricultural practices increased by draining the marshes, an increase in effluent discharge from a citrus processing plant along the southeast shore and domestic sewer waste from the city of Winter Garden dumped into the lake contributed to the nutrient load (Figure 1-2). A hurricane in 1947 destroyed the aquatic macrophytes, decreasing the capacity of the recycling and buffering capacity of the entire ecosystem (Conrow et al., 1989). This excess load of nutrients from point and non-point sources lead to the appearance of the first algal bloom in the 1947.

Eutrophication contributed to a major fish kill in 1963 (Clugston, 1963; Huffstutler et al., 1965), following which other studies reported fish, alligator and turtle deaths (Shotts et al., 1972) because of bacterial infections which were favored by decreasing water levels. Jennings et al. (1988) reported about 90% decline in juvenile alligator population following the TCC spill in 1980. All the above mentioned events made Lake Apopka one of the most polluted lakes in Florida, and contaminants (pesticides and nutrients) lead the U.S. Environmental Protection Agency (U.S.EPA) to include Lake Apopka on the National Priority Site List (NPL). These events made clear that buying out the farms under the Lake Apopka Restoration Act of 1985 would decrease phosphorous or pesticide inputs to the lake (U.S. Fish and Wildlife Service report, 2004).

Under the Lake Apopka Improvement and Management Act, a decision was made to buy out the farms on NSRA and the St. Johns River Water Management District (SJRWMD) was appointed to implement the buyout. The plan included buying the properties and flooding the farms in order for the lake to regain its original size by destroying the levees. To determine the potential risk to wild life, an environmental survey was conducted (ATRA, 1997; 1998) and the outcome suggested that the pesticides were not present at high enough concentrations to harm the wild life and the aquatic fauna. However, higher concentrations of DDx at the site left the piscivorous birds at a higher concern (U.S. Fish and Wildlife Service report, 2004).

SJRWMD completed buying out most of the farms by 1998, and in an effort to restore the site, farmers were asked to flood their farms as they left (U.S. Fish and Wildlife Service report, 2004). This became a major attraction for migratory birds that fed on the fish from the lake, which had high body loads of pesticides. This contributed

to a large bird fatality event (more than 600 birds) at and surrounding the site during the late fall of 1998 and spring of 1999. Among the affected species, the most influenced were the American white pelicans, great blue herons, white storks, and great egrets (U.S. Fish and Wildlife Service report, 2004). The autopsy results showed that DDT, DDD, DDE, toxaphene and dieldrin were present at elevated levels in the tissues of many of the dead birds (U.S. Fish and Wildlife Service report, 2004).

Research Hypotheses

Reductive dehalogenation transforms halogenated compounds to more easily oxidizable and more biodegradable forms by various processes (Adriaens et al., 1994; Beurskens, 1995; Brkovskii, 1996; Ballerstedt, 1997; Albrecht, 1999). Organic compounds may serve as electron donor and halogenated organics may act as terminal electron acceptors; such processes can be stimulated at the field level, but require selective stimulation of desirable organisms. This can be accomplished by careful introduction of suitable electron donor and acceptor combinations (Suflita et al., 1988) along with enough nutrients to sustain growth of the degrading consortia. Our overarching hypothesis was that microbes capable of degrading DDx are already present at the site and careful selection of electron donor: acceptor combination would facilitate enrichment and activities of the degrading consortia.

Research objective one was to investigate impact of different electron donor and acceptor combinations on DDx degradation in microcosms. Objective two was to enrich degrading consortia using different sets of electron donor and acceptor combinations to achieve maximum degradation. Objectives three and four are targeted towards increasing biodegradation by increasing bioavailability. Bioavailability is an

important factor to determine the effectivity of the method, thus any condition that would decrease adsorption to soil would increase DDx bioavailability.

We hypothesized that Na^+ would increase DOC levels and disperse soils and thereby release the bound DDx, making the otherwise non available DDx more accessible based on similar studies done by Kantachote et al. (2001; 2004). Another approach towards increasing bioavailability was the use of the surfactant Tween 80®. Surfactant molecules tend to accumulate at interfaces, thereby decreasing the surface and interfacial tension, and consequently increasing apparent solubility of DDxs (Kile and Chiou, 1989; Edwards et al., 1991; Jafvert et al., 1994; Guha et al., 1998; Yeh and Pavlostathis, 2004). Surfactants act by making the otherwise protected or unavailable hydrophobic organic substances more bioavailable by increase mass transfer from nonaqueous phases and hence substantial degradation (Grimberg et al., 1996).

Specific objectives are:

- Objective 1: Investigate impact of different electron donor: acceptor combinations on DDxs degradation in microcosms.
- Objective 2: Enrichment of degrading species or consortia, using microcosms with different electron donor and acceptor combinations.
- Objective 3: Isolation and identification of degrading species or consortia.
- Objective 4: Investigate effect of Na^+ on DDx degradation in microcosms and mesocosms.
- Objective 5: Investigate effect of surfactant on DDx degradation in microcosms.

Dissertation Overview

Chapter 1 is an introduction chapter that outlines the background of the site and explains the need for research, also stating the specific objectives of this research.

Chapter 2 is a literature review focusing on various aspects of DDx degradation such as biodegradation pathways of DDT under aerobic and anoxic conditions, microbiology of the degraders using organohalides as sole carbon or energy source, cometabolic degradation and syntrophic associations. This chapter also reviews the microbiology, physiology, biochemistry, phylogeny and thermodynamics of dehalorespiring bacteria.

Chapters 3, 4, and 5 describe identification and optimization of the electron donor: acceptor combination giving the highest degradation. Chapter 3 describes initial microcosm experiments the donor: acceptor combination yielding the greatest degradation. Once the optimum target donor: acceptor combination was identified, experiments were scaled up to mesocosm levels as discussed in Chapter 4. In addition, transfers from microcosms provided initial inocula in an attempt to obtain a stable degrading consortium are discussed in Chapter 5.

Chapter 6 describes strategies to enhance bioavailability of DDxs in soils by additions of a range of Na^+ concentrations. In addition, the efficacies of different media were evaluated to investigate potential trace metal and vitamin requirements of the degrading consortia. The optimum conditions were scaled up to mesocosm levels and also described in this chapter.

Chapter 7 focuses on use of surfactants to increase bioavailability, where initial sorption studies evaluated the impact of Tween-80 on solubility of DDT. Following analysis of the impact of Tween-80 on aqueous concentrations of DDT, microcosms were made with Tween-80 to investigate its potential role in increasing biological degradation rates of DDT.

Chapter 8 is the conclusion chapter that states the findings from this research, and recommendations for future studies.

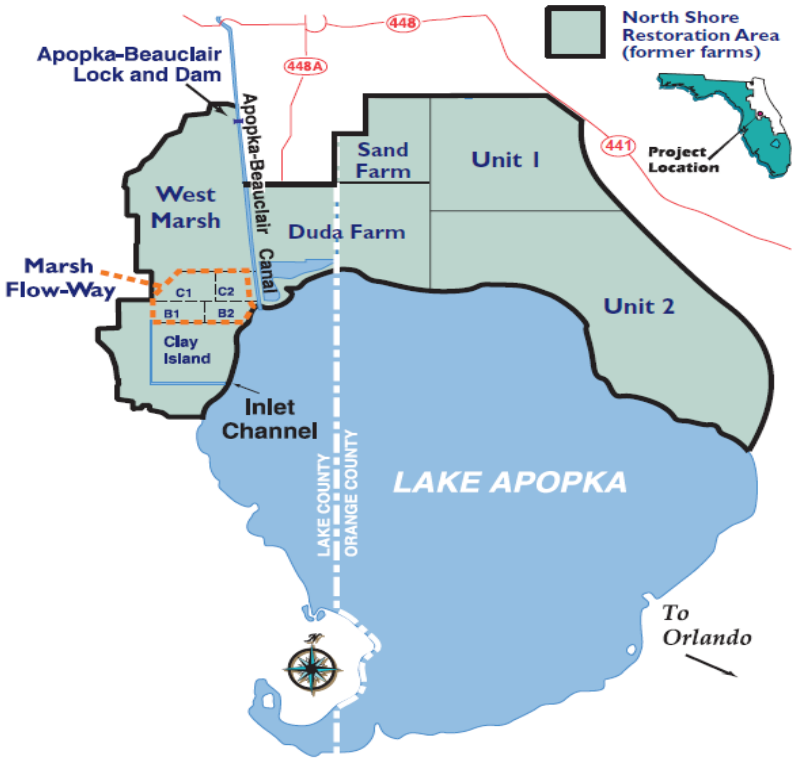


Figure 1-1. North Shore Restoration Area (NSRA) of Lake Apopka. (www.sjrwmd.com).

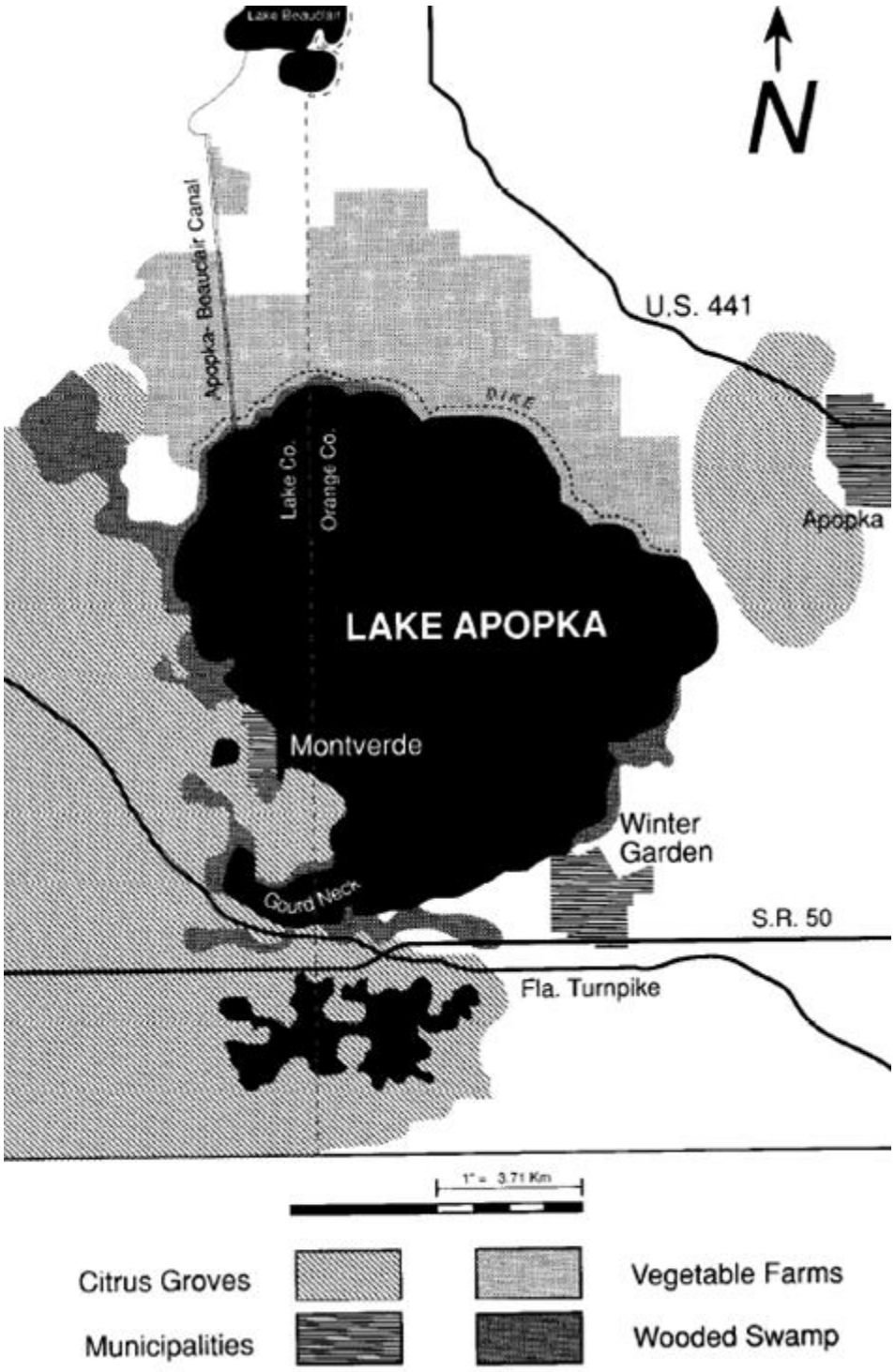


Figure 1-2. Agricultural practices and land uses adjacent to Lake Apopka in early 1980s (reprinted from Woodward et al., 1993).

Table 1-1. Chemical structure, IUPAC and common names for major DDT metabolites (adapted from ATSDR, 2002)

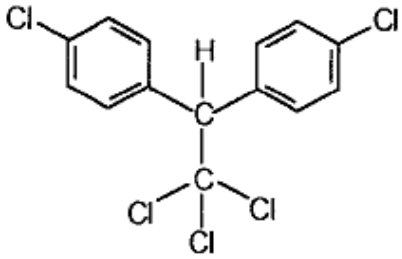
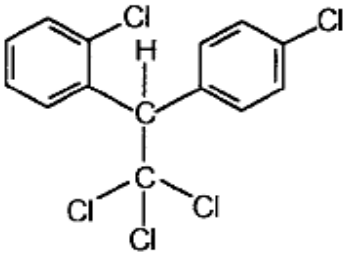
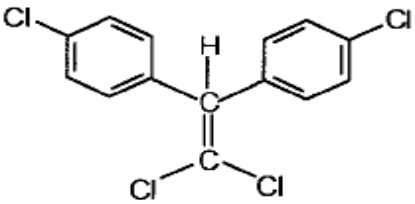
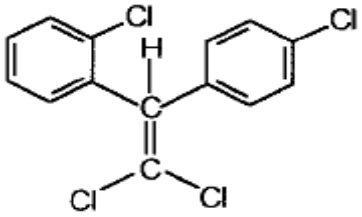
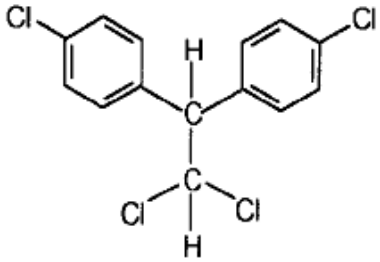
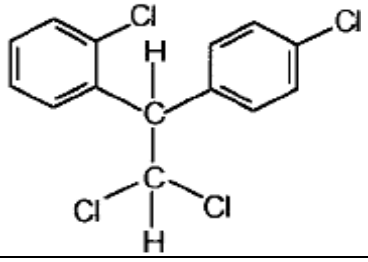
IUPAC name	Common name	Structure
1-chloro-4-[2,2,2-trichloro-1-(4-chlorophenyl)ethyl]benzene	p,p'-DDT	
1-chloro-2-[2,2,2-trichloro-1-(4-chlorophenyl)ethyl]benzene	o,p'-DDT	
1-chloro-4-[2,2-dichloro-1-(4-chlorophenyl)ethenyl]benzene	p,p'-DDE	
1-chloro-2-[2,2-dichloro-1-(4-chlorophenyl)ethenyl]benzene	o,p'-DDE	
1-chloro-4-[2,2-dichloro-1-(4-chlorophenyl)ethyl]benzene	p,p'-DDD	
1-chloro-2-[2,2-dichloro-1-(4-chlorophenyl)ethyl]benzene	o,p'-DDD	

Table 1-2. Physico chemical properties of the p,p′-DDxs (adapted from ATSDR 2002)

Property	p,p′-DDT	p,p′-DDD	p,p′-DDE
Solubility in water (mg/L at 25º C)	0.025 [a]	0.090 [a]	0.12 [a]
Log K_{ow}	6.91 [a]	6.02 [a]	6.51 [a]
Log K_{oc}	5.18 [b]	5.18 [c]	4.70 [c]

K_{ow} =octanol water partition coefficient; K_{OC} = organic carbon coefficients a = Howard and Meylan1997 b = Swann et al., 1981 c = Meylan et al., 1992.

Table 1-3. Physico chemical properties of the o,p′-DDxs (adapted from ATSDR 2002)

Property	o,p′-DDT	o,p′-DDD	o,p′-DDE
Solubility (mg/L at 25º C) in water	0.085 [a]	0.14 [a]	0.1 [a]
Log K_{ow}	6.79 [b]	5.87 [a]	6.00 [a]
Log K_{oc}	5.35 [c]	5.19 [c]	5.19 [c]

K_{ow} =octanol water partition coefficient; K_{OC} = organic carbon coefficients;a = Howard and Meylan1997 b = Swann et al., 1981 c = Meylan et al., 1992

Table 1-4. Half life estimates of DDx and DDT

Metabolite	Environment	Reported half life (years)	Reference
DDT	Maine Forest	20 to 30	Dimond and Owen 1996
DDT reduction to 70%	British Colombia, Canada	19	Aigner et al., 1998
DDT	temperate regions	2.29 to16	Lichtenstein and Schulz 1959; Racke et al., 1997; Stewart and Chisholm et al., 1971
DDT	temperate U.S. soils	5.3	Racke et al., 1997
DDx	Vietnam	6.7	Toan et al., 2009

CHAPTER 2
LITERATURE REVIEW

Foreward

DDT (1,1,1-trichloro-2,2-di(4-chlorophenyl)ethane) was originally synthesized in 1874, and its nonagricultural use began in 1939 (ATSDR, 2002). Paul Mueller was awarded the Nobel Prize in Medicine in 1948 for discovery of its insecticidal properties, and large scale manufacturing began during the Second World War to protect troops from insect-mediated diseases. Post war, it was released for use by civilians (EPA report, 1975), and by the 1950s it had become the highest selling insecticide in the U.S. (Smith et al., 1991).

By the late 1960s, bioaccumulation of DDT and its metabolites, DDD (1-chloro-4-[2,2-dichloro-1-(4-chlorophenyl)ethyl]benzene) and DDE (1,1-*bis*-(4-chlorophenyl)-2,2-dichloroethene) (collectively known as DDx), in wildlife became a concern (WHO, 1979; Turusov, 2002; Ratcliffe, 1967). This created concerns about human safety since measurable quantities were detected not only in wildlife such as birds, fish and mammals, but also in soil and water (EPA, 1975; Carson, 1962). In the early 1970s, DDT was partially banned in many developed countries, except for controlling emergency public health problems (WHO, 1979; Turusov, 2002; Ratcliffe, 1967). Even though it has been banned in the U.S. since the early 1970s, it is still nearly ubiquitous in the environment. Over 30 years after its ban, DDx were found at 305 of 441 hazardous waste sites (HazDat, 2002).

The occurrence of DDx in the environment continues to pose a threat, as they may bioaccumulate in fatty tissues of all life forms, resulting in higher concentrations in higher trophic levels (Le Blanc, 1995). DDx's recalcitrant nature, persistence, and

toxicity have been recognized as a serious environmental and ecological threat (Diamond and Owen, 1996; Kunisue, 2003). Ecological and environmental concerns prompted inclusion of DDx in the U.S.EPA's National Priority List (NPL) (U.S.EPA, 2000) as one of the most recalcitrant and environmentally significant pollutants (U.S.EPA, 2002; Stockholm convention, 2002). This chapter will focus on bioremediation, biochemistry, and microbiology of DDx degradation.

Strategies for DDxs Removal

Various physical, chemical, and biological methods have been employed to remediate DDx contaminated soils. Conventional abiotic methods such as excavation, solvent washing, soil inversion, incineration, use of surfactants, thermal desorption, microwave enhanced treatment, UV irradiation, and sulfuric acid treatment (Foght et al., 2001) have been used. Although such chemical and physical methods have been applied, they are usually intrusive, damaging the health of the soils, and are expensive and labor and energy intensive. Bioremediation is another approach that uses biological agents to remove or transform pollutants to a less or non harmful form. Although bioremediation may include a wide array of biological systems, we will restrict our focus to microbes.

The metabolic diversity of microorganisms empowers them to use extensive array of compounds for growth. We can exploit this capability to promote biologically mediated remediation. Such methods include the use of simple methods such as addition of various amendments to promote cometabolization and stimulation of the indigenous degrading consortia. Amendments ranging from simple carbon compounds such as glucose, yeast extract, peptone (Chacko et al., 1966), glucose, diphenylmethane (Pfaender and Alexander, 1972), octane, hexadecane or glycerol

(Gololeva and Skryabin, 1981) and cellulose (Castro and Yoshida, 1974), to complex amendments such as alfalfa (Guenzi and Beard, 1968; Burge, 1971), green manure (Mitra and Raghu, 1986), rice straw and cellulose (Castro and Yoshida, 1988), have shown to increase the bioremediation potential for DDx. Since soil microbes have been previously shown to cometabolize DDx (Francis et al., 1978; Rao and Alexander, 1985; Focht and Alexander, 1971; Bumpus and Aust, 1987), an alternative carbon source may be important to determine the biodegradation potential.

Another approach to bioremediation is bioaugmentation. This method requires large numbers of an active population to create a measurable change, and may require multiple inoculations (Morgan and Watkinson, 1989). Methods have been developed to improvise and ensure survival of the active degraders, which include encapsulating bacteria (Chen and Mulchandani, 1998) or fungi (Lestan et al., 1996). Another concern as suggested by Linqvist and Enfield (1992) is adsorption of DDx to the introduced microbes which could increase mobility of the contaminants. One more concern is that the active population must be in sufficient amounts to survive the environmental conditions and compete with the indigenous soil population. Research is also required to select potential degraders, such that this area requires extensive research before application.

Analog induction is another approach to remediation in which either non-toxic chemicals or natural amendments with structural similarity to the contaminant are added to the system to increase biodegradation potential. The idea is that growth in the presence of structurally similar chemicals could induce production of DDx degrading enzymes. Pfaender and Alexander (1972) observed an increase in DDT metabolism

with use of diphenylmethane. Another group (Beunink and Rehm, 1988) employed the same analog to enrich DDx metabolizing microorganisms in sewage sludge. Biphenyl was used to induce production of DDx degrading enzymes by Hay and Focht (1998) and Aislabie and coworkers (1999); whereas, another group (Nadeau et al., 1994) used chlorobiphenyl. A major concern with using analogs is the potential toxicity of the analog. Using natural analog is an alternative to chemical analog induction. Natural substances with complex polyaromatic structures such as terpenes are found in orange peels and pine needles, and have been used for inducing biphenyl degradation (Hernandez et al., 1997).

The great versatility of microbial metabolism makes bioremediation a simpler, more environmentally friendly, and more cost effective for clean up of environmental pollution (Jacques et al., 2008) compared with conventional physico-chemical methods. However, before considering bioremediation as a potential option for remediation of DDxs, it is important to understand the transformation pathways of DDx.

Reasons for Recalcitrance of DDxs

One of the major reasons for recalcitrance of DDT is attributed to the presence of chlorine substitutions, since its nonhalogenated analog diphenylmethane is readily degradable (Focht and Alexander, 1971; Subba-Rao and Alexander, 1985; Hay and Focht, 1988; Juhasz and Naidu, 2000). Halogenated aromatic compounds such as DDx are generally resistant to biodegradation because the halogen atom is both larger than the carbon and hydrogen atoms on the DDx molecule and more electron withdrawing. Chlorine is an electronegative substituent, and when present on an aromatic ring lowers the aromaticity by shifting electron density towards itself and hence alters resonance across the ring. As a result, chlorine makes the aromatic ring less succeptible to

oxidative attack. Furthermore, because of the electronegativity and the size of chlorine atom, it can have detrimental electronic effect and steric hindrances for enzyme mediated degradation reactions (Crooks and Copley, 1993; Furukawa, 1986; Sylvestre and Sandossi, 1994). However, the metabolic diversity of soil microorganisms enables them to overcome many of the obstacles and degrade haloaromatics such as DDxs.

Bioavailability

The term “bioavailability” refers to the available or accessible fraction of substrate to biological processes (Foght et al., 2001; Juhasz et al., 2000). Although bioremediation is a promising approach, accessibility of the contaminant to microbes may be a major limiting factor contributing to recalcitrance of most hydrophobic organic compounds (HOCs) such as DDxs (Hunt and Sitar, 1988). Bioavailability may limit remediation by physically sequestering and hence protecting the pollutant from microbial attack (Alexander, 1995; 1997). After entry into soils, HOCs may rapidly combine with the organic matter and a combined effect of diffusion, sequestration to inaccessible sites may lead to tightly binding to and within soil particles (Weissenfels et al., 1992).

With time, the sorbed residues may become resistant to chemical extraction such that they are considered to be tightly bound (Alexander, 2000). Bioavailability of a contaminant depends on various factors, such as physical and chemical properties of soils, chemical nature of the contaminant, duration of contact, and microbes present (Juhasz et al., 1999). Soils with high organic matter contents have higher adsorbed DDxs as compared to sandy soils (Peterson et al., 1971; Castro and Yoshida, 1974; Khan, 1980; Vollner and Klotz, 1994).

Microbial accessibility to a contaminant may be increased by physical dispersion of soils and employing various techniques such as use of surface active agents, cosolvents, surfactants, and monovalent cations have been successfully employed (Keller and Rickabaugh, 1992; Sayles et al., 1997; You et al., 1995; Kantachote et al., 2001; 2004; Juhasz et al., 1999. Approaches such as addition of surfactants have been employed to increase desorption of DDxs from soil particles, thereby increasing bioavailability (Keller and Rickabaugh, 1992). Studies by Parfitt and coworkers (1995) demonstrated that treating DDx contaminated aged soils (30 to 40 years) with Triton X-100® and polypropylene glycolethoxylate not only solubilized but also desorbed DDx. However, biodegradation of the desorbed DDx residues was not studied. A relatively new approach in this area is use of biosurfactants. According to Sayles and coworkers (1997), addition of reducing agents such as zero valent iron may be used as an adjunct to biodegradation to increase conversion of DDT to DDD. Reducing agents such as cysteine or sodium sulfide when amended to anoxic microcosms increased DDT degradation (You et al., 1995).

Aerobic Dehalogenation and Ring Cleavage

Aerobic microorganisms use oxidative catabolic reactions to break down halogenated aromatic compounds (Nadeau et al., 1994; Aislabie et al., 1999; Hay and Focht, 1998), whereas anaerobic microbes use reductive dehalogenation (Quensen et al., 1990; Suflita et al., 1982). Oxidative attack is usually a two step process, although the intermediates may vary depending on the parent compounds, the general strategy for oxidative attack remains similar. The first step typically involves activation of the aromatic ring and the second step is ring fission (Dagley, 1971; Nozaki, 1974).

In the case of oxidative ring cleavage, the initial aim is to remove substituents from the aromatic ring and to introduce hydroxyl groups, following which oxidative cleavage of the aromatic ring occurs. Molecular oxygen serves as a reactant and mono- or dioxygenases introduce either one or both atom/s from molecular oxygen into the substrate. Such enzymatic oxidation reactions would introduce oxygen in the ring of aromatic substrates to form dihydrodiols. Further oxidation of dihydrodiols leads to formation of catechols, which can serve as substrates for other dioxygenases to form ring cleavage products (Juhasz and Naidu, 2000) by either entering the gentisate pathway or ortho or meta cleavage degradation pathways (Dagely, 1977; Harayama and Timmis, 1989; Schink et al., 1992, Fuchs et al., 1994). For example, Nadeau and group (1994) demonstrated aerobic degradation of DDT through a meta cleavage pathway.

Anaerobic Dehalogenation and Ring Cleavage

Anaerobic systems cannot rely on oxygenases, such that anoxic degradation frequently involves reductive dechlorination, which replaces a chlorine atom with a hydrogen atom (Quensen et al., 1990; Suflita et al., 1982). Successive reductive dechlorination reactions decrease the number of chlorines on the aromatic molecule, decreasing resonance and hence making it easier to degrade. The primary reaction for most aromatic ring cleavage under anaerobic conditions is reduction of the ring into different central metabolic pathway intermediates, e.g. CoA thioesters (e.g. Benzoyl CoA). This leads to ring saturation and loss of resonance, followed by ring cleavage, which is eventually incorporated into the tricarboxylic acid cycle (Heider and Fuchs, 1997).

Proposed DDx Degradation Pathways

The initial attack on DDT is on the trichloroalkyl backbone, after which the molecule is often converted to DDD under anoxic, and DDE under aerobic, conditions. Although DDT degradation has occurred at sites contaminated with DDT, the subsequent metabolites (DDD and DDE) accumulate and are limited to further degradation (Aislabie et al., 1997). The rate and concentration of transformation product depends on the soil conditions, water content, and the microbes present in the soil (Aislabie et al., 1997). Under anaerobic conditions, DDT is reduced to form DDD by reductive dechlorination, either microbially (Wedemeyer, 1966) or chemically (Zoro et al., 1974; Baxter, 1990). However, under aerobic conditions, DDT undergoes dehydrodechlorination to form DDE (Pfaender and Alexander, 1972). It is very difficult to account for all DDT transformation products in complex environmental systems such as soils or waste water sludge (Guenzi and Beard, 1967; Burge, 1971; Jensen et al., 1972). The major microbial biotransformation products of DDT are presented in Table 2-1.

It was believed that, although anoxic conditions contribute to successive reductive dechlorination of the aliphatic fragment of DDT, the presence of oxygen is required for ring cleavage and mineralization (Pfander and Alexander, 1972; Golovleva and Skryabin, 1981). However, anaerobic ring cleavage of several aromatic compounds was reviewed by Heider and Fuchs in 1997. Another concern was that anoxic conditions are better for dehalogenation of polyaromatic hydrocarbons (PAHs); however, Nadeau and colleagues (1997) demonstrated aerobic degradation of DDT. We believe that anaerobic conditions would not only lead to reductive dechlorination of the halogens on

the aliphatic backbone of DDT, but also ring cleavage; hence, we will focus our efforts on anaerobic degradation of DDT.

Anaerobic Degradation Pathway

DDT degradation under anaerobic conditions is thought to follow a pathway proposed by Wedemeyer (1967), shown in the Figure 2-1 from University of Minnesota Biocatalysis/Biodegradation Database (UMBBD) website, (http://umbbd.msi.umn.edu/ddt2/ddt2_image_map.html). Anaerobic degradation of DDT is proposed to involve reductive dechlorination. The initial step in the anaerobic biodegradation pathway involves reductive dehalogenation of DDT to form DDD (Johnsen, 1976; Essac and Matsumura, 1980; Lal and Saxena, 1982; Kuhn and Sulflita, 1989; Rochkind-Dubinsky et al., 1987).

One of the first reports demonstrating reduction of DDT to DDD was a pure culture study with *Proteus vulgaris* isolated from a rodent intestine (Barker et al., 1965). Other pure culture studies also demonstrated DDT reduction under anoxic conditions (Table 2-1). Under anaerobic conditions, DDT may be reduced to DDD and successive reductions from DDD to DDMU (1,1-di(p-chlorophenyl)-2-chloroethylene), DDMS 1,1'-(2-chloroethane-1,1-diyl)bis(4-chlorobenzene), DDNU 1-chloro-4-[1-(4-chlorophenyl)ethenyl]benzene, DDOH 2,2,2-trichloro-1,1-bis(4-chlorophenyl)ethanol), DDA 2,2-bis(4-chlorophenyl)acetic acid and DBP bis(4-chlorophenyl)methanone by pure cultures of *Escherichia coli* and *Enterobacter aerogenes* (Langlois et al., 1970; Wedemeyer et al., 1967; Pfaender and Alexander, 1972). DDT undergoes successive reductive dechlorinations, forming DDD, DDMU, DDMS and DDNU. DDNU is oxidized to form DDOH, which is further oxidized to DDA. DDA undergoes decarboxylation, producing DDM, which may go to ring cleavage of one of the rings under aerobic

conditions to form PCPA (4-chlorophenyl) acetic acid (Pfaender and Alexander, 1972). DBP accumulates under anaerobic conditions (Pfaender and Alexander, 1972).

It was thought that the major bacterial dechlorination was efficient under reducing conditions; whereas, oxidative attacks were required for ring cleavage (Pfaender and Alexander, 19723, Golovela and Skryabin, 1981). Pfaender and Alexander (1972) demonstrated that cell-free extracts of *Hydrogenomonas* sp. converted DDT to DDD, DDMS, DBP and other products. However, no ring cleavage products were formed under anoxic conditions. Incorporating oxygen and fresh cells of *Hydrogenomonas* resulted in further metabolism of DDT to PCPA, implying that the enzyme system of a single organism could break down DDT to ring fission products. PCPA could, however, be further metabolized by *Arthrobacter* species. No PCPA was formed under anaerobic conditions, which suggested that aerobic conditions are required for ring cleavage (Pfaender and Alexander, 1972).

Proposed Aerobic DDT Degradation Pathway

The aerobic degradation pathway of DDT is presented in Figure 2-2. Under aerobic conditions, DDT undergoes dehydrochlorination, which involves removal of HCl and formation of a double bond between the carbon atoms on the alkyl chain of DDT. The first report of degradation of DDT under aerobic conditions was presented by Nadeau (Nadeau et al., 1994). The same group obtained the isolates by analog enrichment. Diphenylmethane was used as a primer to enrich and isolate responsible organisms. The first step in this pathway by *Ralstonia eutropha* A5 (earlier referred to as *Alcaligenes eutrophus*) is oxidation of DDT at the ortho and meta positions by dioxygenase to form DDT-dihydrodiol. DDT-dihydrodiol is a transient metabolite which undergoes dehydration by dehydrogenase to form 2,3-dihydroxy-DDT. 2,3-dihydroxy-

DDT undergoes meta cleavage, forming a yellow colored metabolite, finally leading to formation of 4-chlorobenzoic acid. Similarly, using 4-chlorobiphenyl for analog enrichment, other organisms capable of cometabolizing DDT were isolated (Masse et al., 1989; Nadeau et al., 1994; Parsons et al., 1995). *R. eutropha* A5 also metabolizes DDD via meta fission (Hay and Focht, 2000) and other aerobic bacteria namely *Terrabacter sp.* DDE1 (Aislabie et al., 1999) and *Pseudomonas acidovorans M3GY* (Hay and Focht, 1998) metabolizes DDE via meta fission.

Dehalorespiration

As mentioned previously, anaerobic reductive dehalogenation replaces one halogen atom with a hydrogen atom. Organisms that undertake such reactions gain energy in the process, termed dehalorespiration (Dolfing and Harrison, 1992; Mackiewicz and Wiegel, 1998; Suflita et al., 1982). Shelton and Tiedje (1984) enriched a methanogenic consortium that they claimed was growing on 3-chlorobenzoate as sole energy and carbon source. Later efforts to isolate the culture solely on 3-chlorobenzoate failed. These observations suggested that the consortium could be gaining energy from the substrate (Shelton and Tiedje, 1984). Dolfing (1990) demonstrated that energy was conserved via anaerobic reductive dechlorination of 3- chlorobenzoate. Further studies by Mohn and Tiedje (1990; 1991) substantiated this finding.

Biologically diverse environments harbor microbial communities with a broad range of enzymes with wide substrate specificities. This helps organisms use a broad range of substrates, including xenobiotics, for anabolic metabolism or for energy generation. Generally, microbes could use organic or inorganic compounds as an electron donor or as the TEA (terminal electron acceptor). As electron donor, pollutants

are oxidized and the electrons pass through a series of redox reactions, finally going to the TEA.

Conversely, while using such pollutants as TEA, energy is obtained by passing electrons to, and hence reducing the pollutant (Figures 2-3, 2-4). The halo-organic compound is used as a TEA and energy is conserved via electron transport coupled oxidative phosphorylation (Holliger et al., 1999; Smidt and de Vos, 2004). Energy is gained by chemiosmotic gradient along the cell membrane resulting in a proton motive force (PMF) which drives a membrane bound ATPase to generate ATP (Mohn and Teidje, 1991). Such process can be stimulated at the field level, but requires selective stimulation of desirable organisms by intentional introduction of suitable electron donor and acceptor combinations (Suflita et al., 1988) and nutrients to meet requirements of the enriched species. Hence the choice of an electron donor and acceptor combination is crucial to success of such processes.

Electron Transport Chain (ETC) of Dehalorespiring Organisms

ETC of dehalorespiring organisms involves transfer of electrons from donors by hydrogenase to terminal reductive dehalogenase. In the process, an electron gradient forms along the membrane, which creates a proton motive force (PMF) producing ATPase driven ATP synthesis (Holliger et al., 2003). Electron transfer occurs through cytochromes and quinones. Two types of cytochromes have been identified in *Desulfomonile tiedjei* (Louie and Mohn, 1999), *Desulfitobacterium hafniense* (Christiansen and Ahring, 1996), *Desulfitobacterium dehalogenans* (Van de Pas, 2000), and *Desulfuromonas chloroethenica* (Krumholz, 1997) and a soluble type present in *Dehalospirillum multivorans* (Muramatsu et al., 1995). Similarly, menaquinones have been found in membranes of *Dehalospirillum multivorans* (Muramatsu et al., 1995),

Dehalobacter restrictus (Schumacher and Holliger, 1996), and *Desulfitobacterium dehalogenans* (Van de Pas, 2000).

All the studies involved in the electron transport chain (ETC) of dehalorespiring processes have found a membrane associated hydrogenase or formate dehydrogenase (Louie and Mohn, 1999; Magnuson et al., 1998; Miller et al., 1997; Muramatsu et al., 1995; Van de Pas, 2000). By use of membrane impermeable hydrogenase and artificial impenetrable TEA such as methyl viologen, reductive dehalogenases were found in the periplasmic cell fraction whereas hydrogenases were found in the cytoplasmic fraction (Miller et al., 1997; Schumacher and Holliger, 1996; Van de Pas, 2000). Based on previous studies, two schemes for ETC are shown here (Figure 2-5), with dehalogenase facing inside of a cell or (Figure 2-6 dehalogenase facing outside of a cell. Simultaneous activity of hydrogenase and reductive dehalogenase leads to formation of a PMF.

Respirative type of ATP synthesis involves formation of ATP coupled to transport of protons across a semi permeable membrane. Every ATP formed is a result of three protons crossing the membrane (Maloney, 1983). According to this, ATP is not the smallest unit of energy metabolically useful to the cell, it can be as small as one third ATP. This means that a minimum of -20 KJ per mol of substrate converted is what a bacterial cell would need to obtain free energy (Schink and Thauer, 1988; Schink, 1990; Schink and Friedrich, 1994). This explains the low cell yields of dehalorespiring bacteria and it further consolidates studies stating low cell yields observed for *Desulfomonile tiedjei* DCB-1 and *Dehalobacter restrictus* (Mohn and Tiedje, 1991; Schumacher and Holliger, 1996).

Thermodynamic Consideration and Physiology for Dehalorespiration

Thermodymics of a reaction estimate the amount of energy obtained per reaction and control whether the reaction could sustain growth of the cell. One of the prerequisites for halo-organics to support growth is that the reaction they drive has to be exergonic. To check if any reaction would occur spontaneously or not and so yield energy or be endergonic depends on its ∆G values. A negative ∆G value would suggest that the reaction is exergonic and favorable for microbes (Thauer et al., 1977).

Dolfing and Harrison (1992) showed the potential of halogenated aromatics for use as TEA in reduced environments. ∆G values calculated by their group for various haloorganics ranged from -131.3 to -192.6 kJ/mol (Dolfing and Harrison, 1992). Reductive dehalogenation of halo-organics could yield a ∆G between -130 to -180 kJ of Gibbs free energy per mole of halogen removed (Dolfing and Janssen, 1994; Holmes et al., 1993, Huang et al., 1996; Smidt and de Vos, 2004). This ensures that reductive dehalogenation is an exergonic and, hence, thermodynamically favorable process that enables the dehalorespiring bacteria to couple reductive dehalogenation to growth (Smidt and de Vos, 2004). Another example is ∆G value for dehalogenation of 1, 2, 3, 4-tetrachlorodibenzo-p-dioxin with hydrogen as electron donor is -497 KJ/mol (Dolfing, 2003; Huang et al., 1996). This suggests that hydrogenotrophic reductive dehalogenation of polychlorinated dioxins is sufficient to support growth and survival of dehalorespiring organisms. Since ∆G is calculated at standard conditions, under environmental conditions some times, some reactions may go too slow such that they are not sufficient to support life of conducting organisms.

Redox potential (Eh) is the measure of tendency of a substance to gain electrons compared to H_2 (set at 0). The more positive the Eh, the higher is the affinity for

electrons and higher is the energy generation. Redox potential range for haloorganics is similar to nitrate NO_3^-/NO_2^- range which yields $E^{o\prime}$ of +433 mV and higher than sulfate reduction SO_4^{2-}/H_2S with $E^{o\prime}$ of -217 mV (Thauer et al., 1977; Dolfing and Harrison, 1992). $E^{o\prime}$ for halo-organics is relatively higher than bicarbonate (HCO_3^-/CH_4) and SO_4^{2-}/H_2S, such that dehalorespiring organisms would be expected to outcompete methanogens or sulfate reducing bacteria (SRB) for reducing equivalents when they are limiting (Apajalahti et al., 1987; Tweel et al., 1987).

Under anaerobic conditions TEAs are frequently limiting, which means that there is a competition for TEAs between dehalorespiring populations and other indigenous populations. Hence, TEAs under anaerobic conditions will affect the community composition. Therefore, more energy generating TEAs would be preferred over lower energy generating TEAs (Figure 2-7). Susarla and coworkers (1996) hypothesized that microbially mediated dehalorespiration sequentially uses higher to lower energy yielding TEAs. To test this, they conducted anaerobic dechlorination experiments and demonstrated that dechlorination was a preferred reaction with higher redox potential TEAs being used first. TEAs with highest reduction potentials were used preferentially, followed by the lower energy generating TEA in the order $O_2 > NO_3^-$ >halo-organics > $SO_4^{2-} > HCO_3^-$. This indicates that dehalorespiration is feasible thermodynamically when compared to SRBs, acetogens or methanogens. One could predict from the redox potentials that such processes occur under anoxic environments (Dolfing, 2003) and that halo-organic compounds are important TEAs.

Dehalorespiring Bacterial Biodiversity

All of the dehalorespiring organisms known to date belong to the domain Bacteria. They further fall into 3 phylogenetic branches; namely Gram positive low G+C content

(*Firmicutes*), *Proteobacteria* subgroup ε, α, or δ (SRB), and *Chloroflexi* (Figure 2-8) (Holliger et al., 2003; Hiraishi, 2008). Electron donors used by dehalorespiring bacteria can range from electron rich H_2 to organic acids such as formate, pyruvate, acetate, lactate, and butyrate (Table 2-2). Dehalorespiring populations are quite versatile in their ability to use electron donor and TEA.

Electron Donors

There is a competition for electron donors between dehalorespirers and other anaerobic microbes in the soil. Dehalorespiring organisms can use a broad range of electron donors (Table 2-2). H_2 is an important electron donor in dehalogenation, and is used by a wide range of dehalorespiring organisms as well as syntrophs (Schink, 1997). Based on the thermodynamic gains, dehalorespiring organisms can outcompete hydrogenotrophic methanogens, homoacetogens, and SRBs (McCarty et al., 1997; Ballapragada et al., 1997; Fennell et al., 1997; Fennell and Gossett, 1998, Loffler et al., 1999; Smatlak et al., 1996; Yang and McCarty, 1998).

Fermentative Dehalogenation or Syntrophic Dehalogenation

Syntrophy is a symbiotic relation between two or more metabolically different bacteria, that together may degrade a substance and in the process satisfy energy needs of the involved organisms (Atlas and Bartha, 1993). Organisms involved in this process can together perform a reaction which neither can perform individually. Syntrophy plays an important role in reductive dehalogenation. Syntrophs have been previously shown to dehalogenate (Mohn and iedje, 1991; Yang and McCarty, 1998; Drzyzga and Gottschal, 2002; Dolfing, 2003; Sung et al., 2003). Dehalorespiring populations participate in close syntrophic associations with hydrogen producing organisms, which require substantial low hydrogen concentrations for energy gain

(Drzyzga and Gottschal, 2002). Dehalorespirers could keep hydrogen concentrations low to allow growth and sustenance of obligate syntrophs (Dolfing, 2003; Sung et al., 2003; Yang and McCarty, 1998). Dehalorespirers would be favored by slow hydrogen producing obligate syntrophs fermenting on organic acids over methanogens or acetogens when present in adequate concentrations (Dolfing, 2003; Sung et al., 2003; Yang and McCarty, 1998).

Phylogeny of Dehalorespiring Populations

Dehalorespiring organisms, because of their wide metabolic diversity, have been isolated from several different environments. As mentioned previously, all dehalorespirers described to date fall within the domain Bacteria and into three major phyla: *Chloroflexi*, *Firmicutes* and *Proteobacteria* (Figure 2-8, Table 2-2) (Holliger et al., 2003; Hiraishi, 2008). *Desulfomonile tiedjei* DCB1 of the δ-*Proteobacteria* was the first organism described to derive all of its energy needs by dehalorespiration on 3-chlorobenzoate (Di Gioia, 1998; Shelton and Tiedje, 1984; Suflita et al., 1982). Discovery of dehalorespiration lead to intense research in this direction (mid 1990's) towards enrichment, isolation, identification, characterization from a phylogenetic perspective, sequencing and genomics of dehalorespiring isolates (Loffler et al., 2003; Smidt and Vos, 2004).

Although dehalorespiration of haloaliphatics has been studied extensively, little information is available for dehalorespiration on haloaromatic compounds. Reductive dehalogenation of polychlorinated biphenyl (PCB) by a pure culture has so far been reported only for *Dehalococcoides* (Baba et al., 2007; Yan et al., 2006) which belongs to the phylum *Firmicutes*. Based on their metabolic properties, dehalorespirers are further

divided into two physiological types, the facultative and obligate dehalorespiring bacteria (Table 2-3).

Facultative Dehalorespiring Organisms

Facultative dehalorespiring organisms are very versatile in their choice of electron donor and acceptor (Table 2-2, 2-3). Organisms falling under this physiological group belong to Class δ or ε *Proteobacteria*, or the phylum *Firmicutes*. *Desulfovibrio* and *Desulfuromonas* contain facultative dehalorespiring members. The former dehalogenates 2-chlorphenol (Sun et al., 2002), whereas the latter utilizes tetrachloroethene (PCE) and trichloroethene (TCE) (Krumholz, 1997; Sung et al., 2003). *Geobacter* contains metal reducing members, and belongs to the δ *Proteobacteria;* Geobacter *lovleyi* is a metal reducer that is also capable of dehalorespiration (Sung et al., 2006). *Desulfitobacterium* belongs to the low G+C, gram positive bacteria and phylum *Firmicutes*. Most of the strains from this genus have been isolated with either PCE or TCE when grown with H_2 as an electron donor (Table 2-2). *Desulfitobacterium* isolates are so named as the first pure culture isolated grew on sulfite as TEA (Utkin et al., 1994) and most of them could repeat this as well (PCE1, PCE-S, Viet1). Being versatile with their choice of electron donor or TEA, some strains of *Desulfitobacterium* use chlorinated phenolic compounds and chloroethenes, whereas others could use either of the two. This is in accordance with other studies showing that different enzyme systems work with chloroaryl and chloroalkyl reduction, wherein respective substrates induce each enzyme system (Gerritse et al., 1999; Miller et al., 1998).

As mentioned in Table 2-2, the facultative dehalorespiring organisms have adapted very well with respect to the array of utilizable electron donors and acceptors. Even though, there is not enough information regarding use of PCBs by this group. The

only exception to this is *Desulfitobacterium dehalogenans,* which has been reported to dehalogenate hydroxylated PCBs (Wiegel et al., 1999).

Obligate Dehalorespiring Organisms

The obligately dehalorespiring group contains only two genera named to date: *Dehalobacter* and *Dehalococcoides,* which belong to the phyla *Firmicute* and *Chloroflexi,* respectively. Energy is obtained where halogenated aliphatic and aromatic compounds are used as TEA and energy is conserved via electron transport coupled phosphorylation (Holliger et al., 1999; Smidt and de Vos, 2004).

Genus *Dehalobacter* usually fulfills all its energy needs solely from haloorganics as TEA. They grow with H_2 as electron donor and acetate as carbon source (Holliger et al., 1993). *Dehalococcoides* belongs to the phylum *Chloroflexi*. *Dehalococcoides* species are hydrogenotrophs, and may make good partners with syntrophs. Growth linked respiration for PCBs has been described only with *Dehalococcoides* isolates. *Dehalococcoides ethenogenes* 195 was the first isolated organism from a sewage sludge reactor to dehalorespire on PCE to vinyl chloride (VC) to ethane (Maymo-Gatell et al., 1997; 2001). Isolation of *Dehalococcoides* 195 was followed by CBDB1, a strain that dechlorinates chlorinated benzenes (Adrian et al., 2000) and polychlorinated dibenzodioxins (Bunge et al., 2003). BAV1 uses VC and dichloroethene (DCE) isomers as electron acceptors (He et al., 2003), and strain FL2 grows uses TCE, *cis*-DCE, and *trans*-DCE as electron acceptors and dechlorinates them to ethene (He et al., 2005). Uncultured *Chloroflexi* are obligate dehalorespirers that belong to the *Dehalococcoides*-like groups (DLG) cluster of *Dehalococcoides* within phylum *Chloroflexi.* Their presence was detected by several enrichments on PCB, polychlorinated dioxins and chlorobenzene from contaminated sites (Cutter et al., 2001; Wu et al., 2002). Some of

the dehalorespiring populations are obligate dehalorespirers which make them very beneficial to environmental bioremediation efforts.

Outline of this Dissertation

The aim of this research is to selectively enhance growth of DDx degrading bacteria. The extensive metabolic capabilities of microorganisms lead us to hypothesize that organisms capable of DDx degradation are already present in Apopka soils. To attain appreciable bioremediation, the creation of a unique niche for the desired population is required, such that they can be selectively diverted to biodegradation. Careful selection of electron donor and acceptor combination could lead to creating that niche by selective growth of the degrading population (Suflita et al., 1988). We investigated a wide an array of different electron donors and acceptors to find the combination that maximizes degradation in Chapter 3. Once the donor acceptor combination leading to the greatest loss of DDX was defined, lab scale microcosm experiments were scaled up to mesocosm level as discussed in Chapter 4. Chapter 5 describes efforts that targeted enrichment, isolation and characterization microorganisms that could biodegrade DDT from the organic Apopka soils. Isolates from the degrading consortium could be used to study genetics, physiology and biochemistry of these microbes, which could further enhance the microbial processes to achieve bioremediation of DDT with precision and in a short time.

Despite the evidence that microbes capable of DDx degradation exist, DDxs accumulate in soils. One major reason for their prolonged persistence is the decreased bioavailability (Hunt and Sitar, 1988). While bioremediation is a feasible option for remediation of DDx-contaminated lands, the pollutant must be bioavailable (Alexander 1995, 1997). Desorption of contaminants from soils is a major step to increase

biodegradation, and efforts to desorb DDxs from aged contaminated soils were made by use of Na^+ ions and surfactant (Tween-80).

Sodium is known to disperse soils and increase dissolved organic carbon (DOC) levels in soils, both of which could potentially increase DDxs bioavailability (Nelson and Oades, 1998; Wood, 1995; White, 1997; Brady and Weil, 2003; 2007). Na^+ is known not only to enhance physical dispersion of soils (Kantachote et al., 2001; 2004), but also to increase aqueous DDT concentrations. Both of these could increase the biodegradation potential of DDxs from aged contaminated soils, which has been demonstrated by various studies (Kantachote et al., 2001; 2004; Juhasz et al., 1999).

Chapter 6 describes lab scale microcosm experiments established with various Na^+ concentrations to achieve an optimum amount that maximized DDxs degradation. Another approach here was to obtain nutritional requirements of the degrading consortia such as mineral salts, cofactors and vitamins. Once again, when optimum Na^+ concentration and nutritional needs of the degrading consortia were identified, lab scale microcosm experiments were scaled up to mesocosm levels as discussed in Chapter 6.

One means to enhance bioavailability of HOCs such as DDT is use of surfactants (Rouse et al., 1994; Volkering et al., 1998). Surfactants increase the apparent solubility of HOCs simultaneously increasing mass transfer rates from the nonaqueous phase, hence potentially increasing the bioavailability (Kile and Chiou., 1989; Edwards et al., 1991; Jafervert et al., 1994; Guha et al., 1998; Yeh and Pavlosthathis., 2004). To further address the bioavailability issue, we studied Tween-80 (surfactant listed in the U.S. EPA - January 2008 “List of inert ingredients for use in non food use pesticide products”). Initial sorption isotherm studies were targeted to investigate the effect of Tween-80 on

sorption of DDT to soils. Once the isotherm results confirmed that Tween-80 decreased DDT sorption to soils, microcosm experiments were established as discussed in Chapter 7.

Various chemical and physical methods employed for remediation of aged DDx contaminated soils have been discussed earlier in the chapter. Although such methods may be more rapid, they are usually more intrusive, destructive, expensive, and more labor and energy intensive compared to bioremediation. Bioremediation can be considered a viable, more approachable, cost effective, environmentally friendly and a promising option, since soil organisms with the ability to degrade DDxs already exist. Although complex, bioremediation can be applied efficiently and effectively for cleanup of an environmental system.

Table 2-1. Biotransformation products of DDT

Organism	Transformation products	Mechanism	Source	Reference
Proteus vulgaris	DDT→ DDD	Reductive dechlorination	Mouse intestine	Barker et al., 1965
Escherichia coli *Enterobacter*	DDT→ DDD	Reductive dechlorination	Rat feces	Mendel and Walton, 1966
Pseudomonas aeruginosa *Bacillus sp.* *Flavobacterium*	DDT→ DDD	Reductive dechlorination	Activated sludge	Sharma et al., 1987
Enterobacter cloacae	DDT→ DDD	Reductive dechlorination	Sewage sludge	Beunink and Rehm, 1988
Bacillus sp.	DDT→ DDD	Reductive dechlorination	Soil	Katayama et al., 1993
Cyanobacteria	DDT→ DDD	Reductive dechlorination	Soil	Megharaj et al., 2000
E aerogenes	1. DDT→ DDD, DDMU,DDMS, DDNU, DDOH, DDA andDBP 2. DDT → DDE	Anaerobic pathway	Not mentioned	Wedemeyer, 1966; 1967
Bacillus spp., E coli, E aerogenes	1. DDT→ DDD, DDMU,DDMS, DDNU, DDOH, DDA andDBP 2. DDT → DDE	Anaerobic pathway	Not mentioned	Langlois et al., 1970
Pseudomonas isolated as Hydrogenomonas	^{14}C DDT → DDD, DDMS, DDNU andDBP	Anaerobic pathway by cell free extracts	Sewage	Pfaender and Aexander, 1972
Pseudomonas aeruginosa 640X	DDT→ring cleavage metabolites	Reductive dechlorination	Soil	Golovleva and Skryabin, 1981
Strain B-206	DDT→ DDE,DDD, DDMU to hydroxylated metabolites	Transformation of DDT to hydroxylated metabolites	Activated sludge	Masse et al., 1989
Ralstonia eutropha	Meta cleavage	Meta cleavage	Soil	Nadeau et al., 1994, 1998
Alcaligens sp. JB1	Meta cleavage	Meta cleavage	Soil	Parsons et al., 1995
Pseudomonas acidovorans M3GY	Meta cleavage of DDE	Meta cleavage of DDE	Genetically engineered	Hay and Focht, 1998
Terrabacter sp. Strain DDE1	Meta cleavage of DDE	Meta cleavage of DDE	Soil	Aislabie et al., 1999
R.eutropha A5	Meta cleavage of DDD	Meta cleavage of DDD	Soil	Hay and Focht, 2000

Table 2-2. Phylogeny and properties of dehalorespiring bacteria adapted from (Haggblom and Bossert, 2003; Holliger et al., 1999).

Name	Dechlorinated compound	Electron donor	Phylogeny	References
Desulfomonile tiedjei	PCE,TCE,H_2, 3-chlorobenzoate, Pentachlorophenol	H_2, formate, pyruvate	Gram negative, SRB belong to δ-Proteobacteria	Deweerd et al., 1991
Isolate 2CP-1	2-chlorophenol, 2,6-dichlorophenol	acetate, formate, yeast extract	Facultative anaerobe, Gram negative, δ-Proteobacteria	Cole et al., 1994
Desulfitobacterium chlororespirans	2,4,6 trichloro phenol, 3-Cl 4-OH phenylacetate	H_2, formate, pyruvate,lactate, butyrate, crotonate	Low G+C, Gram positive	Sanford et al., 1996
Desulfitobacterium hafniense	3-Cl 4-OH phenylacetate, Pentachloro phenol	pyruvate, formate, tryptophan, lactate, butyrate, crotonate, ethanol	Low G+C, Gram positive	Christiansen and Ahring, 1996
Desulfitobacterium frappieri	2,4,6 trichloro phenol, 3-Cl 4-OH phenylacetate	pyruvate	Low G+C, Gram positive	Bouchard et al., 1996
Desulfitobacterium dehalogenans	PCE, 2,4,6-trichlorophenol	H_2, formate, pyruvate	Low G+C, Gram positive	Utkin et al., 1994
Desulfitobacterium strain *PCE 1*	3-Cl-4-OH-phenylacetate	formate, pyruvate	Low G+C, Gram positive	Gerritse et al., 1996
Desulfitobacterium strain *PCE-S*	PCE,TCE, 2,4,5-trichlorophenol, Pentachloro phenol	pyruvate	Low G+C, Gram positive	Granzow, 1998
Dehalobacter restrictus	PCE,TCE	H_2	Gram positive, affiliated with Desulfitobacterium, derive energy by dehalorespiration	Holliger et al., 1998
Isolate *TEA*	PCE,TCE	H_2	Affiliated with Desulfitobacterium derive energy by dehalorespiration	Wild et al ., 1996
Dehalospirillium multivorans	PCE,TCE	H_2, formate, pyruvate	Gram negative belong to δ-Proteobacteria	Scholz-Muramatsu et al., 1995
Desulfuromonas chloroethenica	PCE,TCE	acetate, pyruvate	Gram negative, SRB	Krumholz et al., 1996
Dehalococcoides ethenogenes	PCE,TCE, DCE, chloroethenes	H_2	Eubacterium, only aerobe isolated so far with energy conservation though dehalorespiration and known to dechlorinate PCE all the way to ethene	Maymo-Gatell et al., 1997

Table 2-2. Continued.

Name	Dechlorinated compound	Electron donor	Phylogeny	References
Enterobacter strain *MS-1*	PCE,TCE	formate, pyruvate, acetate	Facultative anaerobe, Gram negative γ-Proteobacteria, energy conservation with dehalorespiration	Sharma and McCarty, 1996
Dehalococcoides ethenogenes strain *195*	PCE,TCE, cis-DCE,1,1-DCE, 1,2-DCA, VC	H_2	Green non sulfur bacteria (*Chloroflexi*)	Maymo-Gatell et al., 1997; 1999
Dehalococcoides strain *CBDB1*	1,2,3-TCB, 1,2,4-TCB, 1,2,3,4-TeCB, 1,2,3,5-TeCB, 1,2,4,5-TeCB	H_2	Green non sulfur bacteria (*Chloroflexi*)	Adrian et al., 2000

PCE= tetrachloroethene; TCE= trichloroethene; DCE= dichloroethene; TCB= trichlorobenzene; VC= vinyl chloride; DCB= dichlorobenzene; TeCB= tetrachlorobenzene; DCA= dichloroethane.

Table 2-3. Phylogeny of facultative and obligate dehalorespiring bacteria

Genus	Species / strain	Dechlorination substrate or TEA	Microbiology and physiology	Reference
Anaeromyxobacter	*Anaeromyxobacter dehalogens*	2-bromophenol, nitrate, fumarate, oxygen	Facultative anaerobic dehalorespiring myxobacteria, δ Proteobacteria	Sanford et al., 2002
Desulfomonile	*Desulfomonile tiedje DCB-1*	3-chlorobenzoate, fumarate, sulfate, sulfite, thiosulfate, nitrate	Facultative dehalorespirers, δ Proteobacteria	DeWeerd et al., 1990
	Desulfomonile limimaris	3-chlorobenzoate	Facultative dehalorespirer, δ Proteobacteria	Sun et al., 2000
Desulfovibrio	*Desulfovibrio dechloroacetivorans*	2-chlorophenol	Facultative dehalorespirers belonging to group SRB, acetate oxidizers, δ Proteobacteria	Sun et al., 2001
Desulfuromonas	*Desulfuromonas acetooxidans*	PCE, TCE	Facultative dehalorespirers belonging to anaerobic group of SRB and acetate oxidizing bacteria, δ Proteobacteria	Pfennig and Biebl, 1976
	Desulfuromonas michiganesis strain BB1 and strain BRS1	PCE, TCE, fumarate, ferric iron	Facultative dehalorespirers, acetate oxidizers, δ Proteobacteria	Sung et al., 2003
Geobacter	*Geobacter lovleyi strain SZ*	Metals, PCE	Facultative dehalorespirers metal reducers, δ Proteobacteria	Sung et al., 2006
	Trichlorobacter thiogenes	Trichloroacetic acid	Facultative dehalorespirers, δ Proteobacteria metal reducers	Nevin et al., 2007
Sulfurospirillum	*Sulfurospirillum halorespirans*	PCE to cis DCE	Facultative dehalorespirers, ε Proteobacteria	Luijten et al 2003
Desulfitobacterium	*Desulfitobacterium dehalogenans*	2,4dichlorophenol, hydroxylated PCBs and chloroalkenes	Facultative dehalorespirers, phylum Firmicutes	Utkin et al., 1994 ; Wiegel et al., 1999
	Desulfitobacterium sp. strain PCE1, PCE-S, Viet1,	TCE (trichloro ethane) or cis-DCE (tetrachloro ethane)	Facultative dehalorespiring, low G+C, Gram positive, phylum Firmicutes	Miller et al., 1997 ; Löffler et al., 1997 ; Gerritse et al ., 1996 ; 1999
	Desulfitobacterium chlororespirans	3-chloro-4-hydroxybenzoate	Anaerobic lactate oxidizing organism, phylum Firmicutes	Sanford et al., 1996
	Desulfitobacterium hafniense	Pentachlorophenol, PCE, 2,4,6-trichlorophenol	Facultative dehalorespirer, phylum Firmicutes	Bouchard et al., 1996 ; Lanthier et al., 2000 ; Breitenstein et al., 2001 ; Gerritse et al., 1996 ; Suyama, 2001 ; Tartakovsky et al., 1999

Table 2-3. Continued.

Genus	Species / strain	Dechlorination substrate or TEA	Microbiology and physiology	Reference
	Desulfitobacterium metallireducens	PCE, TCE, Fe(III)citrate, Mn(IV)oxide, humic acids, elemental sulfur	Lactate as C source and lactate oxidizing facultative dehalorespirer, phylum Firmicutes	Finneran et al., 2002
Dehalobacter	*Dehalobacter restrictus PER-K23^T*	PCE/ TCE	Obligate dehalorespirers, phylum Firmicutes, low G+C, Gram positive, H_2 as e^- donor and acetate as C source	Holliger et al., 1993
	Dehalobacter sp. Strain TCA1	1,1,1-trichloro ethane	Obligate dehalorespirers, H_2 or formate e^- donor, phylum Firmicutes	Holliger et al., 1998 ; Sun et al., 2002
Dehalococcoides	*Dehalococcoides ethenogenes strain 195*	PCE→VC→Ethene	Obligate dehalorespirers, belong to phylum *Chloroflexi*	Maymo-Gatell et al., 1997; 2001
	Dehalococcoides strain CBDB1	Chlorobenzenes, chlorophenolsand PCDD/Fs	Obligate dehalorespirers, belong to phylum *Chloroflexi*	Adrian et al., 2000 ; 2007 ; Bunge et al., 2003
Uncultured Chloroflexi	*o-17*	2,3,5,6-CB	Obligate dehalorespirers belong to DLG cluster of Dehalococcoides within phylum *Chloroflexi*, acetate oxidizers	Cutter et al., 2001
	DF-1	PCB congeners with doubly flanked chlorines	Obligate dehalorespirers belong to DLG cluster of *Dehalococcoides* within phylum *Chloroflexi* formate or H_2-CO_2 for growth	Wu et al., 2002

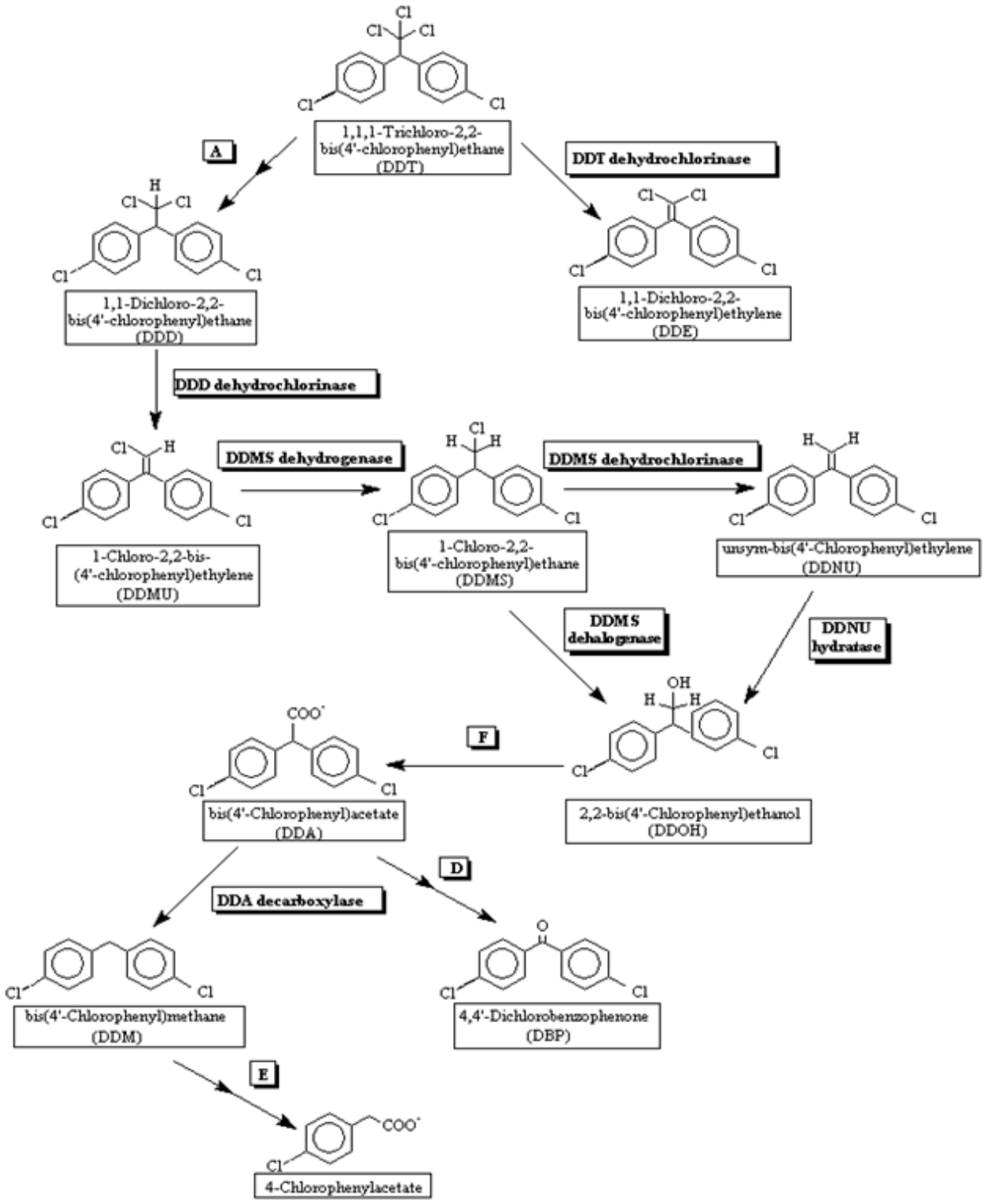


Figure 2-1. Proposed anaerobic DDT degradation pathway from University of Minnesota Biocatalysis/Biodegradation Database (UMBBD) website. (http://umbbd.msi.umn.edu/ddt2/ddt2_image_map.html)

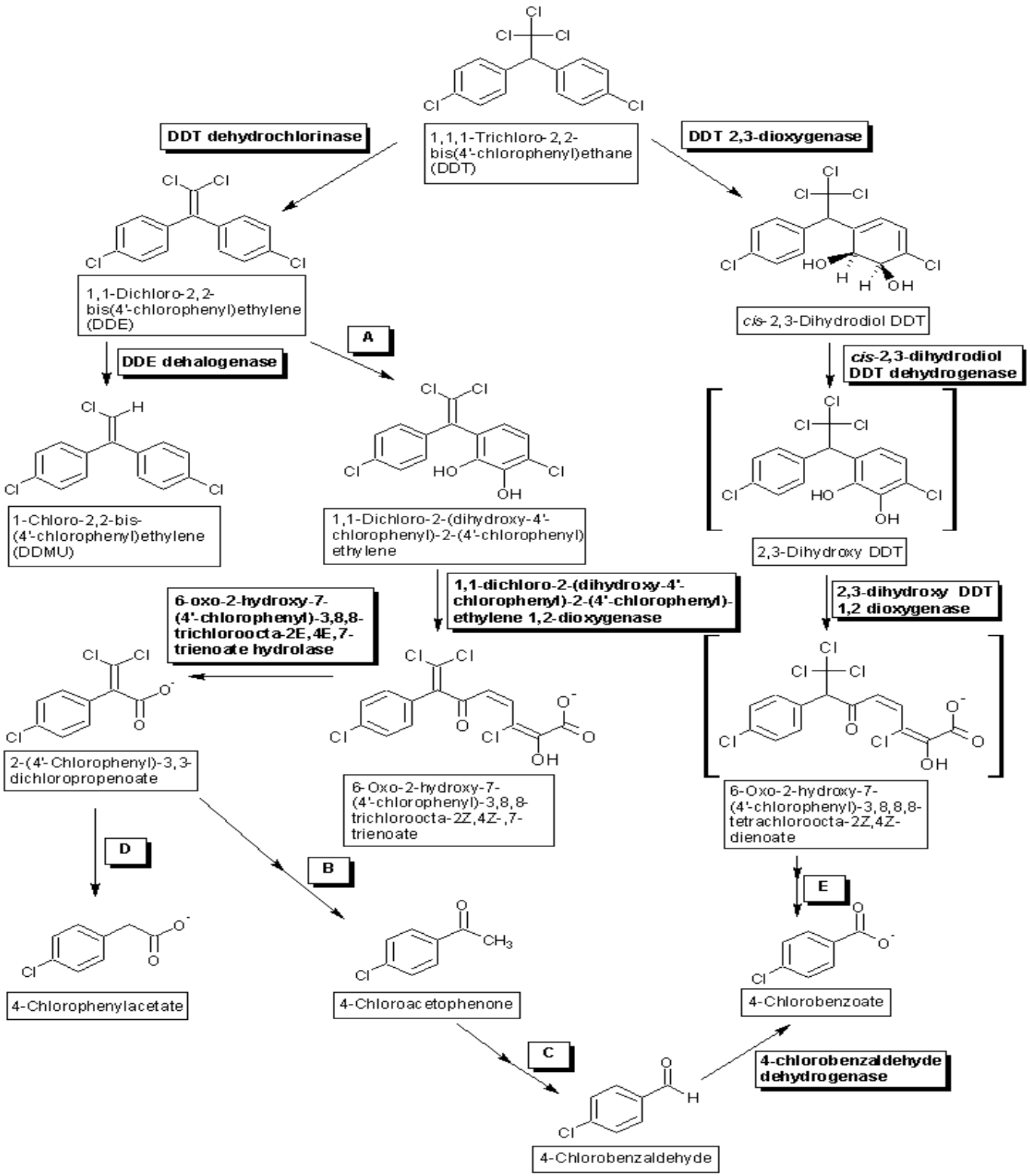


Figure 2-2. Proposed aerobic DDT degradation pathway from UMBBD website. (http://umbbd.msi.umn.edu/ddt/ddt_image_map.html)

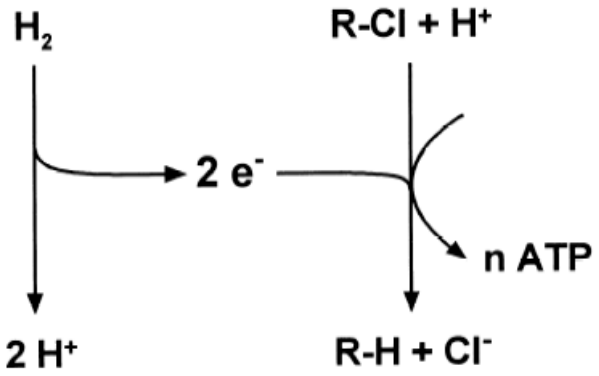


Figure 2-3. Dehalorespiration Schematic (Holliger et al., 1999).

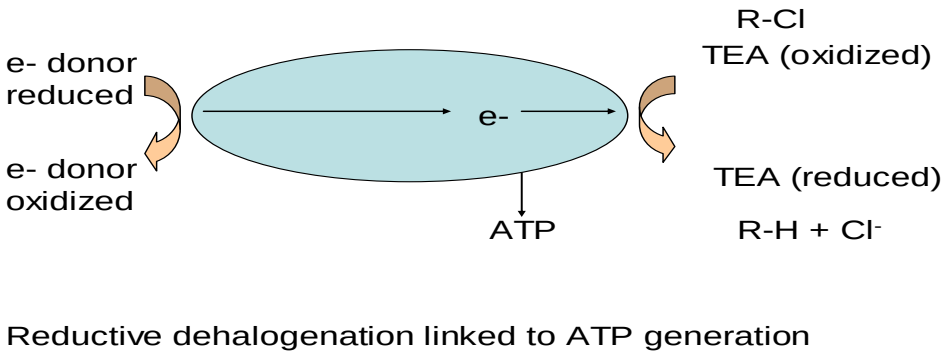


Figure 2-4. Reductive dehalogenation linked to ATP generation (adapted from Haggblom and Bossert, 2003).

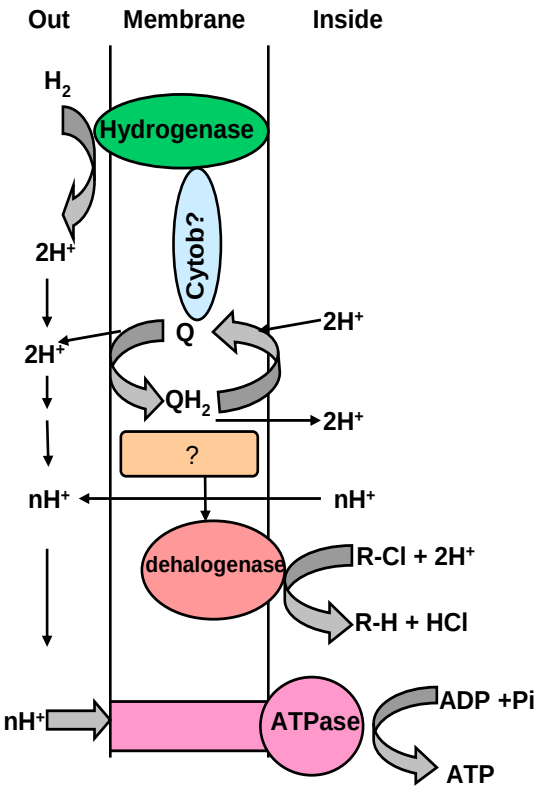


Figure 2-5 Dehalorespiration electron transport chain scheme with dehlogenase inside of a bacterial cell. (Cytob =cytochrome b) (Adapted from Haggblom and Bossert, 2003).

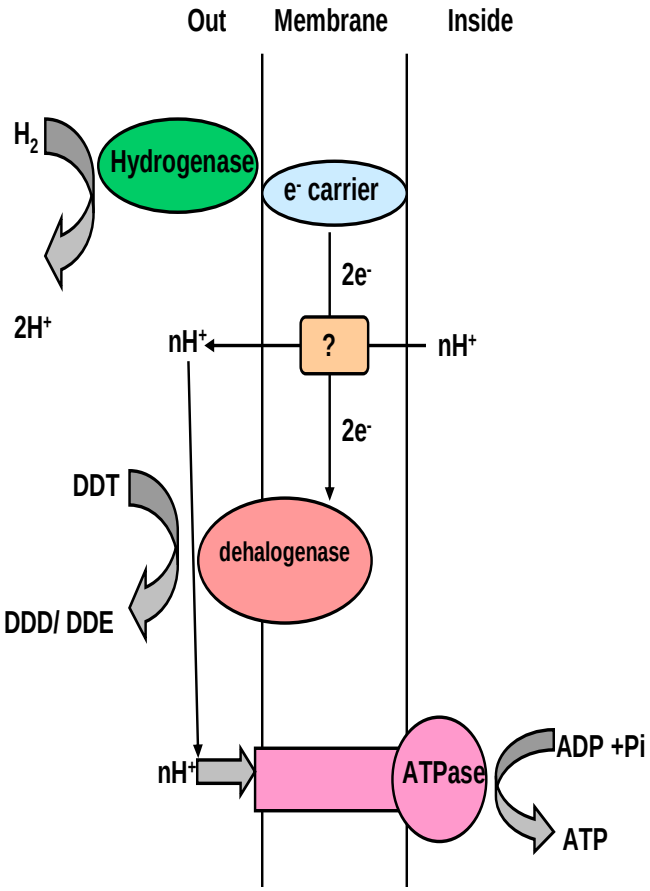


Figure 2-6 Dehalorespiration electron transport chain scheme with dehlogenase outside of a bacterial cell. (Cytob =cytochrome b) (Adapted from Haggblom and Bossert, 2003).

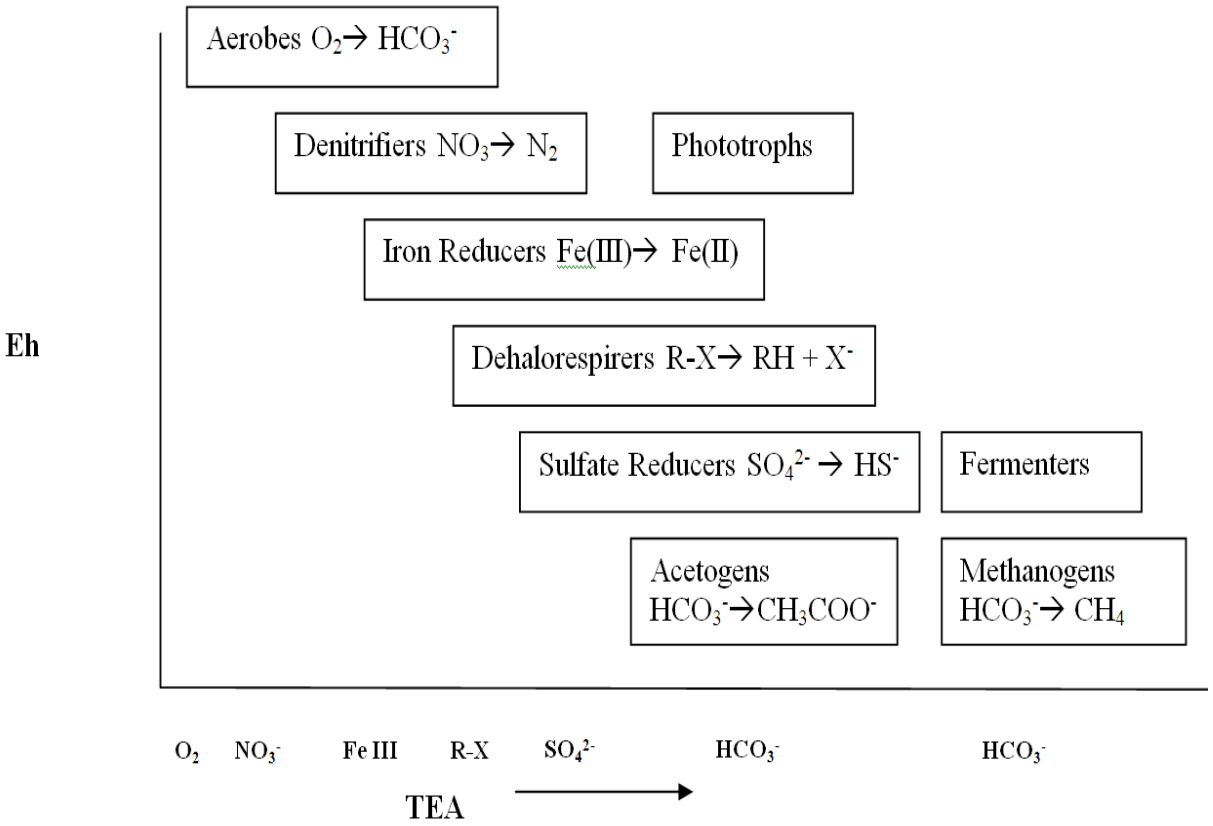


Figure 2-7. Energetics of dehalorespiration (Dehalogenation by Haggblom and Bossert, 2003).

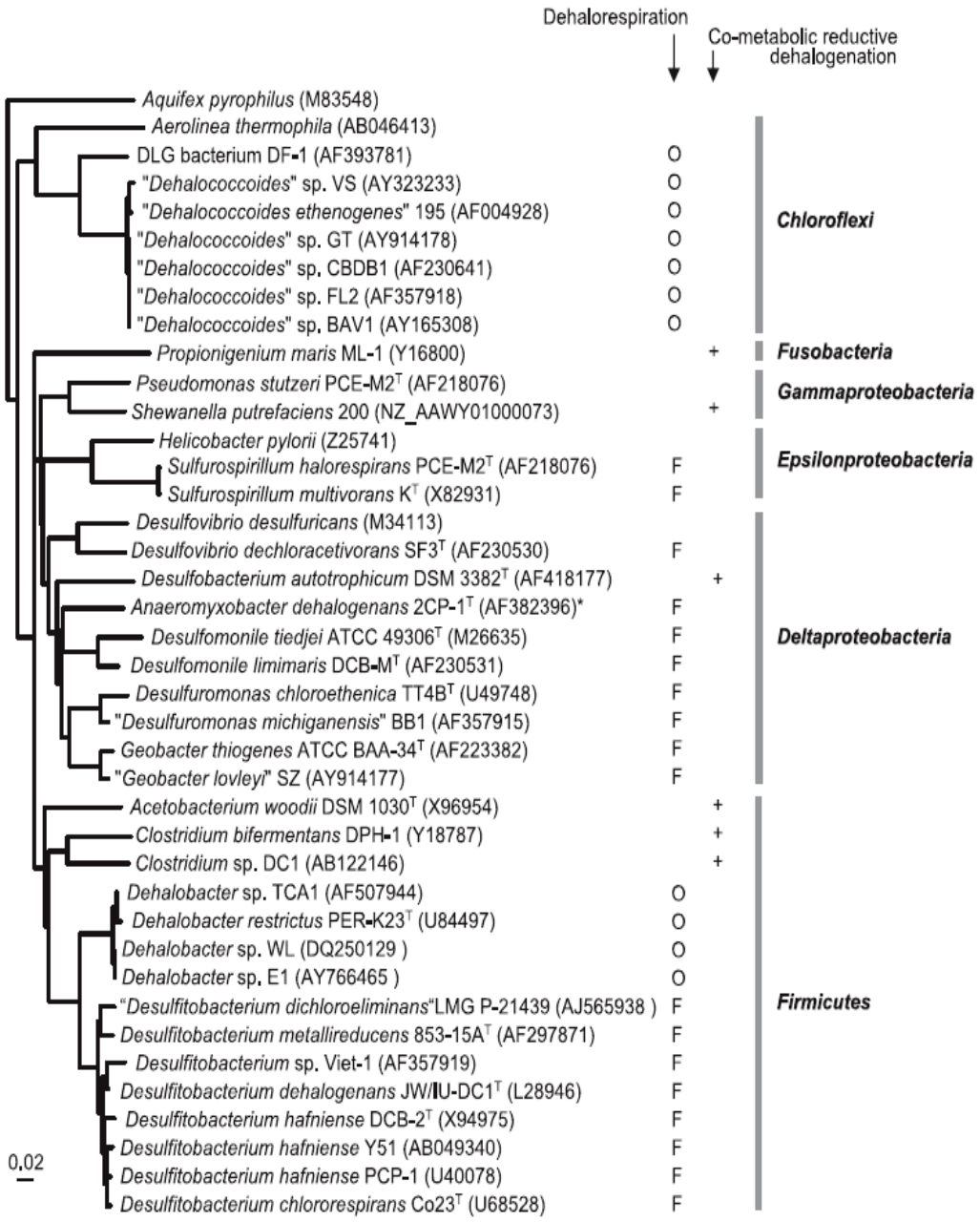


Figure 2-8. Phylogeny of known dehalorespiring organisms reproduced from Hiraishi 2008. key O=obligate dehalorespirers, F=facultative dehalorespirers, +=organisms showing cometabolic reductive dehalogenation.

CHAPTER 3
EVALUATION OF ELECTRON DONOR AND ACCEPTOR COMBINATIONS TO MAXIMIZE DEGRADATION OF DDT AND ITS METABOLITES IN MICROCOSM STUDIES

DDT (1,1,1-trichloro-2,2-di(4-chlorophenyl)ethane)is a synthetic organochlorine pesticide that was introduced into the environment during production and application processes. It was initially used to control insect mediated diseases such as malaria and typhus, and later it became frequently used to control insects in crops, live stock, and for domestic uses (EPA report 1975). Low costs, high effectivity, and prolonged persistence lead to making it the world's most popular and widely used insecticide in the 1960s (Dunlap, 1981).

By the early 1970s, the adverse biological and environmental effects of DDT and its metabolites DDD and DDE (collectively known as DDx) were becoming evident, which led to its ban in the U.S. in 1972 (WHO, 1979; Turusov, 2002; Ratcliffe, 1967). Even though it had been banned in most developed countries in the early 1970s, DDx persists in significant concentrations in many soils and sediments in the U.S..

Biologically diverse environments harbor a broad range of enzyme systems with wide substrate specificities. This enables the microbial community to utilize a broad range of substrates, including xenobiotics, for anabolic metabolism and for energy generation. Microbes use organic or inorganic compounds as electron donor or as the terminal electron acceptor (TEA). As electron donor, pollutants are oxidized and the electrons pass through a series of redox reactions, finally going to the TEA. Conversely, energy is obtained by passing electrons to, and hence reducing, the halogenated pollutant in a process is called reductive dehalogenation. The most important feature of reductive dehalogenation is the gain of energy in the process termed dehalorespiration.

Dehalorespiration is a type of anaerobic respiration where energy conservation is coupled to reduction of a halogenated organic compound (Holliger et al., 1999; Smidt and de Vos, 2004).

Dehalorespiration processes can be stimulated at the field level, but requires selective stimulation of target organisms by addition of a suitable electron donor: acceptor combination (Suflita et al., 1988). Selection of the appropriate donor: acceptor combination is crucial to the success of the degradation process, such that this chapter describes the identification of the combination leading to achieve highest degradation rates in laboratory microcosms.

We formulated our hypothesis based on the assumptions that microbes capable of degrading DDx are already present at the site and that careful selection of electron donor: acceptor combination would facilitate enrichment of the degrading consortia. We did not know if DDT would serve as a better electron donor or acceptor. To test these assumptions, a series of microcosms were established which could broadly be divided into two categories: those that selected for DDT as electron donor; and another that that selected for DDT as TEA.

In addition, microcosms with DDT as TEA were also tested with varying sulfate concentrations. Sulfate reducing bacteria (SRB) have previously been reported to dehalorespire on chlorinated pollutants (Zwiernik et al., 1998; Fava et al., 2003; Zanaroli et al., 2006), such that decreasing concentrations of sulfate may increase the numbers of SRB under the relatively high concentrations, followed by inducing a shift toward use of DDx as TEA under low sulfate concentrations.

Materials and Methods

Soils

Soil used for microcosms was collected from the North Shore Restoration Area (NSRA) at Lake Apopka, FL. Lake Apopka is a shallow lake with 125 km^2 surface area, located in central Florida. Following collection, the soils were shipped to University of Florida, Gainesville. Soils were sieved through a 20 mesh sieve (nominal diameter of 80 µm) and homogenized by mixing before establishing the microcosms. Pertinent soil properties included: average % moisture (on dry weight basis) was 142.8%; average pH was 5.0; average mg NOx-N/kg soil (dry weight) was 165.9; and total organic carbon (TOC) content was 45 percent (as determined by the Environmental Microbiology/Chemistry Laboratory, Soil and Water Sciences Department, University of Florida).

Microcosm

Microcosms were established using NSRA soils, and could broadly be divided into two sets: 1) microcosms testing DDT loss under different electron accepting conditions, where DDT would be the electron donor; and 2) microcosms with various electron donors and DDT as terminal electron acceptor. p,p'-DDT solubilized in methanol was added to sieved and homogenized soils. Upon evaporation of the solvent, soils were mixed manually and 10 g dry weight soil (containing 10 µg p,p'-DDT/g dry soil) was added to 125 mL serum bottles with 45 mL mineral medium (Ou et al., 1978). To maintain anaerobic conditions, all sets were purged with N_2 except the H_2 sets where H_2 and CO_2 was purged, sealed with Teflon-lined butyl rubber stoppers and secured with aluminum crimps. Microcosms were incubated at 27^oC in the dark for about two

months. Autoclaved controls were made for each set, which consisted of soils autoclaved three times over three consecutive days.

DDT as Electron Donor

Four different terminal electron acceptors (TEA) were tested with DDT as electron donor and carbon source as shown in Table 3-1: (1) Sulfate (as K_2SO_4) was applied at a rate of 0.42 mg of SO_4-S/g dry soil (Wright and Reddy, 2001). Sulfate sets were purged with N_2 on a weekly basis to prevent accumulation of sulfide; (2) Nitrate (in the form of KNO_3) applied at a rate 0.221 mg of NO_3-N /g dry soil (Wright and Reddy, 2001); (3) Fe (III) (in the form of Fe(III)hydroxide, γ-phase) at a concentration of 4.42 mg/g of soil (Monserrate and Haggblom, 1997); and (4) control with no exogenous TEA provided. In addition to the autoclaved control these microcosms included another set of control called substrate controls which had no exogenous electron donor, i.e. no DDT added.

DDT as Terminal Electron Acceptor

Four different electron donors were tested (Table 3-2), which included two organic acids (lactate and acetate at 20mM), H_2 at 100kPa, and no exogenous electron donor. Concentrations for the donors chosen here were significantly higher than environmental values to quickly investigate if increased concentrations of such donors would positively correlate with degradation. All the microcosms were purged weekly with N_2 except the H_2 sets which were purged with H_2 and CO_2 during the incubation to remove sulfide and compensate for the used H_2 and CO_2.

DDx Extraction

DDx extraction could be divided into three stages: soil preparation; accelerated solvent extraction (ASE); and florisil extraction. The extraction method was based on U.S.EPA method #3545 (U.S.EPA, 2000) with minor modifications developed by Soil

Microbial Ecology Laboratory, University of Florida, and chemists at Pace Analytical Services, Ormond Beach, FL.

Soil Preparation

Soils were separated from liquid by centrifugation in Beckman J2-21 Floor Model Centrifuge (JA-14 rotor) at 7155 x g for 20 minutes at 4°C. Wet soil samples were allowed to air dry for two to three days following which they were ground. Soil moisture content was determined in the sample. Moisture was adjusted to 50% moisture on a dry weight basis, and then the samples were allowed to equilibrate in 4°C refrigerator for 3 days.

Accelerated Solvent Extraction

Soil was mixed with Hydromatrix, a drying and bulking agent, at a ratio of 1:2, and placed into 34 mL stainless steel extraction cells. The remaining headspace in extraction cells were topped with clean Ottawa sands (Fisher Scientific, Pittsburgh, PA) to decrease the amount of solvent usage. Solvent used for extraction was methylene chloride: acetone (4: 1 v/v). Soils were extracted under high pressure about 1200 to 1400 psi at temperature of 100°C in a Dionex ASE 100 Accelerated Solvent Extractor (Sunnyvale, CA).

The extraction cycle included filling the extraction cell with about 19 mL solvent, followed by heating cycle where the solvent was heated to 100°C. Static extraction followed the heating cycle which performed for 5 minutes, followed by flushing cycle which flushed about 19mL solvent through the cell. The cell was finally purged with N_2 for about 2 minutes, which completed one extraction cycle.

Florisil Extraction

Florisil clean up used prepacked florisil “Bond Elute LRC” columns (200 µm particle size) (Varian Inc., Palo Alto, CA) which contained approximately 1 g Florisil packaged into the plastic holder. The column was conditioned using 5 mL hexane: acetone (9:1v/v), loaded with 1 mL of ASE extract and the column was eluted using 9 mL of solvent.

A volume of 5 mL from the cleanup volume was concentrated to dryness under a gentle stream of air. It was very important to concentrate the samples to complete dryness prior to GC (gas chromatography) analysis to avoid matrix response enhancement (Schmeck and Wenclawiak, 2005). Samples were reconstituted in 1 mL of hexane, and tubes with samples were vortexed and transferred to a 2 mL amber glass GC vials (Fisher Scientific Inc., Atlanta, GA) and crimped with 12 x 32mm aluminum crimp seals with prefitted PTFE lined septa (Fisher Scientific Inc., Atlanta, GA) for subsequent analysis by GC.

GC Conditions

Perkin-Elmer Autosystem Gas Chromatograph (GC) equipped with autosampler and an electron capture detector (ECD) was used for analyzing the extracts. Column conditions were: He as the carrier gas at flow rate of 1.0 mL/min, 5% methane in argon as the make-up gas with a flow rate of 50 mL/min, electron capture detector (ECD) temperature at 350 °C, and injector temperature at 205 °C in splitless mode. Injection volume was 3 µL in slow injection mode. GC temperature was programmed at 110 °C for 0.5 minutes, followed by a ramp at 20 °C/min to 210 °C and hold for 0 minutes, then ramp at 11 °C/min to 280 °C and held for 6.3 minutes. Under these conditions, the detection limit was calculated at the 90% confidence level to be: 0.007 µg/mL for o,p’-

DDE; 0.005 µg/mL for p,p'-DDE; 0.006 µg/mL for o,p'-DDD; 0.009 µg/ mL for p,p'-DDD; 0.005 µg/mL for o,p'-DDT; and 0.010 µg/mL for p,p'-DDT (Hubaux and Vos, 1970). These results are similar to the detection limit of 0.010 µg/mL reported by Pace, Inc.

Statistical Analysis

All the statistical analysis was done using JMP software manufactured by SAS (Cary, NC). One way ANOVA analysis was used for microcosm data analysis. Controls were tested against the treatments using Tukey's post hoc test. Values with $p<0.05$ were considered significantly different and variables in graph not connected by the same letter are significantly different.

Results and Discussion

One of the major reasons for recalcitrance of DDx is chlorine atoms on the molecule (Aislabie et al., 1997). Although, both aerobic (Nadeau et al., 1994) and anaerobic degradation (Langlois et al., 1970; Wedemeyer et al., 1967; Pfaender and Alexander, 1972) of DDx have been reported, anaerobic organisms may have an advantage in dechlorination of the poly chlorinated aromatic DDx (Pfaender and Alexander, 1973; Golovleva and Skryabin, 1981). However, it was not known if anaerobic organisms prefer DDx as electron donor or TEA, such that both possibilities were investigated in lab scale microcosm experiments.

1) DDT as Electron Donor: The first set of microcosms (Figures 3-1 to 3-4) was intended to test the degradation of DDXs under an array of redox conditions, including sulfate, iron and nitrate reducing conditions, and fermenting conditions. The target organisms were sulfate reducing bacteria (SRB), nitrate reducing bacteria (NRB), iron (III) reducing bacteria (FeRB) and mixed acid fermenters in sulfate, nitrate, iron and no exogenous electron donor sets, respectively. The data suggests that following anoxic

incubations, DDx was more extractable from soils. Microcosms (Figures 3-2 to 3-4) intended to stimulate SRB, NRB and FeRB show a statistically significant difference in p,p'-DDT concentrations between treatments and no terminal electron donor sets.

The extractability or availability of p,p'-DDT significantly increased in case of experimental treatments versus fermenters from 86 mmol/ gram of dry soil in case of the latter to 139, 113, and 123 mmol/ gram of dry soil in case of SRB, NRB and FeRB, respectively. Even though DDxs extractability appear to have been increased during anaerobic incubations, there was no evident degradation observed between the controls and the treatments.

Being reduced, the system was already rich in electron donors such that microorganisms could oxidize endogenous electron donors more easily compared to chemically stable DDT. Even though DDxs were bioavailable, proper metabolic requirements were not achieved to sustain and promote growth of the degrading consortia. The system is highly reduced and so there is no evident effect of TEA provided. Regardless, it is unlikely that any of these conditions significantly promote degradation of DDxs.

2) DDT as TEA: The second set of microcosms (Figures 3-5 to 3-8) tested potential degradation of DDx with a range of electron donors, with DDxs serving as the only added potential TEA. These microcosms also tested the influence of sulfate on degradation. Influence of sulfate on degradation was checked because sulfate reducing bacteria (SRB) have previously been reported to dehalorespire chlorinated pollutants (Zwiernik et al., 1998; Fava et al., 2003; Zanaroli et al., 2006). Subsequent transfers to lower sulfate concentrations were conducted with the intention that SRB capable of

utilizing DDT as TEA will shift to dehalorespiration after depletion of sulfate. No significant effect of sulfate was observed in either of the microcosms, which suggests that sulfate reducing bacteria might not be involved in DDx degradation.

p,p'-DDT concentrations ranged from 337 to 382 mmol/ g dry soil in case of control (no electron donor), to 56 to 98 mmol/ g dry soil for acetate sets, about 25 mmol/ g dry soil in case of hydrogen sets to less than 10 mmol/ gram dry soil in case of lactate sets. Hence, there were successively lower concentrations of p,p'-DDT starting from control sets to acetate followed by hydrogen and the lowest concentrations were observed in the lactate sets.

One of the biggest obstacles for degradation of hydrophobic pollutants is bioavailability. Being hydrophobic, the aqueous solubility of compounds is very low and this could hinder bioavailability and hence biodegradation. Anoxic incubations increase the dissolved organic carbon (DOC) fraction of soils and it is the most bioavailable fraction of soil organic matter (Marschner and Kalbitz, 2002). Many researchers showed that DOC can enhance solubility and mobility, and, hence, bioavailability of organic compounds (Blaser, 1994; Piccolo, 1994; Zsolnay, 1996; Marschner et al., 1999).

Previous studies by Pravecek and coworkers (Pravececk et al., 2005) studied PAHs such as benzanthracene and benzapyrene under anaerobic microcosm incubations and demonstrated that the aqueous concentrations of the PAHs increased. Extractability and bioavailability of both the PAHs from soil increased under anaerobic incubations compared to aerobic incubations, and the aqueous solubility for benzapyrene increased by an order of magnitude. They emphasized that microbially mediated changes in oxidation reduction potential caused pH and DOC alteration, which

resulted in direct or indirect PAH release. Similar studies by Kim and Pfander (Kim and Pfaender, 2005) emphasized microbially mediated redox conditions on PAH and soil interactions. They observed that DOM released under highly reduced conditions exhibited higher sorption capacity for pyrene compared to that obtained from aerobic incubations. Another study by Kim et al. (2008), demonstrated that DOM released under anoxic conditions exhibited a higher affinity for PAHs. In these microcosms after anaerobic incubation, the extractability and solubility of PAHs increased by factors as high as 62.8 relative to initial concentrations.

The highest concentrations of p,p'-DDT (ranging from 337 to 382 mmol/ g dry soil) was observed in no-electron donor microcosms (Figure 3-5), which suggests that following anaerobic incubations as the DDxs were desorbed from the soils, there was no utilizable carbon or energy source to sustain the degrading population.

Autoclaving has been shown to alter certain characteristics of soils, which could potentially impact the extractability DDx. No effect of autoclaving was observed with regard to the extractability of DDT in our microcosms with DDx as the electron donor and carbon source (Figure 3-1, and in parallel experiments from Environmental Microbiology/ Chemistry Lab, personal communication, Dr.John Thomas). As can be seen from Figure 3-1, no differences in DDx concentrations was observed between the autoclaved control and the no-electron donor microcosms, indicating that autoclaving did not significantly impact the extractability of the DDx.

Acetate microcosms (Figure 3-6) show significant degradation compared to the no-electron donor sets, but is lower than the hydrogen or lactate sets. This is probably because acetate is not as energy rich as hydrogen or lactate.

Significant loss of p,p'-DDT was observed with H_2/CO_2 as the energy/carbon source (Figure 3-7), with no effect of various sulfate concentrations. This strongly suggests that sulfate reducing bacteria (SRB) are not involved in this process. It is likely that DDx served as terminal electron acceptors in these microcosms in a TEA limiting reduced environment, likely via dehalorespiration. The responsible organisms could have gained energy in the process through electron transport coupled phosphorylation with simultaneous reduction of DDxs as seen with other halogenated compounds (Holliger et al., 1999; Smidt andand de Vos, 2004).

If SRB are responsible for degradation of DDx, they do not use H2 as electron donor. Members of the Chloroflexus group (such as Dehalococcoides) may outcompete SRB for H2. In cases of hydrogen as main electron donor, studies have shown that dehalorespirers would outcompete hydrogenotrophic sulfate reducing bacteria, homoacetogens and methanogens based on energy gains (Fennell andand Gossett, 1998; Loffler et al., 1999). Members of Chloroflexus have previously been shown to dehalorespire on chlorinated compounds (Maymo-Gatell et al., 1997; 1999).

The greatest losses of p,p'-DDT and p,p'-DDE were observed with lactate as electron donor (Figure 3-8). Lactate is fermented to smaller organic acids and hydrogen. Different groups of organisms could be feeding on the lactate fermentation byproducts, syntrophs have previously shown to be involved in dehalogenation processes (Mohn and Tiedje, 1992; Yang and McCarty, 1998; Drzyzga and Gottschal, 2002; Dolfing, 2003; Sung et al., 2003). Syntrophy is a symbiotic relationship between two metabolically different bacteria, where they can together degrade a substance and in

the process satisfy energy needs of both the bacteria which none of them can attain individually (Atlas and Bartha, 1993).

Syntrophs require low hydrogen concentrations for survival and energy gain and they could coexist with dehalorespiring populations (Drzyzga andand Gottschal, 2002). Interspecies hydrogen transfer could efficiently support mutual coexistence of the species involved. Dehalorespirers would be favored by slow hydrogen producing obligate syntrophs fermenting on organic acids over methanogens or acetogens provided they are present in adequate concentrations. They could keep hydrogen concentrations sufficiently low to allow growth of obligate syntrophs (Dolfing, 2003; Sung et al., 2003; Yang and McCarty, 1998).

The much greater loss of DDT observed in lactate microcosms (approximately 83%) relative to the H_2/CO_2 micrososms (approximately 55%) is somewhat surprising since H_2 is a stronger reductant than lactate. This may indicate different groups of microorganisms are stimulated under the two conditions.

Inference

DDx residues persist in soils through various interactions with soil organic matter decreasing the bioavailability; hence, becoming recalcitrant. Many researchers demonstrated that DOM can enhance solubility and mobility, and, hence, bioavailability of organic compounds (Blaser, 1994; Piccolo, 1994; Zsolnay, 1996; Marschner et al., 1999). Anaerobic incubations increase the DOM fraction of soils which is the most bioavailable fraction of soil organic matter. Once the DDx are made bioavailable following anoxic incubations, suitable electron donors and acceptors should be present to facilitate growth of degrading population.

Once bioavailability is addressed, the next challenge is to selectively stimulate desirable population which can be achieved by intentional use of electron donor and acceptor combination. In TEA limited anoxic environment, TEAs generating high energy are exhausted first followed by lower energy yielding TEAs. So the more energy generating TEAs would get a preference over the lower energy generating TEAs. Thus, TEAs under anaerobic conditions govern community composition (Bossert et al., 2003; Susarla et al., 1996).

Our experiments demonstrated that the responsible consortia used DDx more efficiently as TEA than as donor. Observations from this study are in agreement with other studies stating that dehalorespiration is thermodynamically favorable when compared to sulfate or iron reducing organisms, acetogens, or methanogens (Bossert et al., 2003; Ballapragada et al., 1997; Fennell et al., 1997; Fennell and Gossett, 1998; Loffler et al., 1999; Smatlak et al., 1996; Susarla et al., 1996; Yang and McCarty, 1998).

The highest degradation (about 83%) was observed in lactate microcosms, where lactate would have provided the electron and carbon needs, and DDT served as TEA to the degrading population. The responsible consortia could have a syntrophic relationship such that lactate is fermented to smaller organic acids such as formate, acetate, and hydrogen. Different groups of organisms such as dehalorespiring population could be feeding on the lactate fermentation byproducts. Syntrophs have previously been shown to be involved in dehalogenation processes; hence, degradation could be tentatively explained by presence of syntrophic relationships between hydrogen producing lactate fermenting organisms and dehalogenating populations.

Summary

Once the highest degrading donor: acceptor pair (lactate: DDT) was achieved, further research to scale up of microcosm to mesocosm studies would demonstrate its applicability. To better understand the processes it is necessary to enrich and isolate stable consortia and identify organisms involved. This would help to understand the pathways and mechanisms involved and will be used to check degradation reproducibility. Such studies would help determine the bioremediation potential of the dehalogenating community and its applicability at environmental sites.

Table 3-1. Microcosms testing DDT loss under different electron accepting conditions.

TEA	Electron donor
$SO_4^{=}$	DDT
NO_3^-	DDT
Fe^{+3}	DDT
No TEA	DDT

Table 3-2. Microcosms with various electron donors and DDT as terminal electron acceptor (TEA)

TEA	Electron donor	Carbon source
DDT	Acetate(20mM)	Acetate
DDT	Lactate(20mM)	Lactate
DDT	H_2 (100kPa)	CO_2
DDT	No exogenous source	No exogenous source

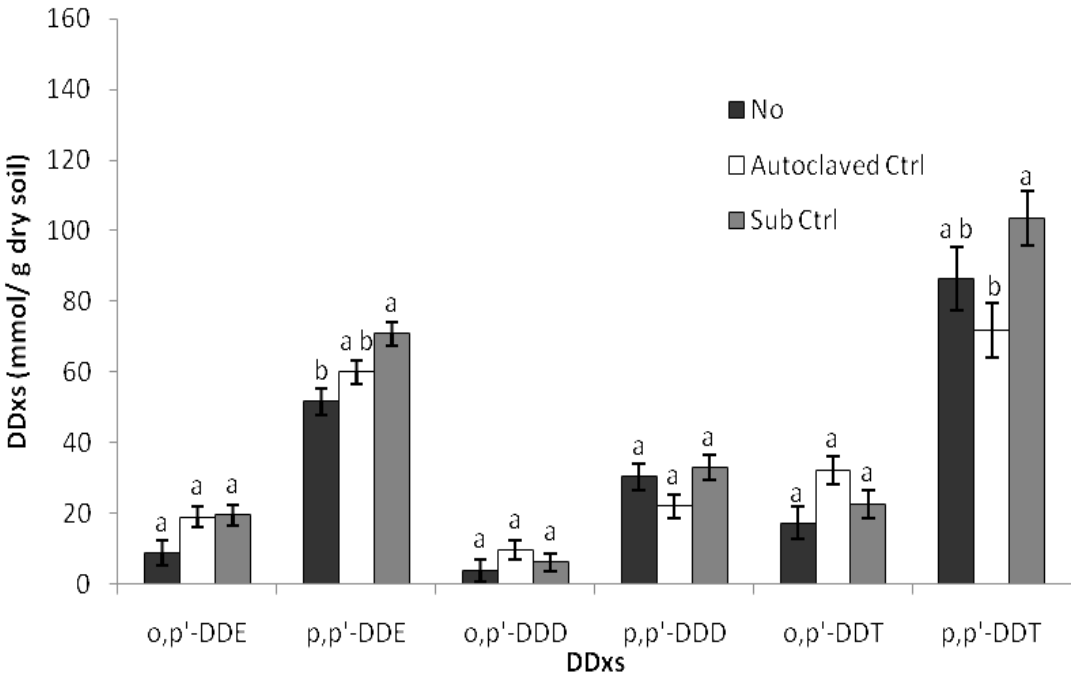


Figure 3-1. Concentration of DDxs with no additional terminal electron donor or electron acceptor added. Error bars represent +/- one standard deviation based on three replicates. No = no exogenous TEA provided, Sub Ctrl = substrate control, i.e., DDT not spiked on these soils. Data labels not connected by same letter are significantly different $p<0.05$.

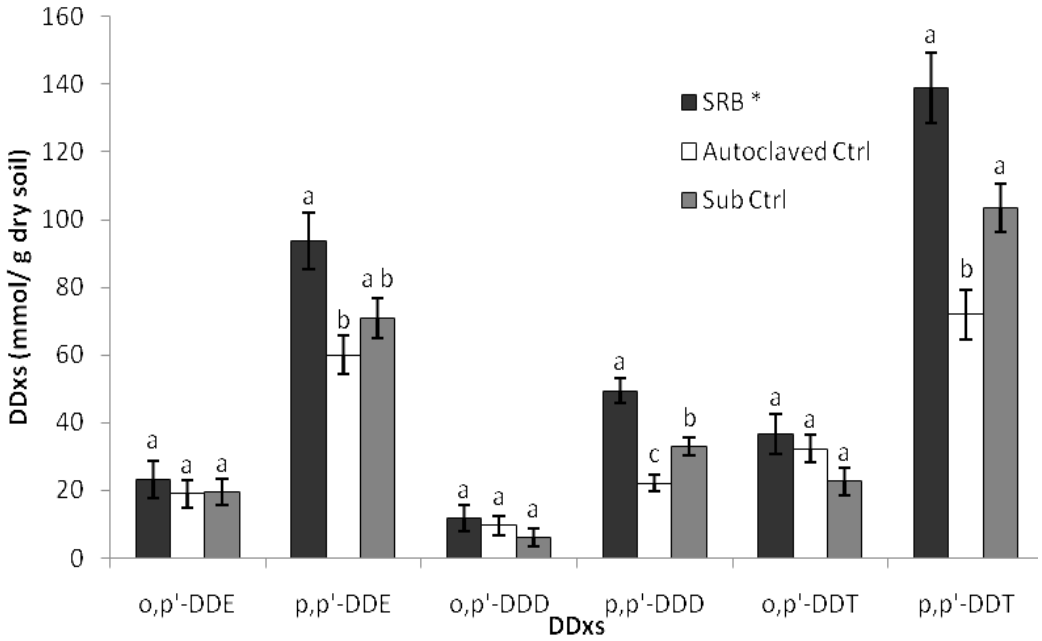


Figure 3-2. Concentrations of DDx under sulfate reducing conditions. Error bars represent +/- one standard deviation based on three replicates. Data labels not connected by same letter are significantly different $p<0.05$.

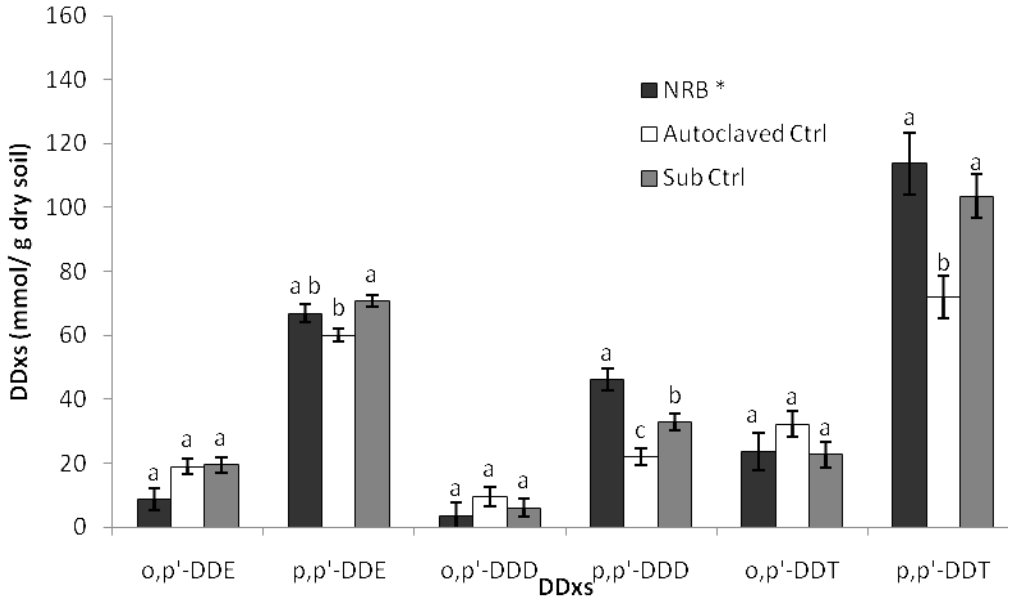


Figure 3-3. Concentrations of DDx under nitrate reducing conditions. Error bars represent +/- one standard deviation based on three replicates. Data labels not connected by same letter are significantly different $p<0.05$.

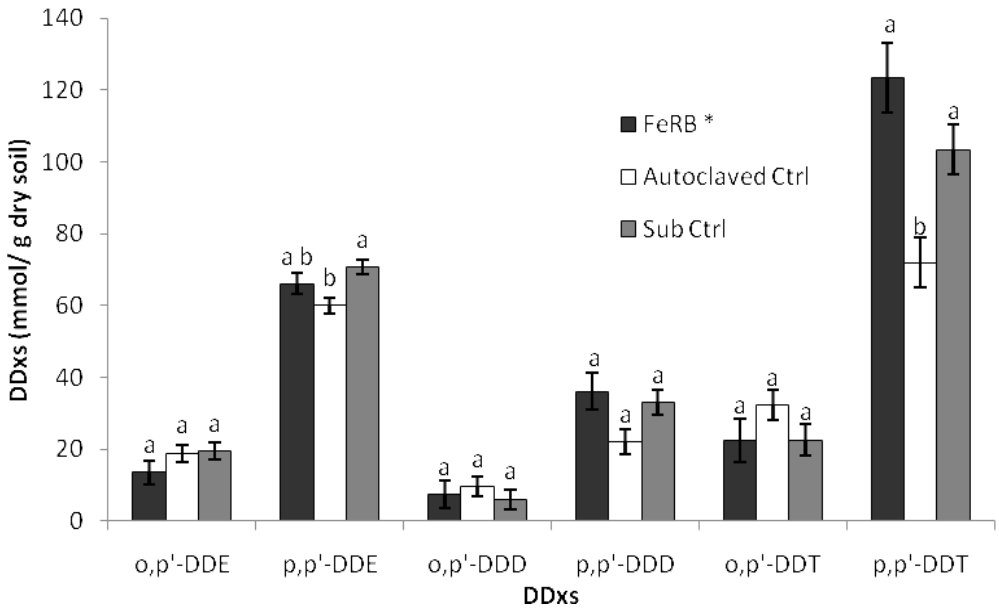


Figure 3-4. Concentrations of DDx under Fe(III) reducing conditions. Error bars represent +/- one standard deviation. Data labels not connected by same letter are significantly different $p<0.05$.

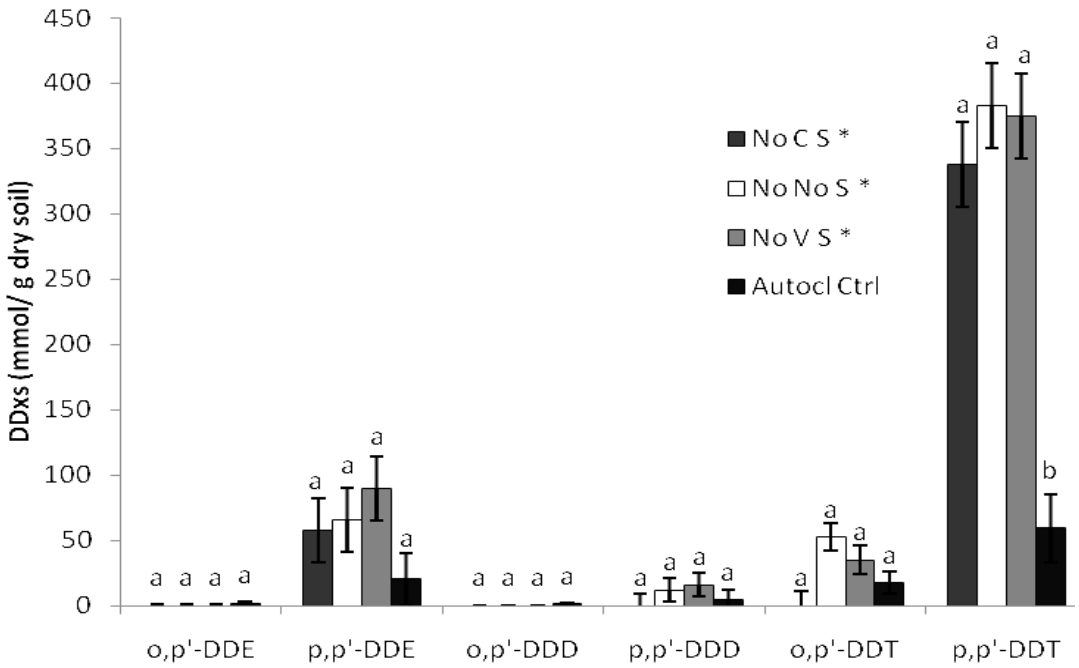


Figure 3-5. Final concentrations of DDx with no external electron/ carbon source or terminal electron acceptor amended. No S, C S, and V S refer to microcosms with no added sulfate, constant sulfate, and variable sulfate, respectively. Error bars represent +/- one standard deviation based on three replicates, except where denoted by *, which indicates 2 replicates. Data labels not connected by same letter are significantly different $p<0.05$.

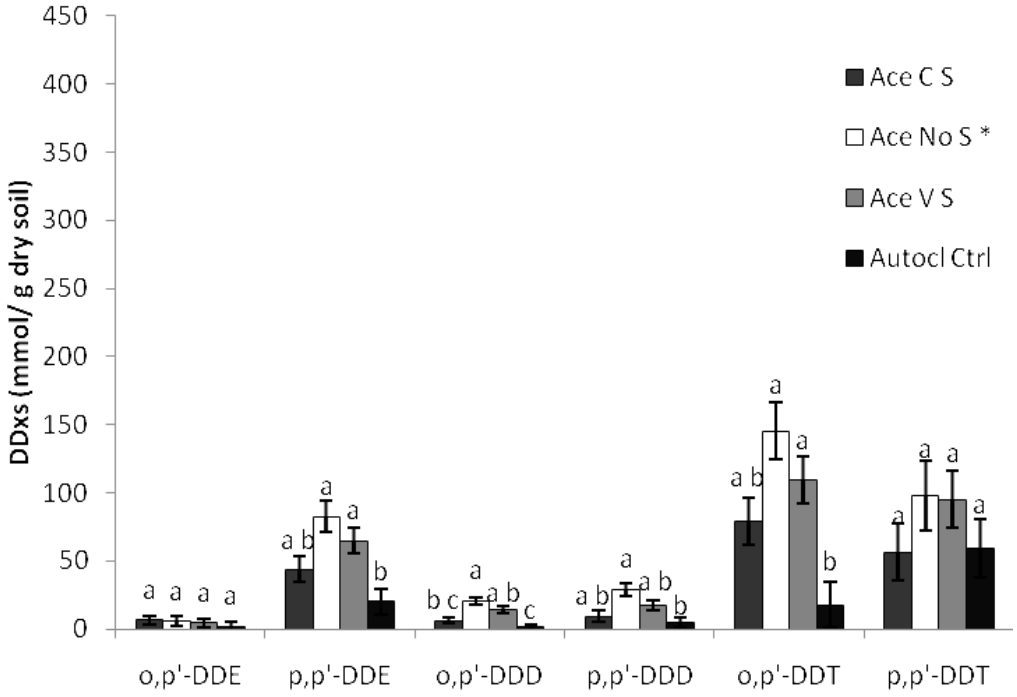


Figure 3-6. Final concentrations of DDx with acetate as electron/ carbon source. No S, C S, and V S refer to microcosms with no added sulfate, constant sulfate, and variable sulfate, respectively. Error bars represent +/- one standard deviation based on three replicates, except where denoted by *, which indicates 2 replicates. Data labels not connected by same letter are significantly different $p<0.05$.

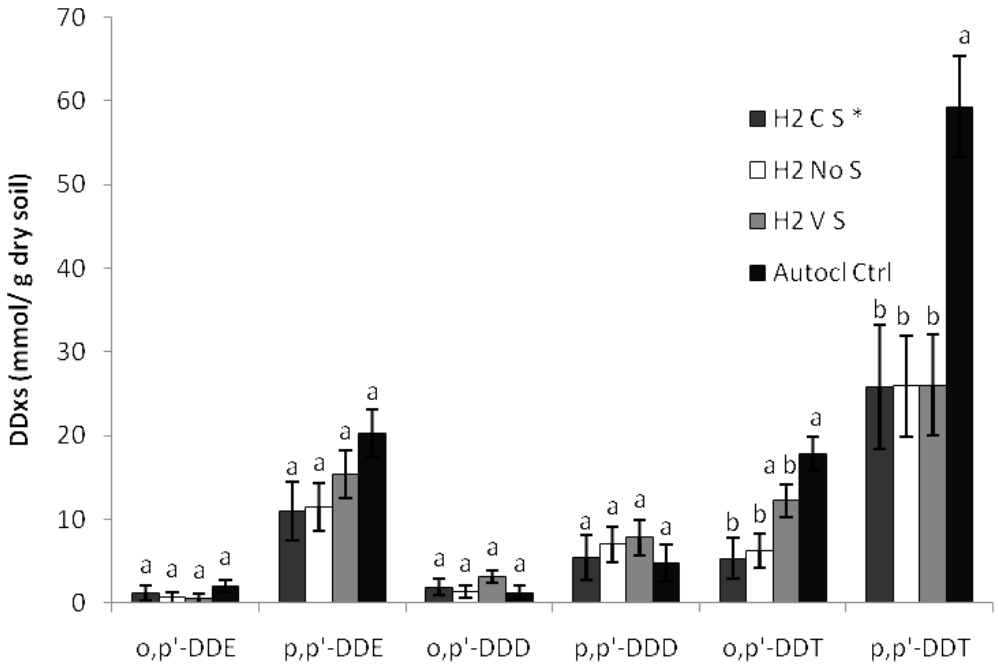


Figure 3-7. Final concentrations of DDx with H_2/CO_2 as electron/ carbon source. No S, C S, and V S refer to microcosms with no added sulfate, constant sulfate, and variable sulfate, respectively. Error bars represent +/- one standard deviation based on three replicates, except where denoted by *, which indicates 2 replicates. Data labels not connected by same letter are significantly different $p<0.05$.

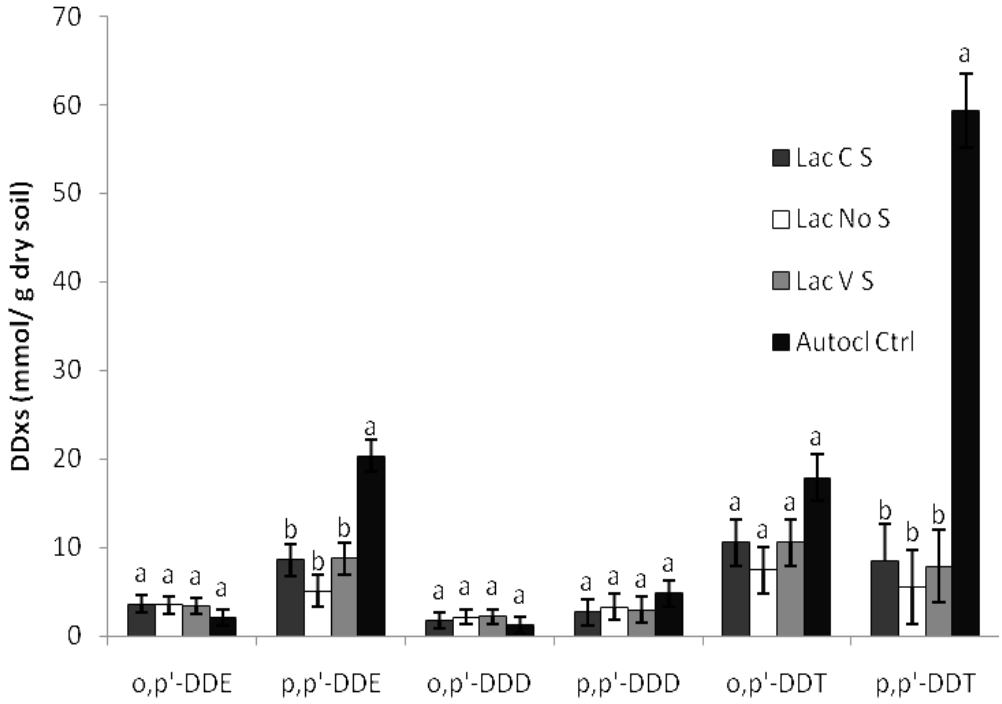


Figure 3-8. Final concentrations of DDx with lactate as electron/ carbon source. No S, C S, and V S refer to microcosms with no added sulfate, constant sulfate, and variable sulfate, respectively. Error bars represent +/- one standard deviation based on three replicates. Data labels not connected by same letter are significantly different $p<0.05$.

CHAPTER 4
ANAEROBIC DEGRADATION OF DDT AND ITS METABOLITES STIMULATED BY LACTATE AMENDMENTS IN MESOCOSM EXPERIMENTS

Biological degradation of chlorinated compounds in anoxic environments might be selectively stimulated by careful selection of electron donors and acceptors (Lee et al., 1998; Suflita et al., 1988). Once target organisms are enriched, reductive dechlorination would promote the dominant process for bacterial dechlorination, and dechlorinating organisms would conserve energy with simultaneous reductive dehalogenation of halogenated compounds in a process termed “dehalorespiration” (El Fantroussi et al., 1998).

In order to optimize the donor:acceptor combinations leading to highest degradation rates in Lake Apopka soils, laboratory microcosm studies were initiated to test an array of electron donor:acceptor combinations. It was not known whether DDT would serve as a better electron donor or acceptor to the responsible consortia, such that both possibilities were investigated in microcosm experiments (Chapter 3). Those results suggested that use of DDT and its metabolites (collectively known as DDx) as terminal electron acceptors (TEA) lead to greater degradation rates in Lake Apopka soils than as electron donors. The greatest degradation was observed in microcosms with lactate as electron donor. It may be that the degrading coupled lactate fermentation with DDx degradation in microcosms.

The specific mechanisms have not yet been investigated, but could be explained by a syntrophic association between lactate fermenting hydrogen producing organisms and a dehalorespiring population. Lactate can be fermented to acetate, formate, and hydrogen. A dehalorespiring population could feed on lactate fermentation byproducts while participating in close syntrophic associations with hydrogen producing organisms

which require low hydrogen concentrations for energy gain and survival (Drzyzga and Gottschal, 2002).

Once the highest degrading donor:acceptor combination (lactate: DDT) was identified, we wanted to investigate its applicability at larger scales. This chapter is dedicated to scaling up of lactate applications to the mesocosm scale. We hypothesized that once DDx are made available during anoxic incubations, lactate would support growth of the dechlorinating consortia. Another intention was to determine if extractability and, hence bioavailability, increase as a result of dissolved organic carbon (DOC) release at mesocosm levels.

Materials and Methods

Mesocosm Soil Collection

Soils from the Lake Apopka, FL sample site ZNS2017 located in the North Shore Restoration Area (NSRA) north boundary were collected from a 16 foot by 16 foot plot east of Laughlin Road (UTM coordinates: X = 440603.7, Y = 3177660.0). The site was selected because of high DDx concentration as determined by Pace Analytical Services, reported as follows: p,p'-DDE was 5990, p,p'-DDD was 564, and p,p'-DDT was 15800 µg/kg soil (on a dry weight basis). Concentrations were determined by analytical method EPA 8081 and sample preparation method EPA 3550 (EPA, 1994). Total Organic Carbon (TOC) was reported by DB Environmental, Inc. to be 37 %. Vegetation on the top layer of the soils was removed and soils were homogenized on site using a trackhoe. Following mixing, the soils were loaded into a dump truck using a front end loader. The dump truck transported the soil to the University of Florida, where the soils were stored in wooden boxes lined with a plastic liner.

Preparation of Anaerobic Mesocosms

Mesocosms were constructed from 100-gallon Rubbermaid^{R} "Farm Tough" stock tanks made of high density polyethylene and were based on a design used by the Wetland Biogeochemistry Laboratory at the University of Florida. The dimensions at the top of the tanks were: length and width of 54 inches (137.16 cm) and 35 inches (88.9 cm), respectively, and the height was 23 inches (58.42 cm) (Figure 4-1). The tanks had a controllable opening at the bottom of tank and a bed of egg rocks to allow drainage.

Six tanks were placed on cement block tables inside a greenhouse (Figure 4-2). The order of the mesocosms tanks, from north to south, was Control-1, Lactate-1; Control-2, Lactate-2; Control-3, Lactate-3. This design was intended to randomize any effects that location in the greenhouse might have, such as temperature.

Soil was added to the mesocosm tanks to a depth of approximately 30 cm (12 inches). Soil from wooden bins was mixed manually using shovels and added to the wheel barrows which were used to fill the tanks sequentially using one wheel barrow load at a time. Once in tanks the soil was mixed again with a shovel, then water was added to height of 10 cm above the soil layer. For lactate tanks, lactate was added to 20 L containers and dissolved in tap water to yield a final concentration of 10 mM. Lactate solution rather than solid addition was used to obtain a more homogenous distribution within the tanks. Approximately 160 L lactate solution was required to fill each treatment tank to 10 cm above soil surface. Ten soil samples were collected randomly from each mesocosm at a depth of 5 cm, mixed to form one composite sample per tank, and frozen at -80^{o}C. Continual replacement of lactate solution was done on a weekly basis to maintain the aqueous phase 10 cm above the soils.

Sample Collection

Upon stabilization of redox potentials in the mesocosms, eight samples were collected from each tank and mixed to obtain a homogenous composite sample. Samples were collected once every two weeks using a 50 mL disposable syringe. The ends of the syringes were cut off and a rubber stopper was used to create suction to hold the sediment sample. A syringe was used for sampling to avoid disturbance and homogenization of the layers in the sample. Samples were stored at -80°C until analysis.

Redox Potential (Eh) and Temperature Measurements

Redox potential was determined at biweekly intervals during the anoxic treatment. Eh was measured with a portable pH/ Eh and Temperature meter (HI 9126 Hanna Instruments; Woonsocket, RI). The probe was calibrated before every use. The Eh probe was submerged in each tank to a depth of 10 cm and allowed to equilibrate before taking the reading; temperature readings were taken at the same depth using a temperature probe.

Dissolved Organic Carbon (DOC) Measurements

DOC was extracted from soils using cold water extraction method. One gram soil on a dry weight basis was diluted to a concentration of 1:10 (soils: distilled water). Sample slurries were incubated for 16 hours at room temperature, followed by centrifugation in Beckman J2-21 Floor Model Centrifuge (GMI Incorporation Ramsey, MN) (JA-14 rotor) at 4472 x g for 15 minutes. The resulting supernatant was filtered through a 0.22 µm filter (Durapore PVDF membrane filters, Fisher Scientific, Pittsburgh, PA) under vacuum. Filtered solutions were analyzed on Shimadzu sample module 5000A Carbon Nitrogen Analyzer (Columbia, MD) in the UF Forest Soils Laboratory.

pH Measurements

pH was analyzed from pore water of soil samples. Soil samples were centrifuged in a Beckman centrifuge Model J2-21 at 7155 x g for ten minutes. pH was measured from supernatant using Orion pH meter model SA720(Cole-Parmer Instrument Company Vernon Hills, IL).

DDx Extraction

DDx extraction could be divided into three stages: soil preparation; accelerated solvent extraction (ASE); and florisil extraction. The extraction method was based on U.S.EPA method #3545 (U.S.EPA, 2000) with minor modifications developed by Soil Microbial Ecology Laboratory, University of Florida, and chemists at Pace Analytical Services, Ormond Beach, FL.

Soil Preparation

Soils were separated from liquid by centrifugation in Beckman J2-21 Floor Model Centrifuge (JA-14 rotor) at 7155 x g for 20 minutes at 4°C. Wet soil samples were allowed to air dry for two to three days following which they were ground. Soil moisture content was determined in the sample. Moisture was adjusted to 50% moisture on a dry weight basis, and then the samples were allowed to equilibrate in 4°C refrigerator for 3 days.

Accelerated Solvent Extraction

Soil was mixed with Hydromatrix, a drying and bulking agent, at a ratio of 1:2, and placed into 34 mL stainless steel extraction cells. The remaining headspace in extraction cells were topped with clean Ottawa sands (Fisher Scientific, Pittsburgh, PA) to decrease the amount of solvent usage. Solvent used for extraction was methylene chloride: acetone (4: 1 v/v). Soils were extracted under high pressure about 1200 to

1400 psi at temperature of 100°C in a Dionex ASE 100 Accelerated Solvent Extractor (Sunnyvale, CA).

The extraction cycle included filling the extraction cell with about 19 mL solvent, followed by heating cycle where the solvent was heated to 100°C. Static extraction followed the heating cycle which performed for 5 minutes, followed by flushing cycle which flushed about 19mL solvent through the cell. The cell was finally purged with N_2 for about 2 minutes, which completed one extraction cycle.

Florisil Extraction

Florisil clean up used prepacked florisil “Bond Elute LRC” columns (200 µm particle size) (Varian Inc., Palo Alto, CA) which contained approximately 1 g Florisil packaged into the plastic holder. The column was conditioned using 5 mL hexane: acetone (9:1v/v), loaded with 1 mL of ASE extract and the column was eluted using 9 mL of solvent.

A volume of 5 mL from the cleanup volume was concentrated to dryness under a gentle stream of air. It was very important to concentrate the samples to complete dryness prior to GC (gas chromatography) analysis to avoid matrix response enhancement (Schmeck and Wenclawiak, 2005). Samples were reconstituted in 1 mL of hexane, and tubes with samples were vortexed and transferred to a 2 mL amber glass GC vials (Fisher Scientific Inc., Atlanta, GA) and crimped with 12 x 32mm aluminum crimp seals with prefitted PTFE lined septa (Fisher Scientific Inc., Atlanta, GA) for subsequent analysis by GC.

GC Conditions

Perkin-Elmer Autosystem Gas Chromatograph (GC) equipped with autosampler and an electron capture detector (ECD) was used for analyzing the extracts. Column

conditions were: He as the carrier gas at flow rate of 1.0 mL/min, 5% methane in argon as the make-up gas with a flow rate of 50 mL/min, electron capture detector (ECD) temperature at 350 °C, and injector temperature at 205°C in splitless mode. Injection volume was 3 µL in slow injection mode. GC temperature was programmed at 110 °C for 0.5 minutes, followed by a ramp at 20°C/min to 210°C and hold for 0 minutes, then ramp at 11°C/min to 280°C and held for 6.3 minutes. Under these conditions, the detection limit was calculated at the 90% confidence level to be: 0.007 µg/mL for o,p'-DDE; 0.005 µg/mL for p,p'-DDE; 0.006 µg/mL for o,p'-DDD; 0.009 µg/ mL for p,p'-DDD; 0.005 µg/mL for o,p'-DDT; and 0.010 µg/mL for p,p'-DDT (Hubaux and Vos, 1970). These results are similar to the detection limit of 0.010 µg/mL reported by Pace, Inc.

Analysis of Organic Acids by HPLC

Sample Preparation

One gram soil on a dry weight basis was diluted to a concentration of 1:10 (soils: distilled water). Sample slurries were incubated for 16 hours at room temperature, followed by centrifugation in Beckman J2-21 Floor Model Centrifuge (JA-14 rotor) at 4472 x g for 15 minutes. The resulting supernatant was filtered through a 0.22 µm filter (Durapore PVDF membrane filters, Fisher Scientific, Pittsburgh, PA) under vacuum.

Derivatization

Samples were derivatized using following steps. Pyridine buffer (0.2 mL) was added to 2 mL sample. The resulting solution was purged with nitrogen to remove CO_2 and O_2 for four minutes. Anoxic solution got amended with 0.2mL of 0.1M 2-nitrophenyl hydrazine (in 0.25M HCl) and 0.2 mL of 0.3 M /-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride, after mixing the vials were incubated at room temperature

for 90 minutes. Following incubation 0.1mL of 40% KOH was added and the samples were heated in a heating block at 70^{o}C for 10 min.

HPLC

Concentrations of fatty acids were determined using HPLC with UV/VIS detector at 400 nm wave length and a C8 reverse phase column (22 cm $\times$ 1.5 cm). Mobile phases included 2 solvents. Solvent A was composed of 2.5% n-butanol, 50 mM Sodium acetate, 2 mM Tetrabutylammonium hydroxide, 2 mM Tetradecyltrimethylammonium bromide (TDTMABr) with pH adjusted to 4.5 using phosphoric acid. Solvent B differed from solvent A only in containing 50 mM TDTMABr. The injection volume was 100 μL. Retention times for lactate, acetate, propionate, formate were 9.95, 12.62, 14.67 and 13.28 minutes, respectively. Standards fatty acids used were lactate, acetate, formate, propionate, butyrate, succinate, iso-butyrate, iso-valerate (Albert and Martens, 1997; Dhillon et al., 2005; Jonkers et al., 2003).

Statistical Analysis

Statistical comparisons were conducted using the one way ANOVA analysis in JMP manufactured by SAS (Cary, NC). Controls were tested against the treatments using Tukey's post hoc test. Mesocosm data sets were compared using studentized t-test. Values with $p<0.05$ were considered significantly different and variables in graph not connected by the same letter are significantly different.

Results and Discussion

Mesocosms were established not only to scale up the microcosms, but also to evaluate reproducibility of the previous results. Hydrophobic organics sequester in the soil or sediment through interactions with solid or sediment phase or they may diffuse into micropores or nonaqueous phase liquids, thereby decreasing their bioavailability

(Luthy et al., 1997). Anoxic incubations release DOC, such that an additional aim of the mesocosm experiments was to confirm DOC release upon anaerobic incubations and to investigate if DOC release facilitated DDxs extractability. The greatest amount of transformation of DDx in microcosms was observed with lactate as an electron donor (Chapter 3), suggesting that lactate stimulated, either directly or indirectly, microbial groups capable of utilizing DDx as a terminal electron acceptor.

Lactate was chosen as it naturally enters anoxic soils via a variety of pathways from decomposition and fermentation of plant matter, particularly from fermentation of carbohydrates such as cellulose (Daeschel et al., 1987). Stimulation of DDx metabolism by addition of lactate in microcosms indicates that the pathways responsible for DDx are limited in electron donors; the addition of electron donors to soils led to increased degradation rates.

As described above, mesocosms were established in an alternating fashion from east to west (control, lactate, control, lactate, control, and lactate) within the greenhouse to average out any temperature effects between the sides of the greenhouse. As shown in (Figure 4-3), no difference between mesocosms was observed in temperature, indicating good temperature regulation throughout the greenhouse.

Redox potential (Eh) (Figure 4-4) is an important indicator of the dominant microbial processes in anaerobic systems, and can be used as a general indicator of the shift between aerobic and anaerobic systems. Redox potentials in control mesocosms decreased more slowly than those with lactate added. Lactate mesocosms exhibited Eh values below -200 mV within 58 days following establishment of the mesocosms; however, control mesocosms required approximately 7 more weeks (41

days) to drop below -200 mV. The more rapid drop in redox potential exhibited by the lactate mesocosms is expected and is characteristic of more bioavailable carbon.

DDx concentrations in control and lactate mesocosms throughout the incubation period are presented in Figures 4-5 and 4-6, respectively. Of significance in both sets of mesocosms is the apparent rapid increase in p,p'-DDT and p,p'-DDE concentrations observed between the 58^{th} and 64^{th} days of incubation. This is likely due to an increase in extractability following anoxic incubations. Significantly, these increases in measured DDx concentrations correlate with the drop in redox potentials and simultaneous increase in DOC in both mesocosms. In Figures 4-5 and 4-6, the phase with rapid increases in DDx concentrations is referred to as the "Release phase."

Experiments with soils and sediments demonstrated that reductive dechlorination decreased with an increase in redox potential (Bradley, 2000; Dolfing and Beurskens, 1995; Gerritse et al., 1997; Haggblom et al., 2000; Kuhlmann and Schottler, 1996; Lee et al., 1997; Pavlostathis and Zhuang, 1993; Stuart et al., 1999). Other investigators observed that with decreases in redox potentials, the dissolved organic carbon concentrations increased, which in turn decreased the adsorption of hydrophobic molecules to soils (Pravecek et al., 2005; Kim and Pfaender, 2005; 2008). Hence, decreases in adsorption not only increase the aqueous solubility but also increase the bioavailability, which in turn increases biodegradation potential. Other researchers have reported that addition of DOC to the aqueous phase reduced sorption of DDT to soils (Suffet and Belton, 1985). Cho and group (2002) stated that DOM can increase the solubility of hydrophobic molecules. In that study, they did a comparison between using

DOM and surfactant versus DOM only and found that apparent solubility of the hydrophobic compounds tested was higher in DOM only.

Hydrophobic molecules associated with soil organic matter determines the solid and aqueous phase concentrations and can help predicting bioavailability and fate of degradation (Chiou et al., 1986; Cho et al., 2002; Gamst et al., 2007; Johnson and Amy, 1995; Mackay and Gschwend, 2001; Rutherford et al., 1992). Pravecek and coworkers (2005) discovered that anoxic conditions stimulate aqueous solubilization of hydrophobic molecules. Under highly reduced conditions, use of the available TEAs results in more reduced conditions, followed by subsequent hydrogen ion consumption. This causes the pH to become more alkaline causing higher dissolution and release of SOM resulting in enhanced aqueous solubilization of hydrophobic moieties.

Dissolved organic carbon (DOC) contents were measured in pore waters for each of the sampling dates (Figure 4-7). As expected, DOC concentrations increase with incubation time with simultaneous decreases in redox potential and increase in p,p'-DDE and p,p'-DDT concentrations. Following the "release phase," p,p'-DDT concentrations in the treatment tanks were consistently lower than in the control tanks ($p < 0.05$). This observed decrease in DDT concentrations coincided with lower Eh (Figure 4-4) and alkaline pH values in treatments (Figure 4-8). This phase is termed the "degradation phase."

Concentrations of organic acids in the DOC were measured to determine if lactate fermentation products were present, or if the dominant DOC was likely to be derived from soil organic matter. Since there were two peaks with significant release of DOC, 64^{th} day and 114^{th} day samples from these days were chosen for analysis. Results from

the 64th and 114th days are presented in Figures 4-9 and 4-10, respectively. The major short chain fatty acids observed in these samples were acetate, lactate, and formate, although formate concentration was low in the 114th day samples, and no butyrate or isobutyrate were detected in either lactate or control samples.

Samples collected on the 64th day have significantly lower concentrations of acetate (34 µg/g soil) and formate (60 µg/g soil) in lactate mesocosm samples while the controls had, 525 µg/g soil acetate and 269 µg/g soil formate, respectively (Figure 4-9). This can be explained as the system was carbon limited before the release of DDx, such that there was not enough carbon to promote growth of the degrading population. In the treatment mesocosms, lactate enriched the degrading population that could use DDx (presumably as TEA) once they were released with DOC. Lower concentrations of these short chain fatty acids suggest that the degrading consortia utilized them for dehalorespiration on DDxs. In the lactate mesocosms, organic acids on 114th day were not significantly different from control Figure 4-10 except acetate because of the poor reproducibility among replicates.

Following release, the DDx were made more bioavailable, and since lactate was present to sustain the degrading population, greater degradation was observed in the lactate treatment. This is in accordance with the microcosm studies, in which the greatest losses of p,p'-DDT and p,p'-DDE were observed with lactate as electron donor. Different groups of organisms could be feeding on the lactate fermentation byproducts; syntrophs have previously shown to be involved in dehalogenation processes (Mohn and Tiedje, 1992; Yang and McCarty, 1998; Drzyzga and Gottschal, 2002; Dolfing, 2003; Sung et al., 2003).

Syntrophs require low hydrogen concentrations for energy gain and can coexist with dehalorespiring populations (Drzyzga and Gottschal, 2002) that aid in maintaining low H_2 concentrations. Dehalorespirers would be favored by slow hydrogen producing obligate syntrophs fermenting organic acids over methanogens or acetogens, provided they are present in adequate concentrations (Dolfing, 2003; Sung et al., 2003; Yang and McCarty, 1998).

Inference

One of the biggest obstacles for degradation of hydrophobic pollutants in soil with a high organic matter content is bioavailability. Anoxic incubations increase the DOM fraction of soils which is the most bioavailable fraction of soil organic matter (Marschner and Kalbitz, 2002). DOM can enhance solubility, mobility, and, hence, bioavailability of organic compounds (Blaser, 1994; Piccolo, 1994; Zsolnay, 1996; Marschner et al., 1999).

Anoxic incubations increased aqueous DDx concentrations both in case of lactate and controls indicating that anaerobic incubations increased extractability of DDx. Increased extractability coincided with drop in Eh and increase in DOC. These reduced conditions lead to higher hydrogen ion consumption, thus, more alkaline environments resulted in conjunction with higher dissolution and release of soil organic matter (SOM). With the release of SOM, the otherwise unavailable hydrophobic molecules are more bioavailable and, hence, prone to biodegradation.

Following the “release phase,” p,p’-DDT concentrations in lactate mesocosms were consistently lower ($p<0.05$) compared to the controls. Lactate selectively enriches the dehalorespiring population. This “degradation phase” can be explained as the system was carbon limited and, being anoxic, it is limited in TEAs; when lactate was

present it sustained the degrading population to dehalorespire on DDxs. Lactate is fermented to small organic acids and hydrogen; hence, hydrogen producing organisms may be involved in syntrophic associations with the dehalorespiring organisms.

Summary

Further research is needed to identify the composition of DOC, organisms, mechanisms, byproducts, and pathways involved. To better understand the processes, it is necessary to enrich and isolate stable consortia and identify organisms involved. The structure of dechlorinating community is very important to understanding their bioremediation potential, as the metabolic pathways such communities use might be completely different from the known paths. Such studies would help determine the bioremediation potential of the dehalogenating community and its applicability at environmental sites. To ensure environmental and ecological safety, another important task would be to study the reactivity and effects of the byproducts before application to field level. Although, anoxic bioremediation of DDx remains an attractive option, one major drawback of anoxic degradation could be release of DOC. This could not only increase the mobility of DDx, since it could increase DDx runoff; hence, contaminating offsite of NSRA. The increase in mobile DDx could also increase uptake and bioaccumulation along the ecosystems. Additionally, another cheap, safe, and environmentally-friendly substitute for lactate, such as plant based materials, should be studied for application to vast sites.

Figure 4-1. Mesocosm tank, used for construction of anaerobic mesocosms. Photo courtesy by Hiral Gohil.

Figure 4-2. Anaerobic mesocosms for bioremediation of DDE, DDD, and DDT in soil from the Lake Apopka North Shore Restoration Area. Photo courtesy by Hiral Gohil.

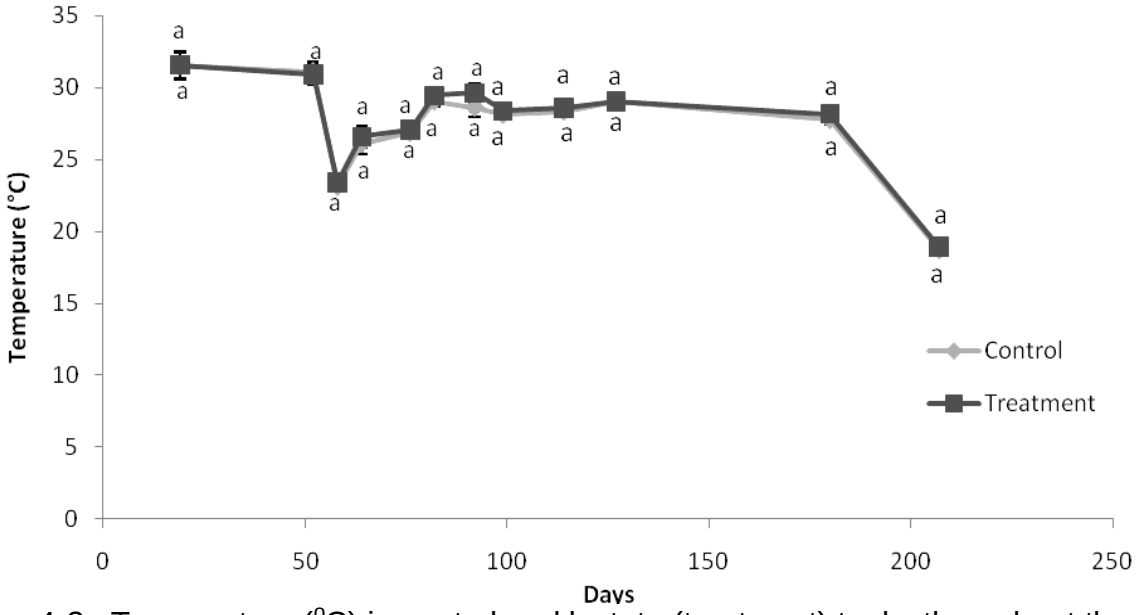


Figure 4-3. Temperature (^{o}C) in control and lactate (treatment) tanks throughout the study period. Error bars represent +/- one standard deviation based on three replicates. Data labels that do not share the same letter are significantly different at $p<0.05$.

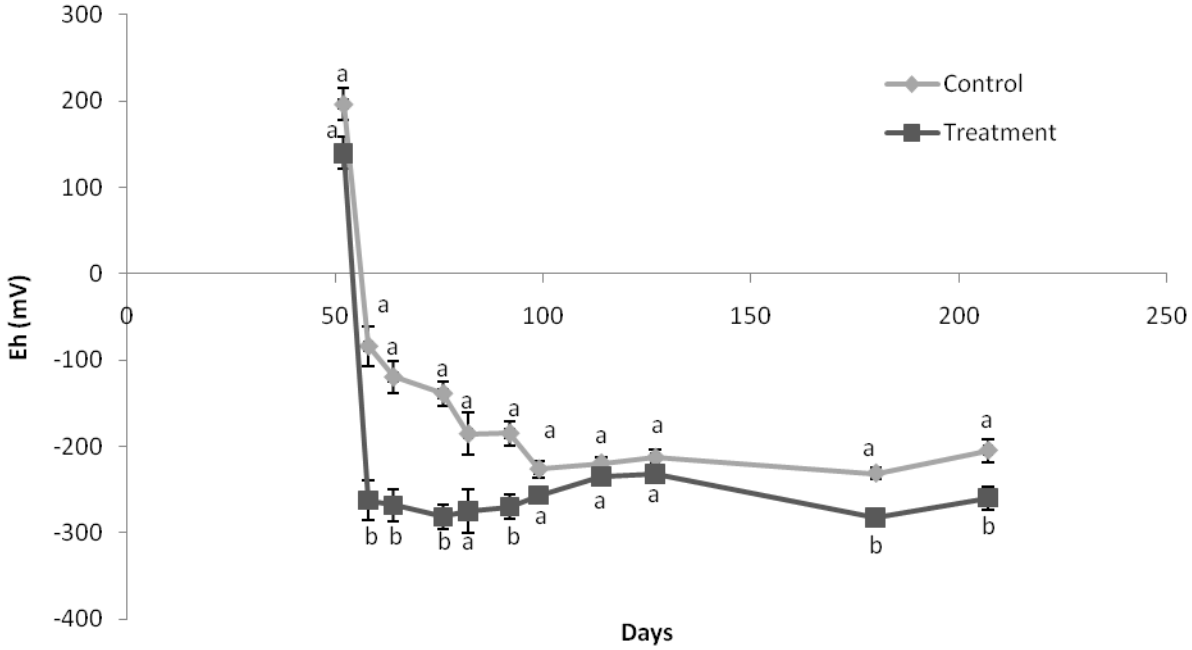


Figure 4-4. Redox potentials (mV) in control and lactate (treatment) tanks throughout the study period. Error bars represent +/- one standard deviation based on three replicates. Data labels that do not share the same letter are significantly different at $p<0.05$.

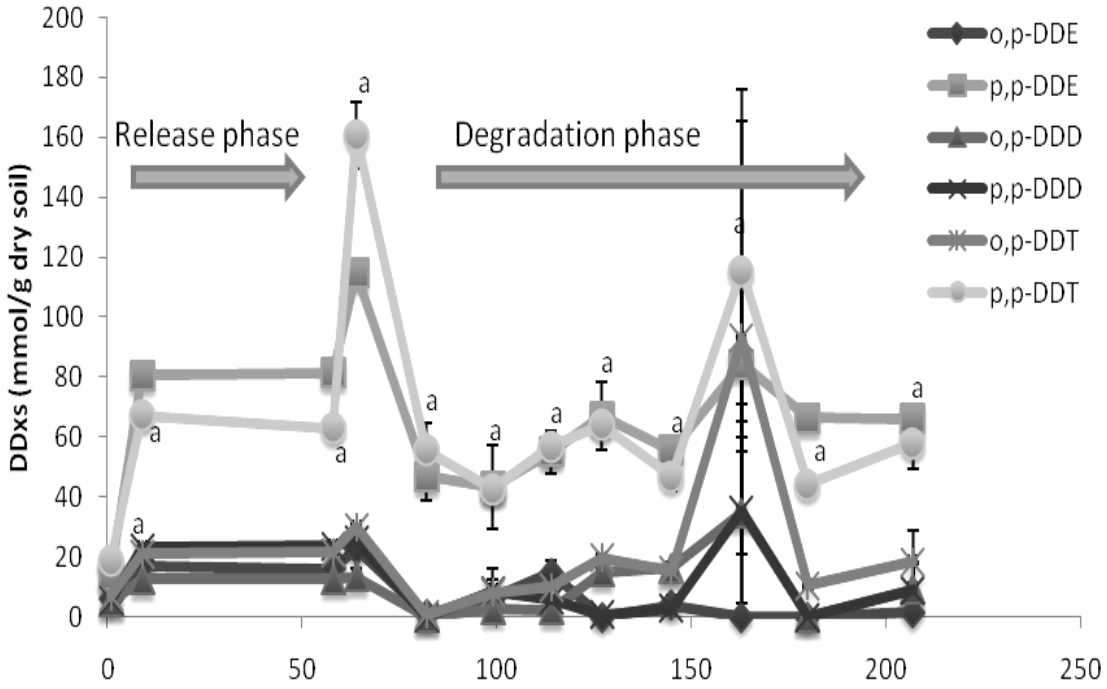


Figure 4-5. DDx concentrations (mmol/g dry soil) in control mesocosm tanks throughout the study period. Error bars represent +/- one standard deviation based on three replicates. Statistical analysis output is presented with data labels. Other than p,p'-DDT, all the other DDxs were not significantly different hence to avoid over crowding in the figure data labels are only for p,p'-DDT. Data labels that do not share the same letter are significantly different at $p<0.05$.

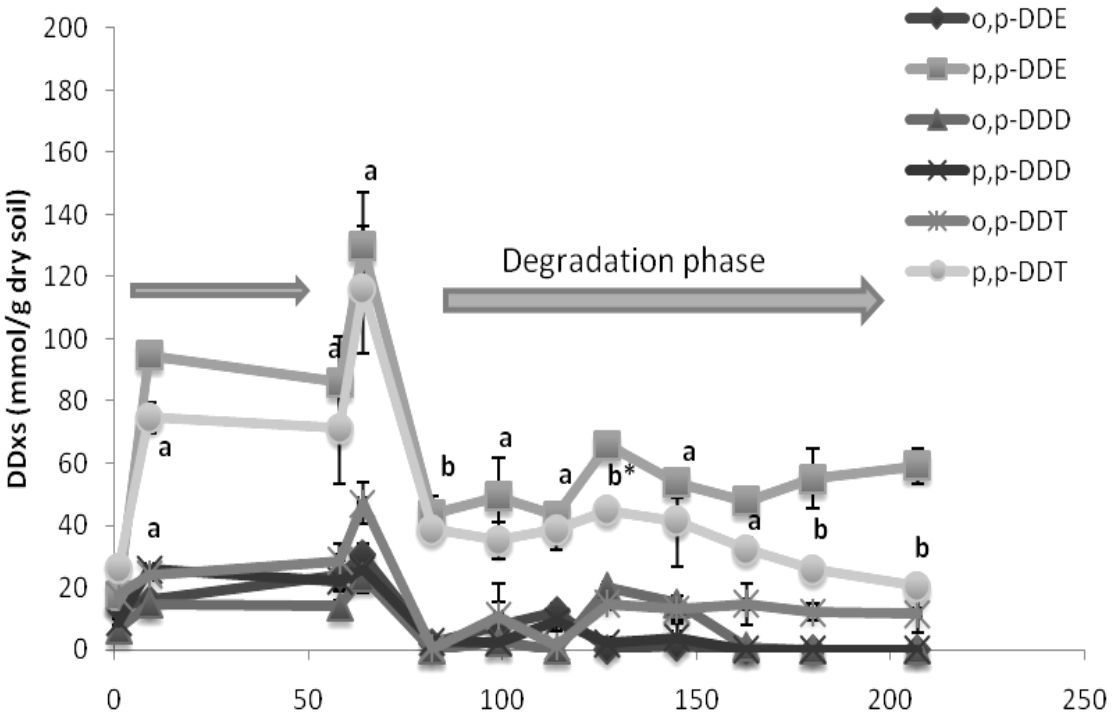


Figure 4-6. DDx concentrations (mmol/g dry soil) in Lactate mesocosm tanks throughout the study period. Error bars represent +/- one standard deviation based on three replicates. * = $p<0.01$. Statistical analysis output is presented with data labels. Other than p,p'-DDT, all the other DDxs were not significantly different hence to avoid over crowding in the figure data labels are only for p,p'-DDT. Data labels that do not share the same letter are significantly different at $p<0.05$.

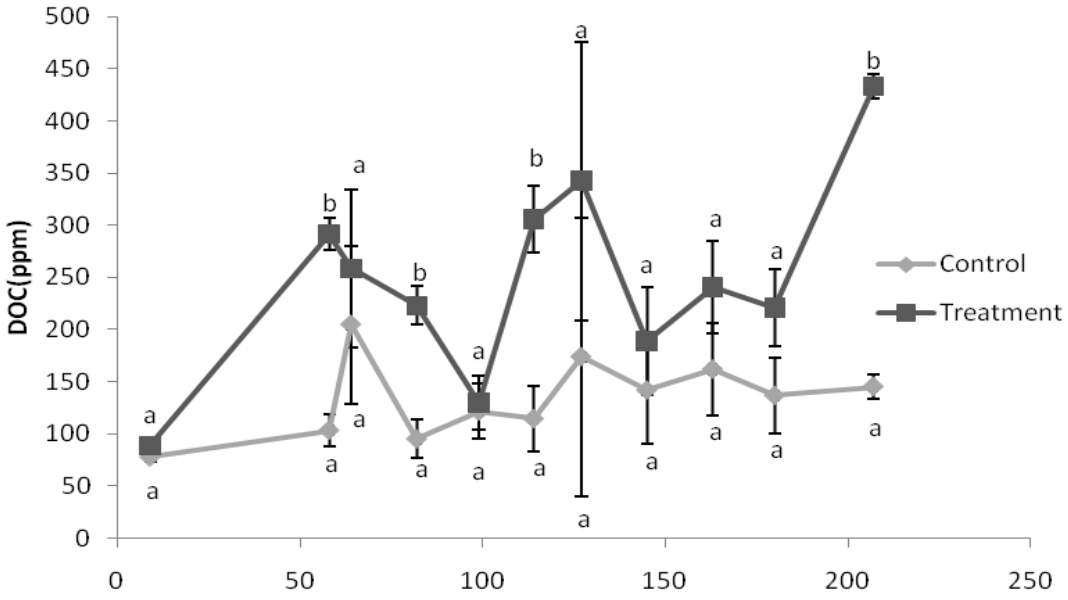


Figure 4-7. Dissolved organic carbon (DOC) ppm in control and lactate (treatment) tanks throughout the study period. Error bars represent +/- one standard deviation based on three replicates. Data labels that do not share the same letter are significantly different at $p<0.05$.

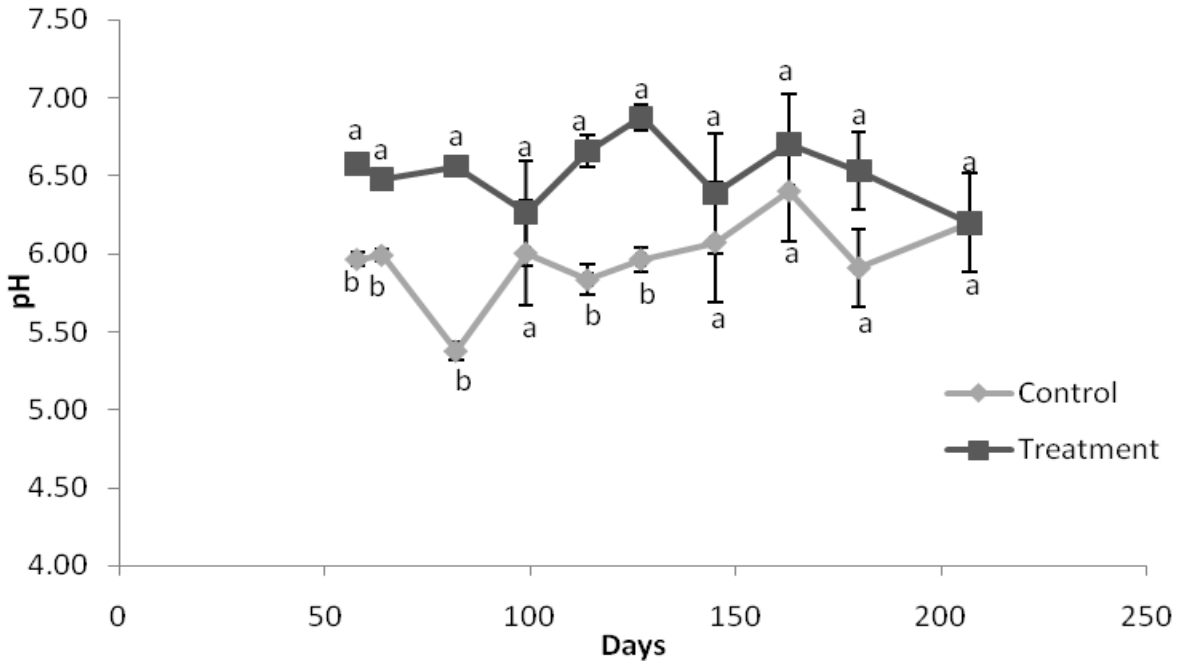


Figure 4-8. pH in control and lactate (treatment) tanks throughout the study period. Error bars represent +/- one standard deviation based on three replicates. Data labels that do not share the same letter are significantly different at $p<0.05$.

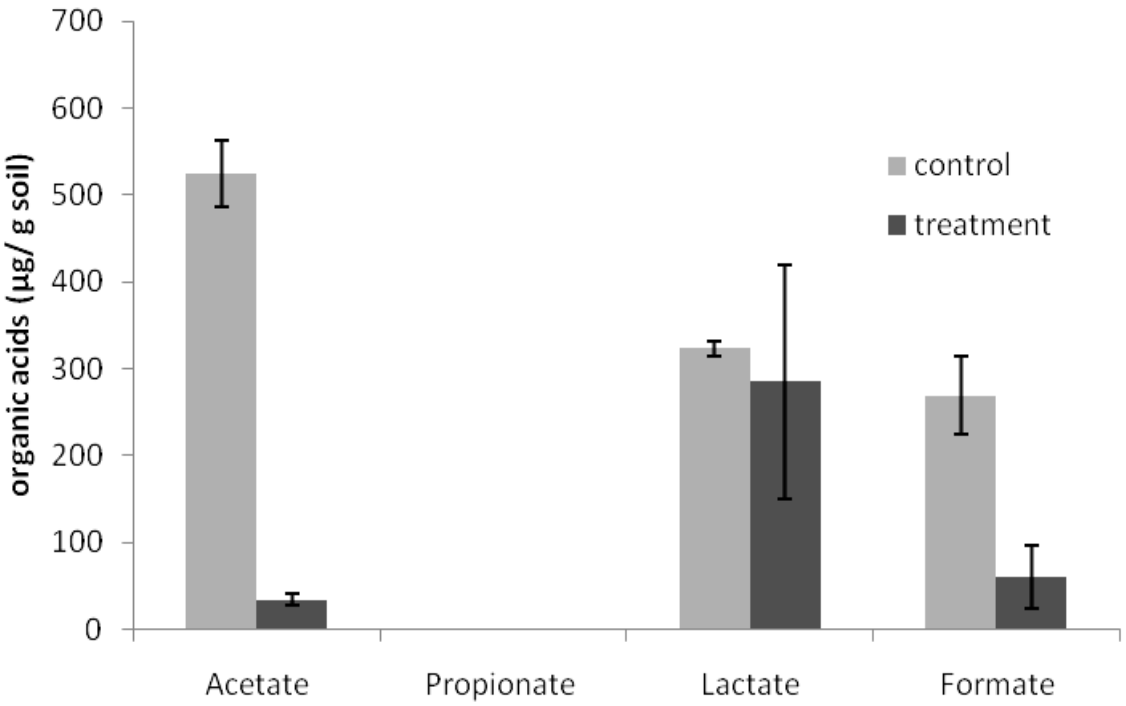


Figure 4-9. Organic acids (µg/g dry soil) in control and lactate (treatment) tanks at 64th day in incubation. Error bars represent +/- one standard deviation based on three replicates.

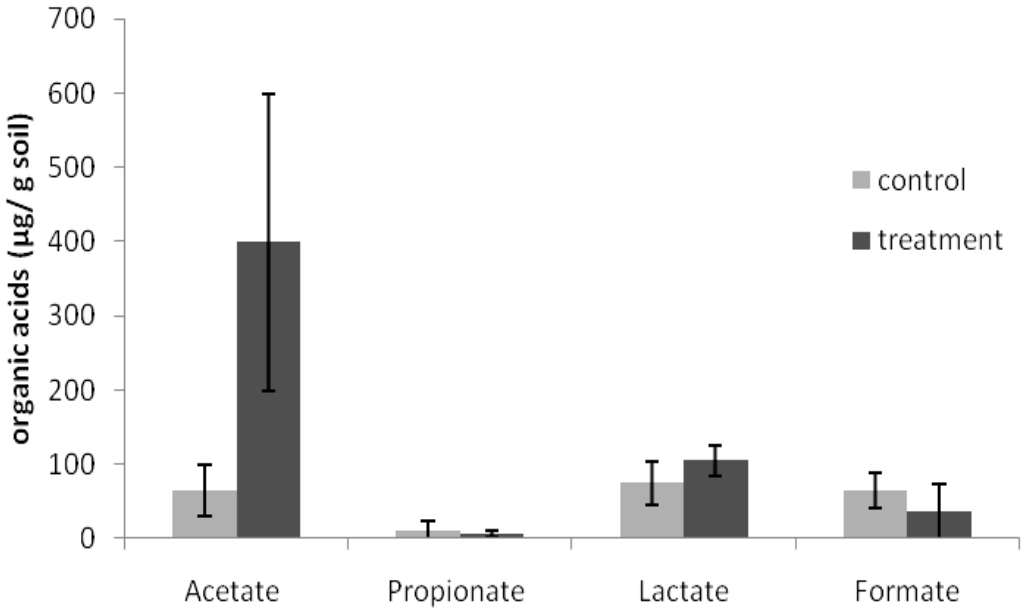


Figure 4-10. Organic acids (µg/g dry soil) in control and lactate (treatment) tanks at 114th day in incubation. Error bars represent +/- one standard deviation based on three replicates.

CHAPTER 5
ENRICHMENT, ISOLATION AND IDENTIFICATION OF LACTATE UTILIZING, DDX DEGRADING CONSORTIA IN LAKE APOPKA

Dehalorespiring populations are attractive targets for stimulation for environmental remediation of chlorinated compounds in anoxic environments. Terminal electron acceptors (TEAs) are typically limiting in these environments, such that donating electrons to the pollutant and gaining energy in the process can be beneficial to the dehalorespiring population. Dehalorespiration may transform halogenated compounds to more easily oxidizable, and hence more easily biodegradable, forms (Adriaens et al., 1994; Beurskens, 1995; Barkovskii, 1996; Ballerstedt, 1997; Albrecht, 1999). Such processes can be stimulated at the field level by intentional introduction of suitable electron donor and acceptor combinations (Suflita et al., 1988). In an attempt to determine the most efficient system for degradation of DDx, we established laboratory microcosms to test a range of electron donor: acceptor systems (Chapter 3).

As demonstrated in Chapter 3, the highest degradation (about 83%) was observed in microcosm sets using lactate as electron donor and DDT as electron acceptor. Once the optimal donor: acceptor combination was identified in microcosm experiments, simultaneous transfers from microcosms were continued to fresh microcosm batches in an attempt to enrich and isolate the responsible consortium. This chapter describes attempts to enrich, isolate, and identify the organisms involved in DDx degradation using standard enrichment, isolation, and molecular approaches. This would enable us to determine the roles, byproducts, and pathways in DDT degradation.

Syntrophs have previously been shown to be involved in dehalogenation processes (Mohn and Tiedje, 1992; Yang and McCarty, 1998; Drzyzga and Gottschal, 2002; Dolfing, 2003; Sung et al., 2003). Syntrophy is a relationship between two

metabolically different bacteria in which they metabolize a substance and, in the process, satisfy energy needs of both the bacteria which neither can attain individually (Atlas and Bartha, 1993). When hydrogen is main electron donor, studies have shown that dehalorespirers outcompete hydrogenotrophic sulfate reducing bacteria, homoacetogens, and methanogens based on energy gains (Fennell and Gossett, 1998; Loffler et al., 1999). Dehalorespiring populations may therefore participate in close syntrophic associations with hydrogen producing organisms, which require substantial low hydrogen concentrations for energy gain (Drzyzga and Gottschal, 2002). Dehalorespirers would be favored by hydrogen producing obligate syntrophs fermenting organic acids over methanogens or acetogens when present in adequate concentrations (Dolfing, 2003; Sung et al., 2003; Yang and McCarty, 1998).

For the lactate enrichments, we hypothesized that a syntrophic consortium with dehalorespiring organisms was responsible for DDT degradation. Lactate is fermented to small organic acids, such as formate and acetate, and hydrogen, such that different groups of organisms may feed on the lactate fermentation byproducts, and serve as an electron donor to the dehalorespiring population.

Materials and Methods

Microcosm

Microcosms were made in an effort to investigate impact of different electron donor: acceptor combinations on DDx degradation and served as inocula for enrichments. Microcosms were established using NSRA soils, and were divided into two sets: 1) microcosms testing DDT loss under different electron accepting conditions where DDT would be the electron donor; and 2) microcosms with various electron donors and DDT as terminal electron acceptor. Irrespective of the microcosm type, p,p'-

DDT solubilized in methanol was added to sieved and homogenized soils. Upon evaporation of the solvent, soils were mixed manually and 10 g dry weight soil (containing 10 µg p,p'-DDT/g dry soil) were added to 125 mL serum bottles with 45 mL mineral medium (Ou et al., 1978). To maintain anaerobic conditions, bottles were purged with either N_2 except the H_2 sets which were purged with or H_2 and CO_2, sealed with Teflon-lined butyl rubber stoppers and secured with aluminum crimps. Microcosms were incubated at 27^{o}C in the dark for approximately two months. These microcosms served as inocula for enrichments following incubation.

DDT as Electron Donor

Four different terminal electron acceptors (TEA) were tested with DDT as electron donor and carbon source (Table 5-1): (1) sulfate (as K_2SO_4 salt) applied at a rate of 0.42 mg of SO_4^{2-}S/gram dry soil (Wright and Reddy, 2001); sulfate sets were purged with N_2 on a weekly basis to prevent accumulation of sulfide; (2) nitrate (in the form of KNO_3) applied at a rate of NO_3-N 0.22 mg/gram dry soil (Wright and Reddy, 2001); (3) Fe (III) (in the form of Fe(III)hydroxide, γ-phase) at a concentration of 4.42 mg/gram of soil (Monserrate and Haggblom, 1997); and (4) control with no exogenous TEA provided.

DDT as Terminal Electron Acceptor (TEA)

Four different electron donors were tested (Table 5-2), including lactate and acetate at 20mM, H_2 at 100kPa, and no exogenous electron donor. Concentrations for the donors chosen here was significantly higher than field values to quickly investigate if increased concentrations of such donors would correlate with degradation. Microcosms were purged weekly with either N_2 or H_2 and CO_2 to remove sulfide and compensate for the used H_2 and CO_2.

Enrichments

Microcosms were used as starter cultures to selectively enrich for cultures with different electron donor (Table 5-1) and acceptor combinations (Table 5-2) yielding significant degradation of DDT. Subsequent transfers were made in two ways, referred to as “liquid transfers” and “membrane transfers.”

For liquid transfers, a homogenous sediment suspension amounting to a 10% of the total volume from all microcosms was transferred into fresh batch of complex medium containing a range of trace nutrients and vitamins (Tanner et al., 2007) and with DDT (10 µg/ membrane) added to Teflon membranes (Bastieans et al., 2000). Standard procedures for enrichment and isolation of specific functional groups, e.g. iron reducing bacteria (Cummings and Magnuson, 2007), sulfate reducing bacteria (Hines et al., 2007), denitrifiers (Song et al., 2000), and dehalorespiring bacteria (Maymo-Gatell et al., 1997) were followed. DDT was spiked on to membranes dissolved in solvent and upon evaporation of solvent the spiked membrane was added to the serum bottles. To facilitate growth of complex microbial communities, a small volume (10%) from the previous batch was transferred to new sets and transfers made in this way are termed “liquid transfers.”

For membrane transfers, spiked Teflon membranes from previous batches were transferred to a fresh batch of Tanner medium. Following approximately two months of incubation, transfers were made. Membrane transfers were conducted to promote growth of biofilm-forming organisms or syntrophs where it is important for different organisms to grow in close proximity to each other for interspecies hydrogen transfer.

Preliminary experiments using a combination of purified ^{14}C-DDT and ^{12}C-DDT indicated approximately 97% recovery of DDT from the Teflon membranes.

Enrichments for each treatment with both membrane and liquid transfers were continued for about 18 months and transfers were made approximately once every two months. The appropriate electron donor/acceptor combinations were maintained throughout the enrichments.

DDT as Electron Donor

The standard procedures (Table 5-3) here were targeted to enrich sulfate reducing bacteria (Hines et al., 2007), iron reducing bacteria (Cummings and Magnuson, 2007), denitrifiers (Song et al., 2000), and mixed acid fermenters. The transfer scheme for enrichments is shown in Figure 5-1. The appropriate electron donor: acceptor combinations were maintained throughout the incubations and transfers were conducted under N2 stream to maintain anaerobic conditions for the first three transfers after which transfers were made in Coy anaerobic glove box.

DDT as TEA

For enrichments with DDT as TEA, four different electron donors were tested: two organic acids at 20 mM; H_2 at 100 kPa; and no exogenous electron donor. Enrichment sets for each electron donor were further divided into three groups: no sulfate; constant sulfate; and variable sulfate (Table 5-4, Figure 5-2). For “variable sulfate,” the concentration of sulfate decreased with subsequent transfers (Table 5-4). Whereas in case of “constant sulfate” sets, sulfate concentrations remained unchanged (200 mg) throughout subsequent transfers, and “no sulfate” sets had no exogenous sulfate added to the medium. Sulfate concentrations in the variable sulfate sets eventually approached zero. This was done to determine critical concentrations of sulfate that promote use of DDT as TEA. Sulfate concentration may have an impact on DDT dechlorination, as some previously identified dehalorespirers are classified as sulfate reducing bacteria

(SRB) (Zwiernik et al., 1998; Fava et al., 2003; Zanaroli et al., 2006). Subsequent transfers, in case of both membrane and liquid transfers, were made to a lower sulfate concentration every two months, with the intention that those SRB capable of utilizing DDT as TEA will shift to dehalorespiration at some critical sulfate concentration (Table 5-4). Transfers were made under strict anaerobic conditions.

Isolation

After five transfers, soil carbon was assumed to be diluted out, such that only those organisms or consortia capable of growth on the appropriate electron donor:acceptor combination would remain. Efforts to isolate the responsible consortia were hence made. Tanner medium plates were made with additional ingredients namely ultra pure agar 15 g/L, resazurin 0.001 g/L, and reducing agents L-cysteine HCl and Na2S.9H2O, both used at concentrations of 0.5 g/L. The appropriate electron donor/acceptor combinations were maintained throughout the isolation process.

p,p'-DDT (AccuStandard Incorporation, New Haven, CT, U.S.A.) was added to sterile Teflon membranes at 10 µg/ membrane by dissolving in diethyl ether, allowed for solvent to evaporate and placed on petri plates with the DDT side down, after the plates were streaked. Tanner medium plates were streaked with an aliquot from lactate and hydrogen for liquid transfer enrichments. For membrane transfers, membranes were added to sterilized test tubes containing sterile distilled water and vortexed to dislodge the biofilm from the Teflon membrane. The resulting inoculum was streaked on Tanner medium plates. Media preparations and inoculation was conducted following standard anaerobic handling techniques, and plates inoculations were performed in a Coy anaerobic glove box (Coy laboratory products, Grass Lake, MI) with an N_2 atmosphere. Isolated colonies were selected, diluted, and streaked onto similar plates for isolation.

DNA Extraction and PCR Amplification of 16S rRNA Genes

DNA was extracted from lactate and hydrogen enrichments using Ultra Clean Soil DNA kit (Mobio Laboratories Incorporation, Solana Beach, CA) following manufacturer's instructions. PCR for community DNA amplification was performed using bacterial universal primers 27 Fp and 1492 Rp for 16S rRNA genes (Lane, 1991) from lactate sets. PCR amplifications were performed in 100 µL PCR tubes with a 50 µL reaction mixture containing 25 µL Go Taq Green Master Mix (Promega Corporation), 10pmol/µL forward primer (27 Fp), 10 pmol/µL reverse primer (1492 Rp), 22 µL nuclease free water and 1 µL DNA sample. The amplification cycle consisted of an initial denaturation at 95°C, followed by 35 cycles consisting of three steps of 30 seconds each: strand separation at 95°C; elongation at 72°C; and annealing step at 58°C. Amplification was completed with a final extension step at 72°C for 7 minutes. Amplification products were confirmed through gel electrophoresis on 1% agarose gel made in trisacetate EDTA buffer and stained using ethidium bromide and observed under UV light.

Cloning and Identification

Upon confirmation, fresh PCR products were ligated into pCRII-TOPO cloning vector and transformed into chemically competent *Escherichia coli* TOP10F cells according to manufacturer's recommendations (Invitrogen, Carlsbad, CA.). Blue, light blue and white colonies were randomly picked from the ampicillin plates and inoculated into 96 well plates containing Luria Bertini(LB) broth with ampicillin (1L LB contained 10 mL of 10 mg/mL filter sterilized ampicillin). After incubation at 37°C overnight, live clones were submitted for sequencing at the Genome Sequencing Laboratory, University of Florida. Phylogenetic analysis of DNA sequences was performed using PHYLIP and aligned with Clustal version 1.81 (Thompson et al., 1997). Phylogenetic

tree was built with a neighbor joining analysis based on similar nucleotide sequence database obtained from other studies at the NCBI website (http://www.ncbi.nlm.nih.gov) using the Basic Local Alignment Search Tool (BLAST).

PCR for Dehalococcoides

Once community DNA was extracted, as mentioned earlier, amplification targeting 16S rRNA genes followed a second nested PCR using *Dehalococcoides* (DHC) specific primers, Fp DHC 587 and Rp DHC1090, (Hendrickson et al., 2002) to screen microcosms showing the greatest loss of DDT (hydrogen and lactate treatment) for the presence of *Dehalococcoides*. Amplifications were performed in 100 µL PCR tubes with a 50 µL reaction volume containing 25 µL Go Tag Green Master Mix ((Promega Corporation), 10 pmol/µL forward primer (587 DHC), 10 pmol/µL reverse primer (1090 DHC), 22 µL nuclease-free water, and 1 µL DNA sample.

PCR was set up for an initial denaturation at 95°C for 2 minutes, followed by 35 cycles of three steps with an initial melting temperature of 94°C for 1 minute, followed by annealing at 55°C for 1 minute, and extension at 72°C for 1 minute. Amplification reactions included a positive control of *Dehalococcoides* DNA obtained from Dr. Donna Fenell's laboratory at Rutgers University, New Brunswick, NJ, and a negative control (deionized water). *Dehalococcoides* specific primer sequence for forward primer 587 DHC was 5'GGACTAGAGTACAGCAGGAGAAAAC3' and that for reverse primer 1090 DHC Rp was 5'GGCAGTCTCGCTAGAAAAT3' (Hendrickson et al., 2002). These primers amplified a product of 503 bp.

Enrichment Experiments with Lactate Isolates using ^{12}C-DDT and ^{14}C-DDT

To confirm isolation of a degrading consortium, sterile autoclaved Teflon membranes were spiked with 100 µg/membrane p,p'-DDT, added to 45 mL Tanner

medium and inoculated with 4.5 mL culture from the previous enrichment for liquid transfer sets. Autoclaved controls had the same volume of inoculum aliquot, but were autoclaved at 121°C for 21 minutes prior to transfer. For membrane transfer sets, membranes from the latest transfer were cut into half, and one half was used to inoculate the sterile medium and the other half was autoclaved and added to the control. Microcosm sets were handled using anaerobic techniques and purged with N_2 / H_2+CO_2 in the head space, followed by incubation in dark for 45 days. Triplicate sets were sacrificed at 15 days, 30 days and 45 days of incubation.

Following sacrifice, membranes were extracted with 10 mL hexane by adding hexane to the membrane in a clean test tube and extracting on a mechanical shaker at high speed for 15 minutes. The extractant was concentrated with nitrogen stream under a fume hood and analyzed using GC-ECD after appropriate dilution. Medium and container walls were analyzed once to check for presence of DDT and daughter products either in the aqueous phase or adsorbed to the container walls in case of ^{12}C-DDT enrichments.

If aqueous concentrations were below detection limits, no further aqueous phase determination would be continued for ^{12}C enrichment sets, whereas all the phases (container walls, growth medium, stoppers, and teflon membrane) were extracted for ^{14}C enrichment sets. ^{14}C data was determined by counting on a Beckman LS 100-C liquid scintillation counter (LSC) (Beckman Coulter, Incorporation, Brea, CA). Preliminary experiments using ^{14} C-DDT revealed 97% recovery of the spiked DDT from the membrane.

GC Conditions

Perkin-Elmer Autosystem Gas Chromatograph (GC) equipped with autosampler and an electron capture detector (ECD) was used for analyzing the extracts. Column conditions were: He as the carrier gas at flow rate of 1.0 mL/min, 5% methane in argon as the make-up gas with a flow rate of 50 mL/min, electron capture detector (ECD) temperature at 350°C, and injector temperature at 205°C in splitless mode. Injection volume was 3 µL in slow injection mode. GC temperature was programmed at 110°C for 0.5 minutes, followed by a ramp at 20°C/min to 210°C and hold for 0 minutes, then ramp at 11°C/min to 280°C and held for 6.3 minutes. Under these conditions, the detection limit was calculated at the 90% confidence level to be: 0.007 µg/mL for o,p'-DDE; 0.005 µg/mL for p,p'-DDE; 0.006 µg/mL for o,p'-DDD; 0.009 µg/ mL for p,p'-DDD; 0.005 µg/mL for o,p'-DDT; and 0.010 µg/mL for p,p'-DDT (Hubaux and Vos, 1970). These results are similar to the detection limit of 0.010 µg/mL reported by Pace, Incorporation.

Liquid Scintillation Counter

Following incubation all the components of enrichments were analyzed for the presence of ^{14}C. Membranes were extracted similarly to ^{12}C enrichments, 10 mL hexanes added to the membrane in clean Teflon lined test tube and extracted on a mechanical shaker for 15 minutes at high speed. Container walls were extracted using 45mL hexane, liquid was extracted using 1:1 (v/v) of aqueous phase: hexane and rubber stoppers from the bottles were extracted using 5mL hexane. All extracts were analyzed on LSC in a scintillation vial with 1 mL extractant and 10 mL scintillation cocktail (Ecoscint A, National diagnostics, Atlanta, GA) for 5 minutes. Background

radioactivity was subtracted from a blank of hexane (1 mL) and 10 mL scintillation cocktail.

Check for Viability

Upon confirmation of degradation using ^{12}C and ^{14}C DDT enrichment studies, efforts would be made to check for viability, identification of the involved population and isolate using molecular approaches. This would help to determine the roles, byproducts, mechanisms and pathway in DDT degradation.

Statistical Analysis

^{12}C and ^{14}C DDT enrichment studies data was analyzed using the one way ANOVA analysis in NCSS (Kaysville, UT). Controls were tested against the treatments using Fisher's LSD test. Values with $p<0.05$ were considered significantly different.

Results and Discussion

Dehalorespiration is an anaerobic process, such that dehalorespirers and other anaerobic microbes compete for electron donors in the soil. Even though dehalorespiring organisms can use a broad range of electron donors (Chapter 2, Table 2-2), H_2 is an important electron donor for dehalogenation. Based on the H_2 utilizing efficiency of dehalorespiring populations and the resulting thermodynamic gains, dehalorespirers typically outcompete hydrogenotrophic methanogens, homoacetogens, and SRBs (Ballapragada et al., 1997; Fennell et al., 1997; Fennell and Gossett, 1998; Loffler et al., 1999; Smatlak et al., 1996; Yang and McCarty, 1998). Thus dehalogenating population could serve as efficient members of a syntrophic association (Smatlak et al., 1996).

The greatest loss of DDT was observed in lactate microcosms (Chapter 3), which could be tentatively explained by the presence of a syntrophic relationship between

hydrogen producing lactate fermenting organisms and dehalogenating populations. Lactate in these enrichments could be fermented to H_2, which could be used by dehalorespirers to dehalogenate DDT.

PCR for *Dehalococcoides* from Hydrogen and Lactate Enrichments

To investigate potential dehalorespiring populations, we initially targeted *Dehalococcoides* because previous studies identified this group in NSRA soils (SJWMD, personal communication). Strains from this genus are known to use hydrogen as an electron donor during dehalorespiration on a variety of halogenated compounds (Hiraishi, 2008). The variety of compounds dehalorespired by this genus range from haloaliphatics, such as TCE, PCE and VC (D. strain BAV1 (He et al., 2003a; 2003b) and strain GT; (Sung et al., 2006), to haloaromatics such as chlorobenzenes, chlorophenols, chloroethenes, polychlorinated dibenzo-p-dioxins, polychlorinated dibenzo-p-furans, polychlorinated biphenyls, polychlorinated dioxins (D. strain CBDB1; Adrian et al., 2000; 2000; Bunge et al., 2003 and D. ethenogenes strain 195 Adrian et al., 2003; Fennell et al., 2004). *Dehalococcoides* is one of only two genera (*Dehalobacter* and *Dehalococcoides*) that have been shown to dehalorespire on polychlorinated biphenyls (*D. ethenogenes* strain 195) (Adrian et al., 1994; 2000; Bunge et al., 2003; Fennell et al., 2004). For these reasons, *Dehalococcoides* species have gained attention in the field of environmental bioremediation (Adrian et al., 1998; Cupples et al., 2003; He et al., 2003; Hendrickson et al., 2002; Maymo´ Gatell et al., 1997; Bunge et al., 2003; Ellis et al., 2000; Fennell et al., 2004; Lendvay et al., 2003; Major et al., 2002).

To check for presence of *Dehalococcoides* in the, lactate and hydrogen enrichments, we used a nested PCR technique (Hendrickson et al., 2002). The first

PCR targeted bacterial 16S rRNA genes and the second round of PCR amplified *Dehalococcoides*-specific genes. The nested PCR approach was used to increase sensitivity. No amplification from the nested PCR with *Dehalococcoides* specific primers (Hendrickson et al., 2002) was observed from the lactate or hydrogen microcosms.

Characterization of Lactate Consortium by 16S rRNA Gene Amplification and Cloning

After five transfers, soil carbon was assumed to be diluted out of the lactate microcosms. Community DNA was isolated from the last lactate microcosm and 16S rRNA genes of the community DNA were amplified using bacterial universal primers (27F and 1492R), cloned and sequenced.

The dominant sequences clustered within the low G+C Gram positive *Firmicutes* (Figure 5-3). *Firmicutes* is comprised of 3 classes: *Bacillus*; *Clostridium*; and *Mollicutes*. Most of the enriched species clustered within the class *Clostridium* (Table 5-6). Representatives of these bacterial groups have been shown to metabolize haloorganics, with close relatedness to the phylum *Firmicutes* sequences shown to use chlorinated compounds as TEA (Tables 5-5 and 5-6).

The genus *Sporomusa* includes homoacetogens, which have previously been shown to form close associations with dehalogenating populations (Yang et al., 2005). In that research, *Sporomusa* was enriched with hydrogen and acetate as electron donors and PCE or cis-1,2-dichloroethene (cDCE) as TEA. Those enrichments harbored a combination of *Dehalococcoides* and *Desulfitobacterium,* and *Sporomusa*-like bacteria. *Sporomusa ovata* has been shown to reductively dechlorinate perchloroethene (PCE) to trichloroethylene TCE (Terzenbach and Blaut, 1994). Members of the genus *Sporotalea* ferment lactate, glucose, lactose, fructose, and

pyruvate to propionate and acetate. *Sporotalea propionica* TM1 was shown to be involved in tetrachloromethane degradation (Penny et al., 2010). Other studies have shown the presence of *Sporotalea* species in dechlorinating enrichment cultures inoculated with sediment samples with TCE and pyruvate *Sporotalea colonica* and uncultured *Sporotalea* sp. clone 175 (accession number FM994641) (Dowideit et al., 2009).

Psychrosinus is a strictly fermentative psychrophile that ferments only lactate and a few related organic acids. *Psychrosinus* strain FCF9 is closely related to *Pelosinus* species. Unlike *Psychrosinus*, *Pelosinus* species such as *P. fermentans* ferment several different substrates (Shelobolina et al., 2007), including sugars and organic acids. *P. fermentans* is also capable of forming endospores (Boga et al., 2007; Shelobolina et al., 2007). *Pelosinus* belongs to *Firmicutes; P. fermentans* may donate electrons to Fe(III) for fermentative growth rather than using it as TEA for anaerobic respiration (Shelobolina et al., 2007). *Anaerovibrio lipolytica, Anaerovibrio glycerini* and *Anaerovibrio burkinabensis* cluster within the *Sporomusa-Pectinatus-Selenomonas* phyletic group (Strompl et al., 1999).

Enrichment Experiments

Based on sequencing results that indicate that *Firmicutes* dominate, we hypothesized that we have enriched degrading syntrophic consortia from the lactate enrichments. The next steps were to isolate the dehalorespiring consortia. To this end, enrichment studies using ^{14}C and ^{12}C DDT were initiated.

Figure 5-4 describes recovery of p,p'-DDT from ^{12}C-DDT enrichment experiments, whereas Figure 5-5 includes recoveries from ^{14}C-DDT experiments. Although there is a significant difference in recoveries from 15 days to 30 and 45 days for ^{12}C-DDT

enrichments, there is no difference between controls and treatments. A decrease in recovery efficiencies from days 15 to 30 and 45 days remains unexplained, although it might be attributed to adsorption to container walls or partitioning into the aqueous phase. However, recoveries could be traced better using ^{14}C.

Extraction efficiencies remain similar on 30^{th} and 45^{th} day for ^{14}C enrichments, which is a more accurate and precise method compared than ^{12}C detection. The lower extraction on 15^{th} day of ^{14}C enrichments was the result of human error. Similar to ^{12}C enrichment, there were no significant differences between the treatment and control, and between liquid and membrane transfers. These results suggested that the enriched culture may no longer be viable; hence, a viability test was performed on the enrichment culture.

When the culture was grown in an environment similar to its enrichment and isolation, i.e. with DDT as TEA and lactate as the only exogenous electron donor under strict anoxic conditions, no growth was observed on minimal media plates. Subsequently, the viability of the culture was checked by attempted cultivation on a rich medium under anoxic conditions. After 24 hours of incubation, growth on the rich medium was observed, which confirmed that the dehalogenating culture or consortia was lost while attempting to isolate and enrich the culture.

We were trying to isolate probable syntrophic consortia from lactate microcosm sets, where lactate provided the electron and carbon needs and DDT served as probable TEA to the degrading population. In case of the lactate microcosms, the greatest amount of degradation could be tentatively explained by a syntrophic relationship between hydrogen producing lactate fermenting organisms and

dehalogenating populations. Although anaerobic reductive dechlorination of halogenated organics has been reported in laboratory microcosms and in the environment (Bedard and Quensen, 1995), many attempts to isolate and identify the responsible microbiota have been unsuccessful. There are several difficulties while trying to cultivate and isolate a syntrophic community, including interdependence of the members involved (Schink, 1997. Isolation of a dehalogenating syntrophic community is even more challenging due to the low aqueous solubility of DDT (Holliger and Schumacher, 1994). Another complication related to isolation and growth is the interdependence of community on the members involved. For example, growth of *Dehalococcoides ethenogenes* strain 195 requires growth factors contained in anaerobic sludge supernatant in addition to H_2 (Maymó-Gatell et al., 1997).

An important step while isolating syntrophic dehalogenating cultures lies in the complexity of the community structure. Several studies with reductively dehalogenating enrichment cultures have reported presence of a community structure. Some examples are in the case of chlorinated ethenes (Duhamel et al., 2002; Richardson et al., 2002; Dennis et al., 2003; Rossetti et al., 2003; Gu et al., 2004), chlorinated propanes (Schlötelburg et al., 2002) and chlorinated benzenes (von Wintzingerode et al., 1999). In the above studies, there are some indications that a syntrophic consortium was linked to reductive dechlorintaion; however, this was not confirmed.

Inference

Microorganisms capable of dehalorespiration are ubiquitous, and their ability to use these compounds and conserve energy in the process makes them an attractive option for remediation. In case of lactate microcosms, lactate could be fermented to H_2 and short chain fatty acids. Hence, reductively dehalogenating population could be

growing in close proximity to these lactate fermenting, hydrogen producing organisms. Hydrogen is an important electron donor for the dehalorespiring population, which could get used in the process of reductive dehalogenation of DDx. Previous studies have demonstrated coexistence of syntrophic and dehalogenating population (Mohn and Tiedje, 1992; Yang and McCarty, 1998; Drzyzga and Gottschal, 2002; Dolfing, 2003; Sung et al., 2003). Reproducibility of DDx reductive dehalogenation was confirmed not only in microcosm experiments, but also with the mesocosm experiments (Chapter 3).

Although we were able to demonstrate degradation of DDx under lactate fermenting conditions, we did not succeed in enrichment and isolation of the organism(s) involved. This is not uncommon, due to the complications involved in enriching, isolating, and growing strains belonging to such complex systems. For a better understanding of biology of dehalogenating population, it is very important to study axenic cultures at molecular level. This may elucidate pathways, mechanisms, and role of the members involved and could be done by traditional enrichment cultures, such as metagenomics studies. A better understanding of the community dynamics of the population involved will help predict the bioremediation potential of those consortia.

Table 5-1. Microcosms testing DDT loss under different electron accepting conditions

TEA	Electron donor
$SO_4^{=}$	DDT
NO_3^-	DDT
Fe^{+3}	DDT
No TEA	DDT

Table 5-2. Microcosms with various electron donors and DDT as terminal electron acceptor

TEA	Electron donor	Carbon source
DDT	Acetate(20mM)	Acetate
DDT	Lactate(20mM)	Lactate
DDT	H_2 (100kPa)	CO_2
DDT	No exogenous source	No exogenous source

Table 5-3. Enrichments with target organisms under different electron accepting conditions

TEA	Target organisms
Sulfate	Sulfate reducing prokaryotes
Nitrate	Nitrate reducing organisms
Iron (III)	Iron reducing organisms
No TEA	Mixed acid fermenters

Table 5-4. Sulfate concentrations (mg/L) in different transfers (membrane and liquid) for variable sulfate replicates

Treatments	1^{st} transfer	2^{nd} transfer	3^{rd} transfer	4^{th} transfer	5^{th} transfer	6^{th} transfer
No $SO_4^{=}$	0	0	0	0	0	0
Constant $SO_4^{=}$	200	200	200	200	200	200
Variable $SO_4^{=}$	200	100	50	25	10	0

Table 5-5. Closest relatives of clone sequences from lactate enrichments

Related to isolate	Accession number	Genus	Metabolism + Phylogeny	Dehalogenation	Reference
A01	FJ269100	iron-reducing bacterium enrichment culture	ribosomal RNA gene, partial sequence,	diversity of dissimilatory ferric iron reducers in arsenic contaminated paddy soil	Wang,X., Zhu,Y., Yang,J. andChen,X Unpublished
B01, C01	DQ833299	uncultured bacterium	16S ribosomal RNA gene, partial	Anaerobic Reductive Dechlorination of Vinyl Chloride in Highly Enriched Communities	Ritalahti,K.M., He,J., Krajmalnik,R. and Loeffler,F.E Unpublished
C01	EF033503	uncultured Anaerovibrio sp Firmicutes	16S ribosomal RNA gene, partial	electron donors lactate and ethanol, denitrifying consortia in dispersed-growth reactors	Gentile et al., 2007
A03	FJ269098	iron-reducing bacterium enrichment culture	16S ribosomal RNA gene, partial	dissimilatory ferric iron reducers in arsenic contaminated paddy soil	Wang,X., Zhu,Y., Yang,J. andChen,X Unpublished
B03	AF275913	anaerobic digester bacteria	16S ribosomal RNA gene, partial	Low GC Gram+clone SJA143 (AJ009494) [trichlorobenzene-transforming consortium]	Moletta and Godon, 2000
C03	AY524569	iron(III)-reducing microbial Uncultured bacterium	16S ribosomal RNA gene, partial	acidic subsurface sediments contaminated with uranium(VI)	North et al., 2003

Table 5-5. Continued

Related to isolate	Accession number	Genus	Metabolism + Phylogeny	Dehalogenation	Reference
A04	FJ269098	dissimilatory ferric iron reducers in arsenic contaminated paddy soil	16S ribosomal RNA gene, partial	diversity of dissimilatory ferric iron reducers in arsenic contaminated paddy soil	Wang,X., Zhu,Y., Yang,J. and Chen,X. Unpublished
B04	DQ833365	Firmicutes bacterium	16S ribosomal RNA gene, partial	isolates obtained through incubation of VC and cis-DCE dechlorinating enrichment cultures	Ritalahti,K.M., Krajmalnik,R., He,J., Sung,Y. and Loeffler,F.E. Unpublished

Table 5-6. Genera related to clone sequences from lactate enrichments (based on clustering presented in Figure 5-3).

Genus	Characteristics/ phylogeny	Dehalogenation	Reference
Sporomusa	Hydrogenotrophic Homoacetogens	PCE dehalogenation	Yang et al., 2005
Anaerobranca	*Clostridium*/*Bacillus* subphylum Thermoalkaliphilic, obligate anaerobe, low G+C	Not known	Prowe and Antranikian, 2001
Sporotalea	*Firmicute*, *Clostridia,* obligate anaerobe, chemoorganotroph with fermentative	Tetrachloromethane and TCE degradation	Boga et al., 2007; Dowideit et al., 2009; Penny et al., 2010
Psychrosinus	Closely related to *Clostridium* species, Lactate-fermenting, psychrophilic, obligate anaerobe, branches within *Sporomusa:Pectinatus:Selenomonas* phyletic group	Not known	Sattley et al., 2008
Pelosinus	*Firmicute* , *Clostridia*-like species, spore forming, obligate anaerobes, mesophilic, fermentative metabolism	Not known	Shelobolina, 2007
Anaerovibrio	*Clostridia*-like species, chemoorganotrophic organisms that clusters within the *Sporomusa:Pectinatus:Selenomonas* phyletic group	Not known	Strompl et al., 1999

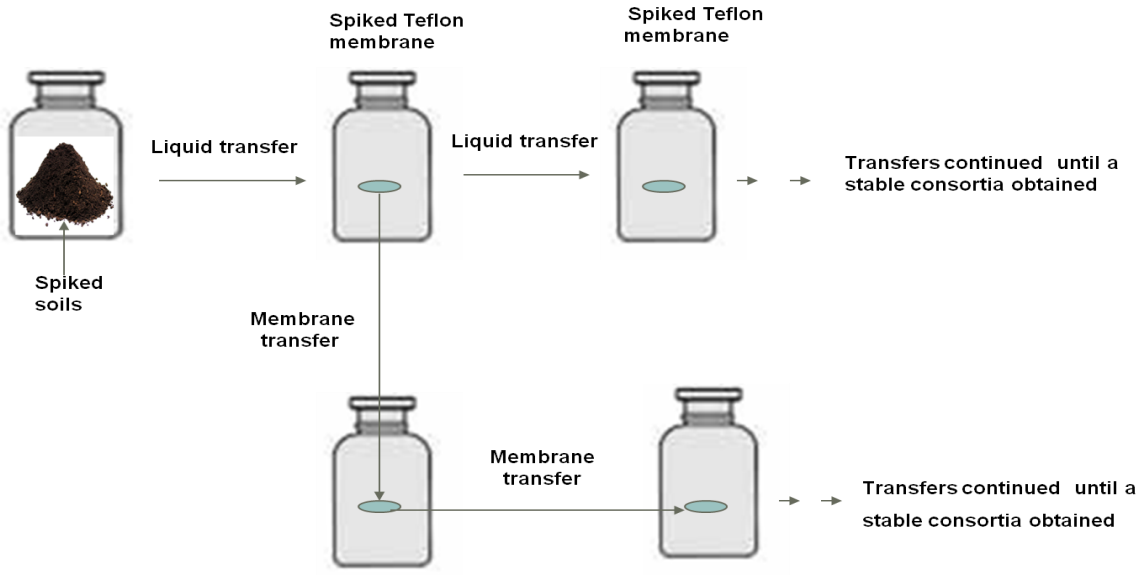


Figure 5-1. Enrichment scheme for different electron accepting conditions

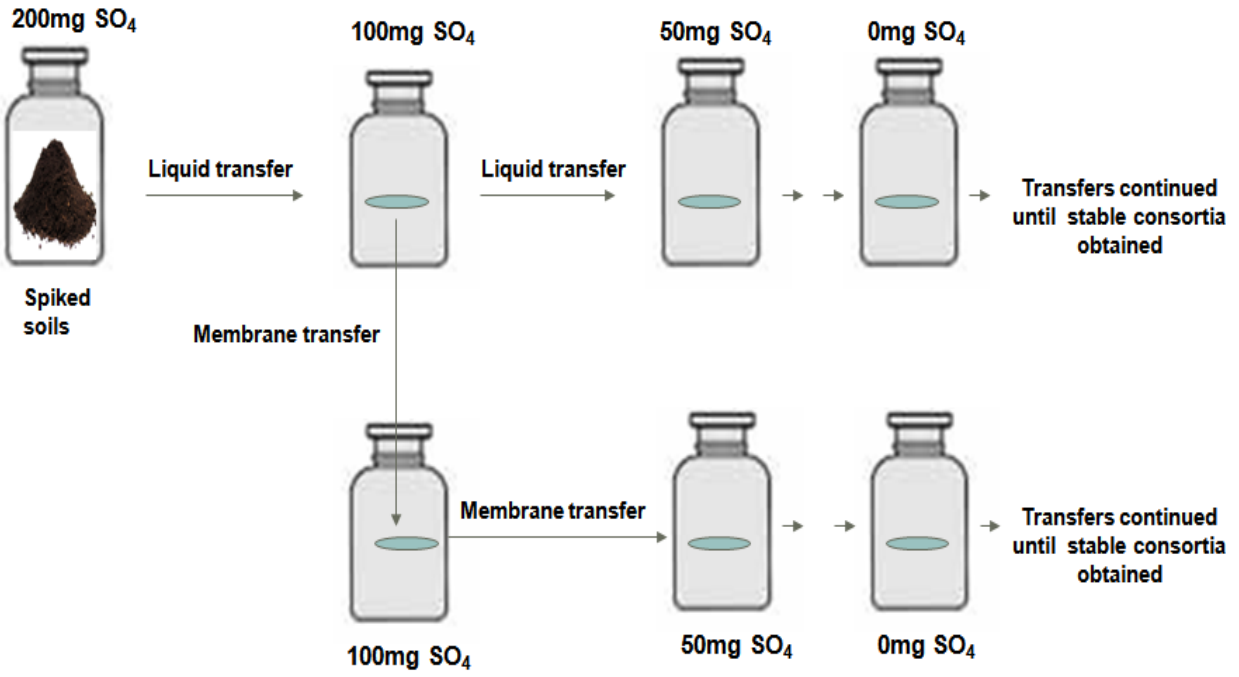


Figure 5-2. Enrichment scheme for DDT as TEA with variable sulfate sets.

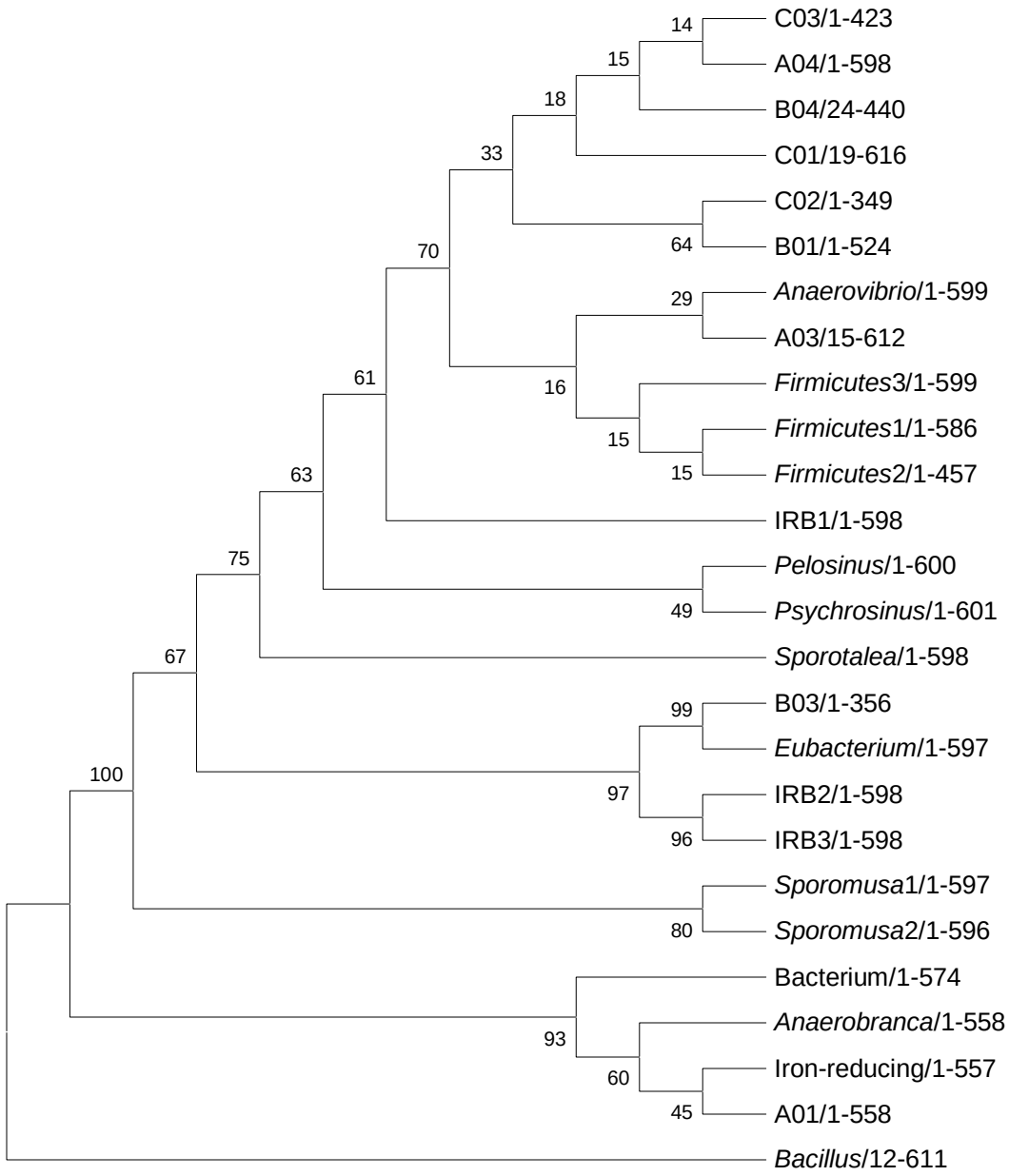


Figure 5-3. Phylogenetic tree of 16S rRNA gene sequences clustering with the Firmicutes (Low G+C Gram positive bacteria).

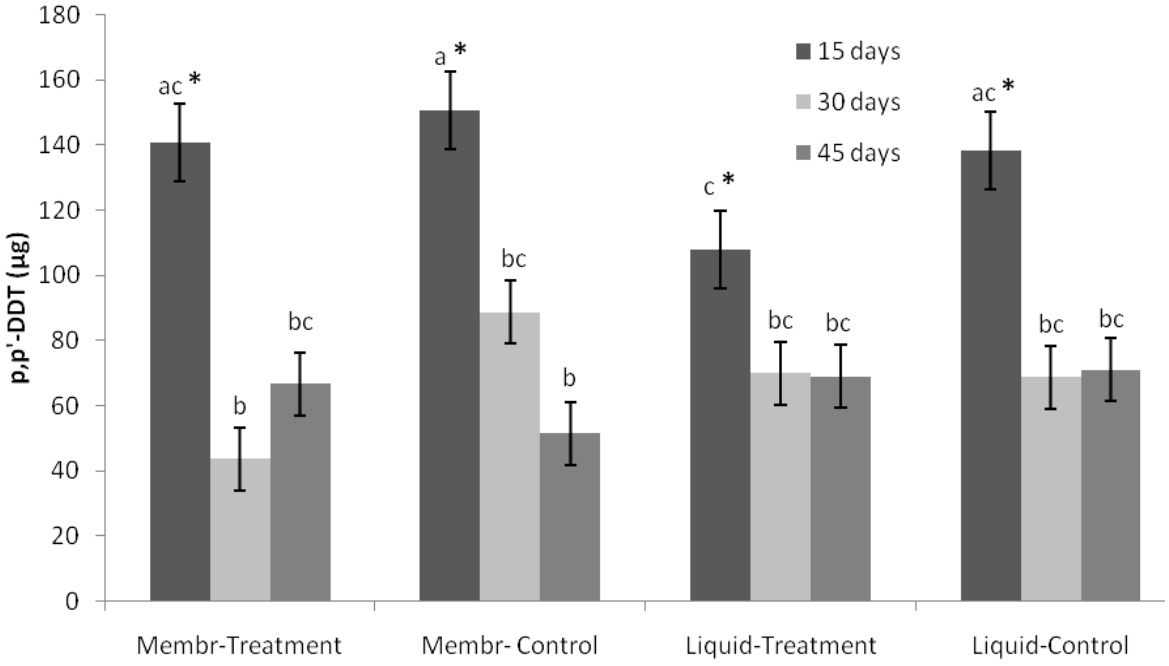


Figure 5-4. p,p'-DDT (µg) recovered from ^{12}C enrichments after 15, 30 and 45 days in incubation (based on three replicates, * =based on two replicates). Data labels not connected by same letter are significantly different $p<0.05$.

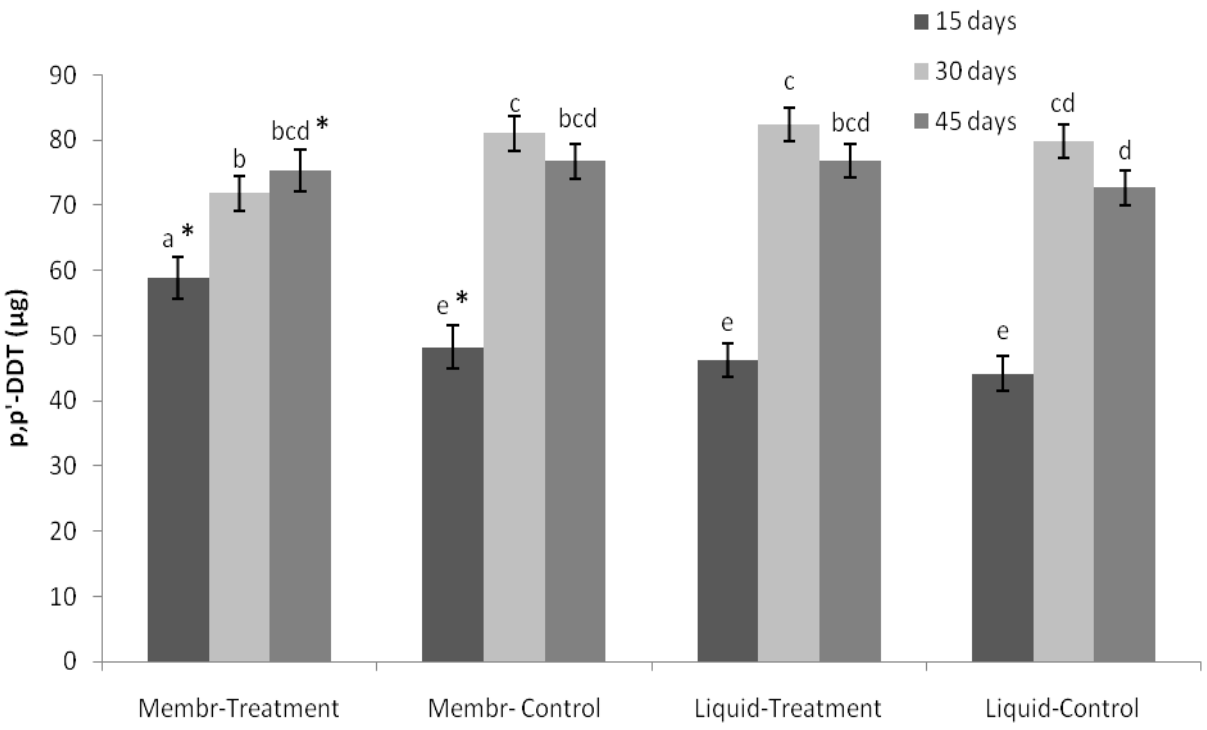


Figure 5-5. p,p'-DDT (µg) recovered from ^{14}C enrichments after 15, 30 and 45 days in incubation (based on three replicates, * =based on two replicates). Data labels not connected by same letter are significantly different $p<0.05$

CHAPTER 6
EVALUATION OF THE POTENTIAL FOR SODIUM IONS TO ENHANCE BIOAVAILABILITY AND BIODEGRADATION OF DDT AND ITS METABOLITES

Bioremediation is an economically attractive option for remediation of soils contaminated with hydrophobic organic contaminants (HOCs). One of the major critical factors that impede bioremediation of HOCs is bioavailability. Bioavailability may be limited because the pollutant may diffuse into the soil with time and become unavailable (Alexander et al., 1995; 1997). There are various theories to explain the prolonged persistence of HOCs in soils, including: sorption of PAH; partitioning of the HOC into non aqueous phase liquids (NAPLs); or diffusion within micropores or entrapment in the physical matrix of the soil (Alexander et al., 1997; Guthrie and Pfaender, 1998; Luthy et al., 1997).

HOCs such as DDT and its major metabolites DDD and DDE (collectively known as DDx) undergo an “aging” process with time in soil, such that the DDx molecules have a decreased bioavailability over time (Alexander et al., 1995; 1997). Aging has been shown to decrease rates of loss of DDx by volatilization, leaching, or biodegradation (Boul et al., 1995). Scibner and coworkers demonstrated that the longer that compounds remain in soil, the more resistant they become to desorption and biodegradation (Scribner et al., 1992).

Soil pollution with recalcitrant DDxs can lead to significant ecosystem damage (WHO, 1979; Ratcliffe, 1967; Megharaj et al., 2000; Turusov, 2002) and, hence, methods to bioremediate such pollutants is a high priority. Since bioavailability is an important issue in degradation of HOCs, a factor that increases accessibility to degrading organisms might increase biodegradation rates. Bioavailability of a compound might be enhanced by physical dispersion of the soil matrix (Juhasz et al.,

1999) leading to release of dissolved organic matter (DOM). Several studies have demonstrated that DOM can increase the solubility and mobility of HOCs (Caron et al., 1985; Chiou et al., 1986; Juhasz et al., 1999), and that DOC may influence the bioavailability of such compounds (Juhasz et al., 1999; McCarthy and Zachara, 1989).

One means of increasing bioavailability is use of sodium (Sumner and Naidu, 1998). Sodium is known to disperse soils and increase DOC levels (Nelson and Oades 1998; Wood, 1995; White, 1997; Brady and Weil, 2003; 2007; Kantachote et al., 2003). Both increased DOC and soil dispersion could potentially increase bioavailability of the otherwise inaccessible and aged DDxs (Kantachote et al., 2001; 2004; Quensen et al., 1998). This happens by releasing soil bound particles or colloids or by making the previously unavailable or protected DDx available to the potential degrading organisms (Kantachote et al., 2004). Kantochote and group (Kantachote et al., 2001; 2004) showed that Na^+ application to long term DDT contaminated soil significantly increased the DOC levels, anaerobic bacterial numbers, and amount of DDT residues in soil solution, followed by biodegradation that resulted in ~95% loss of the HOC.

This chapter focuses on use of Na^+ ions to increase biodegradation rates of DDx in microcosms and mesocosms. To explore the potential for Na^+ to increase bioavailability and degradation of DDxs in NSRA soils, microcosm experiments were performed. Chapters 3 and 4 demonstrated that anoxic incubations increase DOC, which may increase both bioavailability of DDx and subsequently increases biodegradation rates, such that the microcosms in this study were performed under anoxic conditions.

In this study, our overall objective was to optimize Na^+ concentrations to obtain maximum degradation rates. Microcosms were established with a series of different NaCl concentrations and ground cattail (family *Typhaceae*) served as a source of carbon and energy for the degrading species. Chapters 3 and 4 demonstrated that the greatest degradation occurred with lactate as electron donor and DDx as terminal electron acceptor (TEA). By using cattail in this study, we are promoting lactate production because approximately 40% of cattail is cellulose (DeBusk and Reddy, 1998).

Our over arching hypothesis is that the degraders will use DDx as TEA or cometabolize DDx while using cattail fermentation products for carbon and energy sources. The general concept is that Na^+ will disperse the organic carbon polymers associated with soil solids; thereby, increasing bioavailability of the DDx. Another assumption is that the system would be limited in trace metals which may be required for synthesis of certain enzymes. Therefore, two different types of growth media were compared in microcosm systems. Once these conditions were optimized in microcosms for biodegradation of DDx, the optimum conditions were scaled up to the mesocosm levels.

Materials and Methods

Soils Used

Soils used for microcosms were collected from North Shore Restoration Area (NSRA) at Apopka. Lake Apopka is a shallow lake with 125 km^2 surface area, located in Central Florida. Following collection, the soils were shipped to University of Florida, Gainesville and refrigerated at 4°C until use. Soils were sieved through a 20 mesh sieve

(nominal pore size of 80 micrometers) and homogenized by mixing before establishing the microcosms.

NaCl Microcosms

Microcosms were constructed by mixing air-dry soils with cattail powder at a rate of 0.04g cattail powder/g dry soil. Sieved (20 mesh sieve, nominal diameter of 80 µm) and homogenized soils were spiked with 10 µg p,p'-DDT/g dry soil solubilized in methanol. Upon evaporation of solvent, soils were mixed manually and 10g dry weight soil was mixed with cattail (0.04 g cattail powder/g dry soil) and added to 125 mL serum bottles with 45 mL growth medium. Two different types of growth medium were compared for this experiment. The first set was made using minimal growth medium according to Ou et al., (1978) and a second set contained the more complex growth medium according to Tanner et al., (2007). Tanner medium is a richer medium that includes a range of growth factors and inorganic nutrients, cofactors, vitamins and trace metals not present in Ou medium.

Ou medium consisted of (g/L of distilled water) K_2HPO_4, 4.8; KH_2PO_4, 1.2; NH_4NO_3, 1; $CaCl_2.2H_2O$, 0.025; $MgSO_4.7H_2O$, 0.2; $Fe_2(SO_4)_3$, 0.001. Tanner medium consisted of (amount /L of distilled water) mineral solution 10mL, vitamin solution 10mL, trace metal solution 1.5mL, yeast extract 0.1g. Ingredients for the individual components starting with mineral solution (g/L of distilled water)was NaCl, 80; NH_4Cl, 100; KCl, 10; KH_2PO_4, 10; $MgSO_4.7H_2O$, 20; $CaCl_2.2H_2O$, 4. Vitamin solution consisted of (g/L of distilled water) pyridoxin HCl, 10; thaimine HCl, 5; riboflavin, 5; calcium pantothenate, 5; thioctic acid, 5; p-aminobenzoic acid, 5; nicotinic acid, 5; vitamin B_{12}, 5; biotin, 2; folic acid, 2. Trace metal solution ingredients (g/L of distilled water) were nitrilotriacetic acid, 2; $MnSO_4.H_2O$, 1; $Fe(NH_4)_2(SO_4)_2.6H_2O$, 0.8; $CoCl_2.6H_2O$, 0.2; $ZnSO_4.7H_2O$, 0.2;

$CuCl_2.2H_2O$, 0.02; $NiCl_2.6H_2O$, 0.02; $Na_2MoO_4.2H_2O$, 0.02; Na_2SeO_4, 0.02; Na_2WO_4, 0.02.

Both media sets were tested for four Na^+ concentrations, including 0, 30, 50, and 80mg Na^+/kg soil. Sodium was added in the form of NaCl. In case of autoclaved controls, soils that had been autoclaved for three consecutive days at 121°C for 30 minutes were included to account for any abiotic losses. Another set of controls referred to as cattail controls that contained no cattail were used for each set. Cattail controls were constructed to signify use of cattail as carbon and energy source by the indigenous populations.

To maintain anaerobic conditions, all sets were purged with N_2 sealed with Teflon-lined butyl rubber stoppers and secured with aluminum crimps, and incubated at 27^{o}C in the dark for about two months. Following incubation, soils were extracted and DDx concentrations determined.

Mesocosm Soil Collection

Soils from Lake Apopka, FL sample site ZNS2017 from NSRA's north boundary were collected from a 16 foot by 16 foot plot on east of Laughlin Road (UTM coordinates: X = 440603.7, Y = 3177660.0). The site was selected because of high DDx concentration as determined by Pace Analytical Services under contract to the St. Johns River Water Management District. The concentration of p,p'-DDE was 5990, p,p'-DDD was 564 and that for p,p'-DDT was 15800 µg/Kg soil using analytical method EPA 8081 and sample preparation method EPA 3550. Total Organic Carbon (TOC) was determined to be 37% by DB Environmental, Inc. Vegetation was removed and soils were homogenized on site using a trackhoe. Following mixing the soils were loaded into

a dump truck using a front end loader. The soil was transported to the University of Florida where the soils were stored in wooden boxes lined with a plastic liner.

Preparation of Anaerobic Mesocosms

Rubbermaid "Farm Tough" (100-gallon) stock tanks made of high density polyethylene were used for mesocosms. The dimensions at the top of the tanks were length and width of 54 inches (137.16 cm) and 35 inches (88.9 cm), respectively, height was 23 inches (58.42 cm) (Figure 6-1). The tanks had a controllable opening at the bottom of tank for drainage. Prior to filling the tanks with soils, a bed of egg rocks was added to promote drainage.

Four tanks were placed on cement block tables inside a greenhouse (Figure 6-2). The order of the mesocosms tanks from north to south was Control-1, NaCl-1; Control-2, NaCl -2. This design was intended to randomize any effects that location in the greenhouse might have, such as temperature.

Soil was manually mixed with shovels and added to the tanks sequentially, one wheel barrow-full at a time. Once in the tanks, soil was thoroughly mixed with shovels. Water was added to a height of 10 cm above the soil surface (Figure 6-1) to create anaerobic conditions. For all mesocosms, sodium lactate was mixed with tap water in 20 L containers to a final concentration of 10 mM lactate. Approximately 180 L lactate solution was required to fill each tank to 10 cm above soil surface. Ten soil samples were collected randomly from each mesocosm at a depth of 5 cm, mixed to form one composite sample per tank, and frozen at -80^{o}C for DDx analysis. The lactic acid included in these additions was added as sodium lactate, which lead to a final Na^{+} concentration of 0.015 mg Na^{+} per gram dry soil.

Initial microcosm experiments indicate positive correlation of Na^+ additions to degradation, for Tanner medium R^2 values for 0, 30, 50 and 80 mg Na^+ per kg dry soils were 0.42, 0.43, 0.72 and 0.70 respectively. Due to limited availability of NSRA soil, a temporally staggered experimental design was established such that 0.015 mg Na^+ per gram dry soil was added to two experimental tanks and monitored until the redox potential in the tanks stabilize. Two weeks following stabilization, a second addition of Na^+ was made to bring the final concentration to 80 mg Na^+ per kg dry soil. Additions were made in the treatment tanks on 80th day following anoxic incubation.

Tanner medium (Tanner et al., 2007) trace metal solution was added to all mesocosms (control and experimental) at the same time as the additional Na^+ described above. Trace metal solution was added to both Control and NaCl tanks to study the effect of additional NaCl. The additional Na^+ and metals was dissolved in 500 mL distilled water to appropriate concentrations, autoclaved, and manually mixed using a shovel on 80th day and mixed once again on 81st day to ensure homogeneity.

Ingredients amended (mg/g dry soil) were Nitrilotriacetic acid (NTA), 0.0135; $MnSO_4.H_2O$, 0.00675; $Fe(NH_4)_2(SO_4)_2.6H_2O$, 0.0054; $CoCl_2.6H_2O$, 0.00135; $ZnSO_4.7H_2O$, 0.00135; $CuCl_2.2H_2O$, 0.00135; $NiCl_2.6H_2O$, 0.00135; $Na_2MoO_4.2H_2O$, 0.000135; Na_2SeO_4, 0.000135; Na_2WO_4, 0.000135.

Sample Collection

Upon stabilization of redox potentials in the mesocosms, eight samples were collected from each tank and mixed to obtain a homogenous composite sample. Sampling was done once every two weeks using a 50 mL disposable syringe. The ends of the syringes were cut off and a rubber stopper was used to create suction to hold the sediment sample. A syringe was used for sampling to avoid disturbance and

homogenization of the layers in the sample. Samples were stored at -80°C until analysis.

Redox Potential (Eh) and Temperature Measurements

Redox potential was determined every two weeks during the anoxic treatment. Eh was measured with portable pH/ Eh and Temperature meter (HI 9126 Hanna Instruments; Woonsocket, RI). The probe was calibrated before every use. The Eh probe was submerged in each tank to a depth of 10 cm below soil surface and allowed to equilibrate before taking the reading; temperature readings were taken at the same depth using a temperature probe.

Dissolved Organic Carbon (DOC) Measurements

DOC was extracted from soils by a cold water extraction method. One gram soil on a dry weight basis was diluted to a concentration of 1:10 (soils: distilled water). Sample slurries were incubated for 16 hours at room temperature, followed by centrifugation in Beckman J2-21 Floor Model Centrifuge (GMI Incorporation Ramsey, MN**)** (JA-14 rotor) at 4472 x g for 15 minutes. The resulting supernatant was filtered through a 0.22 µm filter (Durapore membrane filters) under vacuum. Filtered solutions were analyzed on Shimadzu sample module 5000A Carbon Nitrogen Analyzer (Columbia, MD) vendor).

pH Measurements

pH was analyzed from pore water of soil samples. Soil samples were centrifuged in a Beckman centrifuge Model J2-21 at 7155 x g for ten minutes. pH was measured from supernatant using Orion pH meter Model SA720 (Cole-Parmer Instrument Company Vernon Hills, IL).

DDx Extraction

DDx extraction could be divided into three stages: soil preparation; accelerated solvent extraction (ASE); and florisil extraction. The extraction method was based on U.S.EPA method #3545 (U.S.EPA, 2000) with minor modifications developed by Soil Microbial Ecology Laboratory, University of Florida, and chemists at Pace Analytical Services, Ormond Beach, FL.

Soil Preparation

Soils were separated from liquid by centrifugation in Beckman J2-21 Floor Model Centrifuge (JA-14 rotor) at 7155 x g for 20 minutes at 4°C. Wet soil samples were allowed to air dry for two to three days following which they were ground. Soil moisture content was determined in the sample. Moisture was adjusted to 50% moisture on a dry weight basis, and then the samples were allowed to equilibrate in 4°C refrigerator for 3 days.

Accelerated Solvent Extraction

Soil was mixed with Hydromatrix, a drying and bulking agent, at a ratio of 1:2, and placed into 34 mL stainless steel extraction cells. The remaining headspace in extraction cells were topped with clean Ottawa sands (Fisher Scientific, Pittsburgh, PA) to decrease the amount of solvent usage. Solvent used for extraction was methylene chloride: acetone (4: 1 v/v). Soils were extracted under high pressure about 1200 to 1400 psi at temperature of 100°C in a Dionex ASE 100 Accelerated Solvent Extractor (Sunnyvale, CA).

The extraction cycle included filling the extraction cell with about 19 mL solvent, followed by heating cycle where the solvent was heated to 100°C. Static extraction followed the heating cycle which performed for 5 minutes, followed by flushing cycle

which flushed about 19mL solvent through the cell. The cell was finally purged with N_2 for about 2 minutes, which completed one extraction cycle.

Florisil Extraction

Florisil clean up used prepacked florisil “Bond Elute LRC” columns (200 µm particle size) (Varian Inc., Palo Alto, CA) which contained approximately 1 g Florisil packaged into the plastic holder. The column was conditioned using 5 mL hexane: acetone (9:1v/v), loaded with 1 mL of ASE extract and the column was eluted using 9 mL of solvent.

A volume of 5 mL from the cleanup volume was concentrated to dryness under a gentle stream of air. It was very important to concentrate the samples to complete dryness prior to GC (gas chromatography) analysis to avoid matrix response enhancement (Schmeck and Wenclawiak, 2005). Samples were reconstituted in 1 mL of hexane, and tubes with samples were vortexed and transferred to a 2 mL amber glass GC vials (, Fisher Scientific Inc., Atlanta, GA) and crimped with 12 x 32mm aluminum crimp seals with prefitted PTFE lined septa (, Fisher Scientific Inc., Atlanta, GA) for subsequent analysis by GC.

GC Conditions

Perkin-Elmer Autosystem Gas Chromatograph (GC) equipped with autosampler and an electron capture detector (ECD) was used for analyzing the extracts. Column conditions were: He as the carrier gas at flow rate of 1.0 mL/min, 5% methane in argon as the make-up gas with a flow rate of 50 mL/min, electron capture detector (ECD) temperature at 350°C, and injector temperature at 205°C in splitless mode. Injection volume was 3 µL in slow injection mode. GC temperature was programmed at 110°C for 0.5 minutes, followed by a ramp at 20°C/min to 210°C and hold for 0 minutes, then

ramp at 11°C/min to 280°C and held for 6.3 minutes. Under these conditions, the detection limit was calculated at the 90% confidence level to be: 0.007 µg/mL for o,p'-DDE; 0.005 µg/mL for p,p'-DDE; 0.006 µg/mL for o,p'-DDD; 0.009 µg/ mL for p,p'-DDD; 0.005 µg/mL for o,p'-DDT; and 0.010 µg/mL for p,p'-DDT (Hubaux and Vos, 1970). These results are similar to the detection limit of 0.010 µg/mL reported by Pace, Inc.

Analysis of Organic Acids by HPLC

Sample Preparation

One gram soil on a dry weight basis was diluted to a concentration of 1:10 (soils: distilled water). Sample slurries were incubated for 16 hours at room temperature, followed by centrifugation in Beckman J2-21 Floor Model Centrifuge (JA-14 rotor) at 4472 x g for 15 minutes. The resulting supernatant was filtered through a 0.22 µm filter (Durapore PVDF membrane filters, Fisher Scientific, Pittsburgh, PA) under vacuum.

Derivatization

Samples were derivatized using following steps. Pyridine buffer (0.2 mL) was added to 2 mL sample. The resulting solution was purged with nitrogen to remove CO_2 and O_2 for four minutes. Anoxic solution got amended with 0.2mL of 0.1M 2-nitrophenyl hydrazine (in 0.25M HCl) and 0.2 mL of 0.3 M *l*-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride, after mixing the vials were incubated at room temperature for 90 minutes. Following incubation 0.1mL of 40% KOH was added and the samples were heated in a heating block at 70^{o}C for 10 min.

HPLC

Concentrations of fatty acids were determined using HPLC with UV/VIS detector at 400 nm wave length and a C8 reverse phase column (22 cm $\times$ 1.5 cm). Mobile phases included 2 solvents. Solvent A was composed of 2.5% n-butanol, 50 mM Sodium

acetate, 2 mM Tetrabutylammonium hydroxide, 2 mM Tetradecyltrimethylammonium bromide (TDTMABr) with pH adjusted to 4.5 using phosphoric acid. Solvent B differed from solvent A only in containing 50 mM TDTMABr. The injection volume was 100 μL. Retention times for lactate, acetate, propionate, formate were 9.95, 12.62, 14.67 and 13.28 minutes, respectively. Standards fatty acids used were lactate, acetate, formate, propionate, butyrate, succinate, iso-butyrate, iso-valerate (Albert and Martens, 1997; Dhillon et al., 2005; Jonkers et al., 2003).

Statistical Analysis

Statistical analyses were conducted with JMP software manufactured by SAS (Cary, NC). One way ANOVA analysis was used for microcosm data analysis. Controls were tested against the treatments using Tukey's post hoc test. Mesocosm data sets were compared using studentized t-test. Values with $p<0.05$ were considered significantly different and variables in graph not connected by the same letter are significantly different.

Results and Discussion

Microcosms

Although a direct role of Na^+ in DDT degradation is not well documented, its role in soil and organic matter dispersion is well recognized (Nelson and Oades, 1998; Wood, 1995; White, 1997; Brady and Weil, 2003; 2007; Kantachote et al., 2003). Dispersion may lead to expose new surfaces thereby increasing accessibility or bioavailability to the degrading consortia. So Na^+ is not only responsible for dispersing the soils but also increasing the DOC following anoxic incubation. Earlier chapters in this thesis (Chapters 3 and 4) discussed the potential for DOC to increase

bioavailability, and studies by Kantachote (Kantachote et al., 2001; 2003) demonstrated the importance of increased DOC on increasing bioavailability of DDxs.

Comparison of Media

Results using the Ou medium are presented in Figure 6-3, and studies using the Tanner medium, rich in mineral salts, trace metals and vitamins, are presented in Figure 6-4. Tanner medium microcosms demonstrated that an increase in NaCl concentration results in a decrease in DDx concentration. With respect to the remaining DDx concentrations, 0 mg Na^+ microcosm soils are consistently significantly different from those in 80 mg Na^+ microcosms. For example, 0 mg Na^+ microcosm soils contain 37.5 and 76.9 mmol of p,p'-DDE and p,p'-DDT when compared to that in case of 80 mg, concentrations are 1.4 and 19.7 mmol, respectively. Similarly, concentrations of o,p'-DDE, o,p'-DDD and o,p'-DDT for 0 mg Na^+ are 4.7, 5.5 and 27.2 mmol per g dry soil, respectively. When compared to o,p'-DDxs in 80 mg microcosms, concentrations are 0 mmol for both o,p'DDE and o,p'DDD and 6.5 mmol for o,p-DDT.

When comparing 0 and 30 mg Na^+ microcosms, no significant difference in DDx concentrations was observed, and little difference was observed between the 50 and 80 mg Na^+ microcosms. However, a significant difference was observed when the sets are divided into two groups (0 and 30 mg, and 50 and 80 mg). Regardless of Na^+ concentrations, it is evident from Figure 6-4 that highest degradation was achieved with 80 mg Na^+ microcosms. Furthermore, Na^+ is positively impacting biological degradation, such that the degradation sequence is 80 mg >50 mg >30 mg >0 mg. Findings from this experiment are in accordance with similar studies by Kantachote and colleagues (Kantachote et al., 2001; 2003).

Similar studies (Kantachote et al., 2003) demonstrated that applications of Na^+ at various concentrations to long-term DDT contaminated soils lead to DDT biotransformation under anoxic conditions. They attributed this transformation to dispersive nature of Na^+ which coincided with increased DOC concentration, anaerobic bacterial numbers and aqueous solubility of DDxs. DDxs transformation ranged from 95% to 72% with maximal transformation at 30 mg Na^+ per kg dry soil. DDT transformation was repressed at higher Na^+ levels due to flocculation and decreased DOC levels. Another study by the same group (Kantachote et al., 2004) increased biodegradation of DDT using seaweed. The basis for this study is that seaweed is a potential source of Na^+ and nutrients, which might not only increase the bioavailability but also support growth of the degrading consortia. The highest degradation rate observed by this group was 80% and ranged from 80% to 34%, such that 0.5% seaweed gave highest degradation. Higher concentrations of seaweed actually retarded degradation probably by binding of DDxs residues to the DOC of seaweed. Another important study demonstrated DDE (isomer not mentioned) (the most recalcitrant of the DDx) was reductively dechlorinated to DDMU in marine sediment microcosms (Quensen et al., 1998).

Since previous microcosm and mesocosm studies demonstrated that the greatest amount of degradation of DDxs was achievable using lactate as carbon and electron source, whereas DDxs served as probable TEAs or were cometabolized (Chapters 3 and 4). These conditions may have promoted growth of a syntrophic community, where lactate fermenting, hydrogen-producing organisms coexisted with a hydrogen-utilizing, DDx dehalorespiring population (Chapter 5). An attempt in this study was to simulate

lactate production by cattail fermentation in order to provide the energy and carbon needs of the degrading consortia. By using cattail, we are promoting lactate production. Using cattail can make the system more sustainable, affordable, and increase the feasibility for field application.

The cattail control (no cattail) exhibited lower concentrations of DDx than the autoclaved control, indicating that the organisms capable of degradation are present, but the system is carbon limited. Hence, careful selection of carbon and electron donor in a TEA limited anoxic environment can shift the system to dehalorespiration (Suflita et al., 1988). Autoclaved control has the highest concentrations of DDxs which indicates that changes in DDxs are caused all microbes mediated transformation.

A high variability among replicates was observed for the Ou Medium microcosms (figure 6-3), and no relationship between Na^+ concentration and degradation was observed. Greater degradation of DDx was observed in the microcosms with Tanner medium than with the Ou medium, likely because Ou medium is a minimal salts medium, containing no vitamins or trace metals. Tanner medium has higher concentrations and numbers of trace metals and cofactors that may be limiting in the peat soils of NSRA.

Microcosms Conclusions

There is a positive correlation between Na^+ concentration and DDx degradation in microcosms with the Tanner medium. A likely explanation is that, upon soil dispersion by the added Na^+, the otherwise unavailable DDx were made available to the degrading consortia. Furthermore, with anoxic incubation there is an increase in DOC concentrations (Chapters 3 and 4), which may also increase the solubility and hence bioavailability of DDx. Both these factors could increase exposure of substrate to the

microbes. Another objective in this study was to meet with the energy and carbon needs of the degrading consortium via decomposition of cattail.

The lowest amount of degradation observed was in case of autoclaved control, which indicates that degradation was microbially mediated. Higher concentration of p,p'-DDT in the no-cattail controls suggests that the system is carbon limited and that cattail fermentation products could feed the degrading populations. Another indication is that although organisms capable of degradation are indigenous to the soils, suitable selection of electron donor and carbon source in TEA limited anaerobic system along with increased bioavailability may result in biodegradation of the otherwise inaccessible aged DDx.

Another observation from this study is that trace metals, cofactors, and vitamins are needed by the degrading population. They may be responsible for triggering the production or activation of degrading enzymes, or may be required as growth factors by the degrading consortia. Further research is needed for confirming the above observations.

Mesocosm Results

In microcosm studies, Na^+ at a rate of 80 mg/kg dry soil with cattail as the sole exogenous carbon and electron source gave the highest degradation of DDx. Another important amendment in the microcosms exhibiting the greatest loss of DDx was a medium rich in trace metals, vitamins, and cofactors, which may have triggered production or activation of degrading enzymes. Mesocosms were established not only to scale up the microcosms but also to check reproducibility of microcosm results. The microcosms studies described in Chapter 3 indicated that the greatest amount of transformation of DDx was observed with lactate as an electron donor, indicating that

lactate stimulated, either directly or indirectly, microbial groups capable of utilizing DDx as a terminal electron acceptor. The objective of these experiments was to investigate the role of Na^+ in mesocosms amended with lactate (10 mM). The only difference between the Na^+ tanks (treatment) and control tanks was the presence of additional Na^+ in treatment tanks resulting in 80 mg/kg dry soil. Both the control and treatment sets received lactate (10mM), and trace metals, cofactors, and vitamins.

Within three weeks after establishing the mesocosms, redox potentials dropped to those characteristic of anoxic soils (Figure 6-6) and remained below -100 mV for the duration of the experiment. We expected that mixing of the amendments on 80th day would incorporate air in the tanks and increase Eh; however, Eh remained low throughout the incubation. A significant decrease in redox was observed in the treatment tank immediately following mixing. This could be because the otherwise accessible lactate was now distributed into the deeper layers in soils. Increased availability of lactate could have driven the Eh to more negative values. Temperatures fluctuated somewhat throughout the experimental period, although temperatures were similar between control and experimental mesocosms (Mean temperature for control and treatment tanks were 18.1°C and 18.5°C with standard deviation of 2.9 and 3.0 respectively) (Figure 6-5). pH trends (Figure 6-8) were similar between treatment and controls throughout the incubation period, with a significant drop in pH observed following addition of trace elements on the 80th day. Following mixing amendments, the pH gradually increased with a significant difference between control and treatment tanks observed by the end of the experiment (Figure 6-8).

DOC contents were measured in pore waters for each of the samples (Figure 6-7). DOC concentrations increased with incubation time, approximately following the observed decreases in redox potential. DOC for treatment tanks is consistently higher than for control tanks, likely due to higher concentrations of Na^+ in the treatment tanks. Following a peak in DOC concentrations on the 39th day, DOC concentrations dropped significantly in both control and treatment tanks. This drop in DOC concentrations may be related to a concomitant drop in temperature (Figure 6-5), which may have decreased production of DOC. DOC concentrations remained relatively constant until 121st day in incubation, when increases in pH and DOC were observed.

Concentrations of organic acids in the DOC were measured to determine if lactate fermentation products were present, or if the dominant DOC was likely to be derived from soil organic matter (SOM). Since there were two peaks with significant release of DOC, samples taken on the 39th and 139th days were chosen for analysis. Results from these days are presented in Figures 6-13 and 6-14, respectively. The major short chain fatty acids observed in these samples were acetate, lactate, and formate, with no butyrate or isobutyrate detected in either treatment or control samples. To check if the dominant DOC was derived from SOM we calculated percent of lactate contributing to DOC, values for which are as follows, control and treatment tanks on 39th day accounted for 10% and 8% respectively, while values for 139 days were 6% and 4%, respectively. These values are considered fairly negligible, which suggests that the lactate amended to the tanks was used up very quickly and that the DOM released originated from anoxic incubations in soils.

DDx concentrations in soils followed similar general trends in both controls and Na^+ tanks prior to addition of Na^+ (Figures 6-9, 6-10, 6-11 and 6-12). Prior to Na^+ addition, these tanks may be regarded as replicates because both contained lactate (10 mM) as the carbon and energy source and DDx as potential terminal electron acceptors. DDx concentrations increased during the initial month approximately for the first 40 days in incubation which correlates with the "Release Phase" in case of lactate mesocosms (Chapter 4). It is interesting to note that the peak in DDx concentrations observed on 39th day corresponds with the peak in DOC concentrations (Figure 6-7), suggesting that release of DOC increases desorption and bioavailability of DDx (Kim et al., 2008). The peak in DDx concentration is followed by a decrease in both the control and treatment tanks. This decrease is in accordance with the "Degradation Phase" in anoxic lactate mesocosm studies (Chapter 4). Furthermore, these studies again support the hypothesis that the system is carbon limited and supplementation with lactate lead to rapid biological degradation.

It is possible that lactate was fermented by syntrophic bacteria to H_2 and organic acids such as formate and acetate, which may have served as electron donors to the dechlorinating population for DDx degradation. The concentrations of all the DDx went to less than 10 mmol/g dry soil by the 50th day and remained below that until amendments were made on the 80th day (Figures 6-9, 6-10, 6-11 and 6-12). On the 80th day, Na^+ and trace metal solutions were manually mixed into the treatment tanks while the control tanks received only trace metal solutions. Following mixing, DDx concentrations increased immediately on 81st day in both control and treatment tanks.

Mixing may have had several effects on measureable DDx concentrations, including: (1) perturbation of soils which could make the otherwise inaccessible soils available for sampling; and (2) dispersion and homogenization of DOC in the more anoxic or inaccessible zones. Both homogenization of soils and DOC dispersion might have increased the extractability by releasing the bound DDx and made the otherwise inaccessible sites now available for sampling. Since DDx concentration prior to mixing was lower than the detectable limits, it might suggest the presence of DDx degrading consortia. When the DDx concentration increases were observed, trace metals and carbon source (lactate) may have facilitated the degrading consortia.

Analysis of time points toward the end of the experiment show a significant difference between the control and treatment tanks. Concentrations of all o,p'-DDx metabolites (o,p'-DDE, o,p'-DDD and o,p'-DDT) measured in treatment tanks were significantly lower ($p<0.05$) from the control tanks by 146th day (Figures 6-11 and 6-12). p,p'-DDT was significantly lower in Na^+ amended than in the control tanks by the 151st day (Figures 6-9 and 6-10). Analysis of further time points would have been useful to determine if the decreases in DDx concentrations would continue to be significant or drop below detectable levels, as was observed during the first phase of incubation (prior to mixing amendments 50th to 80th day); however, the experiment was discontinued due to time constraints. Significantly, the observed increases in DDx in both phases match increases in DOC concentrations, both of which precede a significant loss of measurable DDx.

Inference for Anaerobic Mesocosms

Data presented in Figures 6-9, 6-10, 6-11, and 6-12 indicate loss of the parent and metabolites to less than 10 mmol/g dry soil in all mesocosms within 50 days of

incubation. This is in accordance with anoxic lactate mesocosms (Chapter 4), strongly suggesting that degradation of DDx is limited by available carbon. Following amendments, the observed trends indicated a repeated increase followed by a decrease in DDx concentrations. Data analysis suggested that there was no significant difference between the control and Na^+ treatment tanks, perhaps due to the relatively high variability in some of the data points. Irrespective it is interesting to note that towards the end there is some significant difference between treatment and control tanks with respect to both o,p' and p,p'DDx metabolites.

Greater extraction and degradation of DDx under anaerobic conditions can be explained by earlier studies demonstrating the importance of DOC in increasing the bioavailability of hydrophobic compounds (Kim and Pfaender, 2005; Kim et al., 2008; Pravacek, 2005). They showed that anaerobic incubations stimulated release of bound hydrophobic residues making them more soluble and, hence, more bioavailable. Following the addition of trace metals and Na^+, an increase in DDx concentrations was observed, indicating that as the availability of DDx increased lactate provided energy requirements for the degrading population. Anoxic Eh promotes DOC release (Kim et al., 2008; Pravacek, 2005) and DOC increases the solubility (and hence bioavailability) of DDx. This is in accordance with the DOC data (Figure 6-7), in which higher concentrations of DOC coincide with higher concentrations of DDx, and precede more rapid decreases in DDx concentrations.

Summary

Na^+ addition may be an inexpensive, safe, sustainable, and feasible alternative to the expensive chemical and physical remediation methods. Low concentrations of NaCl increased degradation of aged DDx, in microcosms possibly due to dispersal of soil

polymers, resulting in increased bioavailability. Na^+ may have potential to remove such HOCs from long term aged soils and remediate such soils. Another advantage is that the technique being environmentally friendly may lead to clean up of otherwise contaminated site. More research is needed to confirm its applicability.

Figure 6-1. Mesocosm tank, used for construction of anaerobic mesocosms. Photo courtesy by Hiral Gohil.

Figure 6-2. Anaerobic mesocosms for bioremediation of DDE, DDD, and DDT in soil from the Lake Apopka North Shore Restoration Area. Photo courtesy by Hiral Gohil.

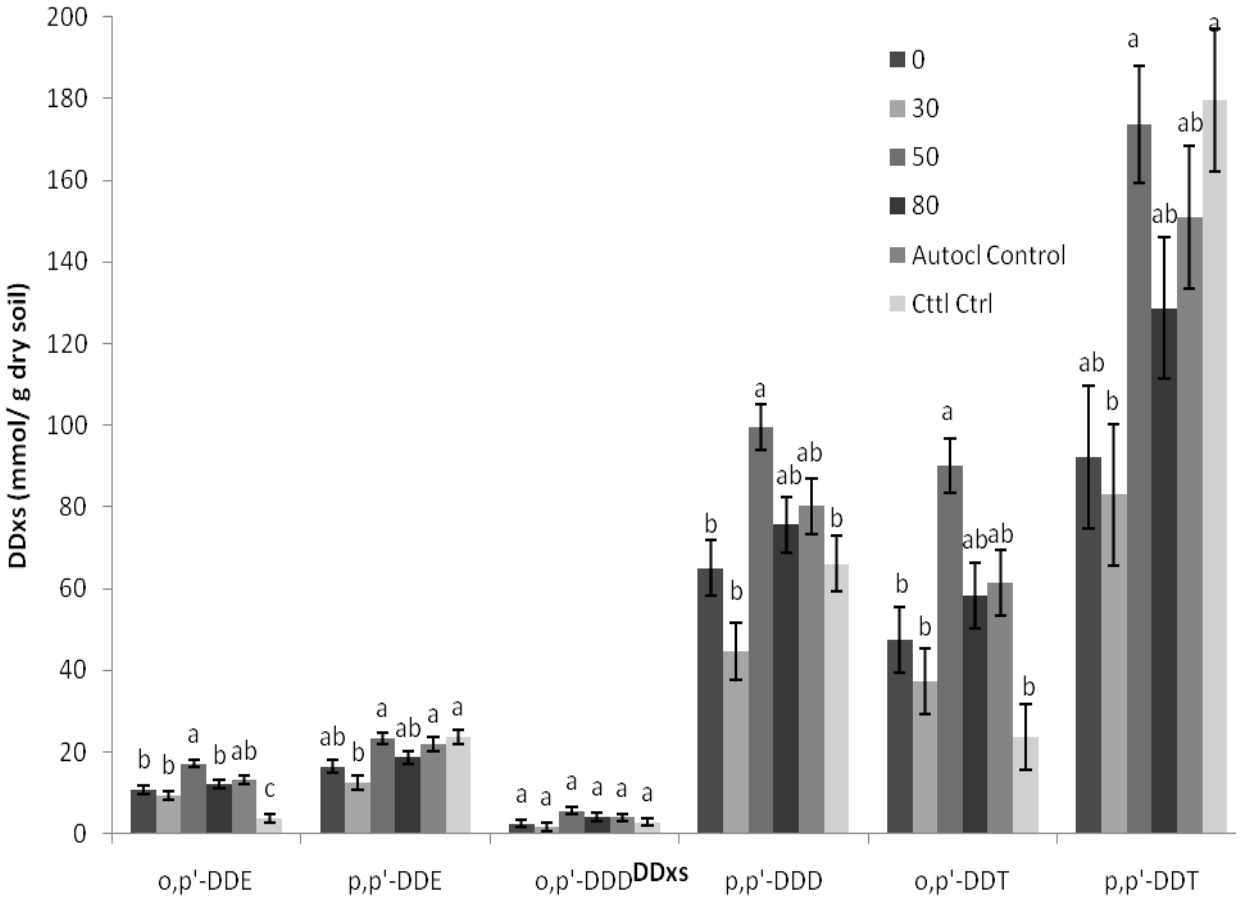


Figure 6-3. Concentration of DDxs measured in NSRA soil following incubation in minimal Ou medium (Ou et al., 1978) with a range of Na^+ concentrations. Error bars represent +/- one standard deviation. Data labels not connected by same letter are significantly different $p<0.05$. "Cttl Ctrl" = cattail control, "Autocl Ctrl"= autoclaved control.

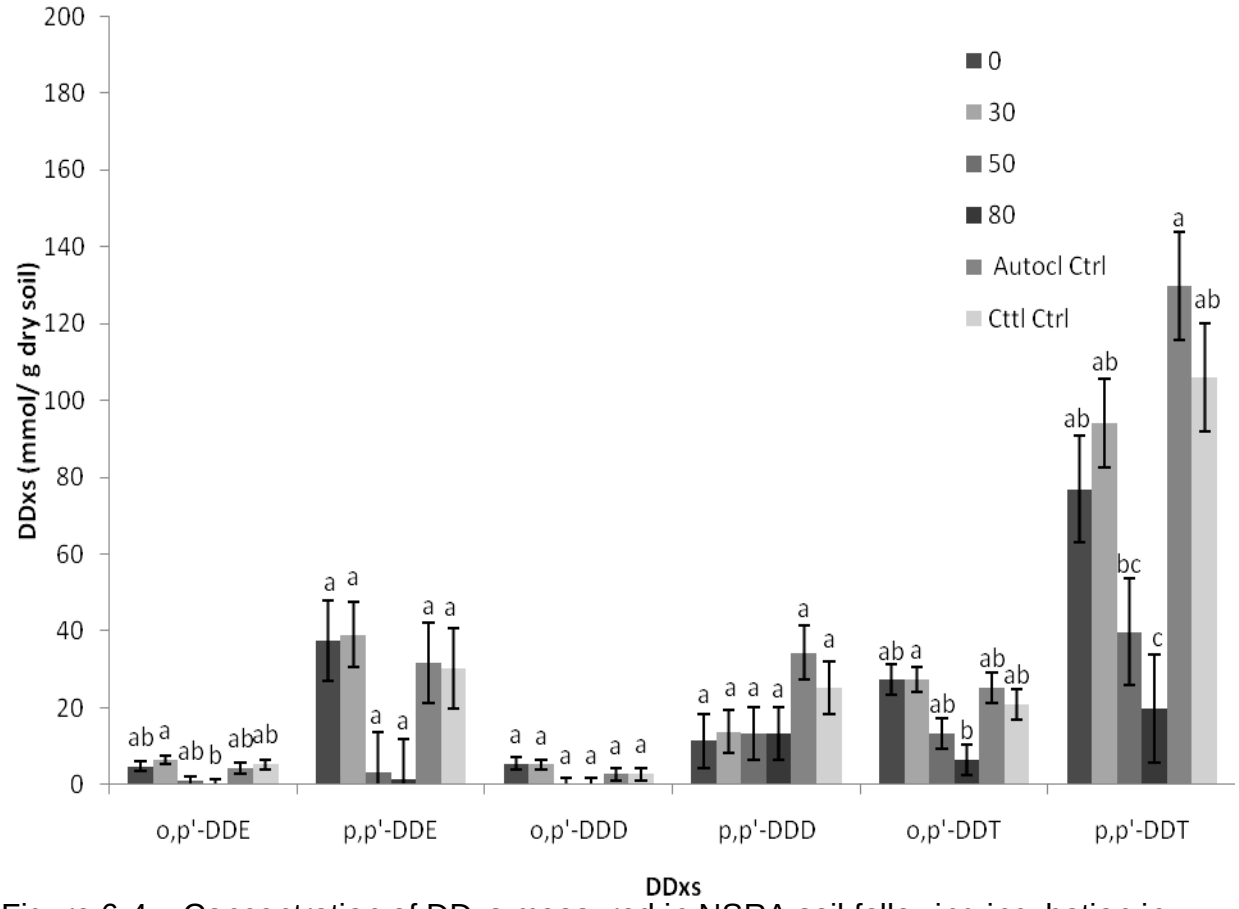


Figure 6-4. Concentration of DDxs measured in NSRA soil following incubation in complex Tanner medium (Tanner et al., 2007) with a range of Na^+ concentrations. Error bars represent +/- one standard deviation. Data labels not connected by same letter are significantly different $p<0.05$. "Cttl Ctrl" = cattail control, "Autocl Ctrl"= autoclaved control.

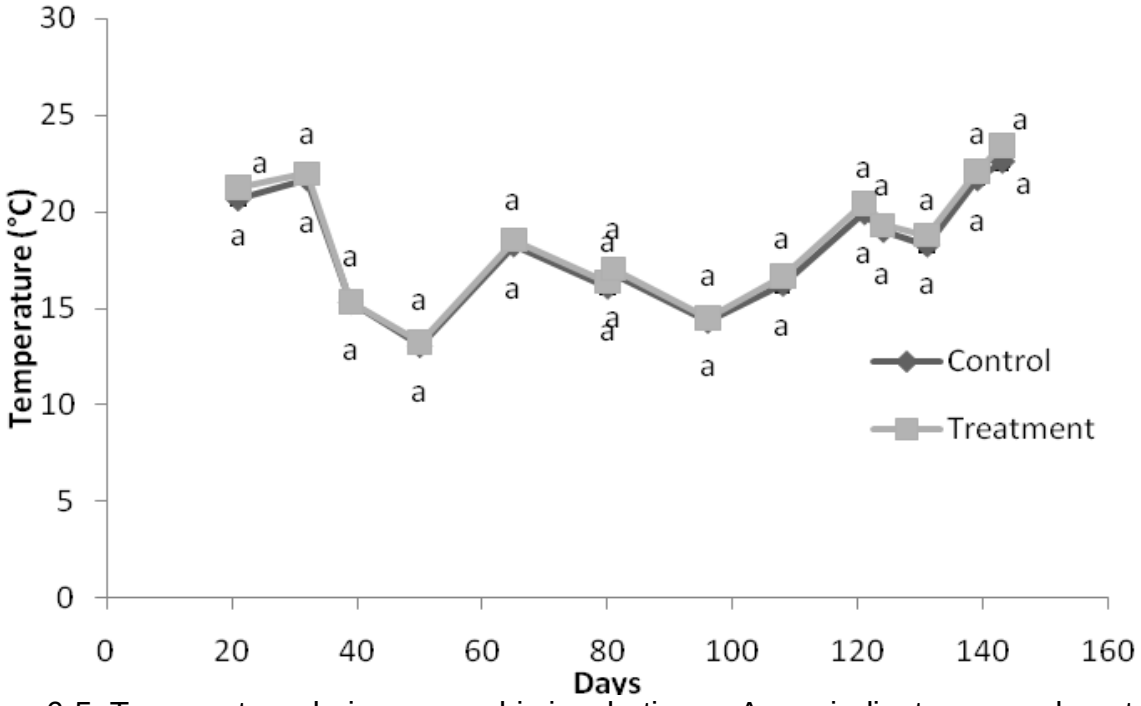


Figure 6-5. Temperature during anaerobic incubations. Arrow indicates amendments made on 80th day. Error bars represent +/- one standard deviation based on two replicates. Data labels not connected by same letter are significantly different $p<0.05$.

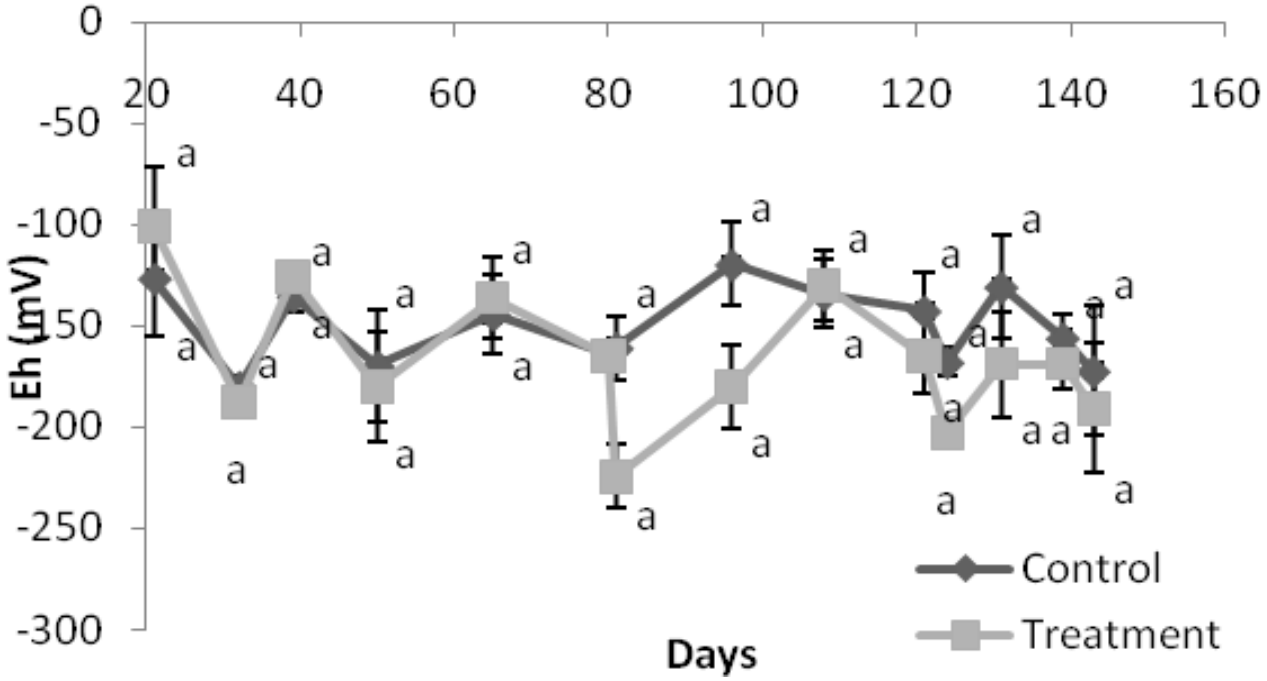


Figure 6-6. Redox (Eh) values measured during anaerobic incubation. Arrow represents amendments added on 80th day. Error bars represent +/- one standard deviation based on two replicates. Data labels not connected by same letter are significantly different $p<0.05$.

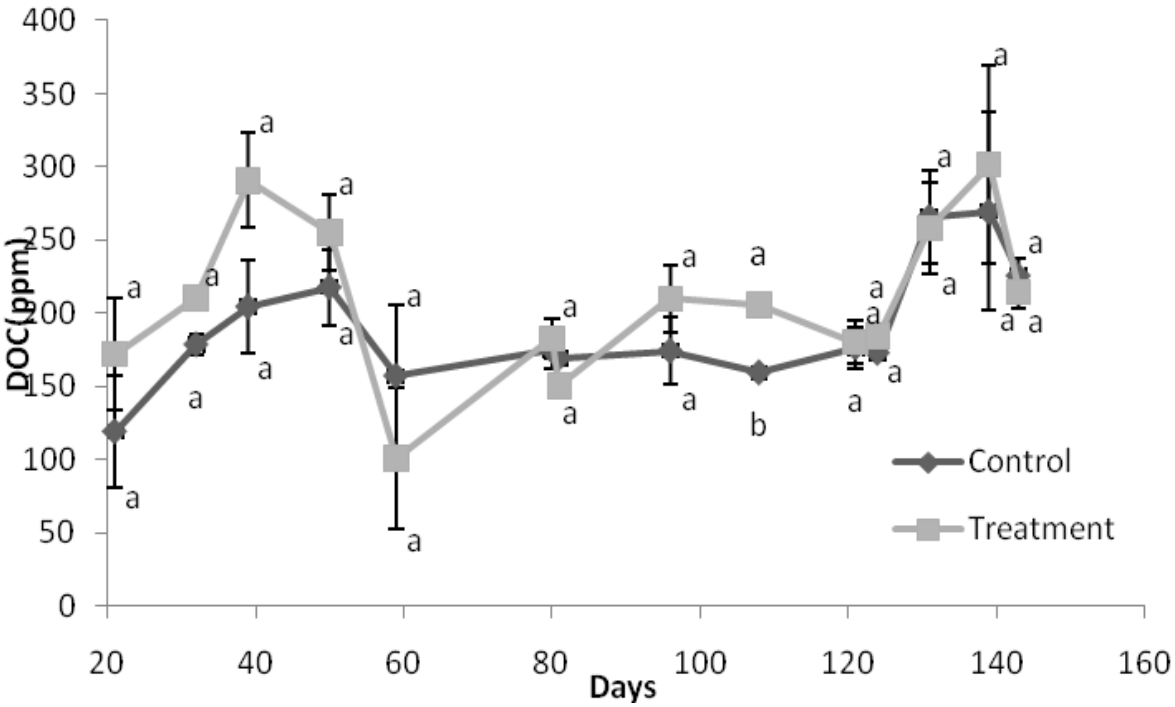


Figure 6-7. Dissolved organic carbon (DOC) during anerobic incubations. Arrow indicates amendments made on 80th day. Error bars represent +/- one standard deviation based on two replicates. Data labels not connected by same letter are significantly different $p<0.05$.

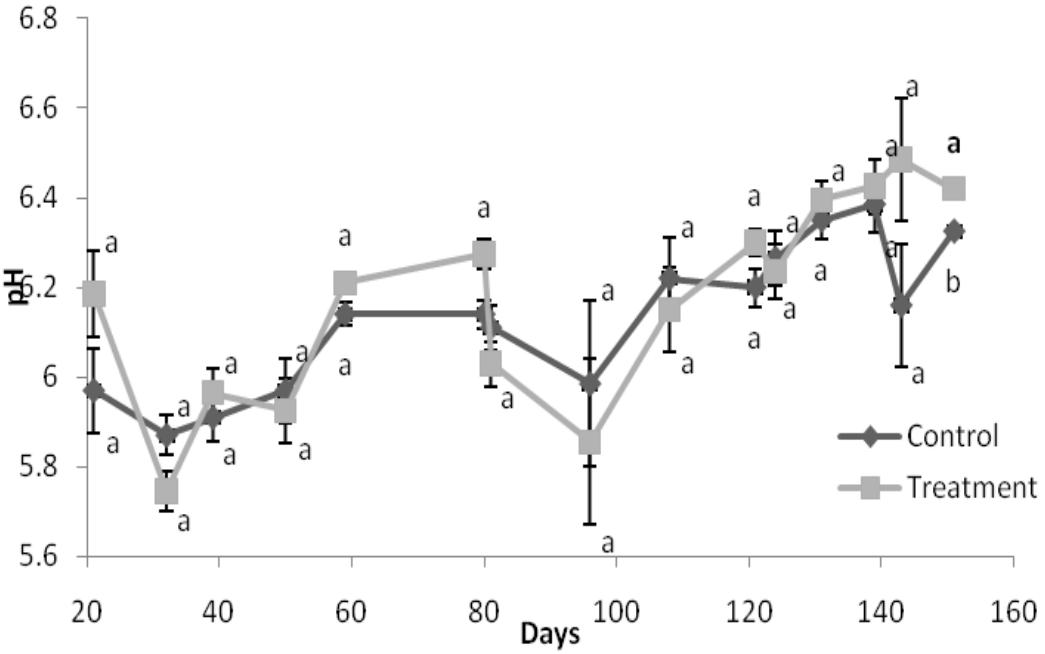


Figure 6-8. pH readings during anaerobic incubations. Arrow indicates amendments made on 80th day. Error bars represent +/- one standard deviation. Data labels not connected by same letter are significantly different $p<0.05$ based on two replicates.

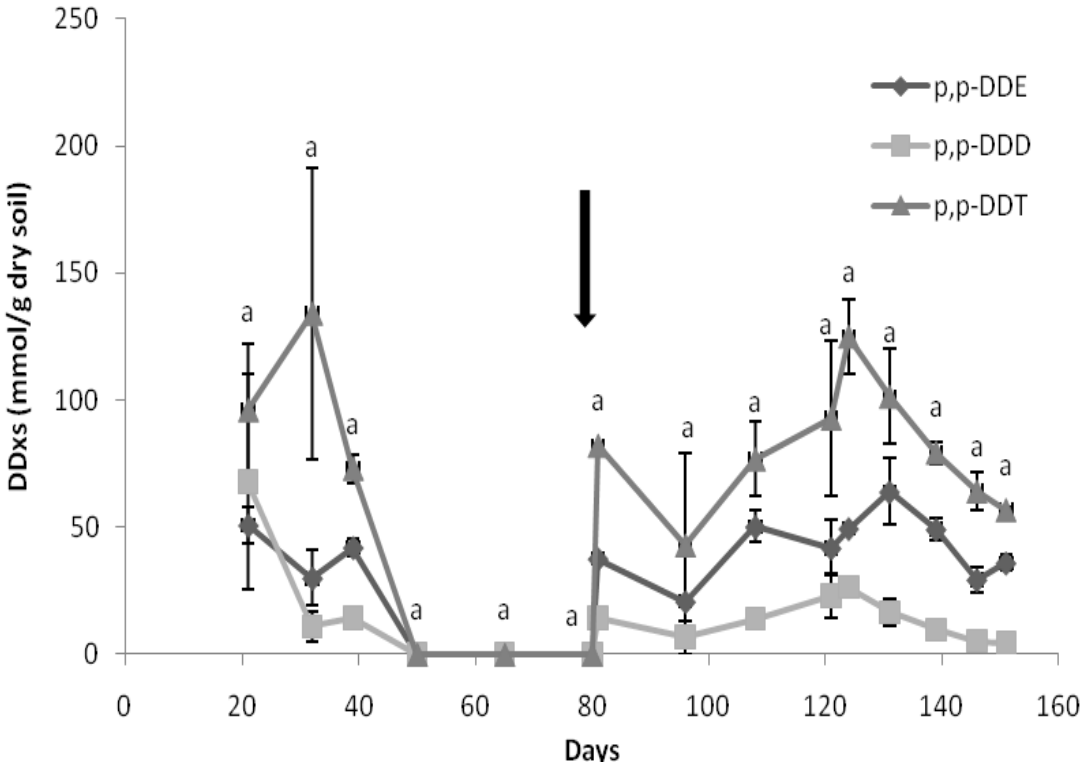


Figure 6-9. p,p'-DDx concentration in control tank soils. Trace metals were amended on 80th day indicated by arrow. To simplify the figure data labels are added only in case of p,p'-DDT where there was a significant difference between control and treatment. Data labels not connected by same letter are significantly different $p<0.05$. Error bars represent +/- one standard deviation based on two replicates.

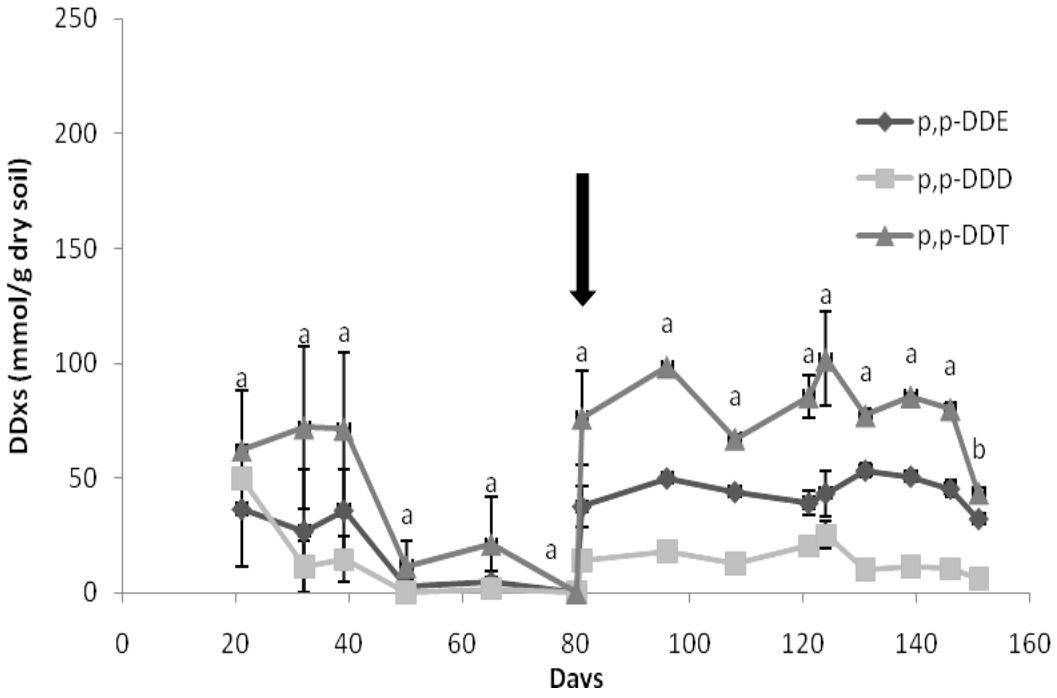


Figure 6-10. p,p'-DDxs concentration in treatment (Na^+) tank soils. Trace metals and Na^+ were amended on 80^{th} day indicated by arrow. To simplify the figure data labels are added only in case of p,p'-DDT where there was a significant difference between control and treatment. Error bars represent +/- one standard deviation based on two replicates. Data labels not connected by same letter are significantly different $p<0.05$.

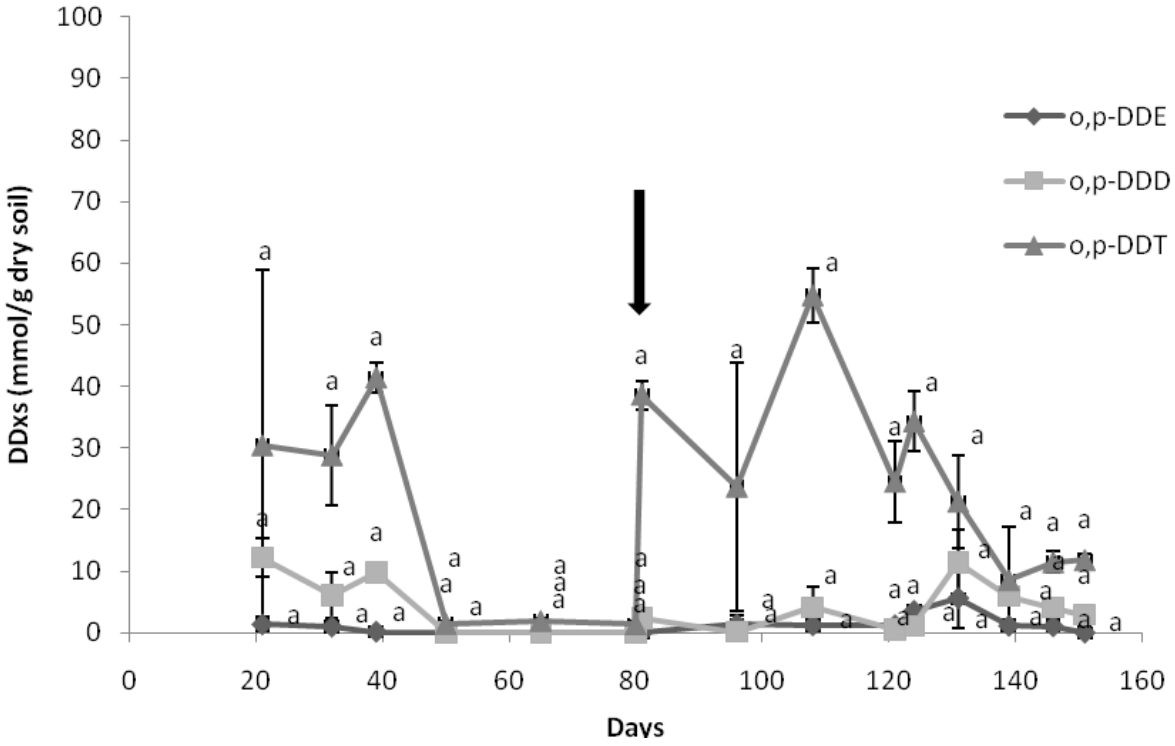


Figure 6-11. o,p'-DDxs concentration in control tank soils. Trace metals were amended on 80th day indicated by arrow. Error bars represent +/- one standard deviation based on two replicates.

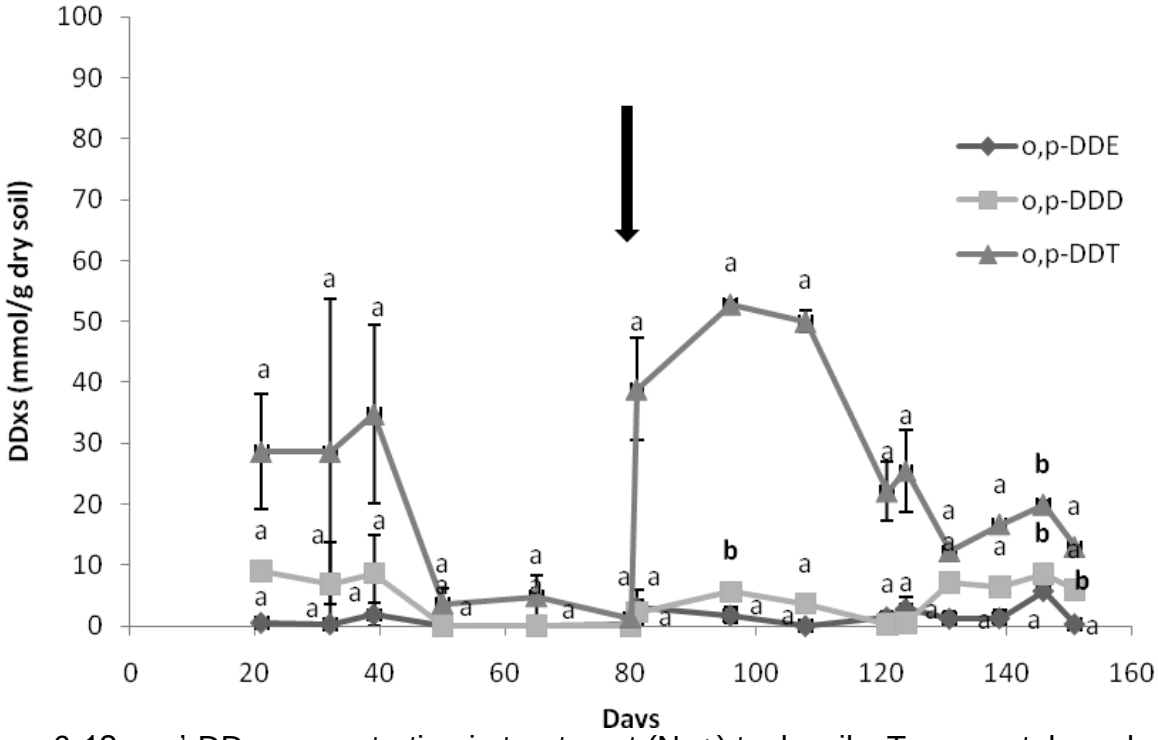


Figure 6-12. o,p'-DDx concentration in treatment (Na+) tank soils. Trace metals and Na+ were amended on 80th day indicated by arrow. Error bars represent +/- one standard deviation based on two replicates. Data labels not connected by same letter are significantly different $p<0.05$.

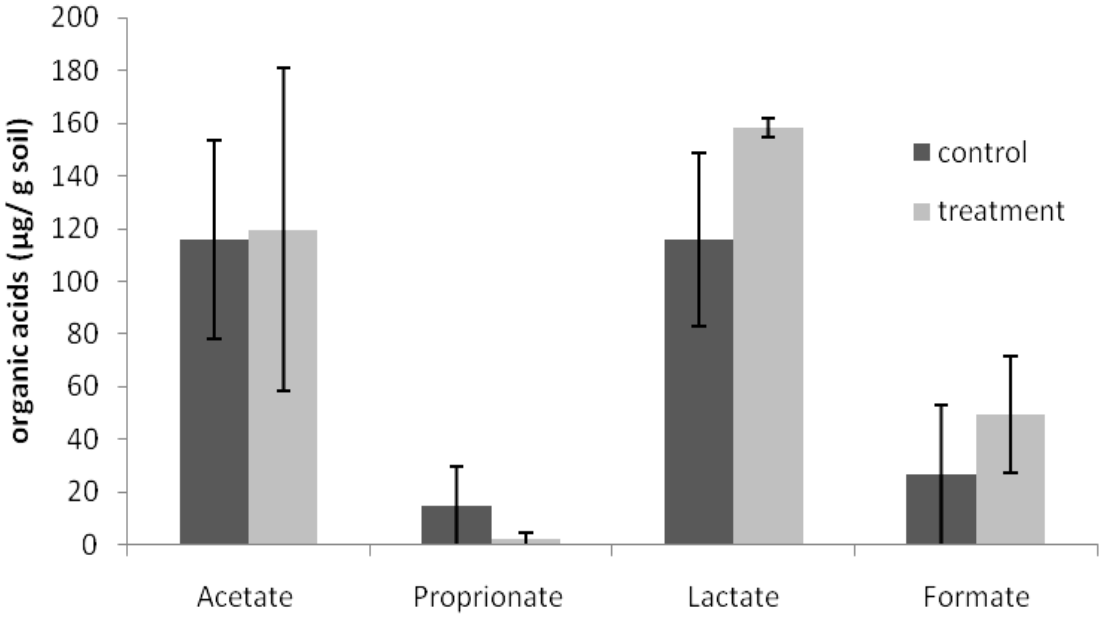


Figure 6-13. Fatty acids (µg/g dry soil) in control and NaCl amended (treatment) tanks throughout the study period.

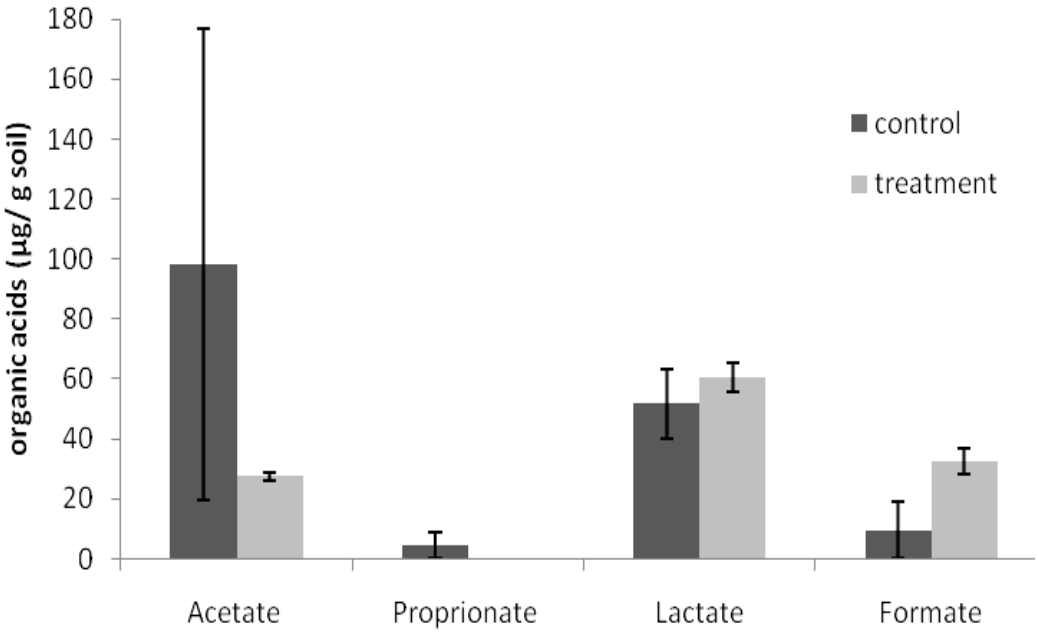


Figure 6-14. Fatty acids (µg/g dry soil) in control and NaCl amended (treatment) tanks throughout the study period.

CHAPTER 7
EVALUATION OF THE POTENTIAL FOR TWEEN-80 TO ENHANCE BIOAVAILABILITY AND BIODEGRADATION OF DDT AND ITS METABOLITES

Previous experiments demonstrated that DDT and its major breakdown products DDD and DDE (collectively known as DDx) have limited bioavailability in the highly organic soils surrounding Lake Apopka (Chapters 3, 4, and 6). Another revelation from these experiments is that the soils are limited in bioavailable carbon. Strategies that would increase bioavailability of DDx and bioavailable carbon could potentially increase biodegradation potential.

One approach to increase bioavailability of aged DDT from organic soils is addition of surfactants (Rouse et al., 1994; Volkering et al., 1998). Surfactant molecules are amphiphilic, i.e., consisting of a hydrophobic part and a hydrophilic part. The hydrophilic part makes them soluble in water and the hydrophobic part promotes concentration at interfaces. At lower concentrations, surfactants tend to accumulate at the interface in an aqueous solution, and as the concentration crosses beyond a threshold known as the critical micelle concentration (CMC), surfactant molecules concentrate and aggregate to form micelles. Micelles have a hydrophobic core and hydrophilic periphery. Reverse micelles or Oil-in-water type of micelles lead to an increase in the apparent solubility of hydrophobic organic compounds (HOCs) (Kile andand Chiou, 1989; Edwards et al., 1991; Jafervert et al., 1994; Guha et al., 1998; Yeh and Pavlosthathis, 2004).

Some surfactants at concentrations exceeding CMC enhance desorption and solubilization of poly aromatic hydrocarbons (PAHs) (Wilson and Jones, 1993). They do so by solubilizing hydrophobic organics into their micelles and by increasing mass transfer rates from solid or nonaqueous phases into the aqueous phase (Aronstein et al., 1991; Ducreux et al., 1995; Volkering et al., 1995; Tiehm, 1994). Although

exceeding the CMC results in enhanced solubilization and degradation, some studies have demonstrated that degradation rates also increased below the CMC of some surfactants (Aronstein et al., 1991; Aronstein and Alexander, 1992; Kile and Chiou, 1989). For example, solubilities of DDT increased by using Triton X-100, Triton X-114, Triton X-405 and Brij 35 at concentrations below their CMC's (Kile and Chiou, 1989).

Many studies demonstrate that surfactants increase the apparent solubility of hydrophobic organics, simultaneously increasing mass transfer rates from the nonaqueous phase; hence, potentially increasing the bioavailability of the hydrophobic organics (Kile andand Chiou, 1989; Edwards et al., 1991; Jafervert et al., 1994; Guha et al., 1998; Yeh and Pavlosthathis, 2004). Nonpolar substrates can reversibly dissolve in the inner hydrophobic core of the micelle and appear to be solubilized (Guha and Jaffe, 1996). Once soluble, the nonpolar substrate now is more bioavailable and, hence, prone to microbial attack.

A study by You and coworkers (1996) demonstrated that non-ionic surfactant application can significantly increase initial degradation of DDT added to soils. Four non-ionic surfactants, Tween-20 (IUPAC name Polyethylene-sorbitan monolaurate, CAS# 9005-64-5, Tween-40 (IUPAC name Polyethylene-sorbitan monopalmitate, CAS# 9005-66-7), Tween-60 (IUPAC name Polyethylene-sorbitan monostearate, CAS# 9005-67-8) and Tween-80 (IUPAC name Polyethylene-sorbitan monooleate, CAS# 9005-65-6), listed in the U.S. EPA (January 2008) "List of inert ingredients for use in non food use pesticide products," were tested by the Environmental Microbiology/Chemistry laboratory at the Soil and Water Science Department, University of Florida. Of the four selected surfactants, Tween-80 exhibited the lowest CMC, which suggests that of the

four selected surfactants, Tween-80 was the most efficient at lower concentration (John Thomas, personal communication). Hence, Tween-80 was used for these experiments.

Sorption coefficients are used to describe the sorption of chemicals such as DDT in soils, which in turn can be used to estimate the relative degree of bioavailability of chemicals in soils. One such coefficient is K_{oc}, used to normalize adsorption to the organic carbon content of soils. K_{oc} of a pollutant is the ratio of sorbed phase concentration on soils to the solution phase concentration that is normalized to total organic carbon content (Karickhoff et al., 1978). K_{oc} is traditionally determined in aqueous systems; however, DDx are extremely hydrophobic and it is very difficult to estimate sorption coefficient of DDx in aqueous systems. Solvophobic theory can be applied to prediction of sorption coefficients of hydrophobic organic compounds (HOCs) such as DDx. According to this model, sorption of HOCs decrease exponentially as the co-solvent fraction increases (Rao et al., 1985). Extrapolating sorption coefficient to estimate the sorption coefficient at a co-solvent fraction of zero can be used to estimate the sorption coefficient in an aqueous system (Nkedi-Kizza et al., 1985).

K_d of a given chemical is the ratio of sorbed phase concentration on soils (Se) to the solution phase concentration (Ce) of a solute at equilibrium (Karickhoff et al., 1978). Initial preliminary sorption experiments were designed to determine the distribution coefficient (K_d) of p,p'-DDT in soil and the aqueous phase with and without Tween-80. Determining K_d both with and without Tween would provide information on the aqueous concentrations of DDT at equilibrium; thereby, providing information on the extent to which Tween increased the aqueous solubility, and by extension, the bioavailability of DDT. Upon confirmation of increased bioavailability of DDT in aqueous phase,

microcosms designed to test the impact of Tween-80 on biodegradation were initiated. The overall objective of this chapter was to determine the effect of surfactant enabled biodegradation of DDx.

Materials and Methods

Sorption of DDT on soils

To ensure minimal sorption to container walls, sorption experiments were conducted in Teflon lined centrifuge tubes with mixed solvents (methanol and 0.01M CaCl2) in a range of co-solvent ratios, from methanol fraction (fc) of 0.5 to 0.8. Stock solutions of both ^{14}C-DDT and ^{12}C-DDT were made in Teflon lined centrifuge tubes with pure methanol to minimize sorption to container walls (Nkedi-Kizza et al., 1985; Muwamba et al., 2009). Prior to the actual sorption experiments, a preliminary experiment was conducted to ensure that a maximum equilibrium concentration was maintained below 0.004 µg/ml at all fc values so as not to exceed the aqueous solubility of DDT. The volume of ^{12}C-DDT standards (concentrations of 2 and 0.2 µg/mL) to be added were calculated from solvophobic theory. The model was also used to estimate sorption coefficients at each fc. Equation 7-1 was used to calculate Co, where Co= initial concentration (µg/ml), Ce= equilibrium concentration (µg/ml), m= mass of soil (g), Kd= soil-water distribution coefficient (mL/g), and v= final volume (mL). The range of Ce at each methanol fraction (fc) was predetermined to be less than 0.004 µg/ml. This was achieved by varying the amount of Co, m and K_d depending on fc at which Ce was measured according to equation 7-2.

$$Co = Ce \times ((m \times K_d / v) + 1) \quad (7\text{-}1)$$

$$Ce = Co / ((m \times K_d / v) + 1) \quad (7\text{-}2)$$

Initial concentrations of ^{12}C-DDT are presented in Table 7-1, and the ratios of soil solutions at different fc's for all the treatments are presented in Table 7-2. Once the volume of ^{12}C-DDT was determined and pipetted into 50 mL Teflon-lined centrifuge tubes, an additional 1mL volume of ^{14}C-DDT (such that it gave a concentration of 6184 cpm/ml) in methanol was also pipetted into the 50 ml tubes. Additional volumes of methanol and 0.01 M $CaCl_2$ were added to the centrifuge tubes to make a final volume of 10 ml that resulted in a range of 0.05 to 0.8 volume fractions of methanol (fc).

Each isotherm was conducted in triplicate and incubated at room temperature with shaking on a mechanical shaker for 24 hours. Following incubation, tubes were centrifuged in Beckman J2-21 Floor Model Centrifuge (GMI Incorporation Ramsey, MN**)** (JA-14 rotor) at 7155 x g for 20 minutes at 4°C. Equilibrium concentrations of DDT (Ce) were determined by counting 1 mL of the supernatant from each tube on Beckman LS 100-C liquid scintillation counter (LSC) (Beckman Coulter, Incorporation, Brea, CA). Prior to scintillation counting, 1mL supernatant was mixed with 5mL scintillation cocktail (Ecoscint A, National Diagnostics, Atlanta, GA) and allowed to sit for 1 hour in the dark. In order to correct for pipetting error, each tube was read three times and an average of the three readings was used to calculate Ce. Reading time in the scintillation counter was 5 minutes and the background activity from a blank of methanol and 0.01M $CaCl_2$ was subtracted from all readings. The decrease in DDT concentration was assumed to be due to adsorption to soils.

Adsorption to soils (Se) was calculated using Equation 7-3. K_d was obtained from Equation 7-4. A graph was plotted with natural log of K_d versus fc at various methanol

fractions. The slope of the graph is extrapolated to estimate the K_d in aqueous systems, i.e. at fc=0. Dividing K_d by soil organic carbon content yields K_{OC} (sorption coefficient).

$$Se = V/m\ (Co-Ce) \quad (7\text{-}3)$$

$$K_d = Se/Ce \quad (7\text{-}4)$$

Tween isotherms were made by adding Tween-80 in 0.01M $CaCl_2$ to obtain stock solutions with final concentrations of 0.2% Tween-80. Tween isotherms were performed similar to the no-Tween isotherms. K_d's for Tween isotherms were obtained similarly using Equation 7-4; however, the slope of the graph would be lower when compared to no-Tween. The slope is smaller because p,p'-DDT is distributed between two phases: soil and the aqueous co-solvent phase that consisted of water, methanol and Tween-80. However, in case of the no-Tween isotherms the distribution phases were soil and aqueous phase which consisted of water and methanol only.

Tween Microcosms

Tween microcosms were created with 0.2% Tween-80. Microcosms were intended to investigate the impact of Tween-80 on DDx biodegradation. Sieved and homogenized soils were spiked with p,p'-DDT (10 µg p,p'-DDT/g dry soil) dissolved in methanol. Upon evaporation of the solvent, soils were mixed manually and 10 g dry weight soil were added to 125 mL serum bottles with 45 mL Tanner medium (recipe see below; Tanner et al., 2007). Two sets of microcosms were created: one of which was aerobic and the other anaerobic. To maintain anaerobic conditions, all anoxic sets were purged with N_2, both in solution and headspace, sealed with Teflon-lined butyl rubber stoppers, and secured with aluminum crimps.

Lactate (20 mM) was added to microcosms to provide carbon and energy to potential degrading consortia. Previous experiments demonstrated that lactate directly

or indirectly promoted degradation of DDx (Chapters 3, 4, and 6). Two controls (a no-Tween control and autoclaved control) were included in the experiment. In case of autoclaved control, soils were autoclaved on three consecutive days at 121°C for 30 minutes, and were included to account for any abiotic losses.

Tanner medium consisted of (amount /L of distilled water): mineral solution 10mL, vitamin solution 10mL, trace metal solution 1.5mL, yeast extract 0.1g. Ingredients for the individual components include: the mineral solution (g/L of distilled water) NaCl, 80; NH_4Cl, 100; KCl, 10; KH_2PO_4, 10; $MgSO_4.7H_2O$, 20; $CaCl_2.2H_2O$, 4; the vitamin solution (g/L of distilled water) pyridoxin HCl, 10; thaimine HCl, 5; riboflavin, 5; calcium pantothenate, 5; thioctic acid, 5; p-aminobenzoic acid, 5; nicotinic acid, 5; vitamin B_{12}, 5; biotin, 2; folic acid, 2; and the trace metal solution (g/L of distilled water) were nitrilotriacetic acid (NTA), 2; $MnSO_4.H_2O$, 1; $Fe(NH_4)_2(SO_4)_2.6H_2O$, 0.8; $CoCl_2.6H_2O$, 0.2; $ZnSO_4.7H_2O$, 0.2; $CuCl_2.2H_2O$, 0.02; $NiCl_2.6H_2O$, 0.02; $Na_2MoO_4.2H_2O$, 0.02; Na_2SeO_4, 0.02; Na_2WO_4, 0.02.

All microcosms were incubated at 27^{o}C in the dark. Following incubation, soils were extracted and analyzed with a gas chromatograph equipped with an electron capture detector (GC-ECD). Of nine replicates from each treatment aerobic, anaerobic, tween control and autoclaved control, triplicate sets were sacrificed at 15th, 30th and 45th days of incubation to determine DDx concentrations.

DDx Extraction

DDx extraction could be divided into three stages: soil preparation; accelerated solvent extraction (ASE); and florisil extraction. The extraction method was based on U.S.EPA method #3545 (U.S.EPA, 2000) with minor modifications developed by Soil

Microbial Ecology Laboratory, University of Florida, and chemists at Pace Analytical Services, Ormond Beach, FL.

Soil Preparation

Soils were separated from liquid by centrifugation in Beckman J2-21 Floor Model Centrifuge (JA-14 rotor) at 7155 x g for 20 minutes at 4°C. Wet soil samples were allowed to air dry for two to three days following which they were ground. Soil moisture content was determined in the sample. Moisture was adjusted to 50% moisture on a dry weight basis, and then the samples were allowed to equilibrate in 4°C refrigerator for 3 days.

Accelerated Solvent Extraction

Soil was mixed with Hydromatrix, a drying and bulking agent, at a ratio of 1:2, and placed into 34 mL stainless steel extraction cells. The remaining headspace in extraction cells were topped with clean Ottawa sands (Fisher Scientific, Pittsburgh, PA) to decrease the amount of solvent usage. Solvent used for extraction was methylene chloride: acetone (4: 1 v/v). Soils were extracted under high pressure about 1200 to 1400 psi at temperature of 100°C in a Dionex ASE 100 Accelerated Solvent Extractor (Sunnyvale, CA).

The extraction cycle included filling the extraction cell with about 19 mL solvent, followed by heating cycle where the solvent was heated to 100°C. Static extraction followed the heating cycle which performed for 5 minutes, followed by flushing cycle which flushed about 19mL solvent through the cell. The cell was finally purged with N_2 for about 2 minutes, which completed one extraction cycle.

Florisil Extraction

Florisil clean up used prepacked florisil “Bond Elute LRC” columns (200 µm particle size) (Varian Inc., Palo Alto, CA) which contained approximately 1 g Florisil packaged into the plastic holder. The column was conditioned using 5 mL hexane: acetone (9:1v/v), loaded with 1 mL of ASE extract and the column was eluted using 9 mL of solvent.

A volume of 5 mL from the cleanup volume was concentrated to dryness under a gentle stream of air. It was very important to concentrate the samples to complete dryness prior to GC (gas chromatography) analysis to avoid matrix response enhancement (Schmeck and Wenclawiak, 2005). Samples were reconstituted in 1 mL of hexane, and tubes with samples were vortexed and transferred to a 2 mL amber glass GC vials (, Fisher Scientific Inc., Atlanta, GA) and crimped with 12 x 32mm aluminum crimp seals with prefitted PTFE lined septa (, Fisher Scientific Inc., Atlanta, GA) for subsequent analysis by GC.

GC Conditions

Perkin-Elmer Autosystem Gas Chromatograph (GC) equipped with autosampler and an electron capture detector (ECD) was used for analyzing the extracts. Column conditions were: He as the carrier gas at flow rate of 1.0 mL/min, 5% methane in argon as the make-up gas with a flow rate of 50 mL/min, electron capture detector (ECD) temperature at 350 °C, and injector temperature at 205 °C in splitless mode. Injection volume was 3 µL in slow injection mode. GC temperature was programmed at 110 °C for 0.5 minutes, followed by a ramp at 20 °C/min to 210 °C and hold for 0 minutes, then ramp at 11 °C/min to 280 °C and held for 6.3 minutes. Under these conditions, the detection limit was calculated at the 90% confidence level to be: 0.007 µg/mL for o,p’-

DDE; 0.005 µg/mL for p,p'-DDE; 0.006 µg/mL for o,p'-DDD; 0.009 µg/ mL for p,p'-DDD; 0.005 µg/mL for o,p'-DDT; and 0.010 µg/mL for p,p'-DDT (Hubaux and Vos, 1970). These results are similar to the detection limit of 0.010 µg/mL reported by Pace, Inc.

Data Analysis

All the data analysis was performed in NCSS (Kaysville, UT), using ANOVA general linear model. Mean DDx concentrations among different treatments were compared using a general linear model. Values with $p<0.05$ were considered significantly different. Similarly, a general linear model was used to compare effects of incubation times.

Results and Discussions

Sorption Studies With and With No Tween-80

Sorption is a major process controlling the fate of contaminants in the environment. The sorption coefficient of a contaminant is the ratio between concentrations sorbed to soils and that dissolved in the aqueous phase (soil solution) at equilibrium. Sorption to soils decreases bioavailability of a contaminant, which may be a major rate limiting step in biodegradation. Sorption to soils can be decreased using various methods, such as application of surfactants. Preliminary isotherm experiments are required to evaluate potential increases in apparent solubility, and hence bioavailability, of DDx through the use of Tween-80.

Since Tween is soluble in aqueous phase of isotherms, parallel experiments with and without Tween were set up. The difference in K_d between these isotherms would provide information on the increase in solubility of DDT by Tween.

Figure 7-1 presents isotherm with no-Tween, and isotherm with 0.2% Tween are presented in Figure 7-2. Calculating K_d from these graphs, K_d without Tween is 15122

mL/g; whereas, K_d with 0.2% Tween is 705mL/g. Plotting a graph of natural log of K_d on Tween against various methanol fractions, and extrapolating the slope to fc=0 will give K_d with Tween in an aqueous system. K_d decreased significantly with the addition of Tween, since KD= Se/Ce, it implies that the aqueous concentration of DDT increased with Tween application. Based on these results, microcosms were created using 0.2% Tween-80 concentration.

However, one probable limitation to this method could be use of methanol, as methanol could disrupt micelle formation at concentrations above CMC. CMC for Tween-80 is 0.012mM (Task 8, SJRWMD), and concentration of Tween-80 used in isotherms was 0.2mM which is 16 times higher than the CMC. This could have lead to over estimation of K_d, although it remains unknown at this time if methanol, would disrupt the micelles.

Tween Microcosms

Microcosms were established with and without 0.2% Tween with lactate as energy and carbon source, such that DDxs may be TEAs and/or substrates for cometabolism. NaCl microcosms and mesocosms (Chapter 6) demonstrated the importance of trace metals and cofactors in degradation of DDx in these soils, Tanner medium was selected for Tween microcosms. Triplicate sets from each treatment were sacrificed following 15, 30, and 45 days in incubation and soils phases were analyzed.

Figure 7-3 compares o,p'-DDx concentrations at different incubation time intervals among various treatments. Straight lines across the graphs separate data sets with respect to incubation time i.e., 15 days from 30 and 45 days. Data analysis compare metabolite concentration mean within one group for example, o,p'-DDE concentration among all the 15 day treatments (aerobic, anaerobic, autoclaved control and Tween

control). To evaluate effect of incubation time, general linear model, Fisher's LSD Multiple-Comparison Test was used to compare 15, 30, and 45 days. Data analysis results reveal that 15 days set was different from the 30 days, but neither 15 days nor 30 days sets were different from the 45 days sets. Overall trend for o,p'-DDx were similar irrespective of incubation condition (i.e., aerobic or anaerobic). Furthermore, no significant difference among treatments was observed (Figure 7-3).

Figure 7-4 compares p,p'-DDx concentrations at different incubation time intervals among different treatments. Similar to the o,p'-DDx, straight lines across the graphs separate data sets with respect to incubation time i.e., 15 days from 30 and 45 days. Also similar to the o,p'-DDx sets, data analysis in case of p,p'-DDx sets compare metabolite concentration mean within one group for example, p,p'-DDE concentration within all the 15 day treatments (aerobic, anaerobic, autoclaved control and Tween control).

No significant difference was observed in case of 45 days p,p'-DDx with respect to incubation condition; similarly, no significant difference among treatments was observed. However, p,p'-DDT concentrations were higher in case of Tween controls (i.e., sets with no Tween) during 15 and 30 days of incubation.

Tween anoxic incubations followed the same trends as previous experiments, including lactate microcosms and lactate mesocosms (Chapters 3 and 4). This is yet another confirmation that following anaerobic incubation, DDx extractability increased. In case of lactate mesocosms (Chapter 4), the increased extractability coincided with an increase in dissolved organic carbon (DOC) and a drop in Eh. Anoxic incubations increase the dissolved organic matter (DOM) fraction of soils which is the most

bioavailable fraction of soil organic matter (Marschner and Kalbitz, 2002). DOM can enhance solubility, mobility, and hence, bioavailability of organic compounds (Blaser, 1994; Piccolo, 1994; Zsolnay, 1996; Marschner et al., 1999).

There could be several reasons for the lack of effect observed in Tween-80 microcosms. Even though isotherms increased the aqueous concentration of DDx, incubation conditions for isotherms were quite different from that of microcosms. Isotherms were continuously shaken which likely increased distribution of DDxs in all the phases, whereas microcosms were not shaken. Another reason could be that Tween-80 could be potentially harmful to the degrading organisms.

Surfactants can have toxic effects on bacteria, mainly in two ways. Primarily, they can interact with the lipid layers in the cell membranes causing them to disrupt and finally lyse a cell. Secondly they can interact with essential proteins that maintain normal functioning of a cell (Helenius and Simons, 1975). Many other factors can lead to lower the biodegradation potential while using surfactants such as depletion of minerals, formation of toxic surfactant intermediates (Holt et al., 1992) or degradation of surfactant. Selective or preferred surfactant degradation could slow pollutant degradation (Tiehm et al., 1994) or decrease the apparent solubility or bioavailability of the pollutant (Holt et al. 1992; Oberbremer et al. 1990).

Summary

Although surfactants have been found to stimulate degradation of HOCs such as DDT, no general trends were found in these experiments. More biodegradation experiments with different incubation conditions (with and without shaking), desorption and mobilization experiments are needed before applying such *in situ* processes at the field level. Therefore, more laboratory scale research is needed to understand the

dynamics of surfactant-mediated DDx biodegradation. Lab scale research can help understand and predict the more complicated field level situations, which is very important in surfactant mediated remediation efforts as surfactants can mobilize the contaminant, spreading it to the otherwise uncontaminated areas. Hence, it is very important to have more knowledge in this aspect before applying surfactant mediated remediation.

Table 7-1. Initial concentrations (Co) of ^{12}C-DDT added to isotherms at different fc's

fc	control	0.2%Tween-80
0.5	0.2	0.2
0.6	0.1	0.1
0.7	0.02	0.02
0.8	0.02	0.02

Table 7-2. Ratios of soil solutions at different fc's for all treatments

fc	control	0.2%Tween-80
0.5	1:2.5	1:2.5
0.6	1:2.5	1:2.5
0.7	1:5	1:5
0.8	1:2.5	1:2.5

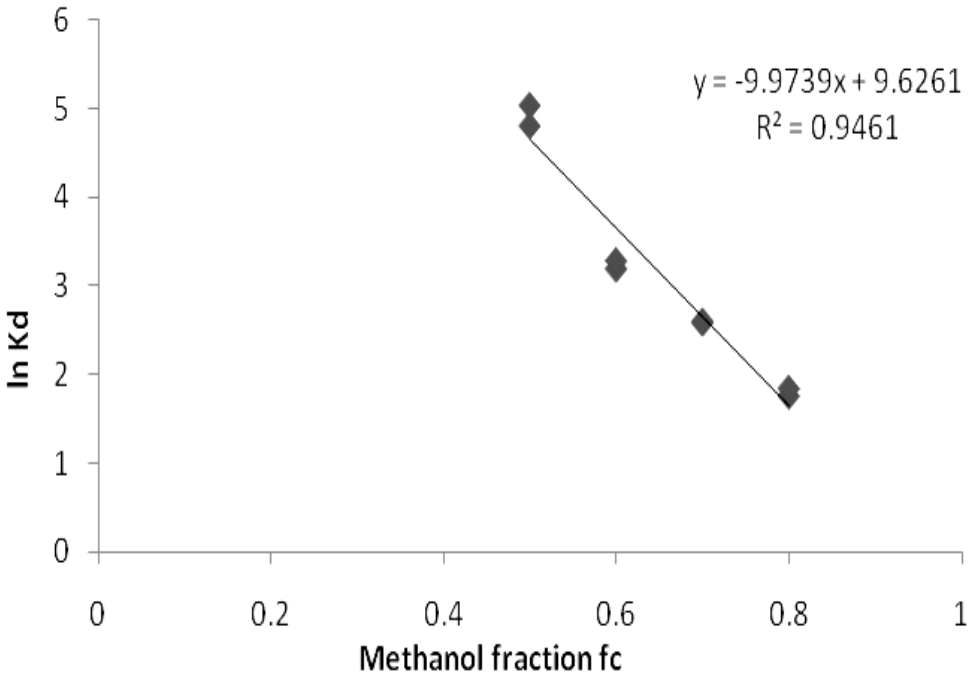


Figure 7-1. Log-linear relationship between sorption coefficient (Kd) and fraction of methanol (fc) for sorption of DDT by soil.

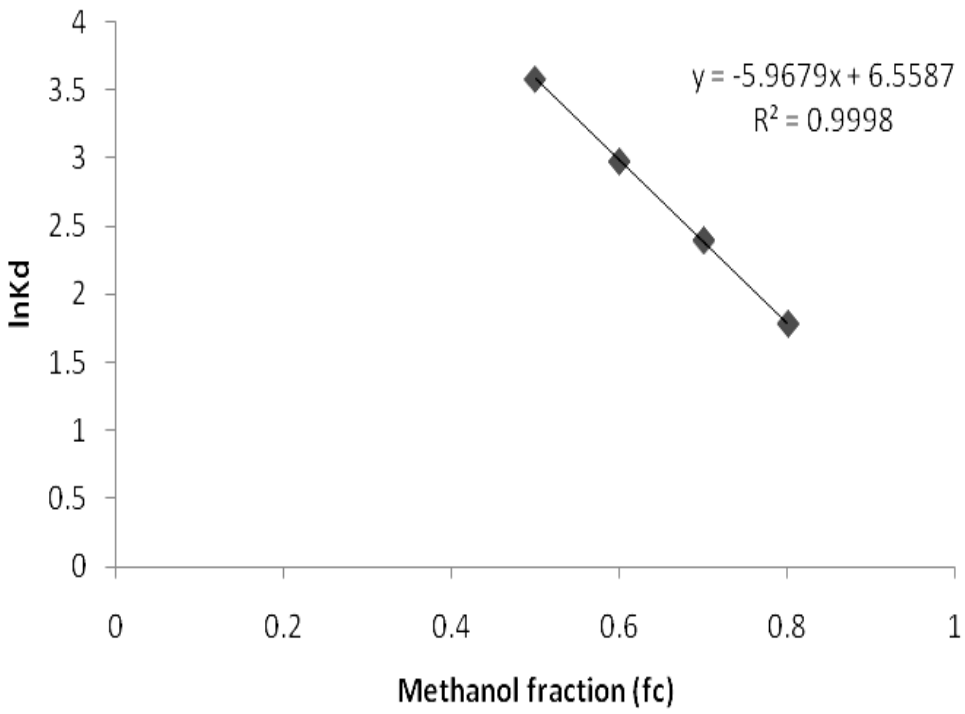


Figure 7-2. Log-linear relationship between sorption coefficient (Kd) and fraction of methanol (fc) for sorption of DDT by soil with 0.2% Tween-80.

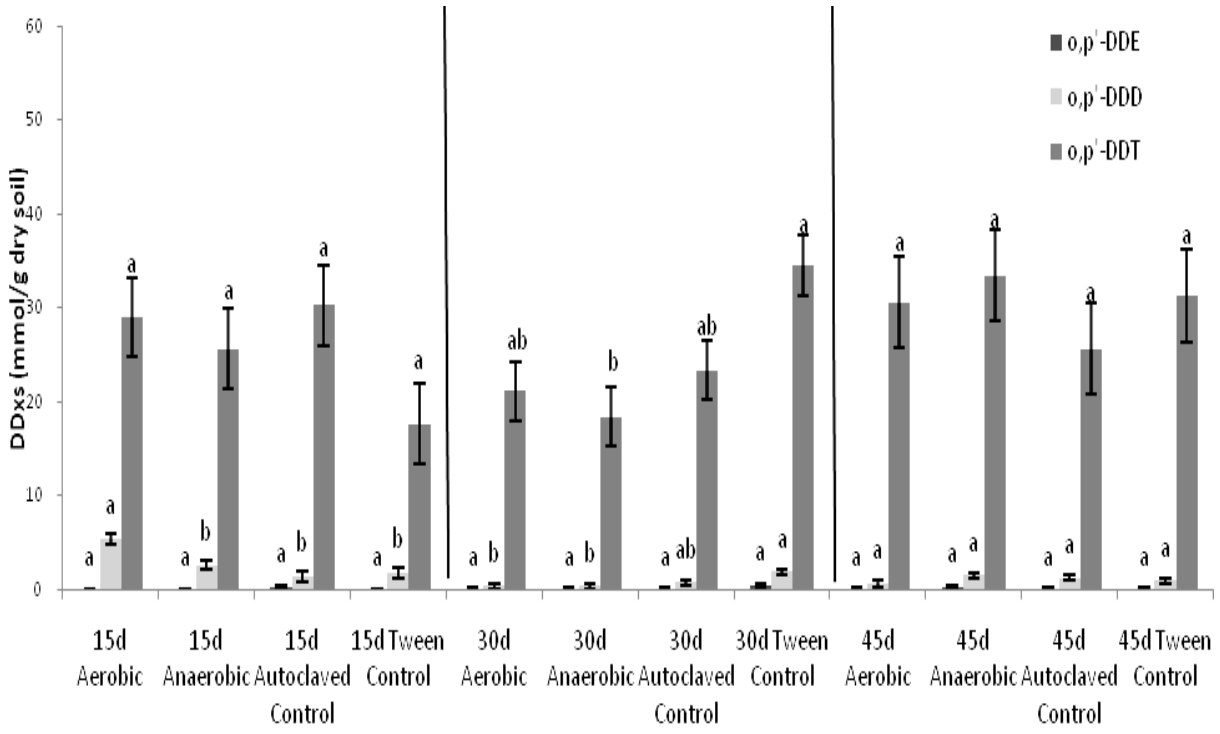


Figure 7-3. o,p'-DDx concentrations at different incubation times in various treatments. Error bars represent +/- one standard deviation based on two replicates. Straight lines across the graphs separate data sets for 15, 30 and 45 days. Data analysis compare metabolite mean within one group (i.e., o,p'-DDE concentration within all the 15 day treatments are compared). Data labels not connected by same letter are significantly different $p<0.05$.

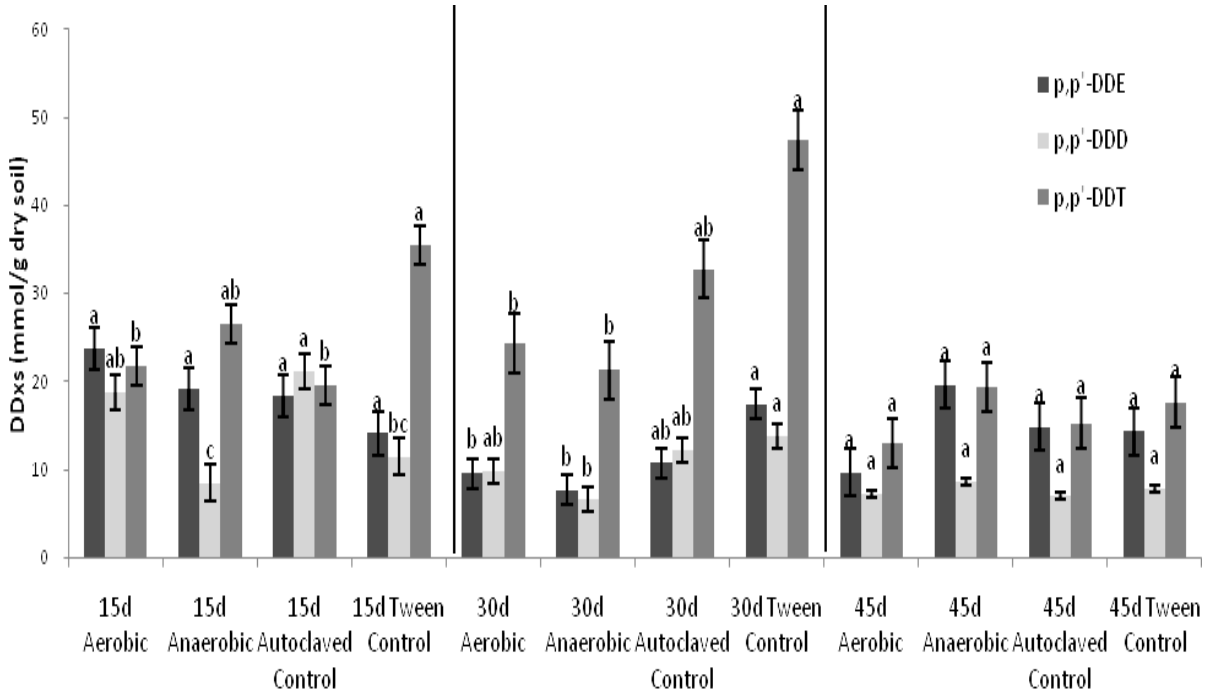


Figure 7-4. p,p'-DDx concentrations at different incubation times in various treatments. Error bars represent +/- one standard deviation based on two replicates. Straight lines across the graphs separate data sets for 15, 30 and 45 days. Data analysis compare metabolite mean within one group (i.e., p,p'-DDE concentration within all the 15 day treatments are compared). Data labels not connected by same letter are significantly different $p<0.05$.

CHAPTER 8
SUMMARY AND CONCLUSIONS

DDT was one of the first synthetic chlorinated organic pesticides to gain wide acceptance (EPA report, 1975). One of the qualities that make a pesticide desirable is its persistence, which would decrease the need for reapplication. A large proportion of DDT and its major metabolites DDD and DDE; collectively known as DDx) are hydrophobic and polychlorinated molecules, properties which contribute to their persistence in the environment. Prior to its partial ban in the U.S. in 1972, a total of approximately 1,350,000,000 pounds of DDT were applied domestically (EPA report, 1975). Owing to the persistent nature, DDx have been found at 305 of the 441 National Priority List (NPL) hazardous waste sites in the U.S. (HazDat, 2002). One such DDx contaminated site is the North Shore Restoration Area (NSRA) adjacent to Lake Apopka, where substantial quantities of DDx were introduced due to extensive agricultural practices in the early 1940s (Woodward et al., 1993; Huffstutler et al., 1965; Florida Dept. Environ. Reg., 1979).

The ecological and environmental effects of DDx were realized by the late 1960s (WHO, 1979; Turusov, 2002; Ratcliffe, 1967). They are a great concern to wildlife and humans because of their toxicity, persistence, and bioaccumulation factors. Being lipophilic, DDx tend to bioaccumulate in fatty tissues of non-target organisms, starting at lower concentrations at the base of the food chain and magnifying as they travel up the trophic levels (Dearth and Hites, 1991; Tanabe et al., 1983; Gray et al., 1992; Vrecl et al., 1996; Longnecker, 1997; Nataka et al., 2002; Turusov et al., 2002; Jaga and Dharmani, 2003; Kunisue et al., 2004). Soil contamination with such recalcitrant organochlorine pesticides and their metabolites is a major problem that may lead to

significant ecosystem damage (Colborn and Smolen, 1996; Meghraj et al., 1999; 2000). In order to restore the site, a decision was made to buy out the farms under the Lake Apopka Improvement and Management Act. However, higher concentrations of DDx at the site left the piscivorous birds at a higher risk (U.S. Fish and Wildlife Service report, 2004). This contributed to the deaths of more than 600 birds at and surrounding the site during the late Winter of 1998 and Spring of 1999. Autopsy results showed that DDT, DDD, DDE, toxaphene and dieldrin were present at elevated levels in the tissues of many of the dead birds (U.S. Fish and Wildlife Service report, 2004).

Soil contamination with recalcitrant DDx can lead to significant ecosystem damage (WHO, 1979; Ratcliffe, 1967; Megharaj et al., 2000; Turusov, 2002), and, hence, a method to remediate these pollutants is a high priority. Physical and chemicals methods employed for remediation have proved to be intrusive, damaging to the health of the soils, and are expensive plus labor and energy intensive. Bioremediation is a potential option that is simpler, less intrusive, more environmentally friendly, and more cost effective for clean up of environmental pollution (Jacques et al., 2008). The main objective of this research was therefore to investigate bioremediation approaches that could be employed at the NSRA.

The great versatility of microbial metabolism makes bioremediation an attractive option. Numerous studies have demonstrated that anaerobic reductive dehalogenation plays a crucial role in initial attack of higher halogenated compounds. Furthermore, many studies have given strong evidence indicating that chlorinated compounds are degraded under reducing environments (Mohn and Tiedje, 1992; Fetzner, 1998; El Fantroussi et al., 1998; Holliger et al, 1999; Middeldorp et al., 1993). Under TEA limiting

anoxic environments, certain iron-, sulfate-, nitrate- reducing organisms, acetogens, methanogens, and syntrophs can catalyze dehalogenation reactions (El Fantroussi et al., 1998; Gantzer and Wackett, 1991; Holliger and Schraa, 1994). The extensive metabolic capabilities of microorganisms lead us to hypothesize that DDx degrading organisms are already present at the site, such that careful selection of the optimum electron donor and acceptor combination would selectively enrich the degrading population (Suflita et al., 1988). To attain appreciable loss it was necessary to divert those organisms to biodegradation, such that they could be selectively directed towards biodegradation hence creating a unique niche for the degrading population. Chapter 3 addresses investigation of such niches, selectively enhancing growth of the DDx degrading consortia. It was not known if DDx would act as electron donor or as terminal electron acceptor (TEA); hence, both possibilities were studied in microcosm experiments, as described in Chapter 3. With DDx as electron donor, a wide array of electron accepting conditions to enrich iron-, sulfate-, nitrate-reducing organisms and mixed acid fermenters were studied. Another set of microcosms tested DDx as TEAs, with various electron donors such as hydrogen and selected organic acids under varying concentrations of sulfate. The effect of sulfate concentrations was studied with the intention that those sulfate reducers capable of utilizing DDT as TEA would shift to dehalorespiration at some critical sulfate concentration. No effect of sulfate was observed, however.

Overall results from Chapter 3 demonstrated that DDx are more efficient as TEAs than as donors in TEA- limiting environments. Observations from this study are in agreement with other studies indicating that dehalorespiration is thermodynamically

favorable compared to sulfate or iron reduction, acetogenesis, or methanogenesis (Bossert et al., 2003; Ballapragada et al., 1997; Fennell et al., 1997; Fennell and Gossett, 1998; Loffler et al., 1999; Smatlak et al., 1996; Susarla et al., 1996; Yang and McCarty, 1998). Another observation is that since the system was already reduced, i.e., rich in electron donors, microorganisms may oxidize endogenous electron donors more readily than the otherwise chemically stable DDx. The highest degradation (about 83%) was observed in lactate microcosms, where lactate likely provided the electron and carbon needs of the degrading consortia, and DDT served as TEA to the initial degrading population. The responsible consortia may have a syntrophic relationship such that lactate is fermented to smaller organic acids such as formate, acetate, and hydrogen. Syntrophs have previously been shown to be involved in dehalogenation processes (Mohn and Tiedje, 1992; Yang and McCarty, 1998; Drzyzga and Gottschal, 2002; Dolfing, 2003; Sung et al., 2003); hence, degradation could tentatively be explained by syntrophic relationships between hydrogen producing lactate fermenting organisms and dehalogenating populations.

Another important finding from this study was that the extractability of DDx increased upon anoxic incubation. DDx residues persist in soils through various interactions with soil organic matter, thereby decreasing bioavailability. Previous studies demonstrated that DOC can enhance solubility and mobility, and hence bioavailability, of organic compounds (Blaser, 1994; Piccolo, 1994; Zsolnay, 1996; Marschner et al., 1999). Once the DDx are made bioavailable following anoxic incubations, suitable electron donors and acceptors facilitated growth of degrading population.

Once a suitable electron donor:acceptor combination was identified, the next objective was to scale up the lab scale microcosms to mesocosms experiments. Chapter 4 addresses mesocosm studies to check its applicability at the field level. Results from Chapter 4 are in agreement with other studies that demonstrated that anoxic incubations increase DOC concentrations. (Marschner and Kalbitz, 2002). Anoxic incubations increased DDx concentration both in both lactate and control tanks, indicating that anaerobic incubations increased extractability of DDx. Increased extractability coincided with a drop in Eh and an increase in DOC. These reduced conditions lead to higher hydrogen ion consumption, creating more alkaline environments that resulted in higher dissolution and release of soil organic matter (SOM). With the release of SOM the otherwise unavailable hydrophobic molecules are more bioavailable and hence prone to biodegradation. DDx in both the control and treatment tanks increased initially, after which the p,p'-DDT concentration in lactate mesocosms was consistently lower than the controls, indicating that lactate selectively enriched the dehalorespiring population.

To better understand the processes involved in DDx degradation, it is necessary to enrich and isolate stable consortia and identify the organisms involved in degradation of DDx. Attempts to isolate degradative consortia are addressed in Chapter 5. Although we demonstrated degradation of DDx occurred under lactate fermenting conditions, we did not succeed in isolation of the organisms involved. This is not uncommon due to the complications involved in enriching, isolating, and growing strains belonging to such complex systems. For a better understanding of the biology of the dehalogenating consortium, it is very important to study axenic cultures at the molecular level. This

could elucidate pathways, mechanisms, and roles of the individual members involved, and could be accomplished by traditional enrichment cultures or metagenomics studies.

Chapter 6 addresses the bioavailability issue, which may limit remediation by physically sequestering and, hence, protecting the pollutant from microbial attack (Alexander, 1995; 1997). Na^+ microcosms suggested that there is a positive correlation between Na^+ concentration and DDx degradation in microcosms with the Tanner medium. A likely explanation is that, upon soil dispersion by the added Na^+, the otherwise unavailable DDx were made available to the degrading consortia. Furthermore, with anoxic incubation there is an increase in DOC concentrations (Chapters 3 and 4), which may also increase the solubility and, hence, bioavailability of DDx. Both these factors could increase exposure of substrate to the microbes. Another objective in this study was to meet with the energy and carbon needs of the degrading consortium by finding an alternative to lactate, which we achieved by promoting lactate formation by decomposition of cattail. Higher concentration of p,p'-DDT in the no-cattail controls suggests that the system is carbon limited and that cattail fermentation products could feed the degrading populations. Another indication is that although organisms capable of degradation are indigenous to the soils, suitable selection of electron donor and carbon source in TEA limited anaerobic system with increased bioavailability may result in biodegradation of the otherwise inaccessible aged DDx. Another observation from this study is that trace metals, cofactors, and vitamins are needed by the degrading population. They may be responsible for triggering the production or activation of degrading enzymes, or may be required as growth factors by the degrading consortia. Further research is needed for confirming the above

observations. Once an optimum DDx degradation promoting Na^+ concentration and trace metals were obtained the microcosms were again upscaled to mesocosm level studies.

Data from the Na^+ mesocosms indicate loss of the parent and metabolites to less than 12 mmol/g dry soil in all mesocosms within 50 days of incubation. This observation is similar to those from the lactate mesocosms (Chapter 4), indicating that degradation of DDx is limited by available carbon. Following amendments, the observed trends indicated a repeated increase followed by a decrease in DDx concentrations. Data analysis indicated that there was no significant difference between the control and Na^+ treatment tanks due to the relatively high variability. Regardless, it is interesting to note that towards the end of the experiment, significant differences were noted between treatment and control tanks with respect to both o,p'- and p,p'-DDx.

Following the addition of trace metals and Na^+, an increase in DDx concentrations was observed, indicating that as the availability of DDx increased lactate provided energy requirements for the degrading population. Anoxic Eh promotes DOC release (Kim et al., 2008; Pravacek, 2005) and DOC increases the solubility (and bioavailability) of DDx. This is in accordance with the DOC data, in which higher concentrations of DOC coincide with higher concentrations of DDx, and precede more rapid decreases in DDx concentrations.

Chapter 7 describes another approach to increase the bioavailability of DDx. Initial trends of sorption isotherms demonstrated that DDx sorption to soils decreased upon Tween-80 addition. Furthermore, surfactants have been found to stimulate degradation of HOCs such as DDT; however, no general trends were observed in microcosm

experiments with Tween-80. Surfactants can be toxic to bacteria, and more biodegradation experiments with different incubation conditions (with and without shaking), desorption and mobilization experiments are needed before applying surfactants at the field level. Lab scale research can help understand and predict the more complicated field level situations, which is very important in surfactant mediated remediation efforts as surfactants can mobilize the contaminant, spreading it to otherwise uncontaminated areas.

The overall conclusion from this research is that NSRA soils have indigenous organisms capable of DDx degradation. These organisms may be diverted to adapt to using such organohalides by careful amendment with appropriate electron and carbon sources, creating an appropriate ecological niche. Of the various amendments that were applied, lactate microcosms and mesocosms indicated creation of such niche for the DDx degraders in NSRA soils. Such amendments would likely increase biodegradation rates in the field. However, an assessment of bioremediation potential will require better understanding of the ecological interactions between the dehalogenating organisms and soil community.

A greater concern while trying to achieve anoxic degradation is greater availability of DDx following anoxic incubations. This can be explained by the importance of DOC in increasing the bioavailability of hydrophobic compounds (Kim and Pfaender, 2005; Kim et al., 2008; Pravacek, 2005). Although anoxic bioremediation of DDx remains an attractive option, one limitation of anoxic degradation is the release of DOC that may not only increase the bioavailability of DDx, but also the mobility of DDx, such that it could increase runoff of DDx. However, anoxic mesocosm studies with lactate strongly

indicated that DDx concentrations dropped as low as 10 mmol/ g dry soil within 50 days of incubation. Furthermore, when the DOC mediated release of DDxs occurred in lactate mesocosms (on 64th day), DDx concentrations dropped to less than 2.5 mmol for all DDx except for p,p'-DDT and p,p'-DDE, with concentrations of 38 and 43 mmol/ g dry soil, respectively, in 18 days. It is quite interesting to note that in case of NaCl mesocosms, following the DOC mediated DDx release by the 32nd day, DDx concentrations were less than 12mmol/ g of dry soils after 18 days. Although mobility of DDx is of concern, anoxic degradation is a promising technique, and with the use and creation of models to predict the release and mobility, and monitoring the system such techniques are applicable.

What makes anaerobic degradation a powerful tool is that it is possible to remove even trace levels of haloaromatics (Lowe et al., 1993). Such technology can be effectively applied at the NSRA site where, because of aging, the DDx are bound such that they are not easily extractable with chemical or physical methods, leading to erroneous evaluation of levels of DDx present. This may impose a serious problem, as it may expose the ecosystem to threatening levels of DDxs, which may otherwise appear to be clean. For example, in the case of DDx as TEA microcosms, only 28 mmol/ g dry soil of p,p'-DDT was applied to the soils initially, following incubation soil concentrations of p,p'-DDT increased to as high as 382 mmol/ g dry soils (Chapter 3). To reconfirm that there was no human, or instrumentation error responsible for the high readings, these samples were extracted and analyzed three times.

Upon anoxic incubations, our studies indicate that the bound or sorbed DDx are released, and further more it suggests that indigenous organisms capable of DDx

degradation are present at the site. If proper measures such as creating a unique niche for the dehalogenating population are taken, anaerobic treatment technology can be a powerful tool. Our studies demonstrated that the dehalogenating organisms can be utilized for bioremediation by application of lactate. Lactate is an expensive amendment, such that use of natural inexpensive alternatives such as dried cattail powder also supported growth DDx degradation. Impoundment could be used to contain of DOC complexed DDx, until the DDx concentrations drop below detection.

APPENDIX A
Ou Medium

Table A-1. Ou medium

Ingridients	g/L distilled water
K_2HPO_4	4.8
KH_2PO_4	1.2
NH_4NO_3	1
$CaCl_2.2H_2O$	0.025
$MgSO_4.7H_2O$	0.2
$Fe_2 (SO_4)_3$	0.001

APPENDIX B
Tanner Medium

Table B-1. Tanner medium

Ingridients	Amount/L distilled water
Mineral solution	10mL
Vitamin solution	10mL
Trace metal solution	1.5mL
Yeast extract	0.1g

Table B-2. Tanner medium Mineral solution

Ingridients	(g/L of distilled water)
NaCl	80
NH_4Cl	100
KCl	10
KH_2PO_4	10
$MgSO_4.7H_2O$	20
$CaCl_2.2H_2O$	4

Table B-3. Tanner medium Vitamin solution

Ingridients	(g/L of distilled water)
Pyridoxin HCl	10
Thaimine HCl	5
Riboflavin	5
Calcium pantothenate	5
Thioctic acid	5
p-aminobenzoic acid	5
Nicotinic acid	5
Vitamin B_{12}	5
Biotin	2
Folic acid	2

Table B-4. Tanner medium Trace metal solution ingredients

Ingridients	(g/L of distilled water)
nitrilotriacetic acid	2
$MnSO_4.H_2O$	1
$Fe(NH_4)_2(SO_4)_2.6H_2O$	0.8
$CoCl_2.6H_2O$	0.2
$ZnSO_4.7H_2O$	0.2
$CuCl_2.2H_2O$	0.02
$NiCl_2.6H_2O$	0.02
$Na_2MoO_4.2H_2O$	0.02
Na_2SeO_4	0.02
Na_2WO_4	0.02

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BIOGRAPHICAL SKETCH

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