EXPLORATION OF BIODEGRADATION OF TOXAPHENE AND OTHER MAJOR PESTICIDES IN THE NORTH SHORE RESTORATION AREA SOILS OF LAKE APOPKA, FL

By

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To my parents

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In order to accurately evaluate the bioremediation strategy of the Lake Apopka North Shore Restoration Area (NSRA) FL, the optimal conditions for extracting toxaphene, 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane (DDT), 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene (DDE), and 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethane (DDD) from this area with high organic carbon were firstly established. Accelerated solvent extraction (ASE) was compared to Soxhlet extraction (SOX) and the effects of solvent systems, and soil characteristics such as volumetric water content, and carbon content on extraction efficiencies were investigated. The results indicated that only the toxaphene recovery in ASE was highly correlated to soil carbon content. Using ASE, methylene chloride/acetone (4:1, v/v) extracted toxaphene, DDE, and DDT more efficiently than hexane/acetone (1:1, v/v); while the extraction for DDD and DDE was extracted by SOX with higher efficiency using hexane/acetone (1:1, v/v) than using methylene chloride/acetone (4:1, v/v). Volumetric water content was significantly correlated to extraction efficiency for DDT by SOX, and for DDE, DDT, and toxaphene by ASE. In both ASE and SOX, intermediate water content yielded the greatest overall efficiencies for extracting toxaphene, DDT and its toxic metabolites simultaneously from the soils. Finally, with the exception of DDT,

toxaphene, DDE, and DDD can be extracted by ASE with statistically significant efficiency in compared with by SOX from highly contaminated soils, while ASE produced at least comparable efficiencies with SOX for extracting DDT family from moderately contaminated soils with a statistically significant higher efficiency for toxaphene.

A series of laboratory microcosms were firstly set up with organochlorine pesticide-contaminated soils taken from NSRA to identify anaerobic processes leading to the decomposition of the recalcitrant pesticides toxaphene and DDT family. In separate replicated (two) microcosms, lactate, butyrate, H_2, formate, acetate, and propionate were used as electron donors in the presence of varying concentrations of sulfate and a constant level of the pesticides. After about one month, all microcosms were sacrificed and organochlorine pesticides (OCPs) were extracted and determined using the established methods. Among the tested electron donors, lactate, butyrate, and hydrogen showed potential capability of stimulating the biodegradation. Their roles in OCPs degradation were further established with another series of laboratory microcosms with three replicates. After a two-month incubation, significant loss of the parent compounds were observed in the lactate, butyrate, and H_2 microcosms relative to autoclaved controls and controls with no exogenous electron donor. In order to understand the characteristics of microorganisms participating the contaminants metabolism, indigenous bacteria with the capability to dechlorinate toxaphene were enriched using lactate as the electron donor in the presence of 20 ppm toxaphene. After five transfers, release of Cl^- was measured by ion chromatography with time. A rapid and significant rise in concentrations of Cl^- was observed in all replicates relative to controls, indicating biological dechlorination mechanism of toxaphene. The possibility that dechlorination was affected by sulfate reducing bacteria was investigated by isolation of DNA followed by polymerase chain reaction (PCR) with primers

specific to the dissimilatory sulfite reductase gene, *dsrA*. No *dsrA* amplicons were observed, which meant other group(s) of bacteria besides sulfate reducing bacteria may be responsible for degrading toxaphene. Phylogenetic analysis demonstrated that some sequences most abundant in the clone libraries were related to the genus *Citrobacter* (99.9% similarity), some closely related to Gram positive *Sporomusa*, (98% similarity).

Finally, the concepts for remediation of the contaminated area were confirmed and extended up to the mesocosm scale using easily available plant materials, cattail, or lactate as the electron donors plus a set of control with a cycle of anoxic followed by oxic conditions promoting degradation. Concentrations of OCPs were monitored every two weeks. Both lactate and cattail treated mesocosms showed significant disappearance of toxaphene and DDT by the end of the anoxic phase. More importantly, biodegradation of OCPs in cattail treated mesocosms was as obvious as that in lactate treated ones, which will greatly decrease the expense without any adverse effect on the efficiency. However, overall concentrations of toxaphene, DDT, DDD and DDE did not show significant difference by the end of the oxic treatment.

The present study contributed to establish a efficient guideline for simultaneously extracting toxaphene, DDD, DDE, and DDT from soils with high organic matter and a greater understanding of bioremediation of toxaphene and DDT family contaminated farmland using a cost efficient strategy.

CHAPTER 1
INTRODUCTION

Toxaphene and DDT in the Environment

Occurrence and Chemical Structure of Toxaphene and DDT

Even though it was first synthesized in 1847, 1, 1, 1-trichloro -2,2-bis(4-chlorophenyl)ethane, (DDT) was not widely used as an insecticide until World War II, when it was used to protect the troops and civilians from some diseases (Parr and Smith, 1974). Afterwards DDT was used for agricultural application to controlling disease vectors. The investigations on toxicological effects of DDT and the persistence of its hazardous metabolites, 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene (DDE), and 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethane (DDD), resulted in some countries banning its use in the 1970s, forcing some countries to turn to alternatives. Toxaphene was one of the most important pesticides in USA following the national ban on DDT, with more than 409,000 metric tons of toxaphene applied over ten years (Hooper et al., 1979). It was registered for use on more than 277 agricultural commodities and crops to control 167 major insect pests. Toxaphene was also applied by fisheries managers in Canada and USA to get rid of undesirable fish (Saleh, 1991). In 1982, EPA banned its use after toxaphene was shown to cause cancer in rats and mice (Reuber, 1979; Saleh, 1991). In addition to being carcinogenic, toxaphene is highly persistent (Nash and Woolson, 1967; Menzie, 1972) in the environment.

Neither toxaphene nor DDT occurs naturally in the environment. Fig. 1-1 and Table 1-1 show the structure, physical and chemical properties of *p,p'*-DDT, *p,p'*-DDD, *p,p'*-DDE, and toxaphene (with the general formula $C_{10}H_{10}Cl_n$, n=5-10). The widest use of DDT was to kill disease vectors such as mosquitoes carrying malaria. It is cheap and efficient, and was used as an insecticide in agricultural applications, particularly for cotton. Toxaphene is a complex

compound with a broad spectrum of pesticidal activity. This compound consists at least of 670 congeners, whose composition varies with the manufacturer. It is generally considered to have an average formula of $C_{10}H_{10}Cl_8$ (Hooper et al., 1979). Both of these two contaminants exhibit low aqueous solubilities (Table 1-1) and dissolve in many organic solvents. Because 80 to 90% of all toxaphene and DDT use was estimated to be used on cotton, their residues are predominantly in the southern and southeastern U.S. (Saleh, 1991), where cotton was primarily planted. Toxaphene was also used on other crops, such as soybeans and peanuts.

Toxicity and Biochemistry of Toxaphene and DDT

Numerous investigations have been carried out on the toxicity of toxaphene to a range of organisms, including mammals, birds, aquatic organisms, insects, and plants. Among the reports, those testifying its carcinogenicity, and mutagenicity gained increasing attention. Until 1979, a few decades after its first production and application, toxaphene was shown to cause not only neoplasms at all sites but also the malignant neoplasms in both female and male rats and mice (Reuber, 1979). Although some researchers (Epstein et al., 1972) reported that there is no significant evidence that shows the mutagenicity of toxaphene, Hooper et al. (Hooper et al., 1979), Zeiger (Zeiger, 1987), and Saleh (Saleh, 1991) confirmed that toxaphene has both mutagenic and carcinogenic effects.

Among the tested organisms, fish are the most sensitive to DDT. Generally, behavioral development of hatched eggs is very vulnerable. For example, very high dose levels of DDT, 50 and 100 μg/L, impaired the balance and delayed the occurrence of normal behavior after 30-day growth after hatching in Atlantic salmon eggs (Dill and Saunders, 1974). Furthermore, several investigations revealed that its best-known adverse effects are those on reproduction. For other wildlife, DDT has an endocrine disrupting effect. And female herring gull (Colborn et al., 1996) in the Great Lakes was observed abnormal in maternal behavior under the stress of DDT. DDT,

especially its metabolite DDE, also alters the ratios of male and female birds by feminizing their male sexual organs (Fox, 1992). Another aspect of significant adverse impact on reproductive success is the eggshell thinning. Similar adverse effects on reproduction and estrogenic development such as advanced puberty and persistent vaginal estrus have been observed in laboratory animals. Because many developing countries still apply DDT to control insects and malaria, the chronic nervous-system effects of long-term occupational exposure to DDT was evaluated and showed significant correlations between time period of exposure and worse neurobehavioural functions and an increase of neuropsychological and psychiatric symptoms, even though the mechanism of action for DDT to affect neurological function was not elucidated (de Joode et al., 2001). Probable carcinogenicity of organochlorine pesticides has also been paid much attention. DDT and its persistent metabolites cause cancer problems in experimental animals. Zheng (Zheng et al., 1999) investigated the relation between DDE and DDT exposure and breast cancer risk and did not find any significance relationship. However, the study is limited by lack of exposure data and a relatively small number of deaths. A possible carcinogenic role in human for DDT is not impossible considering the similarity of structure and chemical properties shared by halogenated aromatic hydrocarbons.

Efforts of demonstrating the toxicological mechanisms of DDT provide a few ways to understand how the organisms responded to this insecticide and its metabolites. Some researchers believed that *o,p'*- or *p, p'* isomers interact with estrogen or androgen receptors of rats (Heinrich et al., 1971) and then cause a series of abnormal effects, as mentioned above. Blocking androgen action at its receptor and altering the expression of androgen-dependent genes (Kelce et al., 1995; Kelce et al., 1997) were considered another likely mechanism for DDE's changing the sex differentiation in male rats. Additionally, the decrease in serum

testosterone levels in rats (Krause, 1977) and alligators in Lake Apopka (Guillette et al., 1994) exposed to DDT is another avenue to affect androgen homeostasis.

The understanding of the toxicological mode of action and the metabolism of toxaphene in various organisms has slow due to its complicated structure and the difficulties in analysis. Generally, toxaphene affects through the central nervous system, liver mixed function oxidases, and other metabolic pathways. Some receptors binding to specific sites in mammal and insect brains were shown to be inhibited competitively by toxaphene and its purified components (Lawrence and Casida, 1984; Matsumura and Tanaka, 1984). Additionally, toxaphene has an effect on the central nervous systems by increasing K^+ and Ca^{2+} and inhibiting ATPase activities in cockroaches (Whitson and Crowder, 1979) and mice (Trottman and Desaiah, 1979). In addition to central nervous system, the process of glycolysis and other energy metabolisms can be inhibited by toxaphene (Trosko et al., 1987).

Environmental Fate of Toxaphene and DDT

With the increasing concerns about environmental health problems resulting from these pesticides, a large number of studies have investigated the environmental fate and environmental characteristics of chlorinated pesticides such as toxaphene and DDT. The physical and chemical characteristics of these pesticides such as low water solubility, high stability and semi-volatility contribute to its relatively high persistence in the environment. The percentage of pesticides remaining in the soil depends on the application rate before the monitoring investigation. The higher concentration applied, the higher percentage remained in the soil. The environmental conditions have a very important effect on the persistence of organochlorine pesticides (OCPs). Organic matter is the most important factor among those affecting the persistence of OCPs, OCPs persisting longer in soils rich in organic matters. For example, in the muck with high organic matter ranging from 27 to 56 %, mean concentration of DDTr was as high as 4685 ppb

dry weight and residues of DDT were still present there, about 70 % decreasing after 16 years (Oloffs et al., 1971; Szeto and Price, 1991). In the silt loam farms with organic matter ranging from 3.7 to 6.5 %, only 433 ppb of DDTr were detected. The dissipation of DDTr in the loamy sand soils, with less than 2% organic matter, was almost complete after 16 years. Organic matters adsorbed DDT as a result of its high lipophilicity so as to increase the persistence by removing DDT from water solution in soils and preventing leaching through soil. Soil acidity, another chemical property of soil, may cause pesticides more persistent (Gong et al., 2004). When the pH was closer to acidity, the changes of structure of the humic acid would lead to bigger cavities in its polymer, resulting in more organochlorine pesticides trapped into these cavities (Alawi et al., 1995). Because of high solar radiation and microorganism diversity in tropical region, degradation of degradative pesticides was remarkable in this kind of area than in temperature regions (Khan, 1994). Additionally, high temperature can also accelerate the volatilization of organochlorines in spite of their low vapor pressure (Farmer et al., 1972; Nash, 1983; Singh and Agarwal, 1995).

Both toxaphene and DDT are the persistent pollutants in the environments, long half-life in soils, sediment and water (Table 1-2). One study revealed the large amount of residue of toxaphene in the soil (Willis and McDowell, 1987). It was estimated that only between 9 and 19% of toxaphene applied to cotton fields by aircraft was partitioned in plants, while most of the toxaphene went to the soil (Willis and McDowell, 1987). Then how did the toxaphene in the soil translocate? Compared with other chlorinated hydrocarbon insecticides, toxaphene exhibited a relatively long half-life of 11 years in a Congaree sandy loam soil, while the half-life for purified aldrin, technical aldrin, dieldrin, chlordane, heptachlor, Dilan, BHC and DDT were respectively 5, 9, 7, 8, 2 to 4, 4, 2, and 10.5 yrs (Table 1-2) (Nash and Woolson, 1967). Even after 20 years,

45.1% of applied toxaphene was detected (Nash et al., 1973) because of the rather slow degradation rate. The extent of chlorination may explain its long half-life though, the chlorination rendering the much difficulties in the biodegradation. Major components of toxaphene detected in sediment, soils, sewage sludge and various mammals (Stern et al., 1996; Buser et al., 2000) come from the reductive dechlorination of toxaphene. The study on the distribution and movement of toxaphene in anaerobic saline marsh soils revealed that the concentration of toxaphene decreased with depth (Gallagher et al., 1979). The translocated toxaphene, therefore, became the pollution source of groundwater. Volatilization is of great importance accounting for the loss of toxaphene from soil. Glotfelty reported (Glotfelty et al., 1989) substantial losses (31 %) of surface-applied toxaphene residues within a 21-day period and a decrease in the volatilization when the soil surface became dry.

On the other hand, DDT has also been detected in the global, most heavily in humid temperate and tropical zones. In general, DDT residues disappear from three primary mechanisms (Woodwell et al., 1971) including volatilization, water runoff, and degradation. Because DDT can be degraded to DDE with the existence of oxygen, DDE maked up the major proportion of the total DDT residues in the superficial or well-aerated soils (Spencer, 1975; Aislabie et al., 1997). Greater vapor pressure (Table 1-1) leads DDE to be the major component found in the atmosphere over a field which was previously applied technical DDT (Spencer, 1975; Aislabie et al., 1997). For the other, in the flooding conditions, a higher percentage of DDD constituted the residues (Spencer, 1975; Aislabie et al., 1997).

Extraction of Toxaphene and DDT from Soils

To date, a variety of extraction methods have been developed for organic compounds. In 1879, a German chemist pioneered the extraction technology with the solid-liquid extraction apparatus that bears his name, Soxhlet (SOX). The Soxhlet extraction process can ensure the

intimate contact of the sample matrix with the extraction solvent. As a traditional method, it cannot overcome the limitation at the point of process period that requires the use of long time cycles e.g. 8 to 24 hours. Alternative methods such as solvent-shake extraction require intensive labor for the complicated steps. In addition, a large quantity of solvent is used in those extractions, thereby increasing the cost and producing much waste to be disposed.

In 1995, accelerated solvent extraction (ASE) was introduced for analysis of organics because it requires only a small amount of solvent and less time than Soxhlet, which has sparked increasing interest in it use in environmental studies (Richter et al., 1996). The solvent is first pumped into the extraction cell containing the sample, and then the temperature and pressure are elevated quickly, respectively, to 100 ºC and 2000 p.s.i. (Fitzpatrick et al., 2000). Increased temperature and pressure allow the solvent to penetrate the sample matrix by decreasing the viscosity of organic solvents (Montgomery et al., 1995). In addition, increased temperature and pressure can keep the solvent liquefied above the boiling point and disrupt the strong interactions between the solute molecules and active site of the matrix, and hence improve extraction. After a few minutes, the extract is purged from the cell to a collection vial for further processing, such as additional cleanup if required or direct analysis.

Due to the reduced sample preparation time and the lower amount of solvent required, ASE has been increasingly used to extract organic contaminants from various environments, including soils and sediments (Montgomery et al., 1995; Fisher et al., 1997; Schantz et al., 1997; Saim et al., 1998). And comparisons between ASE and some traditional techniques such as Soxhlet extraction and fluid extraction for extracting pesticides show an agreement that ASE has similar recoveries to those from traditional ones (Conte et al., 1997; Frost et al., 1997; Fitzpatrick et al., 2000). Additionally, ASE has also been evaluated when applied to extract DDT and its

metabolites from environmental reference materials and good agreement for extracting these organic pollutants was achieved between SOX and ASE (Fitzpatrick et al., 2000).

For choosing a proper extraction technique, it is important to realize that various factors affect the performance of the specific extraction methods to some extent. ASE is sensitive to the soil characteristics; an increasing amount of carbon in the sediments caused chlorinated organic compounds less accessible to the extraction solvent (Josefsson et al., 2006). The choice of extraction solvent has been demonstrated as one of the most significant variables for polycyclic aromatic hydrocarbons (Parr and Smith, 1974). As an important factor, water content was shown critical to obtain high extraction efficiencies for organic pollutants from low organic carbon soils in many investigations (Wall and Stratton, 1991; Heemken et al., 1997; Spack et al., 1998; Berglof et al., 2000).

Microbiology of Toxaphene and DDT Degradation

Reductive Dechlorination of Organochlorine Contaminants

Despite their persistence, long half-life in the environment, and resistance to any chemical, physical and biological degradation, higher halogenated compounds may be dehalogenated when the soil is subjected to a reducing environment. Lower halogenated pollutants may be oxidized aerobically by microbial enzymatic systems, while aerobic microorganisms may not be capable of oxidizing higher halogenated ones. Reductive dechlorination is considered as the most important reaction of the biodegradation of complicated chlorinated compounds, and the participation of transition metal-containing coenzymes (Esaac and Matsumura, 1980; Mohn and Tiedje, 1992) has been documented for this process of an electron transfer with the release of a chlorine and its replacement by hydrogen (Fingerling et al., 1996). Using this alternative electron acceptor benefits the dehalogenating bacteria since the standard free energy change for the

oxidation of hydrogen under this situation can yield more energy than some more common electron acceptors such as methane and sulfate in anaerobic soils. When hydrogen is used as electron donor, the amount of energy available from general dechlorination of halogenated compounds results in $\Delta G'$ values between 101 and 159 kJ/mol of chlorine removed according to a conservative estimate. This is at least 3 times more than the amount of energy available from sulfate reduction or methanogenesis per mole of hydrogen consumed (Dolfing and Harrison, 1992). In general, sulfate reducing bacteria (SRB) widely distribute in the anaerobic environments and relate to such process which is coupled with energy conservation with the haloorganic compounds serving as the terminal electron acceptor instead of sulfate. Some SRB directly dehalogenate the chemicals (Utkin et al., 1994; Bouchard et al., 1996; Dennie et al., 1998; Boyle et al., 1999; Gerritse et al., 1999; Wiegel et al., 1999; Drzyzga and Gottschal, 2002; Zanaroli et al., 2006) or indirectly participate in the dehalogenation by providing dehalogenating bacteria with hydrogen, an electron donor (de Best et al., 1997; Drzyzga et al., 2002; Drzyzga and Gottschal, 2002). However, dehalogenating bacteria were reported to compete with SRB for electron donors under sulfate reducing conditions in some cases (Hoelen and Reinhard, 2004). With the diversities of SRB's role in the dehalogenation of complex organic contaminants, their microbiology and biochemistry related to halorespiration still remain to be elucidated.

Based on our previous study, SRB is present in the NSRA soils (Castro and Ogram, data not shown) so that we assumed that this group of versatile bacteria might be involved in the potential biodegradation of organochlorine pesticides (OCPs) in NSRA.

Microbial Dechlorination and Degradation of toxaphene, DDT, DDD, and DDE

Reductive dechlorination of DDT in anoxic condition to DDD is considered to be the dominant reaction of two major routes (Johnsen, 1976; Esaac and Matsumura, 1980; Lal and Saxena, 1982; Boul, 1996; Aislabie et al., 1997). In most cases, DDT dechlorination is not an

energy-yielding process as DDT does not serve as the terminal electron accepter in a bioenergetic pathway (Foght et al., 2001). Under anaerobic conditions, DDD can be further metabolized (Fig. 1-2). Similar to the first reductive dechlorination reaction, all steps after the first reductive transformation of DDT to DDD until the formation of benzhydrol were found to require co-metabolic substrates (Foght et al., 2001). For another, dehydrochlorination of DDT under aerobic conditions to DDE is the other possible pathway despite not occurring often (Fig. 1-3).

Degradation of DDT by microorganisms in pure culture has been studied for a few decades. As early as 1980, a pure strain, *Pseudomonas aeruginosa* 640X (Golovleva et al., 1980), was isolated to degrade DDT from soils with a series conditions including aeration and alternating co-substrates after the fist step of reductive dechlorination. Till today, many pure cultures and consortia have been reported to play important roles in destroying DDT under aerobic or anaerobic conditions. The physiology and biochemistry of biodegradation of DDT have been investigated on a laboratory scale and reveal many important strategies or processes to control the metabolism process. Some studies showed the toxicity of DDT to microbial degraders. For a DDT-degrading consortium composed of four bacterial strains, the *Serratia marcescens* DT-1P (Bidlan and Manonmani, 2002) was shown to be capable of degrading DDT up to 15 ppm DDT; however, 50 ppm DDT inhibited degradation. DDT and polychlorinated biphenyls (PCBs) share similar aspects of their chemical structures, and some researchers studied degradation of DDT by PCB degrading bacteria. For example, *Alcaligenes eutrophus* A5 was originally enriched from PCB-contaminated sediment and was found to degrade DDT via 4-CB aerobically (Nadeau et al., 1994). With *Alcaligenes denitrificans* ITRC-4 (Ahuja and Kumar, 2003) degrading DDT, sodium acetate and sodium succinate inhibit aerobic metabolism by 38%

and 47%, respectively. And also, biphenyl vapors and 2,2'-bipyridyl inhibit degradation completely by competing with structural analog to DDT to bind at the active site of the metabolizing enzyme and making it unavailable for DDT metabolism or inhibiting the iron-containing enzyme in the metabolic pathway. Nevertheless, glucose shows no inhibition of aerobic dechlorination of DDT, and even enhances the transformation of DDT to DDD by 50%. Since microorganisms in soil co-metabolize DDTr the amendment of suitable carbon source will successfully improve the DDT degradation rate. To date, plenty metabolism explorations resulted in a basically complete degradation pathway under either aerobic or anaerobic condition (Fig. 1-2 and Fig. 1-3).

Because of its complex structure, the degradation pathways of toxaphene have not been studied as much as those of DDT, DDD and DDE, its major products. Most of the investigations about the biodegradation of toxaphene revealed that the major pathway was carried on under anaerobic conditions (Murthy et al., 1984) and follows first-order kinetics, which implicates that the degradation rate depends on the concentration of toxaphene remained in the system. Except for the well-established anaerobic metabolism, the oxidative process plays an important role in the degradation of toxaphene (Chandurkar and Matsumura, 1979; Maiorino et al., 1984). A strain of *Pseudomonas putida* was shown to decompose toxaphene aerobically and anaerobically in that study. Recently, the white-rot fungus *Bjerkandera* sp. strain BOL13 (Romero et al., 2006) was isolated to aerobically degrade toxaphene, while *Dehalospirillum multivorans* (Ruppe et al., 2002) and *Enterobacter cloacae* (Lacayo-Romero et al., 2005) anaerobically degrade toxaphene. Nevertheless, biochemistry of degradation by these microorganisms has not been reported yet.

Two pathways are proposed for biodegradation of toxaphene including reductive dechlorination, a major process, (Fingerling et al., 1996) and dehydrochlorination, a minor

process, (Ruppe et al., 2004) and then lower chlorinated complex are taken over by aerobes. Dechlorination of toxaphene is reported to be stereoselective. The first loss of chlorine atom usually occurs in the C-2_{endo} position, the most labile position in the carbon ring, from each geminal dichloro group, which two chlorines are attached to the same carbon in the ring. However, reasons for anaerobes favoring 2-endo chloro substitution remain unclear. The dechlorination rate, in most of cases, depends on the chlorination stage and follows the order of nonachlorobornanes > octachlororbornanes > heptachlorobornanes (Fig. 1-4) (Fingerling et al., 1996; Buser et al., 2000). Generally, a hexachlorobornane (Hx-Sed) and a heptachlorobornane (Hp-Sed) are the predominant end metabolites of toxaphene in treated environments (Stern et al., 1996; Buser et al., 2000).

Redox Potential and Biodegradation of Organochlorine Compounds

Redox potential (Eh) is a measure of the free electron activity of a system. Redox conditions in vadose zones and saturated environments are related to promoted microorganisms' growth and efficient biodegradation of organic contaminants (Szeto and Price, 1991; Jin and Englande, 1996; Crawford et al., 2000; Davis et al., 2004). A chemical with more positive Eh will have a more affinity for an electron and tendency to be reduced. The poly-chlorination of organic chemicals results in the increase in their electrophilicity and oxidation status (Jin and Englande, 1996), which leads to the instability and biodegradation under the reduction condition and explained why they can be easily broken down under the anaerobic conditions. As shown in Table 1-4, higher halogenated compounds have more positive potentials than lower halogenated chemicals have and the reduction of the halogenated contaminants produce more energy than sulfate reduction or even denitrification in some cases, making these organic compounds the favorable electron acceptor. A high Eh indicates the environment that allows the oxidation reaction with a relatively high tendency, while the reduction reaction more possibly occurs when

the system has a low Eh. It is therefore evident that redox potential in the environments represents a rather important factor that may have an effect on the efficiency of organochlorine compounds such as toxaphene, DDT, DDD and DDE.

To date, many investigations have revealed that various redox potentials influenced the biodegradation efficiency and led to different degradation pathways (Esaac and Matsumura, 1980; Doong and Wu, 1992). Detailed information about the influence of redox potentials on the degradation of chlorinated compounds has been reported in a study by Jin and Englande (Jin and Englande, 1996). Under the different redox potentials the degradation rate of carbon tetrachloride (CT) was investigated, the lowest minimum redox potential corresponding to highest degradation rate, K (Table 1-3), the overall rate constants depending on cell mass and k, specific rate constants independent of cell mass.

Redox potentials can be made negative by the addition of organic matter (Szeto and Price, 1991), carbon source (Davis et al., 2004), electron acceptors, and microbial activities (Crawford et al., 2000). In general, as the organic matter in the soil itself is small and not readily available, the Eh is more positive in the environment without any amendment (Ugwuegbu et al., 2001). A flooded soil can limit the diffusion of oxygen into the soil and the oxygen in both water phase and soils can be removed by microbial respiration. The addition of acetate, glucose, or other energy source is capable of accelerating the process of reducing oxygen and driving the Eh to drop.

Bioremediation of Toxaphene, DDT, DDD and DDE

The management of toxaphene, DDT and its metabolites in the soil, water and air has been concerned especially with remediation of contaminated environments. Conventional techniques for organochlorine contaminated soils involve physical and chemical strategies including excavation and incineration, thermal desorption (Norris et al., 1997), microwave-enhanced

thermal decontamination (Kawala and Atamanczuk, 1998), leaching with surfactant (Kile and Chiou, 1989; Ganeshalingam et al., 1994; Parfitt et al., 1995), and supercritical fluid extraction (Sahle-Demessie and Richardson, 2000). Even though the above technologies are more rapid than bioremediation, they do not change the chemicals themselves but transfer the contamination from one place to another and disturb the original landscape. Bioremediation overcomes these disadvantages and may be more cost-effective than chemical and physical remediation. It utilizes the metabolic diversity of bacteria, fungi, and plants. These powerful cleaning creatures degrade the pollutants, produce less toxic or nontoxic chemicals and allow them to enter the clean geochemical cycles. Therefore, in the recent years, bioremediation has gained a lot of interest in research and application.

As early as the 1970s, the biodegradation of DDT partially succeeded in an Everglades muck (Parr and Smith, 1974) and flooded soil (Guenzi and Beard, 1976). Regardless of the extreme complexity of structure of toxaphene, a few publications have documented toxaphene biodegradation in anaerobic soils (Parr and Smith, 1974; Lacayo-Romero et al., 2006), sewage sludge (Buser et al., 2000), and water (LacayoR et al., 2004). Although the biological process for degradation of toxicants is selective in general, toxaphene degradation doesn't show the specificity (Buser et al., 2000). At this point, toxaphene offers special convenience for microbial attack and thereby improving the degradation rate. For such a recalcitrant pollutant, microbial remedy is probably the most promising solution to the toxaphene environmental topic.

Because highly chlorinated contaminants prefer anaerobic metabolisms and less chlorinated species favor aerobic metabolisms, complete mineralization of these compounds can be achieved by anaerobic treatment followed with aerobic oxidation. More efficient bioremediation of organochlorine compounds has been reported in alternating anaerobic/aerobic

mode. Promising results have been obtained from the complete investigations (Anid et al., 1991) on the destruction of PCBs in river sediment under sequential anaerobic-aerobic processes. During the anaerobic period, reductive dechlorination of PCBs occurred, and tri-, tetra-, penta-, and hexachlorobiphenyl concentrations decreased, whereas mono- and dichlorobiphenyl were accumulated. Under the following aerobic phase, significant degradation of the anaerobic products occurred. By the end of the anaerobic-aerobic treatment, only 43% of the 300 mg of PCB/kg of soil remained. Despite the lack of the quantification of DDT degradation under the sequential anaerobic-aerobic process, prospects of alternating anaerobic and aerobic incubation were indicated in an *in vitro* study of cell free extracts of *Pseudomonas* sp. extensively degrading DDT (Pfaender and Alexander, 1972). *Pseudomonas* sp. can not dechlorinate DDT under the strict aerobic condition but can anaerobically co-metabolize DDT. Under the anaerobic conditions, DDT can be metabolized to 4,4'-dichlorobenzophenone (DBP) as the end product through DDD, 1-chloro-2,2-bis(4-chlorophenyl)ethane (DDMS), 2,2-bis(4-chlorophenyl)ethylene (DDNU) by a *Pseudomonas* sp. (Pfaender and Alexander, 1972). Nevertheless, subsequently aerobic incubation with fresh *Pseudomonas* sp. produced a *p*-chlorophenylacetic acid (PCPA), which was not found in the strict anaerobic conditions. This product was readily further degraded by an *Arthrobacter* sp., producing *p*-chlorophenylglycoaldehyde. It implies that the ring cleavage of DDT needs the existence of oxygen and alternating anaerobic and aerobic incubation conditions can promote more complete DDTr biodegradation.

Although various microorganisms are at the laboratory capable of biologically degrading toxaphene, DDT and its residues, DDTr, their mineralization in soil is very slow. In environments unlike in laboratory, carbon source and electron donors may not be sufficient;

degraders may compete with other strains; bioavailability of contaminants may be low or concentrations of contaminants may be too high as to be toxic for degrading microbes, etc. These difficulties can not overshadow the prospective of bioremediation of toxaphene and DDTr and biodegradation has the potential to be enhanced by some strategies. For example, the addition of co-substrates such as lime (Parr and Smith, 1974) and alfalfa (Parr and Smith, 1974) seemed to increase the bacterial population, thereby increasing the microbial degradation. Among environmental factors, temperature was very important for enhancing the conversion of DDT to DDD (Guenzi and Beard, 1976), while aerating with N_2 was capable of activating the anaerobes responsible for toxaphene degradation (Parr and Smith, 1974). In order to improve the bioavailability of contaminants, surfactant addition is the most common approach (You et al., 1996). Fortunately, all these enhancements can be used in the field and aid in bioremediation during large scale cleanup processes.

Site Characteristics

Lake Apopka is located in northwest Orange and southeast Lake counties (Fig. 1-5), and is one of the most polluted lakes in Florida. Farming operations, wastewater discharged from shoreline communities and citrus processing plants were the main pollution sources. Excessive phosphorus in Lake Apopka resulted in the decline of the vegetation, food for the fish and wildlife under the water. As a result, the State of Florida closed this area to farming in 1998 and is now restoring the lake and surrounding land. During the winter of 1998-1999, over 600 birds, primarily white pelicans, associated with the lake died. The reasons for this large bird kill are still unclear, although the tissues of some of the pelicans contained high concentrations of OCPs. The major pesticides in this area are toxaphene, DDT, DDD, and DDE. According to En Chem, in 1999, Lake Apopka NSRA had 16 ppm of toxaphene, 0.35 ppm of DDT, 2 ppm of DDE, and 0.54 ppm DDD in the moderately contaminated areas. In the highly contaminated sites, the

contaminated pesticides and metabolites had approximate recovery of 48 ppm of toxaphene, 1.2 ppm of DDT, 2.4 ppm of DDE, and 1.6 ppm DDD. The concentrations of these pollutants in the extremely highly contaminated sites were as high as 150 ppm of toxaphene, 2.4 ppm of DDT, 2.8 ppm of DDE, and 1.8 ppm DDD. Obviously, the contamination level of toxaphene exceeds the maximum contaminant level (MCL) of 5 ppb by EPA. The removal of these OCP is of great concern. The data also give a clue that DDT has probably converted to DDD and DDE through intrinsic degradation. We could be confident that the bioremediation of the pesticides in this restoration area is feasible.

Research Hypotheses

Published studies demonstrated that the biodegradation of toxaphene, DDT and its major metabolites under appropriate conditions could occur. As a promising technique for restoring these OCP-contaminated soils, bioremediation has not been studied extensively. Specifically, the microbiology at this site of their biodegradation remains unknown.

The high organic matter in NSRA soils results in the difficulty in the extraction and remediation of OCPs. Our overall objective was to establish the optimal extraction methods of pollutants, the reasonability and applicability of bioremediation in the upper scale and the most efficient conditions for restoring the NSRA. Specific hypotheses were proposed to gain the information for the extraction of OCPs and their biodegradation in both microcosm and mesocosm scales of the NSRA soils.

- **Hypothesis 1:** Compared with SOX, a traditional extraction method, ASE simultaneously extracts toxaphene and DDT family from contaminated soils from NSRA with highest efficiency, and repeatability. Solvent system, moisture content and organic matter impact the performance of the extraction methods.
- **Hypothesis 2:** Among six most commonly used electron donors under sulfate reducing conditions, introduced H_2 will be directly utilized by dehalogenaters. Or dehalogenaters may be stimulated by hydrogen transformed from special donors through syntrophy.

Toxaphene, DDT and its major metabolites can be used as the sole energy source or electron acceptors by stimulated degraders under these conditions.

- **Hypothesis 3:** A pure culture or consortium native in the contaminated soils reductively dechlorinates toxaphene by utilizing toxaphene as electron acceptor when provided with the electron donor that shows the potential effect on stimulating biodegraders in study of Hypothesis 2.
- **Hypothesis 4:** Mesocosms, a scale up study, will extend the concepts about relationship between electron donors and biodegradation developed in the bench scale experiments; readily available plant materials might be fermented and produce nutrients, especially electron donors, which has the equivalent effects on stimulating OCP degraders to technical electron donor (lactate).

Study Overview

Our study was conducted to: 1) optimize the extraction methods for toxaphene, DDT, DDD, and DDE from soils with high organic matter contents; 2) more importantly, establish the feasibility and further the valuable information of bioremediation of OCPs contaminated soils in the bench scale by testing various electron donors and attempting to characterize the responsible organisms; 3) extend the concepts in the microcosm studies to the scale up of mesocosm.

Chapter 2 investigates the most efficient methods for extracting toxaphene, DDT and its major metabolites from NSRA soils. Conditions for the simultaneous determination of these pollutants from different matrices are optimized using both ASE and SOX. The influence of water content in samples on the extraction efficiency is examined. Efficiency of isolating these chemicals using solvents methylene chloride and hexane is compared.

Chapter 3 focuses on whether sulfate reducing conditions would promote the dechlorination of toxaphene and DDT. And also, this chapter compares degradation of the OCPs with important electron donors in dechlorination to find the best combination for degradation of the target OCPs. Finally, this Chapter tries to identify the biodegraders and investigate whether pure culture or a consortium of bacteria cometabolizes toxaphene or DDT.

Chapter 4 tests the concepts developed from the microcosm studies by extending the microcosm studies to a mesocosm scale. Furthermore, this Chapter evaluates the efficacy of produced lactate and hydrogen from the fermentation of readily available plant materials as electron donors in order to decrease the cost and make the process safely or practically handled.

Finally, Chapter 5 summarizes implications of this research toward greater understanding of biodegradation of toxaphene, DDT, DDD and DDE, and factors controlling this process in NSRA soils.

Table 1-1. Physical and chemical properties of toxaphene, *p,p'*-DDT, DDD and DDE

	Toxaphene	*p,p'*-DDT	*p,p'*-DDD	*p,p'*-DDE
Chemical formula	$C_{10}H_{10}Cl_8$	$C_{14}H_9Cl_5$	$C_{14}H_{10}Cl_4$	$C_{14}H_8Cl_4$
Molecular weight	413.82 (avg)	354.49	320.04	318.02
Melting point (°C)	65 - 90	106.5	109.5	89
Boiling point (°C)	decomposes	decomposes	350	336
Density (g/cm³)	1.65	1.6	1.39	No data
Vapor pressure (mm Hg at 20 °C)	0.2 - 0.4	1.5×10^{-7}	1.35×10^{-6}	6×10^{-6}
Partition coefficients:				
Log K_{ow}	3.30	6.91	6.02	6.51
Log K_{oc}	2.47	5.18	5.18	4.70
Water solubility (mg/L at 25 °C)	3	0.023	0.09	0.12

Table 1-2. Half-life of toxaphene, DDT, DDD and DDE in different matrices

	Toxaphene	DDT	DDD	DDE
Soil	11[a] yr	10.5[a] yr	No data	5.7[e] yr
Sediment	No data	12[c] yr	No data	No data
Surface water	No data	350[b] day	No data	No data
Water	20[d] yr			3.3-3.7[f] day
Ground water		31[b] yr	No data	No data

a: Nash 1967
b: Howard 1991
c: Van Metre 2005
d: Miller 1999
e: Beyer 1989
f: ATSDR 2002

Table 1-3. Effect of Eh on CT biodegradation by *Providencia stuartii*

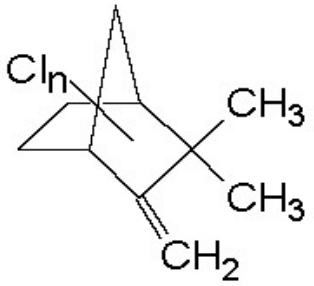
	Min. Eh (mv)	Overall rate C. K (r) (hr^{-1})	Specific rate C. k (r) [hr^{-1}/(mg/l cell mass)]
Control	-4.2	1.29×10^{-3} (0.963)	6.91×10^{-6} (0.959)
Treatment1	-181.7	2.31×10^{-2} (0.900)	1.26×10^{-4} (0.897)
Treatment2	-239.5	2.57×10^{-2} (0.952)	1.47×10^{-4} (0.984)
Treatment3	-208.5	2.29×10^{-2} (0.931)	6.02×10^{-5} (0.939)

Data were taken from Jin and Englande (1996). C: concentration of CT (ppb); r: correlation coefficients.

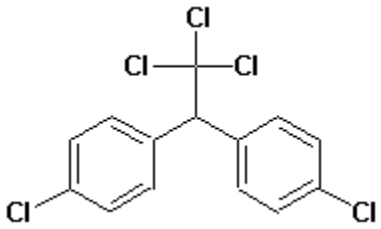
Table 1-4. Gibbs free energy values and redox potentials of selected chlorinated compounds and inorganic redox couples

Electron acceptor	Half-reaction of reductive conversion			$\Delta G_o'$ (kJ/electron)	E_o' (mV)
O_2	$O_2 + 4H^+ + 4e^-$	→	$2H_2O$	-78.7	816
Fe^{3+}	$Fe^{3+} + e^-$	→	Fe^{2+}	-74.4	771
CT	$CCl_4 + H^+ + 2e^-$	→	$CHCl_3 + Cl^-$	-65.0	673
PCE	$C_2Cl_4 + H^+ + 2e^-$	→	$C_2HCl_3 + Cl^-$	-55.4	574
CF	$CHCl_3 + H^+ + 2e^-$	→	$CH_2Cl_2 + Cl^-$	-54.0	560
TCE	$C_2HCl_3 + H^+ + 2e^-$	→	$C_2H_2Cl_2 + Cl^-$	-53.0 to -50.8	550 to 527
hexachlorobenzene	$C_6Cl_6 + H^+ + 2e^-$	→	$C_6HCl_5 + Cl^-$	-171.4	478
pentachlorobenzene	$C_6HCl_5 + H^+ + 2e^-$	→	$C_6H_2Cl_4 + Cl^-$	-163.4 to -167.7	455 to 421
pentachlorophenol	$C_6HCl_5O + H^+ + 2e^-$	→	$C_6H_2Cl_4O + Cl^-$	-167.8 to -156.9	455 to 399
NO_3^-	$NO_3^- + 2H^+ + 2e^-$	→	$NO_2^- + H_2O$	-41.7	432
DCE	$C_2H_2Cl_2 + H^+ + 2e^-$	→	$C_2H_3Cl + Cl^-$	-40.6 to -38.3	420 to 397
tetrachlorophenol	$C_6H_2Cl_4O + H^+ + 2e^-$	→	$C_6H_3Cl_3O + Cl^-$	-157.1 to -132.2	400 to 271
trichlorobenzoate	$C_6H_3Cl_3O_2 + H^+ + 2e^-$	→	$C_6H_4Cl_2O_2 + Cl^-$	-163.1 to -147.4	397 to 331
chlorobenzoate	$C_6H_5ClO_2 + H^+ + 2e^-$	→	$C_6H_6O_2 + Cl^-$	-145.4 to -137.3	339 to 297
$Fe(OH)_3$	$Fe(OH)_3 + 3H^+ + e^-$	→	$Fe^{2+} + 3H_2O$	-11.4	118
SO_4^{2-}	$SO_4^{2-} + 9H^+ + 8e^-$	→	$HS^- + 4H_2O$	+20.9	-217
HCO_3^-	$HCO_3^- + 9H^+ + 8e^-$	→	$CH_4 + 3H_2O$	+23.0	-238
H^+	$2H^+ + 2e^-$	→	H_2	+40.5	-420
Fe^{2+}	$Fe^{2+} + 2e^-$	→	Fe(0)	+42.5	-440

The $\Delta G_o'$ and E_o' values were taken from De Wildeman and Verstraete (2003) and Dolfing and Harrison (1992).



A



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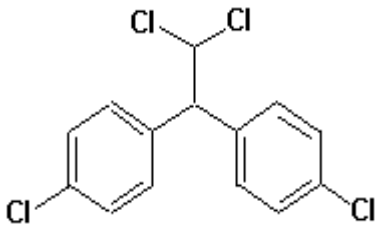

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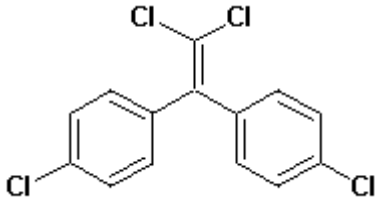

D

Figure 1-1. A: Toxaphene: $C_{10}H_{10}Cl_n$ (n=5-12); B: DDT: $C_{14}H_9Cl_5$; C: DDD: $C_{14}H_{10}Cl_4$; D: DDE: $C_{14}H_8Cl_4$.

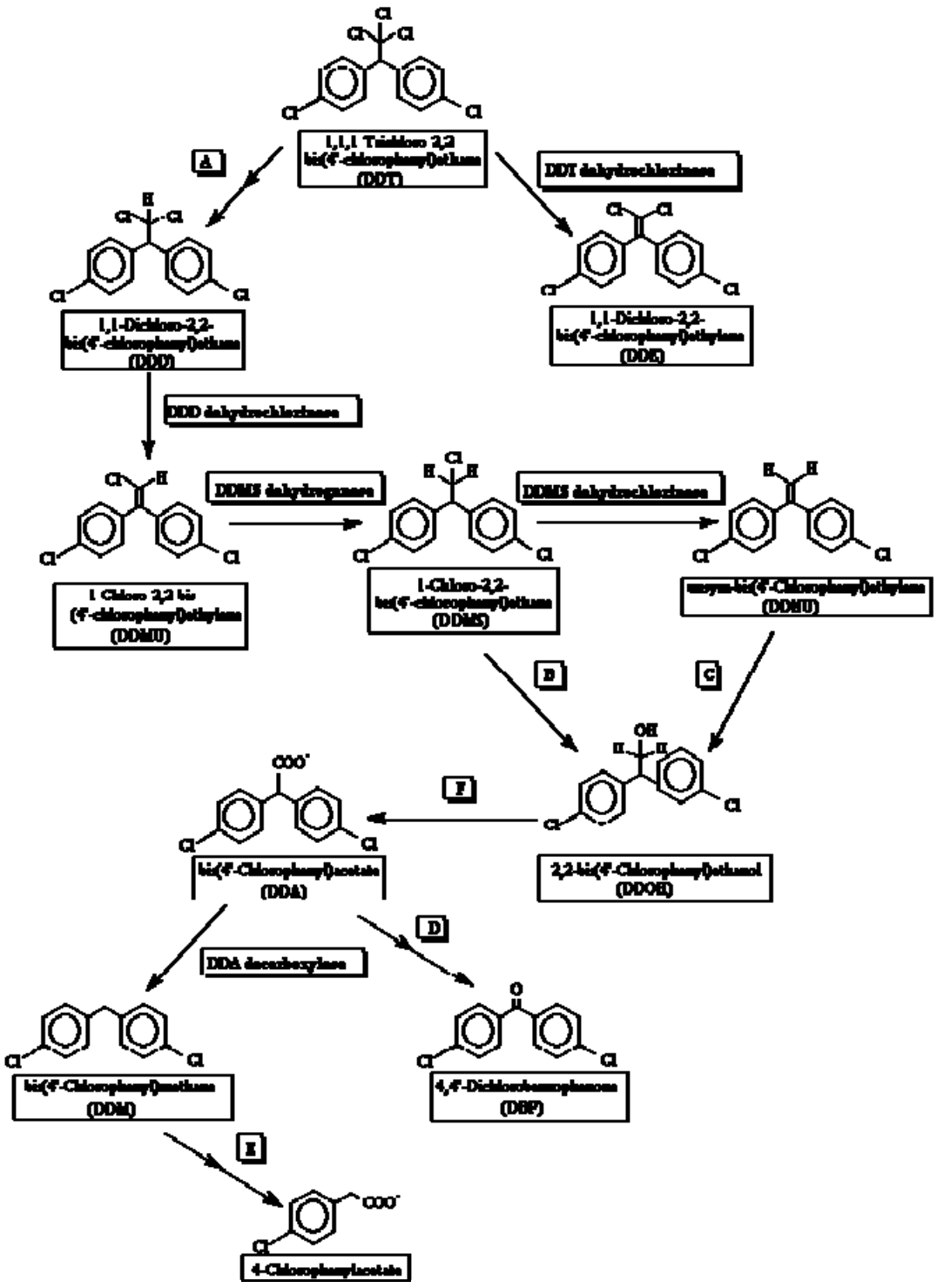

Figure 1-2. Anaerobic degradation pathway of DDT (A, D, E and F are multiple steps whose intermediates are not identified yet.). (http://umbbd.ahc.umn.edu/ddt2/ddt2_map.html)

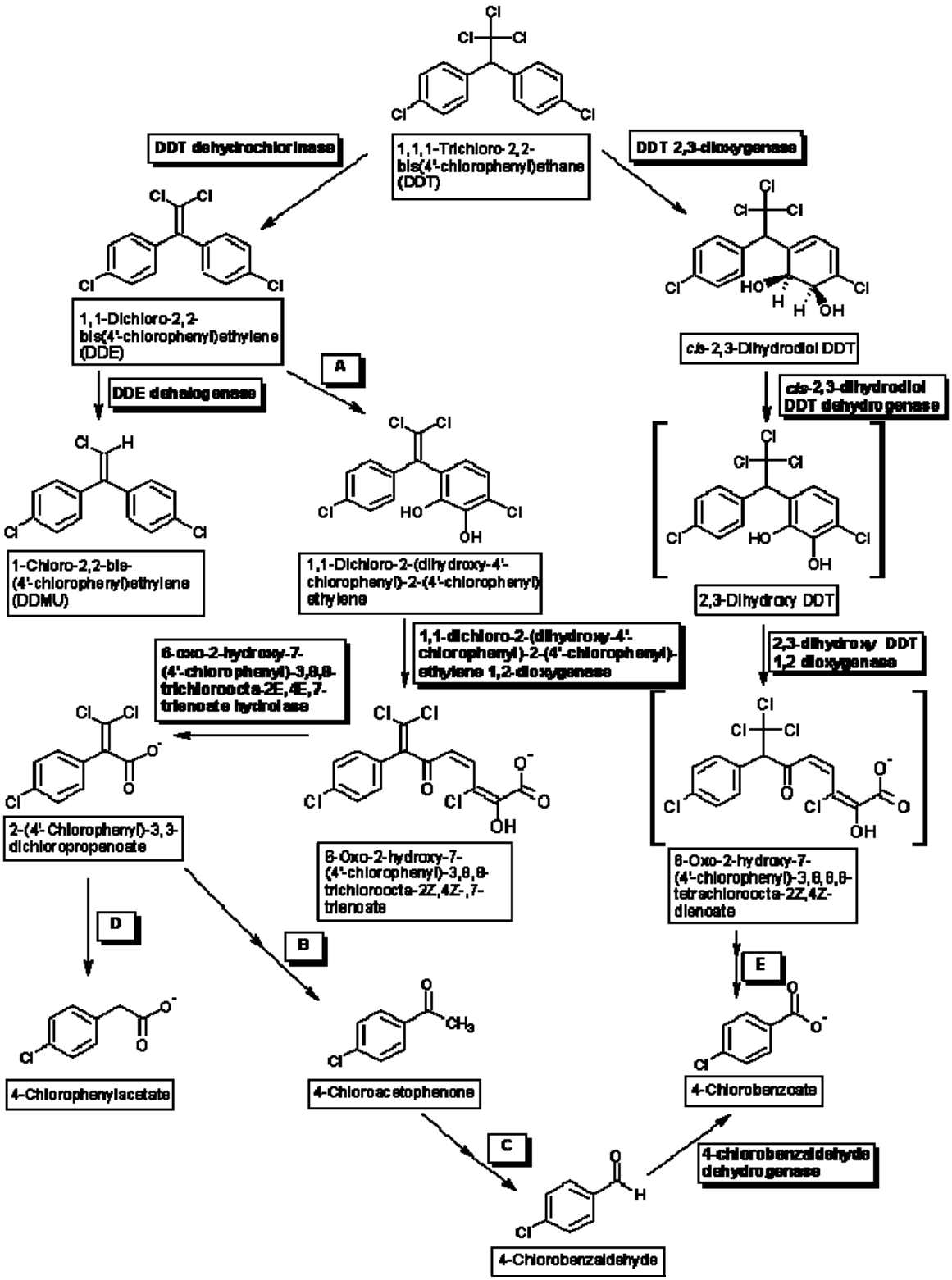

Figure 1-3. Aerobic degradation pathway of DDT (A, B, C, D, and E are multiple steps whose intermediates are not identified yet.). (http://umbbd.ahc.umn.edu/ddt/ddt_map.html)

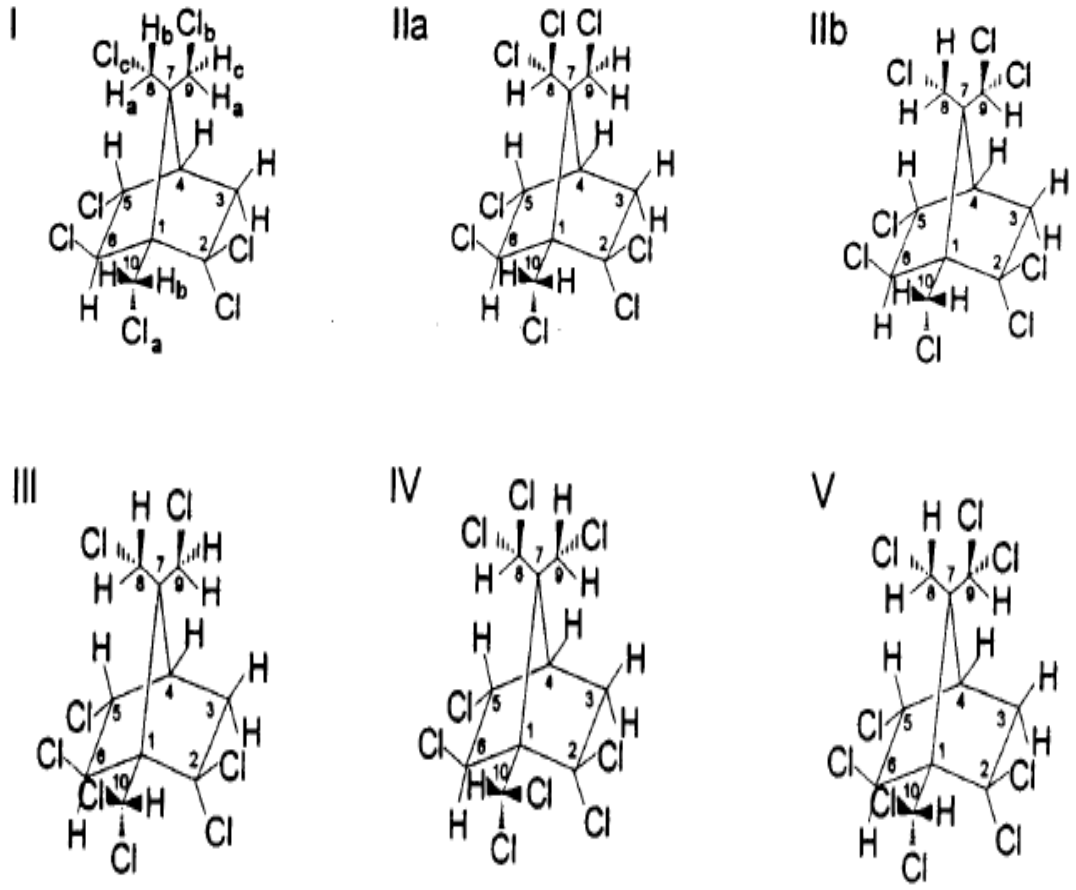

Figure 1-4. Six environmentally relevant toxaphene components; I: 2,2,5-endo,6-exo,8c,9b,10a-heptachlorobornane (Tox B); IIa: 2,2,5-endo,6-exo,8b,8c,9c,10a-octachlorobornane (Tox A_1); IIb: 2,2,5-endo,6-exo,8c,9b,9c,10a-octachlorobornane (Tox A_2); III: 2,2,5-endo,6-exo,8c,9b,10a,10b-octachlorobornane; IV: 2,2,5-endo,6-exo,8b,8c,9c,10a,10c-nonachlorobornane; V: 2,2,5-endo,6-exo,8c,9b,9c,10a,10b-nonachlorobornane.

Figure 1-5. The North Shore Restoration area of Lake Apopka in FL

CHAPTER 2
EVALUATION OF EXTRACTION OF TOXAPHENE, DDT, DDD, AND DDE FROM NSRA SOILS WITH HIGH ORGANIC CARBON CONTENT

Toxaphene (a complex mixture of polychlorinated monoterpenes), 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane (DDT), 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene (DDE), and 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethane (DDD) are organochlorine pesticides (OCP) which have been of environmental concern due to their carcinogenic toxicity, adverse effects on human development, and long environmental persistence (Hill and McCarty, 1967; Nash and Woolson, 1967; Nash et al., 1973; Zeiger, 1987; Saleh, 1991; Sunyer et al., 2006). Adsorption of hydrophobic molecules to organic materials in soils and sediments as a result of low water solubility decreased availability for uptake and transformation by soil microorganisms and limited degradation rates in soils (Alexander, 2000; Ghosh et al., 2003). Adsorption of these contaminants to soils with high organic carbon contents may also make extraction difficult for subsequent quantification. The extraction efficiency of contaminants can be affected by a variety of factors, including soil properties, extraction solvent, and sample moisture (Bandh et al., 2000; Josefsson et al., 2006).

A variety of approaches for extracting organic chemicals from soils have been used over the years, including Soxhlet (SOX) and Accelerated Solvent Extraction (ASE). SOX is based on hot solvent extractions, and has relatively good extraction efficiencies from most soils (Spack et al., 1998). SOX is time consuming (12 to 24 hours), however, and requires at least 100 ml of solvent. ASE is a relatively new approach, and is intended to bypass some of the limitations associated with SOX (Richter et al., 1995). In ASE, approximately about 50 ml of solvent is pumped into an extraction cell containing the sample, at which time the temperature and pressure are elevated. After a few minutes, the extract is purged from the cell to a collection vial for further cleanup or direct analysis. ASE is faster (5 to 15 minutes) and requires less extraction

solvent than SOX, making it a promising alternative for extraction of analytes such as OCPs from soil. Information available comparing extraction efficiencies of hydrophobic OCPs by SOX and ASE from organic soils is limited, however.

We are primarily interested in the fate of OCPs in the organic soils from the northern shore of Lake Apopka, FL. The marshes of the northern shore were drained in 1941 for muck farming (2000). Nutrient runoff from the highly productive farms resulted in the lake becoming highly eutrophic. As a result, the State of Florida closed this area to farming in 1998 and is now restoring the lake and surrounding land. During the winter of 1998-1999, over 600 birds, primarily white pelicans, associated with the lake died over this period. The reasons for this large bird kill are still unclear, although the tissues of some of the pelicans contained high concentrations of OCPs. Toxaphene and DDT concentrations from the northern shore of Lake Apopka have been reported to range from 21 ppb to 150 ppm, and removal of these OCPs is important for the complete restoration of Lake Apopka. In order to evaluate the effectiveness of potential bioremediation strategies in the organic soils of Lake Apopka, effective extraction methods are required for use with established analytical strategies.

In this study, ASE was compared with SOX to extract the toxaphene, DDT and its metabolites from Lake Apopka North Shore Restoration Area (NSRA). Conditions for the simultaneous determination of these pollutants from different matrices were investigated using both ASE and SOX. The influence of water content in samples on the extraction efficiency was examined. Efficiency of extracting these chemicals using solvents methylene chloride and hexane were compared. Except for the effect of soil characteristics on the recovery rate, all the evaluations of the extraction methods and parameters were investigated for extraction of aged residues of toxaphene, DDT, DDD and DDE.USING THE FORMATTING TEMPLATE

Materials and Methods

Samples: Collection and Preparation

Three different levels of contamination, low, moderate, and high based on data reported by En Chem (Green Bay, WI) in 1999 (Table 2-1) were used in this study. The low contaminated soil sample was collected from Lake Apopka NSRA (Lat 28˚ 42'02.439 N, Long 81˚38' 59.113 W). The moderately contaminated sample was from Lat 28˚ 42'38.213N, Long 81˚36' 24.836 W. The highly contaminated soil was initially excavated near a former airstrip approximately 18 months prior to collection for this study, and kept in a large vat under shelter. The definition of contamination levels was based on the concentration of toxaphene in the soil. Upon collection, the samples were placed in an ice cooler and transported to University of Florida campus in Gainesville. Immediately upon arrival in Gainesville, the soils were stored at 4°C until use. Total carbon and nitrogen content was determined in the Teaching Analytical Chemistry Laboratory of the Soil and Water Science Department, the University of Florida, using a TruSpec CN Elemental Determinator (LECO, St. Joseph, MI).

Soil samples were ground and passed through a 20 mesh sieve (850 micrometer) after allowing to air dry for three days at room temperature. Following drying, the soil volumetric water content was adjusted to intermediate or saturated, and allowed to equilibrate for three days in sealed containers. The prepared samples were extracted within seven days after moisture adjustment. A surrogates solution (Decachlorobiphenyl (DCB) and tetrachloromethyl xylene (TCMX), AccuStandard, Inc., USA) and internal standards (Toxaphene, DDD, DDE, and DDT, AccuStandard, Inc., USA) were added to samples prior to extraction. 50 ppm of technical DDD, DDE, DDT, and 100 ppm of technical toxaphene were spiked to low and highly contaminated soils, respectively, to test the recoveries.

Extraction Procedures

SOX. Four grams of soils in triplicate were mixed in cellulose thimbles (25×80mm, Ahlstrom Mt. Holly Springs, LLC, PA, USA) with four grams of anhydrous sodium sulfate, which had been purified at 400°C for four hours. Using methylene chloride/acetone (4:1, v/v) or hexane/acetone (1:1, v/v) as solvent, OCPs were extracted at 5 to 6 cycles per hour for 12 hours.

ASE. A Dionex ASE 100 Accelerated Solvent Extractor (Dionex, Sunnyvale, CA) was used to perform the extractions. Triplicates of soil samples were mixed with hydromatrix (Varian, Palo Alto, CA) in 2:1 proportion and extracted in a 34 ml stainless vessel using methylene chloride/acetone (4:1, v/v) or hexane/acetone (1:1, v/v) as solvent. The cell was filled with the solvent and heated to 100 ºC. The static extraction was performed for 5 min, followed by flushing the vessel with 60% of the 34 ml cell of fresh solvent. Finally, the vessel was purged with nitrogen for 100 sec at 150 psi. The total amount of extraction solvent was about 50 ml, for carrying out one static cycle.

Cleanup and Analysis

All extracts were concentrated to near dryness by a gentle stream of N_2. Repeated this process twice to remove acetone and methylene chloride by adding hexane. The volume of extracts was measured in a glass graduated volumetric cylinder. At this point, the samples were split for analysis of toxaphene and DDT and its metabolites.

For analysis of DDT and its metabolites, one ml of extracts was passed through a florisil cartridge packed with approximately 500 mg of 200 μm Florisil (Varian, Palo Alto, CA). Nine ml of hexane:acetone (9:1, v/v) were used to rinse the column. The resulting eluent was concentrated to 1 ml by an N_2 stream and then transferred to a glass GC vial (2 ml, Fisherbrand, Atlanta, GA) and crimped with a Teflon lined cap for subsequent analysis by gas chromatography.

Another one ml extract was purified by sulfuric acid treatment for further analysis of toxaphene. Equal amounts of concentrated sulfuric acid and sample were mixed in a 10 mL vial. The vial was capped and vortexed for 1 min. Phases were allowed to separate, and the upper (organic) phase transferred to a GC vial for analysis.

Extracts were analyzed with a gas chromatograph (Shimadzu GC-17A) equipped with a ^{63}Ni electron capture detector (ECD). The column was a RTX-5 (0.25 mm ID × 15 m long; Restek Corporation, Bellefonte, PA, USA). In most cases of DDT analyses, 250 ºC oven temperature was used, however toxaphene was considered as the first concern such that we applied the program which Paan and Chen developed (Pearson et al., 1997; Paan et al., 2006). The temperature was programmed as follows: 60 ºC, 3.0 ºC/min to 70 ºC for 1 min, 10 ºC/min to 130 ºC for 3 min, 10 ºC/min to 160 ºC, 20 ºC/min to 200 ºC. A calibration curves with 6 points bracketing the experimental concentrations was used for both analyses. The concentration calculation followed EPA 8000B and methods used by Pearson and Paan (Pearson et al., 1997; Paan et al., 2006).

Statistical Analysis

One-way ANOVA was used to test the difference among treatment groups. Pairwise comparisons for differences in concentration means between different soil characteristics, extraction methods, and extraction solvents were done by Tukey's Studentized test, while correlations were tested by Spearman and Pearson using SAS version 9.1 (SAS Institute, Cary, NC). The null hypothesis was rejected at $p < 0.05$, thus the probability of observing no difference among the pair wise is less than 5%.

Results and Discussion

Correlation of ASE and Soxhlet Recoveries to Soil Characteristics

Since true concentrations of toxaphene, DDT, DDD and DDE in the soils of the NSRA soils are not known, the true extraction recovery is difficult to determine. Recoveries here refer to the rate at which the spiked technical chemicals of these OCPs were extracted. The recoveries of OCPs from the two sample sites were compared to the site characteristics in Fig. 2-1 (organochlorine pesticides concentration, total of carbon, and total of nitrogen, all data from Table 2-1) to examine whether a correlation exists between the soil properties and the recoveries. Table 2-2 gave the Pearson correlation and *p* values.

Table 2-2 indicated that the recovery for toxaphene in ASE was correlated to the site. The correlation between recovery and total of organic carbon (TOC), total of nitrogen (TON) for toxaphene was further illustrated in Table 2-2 by a high correlation (-0.98) and the very low associated *p* value (<0.0001). High TOC and low C/N of sampling site seemed to decrease the toxaphene recovery in ASE by 36.4%. This tendency is consistent with the investigations performed where an increasing amount of carbon in the sediments caused chlorinated organic compounds less accessible to the extraction solvent in ASE (Bandh et al., 2000). However, some studies showed the opposite trend, in which the TOC did not influence pressurized liquid extractions or the recoveries decreased as the C/N ratio increased (Josefsson et al., 2006). In that study, the sediments used for examination had a relatively limited range of low organic carbon contents, ranging from 1.4 to 6.3%.

Recoveries of the two different sites for DDD, DDE, and DDT were similar in both ASE and Soxhlet, while toxaphene in Soxhlet, can be observed from Fig. 2-1. The low Pearson correlations and high *p*-values indicated the lack of correlation between the soil characteristics and the recoveries for the DDT family. These findings agree with a published study on

pendimethalin (Spack et al., 1998). Even though the soil properties varied greatly with regard to soil pH, soil structure, total C, total N, the recovery was not significantly different when using Soxhlet extraction. Even though negative correlations were observed between recoveries of DDT and its metabolites, and water insoluble organic carbon (WIOC) were shown in another study (Tao et al., 2004), the variation in the WIOC was produced by amending the reagent to the soil while the soil without any amendment was used as the control. On the other hand, other major matrix characteristics, such as the C/N ratio, were shown to correlate with decreased recoveries. In a report by Josefsson et al. (2006), the C/N ratio was significantly correlated with extraction efficiency of polychlorinated biphenyls (PCB) by ASE (Josefsson et al., 2006). The higher C/N ratio indicates the possible aging of matrix, which causes the contaminants to intertwine with the matrix affecting the extractability. Nevertheless, the C/N ratio of soils in that study ranged from 15.6 to 32.3, which allowed for observation of the significant correlation of the recovery to the C/N ratio. The C/N ratios in the present study only extended across a 1.2 fold range, such that more research might be of value in revealing a potential correlation between C/N and extractability in ASE and Soxhlet.

Effect of Extraction Solvents

Extraction solvent has been demonstrated as one of the most significant variables for PAH (Parr and Smith, 1974). Methylene chloride/acetone and hexane/acetone are the most commonly used solvent systems for the pesticide residue analysis. Pairwise comparisons of OCP concentrations extracted by these two solvent systems with ASE and Soxhlet are shown in Table 2-3. Using ASE, methylene chloride/acetone (4:1, v/v) provided significantly higher extraction efficiency for toxaphene, DDE, and DDT than did hexane/acetone (1:1, v/v); however, extraction of DDD did not differ between the two solvent systems. Significantly more toxaphene, DDT and DDE were extracted by ASE with methylene chloride/acetone than with hexane/acetone (Table

2-3). The boiling point of the solvents and the extraction temperature of the compared two procedures can explain our findings. For one thing, boiling point for methylene chloride (40 ºC) is much lower than hexane (68 ~ 70 ºC), 30 ºC. Therefore, boiling point for methylene chloride/acetone (4:1, v/v) would be lower than hexane/acetone (1:1, v/v), which acetone has a boiling point of 56 ºC. This fact indicates that hexane/acetone should be better than methylene chloride/acetone since the boiling point of the solvent system is the extraction temperature of SOX. In ASE, extraction temperature is fixed as 100 ºC for both solvent systems. Our study revealed that methylene chloride/acetone is a better solvent than hexane/acetone at the same extraction temperature. However, methylene chloride as the only solvent in the extractions did not extract significantly higher amounts of OCP than hexane/acetone (1:1, v/v) when evaluated pressurized fluid extraction (PFL) and Soxhlet for the extraction of DDT, DDE, and DDD from various environmental matrices, such as urban dust, sediment, and fish (Schantz et al., 1997). On the contrary, they were comparable with PFL for all the tested matrices. The difference may be ascribed to aging and other characteristics of NSRA soil. It suggests that the decision of the appropriate extraction solvent depends on the environmental matrix. Interestingly, the extraction of DDD was rather easily influenced by SOX but not affected much by ASE (Table 2-3). Additionally, the tendency of effects of solvent on other chemicals except for DDT using SOX showed to be opposite to that using ASE (Table 2-3). Pairwise comparisons revealed that extraction efficiencies of both DDE and DDD using hexane/acetone (1:1, v/v) were significantly higher than using methylene chloride/acetone (4:1, v/v) at 95% confidence level. And the effect of hexane/acetone on toxaphene and DDT was significant at 90% confidence level. The means of DDE and DDD concentrations using hexane/acetone as extractant were nearly double those using methylene chloride/acetone. Additionally, the extraction efficiency for toxaphene using

Soxhlet with hexane/acetone is comparable to that using ASE with methylene chloride/acetone. We can conclude, therefore, that the optimum extractant depends on the procedure. Hence, the appropriate combination extraction method with the extraction solvent system is a very important factor to gain optimum extraction efficiency.

Comparison of ASE and Soxhlet

Since methylene chloride/acetone (4:1, v/v) was evaluated for ASE, extraction methods were further compared with this solvent system. The ASE extractions for extracting from the highly contaminated sample generally showed higher extraction efficiencies than SOX, with the exception of DDT (Table 2-4). Means for extracted toxaphene, DDE, and DDD using ASE were 31.69%, 55%, 52.4% higher, respectively, than for SOX. The rather low *p*-values further confirm that ASE extracted these organic compounds more efficiently than Soxhlet at the statistical level ($p < 0.05$). Although ASE did not extract DDT with significantly higher efficiency at the statistical, it was comparable to SOX. Large relative standard deviation (RSD) values indicated SOX extraction was less precise than ASE (Table 2-4).

For the moderate contaminated soil, ASE extractions performed well in comparison with SOX (Table 2-5). ASE was 55.14% more efficient than SOX for toxaphene extraction. A pairwise analysis of extraction methods indicated ASE was significantly more efficient than SOX. Similar to DDT measured from the highly contaminated soil, the means were very similar. Statistical tests did not indicate significant difference in means of DDE and DDD, unlike the observation from the highly contaminated soil. RSD values of means for extracting with ASE were also satisfactory in general.

To date, most, if not all, extraction studies conducted for comparison of ASE with SOX conducted with polyaromatic hydrocarbons, PCBs and other organic compounds (Williams et al., 1993; Heemken et al., 1997; Schantz et al., 1997; Brumley et al., 1998; Bandh et al., 2000)

showed that ASE performed at least as well as SOX. For the first time, the efficiency of ASE for extracting the residue of pesticides from the high organic carbon soils was compared with that of SOX. Our investigation agreed with the previous studies by showing the comparable or better efficiencies.

Effect of Volumetric Water Content

To investigate the influence of water content on extraction efficiency, OCPs were extracted from aged samples by ASE and Soxhlet with methylene chloride/acetone (4:1, v/v) with varying water contents (Table 2-6; Fig. 2-1). As reported in other investigations of water effect on the extraction efficiency for organic pollutants (Wall and Stratton, 1991; Heemken et al., 1997; Spack et al., 1998; Berglof et al., 2000), water content was shown to be an important factor to obtain high extraction efficiencies from low organic carbon soils. Water content was significantly correlated to extraction efficiency for DDT by SOX, and for DDE, DDT, and toxaphene by ASE. The correlation of water content to DDE extraction efficiency was not significant. In both ASE and SOX, intermediate water content (Fig. 2-1) yielded the greatest overall efficiencies for extracting toxaphene, DDT and its metabolites simultaneously from the aged contaminated soils with high organic carbon.

Two explanations have been proposed to explain the effects of water content on the extraction efficiency (Wall and Stratton, 1991; Spack et al., 1998). First, water content may change some properties that are responsible for the adsorption of pesticides to soil. A certain amount of moisture may swell the organic matter in soil, allowing extraction solvents to come in contact with the OCP, and is especially important for NSRA soils that contain high organic carbon contents. Such effect of the swelling or deactivating function of water was proposed to render pendimethalin sorption sites more accessible to supercritical fluid extraction (Spack et al., 1998). When water was not available, soil particles or organic matter collapsed and adsorbed the

OCPs strongly so as to keep them unavailable for solvents. Excessive water still led to extraction difficulties because the soil particles are surrounded by water, which potentially blocks extraction solvent accessed to the OCPs. In addition, water soluble organic carbon (WSOC) may be another factor that influenced the adsorption of pollutants. The increase in the water content reduces the WSOC by partitioning it into the water matrix, which will combine with organic pollutants, thereby improving the extraction efficiency (Tao et al., 2004).

This study compared the traditional and alternative extraction methods for extracting toxaphene, DDT and its major metabolites from the high organic soils. The parameters of interest were evaluated. When using ASE, the recovery of toxaphene was highly correlated to TOC and TON, while SOX was not affected. Both ASE and SOX extracted DDT and its metabolites with similar recoveries despite the different samples. As an important factor in the extraction efficiency, intermediate water content yielded the greatest overall efficiencies for extracting OCP in both ASE and SOX. Significantly more toxaphene, DDT, and DDE were extracted by ASE using methylene chloride/acetone (4:1, v/v) than those with hexane/acetone (1:1, v/v). However, the hexane/acetone system showed statistically higher efficiencies for DDE and DDD at 95% confidence level and toxaphene at 90% confidence level using SOX. Statistical evaluations showed that equivalent results (moderate contaminated soils) or higher efficiencies (highly contaminated soils) were achieved for the simultaneous extraction of residues of toxaphene, DDT and its metabolites by ASE as compared to SOX. Considering much less solvent consumption and extraction time and comparable efficiency, ASE is the preferable extraction technique for the investigation of high organic carbon contaminated soils.

Table 2-1. Soil characteristics for low, moderately, and highly contaminated soils

	Low	Moderate	High
Total organic carbon (TOC %)	26.07(1.14)	41.34(2.30)	18.07(1.59)
Total organic nitrogen (TON %)	2.70(0.26)	3.71(0.10)	1.59(0.12)
C/N	9.66	11.14	11.36
Toxaphene (ppm)	5.1	16	48
DDE (ppb)	160	2000	2400
DDD (ppb)	34	540	1600
DDT (ppb)	21	350	1200

Note: number in the parentheses represents the standard deviation.

Table 2-2. Correlation of soil properties to recovery rate in Soxhlet and ASE

	Soxhlet				ASE			
	toxaphene	DDT	DDE	DDD	toxaphene	DDT	DDE	DDD
ρ[a]	0.11	-0.49	0.33	0.39	-0.98	0.07	0.11	0.62
p[b]	0.7862	0.1681	0.3912	0.2954	<0.0001	0.8671	0.7808	0.0501
r[c]-Low	0.635	1.398	0.845	0.560	0.517	1.396	0.762	0.481
r[c]-High	0.612	1.496	0.814	0.534	0.812	1.377	0.747	0.431

[a] Pearson correlation coefficient.

[b] Probability of significant correlation; statistical significance set at $p < 0.05$.

[c] Recovery rates for low and highly contaminated soils respectively.

Table 2-3. Comparison of extraction solvents for extracting toxaphene, DDT and its metabolites using Soxhlet and ASE from highly contaminated soil

	ASE			Soxhlet		
	Concentration (ppm)			Concentration (ppm)		
	methylene-Cl:acetone	hexane: acetone	*p*-value	methylene-Cl:acetone	hexane: acetone	*p*-value
Toxaphene	40.10	30.03	0.0214	30.45	41.47	0.0680
DDE	5.58	3.41	0.0076	3.60	6.65	0.0039
DDD	3.49	3.41	0.8610	2.29	4.03	0.0096
DDT	9.25	5.12	0.0039	8.73	6.07	0.0640

Table 2-4. Comparison of ASE and Soxhlet for extracting toxaphene, DDT and its metabolites from highly contaminated soil. Methylene chloride/acetone (4:1, v/v) was used as the solvent.

	ASE		Soxhlet		
	Concentration (ppm)	RSD[f]	Concentration (ppm)	RSD	*p*-value
Toxaphene	40.10	0.095	30.45	0.052	0.0153
DDE	5.58	0.071	3.60	0.086	0.0024
DDD	3.49	0.094	2.29	0.178	0.0168
DDT	9.25	0.107	8.73	0.190	0.6618

Table 2-5. Comparison of ASE and Soxhlet for extracting toxaphene, DDT and its metabolites from moderately contaminated soil. Methylene chloride/acetone (4:1, v/v) was used as the solvent.

	ASE		Soxhlet		
	Concentration (ppm)	RSD	Concentration (ppm)	RSD	*p*-value
Toxaphene	2.87	0.114	1.85	0.052	0.0241
DDE	3.88	0.131	4.93	0.086	0.0810
DDD	2.65	0.018	3.48	0.178	0.1048
DDT	10.23	0.013	10.46	0.190	0.8763

Table 2-6. Correlation of water content in the soil to extraction efficiency by Soxhlet and ASE. Methylene chloride/acetone (4:1, v/v) was used as the solvent.

	Soxhlet				ASE			
	toxaphene	DDT	DDE	DDD	toxaphene	DDT	DDE	DDD
ρ^{g}	-0.47	0.90	0.53	0.37	0.79	0.9	-0.47	-0.05
p^{b}	0.0820	<0.0001	0.0524	0.1396	<0.0001	<0.0001	0.0326	0.4506

[g] Spearman correlation coefficient.

[b] Probability of significant correlation; statistical significance for one-side test set at $p < 0.05$.

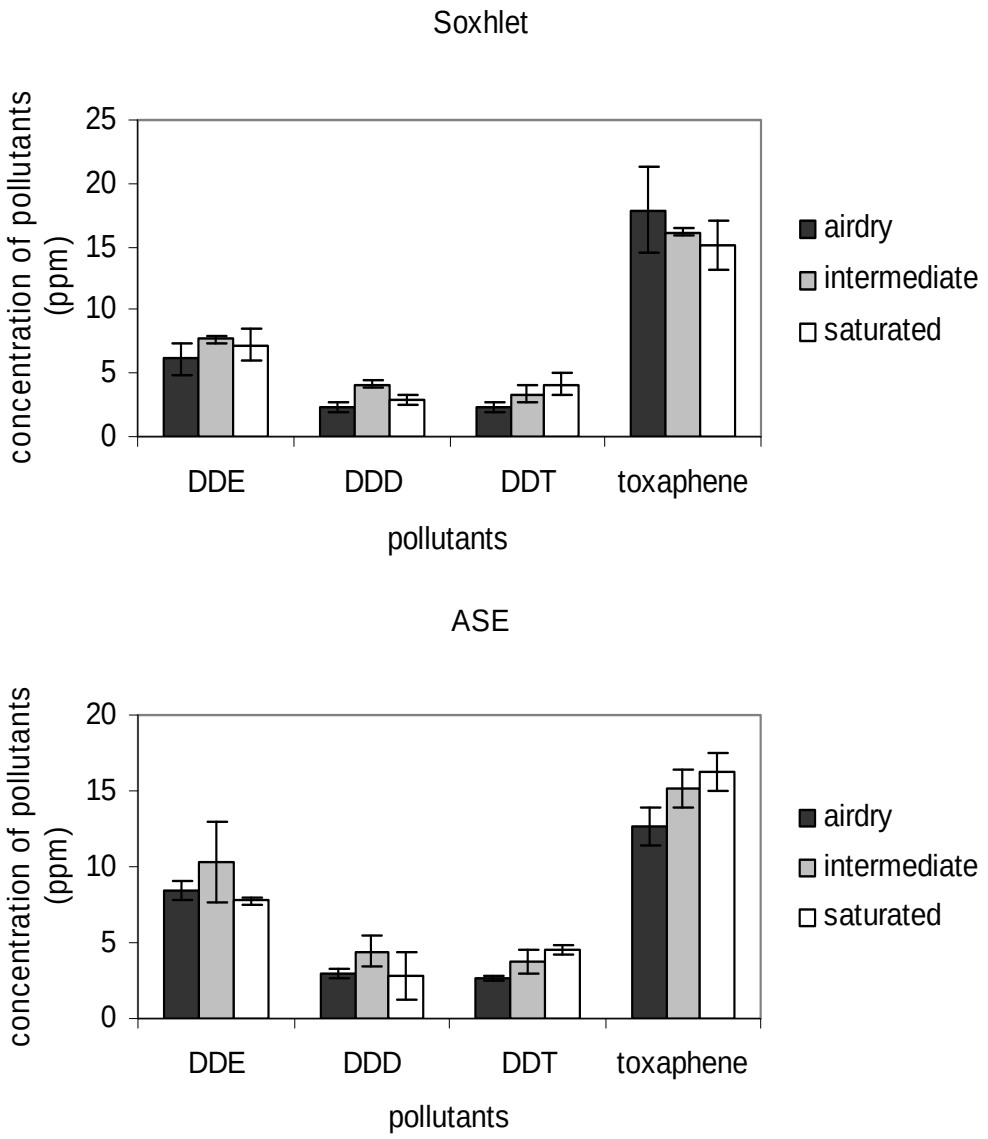

Figure 2-1. Effect of water content on the average means of extracted pesticides in the intermediate level of contaminated soils. Methylene chloride/acetone (4:1, v/v) was used as the solvent.

CHAPTER 3
BIODEGRADATION OF TOXAPHENE, DDT, AND ITS METABOLITES IN MICROCOSMS

Toxaphene is a low volatile organochlorine pesticide consisting of hundreds of chlorobornanes and other bicyclic compounds. Following the ban of toxic 1, 1, 1-trichloro -2,2-bis(4-chlorophenyl)ethane (DDT), toxaphene became one of the world's most commonly used pesticides in agriculture, used on crops such as cotton, grains, fruits, and vegetables (Howdeshell and Hites, 1996). The global use of toxaphene reached as high as 1,330,000 tons (Voldner and Li, 1993). The United States Environmental Protection Agency (EPA) banned toxaphene in 1982 due to its carcinogenic toxicity (Hill and McCarty, 1967; Nash and Woolson, 1967; Nash et al., 1973; Zeiger, 1987; Saleh, 1991), and high persistence under natural environmental conditions (10-14 yrs) (Nash and Woolson, 1967; Menzie, 1972). As a result of agricultural practices in the second half of the 20th century, Lake Apopka has become Florida's most polluted lake. During the winter of 1998-1999, over 600 birds associated with the lake died (2000). Most of them were white pelicans, wood storks, and great blue herons. Even though the reasons for the death of these birds are still unclear, high concentrations of organochlorine pesticides (OCPs) detected in the tissues of some of the pelicans are the primary factor or the cause of the death, these birds probably accumulated OCP by consuming contaminated fish.

In general, techniques for cleaning up OCPs contaminated environments include physical, chemical, and biological methods. Chemical remediation of toxaphene has been investigated (Clark et al., 2005); in particular, physico-chemical treatments by Fe(II)/Fe(III) redox coupling (Khalifa et al., 1976; Williams and Bidleman, 1978) have been applied to clean up toxaphene contamination. In situ bioremediation is an attractive strategy for cleaning contamination of toxic OCP in soils. The metabolic versatility of indigenous microorganisms to metabolize and detoxify

toxic compounds, the low cost, and avoidance of secondary pollution, are attractive properties compared with chemical or physical remediation strategies. Bioremediation of DDT by anaerobic and/or aerobic microbes has been given a great deal of interest (Subbarao and Alexander, 1985; Bidlan and Manonmani, 2002; Chiu et al., 2004). Despite the complex structure (consisting of at least 670), toxaphene has been reported to be degraded under anaerobic conditions (Williams and Bidleman, 1978; Fingerling et al., 1996; Buser et al., 2000; Lacayo-Romero et al., 2006).

Reductive dechlorination is the first and limiting step in the metabolism of many OCP by anaerobic bacteria. Sulfate-reducing bacteria (SRB) are a group of microorganisms reportedly involved in reductive dechlorination of chlorinated pollutants, which may be either a cometabolic process (de Best et al., 1997; Drzyzga et al., 2001; Drzyzga et al., 2002) with the appropriate electron donor/acceptor (sulfate) or a metabolic process (Zwiernik et al., 1998; Fava et al., 2003; Zanaroli et al., 2006) using chlorinated chemicals as the alternative electron acceptor upon the depletion of sulfate.

The objective of this investigation was to investigate whether sulfate reducing conditions would promote the dechlorination of toxaphene and DDT by setting up two batches of experiments: one with low concentration of sulfate; and the other with a higher concentration of sulfate. At the same time, we compared degradation of the OCPs with important electron donors in dechlorination (de Best et al., 1997; Villemur et al., 2006), such as butyrate, propionate, formate, acetate, lactate, and hydrogen to find the best combination for degradation of the target OCPs.

Batch enrichment culture followed by isolation of pure culture is a widely used method to isolate biodegraders despite some limitations. Additional objectives of this study were to identify

the biodegraders and investigate whether pure culture or a bacterial consortium could be isolated that would metabolize toxaphene or DDT. Information gained from this study will be used to design large-scale mesocosm experiments that will provide a valuable test of principles for field scale treatment of contaminated areas of the north shore restoration area (NSRA).

Materials and Methods

Sampling

Two soil samples with different levels of contamination, termed moderate and high as described in Table 3-1, were used in this study. The moderately contaminated sample was from Lat 28˚ 42'38.213N, Long 81˚36' 24.836 W. The highly contaminated soil was initially excavated near the former Lust airstrip approximately 18 months prior to collection for this study, and kept in a large vat under shelter. These soils were placed in an ice cooler and transported to University of Florida campus in Gainesville. Immediately upon arrival, the soils were stored at 4°C until use. Total carbon and nitrogen content was determined in the Teaching Analytical Chemistry Laboratory of the Soil and Water Science Department, the University of Florida, using a TruSpec CN Elemental Determinator (LECO, St. Joseph, MI).

Microcosm

Microcosms were constructed from well-mixed highly contaminated soil. Five g of soil were added to each culture tube and mixed with 45 ml of an autoclaved mineral salts solution consisting of (g/L of distilled water): K_2HPO_4, 4.8; KH_2PO_4, 1.2; NH_4NO_3, 1; $CaCl_2 \cdot 2H_2O$, 0.025; $MgSO_4 \cdot 7H_2O$, 0.2; $Fe_2(SO_4)_3$, 0.001; resazurin, 0.001; pH was adjusted to 7.5. The medium was boiled for 5 minutes and then cooled under nitrogen gas. 200 mM anaerobic stock solution of acetate, lactate, formate, butyrate, and propionate was prepared by gassing the solution and then the headspace with nitrogen gas. Then, 2% cysteine-sodium sulfide and 5mL electron donor solution were added in to serum vials using sterile syringes, plus a 100 kPa H_2

and one control with only 100 kPa N_2 gas in the headspace. A sterile control microcosm was included for each electron donor microcosm to test the importance of each electron donor, and consisted of soil sterilized by three successive autoclavings and all components for each experimental replicate described above. Each microcosm was supplemented with a final of 20 ppm toxaphene (technical mix; Accustandard, New Haven, CT, USA) which was solved in hexane. The hexane was blown off with N_2 before the medium was added. Serum tubes were incubated in the dark at 30 °C without shaking. A second batch of enrichments similar to those described above was established, in additon 20 mg $MgSO_4 \cdot 7H_2O$ per liter was added to in the mineral salt solution. Microcosms were sacrificed after 25 days.

In order to establish OCP biodegradation and increase statistical power, three replicates were constructed using the moderately contaminated soil incubated for two months. The added study only tested three potentially promising electron donors, hydrogen, lactate, and acetate, plus one control. The medium components and procedure remained same.

OCP Determination

Extraction Procedure. OCP concentrations were determined in both soil and solution phases. OCPs in the aqueous phase and the portion adsorbed to the walls and lids of the microcosms were determined via extraction with hexane. Soils were separated from liquid via centrifugation at 8,000 xg for 30 minutes. All the soils were grinded and then passed through a 20 mesh sieve (nominal pore size, 850 micrometers) after three-day air dry. Surrogates solution of 50 ppb (Decachlorobiphenyl and TCMX, AccuStandard, Inc., USA) was spiked to samples prior to extraction.

A Dionex ASE 100 Accelerated Solvent Extractor (Sunnyvale, CA) was used to perform the extractions. Triplicates of soil samples were mixed with hydromatrix (Varian, Palo Alto, CA) in 2:1 proportion and extracted in a 34 ml stainless vessel using methylene chloride/acetone (4:1,

v/v) or hexane/acetone (1:1, v/v) as solvent. The cell was filled with the solvent and heated to 100 ºC. The static extraction was performed for 5 min, followed by flushing the vessel with 60% of the 34 ml cell of fresh solvent. Finally, the vessel was purged with nitrogen for 100 sec at 150 psi. The total amount of extraction solvent was about 50 ml, for carrying out one static cycle.

Cleanup and Analysis. All extracts were concentrated to near dryness by a gentle stream of N_2. Repeated this process twice to remove acetone and methylene chloride by adding hexane. The volume of extracts was measured in a glass graduated volumetric cylinder. At this point, the samples were split for analysis of toxaphene and DDT and its metabolites.

For analysis of DDT and its metabolites, one ml of extracts was passed through a florisil cartridge packed with approximately 500 mg of 200 μm Florisil (Varian, Palo Alto, CA). Nine ml of hexane:acetone (9:1, v/v) were used to rinse the column. The resulting eluent was concentrated to 1ml by an N_2 stream and then transferred to a glass GC vial (2 ml, Fisherbrand, Atlanta, GA) and crimped with a Teflon lined cap for subsequent analysis by gas chromatography.

Another one ml extract was purified by sulfuric acid treatment for further analysis of toxaphene. Equal amounts of concentrated sulfuric acid and sample were mixed in a 10 mL vial. The vial was capped and vortexed for 1 min. Phases were allowed to separate, and the upper (organic) phase transferred to a GC vial for analysis.

Extracts were analyzed with a gas chromatograph (Shimadzu GC-17A) equipped with a ^{63}Ni electron capture detector (ECD). The column was a RTX-5 (0.25 mm ID × 15 m long; Restek Corporation, Bellefonte, PA, USA). In most cases of DDT analyses, 250 ºC over temperature was used, however toxaphene was considered as first concern such that we applied the program which Paan and Chen developed (Pearson et al., 1997; Paan et al., 2006). The

temperature was programmed as follows: 60 ºC, 3.0 ºC/min to 70 ºC for 1 min, 10 ºC/min to 130 ºC for 3 min, 10 ºC/min to 160 ºC, 20 ºC/min to 200 ºC. A calibration curves with 6 points bracketing the experimental concentrations was used for both analyses. The concentration calculation followed EPA 8000B and methods used by Pearson and Paan (Pearson et al., 1997; Paan et al., 2006).

Isolation of Biodegraders

The mineral basal medium used for enrichment (MBME) of degraders consisted of (per L of distilled water) 5.455 g Na_2HPO_4; 0.675 g KH_2PO_4; 0.25 g NH_4NO_3; 0.029 g $Ca(NO_3)_2 \cdot 4H_2O$; 0.2 g $MgSO_4 \cdot 7H_2O$; 0.001g resazurin; and 1mL of trace mineral solution. The trace mineral solution contained (g/L of distilled water): 1 g $Fe_2(SO_4)_3$; 1 g $MnSO_4 \cdot H_2O$; 0.366 g $CuSO_4 \cdot 5H_2O$; 0.25 g $Na_2MoO_4 \cdot 2H_2O$; 0.1 g H_3BO_3 and 5 ml of concentrated H_2SO_4. pH was adjusted to 7.5. Sodium sulfide (0.5%) was used as the anaerobic reductant instead of cysteine-sodium sulfide to avoid the interference of Cl in measurement of dechlorination. Technical toxaphene or DDT (AccuStandard, New Haven, CT, USA) was absorbed to the bottom of the serums to a final concentration of 20 ppm of each. The medium was prepared anaerobically as described above.

Dechlorination of Toxaphene and DDT. Five g moderately and highly contaminated soils were added to 45 ml of MBME using lactate as the electron donor. After one month, 5 ml of the enriched medium were transferred to fresh MBME (45 ml). A total of 5 transfers after the first transfer were made every 14 days before the genetic analysis and chloride ion measurement were conducted. Ten ml of the solution in the serum was centrifuged and filtered through a 0.45 μm syringe filter (Nalge, Rochester, NY) after 5, 8, 11, 14, and 17 days respectively. Chloride ion concentrations were measured by ion chromatography using a Dionex Model LC20 equipped with an AS14 column and an ECD50 conductivity detector (Dionex, Sunnyvale, CA), using 3.5 mM sodium carbonate/1.0 mM sodium bicarbonate as eluent.

Roll Tube Experiment. A modified Hungate roll tube technique was used to isolate potential OCP degraders. Serum tubes, rather than screw capped tubes, of MBME with 1.6% agar were utilized, plus 100kPa N_2 gas. Solid medium was inoculated with 0.2 ml suspension of enriched medium, and was then incubated at 30 °C in the dark until well-formed colonies were observed. Colonies were picked up taking into account morphological differences and transferred to the liquid fresh medium. This process was repeated several times to make sure that only one single strain tolerating or toxaphene had been picked.

DNA Sequencing Analysis

Nucleic acids were extracted with UltraClean Soil DNA kits (Mobio, Solana Beach, CA). Following the manufacturer's instruction, 1 ml of culture was used. PCR for 16S rRNA genes was firstly conducted with SRB primers designed by Wagner et al. (Wagner et al., 1998), which amplifies a 1.9-kb fragment of the DSR gene. This set consists of primers DSR1F (5'-ACSCACTGGAAGCACG-3') and DSR4R (5'-GTGTAGCAGTTACCGCA-3'). And then the universal primer set 27F/1492r (Lane, 1991) was used. One μl of diluted DNA solution was mixed with 10 μl of HotStarTaq Master Mix (Qiagen, Valencia, CA), 7 μl of H_2O provided with Master Mix, 1 μl of each primer (10 pmol/μl). PCR amplification was carried out in a GeneAmp PCR system 2400 (Perkin-Elmer Applied Biosystems, Norwalk, CT). The initial enzyme activation and DNA denaturation was performed at 95°C, followed by 35 cycles of 30s at 95°C, 30s annealing at 58°C, and 30s extension at 72°C for amplification of 16S rRNA genes. A 7 min final extension was performed at 72°C. The amplicons were electrophoresed through 1% agarose gels in TAE buffer.

Fresh PCR amplicons were pretreated and transformed into chemically competent *E. coli* TOP 10F' cells following the manufacturer's instructions (Invitrogen, Carlsbad, CA). Twenty

individual colonies of *E. coli* were picked up for screening by direct PCR amplification using T7 and SP6 primers and electrophoresed as described before. Restriction Fragment Length Polymoprhism analyses performed with the restriction enzyme *Hha*1 were used to classify clones in order to reduce the number of clones sequenced. Digests were electrophoresed through 2% agarose. Selected clones were sequenced at the DNA Sequencing Core Laboratory at the University of Florida using the 27F primer. Phylogenetic analysis based on 16S rDNA sequences was performed with the aid of PHYLIP (version 3.6a2; J.L. Felsenstein, Department of Genetics, University of Washington, Seattle, WA).

Statistical Analysis

One-way ANOVA was used to test the difference among treatment groups. Pairwise comparisons for differences in concentration means between different soil characteristics, extraction methods, and extraction solvents were done by Tukey's Studentized test, while correlations were tested by Spearman and Pearson using SAS version 9.1 (SAS Institute, Cary, NC). Contrast for different treatments for stimulating degradation between sulfate concentration, sterile control, and electron donors were tested using SAS version 9.1 (SAS Institute, Cary, NC). The null hypothesis was rejected at $p<0.05$, thus the probability of observing no difference among the pair wise is less than 5%.

Results and Discussion

Degradation of Toxaphene, DDT and its Metabolites

Our initial hypothesis was that toxaphene, and DDT and its metabolites at NSRA may be anaerobically degraded under sulfate reducing conditions. A preliminary screening of soils from a flooded area of the NSRA indicated sulfate reduction occurred, confirming the presence of active SRB (data not shown).

Under anaerobic conditions, soils containing high levels of recalcitrant contaminants are typically enriched with those microorganisms capable of utilizing the contaminant as either nutrient or terminal electron acceptor, such that higher levels of biodegradation may be expected in these soils than in moderately contaminated soils. Microcosms were established with a range of electron donors, including acetate, lactate, formate, butyrate, propionate, and H_2. These electron donors are major fermentation products that are used by a number of anaerobic bacteria, including those SRB (Villemur et al., 2006) that may dechlorinate organic compounds. Acetate and lactate are used by relatively few anaerobic bacteria, and almost exclusively by SRB when sulfate is in abundance (de Best et al., 1997; Villemur et al., 2006). H_2 is a very electron rich donor that controls a variety of anaerobic processes, although SRB are among the primary utilizers under sulfate reducing conditions. Experimental controls included live soil in which no exogenous electron donors were added to test the importance of individual electron donors, and a sterile autoclaved control for each of the electron donors. The sterile control was used as reference for degradation.

Two concentrations of sulfate were tested, including 200 mg $MgSO_4 \cdot 7H_2O$ per L medium and 20 mg $MgSO_4 \cdot 7H_2O$ per L medium. The higher sulfate concentration was considered sufficient to maintain an actively growing population of SRB with the concentration of electron donors provided. The lower level of sulfate was intended to provide sufficient sulfate to enrich SRB, but not to sustain the growth of SRB with the level of electron donors provided. If SRB capable of utilizing OCP as terminal electron acceptors are present, they might be expected to switch to OCP once sulfate became limiting.

The highest levels of degradation relative to sterile controls were observed for toxaphene (Fig. 3-1), with relatively little degradation observed for DDT, DDE, and DDD (Fig. 3-2 – Fig.

3-4). Very little toxaphene was observed in medium solution or in hexane extracts of the bottles (data not shown), suggesting that hexane was not an efficient extractant of toxaphene from the glass microcosm bottles used in this study.

Statistically significant differences were detected in the concentrations of toxaphene for the 200 mg/L $MgSO_4 \cdot 7H_2O$ ($p<0.001$) amended and 20 mg/L $MgSO_4 \cdot 7H_2O$ ($p<0.0001$) amended treatments compared to the autoclaved soil control, using the least squared means test adjusted to Tukey. Over all, there was no significant difference between the two levels of $MgSO_4$, which means we do not have the evidence that SRB were responsible for the significant decrease in the concentrations of the tested OCP. Among the two different levels of sulfate supplies, however, the choice of electron donors resulted in statistically significant difference in promoting biodegradation. Four electron donors among the six tested ones significantly ($p<0.05$) stimulated the degradation of toxaphene. With 20 mg/L $MgSO_4 \cdot 7H_2O$ supplied media, toxaphene was degraded in the systems with hydrogen, acetate, and lactate as the electron donors, in contrast to the sterile soil. Butyrate had a significant ($p<0.05$) role in the decrease in the toxaphene concentration with the supplement of 200 mg/L $MgSO_4 \cdot 7H_2O$. Formate and propionate were less effective. This observation may be explained because acetate, butyrate, and lactate may be utilized more efficiently by some secondary fermenters than formate and propionate through syntrophy to produce hydrogen, leading to halorespiration with hydrogen as the electron donor. Furthermore, hydrogen amended to the headspace of the microcosm system was directly used as the electron donor for halorespiration. The lack of significant evidence between the amended electron donors and live soil control showed that the natural attenuation of toxaphene occurred by the fact that 51% and 39% of toxaphene was reduced even in the control sets with low and high level of sulfate media. The effect of sulfate was particularly important with lactate and H_2,

in which degradation was higher in low sulfate than in high sulfate. Seemingly, sulfate depletion is necessary for observing the degradation. This observation is consistent to microbial reductive dechlorination of polychlorinated biphenyls (PCBs) by SRB according to Zanaroli (Zanaroli et al., 2006). However, in the presence of butyrate, degradation was higher in high sulfate than in low. Degradation of toxaphene under limiting sulfate conditions with H_2 and lactate raised the possibilities that sulfate inhibited toxaphene degradation because, as initially hypothesized, sulfate was used preferentially as the terminal electron acceptor, or that SRB competed with another group responsible for toxaphene degradation. However, the duplicates exhibited a large relative standard deviation (RSD). For example, even though acetate, lactate, and butyrate were observed to stimulate some microorganisms to degrade toxaphene, the experimental system exhibited RSDs as high as 0.905, 1.315, and 0.632, respectively (Fig. 3-1).

Significant (p<0.05) degradation of DDE was also observed in the 20 mg/L $MgSO_4 \cdot 7H_2O$ group compared to the high sulfate set and sterile control. Among the six electron donors, formate was the only effective compound to be utilized to reduce DDE. In contrast to the control, 44% of DDE disappeared with the existence of formate. Seemingly adequate sulfate did not help reduce DDE (Fig. 3-3) since the average concentration of DDE for all six electron donors plus control was similar to that in the sterile soil set. Some bacteria favored formate and propionate as electron donors to attack DDT. Formate in the 20 mg/L $MgSO_4 \cdot 7H_2O$ medium most efficiently stimulated bacteria that can destroy DDT in comparison with other electron donors when treated by low level of sulfate (Fig. 3-2), which the concentration of DDT decreased 19%. In 200 ppm sulfate treated group propionate played a role in degrading DDT by 20%. There was no statistical evidence to show the degradation of DDD (Fig. 3-4) in all three-treatment groups with various donors after 25-day incubation. Not surprisingly, the high RSD effected the confidence in

determination of DDD and DDT. RSD for DDT ranged from 0.001 to 0.494, while RSD for DDD ranged from 0.006 to 0.244.

A limitation of the experimental design was that only two replicates were included for each electron donor and control, and the time of incubation was relatively short for an anaerobic incubation of this type. Two, rather than three, replicates were used in order to reduce the number of analyses, and hence time required for gas chromatography. The limited number of replicates made statistically significant differences between treatments difficult to observe (Figs. 3-1 – 3-4). Microcosm studies were repeated with three replicates and a two-month incubation period for those treatments that appeared promising.

Another potential limitation of this experiment was that the soil used was excavated approximately 18 months prior to our collection, during which time the soil became air dried. The numbers of non-spore forming microorganisms may have been reduced, thereby likely reducing initial degradation rates. Repeat of this study should use fresh soil that has high endogenous levels of OCP.

Establishment of Degradation of Toxaphene, DDT and its Metabolites

Degradation of toxaphene, DDT and its metabolites was observed to some extent in previous microcosms with H_2, lactate, or butyrate as electron donors. However, the degradation needs to be established by using moderately contaminated soils, the aged OCP contaminated soils with fresh indigenous microorganisms. Additionally, the anaerobic microcosms were stopped after incubating two month for three replicates, thereby gaining greater statistical power. In the second study, anaerobic degradation of OCPs in soils was established (Fig. 3-5 – Fig. 3-8).

After two months of incubation, statistically significant ($p<0.01$) degradation of toxaphene was observed in the non sterile groups (Fig. 3-5). In agreement with the exploratory study,

overall toxaphene degradation was not distinguishable between the low and high levels of sulfate. Lactate and butyrate were capable of stimulating toxaphene degradation at the statistical level $p<0.10$ when the sulfate concentration was relatively high (200 mg/L $MgSO_4 \cdot 7H_2O$). On the contrary, another study of aged and freshly toxaphene contaminated soils in anaerobic bioreactors indicated that lactic acid interfered with the biodegradation (Lacayo-Romero et al., 2006). They reported that toxaphene congeners showed a significantly lower degradation rate after the addition of lactic acid. The structure of microbial community or soil conditions in their investigation and our soils may contribute to such a difference. As discussed above, lactate and other electron donors such as butyrate can not be used by some dehalogenating bacteria such that they have to be fermented to hydrogen through syntrophy. Generally, SRB or other microorganisms participate in such fermentation when sulfate is not sufficient (Drzyzga and Gottschal, 2002). Hydrogen is the preferred electron donors by dechlorinaters. Once dechlorinaters obtain hydrogen, replacement of the chloride of OCP with hydrogen is accomplished. High concentration of sulfate, for the other, will make SRB outnumber dehalogenating bacteria and stop the dehalogenation. In the present study, abundant sulfate salt (200 mg/L $MgSO_4 \cdot 7H_2O$) allowed the consumption of lactate or butyrate to hydrogen, thereby promoting the disappearance of toxaphene. Possibly, other groups of microorganisms used lactate or butyrate as the electron donor and dehalogenating microbes utilized the produced hydrogen to yield energy by preferring dehalogenation, more efficient energy yielding approach, to sulfate reduction (Dolfing and Harrison, 1992). When hydrogen is provided artificially, dehalogenating bacteria would show higher degradation. In our study, we supplied with hydrogen gas, therefore, more degradation was detected with highly statistical significance ($p<0.05$), for each contrast of 200 mg/L $MgSO_4 \cdot 7H_2O$ group vs sterilized 200 mg/L

$MgSO_4 \cdot 7H_2O$ group and 20 mg/L $MgSO_4 \cdot 7H_2O$ group vs sterilized 200 mg/L $MgSO_4 \cdot 7H_2O$ group. Intrinsic disappearance of toxaphene was confirmed in the control systems of both high and low sulfate medium with the statistical evidence ($p<0.05$). With three replicates, RSDs were appreciably uniform and acceptable (within 0.2).

In the second study, RSDs were relatively low and the degradation was confirmed more precisely for DDT and its metabolites. DDT significantly disappeared in non sterile groups. Compared with the sterile group, the concentrations of DDT in the groups treated with 20 ppm and 200 ppm sulfate differed from the sterile control with strong statistical evidence ($p<0.0001$). In both non sterile groups, DDT was degraded ($p<0.05$) notably in all the three chosen electron donors. With limited supplement of sulfate (20 mg/L $MgSO_4 \cdot 7H_2O$), bacteria degraded DDT with the highest efficiency (Fig. 3-8), 47% reduction utilizing hydrogen as the electron donor in contrast to other electron donors. This finding supported our contention that the dechlorinating process can be stimulated under sulfate reducing condition (Zwiernik et al., 1998; Fava et al., 2003; Zanaroli et al., 2006). Additionally, DDT in moderately contaminated soil without any electron donor was degraded intrinsically, an average of 16% and 29% of decrease in the concentration in comparison with the sterile group. Unlike in the highly contaminated soil, DDD was observed degraded in the moderately contaminated soil after incubated for two month. The treatment factors such as the level of sulfate salt, different electron donors, and sterilization had statistically significant effects ($p<0.0001$) on DDD biodegradation. Within 200 ppm sulfate salt group, all these three electron donors participated in the statistically significant ($p<0.05$) disappearance of DDD in comparison with sterile control; degradation even happened greatly in control set. Although intrinsic degradation of DDD was observed in treatments of 20 mg/L $MgSO_4 \cdot 7H_2O$, the applied electron donors significantly promoted the degradation (Table 3-2).

Microcosms in this system seemed to be activated to the highest extent, which degraded 45% of DDD. In contrast to the first set, we didn't observe a significant decrease but an increase in the concentration of DDE in some serum bottles. Because DDT was considered dechlorinated to DDE, accumulation of DDE indicated the degradation of DDT.

Isolation of Toxaphene Degraders

Dechlorination of Toxaphene. In order to characterize the OCPs biodegrader(s) in the NSRA contaminated soils, a batch of enrichment experiments were established. After three months with five transfers, chloride ion was determined to confirm the occurrence of biodegradation in the enrichment media. During the test period of experiment, the concentration of chloride ion increased in both serum bottles enriched from highly and moderate contaminated NSRA samples (Figs. 3-9 and 3-10). But the dynamics of released chloride differed in two different levels of contamination. In both systems, 5 ppm of chloride ion was found following five days after the transfer, whereas bacteria from moderate soils were more active to decompose the organochlorine chemical. Until the 11^{th} day, 15 ppm of chloride ion was produced in the serum bottles originated from the highly contaminated sample. But 18 ppm of chloride was determined only after 8 days, and 28 ppm chloride appeared at the point of 11 days in moderate soil. In contrast to moderately contaminated soil, only 25 ppm of chloride was released after 17-day incubation in high soil. The production of Cl^- accompanying the growth of the bacteria strongly suggested the mechanism of reductive dechlorination of toxaphene (Khalifa et al., 1976; Fingerling et al., 1996; Buser et al., 2000).

Although DDT showed a degradable potential in the previous microcosm study, there was no detectable Cl^-. Possibly, the DDT degrading consortium was lost somewhere in the process of those five transfers. Furthermore, the turbidity of the medium that revealed the growth of bacteria suggested that lactate was the preferred carbon and energy source to DDT for the

bacteria in the medium. We, thereby, drew a conclusion that the strains which would be isolated from the solution might not degrade DDT with the presence of lactate.

Roll Tube, 16S rDNA and Sequencing. In this study, anaerobic roll tube experiments were set up to separate the colonies and transferred to the limited medium with toxaphene as the sole substrate and lactate as the electron donor. Each of very small colonies that developed were picked and cultured in the limited liquid medium. The turbidity in the limited medium was observed and 16S rDNA was sequenced. However, the sequencing analysis still revealed that different species existed in the medium. In addition, the second or third transfer of the culture to the fresh liquid medium with toxaphene and lactate did not show any growth. It seemed that a consortium participated in the degradation of toxaphene. As a powerful tool to isolate the pure strains roll tube is, there are a few limitations. Problems coming with this method include: first, real environmental conditions are very different from those in the enrichment experiments, such as the redox potential; second, the concentration used as the source of carbon and energy to isolate the degraders is usually higher than in the environment; third, many pesticides are shown to be attacked by consortia instead of pure cultures.

In order to identify the bacteria who were capable to grow in the presence of toxaphene and dechlorinate toxaphene, their 16S rRNA genes were sequenced and compared to others in GenBank. As specific primers for SRB were tried to isolate the degraders, no DNA was detected with this type of primers. A range of strains of bacteria was characterized from enrichment cultures when using the universal primers. Phylogenetical analysis demonstrated that some sequences related to the genus *Citrobacter* with 99.9% sequence similarity were most abundant in the clone libraries (Fig. 3-11); others were closely related to the gram positive genus of *Sporomusa,* with 98% similarity (Fig. 3-12). *Citrobacter* species have been previously reported

to tolerate or accumulate heavy metals such as lead (Levinson and Mahler, 1998), cadmium (Montgomery et al., 1995), copper (Williams et al., 1993; Pathak and Gopal, 1994; Pickup et al., 1997) and nickel (Pickup et al., 1997). Additionally, anaerobic degradation a range of organic pollutants such as phenol (Kanekar et al., 1999), biphenyl (Grishchenkov et al., 2002), TNT (Li et al., 1989), 2,4-dichlorophenol (DCP), 2,4,6-trichlorophenol (TCP) and pentachlorophenol (PCP) (Martinez et al., 2000) has been reported to abscribe to *Citrobacter* species. For the first time, this work demonstrated the potential biodegradation of toxaphene at the microbial level and related *Citrobacter* to toxaphene degradation. And also, *Sporomusa ovata* was reported to reductively dechlorinate perchloroethene (PCE) to trichloroethylene (TCE) (Terzenbach and Blaut, 1994) as the sole product with methanol as an electron donor. The reversible inhibition with propyl iodide suggested that Corrinoids in *S. ovata* were involved in the dechlorination reaction of PCE to TCE. The mechanisms that the bacteria naturally occurred in the studied enrichment culture used to dechlorinate toxaphene are attempted to demonstrate. Recently, a white-rot fungus, *Bjerkandera* sp., (Romero et al., 2006) has been reported to degrade toxaphene when supplied with wood chips, wheat husk or cane molasses as cosubstrates in batch culture experiments. Another strain which has been isolated from the aged contaminated soil to degrade toxaphene was analyzed to be related to *Enterobacter cloacae* (Lacayo-Romero et al., 2005) and characterized to be a facultative anaerobic, Gram-negative heterotrophic bacterium. However, both investigations did not reveal the real mechanisms for these microorganisms to use for toxaphene degradation.

Table 3-1. Soil characteristics for moderately and highly contaminated soils

	Moderate	High
Total organic carbon (TOC %)	41.34	18.07
Total organic nitrogen (TON %)	3.71	1.59
C/N	11.14	11.36
Toxaphene (ppm)	16	48
DDE (ppb)	2000	2400
DDD (ppb)	540	1600
DDT (ppb)	350	1200

Table 3-2. Statistical contrast analyses of roles of various electron donors in biodegradation of OCPs in microcosms after two-month incubation

OCPs	Electron donors	Test	Estimate	*p*-value
Toxaphene	Lactate	200 ppm[a] vs S200 ppm[b]	-1.64	0.0872
		20 ppm[c] vs S200 ppm[b]	-1.15	0.2027
	H_2	200 ppm[a] vs S200 ppm[b]	-2.15	0.0317
		20 ppm[c] vs S200 ppm[b]	-2.32	0.0239
	Butyrate	200 ppm[a] vs S200 ppm[b]	-2.58	0.0537
		20 ppm[c] vs S200 ppm[b]	-0.25	0.8077
DDT	Lactate	200 ppm[a] vs S200 ppm[b]	-1.67	0.0014
		20 ppm[c] vs S200 ppm[b]	-1.70	0.0013
	H_2	200 ppm[a] vs S200 ppm[b]	-1.58	0.0002
		20 ppm[c] vs S200 ppm[b]	-2.23	<0.0001
	Butyrate	200 ppm[a] vs S200 ppm[b]	-1.95	0.0059
		20 ppm[c] vs S200 ppm[b]	-1.12	0.0534
DDD	Lactate	200 ppm[a] vs S200 ppm[b]	-2.20	0.0008
		20 ppm[c] vs S200 ppm[b]	-2.26	0.0007
	H_2	200 ppm[a] vs S200 ppm[b]	-1.65	<0.0001
		20 ppm[c] vs S200 ppm[b]	-2.67	<0.0001
	Butyrate	200 ppm[a] vs S200 ppm[b]	-2.69	0.0019
		20 ppm[c] vs S200 ppm[b]	-1.57	0.0215
DDE	Lactate	200 ppm[a] vs S200 ppm[b]	0.21	0.5083
		20 ppm[c] vs S200 ppm[b]	-0.49	0.1495
	H_2	200 ppm[a] vs S200 ppm[b]	-0.10	0.6091
		20 ppm[c] vs S200 ppm[b]	-1.29	0.0004
	Butyrate	200 ppm[a] vs S200 ppm[b]	0.50	0.2085
		20 ppm[c] vs S200 ppm[b]	0.60	0.1441

a: medium with 200 mg/L $MgSO_4.7H_2O$

b: medium with 200 mg/L $MgSO_4.7H_2O$ and sterile soil

c: medium with 20 mg/L $MgSO_4.7H_2O$

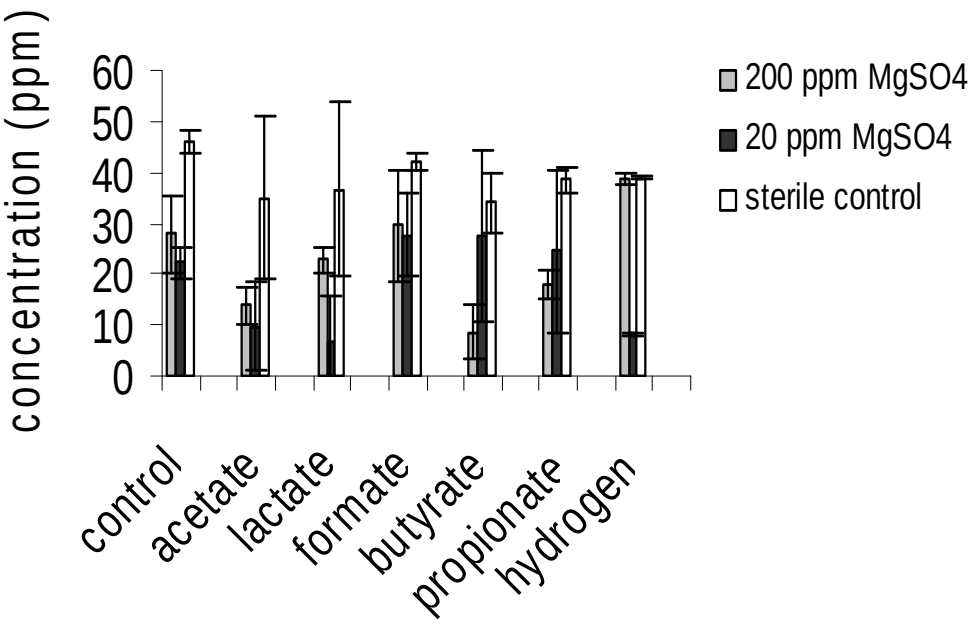

Figure 3-1. Degradation of toxaphene in microcosms containing different electron donors and sulfate concentrations.

Note: $MgSO_4$ was added as the form of $MgSO_4.7H_2O$. Error bars represent +/- one standard deviation.

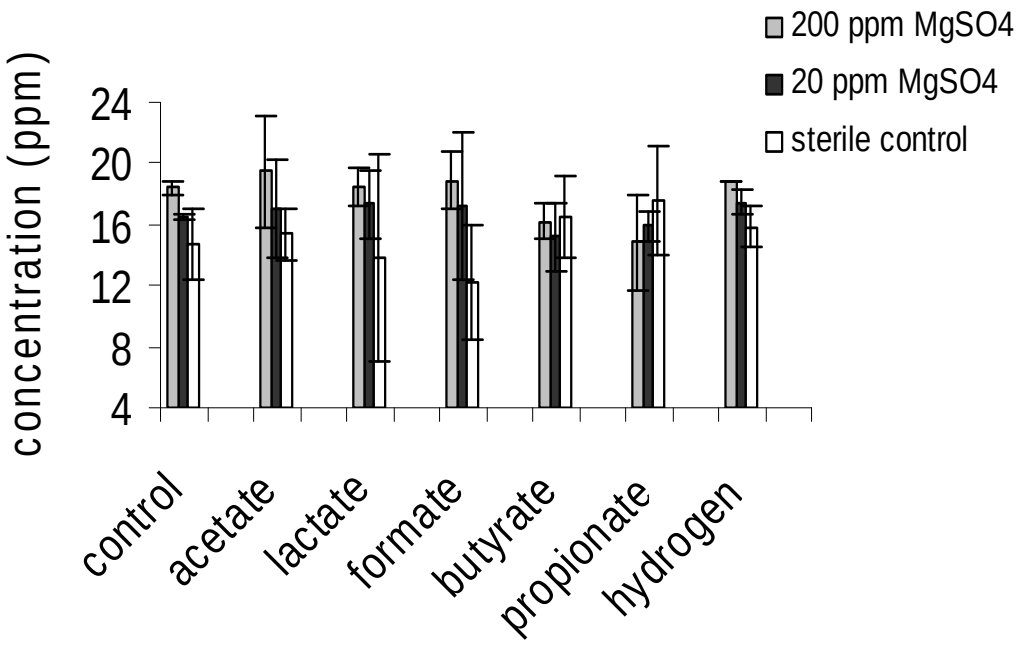

Figure 3-2. Degradation of DDT in microcosms containing different electron donors and sulfate concentrations

Note: $MgSO_4$ was added as the form of $MgSO_4.7H_2O$. Error bars represent +/- one standard deviation.

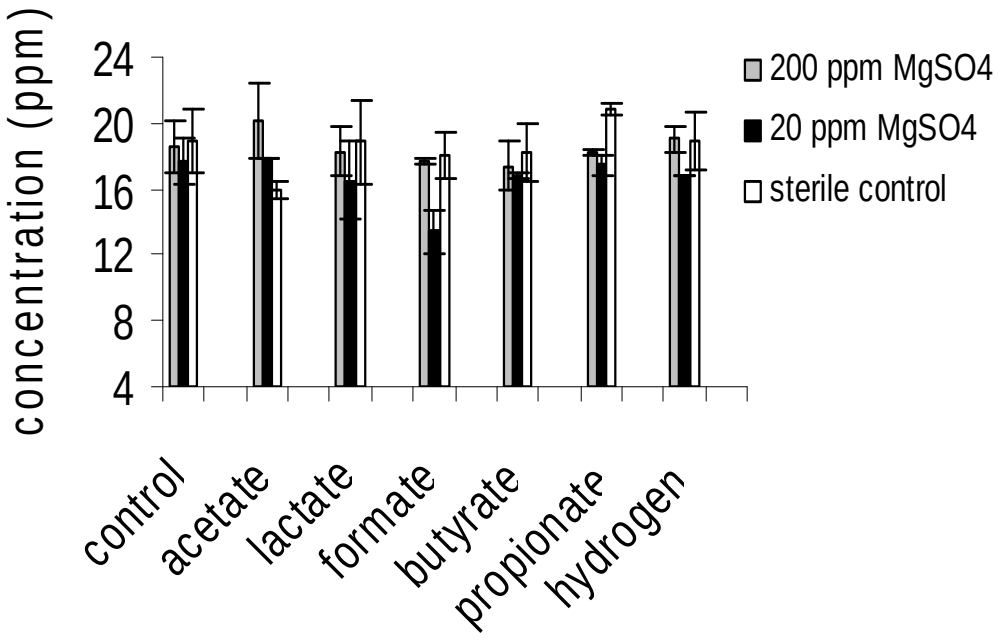

Figure 3-3. Degradation of DDE in microcosms containing different electron donors and sulfate concentrations

Note: $MgSO_4$ was added as the form of $MgSO_4.7H_2O$. Error bars represent +/- one standard deviation.

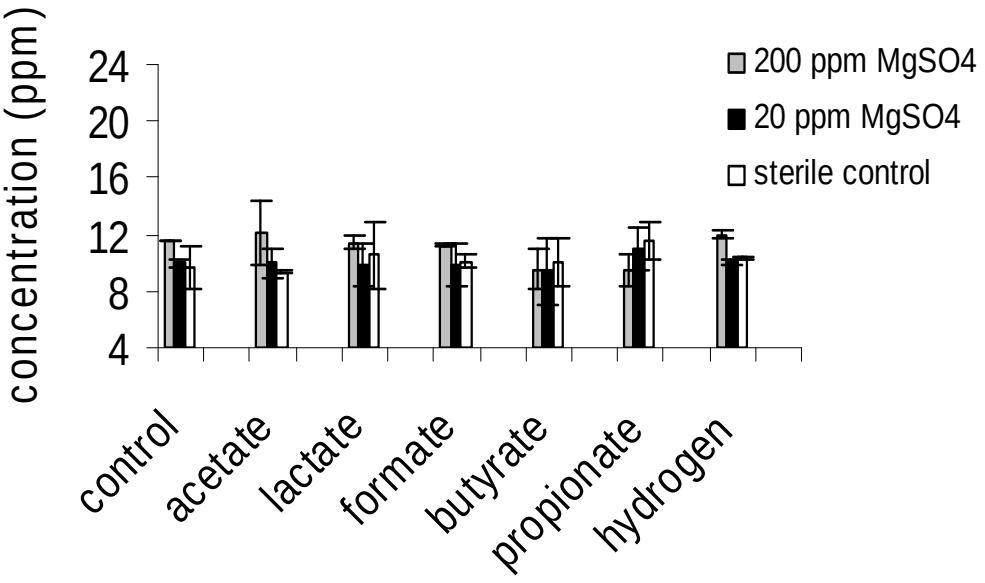

Figure 3-4. Degradation in DDD in microcosms containing different electron donors and sulfate concentrations

Note: $MgSO_4$ was added as the form of $MgSO_4.7H_2O$. Error bars represent +/- one standard deviation.

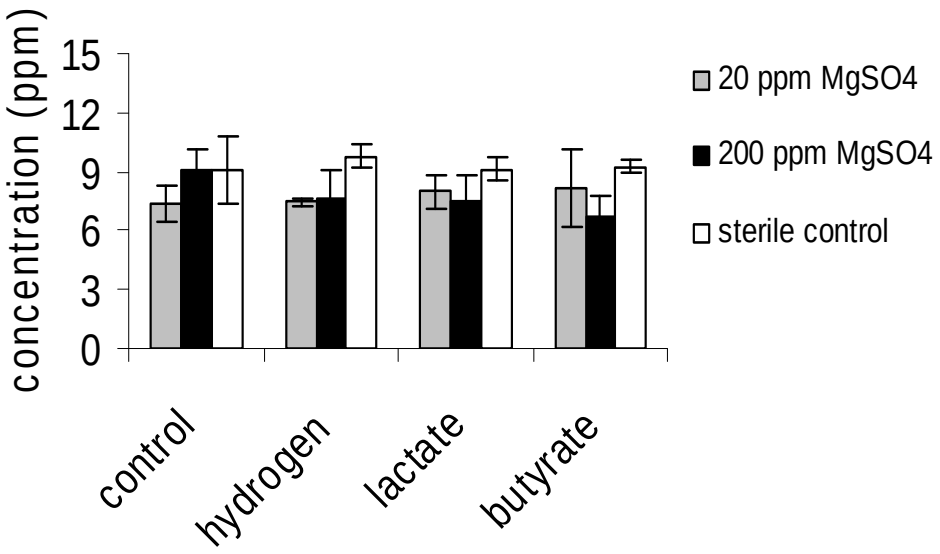

Figure 3-5. Degradation of toxaphene in microcosms containing different electron donors and sulfate concentrations

Note: $MgSO_4$ was added as the form of $MgSO_4.7H_2O$. Error bars represent +/- one standard deviation.

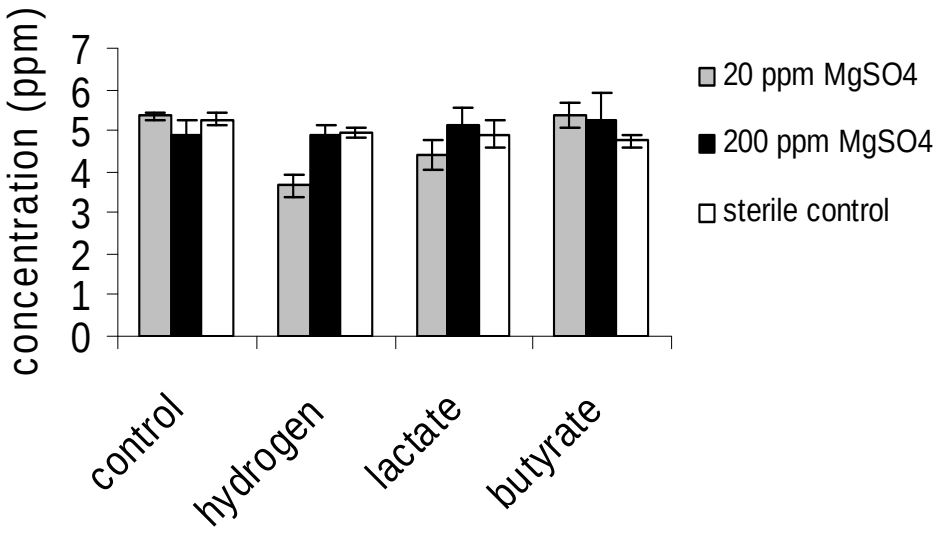

Figure 3-6. Degradation of DDE in microcosms containing different electron donors and sulfate concentrations

Note: $MgSO_4$ was added as the form of $MgSO_4.7H_2O$. Error bars represent +/- one standard deviation.

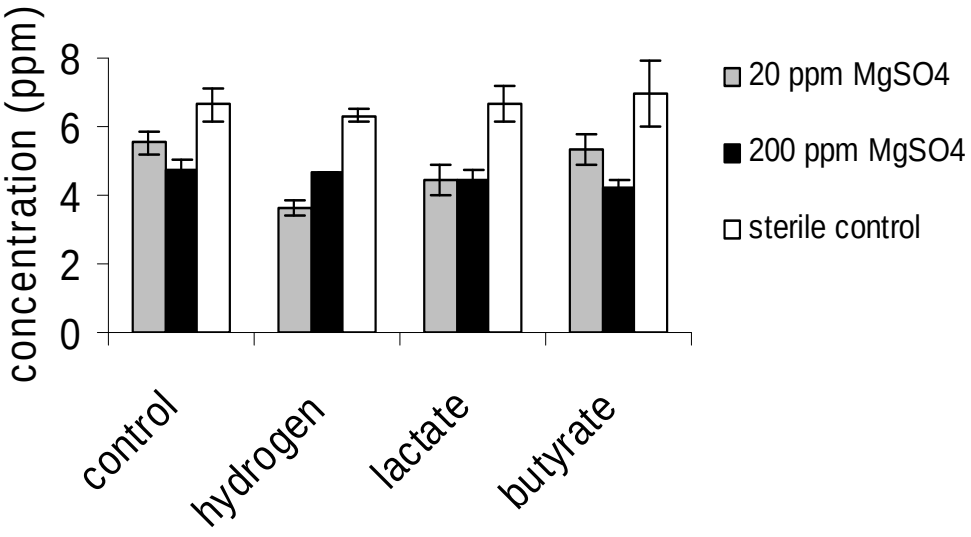

Figure 3-7. Degradation of DDD in microcosms containing different electron donors and sulfate concentrations

Note: $MgSO_4$ was added as the form of $MgSO_4.7H_2O$. Error bars represent +/- one standard deviation.

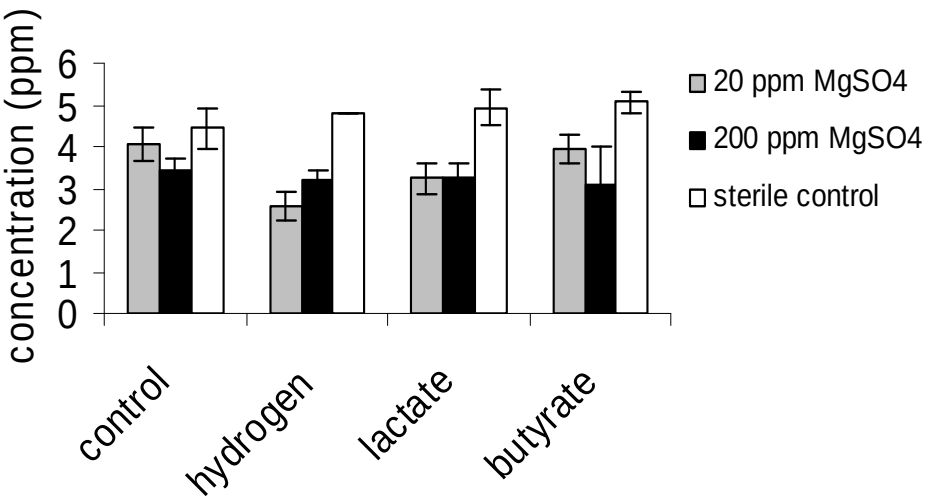

Figure 3-8. Degradation in DDT in microcosms containing different electron donors and sulfate concentrations

Note: $MgSO_4$ was added as the form of $MgSO_4.7H_2O$. Error bars represent +/- one standard deviation.

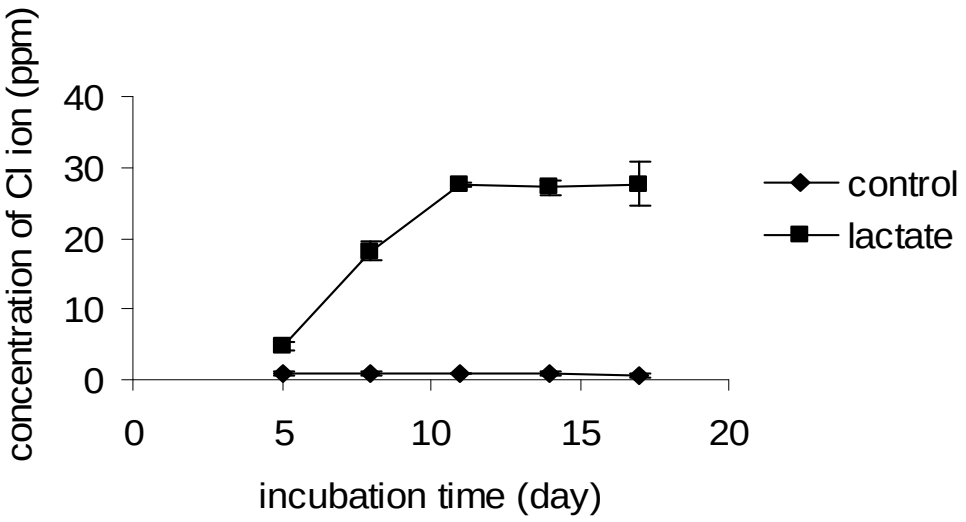

Figure 3-9. Dechlorination of toxaphene with lactate as the electron donors enriched from moderately contaminated soils

Note: Error bars represent +/- one standard deviation.

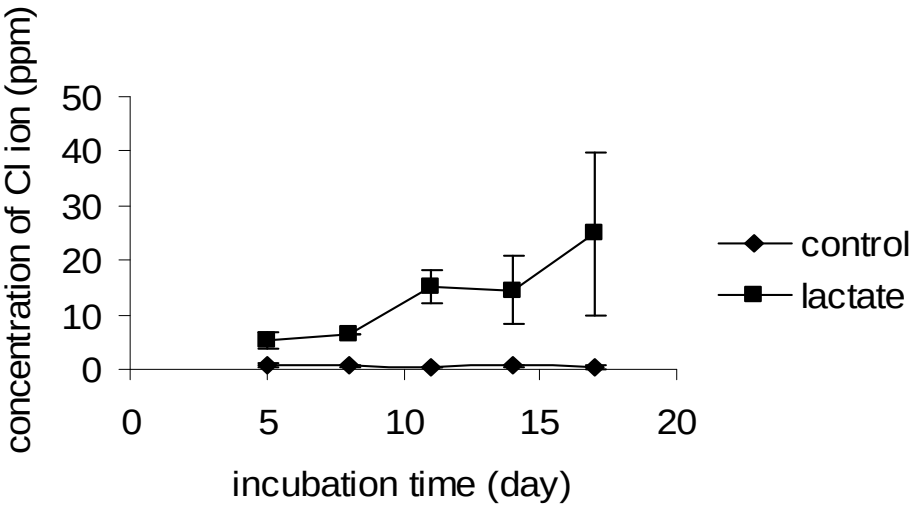

Figure 3-10. Dechlorination of toxaphene with lactate as the electron donors enriched from highly contaminated soils

Note: Error bars represent +/- one standard deviation.

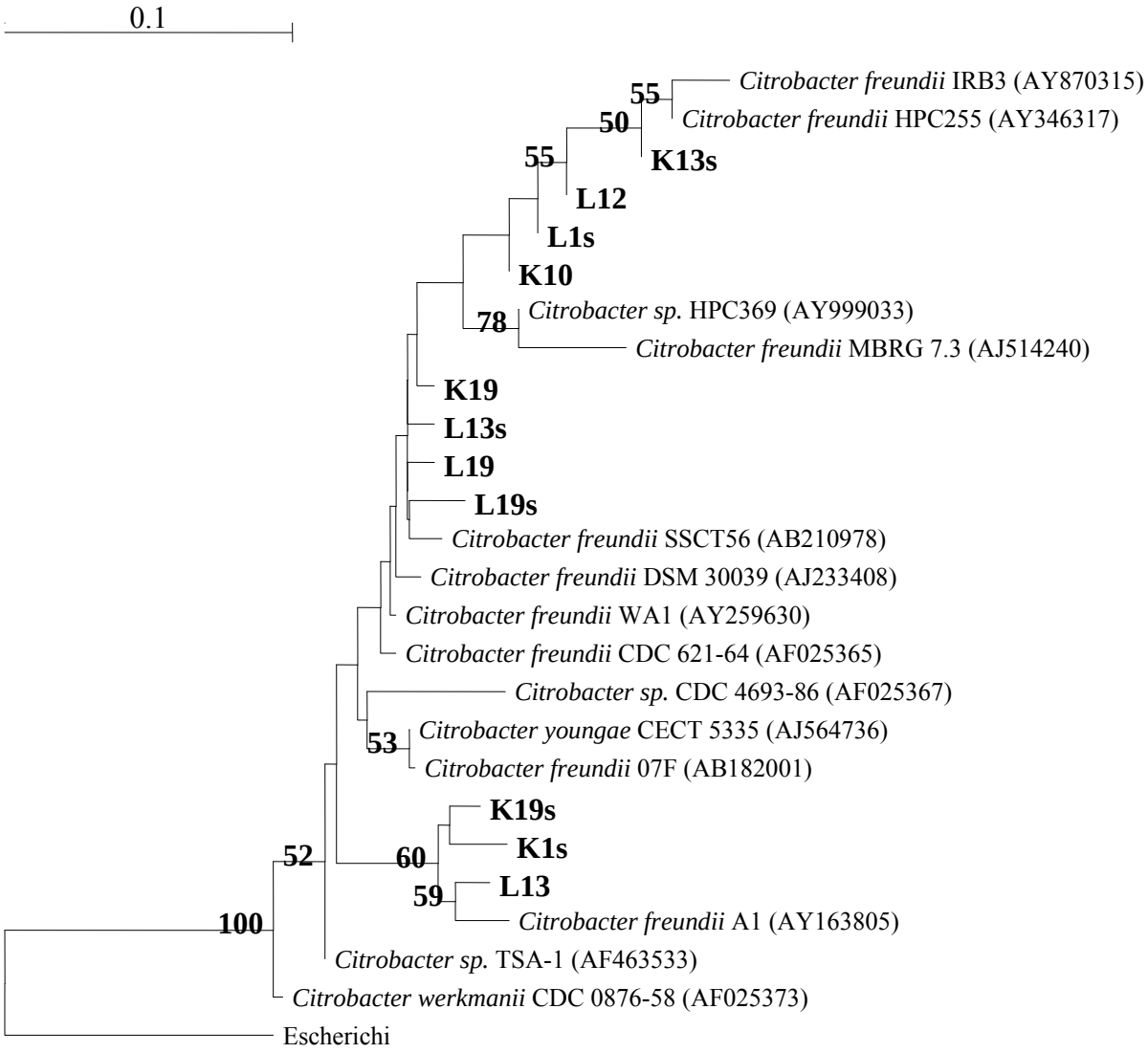

Figure 3-11. Phylogenetic tree of dominant sequences (K13s, L12, L1s, K10, K19, L13s, L19, L19s, K1s, K19s, L13) enriched from NSRA soils

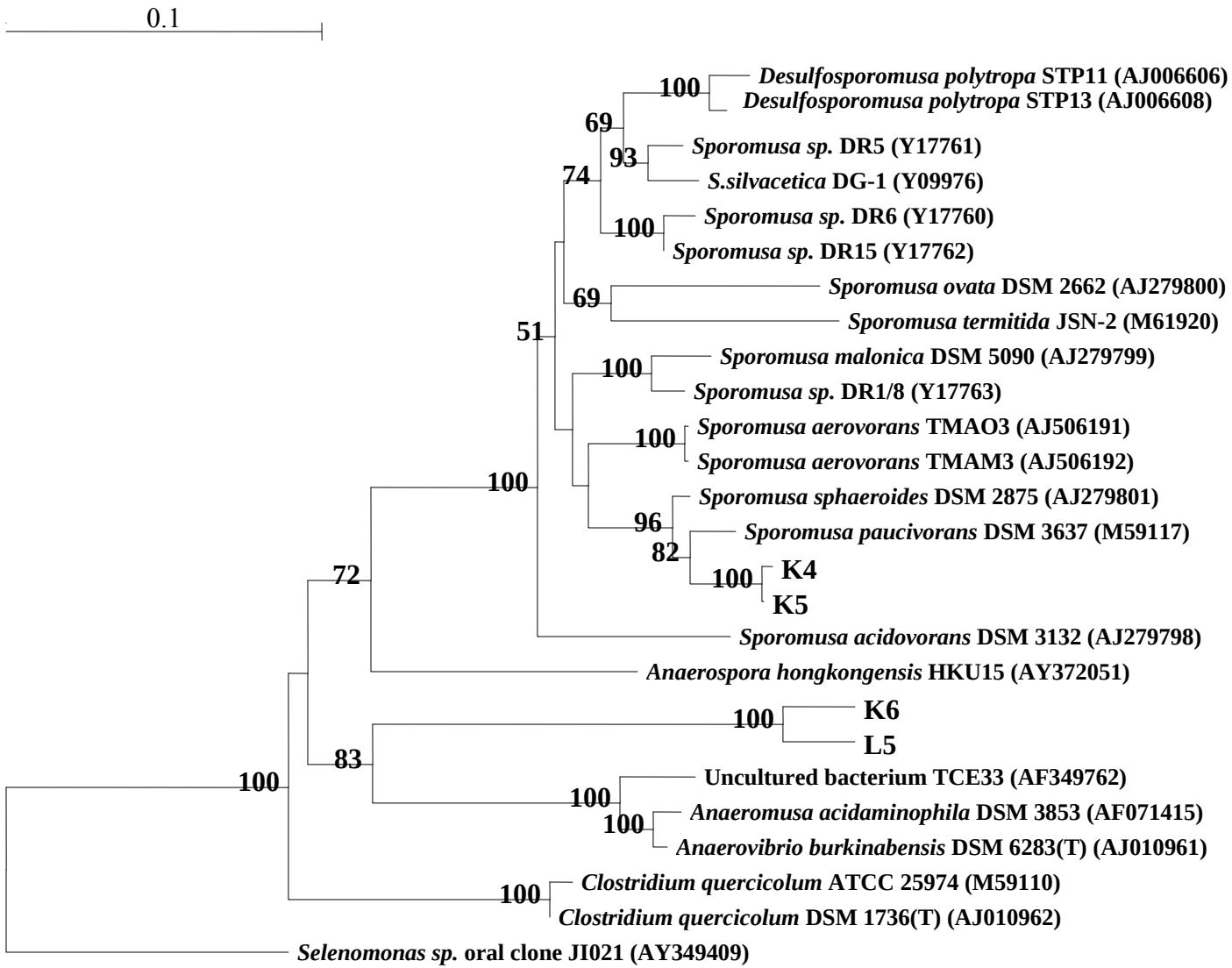

Figure 3-12. Phylogenetic tree of Gram-positive species (K4, K5, K6, L5) enriched from NSRA soils

CHAPTER 4
BIOLOGICAL BREAKDOWN OF PESTICIDES IN LAKE APOPKA NORTH SHORE RESTORATION AREA SOIL IN A MESOCOSM EXPERIMENT

Residues of toxaphene, DDT (1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)-ethane), DDE (1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene), and DDD (1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethane) are frequently detected in soils, surface water supplies, and aquatic sediments (Szeto and Price, 1991; Hargrave et al., 1993; Paasivirta et al., 1993; Pearson et al., 1997; Xie et al., 1997; Kelderman et al., 2000; Shivaramaiah et al., 2002). Since their toxicities and potential for bioaccumulation are significant, removing them from environmental matrices via an efficient and economical strategy is of interest. *In situ* bioremediation of organochlorine pesticides (OCPs) is a particularly attractive option because of its cost efficiency.

The marshes of the northern shore of Lake Apopka, FL were drained in 1941 for muck farming. Nutrient runoff from the highly productive farms resulted in the lake becoming highly eutrophic. The State of Florida closed this area to farming in 1998 and is now restoring the lake and surrounding land. During the winter of 1998-1999, over 600 birds, primarily white pelicans, associated with the lake died over this period. The reasons for this large bird kill are still unclear, although the tissues of some of the pelicans contained high concentrations of OCPs. Toxaphene and DDT concentrations from the northern shore of Lake Apopka have been reported to range from 21 ppb to 150 ppm, and removal of these OCPs is important for the complete restoration of Lake Apopka.

Studies about biodegradation of contaminants usually start from the bench scale microcosm. In this scale, conditions for stimulating the biodegraders responsible for the degradation are easily controlled. Once the feasibility of the bioremediation strategy in the microcosm system is confirmed, a larger scale investigation is preferred to represent the conditions in the field for evaluating such a strategy for cleaning up the contaminated site at both

cost and efficiency aspects as findings in the bench scale can not necessarily occur as exactly as in the field.

To evaluate the feasibility of bioremediation of OCPs, we tested a set of six different electron donors under sulfate reducing conditions and demonstrated that appropriate electron donors stimulated the bacteria which can utilize OCP as a terminal electron acceptor or energy source after incubating one month. In order to test the concepts developed from the microcosm studies, we extended the microcosm studies to a series of replicated tanks containing soil from the NSRA. Furthermore, since hydrogen can not be safely or practically handled, and pure chemicals of lactate applied in the microcosms are relatively expensive, we tested the efficacy of produced lactate and hydrogen from the fermentation of some readily available plant materials as electron donors. DeBusk and Reddy reported that the cellulose content of cattail is approximately 40% (DeBusk and Reddy, 1998), which means 1 kg cattail would contain approximately 400 grams cellulose and therefore would be theoretically transformed to approximately 5 moles of lactate through the fermentation pathway. In addition to cellulose, other fermentable substrates are also present in cattail to be the potential candidates for forming more fermentation products.

Cycling between anoxic and oxic systems is believed to be an efficient way to completely detoxificate persistent OCPs (Gavrilescu, 2005) because complicated pollutants are resistant to the oxidative metabolism such that anoxic process, e.g. dechlorination reactions, has to degrade the chemicals to smaller molecular for the aerobes to take over. Since hydrology at NSRA is simply controlled, such cycling is allowed; one cycle of anoxic/oxic incubations was included in these studies to gain the most possible degradation.

Materials and Methods

Site

Prior to determining the site for soil collection for the mesocosm studies, soil samples were taken from five separate sites at NSRA. Candidate sites were selected, marked, and cleared of vegetation by district personnel. Samples from the individual sites were composited from five randomly selected spots at each site. The soil from each site was initially mixed on site, transported to the laboratory in Gainesville, where soil was thoroughly mixed by hand in the laboratory. Approximately half the soil in each sample was shipped to Pace Analytical Services, Inc. for analysis of OCP residues, and the other half was analyzed at the University of Florida using accelerated solvent extraction (ASE) methods as described below.

The site chosen for soil collection for use in the mesocosms was ZSS0750, located in a field south of Lust Rd (UTM coordinates, X 44411, Y 3171355), and was selected on the basis of OCP concentration and ease of access (Fig. 4-1). The OCP values reported by Pace for this site were: toxaphene, 9200 μg/kg; DDT, 130 μg/kg; and DDXs, 1620 μg/kg (including DDT, DDE, and DDD).

Approximately 6 cubic yards of soil were collected from a 13 ft x 13 ft plot at this site on December 12, 2005. The soil was mixed at the site using a trackhoe by a hazardous materials contractor under the direction of District personnel (Fig. 4-2). The soil was loaded onto a dump truck and transported to the Soil and Water Science Department's greenhouses on the University of Florida campus in Gainesville.

Mesocosm

Once at the University of Florida greenhouses, soil was initially manually mixed with shovels, and added to the tanks sequentially, one wheel barrow at a time. Mixing was deemed to

be satisfactory, as indicated by the relatively low variability within OCP concentrations within and between tanks, as reported by Pace Analytical Services.

Mesocosms were based on a design used by the Wetland Biogeochemistry Laboratory at the University of Florida. Mesocosms were constructed from 150 gallon Rubbermaid "Farm Tough" stock tanks; these are constructed of high density polyethylene and are approximately 35 inches (88.9 cm) wide at the top, 54 inches (137.16 cm) long at top, and 23 inches (58.42 cm) deep, with drains built into the sides of the tanks. Prior to addition of soil, a layer of gravel approximately five inches (12.5 cm) thick was added to the bottom of each mesocosm to facilitate drainage.

Nine mesocosm tanks (Table 4-1) were set on tables reinforced with cement block supports within the greenhouse (Fig. 4-3). The order of the mesocosms, from north to south, is Control-1, Cattail-1, Lactate-1; Control-2, Cattail-2, Lactate-2; Control-3, Cattail-3, Lactate-3. This design is 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 26 cm.

The top 10 cm soil was removed from each of the cattail tanks and mixed with 1 kg dried, ground cattail in a small electric cement mixer. Cattail for this study was harvested from Lake Alice on the University of Florida campus. This level of plant material is not related to standing crop on site (which has little or no cattail), but rather that amount of cattail that could be easily obtained and plowed into the soil. The mixture was then added back to the appropriate tanks. Ten samples were randomly collected from each tank at soil depths of 5 cm, and mixed to form a single composite sample for each mesocosm.

On January 10, 2006, water was added to a height of 10 cm above soil surface. For the lactate mesocosms, lactic acid was mixed with tap water in 20 L containers to form a final

concentration of 10 mM lactate. Approximately 160 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 ˚C.

The redox potentials in all tanks stabilized by February 21, 2006, which was considered the starting point for analyses. Anoxic incubation continued until June 8, 2006, at which time the water was removed from the tanks to begin the aerobic phase. Water was removed by siphon from the top of the tanks, and allowed to drain from the bottom through the drain. Drainage was very slow to ensure that water above soil was siphoned off.

DNA Extraction Procedure

Soils were collected from five sites randomly selected from each tank and then composited. Nucleic acids were extracted with UltraClean Soil DNA kits (Mobio, Solana Beach, CA). Following the manufacturer's instruction, one gram of soil was used. PCR for 16S rRNA genes was conducted with the universal primer set 27F/1492r (Lane, 1991) and DHC primers, Fp DHC 587/Rp DHC 1090 (Hendrickson et al., 2002), for *Dehalococcoides*. One μl of diluted DNA solution was mixed with 10 μl of HotStarTaq Master Mix (Qiagen, Valencia, CA), 7 μl of H_2O provided with Master Mix, 1 μl of each primer (10 pmol/μl). PCR amplification was carried out in a GeneAmp PCR system 2400 (Perkin-Elmer Applied Biosystems, Norwalk, CT). The initial enzyme activation and DNA denaturation was performed at 95°C, followed by 35 cycles of 30s at 95°C, 30s annealing at 58°C, and 30s extension at 72°C for amplification of 16S rRNA genes. A 7 min final extension was performed at 72°C. The amplicons were electrophoresed through 1% agarose gels in TAE buffer.

Redox Potential Measurement

Three redox probes were permanently installed in each tank (one at each end approximately 30 cm from the wall, one in the center) at a soil depth of 5 cm. Redox potential (Eh) was determined at weekly intervals during the anoxic treatment. Eh was measured with permanently installed platinum electrodes; platinum wire (2 mm diameter, 15 mm length) was welded on a copper wire protected with insulation material. Eh was read using ORION SA 230, (Orion Research Inc., Boston). A photograph of permanently installed redox probes is presented in Fig. 4-4.

Temperature Measurement

Temperature was determined every two weeks at the time of sampling at a depth of 5 cm. A glass thermometer was inserted in selected mesocosms close to the two sides of window installed with fans in summer or the middle of the green house where the temperature was relatively high.

Water Content Measurement

The water content of the mesocosms in the aerobic phase was measured at randomly chosen ten locations at 12cm depth in the soil by TDR 300 soil moisture meter (Spectrum Technologies, Inc. East - Plainfield, Illinois).

Sample Collection

When redox potentials in the mesocosms stabilized, ten samples were taken every two weeks from each mesocosm to a depth of approximately 5 cm using a 50 ml disposable syringe with the end cut off. The use of a syringe was necessary because the soils exhibited a slurry-like consistency that precluded the use of more traditional core samplers. The ten samples from each mesocosm were mixed and centrifuged in the laboratory to form composite samples. The separated solution and soils were stored at –80 ^{o}C until analysis.

Organic Acid Analysis

Acetate is an important metabolite of cellulose fermentation; thus, acetate concentrations provide information on the functioning of the system. Acetate and lactate concentrations in pore water were determined by high pressure liquid chromatography equipped with a UV detector (Waters, Co), with Aminex HP 87H column as the separating column (300 X 7.5 mm) and sulfuric acid (5mM) as eluent.

OCP Analysis

Toxaphene, DDT, DDE and DDD concentrations were determined by extraction with accelerated solvent extraction (ASE) and gas chromatography. Preliminary tests conducted to investigate the influence of soil moisture content on OCP extraction by ASE indicated that medium water content yielded the greatest efficiencies of recovery. Samples were air dried, ground, and sieved prior to analysis. Deionized water was added to sieved soils and allowed to equilibrate for three days in a closed dessicator. Water content was adjusted to 50% before extraction. A surrogates solution (50 ppb) (Decachlorobiphenyl and TCMX, AccuStandard, Inc., USA) was added to samples prior to extraction.

ASE A Dionex ASE 100 Accelerated Solvent Extractor (Dionex, PA, USA) was used to perform the extractions. Triplicates of soil samples were mixed with hydromatrix (Varian, Palo Alto, CA) in 2:1 proportion and extracted in a 34 mL stainless vessel using methylene chloride/acetone (4:1, v/v) or hexane/acetone (1:1, v/v) as solvent. The cell was filled with the solvent and heated to 100ºC. The static extraction was performed for 5 min, followed by flushing the vessel with 60% of the cell of fresh solvent. Finally, the vessel was purged with nitrogen for 100 sec at 150 psi. The total amount of extraction solvent was about 50 mL, and one static cycle was conducted.

Cleanup and Analysis All extracted volumes were reduced and then hexane was added by an N_2 stream. Repeated this process twice to remove acetone and methylene chloride. The volume of extracts was measured in a glass graduated volumetric cylinder. At this point, the samples were split for analysis of toxaphene and DDT and metabolites.

For analysis of DDT and its metabolites, one mL of extracts was passed through a florisil cartridge packed with approximately 500 mg of 200μm Florisil (Varian, Palo Alto, CA). Nine mLs of hexane:acetone (9:1, v/v) were used to rinse the column. The resulting eluent was concentrated to 1mL by an N_2 stream and then transferred to a glass vial (2 mL, Fisherbrand, Atlanta, GA) and crimped with a Teflon lined cap for subsequent analysis by gas chromatography.

Another one mL extract was purified by sulfuric acid treatment for further analysis of toxaphene. Equal amounts of concentrated sulfuric acid and sample were mixed in a 10 mL vial. The vial was capped and vortexed for 1 min. Phases were allowed to separate, and the upper (organic) phase transferred to a GC vial for analysis.

Extracts were analyzed with a gas chromatograph (Shimadzu GC-17A) equipped with a ^{63}Ni electron capture detector (ECD). The column was a RTX-5 (0.25mm ID × 15m; Restek Corporation, Bellefonte, PA, USA). The temperature was programmed as follows: 60ºC, 3.0ºC/min to 70ºC for 1 min, 10ºC/min to 130ºC for 3 min, 10ºC/min to 160ºC, 20ºC/min to 200ºC. A calibration curves with 6 points bracketing the experimental concentrations was used for both analyses. The concentration calculation followed EPA 8000B and methods used by Pearson and Paan (Pearson et al., 1997; Paan et al., 2006).

Statistical Analysis

One-way ANOVA was used to test the difference among treatment groups. Comparisons for differences in concentration least square means between sulfate concentration, sterile control,

and electron donors were done by Tukey's adjustment using SAS version 9.1 (SAS Institute, Cary, NC). The null hypothesis was rejected at $p<0.05$, thus the probability of observing no difference among the pair wise is less than 5%.

Results and Discussion

Physical Conditions Related to OCPs' Degradation within Mesocosms

Temperature Temperature was monitored throughout anoxic and oxic phases of the investigation. In treatment of Cattail-1, the increase in temperature from 22ºC in February to above 30ºC after May (Fig. 4-5) was observed. The similar scenario occurred with other monitored mesocosms. It was not difficult to understand the fluctuation in all monitored mesocosms from the overall low temperature to the overall high temperature from the very beginning until the end of the experiment. It likely resulted from the poor cooling system in the greenhouse that could not keep temperature in the hotter days of summer as same as in the cold days of winter. Furthermore, temperature in the mesocosm located in the middle of the row of nine tanks was higher than that in the mesocosms in the two ends of the row. For example, the average temperature in Cattail-2 in summer was generally higher (Fig. 4-5) than that in Control-1. Such variability may be explained by the fact that fans were only installed on the two sides of the window wall so as to cool the air around the ends of row closed to the windows.

Redox Potential Redox potentials were monitored in all tanks following flooding and throughout the anoxic phase by averaging three readings from each mesocosm (Fig. 4-6). The fastest drop of redox potentials to ca. -250 mV, which was reported as the desirable redox potentials for reductive dechlorination (Jin and Englande, 1996), happened in the tanks with added carbon amendments approximately one month after flooding. This indicated that the cattails were decomposed and released the carbon, probably other nutrients, quickly after amended, which were comparable with the pure chemical, lactate, as far as their effect on the

change in the soil conditions was concerned. In contrast, redox potentials in the control mesocosms kept falling throughout the flooded phase. It was not unexpected that the applications of carbon source in the treatments helped reduce the redox potentials in the system and maintain a more reductive environment very efficiently (Ugwuegbu et al., 2001; Davis et al., 2004; Lookman et al., 2005), which would suggest potential for anaerobic biodegradation of OCPs. While in controls limited in carbon and electron energy redox potentials could not decrease rapidly as in treatments. Following drainage of water, the aerobic range of the redox potential was approached within two weeks (Fig. 4-7).

Water Content Volumetric water content was monitored in all mesocosms following draining on June 8, 2006 by averaging 10 readings by TDR 300 from each mesocosm. The surface soils were rather wet after draining and remained water was continually evaporated thanks to the hot summer. However, volumetric water content in some tanks (Lactate-1, Cattail-2, and Lactate-2) was still high, around 50%, (Fig. 4-8) until the end of July. Other mesocosms showed a relatively constant water content.

Degradation of Toxaphene in Mesocosm

Table 4-2 shows similar toxaphene concentrations at the beginning of the flooded phase in both controls and treatments. However, statistical significance of toxaphene concentrations between treatments and controls (Table 4-2) in the end of flooding was revealed by pair-wise comparison as would be expected. Throughout the anoxic phase, toxaphene was continually degraded when supplied with an appropriate electron donor and toxaphene decreased until May 31 (Fig. 4-9). Toxaphene concentrations in control mesocosms did not decrease until May 3 (Fig. 4-9), which seems to correspond with much more reductive redox potential (-200 mv). This phenomenon indicated the metabolism mechanism that highly chlorinated compounds can only be degraded under the anaerobic conditions. On the other hand, intrinsic degradation was

observed in the non sterile group in our previous laboratory studies, explaining why insignificant degradation of toxaphene in controls was found until an anaerobic condition. However, toxaphene concentrations in control mesocosms did not continue decreasing after this time point.

At the end of anoxic period, toxaphene concentrations in lactate and ground cattail reduced to 13.20 ppm and 12.63 ppm respectively, while control mesocosms still had 18.38 ppm of toxaphene. This observation reflected the results of the microcosm studies. In microcosms, the appropriate electron donors including lactate and hydrogen were capable of stimulating the toxaphene feeders during the two months of anaerobic incubation. As expected that cattail can be consumed and produce some electron donors including lactate and then syntrophs utilized lactate to produce hydrogen (Drzyzga et al., 2002), halogenated chemicals such as toxaphene are believed to release the chlorine ion with the replacement by hydrogen accompanied by a two-electron transfer (Fingerling et al., 1996). Fermentation process in all lactate and cattail mesocosms was also confirmed by both gas production and the rapid decrease in the redox potential soon after flooding in comparison with those in control systems. These phenomena demonstrated that the microbial community quickly responded to the amendment of the exogenous carbon source and this became the stimulus for the degradation, although others reported an inhibition of toxaphene biodegradation with the addition of lactic acid as the carbon source (Lacayo-Romero et al., 2006). No obvious production of sulfide confirmed that sulfate reducing bacteria might not take a role in attacking toxaphene in the anoxic phase. Probably, some other group of bacteria were feeding upon toxaphene as the carbon and energy source and electron acceptor. Up to now, two strains of anaerobes, *Enterobacter* sp. strain D1 (Lacayo-Romero et al., 2005) and *Dehalosprillum multivorans* (Ruppe et al., 2002), were isolated to degrade toxaphene. *Dehalococcoides* was detected and in NSRA soils (personal

communication). In fact, reductive dechlorination of certain compounds by *Dehalococcoides* sp., has been shown by Krajmalnik-Brown (Krajmalnik-Brown et al., 2004) to dechlorinate some compounds using lactate as electron donor in consortia, and H_2 as electron donor in pure culture (He et al., 2003). Lactate was thought to be fermented to H_2 in consortia in that study, which then served as the primary electron donor for reduction of trichloroethylene by *Dehalococcoides*. Although both lactate and H_2 were identified as efficient electron donors for OCP degradation in our previous microcosm studies, microorganisms enriched as capable of utilizing toxaphene as electron acceptor did not include any strain with 16S rDNA sequence similarity to *Dehalococcoides* sp. Amplicons could not be obtained using *Dehalococcoides* sp. specific PCR primers from soils used in mesocosms before flooding (data not presented). This evidence was not strong enough to exclude the degrading role of *Dehalococcoides* in NSRA, which probably resulted from the procedures we followed, although it was not detected in the microcosm studies. For most samplings from the beginning to the end of anoxic period, ground cattail and lactate treatments could not be distinguished at the statistical level. This study demonstrated that the cheap and readily available plant materials as amendments can achieve a very high efficacy of degrading toxaphene under anaerobic conditions. During the oxic phase, continued degradation of toxaphene was not observed. On the contrary, all the treatment mesocosms showed the tendency to be constant and no significant loss of toxaphene was observed under the oxic condition. Statistical analyses yielded significantly lower concentration of toxaphene in treatment systems than that determined in the control mesocosms (Table 4-2). No significant difference between the cattail and lactate treatments can be found from the pair-wise comparison analyses. Dehalogenation of toxaphene in our study did not seem to prefer the aerobic incubation. Even if we could not determine the structure of components, products of

dechlorination of toxaphene in the treatment mesocosms, the fact that aerobic soil microorganisms generally fail to metabolize higher halogenated pesticides might be thought to be the reason of no further degradation during the oxic phase.

Degradation of DDT and its Metabolites in Mesocosm

At the beginning of the anaerobic phase, concentrations of DDT and DDD and DDE did not show any statistically significant difference among the tanks (Table 4-2).

In comparison with controls, degradation of DDT was observed in the cattail mesocosms, concentrations of DDT declining constantly throughout the anoxic phase (Fig. 4-10). Even though concentrations of DDT in the lactate mesocosms in the starting of the anaerobic period were not sensitive to the treatment, a decrease was noted after May 3, pending the end of anoxic phase. It is likely that the microbial community in the NSRA soils took some time to respond to the addition of the applied carbon and energy source. By the end of the anoxic phase, concentrations of DDT were significantly lower in the lactate and cattail mesocosms than in the control mesocosms (Table 4-2). The observations of such degradation supported and extended our bench scale investigations. In our previous study, we observed that lactate and hydrogen stimulated the significant disappearance of DDT from NSRA soils under both low and high concentrations of sulfate salt.

Concentrations of DDD and DDE, the major products of transformation of DDT, were also determined. An increase in DDD concentrations in the cattail mesocosms was noted in the first weeks of anaerobic phase (Fig. 4-11), corresponding to a decrease in DDT concentrations despite the big variation (Fig. 4-10), while the concentrations of DDD in the lactate mesocosms kept unchanged at the same period. As flooding condition ended, a general increase in DDD concentrations was observed in the lactate and cattail mesocosms, consistent with the relatively large decrease observed in those tanks for DDT. Such findings led the confidence that the

observed degradation of DDT in the treated mesocosms was real. At the end of the flooded phase, DDD concentrations were significantly higher in the lactate mesocosms than in the control mesocosms. Although the effect of cattail could not be distinguished from controls, the tendency of increase of DDD was not negligible and probably masked by the variabilities of the DDD measurement.

DDE concentrations revealed little information of degradation under anaerobic conditions. No loss of DDE was observed in any treatment during the anoxic phase (Fig. 4-12, Table 4-2), nevertheless not surprising according to the degradation pathway of DDT under the anaerobic condition (Fig. 1-2). Generally, DDE can not be degraded further to any other compounds in the anaerobic phase as would be expected, which was already observed in our bench scale microcosms.

Our results indicated that added electron donors in the form of ground cattail and lactate promoted DDT degradation relative to control mesocosms, and stimulated production of DDD under anoxic conditions. The fact that no lactate or acetate was detected in porewaters of any of the mesocosms (data not shown) confirmed that specific bacteria utilized lactate direct from the chemicals or the fermentation of the plant residues as the electron donors. As well known, many contaminants were degraded under methanogenic conditions or sulfate reducing conditions. However, relatively few microorganisms are capable of directly utilizing lactate as electron donor and largely restricted to secondary fermenters (syntrophs) in consortia with methanogens (Sekiguchi et al., 2006). Sulfate reducing bacteria, a major group of bacteria involved in the anaerobic degradation of halogenated compounds, were possibly not the microorganisms that were capable of utilizing lactate or a fermentation product as electron donor, either directly or indirectly, and using OCP as electron acceptor (van Pee and Unversucht, 2003) based on the

evidence that no noticeable sulfide was produced during the experiments period. In our study, another valuable finding was that mesocosms with lactate and ground cattail were approximately equivalent in stimulating degradation of DDT. The plant residues from the cattail treatments were capable of being transformed to H_2 and lactate, indicated by gas production and then drove DDT degradation as expected. This observation suggested that cheap and easily available plants materials made the cost efficient remediation strategy possible.

At the end of the oxic phase, no significant difference was observed for DDT and DDE, as with toxaphene. On the other hand, the same modes of higher concentrations of DDD in mesocosms treated by lactate and cattail and rather lower in controls were observed at the end of anoxic phase as the end of the oxic phase. The lack of degradation during the oxic phase was somewhat surprising. Potential nitrogen and phosphorus limitations at later stages of the incubation were possible reason responsible for no further degradation throughout the oxic phase.

These investigations have shown that much of the observed degradation of OCPs occurred prior to draining, while continued degradation was not observed during the oxic phase. In this case, the primary and most efficient approach for these OCPs degradation suggested was anaerobic and, therefore, future efforts for NSRA remediation should be attempted for anaerobic process.

Another important factor in degradation is the bioavailability of the contaminants for the organisms responsible for metabolism of the compounds. These degraders in NSRA soils are likely limited to access to the OCPs as a result of their hydrophobic properties and the high organic maters in the soils. Increasing the availability of OCPs to microbial attack may provide an optimal strategy for accelerating their degradation (Walters and Aitken, 2001). Other reported

strategies for increasing availability included the application of surfactants (Kommalapati et al., 1997) (Gao et al., 2000; Lunney et al., 2004). In our project of clean-up of NSRA contaminated soils, application of surfactants with incorporation of plant residue prior to flooding might be desirable by resulting in increased degradation.

Table 4-1. Experimental design of mesocosm studies

Treatment/Control	Description	No. of Tanks
Treatment – Lactate	10 mM lactate	3
Treatment – Cattail	0.04 g ground cattail per gram soil	3
Control- No electron donor	soil and water only	3
Total Mesocosms		9

Table 4-2. Pair-wise Comparison of the treatments on the degradation of pesticides

		Toxaphene	**DDT**	**DDD**	**DDE**
Starting concentrations (Feb. 21, 2006)	Control	22.22(A)	3.17(A)	1.54(A)	3.16(A)
	Cattail	24.52(A)	3.34(A)	1.83(A)	3.53(A)
	Lactate	25.21(A)	3.14(A)	1.47(A)	3.44(A)
Anaerobic period (Ending May 31, 2006)	Control	18.38(A)	3.16(A)	1.42(B)	3.92(A)
	Cattail	12.63(B)	2.08(B)	1.90(B)	4.18(A)
	Lactate	13.20(B)	2.55(AB)	4.10(A)	4.11(A)
Aerobic period (Ending Aug. 21, 2006)	Control	18.60(A)	3.07(A)	1.87(B)	3.93(A)
	Cattail	12.49(B)	2.74(A)	4.02(A)	4.24(A)
	Lactate	14.42(B)	2.23(A)	4.34(A)	3.98(A)

Note: Concentrations are presented in mg/Kg and concentrations within a given time point sharing the same letter did not show significant difference at the $p<0.05$ level.

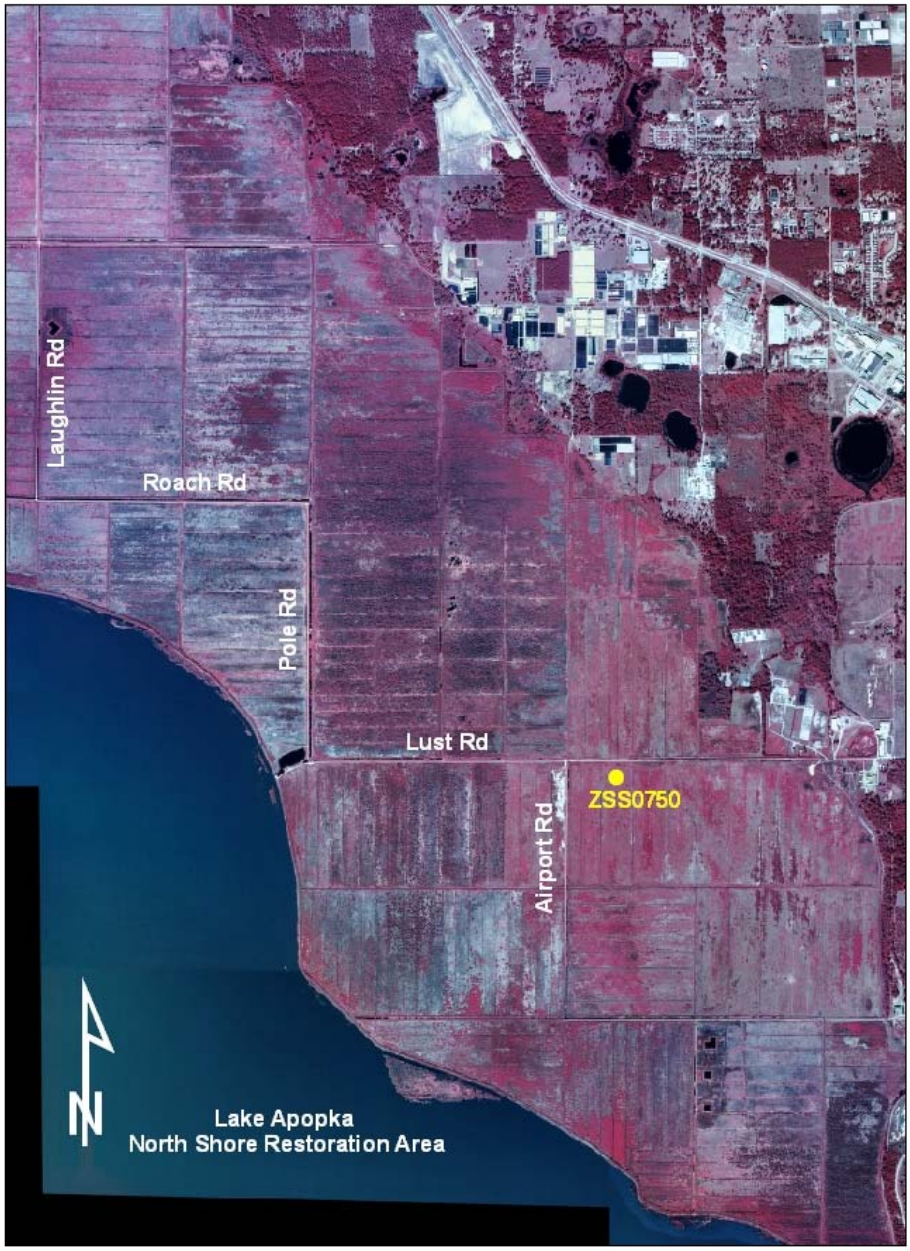

Figure 4-1. Soil collection site ZSS0750 in the North Shore Restoration Area at Lake Apopka, Orange County, Florida.

Figure 4-2. Soil mixed in a 13 ft x 13 ft plot at site ZSS0750 by a trackhoe prior to collection and transport to the greenhouses at the Department of Soil and Water Science, University of Florida, Gainesville.

Figure 4-3. Mesocosm tanks in the greenhouse at the Department of Soil and Water Science, University of Florida, Gainesville.

Figure 4-4. Permanently installed redox electrodes in mesocosm tank.

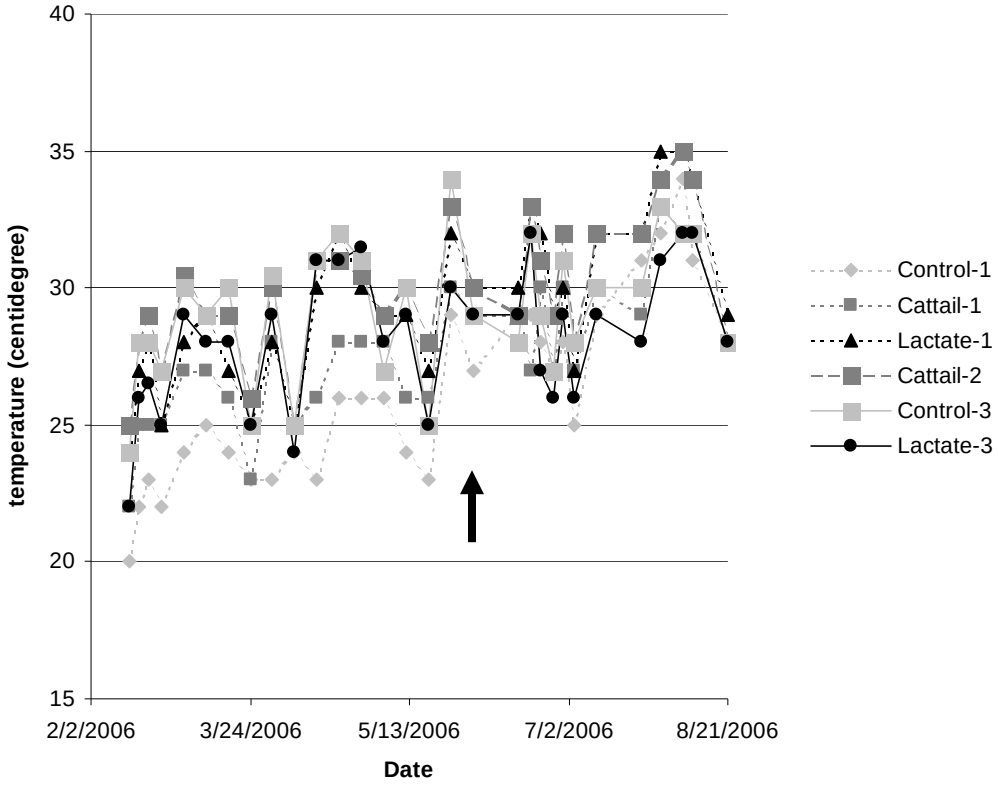

Figure 4-5. Temperatures within mesocosms at a depth of 5 cm.

Note: Vertical black arrow indicates date of draining (June 8).

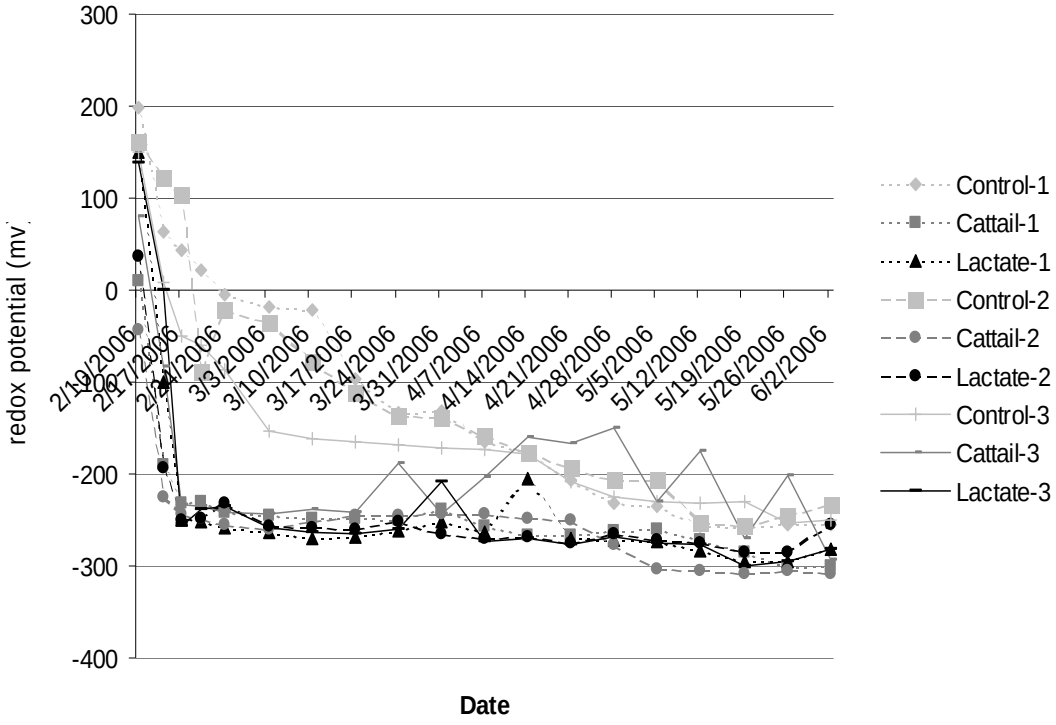

Figure 4-6. Redox potentials with mesocosm tanks during flooded phase.

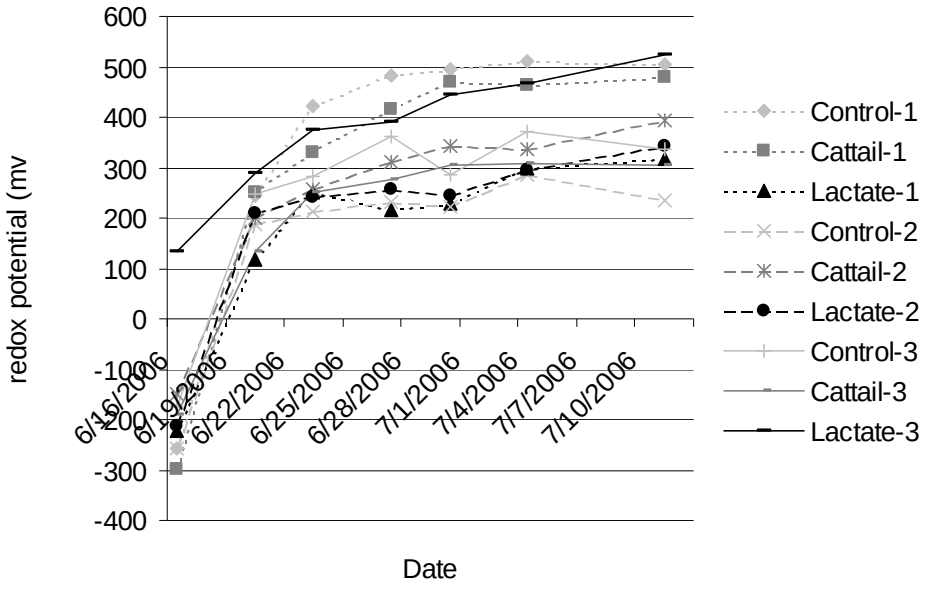

Figure 4-7. Redox potentials following draining.

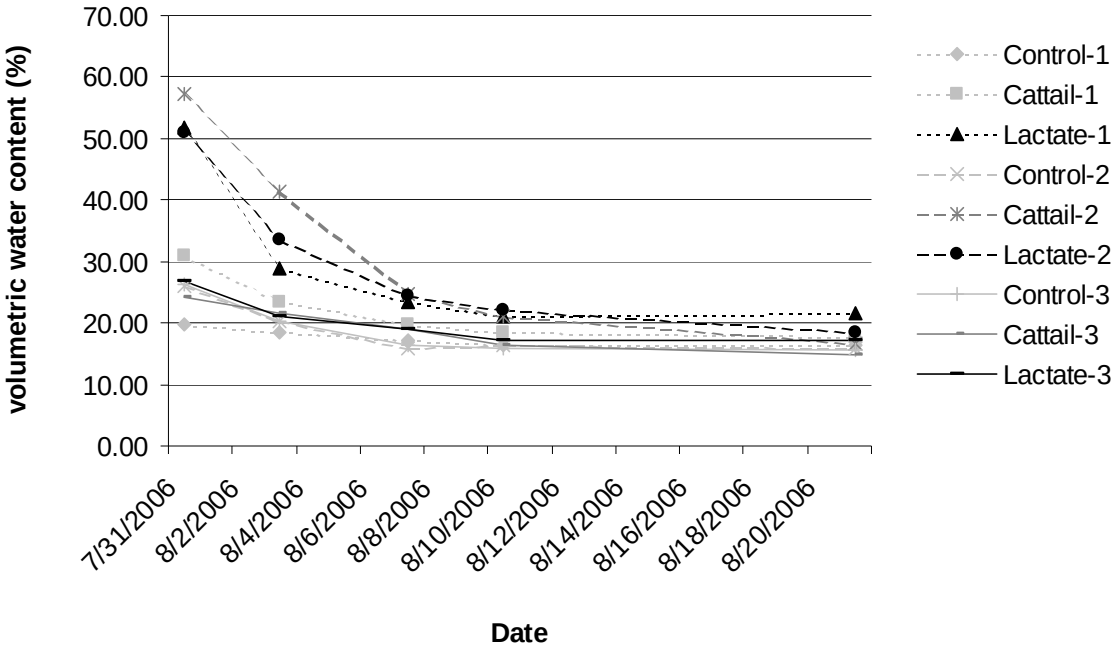

Figure 4-8. Volumetric water contents following draining.

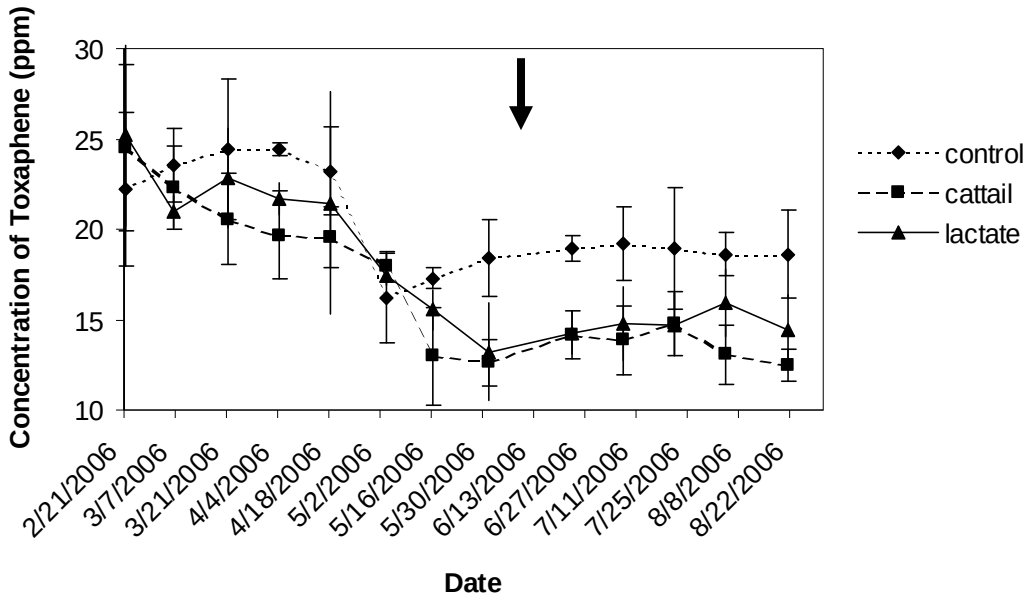

Figure 4-9. Concentrations of toxaphene during study.

Note: Error bars represent +/- 1 standard deviation. Vertical black arrow indicates date of draining (June 8).

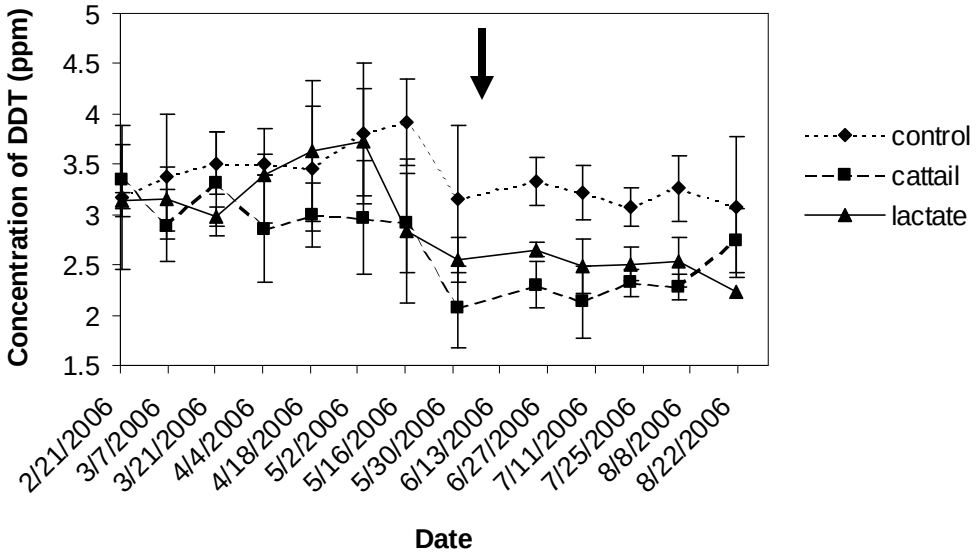

Figure 4-10. Concentrations of DDT during study.

Note: Error bars represent +/- 1 standard deviation. Vertical black arrow indicates date of draining (June 8).

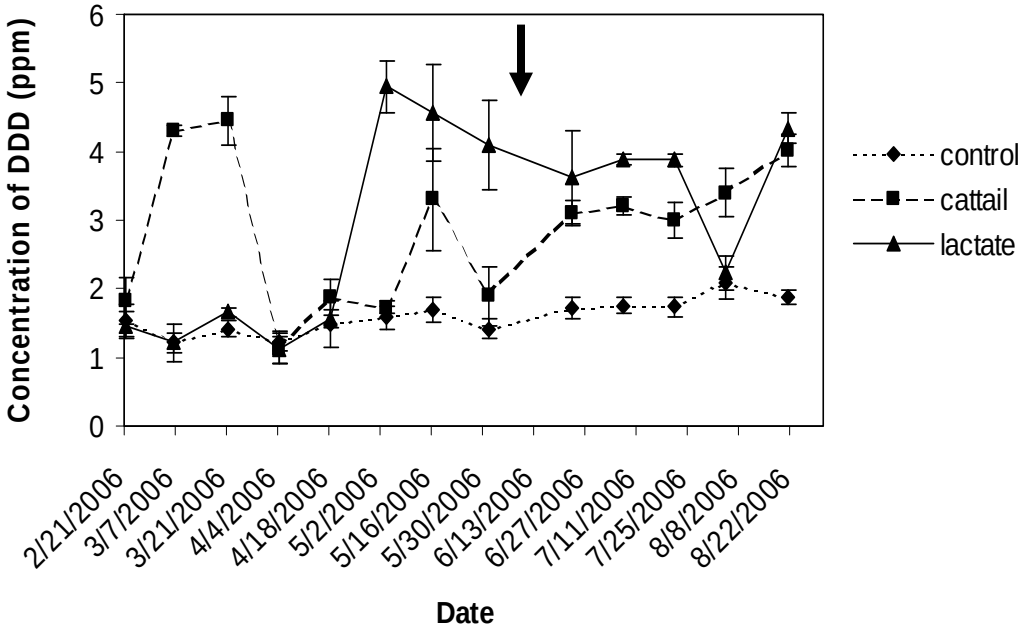

Figure 4-11. Concentrations of DDD during study.

Note: Error bars represent +/- 1 standard deviation. Vertical black arrow indicates date of draining (June 8).

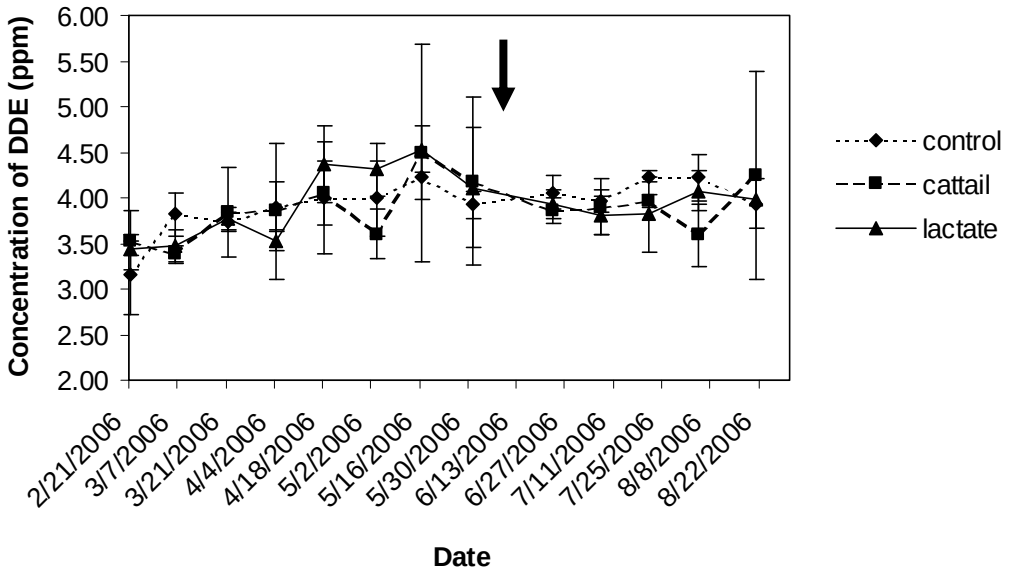

Figure 4-12. Concentrations of DDE during study.

Note: Error bars represent +/- 1 standard deviation. Vertical black arrow indicates date of draining (June 8).

CHAPTER 5
SUMMARY AND CONLUSIONS

OCPs have endangered wildlife and humans as a result of widespread use and their carcinogenic toxicities. Removal of OCPs from contaminated environments has become an environmental priority. Cost efficiency leads bioremediation to be a desirable strategy compared with physical and chemical techniques. The NSRA is such a widely contaminated site by toxaphene and DDTr that the presence of the OCPs constitutes a risk to wildlife, and toxaphene and DDTr may have contributed to the deaths of several hundred white pelicans. The large area of contamination makes the NSRA to be a good candidate for researching environmental behavior and remediation of OCPs.

This research was conducted to understand the microbiological processes of toxaphene and DDTr degradation under appropriate conditions, with special emphasis on anaerobic biodegradation at the bench and mesocosm scales.

Chapter 2 addressed an elementary problem for studying biodegradation of OCPs by evaluating conventional and alternative extraction methods and optimizing factors that affected the extraction efficiency. The correlation of the recovery of toxaphene using ASE with TOC and TON was very negatively high, -0.95, while SOX was not affected much by the sample characteristics. The recovery of DDTr was not correlated to TOC and TON using either ASE or SOX. These results confirmed previous work (Bandh et al., 2000) that indicated that high organic carbon contents more strongly adsorbed toxaphene, making it difficult to remove the toxicant from the soil matrix using ASE. Therefore, choosing ASE for gaining high recovery of toxaphene should consider the soil characteristics, especially amounts of organic matter. Among other soil properties, the water content influenced the extraction efficiency, with intermediate water content yielding the greatest overall efficiencies for extracting OCPs in both ASE and

SOX. Our finding indicated that suitable wet condition, intermediate water content, swelled organic soil structure allowing the OCPs in the soil pores available for solvent to carry out into the bulk extraction liquid, especially important for NSRA soils that contained high organic carbon. When water was not available, soil particles collapsed and adsorbed the OCPs strongly so as to keep then unavailable for solvents. Excessive water still led to extraction difficulties because the soil particles are surrounded by water, which potentially blocks extraction solvent accessed to the OCPs. Water soluble organic carbon could be partitioned into water matrix instead of combining with OCPs, thereby improving the extraction efficiency (Tao et al., 2004), which would be another reason to indicate the effect. Significantly more toxaphene, DDT, and DDE were extracted by ASE using methylene chloride/acetone (4:1, v/v) than using hexane/acetone (1:1, v/v). However, pairwise comparisons revealed that using SOX, the hexane/acetone system showed statistically higher efficiencies for DDE and DDD, at 95% confidence level and toxaphene at 90% confidence level. Reasons responsible for performance of extractant have not been well elucidated based on previous publications. Differences in polarity and boiling point might contribute tbo explain the phenomena. The present study strongly suggested that the choice of extraction solvent system must be dependent on the extraction procedure.

Statistical evaluations showed that equivalent results (moderate contaminated soils) or higher efficiencies (highly contaminated soils) were achieved for the simultaneous extraction of residues of toxaphene, DDT and its metabolites by ASE in contrast to SOX. Considering the solvent consumption and extraction time, ASE is the preferable extraction technique for the investigation of contaminated high organic carbon soils. Although many studies investigated the extraction of similar PAH, none have reported the comparison of toxaphene and DDTr extraction

procedures and evaluated the effects of environmental characteristics and extraction solvents. The present study provided a guideline in practice to gain efficient extraction of toxaphene and DDTr given all parameters of interest.

Chapter 3 offered a biological concept to treating OCP-contaminated soils by making use of OCP-degrading bacteria. In our microcosm study, statistically significant degradation of toxaphene was observed in the non sterile groups. This finding confirmed the feasibility of biodegradation of toxaphene. Among three promising electron donors which showed possible roles in the metabolism of OCP, lactate and butyrate were capable of stimulating toxaphene degradation at the marginal statistical level ($p<0.10$) when the sulfate concentration was relatively high (200 mg/L $MgSO_4 \cdot 7H_2O$). As well known, anaerobic dechlorination is the first step of complex organochlorine pesticides and may be achieved by different pathways. One possibility occurs when lactate or other electron donors except for hydrogen are present in the system. Dechlorination may not be processed without hydrogen such that the lactate or other donors have to be fermented by syntrophs to hydrogen. Generally, SRB or other microorganisms participate in such fermentation when sulfate salt is not sufficient (Drzyzga and Gottschal, 2002). Once dechlorinaters obtain hydrogen, replacement of a chloride of OCP with hydrogen is accomplished. Alternatively, high concentrations of sulfate will make SRB outnumber dehalogenating bacteria and stop the dehalogenation. In our study, abundant sulfate salt (200 mg/L $MgSO_4 \cdot 7H_2O$) allowed the consumption of lactate or butyrate to produce hydrogen, thereby promoting the disappearance of toxaphene. Since no amplicons were detected related to sulfate reduction in enrichment culture, we concluded that other groups of microorganisms used lactate or butyrate as the electron donor and degraders utilized the produced hydrogen to yield energy by preferring dehalogenation, a more efficient energy yielding approach, to sulfate

reduction (Dolfing and Harrison, 1992). In my experiments, when hydrogen gas was supplied in serum bottles, statistically significant degradation of toxaphene, DDT and DDD was detected between high level of sulfate group vs sterilized group, and low level group vs sterilized group. With limited supplement of sulfate (20 mg/L $MgSO_4 \cdot 7H_2O$), the responsible bacteria degraded DDT with the highest efficiency in hydrogen. This finding further indicated that the biological degradation of OCPs was not influenced by the concentration of sulfate only if hydrogen existed in the anaerobic system. Further, dechlorinating microorganisms were the major group of degrading the OCPs based on the dechlorination noticeable observed in enrichment medium.

A second mechanism attributed to dechlorination of OCP is that some dehalogenaters utilize halogenated compounds as energy source and degrade OCP through halorespiration when hydrogen is supplied as the electron donor. The effect of hydrogen added to the serum bottles in the present work demonstrated the presence of dehalogenating bacteria and their metabolizing roles. Additionally, DDT and DDD in non sterile moderate soil without added electron donor were not degraded significantly in comparison with the sterile group after two months incubation. In contrast to the first set, a significant decrease was not observed, but an insignificant increase in the concentration of DDE in some serum bottles, which indicated anaerobic transformation of DDT to DDE. Although degradation of toxaphene, DDT, and DDD were observed in the present work, the biodegradation products are not known at this moment. The limitation of this present study resulted from the lack of instruments such as GC-MS. Further work should confirm the structure and toxicities of metabolic products and conversion rate of parent chemicals to metabolites.

The production of Cl^- during the growth of the bacteria in enrichment cultures strongly suggested the mechanism of reductive dechlorination of toxaphene. The different dynamics of

released chloride confirmed our speculation for microcosms study that microbial inactivity resulted in limited degradation of OCPs in highly contaminated soils. These findings suggested that biodegradation of OCPs should take the microbial activities into account for gaining the largest degradation.

Phylogenetic analysis demonstrated that some sequences most abundant in the clone libraries were related to the genus *Citrobacter* (99.9% similarity), some closely related to Gram positive *Sporomusa*, (98% similarity). *Sporomusa* are homoacetogens capable of producing acetate with CO_2 as the electron acceptor and hydrogen as the electron donor. Both species are involved in the degradation of organic contaminants. *Sporomusa ovata* is capable of reductive dechlorinating PCE to TCE with methanol as an electron donor (Terzenbach and Blaut, 1994). On the other hand, facultative anaerobic *Citrobacter* belongs to *Enterobacteriaceae* and can ferment glucose, glycerol, or other carbon source to acetate, lactate, etc. It has been reported to be capable of utilizing chlorophenol or biphenyl as sole carbon and energy source (Martinez et al., 2000; Grishchenkov et al., 2002). Even though *Citrobacter* can tolerate or accumulate a variety of toxicants and *Sporomusa* was involved in dechlorination, it was not possible to conclude how *Citrobacter* and *Sporomusa* are involved in toxaphene dechlorination in our enrichment experiments. Further work should contribute to isolation of the pure culture or determine the members of consortium which can degrade toxaphene.

Chapter 4 supported and extended the bench scale investigations that concluded that added electron donors promote OCPs degradation under anoxic conditions. By the end of the anoxic phase, both lactate and cattail treatments significantly promoted the degradation of toxaphene and DDT as expected based on the microcosm studies. As flooding condition ended, a general increase in concentrations of DDD, the major products of reductive dechlorination of DDT, was

observed in the lactate and cattail mesocosms, consistent with the relatively large decrease in those tanks for DDT. Such findings led to the confidence that the observed degradation of DDT in the treated mesocosms was real. The fact that no lactate or acetate was detected in porewaters of any of the mesocosms indicated that bacteria immediately metabolized lactate in the systems by aerobic (in the water phase and the soil surface) and anaerobic (responsible for degradation) bacteria. This was not unexpected since our microcosm studies already indicated the significant role of acetate and lactate in degrading toxaphene. This study demonstrated that use of inexpensive and readily available plant materials cattail as amendments can promote degradation of toxaphene under anaerobic conditions at the NSRA.

Alternating anaerobic with aerobic conditions is considered to be an effective strategy for OCP decontamination, but in this case, the primary and most efficient approach for these OCPs degradation suggested was anaerobic and therefore future efforts for NSRA remediation should be attempted for anaerobic process. The fact that aerobic soil microorganisms generally fail to metabolize higher halogenated pesticides might be the reason for no further degradation during the oxic phase. Potential nitrogen and phosphorus limitations at later stages of the incubation may have been a possible reason as well. Finally, degraders in NSRA soils are likely limited to access to the OCPs as a result of their hydrophobic properties and the high organic matter content of the soils. In future, increasing the availability of OCPs to microbial attack, e.g. application of surfactants may provide an optimal strategy for accelerating their degradation.

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BIOGRAPHICAL SKETCH

Yun Cheng was born in Yingkou, P.R. China. She received her B.S. in Biology in 1999 from Shenyang Normal University, P.R. China. At the same year, she was admitted to the graduate school in Chinese Academy of Sciences to pursue her M.S. in Ecology after the nationally competitive exam. In 2002, she completed her M.S. degree and joined the Soil and Water Science Department at the University of Florida to start her Ph.D. studies. In 2003, she joined Dr. Andrew Ogram's research team. Her project focused on bioremediation of contaminated soils in Lake Apopka, one of the most polluted lakes in Florida.