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Influence of Typha latifolia and Phragmites communis root matter on degredation of aged 1,2,3,4-Tetrachlorobenzene in bayou sediments

Elaiza Maria Alvarez
Louisiana State University and Agricultural and Mechanical College, ealvar5@lsu.edu

INFLUENCE OF *TYPHA LATIFOLIA* AND *PHRAGMITES COMMUNIS* ROOT MATTER ON DEGRADATION OF AGED 1,2,3,4-TETRACHLOROBENZENE IN BAYOU SEDIMENTS

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ABSTRACT

The purpose of this study was to investigate the effects of *Typha latifolia* and *Phragmites communis* root matter in degradation of sediments contaminated with aged 1,2,3,4-Tetrachlorobenzene (1,2,3,4-TeCB). In the present study, complete reductive dechlorination of 1,2,3,4-TeCB was observed in root matter systems, contrasting sediment with no roots present. Microcosms amended with *Phragmites communis* roots achieved the greatest first-order reaction rate constant over a 4-week period (0.113 d^{-1}, half-life time of 6.13 days). *Typha latifolia* roots also played an important role in reductive dehalogenation of 1,2,3,4-TeCB, although the first-order reaction rate constant observed within the same period of time (0.097 d^{-1}, half-life time of 7.11 days) was 16% less than that exhibited by *Phragmites* treatment. Higher concentrations of H_2 associated to organic matter in root matter may have caused higher dechlorination activities. Therefore, the wetland plants used in this study represent a very promising alternative for application in phytoremediation.

Molecular techniques including Polymerase Chain Reaction (PCR), Denaturing Gradient Gel Electrophoresis (DGGE) analysis, and cloning of 16S rRNA gene sequences amplified from community DNA were used to determine the diversity of microbial communities present in the sediments. An average size of 570 bp and 180 bp regions of bacterial and archaeal 16S rRNA genes, respectively, were amplified from genomic DNA. A total of 13 bacterial 16S rDNA sequences were derived from DGGE bands and these formed 6 clusters: α, β, and γ subdivisions of *proteobacteria*; low GC gram-positive bacteria; green-nonsulfur bacteria; and an unknown bacteria group located between α-*proteobacteria* and green nonsulfur bacteria. Effective reductive dehalogenation of 1,2,3,4-TeCB and chlorobenzene congeners in vegetated treatments was attributed to the presence of two important bacterial populations, *Desulfitobacterium* sp. and

the chlorobenzene-respiring anaerobe *Dehalococcoides* sp. strain CBDB1. Moreover, methanogenic activity by identified *Methanomicrobiaceae*, *Methanosarcinaceae*, and *Methanosaetaceae* families, confirmed anaerobic conditions and the reduced environment necessary for dehalorespiration of chlorobenzene congeners in microcosm bottles.

CHAPTER 1. INTRODUCTION

For many years now, the organohalides have posed a health risk to humans and have been linked to environmental problems. According to Smidt et al. (2004), more than 3500 organohalides are created naturally and are responsible for chlorinated metabolites predominantly produced in terrestrial systems. Large amounts of halogenated compounds are found in multiple products such as solvents, degreasers, pesticides, and biocides. The release of these compounds into the environment does not only occur from anthropogenic sources but also from biogenic and geogenic sources (Smidt et al., 2004). When natural attenuation of the contaminants is insufficient to meet the risk management goals, a system must be designed to enhance contaminant degradation (Hughes et al., 2002). For that reason government regulations focus efforts to clean up the affected sites and strive to a responsible administration of our natural resources.

The extent of groundwater contamination in the United States is significant and hazardous chemicals may exist at 33,000 to 400,000 sites (Lee et al., 1998). Also, these sites, according to the USEPA, pose an unacceptable risk to human health or the environment requiring some form of remediation. Chlorinated solvents are one of the most commonly cited sediment contaminants after Polychlorinated Biphenyls (PCBs) and Polycyclic Aromatic Hydrocarbons (PAHs) (USEPA, 2004). Compounds such as chlorobenzenes and PAHs represent two important classes of organic contaminants known for their persistence in the environment, mostly present in areas surrounding hazardous waste sites. Chlorobenzenes for example, have been extensively found in groundwater, soil and sediments, and in sensitive areas such as wetlands near contaminated sites (USEPA, 2005).

It has been observed that after a prolonged contact of these contaminants with sediments, contaminant sorption into sediment particles takes place (WSRC, 2004). According to Cornelissen et al. (2001), sequestered compounds remain in place and desorb only slowly becoming mostly unavailable for transport and uptake by microorganisms. Pavlostathis et al. (2003) indicated that sequestration may control the rate and extent of contaminant biotransformation in subsurface systems. In their study, they also addressed how polychlorinated organic compounds, many of which are toxic and carcinogenic, are observed to be especially resistant to biodegradation due to the stability induced by chlorine constituents.

Lee et al. (2003) has also shown that sorption of organic compounds to soil increases as water solubility decreases, as the molecular weight of nonionic organic compounds increases, and as the organic matter in the soil increases. From the ongoing discussion, a slow desorption process has been associated to a resistant fraction of organics chemicals from sediments (Gomez et al., 2006). Even though significant research has been conducted to study the sorption and desorption kinetics of organic compounds and their bioavailability, few studies have focused on the bioavailability of contaminants in soils containing only the desorption-resistant fraction (Lee et al., 2004). Bucheli and Gustafsson (2000) quantified high soot-water distribution coefficients of PAHs which represented on a sorbent carbon-atom basis 25-50 times stronger sorption than predicted for same compounds interactions with bulk natural organic matter. In addition, they implied that such extremely short equilibration times would lend further support to the concept that adsorption sites are mainly limited to the rapidly accessible exterior surface of the soot matter.

Despite of the fact that natural attenuation may overcome the aging effects of contaminants, a combination with other technologies at sites has been object of more conservative approaches, e.g. if the mobility of contaminants exceeds contaminant destruction,

the receptors are threatened before any degradation takes place, or if contaminant concentrations are inhibitory to microbial growth (Lee et al., 1998). Today, environmentalists can choose from a variety of approaches for remediation of contaminated sites (Frick et al., 1999). However, because of the need for more cost-effective approaches, plant-based remediation has emerged as a feasible method for treatment of contaminated sites (WSRC, 2004).

Phytoremediation is the *in situ* use of plants and their associated microorganisms to degrade, contain or render harmless contaminants in soil or groundwater that can be accessed by the roots of plants (Cunningham et al. 1996; Li et al. 2005; Schnoor, 2002). Even though the use of plants in remediation of contaminants is not new, phytoremediation has evolved in the past decade as a technology for the treatment of contaminated soil and groundwater offering low costs and less landscape disruption (Li et al., 2005).

Processes associated with phytoremediation such as plant uptake, translocation, and transformation have been investigated explicitly, which contribute to achieving more effectiveness in the application of this technique (Orchard et al., 2000). The uptake featured by plants is the basis for phytoremediation studies because of their influence on xenobiotic levels in sediments and/or soils (WSRC, 2004). In accordance with scientific evidence, biodegradation studies involving a plant/soil rhizosphere system where microbial activity, diversity and biomass are greater, offer many possibilities of enhanced microbial remediation of contaminants (WSRC, 2004).

Highly lipophilic contaminants characterized by K_{ow}> 3 are likely to be absorbed by plants roots from water, then specific microorganisms developed around the roots known as dehalorespirers tend to render these compounds for further degradation and ultimate mineralization through a process called reductive dechlorination (Li et al., 2005). In nature, reductive and oxidative processes operate together, but in groundwater and sediment

microenvironments usually lacking of oxygen, the key to achieve biodegradation of highly chlorinated compounds is represented by reductive dehalogenation (Lee et al., 1998). The results from recent microbial and microcosm experiments obtained during phytoremediation studies has shown how halorespiring species may play a significant remedial role as well as other dehalogenating species that may involve synergistic interactions (WSRC, 2004). Regarding the role of microorganisms during remediation, Anderson et al. (1993) has shown that microbial consortia, rather than individual microbial species, are likely to be involved in the degradation of numerous toxicants in the rhizosphere.

In the present study, 1,2,3,4-tetrachlorobenzene (1,2,3,4-TeCB) was selected for experimental purposes based on its physical/chemical properties, because it has been described as one of the most hydrophobic chlorobenzene that could be degraded by certain dehalorespirers (Adrian et al., 2000), and because it is expected to persist in sediments. The fate of 1,2,3,4-tetrachlorobenzene in planted systems is of particular importance because of its occurrence as sediment and groundwater contaminant. This study also consisted of providing an update on the current knowledge on metabolic and phylogenetic diversity of dehalorespirers. Therefore, the objective of the work presented herein was to study the influence of Typha *latifolia* and Phragmites *communis* root matter on degradation of aged 1,2,3,4-tetracholorobenzene in bayou sediments focusing on the interaction between roots, microbes and desorption resistant fractions.

CHAPTER 2. LITERATURE REVIEW

2.1 Effects of Aging in Sediments

According to a WSRC report (2004), the aging process is a time dependent sorption which results in sequestration of contaminants in sediments; sequestration that depends on the physical/chemical properties of the contaminant molecule and its surrounding environment in the sediment matrix. The report also pointed out that chemical bond formation with the sediment matrix does not occur for chlorinated hydrocarbons and as a consequence there is no permanent sequestration of the molecule. Instead, the report found that a non-linear release of the neutral hydrocarbon compounds is exhibited as a result of a resistant fraction in the sediment.

Kan et al. (1998) analyzed seven organic compounds for water solubility ranging from 0.005 to 517 mg/L during multiple cycles of adsorption/desorption experiments in natural sediments. The results also showed that fractions of those compounds exhibited irreversible sorption behavior, yet the compounds were not covalently bonded to the sediment. This was taken to indicate the presence of contaminant resistant fractions.

Several studies have reported that aging of soils has increased the resistance of the contaminants to biodegradation, suggesting the possibility of formation of strong bonds between sediment and organic compounds (Hatzinger and Alexander, 1995). This phenomenon, also known as desorption resistance of organic compounds from the sediment, was explained by a finite sized desorption-resistant compartment which, once full, represents reversibility of desorption and adsorption processes (Gomez et al., 2006). Other studies report that hydrophobic contaminants become sequestered in the soil matrix with increasing age, becoming less extractable and bioavailable (Huesemann et al., 2004).

Cornelissen et al. (1997) explained in their work that the aging process leads to two stages for desorption of organic chemicals from sediment. First, a rapid release of a labile fraction and second, a slow release of a nonlabile fraction, with the last being kinetically limited and diffusional retarded within the organic matter matrix or within narrow intraparticle pores or micropores.

Nevertheless, aging processes simulated in the lab may report different results from those where field-contaminated and aged materials are used. Thus, these experimental artifacts must be considered when drawing conclusions (Pavlostathis et al. 2003; Huesemann et al. 2004). Low contaminant bioavailability may control the rate and extent of dechlorination in subsurface systems, especially those with long-term contamination. For example, long residence time of the contaminants and high degree of sequestration was assumed responsible for low rates of soil-sorbed PCE and TCE desorption and dechlorination (Pavlostathis et al., 2003). Braida et al. (2004) on the other hand, has shown how bioremediation ceases before all the contaminants are degraded mainly due to rate-limiting desorption, which is increased by long contaminant-soil contact times.

2.2 Bioavailability of Contaminants from Desorption Resistant Fractions in Sediments

In the Terminology Reference System from the EPA, bioavailability is defined as a measure of the physicochemical access that a toxicant has to the biological processes of an organism. The lower the bioavailability of a toxicant, the less its toxic effect on an organism (USEPA, 2006). Huesemann et al. (2004) investigated the bioavailability of PAHs and alkanes in aged soils using abiotic desorption assays. The study also considered the fate and bioavailability of contaminants in aged soils enriched with microbial populations of aerobic heterotrophs and hydrocarbon degraders in order to determine the extent of biodegradation. Based on this study, they concluded that bioremediation could be limited by microbial reaction rates or otherwise by

mass transfer, suggesting also that it would be inappropriate to assume that residual contaminants are recalcitrant because of bioavailability limitations unless conclusions are supported by a reliable test regarding an abiotic contaminant release.

According to Lee et al. (2003), sorption processes play an important role in biodegradation, bioavailability, and subsurface transport of various organic chemicals. In one of their studies, it was demonstrated that the extent of bioavailability of desorption resistant 1,4-DCB decreased with aging. In other words, aging may decrease bioavailability and reduce biodegradation rates albeit it was evident that aged, sorbed contaminants were indeed bioavailable and subject to environmental fate processes such as biodegradation.

Contaminant aging is presented as a critical factor affecting desorption resistance, and consequently the limited bioavailability. Furthermore, it has been suggested that if sequestration of organic molecules requires entrapment within the soil structure, disruption of the structural integrity might increase the bioavailability of the compounds (Hatzinger and Alexander, 1995).

Studies have demonstrated that the aged fraction of organic compounds may result from the slow diffusion of contaminants within some components of solid organic matter in soils (Brusseau et al., 1991[a,b]). In addition, sediment organic matter heterogeneity in sediments is shown to impact the sorption behavior of contaminants (Karapanagioti et al., 2000). Studies have revealed that slow release of contaminant from soils and sediments is highly dependent on the characteristics of the sediment organic matter (WSRC, 2004). Bucheli and Gustafsson (2000) quantified high soot-water distribution coefficients of PAHs which represented on a sorbent carbon-atom basis 25-50 times stronger sorption than predicted for same compounds interactions with bulk natural organic matter. In addition, they implied that such extremely short equilibration times would lend further support to the concept that adsorption sites are mainly limited to the rapidly accessible exterior surface of the soot matter.

Understanding bioavailability has been considered important during risk assessment of soil contaminants and during the selection of the appropriate remediation technologies for polluted sites especially those where plant uptake may represent a significant fate process (Braida et al., 2004). However, although significant research has been conducted on this topic, few studies have focused on the bioavailability of contaminants in planted sediments containing only the desorption-resistant fraction.

2.3 Desorption Kinetics of Chlorobenzenes

The adsorption and desorption of chemicals into the soil and sediments involves many different binding mechanisms with different strengths and time constants. The mechanisms include ion exchange, van der Waals forces, hydrogen bonding, charge-transfer complexation, hydrophobic interactions and covalent bonding (Jantunen, 2004). In several cases, desorption kinetics have been described by using either a two or a three-compartment first-rate model (Lee et al., 2004; Cornelissen et al., 2001).

Studies using plants have defined desorption of contaminants from as the first step of a three-step process occurring before plant uptake takes place (Gomez, 2001). It has been shown that during compounds desorption, two different sorption behaviors, linear and Langmuir-like sorption, usually occur. A linear isotherm describes sorption of the rapid released or labile fraction, while the Langmuir-type sorption isotherm describes the slow-desorbing or non-labile fraction (Cornelissen et al., 2001; Gomez, 2001).

According to Schnoor (2002), sorption can be reversed using planted systems, and it can be measured using standard sorption isotherms as mentioned above. However, when sorption is not reversible, the contaminant has undergone a chemical and/or biological reaction at the root surface that it cannot be easily translocated within the plant (e.g. hydrophobic chemicals with a log K_{ow}>3.5). Hydrophobic compounds such as tetrachlorobenzenes (log K_{ow}=4.6) are most

likely suitable candidates to undergo two different processes; phytostabilization, which refers to holding contaminated soils in place by use of rooted vegetation and by immobilizing the contaminants within the soil (Schnoor, 2002), or rhizosphere bioremediation which consists of transforming contaminants at the root surface by extracellular enzymes or by membrane-bound enzymes (Schnoor, 2002). Furthermore, these processes are appropriate considering the fact that the more hydrophobic the compound the greater the partitioning of it onto roots.

Studies on bioavailability of reversibly sorbed and desorption-resistant 1,3-DCB from a superfund site soil was done by Lee et al. (2004). They used an empirical model developed by Opdyke and Loehr (1999) where a biphasic desorption behavior (readily desorbing and desorption-resistant fractions) was considered, and found that 70% of the sorbed 1,3-DCB was desorbed in about 5 days whereas 20-30% of the sorbed contaminant remained in the desorption-resistant compartment.

Cornelissen et al. (2001) showed that desorption in a contaminated sediment was represented by three different fractions (rapidly, slowly, and very slowly desorbing) following distinct first-order kinetics. Then, a three-compartment model was applied obtaining rapidly desorbing fractions for chlorobenzenes in the low range of 0.004 to 0.08. These findings supported the fact that magnitude of rapidly desorbing fraction (F_{rap}) may vary considerably due to variation in nonlinear sorption capacity. Nevertheless, for many compound-sediment combinations, F_{rap} was found to be comparable to the slowly desorbing fraction (F_{slow}) while the amount linearly sorbed was about two times the amount desorbed to Tenax on a contact time of 6 h.

Finally, it is important to mention that kinetics for uptake of chlorobenzenes have not only been studied for sediments but also for water. Chlorobenzenes uptake from water by tissues of a soybean plant under artificial laboratory conditions was studied by Tam et al. (1996). The

data was analyzed using the simplest expression for uptake represented by a first-order one-compartment equation (conventionally used for uptake of chemicals by fish from water). The uptake of 1,2-DCB, 1,2,4-TCB, and 1,2,3,5-TeCB was found to range from 30% to 65% in the first 2 h consistently with previous studies. Thus, the results also suggested that the equilibrium partitioning of hydrophobic chemicals from water to plant tissues depends on the hydrophobicity of the chemical (as expressed by K_{ow}).

2.4 Role of Plants in Degradation of Contaminants

The economic, aesthetic, and environmental benefits, in addition to the need for more cost-effective approaches, has converted plant-based remediation into an emerging feasible method for treatment of contaminated sites increasing dramatically in recent years (Aitchison et al., 2000; Braida et al., 2004; Li et al., 2005; WSRC, 2004). Several studies have been carried out in contaminated sites close to wetlands and decreases have been attributed to microbial processes that are supported by vegetation (Pardue et al., 2000). The term of phytoremediation first appeared in technical literature in 1994 (Schnoor, 2002). Phytoremediation (also called green remediation, botano-remediation, agroremediation, and vegetative remediation) is the use of plants to partially or substantially remediate selected contaminants present in soil, sludge, sediment, ground water, surface water, and waste water (Pivetz, 2001). Despite of whether aquatic or terrestrial plants, their contribution involve biological processes and physical characteristics. Based on the preceding discussion, it can be argued that a clear understanding of the contaminants speciation and interactions between contaminants and nutrients may lead to obtaining better results.

In addition to phytostabilization and rhizosphere bioremediation, phytoremediation also encompasses a number of other different mechanisms that can lead to contaminant degradation by various biotic or abiotic processes; removal (through extraction, accumulation or

volatilization); immobilization; hydraulic control; and control of runoff, erosion, and infiltration by vegetative covers (USEPA, 2000; Li et al., 2005). The degradation pathways for uptake of contaminants by plants represent the destruction or alteration of the organic contaminant in the root zone or plant by the release of exudates and enzymes that stimulate microbial activity and biochemical transformations (WSRC, 2004). These two pathways are *rhizodegradation* or enhancement of biodegradation in the below-ground root zone by microorganisms; and *phytodegradation* or contaminant uptake and metabolism above or below ground, within the root, stem, or leaves (Pivetz, 2001). In cases where contaminants are highly hydrophobic, have low solubility and volatility, the first pathway is predominant and it is controlled by the properties of the contaminant, the plant species, and the soil (Briggs et al., 1982; Bell, 1992; Schnoor, 1997). Extremely lipophilic compounds (log K_{ow}>4.0) were so strongly bound to the roots that were not further translocated to other plant tissues (Trapp et al., 1994), in agreement with Pivetz (2001) and Schnoor et al. (1995) suggested that the more hydrophobic the compounds the more bound to root surfaces or partition into root solids, resulting in less translocation within the plant.

In many reported studies, plant roots and the associated microorganisms are presented as one of the most promising *in situ* biological remediation of chemically contaminated soils (Anderson et al., 1993; Schnoor et al., 1995). Generally, during phytoremediation the root system serves as a source of organic substrates that induces substances for microbes in the rhizosphere and therefore stimulates aerobic degradation (Schnoor, 2002). During treatment of moderately hydrophobic contaminants (log K_{ow}=1 to 3.5), phytoremediation has shown the ability to remove significant quantities of organic contaminants from saturated zones and helped to reduce the migration of these soluble contaminants (Schnoor, 2002).

Experiments on chlorobenzene uptake by tissues of a soybean plant showed that the use of plants contributed in enhancing rates of soil remediation by providing a pathway from soil to the atmosphere as well as a substrate for biodegradation (Tam et al., 1996). Furthermore, a study reported by Li et al. (2005), using wheat seedlings demonstrated that plant lipids were primarily responsible for the sorption of LDN and HCB by the seedlings. Because the plant sorption occurs essentially by partition, the consistently higher sorption with shoots than with roots may be related to their compositions. It was evident that there was a predominant role played by plant lipids over other plant constituents in the uptake of high-lipophilic solutes. The results suggested as well that the solute partition with plant lipids (K_{lip}) could be significantly higher than that estimated by the K_{ow}.

Gomez et al. (XXX) studied the uptake of contaminants from the desorption-resistant compartment of sediments by using plants. The influence of shoots and roots of two wetland plants (*Salix nigra* and *Scirpus olneyi*) in the uptake of aged contaminants was analyzed. The two plants were shown to play an important role in the uptake of highly lipophilic and hydrophobic contaminants such as phenanthrene and chlorobenzenes. The uptake of chlorobenzenes from the desorption-resistant treatment increased over time and ranged from 1-12% for *Scirpus* and from 1.5-5.5% for *Salix* over a period of 5 weeks. The distribution of ^{14}C in the plant indicated that the majority of mass remaining in the plant resides in the root zone (roots and stem) in contact with the sediment. Moreover, results from a simple modeling approach demonstrated that uptake of aged contaminants is possible albeit slow and can be predicted using a sorption mechanism and not from a translocation model.

Lin (2003), studied the dechlorination of 1,2,3,4-TeCB in organic matter and mineral dominated soils as well as in sediments amended with roots of a wetland plant called *Typha latifolia*. It was shown that plant roots were responsible for increasing diverse microbial

communities which contributed to the enhancement of biodegradation activities. Although there were no significant differences in the dechlorination pathways between treatments with roots and without roots, dechlorination kinetics increased with increasing amounts of roots, indicating that *Typha* root matter strongly benefited biodegradation of chlorobenzenes.

Other researchers have drawn similar conclusions during degradation studies that have used vegetation. The presence of vegetation serves to increase the amount of organic carbon in the soil which, in turn, stimulates microbial activity (Xu and Jaffe, 2003; Schnoor, 1995). The nature of organic matter has been reported to play a significant role in the sorptive behaviors of sediments, and organic carbon content was able to normalize distribution coefficients of some pollutants such as 1,1-DCA and *cis*-1,2-DCE (Kassenga et al., 2003).

2.5 Role of Microbial Populations in Dechlorination

Haloorganic compounds of natural origin have primed the development of bacteria and novel microorganisms that dehalogenate or completely mineralize them, preventing an uncontrolled spread and accumulation in the environment (Smidt et al., 2004; Lee et al., 1998). Recent studies have focused their attention on chemical compounds' degradation pathways and have also analyzed and quantified developing microbial populations trying to establish a relationship between the microorganisms numbers and the rate of degradation (Adrian et al., 2000; Lee et al., 1998; Lendvay et al., 2003; Lin, 2003; Pavlostathis et al., 2003; Smidt et al., 2004; WSRC, 2004; Wu et al., 2002; Yang et al., 2005).

According to Smidt et al. (2004), quantification of developing dechlorinating populations in vegetated treatments could make it possible to establish a relationship between their number and the rate of chlorobenzene removal. This publication provided an update on metabolic and phylogenetic diversity of anaerobic microorganisms that are capable of dehalogenating-or completely mineralizing-halogenated hydrocarbons by fermentative, oxidative, or reductive

pathways. They focused on dehalorespiring anaerobes, which couple the dehalogenation by dedicated enzyme systems to the generation of energy by electron transport-driven phosphorylation. Hence, the results can be useful for further exploitation of these microorganisms in biological remediation processes.

Pavlostathis et al. (2003) defined halorespiration as a real promise for *in situ* bioremediation applications if the rates of dechlorination are shown to be higher than in co-metabolic dechlorination process. Generally, anaerobic environments enhance halorespiration process whereas in environments such as wetlands, rich in organics and accompanied by intense methanogenic or sulfidogenic respiration, partial co-metabolic dechlorination of solvents can be significant (Lee et al., 1998).

Different perspectives on microbial dehalogenation of chlorinated solvents were addressed by Lee et al. (1998). Reductive dehalogenation was shown to play an important role in the future of engineered *in situ* bioremediation technologies due to its potential for completely dechlorinating fully chlorinated solvents such as PCE. They also observed that chlorinated solvent plumes may be self-enriching for dehalogenating bacteria due to the energy-yielding of the reactions. Additionally, they reported that reducing environments with relatively high concentrations of dissolved organics as well as high concentrations of methane and dissolved iron are more likely to sustain organisms capable of complete reductive dehalogenation resulting in branched biodegradation pathways.

Biodegradation of some sorbed chemicals has relied on the presence of appropriate microorganisms and high mass-transfer rates from the sorbed state to the microorganism. For example when *Pseudomonas* strain R were used as phenanthrene degrader, similarity was found between desorption and mineralization curves suggesting that phenanthrene must leave the sorbed state before being metabolized by the microorganisms. Hence, microbial degradation was

shown to be controlled by desorption, even more, when contaminant-soil contact times were increased (Braida et al., 2004).

The low G+C gram-positive genus *Desulfitobacterium* and the deeply branching *Dehalococcoides* group comprise to date the largest number of reductively dehalogenating pure cultures according to Smidt et al. (2004). Both show a large diversity with respect to the used halogenated electron acceptors. *Desulfitobacterium* includes a large number of versatile strains that reductively dehalogenate chlorinated ethenes and haloalkanes, haloaromatics, or both; and show relatively rapid growth. *Desulfitobacterium dichloroeliminans* strain DCA 1, is the first bacterial isolate that completely dechlorinates vicinal dichloroalkanes, including 1,2-DCA, by dihaloelimination (Smidt et al., 2004; De Wildeman et al., 2003).

Several pure isolates of the genus *Dehalococcoides* are now available growing at the expense of a broad variety of chlorinated benzenes, organohalides, dioxins, chlorinated alkanes and alkenes, among others. Recent findings have shown that *Dehalococcoides* species comprise metabolically and phylogenetically distinct subgroups. *Dehalococcoides* strains, such as CDBD1, which is part of the Pinellas cluster of *Dehalococcoides*, reduces 1,2,3-TCB, 1,2,4-TCB, 1,2,3,4-TeCB, 1,2,3,5-TeCB, or 1,2,4,5-TeCB to dichlorobenzenes or 1,3,5-TCB (Adrian et al., 2000; Hölscher et al., 2003; Smidt et al., 2004; WSRC, 2004).

Halorespirers on the other hand, can unlock halogenated compounds for further consumption by dehalogenation using reductive dehalogenases (RDs) which are the key catalysts in the respiratory chain of halorespiring microorganisms. There are different classes studied extensively from aerobic bacteria. However, in recent years information about these enzymes obtained from anaerobic microorganisms just started to accumulate (Smidt et al., 2004).

Chlorobenzene dehalogenase activity in crude extracts of *Dehalococcoides* sp. strain CBDB1 revealed exceptionally high specific activities for 1,2,3,4-TeCB and QCB, which were

approximately 10-fold higher than other known RD activities. Pre-growth on different congeners however, indicated that distinct RDs might be differentially induced by different electron acceptors (Adrian et al., 2000; Smidt et al., 2004).

Wu et al. (2002) observed that polychlorinated biphenyl (PCB) dechlorinating bacterium DF-1 was able to dechlorinate HCB, QCB, 1,2,3,5-TeCB, 1,3,5-TCB, but it did not dechlorinate other tetrachlorobenzenes or any trichlorobenzenes. They also argued that from tetrachlorobenzenes to hexachlorobenzene, compounds tend to resist aerobic degradation but have been shown to be susceptible to anaerobic dechlorination. Within 56 days of their study, the culture with bacterium DF-1 dechlorinated 100% of HCB, or QCB at equivalent concentration, to 1,3,5-TCB through 1,2,3,5-TeCB. However, isomers 1,2,3-TCB, 1,2,4-TCB, 1,3,5-TCB, 1,2,3,4-TeCB, or 1,2,4,5-TeCB were not dechlorinated within 56 days of incubation. The dechlorinating activity was very restricted and only directed at selected double flanked chlorines (Wu et al., 2002).

As mentioned before, the behavior of halorespiring bacteria can be sensitive to different environmental conditions. From a thermodynamic point of view, hydrogenotrophic halorespiring bacteria should out-compete hydrogenotrophic sulfate reducers, acetogens, and methanogens in environments where hydrogen is the main source of electrons (Smidt et al., 2004). Halorespiring microorganisms can also serve as cellular electron acceptors in tight syntrophic interactions with hydrogen-producing populations. In addition, the slow release of hydrogen by obligate syntrophic fatty acid-fermenting populations favors reductive dehalogenators over methanogens and acetogens. Nevertheless, halorespiring populations must be present in adequate abundance to sufficiently lower the partial hydrogen pressure and win the competition with other hydrogenotrophic microorganisms (Smidt et al., 2004). Acetate on the other hand, is also one of the main intermediates during the anaerobic degradation of organic matter, and has recently been

acknowledged as another highly relevant source of electrons for reductively dehalogenating populations (Adrian et al., 2000).

2.6 Relationships between Trace Gases during Anaerobic Dechlorination

According to Conrad (1996), some of the trace gases are specifically metabolized in the rhizosphere. In wetland soils, a typical environment devoid of O_2 (below 2 to 3 mm deep), CO_2 and H^+ are the predominant electron acceptors. Thus, this zone is dominated by fermentation and methanogenesis, which suggests that production of H_2 by fermentation, is immediately converted by methanogens into CH_4. Hydrogen consumption by sulfate reducers takes place due to the fact that sulfate reducing conditions can be also quite extensive in wetland areas. In steady state, sulfate reducers lower the H_2 concentration to inferior values than found in the methanogenic zone; creating a flux of H_2 from the methanogenic zone into the SO_4^{2-}- reducing zone (Conrad, 1996). A study by Kimura et al. (1991) on trace gases (CO_2, H_2, and CH_4) production in rice rhizosphere also supports the foregoing notion. They found that H_2 was produced by microorganisms on and/or inside roots, and, that not only root exudates (sugars, amino acids, organic acids) but also H_2 and CO_2 produced, contributed to CH_4 production in the rhizosphere. They also observed a competition for root exudates and H_2 between the CH_4-producing bacteria and sulfate-reducing bacteria. However, sulfate reduction was dominant over CH_4 production in the rice rhizosphere.

During a study about dechlorination of 1,2,3,4-TeCB in organic matter-and mineral-dominated soils, Li (2003) obtained H_2 concentration trends that in some treatments suggested that H_2 was probably used as an electron donor during methanogenesis and for driving dechlorination reactions. Nevertheless, methane started to accumulate after 1,2,3,4-TeCB and daughter products were completely degraded in most active control microcosms, indicating a probable responsibility of dechlorinators that may have out competed methanogens for electron

donors. However, the relationship between H_2 concentrations and/or dechlorination was not clearly established in this study. In contrast, previous work presented by Adrian et al. (2000), found that cultures of the oxygen sensitive strain CBDB1, capable of dechlorination of chlorobenzenes, showed high dechlorinating activity when restricted only to TCB, hydrogen and acetate.

Recently, Luijten et al. (2004) reviewed the role of pure cultures of halorespiring bacteria in hydrogen concentrations. They observed that these specific bacteria have a high affinity for H_2 as if H_2 was the most important electron donor for halorespiration in soil. In this report, they also showed that halorespiring organisms were capable of competing methanogenic archaea, acetogenic bacteria, and sulphate-reducing bacteria at low H_2 concentrations. Furthermore, the analyses of H_2 concentrations obtained from a site polluted with chlorinated ethenes showed that only where high concentrations of H_2 were observed, a complete dechlorination had taken place. Hence, the study suggested that *in situ* measurements of H_2 could indicate the extent of anaerobic reductive dechlorination.

CHAPTER 3. MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals

1,2,3,4-Tetrachlorobenzene (1,2,3,4-TeCB) from Aldrich (Aldrich Chemical Company, Inc) was used for contaminating the sediment from bayou. Semivolatiles analyses used internal standard mix supplied by Supelco (Bellefonte, PA) according to the EPA Method 8270. Likewise, Supelco provided internal standards mix and surrogate standards for analysis of volatiles according to the EPA Method 8260. Isopropanol was obtained from Sigma (Sigma-Aldrich Co., St. Louis). An electrolyte solution was made of $CaCl_2*H_2O$ (Mallinckrodt® LabGuard™, Germany) and NaCl crystals (EM Industries, Inc). Solvents such as Hexane HPLC grade (EMD Chemicals Inc., Germany), Acetone Pesticide grade (Fisher Scientific, Canada) and Methanol (Fisher Scientific, Canada) were also used during this study.

3.1.2 Soil

The sediment selected came from a nearby natural water course known as Bayou Fountain (Baton Rouge, LA) during the summer of 2004. The sediment was collected at 30 cm below the sediment's surface. Any kind of leaves and other debris was removed manually from the bulk soil for better particle homogenization. The water content was adjusted to a ratio of 1:1 (soil to water) using natural water collected also from Bayou Fountain to facilitate the preparation of microcosms and further analyses.

Total organic matter (OM) of the test soil was estimated by weighing oven-dried soil before (103ºC for 24 hrs) and after combustion at 550ºC for 24 hrs (as cited by Lin, 2003). Likewise, total organic carbon content (TOC) was calculated, in agreement with Lin (2003), from OM using a division factor of 1.7 (i.e., TOC=OM/1.7). Results obtained from the

laboratory are shown in Table 3.1. It was also assumed that the low amounts of methanol in the 1,2,3,4-TeCB solution did not interfere with the generation of dissolved organic carbon (DOC) because of the low organic carbon affinity of methanol (Cornelissen et al., 1997).

Table 3.1: Total organic matter and total organic carbon from sediment at Bayou Fountain.

Fraction of OM (g/g), %	OM Standard Deviation, %	TOC, %	TOC Standard Deviation, %
7	0.13	4.1	0.07

3.1.3 Plants

Live roots from two different types of plants were considered in this study: a dense rooted plant from the Cattail family (*Typhaceae*), *Typha latifolia* and one plant from the Grass family (*Poaceae*), *Phragmites communis*. These plants were provided by Hydra's wetland plants nursery and grown in greenhouse. Both plants selected have been found indigenous herbaceous wetland vegetation with different detrital pathways. In recent studies, *Typha latifolia* has been used with positive outcomes for enhancement of recalcitrant chlorobenzenes biodegradation (Lin, 2003).

3.2 Experimental Procedure

3.2.1 Slurry Preparation and Aging

The water content in the sediment was adjusted to a ratio of 1:1 (sediment to water) using water from the bayou. The prepared slurry was poured into 1 L polypropylene autoclavable jars. The jars containing the slurry were autoclaved for 15 min at 121ºC. The slurry was allowed to cool off for a period of 30 min. And, after the cooling period was over, 1,2,3,4-TeCB was dissolved in methanol (Lin, 2003). Then, 25 ml of that solution of 1,2,3,4-TeCB at a concentration of 20,000 ppm was added to each jar. The contaminated slurry contained in jars was immediately flushed with nitrogen gas at 1 atm for about 10 min inside a fume hood trying

to limit possible effects of methanol on dechlorination. Later, the jars were placed in a tumbler for 11 days. This period of 11 days was intended to allow a homogenous mix and to affect 1,2,3,4-TeCB biodegradability and the ease of its extraction. The jars were centrifuged using an Avanti® J-20 XPI expanded-performance centrifuge (Beckman Coulter, CA) at 5,000 rpm for 10 min and the supernatant was discarded. Isopropanol extraction was used to accelerate desorption altogether with an electrolyte solution. All discarded volume was substituted with a solution of 50% Isopropanol and 50% electrolyte solution to remove easily desorbed 1,2,3,4-TeCB from the sediment (procedure cited in Gomez, 2001). The slurry in the jars was shaken and placed in the tumbler for 24 hrs. The next step corresponded to centrifugation (same speed mentioned above) followed by discarding supernatant. Two more washes were done following the same procedure as above but in this case all the supernatant volume was discarded and replaced by the electrolyte solution.

3.2.2 Microcosms Preparation

Roots from the two plants were collected from the greenhouse, washed several times with tap water followed by deionized water and 5 g of each root were introduced into the 160 ml microcosm bottles. For this study it was decided that 5 g were appropriate based on the results obtained by Lin (2003), who found that the greatest dechlorination kinetics were obtained using 5 g of roots in root amended treatments. These bottles were filled with 50 ml of aged slurry and 50 ml of natural slurry (similar water to sediment ratio of 1:1) leaving a 40 ml headspace. Natural slurry was unaged and uncontaminated. Its purpose was to reestablish the microbial population in the microcosm. Microcosms were sealed with Teflon lined rubber septa and aluminum crimp seals. The microcosms were neither amended with electron donors nor nutritional supplements to support microbial growth. A total of 3 treatments were prepared inside a glove bag (I^2R, Cheltenham, PA): Control (no roots), treatment amended with *Phragmites*

roots, and treatment amended with *Typha* roots, as it is shown in Table 3.2. Each treatment was prepared in two set of triplicates considering that one set of triplicates was used for gas analyses and the other for chlorobenzenes and molecular analysis. The final step corresponded to the accommodation of the bottles upside down and incubation under static and dark conditions in a constant temperature room (~25ºC).

Table 3.2: List of treatments based on the amounts of *Typha* and *Phragmites* roots.

Treatments	Mass of roots, g	Mass of slurry, g	Ratio of roots to sediment, R/S (g/g), %
Control (no roots)	0	130	0
Amended with *Typha latifolia* roots	5	130	3.85
Amended with *Phragmites communis* roots	5	130	3.85

Slurry characteristics: moisture content=55%, density=1.3 kg/l.

3.2.3 Microcosms Sampling

3.2.3.1 Monitoring of 1,2,3,4-TeCB Degradation

The parent and daughter dechlorination products were monitored by taking contaminated slurry from the bottles under anaerobic conditions for a period of one month on specific days: 0, 3, 6, 10, 14, 20, and 30. The set of bottles corresponding to degradation studies were first taken from the constant temperature room and placed inside of a glove bag (I^2R, Cheltenham, PA). The bottles were shaken before opening and 5 ml of sediment slurry was poured into a pre-weighed Teflon centrifuge tube (in order to minimize the adsorption of chlorinated benzenes). Then, the slurry was mixed with 10 ml of a Hexane/Acetone solution (v/v =1:1). This process was performed on all the 3 treatments and microcosm bottles were capped again under the same

anaerobic conditions. The Teflon centrifuge tubes were closed and shaken, therefore tumbled for 24 hrs facilitating this way the extraction of chlorobenzenes. The suspension was then centrifuged using an Avanti® J-20 XPI expanded-performance centrifuge (Beckman Coulter, CA) at 3,000 rpm for 20 min. One milliliter of supernatant was transferred into an amber 2 ml GC-MS vial for analysis of semivolatile chlorobenzenes using an autosampler on a gas chromatograph-mass selective detector.

The hexane extract was analyzed following the EPA Method 8270 for the measurement of semivolatile chlorinated benzenes (Lin, 2003). Ten µl of semivolatile internal standard mix (6 analytes, 2000 µg/ml each in methylene chloride, containing 1,4-dichlorobenzene-d_4, naphthalene-d_8, acenaphthene-d_{10}, phenanthrene-d_{10}, chrysene-d_{12}, and perylene-d_{12}) (Supelco Chemical Co., Bellefonte, PA) was injected into 1 ml hexane extract. The sample was analyzed then by a GC-MS (Agilent 6890 series gas chromatograph-5972A mass selective detector). The GC was equipped with a capillary column (DB-5, 30 m × 0.25 mm i.d., 0.25 µm film thickness) which was directly interfaced to the mass spectrometer. Ultra-high-pure (UHP) compressed helium (Capitol Welders Supply Co. Inc, Baton Rouge) was used as a carrier gas at a flow rate of 1.8 ml/min. The injector temperature was 250ºC. The GC column was initially held at 37ºC for 2 min, then ramped to 260ºC at 8ºC/min, and finally ramped to 300ºC at 40ºC/min and held for 10 min. The detector temperature was maintained at 280ºC. Remaining samples in Teflon centrifuge tubes were separately weighed wet as well as dry (110ºC for 24 hours) to determine the mass of contaminant per mass of sediment once the supernatant was extracted.

On the other hand, aqueous samples for analysis of benzene were directly withdrawn from microcosms using a gas tight syringe and transferred into autosampler vials. The analysis of benzene was performed by the EPA Method 8260 using a purge and trap volatiles concentrator attached to an Agilent 6890 Series Gas Chromatograph equipped with a 5972A mass selective

detector. A thermal desorption trap (VOCARB 3000; Supelco, Bellefonte, PA) was employed in the purge and trap apparatus. The hexane extract along with 10 µl internal standard (3 analytes, 25 µg/ml each in methanol, containing chlorobenzene-d_5, 1,4-dichlorobenzene-d_4, fluorobenzene) (Supelco Chemical Co., Bellefonte, PA) and 2.5 µl surrogate (Supelco, Bellefonte, PA) was manually injected into the purge and trap autosampler (Tekmar 2016; Tekmar-Dohrmann, Mason, OH), and purged for 11 min with UHP compressed helium at a flow rate of 35 ml/min. Then, the samples were desorbed for 0.5 min and baked for 13 min at 225ºC. Furthermore, samples were introduced onto the GC equipped with a 60 m × 0.32 mm × 3 µm film, Agilent 5MS capillary column (Palo Alto, CA). UHP helium gas was used as a carrier gas at a flow rate of 2.1 ml/min. The GC column temperature program was 35ºC for 5 min, and ramped at 4ºC/min to a final temperature of 200ºC. The temperatures of the injector and detector were 250ºC and 280ºC, respectively.

3.2.3.2 Monitoring of Gases

Two different gases, hydrogen and methane, were monitored using a Gas Chromatograph/Reduction Gas Detector (GC/RGD) (Trace Analytical, Menlo Park, CA) and a Gas Chromatograph/Flame Ionization Detector (GC/FID Agilent 5890 series II) respectively. Hydrogen and methane concentrations were measured in order to evaluate microbial metabolism (e.g., metabolism of methanogens) during 1,2,3,4-TeCB degradation. These gases were analyzed for a period of one month on specific days: 0, 3, 6, 10, 14, 20, and 30; similar to the 1,2,3,4-TeCB degradation analysis. The bottles were placed right side up for at least 30 min before sampling to allow them to reach equilibrium. For the hydrogen analyses, 3 ml of sample were taken using a gas tight syringe from the headspace of each bottle following manually injection into a 1 ml gas sampling loop, and then separated with a molecular sieve analytical column (Trace Analytical, Menlo Park, CA) at an oven temperature of 40ºC. UHP nitrogen (Capitol

Welders Supply Co. Inc, Baton Rouge) was used as a carrier gas; and all hydrogen data were reported as aqueous concentration.

Aqueous concentration of H_2 was calculated following the equation adopted from Löffler et al. (1997):

$$\left[H_{2,aq.}\right] = \frac{LP}{RT} \quad \text{(Eq. 1)}$$

where $H_{2,aq.}$ is the aqueous concentration of H_2 (moles/l);

L is the Ostwald coefficient for H_2 solubility (0.01913 at 25ºC);

P is the partial pressure of H_2 (atm);

R is the universal gas constant (0.0821 liter·atm·K^{-1}·mol^{-1});

And T is the temperature (K).

$$P = \frac{C}{10^6} \quad \text{(Eq. 2)}$$

where C is the gas phase concentration of H_2 (ppm).

Methane present in microcosm bottles on the other hand, was analyzed withdrawing 1 ml of gas from the headspace of bottles using a gas tight syringe, and then the sample was injected into the GC/FID followed by 10 ml of UHP nitrogen. The GC/FID was equipped with a 2.4 m × 0.32 mm i.d. column packed with Carbopack b/1 % SP-1000 (Supelco, Bellefonte, PA). The injector and detector temperatures were 375ºC and 325ºC, respectively. The column temperature was held constant at 50ºC for 6.50 min. UHP nitrogen (Capitol Welders Supply Co. Inc, Baton Rouge) was used as a carrier gas at a flow rate of 12 ml/min. All methane data were reported as aqueous concentrations in μM (μmol/l). Headspace methane concentrations were therefore converted to aqueous phase concentrations using Henry's Law (Henry's constant for methane at 25ºC is 0.6364 atm/mol/m^3). Methane concentrations were calculated assuming that pressure in

the bottles was 1 atm even though it might have been slightly higher. Some pressure building up inside the bottles was noted during sampling after day 14. However, due to the fact that bottles did not break, pressure inside the bottles was less than 2 atm.

3.3 Analysis

3.3.1 Microbial Analyses

The analyses involving characterization of microbial communities that dechlorinate 1,2,3,4-TeCB in the soil amended with roots, require the use of molecular techniques such as DNA extraction, Polymerase Chain Reaction (PCR), Denaturing Gradient Gel Electrophoresis, Ligation, Transformation and Sequencing of 16S rDNA Clones. The samples were taken under anaerobic conditions at the same time samples for degradation analyses were taken. An aliquot of the slurry (~2 ml) was collected in microcentrifuge tubes and stored at -20 ºC for further analyses.

3.3.1.1 DNA Extraction from Samples

Samples from 3 different treatments: Control (no roots), treatment amended with *Phragmites* roots, and treatment amended with *Typha* roots, were collected on days 3, 10, 14, 20, and 30 for DNA extraction. All collected samples were centrifuged using an Eppendorf centrifuge 5415D (Eppendorf North America, Inc.) to discard excess water. Total genomic DNA was then extracted from 0.2-0.3 mg of the samples using the MoBio PowerSoil™ DNA kit (MoBio Laboratories, Inc., Solana Beach, CA). The manufacturer's protocol was slightly modified by including a buffer (120 mM sodium phosphate at a pH=8.0) for washing the soil samples three times before starting with first step on the manufacturer's protocol. Each sample was mixed with 1.5 ml of buffer and shaken in an incubator at room temperature (~25ºC) for 15 min. Samples were then centrifuged for 1 min and supernatant was discarded (this step was repeated two more times to complete the three washings mentioned before). The purified DNA

was suspended in 50 µl of solution S5 (MoBio Laboratories, Inc.) and stored at –20°C until further analyses.

3.3.1.2 16S rDNA Amplification and DGGE Analysis

For denaturing gradient gel electrophoresis (DGGE), initial PCR amplifications of the 16S rRNA gene of bacteria and archaea were conducted by using primers 27F and 1492R, and 25F and 1492R, respectively. The product obtained was used as a template in the second PCR for DGGE analysis. The primers 341F-GC clamp and 907R for 16S rRNA gene of bacteria, and the primers 344F-GC clamp and 518R for archaea 16S rRNA gene were used for DGGE analysis (Muyzer et al., 1998; Raskin et al., 1994). The PCR products contained 0.2 mM of each primer, 0.2 mM of each deoxynucleotide, 2 mM of $MgCl_2$, 1 U of Taq polymerase (Promega, Madison, Wis.), 1 µl of DNA extract, and 10×PCR reaction buffer to a final volume of 50 µl. PCR amplification was performed using a BioRad iCycler as follows: 95°C for 5 min, followed by 30 cycles of 94°C for 45 s, 58°C for 45 s for bacteria or 56°C for 45 s for archaea, and 72°C for 45 s and a final extension at 72°C for 10min. Then, for denaturing gradient gel electrophoresis (DGGE), a "touchdown" PCR modified from that used by Schafer and Muyzer (2001) was used as follows: initial denaturation at 95°C for 10 min, 30 sec at 95°C (denaturation), 30 sec at 65°C (annealing), and 30 sec at 72°C (elongation) with a 1°C touchdown every cycle during annealing for 10 cycles, followed by 20 cycles with an annealing temperature of 55°C in case of bacteria and 50°C for archaea and a final cycle that consisted of 10 min at 72°C. DGGE analysis of 16S rDNA fragments was performed using a DCode™ Universal Mutation Detection System (BioRad Laboratories, Hercules, CA). Gels (16 cm ×16 cm) consisted of 6% bis-acrylamide (37.5:1) and a denaturing gradient ranging from 40% to 60% denaturant (where 100% denaturant contains 7 M urea and 40% v/v formamide). Electrophoresis was performed in 1×TAE buffer (0.04 M Tris base, 0.02 M sodium acetate, and 1 mM EDTA; pH=7.4) at 60°C and 65V for 15 h.

Gels were stained for 10 min with ethidium bromide (EtBr) distained and analyzed using the Gel Doc 2000 system (BioRad Laboratories, Hercules, CA) and Quantity One software (BioRad Laboratories, Hercules, CA).

3.3.1.3 Cloning and Sequence Analysis

Some of the bands obtained in the DGGE profiles were sequenced. The middle portion of selected DGGE band was excised, and bands were transferred to a 1.5 ml microcentrifuge tube containing 50 µl of deionized water (Milli-Q Synthesis System A10, Millipore) and incubated at 4°C for 12 h. After centrifugation at 11,000 x *g* for 60 s, the supernatant was transferred to a new tube, and 1 µl of it was used as the template for subsequent PCR-DGGE analysis to check band position and purity. PCRs were performed with the DNA bands and the 341F (non-GC) and 907R primer pair for bacteria or the 344F (non-GC) and 518R primer pair for archaea. The protocol used was a modified version of that presented by Schafer and Muyzer (2001) for bacteria: initial denaturation for 10 min at 95°C; 30 cycles of 95°C (45 sec), 50°C (45 sec), and 72°C (45 sec); and extension for 10 min at 72°C. However, the same protocol varied for archaea as follows: initial denaturation for 10 min at 95°C; 30 cycles of 94°C (45 sec), 48°C (45 sec), and 72°C (45 sec); and extension for 10 min at 72°C. This procedure was repeated until a single sharp band was detected. PCR products were purified with Ultra Clean PCR Clean-Up kit (MoBio Laboratories, Inc.), and purified PCR products were then cloned into the pGEM-T easy vector, with *Escherichia coli* strain JM109 used for transformation, according to the procedure given by the manufacturer (Promega, Madison, WI.). The clones were cultured and then, plasmids were extracted by using an UltraClean Mini Plasmid Prep Kit (MoBio Laboratories, Inc.).

Purified plasmids were sequenced by using primers T7 or SP6. Sequencing reactions were performed by using an ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction

kit on an ABI 377 automated DNA sequencer (Applied Biosystems, Foster City, CA). Electropherograms were edited by using Chromas "freeware" (version 2.3; School of Health Science, Griffith University, Gold Coast Campus, Southport, Queensland, Australia), and sequences were assembled with the BioEdit version 7.0.5 (Hall, 1999).

Phylogenetic trees were constructed by using calculation of maximum-likelihood distances and by using the neighbor-joining algorithm through the BioEdit program. Likewise, the statistical significance of phylotype groups within the various trees was tested by using bootstrap analysis with the Phylip program SEQBOOT and CONSENSE (Felsenstein, Department of Genetics, University of Washington, Seattle). Finally, trees were drawn from the neighbor output by using the program TreeView (Page, 1996).

As follows is presented a summary of primers used in this study:

Table 3.3: The target positions of the PCR and sequencing primers used in this study.

Primer[a]	Positions[b]	Sequence (5' to 3')	Target
27F	11-27	GTTGATCCTGGCTCAG	Bacteria
1492R	1492-1512	ACGGYTACCTTGTTACGACTT	Prokaryotes
341F GC clamp		CGCCCGCCGCGCCCCGCGCCCGGCCCGCCG CCCCCGCCCCCCTACGGGAGGCAGCAG	Bacteria
907R	907-916	CCGTCAATTCCTTTGAGTTT	Bacteria
341F non GC clamp	341-357	CCTACGGGAGGCAGCAG	Bacteria
25F	19-25	CYGGTTGATCCTGCCRG	Archaea
344F GC clamp		CGCCCGCCGCGCCCCGCGCCCGTCCCGCCG CCCCCGCCCCACGGGGCGCAGCAGGCGCG A	Archaea
518R	518-534	ATTACCGCGGCTGCTGG	Archaea
344F non GC clamp	315-344	ACGGGGCGCAGCAGGCGCGA	Archaea

[a] The number corresponds to the *Escherichia coli* position (cited by Kaksonen et al., 2004) to which the 3' end of the primer anneals. F and R correspond to forward and reverse primer, respectively.
[b] *E. coli* numbering.
The sequence of the 40 bp GC clamp used was: CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC G.

3.3.1.4 Nucleotide Sequence Accession Numbers

Bacteria and archaea 16S rDNA sequences obtained in this study were deposited with GenBank and are available under accession numbers: DQ483028 through DQ483051.

3.3.1.5 Diagram of Molecular Analyses Steps

The complete microbial analysis is explained step by step as follows through an experimental scheme in order to provide a clear picture to the readers (See Figure 3.1).

3.3.2 Data Analysis

3.3.2.1 Kinetic Data Modeling

Degradation data from the three treatments, control, and amended with *Phragmites* and *Typha* roots, were modeled using a first-order kinetic equation by optimization of degradation kinetic data using non-linear regression techniques. The equation used for calculating the first-order reaction rate constant is shown as follows:

$$C_t = C_o e^{-kt} \quad \text{.....(Eq. 3)}$$

where t is the time (day);

C_t is the concentration at anytime t (moles/kg dry soil);

C_o is the initial concentration (moles/kg dry soil);

and k is the pseudo first-order reaction rate constant (day^{-1}).

The characteristic half-life period ($t_{1/2}$) was also calculated by using the first-order reaction rate constant (k) through the following equation (Lin, 2003):

$$t_{\frac{1}{2}} = -\left(\frac{\ln 2}{k}\right) = \frac{0.693}{k} \quad \text{.....(Eq. 4)}$$

where $t_{1/2}$ is the half-life time (days);

k is the pseudo first-order reaction rate constant (day^{-1}).

3.3.2.2 Statistical Analysis

SigmaPlot 2004 for Windows Version 9.01 was used to calculate the first-order kinetic rate constant and standard errors from a non-linear regression of kinetic data (Systat Software, Inc., Richmond, CA). On the other hand, a paired two-sample T-test from Microsoft® Office

Excel 2003 (Microsoft Corporation) was used to compare the performance of different treatments on degradation of parent compound 1,2,3,4-TeCB (moles/kg dry soil) at a significant level of 5%.

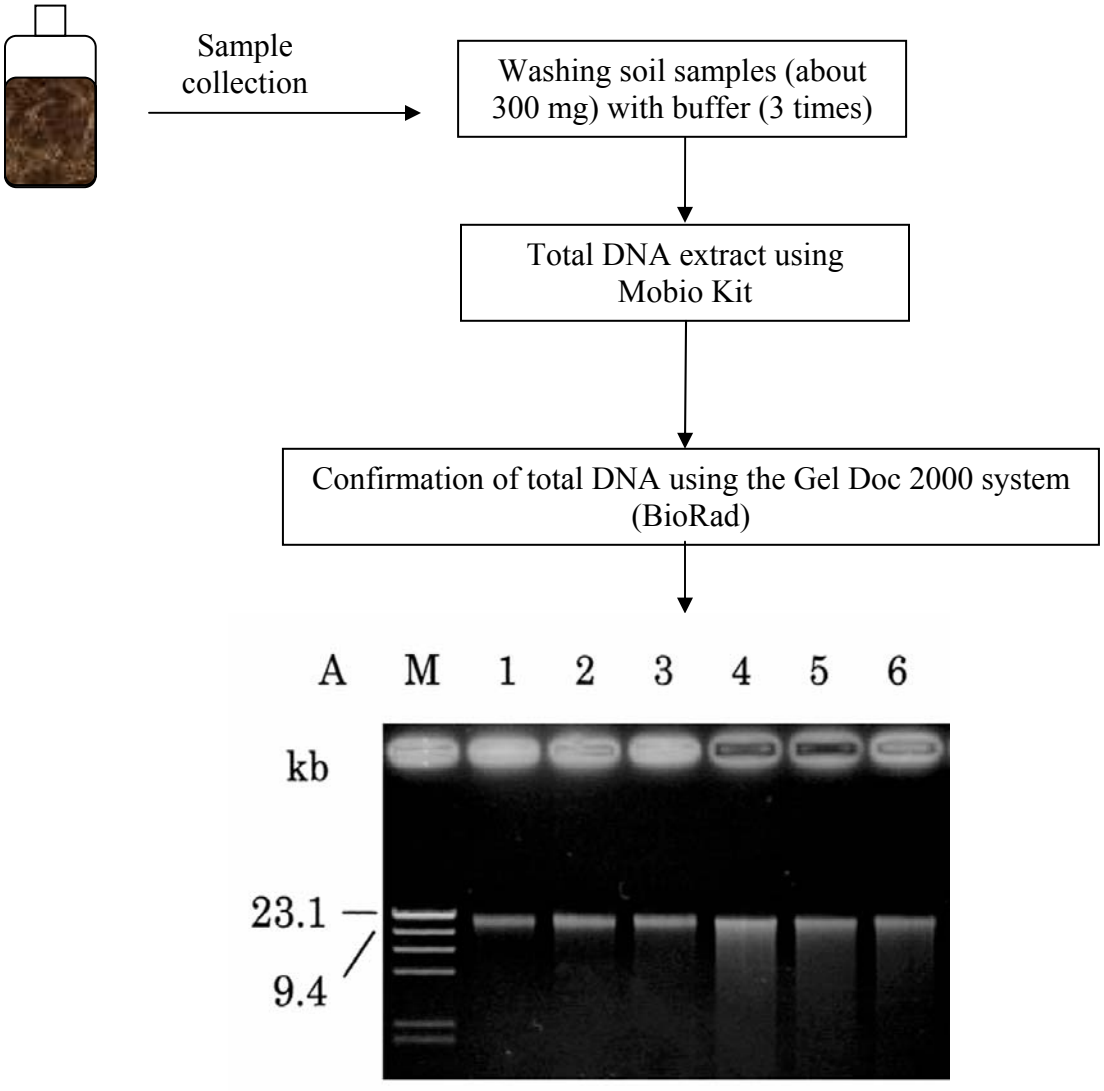
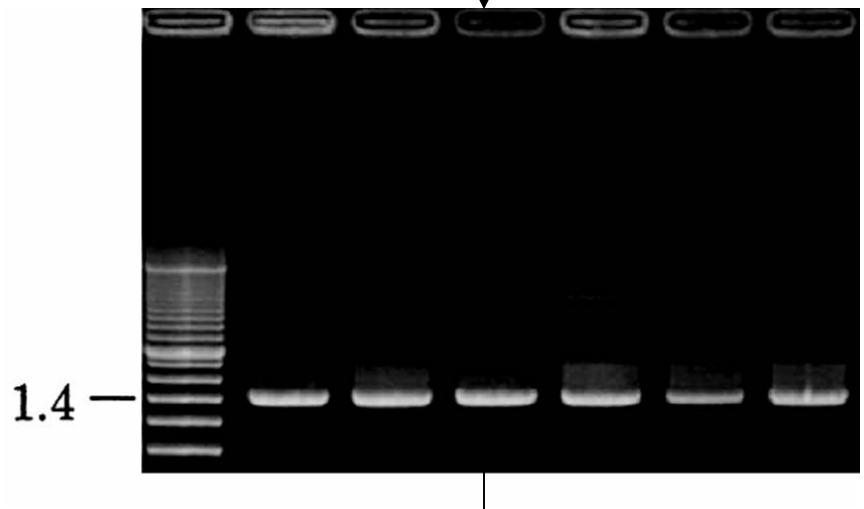


Figure 3.1: Steps for Molecular Analyses (fig. con'd).

⊗Confirmation of bacterial and archaeal 16S rDNA by using the Gel Doc 2000 system (BioRad)
⊗Bacteria 16S rDNA amplification by using 27F and 1492R, archaea 16S rDNA amplification by using 25F and 1492R



⊗Bacteria 16S rDNA amplification by using 341F GC clamp and 907R, archaea 16S rDNA amplification by using 344F GC clamp and 518R for DGGE analysis (second PCR).
⊗The product obtained from bacterial and archaeal 16S rDNA (above) was used as a template in the second PCR for DGGE analysis.

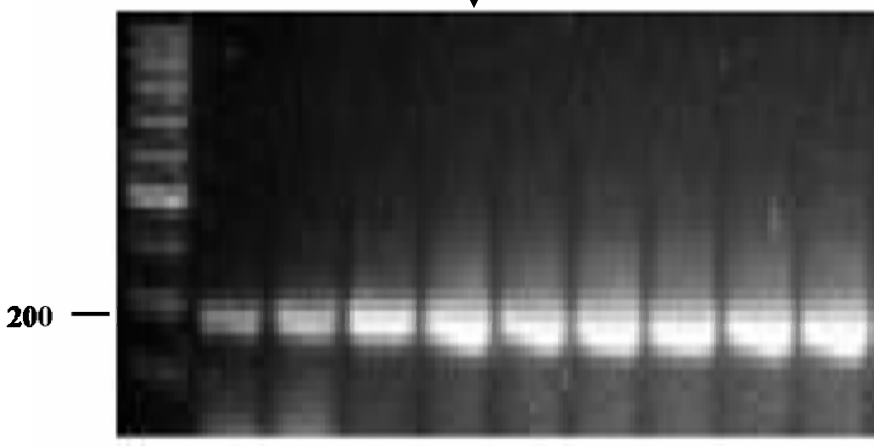
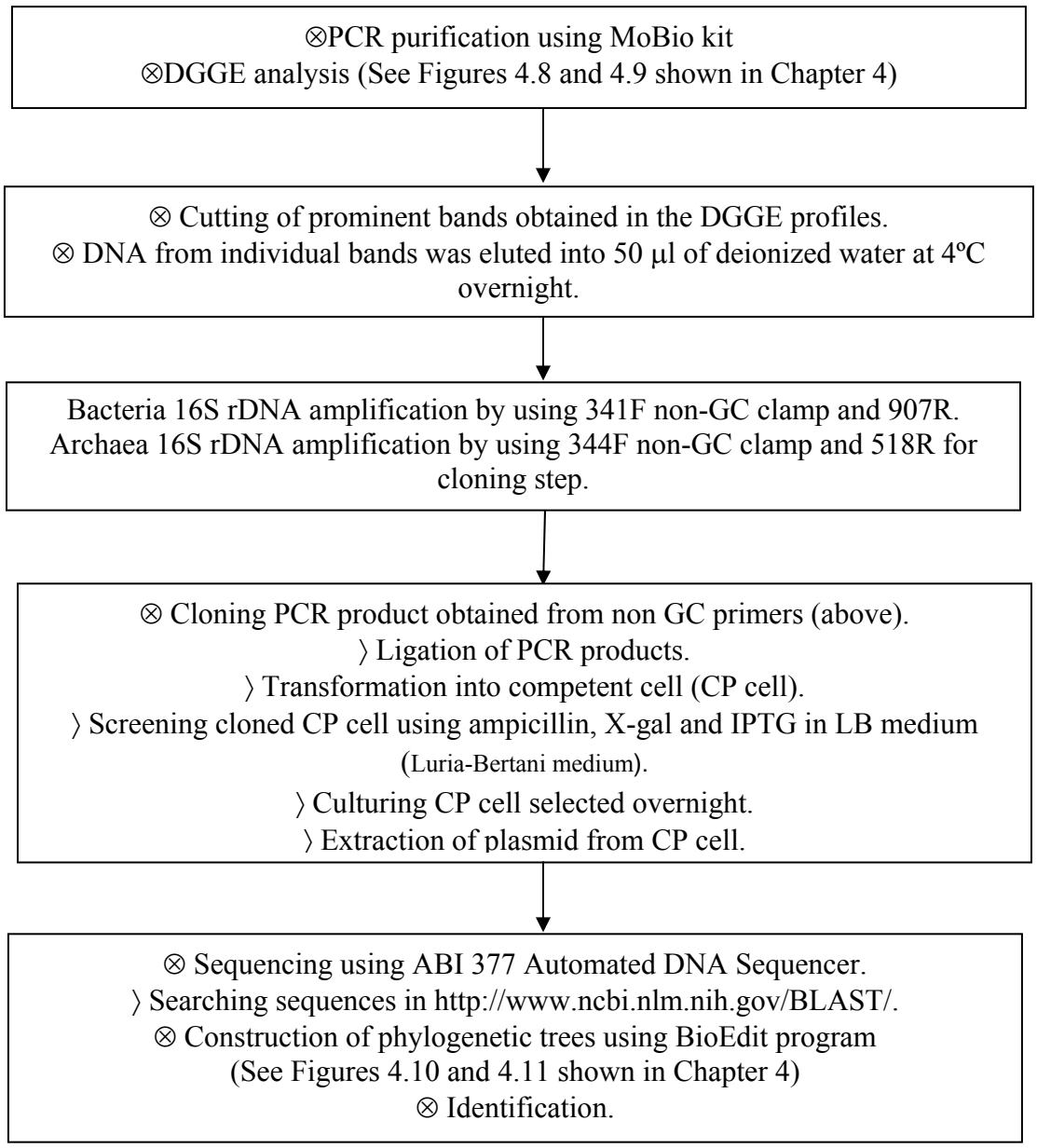


Figure 3.1: (continued).


⊗PCR purification using MoBio kit
⊗DGGE analysis (See Figures 4.8 and 4.9 shown in Chapter 4)
⊗ Cutting of prominent bands obtained in the DGGE profiles.
⊗ DNA from individual bands was eluted into 50 µl of deionized water at 4ºC overnight.
Bacteria 16S rDNA amplification by using 341F non-GC clamp and 907R.
Archaea 16S rDNA amplification by using 344F non-GC clamp and 518R for cloning step.
⊗ Cloning PCR product obtained from non GC primers (above).
〉 Ligation of PCR products.
〉 Transformation into competent cell (CP cell).
〉 Screening cloned CP cell using ampicillin, X-gal and IPTG in LB medium (Luria-Bertani medium).
〉 Culturing CP cell selected overnight.
〉 Extraction of plasmid from CP cell.
⊗ Sequencing using ABI 377 Automated DNA Sequencer.
〉 Searching sequences in http://www.ncbi.nlm.nih.gov/BLAST/.
⊗ Construction of phylogenetic trees using BioEdit program
(See Figures 4.10 and 4.11 shown in Chapter 4)
⊗ Identification.

CHAPTER 4. RESULTS AND DISCUSSION

4.1 Dechlorination Pathways and Kinetics

4.1.1 Dechlorination Pathways

Fate of 1,2,3,4-TeCB and other chlorobenzene congeners was monitored during a period of 4 weeks in aged reactors amended with roots (*Typha latifolia* and *Phragmites communis* roots) as well as in unamended aged reactors. 1,2,3,4-TeCB was reductively dechlorinated to 1,2,3-TCB and 1,2,4-TCB. The final end-products of 1,2,3,4-TeCB dechlorination observed in control treatment were 1,4-DCB, 1,3-DCB, and chlorobenzene; and 1,4-DCB, 1,3-DCB, chlorobenzene, and benzene for treatments using roots. In the route leading to 1,3-and 1,4-DCB via 1,2,4-TCB and 1,2,3-TCB, singly flanked chlorine substituents were removed. It seems that organisms present in the root matter therefore catalyzed different pathways to dechlorinate 1,2,3,4-TeCB even though it was not possible to determine the exact pathways. Dechlorination daughter products in each treatment are listed in Table 4.1.

Table 4.1: List of dechlorination intermediate and daughter products observed in all treatments.

Treatments	Daughter products	Major intermediate products
Control (no roots)	CB	1,2,4-TCB, 1,2,3-TCB, 1,4-DCB, 1,3-DCB
Amended with *Typha latifolia* roots	Benzene	1,2,4-TCB, 1,2,3-TCB, 1,4-DCB, 1,3-DCB, CB
Amended with *Phragmites communis* roots	Benzene	1,2,4-TCB, 1,2,3-TCB, 1,4-DCB, 1,3-DCB, CB

The degradation profile observed in this study is similar to that reported in Lin (2003), although Lin (2003) used freshly contaminated sediments for microcosm's preparation and a different type of sediment. Differences in dechlorination patterns are probably due to differences in the microbial communities present during dechlorination processes and the effects of the aging process performed at the beginning of the experiments.

During the analysis of 1,2,3,4-TeCB degradation, significant amounts of chlorobenzene and trace amounts of benzene in aqueous samples were observed in vegetated treatments unlike in control that benzene was not detected. This observation suggests that over the monitoring period of four weeks, final stages of dechlorination of 1,2,3,4-TeCB from chlorobenzene to benzene were still occurring. The dechlorination profiles corresponding to each treatment are shown in Figures 4.1 through 4.3.

Comparing the profiles of vegetated treatments, it is evident that the same dechlorination products were dominating the degradation of 1,2,3,4-TeCB. Nevertheless, daughter products obtained in the control treatment were not all similar to those observed in vegetated treatments. In other words, dechlorination process in control treatment was not as extensive and complete as in vegetated treatments, suggesting that roots and associated microbial communities were most likely responsible for stimulating dechlorination. In all treatments, dechlorination had occurred completely all the way to monochlorobenzene (CB) by day 30 (Figure 4.1 to 4.3). Furthermore, analysis of aqueous samples for detection of benzene on day 30 showed that treatments amended with roots had accumulated benzene (0.67 mg/l, *Phragmites* treatment; 0.8 mg/l, *Typha* treatment), whereas in control treatment dechlorination process had stopped at CB and benzene was not detected (Figure 4.1).

The mass balances between days 3 and 30 for vegetated treatments were relatively poor (recovery: 57.5%, *Typha*; 49.7%, *Phragmites*). The deficit in the total mass of contaminants can

be attributed to a) benzene that had degraded; b) variations in the extraction efficiencies due to the presence of root matter in microcosms; and c) low extraction efficiency due to aging effects. In control treatment, mass balances closed very well with more than 103.2% recovery, which suggests that aging effects might have not been responsible of deficit in the total mass of contaminants observed in vegetated treatments.

The degradation of benzene under anaerobic conditions has been reported in previous studies since this compound is of concern for its toxicity, water solubility and carcinogenicity (Kazumi, 1997; Weiner and Lovley, 1998). According to Kazumi et al. (1997), benzene transformation into CH_4 and CO_2 was noted in aquifer sediments under methanogenic conditions concomitantly with methane production and microbial activities. Weiner and Lovley (1998) suggested that addition of acetate promoted methane production as the predominant terminal electron-accepting process and benzene mineralized faster than it would be calculated for aerobic degradation alone.

4.1.2 Dechlorination Kinetics

The difference observed in the removal trends was found to be not statistically significant (at α=0.05 level) between treatments using *Typha latifolia* and *Phragmites communis* roots. In treatments using *Phragmites communis* roots, k=0.113 d^{-1} and using *Typha latifolia* roots, k=0.097 d^{-1}. The degradation rate constants for the *Phragmites* and *Typha* treatments differed by about 16% and these rates were lower than those observed with non-aged sediments (Lin, 2003). The *Typha latifolia* and *Phragmites communis* treatments fitted the first order kinetics model (Figure 4.4 and 4.5, respectively) whereas, unlike these trends, the control treatment could not be fitted successfully to any function with an r^2 value greater than 14%.

The half-life of 1,2,3,4-TeCB expected in aged Bayou Fountain sediment planted with *Typha latifolia* and *Phragmites communis* were 7.11 days and 6.13 days, respectively.

4.2 Hydrogen and Methane Concentrations

According to Figure 4.6, the hydrogen concentrations as a result of the breakdown of readily degradable organic matter followed a similar pattern in all treatments. The H_2 concentration decreased with time as it was being used as an electron donor by the dechlorinating organisms to reduce chlorinated solvents (McCarty and Wilson, 1992). The type of sediment used in this study was characterized as having only a small content of readily degradable organic matter (7%) discussed in Section 3.1.2. So, rapid exertion of this readily degradable organic matter with time may have also contributed to limiting H_2 production, and by day 30 H_2 was apparently depleted in all treatments.

Furthermore, substrates that generate hydrogen slowly may result in sustained dechlorination and enrichment for dechlorinating bacteria (Lee et al., 1998). However, if a dechlorinating population is present at a site (as suggested in following sections) almost any fermentable substrate, e.g. acetate, can be effective in stimulating the activity of such population through H_2 that is being produced during fermentation. In other studies, fermentation of acetate (known as a root exudate) to CO_2/H_2 has been observed to increase H_2 concentrations (Lin, 2003). Since acetate is commonly found in vegetated treatments, this may explain the fact that vegetated treatments showed greater concentrations of H_2 compared to non-vegetated treatment during the early period of the experiment when roots were still fresh. Variations in hydrogen and methane concentrations observed in this study may suggest that some competition for electron donors had taken place, in this case competition for H_2 between organisms carrying out the reductive dechlorination and others present in the consortium (Lee et al., 1998).

CH_4 is one of the trace gases used in this study to evaluate any change that might explain variations in microbial activities occurring under highly reduced conditions which are also conducive for dehalogenation. Another reason for monitoring this gas is that CH_4 is formed from

H_2 which is the electron donor driving the dechlorination process under anaerobic conditions. The limiting action of H_2 of overall degradation and CH_4 production by hydrolysis and fermentation was observed by Glissmann et al. (2004). Due to the fact that dechlorinators outcompete methanogens for H_2 when reductive dechlorination progresses, methanogens were affected and CH_4 decreased as it was observed in all treatments by day 30 (Figure 4.7). Since most methanogens are H_2-consuming organisms, comparison of Figures 4.6 and 4.7 denote how increases in CH_4 production seemed to be limited by the H_2 production and its changes.

During the overall process, *Phragmites* treatment seemed to have achieved the highest concentrations of H_2 followed by *Typha* treatment. CH_4 accumulation in vegetated treatments seemed to be greater than in treatment where no roots were used for amending the sediment.

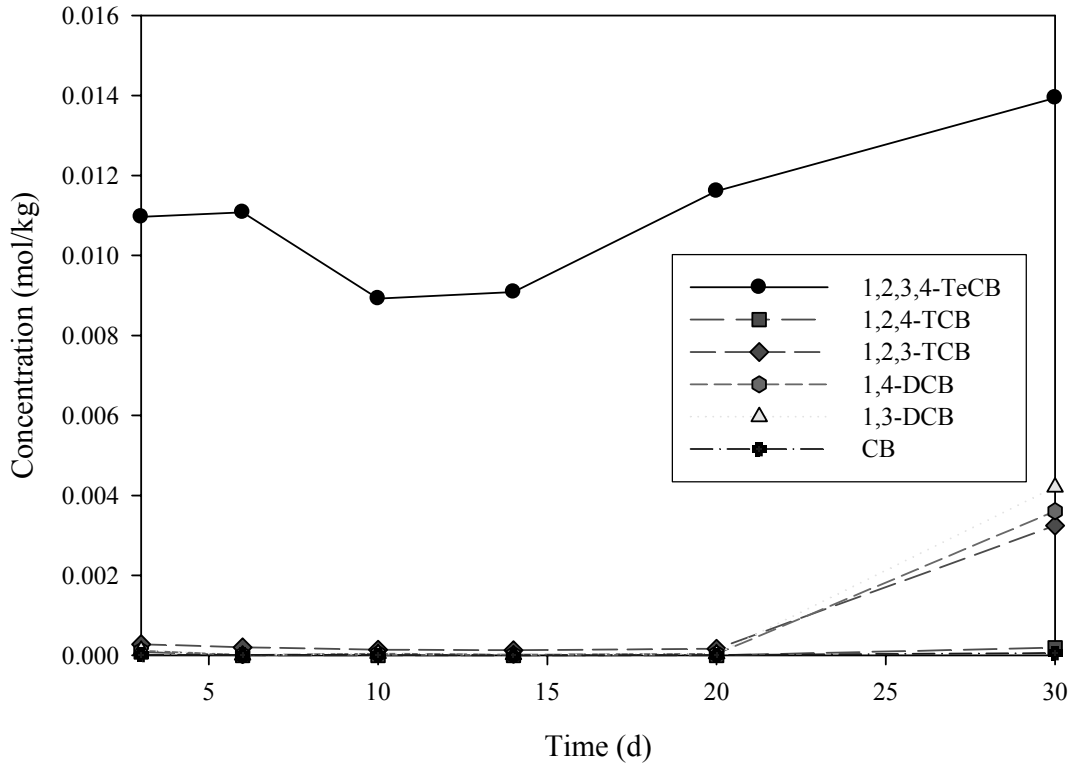


Figure 4.1: Dechlorination profile of 1,2,3,4-TeCB and major intermediate products observed in a representative replicate obtained from Control treatment.

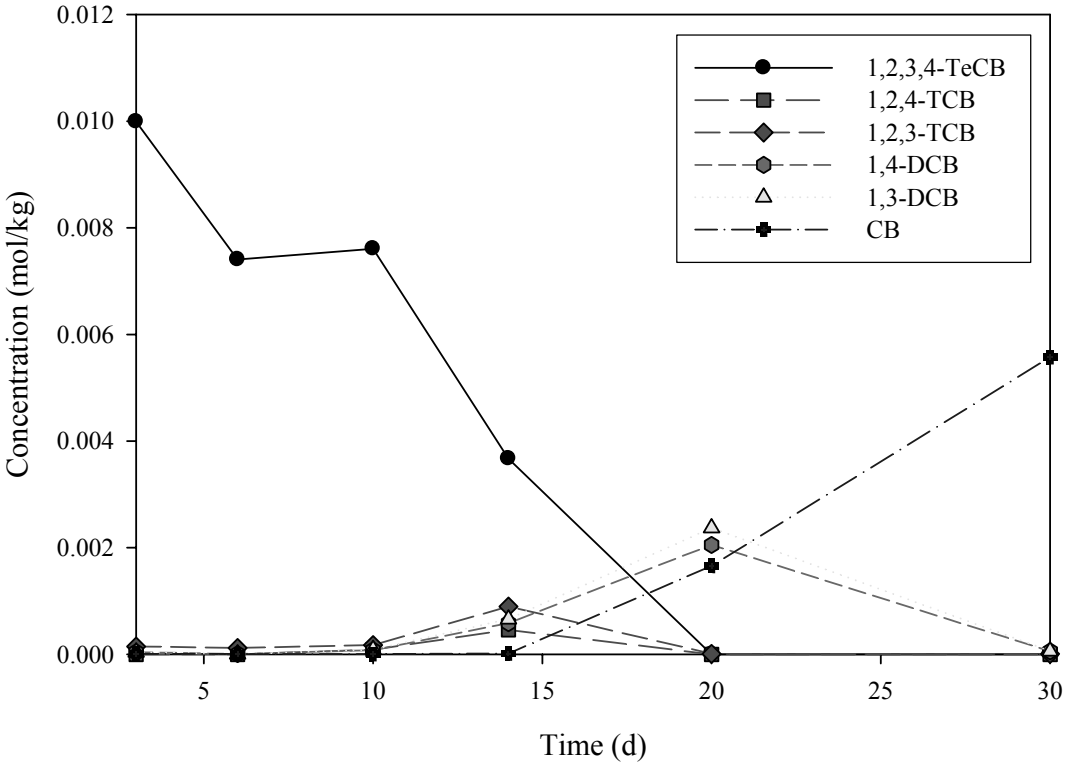


Figure 4.2: Dechlorination profile of 1,2,3,4-TeCB and major intermediate products observed in a representative replicate using *Typha latifolia* roots.

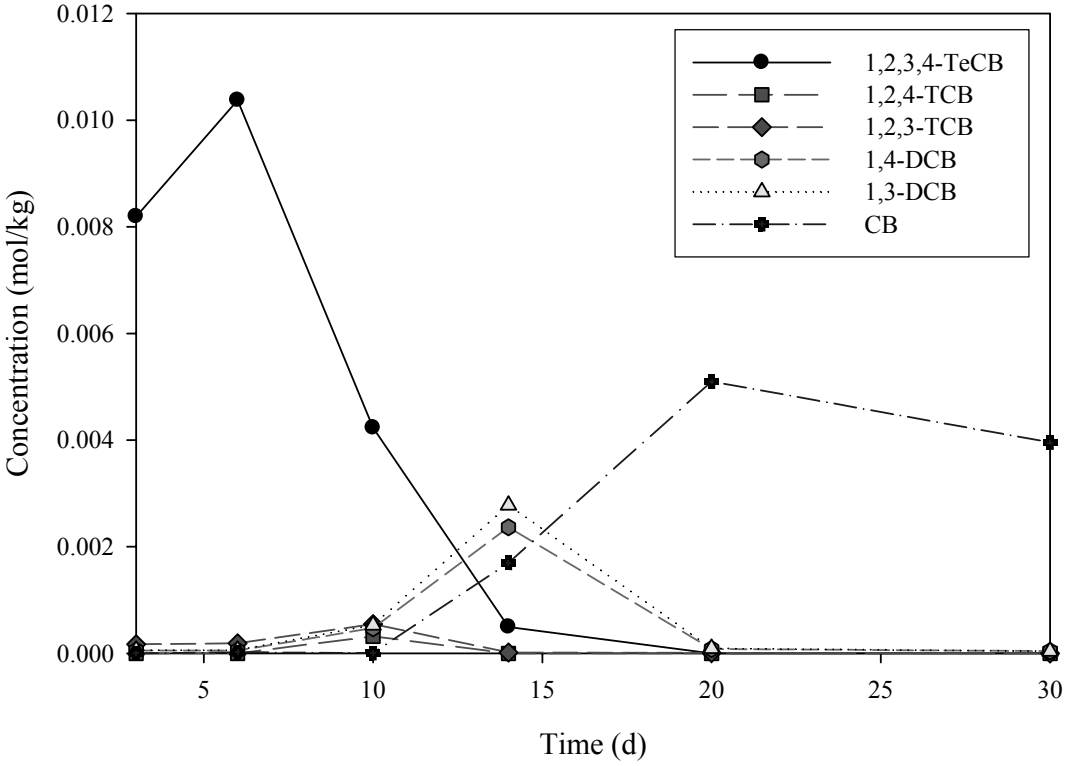


Figure 4.3: Dechlorination profile of 1,2,3,4-TeCB and major intermediate products observed in a representative replicate using *Phragmites communis* roots.

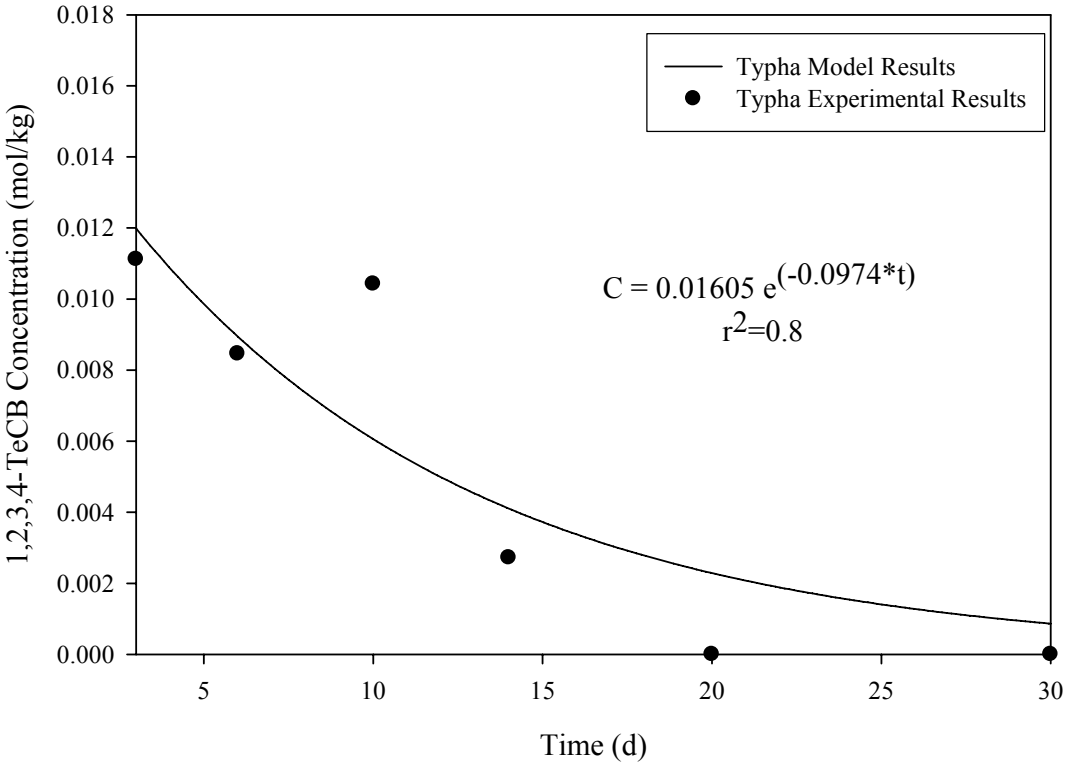


Figure 4.4: 1,2,3,4-TeCB first order degradation kinetics in treatment using *Typha latifolia* roots.

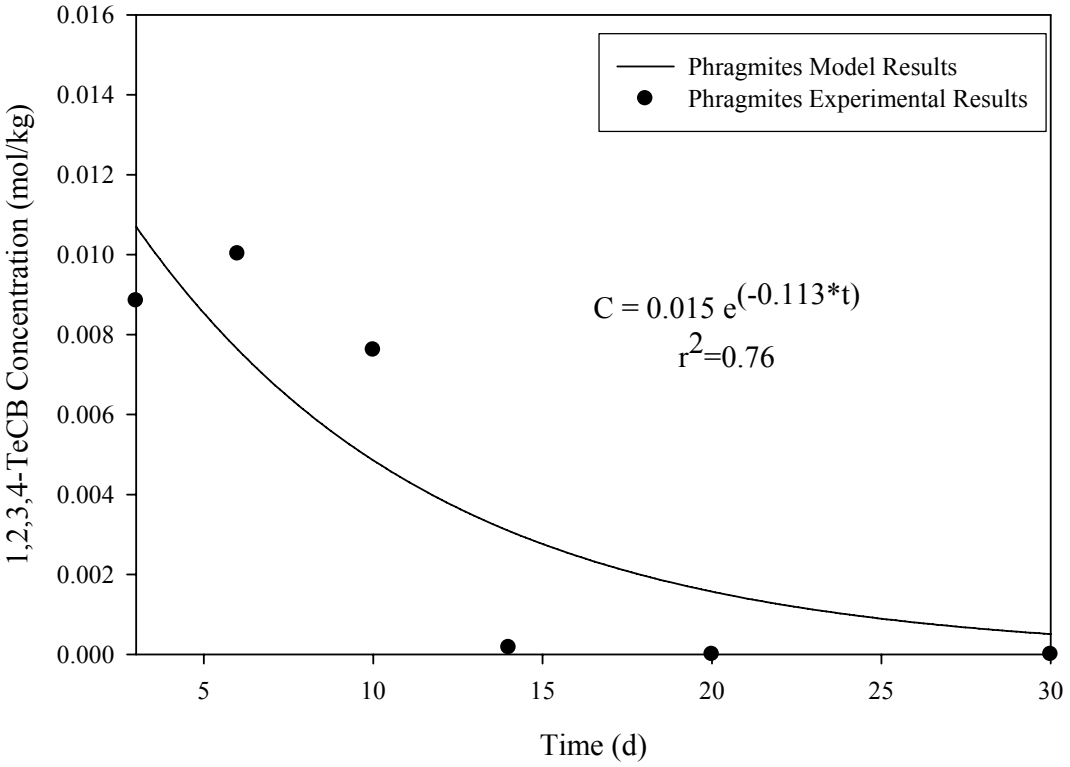


Figure 4.5: 1,2,3,4-TeCB first order degradation kinetics in treatment using *Phragmites communis* roots.

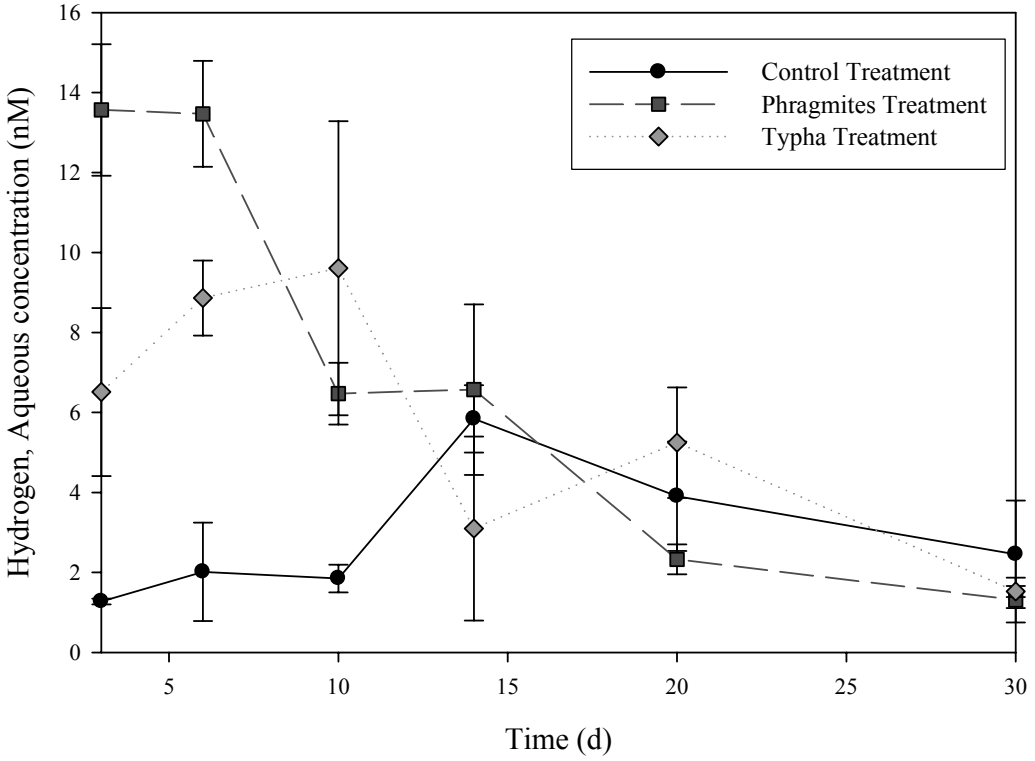


Figure 4.6: Variation in hydrogen concentrations present in the microcosm bottles during a period of 4 weeks.

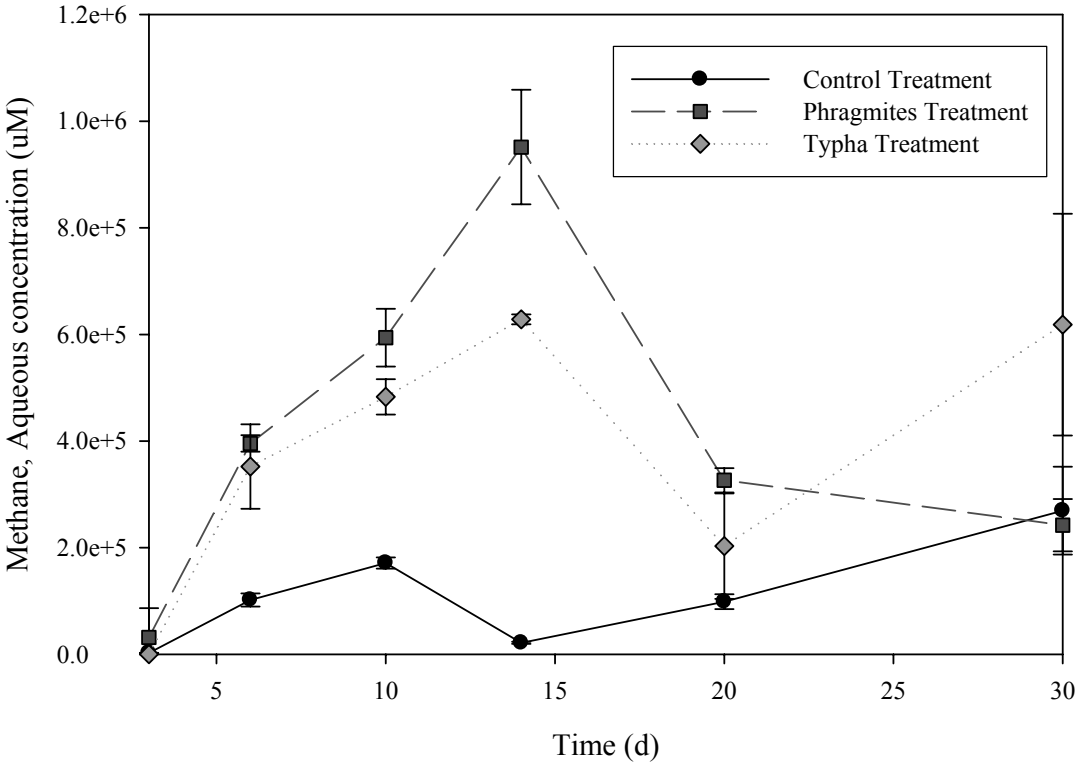


Figure 4.7: Variation in methane concentrations present in the microcosm bottles during a period of 4 weeks.

4.3 Diversities of Microbial Populations

4.3.1 Bacterial Group

A total of 13 bacterial 16S rDNA sequences derived from DGGE bands were obtained from three different treatments (aged sediment using 2 types of wetland roots and a control without roots) and summarized in Table 4.2. Four sequences were 98% or more identical to sequences already deposited in the GenBank database, while nine showed 97% or less identity with sequences in the database. Zhu et al. (2003) addressed that an accurate identification of a 16S rRNA gene to a sequence in the database, requires 97% or better match to the genus level and 98% or better match to the species level. However, they also mentioned that DNA sequences show lower similarity to those in the database may be when they are derived from previously uncultivated or unknown bacterial species.

All thirteen sequences were used as representatives to construct a phylogenetic tree shown in Figure 4.10. The phylogenetic analysis showed that the sequences retrieved in this study formed six major phylogenetic clusters: α-*proteobacteria*, β-*proteobacteria*, γ-*proteobacteria*, green nonsulfur bacteria, low GC gram-positive bacteria, and an unknown bacteria group (Figure 4.10). The unknown bacteria group was located between α-*proteobacteria* and green nonsulfur bacteria (Figure 4.10).

Most bacterial species contain multiple copies of 16S rRNA gene in their chromosomes (Boyer et al., 2001) so that pure bacterial cultures can give rise to several bands with different mobility in DGGE gel analysis. The number of bands shown in DGGE gels may be larger or smaller than the actual number of unique species detected by DNA sequence analysis since there may be co-migration of non-identical sequences. Moreover, individual organisms could potentially contribute multiple bands on a DGGE gel because of the presence in some bacteria of

multiple rDNA operons with sequence heterogeneity (Rainey et al., 1996). In this study, cloning was performed after DGGE analysis to establish that the sequences in band were, in fact unique.

Bands sequenced from the *Phragmites* treatment (BP6, BP8, BP9, BP11, BP12) showed species falling into the 3 major classes of bacteria, the low GC gram-positive bacteria (BP8 and BP9), green nonsulfur bacteria (BP12), and *proteobacteria* (BP6 and BP11, are beta and gamma lineages, respectively). On sequenced bands in *Typha* treatment during days 3, 10, 14 and 20, the sequenced bands in the *Typha* treatment were mainly represented by members of the low GC gram-positive bacteria (BP13, BP14, BP15, BP16, and BP18). *Clostridium* sp. were found in the *Typha* treatment (BP13) and increased with time. In previous studies, similar species had been associated with those found in sulfate-reducing fluidized-bed reactors (Kaksonen et al., 2004). The sequenced bands from the Control treatment was only represented by two groups of bacteria: low GC gram-positive bacteria and a type of unknown bacteria which seems distantly related to the *Dehalococcoides* sp. (Figure 4.10).

As it was mentioned before, bands BP2, BP8, BP9, BP13, BP14, BP15, BP16, and BP18 were identified within the low GC gram-positive bacteria. These bands can be more specifically described as members of the "*Clostridium*" subgroup, division "Endospore-forming gram-positive bacteria", genera "*Clostridium* and *Bacillus*". In all treatments (Control, *Phragmites*, and *Typha*), species from the genera *Bacillus* were detected as it is shown on Table 4.2 and on the phylogenetic tree in Figure 4.10.

Bands BP8 and BP14 were detected in *Typha* and *Phragmites* treatments. These two bands are closely related to uncultured bacterium SJA-118 (Accession No. AJ009489) based on the GenBank database. These bacteria seemed to be related to an anaerobic, trichlorobenzene-transforming population (von Wintzingerode et al., 1999), more specifically to sulfate-reducing bacteria, *Desulfitobacterium hafniense* (Accession No. X94975) and *Desulfosporosinus orientis*

(Accession No. Y11570) supported by bootstrap values >74%. Furthermore, these bacteria found seemed to be also related to dehalorespiring bacteria with vicinal dichlorinated alkanes, more specifically to *Desulfitobacterium dichloroeliminans* strain DCA1 (Accession No. AJ565938) whose exclusive *anti* dichloroelimination makes their dechlorination biochemistry distinct from that of all other known dehalorespiring isolates (De Wildeman et al., 2003). Both bands, BP8 and BP14, revealed similarities to the closest relative in the order of 93-95% (http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi).

Band BP12 was identified as *Dehalococcoides* sp. (Accession numbers AY165308 and AF230641, which represent strains BAV1 and CBDB1 respectively) in *Phragmites* treatment. It was also found that this band also revealed 99% similarity to the closest relatives. From the two species of *Dehalococcoides* identified, strain CBDB1 (Accession No. AF230641) grown with hexachlorobenzene or pentachlorobenzene was found responsible of the reductive dechlorination of those compounds via dehalorespiration (Jayachandran et al., 2003). Even though 1,2,3,4-TeCB was not detected in the process, *Dehalococcoides* sp strain CBDB1 catalyzed two different pathways that dechlorinated these highly chlorinated benzenes. Hexachlorobenzene was degraded to pentachlorobenzene, which was converted to a mixture of 1,2,3,5- and 1,2,4,5-tetrachlorobenzene being the final end-products 1,3,5-trichlorobenzene, 1,3- and 1,4-dichlorobenzene. In the present study, the detection of *Dehalococcoides* sp strain CBDB1 can be linked to the presence of intermediate products such as 1,2,3-trichlorobenzene detected by chromatography analyses (see Table 4.1) as it was reported in past studies (Adrian et al., 2000). Conversely to what Jayachandran et al. (2003) reported, 1,2,3,4-TeCB did not seem to be toxic for strain CBDB1. Hence, this specie might not only be responsible of degrading higher chlorinated benzenes such as hexa- and pentachlorobenzene but also be responsible of degrading 1,2,3,4-TeCB to end products like chlorobenzene and benzene as it was observed in this study.

Jayachandran et al. (2003), reported also that 1,2,3-trichlorobenzene dehalogenase activity is present in cells of *Dehalococcoides* sp. strain CBDB1 regardless of whether growth occurred with 1,2,3-trichlorobenzene, hexa- or pentachlorobenzene, although 1,2,3-trichlorobenzene is not an intermediate in hexachlorobenzene and pentachlorobenzene dechlorination.

On the other hand, *Dehalococcoides* sp. strain BAV1 (Accession No. AY165308) has been reported to dechlorinate DCE and VC but not PCE and TCE (He et al., 2003). It was also observed in their study that the growth of isolate BAV1 depended strictly on reductive dechlorination and the presence of hydrogen as an electron donor, which could not be replaced by organic substrates including formate, acetate, lactate, pyruvate, propionate, glucose, among others. He et al. (2003) also reported that BAV1 was the first isolate capable of the metabolic dechlorination of all DCE isomers. Since degradation of chlorobenzenes by *Dehalococcoides* sp. strain BAV1 has not been observed, it is likely that CBDB1 is the strain present.

These observations suggest that different microbial communities were able to completely dechlorinate 1,2,3,4-TeCB in vegetated treatments. Comparison of numbers and distribution patterns of DGGE bands revealed that diversities of the microbial populations were different for the same type of sediment. Comparing the banding profiles of the bacteria group, different banding patterns were observed in root-amended and unamended microcosms, indicating that the presence of roots changed the bacterial community. Enhancement of degradation has been observed in previous studies as a result of the presence of plants (Pivetz, 2001; Briggs et al., 1982; Bell, 1992; Schnoor, 1997; Schnoor, 2002). Moreover, it has been observed greater bacterial diversity in the rhizosphere soil than in the bulk soil (Lin, 2003; Anderson et al., 1993). However, in some cases the bulk soil showed a greater diversity of bacterial communities as it was observed by Marilley et al. (1998). It has been also demonstrated that phytoremediation systems substantially decreased Total Petroleum Hydrocarbons (TPHs) in soil by increasing

specific bacteria in the bulk and rhizosphere soil (Siciliano et al., 2003). Based on the findings from this study, it might be possible that diverse plants affect in different ways the microbial diversity in the root matter most likely due to the influence of root exudates. Likewise concentration of the compounds used for contaminating the sediments can be responsible of toxicity to certain bacteria or used positively as a substrate for their growth instead.

The most relevant results withdrawn from this study is the presence of bacterial populations members of *Desulfitobacterium* sp. and the chlorobenzene-respiring anaerobe *Dehalococcoides* sp. strain CBDB1, which have been demonstrated to play an important role in reductive dehalogenation of chlorobenzene congeners (Hölscher et al., 2003). The presence of these important species especially in vegetated treatments may suggest the positive effect of roots in specific microbial growth enhancement. These results suggest that plants and the associated rhizosphere microorganisms could be the key for bioremediation of soils contaminated with 1,2,3,4-TeCB or other chlorinated compounds, as it was shown by Nichols et al. (1997). *Typha latifolia* and *Phragmites communis* and the microbial consortium associated to the root matter demonstrated to be capable of complete dechlorination of 1,2,3,4-TeCB and other less chlorinated benzenes.

4.3.2 Archaeal Group

Reduced environments denote the lack of oxygen and presence of indicators of anaerobic microbial activity such as methane; in general complete dechlorination occurs under methanogenic conditions (Lee et al., 1998). In the present study, detected and identified methanogenic populations assure high reduced conditions. As it was shown in Figure 4.7, the accumulation of CH_4 denotes methanogenic species adaptation to the solvent concentrations and apparently no negative effects from 1,2,3,4-TeCB use.

Archaeal sequences observed in three different treatments analyzing aged 1,2,3,4-TeCB degradation are summarized in Table 4.3. Of a total of 11 archaeal 16S rDNA sequences retrieved, 8 were more than 97% identical to the sequences in GenBank, while 3 sequences showed 95 to 96% identity to those in GenBank databases, which indicates that they may be from currently unknown species. The results obtained in this study are related to those of methanogenic archaea. They formed two major phylogenetic clusters as shown in Figure 4.11, corresponding to two orders: order III, *Methanomicrobiales* and order IV, *Methanosarcinales*.

Species found within the two orders, *Methanomicrobiales* and *Methanosarcinales*, has been previously observed in anoxically incubated rice field soil slurries (Lueders and Friedrich, 2000). According to these authors, a typical sequence of reduction processes were exhibited and characterized by reduction of nitrate, Fe^{3+}, and sulfate prior to the initiation of methane production. Moreover, *Methanosarcina* sp. found in Control and *Typha* treatments suggest a favorable environment rich in methanogenic substrates, such as acetate. *Methanosaeta* sp. are not adapted in the same way to high concentrations of acetate, however, *Methanosaeta* sp were observed in *Phragmites* treatment whose sequences revealed 96 and 99% similarities to the closest relatives found in the GenBank databases.

Most of the sequences in order IV belong to the family *Methanosarcinaceae*, whose members can form methane from a variety of substrates, such as acetate, H_2 and CO_2, methanol, and methylamines (Zhu et al., 2003). Most of these species were found in Control and *Typha* treatment according to Figure 4.11. Three other sequences within the same order belong to the family *Methanosaetaceae*, principally observed in *Phragmites* treatment. Members of this family can use only acetate as a substrate to produce methane and CO_2 (Zhu et al., 2003). In the present study, production of acetate probably occurred as a result of washed excised roots used in microcosms preparation as it once was found and reported by Conrad and Klose (2000) but

instead using roots of rice (*Oryza sativa*). Members of the *Methanosaetaceae* family have been also found responsible of benzene conversion to CH_4 and CO_2 without apparent lag (Weiner and Lovley, 1998). Thus, these species could be responsible for the loss of benzene mass observed in the vegetated treatments.

On the other hand, two sequences belong to family *Methanomicrobiaceae* in order III, *Methanomicrobiales*. These two sequences were only detected in vegetated treatments. *Methanomicrobiaceae* are limited to the use of H_2 and CO_2 and formate as substrates to form CH_4 and H_2O (Zhu et al., 2003). Even though a possible competition for H_2 with other bacterial species such as *Dehalococcoides* sp. strain BAV1 occurred, hydrogenotrophic methanogens were indeed present in the sediment. Hence, the presence of methanogens was consistent with the methanogenic activities detected.

Methanosaeta concilii, also known as *Methanothrix concilii* (Accession No. AB212062) according to the NCBI Taxonomy database (http://www.ncbi.nlm.nih.gov/Taxonomy), was observed in previous studies related to acetate and CO_2 assimilation (Ekiel et al., 1985). Ekiel et al. (1985) reported that the major biosynthetic reaction in *Methanothrix* sp. was the formation of pyruvate from acetate and CO_2 as found for those methanogens capable of reducing CO_2 to CH_4. Methanogenic activity represented by identified *Methanomicrobiaceae*, *Methanosarcinaceae*, and *Methanosaetaceae* families, suggest anaerobic conditions and the reduced environment necessary for dehalorespiration of chlorobenzene congeners in microcosm bottles. According to the characteristics of methanogens found in all treatments, acetate and hydrogen seemed to be present in all microcosm bottles acting as substrates for CH_4 production.

Since the lab temperature during the microcosm studies was always at room temperature (~25ºC), it is suggested that the pathway for organic matter degradation was dominated by H_2-dependent methanogenesis (Glissmann et al., 2004). The incubation of sediment at different

temperatures (4-30ºC) did not affect the structure of the methanogenic community primarily affiliated with *Methanosaetaceae* and *Methanomicrobiaceae*, in conjunction with other three deeply branching euryarchaeotal clusters, temperature changes rather affected the activity of them (Glissmann et al., 2004).

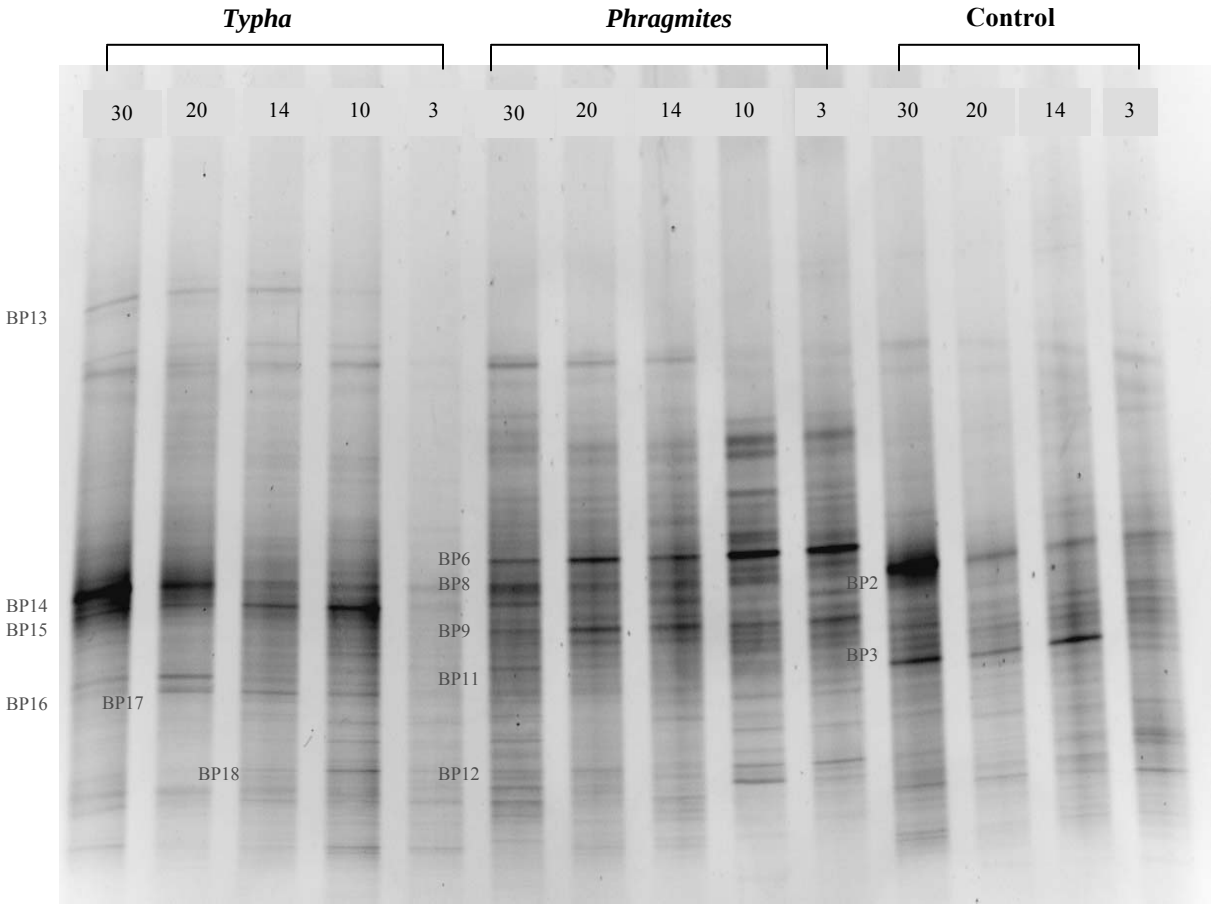


Figure 4.8: Negative image of ethidium bromide-stained DGGE profiles of PCR-amplified 16S rRNA fragment by using universal bacterial primers corresponding to samples taken on days 3, 10, 14, 20, and 30, respectively.

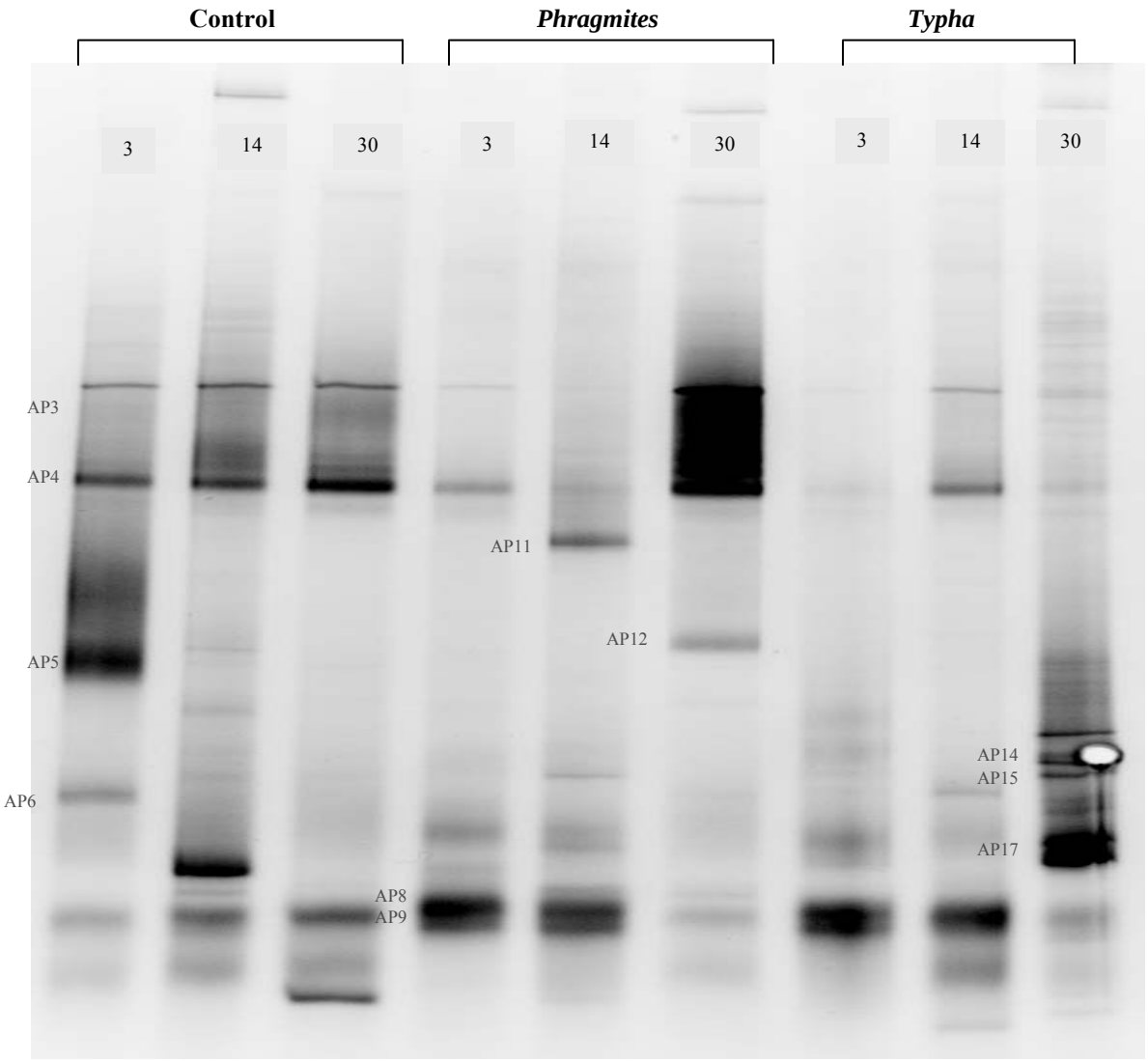


Figure 4.9: Negative image of ethidium bromide-stained DGGE profiles of PCR-amplified 16S rRNA fragment by using universal archaea primers corresponding to samples taken on days 3, 14, and 30, respectively.

Table 4.2: Phyla of the bacterial 16S rRNA sequences obtained from DGGE bands.

Bands	16S rRNA gene sequencing			Subclass
	Closest match	No. of nucleotides compared	% Similarity with Closest match	
BP2	*Bacillus* sp (AJ229238)	576	97	Low GC gram positive[a]
BP3	Uncultured bacterium (AY532555)	542	98	Unknown cluster
BP6	Unidentified bacterium (AJ412674)	609	96	β[b]
BP8	Uncultured bacterium SJA-118 (AJ009489)	589	95	Low GC gram positive
BP9	*Bacillus firmus* (AJ717383)	604	97	Low GC gram positive
BP11	*Photorhabdus luminescens* (AY444555)	586	99	γ[c]
BP12	*Dehalococcoides* sp. CBDB1 (AF230641)	564	99	Green nonsulfur bacteria
BP13	*Clostridium* sp. (AY548783)	607	97	Low GC gram positive
BP14	Uncultured bacterium SJA-118 (AJ009489)	588	97	Low GC gram positive
BP15	*Bacillus asahii*(AB109209)	608	96	Low GC gram positive
BP16	*Desulfosporosinus* sp. (AJ582756)	570	95	Low GC gram positive
BP17	*Sinorhizobium* sp. (AY943388)	560	98	α[d]
BP18	Uncultured bacterium AY160815	618	94	Low GC gram positive

[a] low G+C gram positive bacteria, [b] beta-*proteobacteria*, [c] gamma-*proteobacteria*, [d] alpha-*proteobacteria*.

Table 4.3: Phyla of the archaeal 16S rRNA sequences obtained from DGGE bands.

Bands	16S rRNA gene sequencing			Subclass
	Closest match	No. of nucleotides compared	% Similarity with Closest match	
AP3	Uncultured archaeon AF418929	171	99	Methanosarcinales
AP4	Uncultured Methanosarcina AY607257	173	99	Methanosarcinales
AP5	Unidentified archaeon AJ831023	166	96	Methanosarcinales
AP6	Unidentified archaeon AJ830967	168	95	Methanosarcinales
AP8	Uncultured Methanosaeta DQ201632	164	99	Methanosarcinales
AP9	Uncultured Methanosaeta sp. DQ201630	160	96	Methanosarcinales
AP11	Uncultured Methanomicrobiaceae AY133906	170	98	Methanomicrobiales
AP12	Uncultured archaeon AF225664	168	98	Methanosarcinales
AP14	Uncultured archaeon AF225665	164	98	Methanosarcinales
AP15	Uncultured archaeon AY570681	174	98	Methanosarcinales
AP17	Uncultured archaeon AF050616	172	99	Methanomicrobiales

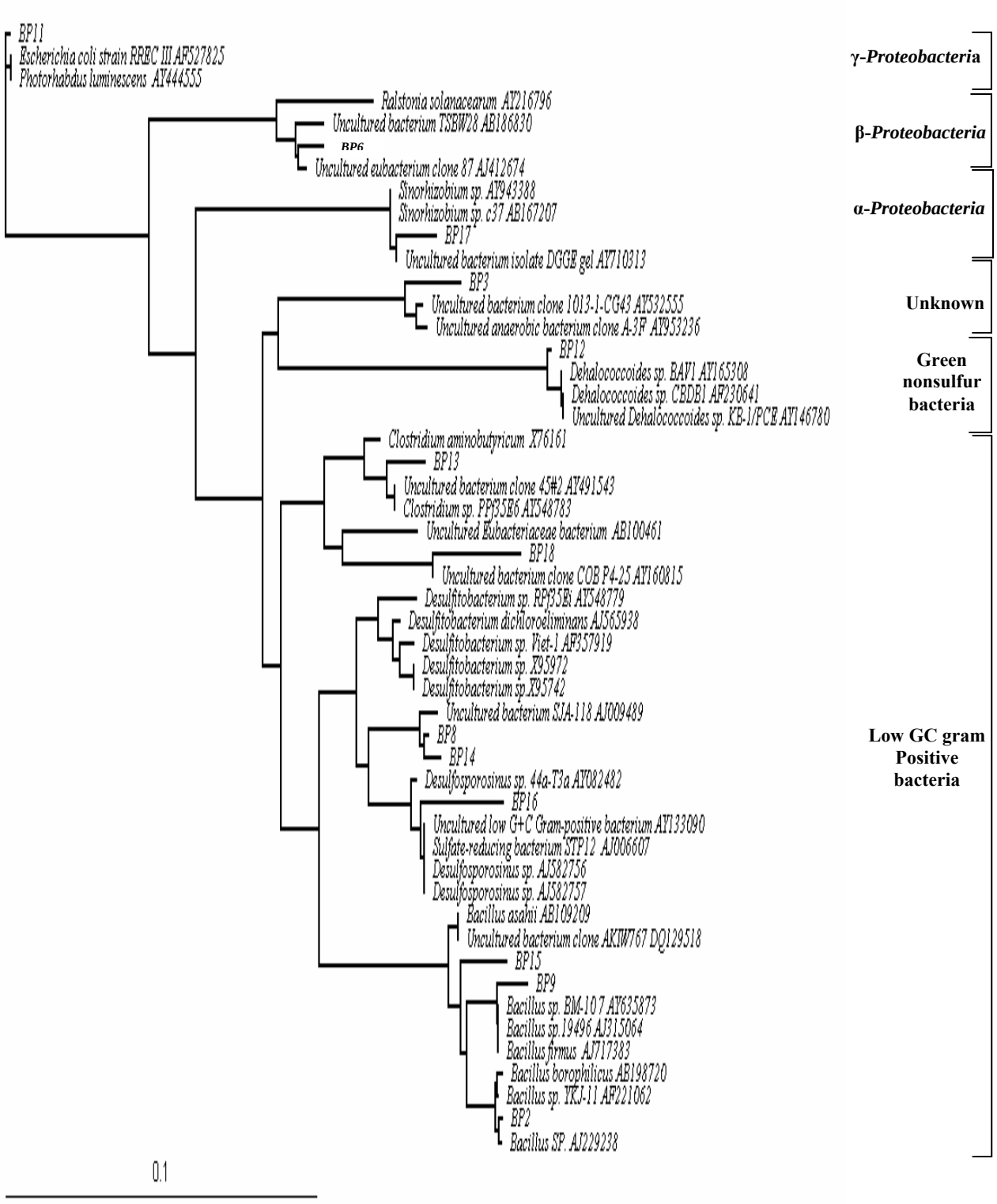


Figure 4.10: Phylogenetic tree of DGGE band sequences of bacteria 16S rDNA. The phylogenetic tree was constructed using neighbor-joining analysis with 1,000 bootstrap replicates. The scale bar indicates 0.1 nucleotide substitutions per site.

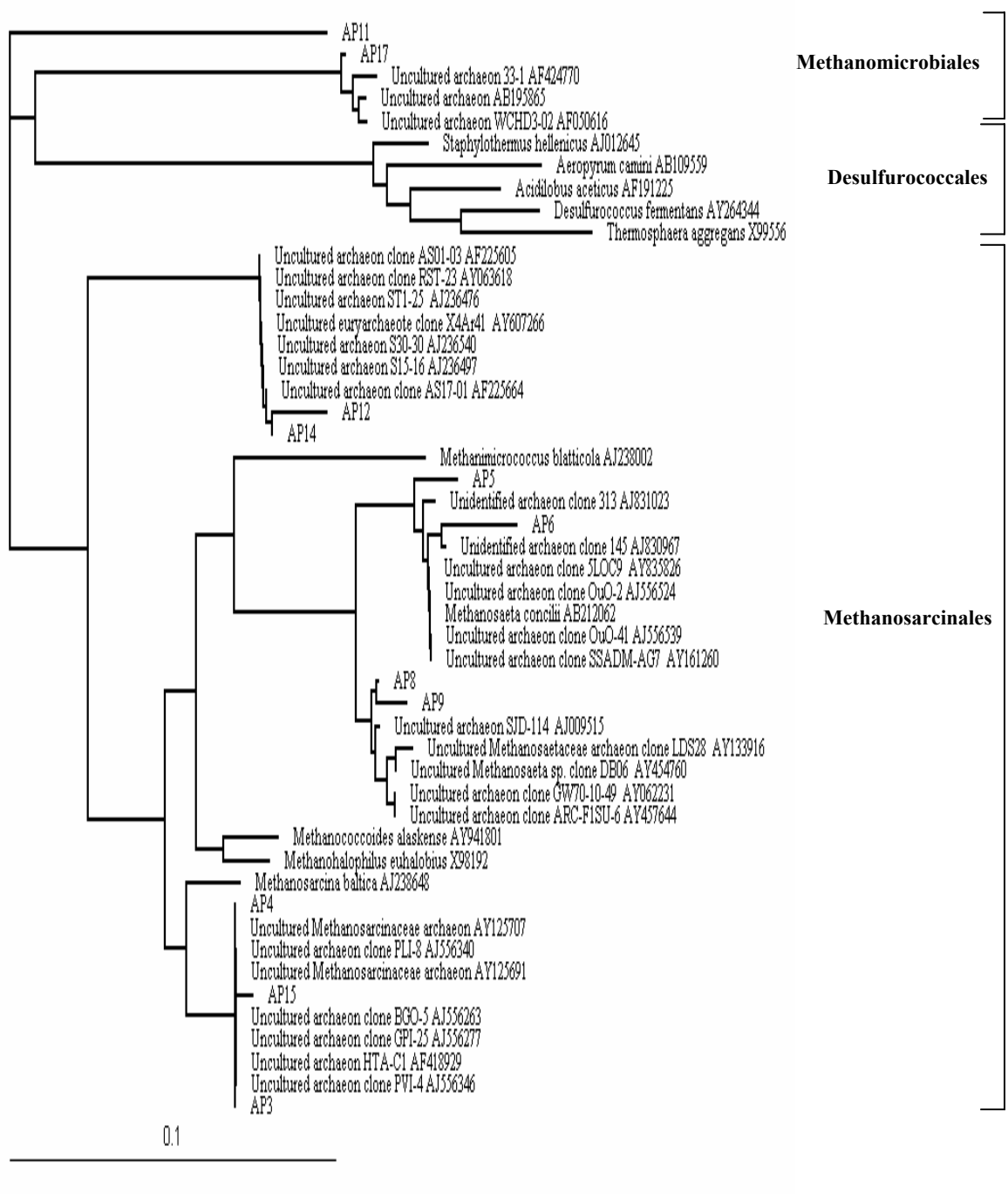


Figure 4.11: Phylogenetic tree of DGGE band sequences of archaea 16S rDNA. The phylogenetic tree was constructed using neighbor-joining analysis with 1,000 bootstrap replicates. The scale bar indicates 0.1 nucleotide substitutions per site.

CHAPTER 5. CONCLUSIONS AND RECOMMENDATIONS

The results obtained from this study corroborate that *Typha latifolia* and *Phragmites communis* roots stimulate the degradation of chlorinated benzenes via reductive dechlorination. Microcosms amended with *Phragmites communis* roots achieved the greatest first-order reaction rate constant over a 4-week period (0.113 d^{-1}, half-life time of 6.13 days). *Typha latifolia* roots also played an important role in reductive dehalogenation of 1,2,3,4-TeCB, although the first-order reaction rate constant observed within the same period of time (0.097 d^{-1}, half-life time of 7.11 days) was 16% less than that exhibited by *Phragmites* treatment. On the other hand, higher concentrations of H_2 (electron donor) associated to organic matter in root matter may have caused higher dechlorination activities observed.

Effective reductive dehalogenation of 1,2,3,4-TeCB and chlorobenzene congeners in vegetated treatments was attributed to the presence of two important bacterial populations, *Desulfitobacterium* sp. and the chlorobenzene-respiring anaerobe *Dehalococcoides* sp. strain CBDB1. Moreover, methanogenic activity by identified *Methanomicrobiaceae*, *Methanosarcinaceae*, and *Methanosaetaceae* families, confirmed anaerobic conditions and the reduced environment necessary for dehalorespiration of chlorobenzene congeners in microcosm bottles.

The results obtained and presented in this study might not be applicable to field studies; hence in case of extrapolating these results special considerations must be taken. Based on the fact that using plants enhances reductive dechlorination of 1,2,3,4-TeCB, it is recommended further investigation involving vegetated mesocosms and/or a pilot scale study using plants to mimic real conditions present in the field.

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APPENDIX. SPECIES OF PLANTS USED IN MICROCOSMS TO ANALYZE THE FATE OF 1,2,3,4-TeCB

Figure A1: *Typha latifolia*, member of the Cattail family (*Typhaceae*).

Figure A2: *Phragmites communis*, member of the Grass family (*Poaceae*).

VITA

Elaiza Maria Alvarez Castellanos was born in Maracay, Aragua, Venezuela, on December 16, 1979. She attended Universidad Nacional Experimental de las Fuerzas Armadas (U.N.E.F.A) where she obtained a Bachelor of Science degree in civil engineering in 2001.

Later in August 2003, she enrolled in the graduate program offered by the Department of Civil and Environmental Engineering at Louisiana State University in Baton Rouge, Louisiana. She will receive the degree of Master of Science in Civil Engineering, majoring in environmental engineering in May 2006. She is planning to work in the environmental engineering field after graduation.