CONTRIBUTION OF METHANOTROPHIC GROUNDWATER AND RHIZOSPHERE BACTERIA TO PHYTOREMEDIATION

By

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This dissertation is dedicated to my parents.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Abstract of Dissertation Presented to the Graduate School
of the University of Florida in Partial Fulfillment of the
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Trichloroethylene (TCE), a widely used solvent and ubiquitous contaminant, is effectively removed from soil and groundwater by the use of plants (phytoremediation). Rapid removal has been reported at the root-zone (rhizosphere), where methanotrophs (methane-oxidizing bacteria) capable of co-oxidizing TCE are present. The objective of the study was to determine, by the development of an adequate protocol, how plant type, system design, and environmental conditions present at two phytoremediation sites impacts methanotroph's biodegradation potential. To develop characterization methods, phenotypic and genotypic analyses of an uncharacterized methanotroph, Strain CSC1, isolated from an uncontaminated groundwater aquifer, were performed. Field sites represented an engineered system, with poplar and willow trees, and a natural loblolly pine re-growth area. Laboratory studies were conducted to assess the ability of methanotrophs to oxidize pine exudates (monoterpenes) and its effects on TCE oxidation. Field samples were analyzed by culture-dependent microbial counts and enrichments, and

culture-independent stable isotope probing (SIP) microcosms and molecular methods. Strain CSC1 possessed a unique spiny S-layer and was shown to be a novel strain of the genus *Methylocystis* and was named *Methylocystis aldrichii* sp. nov. Characterization methods developed with Strain CSC1 were successfully applied to phytoremediation field samples and isolates. Different types of methanotrophs were capable of oxidizing monoterpenes (α-pinene) and, in the presence of TCE; antagonistic and synergistic responses were observed depending on methanotroph type. Rhizosphere samples analyzed by culture-dependent methods confirmed the presence of methanotrophs at both sites; however, enrichments were biased towards type II methanotrophs and did not correspond with the active populations. Active populations were more diverse and abundant in the planted samples and strongly influenced by the design, especially the use of planting material that resulted in a dominance of thermotolerant methanotrophs. Variable results between the engineered and natural settings highlight the importance of measuring oxidation potentials and diversity of rhizosphere methanotrophs at any phytoremediation site, especially if monoterpene-releasing plants are contemplated for use. Also, study of active populations was shown to be the most accurate characterization method. Phylogenetic analysis combined with SIP microcosms offers powerful analytical tools that can ultimately aid practitioners in optimizing phytoremediation for more effective treatment.

CHAPTER 1
INTRODUCTION

Chlorinated solvents, such as trichloroethylene (TCE), are a major source of groundwater and soil pollution throughout the United States. It is well known that soil microorganisms in the presence and absence of oxygen are capable of degrading these compounds (Barrio-Lage et al., 1986; Fox et al., 1990; Hanson and Hanson, 1996). TCE can be metabolized to vinyl chloride, a potent carcinogen, if oxygen is not present (Barrio-Lage et al., 1986; Ensley, 1991). Therefore, this pathway of degradation is undesirable given conditions where vinyl chloride can accumulate with no further breakdown. In contrast, methane-oxidizing bacteria (methanotrophs), aerobic microorganisms known for their bioremediation potential and prevalence in the environment, can co-metabolize TCE to CO_2 at higher rates than other microorganisms (Wilson and Wilson, 1985; Little et al., 1988; Fox et al., 1990). However, the pathway of TCE degradation under aerobic conditions is not without risk of forming toxic intermediates, including chloral hydrate, dichloroacetate, and ethylene glycol (Oldenhuis et al., 1989; Alvarez-Cohen and McCarty, 1991a; Stacpoole et al., 1998; Lash et al., 2000).

Recently, the possibility of using vegetation to enhance degradation of organic contaminants in soil systems (phytoremediation) has received attention as an attractive low-cost alternative to the traditional engineering approaches of soil excavation and incineration, air stripping, and pump-and-treat (EPA, 2000b; EPA, 2001; McCutcheon and Schnoor, 2003). When plants are used for this purpose, a series of mechanisms are

involved, including phyto-volatilization, -accumulation, -degradation, -stabilization, and rhizodegradation. However, the role and contribution of each of these processes to the overall remediation system has not been accurately characterized (Orchard et al., 2000b; Shang et al., 2003).

It is well known that the microenvironment surrounding the root-zone of plants (rhizosphere) is characterized by higher numbers of bacteria and increased microbial activity (Curl and Truelove, 1986). Therefore, rhizosphere metabolism can significantly contribute or govern the remedial potential of vegetation. Pilot studies of phytoremediation systems are being tested in the field in areas contaminated with chlorinated compounds. The two sites studied in this project are the Savannah River Site (SRS) in Aiken, South Carolina, and the former LaSalle Electrical Utilities in LaSalle, Illinois, both involving active phytoremediation of TCE and PCE (Brigmon et al., 2001; EPA, 2002; Lange, 2004). Research conducted at the SRS has demonstrated that TCE degradation occurs faster in the rhizosphere of trees (Walton and Anderson, 1990; Anderson and Walton, 1995; Brigmon et al., 2001). Thus, vegetation may be used to actively promote microbial restoration of contaminated soils and enhanced hazardous contaminant biodegradation.

In these sites several tree species are being studied for their phytoremediation potential. Loblolly pine (*Pinus taeda*) is being tested as a promising species that is capable of up to 90% TCE removal from soils and groundwater at the SRS (Brigmon et al., 2001). This species is characterized by the production of significant quantities of oil extracts, composed mainly of monoterpenes (Amaral et al., 1998; Savithiry et al., 1998; Phillips et al., 1999). Therefore, these compounds may influence the microbial processes

occurring in the rhizosphere either as root exudates or leachate from the decaying foliage in the surface soil layers. The importance and occurrence of these interactions on microbial metabolism have been addressed previously in several studies (White, 1986; Misra et al., 1996; Amaral and Knowles, 1997; Ward et al., 1997). Conflicting findings on the role of plant exudates on microbial natural processes in the upper soil layers, such as nitrification and methane consumption, have claimed inhibition as well as stimulation by these compounds (White, 1986; Misra et al., 1996; Amaral and Knowles, 1997; Ward et al., 1997). In planted soils at phytoremediation sites, there is no evidence that connects the increased microbial mineralization of contaminants with the presence of plant compounds.

The lack of understanding of the potential roles that rhizosphere bacteria can assume in the overall removal of contaminants is hindered by the inability to directly assess the activity and diversity of microorganisms *in situ* using traditional culture-dependent methods (Fry, 2004; Smalla, 2004). The recent development of culture-independent methods that involve soil microcosms and labeled substrates combined with molecular techniques have enabled scientists to more effectively test *in situ* conditions and, more importantly, accurately identify and characterize active microbial populations (Radajewski et al., 2000).

The main purpose of this study is to determine, by the development of an adequate protocol, how plant type, system design, and environmental conditions present at two phytoremediation sites impact the potential ability of methanotrophic bacteria to achieve biodegradation of chlorinated solvents in contaminated rhizosphere soils and groundwater. The characterization of this mechanism and the provision of an adequate

technique specific to the rhizosphere can ultimately lead to more efficient phytoremediation for more effective environmental restoration.

Significance of the Study

Any substance that poses a significant threat to human health and the environment deserves prioritized attention not only to the study of its toxicology profile but also to its environmental fate and development of remediation technologies. The U.S. EPA has classified chlorinated solvents, TCE and PCE, as priority pollutants on the basis of its widespread contamination in groundwater, its possible carcinogenic nature, and its potential to be biologically converted to the more potent carcinogen vinyl chloride under anaerobic conditions. Therefore, the majority of the National Priority List (NPL) sites are dealing with this type of contamination.

Groundwater and soil contamination by chlorinated solvents presents unique challenges to remediation technologies. Due to the chemical and physical properties of these compounds, small amounts of these solvents can contaminate a large volume of groundwater. The remediation of contaminated soil usually involves excavation and disposal of the impacted media. However, if the contaminant has reached the groundwater, the risk to the public, the remedial cost, and the amount of time required to remove the contaminants can increase substantially (Cheremisinoff, 2001).

Alternatively, *in situ* remediation technologies, such as bioremediation and phytoremediation are being tested at several NPL field sites. One example is the SRS where the potential for phytoremediation of chlorinated solvents has been demonstrated with loblolly pines capable of up to 90% TCE removal in soil and groundwater (Walton and Anderson, 1990; Anderson and Walton, 1995). These results are encouraging for the application of more sustainable remediation technologies that do not require large

amounts of inputs and promote the application of biological systems already in nature to environmental problems created by humans.

In order to better understand and monitor these biological processes occurring during phytoremediation, this study combined a laboratory and field approach. The focus of the study is on the potential contribution of methanotrophic bacteria, capable of co-oxidizing chlorinated compounds, to the rhizodegradation mechanism in phytoremediation systems. Additionally, the project concentrates efforts on method development specific to the rhizosphere environment and compares traditionally used methodologies with more recent culture-independent methods. Laboratory-based studies involved characterization of isolated pure and mixed cultures, including a groundwater isolate, Strain CSC1, which served as a means to develop phenotypic and genotypic protocols used in the phytoremediation portion of this study. In addition, the role of plant exudates and their impact on TCE biodegradation was determined with representative methanotrophs in the laboratory. Field-based studies involved characterization of the methanotrophic community in rhizosphere samples from two current TCE- and PCE-contaminated sites undergoing phytoremediation with different tree types.

Literature Review

Chlorinated Compounds

The widespread use of chlorinated hydrocarbons as solvents and degreasers in the metal and dry cleaning industries and their indiscriminate disposal have resulted in a significant adverse effect on the environment. Trichloroethylene (TCE) and tetrachloroethylene (PCE), primary chlorinated solvents found at hazardous waste sites, occupied the 16th and 31st position, respectively, in the Priority List of the United States Comprehensive Environmental Response, Compensation, and Liability Act (CERCLA),

known as Superfund (ATSDR, 2006). The pollutants rank according to their presence at National Priority List facilities, possible carcinogenic nature, and potential to be converted to more toxic byproducts, as vinyl chloride that occupies the 4th position in the CERCLA Priority List (Vogel and McCarty, 1985). A recent report from the National Academies of Science's Committee on Human Health Risks of Trichloroethylene concluded that the evidence of TCE's carcinogenic risk has increased since 2001 (NAS, 2006). Consequently, these compounds are heavily regulated by federal and state standards. The Safe Drinking Water Act regulates the national maximum contaminant level (MCL) of TCE and PCE in drinking water at 5 ppb, with a zero maximum contaminant level goal (MCLG) (EPA, 2000a).

The fate of TCE and PCE released into the environment through a variety of waste streams will be dictated by their physical and chemical properties (Table 1-1). Because their densities are greater than 1 g ml^{-1}, PCE and TCE are considered dense nonaqueous phase liquids (DNAPLs). These compounds, considered volatile organic compounds (VOCs), because of their high values of vapor pressure (74-18.5 mm Hg) and Henry's law constants (0.011-0.018 atm m^3 mol^{-1}), when released into the atmosphere or surface water and soil, will volatilize into the atmosphere. In the atmosphere, both compounds are subjected to photooxidation with a half-life of a couple of months to days. Also, they both would be predicted to reach the groundwater given their low partitioning coefficient values (log K_{oc} and log K_{ow} of 2-3), high specific gravity (>1), and resulting low tendencies to adsorb to sediments or soils and to bioconcentrate in animals and plants. Nevertheless, specific site conditions such as organic soil content can readily contribute to transient sorption of TCE (Brigmon et al., 1998; Sheremata et al., 2000). PCE may

move slower than TCE in soil infiltration processes, because of its lower water solubility (150 mg l^{-1}) compared to TCE (1,366 mg l^{-1}) (ATSDR, 1999).

As a result of their chemical and physical characteristics, groundwater contamination by chlorinated compounds presents several challenges for remediation. When TCE and PCE reach the groundwater, they are anticipated to sink deeper into the subsurface until they reach a less permeable stratum (confining layer). In this layer they will spread out or escape through fractures of the rock or clay (Kueper and McWhorter, 1991). Therefore, remediation is more difficult than spills of light NAPLs (LNAPLs), such as gasoline fuels, that float near the surface of the water table as a compact mass and do not act as a slow-releasing, continuous source of pollution (Cheremisinoff, 2001).

Chlorinated compounds in the environment are prone to microbial degradation; however, the rate and extent of oxidation in the presence of oxygen is inversely related to the chlorine-to-carbon ratio (Hanson and Hanson, 1996). Highly chlorinated hydrocarbons, such as PCE, are not degraded aerobically. PCE is reductively dehalogenated under anaerobic conditions (Uchiyama et al., 1989; Bowman et al., 1993b).

In aerobic environments, TCE and the metabolites of the reductive dehalogenation of PCE and TCE, such as dichloroethylene (DCE) and vinyl chloride (VC), are cometabolically oxidized to CO_2 by bacteria that possess oxygenase enzymes. Some of these enzymes are the methane monooxygenase (MMO) of methanotrophs, toluene dioxygenase (TDO) of *Pseudomonas putida* F1, toluene 2-monooxygenases (TMO) of *Burkholderia cepacia* G4, propane monoxygenase of *Mycobacterium vaccae* JOB5, phenol hydroxylase (PH) of *Alcaligenes eutrophus* JMP134 and *Burkholderia cepacia*

G4, alkene monooxygenase (AMO) of *Alcaligenes denitrificans* spp., ammonia monooxygenase of *Nitrosomonas europaea*, and isopropylbenzene dioxygenase (IPB) of *Pseudomonas* sp. JR1 (Arciero et al., 1989; Wackett et al., 1989; Ewers et al., 1990; Folsom et al., 1990; Fox et al., 1990; Dabrock et al., 1992; Kim et al., 1996; Smith et al., 1997). From this cometabolic process, microorganisms do not gain energy or carbon of the oxidized pollutant. Therefore, an external source of carbon (electron donor) must be present apart from oxygen that serves as the electron acceptor in the reaction.

In anaerobic conditions, pathways of degradation occur via the process of dehalorespiration catalyzed by the reductive dehalogenase enzyme. The chlorinated compound functions as the electron acceptor and, commonly, hydrogen as the electron donor. The only known microorganism that performs reductive dechlorination of TCE and PCE to completion is *Dehalococcoides ethenogenes* strain 195 (Maymo-Gatell et al., 1999). Other anaerobes and facultative anaerobes, such as sulfate reducers and methanogens, degrade TCE and PCE incompletely to cis-DCE and VC (Holliger et al., 1998). *Desulfuromonas chloroethenica*, a sulfur-reducing bacterium utilizes pyruvate or acetate as the electron donor and degrades PCE or TCE to cis-DCE (Krumholz, 1997).

Phytoremediation

Phytoremediation, the use of plants to remediate contaminated sites, takes advantage of the ability of plants to extract, sequester or degrade pollutants by the mechanisms of phyto-extraction, -volatilization, -degradation, -stabilization, and rhizodegradation (Fig. 1-1). The last mechanism is of special interest because it involves plant-microbe interactions occurring in the root system (rhizosphere). The role of rhizosphere microorganisms in the overall breakdown and removal of pollutants is influenced by the type of contaminant and plant species utilized. Rhizodegradation has

been reported as the main process in organic pollutant remediation of toluene, phenol, and TCE (Narayanan et al., 1999).

The use of plants represents an alternative technology to traditional waste management practices, such as incineration, excavation and landfilling, and pump-and-treat-systems. The effectiveness of phytoremediation has been demonstrated in a wide range of applications, such as herbicides, petroleum hydrocarbons, metals, radionuclides, leachates from landfills and sewages, nutrients, pentachlorophenol, polycyclic aromatic hydrocarbons, and chlorinated solvents. Phytoremediation offers multiple advantages, including being a low cost *in situ* technology that is environment-friendly and publicly accepted. Most importantly there is no need to disturb the site and, after the treatment, the soil is left fertile for further use. However, some limitations and concerns dictate the potential applications of this technology, including the time necessary for acceptable effects to take place, the limited depth of the root system, the sensitivity of plants and microbes towards the contaminant, the seasonal variability in the rate of treatment, and the potential of contaminant bioaccumulation or transport into the food chain. Nevertheless, some of these limitations can be overcome by selecting the appropriate plant species or by combining other technologies, such as pumping and irrigating the trees with the deeper contaminated groundwater (EPA, 2000b; McCutcheon and Schnoor, 2003).

Phytoremediation efficiency is still limited by a lack of knowledge of many basic plant processes and interactions with other organisms such as bacteria and fungi. Pollutant degradation by bacteria and fungi have been studied extensively and, even though plants can also express similar metabolic pathways, it is only recently that efforts

have been concentrated towards understanding the plant system. Most enzymes involved in organic xenobiotic degradation, such as cytochrome P450 oxidases, peroxidases, and glutathione-S-transferase, are known to be present in both microorganisms and plants (Sandermann, 1994; Shang et al., 2003; Chaudhry et al., 2005).

Phytoremediation of chlorinated solvents from groundwater and soil have reported up to 90% contaminant removal by the use of different plant species (Walton and Anderson, 1990; Newman et al., 1999; Brigmon et al., 2001; Nevius et al., 2004). However, when assessing the responsible mechanisms of contaminant removal, studies are not consistent. The main contradiction in phytoremediation of chlorinated solvents regards the magnitude of plant uptake, phytovolatilization, and rhizodegradation (Orchard et al., 2000a). Several studies have reported that TCE disappearance in planted systems is mainly due to plant uptake, followed by phytovolatilization and diffusion through the stem and/or metabolism by the plant (Schroll et al., 1994; Anderson and Walton, 1995; Newman et al., 1997; Burken and Schnoor, 1998). On the contrary, other studies have observed TCE degradation occurring mainly as a result of rhizosphere microbial metabolism (Walton and Anderson, 1990; Anderson et al., 1993; Schnabel et al., 1997; Orchard et al., 2000a). Contradictory results may be the outcome of experimental artifacts caused by high exposure to TCE concentrations, use of co-solvents, the short duration of many studies, and plant stress originated by the use of static chambers to assess a mass balance of the system. Additionally, problems exist in the separation of the above- and below-ground compartments, selection of adequate controls, and lack of methods to correlate bench-scale studies to the field (Orchard et al., 2000b; Orchard et al., 2000a).

Poplar and willow trees are the preferred plant species in temperate climates for TCE and PCE phytoremediation. They have also been used for the remediation of heavy metals, salts, pesticides, explosives, radionuclides, hydrocarbons, and landfill leachates (Isebrands and Karnosky, 2001). Valuable poplar and willow characteristics that make them ideal for this application are that they are fast-growing, easily propagated, tolerant to high levels of contaminants (<550 ppm TCE), resistant to saturated conditions, and they are phreatophytes (deep-rooted plant where water uptake is mainly from the groundwater) (Isebrands and Karnosky, 2001; Pilon-Smits, 2005). In particular, willows have been found to consistently utilize groundwater sources even during periods of rainfall (Snyder and Williams, 2000). Additionally, poplar and willow trees possess specialized root vessels (aerenchyma) that may comprise up to 60% of the intracellular volume and mediate oxygen diffusion deeper into the soil profile (Chaudhry et al., 2005). It has been hypothesized that willow trees may contain a higher concentration of oxidative enzymes. When poplar and willow trees were dosed with PCE, only by-products of degradation were found in willow and no TCE was detected, as it was commonly found in poplar tissue and its rhizosphere (Nzengung and Jeffers, 2001).

The large surface area and porous wood of poplar trees allows water transport through the entire cross-section of the stem, which can result in 3 m $year^{-1}$ growth under optimal conditions (Landmeyer, 2001). Transpiration rates can increase from 19 to 200-1000 L of water day^{-1} in young to mature trees (Newman et al., 1997; Pilon-Smits, 2005). These high transpiration rates can extract enough water to depress the water table locally, inducing flow toward the trees and, consequently, containing the contaminant plume (hydraulic control). Additionally, poplar trees possess endophytic bacteria, including

methanotrophs, that live symbiotically within the plant. Some of these bacteria isolated from plants are known for their bioremediation potential, including members of *Pseudomonas* sp., *Enterobacter-Clostridium* species, and methylotroph species such as *Methylobacterium populi* sp. nov. (Brigmon et al., 1999; Van Aken et al., 2004a; Van Aken et al., 2004b).

The effectiveness of chlorinated solvent phytoremediation by poplar and willow trees is strongly influenced by the choice of genotypes (clones). Consideration of the adequate clone is an essential selection criterion, as the choice must be compatible with the intended use, the site characteristics (soil type, microclimate, pests and diseases) and with the local opinion concerning use of native *versus* exotic trees (Isebrands and Karnosky, 2001).

Other potential tree species that have been studied for chlorinated solvent phytoremediation are conifers, in particular the loblolly pine (Anderson and Walton, 1995; Punshon et al., 2002; Brigmon et al., 2003). In a study where pine, willow, and poplar trees were compared for their TCE phytoremediation potential, undegraded TCE was found primarily in the vascular system and leaves of pine, whereas plant metabolites of TCE were found within the leaf tissue of poplar and willow trees, suggesting plant degradation potential of these type of trees (Punshon et al., 2002). Meanwhile, for pines, it has been postulated that rhizodegradation is the main phytoremediation mechanism (Anderson and Walton, 1995).

The Rhizosphere

The rhizosphere is the root-zone under the influence of the plant (Curl and Truelove, 1986). This zone is constantly enriched with a variety of plant-derived compounds, and, as a result, higher microbial densities (5-20 times) and rates of activity

(2-3 orders) occurred in this area compared to non-vegetated soil (Walton et al., 1994). In rhizosphere studies, the "rhizoplane" is defined as the soil adhered to the roots, the "rhizosphere" as the soil under the influence of the plant, and the "rhizosphere effect" (R/S ratio) as the ratio between the abundance of microbial populations in the rhizosphere to that in bulk soil. However, the effect of the plant is not only translated in higher abundance but also in higher activity. Therefore, when plants are present, selective enrichment of populations may or may not translate to higher R/S ratios, although higher degradation activity is observed (Haby and Crowley, 1996).

Up to 10-40% of the assimilated carbon may be exuded by plants into the rhizosphere (rhizodeposition) in the form of compounds that are readily utilized by microorganisms (Whipps and Lynch, 1983). Plant exudates include sugars, amino acids, organic acids, nucleotides, flavonones, phenolic compounds, terpenes, and certain enzymes. The rate of exudation depends on the age of the plant, soil nutrient availability, presence of contaminants, and seasonality. These compounds have been shown to be released into the rhizosphere in greater amounts at the end of the growing season during leaf senescence (Hegde and Fletcher, 1996). During this period, about 58% of the produced fine root biomass dies (root turnover), and, as a result, an increase of up to 2-fold in phenolic compounds has been observed at the rhizosphere (Leigh et al., 2002). These compounds are known to stimulate polychlorinated biphenyl (PCB) biodegradation (Donnelly et al., 1994). Apart from the variety of carbon sources, the rhizosphere provides steady redox conditions and ideal attachment sites for bacterial proliferation (Curl and Truelove, 1986; Shim et al., 2000).

Plants benefit from the presence of rhizosphere microorganisms because they can increase nutrient availability through biosurfactant production (solubilizes soil-bound nutrients) and N_2 fixation, produce hormones that promote plant growth, suppress deleterious microorganisms by the production of antibiotics, and degrade phytotoxic soil contaminants (Smalla et al., 2001). Thus, there is also considerable interest in characterizing the structure and function of rhizosphere microbial communities for the advantageous effects to plants.

Phytoremediation may exploit the beneficial effect of moderate plant stress (Barocsi et al., 2003; Chaudhry et al., 2005). Certain levels of nutrient and water deficiencies and chemical toxicity may induce stress adaptation, root proliferation and exudation, and enhance root hair density. For example, P or K deficiency is known to stimulate exudation of organic acids and certain enzymes. Meanwhile, Fe or Zn deficiency induces the production of metal chelators (phytosiderophores) (Chaudhry et al., 2005). Plant tolerance to heavy metals was enhanced when a synthetic chelate (ethylenediamintetraacetic acid, EDTA), which rapidly increases metal bioavailability, was applied in several low doses avoiding plant detrimental effects and securing time for plant adaptation (Blaylock et al., 1997; Barocsi et al., 2003).

Within the diversity of rhizosphere microorganisms, there are strains capable of degrading xenobiotic compounds (Curl and Truelove, 1986; Walton and Anderson, 1990; Walton et al., 1994; Anderson and Walton, 1995; Brigmon et al., 1999). The diversity of heterotroph microorganisms may enhance stepwise transformation of contaminants by microbial consortium and/or provide an environment that is favorable for genetic exchange and gene rearrangements of the degradative traits. The presence of structural

analogs to contaminants in root exudates, cell wall components, and lysates, as well as secondary products of degradation of these materials, might fortuitously select for microbes that metabolize (accompanied by energy gain) or cometabolize (involving no energy gain) xenobiotics. Terpenes (secondary plant metabolites) and PCBs plant analogs (phenolic compounds) have been reported to play an important role in activating or transforming specific bacterial habitats by inducing biphenyl dioxygenase in PCB-degrading bacteria and increase populations of this degraders by up to 100-fold (Donnelly et al., 1994; Fletcher and Hegde, 1995; Haby and Crowley, 1996).

The rhizosphere provides stable sources of oxygen and methane that can support the activity of methane-oxidizing bacteria (methanotrophs), known to cometabolically oxidize TCE at higher rates than other bacteria (Little et al., 1988; Fox et al., 1990; Brigmon et al., 1999). It has been demonstrated that TCE degradation occurs faster in the rhizosphere of plants (Walton and Anderson, 1990; Anderson and Walton, 1995), where the presence and density of methanotrophs has been shown to play an important role in TCE degradation (Brigmon et al., 1999).

Microbial degradation of contaminants is usually not driven by energy needs, but by a necessity to reduce toxicity for which microbes may experience an energy deficit. Therefore, the process may be assisted and driven by the abundant energy available in the rhizosphere environment as root exudates and accumulated plant biomass. The type of compounds, the species of plant, and the degree of contamination may have the potential to exert pressure and thus select for specialized degrading bacterial populations. Consequently, rhizosphere microbial populations may change considerably with time in response to the type and degree of contamination (Fletcher and Hegde, 1995; Hernandez

et al., 1997; Brigmon et al., 1999; Kozdroj and van Elsas, 2000). However, there is a lack of information on specific plant characteristics that promote microbial degradation of organic pollutants (Chaudhry et al., 2005).

Another unexplored area of rhizosphere microenvironments is the interaction of plant and microbial populations with mycorrhizae. Mycorrhizae, symbiotic root-fungi, play an important role in plant establishment and survival. Some of these symbiotic associations are specific to plant species, such as with loblolly pine trees used in phytoremediation. In these pines higher densities of methanotrophic bacteria were observed to be associated with the fungi (Brigmon et al., 1999). Therefore, mycorrhizae may contribute significantly to the remediation potential of several plant species. The fungi provides the plant and rhizosphere bacteria protection against drought and toxic pollutants because of the physical barrier created by their extensive hyphae network. Also, this network can increase the surface area over which the plants and associated microorganisms explore for water, nutrients, and pollutant uptake. Additionally, mycorrhizae is known for the extraction of heavy metals and degradation of organic pollutants from soil, including 2-4-D, atrazine, and PCBs (Donnelly and Fletcher, 1995; Meharg and Cairney, 2000; Chaudhry et al., 2005).

Rhizosphere-enhanced microbial degradation processes are poorly understood and certainly vary according to soil conditions, plant species, and type of contaminant (Haby and Crowley, 1996). It is relevant to study these processes, as they have the potential to completely mineralize contaminants. As a result, contaminants are not transported into the plant, reducing the possibility of passing the toxic compound into other organisms in the food chain and the release of potentially harmful pollutants into the atmosphere. This

scenario may represent the ideal *in situ* remediation system, where the role of the plant is to support and stimulate microorganisms capable of contaminant degradation.

Monoterpenes

More than 70 hydrocarbons, including isoprene, mono- and sesqui-terpenes and a substantial number of oxygenated organics, are the predominant chemical species emitted by vegetation (Benjamin et al., 1996). Monoterpenes and isoprene are the major natural volatile organic compounds (VOCs) and α- and β-pinene are the representative monoterpenes (Kim, 2001). Monoterpenes are the simplest constituents of the plant essential oils and the major non-methane hydrocarbon emitted to the atmosphere (4.8 X 10^{14} g $year^{-1}$), which contributes to the formation of tropospheric ozone (Zimmerman et al., 1978).

Emissions from conifer forests are predominantly monoterpenes (Amaral and Knowles, 1998; Savithiry et al., 1998). For *Pinus taeda* (loblolly pine), monoterpene emissions are 5.1 μg g leaf dw^{-1} h^{-1} with greater than 60% represented by α-pinene (Benjamin et al., 1996; Kim, 2001). On the contrary, broad leaf species, such as *Populus deltoides* (poplar) and *Salix nigra* (willow), are among the high isoprene-emitting species (Geron et al., 2001) with 37.0 and 25.2 μg leaf dw^{-1} h^{-1}, respectively, with no detected monoterpene emissions (Lamb et al., 1985).

Apart from tree emissions, monoterpenes can be released into the environment from discharge effluents of the pulp-manufacturing industry, as monoterpenes are the predominant component of turpentine (Kleinheinz et al., 1999). Monoterpenes are also being used in the food, perfume, pharmaceutical industries, and, recently, at a larger scale in an effort to substitute for chlorofluorocarbons and halogenated solvents (Amaral et al.,

1998). Therefore, their environmental fate and interactions with other substances are of importance.

These compounds possess a broad range of functions in nature, from ecological interactions that extend from allelopathy agents (antimicrobials and fungicides) to pollinator attractants (Tooker et al., 2002). Potential sources of monoterpenes in soils include leachate from leaf litter and canopy leaves, root exudation, and deposition from the atmosphere. The role of monoterpenes and their effect on soil microbial communities is complex and has not been fully elucidated. It is known that certain microbial enzymes are stimulated by the presence of monoterpenes and that several microorganisms, including *Pseudomonas* sp., *Alcaligenes xylosoxidans,* and *Bacillus* sp., can use these compounds as carbon and energy sources (Vokou et al., 1984; Misra et al., 1996; Vokou and Liotiri, 1999; Yoo et al., 2001). Also, it has been reported that monoterpenes, first introduced as decaying plant material or exudates of monoterpene-releasing plants, may enhance biotransformation of PCBs (Hernandez et al., 1997). However, there are also reports of inhibition of different microbial processes by these compounds (Vokou et al., 1984; White, 1986; Ward et al., 1997).

Nitrogen mineralization and nitrification is inhibited in the presence of monoterpenes, but the precise mode of action has not yet been elucidated (White, 1986; White, 1988; White, 1994). White (1988) proposed that monoterpenes hinder nitrification by inhibiting the enzymatic activity of ammonium monooxygenase (AMO), the first enzyme in the ammonia oxidation pathway, and that the degree of inhibition was determined by the structure of the compound. These results have provoked other studies on the effect of monoterpenes on methane oxidation because of the similarity between the

monooxygenase enzymes of these two groups of bacteria (Amaral and Knowles, 1997; Amaral et al., 1998). The speculation is that monoterpenes inhibit MMO similarly to AMO, resulting in the inhibition of CH_4 uptake. The authors also support this hypothesis by mentioning that methanotrophs are not that commonly found in the surface layers of forest soils, where monoterpene concentrations are the highest (Amaral and Knowles, 1997; Amaral et al., 1998). However, methanotrophs in soil surfaces oxidized atmospheric CH_4 (at concentrations of 1.7 ppm), and their isolation has proven to be difficult because of the competitive advantage of low affinity methanotrophs in generally used laboratory conditions at high CH_4 concentrations (usually 20% (v/v) CH_4). To date, high affinity methanotrophs have been phylogenetically identified but not isolated (Holmes et al., 1999; Jensen et al., 2000). Consequently, the low abundance of methanotrophs in soil surfaces may be the result of inadequate cultivation techniques.

Alpha-pinene, one of the most abundant monoterpenes, exists predominantly in North America as the right enantiomer, (+)-α-pinene (Savithiry et al., 1998). This compound is a bicyclic alkene composed of two isoprene units-C_5H_8. When released into the environment, the fate of this monoterpene is dictated by its physical and chemical properties (Table 1-2). *Alpha*-pinene released into the atmosphere exists solely as a vapor that can be degraded by the reaction with photochemically produced hydroxyl radicals (half-life= 4 h), ozone (half-life= 40 min), and nitrate radicals and in a nighttime reaction (half-life= 6 min). In soils, α-pinene shows low mobility because it adsorbs to soil particles (K_{oc} of 1 200 and log K_{ow} of 4.83). However, in moist soil surfaces, volatilization is expected to be an important process based on its Henry's law constant (0.107 atm m^3 mol^{-1}). In water, α-pinene will adsorb to suspended solids and sediments

and exhibit a high potential for bioconcentration in aquatic organisms (bioconcentration factor of 2, 800) (HSDB, 1999). Biodegradation of α-pinene occurs in soils, whereas a variety of bacteria (*Pseudomona*s sp., *Alcaligenes xylosoxidans*, *Bacillus* sp.) and fungi (*Cladosporium* sp.) partially degrade this compound in both aerobic and anaerobic conditions (Harder and Probian, 1995; Misra et al., 1996; Misra and Pavlostathis, 1997; Kleinheinz et al., 1999; Pavlostathis and Misra, 1999; Yoo et al., 2001).

Methanotrophic Bacteria

Methanotrophs belong to the physiological group of methylotrophs. Methylotrophs are aerobic microorganisms that utilize as their sole source of carbon and energy reduced carbon substrates with no C-C bonds (C_1 compounds) and assimilate carbon via formaldehyde (Fig. 1-2) (Hanson and Hanson, 1996). Methanotrophs are considered obligate methylotrophs because they only grow on C_1 compounds, including methane and methanol (Lidstrom, 2001). However, recently, a new species was described with the capability of facultative growth on multi-carbon compounds, *Methylocella silvestris* BL2 (Theisen et al., 2005).

The ability to grow on CH_4 is almost exclusive to methanotrophs, except for a gram-positive methylotroph of the genus *Mycobacterium* (Reed and Dugan, 1987). Methanotrophs possess complex intracytoplasmic membrane systems, which appear to be involved in CH_4 uptake. The configuration of these membranes apart from other characteristics separates methanotrophs into two groups. Those that possess membranes as bundles of disks stacked throughout the center of the cell (type I) and those with membranes arranged as rings at the periphery of the cell (type II) (Table 1-3). Other characteristics that correlate to the type classification include DNA GC content, pathways of C-assimilation, rosette formation, types of cysts, and ability to fix N_2. A small number

of methanotrophs from the genus *Methylococcus* possess characteristics of both groups; therefore, they have been classified into the type X category (Hanson and Hanson, 1996; Graham et al., 2002). The existence of this type X grouping is, however, a point of debate within the C1 research community.

The mechanism by which methanotrophs oxidize CH_4 to methanol and cometabolize (the microorganism gains no carbon or energy from the substrate it oxidizes, as previously defined) many other compounds including chlorinated solvents is facilitated by the enzyme methane monooxygenase (MMO), unique to methanotrophs. MMO exists in the soluble (sMMO) or particulate (pMMO) form depending on the bioavailability of copper in the environment. pMMO is a Cu- and Fe-containing enzyme bound to the intracytoplasmic membrane (Nguyen et al., 1994; Lieberman and Rosenzweig, 2004), whereas sMMO, with a unique di-iron site at its catalytic center, is located in the cytoplasm (Lipscomb, 1994; Kopp and Lippard, 2002). Although both forms of MMO exhibit a lack of substrate specificity, the soluble form has been shown to display a broader range, including alkanes, alkenes, and aromatic compounds. sMMO is among the most nonspecific enzymes known to date and exhibit high substrate turnover rates. Therefore, sMMO is more suitable for the degradation of a wider variety of contaminants. However, sMMO is only synthesized by type II and X methanotrophs in environments with Cu concentrations less than 50 nM (< 0.89 -1 μmol Cu g^{-1} dw cells) (Oldenhuis et al., 1989; Hanson and Hanson, 1996).

The oxygen and methane levels also influence the expression of either form of MMO. In environments with abundant oxygen and limiting concentrations of CH_4, methanotrophs express pMMO, regardless of whether Cu is limiting. On the contrary, in

oxygen-limited environments with high CH_4 concentrations, the expression of either enzyme is dictated solely by the Cu availability. At low Cu concentrations and high cell densities, sMMO is expressed. Cells that express pMMO have higher growth yields and greater affinity for CH_4 because pMMO employs an abundant high-energy electron donor for CH_4 oxidation. Meanwhile, sMMO possesses a high-energy demand, because of the involvement of $NADH+H^+$ as an electron donor that catalyzes this reaction (Hanson and Hanson, 1996; Sullivan et al., 1998).

Ecology and habitats of methanotrophs

Methanotrophs have been widely studied for their role in the carbon cycle. They intercept and oxidize CH_4 that escapes from anaerobic environments, thus preventing large quantities from escaping into the atmosphere (Hanson and Hanson, 1996). Thus, methanotrophs are considered the principal biological sink of atmospheric CH_4 by regulating the amount of CH_4 present in the atmosphere and, consequently, decreasing the impact that CH_4, 23 times more potent than CO_2, has on global warming (Houghton et al., 2001).

Methanotrophs are widespread in nature, found in any environment where CH_4 and oxygen are present. Under flooded conditions, such as rice paddies, wetland soils, swamps, and bogs, they are restricted to the soil surface layers and to the rhizosphere of plants where they intercept the CH_4 being produced nearby under anaerobic conditions. Meanwhile, in upland soils, non-flooded habitats, such as forest, grasslands, and arable land, methanotrophs are found in the top soil layers where they oxidize atmospheric CH_4 (Hanson and Hanson, 1996; Horz et al., 2002). Also, in these habitats, they are found deeper in the soil profile, stratified in a narrow band at the oxic-anoxic interface where concentrations of CH_4 and oxygen are the highest. Methanotrophs have been isolated

from marine, freshwater, and terrestrial habitats, under conditions of high and low pH, and temperatures up to 55°C. They exist as symbionts with invertebrates and plants. However, little attention has been paid to symbiotic relationships between plants and methanotrophs, even though the first methanotroph isolated was from the leaves of a macrophyte in 1906 by Söhngen (Hanson and Hanson, 1996).

It is well known that different types of methanotrophs adapt better to different environmental conditions. Methane, oxygen, and nitrogen concentrations are the primary determinants of the type of methanotroph present in an environment. Type I methanotrophs outcompete type II species at low CH_4 concentrations (<2 ppmv in soils), whereas growth of type II methanotrophs is favored under low oxygen (<0.2 ppm in deep waters) and high CH_4 conditions (>1 000 ppmv in sediments) (Hanson and Hanson, 1996). However, because of habitat heterogeneity or differences in experimental techniques used, a consistent pattern concerning the competitive dominance of certain types of methanotrophs has been difficult to discern. Type II methanotrophs have been reported to be dominant in soils; however, an abundance of type I or of both types has also been reported in the soil environment (Vecherskaya et al., 1993; Brusseau et al., 1994; Sundh et al., 1995; Hanson and Hanson, 1996; Seghers et al., 2005). On the contrary, type I methanotrophs appear to prevail in aquatic environments such as lake water, sediments, and groundwater. Apart from these contradictory results, some genera, including *Methylobacter* and *Methylocystis*, representatives of type I and II methanotrophs, respectively, have been detected in a wide range of habitats. It has been speculated that their ability to produce resistant cysts enables these strains to persist in a wide range of habitats (Knief et al., 2003; Bodelier et al., 2005).

Environmental factors affecting methanotrophs

The effect of several environmental variables on methanotroph composition, community structure, and activity has been studied in a variety of habitats with some inconsistent results. The outcome of these studies seems to depend on the type of habitat being evaluated and on the variety of methodologies used. Soil type has been reported as the primary determinant of the methanotroph community structure in agricultural soils (Girvan et al., 2003; Seghers et al., 2005). However, in forest soils pH value has been postulated as the primary factor affecting methanotroph distribution (Knief et al., 2003). Atmospheric CH_4 oxidation activity has been reported to depend on plant cover and land use, where activity has been shown to decrease with an increase in degree of disturbance (woodland>grasslands>farmland) (Willison et al., 1995; Knief et al., 2003). Also, management practices, including fertilizer type (organic *versus* mineral) and type of tree in forest stands, have been reported to influence methanotroph activity and abundance (Reay et al., 2001; Girvan et al., 2003). However, some genera, *Methylocaldum*, *Methylosinus*, and *Methylocystis,* are universally observed in different soils, independent of land use or plant cover (Knief et al., 2003).

In the presence of plants, mainly in saturated environments, the spatial distribution of methanotrophs is determined by the soil compartment (rhizosphere> bulk soil> bare soil) or the position of the soil-water interface (Gilbert and Frenzel, 1998; Dubey and Singh, 2001; Macalady et al., 2002). It has been proposed that, because plants differ in their ability to transport oxygen to the rhizosphere, different factors control their associated methanotroph populations (King, 1994; Macalady et al., 2002). Spatial changes in the methanotroph community have also been observed in forest soils depending on season and soil depth (Henckel et al., 2000; Bodelier et al., 2005). In

winter, atmospheric CH_4 oxidation occurs in a well-defined subsurface layer (6-14 cm deep), and, during summer, the complete soil core (0-26 cm deep) is active. However, no seasonal shift in community composition was detected, the same methanotroph population was identified in summer and winter (Henckel et al., 2000).

Other environmental factors known to affect methanotrophs are the potential inhibitory effects of ammonium and/or nitrite that act as competitive substrates for MMO (Dunfield and Knowles, 1995; Hanson and Hanson, 1996). However, recent studies with rice plants have shown that nitrogen fertilization increases CH_4 oxidation in densely rooted soils because rhizosphere methanotrophs face intense plant and microbial competition for nitrogen (Macalady et al., 2002; Eller et al., 2005).

Methanotrophs and chlorinated compounds

Methanotrophs oxidize the less-chlorinated hydrocarbons at very different rates depending on the form of MMO expressed (Leadbetter and Foster, 1959; Little et al., 1988; Fox et al., 1990; Alvarez-Cohen and McCarty, 1991a; Alvarez-Cohen and McCarty, 1991b; Henry and Grbić-Galić, 1991). TCE oxidation by sMMO is comparable to that of CH_4 and up to 700-fold higher than that reported for other MMO microbial enzymes (toluene 4-monoxygenase, ammonia monooxygenase, and propane monooxygenase) (Fox et al., 1990). However, TCE oxidation catalyzed by pMMO occurs at much lower rates than sMMO (DiSpirito et al., 1992).

sMMO oxidizes TCE to TCE epoxide (95%) and chloral hydrate (5%) (Fig. 1-3A) (Oldenhuis et al., 1989; Newman and Wackett, 1991; Fox et al., 1990). TCE epoxide rapidly undergoes spontaneous decomposition; meanwhile, chloral hydrate is more stable and undergoes biological transformation within 1 to 24 h of incubation to trichloroethanol and trichloroacetic acid. At high temperature (60°C) and pH of 9.0, chloral hydrate is

easily decomposed to chloroform and formic acid (Newman and Wackett, 1991). Since TCE degradation is strictly a cometabolic process, no energy or carbon gain results from its oxidation; therefore, the presence of a cosubstrate is necessary to maintain cell biomass and regenerate reductant supply. Although CH_4 oxidation is required for growth and can provide electrons, it also functions as a competitive inhibitor of TCE transformation (Henry and Grbić-Galić, 1991). Byproduct toxicity also occurs as a result of this reaction, with a concomitant decrease in CH_4 oxidation rates, respiratory activity, and TCE degradation rates (Alvarez-Cohen and McCarty, 1991b; Hanson and Hanson, 1996; Chu and Alvarez-Cohen, 1999). Additionally, TCE metabolites can bind nonspecifically to cell proteins and inactivate MMO activity (Fox et al., 1990). TCE epoxide has been postulated as the responsible compound for the observed toxicity due to its reactivity or that of its degradation products (Fox et al., 1990; Chang and Alvarez-Cohen, 1996; Vlieg et al., 1996; Sullivan et al., 1998). Intermediate toxicity can be reduced by the addition of an external supply of reducing equivalent such as formate (Alvarez-Cohen and McCarty, 1991b). However, TCE oxidation toxicity appears to have a selective effect over different species of methanotrophs based on observations of distinct rates of recovery (Henry and Grbić-Galić, 1991).

Under anaerobic conditions chlorinated compounds readily undergo reductive dechlorination (Fig. 1-3B). PCE and TCE are degraded to dichloroethene isomers (*cis*- and *trans*-1,2-DCE), 1,1-DCE, vinyl chloride, ethene, and ethane. DCE isomers and vinyl chloride in the presence of TCE and no oxygen often persist in the environment because their dechloronation yields less energy than that of their parent compound (Fox et al., 1990; Hanson and Hanson, 1996). Considerable concern exists over the biological

production of vinyl chloride, a known human carcinogen; however, this product is readily oxidized by sMMO in aerobic environments (Fox et al., 1990). Additionally, when consortia of bacteria (methanotrophs and heterotrophs) are present, further oxidization of chloral hydrate, chlorinated acetic acids, and vinyl chloride has been observed along with a provision of additional reducing power for the process (Alvarez-Cohen et al., 1992; Uchiyama et al., 1992; Chang and Alvarez-Cohen, 1996).

Methanotrophs and plants

Plant-methanotroph associations studied to date have considered mainly rice fields and wetlands because of their importance as major areas of CH_4 production. DeBont et al. (1978) was the first to report CH_4 oxidation associated with rice roots, he noticed that most of the CH_4 diffused through the rhizosphere was oxidized. This observation was of relevance because any small change in oxidation processes occurring at the rhizosphere could have a global impact because rice fields contribute to approximately 25% of the current CH_4 flux to the atmosphere. However, studies to date on these interactions show high unexplained variability within plant species and between environments. It has been observed that plant species, known to oxidize CH_4 in their rhizospheres, when planted in a different environment, CH_4 consumption ranged from detected to no oxidation (King, 1996).

Root surfaces and their interior, zones where CH_4 is transported from the methanogenic sediments to the atmosphere and where atmospheric oxygen is transported to the sediments, both support methanotroph populations in saturated environments (King, 1996; Gilbert et al., 1998; Eller et al., 2005). However, methanotrophs and methylotrophs have also been detected in these locations in poplar and pine trees in non-saturated environments (Brigmon et al., 1999; Pilon-Smits, 2005). Methylotrophs

permanently associated with the plant are often encountered in the phyllosphere (leaf surface) and rhizosphere. Plants can also benefit from these associations. For example, methanotrophs can excrete or expel by cell lysis phytohormones (cytokinins and auxins) and other bioactive compounds. Additionally, type II and X methanotrophs can fix nitrogen and, therefore, can be considered phytosymbionts on the surface and inside plant tissues (Doronina et al., 2004).

The pattern of methanotroph root colonization has been studied in rice plants (Gilbert et al., 1998; Gilbert and Frenzel, 1998). The colonization is spatially very heterogeneous; some roots are not colonized at all, while others possess microcolonies as clumps or thick bacterial layers. As known for other types of bacteria, methanotroph root colonization followed the pattern of cell wall formation, potentially due to the exudation of organic substrates and oxygen leakage at these sites. While, methanotrophs cannot utilize complex organic substrates for growth, they do utilize some amino acids as nitrogen sources (Gilbert and Frenzel, 1998).

Phylogenetics of methanotrophs

Methylotrophs are scattered among the *Proteobacteria* within the α-, β-, and γ-subdivisions, not forming an evolutionary coherent group. Multi-gene operons appear to be rare among its members, and, on the contrary, plasmids are common. However, no functions have been ascribed to these plasmids, and they are entirely cryptic. Methanotrophs cluster into the α- and γ-*Proteobacteria* and are considered ideal microorganisms for molecular biology studies. Methanotroph phylogeny and their phenotypic and eco-physiology characterization into types I, II, and X validate each other (Lidstrom, 2001). The type classification, initially proposed by Whittenbury et al.

(1970), has been supported by analysis of 5S and 16S rRNA genes. Recently, with the incorporation of molecular methods to methanotroph studies, novel strains are being recognized that do not grow in standard laboratory conditions (enriched solid and liquid media with high concentrations of CH_4). For example, the upland methanotroph soil clusters (USC-α and USC-γ) that oxidize atmospheric CH_4 in forest soils. Another example is the genus *Methylocella*, sensitive to salts in regular cultivation media (Holmes et al., 1999; Henckel et al., 2000; Jensen et al., 2000; Bourne et al., 2001; Knief et al., 2003; Theisen et al., 2005).

Molecular analysis of methanotrophs

Methanotroph phylogenetic studies have been conducted with both phylogenetic and functional gene markers. Functional gene markers detect the active-site subunit of both MMO forms, *pmoA* for pMMO and *mmoX* for sMMO, and of methanol dehydrogenase by the *mxaF* gene. The use of these markers enables assessment of the potential functional diversity of methylotrophs and methanotrophs within an environment. The universal phylogenetic 16S rDNA (rRNA) primer set amplifies the variable V3 region of the gene, extensively studied to enable inference of phylogenetic relationships among microorganisms. Phylogenetic analysis of methanotrophs usually considers both *pmoA* and 16S rDNA analysis due to the fact that most methanotrophs express the pMMO gene and phylogenies between these two primer sets are closely related to each other (Bowman, 2000).

Interpretation of *pmoA* phylogenetic analysis must take into consideration that multiple copies of the gene can exist in one organism; therefore, novel clusters of *pmoA* sequences do not necessarily indicate that novel groups of uncultivated methanotrophs exist. Copies of the *pmoA* gene (*pmoA2*) can show less than 80% identity to the

previously known *pmoA* gene (*pmoA1*). Also, in some cases, there is no correlation between the 16S rDNA and *pmoA* phylogenies, and, for the genus *Methylobacter,* some strains poorly amplify the *pmoA* gene with the standard primer sets, underestimating the methanotroph diversity. Finally, one must keep in mind that the genus *Methylocella*, the only known exception to the universality among methanotrophs of the *pmoA* gene, must be detected using a different primer set. Other primer sets that could be used are the *mmoX* or *mdh* that amplify the sMMO and methanol dehydrogenase active site, respectively (Dedysh et al., 2000).

It is of importance to recognize that the success in gene retrieval from environmental samples depends on the quality of the primer sets used. Different levels of methanotroph diversity have been reported with different primers sets (Bourne et al., 2001). For example, Hutchens et al. (2004) reported that, by using the *pmoA* primer set A189f/A682r, only 8 operational taxonomic units (OTUs) were detected, but, with the A189f/mb661r primer set, 12 OTUs were retrieved (Hutchens et al., 2004). The *pmoA* primer set A189f/mb661r detects almost all methanotrophic bacteria, except sequences of *Methylomonas*, *Methylocaldum*, and the reported forest clone clusters, but it does exclude all known *amoA* sequences of ammonia-oxidizing bacteria, except for *Nitrosococcus* (Kolb et al., 2003).

With the incorporation of molecular techniques to the study of microbial ecology, one of the most intriguing questions is the relationship between what has been reported previously by community assessment based on traditional culturing and what has recently been described by culture-independent techniques. Several methanotroph studies that implemented denaturing gradient gel electrophoresis (DGGE) point out misleading

results of previous traditional culture-dependent methods. For example, in grassland soils the culture-dependent most probable number (MPN) technique was compared to direct soil sample DGGE analysis (Horz et al., 2002). While MPN analysis detected only one methanotroph strain, DGGE revealed a more diverse and dynamic methanotroph community. In a similar matter, enrichments characterized by DGGE were compared to results of morphological observations and strain isolation from agricultural soil (Jensen et al., 1998). The DGGE profile of the enrichments showed higher diversity (13-14 bands) than the morphological observation and isolation, where only 2 to 4 dominant morphological types were detected and only one colony was isolated (Jensen et al., 1998).

Another methodology that is revolutionizing methanotroph studies by linking microbial identity to biological function under conditions approaching those *in situ* is stable isotope probing (SIP) (Radajewski et al., 2000). A labeled substrate, a less naturally frequent isotope, is incorporated into the active microbial biomass. In the case of methanotrophs, $^{13}CH_4$ has been used to label the DNA of active organisms during DNA synthesis and replication. The heavier DNA (^{13}C-DNA) can then be separated from the naturally occurring ^{12}C-DNA. The methodology has been used to study methanotroph communities of peat soils (Morris et al., 2002), acidic forest soils (Radajewski et al., 2002), cave water (Hutchens et al., 2004), and soda lake sediments (Lin et al., 2004).

Results of this SIP method confirmed that most methanotroph communities in the environment are active and constitute a small fraction of the entire population responsible for CH_4 oxidation. Soil community fractions revealed that only a small percentage or

possibly no methanotrophs were present in the ^{12}C-DNA fraction, while the ^{13}C-DNA fraction was composed of 32% or 96% methanotroph, in peat and forest soils, respectively (Morris et al, 2002; Radajewski et al., 2002). Interestingly, sequences have been found that may represent novel methanotrophs and methylotrophs, suggesting that these bacteria most probably assimilated methanol ($^{13}CH_3OH$) excreted by methanotrophs during $^{13}CH_4$ oxidation. However, in some cases, the affiliation to methanotrophs of the retrieved sequences (β-Proteobacteria) can not be explained, suggesting the possibility that bacteria not previously considered to be involved in CH_4 oxidation may derive a significant proportion of their carbon from products of methanotroph metabolism or possibly even from CH_4 itself.

When the functional *pmoA* gene has been examined before and after SIP experiments, the diversity was lower in the ^{13}C-DNA fraction indicating that not all methanotrophs in an environment are active (Morris et al., 2002; Radajewski et al., 2002; Lin et al., 2004). Overall these studies using SIP methods have revealed that the active methanotroph community in peat and acidic forest soils was dominated by type II methanotrophs (Morris et al., 2002; Radajewski et al., 2002) and in soda lake sediments by type I methanotrophs (Lin et al., 2004). These results give insight into the ecological niches occupied by each methanotroph type.

Methods Used to Assess Rhizodegradation Potential in Phytoremediation

The microbial composition of the rhizosphere is known to differ both qualitatively and quantitatively from that in a non-planted soil. However, a precise determination of the microbial diversity in soil or the rhizosphere compartment remains to be established, as only up to 10% of soil microbial species can currently be cultured in the laboratory (Fry, 2004). Although the ability to culture the yet-uncultured bacteria is of importance,

a number of indirect methods are currently used to establish the biodegradation potential of soil microorganisms. By the use of these indirect methods, not only is the microbial composition and structure of a specific habitat being determined but also it is possible to link function to activity by the use of labeled substrates.

Culture-dependent techniques

Microbial counts. Historically, colonies forming units (CFU) and most probable number (MPN) technique have been used to enumerate selected microorganisms and assess the microbial composition of a site. However, it is recognized that only a small portion of bacteria can form colonies when traditional plating techniques are used. The proportion appears to be determined by the oligotrophic extent of the evaluated environment, where the more oligotrophic the environment, the higher portion of bacteria that do not grow under standard cultivation conditions (Smalla, 2004). Culturability, defined as the percentage of culturable bacteria to total cell counts (microscopically assess), has been determined to be around 0.3% in soils (Amann et al., 1995). Further limitations represent organisms that, under environmental stress, enter the viable but nonculturable state and bacteria strongly attached to soil particles that cannot be dislodged (Smalla, 2004). While microbial counts are widely used and easy to prepare, they are time-consuming and require multiple replicates and cultivation periods of weeks or months. Additionally, they do not discern relationships among bacteria, are highly selective, and inaccurate, underestimating the abundance of the microbial populations (Lynch, 2002).

Enrichments. Another commonly used approach for microbial characterization of environmental samples is to obtain enrichments of selected groups of bacteria. Usually, the highest dilution of the MPN technique is used as an inoculum for further cultivation.

This procedure avoids the selection of only the fast-growing and less-numerous bacteria, which benefit from the fact that some abundant bacteria do not grow directly on conventional media (Fry, 2004). Enrichments offer the possibility of preserving syntrophic relationships among bacteria and obtaining environmental isolates from which physiological and phylogenetic characterization can be performed (Wise et al., 1999). Additionally, cultures can be used to assess potential microbial activity, optimum conditions for degradation, and microbial diversity of a particular sample. However, conditions for enrichment do not resemble the field, and they are highly selective, which makes extrapolation of results to the field difficult. Furthermore, obtaining a stable culture can take months, and, when studying the culture's phylogenetics, there is no real indication of gene expression *in situ*, which is ultimately what determines environmental impact at the field. Because this technique relies on the culturability of the members of a particular sample, it is a common finding that isolates represent only a few of the most abundant bacteria. However, in some environments, isolates can represent higher numbers. In seawater, isolates represented 7-69% of the total bacterial clones obtained from culture-independent methods (Fry, 2004).

Culture-independent techniques

Molecular methods. Since 1990, microbial ecologists have been studying bacterial diversity by isolating community DNA, amplifying their 16S rRNA genes, cloning the fragments, and sequencing the clones. The development of molecular methods over the past two decades has helped resolve difficulties inherent in studying diversity using traditional approaches that are based on observations of physiology and morphology. It has led to an increase in the numbers of identified bacteria divisions to greater than 40, in which only 23 divisions are represented by isolates (Smalla, 2004).

Therefore, most of the bacteria in culture collections that grow on conventional media are not the most abundant in natural habitats. For this reason, there is a necessity to isolate the ecologically relevant bacteria, the as-yet-uncultured bacteria, and study their physiology. The basic problem is that many numerically abundant bacteria grow more slowly than the less-dominant bacteria on most laboratory media (Fry, 2004).

In spite of the advances, some challenges still remain for the molecular microbial ecologist. The most pressing challenges are obtaining nucleic acids suitable for molecular analysis and access to sufficiently large, high quality databases (Smalla, 2004). Extraction problems when organics are high in an environment, such as in the rhizosphere, still constitute a major draw back of this technique. Also, the effectiveness of oligonucleotide probes to detect organisms may be uncertain because of the possibility of encountering new genes or genes that are not conserved in a similar matter within related groups of bacteria, and, as a result, genes obtained from cultured organisms may not be sufficiently similar to genes in the environment (Hanson and Hanson, 1996). Also, there is a lack of rDNA sequences of many described species. Another limitation is that some molecular applications do not allow conclusions about the metabolically active populations or on gene expression because they do not distinguish between active and non-active organisms, thus limiting the use of these methods. Nevertheless, this information might be obtained from RNA analysis or by the use of labeled substrates.

DGGE analysis. The technique is based on the separation of PCR fragments of the same length in polyacrylamide gels containing a linearly increasing gradient of chemical denaturants (urea and formamide) (Muyzer et al., 1993; Muyzer et al., 2004). Separation is based on the electrophoretic mobility of the partially melted DNA molecule, which is

lower compared to that of the completely helical form of the molecule. The different fragments melt in discrete melting domains (stretches of base pairs with an identical melting temperature). Once the domain with the lowest melting temperature reaches its denaturing concentration at a particular position in the gel, a transition from helical to partially melted molecule occurs, and the molecule will stop migrating. Therefore, sequence variants (different in base pairs) will stop migrating at different positions from which DNA fragments are differentiated and excised for sequence analysis (Muyzer et al., 1993). A GC-rich sequence (GC clamp) is incorporated into one of the primers to modify its melting behavior to the extent to which close to 100% of all possible sequence variations can be detected. The resulting banding pattern represents a profile of the populations in the sample, and the relative intensity of each band and position represents the relative abundance of a particular member of the community (Muyzer et al., 1993).

The main advantage of DGGE is that it permits high-resolution phylogenetic analysis of a complete community by its diversity pattern in a qualitative and semi-quantitative matter. Large numbers of samples can be quickly analyzed and compared, permitting temporal and spatial analysis within and between communities. The only comparable technique at the moment is terminal restriction fragment length polymorphism (T-RFLP) (Bodelier et al., 2005). However, interpretation of T-RFLP data requires constructing a clone library that can be time-consuming due to the cloning step, and less abundant species are not always detected. On the contrary, DGGE can detect species represented by as low as 1% of the population, and bands are directly excised from the gel, reamplified, and sequenced without the need of cloning (Muyzer et al., 1993). DGGE cagn detect up to 95% of all possible single base substitutions among

sequences of up to 1, 000 bp in length, and it can be adjusted to narrowed denaturant gradients to provide higher resolution. Also, DGGE profiles can be transferred to hybridization membranes and probed with specific oligonucleotides (Vallaeys et al., 1997).

However, the main constraint of DGGE is the amount of phylogenetic information in the length of the commonly amplified fragments (< 500 bp). These partial sequences may not be sufficient to discriminate among strains (Boon et al., 2002; Bodelier et al., 2005). Another limitation is the production of multiple bands by one organism because of multiple heterogeneous operons or copies of the target gene, or due to the use of degenerate primers. Also, if the target sequences in a sample are present at dissimilar concentrations, the less abundant sequences may not amplified sufficiently to be visualized as bands, underestimating the diversity of the sample (Boon et al., 2002). Another problem of DGGE is co-migration of bands and bands at identical positions that are not necessarily derived from the same species; however, these bands can be screened by reducing the denaturant gradient, and, when necessary, bands in similar positions may require multiple sequencing. Finally, in community analysis of highly related phylogenetic clusters, bands can represent heteroduplexes, PCR artifacts from mixed DNA templates that result from two similar, but not corresponding strands, annealing together. These artifacts can be detected because they produce bands at low denaturant concentrations (Wise et al., 1999).

Stable isotope probing microcosms (SIP). SIP microcosms permit the identification of organisms responsible for certain *in situ* transformation processes by the use of a labeled substrate (a less naturally frequent isotope) that will be incorporated into

the active microbial biomass (Radajewski et al., 2000; McDonald et al., 2005). Subsequently, the labeled DNA fraction is separated from the naturally occurring fraction by CsCl density gradient centrifugation. The labeled fraction is then analyzed to identify the active microbial community by cloning, followed by restriction fragment length polymorphism (T-RFLP) or by DGGE analysis.

The major limitation of the SIP methodology is the dilution of the labeled substrate before its assimilation and incorporation into the active organisms, which can happen if simultaneous growth on an unlabeled substrate is occurring in the microcosms. Other constraints may be the relative long incubation periods needed to label sufficient biomass and, consequently, the potential for the use of labeled metabolites by non-target organisms (cross-feeding). Also, the artificial spike and relatively high concentrations used of the labeled substrate, may stimulate microorganisms that were not active *in situ,* and, as a result, the analysis may represent the potential active population, rather than the active microbial community at the time of sampling (Lin et al., 2004; McDonald et al., 2005). Therefore, SIP studies should provide a rational basis for the application of molecular biological techniques to study the role of specific organisms that are likely to be involved in a defined process (Radajewski et al., 2003). Finally, the technique is expensive and requires certain expertise, but it is one of the most powerful molecular techniques available, providing information on the active microbial populations of an environmental sample and linking function to identity.

Study Hypothesis

The diversity and activity of methanotroph populations associated with the rhizosphere of plants used in phytoremediation processes are impacted by plant type, system design, and environmental conditions present at the site.

Study Objectives

Broad Objectives

- Provide full characterization of a well-known, pure methanotroph, Strain CSC1, isolated from a groundwater aquifer known to oxidize TCE as a means of method development of phytoremediation studies.
- Assess the effects of plant exudates, specifically monoterpenes, on TCE cometabolism by methanotroph bacteria.
- Develop a protocol using stable isotope probing (SIP) methods specific to rhizosphere microorganisms.
- Assess differences in methanotroph abundance, activity, and diversity observed in rhizosphere samples from several plant species used in phytoremediation.
- Determine the effectiveness of culture-dependent and culture-independent methods to characterize potential microbial degraders.
- Ultimately provide guidance for the phytoremediation practitioner to more accurately predict the extent of TCE rhizodegradation when using monoterpene- and non-monoterpene releasing plants.

Specific Objectives

- Characterize the methanotroph Strain CSC1 using phenotypic and physiological descriptions, phylogenetics of the 16S rDNA and multiple functional genes (*mmoX*, *pmoA*, *mxaF*), and DNA-DNA hybridization.
- Determine the effect of (R)-α-pinene on TCE cometabolism by pure cultures of representative type I, II, and X methanotrophs using oxygen uptake analysis.
- Combine and implement SIP methods with molecular fingerprints techniques, as denaturing gradient gel electrophoresis (DGGE), using the 16S rDNA and functional *pmoA* genes, to develop a precise methodology for methanotroph rhizosphere studies.
- Determine by culture-dependent microbial counts the abundance of the heterotroph and methanotroph communities from rhizosphere soil compartments of two phytoremediation sites.
- Enrich for and characterize methanotroph mixed cultures from rhizosphere soil compartments of two phytoremediation sites by their oxidation potential using oxygen uptake analysis, presence and activity of soluble methane monoxygenase, and phylogenetics of 16S rDNA-DGGE analysis.

- Determine the effect of several environmental variables (location, time, tree type, contaminant type and concentration, depth, and system design) on the methanotroph populations of different rhizosphere soil compartments at two curent phytoremediation sites.

Table 1-1. Physical and chemical properties of TCE and PCE.[1]

Characteristic	Compound	
	TCE	PCE
Molecular formula	C_2HCl_3	C_2Cl_4
Molecular weight (g mol^{-1})	131.4	165.8
Specific gravity (at 20ºC)	1.465	1.623
Vapor pressure (at 25ºC) (mm Hg)	74	18.5
Water solubility (at 25ºC) (mg l^{-1})	1 366	150
log K_{oc}	2.03-2.66	2.20-2.70
log K_{ow}	2.42	3.40
Henry's law constant (at 25ºC) (atm m^3 mol^{-1})	0.011	0.018

[1](ATSDR, 1999)

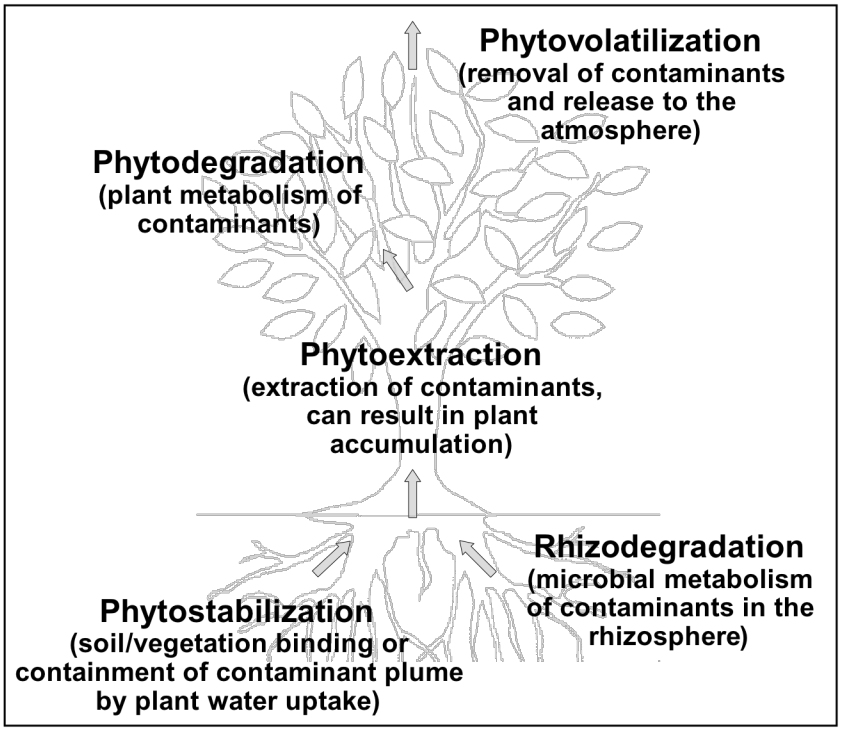

Figure 1-1. Schematic of processes in a phytoremediation system (McCutcheon and Schnoor, 2003).

Table 1-2. Physical and chemical properties of α-pinene.[1]

Property	Value
Molecular formula	$C_{10}H_{16}$
Molecular weight (g mol^{-1})	136.24
Specific gravity (at 20ºC)	0.8592
Vapor pressure (at 25ºC) (mm Hg)	4.75
Water solubility (at 25ºC) (mg l^{-1}) [2]	2.5
K_{oc}	1 200
Log K_{ow}	4.83
Henry's law constant (at 25ºC) (atm m^3 mol^{-1})	0.107

(1HSBD, 1999; 2Li et al., 1998)

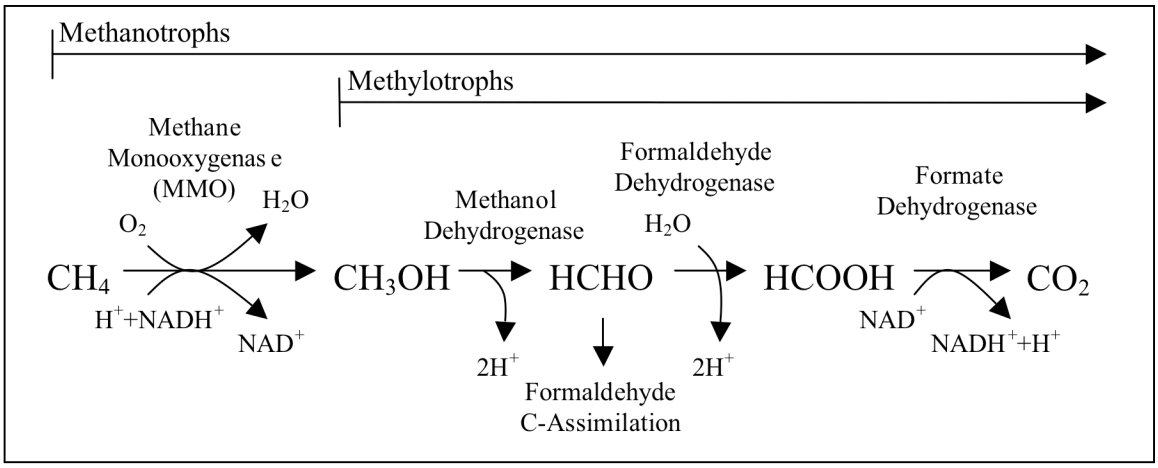

Figure 1-2. C1 metabolism by methanotrophs and methylotrophs as described by Wackett (1995).

Table 1-3. Characteristics of different methanotroph types[1].

Characteristic	Type I	Type II	Type X
Recognized genera	*Methylomonas* *Methylobacter* *Methylomicrobium* *Methylocaldum* *Methylosphaera*	*Methylosinus* *Methylocystis* *Methylocella* *Methylocapsa*	*Methylococcus*
Cellular shape	Short rods, some cocci or ellipsoids	Rods, crescent- or pear-shaped	Cocci
Resting stages	Azotobacter-type cysts	Exospores or lipid cysts	Azotobacter-type cysts
Intracytoplasmic membranes	Disc-shaped bundles of vesicles	Paired, parallel to the cytoplasmatic membrane	Disc-shaped bundles of vesicles
Formaldehyde pathway assimilation (C assimilation)	RuMP[2]	Serine	RuMP (major)/ Serine
TCA cycle[2]	Incomplete (one exception)	Complete	Incomplete
DNA G+C content	50-54%	62.5%	62.5%
Predominant phospholipid fatty acids (PLFAs)	16 C-atoms	18 C-atoms	16 C-atoms
Key enzymes: -Methane monooxygenase	pMMO[2]	pMMO/sMMO[2]	pMMO/sMMO
-3-hexulose phosphate synthase	+	-	+
-Hydroxypyruvate reductase	-	+	+
-Nitrogenase	-	+	+
-Ribulose-biphosphate carboxylase	-	-	+
-Isocitrate dehydrogenase[2]	$NAD^+/NADP^+$	$NADP^+$	NAD^+
Growth temperature	40ºC>	40ºC>	>45ºC
Phylogeny	δ Proteobacteria	α Proteobacteria	δ Proteobacteria

[1](Hanson and Hanson, 1996; Sullivan et al., 1998; Graham et al., 2002).
[2]RuMP= ribulose monophosphate cycle; TCA= ricarboxylic acid cycle; pMMO= particulate methane monooxygenase; sMMO= soluble methane monooxygenase; NAD^+= nicotinamide adenine dinucleotide; $NADP^+$= nicotinamide adenine dinucleotide phosphate.

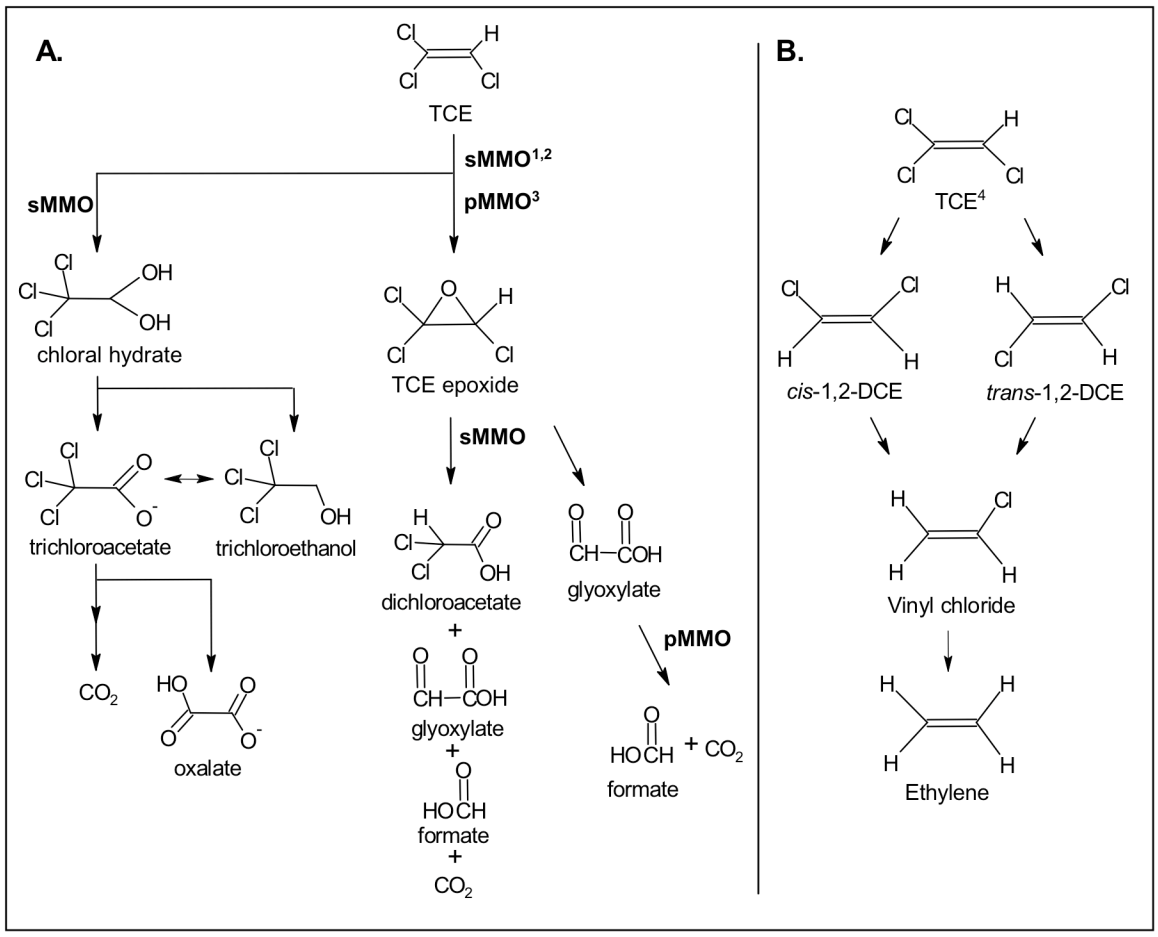

Figure 1-3. Oxidation of TCE by aerobic methanotroph degradation (A) and anaerobic reductive dehalogenation (B) ([4]Barrio-Lage et al., 1986; [3]Fox et al., 1990; [1]Vlieg et al., 1996; [2]Lontoh et al., 2000).

CHAPTER 2
METHYLOCYSTIS ALDRICHII SP. NOV., A NOVEL METHANOTROPH ISOLATED FROM A GROUNDWATER AQUIFER

Note: Manuscript submitted to the International Journal of Systematic and Evolutionary Microbiology

Lindner, A.S., Pacheco, A., Aldrich, H.C., Costello Staniec, A., Uz, I. and Hodson, D.J. 2006. *Methylocystis aldrichii* sp. nov., a novel methanotroph isolated from a groundwater aquifer. *International Journal of Systematic and Evolutionary Microbiology* **XX:** XXX-XXX.

Introduction

Species of the genus *Methylocystis* are strictly aerobic, gram-negative bacteria that are able to grow on one-carbon compounds (e.g., methane or methanol) (Bowman et al., 1993a). The genus *Methylocystis* belongs to the alpha-subclass of the Proteobacteria and currently consists of 2 species with standing in nomenclature, *Methylocystis parvus* and *Methylocystis echinoides* (Whittenbury et al., 1970; Gal'chenko et al., 1977; Bowman et al., 1993a). Numerous *Methylocystis* strains have been identified in a variety of environments, including lake, ocean, marsh, and creek sediments and water, coal mine drainage water, the roots of plants, etc. (Whittenbury et al., 1970; Gal'chenko et al., 1977; Bowman et al., 1993a; Hanson and Hanson, 1996; Calhoun and King, 1998; Heyer et al., 2002).

Species of the genus *Methylocystis* are Type II methanotrophs, classified, in part, by their possession of paired membranes aligned with the cell periphery, the serine pathway, and predominant fatty acids with 18 carbons (Hanson and Hanson, 1996; Graham et al., 2002). All known Type II methanotrophs, including the *Methylocystis* species, express the particulate form of methane monooxygenase (pMMO), and, with the

exception of *Methylocystis parvus*, all express the soluble form of methane monooxygenase (sMMO) under conditions of low copper concentrations (Stanley et al., 1983; Prior and Dalton, 1985; Choi et al., 2003). *Methylocystis parvus* does not possess genes encoding for sMMO (Tsien and Hanson, 1992; McDonald et al., 1997; Lloyd et al., 1999) and is, therefore, incapable of oxidizing aromatic compounds. All *Methylocystis* species produce oxidase and catalase, are nonmotile and are capable of fixing atmospheric nitrogen (Hanson and Hanson, 1996).

The focus of this paper is Strain CSC1, a group II methanotroph previously isolated from an uncontaminated groundwater aquifer at Moffet Naval Air Station in Mountain View, CA, USA (Henry and Grbić-Galić, 1990). This methanotroph expresses sMMO under copper-limiting conditions and is capable of oxidizing aliphatic and aromatic compounds (Henry and Grbić-Galić, 1991; Adriaens and Grbić-Galić, 1994; Adriaens, 1994; Hršak, 1996; Hršak and Begonja, 1998). Despite its being the focus of these numerous studies aimed primarily towards contaminant degradation potential, Strain CSC1 has not been characterized and differentiated from other known Type II methanotrophs. This study provides phenotypic and genotypic analysis of this groundwater isolate. The formal taxonomic description of this novel *Methylocystis* bacterium, *Methylocystis aldrichii* sp. nov. strain CSC1, is reported. Differences in various characteristics of Strain CSC1 compared to other known methanotrophs are described, and its unique surface features broaden the observed physiological traits of methanotrophic bacteria.

Materials and Methods

Strain CSC1 was obtained from Dr. Dubravka Hršak at the Rudjer Boskovic Institute in Zagreb, Croatia, and *Methylosinus trichosporium* was obtained from Dr.

Jeremy Semrau in the Department of Civil and Environmental Engineering at the University of Michigan, Ann Arbor, USA. *Methylocystis parvus* and *Methylocystis echinoides* were obtained from NCIMB (Aberdeen, England). The basal medium used for growth when culturing for sMMO expression was nitrate mineral salts (NMS) medium with no added copper, as described previously (Whittenbury et al., 1970; Lontoh and Semrau, 1998). Ten μmol l^{-1} copper nitrate ($Cu(NO_3)_2$) was added to the NMS medium to provide conditions for pMMO expression. Liquid cultures were routinely grown at 250 rpm and 30°C in either 50- or 500-ml batches in 250-ml Erlenmeyer or 2800-ml Fernbach flasks, respectively. The flasks were fitted with rubber stoppers (Fisher Scientific, Pittsburgh, PA, USA) equipped with a resealable glass tube filled with glass wool to allow headspace removal and filling. A portion of the air headspace was removed and refilled with methane of 99.99% purity (Strate Welding, Jacksonville, FL, USA) using a vacuum pump assembly to achieve a headspace concentration of air with 20% (v/v) methane.

For solid culturing, 1.5% (w/v) of Bacto agar (Difco Laboratories, Detroit, MI, USA) was added to the NMS medium. All plates were incubated in a sealed desiccator, containing anhydrous $CaSO_4$ (Drierite, W.A. Hammond Drierite Company, Xenia, OH, USA) under an atmosphere of 20% methane and 80% air (by volume) at 30°C that was refreshed every four to five days. Purity of the cultures was verified by routine streaking on 2% (w/v) nutrient agar in doubly deionized water.

sMMO expression was qualitatively verified by a naphthalene assay modified from Brusseau et al. (1990). Four negative controls—autoclaved cells, cells cultured with 10 μmol l^{-1} $Cu(NO_3)_2$ (for expression of pMMO), cell-free, and cells that have been

subjected to addition of one to two ml of acetylene gas (a known inhibitor of MMO, Prior and Dalton (1985))—were included with three live samples of active test culture diluted to an absorbance of 0.2 (at a wavelength of 600 nm) and transferred to autoclaved 10-ml capped test tubes. Seventy mg of crushed naphthalene (Sigma, St. Louis, MO, USA) were added to each tube. After incubation at 30°C and 250 rpm for a minimum of one hour, 0.1 ml of freshly prepared 4.21 mmol l^{-1} tetrazotized ortho-dianisidine (Sigma, St. Louis, MO, USA) solution was added. A subsequent pink-to-purple color formation in the tubes indicated positive sMMO activity that was verified using spectrophotometry (Fisher Scientific, Pittsburgh, PA, USA) at 550 nm.

Genomic DNA was isolated from Strain CSC1, grown to exponential phase, by a standard method (Ausubel et al., 1989). 16S rRNA gene was amplified by PCR using the universal bacterial primers 27f and 1492r (Lane, 1991). PCR primers used for sMMO were *mmoXA* (5'-ACCAAGGARCARTTCAAG-3') and *mmoXB* (5'-TGGCACTCRTARCGCTC-3') (Auman et al., 2000); for methanol dehydrogenase (MDH), *mxa* f1003 (5'-GCGGCACCAACTGGGGCTGGT-3') and *mxa* r1561 (5'-GGGCAGCATGAAGGGCTCCC-3') (McDonald and Murrell, 1997); and for pMMO, A189f (5'-GGNGACTGGGACTTCTGG-3') and A682r (5'-GAASGCNGAGAAGAASGC-3') (Holmes et al., 1995).

All PCR reactions were carried out in a PTC-200 Thermo Cycler (MJ Research, MA, USA) using 25 µl reactions and Premix Taq polymerase (Takara, Otusu, Shiga, Japan). Conditions used for the primer sets have been described previously (Holmes et al., 1995; Costello and Lidstrom, 1999; Auman et al., 2000). The PCR amplification products were ligated to vector pCR2.1 (Invitrogen, Carlsbad, CA, USA) and

transformed to competent *E. coli* cells (TOP10F') according to the vendor's instructions. Plasmid DNA from transformants was isolated and the inserts sequenced by the Biotechnology Resource Center at Cornell University (Ithaca, NY, USA).

Sequences were compared with previously identified sequences in the National Center for Biotechnology Information (NCBI) database using BLAST (Altschul et al., 1990). The 16S rRNA gene from Strain CSC1 was also aligned with sequences obtained from the Sequence Match program provided by the Ribosomal Database Project II (RDP-II) (Cole et al., 2005). Phylogenetic trees were generated using PHYLIP version 3.6 (Felsenstein, 2004) and viewed using Treeview (Page, 1996). The GenBank accession numbers for the 16S rRNA, MDH, sMMO and pMMO gene sequences obtained in this study are DQ364433, DQ664499, DQ664498, and DQ364434, respectively.

DNA-DNA hybridization was performed on Strain CSC1 by DSMZ (Braunschweig, Germany) against *Methylocystis echinoides* strain IMET 10491 was performed using 2 x SSC buffer (0.3 M NaCl, 0.03 M sodium citrate, pH 7.0) + 10% (v/v) formamide at an optimal renaturation temperature of 68°C.

Earlier studies reported Strain CSC1 as gram-negative, non-motile, coccobacillus, possessing an internal membrane structure characteristic of Type II methanotrophs (paired membranes inside the periphery of the cell), and forming lipid inclusions (Henry and Grbić-Galić, 1990; Henry and Grbić-Galić, 1991; Hršak and Begonja, 1998). Fang et al. (2000) concluded that the intact phospholipids of Strain CSC1 clustered within the Type II grouping, clearly distinct from groupings of Type I methanotrophs. This study extended the previous phenotypic characterization studies by assessing exospore and rosette formation, growth at 37°C, the presence of a surface (S-) layer, carbon and

nitrogen source utilization, and lysis by 2% (w/v) sodium dodecyl sulfate (SDS), all identified by Bowman et al. (1993a) or Hanson and Hanson (1996) as differentiating characteristics among Type II methanotrophic species.

Exospore formation was determined with one- to two-week-old broth cultures grown as previously described following methods of Smibert and Krieg (1981). Five ml of culture were transferred in duplicate to fresh NMS medium for controls. A second set of duplicates was heated in a water bath at 80°C for 20 min for pasteurization. Growth was monitored after streaking the controls and treated cultures onto solid NMS plates and incubation (as previously described) for 21 days. Exospores were monitored using light microscopy, also used to determine rosette formation (Norris and Ribbons, 1971). Growth in liquid culture was monitored using 250-ml nephlos flasks with the same stopper assembly described above and a spectrophotometer (Fisher Scientific, Pittsburgh, PA, USA) at a wavelength of 600 nm.

Nitrogen and carbon sources were tested using NMS basal medium. To test for alternate nitrogen sources, KNO_3 was replaced with 0.1% (w/v) of anhydrous L-asparagine (MP Biomedicals, Irvine, CA, USA), L-aspartate (Pfaltz & Bauer, Waterbury, CT, USA), or L-glutamine (MP Biomedicals, Irvine, CA, USA) (all shown to support growth of *Methylocystis echinoides* and *Methylocystis parvus*, Bowman et al., 1993a) or L-lysine monohydrochloride (Sigma-Aldrich, St. Louis, MO, USA), L-ornithine hydrochloride (MP Biomedicals, Irvine, CA, USA), or putrescine (MP Biomedicals, Irvine, CA, USA) (all shown to support growth of Methylosinus trichosporium, Bowman et al., 1993a). NMS medium with KNO_3 and without a nitrogen source served as positive and negative controls, respectively, and the latter control also served as a test to fix

atmospheric nitrogen. To test for alternate carbon sources, 0.2% (w/v) of methylamine hydrochloride (Alfa Aesar, Ward Hill, MA, USA), dimethyl sulfoxide, methanol, or glucose (Fisher Scientific, Pittsburgh, PA, USA) was added. Over the 30-day test period, flasks were prepared in duplicate, and transfers were made to fresh medium and nitrogen or carbon source every 4 days. Growth measurements were performed as described previously.

Lysis by 2% (w/v) SDS (Fisher Scientific, Pittsburgh, PA, USA) was determined by direct microscopic observation using cells harvested at ¾-log phase. Cells were centrifuged at 2460 x g for 20 min, resuspended in the 2% SDS stock solution for approximately 2 hours, and observed using an oil immersion phase contrast microscope (Zeiss, Oberkochen, Germany).

Transmission electron microscopy was used to observe cells of Strain CSC1 expressing MMO, lipid inclusions, and other fine structural features, including S-layers. Liquid cultures were incubated for two to three days and were fixed for 30 min at room temperature with cacodylate-buffered glutaraldehyde both with and without 0.1% Alcian blue (Fassel et al., 1992), stained for 30 min at room temperature with 1% cacodylate-buffered osmium tetroxide, and then stained for 50 min in 1% aqueous uranyl acetate. After dehydrating in increasing strengths of ethanol, cells were embedded in both Spurr's and Epon resins (Dykstra, 1993). Thin sections were prepared and stained with lead citrate and examined on a Zeiss EM-10CA transmission electron microscope. *Methylocystis echinoides* was observed by negative stain using 1% aqueous uranyl acetate applied to cell suspensions on Formvar-coated grids.

In order to provide evidence that the observed S-layer is glycoprotein, two additional cytochemical approaches were utilized. Images of Alcian blue-stained specimens were compared to those with no Alcian blue in the glutaraldehyde fixative, since Alcian blue stains polysaccharide moieties (Lewis and Knight, 1977). Secondly, thin Epon sections on Formvar-coated nickel grids were first exposed to 3% hydrogen peroxide (H_2O_2) for 15 min at room temperature to remove osmium and then exposed to 1% aqueous pronase solution (Sigma Chemical Company, St. Louis, MO, USA) for 60-90 min at 35°C to remove protein components from the section (Monneron and Bernhard, 1966; Lewis and Knight, 1977). Controls included H_2O_2 alone and water substituted for the pronase step.

Results

The phylogenies of the 16S rRNA, sMMO, MDH, and pMMO genes, shown in Fig. 2-1 and Fig. 2-2 (a-c), are consistent with placement of Strain CSC1 with other known Type II methanotrophs. The 16S rRNA phylogeny of Strain CSC1 clearly places it within a branch of the *alpha-Proteobacteria* dominated by *Methylocystis* species. This methanotroph shares 98% 16S rRNA gene sequence similarity with its nearest defined relatives, an uncultured member of the *Methylocystaceae* (AF358021), as well as two cultured organisms: *Methylocystis* sp. L32 (AJ831522) and *Methylocystis* sp. SC2 (AJ431384), although 16S rRNA gene similarity is not sufficient to place Strain CSC1 to the species level. The DNA-DNA hybridization results showed that Strain CSC1 possesses a 3.8% DNA-DNA similarity with *Methylocystis echinoides* strain IMET 10491.

As shown in Table 2-1, rosette formation by cells of Strain CSC1 was not observed. No growth was evident after pasteurization, indicating that this methanotroph

is not resistant to heat, and growth was also not observed at 37°C. Optimum growth was observed at approximately 30°C. Strain CSC1 was not lysed by a 2% solution (w/v) of SDS, but a 10% solution (w/v) of SDS did lyse the cells. It was shown to be capable of growing on alternate nitrogen sources of L-asparagine, L-aspartate, L-glutamine, L-ornithine and putrescine; however, no growth was visible in the presence of L-lysine. Of the four alternate carbon sources of methylamine, dimethylsulfide, methanol, and glucose tested, only methanol supported growth of Strain CSC1.

The expression of sMMO upon culturing Strain CSC1 in NMS medium with no copper was confirmed by formation of a purple color after incubation with naphthalene and addition of *ortho-dianisidine*, whereas controls with acetylene and with cells cultured in the presence of copper yielded no color. These results strongly suggest that sMMO was expressed in Strain CSC1 when grown without copper and was responsible for naphthalene oxidation.

Transmission electron micrographs of Strain CSC1 grown in the presence of copper verify the Type II membrane structure of paired membrane lamellae in the peripheral cytoplasm (Fig. 2-3a,b). In thin section, a variety of cell shapes were visible at low magnification (Fig. 2-3a), but elongated or dumbbell shapes of cells predominated. Many of the other profiles could represent dumbbell shapes sectioned in different planes. Cells grown without copper contained only a few internal membranous lamellae (data not shown). Polyphosphate bodies and lipid inclusions were common.

As shown in Figs. 2-3(a) and 2-3(b), distinctively striking S-layers, likely composed of glycoprotein, were revealed with transmission electron microscopy of ultrathin sections of Strain CSC1 fixed with Alcian blue. These spiked S-layer structures,

50-75 nm in height, covered the entire surface of the cell wall. We have seen that the cytoplasm of cells of Strain CSC1 embedded in Spurr resin will sometimes shrink away from the wall, lending support to the idea that the S-layer is more rigid than the rest of the wall (data not shown). This shrinkage does not occur in cells embedded in Epon resin (e.g., cells in Fig. 2-3a,b).

S-layers have been observed in both Type I and II methanotrophs isolated from a wide range of environments, including the genera *Methylomicrobium*, *Methylomonas*, *Methylosinus*, and *Methylocystis* (Fassel et al., 1992; Sorokin et al., 2000; Trotsenko and Khmelenina, 2002). Type II *Methylosinus trichosporium* OB3b was found to have bead-like S-layer structures and occasional filamentous material in the outer envelope (Fassel et al., 1990; Fassel et al., 1992). Similar bead-like S-layer structures were observed in the cell envelope of *Methylocystis* sp. strain Lake Washington but not in *Methylocystis paris*, and the authors concluded that the absence of these structures in the latter species could be a species variation. Both *Methylocystis* species possessed considerable filamentous material, however (Fassel et al., 1992). *Methylocystis echinoides* strain IMET 10491 was reported to have rigid tubular structures arranged radially at the cell surface (Gal'chenko et al., 1977), features that are absent from *Methylocystis parvus* (Heyer et al., 2002). In this study, negative stain preparations of *Methylocystis echinoides* show ellipsoid cells with square-ended tubular projections (Fig. 2-3c) that appear striated at high magnification (Fig. 2-3d). This striation was not reported in previous studies of this strain (Gal'chenko et al., 1977; Bowman et al. 1993a), and the tubular appearance of this S-layer is much different from the solid-sharp spines of Strain CSC1.

To further elucidate the nature of the spiked S-layer in Strain CSC1 cells fixed with glutaraldehyde alone (Fig. 2-4a) were compared with those fixed with an Alcian blue/glutaraldehyde mixture (Fig. 2-4b). Alcian blue is a differential stain for polysaccharide (Lewis and Knight, 1977), and the spines in Fig. 2-4b were considerably darker, longer, and more distinct than the same structures in Fig. 2-4a, even though Fig. 2-4b is at a lower magnification. This strongly indicates polysaccharide content. After treatment with H_2O_2 to remove osmium from the Epon sections, sections treated with pronase, a broad-spectrum protease, lost the entire S-layer (Fig. 2-4c,d), indicating that the layer contains considerable protein.

Discussion

Sequence analysis of the 16S rRNA gene (Fig. 2-1), the sMMO gene (Fig. 2-2a), the methanol dehydrogenase gene (Fig. 2-2b), and the pMMO gene (Fig. 2-2c) supports placement of Strain CSC1 within the closely related genera *Methylocystis* and *Methylosinus*.

Given the suspected placement in the genera *Methylocystis*, DNA-DNA hybridization was performed with *Methylocystis echinoides*. Based on the DNA-DNA hybridization results showing only 3.8% similarity, Strain CSC1 does not belong to the species *Methylocystis echinoides*, following the threshold value recommendation of Wayne et al. (1987). Phenotypic results in Table 2-1 show that Strain CSC1 differentiates from *Methylosinus trichosporium*, *Methylocystis echinoides*, and *Methylocystis parvus*, three closely matching cultured strains in the phylogenetic analysis, in various characteristics. All of the strains shown in Table 2-1 have been reported to be oxidase- and catalase-positive, possess colonies that are of opaque transparency, smooth edge, convex elevation, form poly-β-hydroxybutyrate, grow on

methanol, and capable of fixing atmospheric nitrogen. Unlike the known strains, Strain CSC1 was not capable of growth at 37°C; however, all of the methanotrophs grow optimally near 30°C. It is important to note that Gal'chenko et al. (1977) and Bowman et al. (1993a) report conflicting information concerning the ability of *M. echinoides* to accumulate poly-β-hydroxybutyrate and grow at 37°C, the former reporting positive results for each and the latter reporting negative results. Our TEM and growth studies with this strain agreed with Gal'chenko et al. (1977) (data not shown).

The elongated dumbbell shape of Strain CSC1, lack of motility, and ability to form polyphosphate separate it from the *Methylosinus trichosporium*. Other distinguishing characteristics between Strain CSC1 and *M. trichosporium* include smaller cell size, S-layer morphology, and lack of heat resistance. Also, unlike reported observations of *M. trichosporium*, Strain CSC1 can use L-asparagine, L-aspartate and L-glutamine and cannot use L-lysine as nitrogen sources. Both strains share the ability to use L-ornithine and putrescine.

Most similarities, however, are shared with the two *Methylocystis* strains, including lack of motility, heat resistance, and rosette formation. Strain CSC1's cell shape, ability to form polyphosphate, and colony color of yellow-white differ from *Methylocystis echinoides* and *Methylocystis parvus* (Table 2-1). Unlike *M. echinoides*, Strain CSC1 is capable of using L-ornithine and putrescine as nitrogen sources, whereas, unlike *M. parvus*, Strain CSC1 is not capable of using L-lysine. In addition, as reported for *M. trichosporium*, *M. echinoides*, and *M. parvus*, Strain CSC1 is not lysed by a 2% solution (w/v) of SDS.

While Strain CSC1 has previously been shown by TEM to contain characteristic Type II membranes (Henry and Grbić-Galić, 1990; Hršak and Begonja, 1998), it was revealed here to accumulate both polyphosphate bodies (Fig. 2-2) and poly-β-hydroxybutyrate storage granules, consistent with *Methylocystis parvus* (Bowman et al., 1993). No study has reported the structure of Strain CSC1's cell envelope in comparison to that of other well-characterized methanotrophs. Of special interest are the surface- (S-) layers, regular crystalline surface layers in Archaebacteria and Eubacteria, composed of protein or glycoprotein subunits (Sleytr et al., 1993; Sidhu and Olsen, 1997).

It is not known why S-layers develop in some strains of closely related bacteria and not in others. However, one hypothesis is that formation of these structures reflects adaptation to an ecological niche (Easterbrook and Alexander, 1983; Easterbrook, 1989) or a response to exposure to harsh environments (Minsky et al., 2002). Others suggest that S-layers may provide microorganisms with a selective advantage by serving as a protective coating or as molecular porins or sieves and traps for substrates, in maintaining the rigidity of the cell envelope, or providing a means of cell adhesion and surface recognition (Sára and Sleytr, 1987; Sleytr and Messner, 1988; Sára et al., 1992; Sidhu and Olsen, 1997). Easterbrook and Sperker (1982) hypothesized that spinae may simultaneously fulfill many fortuitous roles, analogous to "arms" with multipotential activities, including attachment, distance-keeping, and protection. However, why some species are prone to spine formation and others not, why S-layers exist in a variety of shapes and symmetries, and why these structures develop among species of methanotrophs is not clearly understood.

Methylocystis echinoides strain IMET 10491 was isolated from lake mud in Russia (Gal'chenko et al., 1977), possibly a more nutrient-rich environment than the sediments of the uncontaminated groundwater aquifer in California where Strain CSC1 was isolated. *Echinoides* is the Latinized adjective derived from the Greek word *echinos*, meaning "hedgehog," named for the hedgehog-like appearance of this bacterium. However, as reported by Gal'chenko et al. (1977), and verified in this study (Fig. 2-3c,d), the spines on this methanotroph appear to be tubular and less dense in comparison to the spikes observed on Strain CSC1, which would be more aptly named for a hedgehog. Despite the different originating environments of these two strains, proximity in the grouping of the *Methylocystis* genus, as strongly suggested by the results of this study, adds credence to the hypothesis that phylogeny and ecology may both play a role in S-layer formation. Similar clustering of S-layer-producing strains of *Bacillus cereus* has been observed, and, similar to these results, strains in this cluster do not possess S-layers, while others do (Mignot et al., 2001). These authors concluded that ecological pressure is associated with the acquisition and maintenance of S-layers in hosts that fall into a phylogenetic cluster.

Phylogenetically, Strain CSC1 is most closely related to *Methylocystis* sp. Its cell size, rosette formation, and presence of surface layers are most similar to *Methylocystis echinoides*. However, Strain CSC1 showed only 3.8% similarity with *Methylocystis echinoides* by DNA-DNA hybridization, and these two strains showed differences in surface-layer morphology, cell shape, colony color, formation of polyphosphate, and ability to use L-ornithine or putrescine as a nitrogen source. Characteristics of cell shape and the presence of surface layers, genes encoding for sMMO expression, and ability to

use L-lysine as a nitrogen source are divergent from those of *Methylocystis parvus*. The lack of polar flagella, smaller cell size, different cell shape, lack of heat resistance, presence of polyphosphate, ability to use L-asparagine, L-aspartate, or L-glutamine and inability to use L-lysine as a nitrogen source differentiate Strain CSC1 from *Methylosinus trichosporium*. Accepting these differences, Strain CSC1 could be described as a new species in the *Methylocystis* genus. We proposed this species be name *Methylocystis aldrichii* sp. nov.

Description of *Methylocystis aldrichii* sp. nov.

Methylocystis aldrichii sp. nov. (al.drich'i.i ML gen. N. *aldrichii* of Aldrich; named after H. C. Aldrich, an American microbiologist, deceased August 9, 2005).

Cells are aerobic, gram-negative, 0.3-0.6 x 0.7-1 μm in size that occur singly or in clusters. Reproduces by normal cell division. Budding division does not occur. Cells are not motile but possess a spiney surface layer composed of polysaccharide. Produces oxidase and catalase. Forms lipid cysts. Poly-β-hydroxybutyrate accumulates. Contains Type II intracytoplasmic membranes which are aligned parallel to the cell wall. Type II methanotroph. Methane and methanol are the sole sources of carbon and energy. Capable of using KNO_3, L-asparagine, L-aspartate, L-glutamine, L-ornithine, and putrescine as nitrogen sources. Capable of fixing atmospheric nitrogen. Expresses sMMO under low copper concentrations. Capable of cometabolically oxidizing a variety of aliphatic and aromatic compounds. Not resistant to pasteurization. Is not lysed by 2% (w/v) SDS. Is lysed by 10% (w/v) SDS. Colonies are white/yellow, slow-growing and 0.8-1.5 mm in diameter after 17-18 days at 30°C on NMS agar plates, incubated in the presence of 20% by volume methane in the headspace of a sealed desiccator. No growth

on complex organic media. Optimal pH for growth is 7.0; does not grow at pH 4.0 or 9.0. Is not capable of growth at 37°C. Optimal temperature for growth is approximately 30°C. The type strain, Strain CSC1 (ATCC BAA-1344), was isolated from an uncontaminated groundwater aquifer in the mid-1980s from Moffett Naval Air Station in Mountain View, CA, USA.

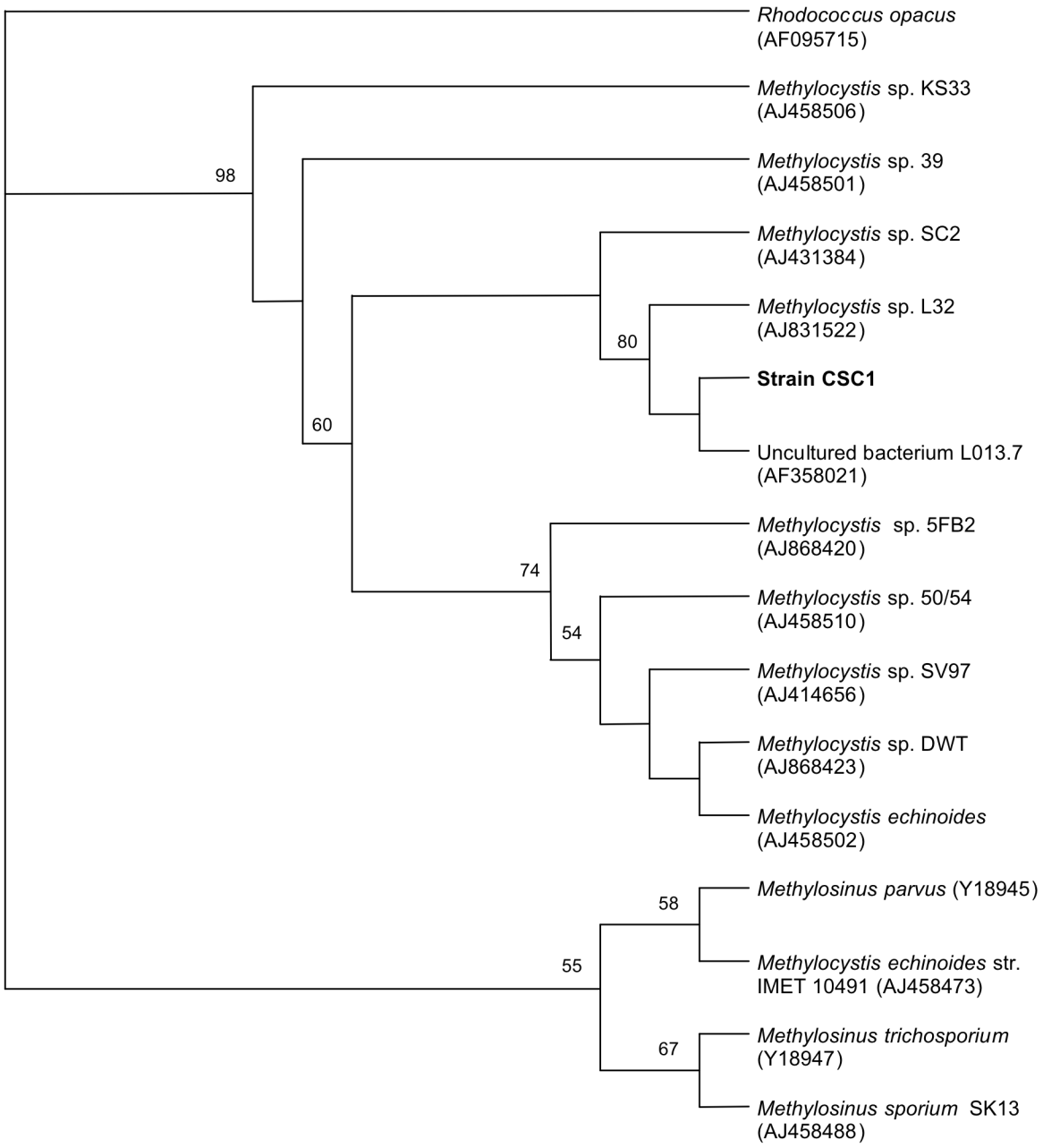

Figure 2-1. 16S rRNA phylogeny of Strain CSC1 and related *Methylosinus* and *Methylocystis* species. Numbers at branch points represent bootstrap values based on 100 replicates.

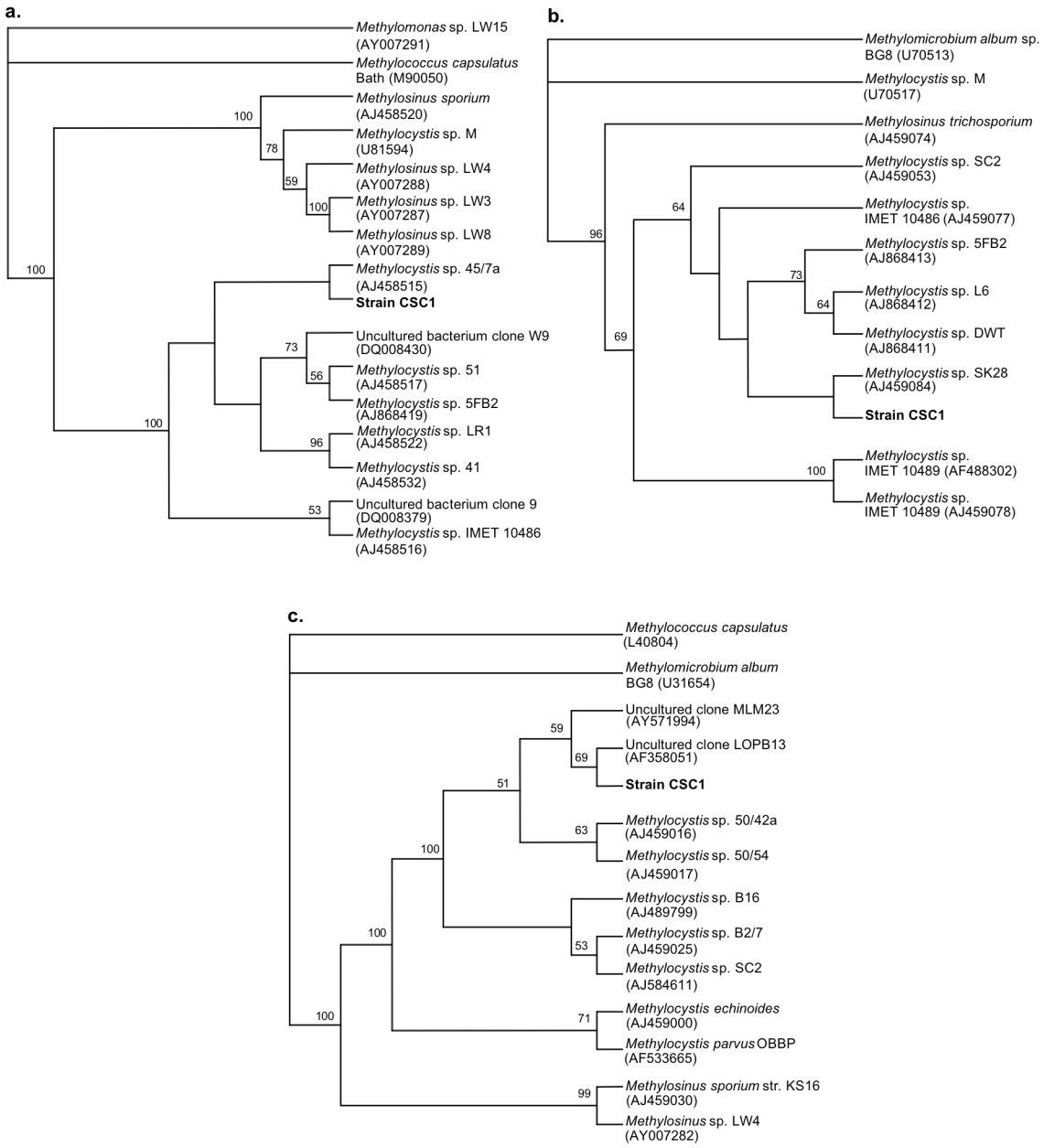

Figure 2-2. Functional genes phylogenies of Strain CSC1. (a) Phylogenetic tree of Strain CSC1 soluble methane monooxygenase (sMMO) gene sequence. (b) Phylogenetic tree of Strain CSC1 methanol dehydrogenase (MDH) gene sequence. (c) Phylogenetic tree of Strain CSC1 particulate methane monooxygenase (pMMO) gene sequence. Numbers at branch points represent bootstrap values based on 100 replicates.

Table 2-1. Phenotypic characteristics differentiating Strain CSC1 from *Methylosinus trichosporium*, *Methylocystis echinoides,* and *Methylocystis parvus*.[1]

Characteristic	Strain CSC1	*M. trichosporium*	*M. echinoides*	*M. parvus*
Colony morphology:				
Color	Yellow/ white	White/buff	White/pale pink	White/pale pink/tan
Cell morphology:				
Width (μm)	0.3-0.6	0.5-1.5	0.6-0.8	0.3-0.5
Length (μm)	0.7-1	2-3	0.8-1.2	0.5-1.5
Shape	Dumbbell	Rods, Pyriform	Pear-shape, Ovoid	Pear-shape, Ovoid
S-layers	Sharp, solid spines†	Bead-like/ filamentous	Tubular spines	-
Polyphosphate	+†	-	-	+
Poly-β-hydroxybutyrate	+	+	+¶	+
Motility	-	Polar flagella	-	-
Rosettes	-†	+	-	-
Heat resistance	-†	+	-	-
2%(w/v) SDS lysed	-†	-	-	-
Growth at 37°C	-†	+	+¶	+
N_2-source and use:				
L-asparagine	+†	-	+	+#
L-aspartate	+†	-	+	+#
L-glutamine	+†	-	+	+#
L-lysine	-†	+	-	+#
L-ornithine	+†	+	-	+#
Putrescine	+†	+	-	+#
NMS no N_2-source	+†	+	+	+
C-source and use:				
Methylamine	-†	-	-†	-
Dimethylsulfide	-†	-	-	-
Methanol	+†	+	+†	+
Glucose	-†	-	-†	-

[1]References: Whittenbury, 1970; Gal'chenko et al., 1977; Fassel et al., 1990, Fassel et al. 1992; Henry and Grbić-Galić, 1991; Bowman et al., 1993a; Hanson and Hanson, 1996; Hršak and Begonja, 1998. †This study. ¶ Gal'chenko et al. (1977) reported that *M. echinoides* forms lipid cysts and does grow to a limited extent at 37°C. Bowman et al. (1993a), however, reported that this strain does not accumulate poly-β-hydroxybutyrate, and only 0-10% of the strains tested grew at 37°C. #Reported by Bowman et al. (1993a) as 75-87% of the strains were positive.

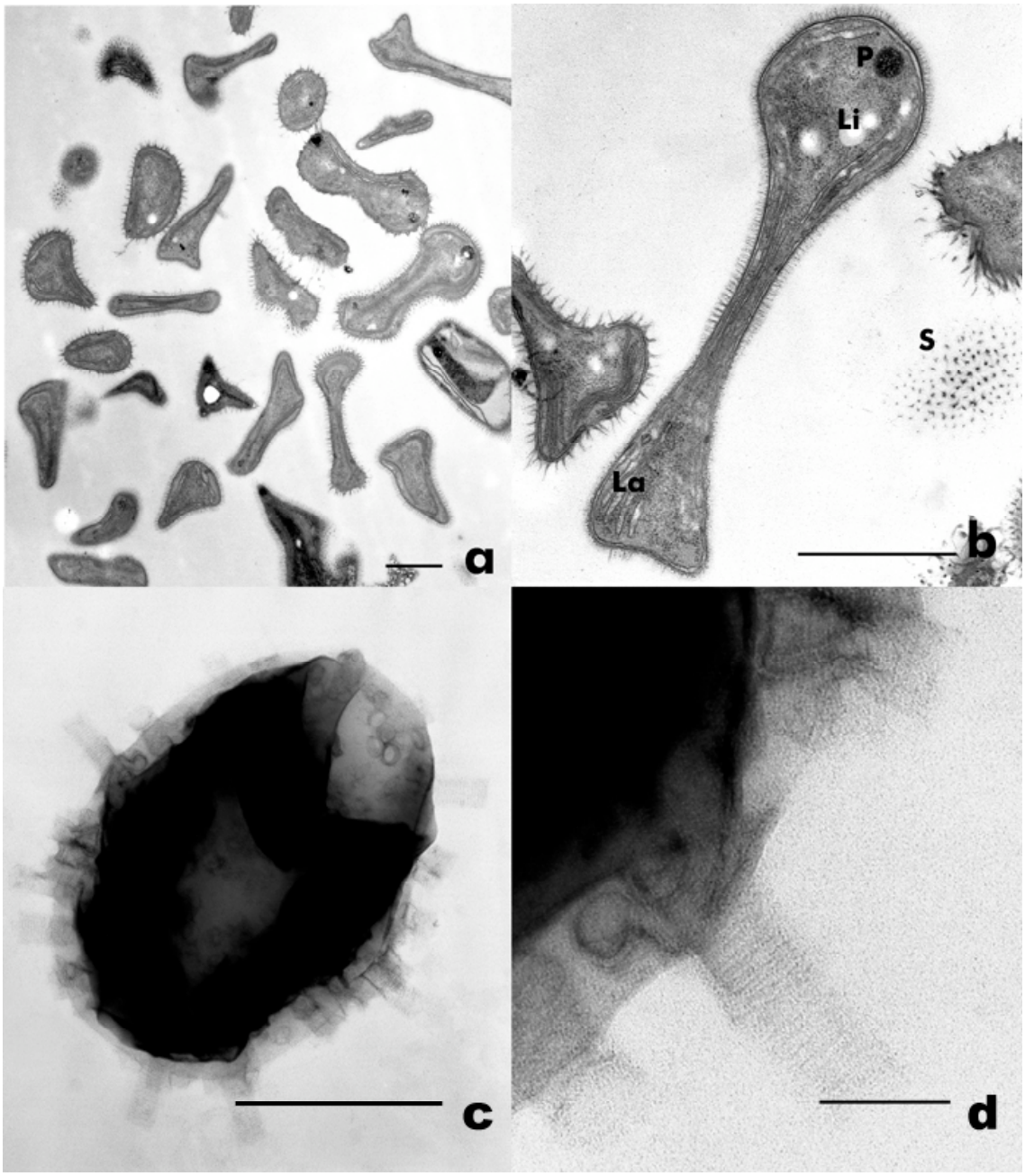

Figure 2-3. Transmission electron microscopy photographs of Strain CSC1 and *Methylocystis echinoides*. Panels (a) and (b) show the morphology of cells of Strain CSC1 grown with 10 μM Cu. In panel (b), numerous lamellae (La) are present. Lipid inclusions (Li) and polyphosphate (P) storage inclusions are also present. S indicates a surface view of the spiny surface of a cell. Cells of *Methylocystis echinoides* viewed with negative stain (panels (c) and (d)) are elliptical in profile and have numerous tubular projections from the surface. Some may be seen in circular end-on profile at the upper right of panel (c). The high magnification view in panel (d) shows that the tubes are striated. Markers in panels (a) and (b) indicate 1 μm; in panel (c), 0.5 μm; in panel (d), 0.1 μm.

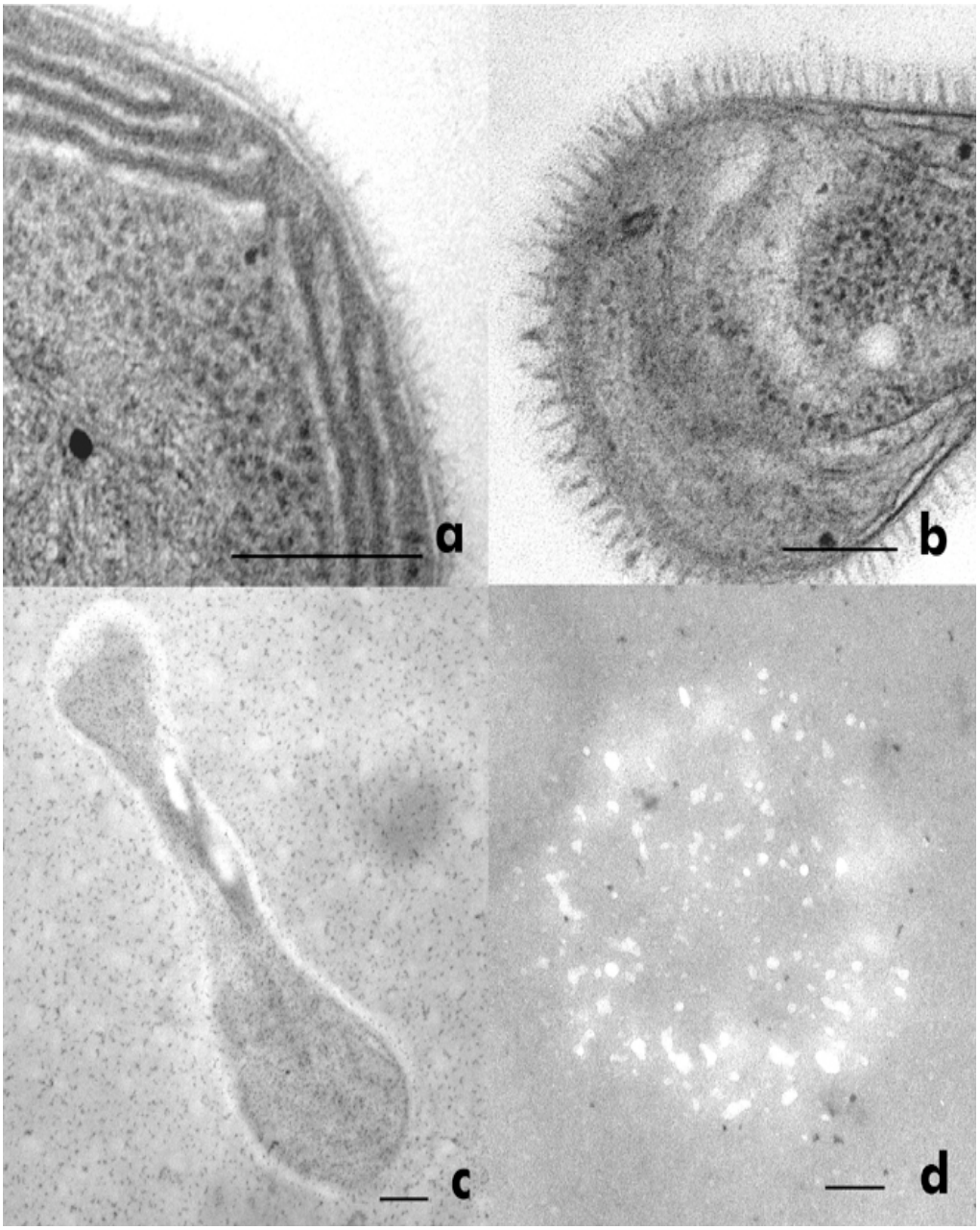

Figure 2-4. Electron microscope cytochemistry of the S-layer of Strain CSC1. Panel (a) shows the surface of a cell fixed initially with glutaraldehyde alone. Spiny layer is indistinct and lightly stained. Panel (b) shows the surface of a cell fixed initially with a glutaraldehyde/Alcian blue mixture to selectively stain polysaccharide. Compared to panel (a), the spiny layer stains darker and is thicker and is more distinct. Panels (c) and (d) show cells after pronase digestion. In panel (c), a cross section, a light layer around the cell has been left where the pronase removed the protein in the S-layer. In panel (d), a grazing section of the spines at the cell surface, numerous light spots in the plastic show where the pronase removed the spikes. Markers represent 0.2 μm.

CHAPTER 3
EFFECTS OF ALPHA-PINENE AND TRICHLOROETHYLENE ON OXIDATION POTENTIALS OF METHANOTROPHIC BACTERIA

Note: Published manuscript (Pacheco and Lindner, 2005)

Pacheco, A., and Lindner, A.S. (2005) Effects of *alpha*-pinene and trichloroethylene on oxidation potentials of methanotrophic bacteria. *Bulletin of Environmental Contamination and Toxicology* **74**:133-140.

Introduction

Trichloroethylene (TCE), a widely used solvent notable for its degreasing properties, is a common environmental contaminant that poses significant risk to public health ((ATSDR), 1999). TCE has been shown to be effectively removed from soil and water by phytoremediation, often favored over other methods because of its effectiveness, low cost, and aesthetic benefits. More rapid TCE removal has been observed in the root zone of plants (rhizosphere) used in phytoremediation (Walton and Anderson, 1990; Anderson and Walton, 1995; Brigmon et al., 1999), and methanotrophs, methane-oxidizing bacteria that thrive on oxygen and methane and are capable of co-oxidizing TCE (Wilson and Wilson, 1985; Little et al., 1988), have been implicated in this increased activity (Brigmon et al., 1999).

Loblolly pines (*Pinus taeda*), shown to support large rhizosphere populations of methanotrophs (Brigmon et al., 1999), have been considered for TCE remediation. These trees produce and release significant quantities of monoterpenes, the most predominant being (R)-α-pinene, composing over 65% of the total oleoresin composition in different plant tissues (Phillips et al., 1999). Since concentrations of (R)-α-pinene have been observed to be as high as 1.4 mg g^{-1} in fresh litter layers of pine forest soils (White,

1994), the probability that soil microorganisms encounter these compounds in nature is high. Previous studies have shown that (R)-*α*-pinene has a concentration-dependent inhibitory effect on methane oxidation by methanotrophs (Amaral and Knowles, 1997; Amaral et al., 1998; Amaral and Knowles, 1998), and, thus, may impact not only the growth of these bacteria in the rhizosphere but also their ability to co-oxidize TCE. While methanotrophs were shown to regain methane oxidation activity one to three days after exposure to (R)-*α*-pinene (Amaral et al., 1998), the implications of the long-term presence of this monoterpene on methanotrophic activity in the rhizosphere, in particular concentration effects of this chemical and its influence on TCE removal potentials, are not clear.

To this end, this study sought to first assess the ability of representative Type I, II, and X methanotrophs, grouped by their differences in carbon assimilation pathways, intracytoplasmic membrane structures, fatty acid carbon lengths, and phylogeny (Bowman et al., 1993a), to oxidize (R)-*α*-pinene over a range of concentrations using oxygen uptake analysis. Secondly, this study sought to gain a better preliminary understanding of the variation in oxygen uptake responses to mixtures of (R)-*α*-pinene and TCE by representative methanotrophs, thus ultimately providing insight into the effect of (R)-*α*-pinene on TCE oxidation potentials of these bacteria and guidance for the phytoremediation practitioner to more accurately predict the extent of TCE rhizodegradation when using monoterpene-releasing plants. We report herein observations of the potential of methanotrophs to oxidize (R)-α-pinene over a broad range of concentrations and (R)-α-pinene/TCE mixture effects on methanotrophic oxygen uptake activity.

Materials and Methods

Methanotroph strains used in this study included Type I *Methylomicrobium album* BG8 (ATCC 33003) and Type II *Methylosinus trichosporium* OB3b (ATCC 35070), obtained from Dr. Jeremy Semrau (University of Michigan, Ann Arbor, MI, USA), and Type X *Methylococcus capsulatus* (Bath) (ATCC 33009), purchased from the American Type Culture Collection (Manassas, VA, USA). Cultures were grown in nitrate mineral salts (NMS) medium (Whittenbury et al., 1970), with or without 10 μM $Cu(NO_3)_2$ to provide conditions for expression of pMMO or sMMO, respectively. With the exception of *M. capsulatus* (Bath), incubated at 45 ^oC with 50% methane (99.99% pure, Strate Welding, Jacksonville, FL, USA) in the headspace, all organisms were routinely subcultured in sealed erlenmeyer flasks containing 20% methane in the headspace and incubated at 30^oC in a rotary shaker at 250 rpm, as previously described (Lindner et al., 2000). Purity of the cultures was verified by routine streaking on 2% (w/v) nutrient agar plates (Difco, Sparks, MD, USA). Expression of sMMO was qualitatively verified by a naphthalene assay modified from Brusseau et al. (1990) and described by Lindner et al. (2000).

Oxygen uptake analysis was performed in this study, as it has been shown to be a rapid, effective means of assessing oxidative potential of whole cells (Lindner et al., 2000; Lindner et al., 2003). (R)-α-pinene was chosen to represent monoterpenes because it is a major component of loblolly pine oleoresin (Phillips et al., 1999). (R)-α-pinene and TCE were obtained in the highest purity available from Aldrich Chemical Co. (Milwaukee, WI, USA). Standard solutions of 10 μmol ml^{-1} were prepared in 1,4-dioxane (Fisher Scientific, Pittsburgh, PA, USA), used as the carrier solvent because it easily solubilized the substrates, was not oxidized by any of the cultures studied, and

caused no probe effects during oxygen uptake analysis (Lindner et al., 2000). Resting-cell suspensions were prepared from 500 ml cultures harvested at ¾-log phase by centrifugation in a J2-HS Beckman floor model centrifuge (Beckman-Coulter, Fullerton, CA, USA) at 2460 *x g*, 4°C, for 20 min. To ensure removal of all methane, the cells were washed with NMS medium, recentrifuged, and resuspended in the NMS medium to a wet cell concentration of 0.2 g ml^{-1}. The oxygen uptake system was composed of a 1.9 ml, well-stirred, enclosed reactor held at room temperature, as described by Lindner et al. (2000). After assessing the ability of methanotrophs to oxidize TCE and (R)-α-pinene alone, the study proceeded to investigate the effect of (R)-α-pinene on TCE oxidation by adding both substrates simultaneously into the oxygen uptake system before addition of the resting cells.

Despite storage of the resuspended cells on ice throughout the oxygen uptake experiments, loss of cell activity over time was observed. To ensure comparability of measurements throughout the 2-3-day testing period, all rates of oxygen uptake were normalized to the rates observed with 4 ml of methane gas, measured just prior to a change to a new substrate concentration. Details of this normalization procedure are presented in Lindner et al. (2000). The electrode was calibrated at least daily with a saturated sodium sulfite solution, and "live" runs were performed at least in triplicate for each concentration tested. All runs were corrected for endogenous metabolism. Controls without cells and with 4 ml of acetylene gas, a known inhibitor of MMO (Prior and Dalton, 1985), were routinely run to verify that depletion of oxygen, hence, oxidation activity, was a result of MMO activity. Initial rates of oxygen uptake were calculated by

linear or polynomial fits to the data points using Microsoft Excel software (Microsoft Corp., Redmond, WA, USA).

Results and Discussion

M. trichosporium OB3b and *M. capsulatus* (Bath), when cultured with no copper, expressed positive sMMO activity, as evidenced by a bright pink-to-purplish color in the assay. All of the strains tested negative for sMMO activity (no color change observed) when cultured with 10 μM $Cu(NO_3)_2$. sMMO and pMMO expression under culturing conditions without and with copper, respectively, was thus assumed, a reasonable conclusion given that enzyme expression in these methanotrophs under these conditions is well characterized. Active resting cells of all three representative methanotrophs consumed oxygen over a range of TCE and (R)-α-pinene concentrations, regardless of the type of MMO expressed (Fig. 3-1, A-E). No oxygen uptake was observed after addition of acetylene or without cells present, verifying MMO activity in all cases. As shown in Figure 3-1, regardless of the methanotroph or substrate tested, a maximum rate of oxygen uptake was observed, followed by a rapid decrease in rates, suggesting toxic effects of either the substrate itself or of oxidation products formed. This oxygen uptake behavior has been reported previously for methanotrophs with aromatic substrates (Lindner et al., 2000), and, while both substrates have been shown to have toxic effects on methanotrophic activity (Fox et al., 1990; Alvarez-Cohen and McCarty, 1991a; Henry and Grbić-Galić, 1991; Oldenhuis et al., 1991; White, 1994; Amaral and Knowles, 1997; Amaral et al., 1998; Amaral and Knowles, 1998), there have been no previous reports on the effects of a range of substrate concentrations on relative activities.

As shown in Figure 3-1, methanotrophs expressing sMMO (plots A, C) oxidized TCE at higher maximum rates than those expressing pMMO (plots B, D, E), as

previously reported (Little et al., 1988; DiSpirito et al., 1992; Lontoh and Semrau, 1998). The maximum normalized rates of oxidation by *M. trichosporium* OB3b and *M. capsulatus* (Bath) expressing sMMO or pMMO were 0.11 ± 0.01 and 0.03 ± 0.00 and 0.05 ± 0.01 and 0.03 ± 0.01, respectively (Fig. 3-1, A-D), while the maximum rate expressed by *M. album* BG8, capable of pMMO expression only, was 0.05 ± 0.01 (Fig. 3-1, E). The TCE concentrations where the observed normalized oxygen uptake rate was the highest ranged from 20 to 35 ppm for the tested strains. *M. trichosporium* OB3b and *M. capsulatus* (Bath) expressing sMMO exhibited oxygen uptake maxima at higher TCE concentrations (35 ppm) than when expressing pMMO (20-25 ppm), and *M. album* BG8 expressing pMMO showed a maximum observed rate at 35 ppm TCE. These results do suggest differing sensitivity levels to TCE, depending on the methanotroph and type of MMO expression.

As observed with TCE, both sMMO-expressing methanotrophs were also capable of oxidizing (R)-α-pinene at higher rates than their pMMO-expressing counterparts (Fig. 3-1, A-D). The maximum normalized rate of oxygen uptake by *M. trichosporium* OB3b expressing sMMO was almost 10 times the rate observed with pMMO-expressing cells (0.28 ± 0.04 and 0.02 ± 0.01, respectively); however, both rate maxima occurred at 20 ppm (R)-α-pinene (Fig. 3-1, A, B). The maximum normalized oxygen uptake rate with *M. capsulatus* (Bath), expressing sMMO, was 0.10 $\pm$ 0.02 at 20 ppm (R)-α-pinene, compared to 0.08 $\pm$ 0.01 at 50 ppm (R)-α-pinene under pMMO expression (Fig. 3-1, C, D). The observed maximum normalized rate of oxygen uptake by M. *album* BG8 was 0.04 ± 0.00, between the values observed for the other two strains under pMMO expression (Fig. 3-1E). Previous studies have reported higher TCE oxidation rates by

pure methanotrophs under sMMO expression (Wilson and Wilson, 1985; Little et al., 1988; DiSpirito et al., 1992; Lontoh and Semrau, 1998); however, this is the first report of such a trend with (R)-α-pinene. These results bring direct relevance to the environment, as sMMO expression in methanotrophs occurs only at very low copper concentrations (Lontoh and Semrau, 1998). Measurement of bioavailable copper is essential, therefore, for effective prediction of methanotrophic activity potential.

The response of each methanotroph in the presence of 20 ppm TCE over a range of (R)-α-pinene concentrations is shown in Figure 3-2, A-E. This plot presents the change in normalized oxygen uptake rate with 20 ppm TCE alone caused by the presence of different concentrations of (R)-α-pinene and thus represents the influence of (R)-α-pinene on TCE oxidation and provides insight into mixture effects on methanotroph activity. The concentration of 20 ppm TCE was chosen because it was not observed to be toxic to any of the methanotrophs tested previously (Fig. 3-1).

The responses to (R)-α-pinene were highly dependent on the type of methanotroph and MMO expression, with *M. trichosporium* OB3b showing decreased rates relative to 20 ppm TCE alone regardless of (R)-α-pinene concentration (Fig. 3-2, A, B) and *M. capsulatus* (Bath) and *M. album* BG8 showing mostly increased rates (Fig. 3-2, C, D, E). With the exception of *M. capsulatus* (Bath) under pMMO expression, the highest observed rates in the presence of the mixture were lower than those observed with (R)-α-pinene alone. *M. trichosporium* OB3b expressing pMMO showed consistently small decreases in oxygen uptake activity in the presence of the mixture compared to 20 ppm TCE alone; however, the activity of this strain when expressing sMMO appeared to be inhibited to a greater extent in the presence of all tested concentrations (2 to 20 ppm) of

(R)-α-pinene in the mixtures (Fig. 3-2, A, B). Regardless of MMO expression, *M. capsulatus* (Bath) yielded increased normalized oxygen uptake rates in the presence of the mixture above approximately 20 ppm (R)-α-pinene relative to its observed rate at 20 ppm TCE alone. The greatest rate increase shown by *M. capsulatus* (Bath) expressing sMMO in the presence of the mixture was observed at 40 ppm (R)-α-pinene. This maximum rate observed with the mixture was 1.8 times the rate with 20 ppm TCE alone, suggesting a lessoning of toxicity effects on the cells. The maximum increase with this strain under pMMO expression was observed at 30 ppm (R)-α-pinene and was approximately 3.5 times higher than with 20 ppm TCE alone and 1.5 times higher than observed at 50 ppm (R)-*α*-pinene alone. Increase in oxidation potential of *M. album* BG8 was also observed when (R)-α-pinene was in the presence of 20 ppm TCE (Fig. 3-2, E). At the highest concentration of (R)-α-pinene tested (30 ppm) with this strain, the increase in normalized oxygen uptake rate was 1.8 times the rate observed with TCE alone.

In conclusion, all of the tested methanotrophs expressing either sMMO or pMMO were capable of oxidizing (R)-α-pinene over a range of environmentally relevant concentrations. However, toxicity effects of this monoterpene, similar to those shown with TCE, were observed. When both (R)-α-pinene and TCE were introduced to the representative methanotrophs, varying responses in the rates—decreases with the Type II methanotroph and increases with the Types I and X methanotrophs—were observed in comparison to those observed in the TCE-only experiments. Whether TCE and/or (R)-α-pinene were oxidized in the mixture is not known, given the indirect measurement method of oxygen uptake analysis; however, it is suggested here that the total oxidation potential of methanotrophs is affected, either antagonistically or synergistically, in the

presence of TCE and (R)-α-pinene mixtures. These results emphasize the importance of not only assessing the concentration levels of both contaminants and monoterpenes and but also of measuring the oxidation potentials and diversity of rhizosphere methanotrophs at phytoremediation sites where plants that release large amounts of monoterpenes are being contemplated for use.

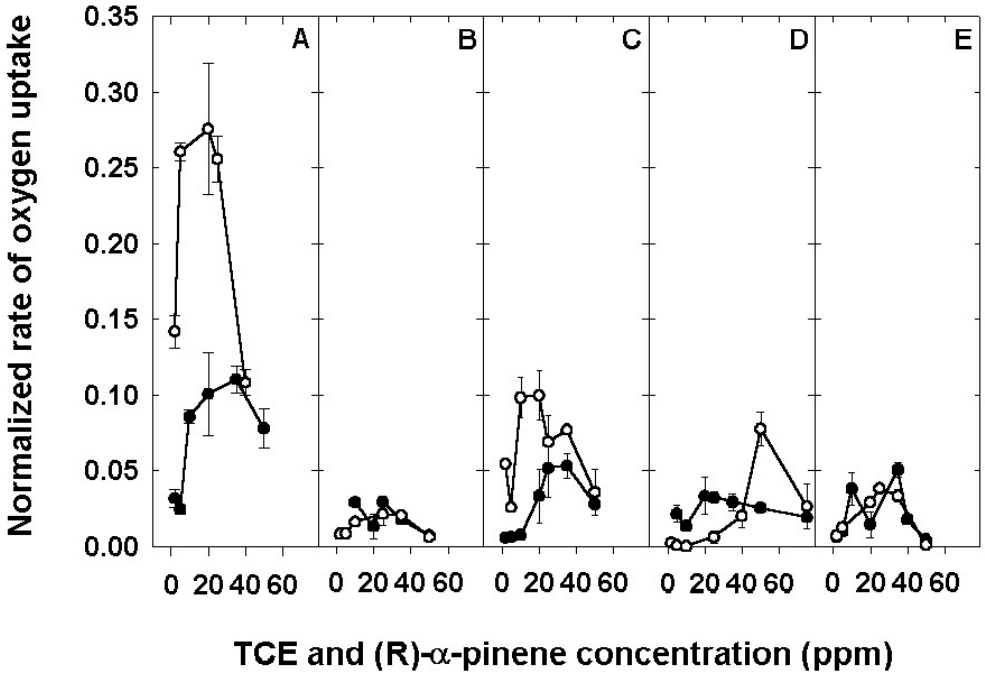

Figure 3-1. Normalized rate of oxygen uptake by the representative methanotrophs in the presence of varying concentrations of TCE (●) and (R)-α-pinene (○). (A), (B): *M. trichosporium* OB3b cultured without and with copper, respectively. (C), (D): *M. capsulatus* (Bath) cultured without and with Cu, respectively. (E): *M. album* BG8 cultured with Cu. Error bars represent the standard deviation for triplicate samples.

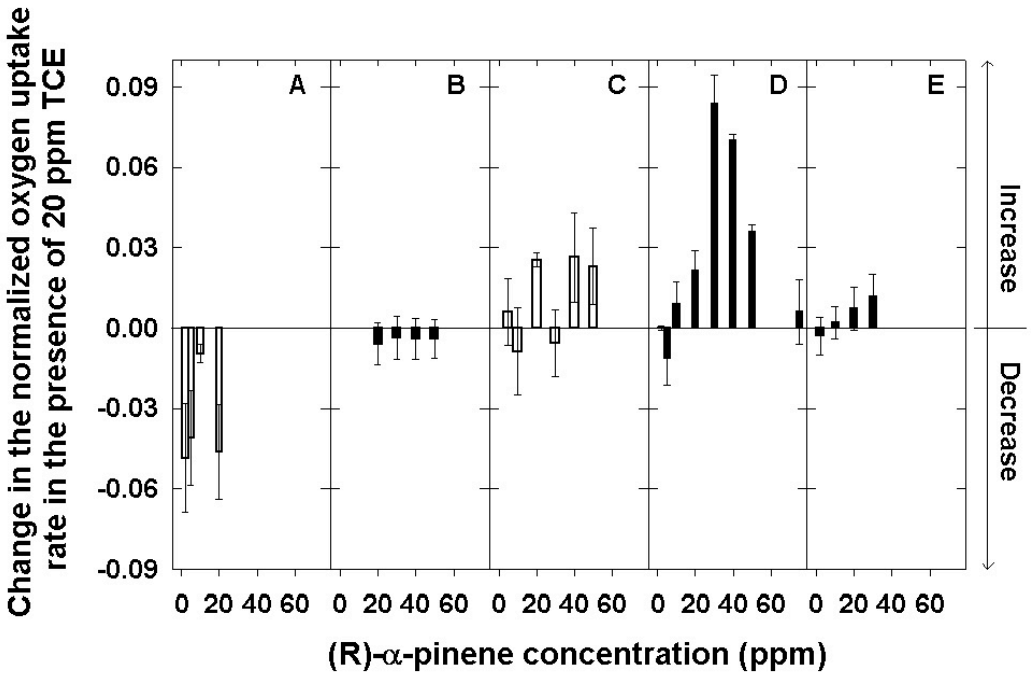

Figure 3-2. Change in the normalized oxygen uptake rate by representative methanotrophs observed in the presence of 20 ppm TCE at varying concentrations of (R)-α-pinene. (A), (B): *M. trichosporium* OB3b cultured without and with Cu, respectively. (C), (D): *M. capsulatus* (Bath) cultured without and with Cu, respectively. (E): *M. album* BG8 cultured with Cu. Error bars represented the standard deviation for triplicate samples.

CHAPTER 4
STABLE ISOTOPE PROBING FOR CHARACTERIZATION OF METHANOTROPHIC BACTERIA IN THE RHIZOSPHERE OF PHYTOREMEDIATING PLANTS

Note: Manuscript to be submitted to Biology Letters

Introduction

Phytoremediation, the use of plants to remove a variety of contaminants from soil and aqueous environments, has been shown to be more economical and aesthetically pleasing than traditional remediation methods, such as pump-and-treat approaches (McCutcheon and Schnoor, 2003). Despite its observed effectiveness in removal of contaminants, including trichloroethylene (TCE) and tetrachloroethylene (PCE), two widely distributed chlorinated solvents that cause concern because of their potential health effects (ATSDR, 2006), phytoremediation is still limited by a lack of understanding of the primary removal processes involved. In particular, the potential roles that root-zone (rhizosphere) bacteria can assume in the overall removal of contaminants is not fully appreciated (Walton and Anderson, 1990; Anderson and Walton, 1995; Brigmon et al., 1998; Brigmon et al., 1999).

One reason for the lack of specific information on the degradation potentials of root-zone bacteria is that traditional culture-dependent methods are not capable of directly assessing the activity and diversity of microorganisms *in situ*. Furthermore, these methods provide limited information because of their associated inherent cultivation bias (Fry, 2004; Smalla, 2004). The development of culture-independent molecular methods such as stable isotope probing (SIP), has enabled scientists to study *in situ* conditions

more effectively and, more importantly, to characterize the active microbial populations. The promising SIP technique relies on the incorporation of a labeled substrate with a less naturally frequent isotope, into the active microbial community of a sample that later can be separated from the unlabeled biomass (Radajewski et al., 2000).

Recent studies of methanotrophic bacteria have shown successful results using the SIP approach in environments, such as peat soils, acidic forest soils, cave water, and soda lake sediments (Morris et al., 2002; Radajewski et al., 2002; Hutchens et al., 2004; Lin et al., 2004). Methanotrophs are among the aerobic bacteria that are known to reside in the rhizosphere of plants and that are capable of oxidizing chlorinated contaminants such as TCE (Wilson and Wilson, 1985; Hanson and Hanson, 1996; Brigmon et al., 1999; Doronina et al., 2004; Pilon-Smits, 2005). Because methane serves as the sole source of carbon and energy for methanotrophs, it is often used as the measure of methanotroph activity in the environment, and is a natural substrate for SIP testing. Labeled methane (^{13}C-CH_4) has been successfully incorporated into the DNA of growing cells of methanotrophs (^{13}C-DNA) and separated from the naturally occurring ^{12}C-DNA by density gradient centrifugation (Radajewski et al., 2000; Morris et al., 2002; Radajewski et al., 2002; McDonald et al., 2005). Molecular fingerprinting techniques, such as denaturing gradient gel electrophoresis (DGGE) with DNA fragments of specific methanotroph enzymes, such as particulate methane monoxygenase (pMMO), can be subsequently used to identify and assess the relative abundance of the active populations (Muyzer et al., 1993).

With the advent of this sophisticated molecular biology method that links identity with function, development of a protocol using SIP methods that is specific to the

rhizosphere holds promise in better understanding the rhizodegradation process of phytoremediation systems. This study provides a first-basis in method development and analysis of the SIP technique for the measurement of potential *in situ* activity and diversity of methanotrophic bacteria in the root-zone of trees used for remediation of TCE from contaminated groundwater and soil.

Materials and Methods

Site Description

This study was located at a Superfund site, the former LaSalle Electrical Utilities in LaSalle, IL (USA). The company manufactured capacitors from 1943 to 1982, resulting in soil and groundwater contamination of mostly polychlorinated biphenyls and the chlorinated solvents, TCE and PCE. Currently, in the final stages of the cleanup process at the site, two phytoremediation plots have been implemented to enhance chlorinated solvent removal (Lange, 2004). The first plot (0.25 ha), contaminated with TCE (0-254 ppb), was installed in September 2002 (labeled as "TCE Site"). Poplar (18 clones) and willow (24 clones) genotypes were planted by lowering 1.8 m rooted cuttings to the bottom of boreholes (0.6 m diameter) lined with high-density polyethylene pipe and filled with an equal mix of sand, soil, bark, and peat (pH 7.8). The second phytoremediation plot (0.21 ha), contaminated with PCE (0-838 ppb), was established in March 2002 (labeled as "PCE Site"). At this plot, poplar trees were planted directly into the improved soil (pH 7.3) with mulch composed of tree chips on the top 0.5 m of the soil surface.

Sampling

All rhizosphere soil samples were collected using a small diameter (1.9 cm) hand soil auger to minimize disturbance in the pots. Samples were taken at three time periods, July 2003, July 2004, and November 2004, in order to compare summer and fall

conditions and time effects on the activity and population diversity of methanotrophs. Each sampling period, mid-summer (July) and early fall (November), fell in the "wet season" of April to December when 642 mm of precipitation were collected at the site during 2004. The average daily air and soil temperatures were 21°C and 11°C, respectively, in the 2004 summer sampling, and 20°C and 9°C, respectively, in the 2004 fall sampling. Groundwater levels below the planted plots fluctuated in 2004 from 2.1 to 3.1 m and 1.8 to 3.3 m from the soil surface at the TCE and PCE Site, respectively.

Root growth of the trees at the TCE and PCE Site was observed to extend from the surface to 90-120 cm below surface. A composite sample, in regions of high contaminant concentration, was taken from two opposite locations around the tree base at a depth of 30-60 cm. This soil layer was chosen as a potential zone of intermediacy rhizosphere activity between surface and deeper soil layers. At the TCE Site, samples were also removed from non-planted pots in the contaminated area to serve as a control when compare to the planted pots. Also different tree clones, showing the greatest vigor, were sampled at the TCE Site. They were one poplar clone I45/51 (*Populus deltoides* x *P. nigra*; origin, North America x Europe) and three willow clones, SX61 (*Salix sachalinensis*; origin, Japan; exotic), S365 (*S. discolor* 18; origin, University of Toronto), and 94014 (*S. purpurea*; origin, State University of New York; exotic).

While it is well known that methanotrophs are not capable of oxidizing PCE, samples were removed from the PCE Site to serve as a mean of comparing methanotroph activity and diversity with the TCE Site samples. One poplar clone, I45/51, was sampled at the PCE Site, along with a non-planted sample removed from outside the plot in an

uncontaminated region that was eventually converted to an irrigated, fertilized soccer field after the first sampling in July 2003.

To prevent cross-contamination, the sampling auger was washed with sterile water, rinsed with 95% ethanol, and washed again before each sample was taken. Samples were immediately placed in sterile bags (Nasco Whirl-Pak, Fort Atkinson, WI, USA), placed on ice, and transported to the UF laboratory where they were subsequently stored at 4ºC until testing. In the laboratory, samples were gently homogenized using a sterile spatula, and fine roots of less than 2 mm diameter were separated from the soil for separate testing.

Stable Isotope Probing (SIP) Soil Microcosms

Experimental conditions. In order to assess activity and diversity of the active methanotroph populations in poplar and willow tree rhizospheres, during TCE remediation, soil microcosms were prepared from samples collected over time. Microcosms consisted of 10 g wet soil (plant material removed) normalized to 16% water content with sterile water. This water content represented approximately 40% of the field capacity of the TCE and PCE Site soils, where the greatest extent of CH_4 oxidation was observed in preliminary experiments, as previously described by Reay et al. (2001). The "wetted" soil was placed in sterile 160 ml serum vials, which were subsequently sealed with gray butyl rubber stoppers and crimp tops. Ten ml (0.4 mmol; ~7%, v/v) of filter-sterilized $^{13}CH_4$ (99.9%; Isotec, Miamisburg, OH, USA) or $^{12}CH_4$ (99.9%; Airco-BOC, Murray Hill, NJ, USA), used in preliminary experiments to optimize conditions and assess any effects of the labeled substrate by comparing to the $^{13}CH_4$ microcosms rates, was then added as previously described (Morris et al., 2002; Radajewski et al., 2002), and each vial was wrapped with aluminum foil for subsequent incubation in the dark at room

temperature (~25°C). Headspace CH_4 depletion was monitored every 2 to 5 days by removal of 25 ul of CH_4 with a gastight syringe and analysis using a gas chromatograph (Model HP5809A GC/TCD; Hewlett Packard, PaloAlto, CA, USA) equipped with a GS-Carbon plot column (Agilent Technology, PaloAlto, CA, USA). The gas chromatograph was maintained at a head pressure of 5 psi and programmed in a 4 min run with temperatures of 25, 120 and 200°C in the oven, injector and detector, respectively.

When more than 90% of the CH_4 was consumed, vials were opened, gently flushed with filter-sterilized air for 5 s to remove any accumulated $^{13}CO_2$ and to maintain aerobic conditions, resealed, and the same initial amount of $^{13}CH_4$ added. The procedure was repeated five times until a total of 2.0 mmol of CH_4 was consumed (Radajewski et al., 2002). A positive control with pure methanotroph, *Methylocystis trichosporium* OB3b, and three negative controls with no CH_4 added, with twice-autoclaved (killed) soil, and with 20% (v/v) each of CH_4 and acetylene (a known inhibitor of methane monooxygenase, Prior and Dalton (1985)) in the headspace, were also included. Additionally, some of the microcosms were set in replicates to assure reproducible results. Initial CH_4 depletion rates were calculated from data taken during incubation after the first CH_4 addition by linear regression analysis of the consumption curve.

DNA extraction and ultracentrifugation. The content of the microcosms (10 g soil) was processed using a PowerMax Soil DNA Extraction Kit (Mo-Bio, Carlsbad, CA, USA). DNA extracts were resolved by CsCl density gradient centrifugation. Briefly, 1 g ml^{-1} of CsCl (Fisher Scientific, Pittsburgh, PA, USA) was dissolved in the DNA solution, and 100 μl of ethidium bromide (10 mg ml^{-1}; Bio-Rad, Hercules, CA, USA) was added before loading the solution into 5.1 ml quick-seal polyallomer ultracentrifuge tubes

(Beckman Coulter, Fullerton, CA, USA). Ultracentrifugation was performed using a VTi65 vertical rotor in a Model L8-80 ultracentrifuge (Beckman Instruments, Fullerton, CA, USA) at 265,000 x *g* for 16 h at 20°C. After ultracentrifugation, fractions were visualized with UV light at 365 nm (Sambrook et al., 1989; Radajewski et al., 2002). Three DNA bands were generally observed and collected: (1) a light-DNA upper band (^{12}C-DNA); (2) a middle band, smear of ^{12}C- and ^{13}C-DNA; and (3) a heavy-DNA lower band (^{13}C-DNA). DNA fractions were collected and purified as described by Sambrook et al. (1989). Ethidium bromide was extracted from the DNA with 1-butanol (Fisher Scientific, Pittsburgh, PA, USA) saturated with water. Following five extractions the DNA solution was diluted in water and precipitated with ethanol overnight at -20°C and dissolved in 100 ul TE buffer (Sambrook et al., 1989). A second ultracentrifugation step was not necessary after confirming that the protocol of DNA band extraction was exact. Re-runs verified the presence of only one distinct band in the new columns.

Polymerase chain reaction (PCR) amplification. The purified DNA fractions (^{12}C- and ^{13}C-DNA) were used as a template for PCR analysis. The phylogenetic analysis was performed with the functional *pmoA* gene, targeted using the primer set A189f (5'-GGNGACTGGGACTTCTGG-3') and mb661 (5'-CCGGMGCAACGTCYTTACC-3') (Integrated DNA Technologies, Coralville, IA, USA), specific to the pMMO active site (Costello and Lidstrom, 1999). A GC-clamp (5'-ccccccccccccgccccccgccccccgccccgccgccc-3') was attached to the A189f primer as described by Henckel et al. (1999).

PCR amplification was performed according to the procedure described by Knief et al. (2003). PCR reactions consisted of the MasterAmp 2X PCR premixture F (Epicentre

Technologies, Madison, WI, USA) containing 100 mM Tris-HCl (pH 8.3), 100 mM KCl, 400 μM each of dNTP, 3-7 mM $MgCl_2$, and the enhancer betaine (0-8 X), combined with 0.5 μM each primer, 1U Taq polymerase, and sterile water to a total reaction volume of 50 μl (Knief et al., 2003). All reactions were assembled on ice, and the cooled tubes were placed in a preheated (94°C) thermal block for PCR initiation (Henckel et al., 1999).

The PCR protocol consisted of a touchdown program using a thermocycler (Mastercycler Personal 5332; Eppendorf, Westbury, NY, USA) with the following parameters: initial denaturation of 5 min at 94°C, followed by 35 cycles of 1 min at 94°C for denaturation, 1.5 min at 62 to 55°C in -0.5°C increments for annealing, 1 min at 72°C for elongation, with a final extension step of 7 min at 72°C (Knief et al., 2003). PCR product size (540 bp) was examined by horizontal agarose electrophoresis. PCR positive controls included representatives of all methanotrophs types (type X *Methylococcus capsulatus* (Bath) (ATCC 33009), type II strains, *Methylosinus trichosporium* OB3b (ATCC 35070), Strain CSC1, *Methylocystis echinoides* (IMET 10491), *Methylocystis parvus* OBBP (NCIMB 11129), and type I *Methylomicrobium album* BG8 (ATCC 33003)).

Denaturing Gradient Gel Electrophoresis Analysis (DGGE), Sequencing, and Phylogenetic Analysis

DGGE. PCR products were separated by DGGE in the DCode System (Bio-Rad, Hercules, CA, USA) as described by Henckel et al. (1999). Briefly, 1 mm thick 6.5% (w/v) polyacrilamide gels (37.5:1 acrylamide-bisacrylamide) (Fisher Scientific, Pittsburgh, PA, USA) were prepared and electrophoresed in 1X TAE buffer at 61°C and 180 V for 5 h in a 35-65% linear denaturant gradient (65% is 4.5 M urea and 26% (v/v)

deionized formamide). Gels were loaded with 25-45 μl of PCR product, according to band intensity in agarose gels, and ¼ volume of loading buffer. Gels were stained with ethidium bromide according to the manufacture's instructions (Bio-Rad, Hercules, CA, USA), visualized at 312 nm on a UV transilluminator (Model 88A, Fisher Scientific, Pittsburgh, PA, USA), and photo-documented with the system DigiDoc-IT ™ (Daigger, Vernon Hill, IL, USA) using the Doc-It software v. 2.4 (UVP, Upland, CA, USA). DGGE bands were excised from the middle part of the band with a sterile scalpel and the DNA eluted according to the protocol described by Chory and Pollard (1999). The eluted DNA was reamplified and reanalyzed on DGGE to verify sample purity. Band reamplification was performed by modifying the PCR protocol to 30 cycles of 30 s at 94°C for denaturation, 45 s at 66°C for annealing (to avoid sequence ambiguity as reported by Dunfield et al. (2002)) and 30 s at 72°C for elongation, with the same initial and final steps. Several bands with the same mobility were excised from different lanes to check for sequence identity.

Sequencing. Reamplified PCR products of excised DGGE bands were purified with a PCR purification kit (Mo-Bio, Carlsbad, CA, USA) before sequencing. PCR product concentration and purity was determined by UV absorption spectrophotometry (1:20 dilution). PCR products were sequenced by the Interdisciplinary Center for Biotechnology Research (ICBR), University of Florida (Gainesville, Florida, USA).

Phylogenetic analysis. Sequences were compared in the National Center for Biotechnology Information (NCBI) database using BLAST (Altschul et al., 1990). Related sequences identified in BLAST, as well as sequences of extant methanotrophs, were aligned and adjusted manually with CLUSTALX v. 1.8 (Thompson et al., 1997).

Phylogenetic trees were generated by the neighbor joining (NJ) method with CLUSTALX and displayed in TreeView v. 1.6.6 (Page, 1996). Nucleotide accession numbers of all obtained sequences were placed in the GenBank for future access (AYXXX-AYXXX).

Statistics

CH_4 depletion rates observed in the SIP microcosms were analyzed by comparing the initial slopes of the linear regression curves fitted to the consumption curve during incubation after the first CH_4 addition. When a set of samples showed no significant differences in rates, an average depletion rate (common regression coefficient) was calculated as an estimate of the CH_4 depletion rate underlying all rates of a particular set of samples (for example, rates among the same plant type in each sampling period). Additionally, differences among sample means and between samples and the control were analyzed by Tukey's and Dunnett's test, respectively (Zar, 1984). SAS software v. 7 (SAS Institute, Cary, NC, USA) was used for all the analysis.

Results

SIP Protocol Implementation

The SIP technique was successfully applied to the rhizosphere soils. The rates observed in the $^{13}CH_4$ and $^{12}CH_4$ microcosms were comparable, and variability among the replicates was low. The *M. trichosporium* OB3b control was effectively labeled by the $^{13}CH_4$ and no $^{13}CH_4$ consumption was observed in the negative controls.

DNA extracts from the microcosms were effectively separated by CsCl density gradients (Fig. 4-1A). When a smear was present between the unlabeled (^{12}C-DNA) and labeled (^{13}C-DNA) fractions, it was collected as a $^{12\text{-}13}$C-DNA combined fraction and was not included in the study. Correct recovery of these fractions was verified by a second

ultracentrifugation under the same protocol, even though band position varied because of changes in the density of the new solution (Fig. 4-1, B-C). The extracted ^{13}C-DNA fractions produced *pmoA* gene fragments of the expected size (540 bp). The methanotroph positive control cultures revealed multiple DGGE bands (Fig. A1, Appendix), in keeping with earlier reports of the high probability of encountering multiple copies of the *pmoA* gene in methanotrophs (Semrau et al., 1995; Dunfield et al., 2002). Because DGGE band purification was difficult in most samples yielding ambiguous positions after sequencing, the annealing temperature was increased from 62 to 66°C and only the reverse primer was used for sequencing (Dunfield et al., 2002). Furthermore, 16S rDNA-DGGE profiles revealed complex band patterns and smears that were difficult to examine (data not shown).

In general, *pmoA*-DGGE profiles of the ^{13}C-DNA fractions of the different rhizosphere soil microcosms did not differ greatly among sites, plant type or sampling period. Consequently, it was useful to set a reference profile for the analysis (Fig. 4-3A, lane 1). Reference bands 1, 3, 9, 10 and 11 were not possible to sequence.

Methanotroph Activity and Composition in the TCE Site

$^{13}CH_4$ added in five additions to a total of approximately 2.0 mmol, was consumed within 31-37 days in all TCE Site soil microcosms. Initial CH_4 depletion rates (Fig. 4-2A), calculated as a measure of the oxidative potential at the time of sampling, showed no significant differences within plant type over the 16-month sampling period. A comparison of average rates per plant type, which represents plant type's overall activity throughout the study, shows also no significant difference in CH_4 depletion rates among plant types and with the non-planted sample ($P<0.05$). The overall average CH_4 depletion rate at the TCE Site was 0.11 μmol h^{-1} g^{-1} dry weight soil (± 0.01, SE).

As shown in Fig. 4-3, TCE Site (panel A to C) and PCE Site (panel D) *pmoA*-DGGE profiles were mostly described by the same group of bands numbered from 1 to 11 in the reference profile (Fig. 4-3A, lane 1). Concentric circles with letters denoted bands different from the reference profile. Profiles were labeled starting with the plot location (TCE or PCE) followed by the tree type (poplar or willow), soil compartment analyzed (rhizosphere, rhizoplane, or non-planted soil), and sampling period. Willow clones SX61 and 94019 were not sampled in the November 2004 sampling period, consequently, they only showed two profiles each (Fig. 4-3C, lanes 1-4). The *pmoA*-amplified sequences from the ^{13}C-DNA fractions of the TCE Site microcosms revealed DGGE profiles composed by 2 to 11 bands (Fig. 4-3, B-C). These profiles were mainly described by three groups of highly similar sequences according to band position and BLAST alignments, indicating that some bands may represent copies of multiple *pmoA* genes. However, some profiles that exhibited these groupings did not reveal all of the bands.

The TCE Site DGGE profiles throughout the study did not vary to a great extent among plant type (Fig. 4-3, B-C), but they were distinct from the non-planted soil (Fig. 4-3B, lane 4 to 6). Rhizosphere DGGE profiles from poplar (Fig. 4-3B, lane 1-2) and all willow clones (Fig. 4-3, panel B, lanes 7-8, and, panel C, lanes 1 to 4) collected in July 2003 and 2004 revealed the same community of organisms. These profiles were composed of two groupings of bands represented by reference bands 4 and 6 to 8, and bands 2 and 5 (Fig. 4-3A, lane 1). Within each grouping, bands shared 100% sequence similarity and aligned in BLAST (>99% similarity) with two clones of uncultured *Methylocaldum* sp. isolated from a landfill cover soil. The phylogenetic tree clustered

these sequences as the described groupings (Fig. 4-4, Group 1 and 2) and within the *Methylocaldum* branch (type X methanotroph). These groupings are closely related to the cultured organism, *M. gracile*. Also, a third group of bands, designated by reference bands 9 to 11 (Fig. 4-3A, lane 1), was detected in all profiles. However, because reamplification of this group was not possible, and since they were the only bands present in the July 2003 TCE Site non-planted profile (Fig. 4-3B, lane 4), it is possible they represent another set of similar *pmoA* genes.

From the planted microcosms at the TCE Site, only the polar and willow clone 94014 exhibited changes in their methanotroph community throughout the study. The poplar tree, in the November 2004 sampling (Fig. 4-3B, lane 3), showed less than half of the bands in the July samplings (Fig. 4-3B, lane 1-2). It only revealed three bands, two at very low intensity and the same major band of previous samplings (reference band 5). Willow clone 94014, in the July 2004 sampling (Fig. 4-3C, lane 4), did not exhibit this major band and showed an extra band of an uncultured methanotroph that closely related (88% similarity) to another type X methanotroph, *Methylococcus capsulatus* (Fig. 4-4).

The TCE Site non-planted microcosms, over the three sampling times, exhibited variable profiles at very low intensity (Fig. 4-3B, lane 4 to 6). In the July 2003 sampling, only the 9 to 11 grouping was retrieved. However, in the July and November 2004 samplings, the 9 to 11 grouping was observed along with some of the bands that described the *Methylocaldum* clones found in the planted samples and uncultured bacteria from rice fields and upland soils (>87% similarity). The uncultured bacteria (Fig. 4-3B, lane 5-6) were placed within the *Methylocaldum* and *Methylococcus* branch (Fig. 4-4),

closely related to the type species *Methylocaldum szegediense* and *Methylococcus capsulatus*, respectively.

Additionally, one of the ^{12}C-DNA fractions from the willow clone S365 microcosms was amplified to compare to the ^{13}C-DNA fraction (Fig. 4-3C, lane 6-7). Only one band was different between these profiles, the first band in the ^{12}C-profile (Fig. 4-3C, lane 7), which aligned in BLAST to an uncultured bacterium from an upland soil (97% similarity). This band was placed in the phylogenetic tree within the type X methanotrophs closely related to *Methylocaldum szegediense* (Fig. 4-4).

Methanotroph Activity and Composition in the PCE Site

At the PCE Site, soil microcosms activity differed greatly between planted and non-planted samples (Fig. 4-2B). However, it was the activity of the non-planted soil that significantly changed among sampling periods (P<0.0005). Non-planted microcosms rates ranged from 0.01 to 0.29 μmol h^{-1} g^{-1} dry weight soil (± 0.00 SE) , from the first to the last sampling period. As mentioned previously, the PCE control was located in a grassy area that was transformed into a soccer field, the shift may have improved conditions for microbial populations to thrive during the last sampling period. As in the TCE Site, planted microcosms rates were comparable during the study (P<0.05) and averaged 0.03 μmol h^{-1} g^{-1} dry weight soil (± 0.01 SE). However, these rates were significantly lower than the rates of the planted microcosms at the TCE Site (Tukey's test, P<0.0005) and resulted in longer periods of exposure to the $^{13}CH_4$ (86 ± 20 days).

The active methanotroph composition of the poplar trees at the PCE Site revealed in each sampling period a distinct DGGE profile composed of 3 to 7 bands (Fig. 4-3D, lane 1-2). In the July 2003 sampling (Fig. 4-3D, lane 1), the profile of the PCE Site

poplar was composed of the same group of bands that described the uncultured *Methylocaldum* sp. clone found at the TCE Site trees (Fig. 4-3, B-C). The placement of these sequences in the phylogenetic tree (Fig. 4-4, Group 2) confirmed that the reference bands found at the PCE Site corresponded to the bands at TCE Site, closely related to *M. gracile*. However, in the July 2004 sampling, the composition of the poplar tree changed, the observed bands (97% similarity) aligned in BLAST to uncultured bacteria (>87% similarity) from upland soils in Thailand (Fig. 4-3D, lane 2). These bands were placed next to the *Methylococcus* branch in the phylogenetic tree, closest to *M. capsulatus* (Fig. 4-4).

The only PCE non-planted soil microcosms analyzed by DGGE was collected during November 2004 (Fig. 4-3D, lane 3), and exhibited bands of the same uncultured *Methylocaldum* sp. clone as the planted sample during July 2003 (Fig. 4-3D, lane 1). In addition, two bands that shared 99% similarity and aligned in BLAST with different *Methylocystis* sp. clones (99% similarity) were retrieved. The phylogenetic analysis confirmed their placement in the *Methylocystis* branch (type II methanotrophs) closely related to *Methylocystis* sp. strain SC2. However, differences between trees and the non-planted soil were difficult to assess at this site because various samples were lost during analysis.

Discussion

In this study the SIP technique was successfully implemented to characterize the active methanotroph populations present in the rhizosphere of different trees types used in two phytoremediation plots with distinct environmental conditions. At the TCE Site, because the trees were grown in pots containing planting material, a "compost effect" governed the activity and composition of the methanotroph populations, which may

explain why no significant trend was observed among clones, time samples, or the non-planted soil. Additionally, the years of establishment of the plots, first and second growing seasons, may have also contributed to the homogeneity of the TCE Site samples. It has been reported that differences between poplar clones can take time to reveal in phytoremediation applications (Eberts et al., 2003). Also, studies on the above ground performance of these trees at the site have not been able to detect any differences in genotypes after two year of establishment (Rockwood et al., 2005). Consequently, differences in community structure and activity of the rhizosphere methanotrophic bacteria may develop with time as the different plant types exerted a greater influence over the system such as root expansion and plant biomass accumulation.

The phylogenetic analysis at the TCE Site revealed that type X methanotrophs (thermotolerant bacteria), of the genus *Methylocaldum* and *Methylococcus,* dominated the active methanotroph populations. This result supports the "compost effect" mentioned above. It is known that *Methylocaldum* strains are commonly found among the methanotroph populations in compost piles (10^9 cells g dw^{-1}) (Eshinimaev et al., 2004; Jackel et al., 2005). Also, increased methanotroph activity has been reported when compost is incorporated to soils (Seghers et al., 2005). Consequently, by utilizing a planting material high activity of type X methanotrophs can be expected as it was observed in this study when both phytoremediation sites were compared. However, *Methylocaldum* strains do not produce the soluble form of MMO (Bodrossy et al., 1997), preferred because of its higher oxidative potential (Oldenhuis et al. 1989; Hanson and Hanson, 1996). Nevertheless, *Methylococcus* strains can express both forms of MMO

and degrade chlorinated compounds at higher rates than other types of methanotrophs (DiSpirito et al., 1992; Hanson and Hanson, 1996).

DGGE fingerprints, which showed greater sensitivity in detecting differences among soil microcosms, revealed that at the TCE Site non-planted profiles were significantly different from the planted. TCE Site non-planted samples varied greatly with time and showed lower relative abundance of its community members than the planted pots, suggesting a plant effect of the trees over the methanotrophic populations at the site. Additionally, DGGE profiles revealed that the TCE Site poplar in the November 2004 sampling exhibited significantly fewer bands than previous samplings (July 2003 and 2004), which may imply that poplar rhizosphere methanotroph population exhibited a seasonal effect (summer *versus* fall). During 2004, the months of July and November experienced soil temperatures of 22 and 9°C, respectively. Therefore, the November sampling conditions may not be the optimum for growth of thermotolerant methanotrophs (Bodrossy et al., 1997). Also, during the second growing season willow clone 94014 did not show the major band as in the previous sampling, which corresponded to the *Methylocaldum* clone closely related to *M. gracile*. However, in the poplar tree, it was this clone the only one that persisted in the last sampling period. This result may suggest certain selection of *Methylocaldum* clones by the type of plant, as for each tree type a different clone of *Methylocaldum* persisted in their DGGE profiles. Also, it was interesting that willow clones S365 and SX61 showed exactly the same profile throughout the study. Willow trees produced higher fine root biomass than poplar trees (data not shown), thus possibly assisting in the maintenance of higher temperatures

in the potted soil compared to the poplar or the non-planted soils, which may have been more susceptible to changes in the environment.

Methylocaldum strains can grow in a wide range of temperatures. *M. gracile* grows in a lower temperature range (20- 47°C) than *M. szegediense* (37-62°C) (Bodrossy et al., 1997), which may explain why *M. szegediense* was only found at low abundance and in the inactive fraction (^{12}C-band) of willow clone S365. However, some thermotolerant methanotrophs as the genus *Methylococcus*, present at both phytoremediation sites, are known to contain additional glycoprotein structures on the outer surface of their cell walls, which apparently provides higher resistance to stress factors such as temperature fluctuations and concentration of solutes (Eshinimaev et al., 2004). However, even if type X methanotrophs possess high adaptability to changes in the environment, conditions at the site are not the optimum for these species. Part of the year the soil freezes and the highest soil and air temperature recorded during the study were 25 and 32°C, respectively. Therefore, the question remains as to whether these active methanotroph populations detected after only one and two years of establishment will persist at these phytoremediation plots.

Contrary to results at the TCE Site, DGGE profiles in the PCE Site poplar trees were distinct for each sampling period. Given that methanotroph populations are not capable of oxidizing PCE, it is probable that they showed higher susceptibility to the toxic effects of the contaminant and, consequently, are more vulnerable to environmental changes at the site (Oldenhuis et al., 1991; Hanson and Hanson, 1996). Additionally, no differences between planted and non-planted microcosms were detected at the PCE Site.

However, it was not possible to determine a time effect of the non-planted soil because of the loss of several samples.

Phylogenetic analysis of the PCE Site poplar samples revealed unique profiles composed of only, clones of the *Methylocaldum* genus or uncultured bacterium closely related to *Methylococcus*. On the contrary, the PCE Site control was more diverse composed of one member of the genera *Methylocaldum* and *Methylocystis.* Consequently, active methanotroph populations of the poplar tree rhizosphere were less diverse and more variable at the PCE Site than at the TCE Site. Also, the PCE Site methanotroph community was not dominated by *Methylocaldum* strains. However, *Methylocaldum* is frequently found in natural soils along with *Methylosinus* and *Methylocystis* (Bodrossy et al., 1997; Knief et al., 2003).

In conclusion, the SIP technique was effectively implemented in rhizosphere soils of phytoremediation sites and the protocol described in detail. By combining the SIP technique with the *pmoA*-DGGE analysis the extent and resolution of the technique was broaden to possibly detect differences over time and among the relative abundance of the active members of the methanotroph community. Although, it is expensive and requires certain expertise the method is a powerful technique that allows the assessment of the potential degraders by assessing the active microbial population at any phytoremediation site. Consequently, by using this protocol the phytoremediation practitioner can adequately monitor and implement their remediation technology to the needs of the assessed active rhizosphere populations.

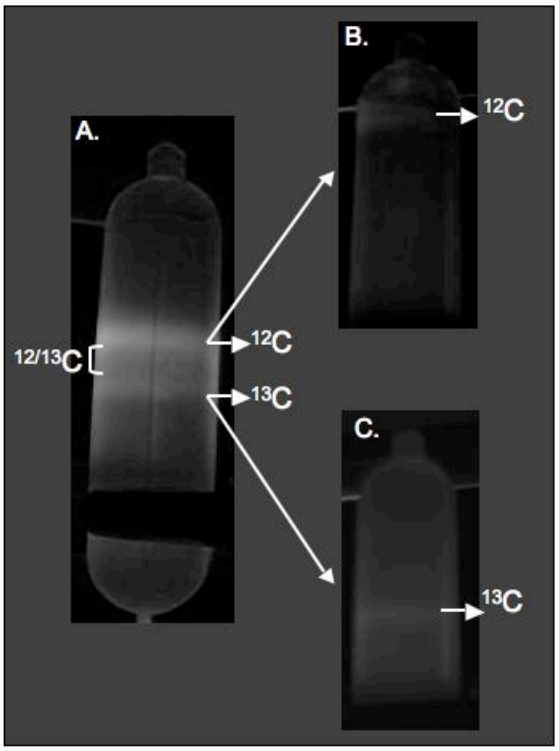

Figure 4-1. Equilibrium centrifugation of isotopically labeled DNA in CsCl density gradient columns. (A) ^{12}C- and ^{13}C-DNA first separation column from TCE Site poplar SIP microcosms, in the July 2003 sampling. (B) ^{12}C-DNA second separation column. (C) ^{13}C-DNA second separation column.

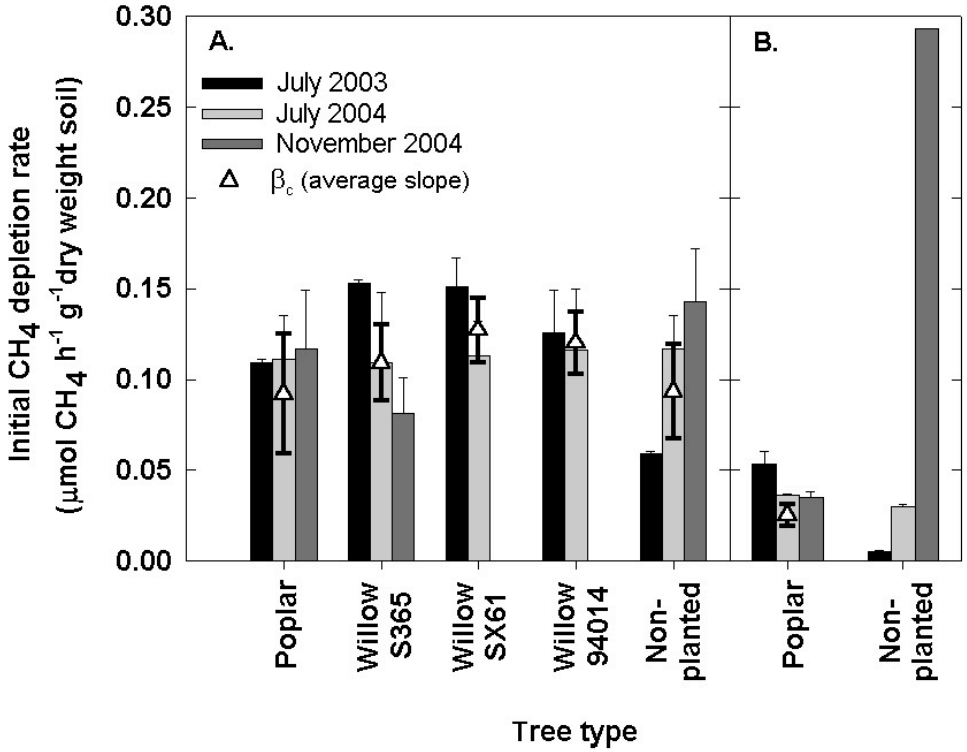

Figure 4-2. Initial $^{13}CH_4$ depletion rates (bars) observed in SIP microcosms after the three sampling periods at the TCE Site (A) and PCE Site (B). Triangles represent average of initial rates over the three time periods. Error bars represent the standard error of the linear regression analysis.

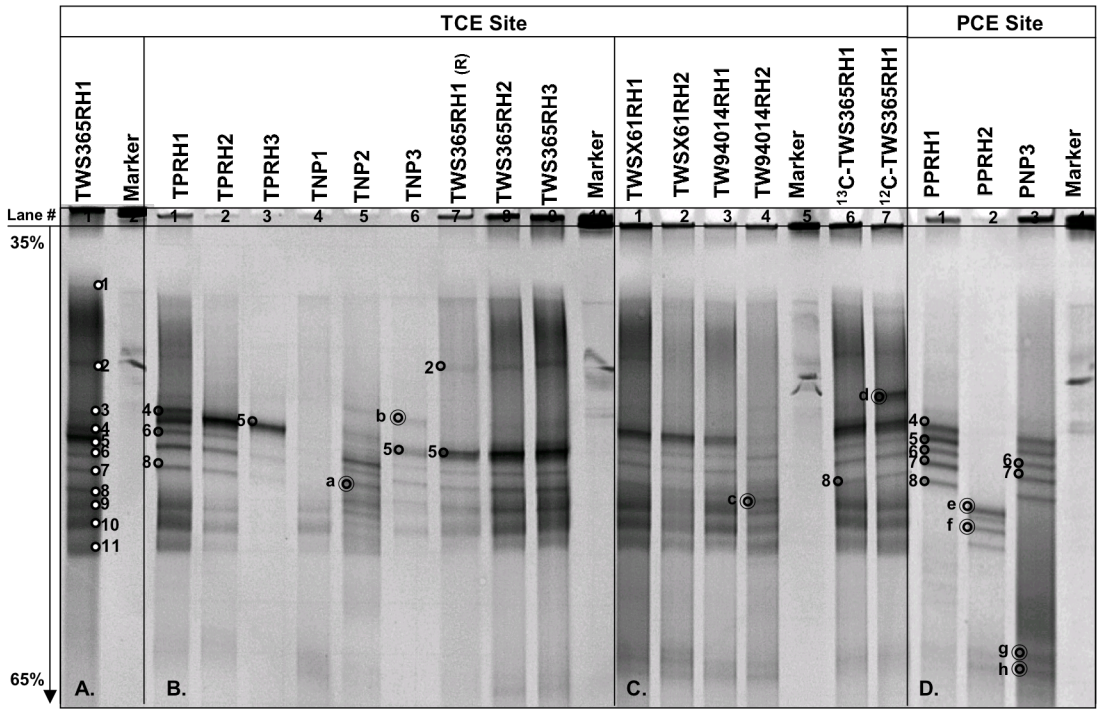

Figure 4-3. DGGE gels of *pmoA* PCR products derived from the ^{13}C-DNA fraction of SIP microcosms at the TCE Site (A-C) and PCE Site (D). Reference bands (white circles), sequenced bands (empty circles) and sequenced bands different from the reference profile (concentric circles) are denoted. Profile names= contaminant type (▲T= TCE, ■P= PCE), tree type (P= poplar, W= willow), willow clone (S365, SX61, 94014), soil compartment (RH= rhizosphere, NP= non-planted), sampling period (1= Jul 2003, 2= Jul 2004, 3= Nov 2004), and replicates (R). Bands from reference profile are denoted by numbers (1-11) and bands different from the reference profile by letters (a-h) and concentric circles.

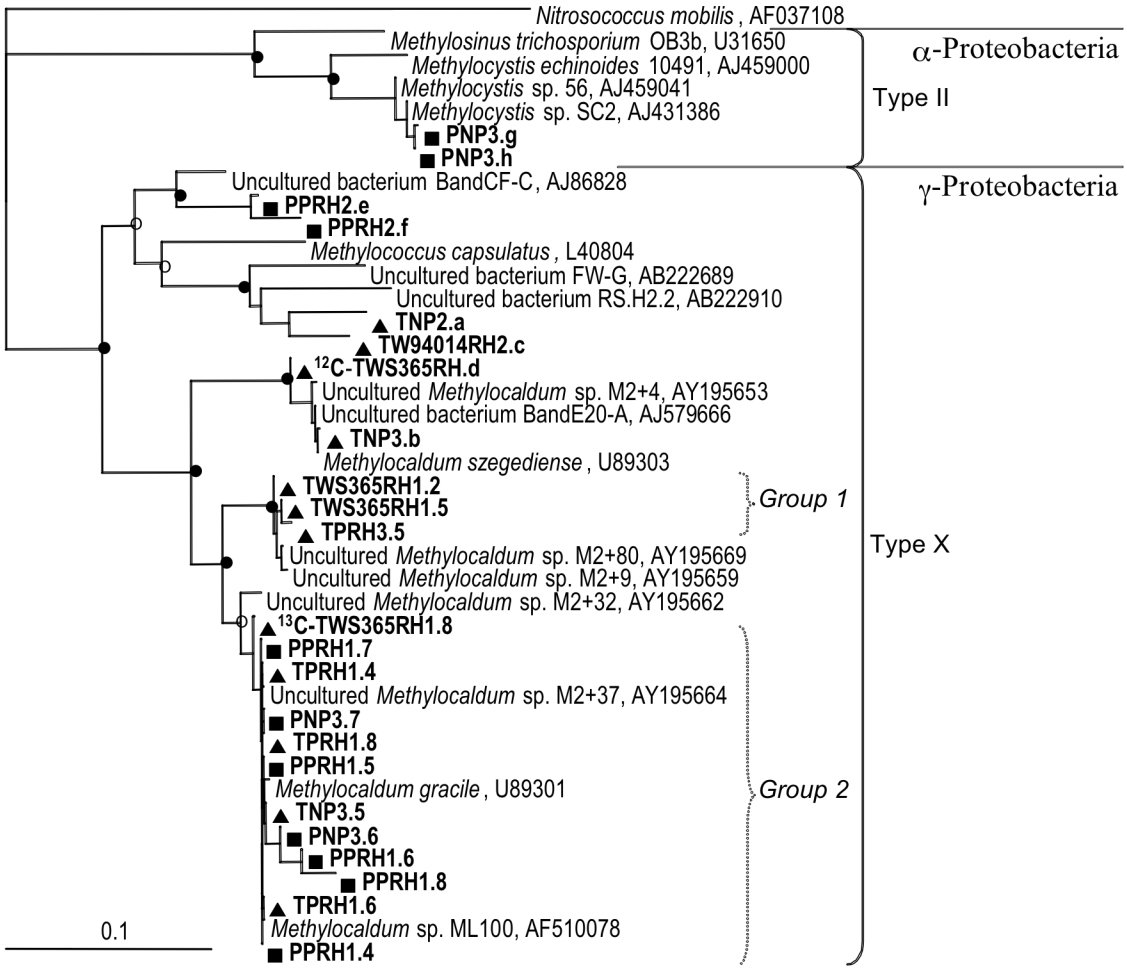

Figure 4-4. Neighbor joining phylogenetic tree of *pmoA* sequences derived from the ^{13}C-DNA fraction of $^{13}CH_4$ SIP microcosms. Tree constructed in relation to extant methanotrophs and highest score BLAST aligned sequences. Length of branches is proportional to % dissimilarity (0.1 substitution per nucleotide site). Branch circles represent ≥90% (●) or ≥68% (O) bootstrap values from 1000 replicates. Sequence name= contaminant type (▲T= TCE, ■P= PCE), tree type (P= poplar, W= willow), willow clone (S365, SX61, 94014), soil compartment (RH= rhizosphere, NP= non-planted), sampling period (1= Jul 2003, 2= Jul 2004, 3= Nov 2004), and band number (1-11= bands from reference profile, a-h= bands different from the reference profile).

CHAPTER 5
CHARACTERIZATION OF RHIZOSPHERE METHANOTROPHIC BACTERIA IN TCE PHYTOREMEDIATION: IMPACT OF THE DESIGN

Note: Manuscript to be submitted to Journal of Applied Microbiology

Introduction

As the use of plants to remediate contaminated sites (phytoremediation) has emerged as an attractive technology, different designs are being tested to improve the effectiveness of the cleanup practice. Given that phytoremediation is the result of multiple, plant-related contaminant removal processes, it is of importance to assess how different designs may affect the extent of contribution of each mechanism. Rhizodegradation, microbial breakdown of pollutants at the root-zone of the plant (rhizosphere), has been proposed as the main removal mechanism contributing to the overall remediation of groundwater and soils contaminated with chlorinated solvents (Anderson and Walton, 1995; Brigmon et al., 1999). Although phytoremediation is being tested in the field, focused studies on rhizodegradation and, even to a lesser extent, the effect of the design on rhizosphere microbial processes are scare.

Plant root-zone processes have long been recognized and evaluated in agricultural fields because of their beneficial effects on plants. Furthermore at a larger scale, this zone of higher microbial activity has a major impact on the global cycling of nutrients (Curl and Truelove, 1986; Walton et al., 1994). Activity, composition, and abundance of rhizosphere microorganisms are driven by complex interactions between the environment (soil type, nutrient status, pH, moisture) as well as plant variables (species, age).

Additionally, since soils are characterized for being oligotrophic environments, plant-related organic compounds (root exudates, cell lysates) can greatly influence soil microbial populations as they contribute to the carbon pool of the soil. The variety of these compounds, determined by the type of plant, has been postulated as a key factor influencing soil microbial communities. Plant species effects may further vary with root zone compartment, plant age, and mycorrhizal fungi interactions. However, contrasting results have been reported on the role that environmental and plant variables play as determinants of soil microbial community composition (Marschner et al., 2001).

Microorganisms have the ability to persist in the environment despite unfavorable growth conditions, and, as a result, an almost universal microbial composition and structure are found in soil (Mahaffee and Kloepper, 1997), including those in microenviroments formed by the influence of the root system of a plant. There is great need, therefore, for experimental approaches that reveal not only the microbial composition of a site but also its relative abundance and *in situ* activity of its components to effectively predict microbial rhizosphere processes and assess differences among planted systems (Mahaffee and Kloepper, 1997). However, a cumulative effect of land-use on the microbial community structure of a site has also been reported (Felske and Akkermans, 1997; Buckley and Schmidt, 2001). For example, sites with a long-term history of agricultural management share similarities despite differences in plant composition, soil type, and management practices.

In the phytoremediation scenario, microorganisms responsible for the breakdown of contaminants are of special interest. Methanotrophs (aerobic methane-oxidizing bacteria) present in the rhizosphere of plants, are known for their capabilities to degrade a

variety of xenobiotic compounds and thus play an important role in chlorinated compound degradation (Hanson and Hanson, 1996; Brigmon et al., 1999). The methanotroph community composition and structure of a soil have been shown to be affected by land-use, soil type, plant type, water content, and management practices (Dubey and Singh, 2001; Reay et al., 2001; Knief et al., 2005; Seghers et al., 2005). However, in saturated habitats, other factors, such as plant type and their ability to transport oxygen to the rhizosphere along with the location of the soil-water interface, gain more importance in determining the methanotroph community (King, 1994; Macalady et al., 2002). Additionally, the methanotroph abundance varies according to the soil compartment. Higher numbers have been observed in the rhizosphere compared to the bulk soil (Brigmon et al., 1999).

Improved practices in phytoremediation systems have included plant and clone type selection, manipulation of the planting method, and addition of soil amendments (McCutcheon and Schnoor, 2003; Negri et al., 2003; Rockwood et al., 2005). To treat sites contaminated with chlorinated solvents, phytoremediation system designs have ranged from natural settings (natural attenuation) to completely engineered systems with conifers, poplars, and willows trees (McCutcheon and Schnoor, 2003; Negri et al., 2003). In the engineered systems, tree cuttings are cast in the soil to restrict water usage to the contaminated groundwater and promote deep root proliferation. Also, trees can be planted in trenches with the incorporation of soil amendments, resulting in a homogenized soil rich in nutrients. The impact of these phytoremediation practices have not yet been fully characterized, especially in terms of their effect on rhizosphere microbial populations and their biodegradation potential.

This study describes the rhizosphere methanotroph community of three different phytoremediation settings as a means of comparing their rhizodegradation potential. The phytoremediation settings ranged from a natural revegetated area to an engineered system, located in two different sites that vary significantly by soil type, climate zone, and plant type. More specifically, this study describes the abundance, activity, and phylogenetic composition of the methanotroph community by culture-based methods, microbial counts and enrichments, combined with culture-independent molecular analysis of phylogenetic and functional genes by denaturing gradient gel electropheresis (DGGE) and stable isotope probing microcosms (SIP). The ultimate goal of this work is to establish for each phytoremediation setting the main factors influencing methanotrophic rhizodegradation potential to further improve phytoremediation efficiency.

Materials and Methods

Site Description

Two Superfund sites, contaminated with chlorinated solvents, were studied. A natural attenuation setting at C-Area of the Savannah River Site (SRS), Aiken, S.C., and two different engineered systems at the former LaSalle Electric Utilities, LaSalle, IL (Fig. 5-1). The primary climate and soil characteristics of each phytoremediation site are presented in Table 5.1 and Table A1 (Appendix), respectively. A detailed description of each site and its phytoremediation design follows.

SRS, S.C. The SRS (80, 289 ha) was constructed in 1950s by the U.S. DOE for the production of basic materials used in nuclear defense programs. Production ceased in 1988 with sufficient chemical and radioactive waste that resulted in soil and groundwater contamination. The study was located in a natural revegetated area dominated by 20 year-old loblolly pine trees (*Pinus taeda*) where a TCE plume (6-20 ppb) resurfaces from

the contaminated groundwater into the Four Mile Branch Creek. Vinyl chloride and *cis*-1,2-dichloroethylene (cis-DCE), both possible TCE anaerobic metabolites, have also been detected in the area (Punshon et al., 2002).

LaSalle, IL. The former LaSalle Electrical Utilities (4 ha) manufactured capacitors from 1943 until 1982, when it filed bankruptcy. The activity caused major contamination of surface soils and groundwater with polychlorinated biphenyls and chlorinated solvents. Two phytoremediation plots were implemented to enhance chlorinated solvent removal, a final stage of the cleanup process. The first plot (0.25 ha), contaminated with TCE, was installed in September 2002 (labeled as "TCE Site"). Poplar and willow trees were planted by lowering 1.8 m rooted whips to the bottom of 0.6 m diameter boreholes lined with high-density polyethylene pipe and filled with an equal mix of sand, soil, bark, and peat (pH 7.8) (Table 5-1). Low and high concentration regions exist at the site, exhibiting a broad range of TCE concentrations (0-254 ppb) (Fig. 5-1). In each region, 18 poplar and 24 willow clones were planted. The second phytoremediation plot (0.21 ha), contaminated with PCE was established in March 2002 ("PCE Site"). The soil (pH 7.3) was improved by incorporating a mulch composed of tree chips on the top 0.5 m of the soil surface and poplar, pine, and willow trees were planted directly into the soil. This site also presents a concentration gradient where high and low PCE regions have been delineated (0-838 ppb) (Fig. 5-1). At both phytoremediation plots, there is evidence that supports tree uptake of the contaminated groundwater apart from complete breakdown of the chlorinated compounds (R. Lange and J. Isebrands, personal communication). Since 2002, contaminant concentrations have decreased substantially. In 2005, TCE concentrations were reduced from 254 to 11 ppb and PCE from 838 to 300

ppb. Only one anaerobic metabolite of degradation has been detected at the site (cis-DCE), and its concentration has also decreased with time (353 to 3.6 ppb). Nevertheless, insufficient data exist to accurately assess the role of phytoremediation because a dual solid-phase water extraction system is also operated at the site (Lange, 2004).

Sampling

Soil sampling occurred in October 2003 at SRS, and in July 2003, July 2004, and November 2004 at LaSalle. At the SRS, three pine trees were sampled with a large diameter (7.6 cm) hand soil auger, all in the same direction (west) and at approximately 1.5 m from the tree base,. The sampled area presents a declining slope towards the seepline outflow; consequently, trees were designated as pine 1 to 3 (pine 1 at the top of the gradient and pine 3 at the bottom) (Fig. 5-1). Pine 2 and pine 3 were under saturated conditions during sampling (>30 cm depth). To test the effect of sampling depth, the complete soil profile influenced by the root system was sampled at depths of 0-15, 15-30, 30-60, 60-90 and 90-120 cm. A control next to the revegetated area was also sampled.

At the LaSalle site, trees were sampled at approximately 0.3 m from the tree base with a small diameter (1.9 cm) hand soil auger to minimize soil disturbance. The same soil depths as at the SRS were sampled; however, the first sampling (July 2003) only extended to the 60-90 cm depth due to limited root expansion. Because of limited sampled area at the PVC pots and to collect enough rhizosphere material to analyzed, the first two soil layers that were 15 cm in depth (0-15 and 15-30 cm) consisted of a composite sample from all four cardinal points around the tree base. The rest of soil layers sampled (30 cm in depth) were collected from two opposite locations around the tree base, to obtain the same sampled volume per soil layer.

To determine if differences exist among the superior tree genotypes (clones with vigorous aboveground growth) at the TCE Site, one poplar clone (clone I45/51; *Populus deltoides* x *P. nigra*; origin, North America x Europe) and three willow clones were studied (clone SX61, *Salix sachalinensis*, Japan, exotic; clone S365, *S. discolor* 18, University of Toronto; clone 94014, *S. purpurea*, State University of New York, exotic). Additionally, a non-planted sample was taken from an established borehole that was not planted. At the PCE Site, the same poplar clone I45/95 was sampled along with a non-planted sample removed from outside the contaminated plot in a grassy area (serving as a control). This control area was transformed to a well-maintained soccer field between July 2003 and 2004. In order to observe effects of the degree of TCE and PCE concentration, sampling was conducted in both the low and high concentration regions at each site (Fig. 5-1).

To prevent cross-contamination, the auger was washed with sterile water, rinsed with 95% ethanol, and washed again several times. Samples were collected in sterile 0.025 mm bags (Nasco Whirl-Pak, Fort Atkinson, WI, USA), placed on ice, transported to the University of Florida within 1 to 2 days, and stored at 4ºC. In order to compare activity and diversity in the soil (in the rhizosphere; denoted as RH), in the soil adhered to the roots (in the rhizoplane; denoted as RP), and in soil not influenced by the plants (in the non-planted soil; denoted as NP), samples were homogenized using a sterile spatula, and fine roots (<2 mm in diameter) were separated from the soil for separate testing.

Soil Characterization

Soil analysis was conducted at the Analytical Research Laboratory in the Institute of Food and Agricultural Science (IFAS) at the University of Florida. Water-extractable P, Ca, Mg, and Cu, and KCl-extracted ammonia were analyzed by inductively coupled

plasma (ICP) in combination with colorimetric analysis for P determination. Organic matter was analyzed by loss-on-ignition, and water content by the gravimetric method. Soil pH was resolved in deionized water, and the soil field capacity was qualitatively determined (Mylavarapu and Kennelley, 2002).

The analysis of the samples was conducted in two separate approaches, by traditional culture-dependent methods, microbial counts and enrichments, and, by a culture-independent method, stable isotope probing (SIP) using soil microcosms. Additionally, phylogenetic analysis of the enrichments and SIP microcosms were performed to characterize the studied microbial populations within each approach.

Microbial Counts

Heterotrophs and methanotrophs present in soil and root samples were enumerated by colony forming units (CFU) and most probable number techniques (MPN), respectively. Counts were performed as described by Baker et al. (2001) and Woomer (1994). Briefly, 1 g wet weight soil or root sample were placed in 9 ml of phosphate buffer (10 mM; pH 7.0) and shaken with 0.8 g glass beads (0.5 mm diameter) at 250 rpm and 30°C for 20 min to disaggregate particles for subsequent dilutions. Serial dilutions were plated in triplicate on tryptic soy agar plates (1/10 strength) and incubated at 30^{o}C for 1 week before counting colonies. MPN vials (10^{-6} dilutions) in triplicate were inoculated with dilution aliquots into nitrate mineral salts medium (NMS) with 10 μM $Cu(NO_3)_2$ and 20% (v/v) CH_4 (Whittenbury et al., 1970). Vials were incubated for a month at room temperature, and growth was recorded as positive where turbidity was observed to increase. Microbial counts were adjusted to a dry weight soil basis and when less than 1 g root material was available.

Characterization of Enrichments

The lowest positive dilution from the MPN technique was subsequently cultured in NMS with and without Cu, incubated on a reciprocal shaker (250 rpm) at 30°C with 20% (v/v) CH_4 for more than two months. Most of the characterization of the enrichments was restricted to enrichments obtained from the 30-60 cm soil layer, to limit samples tested to a manageable number. Enrichments were characterized by growth curves, sMMO activity, and oxygen uptake in the presence of CH_4, as follows.

Growth curves. The culture medium was inoculated with the corresponding enrichment to an initial optical density of 0.035 at 600 nm and incubated at 250 rpm and 30°C with 20% (v/v) CH_4. Cultures in triplicates and a negative control (no CH_4 added) were used to construct curves and determine exponential growth rates by monitoring turbidity at 600 nm in a spectrophotometer (Spectronic 21, Milton Roy Company, PA, USA).

sMMO assay. Presence and activity of soluble methane monooxygenase (sMMO) was qualitatively verified by a naphthalene assay modified from Brusseau et al. (1990). Triplicates of the active test culture and controls were included as described by Lindner et al. (2002). Controls included one heat-killed, one cell-free, and one cultured with live cells in the presence of 1μM $CuSO_4 \bullet 6H_2O$, known to repress sMMO synthesis (Prior and Dalton, 1985). The intensity of the color indicated the degree of sMMO activity.

Oxygen uptake experiments. The oxidation potential of the enrichments was determined by measuring oxygen uptake in the presence of CH_4, employing a Clarke electrode and an automated data acquisition system, as described by Lindner et al. (2000). The substrate was introduced to the test culture by bubbling CH_4 (99.9%; Airco BOC,

Murray Hill, NJ, USA) for 5 min into sterile water and immediately injecting 400 μl into the reactor. In this study, the reactor was set at room temperature (~25°C). Triplicate curves were conducted per sample plus a negative control curve prepared by adding 4 ml of acetylene (99.6%, Praxair, Dambury, CT, USA), an MMO inhibitor (Prior and Dalton, 1985), in the presence of CH_4. Potential rates of oxidation were calculated by regression analysis of the initial linear portion of the curve, after accounting for endogenous metabolism.

DNA extraction. Genomic DNA was extracted from 10 ml of enrichment culture with a commercial microbial DNA isolation kit (Mo-Bio Lab., Carlsbad, CA, USA). The enrichments from all time samples removed from the LaSalle TCE Site poplar tree and the non-planted sample were analyzed, whereas only the November 2004 samples removed from the willow clone S365 and PCE Site poplar tree were analyzed. DNA templates were stored at -40°C until the analysis. Quantification and purity of DNA templates was determined by UV spectrophotometry as described by Ausubel et al. (1992).

Stable Isotope Probing (SIP) Soil Microcosms

The SIP protocol was previously described in Chapter 4. Briefly, 10 g wet weight of soil (16% water content) from the 30-60 cm soil layer and high-contaminant region were placed in 160 ml serum vials, and 10 ml of $^{13}CH_4$ (99.9%, Isotec, Miamisburg, OH, USA) were aseptically added (Morris et al., 2002; Radajewski et al., 2002). Vials were incubated in the dark at room temperature. Headspace CH_4 depletion was monitored every 2-5 days by gas chromatography. After more than 90% of the CH_4 was consumed, vials were opened, gently flushed with air for 5 s, resealed, and replenished with $^{13}CH_4$.

The procedure was repeated five times (Radajewski et al., 2002). Initial CH_4 depletion rates were calculated from data taken during incubation after the first CH_4 addition by linear regression analysis of the consumption curve. DNA extraction from microcosm soils was performed using the PowerMax Soil DNA Extraction Kit (Mo-Bio Lab., Carlsbad, CA, USA). The DNA extracts were resolved by CsCl density gradient centrifugation (Beckman VTi65 rotor, 6 h, 20°C, 265 000 x g) (Sambrook et al., 1989; Radajewski et al., 2002). Three fractions were generally collected as the following: (1) a light-DNA upper band (^{12}C-DNA); (2) a middle band, combine ^{12}C- and ^{13}C-DNA; and (3) a heavy-DNA lower band (^{13}C-DNA). DNA fractions were purified as described by Sambrook et al. (1989).

Phylogenetic Analysis of Enrichments and SIP Microcosms

Primer sets and polymerase chain reaction (PCR) amplification. DNA extracted from enrichments and SIP microcosms was used as templates for PCR amplification as previously described (Chapter 4). The phylogenetic analysis was performed with the 16S rDNA primer set 533f/907r, targeting all life (Henckel et al., 1999). The functional *pmoA* primer set A189f/mb661 was used to specifically target the pMMO active site (Costello and Lidstrom, 1999). GC-clamps were attached to the 907r-16S rDNA primer and A189f- *pmoA* primer as described by Henckel et al. (1999). PCR reactions and amplification protocol for the 16S rDNA and *pmoA* gene were followed according to Henckel et al. (1999) and Knief et al. (2003), respectively. PCR products were verified by 1.5% agarose horizontal electrophoresis.

PCR products were separated by DGGE in the DCode System (Bio-Rad Lab., Hercules, CA, USA) as described by Henckel et al. (1999). Briefly, 1 mm thick 6.5% (w/v) polyacrilamide gels (37.5:1 acrylamide-bisacrylamide) (Fisher Scientific,

Pittsburgh, PA, USA) were prepared and electrophorese in 1X TAE buffer at 60°C and 150 V for 5 h in a 35-80% linear denaturant gradient (80% denaturant represents 5.6 M urea and 32% (v/v) deionized formamide). Different conditions for running the gels (61°C and 180V for 5 h in a 35-65% linear denaturant gradient) were used when working with the *pmoA* primers. Gels were loaded with 25-45 μl of the PCR product according to agarose gel band intensity and 1/4 volume of loading buffer. After, gels were stained with ethidium bromide according to the manufacture's protocol, visualized on a UV transilluminator at 312 nm (Model 88A; Fisher Scientific, Pittsburgh, PA, USA), and photographed with the digital photodocumentation system DigiDoc-IT ™ (Daigger, Vernon Hill, IL, USA). DGGE bands were excised from the middle part of the band with a sterile scalpel, and DNA was eluted according to the protocol described by Chory and Pollard (1999). The eluted DNA was reamplified and reanalyzed on DGGE to verify sample purity. Reamplification of 16S rDNA bands was performed by modifying the PCR protocol to an annealing temperature of 60°C, with no touchdown program and 25 cycles. For *pmoA* band reamplification, the PCR protocol was changed to an initial denaturing step of 5 min at 94°C, followed by 30 cycles of 30 s at 94°C for denaturation, 45 s at 66°C for annealing (to avoid sequence ambiguity as reported by Dunfield et al. (2002)), 30 s at 72°C for elongation, and a final elongation step of 7 min at 72°C. Several bands with the same mobility were excised from different lanes to check for sequence identity.

Sequencing. Reamplified PCR products were purified with a commercial PCR purification kit (Mo-Bio Laboratories, Carlsbad, CA, USA) before sequencing. Quantification and purity of the PCR product (1:20 dilution) was determined as

mentioned above (Ausubel et al., 1992). PCR products were sequenced by the Interdisciplinary Center for Biotechnology Research (ICBR) at the University of Florida in Gainesville, FL.

Phylogenetic analysis. Sequences were compared with database sequences in the National Center for Biotechnology Information (NCBI) using BLAST (Altschul et al., 1990). Related sequences identified in BLAST and sequences of extant methanotrophs were manually aligned with CLUSTALX v. 1.8 (Thompson et al., 1997). Phylogenetic trees were constructed by the neighbor joining (NJ) method in CLUSTALX and displayed in TreeView v. 1.6.6 (Page, 1996). All nucleotide accession numbers of the obtained sequences were placed in the GenBank for future access (AYXXX-AYXXX).

Statistics

Microbial counts ($\log_{10}$ transformed) were analyzed for significant differences ($P<0.05$) among sampling periods and soil depths by repeated measurements and a split-plot ANOVA design, respectively. Additionally, differences among sample means and between controls were analyzed by Tukey's and Dunnett's test, respectively. Also, the Student's t-test was used to compare differences between the rhizosphere and rhizoplane samples.

Methane depletion rates of SIP soil microcosms were analyzed by comparing the initial slopes of the linear regression curves of each plant type and control per sampling period. When a set of samples showed no significant differences in rates, an average depletion rate was calculated as an estimate of their CH_4 depletion rate. Average regression rates were compared by a modified Tukey's test, and differences between the controls by a modified Dunett's test (Zar, 1984). SAS software v. 7 (1998) (SAS Institute Inc., Cary, NC, USA) was used for all analyses.

At each phytoremediation setting, principal component analysis (PCA), , was performed for culture-dependent and -independent data. PCA allows multivariate data to be characterized by a smaller number of variables with no bias from *a priori* assumptions about treatment effects (Balser et al., 2002). To visualize each phytoremediation setting, principal components PC1 and PC2 were plotted in a two-dimensional plot. SPSS v. 8.0.0 (1997) was used as the statistical software for this analysis (SPSS Inc., Chicago, IL, USA).

Results

Description of Sites

The two phytoremediation sites studied vary significantly in climate zone, soil type, and planted system. The SRS is located in a semi-tropical climate with rainfall distributed throughout the year, with relatively high temperatures, humidity, and precipitation (Table 5-1). SRS soils are acidic, frequently flooded, with surface sand layers and loamy subsoil of clays (<52% sand, 28-50% silt, and 7-27% clay) (USDA, 1990) (Table 5-1). Climate at the LaSalle site is temperate with lower temperatures and approximately half the precipitation reported at SRS. Soils in the LaSalle region possess neutral pH and are primarily silty, formed in loess and calcareous lacustrine sediments (>80% silt and <12% clay) (USDA, 1996). However, only the PCE Site phytoremediation plot at LaSalle contained native soil (Table 5-1), whereas the TCE Site contained planting material.

Soil physicochemical characteristics, at the SRS, were influenced by the depth of the soil profile (Table A1, Appendix). Nutrients and organic matter content decreased drastically between the 0-15 and 15-30 cm soil layer, and the non-planted soil exhibited the lowest values compared to that of the planted soil. On the contrary, soil conditions at

LaSalle were relatively homogeneous throughout the soil profile with similar physicochemical values between planted and non-planted areas (Table A1). Macro- and micro-nutrients (P, Ca, Mg, and NH_3-N) at the SRS were lower than at LaSalle, except for Cu concentrations (Table A1). Phosphorus was the only soil nutrient detected below the low range in soil fertility tables (Mylavarapu and Kennelley, 2002). Both the SRS and LaSalle PCE Site soils contained low P levels at 0.4-1.4 and 0.7-1.3, respectively.

Microbial Counts

Savannah River Site (SRS). Microbial abundance in pine trees varied depending on the microbial group studied, tree location, and soil compartment (Fig. 5-2A). However, counts were not significantly affected by soil depth. Rhizosphere heterotroph counts (10^6-10^9 CFU g^{-1} dw soil) were significantly different among the three pine trees sampled (P<0.0001, n=20). However, the heterotroph counts in the non-planted soil sample was not different from Pine 3. Counts appeared to decrease according to the location of the trees on the slope towards the seepline outflow. Pine 1, at the top of the slope, exhibited the highest numbers whereas Pine 3, at the bottom, exhibited the lowest. Conversely, heterotroph counts in the rhizoplane were similar among trees (10^6-10^7 CFU g^{-1} fresh root material).

Methanotroph counts in the rhizosphere (10^1-10^4 cells g^{-1} dw soil) did not vary among trees and only Pine 3 counts differed from those of the other trees and the non-planted bulk soil (P=0.003, n=20). However, at the rhizoplane, methanotroph counts (10^3-10^5 cells g^{-1} fresh root material) were significantly different (P=0.0155, n=8). Interestingly, Pine 3 at the bottom of the slope (under saturated conditions) showed the lowest numbers of methanotrophs in the rhizosphere ($5.0 \pm 4 \times 10^1$ cells g^{-1} dw soil) but the highest at the rhizoplane ($2.5 \pm 5 \times 10^4$ cells g^{-1} fresh root material) (Fig. 5-2A).

LaSalle Site. Although, microbial abundance was determined at LaSalle in different sampling periods (July 2003, July 2004, and November 2004), only the November 2004 data are presented in Figure 5-2 to describe comparable times in the year (October and November) between phytoremediation sites. However, counts at LaSalle did not significantly vary with time, depth, or among the tree types or the non-planted soil. Depth showed an effect on methanotroph abundance only in the TCE Site rhizosphere soil sample removed in the July months (Table A2). Methanotroph counts increased from the 0-15 to the 30-60 cm soil layer, from an average of 4.9 to 5.7 cells g^{-1} dw soil. Also, at the TCE Site, a negative effect on methanotroph counts in the rhizoplane was observed in the high-contaminant exposure zone (Table A2). Although, not significantly different throughout the study, willow clone SX61 rhizosphere samples exhibited both heterotroph and methanotroph counts that were positively affected by the high-contaminant exposure and values that were higher than the non-planted sample.

As shown in Figure 5-2, the highest numbers of both microbial groups studied were observed at the LaSalle-TCE Site with no significant differences between soil compartments (rhizosphere *versus* rhizoplane) or among tree types or the non-planted soil, as previously mentioned (Fig. 5-2B). Heterotrophs ranged from 10^8-10^9 CFU g^{-1} dw soil or fresh root material, and methanotrophs ranged from 10^5-10^6 cells g^{-1} dw soil or fresh root material. The LaSalle PCE Site (Fig. 5-2C) showed a similar pattern as the TCE Site but with lower numbers of methanotrophs (10^2-10^5 cells g^{-1} dw soil or fresh root material).

Root Biomass

Fine root (<2 mm diameter) biomass production and distribution throughout the soil profile differed for each tree type evaluated (data not shown). At the SRS, pine trees

under saturated conditions showed maximum root biomass in the 15-30 cm soil layer. Meanwhile, trees not directly influenced by the seepline outflow exhibited a more homogeneous root distribution in the soil profile, with a maximum at the 30-60 cm soil layer. Tree cuttings at LaSalle, after 2 years of establishment (July 2004 sampling period), exhibited maximum root biomass in the surface soil layer (0-15 cm). Overall, fine root biomass was higher in the willow trees compared to poplars. Additionally, the TCE Site poplar trees exhibited higher root biomass than the PCE Site poplar trees.

Enrichments Activity

Enrichments were successful in NMS medium with Cu added from all soil compartments evaluated at both phytoremediation sites. However, at the LaSalle, various enrichments in NMS medium without Cu added were not obtained from the rhizoplane samples removed from the high TCE and PCE concentration regions throughout the study (data not shown). Figure 5-3 shows the activity of only the enrichments obtained from samples removed during the last sampling at LaSalle. Only these LaSalle enrichment activities are shown in order to compare the respective SRS activities of enrichments obtained from samples removed during the same fall sampling period (November and October, at LaSalle and SRS, respectively). Overall, activity between rhizosphere and rhizoplane enrichments was comparable.

Maximum growth rates for all enrichments, in each culture medium, were comparable. Growth rates ranged from 0.014 to 0.027 h^{-1} and 0.003 to 0.008 h^{-1} in NMS with Cu and without Cu, respectively. Greater differences were observed in oxygen uptake rates in the presence of CH_4 (0.009-0.065 and 0.003-0.023 μmol O_2 s^{-1} for the NMS with Cu and NMS without Cu enrichments, respectively), which was confirmed by the range in sMMO activity as observed by color intensity, as previously described (data

not shown). Enrichments in NMS with Cu showed the highest growth and oxygen uptake rates when compared to the NMS without Cu enrichments (Fig. 5-3, panels A-C *versus* panels D-F). However, CH_4 oxidation rates were always lower than the non-planted enrichments (Fig. 5-3), with the exception of Pine 3 NMS with Cu rhizosphere enrichment (Fig. 5-3A) and PCE Site poplar rhizoplane NMS without Cu enrichment (Fig. 5-3F), each of which showed similar rates to that of the non-planted soil.

Savannah River Site (SRS). Analysis of each pine tree revealed that enrichments activity in NMS with Cu increased according to their position in the slope of the sampled area, from Pine 1 (at the top) to Pine 3 (at the bottom) (Fig. 5-3A). On the contrary, NMS without Cu enrichments showed decreasing oxidation rates and sMMO activity with the decreasing sampling area slope (Fig. 5-3D). Enrichments from the SRS site generally showed higher rates compared to the LaSalle enrichments.

LaSalle Site. Enrichment activity from the different tree types at the LaSalle varied throughout the study with no detectable trends. Only enrichments from the TCE Site poplar trees consistently showed higher rates in the rhizosphere than rhizoplane (Fig. 5-3, B and E). Attempts to enrichment some rhizoplane samples from the high-concentration TCE zone in NMS without Cu medium were not possible, possibly negatively affected by the high-contaminant exposure (data not shown). In both enrichment media, poplar rhizosphere and willow clone S365 rhizoplane showed the highest rates in each soil compartment analyzed. Poplars at the LaSalle PCE Site showed higher rhizoplane rates than poplar trees at the TCE Site, and all NMS without Cu rhizoplane enrichments were successful using these samples.

Activity of the enrichments from samples taken throughout the soil profile was determined for the LaSalle TCE Site poplar trees in NMS with Cu conditions during July 2003 and November 2004 samplings (Fig. A2). A shift in activity was observed between samplings. In July 2003, the highest activity was observed at the surface soil layer (0-15 cm). However, in November 2004 rates increased with depth, and the activity of the non-planted soil exhibited a maximum rate in the middle soil layer (30-60 cm).

Phylogenetics of Enrichments

The universal primer set was successfully amplified from all enrichments. The variability of the PCR and DGGE analysis was low, with almost identical profiles obtained in replicate runs (Fig. A3). DGGE profiles from each site and tree type evaluated were unique and consisted of multiple bands (1-7 bands) (Fig. A3). The least number of bands was found in the rhizoplane enrichments and the greatest, in the non-planted soil enrichments.

Savannah River Site (SRS). The SRS enrichments showed DGGE profiles with a greater number of bands (5-7 bands) compared to the LaSalle sites enrichments (Fig. A3). BLAST alignments confirmed that the most abundant members of the enrichments belonged to type II methanotrophs. Rhizosphere and non-planted enrichment bands aligned with *Methylocystis* sp. strain 18-2 (100% similarity) from a consortium that rapidly degrades TCE. Meanwhile, the dominant member in the rhizoplane enrichment revealed a 100% similarity to an uncultured *Methylosinus* sp. from a mine environment. The rest of the enrichment bands aligned to species of *Sphingobacteria-Bacteroidetes*, β-*Proteobacteria*, and uncultured bacteria. Additionally, one methylotroph (*Hyphomicrobium* sp.) was retrieved from the rhizoplane enrichment.

LaSalle Site. The phylogenetic analysis of TCE Site poplar tree enrichments was conducted throughout the study along with the non-planted samples (Fig. A3). However, willow clone S365 and PCE Site poplar tree enrichments were only analyzed during the last sampling period (November 2004).

The microbial composition of the TCE Site poplar tree enrichments was distinct in soil compartment analyzed and sampling period. A reduction in the components of the rhizosphere enrichment was observed in the November 2004 enrichments (Fig. A3). Profiles from the July 2003 and 2004 enrichments showed 5-6 bands, whereas the November 2004 profile exhibited only two bands. The non-planted enrichment profiles showed no noticeable trend in seasonal effects.

Enrichments from both phytoremediation plots at LaSalle exhibited numerous BLAST alignments with uncultured bacteria that were of particular interest, as most of these sequences belonged to the major bands of the profiles. Only three out of the 14 enrichments analyzed exhibited as dominant members of their community extant methanotrophs. Overall, methanotrophs retrieved from the LaSalle enrichments aligned (>98% similarity) with several strains of *Methylosinus* and *Methylocystis* from a variety of environments (river floodplain soils, forest soils, groundwater, and a consortium that rapidly degrades TCE). Additionally, from the PCE Site poplar rhizosphere enrichment, one methylotroph was retrieved that revealed a 100% similarity to uncultured *Methylobacterium* sp. Still, as in the SRS, only type II methanotrophs from the genus *Methylocystis* and *Methylosinus* were detected at LaSalle.

In general, all enrichments from the different phytoremediation settings exhibited as major phyla, *Proteobacteria* (44-58%), *Bacteroidetes* (13-33%), and uncultured

bacteria (8-38%) (Fig. 5-4). Interestingly, the major class of *Bacteroidetes* at LaSalle was *Flavobacteria* and, at SRS, *Sphingobacteria*. The phylogenetic tree confirmed this grouping; however, it did not show any other specific clustering according to soil compartment, tree type, site or phytoremediation setting (Fig. A4). In each major phylogenetic group, all phytoremediation settings were represented by sequences of their enrichments. The sequences that aligned with uncultured bacteria, mostly from the LaSalle enrichments, were placed in its majority within *Bacteroidetes*.

SIP Soil Microcosms

As previously reported (Chapter 4), SIP microcosms were effectively implemented to rhizosphere studies of phytoremediation settings. In this study, the technique is further applied to a different system, the Savannah River Site, which presents the same contaminant scenario in a more natural setting compared to the engineered LaSalle plots.

SIP activity. Initial CH_4 depletion rates from microcosms of each phytoremediation setting did not significantly vary among sampling periods, tree types, or with the non-planted soil. The only exception was the PCE Site non-planted microcosms that showed increased rates with time, which may correspond to the change of this area to a well-maintained soccer field. Consequently, without considering the PCE Site non-planted samples, an average CH_4 depletion rate was calculated for each phytoremediation setting. Average rates significantly differed among phytoremediation settings and followed the order LaSalle TCE Site (0.52 ± 0.06 μmol g dw^{-1} h^{-1}, n=13) > SRS (0.28 ± 0.05 μmol g dw^{-1} h^{-1}, n=4) > LaSalle PCE Site (0.12 ± 0.03 μmol g dw^{-1} h^{-1}, n=3).

SIP phylogenetic analysis-Savannah River Site (SRS). Labeled DNA (^{13}C-DNA), successfully separated from the non-active portion, was further purified in the SRS microcosms as only weak bands were initially obtained from the *pmoA*-DGGE profiles. Since purification attempts failed and because of the limited amount of DNA template, no further purification was pursued. Consequently, given that an unexplained smear of the PCR products was visualized in agarose gels, PCR fragments were extracted from this gel and reamplified. By using this procedure, detection of two bands in the *pmoA*-DGGE profiles was possible, and these profiles were the same for all pine trees and non-planted samples. These bands aligned in BLAST (98% similarity) with an uncultured *Methylocaldum* sp. clone from a landfill cover soil (Table 5-2). To confirm this finding, the 16S rDNA primer set was also used to reamplify these DNA templates.

The active (^{13}C-DNA) and non-active (^{12}C-DNA) fractions of two SRS microcosms were effectively amplified with the 16S rDNA primer set. This result suggested that the DNA template did not contain any PCR-inhibiting substances (as humic acids). DGGE profiles of the 16S rDNA fragments revealed low DNA diversity of the rhizosphere soil and distinct profiles among the pine tree soils (Fig. 5-5A). However, the pine tree microcoms shared the major bands in the ^{13}C- and ^{12}C-DNA profiles. The major band sequenced from the ^{13}C-DNA profile aligned in BLAST with uncultured α-*Proteobacteria* from a pasture soil (99% similarity) and, secondly, with *Methylocella* sp. BL2 from a forest soil (98% similarity). The phylogenetic tree (Fig. 5-6B) confirmed placement of this sequence along with another major band of the same profile within type II methanotrophs of the genera *Methylocella* and *Methylocapsa*. Additionally, a methylotroph (94% similarity), closely related to the genus *Methylophilus*, was retrieved

from the same profile. This band was observed as the major component of the non-active profiles (Fig. 5-5A, lane 1 and 3), which may suggest cross-feeding of the labeled byproducts of methane oxidation (methanol). Overall, the dominant component of the active methanotroph population at the SRS was closely related to the genus *Methylocella* (Table 5-2), possibly explaining the difficulty in obtaining a *pmoA*-PCR product since this genus does not express pMMO (Dedysh et al., 2000).

SIP phylogenetic analysis-LaSalle. Contrary to the low DNA diversity observed in the SRS microcosms, an attempt to study the LaSalle 16S rDNA-DGGE profiles revealed DNA smears where bands could not be differentiated, probably due to the high content and diversity of the DNA in these soils (data not shown). As already described in Chapter 4, *pmoA*-DGGE analysis of the active LaSalle populations revealed three groups composed of highly similar sequences accordingly to band position and BLAST alignments. Two of the groups described uncultured *Methylocaldum* sp. clones from landfill cover soil and the other group was not possible to amplified (Table 5-3). Additionally, several distinct bands were also observed that aligned in BLAST with uncultured bacteria of diverse environments (rice field, upland soils, and rice straw). These bands clustered in the phylogenetic tree within *Methylococcus* and *Methylocaldum* (Table 5-3). Although, profiles among tree types were comparable, differences between planted and non-planted samples were evident. Non-planted soil microcosms exhibited DGGE profiles that varied greatly with time and showed low relative abundance of its components.

At the LaSalle PCE Site, poplar tree profiles shifted greatly with time. In the first sampling (July 2003), only the *Methylocaldum* clones found at the TCE Site were

retrieved and, in the subsequent sampling (July 2004), only uncultured bacteria closely related to the genus *Methylococcus* were observed (Table 5-3). The non-planted soil exhibited the same *Methylocaldum* sp. clone as the poplar tree and a group of bands that described *Methylocystis* sp. (99% similarity). Differences among trees and the non-planted soil could not be determined because some samples were lost during analyses. Overall, a dominance of thermotolerant methanotrophs (*Methylocaldum* and *Methylococcus*); was observed in the LaSalle TCE Site samples and, even though the same genera were found at the LaSalle PCE Site they were not consistently retrieved in all sampling periods as observed in the TCE Site samples (Table 5-3).

Principal Component Analysis (PCA)

PCA the measurements obtained from the culture-dependent methods, microbial counts and enrichment activity, of the different soil compartments evaluated suggested that the variability among different data sets was explained by the phytoremediation setting (Fig. 5-6). The different phytoremediation settings were separated by the second axis (PC2), which explained 25% of the variability (Fig. 5-6A). However, these groups were not completely separated on the first axis (PC1), which explained a higher variability (38%). The LaSalle TCE Site separated completely from the SRS and the LaSalle PCE Site showed an intermediate position between a completely engineered system (LaSalle TCE Site) and a natural revegetated setting (SRS). Also, it was observed that most of the rhizoplane samples from the TCE Site grouped in the negative quadrant of the first axis, which may indicate further differences accordingly to soil compartment analyzed.

The same PCA results were obtained by incorporating into the analysis the culture-independent CH_4 depletion rates of the SIP soil microcosms (Fig. 5-6B). This time the

first axis, which explained 36% of the variability, separated the LaSalle TCE Site from the group between the LaSalle PCE Site and SRS. The second axis, which explained 27% of the variability, separated completely the TCE Site from the SRS, but again the PCE Site did not separate from either the TCE Site or the SRS. The PCE Site grouped in one axis with the SRS data and in the other with the TCE Site.

Discussion

The three studied phytoremediation settings represent different remediation strategies in distinct environments. However, the ability to discern meaningful trends in populations at these sites varied with the method used.

Microbial Abundance

The microbial count methodology, relying on the culturability of the microorganisms, was not sensitive enough to detect differences among tree types, time, or non-planted samples. At the SRS, where soil physicochemical characteristics drastically changed with the soil profile and where nutrients in the vegetated ecosystem were higher in concentration than in the non-planted areas, no differences were assessed in microbial counts with respect to soil profile or presence of plantings. However, because the soils are acidic and frequently experience saturated conditions, rapid leaching of nutrients may occur, thus explaining the variability observed among the pine trees positioned down the slope towards the contaminated seepline outflow. As water saturation increased, rhizosphere soil heterotroph and methanotroph counts decreased while counts of methanotrophs in the roots increased. Methanotrophs in this environment may benefit from the input of oxygen by the plants (Whipps and Lynch, 1983; Walton et al., 1994) and their proximity to the anoxic zones, where CH_4 is being produced by methanogens (Hanson and Hanson, 1993; Gilbert and Frenzel, 1998). These results may

imply a higher biodegradation potential of the saturated environment since methanotrophs proliferate in the roots of the plants in direct contact with the contaminated groundwater.

As expected, the design at the LaSalle TCE Site (PVC pots filled with planting material) constituted a homogeneous environment, characterized by its neutral pH and adequate nutrient content. These conditions reflected microbial counts that slightly varied throughout the study, by seasons, by tree type, or between planted and non-planted pots, suggesting that the planting material used in this remediation system may have acted as the source of microbial inoculum. However, this trend may depend on plant growth and years of establishment of the site (1-2 years at the time of the study). The willow trees showed higher root biomass than poplars; despite samples from these two tree types not showing higher microbial counts, activity, or soil diversity for the willow trees. Overall, methanotroph abundance was higher at the TCE Site, a result anticipated since growth of methanotrophs was not expected at the PCE Site, given their inability to oxidize PCE (Uchiyama et al., 1989; Bowman et al., 1993b).

Activity and Phylogenetics of Enrichments

All phytoremediation settings contained methanotrophs capable of expressing both forms of MMO. However, enrichments in NMS without Cu from rhizoplane samples at the high-contaminant regions of the LaSalle TCE Site were not successful, probably as the result of a negative effect of the high-contaminant exposure in the roots as they are in direct contact with the contaminant during plant water uptake. NMS with Cu enrichments showed higher oxidation rates than NMS, as expected for pMMO-expressing methanotrophs, which possess a lower energy demand and higher affinity for CH_4 (Hanson and Hanson, 1996). Enrichments in NMS with Cu from the SRS showed the

highest oxygen uptake rates in the presence of CH_4, correlating with the higher bioavailability of Cu found at this site, as it is known that pMMO oxidizes CH_4 faster than sMMO (Semrau et al., 1995). Also, the non-planted soil in most of the enrichments showed higher oxidative activity than the plant-related cultures, even though microbial counts were comparable. The higher activity observed in the non-planted samples may be related to a negative effect of high-contaminant exposure, as previously mentioned. The effect being more pronounced at the TCE Site where concentrations are approximately 100-fold greater than those at the SRS. However, rhizosphere samples from deeper soil layers (>30-60 cm), during the November sampling showed higher activity than the non-planted soil implying that methanotroph activity is benefited by the presence of the plant which extents deeper in the soil profile. Phytoremediation plot differences may relate to the type of methanotroph present at the site and their ability to express either oxidative enzyme as TCE and its metabolites have been shown to have a selective effect on particular groups of bacteria and on different types of methanotrophs (Kanazawa and Filip, 1987; Alvarez-Cohen and McCarty, 1991a; Henry and Grbić-Galić, 1991).

Tree type differences were difficult to assessed by this culture-dependent method; however, poplar trees at the TCE Site consistently exhibited higher oxygen uptake rates in the rhizosphere enrichments compare to the rhizoplane, which may be related to a higher exposure to the contaminant and its by-products, known to be toxic to methanotrophs (Fox et al., 1990; Alvarez-Cohen and McCarty, 1991a). However, the same behavior was not observed on willows, which are reported to possess comparable transpiration and contaminant uptake rates as poplars (Snyder and Williams, 2000).

Though, willow trees showed higher fine root biomass that may have contributed to a higher tolerance to the contaminant in the rhizoplane. Comparable rates between soil compartments may suggest higher resistant to the contaminant by the tree type, as with pine and willow trees.

The phylogenetic analysis of all NMS with Cu enrichments revealed distinct community profiles composed of associations of methanotrophs with other bacterial groups, mainly from the phylum Bacteroidetes and uncultured bacterium. The number of methanotrophs retrieved from each culture did not correlate with the microbial abundance assessed for the site neither with activity. However, all detected methanotrophs were type II, mainly from the genus *Methylocystis* that are known to dominate in soil environments with a broad range of pH (Dedysh et al., 2001; Knief et al., 2003). However, since there is an inherent bias with culture-dependent methods, the high concentration of CH_4 used (20%, v/v) may have promoted the dominance of type II methanotrophs known for their low affinity to CH_4 (Hanson and Hanson, 1996). Cultivation bias towards type II methanotrophs has been reported in several studies (Jensen et al. 1998; Baker et al., 2001; Horz et al., 2002). At the SRS and LaSalle PCE Site, a *Methylocystis* strain described as one that rapidly degrades TCE was retrieved, which may confer a high biodegradation potential to these plots. However, this result does not imply that type II methanotrophs are the active populations at these sites.

The fact that not all of the enrichments showed similar oxidative potential or community profiles, even though all of them possessed the same type of methanotroph populations is intriguing. It does suggest that at the SRS, characterized as an oligotrophic environment with low microbial biomass (Nevius et al., 2004), methanotrophs may be a

dominant bacterial group as the major members of their community profiles were methanotrophs. However, this was not the case in the LaSalle TCE Site, even though it showed the highest abundance of methanotrophs.

Activity and Phylogenetics of SIP Soil Microcosms

SIP soil microcosms of each phytoremediation setting showed, even at normalized laboratory conditions and water content, significant differences in CH_4 depletion rates among plots. However, no differences in activity were observed between planted and non-planted samples within the plots. Although, correlating with the higher methanotroph abundance assessed by culture-dependent methods but not with the activity of the enrichments, the LaSalle TCE Site soil microcosms showed the highest CH_4 depletion rates followed by the SRS and the PCE Site. Only by utilizing the SIP technique combined with culture-independent molecular methods it was possible to accurately discerned on the types of active methanotroph populations present at each phytoremediation plot. Also, this protocol was the only to assessed differences between planted and non-planted samples.

Each phytoremediation setting was composed of different active methanotroph communities. At the SRS, the active methanotroph community was dominated by the *Methylocella-Methylocapsa* group, moderately acidophilic methanotrophs that grow between pH values of 4.5 to 7 (Dedysh et al., 2000). This finding correlates with the environmental conditions present at the site and with previous reports of active methanotrophs present in moderately acidic environments (Radajewski et al., 2002). The *Methylocella* group appears to possess high adaptability to environmental conditions as it has been recently reported that one of its strains is the only known exception to obligate methylotrophy, presenting facultative growth on multicarbon sources (Dedysh et al.,

2005). Also, high bioremediation potential may be expected by this methanotroph genus as it only expresses the high oxidative form of MMO (sMMO), which is not regulated by the presence of Cu (Dunfield et al., 2003; Theisen et al., 2005). Therefore, *Methylocella* may be present in environments with higher Cu concentrations than other sMMO expressing methanotrophs. However, no TCE degradation studies have been performed with these strains due to the difficulty of culturing this microorganism in batch (salt sensitive).

At LaSalle TCE Site, the thermotolerant type X methanotrophs (*Methylocaldum-Methylococcus*) dominated the active populations. It is speculated that the planting material because of its origin in mulch piles at high temperatures possessed a dominance of thermotolerant methanotrophs, which have been commonly isolated from this environment (Eshinimaev et al., 2004; Jackel et al., 2005). Therefore, it is proposed that the planting material functioned as the methanotroph inoculum in this plot ("compost effect"). However, the community profile of the non-planted pot was not identical to that of the planted pots. Low relative abundance and higher variability of its components was observed in the non-planted samples. Consequently, the plant must exert some effect that allowed *Methylocaldum* strains persist in the planted pots, especially in the willow clones, which maintained the same profile throughout the study. As mentioned previously, willow clones produced higher surface root biomass than the poplar trees that may have assisted in keeping higher temperatures in the pots compare to the poplars or the non-planted soil. As a result, the poplar trees and the non-planted pots may have been more susceptible to environmental changes at the site.

The changes observed with time in the TCE Site poplar tree community profiles, during the fall sampling may exemplified the effect of temperature over the active methanotroph populations in this plot. Although *Methylocaldum* strains can grow in a wide range of temperatures (moderate thermophiles), some species can sustain lower temperatures than others (Bodrossy et al., 1997). For example, *M. gracile* can grow between 20-47°C, but *M. szegediense* only grows from 37-62°C. Members of the active methanotroph populations of the trees at the TCE Site closely related to *M. gracile* more than *M. szegediense*, which may suggest that conditions at the site are not the optimum for some of these thermotolerant species. Additionally, the *Methylococcus* genus, also retrieved as an active member of the TCE Site, possess extra glycoprotein structures on the outer surface of the cell wall, which apparently provide higher resistance to stress factors, including temperature fluctuations and concentration of solutes (Eshinimaev et al., 2004). In general, type X methanotrophs exhibit structural and functional adaptability to changes in environmental conditions that may explain their presence after two years of establishment of the sites. However, even if type X methanotrophs possess high adaptability, conditions at the site are not optimum for these species (part of the year the soil freezes). Therefore, the question remains to whether these active methanotroph populations detected only 1-2 years after establishment will persist in this plot. Additionally, the degradative potential of these species may be high as *Methylococcus* species are known to express both forms of the MMO enzyme and showed high TCE biodegradation potential (Hanson and Hanson, 1996). However, *Methylocaldum* species do not produce the soluble form of the MMO that shows higher oxidation capacity (Bodrossy et al. 1997).

At the PCE Site, the genus *Methylocaldum* was also found but not as dominant as in the TCE Site, and *Methylocystis* also describe the active methanotroph populations at this plot. These genera along with *Methylosinus* represent commonly found methanotrophs in soils (Knief et al., 2003). Poplar trees planted in the TCE and PCE plots showed distinct methanotroph communities, therefore, methanotrophs appeared to be influenced at this time of the study more by the phytoremediation design than by the plant type.

Principal Component Analysis (PCA)

PCA of culture-dependent and culture-independent measurements suggested that the variability among measurements might be explained by separating the LaSalle TCE Site from both the LaSalle PCE Site and SRS. The LaSalle PCE Site seemed to have an intermediate position between the TCE Site and the SRS data sets. Consequently, the methanotroph community of these phytoremediation plots may be explained by the degree of departure of the design from a natural setting and not because of location or tree type. However, the use of planting material at LaSalle TCE Site seemed to dictate the active methanotroph populations at the time of the study, which exemplified an early stage of plant development. Methanotroph community shifts as plant grow and exerts higher pressure over the rhizosphere environment may be expected. Results of management practices may take time to affect soil microbial communities as soils show an immense capacity for diversity. Also, differences among tree clones have been shown to take time to develop (Girvan et al., 2003).

In conclusion, the results of this study assessed as important variables for the establishment of different types of methanotroph, the degree of soil saturation, contaminant concentration, soil compartment, and the use of planting material. However,

results were not consistent within the traditional methods used or between culture-dependent and -independent techniques. Limitations inherent in cultivation were evident. Methods that consider the active microbial populations were more effective in assessing differences among planted and non-planted samples with respect to microbial community composition, structure, and activity at each plot, and are recommended for future evaluations. However, at the time of the study there was a strong effect of the phytoremediation design in some of the plots evaluated. The early stage of plant development in these plots may suggest that the observed patterns may potentially change with time, and continued monitoring is recommended.

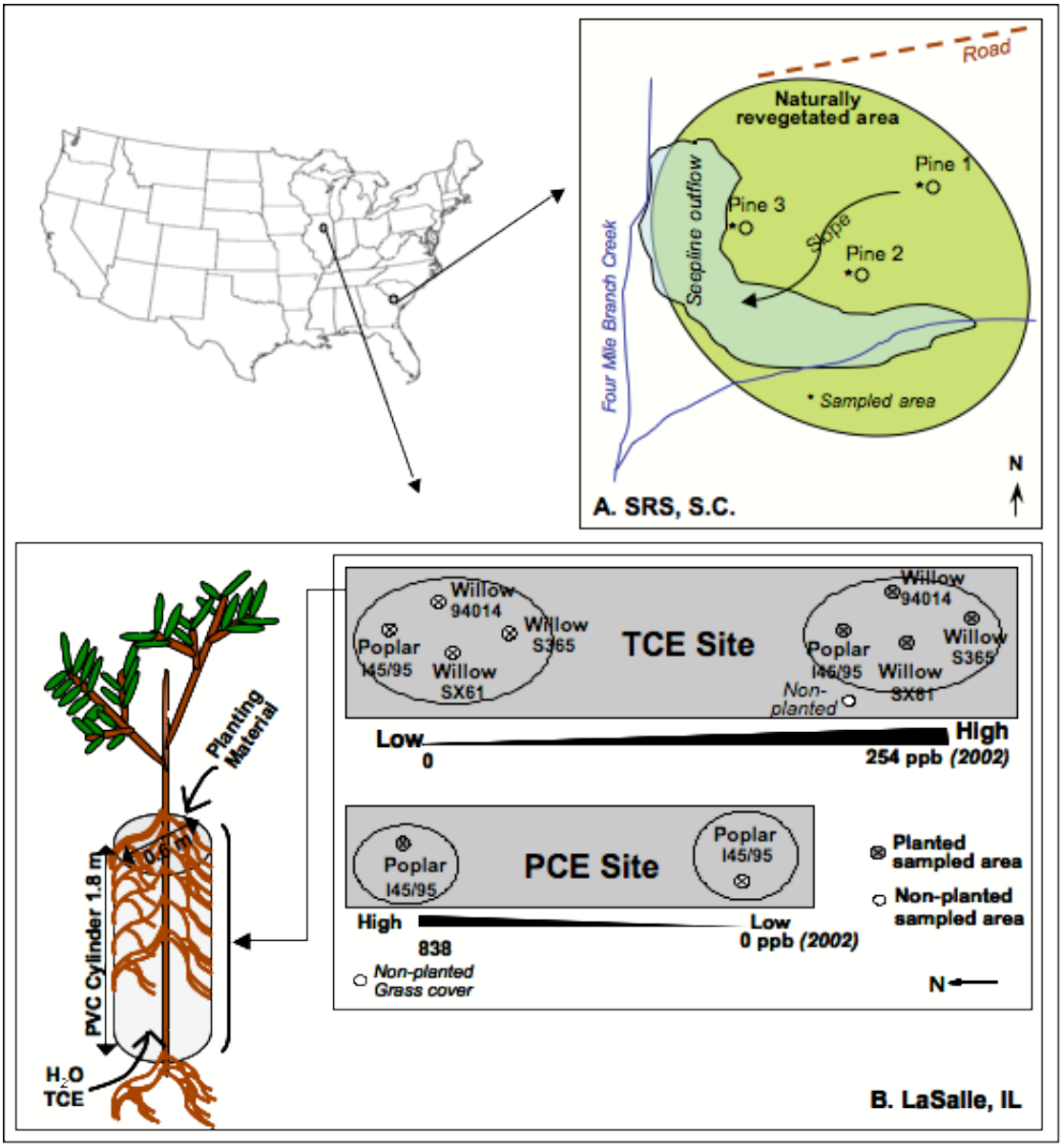

Figure 5-1. Location of phytoremediation sites and diagram of sampling areas at the (A) Savannah River Site (SRS), S.C., and (B) LaSalle, IL.

Table 5-1. General characteristics of the different phytoremediation plots at the SRS and LaSalle sites.

Characteristic	Site		
	SRS[1]	LaSalle	
		TCE Site	PCE Site
Location	33° 15' N; 81° 42' W	41° 20' N; 88° 70' W	
Elevation (m)	53	200-300	
Annual climate data[3]			
• Temperature (°C)	18	11	
• Precipitation (mm year^{-1})	1554	642	
Phytoremediation setting • Plant type	Loblolly pine (mature trees in revegetated area)	Poplar, willow (tree cuttings in PVC pots)	Poplar (tree cuttings in native soil)
• Soil material	Native	Planting material	Native-mulch
Soil type	Fluvaquents, Vaucluse-Ailey Assoc.	sand:soil:bark:peat	Patton-Harco Assoc.[2] and tree bark mulch
Soil pH[4]	5.4	7.6	7.3
Contaminant concentration (ppb)[5]	6-20	4-254	838

[1]Soil and climate data from USDA (1990) and Hunter (2004), respectively; [2]Approximation from the area (USDA, 1996); [3]Year 2004; [4]At the 15-30 cm soil layer; [5]Year 2002.

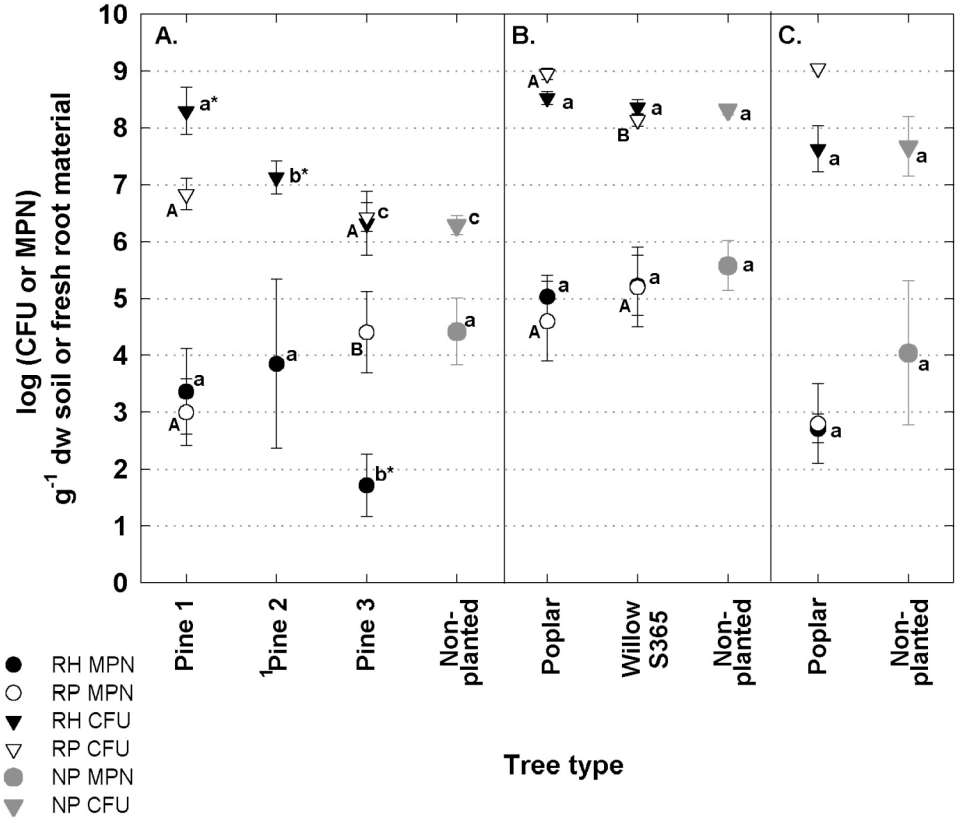

Figure 5-2. Microbial counts per tree type from the different phytoremediation plots at SRS and LaSalle. Methanotroph (circles) and heterotroph (triangles) counts in the rhizosphere (RH- black symbols), rhizoplane (RP- white symbols) and non-planted soil (NP- gray symbols). Data collected in October 2003 and November 2004 at the SRS (A) and LaSalle (B-C) high-contaminant regions, respectively. Values represent average ± SD over the soil profile (SRS: RH-n=15, RP-n=12, NP-n=3; LaSalle: RH-n=15, RP-n=12, NP-n=3). [1]Pine 2 RP counts not determined. Means with the same letter are not significantly different (P<0.05), RH and RP results denoted by lower case and capital letters, respectively. *Significant differences between planted and non-planted samples (Dunnett's test, P<0.05).

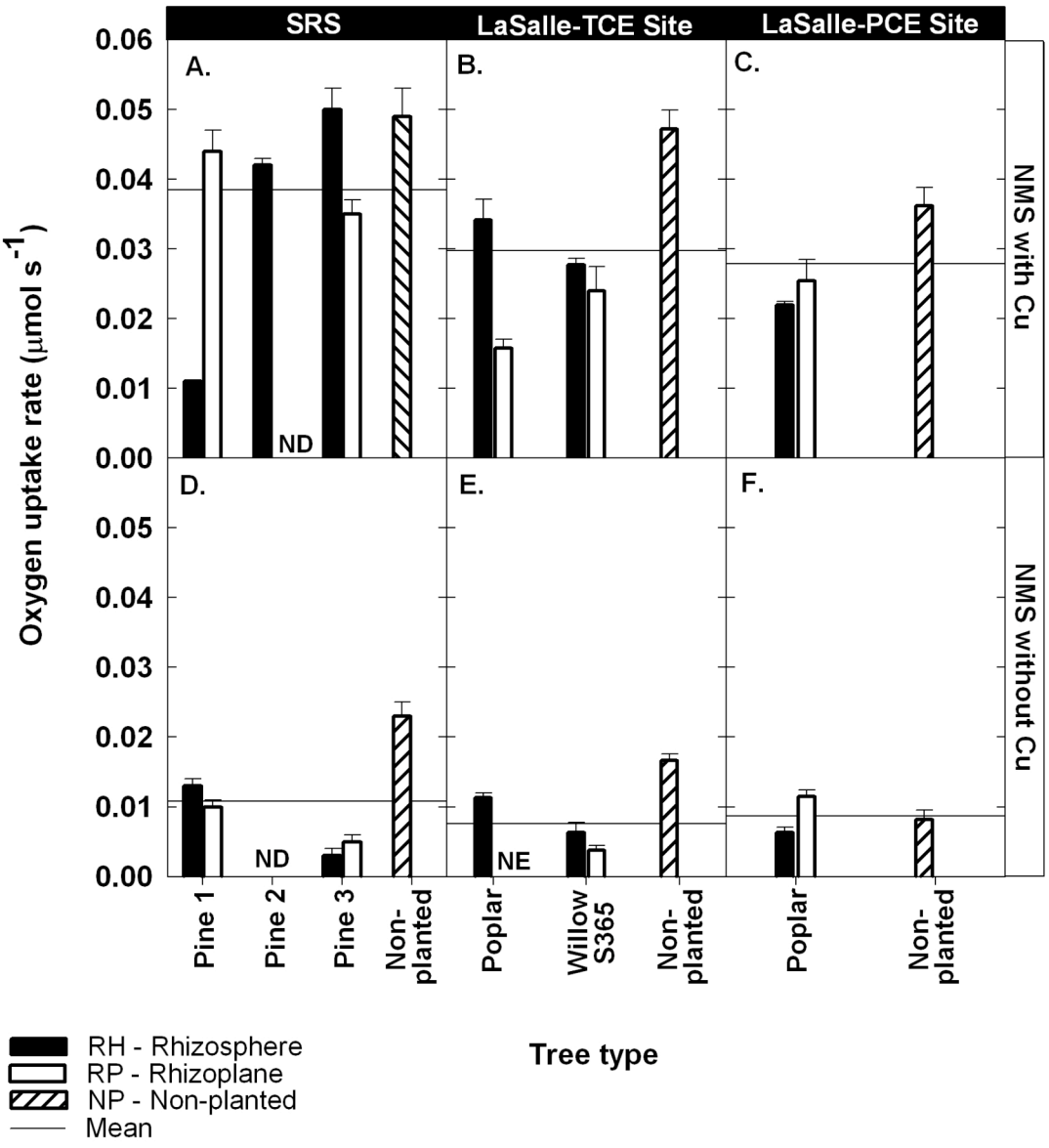

Figure 5-3. Oxygen uptake rates of enrichments from different tree types in the presence of CH_4. Enrichments from the 30-60 cm soil layer in NMS with Cu (A,B,C) and without Cu (D,E,F) at the SRS and LaSalle high-contaminant regions, during October 2003 and November 2004, respectively. ND= Not determined. NE= Not enriched. Values represent average ± standard deviation of triplicate runs.

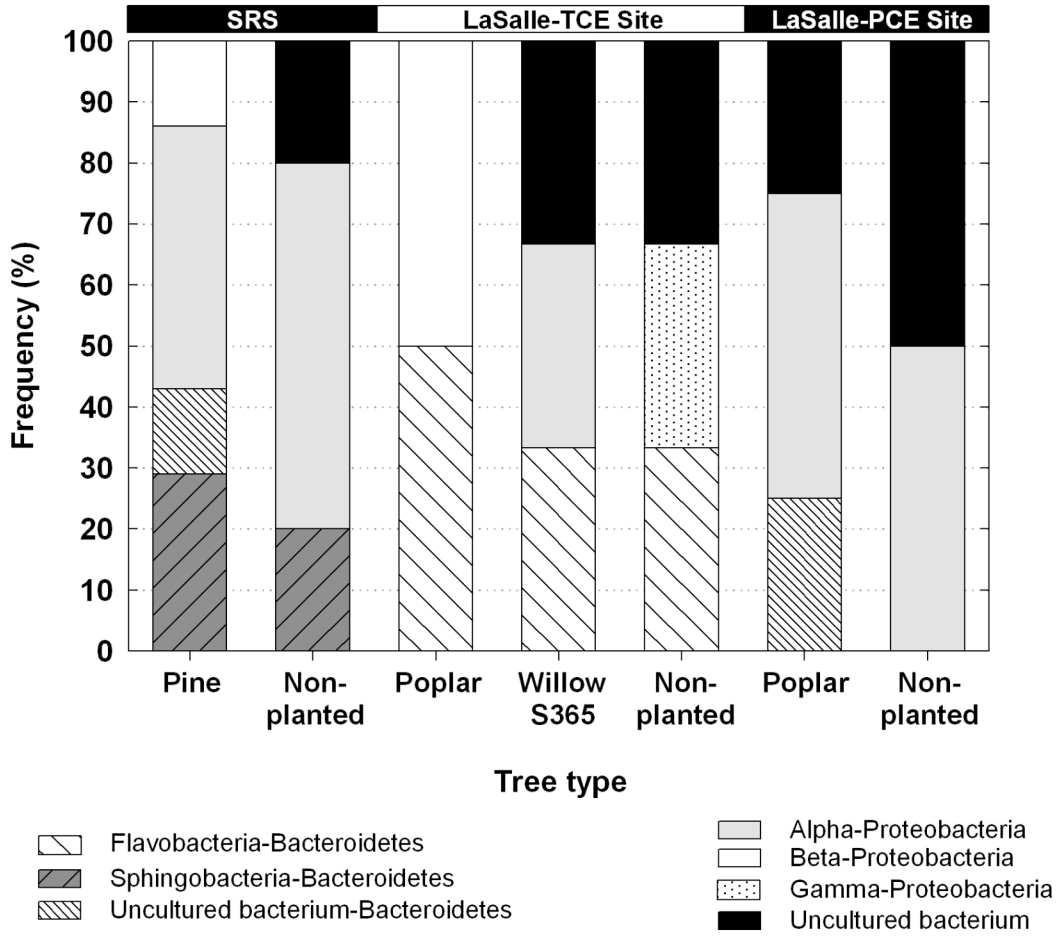

Figure 5-4. Frequency of phylum affiliations per tree type of the NMS with Cu enrichment components. Phylogenetic analysis of the 16S rDNA gene of planted and non-planted sample enrichments according to BLAST alignments. Samples were obtained from the 30-60 cm soil layer during October 2003 and November 2004 at the SRS and LaSalle high-contaminant regions, respectively.

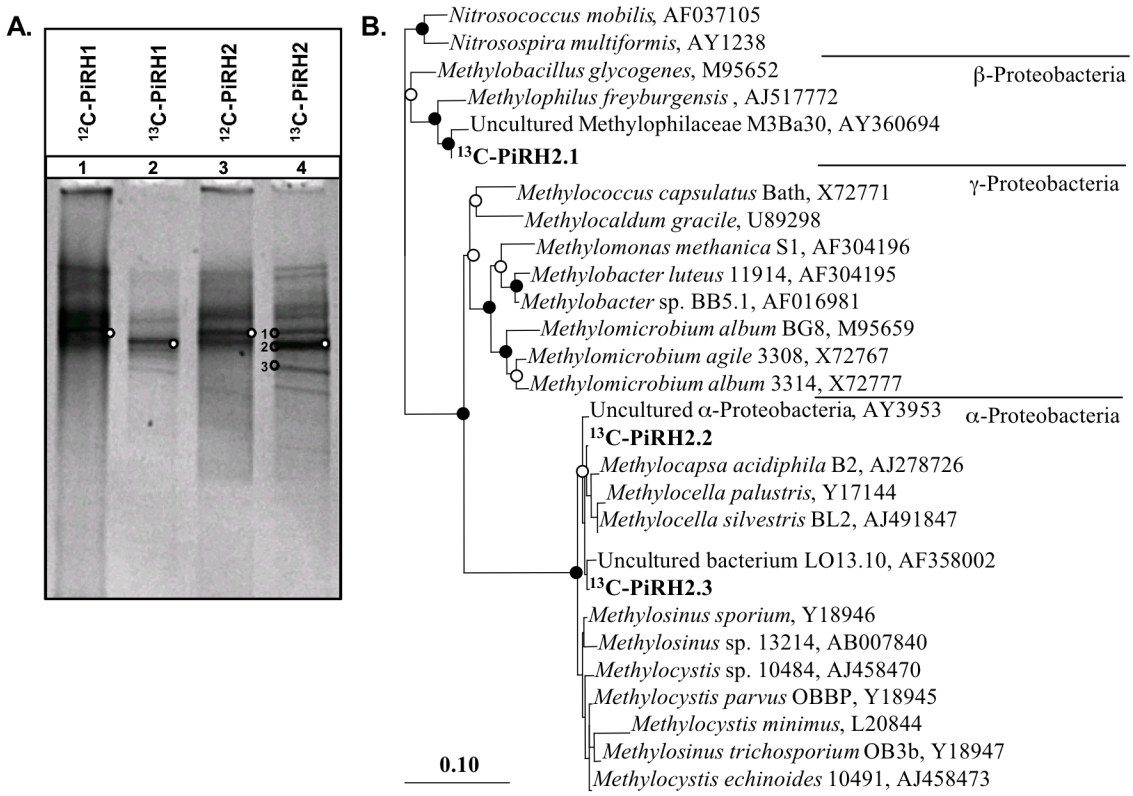

Figure 5-5. Phylogenetic analysis of the SRS SIP soil microcosms. (A) 16S rDNA-DGGE gel of the ^{12}C- and ^{13}C-DNA fractions of pine 1 and 2 at the 30-60 cm soil layer. White and empty circles represent most abundant and sequenced bands, respectively. (B) Rooted phylogenetic tree based on the nucleotide sequence of 16S rDNA partial fragments from the ^{13}C-DNA fraction of pine 2 in relation to type I and II methanotrophs and the highest scoring BLAST aligned sequences. Length of branches is proportional to % dissimilarity (0.1 substitution per nucleotide site). Black (≥90%) and white circles (≥50%) on branches represent % of bootstrap values from 1000 replications. Sequence name= DNA fraction (^{12}C-, ^{13}C-), tree type (Pi= pine), soil compartment (RH= rhizosphere), pine tree number (1,2), band number (1,2,3).

Table 5-2. Summary of phylogenetic assignments (BLAST search) of the *pmoA* gene sequences of the active methanotroph populations (^{13}C-DNA fraction) from the SIP soil microcosms in each phytoremediation plot.*

Methanotroph genus	Phytoremediation plot						
	SRS		LaSalle				
			TCE Site			PCE Site	
	Pine	Non-planted	Poplar	Willow S365	Non-planted[2]	Poplar	Non-planted
Type X							
Methylocaldum	+[1]	+	++	++	+++	++	++
Methylococcus	-	-	-	+	+	+	-
Type II							
Methylocella[4]	++	ND[3]	ND	ND	ND	ND	ND
Methylocystis	-	-	-	-	-	-	+
Unknown group[5]	-	-	+	+	+	-	-

*At the 30-60 cm soil layer and high-contaminant regions.[1]+ = Potential strain or clone; [2]Low relative abundance; [3]ND = Not determined; [4]Determined by 16S rDNA-DGGE analysis; [5]Bands not sequenced.

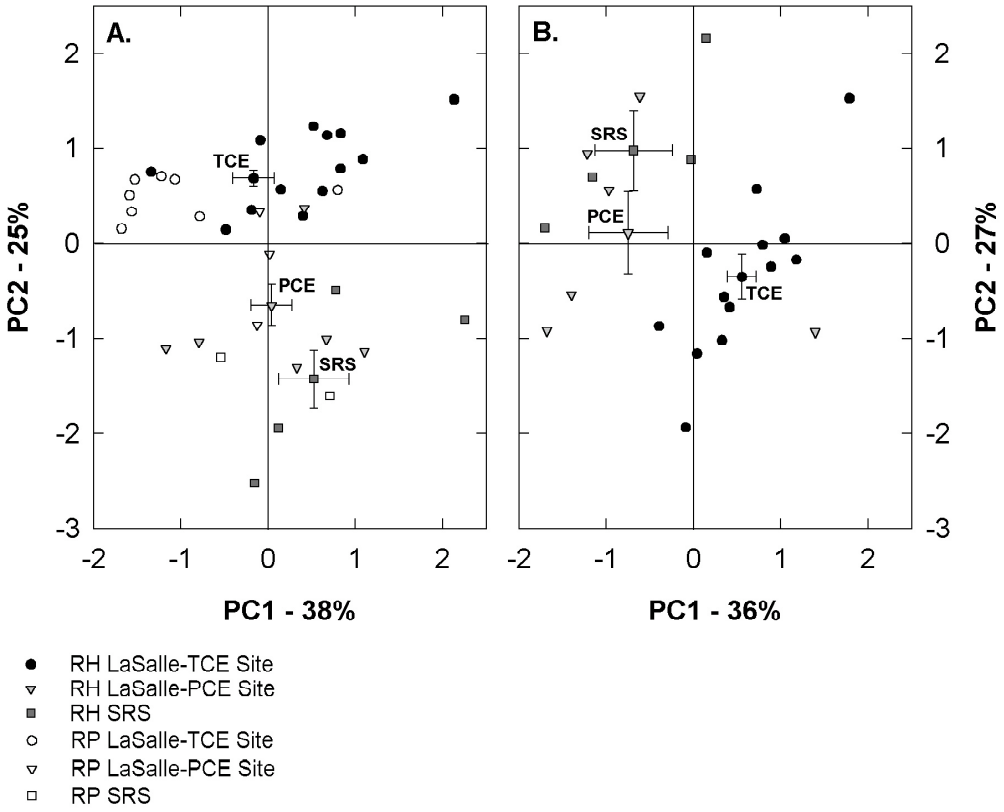

Figure 5-6. Principal component analysis (PCA) of culture-dependent and culture–independent measurements. (A) Data from culture-dependent methods of rhizosphere (filled symbols) and rhizoplane (empty symbols) samples (n=34). (B) Data from culture-dependent and culture–independent methods of only rhizosphere samples (n=23). These methods were used to describe microbial abundance and activity of enrichments and SIP soil microcosms at each phytoremediation plot. Values represent mean of regression coefficients ± standard error.

CHAPTER 6
CONCLUSIONS

Laboratory and field studies were conducted to characterize communities of methanotrophs in the rhizosphere of phytoremediating plants used to remediate soil and groundwater contamination of chlorinated solvents. By using culture-dependent methods and developing culture-independent protocols, specific to the rhizosphere environment, methanotroph abundance, activity, and phylogenetics were determined. Also, variables that potentially affect methanotroph processes in the field were assessed. Ultimately, these results can assist in the development of more efficient remediation technologies.

Laboratory studies with pure cultures served to describe a novel strain of methanotrophs with unique morphological characteristics and known to degrade a wide range of contaminants. Secondly, the ability of methanotrophs to degrade chlorinated and plant-related compounds, the later not previously reported in the literature, was assessed for different types of methanotrophs. Field experiments permitted the development of a protocol using culture-independent stable isotope probing (SIP) methods that holds promise in better characterizing the potentially active rhizosphere methanotroph community. Moreover, due to the combine use of culture-dependent and culture-independent techniques it was possible to confirm the accuracy and relevance of the SIP technique. Additionally, three ongoing phytoremediation plots that varied in their design were characterized accordingly to abundance, activity, and phylogenetics of their methanotroph communities. Field characterizations and laboratory studies revealed the capability of methanotrophic bacteria to adapt to a variety of environments and assessed

the potential factors driving these populations. These findings provide a basis for improving the efficacy of this biological remediation method. The following paragraphs describe in detail the findings of each chapter in this study.

In Chapter 2, a novel methane-oxidizing bacterium isolated from an uncontaminated aquifer was described, *Methylocystis aldrichii* sp. nov. Strain CSC1. This microorganism exemplified the ubiquity of methanotrophs in nature and their capability to degrade xenobiotics, from aromatic to aliphatic compounds. Additionally, no study has reported the structure of Strain CSC1's cell envelope that possesses a unique spiny S-layer. The role of this unique surface feature has not been elucidated to date; however, these results supported the hypothesis that phylogeny and ecology may both play a role in S-layer formation. Overall, this study broadens the observed physiological traits of methanotrophs and brings up topics that could be addressed in future work related to the role of this S-layer in nature and its effect on contaminant degradation potential.

Laboratory studies continued in Chapter 3 with different types of pure methanotroph cultures that were assessed for their ability to oxidize monoterpenes (α-pinene) and determine its effects on TCE oxidation. Methanotrophs expressing either sMMO or pMMO were capable of oxidizing α-pinene, abundant in plants such as pine trees, over a range of environmentally relevant concentrations. Oxidation potential of methanotrophs were affected, either antagonistically or synergistically, in the presence of TCE and α-pinene mixtures. Activity decreased with the type II methanotrophs and increased with the types I and X methanotrophs. These results emphasize the importance of not only assessing concentration levels of contaminants and monoterpenes but also of

measuring the oxidation potentials and diversity of rhizosphere methanotrophs at phytoremediation sites where monoterpene-releasing plants are considered for use. Further research must concentrate on the mechanisms of degradation when TCE and a-pinene are both present in the system. Also, if monoterpenes released by plants do stimulate methanotrophic TCE degradation, then these compounds could be incorporated in the field to potentially increase TCE removal.

In Chapter 4, a protocol using the SIP method specific to rhizosphere studies of phytoremediating plants was developed. By combining this technique with *pmoA*-DGGE analysis the extent and resolution of the SIP method was broadened to detect differences over time and among the relative abundance of the active members of the community. Although it is expensive and requires certain expertise, the power of this method is almost incomparable at the present time. Contrary to traditional methods, which are labor-intensive and dependent on the culturability of the microorganisms, the SIP method allows the assessment of the potentially relevant biodegradating organisms. Consequently, by using this protocol, the phytoremediation practitioner could better monitor and implement the remediation treatment to the needs of the assessed active rhizosphere populations.

Finally, in Chapter 5, the rhizosphere methanotroph community of three different phytoremediation settings was characterized. Culture-dependent and culture-independent techniques were useful in elucidating factors such as soil water content, contaminant concentration, and system design, which greatly affected methanotrophs at the field. However, results between methodologies were not consistent, and limitations inherent to cultivation dependent techniques were evident. Because the ultimate goal of the study

was to assess methanotroph rhizodegradation potential at each phytoremediation setting, the SIP technique combined with molecular community analysis is recommended as the only methodology that effectively described the active methanotroph community of the sites and detected differences between planted and non-planted samples.

Additionally, each of the rhizosphere methanotroph populations in the different phytoremediation settings was distinct from the others. The LaSalle Site presented higher microbial abundance, activity, and diversity of the active methanotroph populations. However, there was a strong effect of the planting material at the time of the study. The SRS methanotroph community showed comparable results to the highly controlled and rich environment of the LaSalle plots, even when microbial diversity and abundance were low. Years of establishment at the SRS plot may have selected for methanotrophs well adapted to the contaminated environment. Phytoremediation plots were separated between natural and completely engineered systems by PCA analysis and, therefore, the system design seemed to be more important than plant species or location in describing the methanotroph rhizosphere community of the different settings. However, because of the early stage of plant development at the LaSalle plots, the observed patterns may potentially change with time.

Future work is needed to effectively assess contaminant degradation rates of the already characterized rhizosphere methanotroph communities at each phytoremediation plot. By assessing the rhizodegradation potential at each plot, the role that this mechanism plays in the overall chlorinated solvent phytoremediation system can be determined. This study contributes to a better knowledge of methanotroph interactions in the rhizosphere of plants and provides a basis for improving the efficacy of this biological

remediation technique. Since this work shows that plant-related compounds affect the oxidation activity of methanotrophs in the presence of TCE, further research is needed to investigate the mechanisms that select and regulate microbial activity in the rhizosphere. Additionally, changes may occur at the recently established LaSalle phytoremediation plots due to long-term contaminant exposure and plant growth and, therefore, continuing monitoring is required to assess such changes in methanotroph populations.

APPENDIX
ADDITIONAL TABLES AND FIGURES

Table A1. Soil characteristics of the SRS and LaSalle phytoremediation plots from high-contaminant regions.*

Site		Sample	Depth (cm)	pH	P	Ca	Mg	Cu	NH_3-N	OM[b]	WC[b]	FC[b]
					mg/kg					%	%	%
SRS		Loblolly pine (Tree #2)	0-15	5.2	1.4	189	22.55	0.25	11.55	7.7	34	-
			15-30	5.4	0.8	42	0.00	0.25	3.62	3.3	20	24
			60-90	4.7	0.5	28	0.00	0.25	0.88	0.5	100*	-
			90-120	4.6	0.6	30	0.00	0.25	0.79	1.0	100*	-
		Control	**15-30**	**4.9**	**0.4**	**66**	**0.00**	**0.19**	**0.82**	**0.8**	**12**	**-**
LaSalle	TCE site	Poplar	0-15	7.6	9.5	1408	160.2	0.14	1.26	8.7	3	-
			15-30	7.6	9.6	1867	223.1	0.06	1.33	7.7	5	36
			60-90	7.5	8.9	1789	216.8	0.08	1.12	12.3	9	-
		Control	**15-30**	**7.6**	**7.7**	**1719**	**175.4**	**0.07**	**1.18**	**8.5**	**14**	**-**
		Planting Material		**7.8**	**10.5**	**1731**	**216.5**	**0.11**	**1.00**	**7.4**	**13**	**-**
	PCE site	Poplar	0-15	6.5	1.3	2503	631.0	0.09	0.81	5.1	12	-
			15-30	7.3	1.1	2551	604.0	0.15	0.93	6.2	9	44
			60-90	7.4	0.7	2281	618.0	0.21	0.49	2.3	11	-
		Control	**15-30**	**7.3**	**0.8**	**2529**	**459.8**	**0.24**	**0.76**	**5.1**	**11**	**-**

*Samples collected in October 2003 and July 2003 at the SRS and LaSalle, respectively.
[b]OM= organic matter; WC= water content; FC= field capacity.

Table A2. Analysis of variance results (*P*-values) for the effect of time and depth on rhizosphere (RH) and rhizoplane (RP) microbial abundance of the LaSalle phytoremediation plots.

Variable	TCE Site				PCESite			
	Heterotrophs		Methanotrophs		Heterotrophs		Methanotrophs	
	RH	RP	RH	RP	RH	RP	RH	RP
Time Analysis[1]								
[TCE/PCE][2]	NS	NS	NS	0.0283	NS	NA	NS	NA
Tree ([TCE/PCE])	NS	NS	0.0002	NS	NS	NA	NS	NA
Time	NS	NS	NS	NS	NS	NA	NS	NA
Time X [TCE/PCE]	NS	NS	NS	NS	NS	NA	NS	NA
Depth Analysis[3]								
[TCE/PCE]	NS/NS/NS	NA	NS/NS/NS	NA	NS/NS/NA	NA	NS	NA
Tree ([TCE/PCE])	-	-	-	-	-	-	-	-
Depth	NS/NS/NS	NA	0.0106/<.0001/NS	NA	0.0175/NS/NA	NA	NS	NA
Depth X [TCE/PCE]	0.0360/NS/NS	NA	NS/NS/NS	NA	0.0462/NS/NA	NA	NS	NA
RH vs RP Analysis								
t-test	<0.0001		NS		0.0531		NS	

NS= not significant (*P*-value>0.05); NA= not analyzed

[1]At the 30-60 cm soil layer – repeated measurement design

[2][TCE/PCE]= Contaminant concentration

[3]At each sampling time (Jul 2003/Jul 2004/Nov 2004) – split-plot design

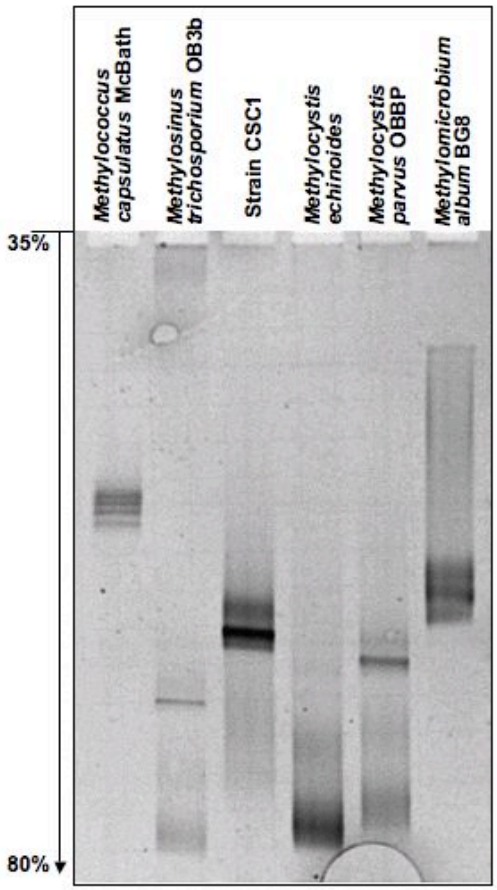

Figure A1. DGGE gel of PCR-amplified partial *pmoA* fragments of different methanotroph types.

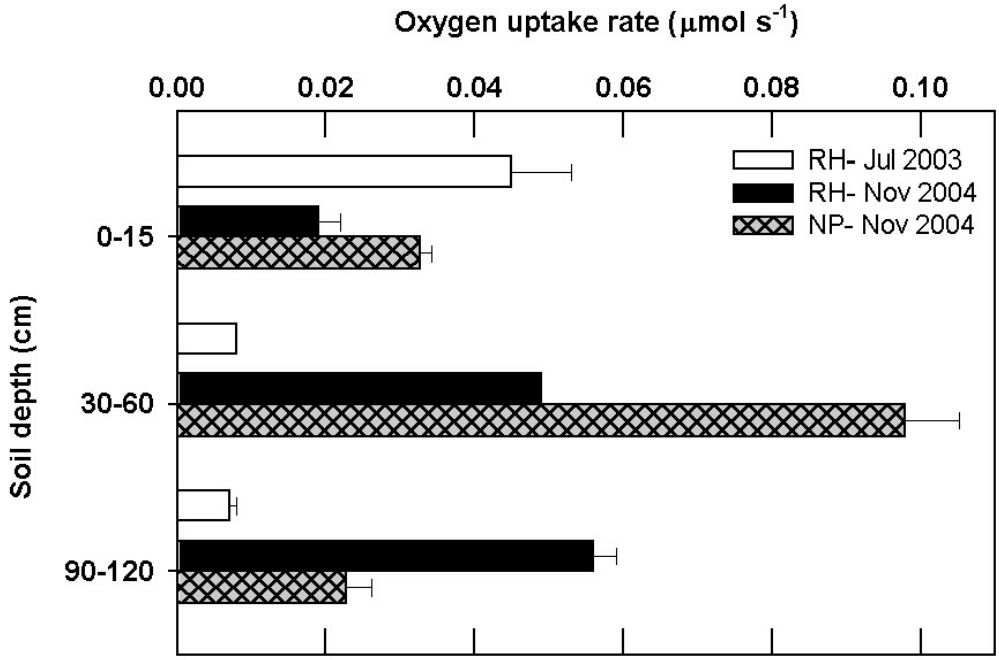

Figure A2. Effect of depth on oxygen uptake rates of NMS with Cu enrichments. Methanotroph activity in the presence of CH_4 for the TCE Site poplar rhizosphere (RH-solid bars) and non-planted soil (NP-crossed bars) at the high-contaminant region and during July 2003 (empty bars) and November 2004 (filled bars). Values represent average ± standard deviation of triplicate runs.

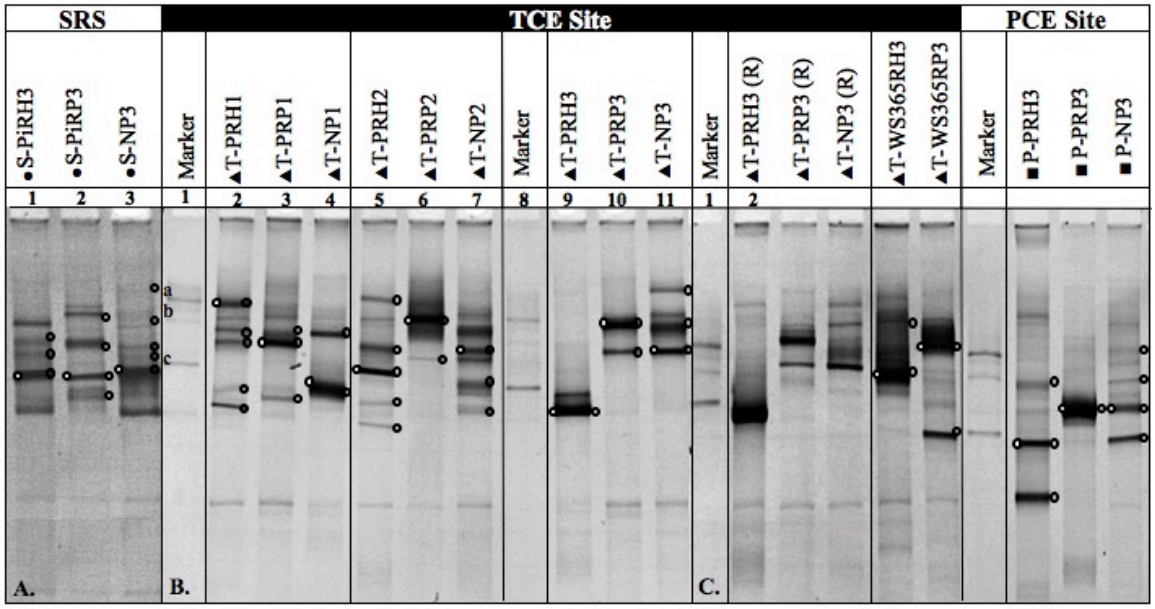

Figure A3. DGGE gels of 16S rDNA partial sequences from NMS with Cu enrichments. Samples collected from the high-contaminant region and 30-60 cm soil layer. Profile name= phytoremediation plot (● S= SRS, ▲T= LaSalle TCE Site, ■P= LaSalle PCE Site), tree type (Pi= pine, P= poplar, W= willow), willow clone (S365), soil compartment (RH= rhizosphere, RP= rhizoplane, NP= non-planted soil), sampling period (1= July 2003, 2= July 2004, 3= October 2003 and November 2004, at the SRS and LaSalle sites, respectively). (R)= replicate profile. White filled circle= most abundant band. Empty circle= sequenced band. Marker (a= *Methylococcus capsulatus* [Bath], b= *Methylomicrobium album* BG8, c= *Methylocystis trichosporium* OB3b).

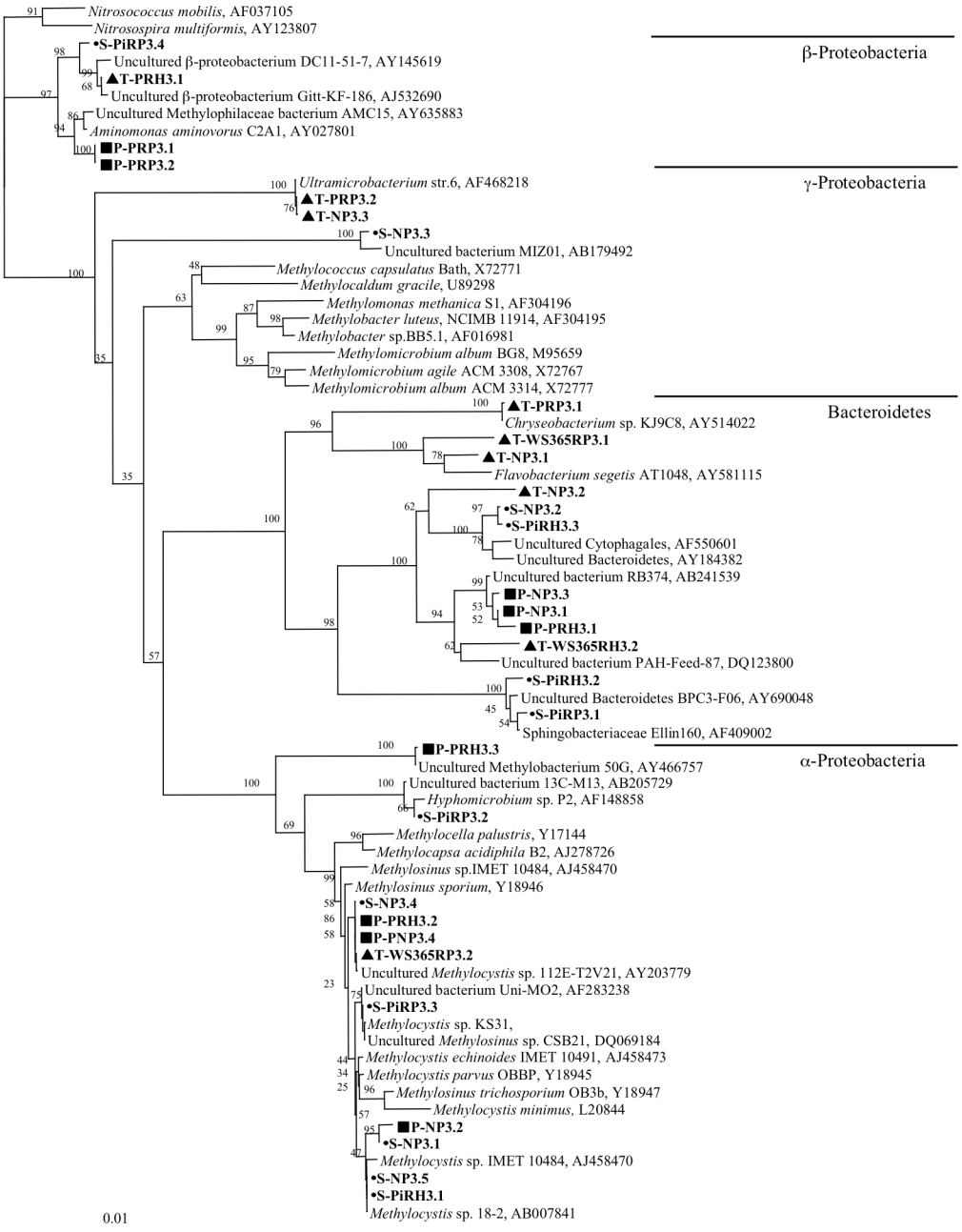

Figure A4. Phylogenetic tree of 16S rDNA partial sequences from NMS with Cu enrichments. Analysis based in relation to extant methanotrophs and highest scoring BLAST aligned sequences. Samples collected from the 30-60 cm soil layer at the high-contaminant regions. Length of branches is proportional to % dissimilarity (0.01 substitution per nucleotide site). Numbers on branches represent % of bootstrap values from 1000 replications. Sequence name= phytoremediation plot (●S=SRS, ▲T=LaSalle TCE Site, ■P=LaSalle PCE Site), tree type (Pi=pine, P=poplar,W=willow), willow clone (S365), soil compartment (RH=rhizosphere, RP=rhizoplane, NP=non-planted soil), sampling period (3= October 2003 and November 2004, at the SRS and LaSalle sites, respectively), band number.

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BIOGRAPHICAL SKETCH

I have always been interested in the relationship between humans and their environment, and how we can offer society sustainable alternatives to utilize the natural surroundings. I began my approach to my professional goal by studying forestry in my home country, Costa Rica, in 1994. As I progressed through my B.S. degree, I realized how important it was to fully understand how natural systems function. That is why I decided to continue my studies and further explore my understanding of the natural systems by pursuing a M.S. degree in biology in 1998 at the University of Costa Rica. When I completed my M.S. degree in 2001, I knew I wanted to apply the knowledge I had acquired in finding ways to remediate the human impacted environment by utilizing natural systems. Therefore, I pursued my Ph.D. degree, at the University of Florida, in environmental engineering sciences in 2001 in the area of bioremediation and phytoremediation, the use of microorganisms and plants to remediate contaminated sites. The skills and experience I have obtained along the way of pursuing my Ph.D. are unparalleled. Now, I look forward to a career in research of remediation technologies to society contamination problems, from the molecular to the ecosystem level approach, in particular, the utilization of natural processes and systems as sustainable solutions.