ABSTRACT

Title: EVALUATING THE IMPACT OF A WETLAND PLANT AND RHIZOSPHERE MICROORGANISMS ON THE FATE OF A MODEL CHLORINATED SOLVENT IN A WETLAND PLANT BIOREACTOR

Ilisa Tawney, Master of Science, 2005

Directed By: Assistant Professor Jennifer G. Becker
Department of Biological Resources Engineering

The ability of wetland plants to impact the removal of *cis*-1,2-dichloroethene (DCE), an intermediate formed during reductive dehalogenation of tetrachloroethene (PCE) and trichloroethene (TCE), either via phytoremediation or rhizosphere oxidation, which enhances aerobic microbial activity, was investigated. To accomplish this goal, a bench-scale bioreactor system was designed to model wetland conditions and evaluate DCE biodegradation. The bioreactor was operated as a continuous-flow, completely-mixed biofilm reactor containing a single *Phragmites australis* individual and root associated microorganisms. Significant removal of DCE in the bioreactor was observed. To elucidate the removal mechanisms, the fate of ^{14}[C]-DCE was determined. The predominant removal mechanism was microbially-mediated oxidation, presumably facilitated by plant-supplied oxygen and growth substrates, followed by phytovolatilization, and incorporation into, and/or sorption to, plant and microbial biomass. Based on these results, wetland plants and their associated microorganisms are expected to contribute to DCE removal in the rhizosphere.

EVALUATING THE IMPACT OF A WETLAND PLANT AND RHIZOSPHERE MICROORGANISMS ON THE FATE OF A MODEL CHLORINATED SOLVENT IN A WETLAND PLANT BIOREACTOR.

By

Ilisa Tawney

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Advisory Committee:
Assistant Professor Dr. Jennifer G. Becker, Chair
Associate Professor Dr. Andrew Baldwin
Associate Professor Dr. Eric Seagren

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Chapter 1: Introduction

The chlorinated solvents tetrachloroethene (PCE) and trichloroethene (TCE) are commonly found groundwater contaminants. Past studies have provided evidence that the sequential dechlorination of PCE and TCE by anaerobic bacteria to ethene, a non-toxic product, is possible (Freedman and Gossett, 1989). However, with each successive dehalogenation, the compounds become less susceptible to reductive dehalogenation due, in part, to kinetic and thermodynamic limitations (Lee et al., 1998). Thus, in practice, intermediates in the anaerobic pathway such as *cis*-1,2-dichloroethene (DCE) and vinyl chloride (VC) often accumulate at sites that are remediating PCE and/or TCE contamination through anaerobic natural attenuation or engineered bioremediation approaches. The accumulation of these intermediates is undesirable because DCE and VC are also considered environmental toxins. Under aerobic conditions, an alternative route for complete detoxification of PCE and TCE is possible. Specifically, the chlorinated ethenes with three or fewer chlorine atoms can be mineralized to CO_2 through cometabolic or, in the case of DCE and VC, metabolic reactions (Coleman et al., 2002). Thus, if both anaerobic reductive dehalogenation and aerobic oxidation could be coupled, it should be possible to completely biodegrade PCE to CO_2.

One environment in which anaerobic and aerobic conditions exist in close proximity is in the wetland rhizosphere, the soil that immediately surrounds the plants' roots and interacts with the plant. Specifically, wetland plants have developed adaptations in response to the anaerobic flooded soils in which they grow. These adaptations

include a porous internal structure that acts as a highly efficient diffusion path to transport oxygen from the atmosphere to the root zone. In the process of oxygen being transported through the roots, some oxygen can diffuse into the rhizosphere.

Groundwater that discharges to wetland systems is frequently contaminated with chlorinated solvents because hazardous waste sites are often in close proximity to surface waters (Lorah and Olsen, 1999). In these situations, the groundwater passes sequentially through anaerobic and aerobic zones in the bulk wetland and rhizosphere soils, respectively. Under these conditions, the potential for complete mineralization of the chlorinated solvents via coupled anaerobic and aerobic biological processes exists.

In addition to microbially-mediated removal of chlorinated compounds in wetlands, phytoremediation of these compounds can occur via several mechanisms including phytovolatilization, transformation and/or incorporation into biomass. However, there are few studies examining phytoremediation with wetland plants, particularly herbaceous species. Thus, the potential impact of wetland plants on the fate of chlorinated compounds is unknown.

The goal of this study was to evaluate the impacts of wetland plants on the fate of chlorinated solvents in contaminated groundwater. Specifically, the ability of plants to affect contaminant levels directly through phytoremediation and indirectly through rhizosphere oxidation, which could enhance microbial biodegradation of

contaminants, were considered. To accomplish this goal, a bench-scale bioreactor system was designed to model wetland conditions and evaluate the biodegradation of a model contaminant, DCE. This information is needed to improve our ability to predict the behavior of these compounds in wetland sites undergoing natural attenuation and to design more effective constructed wetlands for use with flows containing chlorinated solvents.

Chapter 2: Literature Review

Wetlands and constructed wetlands have not been studied at length for their ability to degrade anthropogenic compounds. However, constructed wetlands have been used extensively in the past to treat nutrient and particulate waste streams, such as sanitary waste, agricultural run off, and flows containing heavy metals, including acid mine drainage (Kadlec and Knight, 1996). It is believed that the same processes used to treat these waste streams may have application to the removal of anthropogenic compounds from waste streams. However, a better understanding of microbial degradation processes in wetlands and the influence of plants on these processes is needed before the full treatment potential of wetlands can be realized. In this literature review, a description of known plant and microbially-mediated processes for removal of a model compound, DCE, is provided along with key plant characteristics that could affect these processes.

Plant Response to Wetland Conditions

Wetland environments are ecotones between upland and aquatic systems. Wetlands characteristically contain saturated soils that are rich in organic matter. Metabolism of the organic matter by soil bacteria quickly depletes oxygen in the pore-water (Mitsch and Gosselink, 2000). Oxygen diffusion in water is 10,000 times slower than in air (Drew, 1997); therefore, when the demand for oxygen exceeds the supply, dissolved oxygen is depleted (Mitsch and Gosselink, 2000), giving rise to hypoxic or anoxic conditions in saturated wetland soils.

Anaerobic conditions are undesirable for plants for two reasons, as described by Armstrong (1979): (1) oxygen is required for cell maintenance and growth, especially at the root tip; and (2) soil phytotoxins have a greater accessibility to the plant in anaerobic conditions. As previously mentioned, introduction of oxygen to the rhizosphere via diffusion through flooded soil pores is limited; therefore, plants have developed adaptations that allow them to survive in anaerobic soil. The basis of most of these adaptations is the internal diffusion of oxygen within plants to provide cells with the required amounts of oxygen. Wetland plants have a variety of mechanisms to increase the efficiency of internal oxygen diffusion in response to oxygen deficiencies associated with flooding. Among others, these mechanisms include development of intracellular gas-filled spaces or aerenchyma; development of a barrier to radial oxygen loss (ROL); and changes in root physiology, including root thickness, length of roots and arrangement of roots. The first two adaptations are relevant to this research and are discussed further below.

It is generally accepted that aerenchyma act as a preferential diffusion path for oxygen from shoot to root because they increase porosity and decrease tortuosity within the root. As summarized by Armstrong (1979), previous studies have demonstrated the continuity of the gas space between the shoot and root, and the dependence of the root on the oxygen transfer from the shoot. Aerenchyma formation in certain species may explain why those species fare well in wet soil (Kawase and Whitmoyer 1980). A study by Justin and Armstrong (1987) provided evidence that wetland plants are more prone to having porous structures and the ability to aerate

their roots with atmospheric oxygen. Depending on the species, aerenchyma are either inducible by flooding, as in maize, or formed constitutively without external stimuli, as in rice. However, even in the latter, the overall porosity can be increased above constitutive levels by external stimuli, e.g., flooding (Drew, 1997).

Oxygen transported through aerenchyma can ultimately diffuse radially through, and out of, the root into the rhizosphere, thereby aerating this zone. This phenomenon is known as ROL. Evidence of ROL includes the presence of a thick layer of hydrated ferric oxide, formed by the reaction of reduced iron with oxygen, around the roots of plants growing in reduced soils (Armstrong and Boatman, 1967). ROL can provide several benefits to the plant including protection against phytotoxins that are present in anaerobic soils. Released oxygen oxygenates the rhizosphere and, in turn, phytotoxins, thereby reducing their toxicity while still allowing for uptake of water and nutrients (Armstrong, 1979).

In some cases, a barrier to ROL can prevent oxygen loss; however, not all plants develop an ROL barrier. As summarized by Colmer (2003) it has been suggested that whether or not a plant develops an ROL barrier depends on the effectiveness of longitudinal diffusion and soil redox potential. As the volume of aerenchyma and/or root diameters become larger, there is less need to prevent ROL. Therefore, a plant may develop aerenchyma in response to flooding, but not an ROL barrier, if the aerenchyma provide enough oxygen to the tip even with ROL.

Anatomical and morphological changes, such as the formation of aerenchyma and ROL, are important adaptive mechanisms that plants have developed to overcome oxygen deficiency in the soil. However, the extent to which aerenchyma, ROL, and other physiological changes develop varies with plant species. Therefore, the amount of rhizosphere aeration that occurs as a result of these adaptations is species-specific. Ultimately, these adaptations to flooded soils allow wetland plants, in particular, to provide oxygen to their roots and subsequently release oxygen to the rhizosphere to create an aerobic zone within the bulk anaerobic wetland soil.

Phytoremediation of Chlorinated Compounds

The role of non-wetland plant species, predominately hybrid poplars, in the removal of chlorinated compounds, particularly TCE, has been investigated in past studies. Evidence exists for various fates of TCE within plants, including phytovolatilization, transformation, and incorporation into biomass. Phytovolatilization occurs when the contaminant is taken up by the root system, diffuses upward through the plant and is released through the aerial portions of the plants (leaves and stem). Several lab-based studies have demonstrated that TCE can be released into the atmosphere via phytovolatilization. Although there has been some variability in the extent of phytovolatilization observed in different studies, phytovolatilization appears to be the major removal mechanism in many studies (Burken and Schnoor, 1998; Schnabel et al., 1997). A study by Ma and Burken (2003) used a laboratory reactor system designed specifically to test for phytovolatilization and demonstrated that TCE was taken up by poplars and volatilized to the atmosphere. Most studies have been conducted on hybrid poplars (e.g. Burken and Schnoor, 1998), but there have also

been studies demonstrating TCE phytovolatilization using other types of plants, including edible garden plants (Schnabel et al., 1997) and grasses (Anderson and Walton, 1995).

Phytovolatilization does not transform the contaminant to a non-toxic product; however, there is evidence that some plants can metabolize TCE to compounds that are either released or incorporated into plant biomass. For example, Gordon et al. (1998) detected the TCE oxidative metabolites trichloroethanol (TCOH), trichloroacetic acid (TCAA), and dichloroacetic acid, and the mineralization product CO_2 in tests with poplar cuttings that were supplied with TCE. Testing conducted by Shang et al. (2001) on the fate of TCOH in tobacco plants, revealed a reduction of TCOH in the plant tissue of plants suspended in liquid media containing TCE, indicating further metabolism of TCOH. The authors proposed a pathway in which TCOH would be further metabolized to become incorporated into plant biomass. This pathway included TCOH glycosylation to trichloroethanol glucoside. Trichloroethanol glucoside was detected during the study, but did not persist in the plant tissue once TCE exposure was discontinued, indicating that this compound was metabolized further. The oxidative enzyme cytochrome P450 has been detected in some plants species, but was not detectable in hybrid poplars, although there was transformation of TCE to TCAA indicating that oxidation was occurring (Dietz, 2000). The presence of cytochrome P450 is of significance because it is the primary enzyme involved in TCE oxidative metabolism in mammals, which results in the products described above (e.g. TCOH and TCAA).

The studies discussed above all focused on TCE removal. However, in wetland systems it is expected that TCE will be transformed via microbially-mediated anaerobic reductive dehalogenation in the bulk soil to less chlorinated products, e.g. DCE. Therefore, understanding the fate of these less-chlorinated products is also of great interest. Vroblesky et al. (1999) observed uptake of DCE by several floodplain and wetland tree species in the field, but apparently no studies have been conducted with DCE and herbaceous wetland species. Burken (1996) proposed a relationship between contaminant log K_{ow} values and the percent of contaminant volatilized following uptake by hybrid poplars, based on studies conducted with several volatile compounds including BTEX and TCE:

$$\%\ Volatilized = -0.354(Log K_{ow}) + 1.57 \qquad (1)$$

K_{ow} is the octanol-water partitioning coefficient, which is the ratio of the equilibrium concentrations of a compound between octanol and water and indicates the potential for portioning into soil organic matter. DCE has a log K_{ow} value of 1.86 (EPA, 2005). According to Eq. (1), phytovolatilization of 91% of DCE by hybrid poplars would be expected under the test conditions used by Burken. These results indicate that uptake of DCE by hybrid poplars is likely to occur, but tests have not specifically examined the effects of herbaceous wetland plants on the fate of DCE.

Microbial Biodegradation of Chlorinated Solvents

Under both anaerobic and aerobic conditions, microbially-mediated processes can act to transform anthropogenic compounds in the environment. Some of the most important reactions involved in the biotransformation of chlorinated solvents, and their relevance to this research, are reviewed below.

Anaerobic Reductive Dehalogenation

Reductive dehalogenation involves the addition of two electrons to a molecule and the simultaneous removal of the halogen. When compounds like PCE and TCE undergo reductive dehalogenation, the halogen is replaced with a hydrogen atom in a reaction known as hydrogenolysis. Because it requires an input of reducing equivalents, reductive dehalogenation predominately occurs under anaerobic conditions and requires a suitable electron donor, such as H_2. Sequential reductive dehalogenations of PCE can theoretically result in complete dehalogenation (and detoxification) of the parent compound. Specifically, in the case of PCE, the sequential removal of chlorine atoms can result in the production of TCE, DCE (predominantly the *cis*-1,2 DCE isomer), VC, and, ultimately, ethene (Freedman and Gossett, 1989) (Figure 1-1).

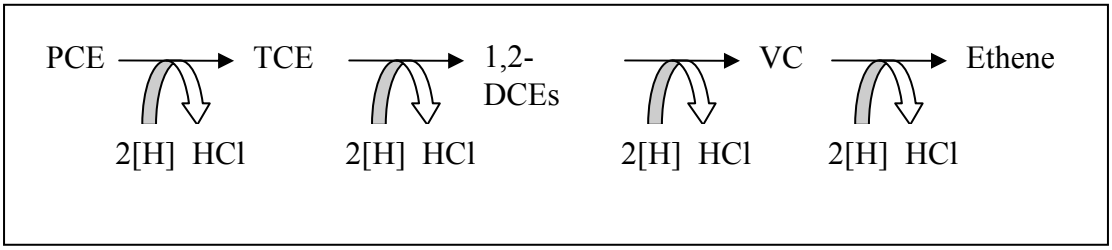

Figure 1-1: Reductive dehalogenation pathway of PCE to ethene (adapted from Freedman and Gossett, 1989)

The complete dechlorination of PCE to ethene is desirable because ethene is non-toxic; however, conversion of PCE to ethene is often not achieved in the field (Yang and McCarty, 1998; Lee et al., 1998). As chlorine atoms are removed, the compound becomes less susceptible to reductive dehalogenation due, in part, to kinetic and thermodynamic limitations. Thus, under anaerobic conditions, PCE is often rapidly

degraded, whereas less chlorinated species (e.g. DCE) are longer-lived (Lee et al., 1998) indicating that the subsequent reduction of the lightly chlorinated compounds is slower than the reduction of the highly chlorinated compounds. Thermodynamic limitations relate to the redox potentials of the reductive dechlorination reactions. For example, the potentials of the redox couples PCE/TCE, TCE/DCE, DCE/VC, and VC/ethene are 0.58, 0.54, 0.36, and 0.49 V, respectively (Vogel et al., 1987). Because of their higher redox potentials, PCE and TCE are somewhat more susceptible to reduction than DCE and VC, which contributes to the frequent accumulation of DCE and VC under anaerobic conditions (Wiedemeier et al., 1999). The accumulation of DCE and VC is undesirable because, similar to the parent compounds, they are considered environmental toxins. In particular, VC is a known carcinogen. One approach to overcoming incomplete reductive dehalogenation is to provide excess electron donor. However, this incurs additional expense, and competition for electron donors between dehalogenating and other organisms and other factors may limit the success of this approach (Yang and McCarty, 1998). Therefore, although the complete detoxification of PCE under anaerobic conditions has been observed, the accumulation of less chlorinated ethenes in practice limits the usefulness of reductive dehalogenation as a stand-alone biodegradation process.

Aerobic Cometabolism

As summarized by Rittmann and McCarty (2001), aerobic oxidation of PCE, which is completely oxidized, is not known to occur and is thermodynamically unfavorable, whereas aerobic oxidation of the less chlorinated ethenes, TCE, DCE, and VC, is known to occur. Oxidation of chlorinated ethenes such as DCE and VC by molecular

oxygen can result in their mineralization to CO_2. Aerobic oxidation of at least some of the chlorinated ethenes can occur via either cometabolic or metabolic processes (Lee et al., 1998). Metabolic reactions lead to energy conservation and/or cell growth, whereas there is no benefit in terms of energy generation or cell synthesis to cells that mediate cometabolism. Cometabolic reactions involving chlorinated ethenes are typically mediated by an oxygenase enzyme system (Arp et al., 2001).

A number of different oxygenase systems can act on chlorinated ethenes, in addition to metabolizing the primary (physiological) growth substrate. These systems include methane monooxygenase (MMO) of methanotrophs, which are found in wetland environments (Lorah et al., 1997). Methanotrophs utilize methane as their primary growth substrate under aerobic conditions. In wetland systems, the methane is produced by methanogens in the bulk anaerobic wetland soil, and from there, it can migrate to the aerobic rhizosphere. Methane is metabolized by MMO to methanol in the presence of oxygen, and subsequent reduction and oxidation reactions regenerate reducing equivalents (NADH) and CO_2 (Figure 1-2).

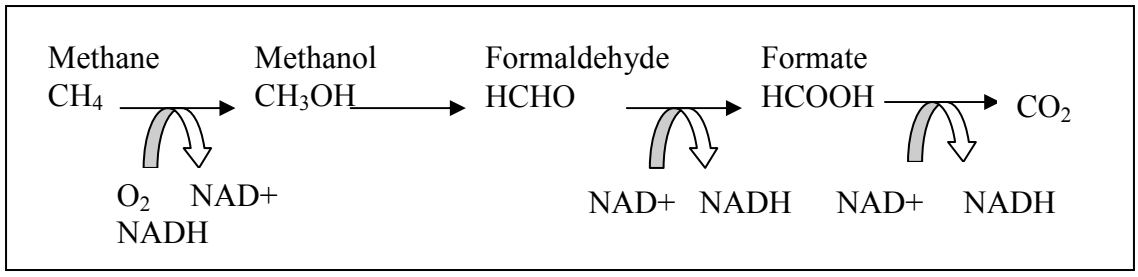

Figure 1-2: MMO metabolism of methane including regeneration of reducing equivalents and production of carbon dioxide

Chlorinated ethenes can also be cometabolized by MMO to their respective epoxides, short-lived unstable compounds that undergo abiotic transformations to various products including aldehydes and acids (Vogel et al., 1987). These products formed from the epoxide are not funneled into core metabolic pathways of methanotrophs resulting in a net loss of energy during cometabolism of chlorinated ethenes (van Hylckama Vlieg et al., 1996). Instead, the products can be metabolized by heterotrophic bacteria to water and CO_2 (Figure 1-3) (Little et al., 1988). Consequently, methanotrophs deplete their reducing equivalent supplies without deriving any benefit from the oxidation reaction.

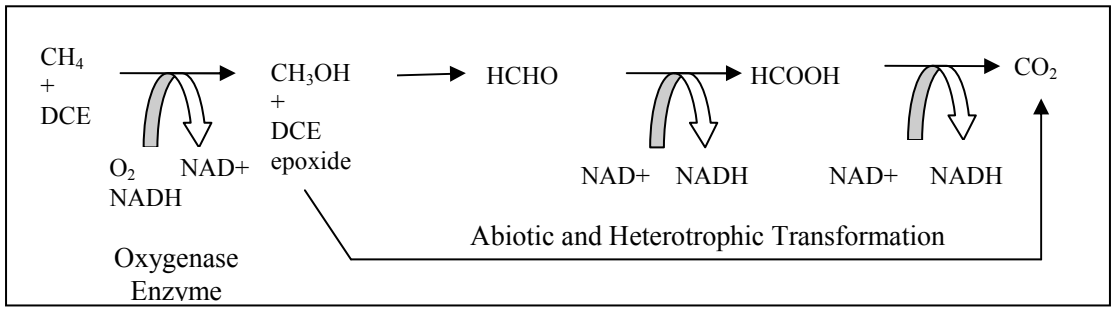

Figure 1-3: Cometabolic transformation of DCE with methane as the primary growth substrate.

Utilizing methanotrophic bacteria to exploit an MMO system for the transformation of chlorinated ethenes may be feasible in contaminated environments that contain methane and oxygen, but there are several aspects of this approach that may limit its application. Potential drawbacks to cometabolic oxidation of chlorinated ethenes include:

- Competition of substrates for enzymes – Competition of substrates for enzymes occurs between the primary growth substrate (e.g., methane in the case of methanotrophs) and the chlorinated ethenes, resulting in reduced rates of degradation. For example, the maximum rate of TCE oxidation for most

organisms is 10 to 100 times less than the oxidation rate of the primary growth substrate (Arp et al., 2001).

- Toxicity – Based on several studies, as summarized by Arp et al. (2001), toxic effects on bacteria cometabolizing chlorinated ethenes have been observed. Observed toxic effects include damage to the oxygenase enzyme system, resulting in enzyme inactivation, as well as to the whole cell. It is thought that the oxidation products of chlorinated ethene metabolism, rather than the parent compound themselves, are primarily responsible for the toxic effects, but this has not been proven.

- Depletion of reducing equivalents – Metabolism of the primary growth substrate regenerates NADH for the bacteria, whereas metabolizing the chlorinated ethenes does not regenerate the reducing equivalents used to transform the chlorinated ethene to an epoxide (Figure 1-3). As a result, the rate and extent of cometabolic transformations may decrease as NADH supplies are depleted (Alvarez-Cohen and Speitel, 2001). The depletion of reducing equivalents can potentially be overcome by adding an intermediate that is converted to NADH in a physiological pathway, e.g., formate (Figure 1-3). For example, in a study by Dolan and McCarty (1995), a methanotroph had a higher capacity for chlorinated ethene degradation in the presence of formate. However, in practice, continuously resupplying a source of reducing equivalents may not be practical.

Aerobic Metabolism

In some cases, aerobic bacteria are able to utilize chlorinated ethenes as a growth substrate (carbon source and electron donor) (Lee et al., 1998). Because the chlorinated solvent is the growth substrate, many of the disadvantages associated with cometabolism are eliminated with metabolic transformations. However, the enzymatic machinery involved in the metabolic transformations is probably similar to that involved in cometabolism. Specifically, an oxygenase enzyme is thought to be involved in the first transformation of a chlorinated ethene to an epoxide, and the chlorinated ethenes are ultimately mineralized to CO_2, as in cometabolic transformation (Figure 1-4) (Coleman et al., 2002; Verce et al., 2002). To date, the substrate range of organisms that are able to grow by aerobic oxidations appears to be limited to chlorinated ethenes that have one or two chlorine atoms. Several species have been shown to grow on VC, e.g., *Pseudomonas aeruginosa* MF1 and *Mycobacterium aurum* L1 (Verce et al., 2002). Recently, strain JS666, a member of the *Comamonadaceae* family that grows on DCE, was isolated (Coleman et al., 2002). Five other strains that belong to the *Bacillus*, *Pseudomonas*, and *Acinetobacter* genera were also identified as being able to grow on DCE (Olaniran et al., 2004). In most cases, the bacteria are able to cometabolize other chlorinated solvents, such as TCE, *trans*-1,2 DCE, VC and/or *cis*- 1,2 DCE, in the presence of their chlorinated growth substrate.

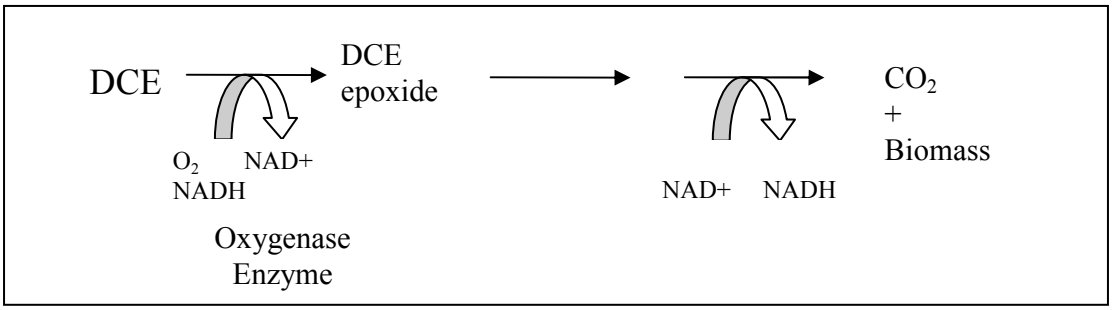

Figure 1-4: Aerobic metabolism of DCE to form biomass and CO_2

It should be noted that little is known about the abundance and significance of aerobic chlorinated ethene degraders in contaminated environments. Coleman et al. (2002) speculated that bacteria able to grow on DCE are rare and may only exist in highly selective environments, because they were only able to isolate two DCE-degraders out of 18 aerobic enrichments despite extended incubations times and the use of inocula from chloroethene-contaminated sites. However, there is growing evidence that aerobic metabolizers are present in various environments. For example, as mentioned above, Olaniran et al., (2004) isolated five strains capable of utilizing DCE isomers as growth substrates from sites contaminated with chlorinated ethenes in Africa. Additionally, other studies have found mixed cultures capable of utilizing DCE without the presence of another growth substrate (Bradley and Chapelle, 2000; Davis et al., 2002). In fact, in a study by Klier et al. (1999), removal of all DCE isomers was observed in mixed cultures from soil that had no previous exposure history to chlorinated compounds without supplementation of exogenous organic nutrients. It is not known how many studies have been devoted to trying to isolate and identify bacteria that can utilize DCE or VC as a growth substrate, but these recent studies indicate that metabolism by pure or mixed cultures does occur in various environments. Thus, the small number of aerobic chlorinated ethene isolates

may mean that not enough effort has been devoted to looking for these organisms (using appropriate culturing techniques), or it could be reflective of their environmental significance. If the latter is true, it could complicate efforts to exploit these organisms for *in-situ* bioremediation.

Coupling Anaerobic and Aerobic Metabolism

Coupling anaerobic and aerobic metabolism offers the potential to enhance the biodegradation rate of organic contaminants with multiple chloro, nitro, or azo groups (Field et al., 1995). In addition to PCE and TCE, examples of environmental contaminants that appear to be well-suited to transformation via sequential anaerobic and aerobic processes include 2,4,6-tricholorophenol, polychlorinated biphenyls, and nitrobenzene (Field et al., 1995). In the case of chlorinated ethenes, reductive dehalogenation is necessary and effective for the removal of the more oxidized highly chlorinated ethenes, while aerobic oxidation can be utilized to effectively metabolize the less chlorinated ethenes that accumulate from reductive dehalogenation. In fact the removal of the lightly chlorinated ethenes, DCE and VC, via aerobic oxidation may sometimes occur at a faster rate compared with biodegradation by anaerobic reductive dehalogenation. A review by Suarez and Rifai (1999) summarized a number of studies examining the degradation of chlorinated solvents under both aerobic and anaerobic conditions. For DCE, the mean first-order removal rates for studies conducted under both field and laboratory conditions were 0.476 and 0.004 day^{-1} for aerobic cometabolism and anaerobic reductive dehalogenation, respectively. The mean first order removal rates for VC were 1.023 and 0.153 day^{-1} for aerobic cometabolism and anaerobic reductive dehalogenation, respectively. Therefore,

exploiting anaerobic reductive dehalogenation and aerobic cometabolism or metabolism in sequence, is expected to result in the complete removal of PCE that occurs more rapidly compared to transformation under entirely anaerobic conditions.

Various studies have been conducted to examine the effectiveness of using sequential anaerobic and aerobic zones for the biological removal of chlorinated ethenes. A study by Devlin et al. (2004) investigated the removal of a mixture of chlorinated compounds, including PCE, from groundwater in an isolated surface section of an aquifer, consisting of an anaerobic zone followed by an aerobic zone. Two sections were hydraulically separated from the bulk of the aquifer. In one section, sequential anaerobic and aerobic conditions were artificially established with reducing agents and biosparging, respectfully. The second section was not modified in any way so that removal via natural attenuation could be monitored. The authors concluded that removal of PCE was significantly better in the sequential treatment section compared to the natural attenuation section. Very little PCE removal was observed within the 339 day test period under natural attenuation conditions, whereas PCE had a half-life of 60 days in the sequential treatment system. In the sequential treatment system, DCE was formed in the anaerobic zone and underwent significant degradation in the aerobic zone (10-15 day half-life). Kao et al. (2003) observed similar results in a flow-through system consisting of several columns in series for treatment of PCE in a 90-day study. TCE, DCE and VC accumulated in the anaerobic zone, but their concentrations were reduced in the aerobic zone. Aerobic degradation was assumed based on the molar PCE recovery, which decreased in each column and by an overall

total of 14%. The results indicated that the initial PCE concentration was not entirely accounted for in the form of TCE, DCE, VC and/or ethene, and, therefore, some oxidation must have occurred. These studies demonstrate the potential benefits of utilizing sequential anaerobic and aerobic zones to increase the extent of chlorinated ethene transformation compared to completely anaerobic conditions.

Bioremediation in Wetland Environments

The studies described above are examples of systems in which sequential or adjacent anaerobic and aerobic zones were mechanically or chemically established. Conversely, in wetland systems, these conditions are naturally established via biogeochemical activity. Interestingly, wetlands are susceptible to contamination with chlorinated solvents, because many hazardous waste sites at military installations and at industrial facilities are located near surface-water bodies where wetlands are a dominant part of the landscape (Lorah and Olsen, 1999).

The concept of using wetland environments for removal of chlorinated ethenes has been examined in field- and bench-scale studies. Work by Lorah and Olsen (1999) examined the natural attenuation of TCE and 1,1,2,2-tetrachloroethane (PCA) in a contaminant plume discharging from an aerobic aquifer through wetland sediments. Results from their study provided evidence that both TCE and PCA were completely mineralized through pathways that included formation of DCE and VC, as a result of reductive dehalogenation of the parent compounds. In addition to transformation of DCE and VC via reductive dehalogenation, aerobic biodegradation of these intermediates at the root interface also appeared to be possible, based on results from

a previous study by Lorah et al. (1997). The earlier study demonstrated the removal of DCE and VC under aerobic conditions using wetland soil in batch microcosms. The authors observed that the rate constants for methanotroph aerobic biodegradation of *cis*-DCE, *trans*-DCE, and VC (0.05 – 0.09, 0.09 – 0.13, and 0.19 – 0.21 day^{-1}, respectively) were as high as TCE and PCA degradation under anaerobic conditions (0.045 – 0.131, and 0.25 day^{-1}, respectively), leading the authors to conclude that the production of DCE and VC by the anaerobic biodegradation of TCE and PCA could be balanced by their aerobic biodegradation in locations where oxygen is available, e.g., at the sediment-water interface and near plant roots. Further, the contribution of aerobic biodegradation to the overall removal may have been significant, based on observations of extensive root systems in the field and evidence of methanotrophic activity.

A bench-scale study was also conducted to examine the fate of TCE under wetland conditions (Bankston et al., 2002). The batch experimental system included wetland soil with indigenous bacteria and the wetland plant, broad-leaf cattail (*Typha latifolia*). Following the addition of radiolabeled TCE, a mass balance on ^{14}C demonstrated the production of $^{14}CO_2$ from [^{14}C]-TCE and the uptake of radiolabeled carbon into biomass after 20 days. This study provides more definitive evidence for detoxification of chlorinated ethenes in wetland systems via oxidation.

A preliminary constructed wetland design approach has been developed specifically for the removal of chlorinated solvents, as summarized by Kassenga et al. (2003).

The approach was designed to have the following treatment stages: (1) after water is pumped into a wetland bed in an upflow mode, chlorinated solvents undergo incomplete reductive dehalogenation in the anaerobic zone at the base of the wetland; (2) as water moves upward, chlorinated ethenes are sorbed onto the organic soil, which results in longer contaminant detention times; and (3) within the plant root zones, the less chlorinated ethenes pass through an aerobic area populated with methanotrophic bacteria. A bench-scale study utilizing a column reactor containing soil and wetland plants was developed to investigate the feasibility of using this type of design (Kassenga et al. 2003). DCE and VC were supplied to the bottom of the column, and the concentration of DCE and VC at various depths was monitored. Results showed that as the distance above the core bottom increased, first DCE levels decreased, then VC was subsequently produced, and finally VC levels decreased. Although there were some operational problems, including breakthrough, the authors concluded that treatment wetlands may be a technically and economically viable option for treatment of groundwater contaminated with chlorinated solvents.

In the two bioreactor studies discussed above the removal of the chlorinated compounds was attributed to microbial activity; however, no tests were conducted with plants only to quantify the effects of the plants on the contaminant removal. For example, the reactor system used by Kassenga et al. (2003) was not a fully closed system and, thus, was not designed to ascertain the plant contribution to DCE removal. In studies that utilized a closed two-phase experimental system, often the aqueous and/or gas phase was maintained in batch mode. Another shortcoming of

many of these studies was the inclusion of a headspace in the aqueous root chamber, thereby allowing considerable abiotic volatilization of the chlorinated compound to occur (e.g. Bankston et al., 2002). Based on the results from these reactor designs, it is difficult to make predictions about the partitioning and transformation of chlorinated ethenes in a real wetland rhizosphere, in which the aqueous-phase contaminants would be introduced more or less continuously and air flows freely past the leaves and stem.

Overall, the studies discussed above suggest that wetland environments provide suitable conditions for the complete microbially-mediated biodegradation of chlorinated solvents via coupled anaerobic and aerobic conditions and also offer the opportunity for removal of these contaminants through phytoremediation. However, the processes controlling complete mineralization and/or transformation of these compounds are not understood, which limits our ability to predict and enhance these processes. The purpose of this research is to extend our understanding of these processes.

Chapter 3: Hypothesis

This research project was designed to evaluate whether wetland ecosystems could provide ideal conditions for the biological removal of chlorinated ethene intermediates formed during the reductive dehalogenation of PCE and TCE, as groundwater or waste streams impacted by these contaminants move through the rhizosphere. Specifically, it was hypothesized that the plants may affect contaminant levels indirectly by generating an aerobic rhizosphere habitat that promotes the activity of aerobic bacteria capable of degrading the intermediates or directly, by facilitating removal via phytovolatilization, transformation to CO_2 or other metabolites, or incorporation into plant biomass. DCE was selected as a model compound for use in this study because DCE often accumulates during biodegradation of PCE and TCE in anaerobic systems, and microbial biodegradation of DCE under aerobic conditions has been observed (Coleman et al., 2002).

Chapter 4: Goals and Objectives

The goals of this research were to obtain information that can be used to design more efficient and cost-effective *in-situ* wetland treatment methods, as compared to existing engineered systems (Lorah and Olsen, 1999), and improve our ability to predict the fate of chlorinated ethenes in engineered and natural attenuation systems. In order to test the research hypotheses and achieve the research goals, the experimental approach was designed to achieve the following specific objectives:

1. Design an experimental bioreactor system that can be used to evaluate the ability of wetland plants to support the transformations of chlorinated ethenes.
2. Construct and characterize a prototype of the bioreactor.
3. Use the bioreactor to characterize the fate of a model contaminant, [^{14}C] DCE, fed to the root zone of the bioreactor.

Chapter 5: Experimental Approach

Conceptual Model of PCE Degradation in Wetland Soil and Plant Rhizosphere

A conceptual model of how PCE biodegradation in anaerobic and aerobic soil regions occurs, as groundwater or waste streams impacted by these contaminants moves through wetland soils, is depicted in Figure 5-1. As previously described, aerobic oxidation of PCE is not possible. Therefore, PCE degradation must be initiated in the anaerobic zones and involves reductive dehalogenation to TCE and DCE. Thereafter, various potential fates are possible for DCE in the conceptual model. Microbial biodegradation via reductive dehalogenation to VC and finally ethene under anaerobic conditions is possible, although degradation to CO_2 via aerobic microbial oxidation in the plant rhizosphere is expected to occur to a greater extent. Oxidation of the DCE formed may be the result of either cometabolic or metabolic processes. It should be noted that aerobic oxidation of TCE and VC are also feasible biodegradation processes. Aerobic oxidation reactions in the rhizosphere are possible due to efficient internal oxygen diffusion through plants from the shoots to the roots, and subsequent loss through the roots into the rhizosphere. Additionally, plants can act upon DCE via phytovolatilization, transformation to CO_2 or other metabolites, or incorporation into biomass.

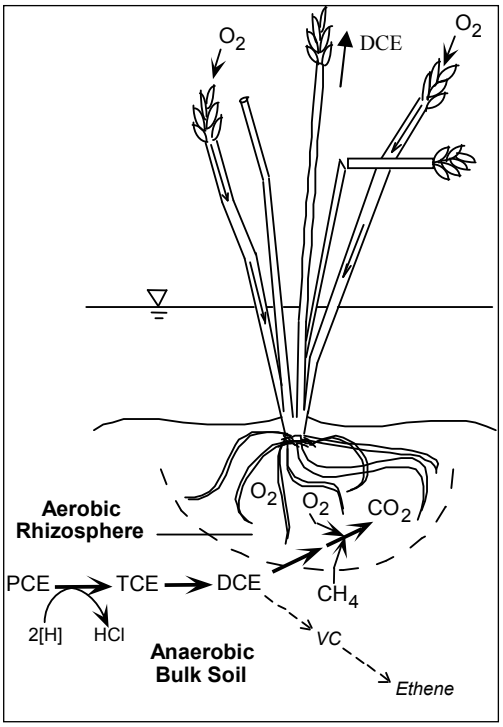

Figure 5-1: Conceptual model of PCE degradation in a wetland environment.

Development of Prototype Bioreactor and Characterization of [^{14}C] DCE fate

In order to test the hypotheses, a physical model of a wetland plant – rhizosphere – soil bacteria system was developed to evaluate the fate of DCE in this type of system. DCE was selected as the model contaminant because it often accumulates following anaerobic reductive dehalogenation and is susceptible to aerobic oxidation, as previously described. Radiolabeled DCE was added to characterize the fate of DCE in the model system and determine the extent of aerobic DCE metabolism. The bioreactor consisted of rhizosphere and foliar chambers that are separated so that the only source of oxygen in the rhizosphere is provided by oxygen transport through the plant, as previously described. The bioreactor was operated in a continuous-flow mode. [^{14}C]-DCE-amended media was supplied to the rhizosphere, and air was continuously drawn through the foliar chamber. Compared to a batch system,

operating the reactor in a continuous-flow mode made the mass balance calculations for evaluating the substrate rate more straight-forward. Further, the flow-through reactor is more representative of wetland conditions in which water is continuously moving and providing a steady supply of contaminants.

Chapter 6: Material and Methods

Bacterial Culture Maintenance

Strain JS666 was obtained from the laboratory of Dr. Jim Spain (Air Force Research Laboratory, Tyndall AFB). The initial culture was grown up on ¼-strength Tryptic soy agar (TSA) plates. Media and solid materials were sterilized by autoclaving at 120°C for 15-20 minutes, unless otherwise noted. Individual colonies were then inoculated into 50 mL of autoclaved minimal salts medium (MSM), which consists of in (g/L) K_2HPO_4 (2.27), KH_2PO_4 (0.95), $(NH_4)_2SO_4$ (0.67), and 0.1 mL of a trace metals solution containing (g/L): $Na_2ETA{\cdot}2H_2O$ (6.37), $ZnSO_4{\cdot}7H_2O$ (1.0), $CaCl_2{\cdot}2H_2O$ (0.5), $FeSO_4{\cdot}7H_2O$ (2.5), $NaMoO_4{\cdot}2H_2O$ (0.1), $CuSO_4{\cdot}5H_2O$ (0.1), $CoCl_2{\cdot}6H_2O$ (0.2), $MnSO_4{\cdot}H_2O$ (0.52), $MgSO_4{\cdot}7H_2O$ (60.0), adjusted to pH 7.2 with 10 M NaOH, and 3 µL 97% DCE (Fisher) to yield a nominal concentration of 70 mg/L (Coleman et al., 2002). Liquid cultures were established in 160-ml serum bottles that were sealed with sterile Teflon-faced butyl septa and aluminum crimp caps and are referred to here as first-generation cultures. All cultures were wrapped in foil and maintained at room temperature (20-25°C) on a platform shaker (90 oscillations per minute, Eberbach). The cultures were re-supplied DCE whenever it was depleted. 0.5-mL headspace samples were obtained from the serum bottles for measurement of DCE concentrations using gas chromatography (described below). After three to five additions of DCE, degradation of DCE slowed or stopped (N. Coleman, 2004 and this study). Therefore, after degradation of several DCE additions, air was added to each culture by first removing the septum and then

flushing the headspace with filter-sterilized (0.2 μm, Air Vent, Pall) lab air with a sterile cannula for five minutes. Typically this was sufficient to restart DCE degradation. However, it was also observed that after several additions of DCE had been degraded, the pH decreased to 6.5, presumably due to the accumulation of HCl produced by the dechlorination reactions. Therefore, when the addition of air alone was not sufficient to restart DCE degradation, the pH was adjusted to 7.2 by adding 3.25 mL of 0.1 M NaOH. These two actions were able to restart DCE degradation over several months.

Several quality control measures were undertaken to help maintain the purity and genetic characteristics of the stock cultures sent by Dr. Spain. First, in order to confirm that JS666 was the dominant organism in solid and liquid cultures, samples of these cultures were examined under a phase contrast microscope and compared to a physical description of the bacteria and a TEM image of JS666 provided by N. Coleman. Second, samples of both the solid and liquid cultures were used to prepare long-term, frozen glycerol stocks of JS666. JS666 was harvested from liquid cultures by collecting 2 mL of the suspended culture and centrifuging it (4000 rpm) for 20 minutes to obtain a pellet. Solid culture samples were obtained by scraping cells from one TSA plate. The collected cells were suspended in 0.4 mL of 20% glycerol stock solution prepared using JS666 MSM for the liquid cultures and ¼-strength Tryptic soy broth (TSB) for the solid cultures. 0.2 mL aliquots of the glycerol stock were transferred to cryogenic tubes and stored in a -80^{o}C freezer. The viability of JS666 in the glycerol stocks was tested by plating with 0.1 mL of glycerol stock on

spread plates one week after storage. In all cases, JS666 colonies formed on the plates.

Evaluation of Biofilm Formation

Second generation liquid cultures (50 mL MSM) were prepared in triplicate to evaluate the ability of JS666 to form a biofilm. Each second generation culture contained enough 2-mm soda lime glass beads to cover the bottom of the serum bottle (approximately 7 g), along with an inoculum of 0.5 mL of first generation culture. After three additions of DCE were degraded, the liquid MSM was aseptically removed, and the beads were gently rinsed with 15 mL of sterile MSM twice to remove any of the original MSM and, more importantly, any suspended biomass. Fresh sterile MSM was then added, and the bottles were resealed and respiked with DCE.

Bacterial Protein Analysis

Protein concentrations of the JS666 batch cultures were determined using a modified Bradford colorimetric method (Bradford, 1976). Culture fluid (500 µL) was mixed with 50 µL of 4.4 N NaOH and heated for 30 minutes at 80^{o}C to affect cell lysis. Following cooling to room temperature, the solution was neutralized with 50 µL of 4.4 N HCl. Each sample was centrifuged (14,000 x g, 10 min) to remove cells. 450 µL of the supernatant was mixed with 450 µL DI water and 900 µL of the Bradford Reagent (Sigma Chemical Co.). The absorbance was measured at 595 nm with a spectrophotometer (HACH, DR/4000, Loveland, CO, USA) and compared to a

calibration curve prepared using bovine serum albumin standards that were treated identically.

Bacterial Plate Counts

Prior to the addition of a batch of JS666 liquid culture to the reactor, a plate count was conducted to determine its bacterial density, as follows. First, 0.1 mL of culture was mixed with 9.9 mL of dilution water (1.25 mL phosphate buffer solution (pH 7.2) diluted to 1 L in DI water). Six serial dilutions were then prepared from this initial dilution, and 0.1 mL of each dilution was plated on a ¼-TSA plate. The plates were incubated at 19^{o}C for three days. The number of colony forming units (CFU) on each plate that contained between 30 and 300 colonies was counted.

Plant Growth

Individuals of common reed (*Phragmites australis* (Cav.) Trin. ex Steud.) that conformed to the bioreactor were grown from samples collected from the environment. The following procedure was developed for collecting and preparing plant material for the reactor. Samples were dug from a wetland site located adjacent to a stormwater retention pond on the University of Maryland campus. These samples were separated from the overall rhizome structure, so that each individual retained a 10 to 15 cm section of the rhizome, from which an extensive root structure extended. The shoots were cut just above the roots and potted in sterile vermiculite (autoclaved 120^{o}C, 60 min). The plants were grown in a walk-in climate controlled environmental chamber (Environmental Control Chambers, Chagrin Falls, Ohio) as follows: The pots were placed in plastic containers, which were filled with several

inches of DI water to maintain saturated conditions. In addition, the humidity level was maintained at 80% to minimize water loss. The environmental chamber was programmed to simulate natural spring to early summer growing conditions in the Mid-Atlantic region for the wetland plants. The chamber was set to provide a photoperiod of twelve hours (average light reading 380 μmol/s m^2 μA) and ten hours of darkness with an hour of gradual light variation between each period. In addition to providing an ideal photoperiod, the chamber temperatures, 30°C during the day and 20°C during the night, presumably resulted in maximum growth. Sterile growth solution, MSM, was added to the pots twice a week. The plants were maintained on MSM, rather than the more typically-used Hoaglands media (Hoagland and Arnon, 1938), because MSM was provided to JS666 and plants maintained in the flow-through reactor (described below). The health and growth of the plants provided with MSM or Hoaglands media was compared to confirm that MSM did not negatively impact the plants before using it to maintain the plants (data not shown).

Prior to use in the bioreactor, the roots of appropriately sized plants were typically treated to reduce levels of indigenous bacteria. The following root sanitization method did not negatively impact plant health and involved five root treatments: (1) shaking for 30 minutes in 0.05 M (pH 7.2) phosphate buffer solution to prevent hypochlorite uptake in the next step (Okon et al., 1977); (2) exposure to a 10% bleach (~5% hypochlorite) solution for 3 minutes; (3) a sterile DI water rinse; (4) shaking for 10 minutes in sterile 0.05 M phosphate buffer solution (pH 5.0) to remove residual bleach; and (5) shaking for 30 minutes in 0.1 M (pH 7.2) phosphate buffer solution to

neutralize pH. All solutions were sterilized by autoclaving. Shaking was conducted with a rotary platform shaker (120 RPM, Baxter S/P® Rotator V). The last four steps were provided by A. Grybauskas (personal communication, 2004).

Design and Construction of Prototype Bioreactor

In this research project, an experimental bioreactor system was designed to characterize the effects of a wetland plant on microbial biodegradation of DCE in a model wetland rhizosphere. A schematic of the final design is shown in Figure 6-1. All components were constructed out of materials that are chemically compatible with DCE including glass, brass fittings (Swagelok), and Teflon tubing covered with Viton tubing to minimize abiotic loss of DCE and oxygen diffusion, respectively.

The bioreactor consisted of two glass chambers that housed a single plant. The roots were in the lower (rhizosphere) chamber, while the leaves and stem were in the upper (foliar) chamber. The two chambers were fitted together as follows: A glass blower fused a glass O-ring to each chamber. The glass O-rings were sealed together with a Viton O-ring, which fit into a channel on each glass O-ring, and held together with a clamp. A tight seal between the two chambers prevented exchange of materials between the two chambers, except via the plant. In particular, it was important that oxygen transfer between the two chambers be limited to that supplied by the plant via internal oxygen diffusion and subsequent ROL. This was accomplished by using a Teflon-lined septum with an opening to accommodate the plant stem and non-toxic silicone caulk to separate, and create a gas-tight seal between, the lower and upper chamber. Oxygen was supplied to the plant via the gas system (described below).

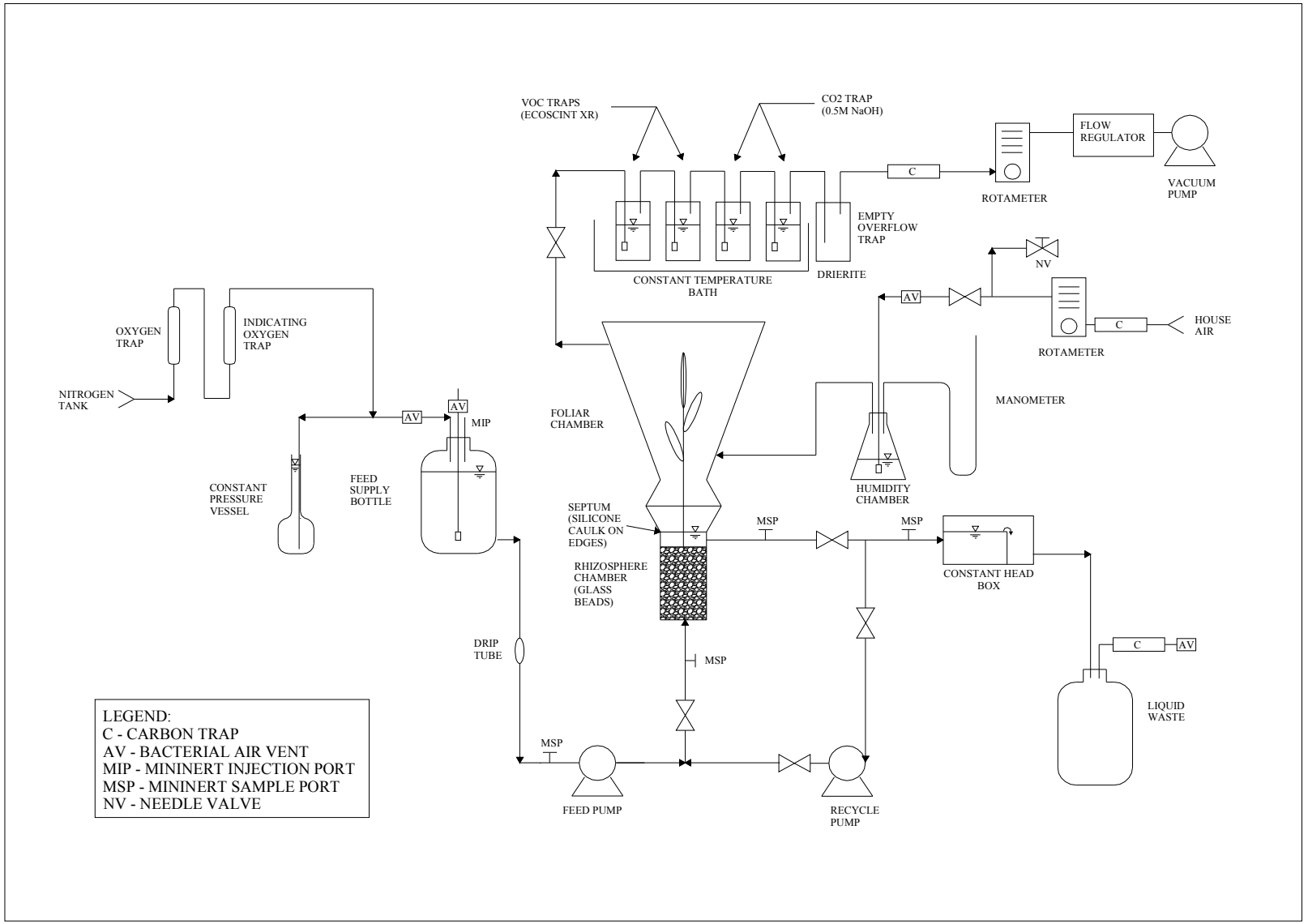

Figure 6-1: Overall design of the continuous-flow, completely-mixed bioreactor

A continuous supply of sterile, deoxygenated MSM amended with DCE (97% purity, Fisher) was pumped in an upflow mode through the rhizosphere chamber to meet the nutritional needs of both the bacteria and plant. A plant growth light (compact fluorescent 125 W, 6400 K wide spectrum, Hydrofarm, Petaluma, CA) was utilized to provide sufficient light (12 hr photoperiod) to the plant while in the bioreactor (average light reading 220 μmol/s m^2 μA). An LCD temperature strip attached to the foliar chamber was used to monitor the temperature inside the chamber. The temperature typically ranged from 20°C (typical nighttime temperature) and 28°C (typical daytime temperature).

The rhizosphere chamber was designed to support an aerobic DCE-degrading biofilm growing on 2-mm acid-washed glass beads and contain the plant roots. The rhizosphere chamber was 5 cm in diameter and had a total height of 140 mm. Glass wool was placed in the bottom 33 mm of the chamber to support the glass beads, which filled the remainder of the chamber (as described below). It should be noted that there was a space between the top of the beads, which fell just below the effluent line, and the septum used to separate the two chambers. This area was not part of the 140 mm reactor zone, but was filled with liquid during the experiments. The total volume of the glass bead zone was 210 mL, with a pore-volume of 88.2 mL. The rhizosphere chamber was completely saturated with media and covered with foil during experiments. The level in the chamber was maintained with a polycarbonate head box located on the waste line (Figure 6-1). During start-up, the height of the weir in the head box was adjusted until the liquid level in the chamber was just below

the septum. Sampling ports (Mininert termination valves) were provided on the rhizosphere chamber feed, influent, effluent, and wasting lines. Both the feed and recycle pumps consisted of a Masterflex (Cole-Parmer) pump drive and Masterflex Teflon tubing pump head (Cole-Parmer).

The rhizosphere chamber was sized on the basis of the physical characteristics of the plant roots and bacterial growth and substrate-utilization kinetics using a completely-mixed biofilm model (Rittmann and McCarty, 2001). In theory, maintaining a hydraulically completely-mixed condition, should have ensured that DCE and oxygen were uniformly distributed throughout the rhizosphere chamber and helped to prevent the formation of DCE hotspots and excessive biomass where substrate concentrations were high. A completely-mixed hydraulic condition was achieved by incorporating a high rate of recycle flow relative to the influent flow (Rittmann, 1982). Rittmann (1982) found that a minimum recycle-to-influent ratio of 10 was sufficient to approximate completely-mixed conditions in a biofilm reactor. The sizing calculations were based on steady-state mass balance equation for a completely-mixed biofilm reactor according to:

$$hV\frac{ds}{dt} = 0 = Q_T(S_{in} - S) - J_{ss}aV \qquad (2)$$

where Q_T is the total volumetric flow rate (L^3T^{-1}) and is equal to Q_R (L^3T^{-1}), the recycle flow rate, plus Q (L^3T^{-1}), the influent flow rate; a is the specific biofilm surface area (L^{-1}); S_{in} is the influent substrate concentration (M_sL^{-3}); S is the effluent substrate concentration (M_sL^{-3}); J_{ss} is the substrate flux into the biofilm ($M_sL^{-2}T^{-1}$); V is the reactor volume (L^3); and h is the pore volume (L^3). Figure 6-2 provides a

simplified schematic of the rhizosphere chamber including some of the above parameters. Eq. (2) was solved for a Q_T that yielded a V, and thus a length, that was suitable for the *P. australis* plant roots when the chamber diameter was fixed at 5 cm to accommodate the plant roots. Specifically, the values for Q_R and Q were adjusted in a trial-and-error process until the length was suitable to contain the plant roots. The design values for S and S_{in} were 1.5 mg/L and 50 mg/L, respectively. The value of S was selected to be approximately four times greater than S_{min} (0.38 mg/L), which is the minimum substrate concentration required to retain biomass at a steady-state concentration. The value for S_{in} was selected based on concentrations found in the environment at contaminated sites. Wrenn (2003) stated that the concentration of chlorinated compounds in contaminated groundwater typically ranges between 10 and 100 mg/L. For example, DCE concentrations of approximately 51.4 mg/L were observed at Plattsburgh Air Force Base (New York) (Wiedemeier et al., 1999). Therefore, an intermediate value of 50 mg/L was selected. This S_{in} was achieved by using a DCE concentration of ~1500 mg/L in the feed (S_o), which is below the solubility limit of 1888 mg/L determined in this study. J_{ss} and a were solved for using the approach described by Rittmann and McCarty (2001) and references cited therein, an assumed porosity of 0.4, and the kinetic constants of the bacterium selected (JS666) for use in the bioreactor (described below). With these constraints, a recycle flow rate of 30 times the feed pump rate (Q = 15 mL/hr and Q_R = 450 ml/hr), yielded a total chamber volume of 210 mL and a length of 107 mm.

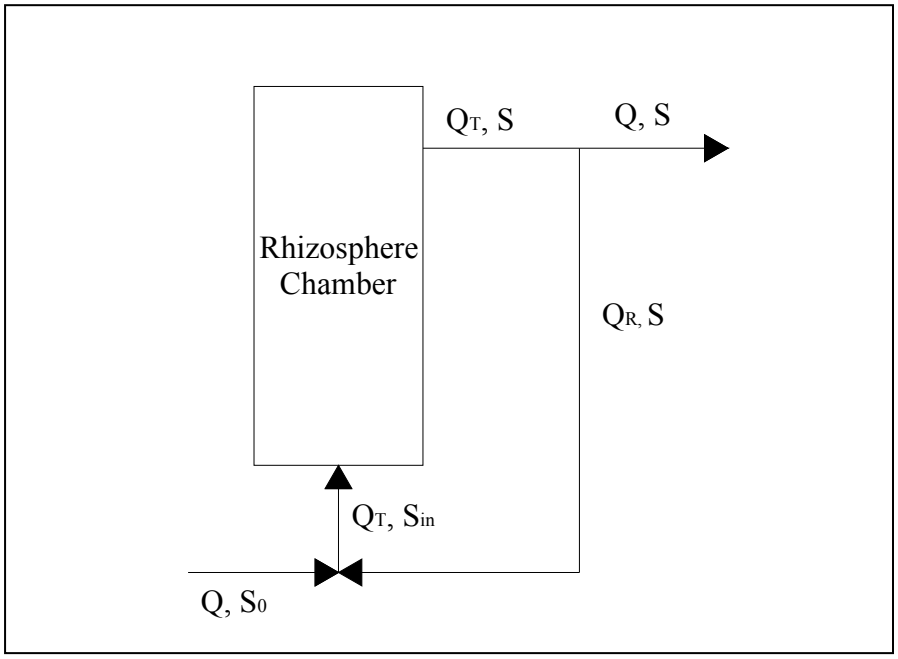

Figure 6-2: Schematic of rhizosphere chamber detailing parameters used in the completely-mixed biofilm reactor mass balance equation (parameters defined in text).

A tracer study was conducted to verify that the rhizosphere chamber behaved as a completely-mixed reactor. Following the method described by Seagren et al. (1999), fluorescein dye was utilized as a non-reactive tracer to monitor breakthrough without microorganisms or a plant in the reactor. Fluorescein dye was provided as a step input to the reactor. Samples were collected continuously by connecting a sample pump to the sampling point on the wasting line. The flow rate of the sample pump was set to 75% of the feed pump flow rate, and the collected fluid was discharged into a fraction collector. The fraction collector was set so that each tube collected 2 mL, which required approximately 10 minutes per tube. Every third sample was analyzed as described below.

The gas system of the bioreactor was designed to continuously supply fresh, sterile (0.2 μm, Air Vent, Pall) moist air to the foliar chamber, which was constructed using

a modified 2-L Erlenmeyer flask. This air provided the only source of oxygen for diffusion to the root chamber in most experiments. Positive pressure alone was initially used to supply humid air to the foliar flask. However, the amount of pressure needed to pump air through the chamber using this approach resulted in air being forced through the plant into the lower chamber, which in turn caused the water level in this chamber to drop. Therefore, a vacuum pump (Schwarzer Precision, SP 700 EC) and a vacuum flow regulator (Cole-Parmer, 32505 Series Flow Controller) were used to draw air from the rhizosphere chamber and through the downstream gas effluent traps. The negative and positive pressures were carefully balanced using a needle valve on the gas influent (Figure 6-1). The needle valve was adjusted so that the reading on the manometer, which measured pressure in the foliar chamber, was just above atmospheric pressure. This provided an influent flow rate that was slightly greater than the vacuum flow rate and did not force air into the lower chamber. The flow rate was maintained at 0.8 to 1.0 LPM (Burken and Schnoor, 1998), as measured by a rotameter on the vacuum line. This flowrate prevented heat and moisture accumulation in the chamber. The effluent traps consisted of 4 oz. glass bottles with Teflon coated caps, through which holes were drilled to accommodate tubing and glass dispersion tubes. These traps were in place in the gas system at all times during the experiments. Before radiolabeled DCE was added, the traps were filled with DI water. After [^{14}C]-DCE was added, volatile organic compounds (VOCs) and CO_2 in the gas effluent were collected by four traps in series (described below). A drop flask containing Drierite desiccant was placed after the VOC and CO_2 traps to remove moisture from the gas flow. Finally, a granular activated carbon (GAC) trap (ORBO

32, Supelco) was placed downstream of the other traps at all times to trap any residual VOCs.

The media supply system was designed so that either anoxic or fully-aerated media could be supplied to the rhizosphere. When anoxic media was required, it was deoxygenated by autoclaving it at 120^{o}C for 30 minutes, which also sterilized the media, and sparging the hot media under filter-sterilized (0.2 μm, Air Vent, Pall) oxygen-free nitrogen (Ultrapure carrier grade, Air Gas) until the media cooled to room temperature. The oxygen content of the cooled media was measured. If oxygen could be detected in the media, it was stored in an anaerobic chamber (85% N_2/ 10% CO_2/ 5% H_2; Coy Laboratories) with a sterile cotton plug to allow any residual oxygen to diffuse out. The media bottle was then connected to the bioreactor system via a quick disconnect (Swagelok). A continuous supply of filter-sterilized (0.2 μm, Air Vent, Pall) nitrogen (High purity grade, Air Gas) and a constant head vessel (1-L volumetric flask) was used to maintain the media bottle under a slightly positive pressure (12″ H_2O or 0.03 atm above atmospheric pressure) and prevent oxygen from entering, even as the liquid level decreased. Any residual oxygen in the nitrogen supply was removed with an oxygen trap and an indicating oxygen trap (Trigon Technologies) in series. When oxygenated media was required, it was prepared by sparging the media with filter-sterilized (0.2 μm, Air Vent, Pall) lab air after being autoclaved and cooled.

Bioreactor Experimental Procedure

A series of experiments were conducted using the bioreactor system. In each case, the fate of DCE under steady-state conditions was evaluated. The experimental system used in each experiment varied, as follows. In the first experiment, a root-only with JS666 experiment was performed to evaluate the removal of DCE in the presence of a plant root. In the second experiment, an abiotic control experiment was performed with the rhizosphere chamber only and aerated media to determine if there was any abiotic loss of unlabeled DCE in the system. In the third experiment, the rhizosphere chamber was operated with JS666 only and aerated media to evaluate the ability of JS666 to transform unlabeled DCE in a continuous-flow biofilm environment without the potential for inhibition due to the presence of preferential substrates or competition with, and/or grazing by, protozoa introduced along with the plant. In the fourth experiment, the foliar and rhizosphere chamber were used with the plant and associated microorganisms to characterize the roles that wetland plants play in affecting the fate of DCE. Two ways in which plants can influence microbial transformation of DCE in the rhizosphere are by releasing oxygen and/or exudates, which can serve as growth substrates through their roots. Therefore, in the fourth experiment, the plant was supplied with anoxic, DCE-free media and influent and effluent DO levels were measured to estimate oxygen release and the influent and effluent soluble chemical oxygen demand (COD) concentrations were measured periodically to obtain an estimate of exudate production. The oxygen levels were measured until they reached steady-state. After steady state oxygen release was observed, unlabeled DCE was added to the media. DCE levels were monitored until

the bioreactor reached steady-state with respect to DCE removal. Then [^{14}C]-DCE was added to the feed along with unlabeled DCE to characterize the contributions of *P. australis* and the root-associated microorganisms to the removal of DCE and the removal mechanisms themselves.

For all experiments, the performance of the bioreactor in terms of unlabeled DCE removal was monitored by measuring the concentration of unlabeled DCE in the liquid effluent and feed daily with a gas chromatograph (GC). Steady-state removal of DCE was evaluated by measuring either the concentration of unlabeled DCE or ^{14}C-activity for radiolabeled DCE in both the influent and effluent. When at least five sequential measurements typically made on a daily basis could be fitted with a line that had a slope not significantly different than zero (using a t-test with a $p > 0.05$), the reactor was said to be at steady-state.

For experiments utilizing JS666, bacteria were added to the bioreactor once the liquid level was stable in the rhizosphere chamber. For these experiments, the system was supplied with oxygenated media to promote the establishment of both the plant (if present) and bacteria. The bacteria were added aseptically with a sample pump through the sampling point on the rhizosphere chamber influent. The bacterial source was a liquid culture of JS666 (described above) containing between 10^6 to 10^8 CFU/ml (method described previously). JS666 cultures used to inoculate the rhizosphere chamber were actively degrading DCE and were harvested within one to two days of degrading a DCE addition. JS666 liquid cultures were pumped (50

mL/hr) into the reactor over a one-hour period. After the culture was added to the bioreactor, aqueous DCE was added to the bioreactor via the effluent line, resulting in a DCE concentration of 10 mg/L within the bioreactor system. Then the culture was recycled through the rhizosphere chamber for 12 hours using the recycle pump. During this time, there was no flow into or out of the bioreactor system. Afterwards, the recycle pump was also turned off and the system was allowed to sit quiescently for 24 hours to allow JS666 to attach to the glass beads. After 24 hours, the reactor was again operated in recycle mode, without any inputs or outputs. The flow rate of the recycle pump was slowly ramped up to 450 mL/hr, in an effort to prevent shearing of the biofilm. The reactor was then operated as designed, i.e., with influent, effluent and recycle flows, and feed and effluent DCE levels were monitored.

[^{14}C]-DCE was used in this study so that the fate of DCE in different phases (aqueous, gas, plant, and microbial biomass) could be quantified and the overall efficiency of the DCE removal could be measured. After [^{14}C]-DCE was added, samples of the liquid, gas, and solid phases were collected and treated in different ways to evaluate the amounts and forms of ^{14}C that they contained. Samples of the liquid effluent were collected approximately every 24 hours to determine aqueous [^{14}C]-DCE levels. This sampling frequency was selected based on the results of the tracer study (described below). ^{14}C-labeled VOCs and $^{14}CO_2$ in the gas system effluent were collected by a series of traps. Traps were maintained in duplicate (in series) and cooled in a 4^{o}C in-line water bath to increase their trapping efficiency (Gage et al., 1959). Each of the first two traps contained 100 mL of the scintillation

cocktail Ecoscint XR to trap both labeled and unlabeled VOCs. The selection of the Ecoscint XR cocktail was based on information provided by the manufacturer (National Diagnostics), which indicated that this scintillation cocktail can be used to trap organic compounds. The traps were not replaced during the experiment, because the amount of phytovolatilized VOCs was not expected to exceed the reported trapping capacity of the cocktail. The remaining two traps contained a 100-mL solution of 0.5 M NaOH, which captured both labeled and unlabeled CO_2. The contents of these traps were exchanged once a day, because they were expected to reach their CO_2 trapping capacity after approximately 24 hours. At the conclusion of the experiment, the amount of ^{14}C associated with the plant material and bacterial biomass was determined as described below.

Preparation of ^{14}[C]-DCE solution

The [^{14}C]-DCE (1 mCi, purity ≥ 96%, specific activity 4 mCi/mmol) was obtained from Moravek. The [^{14}C]-DCE was dissolved in 160 mL of sterile DI water in a 160 mL serum bottle as follows: First the ampoule (washed with 70% ethanol) containing the [^{14}C]-DCE was inserted in the empty serum bottle (placed on ice) and held in place at the bottom of the bottle. Next approximately 140 mL of sterile DI water was added. Then the class ampoule was broken and immediately the bottle was filled to the top with approximately 20-mL of DI water and capped with a Teflon-faced butyl septa and aluminum crimp cap. A 0.1-mL sample was tested to check for [^{14}C]-DCE activity and a second 0.1-mL sample was collected for headspace GC analysis (described below) to check for contamination. The 160-mL stock was divided into seven, 20-mL aliquots in 22-mL sterile serum bottles, and two, 10-mL aliquots in 10-

mL sterile serum bottles and stored at 4°C. For each 2 L of MSM prepared, one 20-mL aliquot of [^{14}C]-DCE stock solution was added, in addition to unlabeled DCE (97%, Fisher), for a specific activity [^{14}C]-DCE of approximately 30 μCi/L.

Analytical Methods

Analysis of Volatile Organics

The concentration of DCE was measured using a GC (Hewlett Packard, Model 5890 Series II Plus) equipped with a flame-ionization detector (FID) and a 3.2 mm x 2.44-meter stainless-steel GC column packed with 1% SP-1000 on 60/80 Carbopak-B (Supelco, Inc). Helium (Ultra Pure Carrier Grade, Air Gas) was used as the carrier gas at a flow rate of 40 ml/min, and the FID was fueled by hydrogen (Ultra Pure Grade, Air Gas) and air (Ultra Pure Grade, Air Gas) provided at flow rates of 40 ml/min and 400 ml/min, respectively. The injector and detector temperatures were set at 200°C and 250°C, respectively. The oven temperature was maintained according to the following operating sequence: (1) 60°C for two minutes, (2) 20°C /min ramp to 150°C, and (3) 10°C C/min ramp to 200°C (Gossett, 1985).

To determine DCE aqueous concentrations in the bioreactor system, 100-μL samples were collected with a 250 μL gastight syringe (Hamilton), injected into a 10-ml sample vial sealed with a Teflon-faced butyl stopper and aluminum crimp cap and equilibrated at 30°C for a minimum of one hour using a Multi-Blok heater (Lab-Line Instruments, Inc.). Headspace samples (0.5 mL) were taken from the sample vials using a 1-ml gas-tight syringe equipped with an on-off push-button valve (Pressure-Lok®, Series A-2) and manually injected into the GC. The concentrations of the

unknown samples were determined based on a calibration curve. The calibration curve was prepared using headspace samples obtained from aqueous DCE standards that were prepared gravimetrically using a stock solution of DCE in methanol (Gossett, 1985) and then transferred to the headspace vials. The linear calibration curve was fit to the data using a relative least-squares (RLS) technique, as described by Saez and Rittmann (1992). This was necessary because the residuals generated using an absolute least-squares (ALS) linear relationship was correlated to the observations. Specifically, their value increased as the dependent variable (concentration) increased. As a result, the higher concentrations had more influence on the ALS parameter estimation compared with the lowest standard concentration. This was not unexpected, because the concentration range of the standards varied over more than one order of magnitude (Saez and Rittmann, 1992).

Tracer Study Analysis

A 1.5 mL aliquot of the samples collected in the tracer study was placed in a quartz cuvette and analyzed in a spectrofluorometer (Shimadzu) at a $\lambda_{excitation}$ of 440-442 nm and a $\lambda_{emission}$ of 514-518 nm (Seagren et al., 1999) to determine their fluorescein content. A calibration curve was prepared with aqueous standards containing known concentrations of fluorescein.

Quantification of Plant Exudates

The overall plant exudate content of the bioreactor effluent was estimated in terms of COD. COD was quantified in 2-mL samples of the reactor feed and effluent using the Hach method (0 - 150 mg/L), which utilizes potassium dichromate oxidation and

reactor digestion based on the approach described by Jirka and Carter (1975). A Hach DR/4000 photometer was used in the analysis.

Dissolved Oxygen Measurement

To measure the plant root oxygen release in the reactor, the oxygen content in the reactor effluent and feed were monitored. A flow-through oxygen probe (Lazar, Model DO-166) was utilized with a polypropylene membrane (2-mm, Fisher). The probe was connected to a pH meter (Orion 940A) to obtain output readings (mV units). The meter output reading was calibrated to read percent oxygen saturation. The probe was calibrated to 21% saturation daily by injecting air through the unit using a syringe. The probe was calibrated once to 0% DO by injecting deoxygenated DI water. The percent saturation readings measured in aqueous samples were converted to DO concentrations using the following equation:

$$DO\ (mg/L) = \frac{ac}{22.414} x \frac{(760 - p)\ mm\ Hg}{760\ mm\ Hg} x \frac{r\%}{100} x \frac{32000 mg\ O_2}{mol\ O_2} \qquad (3)$$

where ac is the absorption coefficient of gas at 20^{o}C, p is the vapor pressure of water at 20^{o}C (mm Hg), and r% is the percent oxygen reading.

Analysis of ^{14}C-labeled fractions

The steady-state distribution of ^{14}C in the different gas, liquid, and solid phases was determined using the approach outlined in Figure 6-3. The radioactivity of all samples was measured using a liquid scintillation counter (Perkin Elmer Tri-Carb Scintillation Counter) with a counting time of 15 minutes using Ecoscint XR scintillation cocktail (National Diagnostics) in 7-mL glass scintillation vials. A counting time of 15 minutes was selected because in a previous study by Seagren

(1994), this time was found to be sufficiently long to keep the uncertainty at the 2σ level to <10% of the total count rate for most samples. An internal standard quench curve was used. All samples containing NaOH were allowed to sit overnight in the dark to reduce chemiluminescence before they were counted (Voroney et al., 1991). This method to reduce chemiluminescence was verified by comparing the disintegrations per minute (dpm) measured in two 5-mL samples consisting of NaOH (2 mL, 0.1 M) and Ecoscint XR (3 mL), one of which had been incubated in the dark overnight and one of which was analyzed within 30 minutes of being prepared. A background sample was also prepared by adding 2-mL of DI water to 3-mL Ecoscint XR. The measured activity for the sample that had not been incubated overnight was 32.06 dpm, whereas the sample that had incubated overnight was 30.50 dpm, compared to the background sample with 30.54 dpm. Comparing the data showed that the radioactivity decreased in the sample that incubated overnight to levels similar to those in the background sample. In contrast, the dpm counted in the sample that had not incubated overnight, were higher than the background sample.

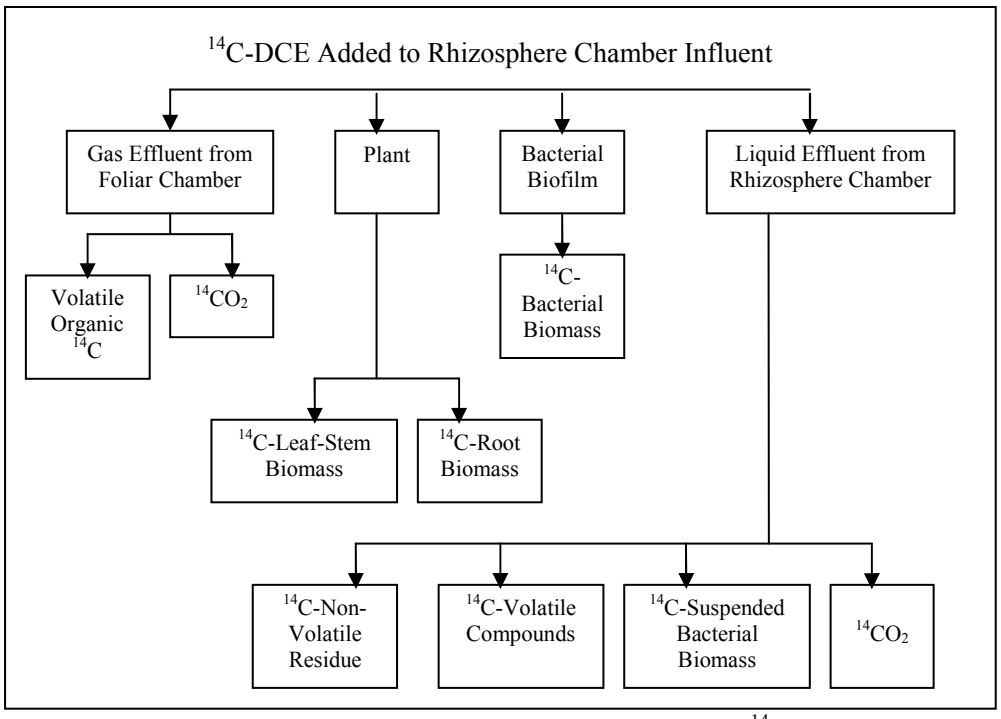

Figure 6-3: Sample fractions to be analyzed for [^{14}C]

Liquid Fractions

Liquid effluent samples (4 mL) were manually collected with a 5-mL gas tight syringe (Hamilton) by pulling a sample from the rhizosphere chamber effluent. NaOH (0.1 mL, 2.25 M) was added to the sample to raise the pH above 10.5 and drive all $^{14}CO_2$ into the aqueous phase (Freedman and Gossett, 1989). Then, the aqueous sample was split into eight sub-samples (0.5 mL each), which were treated in duplicate as follows (following Seagren, 1994): (1) injected directly through a Teflon-lined septum screw cap into a scintillation vial containing scintillation cocktail; (2) injected into an empty scintillation vial and stripped by nitrogen sparging for 30 minutes to remove ^{14}C-labeled volatile compounds; (3) injected into an empty scintillation vial and acidified with two drops of concentrated HCl (pH $\approx$ 1) and stripped to remove the trapped $^{14}CO_2$ plus ^{14}C-labeled volatile compounds; and (4) filtered (0.2 μm Millex® –FG Millipore) with the filtrate treated in the same manner

as sub-sample (3). 5 mL of scintillation cocktail was added to each vial for treatments (2) through (4). All vials were shaken by hand for 30 seconds. Comparison of these four sub-samples yielded the total ^{14}C in the liquid phase (1), ^{14}C-labeled volatile compounds (1) – (2), $^{14}CO_2$ (3) – (2), ^{14}C-labeled biomass (3) – (4), and ^{14}C-labeled non-volatile residues (1) – (2) – (3).

The amount of time required to strip the samples was verified as follows. A solution of unlabeled DCE was added to DI water (0.5 mL) for a final DCE concentration of approximately 10 mg/L. Prior to stripping the DCE with nitrogen, a 0.5 mL headspace sample was obtained from the vial and analyzed on the GC to verify the presence of DCE. The sample was then sparged for 30 minutes with nitrogen (Ultra Pure Carrier Grade, Air Gas) supplied through a distribution manifold. After 10 and 30 minutes a headspace sample was collected and analyzed on the GC. After 10 minutes DCE was still detectable. After 30 minutes, there was no detectable DCE.

The presence of DCE and CO_2 in different liquid fractions were verified following the methods described by Seagren (1994). First, the fraction of DCE in the volatile ^{14}C was determined by collecting a 100 μL effluent sample and analyzing it on the GC, as previously described, for the presence of other volatile compounds such as VC. Second, the $^{14}CO_2$ fraction was confirmed to be CO_2 by barium carbonate ($BaCO_3$) precipitation. A 0.5 mL sample of the liquid effluent was collected and acidified following the method described for subsample (3) above and then inserted into a vial and capped with a septum and screw cap with a hole. The sample was

sparged with nitrogen for 30 minutes through a needle inserted through the septum to the bottom of the vial. A 1/16" OD Teflon tube was inserted through the septum into the headspace of the vial. The other end of this tube was inserted to near the bottom of a test tube containing 4 mL of 0.5 M NaOH and 145.6 mg $BaOH_2$. This test tube also contained a small magnetic stir bar and was placed on a magnetic stirrer to keep solids from settling to the bottom. After the sample was sparged, the $BaCO_3$ was allowed to settle, and a 0.5mL sample of the solution was collected. The sample was filtered through a 0.2 μm Millex® –FG Millipore syringe filter and counted in 5-mL of Ecoscint XR.

Any compounds originally trapped in the basified sample, which became strippable from solution when the sample was acidified (e.g. CO_2), should have been trapped in the NaOH in the second vial. In additions, any of the stripped ^{14}C from the first vial that was CO_2 should have been precipitated as $BaCO_3$ in the second vial, and thus settled or filtered out and not been counted in the solution. Therefore, the counts in the NaOH solution should have been at background levels if the fraction determined by the presumptive test to be CO_2 was indeed all CO_2. The background sample consisted of autoclaved MSM subjected to the same treatment.

Gas Fractions

Gas traps for VOCs, presumptively [^{14}C]-DCE, and $^{14}CO_2$ discharged from the foliar zone were incorporated into the design, as previously described. The trapping medium for [^{14}C]-DCE also served as the scintillation cocktail and was counted

directly by transferring 5 mL of trapping medium to a scintillation vial. For the CO_2 traps, 1.5 mL of the trapping agent (0.5 M NaOH) was added to a scintillation vial containing 5 mL of scintillation cocktail. Three separate samples from each trap were collected for counting to produce an average radioactivity level for a given volume of trapping solution.

Solid Fractions

The amount of ^{14}C taken up or absorbed by the plant was determined at the termination of the experiment. The plant roots were separated from the stem and leaves, and then each portion was weighed. Sub-samples (1g) of the separated plant material were combusted in a biological oxidizer (RJ Harvey, Model OX400) as described by Nair et al. (1993). The $^{14}CO_2$ produced following combustion was trapped in 20 mL of 0.5 M NaOH. Triplicate 1.5 mL aliquots of each 20-ml volume of trapping solution were each combined with 5 mL of scintillation cocktail, and the results of the triplicate analysis were averaged.

The efficiency of $^{14}CO_2$ recovery for the oxidizer was determined by combusting a sample containing a known amount of activity. The chemical selected to determine the recovery efficiency should be representative of the samples combusted; therefore, glucose was selected because plant material is largely composed of glucose. The uniformly ^{14}C-labeled glucose ($\geq$ 97% purity, specific activity 100 μCi/mL) was obtained from Moravek. A ^{14}C-labeled glucose solution with a known activity between 10,000 and 20,000 counts per minute (CPM) was prepared by diluting the

stock ^{14}C-labeled glucose in 5.6 mL of DI water, added to 50-mg mannitol, and combusted. The activity of the recovered $^{14}CO_2$ was determined using the method described above. The recovery efficiency, referred to as the correction factor, was calculated by dividing the known activity by recovered activity. A background sample was prepared by combusting 50 mg of mannitol. The correction factor was used to determine the total activity in the plant material based on the amount of $^{14}CO_2$ recovered from combusting the plant material.

In addition, the ^{14}C incorporated into bacterial biomass was determined by collecting 100 beads from each of the algal, top, middle, and bottom layers (described below) of the packed bed of biofilm at the conclusion of an experiment. The beads were added to 5-mL of Ecoscint XR in a scintillation vial, agitated for 18 hours, and counted (Martin et al., 1996). A background sample was prepared with clean beads.

Biofilm Characterization

After completion of experiments conducted with added JS666 or bacteria associated with the roots of an intact plant, the biofilm thickness (L_f) and density (X_f) were measured following the method described by Raskin (1987). 100 beads from each of the algal, top, middle, and bottom layers (described below) of the packed bed were collected, weighed, dried at 103^oC overnight, and reweighed. The difference in the wet and dry masses (W; M) was used to calculated L_f using the following equation:

$$L_f = \frac{W}{\rho n A(0.99)} \tag{4}$$

where ρ is density of water at 20°C (M/L^3) = 0.9982 g/cm^3, n is the number of glass beads = 100, A is the surface area per glass bead (L^2). The surface area of the 2 mm glass beads was calculated based on the diameter to be 12.56 mm^2.

To determine X_f, the dried beads were then ignited at 500°C overnight, cooled in a desiccator, and weighed. The difference in the dried and ignited masses is the volatile suspended solids (VSS) content of the biofilm and was used to calculate the biofilm density as follows:

$$X_f = \frac{VSS}{nAL_f} \tag{5}$$

Chapter 7: Results and Discussion

Bacterial Selection

The original experimental approach for this research project was based on the idea that plants used in the bioreactor would essentially be cleansed of native bacteria associated with their roots so that a pure DCE-metabolizing bacterial culture could be maintained in the rhizosphere chamber. The rationale underlying the selection of this approach was as follows: (1) it would help ensure that microbial oxidation of DCE would occur in the bioreactor, and (2) the kinetics of DCE oxidation and microbial growth would be defined and therefore could be used to select the reactor size and flow rate and interpret the substrate removal. Therefore, a significant amount of effort was devoted to the selection of a bacterium that would be appropriate for use in the bioreactor. Specifically, it had to be able to oxidize DCE and form a biofilm. Initial literature searches focused on methanotrophs because, as previously discussed, it is known that in a natural wetland system, methanotrophs are able to mineralize PCE daughter products through cometabolism while using methane as a growth substrate. Many studies examining degradation of PCE daughter products with methanotrophic bacteria have focused on *Methylosinus trichosporium* OB3b, because of its high rates of chlorinated ethene degradation (e.g., van Hylckama Vlieg et al. (1996) and Fitch et al. (1996)). Strain OB3b was a potential candidate for use in this project; however, it does not readily form a biofilm in pure culture. Efforts to improve strain OB3b's ability to form a biofilm, including polymer flocculation and application of antibiotics to reduce the competition of contaminating strains with

antibiotic resistant OB3b strains have shown only marginal improvements in biofilm formation (Fitch et al., 1996). In addition, there are general problems with using cometabolic processes to biodegrade DCE. That is, they can be difficult to sustain, and supplying adequate concentrations of methane, which is rather insoluble, in the feed is technically challenging. Therefore, other types of bacteria were considered.

As previously described, a bacterium capable of growing on DCE, strain JS666, was recently identified (Coleman et al., 2002). JS666 was isolated from granular activated carbon used in a pump-and-treat operation that was processing groundwater contaminated with PCE, TCE, and DCE. Therefore, it seemed likely that JS666 would form a biofilm. JS666 oxidizes DCE at a relatively high rate compared to the kinetics of other DCE-degrading organism reported in literature. For example, JS666 has a maximum specific substrate utilization rate (k) of 12.6 nmol/min/mg-protein. In comparison, *Methylomicrobium album* BG8, a bacterium that transforms DCE cometabolically, has a k of 0.12 nmol/min/mg-protein (Han et al., 1999). On the other hand, JS666 appears to have a lower affinity for DCE compared to some methanotrophic DCE degraders. The half-saturation constants for DCE (K_s) of strains JS666 and BG8 are 1.6 μM and 0.8 μM, respectively. Little is known about the mechanism involved in the biodegradation of DCE by JS666. However, Coleman et al. (2002) suggested that the formation of a DCE epoxide by a monooxygenase enzyme system is the first step in DCE oxidation.

Although JS666 had previously been shown to degrade DCE, the first criterion for use in this research, it was not known if it would form a biofilm on the glass beads used in the bioreactor. Therefore, beads placed in triplicate JS666 cultures that were actively degrading DCE were washed with MSM and resupplied with DCE, as previously described, to evaluate JS666's ability to attach to the glass beads. Within three to five weeks of the washing step, which should have removed suspended JS666 cells, DCE was degraded in all three bottles (Figure 7-1 and 7-2). This provided strong evidence that JS666 adhered to the glass beads and was able to degrade DCE in the attached form. Based on the results of this test, strain JS666 was selected for use in the bioreactor because it appeared to be able to both degrade DCE and form a biofilm.

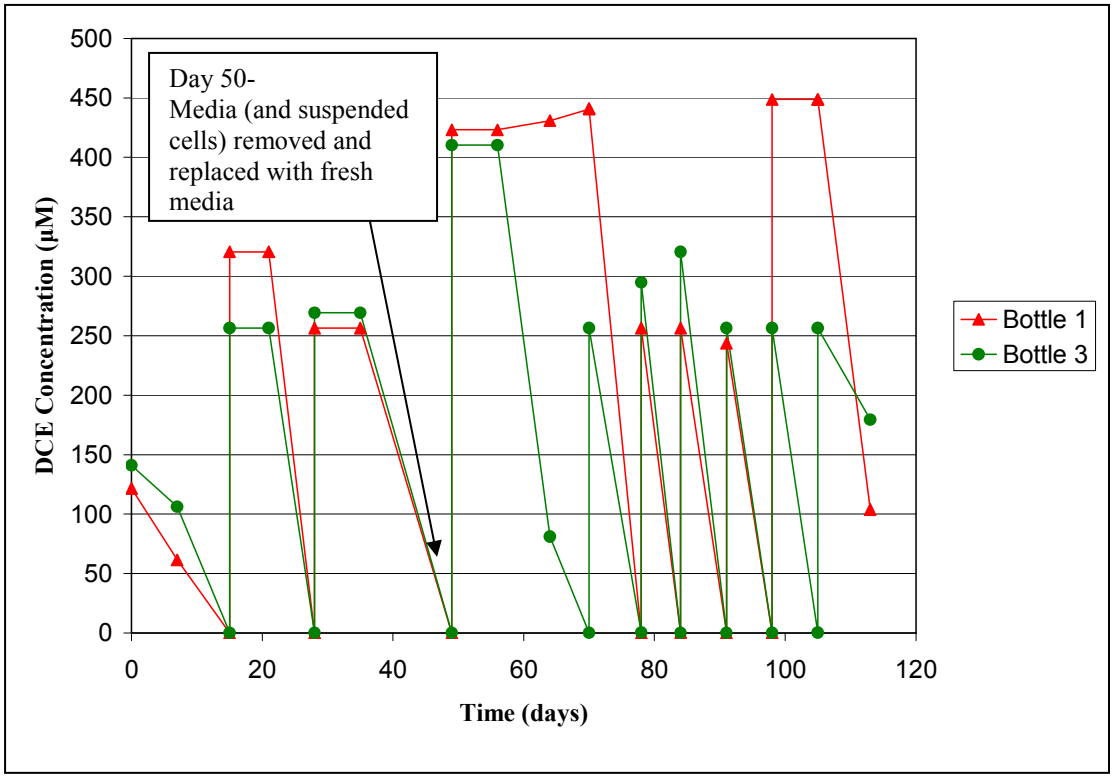

Figure 7-1: Biofilm testing results for Bottles 1 and 3. Concentrations of DCE added on days 15, 29, 49, 70, 78, 84, 91, 98, and 105 were estimated. All other concentrations were measured. Each data point represents a single measurement.

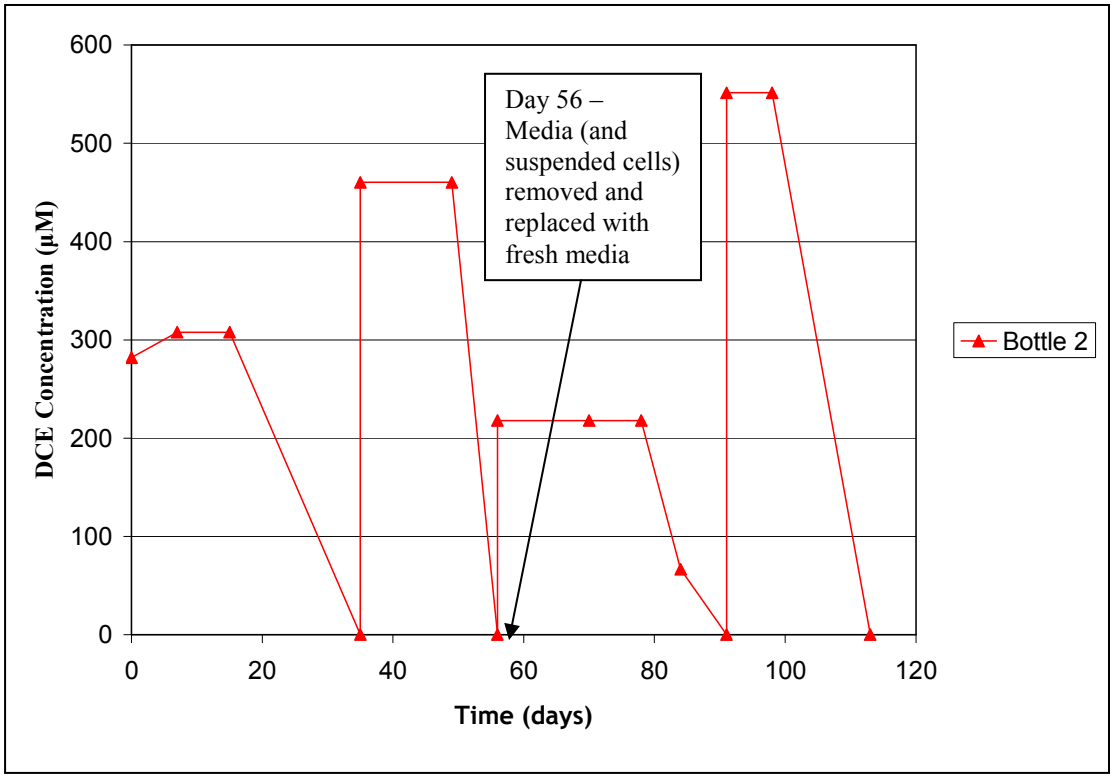

Figure 7-2: Biofilm testing results for Bottle 2. Concentrations of DCE added on days 21, 28, 35, 56, and 91 were estimated. All other concentrations were measured. Each data point represents a single measurement.

Plant Selection

Considerable effort was also devoted to identification of a plant species that demonstrated two key characteristics: (1) an ability to supply sufficient oxygen to the rhizosphere to support aerobic bacterial respiratory needs and DCE oxidation, and (2) a morphology that conformed to the physical conditions of the bioreactor. In particular, the ability to develop an extensive root structure was desirable to maximize the surface area for oxygen release and biofilm growth. A short shoot was also required to fit the plant stem and leaves inside the foliar chamber. It was also thought that it would be desirable from an ecological perspective to choose a Mid-Atlantic native, non-invasive wetland plant that traditionally is included in

constructed wetlands. Therefore, the plants initially considered included softstem bulrush (*Schoenoplectus tabernaemontani* K.C. Gmel. Palla), soft rush *(Juncus effusus L.*), broad-leaf cattail (*Typha latifolia L.*), and Pennsylvania smartweed (*Polygonum pensylvanicum L.*). These plants were also considered in part because they were expected to supply different amounts of oxygen to the rhizosphere because they varied in terms of their classification as obligate or facultative wetland species, their ability to tolerate flooded conditions, morphologies and other factors. However, none of these plants could be grown in a way that developed an extensive root structure with a short shoot.

The evaluation of plant candidates next turned to other species and ultimately resulted in the selection of common reed (*Phragmites australis* (Cav.) Trin. ex Steud.), an obligate wetland species, for use in this project. Common reed was selected because it is known to oxygenate the rhizosphere; in fact, the oxygen concentrations in the proximity of the root can reportedly reach the same concentration as found in the atmosphere (Massacci et al., 2001). As measured by Armstrong et al. (2000), the oxygen release from a 160-mm-long *P. australis* root (approximately 1.3 mm radius) was 60 ng/cm^2 root surface/min at 7 mm from the root tip. In addition, *P. australis* can morphologically conform to the physical requirements of the bioreactor experiments. Plants with mature extensive roots can be pruned to stimulate growth of new, and thus short, shoots from their roots. It is recognized that *P. australis* can become an invasive species, resulting in *P. australis* monoculture wetlands, which is ecologically undesirable. However, this plant can be beneficial because it has been

able to colonize contaminated areas whereas few other species can survive (Massacci et al., 2001).

Plant Treatment

Prior to use in the bioreactor, the roots of appropriately sized *P. australis* plants were treated to significantly reduce levels of indigenous bacteria unless otherwise noted below. Various methods reported in literature were evaluated for use in the study before the final approach (described below) was adopted. The goal of the treatments was to remove as many of the bacteria as possible without causing irreversible damage to the plant. The methods that were tested included various concentrations of hypochlorite treatment, with and without rotary shaking or sonification; UV treatment (two 10 minute exposures, which was based on the typical amount of time required to sterilize inanimate objects); and antibiotic treatment with vancomycin (Mehta et al., 1959). The amount of bacterial inactivation and plant health were evaluated for each treatment. Bacterial inactivation was measured using plate counts of root pressings on full-strength TSA plates made before and after a treatment (Dandurand and Knudsen, 2002). Plant health was evaluated qualitatively. No treatment resulted in complete bacterial inactivation, although a one or two log reduction in the colony counts was observed for most treatments. However, in most cases, the plant yellowed and died shortly after treatment. The only treatment that did not irreversibly impair plant health involved treatment with hypochlorite (10%) and rotary platform shaking (120 RPM, Baxter S/P® Rotator V), as described above. This method resulted in an approximately ten-fold reduction in the number of colony forming units.

Bioreactor Operation

The bioreactor system described above was constructed and mechanically tested without any biological components by eliminating leaks and maintaining a constant water level in the rhizosphere chamber. Following the mechanical testing, a treated *P. australis* individual was placed in the bioreactor to confirm that an individual plant could survive in the reactor. Following insertion of the plant, the bioreactor was operated continuously for over six weeks without major operational difficulties. Oxygenated media was supplied for the first four weeks during which time the plant grew. Four new leaves emerged after the plant was placed in the reactor, although the original leaves turned partially yellow. After three and a half weeks of operation, there was significant yellowing of the newly emerged leaves. However, the top most leaves remained mostly green, and new leaves continued to emerge at the top of the plant. A few days after the emergence of the second round of new leaves, deoxygenated media was supplied to the reactor to determine if the plant could survive on anoxic media. The reactor was operated with deoxygenated media for over two weeks, and during this time the plant survived. Some new growth was observed at the top of the plant. However, the partially yellow leaves became completely yellow. These results showed that the bioreactor could function hydraulically as designed, and that the selected plant could survive in the system.

Tracer Study

A non-reactive tracer study was performed on the rhizosphere chamber (as described above). The experimentally measured effluent concentrations were compared to the

predicted results for a flow-through completely-mixed bioreactor, which were based on a mass balance of the system, according to:

$$V_T \frac{dS}{dt} = QS_o - QS \tag{6}$$

where V_T is the total volume (L^3). The influent concentration of fluorescein (S_o) was 2.3 mg/L. The total volume (V_T) was 142 mL, which included the pore-volume (V_v = 88.2 mL), the volume above the beads (37 mL), and the volume of the recycle tubing (21 mL for 122 cm of 0.47 cm I.D. tubing). The pore volume was determined using the total volume within the bead zone (V = 210 mL as described above) and the measured porosity (0.42 as determined using a displacement method). The flow rate was 16 mL/hr, which yielded a hydraulic detention time of 8.9 hours. As shown in Figure 7-3, the experimental results closely matched the predicted concentrations calculated with Eq. (6). This good fit suggests that the rhizosphere chamber did behave as a hydraulically completely-mixed reactor. Further, the results of the tracer study indicate that steady state was reached in approximately three days when no reactions were occurring.

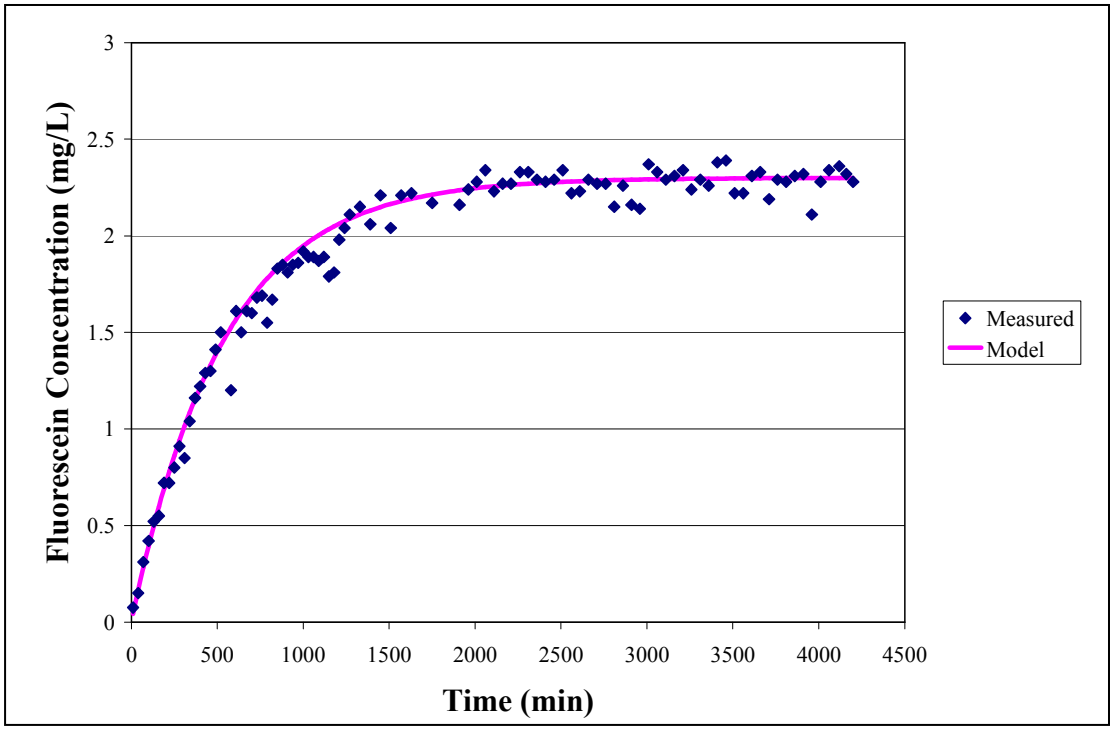

Figure 7-3: Experimental results of the tracer study conducted on the rhizosphere chamber compared to the predicted model for a completely-mixed bioreactor.

Root and JS666 Experiment

An experiment utilizing a plant root and a culture of JS666 was conducted to evaluate removal of DCE by JS666 in the presence of plant material and to determine if DCE transformation under these conditions occurs as predicted by the completely-mixed biofilm model. Table 7-1 summarizes the phases involved with this experiment. Following addition of bacteria to the bioreactor, and allowing the DCE concentration to drop to 1.0 mg/L, a feed concentration of 1505 mg/L DCE was supplied as planned (described above). Within 8 hours the effluent DCE concentration had reached 395 mg/L (4.2 mM), well over the DCE level previously shown to be toxic to JS666 (2 mM) (N. Coleman, personal communication). Therefore, the bioreactor was supplied with unamended feed to flush out the DCE, and then operated in recycle-only mode

for several days. Next a second batch of JS666 was added in the event that the toxic levels of DCE had killed off the first batch of added JS666. The DCE concentration in the feed was reduced to 75 mg/L (790 μM), which was below the toxic level; however, there was still little removal of DCE observed. The feed was then further reduced to 10 mg/L, a level that was still significantly greater than S_{min}. This feed concentration is still of environmental relevance, because many sites are contaminated with concentrations of DCE in this range (Wiedemeier, 1999). Other laboratory studies of microbial DCE biodegradation also used concentrations in this range (e.g. Kassenga et al., 2003; Suarez and Rifai, 1999). At this feed concentration, approximately 1 mg/L removal of DCE was consistently observed over a time period of five days. This removal was assumed to be attributed to JS666 alone.

Table 7-1: Phases of operation incorporated with the root & JS666 experiment

Operation Phase	Days of Operation	Feed Characteristics	Purpose
1	0-4	Aerated, DCE free	Establish system
2	4-11	No Feed	Add JS666
3	12	Aerated, amended with DCE (1500 mg/L)	Measure steady-state removal of DCE
4	13-18	Aerated, DCE free	Reduce DCE concentration
5	19-20	No Feed	Add JS666 (2^{nd} batch)
6	21-28	Aerated, amended with DCE (75 mg/L)	Measure steady-state removal of DCE
7	28-49	Aerated, amended with DCE (10 mg/L)	Measure steady-state removal of DCE

Based on the results of this experiment, it was apparent that there was not sufficient attached biomass to achieve the removal predicted by the biofilm model using JS666 substrate removal kinetics and assumed X_f values (described above) or that the ability of JS666 to degrade DCE was inhibited in the bioreactor environment. In calculating the predicted DCE removal, a typical biofilm density of 40 mg VSS/cm^3 (Rittmann and McCarty, 2001) was assumed. Based on 1 mg/L removal of DCE, Eq. (7) was solved for J_{ss}.

$$J_{SS} = \frac{Q_T\ (S_O - S)}{aV} \tag{7}$$

Eq. (8) was solved for X_f, assuming L_f was equal to the thickness of one bacterium (1μm).

$$X_f L_f = \frac{YJ_{SS}}{b'} \tag{8}$$

Where b' is the overall specific loss rate for the biofilm (T^{-1}) and Y is the JS666 true yield coefficient ($M_x M_s^{-1}$) (6.1 g protein/mol cDCE) (Coleman et al., 2002). Using Eq. (7) and (8), and additional equations provided by Rittmann and McCarty (2001) to solve for b', the biofilm density was calculated as 1.39 mg VSS/cm^3. This value is below the normal range of 10 – 100 mg VSS/cm^3, implying a non-continuous biofilm (Rittmann, 1993). Based on these results, an analysis was conducted using Eq. (7) and (8) to determine if lowering the feed flow rate would improve the removal with a DCE feed concentration of 10 mg/L, while maintaining the other operating parameters (e.g. recycle rate). This analysis showed that reducing the flow rate would reduce the effluent concentration by providing additional time for JS666 to degrade the DCE. Therefore, the feed rate was reduced to the lowest reasonable setting on the

feed pump (approximately 6 ml/hr). At this flow rate, a DCE effluent concentration of 7.5 mg/L was predicted. Table 7-2 summarizes the operating parameters of the bioreactor in subsequent experiments, and reflects these operational changes.

Table 7-2: Operating parameters of the continuous-flow, completely-mixed bioreactor.

Characteristic	Value
Active length of reactor	107 mm
Diameter of reactor	5 cm
Cross-sectional area of reactor, A_{cs}	19.6 cm^2
Volume of reactor, V	210 cm^3
Diameter of glass beads, d_p	2 mm
Surface area of a glass bead, A	12.56 mm^2
Specific surface area, a	18 cm^{-1}
Porosity, $\varepsilon = V_v/V$	0.42
Pore volume, V_v	88.2 cm^3
Feed flow rate, Q	6 cm^3/hr
Detention time, $\theta = V_v/Q$	14.7 hr
Recycle ratio, Q_R/Q	75
Time of one pass through the column, $V_v/ (Q + Q_R)$	11.6 min
Superficial velocity of fluid, $v = (Q + Q_R)/A_{cs}$	556 cm/d

To explain the low amount of DCE removal observed, the possibility that JS666 was washing out and was not attaching to the beads to a significant extent was examined by analyzing the effluent for the presence of JS666. First, 0.1-mL of effluent from the wasting line was plated and showed few colonies of JS666 (< 10 CFU/ml). Additionally, 1-mL samples from the effluent were inoculated in 50 mL MSM to test for DCE removal. It took approximately six weeks for removal of the first spike of

DCE to occur, and little biomass was observed in the cultures. This time period was significantly longer than the time required for removal of a similar concentration of DCE in the batch cultures. This seemed to indicate that few cells of JS666 were washing out and therefore, this did not explain the low DCE removal.

Another potential explanation for the low amount of DCE removal is that JS666 preferentially utilizes alternative substrates, i.e. plant exudates, in lieu of DCE. The tendency of JS666 to use non-chlorinated organic substrates preferentially has previously been observed (N. Coleman, personal communication) and was observed in this study in batch cultures of JS666 supplied with 50 mM ethanol and 400 μM DCE. In these cultures, removal of DCE was not observed although there was significant biomass present. Therefore, the bioreactor effluent in the root and JS666 experiment was tested for possible alternative substrates by measuring its COD content, which was 43 mg/L. The COD measurement suggests that the plant root was releasing exudates that could possibly serve as alternative substrates for JS666. Therefore, it is possible that the presence of the plant may have had a negative impact on DCE removal by JS666.

Additionally, JS666 is highly subject to predation by other microorganisms (N. Coleman, personal communication), which also could potentially have reduced the density of JS666 in the reactor. Spread plates prepared using reactor effluent samples revealed other colony morphologies, indicating that other microorganisms were present in the reactor, in addition to JS666. This was not surprising because the

process used to remove native microorganisms from the plant surface was shown to be less than 100% effective.

Abiotic Control Experiment

The bioreactor was next operated without any biological components (plants or microorganisms) to quantify abiotic loss of DCE in the bioreactor system. The phases of this experiment are outlined in Table 7-3. Because the feed DCE concentration decreased over time as the level in the feed bottle decreased due to volatilization of a decreasing total mass of DCE, feed and effluent samples were compared to determine the removal of DCE in the bioreactor. Once the removal had appeared to level-off, five influent and effluent samples were collected on four consecutive days (3 to 6) to test whether steady state had been achieved (Figure 7-4).

Table 7-3: Phases of operation incorporated in the abiotic control experiment.

Operation Phase	Days of Operation	Feed Characteristics	Purpose	Figure
1	0	Aerated, DCE-Free	Establish System	NA
2	0-6-	Aerated, amended with DCE	Measure steady-state removal of DCE	7-4

Steady-state removal of DCE was confirmed through a statistical analysis. The statistical analysis tested if the slope of a line drawn through the last five data points of DCE removal was equal to zero. A slope of zero would indicate steady-state removal of DCE. The statistical test produced a t-statistic of 0.34 with a corresponding p-value of 0.38; therefore, the system was determined to be at steady

state (95% confidence interval). State-state DCE removal averaged 1.0 mg/L or 12.7% removal.

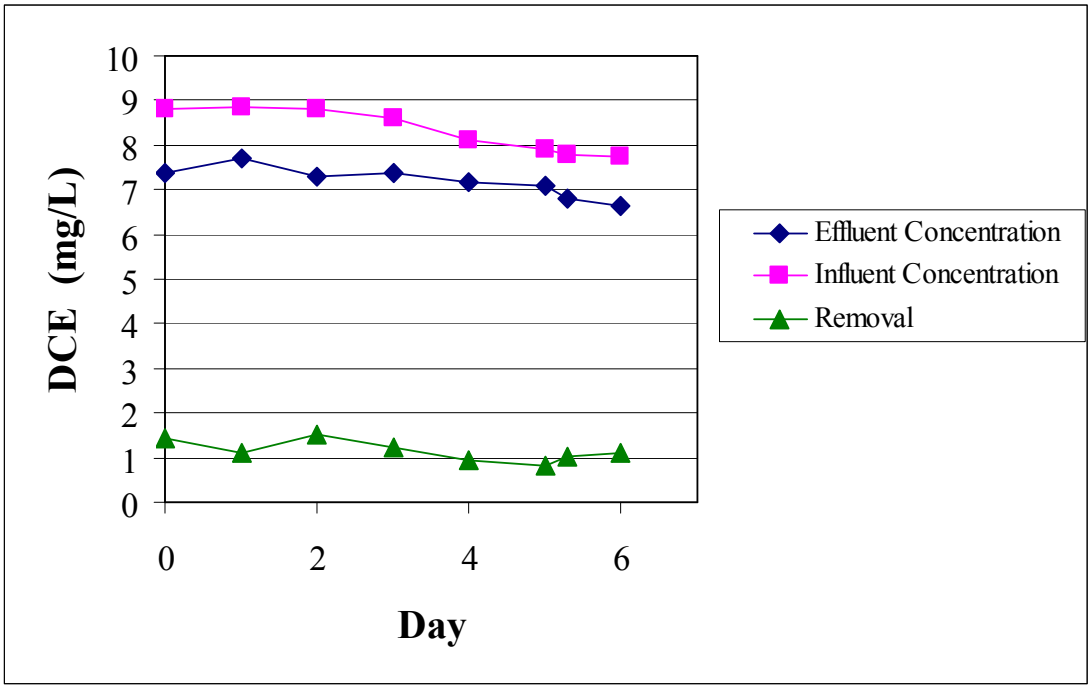

Figure 7-4: Influent and effluent DCE concentrations and DCE removal in a bioreactor without any biological components supplied with DCE-amended aerated media. Each data point represents the concentration in a single influent or effluent sample obtained on the same day (or the difference in these concentrations).

The abiotic removal observed was most likely due to interactions between DCE and the reactor tubing and septum used to seal the top of the rhizosphere chamber. Loss of chlorinated compounds in the presence of Teflon tubing has been observed for TCE (Wrenn, 1992) and polychlorinated biphenyls (Cseh, et al., 1989). In the case of TCE, Wrenn (1992) found that the Teflon tubing became saturated with the chlorinated solvent relatively quickly, and TCE losses became relatively minor after a short contact period. In a study conducted by Kovacs and Kampbell (1999), loss of TCE and PCE was observed in vials containing Teflon coated septum. For a septum

having a surface area approximately equal to that used in this study, there was approximately 12% and 34% loss of TCE and PCE, respectively, after an exposure period of 14 days at 22^{o}C. Based on these observations and others, the authors concluded that larger, less water-soluble molecules were more highly sorbed by the Teflon. Assuming this trend holds for DCE, which is smaller and more water-soluble than TCE or PCE, then it would be expected that less than 12 to 34% DCE in the reactor feed would be sorbed by the Teflon-coated septum used in this study. This is consistent with a total observed loss of 12.7%, which presumably reflects sorption of DCE to both the Teflon-coated septum and the Teflon tubing.

JS666 Only Experiment

After completion of the abiotic experiment, JS666 was added to the bioreactor to specifically evaluate the role of JS666 in removing DCE in the rhizosphere. Table 7-4 outlines the phases incorporated into the JS666-only experiment. 50 mL of a JS666 culture (10^6 CFU/ml) was added, as described above. It was soon observed that there was little DCE removal above abiotic loss, although this can not be examined statistically because steady-state DCE removal never occurred in the JS666 only experiment. In fact, the removal of DCE slowly decreased in the days following the start of the experiment, resulting in less than 10% removal (Figure 7-5). A second 50-mL batch of JS666 (10^5 CFU/ml) was added to the reactor on day nine, in an attempt to increase the biomass concentration and stimulate DCE removal. The process of adding bacteria was modified to allow a longer attachment period (40 hours) in this case, to promote biofilm formation. Further, to ascertain if the bacteria were failing to attach and were instead washing out of the reactor, a 0.1 mL of sample

from the wasting line was collected and plated on ¼-TSA plates for several days after the reactor operation was restored to a flow-through mode. The spread plate results indicated that within a few hours after starting flow, little washout was occurring; however, during the next three consecutive days, there was significant washout, as indicated by a large number of colonies on the plates (> 300 CFU per plate). Concurrent with the washout, little removal of DCE was observed. In fact, the DCE removal was again less than that observed during the abiotic experiment, and it ultimately fell to 0.37 mg/L or 5.1% DCE removed, compared to 1 mg/L or 12.7% removal in the abiotic experiment (Figure 7-5). It is not clear why less DCE removal was observed in the presence of JS666 than in the abiotic experiment. Possible explanations include that the Teflon tubing and/or septum had become saturated with DCE and there was no further abiotic removal of DCE and there may have been DCE contained with the inoculum.

Table 7-4: Phases of operation incorporated into the JS666-only experiment

Operation Phase	Days of Operation	Feed Characteristics	Purpose	Figure
1	0	No Feed	Add JS666	7-5
2	0-6	Aerated, amended with DCE	Measure steady-state removal of DCE	7-5
3	6-8	No Feed	Add JS666	7-5
4	8-10	Aerated, amended with DCE	Measure steady-state removal of DCE	7-5

After flushing the reactor for three days, the bioreactor was disassembled to collect samples of beads for measurement of the biofilm parameters, L_f and X_f. No visible

biofilm was observed on the beads. Three 100 bead samples were collected, from the top, middle, and bottom sections of the bioreactor column. L_f values of 1.4 μm, 0.56 μm and 0.56 μm were measured in the top, middle, and bottom sections, respectively, using Eq. (4). X_f values of 2.997 x 10^7 mg/L, 0 mg/L, and 1.4 x 10^7 mg/L were determined for the top, middle, and bottom sections, respectively using Eq. (5). These results suggested that any attached JS666 biomass was present in a thin, but dense biofilm. In general, thin biofilms tend to be dense, so in general the combination of a small L_f and a large X_f was not surprising. However, the X_f values are orders of magnitude larger than values reported in other studies. For example, Rittmann et al. (1986) measured values of biofilm thickness and density in the range of 18 to 440 μm and 13.1 to 58.0 mg/L, respectively, in an aerobic biofilm reactor containing a mixed culture. Therefore, it seemed likely that the X_f values measured in this study were not accurate. In fact, after reviewing the procedures that were used to measure X_f, it was determined that some mistakes were probably made. Specifically, the beads were not cooled in a desiccator after the first heating period, which may have allowed any biomass and/or the beads to absorb atmospheric water, impacting the weight and, thus, the calculated biofilm parameters. In addition, X_f is a function of L_f (Eq. 5), and because L_f is so small, it is difficult to accurately measure this value, and small changes in L_f can have a large impact on the magnitude of X_f. Despite these problems, it is likely that there was very little biofilm formation on the glass beads and that, at best, it was patchy throughout the bioreactor.

Based on the low DCE removal, the observed washout of bacteria in the JS666-only experiment, and the biofilm measurements, it was concluded that JS666 was unable to form a significant biofilm in the bioreactor. This was unexpected based on the results of the batch studies in which JS666 appeared to attach to the glass beads and the root-JS666 experiment in which little washout was observed. However, the conditions in the batch experiments did not replicate the bioreactor conditions, particularly the shear resulting from the continuous flow and high rate of recycle. This could explain why the bacteria apparently were able to form a biofilm under quiescent batch conditions, but not in the reactor. The fact that little washout was observed in the root-JS666 experiment may suggest that the root provided a more favorable attachment surface compared to the glass beads.

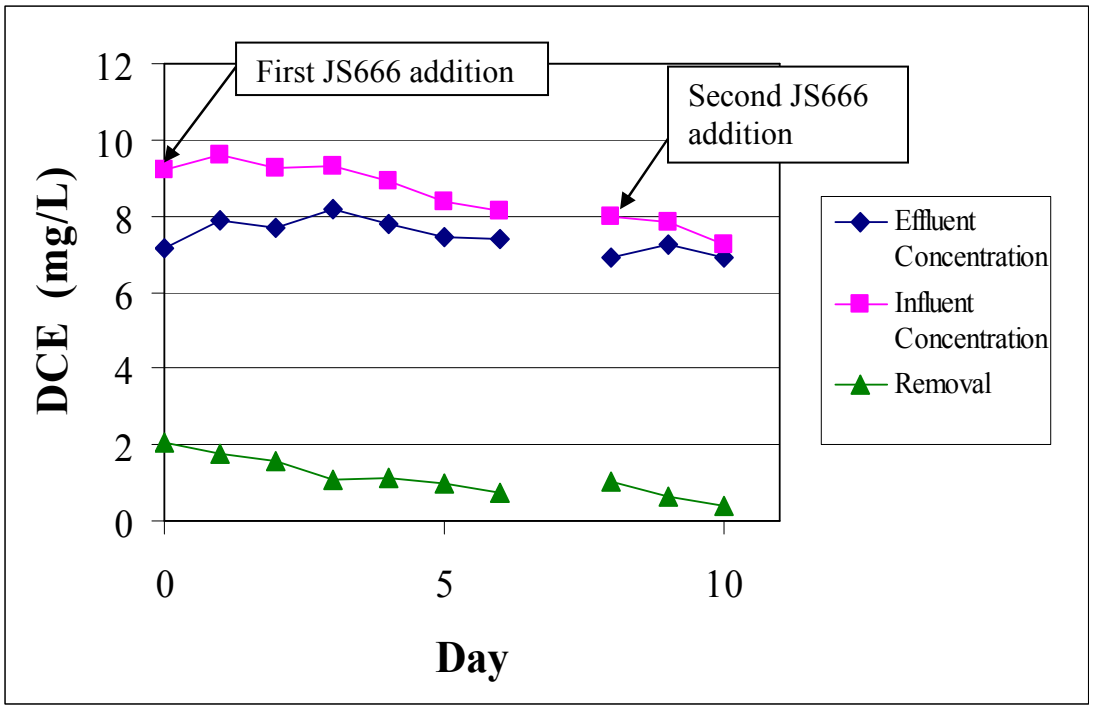

Figure 7-5: Influent and effluent DCE concentrations and DCE removal in a continuous-flow, completely-mixed biofilm reactor containing JS666 only. Each data point represents the concentration in a single influent or effluent sample obtained on the same day (or difference in concentrations of the two samples).

The role of surface roughness in determining whether or not bacteria will attach to a given surface has frequently been reported in the literature. Increased surface roughness probably often enhances biofilm development because microbial attachment initiates in crevices, which are protected from fluid shear and more prevalent in rough surfaces than in smooth surfaces (Annachhatre and Bhamidimarri, 1992). Thus, the glass beads may not have been rough enough to provide sufficient protected crevices for biofilm initiation, although they are commonly used for this purpose in laboratory bioreactor studies. Therefore, an attempt was made to identify a material that would provide a better attachment surface for JS666 and could be purchased in the form of 2-mm beads that could be incorporated into the rhizosphere chamber with the glass beads. Stainless steel is commonly found to be contaminated with bacteria in the food industry (Boulangé-Petermann, 1996) and stainless steel (grade 304L) has a greater surface roughness than glass (Faille et al., 2002). Therefore, it was decided that 2-mm (grade 316) stainless steel beads should be added in future experiments conducted with JS666. The surface of the steel beads was manually scratched with a file to increase surface roughness. 316 grade stainless steel was selected because it is a softer material and was relatively easy to score.

Other factors could have contributed to the inability of JS666 to attach to the glass beads in the absence of a plant root. For example, during the root-JS666 experiment, the reactor operated in recycle-only mode (no feed) for approximately seven days following the addition of the bacteria to allow the DCE levels to decrease. In comparison, the reactor operated in recycle-only mode for approximately two hours

as the recycle rate was ramped up to full speed, following addition of the bacteria in the JS666-only experiment. The extended period of time of operation in recycle-only mode in the root/JS666 experiment may have provided additional opportunities for the planktonic JS666 to attach to surfaces. Therefore, any future experiments conducted with JS666 should incorporate a longer recycle-only period following addition of the bacteria. It should be noted that relatively few effluent samples of the root-JS666 experiment were obtained for analysis of CFU. Therefore, it is possible that significant washout did occur in the root and JS666 experiment but was not detected with the sampling frequency used.

Plant and Associated Microorganisms Experiment (Trial 1)

Given the apparent inability of JS666 to attach to the glass beads under flow-through conditions, no further work with JS666 was conducted in this study. Therefore, an experiment was next conducted with a whole *P. australis* individual in the bioreactor. The surface of the plant root was treated as described above to reduce the size of the native root microorganisms populations before it was inserted into the rhizosphere chamber. In addition to glass beads, stainless steel beads (approximately 1000) were added to the rhizosphere chamber. A series of tests were conducted with this plant to evaluate the importance of different potential roles for wetland plants in removing DCE and related compounds from the rhizosphere. Specifically, the evaluations were designed to determine: (1) the amount of DO released by the plant, (2) the production of plant exudates (measured as COD), (3) the effect of the plant on DCE levels, and (4) the fate of the DCE using radiolabeled substrate. Table 7-5 outlines the phases incorporated into this experiment to accomplish these evaluations.

After the plant was inserted into the rhizosphere chamber, the bioreactor flows, both gas and liquid, were initiated in the start-up phase (Phase 1), which included leak testing the seal between the rhizosphere and foliar chambers. During this time, aerated DCE-free feed was supplied. Once the bioreactor operation was established, an effluent sample was collected to determine plant exudate release under aerated feed conditions (discussed below). Next, anoxic media was supplied to the bioreactor so that DO and exudate production could be measured under environmentally-relevant conditions (Phase 2). In order to best explain the data, the DO data from phase 2 are discussed first, followed by the plant exudate data from phase 1 and 2.

Table 7-5: Phases of operation incorporated into the plant experiment (Trial 1)

Operation Phase	Days of Operation	Feed Characteristics	Purpose	Figure
1	0-15	Aerated, DCE-Free	1) Establish reactor, including seal 2) Measure exudate production	NA 7-7
2	16-22	Anoxic, DCE-Free	1) Measure steady-state DO release 2) Measure exudate production	7-6 7-7
3	23-32	Anoxic, amended with unlabeled DCE	Measure steady-state DCE removal	7-8
4	32-40	Anoxic, amended with labeled DCE	Measure steady-state [^{14}C]-DCE removal	7-9

The percent DO of the bioreactor influent (measured following the break tube [Figure 6-1]) apparently never reached zero, in part because the media supply could not be fully deoxygenated. Media supply DO levels ranged from 0.7 to 1.9% (0.3 to 0.82

mg/L) DO. Even more importantly, the bioreactor could not be assembled without entrapping some air in the break tube in the feed line, which undoubtedly contributed to the DO measured in the reactor influent. Over time the influent DO concentration decreased from 3.8 to 2.8 mg/L, as shown in Figure 7-6, indicating that the air in the break tube was slowly dissolving into the feed and being depleted. When the influent DO level was 3.8 mg/L, it was considered sufficiently low enough to measure the amount of DO released by the plant. Influent and effluent DO levels were measured from day 16 through 22. On day 21 it appeared that steady-state with respect to oxygen release had been reached, based on the statistical test conducted ($p > 0.05$). The results are shown in Figure 7-6 and indicate that there was an average 2.48 mg/L DO net increase in the rhizosphere during the steady-state period (days 18-22).

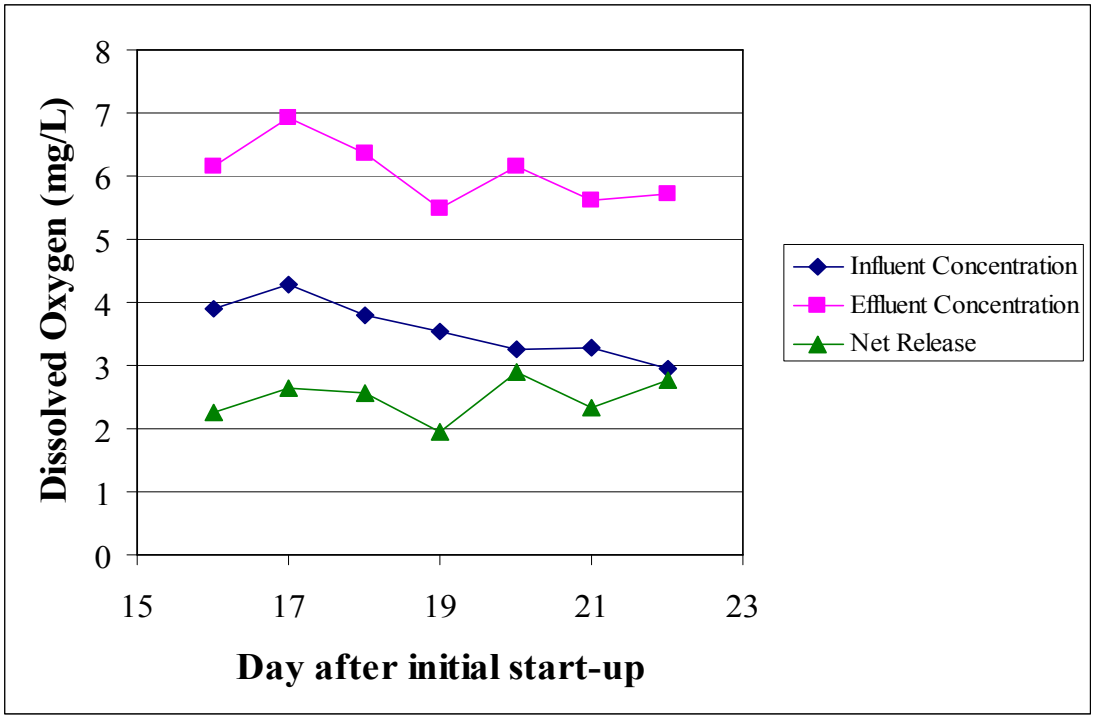

Figure 7-6: Influent and effluent oxygen concentrations and net change in oxygen in a continuous-flow, completely-mixed bioreactor containing a *P. australis* individual and associated native microorganisms and fed DCE-free anoxic media (Trial 1). Each data point represents the concentration in a single influent or effluent sample obtained on the same day (or the difference in the concentrations in these samples).

The net DO increase is the difference between the effluent and influent DO values and takes into account processes occurring in the reactor that can both increase DO (e.g., plant root aeration) and decrease DO (e.g., endogenous microbial respiration). A previous study by Massacci et al. (2001) indicated that the oxygen concentrations in the proximity of a *P. australis* root can reach the same concentration as found in the atmosphere (20.9%). Based on this information, it was anticipated that the effluent DO concentrations would reach levels close to atmospheric; however, the effluent concentration averaged 14.0% (5.74 mg/L) during the DO testing period. Possible explanations for a lower than anticipated DO content include lower than normal aeration by the plant and plant root and respiration by microorganisms and/or roots.

Significant numbers of microorganisms were introduced to the bioreactor with the plant and subsequently disassociated from the plant. To evaluate the role of microorganisms in decreasing the DO levels, spread plates were prepared with effluent samples five days after the initial start-up. Microorganisms were present at concentrations much greater than 300 CFU/ml and therefore were likely consuming a significant amount of the oxygen released by the plant. Respiration by root matter may also have decreased the net oxygen released.

In addition to microbial respiration leading to a lower than anticipated DO concentration in the bioreactor, it is possible that the plant in the bioreactor may not have been able to aerate the root zone to the same extent as a plant in natural

conditions. Several observations support this statement. First, the approximately one foot tall shoot in the bioreactor was considerably shorter than that of a full-grown plant (six to seven feet tall), which reduced the amount of surface area available for intake of oxygen that could be transported to the root zone via passive diffusion. Second, the old shoots, which were cut off before placing the plant in the bioreactor and are capable of generating convective air flow through *P. australis* that contributes to root aeration (Colmer, 2003) were below the septum in the rhizosphere chamber. As a result, convective air flow presumably could not occur in the bioreactor plant, reducing its overall aeration capacity. There was evidence that the cut shoots could contribute to root aeration in the bioreactor because bubbles were released from the cut shoots during the start-up period. However, bubbling was undesirable because it had a negative impact on the hydraulic operation of the reactor; therefore, caulk was applied to the cut shoots to stop the bubbling. This likely reduced or even eliminated their ability to contribute to rhizosphere aeration. Finally, the health of the plant declined after it was placed in the bioreactor, as revealed by the yellowing of the leaves and stems; however, a new green shoot was emerging during this period of testing. Presumably, a green and healthy plant transmits more oxygen to the roots compared to a less healthy plant or a shoot which has not fully developed.

Abiotic factors may also have contributed to the net release of DO; however, this has not yet been tested. Assuming that the DO release observed was due solely to plant activity, the observed release of oxygen into the rhizosphere chamber has important implications. Most importantly, it indicates that *P. australis* has the capability to

generate appropriate redox conditions for aerobic microorganisms. This suggests that if the appropriate aerobic microorganisms are present, the potential exists for oxidation of DCE. Depending on the biofilm density present in the reactor, microbially-mediated oxidation of DCE was expected to represent a significant percent of the ^{14}[C]-DCE removal in phase 4 of the experiment.

Influent and effluent COD concentrations were measured periodically during the period of DO release measurements, in addition to the COD measurement made prior to the period of the DO test, as described above to test for plant exudates. As shown in Figure 7-7, the effluent COD concentration was higher than the influent concentration on two out of the three days when both measurements were made. One pair of measurements was taken when the reactor was supplied with aerated feed (day 8), and the other pair was taken during a period of anoxic feed (day 22). The COD measured in the feed on all sampling days was most likely the result of the oxygen demand exerted by the various inorganic chemicals present in the media. The effluent COD was approximately equal to the influent COD on day 17, which was two days after anoxic feed was initiated (Figure 7-7). This was due to an increase in the influent COD concentration between days 8 and 17, rather than a decrease in effluent COD during this period. A likely explanation for the increase in the influent COD concentration is the switch to anoxic media. To directly test this idea, a fresh batch of deoxygenated media was prepared and analyzed for COD concentration and then the media was aerated and reanalyzed for COD concentration. The deoxygenated media was shown to have a higher COD content, by at least 6 mg/L,

compared to the aerated media. As can be seen in Figure 7-7, this is consistent with the results of the next set of measurements made (day 22) after the switch to anoxic feed. The effluent COD was again significantly higher than the influent COD concentration, resulting in a net COD release of 19.5 mg/L. The overall net increase of COD was presumably a result of plant exudates being released into the rhizosphere chamber. At least some of the exudates could presumably serve as a carbon source (growth substrate) for the native root microorganisms and, thus, cometabolism of DCE by organisms growing on exudates could conceivably occur. However, competition between DCE and the growth substrates (exudates) for the enzymes involved in DCE cometabolism and other factors could decrease the extent of DCE cometabolism actually observed.

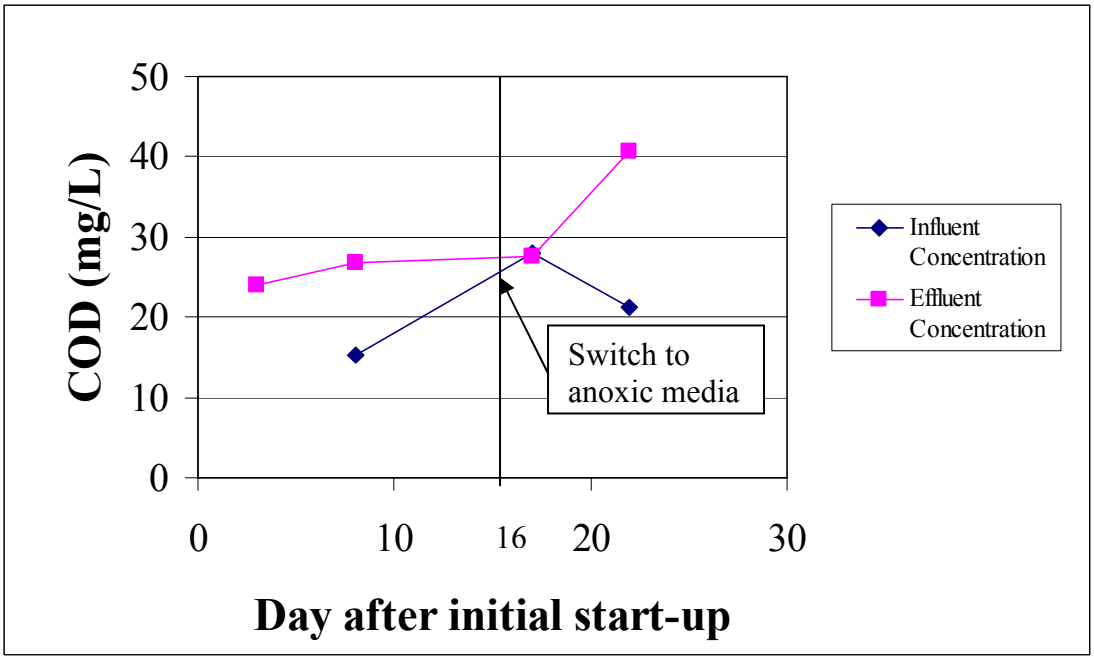

Figure 7-7: Influent and effluent COD concentrations in a continuous-flow, completely-mixed bioreactor containing *P. australis* plant and native microorganisms in a bioreactor supplied with both DCE-free aerated and anoxic media (Trial 1). Each data point represents the concentration in a single influent and single effluent sample obtained on the same day.

On the 23rd day after initial start-up, after the DO and COD measurements were completed, unlabeled DCE was added to the reactor feed. As previously done, the influent and effluent concentrations were monitored to determine the net DCE removal. For the first few days, it was observed that the influent DCE concentration decreased more rapidly than observed in the past. For example, between days 23 and 24, there was a decrease of 2 mg/L (Figure 7-8). In previous experiments, the maximum daily decrease seen was 0.5 mg/L, which is nearly an order of magnitude lower. This "normal" decrease in aqueous DCE concentration was thought to be due to equilibrium of a decreasing total DCE mass in the feed bottle between the gas and aqueous phases. On day 24, additional DCE was added to the feed bottle to compensate for this loss, causing the influent DCE concentration to increase to 9 mg/L on day 25. However, by the following day, the influent DCE concentration had decreased again to 7.6 mg/L. One potential explanation for the higher-than-normal decrease in influent DCE concentrations might have been loss of DCE through the nitrogen supply line that connected the feed supply bottle and the constant pressure vessel (Figure 6-1) and was used to maintain a constant pressure in the feed supply bottle. Up to this point, the constant pressure system had only been used during the period of the DO experiment and not in any previous experiments. The rapid decrease in the influent DCE concentration was undesirable because it complicated measurement of DCE removal. Therefore, the constant pressure system was disconnected on day 26 (Figure 7-8). The constant pressure system was disconnected by using isolation valves so that oxygen could not be introduced to the feed supply bottle. Steady-state removal of DCE began within one day after disconnecting the

constant pressure system, as determined by the statistical analysis ($p > 0.05$). Removal of unlabeled DCE was monitored for six days and averaged 1.88 mg/L, or 30.5% removal, during this period. Based on the results of the abiotic control experiment, removal of 12.7% of the influent DCE was presumably due to abiotic losses. Therefore, removal of 17.8% of the influent DCE was probably attributable to the presence of the plant and/or associated microorganisms.

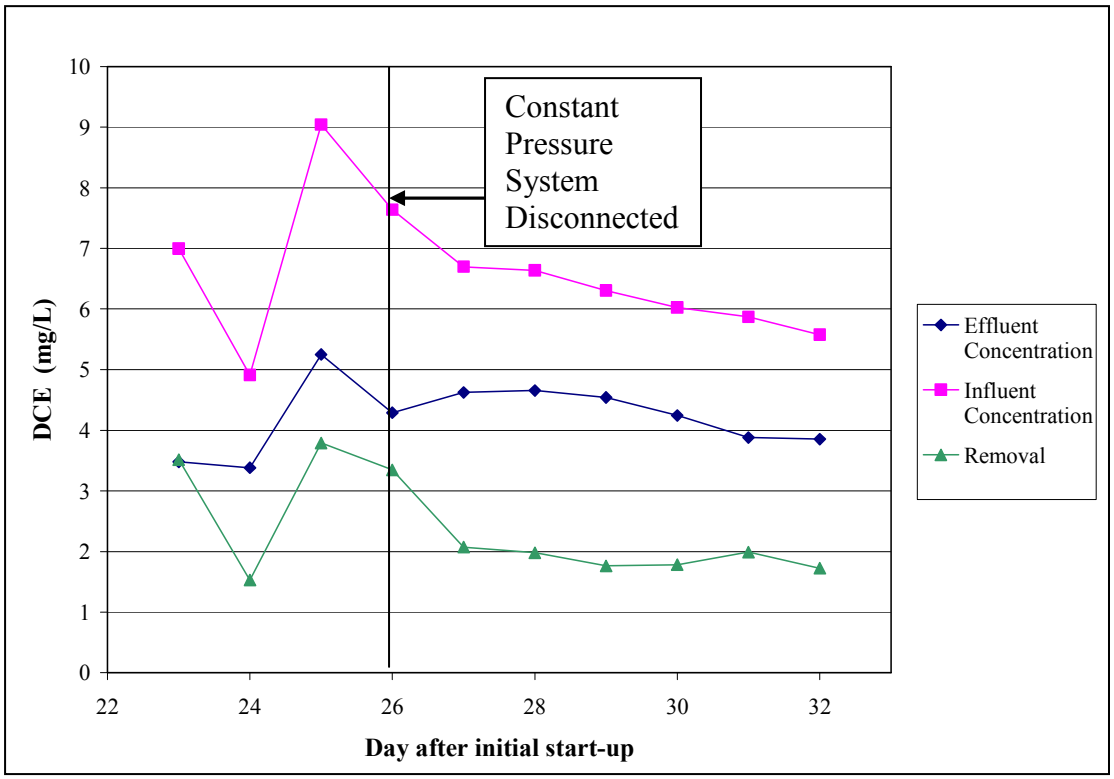

Figure 7-8: Influent and effluent DCE concentrations and DCE removal in a continuous-flow, completely-mixed bioreactor containing a *P. australis* individual and associated microorganisms supplied with DCE (unlabeled)-amended anoxic media (Trial 1). Each data point represents the concentration in a single influent or effluent sample obtained on the same day (or the difference in the concentrations in these samples).

To improve our understanding of the mechanisms contributing to the observed removal of DCE in the presence of *P. australis* and microorganisms introduced with plant, ^{14}[C]-DCE was added to the feed supply bottle on the 32nd day after initial start-

up of the bioreactor. The reactor was sampled as described above to capture the ^{14}C fractions produced in the liquid, gaseous, and solid phases. The ^{14}C content in the liquid fractions was measured daily, the gaseous CO_2 traps were measured and exchanged daily, and the remaining fractions, gaseous DCE and plant and biofilm solid fractions, were measured at the conclusion of the experiment after steady-state ^{14}[C]-DCE removal was reached. Figure 7-9 shows the daily ^{14}C content in the liquid effluent fractions including, volatile ^{14}C (presumably ^{14}[C]-DCE), $^{14}CO_2$, ^{14}C-biomass, and ^{14}C-labeled non-volatile residues; ^{14}C influent activity (presumably ^{14}[C]-DCE); and ^{14}[C]-DCE removal over a eight day period. During this period, steady-state with regard to ^{14}[C]-DCE removal was reached, as determined by statistical analysis ($p > 0.05$). The average ^{14}[C]-DCE removal was 9.28 μCi/L or 31.4% during the steady-state period, consistent with the removal of unlabeled DCE.

The measurement of ^{14}[C]-DCE and $^{14}CO_2$ from the liquid fractions, was presumptive; however, the presence of ^{14}[C]-DCE and $^{14}CO_2$ was confirmed in a single sample through GC analysis and precipitation of $^{14}CO_2$ using $Ba(OH)_2$, respectively. DCE was the only volatile compound detected in both the effluent and influent using GC. The precipitation of $^{14}CO_2$ as $BaCO_3$ resulted in a ^{14}C activity in the NaOH trapping vial (described above) of 36.58 DPM, which was similar to the activity in the blank that was treated in the same manner (27.68 DPM). In comparison, the activity in the presumptive $^{14}CO_2$ fraction collected on the same day of this test was 1083 DPM. Thus 99% of the activity in this fraction could actually be attributed to $^{14}CO_2$.

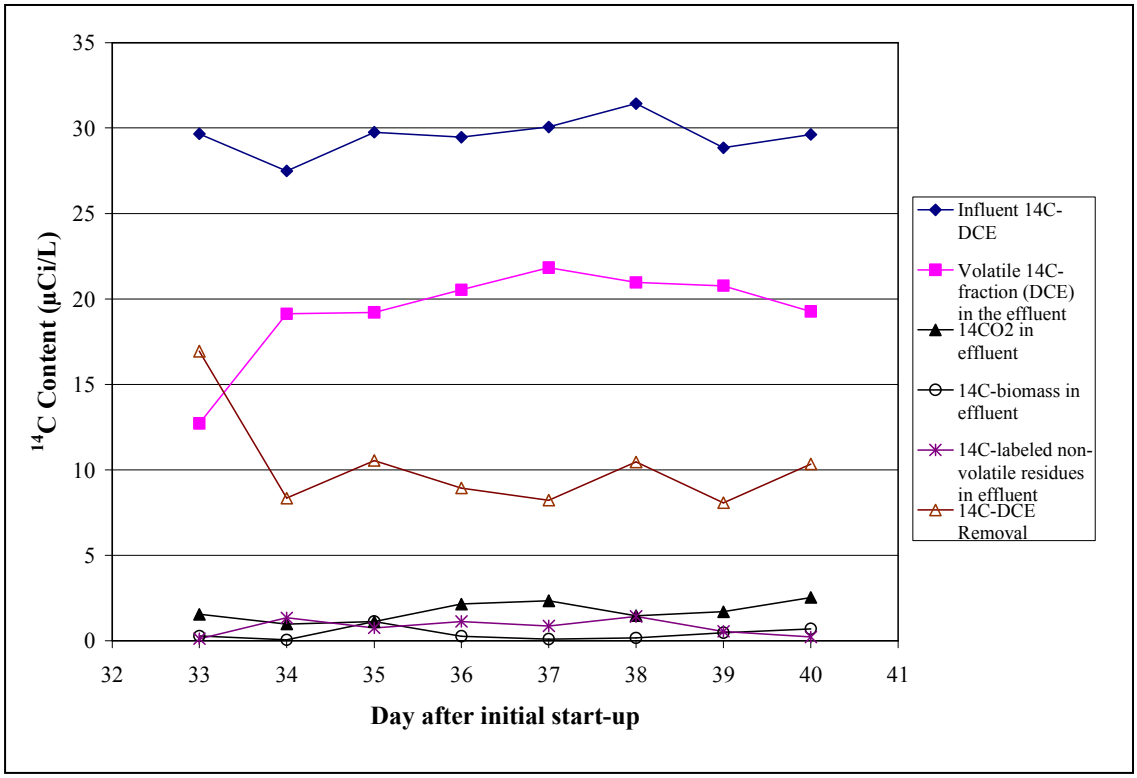

Figure 7-9: Influent ^{14}[C]-DCE and ^{14}C activity associated with volatile, CO_2, biomass, and non-volatile residue fractions in the effluent and ^{14}C-DCE removal, in a continuous-flow, completely-mixed bioreactor containing a *P. australis* individual and associated microorganisms supplied with ^{14}[C]-DCE-amended anoxic media (Trial 1). Each data point represents the average of duplicate analyses.

After steady-state ^{14}[C]-DCE removal had been reached and all fractions had been analyzed for their ^{14}C content, a mass balance on the ^{14}C was conducted to determine the percent ^{14}C recovered in each fraction, the total recovery of the ^{14}C supplied to the reactor, and the percent removal of ^{14}[C]-DCE. This analysis was conducted over the steady-state period (days 34-40). Because those fractions that were collected at the conclusion of the experiment (e.g. biomass) had to be characterized in terms of ^{14}C activity per experiment, the activity in the fractions collected on a daily basis (e.g. liquid fractions) also had to be expressed in terms of ^{14}C activity per experiment. For the liquid fractions, this was done by multiplying the measured activity in a given

volume (μCi/L) by the flow rate (6 mL/hr) and the duration of the steady-state period. Table 7-6 shows the total ^{14}C recovery and the percent of ^{14}C recovered in each fraction analyzed during the experiment. It should be noted that it is not clear if the plant biomass and $^{14}CO_2$ (gas phase) were statistically significant fractions. With a total ^{14}C recovery of 87.7%, 12.3% of the influent activity was unaccounted for in the bioreactor. During the abiotic control experiment, there was 12.7% removal of DCE, presumably due to abiotic losses, as described above. Therefore, the 12.3% of the influent ^{14}C that was unaccounted for was presumably due to abiotic losses.

Table 7-6: Steady-state distribution of ^{14}C activity within each fraction analyzed in a continuous-flow, completely-mixed bioreactor containing a *P. australis* individual and associated microorganisms supplied with ^{14}[C]-DCE-amended anoxic media (Trial 1).

Fraction	Recovered ^{14}C Activity (μCi)	% influent ^{14}C Recovered
Influent	29.8	--
Liquid Effluent[1]		
^{14}[C]-DCE	20.41	68.6
$^{14}CO_2$	1.77	5.9
^{14}C- biomass	0.42	1.4
^{14}C-Non-Volatile residues	0.91	3.1
Gas Effluent		
^{14}[C]-DCE	1.96	6.6
$^{14}CO_2$	0.08	0.25
Biomass		
^{14}C-Biofilm on beads	0.36	1.2
^{14}C-Plant (and root associated biofilm)	0.18	0.60
Total	**26.09**	**87.7**

1: Calculated as the sum of the daily measurement of ^{14}C activity (μCi/L) x flowrate x 24 hr.

The overall removal of [^{14}C]-DCE was 31.4%; however, the removal due to biotic factors alone was approximately 19.1%. These values are in close agreement with the measured removal of unlabeled DCE. The total average removal of unlabeled DCE was 30.5%, with 17.8% removal likely due to biotic factors, because 12.7% of the total was presumably due to abiotic losses (based on the results of the abiotic control experiment).

The daily liquid fraction measurements, shown in Figure 7-9, show that activity in the $^{14}CO_2$, ^{14}C-biomass, and ^{14}C-labeled non-volatile residues fractions was present within one day after introducing ^{14}[C]-DCE. The $^{14}CO_2$ activity was typically the highest of the three, followed by the activity of the ^{14}C-labeled non-volatile residues. Thus, the results of the analysis indicated that some DCE (~10%) was being transformed by microorganisms into CO_2 plus non-volatile residues. The non-volatile residues are suspected to be intermediates in the complete oxidation of DCE. The epoxide formed from DCE oxidation is a short-lived, unstable compound that undergoes abiotic transformations to various products, including aldehydes and acids (Vogel et al., 1987), which would be detected as non-volatile residue. Thus, complete transformation to $^{14}CO_2$ appears to have occurred to a greater extent than incomplete transformation to ^{14}C-labeled non-volatile compounds. The former is more desirable because the non-volatile compounds that form in some instances possess toxic properties. For example, one intermediate transiently formed during TCE oxidation, chloral hydrate, is a controlled substance and mutagen (Newman and Wackett, 1991); however, no attempt was made to identify the non-volatile compounds in this study,

so it is not known if they have any toxic properties. Activity in the effluent biomass may have been due to microbial metabolism of ^{14}[C]-DCE, ^{14}C-labeled non-volatile residues, or $^{14}CO_2$. If the fractions potentially reflecting oxidative pathways, CO_2 (gas and aqueous) and non-volatile residue, are combined, then the most predominant removal mechanism operating in the bioreactor appears to be oxidation, accounting for 9.3% removal of the ^{14}[C]-DCE introduced in the feed. However, the amount of ^{14}[C]-DCE oxidized may be higher because the microbial biomass (suspended and attached) and plant presumably took up $^{14}CO_2$ and/or ^{14}C-labeled non-volatile residues. If activity associated with the biomass is also attributed to oxidation, then up to 12.5% of the ^{14}[C]-DCE removal may be due to oxidation.

^{14}[C]-DCE removal via oxidation was not unexpected because oxygen was present in the system, as demonstrated above, and microorganisms were known to be present, and presumably grew, in the system. However, until the ^{14}C tracer was added, it was not known whether the microorganisms were actually acting upon the DCE. Based on previous studies it was suspected that microorganisms introduced with the plant would be able to transform DCE. For example, Bankston et al. (2002) monitored ^{14}[C]-TCE removal in a batch microcosm containing a *T. latifolia* individual and soil with native microorganisms and recovered 5.3% of the ^{14}C-activity as $^{14}CO_2$. It should be noted that in the study by Bankston et al. (2002), the plant and soil were obtained from a site known to be contaminated with TCE. In contrast, in this study, the *P. australis* plants were collected from a site with no known history of chlorinated solvent contamination, which demonstrates that it is possible for transformation to

occur without previous exposure of plants and microorganisms to DCE. This phenomenon was also observed in a study by Klier et al. (1999), in which removal of all DCE isomers was observed in mixed cultures obtained from soil that had no previous exposure history to chlorinated compounds. Overall, the results obtained in this study demonstrate that the native microorganism populations associated with the *P. australis* collected in this study have the ability to transform DCE using plant-supplied oxygen. Thus, in a natural wetland containing *P. australis*, a significant amount of oxidation of DCE is also expected to occur.

To determine the ^{14}C-activity associated with the attached biomass along the rhizosphere chamber, the rhizosphere chamber was vertically divided into four sections. 100 glass beads were obtained from each section and analyzed for ^{14}C-activity and the total amount of biomass (measured as L_f and X_f as described in Eq. (4) and (5)). It should be noted that the stainless steel beads were excluded from this analysis. The first section consisted of the top 10% of the chamber, which contained a green algal biofilm. The remaining beads were vertically divided (approximately into thirds) to create the top, middle, and bottom sections. As shown in Table 7-7, there was a thin dense biofilm throughout the reactor, similar to the biofilm characteristics in the experiment containing JS666 only. The biofilm density in the bottom layer was more than an order of magnitude higher than in the rest of the reactor, although there was the least amount of ^{14}C-activity recovered in this section. The variability in biofilm density may be the result of measurement error, because the difference in weight between the various treatments used to determine the solids

concentration (described above) was small (in the mg range). Uneven mixing of the substrates (e.g., oxygen and/or ^{14}[C]-DCE) on a micro-scale could also have contributed to a heterogeneous biofilm formation. The ^{14}C activity in the biofilm was also not found to be uniformly distributed in the rhizosphere chamber. Instead, there was a slight gradient in the activity of ^{14}C-labeled biomass along the length of the chamber. The most activity was recovered in the algal layer, which could be the result of uptake of $^{14}CO_2$ through photosynthesis. The amounts of activity measured in the remaining sections were similar and may reflect metabolism of ^{14}[C]-DCE oxidation products and/or ^{14}[C]-DCE. Sorption of ^{14}[C]-DCE and/or its oxidation products to biomass also could have contributed to the activity in each of these sections.

Table 7-7: Activity of ^{14}C associated with the attached biomass and biofilm thickness and density as a function of depth in the rhizosphere chamber in a continuous-flow, completely-mixed bioreactor containing a *P. australis* individual and associated microorganisms supplied with ^{14}[C]-DCE-amended anoxic media (Trial 1).

Reactor Section	L_f (μm)	X_f (mg/mL)	Total Activity (μCi)
Algal	0.21	2305.84	0.15
Top	0.07	3952.87	0.09
Middle	0.18	2371.72	0.07
Bottom	0.11	38,540.50	0.05

The microorganisms responsible for oxidation were present as a result of incomplete root surface sterilization and became established in the bioreactor in the weeks leading up to the addition of DCE to the reactor following initial start-up. In fact during this period, algae growth was visually observed in the upper layers of the beads and along the plant stem below the septum, which demonstrated the ability of

at least some of the native plant root microorganisms to survive in the reactor. The algal source in the rhizosphere chamber was the plant because algae were seen on the plant roots prior to insertion into the rhizosphere chamber (even after surface treatment). Additionally algae were never visually observed in the feed supply bottle or the feed tubing, only in the upper portion of the rhizosphere chamber, indicating that algae were not growing in the feed supply bottle.

It seems likely that the microbial transformation of DCE in the bioreactor was the result of cometabolic rather than metabolic activity, for several reasons: (1) the extent of DCE oxidation and incorporation into microbial biomass was not extensive (Figure 7-9 and Table 7-6), as expected for cometabolic transformations (Alexander, 1981); (2) there are more known aerobic chlorinated ethene cometabolizers compared with characterized aerobic organisms that transform these compounds through metabolic processes (Coleman et al., 2002); and (3) the plant exudates presumably were available as growth substrates that could support cometabolic processes. However, it should be noted that all of this is speculative and the exact nature of the DCE oxidation mechanism is unknown.

Phytovolatilization of DCE was also an important process in the bioreactor as revealed by the presence of activity in the ^{14}[C]-VOC traps (presumably ^{14}[C]-DCE). Phytovolatilization accounted for 6.6% (Table 7-6) of the removal of the ^{14}[C]-DCE introduced in the feed. It should be noted that occasionally the pressure in the foliar chamber fell below atmospheric, creating a slight vacuum (max 3" H_2O or 0.0074

atm). It is possible that the vacuum may have enhanced phytovolatilization by "pulling" ^{14}[C]-DCE through the plant; however, because the vacuum pressure was very small, it is expected that any enhancement was negligible. Phytovolatilization is a less desirable removal mechanism compared to oxidation, because unlike oxidation, phytovolatilization is a non-destructive removal mechanism. The ability of *P. australis* to volatilize DCE was not unexpected because phytovolatilization of TCE has been found to occur with hybrid poplars (e.g., Burken and Schnoor, 1998). In addition, *P. australis* contains an internal diffusion pathway that, in addition to increasing oxygen diffusion into the roots, should allow for diffusion of volatile compounds up from the roots and into the shoots.

Interestingly, there was little evolution of gaseous $^{14}CO_2$, as shown in Table 7-6. This may be the result of little $^{14}CO_2$ uptake by the plant roots and/or funneling of $^{14}CO_2$ taken up by the roots into photosynthetic pathways in the shoots. The gaseous $^{14}CO_2$ data collected on a daily basis are shown in Figure 7-10 and reveal that there were fluctuations in the daily release of $^{14}CO_2$, which may reflect changing rates of photosynthesis in the plants and/or daily differences in trapping efficiencies as a result of variability in the gas flow rate through the traps.

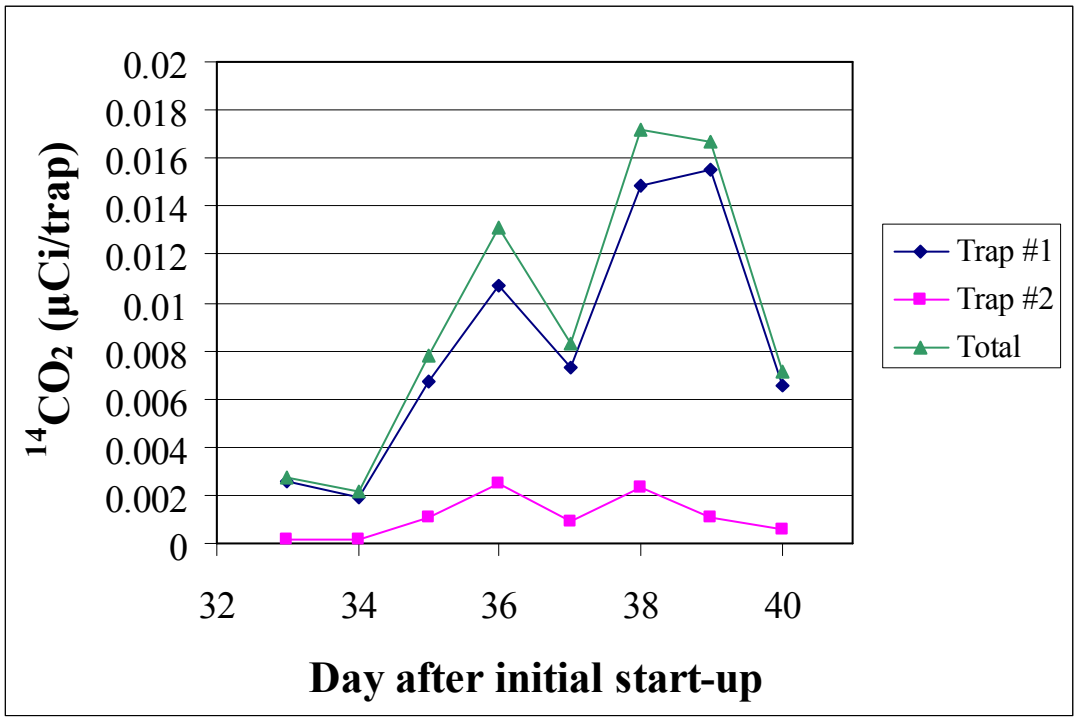

Figure 7-10: ^{14}C activity associated with two CO_2 traps in series used to capture $^{14}CO_2$ from the gas phase of a continuous-flow, completely-mixed bioreactor containing a *P. australis* individual and associated microorganisms supplied with $^{14}[C]$-DCE-amended anoxic media (Trial 1). Each data point represents the average of four analyses.

The smallest fraction of ^{14}C recovered (excluding gaseous CO_2) was in the plant biomass, indicating that there was limited incorporation of DCE into the biomass and limited plant metabolism of DCE. When the plant was oxidized to quantify its total ^{14}C-activity, it was also separated into various sections to determine how the ^{14}C-activity was distributed throughout the plant. The following sections were oxidized and analyzed for ^{14}C-activity separately: (1) the new shoot that developed during the course of the experiment; (2) the original shoot, which was further divided into two categories (pieces that had yellowed during the experiment and pieces that had remained green); and (3) the root, which was further divided into the rhizome and the root hairs extending from the rhizome. The activity of each section was measured,

and the activity per mass of each section was calculated. The pieces were weighed after the plant had air-dried overnight. As can be seen from Table 7-8, the highest ^{14}C-activity was observed in the new shoot, followed by the ^{14}C-activity in the root hairs. If the ^{14}C-activity in the new shoot was primarily the result photosynthetic uptake of $^{14}CO_2$, then it could explain why there was more ^{14}C-activity in the new shoot compared to the other sections. As previously mentioned, photosynthetic $^{14}CO_2$ uptake could also explain why there was little evolution of $^{14}CO_2$ by the plant. There was less ^{14}C-activity in the yellowed sections of the leaves compared to the new green shoot, presumably because green shoots carry out more photosynthesis than yellow shoots. However, the detection of some activity in the yellow shoots suggests that these shoots may have also played a role in DCE removal. This is important because it may mean that some removal of DCE due to plant effects could continue in the field after the growing season into the fall and winter, although the amount of DCE removal may decrease significantly when photosynthesis stops. ^{14}C-activity per unit biomass in the root hairs exceeded that in the rhizome by two orders of magnitude, which may be the result of less surface area for DCE uptake in the rhizome compared with the root hairs. Detention of ^{14}C in the rhizome also might be shorter than in the root hairs because the rhizome appears to have more internal porosity compared to the root hairs, which could result in quick passage of ^{14}C through the rhizome to the shoot. The root hairs presumably grew during the course of the experiment, whereas no growth of the rhizome was observed. Thus, the higher ^{14}C content in the root hairs could be the result of a higher rate of metabolism resulting in incorporation of ^{14}C into biomass in the root hairs compared with the rhizome. It is important to note that

the ^{14}C-activity in the root hairs and the rhizome could be the result of both plant and root-associated microbial activity; therefore, it is difficult to say if the ^{14}C recovered in these biomass fractions is the result of plant or microbial metabolism. However, overall, the highest activity was found in those sections where growth was occurring, new shoot and root hairs, which supports the idea that the greatest amount of DCE removal in the field probably will occur when wetland plants are growing. Although the amount of ^{14}C recovered in the plant was relatively small, it should be noted that the plant shoot (approx 1 foot tall) used in the bioreactor was considerably smaller than a full-grown plant, which can reach heights of at least 6 to 7 feet if not more. Because the highest concentration of activity was found in the new shoot, it is possible that a taller plant would remove much greater amounts of DCE than observed in the laboratory experiment.

Table 7-8: Activity of ^{14}C associated with the *P. australis* and associated root microorganisms as a function of section in a bioreactor containing a *P. australis* individual and associated microorganisms supplied with $^{14}[C]$-DCE-amended anoxic media (Trial 1).

Section	Dry Biomass (g)	Activity (μCi)	Activity/ biomass (μCi/g)
New Shoot	0.064	0.031	0.488
Old Shoot-Yellow	0.065	0.008	0.115
Old Shoot-Green	0.077	0.006	0.072
Root Hair (average of 5 samples)	0.091	0.020	0.23 ± 0.19
Rhizome (average of 4 samples)	0.980	0.008	0.01 ± 0.02

The growth of a new shoot during the experiment indicates that the concentrations of DCE used during the experiment, 5 mg/L (0.05 mM) to 10 mg/L (0.1 mM), did not completely inhibit plant growth and the plant was able to maintain normal functions in the presence of DCE. These results are in good agreement with a previous study by Dietz (2000) that examined the phytotoxic effects of various chlorinated compounds, including DCE, on poplars. In that study, the author found that there was zero growth above 6.0 mM DCE. Growth occurred at lower DCE concentrations, although to a lesser extent than in the absence of DCE, and decreased linearly from 0 to 6.0 mM DCE.

Plant and Associated Microorganisms Experiment (Trial 2)

A second plant-only experiment was conducted to determine if the removal of DCE by various processes in the presence of a *P. australis* individual with root-associated microorganisms observed in Trial 1 was reproducible. There were some minor differences in the experimental procedures used in the two experiments. Most importantly, the root surface was not sanitized in Trial 2 because it was found in the previous experiment that the native microorganisms contributed significantly to DCE removal. Instead, a second *P. australis* individual was placed directly in a second rhizosphere chamber. When the rhizosphere chamber used in Trial 1 was removed from the bioreactor set-up for analysis of plant and attached microbial biomass, it was replaced with the second rhizosphere chamber and plant without resterilizing the various sections of tubing and other bioreactor components. Unlabeled DCE was introduced the following day, once it was confirmed that the seal between the rhizosphere and foliar chambers was sound. Table 7-9 outlines the phases

incorporated into the Trial 2 experiment with the plant and associated microorganisms.

Table 7-9: Phases of operation incorporated into the plant experiment (Trial 2)

Operation Phase	Days of Operation	Feed Characteristics	Purpose	Figure
1	1	Anoxic, DCE-Free	Establish reactor, including seal	NA
2	1-10	Anoxic, amended with unlabeled DCE	1) Monitor steady-state DO release 2) Measure steady-state DCE removal	7-11 7-12
3	10-14	Anoxic, amended with labeled DCE	Measure steady-state [^{14}C]-DCE removal	7-13

DO measurements were made concurrently with DCE measurements. Steady-state with respect to net DO change was established within the five-day period, as determined through statistical analysis ($p>0.05$). A net loss of DO, averaging 0.18 mg/L, was observed over the five-day period when DO measurements were taken, as shown in Figure 7-11. In contrast, a net increase in DO levels was observed in Trial 1 conducted with a plant and associated microorganisms. Two factors probably were primarily responsible for the different DO results in the two experiments. First, DCE was not included in the feed while changes in DO were monitored in Trial 1, as it was in Trial 2. As discussed below, DCE removal did occur during phase 2 of Trial 2. Presumably a significant amount of the DO removal was due to DCE oxidation, an oxygen-demanding process that was not active during the period of DO measurements (Phase 2) in Trial 1. Second, the net DO loss was presumably due in

part to a large microbial population associated with the root, because no attempt was made to reduce the abundance of root microorganisms as in Trial 1. In fact, it was noted that during phase 2 of Trial 2 the water above the beads in the rhizosphere chamber was cloudy, but cleared a few days later, presumably due to washout of the high concentration of suspended microorganisms from the rhizosphere chamber. No cloudiness was ever observed in the reactor during Trial 1, in which a net increase in DO was observed. In addition to oxidizing DCE, these root-associated microorganisms in Trial 2 could have exerted a greater oxygen demand than the root microorganisms in Trial 1 by oxidizing larger amounts of exudates and through increased levels of endogenous respiration. Thus, overall, the DO results obtained in Trial 2 support the idea that root-associated microorganisms are able to utilize oxygen supplied by the plants for DCE oxidation and other processes.

It was expected that there would be increased DCE removal in Trial 2 compared with the previous experiment because of the larger population of root-associated microorganisms in Trial 2. This appeared to be the case initially. As shown in Figure 7-2 in the first three to four days, DCE removals of ~4 mg/L were observed (except for on day 2 at which time the DCE effluent concentration should have still been ramping up based on the tracer study). In comparison removals of ~2 mg/L were observed in Trial 1 (Figure 7-8). However, the amount of DCE removal in Trial 2 decreased steadily until day eight. During this period, a significant amount of suspended biomass was washed out of the rhizosphere chamber, as indicated by the decrease in cloudiness and may explain why the DCE removal decreased. By day

eight, steady-state with respect to DCE removal was reached, as determined with the statistical test ($p>0.05$). However, it took seven days after the initial addition of DCE before steady-state was reached, whereas in the previous experiment, steady-state DCE removal was observed within one day after the influent DCE concentration stabilized.

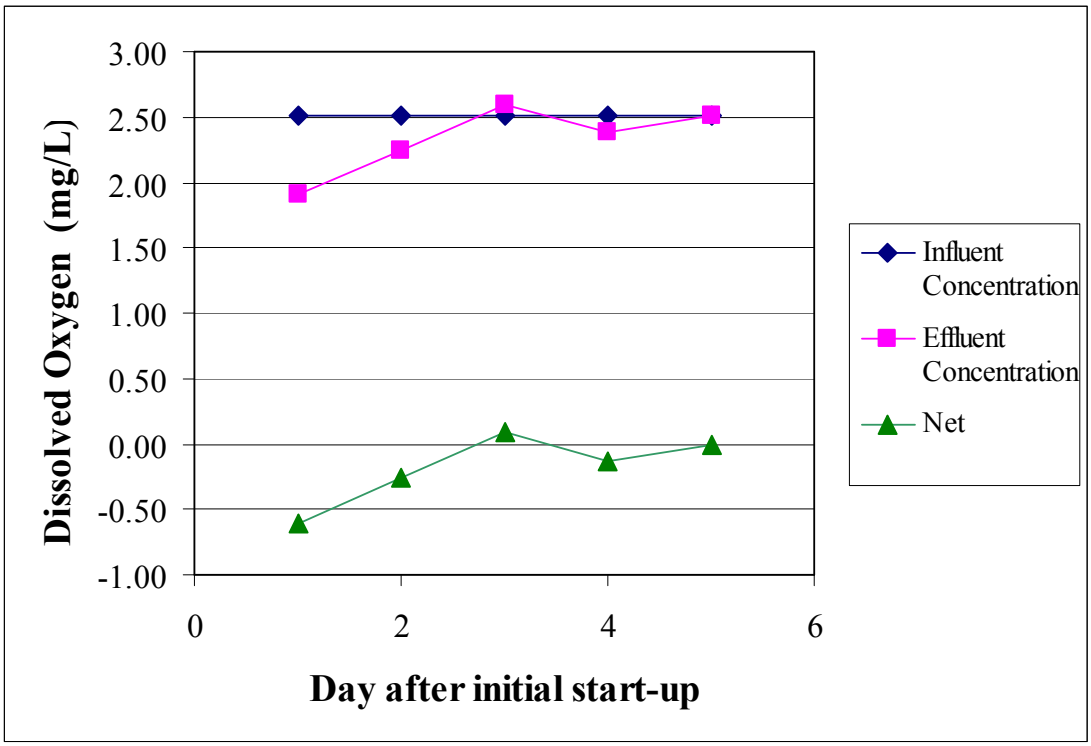

Figure 7-11: Influent and effluent oxygen concentrations and net change in oxygen in a continuous-flow, completely-mixed bioreactor containing a *P. australis* individual and associated native microorganisms and fed DCE-amended anoxic media (Trial 2). Each data point represents the concentration in a single influent effluent sample obtained on the same day (or difference in the concentrations in these samples).

Steady-state removal of unlabeled DCE was monitored from day 8 to 10 and averaged 2.05 mg/L (22.7%) during this period. Five sets of samples were collected over this period, during which three pore volumes passed through the reactor, and averaged 2.05 mg/L (22.7%) during this period. Based on the results of the abiotic

control experiment, removal of 12.7% of the influent DCE was presumably due to abiotic losses. Therefore, removal of 10% of the influent DCE was probably attributable to the presence of the plant and/or associated microorganisms.

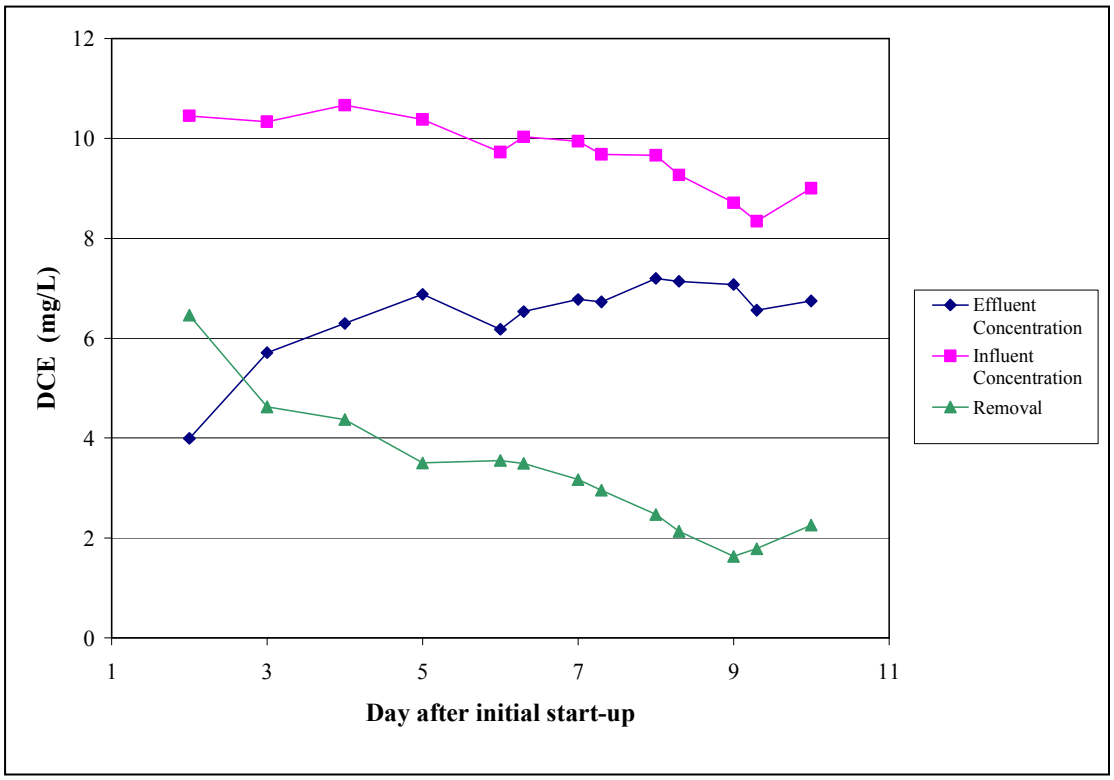

Figure 7-12: Influent and effluent DCE concentrations and DCE removal in a continuous-flow, completely-mixed bioreactor containing a *P. australis* individual and associated microorganisms supplied with DCE (unlabeled)-amended anoxic media (Trial 2). Each data point represents the concentration in a single influent or effluent sample obtained on the same day (or difference in the concentrations in these samples).

Initially, it was expected that DCE removal in Trial 2 would be greater than that observed in the previous experiment (30.5%) because of a larger microbial population size. Thus, the removal of unlabeled DCE in Trial 2 (22.7%), statistically lower ($p>0.05$) than the 30.5% removal observed in Trial 1, was somewhat surprising. However, there are several factors that may explain the lower than expected removal. First, plant health declined over the course of the second experiment as indicated by

complete yellowing of all leaves. In comparison, in the previous experiment, only partial yellowing of the original leaves occurred. The complete yellowing was likely the result of stress associated with being placed in the rhizosphere chamber without an initial aerated feed supply and not a phytotoxic response to DCE. Providing aerated media may help get plants established in the bioreactor and this was done in all previous experiments conducted with intact plants. In contrast, anoxic feed was supplied throughout the Trial 2 experiment. Another difference between the condition of the plants in the two experiments is that a new shoot developed in the foliar chamber during the first experiment, but not in the second experiment. This is significant because the highest amount of plant-associated ^{14}C-activity in the first experiment was found in the new shoot. In Trial 2 a new shoot did develop below the septum in the rhizosphere chamber, but because it could not penetrate the septum and continue growing into the foliar chamber, it most likely was unable to significantly contribute to DCE removal because it could not function as an inlet for diffusion of oxygen to the roots and as an outlet for diffusion of DCE from the roots. The development of a new shoot indicates that growth was not inhibited by DCE and/or the anoxic conditions.

Another potential reason for lower than expected DCE removal is related to the microbial populations, which may have been decreasing through this test period as a result of washout. The impact of suspended microorganisms prior to washout can be seen in the first few days (3-6) in Figure 7-12, when the overall removal of DCE exceeded 30.5%, ranging from 35 to 45% (3.5 to 4.6 mg/L). During this time a net

loss of DO was also measured (Figure 7-11), which was indicative of relatively high biological activity and consistent with the relatively high DCE removal observed. After washout of the suspended microorganisms occurred, there may not have been a sufficient biofilm to maintain the relatively high DCE removal because there may have been insufficient time, compared to Trial 1, to form a sizable biofilm before DCE was supplied to the bioreactor. Compared with Trial 1 where DCE was added to the feed on the 23rd day after the initial start-up, DCE was added to the feed on the 2nd day in Trial 2. Thus, where initial biomass levels may have been lower in the first experiment, they could have formed a more stable biofilm that could achieve greater substrate removal before DCE was added compared with the population present in Trial 2.

On day 10, after the DO and unlabeled DCE measurements were completed, labeled DCE was added to the reactor feed, as in the previous experiment. ^{14}[C]-DCE was supplied in the feed for a total of four days. The distribution of ^{14}C was analyzed as before by collecting daily samples of the liquid fractions and measuring the activity in the biomass phases and the gas phase ^{14}[C]-VOC traps at the conclusion of the experiment. However, the gas phase CO_2 traps used in Trial 1 were not incorporated into this experiment, because gas phase $^{14}CO_2$ represented only a small fraction of the total ^{14}C recovered in the previous experiment, and the daily exchange of these traps caused major reactor operational problems.

The results of the daily analyses of the liquid fractions are shown in Figure 7-13. During the first two days effluent ^{14}[C]-DCE levels increased as the feed containing unlabeled and labeled DCE gradually replaced the media containing only unlabeled DCE in the bioreactor. Effluent ^{14}[C]-DCE activity and ^{14}[C]-DCE removal leveled off on day 13, three days after adding the ^{14}[C]-DCE feed (day 10), and presumably the bioreactor was at steady-state with respect to ^{14}[C]-DCE removal from this point on. Over the two day period (day 13 and 14) when the ^{14}[C]-DCE removal leveled off, the percent ^{14}[C]-DCE removal averaged 20%, which closely matches the percent removal observed during the unlabeled DCE feed (22.7%). Further, in Trial 1, ^{14}[C]-DCE removal appeared to be at steady-state within two days of adding ^{14}[C]-DCE (Figure 7-9). It should be noted that between the two sampling events on days 13 and 14, more than three pore volumes passed through the reactor. Constant substrate removal over three pore volumes is generally though to be a sufficient indicator of stable reactor performance.

As in the previous experiment, ^{14}C-activity was measured in all of the fractions, and a mass balance analysis was conducted. In this case, the mass balance analysis was conducted using only effluent ^{14}C data collected over the two-day period during which the reactor appeared to be at steady-state (i.e. days 13 and 14), and ^{14}C biomass. Table 7-10 shows the total recovery and the percent of ^{14}C recovered in each fraction analyzed during the experiment.

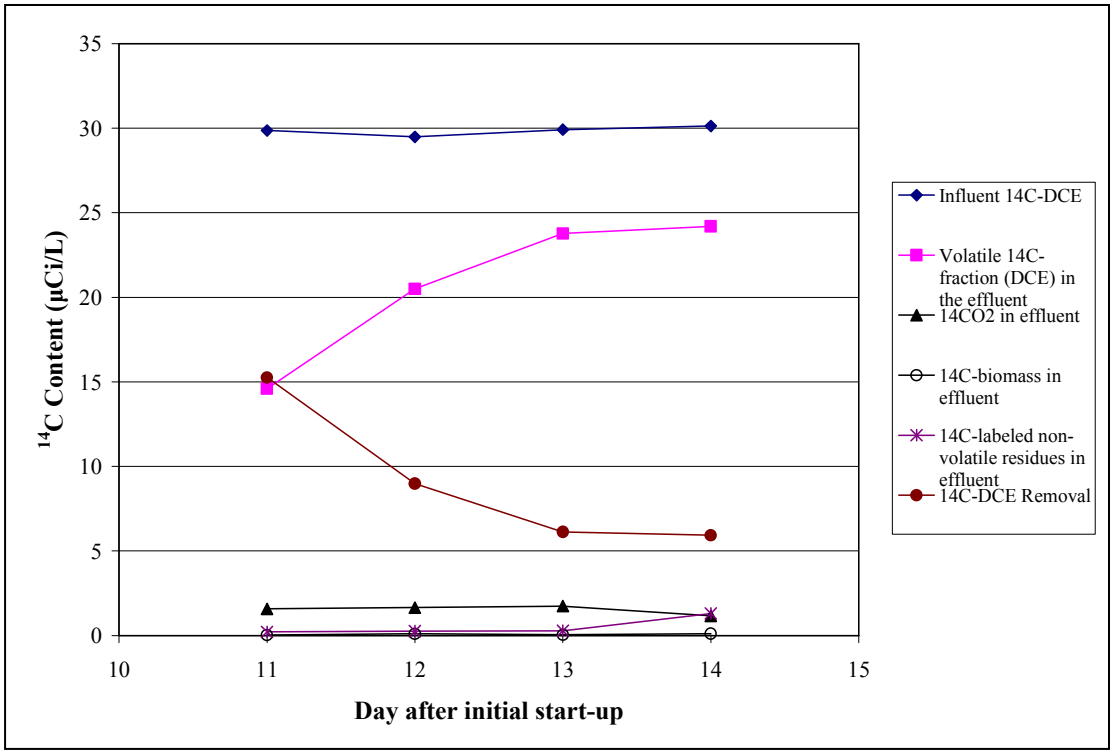

Figure 7-13: Influent ^{14}[C]-DCE and ^{14}C activity associated with volatile, CO_2, biomass, and non-volatile residue fractions in the effluent and ^{14}C-DCE removal, in a continuous-flow, completely-mixed bioreactor containing a *P. australis* individual and associated microorganisms supplied with^{14}[C]-DCE-amended anoxic media (Trial 2). Each data point represents the average of duplicate analyses.

In the previous experiment the mass balance analysis was performed by multiplying the activity measured daily in the liquid fractions times the flow rate over a 24 hour period and summing these values. The activity in the biomass fractions and ^{14}[C]-DCE traps, which were analyzed only at the conclusion of the experiment, were assumed to have accumulated only during the steady-state period. Although this assumption clearly introduced some error into the mass balance analysis, it was not thought to be too significant because the period of steady-state analysis accounted for most of the time that ^{14}[C]-DCE was supplied to the reactor in Trial 1 (seven out of eight days). In contrast, in Trial 2, only data collected on two of the four days that ^{14}[C]-DCE was supplied to the reactor was used in the mass balance analysis.

Therefore, 50% of the measured activity in the biomass and ^{14}[C]-DCE trap fractions was assumed to have accumulated during the steady-state period and used in the mass balance analysis. Analysis of ^{14}C distribution in the liquid effluent fractions was handled the same way as in Trial 1. Based on these assumptions, a total ^{14}[C]-DCE removal of 20.1% was calculated, 12.6% of this removal was attributed to biotic factors.

Table 7-10: Steady-state distribution of ^{14}C activity within each fraction analyzed in a continuous-flow, completely-mixed bioreactor containing a *P. australis* individual and associated microorganisms supplied with ^{14}[C]-DCE-amended anoxic media (Trial 2).

Fraction	Recovered ^{14}C Activity (μCi)	% influent ^{14}C Recovered
Influent	8.6	--
Liquid Effluent[1]		
^{14}[C]-DCE	6.9	79.9
$^{14}CO_2$	0.42	4.8
^{14}C- biomass	0.02	0.26
^{14}C-Non-Volatile residues	0.23	2.6
Gas Effluent		
^{14}[C]-DCE[2]	0.04	0.48
Biomass		
^{14}C-Biofilm on beads[2]	0.33	3.9
^{14}C-Plant (and root associated biofilm)[2]	0.05	0.51
Total	**7.99**	**92.5**

1: Calculated as the sum of the daily measurement of ^{14}C activity (μCi/L) x flowrate x 24 hr.
2: Accounts for only 50% of the total ^{14}C-activity recovered

The total recovery of influent ^{14}[C]-DCE was 92.5%. The balance of 7.5% unaccounted for in the bioreactor and presumably reflects abiotic ^{14}[C]-DCE loss. A significantly greater fraction of the influent ^{14}[C]-DCE (12.3%) was unaccounted for in the first experiment. The difference in the calculated recoveries could reflect sampling error that causes excessively high activity to be measured resulting in higher total recovery. It is also possible that less than 50% of the activity in the biomass fractions(s)/ ^{14}[C]-DCE trap were attributable to the steady-state period in Trial 2. Finally, it is conceivable that the differences in the calculated recoveries are real and there was less abiotic loss occurring during the second experiment.

The presence of ^{14}C-activity in all of the fractions demonstrates that the DCE removal mechanisms that occurred in Trial 1 in the presence of *P. australis* and associated root microorganisms were reproducible. There are some similarities and dissimilarities with respect to the percent ^{14}C recovered in each fraction in the two plant and associated root microorganism experiments. First, the percent aqueous $^{14}CO_2$ recovered was similar (5.9% and 4.8% for the first and second experiment, respectively). The percent ^{14}C recovered as non-volatile residues was also fairly constant for the two experiments. The distribution of ^{14}C in microbial biomass was different in the two experiments. Compared to the first experiment there was less ^{14}C recovered in liquid effluent biomass but greater ^{14}C recovery in the biofilm. This may be an indication that there might have been more opportunity for the DCE-removing microorganisms from the established biofilm to washout in the first experiment compared to the second experiment because the ^{14}C analysis was

conducted over a longer time period in the first experiment. If the ^{14}C-activity associated with both the suspended and biofilm microorganisms are combined, 2.6% and 4.1% for the first and second experiment, respectively, then there is a similar recovery of the ^{14}C-activity in all of the microorganism fractions.

Overall the fractions that might be attributable to bacterial oxidation (as discussed above for Trial 1) account for 7.5 to 12.1% of the total recovery, compared to 9.3 to 12.5% in the previous experiment. Because these two ranges are similar, it appears that oxidation occurred to a similar extent in the second experiment compared with the first experiment. However, less phytovolatilization occurred in Trial 2 than in Trial 1. ^{14}[C]-DCE in the gas phase accounted for only 0.96% of the total ^{14}C recovered in Trial 2 (0.48% over the steady-state period), whereas it accounted for 6.7% of the ^{14}C recovered in the previous experiment. Decreased phytovolatilization likely explains the overall decrease in ^{14}[C]-DCE removal observed in Trial 2 compared with the previous experiment. The reason for reduced phytovolatilization may be related to plant health. As described above, the plant shoot became completely yellow during the experiment, and there were no growing parts of the plant in the foliar chamber. Another possible explanation for the reduced gas phase^{14}[C]-DCE recovered is related to the trapping efficiency of the ^{14}[C]-DCE traps. In Trial 2 there was a consistent high gas flow rate through the ^{14}[C]-DCE traps which may have reduced the contact time between the ^{14}[C]-DCE and trapping medium and thus the amount of ^{14}[C]-DCE trapped. A high flow rate was only occasionally observed in Trial 1.

Samples of the biofilm were collected as done before to measure ^{14}C for activity and estimate the biofilm parameters L_f and X_f. As in the previous experiment, the rhizosphere chamber was vertically divided into four sections. The top-most layer was predominately an algal biofilm and comprised ~ 20% of the bed height. The remaining bed height was evenly divided into a top, middle, and bottom layer. Table 7-11 lists the biofilm parameters and total activity for each section. 100 bead samples were obtained from each section and analyzed for ^{14}C-activity and the total amount of biomass. As in the previous experiment, the biofilm was present as a thin but dense biofilm; however, the highest density was within the algal layer, unlike in the previous experiment in which the bottom layer had the highest density. However, as in the previous experiment, the highest activity was found in the algal section, with lower activity in the remaining sections. Overall the total activity recovered in the biofilm was a similar order of magnitude in the two experiments.

Table 7-11: Activity of ^{14}C associated with the attached biomass and biofilm thickness and density as a function of depth in the rhizosphere chamber in a continuous-flow, completely-mixed bioreactor containing a *P. australis* individual and associated microorganisms supplied with ^{14}[C]-DCE-amended anoxic media (Trial 2).

Reactor Section	L_f (μm)	X_f (mg/mL)	Total Activity (μCi)
Algal	0.04	24,705	0.51
Top	0.04	6917	0.08
Middle	0.18	1185	0.04
Bottom	0.11	2305	0.04

The plant material was divided into the following sections for oxidation: (1) the initial shoot; (2) the new shoot that developed below the septum, and (3) the root, which

was further divided into rhizome and root hairs as before. The activity of each section was measured, and the activity per mass of each section was calculated to determine the greatest concentration of ^{14}C (Table 7-12). As can be seen from these results, the highest ^{14}C-activity was observed in the root hairs, followed by the ^{14}C-activity levels in the new shoot. As before, there was more activity in the root hairs than in the rhizome, and, overall the highest activity was found in those sections where growth was occurring (i.e., the root hairs). Interestingly, a significant amount of activity was found in the new shoot. This could be the result of absorption because this shoot was submerged in the rhizosphere chamber and thus in direct contact with the aqueous ^{14}[C]-DCE. Alternatively, it is possible that the new shoot did in fact contribute to a limited extent to ^{14}[C]-DCE removal, perhaps as the result of either ^{14}[C]-DCE or $^{14}CO_2$ metabolism.

Table 7-12: Activity of ^{14}C associated with the *P. australis* and associated root microorganisms as a function of section in a bioreactor containing a *P. australis* individual and associated microorganisms supplied with ^{14}[C]-DCE-amended anoxic media (Trial 2).

Section	Biomass (g)	Activity (μCi)	Activity/biomass (μCi/L)
Old Shoot	0.2726	0.001	0.004
New Shoot	0.6579	0.007	0.011
Root Hair (average of 3 samples)	0.504	0.016	0.031 ± 0.005
Rhizome (average of 5 samples)	1.388	0.008	0.005 ± 0.001

Chapter 8: Conclusion

This study was designed to test the hypothesis that wetland plants could impact the fate of DCE in the rhizosphere via direct and/or indirect mechanisms. The results of this study demonstrate that *P. australis* and root-associated microorganisms significantly reduced DCE levels. The removal mechanisms included oxidation of DCE by the microorganisms, which was likely possible because of plant-supplied oxygen and exudates; DCE phytovolatilization; and, to a lesser extent, incorporation of DCE or its metabolites into biomass. These results indicate that wetland plants have both direct and indirect impacts on the fate of DCE in the rhizosphere.

All of the removal processes observed in this study, including oxidation and phytovolatilization would be expected to occur in natural wetlands. The DCE removal mechanisms observed in this study and their relative extent are shown graphically in a revised conceptual model of biological processes that can affect the fate of chlorinated solvents in the rhizosphere (Figure 8-1). Understanding the DCE removal mechanisms should improve our ability to predict the fate of DCE discharged to a wetland and perhaps provide a basis to enhance the removal of DCE from wetlands. Based on this study, it would be expected that most DCE removal would occur via microbially-mediated oxidation to CO_2 and, to a lesser extent, non-volatile compounds, as depicted in Figure 8-1. Phytovolatilization, to a lesser extent than oxidation, would also be expected to contribute to removal of DCE from the groundwater, but this is a less desirable removal mechanism because it does not result in the destruction of DCE. However, it might be possible to increase the extent of

oxidation by using engineered approaches to increase microbial activity. For example, if methanotrophs were found to be largely responsible for facilitating the DCE oxidation, providing an optimal amount of methane, that could serve as their growth substrate, could increase the cometabolic rate of DCE oxidation. Conceivably, if microbial activity is optimized, DCE could be largely oxidized to either CO_2 or non-volatile compounds before it comes into contact with the plant roots, resulting in reduced DCE phytovolatilization. Finally, as shown in Figure 8-1, the model includes some plant and microbial DCE metabolism, resulting in incorporation of DCE or its metabolites into biomass; however, this is expected to occur to a lesser extent compared to the other removal mechanisms.

Interestingly, Kassenga et al. (2003) proposed a wetland design conceptual model targeted at the removal of chlorinated ethenes. The removal process included formation of less chlorinated ethenes (e.g. DCE and VC), through reductive transformations of the more highly-chlorinated ethenes in the anaerobic zone, and microbially-mediated aerobic oxidation of the DCE and VC within the rhizosphere. The current study demonstrates that microbially-mediated aerobic oxidation of DCE can occur in the rhizosphere, but in addition, other removal mechanisms, namely phytovolatilization, should be included in the conceptual model as noted above. These are important findings because the accumulation of incompletely dehalogenated ethenes, especially DCE and VC, is a major problem in many TCE- and PCE-contaminated groundwater aquifers. This study shows that if the groundwater flow path includes passage through a wetland containing plants like *P.*

australis that deliver significant amounts of oxygen to the rhizosphere, DCE accumulated in anaerobic zones may be removed through several mechanisms that do not involve formation of VC, which is highly toxic. Therefore, complete detoxification of PCE may be feasible at these sites.

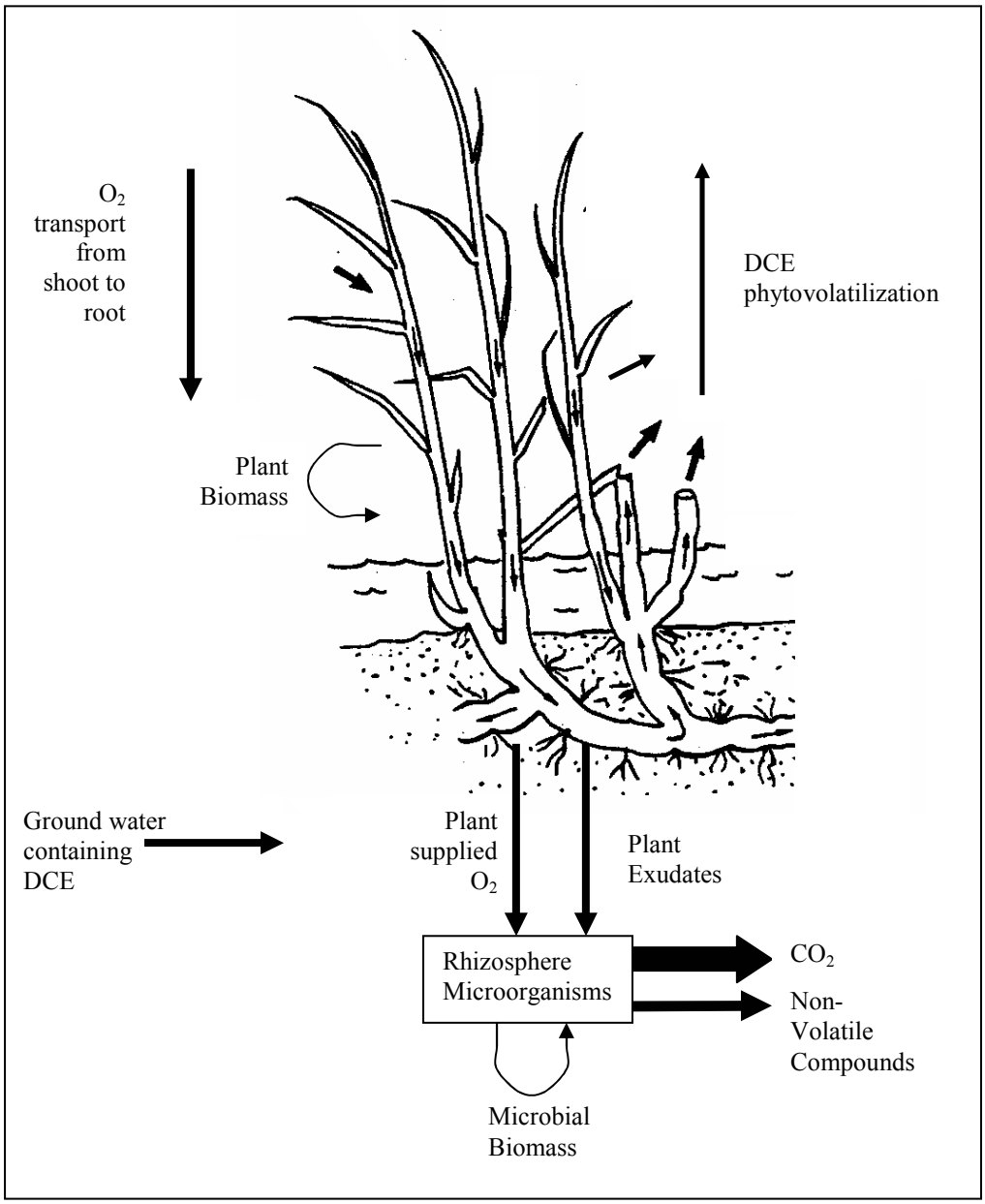

Figure 8-1: Potential uptake and transformation pathways of DCE in a plant-rhizosphere microorganism bioremediation system. The size of the outflow arrows reflects the likely relative extent of DCE transformation among the possible removal processes. (Plant image adapted from Brix et al., 1992)

The results of this study provide valuable insight into potentially important removal mechanisms that could influence the fate of chlorinated ethenes in the wetland rhizosphere. However, because the conditions used in the lab are different from those encountered throughout the year in wetland ecosystems, further research is required before accurate predictions about the fate of chlorinated ethenes in ground water that discharges into wetlands can be made. Field studies involving wetlands, in particular those containing *P. australis*, could provide information on the extent of DCE removal that could be expected throughout the year as conditions change in natural or constructed wetlands. Even greater removal of DCE might occur in a real wetland, at least during certain times of the year. For example, during the summer months *P. australis* will grow considerably larger than the plant size used in this experiment, which may result in a greater amount of DCE phytovolatilization and/or increased oxidation of DCE by microorganisms thru increased delivery of oxygen to the rhizosphere. Additionally, summer time temperatures will typically be higher than that used in the lab (approximately 20^{o}C), which would further increase microbial activity including, presumably the extent of microbially-mediated oxidation of DCE. Conversely, during the winter months, when there are no active growing shoots and microbial activity is decreased, there may be less DCE removal than observed in this study. The effects of these environmental changes would have to be factored into any assumptions about chlorinated ethene removal in either natural or constructed wetlands.

Overall, the research conducted provided information on the relationships between wetland plants and rhizospheric microorganisms and their roles in achieving the biodegradation of chlorinated ethenes. Specifically, this study demonstrated that the wetland plant *P. australis* affected contaminant levels indirectly, by helping to generate an aerobic rhizosphere habitat that promoted the activity of aerobic root-associated microorganisms capable of degrading DCE, and directly, by facilitating removal via phytovolatilization, and to a lesser extent incorporation into plant and microbial biomass. This information should enhance our ability to predict and improve the fate of groundwater contaminants that are discharged to natural wetlands. In addition, it should improve our ability to apply constructed wetlands in the treatment of waste streams containing chlorinated solvents and, potentially, other anthropogenic compounds, in order to improve our water resources. Further work would be required prior to application to large-scale industrial waste or contaminated ground water sites, but this work provides a fundamental first step in understanding the processes occurring in wetlands and their application to bioremediation.

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