COMETABOLIC BIODEGRADATION OF HALOGENATED ALIPHATIC HYDROCARBONS BY AMMONIA-OXIDIZING MICROORGANISMS NATURALLY ASSOCIATED WITH WETLAND PLANT ROOTS

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By

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ABSTRACT


Bench-scale microcosms with wetland plant roots were investigated to characterize the microbial contributions to contaminant degradation of halogenated aliphatic hydrocarbons (HAHs) with ammonium. The batch system microcosms consisted of a known mass of wetland plant roots in aerobic growth media where the roots provided both an inoculum of root-associated ammonium-oxidizing microorganisms and a microbial habitat. Aqueous growth media, ammonium, and HAHs including trichloroethene (TCE), cis-1,2-dichloroethene (cis-DCE), chloroform (CF), 1,1,2-trichloroethane (1,1,2-TCA), ethylene dibromide (EDB, or 1,2-dibromoethane, 1,2-DBA) and 1,2-dichloroethane (1,2-DCA) were replaced weekly in batch microcosms while retaining roots and root-associated biomass. Molecular biology results indicated that ammonium-oxidizing bacteria (AOB) were enriched from wetland plant roots while analysis of contaminant and oxygen concentrations showed that those microorganisms can degrade HAHs by aerobic cometabolism.

Cometabolism of TCE, at 29 and 46 µg/L, was sustainable over the course of 9 weeks, with 20-30 mg/L ammonium-N. However, at 69 µg/L of TCE, ammonium
oxidation and TCE cometabolism were completely deactivated in two weeks. This indicated that between 46 and 69 µg/L TCE with 30 mg/L ammonium-N there is a threshold [TCE] below which sustainable cometabolism can be maintained with ammonium as the primary substrate. However, cometabolism-induced microbial deactivation of ammonium oxidation and TCE degradation at 69 µg/L TCE did not result in a lower abundance of the amoA gene in the microcosms.

In the following experiments with TCE and elevated concentration of 75 mg/L ammonium-N, the deactivation of cometabolism with TCE was observed again when TCE reached from ~50 to ~70 µg/L. The cometabolic system was activated again one week after the system was replaced by a TCE-free medium culture. Rate constants did not change significantly during the inactivation cycle if normalized by X. It can be inferred that the drop in ammonium and TCE degradation at certain [TCE] are due to the activity shut down by ammonia oxidizers. Similar deactivation trends were observed in microcosm amended with cis-DCE, 1,1,2-TCA and EDB when HAHs concentration increased above ~150 µg/L. No deactivation was observed in the reactors with CF and 1,2-DCA.

A shift of ammonium oxidation production from nitrite of nitrate after HAHs were added was observed in all the HAHs batch systems and those shifts all coincided with a moderate decrease in ammonium and HAHs degradation kinetics. Following sequencing analysis on a cometabolic system with TCE showed that the relative
abundance of nitrite oxidizers (especially *Nitrospira*) changed significantly after 14 weeks, which suggest that the microbial community changed with the addition of elevated concentration of TCE. The relative abundance of previous dominance genera *Nitrosomonas* decreased at higher TCE concentrations while the abundance of nitrite oxidizers *Nitrospira* increased significantly due to the weakened competence for oxygen from *Nitrosomonas*.

This research indicates that microorganisms associated with wetland plant roots can assist in the natural attenuation of HAHs in contaminated aquatic environments, such as urban or treatment wetlands, and wetlands impacted by industrial solvents.
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1. BACKGROUND

It is noticed that the persistence of halogenated aliphatic hydrocarbons (HAHs) in different kinds of environments, especially shallow, vegetated aquatic environments (wetlands) has been an important and impending issue. They have been a threat to human health as suspected carcinogens (Powell et al., 2011). Common HAHs include halogenated methanes, halogenated ethenes, and halogenated ethanes (Table 1.1 (Arp et al., 2001)). Various industrial and domestic sources account for the wide spread of HAHs. Traditional wastewater treatment systems require large capital investments and operating costs, thus they are not realistic solutions for HAHs producers that cannot afford such expensive treatment systems, especially in less-developed countries (Kantawanichkul et al., 2009), while bioremediation is a promising approach that has been proved effective at cleaning sites contaminated by HAHs (Field and Sierra-Alvarez, 2004). Bioremediation can happen in both reductive and oxidative environments with different pathways (in this review, the oxidative pathway is the topic of interest, therefore reductive pathways will not be discussed). By respiration and fermentation, widely distributed microorganisms can break down organically-derived pollutants into presumed harmless substances such as carbon dioxide (CO₂), nitrogen gas (N₂) and water (H₂O) (Faulwetter et al., 2009).

In microbial respiration, electrons from donor compounds (including the HAHs of concern) at higher energy state are transferred to an electron acceptor at a
lower state. This energy difference is used for microbial growth and energy production. Thus, the pollutant removal process by microorganisms is highly dependent on the oxidation-reduction (redox) conditions in the surrounding environments (Faulwetter et al., 2009). In high redox potential (thus oxidized) environments, nitrification is probably one of the most common ones. Nitrification means that the removal of reduced nitrogen compounds in the nitrogen cycle is initiated by microorganisms that carry out the oxidation of ammonia to nitrate. Ammonia oxidizing bacteria (AOB) and/or ammonia oxidizing archaea (AOA) play an important role by oxidizing urea and ammonia fertilizers to nitrite and subsequently converting nitrite to nitrate with the help of nitrite oxidizers (Purkhold et al., 2000; You et al., 2009; Sayavedra-Soto et al., 2010). Through cometabolism, AOB and/or AOA can oxidize alternative substrates (HAHs) which cannot support their growth (Wallack, 1996). The cometabolism process generally requires the growth substrate to both induce enzymes and supply reductant necessary for the alternative/secondary substrate oxidization (Sayavedra-Soto et al., 2010). In this work cometabolism refers to bacterial transformation of a compound (e.g. HAHs) in the presence of growth-supporting substrates (Arp et al., 2001).

Generally, ammonia is produced and consumed by microorganisms primarily in a healthy ecosystem. Under aerobic conditions, AOB and/or AOA, which belong to chemolithoautotrophs, consume reduced nitrogen (mainly ammonia) as both an energy source and reductant, use CO₂ for carbon requirements, and molecular oxygen as electron acceptor (You et al., 2009; Sayavedra-Soto et al., 2010).

The effects of AOB that can degrade various HAHs are summarized in Arp et al.’s work (Arp et al., 2001). Thus, using AOB in bioremediation has become an
intriguing topic in recent years. Studies have been focused on both contaminants in soils and wastewater. Due to the usually high concentration of ammonia present in wastewater, this cometabolic process can be more attractive because it can degrade both types of harmful contaminants (HAHs and ammonia) together (Sayavedra-Soto et al., 2010). Another advantage is that by aerobic cometabolism, HAHs such as TCE can be totally mineralized, without the accumulation of toxic intermediates as observed in reductive dehalogenation (Vogel and McCarty, 1985; Sayavedra-Soto et al., 2010).

Wetlands can remove pollutants by a complex variety of physical, chemical, and biological processes (Gersberg et al., 1986). Treatment wetlands include surface and subsurface-flow systems. The former is often found in natural environments due to the permanent standing water and conditions favorable for wetland plant species (Truu et al., 2009), and is the topic of concern in this article. Aquatic plants transfer atmospheric or photosynthetic oxygen from shoots to roots by porous aerenchymatous tissue functioning as a gas channel, making the rhizosphere (root zone) an oxidized environment in the typically anaerobic zone, thus stimulating a great number of aerobic chemical and biological processes, including the growth of nitrifying bacteria favored in aerobic ammonia oxidization (Gersberg et al., 1986; Bodelier et al., 1996; Kowalchuk et al., 1998; Venter et al., 2004; Wiessner et al., 2005; Chen et al., 2008). Therefore, in contrast to traditional water treatment methods, bioremediation in wetlands can be efficient, using renewable energy, without forced aeration or need to add chemicals (Gersberg et al., 1986; Tietz et al., 2007; Baptista et al., 2008). Also, constructed wetlands have the ability to remove relatively high pollutant concentrations (Ibekwe et al., 2003). Up to now many natural or constructed aquatic environments with the vegetation species Carex comosa (longhair sedge), Glyceria
maxima (reed sweetgrass), Oryza sativa (Asian rice), Typha latifolia (common bulrush), Spartina alterniflora (smooth cordgrass), Spartina patens (saltmeadow cordgrass), Scirpus validus (great bulrush), Phragmites australis (also known as *Phragmites communis*, common reed) and mangrove have been studied (Gersberg et al., 1986; Bodelier et al., 1996; Chen et al., 2008; Wang et al., 2008; Moin et al., 2009; Ruiz-Rueda et al., 2009; Li et al., 2011; Powell et al., 2011).

This dissertation will mainly focus on redox transformation potential of HAHs in wetlands by various ammonia-oxidizing microorganisms. Key oxidative pathways and the roles of important microorganisms involved in a series of processes will be discussed in this article.

1.1 MICROORGANISMS INVOLVED

As a widely distributed group of bacteria, ammonium oxidizing bacteria (AOB) are ubiquitous and can be found mainly in freshwater sediments, sewage/wastewater, marine environments and soils (Arp et al., 2007). The AOB all belong to two phylogenetic groups: *Nitrosococcus oceani* and *Nitrosococcus halophilus* within the class γ-Proteobacteria, and *Nitrosomonas* and *Nitrosospira* spp. within the class β-Proteobacteria (Purkhold et al., 2000; Silyn-Roberts and Lewis, 2001). The species can be easily identified through 16S r-RNA based approaches (Kowalchuk et al., 1998). The β-subgroup AOB comprise at least seven phylogenetically distinguishable sequence clusters within the *Nitrosomonas* and *Nitrosospira* genera, with the latter encompassing the previous genus *Nitrosolobus* and *Nitrosovibrio* (Head et al., 1993; Stephen et al., 1996), and is usually regarded as the dominant AOB subgroup in natural and engineered systems (Nold et al., 2000; Park et al., 2002). Purkhold et al. conducted the 16S r-DNA sequence analysis of 10 *Nitrosomonas* species and
Nitrosococcus mobilis (Purkhold et al., 2000). Establishing a robust phylogenetic framework of AOB, they found similar but not identical evolutionary relationships of those AOB studied by comparative phylogenetic analysis using 16S rRNA and ammonia monooxygenase (AMO) as marker molecules (Purkhold et al., 2000). Figure 1.1 shows the details of classified AOB clusters as well as also microorganisms and the microorganisms identified to those clusters in Purkhold et al.’s work (Purkhold et al., 2000). Those Nitrosospira-like and Nitrosomonas-like AOB sequences were detected in a great diversity of environments (Silyn-Roberts and Lewis, 2001; Ibekwe et al., 2003; Bowatte et al., 2006; Tietz et al., 2007; Dorador et al., 2008; Kantawanichkul et al., 2009; Moin et al., 2009; Ruiz-Rueda et al., 2009; Allen et al., 2010). Once established, AOB communities can maintain themselves at a stable level, varying very slightly in community composition over time (Dionisi et al., 2002; Hallin et al., 2005). Note that the oxidation of ammonia via nitrite to nitrate is actually completed by two phylogenetically different groups of bacteria, ammonia oxidizing bacteria and nitrite oxidizing bacteria (NOB, belongs to Nitrobacter spp. and Nitrospira spp.) (Degrange and Bardin, 1995; Silyn-Roberts and Lewis, 2001; Allen et al., 2010).

AOB have undergone intense genome analysis with many researchers finding diverse dominant species in different sites. Some found that in aquatic environments, mainly Nitrosomonadaceae will be present due to their gene function (Abeliovich, 1987). In high oxygen demand and high solute concentration aquatic environments, Nitrosomonas eutropha has genes that encode proteins involved in microaerophilic respiration, competing for oxygen and Nitrosomonas europaea and N. eutropha contain genes for metal efflux, making them adaptive in such environments (Stein et al., 2007). While in soils, Nitrosospira multiformis is dominant due to its gene ability
to metabolize urea in areas where fertilizers are utilized (Norton et al., 2008). Some also reported this difference at the level of genus, stating that a higher percentage of Nitrosospira-like sequences were found in the wetland samples while a higher percentage of Nitrosomonas-like sequences were present in other environments such as raw freshwater and facultative ponds (Ibekwe et al., 2003). Generally, Nitrosomonas spp. have a lower substrate affinity but higher maximum activity than Nitrosospira spp. (Schramm et al., 1996), thus the latter are more likely to be dominant in low ammonia environments and better at withstanding physicochemical variations (Kowalchuk et al., 2000; Purkhold et al., 2000; Backman et al., 2003). While Nitrosomonas spp. are usually prevalent in high ammonia concentration systems and can sometimes dominate such systems with a single species (Schramm et al., 1996; Okabe et al., 1999). Nitrosomonas spp. may also be one of the initial colonizers of wetland’s substrata (Silyn-Roberts and Lewis, 2001). Depending on wetland type and environmental conditions, the abundance of Nitrosomonas spp. can range from 0–1.5% in subsurface wetlands and up to 4% in surface wetlands (Flood et al., 1999; Silyn-Roberts and Lewis, 2001), while the bacteria from genus Nitrospira and Nitrobacter (nitrite oxidizing bacteria) may reach 1.5% and 4.3%, respectively in surface wetlands (Shipin et al., 2005). It was also noted that in a treatment wetland high in ammonia concentration, a robust microcommunity containing a Nitrosomonas oligotropha-like sequence, a Nitrosococcus mobilis-like sequence, and other Nitrosospira-like sequences was also found (Sundberg et al., 2007a). A treatment wetland containing a filter bed connected to an open pond had a less diverse AOB community with Nitrosospira-like sequences (Sundberg et al., 2007b).

Though these AOB are adaptive in either aquatic or soil environments, they are sensitive to a series of factors, like pollutants (organic solvents and metals), redox
condition, pH, salinity, temperature, day/night rhythm, seasonal variability, plant density, flow rate of various gases including O₂, CO₂ and CH₄ (Armstrong and Armstrong, 1990; Wagner et al., 1996; Bernhard et al., 2007; Truu et al., 2009; Lage et al., 2010; Sayavedra-Soto et al., 2010; Wang et al., 2011b; Zhang et al., 2011), and even are considered to be the most sensitive organisms in ammonia removal (Radniecki et al., 2008). The optimal pH value for AOB is between 7.0 and 8.0 (Paredes et al., 2007). Ammonia oxidizers grow faster than nitrite oxidizers at temperatures above 15 °C, and at 25 °C the nitrite oxidizers can be dislodged by ammonia oxidizers (Paredes et al., 2007). Generally speaking, low temperatures have negative impacts on the growth and activity of AOB and the ideal temperature for AOB growth ranges from 20 to 35 °C (Abeliovich, 1992; Truu et al., 2009). Based on one research, at 15 °C, *Nitrosomonas europaea* can oxidize 165 mg/g/h of ammonia, while at 30°C, the oxidation rate was 260 mg/g/h (Groeneweg et al., 1994). Some reported the amount of AOB in summer was 10 to 30 fold higher than in spring (Wang et al., 2008). Nitrite production is found to increase from February to August and decrease during other times of the year (Zhang et al., 2011). However, a robust mixed AOB community has been found in a vertical flow treatment wetland which can withstand strong temperature changes (as low as 5.4 °C) (Tietz et al., 2007). Optimal ammonia concentrations for AOB are found to be from 100 to 1400 mg/L (Silyn-Roberts and Lewis, 2001). For the dissolved oxygen (DO), *Nitrosomonas* spp. was reported to have a slow growth at DO levels less than 4 mg/L but still be active even when DO levels went down to 0.5 mg/L (Silyn-Roberts and Lewis, 2001). Another finding is a negative relationship between the Shannon biodiversity index for AOB and ammonia concentration, indicating the ammonia removal is usually performed by dominant species (Wang et al., 2011a). A comprehensive report stated
the maximum nitrifier activity in two sites was achieved at 35 °C, pH 8.5, salinities of 0.3 to 0.5% Na\(^+\) and K\(^+\), ammonia concentration greater than 0.5g/L, and at 40 °C, pH 8.0, salinities of 0.5 to 1.0% Na\(^+\) and K\(^+\), ammonia concentration greater than 0.2g/L, respectively. They also found nitrite at a level greater than 5 mg/L can inhibit ammonia oxidation while nitrate had no significant effect on that process (Jones and Hood, 1980). Compared to nitrite oxidizers, AOB usually develop quickly and can be one order of magnitude more abundant (Bahgat et al., 1999). Distribution of AOB community was also found to be related to filter material, microbial biomass, and total nitrogen and phosphorus concentration (Truu et al., 2009; Li et al., 2011). Studies found zeolite was an ideal growth media for AOB (Gorra et al., 2007).

Currently, the research on the effects of AOB degrading pollutants is mainly on N. europaea from Nitrosomonadaceae as they can cometabolize various HAHs (Vannelli et al., 1990; Arp et al., 2007). A number of studies have documented it at physiological, biochemical, and molecular levels (Rasche et al., 1991a; Keener and Arp, 1993; Hyman et al., 1995; Sayavedra-Soto et al., 1998; Vader et al., 2000). Other AOB, e.g. Nitrosococcus oceani (Klotz et al., 2006; El Sheikh and Klotz, 2008), N. eutropha (Schmidt and Bock, 1997; Stein et al., 2007), Nitrosococcus mobilis (Purkhold et al., 2000), Nitrosomonas marina, Nitrosomonas ureae (Ruiz-Rueda et al., 2009), Nitrosomonas oligotropha (Allen et al., 2010), and Nitrosospira sp. (Klotz and Norton, 1995) have also been investigated in peer-reviewed studies. Genomic tools have been developed based on those researches to understand the mechanisms of bioremediation of pollutants.

Besides AOB, ammonia oxidizing archaea (AOA) are also important microbial groups involved in ammonia oxidation (Konneke et al., 2005; You et al.,
Nitrosopumilus maritimus, which has the shape of a straight rod with a diameter of 0.17–0.22 μm and a length of 0.5–0.9 μm, is the first isolated AOA. It comes from a tropic marine aquarium tank, represents the ubiquitous marine group 1 Crenarchaeota, and contains putative genes for all three subunits (amoA, amoB, amoC) of AMO (Konneke et al., 2005). It can use ammonia as the sole energy source and has a similar growth rate and cell production as AOB (Konneke et al., 2005). The maximum growth rate of Nitrosopumilus maritimus pure culture is 0.78 day⁻¹ (You et al., 2009).

Cenarchaeum symbiosum was also found to bear the genes for ammonia oxidation, though a pure culture of that species is extremely hard to isolate (Hallam et al., 2006a; Hallam et al., 2006b). Nitrososphaera viennensis was isolated from soil and found to have the ability to use ammonia or urea as an energy source, and is capable of using even higher ammonia concentrations than Nitrosopumilus maritimus (Tourna et al., 2011). Nitrososphaera gargensis, which has high activity at ammonia concentrations up to 0.79 mMol/L, was found from a hot spring and believed to be the first described thermophilic ammonia oxidizer (Hatzenpichler et al., 2008).

Recently studies have listed those AOA (including Nitrosopumilus maritimus, Cenarchaeum symbiosum, Nitrososphaera viennensis, and Nitrososphaera gargensis) in a new archaeal phylum Thaumarchaeota, which is marked by the glycerol dialkyl glycerol tetraether lipid crenarchaeol (Brochier-Armanet et al., 2008; Spang et al., 2010; Bannert et al., 2011; Tourna et al., 2011)

AOA are widely distributed and can even be found in many extreme environments. Common habitats for AOA include hot/thermal springs, marine and fresh waters, soils, wastewater treatment facilities, and wetlands (Park et al., 2006; Hoefferle et al., 2010; Sims et al., 2012).
Caffrey et al., 2007; Francis et al., 2007; Hatzenpichler et al., 2008; You et al., 2009). In some scenarios, AOA can be the overwhelming prokaryote (Herrmann et al., 2008; You et al., 2009). In paddy soils, rice cultivation resulted in greater abundance of AOA than AOB ammonia monooxygenase a-subunit (amoA) gene copies, which directly has the ability to aerobically oxidize ammonia and thus leads to a strong nitrification in rice rhizosphere (Chen et al., 2008; Li et al., 2008). In an aquatic environment with the ammonia oxidizing community in the rhizosphere of Littorella uniflora (shoreweed), AOA outnumbered AOB by 8000-fold in amoA gene copy numbers (Herrmann et al., 2008). It seems AOA may be better adapted than AOB to rhizosphere oxygenation and/or plant root exudation, and when AOA and β-AOB were both studied, AOA were typically more abundant (Herrmann et al., 2008; Moin et al., 2009; Sims et al., 2012). In wetland soils and wetlands featured by mangrove, some reported that AOB was more abundant than AOA in terms of amoA gene (Hoefferle et al., 2010; Li et al., 2011). In a constructed wetland, the nitrification was positively correlated to the increase of AOB population while AOA abundance remained unchanged, suggesting the nitrification process was more affected by AOB (Sims et al., 2012). The AOA abundance is strongly affected to ammonia concentration, pH, and salinity, and is also highly related to AOB in terms of the amoA gene (Moin et al., 2009; Li et al., 2011; Wang et al., 2011b). In wetland soils, AOA community structure is more stratified with depth than AOB, with higher diversity and abundance in the surface layer sediments (Hoefferle et al., 2010; Li et al., 2011).

1.2 AEROBIC AMMONIA OXIDIZATION

Aerobic ammonia oxidation generally involves several steps. First, the
membrane-bound enzyme ammonia monooxygenase (AMO) helps oxidize ammonia to hydroxylamine. Then hydroxylamine is further oxidized to nitrite in the presence of the periplasmic enzyme hydroxylamine oxidoreductase (HAO) (Figure 1.2; (Sayavedra-Soto et al., 2010)). During that step (hydroxylamine to nitrite), two of the four electrons released would function to maintain AMO activity, while the other two would pass through the respiratory chain to produce the proton motive force and to provide necessary reductant for growth (Arp et al., 2002). Usually nitrite can be further oxidized to nitrate in the presence of nitrite oxidizing bacteria (Wagner et al., 1996). Other studies reported denitrification, where ammonia oxidation products were denitrified to N₂ or N₂O and released (Gersberg et al., 1986). The “classic” nitrification and denitrification process therefore can be shown as NH₃→NH₂OH→NO₂⁻→NO₃⁻, and NO₃⁻→NO₂⁻→NO→N₂O→N₂, respectively (Tanner et al., 2002).

Besides the ability to catalyze the first step of aerobic ammonia oxidation, AMO is also capable of carrying out the oxidation (this process is also known as cometabolism) of variety of hydrocarbons (including HAHS, Table 1.1 (Arp et al., 2001)). HAHS that can be cometabolized by AMO include vinyl chloride (VC), trichloroethylene (TCE), chloroform (CF), bromodichloromethane (BDCM), dibromochloromethane (DBCM), bromoform (BF) (Wahman et al., 2005), etc. Full details and pathways are listed in Figure 1.3 (Arp and Stein, 2003) and Table 1.1 (Arp et al., 2001). Compounds such as chloromethane (CM) and dichloromethane (DCM) can be used as primary growth substrates (the process is also known as metabolism), and also can be used by a variety of aerobic or anaerobic microorganisms other than AOB. Usually CF is considered to be degraded exclusively through cometabolism (by AOB or methane oxidizers) (Field and Sierra-Alvarez, 2004). Generally, the oxidation
potential of HAHs depends on the oxidation number of the carbon atom: the lower the oxidation number the carbon has, the higher its oxidation potential and the more easily it’s oxidized (Vogel et al., 1987). Studies found, in term of enzyme affinities for AMO, 1,1-DCE (1,1-Dichloroethene) = TCE > CT (Carbon tetrachloride) > CF > 1,2-DCA (1,2-Dichloroethane), while for maximum substrate transformation rates, it is NH₃ > 1,2-DCA > CF > TCE = 1,1-DCE > CT (=0) (Ely et al., 1997). For brominated HAHs, the degradation rates increased with bromine substitution, i.e., BF > DBCM > BDCM > CF (Wahman et al., 2005).

The aerobic degradation of HAHs by AMO, literally, requires oxygen. During this process, one oxygen atom is inserted into the molecule, the halogen is released, and an aldehyde or epoxide formed. The other oxygen atom is reduced to water (Field and Sierra-Alvarez, 2004). Those hydrocarbon products are usually unstable and can be further transformed to alcohol, organic acids, or even carbon dioxide. AMO obtains energy for carbon dioxide fixation through the oxidation of ammonia (Erwin et al., 2005). The oxidation of alternative substrates by AMO also requires reductant and thus electrons are also needed from other processes (Sayavedra-Soto et al., 2010). Oxygen can serve as both an electron acceptor of the electron released and a direct reactant with HAHs (Bouwer and Zehnder, 1993). AMO began to synthesize when an intruder (typically enzyme substrate) triggered the relevant response of cells. Then transcription of the structural genes for AMO was upregulated, leading to the increase the synthesis of AMO and other relevant enzymes (Arp et al., 2001).

AMO is a multicomponent enzyme and almost impossible to be purified with sustained activity, usually losing its activity in hours where no cells are present (Ensign et al., 1993). Also, AMO is strongly associated with the membrane fraction
upon cell breakage and can only be released by treatment with detergents (Arp et al., 2001). Thus, to date, all kinetic information of AMO is based on whole-cell analysis, which however, been proved to be still an effective way (Sayavedra-Soto et al., 2010). AMO consists of three polypeptides, *amoA, amoB, amoC* (Arp et al., 2001). The *amoA* gene is a 27 kDa polypeptide which has been identified as responsible for catalyzing autotrophic bacterial ammonia oxidation (Arp et al., 2001; Chen et al., 2008). Both AOB and AOA can carry these genes (Venter et al., 2004). *amoB* is a 40 kDa polypeptide which cannot be labeled upon inactivation of ammonia oxidation by acetylene like *amoA* (Hyman and Wood, 1985; Arp et al., 2001). *amoC* is suggested through genetic characterizations despite scarce physical evidence (Klotz et al., 1997; Arp et al., 2001). Those genes share high sequence identity with particulate methane monooxygenase (pMMO) and are believed to be evolutionarily related to methane oxidizers (Erwin et al., 2005).

The enzyme that stimulates the second step, HAO, is usually unaffected during cometabolism by alternative substrates. Thus, HAO alternative substrates can be added during cometabolism to provide additional reductants (Keener and Arp, 1993). Those alternative HAO substrates, such as hydrazine, can release electrons, thus stimulating cometabolism. Note, however, that, those substrates themselves cannot support AOB and are usually harmful to AOB (Sayavedra-Soto et al., 2010).

Cometabolism was regarded as a highly complex phenomenon in their kinetic studies (Alvarez-Cohen and Speitel, 2001). In aquatic environments, various pathways, including damage to AMO (either reversible or irreversible), multiple cosubstrates, byproducts, and other microorganisms will simultaneously interact (Figure 1.4; Sayavedra-Soto et al., 2010). In most situations, cometabolism can
result in the decrease of both AMO activity and cell viability (Rasche et al., 1991a; Ely et al., 1997). Thus to have sustainable bioremediation, it is important to maintain a stable population of AOB.

Many previous studies have proved the feasibility of bioremediation of HAHs through aerobic cometabolism with AOB. In a mixed microbial community in Istanbul, Turkey, TCE degradation was found to occur at rates similar to rates in pure cultures with an unlimited oxygen supply (Kocamemi and Cecen, 2007). Mixed cultures including *Nitrosomonas oligotropha* were also found to have effects similar to pure cultures, like *N. europaea*, which can degrade up to 800 μg/L of trihalomethanes commonly found as byproducts of chlorination in drinking water (Wahman et al., 2006). It is also reported that by actively manipulating the biodegradation process, a continuous bioremediation of TCE was achieved (Yang et al., 1999). Powell et al. (2011) reported the possibility of bioremediation of TCE using AOB attached on the roots of wetland plant *Carex comosa*. Kocamemi and Cecen (2009) reported a 70-90% removal efficiency of 1,2-DCA in a nitrifying biofilm reactor. The ability of *N. europaea*, *Nitrosomonas oligotropha*, and mixed culture nitrifiers to degrade low concentrations (50 to 800 μg/L) of bromide HAHs were continuously reported by a research group (Wahman et al., 2005; Wahman et al., 2006; Wahman et al., 2011b). They observed a 75% removal for DBCM in mixed nitrifying cultures (Wahman et al., 2011a).

Currently, little is known about the ammonia oxidation pathways by AOA (Hallam et al., 2006a; Hallam et al., 2006b). Though the metabolism by AOA is similar to that of AOB in many aspects, there are still some distinct aspects exhibited by AOA (You et al., 2009). In the terms of autotrophic carbon fixation, AOA usually
uses a 3-hydroxypropionate pathway or a reductive tricarboxylic acid (TCA) cycle, rather than the Calvin cycle used by AOB (Ward et al., 2007). Due to difficulty culturing AOA in laboratory, studies clearly describing the cometabolism of AOA are scarce (You et al., 2009; Sayavedra-Soto et al., 2010).

1.3 Inhibition by HAHS and Recovery from the Inhibition

Cometabolism can cause loss of certain HAH biodegradation capacity, ammonia oxidizing capacity, and oxygen uptake (Rasche et al., 1991a). Generally, the mechanism and extent of inhibition and inactivation highly depend on the type of the compound (Sayavedra-Soto et al., 2010). HAHS can inhibit AMO activity by competitive (Keener and Arp, 1993; Hyman et al., 1995), noncompetitive, or mixed noncompetitive modes (Keener and Arp, 1993). In competitive modes, the growth and alternative/secondary substrates will compete for active sites, leading to a reduced oxidation rate since AMO is non-specific (Hyman et al., 1995; Sayavedra-Soto et al., 2010). The inhibition by TCE is typically competitive, and TCE was found to have an affinity for AMO four times greater than that of ammonia for the enzyme (Ely et al., 1995a; Yang et al., 1999; Sayavedra-Soto et al., 2010). TCE can also inhibit the formation of hydroxylamine, thus decreasing the overall electron flux rate through both HAO and AMO (Hyman et al., 1995). These competitive phenomena are observed in many studies. Alvarez-Cohen and Speitel (2001) mentioned a competitive inhibition found in the cometabolism of TCE in pure cultures of *N. europaea*, which could tolerate up to 3.3 mg/l of TCE with a maximum specific degradation rate of $k_c=1$ to 1.6 mg/mg dwt cells-day (Ely et al., 1997). Some found the inhibition constant ($K_i$, the concentration of the inhibitor that is required in order to decrease the maximal rate of the reaction by half) of TCE to ammonia is 666-802 μg/L and TCE
showed first order degradation at the initial concentration up to 845 μg/L (Kocamemi and Cecen, 2007; Kocamemi and Cecen, 2010). In their previous studies, Kocamemi and Cecen (2005) observed specific oxygen uptake rate with ammonia oxidation decreased by about 30% at 50–100 ppb TCE, 45% at 500–1000 ppb TCE, 50% at 2500 ppb TCE, 56% at 5000 ppb TCE, 65% at 10,000 ppb TCE and 88% at 50,000 ppb TCE. Powell et al. (2011) observed a rapid and complete inhibition of ammonia oxidation with 150 μg/L of TCE at 20 mg/L of ammonia in batch experiments, showing the great sensitivity of AOB to TCE. Yang et al. (1999) reported TCE at the levels higher than 200 μg/L can inhibit ammonia oxidation. They also suggested the NH$_3$/TCE ratio, rather than just TCE concentration, determined growth substrate (NH$_3$) utilization rate (qNH$_3$). Hyman et al. (1995) found the $K_i$ value for TCE (30 mmol/L) is similar to the $K_m$ (Michaelis constant, the substrate concentration at which the reaction rate is half of the maximum rate achieved by the system) value for ammonia (40 mmol/L) in the cometabolism process by N. europaea.

Yang et al. (1999) summarized the models behind this competitive mode: For the pure growth substrate without TCE added, the growth substrate utilization rate (qNH$_3$, mg/mg VSS day) related to the growth substrate (NH$_3$) concentration as follows:

$$q_{NH_3} = \frac{(kS)}{(K_m + S)}$$

where S (mg/L) is NH$_3$ concentration, k (day$^{-1}$) is the maximum specific utilization rate of NH$_3$, and $K_m$ (mg/L) is the half-saturation constant of the growth substrate. At low S, $K_m >> S$, and qNH$_3$ is proportional to S. When TCE is present, the equation changes to:

$$q_{NH_3} = \frac{(kS)}{[K_m (1 + I/K_I) + S]}$$
where I (mg/L) is the competitive substrate (TCE) concentration and $K_I$ (mg/L) is the half-saturation constant of TCE. This equation shows that when TCE is added, qNH$_3$ was affected by both NH$_3$ and TCE concentrations. And also when TCE concentration was low (compared to $K_I$ value of 1.4 mg/L according to Ely et al. (Ely et al., 1995b)), qNH$_3$ was only proportional to NH$_3$ concentration as well. And for TCE utilization rate (qTCE), the model is:

$$q_{TCE} = \frac{(k_I I)}{[K_I (1 + S/K_m) + I]}$$

where $k_I$ (day$^{-1}$) is the maximum specific utilization rate of TCE. After a series of mathematical transformations, they got

$$q_{NH_3} = (S/I) \times (q_{TCE} \times \varepsilon)$$

at lower TCE and NH$_3$ levels, where $\varepsilon$ is the dimensionless first-order reaction rates ratio and equals to $((k/K_m) / (k_I/K_I))$. Thus, when qTCE did not change significantly, qNH$_3$ was only determined by the ratio of growth substrate and competitive substrate (Yang et al., 1999). The transformation yield ($T_y$) of TCE, i.e., the amount of TCE degraded per unit mass of NH$_4$–N, was strongly depended on the initial concentrations of those two and a linear relationship between $1/T_y$ and the initial NH$_4$–N/TCE ratio was observed by Kocamemi and Cecen (Kocamemi and Cecen, 2010).

In the noncompetitive scenarios, growth substrates and secondary substrates bind to different sites on the enzyme, and a decrease in overall oxidation rates is resulted. According to Sayavedra-Soto et al. (2010), cometabolism of CM, bromomethane (BM), DCM, and CF with AMO is noncompetitive. CF, for example, was observed to be degraded by N. europaea with a maximum specific degradation
rate $k_c = 10 \text{ mg/mg dwt cells-day}$ with the tolerance of 0.65 to 1.6 mg/l (Ely et al., 1997; Alvarez-Cohen and Speitel, 2001). The inhibition effects can increase along with the halogen’s size and number of substituents, but decrease with the inhibitor’s molecular weight (Keener and Arp, 1993).

As discussed above, the products of cometabolism can also cause cellular damage, which can inhibit further cometabolism and nitrification. This process is usually irreversible (Hyman et al., 1995). Studies using $^{14}$C labeled TCE found those labels in proteins, indicating cellular damage was caused by a reactive product of TCE (Rasche et al., 1991a). Those reactive products (acyl chlorides) may be short-lived intermediates caused by the oxidation of TCE. And those intermediates mainly impact DNA repair mechanisms, as shown for Burkholderia cepacia G4 (Yeager et al., 2001), deactivate key enzymes, or lead to general cumulative damage to cells (Sayavedra-Soto et al., 2010).

The extent of inhibition and inactivation also varied by substrate. For example, the inactivation caused by DCM and CF is much more substantial than that caused by CM (Rasche et al., 1990; Vannelli et al., 1990; Rasche et al., 1991a). For, 1,2-DCA, minimal inactivation was documented by Kocamemi and Cecen (2009), who reported in their nitrifying biofilm reactors, long-term input of 1,2-DCA did not change the quantities and diversities of Nitrosomonas, Nitrobacter and Nitrospira species significantly. For brominated HAHs, product toxicity (measured by transformation capacity, $\text{Tc}$) also increased with bromine substitution, i.e., BF > DBCM > BDCM > CF (Wahman et al., 2005). In general, HAHs can be divided into three groups: 1) not biodegradable by AOB and thus not toxic to cells; 2) biodegradable and having little or no toxic effect on cells; 3) can be cometabolized by AOB and can inhibit or
inactivate ammonia oxidation by AOB (Table 1.2) (Rasche et al., 1991a). The first group includes compounds which do not bind to AMO or bind to AMO but are not cooxidized, (e.g., carbon tetrachloride and tetrachloroethene). Group 2 includes chloromethane, chloroethane, and 1,2-dichloroethane. In this group, a single chloride ion is removed from a monochlorinated carbon and forms the corresponding aldehyde (formaldehyde, acetaldehyde, and chloroacetaldehyde). Group 3 includes various HAHs (see Table 1.2). Those compounds usually carry a dichlorinated carbon. During the hydroxylation of the dichlorinated carbon, acyl chloride will form by the following mechanism:

\[
\begin{array}{c}
\text{Cl} & \text{Cl} & \text{Cl} \\
\mid & \text{O}_2 & \mid \\
\text{R} - \text{C} - \text{H} & \rightarrow & \text{R} - \text{C} - \text{O} - \text{H} & \rightarrow & \text{R} - \text{C} \equiv \text{O} + \text{Cl}^- + \text{H}^+ \\
\mid & \text{AMO} & \mid \\
\text{Cl} & \text{Cl} & \text{Cl}
\end{array}
\]

Acyl chloride can act as a protein-modifying agent to inactivate enzymes or proteins such as electron carriers through covalent modification, and thus lead to inactivation and inhibition (Rasche et al., 1991a).

Currently, the molecular responses of AOB to cometabolism have been studied at proteome and transcriptome levels, leading to the identification of gene promoters that can serve as indicators of cometabolic stress (Gvakharia et al., 2007; Park and Ely, 2008a, b). For example, amoA mRNA showed a sensitive response to ammonia oxidation and thus can be a good indicator of ammonia oxidizing activity in a mixed culture water treatment facility (Aoi et al., 2004). The functional amoA gene of AOB as an effective molecular marker indicating ammonia oxidizing bacteria was reported in a number of studies (Ibekwe et al., 2003; Ma et al., 2008), and was also found to
work well in the current study. Whole genome expression analysis of *N. europaea* has been carried out to examine its response to many cometabolic substrates, including HAHs such as CF and CM (Gvakharia et al., 2007). The introduction of HAHs such as CF and CM to a microbial community can cause downregulation of “housekeeping” genes (e.g., genes that encode ribosomal proteins), and thus result in the inhibition of cometabolism. Those downregulation responses are usual in prokaryotes (Betts et al., 2002). Also, some “sentinel” genes were upregulated after exposure to alternative substrates and thus maintain the oxidizing ability (Gvakharia et al., 2007). These molecular responses can thus be served as indicators of the presence of pollutants (Bott and Love, 2002).

Damages can be recovered after a period of TCE (or other HAHs) free medium culture (Ely et al., 1995b; Yang et al., 1999). The mechanism of this recovery is due to a de novo protein synthesis, including AMO, to restore the ammonia oxidizing activity (Rasche et al., 1991a). The kinetics of recovery seem to be related to both the extent of inactivation and/or inhibition and the degree of specificity of the inactivating treatment (Hyman et al., 1995). At increased inactivation levels, the inactivated cells did not recover as rapidly as those lightly inactivated cells (Hyman et al., 1995). Recovery is important to long-term sustainable bioremediation. When TCE oxidation is balanced by recovery, the maximal sustainable rate of TCE oxidation is achieved (Hyman et al., 1995).

### 1.4 RESEARCH GOALS

- Confirm the presence of AOB in the rhizosphere of a common wetland plant *Carex comosa* (‘longhair sedge, or bristly sedge’), by both a molecular method (PCR) and an analytical method (degradation of ammonium).
• Characterize aerobic, cometabolic HAH degradation (TCE, CF, 1,1,2-TCA, cis-DCE, EDB and 1,2-DCA) by the AOB associated with Carex comosa roots.

• Investigate the effects of HAH concentration (TCE, CF, 1,1,2-TCA, cis-DCE, EDB and 1,2-DCA) on the transformation of HAHs by AOB naturally associated with the roots of C. comosa.

• Evaluate the feasibility of sustainable HAH removal with Carex comosa roots at bench-scale with ammonium.

• Evaluate the potential that wetlands have for complete mineralization of harmful pollutants.

• Investigate the relationships between degradation kinetics and microbes associated in root zones.

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Figure 1.1. Phylogenetic 16S rRNA tree reflecting the relationships of AOB and several non-AOB reference organisms based on neighbor-joining tree (MOB, methane oxidizing bacteria) from (Purkhold et al., 2000).
Figure 1.2. Reactions catalyzed by AMO and HAO. During cometabolism of HAHs (using chloromethane for example), there is an energy drain as it is oxidized (Sayavedra-Soto et al., 2010).
Figure 1.3. Pathways of HAHs and halogenated aromatic hydrocarbons can be oxidized by AMO (Arp and Stein, 2003).
Figure 1.4. Possible pathways for AOB during cometabolism of HAhs in an aquatic environment. HC, any halogenated hydrocarbon. HC=O, a byproduct resulting from the oxidation of HC (Sayavedra-Soto et al., 2010).
Table 1.1. HAHs that can be metabolized or cometabolized from (Arp et al., 2001)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
<th>Abbreviation</th>
<th>Metabolism</th>
<th>Cometabolism</th>
<th>Substrates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloromethane</td>
<td>CH₃Cl</td>
<td>CM</td>
<td>Y</td>
<td>Y</td>
<td>A, M</td>
</tr>
<tr>
<td>Methylene chloride</td>
<td>CH₂Cl₂</td>
<td>DCM</td>
<td>Y</td>
<td>Y</td>
<td>A, M</td>
</tr>
<tr>
<td>Chloroform</td>
<td>CHCl₃</td>
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<td>N</td>
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<tr>
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</tr>
<tr>
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<td>CH₂CHCl₂</td>
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<td>A, M</td>
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<tr>
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<td>1,2-DCA</td>
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<td>Y</td>
<td>A, C, M, T</td>
</tr>
<tr>
<td>1,1,1-Trichloroethane</td>
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<td>A, M</td>
</tr>
<tr>
<td>1,1,2-Trichloroethane</td>
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<tr>
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<td>A</td>
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<tr>
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<td>–</td>
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<tr>
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<td>Vinyl chloride</td>
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<td>Y</td>
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<tr>
<td>1,1-Dichloroethene</td>
<td>CCl₁=CH₂</td>
<td>1,1-DCE</td>
<td>N</td>
<td>Y</td>
<td>A, C, M, Pr, T, V</td>
</tr>
<tr>
<td>1,2-Dichloroethene (cis)</td>
<td>CHCl=CHCl</td>
<td>cis-DCE</td>
<td>N</td>
<td>Y</td>
<td>A, B, C, M, Pr, Py, T, V</td>
</tr>
<tr>
<td>1,2-Dichloroethene (Skinner et al.)</td>
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<td>trans-DCE</td>
<td>N</td>
<td>Y</td>
<td>A, C, M, Py, T, V</td>
</tr>
<tr>
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<td>TCE</td>
<td>N</td>
<td>Y</td>
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<tr>
<td>Tetrachloroethene</td>
<td>CCl₂=CCl₂</td>
<td>PCE</td>
<td>N</td>
<td>Y</td>
<td>T</td>
</tr>
</tbody>
</table>

Metabolism: Y, compound will serve as growth substrate; N, compound will not serve as growth substrate.

Cometabolism: Y, compound transformed through cometabolic processes; N, compound not transformed through cometabolic processes.

Growth substrates: A, ammonia; M, methane; B, butane; C, chloroethane; T, toluene; Pr, propane; Py, propylene; V, vinyl chloride; D, 2,4-dichlorophenoxyacetic acid; I, isoprene; Ph, phenol.
Table 1.2 HAH groups in term of the biodegradability of AOB and inactivation to ammonia oxidation (Rasche et al., 1991b).

<table>
<thead>
<tr>
<th>HAHs</th>
<th>HAHs Degradability</th>
<th>Products</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Group 1 (not degradable, no inactivation)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carbon tetrachloride</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>Tetrachloroethene</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td><strong>Group 2 (degradable with minimal inactivation)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chloromethane</td>
<td>Yes</td>
<td>Formaldehyde</td>
</tr>
<tr>
<td>Chloroethane</td>
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<td>Acetaldehyde</td>
</tr>
<tr>
<td>1,2-Dichloroethane</td>
<td>Yes</td>
<td>Chloroacetaldehyde</td>
</tr>
<tr>
<td><strong>Group 3 (degradable with substantial inactivation)</strong></td>
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<td></td>
</tr>
<tr>
<td>Dichloromethane</td>
<td>Yes</td>
<td>Carbon monoxide</td>
</tr>
<tr>
<td>Chloroform</td>
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</tr>
<tr>
<td>1,1-Dichloroethane</td>
<td>Yes</td>
<td>Acetic acid</td>
</tr>
<tr>
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<td>Yes</td>
<td>2,2,2-Trichloroethanol</td>
</tr>
<tr>
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</tr>
<tr>
<td>Trichloroethene</td>
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</tbody>
</table>
2 NATURAL ATTENUATION POTENTIAL OF TRICHLOROETHENE IN WETLAND PLANT ROOTS: ROLE OF NATIVE AMMONIUM-OXIDIZING MICROORGANISMS

2.1 INTRODUCTION

2.1.1 Ammonium Availability in Wetlands

Ammonium is a naturally occurring pore-water constituent, participant in nitrogen (N)-cycling in aquatic environments including wetlands, and an end product of polymeric organic matter degradation in anaerobic soil (Chapin et al., 2002). Ammonium may also be introduced into treatment wetlands by nitrogen-rich effluents such as from wastewater plants, septic tanks, and landfill leachates (Nivala et al., 2007). Many biological molecules and polymers contain notable quantities of organic nitrogen, particularly proteins, chitin, porphyrins, Flavin Adenine Dinucleotide (FAD), Nicotinamide Adenine Dinucleotide (NAD), Deoxyribo Nucleic Acid (DNA), and Ribo Nucleic Acid (RNA) (Reddy and DeLaune, 2008), which may be degraded into their monomeric subunits (amino acids, amino sugars, nucleic acids) by extracellular enzymes and are made bioavailable to the soil microorganisms associated with plants. A new paradigm of N-cycling in soils suggests that such depolymerization of organic matter to N-monomers
may be the limiting step in nitrogen availability (Schimel and Bennett, 2004); further mineralization of N-monomers to ammonium or nitrate is analogous to the degradation of the carbonaceous fraction of complex organic matter to methane (CH₄) during anaerobic decay or to carbon dioxide (CO₂) during aerobic decomposition (Atlas and Bertha, 1998). As such, ammonium concentration in soils is both a function of its production facilitated by microorganisms, and its uptake by both microbes and plants (Schimel and Bennett, 2004).

In many cases, the bulk of the wetland soil/sediment below the water table is anoxic, which creates a strongly reducing environment (Askaer et al., 2010). Yet, the soil at shallow depths in a vegetated wetland and similar aquatic environments can be relatively oxidizing due to aerenchymous gas transport and O₂ leakage from wetland plant roots (Laan et al., 1989; Armstrong et al., 1994). While ammonium can accumulate in the anaerobic zones of a wetland it will also diffuse along the concentration gradient or flow with the bulk water and pass through the shallow, more aerobic, depths of the vegetated wetland. Once there, ammonium has been shown to be oxidized by microbes at the root surface, with subsequent denitrification occurring in the bulk soil and N₂ gas released to the atmosphere (Reddy et al., 1989). The pathway of microbial ammonium oxidation involves two reactions in the presence of molecular oxygen (Fernández et al., 2008; Gilch et al., 2009); ammonium is oxidized to hydroxylamine by the enzyme ammonium monoxygenase (AMO) that is further oxidized to nitrite by the enzyme hydroxylamine oxidoreductase (HAO). Nitrite can be subsequently transformed to nitrate, which completes the process known as nitrification.
Ammonium oxidizing bacteria (AOB) have been identified and studied in various environments including the rhizosphere from rice (Briones et al., 2003), G. maxima (Bodelier et al., 1996; Kowalchuk et al., 1998), and barley (Glaser et al., 2010). A greater diversity of the ammonium monoxygenase (amoA) gene on rice root samples compared to unplanted soil (Briones et al., 2003) may indicate the presence of well-established ammonium-oxidizing microbial communities on certain plant roots. Glyceria maxima, an aerenchymatous plant species that inhabits marsh environments, has provided numerous insights into the distribution of AOB; for example, a significantly greater number and higher activities of AOB were reported in spring and early summer (Bodelier et al., 1996); and genetic evidence of Nitrosospira, an AOB, was found in root-associated soil samples (Kowalchuk et al., 1998). AOB associated with wetland plant root zone has been identified either taxonomically (Kowalchuk et al., 1998) or by studying the functional group ammonium monoxygenase (amoA) gene (Briones et al., 2003; Glaser et al., 2010).

2.1.2 Aerobic Cometabolism of Tricholoethene

According to National Resource Council or NRC (2006), chlorinated ethenes pose a significant threat to groundwater as a pollutant throughout the United States. Trichloroethene (TCE) was considered as a potential carcinogen, even if the concentration is below maximum contaminant level of 5 μg/L, as per NRC (2006). The natural attenuation approaches to treat groundwater contaminated with chlorinated ethenes (e.g., TCE) has received much attention in recent years, mainly because the costs may be lower in comparison to other methods for cleaning-up of contaminated aquifers, and also due to the universal distribution of the bacteria that can facilitate TCE
degradation (Hagopian and Riley, 1998). Among various approaches for the treatment of TCE and similar recalcitrant halogenated hydrocarbons found in shallow, aerobic groundwater plumes, a treatment approach based on cometabolic biodegradation can be effective (McCarty et al., 1998; Gandhi et al., 2002), since no aerobic microorganism that can grow on TCE as a sole carbon or energy source has been isolated (Hyman et al., 1995). Cometabolism is the fortuitous microbial transformation of a non-growth supporting compound that is catalyzed by an enzyme or cofactor with a nonspecific active site, and supported by the metabolism of a growth-supporting substrate or another transformable compound (Dalton and Stirling, 1982; Hyman et al., 1995). In aerobic cometabolism, TCE is mineralized to CO₂, H₂O, and chloride ions, without the accumulation of stable and carcinogenic intermediates such as vinyl chloride (Vogel and McCarty, 1985).

Numerous compounds have been shown to act as growth substrates to support TCE biodegradation by aerobic cometabolism, including toluene, phenol, methane, propane, propene, butane, cumene, isoprene, and ammonium (Hyman et al., 1995; Arp et al., 2001). In fact, many mono, di, and tri-chlorinated ethanes and ethenes can be degraded by cometabolic process with ammonium as a growth supporting substrate (Arp et al., 2001). AMO enzyme that is produced by ammonia oxidizing microbes has been shown to have greater affinity for TCE and 1,1-dichloroethene than for ammonium, though the maximum substrate transformation rate was highest for ammonium (Ely et al., 1997). TCE may be a potent competitor for AMO enzyme in ammonium oxidation, suggested by the similar inhibition constant for TCE (30 mM) and the half-saturation constant (Kₘ) for ammonium (40 mM) (Hyman et al., 1995); TCE may also be quite toxic
to ammonium oxidizers as 60 nmol of TCE per mg of protein from *Nitrosomonas europaea* could completely inactivate the bacteria, although the cells recovered rapidly if the inactivation level was 40% or less.

Much of the impetus for the present work came from recent studies at an upward-flow treatment wetland at Wright-Patterson AFB (Amon et al., 2007; Powell, 2010), which showed increasing ammonium concentrations with decreasing depth, i.e. along the water flow path. However, in the shallower, vegetated zone of the wetland that is influenced by plant roots, the ammonium concentration decreased quickly suggesting a significant role of rhizospheric ammonium oxidation by microbial processes (Powell, 2010). In this study, we examined the ammonium oxidation potential of the bacteria that are naturally associated with the roots of a common wetland plant, *Carex comosa* (‘bristly sedge’). The key objectives of this study were the following: (1) confirm the presence of AOB in the rhizosphere of *Carex comosa*, by both molecular method (PCR) and analytical method (degradation of ammonium); (2) characterize aerobic, cometabolic TCE degradation by the AOB associated with *Carex comosa* roots; and (3) evaluate the feasibility of sustainable TCE removal with *Carex comosa* roots at bench-scale with ammonium.

### 2.2 MATERIALS AND METHOD

#### 2.2.1 Experimental Set-up

*2.2.1.1 Collection of wetland plants*
Wetland plants (*Carex comosa*) were collected from a natural fen site in Dayton, OH (USA). After collection, the plants were placed in individual pots containing a 50:50 mix of peat moss and wetland soil, and maintained in a greenhouse until use. The roots from potted *Carex comosa* plant were prepared for bench-scale experiments by first clipping from the plant shoot, washing with de-ionized water thoroughly to remove attached soil, drying in air, and then weighing for individual bottles.

### 2.2.1.2 Microbial enrichment with ammonium (in absence of TCE)

Six microcosms (112 mL amber borosilicate glass serum bottles; Wheaton, Millville, NJ) were used for the microcosm experiments with ammonium as a substrate. Three bottles were prepared as live microcosms with 0.7 g of fresh plant roots and 70 mL of growth medium (containing either 20 or 30 mg/L of aqueous NH$_4$-N), and the three remaining bottles were prepared as inhibited controls prepared as above but with 10 mg/L of allylthiourea (ATU) to minimize the activity of ammonium-oxidizing bacteria (Rasche *et al.*, 1991; Ginestet *et al.*, 1998). After these additions, a headspace of 42 mL remained in each microcosm. All microcosms were bubbled with air for ~30 min to start the experiment with enough dissolved oxygen (DO) to maintain aerobic conditions during individual cycles. The microcosms were capped with Teflon-lined grey butyl rubber stoppers (20 mm dia.; Wheaton, Millville, NJ) and sealed with aluminum crimps (Wheaton, Millville, NJ). The bottles were then wrapped in aluminum foil to exclude light and placed on a rotary shaker (Glas-Col, Terre Haute, IN) at 30 rpm for gentle horizontal mixing in an upside down position at 22±1°C. There were three cycles of microbial enrichment (AME1 through AME3) with ammonium as a substrate. The first enrichment cycle (AME1) required 21 days to achieve a significant decrease in
ammonium concentration, but each of the subsequent cycles were only 7 days long. Each cycle corresponds to a period during which ammonium was degraded in the live microcosms. The cycles ended after sufficient ammonium removal in the microcosms, and the batch microcosms were opened to carefully decant and replace the aqueous growth medium without removing the roots (control microcosms again amended with 10 mg/L of ATU), bubbled with air, resealed, and placed on the rotary shaker, as described earlier. Each new cycle began with ammonium substrate at 20 or 30 mg/L of aqueous NH$_4^+$-N. The decanted media were saved at the end of each cycle for molecular biological analysis (described later). Repeated measured analysis of variance (RM ANOVA) tests were performed using software SPSS 19.0 (IBM, Armonk, NY, USA) to evaluate kinetics changes along through time by treatment. The composition of the growth medium, methods of chemical analysis of microcosm samples, and data treatment (including approach for estimating active biomass) are provided in Appendix.

2.2.1.3 Cometabolic TCE degradation with ammonium

Following the 3 cycles of microbial enrichment with ammonium (AME 1 through AME 3, described above), TCE degradation was examined in these microcosms (in triplicate) for 11 additional cycles (AMT 1 through AMT 11). Ammonium substrate was nominally either 20 mg/L of aqueous NH$_4$-N (cycles AMT 1 and AMT 2) or 30 mg/L of aqueous NH$_4$-N (cycles AMT 3 through AMT 11), and TCE was varied in order to assess the effect of its concentration on the degradation process. TCE concentration was varied as follows: nominally at 30 μg/L in AMT 1 to AMT 4, 45 μg/L in AMT 5 to AMT 7, and 70 μg/L in AMT 8 to AMT 11. Ammonium was increased in the latter cycles to examine whether TCE degradation would change with higher [ammonium] and constant [TCE]
Triplicate ATU-inhibited control microcosms were operated at ammonium and TCE concentrations equivalent to the live microcosms during all cycles.

### 2.2.2 DNA Extraction and PCR Amplification

The growth media that was decanted at the end of each cycle (described earlier) was centrifuged for 30 minutes at 1500 g to separate the bacterial biomass from the liquid. The pellets (biomass) were subjected to DNA extraction using the Ultraclean DNA Isolation kit (MOBIO, Carlsbad, CA). The DNA extracts were analyzed using the polymerase chain reaction (PCR) technique; the PCR primer pair amoA 151f and amoA 359rC were chosen to target the amoA gene encoding the ammonium monooxygenase enzyme in bacteria (Junier et al., 2008). PCR conditions were 94 °C for 2 minutes followed by up to 35 cycles of 94 °C for 30 seconds, 56 °C for 30 seconds, and 72 °C for 30 seconds. A final extension of 72 °C for 5 minutes was followed by holding the block at 4 °C until the samples could be transferred to cold storage. PCR reaction products were run on 1.5% agarose gels with 1X TAE buffer after amplification to verify amplicon size and primer specificity. A 100 base pair DNA size standard (ladder) was also run on each gel to verify product size.

### 2.3 RESULTS AND DISCUSSION

#### 2.3.1 Ammonium-Oxidizing Microbes in Wetland Plant Roots

##### 2.3.1.1 Molecular evidence

Ammonium was previously shown to degrade in the subsurface of a treatment wetland at metabolically significant levels (Powell, 2010). The same study also indicated
the potential to couple the ammonium oxidation to TCE biodegradation by aerobic cometabolism in shallow vegetated subsurface. Of interest then was the question of whether ammonium-oxidizing bacteria (AOB) are present in association with wetland plant roots in the shallow subsurface. Two approaches were used to gather evidence of AOB presence and activity. The molecular biology approach yielded evidence based on DNA presence. PCR primers were chosen to target the gene encoding the α-subunit of the amoA enzyme (Junier et al., 2008; Park et al., 2008). PCR results demonstrated that amoA gene was present in DNA extracts from the reactors described above (Figure B.2.1 in appendix). As expected, an abundance of the amoA target gene was present in the three live microcosms at both sampling times, while no amoA was detected in the triplicate control microcosms. This provides substantial evidence that AOB were present in live microcosms and inhibited in control microcosms, as intended. A similar approach was taken by Bollman and Laanbroek (2001), who amplified 16S rDNA from ammonium-oxidizing bacteria cultures, and followed PCR with other techniques to quantify the diversity and microbial community structure. In the present study, techniques other than PCR were not employed since determining the presence or absence of the amoA gene was the desired outcome. By targeting the functional gene (amoA) responsible for ammonium oxidation rather than 16S, as is often done, the results could be relied upon to positively demonstrate the presence of ammonium oxidizers. Targeting 16S ribosomal DNA can be less specific when the goal is to demonstrate the presence of a metabolic function. Additionally, an improved resolution in the ecology of ammonium-oxidizing bacteria can be achieved if amoA gene is targeted rather than 16S (Junier et al., 2008). Briones et al. (2003) demonstrated the efficacy of targeting functional genes, as
they studied PCR of the *amoA* gene along with denaturing gradient gel electrophoresis to compare the diversity of ammonium monooxygenase between planted and unplanted samples; their study reported that planted conditions did generally favor greater diversity of *amoA* gene.

2.3.1.2 Analytical evidence

In addition to the strong evidence presented by PCR analysis (*amoA* gene) in this study that support the presence of AOB on *Carex comosa* roots, the evidence based on chemical analysis also supports AOB activity through conversion of ammonium to nitrite and nitrate with a simultaneous decrease in oxygen (Figures 2.1 and B.2.2). During the initial enrichment period (cycle AME 1 for three weeks; Figures B.2.2-B.2.4, in Appendix), significant ammonium removal and nitrate production were measured in the absence of TCE. This compares well to previous reports of nitrification and denitrification in wetland mesocosms using $^{15}$N radiolabeled ammonium (Reddy et al., 1989); in that study, planted, therefore oxygenated, reactors demonstrated significantly greater $^{15}$N$_2$ in the reactor headspace. The results of our investigation are also comparable with another study where soil inocula from the rhizosphere of *Glyceria maxima* were used to establish continuous cultures of ammonium oxidizers at very low NH$_4^+$ concentrations (Bollmann and Laanbroek, 2001). In the present investigation, ammonium oxidation was sustainable with the excised root samples for over 9 weeks (with periodic replenishment of growth medium, described above), showing no significant decline in NH$_4^+$ removal (Figure 1a), even with TCE amendments.

2.3.2 Degradation of TCE and Ammonium
AOB have been shown to degrade TCE through aerobic cometabolism with ammonium as the primary energy source (Arciero et al., 1989; Ely et al., 1997). The results discussed here demonstrate that the population of ammonium-oxidizing organisms closely associated with Carex comosa roots obtained from a local wetland can also be stimulated to degrade TCE. At 29 µg/L TCE, a relatively sustained TCE degradation was observed with either 20 mg/L or 30 mg/L NH₄⁺-N for four weeks (cycles AMT 1-4; Figure 2.1). Microbial TCE oxidation remained largely unaffected in three subsequent weeks (cycles AMT 5-7) even after TCE concentration increased to 46 µg/L (with NH₄⁺-N at 30 mg/L). However, as TCE concentration was increased to 64 µg/L in four subsequent weeks (cycles AMT 8-11; Figure 2.1), a gradual microbial deactivation may have set in, and the AOB activity nearly ceased in cycle AMT 11. Microbial deactivation in cycles AMT 8-11 was also evident as gradually declining ammonium degradation, oxygen consumption and NOx production as TCE concentration increased to 69 µg/L (cycles AMT 8-11; Figures 2.1 and 2.2). A modest decrease in O₂ consumption in cycle AMT 11 may suggest some heterotrophic activity still persisting at that time (Figures B.2.2 and B.2.5, in Appendix).

The pH decline in control reactors was presumably caused by H⁺ production upon heterotrophic oxidation of root-derived DOC in the reactors (Figure B.2.5). In comparison, the pH decline in live reactors was greater than in control reactors through the cycles due to combined H⁺ contributions from ammonia (equation A.2.1 in Appendix) and DOC oxidation. However, at 69 µg/L TCE (cycle AMT 11) the decline in pH in the live and control reactors were nearly identical arguably because of inhibition in metabolic AMO oxidation in live reactors, and only heterotrophic activity continued. Further, the changes
in Total Inorganic Carbon (TIC) through the cycles in live vs. control reactors do not show a clear trend (Figure B.2.6 in Appendix); a lower initial TIC (~2 mmol/L) in batch system did not affect nitrification activity, as suggested for systems below a threshold value of 3 mmol/L TIC (Guisasola et al., 2007).

2.3.3 Degradation Kinetics and Transformation Yields

The pseudo first-order rate constants (Table 2.1) and the zero-order initial rates (Table B.2.1 in Appendix) of ammonium and TCE degradation were calculated for each experimental cycle to assess the activities of the ammonium-oxidizing microorganisms in this study. The mean TCE pseudo first-order rate constant \( k_{\text{obs-TCE}} \) for all cycles was 0.22 (±0.12) \( \text{d}^{-1} \); the lowest value was 0.10 \( \text{d}^{-1} \) while the highest was 0.50 \( \text{d}^{-1} \) (Table 2.1). The mean ammonium pseudo first-order rate constant \( k_{\text{obs-NH4}} \) for all cycles was 0.43 (±0.35) \( \text{d}^{-1} \) and ranged from 0.15 to 1.43 \( \text{d}^{-1} \). The mean of the cellular biomass concentration, \( X \), across all cycles was estimated to be 0.20 (±0.07) mmol/L, based on stoichiometry of biomass synthesis (equation A.2.9 in Appendix). The pseudo first-order rate constants of TCE \( k_{\text{obs-TCE}} \) and ammonium \( k_{\text{obs-NH4}} \) for individual cycles were normalized by dividing with biomass concentration, \( X \), and reported as \( k_{1\text{-TCE}} \) and \( k_{1\text{-NH4}} \) respectively (Table 2.1).

The biomass-normalized degradation kinetics of ammonium \( (k_{1\text{-NH4}}) \) shows variations through 13 cycles that may be reflective of experimental conditions, particularly initial \( \text{NH}_4^+ \) and initial TCE concentrations (Table 2.1). During ammonia enrichment (cycles AME 1-3) with initial \( \text{NH}_4^+ \)-N concentration nominally at 20 mg/L and no TCE, the mean \( k_{1\text{-NH4}} \) was highest at 4.320 ± 0.502 L/mmol/d, but it declined to
2.843 ± 0.577 L/mmol/d upon TCE amendment (initial TCE concentration at 29 µg/L) in cycles AMT 1-2. After ammonia increased (initial NH$_4^+$-N concentrations nominally at 30 mg/L) without any change in TCE concentration in cycles AMT 3-4, the mean $k_{1,\text{NH}_4}$ declined further to 1.380 ± 0.280 L/mmol/d. The $k_{1,\text{NH}_4}$ did not vary much in subsequent cycles; during cycles AMT 5-7 (initial TCE concentration at 46 µg/L), the mean $k_{1,\text{NH}_4}$ was slightly lower at 1.180 ± 0.315 L/mmol/d, but it was higher again at 1.373 ± 0.978 L/mmol/d during cycles AMT 8-10 (initial TCE concentration at 69 µg/L). On the other hand, the mass-normalized TCE degradation kinetics ($k_{1,\text{TCE}}$) through cycles (AMT 1-10) showed smaller variations as a function of initial NH$_4^+$ and initial TCE concentrations (Table 2.1); the mean $k_{1,\text{TCE}}$ value ranged from 0.673 ± 0.237 L/mmol/d during cycles AMT 5-7 to 1.450 ± 0.517 during cycles AMT 3-4. The overall means for $k_{1,\text{NH}_4}$ and $k_{1,\text{TCE}}$ during 10 TCE cycles are 1.61 (±0.84) and 1.10 (±0.69) L/mmol/d respectively. The overall mean $k_{1,\text{TCE}}$ is similar to a recently published value from a suspended nitrifying culture (Kocamemi and Cecen 2010); assuming that biomass is 113 g VSS/mole, their $k_{1,\text{TCE}}$ at 30 mg/L NH$_4^+$-N would convert to 0.73 L/mmol/d.

In a similar experimental set-up with excised Carex comosa roots and growth media, but with methane as the growth substrate (Powell, 2010) instead of ammonia, the kinetics of TCE degradation by aerobic cometabolism were considerably lower than previously reported values for suspended cultures. This was attributed to mass transfer limitations in these microcosms since the biomass was most likely located primarily on the root surface as biofilm, and not homogeneously dispersed throughout the solution. Further, the calculated mean biomass concentration for all cycles in this study (~29 mg/L) is near half the biomass concentration reported in other, suspended culture,
systems (Ely et al., 1997). It is possible that the degradation rate constants reported by (Kocamemi and Cecen 2010) are somewhat lower than that of other suspended cultures as well because their cultures were derived from a wastewater treatment plant and may have a large proportion of non-nitrifying biomass that competes with TCE degradation and mass transfer.

In the present study, the total mass of TCE degraded in cycles was related to the initial TCE concentration; as TCE concentrations increased, so did the total mass of TCE degraded. The effect on transformation yield ($T_y$) was similar, except that $T_y$ was affected by both TCE and ammonium concentrations (Figure 2.3). At 26 µg/L initial TCE concentration and 30 mg/L initial NH$_4^+$-N concentrations, the mean $T_y$ was significantly lower with initial NH$_4^+$-N concentration of 30 mg/L than it was at 20 mg/L (Table B.2.2 in Appendix). On the other hand, as initial TCE concentration increased from 26 to 69 µg/L in AMT 3-10 with constant ammonium-N at 30 mg/L, the $T_y$ also increased (Figure 2.3; also mean $T_y$ in Table B.2.2). From a competitive inhibition standpoint, this is reasonable since AMO is non-specific (Hyman et al., 1995); as the numbers of TCE molecules increases compared to the same number of ammonium molecules, the odds will increase that TCE encounters AMO and is oxidized by the enzyme. Since TCE shows greater affinity for AMO than that of NH$_4^+$ for the enzyme (Ely et al., 1995), increasing TCE concentration is likely to have more significant effects on $T_y$ than decreasing ammonium. Similar impacts on transformation yield resulting from changes in concentration have been reported in other cometabolic systems (Anderson and McCarty, 1997; Powell et al., 2011). Similarly, at a higher concentration of NH$_4^+$, the chances that an enzyme active site to be occupied by an ammonium molecule rather than a TCE
molecule are increased, which causes a decrease in $T_y$ with increasing $\text{NH}_4^+$ concentration.

### 2.3.4 Deactivation of TCE and Ammonium Degradation

It has been shown previously that TCE cometabolism can be toxic/inhibitory towards ammonium oxidizing bacteria (Ely et al., 1997; Powell et al., 2011). In the results reported here, the microbial deactivation was not observed at low TCE concentrations; at 29 and 46 $\mu$g/L TCE, the mean $k_{1,\text{TCE}}$ (Tables 2.1) and TCE initial degradation rate (Tables B.2.1 in Appendix) stayed relatively stable through 7 weeks (cycles AMT 1-7). However, keeping the ammonium concentration constant and increasing the TCE concentration to 69 $\mu$g/L yielded apparent microbial deactivation beginning from Cycle AMT 9 ($p<0.05$, one-way RM ANOVAs of $k_{1,\text{TCE}}$, [TCE] level effect, exclude outlier AMT 8). The first cycle nominally at 69 $\mu$g/L TCE (AMT #8) yielded modest increase in TCE removal (Table 2.1, and Table B.2.1). However, in the next three consecutive cycles (AMT 9-11), the TCE degradation rate declined sharply compared to the preceding cycle until eventually TCE degradation halted (Table B.2.1). A similar trend was observed with ammonium degradation (Table B.2.1), oxygen uptake and NO$_x$ production (Figure 2.2), all suggesting rapid progressive microbial deactivation at 69 $\mu$g/L TCE (AMT 9-11). The decrease in ammonium degradation rate in consecutive cycles was less pronounced, but the removal essentially halted in the same cycle, as did TCE degradation (Figure 2.1). These results indicate that at 30 mg/L of ammonium-N, there is a threshold concentration of TCE between 46 and 69 $\mu$g/L, where the energy gained from oxidation of ammonium is balanced by the cellular damage caused by TCE cometabolism. The results also show that overall conversion of ammonium to NO$_x$ was relatively constant in the first 7 cycles.
(Figure 2.2), but also that the conversion is increasingly inhibited in cycles AMT 8-10. This is less likely due to competitive inhibition between TCE and ammonium since $T_y$ did not decrease but increase instead. For competitive inhibition, the threshold TCE concentration usually ranged from 200 to 3000 μg/L (Yang et al., 1999; Alvarez-Cohen and Speitel, 2001; Kocamemi and Cecen, 2007; Kocamemi and Cecen, 2010). It’s more likely in the current scenario that the inhibition was due to cellular damage of AOB, caused by toxic intermediate products, acyl chlorides, produced from TCE oxidation (Rasche et al. 1991). On a positive note, however, previous reports have shown that the bacteria can recover from substantial inactivation, given sufficient time, ammonium, and oxygen (Hyman et al., 1995). The PCR results from this study depicted in Figure B.2.1 lend credence to those previous reports. Even though ammonium and TCE degradation had essentially halted by cycle 10, likely due to microbial deactivation at high TCE concentration, the intensity of the PCR bands for the amoA gene is just as high at the end of cycle AMT 10 as it was after cycle AMT 7. Even though the AMO enzyme had been inactivated by TCE oxidation before cycle AMT 10 ended, the AMO gene required to synthesize more enzyme was present in abundance.

2.4 CONCLUSIONS

Our recent field study has shown a near complete removal of chlorinated ethenes in the groundwater passing through the research wetland site constructed at Wright-Patterson Air Force Base in Dayton, OH (Amon et al., 2007). The present bench-scale study provides a validation of simultaneous degradation of ammonium and chlorinated ethenes in the shallow, vegetated zone of the wetland (Amon et al., 2007), and offers a mechanism for destruction of chlorinated ethenes through aerobic cometabolism. This
study provides good evidence for an oxidative degradation pathway of TCE in the root zone of wetland plants with ammonium, a natural product of organic matter biodegradation, as the driving energy source. PCR amplification of the AMO gene showed that ammonium-oxidizing bacteria were associated with washed root samples of *Carex comosa*, and upon stimulation, they were able to cometabolize TCE in the presence of ammonium and oxygen. Between 29 and 69 μg/L, TCE was significantly degraded along with a subsequent conversion of ammonium to nitrite and nitrate. Over the course of three cycles at 69 μg/L TCE, the ammonium and TCE oxidation declined rapidly and shut down in cycle AMT 11, whereas 7 prior cycles at 29-46 μg/L TCE did not show such microbial deactivation. It appears that at 30 mg/L of NH$_4^+$-N there is a threshold TCE concentration between 46 and 69 μg/L where cellular damage overcomes the ability of the organism to enact repairs. The data presented here suggest that in a wetland with a nominal ammonium concentration of 30 mg/L and with wetland plants that provide oxygen to the subsurface, more than 46 μg/L of TCE may not be sustainably treated. Our study indicates that microorganisms associated with wetland plant roots can assist in the natural attenuation of TCE in contaminated aquatic environments, such as urban wetlands, or wetlands impacted by industrial solvents. The results may be helpful in applying constructed or restored wetlands towards pollutant mitigation and site management, and a sustainable TCE degradation at concentrations much greater than the US-EPA drinking water maximum contaminant level can occur in a treatment wetland.

2.5 REFERENCES


contribution to N use efficiency of different rice cultivars. Plant Soil 250, 335-348.


Figure 2.1 Variations in (A) TCE, and (B) NH₄⁺-N concentrations show ammonium-dependent cometabolic degradation of TCE. Data shown represent average of triplicate microcosms through 11 successive cycles (AMT 1 through AMT 11). Solid symbols represent average values in live microcosms while open symbols represent average values in control microcosms. Individual cycle numbers are shown above the charts.
Figure 2.2 Variations in (A) dissolved oxygen (DO), and (B) NO$_x$ (i.e., NO$_2^-$ + NO$_3^-$) concentrations shows oxygen consumption and nitrification of ammonium during TCE cometabolism. Data shown represent average of triplicate microcosms through 11 successive cycles (AMT 1 through AMT 11). Solid symbols represent average values in live microcosms while open symbols represent average values in control microcosms. Individual cycle numbers are shown above the charts.
Figure 2.3 Scatter plot of (A) Regression of TCE Transformation Yield ($T_y$) vs. initial TCE concentrations ($\mu$g L$^{-1}$) for average of triplicate microcosms, and (B) TCE Transformation Yield ($T_y$) vs. initial NH$_4^+$-N concentrations in individual microcosms, for 11 successive cycles (AMT 1 through AMT 11). Transformation Yield ($T_y$) is defined as the quotient of net TCE degraded (μmoles) divided by net NH$_4^+$ consumed (mmoles).
Table 2.1 Steady state biomass concentration ($X$, mmol/L); initial pseudo first-order rate constants of NH$_4^+$-N removal ($k_{\text{obs-NH}_4}$, d$^{-1}$) and TCE degradation ($k_{\text{obs-TCE}}$, d$^{-1}$), and their corresponding coefficients of determination ($r^2$); biomass normalized NH$_4^+$-N removal rate constants ($k_{1-\text{NH}_4}$, L mmol$^{-1}$ d$^{-1}$) in 3 enrichment cycles (AME 1-3) and 10 experimental cycles (AMT 1-10); and biomass normalized TCE degradation rate constants ($k_{1-\text{TCE}}$, L mmol$^{-1}$ d$^{-1}$) in 10 experimental cycles (AMT 1-10). Cycle AMT 11 was excluded due to deactivation.

<table>
<thead>
<tr>
<th>Cycle</th>
<th>Initial [TCE] (µg L$^{-1}$)</th>
<th>Initial [NH$_4^+$] (mg L$^{-1}$)</th>
<th>[Calculated Biomass], $X$ (mmol/L)</th>
<th>NH$<em>4^+$ removal rate constant $k</em>{\text{obs-NH}_4}$ (d$^{-1}$)</th>
<th>$r^2$</th>
<th>$k_{1-\text{NH}_4}$</th>
<th>TCE degradation rate constant $k_{\text{obs-TCE}}$ (d$^{-1}$)</th>
<th>$r^2$</th>
<th>$k_{1-\text{TCE}}$</th>
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<td>0</td>
<td>18.84</td>
<td>0.05</td>
<td>0.24</td>
<td>0.81</td>
<td>4.320 ± 0.502</td>
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<td>0.17</td>
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<td>0.94</td>
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<td>0.32</td>
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<td>19.59</td>
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<td>0.61</td>
<td>0.99</td>
<td>2.843 ± 0.577</td>
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<td>0.71</td>
<td>0.98</td>
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<td>0.29</td>
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<td>0.99</td>
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<td>29.06</td>
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<td>0.21</td>
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3 NATURAL ATTENUATION POTENTIAL OF COMMON CHLORINATED VOLATILE ORGANIC COMPOUNDS IN WETLAND PLANT ROOTS BY NATIVE AMMONIA-OXIDIZING MICROORGANISMS

3.1 INTRODUCTION

Chlorinated aliphatic hydrocarbons (CAHs) are common groundwater pollutants and pose a significant threat to human health as suspected carcinogens through skin absorption and lung inhalation (Matamoros et al., 2007). Trichloroethene (TCE), cis-dichloroethene (cis-DCE) and chloroform (CF) are among the most frequent chlorinated volatile organic compounds (CVOCs) in drinking water aquifers in the US (Carter et al., 2008; Carter et al., 2012). Various industrial and domestic sources account for the widespread occurrence of CAHs, which includes chlorine disinfection of treated wastewater which results in the oxidative breakdown of numerous organic substances present in treated effluents (Matamoros et al., 2007), and the use of chlorinated reclaimed water for agricultural applications (irrigation) (Levine and Asano, 2004).

Traditional wastewater treatment systems generally require large capital investments and operating costs, and are thus not realistic solutions for producers that cannot afford such expensive treatment systems, especially in less-developed countries.
(Kantawanichkul et al., 2009). However, natural attenuation has been proved to be effective to clean sites contaminated by CAHs (Field and Sierra-Alvarez, 2004). Through cometabolism, the ammonia-oxidizing bacteria (AOB) can oxidize alternative substrates (CAHs) in the presence of growth-supporting substrates (ammonium) (Wackett, 1996; Arp et al., 2001). In this process, AOB consume ammonia (or ammonium), which naturally occurs in wetlands as a participant in nitrogen cycling, a content in nitrogen-rich effluents (e.g. wastewater plants or septic tanks), or an end product of anaerobic polymeric organic matter degradation (Chapin et al., 2003; Nivala et al., 2007), as both energy source and reductant, CO₂ for carbon requirements, and molecular oxygen as electron acceptor (Sayavedra-Soto et al., 2010).

In wetlands, aquatic plants can transfer atmospheric oxygen from shoots to roots by porous aerenchyma tissue functioning as gas channel, making the plant rhizosphere an oxidizing environment in the otherwise anaerobic soil. This can stimulate a great number of aerobic biogeochemical processes, including the growth of ammonia-oxidizing bacteria that facilitate aerobic ammonium oxidation (Gersberg et al., 1986; Bodelier et al., 1996; Kowalchuk et al., 1998; Venter et al., 2004; Wiessner et al., 2005). It’s thus possible to direct groundwater contaminated by CAHs through wetlands for natural attenuation.

The aquatic plant *Carex comosa* (bristly sedge) was chosen for batch scale
experiments. This is a follow-up to previous investigations on TCE which were described in Chapter 2 and also can be found in (Qin et al., 2014) where degradation stopped when TCE concentrations exceeded 70 µg/L. In the current study, TCE (at higher concentration), cis-1,2 DCE and CF (Table 3.1 - 3.3) were investigated in batch experiments at varying concentrations. The main objective of the current study was to investigate the effects of CAH concentration on the transformation of several CAHs by AOB naturally associated with the roots of a common wetland plant, C. comosa.

3.2 MATERIALS AND METHOD

3.2.1 Experiment Setup

In this study, the procedures for collection of wetland plant (Carex comosa) from a natural wetland site, microcosm preparation containing washed (soil-free) plant roots, preparation of growth medium and reagents, enrichment for ammonia-oxidizing bacteria, gas chromatographic analysis, approaches for biomass and transformation yield calculations were identical to our previous investigation (Qin et al., 2014) which was also described in Chapter 2. Microcosm experiments with individual CAH treatments (TCE, cis 1,2-DCE, and CF) were completed in this study; a similar study was completed earlier only with TCE at a lower concentration rage (Qin et al., 2014); the key findings of that study with TCE are included here for comparison and further analysis.

3.2.2 Cometabolic Degradation of CAHs with Ammonium
The degradation potential of individual CAHs (cis-DCE, TCE, and CF) were examined in separate sets of triplicate microcosms (Tables 3.1-3.3). For each microcosm set, after one to three cycles of microbial enrichment, the CAH degradation studies began as follows: Set 1: TCE for 19 cycles (Table 3.1); set 2: cis-DCE for 16 cycles (Table 3.2); and Set 3: CF for 9 cycles (Table 3.3). The microcosms were amended with ammonium nominally at 75 mg/L NH₄⁺-N in each cycle of all three sets; the concentration of individual CAH, however, was gradually increased starting from 30-50 µg/L (Tables 3.1-3.3). The degradation experiment with TCE continued for 19 cycles, and its nominal concentration increased gradually (Table 3.1) starting at 40 µg/L in cycles T1-T3, 60 µg/L in cycles T4-T7, 100 µg/L in cycles T8-T12, 150 µg/L in T13-T16, and 200 µg/L in cycles T17-T19. The degradation experiment with cis 1,2-DCE continued for 17 cycles, and its nominal concentration increased as follows (Table 3.2): 30 µg/L in cycles D1-D2, 110 µg/L in cycles D3-D8, 160 µg/L in cycles D9-D11, 190 µg/L in cycles D10-D12, and 220 µg/L in cycles D13-D17. The experiments with chloroform (Table 3.3) began at 50 µg/L in cycles C1-C2, and increased to 100 µg/L in cycles C3-C5, 150 µg/L in cycles C6-C7, and 190 µg/L in cycles C8-C9. Triplicate ATU-inhibited control microcosms (to minimize ammonium oxidation activity) were also prepared at ammonium and CAHs concentrations equivalent to the live microcosms during all cycles.

3.3 RESULTS AND DISCUSSION

3.3.1 Aerobic TCE Cometabolism: Concentration Effect
3.3.1.1 Sustained TCE degradation with ammonium

Sustained aerobic cometabolic TCE degradation was observed for ~200 days (Figure 3.1; Table 3.1) in the presence of ammonium and AOB associated with Carex comosa roots, with the exception in cycle T5 when both ammonium and TCE degradation nearly ceased. Similar long-term, sustained TCE and ammonium degradation was reported by others (Arciero et al., 1989; Ely et al., 1997; Kocamemi and Cecen, 2010), and also in our earlier investigation of TCE degradation at lower concentration (Qin et al., 2014). In this study, sustained TCE degradation was observed for 3 weeks at 40 µg/L TCE (T1-T3). Soon after [TCE] increased from ~40 to ~60 µg/L in cycle T4, a temporary loss in microbial activity was evident in cycle T5, indicated by lack of ammonium removal, TCE degradation, oxygen consumption and NOx (mainly nitrite) production in cycle T5 (Table 3.1; Figures 3.1, and B.3.1 in Appendix). In our recent work (Qin et al., 2014), a similar microbial deactivation was evident at a similar TCE level (between 46 to 69 µg/L) with 30 mg/L [NH₄⁺-N], in comparison to 75 mg/L NH₄⁺-N in the present study. It is then assumed that cometabolism can be deactivated at a relatively fixed [TCE] level, and this deactivation had little to do with [NH₄⁺]. However, incubation of the microcosms in a TCE-free growth medium for a few days restored the microbial activity towards ammonium removal. It is plausible that the observed deactivation in cycle T5 may have been due to cellular damage of AOB caused by toxic intermediate acyl chloride produced from TCE oxidation; however, the AOB recovered after TCE was removed for sufficient time, and ammonium, and oxygen were available (Rasche et al., 1991; Hyman et al.,
The degradation of both ammonium and TCE resumed after recovery in cycles 6-7 (Table 3.1). Also, a transition in the product of ammonium oxidation was observed starting in cycle T8; the oxidation of ammonium thus far produced mainly nitrite (in enrichment cycles E1-E3, and also in TCE cycles T1-T7). However, nitrite produced from ammonium oxidation declined in cycle T8, and it was replaced by nitrate production. Such transition in oxidation product from nitrite to nitrate was also documented by others, for example Schramm et al., (1999), who attributed this to ammonium limitation, that inhibited AOB growth and thus favoring nitrite-oxidizing bacteria (Schramm et al., 1999). It was shown that AOB can inhibit ordinary heterotrophic organisms and nitrite-oxidizers by outcompeting them for oxygen (Schramm et al., 1999; Shen et al., 2014). In our study, the transition from nitrite to nitrate production seemed to have followed the deactivation of AOBs in cycle T5. It seems possible that the population of nitrite oxidizers began to increase and became a part of the total biomass, such that nitrite produced by AOBs was used by nitrite oxidizers. The TCE degradation and ammonium removal was sustained for another 12 cycles when [TCE] gradually increased and reached 100, 150, and eventually 200 µg/L without indication of further microbial deactivation. Similar recovery was observed in oxygen consumption as well (Figure B.3.1 in Appendix).

### 3.3.1.2 Variations in cell biomass

The steady-state biomass concentration, $X$, and the pseudo first-order rate constants for ammonium removal ($k_{\text{obs-NH}_4}$) and TCE degradation ($k_{\text{obs-TCE}}$) for individual
cycles for set 1 triplicate microcosms are summarized in Table 3.1. During initial AOB enrichment without TCE, the average biomass concentration, $X$, in cycles E1-E3 was 0.35±0.11 mmol/L, and it remained stable at 0.34±0.02 mmol/L in the 3 first experiment cycles (T1-T3) at 40 µg/L TCE. After [TCE] increased to 60 µg/L, $X$ decreased slightly to 0.31 mmol/L in cycle T4, and plummeted to 0.02 mmol/L in cycle T5 showing a major loss in biomass presumably due to cellular damage at higher [TCE]. The recovery of AOB biomass was modest in the following 2 cycles (T6-T7), with an average $X$ at 0.23±0.03 mmol/L. A further increase in $X$ was evident in cycle T8 at 100 µg/L TCE, and it remained fairly stable thereafter; at 100 µg/L TCE in cycles T8-T12, the mean $X$ is 0.48±0.12 mmol/L. With further increase in [TCE] to 150 µg/L in cycles T13-T16, $X$ declined to 0.37±0.07 mmol/L, and then to 0.28±0.01 mmol/L in cycles T17-T19 at 200 µg/L TCE. Overall, the average $X$ before deactivation was 0.35±0.07 mmol/L; however, after recovery the average $X$ in cycles T8-T19 was 0.39±0.12 mmol/L suggesting AOB regaining biomass.

3.3.1.3 Variation in degradation kinetics of ammonium and TCE

The average $k_{\text{obs-NH4}}$ was generally low (~0.13±0.04 d$^{-1}$) during enrichment cycles (E1-E3) without TCE and the first 3 experiment cycles (T1-T3) at 40 µg/L TCE. In cycle T5 where deactivation was observed at 60 µg/L, ammonium removal nearly ceased ($k_{\text{obs-NH4}}<0.01$ d$^{-1}$). After recovery in cycle T6-T7 at 60 µg/L TCE, the $k_{\text{obs-NH4}}$ gradually increased to 0.35 d$^{-1}$ in cycle T9 at 100 µg/L TCE, and remained elevated for 8 weeks.
without expressing toxicity; at 100-150 µg/L TCE in cycles T9-T16, the mean $k_{\text{obs-NH}_4}$ is $0.31\pm0.06$ d$^{-1}$. The $k_{\text{obs-NH}_4}$ declined only slightly ($p>0.05$, one-way RM ANOVAs, [TCE] level effect) thereafter to $0.28\pm0.14$ d$^{-1}$ at 200 µg/L TCE in cycles T17-T19. Overall, the average $k_{\text{obs-NH}_4}$ before deactivation (cycles T1-T3) was $0.11\pm0.02$ d$^{-1}$, but after recovery (cycles T8-T19), the average $k_{\text{obs-NH}_4}$ increased significantly ($P<0.001$, one-way RM ANOVAs, [TCE] level effect) to $0.30\pm0.09$ d$^{-1}$, suggesting a behavioral change in AOB activity. Similarly, the average $k_{1-\text{NH}_4}$ during enrichment cycles (E1-E3) without TCE was generally low ($\sim0.4\pm0.14$ L/mmol/d). After amending the reactors with 40 µg/L TCE, the mean $k_{1-\text{NH}_4}$ declined somewhat to $0.34\pm0.08$ L/mmol/d in the first 3 experiment cycles (T1-T3). After [TCE] increased to 60 µg/L in cycles T4-T7, where ammonium removal kinetics ($k_{\text{obs-NH}_4}$) nearly ceased due to a sharp decline in AMO biomass, the $k_{1-\text{NH}_4}$ was not affected and its mean remained stable at $0.38\pm0.07$ L/mmol/d (Table 3.1). After biomass recovery, however, the mean $k_{1-\text{NH}_4}$ jumped to $0.65\pm0.2$ L/mmol/d in cycles T8-12 at 100 µg/L TCE, and remained elevated thereafter without expressing toxicity. In cycles T13-T16 (at 150 µg/L TCE), the mean $k_{1-\text{NH}_4}$ increased to $0.89\pm0.06$ L/mmol/d, and it further increased to $0.98\pm0.50$ L/mmol/d in cycles T17-T19 (at 200 µg/L TCE). Overall, the average $k_{1-\text{NH}_4}$ in the first 6 cycles (E1-T3) was $0.37\pm0.11$ L/mmol/d, but after microbial deactivation/recovery (cycles T4-T7), the average $k_{1-\text{NH}_4}$ more than doubled to $0.81\pm0.30$ L/mmol/d in cycles T8-T19, suggesting a fundamental shift ($P<0.05$, one-way RM ANOVAs, [TCE] level effect) in ammonium removal efficiency. The increase in ammonium removal kinetics starting in cycle T8 agrees well with the onset of
nitrate production over nitrite suggesting changes in the biogeochemical pathway, which may be attributed to changes in AOB community.

The mean pseudo first-order TCE degradation rate constant ($k_{\text{obs-TCE}}$) was 0.2±0.03 d$^{-1}$ during the first 3 experiment cycles (T1-T3) at 40 µg/L TCE, but it declined somewhat to 0.14 d$^{-1}$ as [TCE] increased to 60 µg/L in cycle T4. In cycle T5, TCE degradation and ammonium removal nearly ceased ($k_{\text{obs-TCE}}$ <0.01 d$^{-1}$). The recovery in AOB activity in cycle T6-T7 still at 60 µg/L TCE was modest with $k_{\text{obs-TCE}}$ at 0.11±0.07 d$^{-1}$. The TCE degradation kinetics increased significantly after recovery in cycle T8 at 100 µg/L TCE, and remained elevated for the remainder of the study; this increase in TCE degradation kinetics is similar to ammonium removal kinetics described earlier. At 100 µg/L TCE in cycles T8-T12, the mean $k_{\text{obs-TCE}}$ was 0.69±0.25 d$^{-1}$. As [TCE] increased further to 150 µg/L in cycles T13-T16, $k_{\text{obs-TCE}}$ declined to 0.53±0.08 d$^{-1}$, and further to 0.40±0.05 d$^{-1}$ in cycles T17-T19 at 200 µg/L TCE. Overall, the average $k_{\text{obs-TCE}}$ before deactivation (cycles T1-T3) was 0.2±0.03 d$^{-1}$, but after recovery (T8-T19), the average $k_{\text{obs-TCE}}$ increased significantly (P<0.005, one-way RM ANOVAs, [TCE] level effect) to 0.56±0.20 d$^{-1}$ suggesting a behavioral change in microbial activity.

The biomass-normalized pseudo first-order TCE degradation rate constants ($k_{1\text{-TCE}}$) through 19 cycles are summarized in Table 1. Initially at 40 µg/L TCE (cycles T1-T3), the mean $k_{1\text{-TCE}}$ was 0.58±0.05 L/mmol/d. After [TCE] increased to 60 µg/L in cycles T4-T7, $k_{\text{obs-TCE}}$ declined sharply, due mainly to decline in $X$, but the decline in $k_{1\text{-TCE}}$ much
less (mean: 0.42±0.13 L/mmol/d) and temporary. Following the recovery of AOB biomass during cycles T6-T7, the mean $k_{1\text{-TCE}}$ more than doubled to 0.88 L/mmol/d in cycle T8 (at 100 μg/L TCE); the mean $k_{1\text{-TCE}}$ for cycles T8-T12 was 1.40±0.34 L/mmol/d, and it remained elevated with a mean value of 1.47±0.29 in cycles T13-T16 (at 150 μg/L TCE) and 1.43±0.17 in cycles T17-T19 (at 200 μg/L TCE). Overall, the mean $k_{1\text{-TCE}}$ before deactivation was 0.55±0.07 L/mmol/d in cycles T1-T4; however, the mean $k_{1\text{-TCE}}$ more than doubled in cycles T8-T19 to 1.43±0.26 L/mmol/d suggesting a major increase (P < 0.001, one-way RM ANOVAs, [TCE] level effect) in TCE degradation efficiency by AOB.

Similarly, the average $k_{1\text{-NH4}}$ values before and after microbial deactivation are 0.37±0.11 and 0.81±0.30 L/mmol/d, respectively. However, they are significantly lower (P < 0.005, one-way RM ANOVAs, [TCE] level effect; P < 0.005 and P < 0.05 respectively, Bonferroni Pairwise Comparions, [TCE] level effect) than the average $k_{1\text{-NH4}}$ of 1.61±0.64 L/mmol/d for the investigation at lower [TCE] and ammonium (Qin et al., 2014) where both initial ammonium (20 to 30 mg/L compared to 75 mg/L) and TCE (64 μg/L compared to 200 μg/L at maximum) were lower. It seems possible that the competition effect of increased TCE caused $k_{1\text{-NH4}}$ to decrease. $k_{1\text{-TCE}}$ values before and after deactivation are 0.55±0.07 and 1.43±0.26 L/mmol/d, (or 0.004±0.001 L/mg/d and 0.012±0.002 L/mg/d assuming that biomass is 113 g VSS/mole) respectively that are comparable (P < 0.005, one-way RM ANOVAs, [TCE] level effect; P < 0.05 and P > 0.05.
respectively, Bonferroni Pairwise Comparisons, [TCE] level effect) to the average $k_{1,TCE}$ of 1.1±0.69 L/mmol/d (or 0.008±0.005 L/mg/d) for the investigation at lower [TCE] (ranging from 30 to 70 µg/L) reported earlier (Qin et al., 2014). The $k_{1,TCE}$ are also comparable to a recently published value from a mixed suspended nitrifying culture (Kocamemi and Cecen, 2010) whose values are between 0.007 and 0.012 L mg$^{-1}$ d$^{-1}$ when initial N-NH$_4^+$ were 50 mg/L and 100 mg/L, respectively. The values were significantly lower than those observed with pure cultures of AOB with 0.74 (Ely et al., 1995) and 1.02 (Ely et al., 1997) L/mmol/d. This appears reasonable, since a mixed culture was studied here and the biomass did not consist of ammonium oxidizers only and not all the biomass was responsible for the cometabolic degradation. The values are also comparable to several studies with other growth substrates, which were mostly mixtures. The values are significantly lower than most experiments with pure cultures (Table B.3.7, in Appendix).

### 3.3.1.4 Variation in TCE Transformation Yield

A positive linear relation between TCE transformation yield ($T_y$) and actual initial [TCE] is shown in Figure 3.2(A) for all cycles, except T5. At [TCE] > 80 µg/L, $T_y$ values showed greater variations. In general, at 40, 60, 100 and 150 µg/L TCE, the correlation between $T_y$ and initial [NH$_4^+$-N] was weak (Figure 3.2(B), and B.3.4 in Appendix), and the effect of initial [NH$_4^+$-N] on $T_y$ was not significant. However, at 200 µg/L TCE, $T_y$ declined from 0.6 to 0.3 apparently due to competitive inhibition as [N-NH$_4^+$] increased.
from 60 to 70 mg/L (Figure 3.2(B), and B.3.4 in Appendix). This is reasonable since
AMO is a non-specific enzyme (Hyman et al., 1995), and while both TCE and
ammonium compete for the AMO, TCE may have a greater affinity for the enzyme than
ammonium does (Ely et al., 1995). As such, increasing [TCE] can have a more significant
effects on $T_y$ than decreasing $[\text{NH}_4^+]$. At lower [TCE], the competition between TCE and
ammonium is lower, and $T_y$ was unaffected by small variations in $[\text{NH}_4^+]$. At 200 $\mu$g/L
[TCE], however, the competition reached a threshold that ammonium and TCE competed
for limited AMO, and thus small variations in $[\text{NH}_4^+]$ had a significant effect on $T_y$
(described above). This may also imply that the system may be close to its TCE tolerance
limit, and further increase in [TCE] may cause shutdown in ammonium oxidation,
expressed as microbial deactivation (similar to cycle T5), but due to competition between
TCE and ammonium, not toxicity of TCE. Those impacts were reported in cometabolic
systems with other substrates (Anderson and McCarty, 1997; Powell et al., 2011).

The $T_y$ values in our study (0.084, 0.179, and 0.433 $\mu$mol TCE / mmol N-$\text{NH}_4^+$ at
40, 110 and 200 /L initial TCE concentration, respectively) were slightly lower but
comparable to a recently published report (Kocamemi and Cecen, 2010), whose values
were 0.149, 0.245 and 0.351 $\mu$mol TCE / mmol N-$\text{NH}_4^+$ at 40, 110 and 325 $\mu$g/L initial
TCE concentration, respectively and our previous research (Qin et al., 2014) whose value
was 0.153 $\mu$mol TCE / mmol N-$\text{NH}_4^+$ with initial TCE concentration ranged from 30 to
70 $\mu$g/L. It is quite possible the higher ammonium concentration (75 mg/L compared to
40 mg/L and less than 30 mg/L) caused \(T_Y\) in our scenario to be a bit lower due to the competition discussed above. The published report by Kocamemi and Cecen (2010) also documented a \(T_Y\) value (2.6 \(\mu\)mol TCE / mmol NH\(_4^+\)-N) at 845 \(\mu\)g/L initial [TCE]. This suggests that as [TCE] increased further, the AMO enzyme tends to bind with TCE rather than ammonium. Actually the value reported by Kocamemi and Cecen (2010) were within the same order of magnitude as several reported studies on cometabolism with TCE (higher than 500 \(\mu\)g/L) and other growth substrates including methane, propane, phenol and toluene (Table B.3.7).

3.3.2 Effect of cis-1,2-Dichloroethene (cis-DCE) Concentration

3.3.2.1 cis-DCE Degradation with ammonium and degradation deactivation

\(cis\)-DCE was continuously degraded for more than 120 days through cometabolism with the presence of ammonium and AOB associated with the rhizosphere of Carex comosa (Figure 3.3). Despite the fact that the cometabolism of other chlorinated ethenes like TCE and 1,1-DCE by AOB have already been studied and reported in detail (Ely et al., 1997), observation of sustainable \(cis\)-DCE cometabolic degradation was less studied and prior work investigated cometabolic enzymes other than AMO (e.g. methane monooxygenase, MMO) (Han et al., 1999). The results here demonstrated that the populations of AOB associated with Carex comosa collected from the local wetland, which is capable of cometabolizing TCE, can also degrade \(cis\)-DCE. Sustained \(cis\)-DCE
degradation lasted for 10 weeks with 75 mg/L initial ammonium concentration when cis-DCE concentration was at 30 µg/L, 100 µg/L, and 160 µg/L. As cis-DCE concentration increased to 190 µg/L, cis-DCE degradation was gradually affected and totally inhibited in Cycle 16 at 220 µg/L (Figure 3.3). Gradual decline of ammonium degradation, DO loss, and NO\textsubscript{x} production was also observed starting from Cycle 10 (Figure 3.3, Figure B.3.2, in Appendix). According to previous findings ([Rasche et al., 1991], cis-DCE and TCE fell into same “group” in which they can be degraded cometabolically by AMO but the degradation was inhibited at higher DCE or TCE concentrations since acyl chloride would form as an intermediate which functions as a protein-modifying agent to inactivate key enzymes or proteins such as electron carriers through covalent modification. So at 75 mg/L of ammonium-N, continuous exposure of cis-DCE higher than 120 µg/L may cause cometabolic deactivation ([Rasche et al., 1991]). Theoretically, the damage can be reversed after depleting cis-DCE for a period of time ([Ely et al., 1997]).

Similarly as described in the TCE microcosms, a shift of ammonium oxidation production from nitrite to nitrate was also observed, but at an earlier stage, right after the first cis-DCE injection. Although the toxicity of cis-DCE was not focused on in this or other studies, another dichloroethene (1,1-DCE) was found to be three times more potent in deactivating cells than TCE in ammonium cometabolism systems ([Ely et al., 1997]). Thus the possible higher toxicity of cis-DCE may explain this earlier transition than in the TCE microcosms. This transition also came along with a decrease in calculated
biomass in the first two cycles Cycle D1-D2, which will be discussed in following sections.

3.3.2.2 Variations in biomass

Pseudo first-order degradation of ammonium and cis-DCE in relation to biomass was calculated for each cycle in order to evaluate AOB activities (Table 3.2). The mean of the steady state cellular biomass concentration, $X$, through all the cycles, was $0.119 \pm 0.122$ mmol/L, substantially less ($P < 0.005$, one-way RM ANOVAs, group effect) than to $0.347 \pm 0.156$ mmol/L for TCE. The relatively large standard deviation suggested that biomass shifted significantly between different cis-DCE concentrations. Biomass was lower ($0.133 \pm 0.038$ mmol/L) in the beginning of the experiment, Cycle D1-D2, when cis-DCE concentration was at 30 µg/L, which coincided with the shift of the ammonium oxidation product from nitrite to nitrate. Similar like we discussed in the TCE section, substantial amount of ammonium oxidizers may be inhibited by cis-DCE. This inhibition happened right after the first injection of cis-DCE. $X$ then increased to $0.338 \pm 0.027$ mmol/L when cis-DCE concentrations rose to 100 µg/L at Cycle D3-D4. Biomass showed great variations at 120 µg/L cis-DCE from Cycle D5 to D8, with the average of 0.172 mmol/L and a standard deviation of 0.135 mmol/L, which suggests the major microbial community may be inhibited at the elevated cis-DCE concentrations. When cis-DCE concentration further increased above 160 µg/L after Cycle D10, biomass further decreased to $0.034 \pm 0.019$ mmol/L. The low biomass eventually slowed the rate of
degradation and lead to cometabolism shutdown.

3.3.2.3 Variation in degradation kinetics of ammonium and cis-DCE

For the pseudo first-order rate constants, the mean of $k_{\text{obs-DCE}}$ for all cycles was $0.475 \pm 0.359 \text{ d}^{-1}$, ranging from 0.033 (Cycle 16) to 1.042 (Cycle 3) d$^{-1}$. The first order rate constant was similar ($P > 0.05$, one-way RM ANOVAs, group effect) to $k_{\text{obs-TCE}}$ ($0.406 \pm 0.245 \text{ d}^{-1}$) and clearly descended as cis-DCE concentration increased. The calculated means of biomass normalized pseudo first-order rate constants of cis-DCE ($k_1$) were $6.542 \pm 7.043 \text{ L mmol}^{-1} \text{ d}^{-1}$, or $0.058 \pm 0.062 \text{ L mg}^{-1} \text{ d}^{-1}$ assuming the biomass of 113 g VSS/mole. This value was significantly higher ($P < 0.01$, one-way RM ANOVAs, group effect) than $k_{1\text{-TCE}}$ in this study. The value ($0.058 \text{ L mg}^{-1} \text{ d}^{-1}$) was lower than those reported with pure (7.1 L mg$^{-1}$ d$^{-1}$) (Chang and Alvarez-Cohen, 1996; Kim and Graham, 2003) or mixed cultures (2.7 L mg$^{-1}$ d$^{-1}$) (Chang and Alvarez-Cohen, 1996) of methanotrophs, but very similar to values reported with propane (0.06 L mg$^{-1}$ d$^{-1}$) (Frascari et al., 2008) and phenol (0.8 – 1.0 L mg$^{-1}$ d$^{-1}$) (Segar, 1994) as primary growth substrates.

The mean of $k_{\text{obs-NH4}}$ for all cycles was $0.074 \pm 0.051 \text{ d}^{-1}$, ranging from 0.001 (Cycle 15) to 0.153 (Cycle 1) d$^{-1}$. The rate constant for ammonium was lower ($P < 0.005$, one-way RM ANOVAs, group effect) than in the TCE experiments, suggesting a more intense competition between cis-DCE and ammonium as electron donors. The rate
constants kept relatively stable until Cycle 11, then dropped to 0.023 d\(^{-1}\) at Cycle 12 and decreased further afterwards. The biomass-normalized rate constants \(k_{1,\text{NH4}}\) had the average of 0.978±0.952 L mmol\(^{-1}\)d\(^{-1}\), and ranged from 0.040 (Cycle 15) to 3.717 (Cycle 9) L mmol\(^{-1}\)d\(^{-1}\). These values were not significant (\(P > 0.05\), one-way RM ANOVAs, group effect) from the microcosms for TCE degradation. Like \(k_{1,\text{DCE}}\), extremely high values were again observed at Cycle 8, 9, and Cycle 10 for \(k_{1,\text{NH4}}\). From the pseudo first-order rate constants, it can be inferred that the ammonium oxidizing group in cis-DCE reactors had lower affinities to ammonium than those in TCE reactors, with lower rate constants for ammonium and higher rate constants for chlorinated ethenes. Based on rate constants, biomass and biomass normalized rate constants, it can be speculated that biomass increased under 120 \(\mu\)g/L cis-DCE, and the high biomass caused higher cometabolism rates; the loss of biomass when cis-DCE went above 120 \(\mu\)g/L does not lead to a sudden drop in kinetics, but to the contrary increased the biomass normalized rate constants remarkably and the biomass concentration became unstable since then; after continuous exposure to high concentrations of chlorinated ethenes, the biomass further decreased and finally led to decreased rate constants and eventually, cometabolism shutdown.

### 3.3.2.4 Variation in cis-DCE transformation yield

Similar to the results of TCE, there was also a positive relationship between increased cis-DCE concentration and the mass of cis-DCE removed. The trends were similar for transformation yield \(T_y\); as cis-DCE concentration increased, \(T_y\) increased.
correspondingly (Figure 3.4(A)). However, unlike the linear trends between $T_y$ and [TCE], there appeared to be an exponential relation between $T_y$ and the initial cis-DCE concentration ($T_y = 0.1019e^{0.0151[cis-DCE]}$, R² = 0.92). Based on the discussion about TCE and $T_y$ in the previous section, it can be inferred that cis-DCE has an even greater affinity for the enzyme AMO, so increasing the cis-DCE concentration will tremendously raise the odds of encountering AMO and therefore being oxidized. The effects of elevated ammonium concentration on $T_y$ were less (Figure 3.4(B) and Figure B.4.5 in Appendix), and the negative effect of elevated ammonium concentration on $T_y$ was only observed at 220 µg/L initial cis-DCE concentration, where the system reached its degradation limit, thus leading to intense competition between cis-DCE and ammonium for the limited AMO available.

3.3.3 Effect of Chloroform (CF) Concentration

3.3.3.1 Sustained CF degradation with ammonium

CF was also continuously degraded for more than 60 days through cometabolism in the presence of ammonium and AOB associated with the rhizosphere of Carex comosa without significant inactivation (Figure 6). The cometabolism of CF by AOB has been reported in several studies (Ely et al., 1997; Wahman et al., 2005; Wahman et al., 2006). The results in this study also demonstrated the ability of AOB naturally associated with Carex comosa to degrade CF via cometabolism. Sustained CF degradation lasted for 9
weeks with 75 mg/L initial [N-NH$_4^+$] when CF concentration was at 50 µg/L, 100 µg/L, 150 µg/L and 190 µg/L. No significant deactivation was observed during the 9-week cometabolic degradation process. Although CF was also in the same “Group 3” as TCE and cis-DCE with substantial deactivation at higher initial concentrations (Rasche et al., 1991), the amount of CF added in this research was lower compared to other studies (Wahman et al., 2005) and thus did not reach the threshold value of deactivation.

The shift of ammonium oxidation product from nitrite to nitrate was also observed in the microcosms in Cycles C1-C3, which is similar to what was observed in the cis-DCE microcosms where the shift happened right after the first injection of cis-DCE. However, instead of a sudden transition from nitrite to nitrate as we observed in the cis-DCE microcosms, the transition in the CF microcosm is similar to the TCE study where both nitrite and nitrate coexist in the system for several cycles. Then beginning from Cycle C4, the majority of ammonium oxidation products changed to nitrate.

3.3.3.2 Variations in Biomass

Within the concentration range from 50 to 190 µg/L of CF, biomass $X$ (0.217±0.071 mmol/L) remained relatively stable during the nine-week experiment, indicating sustainable degradation without significant deactivation (Table 3.3). $X$ increased during the first three cycles where, as mentioned earlier, there was a gradual shift of ammonium oxidation product from nitrite to nitrate. $X$ then dropped in Cycle C4.
from 0.316 (Cycle C3) to 0.159 mmol/L. It could be inferred that when CF concentration was 50 µg/L in Cycle C1-C2 and at the first cycle of 100 µg/L in Cycle C3, although nitrite oxidizers increased, the majority of ammonia oxidizers was not significantly inhibited. However, in the second cycle of 100 µg/L CF (Cycle C4), a certain amount of ammonium oxidizers were inhibited and the nitrite production was balanced again. X then gradually rose and in Cycle C9 reached 0.343 mmol/L, accompanied by a tiny amount of nitrite in the first days of the cycle. That indicated the microcosm was sustainable and had the potential to operate further since the majority of biomass was not affected by the elevated concentration of CF, while the production and consumption of nitrite was in equilibrium.

3.3.3.3 Variation in degradation kinetics of ammonium and CF

The pseudo first-order rate constants $k_{\text{obs-CF}}$ (0.70±0.39 d$^{-1}$) and $\text{NH}_4^+$ $k_{\text{obs-NH4}}$ (0.14±0.05 d$^{-1}$) were relatively stable throughout the experiment (Table 3.3). Initially, in cycles C1-C2, the $k_{\text{obs-CF}}$ values (0.09 and 0.14 d$^{-1}$, respectively) were significantly lower than average $k_{\text{obs-CF}}$, which suggests that the system had not fully established steady CF degradation after the addition of CF. The calculated means of biomass normalized pseudo first-order rate constants of CF ($k_{1-\text{CF}}$) were 3.30±1.97 L mmol$^{-1}$ d$^{-1}$ (or 0.03±0.02 L/mg/d assuming biomass is 113 g VSS/mole). This value was similar to results from several previous reports using pure cultures of propane oxidizers (0.05 L mg$^{-1}$ d$^{-1}$) (Frascari et al., 2008), butane oxidizers (0.026 L mg$^{-1}$ d$^{-1}$) (Frascari et al., 2005) and mixed culture of
methane oxidizers (0.014 L mg\(^{-1}\) d\(^{-1}\)) (Leeson and Bouwer, 1989). The average \(k_{obs-NH4}\) was lower than in the TCE experiments but higher than in the \textit{cis}-DCE microcosms. The biomass-normalized rate constants \(k_{1-NH4}\) had the average of 0.68±0.17 L mmol\(^{-1}\) d\(^{-1}\), which was lower than those in TCE and \textit{cis}-DCE experiments. It can be inferred that initially the AOBs in CF reactors had a stable yet lower affinity towards ammonium, but the affinity to CAHs (CF) was higher once the AOB community was fully established.

\textbf{3.3.3.4 Variation in CF transformation yield} \(T_y\)

\(T_y\) increased linearly (\(R^2 = 0.92\)) as CF concentration increased, which is similar to TCE (Figure 3.6(A)). \(T_y\) (0.63±0.16 μmol mmol\(^{-1}\)) when initial [CF] was at \(\sim\)190 μg/L, however, was higher than in the TCE system (0.43±0.02 μmol mmol\(^{-1}\)) when initial [TCE] was around 200 μg/L. Combined with the results of rate constants, it can be speculated that the cometabolic degradation mechanism of CF and TCE was similar. But CF had greater affinity to the AMO enzyme. No distinct relation was observed between transformation yield and [NH\(_4^+\)-N] except for at highest [CF], around 190 μg/L, where \(T_y\) slightly decreased with elevated [NH\(_4^+\)-N] (Figure 35.6(B), and Figure B.3.6, in Appendix). Also, from Figure 3.6(A), \(T_y\) at 150 μg/L and 190 μg/L did not show great variance (0.60±0.09 and 0.63±0.16 μmol mmol\(^{-1}\), respectively). This indicates the system was in stable and could potentially sustain further degradation.

\textbf{3.4 CONCLUSIONS}
Sustainable cometabolic degradation of all three CAHs was observed in microcosms enriched with ammonium, which indicates that microorganisms associated with wetland plant roots can assist in the natural attenuation of several CAHs including TCE in contaminated aquatic environments (wetlands). Deactivation of cometabolic degradation of CAHs and ammonium were observed in TCE and cis-DCE microcosms when the respective CAHs concentration reached a certain level. The cometabolic system for TCE was activated again one week after the system was replaced by a TCE-free medium culture. Rate constants did not change significantly during the inactivation cycle if normalized by $X$. It can be inferred that the drop in ammonium and TCE degradation at certain [TCE] are due to the activity shut down by ammonia oxidizers. A shift of ammonium oxidation production from nitrite of nitrate after CAHs were added was observed in all three systems. It can be inferred that the addition of the CAHs will change the microbial community by inhibiting ammonia oxidizers and this inhibition may lead to degradation deactivation. Future microbial community analysis may help to further understand the mechanisms by which the systems respond to the addition of CAHs.

3.5 REFERENCES


Figure 3.1 Degradation of NH$_4^+$-N (A) and TCE (C), and production of NO$_x$-N (B, individual NO$_2$-N and NO$_3$-N); error bars represent one standard deviation in triplicate reactors through 19 successive cycles (T1-T19). Solid symbols in (A) and (C) represent average values in live microcosms while open symbols represent average values in control microcosms. Solid symbols in (B) represent average values of NO$_3$-N while open symbols represent average values of NO$_2$-N.
Figure 3.2 Scatter plot of (A) Regression of TCE Transformation Yield ($T_y$) vs. initial TCE concentrations ($\mu$g L$^{-1}$) for average of triplicate microcosms, and (B) TCE Transformation Yield ($T_y$) vs. initial NH$_4^+$-N concentrations in individual microcosms, for 19 successive cycles (T1-T19). Transformation Yield ($T_y$) is defined as the quotient of net TCE degraded ($\mu$moles) divided by net NH$_4^+$ consumed (mmoles).
Figure 3.3 Degradation of NH$_4^+$ (A) and cis-DCE (C) and production of NO$_x$ (B, individual NO$_2^-$ and NO$_3^-$); error bars represent one standard deviation in triplicate reactors through 16 successive cycles (D1-D16) except for in (B) the data in enrichment cycle (in negative value) was listed to show the shift of NO$_x$ production. Solid symbols in (A) and (C) represent average values in live microcosms while open symbols represent average values in control microcosms. Solid symbols in (B) represent average values of NO$_3^-$ while open symbols represent average values of NO$_2^-$.
Figure 3.4 Scatter plot of (A) Regression of cis-DCE Transformation Yield ($T_y$) vs. initial cis-DCE concentrations ($\mu$g L$^{-1}$) for average of triplicate microcosms, and (B) cis-DCE Transformation Yield ($T_y$) vs. initial NH$_4^+$-N concentrations in individual microcosms, for 16 successive cycles (D1-D16). Transformation Yield ($T_y$) is defined as the quotient of net cis-DCE degraded (µmoles) divided by net NH$_4^+$ consumed (mmoles).
Figure 3.5 Degradation of NH$_4^+$-N (A) and CF (C), and production of NO$_x$-N (B, individual NO$_2^-$-N and NO$_3^-$-N); error bars represent one standard deviation in triplicate reactors through 9 successive cycles (C1-C9). Solid symbols in (A) and (C) represent average values in live microcosms while open symbols represent average values in control microcosms. Solid symbols in (B) represent average values of NO$_3^-$-N while open symbols represent average values of NO$_2^-$-N.
Figure 3.6 Scatter plot of (A) Regression of CF Transformation Yield ($T_y$) vs. initial CF concentrations ($\mu$g L$^{-1}$) for average of triplicate microcosms, and (B) CF Transformation Yield ($T_y$) vs. initial NH$_4^+$-N concentrations in individual microcosms, for 9 successive cycles (C1-C9). Transformation Yield ($T_y$) is defined as the quotient of net CF degraded (µmoles) divided by net NH$_4^+$ consumed (mmoles).
Table 3.1 Average parameters in Set 1 triplicate microcosms for cycles: initial concentrations of TCE ([TCE]₀, μg/L), and ([NH₄⁺-N]₀, mg/L); steady state cell biomass concentration (X, mmol/L); initial pseudo-first-order rate constants of NH₄⁺-N removal (kₘ₉-NH₄, d⁻¹) and TCE degradation (kₘ₉-TCE, d⁻¹), and their corresponding coefficients of determination (R²); biomass-normalized NH₄⁺-N removal rate constants (k₁-NH₄, L mmol⁻¹ d⁻¹) in 3 enrichment cycles (E1-E3) and 19 TCE cycles (T1-T19); biomass-normalized TCE degradation rate constants (kₙ-TCE, L mmol⁻¹ d⁻¹) in 19 TCE cycles (T1-T19); and transformation yield (Tₚ, umol TCE/ mmol NH₄⁺-N). The degradation experiment started at 40 μg/L in cycles T1-T3, 60 μg/L in cycles T4-T7, 100 μg/L in cycles T8-T12, 150 μg/L in T13-T16, and 200 μg/L in cycles T17-T19.

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<th>Cycle</th>
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<th>X</th>
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<th>kₘ₉-TCE; (R²)</th>
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<td>0.17; (0.98)</td>
<td>0.30</td>
<td>0.34±0.08</td>
<td>0.56</td>
<td>0.58±0.05</td>
<td>0.08</td>
<td>0.08±0.00</td>
</tr>
<tr>
<td>T2</td>
<td>41.4</td>
<td>65.0</td>
<td>0.36</td>
<td>0.10; (0.84)</td>
<td>0.20; (0.97)</td>
<td>0.28</td>
<td>0.38±0.07</td>
<td>0.42</td>
<td>0.42±0.13</td>
<td>0.06</td>
<td>0.09±0.03</td>
</tr>
<tr>
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<td>41.5</td>
<td>72.3</td>
<td>0.35</td>
<td>0.15; (0.99)</td>
<td>0.22; (0.98)</td>
<td>0.43</td>
<td>0.16±0.08</td>
<td>0.14</td>
<td>0.14±0.06</td>
<td>0.09</td>
<td>0.09±0.05</td>
</tr>
<tr>
<td>T4</td>
<td>61.4</td>
<td>71.5</td>
<td>0.30</td>
<td>0.11; (0.99)</td>
<td>0.14; (0.99)</td>
<td>0.36</td>
<td>0.17±0.07</td>
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<td>0.17±0.07</td>
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<td>0.17±0.07</td>
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<tr>
<td>T5</td>
<td>61.2</td>
<td>76.9</td>
<td>0.02</td>
<td>0.01; (0.14)</td>
<td>0.01; (0.26)</td>
<td>0.32</td>
<td>0.16±0.08</td>
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<td>0.14±0.06</td>
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<td>57.3</td>
<td>62.8</td>
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<td>0.11; (0.97)</td>
<td>0.14; (0.98)</td>
<td>0.48</td>
<td>0.18±0.08</td>
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<td>0.18±0.06</td>
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<td>0.18±0.06</td>
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<td>T7</td>
<td>64.8</td>
<td>68.3</td>
<td>0.27</td>
<td>0.09; (0.98)</td>
<td>0.09; (0.93)</td>
<td>0.32</td>
<td>0.16±0.08</td>
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<td>0.16±0.06</td>
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<td>0.16±0.06</td>
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<td>103.8</td>
<td>77.2</td>
<td>0.40</td>
<td>0.16; (0.98)</td>
<td>0.35; (0.85)</td>
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<td>1.40±0.34</td>
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<td>0.22±0.06</td>
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<td>106.0</td>
<td>73.9</td>
<td>0.37</td>
<td>0.35; (0.99)</td>
<td>0.66; (0.98)</td>
<td>0.95</td>
<td>0.98±0.03</td>
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<td>0.98±0.03</td>
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<td>T10</td>
<td>107.3</td>
<td>74.3</td>
<td>0.44</td>
<td>0.26; (0.98)</td>
<td>0.57; (0.98)</td>
<td>0.58</td>
<td>1.31±0.07</td>
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<td>1.31±0.07</td>
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<tr>
<td>T11</td>
<td>116.8</td>
<td>82.6</td>
<td>0.65</td>
<td>0.39; (0.99)</td>
<td>0.97; (0.98)</td>
<td>0.59</td>
<td>1.48±0.07</td>
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<td>1.48±0.06</td>
<td>1.48</td>
<td>1.48±0.06</td>
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<tr>
<td>T12</td>
<td>62.3</td>
<td>80.2</td>
<td>0.56</td>
<td>0.41; (0.96)</td>
<td>0.89; (0.93)</td>
<td>0.72</td>
<td>1.57±0.07</td>
<td>1.57</td>
<td>1.57±0.07</td>
<td>1.57</td>
<td>1.57±0.07</td>
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<td>T13</td>
<td>150.3</td>
<td>64.8</td>
<td>0.30</td>
<td>0.29; (0.99)</td>
<td>0.44; (0.97)</td>
<td>0.96</td>
<td>0.89±0.22</td>
<td>0.89</td>
<td>0.89±0.22</td>
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<td>0.89±0.22</td>
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<td>T14</td>
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<td>0.35; (0.98)</td>
<td>0.51; (0.95)</td>
<td>1.02</td>
<td>1.47±0.06</td>
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<td>1.47±0.06</td>
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<td>1.47±0.06</td>
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<tr>
<td>T15</td>
<td>147.3</td>
<td>85.7</td>
<td>0.46</td>
<td>0.26; (0.97)</td>
<td>0.53; (1.0)</td>
<td>0.56</td>
<td>1.14±0.06</td>
<td>1.14</td>
<td>1.14±0.06</td>
<td>1.14</td>
<td>1.14±0.06</td>
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<tr>
<td>T16</td>
<td>88.0</td>
<td>67.9</td>
<td>0.35</td>
<td>0.36; (0.97)</td>
<td>0.63; (0.98)</td>
<td>1.01</td>
<td>1.78±0.06</td>
<td>1.78</td>
<td>1.78±0.06</td>
<td>1.78</td>
<td>1.78±0.06</td>
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<tr>
<td>T17</td>
<td>212.6</td>
<td>68.0</td>
<td>0.27</td>
<td>0.17; (0.67)</td>
<td>0.40; (0.95)</td>
<td>0.63</td>
<td>0.98±0.06</td>
<td>0.98</td>
<td>0.98±0.06</td>
<td>0.98</td>
<td>0.98±0.06</td>
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<tr>
<td>T18</td>
<td>212.7</td>
<td>63.2</td>
<td>0.29</td>
<td>0.22; (0.92)</td>
<td>0.46; (0.96)</td>
<td>0.76</td>
<td>1.56±0.07</td>
<td>1.56</td>
<td>1.56±0.07</td>
<td>1.56</td>
<td>1.56±0.07</td>
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<td>T19</td>
<td>206.9</td>
<td>65.4</td>
<td>0.28</td>
<td>0.44; (0.99)</td>
<td>0.35; (0.91)</td>
<td>1.56</td>
<td>1.24±0.06</td>
<td>1.24</td>
<td>1.24±0.06</td>
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<td>1.24±0.06</td>
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Table 3.2: Average parameters in Set 2 triplicate microcosms for cycles: initial concentrations of cis-DCE ([cis-DCE]₀, μg/L), and ([NH₄⁺-N]₀, mg/L); steady state cell biomass concentration (X, mmol/L); initial pseudo first-order rate constants of NH₄⁺-N removal (k_{obs-NH₄} ᵃ₁, d⁻¹) and cis-DCE degradation (k_{obs-DCE}, d⁻¹), and their corresponding coefficients of determination (R²); biomass-normalized NH₄⁺-N removal rate constants (k₁-NH₄, L mmol⁻¹ d⁻¹) in the enrichment cycle (E1) and cis-DCE cycles (D1-D16); biomass-normalized cis-DCE degradation rate constants (k₁-DCE, L mmol⁻¹ d⁻¹) in cis-DCE cycles (D1-D16); and transformation yield (T_y, umol cis-DCE / mmol NH₄⁺-N). cis-1,2-DCE concentration increased as follows: 30 μg/L in cycles D1-D2, 110 μg/L in cycles D3-D8, 160 μg/L in cycles D9-D11, 190 μg/L in cycles D10-D12, and 220 μg/L in cycles D13-D17.

<table>
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<th>Cycle</th>
<th>[cis-DCE]₀</th>
<th>[NH₄⁺-N]₀</th>
<th>X</th>
<th>𝑘_{obs-NH₄} ( (R^2) )</th>
<th>𝑘_{obs-DCE} ( (R^2) )</th>
<th>𝑘₁-NH₄</th>
<th>Avg. 𝑘₁-NH₄</th>
<th>𝑘₁-DCE</th>
<th>Avg. 𝑘₁-DCE</th>
<th>𝑇_𝑦</th>
<th>Avg. 𝑇_𝑦</th>
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</thead>
<tbody>
<tr>
<td>E1</td>
<td>--</td>
<td>68.0</td>
<td>0.35</td>
<td>0.21; (0.90)</td>
<td>--</td>
<td>0.59</td>
<td>0.59</td>
<td>--</td>
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</tr>
<tr>
<td>D1</td>
<td>30.5</td>
<td>64.6</td>
<td>0.16</td>
<td>0.15; (0.95)</td>
<td>1.03; (0.99)</td>
<td>0.96</td>
<td>0.98±0.22</td>
<td>6.46</td>
<td>8.04±2.23</td>
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<td>0.13±0.01</td>
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<td>D2</td>
<td>32.3</td>
<td>60.8</td>
<td>0.11</td>
<td>0.11; (0.98)</td>
<td>1.02; (0.98)</td>
<td>0.99</td>
<td>3.26</td>
<td>1.92</td>
<td>2.59±0.95</td>
<td>0.45</td>
<td>0.45±0.01</td>
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<td>D3</td>
<td>99.3</td>
<td>66.4</td>
<td>0.32</td>
<td>0.14; (0.92)</td>
<td>1.04; (0.99)</td>
<td>0.44</td>
<td>0.38±0.08</td>
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<td>0.91±0.82</td>
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<td>0.67</td>
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<tr>
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<td>100.7</td>
<td>68.4</td>
<td>0.36</td>
<td>0.12; (0.89)</td>
<td>0.69; (0.96)</td>
<td>0.33</td>
<td>1.41</td>
<td>1.41</td>
<td>5.87±6.14</td>
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<td>0.88±0.29</td>
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<tr>
<td>D5</td>
<td>118.6</td>
<td>70.7</td>
<td>0.31</td>
<td>0.09; (0.94)</td>
<td>0.52; (0.88)</td>
<td>0.28</td>
<td>1.2</td>
<td>1.2</td>
<td>18.62±15.57</td>
<td>0.80</td>
<td>1.14±0.48</td>
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<tr>
<td>D6</td>
<td>126.0</td>
<td>69.4</td>
<td>0.09</td>
<td>0.07; (0.81)</td>
<td>0.50; (0.95)</td>
<td>0.86</td>
<td>2.29</td>
<td>2.29</td>
<td>7.61</td>
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<td>1.80</td>
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<tr>
<td>D7</td>
<td>117.8</td>
<td>66.3</td>
<td>0.27</td>
<td>0.11; (0.86)</td>
<td>0.38; (0.98)</td>
<td>0.42</td>
<td>2.2</td>
<td>2.2</td>
<td>18.62±15.57</td>
<td>0.80</td>
<td>1.14±0.48</td>
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<tr>
<td>D8</td>
<td>127.7</td>
<td>65.2</td>
<td>0.03</td>
<td>0.07; (0.79)</td>
<td>0.45; (0.98)</td>
<td>2.09</td>
<td>2.97±1.06</td>
<td>29.63</td>
<td>4.54±0.41</td>
<td>1.48</td>
<td>1.67±0.19</td>
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<tr>
<td>D9</td>
<td>158.5</td>
<td>67.9</td>
<td>0.03</td>
<td>0.11; (0.94)</td>
<td>0.85; (0.99)</td>
<td>3.72</td>
<td>2.97±1.06</td>
<td>29.63</td>
<td>2.45</td>
<td>1.48</td>
<td>1.53</td>
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<tr>
<td>D10</td>
<td>190.6</td>
<td>66.6</td>
<td>0.05</td>
<td>0.12; (0.97)</td>
<td>0.41; (0.98)</td>
<td>2.22</td>
<td>2.97±1.06</td>
<td>7.61</td>
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<tr>
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<td>60.3</td>
<td>0.07</td>
<td>0.05; (0.79)</td>
<td>0.28; (0.90)</td>
<td>0.81</td>
<td>0.96±0.21</td>
<td>4.25</td>
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<td>0.10; (0.93)</td>
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<td>0.36±0.27</td>
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<td>0.01; (0.28)</td>
<td>0.17; (0.95)</td>
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<td>2.33</td>
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<tr>
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<td>67.5</td>
<td>0.03</td>
<td>0.01; (0.64)</td>
<td>0.08; (0.98)</td>
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<td>0.36±0.27</td>
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<td>65.5</td>
<td>0.03</td>
<td>0.001; (0.01)</td>
<td>0.05; (0.76)</td>
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<td>0.36±0.27</td>
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Table 3.3 Average parameters in Set 3 triplicate microcosms for cycles: initial concentrations of CF ([CF]₀, μg/L), and ([NH₄⁺-N]₀, mg/L); steady state cell biomass concentration (X, mmol/L); initial pseudo first-order rate constants of NH₄⁺-N removal (k_{obs-NH₄⁺}, d⁻¹) and CF degradation (k_{obs-CF}, d⁻¹), and their corresponding coefficients of determination (R²); biomass-normalized NH₄⁺-N removal rate constants (k_{1-NH₄⁺}, L mmol⁻¹ d⁻¹) in 2 enrichment cycles (E1-E2) and 9 CF cycles (C1-C9); biomass-normalized CF degradation rate constants (k_{1-CF}, L mmol⁻¹ d⁻¹) in 9 CF cycles (C1-C9); and transformation yield (T_y, umol CF/ mmol NH₄⁺-N). The experiments of CF began at 50 μg/L in cycles C1-C2, and increased to 100 μg/L in cycles C3-C5, 150 μg/L in cycles C6-C7, and 190 μg/L in cycles C8-C9.

<table>
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<th>Cycle</th>
<th>[CF]₀</th>
<th>[NH₄⁺-N]₀</th>
<th>X</th>
<th>k_{obs-NH₄⁺} (R²)</th>
<th>k_{obs-TCE} (R²)</th>
<th>k_{1-NH₄⁺}</th>
<th>Avg. k_{1-NH₄⁺}</th>
<th>k_{1-CF}</th>
<th>Avg. k_{1-CF}</th>
<th>T_y</th>
<th>Avg. T_y</th>
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<td>0.31</td>
<td>0.43±0.17</td>
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<td>--</td>
<td>0.14</td>
<td>0.14±0.01</td>
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<tr>
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<td>68.1</td>
<td>0.17</td>
<td>0.09; (0.89)</td>
<td>--</td>
<td>0.55</td>
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<td>0.13</td>
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<td>0.09; (0.98)</td>
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<td>0.14; (0.99)</td>
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<td>0.69</td>
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<td>0.13</td>
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<td>68.1</td>
<td>0.32</td>
<td>0.13; (0.96)</td>
<td>0.92; (0.98)</td>
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<td>2.92</td>
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<td>0.36; (0.96)</td>
<td>0.89</td>
<td>0.69±0.25</td>
<td>2.25</td>
<td>3.53±1.67</td>
<td>0.31</td>
<td>0.33±0.03</td>
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<tr>
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<td>91.1</td>
<td>69.0</td>
<td>0.19</td>
<td>0.14; (0.99)</td>
<td>1.03; (0.96)</td>
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<td>5.43</td>
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<td>C6</td>
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<td>0.90; (0.99)</td>
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<td>0.13; (0.97)</td>
<td>0.80; (0.99)</td>
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<td>4.85</td>
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<td>3.50±0.66</td>
<td>0.60</td>
<td>0.63±0.05</td>
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<td>62.2</td>
<td>0.34</td>
<td>0.25; (0.99)</td>
<td>1.04; (0.97)</td>
<td>0.74</td>
<td></td>
<td>3.03</td>
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<td>0.67</td>
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# 4 NATURAL ATTENUATION POTENTIAL OF EMERGING HALOGENATED ALIPHATIC HYDROCARBONS IN WETLAND PLANT ROOTS BY NATIVE AMMONIA-OXIDIZING MICROORGANISMS

## 4.1 INTRODUCTION

Three halogenated ethane species including 1,1,2-trichloroethane (1,1,2-TCA), 1,2-dibromoethane (1,2-DBA, or EDB) and 1,2-dichloroethane (1,2-DCA) are regarded as pollutants under National Primary Drinking Water Regulations (NPDWRs) (U.S. Environmental Protection Agency, 2003). All of the three contaminants pose hazards to human health: 1,1,2-TCA which has potential adverse effects on liver, kidney, or immune system has a Maximum Contaminant Level (MCL) of 0.005 mg/L; EDB has a MCL of 0.00005 mg/L as a potential carcinogen and can also cause liver, stomach, reproductive system, or kidney problems; The MCL of 1,2-DCA is 0.005 mg/L and long exposure to 1,2-DCA can also increase the risk of human cancer (U.S. Environmental Protection Agency, 2003). These halogenated aliphatic hydrocarbons (HAHs) were previously widely used and thus can enter the environments from various sources: 1,1,2-TCA was primarily used in producing 1,1-dichloroethene and also served as an organic solvent.
in limited scenarios (ATSDR, 1989); EDB was once a lead scavenger in antiknock mixtures added to gasolines (IPCS, 1996) and also used as a pesticide (ATSDR, 1992); 1,2-DCA is currently used in producing vinyl chloride and was also used as an organic solvent, fumigant, lead scavenger and anesthetic (USDHSS, 2014). The compounds can be detected in ambient air, soil, groundwater, and food (ATSDR, 1989, 1992, 2001) and could be very mobile in groundwater due to their physical properties (Henderson et al., 2008). One concern is the fate and transport of HAHs in shallow, vegetated aquatic environments (wetlands) (Powell et al., 2011). It was even practicable to actively direct HAHs contaminated groundwater to wetlands for treatment. Our recent field study has shown a near complete removal of chlorinated ethenes in the groundwater passing through the research wetland site constructed at Wright-Patterson Air Force Base in Dayton, OH (Amon et al., 2007).

Bioremediation, as a promising approach, has been proved to be effective at cleaning sites contaminated by HAHs (Field and Sierra-Alvarez, 2004). While bioremediation of HAHs typically involve reduction (Aulenta et al., 2007), bioremediation can also happen under oxidative pathways through cometabolism. For example, the enzyme ammonia monooxygenase (AMO) produced by ammonia oxidizing bacteria (AOB) can oxidize alternative substrates (HAHs) in the presence of a growth-supporting substrate (ammonium) (Wackett, 1996; Arp et al., 2001). In this process, AOB consume ammonia, which naturally occurs in wetlands as a participant in nitrogen cycling, a content in nitrogen-rich effluents (e.g. wastewater plants or
septic tanks), or an end product of anaerobic polymeric organic matter degradation (Chapin et al., 2003; Nivala et al., 2007). Ammonia served as both energy source and reductant, CO₂ for carbon requirements, and molecular oxygen is the electron acceptor (Sayavedra-Soto et al., 2010).

In wetlands, aquatic plants can transfer atmospheric oxygen from shoots to roots by porous aerenchyma tissue functioning as a gas channel, making the plant rhizosphere an oxidizing environment in the otherwise anaerobic soil. This can stimulate a great number of aerobic biogeochemical processes, including the growth of nitrifying bacteria that facilitate aerobic ammonia oxidization to nitrite (Gersberg et al., 1986; Bodelier et al., 1996; Kowalchuk et al., 1998; Venter et al., 2004; Wiessner et al., 2005). Usually nitrite can be further oxidized to nitrate in the presence of nitrite oxidizing bacteria (Wagner et al., 1996). It’s thus possible to direct groundwater contaminated by HAHs through wetlands for natural attenuation. In this study, the aquatic plant Carex comosa (bristly sedge) was chosen for use in batch scale experiments. This is a follow-up investigation of our recent work (Qin et al., 2014) on TCE degradation. In this work, 1,1,2-TCA, EDB (1,2-DBA) and 1,2-DCA (Tables 4.1-4.3) were investigated in batch experiments at varying concentrations.

Unlike 1,2-DCA degradation by ammonia oxidizers, which has been studied relatively intensively (Ely et al., 1997; Kocamemi and Cecen, 2010), research on cometabolic biodegradation by ammonia oxidizers of EDB and 1,1,2-TCA was limited (Frascari et al., 2008; Baek et al., 2014a). The records of aerobic
cometabolism of 1,1,2-TCA were mainly with other substrates like propane (Frascari et al., 2008) and butane (Kim et al., 2000). Several studies focused on aerobic cometabolic degradation of EDB, but generally with other substrates like phenol (Baek et al., 2014a), methane (McKeever et al., 2012), and pentane (Danko et al., 2012). The cometabolic biodegradation of 1,1,2-TCA and EDB was also reported by pure cultures of ammonium oxidizers, *Nitrosomonas europaea* (Vannelli et al., 1990).

The main objective of this study was to investigate the effects of HAH (1,1,2-TCA, EDB, and 1,2-DCA) concentration on their transformation by AOB naturally associated with the roots of a common wetland plant, *C. comosa*. Results of this study may have implications for pollutant mitigation and site management using constructed or restored wetlands.

4.2 MATERIALS AND METHOD

4.2.1 Experiment Setup

In this study, the procedures for collection of wetland plant (*Carex comosa*) from a natural wetland site, microcosm preparation containing washed (soil-free) plant roots, preparation of growth medium and reagents, enrichment for ammonia-oxidizing bacteria, gas chromatographic analysis, approaches for biomass and transformation yield calculations were identical to our previous investigation (Qin et al., 2014) and also described in Chapter 2. Microcosm experiments with individual HAH treatments (1,1,2-TCA, EDB, and 1,2-DCA) were completed in this study.

4.2.2 Cometabolic Degradation of CAHs with Ammonium
The degradation potential of individual HAHs (1,1,2-TCA, EDB, and 1,2-DCA) were examined in separate sets of triplicate microcosms (Tables 4.1-4.3). For each microcosm set, the HAH degradation studies began after multiple cycles of microbial enrichment, as follows: Set 1: 1,1,2-TCA for 9 cycles (Table 4.1); set 2: EDB (1,2-DBA) for 9 cycles (Table 4.2); and Set 3: 1,2-DCA for 3 cycles (Table 4.3). The microcosms were amended with ammonium nominally at 75 mg/L NH₄⁺-N in each cycle of all three sets; the concentration of individual HAH, however, was gradually increased starting from 40-50 µg/L except for the 1,2-DCA added microcosms where the initial concentration was around 350 µg/L due to its less toxicity to AOB (Rasche et al., 1991) (Tables 4.1-4.3). Triplicate ATU-inhibited control microcosms in order to minimize ammonium oxidation activity (Rasche et al., 1991; Ginestet et al., 1998) were also prepared at ammonium and HAHs concentrations equivalent to the live microcosms during all cycles.

4.3 RESULTS AND DISCUSSION

4.3.1 Aerobic 1.1.2-TCA Cometabolism: Concentration Effect

4.3.1.1 Sustained 1,1,2-TCA degradation with ammonium and degradation deactivation

Continuous degradation of 1,1,2-TCA for more than 60 days (Figure 4.1) was observed in the presence of ammonium and AOB associated with the rhizosphere of Carex comosa. Observation of aerobic cometabolic TCA degradation is scarce in the literature, and mainly with substrates other than ammonium (Strand et al., 1991; Kim
et al., 2002; Frascari et al., 2008). The results in this study demonstrated that AOB associated with Carex comosa naturally associated with local wetlands can also degrade 1,1,2-TCA. Sustained 1,1,2-TCA degradation lasted for five weeks with 75 mg L\(^{-1}\) initial [N-NH\(_4^+\)] and 40 μg L\(^{-1}\) initial [1,1,2-TCA]. As initial [1,1,2-TCA] increased to 90 μg L\(^{-1}\), 1,1,2-TCA and ammonium degradation were gradually affected and finally stopped one week after [1,1,2-TCA] reached 150 μg L\(^{-1}\) (Figure 4.1). Gradual decline of DO loss and NO\(_x\) production was also observed starting from Cycle T7, the second cycle when [1,1,2-TCA] increased to 90 μg L\(^{-1}\) (Figure B.4.1, in Appendix). There was also a shift of ammonium product from nitrite to nitrate observed beginning with Cycle T2, and after one week in Cycle T3 no nitrite was detected in the system (Figure 4.1(B)). 1,1,2-TCA was a “Group 3” chlorocarbon that is biodegradable but with substantial deactivation at higher concentrations due to the toxic intermediates acyl chlorides (Rasche et al., 1991). So at 75 mg L\(^{-1}\) of ammonium-N, continuous exposure of 1,1,2-TCA higher than 90 ug L\(^{-1}\) may cause cometabolic deactivation.

4.3.1.2 Variations in biomass

Pseudo first-order degradation of ammonium and 1,1,2-TCA in relations to biomass was calculated for each cycle in order to evaluate AOB activities (Table 4.1). The mean of calculated steady-state biomass \(X\) through all the nine cycles was 0.194±0.130 mmol L\(^{-1}\). The relatively large standard deviation indicated the biomass shifted significantly between different 1,1,2-TCA concentrations. \(X\) decreased in
Cycle T3 from 0.29 to 0.10 mmol L\(^{-1}\), this coincides with the ammonium oxidation product shift from nitrite to nitrate. This transition in oxidation product from nitrite to nitrate was also documented by others, for example Schramm et al., (1999), who attributed this to ammonium limitation, that inhibited AOB growth and thus favoring nitrite-oxidizing bacteria (Schramm et al., 1999). It was shown that AOB can inhibit ordinary heterotrophic organisms and nitrite-oxidizers by outcompeting them for oxygen (Schramm et al., 1999; Brockmann and Morgenroth, 2010; Shen et al., 2014). It was reasonable to infer that a certain number of ammonium oxidizers were inhibited and the nitrite produced by ammonium was balanced by nitrite oxidizers. This decline in \(X\) did not affect the rate constants though (discussed later). \(X\) then gradually increased to 0.31 mmol L\(^{-1}\), and then dropped sharply in Cycle T7 (0.04 mmol L\(^{-1}\)) where deactivation began to be observed. \(X\) increased a bit to 0.08 and 0.09 mmol L\(^{-1}\) respectively in the following two cycles, but the rate constants decreased further, ultimately being totally deactivated.

4.3.1.3 Variation in degradation kinetics of ammonium and 1,1,2-TCA

The mean of \(k_{\text{obs-TCA}}\) for all cycles was 0.364±0.358 d\(^{-1}\), ranging from 0 (Cycle T9) to 1.02 (Cycle T3) d\(^{-1}\). Biomass-normalized rate constants \(k_{1\text{-TCA}}\) for all cycles was 2.352±3.258 L mmol\(^{-1}\) d\(^{-1}\) (or 0.021±0.029 L mg\(^{-1}\) d\(^{-1}\) assuming the biomass of 113 g VSS/mole), ranging from 0 (Cycle T9) to 10.20 (Cycle T3) L mmol\(^{-1}\) d\(^{-1}\). Reported kinetics values for 1,1,2-TCA degradation was limited, but the value was comparable to previous research using a collection of pure propane oxidizers with \(k_{1\text{-TCA}}\) equal to
0.007 L mg\(^{-1}\) d\(^{-1}\) (Frascari et al., 2008). There were two extraordinarily high of \(k_{1\text{-TCA}}\) values in Cycle T3 and Cycle T4, where \(X\) decreased temporally without affecting the rate constants. It was possible that a certain number of ammonium oxidizers were gradually inhibited once 1,1,2-TCA was added to the system, but some groups that can tolerate 1,1,2-TCA survived and were also capable of degrading 1,1,2-TCA more efficiently, i.e., the AMO enzyme affinity to 1,1,2-TCA was significantly higher in those groups. The mean of \(k_{\text{obs-NH4}}\) for all cycles was 0.136±0.092 d\(^{-1}\), ranging from 0.005 (Cycle T9) to 0.24 (Cycle T5) d\(^{-1}\). Biomass-normalized rate constants \(k_{1\text{-NH4}}\) had an average of 0.756±0.852 L mmol\(^{-1}\) d\(^{-1}\), and ranged from 0.06 (Cycle T9) to 2.03 (Cycle T3) L mmol\(^{-1}\) d\(^{-1}\).

In Cycle T7, a sudden drop of \(k_{\text{obs-TCA}}\) (0.37 to 0.05 d\(^{-1}\)) and \(k_{\text{obs-NH4}}\) (0.17 to 0.03 d\(^{-1}\)) was observed. \(k_{1\text{-NH4}}\) (0.85 to 0.21 L mmol\(^{-1}\) d\(^{-1}\)) and \(k_{1\text{-TCA}}\) (1.45 to 0.10 L mmol\(^{-1}\) d\(^{-1}\)) decreased in the following Cycle T8 (Table 4.1). Based on rate constants, biomass and biomass normalized rate constants, it can be speculated that ammonium oxidizers survived the first addition of 1,1,2-TCA and increased when the concentration of 1,1,2-TCA was under 50 µg/L 1,1,2-TCA; however when 1,1,2-TCA increased to 90 µg/L, the majority of ammonium oxidizers could not tolerate that high 1,1,2-TCA concentration, and a sharp loss of biomass was observed in Cycle T7. This large loss of biomass also caused the pseudo first-order rates of ammonium and 1,1,2-TCA degradation to decrease. Biomass was elevated again in Cycle T8, but this biomass may contribute little to ammonium and 1,1,2-TCA degradation, thus the biomass-normalized kinetics decreased tremendously in Cycle T8. Eventually
cometabolism shut down due to the inability of the ammonium oxidizers to tolerate the high 1,1,2-TCA concentrations.

**4.3.1.4 Variation in 1,1,2-TCA transformation yield ($T_y$)**

$T_y$ increased as initial [1,1,2-TCA] increased, but there was no linear relation between $T_y$ and initial [1,1,2-TCA] (Figure 4.4(A)). $T_y$ values showed greater variations at higher [1,1,2-TCA], suggesting this linear relation became unstable when the initial [1,1,2-TCA] reached to 90 µg L$^{-1}$. Average $T_y$ values at 50 µg/L (0.10±0.03 µmol TCA/mmol NH$_4^+$) and 90 µg/L (0.28±0.19 µmol TCA/mmol NH$_4^+$) initial [1,1,2-TCA] were significantly lower than a published report (4.237 µmol TCA/mmol propane) using propane as the growth media (Frascari et al., 2008). No distinct relation was observed between transformation yield and [NH$_4^+$-N] (Figure 4.4(B)). This implied that the deactivation was more likely caused by the toxicity of elevated 1,1,2-TCA, not due to the competition between ammonium and 1,1,2-TCA.

**4.3.2 Aerobic EDB (1,2-DBA) Cometabolism: Concentration Effect**

**4.3.2.1 Sustained EDB degradation with ammonium and degradation deactivation**

Continuous degradation of EDB for more than 60 days (Figure 4.2) was also observed in the presence of ammonium and AOB associated with the rhizosphere of *Carex comosa*. As an emerging contaminant, aerobic cometabolism of EDB has received attention recently, but with substrates other than ammonium (Danko et al., 2012; McKeever et al., 2012; Baek et al., 2014b). The results in this study demonstrated that AOB associated with *Carex comosa* naturally associated with local
wetlands can also degrade the emerging contaminant EDB. Sustained EDB degradation lasted for three weeks with 75 mg L\(^{-1}\) initial \([\text{NH}_4^+ - N]\) and 50 μg L\(^{-1}\) initial [EDB]. As initial [EDB] increased to 100 μg L\(^{-1}\), EDB and ammonium degradation were gradually affected and finally stopped in the third cycle (Cycle B9) after [EDB] reached 150 μg L\(^{-1}\) (Figure 4.2). Gradual decline of DO loss and NO\(_x\) production was also observed starting with Cycle B5, the second cycle when [EDB] increased to 100 μg L\(^{-1}\) (Figure S2, SM). 75 mg L\(^{-1}\) of ammonium-N, continuous exposure to EDB concentrations higher than 100 μg L\(^{-1}\) may cause cometabolic deactivation. Similar to what was observed for 1,1,2-TCA cometabolic degradation, the oxidation product of ammonium began to shift from nitrite to nitrate in Cycle B3, and in Cycle B5 almost no nitrite could be detected (Figure 4.2(B)).

**4.3.2.2 Variations in biomass**

In terms of reaction kinetics (Table 4.2), the mean of \(X\) through all the nine cycles was 0.164± 0.096 mmol L\(^{-1}\). \(X\) was not significantly (\(P > 0.05\), one-way RM ANOVAs, group effect) different from the value in the 1,1,2-TCA systems. The relatively large standard deviation indicated the biomass shifted significantly between different [EDB]. \(X\) decreased (\(P < 0.05\), one-way RM ANOVAs, [EDB] effect) when initial [EDB] increased from 50 μg L\(^{-1}\), 100 μg L\(^{-1}\) to 150 μg L\(^{-1}\) and the significant differences were observed at 150 μg L\(^{-1}\) [EDB] compared to 50 μg L\(^{-1}\) (\(P < 0.05\), Bonferroni Pairwise Comparisons, [EDB] effect) and 100 μg L\(^{-1}\) (\(P < 0.05\), Bonferroni Pairwise Comparisons, [EDB] effect) [EDB]. The average values were 0.268±0.044.
mmol L⁻¹, 0.155±0.054 mmol L⁻¹, and 0.069±0.046 mmol L⁻¹, respectively. X gradually increased from enrichment cycle E1 (0.02 mmol L⁻¹) to the second experimental cycle B2 (0.30 mmol L⁻¹). X then dropped moderately in Cycle B3 and Cycle B4, which also coincided with the oxidation product of ammonium beginning to shift from nitrite to nitrate. X then sharply dropped from 0.21 to 0.10 mmol L⁻¹ in Cycle B5, again suggesting a certain number of ammonium oxidizers was inhibited and increasing nitrite oxidizers consumed all the nitrite produced. X elevated a bit in Cycle B6, then further decreased until Cycle B9 when the cometabolic system totally shut down.

4.3.2.3 Variation in degradation kinetics of ammonium and EDB

The mean of $k_{obs-EDB}$ for all cycles was 0.104±0.085 d⁻¹, ranging from 0.03 (Cycle B9) to 0.27 (Cycle B3) d⁻¹. This value is significantly lower (P < 0.05, one-way RM ANOVAs, group effect) than the value for 1,1,2-TCA, indicating that EDB was more difficult to degrade. Biomass-normalized rate constants $k_{1-EDB}$ for all cycles was 0.709±0.368 L mmol⁻¹ d⁻¹ (or 0.006±0.003 L mg⁻¹ d⁻¹ assuming the biomass of 113 g VSS/mole), ranging from 0.21 (Cycle B2) to 1.26 (Cycle B3) L mmol⁻¹ d⁻¹. This value was not significantly different from (P > 0.05, one-way RM ANOVAs, group effect) the 1,1,2-TCA microcosms. Prior studies of aerobic degradation of EDB were very limited (Danko et al., 2012; Baek et al., 2014b), and to the best of our knowledge, no kinetics were reported except for a report recording a slow natural attenuation process with a rate constant of 2.97 yr⁻¹ (Baek et al., 2012) compared to
0.104 d\(^{-1}\), or 37.92 yr\(^{-1}\) in this study. The mean of \(k_{\text{obs-NH}_4}\) for all cycles was 0.069±0.041 d\(^{-1}\), ranging from 0.02 (Cycle B9) to 0.12 (Cycle B2) d\(^{-1}\).

Biomass-normalized rate constants \(k_{1-\text{NH}_4}\) had an average of 0.451±0.141 L mmol\(^{-1}\) d\(^{-1}\), and ranged from 0.23 (Cycle B7) to 0.60 (Cycle B5) L mmol\(^{-1}\) d\(^{-1}\). The values of \(k_{\text{obs-NH}_4}\) were also lower (\(P < 0.05\), one-way RM ANOVAs, group effect) compared to those found for 1,1,2-TCA. This implies that the high toxicity of EDB to AOB fundamentally affected the kinetics of both ammonium and EDB degradation.

Like the scenario for 1,1,2-TCA deactivation, in Cycle B5, a sudden drop of \(k_{\text{obs-NH}_4}\) (0.11 to 0.06 d\(^{-1}\)) and \(k_{\text{obs-TCA}}\) (0.23 to 0.04 d\(^{-1}\)) was observed. Biomass normalized rate constants \(k_{1-\text{EDB}}\) (1.05 to 0.45 L mmol\(^{-1}\) d\(^{-1}\)) decreased at Cycle B5, but \(k_{1-\text{NH}_4}\) (0.60 to 0.29 L mmol\(^{-1}\) d\(^{-1}\)) decreased in the following Cycle 6 (Table 4.2). It could be speculated that the exposure of AOB to EDB at 100 μg L\(^{-1}\) may inhibit a substantial portion of ammonium oxidizers, which caused biomass and degradation rate constants for both substrates to decrease tremendously. However, unlike the 1,1,2-TCA microcosm, \(k_{1-\text{EDB}}\) and \(k_{1-\text{NH}_4}\) gradually increased again in the last three cycles, which suggests that there might be a very tiny portion of AOB that survived, and they might be able to more efficiently degrade both substrates.

**4.3.2.4 Variation in EDB transformation yield \((T_y)\)**

The relation between initial [EDB] and \(T_y\) appears to be exponential (Figure 5a.). Again, as seen with the 1,1,2-TCA system, due to cometabolic deactivation, the variances of this relationship became bigger at higher initial [EDB]. Average \(T_y\)
(0.169±0.227 μmol EDB/mmol ammonium) was comparable to a natural attenuation study with methane oxidizers where $T_y$ was 0.105 μmol EDB/mmol methane with an initial EDB concentration of 82 μg L$^{-1}$ (McKeever et al., 2012). No relation was observed between transformation yield and $[\text{NH}_4^+-\text{N}]$ (Figure 4.5(B)). It seems the deactivation was more likely due to the toxicity of EDB rather than the competition between EDB and ammonium.

### 4.3.3 Aerobic 1,2-DCA Cometabolism: Concentration Effect

#### 4.3.3.1 Sustained 1,2-DCA degradation with ammonium

In microcosms with ammonium, 1,2-DCA was added at concentrations of 340 and 400 μg L$^{-1}$ (Figure 4.3). 1,2-DCA was continuously degraded in three cycles for more than 20 days without signs of deactivation even at such a high initial concentration. The cometabolism of 1,2-DCA by ammonia oxidizers was also studied by others (Kocamemi and Cecen, 2010), and the concentrations in this scenario were significantly lower than those observed in their studies, where initial [1,2-DCA] ranged from 1600 μg L$^{-1}$ to 100,000 μg L$^{-1}$. The results demonstrated that the AOB which are capable of degrading 1,1,2-TCA and EDB can also degrade 1,2-DCA, and the tolerance to 1,2-DCA was significantly higher. There was no indication of reduction in oxygen consumption and NOx production (Figure S3, SI). Unlike 1,1,2-TCA, 1,2-DCA was classified as a “Group 2” chlorocarbon which was biodegradable with minimal inactivation (Rasche et al., 1991). That can explain why 1,2-DCA can survive at higher initial concentrations without deactivation. Although no obvious deactivation was observed, the transition of ammonium oxidation produc
was also observed during the three cycles (Figure 4.3(B)). Yet this transition was not fully complete since there was still nitrite detected in Cycle C3.

4.3.3.2 Variations in biomass

The average value of the steady state biomass calculated during the three cycles was 0.460±0.146 mmol L⁻¹, which was significantly higher than found in any initial concentration of other two microcosm systems, suggesting a steady sustainable microbial community. X dropped in Cycle C2 drom 0.51 to 0.30 mmol L⁻¹, and again, this coincided with the change in ammonium oxidation products. Based on the investigation of all three HAHs, it can be inferred that the addition of HAHs will inevitably inhibit a certain portion of AOB and thus favor the growth of nitrite oxidizers, or NOB, which will change the dominant ammonium oxidation products from nitrite to nitrate. X recovered in Cycle C3 (0.58 mmol L⁻¹) and degradation rates also increased (discussed in the following section).

4.3.3.3 Variation in Degradation Kinetics of Ammonium and 1,2-DCA

The pseudo first-order rate constants for both 1,2-DCA $k_{\text{obs-DCA}}$ (0.490±0.116 d⁻¹) and NH₄ $k_{\text{obs-NH₄}}$ (0.170±0.036 d⁻¹) were relatively stable for the three cycles (Table 4.3). Biomass-normalized rate constants also varied less (1.087±0.119 L mmol⁻¹ d⁻¹ for $k_{1\text{-DCA}}$ and 0.386±0.090 L mmol⁻¹ d⁻¹ for $k_{1\text{-NH₄}}$) than those for any level of initial concentration of the 1,1,2-TCA and EDB systems, exhibiting steady degradation at high initial [1,2-DCA] levels. The average $k_{\text{obs-DCA}}$ was higher than for any concentration level of the other two systems. Biomass-normalized rate constant
$k_{1-DCA}$ was significantly higher than those in the EDB reactors, but significantly lower than those of the 1,1,2-TCA system since there were two extraordinarily higher values in that system. The calculated mean $k_{1-DCA}$ (1.087±0.119 L mmol$^{-1}$ d$^{-1}$, or 0.008±0.001 L mg$^{-1}$ d$^{-1}$ assuming the biomass of 113 g VSS/mole) was comparable to a mixed nitrifying systems which reported values between 0.01 to 0.02 L mg$^{-1}$ d$^{-1}$ (Kocamemi and Cecen, 2010), but was lower than a pure *Nitrosomonas europaea* cultures, with the value of 0.37 L mg$^{-1}$ d$^{-1}$ (Ely et al., 1997). This is expected since the mixed culture studied here did not consist only of ammonium oxidizers and not all the biomass was responsible for cometabolic degradation. $k_{obs-NH4}$ was also significantly higher than in the other two systems (1,1,2-TCA and EDB) but $k_{1-NH4}$ was significantly lower. It was possible that since there was no deactivation observed, the whole microbial community did not encounter selection and lots of low-efficiency ammonium oxidizers survived.

**4.3.3.4 Variation in 1,2-DCA transformation yield ($T_y$)**

No clear trend of $T_y$ vs. initial [1,2-DCA] was observed, probably due to the narrow concentration range of 1,2-DCA investigated (Figure 4.6). The average $T_y$ of 1,2-DCA (1.153±0.240 μmol DCA/ mmol NH$_4^+$) was significantly higher than found in the other two systems (1,1,2-TCA and EDB). Based on the kinetics and $T_y$ analysis, the cometabolic mechanisms in 1,2-DCA were different; without clear signs of cometabolic deactivation caused by competition between HAHs and ammonium or the toxicity of the HAH degradation products.
4.4 CONCLUSIONS

The present bench-scale study confirms the potential for simultaneous degradation of ammonium and emerging HAHs contaminants in the shallow, vegetated zone of the wetland. The study also offers a mechanism for destruction of chlorinated ethenes through aerobic cometabolism (Amon et al., 2007). This study provides good evidence for an oxidative degradation pathway of HAHs in the root zone of wetland plants with ammonium, a natural product of organic matter biodegradation, as the driving energy source. Deactivation of cometabolic degradation of HAHs and ammonium was observed in 1,1,2-TCA and EDB microcosms when the respective HAH concentrations reached a certain level. A shift of ammonium oxidation product from nitrite of nitrate after HAHs were added was observed in all the three systems. It can be inferred the addition of the HAHs will change the microbial community by inhibiting ammonia oxidizers and this inhibition may lead to deactivation. Future microbial community analysis may help to further understand the mechanisms how the systems respond to the addition of HAHs.

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Figure 4.1 Degradation of NH$_4^+$-N (A) and 1,1,2-TCA (C), and production of NO$_x$-N (B, individual NO$_2$-N and NO$_3$-N); error bars represent one standard deviation in triplicate reactors through 9 successive cycles (T1-T9). Solid symbols in (A) and (C) represent average values in live microcosms while open symbols represent average values in control microcosms. Solid symbols in (B) represent average values of NO$_3$-N while open symbols represent average values of NO$_2$-N.
Figure 4.2 Degradation of NH$_4^+$-N (A) and EDB (1,2-DBA) (C), and production of NO$_2$-N (B, individual NO$_2$-N and NO$_3$-N); error bars represent one standard deviation in triplicate reactors through 9 successive cycles (B1-B9). Solid symbols in (A) and (C) represent average values in live microcosms while open symbols represent average values in control microcosms. Solid symbols in (B) represent average values of NO$_3$-N while open symbols represent average values of NO$_2$-N.
Figure 4.3 Degradation of NH$_4^+$-N (A) and 1,2-DCA (C), and production of NO$_x$-N (B, individual NO$_2^-$-N and NO$_3^-$-N); error bars represent one standard deviation in triplicate reactors through 3 successive cycles (C1-C3). Solid symbols in (A) and (C) represent average values in live microcosms while open symbols represent average values in control microcosms. Solid symbols in (B) represent average values of NO$_3^-$-N while open symbols represent average values of NO$_2^-$-N.
Figure 4.4 Scatter plot of (A) Regression of 1,1,2-TCA Transformation Yield ($T_y$) vs. initial 1,1,2-TCA concentrations ($\mu$g L$^{-1}$) for average of triplicate microcosms, and (B) 1,1,2-TCA Transformation Yield ($T_y$) vs. initial $\text{NH}_4^+$-$\text{N}$ concentrations in individual microcosms, for 9 successive cycles (T1-T9). Transformation Yield ($T_y$) is defined as the quotient of net 1,1,2-TCA degraded ($\mu$moles) divided by net $\text{NH}_4^+$-$\text{N}$ consumed (mmoles).
Figure 4.5 Scatter plot of (A) Regression of EDB (1,2-DBA) Transformation Yield ($T_y$) vs. initial EDB concentrations ($\mu$g L$^{-1}$) for average of triplicate microcosms, and (B) EDB Transformation Yield ($T_y$) vs. initial NH$_4^+$ concentrations in individual microcosms, for 9 successive cycles (B1-B9). Transformation Yield ($T_y$) is defined as the quotient of net EDB degraded ($\mu$moles) divided by net NH$_4^+$ consumed (mmoles).
Figure 4.6 Scatter plot of (A) Regression of 1,2-DCA Transformation Yield ($T_y$) vs. initial 1,2-DCA concentrations (µg L$^{-1}$) for average of triplicate microcosms, and (B) 1,2-DCA Transformation Yield ($T_y$) vs. initial NH$_4^+$-N concentrations in individual microcosms, for 3 successive cycles (C1-C3). Transformation Yield ($T_y$) is defined as the quotient of net 1,2-DCA degraded (µmoles) divided by net NH$_4^+$ consumed (mmoles).
Table 4.1 Average parameters in Set 1 triplicate microcosms for cycles: initial concentrations of 1,1,2-TCA ([TCA]₀, µg/L), and ([NH₄⁺-N]₀, mg/L); steady state cell biomass concentration (X, mmol/L); initial pseudo first-order rate constants of NH₄⁺-N removal (k_{obs-NH₄}, d⁻¹) and 1,1,2-TCA degradation (k_{obs-TCA}, d⁻¹), and their corresponding coefficients of determination (R²); biomass-normalized NH₄⁺-N removal rate constants (k_{1-NH₄}, L.mmol⁻¹.d⁻¹) in 2 enrichment cycles (E1-E2) and 9 1,1,2-TCA cycles (T1-T9); biomass-normalized 1,1,2-TCA degradation rate constants (k_{1-TCA}, L.mmol⁻¹.d⁻¹) in 9 1,1,2-TCA cycles (T1-T9); and transformation yield (T_y, umol 1,1,2-TCA / mmol NH₄⁺-N). 1,1,2-TCA concentration increased gradually starting at 40 µg/L in cycles T1-T5, 90 µg/L in cycles T6-T8, and 150 µg/L in cycle T9.

<table>
<thead>
<tr>
<th>Cycle</th>
<th>[TCA]₀</th>
<th>[NH₄⁺-N]₀</th>
<th>X</th>
<th>k_{obs-NH₄}; (R²)</th>
<th>k_{obs-TCA}; (R²)</th>
<th>k_{1-NH₄}</th>
<th>Avg. k_{1-NH₄}</th>
<th>k_{1-TCA}</th>
<th>Avg. k_{1-TCA}</th>
<th>T_y</th>
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<tbody>
<tr>
<td>E1</td>
<td>--</td>
<td>69.6</td>
<td>0.04</td>
<td>0.07; (0.91)</td>
<td>--</td>
<td>1.67</td>
<td>1.18±0.68</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>E2</td>
<td>67.3</td>
<td></td>
<td>0.22</td>
<td>0.15; (0.99)</td>
<td>--</td>
<td>0.70</td>
<td>0.52</td>
<td>--</td>
<td>--</td>
<td>--</td>
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<tr>
<td>T1</td>
<td>46.9</td>
<td>67.1</td>
<td>0.40</td>
<td>0.20; (0.99)</td>
<td>0.21; (0.71)</td>
<td>0.51</td>
<td>1.03±0.61</td>
<td>3.69±3.98</td>
<td>0.14</td>
<td>0.08</td>
<td>0.10±0.03</td>
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<tr>
<td>T2</td>
<td>42.4</td>
<td>65.2</td>
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<td>0.18; (0.98)</td>
<td>0.26; (0.91)</td>
<td>0.62</td>
<td>0.88</td>
<td>0.06</td>
<td>0.12</td>
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<tr>
<td>T3</td>
<td>37.8</td>
<td>67.0</td>
<td>0.10</td>
<td>0.20; (0.95)</td>
<td>1.02; (0.87)</td>
<td>2.03</td>
<td>10.20</td>
<td>0.14</td>
<td>1.23</td>
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<tr>
<td>T4</td>
<td>40.4</td>
<td>60.5</td>
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<td>0.18; (0.99)</td>
<td>0.71; (0.97)</td>
<td>1.17</td>
<td>4.58</td>
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<td>T5</td>
<td>45.1</td>
<td>64.5</td>
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<td>0.24; (0.99)</td>
<td>0.66; (0.95)</td>
<td>0.83</td>
<td>2.26</td>
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<td>T6</td>
<td>89.5</td>
<td>67.2</td>
<td>0.31</td>
<td>0.17; (0.97)</td>
<td>0.37; (0.93)</td>
<td>0.54</td>
<td>0.53±0.32</td>
<td>1.18</td>
<td>0.23</td>
<td>0.23</td>
<td>0.28±0.19</td>
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<tr>
<td>T7</td>
<td>92.2</td>
<td>72.0</td>
<td>0.04</td>
<td>0.03; (0.91)</td>
<td>0.05; (0.86)</td>
<td>0.85</td>
<td>0.91±0.71</td>
<td>0.49</td>
<td>0.28±0.19</td>
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<td>T8</td>
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<td>0.08</td>
<td>0.02; (0.77)</td>
<td>0.008; (0.87)</td>
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<td>0.10</td>
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<tr>
<td>T9</td>
<td>145.9</td>
<td>74.3</td>
<td>0.09</td>
<td>0.005; (0.76)</td>
<td>0; (0)</td>
<td>0.06</td>
<td>0.06</td>
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Table 4.2 Average parameters in Set 2 triplicate microcosms for cycles: initial concentrations of EDB ([EDB]₀, μg/L), and ([NH₄⁺-N]₀, mg/L); steady state cell biomass concentration (X, mmol/L); initial pseudo first-order rate constants of NH₄⁺-N removal (k₁-NH₄, d⁻¹) and EDB degradation (k₁-EDB, d⁻¹), and their corresponding coefficients of determination (R²); biomass-normalized NH₄⁺-N removal rate constants (k₁,NH₄ L mmol⁻¹ d⁻¹) in 2 enrichment cycles (E1-E2) and 9 EDB cycles (B1-B9); biomass-normalized EDB degradation rate constants (k₁-EDB, L mmol⁻¹ d⁻¹) in 9 EDB cycles (B1-B9); and transformation yield (T_y, umol EDB / mmol NH₄⁺-N). EDB concentration increased as follows: 50 μg/L in cycles B1-B3, 100 μg/L in cycles B4-B6, and 150 μg/L in cycles B7-B9.

<table>
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<tr>
<th>Cycle</th>
<th>[EDB]₀</th>
<th>[NH₄⁺-N]₀</th>
<th>X</th>
<th>k₁-NH₄; (R²)</th>
<th>k₁-EDB; (R²)</th>
<th>Avg. k₁-NH₄</th>
<th>Avg. k₁-EDB</th>
<th>T_y</th>
<th>Avg. T_y</th>
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<td>E1</td>
<td>--</td>
<td>71.5</td>
<td>0.02</td>
<td>0.02; (0.74)</td>
<td>--</td>
<td>0.79</td>
<td>--</td>
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<tr>
<td>E2</td>
<td>71.2</td>
<td>0.06</td>
<td>0.11</td>
<td>(0.98)</td>
<td>1.74</td>
<td>1.27±0.67</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>B1</td>
<td>52.4</td>
<td>84.6</td>
<td>0.28</td>
<td>0.10; (0.99)</td>
<td>0.07; (0.96)</td>
<td>0.34</td>
<td>0.23</td>
<td>0.57±0.60</td>
<td>0.04</td>
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<tr>
<td>B2</td>
<td>54.4</td>
<td>77.8</td>
<td>0.30</td>
<td>0.12; (0.98)</td>
<td>0.06; (0.98)</td>
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<td>0.04</td>
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<td>49.3</td>
<td>69.5</td>
<td>0.22</td>
<td>0.11; (0.99)</td>
<td>0.27; (0.97)</td>
<td>0.52</td>
<td>0.55</td>
<td>1.26</td>
<td>0.07</td>
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<tr>
<td>B4</td>
<td>99.1</td>
<td>74.8</td>
<td>0.21</td>
<td>0.11; (0.99)</td>
<td>0.22; (0.92)</td>
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<td>0.04; (0.61)</td>
<td>0.60</td>
<td>0.70±0.32</td>
<td>0.10</td>
<td>0.13±0.03</td>
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<td>99.4</td>
<td>72.2</td>
<td>0.16</td>
<td>0.05; (0.96)</td>
<td>0.09; (0.82)</td>
<td>0.29</td>
<td>0.46±0.20</td>
<td>0.74</td>
<td>0.33±0.37</td>
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<td>B7</td>
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<td>0.03; (0.93)</td>
<td>0.11; (0.93)</td>
<td>0.23</td>
<td>0.88</td>
<td>0.87±0.12</td>
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<td>B8</td>
<td>155.7</td>
<td>60.3</td>
<td>0.06</td>
<td>0.03; (0.86)</td>
<td>0.04; (0.96)</td>
<td>0.55</td>
<td>0.98</td>
<td>0.98</td>
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<tr>
<td>B9</td>
<td>140.3</td>
<td>66.0</td>
<td>0.03</td>
<td>0.02; (0.71)</td>
<td>0.03; (0.73)</td>
<td>0.59</td>
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Table 4.3 Average parameters in Set 3 triplicate microcosms for cycles: initial concentrations of 1,2-DCA ([DCA]₀, µg/L), and ([NH₄⁺-N]₀, mg/L); steady state cell biomass concentration (X, mmol/L); initial pseudo first-order rate constants of NH₄⁺-N removal (k_{obs-NH₄}, d⁻¹) and 1,2-DCA degradation (k_{obs-DCA}, d⁻¹), and their corresponding coefficients of determination (R²); biomass-normalized NH₄⁺-N removal rate constants (k_{1-NH₄}, L mmol⁻¹ d⁻¹) in 3 1,2-DCA cycles (C1-C3); biomass-normalized 1,2-DCA degradation rate constants (k_{1-DCA}, L mmol⁻¹ d⁻¹) in 3 1,2-DCA cycles (C1-C3); and transformation yield (T_y, umol 1,2-DCA / mmol NH₄⁺-N). The experiments with 1,2-DCA began at 350 µg/L in cycles C1-C3.

<table>
<thead>
<tr>
<th>Cycle</th>
<th>[DCA]₀</th>
<th>[NH₄⁺-N]₀</th>
<th>X</th>
<th>k_{obs-NH₄}; (R²)</th>
<th>k_{obs-DCA}; (R²)</th>
<th>Avg. k_{1-NH₄}</th>
<th>k_{1-DCA}</th>
<th>Avg. k_{1-DCA}</th>
<th>T_y</th>
<th>Avg. T_y</th>
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<tr>
<td>C1</td>
<td>340.0</td>
<td>56.8</td>
<td>0.51</td>
<td>0.21; (0.88)</td>
<td>0.52; (0.86)</td>
<td>0.41</td>
<td>1.01</td>
<td>1.09±0.12</td>
<td>1.292</td>
<td>1.13±0.16</td>
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<td>C2</td>
<td>345.5</td>
<td>61.6</td>
<td>0.30</td>
<td>0.14; (0.96)</td>
<td>0.36; (0.98)</td>
<td>0.46</td>
<td>0.39±0.09</td>
<td>1.23</td>
<td>0.982</td>
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<tr>
<td>C3</td>
<td>394.6</td>
<td>72.2</td>
<td>0.58</td>
<td>0.17; (0.97)</td>
<td>0.59; (0.99)</td>
<td>0.29</td>
<td>1.03</td>
<td>1.125</td>
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5 COMMUNITY ANALYSIS OF AMMONIUM OXIDIZING BACTERIA AND
NITRITE OXIDIZING BACTERIA IN WETLAND PLANT ROOTS

5.1 INTRODUCTION

As a naturally occurring pore-water constituent, ammonium can accumulate in
wetlands as an end product of polymeric organic matter degradation or from nitrogen-rich
effluents (Chapin et al., 2002; Nivala et al., 2007). Although the bulk of the wetland
soil/sediment below the water table is usually anoxic and thus strongly reductive (Askaer
et al., 2010), in some cases, especially in the plant rhizosphere at shallow vegetated
wetland, the environment can be relatively oxidative due to aerenchymous gas transport
and O$_2$ leakage from wetland plant roots (Laan et al., 1989; Armstrong et al., 1994).
Therefore microbial ammonium oxidation can happen at such rhizosphere in wetland
when ammonium source and oxygen are both present. The pathway of microbial
ammonium oxidation involves several reactions requiring molecular oxygen (Fernández
et al., 2008; Gilch et al., 2009); ammonium is oxidized to hydroxylamine by the enzyme
ammonium monooxygenase (AMO) that is further oxidized to nitrite by the enzyme
hydroxylamine oxidoreductase (HAO) (eqs. 5.1 and 5.2) with the participation of
ammonium oxidizing bacteria (AOB). Nitrite can be subsequently transformed to nitrate
in the presence of nitrite oxidizing bacteria (NOB), which completes the process known
as nitrification (eq. 5.3).
\[ \text{NH}_3 + \text{O}_2 + 2[\text{H}] \rightarrow \text{NH}_2\text{OH} + \text{H}_2\text{O} \quad (5.1) \]
\[ \text{NH}_2\text{OH} + \text{O}_2 \rightarrow \text{NO}^- + \text{H}_2\text{O} + \text{H}^+ \quad (5.2) \]
\[ \text{NO}^- + \text{H}_2\text{O} \rightarrow \text{NO}_3^- + \text{H}^+ + 2\text{e}^- \quad (5.3) \]

AOB containing ammonium monoxygenase gene (amoA) accounts for the oxidization of ammonium. The majority of AOB found in wetlands belongs to two genera: *Nitrosospira* and *Nitrosomonas*, whose presences were identified in several case studies (Bodelier et al., 1996; Kowalchuk et al., 1998), as well as in our previous researches. Once established, AOB communities can maintain at a stable level, varying very slightly in community composition over time (Dionisi et al., 2002; Hallin et al., 2005). NOB belong to *Nitrobacter* spp. and *Nitrospira* spp. (Degrange and Bardin, 1995; Silyn-Roberts and Lewis, 2001; Allen et al., 2010). Depending on various wetland types and environmental conditions, the abundance of *Nitrosomonas* spp. can range from 0–1.5% in subsurface wetlands and up to 4% in surface wetlands (Flood et al., 1999; Silyn-Roberts and Lewis, 2001), while the percentage numbers of bacteria from genus *Nitrospira* and *Nitrobacter* (nitrite oxidizing bacteria) may reach 1.5% and 4.3%, respectively in surface wetlands (Shipin et al., 2005).

Through the process of cometabolism defined as “the fortuitous microbial transformation of a non-growth supporting compound that is catalyzed by an enzyme or cofactor with a nonspecific active site, and supported by the metabolism of a growth-supporting substrate or another transformable compound” (Dalton and Stirling, 1982; Hyman et al., 1995), AOB can also oxidize other substrates (e.g., Trichloroethene, TCE) with the presence of ammonium and oxygen. TCE was considered as a persistent pollutant and a potential carcinogen (Council, 2006), and no aerobic microorganism that
can grow on TCE as a sole carbon or energy source has been isolated so far (Hyman et al., 1995). Through cometabolism, however, TCE is mineralized to CO$_2$, H$_2$O, and chloride ions, without the accumulation of stable and carcinogenic intermediates such as vinyl chloride (Vogel and McCarty, 1985).

The cometabolic degradation of ammonium and TCE can be inhibited at a certain TCE concentration due to the competition of enzyme between TCE and ammonium and also by the toxicity of intermediate products acyl chlorides of TCE oxidation (Rasche et al., 1991; Hyman et al., 1995). The inhibition can be recovered after TCE was removed and sufficient time, ammonium, and oxygen were given (Rasche et al., 1991). In our previous researches, the inhibition and recovery of cometabolic degradation activity were also observed. After recovery, biomass-normalized ammonia and TCE oxidation rate constants increased significantly. It may suggest that the microbial community may have shifted during the inactivation-recovery stage. Those species that had greater ammonium and TCE removal efficiency can survive higher [TCE] and became the dominance group in the recovered community. So here in this study, we evaluated a possible microbial community shift by both analytical methods which investigated the product of ammonium and nitrite oxidation and molecular microbiology method which studied microbial community composition through the inhibition-recovery cycle.

5.2 MATERIALS AND METHODS

5.2.1 Experiment Setup

In this study, the procedures for collection of plant (Carex comosa) from a natural wetland site, microcosm preparation containing washed (soil-free) plant roots,
preparation of growth medium and reagents, enrichment for ammonia oxidizing bacteria, gas chromatographic analysis, approaches for biomass and transformation yield calculations were identical to our previous investigation (Qin et al., 2014) and also described in Chapter 2.

5.2.2 Cometabolic TCE Degradation with Ammonium

Following 2 cycles of microbial enrichment with ammonium (AME1 and AME2, described in Appendix), TCE degradation was examined in these microcosms (in triplicate, Exp) for 14 additional cycles (AMT1 through AMT14). Ammonium \([\text{(NH}_4\text{)}_2\text{SO}_4]\) concentration was nominally 75 mg/L of aqueous \(\text{NH}_4^+\)-N, and TCE concentration was varied in order to assess the effect of concentration on the degradation process as follows: nominally 50 μg/L in AMT1 to AMT3, 100 μg/L in AMT4 and AMT5, 150 μg/L in AMT6 to AMT8, 200 μg/L in AMT9 and AMT10, 250 μg/L in AMT11 and AMT12, and 300 μg/L in AMT13 and AMT14. Triplicate ATU-inhibited control microcosms (Con) were set up at [ammonium] and [TCE] equivalent to the live microcosms. No TCE positive controls (Pos) were also set up to evaluate differences in microbial community structures. Additional triplicate of sacrificial groups (Sac) were prepared at the same condition of live microcosms (Exp), and a tiny amount (1 mm) of roots were cut and preserved for RNA/DNA extraction to collect the microbial RNA/DNA attached to roots (while in the other three groups, RNA/DNA were extracted from filtered liquid medium). Total number of microcosms was 12, with the four treatment groups (Exp, Con, Pos, and Sac) mentioned above in triplicates.

5.2.3 RNA/DNA Extraction
RNA and DNA were extracted from the growth media that was decanted at the end of each cycle (described in Appendix) using the combination of MoBio RNA PowerSoil Total RNA Isolation Kit and MoBio RNA PowerSoil DNA Elution Accessory Kit (MoBio, Carlsbad, CA). The sampling intervals for RNA and DNA were two or three weeks and details can be found in Table 5.1. rRNA was then reverse transcribed to complementary DNA (cDNA) using RETROscript® Kit (Life Technologies, Grand Island, NY) with oligo(dT) primers according to the products instruction.

5.2.4 Barcoded Next-Generation Sequencing and Data Analysis

The V2 variable region of the 16S rRNA gene fragments from extracted DNA and cDNA (Table 5.1) was PCR-amplified with the universal primer set 515F (GTGCCAGCMGCGGTAA) and 806R (GGACTACHVGGGTWTCTAAT) (Caporaso et al., 2012). Multiple samples were pooled into one run for paired-end 16S community sequencing on the Illumina MiSeq platform using a sample tagging approach (Caporaso et al., 2012). Followed by reverse complement of 3' Illumina adapter (CAAGCAGAAGACGGCATACGAGAT), a unique 12bp Golay barcode (with reverse primer pad (AGTCAGTCAG) and reverse primer linker (CC)) was added to the 5’ - end of reverse primers for each individual sample (Caporaso et al., 2012). The 1X PCR master mix (25 μL) contained the following reagents: 12.5 μL of PCR grade H₂O (MoBio Laboratories), 10.0 μL of five prime master mix, 0.5 μL of 10 μM forward primer sequence, 1.0 μL of 5 μM reverse primer sequence, and 1.0 μL of template DNA/cDNA. Triplicates were used for each DNA sample to yield enough amplicons. The PCR started with an initial denaturation step at 94 °C for 3 min, followed by 35 cycles of 94 °C for 45 s, 50 °C for 60 s, and 72 °C for 90 s, and a final extension at 72 °C for 10
min. The amplicons were run on a 2% agarose gel with 5 μL of samples and 1 μL of 2X loading dye. Amplicons were then quantified on a Nanodrop 2000 UV-Vis Spectrophotometer (Thermo Scientific, Waltham, MA). Then 500 ng of DNA per sample were combined into a single sterile tube and the final pool was cleaned using MoBio UltraClean PCR Clean-Up Kit #12500.

Sequencing was performed on the Illumina MiSeq platform using the 2 x 150 base pairs (bp) V2 reagent kit. Quality control and sequencing results were analyzed through the web-based Galaxy project. Sequences with a quality score <20 were removed. The average length of sequences is 230 bp with a minimum length of 200 bp and maximum length of 300 bp. Paired-end reads were merged if a 20 bp overlap existed between the two reads. Merged reads were clustered at the 97% identity level and representative sequences were assigned a taxonomy based on the ribosome database project (RDP) classifier.

5.2.5 Statistical Analysis

The software RStudio (an open source integrated development environment for software R, RStudio Inc, 2014) was used to study the microbial community by multivariate statistical analysis of the OTU data generated from Galaxy. Nonmetric multidimensional scaling (NMDS, using metaMDS function in the ‘vegan’ package) was used to group similar communities based on the distribution of OTU (Bray and Curtis, 1957; Briggs et al., 2013). Also, with the “envfit” function in the same package, any correlations between microbial community composition and environmental variables can be displayed (Huang et al., 2013).
5.3 RESULTS AND DISCUSSION

5.3.1 TCE and Ammonium Degradation

Similar to our previous researches (Qin et al., 2014), TCE and ammonium was continuously degraded in a 125-day period with 14 cycles (AMT1 to AMT14) (Figure 5.1, Figure B.5.1, in Appendix). Both the exp and sac treatment groups degraded sustainably with initial TCE concentration ranging from 50 to 300 μg L\(^{-1}\). However, unlike our previous reports (Qin et al., 2014), no inactivation was observed during the 14-cycle cometabolism process. However, a certain level of deactivation did occur in several cycles of the exp and sac groups (Table 5.2). In the exp group, the pseudo first-order rate constants for ammonium degradation (\(k_{\text{obs-NH}_4}\)) decreased from 0.094 (AME2) to 0.029 (AMT1) d\(^{-1}\) when TCE was added; and after a four-week gradual increase, \(k_{\text{obs-NH}_4}\) decreased again from 0.438 d\(^{-1}\) (AMT4) to 0.162 d\(^{-1}\) (AMT5) and further to 0.053 d\(^{-1}\) in Cycle AMT6. The rate constants continued to decrease until Cycle AMT9, and never returned to the same value as in Cycle AMT5. Similar trends happened to the pseudo first-order rate constants for TCE degradation (\(k_{\text{obs-TCE}}\)) from 0.416 d\(^{-1}\) in AMT4 to 0.103 d\(^{-1}\) in Cycle AMT5. The value continued to decrease till Cycle AMT9 and did not recover to previous levels. In the sac group, two decrease of kinetic rates (\(k_{\text{obs-NH}_4}\) and \(k_{\text{obs-TCE}}\)) also happened separately in Cycle AMT1 and Cycle AMT6, with the exception that in Cycle 10, \(k_{\text{obs-TCE}}\) (0.722 d\(^{-1}\)) exceeded the value in Cycle AMT5 (0.475 d\(^{-1}\)). For the calculated biomass, in both the exp and sac groups, the values decreased at Cycle AMT1, i.e., after TCE was added to the batch systems. There was also a significantly decrease in Cycle AMT6 (0.054 mmol L\(^{-1}\)) compared to Cycle AMT5 (0.350 mmol L\(^{-1}\)) in exp group. The decrease of biomass in sac group happened later at Cycle AMT9. It was quite
possible that the decline in kinetics was due to the decrease of biomass, as recorded in our previous researches (Qin et al., 2014).

5.3.2 Nitrite and Nitrate Production

It was observed that in the pos group where no TCE was added throughout the entire experiment, there was no NO$_3$ production in any of the two enrichment cycles and fourteen experimental cycles. It implied that without the alternative substrate TCE, the microbes naturally associated in the rhizosphere were capable of oxidizing NH$_4^+$ to NO$_2$, but unlikely to further oxidize NO$_2$ to NO$_3$. It was documented that AOB may inhibit ordinary heterotrophic organisms and nitrite-oxidizing bacteria by out-competition for oxygen (Schramm et al., 1999; Shen et al., 2014). In the exp and sac groups as well, no NO$_3$ production was tracked in the enrichment cycles and even when low concentrations of TCE was added. However, when initial TCE concentration reached around 150 to 200 μg L$^{-1}$, NO$_3$ began to present in the systems (Figure 5.2).

These changes in the production of NO$_3$ were also documented previously by other researchers who contributed this change to ammonium limitation which inhibited the growth of ammonium oxidizers in their scenario (Schramm et al., 1999). In our results, the changes coincided with the moderate deactivation of cometabolic kinetics and decreased biomass as described in the previous sections. In the exp group, NO$_2$ production decreased from 46.59 ($\pm$ 8.48) mg L$^{-1}$ (Cycle AMT5, 100 μg L$^{-1}$ initial [TCE]) to 22.07 ($\pm$ 8.81) mg L$^{-1}$ (Cycle AMT6, 100 μg L$^{-1}$ initial [TCE]) with a drop in $k_{\text{obs-NH}_4}$, $k_{\text{obs-TCE}}$ and biomass occurring at the same time (see previous discussion). In Cycle AMT9, NO$_3$ production began to be detectable and at the system, $k_{\text{obs-NH}_4}$ and $k_{\text{obs-TCE}}$
increased from 0.038 d\(^{-1}\) (AMT8) to 0.102 d\(^{-1}\) (AMT9) and from 0.058 d\(^{-1}\) (AMT8) to 0.194 d\(^{-1}\) (AMT9), respectively. Biomass also increased from 0.116 mmol L\(^{-1}\) (AMT8) to 0.244 mmol L\(^{-1}\) (AMT9) as well. The production of NO\(_3\) in Cycle AMT9 terminated the decline of cometabolism kinetics, though after Cycle AMT9 the kinetic rate was not recovered to the previous level before the moderate deactivation. In the sac group, similar decline of NO\(_2\) production happened at Cycle AMT6 as well, along with a drop in \(k_{\text{obs-NH4}}\), \(k_{\text{obs-TCE}}\) and biomass discussed in the previous section. However, different from the scenarios in the exp group, NO\(_3\) production was detected as early as in Cycle AMT6, which explained why the decrease in biomass (from 0.473 (AMT5) to 0.313 (AMT6) mmol L\(^{-1}\)) were less intensive compared to the exp group. It was quite possible that nitrite oxidizers began to increase and contributed to the total biomass. That posed a possible explanation to the fact that the sac group recovered faster than the exp group and the kinetics and biomass in the sac groups were higher.

5.3.3 Sequencing Results of Ammonium Oxidizers and Nitrite Oxidizers

The sequencing results for cDNA did not yield good results and showed a sign of RNA contamination, so they were not discussed here. For DNA, a total number of 4 millions sequences from more than 50,000 OTUs were generated based on the sequencing results from Exp, Pos and Sac groups in Cycle AMT3 (**d2), Cycle AMT6 (**d3), and Cycle AMT14 (**d6), where “**” means the treatment group and duplicates (e1 for duplicate 1 in exp group, for example, see Table 5.1). The five most abundance OTUs, in relative abundance to total sequences, were OTU_25749 (4.07%, from genus Brevundimonas), OTU_24393 (3.96%, from genus Nitrosomonas), OTU_16914 (3.80%, from genus Thermomonas), OTU_28996 (3.42%, from family Xanthomonadaceae), and
OTU_33351 (2.51%, from genus *Chryseobacterium*). Note that the sequencing were to the genus level, so generally one genus can have multiple OTUs since there were usually more than one species within one genus. Other than *Nitrosomonas* who is a common ammonia oxidizer, one species of *Brevundimonas* was related to nitrogen fixing (Prakamhang et al., 2009); *Thermomonas* and *Xanthomonadaceae* were related to the reduction of nitrite and nitrate (Mergaert et al., 2003; Wu et al., 2013); and a species of *Chryseobacterium* was a heterotrophic carbon utilizing microbe also capable of simultaneous nitrification and denitrification (Kundu et al., 2014).

There were four genera putatively associated with ammonium (*Nitrosomonas* and *Nitrosospira*) and nitrite (*Nitrobacter* and *Nitrospira*) oxidization. The relative fractions (relative abundance) of each individual genus in the four genera samples were described in Figure 5.3 (A-C). For the exp group, the percentage of NOBs was low in Cycle AMT3 (0.4%) where no nitrate production was identified; in Cycle AMT6 where a modest decrease in cometabolism kinetics was observed, the percentage of NOBs increased to 2.9% and in Cycle AMT14 after the system recovered from moderate deactivation and the majority of nitrite was oxidized to nitrate, the percentage of NOBs increased to 45.1% (in which *Nitrospira* made 38.9%) of the four genera. It implied that the ammonium oxidizers were still present in the system, but the nitrite produced from ammonium oxidation was promptly used up by nitrite oxidizers. Similar trend happened to the sac group with the NOB percentage of 1.6%, 4.1%, and 53.8% in Cycle AMT3, AMT6 and AMT14, respectively. Also in Cycle AMT14, *Nitrospira* made 51.4% of the total numbers from the four genera. It can be speculated that *Nitrospira* may account for the nitrate production in the systems. Note that there was a little amount of nitrate production
in Cycle 6 in the sac group while there was no nitrate in that cycle in Cycle 3, not only because the percentage of NOB was slightly higher in sac group than in the exp group (4.1% compared to 2.9%), but also there was a significant difference in the percentage of Nitrospira (3.3% compared to 0.0%). For the pos group where no nitrate was produced at all, the percentages of NOBs were 0.1%, 0.2%, and 15.2% in Cycle AMT3, AMT6, and AMT14 respectively. However, the percentage of Nitrospira was consistently at 0.0% in the three cycles. It was quite possible that the kinetics of nitrite oxidation was catalyzed by Nitrospira and Nitrospira had the potential to serve as an indicator responding to the load of TCE. Compared to Nitrobacter who is a relatively fast-growing r-strategist with low affinities to nitrite and oxygen and thus requires adequate nitrite and oxygen resources, Nitrospira is K-strategist that is well adapted to nitrite and oxygen limited environments like the batch systems in this study, but with a lower maximum specific growth rate (Schramm et al., 1999; Kim and Kim, 2006; Nogueira and Melo, 2006). The genus Nitrosomonas was found to be adaptive to various conditions like temperature, DO, and ammonium load (Shen et al., 2014) and the abundance of Nitrosomonas in our pos group was also stable although DO and ammonium concentration varied on a weekly basis. So it was quite possible that high concentrations of TCE may have changed the community of ammonium and nitrite oxidizers by affecting the oxidation capability of ammonium oxidizers (Nitrosomonas), and thus favor nitrite oxidizers in genus Nitrospira which would otherwise inhibited by ammonium oxidizers.

The non-metric multidimensional scaling (NMDS) of the sequences for genus Nitrosomonas, Nitrosospira, Nitrobacter and Nitrospira was shown in Figure 5.4. It was clear in the plot that all data from pos group and data of exp and sac groups in Cycle
AMT14 clustered separately. The exp and sac group in AMT3 and AMT6 were present close to the pos group. That indicated a community shift in AMT14 of exp and sac groups. The two samples in ATU-inhibited group were present far away from the two clusters. The sequencing data were classified at species level, it was inferred that the community shifted after recovering from moderate deactivation.

The nitrifying bacterial community can shift during a long-term process mainly due to the limitation of ammonium, and this shift has been documented in several publications (Schramm et al., 1999; Ruiz et al., 2003; Hirayama et al., 2005). The study here, however, might be the first to report a potential nitrifying bacteria community shift simulated by a certain concentration of TCE. To further investigate the ammonium and nitrite oxidation processes, metagenomics can be performed to reveal novel genes involved in these processes.

5.4 CONCLUSIONS

This study evaluated the relationships between ammonium and TCE degradation kinetics and the shifts of microbial community structure in response to varying levels of ammonium and TCE. Similar to our previous studies, the shifts of ammonium oxidation products from nitrite to nitrate happened with a modest decline in the kinetics. The kinetics recovered after ammonium oxidizers and nitrite oxidizers reached a balanced status. The 16S rRNA sequencing results suggest a microbial community change with the addition of elevated concentration of TCE. The relative abundance of previous dominant genera Nitrosomonas decreased at higher TCE concentrations while the abundance of
nitrite oxidizers *Nitrospira* increased significantly due to the weakened competence for oxygen from *Nitrosomonas*.

### 5.5 REFERENCES


Figure 5.1 Degradation of TCE (A) and NH$_4^+$-N (B); error bars represent one standard deviation in triplicate reactors. The explanations for labels can be found in Table 5.1 as well as in the method parts.
Figure 5.2 Production of NO$_2$$^-$-N and NO$_3$$^-$-N, and degradation of TCE and NH$_4^+$-N for live experimental groups (Exp) and live sacrificial groups (Sac); error bars represent one standard deviation in triplicate reactors.
Figure 5.3 The percentages of reads that belong to each genus in total ammonia oxidizers (*Nitrosomonas* and *Nitrosospira*) and nitrite oxidizers (*Nitrobacter* and *Nitrospira*) in experimental (A), sacrificial (B), and positive controls (C) before and after the community shift in Cycle 3, Cycle 6 and Cycle 14.
Figure 5.4 NMDS plots regarding to ammonium oxidizers (*Nitrosomonas* and *Nitrosospira*) and nitrite oxidizers (*Nitrobacter* and *Nitrospira*). Explanation for labels can be found in Table 5.1.
Table 5.1 Labels of sequencing analysis (in duplicates). p, n, e, and s stands for different treatment groups. d (or c, not shown) stands for DNA or cDNA. The numbers behind d (or c) stands for the time when the DNA/RNA were sampled.

<table>
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<tr>
<th>Sampling Cycle Number</th>
<th>Cycle 1</th>
<th>Cycle 3</th>
<th>Cycle 6</th>
<th>Cycle 9</th>
<th>Cycle 12</th>
<th>Cycle 14</th>
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<td>Treatment Groups</td>
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<td></td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>Positive Controls (Pos)</td>
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<td>p1d2,p2d2</td>
<td>p1d3,p2d3</td>
<td>p1d4,p2d4</td>
<td>p1d5,p2d5</td>
<td>p1d6,p2d6</td>
</tr>
<tr>
<td>Negative Controls (Neg)</td>
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<td>n1d2,n2d2</td>
<td>n1d3,n2d3</td>
<td>n1d4,n2d4</td>
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<td>e1d4,e2d4</td>
<td>e1d5,e2d5</td>
<td>e1d6,e2d6</td>
</tr>
<tr>
<td>Live Sacrificial Samples (Sac)</td>
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<td>s1d2,s2d2</td>
<td>s1d3,s2d3</td>
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<td>s1d5,s2d5</td>
<td>s1d6,s2d6</td>
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Table 5.2 Average parameters in triplicate microcosms of live experimental (Exp), live sacrificial (Sac) and positive samples (Pos) for cycles: initial concentrations of TCE ([TCE]₀, µg/L); steady state cell biomass concentration (X, mmol/L); initial pseudo first-order rate constants of NH₄⁻-N removal (kᵦobs-NH₄, d⁻¹) and TCE degradation (kᵦobs-TCE, d⁻¹); biomass-normalized NH₄⁻-N removal rate constants (kᵦ₁-NH₄, L mmol⁻¹ d⁻¹) in 2 enrichment cycles (AME1-2) and 14 TCE cycles (AMT1-14); and biomass-normalized TCE degradation rate constants (kᵦ₁-TCE, L mmol⁻¹ d⁻¹) in 14 TCE cycles (AMT 1-14).

<table>
<thead>
<tr>
<th>Cycle</th>
<th>[TCE]₀</th>
<th>kᵦobs-NH₄</th>
<th>kᵦobs-TCE</th>
<th>X</th>
<th>kᵦ₁-NH₄</th>
<th>kᵦ₁-TCE</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Exp</td>
<td>Sac</td>
<td>Pos</td>
<td>Exp</td>
<td>Sac</td>
</tr>
<tr>
<td>AME1</td>
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<td>0.501</td>
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<td>0.166</td>
<td>0.475</td>
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APPENDIX A  ANALYSIS AND CALCULATIONS

A.1 ADDITIONAL INFORMATION ON EXPERIMENT SETUP

A.1.1 Growth Media

The following growth medium was prepared for the enrichment of ammonium-oxidizing microbes in microcosms [modified after (Powell et al., 2011)]. The ammonium-oxidizing microorganisms that are naturally associated with Carex comosa roots were enriched with media containing 100 mg MgSO$_4$, 50 mg KH$_2$PO$_4$, 445 mg Na$_2$HPO$_4$•12H$_2$O, 30 mg KCl, 50 mg CaCl$_2$•2H$_2$O, 3 mg FeSO$_4$•7H$_2$O, 0.02 mg MnCl$_2$, 0.7 mg ZnSO$_4$•H$_2$O, 0.02 mg H$_3$BO$_4$, 0.01 mg CuCl$_2$, 0.03 mg Na$_2$MoO$_4$•2H$_2$O, 0.02 mg NiCl$_2$•6H$_2$O, 0.1 mg CoCl$_2$•6H$_2$O dissolved in de-ionized water to a final volume of 1 L (final pH 8.7). Microcosms were amended with 20 or 30 mg L$^{-1}$NH$_4$-N (as 94 or 141 mg L$^{-1}$ ammonium sulfate, respectively). The growth media were amended with 400 mgL$^{-1}$ of NaHCO$_3$ (with 20 mg L$^{-1}$ NH$_4$-N) or 600 mg L$^{-1}$ NaHCO$_3$ (with 30 mg L$^{-1}$NH$_4$-N).

A.1.2 Chemical Analysis

Following analyses were performed to determine the activity of ammonium-oxidizing microorganisms naturally associated with fresh plant roots in the batch microcosms. Aqueous concentrations of NH$_4^+$, nitrite (NO$_2^-$), nitrate (NO$_3^-$), and pH were measured frequently in each cycle. Aqueous samples were processed through 13
mm dia. syringe filters with 0.22 µm pore size (Xpertek, Cobert Associates, St. Louis, MO) before analysis. NO$_2^-$ and NO$_3^-$ were quantified by ion chromatography (DX2500, Dionex Corporation, Sunnyvale, CA), and NH$_4^+$ was quantified by a colorimetric phenate method (Greenberg et al., 1992) using a spectrophotometer (Lambda 45 UV/Vis, Perkin Elmer; Waltham, MA). The pH measurements were made using an AP10 meter (Denver Instrument, Bohemia, NY) by collecting ~2.1 mL aqueous samples from each microcosm, after gas sampling. Headspace samples from the microcosms were analyzed daily as described previously (Powell, 2010; Powell et al., 2011). TCE was analyzed by a HP 6890 Series gas chromatograph with electron capture detector (ECD) connected to a capillary column (HP-624, 30 m x 0.32 mm; Agilent Technologies), while O$_2$ and CO$_2$ were analyzed by a HP 5890 GC system with a thermal conductivity detector (TCD) connected to a packed column (Shin Carbon 100/120, 2 m x1 mm; Restek, Bellefonte, PA).

A.2 ESTIMATING BIOMASS OF AMMONIA OXIDIZING MICROORGANISMS FOR ENRICHMENT AND TCE CYCLES USING CELL GROWTH EQUATIONS [MODIFIED AFTER (Sawyer et al., 2003)]

Ammonium is the electron donor,

$$\frac{1}{8}NH_4^+ + \frac{3}{8}H_2O = \frac{1}{8}NO_3^- + \frac{5}{4}H^+ + e^-$$  \hspace{1cm} (A1)

while oxygen is the electron acceptor.

$$\frac{1}{4}O_2 + H^+ + e^- = \frac{1}{2}H_2O$$  \hspace{1cm} (A2)
Adding equations A1 and A2 together and multiplying by eight to normalize for ammonium provides the energy equation for the cell:

$$NH_4^+ + 2O_2 = NO_3^- + 2H^+ + H_2O$$  \hspace{1cm} (A3)

Likewise, the formula for cell synthesis using ammonium as the nitrogen source is,

$$\frac{1}{5}CO_2 + \frac{1}{20}HCO_3^- + \frac{1}{20}NH_4^+ + H^+ + e^- = \frac{1}{20}C_5H_7O_2N + \frac{9}{20}H_2O$$  \hspace{1cm} (A4)

Combining equations A4 and A1 (the electron donor equation) will then provide the growth equation.

$$\frac{7}{40}NH_4^+ + \frac{1}{5}CO_2 + \frac{1}{20}HCO_3^- = \frac{1}{20}C_5H_7O_2N + \frac{3}{8}NO_3^- + \frac{3}{40}H_2O + \frac{1}{4}H^+$$  \hspace{1cm} (A5)

The equation is multiplied by $\frac{40}{7}$ to normalize with respect to ammonium to obtain the final cell synthesis equation,

$$NH_4^+ + \frac{8}{7}CO_2 + \frac{2}{7}HCO_3^- = \frac{2}{7}C_5H_7O_2N + \frac{5}{7}NO_3^- + \frac{3}{7}H_2O + \frac{10}{7}H^+$$  \hspace{1cm} (A6)

The assumption here is that all electron equivalents for the cell come from ammonium conversion to nitrate. Thus, there is a fraction of electrons that go to cell synthesis (equation A6) and a fraction that go to cell energy (equation A3). To account for these fractions, $f_s$ is used to delineate the fraction to synthesis and $f_e$ is used for cell energy. The sum of $f_s$ and $f_e$ is therefore one. The cell energy equation is multiplied by $f_e$ because the fraction of ammonium used for energy is $f_e$,

$$f_eNH_4^+ + 2f_eO_2 = f_eNO_3^- + 2f_eH^+ + f_eH_2O$$  \hspace{1cm} (A7)

And the synthesis equation is multiplied by $f_s$ because the fraction of ammonium used for synthesis is $f_s$. 

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Combining equations A7 and A8 provides the overall cell growth equation below:

\[
N\text{H}_4^+ + 2f_eO_2 + \frac{8}{7}f_sCO_2 + \frac{2}{7}f_sHCO_3^- = (f_e + \frac{5}{7}f_s)NO_3^- + (2f_e + \frac{10}{7}f_s)H^+ + (f_e + \frac{3}{7}f_s)H_2O + \frac{2}{7}f_sC_5H_7O_2N
\]  

(A9)

### A.3 ADDITIONAL INFORMATION ON DATA TREATMENT

The measurements of NO\textsubscript{2}\textsuperscript{-} and NO\textsubscript{3}\textsuperscript{-} in the reactors were converted to molar units, combined together, and are referred to henceforth as NO\textsubscript{x}. NO\textsubscript{x} and NH\textsubscript{4}\textsuperscript{+} masses were summed for aqueous phase N mass-balance, and referred to as dissolved inorganic nitrogen (DIN). The initial pseudo first-order degradation rate constants for ammonium and individual HAHS, \( k_{1,NH4} \) and \( k_{1-HAH} \) respectively, were determined for each cycle based on an exponential fit to the first few values of NH\textsubscript{4}\textsuperscript{+} or the HAHS concentration over time in each cycle (Jenal-Wanner and McCarty, 1997). Transformation yield (\( T_y \)) was calculated for the HAHS cycles by dividing HAHS mass degraded by ammonium mass oxidized (Table B.2.2 for example). Further, the initial (zero-order) rate of HAHS removal and consumption/production of solutes (i.e., DO, Total Inorganic Carbon or TIC (Powell et al., 2011), NH\textsubscript{4}\textsuperscript{+}, and NO\textsubscript{x}) were calculated using the difference between their respective mass on day 2 subtracted from their mass on day 1 for each treatment then divided by the time elapsed between the two analyses (Table B.2.1 for example). The initial rates were taken between day 1 and day 2, rather than day 0 to day 1, to avoid perturbations caused by microcosm reset for each cycle.
A balanced equation for bacterial growth and energy with ammonium as nitrogen and energy source and CO₂ as carbon source (equation A9) can be derived by adding together the equation for oxidation of ammonium to nitrate (equation A7) multiplied by \( f_e \), and the equation for bacterial growth (equation S8) multiplied by \( f_s \), where \( f_s \) is the fraction of electron equivalents derived from ammonium oxidation used for cellular (biomass) synthesis, \( f_e \) is the fraction of electron equivalents used for cellular energy, and the sum of \( f_s + f_e = 1 \) (Sawyer et al., 2003).

Equations A7, A8 and A9 (in section III above) were used to estimate dry biomass for each cycle of TCE degradation experiments. The relationship between ammonium and oxygen removal in equation 6 can be used to calculate \( f_e \):

\[
f_e = \frac{1}{2} \frac{\Delta O_2}{\Delta NH_4^+} \tag{A10}
\]

Not all oxygen use in the microcosms was due to ammonium oxidation, because no effort was taken to control heterotrophic organisms that could also be growing on the roots. In order to correct for oxygen and ammonium uptake by heterotrophs, the \( \Delta O_2 \) and \( \Delta NH_4^+ \) values in equation A9 were calculated as the difference between control and experimental microcosms. Oxygen values were not limiting in either set of microcosms, so it is unlikely that there would be a significant difference in heterotrophic populations between them. The only difference between live and control microcosms was ATU amendment to control reactors, which specifically inhibits the ammonium monooxygenase enzyme.

Using the result of equation A10, \( f_s \) could then be calculated as the difference between 1 and \( f_e \). From equation A9, a relationship between \( f_s \), total ammonium removal, biomass synthesized, and biomass yield, \( Y \), (mole biomass / mole NH₄⁺) was derived:
\begin{equation}
Yield (Y) = \frac{\Delta \text{Biomass}}{\Delta \text{NH}_4^+} = \frac{2}{7} f_s
\end{equation}

Equation A11 above proved useful to calculate both yield (Y) and \( \Delta \) biomass (moles) from the known quantity of ammonium removal. The yield, \( Y \), could then be used to estimate active steady-state biomass concentration (\( X \)) of the ammonium oxidizer that formed during the cycle as follows:

\begin{equation}
X \left( \frac{\text{mol}}{L} \right) = \frac{Y \Delta \text{NH}_4^+}{V t b}
\end{equation}

where \( V \) is the microcosm volume (L), \( t \) is the total cycle time (d), and \( b \) is the endogenous decay coefficient, taken to be 0.19 d\(^{-1}\) (Melcer, 2003); this value of \( b \) was determined for nitrifying sequencing batch reactors at 22 °C in which the fill-and-draw nature of the reactor approximates the ‘experimental cycles’ of these experiments. Finally, the biomass concentration, \( X \) (mol/L), was used to normalize the pseudo first-order rate constants with respect to biomass, which was done by dividing \( k_{1,TCE} \) and \( k_{1,NH_4} \) by \( X \).

A.5 REFERENCES


Figure B.2.1 Negative image of agarose gel showing PCR results of \textit{amoA} on samples from experimental and control microcosms. Different PCR cycle counts were used to determine relative concentrations of the \textit{amoA} gene in the gene extracts. The DNA size standard on the left is in 100 base pair increments. Minor image alterations were performed to make the figure more organized and care was taken to ensure that the relative band intensities were not affected.
Figure B.2.2 Changes in $\text{NH}_4^+$ in enrichment cycles described in Chapter 2. Solid squares represent average $[\text{NH}_4^+-\text{N}]$ in live microcosms while open squares represent average $[\text{NH}_4^+]$ for control microcosm.
Figure B.2.3 Changes in NO\textsubscript{x} (i.e., NO\textsubscript{2} + NO\textsubscript{3}), Dissolved Inorganic Nitrogen or DIN (i.e., NH\textsubscript{4} + NO\textsubscript{x}) in enrichment cycles described in Chapter 2. Open diamonds represent average [NO\textsubscript{x}] while solid circles represent average DIN, both in live microcosm. Values for corresponding control microcosms not shown.
Figure B.2.4 Changes in Dissolved Inorganic Oxygen (DO) in enrichment cycles described in Chapter 2. Solid diamonds represent average DO in live microcosms while open diamonds represent average DO in control microcosm.
Figure B.2.5 Changes in pH during enrichment and TCE cycles in Chapter 2. Solid circles represent average pH in live microcosms while open circles represent average pH in control microcosms.
Figure B.2.6 Changes in Total Inorganic Carbon (TIC) in enrichment and TCE cycles in Chapter 2. Solid triangles represent average TIC in live microcosms while open triangles represent average TIC in control microcosms.
Figure B.3.1 Degradation of total oxygen (TO) and production of N-NO$_x$ in the cometabolic system with TCE (Chapter 3); error bars represent one standard deviation in triplicate reactors. Initial TO was around 12 mg. [N-NO$_x$] equals the sum of [N-NO$_2$] and [N-NO$_3$]. Live experimental measurements were shown in dark color. Decline of both TO consumption and NO$_x$ production was observed in cycle 5. After recovery, both oxygen consumption and NO$_x$ accumulation increased significantly.
Figure B.3.2 Degradation of dissolved oxygen (DO) and production of N-NO₃ in the cometabolic system with cis-DCE; error bars represent one standard deviation in triplicate reactors. Initial DO was around 8 mg/L. [N-NO₃] equals the sum of [N-NO₂] and [N-NO₃]. Live experimental measurements were shown in dark color. Gradual decline of both DO consumption and NO₃ production was observed.
Figure B.3.3 Degradation of total oxygen (TO) and production of N-NO$_x$ in the cometabolic system with CF; error bars represent one standard deviation in triplicate reactors. Initial TO was around 12 mg. [N-NO$_x$] equals the sum of [N-NO$_2$] and [N-NO$_3$]. Live experimental measurements were shown in dark color.
(A) $y = -0.0248x + 2.0505$
$R^2 = 0.7621$

(B) $y = -0.001x + 0.31$
$R^2 = 0.05834$

(C) $y = -0.0051x + 0.5702$
$R^2 = 0.8302$
Figure B.3.4 Scatter plot of TCE (Chapter 3) Transformation Yield ($T_y$) vs. initial NH$_4^+$ concentrations in individual microcosms, under different initial TCE concentration (µg/L) (A-E). Transformation Yield ($T_y$) is defined as the quotient of net TCE degraded (µmoles) divided by net NH$_4^+$ consumed (mmoles).
(A) 220 ug/L cis-DCE

\[ y = 0.0163x + 1.3698 \]
\[ R^2 = 0.0026 \]

(B) 190 ug/L cis-DCE

\[ y = 0.1132x - 6.0196 \]
\[ R^2 = 0.284 \]

(C) 150 ug/L cis-DCE

\[ y = -0.0468x + 4.4154 \]
\[ R^2 = 0.0405 \]
Figure B.3.5 Scatter plot of cis-DCE Transformation Yield ($T_y$) vs. initial NH$_4^+$-N concentrations in individual microcosms, under different initial cis-DCE concentration (µg/L) (A-F). Transformation Yield ($T_y$) is defined as the quotient of net cis-DCE degraded (µmoles) divided by net NH$_4^+$ consumed (mmoles).
(A) $y = -0.0159x + 1.6742$  
$R^2 = 0.2537$

(B) $y = 0.0241x - 1.0901$  
$R^2 = 0.704$

(C) $y = -0.0025x + 0.5194$  
$R^2 = 0.0927$

(D) $T_y$ (umol CF/mmol NH$_4^+$)

Initial [N-NH$_4^+$] (mg L$^{-1}$)

- 200 ug/L CF
- 150 ug/L CF
- 100 ug/L CF
Figure B.3.6. Scatter plot of CF Transformation Yield ($T_y$) vs. initial NH$_4^+$-N concentrations in individual microcosms, under different initial CF concentration (µg/L) (A-DF). Transformation Yield ($T_y$) is defined as the quotient of net CF degraded (µmoles) divided by net NH$_4^+$ consumed (mmoles).
Figure B.4.1 Degradation of dissolved oxygen (DO) and production of N-NO₃ in the cometabolic system with 1,1,2-TCA; error bars represent one standard deviation in triplicate reactors. Initial DO was around 8 mg L⁻¹. [N-NO₃] equals the sum of [N-NO₂] and [N-NO₃]. Live experimental measurements were shown in dark color.
Figure B.4.2 Degradation of dissolved oxygen (DO) and production of N-NOₓ in the cometabolic system with EDB; error bars represent one standard deviation in triplicate reactors. Initial DO was around 8 mg L⁻¹. [N-NOₓ] equals the sum of [N-NO₂] and [N-NO₃]. Live experimental measurements were shown in dark color.
Figure B.4.3 Degradation of dissolved oxygen (DO) and production of N-NO\(_x\) in the cometabolic system with 1,2-DCA; error bars represent one standard deviation in triplicate reactors. Initial DO was around 8 mg L\(^{-1}\). [N-NO\(_x\)] equals the sum of [N-NO\(_2\)] and [N-NO\(_3\)]. Live experimental measurements were shown in dark color.
Figure B.4.4 Scatter plot of 1,1,2-TCA Transformation Yield ($T_y$) vs. initial NH$_4^+$-N concentrations in individual microcosms, under different initial 1,1,2-TCA concentration (µg/L) (A-B). Transformation Yield ($T_y$) is defined as the quotient of net 1,1,2-TCA degraded (µmoles) divided by net NH$_4^+$ consumed (mmoles).
Figure B.4.5 Scatter plot of EDB Transformation Yield \( (T_y) \) vs. initial \( \text{NH}_4^+ \)-N concentrations in individual microcosms, under different initial EDB concentration (µg/L) (A-C). Transformation Yield \( (T_y) \) is defined as the quotient of net EDB degraded (µmoles) divided by net \( \text{NH}_4^+ \) consumed (mmoles).
Figure B.5.1 Degradation of total oxygen (TO) and production of NO\textsubscript{x}−-N; error bars represent one standard deviation in triplicate reactors. The explanations for labels can be found in Table 5.1 as well as in the method parts. NO\textsubscript{x}−-N refers to the combination of NO\textsubscript{2}−-N and NO\textsubscript{3}−-N.
Table B.2.1 Chapter 2: Initial (zero-order) rates of TCE degradation/ NH$_4^+$ removal/ O$_2$ consumption/ NO$_x$ production in control and live microcosms through the cycles (mean ± SD, n = 3). The initial rate is calculated between day 1 and day 2 (~24 hr period) for all TCE cycles. For enrichment cycles, the initial rate was calculated from the first sampling point.

<table>
<thead>
<tr>
<th>Cycle</th>
<th>Initial [TCE] (µg L$^{-1}$)</th>
<th>TCE degradation rate (nmol d$^{-1}$)</th>
<th>Initial [NH$_4^+$] (mg L$^{-1}$)</th>
<th>NH$_4^+$ removal rate (µmol d$^{-1}$)</th>
<th>O$_2$ consumption rate (µmol d$^{-1}$)</th>
<th>NO$_x$ production rate (µmol d$^{-1}$)</th>
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<tbody>
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<td>Live</td>
<td>Control</td>
<td>Live</td>
<td>Control</td>
<td>Live</td>
</tr>
<tr>
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<td>0</td>
<td>NA</td>
<td>NA</td>
<td>18.84</td>
<td>0.21 ± 0.0</td>
<td>14.9 ± 3.1</td>
</tr>
<tr>
<td>AME 2</td>
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<td>NA</td>
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<td>NA</td>
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<td>0.60 ± 0.0</td>
<td>50.6 ± 11</td>
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<td>27.35</td>
<td>3.23 ± 2.1</td>
<td>1.23 ± 1.8</td>
<td>19.59</td>
<td>3.40 ± 0.0</td>
<td>11.9 ± 5.6</td>
</tr>
<tr>
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<td>5.37 ± 0.7</td>
<td>1.35 ± 1.0</td>
<td>18.67</td>
<td>4.82 ± 0.0</td>
<td>35.6 ± 20</td>
</tr>
<tr>
<td>AMT 3</td>
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<td>3.16 ± 0.5</td>
<td>0.77 ± 4.2</td>
<td>30.35</td>
<td>4.35 ± 0.0</td>
<td>19.7 ± 4.7</td>
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<tr>
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<td>30.38</td>
<td>4.95 ± 0.0</td>
<td>53.7 ± 9.7</td>
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<tr>
<td>AMT 5</td>
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<td>3.55 ± 1.1</td>
<td>-0.87 ± 1.3</td>
<td>29.06</td>
<td>6.58 ± 0.0</td>
<td>35.6 ± 20</td>
</tr>
<tr>
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<td>5.07 ± 0.0</td>
<td>42.9 ± 15</td>
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<td>2.83 ± 0.0</td>
<td>30.6 ± 1.7</td>
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<tr>
<td>AMT 8</td>
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<td>AMT 11</td>
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<td>0.64±0.0</td>
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Table B.2.2 Chapter 2: Variations in initial ammonium and TCE concentrations and transformation yields ($T_y$) in live microcosms.

<table>
<thead>
<tr>
<th>Cycles</th>
<th>Live - [NH$_4^+$-N]$_0$ (mmoles)</th>
<th>Live - [NH$_4^+$-N]$_0$ (mg L$^{-1}$)</th>
<th>Live - [TCE]$_0$ (µmoles)</th>
<th>Live - [TCE]$_0$ (µg L$^{-1}$)</th>
<th>Transformation Yield, $T_y$ [Net ΔTCE (µmoles)/Net ΔNH$_4^+$ (mmoles)]</th>
<th>Mean $T_y$</th>
</tr>
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<tbody>
<tr>
<td>AME 1</td>
<td>0.100</td>
<td>18.84</td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>AME 2</td>
<td>0.100</td>
<td>18.88</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AME 3</td>
<td>0.100</td>
<td>18.07</td>
<td></td>
<td></td>
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</tr>
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<tr>
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<td>0.015</td>
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<td>0.105</td>
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Table B.3.1 Chapter 3: Initial (zero-order) rates of TCE degradation/ NH$_4^+$ removal/ O$_2$ consumption/ NO$_x$ production in control and live microcosms through the cycles (mean ± SD, n = 3). The initial rate is calculated between day 1 and day 2 (~24 hr period) for all TCE cycles. For enrichment cycles, the initial rate was calculated from the first sampling point.

<table>
<thead>
<tr>
<th>Cycle</th>
<th>Initial [TCE] (µg/L)</th>
<th>TCE degradation rate (nmol d$^{-1}$)</th>
<th>Initial [NH$_4^+$] (mg/L)</th>
<th>NH$_4^+$ removal rate (µmol d$^{-1}$)</th>
<th>O$_2$ consumption rate (µmol d$^{-1}$)</th>
<th>NO$_x$ production rate (µmol d$^{-1}$)</th>
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<td>AME1</td>
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<td>NA</td>
<td>59.95</td>
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<tr>
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<td>NA</td>
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<tr>
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<td>1.37 ± 0.8</td>
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<td>3.74 ± 3.6</td>
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<td>0.88 ± 0.4</td>
<td>80.15</td>
<td>22.9 ± 1.3</td>
<td>-0.82 ± 2.2</td>
</tr>
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<td>2.44 ± 0.6</td>
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<td>61.7 ± 20.5</td>
<td>11.0 ± 5.1</td>
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<td>24.9 ± 6.5</td>
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<td>74.6 ± 20.1</td>
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<td>107.31</td>
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<td>5.19 ± 1.7</td>
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<td>90.8 ± 21.4</td>
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<tr>
<td>AMT</td>
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<td>Value 2</td>
<td>Value 3</td>
<td>Value 4</td>
<td>Value 5</td>
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<td>60.4 ± 26.4</td>
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<td>39.3 ± 11.8</td>
<td>5.00 ± 5.8</td>
<td>64.82</td>
<td>120.9 ± 24.1</td>
<td>17.2 ± 8.6</td>
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<td>AMT14</td>
<td>152.4</td>
<td>13.3 ± 1.3</td>
<td>1.10 ± 0.8</td>
<td>70.6</td>
<td>44.9 ± 2.8</td>
<td>16.4 ± 11.4</td>
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<td>4.83 ± 1.7</td>
<td>1.24 ± 1.8</td>
<td>85.74</td>
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<td>64.8 ± 47.1</td>
<td>-2.82 ± 4.7</td>
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Table B.3.2 Initial (zero-order) rates of cis-DCE degradation/ NH₄⁺ removal/ O₂ consumption/ NOₓ production in control and live microcosms through the cycles (mean ± SD, n = 3). The initial rate is calculated between day 1 and day 2 (~24 hr period) for all cis-DCE cycles. For enrichment cycles, the initial rate was calculated from the first sampling point.

<table>
<thead>
<tr>
<th>Cycle</th>
<th>Initial [DCE] (µg/L)</th>
<th>DCE degradation rate (nmol d⁻¹)</th>
<th>Initial [NH₄⁺] (mg/L)</th>
<th>NH₄⁺ removal rate (µmol d⁻¹)</th>
<th>O₂ consumption rate (µmol d⁻¹)</th>
<th>NOₓ production rate (µmol d⁻¹)</th>
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<tr>
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<td>Control</td>
<td>Live</td>
<td>Control</td>
<td>Live</td>
<td>Control</td>
</tr>
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<td>AME1</td>
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<td>NA</td>
<td>68.00</td>
<td>71.9 ± 23.2</td>
<td>-10.4 ± 3.4</td>
<td>76.2 ± 5.7</td>
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<td>AMD1</td>
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<td>3.83 ± 1.3</td>
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<td>64.62</td>
<td>14.5 ± 1.0</td>
<td>2.11 ± 3.1</td>
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<td>18.6 ± 3.3</td>
<td>3.44 ± 2.6</td>
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<tr>
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195
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<td>3.04 ± 1.6</td>
<td>2.71 ± 1.7</td>
<td>4.09 ± 1.5</td>
<td>0.55 ± 0.6</td>
<td>2.03 ± 5.2</td>
<td>0.0 ± 0.0</td>
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Table B.3.3 Initial (zero-order) rates of CF degradation/ NH$_4^+$ removal/ O$_2$ consumption/ NO$_x$ production in control and live microcosms through the cycles (mean ± SD, n = 3). The initial rate is calculated between day 1 and day 2 (~24 hr period) for all CF cycles.

<table>
<thead>
<tr>
<th>Cycle</th>
<th>Initial [TCE] (µg/L)</th>
<th>CF degradation rate (nmol d$^{-1}$)</th>
<th>Initial [NH$_4^+$] (mg/L)</th>
<th>NH$_4^+$ removal rate (µmol d$^{-1}$)</th>
<th>O$_2$ consumption rate (µmol d$^{-1}$)</th>
<th>NO$_x$ production rate (µmol d$^{-1}$)</th>
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</thead>
<tbody>
<tr>
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<td>Control</td>
<td>Live</td>
<td>Control</td>
<td>Live</td>
<td>Control</td>
</tr>
<tr>
<td>AME1</td>
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<td>NA</td>
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<td>9.66 ± 3.1</td>
<td>1.21 ± 6.2</td>
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<td>AME2</td>
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<td>NA</td>
<td>NA</td>
<td>68.05</td>
<td>13.1 ± 17.4</td>
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<td>20.8 ± 6.7</td>
</tr>
<tr>
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<td>20.3 ± 11.5</td>
<td>7.69 ± 1.4</td>
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<td>-3.28 ± 2.7</td>
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<td>-2.82 ± 4.7</td>
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Table B.3.4 Kinetics parameters compared to other’s works

<table>
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<tr>
<th>Compound/strain or culture</th>
<th>Growth Substrate</th>
<th>Temperature (°C)</th>
<th>Initial concentration (μg/L)</th>
<th>$k_1$ (L mg$^{-1}$ d$^{-1}$)</th>
<th>$T_y$ (μmol / mmol)</th>
<th>Reference</th>
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<tr>
<td>TCE</td>
<td>Mix culture, batch</td>
<td>Ammonium</td>
<td>22</td>
<td>40-60</td>
<td>0.004 ± 0.001</td>
<td>0.091 ± 0.020</td>
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<td>30-70</td>
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<td>Mix culture, batch</td>
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<td>25</td>
<td>40</td>
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<td>3.531</td>
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<td>0.53</td>
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cDCE
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<td>600-30000</td>
<td>1.9</td>
<td>r,u</td>
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<td>130-15000</td>
<td>1.0</td>
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</tr>
<tr>
<td>Mixed culture, chemostat</td>
<td>Methane</td>
<td>21</td>
<td>22000</td>
<td>0.56</td>
<td>w</td>
</tr>
<tr>
<td>Rhodococcus sp. PB1</td>
<td>Propane</td>
<td>30</td>
<td>60000</td>
<td>0.09</td>
<td>21.74</td>
</tr>
<tr>
<td>Rhodococcus aetherovorans BCP1</td>
<td>Butane</td>
<td>30</td>
<td>3580-14200</td>
<td>0.026</td>
<td>v</td>
</tr>
<tr>
<td>Mixed culture, biofilm</td>
<td>Phenol</td>
<td>23</td>
<td>1200</td>
<td>0.0031</td>
<td>e</td>
</tr>
</tbody>
</table>

References:
a (Kocamemi and Cecen, 2010); b (Ely et al., 1995); c (Ely et al., 1997); d (Tsien et al., 1989); e (Segar, 1994); f (Aziz et al., 1999); g (Henry and Grbicgalic, 1990); h (Leeson and Bouwer, 1989); i (Strand et al., 1991); j (Anderson and McCarthy, 1996); k (Anderson and McCarty, 1997); l (Chang and Criddle, 1997); m (Arvin, 1991); n (Chang and Alvarez-Cohen, 1995); o (Wilcox et al., 1995); p (Dabrock et al., 1992); q (Jenal-Wanner and McCarty, 1997); r (Chang and Alvarez-Cohen, 1996); s (Heald and Jenkins, 1994); t (Arcangeli and Arvin, 1997); u (Van Hylckama Vlieg et al., 1996); v (Frascari et al., 2008); w (Alvarez-Cohen and McCarthy, 1991); x (Frascari et al., 2005); y (Qin et al., 2014), also results in Chapter 2.
Table B.4.1 Initial (zero-order) rates of 1,1,2-TCA degradation/ NH$_4^+$ removal/ O$_2$ consumption/ NO$_x$ production in control and live microcosms through the cycles (mean ± SD, n = 3). The initial rate is calculated between day 1 and day 2 (~24 hr period) for all TCE cycles. For enrichment cycles, the initial rate was calculated from the first sampling point.

<table>
<thead>
<tr>
<th>Cycle</th>
<th>Initial [TCA] (µg L$^{-1}$)</th>
<th>TCA degradation rate (nmol d$^{-1}$)</th>
<th>Initial [NH$_4^+$] (mg L$^{-1}$)</th>
<th>NH$_4^+$ removal rate (µmol d$^{-1}$)</th>
<th>O$_2$ consumption rate (µmol d$^{-1}$)</th>
<th>NO$_x$ production rate (µmol d$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Live</td>
<td>Control</td>
<td>Live</td>
<td>Control</td>
<td>Live</td>
</tr>
<tr>
<td>AME1</td>
<td>0</td>
<td>NA</td>
<td>NA</td>
<td>69.63</td>
<td>14.3 ± 6.9</td>
<td>15.7 ± 8.2</td>
</tr>
<tr>
<td>AME2</td>
<td>0</td>
<td>NA</td>
<td>NA</td>
<td>67.26</td>
<td>46.1 ± 10.4</td>
<td>23.8 ± 27.7</td>
</tr>
<tr>
<td>AMT 1</td>
<td>46.9</td>
<td>0.13 ± 1.7</td>
<td>-0.33 ± 1.9</td>
<td>67.14</td>
<td>40.5 ± 5.7</td>
<td>11.9 ± 5.7</td>
</tr>
<tr>
<td>AMT 2</td>
<td>42.4</td>
<td>2.41 ± 1.7</td>
<td>0.54 ± 0.2</td>
<td>65.15</td>
<td>36.0 ± 16.0</td>
<td>9.42 ± 8.7</td>
</tr>
<tr>
<td>AMT 3</td>
<td>37.8</td>
<td>3.96 ± 4.1</td>
<td>-2.15 ± 0.9</td>
<td>67.04</td>
<td>34.8 ± 26.8</td>
<td>-6.24 ± 10.2</td>
</tr>
<tr>
<td>AMT 4</td>
<td>40.4</td>
<td>11.8 ± 4.0</td>
<td>2.74 ± 2.5</td>
<td>60.54</td>
<td>39.0 ± 35.3</td>
<td>7.11 ± 5.6</td>
</tr>
<tr>
<td>AMT 5</td>
<td>45.1</td>
<td>14.0 ± 4.4</td>
<td>-0.42 ± 1.6</td>
<td>64.45</td>
<td>45.7 ± 9.1</td>
<td>6.88 ± 4.1</td>
</tr>
<tr>
<td>AMT 6</td>
<td>89.5</td>
<td>3.85 ± 4.6</td>
<td>-1.12 ± 2.2</td>
<td>67.2</td>
<td>41.8 ± 13.2</td>
<td>1.10 ± 1.9</td>
</tr>
<tr>
<td>AMT 7</td>
<td>92.2</td>
<td>0.80 ± 2.0</td>
<td>-0.91 ± 0.4</td>
<td>72.04</td>
<td>8.23 ± 0.7</td>
<td>7.66 ± 6.1</td>
</tr>
<tr>
<td>AMT 8</td>
<td>95.9</td>
<td>0.70 ± 0.1</td>
<td>-0.11 ± 0.2</td>
<td>66.87</td>
<td>1.90 ± 1.4</td>
<td>6.49 ± 2.9</td>
</tr>
<tr>
<td>AMT 9</td>
<td>145.9</td>
<td>-0.77 ± 1.6</td>
<td>-1.12 ± 1.1</td>
<td>74.28</td>
<td>2.48 ± 7.4</td>
<td>2.10 ± 3.0</td>
</tr>
</tbody>
</table>
Table B.4.2 Initial (zero-order) rates of EDB degradation/ NH$_4^+$ removal/ O$_2$ consumption/ NO$_x$ production in control and live microcosms through the cycles (mean ± SD, n = 3). The initial rate is calculated between day 1 and day 2 (~24 hr period) for all EDB cycles. For enrichment cycles, the initial rate was calculated from the first sampling point.

<table>
<thead>
<tr>
<th>Cycle</th>
<th>Initial [EDB] (µg L$^{-1}$)</th>
<th>EDB degradation rate (nmol d$^{-1}$)</th>
<th>Initial [NH$_4^+$] (mg L$^{-1}$)</th>
<th>NH$_4^+$ removal rate (µmol d$^{-1}$)</th>
<th>O$_2$ consumption rate (µmol d$^{-1}$)</th>
<th>NO$_x$ production rate (µmol d$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Live</td>
<td>Control</td>
<td>Live</td>
<td>Control</td>
<td>Live</td>
</tr>
<tr>
<td>AME1</td>
<td>0</td>
<td>NA</td>
<td>NA</td>
<td>71.51</td>
<td>-4.07 ± 7.1</td>
<td>3.36 ± 3.3</td>
</tr>
<tr>
<td>AME2</td>
<td>0</td>
<td>NA</td>
<td>NA</td>
<td>71.15</td>
<td>32.2 ± 4.4</td>
<td>40.4 ± 16.9</td>
</tr>
<tr>
<td>AMT 1</td>
<td>52.4</td>
<td>0.78 ± 1.0</td>
<td>0.25 ± 2.5</td>
<td>84.60</td>
<td>31.6 ± 19.8</td>
<td>1.31 ± 0.5</td>
</tr>
<tr>
<td>AMT 2</td>
<td>54.35</td>
<td>1.10 ± 1.7</td>
<td>0.30 ± 0.1</td>
<td>77.83</td>
<td>32.2 ± 8.8</td>
<td>1.66 ± 4.4</td>
</tr>
<tr>
<td>AMT 3</td>
<td>49.32</td>
<td>4.04 ± 3.9</td>
<td>0.19 ± 0.6</td>
<td>69.51</td>
<td>38.7 ± 27.3</td>
<td>-6.61 ± 5.1</td>
</tr>
<tr>
<td>AMT 4</td>
<td>99.08</td>
<td>7.11 ± 5.6</td>
<td>1.23 ± 0.5</td>
<td>74.77</td>
<td>26.7 ± 17.6</td>
<td>-7.58 ± 13.6</td>
</tr>
<tr>
<td>AMT 5</td>
<td>98.06</td>
<td>1.35 ± 1.1</td>
<td>0.44 ± 0.9</td>
<td>71.41</td>
<td>11.5 ± 21.1</td>
<td>-7.89 ± 13.6</td>
</tr>
<tr>
<td>AMT 6</td>
<td>99.41</td>
<td>4.71 ± 8.5</td>
<td>-0.08 ± 0.8</td>
<td>72.16</td>
<td>20.7 ± 31.5</td>
<td>4.31 ± 6.8</td>
</tr>
<tr>
<td>AMT 7</td>
<td>144.65</td>
<td>2.79 ± 2.7</td>
<td>-1.39 ± 0.6</td>
<td>70.02</td>
<td>2.44 ± 12.1</td>
<td>-7.34 ± 9.9</td>
</tr>
<tr>
<td>AMT 8</td>
<td>155.73</td>
<td>3.27 ± 11.9</td>
<td>0.80 ± 1.7</td>
<td>60.3</td>
<td>4.27 ± 6.8</td>
<td>-5.0 ± 6.8</td>
</tr>
<tr>
<td>AMT 9</td>
<td>140.27</td>
<td>0.66 ± 1.4</td>
<td>-0.11 ± 1.2</td>
<td>65.96</td>
<td>-1.46 ± 3.8</td>
<td>0.65 ± 6.4</td>
</tr>
</tbody>
</table>
Table B.4.3 Initial (zero-order) rates of 1,2-DCA degradation/ \( \text{NH}_4^+ \) removal/ \( \text{O}_2 \) consumption/ \( \text{NO}_x \) production in control and live microcosms through the cycles (mean ± SD, \( n = 3 \)). The initial rate is calculated between day 1 and day 2 (~24 hr period) for all 1,2-DCA cycles.

<table>
<thead>
<tr>
<th>Cycle</th>
<th>Initial [DCA] (( \mu \text{g L}^{-1} ))</th>
<th>DCA degradation rate (nmol d(^{-1}))</th>
<th>Initial [( \text{NH}_4^+ )] (mg L(^{-1}))</th>
<th>( \text{NH}_4^+ ) removal rate (( \mu \text{mol d}^{-1} ))</th>
<th>( \text{O}_2 ) consumption rate (( \mu \text{mol d}^{-1} ))</th>
<th>( \text{NO}_x ) production rate (( \mu \text{mol d}^{-1} ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMT 1</td>
<td>340</td>
<td>126.1 ± 84.3</td>
<td>-6.96 ± 0.5</td>
<td>56.79</td>
<td>13.3 ± 7.8</td>
<td>51.1 ± 9.3</td>
</tr>
<tr>
<td>AMT 2</td>
<td>345.5</td>
<td>52.7 ± 24</td>
<td>15.2 ± 10.5</td>
<td>61.57</td>
<td>29.8 ± 11.2</td>
<td>87.7 ± 25.9</td>
</tr>
<tr>
<td>AMT 3</td>
<td>394.6</td>
<td>64.5 ± 29.3</td>
<td>34.6 ± 13.5</td>
<td>72.21</td>
<td>44.0 ± 23.1</td>
<td>83.2 ± 11.5</td>
</tr>
</tbody>
</table>
APPENDIX B REFERENCES


