Biomineralization of atrazine and analysis of 16S rRNA and catabolic genes of atrazine-degraders in a former pesticide mixing and machinery washing area at a farm site and in a constructed wetland

DISSERTATION

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By

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Abstract

Atrazine is one of the most widely used herbicides in the world. It is primarily used in the production of corn in the United States. Although it may marginally increase crop yields, atrazine is also an endocrine disruptor in non-target organisms. Its moderate solubility in water allows for atrazine to contaminate surface and ground waters far removed from the point of application to soil. Although atrazine can be degraded abiotically, its primary mode of attenuation in natural environments is through bacterial degradation. Full mineralization of atrazine to CO_2, H_2O, Cl^- and NH_4^+ has been demonstrated in *Pseudomonas* ADP, which contains the complete suite of *atz* atrazine catabolic genes.

The overall hypothesis of this study is that the microorganisms and catabolic pathways reported in the literature do not universally account for the atrazine biodegradation observed in different natural environments. Furthermore, it is hypothesized that in situ pre-enrichment methods yield atrazine degraders uncultivable by classical laboratory enrichment, including anaerobic bacteria. The discovery of atrazine catabolic genes other than those in the *atz* pathway and the demonstrated involvement of consortia of bacteria in atrazine biodegradation suggest that the full diversity of environmental atrazine biodegradation has yet to be elucidated.

In order to further elucidate the bacteria and genes responsible for atrazine biodegradation in different environments, locations with different exposures to atrazine were chosen for study. Among them were a farm location that was the site of multiple pesticide spill events throughout its history and a constructed wetland that receives river water containing agricultural runoff. Samples from these sites were assessed for atrazine biomineralization via biometer studies. In addition to traditional environmental sampling, these sites were also sampled with Bio-Sep beads to allow for in situ pre-enrichment for atrazine-degrading microbes. Soil and sediment samples were enriched for atrazine-degraders in liquid media, which included atrazine as the sole source of energy, C and N. Atrazine-degrading mixed cultures were screened via 16S-rDNA-PCR-DGGE, and mixed cultures displaying unique banding patterns were chosen for 16S rDNA clone library formation and sequencing. These isolates were also screened for eight atrazine catabolic genes via PCR.

Atrazine mineralization was demonstrated in the sampling sites, but never exceeded 30% total mineralization of the added [U-ring-^{14}C]-atrazine. Atrazine degrading pure and mixed cultures were successfully retrieved, with *Acidovorax*, *Hydrogenophaga, Ralstonia*, *Variovorax*, and *Xanthobacter* most prevalent among the mixed culture clone libraries and *Arthrobacter* spp. dominating the isolate collection.

Dedication

This document is dedicated to Laura Ellen Douglass.

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I would like to thank my dissertation committee: Dr. Boehm, Dr. Daniels, and Dr. Ibba. This entire process would have been impossible without their support and guidance, and their patience has been near limitless. Thank you also to every other faculty member that has been a part of prior incarnations of my committee, specifically Dr. Warren Dick, Dr. Mark Morrison and Dr. Zhongtang Yu. Thanks to Dr. Mark Radosevich and his lab at the University of Tennessee for helping me with all of the molecular biology techniques used in this study. My project and I benefitted greatly from your kind help. The work was funded through the USDA National Research Initiative, Grant no. 2004-35107-14884 and fellowship support from the Robert H. Edgerley Environmental Toxicology Fund, Division of Natural and Mathematical Sciences of the College of Arts and Sciences, Ohio State University. Finally, I would like to thank my advisor, Dr. Tuovinen, for letting me be a part of his lab and this project and for not giving up on me.

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Field of Study

Major Field: Microbiology

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Chapter 1: Introduction

1.1 Background

Atrazine, an *s*-triazine (Fig. 1.1), is one of the most widely used herbicides in the world (Bonora et al., 2013), applied in the continental United States in an amount exceeding 27 million kg in 2012 according to United States Geological Survey estimates (Baker and Stone, 2015). Influenced by many complex environmental factors, the half-life of atrazine in agricultural soils varies greatly (Fenner et al., 2007), ranging from several days (Aguilera et al., 2009) to approximately a year (Accinelli et al., 2001). Atrazine has even been found in detectable amounts in soils 22 years after its last application (Jablonowski et al., 2009).

Due to the variable persistence of atrazine in the environment and moderate solubility in water (33 mg/L), it contributes to the point and non-point source pollution of surface water (Kalkhoff et al, 2003; Warnemuende et al., 2007), groundwater (Leterme et al., 2005; Potter et al., 2007), and wetlands (Rice et al., 2004; King et al., 2008; Rohr et al., 2008; Xu et al., 2009). This is problematic because atrazine has been shown to be an endocrine disruptor in non-target organisms, such as frogs (Hayes et al., 2010), and is a suspected human carcinogen (Fan et al., 2007). For these reasons, the United States Environmental Protection Agency has placed a maximum contaminant level of 3.0 ppb for atrazine in drinking water (Safe Drinking Water Act). This puts the United States on

par with Chinese drinking water standards (Chen et al., 2009), but behind the European Union standard of 0.1 ppb (Sass and Colangelo, 2006). Atrazine concentrations in surface waters regularly exceed the limit for drinking water as imposed by the US EPA, particularly in the Midwestern US (Thurman et al., 1991) and especially after rainfall (Hatfield et al., 1996).

Abiotic degradation of atrazine in the environment is subject to several conditions. Hydrolysis of atrazine generally only occurs at pH levels below 4; photolysis occurs at pH $\leq$ 4 under a wide range of wavelengths of light, or at higher pH levels (~6-8) under wavelengths of light < 300 nm (Comber, 1999). Extrapolating from these facts, abiotic degradation of atrazine would proceed on a scale of days in well-lit acidic waters, on a scale of months in higher pH (~6-8) waters, and on a scale of years in groundwater (Comber, 1999).

The principal means of atrazine degradation in soils and waters is bacterial degradation (Shapir et al., 2007). Although fungal biodegradation of atrazine does occur (particularly as demonstrated with *Phanerochaete chrysosporium*), the ultimate fate of the parent compound is dechlorination and/or dealkylation by fungi, not cyanuric acid ring mineralization (Donnelly et al., 1993; Mougin et al., 1994; Masaphy et al., 1996). Depending on the history of atrazine application and exposure, bacterial degradation can account for nearly complete removal of atrazine within a month (Anderson et al., 2002). For approximately the first four decades of atrazine use, the evidence suggested that bacteria were unable to completely mineralize atrazine (Shapir et al., 2007). The most prominent atrazine metabolites detected in soils were deisopropylatrazine and

deethylatrazine, which suggested that oxidative dealkylation was the main pathway of atrazine biodegradation (Wackett et al., 2002). The enzymes found to be responsible for the reactions producing these metabolites included a cytochrome P450 from *Rhodococcus* sp. N186/21 (Nagy et al., 1995) and AtrA from *Rhodococcus* sp. TE1 (Shao et al., 1995). In a recombinant *Rhodococcus* strain containing *atrA* and *trzA* (*s*-triazine chlorohydrolase), atrazine was degraded to *N*-isopropylammelide and *N*-ethylammelide. To date, no one microorganism has displayed an assortment of oxidative pathway enzymes capable of degrading atrazine to cyanuric acid, but it is likely that consortia of microorganisms would contain such oxidative atrazine degradative capacity (Govantes et al., 2009).

Today, bacterial atrazine mineralization is understood to be achieved primarily through a series of hydrolytic enzymes, none of which require oxygen. Ammonia, chloride, and carbon dioxide are the end products of this mineralization (Devers et al., 2003; Cheng et al., 2005). A complete atrazine mineralization pathway has been elucidated in *Pseudomonas* ADP, an atrazine-mineralizing bacterium isolated from a pesticide spill site in Madison, Minnesota (Mandelbaum et al., 1995). The pathway involves six proteins, encoded by six genes on self-transmissible plasmid pADP-1 (Martinez et al., 2001): AtzA, AtzB, AtzC, AtzD, AtzE, and AtzF. (de Souza et al., 1995; Boundy-Mills et al., 1997; Sadowsky et al., 1998; Cheng et al., 2005). These enzymes act strictly in this order, and rely on the metabolites from the preceding reactions (Shapir et al., 2007). AtzA (atrazine chlorohydrolase) dechlorinates atrazine (1-chloro-3-ethylamino-5-isopropylamino-2,4,6-triazine) to hydroxyatrazine (4-(ethylamino)-2-

hydroxy-6-(isopropylamino)-1,3,5-triazine). AtzB (hydroxyatrazine ethylaminohydrolase) removes the ethylamino side chain, forming *N*-isopropylammelide (6-(propan-2-ylamino)-1H-1,3,5-triazine-2,4-dione); the isopropylamino side chain is in turn removed by AtzC (*N*-isopropylammelide isopropylaminohydrolase), resulting in cyanuric acid (1,3,5-triazine-2,4,6-triol). These two liberated alkylamino side chains can be utilized by bacteria as carbon, energy and nitrogen sources. Collectively, these three steps are referred to as the upper pathway of atrazine mineralization (Figure 1.2). The cyanuric acid ring is cleaved by AtzD (cyanuric acid aminohydrolase), liberating a molecule of carbon dioxide and creating biuret (2-imidodicarbonic diamide). AtzE (biuret hydrolase) removes an amino group from biuret, creating allophanate (urea-1-carboxylate), which is completely mineralized by AtzF (allophanate hydrolase). This sequential mineralization of cyanuric acid by *atzD*, *atzE*, and *atzF* is referred to collectively as the lower pathway of atrazine mineralization (Figure 1.2). The *atzA*, *atzB*, and *atzC* genes are non-contiguous on the plasmid, and are constitutively expressed (Martinez et al., 2001; Devers et al., 2004). The *atzDEF* genes, however, comprise an operon and are regulated by nitrogen limitation and cyanuric acid availability. The effects of exogenous nitrogen sources vary from no effect on cyanuric acid mineralization to complete inhibition (Govantes et al., 2009).

Several alternative enzymes with similar functions to the Atz pathway enzymes have been identified. TrzN, which dechlorinates atrazine and other substrates, has been identified in the Canadian corn field isolate *Nocardioides* sp. strain C190 (Topp et al., 2000a,b; Mulbry et al., 2002) and the South Dakota pesticide spill site isolate

Arthrobacter aurescens TC1 (Strong et al., 2002; Sajjaphan et al., 2004). TrzD, a cyanuric acid amidohydrolase similar in function to AtzD, has been characterized in the Swiss municipal sewage sludge isolate *Pseudomonas* sp. strain NRRLB-12227 (Cook and Hütter, 1981; Eaton and Karns 1991) and the Swiss soil isolate *Klebsiella pneumoniae* strain 99 (now known as *Enterobacter cloacae* strain 99) (Cook and Hütter, 1981; Karns and Eaton 1997). TrzF, which also hydrolyzes allophanate like AtzF, was also identified in *Klebsiella pneumoniae* strain 99 (Cheng et al., 2005). Analyses of catabolic genes in soil bacterial isolates suggest that the complete *atz* pathway is relatively rare and various combinations of *atz* and *trz* genes are more common in environmental isolates (Devers et al., 2007; Vaishampayan et al., 2007; El Sebaï et al., 2011).

Historically, bacteria known to degrade atrazine have belonged to the high-GC Gram-positive group and the α-, β-, and γ-*Proteobacteria*. In a recent study by Fang et al. (2015), a database of bacteria containing known atrazine catabolic genes was constructed. Sequence data for *atzA*, *atzB*, *atzC*, *atzD*, *atzE*, *atzF*, and *trzN* were retrieved from Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway maps. These sequences were queried in NCBI GenBank, resulting in 37 annotated bacterial genera that harbored atrazine catabolic genes. All of these genera belonged to three bacterial phyla: *Actinobacteria*, *Firmicutes*, and *Proteobacteria*. Geographically, bacteria containing atrazine catabolic genes have been found on four continents (Wackett et al., 2002).

1.2 Objectives

The overall hypothesis of this study is that the microorganisms and catabolic pathways reported in the literature do not uniformly account for the atrazine biodegradation observed in different natural environments. When this project was started, a large percentage of the available atrazine biodegradation literature was devoted to mineralization kinetics, enzymology based on a collection of environmental isolates, and piecemeal attempts at culturing atrazine-degrading bacteria. The discovery of atrazine catabolic genes other than the *atz* pathway, and the implication of bacteria other than *Pseudomonas* and *Arthrobacter* in atrazine biodegradation, suggest that the full genetic and microbial diversity involved in environmental atrazine biodegradation had yet to be elucidated. Furthermore, little was known about the anaerobic biodegradation of atrazine. As the predominantly reported atrazine catabolic enzymes did not require oxygen for their functionality, anaerobic as well as microaerophilic biodegradation of atrazine in natural environments was not only likely but perhaps substantial depending on the site being assessed.

In order to further elucidate the genes and bacteria responsible for atrazine biodegradation, a variety of environments with differing exposures to atrazine were chosen for study. Among them were a farm location that was the site of multiple pesticide spill events throughout its history and a constructed wetland that received river water containing agricultural runoff. Samples from these sites were assessed for atrazine biomineralization via biometer studies. In addition to traditional environmental sampling, these sites were also sampled with Bio-Sep beads pre-enrich for atrazine-degrading

microbes. Soil and sediment samples were enriched for atrazine-degraders in liquid media, which included atrazine as the sole source of energy, C and N source. Atrazine-degrading mixed cultures were screened via 16S-rDNA-PCR-DGGE, and mixed cultures displaying unique banding patterns were chosen for 16S rDNA clone library formation and sequencing. Atrazine-degrading bacteria were isolated from mixed cultures and identified via 16S rDNA clone library formation and sequencing; these isolates were also screened for eight atrazine catabolic genes via PCR. Additional results on biometers and atrazine degraders were obtained for agricultural soil samples from the Molly Caren Farm site (London, Madison County, Ohio) and these are presented in Appendix A.

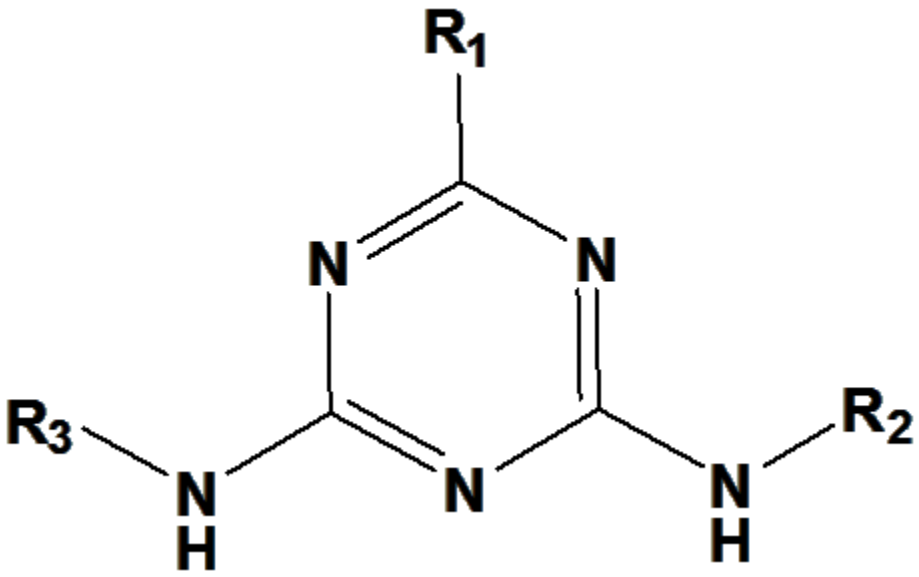

Figure 1.1. Generic structural formula of an *s*-triazine.

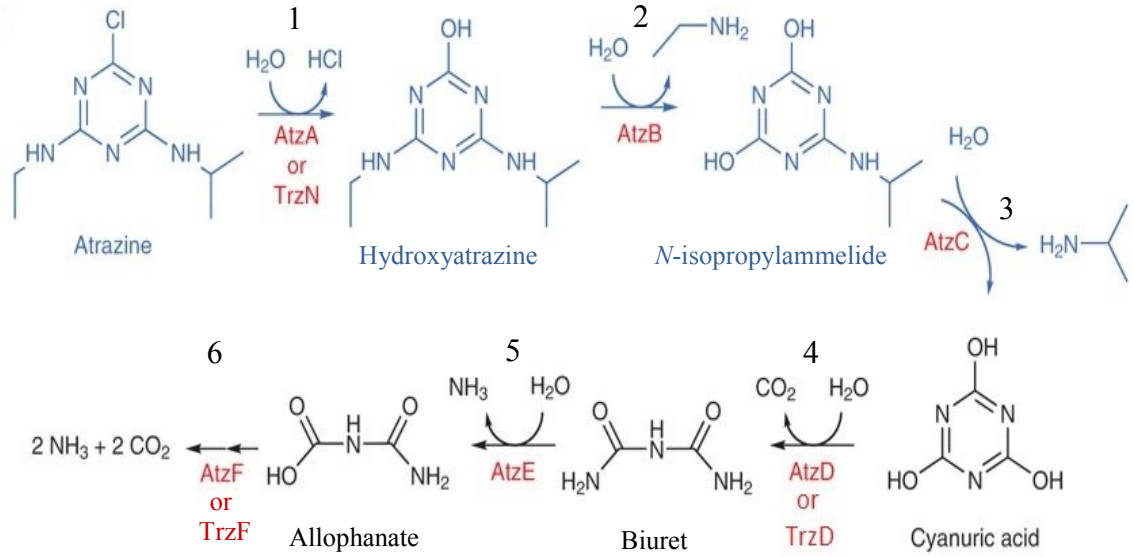

Figure 1.2. Pathway of bacterial atrazine mineralization. Steps 1-3 represent the upper pathway of atrazine mineralization. Steps 4-6 represent the lower pathway of atrazine mineralization. Adapted from Copley et al. (2009).

Chapter 2: Mineralization of atrazine in the river water intake and sediments of a constructed flow-through wetland

2.1 Introduction

Atrazine (2-chloro-4-(ethylamino)-6-(isopropylamino)-*s*-triazine) is widely used as a pre-emergence broadleaf herbicide in cornfields, especially in the American Midwest (Thelin and Stone, 2010). Its persistence in soils and sediments varies widely. The primary mode of atrazine attenuation in soil is by microbial biodegradation (Udiković-Kolić et al., 2012). Numerous soil borne bacteria have been shown to be capable of partial atrazine biotransformation or complete mineralization (Sadowsky, 2010; Udiković-Kolić et al., 2012). Atrazine at environmentally relevant concentrations of 5-10 ppb has been demonstrated to have endocrine disruptor effects in experimental animals and human cells and thus could potentially pose adverse ecological impacts to aquatic wildlife (Colborn et al. 1993; Kucka et al., 2012). In contrast to the much higher soil application rates of atrazine, these trace levels may result in much slower biodegradation rates or failure to induce catabolic pathways in atrazine-degrading microorganisms that maybe present in aquatic environments. Atrazine can run off from agricultural soils with storm water and erosion (Glenn and Angle, 1987; Seta et al., 1993) and thus its residues are invariably present in rivers within agricultural watersheds. Atrazine biodegradation studies have demonstrated the presence of atrazine-degraders in river water in many different geographical locations (Satsuma et al., 2002; Tappin et al., 2012). Wetlands and

riparian strips are important catchment systems in pesticide mitigation in agricultural watersheds (Passeport et al., 2103) and thus knowledge of the overall persistence of atrazine in these ecosystems is relevant to predicting adverse ecological impacts of this widely used herbicide. Atrazine half-lives have been reported to be between one and two weeks in wetland mesocosms (Detenbeck et al., 1996). Moore et al. (2000) presented estimates of the size of the wetland in relation to attenuation of atrazine in simulated agricultural runoff but the relative contribution of biological degradation to the overall attenuation was not determined.

Our previous studies of pesticide biodegradation demonstrated that atrazine was mineralized by microorganisms in a constructed wetland system receiving water from a river receiving agricultural runoff (Anderson et al., 2002). This flow-through Olentangy River wetland was constructed in 1993-1994 and is now well established (Mitsch et al. 2012) with water inlet from and outlet to the Olentangy River. The wetland has an influx of runoff fertilizers and other agrochemicals that vary in concentration throughout the year. As a follow-up to our previous study, we determined atrazine mineralization in intake river water and wetland sediment samples from the Olentangy River wetland under aerobic and anaerobic incubation conditions. Anaerobic incubation studies with a variety of external electron acceptors were performed in an effort to account for the wide variation in atrazine degradation rates previously reported in wetland sediments and overlying waters.

2.2 Materials and methods

2.2.1 Sampling

Surface water was collected from the water intake of the Olentangy River by grab sampling. The samples were stored in quart size glass jars (0.95 l) with lids, each representing a composite of three sub-samples. Sediment samples were taken with a core sampler (4.2 cm inner diameter) from the inlet (water inflow), middle section, and outlet from the Olentangy River wetland (Figure 2.1). Top 7-cm sediment sections (1 per site) were placed in sterile plastic sample bags at each sampling location and combined to a composite for the laboratory incubation experiments. All samples were collected on September 9, 2004. Atrazine in agricultural fields in the watershed had been applied in the last week of April through the first week of May.

2.2.2 Atrazine mineralization in biometer experiments

Initial biometer experiments demonstrated that atrazine biomineralization was negligible in water samples. Subsequently, suspended solids in the river water were pre-concentrated by centrifugation (13,700 x *g*, 10 min), and at a 60-fold pre-concentration of suspended solids atrazine biomineralization was readily detected. The pre-concentrated suspended solids were dispensed in 1 mL aliquots and sediment samples in 5 g wet weight aliquots into 50 mL serum bottles used as biometers (Radosevich et al., 1996). Duplicate aerobic biometers for each sample were set up either with or without the addition of glucose (11.1 mM). Each biometer received 0.1 μCi (0.064 μmol) of [U-ring-^{14}C]-atrazine. For trapping $^{14}CO_2$, each biometer had a 2-mL vial suspended from a rubber septum with copper wire. The vials contained 1 mL of 0.5 M KOH as an alkaline

trap. Biometers were closed with crimp-sealed septa.

Duplicate anaerobic biometers were set up with sterile double distilled H_2O instead of an external electron acceptor or one of the following: sulfate as Na_2SO_4 (15 μmol/biometer; 1.44 mg SO_4^{2-}), nitrate as KNO_3 (25 μmol; 1.55 mg NO_3^-), or Fe(III) as ferrihydrite (125 μmol; 14 mg Fe(III)). Each anaerobic biometer contained 0.1 μCi (0.064 μmol) of [U-ring-^{14}C]-atrazine and an alkaline trap. The headspace of these biometers was flushed with oxygen-free N_2 gas prior to sealing the rubber septa with crimp tops.

All biometers contained 13.8 μg atrazine. For the biometers with 1 mL pre-concentrated suspended solids, the total volume was 2.32 mL. For the biometers with 5 g composite sediment samples, the total liquid volume was 1.32 mL.

At 3-5 day intervals, the alkaline traps were removed and replaced with fresh KOH. The headspace conditions were re-established after each sampling event. The radioactivity in the alkaline trapping solution was measured by liquid scintillation counting using Scintiverse BD (Fisher Scientific, Fair Lawn, NJ) as scintillant. The concentration of atrazine remaining in each biometer was not analyzed. We have previously determined that the ^{14}C mass balance in field samples using the biometer technique is within the range of 97±7% and 95-139% depending on the soil type and incubation conditions (Radosevich et al., 1966; Ostrofsky et al. 1996).

Atrazine mineralization was calculated as the percentage of the total amount of added [U-ring-^{14}C]-atrazine that was mineralized to $^{14}CO_2$. Where the data would allow, half-lives and rate constants of atrazine mineralization were determined by fitting graphs to a first-order rate function, using the equation $P = P_{max} (1-e^{-kt})$, where P = observed

percentage of $^{14}CO_2$ evolved, P_{max} = maximum average extent of mineralization for that biometer pair, t = time (d), and k = the rate constant (d^{-1}). Half-lives ($t_{1/2}$) were then calculated using the rate constant in the equation $t_{1/2} = \ln 2/k$ (Guerin, 1992). Where data would not fit to a first-order rate function, a linear regression ($y = mx + b$) of the data was performed to determine the slope ($m = k$) and y-intercept (b). Half-lives were then calculated as $t_{1/2} = [(y_{max}/2) - b]/m$. For anaerobic incubations of river water samples, the biometer time courses had large variation among the duplicates. These are separated into single biometer data in graphs but the kinetic parameters were calculated as averages.

2.3 Results and discussion

2.3.1 Mineralization of atrazine in river water samples from the intake to the wetland

The concentrations of atrazine in Olentangy River water fluctuate annually between <1 to >5 µg/l reported as monthly averages (King et al., 2012). Peak concentrations may go up to >10 µg/l in early summer, followed by decline to 1 to 5 µg/l range through the summer and the fall. Atrazine concentrations in the Olentangy river water were not measured in the wetland samples in this study.

Atrazine mineralization in Olentangy River water samples under aerobic conditions was not detectable without prior sample pre-concentration. With a 60-fold pre-concentration of water samples the mineralization of atrazine could be readily demonstrated (Figure 2.2). The active phase of mineralization was about two weeks and thereafter the activity leveled off (Figure 2.2). The slow rate during this subsequent phase of mineralization may have resulted from atrazine sequestration by particulate organic matter such as humic substances in the concentrated water samples or decline in

microbial activity due to adverse changes in the biometer content over the three month incubation. For example, a decline in the atrazine concentration below a threshold level is needed to maintain induction/expression of catabolic pathways in atrazine degrading bacteria. The kinetics parameters were calculated for the active phase of biodegradation (Table 2.1). The first order rate constant was 0.13 d^{-1} for the aerobic biometers but this was almost halved when glucose was included in the biometers suggesting that the microbial metabolism was carbon and not nitrogen limited. Anaerobic rate constants varied in the range of 0.02-0.05 d^{-1} (Table 2.1) depending on the electron acceptors present. Duplicate biometers varied greatly and some showed no activity, comparable to the sterile controls (Figure 2.3). If the rate constants are back calculated for unconcentrated samples (i.e., raw water), allowing for the 60-fold concentration, they represent half-lives of atrazine mineralization ranged from 10 months to 5 years depending on incubation conditions. While these results with concentrated samples demonstrate potential biodegradation of atrazine in the water column, the in-situ rate in the river is subject to complex environmental factors that were not possible to simulate in the experimental design.

2.3.2 Mineralization of atrazine in sediments of the constructed wetland

Sediment samples from the wetland showed considerable variation in the extent of mineralization (Figures 2.2 and 2.3). Aerobic mineralization of atrazine in wetland sediment samples had a half-life of about a week with or without glucose amendment (Table 2.1). With or without sulfate or nitrate amendment, the half-life of anaerobic mineralization of atrazine was about 50 days. Ferrihydrite in anaerobic sediment samples

as the electron acceptor decreased the lag period as compared to nitrate and sulfate, but the half-life was extended to almost 6 months. Ferric iron-coupled atrazine biodegradation has not been previously demonstrated. Delaune et al. (1997) reported for soil samples that anaerobic biotransformation of atrazine was much slower as compared to the aerobic biodegradation, as differentiated by contrasting redox zones. Seybold et al. (2001) also demonstrated atrazine biodegradation in wetland sediments under strongly reducing conditions. The rates and half-lives of atrazine biotransformation cannot be compared among wetlands or mesocosms because of numerous differences in anhydric soil types, hydrology, and history of use and runoff of atrazine in the watershed area.

Thus the sediment layer of the flow-through wetland had an active atrazine mineralizing microbial community. Residual atrazine in agricultural runoff reaching the river is partitioned in the aqueous as well as particulate phase. As it enters the wetland the particulate fraction can import atrazine by settling in the sediment layer. Atrazine and metabolites tend to sorb moderately on wetland sediments (Chung et al. 1996; Mersie and Seybold, 1996; Runes et al., 2003). Atrazine biomineralization was typically incomplete, reaching a maximum of 25-30% of the total atrazine added to the microcosms. The incomplete mineralization may also have been due to slow desorption of the parent compound and/or metabolites from sediment organic matter but this seems unlikely given relatively moderate K_{oc} value for atrazine (~50-100) and the slow degradation rates observed even in the concentrated suspended solids. The incomplete mineralization may be the result of limitation of atrazine-metabolizing microbes due to environmental or intrinsic factors such as low residual atrazine concentration, loss of induction or

expression of catabolic enzymes, or micronutrient limitation.

The biodegradation and biomineralization of atrazine represent active microbial metabolism whereby the ethyl- and propionyl-side chains can serve as a source of energy and carbon. Preceding the cleavage of the aliphatic chains is the hydrolytic dechlorination step that is not believed to be coupled with energy transduction. Nitrogen in the chemical structure is ultimately released as NH_4^+ and can be assimilated by microorganisms. The atrazine ring carbon (^{14}C-labeled in this study) is an unlikely carbon source because the carbon atoms already are in an oxidized state (C^{+IV}) (Sadowsky, 2010; Udiković-Kolić et al., 2012). As demonstrated by this study, partial mineralization of this imported particulate phase atrazine by sediment layer microbial communities can be achieved under a variety of aerobic and anaerobic growth conditions. Differences in the kinetics were apparent depending on the external electron acceptor conditions. However, the mineralization rates observed under most conditions tested were relatively slow, resulting in estimated half-lives that exceeded the hydraulic residence time of the wetland. Therefore, if these rates closely approximate those of actual in-situ conditions the continuous input of atrazine from river water combined with slow degradations rates may lead to accumulation of atrazine in the wetland sediments and subsequent adverse impact on aquatic life. These concerns overlook the annual fluctuations in the runoff atrazine concentrations that can lessen the impact. Nutrient and temperature conditions as well as wetland plants also affect the biomineralization of atrazine. The foremost issue in this extrapolation is the unknown validity of biometer experiments to represent in-situ conditions in the wetland. An annual mass balance analysis of atrazine input, output and

sorption and sedimentation in the wetland is needed to validate the laboratory measurements of atrazine biomineralization.

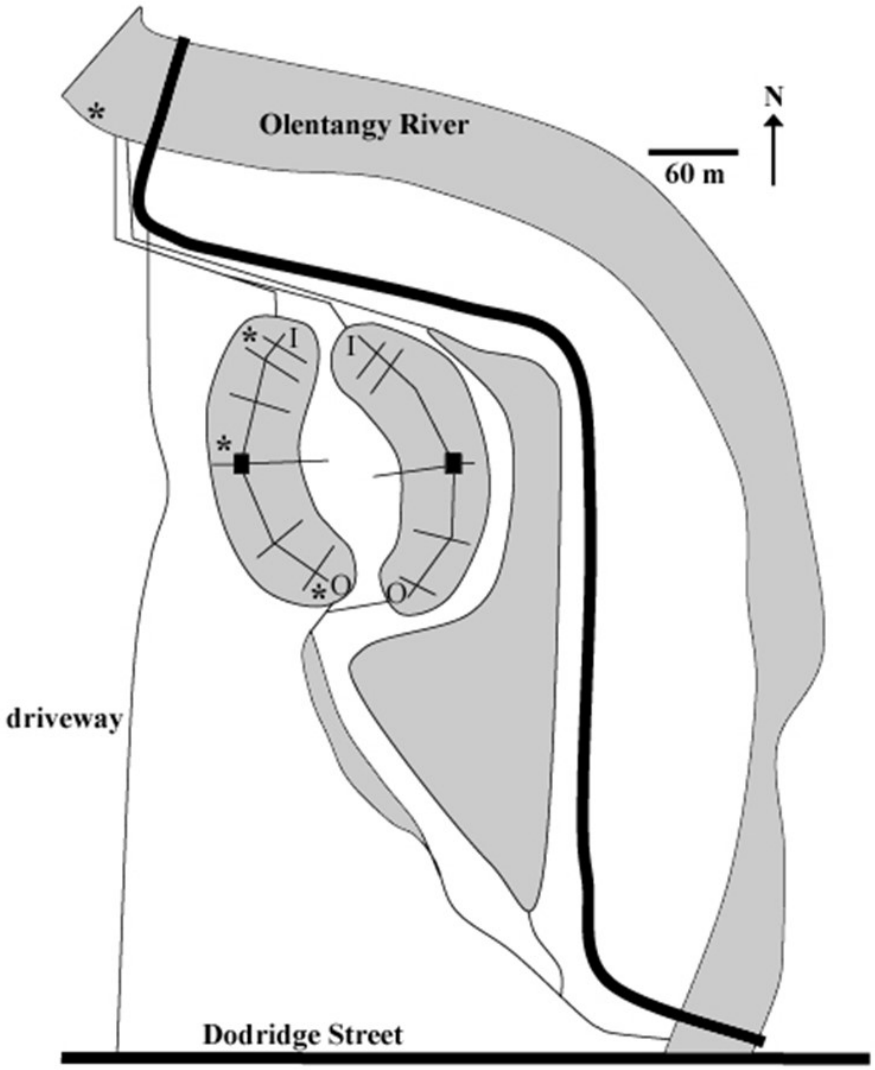

Figure 2.1. Sampling sites at the Olentangy River wetland. I = water intake from the river (water inflow); O = outlet to the river; * = sampling site. Wetland sediments from the three sampling sites were used as a composite for this study. Lines within wetland indicate boardwalks, and the squares indicate platforms. The heavy line denotes a bike and walking trail.

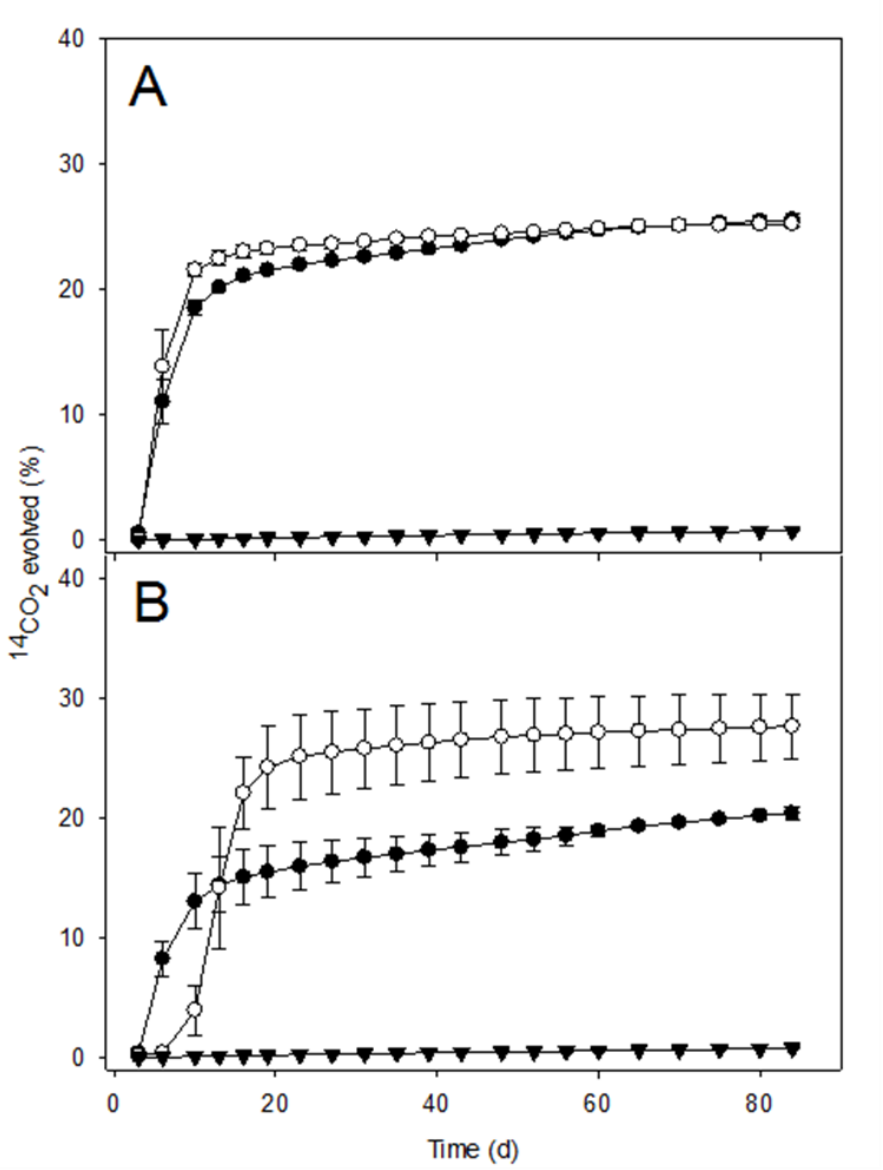

Figure 2.2. Aerobic mineralization of [U-ring-14 C]-atrazine in biometers in the absence (A) and presence (B) of glucose amendment. Symbols: ●, Olentangy River wetland sediment; ○, 60-fold pre-concentrated suspended solids of Olentangy River water; ▼, sterile control. Data points represent mean cumulative percentages obtained from duplicate biometers. Bars indicate actual percentages obtained from each replicate, and in some cases are smaller than the data point symbols.

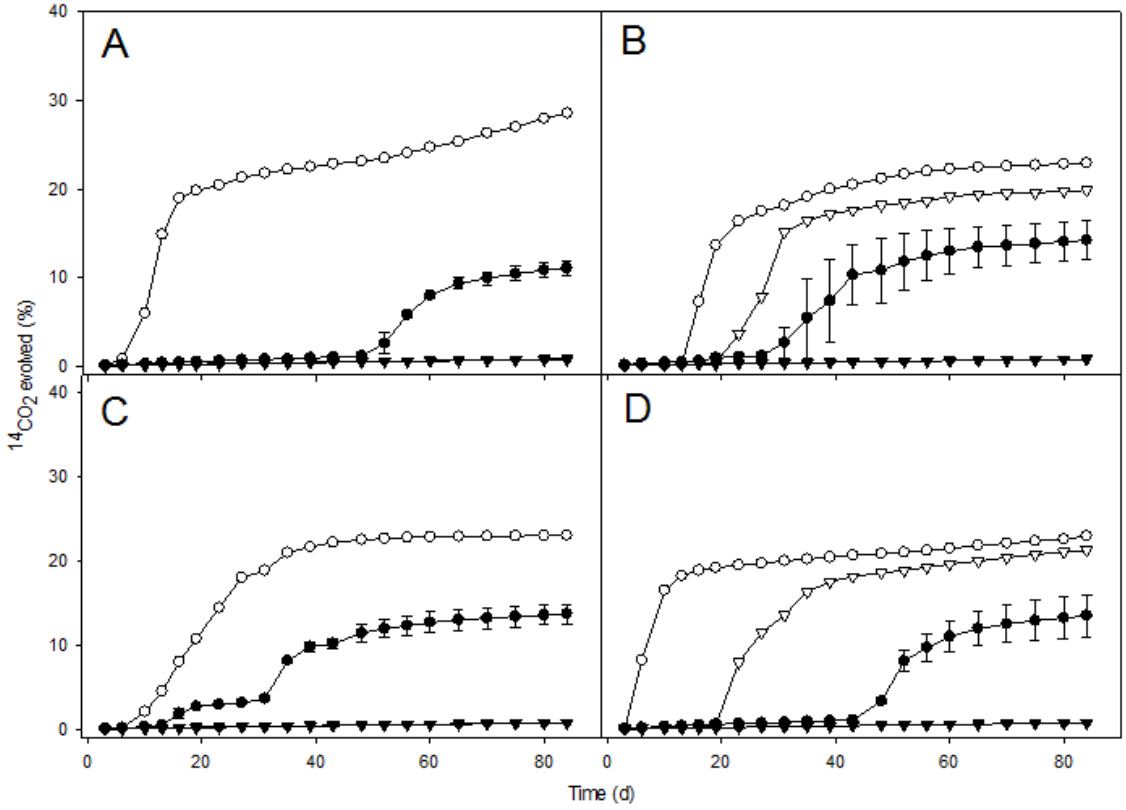

Figure 2.3. Anaerobic mineralization of [U-ring-14 C]-atrazine in biometers. The biometers were amended with (A) no electron acceptor, (B) potassium nitrate, (C) ferrihydrite, and (D) sodium sulfate. Symbols: ●, Olentangy River wetland sediment; ○ and ∇, 60-fold concentrated suspended solids of Olentangy River water; ▼, sterile control. Data points represent mean cumulative percentages obtained from duplicate biometers with the exception of river water biometers, where the duplicates are separated due to large differences. In A and C, the second suspended solids sample was comparable to the sterile control. Bars indicate actual percentages obtained from each replicate, and in some cases are smaller than the data point symbols.

Table 2.1. Kinetic parameters and half-lives of atrazine mineralization in wetland sediment and river water samples. Samples were collected on September 9, 2004. Calculations are based on the biometer experiments shown in Figures 2.2 and 2.3.

Sample	**Additional Amendment**	**Incubation conditions**	**k (d^{-1})**	**R^2**	**$t_{1/2}$ (d)**
River water[a]	None	Aerobic	0.131	0.871	5.3
River water	Glucose	Aerobic	0.059	0.856	11
River water	None	Anaerobic	0.048	0.910	14
River water	Sulfate	Anaerobic	0.039	0.980	18
River water	Nitrate	Anaerobic	0.020	0.891	35
River water	Ferrihydrite	Anaerobic	0.031	0.916	22
Wetland sediment	None	Aerobic	0.104	0.917	6.7
Wetland sediment	Glucose	Aerobic	0.088	0.919	7.9
Wetland sediment	None	Anaerobic	0.153[a]	0.819	53[b]
Wetland sediment	Sulfate	Anaerobic	0.200[a]	0.853	49[b]
Wetland sediment	Nitrate	Anaerobic	0.222[a]	0.916	40[a]
Wetland sediment[c]	Ferrihydrite	Anaerobic	0.015	0.787	45

[a]All river water samples = 60-fold concentrated suspended solids of river water.

[b]The rate constants and half-lives were calculated from linear regression.

[c]Because of the unusual biphasic shape of the time course curve, the k and $t_{1/2}$ values based on the first order rate expression were calculated for the time course of 30-84 days (Figure 2.3).

Chapter 3: Molecular analysis of atrazine-mineralizing bacteria and catabolic genes in the water column and sediment of a constructed wetland in an agricultural watershed

3.1 Introduction

Atrazine, a pre-emergent weed killer in corn fields especially, is commonly found in surface runoff and subsurface drainage in Midwest agricultural watersheds. It is relatively readily biodegradable in soils with annual applications, but residuals and metabolites are detectable waterways and groundwater (Thurman et al., 1991; Burkart et al., 1999; Moore et al., 2001). Riparian zones can attenuate atrazine concentrations as well as other agrochemicals (Angier et al., 2002), but seasonal fluxes can exceed the biodegradation and sorption capacity in soils and riparian soils. Wetlands have been shown to mitigate atrazine due to microbial degradation (Moore et al., 2000; Anderson et al., 2002; Gregoire et al., 2009; Douglass et al., 2014). Simulating agricultural stormwater runoff, Lizotte Jr. et al. (2009) spiked atrazine to a wetland system, demonstrating that about 80% of the atrazine spike was removed from the water column within a week and was undetectable after four weeks. While the data demonstrated the removal of atrazine (and other pesticides) from the wetland water column, they do not differentiate between the biodegradation and partitioning in the sediment. Flow-through wetlands especially have proven effective in the retention of agrochemicals mostly via sorption of chemicals, nutrient assimilation, and biodegradation of runoff pesticides. Aerobic wetland environment appears to promote atrazine biodegradation as compared to

anaerobiosis (DeLaune et al., 1997). Several studies have addressed the phylogenetic diversity of atrazine-degrading bacteria from agricultural soils (Topp et al., 2000a,b; Rousseaux et al., 2001; Aislabie et al., 2005; Zablotowicz et al., 2009). Clones matching with genera such as *Arthrobacter*, *Bacillus*, *Chelatobacter, Pseudaminobacter*, and *Nocardioides* have been abundantly identified in these studies. However, the diversity of bacteria involved in atrazine biotransformations in wetland systems has yet to be addressed.

The flow-through wetland at the Olentangy River Wetland Research Park was constructed in 1993-1994. Because Olentangy River has a highly agricultural watershed, the wetland receives a varying influx of fertilizers and agricultural pesticides (Mitsch et al., 2012). Atrazine in agricultural fields is applied normally during the first week of May or late April depending on the weather conditions. The wetland receives river water from Olentangy River with an outlet back to the river. The inflow of river water to the wetland is pumped continuously from the Olentangy River according to a formula relating pumping rates to river stages. The peak runoff of atrazine from agricultural fields in the river water arrives in the Columbus region usually late June or the first half of July depending on hydrological conditions. The water level varies between two extremes in the wetland when the river is at base flow or flooding. On average, the hydraulic residence time of river water in the wetland system is 3 to 5 days (Mitsch et al., 2012) and atrazine concentrations seasonally reach up to 10 to 12 ppb levels depending on the flow conditions of the Olentangy River.

A previous study demonstrated the presence of *trzD* (cyanuric acid amidohydrolase) and low levels of *atzA* (atrazine chlorohydrolase) in microbial communities of the Olentangy River wetland after six years of wetland construction (Anderson et al., 2002), but the diversity of bacteria was not addressed. Douglass et al. (2014) reported that atrazine was mineralized in sediment samples of the Olentangy River wetland under both aerobic and anaerobic conditions. The rate constants varied with electron acceptor conditions (NO_3^-, Fe(III), SO_4^{2-} or none) but the coupling of atrazine biomineralization with anaerobic respiration was not ascertained. Atrazine mineralization could not be demonstrated in the Olentangy River water column without a prior concentration of the suspended solids.

The present study was undertaken to assess the phylogenetic and catabolic gene diversity of atrazine degraders in samples of the Olentangy River wetland. Both enrichment and pure culture isolates were characterized based on the 16S rRNA gene sequence analysis. Catabolic genes of atrazine metabolism were screened in bacterial isolates from the wetland. In addition to sediment samples, bacteria trapped on activated carbon beads (Bio-Sep beads) from the wetland were tested as additional sources of atrazine-degrading inocula for enrichment cultures and biomineralization assays.

3.2 Materials and methods

3.2.1 Sampling

Samples of sediments were taken from the inflow of river water, the middle and outlet of the Olentangy River wetland in September and November, 2004 and March and July, 2005 (Table 3.1). A sterile, transparent plastic cylinder (Ø 4.2 cm) was used for the

sampling of the top 7 cm of sediment. Samples were placed in sterile zip lock plastic bags and kept on ice for transportation to the laboratory. The sampling was coordinated with testing of atrazine biomineralization in Olentangy River water and wetland sediment samples (Douglass et al., 2014).

Atrazine-degraders in the wetland were probed with porous Bio-Sep beads. The beads were made of granular activated carbon compressed into size and shape of lentils (Sublette et al., 1996; Peacock et al., 2004; Williams et al., 2013). The technique is based on sorption of atrazine in aqueous solution on the beads followed by *in-situ* incubation to enrich for atrazine-degrading bacteria, possibly in the form of biofilm growth (Mehta et al., 2004; Peacock et al., 2004; Ghosh et al., 2009; Omotayo et al., 2011). The beads were equilibrated in solution of 2 mg atrazine/l or, for control beads, the same volume of distilled water for at least three days. Beads equilibrated in atrazine solution contained 24 mg atrazine per kg Bio-Sep beads. Equilibrated beads were placed to sterile nylon mesh bags (~30 beads per bag), and the bags were sealed with plastic clips. Three bags of atrazine-equilibrated beads and three bags of water-equilibrated beads were deployed at each site. Bead bags were clipped to lines, which were tied to weights at one end and Styrofoam floats at the other end, allowing the bags to float usually about 10-20 cm above the sediment. However, bags sometimes ended lying on sediment surface due to fluctuations in water level. Some bead nets were also buried in the top 10 cm layer of wetland sediments. After 4-12 weeks, the bead bags were retrieved and processed in the laboratory as inocula for biometer assays and enrichment cultures.

3.2.2 [U-ring-^{14}C]-atrazine mineralization

The Bio-Sep bead bags were incubated *in-situ* in the water column and the sediment layer of the Olentangy River wetland for either 28 days (March-April) or 80 days (November-February) before testing in biometer assays. The mineralization of atrazine was tested in aerobic 60 mL biometer bottles fitted with rubber septa, as previously described (Radosevich et al., 1996). The biometers were inoculated with 6-10 diced Bio-Sep beads. Each biometer received 0.1 µCi (0.064 µmol) [U-ring-^{14}C]-atrazine (Sigma-Aldrich, St. Louis, MO) and 5 mL basal mineral salt solution, which contained (per liter): K_2HPO_4, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; $FeCl_3 \cdot H_2O$, 0.1 mg; $CaCl_2 \cdot H_2O$, 1 mg; $MnCl_2$, 0.01 mg; $ZnCl_2$, 1 µg; pH 6.8. Alkaline trap solution (1 mL 0.5 M KOH) was employed to trap $^{14}CO_2$ evolved from the mineralization of ^{14}C-labelled atrazine. The alkaline trap solutions were in small vials suspended with Cu-wire inside from the septa. The septa/vial units were crimp-sealed onto the serum bottles. The biometers were incubated at 22±2 °C. Biometer sampling and counting of radioactivity have been previously described (Douglass et al., 2014). $^{14}CO_2$ evolution was calculated as a percentage of the total amount of [U-ring-^{14}C]-atrazine added to the biometers at the beginning of the time course. Cumulative percentages were calculated by adding the values of each data point to the previous data point.

3.2.3 Enrichment culture conditions

Sediment and bead samples were used as inocula for aerobic and anaerobic enrichment cultures with 30 mg/l atrazine in basal mineral salts solution. Aerobic cultures were constructed with or without glucose amendment (2 g glucose/l) and were incubated

in shake flasks (150 rev/min) at 22±2 °C. Glucose was used as a supplement because it was considered a general purpose, non-selective substrate. It was speculated that glucose metabolism in enrichment cultures could yield intermediates that may promote growth of atrazine degraders. To date, there is no universal carbon source that can support growth of all known atrazine degraders. Anaerobic cultures were statically incubated at 22±2 °C in crimp-sealed 60 mL serum bottles in which headspace gas was replaced with argon gas. Aliquots of aerobic enrichment cultures were transferred to the corresponding fresh media at intervals of 2-4 weeks. Aliquots of anaerobic enrichment cultures were transferred to the corresponding fresh media at intervals of 4-6 weeks.

3.2.4 HPLC analysis of enrichment cultures

After at least six transfers to fresh basal mineral salt solution with 30 mg/l atrazine, enrichment cultures were screened for residual atrazine concentration. Aliquots (200 µl) of cultures were mixed with 1.8 mL of mobile phase (65% HPLC-grade acetonitrile in HPLC-grade distilled water) followed by centrifugation at 16,000 x g for 15 min. The supernatant was transferred to an HPLC vial in a Thermo Separation Products AS100 autosampler (Thermo Fisher Scientific, Waltham, MA). Samples were pumped through an Adsorbosphere C18 HPLC column at a flow rate of 1.0 mL/min for 9 min using a Shimadzu LC-10AT pump, and detected by a Shimadzu SPD-10 AV UV/Vis detector at 220 nm. Chromatographs were generated using a Shimadzu CBM-20A communications bus module and recorded with EZStart software (Shimadzu, Columbia, MD). Reference samples containing uninoculated sterile media and varying concentrations of atrazine dissolved in mobile phase were included with every HPLC run.

Enrichments that exhibited at least 80% decrease in atrazine concentration were deemed to contain atrazine-degrading mixed cultures.

3.2.5 Isolation of atrazine-degrading pure cultures

Prospective enrichment cultures of atrazine-degraders were inoculated to solid media. Dilutions of 10^{-3} to 10^{-5} for cultures enriched without glucose and dilutions of 10^{-6} to 10^{-8} for cultures enriched with glucose were spread-plated in atrazine + mineral salts media solidified with 1.5% Noble agar. All plates were incubated at 22±2 °C. Plates were incubated for up to 2 weeks. Discreet colonies from the spread plates were subcultured twice on streak plates before transferring them into 5 mL of corresponding liquid media. Changes in atrazine concentration were monitored during subsequent incubation in liquid media. Pure cultures were stored at 4 °C. Liquid cultures were stored as 20% glycerol stocks at -55 °C.

3.2.6 DNA isolation and 16S rRNA gene amplification

Genomic DNA was isolated from pure and mixed cultures with MoBio UltraClean Microbial DNA Isolation Kits per manufacturer's instructions (MoBio Laboratories, Carlsbad, CA). The V3 region of the 16S rRNA gene from atrazine-degrading mixed cultures was amplified using universal bacterial PCR primers in 50 µl total volume, consisting of 1X TaKaRa Premix Taq (1.25 U *Taq*; 0.2 mM dATP; 0.2 mM dTTP; 0.2 mM dCTP; 0.2 mM dGTP; 10 mM tris-HCl [pH 8.3], 50 mM KCl, 1.5 mM $MgCl_2$), 1 µM primer 357fGC (5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CAG-3'), 1 µM primer 518r (5'-ATT ACC GCG GCT GCT GG-3') (Muyzer et al., 1993), and 20 ng of template DNA.

The PCR amplification involved (1) 5 min at 94 °C, (2) 30 sec at 94 °C, (3) 30 sec at 61 °C -0.5C/cycle, (4) 1 min at 72 °C, (5) 9 times to (2), (6) 30 seconds at 94 °C, (7) 30 sec at 56 °C, (8) 1 min at 72 °C, (9) 24 times to (6), (10) 7 min at 72 °C, and (11) storage at 4 °C.

PCR products were analyzed via denaturing gradient gel electrophoresis (DGGE) using the DCode Universal Mutation Detection System (Bio-Rad, Hercules, CA) per manufacturer's instructions. Gels consisted of 8% acrylamide and a 40%-60% denaturant gradient. Gels were electrophoresed at 83 V for 16 h in 0.5X TAE buffer solution (20 mM Tris base, 10 mM acetic acid, and 0.5 mM EDTA) at 60 °C. After electrophoresis, gels were stained in 1X SYBR Gold (Molecular Probes, Eugene, OR) for 30 min on a rocking platform and visualized on a UV transilluminator. Mixed cultures corresponding to various denaturing gradient gel electrophoresis banding patterns were chosen for further investigation via 16S rDNA clone library sequencing (Appendix B).

3.2.7 16S rDNA clone library formation and analysis

Genomic DNA from atrazine-degrading mixed cultures and isolates was amplified for the approximately 1,500 bp region of the 16S rDNA using universal bacterial PCR primers. PCR reactions were conducted as outlined in section *2.5.* with 1 μM primer 27f (5'- AGA GTT TGA TCM TGG CTC AG -3'), 1 μM primer 1525r (5'- AAG GAG GTG WTC CAR CC-3') (Lane, 1991), and 20 ng of template DNA. The protocol with the thermal cycler involved (1) 2 min at 94 °C; (2) 50 sec at 94 °C; (3) 50 sec at 55 °C; (4) 1 min 30 sec at 72 °C; (5) 31 times to (2); (6) 5 min at 72 °C; and (7) storage at 4 °C.

PCR products were used to construct 16S rDNA clone libraries. The clones were made using the Promega pGEM–T vector system and JM109 high efficiency competent cells per manufacturer's instructions (Promega, Madison, WI). Overnight cultures of clones were plated onto 96-well plates and sent to the Clemson University Genomics Institute for sequencing. For each pure culture, three clones were sequenced; for each mixed culture, 25 clones were sequenced. Sequences were edited in Geneious. A consensus sequence was generated for each set of 3 clones resulting from pure cultures when possible, and these consensus sequences were entered into the NCBI-BLASTN (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch &LINK_LOC=blasthome) and Ribosomal Database Project (RDP) (Cole et al., 2014) databases. Otherwise, individual edited clone sequences or consensus sequences constructed from two clones were entered as queries into the aforementioned databases. Likewise, each individual edited clone sequence resulting from mixed cultures was searched in these databases.

The 16S rRNA gene sequences were deposited with GenBank. The accession numbers for aerobic mixed culture clones are KP115624-KP115683. The accession numbers for anaerobic mixed culture clones are KP719004-KP719015. The accession numbers for aerobic isolates are KM492766- KM492811.

3.2.8 Atrazine catabolic gene detection in atrazine-degrading pure cultures

Genomic DNA from atrazine-degrading isolates was amplified for known atrazine catabolic genes *atzA*, *atzB*, *atzC*, *atzD*, *atzE*, *atzF*, *trzD*, and *trzN*. *Escherichia coli* genomic DNA was also amplified as a negative control for all catabolic genes.

Pseudomonas ADP was used as a positive control for *atzA*, *atzB*, *atzC*, *atzD*, *atzE* and *atzF*, and as a negative control for *trzD* and *trzN*. *Arthrobacter aurescens* TCI was used as a positive control for *trzN* as well as an additional positive control for *atzB* and *atzC* and a negative control for *atzA*, *atzD*, *atzE*, and *atzF*. *Klebsiella pneumoniae* 99 was used as a positive control for *trzD*.

PCR reactions were conducted with 1 μM forward primer and 1 μM reverse primer as specified in Table 3.2 and 20 ng template DNA. The protocol with the thermal cycler involved (1) 4 min at 94 °C; (2) 30 sec at 94 °C; (3) 1 min at each primer's annealing temperature from Table 3.2; (4) 2 min at 72 °C; (5) 33 times to (2); (6) 15 minutes at 72 °C; and (7) storage at 4 °C. PCR products were loaded onto agarose gel, and after electrophoresis the gel was stained with ethidium bromide and visualized with a UV gel imager. Detection of a band of anticipated size was treated as preliminary evidence of the presence of the given catabolic gene, and a random assortment of these PCR reactions were used to create clone libraries as outlined in section 2.7. For each PCR reaction, three clones were sequenced. When possible, a consensus sequence for these three clone sequences was generated and used for bioinformatics analysis; otherwise, a consensus sequence of 2 clones was generated, or a single edited clone sequence was used. Query sequences were entered into NCBI-BLASTN and aligned with their corresponding catabolic gene sequences. Catabolic gene sequences were deposited with GenBank, and the accession numbers are KP339955- KP339976.

3.3 Results and discussion

3.3.1 Measurement of atrazine mineralization with [U-ring-^{14}C]-atrazine and Bio-Sep beads

Biometers were incubated with [U-ring-^{14}C]-atrazine and Bio-Sep beads (6-10 beads/biometer), which had been exposed to the water column and sediments in the Olentangy River wetland for up 80 days. The mineralization of atrazine (Figure 3.1A, B) in the biometers was minimal and mostly indistinguishable from sterile controls. In one case (Figure 3.1B), there was an anomalous $^{14}CO_2$ evolution from [U-ring-^{14}C]-atrazine in a sample of beads previously exposed to water column. This outlier activity was not detected in any other samples of Bio-Sep beads, and it may represent an aggregation of atrazine-mineralizing cells that had formed on the bead during exposure to the water column. All other results suggested that atrazine-mineralizing microbes were associated with beads in insufficient numbers to register a noticeable amount of biomineralization of atrazine in the biometer assay. In subsequent incubation experiments in liquid media, atrazine-degrading bacteria could, however, be enriched from in-situ incubated beads. It is also conceivable that ^{14}C-labelled atrazine may sorb to the activated carbon Bio-Sep bead and thus become biologically unavailable for biodegradation. However, previously published experiments with atrazine and Bio-Sep beads do not suggest that atrazine sorption on the beads retards biomineralization (Ghosh et al., 2009; Omotayo et al., 2011).

3.3.2 16S rRNA gene sequence based analysis of atrazine-degrading enrichment cultures and bacterial isolates from wetland samples

Sequence data from 16S rDNA clones resulting from atrazine-degrading, wetland-sourced mixed cultures was queried in the RDP database for nearest matches. Except where noted, Figure 3.2 summarizes aerobic mixed culture query results at the genus level. All results reported in this study returned s_ab scores of 0.90 or greater. Of the 60 sequences queried, the nearest matches for the dominant genera were *Acidovorax* (33% of the clones), *Ralstonia* (22%), *Azorhizobium* (12%), and *Xanthobacter* (12%). Forty-three of the 60 clones originated from Bio-Sep bead enrichment cultures.

Figure 3.3 summarizes anaerobic mixed culture query results at the genus level, except where noted otherwise. All results returned s_ab scores of 0.90 or greater. Of the 12 sequences sequenced, the nearest matches for the dominant genera were *Hydrogenophaga* (42%) and *Rhizobium* (25%). All 12 clones originated from Bio-Sep bead enrichment cultures and none matched obligate anaerobes in the RDP database. *Hydrogenophaga* spp. have been previously identified in atrazine-degrading microbial communities and some have homologues of AtzD involved in cyanuric acid metabolism (Udiković-Kolić et al., 2010; Peat et al., 2013). Several studies have reported atrazine metabolism by *Rhizobium* spp., some through interspecies catabolism (e.g., Bouquardet al., 1997; Mehmannavaz et al., 2001; Smith et al., 2005).

Sequence data from 16S rDNA clones resulting from atrazine-degrading, wetland-sourced pure cultures were also queried in the RDP database for nearest matches. With the exception of an uncultured *Methyloversatilis*, Figure 3.4 summarizes these query

results at the species level. Isolates were derived from mixed cultures enriched from harvested Bio-Sep beads. Not all genera represented in the mixed culture 16S rDNA clone library are represented in the collection of isolates, nor are all isolates represented in the mixed culture clone library collection. All results reported here returned s_ab scores of 0.97 or greater. Of the 18 sequences queried, 89% of the nearest matches were assigned to *Arthrobacter* spp. and the other two were consistent with a *Sphingomonas* sp. SaMR12 and an uncultured *Methyloversatilis*. Thus the 16S rDNA sequence data demonstrated a main cluster of isolates that was consistent with *Arthrobacter* spp. Members of this genus have been previously isolated in different parts of the world from agricultural soils with various management practices, all involving atrazine application (e.g., Aislabie et al., 2005; Vaishampayan et al., 2007; Govantes et al., 2007; Sajjaphan et al., 2010; El Sebai et al., 2011; Zhang et al., 2011; Wang and Xie, 2012; Fan and Song, 2014). Atrazine biodegradation by *Sphingomonas* spp. has been reported in multiple studies (e.g., Smith and Crowley, 2005; Macías-Flores et al., 2009; Sene et al., 2010). A *Methyloversatilis* isolate has been previously characterized that can degrade s-triazines (Iwasaki et al., 2007; Takagi et al., 2012). The 16S rDNA clone sequences of two isolates from Bio-Sep beads returned database matches with similarity scores of less than 0.97 (0.91 = *Nocardioides* sp. HKT9991, 0.94 = *Nocardioides* sp. ATD6).

3.3.3 Detection of atz *and* trz *catabolic genes*

The presence of atrazine catabolic genes was determined for nine bacterial isolates selected randomly from a pool of atrazine-degraders. A typical pattern of occurrence of catabolic genes was the combination of *trzN* and *atzBC* (Table 3.3).

Neither *atzD* nor *trzD* was detected in the nine isolates. The isolate with 1.00 similarity match with *Sphingomonas* sp. SaMR12 contained most of the Atz pathway genes. The similarity of nucleotide sequences of the PCR products against respective database catabolic gene sequences varied between 99-100% (Table 3.4). The score of recoverage was in the range of 95-100%. Genes associated with atrazine biodegradation are often in conjugative plasmids or transposons and are generally believed to have been transmitted in bacterial communities through lateral gene transfer involving multiple, independent conjugation, transposition and other gene recruitment events (Sadowsky, 2010; Liang et al., 2012).

Arthrobacter spp. in soil microbial communities are generally known to include atrazine-degraders (Fan and Song, 2014). The *trzN* and *atzBC* combination found in this study has been previously reported for many *Arthrobacter* isolates from soils with various histories of atrazine application (e.g., Devers et al., 2004; Sajjaphan et al., 2010; El Sebai et al., 2011; Zhang et al., 2011, Wang and Xie, 2012). In general, atrazine-degrading soil borne bacteria, including some *Arthrobacter* spp., display considerable variation in clusters and arrangements of atrazine catabolic genes. Whether *Arthrobacter* spp. are particularly culturable in atrazine-selective media and represent much of the genetic potential of atrazine biodegradation in soil and wetland environments is not known at this time. The apparent bias for *Arthrobacter* spp. is not in discrepancy with the general finding reported in the literature that atrazine metabolism typically involves soil microbial consortia, which cannot be captured and deciphered with cultural isolation techniques based on atrazine mineralization.

3.4 Conclusions

Active mineralization of atrazine has been previously demonstrated with the biometer technique in the water column and sediment layers of the Olentangy River wetland system. Bio-Sep beads that were initially held in-situ in the wetland water and sediment for 28 and 80 days yielded negligible $^{14}CO_2$ evolution from [U-ring-^{14}C]-atrazine in biometer incubations. Atrazine-degrading bacterial populations were enriched from wetland sediment samples and from Bio-Sep beads under aerobic and anaerobic conditions using 30 mg/l atrazine ± glucose as the substrate. *Arthrobacter* was the main group of atrazine-degrading aerobic isolates that could be recovered in atrazine-selective media from enrichment cultures. Anaerobic enrichment cultures did not yield pure culture isolates. The occurrence of eight catabolic genes representing two atrazine degradative pathways was determined in selected bacterial isolates. Neither *atzA* nor *trzD* were detected in any of the isolates. None of the isolates had a complete Atz or Trz pathway, suggesting mosaics of genes and enzymes acquired through lateral recombination events. The lack of complete assemblage of pathway genes among the isolates suggests that some *atz* and *trz* genes could not be amplified due to differences in nucleotide sequences.

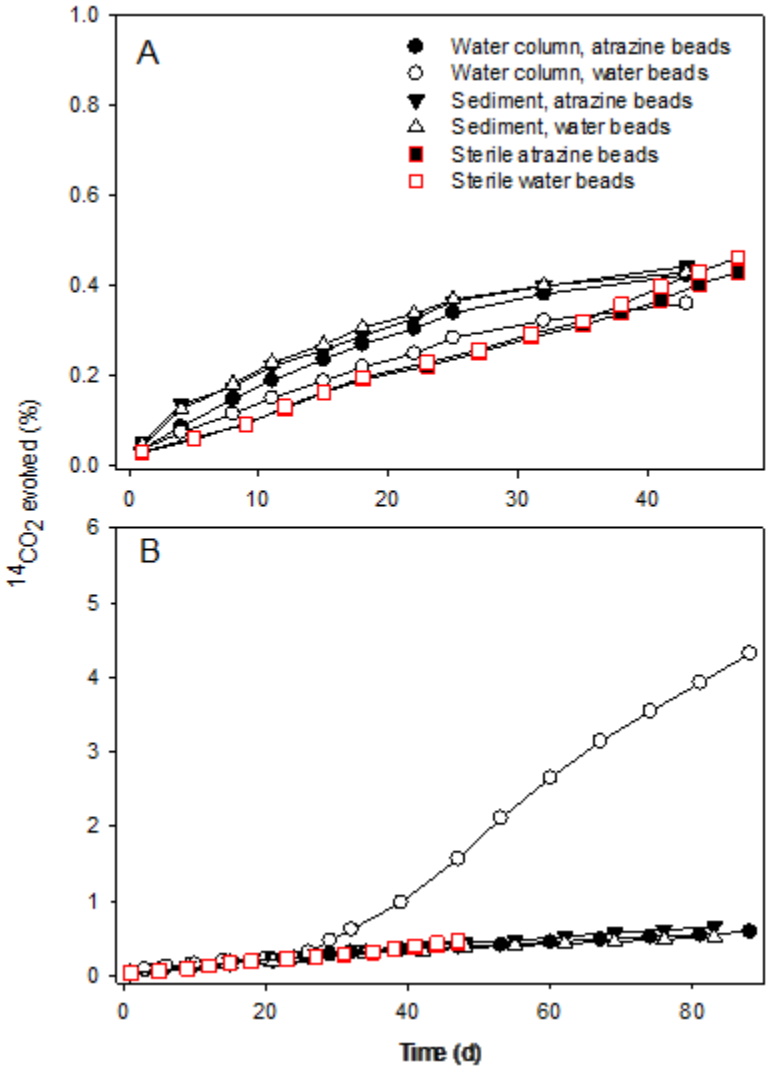

Figure 3.1. Aerobic mineralization of [U-ring-^{14}C]-atrazine in biometers inoculated with Bio-Sep beads. The beads had been submerged in the water column and top sediment layer of the Olentangy River wetland (A) in March – April 2005 and (B) in November 2004 – February 2005 before retrieval. Note the different scale in the time courses. Data points represent cumulative percentages of $^{14}CO_2$ obtained from single biometers. Atrazine Bio-Sep beads were equilibrated in sterile 2 mg atrazine/l solution, and water beads were equilibrated in sterile water.

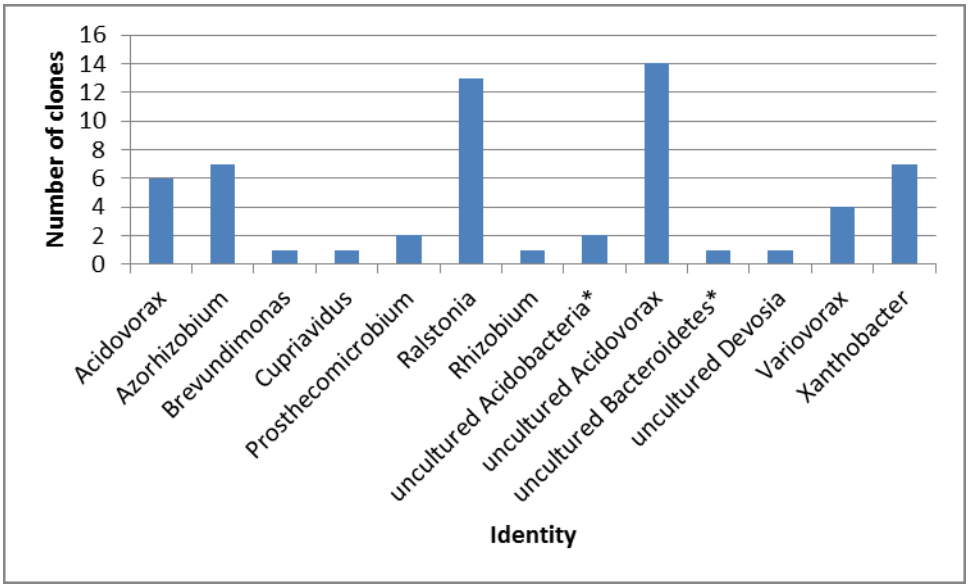

Figure 3.2. Identification of 16S rDNA clones from atrazine-degrading wetland aerobic mixed cultures. Sixty clones in total are represented. All sequence queries returned similarity scores of 0.90 or greater. All identifications are made at the genus level with the exception of those marked with an asterisk (*), which are made at the phylum level.

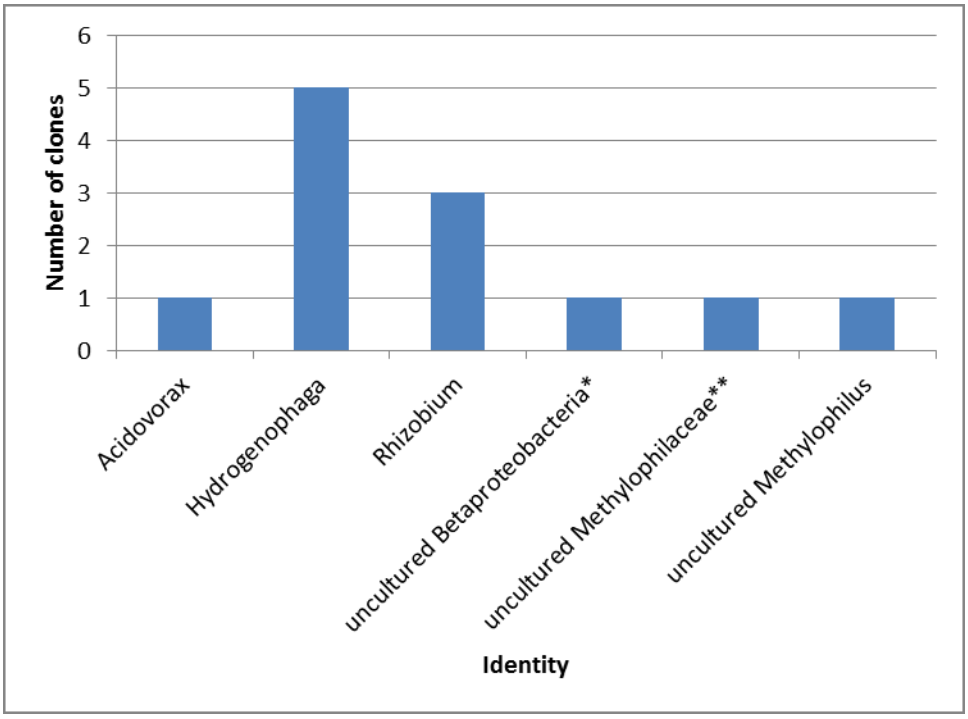

Figure 3.3. Identification of 16S rDNA clones from atrazine-degrading wetland anaerobic mixed cultures. Twelve clones in total are represented. All sequence queries returned similarity scores of 0.90 or greater. Identifications are made at the genus level with the exception of those marked with asterisks, which are made at the class (*) and family (**) levels.

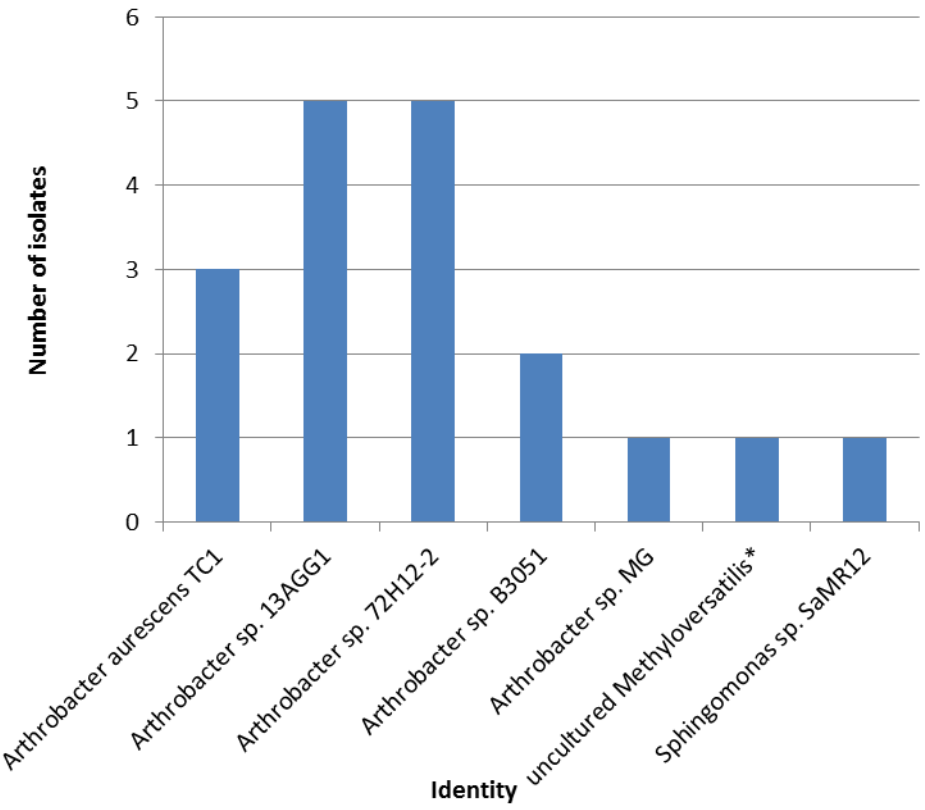

Figure 3.4. Identification of 16S rDNA clones from atrazine-degrading wetland aerobic pure cultures. Eighteen consensus sequences in total are represented. All sequence queries returned similarity scores of 0.97 or greater. All identifications are made at the species level except for the uncultured *Methyloversatilis* (*), which is made at the genus level.

Table 3.1. Sampling sites and dates for enrichment cultures and biometer inocula.

Sampling site	Sediment collected	First Bio-Sep batch		Second Bio-Sep batch		Third Bio-Sep batch	
		Deployed	Harvested	Deployed	Harvested	Deployed	Harvested
Wetland sediment	09/09/2004[a]	11/28/2004	02/15/2005[ab]	03/10/2005	04/07/2005[ab]	7/27/2005	9/9/2005[a]
Wetland water column	No sampling	11/28/2004	02/15/2005[b]	03/10/2005	04/07/2005[ab]	7/27/2005	9/9/2005

[a]Inoculum for enrichment cultures.

[b]Inoculum for biometers.

Table 3.2. Genes and PCR primers used in the study.

Gene	Gene size (bp)	Protein	Primers	PCR product size (bp)	Annealing temperature (°C)	Reference
atzA	1,425	Atrazine chlorohydrolase	atzAf-CCATGTGAACCAGATCCT atzAr-TGAAGCGTCCACATTACC	500	55	de Souza et al. (1998)
atzB	1,446	Hydroxyatrazine ethylaminohydrolase	atzBf-TCACCGGGGATGTCGCGGGC atzBr-CTCTCCCGCATGGCATCGGG	500	68	de Souza et al. (1998)
atzC	1,212	*N*-isopropylammelide isopropylamidohydrolase	atzCf-GCTCACATGCAGGTACTCCA atzCr-GTACCATATCACCGTTTGCCA	600	61	de Souza et al. (1998)
atzD	1,092	Cyanuric acid amidohydrolase	atzDf-TCCCACCTGACATCACAAAC atzDr-GGGTCTCGAGGTTTGATTG	200	61	Devers et al. (2004)
atzE	1,374	Biuret hydrolase	atzEf-GAGCCTCTGTCCGTAGATCG atzEr-GATGGCGTGTACCGTTTACC	200	63	Devers et al. (2004)
atzF	1,818	Allophanate hydrolase	atzFf-ACCAGCCCTTGAATCATCAG atzFr-TATTGTCCCGATACCCAACG	200	63	Devers et al. (2004)
trzD	1,125	Cyanuric acid amidohydrolase	trzDf-CCTCGCGTTCAAGGTCTACT trzDr-TCGAAGCGATAACTGCATTG	800	63	Rousseaux et al. (2001)
trzN	1,371	Atrazine chlorohydrolase	trzNf-CACCAGCACCTGTACGAAGG trzNr- GATTCGAACCATTCCAAACG	420	64	Mulbry et al. (2002)

Table 3.3. Occurrence of atrazine catabolic genes in a selection of atrazine-degrading pure cultures from the wetland samples, based on PCR amplification of genomic DNA followed by agarose gel electrophoresis. Similarity refers to 16S rDNA sequence similarity of isolate consensus sequence to nearest match. (+) indicates the presence of a band of the expected size for the catabolic gene being amplified; (-) indicates the absence of a band of the expected size for the catabolic gene being amplified. A question mark denotes a prominent band of an unexpected size for the catabolic gene being screened.

Isolate and accession nos.	Nearest match and accession no.	Similarity (%)	*atzA*	*atzB*	*atzC*	*atzD*	*atzE*	*atzF*	*trzD*	*trzN*
TN196, KM492776-7	*Arthrobacter aurescens* TC1, CP000474	100	-	+	+	-	-	-	-	+
TN197, KM492778-80	*Arthrobacter aurescens* TC1, CP000474	100	-	+	+	-	-	-	-	+
TN92, KM492796-8	*Arthrobacter* sp. 13AGG1, KJ722486	100	-	+	+	-	-	-	-	+
TN98, KM492808	*Arthrobacter* sp. 13AGG1, KJ722486	100	-	+	+	-	-	-	-	+
TN89, KM492792	*Arthrobacter* sp. B3051, JX266390	99	-	+	+	-	-	-	-	+
TN233, KM492772	*Nocardioides* sp. ATD6, AB638612	93	-	-	+	-	-	-	-	+
TN232, KM492771	*Nocardioides* sp. HKT9991, AB498058	91	?	-	+	-	-	-	-	+
TN119, KM492766-7	*Sphingomonas* sp. SaMR12, JN573357	100	+	+	+	-	+	+	-	-
TN231, KM492768-70	uncultured *Methyloversatilis* sp. clone K2S193, JN177630	99	?	-	+	-	-	-	-	+

Table 3.4. Sequence similarities of catabolic genes cloned from atrazine-degrading pure cultures to respective database gene sequences. Query culture numbers correspond to those in Table 3.3. All query lengths reflect the lengths of consensus sequences generated from triplicate clones, unless otherwise noted.

Gene and subject accession no.	Query culture	Query length	Similarity (%)	Query accession no.
atzA, U66917.2	TN119	226	100	KP339971-3
atzA, U66917.2	TN231	244 (2 sequences)	99	KP339958-9
atzA, U66917.2	TN232	253 (1 sequence)	99	KP339955
atzB, U66917.2	TN197	295 (2 sequences)	99	KP339960-1
atzC, U66917.2	TN196	557	99	KP339964-6
atzC, U66917.2	TN231	314 (2 sequences)	100	KP339956-7
atzE, U66917.2	TN119	202 (1 sequence)	100	KP339970
atzF, U66917.2	TN119	213	100	KP339967-9
trzN, CP000475.1	TN196	417 (2 sequences)	99	KP339962-3
trzN, CP000475.1	TN98	429	100	KP339974-6

Chapter 4: Mineralization of atrazine and bacterial diversity in a former pesticide mixing and machinery washing site

4.1 Introduction

Atrazine is in common use as an agricultural herbicide in the U.S. As a consequence, atrazine-degrading microorganisms are widely found in surface soils, rivers and wetlands in agricultural watersheds. Half-lives of atrazine biodegradation can be shorter than 10 days in agricultural soils that have decades of annual atrazine applications (Pussemier et al., 1997). Soil microbial communities vary greatly in atrazine biodegradation potential. The biodegradative pathway has been elucidated in *Pseudomonas* ADP, involving six proteins (AtzA, AtzB, AtzC, AtzD, AtzE, and AtzF) (Souza et al., 1995; Boundy-Mills et al., 1997; Sadowsky et al., 1998; de Martinez et al., 2001; Cheng et al., 2005). Other degradative enzymes have been characterized, such as trzN in *Nocardioides* strain C190 (Mulbry et al., 2002) and *Arthrobacter aurescens* TC1 (Sajjaphan et al., 2004) and trzD in *Pseudomonas* strain NRRLB-12227 (Eaton and Karns 1991) and *Klebsiella pneumonia* strain 99 (Karns and Eaton 1997). Analyses of catabolic genes in soil bacterial isolates suggest that the complete *atz* pathway is relatively rare and various combinations of *atz* and *trz* genes are more common in environmental isolates (Devers et al., 2007; Vaishampayan et al., 2007; El Sebaï et al., 2011). It is likely that pathway intermediates feed into other degradative pathways in microbial communities and the complete mineralization through the Atz pathway may not be dominant.

Examples could include the hydrolytic release of the ring side chains ethanolamine and isopropyl amine, both biodegradable by many microorganisms that cannot degrade the parent molecule atrazine, or ring cleavage metabolites such as urea, an excellent N-source for soil microorganisms. Cooperative pathways and synergistic metabolism of intermediates are central in the attenuation of atrazine by soil microorganisms.

For this study, the potential of biomineralization of atrazine in an agricultural farm setting in central Ohio was examined. Two sites at this farm were of specific interest: (i) a pesticide mixing area that had been abandoned in the mid-1990's, about 10 years prior to this study, and (ii) an adjacent (about 30 m) area that has received spills from washing of pesticide formulations from application equipment in a shed. In general, previous studies have shown that pesticide mixing and spill areas have microbial populations that can actively degrade residual pesticide mixtures (Udiković-Kolić et al., 2010). Potential problems of point source pesticide contamination in agricultural farm sites have been mitigated with systems whereby the spray or washing area is covered with rich organic material which supports pesticide retention and microbial degradation. Several configurations and performance data have been discussed in the literature (Arthur et al., 2000; De Wilde et al., 2007; del Pilar Castillo et al., 2008; Spanoghe et al., 2009).

At the farm site in this study, atrazine and other pesticides have been used on and off depending on crop rotation and integrated pesticide management practices. No special remediation was undertaken over the years of practicing pesticide disposal until the mid-1990's, at which time the collection of liquid pesticide residues and storage was initiated. The purpose of this study was to determine the half-lives of aerobic and anaerobic biomineralization of atrazine in soil samples from the past pesticide mixing and

machinery washing areas. Attempts were also made to enrich for atrazine degraders from the soil samples and to isolate pure cultures with atrazine as the selective substrate. Phylogenetic analyses based on 16S rRNA gene sequences and the screening of known catabolic genes of atrazine metabolism were included in the scope of this study.

4.2 Materials and Methods

4.2.1 Sampling

The Western Agricultural Research Station of the Ohio Agricultural Research and Development Center is located 5.6 km northwest of South Charleston, Ohio. The farm (est. 1958) is a 173 ha agricultural research facility that supports agronomic and specialty crop programs, including corn and soy, important to farmers in the western part of Ohio. As part of the weed control programs, the management practice has involved the application of atrazine as a pre-emergence weed killer in corn fields.

The farm site has a shed building where herbicide formulations were stocked and prepared for transportation to the field. Next to the shed was a grassy area (Figure 4.1) where pesticides were mixed, and this area received numerous pesticide spills over the years. A second adjacent grassy area was at the base of the ramp to the shed where pesticide application machinery was rinsed. About 10 years prior to this study, the practice of allowing spills and rinsing outdoors was discontinued and liquid waste was contained to a special holding tank for further treatment. A previous study of enrichment of atrazine-degraders from the farm yielded a soil bacterial isolate M91-3 (Radosevich et al., 1995), subsequently identified as *Ralstonia basilensis* (Stamper et al., 2002), obtained originally from samples collected from the spill site.

Top soil samples (0-7 cm) were removed with a sterile trowel and stored in sterile plastic bags until processed in the laboratory (Table 4.1). The sampling avoided grass roots. Soils at the sites sampled are primarily Strawn-Crosby complex (0% to 2% slopes) (http://websoilsurvey.nrcs.usda.gov/app/WebSoilSurvey.aspx). Soil-borne atrazine-degraders were also probed with porous Bio-Sep beads (Ghosh et al., 2009; Omotayo et al., 2011). The beads were equilibrated in solution of 2 mg atrazine/l or distilled water for 3 days before they were closed in sterile nylon mesh bags (~30 beads per bag) and sealed with plastic clips. Bead bags were buried in the top 10 cm layer of soil at the former pesticide mixing area and retrieved after 33 days.

4.2.2 [U-ring-^{14}C]-atrazine mineralization

Aerobic and anaerobic mineralization of atrazine in soil samples and Bio-Sep beads was measured in biometers, which were constructed as previously described (Radosevich et al., 1996). The biometers contained 5 g soil samples or 6-10 diced Bio-Sep beads and 0.064 μmol [U-ring-^{14}C]-atrazine ± glucose (11.1 mM). In order to prevent possible microelement limitation, Bio-Sep bead inoculated biometers received 5 mL basal mineral salt solution, which contained (per liter): K_2HPO_4, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; $FeCl_3 \cdot H_2O$, 0.1 mg; $CaCl_2 \cdot H_2O$, 1 mg; $MnCl_2$, 0.01 mg; $ZnCl_2$, 1 μg; pH 6.8. Anaerobic incubations under N_2 head space involved 25 μmol nitrate as KNO_3, 125 μmol Fe(III) as ferrihydrite, or 15 μmol sulfate as Na_2SO_4, or distilled H_2O as a blank per biometer as external electron acceptors. All biometers were incubated at 22±2 °C in the laboratory. Biometer sampling and counting of radioactivity have been previously described (Douglass et al., 2014). $^{14}CO_2$ evolution was calculated as a percentage of the total amount of [U-ring-^{14}C]-atrazine added to the biometers at the

beginning of the time course. Cumulative percentages were calculated by adding the values of each data point to the previous data point. Rate constants and half-lives of atrazine mineralization were determined with a pseudo first-order rate expression (Guerin and Boyd, 1992).

4.2.3 Enrichment culture conditions

Aerobic enrichment cultures were set up with 2.5 g of soil or 6-10 Bio-Sep beads in mineral salt media containing 30 mg/l atrazine ± glucose amendment (2 g/l) and were incubated in shake flasks (150 rev/min) at 22±2 °C. Aliquots of enrichment cultures were transferred to the corresponding fresh media at intervals of 2-4 weeks.

4.2.4 HPLC analysis of enrichment cultures

After at least six transfers to fresh basal mineral salt solution with 30 mg/l atrazine, enrichment cultures were screened for residual atrazine concentration. Aliquots (200 µl) of cultures were mixed with 1.8 mL of mobile phase (65% HPLC-grade acetonitrile in HPLC-grade distilled water) followed by centrifugation at 16,000 x g for 15 min. The supernatant was transferred to an HPLC vial in a Thermo Separation Products AS100 autosampler (Thermo Fisher Scientific, Waltham, MA). Samples were pumped through an Adsorbosphere C18 HPLC column at a flow rate of 1.0 mL/min for 9 min using a Shimadzu LC-10AT pump, and detected by a Shimadzu SPD-10 AV UV/Vis detector at 220 nm. Chromatographs were generated using a Shimadzu CBM-20A communications bus module and recorded with EZStart software (Shimadzu, Columbia, MD). Reference samples containing uninoculated sterile media and varying concentrations of atrazine dissolved in mobile phase were included with every HPLC run.

Enrichments that exhibited at least 80% decrease in atrazine concentration were deemed to contain atrazine-degrading mixed cultures.

4.2.5 Isolation of atrazine-degrading pure cultures

Prospective enrichment cultures of atrazine-degraders were inoculated to solid media. Dilutions of 10^{-3} to 10^{-5} for cultures enriched without glucose and dilutions of 10^{-6} to 10^{-8} for cultures enriched with glucose were spread-plated in atrazine + mineral salts media solidified with 1.5% Noble agar. All plates were incubated at 22±2 °C. Plates were incubated for up to 2 weeks. Discreet colonies from the spread plates were subcultured twice on streak plates before transferring them into 5 mL of corresponding liquid media. Changes in atrazine concentration were monitored during subsequent incubation in liquid media. Pure cultures were stored at 4 °C. Liquid cultures were stored as 20% glycerol stocks at -55 °C.

4.2.6 DNA isolation and 16S rRNA gene amplification

Genomic DNA was isolated from pure and mixed cultures with MoBio UltraClean Microbial DNA Isolation Kits per manufacturer's instructions (MoBio Laboratories, Carlsbad, CA). The V3 region of the 16S rRNA gene from atrazine-degrading mixed cultures was amplified in 50 µl total volume, consisting of 1X TaKaRa Premix Taq (1.25 U *Taq*; 0.2 mM dATP; 0.2 mM dTTP; 0.2 mM dCTP; 0.2 mM dGTP; 10 mM tris-HCl [pH 8.3], 50 mM KCl, 1.5 mM $MgCl_2$), 1 µM primer 357fGC (5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CAG-3'), 1 µM primer 518r (5'-ATT ACC GCG GCT GCT GG-3') (Muyzer et al., 1993), and 20 ng of template DNA. The PCR amplification involved (1) 5 min at 94 °C, (2) 30 sec at 94 °C, (3) 30 sec at 61 °C -0.5C/cycle, (4) 1 min at 72 °C, (5) 9 times

to (2), (6) 30 seconds at 94 °C, (7) 30 sec at 56 °C, (8) 1 min at 72 °C, (9) 24 times to (6), (10) 7 min at 72 °C, and (11) storage at 4 °C.

PCR products were analyzed via denaturing gradient gel electrophoresis (DGGE) using the DCode Universal Mutation Detection System (Bio-Rad, Hercules, CA) per manufacturer's instructions. Gels consisted of 8% acrylamide and a 40%-60% denaturant gradient. Gels were electrophoresed at 83 V for 16 h in 0.5X TAE buffer solution (20 mM Tris base, 10 mM acetic acid, and 0.5 mM EDTA) at 60 °C. After electrophoresis, gels were stained in 1X SYBR Gold (Molecular Probes, Eugene, OR) for 30 min on a rocking platform and visualized on a UV transilluminator. Mixed cultures corresponding to various denaturing gradient gel electrophoresis banding patterns were chosen for further investigation via 16S rDNA clone library sequencing (Appendix B).

4.2.7 16S rDNA clone library formation and analysis

Genomic DNA from atrazine-degrading mixed cultures and isolates was amplified for the approximately 1,500 bp region of the 16S rDNA. PCR reactions were conducted as outlined in section *4.2.6.* with 1 μM primer 27f (5'- AGA GTT TGA TCM TGG CTC AG -3'), 1 μM primer 1525r (5'-AAG GAG GTG WTC CAR CC-3') (Lane, 1991), and 20 ng of template DNA. The protocol with the thermal cycler involved (1) 2 min at 94 °C; (2) 50 sec at 94 °C; (3) 50 sec at 55 °C; (4) 1 min 30 sec at 72 °C; (5) 31 times to (2); (6) 5 min at 72 °C; and (7) storage at 4 °C.

PCR products were used to construct 16S rDNA clone libraries. The clones were made using the Promega pGEM–T vector system and JM109 high efficiency competent cells per manufacturer's instructions (Promega, Madison, WI). Overnight cultures of clones were plated onto 96-well plates and sent to the Clemson University Genomics

Institute for sequencing. For each pure culture, three clones were sequenced; for each mixed culture, 25 clones were sequenced. Sequences were edited in Geneious. A consensus sequence was generated for each set of 3 clones resulting from pure cultures when possible, and these consensus sequences were entered into the NCBI-BLAST and RDP databases. Otherwise, individual edited clone sequences or consensus sequences constructed from two clones were entered as queries into the aforementioned databases. Likewise, each individual edited clone sequence resulting from mixed cultures was searched in these databases.

The 16S rRNA gene sequences were deposited with GenBank. The accession numbers for aerobic mixed culture clones are KP779907- KP779962. The accession numbers for aerobic isolates are KP779963- KP779997.

4.2.8 Atrazine catabolic gene detection in atrazine-degrading pure cultures

Genomic DNA from atrazine-degrading isolates was amplified for known atrazine catabolic genes *atzA*, *atzB*, *atzC*, *atzD*, *atzE*, *atzF*, *trzD*, and *trzN*. *Escherichia coli* genomic DNA was also amplified as a negative control for all catabolic genes. *Pseudomonas* ADP was used as a positive control for *atzA*, *atzB*, *atzC*, *atzD*, *atzE* and *atzF*, and as a negative control for *trzD* and *trzN*. *Arthrobacter aurescens* TCI was used as a positive control for *trzN* as well as an additional positive control for *atzB* and *atzC* and a negative control for *atzA*, *atzD*, *atzE*, and *atzF*. *Klebsiella pneumoniae* 99 was used as a positive control for *trzD*.

PCR reactions were conducted with 1 μM forward primer and 1 μM reverse primer as specified in Table 3.2 and 20 ng template DNA. The protocol with the thermal cycler involved (1) 4 min at 94 °C; (2) 30 sec at 94 °C; (3) 1 min at each primer's

annealing temperature from Table 3.2; (4) 2 min at 72 °C; (5) 33 times to (2); (6) 15 minutes at 72 °C; and (7) storage at 4 °C. PCR products were loaded onto agarose gel, and after electrophoresis the gel was stained with ethidium bromide and visualized with a UV gel imager. Detection of a band of anticipated size was treated as preliminary evidence of the presence of the given catabolic gene, and a random assortment of these PCR reactions were used to create clone libraries as outlined in *4.2.7*. For each PCR reaction, three clones were sequenced. When possible, a consensus sequence for these three clone sequences was generated and used for bioinformatics analysis; otherwise, a consensus sequence of 2 clones was generated, or a single edited clone sequence was used. Query sequences were entered into NCBI-BLASTN and aligned with their corresponding catabolic gene sequences. Catabolic gene sequences were deposited with GenBank, and the accession numbers are KP863790- KP863798.

4.3 Results and Discussion

4.3.1 Measurement of atrazine mineralization with [U-ring-^{14}C]-atrazine

Under aerobic conditions, mineralization of atrazine in both sites reached ~20-25% (Figure 4.2). Glucose amendment had no discernible effect on mineralization. Soil from the pesticide mixing area mineralized slightly more atrazine than did soil from the machinery washing area. Mineralization in biometers inoculated with Bio-Sep beads was comparable to sterile controls (Figure 4.2). Failure to observe significant $^{14}CO_2$ evolution from Bio-Sep bead biometers was most likely due to an insufficient quantity of atrazine-degrading microbial inoculum provided by the small number of beads used in this assay. This assertion is supported by the fact that, after enrichment in liquid medium, mixed cultures derived from Bio-Sep beads readily degraded atrazine as confirmed via HPLC

analysis. Atrazine-degrading pure cultures were also successfully isolated from mixed cultures derived from Bio-Sep beads.

Under anaerobic conditions, pesticide mixing area soil outperformed machinery washing area soil in biometers with iron amendment (~20% vs. 6%) and no electron acceptor amendment (~18% vs. 7%). The opposite trend was true in the nitrate amended biometers (~26% vs. 22%). Microbial communities in both soils mineralized atrazine to a similar extent (~20%) under sulfate amendment (Figure 4.3). The wash area soil mineralized less than 10% of the added atrazine in the absence of external electron acceptors as well as in ferrihydrite-amended biometers. The reduction of nitrate, Fe(III) and sulfate was not tested in the biometer experiments. In general, anaerobiosis was not prohibitive to atrazine biodegradation, but biometers constructed with samples from the former pesticide mixing area had large variation between duplicate anaerobic biometers.

The half-lives of aerobic mineralization of atrazine calculated from the first order rate constants indicated relatively fast atrazine attenuation, about 3 days in the soil samples from the washing area and 2 days in the pesticide mixing area (Table 4.2). These half-lives clearly indicate that soil microorganisms at these sites have previously acclimated to atrazine. The half-lives of anaerobic mineralization in the washing area were 4 days under nitrate amended conditions, but were extended to 11-34 days in the absence of external electron acceptors or in the presence of Fe(III) or sulfate. In contrast, anaerobic mineralization in soil samples from the pesticide mixing area were in the range of 3 to 5 days (Table 4.2).

4.3.2 16S rRNA gene sequence based analysis of atrazine-degrading enrichment cultures and bacterial isolates

Aerobic enrichment of atrazine degraders from the soil and Bio-Sep bead samples from the washing and mixing areas yielded mixed cultures which consistently depleted atrazine in the growth media upon subcultures. Three of these mixed cultures that were derived from soil samples were selected for further analysis based upon DGGE profiles. Subsequent amplification of 16S rRNA genes from the mixed cultures yielded 49 clones for sequencing. Sequence data from 16S rDNA clones was queried in the RDP database for nearest matches. As shown in Figure 4.4, most clones (≥90% similarity match) were consistent with *Variovorax* (51%) and *Schlesneria* (16%) spp. Other clusters included *Acidovorax* and *Legionella* spp. (8% each). Some clones could only be matched at a family (*Phyllobacteriaceae*, 2%) or class (*Alphaproteobacteria*, 6%) level. *Variovorax* species have previously been isolated from atrazine-degrading mixed cultures constructed from agricultural soils (Smith et al., 2005; Zhang et al., 2012).

Mixed cultures that demonstrated atrazine degradation via HPLC analysis were also subsequently plated onto analogous solidified media. Twelve pure cultures were isolated from these plates. DNA was extracted and amplified for 16S rRNA genes; amplification products were cloned and sequenced. Figure 4.5 summarizes the RDP nearest matches (≥96% similarity) of the resulting 16S rDNA consensus sequences. *Arthrobacter* spp. dominated the pure culture collection (83%; 10/12), with *Arthrobacter* sp. B3051 matching the largest number of isolates (25%; 3/12). *Arthrobacter* spp. have previously been isolated from soils from around the world that have been exposed to atrazine (Aislabie et al., 2005; Vaishampayan et al., 2007; Vibber et al., 2007; Zhang et

al., 2011). A single match was also made with each of *Hydrogenophaga* sp. Gsoil 1545 and *Sinorhizobium* sp. TJ170.

4.3.3 Detection of atz *and* trz *catabolic genes*

The presence of atrazine catabolic genes was investigated in 7 of the 12 pure culture isolates by PCR amplification and agarose gel visualization (Table 4.3). All of the isolates investigated were shown to contain *atzBC*. Additionally, an *Arthrobacter* sp. M2012083 was demonstrated to contain *atzBC-trzN*, a pattern which has been previously reported in the literature (Devers et al., 2004; Sajjaphan et al., 2010; Zhang et al., 2011). *Hydrogenophaga* sp. Gsoil 1545 was shown to contain *atzBC-trzD*, *atzA* and *trzN*. *Hydrogenophaga* spp. have previously been isolated from atrazine-degrading river sediment samples (Vargha et al., 2005) and have been detected along with a *trzN-atzABC-trzD* pattern of catabolic genes in soil from an agrochemical factory (Udiković-Kolić et al., 2010). No isolates were shown to contain *atzDEF*. Three of these successfully amplified catabolic genes were chosen for subsequent cloning and sequencing (Table 4.4). BLASTN searches against respective database catabolic gene sequences yielded similarity scores in the range of 99-100% and query coverage values of 100%.

4.4 Conclusions

The biomineralization of atrazine was comparable in both sites of soil samples, demonstrating that the soils contained active atrazine-metabolizing microbial communities. While the reduction of external electron acceptors was not confirmed in ^{14}C-atrazine containing soil biometers, the data showed response of atrazine biomineralization to electron acceptor conditions. The results from the anaerobic

biometer incubations displayed considerable differences between duplicates, suggesting uneven distribution of atrazine-metabolizing cells. *Variovorax* spp. in enrichment cultures and isolates of *Arthrobacter* spp. were particularly dominant. Arthrobacteria may have been selected due to their resistance to possible desiccation and nutrient deprivation conditions in soil and sampling, and stringent growth conditions for isolation in the laboratory. There was no evidence for the existence of the complete Atz pathway among the isolates, suggesting that atrazine metabolism involves multiple microorganisms that have pathways diverging from atrazine metabolites.

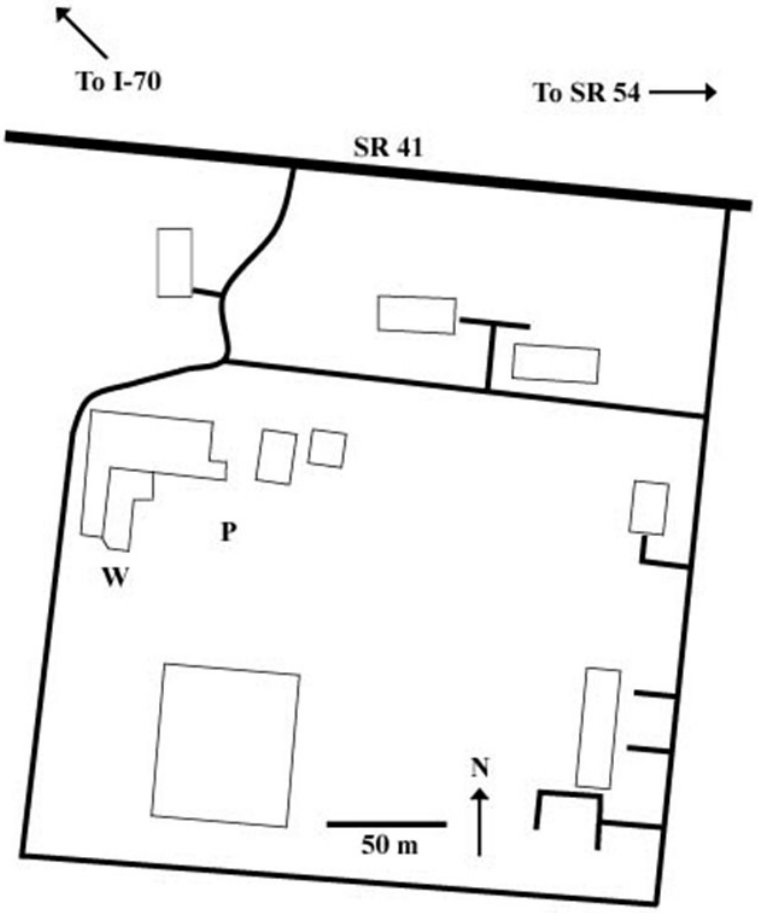

Figure 4.1. Sampling sites at the Western Agricultural Research Station. P = former pesticide mixing area, W = former machinery washing area.

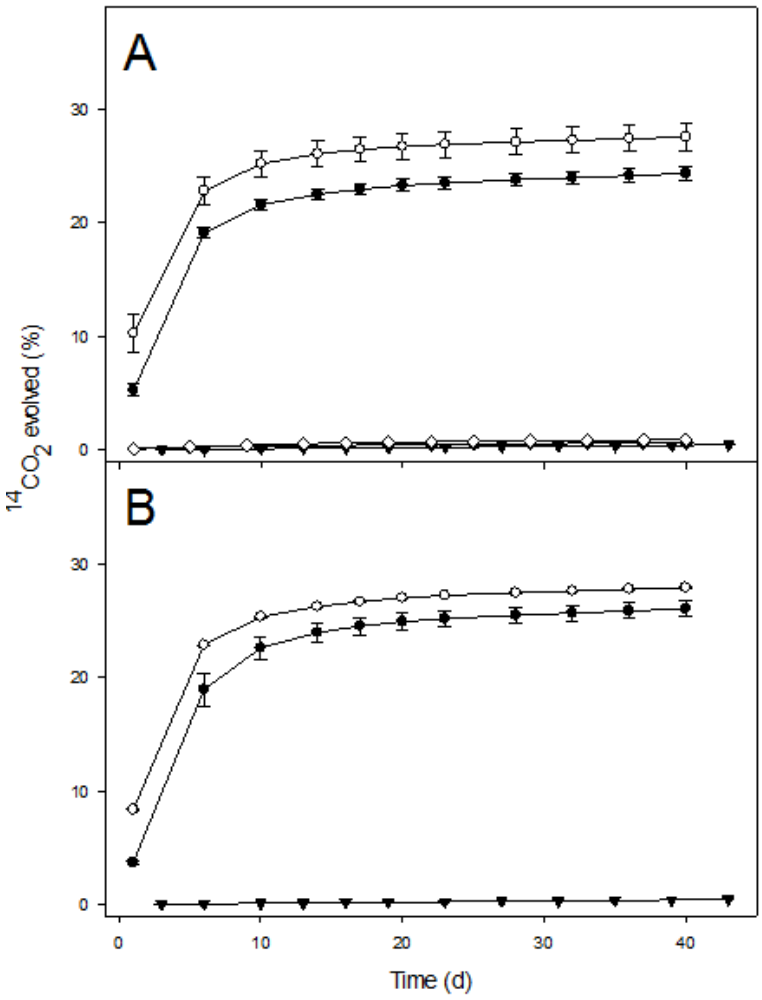

Figure 4.2. Aerobic mineralization of [U-ring-14 C]-atrazine in biometers in the absence (A) and presence (B) of glucose amendment. Symbols: (●), soil from a former machinery washing area; (○), soil from a former pesticide mixing area; (▼), sterile soil control; (◆), atrazine-soaked Bio-Sep beads retrieved from a former pesticide mixing area; (◇), water-soaked Bio-Sep beads retrieved from a former pesticide mixing area. Bio-Sep beads were only used to inoculate biometers without glucose. Data points represent mean cumulative percentages obtained from duplicate biometers, with the exception of those representing Bio-Sep biometers, which represent results from single biometers. Bars indicate actual percentages obtained from each replicate, and in some cases are smaller than the data point symbols.

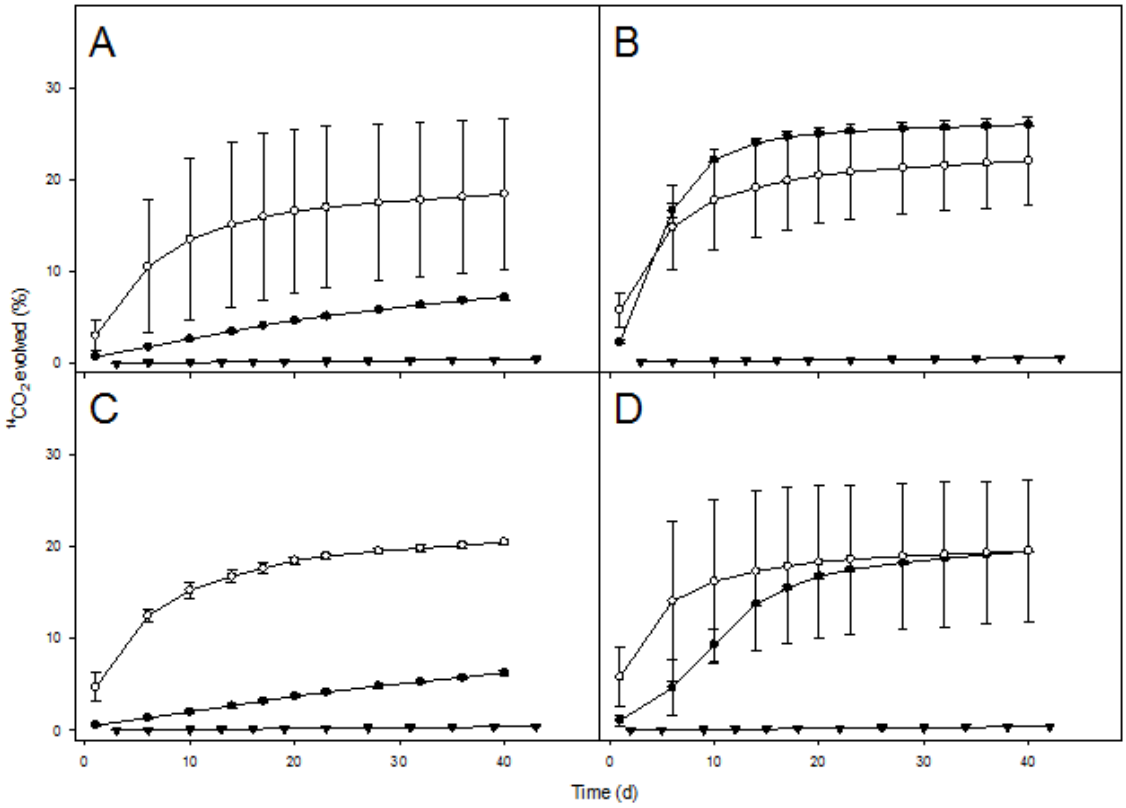

Figure 4.3. Anaerobic mineralization of [U-ring-14 C]-atrazine in biometers. The biometers were amended with (A) no electron acceptor, (B) K-nitrate, (C) ferrihydrite, and (D) Na-sulfate. Symbols: (●), soil from a former machinery washing area; (○), soil from a former pesticide mixing area; (▼), sterile soil control. Data points represent mean cumulative percentages obtained from duplicates. Bars indicate actual percentages obtained from each replicate, and in some cases are smaller than the data point symbols.

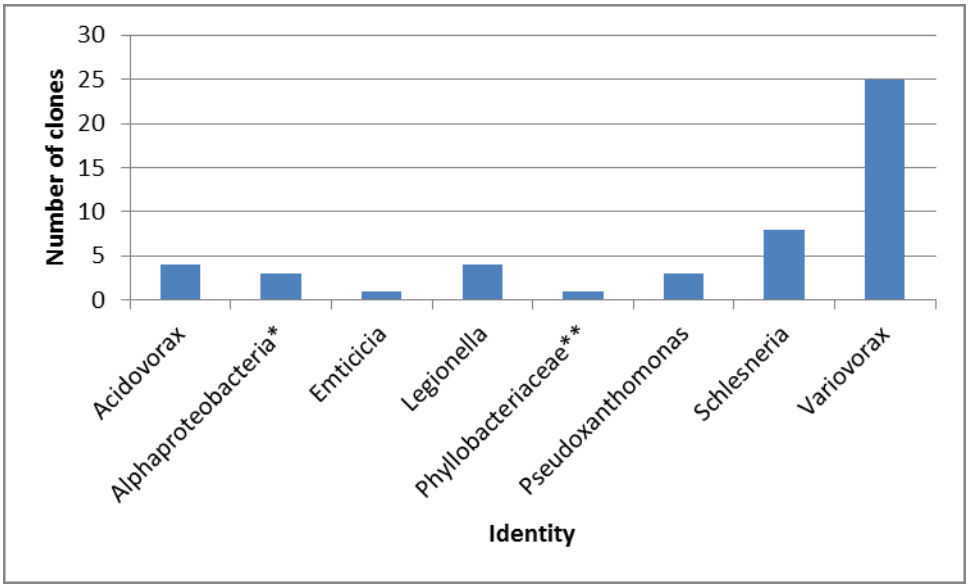

Figure 4.4. Identification of 16S rDNA clones from atrazine-degrading aerobic mixed cultures. Forty-nine clones in total are represented. All sequence queries returned similarity scores of 0.90 or greater. All identifications are made at the genus level except for *Alphaproteobacteria* (*) at the class level and *Phyllobacteriaceae* (**) at the family level.

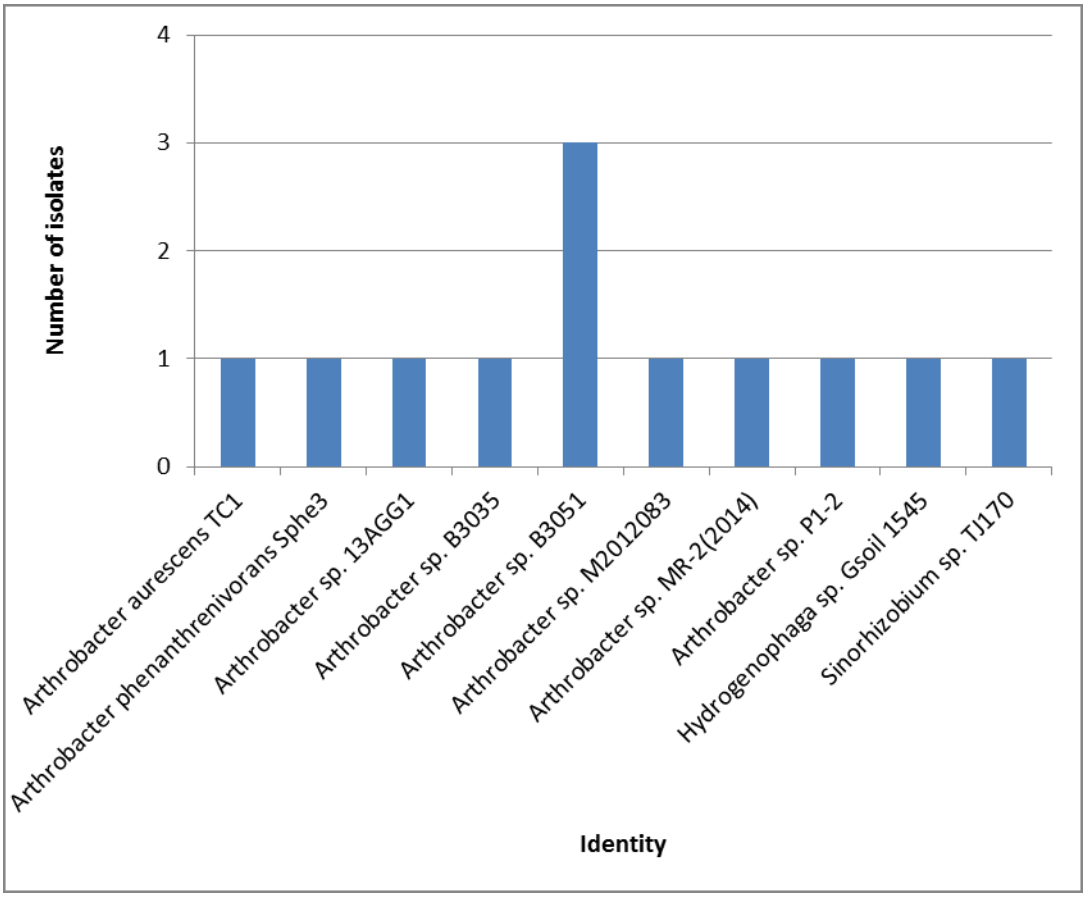

Figure 4.5. Identification of 16S rDNA clones from atrazine-degrading aerobic pure cultures. Eleven consensus sequences in total are represented. All sequence queries returned similarity scores of 0.97 or greater. All identifications are made at the species level.

Table 4.1. Sampling sites and dates of sampling for biometer inocula. Hyphens denote no sampling occurred. Mix area = former pesticide mixing area at the OSU Western Agricultural Research Station (WARS). Wash area = machinery washing area at the OSU Western Agricultural Research Station (WARS).

Sample	Soil collected	Bio-Sep beads deployed	Bio-Sep beads harvested
Former machinery washing area	05/17/2005	-	-
Former pesticide mixing area	05/17/2005	05/17/2005	6/18/2005

Table 4.2. Kinetic parameters and half-lives of atrazine in soils collected from a former farm machinery washing area and a former pesticide mixing area at the Western Agricultural Research Station. Calculations are based on the biometer experiments shown in Figures 4.2 and 4.3.

Sample	Additional amendment	Incubation conditions	k (d^{-1})	R^2	$t_{1/2}$ (d)
Former machinery washing area	None	Aerobic	0.256	0.994	2.7
Former machinery washing area	Glucose	Aerobic	0.208	0.995	3.3
Former machinery washing area	Nitrate	Anaerobic	0.172	0.991	4.0
Former machinery washing area	Sulfate	Anaerobic	0.065	0.973	11
Former machinery washing area	Iron	Anaerobic	0.020	0.995	34
Former machinery washing area	None	Anaerobic	0.031	0.995	23
Former pesticide mixing area	None	Aerobic	0.397	0.971	1.7
Former pesticide mixing area	Glucose	Aerobic	0.317	0.990	2.2
Former pesticide mixing area	Nitrate	Anaerobic	0.195	0.973	3.5
Former pesticide mixing area	Sulfate	Anaerobic	0.235	0.961	2.9
Former pesticide mixing area	Iron	Anaerobic	0.155	0.972	4.5
Former pesticide mixing area	None	Anaerobic	0.139	0.993	5.0

Table 4.3. Occurrence of atrazine catabolic genes in a selection of atrazine-degrading pure cultures from the Western Agricultural Research Station samples, based on PCR amplification of genomic DNA followed by agarose gel electrophoresis. The designation "n/a" indicates the given column heading is not applicable. Similarity refers to 16S rDNA sequence similarity of isolate consensus sequence to nearest match. (+) indicates the presence of a band of the expected size for the catabolic gene being amplified; (-) indicates the absence of a band of the expected size for the catabolic gene being amplified; (+/-) indicates the presence of a band of the expected size for the catabolic gene being amplified, the intensity of which is approximately less than one quarter that of the positive control band for the corresponding gene.

Isolate and accession nos.	Nearest match and accession no.	Similarity (%)	*atzA*	*atzB*	*atzC*	*atzD*	*atzE*	*atzF*	*trzD*	*trzN*
TN134, KP779963-5	*Arthrobacter* sp. M2012083, KF893303	100	-	+	+	-	-	-	-	+
TN151, KP779972-4	*Arthrobacter* sp. B3051, JX266390	99	-	+	+	-	-	-	-	-
TN154, KP779981-3	*Arthrobacter* sp. B3035, JX266392	99	-	+	+	-	-	-	-	-
TN155, KP779984-5	*Arthrobacter* sp. B3051, JX266390	99	-	+	+	-	-	-	-	-
TN159, KP779986-8	*Sinorhizobium* sp. TJ170, AJ505297	96	-	+	+	-	-	-	-	-
TN161, KP779989-91	*Arthrobacter phenanthrenivorans* Sphe3, NR_074770	98	-	+	+	-	-	-	-	-
TN164, KP779995-7	*Hydrogenophaga* sp. Gsoil 1545, AB271047	99	+/-	+	+	-	-	-	+	+/-
Escherichia coli, n/a	n/a	n/a	-	-	-	-	-	-	-	-
Pseudomonas ADP, n/a	n/a	n/a	+	+	+	+	+	+	-	-
Arthrobacter aurescens TCI, n/a	n/a	n/a	-	+	+	-	-	-	-	+
Klebsiella pneumoniae 99, n/a	n/a	n/a	-	-	-	-	-	-	+	-

Table 4.4. Sequence similarities of catabolic genes cloned from atrazine-degrading pure cultures to respective database gene sequences. Query culture numbers correspond to those in Table 4.4. All query lengths reflect the lengths (in nucleotides) of consensus sequences generated from triplicate clones.

Gene and subject accession no.	Query culture	Query length (nt)	Similarity (%)	Query accession nos.
atzB, U66917.2	TN164	454	100	KP863793-5
atzC, U66917.2	TN155	574	99	KP863790-2
trzD, AF086815.2	TN164	584	100	KP863796-8

Chapter 5: General conclusions and future directions

Biometer experiments conducted in the course of this study differed in source of inoculum and culture conditions. Samples used as inoculum for biometers included soils from pesticide spill sites (including a former pesticide mixing area and a former pesticide-spreading machinery washing area), wetland sediments, and river water. In addition, Bio-Sep beads were tested in biometers after their pre-incubation in the aforementioned locations. Biometers constructed from each of these sample types were incubated both in the presence and absence of oxygen. Aerobically incubated biometers were constructed with and without the addition of glucose to the samples. Glucose as well as acetate and citrate have been used as a non-selective substrate in atrazine biodegradation experiments in previously published studies (e.g., Chung et al., 1995, 1996). Glucose was selected in this study because it is a general purpose, non-selective substrate and may yield a flux of metabolites and is also readily fermentable under anaerobic conditions. Anaerobically incubated biometers were not amended with glucose, but were amended with either water or potassium nitrate, sodium sulfate, or ferric iron in order to provide terminal electron acceptors for possible anaerobic atrazine-mineralizing microbes. Because of the safety measures due to the ^{14}C-labelled atrazine no effort was made to determine whether these electron acceptors were reduced during the time course.

Amendment of aerobic biometers with glucose produced varying results in different samples. While total atrazine mineralization slightly increased with glucose amendment in the former machinery washing area soil and the river water, there was virtually no difference in total mineralization associated with glucose amendment in the soil from the former pesticide mixing area. Glucose amendment corresponded with a decrease in total mineralization in the wetland sediment. The first order rate constant for mineralization decreased ~50%, ~20%, and ~10% upon glucose amendment of the river water, pesticide spill site soils, and wetland sediment, respectively.

Anaerobic biomineralization varied greatly across sampling sites. Biodegradation in the wetland and in the spill sites was moderately sensitive to the electron acceptor provided. Wetland sediment biometers reached maximum biomineralization in a shorter period of time in the presence of ferrihydrite than under any other electron acceptor treatment. Biomineralization in soil from the former machinery washing area was retarded in the absence of additional electron acceptors and in the presence of ferrihydrite, but was augmented in the presence of potassium nitrate and sodium sulfate. Because final accounting of terminal electron acceptor usage was not performed, the changes in biomineralization cannot be conclusively attributed to the use of the supplemental as electron acceptors. It is plausible that they were providing for nutritional deficiencies in the samples or were relatively fast depleted by other soil microorganisms.

While atrazine biodegradation occurred in most samples, 100% mineralization was not achieved in the biometers. There can be a variety of reasons for this lack of total mineralization. Atrazine or its metabolites may have become unavailable due to sorption

on soil particles and partitioning to nonpolar phases. Microbes may have exhausted critical nutrients necessary to sustain atrazine biomineralization. This is unlikely, however, because it has been previously reported that trace nutrient solutions do not enhance the biomineralization in biometers. As discovered in the catabolic gene screening assays, very few isolates in this study contained the known lower pathway atrazine catabolic genes. The only gene detected from the lower pathway was *trzD*. Since $^{14}CO_2$ is only evolved from the steps including and following ring cleavage of cyanuric acid in [U-^{14}C-ring]-atrazine, the biometer assay does not report on upper pathway biodegradative activity. It is likely that the ~30% maximum CO_2 evolution in the biometer experiments is the result of only 1/3 of the ^{14}C was liberated from the cyanuric acid ring.

In order to fully appreciate the information provided by the biometer assays in this study, two main components would need to be incorporated in future experiments. First of all, a carbon mass balance would need to be performed to ensure that all radiolabeled carbon can be attributed to either CO_2 or atrazine that has not been fully mineralized. Secondly, metabolite analysis in the biometers would need to be performed to elucidate degradation short of complete mineralization of the parent compound. These data could then be matched with the presence of known pathway genes of atrazine mineralization in an effort to assess whether the full pathway is the main route of carbon from atrazine. Interactions of Atz with other catabolic pathways leading to additional anabolic or catabolic reactions of ^{14}C could also help explain the lack of total biomineralization as measured by the biometer technique with [U-^{14}C-ring]-atrazine.

The same samples that were used to construct the biometers in this study were also enriched for atrazine degraders in liquid culture. Enrichment cultures conditions resembled biometer conditions with regards to absence or presence of additional carbon source and electron acceptors, and were maintained aerobically and anaerobically. A collection of mixed cultures was obtained that was deemed to degrade atrazine based upon HPLC confirmation of the removal of the parent compound (atrazine) from the growth media. The metabolites remaining in the spent growth media (and therefore the extent to which atrazine was degraded in these cultures) were not within the scope of the study. For a selection of these atrazine-degrading mixed cultures, 16S rDNA clone libraries were constructed to determine the identities of community members.

The atrazine-degrading mixed culture collection amassed in this study is dominated by a handful of genera, most of which have been previously associated with atrazine biodegradation. *Acidovorax*, *Hydrogenophaga, Ralstonia*, *Variovorax*, and *Xanthobacter* were among the most frequently occurring genera. The detection of a genus in the atrazine-degrading mixed cultures does not necessarily imply that this genus is responsible for the atrazine biodegradation exhibited by the mixed culture. Members of an identified genus may utilize metabolites from other mixed culture community members, or may represent non-growing or dead cells carried over from previous enrichments.

In part to determine whether these genera contributed to the atrazine biodegradation exhibited by these mixed cultures, the mixed cultures were plated, and resulting isolates were screened using the same HPLC criteria for atrazine biodegradation

as was used for mixed cultures. Atrazine-degrading isolates were identified via 16S rDNA sequence analysis. Almost the entire collection was identified as various *Arthrobacter* species. This is a significant finding, since *Arthrobacter* spp. are entirely absent from all mixed culture 16S rDNA clone library search results. One possible scenario explaining these results is that a relatively small number of *Arthrobacter* spp. in the mixed cultures is responsible for creating atrazine metabolites that were in turn used by a larger number of miscellaneous community members. When subjected to plating and isolation on solid media, bacteria possessing the upper atrazine catabolic pathway (as many *Arthrobacter* spp. do (Devers et al., 2004; Sajjaphan et al., 2010; El Sebai et al., 2011; Zhang et al., 2011, Wang and Xie, 2012)) would be better equipped to survive on media containing atrazine as the sole carbon and energy source and therefore would dwarf the detection of bacteria containing only the lower atrazine catabolic pathway or no atrazine catabolic genes at all. Another possible explanation for this inconsistency between the mixed culture and isolate collections is that *Arthrobacter* spp. are both ubiquitous environmental bacteria and generally considered to be hardy. While *Arthrobacter* numbers may have been dwarfed in the mixed culture 16S rDNA clone libraries, these attributes may have allowed arthrobacters to achieve its high representation in the isolate collection at the expense of less hardy bacteria.

Attempts were made at isolating fungal atrazine degraders. Enrichment cultures were constructed in a mineral salts medium modified to optimize fungal growth and with chloramphenicol added to suppress bacterial growth and favor indigenous fungal atrazine degraders. Samples were also plated on solidified chloramphenicol-containing media.

Neither approach led to the detectable cultivation of atrazine-degrading fungi. Either fungi capable of atrazine degradation were not present in these samples, or they were not readily cultivated with the media utilized.

Atrazine-degrading anaerobic mixed cultures were plated on comparable solid media in an attempt to isolate atrazine-degrading bacteria. Plates were incubated in anaerobic Gas-Pak jars. Although isolates did grow on some of these plates, subsequent HPLC screening did not reveal that any isolates were degrading atrazine. It is speculated that these isolates may have involved oligotrophic bacteria that were living off of impurities in the media. Given that anaerobic atrazine biodegradation was exhibited by the originating mixed cultures, the inability to isolate anaerobic atrazine-degrading pure cultures must be a result of a synergistic microbial relationship occurring in the mixed cultures that becomes unstable when community members are separated from one another. Moreover, denitrifiers are facultative anaerobes and may have included some arthrobacters, but this was not investigated any further. One population that was not sufficiently accounted for in this study was microaerophiles. Since relatively shallow soil samples were used in this study, and since Bio-Sep beads were similarly shallowly buried, it stands to reason that the oxygen availability in the sampled environments would favor the growth of microaerophilic bacteria. Future studies involving such samples should include incubation conditions conducive to the cultivation of microaerophiles, such as the use of a controlled atmosphere growth chamber. A gradient enrichment system, perhaps in vertical simulation of soil horizons O and A, may be ideal for this purpose, with pO_2 ranging from ambient to microaerophilic and anoxic conditions.

Serial dilutions of in situ incubated Bio-Sep beads were also plated on solid media and incubated aerobically in an attempt to isolate atrazine-degrading bacteria. Many isolates were obtained in this plating scheme; however, none was found to degrade atrazine. If any atrazine degraders existed on these plates, the background growth of non-degraders made the direct plating of Bio-Sep bead serial dilutions an inefficient way of culturing them.

A selection of atrazine-degrading isolates obtained in this study was screened for the atrazine catabolic genes *atzABCDEF* and *trzDN*. Isolate genomic DNA was PCR amplified with primers culled from the atrazine literature and visualized on agarose gel as preliminary proof of the presence of each catabolic gene (Appendix B). A subset of these PCR reactions that produced a visible band upon UV illumination of stained gels were cloned, sequenced, and aligned to database sequences of the respective catabolic genes.

Not a single isolate was shown to contain all of the aforementioned genes, nor was any isolate shown to contain an assemblage of these genes that would be necessary to completely mineralize atrazine. The KEGG ortholog information for the atrazine degradation pathway suggests that this is not an anomalous discovery (http://www.kegg.jp/kegg-bin/view_ortholog_table?map=00791). Of the genomes contained in KEGG, none contain the entire suite of atrazine catabolic genes. At best, there are a handful of *Rhizobium* spp. that contain *atzBDF,* and an *Arthrobacter* that contains *atzBCF*.

One *Sphingomonas* isolate presumptively contained *atzABCEF* but not *atzD*. A *Hydrogenophaga* isolate presumptively contained *atzABC* and *trzDN*, but not *atzDEF*.

Another noteworthy catabolic gene pattern uncovered in this assay was an *atzAC-trzN* pattern observed in one each of *Nocardioides* and *Methyloversatilis*. In these 2 isolates, the *atzA* PCR product was approximately 200 bp smaller than the anticipated size; subsequent cloning and sequencing of these two PCR reactions revealed 99% sequence similarity to respective database sequences. The smaller size of these PCR products, therefore, is likely the result of a truncated version of the *atzA* gene present in these isolates. This could conceivably be an effect of the redundancy of atrazine chlorohydrolase genes observed in these isolates, since both *atzA* and *trzN* were detected by this assay. A similar truncation of the *atzA* gene was not detected in the *Hydrogenophaga* isolate despite *trzN* also being detected in this assay; both the *atzA* and *trzN* bands were of greatly diminished intensity, however. The *Nocardioides* and *Methyloversatilis* isolates were not shown to contain *atzB*, which would be necessary in utilizing the ethylamine side chain and thus the formation of *N*-isopropylammelide (the substrate for *atzC*). Either these isolates possessed other catabolic genes allowing for survival on media containing atrazine as the sole carbon and nitrogen sources, or the *atzBC* genes contained by these isolates were sufficiently divergent to escape detection by this PCR assay.

As was previously mentioned, the most prominent genus within the isolate collection was *Arthrobacter*. All of the *Arthrobacter* isolates presumptively contained *atzBC*. The majority of the *Arthrobacter* isolates also presumptively contained *trzN*. The *atzBC-trzN* catabolic gene combination has been widely reported in the literature, especially in *Arthrobacter* spp. (Devers et al., 2004; Sajjaphan et al., 2010; El Sebai et al.,

2011; Zhang et al., 2011, Wang and Xie, 2012). This combination of genes would allow isolates to degrade atrazine to cyanuric acid, thus allowing for utilization of the carbon and nitrogen containing side chains of the parent compound. The absence of both of the atrazine chlorohydrolase genes and/or *atzB* from an isolate may indicate that this isolate is using a thus uncharacterized enzyme; the isolate is using some combination of previously described monooxygenases that were not screened for in this assay; or that divergence in the catabolic genes contained in these isolates were sufficient to prevent proper annealing of the primers used in this assay.

The atrazine catabolic screening assay utilized in this study was limited by the set of primers used. By only using published primers directly designed off of the known catabolic genes, the only possible positive results in the assay would be for genes identical or nearly identical to these sequences. In order to overcome this bias, degenerate primers should be used to account for mutations that would otherwise keep these primers from annealing. The justification for using these primers is that, at least in the case of *atzABC* and *trzN*, the atrazine catabolic genes are highly conserved. While not universally present in the isolates screened in this study, *atzBC* and *trzN* were readily detected, thus lending credibility to the use of these primers in this study. Isolates where neither *atzA* nor *trzN* were detected suggest either that these primers were truly inappropriate for detection in these isolates, or that other catabolic genes were utilized.

The lower pathway genes *atzDEF* and *trzD* were not as readily detected in this assay. The degradation of a wide variety of compounds leads to the formation of cyanuric acid; it is conceivable that sufficient variation exists in these gene sequences to make

these lower pathway primers insufficient for atrazine catabolic gene screening in environmental isolates. It is not surprising that lower pathway genes were not detected in this assay, as the upper pathway of atrazine biodegradation would be sufficient for growth of these isolates on media containing atrazine as the sole carbon and nitrogen source. Gaps detected in the upper and lower pathway genes would need to be elucidated first by determining the atrazine metabolites remaining in spent media. Based on the metabolites detected, the enzymes necessary for this level of degradation could be determined. A more rigorous screening assay could then be constructed.

Techniques in microbial ecology have evolved dramatically since the start of this project. If this project were to be continued into the future, these techniques could be harnessed to remedy the shortfalls addressed in this chapter. The two parts of the current project where bias is most prominently introduced are the culturing of atrazine-degrading microorganisms, and the screening assay for the genes responsible for this biodegradation. It is, therefore, desirable to alleviate or completely eliminate the biases imposed by these processes.

The primary objectives of this project were to assess the atrazine biodegradative capacity of a variety of environments; to determine which microorganisms were involved with this biodegradation; and to determine the enzymatic pathways that were utilized to achieve this biodegradation. These research goals remain worthy of pursuit. Weed scientists would like to know how long atrazine applied to crops will retain its pesticidal effect. Environmental conservationists would like to know how quickly and to what extent this potential pollutant will be removed from soils. Biochemists would be

interested in the evolution of atrazine catabolic pathways, the substrate ranges associated with these pathways, and the transmission of catabolic genes among bacterial community members. As the environments in which atrazine biodegradation occurs are dynamic and multi-faceted, the answers to these questions will be ever-changing and will require constant environmental monitoring to address. However, the methods used to achieve these objectives can be improved upon in order to deliver more insightful information.

An assessment of the atrazine biodegradative activity of an environment under study is necessary to put the results obtained from other experiments into perspective. The biometer assays used in this project, with the alterations outlined earlier in this chapter, would still provide useful information in this regard. In order to obtain the most physiologically appropriate biodegradation data, it would be desirable to keep these biometer studies as close to the environment under study as possible, such as by using mesocosms instead of benchtop experiments. However, the physical difficulty of data collection in this type of experimental setup would be impractical to the point of rendering the results ambiguous. Extensive cataloging of atrazine metabolites in these sites via HPLC and/or GC-MS would be required to piece together catabolic pathways in latter experiments.

The cultivation-dependent approach used in this study was fundamentally limited to the diversity of bacteria that could be grown in laboratory conditions, and therefore likely underestimated the true diversity of bacteria involved in atrazine biodegradation in the environments sampled. Likewise, the catabolic gene screening assay used in this project was limited to detecting atrazine catabolic genes sufficiently similar in sequence

to those from which the PCR primers were designed. In order to sidestep these limitations, a cultivation-independent approach should be used. The same variety of environmental sampling sites could be revisited, with the goal being to answer the same kinds of questions more accurately and without the biases of this project. In order to gain a broad picture of what microbes exist in these environments and what genes they contain, environmental DNA could be sequenced via any number of high-throughput sequencing technologies (Shokralla et al., 2012). These metagenomic sequence data can then be assembled and binned to provide insight into what bacteria exist in the community, and what genes are present among them that would account for the biodegradation observed from the experiments described in the preceding paragraph. Given the short sequence reads employed by high-throughput sequencing technologies, errors will inevitably be made in assembling genomes that will need to be thoroughly vetted by appropriate software. Additionally, the sequence data obtained can be compared across sampling sites in a comparative metagenomics approach, allowing for elucidation of the effects of each habitat on microbial community structure and genetic composition.

While this metagenomics information would be useful in answering these questions, it is limited in that it can only show the genes present in a given microbial community, as opposed to the catabolic pathways actually utilized by these communities. In order to determine exactly what genes are being used to degrade atrazine in these environments, a metatranscriptomics (Gosalbes et al., 2011) and/or metaproteomics (Herbst et al., 2013) approach would need to be employed. The metatranscriptomics

approach would be limited by the short half-life of mRNA in environmental samples, whereas the limitations of the metaproteomic approach would largely rest upon the difficulty of separating an entire microbial community's protein content, identifying proteins of interest, and successfully determining their amino acid sequence. Combining the metagenomics and metatranscriptomics/metaproteomics approaches, a complete map of the atrazine catabolic processes of a microbial community can be constructed, and this metabolic map can be compared with those culled from other sampling sites to ascertain what effects each habitat has on atrazine biodegradation.

While all of this cultivation-independent methodology allows for the voluminous collection of information about a sampled environment, the need to cultivate atrazine-degrading bacteria still exists. In order to definitively demonstrate that atrazine biodegradation is actually occurring in the manner inferred, the actual bacteria will still ultimately need to be isolated. While the Bio-Sep beads utilized in this study provided for the cultivation of some atrazine degraders, other more effective cultivation methods such as the iChip utilized by NovoBiotic Pharmaceuticals could be utilized in an attempt to isolate the bacteria detected in the cultivation-independent methods (Katsnelson, 2015).

The present study demonstrated varying levels of atrazine mineralization in several different settings. A handful of genera were cultivated in mixed and pure culture that degraded atrazine. Pure culture isolates were screened for atrazine catabolic genes, and presumptively contained different patterns of catabolic genes. The assessments of biomineralization and the minimal genetic analyses of atrazine-degraders in the sampling locations provide a baseline of expectations for further atrazine biodegradation research

in atrazine history soils. By demonstrating that atrazine biodegradation does occur and that various bacteria are responsible for this biodegradation, this project has primed these environments for further research. The fate of atrazine as influenced by soil history and other environmental variables is a relevant factor when the use of atrazine is reviewed for re-certification in 2018. Future studies of atrazine biodegradation would benefit from the usage of a metagenomics and metatranscriptomics/metaproteomics approach, coupled with more robust cultivation techniques, to fill in the gaps in knowledge underscored by the present study.

Non-Standard Abbreviations and Definitions

BLASTN – Basic Local Alignment Search Tool (Nucleotide-nucleotide)

DGGE – Denaturing Gradient Gel Electrophoresis

HPLC – High Performance Liquid Chromatography

KEGG – Kyoto Encyclopedia of Genes and Genomes

Koc – Soil Organic Carbon-Water Partitioning Coefficient

MCAC – Molly Caren Agricultural Center

NCBI – National Center for Biotechnology Information

ORWRP – Olentangy River Wetland Research Park

RDP – Ribosomal Database Project

WARS – Western Agricultural Research Station

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Appendix A: Soil sampling at the Molly Caren Agricultural Center: biomineralization data, 16S rDNA-based culture identifications, clone sequence accession numbers and catabolic gene detection

The Molly Caren Agricultural Center (MCAC) is an 850-ha Ohio State University facility located approximately 3 km north of London, Ohio. In addition to housing a 26-ha conservation area, approximately 566 ha of the facility are actively farmed for corn, wheat and soybeans (Figure A.1). The agricultural fields routinely receive annual applications of atrazine. Table A.1 displays atrazine application histories for fields Peninsula, 2A and 3A. At the time of initial sampling (Table A.2), these fields shared application histories with fields 1C, 3B and 10A, respectively. All field designations are cross-referenced with those appearing in Figure A.1. The soils sampled at the MCAC primarily consisted of mixtures of Crosby-Lewisburg silt loam (2% to 6% slopes) and Kokomo silty clay loam (0% to 2% slopes) (http://websoilsurvey.nrcs.usda.gov/app/WebSoilSurvey.aspx). Fields 2A and 3B had received 2 years of prior atrazine application at sampling time in 2004; fields 1C and Peninsula had received one year of prior atrazine application; and fields 3A and 10A had not received atrazine application for at least 3 years prior to being sampled for this study.

Samples of top soil (0-7 cm) were removed with a sterile trowel and stored in sterile plastic bags until processed in the laboratory (Table A.2). The sampling avoided plant roots. Soils 1C and Peninsula, 2A and 3B, and 3A and 10A were each combined

together upon arrival at the laboratory and were treated as composite soils. Soil-borne atrazine-degraders were also probed with porous Bio-Sep beads that were equilibrated in either a solution of 2 mg atrazine/l (atrazine Bio-Sep beads) or distilled water for 3 days (water Bio-Sep beads) before they were enclosed in sterile nylon mesh bags (~30 beads per bag) and sealed with plastic clips. Bio-Sep bead bags were buried in the top 10 cm layer of soil in fields Peninsula, 2A and 3A and retrieved according to the timelines specified in Table A.2.

Aerobic and anaerobic mineralization of atrazine in soil samples and aerobic mineralization of atrazine in Bio-Sep beads was measured in biometers (Figures A.2-A.3; Table A.3), which were constructed and sampled as in section *4.2.2*. The Bio-Sep bead bags were incubated in-situ in the soils for 79 days (November-February) before testing in biometer assays.

Soil and bead samples were used as inocula for aerobic and anaerobic enrichment cultures as described in section *3.2.3*. After at least six transfers to fresh basal mineral salt solution with 30 mg/l atrazine, enrichment cultures were screened for residual atrazine concentration as described in section *3.2.4*. Enrichments that exhibited at least 80% decrease in atrazine concentration were inoculated to solid media as described in section *3.2.5*. Resulting isolates were subcultured, screened for residual atrazine concentration, and stored as described in *3.2.5.*

Genomic DNA was isolated from pure and mixed cultures as described in section *3.2.6*. The V3 region of the 16S rRNA gene from atrazine-degrading mixed cultures was

amplified as described in section *3.2.6.* PCR products were analyzed via denaturing gradient gel electrophoresis (DGGE) as described in section *3.2.6* (Appendix B).

Genomic DNA from atrazine-degrading mixed cultures and isolates was amplified for the full-length rRNA gene as described in section *3.2.7*. PCR products were used to construct 16S rDNA clone libraries as described in section *3.2.7*. Clones were sequenced, and the resulting sequences were analyzed as described in section *3.2.7* (Figures A.4-A.6).

The 16S rRNA gene sequences were deposited with GenBank. The accession numbers for aerobic mixed culture clones are KT225023-KT225026, KT225028-KT225067, KT225069, KT225071, KT225077-KT225079, KT225086- KT225091, KT225093-KT225094, KT225097-KT225098, KT225100, KT225103-KT225105, and KT225109. The accession numbers for anaerobic mixed culture clones are KT225110-KT225119. The accession numbers for aerobic isolates are KT225120-KT225155, KT225159-KT225182, KT225186-KT225216, KT225220-KT225228, and KT225232-KT225258.

Genomic DNA from atrazine-degrading isolates was amplified for atrazine catabolic genes *atzA*, *atzB*, *atzC*, *atzD*, *atzE*, *atzF*, *trzD*, and *trzN*. *Escherichia coli* genomic DNA was also amplified as a negative control for all catabolic genes. *Pseudomonas* ADP was used as a positive control for *atzA*, *atzB*, *atzC*, *atzD*, *atzE* and *atzF*, and as a negative control for *trzD* and *trzN*. *Arthrobacter aurescens* TCI was used as a positive control for *trzN* as well as an additional positive control for *atzB* and *atzC* and a negative control for *atzA*, *atzD*, *atzE*, and *atzF*. *Klebsiella pneumoniae* 99 was used

as a positive control for *trzD*. PCR reactions were conducted as described in section *3.2.8*. PCR products were further visualized, cloned, and sequenced as described in section *3.2.8* (Table A.4). Clone sequences were analyzed as described in section *3.2.8* (Table A.5). Catabolic gene sequences were deposited with GenBank, and the accession numbers are KT346377- KT346394.

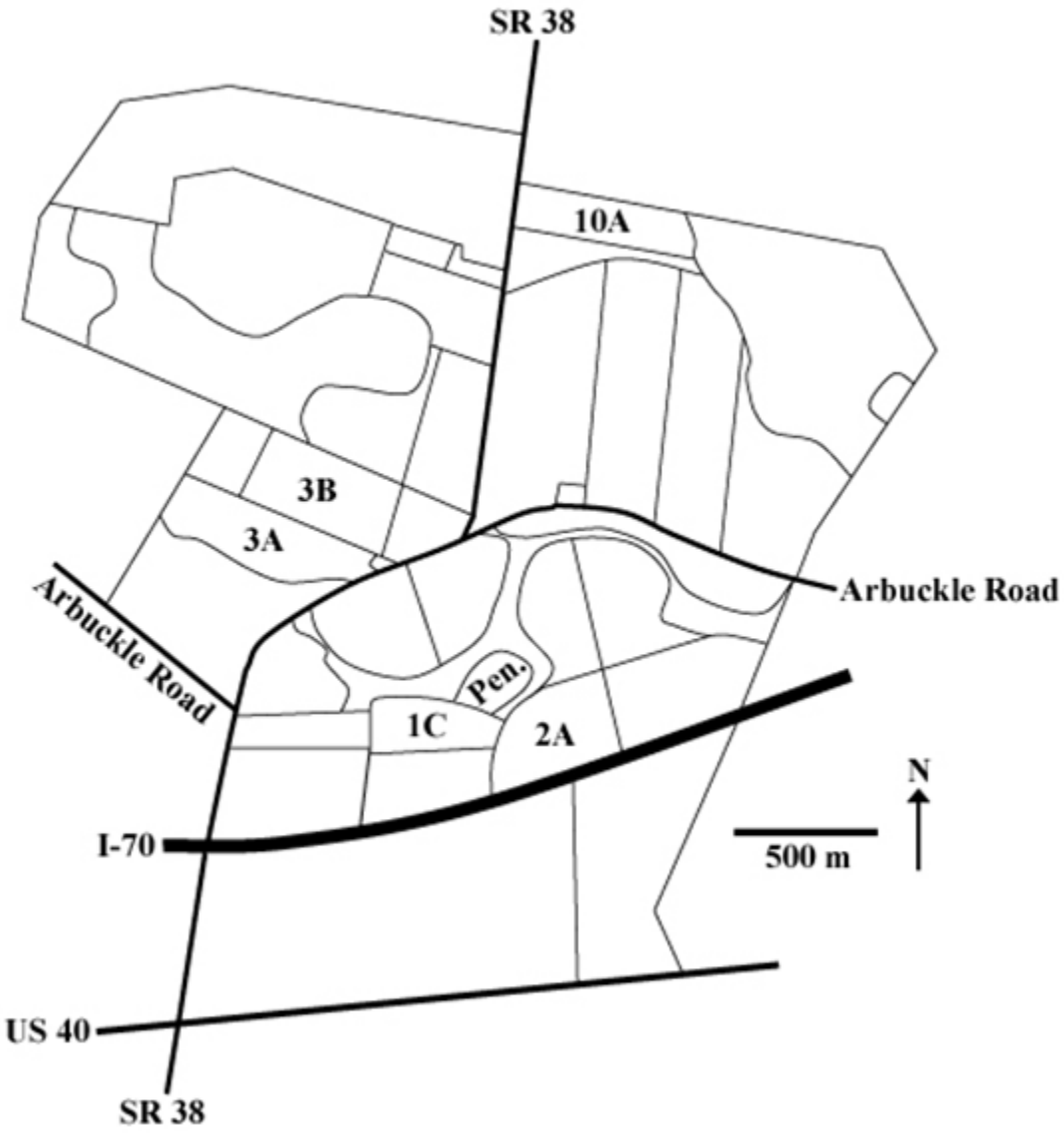

Figure A.1. Locations of fields sampled at the Molly Caren Agricultural Center. Pen. = Peninsula.

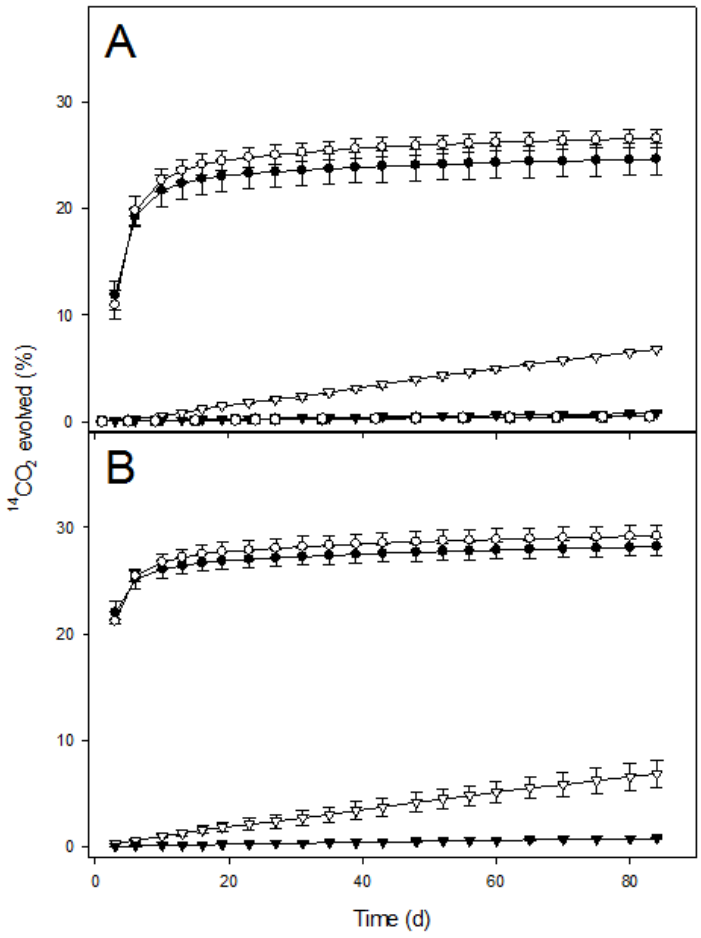

Figure A.2. Aerobic mineralization of [U-ring-14 C]-atrazine in MCAC sample biometers in the absence (A) and presence (B) of glucose amendment. Symbols: (●), composite soil from fields 1C and Peninsula; (○), composite soil from fields 2A and 3B; (▽), composite soil from fields 3A and 10A; (◆), 3A atrazine Bio-Sep beads; (◇), 3A water Bio-Sep beads; (■), Peninsula atrazine Bio-Sep beads; (□), Peninsula water Bio-Sep beads; (⬢), 2A atrazine Bio-Sep beads; (⬡), 2A water Bio-Sep beads; (▼), sterile soil control. Bio-Sep beads were only used to inoculate biometers without glucose. Data points represent mean cumulative percentages obtained from duplicate biometers, with the exception of those representing Bio-Sep biometers, which represent results from single biometers. Bars indicate actual percentages obtained from each replicate, and in some cases are smaller than the data point symbols.

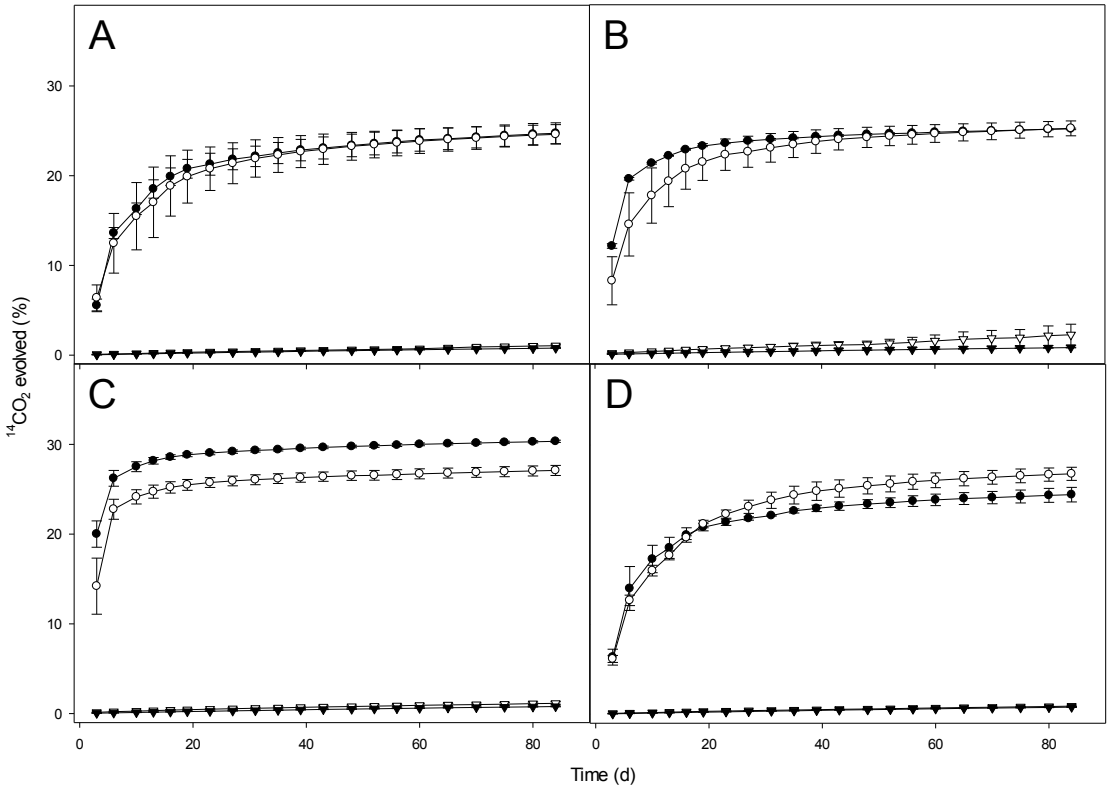

Figure A.3. Anaerobic mineralization of [U-ring-14 C]-atrazine in MCAC soil biometers. The biometers were amended with (A) no electron acceptor, (B) K-nitrate, (C) ferrihydrite, and (D) Na-sulfate. Symbols: (●), composite soil from fields 1C and Peninsula; (○), composite soil from fields 2A and 3B; (∇), composite soil from fields 3A and 10A; (▼), sterile soil control. Data points represent mean cumulative percentages obtained from duplicates. Bars indicate actual percentages obtained from each replicate, and in some cases are smaller than the data point symbols.

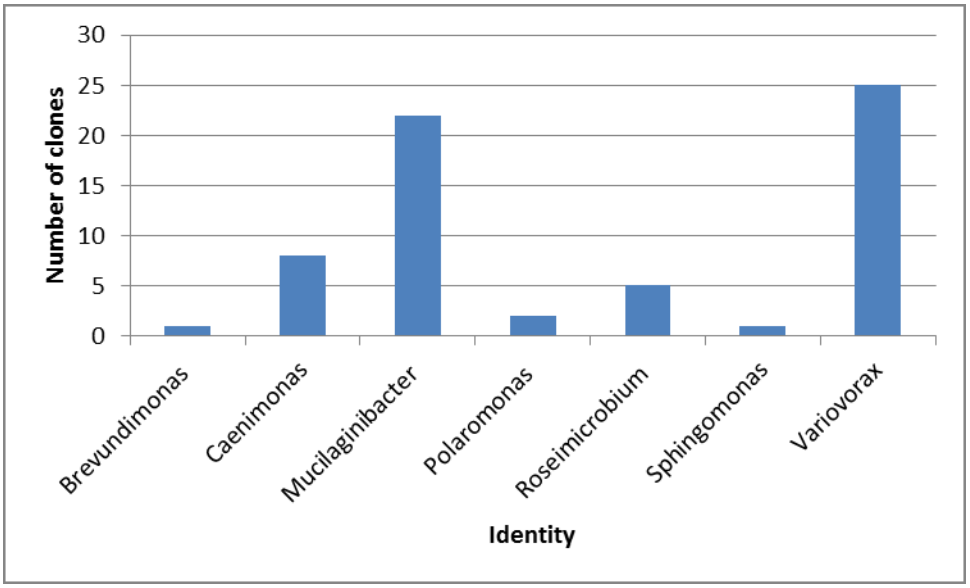

Figure A.4. Identification of 16S rDNA clones from atrazine-degrading MCAC soil aerobic mixed cultures. Sixty four clones in total are represented. All sequence queries returned similarity scores of 0.90 or greater. All identifications are made at the genus level.

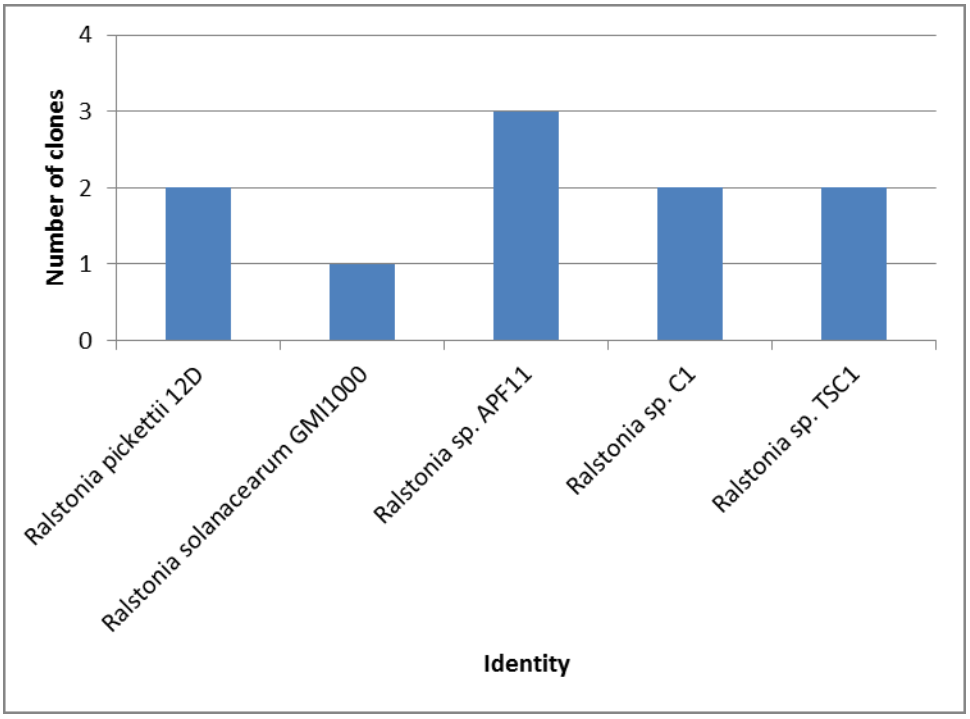

Figure A.5. Identification of 16S rDNA clones from atrazine-degrading MCAC anaerobic mixed cultures. Ten clones in total are represented. All sequence queries returned similarity scores of 1.0. Identifications are made at the species level.

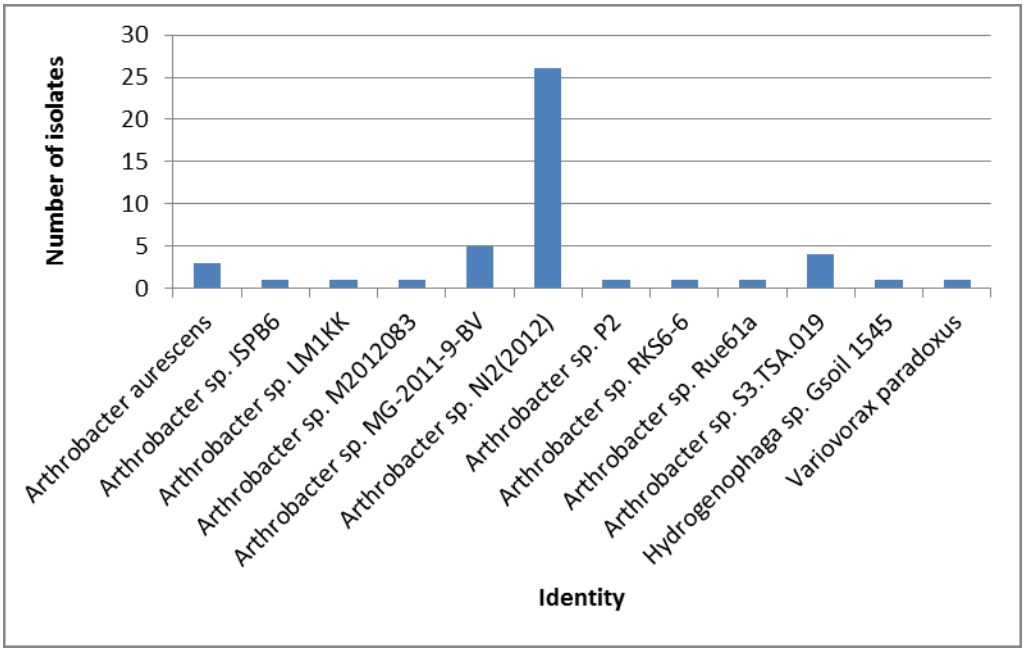

Figure A.6. Identification of 16S rDNA clones from atrazine-degrading MCAC soil aerobic pure cultures. Forty six consensus sequences in total are represented. All sequence queries returned similarity scores of 0.97 or greater. All identifications are made at the species level.

Table A.1. Atrazine application dates and rates for MCAC fields Peninsula, 2A, and 3A. Hyphens denote periods of time without atrazine application. Unknown atrazine application dates are marked n/d.

Field	Year	Application date (mm/dd)	Atrazine applied (kg/ha)
Peninsula	2002	n/d	2.08
	2003	-	-
	2004	04/29	2.08
	2005	04/22	2.08
	2006	-	-
	2007	04/28	2.08
2A	2002	-	-
	2003	n/d	1.12
	2004	04/29	2.08
	2005	-	-
	2006	04/28	2.08
	2007	04/28	2.08
3A	2002	-	-
	2003	-	-
	2004	-	-
	2005	-	-
	2006	04/28	2.08
	2007	-	-

Table A.2. MCAC sampling sites and dates of sampling for enrichment culture and biometer inocula. Hyphens denote no sampling occurred. Fields 1C, Peninsula, 2A and 3B were planted with corn prior to the first sampling date. Fields 3A and 10A were planted with wheat prior to the first sampling date.

Sampling site	Soil collected	First Bio-Sep batch		Second Bio-Sep batch		Third Bio-Sep batch	
		Deployed	Harvested	Deployed	Harvested	Deployed	Harvested
1C	09/02/2004[b]	-	-	-	-	-	-
Peninsula	09/02/2004[b]	11/28/2004	02/15/2005[b]	04/07/2005	05/017/2005[a]	7/27/2005	9/9/2005[a]
2A	09/02/2004[ab]	11/28/2004	02/15/2005[ab]	-	-	-	-
3B	09/02/2004[ab]	-	-	-	-	-	-
3A	09/02/2004[b]	11/28/2004	02/15/2005[b]	04/07/2005	05/017/2005	7/27/2005	9/9/2005[a]
10A	09/02/2004[b]	-	-	-	-	-	-

[a]Inoculum for enrichment cultures.

[b]Inoculum for biometers.

Table A.3. Kinetic parameters and half-lives of atrazine mineralization in MCAC soil samples. Samples were collected on September 2, 2004. Calculations are based on the biometer experiments shown in Figures A.2 and A.3.

Sample	**Additional Amendment**	**Incubation conditions**	**k (d^{-1})**	**R^2**	**$t_{1/2}$ (d)**
1C/Peninsula	None	Aerobic	0.238	0.9682	2.9
1C/Peninsula	Glucose	Aerobic	0.508	0.8082	1.4
1C/Peninsula	None	Anaerobic	0.113	0.973	6.1
1C/Peninsula	Sulfate	Anaerobic	0.122	0.9734	5.7
1C/Peninsula	Nitrate	Anaerobic	0.230	0.9524	3.0
1C/Peninsula	Ferrihydrite	Anaerobic	0.360	0.9281	1.9
2A/3B	None	Aerobic	0.204	0.9654	3.4
2A/3B	Glucose	Aerobic	0.425	0.8672	1.6
2A/3B	None	Anaerobic	0.101	0.9807	6.9
2A/3B	Sulfate	Anaerobic	0.090	0.9881	7.7
2A/3B	Nitrate	Anaerobic	0.128	0.9753	5.4
2A/3B	Ferrihydrite	Anaerobic	0.274	0.9517	2.5
3A/10A	None	Aerobic	0.085[a]	0.999	42[a]
3A/10A	Glucose	Aerobic	0.004	0.9994	191
3A/10A	None	Anaerobic	0.005	0.9842	129
3A/10A	Sulfate	Anaerobic	0.014	0.9917	51
3A/10A	Nitrate	Anaerobic	0.002	0.9844	354
3A/10A	Ferrihydrite	Anaerobic	0.018	0.9889	38

[a]The rate constants and half-lives were calculated from linear regression.

Table A.4. Occurrence of atrazine catabolic genes in a selection of atrazine-degrading pure cultures from the MCAC samples, based on PCR amplification of genomic DNA followed by agarose gel electrophoresis. The designation "n/a" indicates the given column heading is not applicable. Similarity refers to 16S rDNA sequence similarity of isolate consensus sequence to nearest match. (+) indicates the presence of a band of the expected size for the catabolic gene being amplified; (-) indicates the absence of a band of the expected size for the catabolic gene being amplified; (+/-) indicates the presence of a band of the expected size for the catabolic gene being amplified, the intensity of which is approximately less than one quarter that of the positive control band for the corresponding gene. A question mark denotes a prominent band of an unexpected size for the catabolic gene being screened.

Isolate and accession nos.	Nearest match and accession no.	Similarity (%)	*atzA*	*atzB*	*atzC*	*atzD*	*atzE*	*atzF*	*trzD*	*trzN*
TN69, KT225238-40	*Arthrobacter* sp. NI2(2012), JQ435704	99	-	+	+	-	-	-	-	+
TN70, KT225241-3	*Arthrobacter* sp. NI2(2012), JQ435704	98	-	?	+	-	-	-	-	+
TN2, KT225128-9	*Arthrobacter* sp. LM1KK, LC008302	98	-	+	+	-	-	-	-	+
TN3, KT225151-3	*Arthrobacter* sp. NI2(2012), JQ435704	99	-	+	+	-	-	-	-	+
TN67, KT225235-7	*Arthrobacter* sp. NI2(2012), JQ435704	99	-	+	+	-	-	-	-	+
TN58, KT225212-3	*Arthrobacter* sp. MG-2011-9-BV, FR872498	98	-	+	+	-	-	-	-	+
TN53, KT225201-2	*Arthrobacter* sp. MG-2011-9-BV, FR872498	97	-	+	+	-	-	-	-	+

Continued

Table A.4 continued

TN83, KT225247-9	*Arthrobacter* sp. NI2(2012), JQ435704	99	-	+	+	-	-	-	-	+
TN26, KT225148-50	*Arthrobacter* sp. JSPB6, JQ308619	99	-	+	+	-	-	-	-	+
TN85, KT225253-5	*Arthrobacter aurescens*, JQ435703	97	-	+	+	-	-	-	-	+
TN43, KT225168-70	*Arthrobacter* sp. MG-2011-9-BV, FR872498	97	-	+	+/-	-	-	-	-	+
TN81, KT225244-6	*Arthrobacter* sp. NI2(2012), JQ435704	99	-	+	+/-	-	-	-	-	+
TN61, KT225223-5	*Arthrobacter aurescens*, JQ435703	99	-	+	+	-	-	-	-	+
TN84, KT225250-2	*Arthrobacter* sp. MG-2011-9-BV, FR872498	98	-	+	+	-	-	-	-	+
TN253, KT225146-7	*Arthrobacter* sp. RKS6-6, GQ477173	100	-	+	+/-	-	-	-	-	+
Escherichia coli, n/a	n/a	n/a	-	-	-	-	-	-	-	-
Pseudomonas ADP, n/a	n/a	n/a	+	+	+	+	+	+	-	-
Arthrobacter aurescens TCI, n/a	n/a	n/a	-	+	+	-	-	-	-	+
Klebsiella pneumoniae 99, n/a	n/a	n/a	-	-	-	-	-	-	+	-

Table A.5. Sequence similarities of catabolic genes cloned from MCAC atrazine-degrading pure cultures to respective database sequences. Query culture numbers correspond to those in Table A.4. All query lengths reflect the lengths (in nucleotides) of consensus sequences generated from triplicate clones.

Gene and subject accession no.	Query culture	Query length (nt)	Similarity (%)	Query accession nos.
atzB, U66917.2	TN43	469	100	KT346377-9
atzB, U66917.2	TN67	469	100	KT346380-2
atzB, U66917.2	TN69	454	99	KT346383-5
atzB, U66917.2	TN81	466	99	KT346386-8
atzC, U66917.2	TN67	625	100	KT346389-91
trzN, CP000475.1	TN43	432	100	KT346392-4

Appendix B: DGGE and agarose gel images

In the course of this study, a collection of aerobic and anaerobic atrazine-degrading mixed cultures were obtained as described in sections *3.2.3-4* and *4.2.3-4* and Appendix A. Subcultures of anaerobic atrazine-degrading mixed cultures were also incubated aerobically in parallel. Genomic DNA was extracted from all atrazine-degrading mixed cultures in this study as described in sections *3.2.6* and *4.2.6* and Appendix A. The DNA samples were PCR amplified for the V3 region of the 16S rRNA gene as described in sections *3.2.6* and *4.2.6* and Appendix A. PCR products were visualized using DGGE as described in section *3.2.6*. Mixed cultures associated with unique DGGE banding patterns were chosen for further molecular analysis via 16S rDNA clone library construction and sequence analysis.

A collection of atrazine-degrading isolates was obtained as described in sections *3.2.5* and *4.2.5* and Appendix A. Genomic DNA was extracted from all atrazine-degrading isolates in this study as described in sections *3.2.6* and *4.2.6* and Appendix A.. This DNA was PCR amplified for atrazine catabolic genes *atzA*, *atzB*, *atzC*, *atzD*, *atzE*, *atzF*, *trzD*, and *trzN* and visualized on agarose gel as described in sections *3.2.8* and *4.2.8* and Appendix A.

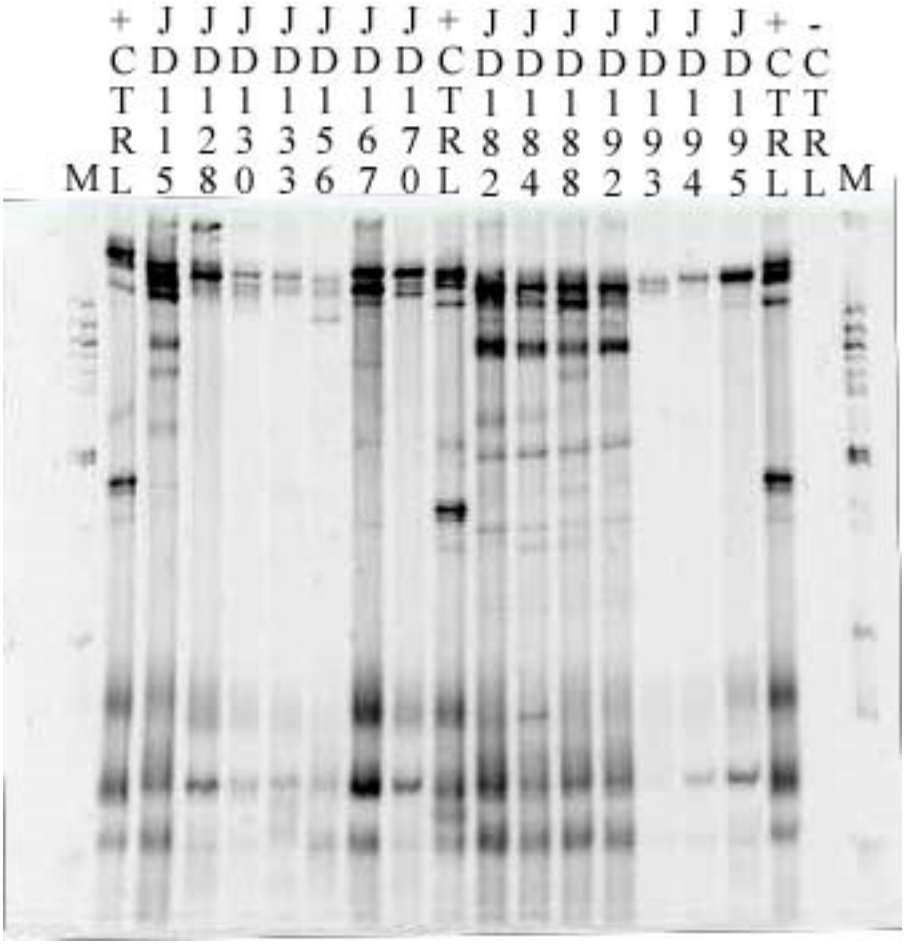

Figure B.1. DGGE gel image of mixed culture 16S rDNA region V3 PCR products. M = 100 bp DNA ladder. + CTRL = mixture of 16S rDNA region V3 PCR products from *Escherichia coli*, *Pseudomonas* strain ADP, *Klebsiella pneumoniae* strain 99, and *Arthrobacter aurescens* TCI. CTRL = PCR reaction without template DNA. All other lane designations correspond to the mixed culture serial numbers appearing in Table B.1.

Table B.1. Mixed cultures appearing in Figure B.1. ORWRP = Olentangy River Wetland Research Park. WARS = Western Agricultural Research Station. No TEA = no terminal electron acceptor amendment. Sulfate = sodium sulfate amendment.

Serial number	Sample location	Sample type	Culture conditions	Relevant chapter/appendix
JD115	ORWRP sediment	atrazine Bio-Sep beads	anaerobic, no TEA	Chapter 3
JD128	ORWRP sediment	atrazine Bio-Sep beads	anaerobic, no TEA	Chapter 3
JD130	ORWRP water	atrazine Bio-Sep beads	anaerobic, no TEA	Chapter 3
JD133	WARS former wash area	soil	anaerobic, no TEA	Chapter 4
JD156	Molly Caren Peninsula	atrazine Bio-Sep beads	anaerobic, sulfate	Appendix A
JD167	ORWRP water	water Bio-Sep beads	anaerobic, sulfate	Chapter 3
JD170	Molly Caren Peninsula	water Bio-Sep beads	anaerobic, sulfate	Appendix A
JD182	WARS former wash area	soil	anaerobic, sulfate	Chapter 4
JD184	Molly Caren Peninsula	atrazine Bio-Sep beads	anaerobic, sulfate	Appendix A
JD188	Molly Caren 3A	atrazine Bio-Sep beads	anaerobic, sulfate	Appendix A
JD192	ORWRP sediment	atrazine Bio-Sep beads	anaerobic, sulfate	Chapter 3
JD193	ORWRP sediment	water Bio-Sep beads	anaerobic, sulfate	Chapter 3
JD194	ORWRP water	atrazine Bio-Sep beads	anaerobic, sulfate	Chapter 3
JD195	ORWRP water	water Bio-Sep beads	anaerobic, sulfate	Chapter 3

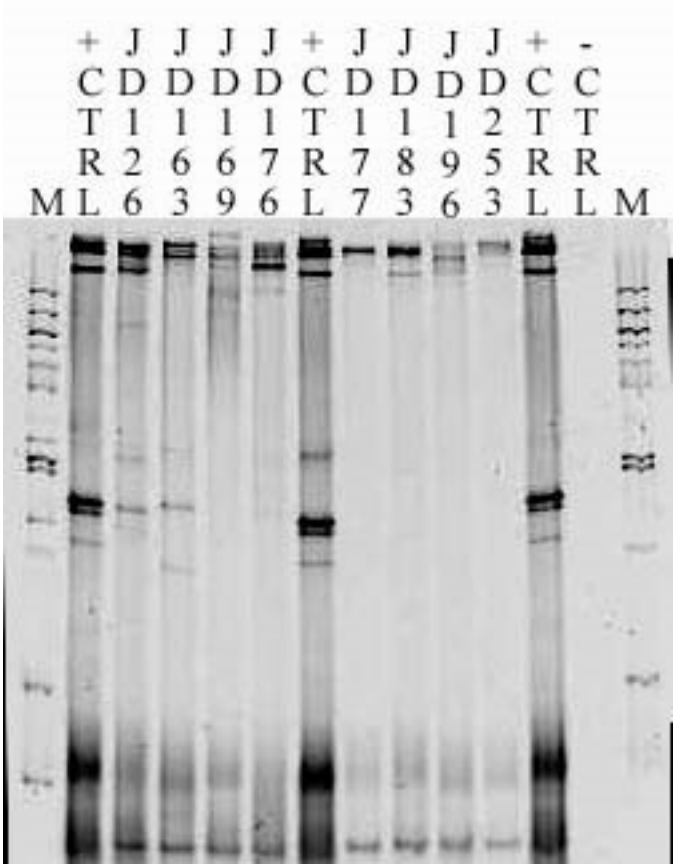

Figure B.2. DGGE gel image of mixed culture 16S rDNA region V3 PCR products. M = 100 bp DNA ladder. + CTRL = mixture of 16S rDNA region V3 PCR products from *E. coli*, *P.* strain ADP, *K. pneumoniae* strain 99, and *A. aurescens* TCI. CTRL = PCR reaction without template DNA. All other lane designations correspond to the mixed culture serial numbers in Table B.2.

Table B.2. Mixed cultures appearing in Figure B.2. ORWRP = Olentangy River Wetland Research Park. WARS = Western Agricultural Research Station. No TEA = no terminal electron acceptor amendment. Sulfate = sodium sulfate amendment. Iron = ferrihydrite amendment. N/a = data not applicable to this study.

Serial number	**Sample location**	**Sample type**	**Culture conditions**	**Relevant chapter/appendix**
JD126	Campus dairy farm	atrazine Bio-Sep beads	anaerobic, no TEA	N/a
JD163	Campus dairy farm	water Bio-Sep beads	anaerobic, sulfate	N/a
JD169	Molly Caren Peninsula	atrazine Bio-Sep beads	anaerobic, sulfate	Appendix A
JD176	Campus dairy farm	water Bio-Sep beads	anaerobic, sulfate	N/a
JD177	ORWRP sediment	atrazine Bio-Sep beads	anaerobic, sulfate	Chapter 3
JD183	WARS former mixing area	soil	anaerobic, sulfate	Chapter 4
JD196	ORWRP water	water Bio-Sep beads	anaerobic, sulfate	Chapter 3
JD253	ORWRP sediment	atrazine Bio-Sep beads	anaerobic, iron	Chapter 3

Figure B.3. DGGE gel image of mixed culture 16S rDNA region V3 PCR products. M = 100 bp DNA ladder. + CTRL = mixture of 16S rDNA region V3 PCR products from . *coli*, *P.* strain ADP, *K. pneumoniae* strain 99, and *A. aurescens* TCI. CTRL = PCR reaction without template DNA. All other lane designations correspond to the mixed culture serial numbers in Table B.3.

Table B.3. Mixed cultures appearing in Figure B.3. ORWRP = Olentangy River Wetland Research Park. WARS = Western Agricultural Research Station. No TEA = no terminal electron acceptor amendment. Sulfate = sodium sulfate amendment. Nitrate = potassium nitrate amendment. N/a = data not applicable to this study.

Serial number	**Sample location**	**Sample type**	**Culture conditions**	**Relevant chapter/appendix**
JD129	ORWRP sediment	water Bio-Sep beads	anaerobic, no TEA	Chapter 3
JD162	Campus dairy farm	atrazine Bio-Sep beads	anaerobic, sulfate	N/a
JD191	WARS former mixing area	water Bio-Sep beads	anaerobic, sulfate	Chapter 4
JD277	ORWRP sediment	atrazine Bio-Sep beads	anaerobic, sulfate	Chapter 3
JD282	Campus dairy farm	water Bio-Sep beads	anaerobic, sulfate, glucose	N/a
JD299	ORWRP water	atrazine Bio-Sep beads	anaerobic, nitrate	Chapter 3
JD338	ORWRP sediment	atrazine Bio-Sep beads	anaerobic, nitrate	Chapter 3
JD363	ORWRP sediment	atrazine Bio-Sep beads	anaerobic, no TEA	Chapter 3
JD365	ORWRP water	atrazine Bio-Sep beads	anaerobic, no TEA	Chapter 3

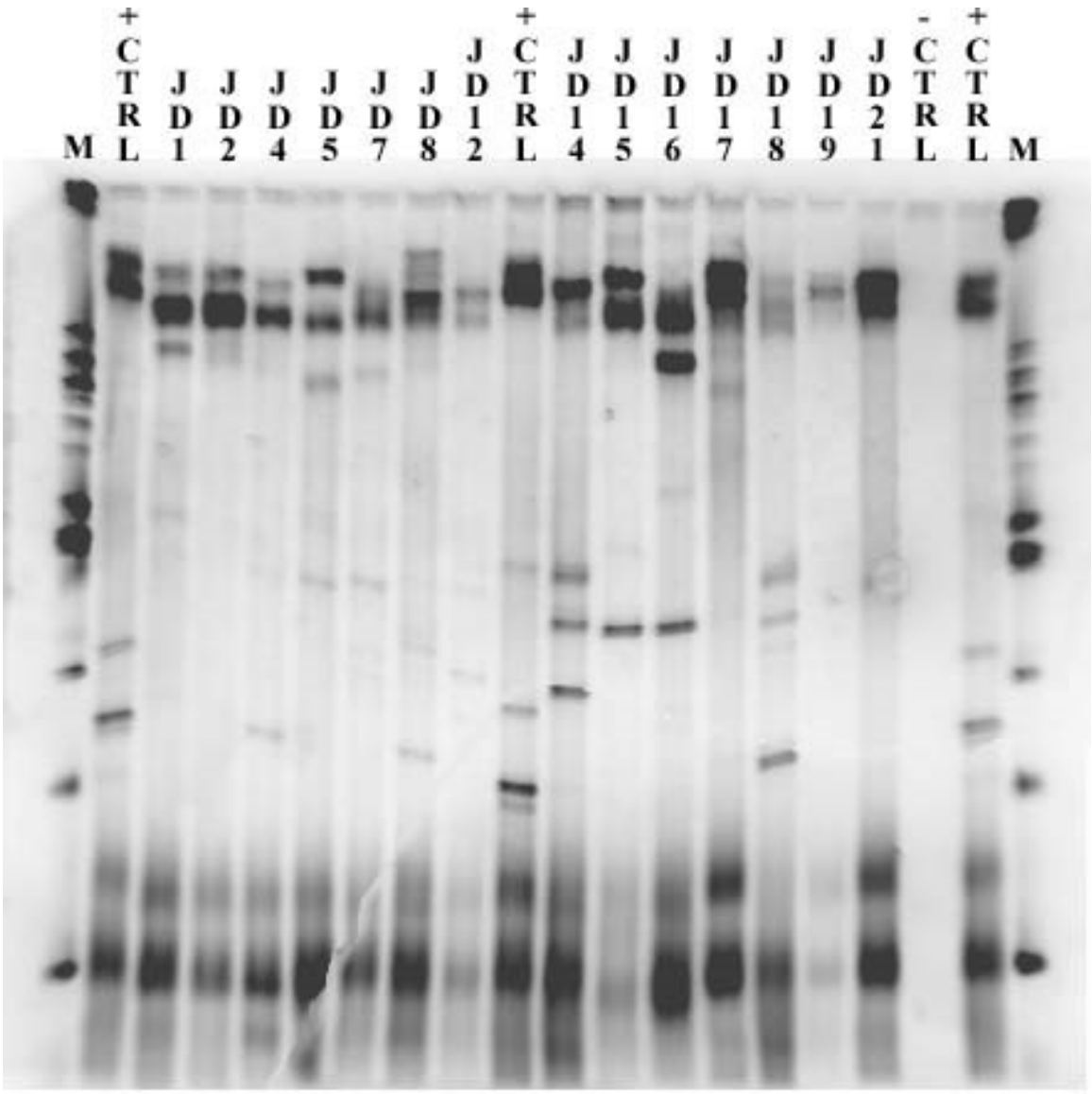

Figure B.4. DGGE gel image of mixed culture 16S rDNA region V3 PCR products. M = 100 bp DNA ladder. + CTRL = mixture of 16S rDNA region V3 PCR products from *E. coli*, *P.* strain ADP, *K. pneumoniae* strain 99, and *A. aurescens* TCI. CTRL = PCR reaction without template DNA. All other lane designations correspond to the mixed culture serial numbers appearing in Table B.4.

Table B.4. Mixed cultures appearing in Figure B.4. ORWRP = Olentangy River Wetland Research Park. WARS = Western Agricultural Research Station. N/a = data not applicable to this study.

Serial number	Sample location	Sample type	Culture conditions	Relevant chapter/appendix
JD1	Molly Caren 1C/Peninsula	soil	aerobic	Appendix A
JD2	Molly Caren 2A/3B	soil	aerobic	Appendix A
JD4	Campus dairy farm	soil	aerobic	N/a
JD5	Campus orchard wetland	sediment	aerobic	N/a
JD7	ORWRP sediment	sediment	aerobic	Chapter 3
JD8	Molly Caren 1C/Peninsula	soil	aerobic, glucose	Appendix A
JD12	Campus orchard wetland	sediment	aerobic, glucose	N/a
JD14	ORWRP sediment	sediment	aerobic, glucose	Chapter 3
JD15	WARS former mixing area	soil	aerobic	Chapter 4
JD16	WARS former wash area	soil	aerobic	Chapter 4
JD17	Molly Caren 1B	soil	aerobic	N/a
JD18	WARS former mixing area	soil	aerobic, glucose	Chapter 4
JD19	WARS former wash area	soil	aerobic, glucose	Chapter 4
JD21	Molly Caren Peninsula	atrazine Bio-Sep beads	aerobic	Appendix A

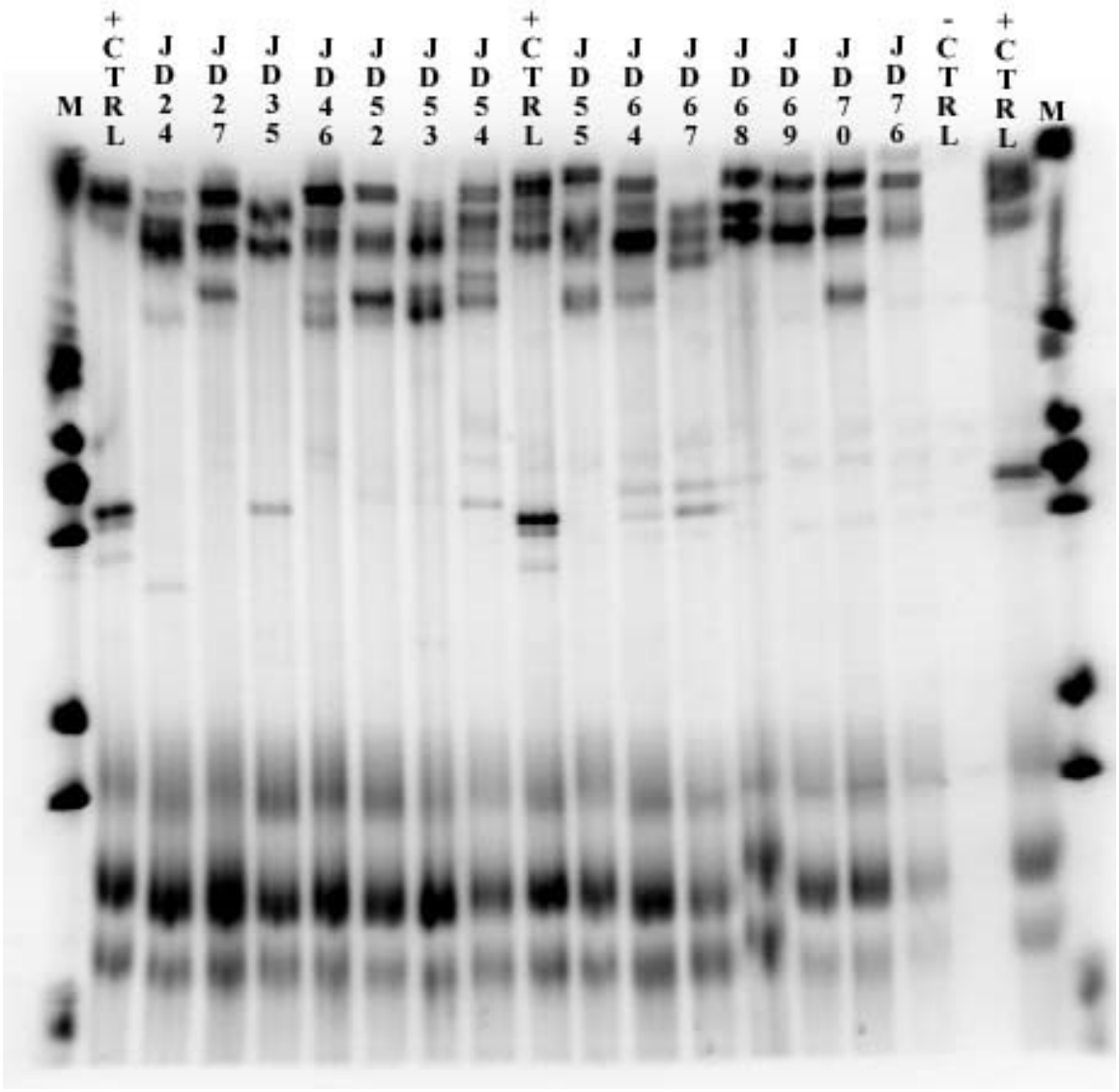

Figure B.5. DGGE gel image of mixed culture 16S rDNA region V3 PCR products. M = 100 bp DNA ladder. + CTRL = mixture of 16S rDNA region V3 PCR products from *E. coli*, *P.* strain ADP, *K. pneumoniae* strain 99, and *A. aurescens* TCI. CTRL = PCR reaction without template DNA. All other lane designations correspond to the mixed culture serial numbers in Table B.5.

Table B.5. Mixed cultures appearing in Figure B.5. ORWRP = Olentangy River Wetland Research Park. N/a = data not applicable to this study.

Serial number	Sample location	Sample type	Culture conditions	Relevant chapter/appendix
JD24	Molly Caren 2A	water Bio-Sep beads	aerobic	Appendix A
JD27	Campus dairy farm	atrazine Bio-Sep beads	aerobic	N/a
JD35	Molly Caren 2A	atrazine Bio-Sep beads	aerobic	Appendix A
JD46	Molly Caren Peninsula	water Bio-Sep beads	aerobic	Appendix A
JD52	Campus dairy farm	atrazine Bio-Sep beads	aerobic	N/a
JD53	ORWRP sediment	atrazine Bio-Sep beads	aerobic	Chapter 3
JD54	ORWRP sediment	water Bio-Sep beads	aerobic	Chapter 3
JD55	ORWRP water	atrazine Bio-Sep beads	aerobic	Chapter 3
JD64	Campus dairy farm	water Bio-Sep beads	aerobic, glucose	N/a
JD67	ORWRP water	atrazine Bio-Sep beads	aerobic, glucose	Chapter 3
JD68	ORWRP water	water Bio-Sep beads	aerobic, glucose	Chapter 3
JD69	Molly Caren Peninsula	atrazine Bio-Sep beads	aerobic	Appendix A
JD70	Molly Caren Peninsula	water Bio-Sep beads	aerobic	Appendix A
JD76	ORWRP sediment	water Bio-Sep beads	aerobic	Chapter 3

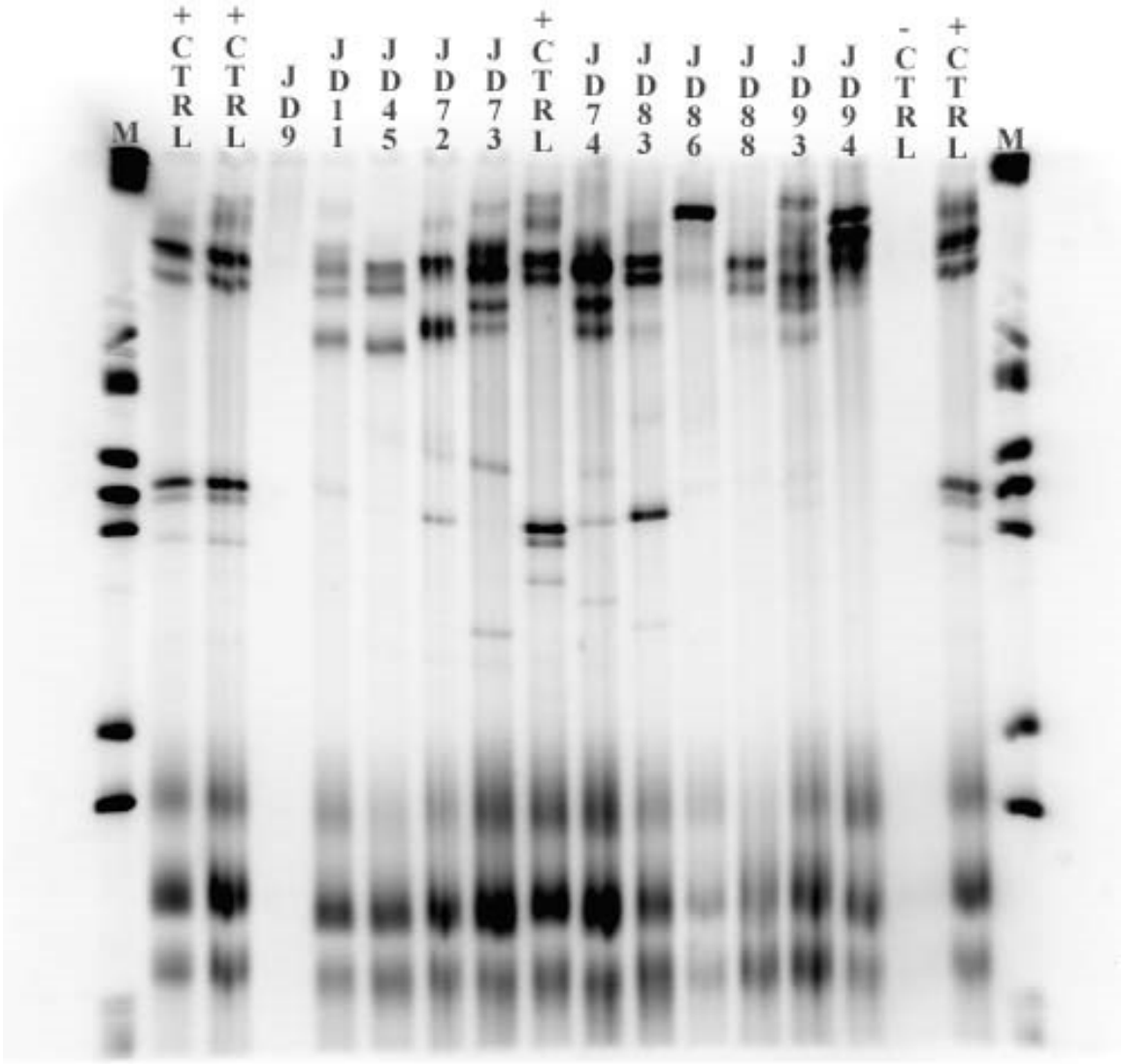

Figure B.6. DGGE gel image of mixed culture 16S rDNA region V3 PCR products. M = 100 bp DNA ladder. + CTRL = mixture of 16S rDNA region V3 PCR products from *E. coli*, *P.* strain ADP, *K. pneumoniae* strain 99, and *A. aurescens* TCI. CTRL = PCR reaction without template DNA. All other lane designations correspond to the mixed culture serial numbersin Table B.6.

Table B.6. Mixed cultures appearing in Figure B.6. ORWRP = Olentangy River Wetland Research Park. WARS = Western Agricultural Research Station. N/a = data not applicable to this study.

Serial number	Sample location	Sample type	Culture conditions	Relevant chapter/appendix
JD9	Molly Caren 2A/3B	soil	aerobic, glucose	Appendix A
JD11	Campus dairy farm	soil	aerobic, glucose	N/a
JD45	Molly Caren Peninsula	atrazine Bio-Sep beads	aerobic	Appendix A
JD72	Molly Caren 1B	water Bio-Sep beads	aerobic	N/a
JD73	Molly Caren 3A	atrazine Bio-Sep beads	aerobic	Appendix A
JD74	Molly Caren 3A	water Bio-Sep beads	aerobic	Appendix A
JD83	Molly Caren 3A	atrazine Bio-Sep beads	aerobic, glucose	Appendix A
JD86	ORWRP sediment	water Bio-Sep beads	aerobic, glucose	Chapter 3
JD88	ORWRP water	water Bio-Sep beads	aerobic, glucose	Chapter 3
JD93	WARS former mixing area	atrazine Bio-Sep beads	aerobic, glucose	Chapter 4
JD94	WARS former mixing area	water Bio-Sep beads	aerobic, glucose	Chapter 4

Figure B.7. DGGE gel image of mixed culture 16S rDNA region V3 PCR products. M = 100 bp DNA ladder. + CTRL = mixture of 16S rDNA region V3 PCR products from *E. coli*, *P.* strain ADP, *K. pneumoniae* strain 99, and *A. aurescens* TCI. CTRL = PCR reaction without template DNA. All other lane designations correspond to the mixed culture serial numbers in Table B.7.

Table B.7. Mixed cultures appearing in Figure B.7. ORWRP = Olentangy River Wetland Research Park. Sulfate = sodium sulfate amendment. Nitrate = potassium nitrate amendment. No TEA = no terminal electron acceptor amendment. N/a = data not applicable to this study.

Serial number	Sample location	Sample type	Culture conditions	Relevant chapter/appendix
JD9	Molly Caren 2A/3B	soil	aerobic, glucose	Appendix A
JD20	Molly Caren 1B	soil	aerobic, glucose	N/a
JD193	ORWRP sediment	water Bio-Sep beads	anaerobic, sulfate	Chapter 3
JD193 AER	ORWRP sediment	water Bio-Sep beads	aerobic	Chapter 3
JD194	ORWRP water	atrazine Bio-Sep beads	anaerobic, sulfate	Chapter 3
JD194 AER	ORWRP water	atrazine Bio-Sep beads	aerobic	Chapter 3
JD277	ORWRP sediment	atrazine Bio-Sep beads	anaerobic, sulfate	Chapter 3
JD277 AER	ORWRP sediment	atrazine Bio-Sep beads	aerobic	Chapter 3
JD282	Campus dairy farm	water Bio-Sep beads	anaerobic, sulfate, glucose	N/a
JD282 AER	Campus dairy farm	water Bio-Sep beads	aerobic	N/a
JD338	ORWRP sediment	atrazine Bio-Sep beads	anaerobic, nitrate	Chapter 3
JD338 AER	ORWRP sediment	atrazine Bio-Sep beads	aerobic	Chapter 3
JD363	ORWRP sediment	atrazine Bio-Sep beads	anaerobic, no TEA	Chapter 3
JD363 AER	ORWRP sediment	atrazine Bio-Sep beads	aerobic	Chapter 3

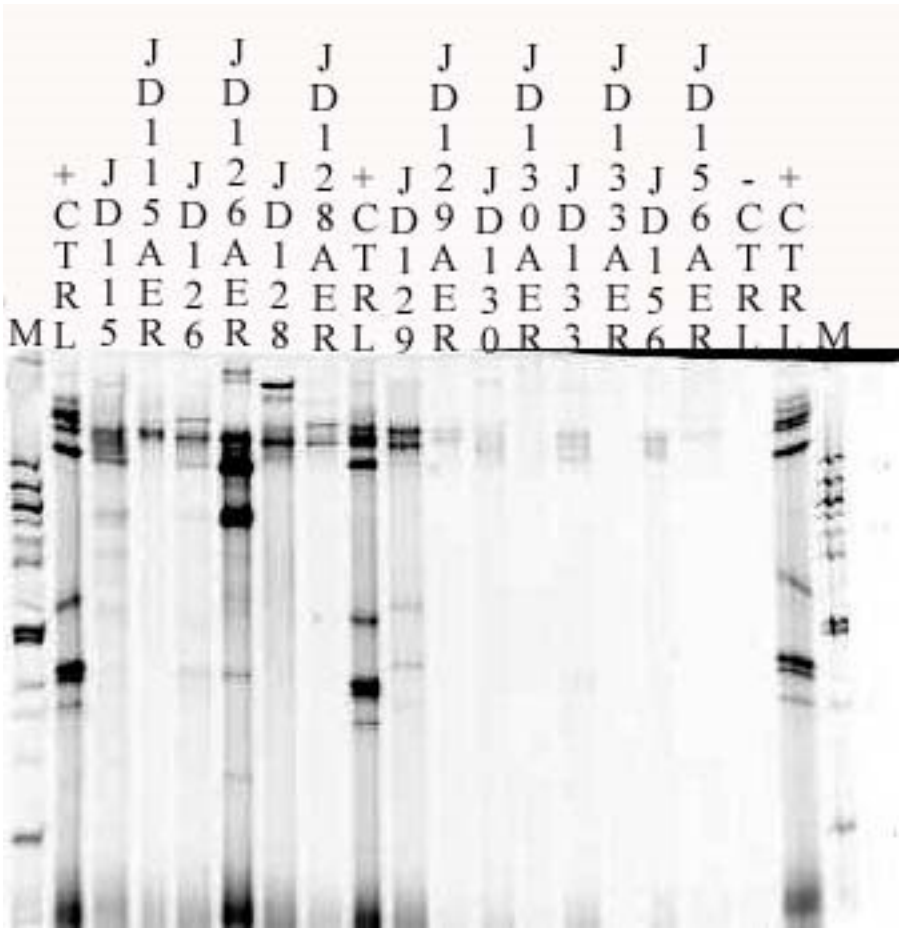

Figure B.8. DGGE gel image of mixed culture 16S rDNA region V3 PCR products. M = 100 bp DNA ladder. + CTRL = mixture of 16S rDNA region V3 PCR products from *E. coli*, *P.* strain ADP, *K. pneumoniae* strain 99, and *A. aurescens* TCI. – CTRL = PCR reaction without template DNA. All other lane designations correspond to the mixed culture serial numbers in Table B.8.

Table B.8. Mixed cultures appearing in Figure B.8. ORWRP = Olentangy River Wetland Research Park. WARS = Western Agricultural Research Station. No TEA = no terminal electron acceptor amendment. Sulfate = sodium sulfate amendment. N/a = data not applicable to this study.

Serial number	Sample location	Sample type	Culture conditions	Relevant chapter/appendix
JD115	ORWRP sediment	atrazine Bio-Sep beads	anaerobic, no TEA	Chapter 3
JD115 AER	ORWRP sediment	atrazine Bio-Sep beads	aerobic	Chapter 3
JD126	Campus dairy farm	atrazine Bio-Sep beads	anaerobic, no TEA	N/a
JD126 AER	Campus dairy farm	atrazine Bio-Sep beads	aerobic	N/a
JD128	ORWRP sediment	atrazine Bio-Sep beads	anaerobic, no TEA	Chapter 3
JD128 AER	ORWRP sediment	atrazine Bio-Sep beads	aerobic	Chapter 3
JD129	ORWRP sediment	water Bio-Sep beads	anaerobic, no TEA	Chapter 3
JD129 AER	ORWRP sediment	water Bio-Sep beads	aerobic	Chapter 3
JD130	ORWRP water	atrazine Bio-Sep beads	anaerobic, no TEA	Chapter 3
JD130 AER	ORWRP water	atrazine Bio-Sep beads	aerobic	Chapter 3
JD133	WARS former wash area	soil	anaerobic, no TEA	Chapter 4
JD133 AER	WARS former wash area	soil	aerobic	Chapter 4
JD156	Molly Caren Peninsula	atrazine Bio-Sep beads	anaerobic, sulfate	Appendix A
JD156 AER	Molly Caren Peninsula	atrazine Bio-Sep beads	aerobic	Appendix A

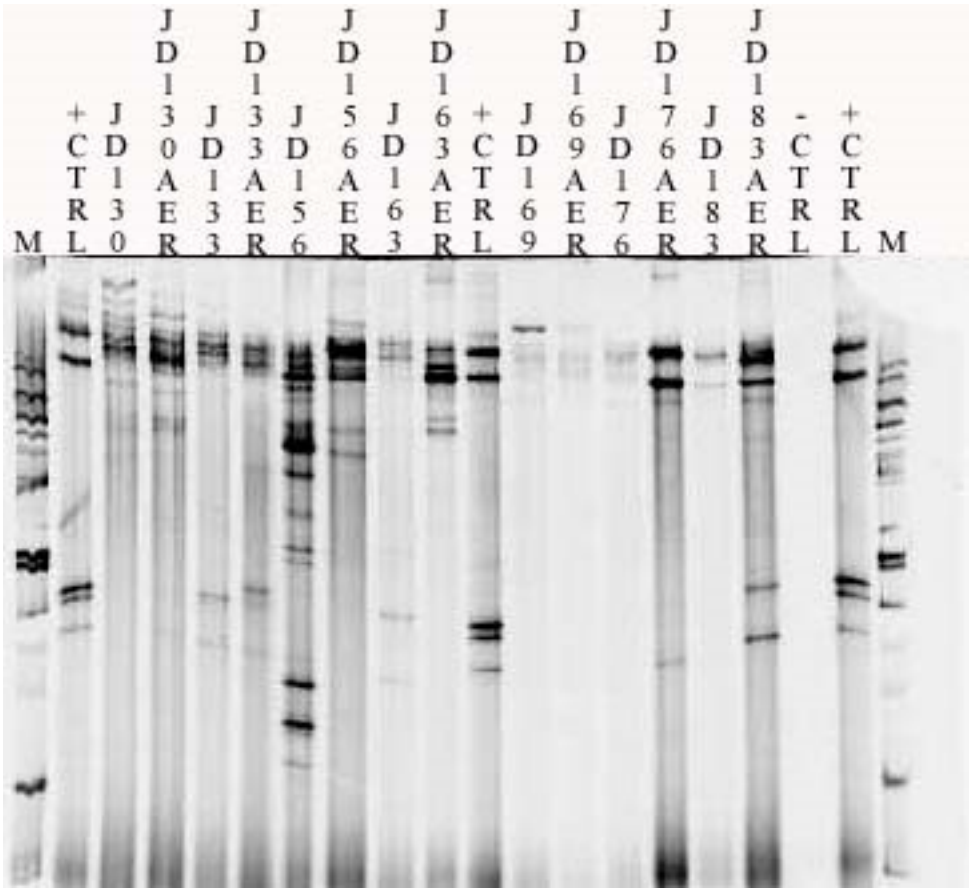

Figure B.9. DGGE gel image of mixed culture 16S rDNA region V3 PCR products. M = 100 bp DNA ladder. + CTRL = mixture of 16S rDNA region V3 PCR products from *E. coli*, *P.* strain ADP, *K. pneumoniae* strain 99, and *A. aurescens* TCI. – CTRL = PCR reaction without template DNA. All other lane designations correspond to the mixed culture serial numbers in Table B.9.

Table B.9. Mixed cultures appearing in Figure B.9. ORWRP = Olentangy River Wetland Research Park. WARS = Western Agricultural Research Station. No TEA = no terminal electron acceptor amendment. Sulfate = sodium sulfate amendment. N/a = data not applicable to this study.

Serial number	Sample location	Sample type	Culture conditions	Relevant chapter/appendix
JD130	ORWRP water	atrazine Bio-Sep beads	anaerobic, no TEA	Chapter 3
JD130 AER	ORWRP water	atrazine Bio-Sep beads	aerobic	Chapter 3
JD133	WARS former wash area	soil	anaerobic, no TEA	Chapter 4
JD133 AER	WARS former wash area	soil	aerobic	Chapter 4
JD156	Molly Caren Peninsula	atrazine Bio-Sep beads	anaerobic, sulfate	Appendix A
JD156 AER	Molly Caren Peninsula	atrazine Bio-Sep beads	aerobic	Appendix A
JD163	Campus dairy farm	water Bio-Sep beads	anaerobic, sulfate	N/a
JD163 AER	Campus dairy farm	water Bio-Sep beads	aerobic	N/a
JD169	Molly Caren Peninsula	atrazine Bio-Sep beads	anaerobic, sulfate	Appendix A
JD169 AER	Molly Caren Peninsula	atrazine Bio-Sep beads	aerobic	Appendix A
JD176	Campus dairy farm	water Bio-Sep beads	anaerobic, sulfate	N/a
JD176 AER	Campus dairy farm	water Bio-Sep beads	aerobic	N/a
JD183	WARS former mixing area	soil	anaerobic, sulfate	Chapter 4
JD183 AER	WARS former mixing area	soil	aerobic	Chapter 4

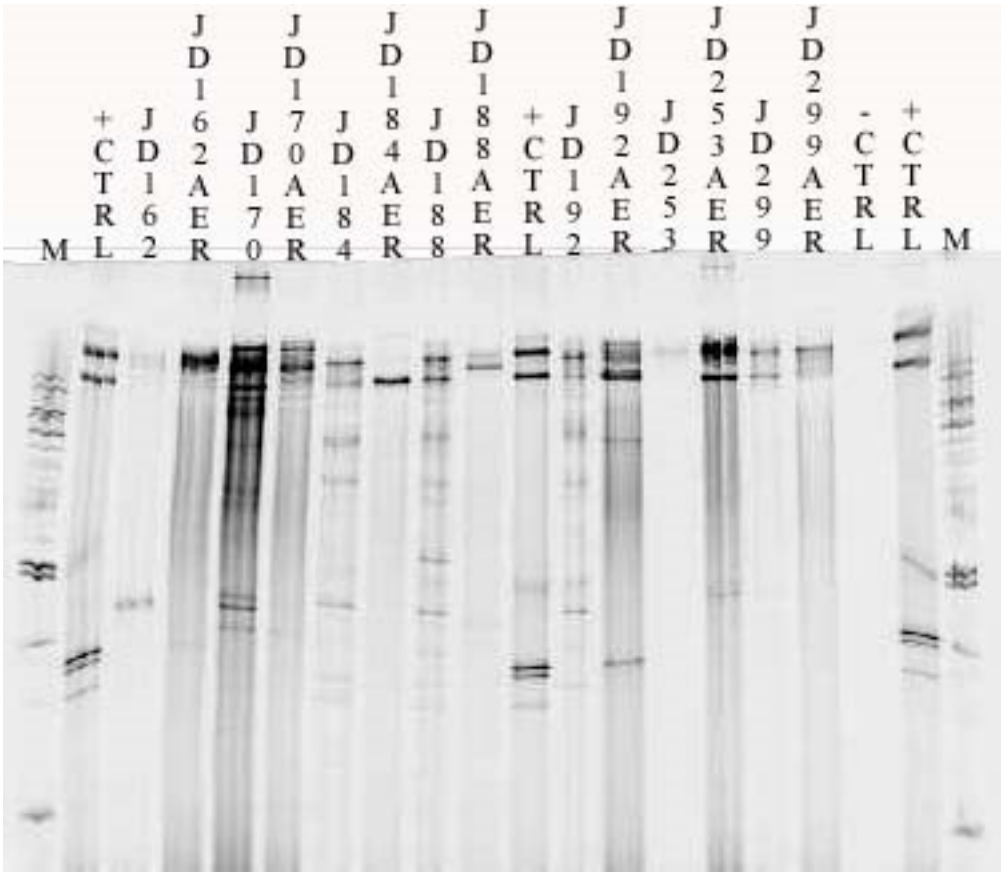

Figure B.10. DGGE gel image of mixed culture 16S rDNA region V3 PCR products. M = 100 bp DNA ladder. + CTRL = mixture of 16S rDNA region V3 PCR products from *E. coli*, *P.* strain ADP, *K. pneumoniae* strain 99, and *A. aurescens* TCI. CTRL = PCR reaction without template DNA. All other lane designations correspond to the mixed culture serial numbers in Table B.10.

Table B.10. Mixed cultures appearing in Figure B.10. ORWRP = Olentangy River Wetland Research Park. Sulfate = sodium sulfate amendment. Iron = ferrihydrite amendment. Nitrate = potassium nitrate amendment. N/a = data not applicable to this study.

Serial number	Sample location	Sample type	Culture conditions	Relevant chapter/appendix
JD162	Campus dairy farm	atrazine Bio-Sep beads	anaerobic, sulfate	N/a
JD162 AER	Campus dairy farm	atrazine Bio-Sep beads	aerobic	N/a
JD170	Molly Caren Peninsula	water Bio-Sep beads	anaerobic, sulfate	Appendix A
JD170 AER	Molly Caren Peninsula	water Bio-Sep beads	aerobic	Appendix A
JD184	Molly Caren Peninsula	atrazine Bio-Sep beads	anaerobic, sulfate	Appendix A
JD184 AER	Molly Caren Peninsula	atrazine Bio-Sep beads	aerobic	Appendix A
JD188	Molly Caren 3A	atrazine Bio-Sep beads	anaerobic, sulfate	Appendix A
JD188 AER	Molly Caren 3A	atrazine Bio-Sep beads	aerobic	Appendix A
JD192	ORWRP sediment	atrazine Bio-Sep beads	anaerobic, sulfate	Chapter 3
JD192 AER	ORWRP sediment	atrazine Bio-Sep beads	aerobic	Chapter 3
JD253	ORWRP sediment	atrazine Bio-Sep beads	anaerobic, iron	Chapter 3
JD253 AER	ORWRP sediment	atrazine Bio-Sep beads	aerobic	Chapter 3
JD299	ORWRP water	atrazine Bio-Sep beads	anaerobic, nitrate	Chapter 3
JD299 AER	ORWRP water	atrazine Bio-Sep beads	aerobic	Chapter 3

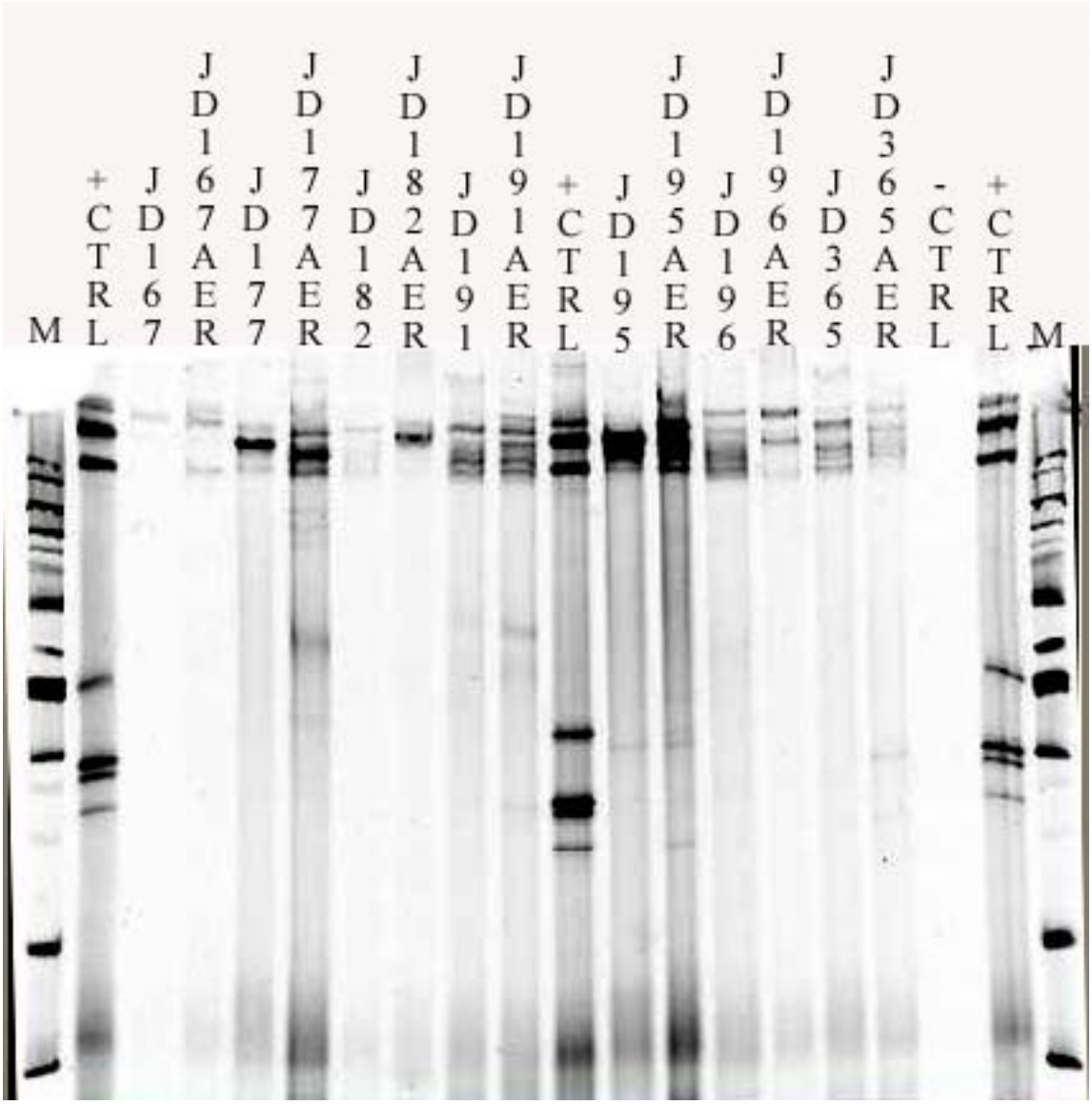

Figure B.11. DGGE gel image of mixed culture 16S rDNA region V3 PCR products. M = 100 bp DNA ladder. + CTRL = mixture of 16S rDNA region V3 PCR products from *E. coli*, *P.* strain ADP, *K. pneumoniae* strain 99, and *A. aurescens* TCI. CTRL = PCR reaction without template DNA. All other lane designations correspond to the mixed culture serial numbers in Table B.11.

Table B.11. Mixed cultures appearing in Figure B.11. ORWRP = Olentangy River Wetland Research Park. WARS = Western Agricultural Research Station. Sulfate = sodium sulfate amendment. No TEA = no terminal electron acceptor amendment.

Serial number	Sample location	Sample type	Culture conditions	Relevant chapter/appendix
JD167	ORWRP water	water Bio-Sep beads	anaerobic, sulfate	Chapter 3
JD167 AER	ORWRP water	water Bio-Sep beads	aerobic	Chapter 3
JD177	ORWRP sediment	atrazine Bio-Sep beads	anaerobic, sulfate	Chapter 3
JD177 AER	ORWRP sediment	atrazine Bio-Sep beads	aerobic	Chapter 3
JD182	WARS former wash area	soil	anaerobic, sulfate	Chapter 4
JD182 AER	WARS former wash area	soil	aerobic	Chapter 4
JD191	WARS former mixing area	water Bio-Sep beads	anaerobic, sulfate	Chapter 4
JD191 AER	WARS former mixing area	water Bio-Sep beads	aerobic	Chapter 4
JD195	ORWRP water	water Bio-Sep beads	anaerobic, sulfate	Chapter 3
JD195 AER	ORWRP water	water Bio-Sep beads	aerobic	Chapter 3
JD196	ORWRP water	water Bio-Sep beads	anaerobic, sulfate	Chapter 3
JD196 AER	ORWRP water	water Bio-Sep beads	aerobic	Chapter 3
JD365	ORWRP water	atrazine Bio-Sep beads	anaerobic, no TEA	Chapter 3
JD365 AER	ORWRP water	atrazine Bio-Sep beads	aerobic	Chapter 3

Table B.12. Key to Figures B.12-B.27. Originating mixed culture serial numbers are cross-referenced with Tables B.1-B.11. ORWRP = Olentangy River Wetland Research Park. WARS = Western Agricultural Research Station.

Lane	Isolate	Mixed culture	Location	Identity
1	TN196	JD67	ORWRP water	*Arthrobacter aurescens* TC1
2	TN134	JD93	WARS former mixing area	*Arthrobacter* sp. M2012083
4	TN197	JD67	ORWRP water	*Arthrobacter aurescens* TC1
5	TN69	JD23	Molly Caren 2A	*Arthrobacter* sp. NI2(2012)
6	TN83	JD2	Molly Caren 2A/3B	*Arthrobacter* sp. NI2(2012)
7	TN26	JD2	Molly Caren 2A/3B	*Arthrobacter* sp. JSPB6
8	TN70	JD23	Molly Caren 2A	*Arthrobacter* sp. NI2(2012)
9	TN85	JD2	Molly Caren 2A/3B	*Arthrobacter aurescens* TC1
10	TN2	JD23	Molly Caren 2A	*Arthrobacter* sp. LM1KK
11	TN3	JD23	Molly Caren 2A	*Arthrobacter* sp. NI2(2012)
12	TN43	JD2	Molly Caren 2A/3B	*Arthrobacter* sp. MG-2011-9-BV
13	TN67	JD23	Molly Caren 2A	*Arthrobacter* sp. NI2(2012)
14	TN81	JD2	Molly Caren 2A/3B	*Arthrobacter* sp. NI2(2012)
15	TN89	JD76	ORWRP sediment	*Arthrobacter* sp. B3051
17	TN151	JD15	WARS former mixing area	*Arthrobacter* sp. B3051
18	TN154	JD15	WARS former mixing area	*Arthrobacter* sp. B3035
19	TN61	JD2	Molly Caren 2A/3B	*Arthrobacter aurescens*
20	TN58	JD23	Molly Caren 2A	*Arthrobacter* sp. MG-2011-9-BV
21	TN53	JD24	Molly Caren 2A	*Arthrobacter* sp. MG-2011-9-BV
22	TN92	JD76	ORWRP sediment	*Arthrobacter* sp. 13AGG1
23	TN155	JD15	WARS former mixing area	*Arthrobacter* sp. B3051
25	TN231	JD54	ORWRP sediment	uncultured *Methyloversatilis* sp. clone K2S193
26	TN164	JD16	WARS former wash area	*Hydrogenophaga* sp. Gsoil 1545
28	TN159	JD15	WARS former mixing area	*Sinorhizobium* sp. TJ170
29	TN98	JD76	ORWRP sediment	*Arthrobacter* sp. 13AGG2
30	TN119	JD86	ORWRP sediment	*Sphingomonas* sp. SaMR12
32	TN84	JD2	Molly Caren 2A/3B	*Arthrobacter* sp. MG-2011-9-BV
33	TN161	JD15	WARS former mixing area	*Arthrobacter phenanthrenivorans* Sphe3
34	TN253	JD73	Molly Caren 3A	*Arthrobacter* sp. RKS6-6
35	TN232	JD54	ORWRP sediment	*Nocardioides* sp. HKT9991
36	TN233	JD54	ORWRP sediment	*Nocardioides* sp. ATD6

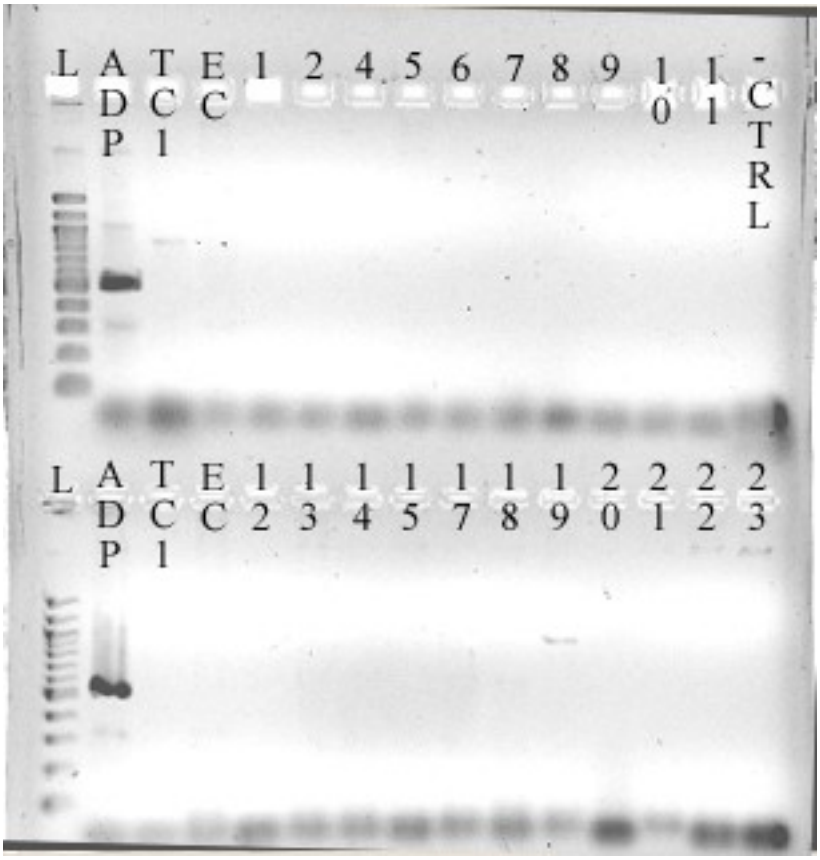

Figure B.12. Agarose gel image of *atzA* PCR products. L = 100 bp DNA ladder. ADP = *P.* strain ADP. TC1 = *A. aurescens* TC1. EC = *E. coli*. CTRL = PCR reaction without template DNA. All other lane designations correspond to the lane number designations in Table B.12.

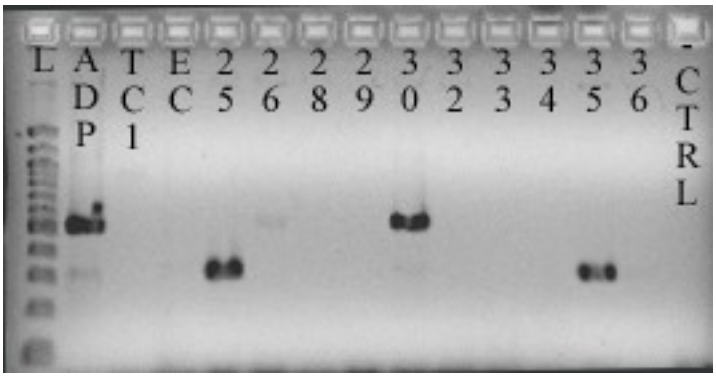

Figure B.13. Agarose gel image of *atzA* PCR products. L = 100 bp DNA ladder. ADP = . strain ADP. TC1 = *A. aurescens* TC1. EC = *E. coli*. CTRL = PCR reaction without template DNA. All other lane designations correspond to the lane number designations in Table B.12.

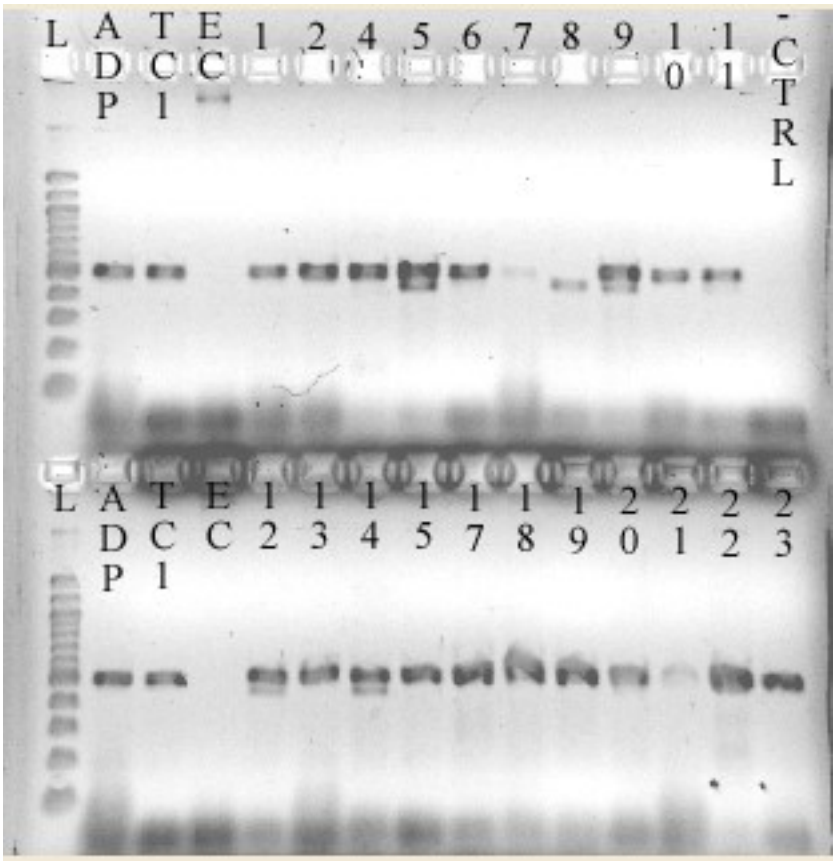

Figure B.14. Agarose gel image of *atzB* PCR products. L = 100 bp DNA ladder. ADP = *P.* strain ADP. TC1 = *A. aurescens* TC1. EC = *E. coli*. CTRL = PCR reaction without template DNA. All other lane designations correspond to the lane number designations in Table B.12.

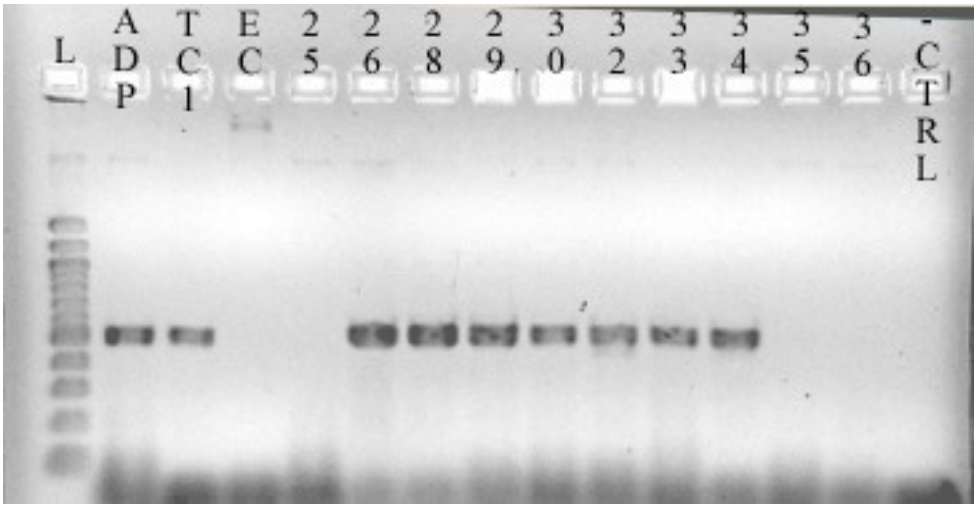

Figure B.15. Agarose gel image of *atzB* PCR products. L = 100 bp DNA ladder. ADP = *P.* strain ADP. TC1 = *A. aurescens* TC1. EC = *E. coli*. CTRL = PCR reaction without template DNA. All other lane designations correspond to the lane number designations in Table B.12.

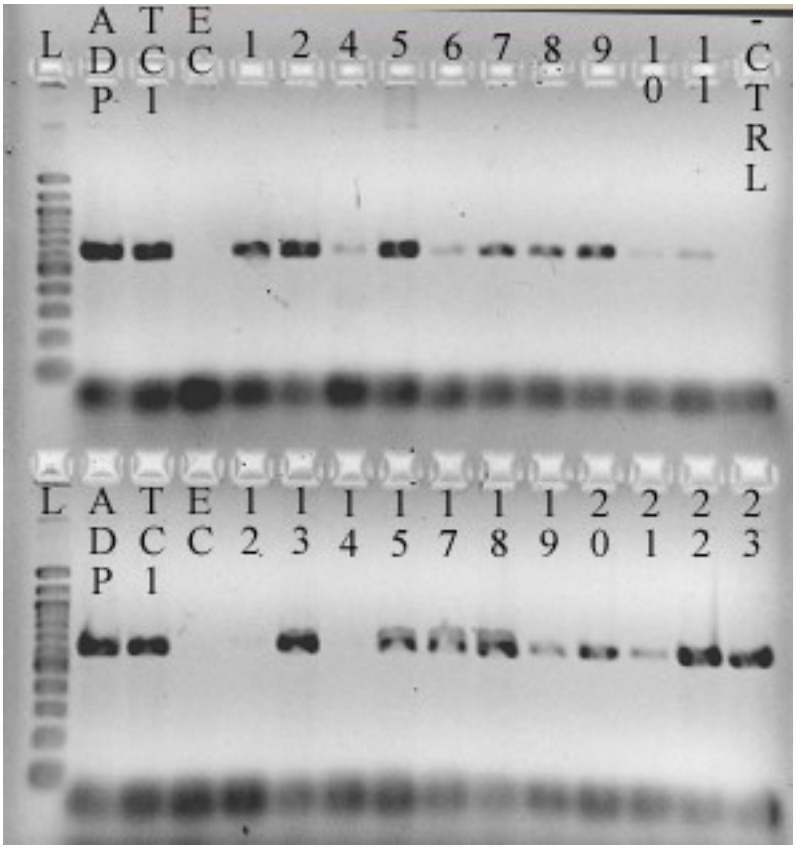

Figure B.16. Agarose gel image of *atzC* PCR products. L = 100 bp DNA ladder. ADP = *P.* strain ADP. TC1 = *A. aurescens* TC1. EC = *E. coli*. CTRL = PCR reaction without template DNA. All other lane designations correspond to the lane number designations in Table B.12.

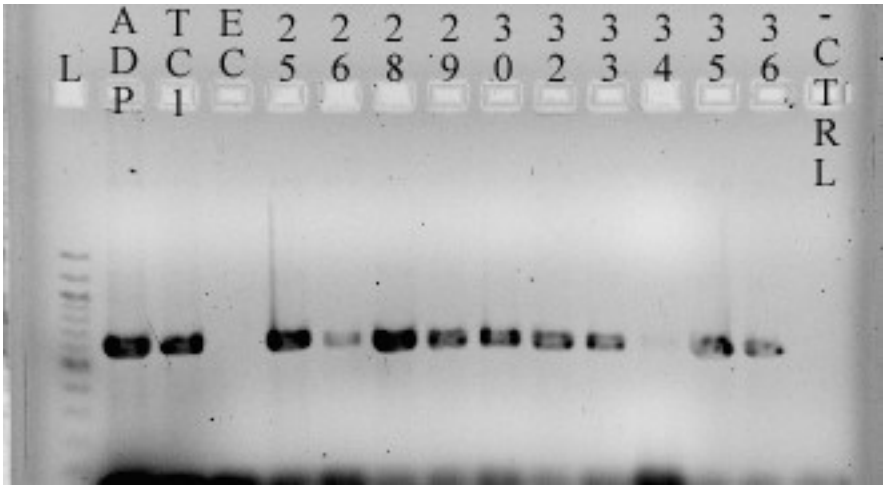

Figure B.17. Agarose gel image of *atzC* PCR products. L = 100 bp DNA ladder. ADP = *P.* strain ADP. TC1 = *A. aurescens* TC1. EC = *E. coli*. CTRL = PCR reaction without template DNA. All other lane designations correspond to the lane number designations in Table B.12.

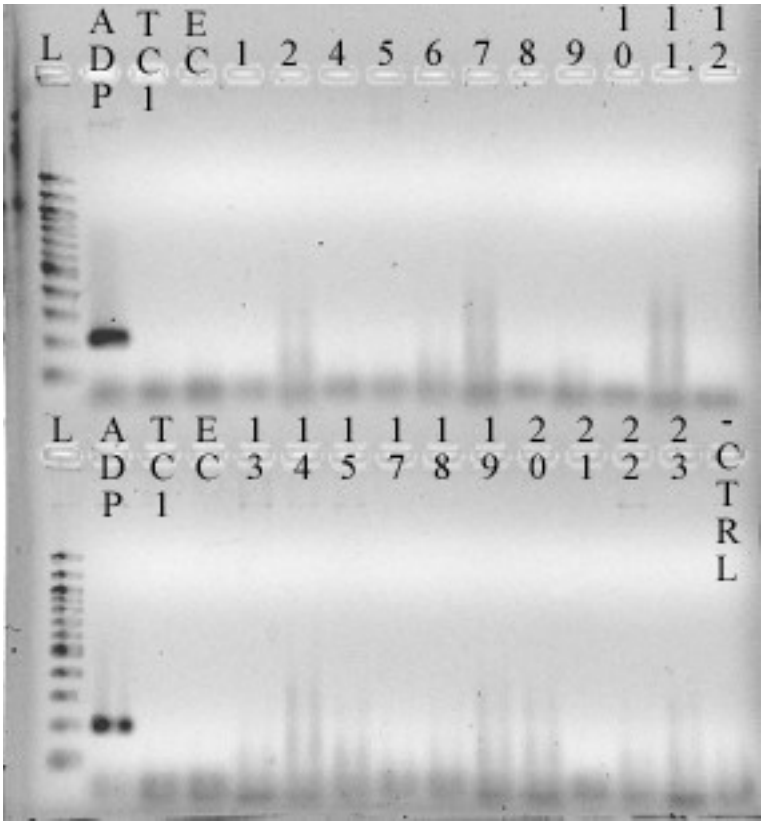

Figure B.18. Agarose gel image of *atzD* PCR products. L = 100 bp DNA ladder. ADP = *P.* strain ADP. TC1 = *A. aurescens* TC1. EC = *E. coli*. CTRL = PCR reaction without template DNA. All other lane designations correspond to the lane number designations in Table B.12.

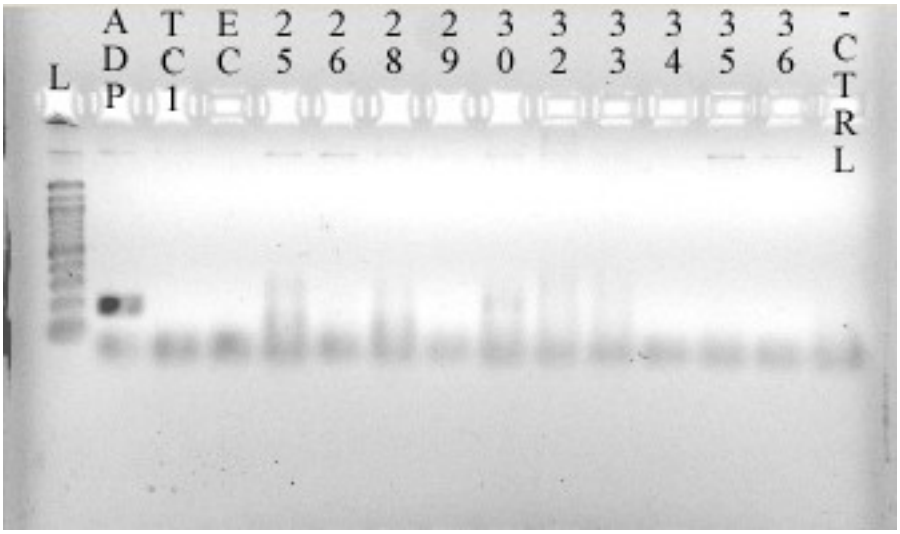

Figure B.19. Agarose gel image of *atzD* PCR products. L = 100 bp DNA ladder. ADP = *P.* strain ADP. TC1 = *A. aurescens* TC1. EC = *E. coli*. CTRL = PCR reaction without template DNA. All other lane designations correspond to the lane number designations in Table B.12.

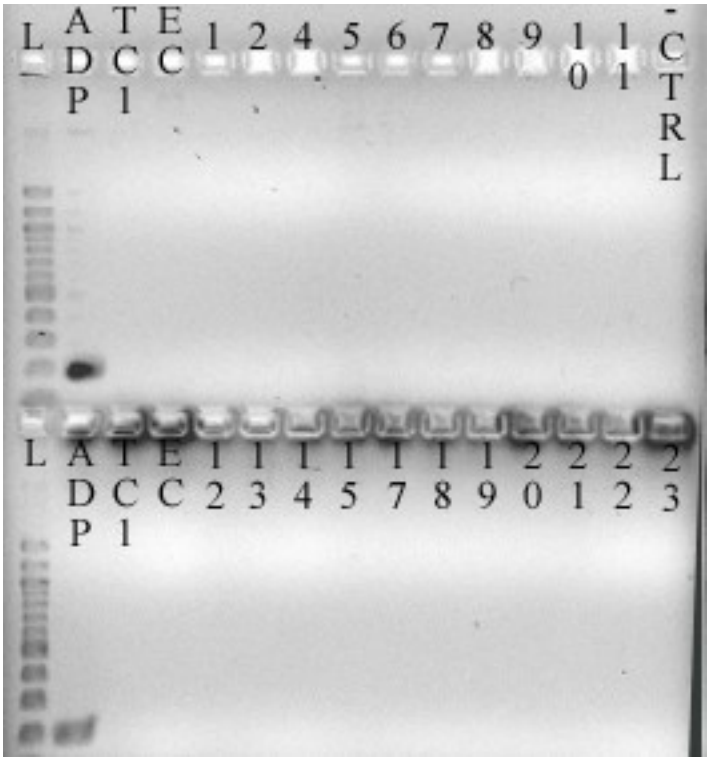

Figure B.20. strain Agarose gel image of *atzE* PCR products. L = 100 bp DNA ladder. ADP = *P.* ADP. TC1 = *A. aurescens* TC1. EC = *E. coli*. CTRL = PCR reaction without template DNA. All other lane designations correspond to the lane number designations in Table B.12.

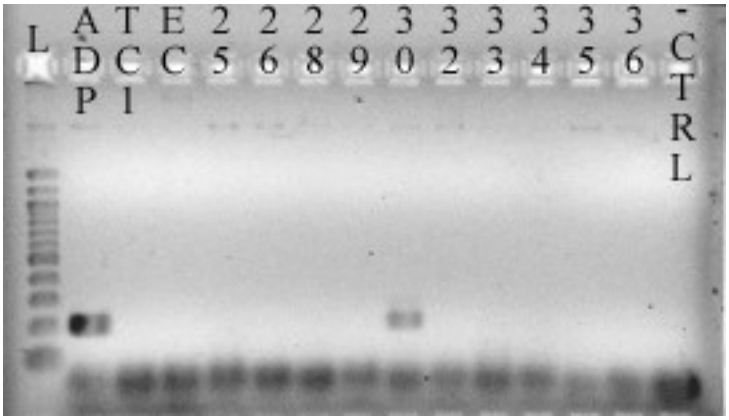

Figure B.21. Agarose gel image of *atzE* PCR products. L = 100 bp DNA ladder. ADP = *P.* strain ADP. TC1 = *A. aurescens* TC1. EC = *E. coli*. CTRL = PCR reaction without template DNA. All other lane designations correspond to the lane number designations in Table B.12.

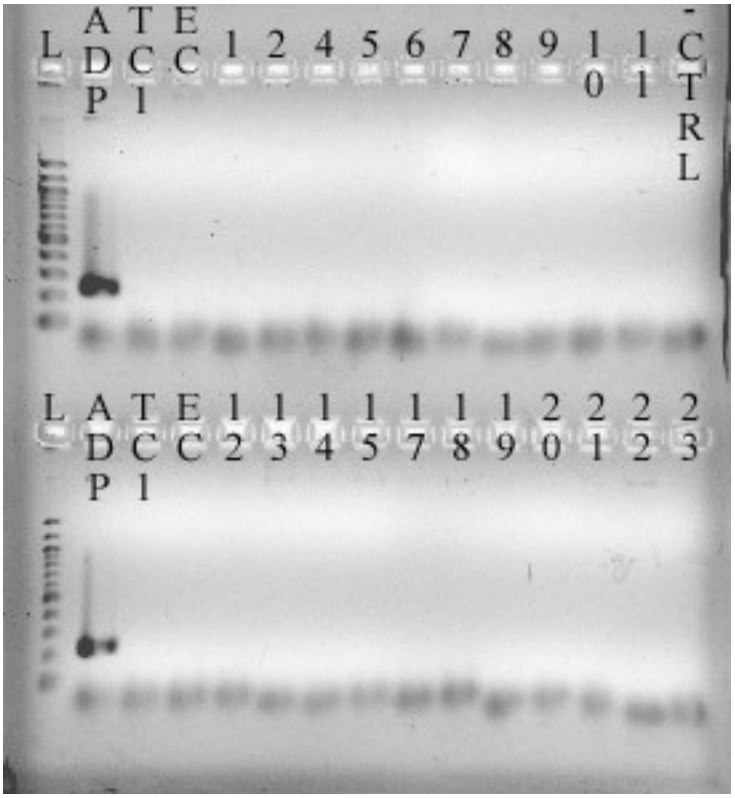

Figure B.22. Agarose gel image of *atzF* PCR products. L = 100 bp DNA ladder. ADP = . strain ADP. TC1 = *A. aurescens* TC1. EC = *E. coli*. CTRL = PCR reaction without template DNA. All other lane designations correspond to the lane number designations in Table B.12.

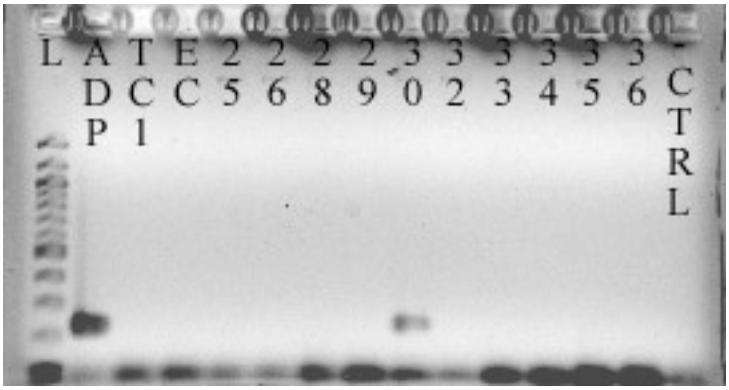

Figure B.23. Agarose gel image of *atzF* PCR products. L = 100 bp DNA ladder. ADP = *P.* strain ADP. TC1 = *A. aurescens* TC1. EC = *E. coli*. CTRL = PCR reaction without template DNA. All other lane designations correspond to the lane number designations in Table B.12.

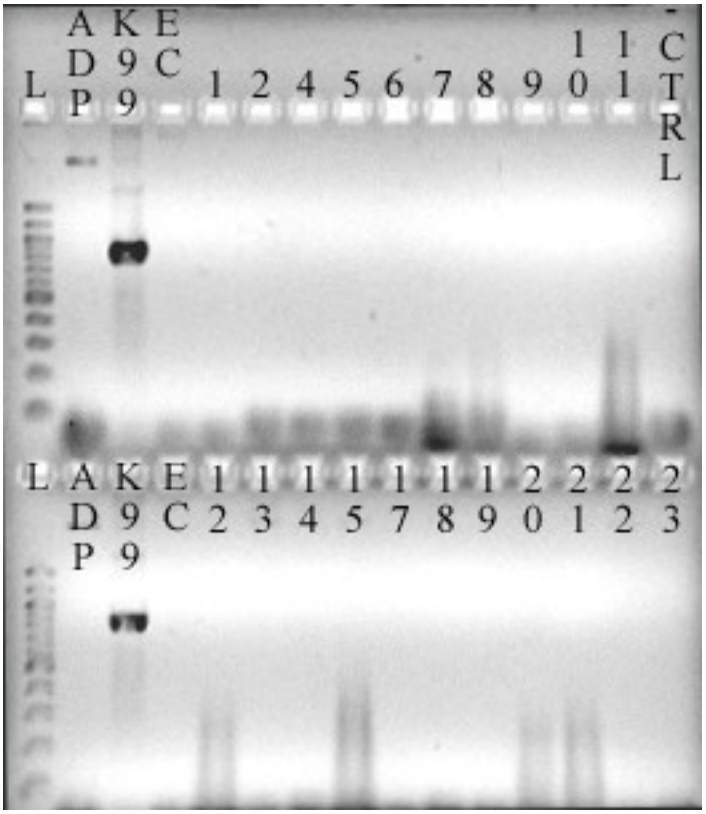

Figure B.24. Agarose gel image of *trzD* PCR products. L = 100 bp DNA ladder. ADP = *P.* strain ADP. TC1 = *A. aurescens* TC1. EC = *E. coli*. CTRL = PCR reaction without template DNA. All other lane designations correspond to the lane number designations in Table B.12.

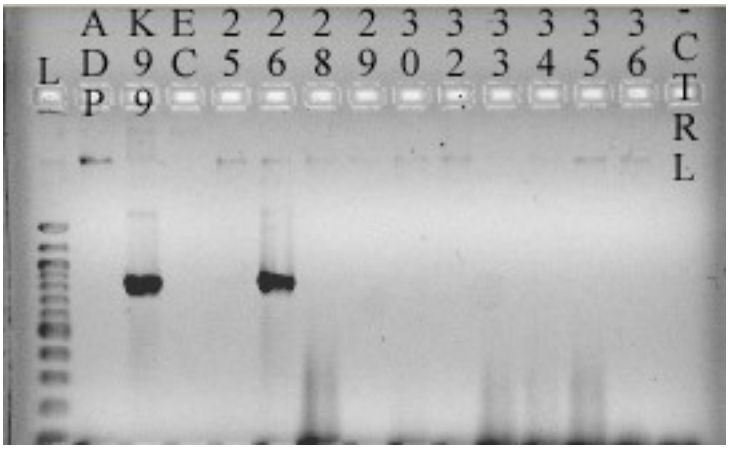

Figure B.25. Agarose gel image of *trzD* PCR products. L = 100 bp DNA ladder. ADP = *P.* strain ADP. TC1 = *A. aurescens* TC1. EC = *E. coli*. CTRL = PCR reaction without template DNA. All other lane designations correspond to the lane number designations in Table B.12.

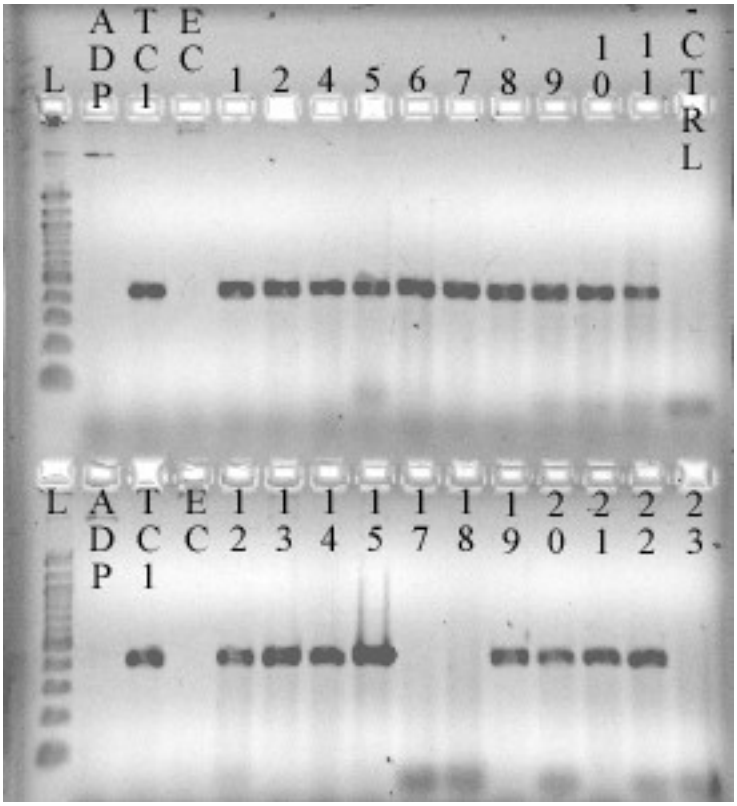

Figure B.26. Agarose gel image of *trzN* PCR products. L = 100 bp DNA ladder. ADP = *P.* strain ADP. TC1 = *A. aurescens* TC1. EC = *E. coli*. CTRL = PCR reaction without template DNA. All other lane designations correspond to the lane number designations in Table B.12.

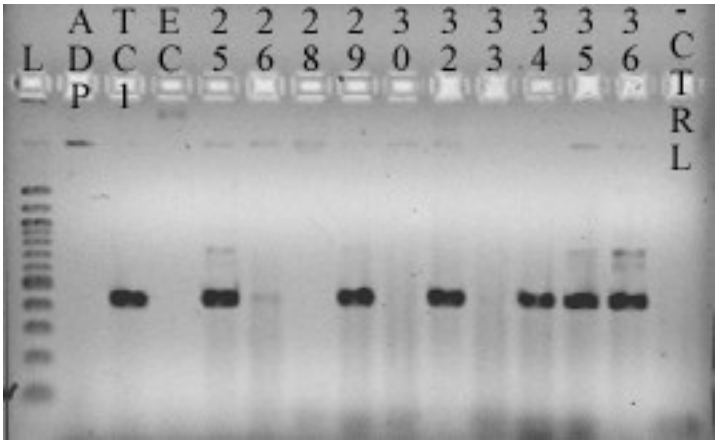

Figure B.27. Agarose gel image of *trzN* PCR products. L = 100 bp DNA ladder. ADP = *P.* strain ADP. TC1 = *A. aurescens* TC1. EC = *E. coli*. CTRL = PCR reaction without template DNA. All other lane designations correspond to the lane number designations in Table B.12.