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Cultivation of Phylogenetically Diverse and Metabolically Novel Atrazine Degrading Soil Bacteria using Bio-Sep® Beads

Emily Catherine Martin
University of Tennessee - Knoxville

To the Graduate Council:

I am submitting herewith a thesis written by Emily Catherine Martin entitled "Cultivation of Phylogenetically Diverse and Metabolically Novel Atrazine Degrading Soil Bacteria using Bio-Sep® Beads." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Environmental and Soil Sciences.

Mark Radosevich, Major Professor

We have read this thesis and recommend its acceptance:

Neal Eash, Susan Pfiffner

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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Linda Painter_____
Interim Dean of Graduate Studies

(Original Signatures are on file with official student records.)

Cultivation of Phylogenetically Diverse and Metabolically Novel Atrazine Degrading Soil Bacteria using Bio-Sep® Beads

A Thesis
Presented for the
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Emily Catherine Martin
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Abstract

The s-triazine herbicide atrazine is among the most widely used herbicides worldwide. The human health effects of atrazine exposure remain unclear, but atrazine and its metabolites appear to cause developmental abnormalities in amphibians. A mounting body of knowledge concerning the ecology of atrazine degradation suggests the current collection of microorganisms and genetic biomarkers of atrazine degradation cannot accurately predict the natural attenuation of atrazine. To this end, a novel in situ enrichment approach using highly porous, atrazine-impregnated Bio-Sep® beads was employed to isolate a taxonomically diverse group of atrazine-degrading bacteria from soil and wetland environments in Tennessee and Ohio. The study greatly increased the scope and diversity of organisms previously shown to degrade atrazine. Most notable, a novel lineage within the Bacteriodetes phylum, *Dyadobacter* sp. was obtained, constituting the first report of the atrazine-degrading phenotype within this division. Although not taxonomically novel, previously unreported atrazine-degrading taxa from Actinobacteria (*Catellatospora*, *Microbacterium*, and *Glycomyces*), Alpha-Proteobacteria (*Methylobacterium*, *Methylopila,* and *Sphingomonas*), Beta-Proteobacteria (*Variovorax* and *Acidovorax*), and Gamma-Proteobacteria (*Acinetobacter, Rahnella,* and *Pantoea*) were also isolated. Evidence for metabolic diversity in atrazine catabolism was observed in the collection. Most significantly, the atrazine-chlorohydrolase gene, encoded by *trz*N, was the only known catabolic gene detected in our collection with the exception of the *Arthrobacter* strains which typically also possessed *atzB* and *atzC*, that code for enzymes needed for sequential dealkylation of 2-hydroxy atrazine. No other known genes for the intermediate metabolism were detected in many of the isolates suggesting the presence of

alternative degradative pathways for atrazine among soil bacteria. Previously, *trz*N has only been reported in high G+C Gram-positive bacteria but our results revealed that this catabolic gene is much more broadly distributed among classes including the Alpha and Beta Proteobacteria. The results demonstrate that Bio-Sep® beads are a suitable matrix for recruiting a highly diverse subset of the bacterial community involved in atrazine degradation.

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I. Introduction and Background

A. Pesticide Use and S-triazines

Rapid human population grown has necessitated the development of tools and technologies to provide an abundant and affordable food supply. These technologies include soil cultivation, development of higher-yielding crop species, soil conservation techniques, and many, many more, but maybe none more important than the development of pesticides. Pesticides have exponentially increased our ability to manage pests including weeds, insects, and microbial pathogens. The Environmental Protection Agency (EPA) estimates total worldwide expenditures for all pesticides at over $31 billion accounting for 5,046 million pounds of active ingredients in 2001. The United States (US) purchased in the same year $11 billion and 1,203 million pounds of pesticides as active ingredient. Over 40% of the US expenditure was on herbicides, compounds specifically targeted toward weed species. Eighty percent of these herbicides were utilized in agriculture (United States Environmental Protection Agency, 2004). The staggering rate of herbicide use, their detection in water supplies, and possible harm to the environment, has prompted a substantial effort to further determine their environmental fate. One such class of herbicides that has received much attention in recent years is the s-triazines.

S-triazines were first discovered and developed in 1952 by a group of researchers at the J.R. Geigy group in Basel, Switzerland (Esser et al., 1975). The s-triazines consist of a six member ring with three symmetrical nitrogen atoms at the 1,3, and 5 positions and two nitrogen-containing alkyl groups at the 4 and 6 ring carbon atom positions

(Wackett, 2004b). The most widely used s-traizines are atrazine and simazine. The chemical structure of atrazine is shown in Figure 1.

B. Atrazine Use and Mode of Action

Atrazine is one of the most heavily used herbicides in the US with over 76 million pounds used in the US in 2001. Second only to glyphosate, in 2001, the EPA estimated between 74 and 80 million pounds of atrazine were applied in the US (United States Environmental Protection Agency, 2004). Between 1980 and 1990, 800 million pounds of atrazine were applied in the US alone (Ralebitso et al., 2002). Atrazine provides control of broad-leaf and grassy weeds and may be used both pre and post emergence. This class of herbicide acts as a photosystem II inhibitor by blocking electron transport in the production of oxygen leading to a build up of carbon dioxide in the cell and eventually plant death (Esser et al., 1975; Solomon et al., 1996). Atrazine's labeled uses include corn, sorghum, sugarcane, guavas, and macadamia nuts. Non-agriculturally, it is labeled for use on conifer forests, sod farms, some rangeland, and golf courses. Agriculture is the largest source of atrazine use with over 75% of field corn acreage, 60% of sorghum, and 76% of all sugarcane receiving the herbicide (United States Environmental Protection Agency, 2003a). Atrazine is formulated as a flowable concentrate, granular, and ready-to-use product (United States Environmental Protection Agency, 2003a). The most common trade name for atrazine is Aatrex® and currently registered for use by Syngenta Corporation, but is also sold as a tank mix with many other herbicides.

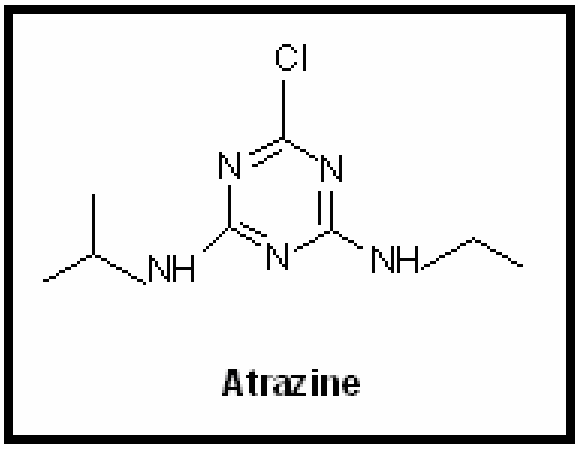

Figure 1: Chemical structure of atrazine.

C. Regulatory History

Frequent detection of atrazine and its metabolites in water supplies especially in = agricultural regions including the Midwestern 'Corn Belt' and the Mississippi Delta has driven regulatory agencies and researchers to further examine potential consequences of s-triazine use in the environment. Atrazine was first registered in 1958 by Ciba-Geigy Corporation and for almost forty years has been a very effective and inexpensive herbicide for weed control. But atrazine became a frequently detected pesticide in water supplies especially in the Midwestern corn producing areas in the early 1990's and prompted the EPA to regulate atrazine under the Safe Drinking Water Act. An maximum contamination level (MCL) was set at 3 ppb and a one-time health advisory level was set at 100 ppb (United States Environmental Protection Agency, 2003a). In addition, other voluntary changes were made by the manufacturers of atrazine that included labeling the herbicide as a restricted use pesticide, meaning it could only be sold and administered by certified chemical applicators, and limited use within specific distances of water sources to reduce direct contamination. The rate of atrazine use was decreased from 4 lbs active ingredient per acre per year first to 3 lbs. and then to 2.5 lbs. In 1994, in response to possible carcinogenic effects from exposure to atrazine from water and food supplies, a special review of all triazine herbicides was launched by the EPA to monitor environmental impact. At the inception of the review, atrazine was listed as a possible human carcinogen, Class C, because of concerns raised about overexposure to atrazine through food, water, or occupational use (United States Environmental Protection Agency, 2003a).

A landmark publication by Hayes et al. (2002) raised great concerns regarding atrazine and amphibian development. Hayes reported African clawed frogs (Xenopus laevis) exposed to atrazine doses as low as 0.1 ppb during larval development exhibited signs of hermaphrodism, having both male and female sex organs, and at doses of 1 ppb male frogs showed demasculinization characterized by decreased larynx size (Hayes et al., 2002). This was the first study to show effects of atrazine exposure at rates well below MCL of 3 ppb, as set by the EPA as safe in drinking water. The author went on to publish field studies of frogs obtained from atrazine contaminated areas reporting the same symptoms in as high as 92% of frogs tested in an area of Wyoming fed by the North Platte river known to be contaminated with atrazine (Hayes et al., 2003). Teavera-Mendosa et al. published similar results showing single exposures to atrazine during tadpole development had significant effects on the development of reproductive organs (Tavera-Mendoza et al., 2002a,b).

In 2003, the EPA released an Atrazine Interim Registration Eligibility Decision (IRED) containing a review of atrazine, its uses, and environmental effects deeming it safe for re-registration with some label amendments (United States Environmental Protection Agency, 2003a). The IRED lists seasonal dietary risk from drinking water in 34 community water sheds concentrated in the Midwest, but acute risks from dietary consumption were below levels of concern. In response, 34 watersheds, in addition to three others previously identified as atrazine contaminated watersheds, were placed under strict monitoring procedures to ensure atrazine was promptly detected and removed from drinking waters in these areas especially in times of heaviest atrazine use. Other label changes were implemented to ensure greater protection for applicators and protection of

families, especially children, in residential areas of atrazine use. Ecological risks were also addressed and concluded that effects could be seen at community and population levels with atrazine concentrations between 10 and 20 ppb (United States Environmental Protection Agency, 2003a). A key ecological impact of atrazine was seen to aquatic organisms from indirect losses of plant biomass in their ecosystems resulting from exposure to atrazine in water supplies (United States Environmental Protection Agency, 2003b). Research efforts concerning amphibian development in the presence of atrazine were underway when the IRED was published that could not be included due to time constraints, so to address these findings, the EPA developed a 'white paper' to critically review the relevant amphibian ecological studies, including the ones described above in Hayes (2002,2003) and Teavera-Mendosa et al. (2002a,b). The review included seven independent studies published in the literature and twelve registrant-sponsored submitted studies. The review board concluded the current body of evidence was inconclusive in determining whether atrazine has significant effects on gonadal and laryngeal development in frog stating inconsistent and non-reproducible effects across all studies based largely on a lack of available dose-response data. But in conclusion, the panel left open the possibility that atrazine may in fact have developmental effects on amphibians and in an effort to gain a greater sense of certainty on the matter, more research was needed (United States Environmental Protection Agency, 2002, 2003b). Ultimately, atrazine was cleared for re-registration.

D. Detection in the Environment

1. Ground and Surface Water

Widespread use, mobility, and moderate persistence make atrazine and its metabolites the most common agriculture pesticide detected in water supplies in the US. Ground water pollution is of great concern as 40 to 50% of domestic drinking water is pumped from groundwater supplies, especially in rural areas (Jayachandran et al., 1994; Mills et al., 2005). Many of the affected areas are in closest proximity to the major agricultural use areas for atrazine such as the Midwestern corn producing areas, but recent studies have shown atrazine moving into urban watersheds as well (Burkart and Kolpin, 1993; Kolpin et al., 1998). In 1991, the United States Geological Survey began the National Water Quality Assessment to monitor water quality across the US and continues to serve as a major source of water quality data (Kolpin et al., 2000). Thurman et al. (1992) reported levels of atrazine 3-5 times greater than its EPA standard maximum contaminant level (MCL) of 3 ppb in surface waters of the Midwestern US just after spring planting, which Thurman coined, the 'spring flush'. In a study of near surface aquifers in the mid-continent US, atrazine and its two main metabolites, deethylatrazine (DEA) and deisopropylatrazine (DIA), were the most frequently detected compounds. In this survey, the MCL for atrazine was not exceeded, and MCL values for DEA and DIA have not been established. In combination, the three compounds were above the MCL for atrazine. The same study also showed that aquifer depth was inversely related to herbicide concentrations; deeper aquifers had more time to dissipate, transform, or adsorb the herbicides while shallow ones did not. Sampled wells within 30 m of a stream contained more than two times the amount of herbicides than ones greater than 30 m

away (Burkart and Kolpin, 1993). Jayachandran et al. (1994) conducted a study to examine atrazine and its metabolites in subsurface drainage and shallow groundwater in conjunction with long-term corn production at the Till Hydrology Site in Ames, Iowa. Forty percent of the samples tested contained concentrations of atrazine above the MCL in shallow groundwater and tile drainage, and DEA and DIA were detected in all samples (Jayachandran et al., 1994).

Kopin and associates have published multiple data sets of water quality data obtained in the mid-1990's of more than 3000 groundwater samples from 2485 sites across the US. Overall, the data indicated that atrazine and DEA were the two most common compounds detected in all of the studies. In many cases, atrazine by itself was not above the MCL, but the total s-triazine residues including DEA and DIA was often times above the MCL for atrazine alone. N-dealkylated degradation products of atrazine have similar toxicities as atrazine, with no EPA regulated MCL, and the authors suggested that in tandem, the parent and metabolites, pose a greater risk to environmental health and should be considered when determining overall effects on the environment (Kolpin et al., 1997, 1998, 2000, 2002; Barbash et al., 2001).

Soil tillage effects on atrazine loss were assessed in Southwestern Ontario, Canada. The MCL of atrazine was exceeded in an average of 60% of surface water runoff samples and 56% of tile drainage samples (n=42) from conventional, no-till, and ridge tillage combined, with no significant differences seen between tillage treatments. The incidence of a surface runoff event within a week of application resulted in up to a 7% loss of applied atrazine (Gaynor et al., 1995).

2. Urban Areas

Even though urban areas are subject to much less pressure from direct application of agricultural pesticides, urban streams are still susceptible to non-point source contamination by many other compounds, including atrazine. Large areas of impermeable land surfaces with storm drains provide a direct route to main water sources in urban areas, and are often times located downstream of major agricultural areas. In a study of pesticides in eight urban streams across the country, Hoffman et al. reported that atrazine was seen in 57% of the streams with a maximum concentration of 0.77 ppb. This is of note, as atrazine use is limited in non-agricultural areas. Atrazine was detected in urban areas with water drainage basins whose area containing less than 2% agricultural use indicating possible atmospheric deposition, which has been seen regarding atrazine and is described below. Unfortunately, only atrazine and none of the degradation products were monitored in this study (Hoffman et al., 2000).

3. Air, Rain, and Fog

Air and rain are also agents of transport for pesticides in the environment. Atrazine is thought to reach the atmosphere through volatilization, particle movement, and drift. Volatilization loss can be approximately 2% of the applied mass, is greatest immediately after application, and can be increased with warm weather and wind. Particle movement and drift may occur when atrazine or soil containing atrazine are picked up by wind during or shortly after application (Thurman and Cromwell, 2000). Atrazine has been seen in rain at low levels across the US throughout the year with maximum concentrations occurring in the months of highest use in April, May, and June (Wu, 1981; Glotfelty et al., 1990; Nations and Hallberg, 1992;). Majewski and

colleagues at the USGS studied pesticides in air and rain in the Mississippi River Valley in both urban and agricultural areas for a six-month period in 1995. Atrazine and DEA were detected in all the rain samples and in an average of 75% of air samples in both urban and agricultural sites with depositional amounts greatest in urban areas, posing questions about the total atmospheric transport of triazine herbicides and its lack of deposition in agricultural areas (Majewski et al., 1998, 2000; Foreman et al., 2000). Atrazine has also been detected at low levels in fog (Glotfelty et al., 1987). The herbicide has also been seen in pristine areas far from agricultural or urban inputs like the Isle Royale National Park in Lake Superior (Thurman and Cromwell, 2000) and in remote Swiss mountain lakes (Nations and Hallberg, 1992).

Atrazine occurrence in the environment has been well documented and in general is thought to be ubiquitous. Generally, the highest amounts of atrazine documented occur at times and in regions of greatest agricultural use, but are also seen deposited in urban areas indicating long-term persistence, and a year-round baseline level of subsistence in the atmosphere including water, rain, fog, and air.

E. Transformations in the Environment

Atrazine's ubiquitous nature and possible environmental effects has stimulated considerable research towards understanding its fate and attenuation in the environment. Many biotic and abiotic factors affect its transformations in the environment and will be discussed further.

1. Contact and Movement in Soil

Initial soil contact with atrazine can be achieved by direct application during pre-emergence of weeds or pre-plant of the crop, displacement from the foliar surface of the plant population, or drift during application. Atrazine is characterized as mobile and moderately persistent in the environment, which is confirmed by its ubiquitous nature in ground and surface water (Kolpin et al., 1998). The chemical and physical attributes of atrazine are listed in Table 1. Atrazine's low vapor pressure and Henry's Law Constant render volatilization of atrazine low, with minimal decrease in activity (Glotfelty et al., 1989; Clendening et al., 1990; Solomon et al., 1996; Weber, et al., 2002). Atrazine is moderately soluble in water (33 mg/L at 22ºC) and in combination with its low sorption coefficients, atrazine is considered relatively mobile in soil (Solomon et al., 1996; United States Environmental Protection Agency, 2002, 2003a). The half-life in soil varies from days to months, due to the multitude of physical and biological conditions in soils. Soil parameters such as pH, temperature, oxygen status, soil preparation, soil structure, initial water content, preferential flow pathways, time of atrazine application, history of atrazine exposure, and microbial activity account for some of the variability of atrazine movement and persistence in soils and groundwater (Jayachandran et al., 1994; Vanderheyden et al., 1997; Sadowsky and Wackett, 2001). Surface water may receive atrazine from run-off, atmospheric deposition, or spray drift but in many cases groundwater becomes contaminated from preferential transport and leaching (United States Environmental Protection Agency, 2003a). Once in water supplies, atrazine is much more recalcitrant with half lives as long as a year (Solomon et al., 1996).

Table 1: Physical and Chemical Characteristics of Atrazine

Chemical Accession Number	1912-24-9
Chemical Name	2-chloro-3-ethylamino-6-isopropyl-amino-1-s-triazine
Molecular Weight	215.70 g/mol
Molecular Formula	$C_8H_{14}N_5Cl$
Water Solubility	33 µg/ml at 22˚C
Vapor Pressure	2.89×10^{-7} mm Hg at 25˚C
Henry's Law Constant	2.48×10^{-9} atm m^3 mol^{-1}
Log K_{ow}	2.68 at 25˚C
Hydrolysis	Stable for 30 d at ph 5-9 at 25˚C
Aqueous Photolysis	Natural Light $t_{1/2}$ 335 d at pH 7
	Mercury Lamp $t_{1/2}$ 17.5 d at pH 7
Soil Photolysis	Natural Light $t_{1/2}$ 12 d
	Mercury Lamp $t_{1/2}$ 5 d
	Xenon Lamp $t_{1/2}$ 45 d

Note: Adapted from Solomon (Solomon *et al.* 1996).

2. Degradation

Abiotically, atrazine can be degraded in soils to hydroxyatrazine (HA), a degradate considered non-phytotoxic in soils, via in a hydrolytic dechlorination enhanced by adsorption of atrazine to organic matter or clay and an acidic soil pH. In early studies of atrazine dissapation, this abiotic transformation was thought to be very important, but eventually was shown to be less significant than biodegradation (Armstrong et al., 1967; Skipper et al., 1967,1972). Although atrazine is transformed abiotically to some extent, microbial degradation is considered the key attenuation pathway (Kaufman and Kearney, 1970; Cook and Hutter, 1981; Erickson and Lee, 1989).

Biological degradation of atrazine has been shown to involve three main steps: hydroxylation, N-dealkylation, and ring cleavage (Kaufman and Kearney, 1970; Behki and Khan, 1986). Atrazine can serve as both a carbon and nitrogen source for microorganisms. It is a poor carbon source as the only available carbon is located on the substituted alkylamino groups. The ring carbons are nearly fully oxidized, rendering them unavailable for energy yielding metabolism (Radosevich et al., 1997; Shapir and Mandelbaum, 1997). Hydroxylation in the case of atrazine involves dechlorination in either an oxidation or hydrolytic reaction to form hydroxyatrazine (Armstrong et al., 1967; Kaufman and Kearney, 1970; Mandelbaum et al., 1993b). Fungi were thought to be the only organisms capable of hydroxylation, until the bacteria were characterized with the same function in the mid-90's (Kaufman and Kearney, 1970; Mandelbaum et al., 1993a). N-dealkylation is thought to be the main microbial mechanism of transformation in soils and water (Zablotowicz et al., 2006). The metabolites DEA and DIA are the most commonly observed in soils and water and are not thought to be formed abiotically

(Kaufman and Kearney, 1970; Zablotowicz et al., 2006). N-dealkylation involves use of the alkyl group as a source of carbon and energy for heterotrophic growth (Erickson and Lee, 1989). The ethyl side group is thought to be preferred over the isopropyl group in microbial metabolism (Mills and Thurman, 1994). Ring cleavage of s-triazines involves the release of carbon dioxide from the breakage of the triazine ring (Erickson and Lee, 1989). The most common routes of degradation include combinations of these metabolic processes and their products have been thoroughly reviewed (Erickson and Lee, 1989). The two most common upper pathways of atrazine degradation are shown in Figure 2.

The main microbial degradation route in soils traditionally has been recognized as sequential N-dealykation to produce DEA and DIA, which are the two major atrazine derivatives seen in soils and water (Shapir and Mandelbaum, 1997; Kolpin et al., 2000). The first organisms identified with the ability to degrade atrazine all via N-dealkylation were first reviewed by Kaufman and Kearney and include many fungi, including *Aspergillus* and *Fusarium* sp. and bacteria, *Arthrobacter* and *Bacillus* sp (Kaufman and Kearney, 1970). Cook and associates identified s-triazines, including cyanuric acid, a central intermediate in the degradation of all s-triazines, as sources of nitrogen for several species of *Pseudomonas* and *Klebsiella*, but none of their enrichments were able to identify isolates capable of degrading atrazine (Cook and Hutter, 1981).

Our understanding of microbial degradation pathways of atrazine was greatly advanced in 1993 when Mandelbaum and colleagues were able to isolate a bacterial consortium from a herbicide spill site with the ability mineralize atrazine. The initial degradatoion step was hydrolysis of atrazine to HA, and atrazine was the sole nitrogen

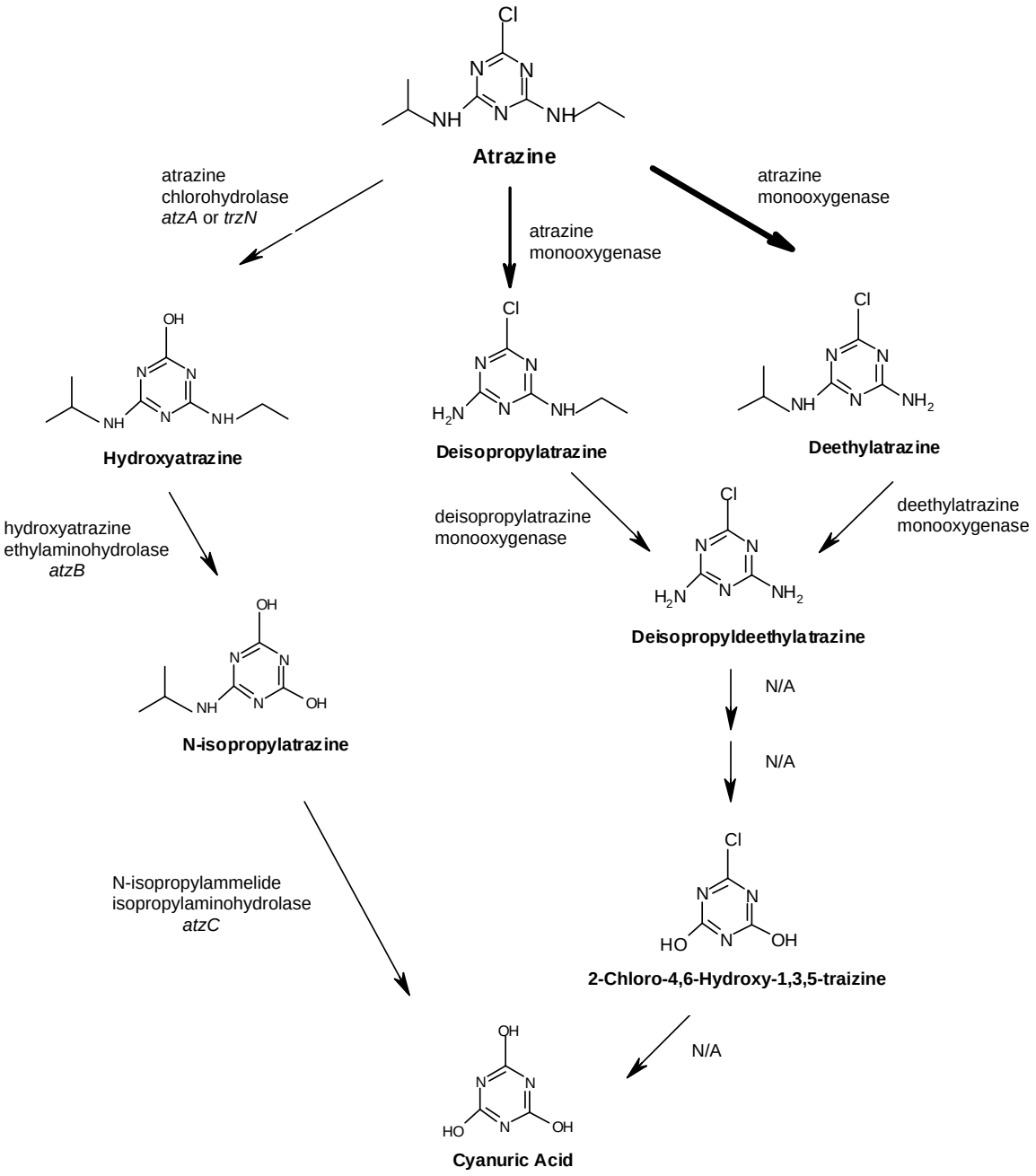

Figure 2: Common upper pathways of atrazine degradation.
Arrows in bold represent major pathways of metabolite formation in Soil. Enzymes associated with each step are listed on the arrow. N/A: Not available.

source (Mandelbaum et al., 1993a,b). The was a substantial finding in that the dogma at the time was substantial finding in that the dogma at the time was that HA was only formed abiotically in soils, and the process was greatly dependent on soil pH and organic matter content. The consortium obtained by Mandelbaum et al. however, was capable of atrazine hydrolysis at a neutral pH. Prior to this finding, Behki and Kahn isolated three species of *Pseudomonas* capable of utilizing atrazine as a sole carbon source that showed some biological accumulation of HA in addition to DEA and DIA (Behki and Khan, 1986). Multiple strains of *Rhodococcus* sp. were also isolated with the ability to dealkylate atrazine as a source of carbon, but were unable to utilize the amino nitrogens (Behki et al., 1993; Behki and Khan, 1994; Shao and Behki, 1995; BoundyMills et al., 1997). In contrast, the Mandelbaum consortium used atrazine as a sole nitrogen source (Mandelbaum et al., 1993a,b).

Biological production of HA is of great interest from a remediation standpoint. The dealkylated products, DEA and DIA, remain phytotoxic. DEA toxicity is very close to atrazine itself, and DIA is five times less toxic. But the dechlorinated degradation products, including HA, are nonphytotoxic and are considered an important step in the deactivation and remediation of atrazine (Kaufman and Kearney, 1970; Winkelmann and Klaine, 1991).

3. Bacterial Mineralization

The first report of a pure bacterial isolate capable of atrazine mineralization was published in 1994. First, Yanze-Kontchou and Gschwind working at Ciba-Geigy Corporation in Switzerland were able to enrich an atrazine consortium from soil collected near an atrazine production facility and subsequently isolated a *Pseudomonas* sp. strain

YAYA6 capable of metabolizing atrazine as a sole carbon and energy source. Aerobic degradation by this strain proceeded through two pathways, dechlorination to form HA and N-dealyklation to form DEA, and then into cyanuric acid and ultimately carbon dioxide and ammonium (Yanzekontchou and Gschwind, 1994). Mandelbaum et al. (1995) isolated a member of the important dechlorinating consortium mentioned earlier. *Pseudomonas* sp. strain ADP mineralized atrazine as a sole nitrogen source with citrate as a carbon source. Finally, Radosevich et al. (1995) enriched mixed bacterial communities from agricultural soils influenced by pesticide spills to isolate a *Ralstonia* sp. strain M91-3. This strain utilized atrazine under several conditions including (a) atrazine as the sole nitrogen source, (b) atrazine as the sole carbon and nitrogen source, and (c) atrazine as a secondary nitrogen source to nitrate with glucose as the carbon source under both aerobic and anaerobic conditions (Radosevich et al., 1995; Stamper et al., 2002). All three of these studies used selective enrichment for isolating bacteria and demonstrated atrazine mineralization via release of $^{14}CO_2$ from ^{14}C-ring-labeled atrazine and release of the chlorine ion and ammonium in the culture medium.

After isolation of the first three atrazine-degrading organisms, many others have been characterized surprisingly with similar degradation pathways, and many without the capability to mineralize atrazine. A list of these isolates, phylogenetic affiliations, degradation end products, and catabolic genes possessed is provided in Table 2. Genera capable of atrazine degradation include: *Rhizobium* (Bouquard et al., 1997), *Agrobacterium* (Struthers et al., 1998), *Nocardiodes* (Topp et al., 2000),

Table 2: Atrazine-Degrading Bacteria

Microorganism	Taxonomic Classification	End Products	Catabolic Genes	Reference
Rhodococcus corrallinus strain NRRL-15444R	Actinobacteria	DIA, DEA, and Hydroxyisopropylatrazine	TrzA (Cytochrome P-450)	Shao et al., 1995
Rhodococcus strain TE1	Actinobacteria	DEA, DIA	AtrA Cytochrome P-450	(Shao and Behki, 1995)
Streptomyces sp. strain PS1/5	Actinobacteria	DEA, DIA	Peroxidases	(Shelton et al., 1996)
Pseudomonas sp. Strain YAYA6	Gamma Proteobacteria	CO_2, NH_3, Cyanuric Acid	N/A	(Yanzekontchou and Gschwind, 1994)
Pseudomonas sp. strain ADP	Gamma Proteobacteria	CO_2, NH_3	*atzA,B,C,D,E,F*	(Martinez et al., 2001)
Ralstonia basilensis sp. M-91	Beta Proteobacteria	CO_2, NH_3	*atzA,B,C*	(deSouza et al., 1998a)
Rhizobium sp. strain PATR	Alpha Proteobacteria	HA	N/A	(Bouquard et al., 1997)
Alcaligenes strain SGI	Beta Proteobacteria	CO_2, NH_3	*atzA,B,C*	(deSouza et al., 1998a)
Clavibacter michiganese ATZ1	Actinobacteria	CO_2, NH_3	*atzA,B,C*	(deSouza et al., 1998a,b)
Agrobacterium radiobacter strain J14A	Alpha Proteobacteria	CO_2, NH_3	*atzA,B,C*	(deSouza et al., 1998a,b; Struthers et al., 1998)
Pseudaminobacter sp. Strain C147	Alpha Proteobacteria	CO_2, NH_3	*atzA,B,C*	(Topp et al., 2000b)
Nocardia sp. C190	Actinobacteria	N-ethylamide	*trzN*	(Topp et al., 2000a)
Chealobacter heintzii	Alpha Proteobacteria	CO_2, NH_3	*atzA,B,C* and *trzD*	(Rousseaux et al., 2001)
Arthrobacter crystallopoites	Acinobacteria	Cyanuric Acid	*atzB,C*	(Rousseaux et al., 2001)
Stenotrophomonas maltophilia	Gamma Proteobacteria	HA	*atzA*	(Rousseaux et al., 2001)
Arthrobacter aurescens strain TC1	Acinobacteria	Cyanuric Acid	*trzN, atzB,C*	(Sajjaphan et al., 2004)
Arthrobacter nictinovorans HIM	Acinobacteria	Cyanuric Acid	*atzA,B,C*	(Aislabie et al., 2005)

Note: N/A – Not available.

P*seudominobacter* (Topp et al., 2000a), *Chelatobacter*, *Stentrophomonas*, *Arthrobacter* (Rousseaux et al., 2001; Strong et al., 2002), and *Streptomyces* (Shelton et al.,1996).

4. Genes and Enzymes

As microbes capable of degrading atrazine were isolated and characterized, so too were the enzymes and genes that mediate pathway reactions. Enzymatic attack has been shown to be both specific and non-specific (Wackett, 2004a).

(a.) Cytochrome P-450

Enzymes and genes encoding the N-dealkyation and susbsequent dechlorination-hydroxylation, named *atrA* and *trzA*, were identified in *Rhodococcus* strains TE1 and NRRL-B1544R respectively. Recombinant strains containing the two genes were successfully developed for bioremediation purposes, but transformation was slow and did not fully mineralize atrazine. The TrzA enzyme was shown to have activity in dechlorinating DEA and DIA, but had no activity with atrazine (Mulbry, 1994; Shao and Behki, 1995; Shao, et al., 1995). It was later found that N-dealkylation of atrazine by *Rhodococcus* strain NI86/21, in addition to strains TE1 and B30, was catalyzed by a non-specific cytochrome P-450 monooxygenase enzyme. A suite of monooxygenase genes, *thcB,C,D*, together in tandem impart atrazine degradation (Behki et al., 1993; Nagy et al., 1995; Topp et al., 2004). The P-450 monooxygenase enzyme is non-specific and capable of degrading other herbicides besides atrazine inferring that it is not an enzyme evolved specifically to metabolize atrazine, but may be very intimately involved in the production of atrazine metabolites in soils (Seffernick and Wackett, 2001). An actinomycete, *Streptomyces* sp. strain PS1/5 and white rot fungus, *Phanerochaete*

chrisosporium strain ATCC24725, have been shown to dealkylate atrazine along with other herbicides via peroxidases (Shelton et al., 1996; Topp et al., 2004). It is important to note, organisms isolated and characterized with the ability to degrade atrazine via N-dealkylation do not have the ability to completely mineralize atrazine. These organisms only produce the metabolites DEA and DIA, which still retain environmental toxicities (Topp et al., 2004).

(b.) *Pseudomonas* sp. ADP -- *atzA,B,C,D,E,F*

Pseudomonas strain ADP (ADP) is the most widely known and comprehensively studied of the known atrazine-degrading bacteria. Its pathway of mineralization proceeds first via hydrolysis, then N-dealklation in the upper pathway, and finally ring cleavage in the lower pathway (Figure 3). The suite of genes involved in atrazine degradation in this organism, *atzA,B,C,D,E,F,* have been exhaustively characterized.

The first step in the biodegradation pathway of *Pseudomonas* sp. ADP is encoded by, *atzA*, and subsequent enzyme, AtzA, an atrazine chlorohydrolase that transforms atrazine to hydroxyatrazine. Gene identification was determined by constructing a 2000 member clone library from genomic DNA of ADP, which were screened for atrazine degradation. A clone containing the large plasmid pMD1, 21.5 kb fragment, degraded atrazine and was further subcloned to isolate a smaller 1.9 kb AvaI DNA fragment capable of dechlorinating atrazine. Tn5 mutagenesis studies confirmed that insertions and deletions to this 1.9 kb fragment eliminated atrazine dechlorinating capabilities (deSouza et al., 1995). The actual sequence of *atzA* was found to be 1419 nucleotides encoding a 473 amino-acid protein. To confirm that the chlorohydrolase was not an oxygenase like those previously characterized in N-dealkylation, enzyme assays were

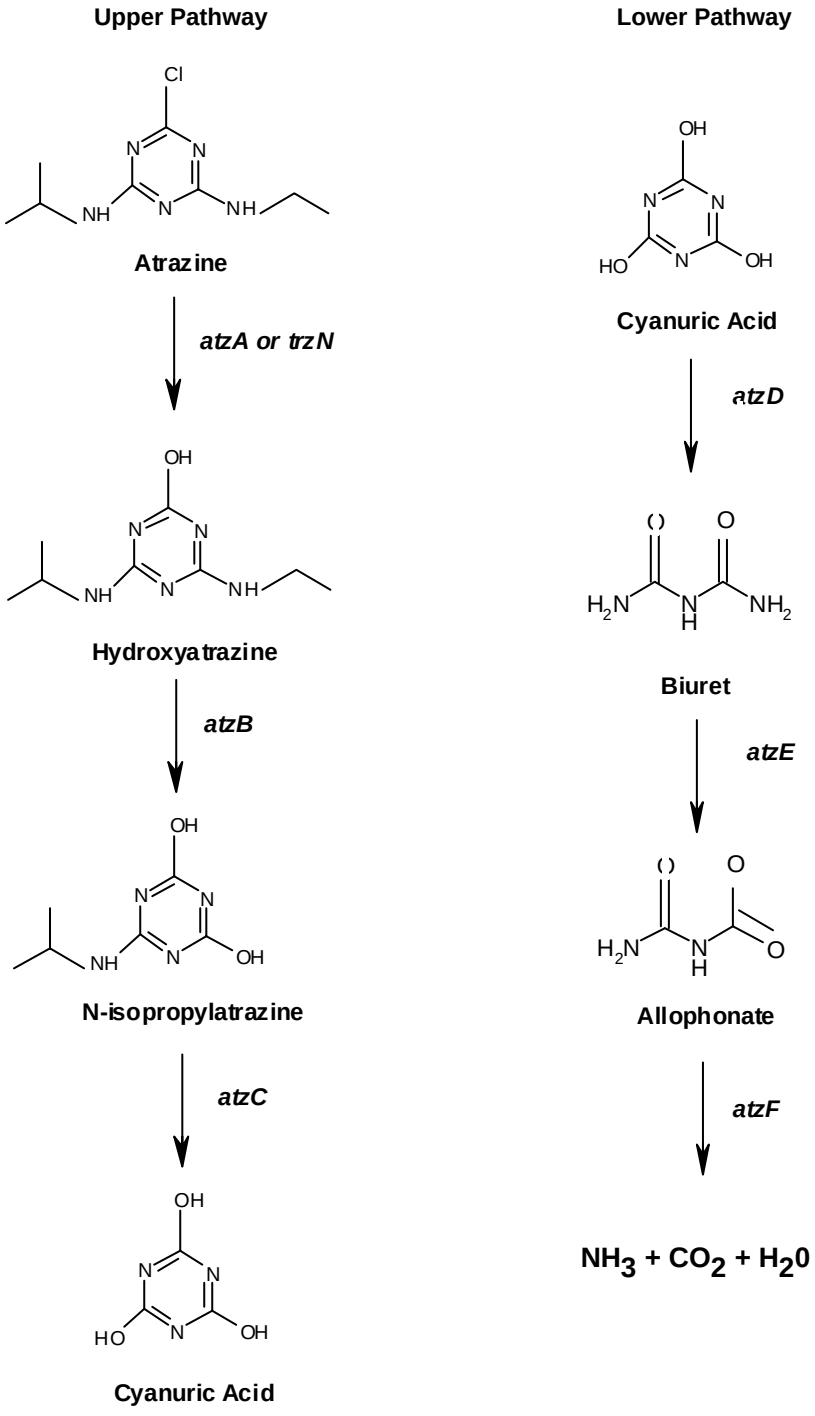

Figure 3: Upper and lower pathway of atrazine degradation characterized in *Pseudomonas* sp. ADP.

conducted in the presence of $H_2{}^{18}O$ and $H_2{}^{16}O$. [^{18}O] or [^{16}O]-hydroxyatrazine was produced in both aerobic and oxygen-limited environments, demonstrating a hydrolytic reaction (deSouza et al., 1996). The isolation and characterization of an enzyme capable of dechlorinating atrazine to hydroxyatrazine has led to important bioremediation schemes for atrazine in soils and water (Wackett et al., 2002).

The second step in *Pseudomonas* sp. ADP atrazine degradation pathway was characterized, *atzB* and the enzyme AtzB, named hydroxyatrazine ethylaminohydrolase, which transforms HA to N-isopropylamelide in an N-dealkylation reaction. Subcloning of the large plasmid, pMD1 revealed that it also contained the *atzB* gene, encoding a 481-amino-acid polypeptide, active in removing only the N-ethylamino side chain of hydroxyatrazine. The enzyme showed great specificity as it did not remove the N-isopropylamino side chain from hydroxyatrazine and had no activity with atrazine (Boundy-Mills et al., 1997).

The third step in *Pseudomonas* sp. ADP atrazine degradation was characterized as *atzC* and AtzC. The gene encodes the enzyme N-isopropylammelide isopropylaminohydrolase, capable of transforming N-isopropylammelide into cyanuric acid and isopropylamine. The N-isopropyl group of N-isopropylammelide is replaced by a hydroxyl yielding cyanuric acid. The *atzC* gene was cloned and sequenced and shown to consist of 1290 nucleotides with a predicted protein encoded by 403 amino acids.

The suite of enzymes, AtzA,B,C, due to sequence homology and similar function, were assigned to an amidohydrolase superfamily. These enzmes all catalyze hydrolytic reactions with nitrogen heterocyclic rings, contain metal centers essential for catalytic activity, and have great specificity (Sadowsky et al., 1998). Zinc was shown as the

coordinated metal center for the active site in AtzC (Shapir et al., 2002). Subsequent studies also identified AtzA as a metalloenzyme, which requires iron(II) to catalyze hydrolytic dechlorination of atrazine (Seffernick et al., 2002).

All three of the catabolic genes, *atzA,B,C* are located on a self-transmissible Plasmid, and it has been shown that these genes are horizontally transferred via conjugation and/or transposition (deSouza et al., 1998a). In order to fully understand its organization and function, the plasmid p-ADP1 was completely sequenced and shown to be similar to the broad host range plasmid IncPß plasmid pR751 with many of the same basic functions such as replication, transfer, and maintenance. The genes for the upper atrazine degradation pathway were not organized into an operon, but segregated on the plasmid indicating recent addition to the genome. In addition, the upper pathway genes are flanked by transposable elements that were identified with great homology to a very diverse group of other microorganisms. The upper pathway genes, *atzA* and *B* are only around 8 kilobases apart and share common G+C content indicative of a similar evolutionary history possibly from other *Pseudomonas* sp., but *atzC* is 34 kb away and shows a much lower G+C content, suggesting acquisition from a very different organism. It was also demonstrated that the *atzA,B,C* genes are expressed constitutively in the presence or absence of other nitrogen sources, and it was speculated the capability was recently acquired due mainly to its lack of organization and the presence of many transposable elements (Sadowsky et al., 1998; Martinez et al., 2001).

The lower pathway leading to complete mineralization of atrazine in ADP consists of three enzymatic reactions and was revealed after plasmid sequencing, earlier not thought to be included on the plasmid. But unlike the upper pathway, the lower

pathway (Figure 3), consisting of *atzD,E,F* was found to be organized into an operon and real-time PCR revealed these genes are cotranscribed on a single mRNA. The lower pathway begins with cyanuric acid, which has been shown to be a nitrogen source for many bacteria (Martinez et al., 2001). A ring-cleaving enzyme, cyanuric amidohydrolase, encoded by t*rzD*, from *Pseudomonas* sp. strain NRRLB-12227, capable of using cyanuric acid and other s-triazines, but not atrazine had previously been described. The gene had very little homology to any of the *atz* genes, did not require metal co-factors to catalyze the reaction, and was thought to have a totally different evolutionary history than the *atz* suite of genes (Karns, 1999). But PCR amplification using *trzD* specific primers was unsuccessful with *Pseudomonas* sp. ADP. The actual enzymes possessed by *Pseudomonas* sp. ADP were subsequently characterized as *atzD.* With 58% amino-acid level homology to TrzD, AtzD also is a cyanuric acid amidohydrolase, which transforms cyanuric acid to biuret (Martinez et al., 2001). A survey of bacteria known to degrade s-triazines showed bacteria have either one or the other but not both enzymes available for cyanuric acid degradation. The authors concluded cyanuric acid degradation via these two enzymes may be widespread in the environment (Fruchey et al., 2003).

The second two steps of the lower pathway of atrazine degradation in ADP proceed via two amidases, *atzE,F* which confer degradation of biuret to allophonate and finally to carbon dioxide and ammonia (Martinez et al., 2001). The last step in the pathway was considered to be novel, as the previous research indicated that cyanuric acid degradation to carbon dioxide and ammonia was through biuret and then urea, but studies

with *Pseudomonas* sp. ADP revealed allophonate was the intermediate, not urea (Cheng et al., 2005; Shapir et al., 2005).

(c.) *Nocardia* sp. C190 -- *trzN*

In addition to *atzA*, another chlorohydrolase gene has also been identified and characterized from *Nocardia* sp. strain C190. Unlike *Pseudomonas* sp. ADP, *Nocardia* sp. strain C190 degrades atrazine to HA and then to N-ethylammelide, not N-isopropylammelide. Even though the dehalogenation step is the same, the *atzA,B,C* genes from *Pseudomonas* sp. ADP do not catalyze degradation in *Nocardia* sp. strain C190, indicating heterogeneity in the genetic mechanisms of degradation. A novel hydrolase enzyme encoded by the gene, *trzN*, was identified to hydrolyse dechlorination of atrazine in *Nocarardia* sp. strain C190 and consists of 1356 nucleotides translated to 456 amino acids. *trzN* was shown to be flanked by transposable elements indicative of horizontal transfer from another organism. *Nocarardia* sp. strain C190 showed a wide range of s-triazine metabolism including methio-substituted s-triazines, due to the broad substrate specificity of *trzN* (Topp et al., 2000a; Mulbry et al., 2002).

5. Commonality and Difference in Degradation Pathways

Atrazine has been on the market since the 1950's and has proven to be one of the most useful herbicides to date with over a billion pounds of active ingredient applied worldwide. Other asymmetrical nitrogenous heterocyclic compounds are ubiquitous in nature and microbes have developed pathways to use them in energy yielding metabolism. Because it is a relatively new compound to the environment with tremendous application rates and no naturally occurring homolog, atrazine catabolism is an ideal model system for the evolutionary study of catabolic pathways (Wackett, 2004a).

Degradation pathways and mechanisms had been reported for the dealkylation of atrazine for many years, but not until the mid-1990's were organisms with the ability to completely mineralize the herbicide identified. Reasons for this lack of identification may very well be due to lack of cultivability, but more likely they are due to the actively evolving evolutionary path of atrazine degradation. There are both similarities and differences in the degradative schemes in the group of currently isolated atrazine-degrading organisms leading to the hypothesis of an evolving degradative mechanism. Several of the known atrazine degrading bacteria have been shown to have similar degradation pathways with great a high degree of conservation among the genes (Table 2), but others show variation in the gene sets utilized. A majority of the isolated organisms belong to the Alpha, Beta, and Gamma classes of the Proteobacteria phylum, which are Gram-negative and have been shown to dechlorinate atrazine via *atzA*. It is important to note again that most of these organisms were isolated under selective enrichment that poorly mimics the environmental conditions of the soil habitat. Thus, this collection of bacteria is likely biased towards those bacteria that grow fastest under enrichment conditions. On the other hand, Gram-positive bacteria, namely Actinobacteria have also been isolated and generally degrade atrazine via *trzN*, but in general have been isolated using direct plating procedures.

Combinations of these gene sets also exist. For example, *Arthrobacter aurescencens* TC1 and a strain of *Arthrobacter crystallopoites* have been shown to degrade atrazine via *trzN*, *atzB*, and *atzC* (Rousseaux et al., 2002; Sajjaphan et al., 2004). Even though *Pseudomonas* sp. ADP and some other degraders seem to have the same pathway, the arrangement, organization, and size of their genes on different plasmids is

often times very different suggesting the evolutionary track of atrazine degradation is still in progress. Topp et al. (2000b) showed several strains of *Pseudaminobacter* with varying sized plasmids and gene sets. The differences in plasmid size were attributed to a transposable element IS1071, which is linked with *atzB* and *atzC*. Great instability was seen in their culture collection as subculturing efforts often resulted in strains that lost one or more of the intermediate genes, thus losing the ability to mineralize atrazine. The authors speculated that with such great instability and variation in the size of the plasmids carrying atrazine catabolic genes, the organisms studied most likely assembled their pathways from plasmid transfer independently and randomly instead of acquiring them at one time in a stable operon containing the entire pathway (Topp et al., 2000b). Rouseaux et al. (2002) characterized the plasmid size and location of different atrazine-degrading bacteria including many strains of Gram-negative *Chelatobacter heintzii* and Gram-positive strains of *Arthrobacter crystallopoites* and also found considerable variation in the number and size of the plasmids involved. The *Chelatobacter* strains containing *atzABC* and *trzD* contained anywhere from two to six plasmids each with varying sizes. In keeping with Topp's results, the *Arthrobacter* strains also possess the *atzB* and *atzC* genes on a single plasmid in association with the IS1071 insertion elements. Again, the authors speculated the importance of plasmid transfer in the dissemination of atrazine catabolic genes (Rousseaux et al., 2002). Researchers involved in the identification of the *Pseudomonas* sp. ADP catabolic pathway argue for a recent evolutionary development and worldwide distribution of these catabolic genes, based on the fact that many atrazine-degrading bacteria have very high sequence identity to the genes seen in *Pseudomonas* sp. ADP and have been isolated from geographically diverse locations

(deSouza et al., 1998a). But the evidence presented on the variability of plasmid size, organization, and diversity of genes leads others to believe the distribution and occurrence of the catabolic genes is much more complicated and in great flux.

F. Attenuation

Atrazine attenuation in soils is greatly dependent on microbial degradation, and thus the half-life of atrazine in soil is in turn very dependent on environmental factors that influence microbial activity and the availability of atrazine. Variability in these factors makes predicting the persistence of atrazine in the environment very difficult. Much research has been undertaken to assess degradation potential in soils, but the current body of knowledge has not been very successful in adequately predicting atrazine fate and degradation potential in soils.

1. Nutrient Availability and Degradation

The organisms currently characterized as atrazine degraders have been discussed, and some of the environmental conditions critical for the most efficient conditions for the microbial community to adapt and mineralize atrazine have been studied. Even though atrazine is a poor carbon source, it contains nitrogen in both the substituted side chains and the triazine ring; regardless, organisms capable of using atrazine as both a carbon and nitrogen source have been isolated and characterized (Table 2). It could then be postulated that in environmental conditions with high carbon to nitrogen ratios, nitrogen-limiting conditions, may lead to selective enrichment for a community of microbes that can utilize atrazine. Multiple studies have shown that introduction of inorganic or mineral nitrogen can inhibit the degradation of atrazine because it provides a more

metabolically efficient source of nitrogen in the soil system (Alvey and Crowley, 1995; Abdelhafid et al., 2000a,b; Rhine et al., 2003).

In a study to examine the acclimation effects of atrazine exposure with varying nutrient availabilities, Rhine et al. (2003) showed no enhanced mineralization in soils with a history of atrazine exposure in nitrogen-limiting conditions with the addition of carbon in the form of pectin or acetate. But in non-history soils, with no previous exposure to atrazine, these carbon additions greatly enhanced mineralization. After atrazine acclimation, there was no significant impact of organic amendments in either the history or non-history acclimated soil. The authors stated that once the soils were adapted to atrazine, mineralization capacity was limited by carbon to nitrogen ratios as the soils were most likely not carbon limited (Rhine et al., 2003).

Soils with high organic matter or addition of organic amendments in soil often result in atrazine becoming chemically bound in the organic residue. When atrazine becomes bound in these organic residues, it is essentially unavailable for microbial degradation resulting in a decrease in atrazine mineralization (Assaf and Turco, 1994; Barriuso and Houot, 1996; Radosevich et al., 1997; Abdelhafid et al., 2000a; Houot et al., 2000). In general, organic and carbon amendments increased the overall abundance of the microbial community in soils with no history of atrazine exposure, and thus in time led to increased mineralization through the adaptation of the community to degrade atrazine. However, in soils a history of atrazine application presumably with an established community of degraders, no major effects were seen from organic or carbon amendments. It seems the most important factor in nutrient availability and the degradation of atrazine is the presence of more readily available sources of nitrogen,

which generally decrease mineralization rates (Alvey and Crowley, 1995; Pussemier et al., 1997; Rhine et al., 2003).

Nitrogen effects on pure cultures of atrazine-degraders have also been characterized for some of the known atrazine degrading bacteria in an attempt to identify atrazine regulation at the cellular level. Bichat et al. (1999) used ^{15}N-labeled atrazine to support earlier reports that *Pseudomonas* sp. ADP and *Agrobacterium radiobacter* J14A constitutively express the atrazine degradation pathway. Exogenous nitrogen had no effect on the uptake and incorporation of atrazine by these bacteria. Atrazine mineralization by *Ralstonia balensis* M91-3 on the other hand was suppressed by amendment with ammonium, and found to be inducible by atrazine (Bichat et al., 1999; Gebendinger and Radosevich, 1999; Rhine et al., 2003).

2. pH

Several studies have shown a positive correlation between alkaline soil pH values and increased atrazine mineralization. For example, Pussemier et al. (1997) showed increased atrazine mineralization with soil pH values above 7. Until this report, pH influence was only addressed when discussing increased abiotic hydrolysis of atrazine, but this study indicated an adapted microbial community for atrazine degradation at alkaline pHs (Pussemier et al., 1997). In a separate study, less than 25% of ^{14}C-atrazine was mineralized in soils with a pH less than 6.5, but up to 80% could be mineralized with pH values between 7.5 and 8.0 (Houot et al., 2000).

3. The Atrazine History Effect

Like other herbicides with microbially mediated degradation, atrazine degradation has been positively and definitively linked to a history effect (Rhine et al., 2003). The

history effect in essence involves an acclimation process of the microbial community in soils. Thus, through repeated exposure, the community acquires the capability to degrade atrazine and use it as a growth substrate, either as a nitrogen source or both a nitrogen and carbon source. Upon repeated exposure to atrazine, the half-life of atrazine typically decreases in soils and subsoils and mineralization rates are generally very high (Aislabie et al., 2004). In many cases only one or two applications can lead to increases in mineralization rates (Pussemier et al., 1997). Even in cases where application on corn is in a crop rotation, the ability of a soil to degrade atrazine was directly linked to its history of exposure, as enhanced mineralization persists even through crop rotations. Although other chemical and physical soil characteristics can influence atrazine degradation, it is the history of prior exposure that appears to be most important in determining atrazine degradation capacity. Thus, understanding the mechanisms leading to the establishment of an acclimated soil community with the ability to degrade atrazine is essential in predicting the environmental fate of this important herbicide (Barriuso and Houot, 1996; Pussemier et al., 1997; Shapir and Mandelbaum, 1997; Vanderheyden et al., 1997; Yassir et al., 1999; Rhine et al., 2003; Aislabie et al., 2004; Zablotowicz et al., 2006).

4. Linking Degradation to Cultivability and Characterized Pathway Genes

The catabolic pathways of a growing number of atrazine degrading organisms have been elucidated and the genes and enzymes involved have been characterized. Since these biomarkers are the only ones available, the use of these genes and organisms as a means of predicting degradation rates have been explored. A most probable number (MPN) assessment has been used to quantify in a culture-dependent manner the microbial population size capable of metabolizing ^{14}C-atrazine (Jayachandran et al., 1998). In

history and non-history soils, total atrazine mineralization rates could not be correlated with an MPN assessment of atrazine-degrading organisms (Yassir et al., 1999). Ostrofsky et al. (2001) attempted to correlate atrazine degradation kinetics with the number of cultivable atrazine degrading bacteria and the presence of known atrazine degration genes using dot-blot hybridization. Characterized genes of interest at the time of publication were *atzA*, atrazine chlorohydrolase from *Pseudomonas* sp. ADP, *atrA*, cytochrome P-450 from *Rhodococcus* TE1, and *trzD*, cyanuric acid amidohydrolase from *Pseudomonas* NRRLB-12228. Results from this study also revealed that cultivable atrazine degraders did not correlate to enhanced mineralization. None of the three gene probes, especially *trzD*, showed consistent positive signals even in the history soils suggesting the current collection of bacteria and the genes and enzymes used to assess atrazine degradation were not active in the soils studied and may not be the most environmentally significant (Ostrofsky et al., 2001, 2002).

Culture independent community analyses using denaturing gradient gel electrophoresis (DGGE) and fatty acid methyl ester analysis (FAME) demonstrated definite shifts in the bacterial community showing an adaptive response to atrazine exposure (Rhine et al., 2003). But distinctly different bacterial populations were seen with varying carbon to nitrogen ratio additions and varying atrazine exposure rates. Although MPN values for the number of atrazine-degrading organisms in several history soil samples were high in the study, a correlation could not be made for increased MPN with enhanced mineralization rates, as some soils with high mineralization rates post acclimation had negligible MPN values. Since very different communities were established, it was suggested that very diverse groups of bacteria capable of atrazine

degradation could be developed under different circumstances (Radosevich and Tuovinen, 2002; Rhine et al., 2003). Aislabie et al. (2004) showed similar results while also examining length of time and application rate until an adaptive response was seen. Results of this study showed that a single application of atrazine could enhance the degradation rate of atrazine and no increase in MPN values were observed in soils exhibiting enhanced mineralization (Aislabie et al., 2004). In another study, Mississippi soils with very short history of exposure to atrazine showed accelerated degradation leading to a concern regarding a loss of efficacy of atrazine due to rapid mineralization. Neither specific PCR amplification nor nested PCR could amplify *atzA* from DNA extracted from soils with rapid mineralization, suggesting diversity in the degradative mechanisms of atrazine degradation in soils (Zablotowicz et al., 2006).

Devers et al. (2004) were interested in the long-term effects of herbicides on the microbial community and sought to examine the pathway regulation of two different atrazine degrading bacteria. Through a real-time reverse transcriptase PCR method, the expression rates *atzA,B,C,D* of *Pseudomonas* sp. ADP and *atzA,B,C* and *trzD* of *C. hentzii* were monitored. It was shown that *Pseudomonas* sp. ADP basally expresses the entire suite of genes, but that the maximum expression was seen only when minimal amounts of atrazine (5 ppm) were present in the medium. The authors postulated that the basal expression of these genes was not necessarily due to atrazine concentration but maybe intracellular atrazine concentration, the presence of a metabolite, or even some method of cell-to-cell signaling. *C. hentzii*, on the other hand, having the same upper pathway displayed basal expression of only the *atzA* gene. The *atzB* gene was

up-regulated in response to atrazine concentration, and *atzC* was not expressed at all. Results suggest that although the known atrazine degradation genes may be conserved in some microbial species, their regulation patterns may be quite different (Devers et al., 2004).

5. Existence of Greater Diversity in Atrazine-Degrading Bacteria and Catabolic Genes

All of these studies collectively demonstrate (i) lack of correlation between MPN and atrazine degradation, (ii) lack of consistent detection of known biomarkers (genes) in high mineralizing soils, and (iii) most highly studied and accepted pathways of degradation do not account for the most commonly seen metabolites in soils and water. The totality of these findings suggests the existence of much greater diversity in the biologically mediated scope of atrazine degradation in situ. The majority of the known atrazine-degrading bacteria were isolated using very classical techniques, and attempts to identify their importance through the amplification of their degradation genes in soils has not been very informative. The current knowledge base of atrazine degradation revolves around one species, *Pseudomonas* sp. ADP, and its degradative pathway, genes, and enzymes mainly because of its exhaustive characterization. And even though the current collection of atrazine-degrading bacteria has grown substantially in the last decade, it appears the current collection of bacteria and their genes cannot adequately predict atrazine degradation in situ and have not been effectively probed in the environment.

G. Culture-Dependent and Independent Means of Assessing Diversity

Culture-independent identification has allowed scientists to identify a lack of cultivability using traditional means and quantify the immense genetic diversity present in natural environments. These findings have stimulated the development of more inventive techniques to cultivate more ecologically important organisms identified by cultivation-independent means.

1. Traditional Methods

Traditional microbiology identified and classified organisms based on their physical and metabolic characteristics, and thus were totally dependent on the capability to cultivate the organisms in pure culture. Hence, much of the historical perspective of microbial diversity was based solely on these cultivated organisms with no real understanding of their evolutionary history (Hugenholtz, 2002). Brock established the existence of culture biases and began the argument for in situ studies in the late 1980's (Brock, 1987). The 'great plate-count anomaly' was coined by Staley and Konopka to describe the discrepancy between direct microscopic counts and the formation of visible colonies on agar plates (Staley and Konopka, 1985).

2. 16S rRNA

Pioneering work by Carl Woese and colleagues for the first time allowed for a conceptual framework for determining evolutionary relationships between organisms based on the molecular sequences of the small subunit of the ribsosomal RNA (rRNA). Woese used these relationships to establish a phylogenetic tree that could be used to relate all organisms and found three basic lines of evolutionary descent for all cellular life: *Eucarya*, *Bacteria*, and *Archea* (Reviewed by Pace, 1997). The rRNA molecules in

the ribosome are excellent biomarkers for inferring evolutionary relationships because (i) they are ancient and universally distributed among all organisms, (ii) rRNAs have regions of high structural and functional conservancy but also contain regions of considerable sequence variation even within very closely related taxa, (iii) the molecules are large enough size to give a strong phylogenetic signal, and (iv) the sequence changes or mutations in these molecules are random and can be identified (Woese, 1987). Pairs of rRNA sequences of organisms were aligned and their differences were considered 'evolutionary distances' between the organisms with no consideration of time, just differences in nucleotide sequences. Differences were then used to infer phylogeny, which provide roadmaps representing the evolutionary pathway to modern-day sequences (Pace, 1997). Taxonomy would forever be changed from physical identification and classification to a molecular-evolutionary based platform (Liesack et al., 1997). The original characterization of the rRNA molecules involved dideoxy sequencing using reverse transcriptase and was very cumbersome, but became considerably easier with the revolutionary discovery of the polymerase chain reaction (PCR). Universal oligonucleotide primers targeting the most highly conserved regions of the rRNA genes were designed to PCR-amplify rRNA from a template genomic DNA (Liesack et al., 1997). Since its conception, well-established caveats of PCR amplification have been identified including differential amplification of specific templates, sensitivity to rRNA copy number and rRNA template concentration, PCR primer specificity, and formation of chimeric sequences. Even with some shortcomings, PCR amplification has become the scientific standard to amplify a specific designed probe or biomarker from both pure cultures and mixed community DNA, and is thought to amplify the most abundant,

metabolically active bacterial sequences in an easy, fast, and cost efficient manner (Hugenholtz et al., 1998). PCR-amplified products are then available for cloning into recombinant *E.coli* and then sequencing to determine the exact nucleotide makeup of the amplified region. Today, massive databases and computer programs have been developed in order to facilitate the alignment of sequence data, identification of the phylogenetic affiliation through comparison to other sequences in the database, and subsequent formation of phylogenetic trees to view the relatedness and evolutionary descent of organisms of interest. Major databases for these comparisions include the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/) and the Ribosomal Database Project (RDP) (http://rdp.cme.msu.edu/).

The use of 16S rRNA technologies has greatly improved our knowledge of diversity of bacteria in many environments, especially in soils, and has uncovered the staggering fact that over 99% of this diversity cannot or has previously resisted cultivation by traditional means. Often times the most easily cultivable organisms in a lab setting are not the most numerically dominant in the environments in which they were obtained (Hugenholtz et al., 1998).

3. Cultivation Strategies

Different cultivation strategies, from very simple to very complicated, have been employed to isolate organisms. At the time of its conception in the early 1900's, the enrichment culture technique allowed, for the first time, the selection of a particular subset of organisms capable of utilizing a specific substrate. Through sequential enrichments, populations of selected organisms increase in biomass to facilitate isolation in pure culture. Enrichment cultures leading to pure isolates have been instrumental in

identifying many enzymes and genes essential in important microbial driven cycles (Wackett, 2001). However, traditional culturing techniques have proven inadequate at mimicking the conditions in situ where organisms naturally reside. Cultivation media often times provides an endless amount of the macronutrients like carbon and nitrogen, and is usually maintained in an either fully aerobic or anaerobic aeration regime, but in soils, those extremes are rarely present. So instead of enriching for bacteria that are most abundant and efficient in soils, selections often yield the organisms that grow fast and are specialized to respond quickly to high concentrations of nutrients (Dunbar et al., 1997). Hugenholtz coined these easily isolated organisms 'weeds' of the microbial world which are estimated to constitute less than 1% of all microbial species (Hugenholtz, 2002).

16S rRNA technologies have allowed scientists to make great strides in identifying organisms and their diversity across many environments without the traditional means of cultivation, but fail at divulging their role in the community. As more and more 16S rRNA data becomes available, the actual community makeup of many different environments and situations are being elucidated. In order to gain a holistic view of the mechanisms and organisms involved, cultivation, again, must come back into the spotlight to identify the roles these organisms play in situ (Liesack et al., 1997). Thus, current efforts in environmental microbiology will focus on linking phylogeny to function. A scientific community-wide acknowledgement of a lack of cultivability has taken place over the last decade, and thus, methods to improve overall cultivability have driven research at very large scales. Some innovations for improving cultivability include use of microchambers (Kaeberlein et al., 2002), single- cell gel-encapsulation (Zengler et al., 2002), and other high throughput technologies to screen a

wide variety of samples and media formulations. In addition, other researchers have identified novel taxonomic groups through 16S rRNA and directed their isolation conditions towards these groups through use of very low nutrient media regimes, longer incubation times, and various aeration regimes (Joseph et al., 2003; Davis et al., 2005).

H. Objective

Cultivation studies directed at isolating atrazine-degrading bacteria have succumbed to the same pitfalls as traditional microbiology. Most of the reported atrazine-degrading bacteria were isolated using classical cultivation techniques and were characterized because of their isolation in pure culture. One might assume that using similar techniques and culturing medias would garner similar organisms and pathways involved in atrazine degradation owing to the same culturing biases as noted earlier (Table 2). And again, like in other historical instances, groundwork has been laid for the occurrence of a more broad community involved in the in situ degradation of atrazine in soils. A complete understanding of its degradation in situ is of utmost importance in not only remediating atrazine contamination, but also in being able to predict its fate. Traditional techniques have resulted in the isolation or organisms that have very little relevance in the overall fate of atrazine in soils. But new technologies and approaches may offer a means of isolating the most pertinent organisms involved in the process. **The objective of this research was to utilize a novel enrichment technique utilizing Bio-Sep® beads to recruit, and then cultivate a diverse group of organisms capable of degrading atrazine in soil.**

I. 'Magic Beads'

Bio-Sep® beads were developed by the Dupont Company and subsequently transferred to Dr. K.L. Sublette at the University of Tulsa and have been utilized in bioremediation efforts as an powder activated carbon immobilizing agent for microbial communities. Most notably, the beads have been used as a matrix to deliver biological species active in the remediation of sulfide-rich wastewaters, especially in association with petroleum refinery waste systems (Sublette et al., 1998; Conner et al., 2000). Bio-Sep® beads have proven successful in other studies as convenient 'bug-traps' to recruit active biofilms (Peacock et al., 2004). Other efforts utilizing Bio-Sep® beads thus far have involved monitoring changes in microbial communities in groundwater, sediments, and another site undergoing hydrocarbon bioremediation (Geyer et al., 2005). Active communities of bacteria form biofilms in the beads and serve as a snapshot of the microbial community in the system (White et al., 2003) (Figure 4). Changes in viable biomass, community composition, and lipid profiles are easily characterized from the bead communities without the typical inhibitory PCR problems associated with organic matter and clays from whole sediment or soil samples. Bio-Sep® beads were proven to allow for greater colonization than glass wool samplers (Peacock et al., 2004). We hypothesized the beads would be useful to sample soil microbial communities as they mimic the soil environment with an array of pore sizes and microhabitats. Our general approach was to impregnate or 'bait' the beads with atrazine to 'enrich' or stimulate atrazine-degrading bacteria in situ in an effort to avoid previously documented enrichment bias associated with classical techniques. The results presented in this thesis suggest that the Bio-Sep® beads offer a very promising method to sample the most active

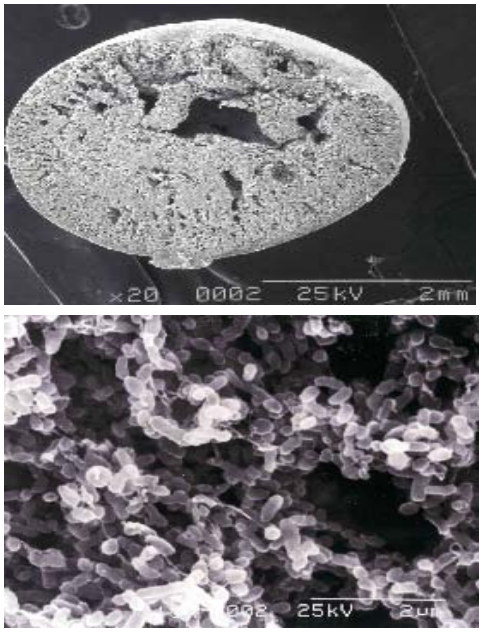

Figure 4: Scanning electron micrograph of a dissected Bio-Sep® bead before deployment in the environment showing the porous network and large surface area for microbial colonization (upper panel). A bead colonized by active indigenous microbial biofilms (lower panel).
Image courtesy of Dr. K.L. Sublette, University of Tulsa.

microbial community with a specific metabolic function while avoiding traditional enrichment culturing biases.

II. Materials and Methods

A. Sample Locations

Soils from sites in Tennessee, Ohio, and Delaware were selected for deployment of Bio-Sep® beads due to having a previous history of atrazine exposure and thus, containing acclimated bacterial communities. Locations outside of Tennessee were overseen by collaborators and shipped to the Radosevich lab for cultivation studies.

Tennessee soils sampled were located at The University of Tennessee Agricultural Research and Education Center located in Milan, Tennessee. The station site was chosen due to its close proximity to a large area of atrazine use in the agriculturally dominant loess bluffs of West Tennessee, where corn acreage and thus atrazine use is substantial. The plot used at this site was classified as a Typic Hapludalf and has been in long-term no-till for over thirty years in a wheat, soybean, corn, crop rotation with corn back-to-back years in 2004 and 2005 during time of bead deployment. Atrazine was applied during all corn rotations in the form of Bicep II® at the labeled rate.

Sites in Ohio included agricultural soils at the Molly Caren Agricultural Center, London, Ohio. Four sites were selected and include fields also in crop rotations of wheat, soybeans, and corn in addition to a site with no atrazine history within the last three years. In addition, a pesticide mix/load site at the South Charleston Extension Farm known to be an area of atrazine accumulation was also chosen for cultivation studies. Two sites were also selected at the Olentangy River Wetland Research Park (ORWRP), Columbus, Ohio. The wetland ecological research site is fed by water from the Olentangy River, which receives agricultural runoff upstream from the site. Both sediment and water column were sampled. Ohio site selection and actual bead deployment was

graciously supervised by collaborator Dr. Olli Tuovinen, Professor of Microbiology, The Ohio State University, Columbus, Ohio.

One additional site at the University of Delaware Agricultural Research Farm, in a long-term corn research plot, was also selected. Table 3 outlines the deployment sites, abbreviations used for isolate identification, and comments pertaining to plot history.

B. Bio-Sep® Bead Soil Samplers

1. Bio-Sep® Description

Bio-Sep® beads were used as a solid phase matrix to recruit active biofilms from soil known to have a history of atrazine exposure. The spherical beads are 2-3 mm in diameter and formed from a composite of 25% aramid polymer (Nomex) and 75% powdered activated carbon (PAC). The porosity is 74% with a median pore diameter of 1.9 microns; however, large macropores of greater than 20 microns are also present. Bulk density of the beads is approximately 0.16 g cm^{-3}. The beads have a substantial internal adsorptive capacity and a specific surface area of > 600 m^2 g^{-1}. Bio-Sep® beads are surrounded by an ultrafiltration-like membrane with pores of 1-10 microns. The beads are purged of fossil organic residues by incineration at 300^{o}C for 4 hrs (Peacock et al., 2004). Sterile beads were provided by Dr. K.L. Sublette, University of Tulsa.

In order to achieve an in situ enrichment for the bacterial community with the ability to degrade atrazine, the Bio-Sep® beads were baited or impregnated with atrazine before deployment. To avoid cultivation biases of classical techniques, enrichment for the most active soil microbial community took place in situ as the beads served as a source of atrazine and a habitat for the colonization by the indigenous microbial

Table 3: Deployment Sites, Abbreviations, and Deployment

	Site Abbreviations	Deployment	Plot History	Comments
West Tennessee Research and Education Center, University of Tennessee, Milan Tennessee				
West TN Enriched Beads	WTEC	Winter 2004	Corn 2004, 2005 Bicep II®	West Tennessee Enrichment Cultures (WTEC) Atrazine- Equilibrated Enrichment Culture Beads
West TN Enrichment Culture	SubA-D	Winter 2004	Corn 2004, 2005 Bicep II®	Subcultures of the WTEC beads inoculated from spent culture media (SubA-D)
West TN Field Incubated Beads	WTFIB-Atz WTFIB-H20	Summer 2005	Corn, 2004, 2005 Bicep II®	Atrazine and Water Equilibrated Beads
Molly Caren Agricultural Center, Ohio State University, London, Ohio				
1B	1B-Atz 1B-H20	Spring 2005	Corn, 2005 1.83#/acre atrazine	Atrazine and Water Equilibrated Beads
2A	2A-Atz	Spring 2005	Corn 2004, 2005 1.83#/acre atrazine	Atrazine Equilibrated Beads
3A	3A-Atz	Spring 2005 Summer 2005	Wheat/Soybean Rotation: 2 years without atrazine	Atrazine (Atz) Equilibrated Beads
Penn	Penn-Atz Penn-H20	Spring 2005	Corn, 2005 1.83#/acre atrazine	Atrazine (Sp) and Water Equilibrated Beads (Sp & S)
Cl	Cl-Atz	Spring 2005	Corn, 2000-2005 0.75#/acre atrazine	Atrazine Equilibrated Beads
Olentangy River Wetland Research Park, Ohio State University, Columbus, Ohio				
ORWRP-S	S-Atz	Spring 2005	Wetland Sediment atrazine runoff	Atrazine Baited Beads deployed in wetland sediment known to receive atrazine runoff
ORWRP-W	W-Atz	Spring 2005	Water Column atrazine runoff	Atrazine-Baited Beads deployed in wetland water column known to receive atrazine runoff
Other Sites				
Pesticide Spill (Waste) Site	PWS-Atz PWS-H20	Summer, 2005	Chemical mix/load site	Water and Atrazine Baited Beads Pesticide Mix and Distribution Site, Ohio
University of Delaware Agricultural Research Station	UDEL-Atz	Spring 2005	Long-term corn plot atrazine at labeled rate	Atrazine-Baited Beads Bead bag destroyed during planting, salvaged some beads as a test

Note: Common nomenclature for all site abbreviations: Site - Bead Equilibration Type.
Example, WTFIB-Atz stands for West Tennessee Field Incubated Beads equilibrated with atrazine. All beads unless specified in the comments were field incubated only with no further enrichment.

population capable of atrazine degradation. For each sampling location, 5 grams of beads were equilibrated or impregnated with atrazine in 60 ml of a 2 ppm solution of atrazine (98% purity, ChemService, West Chester, PA) for 48 hours, until the beads had adsorbed all of the atrazine resulting in a final concentration of 20 mg of atrazine per gram of beads. In addition, during some deployments, water equilibrated beads were also buried to determine if the baited beads actually selected for a different microbial community than the beads containing atrazine.

2. Deployment

Beads were deployed in spring and summer of 2005 to coincide with atrazine application. A preliminary deployment was also carried out as a trial test for the bead deployment in soil in the winter of 2004. In previous Bio-Sep® studies, when assessing groundwater communities, beads were packed into a tube with holes for water movement across the beads and suspended down well below the water table (Peacock et al., 2004). For soils, another simple approach was used. Upon arrival at each location, baited and unbaited beads were secured in nylon mesh bags. The mesh bag, like the bead tube, provides for ample aeration and water movement around the beads in the soil profile to promote bacterial colonization, as well as means for easy recovery post deployment. The bead bags were buried 4-6 inches below the soil surface. Temporal changes in atrazine degraders have been examined, and the most active communities have been identified in the closest to the surface, thus bead deployment was in the top few inches of soil (Radosevich et al., 1996). ORWRP samples were placed at the water/sediment interface at the sediment site and another sample was suspended in the water column. Beads were left to equilibrate for approximately one month. When

utilized in water situations, the beads only take a few days to form biofilms, but as the task was to enrich for a specific bacterial community, ample time was given for generous colonization of the beads (Peacock et al., 2004).

C. Isolation and Cultivation

1. Cultivation Conditions

Once recovered, beads were immediately processed for cultivation studies. Beads were rinsed with sterile water to remove any excess soil and debris, and then diced using a sterile scalpel and extracted in 5 mM phosphate buffer to facilitate recovery of bacteria. Extract from approximately one gram of beads was serially diluted in 5 mM phosphate buffer. The dilution series was plated onto two atrazine minimal salts medias, one with atrazine as the sole nitrogen source, AMS with glucose, and one with atrazine as the sole nitrogen and carbon source, AMS without glucose. The minimal salts media was adapted from media used to isolate *Ralstonia balensis* M91-3 (Radosevich et al., 1995). AMS was composed of the following per liter: 486.5 mg of KH_2PO_4, 248.2 mg of K_2HPO_4, 2 g glucose (only for AMS with glucose), 0.5 g $MgSO_4*7H_20$, 25 mg atrazine (98% purity, Chem-Service, West Chester, PA), and 10 ml of a 100X trace element solution that consisted of: 10 mg $FeCl_3* H_20$, 100 mg $CaCl_2*H_2O$, 1 mg $MnCl_2$, and 0.1 mg $ZnCl_2$. Media pH was adjusted to 7 for all experiments.

Two aeration regimes were utilized in the cultivation studies, room temperature and micro-aerophillic. Soils often exhibit increased levels of carbon dioxide resulting from microbial respiration, but this fact is often neglected in cultivation studies. So, in addition to room temperature plates, a dilution series in both AMS with and without

glucose was also incubated in a chamber containing 2.5% oxygen, 5% carbon dioxide and balance nitrogen.

Other researchers have used a plate-clearing assay to initially assess atrazine degradation by occluding the plate with a very high concentration of atrazine and collecting isolates with zones of clearing (Mandelbaum et al., 1995), but that approach was not used in this study because (1) high concentrations of atrazine are not representative of field conditions and (2) many of the colonies appearing on the plates were very small and unable to produce visible clearing zones. Therefore, 25 mg/L of atrazine was used in the media as it is close to atrazine quantities seen in soils at the labeled rate of atrazine application. Consequently, each isolate collected was screened separately for atrazine degradation by high performance liquid chromatography (HPLC) (Radosevich et al., 1995). Additional amendments to the traditional isolation media included the use of Noble agar as the gelling agent for solid media preparation to minimize growth of non-atrazine degrading bacteria on organic impurities in the agar.

2. Bacterial Isolation and Screening for Atrazine Degradation

Plates were incubated at both room temperature and in the microaerophillic chamber. Isolations were initiated at seven days of incubation and continued as long as new colonies were formed up to seventy days after dilution plating. Random colonies appearing on the dilution plates were isolated and purified on the same media in which they were originally plated. The entire culture collection was then screened for atrazine-degradation via HPLC. The bacteria were initially isolated and purified on agar plates, but in order to evaluate atrazine degradation had to be grown in liquid culture in analyze for loss of atrazine in the culture medium. Due to a large number of isolates, 96-well-

plates were used in cultivating the microbes in liquid culture for degradation-screening to facilitate a more high-throughput method. These plates became especially useful in growing the isolates in liquid culture as many of the collection did not grow well in a fully aerated, large culture flask, but grew well in the small volume, high surface area 96-well-plate. Each isolate was screened in both AMS with glucose media and AMS without glucose media regardless of their original isolation media to evaluate atrazine use as both carbon and nitrogen sources. Isolates were grown in plates for two weeks in a humid chamber to decrease evaporation from the plate at room temperature. HPLC analysis was performed using a Shimadzu VP system. Atrazine was resolved with an Alltech® C_{18} column using an isocratic elution with a flow rate of 1ml per minute and elution make up of 55% acetonitrile and 45% water. Isolates were scored positive for atrazine degradation if no atrazine was seen in the culture supernatant. Negative, uninoculated control wells were included on each plate.

Since a transient loss of the atrazine degradation phenotype has been reported, isolates scoring positive were reconfirmed a second time (Topp et al., 2000a). It was very difficult to discern purity of the isolates on the minimal salts agar medium, because it offers bacteria low concentrations of nutrients, and thus phenotypic characteristics were often difficult to observe as bacteria produced little biomass. In an effort to ensure pure culture isolates, the positive culture collection was then streaked onto a non-selective general-purpose media, $1/10^{th}$ strength trypticase soy, also containing 25 ppm of atrazine, to ensure positive selection pressure on the atrazine-degrading phenotype. Plating the bacteria on a non-selective media allowed for the separation and purification of many of the isolates from two or three member consortia. The positive subset of the culture

collection was then screened a second time for a loss of atrazine to ensure a stable atrazine degrading phenotype.

D. 16S rRNA Phylogenetic Identification

1. DNA Extraction

Phylogenetic affiliation of the positive isolates were determined by sequencing the, 16S rDNA, a well known molecular marker used for bacterial phylogeny. Genomic DNA was extracted from liquid cultures of the positive isolates using a MoBio® Soil DNA extraction kit per manufacturers instructions. The general extraction protocol includes placing a liquid culture of bacteria into a bead beating tube containing beating beads, lysis solution, and an inhibitor removal solution. The cells were lysed in the solution during a 10-minute vortex period. DNA from the lysed cells was then bound on a silica spin filter, where it was washed, and then recovered in PCR quality form. Genomic DNA was frozen at –20˚C for future use.

2. PCR Amplification of 16S rRNA gene

Extracted genomic DNA (25-100 ng) was used as template in PCR reactions to amplify the bacterial 16S rRNA gene utilizing universal 16S rRNA primers, 11f an 1327r, refereeing to the positions in 16S rRNA of *E.coli* (Table 4). Amplification reactions of 50 µL included: 25 µl of 2X Premium Taq™ (Fisher Scientific) including *TaRaTa* Taq™ polymerase, $MgCl_2$ buffer, and DNTPs, 1µL of 50 pmol forward 11f primer, 1µL of 50 pmol reverse 1327r primer, 3 µl of genomic, template DNA, and 20 µl

Table 4: Primers used in PCR amplification.

Primers	
16S rRNA (11 F)	GGGGAAGAAATNG
16S rRNA (1327 R)	CCTTGTTACGACTT
atzA (F)	TGAGACCGGAGGACG
atzA (R)	CGTCCACATTACC
atzB (F)	TCCGCAATCTTGCC
atzB (R)	CCATCGGCAGGGT
atzC (F)	TTGGATATGGG
atzC (R)	TCTGATATTGTCCGAAG
trzN (F)	GGGAAGTTCGGTC
trzN (R)	GTCATCGATGACCT

Note: All primers were synthesized by Operon Biotechnologies, Huntsville, AL.
F – Forward Primer
R – Reverse Primer

of sterile water. DNA amplification was carried out in an Eppendorf Mastercycler under the following conditions: 2 minute 'hot start' at 95˚C, then 35 cycles of 95˚C for 50 sec, 55˚C for 50 sec, and 72˚C for 1 minute, 45 sec. PCR amplified products were stored at 4˚C for long-term storage and future use.

3. TA Cloning

Initial cloning and sequencing efforts revealed that many of the bacterial isolates were actually mixed cultures of two or three bacterial species. Thus, multiple clones of each isolate were sequenced. A pGEM-T cloning vector kit (Promega, Madison, WI) was used in the cloning experiments and conducted per manufacturers instructions. 16S rDNA PCR products were first size verified by agarose gel electrophoresis. Bands were excised and purified from the gel via a Wizard PCR clean-up kit (Promega, Madison, WI) per manufacturers instructions to remove any unincorporated nucleotides, unannealed primers, non-specifically amplified product, and agarose. TA cloning was initiated by ligating purified fragments into the cloning vector p-GEMT Easy Vector system. After overnight ligation at 4˚C, the ligated vectors were then transformed into *E. coli* strain JM109 competent cells through a heat-shock process. Transformed cells were plated on luria broth/ampicillin plates containing IPTG and X-Gal for blue/white screening of the transformed cells. Transformed cells able to grow in the ampicillin plates that did not receive the plasmid have the ability to synthesize ß-galactosidase and thus degrade XGAL, turning the colonies blue. Cells on the other hand that received the insert can no longer synthesis ß –galactosidase and remain white. White, transformed clones were isolated after overnight incubation at 37˚C. Five clones were sequenced for each isolate to ensure full coverage of the culture. Plasmids from recombinant strains were extracted

using a Wizard *Plus* miniprep DNA purification system (Eppendorff, San Diego, CA) and submitted for sequencing.

4. Plasmid Sequencing

Plasmid sequencing was completed by Joe May, Molecular Biology Resources Facility, at the University of Tennessee. Plasmid inserts were cycle sequenced with a Big Dye sequencing kit (Applied Biosystems, Foster City, Calif.) by using M13 forward/reverse primers and approximately 400 ng of plasmid template. Products were purified by using Autoseq G-50 columns (Amersham Pharmacia Biotech Inc., Piscataway, N.J.) and then separated and analyzed with an automated capillary DNA sequencer (ABI model 377XL; Applied Biosystems).

5. Phylogenetic Affiliations

In order to phylogenetically identify 16S rDNA sequences, one must compare the sequences in a database of known species. Worldwide, there are a number of publicly available sequence databases that build massive sequence databases compiled from submissions from individual laboratories and large batch sequencing projects. In the US, the most widely used and comprehensive of these electronic databases is GenBank hosted by National Center for Biotechnology Information (NCBI) (Benson, Karsch-Mizrachi, Lipman, Ostell, & Wheeler, 2006). Within the NCBI system, BLAST (Basic Local Alignment Search Tool) is an algorithm tool used to align and then compare a sequence to all sequences in the GenBank database to identify closely related sequences. The search tool then assigns scores and probability values to quantify the sequence alignment matches (Wheeler et al., 2003). The Ribosomal Database Project (RDP) hosted by Michigan State University is another public database of 16S rRNA sequences also having

classification tools, and additionally hosts chimera check programs, and alignment software for the preparation of phylogenetic trees (Cole et al., 2005).

Sequence files received in ABI format from the sequencing facility were first opened and viewed in Bio-Edit® (Hall, 1997). Sequence chromatograms were examined for overlapping nucleotide sequences and clarity of resolution. Once the sequence was determined to be of good quality, it was saved in the FASTA format. The sequence was then copied into BLASTn, nucleotide database, and searched for closest relatives. Once identified, the sequence was then compared with its closest match and phylogenetic affiliations were made based on these matches. BLAST has the capability of reverse complementing a sequence for comparison analysis, but other databases do not. So if a sequence match was made in the reverse complement, the original sequence was opened again in Bio-Edit® and reverse-complemented for future use, to ensure the correct alignment. Sequences from closest relatives to each isolate were downloaded for use in phylogenetic tree construction. The sequences were also searched in RDP to confirm phylogenetic affiliation assignments in a second database. In the RDP database, all sequences have been verified and published in journal articles. Gen-Bank is more extensive and allows direct submission of sequences without publication. Gen-Bank served as the final determination for phylogenetic affiliation of the sequences analyzed in this study.

6. Creation of Phylogenetic Trees

The PHYLIP tool within RDP database was used to create phylogenetic trees to produce graphical interpretations of phylogenetic affiliations and relatedness of the positive atrazine-degrading isolates. Sequences of interest and close relatives were

uploaded into the program and aligned, distance matrixes were calculated based on a specific algorithm of calculating evolutionary differences, and phylogenetic trees were created. Relatedness and evolutionary distances can be inferred from branch lengths and relative distance on the tree. Three different algorithms for calculating evolutionary distances are available in PHYLIP, which account differently for transition and transversion rates of mutations, and include Kumura-2-parameter, maximum likelihood, and Jukes-Cantor. Comparison trees were produced from each of the three parameters and found to be very similar for our datasets. Neighbor joining trees using the distance matrix algorithm for maximum likelihood are commonly used when comparing organisms of phylogenetically diverse taxonomic hierarchy and were used in this study.

E. Gene Screen with Known Atrazine- Degrading Bacteria

Specific enzymes and the genes encoding an atrazine degradation pathway have been characterized and specific PCR primers have been designed for amplification from bacteria and environmental samples. In an effort to determine the occurrence of these known biomarkers of atrazine degradation in the atrazine-degrading culture collection, genomic DNA was amplified using primers for four of these known genes as probes, *atzA,B,C* and *trzN* (Table 3). Specific primers for the genes of interest were previously published, but more atrazine-degrading bacteria have been isolated since the time of their creation, so in an effort to probe with the most appropriate primer sets, all of the known atrazine-degrading bacteria shown to have the gene of interest were re-aligned in Bio-Edit®, and a new set of primers was created based on the homology of the gene between the organisms.

The same genomic DNA was used in both 16S rRNA gene amplification and biomarker amplification under the same amplification conditions as listed above. *Arthrobacter aurescens* strain TC1 was used as a positive control for *trzN*, and *Pseudomonas* sp. ADP was used for *atzA,B,C*. Stock cultures of both strains were graciously supplied by Dr. Mike Sadowsky at the University of Minnesota. Positive and negative controls were used with each gene screen. In addition, 16S rRNA was re-amplified using genomic DNA from each isolate to ensure the quality of DNA and optimum PCR conditions. PCR products were analyzed by agarose gel electrophoresis and scored positive/negative based on amplification and correct size of the product as compared to the positive controls.

III. Results and Discussion

Atrazine's ubiquity in water supplies and potential adverse environmental effects continue to drive research toward understanding its fate in the environment, especially in soils. The heterogeneous nature of soils provides for a multitude of variables that potentially influence atrazine fate. But even with such great diversity in physical properties of soils, it appears that an acclimated microbial community capable of atrazine degradation is the most influential parameter of atrazine attenuation in the environment. As such, much research has taken place to isolate and characterize organisms from soils capable of mediating this process. Unfortunately, the organisms most easily cultivated in a laboratory enrichment situation may not be the most ecologically relevant species. Culture-independent methodologies have revealed the vast majority of organisms are difficult to cultivate using classical techniques. In the case of atrazine, the available evidence suggests the current collection of atrazine-degrading bacteria, and their pathways of degradation do not adequately reflect the extant diversity of bacteria or catabolic genes involved in atrazine degradation. In an attempt to avoid classical enrichment biases, this study employed a novel in situ enrichment strategy to isolate the most active atrazine-degrading populations by using atrazine baited Bio-Sep® beads to facilitate the recruitment of active bacteria from soils with previously exposed to atrazine. Bacterial communities that colonized the beads were then used to isolate a diverse group of atrazine-degrading bacteria.

A. Culture Collection

1. Atrazine-Degrading Bacteria

Beads were deployed at a total of ten sites, some with both atrazine and water equilibrated beads (Table 5). Bacterial communities extracted from all of the bead sites were plated on both AMS with and without glucose in triplicate and bacterial colonies were isolated and purified from each site at all sampling times. A total of 582 cultures were isolated and screened for atrazine-degradation on both AMS with glucose and without glucose regardless of the media in which they were initially isolated. Subsequently, 47 isolates (8% of the collection) were identified as atrazine degrading bacteria that fell into three categories: (1) closely related to previously reported atrazine-degrading bacteria, (2) closely related to taxa not previously known to degrade atrazine, and (3) phylogenetically novel bacteria from rarely isolated groups. In addition, 305 of the non-degrading isolates were frozen as culture stocks for future use (Table 5). It was unfortunate that the isolation media was not more selective for atrazine-dependent growth. Many organisms were able to grow on the selective media despite the fact that atrazine was the only source of nitrogen. Seemingly the organisms either fixed nitrogen or extracted it from impurities in the culture media. Additionally, the powder activated carbon in the Bio-Sep® beads is capable of sorbing other compounds from the soil environment including other carbon and nitrogen sources, humic and fulvic acids, and others. These additional nutrient sources may have attributed to the colonization of a broad group of organisms.

The micro-aerophillic aeration regime was unsuccessful in identifying any positive atrazine-degrading organisms. Approximately fifty isolates were screened, but

Table 5: Summary of atrazine-degrading bacteria isolated from the various study sites.

Site Location and Bead Type	# Total Screened Isolates	# Positive Atrazine-Degrading Isolates	% of Positive Isolates	# Non-Degraders Retained for Future Use
WTEB	126	19	15%	37
WTFIB-Atz	65	0	0%	56
WTFIB-H20	43	0	0%	32
1B-Atz	19	0	0%	12
2A-Atz	36	5	14%	11
3A-Atz	55	9	16%	23
Penn-Atz	70	6	9%	51
Penn-H20	24	0	0%	21
Cl-Atz	26	2	8%	4
S-Atz	26	0	0%	2
W-Atz	28	3	11%	15
PWS-Atz	14	0	0%	13
PWS-H20	18	1	6%	13
UDEL-Atz	32	2	6%	15
TOTAL	**582**	**47**	**Avg 6%**	**305**

were all negative for atrazine-degradation. A small subset of the later appearing isolates were re-introduced to ambient conditions and are still in the process of being screened for atrazine-degradation.

The largest number of positive degraders from one site came from additional lab-enrichment cultures of West Tennessee beads in a preliminary winter deployment. The greatest percentage of positive-atrazine degraders came from Ohio-3A site, which incidentally had not received atrazine in two years. Possibly, the atrazine-degrading phenotype was well established in the community, and the beads provided a renewed source of atrazine, and thus the beads were able to recruit the most active community capable of atrazine degradation. Other Ohio sites, 2A and Penn, also produced high percentages of atrazine-degrading bacteria, 14 and 16%, respectively.

2. Consortia Isolations

It is widely accepted that many bacteria are not planktonic organisms, but rather they like to live in biofilms and mixed communities where the organisms use byproducts from the cellular metabolism of neighboring cells and cell-to-cell signaling to aid in their growth and reproduction. The interconnectedness of microbial communities often times makes isolating organisms in pure culture an arduous task, as the reasons and mechanisms behind the organisms' associations are often unknown, and thus are difficult to replicate in a selective medium. Our culture collection was no exception. The original isolation of bacterial cultures was carried out on a minimal nutrient media to select for atrazine-dependent growth of bacteria. Unfortunately many bacteria that were presumably enriched in the beads were not able to grow well, so phenotypic

discrimination was difficult as many of the colonies were very small. Once isolates were purified through sequential passages, the bacteria were screened for atrazine degradation. Once shown as positive atrazine-degraders, the organisms were then plated onto a more general-purpose media to verify purity. Allowing the organisms to produce more biomass, phenotypic characteristics allowed for the separation of many thought to be pure atrazine-degrading isolates from one organism into two or even three distinct colony types. The isolates were then separated into what hopefully were pure cultures on the general-purpose media. The separated isolates were once again tested for atrazine degradation to ensure a stable atrazine-degrading phenotype and identify which members of the consortium were degrading atrazine. The re-screening process resulted in the loss of many positive-degrading isolates, which was somewhat expected, albeit disappointing, as the atrazine-degrading phenotype has been known to be very unstable especially during purification and sub-culturing efforts needed here to purify the isolates. But many of the consortia retained their degradative capacity even when separated and purified. Different isolation categories resulting in at least one atrazine-degrading bacterial isolate are presented in Figure 5. Sixty-four percent of all positive-atrazine degrading bacteria were first isolated in consortia and subsequently separated into pure cultures. Many were co-cultures that when separated only one of the two isolates degraded atrazine.

3. Increased Incubation Time

In studies to increase cultivability of rarely isolated groups of organisms, the simple task of increased incubation times has been very successful. Davis et al. (2005) showed that in combination with newly developed complex media, increased incubation times from two week time periods to over 30 days greatly improved their ability to isolate

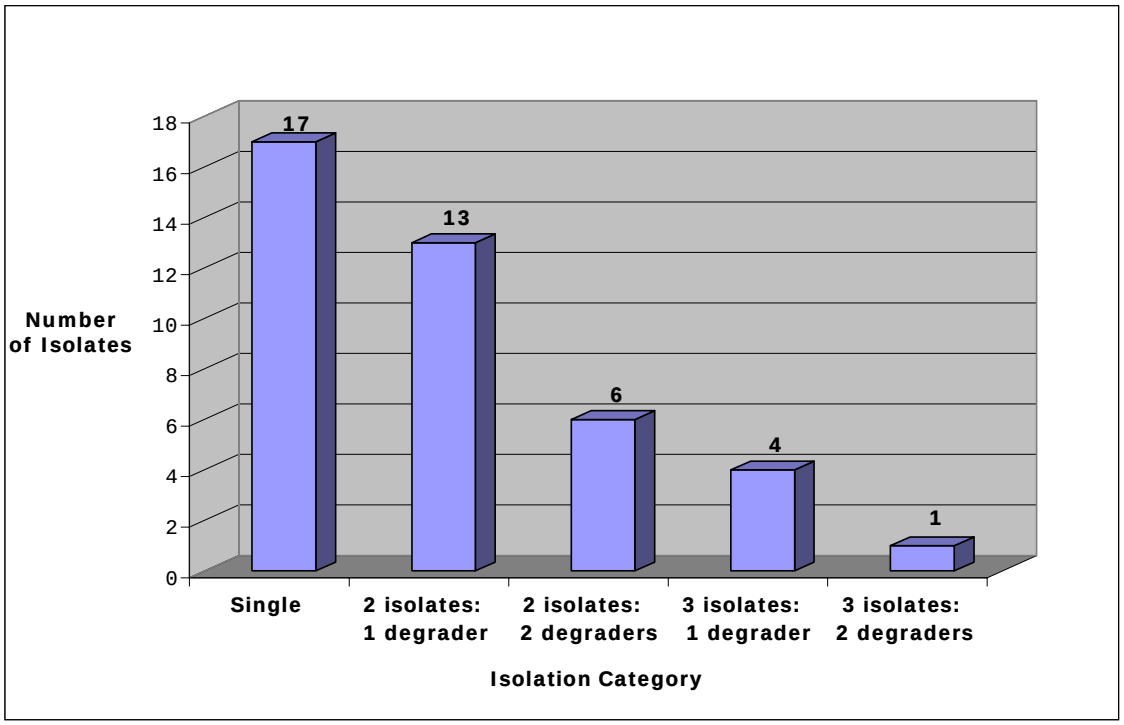

Figure 5: Distribution of atrazine-degrading bacteria that were initially isolated as consortia of two or more bacteria.

rare groups from soils including *Firmicutes*, *Acidobacteria*, and *Proteobacteria*, suggesting these groups are particularly slow growing and may have long lag phases (Davis et al., 2005). Uncultured *Acidobacteria* and *Verucomicrobia* were isolated through a combination of techniques including over 30 day incubation times (Stevenson et al., 2004). In the current study, cultures were isolated at incubation times of up to 70 days. Many isolates were screened in excess of 30 days of incubation, but 72% of the 47 atrazine-degrading bacteria were isolated in the first few weeks of incubation: 34 isolated at 7 days, 10 isolated at 4 days, and 3 isolated at 28 days. In this particular collection, increased length of time did not increase our ability to isolate atrazine-degrading organisms, but many of the latter forming colonies were frozen as culture stocks for possible future identification and characterization (Table 5).

B. Phylogenetic Identification of the Atrazine-Degrading Bacteria

All 47 of the atrazine degrading bacteria were phlogenetically identified through analysis of 16S rRNA genes. Recombinant clone libraries were constructed and five clones from each isolate were sequenced to ensure purity of the isolate in question. Most closely related species were identified through BLAST homology searches and phylogenetic trees were constructed to produce graphical interpretations of phylogenetic affiliations and relatedness of the positive atrazine-degrading isolates. In general, if the 16S rRNA sequence of an isolate was greater than or equal to 97% similar to the best BLAST hit, it was considered to be the same genus and if greater than or equal to 99%, the same species. Further delineations, such as at the family level, from the partial sequence of 16S rRNA is not consistent and must be characterized in more detail to

determine possible novelty. A complete list of the atrazine-degrading isolates sorted by site is listed in Table 6, and include their UTK identification number and closest phylogenetic affiliations.

1. West Tennessee Isolates

Beads were deployed at the West Tennessee Research and Education Center at a long-term no-till plot in a crop rotation with corn every other year, in the winter of 2004 as a trial deployment of the beads in soils. Once recovered from the field, the beads were further enriched in liquid AMS media until the spring deployments were ready for cultivation studies, to obtain a highly enriched atrazine-degrading community. After several passages in fresh AMS media, copious amounts of biomass began to accumulate on the surface of the enriched beads and began sloughing off into the culture medium causing turbidity. The bacteria growing in the spent media were used to start secondary enrichment cultures. Every two weeks, the spend media was decanted from the bead enrichments and fresh media was added; in addition, the liquid enrichments were re-inoculated into fresh media as well.

From these two cultivation experiments, 19 atrazine-degrading bacteria were isolated. The cultures were highly enriched, as 13 of the 19 isolates most closely related to various *Arthrobacter* sp. in the Actinobacteria phylum and class. Many of these were at or above the cutoff for genus similarity percentage. Ten of the isolates most closely matched a strain of *Arthrobacter aurescens*, a genus and species already shown to degrade atrazine. UTK020 was only a 94% match to *Arthobacter nitroguajacolicus* strain Rue 61 and may possibly be a novel species of *Arthrobacter*. In addition,

Table 6: Phylogenetic affiliations of atrazine-degrading isolates based on BLAST alignment of 16S rDNA sequences.

UTK ID	Original ID	Closest Phylogenetic Affiliation
West TN Research and Education Center, Milan, TN (n=19)		
UTK003	WTEC w/o 3	*Arthrobacter* (96%)
UTK013B	WTFIB-2	*Nocardioides* (96%)
UTK015	A1-1	*Arthrobacter* (99%)
UTK016A	A2 w/ 1	*Arthrobacter* (99%)
UTK017	A2 w/ 2	*Arthrobacter (*97%)
UTK020	A2 w/ 9	*Arthrobacter* (94%)
UTK021	A2 w/o 1	*Arthrobacter* (97%)
UTK022A	B w/ 5	*Arthrobacter* (97%)
UTK023A	B w/ 6	*Arthrobacter* (97%)
UTK024A	C w/ 6	*Arthrobacter* (98%)
UTK024B	C w/ 6	*Variovorax* (97%)
UTK027C	D w/ 5	*Arthrobacter* (99%)
UTK028B	D w/ 7	*Sphingomonas* (99%)
UTK029	D w/ 11	*Arthrobacter* (96%)
UTK030A	D w/ 12	*Arthrobacter* (99%)
UTK032A	D w/o 5	*Arthrobacter* (96%
UTK032B	D w/o 5	*Methylopila* (95%)
UTK033B	C w/o 8	*Dyadobacter* (95%)
UTK037	WTEC w/o 2	*Variovorax* (97%)
Molly Caren Agricultural Center, OSU, London, OH (n=22)		
2A (n=5)		
UTK034	2A w/o 7B	*Rahnella (*97%)
UTK044	2A w/ 11B	*Rahnella (*99%)
UTK053	2A w/o 6A	*Variovorax* (99%)
UTK054	2A w/o 6B	*Arthrobacter* (99%)
UTK058	2A w/o 15A	*Glycomyces* (99%)
3A (n=9)		
UTK036	3A w/ 9B	*Acidovorax* (99%)
UTK039	3A w/o 2A	*Streptomyces* (100%)
UTK040	3A w/o 2B	*Pseudomonas* (99%)
UTK050	3A w/ 11	*Arthrobacter* (98%)
UTK051	3A w/ 10A	*Pseudomonas* (98%)
UTK052	3A w/ 10B	*Acidovorax* (99%)
UTK056	3A w/ 13	*Acidovorax* (99%)
UTK059	3A w/ 2A	*Pseudomonas* (99%)
UTK060	3A w/ 2B	*Rahnella* (98%)

Note: Similarities are based on percentage match to best BLAST hit.

Table 6: Continued

UTK ID	Original ID	Closest Phylogenetic Affiliation
Penn (n=6)		
UTK009A	Penn w/ 1	*Streptomyces* (98%)
UTK011A	Penn w/ 4	*Arthrobacter* (98%)
UTK038	Penn w/o 7A	*Catellatospora* (99%)
UTK041	Penn w/ 2	*Methylobacterium* (99%)
UTK048	Penn w/o 4A#1	*Microbacterium* (99%)
UTK049	Penn w/o 4A#3	*Variovorax* (98%)
Cl (n=2)		
UTK055	Cl w/ 12	*Acidovorax* (99%)
UTK064	Cl w/o 12	*Rahnella* (96%)
Olentangy River Wetland Research Park, Columbus, OH (n=3)		
UTK046	W w/3	*Acinetobacter* (96%)
UTK062	W w/o 7	*Acinetobacter* (97%)
UTK063	W w/o 5C	*Pseudomonas* (98%)
Other Sites (n=3)		
UTK047	PWS-H20-24	*Pantoea* (98%)
UTK045	UDEL w/ 9	*Pseudomonas* (97%)
UTK057	UDEL w/o 4A	*Arthrobacter* (99%)

Note: Similarities are based on percentage match to best BLAST hit.

UTK013B, was identified as a *Nocardioides* sp. Several *Nocardioides* sp. have been reported to degrade atrazine and all possess *trzN*.

But the enrichment cultures did yield some very novel groups of atrazine-degrading bacteria. UTK024B and UTK037 were closely related to *Variovorax* species in the Beta-Proteobacteria class. A *Variovorax* species was characterized as a member of a cooperative atrazine-degrading bacterial consortium, but was only able to degrade cyanuric acid, not atrazine (Smith et al., 2005). A *Sphingomonas* sp., UTK028B, and a *Methylopila* sp., UTK032B, from the Alpha-Proteobacteria class were also isolated. Both the phylum and two classes have recognized atrazine-degrading organisms, but the genus have never been shown to degrade atrazine. The UTK032B isolate was only 95% similar to *Methylopila* sp. suggesting the possibility of a new genus in this class of bacteria. In addition, one of the most novel isolates in the collection was obtained from the West Tennessee site. UTK033B, 95% similarity to a *Dyadobacter* sp. is classified in the Bacteroidetes phylum, which has no representatives known to degrade xenobiotics. A phylogenetic tree containing all of the positive isolates from the West Tennessee site is shown in Figure 6.

2. Ohio Sites

(a.) 2A

Molly Caren Agricultural Center, OSU site, 2A, a corn plot in 2004 and 2005, with a spring 2005 deployment yielded 5 positive isolates, 4 of which are from novel atrazine-degrading lineages. Most significant of these novel lineages include: UTK034 and UTK044, *Rahnella* sp., a member of the Gamma-Proteobacteria class; UTK058, *Glycomyces* sp. in the Actinobacteria phylum and class; and UTK053, a *Variovorax* sp.

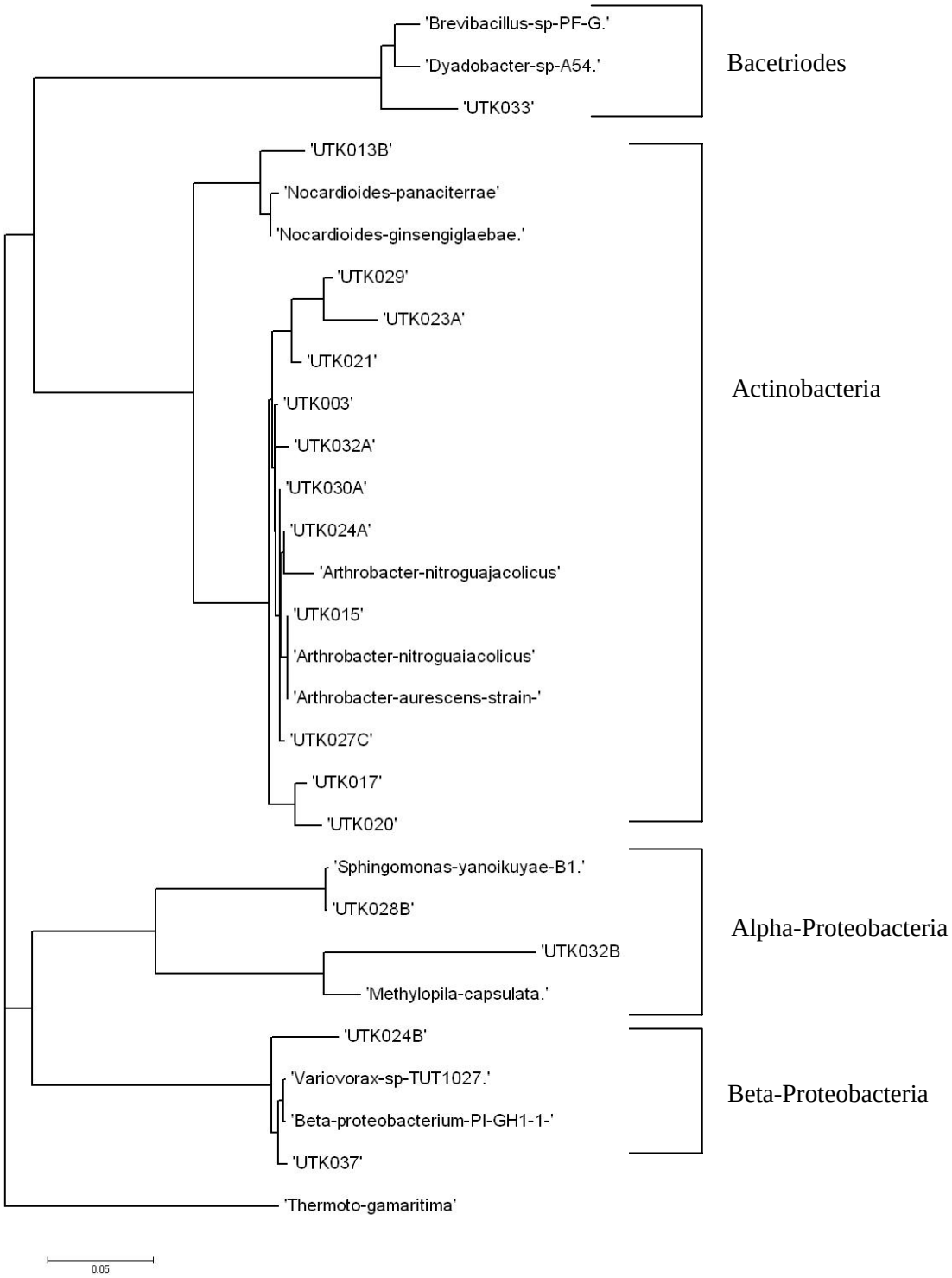

Figure 6: 16S rRNA based phylogram of West Tennessee isolates and their closest relatives.

(b.) 3A

Molly Caren Agricultural Center, OSU site, 3A, a plot in a wheat-soybean crop rotation generated 9 atrazine-degrading isolates, all from the spring 2005 deployment. *Acidovorax* sp. dominated the cultivable community at this site with one-third of the isolates. Like the *Variovorax* sp., an *Acidovorax* sp. has also been identified as a member of an atrazine-degrading consortia, but could not degrade atrazine, only some of the intermediate metabolites (Smith et al., 2005). Previously mentioned Gamma-Protebacteria and Actinobacteria complete the isolates at this site.

(c.) Penn

Another Molly Caren Agricultural Center, OSU site, Penn, planted with corn in 2005, yielded 6 atrazine-degrading bacteria. Two Alpha-Proteobacteria isolates most closely related to *Methylobacterium* and *Microbacterium* sp. were characterized. Both genus are novel atrazine-degraders, never having been reported to degrade atrazine. Additionally, an Actinobacteria, *Catellatospora* sp. also novel was identified.

(**d.) Cl**

The final Molly Caren Agricultural Center, OSU site, was Cl, a five-year corn plot generated 2 atrazine-degrading bacteria from a spring 2005 deployment of beads. *Acidovorax* and *Rahnella* sp. were isolated.

3. Olentangy River Wetland Research Park (ORWRP)

Water samples from the ORWRP yielded positive atrazine degrading bacteria from a spring 2005 deployment. Three Gamma-Proteobacteria, including a *Pseudomonas* sp. and the only *Acinetobacter* sp. in the characterized collection were isolated from the sediment samples.

4. Other Sites

The University of Delaware bead bag was destroyed in the field, but some beads were recovered loose in the soil and plated just to see what could be cultivated. The two isolates identified were from taxa previously known to degrade atrazine, *Arthrobacter* and *Pseuodomonas*. Many isolates from both water and atrazine baited beads were screened from the pesticide-waste-site, but only yielded one positive-degrader, albeit a genus never previously shown to degrade atrazine, a Gamma-Proteobacteria *Pantoea* sp. Positive-isolates from the Ohio sites and their closest phylogenetic affiliates are shown in Figure 7.

It is very interesting to look collectively at the Ohio sites and the organisms isolated from each one. It might be hypothesized that the Ohio soils being from the same general area with somewhat similar agricultural backgrounds would have selected for similar cultivable atrazine-degrading communities, but that was not necessarily the case. Even though the groups of organisms were generally the same, the most abundant species were not. For instance, *Rahnella* sp. dominated the site 2A, but were absent in the Penn site, and only one *Rahnella* isolate was seen in the site 3A. Gamma-Proteobacteria dominated the site 3A, but none were isolated from the Penn site. Bacteria isolated from the water column at the ORWRP consisted of the only *Actinobacteria* in the collection, but since this was the only aquatic environment sampled, one might expect different species. It was also interesting to note, that in bead communities not further enriched in the lab, like the West Tennessee beads, only simple in situ enrichment, very few

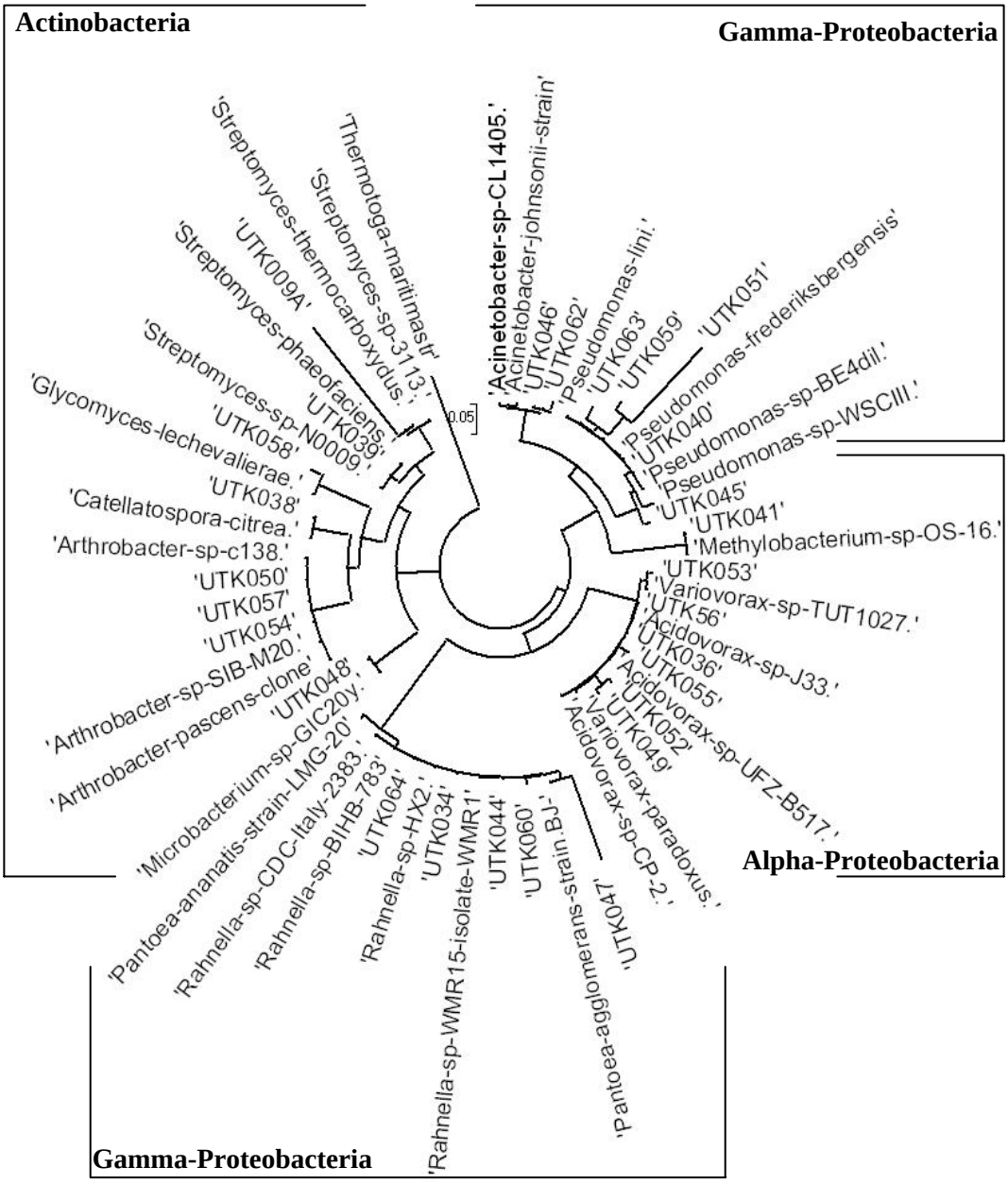

Figure 7: 16S rRNA based phyogram of Ohio sites isolates and their closest relatives.

Arthrobacter sp. were cultivated as atrazine-degraders from other sites nor within the West Tennessee field enriched beads (Table 6). These results further suggest that enrichment in laboratory conditions are biased toward commonly isolated organisms, but more novel cultivation and enrichment techniques can yield a much broader range of bacteria capable of degrading atrazine.

5. Bio-Sep® Beads Increased Cultivable Atrazine-Degrading Bacteria

The objective of this study was to utilize a novel enrichment technique to broaden the number and phylogeny of atrazine-degrading bacteria, and in essence test Bio-Sep® bead utility as a suitable tool in recruiting active microbial communities with a specific metabolic pathway in mind. Admittedly, very few changes in the actual culturing technique were made during cultivation experiments. Most notably, atrazine concentrations were kept closer to rates seen in soils from typical atrazine applications to stimulate natural environments, attempting to avoid yet another culture bias towards the organisms with the fastest and most abundant growth and degradation rates. Traditional selective, minimal nutrient media was used with a widely accepted carbon source, glucose. In effect, the only truly novel innovation in this cultivation experiment was the use of the Bio-Sep® beads. Instead of taking a soil sample from the field, further enriching the soil in a culture flask at high atrazine concentrations, then using traditional cultivation methods to identify atrazine degrading bacteria, enrichment took place in situ, in the field, where the most active community could colonize the beads and then be further cultivated and isolated in a very traditional way.

Table 7 shows a breakdown of the atrazine-degrading collection at a phylum and class level. Forty-nine percent of the culture collection was placed in the class

Table 7: Phylum and class level grouping of atrazine-degrading bacteria.

Phylum	Original ID	Phylogenetic Affiliation
Actinobacteria (n=23)		
***Arthrobacter* (n=17)**		
UTK003	**WTEC w/o 3**	***Arthrobacter* (96%)**
UTK011A	Penn w/ 4	*Arthrobacter* (98%)
UTK015	A1-1	*Arthrobacter* (99%)
UTK016A	A2 w/ 1	*Arthrobacter* (99%)
UTK017	**A2 w/ 2**	***Arthrobacter* (97%)**
UTK020	**A2 w/ 9**	***Arthrobacter* (94%)**
UTK021	**A2 w/o 1**	***Arthrobacter* (97%)**
UTK022A	**B w/ 5**	***Arthrobacter* (97%)**
UTK023A	**B w/ 6**	***Arthrobacter* (97%)**
UTK024A	C w/ 6	*Arthrobacter* (98%)
UTK027C	D w/ 5	*Arthrobacter* (99%)
UTK029	**D w/ 11**	***Arthrobacter* (96%)**
UTK030A	D w/ 12	*Arthrobacter* (99%)
UTK032A	**D w/o 5**	***Arthrobacter* (96%)**
UTK050	3A w/ 11	*Arthrobacter* (98%)
UTK054	2A w/o 6B	*Arthrobacter* (99%)
UTK057	**UDEL w/o 4A**	***Arthrobacter* (97%)**
Streptomyces (n=2)		
UTK009A	Penn w/ 1	*Streptomyces* (98%)
UTK039	3A w/o 2A	*Streptomyces* (100%)
Others (n=4)		
UTK038	Penn w/o 7A	*Catellatospora* (99%)
UTK058	2A w/o 15A	*Glycomyces* (99%)
UTK048	Penn w/o 4A#1	*Microbacterium* (99%)
UTK013B	**WTFIB-2**	***Nocardioides* (96%)**
Bacteroidetes (n=1)		
UTK033B	**C w/o 8**	***Dyadobacter* (95%)**

Note: Similarities are based on percentage match to best BLAST hit.
Isolates noted in **BOLD** are below the 98% identity match and may possibly represent a new genus.

Table 7: Continued

Phylum	Original ID	Phylogenetic Affiliation
Proteobacteria (n=23)		
Alpha-Proteobacteria (n=3)		
UTK041	Penn w/ 2	99% *Methylobacterium*
UTK032B	**D w/o 5**	**95% *Methylopila***
UTK028B	D w/ 7	99% *Sphingomonas*
Beta-Proteobacteria (n=8)		
***Acidovorax* (n=4)**		
UTK036	3A w/ 9B	99% *Acidovorax*
UTK052	3A w/ 10B	99% Acidovorax
UTK055	Cl w/ 12	99% *Acidovorax*
UTK056	3A w/ 13	99% Acidovorax
***Variovorax* (n=4)**		
UTK024B	**C w/ 6**	**97% *Variovorax***
UTK037	**WTEC w/o 2**	**97% *Variovorax***
UTK049	Penn w/o 4A#3	98% *Variovorax*
UTK053	2A w/o 6A	99% *Variovorax*
Gamma-Proteobacteria (n=8)		
***Acinetobacter* (n=2)**		
UTK046	**W w/3**	**96% *Acinetobacter***
UTK062	**W w/o 7**	**97% *Acinetobacter***
***Pseudomonas* (n=5)**		
UTK040	3A w/o 2B	*99% Pseudomonas*
UTK045	**UDEL w/ 9**	**97% *Pseudomonas***
UTK051	3A w/ 10A	98% *Pseudomonas*
UTK059	3A w/ 2A	99% *Pseudomonas*
UTK063	W w/o 5C	98% *Pseudomonas*
Rahnella (n=4)		
UTK034	**2A w/o 7B**	**97% *Rahnella***
UTK044	2A w/ 11B	99% *Rahnella*
UTK060	3A w/ 2B	98% *Rahnella*
UTK064	**Cl w/o 12**	**96% *Rahnella***
Other (n=1)		
UTK047	PWS-H20-24	98% Pantoea

Note: Similarities are based on percentage match to best BLAST hit.
Isolates noted in **BOLD** are below the 98% identity match and may possibly represent a new genus.

Actinobacteria and included *Arthrobacter* sp. (n=17), *Streptomyces* sp. (n=2), *Catellatospora* sp. (n=1) *Microbacterium* sp. (n=1), *Glycomyces* sp. (n=1) and *Nocardioides* sp. (n=1). Twenty-six percent of the collection were placed in the Gamma-Proteobacteria class and consisted of *Pseudomonas* sp. (n=5), *Rahnella* sp. (n=4), *Acinetobacter* sp. (n=2), and *Pantoea* sp. (n=1). Seventeen percent of the positive atrazine-degrading bacteria were of the Beta-Proteobacteria class and included *Variovorax* sp. (n=4) and *Acidovorax* sp. (n=4). Alpha-Proteobacteria comprised 6% of the collection, and included *Sphingonomas* sp. (n=1), *Methylopila* sp. (n=1), and *Methylobacterium* sp. (n=1). The Bacteriodes class rounds out the phylum isolated with *Dyadobacter* sp. (n=1).

Previously described atrazine-degrading bacteria all belong to the Acinobacteria and Proteobacteria phylums. Even though many of the isolates were not taxonomically novel, we were able to isolate atrazine-degraders from a wide range of phylums, and greatly increase the scope and diversity of atrazine-degrading bacteria within these groups. A novel bacterial phylum to atrazine-degradation was also isolated. UTK033B, only a 95% similarity to a *Dyadobacter* sp., is a member of the Bacteroidetes phylum, never shown to be involved in xenobioic degradation. Both new and previous atrazine-degrading bacteria classes were isolated. New atrazine-degrading genus from the Actinobacteria class include, *Catellatospora*, *Microbacterium*, and *Glycomyces*. From the Gamma-Protobacteria, new atrazine-degrading genera, *Rahnella*, *Acinetobacter*, and*Pantoea*, were isolated. Both species isolated from the Beta-Proteobacteria are new atrazine degrading lineages, *Variovorax* and *Acidovorax*. Finally, in the Alpha-Proteobacteria class, new genera include *Sphingobium*, *Methylobacteria*, and

Methylopila. A phylogenetic tree including the known atrazine-degrading bacteria in addition to the isolates identified in this study is shown in Figure 8. It is clear to see how this study widens our view of the cultivable organisms involved in atrazine-degradation.

The use of Bio-Sep® beads clearly lead to the cultivation of atrazine-degrading bacteria not typically isolated by conventional methods. Both field-incubated beads and lab-enriched beads were used to cultivate atrazine-degrading organisms. It was astounding to see the vast difference in diversity of the flask-enriched beads versus the in situ enriched beads. The flask-enriched beads isolated very few groups of organisms, but the in situ enriched beads yielded a much more diverse group of atrazine-degrading organisms. It is clear to see the utility of in situ enrichment when attempting to cultivate a broad group of organisms, not the ones most adaptive to competitive, fast growth.

In conclusion, even though many of the species isolated in this collection may not have been novel from a cultivability standpoint, the collection greatly enhances the scope and diversity of atrazine-degrading organisms. It is important to note that only the organisms with the ability to degrade atrazine were phylogenetically identified. As an aside, a few organisms were sequenced prior to purification or lost their ability to degrade atrazine in the purification process, but put forth the notion that we were able to cultivate some novel organisms along side atrazine degraders. For instance, UTK003 was initially isolated as a pair of organisms. The *Arthrobacter* sp. (UTK003) was separated from a novel Beta-Proteobacteria. Many of the isolated *Arthrobacter* sp. especially from the West Tennessee enrichment cultures were separated from very phenotypically interesting organisms, but were not sequenced due to the large number of organisms in the collection. Additionally, UTK028B, a *Sphingomonas* sp. was separated from a very

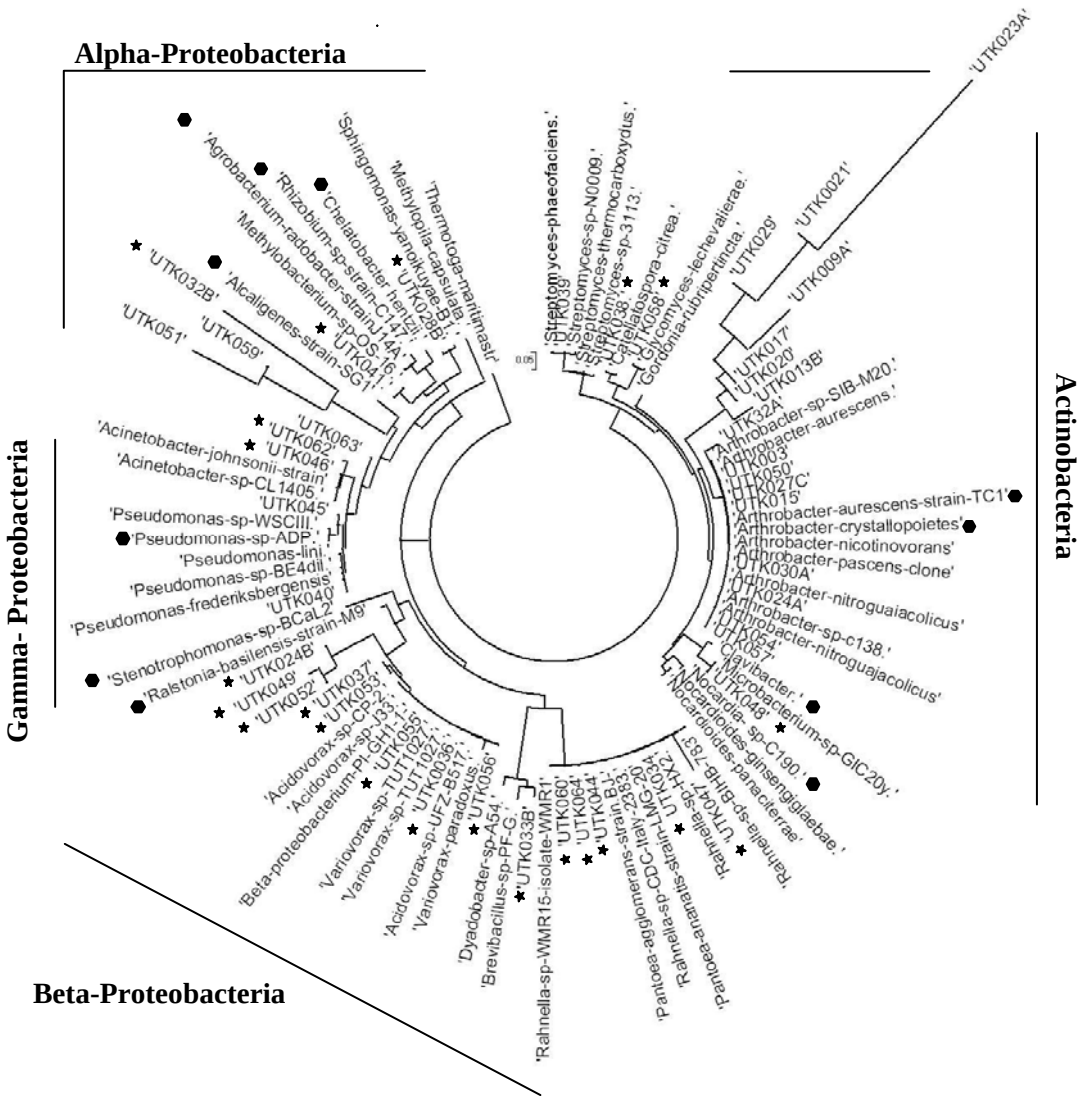

Figure 8: 16S rRNA based phylogram of atrazine-degrading collection in addition to known atrazine-degrading bacteria.

Isolates denoted with a hexagon, ⬢, represent previously characterized atrazine-degrading bacteria. Isolates starred, ★, represent taxa not previously reported to degrade atrazine.

phylogenetically novel *Aquabacterium* sp. which did not degrade atrazine, but in a prelimary growth and degradation study greatly enhanced the degradation of UTK028B. The two in tandem may be an important consortium in the West Tennessee soil in which they were isolated. A growth and degradation curve of the UTK028 consortium is shown in Figure 9. The two organisms in tandem were able to degrade 25 ppm of atrazine in just over 24 hours.

C. Catabolic Gene Detection

Only one major pathway of microbial degradation characterized in *Pseudomonas* sp. ADP in soils has been extensively described and currently stands as the benchmark for all other atrazine-degrading bacteria. The upper pathway proceeds via a dechlorination reaction followed by sequential N-dealkylation reactions to yield cyanuric acid (Figure 3). The suite of genes from *Pseudomonas* sp. ADP encoding enzymatic attack include *atzA,B,C* (Martinez et al., 2001). In addition, another chlorohydrolase gene encoding the same function of *atzA*, *trzN* from *Nocardioides* sp. C190 has also been identified (Mulbry et al., 2002). Many of the current collection of atrazine-degrading bacteria all have some version of these genes, either the *atz* suite or a combination of genes including *trzN* and *atzBC* (Table 2). But these organisms were isolated using similar techniques, and only represent the cultivable portion of the atrazine-degrading community to date. The *atzA,B,C,* suite of genes have been used as genetic biomarkers to probe atrazine-degradation in soils, but a lack of correlation between the frequency of these genes and atrazine degradation has been shown (Ostrofsky et al., 2002). The *trzN* gene was recently characterized in 2002, and has not been probed in the environment to

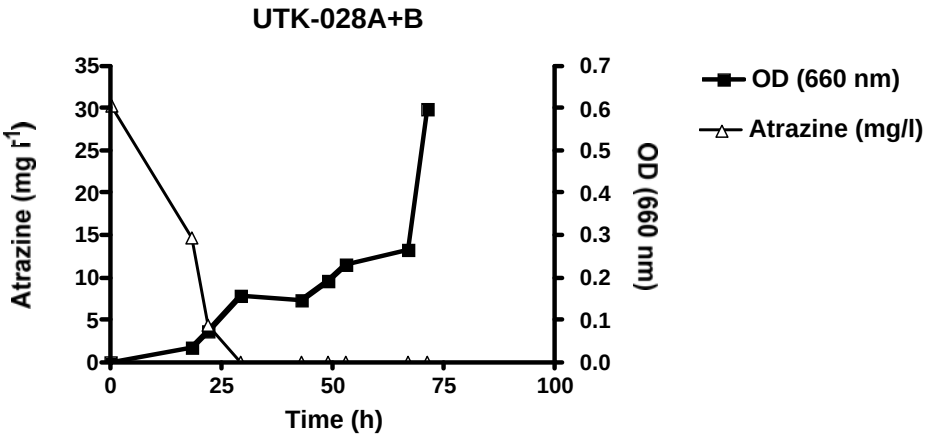

Figure 9: UTK028 growth and degradation curve.

date (Mulbry et al., 2002). As these are the only characterized catabolic genes, they are used as a standard against which all atrazine-degrading bacteria are measured. In order to assess the atrazine-degradation metabolic diversity, the collection was screened for the presence of the upper pathway genes, *atzA,B,C* and *trzN* by PCR with primer sets developed in this study (Table 3). *Pseudomonas* sp. ADP served as a positive control for *atzA,B,C* and *Arthrobacter aurescens* strain TC1 served as a positive control for *trzN* and *atzB,C*.

Many of the isolates in the collection did not amplify with any of the upper pathway genes, especially *atzA,B,C*, suggesting the existence of novel catabolic pathways of atrazine degradation, especially in the novel lineages seen exclusively in this study (Table 8). One of the most exciting aspects of the gene screen, was the fact that *trzN*, not *atzA* was the most commonly detected chlorohydrolase gene in this collection. Previous reports have shown *atzA* is very prevalent among known-atrazine degrading bacteria (deSouza et al., 1998a), but was not in our collection, further solidifying the novelty and uniqueness of the collection of atrazine-degrading bacteria. In addition, *trzN*, has only previously been reported in Actionobacteria, but was detected in *Sphingomonas* sp. (Alpha-Proteobacteria class), three *Pseudomonas* sp. (Gamma-Proteobacteria class), *Variovorax* sp. (Beta-Proteobacteria), and *Dyabobacter* sp. (Bacteriodes class) in our collection. Preliminary metabolite utilization studies have suggested the *Sphingomonas* sp., UTK028B, has a complete pathway of atrazine degradation. *trzN* was also detected in UTK037, *Varivorax* sp, as noted before, a *Variovorax* sp was identified in an atrazine-degrading consortium, but only contained the *atzC* gene used strictly in conversion of N-isopropylammelide to cyanuric acid, and trzD, ring cleaving enzyme (Smith et al., 2005).

Table 8: Detection of known atrazine-degrading genes.

UTK ID	Original ID	Closest Phylogenetic Affiliation	*trzN*	*AtzA*	*atzB*	*AtzC*
West TN Research and Education Center, Milan (n=19)						
UTK003	WTEC w/o 3	*Arthrobacter*(96%)	+	–	+	+
UTK013B	WTFIB-2	*Nocardia* (96%)	–	–	–	–
UTK015	A1-1	*Arthrobacter* (99%)	+	–	+	–
UTK016A	A2 w/ 1	*Arthrobacter*(99%)	+	–	+	+
UTK017	A2 w/ 2	*Arthrobacter* (97%)	+	–	+	+
UTK020	A2 w/ 9	*Arthrobacte* (94%)	+	–	+	+
UTK021	A2 w/o 1	*Arthrobacter* (97%)	+	–	+	+
UTK022A	B w/ 5	*Arthrobacter* (97%)	+	–	+	–
UTK023A	B w/ 6	*Arthrobacter* (97%)	+	–	+	+
UTK024A	C w/ 6	*Arthrobacter* (98%)	+	–	+	–
UTK024B	C w/ 6	*Variovorax* (97%)	–	–	–	+
UTK027C	D w/ 5	*Arthrobacter* (99%)	+	–	–	+
UTK028B	D w/ 7	*Sphingomonas* (99%)	+	–	–	N/A
UTK029	D w/ 11	*Arthrobacter* (96%)	+	–	+	+
UTK030A	D w/ 12	*Arthrobacter* (99%)	+	–	+	–
UTK032A	D w/o 5	*Arthrobacter* (96%)	+	–	+	+
UTK032B	D w/o 5	*Methylopila* (95%)	–	–	–	–
UTK033B	C w/o 8	*Dyadobacter* (95%)	+	–	N/A	–
UTK037	WTEC w/o 2	*Variovorax* (97%)	+	–	–	–
Molly Caren Agricultural Center, OSU, London (n=22)						
2A (n=5)						
UTK034	2A w/o 7B	*Rahnella* (97%)	–	–	N/A	N/A
UTK044	2A w/ 11B	*Rahnella* (99%)	–	–	–	–
UTK053	2A w/o 6A	*Variovorax* (99%)	–	–	–	–
UTK054	2A w/o 6B	*Arthrobacter*(99%)	–	N/A	–	–
UTK058	2A w/o 15A	*Glycomyces*(99%)	–	–	–	–
3A (n=9)						
UTK036	3A w/ 9B	*Acidovorax* (99%)	–	–	–	–
UTK039	3A w/o 2A	*Streptomyces* (100%)	–	–	–	–
UTK040	3A w/o 2B	*Pseudomonas* (99%)	+	–	–	–
UTK050	3A w/ 11	*Arthrobacter* (98%)	–	–	–	–
UTK051	3A w/ 10A	*Pseudomonas* (98%)	–	–	–	–
UTK052	3A w/ 10B	*Acidovorax* (99%)	–	–	–	–
UTK056	3A w/ 13	*Acidovorax* (99%)	–	–	–	–
UTK059	3A w/ 2A	*Pseudomonas* (99%)	+	–	–	–
UTK060	3A w/ 2B	*Rahnella (*98%)	–	–	–	–

Note: Phylogenetic affiliations based upon most significant BLAST alignments.
Pseudomonas sp. ADP positive control for *atzA,B,C.*
Arthrobacter aurescens strain TC1 positive control for *trzN, atzB,C.*
N/A: Not available

Table 8: Continued

UTK ID	Original ID	Closest Phylogenetic Affiliation	*trzN*	*atzA*	*atzB*	*atzC*
Penn (n=6)						
UTK009A	Penn w/ 1	*Streptomyces* (98%)	–	–	–	–
UTK011A	Penn w/ 4	*Arthrobacter* (98%)	N/A	N/A	N/A	N/A
UTK038	Penn w/o 7A	*Catellatospora* (99%)	–	–	–	–
UTK041	Penn w/ 2	*Methylobacterium* (99%)	–	–	–	–
UTK048	Penn w/o 4A#1	*Microbacterium* (99%)	–	–	–	–
UTK049	Penn w/o 4A#3	*Variovorax* (98%)	–	–	–	–
Cl (n=2)						
UTK055	Cl w/ 12	*Acidovorax* (99%)	N/A	N/A	N/A	N/A
UTK064	Cl w/o 12	*Rahnella* (96%)	–	–	–	–
Olentangy River Wetland Research Park, OSU, Columbus (n=3)						
UTK046	W w/3	Acinetobacter (96%)	–	–	–	–
UTK062	W w/o 7	*Acinetobacter* (97%)	–	–	–	–
UTK063	W w/o 5C	*Pseudomonas* (98%)	–	–	–	–
Other Sites (n=3)						
UTK047	PWS-H20-24	*Pantoea* (98%)	–	–	–	–
UTK045	UDEL w/ 9	*Pseudomonas* (97%)	+	–	–	–
UTK057	UDEL w/o 4A	*Arthrobacter* (97%)	–	–	–	–
Control		*Pseudomonas* sp. ADP	–	+	+	+
Control		*Arthrobacter aurescens*	+	–	+	+

Note: Phylogenetic affiliations based upon most significant BLAST alignments.
Pseudomonas sp. ADP positive control for *atzA,B,C.*
Arthrobacter aurescens strain TC1 positive control for *trzN, atzB,C.*
N/A: Not available

Both UTK028B and UTK037 hold great promise for the characterization of novel atrazine-catabolic genes and enzymes.

It is interesting to note the majority of the West Tennessee enrichment culture isolates, predominantly *Arthrobacter* sp., amplified at least one or more of the catabolic genes examined. Plasmid transfer of the atrazine-catabolic genes is thought to be the leading force in the dissemination of these genes, and in a highly-enriched community like this one, the selection pressure for horizontal gene transfer is very high. Their existence after long-term enrichment may not be representative of their true nature in situ, but rather a function of horizontal gene transfer within the bead community due to the selection pressure to utilize atrazine as a nitrogen source.

It is also important to note when explaining the gene detection data that these organisms were only identified as atrazine-degraders by a loss of atrazine from the culture medium. Their degradative pathways have yet to be determined, thus, negative PCR results may simply reflect an organism with an incomplete pathway and not a novel set of catabolic genes. Phylogenetic determinations and a catabolic gene screen were used to identify the most novel of the collection first. Hopefully many in the collection have the ability to fully mineralize atrazine, but it would also not be surprising that many of them only dealkylate or dechlorinate atrazine.

Many of the known atrazine-degrading bacteria isolated from diverse locations have been shown to contain many of the upper pathway genes, so other researchers have concluded that they must be widespread and highly conserved (deSouza et al., 1998a). But others, including myself, are not convinced, especially when viewing the results of this study. The Bio-Sep® beads offer a way to cultivate organisms without some of the

traditional cultivation biases, and in this study have enabled the identification of a diverse collection of atrazine-degrading bacteria many of which represent novel species that potentially possess unique atrazine-degrading metabolic functions.

IV. Final Remarks and Future Studies

A. Final Remarks

Xenobiotics, including pesticides like atrazine, are a driving force in increasing productivity and efficiency in industry, medicine, agriculture, and beyond. If these products are cost-effective and useful they will continue to be utilized, even if they are not particularly positive inputs to the environment, and their environmental impacts are not fully understood. Embracing these realities and identifying environmental hazards drives research to more fully understand these compounds and their interactions in the environment.

In the case of atrazine, even with its ubiquitous nature in Midwestern water supplies, scientists are encouraged by the fact that microorganisms in soil hold the key for its attenuation. Learning and characterizing the natural biological attenuation of this very important herbicide may lead to remediation strategies in contaminated areas and increase our ability to predict its fate in the environment. Even though atrazine may very well be one of the most highly studied herbicides on the market, there are still many areas of uncertainty. It is an accepted fact that microorganisms play a crucial role in rapid mineralization of the herbicide, but the pathways and organisms identified and characterized as important organisms in this process continue to fail at adequately predicting its fate in the environment.

Modern molecular technologies have revealed the immense diversity in the environment, especially in soils, where diversity most certainly accounts for the great ability to drive biogeochemical cycles necessary for life on earth. Atrazine degradation may not be an exception to the rule. A critical review reveals that most of the current

collection of characterized atrazine-degrading bacteria were isolated using the same biased techniques. This understanding in addition to the lack of predictability lead to the possibility that more innovative culturing techniques in tandem with molecular methods may very well be able to lead scientists closer to identifying and characterizing microbes that more adequately reflect the population of microorganisms responsible for these process in situ and thus allow for the creation of more appropriate benchmarks or biomarkers of degradation to better predict mineralization, and hence attenuation of atrazine. The first step in this new level of understanding lies in cultivating novel organisms and was the overall objective of this study. Attempting to circumvent some of the traditional culturing biases, Bio-Sep® beads were used as an in situ enrichment technique to cultivate a very diverse and possibly metabolically novel group of atrazine-degrading organisms. This study yielded novel atrazine-degrading species in many new classes including Bacteriodes, Actinobacteria, Alpha, Beta and Gamma Proteobacteria. In addition, many of these organisms did not contain the upper-catabolic pathway genes of atrazine-degradation, suggesting the possibility of novel degradative pathways, enzymes, and genes.

B. Future Studies

The objectives of this study were only the first in a larger project still in progress to characterize atrazine degradation in soils and wetlands with culture-independent and dependent methods. The project hopes to merge the traditional cultivation-dependent techniques and culture-independent techniques to more closely characterize atrazine degradation at the community level. The other objectives of the project include using the

culture collection identified in this study to identify novel biomarkers of atrazine degradation and then use those biomarkers as environmental probes to assess atrazine biodegradation in situ. In addition, the project will use cultivation-independent measures such as denaturant gradient gel eletrophoresis, lipid analysis, microautoradiography and fluorescent in situ hybridization to characterize the atrazine-degradation on a community level in a culture-independent manner to better understand the process in situ.

The collection of atrazine-degrading organisms isolated in this study greatly expand the scope of organisms capable of atrazine degradation and hold promise for even greater discoveries involving their catabolic genes and enzymes as well as the possibility of novel degradation pathways. The first step in their characterization is to identify the metabolites of degradation pathways in these organisms. Even though complete mineralization is important for complete attenuation, it is also pertinent to identify organisms in soils which aid in the creation and subsequent accumulation of atrazine metabolites like DEA and DIA in soils. Once degradation endpoints are known isolation of the specific genes and enzymes involved can be completed.

In addition, once degradation endpoints are made for the current collection, looking back at the consortia in which it was initially isolated would be very informative. It would be interesting to see if when recombined with its original consortia, members of the group in tandem could degrade atrazine to a later end point or increase degradation rate. As organisms like to grow in groups and biofilms in nature, it would seem natural that they also work in small groups or consortia in tandem to degrade atrazine as well. If patterns in these consortia could be identified they could be used to recognize soils with the capacity to degrade atrazine.

On a different front, the organisms that did not degrade atrazine may include novel or rarely isolated groups of organisms from soil, as many of them were isolated at up to 70 days since original dilution plating. Response curves of cultivation could readily be put together for the k vs r selected populations from many of the sites used in the study.

To conclude, this project was very rewarding and yielded what seems to be a very diverse and novel group of atrazine-degrading organisms, which hopefully will leave the Radosevich lab with a plethora of different avenues of which to explore in the future.

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Vitae

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