Isolation and Identification of Possible Atrazine Degraders from Middle Tennessee Soils

By

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ABSTRACT

Organisms that were potential degraders of atrazine were tested for atrazine tolerance by inoculation onto tryptic soy atrazine agar (TSAA). The atrazine-tolerant organisms were plated onto minimal salts atrazine agar, and compared to growth on minimal salts agar. Six soil samples were taken from various farm land sites in middle Tennessee. Soils had known repeated atrazine use, but had not been sampled for atrazine degraders. Soil isolates were identified using the BBL Crystal^{TN} Identification System. Carbon dioxide (CO_2) evolution studies and atrazine enrichment studies were conducted. Six isolates and four known bacteria showed promising 5 day averages of CO_2 production, all above 20 μM of CO_2 while in the presence of atrazine. Enrichment procedures showed growth, for 5 out of 6 soil isolates with no glucose and 2.65 mL per 250 mL of medium, indicating their reliance on atrazine for growth. Soil samples taken from middle Tennessee showed organisms with atrazine tolerance and potential atrazine degradation.

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CHAPTER I

Introduction

Atrazine (2-chloro-4-ethylamino-6-isopropylamino-s-tirazine) (Figure 1) is one of the most popular herbicides in the United States and was first introduced to the market in 1958. Atrazine and other similar synthetic herbicides such as simazine have been used and studied for over 50 years (Dinamarca *et al*. 2007). These chemicals were purchased by consumers at the rate of 86,000 tons per year in the United States alone in 2001. The United States Environmental Protection Agency (U.S. EPA) reported that 76.4 million pounds of atrazine are applied annually in the United States (2013). Atrazine is widely used because it has the ability to deter the growth of broadleaf weeds and some grassy weeds by inhibiting photosynthesis. Atrazine is used with crops such as sorghum, maize, sweet corn, sugarcane, lupins, lucerne, grass seed, pasture, and potato (Vancov *et al*. 2005). In addition to these crops, atrazine is also an herbicide used extensively in forestry, in particular, to aid in the establishment of plantations, such as *Pinus radiata* and *Eucalyptus* spp. Atrazine can be applied before the planting process or after harvest and is most widely used in the midwest United States. The National Registration Authority for Agricultural and Veterinary Chemicals (2002) also reported that atrazine was starting to emerge as a much used herbicide in triazine-tolerant canola and chickpea crops as well. Of the atrazine applied in the United States, 86 % is used on field and sweet corn (U.S.

EPA 2013). It is reported that 75% of all domesticated field corn is grown with the aid of atrazine. Sorghum and sugarcane follow corn with 10% and 3%, respectively, with the remaining use divided among the remaining crops. Atrazine is widely used, partly because it is not only effective against weed varieties, but it also has a relatively low treatment cost, application flexibility, and a very low risk of damaging the crops.

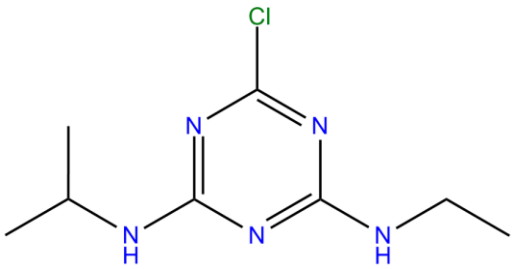

Figure 1. Atrazine: 2-chloro-4-ethylamino-6-isopropylamino-s-tirazine (National Institute of Standards and Technology [NIST])

Although atrazine is a highly selective systemic herbicide, it also has residual properties associated with its ability to bind to water (Vancov *et al.* 2005). Atrazine is considered to persist moderately in natural environments, with a half-life ranging from a few days to several months (Devers *et al*. 2003). Because of its persistence in the environment, according to the U.S. EPA (2013),

the Safe Drinking Water Act established the “Maximum Contaminant Level” in 1991 to be less than 3.0 parts per billion (ppb). The ability of atrazine to migrate off-site and contaminate ground and surface water can be perpetuated by many things. Stackelberg *et al*. (2005) showed positive correlation between the types of soil treated with the herbicide and increased atrazine levels in watersheds. In addition to some soil types being prone to allow atrazine to reach watersheds, environmental factors, such as precipitation levels, also greatly affect atrazine levels. Frequencies of high atrazine levels in watersheds were perpetuated at sites with less permeable soils. These soils increase the likelihood of atrazine reaching watersheds, decrease the ability of soil percolation, and slow the contaminated water. Various restrictions have been placed on atrazine use in order to reduce water contamination (NRA 2002). Reduced application rates have been enforced. Some members of the European Union, such as Germany, Italy, Norway and Sweden, have banned the use of atrazine completely. However, in 2006, the U.S. EPA concluded that the general population would not be harmed, because of the accumulation of atrazine, when exposed at normal levels to food products and water consumption (2013). These findings upheld the prior decision and requirements determined in the October 31, 2003, evaluation of atrazine.

Currently, atrazine toxicity levels are monitored by sampling common watersheds, and the results are classified into one of three levels: short-term

exposure, intermediate-term exposure, and long-term exposure (U.S. EPA 2013). The short-term exposure is based on, and is considered to be, a “Drinking Water Level of Concern” when a one day reading reaches 298 ppb. An intermediate-term of exposure of concern is reached when an average taken over a 90 day sampling period reaches 37.5 ppb. If a sample reading average reaches 3 ppb over a year period, this is concerning. When these limits are reached, licensed users are contacted to decrease the use of atrazine.

The U.S. EPA regulates the use of atrazine based on levels that may alter human hormones, because this is the most sensitive health effect documented (2013). Hayes *et al.* (2002) showed that atrazine levels in the environment, much lower than allowable concentrations, were affecting the hormones of frogs. He showed through laboratory testing that atrazine was able to turn tadpoles into hermaphrodites, decreasing their ability to reproduce. At levels as low as 0.1 ppb, the gonadal development of exposed larva were altered. This experiment was conducted on 30 animals and replicated 3 times. The U.S. EPA (2013), however, could not find reproducible results. The agency released a statement saying, based on negative results, the hypothesis that atrazine exposure affected the development of amphibian gonads was rejected. Endocrine effects on *Rana pipiens* (leopard frog) have been investigated in association with atrazine exposure (De Solla *et al*. 2006). Leopard frog tadpoles, twenty per tank and replicated 4 times, were exposed to atrazine at a rate of 10 ppb. At 10 ppb the

frogs exhibited a higher female to male ratio as compared to the control (De Solla *et al*. 2006). Suzawa and Ingraham showed evidence that an endocrine encoding gene, that controls expression of aromatase, was affected at 2.2 ppb (2008). Zebrafish that were exposed to increased atrazine (22 ppb) had a higher rate of females than the control. Hayes *et al.* (2011) showed statistically significant evidence that not only could atrazine demasculinize *Xenopus laevis,* but complete feminization was also recorded. After exposing the larval and post metamorphic stages of the frogs, at an atrazine concentration of 2.5 ppb, a histological section of the testes was taken. Feminization was concluded based on the observation of female oocyte development in the male testes.

In addition to the hormonal effects, other health effects have been reported. In the late 1980's researchers found a higher incidence of tumors in female Sprague-Dawley rats when exposed to high atrazine concentrations and atrazine was classified as a possible human carcinogen (U.S. EPA 2013). By early 2000, the U.S. EPA and World Health Organization's International Agency for Research on Cancer (IARC) had released a statement saying the herbicide was not likely to be a human carcinogen. Atrazine is currently classified as a group 2B carcinogen (Dehgani *et al.* 2013). Atrazine currently stands as a restricted pesticide, and one must have a license to purchase and use this chemical. Atrazine and other chlorinated triazine pesticides were scheduled for review again by the U.S. EPA in 2013 and reviews are currently being conducted (2013).

Because atrazine has been shown to persist in nature (Devers *et al*. 2003) and affect wildlife (De Solla *et al*. 2006, Hayes *et al.* 2011), faster mineralization will decrease adverse effects. Bioremediation is a process that occurs in nature, and is a way in which chemicals can be recycled. During this process, contaminants are broken down into natural and harmless compounds by organisms such as bacteria, fungi, and plants. The herbicide atrazine has potential to be a substance that can be readily degraded through the process of bioremediation. The most common methods for the degradation of atrazine occur by the biological processes of N-dealkylation, dechlorination, and ring cleavage (Struthers *et al*. 1998). N-dealkylation of atrazine is thought to be a substrate for cytochrome P-450 like monooxygenase (Rousseaux *et al*. 2001). Deethylatrazine (DEA) or deisopropylatrazine (DIA) are two of the most common metabolites of atrazine produced during degradation, which is initiated by the N-dealkylation of the ethyl or isopropyl side chains. Devers *et al*. (2003) described that atrazine can be degraded first into hydroxyatrazine, which can be hydroxylated into deethylatrazine and deisopropylatrazine. These metabolites can then be broken down further or mineralized by bacterial enzymes into carbon dioxide and ammonium.

Many organisms that have the ability to break down atrazine have been recorded (Qingyan *et al*. 2008). Examples of microbes with degradation abilities include: *Agrobacterium, Rhodococcus, Rhizobium, Pseudaminobacter,*

Chelatobacter, Aminobacter, Stenotrophomonas, Arthrobacter and *Pseudomonas.*

Agrobacterium radiobacter J14a is a Gram-negative, motile, rod-shaped, bacterium isolated from surface soil collected from an atrazine-treated corn field near Sheldon, Nebraska, which is capable of utilizing atrazine as a sole nitrogen source (Struthers *et al.* 1998). Using HPLC, *Agrobacterium radiobacter* J14a was shown to be 94 % effective at mineralization of atrazine in culture, after 72 h. *Rhodococcus* species are ubiquitous soil microbes with a history of biodegradation (Behki *et al.* 1993). *Rhodococcus* strain TE1 was originally isolated from a corn field in Brandon, Manitoba, by Tam *et al. (*1987). These strains were shown to metabolize atrazine under aerobic conditions; however, they could only catabolize atrazine into the metabolites DEA and DEIA, but no further. *Rhizobium* sp. strain PATR was isolated from agricultural fields in Doubs, France (Bouquard *et al*.1997). *Rhizobium* sp. strain PATR was shown to consume atrazine in a basal liquid medium. This organism produces a hydrolase enzyme to degrade atrazine into hydroxyatrazine by the process of dechlorination. Hydroxyatrazine was shown to accumulate in the medium, but mineralization was not shown to occur. *Pseudaminobacter* is a Gram-negative, non-motile, rod that was isolated from a farm in Ottawa, Canada (Topp *et al*. 2000). This organism also uses hydrolytic reactions in the atrazine degradation process. Researchers were able to show that dechlorination was followed by

dealkylation and if the *atzABC* genes were present, mineralization to CO_2 occurred in resting cell preparations using HPLC. In 2001, twelve soil samples were taken from various plots in France (Rousseaux *et al*. 2001). From the soil samples, *Chelatobacter heintzii*, *Aminobacter aminovorans*, *Stenotrophomonas maltophilia,* and *Arthrobacter crystallopoietes* were isolated and identified using the sequence of the 16S rRNA gene. By using radioactive labeled atrazine, it was determined that these organisms were able to degrade atrazine to some level. The Gram-negative bacteria, *Chelatobacter*, *Aminobacter*, and *Stenotrophmonas* were able to perform complete mineralization of atrazine into CO_2. In contrast, the Gram-positive *Arthrobacter* was unable to cleave the ring structure to achieve mineralization. However with further investigation, *Arthrobacter* sp. AD26 was found to catabolize atrazine as a sole nitrogen source and could degrade atrazine more efficiently than other known degraders (Qingyan *et al*. 2008). Finally, one organism that is frequently found to be an excellent tool for bioremediation is *Pseudomonas*. *Pseudomonas* sp. strain ADP contains genes that encode enzymes which allow for the complete mineralization of atrazine (Rousseaux *et al*. 2001). The atrazine catabolic genes *atzABC* have been well studied. These three genes have been shown to encode for three hydrolases that catalyze individual steps in the degradation of atrazine to cyanuric acid. These genes are highly conserved among Gram-negative bacteria and are located on a large plasmid, pADP1. This plasmid is self-transmissible to Gram-negative bacteria. The *atzA* gene of *Pseudomonas* sp. strain ADP codes

for the enzyme atrazine chlorohydrolase which breaks atrazine down to hydroxyatrazine (Devers *et al*. 2003). The *atzB* gene codes for the enzyme hydroxyatrazine hydrolase that breaks hydroxyatrazine down to N-isopropylammelide. The *atzC* gene encodes the enzyme N-isoproplyammelide isopropylamidohydrolase which converts N-isoproplyammelide to cyanuric acid. The *atzD* gene encodes the enzyme cyanuric acid amidohydrolase, which cleaves the ring structure of cyanuric acid creating biuret. This is further broken down by a product of *atzE*, biuret hydrolase. The gene *atzF* encodes allophanate hydrolase, which breaks allophanate into CO_2 + NH_4^+. *Pseudomonas* ADP has been shown to up-regulate expression of atrazine degrading genes in the presence of atrazine (Devers *et al*. 2003). More genes have been documented in other *Pseudomonas* species as well. The *trzD* gene was found in *Pseudomonas* NRRLB-12227 (Karns 1999) and encodes an enzyme that degrades an atrazine metabolite. The *trzD* gene has been shown to code for the cleavage of cyanuric acid. By finding organisms that have the ability to express these genes, which degrade atrazine and atrazine metabolites, the process of bioremediation can be improved.

Objective

The ability for microorganisms to evolve is well documented (Noor *et al*. 2012). Due to their ability to adapt and to share genetic information, the discovery of new strains capable of degrading atrazine is valid. Bioremediation of

atrazine by soil microorganisms has not been fully exploited in regards to the application of the herbicide in middle Tennessee. The soils of middle Tennessee farmlands have not, in known literature, been sampled or explored. The objective of this experiment was to identify atrazine degrading bacteria in middle Tennessee soils that had been exposed to the herbicide, but not yet examined, and to determine the extent to which bacteria are able to degrade atrazine.

CHAPTER II

Materials and Methods

Media Preparation

In order to test the toxicity of atrazine, known bacterial isolates were plated onto tryptic soy agar (TSA) with atrazine which will be referred to as tryptic soy atrazine agar (TSAA). Tryptic soy agar was prepared by heating 20 g of TSA from Difco Laboratories (Detroit, MI) with 450 mL of distilled water until clear in a 1000 mL Erlenmeyer flask covered with aluminum foil. The solution was then sterilized in an autoclave at 121 C and 15 psi for 15 min. The solution was placed in a 55 C water bath until the atrazine was prepared. Atrazine was derived from Sipcam Agro USA, Inc. (Roswell, GA) and contained 41.9 % active ingredient, 1.1 % related compounds, and 57.0% other ingredients per gallon. Atrazine was prepared by placing 25 mL of atrazine in each of two 50 mL tubes for a total of 50 mL of atrazine and boiling for 10 minutes. After the atrazine was shaken by hand, it was added to the TSA. The atrazine and TSA mixture was placed onto a heating plate with a stir rod for mixing and poured into petri dishes. After cooling, the plates were inoculated. The growth on many, but not all, plates indicated that the concentration was selective, but not toxic, for all tested organisms. This process was replicated with 35 known bacterial and fungal strains.

Minimal salts atrazine agar (MSAA) was prepared by heating until clear 5.3 g of minimal salts broth without dextrose, 7.5 g of Bacto agar, 5 mL glycerol, 0.003 g of phenol red, and 450 mL of distilled water in an Erlenmeyer flask covered with aluminum foil. Atrazine preparations, sterilization procedures, and plating were followed as above. MSAA plates were inoculated with organisms that were able to grow on TSAA.

Minimal salt agar (MSA) was prepared as above replacing 50 mL of atrazine and with 50 mL of distilled water. The MSA plates were inoculated with the organisms that had growth on the MSAA. All isolates were incubated at room temperature for five days.

MSA and MSAA, were prepared as above with the exclusion of 5 mL of glycerol, were used to determine if glycerol. This exclusion was performed to determine if glycerol was a possible energy source.

Minimal salts broth (MSB) was prepared using 500 mL of deionized water, 5.3 g of minimal broth without dextrose from Difco laboratories (Detroit MI) and 0.003 g of phenol red indicator. Minimal salts atrazine broth (MSAB) was prepared using 450 mL of deionized water, 5.3 g of minimal broth without dextrose and 0.003 g of phenol red indicator. Culture tubes (16×125 mm) were filled with 5 mL of the MSB. Twenty five mL Erlenmeyer flasks (50 mL) were filled with 20 mL of MSB. After sterilization, 50 μL of boiled atrazine was added to the

culture tubes, while 200 μL was added to the Erlenmeyer flasks. Both the culture tubes and the Erlenmeyer flasks were placed on a vortexer for 15 s to homogenize the solution.

MSAA agar, excluding phenol red, was prepared using special cleaning procedures for the glassware. Glassware was cleaned, and then rinsed 3 times with deionized water, to reduce extraneous organics.

Soil Samples

Soil samples were collected using a three inch trowel while avoiding foliage and rocks. Each sample of approximately 200 g was placed in a plastic bag at 4 C until analyzed. All samples were taken from farms with a corn/soybean rotation and known to have been exposed to atrazine within the year of sampling. Sample one was taken from Bonner Farms in Viola, TN. $35^0$32’28.39” N $85^0$52’14.55 W. Sample two was taken from McColloch and Sons Farm in Coffee County, TN: $35^0$30’54.63” N $85^0$53’51.68” W. Sample three was taken from Dewayne Lusk Farm in Coffee County, TN: $35^0$29’22.07” N $85^0$53’17.69” W. Sample four was taken from St. John Farm in Warren County, TN: 35^0 32’55.17” N 85^0 54’32.73” W. The fifth sample was taken from Helton Farms in Warren County, TN: $35^0$33’11.75” N $85^0$53’38.87” W. The sixth sample was taken from Shangri La Lane McMinnville, TN: $35^0$40’26.39” N $85^0$49’46.98” W.

Soil Dilutions

For each soil sample, 10g of soil were diluted with 90 mL of deionized water, then shaken by hand. A volume of 1 mL of the slurry was removed and added to 9 mL of deionized water. A total of five 1:10 serial dilutions were performed. A 0.1 mL aliquot of each sample was plated onto MSAA and spread using a sterilized bent glass rod. Plates were incubated at room temperature (25^0C). Colonies with differing morphology were chosen and plated onto TSA for identification.

Gram Stain and KOH

In order to ensure proper identification using the BBL Crystal Identification method, Gram stains and KOH procedures were performed. The Gram stain procedure was performed by taking a sample using a heat sterilized loop from a culture incubated for 24 hour and smearing onto a glass slide that contained one loop full of water. Once the thin smear was dry and heat fixed, the smear was flooded with crystal violet and allowed to sit for one minute and then rinsed with water. Next, the same steps were performed using Gram's iodine. Furthermore, 95 % (v/v) ethyl alcohol was applied for 5 s and then rinsed with water. Finally the slide was flooded with the counterstain, safranin, for 1 min, rinsed with water, blotted dry and viewed under the microscope for the Gram stain results.

The KOH test was performed by preparing a 3 % solution of potassium hydroxide (Frea *et al*. 1969). A 3 g amount of KOH was dissolved into 100 mL of distilled water. Next a sample from a 24 h old culture was taken with a heat sterilized loop and agitated in a loop full of KOH on a glass slide. If the bacteria created a string-like or mucoid consistency, the bacteria were labeled Gram-negative. If there was no consistency change after thirty seconds, the bacteria were presumed Gram-positive.

BBL Crystal^{TN} Identification

After the Gram stain procedure and KOH tests were performed to confirm the Gram reaction, the organisms were then further investigated using the BBL Crystal^{TN} Identification System (Becton Dickinson and Company, Sparks, MD). The Gram-negative organisms were inoculated using the BBL Crystal^{TN} Enteric/Nonfermenter Identification Systems. Alternatively, the Gram-positive bacteria were inoculated into the BBL Crystal^{TN} Gram-positive Identification Systems. Each organism was inoculated aseptically with a nonpolyester swab into the BBL inoculating fluid and agitated with a vortexer for 10 s until the suspension was slightly cloudy. The cloudy liquid was then poured into the BBL crystal tray and a rolling motion was applied to ensure saturation of all biochemical wells with any excess rotated back to the target area of the base. The lid was placed on the base with the labeled end aligned with the target area. Inoculated BBL trays were incubated, label down, at 37 C for 24 h in an incubator

with 40 to 60 % humidity. Results were read using the BBL Crystal Panel Viewer. The interpreted results were then entered into the BBL Crystal ID System Electronic Codebook for scientific name identification (Version 4.0, Copyright 1996).

Titration and CO_2 Evolution

With some modifications, titrations for CO_2 evolution were performed following the protocol of Atlas *et al*. (1988). The process of measuring CO_2 evolution was further validated by the atrazine mineralization process of *Stenotrophomonas* (Rousseaux *et al*. 2001). Before CO_2 evolution was measured, all growth media and incubation times were standardized (Figure 2). All isolates were grown on TSAA for 48 h at room temperature. Isolated colonies from each soil sample that grew on TSAA were then inoculated in the MSAB culture tubes for 48 h at room temperature. From the MSAB culture tubes, 0.1 mL from each culture was, once again, inoculated for 48 h in fresh MSAB to ensure there were no remaining nutrients from the TSAA.

Once the standardization of inoculation was complete, 0.1mL from each of the 48 h MSAB culture tubes was inoculated into 50 mL Erlenmeyer flasks, more specifically known as Kontes incubation flasks (Figure 3), containing MSAB prepared as described above. Once the Kontes incubation flasks (50 mL) were inoculated, plastic wells from Kontes Scientific Glassware Instruments (Vineland

NJ) were filled with 0.5 mL of 0.1 M KOH, placed in the stopper attachment and the stopper placed on the flasks.

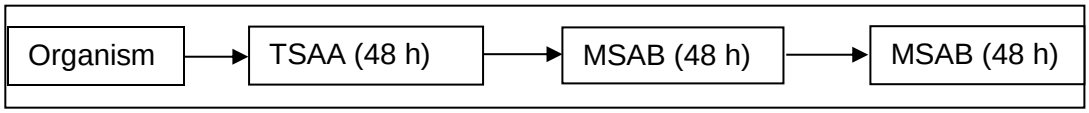

Figure 2. Standardized inoculation and incubation process to prepare for CO_2 evolution measurements.

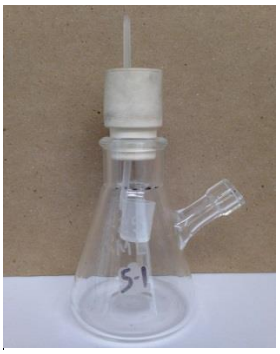

Figure 3. Kontes incubation flasks.

Three flasks were established as controls. One flask that served as a control did not receive bacterial inoculum. The second and third controls, inoculated with *Bacillus subtilis* and *Staphylococcus aureus*, served as negative controls because they did not grow on TSAA and, therefore, did not tolerate

atrazine. All flasks were incubated at 25 C for a total of 5 days. At 24 hour intervals, the 0.1 M KOH was removed and placed into a sterile tube. Fresh KOH solution was added to the wells and the flasks again were topped with a stopper. The KOH that had been allowed to incubate for 24 h was then mixed with 50 μL of saturated barium chloride and 5 μL of 1 % (w/v) phenolphthalein, a pH indicator. Then, 0.05 M HCl was added until neutral pH was obtained. The amount of CO_2 produced by the microbes was determined by calculating the difference in the volume of HCl required for the uninoculated control as compared to the samples. The difference was then multiplied by 25. Growth and measurement procedures were repeated for each sample five times.

Enrichment Procedures

Enrichment procedures were performed using decreasing levels of glucose and increasing levels of atrazine added to the growth medium. MSAB was made using 250 mL ultra-pure deionized water, 2.65 g minimal salts broth without dextrose, and 0.60 g glucose. Water and dry components were combined and heated to dissolve before sterilization using an autoclave. After cooling, 0.25 mL of boiled (10 min) atrazine was added and stirred. Twenty mL of MSAB was added to borosilicate glass disposable culture tubes (16 x 150 mm) and capped. Next, 0.20 g of soil was inoculated into media excluding the control. After growth was detected using a spectrophotometer at an absorbance of 600 nm, 1mL of the sample was inoculated into fresh medium with increasing atrazine and

decreasing glucose. Atrazine was increased in 0.20 mL increments while glucose was decreased in 0.05 g increments until the final medium contained no glucose and 2.65 mL of atrazine. MSAA was prepared and sterilized. A 2.65 mL volume of sterilized atrazine was stirred into MSAA (250 mL) and poured into petri dishes. To confirm growth of the organisms as detected by spectrophotometry, MSAA plates were inoculated with 0.10 mL that was taken from MSAB that contained no glucose and 2.65 mL of atrazine. After inoculation and incubation, positive or negative growth was observed.

CHAPTER III

Results

Known organisms were initially utilized in order to test for toxicity of atrazine. Of the 35 known organisms inoculated onto TSAA, 21 grew in the presence of atrazine. *Enterococcus faecalis* grew, but in a very limited way. The 14 remaining organisms were unable to grow in the presence of atrazine and were discarded as atrazine-sensitive and, therefore, not able to degrade atrazine (Table 1).

In order to gain a broader understanding of the growth requirements of atrazine-tolerant degraders, growth of 21 organisms was compared on MSA with and without atrazine (Table 2). Nine organisms that were able to grow on TSAA did not grow on either MSAA or MSA. Twelve organisms were able to grow on MSAA. Three organisms showed better growth on MSAA than MSA. *Enterococcus faecalis* had limited growth on MSAA, but was unable to grow on MSA. *Rhodotorula rubra* had growth on MSAA and very limited growth on MSA. *Proteus mirabilis* was the only organism that grew on MSAA, but was unable to grow on MSA. Since the only difference between MSAA and MSA was the addition of atrazine, this implies a possibility that the atrazine was providing essential nutrients *Enterococcus faecalis*, *Rhodotorula rubra*, and *Proteus mirabilis.*

Table 1. Growth of known organisms in the presence of atrazine on TSAA

Organism	Growth on TSAA	*Phenol Red Indication Result
Alcaligenes faecalis	+	Alkaline
Aspergillus bisc	+	Acidic
ARSA	-	
Bacillus pumilis	-	
Bacillus sphaericus	-	
Bacillus subtilis	-	
Bacillus subtilis strR	-	
Bacillus subtilis strS	-	
Bacillus thuringiensis	-	
Escherichia coli B	+	Alkaline
Escherichia coli wt	+	Acidic
Escherichia coli C606	+	Alkaline
Escherichia coli 3104	+	Alkaline
Enterobacter aerogenes	-	
Enterococcus faecalis	+	Acidic
Klebsiella pneumoniae	+	Acidic
Micrococcus luteus	-	
Morganella morganii	+	Alkaline
Mycobacterium smegmatis	-	
Penicillium notatum	+	Alkaline
Providencia rettgeri	+	Alkaline
Proteus mirabilis	+	Alkaline
Pseudomonas aeruginosa	+	Alkaline
Pseudomonas fluorescens	+	Alkaline
Pseudomonas putida	+	Alkaline
Rhizopus stolonifer	-	
Rhodotorula rubra	+	Alkaline
Saccharomyces cerevisiae	+	Acidic
Salmonella enteriditis	+	Alkaline
Serratia marcescens	+	Acidic
Shigella sonnei	+	Alkaline
Staphylococcus aureus	-	
Staphylococcus epidermidis	-	
Staphylococcus simuleus	+	Alkaline
Streptomyces griseus	-	

* Indicates color change to the acidic or alkaline indicator color following growth.

Table 2. Organism growth on MSAA as compared to growth on MSA.

Organism	Growth on MSAA	Growth on MSA	*Phenol Red Indication Result
Alcaligenes faecalis	-	-	
Aspergillus bisc	+	+	No Color Change
E. coli 3104	-	-	
Escherichia coli B	+	+	Acidic
Escherichia coli C606	-	-	
Enterococcus faecalis	+	-	No Color Change
Escherichia coli wt	+	+	Acidic
Klebsiella pneumoniae	+	+	Acidic
Morganella morganii	-	-	
Penicillium notatum	-	-	
Proteus mirabilis	+	-	No Color Change
Providencia rettgeri	-	-	
Pseudomonas aeruginosa	+	+	Slightly Acidic
Pseudomonas fluorescens	+	+	Slightly Acidic
Pseudomonas putida	+	+	No Color Change
Rhodotorula rubra	+	+	No Color Change
Saccharomyces cerevisiae	-	-	
Salmonella enteriditis	+	+	No Color Change
Serratia marcescens	+	+	Acidic
Shigella sonnei	-	-	
Staphylococcus simuleus	-	-	

* Indicates color change to the acidic or alkaline indicator color following growth.

Glycerol can be used as an energy source, reducing the need of the organisms to utilize atrazine as a food source, so growth in medium lacking glycerol was determined useful. To compare the ability of the organisms to grow on the two agars, MSAA without glycerol and MSA without glycerol, an isolation streak technique utilizing 4 quadrants of the plate was used and growth was

compared at day 7 (Table 3). *Escherichia coli* B, *Enterococcus faecalis*, and *Proteus mirabilis* grew on MSAA without glycerol but did not grow on MSA without glycerol. *Escherichia coli* wt, *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Serratia marcescens*, and *Pseudomonas putida* grew better on MSAA without glycerol than MSA without glycerol. The remaining four organisms showed no growth difference between the MSAA without glycerol and the MSA without glycerol. Since the glycerol negatively affected the growth of the organisms, all minimal salts media for the remainder did not include glycerol.

Table 3. Organism growth on MSAA without glycerol as compared to growth on MSA without glycerol.

Organism	*Quadrant Growth	*Quadrant Growth
Aspergillus bisc	4+	4+
Eschericia coli B	4+	No growth
Enterococcus faecalis	2+	No growth
Eschericia coli wt	4+	2+
Klebsiella pneumoniae	2+	2+
Proteus mirabilis	3+	No growth
Pseudomonas aeruginosa	4+	2+
Pseudomonas fluorescens	4+	2+
Pseudomonas putida	4+	2+
Rhodotorula rubra	2+	2+
Salmonella enteriditis	4+	4+
Serratia marcescens	4+	3+

*Quadrant growth refers to whether organisms grew on the first (1+), second (2+), third (3+), or fourth (4+) quadrants.

To identify additional atrazine-tolerant organisms that are capable of degrading atrazine, six soil samples from middle Tennessee were collected. For each of the six soil samples taken from farms with a corn/soybean rotation and known atrazine exposure with the ability to grow on MSAA, colonies with differing colony morphology were chosen and inoculated onto TSA for identification. Thirteen organisms were identified with differing confidence levels. *Sphingomonas paucimobilis* had a very low confidence level of only 13.3% additional tests for confirmation (Table 4).

To further test the degradation of atrazine by known and isolated microorganisms, the amount of CO_2 produced by the microbes was determined (Table 5). By removing glycerol and taking measure to have clean glassware, extraneous carbon was greatly reduced. Therefore, the CO_2 being produced is most likely being produced by the organisms breaking down and utilizing atrazine as a Carbon source. This type of CO_2 production, in association with atrazine degraders, has been previously documented (Rousseaux *et al.* 2001) increasing the validity of this test. By calculating the difference in the volume of HCl (mL) required for the control as compared to the samples, CO_2 evolution was evaluated. The difference was then multiplied by 25. This procedure was repeated for each sample five times (Table 6).

Table 4. BBL Crystal^{TN} Identification System Results for organisms isolated from middle Tennessee soils.

*Identification Number	Organism Name	Confidence Interval
1.3.1	Miscellaneous Gram Negative *Bacillus*	90.0%
1.3.3	*Bacillus sphaericus*	79.0%
2.3.1	*Pseudomonas aeruginosa*	96.0%
2.3.3	*Sphingomonas paucimobilis*	**13.5%
3.3	*Bacillus cereus*	99.0%
4.3.1	*Pseudomonas fluorescens*	63.6%
4.3.2	Miscellaneous Gram Negative *Bacilli*	83.5%
4.4.2	*Agrobacterium tumefaciens*	98.5%
5.3.2	*Pseudomonas aeruginosa*	84.4%
5.3.3	*Klebsiella pneumoniae*	98.0%
5.4.3	*Pseudomonas putida*	95.0%
5.5	*Cornebacterium aquaticum*	99.0%
6.2	*Stenotrophomonas maltophilia*	94.2%

*Assigned numbers based on soil location, plate sample was taken from and number assigned to colonies on the same plate.
**Additional tests: Motility, Nitrate and Oxidase were performed to further support the findings of the organism's original identification.

Table 5. HCl added until pH change takes place indicating CO_2 evolution.

Inoculum	**HCl mL added**	**HCl mL added**	**HCl mL added**	**HCl mL added**	**HCl mL added**
	Day 1	**Day 2**	**Day 3**	**Day 4**	**Day 5**
C1 Uninoculated control	1.50	1.50	1.50	1.50	1.50
	1.60	1.50	1.50	1.50	1.50
	1.50	1.50	1.50	1.40	1.45
	0.57	0.90	1.00	1.00	1.00
	0.64	0.90	0.90	0.90	0.90
C2 *Bacillus subtilis*	1.50	1.50	1.50	1.50	1.50
	1.50	1.50	1.50	1.50	1.50
	1.50	1.50	1.40	1.40	1.45
	0.35	1.00	1.00	1.00	1.00
	0.90	0.90	0.90	0.90	1.00
C3 *Staphylococcus aureus*	1.40	1.40	1.50	1.50	1.40
	1.50	1.50	1.45	1.50	1.50
	1.40	1.40	1.40	1.45	1.45
	0.41	0.90	0.90	0.90	0.90
	0.36	1.00	0.90	1.00	1.00
1.2.1 *Fusarium*	1.34	1.45	1.40	1.45	1.45
	1.45	1.40	1.00	0.00	0.00
	1.40	1.33	1.34	1.23	1.27
	0.36	0.86	0.87	0.80	0.80
	0.31	0.90	0.87	0.90	0.90
1.3.1 Miscellaneous Gram Negative *Bacilli*	1.35	0.63	0.00	0.00	0.00
	1.45	0.86	0.20	0.00	0.00
	1.40	0.40	0.00	0.20	0.40
	0.32	0.41	0.39	0.39	0.40
	0.80	0.23	0.06	0.16	0.00

Table 5. HCl added until pH change takes place indicating CO2 evolution. (cont.)

Inoculum	**HCl mL added**	**HCl mL added**	**HCl mL added**	**HCl mL added**	**HCl mL added**
	Day 1	**Day 2**	**Day 3**	**Day 4**	**Day 5**
1.3.3 *Bacillus sphaericus*	1.20	1.45	1.40	1.42	1.35
	1.45	1.38	1.32	0.00	0.00
	1.45	1.45	1.45	1.50	1.42
	0.90	0.90	0.92	0.90	0.90
	0.47	0.25	0.23	0.26	0.25
2.3.1 *Pseudomonas aeruginosa*	1.14	0.80	0.00	0.00	0.00
	1.45	1.10	0.44	0.34	0.17
	1.35	0.40	0.10	0.06	0.16
	0.87	0.90	1.00	1.00	0.90
	0.35	0.85	1.00	1.00	0.90
2.3.3 *Sphingomonas paucimobilis*	1.00	1.14	0.65	0.55	0.30
	1.50	1.39	1.38	1.35	1.38
	1.50	1.36	1.24	1.46	1.42
	0.55	0.90	0.88	0.90	0.90
	0.25	0.90	0.86	1.00	0.90
3.3 *Bacillus cereus*	1.40	1.46	1.45	1.45	1.35
	1.50	1.25	1.30	1.15	1.27
	1.50	1.33	1.25	1.26	1.21
	0.67	0.87	0.80	0.60	0.69
	0.47	0.81	0.77	0.60	0.62
3.4 *Penicillium*	1.24	1.35	1.40	1.35	1.30
	1.00	1.60	1.50	1.45	1.46
	1.50	1.45	1.42	1.50	1.38
	0.40	0.90	0.90	1.00	0.90
	0.27	0.86	1.00	1.00	0.90

Table 5. HCl added until pH change takes place indicating CO2 evolution. (cont.)

Inoculum	**HCl mL added**	**HCl mL added**	**HCl mL added**	**HCl mL added**	**HCl mL added**
	Day 1	**Day 2**	**Day 3**	**Day 4**	**Day 5**
4.3.1 *Pseudomonas fluorescens*	1.00	0.00	0.00	0.00	0.00
	0.30	0.00	0.00	0.00	0.00
	0.85	0.00	0.00	0.00	0.00
	0.15	0.00	0.00	0.00	0.00
	0.23	0.00	0.00	0.02	0.00
4.3.2. Miscellaneous Gram Negative *Bacillus*	1.05	0.35	0.00	0.00	0.00
	0.30	0.54	0.38	0.40	0.18
	1.30	0.35	0.00	0.19	0.25
	0.80	0.25	0.17	0.20	0.00
	0.30	0.22	0.30	0.20	0.00
4.4.2. *Agrobacterium tumefaciens*	1.30	1.40	1.30	1.30	0.00
	1.45	1.32	1.35	1.44	1.35
	1.43	1.30	1.44	1.50	1.44
	0.53	0.33	0.27	0.23	0.20
	0.13	0.80	0.87	0.87	0.87
5.2 *Fusarium*	1.30	1.40	1.20	1.00	0.00
	1.45	1.33	1.35	1.23	0.00
	1.45	1.37	1.50	1.45	1.40
	0.45	0.85	0.90	0.90	0.90
	0.70	0.80	0.47	0.50	0.40
5.3.2 *Pseudomonas aeruginosa*	0.54	0.00	0.00	0.00	0.00
	0.40	0.00	0.00	0.00	0.00
	0.00	0.00	0.00	0.00	0.00
	0.30	0.00	0.00	0.00	0.00
	0.25	0.00	0.00	0.02	0.02

Table 5. HCl added until pH change takes place indicating CO2 evolution. (cont.)

Inoculum	**HCl mL added**	**HCl mL added**	**HCl mL added**	**HCl mL added**	**HCl mL added**
	Day 1	**Day 2**	**Day 3**	**Day 4**	**Day 5**
5.3.3 *Klebsiella pneumoniae*	1.24	1.40	1.20	1.30	0.00
	1.33	0.00	0.00	0.00	0.00
	1.35	0.00	0.00	0.00	0.00
	0.53	0.50	0.40	0.38	0.35
	0.25	0.55	0.90	0.90	0.90
5.4.3. *Pseudomonas putida*	1.30	0.00	0.00	0.00	0.00
	0.80	0.00	0.00	0.00	0.00
	1.25	0.00	0.00	0.00	0.00
	0.37	0.00	0.00	0.00	0.00
	0.26	0.12	0.11	0.20	0.08
5.5 *Cornebacterium aquaticum*	1.40	1.50	1.45	1.45	1.45
	1.33	0.00	0.00	0.00	0.00
	1.42	0.00	0.00	0.00	0.16
	0.37	0.90	0.90	0.90	0.85
	0.62	0.64	0.52	0.45	0.43
6.2 *Stenotrophomonas maltophilia*	0.90	0.35	0.00	0.00	0.00
	1.20	0.25	0.00	0.00	0.00
	1.00	0.00	0.00	0.00	0.00
	0.95	0.90	0.90	0.90	0.90
	0.47	0.34	0.34	0.35	0.28
K1 *Aspergillus bisc*	1.00	0.90	0.00	0.00	0.00
	1.45	1.00	0.00	0.00	0.00
	1.50	1.38	0.00	0.00	0.00
	0.42	0.83	0.85	0.85	0.90
	0.90	0.90	0.90	0.90	0.90

Table 5. HCl added until pH change takes place indicating CO2 evolution. (cont.)

Inoculum	**HCl mL added**	**HCl mL added**	**HCl mL added**	**HCl mL added**	**HCl mL added**
	Day 1	**Day 2**	**Day 3**	**Day 4**	**Day 5**
K2 *Escherichia coli* B	1.21	1.30	1.10	0.10	0.00
	1.30	1.30	1.30	1.30	1.30
	1.50	1.36	0.00	0.00	0.00
	0.63	0.64	0.68	0.59	0.58
	0.55	0.46	0.50	0.50	0.40
K3 *Escherichia coli* wt	1.14	1.10	1.00	0.70	0.10
	1.40	1.36	1.40	1.40	1.00
	1.50	1.45	1.50	1.50	1.50
	0.38	0.87	0.92	0.90	1.00
	0.43	0.30	0.18	0.38	0.24
K4 *Klebsiella pneumoniae*	1.40	0.70	0.50	0.00	0.00
	1.30	0.00	0.00	0.00	0.00
	1.30	0.00	0.00	0.00	0.00
	0.77	0.00	0.00	0.02	0.39
	0.68	0.00	0.00	0.10	0.20
K5 *Rhodotorula rubra*	0.75	1.00	0.00	0.00	0.00
	1.45	1.38	1.36	1.36	1.36
	1.45	1.33	1.45	1.35	0.84
	0.44	0.85	0.90	0.90	0.85
	0.27	0.90	0.90	0.90	0.90
K6 *Proteus mirabilis*	1.40	1.40	1.35	1.30	1.25
	1.30	1.10	0.60	0.27	0.60
	1.45	1.14	0.76	0.60	0.55
	0.37	0.76	0.75	0.67	0.55
	0.56	0.45	0.21	0.20	0.20

Table 5. HCl added until pH change takes place indicating CO2 evolution. (cont.)

Inoculum	**HCl mL added**	**HCl mL added**	**HCl mL added**	**HCl mL added**	**HCl mL added**
	Day 1	**Day 2**	**Day 3**	**Day 4**	**Day 5**
K7 *Pseudomonas aeruginosa*	1.30	0.00	0.00	0.00	0.00
	1.30	0.00	0.00	0.00	0.00
	1.30	0.00	0.00	0.00	0.00
	0.20	0.00	0.00	0.00	0.00
	0.53	0.00	0.00	0.21	0.20
K8 *Pseudomonas fluorescens*	1.25	0.00	0.00	0.00	0.00
	1.00	0.00	0.00	0.00	0.00
	1.30	0.00	0.00	0.00	0.00
	0.12	0.00	0.20	0.20	0.30
	0.30	0.00	0.00	0.00	0.00
K9 *Pseudomonas putida*	1.22	0.00	0.00	0.00	0.00
	0.90	0.00	0.00	0.00	0.00
	0.90	0.00	0.00	0.00	0.00
	0.56	0.00	0.00	0.02	0.30
	0.18	0.00	0.00	0.27	0.20
K10 *Serratia marcescens*	1.30	0.00	0.00	0.00	0.00
	1.34	0.00	0.00	0.00	0.00
	1.30	0.00	0.00	0.00	0.00
	0.13	0.00	0.00	0.00	0.00
	0.28	0.00	0.00	0.00	0.00

Table 5. HCl added until pH change takes place indicating CO2 evolution. (cont.)

Inoculum	**HCl mL added**	**HCl mL added**	**HCl mL added**	**HCl mL added**	**HCl mL added**
	Day 1	**Day 2**	**Day 3**	**Day 4**	**Day 5**
K11*Enteroococcus faecalis*	1.30	1.40	1.35	0.00	0.00
	1.40	1.40	1.45	1.45	1.35
	1.50	1.30	0.00	0.00	0.00
	1.00	0.90	0.90	0.90	0.90
	0.90	0.90	0.90	1.00	1.00

*C=Control
**Numbers assigned based on soil location, dilution tube used and colonies isolated from each.
***K=Known organism not from soil samples

The average μmoles of CO_2 of all five days was calculated (Table 6). *Pseudomonas fluorescens*, Miscellaneous Gram Negative *Bacillus*, *Pseudomonas aeruginosa*, *Pseudomonas putida*, *Stenotrophomonas maltophilia*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* (K7), *Pseudomonas fluorescens* (K8), *Pseudomonas putida* (K9) and *Serratia marcescens* (K10) showed promising CO_2 production, all above 20 μmoles of CO_2 while in the presence of atrazine. The isolate *Pseudomonas aeruginosa* (2.3.1) from soil sample 2 showed less CO_2 production than the known organism *Pseudomonas aeruginosa* (K7), the isolate from soil sample 5 (5.3.2) produced 3.51 more μmoles of CO_2 on average after 5 days of observation. The isolate from soil

sample 4 *Pseudomonas fluorescens* (4.3.1) produced an average of 28.61 μmoles of CO_2, while *Pseudomonas fluorescens* (K8) produced 26.49 μmoles of CO_2 for a difference of 2.12 μmoles of CO_2. From soil sample 5 *Klebsiella pneumoniae* (5.3.3) produced on average 10.55 μmoles of CO_2 less than *Klebsiella pneumonia* (K4). The isolate from soil sample 5 *Pseudomonas putida* (5.4.3) produced an average of 26.67 μmoles of CO_2, while *Pseudomonas putida* (K9) produced 26.1 μmoles of CO_2 for a difference of 0.06 μmoles of CO_2. A correlation can be drawn to the increased rates of CO_2 production by the organisms and the utilization of atrazine, since atrazine is the only known source of Carbon in the growth media.

Enrichment procedures were performed using decreasing levels of glucose and increasing levels of atrazine added to MSAB. After growth was seen using a spectrophotometer at an absorbance of 600 nm, atrazine was increased in 0.20 mL increments, while glucose was decreased in 0.05 g increments until the final medium contained no glucose and 2.65 mL of atrazine (Table 7). Although there was no sample showing growth after 24 h, at 48 h, 5 out of 6 samples showed growth at zero glucose and an atrazine level of 2.65 mL.

Table 6. Total average CO_2 evolution over a 5 day period

Inoculum/Organism	μmoles CO_2
C1 Uninoculated control	0
C2 *Bacillus subtilis*	-0.04
C3 *Staphylococcus aureus*	1.14
1.2.1 Fusarium	6.08
1.3.1 Miscellaneous Gram Negative *Bacillus*	-9.39
1.3.3 *Bacillus sphaericus*	6.94
2.3.1 *Pseudomonas aeruginosa*	14.88
2.3.3 *Sphingomonas paucimobilis*	5.5
3.3 *Bacillus cereus*	4.13
3.4 *Penicillium*	2.13
4.3 1 *Pseudomonas fluorescens*	28.61
4.3.2 Miscellaneous Gram Negative *Bacillus*	23.43
4.4.2 *Agrobacterium tumefaciens*	6.74
5.2 *Fusarium*	6.86
5.3.2 *Pseudomonas aeruginosa*	29.63
5.3.3 *Klebsiella pneumoniae*	13.25
5.4.3 *Pseudomonas putida*	26.67
5.5 Cornebacterium aquaticum	14.42
6.2 *Stenotrophomonas maltophilia*	21.13
K1 *Aspergillus bisc*	15.58
K2 *Escherichia coli* B	12.56
K3 *Escherichia coli* wt	7.51
K4 *Klebsiella pneumoniae*	23.80
K5 *Rhodotorula rubra*	8.27
K6 *Proteus mirabilis*	11.37
K7 *Pseudomonas aeruginosa*	26.12
K8 *Pseudomonas fluorescens*	26.49
K9 *Pseudomonas putida*	26.61
K10 *Serratia marcescens*	26.81
K11 *Enterococcus faecalis*	7.96

Absorbance levels of 0.02, 0.12, 0.16, 0.00, 0.27, and 0.25 were recorded and growth was confirmed by inoculation onto a MSAA. There was no growth on MSAA of the samples with absorbance levels of zero or below.

Table 7. Absorbance at 600 nm 24, 48, and 72 h for isolates from middle Tennessee soils that were exposed to decreasing glucose/increasing atrazine levels.

Location	glucose (g) /250 mL H20	Atrazine (mL)/250 mL H20	A_{600} at 24 h	48 h	72 h
Bonner	0.60	0.25	0.88	n.d.	n.d.
McColloch			0.09	n.d.	n.d.
Helton			0.08	n.d.	n.d.
Dewayne			0.06	n.d.	n.d.
Shangri La			0.57	n.d.	n.d.
St John			0.35	n.d.	n.d.
Bonner	0.55	0.45	0.01	n.d.	n.d.
McColloch			0.15	n.d.	n.d.
Helton			0.01	n.d.	n.d.
Dewayne			0.17	n.d.	n.d.
Shangri La			0.30	n.d.	n.d.
St John			0.09	n.d.	n.d.

Table 7. Absorbance at 600 nm 24, 48, and 72 h for isolates from middle Tennessee soils that were exposed to decreasing glucose/increasing atrazine levels. (cont.)

Location	glucose (g) /250 mL H20	Atrazine (mL)/250 mL H20	A_{600} at 24 h	48 h	72 h
Bonner	0.50	0.65	0.25	n.d.	n.d.
McColloch			0.33	n.d.	n.d.
Helton			0.32	n.d.	n.d.
Dewayne			0.26	n.d.	n.d.
Shangri La			0.39	n.d.	n.d.
St John			0.35	n.d.	n.d.
Bonner	0.45	0.85	0.09	0.04	n.d.
McColloch			0.02	0.00	n.d.
Helton			0.12	0.01	n.d.
Dewayne			0.20	0.00	n.d.
Shangri La			0.20	0.20	n.d.
St John			0.05	0.05	n.d.
Bonner	0.40	1.05	-0.09	-0.07	-0.28
McColloch			-0.16	-0.08	-0.08
Helton			-0.16	-0.22	-0.13
Dewayne			-0.15	-0.03	-0.27
Shangri La			-0.01	-0.04	-0.11
St John			-0.14	-0.20	-0.12

Table 7. Absorbance at 600 nm 24, 48, and 72 h for isolates from middle Tennessee soils that were exposed to decreasing glucose/increasing atrazine levels. (cont.)

Location	glucose (g) /250 mL H20	Atrazine (mL)/250 mL H20	A_{600} at 24 h	48 h	72 h
Bonner	0.35	1.25	-0.05	-0.01	-0.14
McColloch			-0.10	0.00	-0.13
Helton			-0.11	0.15	-0.24
Dewayne			0.02	0.20	0.02
Shangri La			0.04	0.31	-0.13
St John			-0.01	0.29	-0.03
Bonner	0.30	1.45	-0.17	-0.05	n.d.
McColloch			-0.08	0.11	n.d.
Helton			-0.04	0.02	n.d.
Dewayne			-0.14	0.07	n.d.
Shangri La			-0.16	0.28	n.d.
St John			-0.07	0.06	n.d.
Bonner	0.25	1.65	0.25	0.16	n.d.
McColloch			0.21	0.06	n.d.
Helton			0.10	0.19	n.d.
Dewayne			0.28	0.21	n.d.
Shangri La			0.13	0.09	n.d.
St John			0.09	0.13	n.d.

Table 7. Absorbance at 600 nm 24, 48, and 72 h for isolates from middle Tennessee soils that were exposed to decreasing glucose/increasing atrazine levels. (cont.)

Location	glucose (g) /250 mL H20	Atrazine (mL)/250 mL H20	A_{600} at 24 h	48 h	72 h
Bonner	0.20	1.85	0.05	0.12	n.d.
McColloch			-0.13	0.08	n.d.
Helton			-0.10	0.01	n.d.
Dewayne			0.03	0.05	n.d.
Shangri La			-0.05	0.04	n.d.
St John			-0.06	0.23	n.d.
Bonner	0.15	2.05	-0.17	-0.09	n.d.
McColloch			0.07	-0.22	n.d.
Helton			0.04	-0.28	n.d.
Dewayne			0.09	-0.02	n.d.
Shangri La			0.09	-0.08	n.d.
St John			0.14	-0.27	n.d.
Bonner	0.10	2.25	0.11	0.12	n.d.
McColloch			0.00	0.15	n.d.
Helton			0.50	0.07	n.d.
Dewayne			0.16	0.08	n.d.
Shangri La			0.08	-0.1	n.d.
St John			0.28	-0.03	n.d.

Table 7. Absorbance at 600 nm 24, 48, and 72 h for isolates from middle Tennessee soils that were exposed to decreasing glucose/increasing atrazine levels. (cont.)

Location	glucose (g) /250 mL H20	Atrazine (mL)/250 mL H20	A_{600} at 24 h	48 h	72 h
Bonner	0.05	2.45	-0.11	0.06	0.01
McColloch			-0.05	0.05	0.04
Helton			-0.18	0.01	0.04
Dewayne			-0.22	-0.01	-0.02
Shangri La			-0.18	0.02	0.03
St John			-0.18	-0.02	-0.05
Bonner	0.00	2.65	-0.06	0.02	n.d.
McColloch			-0.04	0.12	n.d.
Helton			-0.02	0.16	n.d.
Dewayne			-0.06	0	n.d.
Shangri La			-0.04	0.27	n.d.
St John			0.00	0.25	n.d.

CHAPTER IV

Discussion

Atrazine is one of the most widely used herbicides in the United States (Dinamarca *et al*. 2007) and has a high possibility of being a chemical that can be applied successfully as an agent for bioremediation (Qingyan *et al*. 2008). Bioremediation of atrazine by soil microorganisms can be further perpetuated by finding additional degraders. Tolerance levels were tested with the inoculation of organisms onto TSAA. Of the 35 organisms inoculated on TSAA, 21 grew in the presents of atrazine. This shows that the organisms that grew were able to tolerate atrazine. In order to degrade a chemical, the organisms first have to be able to tolerate the chemical. The 14 remaining organisms that were unable to grow in the presence of atrazine were discarded as non-atrazine tolerant and, therefore, are not able to degrade atrazine.

After tolerance was determined, the 21 organisms that grew on TSAA were inoculated onto MSAA and compared to the growth of the same organisms inoculated on MSA. Since MSA is a minimal nutrient medium, the organisms that could grow on the TSAA may be only tolerating atrazine and not utilizing it as a carbon source and, therefore, are incapable of degradation. Since the only difference between MSAA and MSA is the addition of atrazine, growth on MSAA and lack of growth on MSA would further support that the organisms had the

ability to utilize atrazine in some way. Nine organisms that were able to grow on TSAA did not grow on either MSAA or MSA. This is most likely because these organisms were only tolerating atrazine and were using the nutrition in the TSA. Twelve organisms were able to grow on MSAA. Three organisms showed better growth on MSAA than MSA. Utilization of atrazine by *Enterococcus faecalis*, *Rhodotorula rubra*, and *Proteus mirabilis* is plausible since the only difference between MSAA and MSA was the addition of atrazine, implying some growth advantage in the presence of atrazine.

To further test the hypothesis that the organisms were utilizing atrazine, glycerol, a possible energy source, was removed from the preparation of the MSAA and the MSA. *Escherichia coli* B, *Enterococcus faecalis*, and *Proteus mirabilis* grew on MSAA without glycerol, but did not grow on MSA without glycerol. *Escherichia coli* wt, *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Serratia marcescens*, and *Pseudomonas putida* grew better on MSAA than MSA when the glycerol was removed. Since glycerol can serve as an energy source, once that is removed, atrazine serves this purpose. *Escherichia coli* has been described as useful atrazine degrader when expressing the enzyme chlorohydrolase (Strong *et al.* 2000). *Escherichia coli* and *Enterococcus faecalis* were described as having the ability to degrade atrazine (Koutsotoli 2005). The identification of a *Pseudomonas* degrader, in this study, is consistent with previous reports, where the organisms found to grow with atrazine alone

were found to have atrazine degrading abilities. The remaining four organisms showed no growth difference between the MSAA and the MSA. Since the added glycerol does seem to be affecting the growth of the organisms, this gives validation to the decision to use minimal salts without the addition of glycerol for the remainder of experiments.

Stenotrophomonas, as described earlier, has been shown to fully mineralize atrazine into CO_2 (Rousseaux *et al.* 2001). The ability of *Stenotrophomonas* to produce CO_2 was supported by this experiment and recorded with a 5 day average of 21.13 micromoles. When the 5 day average of CO_2 production was determined, *Pseudomonas fluorescens*, Miscellaneous Gram Negative *Bacillus*, *Pseudomonas aeruginosa*, *Pseudomonas putida*, *Stenotrophomonas maltophilia*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* (K7), *Pseudomonas fluorescens* (K8), *Pseudomonas putida* (K9) and *Serratia marcescens* (K10) showed promising results, all above 20 micromoles of CO_2 while in the presence of atrazine.

Enrichment procedures, in order to pressure the organisms to utilize atrazine as a carbon and/or Nitrogen source, have been documented and were performed to isolate degraders (Rousseaux *et al.* 2001). Although there was no absorbance showing growth after 24 hours, at 48 hours 5 out of 6 samples showed growth at zero glucose and an atrazine level of 2.65 mL. Absorbance levels of 0.02, 0.12, 0.16, 0.00, 0.27, and 0.25 were recorded and growth was

confirmed by inoculation onto a MSAA. There was no growth on MSAA of the sample with 0.00 absorbance levels further confirming spectrophotometer reading.

Growth on MSAA and none on MSA is promising results for discovering atrazine degraders. Even though some organisms that grew on MSA are minimalist bacteria and need very little nutrition to grow, many of these are also known atrazine degraders (Qingyan *et al*. 2008). Organisms that showed promising results of carbon dioxide production have been well documented in past literature helping to solidify the hypothesis of atrazine degraders (Strong *et al.* 2000, Koutsotoli 2005).

Atrazine continues to be a very prevalent herbicide (U.S. EPA 2013). As with all chemicals, certain levels are dangerous and management of toxicity is paramount. Microorganisms that have the ability to degrade atrazine have been discovered in watersheds and soil plots all over the world including: Manitoba, Nebraska, France, and Canada, Iran, and Egypt just to name a few (Tam *et al.* 1987; Bouquard *et al.* 1997; Struthers *et al.* 1998; Topp *et al.* 2000; Dehghani *et al.* 2013; El-Bestawy et al. 2014). This study focused on organisms that were isolated from soils in middle Tennessee. Because application of atrazine in comparison to other areas in the world is not the highest, there have been very few soil studies in search of atrazine degraders performed in Tennessee especially middle Tennessee. Soil samples utilized in this experiment are not

known to have been sampled in the past for degraders. However, the soils tested in this study have had years of exposure to atrazine. Bioremediation continues to be a promising avenue of research, and microorganisms with their fantastic degradation processes should be explored further.

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