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MONITORING ATRAZINE-DEGRADING AND ATRAZINE-TOLERANT BACTERIAL POPULATIONS IN LOWER RIO GRANDE VALLEY AGRICULTURAL CANALS USING QUANTITATIVE-PCR AND INTERNAL *atzA* PRIMERS

A Thesis

by

IBDANELO CORTEZ

Submitted to the Graduate School of the
University of Texas-Pan American
In partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

August 2011

Major Subject: Biology

MONITORING ATRAZINE-DEGRADING AND ATRAZINE-TOLERANT BACTERIAL POPULATIONS IN LOWER RIO GRANDE VALLEY AGRICULTURAL CANALS USING QUANTITATIVE-PCR AND INTERNAL *atzA* PRIMERS

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ABSTRACT

Cortez, Ibdanelo, Monitoring atrazine-degrading and atrazine-tolerant bacterial populations in Lower Rio Grande Valley agricultural canals using quantitative-PCR and internal atzA primers. Master of Science (MS), August, 2011, 58 pp., 18 figures.

The research hypothesis was that atrazine-degrading bacteria would be detectable during the planting and rainy seasons with their populations dissipating during non-planting and dry seasons in 2010-2011. Atrazine concentration was detected in March, May, June and August for all canals with June having the highest average concentration of 0.77ppb. Gene *atzA* abundance was detected from June through August with July samples having the highest gene abundance of 250.6 (pg). Tolerant bacterial densities were detectable from March through June. Atrazine concentration and population abundance were not statistically different during planting and non-planting, rainy and dry seasons, although there was a trend of increased atrazine concentration, denser atrazine-tolerant bacterial populations and increased *atzA* abundance during rainy months. These results indicate atrazine-degrading and atrazine- tolerant bacteria are present in these canals; these organisms potentially degrade excess atrazine in the Lower Rio Grande Valley.

ACKNOWLEDGEMENTS

I would like to thank Dr. Kristine Lowe for her guidance throughout this project. Her vast knowledge of both Microbial Ecology and Molecular techniques were crucial in the completion of this project. Also, I would like to thank the committee members for this thesis: Drs. Jonathan Lieman, Michael Persans, and Christopher Vitek for their recommendations during this project.

I would also like to thank Drs. Frederic Zaidan III, Zen Faulkes, and Robert Edwards for their help with the statistical analysis part of this project. I would like to thank Tom Eubanks and Dr. Matthew Terry for helping me with DNA preparations and DNA sequencing. Lastly I would like to thank UTMB's Bridge to PhD program for their generous funding throughout this project.

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PREFACE

In the last fifty years, aquatic ecosystems have received more than just autochthonous material. Pesticides, due to widespread and long-term use in agriculture, have also found their way into aquatic environments. The toxic and persistent nature of pesticides detrimentally impacts the environment and their use continues to grow with increasing population demands (Ribaudo, 1994). Climatic conditions, like high temperatures and dry seasons, along with large volumes applied to crops contribute to pesticide persistence in aquatic environments and groundwater. Seventy percent of the world's fresh water supply is held as groundwater; thus, regulating and monitoring contaminants in groundwater is very important to humanity (www.groundwater.org).The pesticide atrazine is the most heavily used herbicide in the U.S.A with an estimated 76.4 million pounds applied annually (www.epa.gov). Atrazine and its metabolites are the most commonly detected pesticide in the environment with a frequency of 10 to 20 times higher than other herbicides (Belluck, 1991). Recent studies have shown atrazine concentrations exceeding maximum contaminant levels set by regulatory agencies in groundwater (Garcia and Gonzales, 2001; Russell, 1991). Atrazine has also been shown to affect amphibian and fish populations by causing hormone irregularities that ultimately reduce reproductive success (Hayes, 2002; Tillit, 2010).

The primary mechanism for the rapid degradation of xenobiotics in the environment is through microbial catabolism. In particular, certain bacterial species have been found to preferentially degrade pesticides in areas of heavy use. Agriculture practices used in fields along with the pesticide's tendency to persist, directly govern the metabolic strategy bacteria utilize to degrade them. Microbiological interactions with pesticides have been intensely studied for many years, mainly because bacteria have the unique ability to rapidly adapt to their environment and use a variety of nutrients to survive (Trun, 2000).

Bacteria can catabolize toxic compounds as sources of nutrition and release less harmful chemicals back into their environment. This is highly advantageous for bacteria that possess these catabolizing genes. For example, the bacterium *Pseudomonas* sp. ADP harbors genes *atzABC*, which encode catabolic enzymes specific for the break-down of atrazine into cyanuric acid (De Souza, 1998). Subsequent studies have shown these genes are highly conserved and exist in many genera of bacteria that are geographically distributed (De Souza, 1998). To determine atrazine's influence on atrazine-degrading bacteria, Martin-Laurent (2003) quantified the *atzC* gene after treatments of atrazine and concluded these genes increase in the presence of atrazine. Genes *atzABC* can be found in bacteria inhabiting atrazine contaminated areas (DeSouza, 1998); in the presence of atrazine, atrazine-degrading genes increase (Martin-Laurent, 2003). Studies such as these can be useful for discovering atrazine-degrading bacteria in other atrazine-contaminated areas.

Major crops in the Lower Rio Grande Valley, the site of this study, are heavily treated with atrazine with over 3million pounds applied annually (National Center for Food and Agricultural Policy, 1997). A recent study showed bacterial strains isolated from South Texas canals were able to tolerate atrazine, suggesting that the microflora has come into contact with this pesticide and has developed strategies to resist its toxicity (Puente and Lowe, 2010).

Statement of the purpose

While atrazine-degrading bacteria have been isolated from atrazine contaminated areas, there is still little research of their persistence during the growing and non-growing seasons especially in the lower Grande Valley. Thus, the aim of the current research project was to monitor atrazine-degrading and atrazine-tolerant bacterial populations for one year in the Lower Rio Grande Valley by quantifying *atz* genes, specifically *atzA* using Quantitative Polymerase Chain Reaction (Q-PCR) assays and culturing techniques.

CHAPTER I

INTRODUCTION

Atrazine history

Atrazine has been a major herbicide used in agricultural practices for over 40 years, primarily on corn and sorghum crops (Ribaudo, 1994). Manufactured by CIBA-GEIGY in 1958 to be primarily used for corn production, atrazine quickly became a broad-range herbicide and is now applied to a variety of crops including sugarcane, rangeland, Macadamia orchards, turf grass sod, forestry, and grasslands (EPA, 2003). USEPA has recently eliminated non-agricultural usage of atrazine and ordered farmers to create buffer zones between application sites and surface water areas. For states in which corn and sorghum are major crops, atrazine is applied at rate of 1-1.5 pounds per acre depending on the soil type. Less than 1 pound of atrazine is applied per acre to sorghum crops with sandy soil; this is because atrazine is more susceptible to leaching in this soil type (Ribaudo, 1994).

Atrazine targets broadleaf and grassy weeds by inhibiting their electron transport chain and ultimately photosynthesis (Ribaudo, 1994). Atrazine blocks the ferricyanide reduction and non-cyclic phosphorylation dependent step in the Hill reaction of photosynthesis, an

important step involved in oxidizing water (Shimabukro, 1969). Chloroplasts are the main sites of inhibition in all plants; susceptible plants fail to metabolize atrazine at fast rates therefore allowing more of the active ingredient to reach chloroplasts. Tolerant crops use two strategies to decompose atrazine: dechlorination into hydroxyatrazine by 2,4-dihydroxy-3-keto-7-methoxy-l,4-benzoxazine (benzoxazinone), or an N-dealkylation pathway which converts atrazine into 2-chloro-4-amino-6-isopropylamino-s-triazine (Shimabukuro, 1969). The latter mechanism is more common; however, it only partially detoxifies plant cells. Figure 1 shows the fate of atrazine after it enters a plant cell (Shimabukuro, 1969).

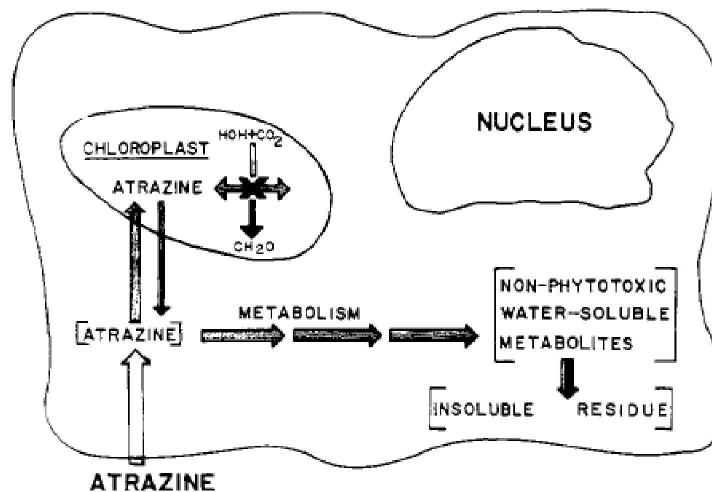

Figure 1. This illustration shows the destination of atrazine after penetration into a plant cell. Atrazine is metabolized in the cytoplasm into water-soluble and insoluble compounds which reduces atrazine presence in the chloroplast. Atrazine not transformed in the cytoplasm enters the chloroplast, the target site for atrazine. Complete photoinhibition occurs when atrazine concentration in the chloroplast equals cytoplasm concentration, a consequence exhibited by susceptible plants.

Atrazine that is not taken up by plants remains in the environment and can travel to other areas beyond its original site of application. Atrazine's mobility is primarily due to its moderate

solubility of 30 mg/l in water (EPA, 2003). During heavy irrigation and rain fall, these properties contribute to the leaching and run-off of atrazine. The half-life of atrazine varies with different soil types but on average can persist from 60 to150 days in soil (EPA, 2003). Goolsby et al. (1995) reported atrazine concentrations peaked and persisted from May through July in small streams, which roughly corresponds to the growing seasons in many locations. In sub-surface environments, atrazine degrades more slowly because less oxygen and light to degrade the parent compound (Bouzaher & Ribaudo, 1994). Goolsby (1993) observed this phenomena and showed atrazine's half-life persisting for one to two years in reservoirs.

Atrazine toxicity

A major concern of atrazine usage is the acute and chronic health effects experienced when animals are exposed to this pesticide at levels higher than the maximum contaminant level (MCL) of 3 ppb. In some areas of the U.S., atrazine has been detected at levels equal to or higher than the MCL during months of heavy irrigation and rainfall. The National Resources Defensive Council (NRDC) reported that several watersheds in the Midwest contained dangerously high concentrations of atrazine, some as high as 237.5 ppb (Wu, 2009). Another study reported that 640 metric tons of atrazine was deposited in the Gulf of Mexico in 1993 (Clark, G. 2000). Atrazine has been detected in 80% of drinking water samples in some Midwestern states (i.e. Missouri, Indiana, Ohio ect.) (Wu, 2009). Peak concentrations were well above 3 ppb. These reports are even more alarming since atrazine monitoring programs are only implemented for susceptible communities (populations near heavy atrazine use) and community water systems that have tested positive for dangerous levels of atrazine and its metabolites. However, even with

an atrazine monitoring program, atrazine is difficult to remove from drinking water unless the city includes an additional filtration step through activated carbon, but this still may leave humans susceptible to health risks associated with atrazine contamination. Short term atrazine exposure can lead to acute symptoms including major organ congestion, muscle spasms and adrenal degeneration (EPA, 2003). Long term exposure to atrazine can cause muscle degeneration, weight loss and mammary tumors (EPA, 2003). Atrazine may also cause populations of non-target organisms to decline, including micro-photosynthetic organisms like algae and cyanobacteria, which are major contributors of atmospheric oxygen.

Many aquatic animals begin their reproductive and development activities during rainy seasons which, is when peak concentrations of atrazine have been detected in reservoirs and streams (Ribaudo, 1994). This common occurrence has adversely affected both fish and amphibians reproductive success. Hayes, et al. (2001) intensely studied the effects of atrazine on frog development and have observed that atrazine feminized male frogs turning them into hermaphrodites. According to this study, atrazine induced the development of ovaries in male frogs by lowering testosterone levels. Even with frogs exposed to atrazine at ≤ 1ppb, a concentration lower than the allowable level set by USEPA, male frogs experienced hermaphroditism and had lower levels of testosterone compared to unexposed females. Further complicating reproductive success of atrazine-exposed frogs, is that they also have reduced vocal cords, which play a major role in courtship strategies (Hayes 2008, 2010a, 2010b). Similar results were seen in fish exposed to atrazine; however, researchers also reported a decline in egg production by females. Furthermore, dissections of contaminated fish revealed abnormalities in the testes of male fish after atrazine-exposure (Tillet, D. 2010). Other animals have been used to demonstrate the effects of atrazine contamination including salamanders and rats, which have all

shown results consistent to the previously discussed studies. These results raise concern of atrazine possibly disrupting hormones levels and causing gonad deformities in humans, as well.

Fate of atrazine in the environment

The movement of atrazine in the environment is a serious problem in natural ecosystems. Atrazine's mobility is strongly influenced by its solubility in water and soil adsorption coefficient. The average organic adsorption value for atrazine is 122 (Koc), which puts it in the moderate to high risk class for mobility (atrazinefactsheet, 2003). The adsorption coefficient value measures a pollutant's potential to bind to organic material in soil. Adsorption (retention) of atrazine is higher in high organic material surface soil (between 0-30cm of the surface horizon) with adsorption decreasing with lower organic material associated with lower soil depths (Jenks, 1998). Years of heavy atrazine use can intensify its concentration in the soil; a ten year study reported 50% of radiolabeled atrazine was recovered from soil nine years after original application (Capriel, 1985). Thus, desorption of atrazine from soil particles is not common; however, the leading cause of atrazine's presence in groundwater is the excessive applications of atrazine and heavy rainfall. A reconnaissance study monitoring 303 groundwater sites revealed the detection frequency for atrazine was over 22% with a maximum contamination level reaching 2.09 ug/L (Koplin, 1996). Heavy application periods of the year coupled with the rainy season (late spring through early summer) tend to lead to pesticide levels exceeding health-based levels in surface water. This was observed in a study monitoring surface water reservoirs which showed atrazine being the most frequently detected herbicide; the average detection level exceeded the MCL at 3.8 ug/L (Goolsby, 1995). Although transportation of atrazine into surface

water reservoirs and groundwater is common, especially in areas where this pesticide is heavily used, atrazine can also be distributed to very distant areas from the original site of application through rainfall. Atmospheric atrazine can travel by wind currents and precipitate at considerable distances. Goolsby et al. (1997) documented the presence of atrazine in rainfall samples from 26 different states for 19 months and discovered atrazine was the most detected and widespread herbicide with a 30% detection percentage and a maximum concentration level of 10 ug/L.

Quantitative-Polymerase Chain Reaction (Q-PCR)

Quantitative-Polymerase Chain Reaction (Q-PCR) is a laboratory technique used for its ability to quantify gene copies and gene expression levels using small amounts of template. Unlike conventional PCR, where the products of a reaction can only be detected with further techniques like electrophoresis, Q-PCR can measure amplified products while the reaction is in progress, also known as “real-time.” The basis of Q-PCR is the ability to measure the initial amount of DNA according to its threshold cycle (C_t) value. Threshold cycle is reached when the fluorescence of amplified products exceeds background fluorescence. A Dilution series for a known concentration of template DNA is run in order to produce a standard curve. The standard curve is created using the log of the quantity of DNA against its C_t value for each dilution. The more template in the sample, the lower the Ct value; in contrast, the lower the quantity of template, the higher the cycle value. The standard curve aids in determining the relative concentration of DNA in samples of unknown concentration. Amplified products are measured using molecules which fluoresce when DNA is amplified in the reaction. The intensity of fluorescence is proportional to the DNA concentration; thus, fluorescence reflects the abundance

of DNA in the reaction. For this study, the commonly used binding dye, SYBR Green, was used as the fluorescent reporter molecule. SYBR Green works by intercalating between double-stranded DNA (dsDNA) nonspecifically. In a solution with unbound SYBR Green fluorescence is minimal; this is known as the background fluorescence. Only when bound SYBR Green exceeds the background fluorescence is there a detectable signal. DNA binding dyes are popular because they permit easy reaction set-up that affords the user the ability to quickly test multiple genes, and they allow specificity of the amplified products to be achieved through melt-curve analysis. Melt-curves add precision to the reaction by distinguishing between illegitimate amplification products like primer-dimers, from target sequences. During a melt-curve, the temperature of the reaction incrementally increases while the fluorescence generated in the amplification step is monitored. As amplified DNA in the reaction denatures or melts, the reduction of fluorescence is measured and plotted as a function of temperature. Melt-curve analysis helps define the target sequence's melting temperature which is unique from other nonspecific products (primer-dimers) that might be amplified in the reaction.

The development of Q-PCR has become invaluable to molecular biologists and changed the way microbial ecologists characterize the diversity of microbial communities in the environment. Along with gene markers and DNA sequencing analysis, microbial ecologists are able to use Q-PCR to measure population abundance of native and non-native microbes from environmental samples. Desouza et al. (1998) evaluated the effect of atrazine on atrazine-degrading microflora; populations were monitored using designed primers for *atzC* and Q-PCR. Abundance of *atzC* increased in response to increased atrazine treatments suggesting a positive feedback process between atrazine concentration and atrazine-degrading microflora populations (Martin-Laurent, 2003). This and several other molecular applications have utilized Q-PCR to

quantify nucleic acid samples. Over 25,000 publications in the last 10 years make reference to Q-PCR (Taylor, 2010).

Atrazine-degrading bacteria

Environmental bacteria are important decomposers “of organic material” and play a major role in recycling organic waste that provides nourishment for plants and other organisms such as protozoans and arthropods. As a result of anthropogenic activities, bacteria are frequently exposed to toxic compounds like pesticides and detergents, which negatively affect their success in the environment. Several studies have reported that microbial populations are negatively affected by long periods of pesticide treatments, which can devastate the nutrient cycle (Moorman, 1989; Malkomes, 1985). For example, after initial pesticide application, microbial population size may increase; however, after subsequent applications some populations decline and disappear. This suggests that pesticides can serve as nutrients for some microbes but persistent treatments may be lethal to others (Taiwo, 1997). However, studies have also shown that some bacterial species can utilize pesticides as a source of nutrition by producing catabolic enzymes during periods of heavy applications (Mendelbaum, 1993; Brimecombe, 2006).

Atrazine-degrading bacteria have been intensely studied for their ability to break down atrazine and metabolize it through enzymatic catabolism. However, most early studies were unable to isolate atrazine-degrading bacteria or could only isolate bacteria that transform atrazine through the N-dealkylation pathway. This still leaves the compound chlorinated and toxic (Erickson, 1989; Geller, 1980). Subsequent studies revealed several mixed cultures that could partially reduce atrazine (Behki, 1986; Eaton, 1990) but it wasn’t until 1993 that a pure culture,

atrazine-degrading organism was isolated (Mendelbaum et al. 1993). This isolate was later designated as *Pseudomonas* sp. strain ADP (Mendelbaum, 1995); the ADP strain was unique in that it rapidly transformed more than 80% of atrazine into CO_2 within 48 hours of treatment. More importantly, this strain was able to thrive in minimal media with only atrazine as a nitrogen source. The results showed for the first time a pure bacterial culture that could reduce atrazine's half-life from months to just a few days. This strain could also completely metabolize atrazine into CO_2, NH_3 and H_2O. The *Pseudomonas* strain ADP is now widely used as a positive control for atrazine degradation. Since the discovery of strain ADP, several bacterial species capable of metabolizing atrazine as a carbon and nitrogen source have been identified (Yanze-Kontchou, 1994; Radosevich, 1995). Additionally, atrazine degrading bacteria are now known to be globally distributed and encompass a diverse group of organisms that includes several other genera (e.g. *Chelatobacter, Arthrobacter* and *Ralstonia*) (De Souza, 1998; Rousseaux, 2001).

Atrazine-degrading genes *atzA, B* and *C*

High Performance Liquid Chromotography (HPLC) revealed that the product hydroxyatrazine (2-ethylamino-6-propan-2-ylamino-1H-1,3,5-triazin-4-one) was the first intermediate formed in the degradation of atrazine by strain ADP (Mendlbaum, 1995). The enzyme responsible for transforming atrazine into hydroxyatrazine is the most crucial step in the pathway since this enzyme dechlorinates atrazine and reduces the pesticides properties including, its toxicity (Kross, 1992). Gene *atzA* was the first gene to be sequenced from strain ADP. Its corresponding protein, Atrazine chorohydrolase (AtzA), was the first enzyme extracted from bacteria to dechlorinate atrazine (De Souza, 1996). The gene *atzA* is composed of 1.4 kilobases

(kb) which encodes a 473 amino acid polypeptide (Desouza, 1996). Atrazine chlorohydrolase's amino acid sequence showed 41% similarity to an enzyme from *Rhodococcus corallines* NRRL B-15444R called TrzA. TrzA catalyzes the dechlorination and deamination of a triazine pesticide; however, *Rhodococucus* lacks the ability to metabolize triazine pesticides (Shao, Z. 1994; Desouza, M. 1996). Comparison studies between the two enzymes showed TrzA had an affinity for triazine rings structurally similar to atrazine such as desethylsimizine, desethylatrazine, and melamine. In contrast, Atrazine chlorohydrolase (AtzA) was specific to only atrazine (DeSouza, 1996). The gene *atzA* removes the chlorine substituent from atrazine via a hydrolytic mechanism (DeSouza, 1996). Since this gene is the most crucial reaction in detoxifying atrazine, the gene was amplified using designed primers by Desouza et al. (1998), and transformed into native bacteria. Transconjugants efficiently degraded simazine, a chlorinated pesticide structurally identical to atrazine (Garcia, M. 2008).

Further investigation of the atrazine metabolic pathway possessed by strain ADP revealed a second gene important in atrazine transformation. The gene *atzB* is made up of 2.6 kilobases with an open reading frame that encodes the enzyme Hydroxyatrazine ethylaminohydrolase (Boundy-Mills, 1996). This enzyme catalyzes the deamidation (removal of the ethylamino group) of hydroxyatrazine to yield isopropylammelide (6-propan-2-ylamino-1H-1,3,5-triazine-2,4-dione). Like the corresponding protein for *atzA* gene, the amino acid sequence for AtzB has low similarity with other proteins like TrzA, which catalyzes the deamination of melamine; therefore, both AtzA and AtzB specifically target the transformation of atrazine (Boundy-Mills, 1996). The gene *atzC* was later isolated from strain ADP and found to be a part of the catabolic pathway of atrazine. This gene is composed of 1.2 kb which encodes an enzyme of 403 amino acids. This enzyme, N-isopropylammelide isopropylaminohydrolase, catalyzes the

transformation of isopropylammelide, the product of the AtzB enzymatic reaction, into cyanuric acid (1,3,5-triazine-2,4,6-triol) and isopropylamine (2-aminopropane) (Sadowsky, 1997). The catabolic genes *atzA, B,* and *C* encode for enzymes that sequentially remove side groups from atrazine with the gene *atzC* hydrolyzing the final side group N-isopropylamine. The enzyme AtzC is less than 30% identical to other proteins with the most similar being Cytosine deaminase, an enzyme that's functionally similar to AtzC (Sadowsky, 1997). Cyanuric acid, the end product of this catabolic pathway, is hastily degraded by bacteria in a manner similar to urea degradation (Cook, 1985). The catalytic enzymes AtzA, B and C undergo hydrolytic reactions with only atrazine and belong to the amidohydrolase protein family which includes proteins like urease, dihydroorotase and cytosine deaminase (Sadowsky, 1997). However, *atz* enzymes show low similarity to other proteins in the amidohydrolase and even less similarity to each other, therefore suggesting that these proteins may have recently evolved but don't have a common evolutionary origin (Sadowsky, 1997). The complete catabolic pathway of atrazine into cyanuric acid by the products of the *atzA*, *B* and *C* genes is illustrated in Figure 2.

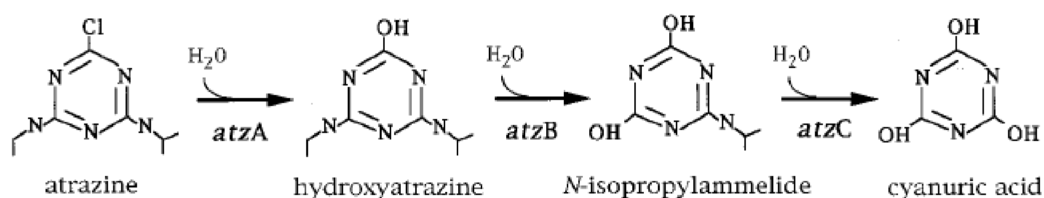

Figure 2. This illustration shows the catabolic pathway of atrazine into cyanuric acid via atrazine-degrading genes *atzABC.*

The catabolic genes *atzA*, *B*, and *C* are widespread and highly conserved amongst atrazine degrading bacteria that are geographically separated (DeSouza, 1998; Topp, 2000). Horizontal gene transfer of these genes is possible due to insertion elements that flank each gene

and also via the self-transmissible plasmid pADP-1, from which these genes originate (Devers, 2005). The flanking insertion elements are mobile pieces of DNA that move the centrally located pieces of DNA along with them into other chromosomal molecules (Trun, 2004). Some bacterial species lack the complete set of atrazine-degrading genes; for example, *Rhizobium sp.* only possesses *atzA* (Bouquard, 1997). Some atrazine degrading bacteria harbor all three genes; however, they can be located on multiple plasmids in the same host (Topp, 2000; Rousseaux, 2002). Moreover, individuals of a bacterial consortium may harbor different combinations of these genes and only as a collective can metabolize atrazine (DeSouza, 1998).

The 100 kb plasmid pADP-1that was purified from strain ADP, carries all the necessary genes to transform atrazine into cyanuric acid, including genes *atzD, E*, and *F*.These latter genes complete the mineralization of atrazine by expressing enzymes that convert cyanuric acid to carbon dioxide and ammonia (Martinez, 2001). Genes *atzD, E,* and *F* encode proteins that are functionally similar to other enzymes involved in the catabolism of cyanuric acid (Martinez, 2001), a pathway frequently detected in common soil bacteria (Cook, 1985) and are therefore not unique to the degradation of atrazine. Atrazine-degrading genes have been detected in Gram-negative and Gram-positive bacteria detached from plasmid pADP-1 (Rousseaux, 2002). This suggests that the insertion elements may play a larger role in the dissemination of these genes than the plasmid on which they were first isolated.

Rationale and objectives

Approximately 90% of corn crops and 65% of sorghum crops in Texas receive atrazine annually (Anciso, personal communication; NCFAP, 1997). The course of atrazine application

depends mainly on climate and soil type but typically is applied to crops pre- or post-emergence. Major crops exposed to atrazine like corn and sorghum are planted twice a year in spring and fall in Texas. Spring planting for corn begins January through February; fall planting for corn begins July through August (Anciso, personal communication).

Due to the high use of atrazine in Texas, the objective of this study was to detect and isolate atrazine-degrading bacteria in South Texas and monitor the abundance of atrazine-degrading genes throughout the year. Specifically, the research hypothesis was that atrazine-tolerant bacteria and atrazine-degrading genes (e.g., *atzA*) would be more abundant during South Texas growing seasons and decrease during non-growing seasons. To test this, culture techniques and Q-PCR monitoring of *atzA* was performed over a 1-year period in 2010-2011.

CHAPTER II

MATERIALS AND METHODS

Sample collection and processing

Water samples were taken from four agricultural canals in Weslaco, TX. Canal sites were chosen due to their close proximity to agricultural fields known to grow crops of corn. Canals were sampled once a month for one year during 2010-2011. Three (3) sterile 50 ml conical tubes were used to collect water from each canal site and transferred to the Biology Department of UTPA on ice. Water samples were processed within 7 days of collection and stored at room temperature during that week.

Enzyme-Linked Immunosorbent Assay (ELISA) testing

Atrazine concentration was measured monthly for each canal sample. An Abraxis Atrazine-ELISA (Enzyme-Linked Immunosorbent Assay) micro-plate kit (Abraxis, Warminster, PA) was used to test each canal sample for atrazine concentration. Assays were performed in triplicate. The ELISA assay worked as follows. Atrazine present in the sample and atrazine-enzyme-conjugate compete for antibody binding sites on the plate. The linked enzyme activates a

dye; the intensity of the dye color is inversely proportional to the concentration of atrazine present in the sample. The color intensity concentration for each site was measured at a wavelength of 450 nm using a micro-plate reader. Mean absorbance values of triplicate assays were used to estimate the atrazine concentration by comparing the sample absorbance to a standard curve generated using known atrazine concentrations.

Media and plating

To estimate the density of bacterial populations tolerant to atrazine, canal samples were plated on minimal media amended with 5 ppm of atrazine. Minimal agar media contained the following ingredients: CH_3COO Na [15 mM],$(NH_4)_2SO_4$ [0.9 mM], $KH_2PO_4 \cdot 3H2O$ [0.57 mM], K_2HPO_4 [0.33 mM], $NaHCO_3$ [0.2 mM], $Na_2EDTA \cdot 2H_2O$ [7 μM], H_3BO_3 [6 μM], $FeSO_4 \cdot 7H_2O$ [0.6 μM], $CoCl_2 \cdot 6H_2O$ [0.5 μM], $Ni(NH_4)SO_4^{2-} \cdot 6H_2O$ [0.5 μM], $Na_2MoO_4 \cdot 2H_2O$ [0.4μM], Na_2SeO_4(anhyd) [0.15 μM], $MnSO_4 \cdot H_2O$ [0.13 μM], $ZnSO_4 \cdot 7H_2O$ [0.1 μM], $CuSO_4 \cdot 5H_2O$ [0.02 μM], casamino acids [0.01% w/v], Vitamin B1 [0.001 mg mL^{-1}], L-arginine HCl [0.02 mg mL^{-1}], L-glutamic acid [0.02 mg mL^{-1}], L-glutamine [0.02 mg mL^{-1}], L-serine [0.04 mg mL^{-1}], MgSO4 $\cdot 7H_2O$ [1 mM], $CaCl_2 \cdot 2H_2O$ [0.5 mM] and NaCl [3% w/v]. Bacto Agar (Difco BBL, Detroit, MI) was added at a ratio of 15 g L^{-1}. One gram of atrazine (2-Chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine) was dissolved in 10 ml of methanol and vortexed for two minutes to mix thoroughly. Then 5 ml of atrazine solution was added per liter of media after autoclaving. Aliquots of 100 μl of canal water sample were serially diluted to 10^{-1}, 10^{-2}, and 10^{-3} and plated in triplicate on agar plates. 100 μl aliquots of each canal sample were spread on prepared media plates and incubated at room temperature for 5 days. After the incubation period,

atrazine tolerant bacteria were observed and colony forming units per milliliter (cfu ml^{-1}) were counted to estimate cell density for each canal. Average cell densities of atrazine tolerant bacteria for each canal were measured monthly for one year.

DNA extraction from *Pseudomonas sp.* strain ADP

Positive control data was achieved by extracting genomic DNA from *Pseudomonas sp*. strain ADP cells. Cells were pelleted by centrifugation and lysed using lysing buffer, and incubated at 60°C for 30 minutes. The lysate was filtered through a spin column and DNA was eluted by centrifuging at 14,500 rpm for 1 minute with 30 µls of nuclease-free water. DNA was quantified using a nano-drop spectrophotometer. The final concentration of DNA from the positive control was 24 ng μl^{-1}. DNA was serially diluted to 10^{-6}; an aliquot of 10 µl of diluted DNA was used to construct a 50 µl Q-PCR reaction that included: 2.5 µl of each forward and reverse primers at a concentration of 1 µM, 25 µl of SYBR Green Master Mix (BioRad; Hercules, CA), and 10 µl of nuclease-free water. Standard curves were constructed by taking the log of the known amount of DNA for each dilution and plotting against their measured threshold value (C_t value). Internal *atzA* primers (DeSouza, 1998) were used to amplify the *atzA* gene from DNA from water canal samples. The forward primer sequence was 5´CCA-TGT-GAA-CCA-GAT-CCT3´ and the reverse primer sequence is 5´TGA-AGC-GTC-CAC-ATT-ACC3´.

DNA extraction from canal samples

The protocol to extract DNA from water samples collected from Weslaco, TX was as follows: 50 ml of canal water was spun down using a centrifuge at 14,500 rpm for 30 seconds, 100 µl of lysing buffer was added to the cell pellet and incubated for 30 mins at 60°C. Lysate was filtered through a genomic DNA spin column. DNA was eluted into a 1.5 ml centrifuge tube with 30 µl of nuclease-free water. DNA was measured using a nano-drop spectrophotometer. DNA yield was at least 500 pg µl^{-1} of from each water sample. Extracted DNA was used in Q-PCR reactions with *atzA* gene primers. The proportions for each reaction were: 2.5 µl of each forward and reverse primers, 25 µl of SYBR Green Master Mix, 10 µl of DNA extracted from canals, and 10 µl of nuclease-free water.

PCR amplification of *atzA* and *atzC* genes from atrazine-tolerant bacteria

Genomic DNA was isolated from atrazine-tolerant isolates using commercial kits (Promega Co. Madison, WI) and amplified with primers for *atzA* and *atzC*. DNA (pADP) from *Pseudomonas* sp. Strain ADP was used as a positive control; sterile water or genomic *Escherichia coli* DNA was used as negative control. PCR conditions were as follows: initial denaturing 95°C for 2 mins, followed by 40 cycles of denaturing for 30 sec at 90°C; annealing at 60°C for 1 min, an extension at 1 min and a final extension at 72°C for 1 min. Samples were then held infinitely at 4°C.

Q-PCR analysis of atrazine-degrading gene *atzA*

Dilution series were performed using 24 ng μl^{-1} of positive control DNA to create a standard curve for *atzA* gene; DNA from water samples were diluted to 500 pg μl^{-1} before performing Q-PCR reactions. SYBR Green Super Mix (BioRad; Hercules, CA) was used for all Q-PCR reactions at a concentration 0 .1 M. Log DNA was measured in fg and plotted against the cycle threshold (C_t). A standard curve for *atzA* gene was used to estimate the gene copy number in each canal once. The manufactures protocol for SYBR Green was followed and the annealing temperature for *atzA* was confirmed by a temperature gradient. Q-PCR conditions were as follows: initial denaturing, 95°C for 2 min; followed by 40 cycles of denaturing, 90°C for 30 sec, and annealing at 60°C for 1 min. Quantification was performed after this step. For the melt curve analysis, the temperature minimum was 55°C and increased by 5°C until the melting point of DNA was reached.

Statistical analysis

Atrazine concentrations, *atzA* gene abundance and atrazine-tolerant population densities were analyzed using the Statistical Package for the Social Sciences (SPSS) computer software (SPSS Inc. Chicago, IL). The Non-parametric Mann-Whitney U test was used for seasonal comparisons for each measurement.

CHAPTER III

RESULTS

All data points for each measurement, i. e. atrazine concentrations, gene *atzA* abundance and bacterial densities tolerant to atrazine were normalized. This was done to show relative increase of data points from their lowest detectable levels. Traces of these measurements may have been present during 2010-2011 but the methods used were not able to measure lower than the lowest data points reported in this study. Therefore, months with a zero reported were treated as measurements that are outside detectable limits.

Atrazine concentration in canals

Atrazine concentrations were measured in Texas during 2010-2011. In general, atrazine concentrations were below the MCL of atrazine (3ppb) as prescribed by the USEPA. Atrazine levels in canal 1 ranged from below detection to 6 times the lowest detectable concentration. (Figure 3, Panel A). The highest concentration was observed in June 2010; however, for much of the year, atrazine concentration was below detection in this canal. Atrazine concentrations in canal 2 ranged from below detection to more than 8 times the lowest detectable concentration ,

which was observed in March (2010). The peak concentration of atrazine in this canal was much earlier in the year compared to the other canals, which typically had peak atrazine levels in summer (e.g. June). As shown in Figure 3B, the March peak of atrazine was followed by another peak later in the year in June, which coincided with the highest levels of atrazine in other canals. Atrazine in canal 3 (Figure 3C) was lower; overall, levels never exceeded the concentrations reported in canals 1 and 2. The lowest detectable level of atrazine was reported in canal 4 which occurred in May 2010, the only time atrazine was detectable in this canal during 2010-2011.

Comparing normalized atrazine concentrations in all canals during planting seasons (January-February and July-August) versus non-planting periods (May-June and September – December) revealed that there was no significant difference (P=0.479) in normalized atrazine concentration during the planting and non-planting times of the year (Figure 4). However, the average normalized atrazine concentration during the non-planting season tended to be higher than planting periods. The average atrazine level in the non-planting season was 4 times higher than the planting season. This may be due to the application of atrazine post-emergence of crops planted in earlier months.

Precipitation amounts may play a role in runoff of atrazine from fields into canals; therefore, normalized atrazine concentration data from all canals was compiled and compared during months with at least 1 inch of rainfall (May-September 2010) versus months with less than 1 inch of rainfall (January-April 2011 and October-December 2010). The average normalized atrazine concentration in the canals during rainy months was nearly 5 times higher than the concentration measured in canals during dry months (Figure 5). However, due to large sampling error, the concentration differences in rainy vs. dry seasons was not overall statistically significant (P=0.219). Despite this, an observed distinction could be made between rainy seasons

and dry seasons. This was further observed when data from planting seasons and rainy seasons were combined (Figure 6) and when non-planting season data were combined with rainy season data (Figure 7). Normalized concentrations in planting months with higher rainfall tended to be higher (July-August) compared to planting months with low rainfall in which the concentration was below detection (January &February; Figure 6). During non-planting months, those with higher rainfall (April & May) had higher normalized concentration compared to non-planting, dry months (Figure 7). Although the normalized concentration levels were not statistically different, in all cases where precipitation was included the atrazine concentrations were measurably higher than periods when rainfall was less. This suggests that precipitation and run-off were a factor in the presence of atrazine in the canals.

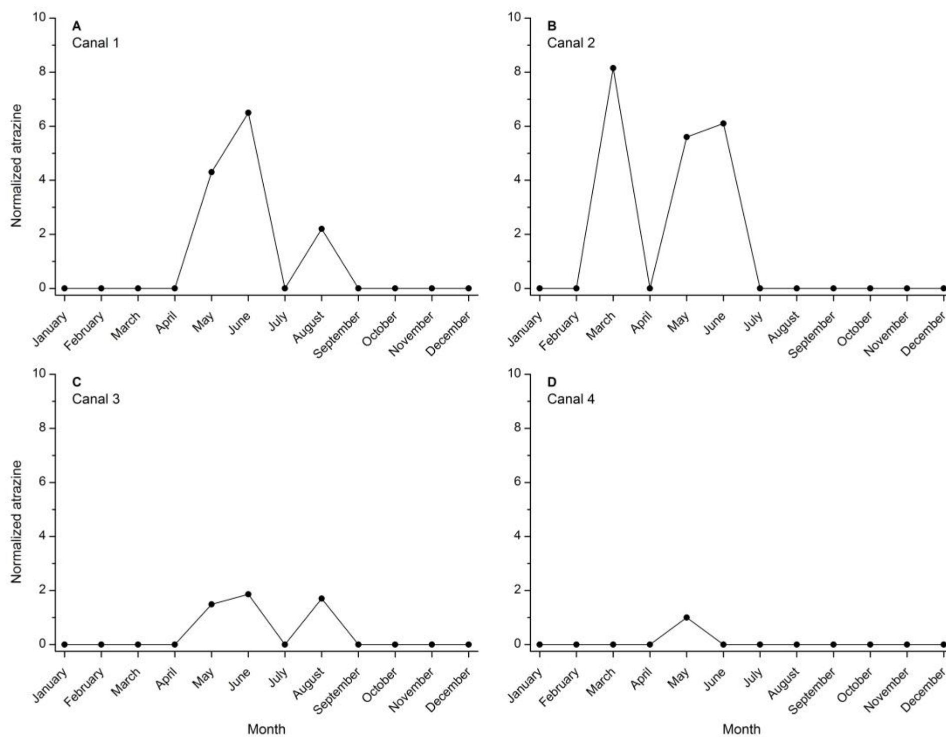

Figure 3. Normalized atrazine concentrations in each canal during 2010-2011 in Weslaco, TX. Y-axis represents normalized parts per billion of the pesticide atrazine and X-axis represent months atrazine was tested. Months with a zero reported indicate times when traces of atrazine were below detection.

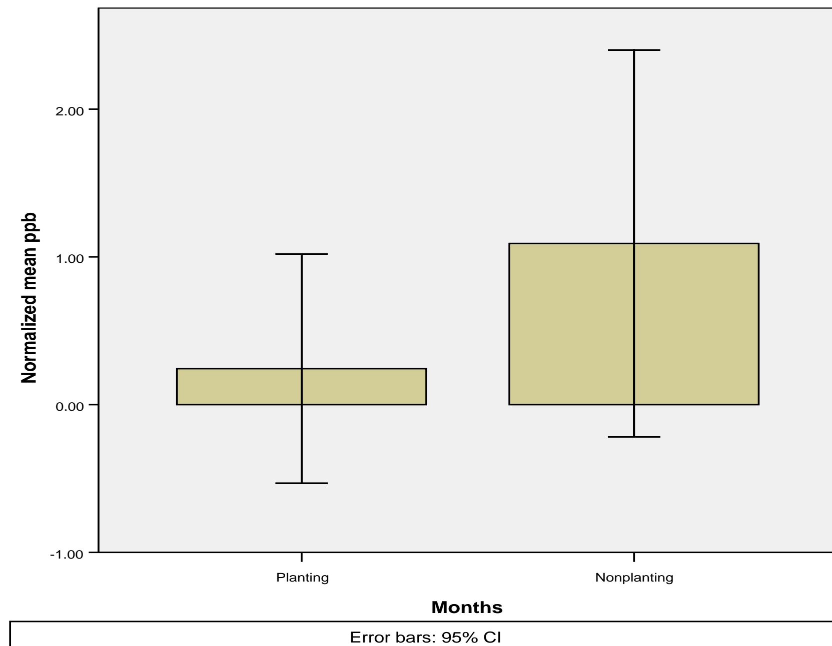

Figure 4. Average normalized atrazine concentrations during the Planting season (January-February and July-August) versus Non-planting seasons (March-June and September-December). Normalized atrazine concentrations were higher in the Non-planting season; however, there was no statistical difference between planting seasons (P=0.49)

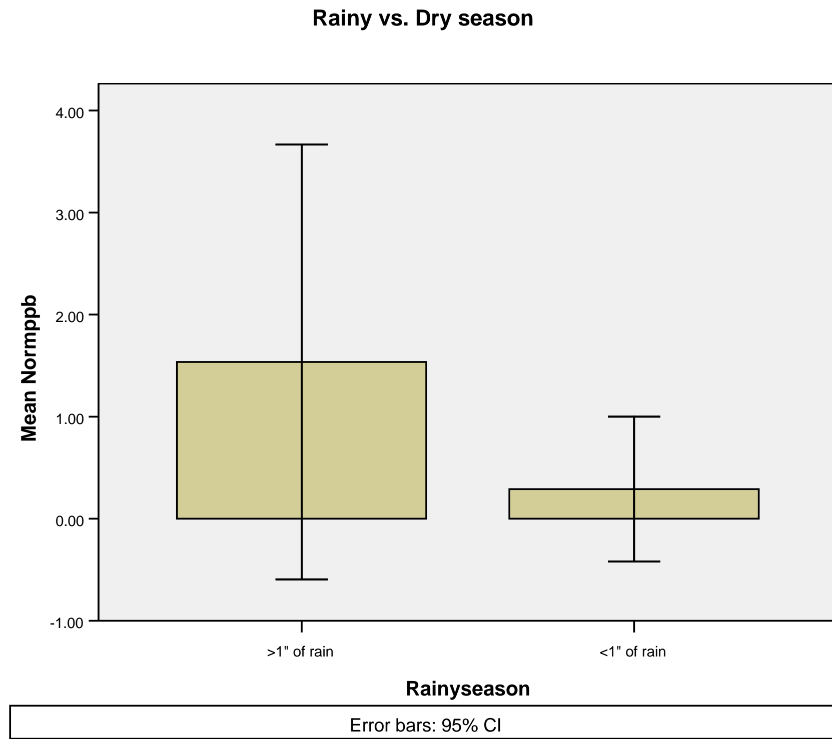

Figure 5. Average normalized atrazine concentrations during months with greater than one inch of rain (May-September 2010) vs. months with less than one inch of rain (January-April 2011, October-December 2010). The season with greater than one inch of rain tended to have a higher average ppb than the season with less than one inch of rain. However there was not a statistical difference between the rainy and dry seasons (P=0.10).

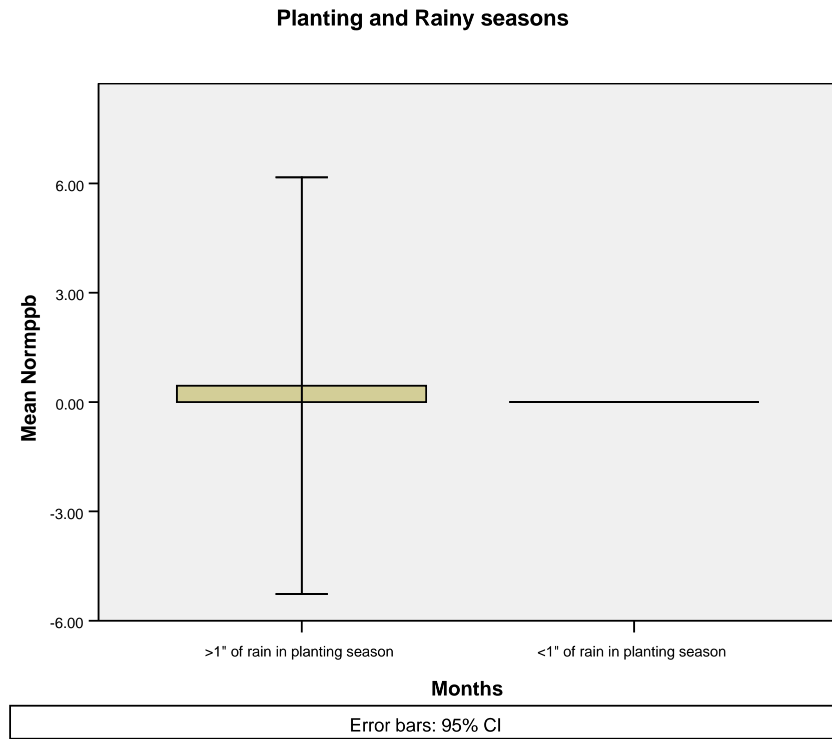

Figure 6. Average normalized atrazine concentrations during the planting months with greater than one inch of rain (July-August 2010) vs. planting months with less than one inch of rain (January-February 2011). Average normalized ppb in the planting months with greater than one inch of rain tended to be higher than planting months with less than one inch of rain; however, there was no statistical difference (P=0.317).

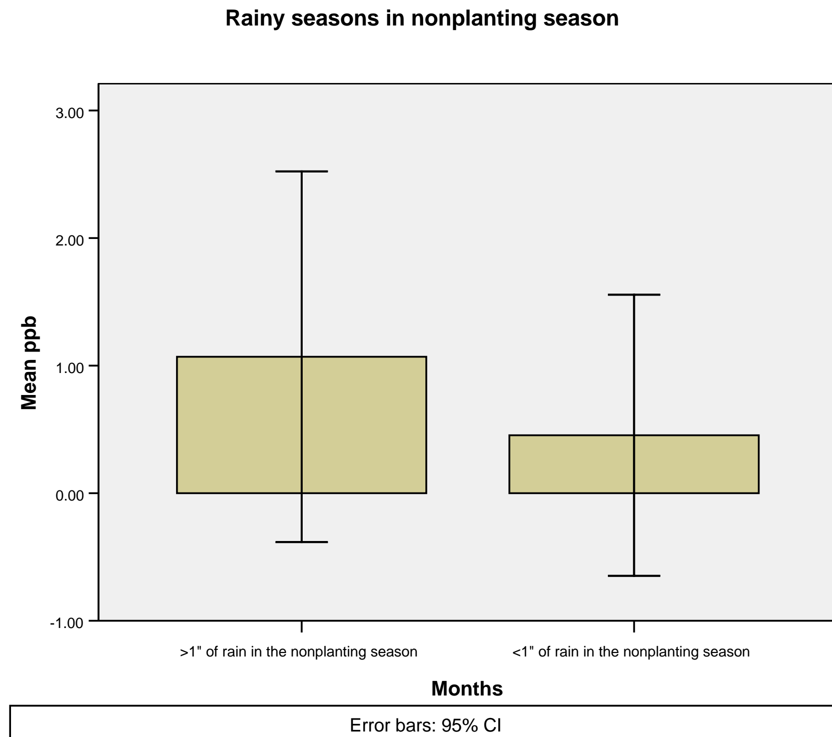

Figure 7. Average normalized atrazine concentrations during non-planting months with greater than one inch of rain (May-June 2010 and September 2010) vs. non-planting months with less than one inch of rain (March-April 2011 and October-December 2010). Average ppb tended to be higher in the non-planting months with greater than one inch of rain compared to non-planting months with less than one inch of rain; however, there was no statistical difference (P=0.386).

Atrazine-tolerant population densities

Isolation and enrichment techniques used in a similar study, (Puent, 2010) were followed to determine the existence of atrazine-tolerant bacteria in agricultural canals during 2010-2011. Culturing experiments to isolate and enumerate atrazine-tolerant microorganisms were

performed monthly. The results showed that atrazine tolerant microbial populations followed a similar yearly pattern in all agricultural canals. Populations were generally low in winter and spring, increased and peaked in early to mid-summer, then declined back to low levels in fall (Figure 8). The largest population size was observed in water from canal 3 during June, which was estimated to be approximately 84 times higher than the lowest detectable level (Figure 8, panel C). For all canals, the largest population sizes were observed in either May or June, which was the time between the bi-annual planting periods in South Texas (Figure 8). During non-planting season (March –June and September-December), average normalized CFUs in canals was 7 times higher compared to normalized CFUs averaged in the planting months (January-February and July-August) (Figure 9). Similar to atrazine chemical concentrations in the canals, precipitation seemed to play a role in the population sizes of atrazine-tolerant organisms in the canals. Overall, population sizes were typically higher during months with more than 1 inch of rain fall compared to months with less than 1 inch of rain (a.k.a. dry season) (Figure 10). This may have been due to increased atrazine in the canals during wetter months, possibly due to run-off from fields where atrazine ws applied post-emergence to the plants and not when the fields were initially planted. Furthermore, precipitation had a greater effect on population size during the non-planting months of the year (Figure 12) versus the planting months (Figure 11). However, in call cases there was no statistical difference when normalized CFUs were averaged and compiled for seasonal comparison (Figures 9-12).

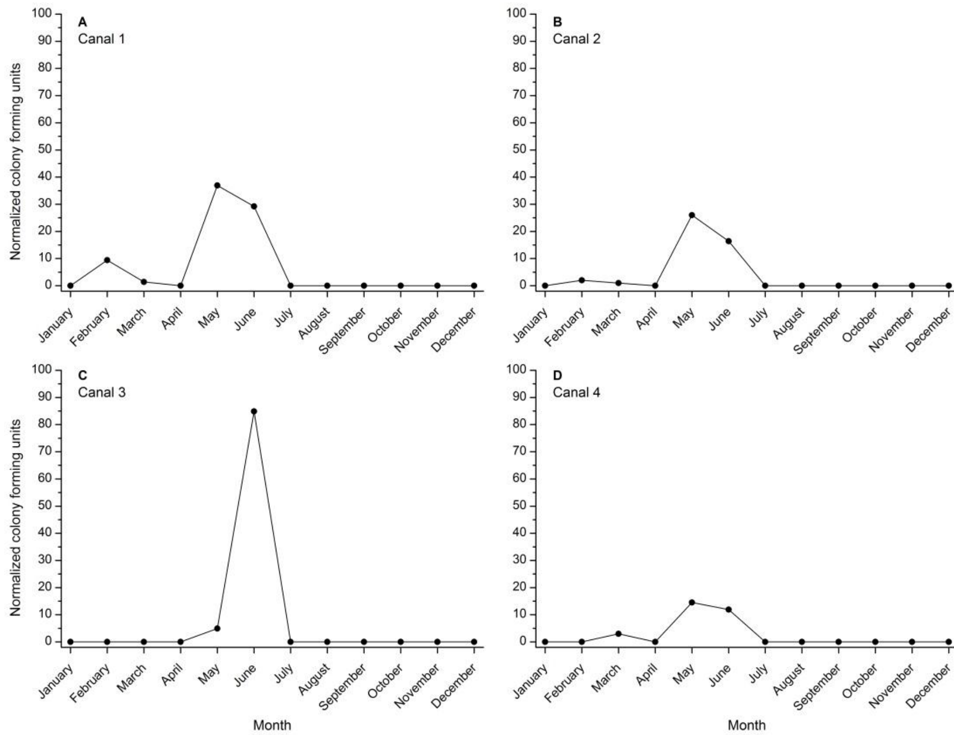

Figure 8. Normalized colony forming units (CFU) for each canal in Weslaco, TX during 2010-2011. Colony forming units were normalized for each canal and plotted as the Y-axis against the months tested during 2010-2011. In general, bacterial densities were below detection but peaked in May and June for all canals. Bacterial densities were observed early in the year in canals 1, 2 and 4 but never exceeded bacterial densities detected in May and June.

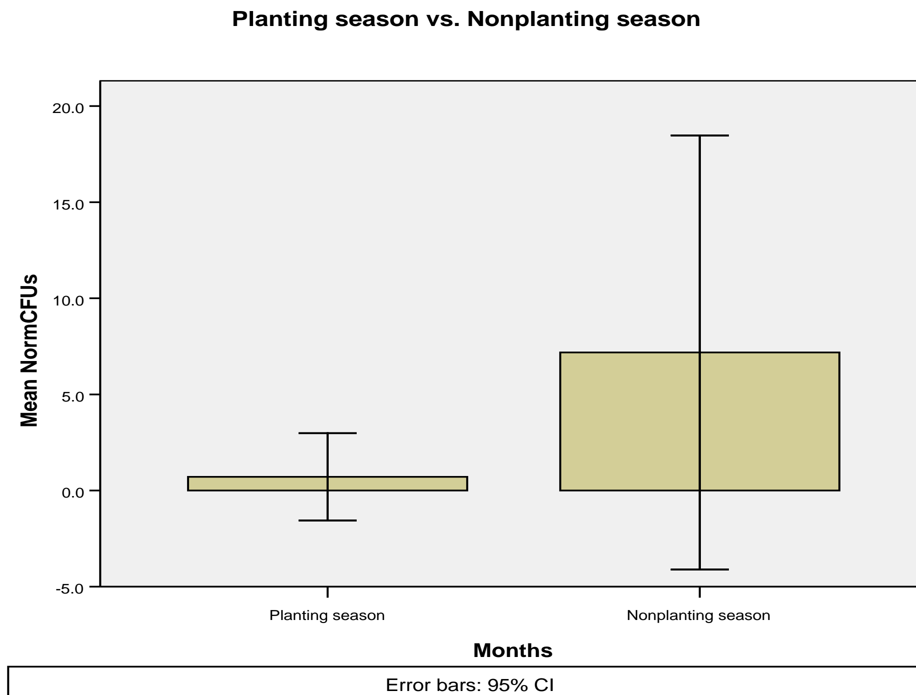

Figure 9. Average normalized colony forming units were averaged and compiled during planting months and non-planting months during 2010-2011. Average CFUs were higher in the non-planting months; however, there was no statistical difference between planting and non-planting seasons (P=0.617).

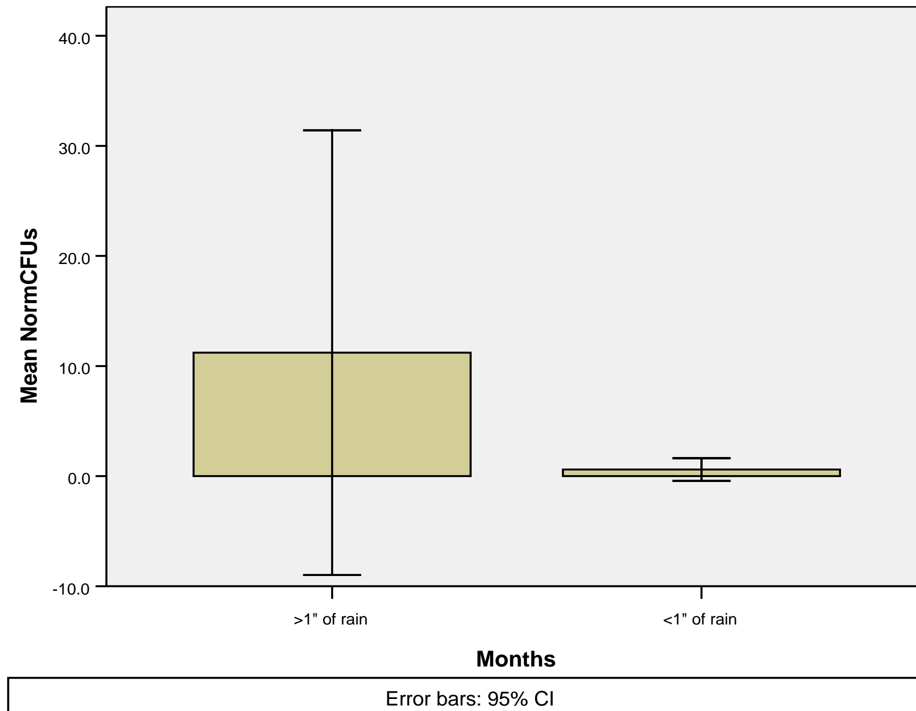

Figure 10: Average normalized CFUs were collected during months with more than one inch of rain (May-September) and compared against months with less than one inch of rain (January-April and October-December). Average CFUs were tended to be higher in months more rainfall. A statistical difference between months with one inch of rainfall and month less than one inch of rainfall was observed (P=0.044).

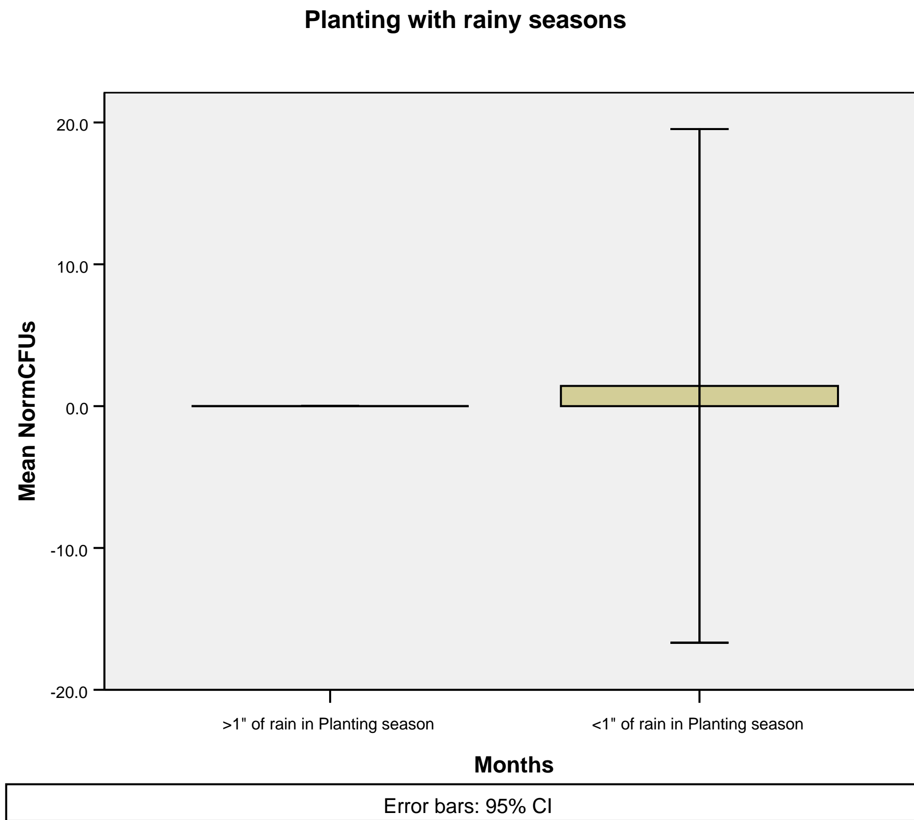

Figure 11. Average CFUs were normalized and compiled during planting months with more than one inch of rain (January-February) and compared against planting months with less than one inch of rain (July-August). Average CFUs tended to be higher in planting months with less than one inch of rain; however, there was no significant difference between planting months with rainfall included (P=0.317).

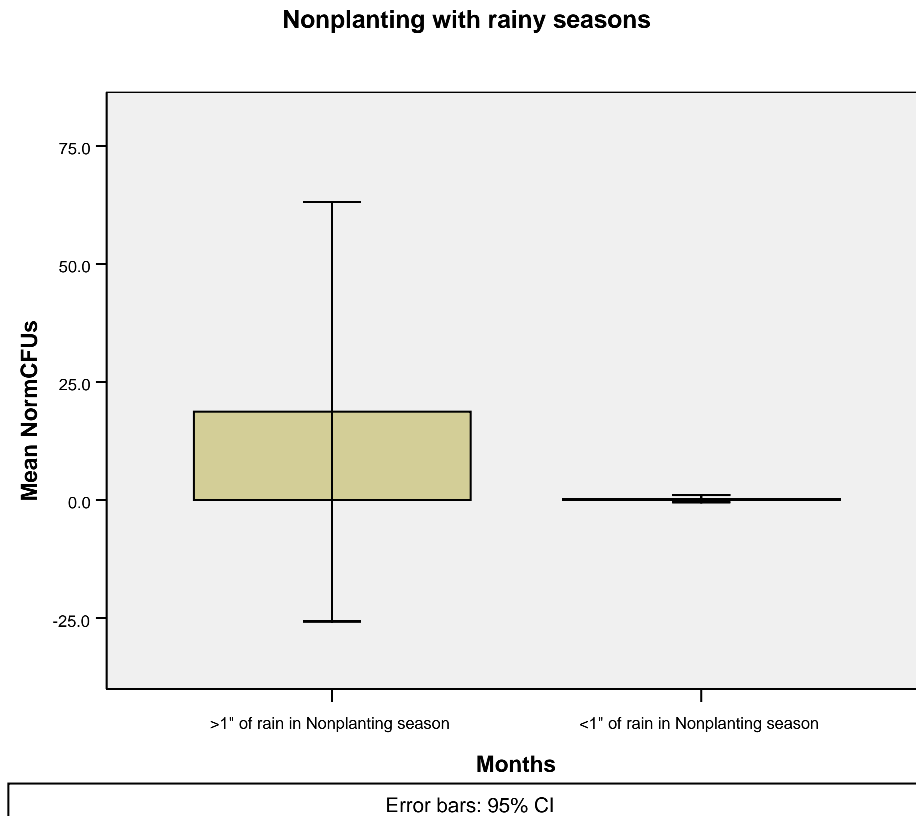

Figure 12. Average normalized CFUs were compiled during non-planting months with more than one inch of rain (May-June and September) and compared against non-planting months with less than one inch of rain (March-April and October-December). The average colony forming units were higher in the non-planting months with more rainfall than in the non-planting months with less than one inch of rain; however, there was no statistical difference between these groups (P=0.124).

Standard PCR amplification of *atzA* and *atzC* in canal bacteria

To determine genes for atrazine degradation were present in LRGV samples, atrazine-tolerant microorganisms isolated from canals were screened for the presence of atzA, B, and C on genomic by PCR amplification using known primers. Amplification of atzB was not successful (data not shown) possibly because the canal isolates did not possess the gene on plasmids or because the gene sequence of the canal isolates was not amplified with the used primer set due to slight sequence differences. Genes atzA and atzC were successfully amplified from a number a number of isolates (Figure 13). These results made it possible for these genes to be used in Quantitative PCR (Q-PCR) assays.

Figure 13. PCR amplification of *atzA* and *atzC* genes from canal bacteria. Plasmids were isolated from atrazine-tolerant isolates using commercial kits and amplified with primers for *atzA* and *atzC*. Genomic DNA from *Pseudomonas* sp. Strain ADP was used as a positive control; genomic *E. coli* DNA was used as negative control. Top panel: positive amplification with *atzA*. Bottom panel: positive amplification with *atzC*.

Lane 1: 1kb size ladder

Lane 2: Positive control (*Pseudomonas* sp. strain ADP): *atzA* gene top panel, *atzC* gene bottom panel

Lane 3: Negative control (E. coli)

Lane 4-15: Atrazine-tolerant bacterial isolates

Gene *atzA* abundance in canals

Q-PCR was used to estimate the monthly abundance of atrazine-degrading genes in South Texas agricultural canals. Gene atzA was used as the representative gene marker for atrazine degradation because it had been detected in isolated bacteria (Figure 14) and is the first, and most important step in the degradation pathway (Kross 1992). Gene *atzA* was detected in all 4 canals and was highest in summer months (Figure 14). The greatest amount of atzA was observed was observed in canal 4 in July; the amount of atzA was over 220 times the lowest detectable level. Gene atzA abundance was much lower in the other canals, although all had their peak abundance in July (Canals 1, 3 and 4) or August (Canal 2) (Figure 14). The greatest concentration for canals 1, 2 and 3 were approximately 20 times higher than lowest detectable level, respectively.

Data from the Q-PCR assays was compiled into planting versus non-planting, rainy vs. dry, and planting and non-planting months with rainfall included. Amplicon abundance of atzA was, on average, greater during planting months (January-February and July-August) compared to non-planting months (March-June and September-December) (Figure 15). Abundance of atzA was on average greater overall during months with more than 1 inch of rain (Figure 16) regardless of whether planting or non-planting was associated with the rainy seasons (Figure 17 and 18). Just as what was observed in atrazine concentration, and atrazine-tolerant microbial density, abundance of gene atzA was greatest during periods of the year when months received more than 1 inch of rainfall. Although data were not statistically different within the error of the measurements, there was a clear, obvious trend that precipitation plays a role in potential atrazine degradation in the canals.

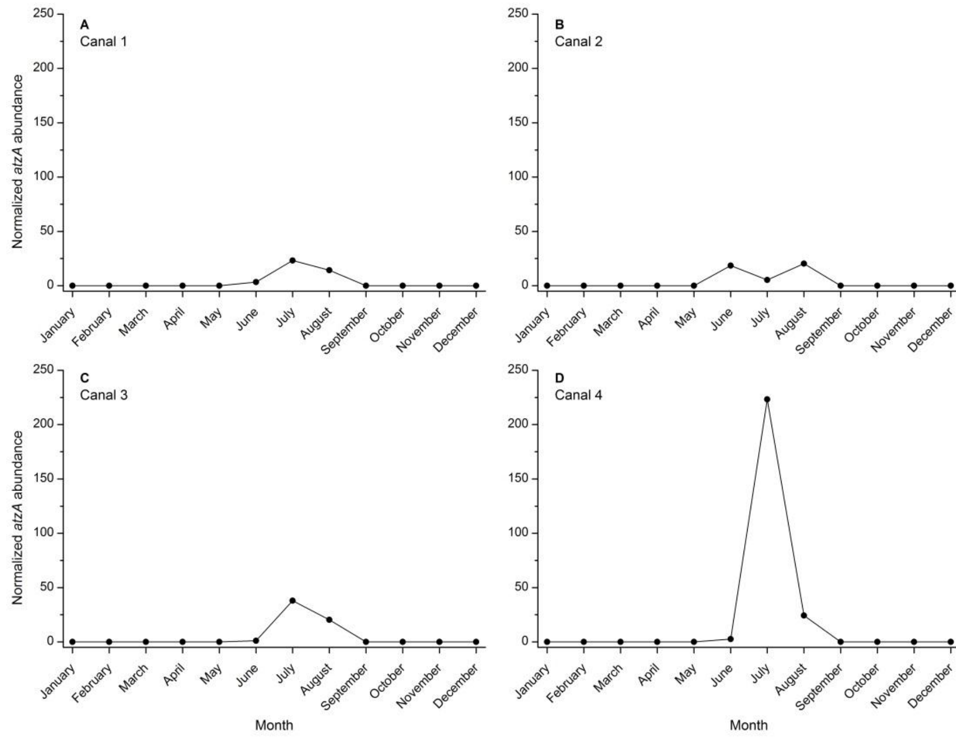

Figure14. Normalized DNA concentrations for each canal in Weslaco, TX during 2010-2011. DNA abundance was originally measured in picograms then normalized and plotted on the Y-axis against months tested for *atzA* amlpicons. DNA abundance was detected from July through August for all canals during 2010-2011.

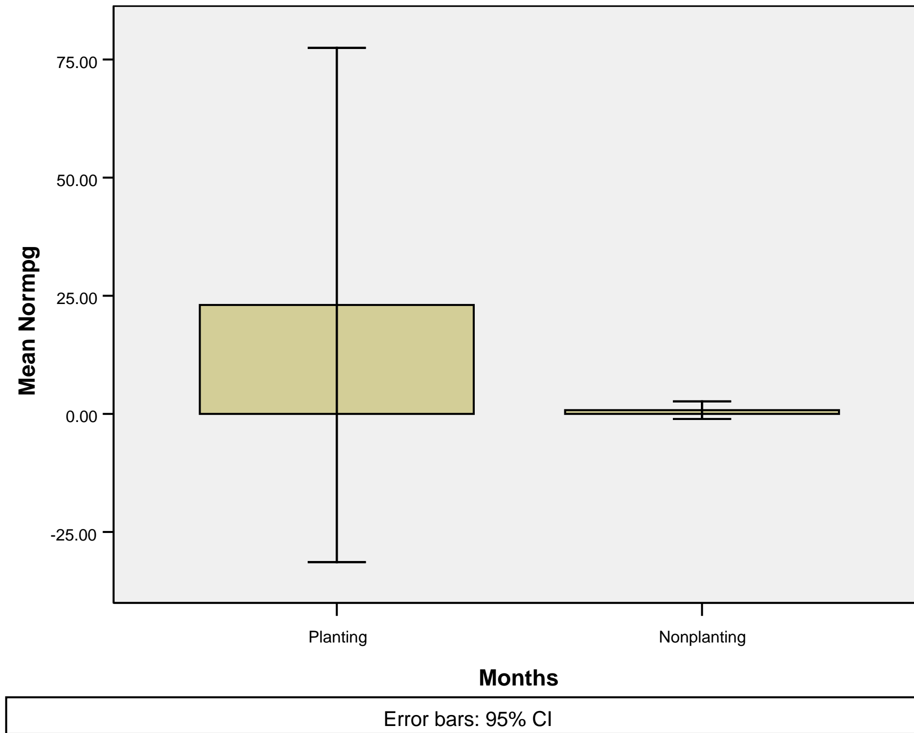

Figure 15: Average normalized *atzA* amplicon abundance during the planting (January-February 2011 and July-August 2010) and the nonplanting seasons (March-June 2010 and September-December). There was not a statistical difference between the planting seasons however, average amplicon levels were higher in the planting season.

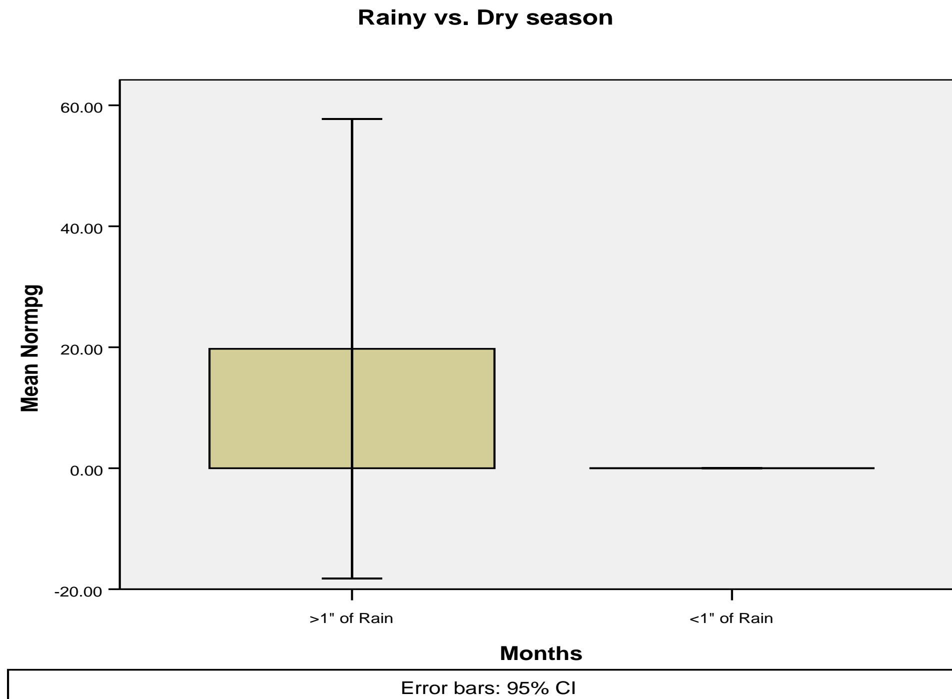

Figure 16: Average normalized amplicon abundance during months with more than one inch of rain (May-September) and months with less than one inch of rain (January-April and October-December). There was no statistical difference between groups (P=0.119); however, average amplicon abundance was higher in months with more than one inch of rain than in months with less than one inch of rain.

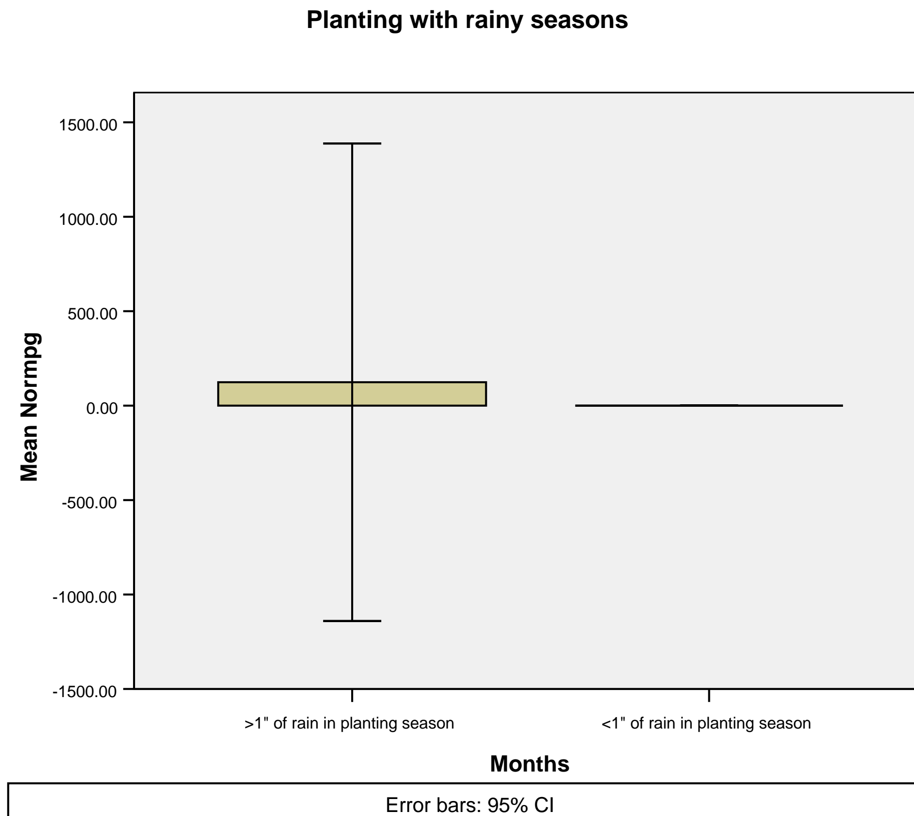

Figure 17. Average normalized *atzA* amplicon abundance during planting months with more than one inch of rain (July-August) and planting months with less than one inch of rain (January-February). A statistical difference (P=0.025) was observed between these groups with higher average amplicon levels in planting months with higher rainfall.

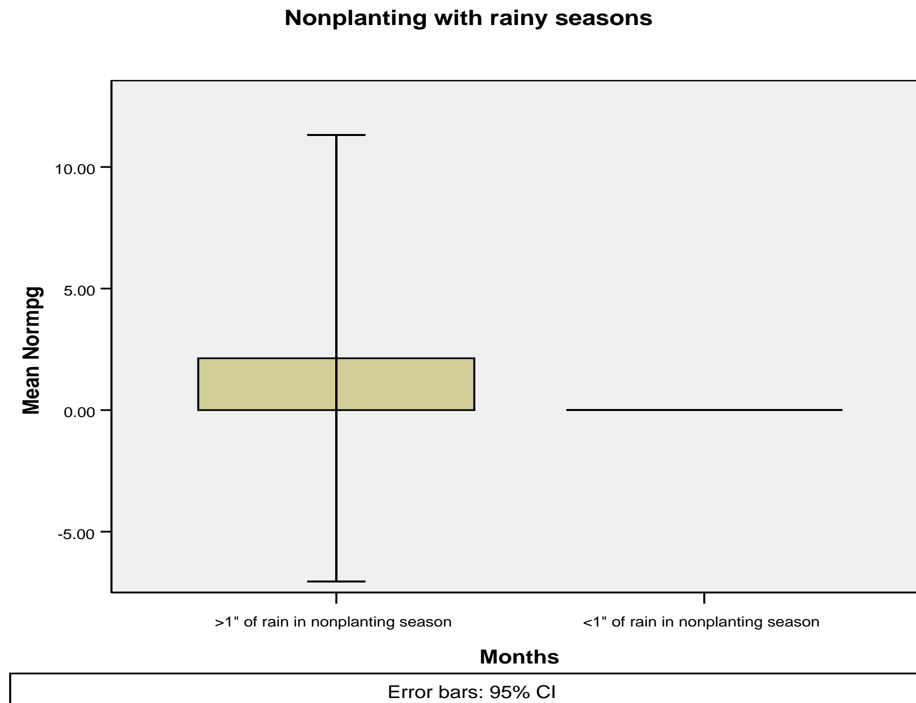

Figure 18. Average normalized *atzA* amplicon abundance was compiled during non-planting months with more than one inch of rain (May-June and September) and non-planting months with less than one inch of rain (March-April and October-December).There was no significant difference between these seasons (P=0.197); however, average amplicon abundance was higher in non-planting months with more than one inch of rain than in non-planting months with less than one inch of rain.

CHAPTER IV

DISCUSSION AND CONCLUSION

Atrazine-ELISA test

In this study, the Atrazine ELISA kit was used to quantify the concentration of atrazine in parts per billion from water samples taken from agriculture canals. Over 90% of corn crops are treated with the pesticide atrazine and therefore should be present in proximal reservoirs during planting seasons and times of precipitation. This kit was chosen because: it requires only a few milliliters of sample, the kit contains antibodies with a high affinity for atrazine thus limiting cross contamination from analogous chemicals and lastly, results are parallel to HPLC results. In general, atrazine levels peaked in spring and summer months (March, May-June and August) for all canals during 2010-2011. Atrazine levels were not detected during both planting seasons (January-February and July-August) indicating pre and post planting applications are peak times for atrazine presence in agricultural canals (Figure 3). This was further observed when average atrazine concentrations tended to be higher in the non-planting months than planting months (Figure 4). Although the concentration of atrazine never exceeded atrazine's maximum

contaminant levels of 3ppb for any canal; highest concentrations of atrazine were detected in canals 1 and 2. This may be due to their rural location compared to canals 3 and 4 which were located in an urban setting. Canals 1 and 2 possibly received more atrazine in the rural area considering more agricultural crops surrounded these canals. Unlike canals 3 and 4 which were surrounded by housing developments. Thus, atrazine concentrations in agricultural canals vary throughout the year and contamination is not restricted to planting seasons but possibly due to trend of pesticide application and environmental settings e. g. urban or rural areas.

The average rainfall for Hidalgo County was highest during May-September (months with more than one inch of rain) with June receiving the most precipitation (8-10”) out of the year (NOAA 2011). In general, this study reported that average atrazine concentrations in canals peaked during months with more than 1 inch of rain (Figure5). This remained apparent when average atrazine concentrations in both planting and non-planting seasons were higher only during months with more than one 1 inch of rainfall (Figures 6 and 7). Similar results, from previous studies observed peak atrazine concentrations in May and June, months receiving more rainfall (Goolsby 1995 and Chung 2009). Average atrazine levels may be high during these months due to the combination of pre-emergent fall application of atrazine and heavy rainfall in the Lower Rio Grande Valley. Clearly though, precipitation has a stronger influence on the presence of atrazine in agricultural canals than the trend of pesticide applications. A report by Sprague et al. (2000) also observed that the mobilization of atrazine is dependent on the rate of precipitation.

Though, the highest concentration of atrazine was detected in canal 2 which was outside months of rainfall and planting seasons. This suggests a heavy treatment of atrazine may have been applied to fields surrounding this canal after the planting season along with the usage of

irrigation techniques. Farmers tend to apply atrazine during times of precipitation in order to activate the pesticide; however, in times of drought, farmers will activate atrazine by irrigating their fields. Just as rainfall can mobilize atrazine, irrigation can also influence atrazine's presence in surface waters via run-off (Sprague 2000 and Atrazine's factsheet 2003). Overall, atrazine levels were detected during spring and summer months in agricultural canals in the LRGV. The location of these canals in rural or urban environment make them more susceptible to atrazine contamination during months of heavy rainfall.

Q-PCR analysis of *atzA* abundance

Quantitative PCR is a very sensitive technique capable of detecting the smallest amounts of DNA in a sample. This makes it a perfect candidate for measuring atrazine-degrading gene *atzA* in the environment. The gene *atzA* was chosen as an indicator for atrazine-degrading populations inhabiting agricultural canals in the LRGV. This gene is involved in the most important step in the metabolism and detoxification of atrazine and thus serves as a unique characteristic for atrazine-degrading populations.

For most of the year, amplicon abundance was below detection; however, *atzA* levels peaked drastically in mid to summer months. In this study, *atzA* amplicons were detected from June through August for all 4 canals with canal 4 containing the highest level of DNA detected during 2010-2011 (Figure 8). Furthermore, both planting and non-planting seasons contained higher average amplicon abundance during months with more than 1 inch of precipitation. This suggests rainfall, during summer months, have a strong influence on the presence of atrazine-degrading populations in agricultural canals, rather than atrazine applications during planting

seasons. Atrazine degrading amplicons were undetectable most of the year; this suggests atrazine-degrading populations may be present in agricultural fields' year around however become abundant enough to be carried by run-off into agricultural canals during months of rainfall. Although some pesticides become toxic to different genera of bacteria, atrazine can promote the growth of bacterial populations possessing these genes (Martin-laurent 2003). This can lead to shifts in the bacterial community with atrazine-degrading populations diluting the community during times of applications. Though the data presented in this report indicates the peak in *atzA* possessing populations is temporary and can decline as quickly as they had arisen. In addition, atrazine-degrading gene populations reflect atrazine levels in canals during 2010-2011 (Figures 3 and 8). In general, high atrazine concentrations were observed in May and June for all canals; conversely, amplicon abundance arises in June, and persisted until August. Data shows that the detection of amplicon levels was delayed until traces of atrazine reached measurable levels. Thus, this report demonstrates that atrazine contamination in the environment may have an influence on atrazine-degrading gene populations. Though, atrazine-degrading populations didn't respond every time atrazine was detected, however amplicon levels spiked when atrazine concentrations were detected for two consecutive months (Figures 3 and 8). This suggests the duration of atrazine contamination in the environment has a stronger influence on atrazine-degrading gene population's abundance than 1 month with high atrazine levels. In previous studies, atrazine-degrading isolates didn't increase until nearly a week after treatment of atrazine (Martin-Laurent 2003); this is known as the lag stage, the period before exponential growth with little to no growth. Following their initial detection, atrazine levels dissipate in July while atrazine-degrading gene populations peak in this month. This suggests a negative relationship between atrazine levels and atrazine-degrading populations in which the reduction of

atrazine is dependent on the abundance of atrazine-degrading gene populations. Several *in vitro* studies have demonstrated a consortium of atrazine-degrading populations can quickly reduce atrazine levels (Mendelbaum 1993, De Souza 1996, Downing 2003).

Overall, the results support previous data from studies showing that atrazine degrading genes increase in the presence of atrazine (Martin-laurent 2003). Moreover, in the rainfall season, atrazine degrading bacterial populations are likely to be found in abundance when atrazine is detected in consecutive months. The atrazine-degrading gene *atzA* is involved in an essential step in the biodegradation of atrazine and is abundant during times of atrazine contamination in the LRGV.

Atrazine-tolerant bacterial Colony Forming Units (CFUs)

Atrazine treatments in the environment can adversely affect the size of bacterial populations (Downing, 2004). Furthermore, pesticides change the diversity of bacterial communities, negatively affecting susceptible populations and increasing tolerant populations (Johnsen, 2001). Since total DNA from environmental samples cannot be extracted, isolating and culturing bacterial communities can reveal the level of abundance of atrazine-tolerant bacterial populations in agricultural canals. Atrazine-tolerant bacterial populations were isolated from water samples taken from agriculture canals. Abundance of CFUs differed for each canal and was higher in the summer months, May and June than in February and March. Higher CFU abundance in summer corresponds with atrazine presence during these months. As seen in figure 13, CFUs were nearly 10^2 higher in May and June than in February and March .This confirms previous studies that pesticide-tolerant populations increase at times of pesticide exposure.

Comparing populations isolated during planting and nonplanting seasons show on average CFUs were higher in the nonplanting season. However CFUs were higher in the rainy season than the dry season, suggesting rainfall may have a stronger influence on their presence in agriculture canals. The combination of planting+rainy season and planting+dry season showed the dry planting season had higher average CFU. However the rainy nonplanting season had a higher abundance of atrazine-tolerant populations than the dry nonplanting season therefore confirming rainfall has a stronger effect on these populations than the planting seasons. The data reported in this study suggest atrazine tolerant bacterial populations are present in Lower Rio Grande Valley agriculture canals, as seen in a previous study (Puente, 2010), and their abundance may be due to rainfall events.

Concluding remarks

The toxic herbicide atrazine was detected in agriculture canals tested in Weslaco, Texas with higher concentrations during the rainy season. Atrazine-tolerant bacterial populations were isolated from these canals and like atrazine abundance were higher during the rainy months May and June. The gene used to report the presence of atrazine-degrading bacteria *atzA* was detected only during summer months that are associated with the rainy and planting season. In conclusion, atrazine abundance was detected in agricultural canals during the rainy season in the Lower Rio Grande Valley which suggests canals receive atrazine which increase atrazine-degrading and atrazine-tolerant bacterial populations. Since atrazine-degrading and atrazine-tolerant populations were not found in planting months then the new hypothesis must be reformulated

and stated as: during months of heavy rainfall, atrazine-degrading and atrazine-tolerant populations will be found in agricultural canals in the LRGV.

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BIOGRAPHICAL SKETCH

The author of this thesis, Ibdanelo Cortez, has completed a Bachelor and Master of Science in Biology at The University of Texas-Pan American. The Bachelors of Science was achieved in May 2008. The Masters of Science was achieved in May 2011. As an undergraduate, Mr. Cortez began working as a Research Assistant for Microbiologist, Dr. Kristine Lowe in June 2007-May 2008. In this time he was trained to process environmental samples, culture bacteria and isolate DNA from bacterial isolates using commercial kits. As a graduate student, he was mentored by Dr. Kristine Lowe throughout his graduate thesis project from August 2008-May 2011. Ibdanelo was awarded a position in UTMB's Bridge to PhD program in May 2009 in which recipients were awarded annual fellowships throughout the duration of their project. Mr. Cortez plans to attend UTMB in the Fall of 2011 where he will pursue a graduate degree in Neuro-Cell Biology.